705-P

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

No. 14-117672- -00000-0000

Fecha: 2014-05-30 16:22:02 Dep. 2020 DIR.NUEVASCR
Tra. 11 PATENTEIYII Eve: 378 FASENACIONALI
Act. 411 PRESENTACION Folios: 5

TM TAMAYO
· PATENTS · TRADEMARKS

Industria y Comercio
SUPERINTENDENCIA

DELEGATURA PROPIEDAD INDUSTRIAL

División de Nuevas Creaciones

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO
INVENTARIO

SOLICITUD DE:

PATENTE DE INVENCION

PCT FASE NACIONAL

21. EXPEDIENTE No. _____

54. TITULO: **ANTÍGENOS Y VACUNAS DIRIGIDOS CONTRA ENTEROVIRUS HUMANOS**

71. SOLICITANTE : SENTINEXT THERAPEUTICS SDN BHD

DOMICILIO: **MENARA NORTHAM, PENANG, MALASIA**

74.- APODERADO**: JOHAN SEBASTIAN GOMEZ VILLA**

22.- **Bogotá, MAYO 30 DE 2014**

Industria y Comercio
SUPERINTENDENCIA

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:ación

DIRECCIÓN DE NUEVAS CREACIONES
SOLICITUD FASE NACIONAL -PCT

1 TIPO DE SOLICITUD

☒ Patente de invención ☐ Patente de Modelo de Utilidad
☒ Capítulo I ☐ Capitulo II

2 DATOS SOLICITUD INTERNACIONAL PCT (86)

Solicitud Internacional No.	PCT/IB2012/003114	**Fecha**	2012/11/01
Publicación Internacional No.	WO 2013/098655 A2	**Fecha**	2013/07/04

3 TÍTULO DE LA INVENCIÓN (200 caracteres o espacios máximos)

ANTÍGENOS Y VACUNAS DIRIGIDOS CONTRA ENTEROVIRUS HUMANOS

4 CLASIFICACIÓN INTERNACIONAL (CIP)

5 SOLICITANTE (S) ☐ Esta persona también es inventor. Para datos adicionales utilizar hoja de información complementaria

APELLIDOS O RAZÓN SOCIAL	NOMBRE	IDENTIFICACIÓN	TIPO
1. SENTINEXT THERAPEUTICS SDN BHD		EXTRANJERA	

6 DATOS DEL SOLICITANTE

DIRECCIÓN	Suite 19-h, Level 19, Menara Northam, 55 Jalan Sultan Ahmad Shah, Penang, 10050	No. TELÉFONO	
CIUDAD	Menara Northam	CORREO ELECTRÓNICO	
DEPARTAMENTO/ESTADO	Penang	NACIONALIDAD O LUGAR DE CONSTITUCIÓN	
PAÍS DE RESIDENCIA	MALASIA		

7 INVENTOR (ES) Para datos adicionales utilizar hoja de información complementaria

APELLIDOS	NOMBRES	NACIONALIDAD
1. CARDOSA	Mary, Jane	MY
2. JAMILUDDIN	Mohamad, Fakruddin	US
3. HAMID	Sharifah, Binti	MY
4.		
5.		

DIRECCIÓN DE CORREO ELECTRÓNICO:

8 DATOS INVENTOR (ES)

PAÍS RESIDENCIA	DEPARTAMENTO/ESTADO	CIUDAD	DIRECCIÓN
1. Malasia	Sarawak	Kuching	No. 8, Richmond Hill, Lorong Stampin Tengah 5g2, Kuching, Sarawak, 93250
2. Malasia	Gelugor	Penang	10-13-p2 Sri Saujana Apts., Lorong Sungai Dua, Gelugor, Penang 11700.
3. Malasia	Selangor	Shah Alam	No. 39 Jalan Anggerik Tainia, 31/120 Kota Kemuning, Seksyen 31, Shah Alam Selangor, 40460
4.			
5.			

OTRO(S) SOLICITANTE(S) Y/O (OTRO(S)) INVENTOR(ES)

☐ Los demás solicitantes y/o (demás) inventores se indican en una hoja a continuación.

9 ☐ **REPRESENTANTE LEGAL** ☒ **APODERADO**

APELLIDOS	NOMBRES	IDENTIFICACIÓN
GÓMEZ VILLA	Johan Sebastian	C.C .9868640 T.P .202.892

DIRECCIÓN	Cra. 13 A No. 28-38 Ofc. 230 MZ 2 PARQUE BAVARIA	No. TELÉFONO	3 419841
CIUDAD	BOGOTÁ D.C.	CORREO ELECTRÓNICO	consultas@tmtamayo.net
PAÍS	COLOMBIA	No. RADICACIÓN DE PROTOCOLO DE PODER GENERAL	

10 DECLARACIONES DE PRIORIDAD ☒ SI ☐ NO

(33) PAÍS DE ORIGEN	CÓDIGO PAÍS	(31) NÚMERO	(32) FECHA (AAAA/MM/DD)
1. MALASIA	MY	PI2011005318	2011/11/03
2.			
3.			

11 DECLARACIÓN SOBRE USO DE RECURSOS GENÉTICOS O BIOLÓGICOS

Declaro que el objeto de la presente solicitud de patente fue obtenido a partir de recursos genéticos o biológicos de los que cualquiera de los países miembros de la Comunidad Andina es país de origen.

☐ SI ☒ NO

Nota: En caso afirmativo deberá anexar copia del contrato de acceso de recursos genéticos o productos derivados, o certificado o número de registro, expedido por la Autoridad competente.

12 DECLARACIÓN SOBRE USO DE CONOCIMIENTOS TRADICIONALES

Declaro que el objeto de la presente solicitud de patente fue obtenido a partir de conocimientos tradicionales de comunidades indígenas, afroamericanas o locales de países miembros de la Comunidad Andina.

☐ SI ☒ NO

Nota: En caso afirmativo deberá anexar la licencia o autorización de uso de conocimiento tradicional, o certificado, o número de registro expedido por la Autoridad competente.

13 REDUCCIÓN DE TASAS

Declaro que carezco de medios económicos para presentar la solicitud de patente.

☐ SI ☒ NO

Nota: En caso de ser persona natural y carecer de medios económicos, y por lo tanto, aplique la reducción de tasas a que se refiere la resolución vigente en tarifas, debe firmar la presente solicitud bajo la gravedad de juramento.

Micro, pequeñas y medianas empresas ☐
Universidades públicas o privadas ☐
Entidades sin ánimo de lucro ☐

Debe aportar los documentos que se indican en el numeral 17 de anexos

14 AUTORIZACIÓN DE NOTIFICACIÓN EN LÍNEA ☐ SI ☒ NO

Manifiesto que he leído y entendido perfectamente los términos y condiciones de uso de medios electrónicos para las notificaciones en línea a través de Internet de los actos administrativos proferidos por la Superintendencia de Industria y Comercio que deben ser notificados personalmente y, en consecuencia, autorizo el servicio de notificación a través de internet.

15	**COMPROBANTE DE PAGO O PAGO ELECTRÓNICO**	**N° 14-0056136**	**Fecha 28-05-2014**
	COMPROBANTE POR REIVINDICACIÓN ADICIONAL A 10	**No**	**Fecha**

16 FIRMA DEL SOLICITANTE, DEL APODERADO O DEL REPRESENTANTE LEGAL

Junto a cada firma, indicar el nombre del firmante y su calidad (si tal calidad no es obvia al leer el petitorio)

JOHAN SEBASTIÁN GÓMEZ VILLA (Apoderado)

17 ANEXOS

Documentación Técnica
Solicitud Internacional en castellano

1. ☒ Descripción — N° de folios: 109
2. ☒ Reivindicaciones — N° Reivindicaciones: 55
3. ☒ Dibujos y/o figuras — N° folios: 25
4. ☒ Resumen.
5. ☐ Certificado de depósito de material biológico si fuera el caso.
6. ☒ Listado de secuencias de nucleótidos y/o aminoácidos en forma digital si fuera el caso.
7. ☐ Arte final 12 x 12.
8. ☒ Anexo formato digital.

Documentación Jurídica

9. ☐ Poderes, si fuera el caso.
10. ☐ Documento que legalmente pruebe la cesión del inventor al solicitante o a su causante.
11. ☐ Copia del contrato de acceso de recursos genéticos o productos derivados, certificado o número de registro, si fuera el caso.
12. ☐ Copia de la licencia o autorización de Conocimientos Tradicionales, certificado o número de registro, si fuera el caso.
13. **Reducción de tasas**

Micro, pequeñas o medianas empresas

☐ Copia simple de la declaración de renta del año inmediatamente anterior, o en su defecto prueba documental idónea.

☐ Documento de constancia de cumplimiento con lo establecido en la ley 905 de 2004.

Universidades públicas o privadas

☐ Copia acto de reconocimiento institucional emitido por el Ministerio de Educación

Entidades sin ánimo de lucro

☐ Copia de registro vigente en Cámara de comercio.
☐ Hoja de información complementaria.
☐ Otros, especificar

14. ☒ Comprobante de pago de la tasa de presentación de la solicitud.
15. ☐ Comprobante de pago por reivindicación de prioridad.
16. ☐ Comprobante de pago de la tasa por concepto de excedente de palabras en la publicación.
17. ☐ Comprobante de pago por reivindicación adicional a 10.

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO
NIT : 800.176.089-2
- / -

Industria y Comercio
SUPERINTENDENCIA

RECIBO DE CAJA

No. 14 - 0056136

Bogotá D.C., Mayo 28 de 2014 - 11:04:47

RECIBIDO DE : TM TAMAYO Y ASOCIADOS LTDA NI 830.091.650 RE

*** Soporte del Pago ***

TIPO PAGO	BANCO	CUENTA	No. PAGO	FECHA PAGO	VR.PAGO
RECIBO DE CA	999999999	57432	08/06/2012		140.000.00
CONSIGNACION	BANCO DE BOGOTA	062754387	601648342	27/05/2014	596.000.00

*** Conceptos Pagados ***

CANT.	RENTISTICO	CONCEPTO	Vr.UNDITARIO	Vr.CONCEPTO
1	50005-01-01 SOLICITUDES	1 TRAMITES DE SOL. DE PATENTE DE INVENCION	515.000.00	515.000.00
				$515.000.00

SON: **QUINIENTOSQUINCE MIL PESOS MONEDA CORRIENTE***

Responsable: _____
-*-

Recibo de Caja Aplicado al Expediente No. _____

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4

Industria y Comercio
SUPERINTENDENCIA

PLANILLA DE RADICACIÓN - DIGITALIZACIÓN DIARIA
RELACIÓN DE ANEXOS

Dependencia:	Fecha: 19 / 06 / 11 (AAAA / MM / DD)	Recorrido:
2020		

Tipo de Radicación: Entrada: X Salida: ☐ Traslado: ☐ Convenio: ☐

Radicador: | Planilla:

ID	No. Radicación	Consecutivo	Item
1	14-117672	0	
2			
3			
4			
5			
6			
7			
8			
9			
10			
11			
12			
13			
14			
15			
16			
17			

Item	Unidad de Conservación
1	Bolsa
2	Caja
3	Libro
4	Sobre
5	Tomo
6	Otro
Item	*Soporte Documental*
7	CD
8	Disquete
9	DVD
10	Folleto
11	Foto
12	Periódico
13	Plano
14	Publicación Periódica
15	USB
16	Otro

Observaciones:
Folio (4) ANEXA CD, el cual contiene un total de (11) Archivos en formato PDF. y fueron procesados correctamente.

Yenny.

Industria y Comercio
SUPERINTENDENCIA

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PATENTE DE INVENCIÓN ☐ **MODELO DE UTILIDAD** ☐ **Art 33 Decisión 486/00**

- ☐ Indicación que se solicita una patente.
- ☐ Datos de identificación del solicitante o de la persona que presenta la solicitud
- ☐ Descripción de la invención
- ☐ Dibujos de ser estos pertinentes
- ☐ Comprobante de pago de las tasas establecidas (De ser el caso formato de descuento)

Completa ☐ Incompleta ☐

PATENTE DE INVENCIÓN PCT ☐ **MODELO DE UTILIDAD PCT** ☐ **Art.33 Decisión 486/00, Circular Única**

- ☐ Indicación que se solicita una PCT
- ☐ Copia de la solicitud en español, tal como fue presentada inicialmente (capítulo descriptivo, reivindicatorio, resumen)
- ☐ Dibujos de ser estos pertinentes
- ☐ Comprobante de pago de las tasas establecidas (de ser el caso formato de descuento)

Completa ☐ Incompleta ☐

DISEÑO INDUSTRIAL ☐ **(Art. 119 Decisión 486/00)**

- ☐ Indicación que se solicita Diseño industrial
- ☐ Datos de identificación del solicitante o de la persona que presenta la solicitud
- ☐ Representación gráfica y fotográfica del Diseño industrial o muestra del material que incorpora el diseño
- ☐ Comprobante de pago de las tasas establecidas

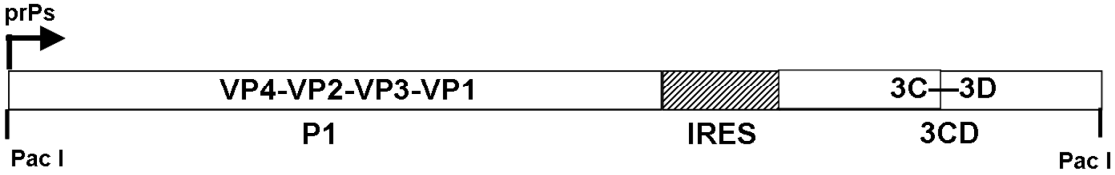
Completa ☐ Incompleta ☐

ESQUEMA DE TRAZADO ☐ **(Art. 92 Decisión 486/00)**

- ☐ Indicación que se solicita un esquema de trazado
- ☐ Datos de identificación del solicitante o de la persona que presenta la solicitud
- ☐ Representación gráfica de un esquema de trazado
- ☐ Comprobante de pago de las tasas establecidas

Completa ☐ Incompleta ☐

Figure 1. HEV71 VLP expression cassette [P1+IRES+3CD] and the pSN01 plasmid.



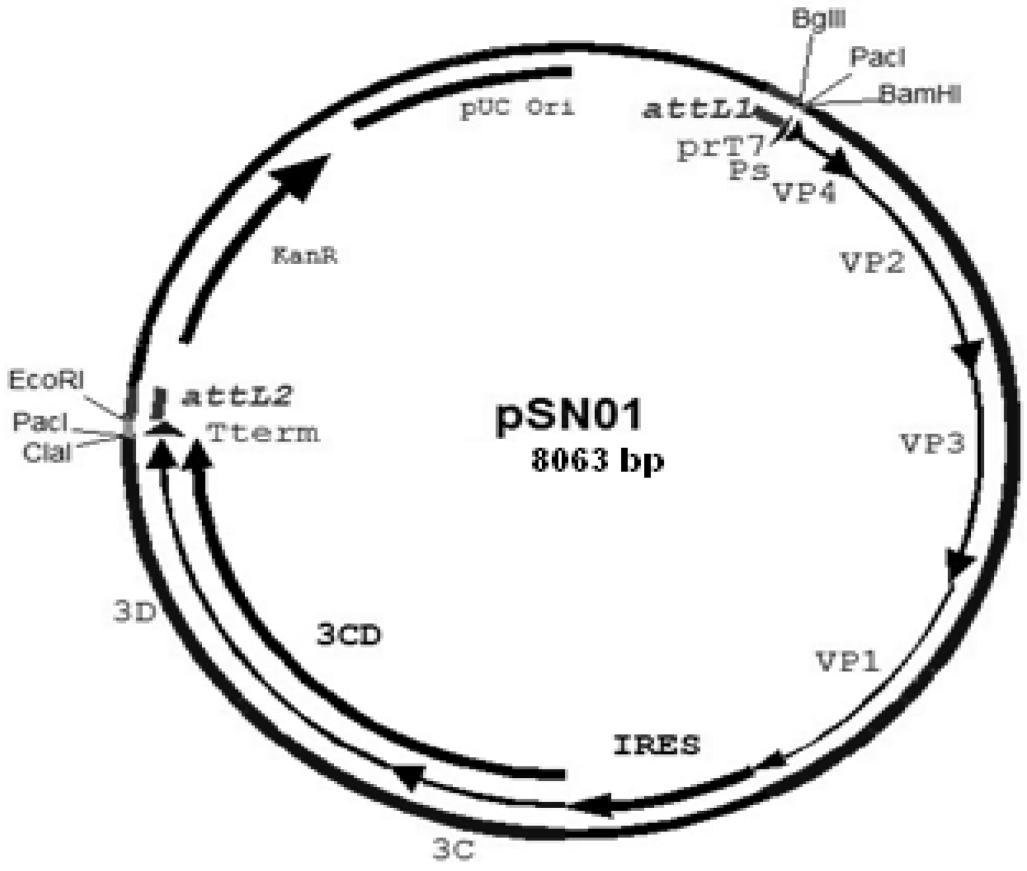
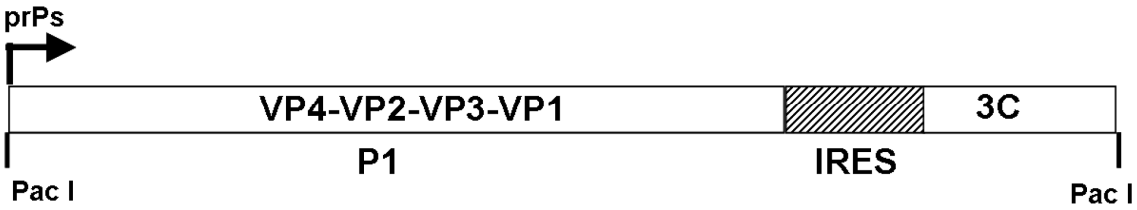


Figure 2. HEV71 VLP expression cassette [P1+IRES+3C] and the pSN03 plasmid.



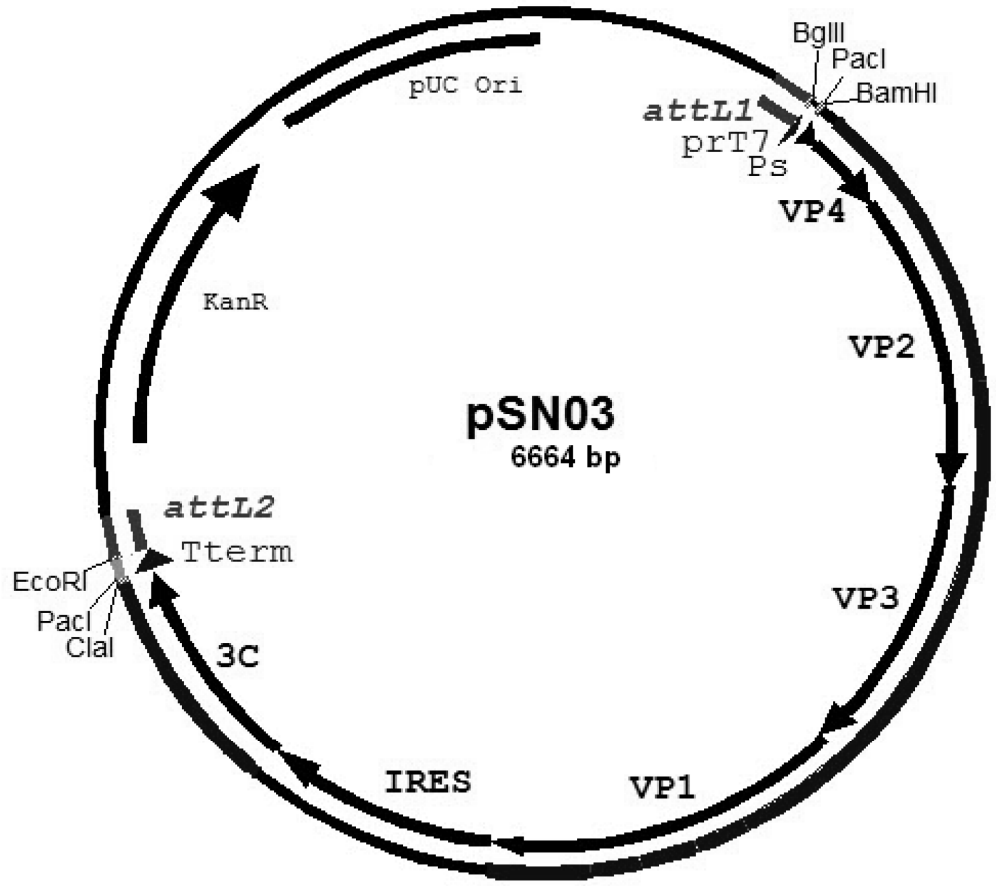


Figure 3. Expression of VP1 in supernatant of SN07 infected Sf9 cells.

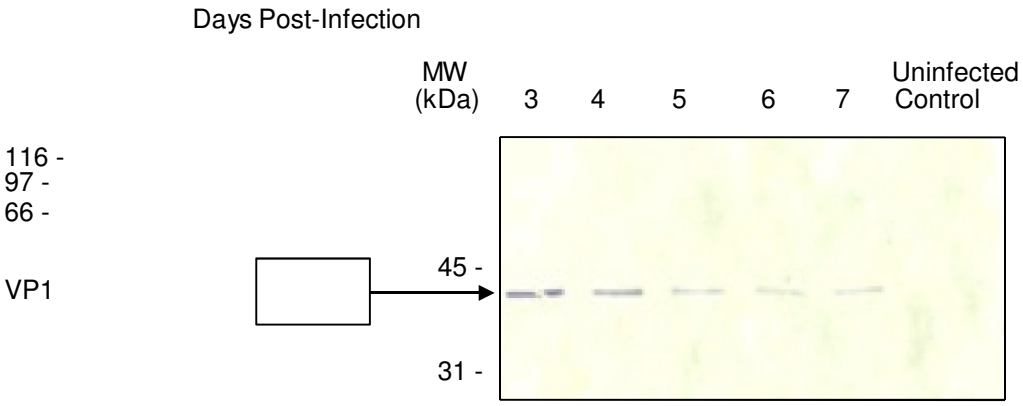


Figure 4. Processed VP1 in both the supernatants and the lysates.

Lanes 1 and 5: bacSN07p3

Lanes 2 and 6: bacSN08p3

Lanes 3 and 7: bacGUSd2

Lanes 4 and 8: Sf9 control

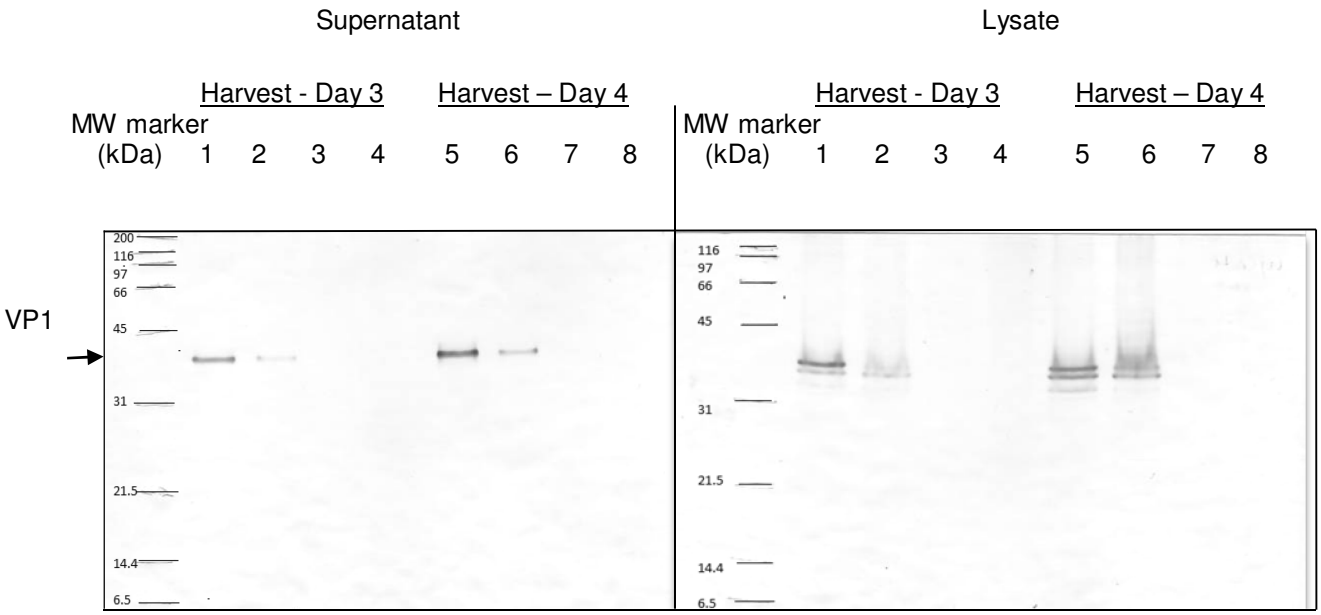


Figure 5. VP1 and VP0 are in the retentate after ultrafiltration over a 100kDa molecular weight cut off (MWCO) membrane.

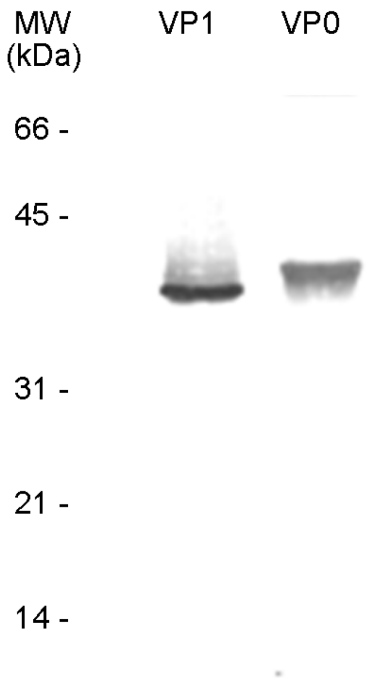


Figure 6.

FIRST PROOF OF CONCEPT OF OUR STRATEGY: TWO IMMUNIZATION SCHEDULES (4 mice per group).

The immunogen was prepared in Freund's Complete Adjuvant (FCA) or Freund's Incomplete Adjuvant (FIA) and administered via subcutaneous injection (sc) or intraperitoneal injection (ip).

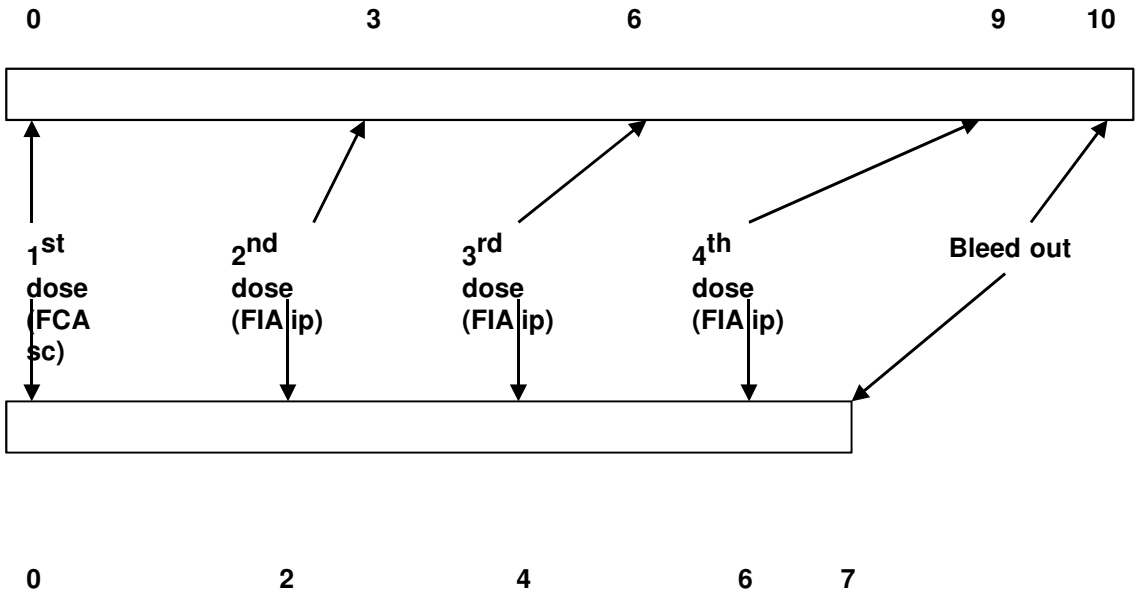


Figure 7. Immunoblots probed with rabbit polyclonal antisera against VP0 (arrow) at 3 different times of harvest.

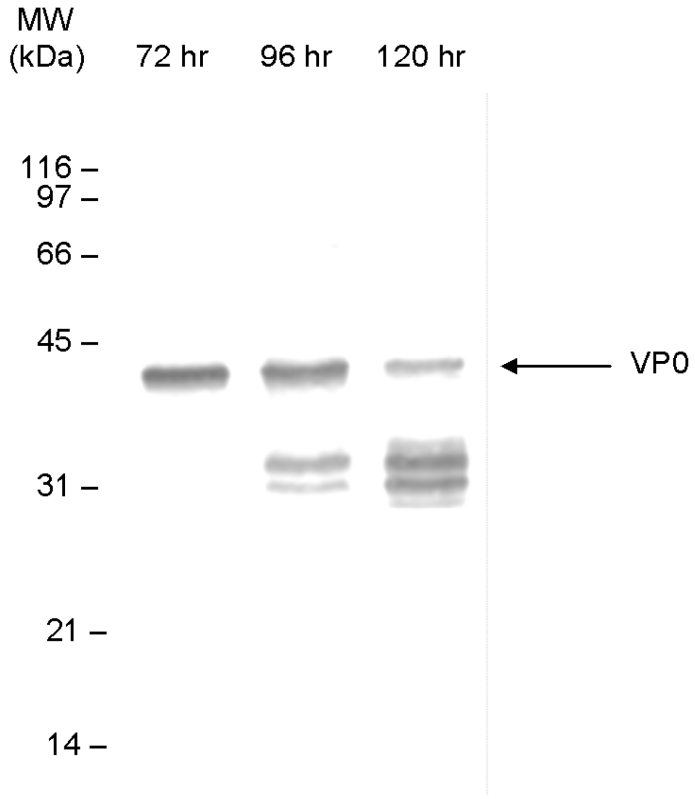


Figure 8. Pooled neutralizing sera from mice immunized with the oligomeric antigens in the supernatant of SN07 infected Sf9 cells have high titres against recombinant VP2 in ELISA.

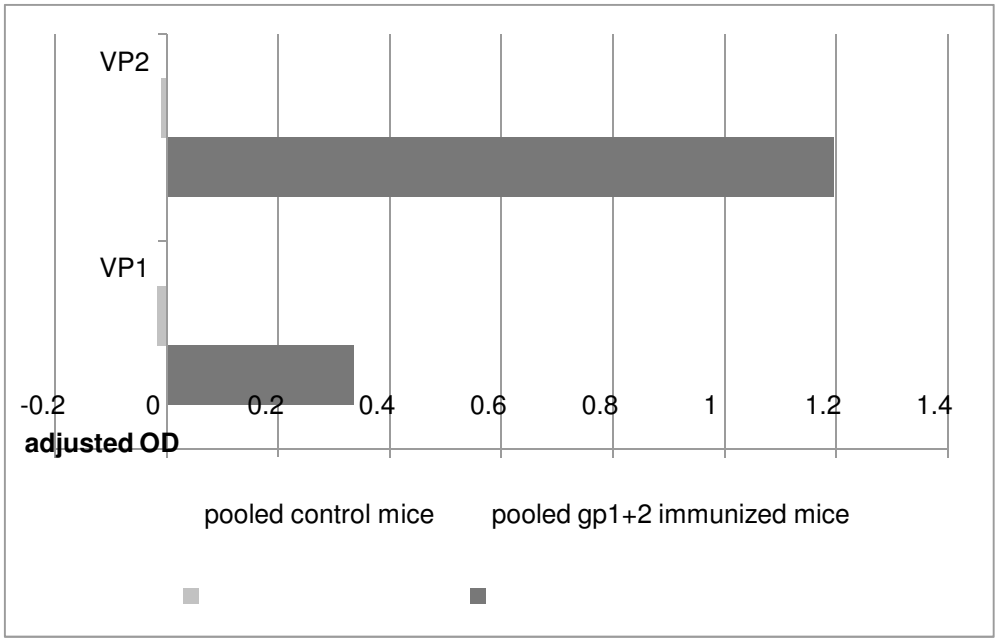
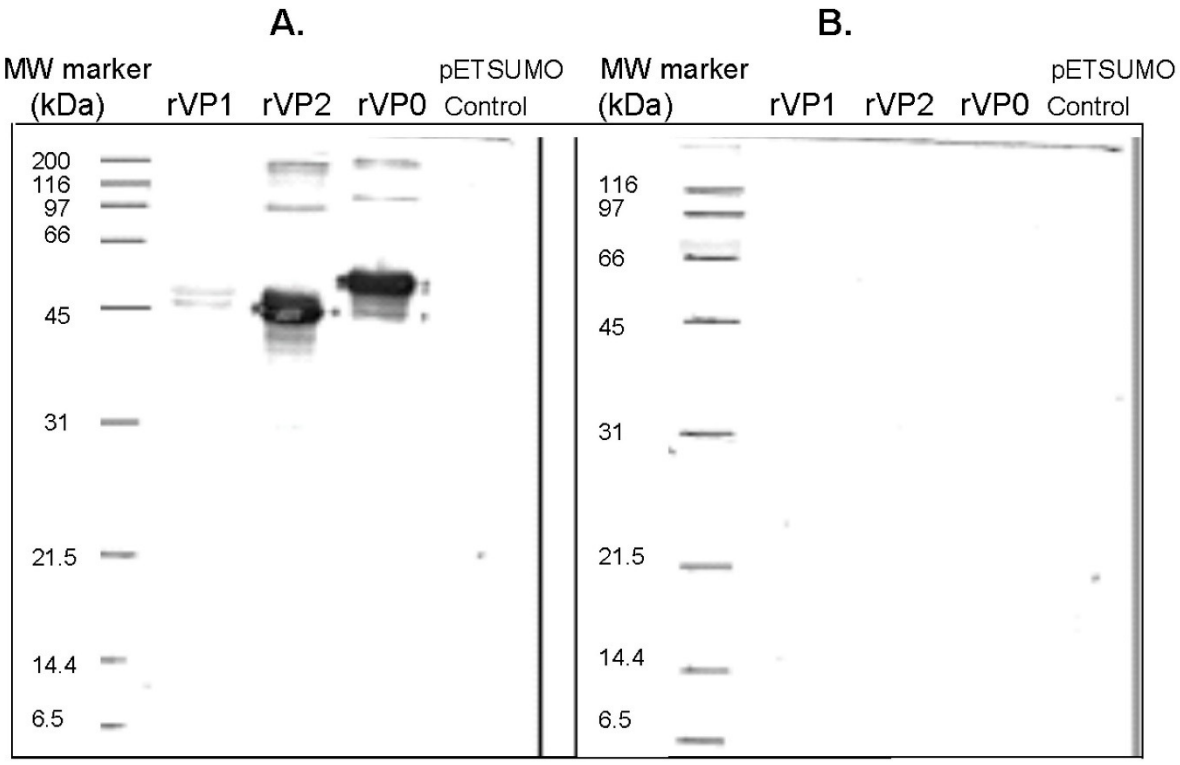


Figure 9. Pooled neutralizing sera from mice immunized with oligomeric antigens in the supernatant of SN07 infected Sf9 cells bind more strongly to VP2 and VP0 than to VP1.



A. Probed with mice serum anti-bacSN07p3 VLPs at 1/2000 dilution.
B. Probed with mice serum anti-SF9 control cells at 1/500 dilution.

Figure 10. IRES region of the EMCV genome (SEQ ID NO:1). The IRES region represents the EMCV genomic sequence in GenBank accession number AF113968.2 ; nucleotides1666 to 2251.

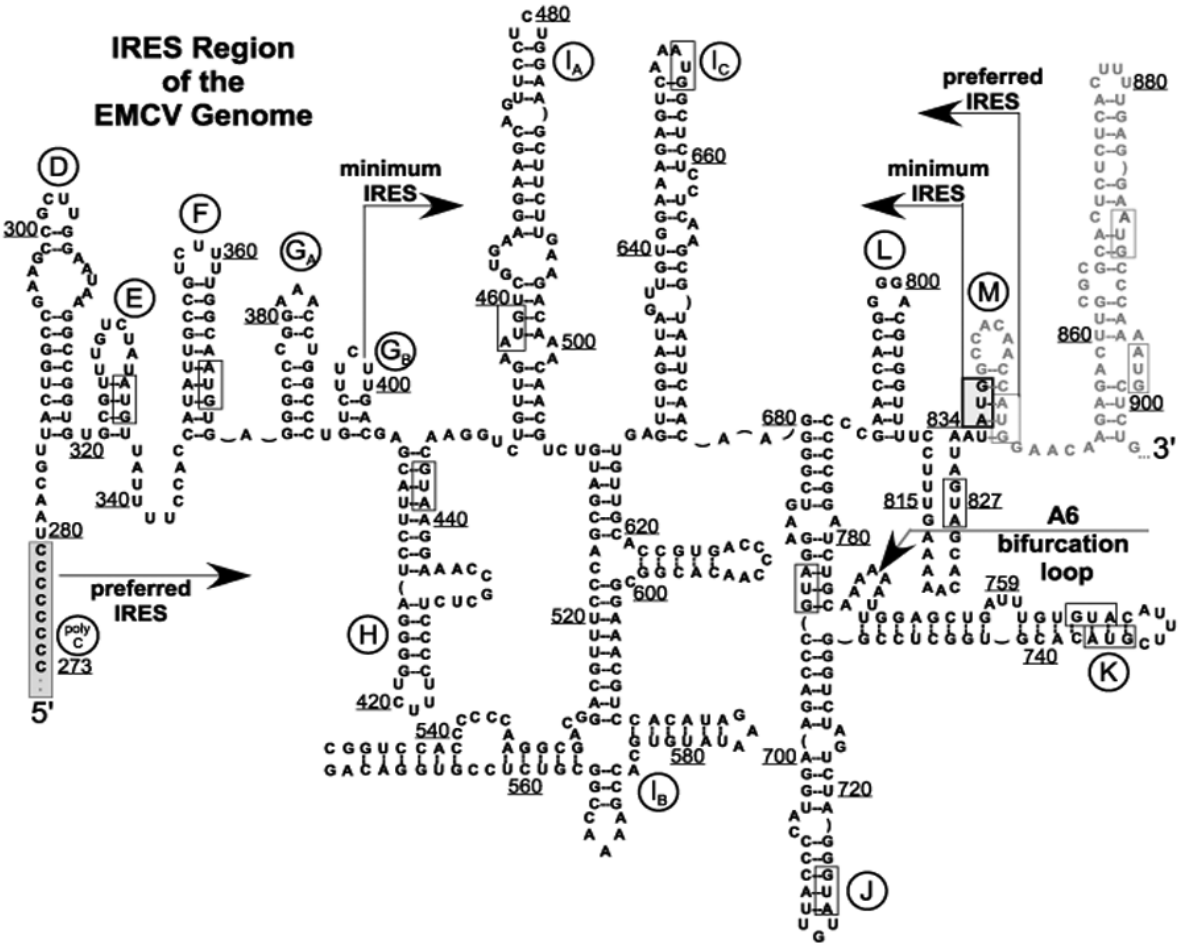


Figure 11. Out framing of the EMCV start codon with the 3CD protease coding sequence; Native EMCV IRES coding sequence (SEQ ID NO:2) is compared to a mutant EMCV IRES sequence coding sequence (SEQ ID NO:3).

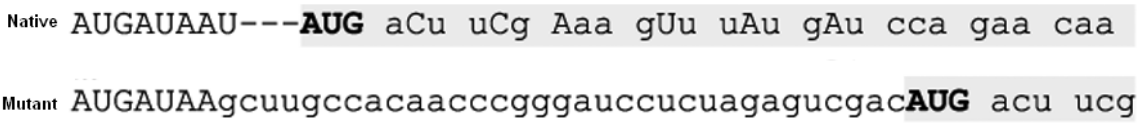


Figure 12. Plasmid pSN01-M1.

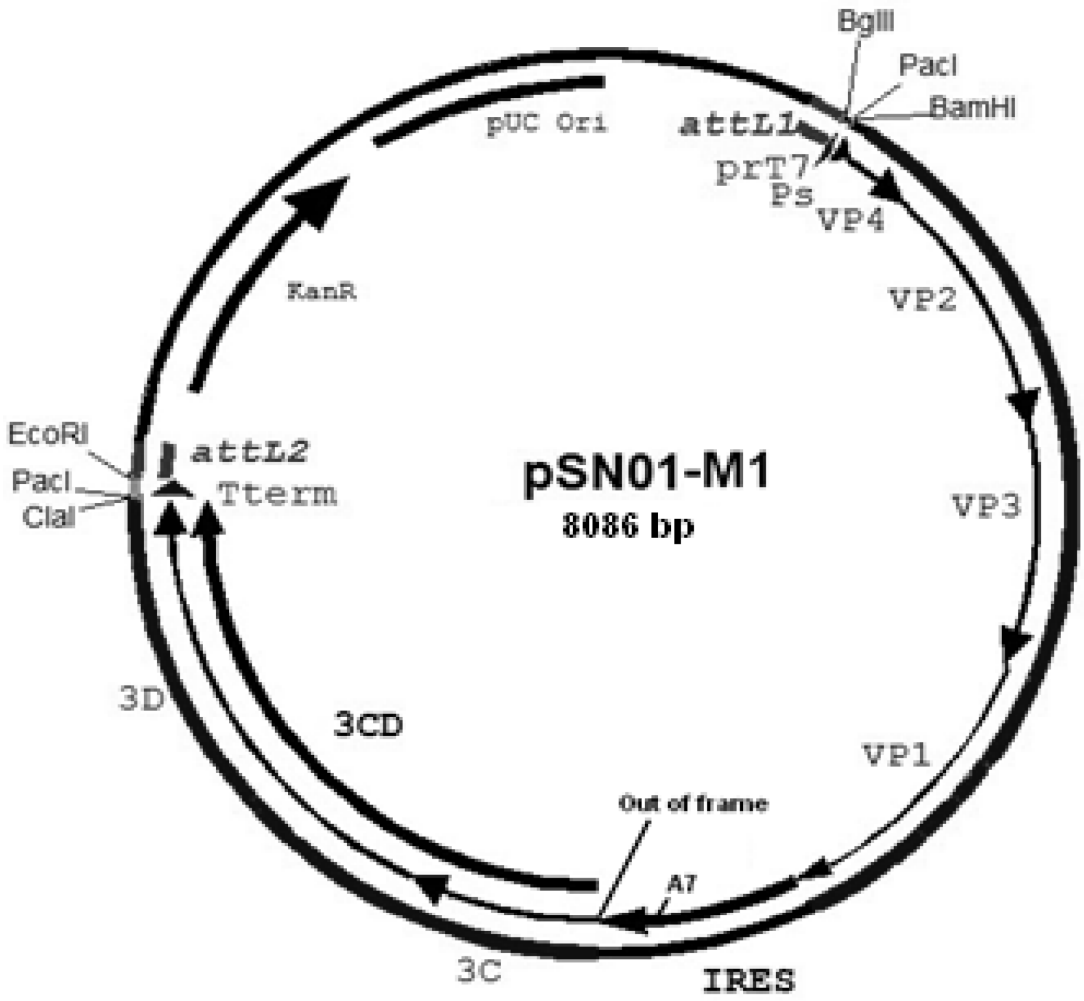


Figure 13. Plasmid pSN01-M2

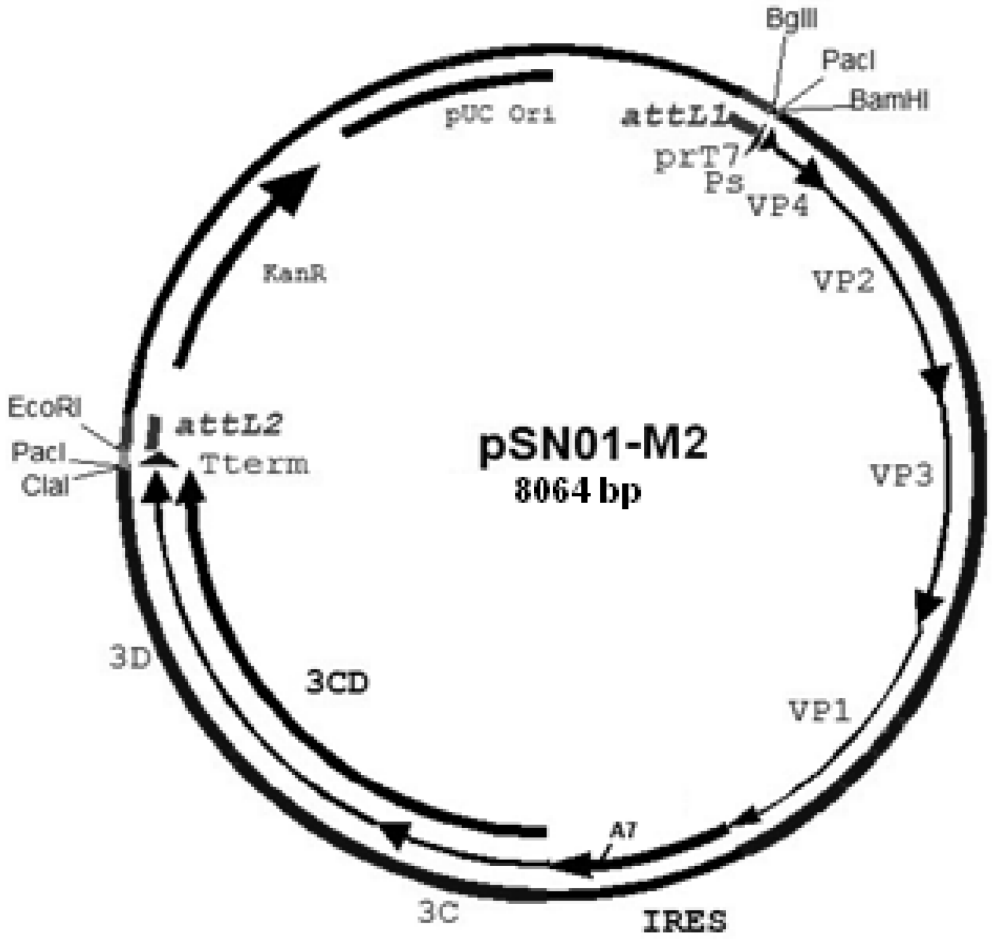


Figure 14. Plasmid pSN01-M3.

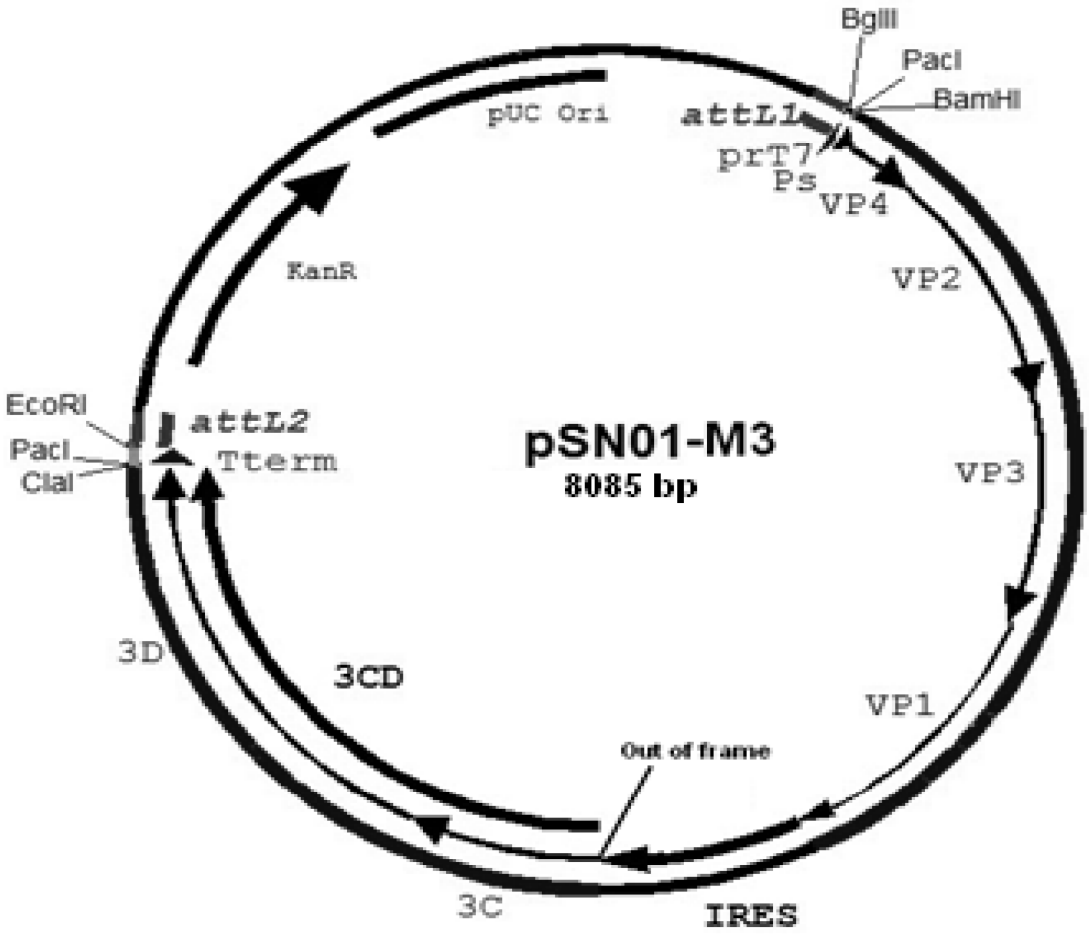


Figure 15. Comparison of expression of different recombinant baculovirus designs for expression of HEV71 VLP's.

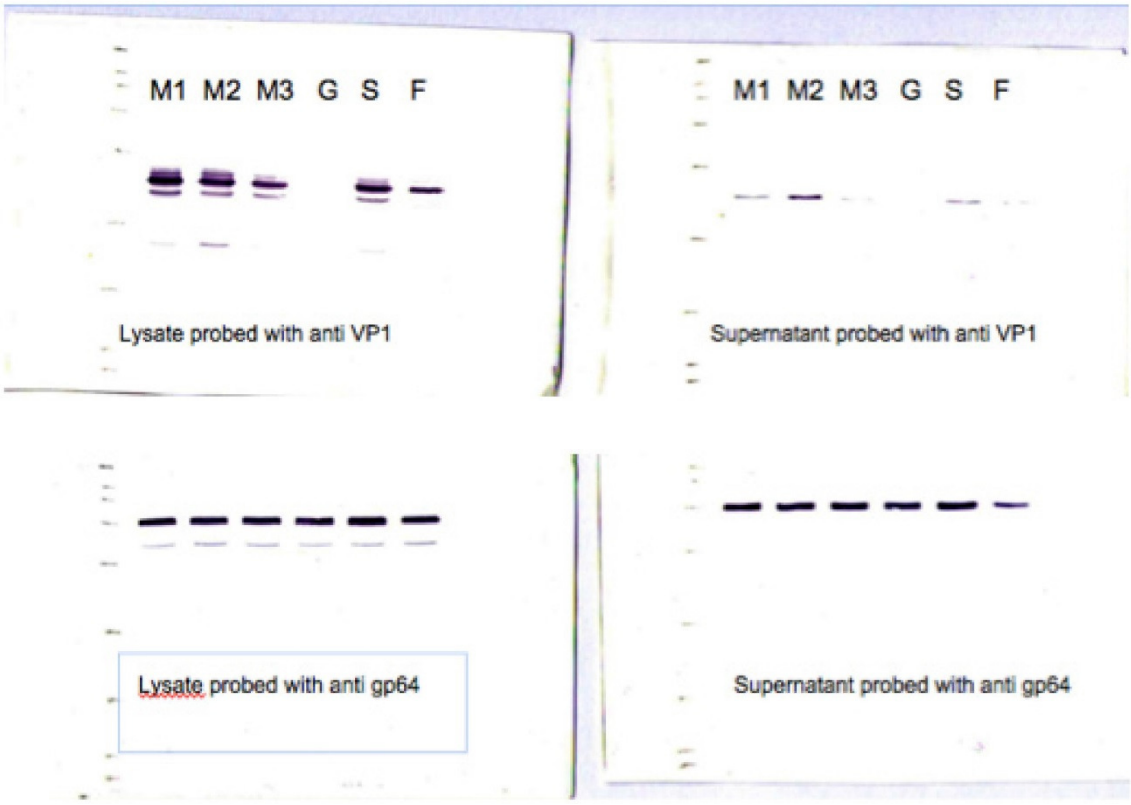


Figure 16. Plasmid pFastBac^{TM} HT

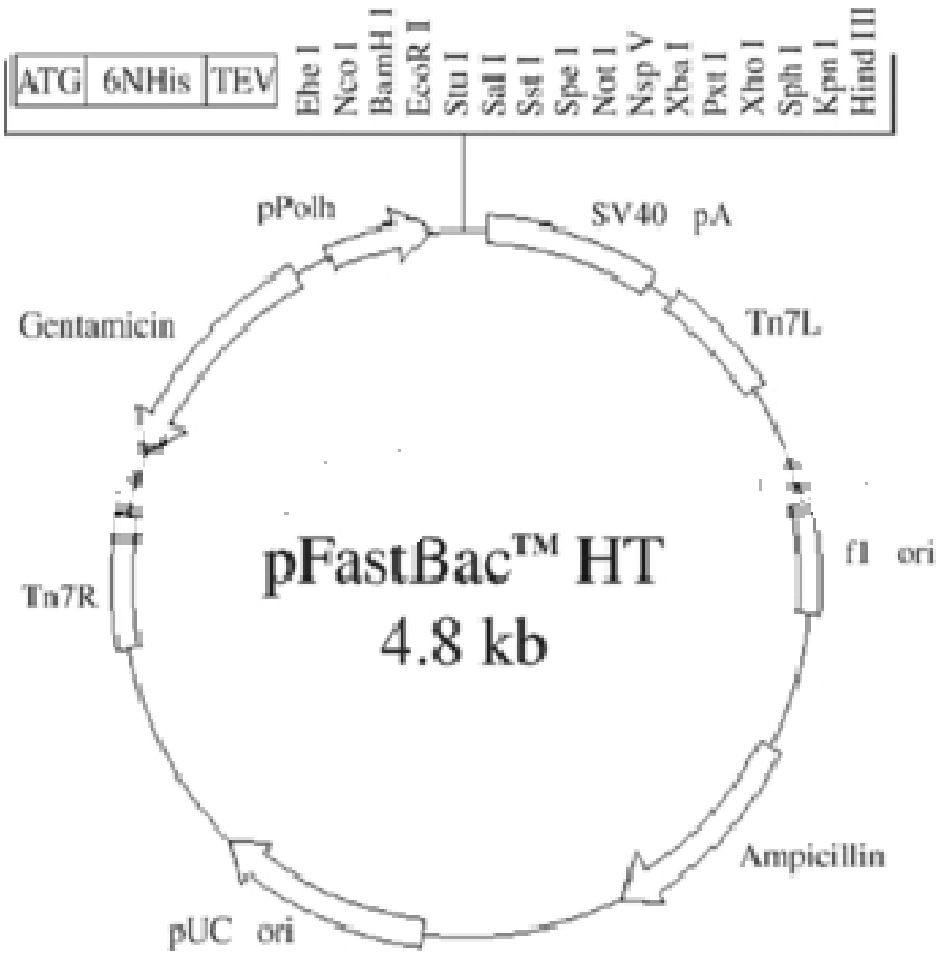


Figure 17. Prokaryotic expression construct for antigenic fusion proteins of *Human enterovirus A* and *Human enterovirus C*.

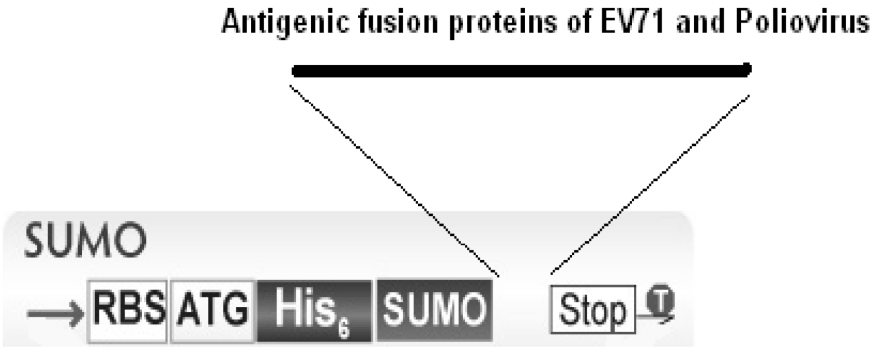


Figure 18. Antibodies from pooled neutralizing sera from mice immunized with SN07 retentate binds to all the components of HEV71 VLPs.

1. 1 ug of rEV71-VP0
2. 1 ug of rEV71-VP1
3. 1 ug of rEV71-VP2
4. 1 ug of rEV71-VP3

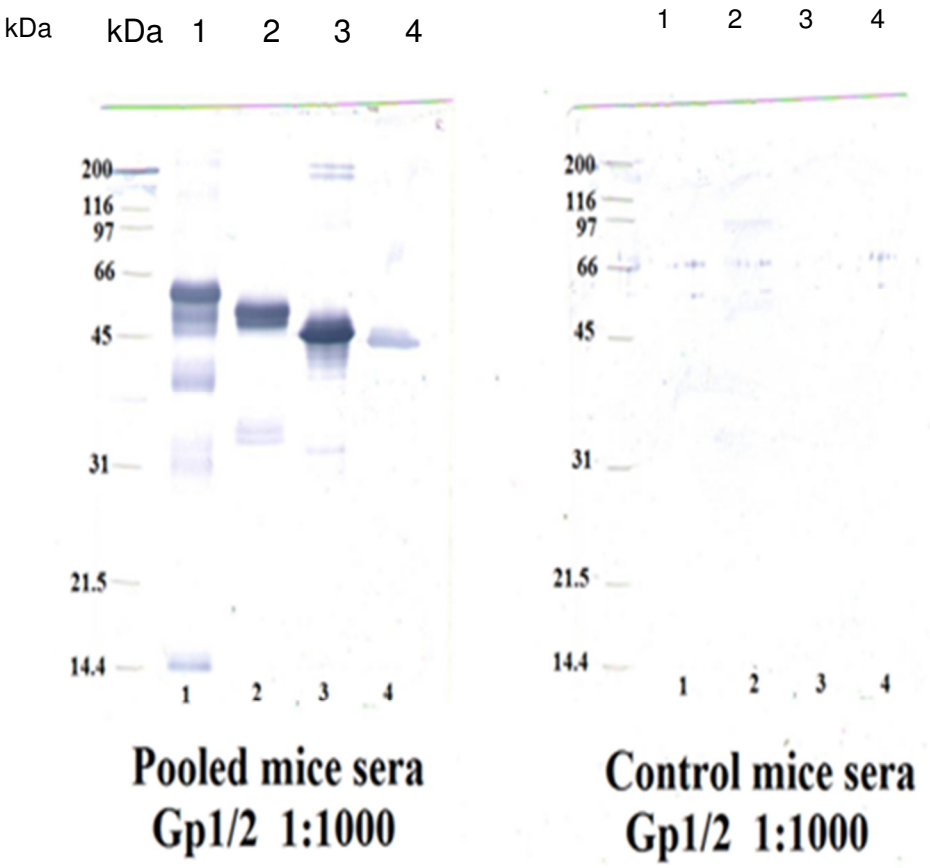


Figure 19. Characterization of HEV71 VLPs, pull-down of HEV71 VLPs from the culture supernatant. The VLP components (VP0, VP1 and VP3) are visible in the Coomassie stained SDS-PAGE gel. (MW- molecular weight marker; CB- Coomassie blue staining)

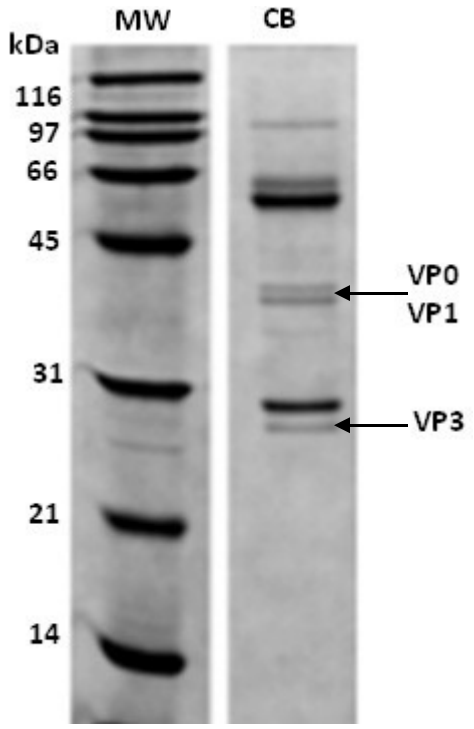


Figure 20. Analysis of affinity column (AFC) purified HEV71 VLPs.

AFC was prepared with a neutralizing monoclonal antibody (EV18/4/D6- 1/F1/G9), which monoclonal antibody was developed using a SN07 retentate. The eluted fraction was analyzed by Western blotting. The eluted fraction in Lanes 1 and 2, probed using an anti-VP1 antibody, shows that VP1 is present in the AFC purified VLPs. The eluted fraction in Lanes 3 and 4, probed using an anti-VP2 antibody, shows that VP2 is also present in the AFC purified VLPs. The eluted fraction in Lanes 5 and 6, probed using an anti-VP2 monoclonal antibody, shows that VP2 is present in the AFC purified VLPs.

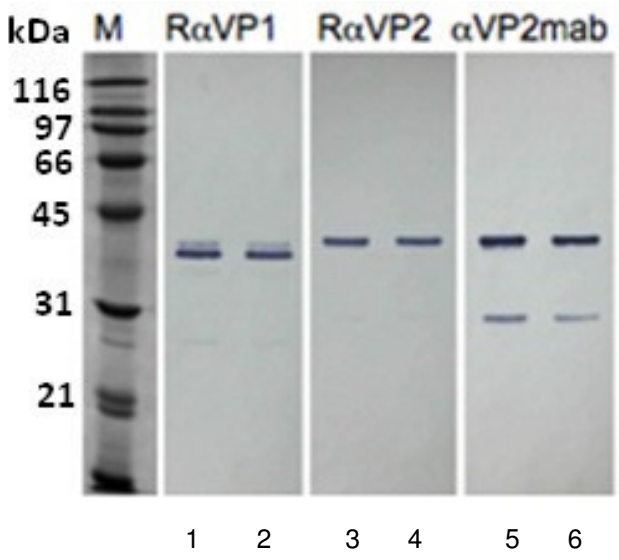


Figure 21. An electron micrograph picture of AFC purified HEV71 VLPs. AFC was prepared with a neutralizing monoclonal antibody (EV18/4/D6-1/F1/G9), which monoclonal antibody was developed using a SN07 retentate. The eluted fraction was analyzed by Western blotting (Figure 20) and electron microscopy. The arrows point to virus-like particles.

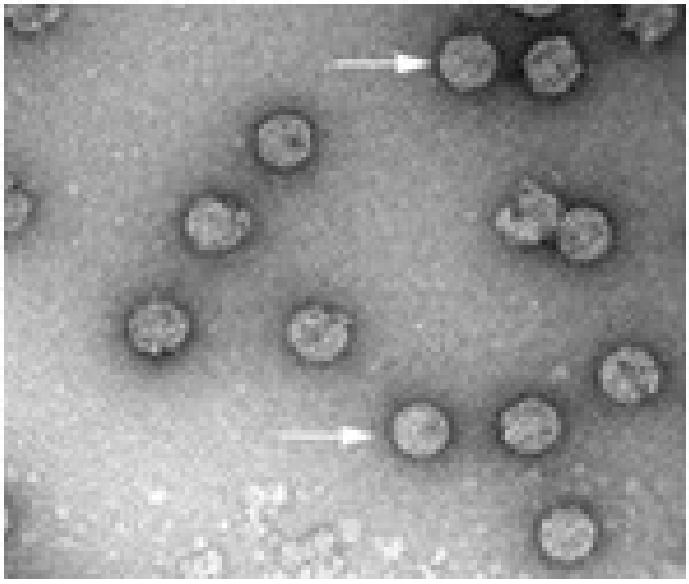
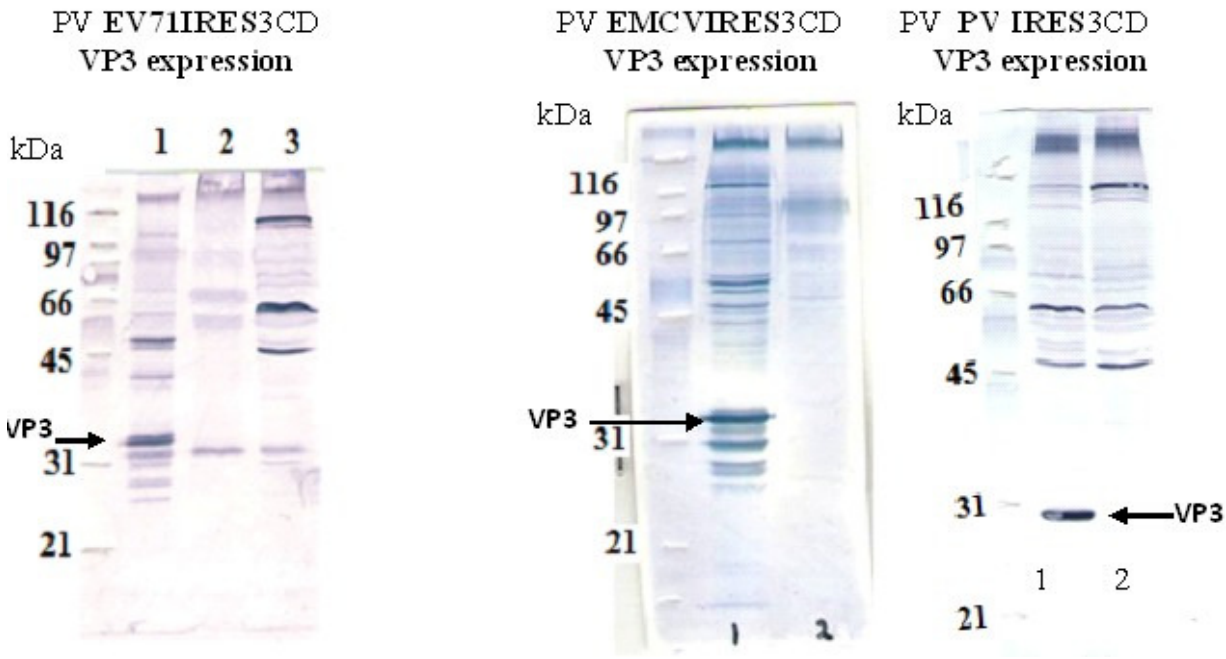


Figure 22. *Human enterovirus C* (poliovirus-PV) VL expression. Three different expression cassettes to generate poliovirus VLPs were constructed. The expression cassettes all comprise a poliovirus P1 polypeptide and differed with respect to the IRES, which IRES directs the expression of a poliovirus 3CD protease. Recombinant baculoviruses harboring the poliovirus VLP expression cassettes were tested. Lysates from the baculovirus infected cells were harvested on day 3 post infection and expression of the poliovirus VP3 was evaluated using rabbit anti-PVP3 antibodies (1:2000).



1. HEV71IRES-lysate
2. HEV71IRES-supernatant
3- bacGus-lysate

1. EMCV IRES-lysate
2. bacGus-lysate

1. PV IRES-lysate
2. bacGus-lysate

Figure 23. Poliovirus-VLP VP3-VP1 ELISA. To demonstrate poliovirus VLP generation, a two sites ELISA was performed using lysates and supernatants from recombinant baculoviruses carrying a poliovirus VLP expression cassette wherein the poliovirus 3CD protease is under the control of a poliovirus IRES. Purified rabbit anti-poliovirus VP3 antibodies were used as capture antibodies. Poliovirus VLPs were detected using mouse anti-VP1monoclonal antibodies.

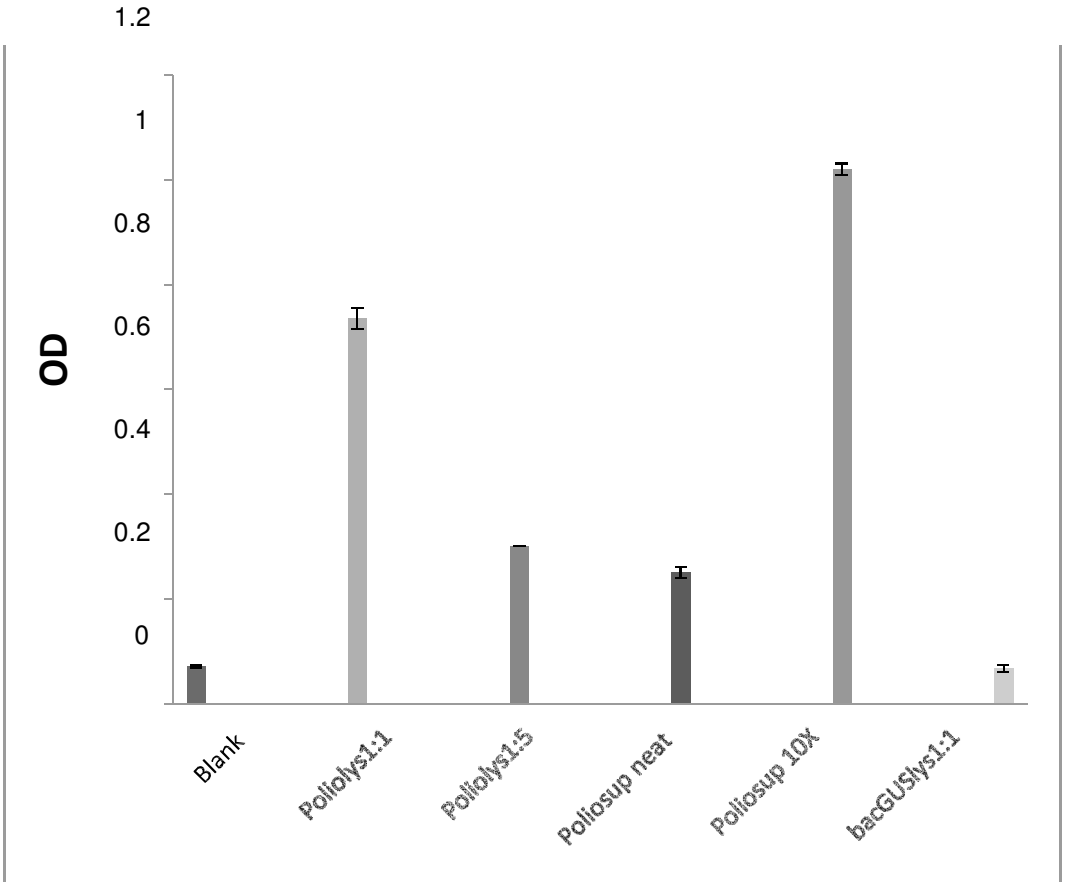
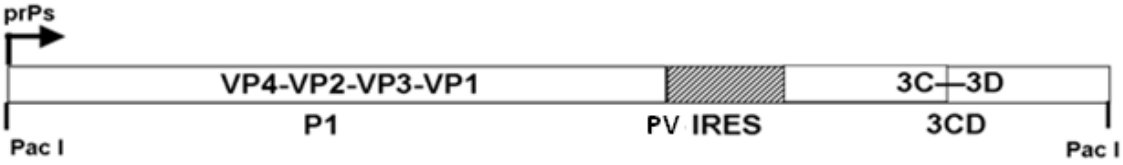


Figure 24. HEV71 VLP Expression Cassette with HEV71 IRES (P1+HEV71 IRES+3CD).

Figure 25. HEV71 VLP Expression Cassette with Poliovirus (PV) IRES (P1+PV IRES+3CD).



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(54) Title: ANTIGENS AND VACCINES DIRECTED AGAINST HUMAN ENTEROVIRUSES

(57) Abstract: The instant invention provides materials and methods for producing immunologically active antigens derived from members of the *Picornaviridae* virus family. The picornavirus antigens of the invention may be in a form for use as a vaccine administered to a subject in a therapeutic treatment or for the prevention of a picornavirus infection. The picornavirus antigens of the invention may be in the form of an immunogenic composition for use in vaccines which are administered for the prevention of an *Enterovirus* infection. The instant invention further encompasses immunogenic compositions comprising *Human enterovirus A, Human enterovirus B, Human enterovirus C, Human enterovirus D* antigens and their use in vaccines for the prevention of an Enterovirus infection.

ANTIGENS AND VACCINES DIRECTED AGAINST HUMAN ENTEROVIRUSES

FIELD OF THE INVENTION

[0001] This invention relates to viruses of the *Picornaviridae* family, and in particular antigens and vaccines that may be effective in preventing and treating infections caused by such viruses.

BACKGROUND OF THE INVENTION

[0002] Picornaviruses are a diverse family of viruses which cause a number of common illnesses. Of the *Picornaviridae* family, viruses of the genus *Enterovirus*, which are all very closely related, are significant for the number of diseases they cause.

[0003] Viruses of the genus *Enterovirus* affect millions of people worldwide each year, and are often found in the respiratory secretions (e.g., saliva, sputum, or nasal mucus) and stool of an infected person. *Enterovirus* infects the gut, thus the derivation of their name from the root "enteric". Historically, poliomyelitis was the most significant disease caused by an enterovirus, that is, poliovirus. There are 62 non-polio enteroviruses that can cause disease in humans: 23 Coxsackie A viruses, 6 Coxsackie B viruses, 28 echoviruses, and 5 other enteroviruses. Polioviruses, as well as Coxsackie viruses and echoviruses, are spread through the fecal-oral route. Infection can result in a wide variety of symptoms ranging from mild respiratory illness (common cold), hand, foot and mouth disease, acute hemorrhagic conjunctivitis, aseptic meningitis, myocarditis, severe neonatal sepsis-like disease, and acute flaccid paralysis.

[0004] Of the picornaviruses, *Enterovirus* represents a genus of a large and diverse group of small RNA viruses characterized by a single positive-strand genomic RNA. All enteroviruses contain a genome of approximately 7,500 bases and are known to have a high mutation rate due to low-fidelity replication and frequent recombination. After infection of the host cell, the genome is translated in a cap-independent manner into a single polyprotein, which is subsequently processed by virus-encoded proteases into

the structural capsid proteins and the nonstructural proteins, which are mainly involved in the replication of the virus.

[0005] The enteroviruses are associated with several human and mammalian diseases. Serologic studies have distinguished 66 human *Enterovirus* serotypes on the basis of antibody neutralization tests. Additional antigenic variants have been defined within several of the serotypes on the basis of reduced or nonreciprocal cross-neutralization between variant strains. On the basis of their pathogenesis in humans and animals, enteroviruses were originally classified into four groups, polioviruses, Coxsackie A viruses (CA), Coxsackie B viruses (CB), and echoviruses, but it was quickly realized that there were significant overlaps in the biological properties of viruses in the different groups.

[0006] The *Enterovirus* genus includes the following ten species:

- *Bovine enterovirus*
- *Human enterovirus A*
- *Human enterovirus B*
- *Human enterovirus C*
- *Human enterovirus D*
- *Human rhinovirus A*
- *Human rhinovirus B*
- *Human rhinovirus C*
- *Porcine enterovirus B*
- *Simian enterovirus A*

[0007] Within these ten species are there are various serotypes, for example:

Enterovirus serotypes HEV71, EV-76, EV-89, EV-90, EV-91, EV-92 and Coxsackievirus A16 are found under the species *Human enterovirus A*.

Serotypes *Coxsackievirus* B1 (CV-B1), CV-B2, CV-B3, CV-B4, CV-B5 (incl. swine vesicular disease virus [SVDV]), CV-B6, CV-A9, echovirus 1 (E-1; incl. E-8), E-2, E-3, E-4, E-5, E-6, E-7, E-9 (including CV-A23), E-11,

E-12, E-13, E-14, E-15, E-16, E-17, E-18, E-19, E-20, E-21, E-24, E-25, E-26, E-27, E-29, E-30, E-31, E-32, E-33, enterovirus B69 (EV-B69), EV-B73, EV-B74, EV-B75, EV-B77, EV-B78, EV-B79, EV-B80, EV-B81, EV-B82, EV-B83, EV-B84, EV-B85, EV-B86, EV-B87, EV-B88, EV-B93, EV-B97, EV-B98, EV-B100, EV-B101, EV-B106, EV-B107, EV-B110 (from a chimpanzee) and the simian enterovirus SA5, are found under the species *Human enterovirus B*.

Serotypes EV-95, EV-96, EV-99, EV-102, EV-104, EV-105, and EV-109 are found under the species *Human enterovirus C.*

Serotypes EV-68, EV-70, & EV-94 are found under the species *Human enterovirus D*.

Poliovirus serotypes PV-1, PV-2, and PV-3 are found under the species *Human enterovirus C*.

[0008] Diseases caused by enterovirus infection include poliomyelitis which is the most notable disease caused by an enterovirus infection. Nonspecific febrile illness is, however, the most common presentation of an enterovirus infection.

[0009] Enteroviruses are the most common causes of aseptic meningitis in children. In the United States, enteroviruses are responsible for 30,000 to 50,000 cases of meningitis. Encephalitis is a rare manifestation of an enterovirus infection; when it occurs, the most frequent *Enterovirus* found to be causing the encephalitis is echovirus 9.

[0010] Pleurodynia caused by enteroviruses is characterized by severe paroxysmal pain in the chest and abdomen, along with fever, and sometimes nausea, headache, and emesis.

[0011] Pericarditis and/or myocarditis are typically caused by enteroviruses. Arrythmias, heart failure, and myocardial infarction have also been reported.

[0012] Acute hemorrhagic conjunctivitis can be caused by enteroviruses.

[0013] Hand, foot and mouth disease is a childhood illness most commonly caused by infection by Coxsackie A virus or HEV71.

[0014] A 2007 study suggested that acute respiratory or gastrointestinal infections associated with enteroviruses may be a factor in chronic fatigue syndrome.

[0015] All members of the genus *Enterovirus*, including HEV71, polioviruses and *Coxsackievirus* A16 have a single stranded positive sense RNA genome which has a single open reading frame encoding a polyprotein, P1, consisting of the capsid proteins VP4, VP2, VP3 and VP1 and several non-structural proteins including the viral proteases 3C and 3CD which are responsible for cleaving the polyprotein P1 into individual capsid proteins VP1, VP3 and VP0, which VP0 is eventually cleaved into VP2 and VP4. The capsid proteins may assemble into virus like particles (VLPs).

[0016] *Human enterovirus* 71 (HEV71) and *Coxsackievirus* A16 are *Enterovirus* serotypes notable as the major causative agents for hand, foot and mouth disease (HFMD), and HEV71 is sometimes associated with severe central nervous system diseases. HEV71 was first isolated and characterized from cases of neurological

disease in California in 1969. To date, little is known about the molecular mechanisms of host response to HEV71 infection, but increases in the level of mRNAs encoding chemokines, proteins involved in protein degradation, complement proteins, and pro-apoptotic proteins have been implicated.

[0017] Hand Foot and Mouth Disease (HFMD) is a common, self-limiting illness of children caused by a group of species A enteroviruses (Picornaviridae family) such as human *Coxsackievirus* A16 (CVA16), *Coxsackievirus* A10 (CVA10) and *Human enterovirus A* 71 (HEV71). The virus is excreted in feces and is also found in pharyngeal secretions. Transmission is associated with close contact among children and through environmental contamination. The disease is characterized by an acute onset of fever with a rash on the palms, soles, buttocks, and knees, and vesicles on buccal membranes that usually resolve in 7-10 days. Only a small proportion of children with HFMD develop severe disease.

[0018] Severe disease involving primarily the neurologic and cardiovascular systems manifesting as syndromes such as meningitis, encephalitis, acute flaccid paralysis, pulmonary edema and cardiac failure generally occur only with HEV71 infection. In the Asia-Pacific Region the most devastating neurological syndrome is brainstem encephalitis, which has a mortality rate of 40-80 percent. Children with severe HFMD may take months to recover, and in some cases the neurologic damage may be permanent. Currently, there is no specific antiviral treatment for HFMD and no vaccines to prevent enterovirus infection other than polio.

[0019] HEV71 was first isolated from a child who died of encephalitis in California in 1969, and first reported in 1974. Although the virus has been detected worldwide since then, the recent regional epidemics of HFMD in Asia has raised concern that more pathogenic forms of HEV71 may be emerging in the region. The first recognition of a HFMD outbreak with a high number of fatalities was in Sarawak, Malaysia in 1997. The

virus associated with the outbreak then was HEV71. Taiwan reported 129,106 HFMD cases in a 1998 epidemic with 405 having severe disease with 78 deaths. Singapore reported an epidemic of 9000 cases with 7 deaths during 2000-2001, and since then has experienced recurrent epidemics every two to three years. During the first 8 months of 2008, Singapore reported 19,530 cases and one death due to HFMD. Since then HEV71 outbreaks have been reported regularly in Singapore, Thailand, Malaysia, Taiwan, Japan, Korea and Vietnam.

[0020] China reported 83,344 cases with 17 deaths in 2007, and in 2008 experienced a large outbreak in Fuyang City in Anhui Province spreading throughout many parts of China. These large outbreaks were widely covered by the press, which highlighted parental concerns about the health of their children and the social disruption from closing of schools and day care centers by public health departments in an attempt to break the chain of transmission. Since then China has reported large outbreaks annually.

[0021] With regard to disease caused by other members of the *Picornaviridae* family, natural infection and prevalence of polio have occurred exclusively in the human being since ancient times as an infectious disease. A large number of humans still become infected with polio every year in developing countries. Hence, the eradication of polio is an ongoing process.

[0022] Polioviruses were formerly classified as a species belonging to the genus *Enterovirus* in the family *Picornaviridae*. The Poliovirus species has been eliminated from the genus *Enterovirus*. The poloviruses are classified as serotypes, Human poliovirus 1 (PV-1), Human poliovirus 2 (PV-2), and Human poliovirus 3 (PV-3), and are considered to be subtypes of species *Human enterovirus C*, in the genus *Enterovirus* in the family *Picornaviridae*. The type species of the genus *Enterovirus* was changed from Poliovirus to *Human enterovirus C* in 2008.

[0023] The three subtypes of species *Human enterovirus C*, PV-1, PV-2 and PV-3, are characterized by a slightly different capsid protein. Capsid proteins define cellular receptor specificity and virus antigenicity. PV-1 is the most common form encountered in nature; however, all three forms are extremely infectious and can affect the spinal cord and cause poliomyelitis.

[0024] Infection with *Human enterovirus C* has been a widespread problem and inactivated whole virus vaccines have been used for mass immunization and are currently available. Good results have been obtained with inactivated poliomyelitis vaccines which may be prepared according to a method which has been developed by Salk and has been improved later in several aspects. Generally, these vaccines contain a mixture of inactivated polio virus of strains Mahoney, MEF1 and Saukett.

[0025] Although an attenuated *Human enterovirus C* has been produced and used as an attenuated oral polio vaccine, the attenuated *Human enterovirus C* may be dangerous because of the possible reversion of pathogenicity (paralysis-based neurovirulence) in persons administered to, or in contact with, whole viruses. Hence, there is a need for a safe and effective polio vaccine which is free of such pathogenicity.

[0026] Like all enteroviruses, four different *Human enterovirus C* coat/capsid polypeptides have been identified and are designated as VP1, VP2, VP3 and VP4, which associate to form an icosahedral virus capsid. Typically, vaccination with the individual polypeptides of *Human enterovirus C* has shown that the isolated polypeptides are not capable of raising neutralizing antibodies in humans and animals (Meloen, et al., J. Gen. Virol. 45:761-763, 1979).

[0027] U.S. Patent No. 4,508,708 teaches that individual polypeptides of polio virus and hand, foot and mouth disease virus, VP1, VP2, VP3 and VP4, are not capable of raising neutralizing antibodies in humans and animals and that, among the individual polypeptides of the hand, foot and mouth disease virus, only VP1 possesses this

capability. U.S. Patent No. 4,508,708 demonstrates that, among the *Human enterovirus C* type 2 MEF1 virion VP1, VP2 and VP3 polypeptides, only the VP3 is capable of inducing neutralizing antibodies, although the antibody titer is low. It was found, however, that VP1, VP2 and VP3 are capable of inducing neutralizing antibodies only when the immunization is carried out with a preparation containing arildone, a broad spectrum antiviral agent that has been shown to selectively inhibit replication of picornaviruses (Langford, et al. Antimicrobial Agents and Chemotherapy 28:578-580, 1985).

[0028] Thus, the problem to be solved is the preparation of an effective vaccine which provides protective immunity against a human enterovirus infection, and without the use of antiviral compounds. The human enteroviruses for which protection is desired are, for example *Human enterovirus A*, including *Coxsackievirus* A16 and *Human enterovirus* 71; *Human enterovirus B*, including Coxsackievirus B serotypes, echoviruses and enterovirus serotypes; *Human enterovirus C*, including *Human poliovirus* 1, *Human poliovirus* 2 and *Human poliovirus* 3; as well as *Human enterovirus D*, including EV 68.

[0029] For the purposes of the instant invention, a vaccine is understood by those skilled in the art and may further be defined as a prophylactic or therapeutic material containing antigens derived from one more pathogenic organisms which, upon administration to a human subject or animal, will stimulate active immunity and protect against infection with these or related organisms (i.e., produce protective immunity).

[0030] Furthermore, protective immunity may be well understood by those skilled in the art. Nonetheless, protective immunity comprises, at least, the induction, or elicitation of neutralizing antibodies and/or T-cell immune response which will neutralize the virus.

[0031] It is well recognized in the vaccine art, that it is unclear whether an antigen derived from a pathogen will elicit protective immunity. Ellis (Chapter 29 of Vaccines, Plotkin, et al. (eds) WB Saunders, Philadelphia, at page 571,1998) exemplifies this

problem in the recitation that "the key to the problem (of vaccine development) is the identification of that protein component of a virus or microbial pathogen that itself can elicit the production of protective antibodies…, and thus protect the host against attack by the pathogen."

[0032] An approach to making improved vaccines against picornaviruses would be to mimic the virus capsid structure or its components which may elicit protective antibodies such as are produced with a killed whole virus vaccine. This kind of approach is safer than a killed, inactivated or attenuated vaccine approach because there is no opportunity for reversion.

[0033] All picornaviruses share the same genomic structure, including 4 structural genes within the P1 gene: VP1, VP2, VP3, and VP4, the VP4 and VP2 being expressed together as VP0, and viral proteases within the 3C and 3D genes. The viral protease will cleave the P1 gene, thereby allowing the virus to assemble into virus like particles (VLPs), virus capsomers, complexes and/or antigens of enteroviruses.

[0034] Vaccines have been proposed with indifferent success. It has been proposed to use subunit vaccines comprising the major capsid protein, VP1, of enteroviruses, as the basis of vaccines for the prevention and treatment of enterovirus infections, including HEV71 infections (Wu, et al., 2001).

[0035] With regard to prophylaxis against enterovirus infection, it is possible to envisage a killed virus vaccine approach, which has been shown to elicit protective antibodies. The low virus titres achieved in general makes manufacturing of such enterovirus vaccines a challenge.

[0036] The present invention pertains to vaccines including antigenic coat/capsid proteins of viruses of the *Picornaviridae* family and which vaccines are devoid of virus RNA which may contribute to neurovirulence. The vaccines of the invention may comprise as antigens, the polypeptides P1 or VP0, or capsid proteins (VP's), designated

as VP1, VP2, VP3 and/or VP4, or immunologically or biologically active fragments thereof which elicit neutralizing antibodies against enteroviruses.

[0037] The present invention relates to vaccines in which the picornavirus antigens are present in the form of one or more picornavirus polypeptides, especially human *Enterovirus* peptides VP2, VP3 or VP0, immunogenic fragments thereof, and/or antigenic determinants thereof. The picornavirus polypeptides may be obtained by chemical synthesis or by means of recombinant DNA techniques using known human *Enterovirus* amino acid or nucleic acid sequences.

SUMMARY OF THE INVENTION

[0038] A vaccine comprising one or more immunologically active antigens comprising one or more human *Enterovirus* polypeptides selected from VP0, VP1, VP2, VP3, VP4, and immunologically active fragments thereof, such a

[0039] vaccine, which elicits a protective and/or neutralizing immune response directed against a human *Enterovirus*, such a

[0040] vaccine, wherein the human *Enterovirus* is selected from *Human enterovirus A, Human enterovirus B*, *Human enterovirus C* and *Human enterovirus D*, such a

[0041] vaccine, wherein the *Human enterovirus A* is selected from *Human enterovirus* 71 (HEV71) and *Coxsackievirus* A16, wherein the *Human enterovirus B* is selected from *Coxsackievirus* B and echovirus, wherein the *Human enterovirus C* is selected from *Human poliovirus* 1, *Human poliovirus* 2 and *Human poliovirus* 3, and wherein the *Human enterovirus D* is *EV* 68, such a

[0042] vaccine, wherein the vaccine comprises an immunologically active *Enterovirus* VP0 polypeptide, such a

[0043] vaccine, wherein the vaccine comprises an immunologically active *Enterovirus* VP2 polypeptide, such a

[0044] vaccine, wherein the vaccine comprises an immunologically active *Enterovirus* VP3 polypeptide, such a

[0045] vaccine, wherein the vaccine comprises an immunologically active *Enterovirus* VP1 polypeptide, such a

[0046] vaccine, wherein the vaccine comprises polypeptides from one or more *Enterovirus* species or serotype, such a

[0047] vaccine, wherein the species or serotype may be *Human enterovirus A* selected from HEV71 and *Coxsackievirus* A16, such a

[0048] vaccine, wherein the species or serotype may be *Human enterovirus C* selected from PV-1, PV-2 and PV-3, such a

[0049] vaccine, wherein the one or more immunologically active antigens comprising one or more human *Enterovirus* polypeptides selected from VP0, VP1, VP2, VP3, VP4, and immunologically active fragments thereof, are in the form of a virus-like particle, capsomer, complex and/or aggregate, such a

[0050] vaccine, wherein the virus-like particle comprises an immunologically active *Enterovirus* VP0 polypeptide, such a

[0051] vaccine, wherein the virus-like particle comprises an immunologically active *Enterovirus* VP2 polypeptide, such a

[0052] vaccine, wherein the virus-like particle comprises an immunologically active *Enterovirus* VP3 polypeptide, such a

[0053] vaccine, wherein the virus-like particle comprises an immunologically active *Enterovirus* VP1 polypeptide, a

[0054] method of vaccinating a subject against an *Enterovirus* infection, comprising administering to the subject a vaccine comprising one or more immunologically active antigens comprising one or more human *Enterovirus* polypeptides selected from VP0, VP1, VP2, VP3, VP4, and immunologically active fragments thereof, in an amount effective to elicit a protective and/or neutralizing immune response when administered to the subject, such a

[0055] method, wherein the vaccine comprises an immunologically active *Enterovirus* VP0 polypeptide, such a

[0056] method, wherein the vaccine comprises an immunologically active *Enterovirus* VP1 polypeptide, such a

[0057] method, wherein the vaccine comprises an immunologically active *Enterovirus* VP3 polypeptide, such a

[0058] method, wherein the vaccine comprises an immunologically active *Enterovirus* VP2 polypeptide, such a

[0059] method, wherein the one or more immunologically active antigens comprising one or more human *Enterovirus* polypeptides selected from VP0, VP1, VP2, VP3, VP4, and immunologically active fragments thereof, are in the form of a virus-like particle, capsomer, complex and/or aggregate, such a

[0060] method, wherein the virus-like particle comprises an immunologically active *Enterovirus* VP0 polypeptide, such a

[0061] method, wherein the virus-like particle comprises an immunologically active *Enterovirus* VP2 polypeptide, such a

[0062] method, wherein the virus-like particle comprises an immunologically active *Enterovirus* VP3 polypeptide, such a

[0063] method, wherein the virus-like particle comprises an immunologically active *Enterovirus* VP1 polypeptide, such a

[0064] method, wherein the vaccine comprises polypeptides from one or more *Enterovirus* species or serotype, such a

[0065] method, wherein the Enterovirus species or serotype may be *Human enterovirus A* selected from HEV71 and Coxsackievirus A16, such a

[0066] method, wherein the Enterovirus species or serotype may be *Human enterovirus C* selected from PV-1, PV-2 and PV-3, an

[0067] expression cassette comprising a promoter operably linked to a nucleic acid encoding a human *Enterovirus* P1 polypeptide, an Internal Ribosome Entry Site (IRES), and a nucleic acid encoding a human *Enterovirus* 3CD protease, such an

[0068] expression cassette, wherein the polypeptide is human *Enterovirus* P1, such an

[0069] expression cassette, wherein the human *Enterovirus* 3CD protease processes the human *Enterovirus* P1 polypeptide, such an

[0070] expression cassette, wherein the processed human *Enterovirus* P1 polypeptides associate to form virus like particles, capsomers, complexes and/or aggregates, such an

[0071] expression cassette, wherein the human *Enterovirus* P1 polypeptide comprises combinations of polypeptides selected fromVP0, VP1, VP2, VP3, VP4, and immunologically active fragments thereof, such an

[0072] expression cassette, wherein the human *Enterovirus* is selected from *Human enterovirus A* and *Human enterovirus C*, such an

[0073] expression cassette, wherein the *Human enterovirus A* is selected from HEV71 and Coxsackievirus A16, such an

[0074] expression cassette, wherein the *Human enterovirus C* is selected from *Human poliovirus* 1 (PV-1), *Human poliovirus* 2 (PV-2) and *Human poliovirus* 3 (PV-3), such an

[0075] expression cassette, wherein the IRES is derived from *Encephalomyocarditis virus* (EMCV), such an

[0076] expression cassette, wherein the IRES derived from *Encephalomyocarditis virus* (EMCV) has been genetically modified to reduce IRES activity, such an

[0077] expression cassette, wherein the IRES derived from EMCV has been genetically modified by adding one nucleotide (A7) to the A6 bifurcation loop in the JK segment, such an

[0078] expression cassette, wherein the IRES is derived from a human *Enterovirus,* such an

[0079] expression cassette, wherein the promoter is a eukaryotic promoter, such an

[0080] expression cassette, wherein the eukaryotic promoter is a polyhedrin promoter, such an

[0081] expression cassette, wherein the promoter is operably linked to a nucleic acid encoding a *Human enterovirus A* P1 polypeptide, an EMCV IRES, and a *Human enterovirus A* 3CD protease, such an

[0082] expression cassette, wherein the EMCV IRES has been mutated to reduce IRES activity, such an

[0083] expression cassette, wherein the promoter is operably linked to a nucleic acid encoding a *Human enterovirus A* P1 polypeptide, an HEV71 IRES, and a *Human enterovirus A* 3CD protease, such an

[0084] expression cassette, wherein the promoter is operably linked to a nucleic acid encoding a *Human enterovirus A* P1 polypeptide, a *Human enterovirus C* IRES, and a *Human enterovirus A* 3CD protease, such an

[0085] expression cassette, wherein the promoter is operably linked to a nucleic acid encoding a Human enterovirus C P1 polypeptide, a Human enterovirus C IRES, and a Human enterovirus C 3CD protease, such an

[0086] expression cassette, wherein the promoter is operably linked to a nucleic acid encoding a *Human enterovirus C* P1 polypeptide, an HEV71IRES, and a *Human enterovirus C* 3CD protease, such an

[0087] expression cassette, wherein the promoter is operably linked to a nucleic acid encoding a *Human enterovirus C* P1 polypeptide, an EMCV IRES, and a *Human enterovirus C* 3CD protease, such a

[0088] method of making a vaccine comprising introducing an VLP expression cassette into a host cell, culturing the host cell for a period of time sufficient to produce the polypeptides of the expression cassette and recovering human *Enterovirus* polypeptides from the host cell and/or culture supernatant, such a

[0089] method, wherein the host cell is a eukaryotic cell, such a

[0090] method, wherein the eukaryotic cell is selected from insect cells, mammalian cell lines and yeast cells, such a

[0091] method, wherein the insect cells are selected from *Spodoptera frugiperda*, *Trichoplusia ni*, drosophila, and mosquito cells derived from *Aedes albopictus*, such a

[0092] method, wherein the mammalian cell lines are selected from CHO, HEK 293, COS-1, HeLa, Vero and NIH3T3.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. HEV71 VLP expression cassette [P1+IRES+3CD] and the pSN01 plasmid.

Figure 2. HEV71 VLP expression cassette [P1+IRES+3C] and the pSN03 plasmid.

Figure 3. Expression of VP1 in supernatant of SN07 infected Sf9 cells.

Figure 4. Processed VP1 in both the supernatants and the lysates.

Figure 5. VP1 and VP0 are in the retentate after ultrafiltration over a 100kDa molecular weight cut off (MWCO) membrane.

Figure 6. Immunization schedules to produce neutralizing antibodies.

Figure 7. Immunoblots probed with rabbit polyclonal antisera against VP0 (arrow) at 3 different times of harvest.

Figure 8. Pooled neutralizing sera from mice immunized with the oligomeric antigens in the supernatant of SN07 infected Sf9 cells have high titres against recombinant VP2 in ELISA.

Figure 9. Pooled neutralizing sera from mice immunized with oligomeric antigens in the supernatant of SN07 infected Sf9 cells bind more strongly to VP2 and VP0 than to VP1.

Figure 10. EMCV IRES (SEQ ID NO:1) region of the EMCV genome.

Figure 11. Out framing of the EMCV start codon with 3CD protease coding sequence; native IRES sequence (SEQ ID NO:2) versus mutant IRES sequence (SEQ ID NO:3).

Figure 12. Plasmid pSN01-M1.

Figure 13. Plasmid pSN01-M2

Figure 14. Plasmid pSN01-M3.

Figure 15. Comparison of expression of different recombinant baculovirus designs for expression of HEV71 VLP's.

Figure 16. Plasmid pFastBac™ HT.

Figure 17. Prokaryotic expression construct for antigenic fusion proteins of *Human enterovirus A* and *Human enterovirus C*.

Figure 18. Antibodies from pooled neutralizing sera from mice immunized with SN07 retentate binds to all the components of HEV71 VLPs.

Figure 19. Characterization of HEV71 VLPs, pull-down of HEV71 VLPs from the culture supernatant.

Figure 20. Analysis of affinity column (AFC) purified HEV71 VLPs.

Figure 21. An electron micrograph picture of AFC purified HEV71 VLPs.

Figure 22. *Human enterovirus C* (poliovirus-PV) VLP expression.

Figure 23. Poliovirus-VLP VP3-VP1 ELISA.

Figure 24. HEV71 VLP expression cassette with HEV71-IRES and HEV71 3CD protease.

Figure 25. HEV71 VLP expression cassette with PV-IRES and HEV71 3CD protease.

DETAILED DESCRIPTION OF THE INVENTION

[0093] The invention provides virus like particles (VLPs), virus capsomers, aggregates, and complexes of antigens from viruses of the *Picornaviridae* family as an immunogenic composition and/or vaccine for the protection against and/or treatment of a picornavirus infection. Representative examples may include an *Enterovirus*, a *Coxsackie virus*, and a poliovirus.

[0094] The invention in another aspect provides virus proteins for example, a P1 protein or a combination of picornavirus VP0 proteins, VP1 proteins, VP2 proteins, VP3 proteins, and/or VP4 proteins, or immunologically or biologically active fragments thereof, which elicit neutralizing antibodies. The invention includes fusion-proteins comprising the aforementioned virus proteins and/or fragments thereof, which fusion proteins are immunologically active or biologically active to elicit production of neutralizing antibodies which are protective.

[0095] In an embodiment, an *Enterovirus* antigen may be a combination of *Enterovirus* coat/capsid proteins, or immunologically active fragments thereof. The virus coat/capsid proteins may be any combination of VP0, VP1, VP2, VP3 and/or VP4 proteins, and may take the form of a virus-like particle (VLP), capsomer, complex and/or aggregate. The combination may be in the form of a fusion protein.

[0096] The invention in an additional aspect includes a method for production of *Picornaviridae* virus like particles (VLPs), capsomers, complexes and/or aggregates which may include the steps of: (i) constructing an expression cassette operably linked to a promoter comprising one or more nucleic acids which each encode a picornavirus protein, for example, a P1 protein or a combination of picornavirus VP0 protein, VP1 protein, VP2 protein, VP3 protein, and/or a VP4 protein, which is/are operably linked to an internal ribosome entry site (IRES), which IRES is operably linked to a 3C or 3CD protease; (ii) transfecting or transforming a suitable host cell with the expression cassette; (iii) culturing the host cells under conditions in which virus like particles (VLPs) and/or capsomers and/or antigens are produced by the cell after expression of the nucleic acids comprised in the cassette.

[0097] A nucleic acid or recombinant DNA molecule may be obtained whereby open reading frames which encode *Coxsackievirus* A16, HEV71, *Human enterovirus C* (human polioviruses PV1, PV2 and/or PV3), EV 68, or any other picornavirus proteins and proteases may be amplified by PCR amplification using suitably designed primers complementary to nucleic acid sequences of *Coxsackievirus* A16, HEV71 or *Human enterovirus C* or any other picornavirus. Suitable primers may be designed according to standard techniques from publicly available nucleic acid sequences of enteroviruses, including *Coxsackievirus* A16, HEV71 and *Human enterovirus C* or any other picornavirus. Complete genome sequences are available in GenBank and are accessible at the National Center for Biotechnology Information (NCBI).

[0098] In an embodiment, a picornavirus P1 protein, or any *Enterovirus* P1 protein, is expressed as a polypeptide which is subsequently cleaved by the 3C or 3CD protease

into VP0, VP1 and VP3 virus protein, or immunologically or biologically active fragments thereof, which *Enterovirus* proteins elicit neutralizing antibodies directed against enteroviruses. The VP0 protein may be further cleaved into VP2 and VP4 proteins, or immunologically or biologically active fragments thereof which elicit neutralizing antibodies directed against enteroviruses. The virus proteins may self-assemble into VLPs, capsomers and/or aggregates of enterovirus proteins. Further it will be appreciated that the protease genes may be included in the same DNA recombinant molecule of the VLP expression cassette or in different DNA recombinant molecules, and/or expressed from different promoters or translation elements.

[0099] Recombinant DNA molecules and nucleic acids of the VLP expression cassettes may be devised whereby open reading frames which encode picornavirus structural proteins or proteases may be obtained by PCR amplification using suitably designed primers complementary to nucleic acid sequences of human picornaviruses.

[00100] In a further embodiment, the recombinant DNA molecule may encode a fusion protein having at least two enterovirus structural proteins, or portions thereof, which are expressed as a single polypeptide antigen.

[00101] The present invention encompasses a VLP expression cassette which harbors the gene sequences for *Enterovirus* structural proteins (P1 region) with a protease (3CD) which is necessary for the processing of P1 proteins into the proteins of the virus capsid, thus allowing the self-assembly of *Enterovirus* VLPs. The expression cassette is a bicistronic vector which uses a promoter upstream of the nucleic acid coding sequence for an *Enterovirus* P1 protein. Downstream from the cistron encoding the P1 protein is an internal ribosome entry site (IRES) sequence followed by the cistron containing a nucleotide sequence encoding the 3CD protease.

[00102] Expression of the P1 region and the 3CD protease proceeds from a single bicistronic message wherein the 3CD protease gene is translated in a cap-independent fashion under the control of the IRES. It is observed that expression of the protease

3CD may be moderately toxic leading to premature death of the host cells, thereby lowering the yield of the *Enterovirus* capsid proteins and VLPs. The activity of the protease may be reduced while maintaining the high level of P1 protein expression from the cassette. Different IRESs and IRES sequences comprising mutations were inserted into the expression cassettes to control expression/activity of the 3CD protease and to identify effective IRES to properly process the P1 without being toxic to the cell. For efficient production of VLPs, a number of recombinant baculoviruses which have the complete P1 coding sequence and the complete 3CD protease coding sequence whose expression is under the control of IRESs from different species or serotypes of viruses, were tested for efficient production of VLPs.

[00103] For example, the expression cassette of invention may comprise a promoter which is operably linked to a nucleic acid encoding a *Human enterovirus A* P1 polypeptide, an EMCV IRES, and a *Human enterovirus A* 3CD protease.

[00104] The expression cassette of invention may comprise a promoter which is operably linked to a nucleic acid encoding a *Human enterovirus A* P1 polypeptide, a *Human enterovirus C* IRES, and a *Human enterovirus A* 3CD protease.

[00105] The expression cassette of invention may comprise a promoter which is operably linked to a nucleic acid encoding a *Human enterovirus C* P1 polypeptide, an HEV71 IRES, and a *Human enterovirus C* 3CD protease.

[00106] Furthermore, the expression cassette of invention may comprise a promoter which is operably linked to a nucleic acid encoding a *Human enterovirus C* P1 polypeptide, an EMCV IRES, and a *Human enterovirus C* 3CD protease.

[00107] Moreover, making truncations and mutations of the 3CD protease in the expression cassette which comprises efficient IRES may achieve maximum yield of VLPs. For example, the Glycine of the HEV71 3C protease which is amino acid 1671 of GenBank accession number DQ341362.1 is changed to an Alanine using site directed

mutagenesis for the expression of mutant HEV71 3C and subsequent processing of an HEV71 P1 polypeptide.

[00108] Counter to conventional wisdom in the art with respect to the goal of achieving high levels of expression and activity of a protein from an expression cassette, in an embodiment, the instant invention actually seeks to reduce the activity of a protein to achieve a maximum protein yield. Mutation of the IRES or 3C protease nucleic acid to reduce activity unexpectedly results in an increased yield of *Enterovirus* capsid proteins and VLPs.

[00109] The expression cassettes may be cloned into suitable vectors and transformed/transfected into appropriate host cells for expression and purification of antigens for vaccines and protection against infections from picornaviruses, including enteroviruses.

[00110] The expression cassettes encoding picornavirus antigens may be comprised in plasmids which may be transfected into eukaryotic host cells and expressed under the appropriate growth conditions. Suitable eukaryotic expression systems are known to those skilled in the art and include inducible expression systems and appropriate eukaryotic host cells.

[00111] Mammalian cell expression vectors comprising an expression cassette of the invention include those which may be transiently transfected into host cells and cell lines. Moreover, mammalian cell expression vectors may be vectors which are stably maintained within the host cell following transfection.

[00112] Furthermore, mammalian cell expression vectors may include vectors which are stably or transiently transfected into mammalian host cells or cell lines wherein expression of the protein of interest is induced by the addition of an inducing agent into the culture medium. Mammalian host cells and cell lines include, for example, CHO, HEK 293, COS-1, HeLa, Vero and NIH3T3 cells. It will also be

appreciated that other eukaryotic host cells may include yeast cells or other mammalian cell lines.

[00113] The expression cassette may be contained in recombinant viruses which may transfect the host cell. Suitable viruses that may be used for this purpose include baculovirus, vaccinia, sindbis virus, SV40, Sendai virus, retrovirus and adenovirus. Suitable host cells may include host cells that are compatible with the above viruses and these include insect cells such as *Spodoptera frugiperda* (e.g. Sf9 cells) *Trichoplusia ni*, CHO cells, chicken embryo fibroblasts, BHK cells, human SW13 cells, drosophila, mosquito cells derived from *Aedes albopictus*.

[00114] The expression cassette comprising *Enterovirus* nucleic acids may be introduced into an appropriate host cell by means known to those skilled in the art. The host cells are propagated and cultured under conditions which allow expression of *Enterovirus* genes and proteins.

[00115] A gene encoding an enterovirus VP2 protein, or immunologically or biologically active fragments thereof which elicit neutralizing antibodies against enteroviruses, may be inserted in a plasmid containing a suitable promoter and expressed in a host cell. The produced enterovirus VP2 protein will be isolated and used as the basis of an immunogenic composition for use as a vaccine or for diagnostic use.

[00116] A gene encoding an enterovirus VP4 protein, or immunologically or biologically active fragments thereof which elicit neutralizing antibodies against enteroviruses, may be inserted in a plasmid containing a suitable promoter and expressed in a host cell. The produced enterovirus VP4protein will be isolated and used as the basis of an immunogenic composition for use as a vaccine or for diagnostic use.

[00117] A gene encoding an enterovirus VP0 protein, or immunologically or biologically active fragments thereof which elicit neutralizing antibodies against enteroviruses, may be inserted in a plasmid containing a suitable promoter and expressed in a host cell. The produced enterovirus VP0 protein will be isolated and used as the basis of an immunogenic composition for use as a vaccine or for diagnostic use.

[00118] A gene encoding an enterovirus VP0 protein may be operably linked to a suitable promoter and inserted into a plasmid, which plasmid exhibits an enterovirus protease linked to a suitable promoter to provide a doubly recombinant plasmid, which doubly recombinant plasmid may ultimately be expressed in a eukaryotic or prokaryotic cell expression system.

[00119] Suitable vectors for the cloning of genes and expression of enterovirus polypeptide antigens include cosmids or plasmids. Suitable expression systems include prokaryotic expression systems known to those skilled in the art and prokaryotic host cells, including *E. coli,* transformed with the cosmids or plasmids for expression of proteins in prokaryotic cells.

[00120] Suitable expression systems include eukaryotic expression systems known to those skilled in the art and eukaryotic host cells transformed with plasmids for expression of proteins in various eukaryotic host cells and cell lines.

[00121] Moreover, the *Enterovirus* polypeptide antigens may be obtained from host cells or culture supernatants by means known to those skilled in the art.

[00122] The *Enterovirus* VLPs, capsomers, antigens, immunologically active components thereof, and/or aggregates thereof, may be obtained from transfected and/or transformed host cells, or host cell culture medium, supernatants and lysates by any suitable means of purification known to those skilled in the art. Isolation of proteins released into the culture medium is a facile method of obtaining *Enterovirus* VLPs,

capsomers, antigens and/or aggregates. The *Enterovirus* VLPs, capsomers, antigens, immunologically active components thereof, and/or aggregates thereof, may be further concentrated and purified by means known to those skilled in the art.

[00123] The invention in another aspect includes a vaccine containing picornavirus antigens, such as *Enterovirus* antigens, VLPs and/or capsomers in combination with a suitable adjuvant. The picornavirus antigens, immunologically active fragments thereof, VLPs and/or capsomers may be combined with any suitable adjuvant such as Modified Vaccinia Virus, ISCOMS, alum, aluminum hydroxide, aluminum phosphate, Freund's Incomplete or Complete Adjuvant, Quil A and other saponins or any other adjuvant as described for example in Vanselow (1987) S. Vet. Bull. 57 881-896.

[00124] The meaning of the terms "aluminum phosphate" and "aluminum hydroxide" as used herein includes all forms of aluminum hydroxide or aluminum phosphate which are suitable for adjuvanting vaccines.

[00125] Moreover, picornavirus antigens may be prepared by chemical synthesis of polypeptides based on the publicly available nucleic acid or protein sequences of human poliovirus or by chemical synthesis.

[00126] In an aspect, the invention provides a vaccine comprising one or more *Human enterovirus C* antigen(s). As used herein the expression "poliovirus antigen" or "*Human enterovirus C* antigen" refers to any antigen capable of stimulating neutralizing antibodies to *Human enterovirus C*. The virus antigen may comprise a coat/capsid protein, or fragments thereof, antigenic determinants, and/or other *Human enterovirus C* proteins.

[00127] The invention in one aspect includes *Human enterovirus C* virus subunit vaccines comprising a single *Human enterovirus C* virus coat/capsid protein as an antigen. More specifically the invention pertains to vaccines comprising a *Human enterovirus C* VP2 coat/capsid protein, or immunogenic fragment thereof, as antigen.

[00128] The invention in another aspect includes a vaccine comprising *Human enterovirus C* virus like particles (VLPs), capsomers, complexes and/or aggregates, comprising *Human enterovirus C* VP1, VP2, VP3 and/or VP4, or VP0 proteins.

[00129] A recombinant DNA molecule may be obtained whereby nucleic acids comprising open reading frames which encode *Human enterovirus C* structural proteins or proteases may be obtained by PCR amplification using suitably designed primers complementary to nucleic acid sequences of human *Human enterovirus C*. Suitable primers may be designed according to standard techniques from publicly available nucleic acid sequences of *Human enterovirus C*, such as those complete genome sequences available in GenBank and accessible at the National Center for Biotechnology Information (NCBI). Accession numbers for the complete genome of the *Human enterovirus C* poliovirus type I genome include V01149 and V01150.

[00130] The expression cassettes of the invention may comprise nucleic acids which encode a *Human enterovirus C* P1 polypeptide. The P1 polypeptide is processed (cleaved) by the 3CD protease translated under the control of the IRES of the expression cassette to yield VP1, VP3 and/or VP0 polypeptides. Combinations of VP0, VP1 and VP3 may self-associate into virus-like particles.

[00131] The *Human enterovirus C* polypeptide antigen may comprise a *Human enterovirus C* coat/capsid VP2 protein, a product of further processing of VP0, in combination with another poliovirus VP0, VP1, VP3 and/or VP4 coat/capsid proteins. The combination of *Human enterovirus C* coat/capsid proteins may take the form of a virus-like particle (VLP), capsomer, complex and/or aggregate.

[00132] A gene encoding a *Human enterovirus C* coat/capsid VP2 protein may be inserted into a plasmid containing a suitable promoter and expressed in a host cell, the protein isolated and used as the basis of an immunogenic composition for use as a vaccine. Furthermore, the gene encoding human poliovirus VP2 protein may be

inserted into a plasmid containing a suitable promoter and expressed in a host cell, the protein isolated, and used as the basis of an immunogenic composition for use as a vaccine.

[00133] A gene encoding a *Human enterovirus C* coat/capsid VP4 protein may be inserted into a plasmid containing a suitable promoter and expressed in a host cell, the protein isolated and used as the basis of an immunogenic composition for use as a vaccine. Furthermore, the gene encoding human poliovirus VP4 protein may be inserted into a plasmid containing a suitable promoter and expressed in a host cell, the protein isolated, and used as the basis of an immunogenic composition for use as a vaccine.

[00134] A gene encoding a *Human enterovirus C* coat/capsid VP0 protein may be inserted into a plasmid containing a suitable promoter and expressed in a host cell, the protein isolated and used as the basis of an immunogenic composition for use as a vaccine. Furthermore, the gene encoding human poliovirus VP0 protein may be inserted into a plasmid containing a suitable promoter and expressed in a host cell, the protein isolated, and used as the basis of an immunogenic composition for use as a vaccine.

[00135] The invention encompasses a vaccine comprising one or more immunologically active antigens comprising one or more *Human enterovirus C* VP0, VP1, VP2, VP3, VP4 polypeptides, and immunologically active fragments thereof, which vaccine elicits a protective and/or neutralizing immune response directed against a human *Enterovirus*.

[00136] In an embodiment, the expression cassette consists essentially of a nucleic acid encoding a *Human enterovirus C* P1 polyprotein, an IRES and an enterovirus 3CD protease under the translational control of the IRES, which protease processes the *Human enterovirus C* P1 polyprotein into structural capsid proteins.

[00137] In another aspect, the invention provides a vaccine comprising *Human enterovirus A* antigen(s), derived from a P1 polyprotein including, VP2, VP4 and/or VP0 proteins, and/or biologically or immunologically active fragments thereof. The *Human enterovirus A* antigens may be derived from HEV71 and/or C*oxsackievirus* A16. The HEV71 antigen may be a single human enterovirus virus coat/capsid protein. In particular, the HEV71 antigen may be an HEV71 P1 polyprotein, a VP4, VP2 or VP0 polypeptide, or a fragment thereof, which elicits an immune response upon administration to a human.

[00138] In an embodiment, the *Human enterovirus A* antigen may be a combination of *Human enterovirus A* coat/capsid proteins, or immunologically active fragments thereof. For example, the *Human enterovirus A* antigen may comprise a poliovirus VP2 protein, in combination with another poliovirus coat/capsid proteins selected from VP1, VP3 and/or VP4 polypeptides. The combination of a VP2 polypeptide with other poliovirus coat/capsid proteins may take the form of a virus-like particle (VLP), capsomer, complex and/or aggregate. The combination may be in the form of a fusion protein.

[00139] More specifically the invention pertains to vaccines comprising a *Human enterovirus A* VP2 coat/capsid protein, or immunogenic fragment thereof, as antigen.

[00140] The invention in another aspect includes a vaccine comprising *Human enterovirus A* virus like particles (VLPs) and/or capsomers comprising VP1, VP2, VP3 and/or VP4, or VP0 *Human enterovirus A* proteins.

[00141] A recombinant DNA molecule may be obtained whereby open reading frames which encode *Human enterovirus A* structural proteins and/or proteases may be amplified by PCR amplification using suitably designed primers complementary to nucleic acid sequences of *Human enterovirus A.* Suitably designed primers may be designed according to standard techniques from publicly available nucleic acid

sequences of HEV71. Accession numbers for the complete genome of HEV71 include DQ341362, AB204852, AF302996 and AY465356.

[00142] A recombinant DNA molecule may be obtained whereby open reading frames which encode *Human enterovirus A* P1, VP1, VP2, VP3 and/or VP4, or VP0 proteins, and immunologically active fragments thereof, and proteases, may be obtained by PCR amplification using suitably designed primers complementary to nucleic acid sequences of *Human enterovirus A*.

[00143] In an aspect of the invention, a *Human enterovirus A* P1 protein is expressed to form a polyprotein or polypeptide which is subsequently cleaved by the 3C or 3CD protease into VP0, VP1 and VP3 proteins. VP0 proteins may be further cleaved into VP2 and VP4 proteins. The enterovirus proteins may self-assemble into VLPs, capsomers, complexes and/or aggregates of enterovirus proteins. Further it will be appreciated that the non-structural genes and the protease genes may be included in the same DNA recombinant molecule or in different DNA recombinant molecules, and or expressed from different promoters or translation elements.

[00144] The expression cassettes of the invention may comprise nucleic acids which encode a *Human enterovirus A* P1 polypeptide. The P1 polypeptide is processed (cleaved) by the 3CD protease translated under the control of the IRES of the expression cassette to yield VP1, VP3 and VP0 polypeptides and immunologically active fragments thereof. Combinations of VP0, VP1 and VP3 polypeptides may self-associate into virus-like particles

[00145] A gene encoding a *Human enterovirus A* VP2 protein, or immunologically active fragment thereof, may be inserted in a plasmid containing a suitable promoter and expressed in a host cell. The isolated *Human enterovirus A* antigen, for example, an HEV71 VP2 protein, may be isolated and used as the basis of an immunogenic composition for use as a vaccine or for diagnostic use.

[00146] A gene encoding a *Human enterovirus A* VP4 protein, or immunologically active fragment thereof, may be inserted in a plasmid containing a suitable promoter and expressed in a host cell. The isolated VP4 protein may be isolated and used as the basis of an immunogenic composition for use as a vaccine or for diagnostic use.

[00147] A gene encoding a *Human enterovirus A* VP0 protein, or immunologically active fragment thereof, may be inserted in a plasmid containing a suitable promoter and expressed in a host cell. The isolated VP0 protein may be isolated and used as the basis of an immunogenic composition for use as a vaccine or for diagnostic use.

[00148] A gene encoding a *Human enterovirus A* VP0 protein, or immunologically active fragment thereof, may be operably linked to a suitable promoter and inserted into a plasmid, which plasmid exhibits a *Human enterovirus A* protease linked to a suitable promoter to provide a doubly recombinant plasmid, which doubly recombinant plasmid may ultimately be expressed in a eukaryotic or prokaryotic cell expression system.

[00149] The *Human enterovirus A* genes and nucleic acids comprised in the expression cassette may be introduced into an appropriate host cell by means known to those skilled in the art. The host cells are propagated and cultured under conditions which allow expression of *Human enterovirus A* genes and proteins.

[00150] The invention encompasses a vaccine comprising one or more immunologically active antigens comprising one or more *Human enterovirus A* VP0, VP1, VP2, VP3, VP4 polypeptides, and immunologically active fragments thereof, which vaccine elicits a protective and/or neutralizing immune response directed against a human Enterovirus.

[00151] The in an embodiment, the expression cassette consists essentially of a nucleic acid encoding a *Human enterovirus A* P1 polyprotein, an IRES and an enterovirus 3CD protease under the translational control of the IRES, which protease processes the *Enterovirus* P1 polyprotein into enterovirus structural capsid proteins.

The structural proteins may take the form of VLPs, capsomers, complexes and/or aggregates.

[00152] Indeed, the expression of one or more of the *Enterovirus* proteins as described herein provides antigens which elicit antibodies, which antibodies are functional and able to neutralize enteroviruses selected from HEV71, *Coxsackievirus* A16, *Human enterovirus C* or any other picornavirus to high titre.

[00153] The expression of one or more of the *Enterovirus* proteins suggests that VP2 and/or VP0 polypeptides contain epitopes recognized by neutralizing antisera.

[00154] These functional antibodies surprisingly bind more strongly to *Enterovirus* VP2 and VP0 polypeptides than to VP1 polypeptides, which VP1 polypeptide is understood in the art to be the major capsid protein required for the generation of neutralizing antibodies. It is unexpected that VP2 polypeptides are, in fact, important for generating neutralizing antibodies against HEV71 infection.

[00155] Thus, it is surprising that an *Enterovirus* VP2 polypeptide is the dominant epitope, or antigenic determinant, of the capsid proteins for the generation of neutralizing antibodies against enterovirus infection. HEV71 VP2 polypeptides, either alone, or in combination with other HEV71 capsid proteins, for example VP0 polypeptides, is the dominant antigen which elicits neutralizing antibodies directed against HEV71.

[00156] In an aspect of the invention, prophylactic vaccinations for prevention of enterovirus infection are contemplated which vaccines incorporate VP0 and/or VP2 structural proteins of human enteroviruses into an immunogenic composition. The immunogenic composition or vaccine may comprise VP0 or VP2 structural proteins from *Human enterovirus A*, including HEV71 and *Coxsackievirus* A16, or a combination thereof. The immunogenic composition may be administered to a subject to elicit neutralizing antibodies directed against human enteroviruses. The immunogenic

composition may be comprised in a vaccine which is administered to a subject for the prevention of hand, foot and mouth disease infection caused by *Human enterovirus A*, such as from viruses HEV71 and/or *Coxsackievirus* A16.

[00157] In another aspect of the invention, therapeutic vaccinations are provided to prevent and/or relieve complications of HEV71 and/or *Coxsackievirus* A16 infection, for example, the neurologic and cardiovascular complications manifesting as syndromes such as meningitis, encephalitis, acute flaccid paralysis, pulmonary edema and cardiac failure.

[00158] In an aspect of the invention, prophylactic vaccinations for prevention of enterovirus infection are contemplated which vaccines incorporate VP0 or VP2 structural proteins from *Human enterovirus C,* for example PV1, PV2, PV3 structural proteins, or combinations thereof, or biologically or immunologically active fragments thereof. The immunogenic composition may be comprised in a vaccine which is administered to a subject for the prevention of polio caused by *Human enterovirus C,* including PV1, PV2, and PV3.

[00159] Furthermore, the immunogenic composition or vaccine may comprise a combination of antigens derived from both *Human enterovirus C and Human enterovirus A.*

[00160] Reference may now be made to various embodiments of the invention as illustrated in the attached figures. In these embodiments it should be noted that the *Enterovirus* VLPs, capsomers, antigens, and aggregates and specific constructs of DNA recombinant molecules are given by way of example.

[00161] It may be concluded that *Enterovirus* VP2 polypeptides are important to achieve neutralizing antibodies. VP2 polypeptides may be sufficient for formulating a vaccine against an infection with picornaviruses, such as *Human enterovirus A,* types

HEV71 and *Coxsackievirus* A16; *Human enterovirus C* types 1, 2 and 3: and *Human enterovirus D* type EV68.

[00162] In an aspect of the invention, prophylactic vaccinations for prevention of picornavirus infection are contemplated which vaccinations incorporate at least VP0 and/or VP2 and VP4 structural proteins of the virus into an immunogenic composition. The immunogenic composition may be administered to a subject to elicit neutralizing antibodies directed against a picornavirus. The immunogenic composition may be comprised in a vaccine which is administered to a subject for the prevention of picornavirus infection.

[00163] In another aspect of the invention, therapeutic vaccinations are provided to prevent and/or relieve complications of picornavirus infection, for example, the neurologic and cardiovascular complications manifesting as syndromes such as meningitis, encephalitis, acute flaccid paralysis, pulmonary edema and cardiac failure.

[00164] In a further aspect of the invention there is provided a vaccine composition according to the invention for use in medicine.

[00165] In yet another aspect, the invention provides a bivalent, or multivalent vaccine comprising enterovirus VP0 and/or VP2 and/or VP4 antigens. For example, *Human enterovirus A* VP0 and/or VP2 and/or VP4 antigens may be combined. Moreover, the aforementioned antigens from different serotypes of *Human enterovirus A*, such as antigens from *Coxsackievirus* A16 and HEV71, may be combined in a vaccine, for example, directed against human foot-and-mouth disease.

[00166] The enterovirus antigens of bivalent or multivalent vaccines may be produced from the expression cassettes described herein. The enterovirus antigens may be in the form of virus-like particles, capsomers, complexes, and/or aggregates.

[00167] In yet another aspect, the invention provides a bivalent, or multivalent vaccine comprising enterovirus antigen(s), and an antigen providing immunity against one or more of the following pathogens: diphtheria (D); tetanus (T); pertussis (P); *Haemophilus influenzae* b (Hib); Hepatitis A (HA) Hepatitis B (HB), and Human Enterovirus 71.

[00168] In a pediatric vaccine, other compatible antigens may also be included, e.g., antigens known to be effective against meningitis B, meningitis A and C, and otitis media.

[00169] The amount of picornavirus antigen in each vaccine dose is selected as an amount which induces an immunoprotective response without significant adverse side effects in typical vaccinees. Such amount will vary depending on which specific immunogens are employed. An optimal amount for a particular vaccine can be ascertained by standard studies involving observation of antibody titers and other responses in subjects. A primary vaccination course may include 2 or 3 doses of a vaccine, given at intervals optimal for providing an immunoprotective response.

[00170] The invention thus provides a method for preventing picornavirus infections in humans, which method comprises treating a human subject in need thereof with an immunologically effective dose of a vaccine according to any aspect of the invention as hereinabove described.

[00171] As used herein and in the claims, the terms and phrases set out below have the meanings which follow.

[00172] "Antibody" refers to an immunoglobulin molecule produced by B lymphoid cells with a specific amino acid sequence evoked in humans or other animals by an antigen (immunogen). These molecules are characterized by reacting specifically with the antigen.

[00173] "Antibody response" or "humoral response" refers to a type of immune response in which antibodies are produced by B lymphoid cells and are secreted into the blood and/or lymph in response to an antigenic stimulus. In a properly functioning immune response, the antibody binds specifically to antigens on the surface of cells (e.g., a pathogen), marking the cell for destruction by phagocytotic cells and/or complement-mediated mechanisms.

[00174] "Antigen" refers to any substance that, as a result of coming in contact with appropriate cells, induces a state of sensitivity and/or immune responsiveness and that reacts in a demonstrable way with antibodies and/or immune cells of the sensitized subject in vivo or in vitro.

[00175] "Epitope" refers to the simplest form of an antigenic determinant, on a complex antigen molecule. This is the specific portion of an antigen that is recognized by an immunoglobulin or T-cell receptor.

[00176] "Fusion protein" refers to a protein antigen formed by expression of a polypeptide made by combining two or more gene sequences derived from different enterovirus structural proteins.

[00177] “Immunologically active fragments” or “biologically active fragments” are fragments of *Enterovirus* structural proteins which elicit neutralizing antibodies directed against enteroviruses. Accordingly, in the context of the invention, such immunologically active fragments are presented to the immune system of an organism in order to affect, or more preferably to induce, a specific immune response and, thereby, vaccinate or prophylactically protect the organism against an infection with an *Enterovirus*.

[00178] Neutralizing antibody immune response is where specialized cells of the immune system recognize the presentation of such heterologous proteins, peptides or epitopes and launch a specific immune response.

[00179] In an embodiment, the vaccine antigen according to the invention can induce a protective immune response. The term " protective immune response" and/or “neutralizing immune response” as used herein is intended to mean that the vaccinated subject may resist or protect itself against an infection with the pathogenic agent against which the vaccination was done.

[00180] "Cellular response" or "cellular host response" refers to a type of immune response mediated by specific helper and killer T-cells capable of directly eliminating virally infected or cancerous cells.

[00181] "Antigen-presenting cell" refers to the accessory cells of antigen inductive events that function primarily by handling and presenting antigen to lymphocytes. The interaction of antigen presenting cells (APC) with antigens is an essential step in immune induction because it enables lymphocytes to encounter and recognize antigenic molecules and to become activated. Exemplary APCs include macrophages, Langerhans-dendritic cells, Follicular dendritic cells, and B cells.

[00182] "B-cell" refers to a type of lymphocyte that produces immunoglobulins or antibodies that interact with antigens.

[00183] "Cytotoxic T-lymphocyte" is a specialized type of lymphocyte capable of destructing foreign cells and host cells infected with the infectious agents which produce viral antigens.

[00184] The language “consisting essentially of” means that in addition to those components which are mandatory, other components may also be present in compositions, provided that the essential, basic and/or novel characteristics of the compositions are not materially affected by their presence.

[00185] The language “operably linked” means that the components described are in a relationship permitting them to function in their intended manner. Thus, for example, a promoter "operably linked" to a nucleic acid means that the promoter and the nucleic acids of a cistron, or more than one cistron, are combined in such a manner that a single cistronic, a single bicistronic, or a single multicistronic messenger RNA (mRNA) may be produced. Protein expression of the messenger RNA may be regulated according to transcriptional/translational elements of the nucleic acid sequence. An IRES sequence which is inserted into an expression cassette in an orientation which is upstream (5’) to a cistron means that the IRES sequence and the nucleic acids of the cistron are ligated in such a manner that translation of the cistronic mRNA is regulated under the control of the IRES.

[00186] Reference may now be made to various embodiments of the invention as illustrated in the attached figures. In these embodiments it should be noted that the picornavirus VLPs, capsomers, antigens, and aggregates and specific constructs of DNA recombinant molecules are given by way of example.

EXAMPLE 1. Description of HEV71 VLP Expression Cassettes and Vectors.

[00187] Expression cassettes may be constructed through means understood in the art.

1.1 HEV71 VLP expression cassette [P1+IRES+3CD]

Features:

Cassette size: 5172 bp
prPs: Pox virus strong early/late synthetic promoter, 43 bp
P1: P1 protein coding sequence from EV71-SB12736-SAR-03 (GenBank Accession: DQ341362) with the addition of a stop codon, 2588 bp
IRES: Internal Ribosome Binding site, 585 bp

3CD: C and D protein coding sequence of P3 from EV71-SB12736-SAR-03 (GenBank Accession: DQ341362) with the addition of a ATG start codon and stop codon, 1940 bp

Pac I: Rare cutters, enables cassette to be cloned into pSNX01 (MVA del 3 integration vector)

The cassette was cloned into pDONR221 Gateway entry vector (Invitrogen) to produce pSN01.

See Figure 1 for a diagram of the expression cassette and the pSN01 plasmid.

1.2 HEV71 VLP expression cassette [P1+IRES+3C]

Features:

Cassette size: 3773 bp

prPS: Pox virus strong early/late synthetic promoter, 43 bp

P1: P1 protein coding sequence from EV71-SB12736-SAR-03 (GenBank Accession: DQ341362) with the addition of a stop codon, 2588 bp

IRES: Internal Ribosome Binding site, 585 bp

3CD: C protein coding sequence of P3 from EV71-SB12736-SAR-03 (GenBank Accession: DQ341362) with the addition of a ATG a start codon and stop codon, 551 bp

Pac I: Rare cutters, enables cassette to be cloned into pSNX01 (MVA del 3 integration vector)

The cassette was cloned into pDONR221 Gateway entry vector (Invitrogen) to produce pSN03.

See Figure 2 for a diagram of the expression cassette and the pSN03 plasmid.

1.3 Methods for obtaining Recombinant Baculoviruses containing the insert HEV71[P1+IRES+3CD] or [P1+IRES+3C]

The source material for HEV71 P1 plus 3CD was pSN01 and for P1 plus 3C was pSN03. The aim was to introduce the HEV71-VLP cassette from pSN01 and pSN03 (Entry vectors) into the baculovirus expression plasmid pDEST8 (Destination vector) by attL/aaR *in vitro* recombination using LR CLONASE®, following the instructions in the Invitrogen BAC-TO-BAC® manual (2009).

Two recombinase reactions were set up:

1. pSN01 (EV71-P1+3CD) x pDEST8 to produce pSN07;
2. pSN03 (EV71-P1+3C) x pDEST8 to produce pSN08

pSN07 and pSN08 were used to produce recombinant bacmids bacSN07 and bacSN08 by transforming DH10bac as described in the Invitrogen BAC-TO-BAC® manual. The recombinant bacmids were transfected into Sf9 cells to rescue recombinant baculoviruses SN07 and SN08.

The recombinant baculoviruses SN07 and SN08 were used to further infect Sf9 cells in 6 well plates to evaluate for expression of processed capsid proteins. A polyclonal rabbit antiserum specific for VP1 was used to identify VP1 protein in Western blots of lysates and supernatants from recombinant baculovirus infected Sf9 cells.

1.4 Expression of VP1 in the supernatant of SN07 infected Sf9 cells.

Supernatants of infected Sf9 cells were harvested daily from day 3 to day 7 post infection and the proteins were resolved on a 12% SDS-PAGE and transferred to nitrocellulose membranes which were then probed with polyclonal rabbit anti-VP1 antisera (1:4000 dilution) overnight followed by anti-rabbit conjugated with HRP (1:1000 dilution) for 1 hour at room temperature. The Western blots were subsequently developed using TMB. The results are shown in Figure 3. It was observed that the HEV71 VP1 was processed and that expression of the protein was observed in the supernatant at day 3 and day 4 post infection and that the amount of VP1 expression diminished thereafter. Recombinant baculovirus SN07 on infection of Sf9 cells generates antigens which are found in the supernatant.

EXAMPLE 2. Processed VP1 in both the supernatants and the lysates.

[00188] Sf9 cells were infected at a Multiplicity of Infection (MOI) of 10 with different recombinant baculovirus isolates, including SN07, SN08, a control baculovirus bacGUS and mock infected. Supernatants and lysates were harvested on days 3 and 4 post infection and expression of the proteins evaluated by Western blots using rabbit anti-VP1 antisera (1:4000 dilution) to compare yields of proteins produced by SN07 and SN08. As shown in Figure 4, expression construct SN07 produced more cleaved VP1 than expression construct SN08 both in the supernatant and in the lysate on both days 3 and 4 post infection.

EXAMPLE 3. VP1 and VP0 is in the retentate after ultrafiltration over a 100kD molecular weight cut off (MWCO) membrane.

[00189] Supernatants from SN07 infected Sf9 cells at day 3 post-infection were clarified and were passed through AMICON® filters (Millipore Corp.) with a 100 kDa MWCO. The retentate was tested for the presence of processed VP1 and VP0. Since the molecular weight of VP1 is approximately 33 kDa, and the molecular weight of VP0

is 36 KDa, these proteins would not be expected to remain in the retentate unless they were in an oligomeric form. As shown in Figure 5, these antigens remain in the retentate on passing the supernatants through a 100kDa MWCO ultrafilter. This suggests that the antigens are associated in an oligomeric form. Thus, it may be concluded that VP1 and VP0 are processed and are in an oligomeric association with other capsid proteins.

EXAMPLE 4. The retentate, when used to immunize mice, elicits strong neutralizing antibodies against HEV71.

[00190] Two groups of outbred white mice were immunized with the supernatant concentrate from Sf9 infected with SN07 as prepared in Example 3. The immunization schedules used are shown in the diagram in Figure 6.

[00191] All mice in the immunized group produced antibodies that neutralized HEV71 as shown in Table 1. The retentate, when used to immunize mice, elicits strong neutralizing antibodies against HEV71.

Table 1. Neutralizing antibodies directed against HEV71.

mouse ID	**neut reciprocal titre**
m1-1	160
m1-2	640
m1-3	>=2560
m1-4	640
m2-1	80
m2-2	640
m2-3	640
m2-4	160
control1	<10
control2	<10

[00192] Thus, it may be concluded that the oligomeric proteins are able to elicit neutralizing antibodies in mice.

[00193] The oligomeric proteins may be utilized in immunogenic compositions and/or comprised in vaccines for administration and prophylaxis of enterovirus infection.

EXAMPLE 5. VP0 is expressed in the lysates of SN07 infected Sf9 cells.

[00194] Sf9 cells were infected with SN07 and the lysates were harvested at 72, 96 and 120 hours. Immunoblots were probed with rabbit polyclonal antisera directed against EV71 VP0 and it was shown that VP0 is expressed at 72 hours post infection. As shown in Figure 7, the VP0 was partially cleaved to VP2 starting at 96 hours post infection. Thus both VP0 and VP2 are present in the lysates of SN07 infected cells.

EXAMPLE 6. Pooled neutralizing sera from mice immunized with the oligomeric antigens in the supernatant of SN07 infected Sf9 cells have high titres against recombinant VP2 in ELISA.

[00195] ELISA plates were coated with equal amounts of recombinant VP1 and VP2 and used to test pooled neutralizing mouse antisera. Figure 8 shows that the mice which received the supernatant retentate antigen preparation were able to bind VP2 better than VP1. Thus, it may be concluded that the antibodies are functional and able to neutralize HEV71 to high titre.

EXAMPLE 7. Pooled neutralizing sera from mice immunized with oligomeric antigens in the supernatant of SN07 infected Sf9 cells bind more strongly to VP2 and VP0 than to VP1.

[00196] The Western blots in Figure 9 show that the neutralizing pooled mouse antisera bound more strongly to VP2 and VP0 than to VP1 even though the same amount of total protein was added to each well. This suggests that VP2 and VP0 contain epitopes recognized by neutralizing antisera.

[00197] These functional antibodies surprisingly bind more strongly to VP2 and VP0 than to VP1 which is considered to be the major capsid protein required for the generation of neutralizing antibodies. It is thus relevant to consider that VP2 is in fact important for generating neutralizing antibodies.

[00198] It may be concluded that the presence of VP2 in a vaccine formulation is important to achieve neutralizing antibodies. VP2 may be sufficient for formulating a vaccine against HEV71 and other species A, B and C enteroviruses including human *Coxsackievirus* A16, Echovirus 30,and poliovirus types 1, 2 and 3.

[00199] It will further be appreciated that the invention includes within its scope a method of generating an immune response directed against poliovirus including the step of administering an effective amount of a vaccine comprising a poliovirus antigen.

EXAMPLE 8. Comparison of the levels of neutralizing antibodies.

[00200] Immunogenic compositions comprising HEV71 VP1 are used to immunize mice. Similarly, immunogenic compositions comprising HEV71 VP2 and/or VP0 are used to immunize mice. The neutralizing antibody levels in the mice immunized with HEV71 VP2 and/or VP0 are compared with the neutralizing antibody levels in the mice immunized with HEV71 VP1. The neutralizing antibody levels in the mice immunized with HEV71 VP2 and/or VP0 are significantly higher than the neutralizing antibody levels in the mice immunized with HEV71 VP1.

EXAMPLE 9. Construction of recombinant baculovirus vector for expression of the *Human enterovirus C* P1 region and the protease 3CD from a single bicistronic message.

[00201] This example provides a method that will result in the efficient production of VLPs of *Human enterovirus C* (poliovirus) by reducing the protease 3CD mediated killing of baculovirus infected cells.

[00202] The construction of a recombinant baculovirus vector for the expression of the P1 region and the protease 3CD from a single bicistronic message is shown, for example, in Figure 1. The 3CD protease gene is translated in a cap-independent fashion under control of the EMCV IRES. This system provides the leverage to regulate the expression of protease 3CD, i.e., evaluate the mutant IRES sequences to find the weakest IRES so that a lesser amount of protease is produced compared to the P1 proteins.

[00203] A bicistronic vector is constructed in which the plasmid contains a polyhedrin promoter upstream of the coding sequence for the P1. Downstream from the cistrons encoding P1 is an *Encephalomyocarditis virus* (EMCV) internal ribosome entry site (IRES) sequence (GenBank accession number AF113968.2; nucleotides1666 to 2251) followed by the cistrons containing the nucleotide sequence encoding the protease 3CD. The source material for the *Human enterovirus C* P1 with 3CD may be accessed at the American Type Culture Collection (ATCC) and synthesized from known *Human enterovirus C* sequences (GenBank available through the National Center for Biotechnology Information (NCBI)).

[00204] Recombinant baculoviruses are generated using the BAC-TO-BAC® system according to the manufacturer's instructions (Invitrogen). Briefly, LR CLONASE® is used to introduce the *Human enterovirus C* VLP cassette into the baculovirus expression plasmid pDEST8 (Destination using vector) by attL/aaR *in vitro* recombination. The LR CLONASE® reaction is carried out at 25°C for 1 hour followed by incubation with proteinase K. The LR CLONASE® reaction mix is transformed into Library Efficiency DH5α competent cells to obtain expression clones. DNA is isolated from the resultant colonies and are confirmed for the presence of the *Human enterovirus C* cassette by restriction enzyme analysis. Recombinant bacmids are constructed by introducing the expression cassette, into the baculovirus genome harbored in DH10bac cells by T7 transposition recombinase. The recombinant bacmids are verified by their white phenotype on LB agar plates supplemented with 50 µg/ml

kanamycin, 7 μg/ml gentamicin, 10 μg/ml tetracycline, 100 μg/ml X-gal, and 40 μg/ml IPTG. The PureLink HiPure Plasmid DNA Miniprep Kit (Invitrogen) is used to purify high quality bacmid DNA from DH10Bac *E. coli*. M13 forward, M13 reverse and internal primers from the insert are used to confirm the existence of the *Human enterovirus C* cassette. EFFECTENE® transfection reagent (Qiagen) is used to rescue recombinant baculoviruses by transfecting the DNAs into Sf9 insect cells. Briefly, Sf9 cells are seeded at 2 million per T25 flask and incubated to adhere for 6hr at 28°C. One microgram of recombinant bacmid DNA is resuspended in 150 μl of DNA condensation buffer and 8μl of enhancer solution is mixed and incubated at room temperature for 5 minutes. Then 25μl of EFFECTENE® reagent is added into the DNA mix and incubated for 10 minutes at room temperature. One ml culture medium is added into the tubes containing the transfection complexes and transferred into cell culture flasks and uniformly distributed. At day 3, the supernatant is harvested by centrifugation at 500g for 5 minutes. Following transfection, a high titer viral stock is prepared. Once a high viral stock is obtained, it is employed to determine the optimal times for target protein expression.

EXAMPLE 10. Production of VLPs of HEV71, as well as *Human enterovirus C*, by means of reducing the protease 3CD mediated killing of baculovirus infected cells.

[00205] This experiment describes the construction of recombinant baculovirus vector for the expression of the P1 region and the protease 3CD from a single bicistronic message. The protease gene 3CD is translated in a cap-independent fashion under control of the EMCV IRES, shown in Figure 10. This system provides the leverage to regulate the expression of protease 3CD, i.e., evaluate the mutant IRES sequences to find weakest IRES so that a lesser amount of the protease is produced compared to the P1 proteins.

[00206] A bicistronic vector was constructed in which a plasmid contains a polyhedrin promoter upstream of the coding sequence for the P1. Downstream from the cistrons encoding P1 is an *Encephalomyocarditis virus* (EMCV) internal ribosome entry

site (IRES) sequence followed by the cistrons containing nucleotide sequence encoding the protease 3CD, see Figure 1. The IRES used in Example 1 contains native EMCV IRES sequence as there are altered forms. The native EMCV IRES sequence in Figure 10 shows the A6 bifurcation loop in the JK segment. Adding one nucleotide, for example an adenine (A7), reduces the expression. Also, in the construct used in Example 1, the 3CD protease is fused with *Encephalomyocarditis virus* IRES at the amino-terminus. Importantly out framing the EMCV start codon with 3CD protease coding sequence should considerably reduce the expression of downstream genes, see Figure 11. Both modifications are incorporated into the EMCV IRES sequence of pSN01and named pSN01-M1, as shown in Figure 12, and synthesized by DNA2.0.

[00207] Experiments were designed to characterize the VLPs expressed from the mutant EMCV IRES of pSN01-M1. Recombinant baculoviruses were generated using the BAC-TO-BAC® system according to the manufacturer's instructions (Invitrogen). Briefly, LR CLONASE® was used to introduce the HEV71 VLP cassette into the baculovirus expression plasmid pDEST8 (Destination using vector) by attL/aaR *in vitro* recombination. The LR CLONASE® reaction was carried out at 25°C for 1 hr followed by incubation with proteinase K. The LR CLONASE® reaction mix was transformed into Library Efficiency DH5α competent cells to obtain expression clones. DNA was isolated from the resultant colonies and confirmed for the presence of the HEV71/poliovirus cassette by restriction enzyme analysis. Recombinant bacmids are constructed by introducing the expression cassette of pSN07-M1, into the baculovirus genome harbored in DH10bac cells by T7 transposition recombinase to give bacSN07-M1. The recombinant bacmids are verified by their white phenotype on LB agar plates supplemented with 50 µg/ml kanamycin, 7 µg/ml gentamicin, 10 µg/ml tetracycline, 100 µg/ml X-gal, and 40 µg/ml IPTG. The PureLink HiPure Plasmid DNA Miniprep Kit (Invitrogen) was used to purify high quality bacmid DNA from DH10Bac *E. coli*. M13 forward, M13 reverse and internal primers from the insert were used to confirm the existence of the HEV71/poliovirus cassette. EFFECTENE® transfection reagent (Qiagen) was used to rescue recombinant baculoviruses by transfecting the DNAs into Sf9 insect cells. Briefly, Sf9 cells were seeded at 2 million per T25 flask and incubated

to adhere for 6hr at 28°C. One microgram of recombinant bacmid DNA was resuspended in 150 µl of DNA condensation buffer and 8µl of enhancer solution is mixed and incubated at room temperature for 5 minutes. Then 25µl of EFFECTENE® reagent was added into DNA mix and incubated for 10 minutes at room temperature. One ml culture medium was added into the tubes containing the transfection complexes and transferred into cell culture flasks and uniformly distributed. At day 3 the supernatant was harvested by centrifugation at 500g for 5 minutes. Following transfection, a high titer viral stock is prepared. Once a high viral stock is obtained, it is employed to determine the optimal times for target protein expression. For the analysis of the protein of interest, Sf9 cells grown in 10% Grace's insect cell medium (Invitrogen) were resuspended in 1% FBS Sf900-II SFM medium (Invitrogen) to get single cells and were seeded at a million per ml density in the flasks and incubated for 4 hr at 28°C. Viral stocks were added into the PBS washed cells at an MOI of 10 and rocked gently for 1 hr. The infected cells were washed three times with PBS and the cells were grown in Sf900II-SFM for different time points. Cells were lysed with hypotonic douncing buffer /1%TRITON® X-100 (TX-100) (1.5mM $MgCl_2$, 50mM KCl, 20mM HEPES, 1% TX-100) by rocking the flask for 30 minutes at room temperature and cell lysates were prepared by collecting the lysed cells from the flask and centrifuging at 4°C for 30 minutes at 7000 rpm. The components of the cell lysates and supernatants were analyzed by immunoblotting and ELISA using specific antibodies.

EXAMPLE 11. Efficient production of VLPs of HEV71, as well as *Human enterovirus C* (poliovirus) by means of reducing the protease 3CD mediated killing of baculovirus infected cells.

[00208] The construction of a recombinant baculovirus vector for expression of the P1 region and the protease 3CD from a single bicistronic message is shown in Figure 1. The 3CD protease gene is translated in a cap-independent fashion under control of the EMCV IRES, shown in Figure 10. This system provides the leverage to regulate the expression of 3CD protease, i.e., evaluate the mutant IRES sequences to find the

weakest IRES so that a lesser amount of protease is produced compared to the P1 proteins.

[00209] A bicistronic vector was constructed and the plasmid contains a polyhedrin promoter upstream of the coding sequence for the P1. Downstream from the cistrons encoding P1 is an *Encephalomyocarditis virus* (EMCV) internal ribosome entry site (IRES) sequence followed by the cistrons containing a nucleotide sequence encoding the 3CD protease, see Figure 1. The IRES used in Example 1 contains native EMCV IRES sequence as there are altered forms. The native EMCV IRES sequence has the A6 bifurcation loop in the JK segment, indeed by adding one nucleotide (A7) known to reduce the expression, see Figure 10. The A6 bifurcation loop was modified into A7 in the EMCV IRES sequence of pSN01 and named pSN01-M2, see Figure 13, which is synthesized by DNA2.0.

[00210] VLPs expressed by the mutant EMCV IRES of pSN01-M2 were characterized. Recombinant baculoviruses were generated using the BAC-TO-BAC® system according to the manufacturer's instructions (Invitrogen). Briefly, LR CLONASE® was used to introduce the HEV71 VLP cassette into the baculovirus expression plasmid pDEST8 (Destination using vector) by attL/aaR *in vitro* recombination. The LR CLONASE® reaction was carried out at 25°C for 1 hr followed by incubation with proteinase K. The LR CLONASE® reaction mix was transformed into Library Efficiency DH5α competent cells to obtain expression clones. DNA was isolated from the resultant colonies and confirmed for the presence of the HEV71/poliovirus cassette by restriction enzyme analysis. Recombinant bacmids were constructed by introducing the expression cassette of pSN07-M2, into the baculovirus genome harbored in DH10bac cells by T7 transposition recombinase to give bacSN07-M2. The recombinant bacmids were verified by their white phenotype on LB agar plates supplemented with 50 µg/ml kanamycin, 7 µg/ml gentamicin, 10 µg/ml tetracycline, 100 µg/ml X-gal, and 40 µg/ml IPTG. The PureLink HiPure Plasmid DNA Miniprep Kit (Invitrogen) was used to purify high quality bacmid DNA from DH10Bac *E. coli*. M13 forward, M13 reverse and internal primers from the insert were used to confirm the

existence of the HEV71/poliovirus cassette. EFFECTENE® transfection reagent (Qiagen) was used to rescue recombinant baculoviruses by transfecting the DNAs into Sf9 insect cells. Briefly, Sf9 cells were seeded at 2 million per T25 flask and incubated to adhere for 6hr at 28°C. One microgram of recombinant bacmid DNA was resuspended in 150 µl of DNA condensation buffer and 8µl of enhancer solution is mixed and incubated at room temperature for 5 minutes. Then 25µl of EFFECTENE® reagent was added into the DNA mix and incubated for 10 minutes at room temperature. One ml culture medium was added into the tubes containing the transfection complexes and transferred into cell culture flasks and uniformly distributed. At day 3, the supernatant was harvested by centrifugation at 500g for 5 minutes. Following transfection, a high titer viral stock was prepared. Once a high viral stock was obtained, it was employed to determine the optimal times for target protein expression. For the analysis of the protein of interest, Sf9 cells grown in 10% Grace's insect cell medium (Invitrogen) was resuspended in 1% FBS Sf900-II SFM medium (Invitrogen) to get single cells and were seeded at a million per ml density in the flasks and incubated for 4 hr at 28°C. Viral stocks were added into the PBS washed cells at an MOI of 10 and rocked gently for 1 hr. The infected cells were washed three times with PBS and cells were grown in Sf900II-SFM for different time points. Cells were lysed with hypotonic douncing buffer /1%TX-100 (1.5mM $MgCl_2$, 50mM KCl, 20mM HEPES, 1% TX-100) by rocking the flask for 30 minutes at room temperature and the cell lysates were prepared by collecting the lysed cells from the flask and centrifuging at 4°C for 30 minutes at 7000rpm. The components of the cell lysates and supernatants were analyzed by immunoblotting and ELISA using specific antibodies.

EXAMPLE 12. Efficient production of VLPs of HEV71 as well as *Human enterovirus C* (poliovirus) by means of reducing the protease 3CD mediated killing of baculovirus infected cells.

[00211] The construction of a recombinant baculovirus vector for expression of the P1 region and the protease 3CD from a single bicistronic message is shown in

Figure 1. The 3CD protease gene is translated in a cap-independent fashion under control of the EMCV IRES, shown in Figure 10. This system provides the leverage to regulate the expression of protease 3CD, i.e., evaluate the mutant IRES sequences to find the weakest IRES so that a lesser amount of protease is produced compared to the P1 proteins.

[00212] A bicistronic vector was constructed in which the plasmid contains a polyhedrin promoter upstream of the coding sequence for the P1. Downstream from the cistrons encoding P1 is an *Encephalomyocarditis virus* (EMCV) internal ribosome entry site (IRES) sequence followed by the cistrons containing a nucleotide sequence encoding the protease 3CD (Figure 1). The IRES used in Example 1 contains native EMCV IRES sequence as there are altered forms. In the pSN01 construct the 3CD protease is fused with *Encephalomyocarditis virus* polyprotein at the amino-terminus. Out framing the EMCV start codon with 3CD protease coding sequence should considerably reduce the expression of downstream genes, see Figure 11. This modification was incorporated into EMCV IRES sequence of pSN01 and named pSN01-M3, see Figure 14, and is synthesized by DNA2.0.

[00213] The VLPs expressed by the mutant EMCV IRES of pSN01-M3 were analyzed. Recombinant baculoviruses were generated using the BAC-TO-BAC® system according to the manufacturer's instructions (Invitrogen). Briefly, LR CLONASE® was used to introduce the HEV71 VLP cassette into the baculovirus expression plasmid pDEST8 (Destination using vector), by attL/aaR *in vitro* recombination. The LR CLONASE® reaction was carried out at 25°C for 1 hr followed by incubation with proteinase K. The LR CLONASE® reaction mix was transformed into Library Efficiency DH5α competent cells to obtain expression clones. DNA was isolated from the resultant colonies and confirmed for the presence of HEV71/poliovirus cassette by restriction enzyme analysis. Recombinant bacmids were constructed by introducing the expression cassette of pSN07-M3, into the baculovirus genome harbored in DH10bac cells by T7 transposition recombinase to give bacSN07-M3. The recombinant bacmids were verified by their white phenotype on LB agar plates supplemented with 50

μg/ml kanamycin, 7 μg/ml gentamicin, 10 μg/ml tetracycline, 100 μg/ml X-gal, and 40 μg/ml IPTG. The PureLink HiPure Plasmid DNA Miniprep Kit (Invitrogen) was used to purify high quality bacmid DNA from DH10Bac *E. coli*. M13 forward, M13 reverse and internal primers from the insert were used to confirm the existence of the HEV71/poliovirus cassette. EFFECTENE® transfection reagent (Qiagen) was used to rescue recombinant baculoviruses by transfecting the DNAs into Sf9 insect cells. Briefly, Sf9 cells were seeded at 2 million per T25 flask and incubated to adhere for 6hr at 28°C. One microgram of recombinant bacmid DNA was resuspended in 150 μl of DNA condensation buffer and 8μl of enhancer solution is mixed and incubated at room temperature for 5 minutes. Then 25μl of EFFECTENE® reagent was added into the DNA mix and incubated for 10 minutes at room temperature. One ml culture medium was added into the tubes containing the transfection complexes and transferred into cell culture flasks and uniformly distributed. After day 3, supernatant was harvested by centrifugation at 500g for 5 minutes. Following transfection, a high titer viral stock is prepared. Once a high viral stock is obtained, it is employed to determine the optimal times for target protein expression.

[00214] For the analysis of the protein of interest, Sf9 cells grown in 10% Grace's insect cell medium (Invitrogen) were resuspended in 1% FBS Sf900-II SFM medium (Invitrogen) to get single cells and were seeded at a million per ml density in the flasks and incubated for 4 hr at 28°C. Viral stocks were added into the PBS washed cells at a MOI of 10 and rocked gently for 1 hr. The infected cells were washed three times with PBS and cells were grown in Sf900II-SFM for different time points. Cells were lysed with hypotonic douncing buffer /1%TX-100 (1.5mM $MgCl_2$, 50mM KCl, 20mM HEPES, 1%TX-100) by rocking the flask for 30 minutes at room temperature and cell lysates were prepared by collecting the lysed cells from the flask and centrifuging at 4°C for 30 minutes at 7000 rpm. The components of the cell lysates and supernatants were analyzed by immunoblotting and ELISA using specific antibodies.

EXAMPLE 13. Mutant IRES construct M2 expresses higher levels of VP1 in the supernatant.

[00215] Recombinant baculoviruses expressing HEV71 capsid proteins under the control of the wild type or mutant EMCV IRES's were evaluated with respect to the level of expression of the HEV71 capsid proteins from the ECMV IRES's. Baculovirus produced VLPs which are expressed under the control of the wild type EMCV IRES from SN07 of Example 1, and the 3 mutant IRES's, M1, M2 and M3 from Examples 10, 11, and 12, respectively, were analyzed with respect to the level of baculovirus expression of the HEV71 capsid proteins. A recombinant baculovirus expressing P1 and 3CD under different promoters (F) and a control recombinant baculovirus expressing bacGUS (G) were also included in the study. Sf9 cells were infected at an MOI of 5 and both lysates and supernatants were harvested on day 3 as described in Examples 10-12 above. The lysates and supernatants were probed with an anti-VP1 antibody to detect the expression of HEV71 capsid proteins.

[00216] The immunoblots of Figure 15 show that when lysates were probed with antibodies to VP1, the mutants M1 and M2 express higher levels of VP1 than the mutant M3 or the construct driven by 2 promoters. However, the mutant M2 produced more VP1 in the supernatant. These data are shown in the top panel.

[00217] The bottom panel of Figure 15 shows the blots which were probed with a control anti-gp64 antibody which is directed to the coat protein of baculovirus. The immunoblot shows that equivalent amounts of baculovirus were produced in each sample.

EXAMPLE 14. Cloning, expression and purification of subunit vaccines using a baculovirus expression system.

[00218] The present invention is intended for the generation and use of recombinant HEV71 and poliovirus structural proteins which are fused as single

immunogens to elicit a protective immune response in vaccinated individuals. The present invention relates generally to preparing recombinant HEV71 and/or poliovirus fusion protein vaccine compositions comprising HEV71 and/or poliovirus subunit protein, or an immunogenic fragment thereof, and an adjuvant in combination with the recombinant HEV71 and/or poliovirus subunit fusion protein. HEV71 and poliovirus subunit fusion proteins may comprise capsid proteins selected from VP1, VP2, VP3, and VP4, combinations thereof, and combinations of immunogenic fragments thereof. In one aspect of this embodiment, the recombinant HEV71 and/or poliovirus fusion protein comprises HEV71 or poliovirus subunit protein and a fusion partner protein in genetic association with the HEV71 or poliovirus subunit protein. The present invention contemplates methods to generate the constructs to express the following subunit vaccines in *E.coli* as well as baculovirus: VP0, VP4-VP2-VP3 fusion, VP2-VP3-VP1 fusion.

[00219] To generate cDNAs from HEV71 and/or poliovirus encoding VP0, VP4-VP2-VP3 fusion, or VP2-VP3-VP1, reverse transcription/polymerase chain reaction (The High Pure Nucleic Acid Kit; Roche) was carried out using purified genomic viral RNA. A forward and a reverse primer was made from the 5'and 3' end of the genes and which primers incorporate a start and stop codon. The amplified PCR products were digested with EcoRI and NotI restriction enzymes and cloned into the pFastBac HT vector (Invitrogen) as shown in Figure 16. The BAC-TO-BAC® expression system from Invitrogen is commercially available and methods were used according to the manufacturer's instructions. The fusion genes are cloned into pFastBac HT donor plasmid and the production of recombinant proteins was based upon the BAC-TO-BAC® to baculovirus expression system (Invitrogen). The pFastBac HT donor plasmid carrying the fusion genes was transferred into a baculovirus shuttle vector (bacmid) by site-specific recombination by T7 transposition recombinase. This was accomplished in *E. coli* strain DH10Bac. The DH10Bac cells contain the bacmid, which conferred kanamycin resistance and a helper plasmid, which encoded the transposase and conferred resistance to tetracycline. The recombinant pFastBac HT plasmids with the gene of interest were transformed into DH10Bac cells for the transposition to generate

recombinant bacmids. The transformed cells were serially diluted and each dilution was plated on LB agar plates supplemented with 50µg/ml kanamycin, 7µg /ml gentamicin, 10µg/ml tetracycline, 100 µg/ml X-gal, and 40 µg/ml IPTG and incubated for at least 48 hours at 37°C. The white colonies were picked and re-streaked to confirm a white phenotype. Recombinant bacmids were isolated by the PureLink HiPure Plasmid DNA Miniprep Kit (Invitrogen) and the DNA samples were dissolved in 40µlof TE (10 mM Tris-HCl pH 8, 1 mM EDTA) and used for transfections.

[00220] The isolated bacmid DNA was screened for the inserted gene of interest by PCR. EFFECTENE® transfection reagent (Qiagen) was used to rescue recombinant baculoviruses by transfecting the DNAs into Sf9 insect cells. Briefly, Sf9 cells were seeded at 2 million per T25 flask and incubated to adhere for 6hr at 28°C. One microgram of recombinant bacmid DNA was resuspended in 150 µl of DNA condensation buffer and 8µl of enhancer solution is mixed and incubated at room temperature for 5 minutes. Then 25µl of EFFECTENE® reagent was added into DNA mix and incubated for 10 minutes at room temperature. One ml culture medium was added into the tubes containing the transfection complexes and transferred into cell culture flasks and uniformly distributed. At day 3, the supernatant was harvested by centrifugation at 500g for 5 minutes. Following transfection, a high titer viral stock is prepared. Once a high viral stock is obtained, it is employed to determine the optimal times for target protein expression. For the analysis of the protein of interest, Sf9 cells grown in 10% Grace's insect cell medium (Invitrogen) was resuspended in 1% FBS Sf900-II SFM medium (Invitrogen) to get single cells and are seeded at a million per ml density in the flasks and incubated for 4 hr at 28°C. Viral stocks were added into PBS washed cells at a MOI of 10 and rocked gently for 1 hr. The infected cells were washed three times with PBS and cells are grown in Sf900II-SFM for different time points. Cells were lysed with hypotonic douncing buffer /1%TX-100 (1.5mM $MgCl_2$, 50mM KCl, 20mM HEPES, 1%TX-100) by rocking the flask for 30 minutes at room temperature and the cell lysates were prepared by collecting the lysed cells from the flask and centrifuging at 4°C for 30 minutes at 7000rpm. The expression of the heterologous protein in the cells was verified by SDS polyacrylamide gel electrophoresis (SDS-

PAGE) and Western blots using the His Probe-HRP antibody (Thermo Scientific) as the probe. Once production of baculovirus and the expression of protein were confirmed, the virus stock was amplified to produce a concentrated stock of the baculovirus that carry the gene of interest. The most appropriate concentration of the virus to infect insect cells and the optimum time point for the production of the desired protein was also established. For purification under denaturing conditions, the cells were lysed in a lysis buffer containing 6 M guanidinium-HCl in 100 mM NaH_2PO_4, 10 mM Tris, 300mM NaCl, 10 mM imidazole, pH 8.0 (lysis buffer). The suspension was sonicated on ice with 5 pulses of 1 minute per pulse at a power setting of 60 watts, and was mixed at room temperature for 1 hour. The lysate was centrifuged at 27K g for 30 min to eliminate cell debris. The supernatant was loaded on to a HisTrap (GE healthcare life sciences) column pre-equilibrated with lysis buffer. Following loading, the column was washed with 20 column volumes of 6 M guanidinium-HCl in 100 mM NaH_2PO_4, 10 mM Tris, 300 mM NaCl, 40 mM Imidazole, pH 8.0 (wash buffer 1), followed by washes with 20 column volumes of 8 M urea in 100 mM NaH_2PO_4,10 mM Tris, 300 mM NaCl, 40 mM imidazole, pH 8.0 (wash buffer 2). The bound protein was eluted with a buffer containing 8 M urea, 100 mM NaH_2PO_4, 10 mM Tris, 300 mM NaCl, 250 mM imidazole, pH 8 (Elution Buffer). The fractions containing the protein were pooled and dialyzed against PBS, overnight at 4° C. TEV protease was used for removal of the histidine tag following protein purification according to manufacturer's instructions.

EXAMPLE 15. Expression and purification of *Human enterovirus A* and *Human enterovirus C* (poliovirus) subunit vaccines in *E.coli*.

[00221] The Champion™ pET SUMO Expression System (Invitrogen) produces the highest levels of soluble protein in *E. coli.* It utilizes a small ubiquitin-related modifier (SUMO) fusion to enhance the solubility of expressed fusion proteins. After expression, the 11 kD SUMO moiety can be cleaved by the highly specific and active SUMO (ULP-1) protease at the carboxyl terminal, producing a native protein. Also it contains N-terminal 6xHis tag for protein detection and purification.

[00222] The construction of pET SUMO-VP0, pET SUMO- VP4-VP2-VP3 and pET SUMO-VP2-VP3-VP1 expression vector for antigenic fusion proteins of HEV71 and poliovirus, as shown in Figure 17, is as follows. The fragments of VP0, VP4-VP2-VP3 fusion and VP2-VP3-VP1 fusion were used as the antigens for HEV71 and poliovirus subunit vaccines. A SUMO motif and the 6XHistag were conjugated to the N-terminus of fusions to aid in solubilization of the protein and purification of the protein, respectively. The antigenic fusion proteins were created by a gene cloning technology comprising cloning cDNA sequences encoding respective proteins into an expression vector to form expression vectors of pET SUMO- VP0, pET SUMO- VP0, pET SUMO-VP4-VP2-VP3 and pET SUMO-VP2-VP3-VP1. The DNA fragments encoding fusion partners were PCR amplified using specific primers which consist of a start codon and a stop codon in the forward and reverse primers, respectively. Ligation of the PCR product was carried out as follows: fresh PCR product, 10X ligation buffer , pET SUMO vector (25 ng/µl) 2 µl, sterile water added to a total volume of 9 µl , and 1 µl T4 DNA ligase (4.0 Weiss units) was added and the ligation reaction incubated at 15ºC for overnight then proceeded to transforming One Shot® Mach1™-T1R (Invitrogen) competent cells. Ten (10) colonies were selected and plasmid DNA isolated from them using the PureLink™ HQ Mini Plasmid Purification Kit (Invitrogen). The plasmids were analyzed by restriction analysis to confirm the presence and the correct orientation of the insert. From the recombinants, plasmid DNA was isolated as earlier and the plasmids were transformed into BL21 (DE3) One Shot® cells (Invitrogen). The transformants were grown and induction of expression with IPTG at several time points was carried out to determine the optimal time of expression. For each time point, 500 µl was removed from the induced and uninduced cultures and each cell pellet was resuspended in 80 µl of SDS-PAGE sample buffer. After centrifuging the boiled samples, 10 µl of each sample was loaded onto an SDS-PAGE gel and electrophoresed.

[00223] To scale-up the purification of recombinant fusion protein using a HisTrap nickel column (GE Healthcare Life Sciences), the following procedure was adapted. An overnight culture (5%) was inoculated into 100-300 ml LB plus 50µg/ml kanamycin and

induced after 2hrs with 1mM IPTG. After 2hrs the cells were harvested by centrifuging at 3000g for 10 minutes. The pellet was resuspended in 10% (total volume of the culture) the binding buffer (20mM sodium phosphate, 0.5M NaCl and 20mM imidazole at pH7.4). The cells were sonicated with Misonic UltraSonicate Liquid processor for five times for a minute with a minute gap in an ice bucket. The sonicated samples were separated into soluble and insoluble form by centrifuging at 4000 rpm for 1hr at 4°C. The insoluble fraction was resuspended with binding buffer containing 6M urea. Both the soluble and insoluble fractions were centrifuged at 4000 rpm for 1hr at 4°C then filtered through 0.22µm filter unit. A HisTrap column was equilibrated with the binding buffer and filtered samples were loaded onto the column. Next, the column was washed with binding buffer with 40mM imidazole and the recombinant protein was eluted with binding buffer containing 0.5M imidazole (6M urea for insoluble fraction). All the collected samples were tested using Coomassie blue staining protocol for proteins. Pure recombinant protein containing eluted fraction were dialysed using Merck tubing in Tris-HCl buffer. After the dialysis protein concentrations were estimated using a Bradford reagent. The native protein was generated by using SUMO protease to cleave the N-terminal peptide containing the 6xHis tag and SUMO according to manufacturer's instructions.

EXAMPLE 16. Antibodies from pooled neutralizing sera from mice immunized with SN07 retentate binds to all the components of EV71VLPs.

[00224] The coding sequences of VP1, VP2 and VP0 were separately cloned into the pET SUMO vector as described in Example 15. The individual capsid proteins were expressed in *E.coli* and purified as described in Example 15. The purified proteins VP0, VP1, VP2 and VP3 were subjected to Western blotting and probed with pooled neutralizing sera used in Example 4 at the dilution of 1:1000. Control mice sera used in Example 4 is also included in the studies.

[00225] Western blotting results in Figure 18 show that pooled neutralizing sera from mice immunized with oliogomeric antigens in the supernatant of SN07 infected Sf9 cells bind to all of the VLP components, VP0, VP1, VP2 and VP3.

EXAMPLE 17. Characterization of HEV71 VLPs - Pull-down of HEV71 VLPs from culture supernatants.

[00226] 20ml of supernatant from cells infected with recombinant baculovirus SN07 was mixed with 10ml neutralizing monoclonal antibody (EV18/4/D6-1/F1/G9) and left at room temperature for 1hr. The mixture was loaded slowly through a 1ml column of MabSelect SuRe™ (GE Health Care) recombinant Protein A, 85um agarose bead size, which was pre-equilibrated with PBS, then washed with 20mls PBS and then eluted with 0.5 ml of 0.1M glycine-HCl, pH3.0. Fractions were neutralized with 30µl of Tris-HCl, pH8.8. Elution fractions were run on SDS-PAGE followed by Coomassie blue staining and destaining.

[00227] Figure 19 shows that all of the VLP components (VP0, VP1 and VP3) are visible in the Coomassie blue stained SDS-PAGE gel.

EXAMPLE 18. Analysis of affinity column (AFC) purified HEV71VLPs.

[00228] An affinity column (AFC) was prepared with a neutralizing monoclonal antibody (EV18/4/D6-1/F1/G9), which was developed using a baculovirus SN07 supernatant retentate. The eluted fraction was analyzed by Western blotting and is shown in Figure 20. The eluted fraction in Lanes 1 and 2, probed using an anti-VP1 antibody, shows that VP1 is present in the AFC purified VLPs. The eluted fraction in Lanes 3 and 4, probed using an anti-VP2 antibody, shows that VP2 is also present in the AFC purified VLPs. The eluted fraction in Lanes 5 and 6, probed using an anti-VP2 monoclonal antibody, shows that VP2 is present in the AFC purified VLPs.

EXAMPLE 19. Electron micrograph of AFC purified HEV71 VLPs.

[00229] The fraction eluted from the affinity column (AFC) of Example 18 was evaluated by electron microscopy. An electron micrograph picture of AFC purified VLPs is shown in Figure 21. The results of the electron microscopy confirm that HEV71 structural proteins are assembled into VLPs.

EXAMPLE 20. Mice protection studies using retentate.

[00230] Antigen used was a 20x concentrated crude retentate of recombinant baculovirus SN07 prepared as described above. Vaginal plugs indicative of pregnancy was determined the morning after mating, designated as Embryonic Day E0.5. Two doses of 100 µL of retentate mixed with IMJECT® (alum) given i.p. to pregnant dams at E3.5 and, on confirmation of pregnancy by weight measurement, a second dose was given on E17.5. Initial blood sample was collected from each mouse prior to the administration of the first vaccine dose, samples were collected weekly thereafter until 14 days after viral challenge of pups. Three groups of Balb/c mice 20 mice were immunized and then challenged (20 mice), 20 mice were mock-immunised and challenged (20 mice), 5 mice non-immunised, non-challenged Five-day-old pups were infected with 50 µl of MP-26M virus containing 100 x HD_{50} of MP-26M. All infected animals were observed twice daily for clinical signs of illness until 14 days post-inoculation. Abnomal signs included failure to thrive; weight loss; runting; stomach empty of milk; lethargy; head tilt; hunched posture; ruffled fur; dehydration, hypothermia; limb paralysis. Paralysis was scored according to the following grading system:

0 – normal
1 – limb weakness but can still move limbs
2 – inability to move affected limbs
3 – quadriplegia i.e. inability to move limbs

[00231] Animals suffering grade 3 paralysis were euthanased by cervical dislocation under anaesthetic (HD_{50}). Histopathology was performed on sections from a variety of target organs.

[00232] The results of the protection study are shown in Table 2 below.

Table 2.

83% PROTECTION IN PASSIVE PROTECTION

VACCINE	NUMBER OF MATED MICE	NUMBER OF PREGNANT MICE	NUMBER OF PUBS/ LITTER	TOTAL NUMBER OF PUBS	NUMBER OF SURVIVED PUBS	SURVIVAL RATE	MEDIAN SURVIVAL (DAYS)
RETENTATE	20	2	1,5	6	5*	83%	14
ALUM/PBS	20	2	5,7	12	0	0%	5
NO VACCINE/ NO CHALLENGE	5+5	2+1	5,7,6	18	18	100%	14

[00233] Two litters of mice were born to VLP vaccinated mothers. One mother had 5 pups and showed good maternal care. The second mother had one pup that was very weak and did not show good maternal care.

[00234] It may be concluded that 83% of mice born to immunized mothers were protected from virus challenge.

[00235] A summary of the histopathological observations is shown in Table 3.

Table 3. SUMMARY OF HISTOPATHOLOGICAL OBSERVATIONS
(N= no abnormalities detected)

VACCINE	AGE	DAYS P1	MOUSE	SYMPTOMS	SPLEEN	LIVER	HEART	SKELETAL MUSCLE	SPINAL CORD	BRAIN
VLP	19	14	V87P4	NO CLINICAL SIGNS OF INFECTION	N	N(1)	N	0.5	N	N
	19	14	V87P5	NO CLINICAL SIGNS OF INFECTION	N	N(1)	N	0.5	N	N
	19	14	V87P6	NO CLINICAL SIGNS OF INFECTION	N	N(1)	N	N	N	N
ALUM/PBS	10	5	DC4	GRADE3 PARALYSIS	N	N(2)	N	3	N	N
			DC5	GRADE3 PARALYSIS	N	N(2)	N	3	N	N
			DC6	GRADE3 PARALYSIS	N	N(2)	N	03	N	N
NO VACCINE	19	NI	V116P4	NO CLINICAL SIGNS OF INFECTION	N	N(0.5)	N	N	N	N
			V116P5	NO CLINICAL SIGNS OF INFECTION	N	N(0.5)	N	N	N	N

[00236] Five mice gave birth to litters and were included in the vaccine protection study (11% pregnancy rate). The value of the humane endpoint (HD_{50}), as calculated by the method of Reed and Muench, 1938, was determined to be 2.0×10^2 $TCID_{50}$ of MP-26M. 83% of mice born to immunized mothers were protected from challenge.

[00237] The histopathology results showed that 3 mice from mothers in the HEV71-VLP immunized group which were then challenged with HEV71 virus showed no clinical signs of infection.

[00238] However, 3 mice from mothers in the mock-immunized (Alum/PBS) and which were then challenged with HEV71 virus succumbed to infection. Two mice from

mothers in the non-immunized, non-challenged group showed no clinical signs of infection.

[00239] The VLP vaccine protected one litter of infant mice (5/6 pups from immunized mothers vs 0/12 pups from mock-immunized mothers) against lethal challenge with a B3 genotype mouse-adapted strain of HEV71.

EXAMPLE 21. *Human enterovirus C* (poliovirus-PV) VLP expression.

[00240] Variations on poliovirus expression cassettes were constructed to generate poliovirus VLPs. The expression cassettes all comprised a poliovirus P1 polypeptide and differed with respect to the IRES, which IRES directs the expression of a poliovirus 3CD protease: (PV-P1+HEV71-IRES+PV-3CD); (PV-P1+EMCV-IRES+PV-3CD); (PV-P1+PV-IRES+PV-3CD). Recombinant baculoviruses harboring the poliovirus VLP expression cassettes were tested. Lysates from the baculovirus infected cells were harvested on day 3 post infection and expression of the poliovirus VP3 was evaluated using rabbit anti-PVP3 antibodies (1:2000).

[00241] Only the PV-IRES-containing construct produced a VP-3 protein of the expected size. Unlike in the PV-IRES construct, the poliovirus VP3 protein is not processed properly when the 3CD protease is under the control of HEV71-IRES or EMCV –RES and, consequently, adversely affects the production of poliovirus VLPs. Thus, the poliovirus PV-VLP expression cassette harboring the PV-IRES is very efficient for poliovirus VLP production.

EXAMPLE 22. ELISA to demonstrate poliovirus PV-VLP assembly.

[00242] To demonstrate poliovirus VLP generation from the PV-VLP expression cassette, a two sites ELISA was performed using lysates and supernatants from recombinant baculoviruses carrying a PV-VLP expression cassette wherein the PV-3CD protease is under the control of a poliovirus IRES. Sf9 cells were infected with the

recombinant baculoviruses carrying a PV-VLP expression cassette including PV-IRES. Lysates and supernatants were harvested on day 3 post infection. Formation of the poliovirus VLPs was evaluated using a two sites ELISA.

[00243] The two sites ELISA procedure was conducted by preparing protein A-purified rabbit anti-poliovirus VP3 antibodies as capture antibodies and diluting in coating buffer, 0.05M Carbonate-bicarbonate buffer, pH 9.6, to 50µg/mL. 100 µl/well of the antibodies was dispensed into NUNC immunoplates and stored at 4°C overnight. After washing with PBST (0.05%), the immunoplates were blocked with 200 µl/well blocking buffer, 1% casein in PBS, at room temperature for 2 hr. Samples of lysates and supernatants from recombinant baculoviruses carrying a PV-VLP expression cassette wherein the PV-3CD protease is under the control of a poliovirus IRES were diluted in diluents, 0.2% casein in PBS, dispensed at 100µl/well, and were incubated at room temperature for 1 hr and then washed with PBST (0.05%).

[00244] For detection of VLPs, mouse monoclonal antibodies, anti-VP1 (Clone 5-D8/1; Dako), were prepared in 0.2% casein in PBS diluents at a 1:250 dilution. The diluted detecting monoclonal antibodies were dispensed at 100µl/well and then incubated at room temperature for 1 hr. Anti-mouse HRP conjugated 2° antibodies were prepared in 0.2% casein in PBS diluents at a 1:1000 dilution. After washing the plate with PBST (0.05%), 100µl/well of the diluted 2° antibodies were dispensed and the plate incubated at room temperature for 1 hr. The plates were washed with washing buffer, PBST (0.05%). SureBlue^{TM} TMB 1(KPL) component microwell peroxidase substrate was dispensed at 100µl/well and incubated at room temperature for 10 min for development. 100µl/well of stop solution, 5mM NaOH, was added to stop the reaction. The absorbance of each well was read at an OD of 650nm.

[00245] The results of the two sites ELISA shown in Figure 23 demonstrate that the VP1 and VP3 proteins are in association with each other indicating that VLPs are indeed formed from the PV-VLP expression cassette.

EXAMPLE 23. Construction of HEV71 VLP Expression Cassettes with HEV71 IRES (P1+HEV71 IRES+3CD).

[00246] The schematic structure of a HEV71 VLP cassette with HEV71-IRES is shown in Figure 24. The expression cassette is similar to the construct shown in Example 1 (pSN01) except that the expression of the 3CD protease is driven by the HEV71 IRES rather than the EMCV IRES. The HEV71 IRES sequence is found in GenBank, Accession Number DQ341362.1; nucleotides 1 to 747. An HEV71 expression cassette containing vector is introduced into the baculovirus expression plasmid pDEST8 (Destination vector) by attL/aaR *in vitro* recombination using LR CLONASE®, following the instructions in the Invitrogen BAC-TO-BAC® manual (2009). Recombination between the entry vector and pDEST8 produces an expression clone. Expression clones give rise to recombinant bacmid by transforming DH10bac as described in the Invitrogen BAC-TO-BAC® manual. Transfection of the recombinant bacmid into Sf9 cells rescues the recombinant baculovirus carrying expression cassette which harbors P1, HEV71 IRES and 3CD.

[00247] Further infection of Sf9 cells can be used to evaluate expression of processed capsid proteins with rescued recombinant baculoviruses in 6 well plates. A polyclonal rabbit antiserum specific for VP1, VP0 and VP3 will identify the assembled VLPs by Western blotting of lysates and supernatants from recombinant baculovirus infected Sf9 cells.

EXAMPLE 24. Construction of HEV71 VLP Expression Cassettes with PV-IRES (P1+PV-IRES+3CD).

[00248] The expression cassette is similar to the expression cassette in Example 24 except that the expression of the 3CD protease is driven by a poliovirus IRES (PV-IRES) rather than an HEV71-IRES. The poliovirus IRES sequence is found in GenBank, Accession Number V01150.12 ; nucleotides 1 to 628.

[00249] An HEV71 expression cassette containing vector is introduced into the baculovirus expression plasmid pDEST8 (Destination vector) by attL/aaR *in vitro* recombination using LR CLONASE®, following the instructions in the Invitrogen BAC-TO-BAC® manual (2009). Recombination reaction between entry vector and pDEST8 is set up to produce an expression clone. An expression clone give rises to recombinant bacmid by transforming DH10bac as described in the Invitrogen BAC-TO-BAC® manual. Transfection of the recombinant bacmid into Sf9 cells rescues the recombinant baculovirus carrying expression cassette which harbors P1, PV IRES and 3CD.

[00250] Further infection of Sf9 cells can be used to evaluate for expression of processed capsid proteins with rescued recombinant baculoviruses in 6 well plates. A polyclonal rabbit antiserum specific for VP1, VP0 and VP3 will identify the assembled VLPs by Western blotting of lysates and supernatants from recombinant baculovirus infected Sf9 cells.

CLAIMS

1. A vaccine comprising one or more immunologically active antigens comprising one or more human *Enterovirus* polypeptides selected from VP0, VP1, VP2, VP3, VP4, and immunologically active fragments thereof.

2. The vaccine of Claim 1, which elicits a protective and/or neutralizing immune response directed against a human *Enterovirus*.

3. The vaccine of Claim 1, wherein the human *Enterovirus* is selected from *Human enterovirus A*, *Human enterovirus B, Human enterovirus C* and *Human enterovirus D*.

4. The vaccine of Claim 3, wherein the *Human enterovirus A* is selected from *Human enterovirus* 71 (HEV71) and *Coxsackievirus* A16, wherein the *Human enterovirus B* is selected from *Coxsackievirus* B and echovirus, wherein the *Human enterovirus C* is selected from *Human poliovirus* 1, *Human poliovirus* 2 and *Human poliovirus* 3, and wherein the *Human enterovirus D* is *EV* 68.

5. The vaccine of Claim 1, wherein the vaccine comprises an immunologically active *Enterovirus* VP0 polypeptide.

6. The vaccine of Claim 1, wherein the vaccine comprises an immunologically active *Enterovirus* VP2 polypeptide.

7. The vaccine of Claim 1, wherein the vaccine comprises an immunologically active *Enterovirus* VP3 polypeptide.

8. The vaccine of Claim 1, wherein the vaccine comprises an immunologically active *Enterovirus* VP1 polypeptide.

9. The vaccine of Claim 1, wherein the vaccine comprises polypeptides from one or more *Enterovirus* species or serotype.

10. The vaccine of Claim 9, wherein the species or serotype may be *Human enterovirus A* selected from HEV71 and *Coxsackievirus* A16.

11. The vaccine of Claim 9, wherein the species or serotype may be *Human enterovirus C* selected from PV-1, PV-2 and PV-3.

12. The vaccine of Claim 1, wherein the one or more immunologically active antigens comprising one or more human *Enterovirus* polypeptides selected from VP0, VP1, VP2, VP3, VP4, and immunologically active fragments thereof, are in the form of a virus-like particle, capsomer, complex and/or aggregate.

13. The vaccine of Claim 12, wherein the virus-like particle comprises an immunologically active *Enterovirus* VP0 polypeptide.

14. The vaccine of Claim 12, wherein the virus-like particle comprises an immunologically active *Enterovirus* VP2 polypeptide.

15. The vaccine of Claim 12, wherein the virus-like particle comprises an immunologically active *Enterovirus* VP3 polypeptide.

16. The vaccine of Claim 12, wherein the virus-like particle comprises an immunologically active *Enterovirus* VP1 polypeptide.

17. A method of vaccinating a subject against an *Enterovirus* infection, comprising administering to the subject a vaccine comprising one or more

immunologically active antigens comprising one or more human *Enterovirus* polypeptides selected from VP0, VP1, VP2, VP3, VP4, and immunologically active fragments thereof, in an amount effective to elicit a protective and/or neutralizing immune response when administered to the subject.

18. The method of Claim 17, wherein the vaccine comprises an immunologically active *Enterovirus* VP0 polypeptide.

19. The method of Claim 17, wherein the vaccine comprises an immunologically active *Enterovirus* VP1 polypeptide.

20. The method of Claim 17, wherein the vaccine comprises an immunologically active *Enterovirus* VP3 polypeptide.

21. The method of Claim 17, wherein the vaccine comprises an immunologically active *Enterovirus* VP2 polypeptide.

22. The method of Claim 17, wherein the one or more immunologically active antigens comprising one or more human *Enterovirus* polypeptides selected from VP0, VP1, VP2, VP3, VP4, and immunologically active fragments thereof, are in the form of a virus-like particle, capsomer, complex and/or aggregate.

23. The method of Claim 22, wherein the virus-like particle comprises an immunologically active *Enterovirus* VP0 polypeptide.

24. The method of Claim 22, wherein the virus-like particle comprises an immunologically active *Enterovirus* VP2 polypeptide.

25. The method of Claim 22, wherein the virus-like particle comprises an immunologically active *Enterovirus* VP3 polypeptide.

26. The method of Claim 22, wherein the virus-like particle comprises an immunologically active *Enterovirus* VP1 polypeptide.

27. The method of Claim 17, wherein the vaccine comprises polypeptides from one or more *Enterovirus* species or serotype.

28. The method of Claim 27, wherein the *Enterovirus* species or serotype may be *Human enterovirus A* selected from HEV71 and C*oxsackievirus* A16.

29. The method of Claim 27, wherein the *Enterovirus* species or serotype may be *Human enterovirus C* selected from PV-1, PV-2 and PV-3.

30. An expression cassette comprising a promoter operably linked to a nucleic acid encoding a human *Enterovirus* P1 polypeptide, an Internal Ribosome Entry Site (IRES), and a nucleic acid encoding a human *Enterovirus* 3CD protease.

31. The expression cassette of Claim 30, wherein the polypeptide is human *Enterovirus* P1.

32. The expression cassette of Claim 30, wherein the human *Enterovirus* 3CD protease processes the human *Enterovirus* P1 polypeptide.

33. The expression cassette of Claim 32, wherein the processed human *Enterovirus* P1 polypeptides associate to form virus like particles, capsomers, complexes and/or aggregates.

34. The expression cassette of Claim 30, wherein the human *Enterovirus* P1 polypeptide comprises combinations of polypeptides selected fromVP0, VP1, VP2, VP3, VP4, and immunologically active fragments thereof.

35. The expression cassette of Claim 30, wherein the human *Enterovirus* is selected from *Human enterovirus A* and *Human enterovirus C*.

36. The expression cassette of Claim 35, wherein the *Human Enterovirus* A is selected from HEV71 and *Coxsackievirus* A16.

37. The expression cassette of Claim 35, wherein the *Human Enterovirus* C is selected from Human poliovirus 1 (PV-1), Human poliovirus 2 (PV-2) and Human poliovirus 3 (PV-3).

38. The expression cassette of Claim 30, wherein the IRES is derived from *Encephalomyocarditis virus* (EMCV).

39. The expression cassette of Claim 38, wherein the IRES derived from *Encephalomyocarditis virus* (EMCV) has been genetically modified to reduce IRES activity.

40. The expression cassette of Claim 39, wherein the IRES derived from EMCV has been genetically modified by adding one nucleotide (A7) to the A6 bifurcation loop in the JK segment.

41. The expression cassette of Claim 30, wherein the IRES is derived from a human *Enterovirus*.

42. The expression cassette of Claim 30, wherein the promoter is a eukaryotic promoter.

43. The expression cassette of Claim 42, wherein the eukaryotic promoter is a polyhedrin promoter.

44. The expression cassette of Claim 30, wherein the promoter is operably linked to a nucleic acid encoding a *Human enterovirus A* P1 polypeptide, an EMCV IRES, and a *Human enterovirus A* 3CD protease.

45. The expression cassette of Claim 44, wherein the EMCV IRES has been mutated to reduce IRES activity.

46. The expression cassette of Claim 30, wherein the promoter is operably linked to a nucleic acid encoding a *Human enterovirus A* P1 polypeptide, an HEV71 IRES, and a *Human enterovirus A* 3CD protease.

47. The expression cassette of Claim 30, wherein the promoter is operably linked to a nucleic acid encoding a *Human enterovirus A* P1 polypeptide, a *Human enterovirus C* IRES, and a *Human enterovirus A* 3CD protease.

48. The expression cassette of Claim 30, wherein the promoter is operably linked to a nucleic acid encoding a *Human enterovirus C* P1 polypeptide, a *Human enterovirus C* IRES, and a *Human enterovirus C* 3CD protease.

49. The expression cassette of Claim 30, wherein the promoter is operably linked to a nucleic acid encoding a *Human enterovirus C* P1 polypeptide, an HEV71IRES, and a *Human enterovirus C* 3CD protease.

50. The expression cassette of Claim 30, wherein the promoter is operably linked to a nucleic acid encoding a *Human enterovirus C* P1 polypeptide, an EMCV IRES, and a *Human enterovirus C* 3CD protease.

51. A method of making a vaccine comprising introducing the expression cassette of Claim 30 into a host cell, culturing the host cell for a period of time sufficient to produce the polypeptides of the expression cassette and recovering human *Enterovirus* polypeptides from the host cell and/or culture supernatant.

52. The method of Claim 51, wherein the host cell is a eukaryotic cell.

53. The method of Claim 52, wherein the eukaryotic cell is selected from insect cells, mammalian cell lines and yeast cells.

54. The method of Claim 53, wherein the insect cells are selected from *Spodoptera frugiperda*, *Trichoplusia ni*, drosophila, and mosquito cells derived from *Aedes albopictus*.

55. The method of Claim 53, wherein the mammalian cell lines are selected from CHO, HEK 293, COS-1, HeLa, Vero and NIH3T3.

Figure 1. HEV71 VLP expression cassette [P1+IRES+3CD] and the pSN01 plasmid.

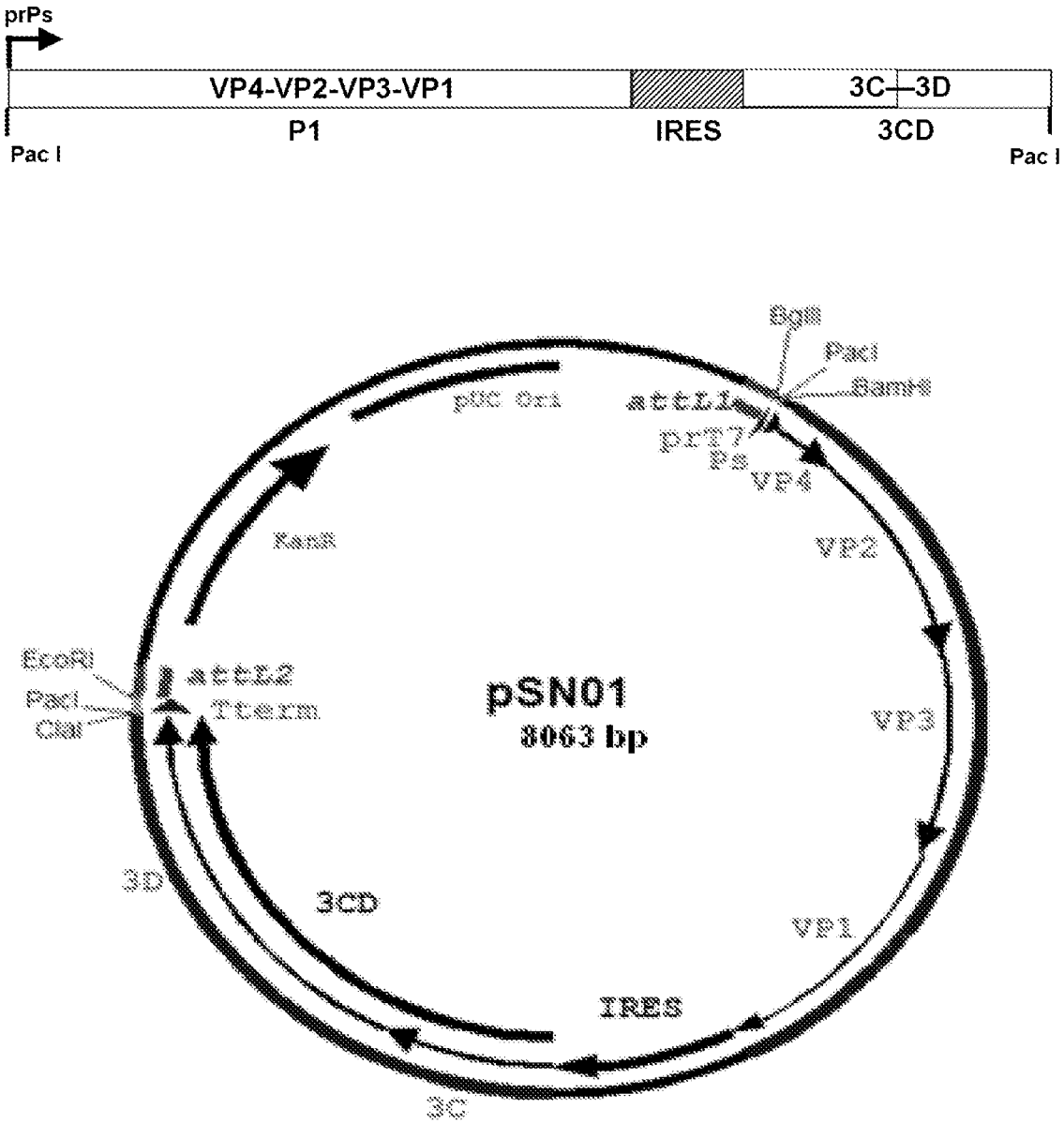


Figure 2. HEV71 VLP expression cassette [P1+IRES+3C] and the pSN03 plasmid.

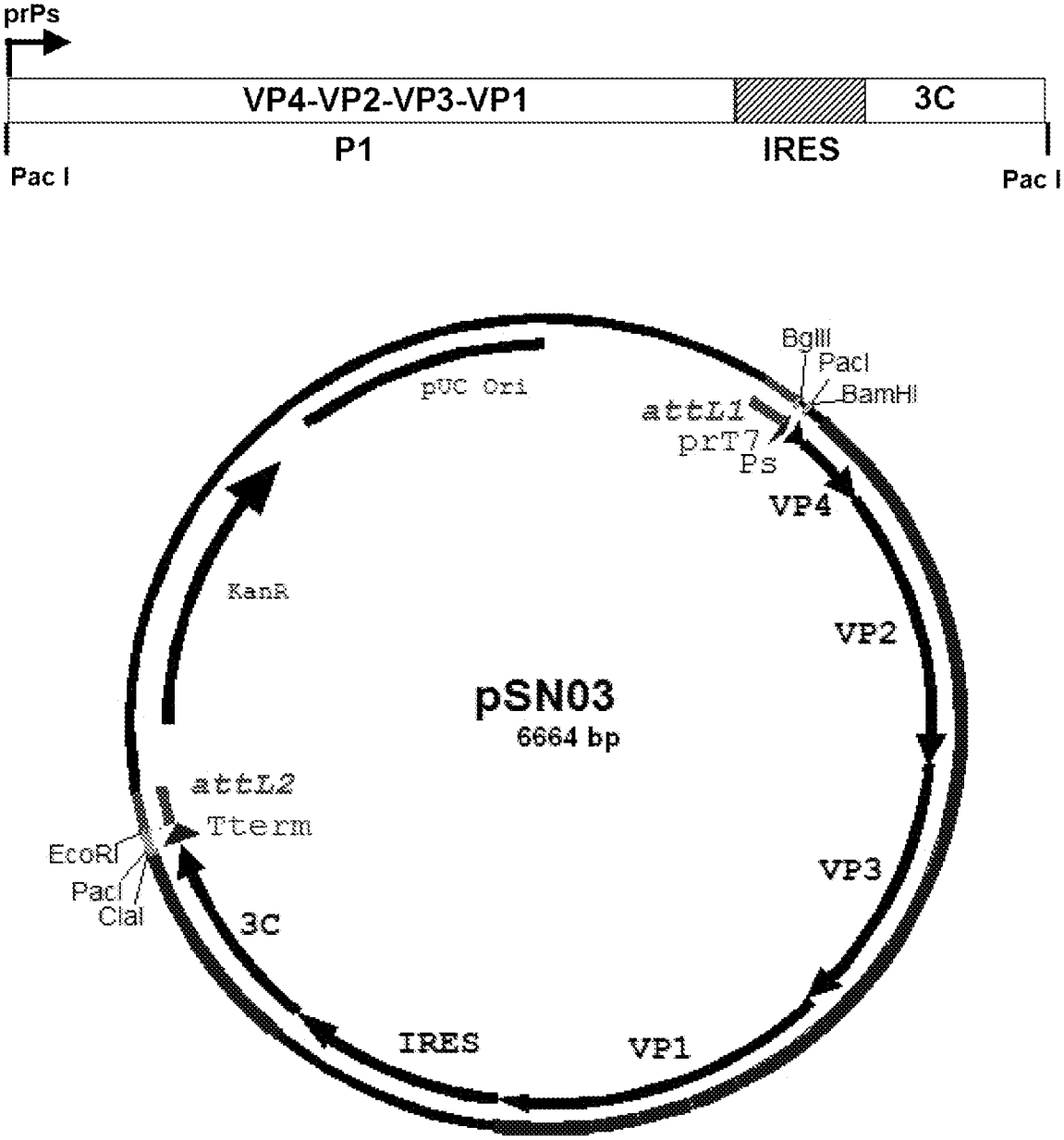


Figure 3. Expression of VP1 in supernatant of SN07 infected Sf9 cells.

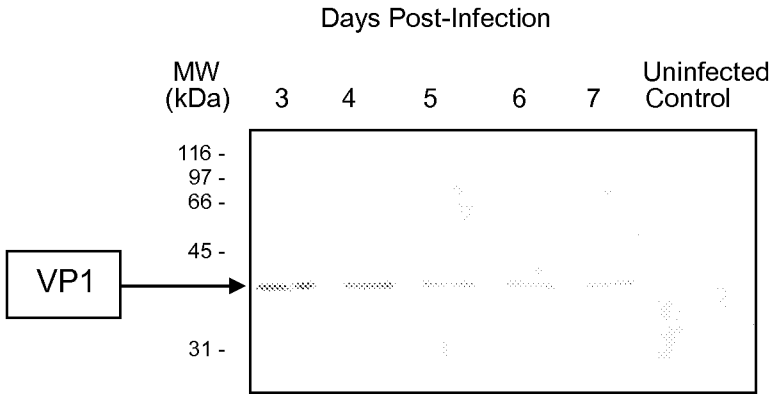


Figure 4. Processed VP1 in both the supernatants and the lysates.

Lanes 1 and 5: bacSN07p3

Lanes 2 and 6: bacSN08p3

Lanes 3 and 7: bacGUSd2

Lanes 4 and 8: Sf9 control

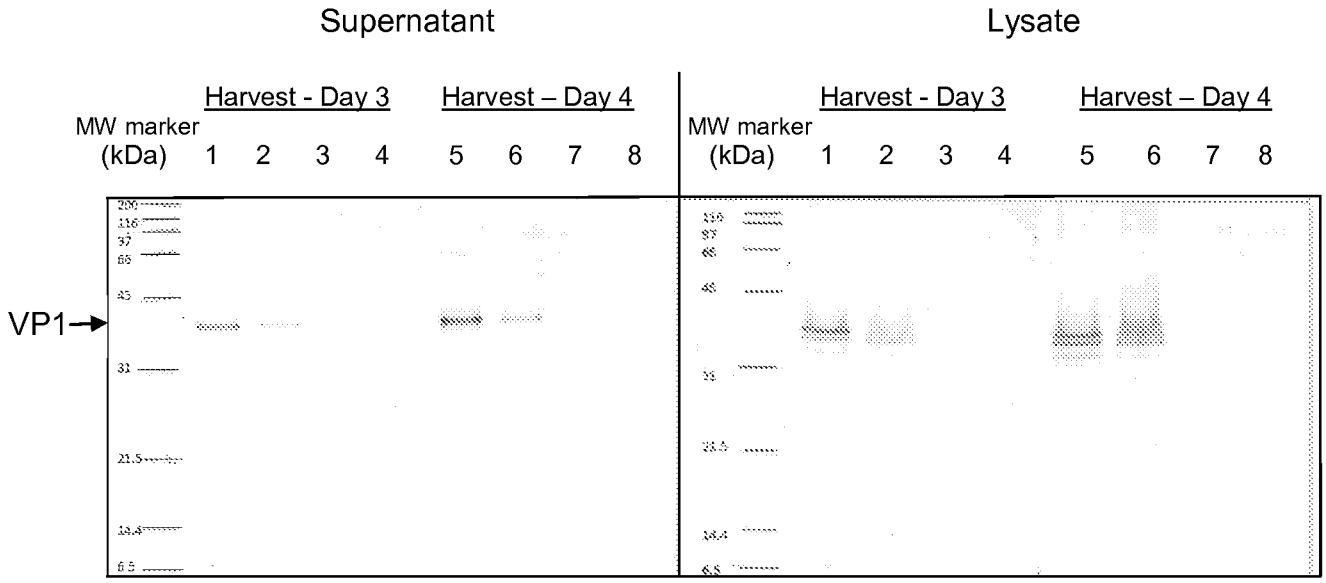


Figure 5. VP1 and VP0 are in the retentate after ultrafiltration over a 100kDa molecular weight cut off (MWCO) membrane.

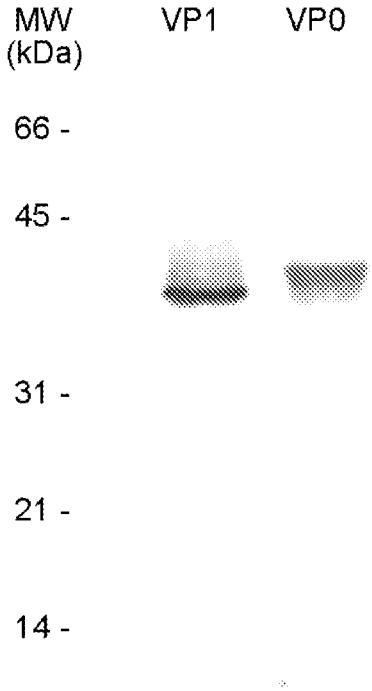


Figure 6.

FIRST PROOF OF CONCEPT OF OUR STRATEGY:
TWO IMMUNIZATION SCHEDULES (4 mice per group).

The immunogen was prepared in Freund's Complete Adjuvant (FCA) or Freund's Incomplete Adjuvant (FIA) and administered via subcutaneous injection (sc) or intraperitoneal injection (ip).

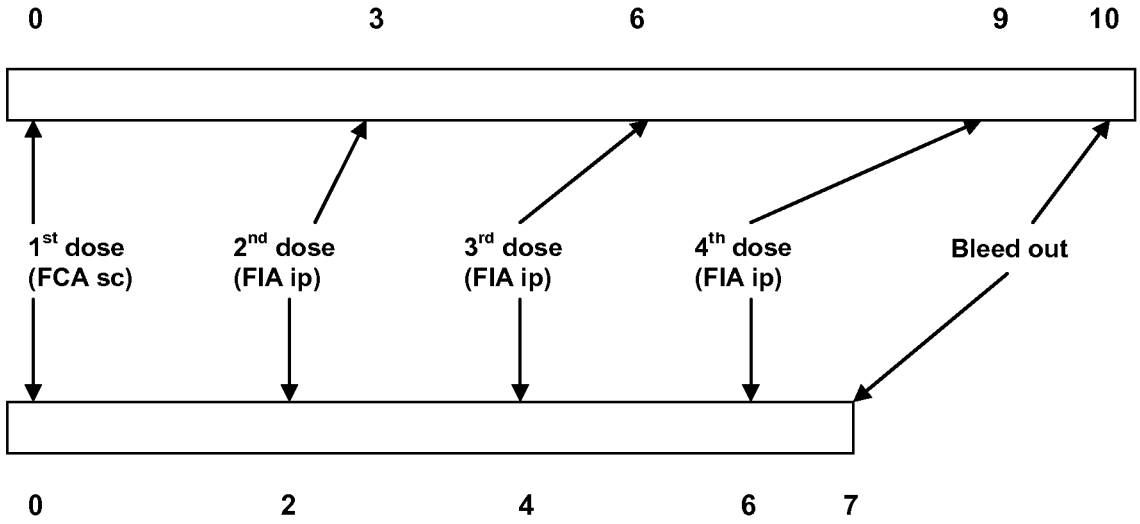


Figure 7. Immunoblots probed with rabbit polyclonal antisera against VP0 (arrow) at 3 different times of harvest.

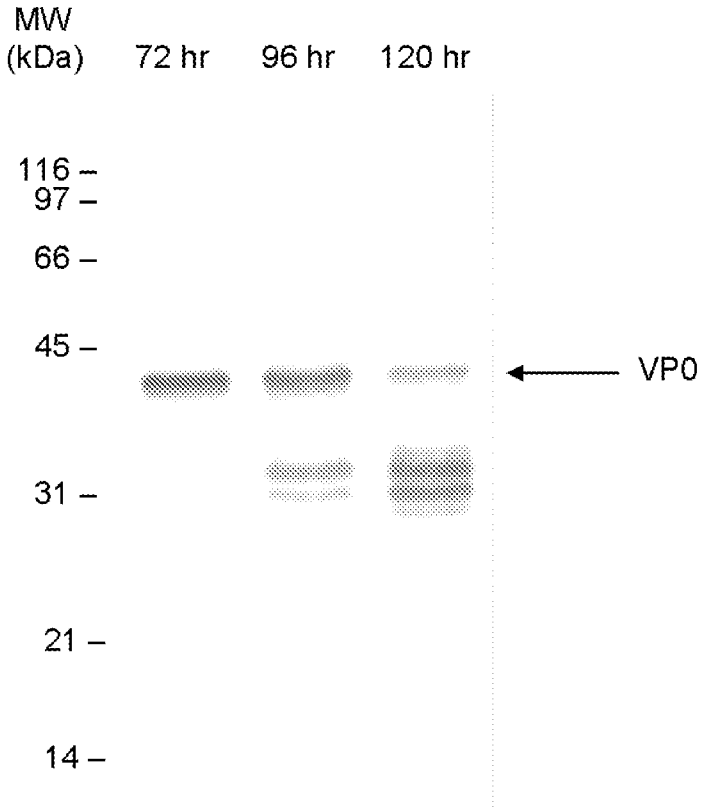


Figure 8. Pooled neutralizing sera from mice immunized with the oligomeric antigens in the supernatant of SN07 infected Sf9 cells have high titres against recombinant VP2 in ELISA.

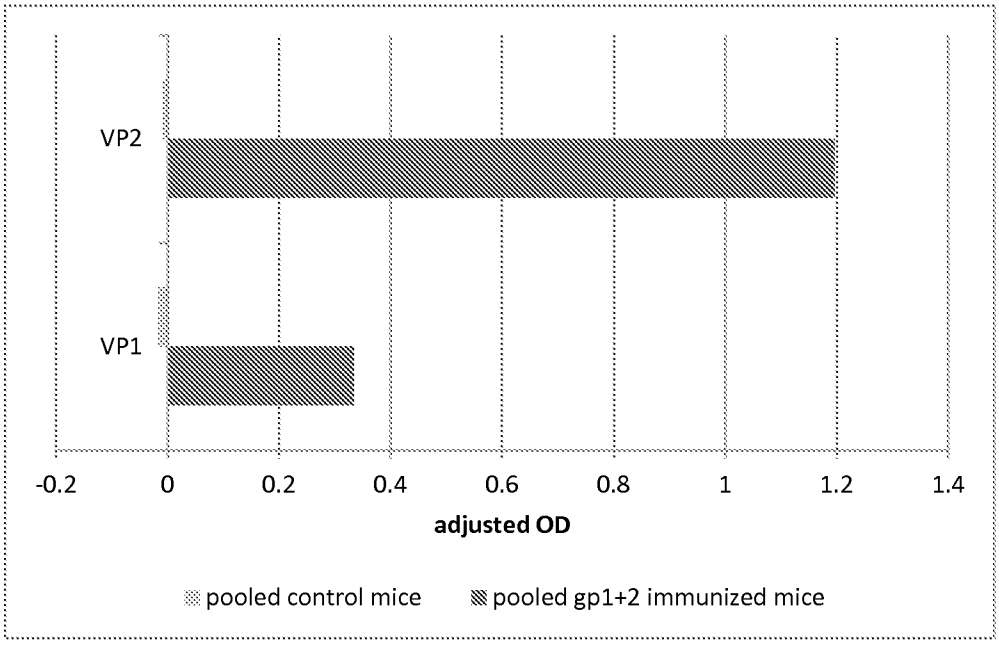
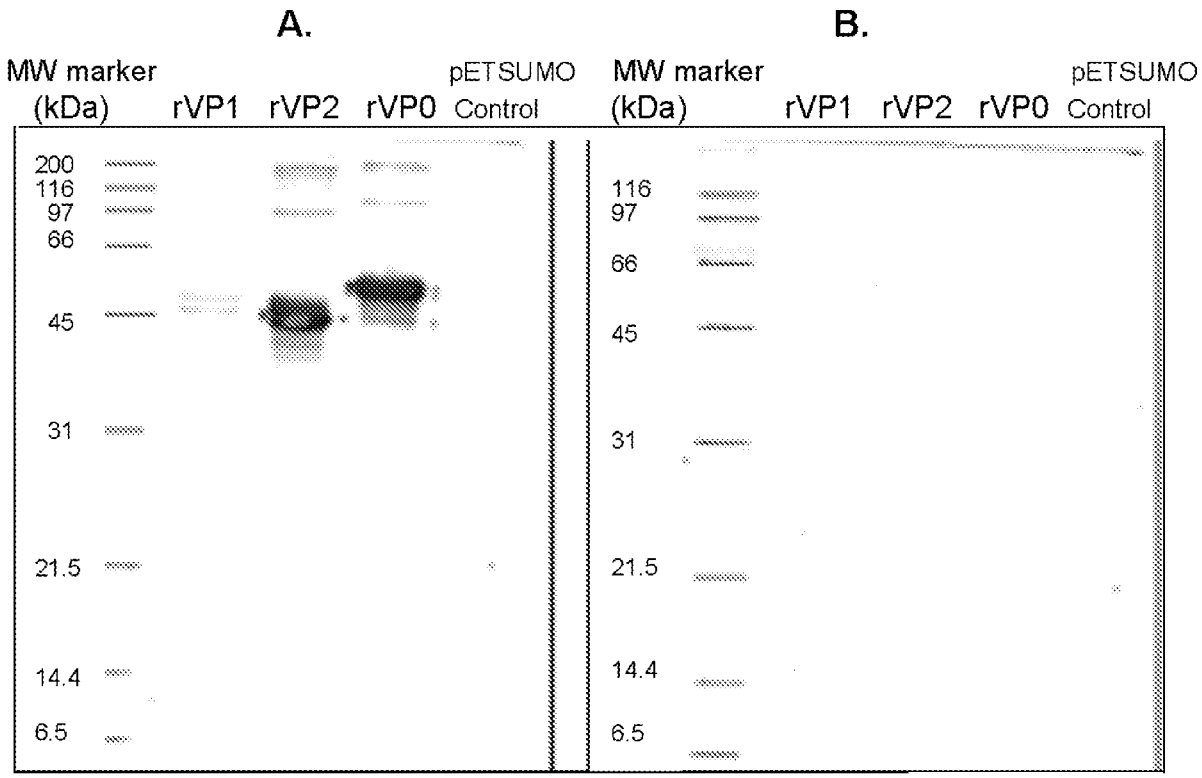


Figure 9. Pooled neutralizing sera from mice immunized with oligomeric antigens in the supernatant of SN07 infected Sf9 cells bind more strongly to VP2 and VP0 than to VP1.



A. Probed with mice serum anti-bacSN07p3 VLPs at 1/2000 dilution.
B. Probed with mice serum anti-SF9 control cells at 1/500 dilution.

Figure 10. IRES region of the EMCV genome (SEQ ID NO:1). The IRES region represents the EMCV genomic sequence in GenBank accession number AF113968.2 ; nucleotides1666 to 2251.

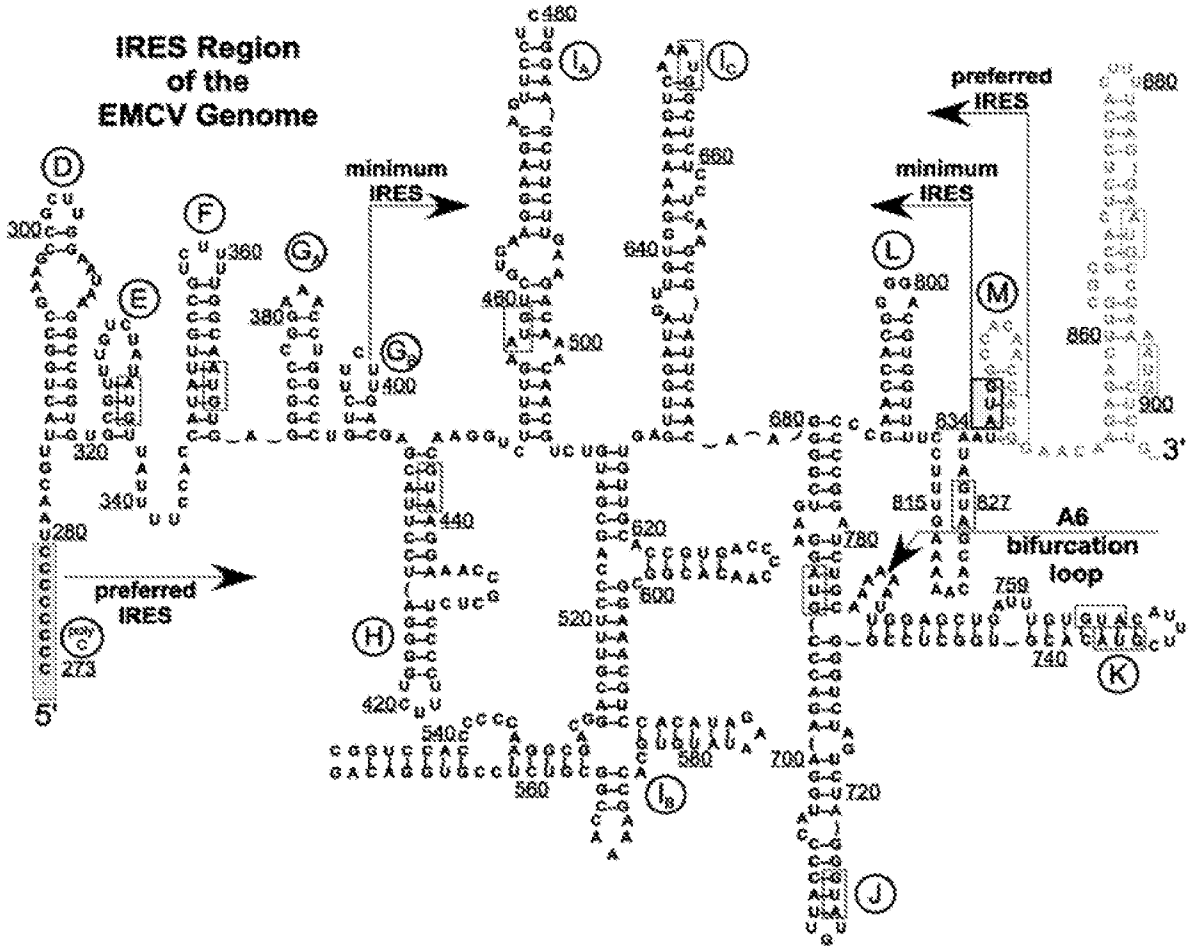


Figure 11. Out framing of the EMCV start codon with the 3CD protease coding sequence; Native EMCV IRES coding sequence (SEQ ID NO:2) is compared to a mutant EMCV IRES sequence coding sequence (SEQ ID NO:3).

Native AUGAUAAU---**AUG** aCu uCg Aaa gUu uAu gAu cca gaa caa

Mutant AUGAUAAgcuugccacaacccgggauccucuagagucgac**AUG** acu ucg

Figure 12. Plasmid pSN01-M1.

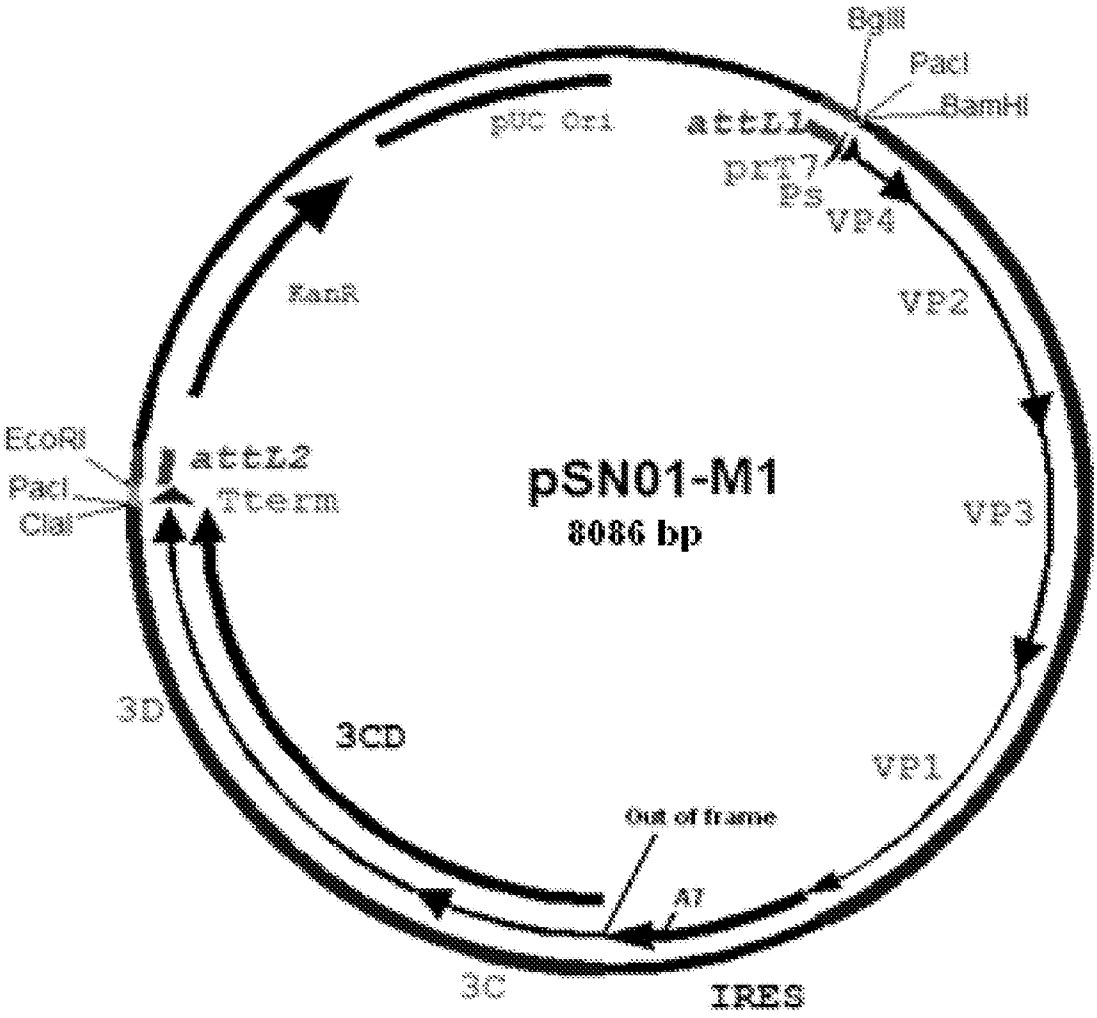


Figure 13. Plasmid pSN01-M2

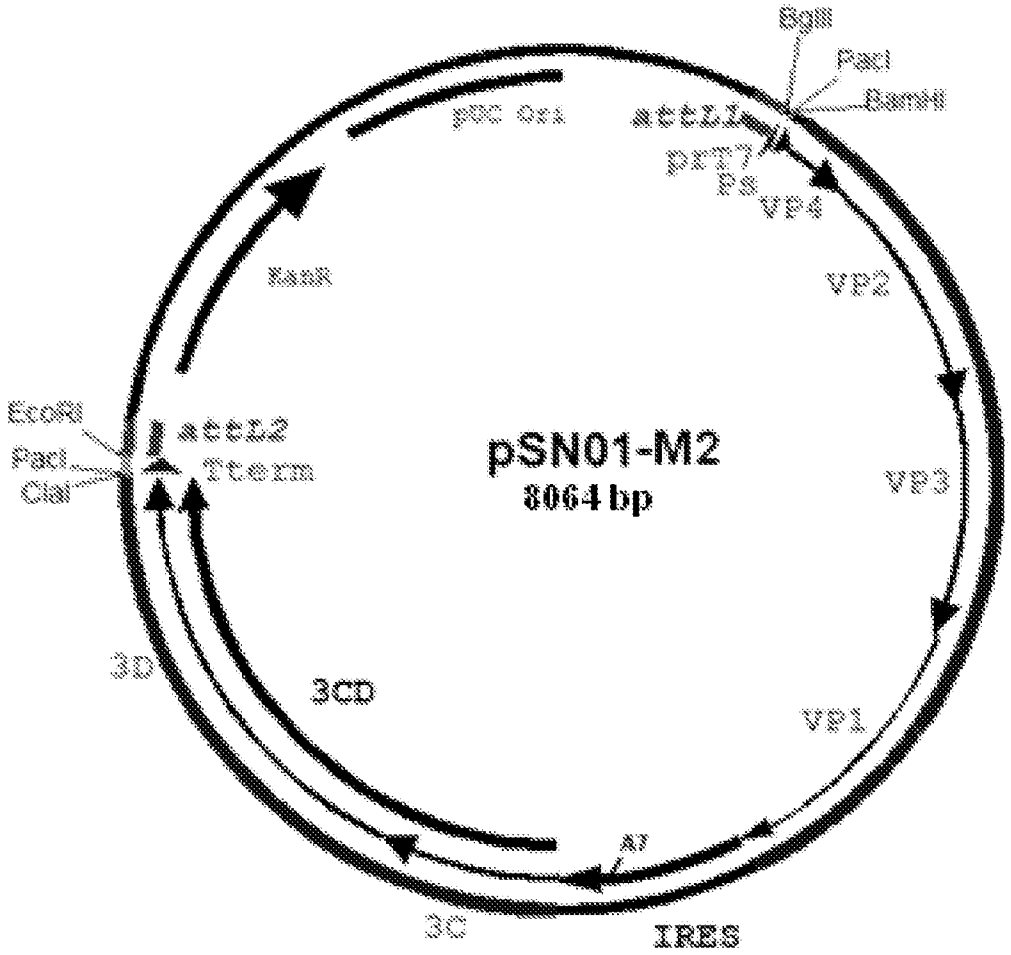


Figure 14. Plasmid pSN01-M3.

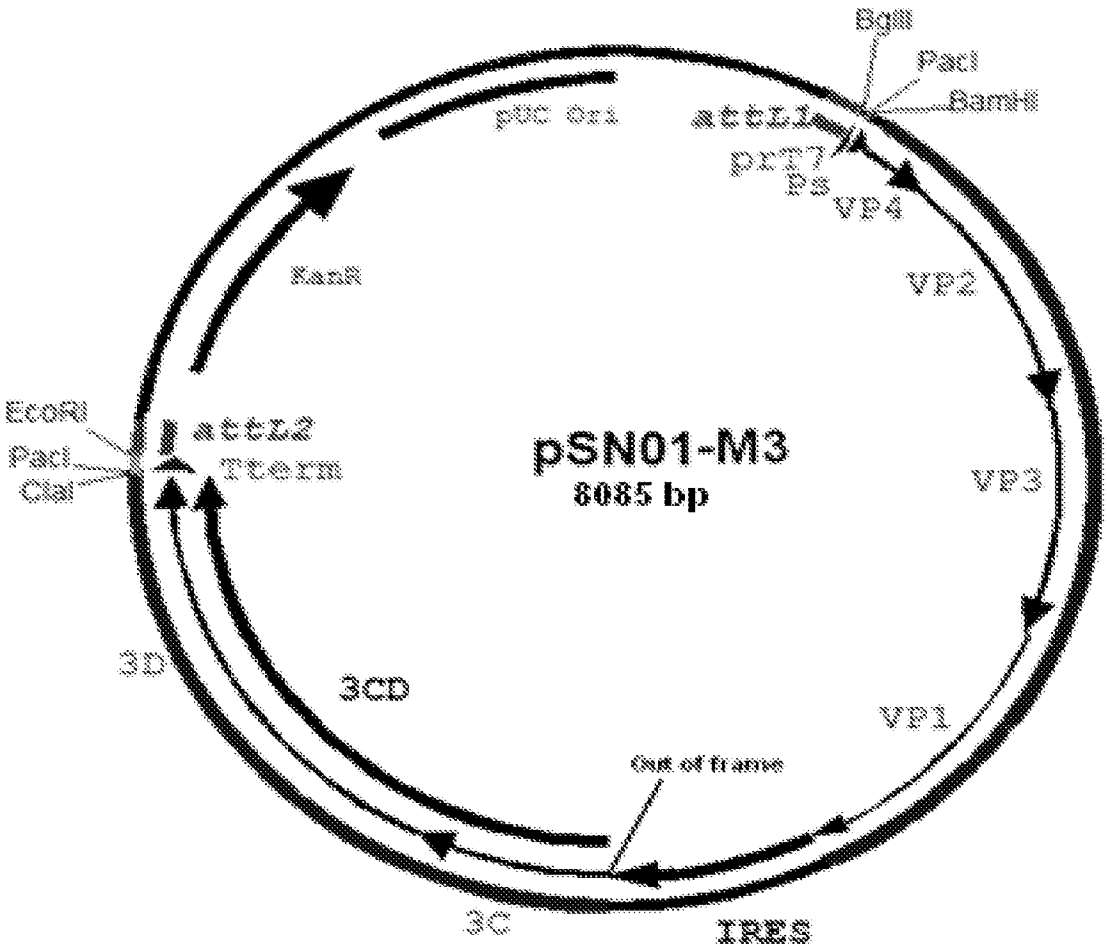


Figure 15. Comparison of expression of different recombinant baculovirus designs for expression of HEV71 VLP's.

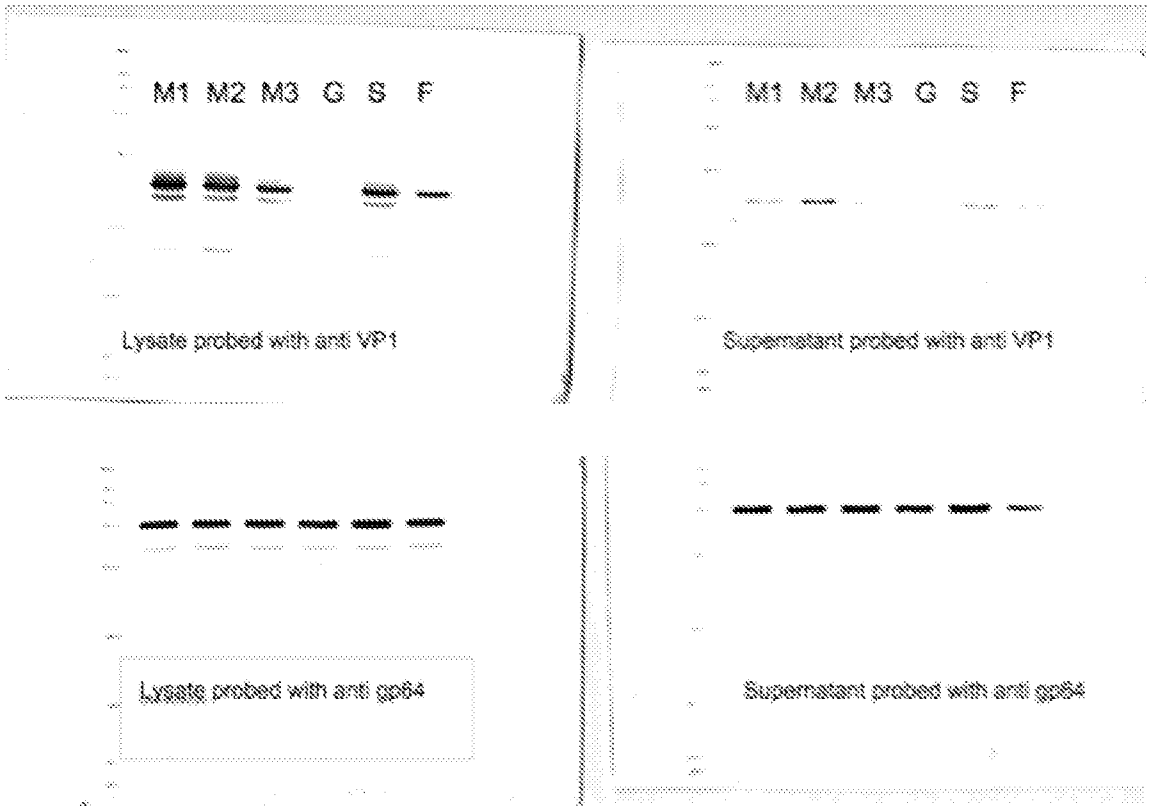


Figure 16. Plasmid pFastBac™ HT

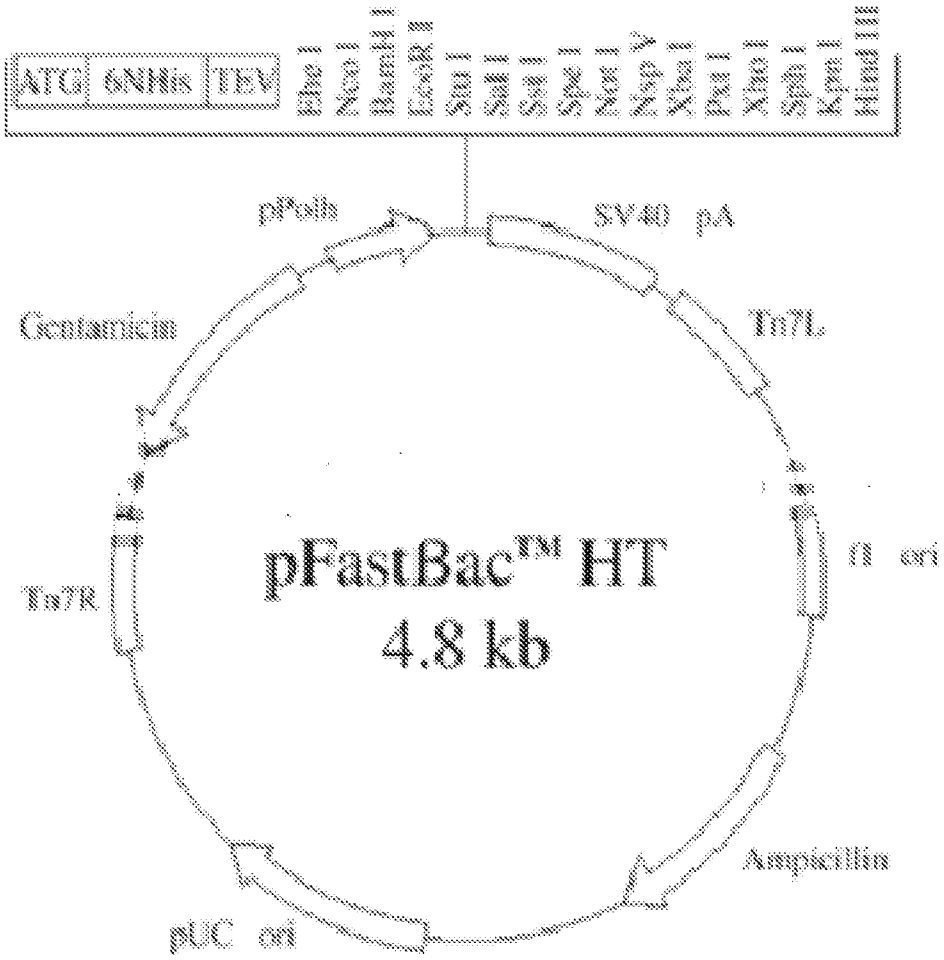


Figure 17. Prokaryotic expression construct for antigenic fusion proteins of *Human enterovirus A* and *Human enterovirus C*.

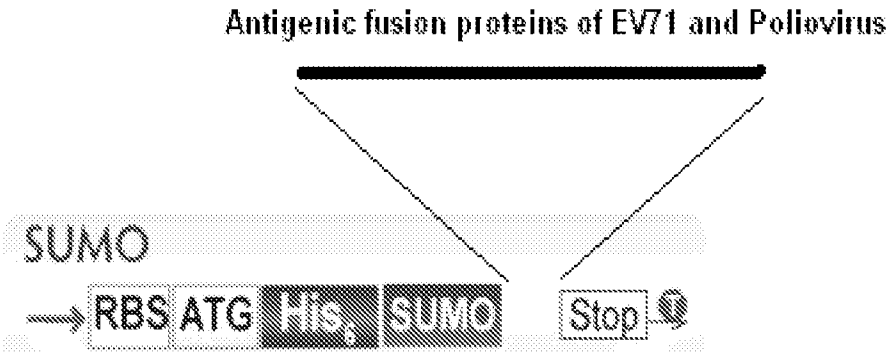


Figure 18. Antibodies from pooled neutralizing sera from mice immunized with SN07 retentate binds to all the components of HEV71 VLPs.

1. 1 ug of rEV71-VP0
2. 1 ug of rEV71-VP1
3. 1 ug of rEV71-VP2
4. 1 ug of rEV71-VP3

Figure 19. Characterization of HEV71 VLPs, pull-down of HEV71 VLPs from the culture supernatant. The VLP components (VP0, VP1 and VP3) are visible in the Coomassie stained SDS-PAGE gel. (MW- molecular weight marker; CB- Coomassie blue staining)

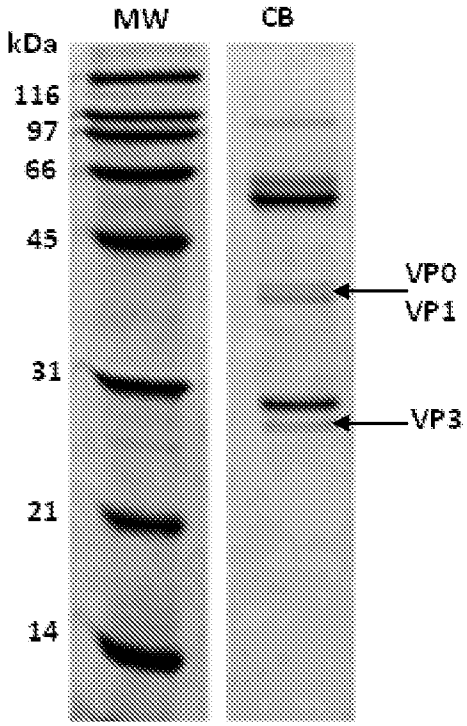


Figure 20. Analysis of affinity column (AFC) purified HEV71 VLPs.
AFC was prepared with a neutralizing monoclonal antibody (EV18/4/D6-1/F1/G9), which monoclonal antibody was developed using a SN07 retentate. The eluted fraction was analyzed by Western blotting. The eluted fraction in Lanes 1 and 2, probed using an anti-VP1 antibody, shows that VP1 is present in the AFC purified VLPs. The eluted fraction in Lanes 3 and 4, probed using an anti-VP2 antibody, shows that VP2 is also present in the AFC purified VLPs. The eluted fraction in Lanes 5 and 6, probed using an anti-VP2 monoclonal antibody, shows that VP2 is present in the AFC purified VLPs.

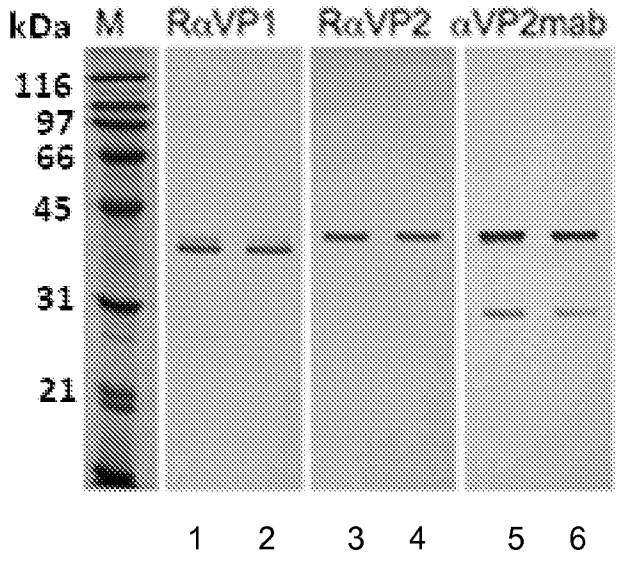


Figure 21. An electron micrograph picture of AFC purified HEV71 VLPs. AFC was prepared with a neutralizing monoclonal antibody (EV18/4/D6-1/F1/G9), which monoclonal antibody was developed using a SN07 retentate. The eluted fraction was analyzed by Western blotting (Figure 20) and electron microscopy. The arrows point to virus-like particles.

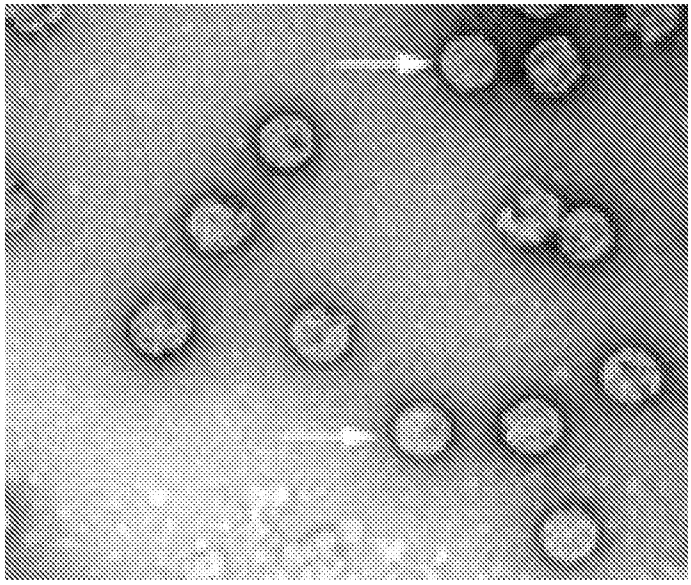
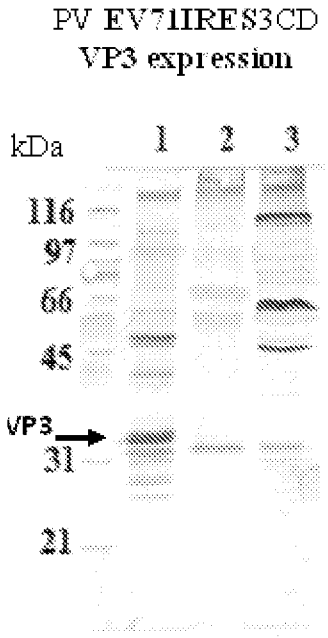
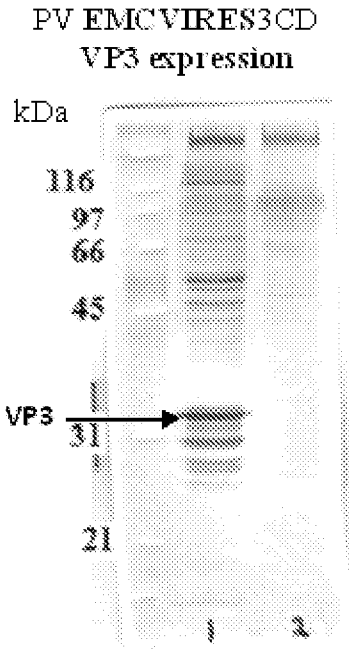


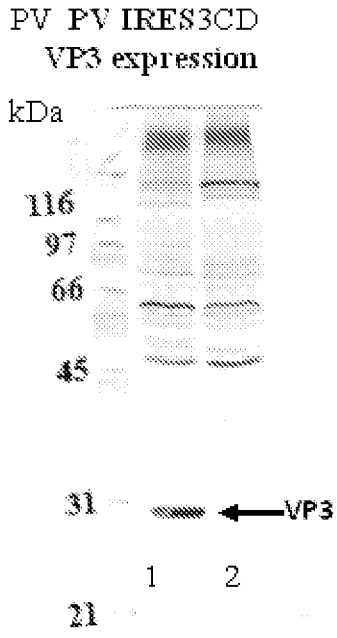
Figure 22. *Human enterovirus C* (poliovirus-PV) VL expression. Three different expression cassettes to generate poliovirus VLPs were constructed. The expression cassettes all comprise a poliovirus P1 polypeptide and differed with respect to the IRES, which IRES directs the expression of a poliovirus 3CD protease. Recombinant baculoviruses harboring the poliovirus VLP expression cassettes were tested. Lysates from the baculovirus infected cells were harvested on day 3 post infection and expression of the poliovirus VP3 was evaluated using rabbit anti-PVP3 antibodies (1:2000).



1. HEV71IRES-lysate
2. HEV71IRES-supernatant
3- bacGus-lysate



1. EMCV IRES-lysate
2. bacGus-lysate



1. PV IRES-lysate
2. bacGus-lysate

Figure 23. Poliovirus-VLP VP3-VP1 ELISA. To demonstrate poliovirus VLP generation, a two sites ELISA was performed using lysates and supernatants from recombinant baculoviruses carrying a poliovirus VLP expression cassette wherein the poliovirus 3CD protease is under the control of a poliovirus IRES. Purified rabbit anti-poliovirus VP3 antibodies were used as capture antibodies. Poliovirus VLPs were detected using mouse anti-VP1monoclonal antibodies.

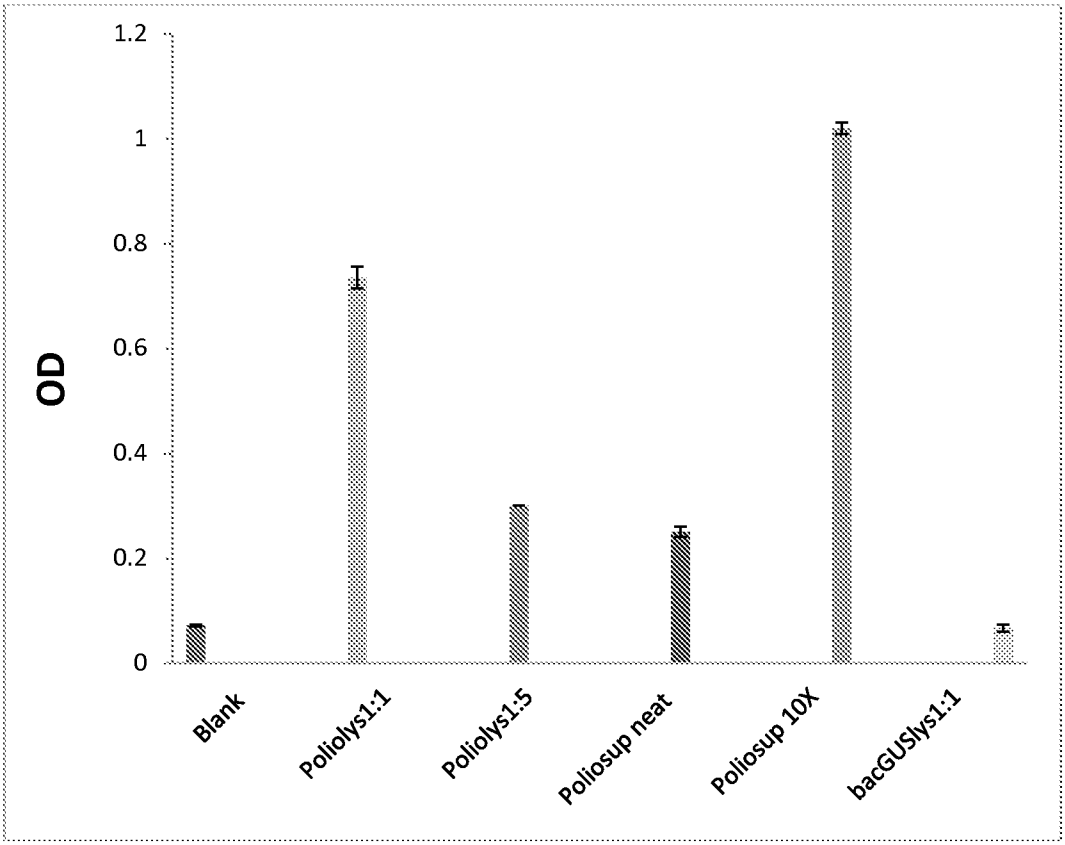


Figure 24. HEV71 VLP Expression Cassette with HEV71 IRES (P1+HEV71 IRES+3CD).

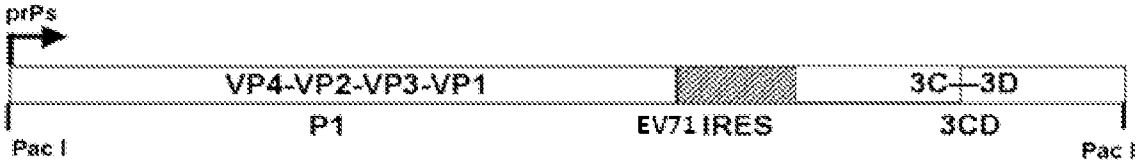
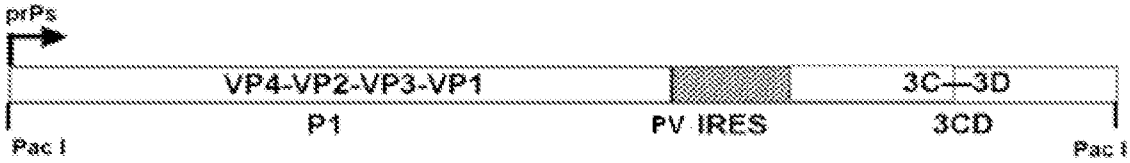


Figure 25. HEV71 VLP Expression Cassette with Poliovirus (PV) IRES (P1+PV IRES+3CD).



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ANTIGENS AND VACCINES DIRECTED AGAINST HUMAN ENTEROVIRUSES

FIELD OF THE INVENTION

[0001] This invention relates to viruses of the *Picornaviridae* family, and in particular antigens and vaccines that may be effective in preventing and treating infections caused by such viruses.

BACKGROUND OF THE INVENTION

[0002] Picornaviruses are a diverse family of viruses which cause a number of common illnesses. Of the *Picornaviridae* family, viruses of the genus *Enterovirus*, which are all very closely related, are significant for the number of diseases they cause.

[0003] Viruses of the genus *Enterovirus* affect millions of people worldwide each year, and are often found in the respiratory secretions (e.g., saliva, sputum, or nasal mucus) and stool of an infected person. *Enterovirus* infects the gut, thus the derivation of their name from the root “enteric”. Historically, poliomyelitis was the most significant disease caused by an enterovirus, that is, poliovirus. There are 62 non-polio enteroviruses that can cause disease in humans: 23 Coxsackie A viruses, 6 Coxsackie B viruses, 28 echoviruses, and 5 other enteroviruses. Polioviruses, as well as Coxsackie viruses and echoviruses, are spread through the fecal-oral route. Infection can result in a wide variety of symptoms ranging from mild respiratory illness (common cold), hand, foot and mouth disease, acute hemorrhagic conjunctivitis, aseptic meningitis, myocarditis, severe neonatal sepsis-like disease, and acute flaccid paralysis.

[0004] Of the picornaviruses, *Enterovirus* represents a genus of a large and diverse group of small RNA viruses characterized by a single positive-strand genomic RNA. All enteroviruses contain a genome of approximately 7,500 bases and are known to have a high mutation rate due to low-fidelity replication and frequent recombination. After infection of the host cell, the genome is translated in a cap-independent manner into a single polyprotein, which is subsequently processed by virus-encoded proteases into

the structural capsid proteins and the nonstructural proteins, which are mainly involved in the replication of the virus.

[0005] The enteroviruses are associated with several human and mammalian diseases. Serologic studies have distinguished 66 human *Enterovirus* serotypes on the basis of antibody neutralization tests. Additional antigenic variants have been defined within several of the serotypes on the basis of reduced or nonreciprocal cross-neutralization between variant strains. On the basis of their pathogenesis in humans and animals, enteroviruses were originally classified into four groups, polioviruses, Coxsackie A viruses (CA), Coxsackie B viruses (CB), and echoviruses, but it was quickly realized that there were significant overlaps in the biological properties of viruses in the different groups.

[0006] The *Enterovirus* genus includes the following ten species:

- *Bovine enterovirus*
- *Human enterovirus A*
- *Human enterovirus B*
- *Human enterovirus C*
- *Human enterovirus D*
- *Human rhinovirus A*
- *Human rhinovirus B*
- *Human rhinovirus C*
- *Porcine enterovirus B*
- *Simian enterovirus A*

[0007] Within these ten species are there are various serotypes, for example:

Enterovirus serotypes HEV71, EV-76, EV-89, EV-90, EV-91, EV-92 and Coxsackievirus A16 are found under the species *Human enterovirus A*.

Serotypes *Coxsackievirus* B1 (CV-B1), CV-B2, CV-B3, CV-B4, CV-B5 (incl. swine vesicular disease virus [SVDV]), CV-B6, CV-A9, echovirus 1 (E-1; incl. E-8), E-2, E-3, E-4, E-5, E-6, E-7, E-9 (including CV-A23), E-11,

E-12, E-13, E-14, E-15, E-16, E-17, E-18, E-19, E-20, E-21, E-24, E-25, E-26, E-27, E-29, E-30, E-31, E-32, E-33, enterovirus B69 (EV-B69), EV-B73, EV-B74, EV-B75, EV-B77, EV-B78, EV-B79, EV-B80, EV-B81, EV-B82, EV-B83, EV-B84, EV-B85, EV-B86, EV-B87, EV-B88, EV-B93, EV-B97, EV-B98, EV-B100, EV-B101, EV-B106, EV-B107, EV-B110 (from a chimpanzee) and the simian enterovirus SA5, are found under the species *Human enterovirus B*.

Serotypes EV-95, EV-96, EV-99, EV-102, EV-104, EV-105, and EV-109 are found under the species *Human enterovirus C.*

Serotypes EV-68, EV-70, & EV-94 are found under the species *Human enterovirus D*.

Poliovirus serotypes PV-1, PV-2, and PV-3 are found under the species *Human enterovirus C*.

[0008] Diseases caused by enterovirus infection include poliomyelitis which is the most notable disease caused by an enterovirus infection. Nonspecific febrile illness is, however, the most common presentation of an enterovirus infection.

[0009] Enteroviruses are the most common causes of aseptic meningitis in children. In the United States, enteroviruses are responsible for 30,000 to 50,000 cases of meningitis. Encephalitis is a rare manifestation of an enterovirus infection; when it occurs, the most frequent *Enterovirus* found to be causing the encephalitis is echovirus 9.

[0010] Pleurodynia caused by enteroviruses is characterized by severe paroxysmal pain in the chest and abdomen, along with fever, and sometimes nausea, headache, and emesis.

[0011] Pericarditis and/or myocarditis are typically caused by enteroviruses. Arrythmias, heart failure, and myocardial infarction have also been reported.

[0012] Acute hemorrhagic conjunctivitis can be caused by enteroviruses.

[0013] Hand, foot and mouth disease is a childhood illness most commonly caused by infection by Coxsackie A virus or HEV71.

[0014] A 2007 study suggested that acute respiratory or gastrointestinal infections associated with enteroviruses may be a factor in chronic fatigue syndrome.

[0015] All members of the genus *Enterovirus*, including HEV71, polioviruses and *Coxsackievirus* A16 have a single stranded positive sense RNA genome which has a single open reading frame encoding a polyprotein, P1, consisting of the capsid proteins VP4, VP2, VP3 and VP1 and several non-structural proteins including the viral proteases 3C and 3CD which are responsible for cleaving the polyprotein P1 into individual capsid proteins VP1, VP3 and VP0, which VP0 is eventually cleaved into VP2 and VP4. The capsid proteins may assemble into virus like particles (VLPs).

[0016] *Human enterovirus* 71 (HEV71) and *Coxsackievirus* A16 are *Enterovirus* serotypes notable as the major causative agents for hand, foot and mouth disease (HFMD), and HEV71 is sometimes associated with severe central nervous system diseases. HEV71 was first isolated and characterized from cases of neurological

disease in California in 1969. To date, little is known about the molecular mechanisms of host response to HEV71 infection, but increases in the level of mRNAs encoding chemokines, proteins involved in protein degradation, complement proteins, and pro-apoptotic proteins have been implicated.

[0017] Hand Foot and Mouth Disease (HFMD) is a common, self-limiting illness of children caused by a group of species A enteroviruses (Picornaviridae family) such as human *Coxsackievirus* A16 (CVA16), *Coxsackievirus* A10 (CVA10) and *Human enterovirus A* 71 (HEV71). The virus is excreted in feces and is also found in pharyngeal secretions. Transmission is associated with close contact among children and through environmental contamination. The disease is characterized by an acute onset of fever with a rash on the palms, soles, buttocks, and knees, and vesicles on buccal membranes that usually resolve in 7-10 days. Only a small proportion of children with HFMD develop severe disease.

[0018] Severe disease involving primarily the neurologic and cardiovascular systems manifesting as syndromes such as meningitis, encephalitis, acute flaccid paralysis, pulmonary edema and cardiac failure generally occur only with HEV71 infection. In the Asia-Pacific Region the most devastating neurological syndrome is brainstem encephalitis, which has a mortality rate of 40-80 percent. Children with severe HFMD may take months to recover, and in some cases the neurologic damage may be permanent. Currently, there is no specific antiviral treatment for HFMD and no vaccines to prevent enterovirus infection other than polio.

[0019] HEV71 was first isolated from a child who died of encephalitis in California in 1969, and first reported in 1974. Although the virus has been detected worldwide since then, the recent regional epidemics of HFMD in Asia has raised concern that more pathogenic forms of HEV71 may be emerging in the region. The first recognition of a HFMD outbreak with a high number of fatalities was in Sarawak, Malaysia in 1997. The

virus associated with the outbreak then was HEV71. Taiwan reported 129,106 HFMD cases in a 1998 epidemic with 405 having severe disease with 78 deaths. Singapore reported an epidemic of 9000 cases with 7 deaths during 2000-2001, and since then has experienced recurrent epidemics every two to three years. During the first 8 months of 2008, Singapore reported 19,530 cases and one death due to HFMD. Since then HEV71 outbreaks have been reported regularly in Singapore, Thailand, Malaysia, Taiwan, Japan, Korea and Vietnam.

[0020] China reported 83,344 cases with 17 deaths in 2007, and in 2008 experienced a large outbreak in Fuyang City in Anhui Province spreading throughout many parts of China. These large outbreaks were widely covered by the press, which highlighted parental concerns about the health of their children and the social disruption from closing of schools and day care centers by public health departments in an attempt to break the chain of transmission. Since then China has reported large outbreaks annually.

[0021] With regard to disease caused by other members of the *Picornaviridae* family, natural infection and prevalence of polio have occurred exclusively in the human being since ancient times as an infectious disease. A large number of humans still become infected with polio every year in developing countries. Hence, the eradication of polio is an ongoing process.

[0022] Polioviruses were formerly classified as a species belonging to the genus *Enterovirus* in the family *Picornaviridae*. The Poliovirus species has been eliminated from the genus *Enterovirus*. The poloviruses are classified as serotypes, Human poliovirus 1 (PV-1), Human poliovirus 2 (PV-2), and Human poliovirus 3 (PV-3), and are considered to be subtypes of species *Human enterovirus C*, in the genus *Enterovirus* in the family *Picornaviridae*. The type species of the genus *Enterovirus* was changed from Poliovirus to *Human enterovirus C* in 2008.

[0023] The three subtypes of species *Human enterovirus C*, PV-1, PV-2 and PV-3, are characterized by a slightly different capsid protein. Capsid proteins define cellular receptor specificity and virus antigenicity. PV-1 is the most common form encountered in nature; however, all three forms are extremely infectious and can affect the spinal cord and cause poliomyelitis.

[0024] Infection with *Human enterovirus C* has been a widespread problem and inactivated whole virus vaccines have been used for mass immunization and are currently available. Good results have been obtained with inactivated poliomyelitis vaccines which may be prepared according to a method which has been developed by Salk and has been improved later in several aspects. Generally, these vaccines contain a mixture of inactivated polio virus of strains Mahoney, MEF1 and Saukett.

[0025] Although an attenuated *Human enterovirus C* has been produced and used as an attenuated oral polio vaccine, the attenuated *Human enterovirus C* may be dangerous because of the possible reversion of pathogenicity (paralysis-based neurovirulence) in persons administered to, or in contact with, whole viruses. Hence, there is a need for a safe and effective polio vaccine which is free of such pathogenicity.

[0026] Like all enteroviruses, four different *Human enterovirus C* coat/capsid polypeptides have been identified and are designated as VP1, VP2, VP3 and VP4, which associate to form an icosahedral virus capsid. Typically, vaccination with the individual polypeptides of *Human enterovirus C* has shown that the isolated polypeptides are not capable of raising neutralizing antibodies in humans and animals (Meloen, et al., J. Gen. Virol. 45:761-763, 1979).

[0027] U.S. Patent No. 4,508,708 teaches that individual polypeptides of polio virus and hand, foot and mouth disease virus, VP1, VP2, VP3 and VP4, are not capable of raising neutralizing antibodies in humans and animals and that, among the individual polypeptides of the hand, foot and mouth disease virus, only VP1 possesses this

capability. U.S. Patent No. 4,508,708 demonstrates that, among the *Human enterovirus C* type 2 MEF1 virion VP1, VP2 and VP3 polypeptides, only the VP3 is capable of inducing neutralizing antibodies, although the antibody titer is low. It was found, however, that VP1, VP2 and VP3 are capable of inducing neutralizing antibodies only when the immunization is carried out with a preparation containing arildone, a broad spectrum antiviral agent that has been shown to selectively inhibit replication of picornaviruses (Langford, et al. Antimicrobial Agents and Chemotherapy 28:578-580, 1985).

[0028] Thus, the problem to be solved is the preparation of an effective vaccine which provides protective immunity against a human enterovirus infection, and without the use of antiviral compounds. The human enteroviruses for which protection is desired are, for example *Human enterovirus A*, including *Coxsackievirus* A16 and *Human enterovirus* 71; *Human enterovirus B*, including Coxsackievirus B serotypes, echoviruses and enterovirus serotypes; *Human enterovirus C*, including *Human poliovirus* 1, *Human poliovirus* 2 and *Human poliovirus* 3*;* as well as *Human enterovirus D*, including EV 68.

[0029] For the purposes of the instant invention, a vaccine is understood by those skilled in the art and may further be defined as a prophylactic or therapeutic material containing antigens derived from one more pathogenic organisms which, upon administration to a human subject or animal, will stimulate active immunity and protect against infection with these or related organisms (i.e., produce protective immunity).

[0030] Furthermore, protective immunity may be well understood by those skilled in the art. Nonetheless, protective immunity comprises, at least, the induction, or elicitation of neutralizing antibodies and/or T-cell immune response which will neutralize the virus.

[0031] It is well recognized in the vaccine art, that it is unclear whether an antigen derived from a pathogen will elicit protective immunity. Ellis (Chapter 29 of Vaccines, Plotkin, et al. (eds) WB Saunders, Philadelphia, at page 571,1998) exemplifies this

problem in the recitation that “the key to the problem (of vaccine development) is the identification of that protein component of a virus or microbial pathogen that itself can elicit the production of protective antibodies…, and thus protect the host against attack by the pathogen.”

[0032] An approach to making improved vaccines against picornaviruses would be to mimic the virus capsid structure or its components which may elicit protective antibodies such as are produced with a killed whole virus vaccine. This kind of approach is safer than a killed, inactivated or attenuated vaccine approach because there is no opportunity for reversion.

[0033] All picornaviruses share the same genomic structure, including 4 structural genes within the P1 gene: VP1, VP2, VP3, and VP4, the VP4 and VP2 being expressed together as VP0, and viral proteases within the 3C and 3D genes. The viral protease will cleave the P1 gene, thereby allowing the virus to assemble into virus like particles (VLPs), virus capsomers, complexes and/or antigens of enteroviruses.

[0034] Vaccines have been proposed with indifferent success. It has been proposed to use subunit vaccines comprising the major capsid protein, VP1, of enteroviruses, as the basis of vaccines for the prevention and treatment of enterovirus infections, including HEV71 infections (Wu, et al., 2001).

[0035] With regard to prophylaxis against enterovirus infection, it is possible to envisage a killed virus vaccine approach, which has been shown to elicit protective antibodies. The low virus titres achieved in general makes manufacturing of such enterovirus vaccines a challenge.

[0036] The present invention pertains to vaccines including antigenic coat/capsid proteins of viruses of the *Picornaviridae* family and which vaccines are devoid of virus RNA which may contribute to neurovirulence. The vaccines of the invention may comprise as antigens, the polypeptides P1 or VP0, or capsid proteins (VP's), designated

as VP1, VP2, VP3 and/or VP4, or immunologically or biologically active fragments thereof which elicit neutralizing antibodies against enteroviruses.

[0037] The present invention relates to vaccines in which the picornavirus antigens are present in the form of one or more picornavirus polypeptides, especially human *Enterovirus* peptides VP2, VP3 or VP0, immunogenic fragments thereof, and/or antigenic determinants thereof. The picornavirus polypeptides may be obtained by chemical synthesis or by means of recombinant DNA techniques using known human *Enterovirus* amino acid or nucleic acid sequences.

SUMMARY OF THE INVENTION

[0038] A vaccine comprising one or more immunologically active antigens comprising one or more human *Enterovirus* polypeptides selected from VP0, VP1, VP2, VP3, VP4, and immunologically active fragments thereof, such a

[0039] vaccine, which elicits a protective and/or neutralizing immune response directed against a human *Enterovirus*, such a

[0040] vaccine, wherein the human *Enterovirus* is selected from *Human enterovirus A, Human enterovirus B*, *Human enterovirus C* and *Human enterovirus D*, such a

[0041] vaccine, wherein the *Human enterovirus A* is selected from *Human enterovirus* 71 (HEV71) and *Coxsackievirus* A16, wherein the *Human enterovirus B* is selected from *Coxsackievirus* B and echovirus, wherein the *Human enterovirus C* is selected from *Human poliovirus* 1, *Human poliovirus* 2 and *Human poliovirus* 3, and wherein the *Human enterovirus D* is *EV* 68, such a

[0042] vaccine, wherein the vaccine comprises an immunologically active *Enterovirus* VP0 polypeptide, such a

[0043] vaccine, wherein the vaccine comprises an immunologically active *Enterovirus* VP2 polypeptide, such a

[0044] vaccine, wherein the vaccine comprises an immunologically active *Enterovirus* VP3 polypeptide, such a

[0045] vaccine, wherein the vaccine comprises an immunologically active *Enterovirus* VP1 polypeptide, such a

[0046] vaccine, wherein the vaccine comprises polypeptides from one or more *Enterovirus* species or serotype, such a

[0047] vaccine, wherein the species or serotype may be *Human enterovirus A* selected from HEV71 and *Coxsackievirus* A16, such a

[0048] vaccine, wherein the species or serotype may be *Human enterovirus C* selected from PV-1, PV-2 and PV-3, such a

[0049] vaccine, wherein the one or more immunologically active antigens comprising one or more human *Enterovirus* polypeptides selected from VP0, VP1, VP2, VP3, VP4, and immunologically active fragments thereof, are in the form of a virus-like particle, capsomer, complex and/or aggregate, such a

[0050] vaccine, wherein the virus-like particle comprises an immunologically active *Enterovirus* VP0 polypeptide, such a

[0051] vaccine, wherein the virus-like particle comprises an immunologically active *Enterovirus* VP2 polypeptide, such a

[0052] vaccine, wherein the virus-like particle comprises an immunologically active *Enterovirus* VP3 polypeptide, such a

[0053] vaccine, wherein the virus-like particle comprises an immunologically active *Enterovirus* VP1 polypeptide, a

[0054] method of vaccinating a subject against an *Enterovirus* infection, comprising administering to the subject a vaccine comprising one or more immunologically active antigens comprising one or more human *Enterovirus* polypeptides selected from VP0, VP1, VP2, VP3, VP4, and immunologically active fragments thereof, in an amount effective to elicit a protective and/or neutralizing immune response when administered to the subject, such a

[0055] method, wherein the vaccine comprises an immunologically active *Enterovirus* VP0 polypeptide, such a

[0056] method, wherein the vaccine comprises an immunologically active *Enterovirus* VP1 polypeptide, such a

[0057] method, wherein the vaccine comprises an immunologically active *Enterovirus* VP3 polypeptide, such a

[0058] method, wherein the vaccine comprises an immunologically active *Enterovirus* VP2 polypeptide, such a

[0059] method, wherein the one or more immunologically active antigens comprising one or more human *Enterovirus* polypeptides selected from VP0, VP1, VP2, VP3, VP4, and immunologically active fragments thereof, are in the form of a virus-like particle, capsomer, complex and/or aggregate, such a

[0060] method, wherein the virus-like particle comprises an immunologically active *Enterovirus* VP0 polypeptide, such a

[0061] method, wherein the virus-like particle comprises an immunologically active *Enterovirus* VP2 polypeptide, such a

[0062] method, wherein the virus-like particle comprises an immunologically active *Enterovirus* VP3 polypeptide, such a

[0063] method, wherein the virus-like particle comprises an immunologically active *Enterovirus* VP1 polypeptide, such a

[0064] method, wherein the vaccine comprises polypeptides from one or more *Enterovirus* species or serotype, such a

[0065] method, wherein the Enterovirus species or serotype may be *Human enterovirus A* selected from HEV71 and Coxsackievirus A16, such a

[0066] method, wherein the Enterovirus species or serotype may be *Human enterovirus C* selected from PV-1, PV-2 and PV-3, an

[0067] expression cassette comprising a promoter operably linked to a nucleic acid encoding a human *Enterovirus* P1 polypeptide, an Internal Ribosome Entry Site (IRES), and a nucleic acid encoding a human *Enterovirus* 3CD protease, such an

[0068] expression cassette, wherein the polypeptide is human *Enterovirus* P1, such an

[0069] expression cassette, wherein the human *Enterovirus* 3CD protease processes the human *Enterovirus* P1 polypeptide, such an

[0070] expression cassette, wherein the processed human *Enterovirus* P1 polypeptides associate to form virus like particles, capsomers, complexes and/or aggregates, such an

[0071] expression cassette, wherein the human *Enterovirus* P1 polypeptide comprises combinations of polypeptides selected fromVP0, VP1, VP2, VP3, VP4, and immunologically active fragments thereof, such an

[0072] expression cassette, wherein the human *Enterovirus* is selected from *Human enterovirus A* and *Human enterovirus C*, such an

[0073] expression cassette, wherein the *Human enterovirus A* is selected from HEV71 and Coxsackievirus A16, such an

[0074] expression cassette, wherein the *Human enterovirus C* is selected from *Human poliovirus* 1 (PV-1), *Human poliovirus* 2 (PV-2) and *Human poliovirus* 3 (PV-3), such an

[0075] expression cassette, wherein the IRES is derived from *Encephalomyocarditis virus* (EMCV), such an

[0076] expression cassette, wherein the IRES derived from *Encephalomyocarditis virus* (EMCV) has been genetically modified to reduce IRES activity, such an

[0077] expression cassette, wherein the IRES derived from EMCV has been genetically modified by adding one nucleotide (A7) to the A6 bifurcation loop in the JK segment, such an

[0078] expression cassette, wherein the IRES is derived from a human *Enterovirus,* such an

[0079] expression cassette, wherein the promoter is a eukaryotic promoter, such an

[0080] expression cassette, wherein the eukaryotic promoter is a polyhedrin promoter, such an

[0081] expression cassette, wherein the promoter is operably linked to a nucleic acid encoding a *Human enterovirus A* P1 polypeptide, an EMCV IRES, and a *Human enterovirus A* 3CD protease, such an

[0082] expression cassette, wherein the EMCV IRES has been mutated to reduce IRES activity, such an

[0083] expression cassette, wherein the promoter is operably linked to a nucleic acid encoding a *Human enterovirus A* P1 polypeptide, an HEV71 IRES, and a *Human enterovirus A* 3CD protease, such an

[0084] expression cassette, wherein the promoter is operably linked to a nucleic acid encoding a *Human enterovirus A* P1 polypeptide, a *Human enterovirus C* IRES, and a *Human enterovirus A* 3CD protease, such an

[0085] expression cassette, wherein the promoter is operably linked to a nucleic acid encoding a Human enterovirus C P1 polypeptide, a Human enterovirus C IRES, and a Human enterovirus C 3CD protease, such an

[0086] expression cassette, wherein the promoter is operably linked to a nucleic acid encoding a *Human enterovirus C* P1 polypeptide, an HEV71IRES, and a *Human enterovirus C* 3CD protease, such an

[0087] expression cassette, wherein the promoter is operably linked to a nucleic acid encoding a *Human enterovirus C* P1 polypeptide, an EMCV IRES, and a *Human enterovirus C* 3CD protease, such a

[0088] method of making a vaccine comprising introducing an VLP expression cassette into a host cell, culturing the host cell for a period of time sufficient to produce the polypeptides of the expression cassette and recovering human *Enterovirus* polypeptides from the host cell and/or culture supernatant, such a

[0089] method, wherein the host cell is a eukaryotic cell, such a

[0090] method, wherein the eukaryotic cell is selected from insect cells, mammalian cell lines and yeast cells, such a

[0091] method, wherein the insect cells are selected from *Spodoptera frugiperda*, *Trichoplusia ni*, drosophila, and mosquito cells derived from *Aedes albopictus*, such a

[0092] method, wherein the mammalian cell lines are selected from CHO, HEK 293, COS-1, HeLa, Vero and NIH3T3.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. HEV71 VLP expression cassette [P1+IRES+3CD] and the pSN01 plasmid.

Figure 2. HEV71 VLP expression cassette [P1+IRES+3C] and the pSN03 plasmid.

Figure 3. Expression of VP1 in supernatant of SN07 infected Sf9 cells.

Figure 4. Processed VP1 in both the supernatants and the lysates.

Figure 5. VP1 and VP0 are in the retentate after ultrafiltration over a 100kDa molecular weight cut off (MWCO) membrane.

Figure 6. Immunization schedules to produce neutralizing antibodies.

Figure 7. Immunoblots probed with rabbit polyclonal antisera against VP0 (arrow) at 3 different times of harvest.

Figure 8. Pooled neutralizing sera from mice immunized with the oligomeric antigens in the supernatant of SN07 infected Sf9 cells have high titres against recombinant VP2 in ELISA.

Figure 9. Pooled neutralizing sera from mice immunized with oligomeric antigens in the supernatant of SN07 infected Sf9 cells bind more strongly to VP2 and VP0 than to VP1.

Figure 10. EMCV IRES (SEQ ID NO:1) region of the EMCV genome.

Figure 11. Out framing of the EMCV start codon with 3CD protease coding sequence; native IRES sequence (SEQ ID NO:2) versus mutant IRES sequence (SEQ ID NO:3).

Figure 12. Plasmid pSN01-M1.

Figure 13. Plasmid pSN01-M2

Figure 14. Plasmid pSN01-M3.

Figure 15. Comparison of expression of different recombinant baculovirus designs for expression of HEV71 VLP's.

Figure 16. Plasmid pFastBac^{TM} HT.

Figure 17. Prokaryotic expression construct for antigenic fusion proteins of *Human enterovirus A* and *Human enterovirus C*.

Figure 18. Antibodies from pooled neutralizing sera from mice immunized with SN07 retentate binds to all the components of HEV71 VLPs.

Figure 19. Characterization of HEV71 VLPs, pull-down of HEV71 VLPs from the culture supernatant.

Figure 20. Analysis of affinity column (AFC) purified HEV71 VLPs.

Figure 21. An electron micrograph picture of AFC purified HEV71 VLPs.

Figure 22. *Human enterovirus C* (poliovirus-PV) VLP expression.

Figure 23. Poliovirus-VLP VP3-VP1 ELISA.

Figure 24. HEV71 VLP expression cassette with HEV71-IRES and HEV71 3CD protease.

Figure 25. HEV71 VLP expression cassette with PV-IRES and HEV71 3CD protease.

DETAILED DESCRIPTION OF THE INVENTION

[0093] The invention provides virus like particles (VLPs), virus capsomers, aggregates, and complexes of antigens from viruses of the *Picornaviridae* family as an immunogenic composition and/or vaccine for the protection against and/or treatment of a picornavirus infection. Representative examples may include an *Enterovirus*, a *Coxsackie virus*, and a poliovirus.

[0094] The invention in another aspect provides virus proteins for example, a P1 protein or a combination of picornavirus VP0 proteins, VP1 proteins, VP2 proteins, VP3 proteins, and/or VP4 proteins, or immunologically or biologically active fragments thereof, which elicit neutralizing antibodies. The invention includes fusion-proteins comprising the aforementioned virus proteins and/or fragments thereof, which fusion proteins are immunologically active or biologically active to elicit production of neutralizing antibodies which are protective.

[0095] In an embodiment, an *Enterovirus* antigen may be a combination of *Enterovirus* coat/capsid proteins, or immunologically active fragments thereof. The virus coat/capsid proteins may be any combination of VP0, VP1, VP2, VP3 and/or VP4 proteins, and may take the form of a virus-like particle (VLP), capsomer, complex and/or aggregate. The combination may be in the form of a fusion protein.

[0096] The invention in an additional aspect includes a method for production of *Picornaviridae* virus like particles (VLPs), capsomers, complexes and/or aggregates which may include the steps of: (i) constructing an expression cassette operably linked to a promoter comprising one or more nucleic acids which each encode a picornavirus protein, for example, a P1 protein or a combination of picornavirus VP0 protein, VP1 protein, VP2 protein, VP3 protein, and/or a VP4 protein, which is/are operably linked to an internal ribosome entry site (IRES), which IRES is operably linked to a 3C or 3CD protease; (ii) transfecting or transforming a suitable host cell with the expression cassette; (iii) culturing the host cells under conditions in which virus like particles (VLPs) and/or capsomers and/or antigens are produced by the cell after expression of the nucleic acids comprised in the cassette.

[0097] A nucleic acid or recombinant DNA molecule may be obtained whereby open reading frames which encode *Coxsackievirus* A16, HEV71, *Human enterovirus C* (human polioviruses PV1, PV2 and/or PV3), EV 68, or any other picornavirus proteins and proteases may be amplified by PCR amplification using suitably designed primers complementary to nucleic acid sequences of *Coxsackievirus* A16, HEV71 or *Human enterovirus C* or any other picornavirus. Suitable primers may be designed according to standard techniques from publicly available nucleic acid sequences of enteroviruses, including *Coxsackievirus* A16, HEV71 and *Human enterovirus C* or any other picornavirus. Complete genome sequences are available in GenBank and are accessible at the National Center for Biotechnology Information (NCBI).

[0098] In an embodiment, a picornavirus P1 protein, or any *Enterovirus* P1 protein, is expressed as a polypeptide which is subsequently cleaved by the 3C or 3CD protease

into VP0, VP1 and VP3 virus protein, or immunologically or biologically active fragments thereof, which *Enterovirus* proteins elicit neutralizing antibodies directed against enteroviruses. The VP0 protein may be further cleaved into VP2 and VP4 proteins, or immunologically or biologically active fragments thereof which elicit neutralizing antibodies directed against enteroviruses. The virus proteins may self-assemble into VLPs, capsomers and/or aggregates of enterovirus proteins. Further it will be appreciated that the protease genes may be included in the same DNA recombinant molecule of the VLP expression cassette or in different DNA recombinant molecules, and/or expressed from different promoters or translation elements.

[0099] Recombinant DNA molecules and nucleic acids of the VLP expression cassettes may be devised whereby open reading frames which encode picornavirus structural proteins or proteases may be obtained by PCR amplification using suitably designed primers complementary to nucleic acid sequences of human picornaviruses.

[00100] In a further embodiment, the recombinant DNA molecule may encode a fusion protein having at least two enterovirus structural proteins, or portions thereof, which are expressed as a single polypeptide antigen.

[00101] The present invention encompasses a VLP expression cassette which harbors the gene sequences for *Enterovirus* structural proteins (P1 region) with a protease (3CD) which is necessary for the processing of P1 proteins into the proteins of the virus capsid, thus allowing the self-assembly of *Enterovirus* VLPs. The expression cassette is a bicistronic vector which uses a promoter upstream of the nucleic acid coding sequence for an *Enterovirus* P1 protein. Downstream from the cistron encoding the P1 protein is an internal ribosome entry site (IRES) sequence followed by the cistron containing a nucleotide sequence encoding the 3CD protease.

[00102] Expression of the P1 region and the 3CD protease proceeds from a single bicistronic message wherein the 3CD protease gene is translated in a cap-independent fashion under the control of the IRES. It is observed that expression of the protease

3CD may be moderately toxic leading to premature death of the host cells, thereby lowering the yield of the *Enterovirus* capsid proteins and VLPs. The activity of the protease may be reduced while maintaining the high level of P1 protein expression from the cassette. Different IRESs and IRES sequences comprising mutations were inserted into the expression cassettes to control expression/activity of the 3CD protease and to identify effective IRES to properly process the P1 without being toxic to the cell. For efficient production of VLPs, a number of recombinant baculoviruses which have the complete P1 coding sequence and the complete 3CD protease coding sequence whose expression is under the control of IRESs from different species or serotypes of viruses, were tested for efficient production of VLPs.

[00103] For example, the expression cassette of invention may comprise a promoter which is operably linked to a nucleic acid encoding a *Human enterovirus A* P1 polypeptide, an EMCV IRES, and a *Human enterovirus A* 3CD protease.

[00104] The expression cassette of invention may comprise a promoter which is operably linked to a nucleic acid encoding a *Human enterovirus A* P1 polypeptide, a *Human enterovirus C* IRES, and a *Human enterovirus A* 3CD protease.

[00105] The expression cassette of invention may comprise a promoter which is operably linked to a nucleic acid encoding a *Human enterovirus C* P1 polypeptide, an HEV71 IRES, and a *Human enterovirus C* 3CD protease.

[00106] Furthermore, the expression cassette of invention may comprise a promoter which is operably linked to a nucleic acid encoding a *Human enterovirus C* P1 polypeptide, an EMCV IRES, and a *Human enterovirus C* 3CD protease.

[00107] Moreover, making truncations and mutations of the 3CD protease in the expression cassette which comprises efficient IRES may achieve maximum yield of VLPs. For example, the Glycine of the HEV71 3C protease which is amino acid 1671 of GenBank accession number DQ341362.1 is changed to an Alanine using site directed

mutagenesis for the expression of mutant HEV71 3C and subsequent processing of an HEV71 P1 polypeptide.

[00108] Counter to conventional wisdom in the art with respect to the goal of achieving high levels of expression and activity of a protein from an expression cassette, in an embodiment, the instant invention actually seeks to reduce the activity of a protein to achieve a maximum protein yield. Mutation of the IRES or 3C protease nucleic acid to reduce activity unexpectedly results in an increased yield of *Enterovirus* capsid proteins and VLPs.

[00109] The expression cassettes may be cloned into suitable vectors and transformed/transfected into appropriate host cells for expression and purification of antigens for vaccines and protection against infections from picornaviruses, including enteroviruses.

[00110] The expression cassettes encoding picornavirus antigens may be comprised in plasmids which may be transfected into eukaryotic host cells and expressed under the appropriate growth conditions. Suitable eukaryotic expression systems are known to those skilled in the art and include inducible expression systems and appropriate eukaryotic host cells.

[00111] Mammalian cell expression vectors comprising an expression cassette of the invention include those which may be transiently transfected into host cells and cell lines. Moreover, mammalian cell expression vectors may be vectors which are stably maintained within the host cell following transfection.

[00112] Furthermore, mammalian cell expression vectors may include vectors which are stably or transiently transfected into mammalian host cells or cell lines wherein expression of the protein of interest is induced by the addition of an inducing agent into the culture medium. Mammalian host cells and cell lines include, for example, CHO, HEK 293, COS-1, HeLa, Vero and NIH3T3 cells. It will also be

appreciated that other eukaryotic host cells may include yeast cells or other mammalian cell lines.

[00113] The expression cassette may be contained in recombinant viruses which may transfect the host cell. Suitable viruses that may be used for this purpose include baculovirus, vaccinia, sindbis virus, SV40, Sendai virus, retrovirus and adenovirus. Suitable host cells may include host cells that are compatible with the above viruses and these include insect cells such as *Spodoptera frugiperda* (e.g. Sf9 cells) *Trichoplusia ni*, CHO cells, chicken embryo fibroblasts, BHK cells, human SW13 cells, drosophila, mosquito cells derived from *Aedes albopictus*.

[00114] The expression cassette comprising *Enterovirus* nucleic acids may be introduced into an appropriate host cell by means known to those skilled in the art. The host cells are propagated and cultured under conditions which allow expression of *Enterovirus* genes and proteins.

[00115] A gene encoding an enterovirus VP2 protein, or immunologically or biologically active fragments thereof which elicit neutralizing antibodies against enteroviruses, may be inserted in a plasmid containing a suitable promoter and expressed in a host cell. The produced enterovirus VP2 protein will be isolated and used as the basis of an immunogenic composition for use as a vaccine or for diagnostic use.

[00116] A gene encoding an enterovirus VP4 protein, or immunologically or biologically active fragments thereof which elicit neutralizing antibodies against enteroviruses, may be inserted in a plasmid containing a suitable promoter and expressed in a host cell. The produced enterovirus VP4protein will be isolated and used as the basis of an immunogenic composition for use as a vaccine or for diagnostic use.

[00117] A gene encoding an enterovirus VP0 protein, or immunologically or biologically active fragments thereof which elicit neutralizing antibodies against enteroviruses, may be inserted in a plasmid containing a suitable promoter and expressed in a host cell. The produced enterovirus VP0 protein will be isolated and used as the basis of an immunogenic composition for use as a vaccine or for diagnostic use.

[00118] A gene encoding an enterovirus VP0 protein may be operably linked to a suitable promoter and inserted into a plasmid, which plasmid exhibits an enterovirus protease linked to a suitable promoter to provide a doubly recombinant plasmid, which doubly recombinant plasmid may ultimately be expressed in a eukaryotic or prokaryotic cell expression system.

[00119] Suitable vectors for the cloning of genes and expression of enterovirus polypeptide antigens include cosmids or plasmids. Suitable expression systems include prokaryotic expression systems known to those skilled in the art and prokaryotic host cells, including *E. coli,* transformed with the cosmids or plasmids for expression of proteins in prokaryotic cells.

[00120] Suitable expression systems include eukaryotic expression systems known to those skilled in the art and eukaryotic host cells transformed with plasmids for expression of proteins in various eukaryotic host cells and cell lines.

[00121] Moreover, the *Enterovirus* polypeptide antigens may be obtained from host cells or culture supernatants by means known to those skilled in the art.

[00122] The *Enterovirus* VLPs, capsomers, antigens, immunologically active components thereof, and/or aggregates thereof, may be obtained from transfected and/or transformed host cells, or host cell culture medium, supernatants and lysates by any suitable means of purification known to those skilled in the art. Isolation of proteins released into the culture medium is a facile method of obtaining *Enterovirus* VLPs,

capsomers, antigens and/or aggregates. The *Enterovirus* VLPs, capsomers, antigens, immunologically active components thereof, and/or aggregates thereof, may be further concentrated and purified by means known to those skilled in the art.

[00123] The invention in another aspect includes a vaccine containing picornavirus antigens, such as *Enterovirus* antigens, VLPs and/or capsomers in combination with a suitable adjuvant. The picornavirus antigens, immunologically active fragments thereof, VLPs and/or capsomers may be combined with any suitable adjuvant such as Modified Vaccinia Virus, ISCOMS, alum, aluminum hydroxide, aluminum phosphate, Freund's Incomplete or Complete Adjuvant, Quil A and other saponins or any other adjuvant as described for example in Vanselow (1987) S. Vet. Bull. 57 881-896.

[00124] The meaning of the terms "aluminum phosphate" and "aluminum hydroxide" as used herein includes all forms of aluminum hydroxide or aluminum phosphate which are suitable for adjuvanting vaccines.

[00125] Moreover, picornavirus antigens may be prepared by chemical synthesis of polypeptides based on the publicly available nucleic acid or protein sequences of human poliovirus or by chemical synthesis.

[00126] In an aspect, the invention provides a vaccine comprising one or more *Human enterovirus C* antigen(s). As used herein the expression "poliovirus antigen" or "*Human enterovirus C* antigen" refers to any antigen capable of stimulating neutralizing antibodies to *Human enterovirus C*. The virus antigen may comprise a coat/capsid protein, or fragments thereof, antigenic determinants, and/or other *Human enterovirus C* proteins.

[00127] The invention in one aspect includes *Human enterovirus C* virus subunit vaccines comprising a single *Human enterovirus C* virus coat/capsid protein as an antigen. More specifically the invention pertains to vaccines comprising a *Human enterovirus C* VP2 coat/capsid protein, or immunogenic fragment thereof, as antigen.

[00128] The invention in another aspect includes a vaccine comprising *Human enterovirus C* virus like particles (VLPs), capsomers, complexes and/or aggregates, comprising *Human enterovirus C* VP1, VP2, VP3 and/or VP4, or VP0 proteins.

[00129] A recombinant DNA molecule may be obtained whereby nucleic acids comprising open reading frames which encode *Human enterovirus C* structural proteins or proteases may be obtained by PCR amplification using suitably designed primers complementary to nucleic acid sequences of human *Human enterovirus C*. Suitable primers may be designed according to standard techniques from publicly available nucleic acid sequences of *Human enterovirus C*, such as those complete genome sequences available in GenBank and accessible at the National Center for Biotechnology Information (NCBI). Accession numbers for the complete genome of the *Human enterovirus C* poliovirus type I genome include V01149 and V01150.

[00130] The expression cassettes of the invention may comprise nucleic acids which encode a *Human enterovirus C* P1 polypeptide. The P1 polypeptide is processed (cleaved) by the 3CD protease translated under the control of the IRES of the expression cassette to yield VP1, VP3 and/or VP0 polypeptides. Combinations of VP0, VP1 and VP3 may self-associate into virus-like particles.

[00131] The *Human enterovirus C* polypeptide antigen may comprise a *Human enterovirus C* coat/capsid VP2 protein, a product of further processing of VP0, in combination with another poliovirus VP0, VP1, VP3 and/or VP4 coat/capsid proteins. The combination of *Human enterovirus C* coat/capsid proteins may take the form of a virus-like particle (VLP), capsomer, complex and/or aggregate.

[00132] A gene encoding a *Human enterovirus C* coat/capsid VP2 protein may be inserted into a plasmid containing a suitable promoter and expressed in a host cell, the protein isolated and used as the basis of an immunogenic composition for use as a vaccine. Furthermore, the gene encoding human poliovirus VP2 protein may be

inserted into a plasmid containing a suitable promoter and expressed in a host cell, the protein isolated, and used as the basis of an immunogenic composition for use as a vaccine.

[00133] A gene encoding a *Human enterovirus C* coat/capsid VP4 protein may be inserted into a plasmid containing a suitable promoter and expressed in a host cell, the protein isolated and used as the basis of an immunogenic composition for use as a vaccine. Furthermore, the gene encoding human poliovirus VP4 protein may be inserted into a plasmid containing a suitable promoter and expressed in a host cell, the protein isolated, and used as the basis of an immunogenic composition for use as a vaccine.

[00134] A gene encoding a *Human enterovirus C* coat/capsid VP0 protein may be inserted into a plasmid containing a suitable promoter and expressed in a host cell, the protein isolated and used as the basis of an immunogenic composition for use as a vaccine. Furthermore, the gene encoding human poliovirus VP0 protein may be inserted into a plasmid containing a suitable promoter and expressed in a host cell, the protein isolated, and used as the basis of an immunogenic composition for use as a vaccine.

[00135] The invention encompasses a vaccine comprising one or more immunologically active antigens comprising one or more *Human enterovirus C* VP0, VP1, VP2, VP3, VP4 polypeptides, and immunologically active fragments thereof, which vaccine elicits a protective and/or neutralizing immune response directed against a human *Enterovirus*.

[00136] In an embodiment, the expression cassette consists essentially of a nucleic acid encoding a *Human enterovirus C* P1 polyprotein, an IRES and an enterovirus 3CD protease under the translational control of the IRES, which protease processes the *Human enterovirus C* P1 polyprotein into structural capsid proteins.

[00137] In another aspect, the invention provides a vaccine comprising *Human enterovirus A* antigen(s), derived from a P1 polyprotein including, VP2, VP4 and/or VP0 proteins, and/or biologically or immunologically active fragments thereof. The *Human enterovirus A* antigens may be derived from HEV71 and/or C*oxsackievirus* A16. The HEV71 antigen may be a single human enterovirus virus coat/capsid protein. In particular, the HEV71 antigen may be an HEV71 P1 polyprotein, a VP4, VP2 or VP0 polypeptide, or a fragment thereof, which elicits an immune response upon administration to a human.

[00138] In an embodiment, the *Human enterovirus A* antigen may be a combination of *Human enterovirus A* coat/capsid proteins, or immunologically active fragments thereof. For example, the *Human enterovirus A* antigen may comprise a poliovirus VP2 protein, in combination with another poliovirus coat/capsid proteins selected from VP1, VP3 and/or VP4 polypeptides. The combination of a VP2 polypeptide with other poliovirus coat/capsid proteins may take the form of a virus-like particle (VLP), capsomer, complex and/or aggregate. The combination may be in the form of a fusion protein.

[00139] More specifically the invention pertains to vaccines comprising a *Human enterovirus A* VP2 coat/capsid protein, or immunogenic fragment thereof, as antigen.

[00140] The invention in another aspect includes a vaccine comprising *Human enterovirus A* virus like particles (VLPs) and/or capsomers comprising VP1, VP2, VP3 and/or VP4, or VP0 *Human enterovirus A* proteins.

[00141] A recombinant DNA molecule may be obtained whereby open reading frames which encode *Human enterovirus A* structural proteins and/or proteases may be amplified by PCR amplification using suitably designed primers complementary to nucleic acid sequences of *Human enterovirus A.* Suitably designed primers may be designed according to standard techniques from publicly available nucleic acid

sequences of HEV71. Accession numbers for the complete genome of HEV71 include DQ341362, AB204852, AF302996 and AY465356.

[00142] A recombinant DNA molecule may be obtained whereby open reading frames which encode *Human enterovirus A* P1, VP1, VP2, VP3 and/or VP4, or VP0 proteins, and immunologically active fragments thereof, and proteases, may be obtained by PCR amplification using suitably designed primers complementary to nucleic acid sequences of *Human enterovirus A*.

[00143] In an aspect of the invention, a *Human enterovirus A* P1 protein is expressed to form a polyprotein or polypeptide which is subsequently cleaved by the 3C or 3CD protease into VP0, VP1 and VP3 proteins. VP0 proteins may be further cleaved into VP2 and VP4 proteins. The enterovirus proteins may self-assemble into VLPs, capsomers, complexes and/or aggregates of enterovirus proteins. Further it will be appreciated that the non-structural genes and the protease genes may be included in the same DNA recombinant molecule or in different DNA recombinant molecules, and or expressed from different promoters or translation elements.

[00144] The expression cassettes of the invention may comprise nucleic acids which encode a *Human enterovirus A* P1 polypeptide. The P1 polypeptide is processed (cleaved) by the 3CD protease translated under the control of the IRES of the expression cassette to yield VP1, VP3 and VP0 polypeptides and immunologically active fragments thereof. Combinations of VP0, VP1 and VP3 polypeptides may self-associate into virus-like particles

[00145] A gene encoding a *Human enterovirus A* VP2 protein, or immunologically active fragment thereof, may be inserted in a plasmid containing a suitable promoter and expressed in a host cell. The isolated *Human enterovirus A* antigen, for example, an HEV71 VP2 protein, may be isolated and used as the basis of an immunogenic composition for use as a vaccine or for diagnostic use.

[00146] A gene encoding a *Human enterovirus A* VP4 protein, or immunologically active fragment thereof, may be inserted in a plasmid containing a suitable promoter and expressed in a host cell. The isolated VP4 protein may be isolated and used as the basis of an immunogenic composition for use as a vaccine or for diagnostic use.

[00147] A gene encoding a *Human enterovirus A* VP0 protein, or immunologically active fragment thereof, may be inserted in a plasmid containing a suitable promoter and expressed in a host cell. The isolated VP0 protein may be isolated and used as the basis of an immunogenic composition for use as a vaccine or for diagnostic use.

[00148] A gene encoding a *Human enterovirus A* VP0 protein, or immunologically active fragment thereof, may be operably linked to a suitable promoter and inserted into a plasmid, which plasmid exhibits a *Human enterovirus A* protease linked to a suitable promoter to provide a doubly recombinant plasmid, which doubly recombinant plasmid may ultimately be expressed in a eukaryotic or prokaryotic cell expression system.

[00149] The *Human enterovirus A* genes and nucleic acids comprised in the expression cassette may be introduced into an appropriate host cell by means known to those skilled in the art. The host cells are propagated and cultured under conditions which allow expression of *Human enterovirus A* genes and proteins.

[00150] The invention encompasses a vaccine comprising one or more immunologically active antigens comprising one or more *Human enterovirus A* VP0, VP1, VP2, VP3, VP4 polypeptides, and immunologically active fragments thereof, which vaccine elicits a protective and/or neutralizing immune response directed against a human Enterovirus.

[00151] The in an embodiment, the expression cassette consists essentially of a nucleic acid encoding a *Human enterovirus A* P1 polyprotein, an IRES and an enterovirus 3CD protease under the translational control of the IRES, which protease processes the *Enterovirus* P1 polyprotein into enterovirus structural capsid proteins.

The structural proteins may take the form of VLPs, capsomers, complexes and/or aggregates.

[00152] Indeed, the expression of one or more of the *Enterovirus* proteins as described herein provides antigens which elicit antibodies, which antibodies are functional and able to neutralize enteroviruses selected from HEV71, *Coxsackievirus* A16, *Human enterovirus C* or any other picornavirus to high titre.

[00153] The expression of one or more of the *Enterovirus* proteins suggests that VP2 and/or VP0 polypeptides contain epitopes recognized by neutralizing antisera.

[00154] These functional antibodies surprisingly bind more strongly to *Enterovirus* VP2 and VP0 polypeptides than to VP1 polypeptides, which VP1 polypeptide is understood in the art to be the major capsid protein required for the generation of neutralizing antibodies. It is unexpected that VP2 polypeptides are, in fact, important for generating neutralizing antibodies against HEV71 infection.

[00155] Thus, it is surprising that an *Enterovirus* VP2 polypeptide is the dominant epitope, or antigenic determinant, of the capsid proteins for the generation of neutralizing antibodies against enterovirus infection. HEV71 VP2 polypeptides, either alone, or in combination with other HEV71 capsid proteins, for example VP0 polypeptides, is the dominant antigen which elicits neutralizing antibodies directed against HEV71.

[00156] In an aspect of the invention, prophylactic vaccinations for prevention of enterovirus infection are contemplated which vaccines incorporate VP0 and/or VP2 structural proteins of human enteroviruses into an immunogenic composition. The immunogenic composition or vaccine may comprise VP0 or VP2 structural proteins from *Human enterovirus A*, including HEV71 and *Coxsackievirus* A16, or a combination thereof. The immunogenic composition may be administered to a subject to elicit neutralizing antibodies directed against human enteroviruses. The immunogenic

composition may be comprised in a vaccine which is administered to a subject for the prevention of hand, foot and mouth disease infection caused by *Human enterovirus A*, such as from viruses HEV71 and/or *Coxsackievirus* A16.

[00157] In another aspect of the invention, therapeutic vaccinations are provided to prevent and/or relieve complications of HEV71 and/or *Coxsackievirus* A16 infection, for example, the neurologic and cardiovascular complications manifesting as syndromes such as meningitis, encephalitis, acute flaccid paralysis, pulmonary edema and cardiac failure.

[00158] In an aspect of the invention, prophylactic vaccinations for prevention of enterovirus infection are contemplated which vaccines incorporate VP0 or VP2 structural proteins from *Human enterovirus C,* for example PV1, PV2, PV3 structural proteins, or combinations thereof, or biologically or immunologically active fragments thereof. The immunogenic composition may be comprised in a vaccine which is administered to a subject for the prevention of polio caused by *Human enterovirus C,* including PV1, PV2, and PV3.

[00159] Furthermore, the immunogenic composition or vaccine may comprise a combination of antigens derived from both *Human enterovirus C and Human enterovirus A.*

[00160] Reference may now be made to various embodiments of the invention as illustrated in the attached figures. In these embodiments it should be noted that the *Enterovirus* VLPs, capsomers, antigens, and aggregates and specific constructs of DNA recombinant molecules are given by way of example.

[00161] It may be concluded that *Enterovirus* VP2 polypeptides are important to achieve neutralizing antibodies. VP2 polypeptides may be sufficient for formulating a vaccine against an infection with picornaviruses, such as *Human enterovirus A,* types

HEV71 and *Coxsackievirus* A16; *Human enterovirus C* types 1, 2 and 3: and *Human enterovirus D* type EV68.

[00162] In an aspect of the invention, prophylactic vaccinations for prevention of picornavirus infection are contemplated which vaccinations incorporate at least VP0 and/or VP2 and VP4 structural proteins of the virus into an immunogenic composition. The immunogenic composition may be administered to a subject to elicit neutralizing antibodies directed against a picornavirus. The immunogenic composition may be comprised in a vaccine which is administered to a subject for the prevention of picornavirus infection.

[00163] In another aspect of the invention, therapeutic vaccinations are provided to prevent and/or relieve complications of picornavirus infection, for example, the neurologic and cardiovascular complications manifesting as syndromes such as meningitis, encephalitis, acute flaccid paralysis, pulmonary edema and cardiac failure.

[00164] In a further aspect of the invention there is provided a vaccine composition according to the invention for use in medicine.

[00165] In yet another aspect, the invention provides a bivalent, or multivalent vaccine comprising enterovirus VP0 and/or VP2 and/or VP4 antigens. For example, *Human enterovirus A* VP0 and/or VP2 and/or VP4 antigens may be combined. Moreover, the aforementioned antigens from different serotypes of *Human enterovirus A*, such as antigens from *Coxsackievirus* A16 and HEV71, may be combined in a vaccine, for example, directed against human foot-and-mouth disease.

[00166] The enterovirus antigens of bivalent or multivalent vaccines may be produced from the expression cassettes described herein. The enterovirus antigens may be in the form of virus-like particles, capsomers, complexes, and/or aggregates.

[00167] In yet another aspect, the invention provides a bivalent, or multivalent vaccine comprising enterovirus antigen(s), and an antigen providing immunity against one or more of the following pathogens: diphtheria (D); tetanus (T); pertussis (P); *Haemophilus influenzae* b (Hib); Hepatitis A (HA) Hepatitis B (HB), and Human Enterovirus 71.

[00168] In a pediatric vaccine, other compatible antigens may also be included, e.g., antigens known to be effective against meningitis B, meningitis A and C, and otitis media.

[00169] The amount of picornavirus antigen in each vaccine dose is selected as an amount which induces an immunoprotective response without significant adverse side effects in typical vaccinees. Such amount will vary depending on which specific immunogens are employed. An optimal amount for a particular vaccine can be ascertained by standard studies involving observation of antibody titers and other responses in subjects. A primary vaccination course may include 2 or 3 doses of a vaccine, given at intervals optimal for providing an immunoprotective response.

[00170] The invention thus provides a method for preventing picornavirus infections in humans, which method comprises treating a human subject in need thereof with an immunologically effective dose of a vaccine according to any aspect of the invention as hereinabove described.

[00171] As used herein and in the claims, the terms and phrases set out below have the meanings which follow.

[00172] "Antibody" refers to an immunoglobulin molecule produced by B lymphoid cells with a specific amino acid sequence evoked in humans or other animals by an antigen (immunogen). These molecules are characterized by reacting specifically with the antigen.

[00173] "Antibody response" or "humoral response" refers to a type of immune response in which antibodies are produced by B lymphoid cells and are secreted into the blood and/or lymph in response to an antigenic stimulus. In a properly functioning immune response, the antibody binds specifically to antigens on the surface of cells (e.g., a pathogen), marking the cell for destruction by phagocytotic cells and/or complement-mediated mechanisms.

[00174] "Antigen" refers to any substance that, as a result of coming in contact with appropriate cells, induces a state of sensitivity and/or immune responsiveness and that reacts in a demonstrable way with antibodies and/or immune cells of the sensitized subject in vivo or in vitro.

[00175] "Epitope" refers to the simplest form of an antigenic determinant, on a complex antigen molecule. This is the specific portion of an antigen that is recognized by an immunoglobulin or T-cell receptor.

[00176] "Fusion protein" refers to a protein antigen formed by expression of a polypeptide made by combining two or more gene sequences derived from different enterovirus structural proteins.

[00177] “Immunologically active fragments” or “biologically active fragments” are fragments of *Enterovirus* structural proteins which elicit neutralizing antibodies directed against enteroviruses. Accordingly, in the context of the invention, such immunologically active fragments are presented to the immune system of an organism in order to affect, or more preferably to induce, a specific immune response and, thereby, vaccinate or prophylactically protect the organism against an infection with an *Enterovirus*.

[00178] Neutralizing antibody immune response is where specialized cells of the immune system recognize the presentation of such heterologous proteins, peptides or epitopes and launch a specific immune response.

[00179] In an embodiment, the vaccine antigen according to the invention can induce a protective immune response. The term " protective immune response" and/or “neutralizing immune response” as used herein is intended to mean that the vaccinated subject may resist or protect itself against an infection with the pathogenic agent against which the vaccination was done.

[00180] "Cellular response" or "cellular host response" refers to a type of immune response mediated by specific helper and killer T-cells capable of directly eliminating virally infected or cancerous cells.

[00181] "Antigen-presenting cell" refers to the accessory cells of antigen inductive events that function primarily by handling and presenting antigen to lymphocytes. The interaction of antigen presenting cells (APC) with antigens is an essential step in immune induction because it enables lymphocytes to encounter and recognize antigenic molecules and to become activated. Exemplary APCs include macrophages, Langerhans-dendritic cells, Follicular dendritic cells, and B cells.

[00182] "B-cell" refers to a type of lymphocyte that produces immunoglobulins or antibodies that interact with antigens.

[00183] "Cytotoxic T-lymphocyte" is a specialized type of lymphocyte capable of destructing foreign cells and host cells infected with the infectious agents which produce viral antigens.

[00184] The language “consisting essentially of” means that in addition to those components which are mandatory, other components may also be present in compositions, provided that the essential, basic and/or novel characteristics of the compositions are not materially affected by their presence.

[00185] The language “operably linked” means that the components described are in a relationship permitting them to function in their intended manner. Thus, for example, a promoter "operably linked" to a nucleic acid means that the promoter and the nucleic acids of a cistron, or more than one cistron, are combined in such a manner that a single cistronic, a single bicistronic, or a single multicistronic messenger RNA (mRNA) may be produced. Protein expression of the messenger RNA may be regulated according to transcriptional/translational elements of the nucleic acid sequence. An IRES sequence which is inserted into an expression cassette in an orientation which is upstream (5’) to a cistron means that the IRES sequence and the nucleic acids of the cistron are ligated in such a manner that translation of the cistronic mRNA is regulated under the control of the IRES.

[00186] Reference may now be made to various embodiments of the invention as illustrated in the attached figures. In these embodiments it should be noted that the picornavirus VLPs, capsomers, antigens, and aggregates and specific constructs of DNA recombinant molecules are given by way of example.

EXAMPLE 1. Description of HEV71 VLP Expression Cassettes and Vectors.

[00187] Expression cassettes may be constructed through means understood in the art.

1.1 HEV71 VLP expression cassette [P1+IRES+3CD]

Features:

Cassette size: 5172 bp

prPs: Pox virus strong early/late synthetic promoter, 43 bp

P1: P1 protein coding sequence from EV71-SB12736-SAR-03 (GenBank Accession: DQ341362) with the addition of a stop codon, 2588 bp

IRES: Internal Ribosome Binding site, 585 bp

3CD: C and D protein coding sequence of P3 from EV71-SB12736-SAR-03 (GenBank Accession: DQ341362) with the addition of a ATG start codon and stop codon, 1940 bp

Pac I: Rare cutters, enables cassette to be cloned into pSNX01 (MVA del 3 integration vector)

The cassette was cloned into pDONR221 Gateway entry vector (Invitrogen) to produce pSN01.

See Figure 1 for a diagram of the expression cassette and the pSN01 plasmid.

1.2 HEV71 VLP expression cassette [P1+IRES+3C]

Features:

Cassette size: 3773 bp

prPS: Pox virus strong early/late synthetic promoter, 43 bp

P1: P1 protein coding sequence from EV71-SB12736-SAR-03 (GenBank Accession: DQ341362) with the addition of a stop codon, 2588 bp

IRES: Internal Ribosome Binding site, 585 bp

3CD: C protein coding sequence of P3 from EV71-SB12736-SAR-03 (GenBank Accession: DQ341362) with the addition of a ATG a start codon and stop codon, 551 bp

Pac I: Rare cutters, enables cassette to be cloned into pSNX01 (MVA del 3 integration vector)

The cassette was cloned into pDONR221 Gateway entry vector (Invitrogen) to produce pSN03.

See Figure 2 for a diagram of the expression cassette and the pSN03 plasmid.

1.3 Methods for obtaining Recombinant Baculoviruses containing the insert HEV71[P1+IRES+3CD] or [P1+IRES+3C]

The source material for HEV71 P1 plus 3CD was pSN01 and for P1 plus 3C was pSN03. The aim was to introduce the HEV71-VLP cassette from pSN01 and pSN03 (Entry vectors) into the baculovirus expression plasmid pDEST8 (Destination vector) by attL/aaR *in vitro* recombination using LR CLONASE®, following the instructions in the Invitrogen BAC-TO-BAC® manual (2009).

Two recombinase reactions were set up:

1. pSN01 (EV71-P1+3CD) x pDEST8 to produce pSN07;
2. pSN03 (EV71-P1+3C) x pDEST8 to produce pSN08

pSN07 and pSN08 were used to produce recombinant bacmids bacSN07 and bacSN08 by transforming DH10bac as described in the Invitrogen BAC-TO-BAC® manual. The recombinant bacmids were transfected into Sf9 cells to rescue recombinant baculoviruses SN07 and SN08.

The recombinant baculoviruses SN07 and SN08 were used to further infect Sf9 cells in 6 well plates to evaluate for expression of processed capsid proteins. A polyclonal rabbit antiserum specific for VP1 was used to identify VP1 protein in Western blots of lysates and supernatants from recombinant baculovirus infected Sf9 cells.

1.4 Expression of VP1 in the supernatant of SN07 infected Sf9 cells.

Supernatants of infected Sf9 cells were harvested daily from day 3 to day 7 post infection and the proteins were resolved on a 12% SDS-PAGE and transferred to nitrocellulose membranes which were then probed with polyclonal rabbit anti-VP1 antisera (1:4000 dilution) overnight followed by anti-rabbit conjugated with HRP (1:1000 dilution) for 1 hour at room temperature. The Western blots were subsequently developed using TMB. The results are shown in Figure 3. It was observed that the HEV71 VP1 was processed and that expression of the protein was observed in the supernatant at day 3 and day 4 post infection and that the amount of VP1 expression diminished thereafter. Recombinant baculovirus SN07 on infection of Sf9 cells generates antigens which are found in the supernatant.

EXAMPLE 2. Processed VP1 in both the supernatants and the lysates.

[00188] Sf9 cells were infected at a Multiplicity of Infection (MOI) of 10 with different recombinant baculovirus isolates, including SN07, SN08, a control baculovirus bacGUS and mock infected. Supernatants and lysates were harvested on days 3 and 4 post infection and expression of the proteins evaluated by Western blots using rabbit anti-VP1 antisera (1:4000 dilution) to compare yields of proteins produced by SN07 and SN08. As shown in Figure 4, expression construct SN07 produced more cleaved VP1 than expression construct SN08 both in the supernatant and in the lysate on both days 3 and 4 post infection.

EXAMPLE 3. VP1 and VP0 is in the retentate after ultrafiltration over a 100kD molecular weight cut off (MWCO) membrane.

[00189] Supernatants from SN07 infected Sf9 cells at day 3 post-infection were clarified and were passed through AMICON® filters (Millipore Corp.) with a 100 kDa MWCO. The retentate was tested for the presence of processed VP1 and VP0. Since the molecular weight of VP1 is approximately 33 kDa, and the molecular weight of VP0

is 36 KDa, these proteins would not be expected to remain in the retentate unless they were in an oligomeric form. As shown in Figure 5, these antigens remain in the retentate on passing the supernatants through a 100kDa MWCO ultrafilter. This suggests that the antigens are associated in an oligomeric form. Thus, it may be concluded that VP1 and VP0 are processed and are in an oligomeric association with other capsid proteins.

EXAMPLE 4. The retentate, when used to immunize mice, elicits strong neutralizing antibodies against HEV71.

[00190] Two groups of outbred white mice were immunized with the supernatant concentrate from Sf9 infected with SN07 as prepared in Example 3. The immunization schedules used are shown in the diagram in Figure 6.

[00191] All mice in the immunized group produced antibodies that neutralized HEV71 as shown in Table 1. The retentate, when used to immunize mice, elicits strong neutralizing antibodies against HEV71.

Table 1. Neutralizing antibodies directed against HEV71.

mouse ID	**neut reciprocal titre**
m1-1	160
m1-2	640
m1-3	>=2560
m1-4	640
m2-1	80
m2-2	640
m2-3	640
m2-4	160
control1	<10
control2	<10

[00192] Thus, it may be concluded that the oligomeric proteins are able to elicit neutralizing antibodies in mice.

[00193] The oligomeric proteins may be utilized in immunogenic compositions and/or comprised in vaccines for administration and prophylaxis of enterovirus infection.

EXAMPLE 5. VP0 is expressed in the lysates of SN07 infected Sf9 cells.

[00194] Sf9 cells were infected with SN07 and the lysates were harvested at 72, 96 and 120 hours. Immunoblots were probed with rabbit polyclonal antisera directed against EV71 VP0 and it was shown that VP0 is expressed at 72 hours post infection. As shown in Figure 7, the VP0 was partially cleaved to VP2 starting at 96 hours post infection. Thus both VP0 and VP2 are present in the lysates of SN07 infected cells.

EXAMPLE 6. Pooled neutralizing sera from mice immunized with the oligomeric antigens in the supernatant of SN07 infected Sf9 cells have high titres against recombinant VP2 in ELISA.

[00195] ELISA plates were coated with equal amounts of recombinant VP1 and VP2 and used to test pooled neutralizing mouse antisera. Figure 8 shows that the mice which received the supernatant retentate antigen preparation were able to bind VP2 better than VP1. Thus, it may be concluded that the antibodies are functional and able to neutralize HEV71 to high titre.

EXAMPLE 7. Pooled neutralizing sera from mice immunized with oligomeric antigens in the supernatant of SN07 infected Sf9 cells bind more strongly to VP2 and VP0 than to VP1.

[00196] The Western blots in Figure 9 show that the neutralizing pooled mouse antisera bound more strongly to VP2 and VP0 than to VP1 even though the same amount of total protein was added to each well. This suggests that VP2 and VP0 contain epitopes recognized by neutralizing antisera.

[00197] These functional antibodies surprisingly bind more strongly to VP2 and VP0 than to VP1 which is considered to be the major capsid protein required for the generation of neutralizing antibodies. It is thus relevant to consider that VP2 is in fact important for generating neutralizing antibodies.

[00198] It may be concluded that the presence of VP2 in a vaccine formulation is important to achieve neutralizing antibodies. VP2 may be sufficient for formulating a vaccine against HEV71 and other species A, B and C enteroviruses including human *Coxsackievirus* A16, Echovirus 30,and poliovirus types 1, 2 and 3.

[00199] It will further be appreciated that the invention includes within its scope a method of generating an immune response directed against poliovirus including the step of administering an effective amount of a vaccine comprising a poliovirus antigen.

EXAMPLE 8. Comparison of the levels of neutralizing antibodies.

[00200] Immunogenic compositions comprising HEV71 VP1 are used to immunize mice. Similarly, immunogenic compositions comprising HEV71 VP2 and/or VP0 are used to immunize mice. The neutralizing antibody levels in the mice immunized with HEV71 VP2 and/or VP0 are compared with the neutralizing antibody levels in the mice immunized with HEV71 VP1. The neutralizing antibody levels in the mice immunized with HEV71 VP2 and/or VP0 are significantly higher than the neutralizing antibody levels in the mice immunized with HEV71 VP1.

EXAMPLE 9. Construction of recombinant baculovirus vector for expression of the *Human enterovirus C* P1 region and the protease 3CD from a single bicistronic message.

[00201] This example provides a method that will result in the efficient production of VLPs of *Human enterovirus C* (poliovirus) by reducing the protease 3CD mediated killing of baculovirus infected cells.

[00202] The construction of a recombinant baculovirus vector for the expression of the P1 region and the protease 3CD from a single bicistronic message is shown, for example, in Figure 1. The 3CD protease gene is translated in a cap-independent fashion under control of the EMCV IRES. This system provides the leverage to regulate the expression of protease 3CD, i.e., evaluate the mutant IRES sequences to find the weakest IRES so that a lesser amount of protease is produced compared to the P1 proteins.

[00203] A bicistronic vector is constructed in which the plasmid contains a polyhedrin promoter upstream of the coding sequence for the P1. Downstream from the cistrons encoding P1 is an *Encephalomyocarditis virus* (EMCV) internal ribosome entry site (IRES) sequence (GenBank accession number AF113968.2; nucleotides1666 to 2251) followed by the cistrons containing the nucleotide sequence encoding the protease 3CD. The source material for the *Human enterovirus C* P1 with 3CD may be accessed at the American Type Culture Collection (ATCC) and synthesized from known *Human enterovirus C* sequences (GenBank available through the National Center for Biotechnology Information (NCBI)).

[00204] Recombinant baculoviruses are generated using the BAC-TO-BAC® system according to the manufacturer's instructions (Invitrogen). Briefly, LR CLONASE® is used to introduce the *Human enterovirus C* VLP cassette into the baculovirus expression plasmid pDEST8 (Destination using vector) by attL/aaR *in vitro* recombination. The LR CLONASE® reaction is carried out at 25℃ for 1 hour followed by incubation with proteinase K. The LR CLONASE® reaction mix is transformed into Library Efficiency DH5α competent cells to obtain expression clones. DNA is isolated from the resultant colonies and are confirmed for the presence of the *Human enterovirus C* cassette by restriction enzyme analysis. Recombinant bacmids are constructed by introducing the expression cassette, into the baculovirus genome harbored in DH10bac cells by T7 transposition recombinase. The recombinant bacmids are verified by their white phenotype on LB agar plates supplemented with 50 µg/ml

kanamycin, 7 µg/ml gentamicin, 10 µg/ml tetracycline, 100 µg/ml X-gal, and 40 µg/ml IPTG. The PureLink HiPure Plasmid DNA Miniprep Kit (Invitrogen) is used to purify high quality bacmid DNA from DH10Bac *E. coli*. M13 forward, M13 reverse and internal primers from the insert are used to confirm the existence of the *Human enterovirus C* cassette. EFFECTENE® transfection reagent (Qiagen) is used to rescue recombinant baculoviruses by transfecting the DNAs into Sf9 insect cells. Briefly, Sf9 cells are seeded at 2 million per T25 flask and incubated to adhere for 6hr at 28℃. One microgram of recombinant bacmid DNA is resuspended in 150 µl of DNA condensation buffer and 8µl of enhancer solution is mixed and incubated at room temperature for 5 minutes. Then 25µl of EFFECTENE® reagent is added into the DNA mix and incubated for 10 minutes at room temperature. One ml culture medium is added into the tubes containing the transfection complexes and transferred into cell culture flasks and uniformly distributed. At day 3, the supernatant is harvested by centrifugation at 500g for 5 minutes. Following transfection, a high titer viral stock is prepared. Once a high viral stock is obtained, it is employed to determine the optimal times for target protein expression.

EXAMPLE 10. Production of VLPs of HEV71, as well as *Human enterovirus C*, by means of reducing the protease 3CD mediated killing of baculovirus infected cells.

[00205] This experiment describes the construction of recombinant baculovirus vector for the expression of the P1 region and the protease 3CD from a single bicistronic message. The protease gene 3CD is translated in a cap-independent fashion under control of the EMCV IRES, shown in Figure 10. This system provides the leverage to regulate the expression of protease 3CD, i.e., evaluate the mutant IRES sequences to find weakest IRES so that a lesser amount of the protease is produced compared to the P1 proteins.

[00206] A bicistronic vector was constructed in which a plasmid contains a polyhedrin promoter upstream of the coding sequence for the P1. Downstream from the cistrons encoding P1 is an *Encephalomyocarditis virus* (EMCV) internal ribosome entry

site (IRES) sequence followed by the cistrons containing nucleotide sequence encoding the protease 3CD, see Figure 1. The IRES used in Example 1 contains native EMCV IRES sequence as there are altered forms. The native EMCV IRES sequence in Figure 10 shows the A6 bifurcation loop in the JK segment. Adding one nucleotide, for example an adenine (A7), reduces the expression. Also, in the construct used in Example 1, the 3CD protease is fused with *Encephalomyocarditis virus* IRES at the amino-terminus. Importantly out framing the EMCV start codon with 3CD protease coding sequence should considerably reduce the expression of downstream genes, see Figure 11. Both modifications are incorporated into the EMCV IRES sequence of pSN01and named pSN01-M1, as shown in Figure 12, and synthesized by DNA2.0.

[00207] Experiments were designed to characterize the VLPs expressed from the mutant EMCV IRES of pSN01-M1. Recombinant baculoviruses were generated using the BAC-TO-BAC® system according to the manufacturer's instructions (Invitrogen). Briefly, LR CLONASE® was used to introduce the HEV71 VLP cassette into the baculovirus expression plasmid pDEST8 (Destination using vector) by attL/aaR *in vitro* recombination. The LR CLONASE® reaction was carried out at 25℃ for 1 hr followed by incubation with proteinase K. The LR CLONASE® reaction mix was transformed into Library Efficiency DH5α competent cells to obtain expression clones. DNA was isolated from the resultant colonies and confirmed for the presence of the HEV71/poliovirus cassette by restriction enzyme analysis. Recombinant bacmids are constructed by introducing the expression cassette of pSN07-M1, into the baculovirus genome harbored in DH10bac cells by T7 transposition recombinase to give bacSN07-M1. The recombinant bacmids are verified by their white phenotype on LB agar plates supplemented with 50 µg/ml kanamycin, 7 µg/ml gentamicin, 10 µg/ml tetracycline, 100 µg/ml X-gal, and 40 µg/ml IPTG. The PureLink HiPure Plasmid DNA Miniprep Kit (Invitrogen) was used to purify high quality bacmid DNA from DH10Bac *E. coli*. M13 forward, M13 reverse and internal primers from the insert were used to confirm the existence of the HEV71/poliovirus cassette. EFFECTENE® transfection reagent (Qiagen) was used to rescue recombinant baculoviruses by transfecting the DNAs into Sf9 insect cells. Briefly, Sf9 cells were seeded at 2 million per T25 flask and incubated

to adhere for 6hr at 28℃. One microgram of recombinant bacmid DNA was resuspended in 150 µl of DNA condensation buffer and 8µl of enhancer solution is mixed and incubated at room temperature for 5 minutes. Then 25µl of EFFECTENE® reagent was added into DNA mix and incubated for 10 minutes at room temperature. One ml culture medium was added into the tubes containing the transfection complexes and transferred into cell culture flasks and uniformly distributed. At day 3 the supernatant was harvested by centrifugation at 500g for 5 minutes. Following transfection, a high titer viral stock is prepared. Once a high viral stock is obtained, it is employed to determine the optimal times for target protein expression. For the analysis of the protein of interest, Sf9 cells grown in 10% Grace's insect cell medium (Invitrogen) were resuspended in 1% FBS Sf900-II SFM medium (Invitrogen) to get single cells and were seeded at a million per ml density in the flasks and incubated for 4 hr at 28℃. Viral stocks were added into the PBS washed cells at an MOI of 10 and rocked gently for 1 hr. The infected cells were washed three times with PBS and the cells were grown in Sf900II-SFM for different time points. Cells were lysed with hypotonic douncing buffer /1%TRITON® X-100 (TX-100) (1.5mM $MgCl_2$, 50mM KCl, 20mM HEPES, 1% TX-100) by rocking the flask for 30 minutes at room temperature and cell lysates were prepared by collecting the lysed cells from the flask and centrifuging at 4℃ for 30 minutes at 7000 rpm. The components of the cell lysates and supernatants were analyzed by immunoblotting and ELISA using specific antibodies.

EXAMPLE 11. Efficient production of VLPs of HEV71, as well as *Human enterovirus C* (poliovirus) by means of reducing the protease 3CD mediated killing of baculovirus infected cells.

[00208] The construction of a recombinant baculovirus vector for expression of the P1 region and the protease 3CD from a single bicistronic message is shown in Figure 1. The 3CD protease gene is translated in a cap-independent fashion under control of the EMCV IRES, shown in Figure 10. This system provides the leverage to regulate the expression of 3CD protease, i.e., evaluate the mutant IRES sequences to find the

weakest IRES so that a lesser amount of protease is produced compared to the P1 proteins.

[00209] A bicistronic vector was constructed and the plasmid contains a polyhedrin promoter upstream of the coding sequence for the P1. Downstream from the cistrons encoding P1 is an *Encephalomyocarditis virus* (EMCV) internal ribosome entry site (IRES) sequence followed by the cistrons containing a nucleotide sequence encoding the 3CD protease, see Figure 1. The IRES used in Example 1 contains native EMCV IRES sequence as there are altered forms. The native EMCV IRES sequence has the A6 bifurcation loop in the JK segment, indeed by adding one nucleotide (A7) known to reduce the expression, see Figure 10. The A6 bifurcation loop was modified into A7 in the EMCV IRES sequence of pSN01 and named pSN01-M2, see Figure 13, which is synthesized by DNA2.0.

[00210] VLPs expressed by the mutant EMCV IRES of pSN01-M2 were characterized. Recombinant baculoviruses were generated using the BAC-TO-BAC® system according to the manufacturer's instructions (Invitrogen). Briefly, LR CLONASE® was used to introduce the HEV71 VLP cassette into the baculovirus expression plasmid pDEST8 (Destination using vector) by attL/aaR *in vitro* recombination. The LR CLONASE® reaction was carried out at 25℃ for 1 hr followed by incubation with proteinase K. The LR CLONASE® reaction mix was transformed into Library Efficiency DH5α competent cells to obtain expression clones. DNA was isolated from the resultant colonies and confirmed for the presence of the HEV71/poliovirus cassette by restriction enzyme analysis. Recombinant bacmids were constructed by introducing the expression cassette of pSN07-M2, into the baculovirus genome harbored in DH10bac cells by T7 transposition recombinase to give bacSN07-M2. The recombinant bacmids were verified by their white phenotype on LB agar plates supplemented with 50 µg/ml kanamycin, 7 µg/ml gentamicin, 10 µg/ml tetracycline, 100 µg/ml X-gal, and 40 µg/ml IPTG. The PureLink HiPure Plasmid DNA Miniprep Kit (Invitrogen) was used to purify high quality bacmid DNA from DH10Bac *E. coli*. M13 forward, M13 reverse and internal primers from the insert were used to confirm the

existence of the HEV71/poliovirus cassette. EFFECTENE® transfection reagent (Qiagen) was used to rescue recombinant baculoviruses by transfecting the DNAs into Sf9 insect cells. Briefly, Sf9 cells were seeded at 2 million per T25 flask and incubated to adhere for 6hr at 28℃. One microgram of recombinant bacmid DNA was resuspended in 150 µl of DNA condensation buffer and 8µl of enhancer solution is mixed and incubated at room temperature for 5 minutes. Then 25µl of EFFECTENE® reagent was added into the DNA mix and incubated for 10 minutes at room temperature. One ml culture medium was added into the tubes containing the transfection complexes and transferred into cell culture flasks and uniformly distributed. At day 3, the supernatant was harvested by centrifugation at 500g for 5 minutes. Following transfection, a high titer viral stock was prepared. Once a high viral stock was obtained, it was employed to determine the optimal times for target protein expression. For the analysis of the protein of interest, Sf9 cells grown in 10% Grace's insect cell medium (Invitrogen) was resuspended in 1% FBS Sf900-II SFM medium (Invitrogen) to get single cells and were seeded at a million per ml density in the flasks and incubated for 4 hr at 28℃. Viral stocks were added into the PBS washed cells at an MOI of 10 and rocked gently for 1 hr. The infected cells were washed three times with PBS and cells were grown in Sf900II-SFM for different time points. Cells were lysed with hypotonic douncing buffer /1%TX-100 (1.5mM $MgCl_2$, 50mM KCl, 20mM HEPES, 1% TX-100) by rocking the flask for 30 minutes at room temperature and the cell lysates were prepared by collecting the lysed cells from the flask and centrifuging at 4℃ for 30 minutes at 7000rpm. The components of the cell lysates and supernatants were analyzed by immunoblotting and ELISA using specific antibodies.

EXAMPLE 12. Efficient production of VLPs of HEV71 as well as *Human enterovirus C* (poliovirus) by means of reducing the protease 3CD mediated killing of baculovirus infected cells.

[00211] The construction of a recombinant baculovirus vector for expression of the P1 region and the protease 3CD from a single bicistronic message is shown in

Figure 1. The 3CD protease gene is translated in a cap-independent fashion under control of the EMCV IRES, shown in Figure 10. This system provides the leverage to regulate the expression of protease 3CD, i.e., evaluate the mutant IRES sequences to find the weakest IRES so that a lesser amount of protease is produced compared to the P1 proteins.

[00212] A bicistronic vector was constructed in which the plasmid contains a polyhedrin promoter upstream of the coding sequence for the P1. Downstream from the cistrons encoding P1 is an *Encephalomyocarditis virus* (EMCV) internal ribosome entry site (IRES) sequence followed by the cistrons containing a nucleotide sequence encoding the protease 3CD (Figure 1). The IRES used in Example 1 contains native EMCV IRES sequence as there are altered forms. In the pSN01 construct the 3CD protease is fused with *Encephalomyocarditis virus* polyprotein at the amino-terminus. Out framing the EMCV start codon with 3CD protease coding sequence should considerably reduce the expression of downstream genes, see Figure 11. This modification was incorporated into EMCV IRES sequence of pSN01 and named pSN01-M3, see Figure 14, and is synthesized by DNA2.0.

[00213] The VLPs expressed by the mutant EMCV IRES of pSN01-M3 were analyzed. Recombinant baculoviruses were generated using the BAC-TO-BAC® system according to the manufacturer's instructions (Invitrogen). Briefly, LR CLONASE® was used to introduce the HEV71 VLP cassette into the baculovirus expression plasmid pDEST8 (Destination using vector), by attL/aaR *in vitro* recombination. The LR CLONASE® reaction was carried out at 25℃ for 1 hr followed by incubation with proteinase K. The LR CLONASE® reaction mix was transformed into Library Efficiency DH5α competent cells to obtain expression clones. DNA was isolated from the resultant colonies and confirmed for the presence of HEV71/poliovirus cassette by restriction enzyme analysis. Recombinant bacmids were constructed by introducing the expression cassette of pSN07-M3, into the baculovirus genome harbored in DH10bac cells by T7 transposition recombinase to give bacSN07-M3. The recombinant bacmids were verified by their white phenotype on LB agar plates supplemented with 50

µg/ml kanamycin, 7 µg/ml gentamicin, 10 µg/ml tetracycline, 100 µg/ml X-gal, and 40 µg/ml IPTG. The PureLink HiPure Plasmid DNA Miniprep Kit (Invitrogen) was used to purify high quality bacmid DNA from DH10Bac *E. coli*. M13 forward, M13 reverse and internal primers from the insert were used to confirm the existence of the HEV71/poliovirus cassette. EFFECTENE® transfection reagent (Qiagen) was used to rescue recombinant baculoviruses by transfecting the DNAs into Sf9 insect cells. Briefly, Sf9 cells were seeded at 2 million per T25 flask and incubated to adhere for 6hr at 28℃. One microgram of recombinant bacmid DNA was resuspended in 150 µl of DNA condensation buffer and 8µl of enhancer solution is mixed and incubated at room temperature for 5 minutes. Then 25µl of EFFECTENE® reagent was added into the DNA mix and incubated for 10 minutes at room temperature. One ml culture medium was added into the tubes containing the transfection complexes and transferred into cell culture flasks and uniformly distributed. After day 3, supernatant was harvested by centrifugation at 500g for 5 minutes. Following transfection, a high titer viral stock is prepared. Once a high viral stock is obtained, it is employed to determine the optimal times for target protein expression.

[00214] For the analysis of the protein of interest, Sf9 cells grown in 10% Grace's insect cell medium (Invitrogen) were resuspended in 1% FBS Sf900-II SFM medium (Invitrogen) to get single cells and were seeded at a million per ml density in the flasks and incubated for 4 hr at 28℃. Viral stocks were added into the PBS washed cells at a MOI of 10 and rocked gently for 1 hr. The infected cells were washed three times with PBS and cells were grown in Sf900II-SFM for different time points. Cells were lysed with hypotonic douncing buffer /1%TX-100 (1.5mM $MgCl_2$, 50mM KCl, 20mM HEPES, 1%TX-100) by rocking the flask for 30 minutes at room temperature and cell lysates were prepared by collecting the lysed cells from the flask and centrifuging at 4℃ for 30 minutes at 7000 rpm. The components of the cell lysates and supernatants were analyzed by immunoblotting and ELISA using specific antibodies.

EXAMPLE 13. Mutant IRES construct M2 expresses higher levels of VP1 in the supernatant.

[00215] Recombinant baculoviruses expressing HEV71 capsid proteins under the control of the wild type or mutant EMCV IRES's were evaluated with respect to the level of expression of the HEV71 capsid proteins from the ECMV IRES's. Baculovirus produced VLPs which are expressed under the control of the wild type EMCV IRES from SN07 of Example 1, and the 3 mutant IRES's, M1, M2 and M3 from Examples 10, 11, and 12, respectively, were analyzed with respect to the level of baculovirus expression of the HEV71 capsid proteins. A recombinant baculovirus expressing P1 and 3CD under different promoters (F) and a control recombinant baculovirus expressing bacGUS (G) were also included in the study. Sf9 cells were infected at an MOI of 5 and both lysates and supernatants were harvested on day 3 as described in Examples 10-12 above. The lysates and supernatants were probed with an anti-VP1 antibody to detect the expression of HEV71 capsid proteins.

[00216] The immunoblots of Figure 15 show that when lysates were probed with antibodies to VP1, the mutants M1 and M2 express higher levels of VP1 than the mutant M3 or the construct driven by 2 promoters. However, the mutant M2 produced more VP1 in the supernatant. These data are shown in the top panel.

[00217] The bottom panel of Figure 15 shows the blots which were probed with a control anti-gp64 antibody which is directed to the coat protein of baculovirus. The immunoblot shows that equivalent amounts of baculovirus were produced in each sample.

EXAMPLE 14. Cloning, expression and purification of subunit vaccines using a baculovirus expression system.

[00218] The present invention is intended for the generation and use of recombinant HEV71 and poliovirus structural proteins which are fused as single

immunogens to elicit a protective immune response in vaccinated individuals. The present invention relates generally to preparing recombinant HEV71 and/or poliovirus fusion protein vaccine compositions comprising HEV71 and/or poliovirus subunit protein, or an immunogenic fragment thereof, and an adjuvant in combination with the recombinant HEV71 and/or poliovirus subunit fusion protein. HEV71 and poliovirus subunit fusion proteins may comprise capsid proteins selected from VP1, VP2, VP3, and VP4, combinations thereof, and combinations of immunogenic fragments thereof. In one aspect of this embodiment, the recombinant HEV71 and/or poliovirus fusion protein comprises HEV71 or poliovirus subunit protein and a fusion partner protein in genetic association with the HEV71 or poliovirus subunit protein. The present invention contemplates methods to generate the constructs to express the following subunit vaccines in *E.coli* as well as baculovirus: VP0, VP4-VP2-VP3 fusion, VP2-VP3-VP1 fusion.

[00219] To generate cDNAs from HEV71 and/or poliovirus encoding VP0, VP4-VP2-VP3 fusion, or VP2-VP3-VP1, reverse transcription/polymerase chain reaction (The High Pure Nucleic Acid Kit; Roche) was carried out using purified genomic viral RNA. A forward and a reverse primer was made from the 5'and 3' end of the genes and which primers incorporate a start and stop codon. The amplified PCR products were digested with EcoRI and NotI restriction enzymes and cloned into the pFastBac HT vector (Invitrogen) as shown in Figure 16. The BAC-TO-BAC® expression system from Invitrogen is commercially available and methods were used according to the manufacturer's instructions. The fusion genes are cloned into pFastBac HT donor plasmid and the production of recombinant proteins was based upon the BAC-TO-BAC® to baculovirus expression system (Invitrogen). The pFastBac HT donor plasmid carrying the fusion genes was transferred into a baculovirus shuttle vector (bacmid) by site-specific recombination by T7 transposition recombinase. This was accomplished in *E. coli* strain DH10Bac. The DH10Bac cells contain the bacmid, which conferred kanamycin resistance and a helper plasmid, which encoded the transposase and conferred resistance to tetracycline. The recombinant pFastBac HT plasmids with the gene of interest were transformed into DH10Bac cells for the transposition to generate

recombinant bacmids. The transformed cells were serially diluted and each dilution was plated on LB agar plates supplemented with 50µg/ml kanamycin, 7µg /ml gentamicin, 10µg/ml tetracycline, 100 µg/ml X-gal, and 40 µg/ml IPTG and incubated for at least 48 hours at 37℃. The white colonies were picked and re-streaked to confirm a white phenotype. Recombinant bacmids were isolated by the PureLink HiPure Plasmid DNA Miniprep Kit (Invitrogen) and the DNA samples were dissolved in 40µlof TE (10 mM Tris-HCl pH 8, 1 mM EDTA) and used for transfections.

[00220] The isolated bacmid DNA was screened for the inserted gene of interest by PCR. EFFECTENE® transfection reagent (Qiagen) was used to rescue recombinant baculoviruses by transfecting the DNAs into Sf9 insect cells. Briefly, Sf9 cells were seeded at 2 million per T25 flask and incubated to adhere for 6hr at 28℃. One microgram of recombinant bacmid DNA was resuspended in 150 µl of DNA condensation buffer and 8µl of enhancer solution is mixed and incubated at room temperature for 5 minutes. Then 25µl of EFFECTENE® reagent was added into DNA mix and incubated for 10 minutes at room temperature. One ml culture medium was added into the tubes containing the transfection complexes and transferred into cell culture flasks and uniformly distributed. At day 3, the supernatant was harvested by centrifugation at 500g for 5 minutes. Following transfection, a high titer viral stock is prepared. Once a high viral stock is obtained, it is employed to determine the optimal times for target protein expression. For the analysis of the protein of interest, Sf9 cells grown in 10% Grace's insect cell medium (Invitrogen) was resuspended in 1% FBS Sf900-II SFM medium (Invitrogen) to get single cells and are seeded at a million per ml density in the flasks and incubated for 4 hr at 28℃. Viral stocks were added into PBS washed cells at a MOI of 10 and rocked gently for 1 hr. The infected cells were washed three times with PBS and cells are grown in Sf900II-SFM for different time points. Cells were lysed with hypotonic douncing buffer /1%TX-100 (1.5mM $MgCl_2$, 50mM KCl, 20mM HEPES, 1%TX-100) by rocking the flask for 30 minutes at room temperature and the cell lysates were prepared by collecting the lysed cells from the flask and centrifuging at 4℃ for 30 minutes at 7000rpm. The expression of the heterologous protein in the cells was verified by SDS polyacrylamide gel electrophoresis (SDS-

PAGE) and Western blots using the His Probe-HRP antibody (Thermo Scientific) as the probe. Once production of baculovirus and the expression of protein were confirmed, the virus stock was amplified to produce a concentrated stock of the baculovirus that carry the gene of interest. The most appropriate concentration of the virus to infect insect cells and the optimum time point for the production of the desired protein was also established. For purification under denaturing conditions, the cells were lysed in a lysis buffer containing 6 M guanidinium-HCl in 100 mM NaH_2PO_4, 10 mM Tris, 300mM NaCl, 10 mM imidazole, pH 8.0 (lysis buffer). The suspension was sonicated on ice with 5 pulses of 1 minute per pulse at a power setting of 60 watts, and was mixed at room temperature for 1 hour. The lysate was centrifuged at 27K g for 30 min to eliminate cell debris. The supernatant was loaded on to a HisTrap (GE healthcare life sciences) column pre-equilibrated with lysis buffer. Following loading, the column was washed with 20 column volumes of 6 M guanidinium-HCl in 100 mM NaH_2PO_4, 10 mM Tris, 300 mM NaCl, 40 mM Imidazole, pH 8.0 (wash buffer 1), followed by washes with 20 column volumes of 8 M urea in 100 mM NaH_2PO_4,10 mM Tris, 300 mM NaCl, 40 mM imidazole, pH 8.0 (wash buffer 2). The bound protein was eluted with a buffer containing 8 M urea, 100 mM NaH_2PO_4, 10 mM Tris, 300 mM NaCl, 250 mM imidazole, pH 8 (Elution Buffer). The fractions containing the protein were pooled and dialyzed against PBS, overnight at 4° C. TEV protease was used for removal of the histidine tag following protein purification according to manufacturer's instructions.

EXAMPLE 15. Expression and purification of *Human enterovirus A* and *Human enterovirus C* (poliovirus) subunit vaccines in *E.coli*.

[00221] The Champion™ pET SUMO Expression System (Invitrogen) produces the highest levels of soluble protein in *E. coli.* It utilizes a small ubiquitin-related modifier (SUMO) fusion to enhance the solubility of expressed fusion proteins. After expression, the 11 kD SUMO moiety can be cleaved by the highly specific and active SUMO (ULP-1) protease at the carboxyl terminal, producing a native protein. Also it contains N-terminal 6xHis tag for protein detection and purification.

[00222] The construction of pET SUMO-VP0, pET SUMO- VP4-VP2-VP3 and pET SUMO-VP2-VP3-VP1 expression vector for antigenic fusion proteins of HEV71 and poliovirus, as shown in Figure 17, is as follows. The fragments of VP0, VP4-VP2-VP3 fusion and VP2-VP3-VP1 fusion were used as the antigens for HEV71 and poliovirus subunit vaccines. A SUMO motif and the 6XHistag were conjugated to the N-terminus of fusions to aid in solubilization of the protein and purification of the protein, respectively. The antigenic fusion proteins were created by a gene cloning technology comprising cloning cDNA sequences encoding respective proteins into an expression vector to form expression vectors of pET SUMO- VP0, pET SUMO- VP0, pET SUMO-VP4-VP2-VP3 and pET SUMO-VP2-VP3-VP1. The DNA fragments encoding fusion partners were PCR amplified using specific primers which consist of a start codon and a stop codon in the forward and reverse primers, respectively. Ligation of the PCR product was carried out as follows: fresh PCR product, 10X ligation buffer , pET SUMO vector (25 ng/µl) 2 µl, sterile water added to a total volume of 9 µl , and 1 µl T4 DNA ligase (4.0 Weiss units) was added and the ligation reaction incubated at 15ºC for overnight then proceeded to transforming One Shot® Mach1™-T1R (Invitrogen) competent cells. Ten (10) colonies were selected and plasmid DNA isolated from them using the PureLink™ HQ Mini Plasmid Purification Kit (Invitrogen). The plasmids were analyzed by restriction analysis to confirm the presence and the correct orientation of the insert. From the recombinants, plasmid DNA was isolated as earlier and the plasmids were transformed into BL21 (DE3) One Shot® cells (Invitrogen). The transformants were grown and induction of expression with IPTG at several time points was carried out to determine the optimal time of expression. For each time point, 500 µl was removed from the induced and uninduced cultures and each cell pellet was resuspended in 80 µl of SDS-PAGE sample buffer. After centrifuging the boiled samples, 10 µl of each sample was loaded onto an SDS-PAGE gel and electrophoresed.

[00223] To scale-up the purification of recombinant fusion protein using a HisTrap nickel column (GE Healthcare Life Sciences), the following procedure was adapted. An overnight culture (5%) was inoculated into 100-300 ml LB plus 50µg/ml kanamycin and

induced after 2hrs with 1mM IPTG. After 2hrs the cells were harvested by centrifuging at 3000g for 10 minutes. The pellet was resuspended in 10% (total volume of the culture) the binding buffer (20mM sodium phosphate, 0.5M NaCl and 20mM imidazole at pH7.4). The cells were sonicated with Misonic UltraSonicate Liquid processor for five times for a minute with a minute gap in an ice bucket. The sonicated samples were separated into soluble and insoluble form by centrifuging at 4000 rpm for 1hr at 4℃. The insoluble fraction was resuspended with binding buffer containing 6M urea. Both the soluble and insoluble fractions were centrifuged at 4000 rpm for 1hr at 4℃ then filtered through 0.22µm filter unit. A HisTrap column was equilibrated with the binding buffer and filtered samples were loaded onto the column. Next, the column was washed with binding buffer with 40mM imidazole and the recombinant protein was eluted with binding buffer containing 0.5M imidazole (6M urea for insoluble fraction). All the collected samples were tested using Coomassie blue staining protocol for proteins. Pure recombinant protein containing eluted fraction were dialysed using Merck tubing in Tris-HCl buffer. After the dialysis protein concentrations were estimated using a Bradford reagent. The native protein was generated by using SUMO protease to cleave the N-terminal peptide containing the 6xHis tag and SUMO according to manufacturer's instructions.

EXAMPLE 16. Antibodies from pooled neutralizing sera from mice immunized with SN07 retentate binds to all the components of EV71VLPs.

[00224] The coding sequences of VP1, VP2 and VP0 were separately cloned into the pET SUMO vector as described in Example 15. The individual capsid proteins were expressed in *E.coli* and purified as described in Example 15. The purified proteins VP0, VP1, VP2 and VP3 were subjected to Western blotting and probed with pooled neutralizing sera used in Example 4 at the dilution of 1:1000. Control mice sera used in Example 4 is also included in the studies.

[00225] Western blotting results in Figure 18 show that pooled neutralizing sera from mice immunized with oliogomeric antigens in the supernatant of SN07 infected Sf9 cells bind to all of the VLP components, VP0, VP1, VP2 and VP3.

EXAMPLE 17. Characterization of HEV71 VLPs - Pull-down of HEV71 VLPs from culture supernatants.

[00226] 20ml of supernatant from cells infected with recombinant baculovirus SN07 was mixed with 10ml neutralizing monoclonal antibody (EV18/4/D6-1/F1/G9) and left at room temperature for 1hr. The mixture was loaded slowly through a 1ml column of MabSelect SuRe™ (GE Health Care) recombinant Protein A, 85um agarose bead size, which was pre-equilibrated with PBS, then washed with 20mls PBS and then eluted with 0.5 ml of 0.1M glycine-HCl, pH3.0. Fractions were neutralized with 30µl of Tris-HCl, pH8.8. Elution fractions were run on SDS-PAGE followed by Coomassie blue staining and destaining.

[00227] Figure 19 shows that all of the VLP components (VP0, VP1 and VP3) are visible in the Coomassie blue stained SDS-PAGE gel.

EXAMPLE 18. Analysis of affinity column (AFC) purified HEV71VLPs.

[00228] An affinity column (AFC) was prepared with a neutralizing monoclonal antibody (EV18/4/D6-1/F1/G9), which was developed using a baculovirus SN07 supernatant retentate. The eluted fraction was analyzed by Western blotting and is shown in Figure 20. The eluted fraction in Lanes 1 and 2, probed using an anti-VP1 antibody, shows that VP1 is present in the AFC purified VLPs. The eluted fraction in Lanes 3 and 4, probed using an anti-VP2 antibody, shows that VP2 is also present in the AFC purified VLPs. The eluted fraction in Lanes 5 and 6, probed using an anti-VP2 monoclonal antibody, shows that VP2 is present in the AFC purified VLPs.

EXAMPLE 19. Electron micrograph of AFC purified HEV71 VLPs.

[00229] The fraction eluted from the affinity column (AFC) of Example 18 was evaluated by electron microscopy. An electron micrograph picture of AFC purified VLPs is shown in Figure 21. The results of the electron microscopy confirm that HEV71 structural proteins are assembled into VLPs.

EXAMPLE 20. Mice protection studies using retentate.

[00230] Antigen used was a 20x concentrated crude retentate of recombinant baculovirus SN07 prepared as described above. Vaginal plugs indicative of pregnancy was determined the morning after mating, designated as Embryonic Day E0.5. Two doses of 100 µL of retentate mixed with IMJECT® (alum) given i.p. to pregnant dams at E3.5 and, on confirmation of pregnancy by weight measurement, a second dose was given on E17.5. Initial blood sample was collected from each mouse prior to the administration of the first vaccine dose, samples were collected weekly thereafter until 14 days after viral challenge of pups. Three groups of Balb/c mice 20 mice were immunized and then challenged (20 mice), 20 mice were mock-immunised and challenged (20 mice), 5 mice non-immunised, non-challenged Five-day-old pups were infected with 50 µl of MP-26M virus containing 100 x HD_{50} of MP-26M. All infected animals were observed twice daily for clinical signs of illness until 14 days post-inoculation. Abnomal signs included failure to thrive; weight loss; runting; stomach empty of milk; lethargy; head tilt; hunched posture; ruffled fur; dehydration, hypothermia; limb paralysis. Paralysis was scored according to the following grading system:

0 – normal

1 – limb weakness but can still move limbs

2 – inability to move affected limbs

3 – quadriplegia i.e. inability to move limbs

[00231] Animals suffering grade 3 paralysis were euthanased by cervical dislocation under anaesthetic (HD_{50}). Histopathology was performed on sections from a variety of target organs.

[00232] The results of the protection study are shown in Table 2 below.

Table 2.

83% PROTECTION IN PASSIVE PROTECTION

VACCINE	NUMBER OF MATED MICE	NUMBER OF PREGNANT MICE	NUMBER OF PUBS/ LITTER	TOTAL NUMBER OF PUBS	NUMBER OF SURVIVED PUBS	SURVIVAL RATE	MEDIAN SURVIVAL (DAYS)
RETENTATE	20	2	1,5	6	5*	83%	14
ALUM/PBS	20	2	5,7	12	0	0%	5
NO VACCINE/ NO CHALLENGE	5+5	2+1	5,7,6	18	18	100%	14

[00233] Two litters of mice were born to VLP vaccinated mothers. One mother had 5 pups and showed good maternal care. The second mother had one pup that was very weak and did not show good maternal care.

[00234] It may be concluded that 83% of mice born to immunized mothers were protected from virus challenge.

[00235] A summary of the histopathological observations is shown in Table 3.

Table 3. SUMMARY OF HISTOPATHOLOGICAL OBSERVATIONS
(N= no abnormalities detected)

VACCINE	AGE	DAYS P1	MOUSE	SYMPTOMS	SPLEEN	LIVER	HEART	SKELETAL MUSCLE	SPINAL CORD	BRAIN
VLP	19	14	V87P4	NO CLINICAL SIGNS OF INFECTION	N	N(1)	N	0.5	N	N
	19	14	V87P5	NO CLINICAL SIGNS OF INFECTION	N	N(1)	N	0.5	N	N
	19	14	V87P6	NO CLINICAL SIGNS OF INFECTION	N	N(1)	N	N	N	N
ALUM/PBS	10	5	DC4	GRADE3 PARALYSIS	N	N(2)	N	3	N	N
			DC5	GRADE3 PARALYSIS	N	N(2)	N	3	N	N
			DC6	GRADE3 PARALYSIS	N	N(2)	N	03	N	N
NO VACCINE	19	NI	V116P4	NO CLINICAL SIGNS OF INFECTION	N	N(0.5)	N	N	N	N
			V116P5	NO CLINICAL SIGNS OF INFECTION	N	N(0.5)	N	N	N	N

[00236] Five mice gave birth to litters and were included in the vaccine protection study (11% pregnancy rate). The value of the humane endpoint (HD_{50}), as calculated by the method of Reed and Muench, 1938, was determined to be 2.0×10^2 $TCID_{50}$ of MP-26M. 83% of mice born to immunized mothers were protected from challenge.

[00237] The histopathology results showed that 3 mice from mothers in the HEV71-VLP immunized group which were then challenged with HEV71 virus showed no clinical signs of infection.

[00238] However, 3 mice from mothers in the mock-immunized (Alum/PBS) and which were then challenged with HEV71 virus succumbed to infection. Two mice from

mothers in the non-immunized, non-challenged group showed no clinical signs of infection.

[00239] The VLP vaccine protected one litter of infant mice (5/6 pups from immunized mothers vs 0/12 pups from mock-immunized mothers) against lethal challenge with a B3 genotype mouse-adapted strain of HEV71.

EXAMPLE 21. *Human enterovirus C* (poliovirus-PV) VLP expression.

[00240] Variations on poliovirus expression cassettes were constructed to generate poliovirus VLPs. The expression cassettes all comprised a poliovirus P1 polypeptide and differed with respect to the IRES, which IRES directs the expression of a poliovirus 3CD protease: (PV-P1+HEV71-IRES+PV-3CD); (PV-P1+EMCV-IRES+PV-3CD); (PV-P1+PV-IRES+PV-3CD). Recombinant baculoviruses harboring the poliovirus VLP expression cassettes were tested. Lysates from the baculovirus infected cells were harvested on day 3 post infection and expression of the poliovirus VP3 was evaluated using rabbit anti-PVP3 antibodies (1:2000).

[00241] Only the PV-IRES-containing construct produced a VP-3 protein of the expected size. Unlike in the PV-IRES construct, the poliovirus VP3 protein is not processed properly when the 3CD protease is under the control of HEV71-IRES or EMCV –RES and, consequently, adversely affects the production of poliovirus VLPs. Thus, the poliovirus PV-VLP expression cassette harboring the PV-IRES is very efficient for poliovirus VLP production.

EXAMPLE 22. ELISA to demonstrate poliovirus PV-VLP assembly.

[00242] To demonstrate poliovirus VLP generation from the PV-VLP expression cassette, a two sites ELISA was performed using lysates and supernatants from recombinant baculoviruses carrying a PV-VLP expression cassette wherein the PV-3CD protease is under the control of a poliovirus IRES. Sf9 cells were infected with the

recombinant baculoviruses carrying a PV-VLP expression cassette including PV-IRES. Lysates and supernatants were harvested on day 3 post infection. Formation of the poliovirus VLPs was evaluated using a two sites ELISA.

[00243] The two sites ELISA procedure was conducted by preparing protein A-purified rabbit anti-poliovirus VP3 antibodies as capture antibodies and diluting in coating buffer, 0.05M Carbonate-bicarbonate buffer, pH 9.6, to 50µg/mL. 100 µl/well of the antibodies was dispensed into NUNC immunoplates and stored at 4℃ overnight. After washing with PBST (0.05%), the immunoplates were blocked with 200 µl/well blocking buffer, 1% casein in PBS, at room temperature for 2 hr. Samples of lysates and supernatants from recombinant baculoviruses carrying a PV-VLP expression cassette wherein the PV-3CD protease is under the control of a poliovirus IRES were diluted in diluents, 0.2% casein in PBS, dispensed at 100µl/well, and were incubated at room temperature for 1 hr and then washed with PBST (0.05%).

[00244] For detection of VLPs, mouse monoclonal antibodies, anti-VP1 (Clone 5-D8/1; Dako), were prepared in 0.2% casein in PBS diluents at a 1:250 dilution. The diluted detecting monoclonal antibodies were dispensed at 100µl/well and then incubated at room temperature for 1 hr. Anti-mouse HRP conjugated 2° antibodies were prepared in 0.2% casein in PBS diluents at a 1:1000 dilution. After washing the plate with PBST (0.05%), 100µl/well of the diluted 2° antibodies were dispensed and the plate incubated at room temperature for 1 hr. The plates were washed with washing buffer, PBST (0.05%). SureBlue™ TMB 1(KPL) component microwell peroxidase substrate was dispensed at 100µl/well and incubated at room temperature for 10 min for development. 100µl/well of stop solution, 5mM NaOH, was added to stop the reaction. The absorbance of each well was read at an OD of 650nm.

[00245] The results of the two sites ELISA shown in Figure 23 demonstrate that the VP1 and VP3 proteins are in association with each other indicating that VLPs are indeed formed from the PV-VLP expression cassette.

EXAMPLE 23. Construction of HEV71 VLP Expression Cassettes with HEV71 IRES (P1+HEV71 IRES+3CD).

[00246] The schematic structure of a HEV71 VLP cassette with HEV71-IRES is shown in Figure 24. The expression cassette is similar to the construct shown in Example 1 (pSN01) except that the expression of the 3CD protease is driven by the HEV71 IRES rather than the EMCV IRES. The HEV71 IRES sequence is found in GenBank, Accession Number DQ341362.1; nucleotides 1 to 747. An HEV71 expression cassette containing vector is introduced into the baculovirus expression plasmid pDEST8 (Destination vector) by attL/aaR *in vitro* recombination using LR CLONASE®, following the instructions in the Invitrogen BAC-TO-BAC® manual (2009). Recombination between the entry vector and pDEST8 produces an expression clone. Expression clones give rise to recombinant bacmid by transforming DH10bac as described in the Invitrogen BAC-TO-BAC® manual. Transfection of the recombinant bacmid into Sf9 cells rescues the recombinant baculovirus carrying expression cassette which harbors P1, HEV71 IRES and 3CD.

[00247] Further infection of Sf9 cells can be used to evaluate expression of processed capsid proteins with rescued recombinant baculoviruses in 6 well plates. A polyclonal rabbit antiserum specific for VP1, VP0 and VP3 will identify the assembled VLPs by Western blotting of lysates and supernatants from recombinant baculovirus infected Sf9 cells.

EXAMPLE 24. Construction of HEV71 VLP Expression Cassettes with PV-IRES (P1+PV-IRES+3CD).

[00248] The expression cassette is similar to the expression cassette in Example 24 except that the expression of the 3CD protease is driven by a poliovirus IRES (PV-IRES) rather than an HEV71-IRES. The poliovirus IRES sequence is found in GenBank, Accession Number V01150.12 ; nucleotides 1 to 628.

[00249] An HEV71 expression cassette containing vector is introduced into the baculovirus expression plasmid pDEST8 (Destination vector) by attL/aaR *in vitro* recombination using LR CLONASE®, following the instructions in the Invitrogen BAC-TO-BAC® manual (2009). Recombination reaction between entry vector and pDEST8 is set up to produce an expression clone. An expression clone give rises to recombinant bacmid by transforming DH10bac as described in the Invitrogen BAC-TO-BAC® manual. Transfection of the recombinant bacmid into Sf9 cells rescues the recombinant baculovirus carrying expression cassette which harbors P1, PV IRES and 3CD.

[00250] Further infection of Sf9 cells can be used to evaluate for expression of processed capsid proteins with rescued recombinant baculoviruses in 6 well plates. A polyclonal rabbit antiserum specific for VP1, VP0 and VP3 will identify the assembled VLPs by Western blotting of lysates and supernatants from recombinant baculovirus infected Sf9 cells.

CLAIMS

1. A vaccine comprising one or more immunologically active antigens comprising one or more human *Enterovirus* polypeptides selected from VP0, VP1, VP2, VP3, VP4, and immunologically active fragments thereof.

2. The vaccine of Claim 1, which elicits a protective and/or neutralizing immune response directed against a human *Enterovirus*.

3. The vaccine of Claim 1, wherein the human *Enterovirus* is selected from *Human enterovirus A*, *Human enterovirus B, Human enterovirus C* and *Human enterovirus D*.

4. The vaccine of Claim 3, wherein the *Human enterovirus A* is selected from *Human enterovirus* 71 (HEV71) and *Coxsackievirus* A16, wherein the *Human enterovirus B* is selected from *Coxsackievirus* B and echovirus, wherein the *Human enterovirus C* is selected from *Human poliovirus* 1, *Human poliovirus* 2 and *Human poliovirus* 3, and wherein the *Human enterovirus D* is *EV* 68.

5. The vaccine of Claim 1, wherein the vaccine comprises an immunologically active *Enterovirus* VP0 polypeptide.

6. The vaccine of Claim 1, wherein the vaccine comprises an immunologically active *Enterovirus* VP2 polypeptide.

7. The vaccine of Claim 1, wherein the vaccine comprises an immunologically active *Enterovirus* VP3 polypeptide.

8. The vaccine of Claim 1, wherein the vaccine comprises an immunologically active *Enterovirus* VP1 polypeptide.

9. The vaccine of Claim 1, wherein the vaccine comprises polypeptides from one or more *Enterovirus* species or serotype.

10. The vaccine of Claim 9, wherein the species or serotype may be *Human enterovirus A* selected from HEV71 and *Coxsackievirus* A16.

11. The vaccine of Claim 9, wherein the species or serotype may be *Human enterovirus C* selected from PV-1, PV-2 and PV-3.

12. The vaccine of Claim 1, wherein the one or more immunologically active antigens comprising one or more human *Enterovirus* polypeptides selected from VP0, VP1, VP2, VP3, VP4, and immunologically active fragments thereof, are in the form of a virus-like particle, capsomer, complex and/or aggregate.

13. The vaccine of Claim 12, wherein the virus-like particle comprises an immunologically active *Enterovirus* VP0 polypeptide.

14. The vaccine of Claim 12, wherein the virus-like particle comprises an immunologically active *Enterovirus* VP2 polypeptide.

15. The vaccine of Claim 12, wherein the virus-like particle comprises an immunologically active *Enterovirus* VP3 polypeptide.

16. The vaccine of Claim 12, wherein the virus-like particle comprises an immunologically active *Enterovirus* VP1 polypeptide.

17. A method of vaccinating a subject against an *Enterovirus* infection, comprising administering to the subject a vaccine comprising one or more

immunologically active antigens comprising one or more human *Enterovirus* polypeptides selected from VP0, VP1, VP2, VP3, VP4, and immunologically active fragments thereof, in an amount effective to elicit a protective and/or neutralizing immune response when administered to the subject.

18. The method of Claim 17, wherein the vaccine comprises an immunologically active *Enterovirus* VP0 polypeptide.

19. The method of Claim 17, wherein the vaccine comprises an immunologically active *Enterovirus* VP1 polypeptide.

20. The method of Claim 17, wherein the vaccine comprises an immunologically active *Enterovirus* VP3 polypeptide.

21. The method of Claim 17, wherein the vaccine comprises an immunologically active *Enterovirus* VP2 polypeptide.

22. The method of Claim 17, wherein the one or more immunologically active antigens comprising one or more human *Enterovirus* polypeptides selected from VP0, VP1, VP2, VP3, VP4, and immunologically active fragments thereof, are in the form of a virus-like particle, capsomer, complex and/or aggregate.

23. The method of Claim 22, wherein the virus-like particle comprises an immunologically active *Enterovirus* VP0 polypeptide.

24. The method of Claim 22, wherein the virus-like particle comprises an immunologically active *Enterovirus* VP2 polypeptide.

25. The method of Claim 22, wherein the virus-like particle comprises an immunologically active *Enterovirus* VP3 polypeptide.

26. The method of Claim 22, wherein the virus-like particle comprises an immunologically active *Enterovirus* VP1 polypeptide.

27. The method of Claim 17, wherein the vaccine comprises polypeptides from one or more *Enterovirus* species or serotype.

28. The method of Claim 27, wherein the *Enterovirus* species or serotype may be *Human enterovirus A* selected from HEV71 and C*oxsackievirus* A16.

29. The method of Claim 27, wherein the *Enterovirus* species or serotype may be *Human enterovirus C* selected from PV-1, PV-2 and PV-3.

30. An expression cassette comprising a promoter operably linked to a nucleic acid encoding a human *Enterovirus* P1 polypeptide, an Internal Ribosome Entry Site (IRES), and a nucleic acid encoding a human *Enterovirus* 3CD protease.

31. The expression cassette of Claim 30, wherein the polypeptide is human *Enterovirus* P1.

32. The expression cassette of Claim 30, wherein the human *Enterovirus* 3CD protease processes the human *Enterovirus* P1 polypeptide.

33. The expression cassette of Claim 32, wherein the processed human *Enterovirus* P1 polypeptides associate to form virus like particles, capsomers, complexes and/or aggregates.

34. The expression cassette of Claim 30, wherein the human *Enterovirus* P1 polypeptide comprises combinations of polypeptides selected fromVP0, VP1, VP2, VP3, VP4, and immunologically active fragments thereof.

35. The expression cassette of Claim 30, wherein the human *Enterovirus* is selected from *Human enterovirus A* and *Human enterovirus C*.

36. The expression cassette of Claim 35, wherein the *Human Enterovirus* A is selected from HEV71 and *Coxsackievirus* A16.

37. The expression cassette of Claim 35, wherein the *Human Enterovirus* C is selected from Human poliovirus 1 (PV-1), Human poliovirus 2 (PV-2) and Human poliovirus 3 (PV-3).

38. The expression cassette of Claim 30, wherein the IRES is derived from *Encephalomyocarditis virus* (EMCV).

39. The expression cassette of Claim 38, wherein the IRES derived from *Encephalomyocarditis virus* (EMCV) has been genetically modified to reduce IRES activity.

40. The expression cassette of Claim 39, wherein the IRES derived from EMCV has been genetically modified by adding one nucleotide (A7) to the A6 bifurcation loop in the JK segment.

41. The expression cassette of Claim 30, wherein the IRES is derived from a human *Enterovirus*.

42. The expression cassette of Claim 30, wherein the promoter is a eukaryotic promoter.

43. The expression cassette of Claim 42, wherein the eukaryotic promoter is a polyhedrin promoter.

44. The expression cassette of Claim 30, wherein the promoter is operably linked to a nucleic acid encoding a *Human enterovirus A* P1 polypeptide, an EMCV IRES, and a *Human enterovirus A* 3CD protease.

45. The expression cassette of Claim 44, wherein the EMCV IRES has been mutated to reduce IRES activity.

46. The expression cassette of Claim 30, wherein the promoter is operably linked to a nucleic acid encoding a *Human enterovirus A* P1 polypeptide, an HEV71 IRES, and a *Human enterovirus A* 3CD protease.

47. The expression cassette of Claim 30, wherein the promoter is operably linked to a nucleic acid encoding a *Human enterovirus A* P1 polypeptide, a *Human enterovirus C* IRES, and a *Human enterovirus A* 3CD protease.

48. The expression cassette of Claim 30, wherein the promoter is operably linked to a nucleic acid encoding a *Human enterovirus C* P1 polypeptide, a *Human enterovirus C* IRES, and a *Human enterovirus C* 3CD protease.

49. The expression cassette of Claim 30, wherein the promoter is operably linked to a nucleic acid encoding a *Human enterovirus C* P1 polypeptide, an HEV71IRES, and a *Human enterovirus C* 3CD protease.

50. The expression cassette of Claim 30, wherein the promoter is operably linked to a nucleic acid encoding a *Human enterovirus C* P1 polypeptide, an EMCV IRES, and a *Human enterovirus C* 3CD protease.

51. A method of making a vaccine comprising introducing the expression cassette of Claim 30 into a host cell, culturing the host cell for a period of time sufficient to produce the polypeptides of the expression cassette and recovering human *Enterovirus* polypeptides from the host cell and/or culture supernatant.

52. The method of Claim 51, wherein the host cell is a eukaryotic cell.

53. The method of Claim 52, wherein the eukaryotic cell is selected from insect cells, mammalian cell lines and yeast cells.

54. The method of Claim 53, wherein the insect cells are selected from *Spodoptera frugiperda*, *Trichoplusia ni*, drosophila, and mosquito cells derived from *Aedes albopictus*.

55. The method of Claim 53, wherein the mammalian cell lines are selected from CHO, HEK 293, COS-1, HeLa, Vero and NIH3T3.

ABSTRACT

The instant invention provides materials and methods for producing immunologically active antigens derived from members of the *Picornaviridae* virus family. The picornavirus antigens of the invention may be in a form for use as a vaccine administered to a subject in a therapeutic treatment or for the prevention of a picornavirus infection. The picornavirus antigens of the invention may be in the form of an immunogenic composition for use in vaccines which are administered for the prevention of an *Enterovirus* infection. The instant invention further encompasses immunogenic compositions comprising *Human enterovirus A, Human enterovirus B*, *Human enterovirus C*, *Human enterovirus D* antigens and their use in vaccines for the prevention of an *Enterovirus* infection.

ANTÍGENOS Y VACUNAS DIRIGIDOS CONTRA ENTEROVIRUS HUMANOS

Campo de la invención

La presente invención se relaciona con virus de la familia de *Picornaviridae* y específicamente, con antígenos y vacunas que pueden ser efectivos en la prevención y en el tratamiento de infecciones causadas por tales virus.

Antecedentes de la invención

Los picornavirus son una familia diversa de virus que causan numerosas en enfermedades comunes. De la familia de los *Picornaviridae*, los virus del género *Enterovirus*, que están todos estrechamente relacionados, son importantes para las numerosas enfermedades que causan.

Los virus del género *Enterovirus* afectan a millones de personas en todo el mundo anualmente y suelen hallarse en las secreciones respiratorias (por ejemplo, la saliva, el esputo, o el moco nasal) y las deposiciones de una persona infectada. El *enterovirus* infecta el intestino, de allí la derivación de su nombre de la raíz “entérico”. Históricamente, la poliomielitis fue la enfermedad más importante causada por un enterovirus, es decir, el poliovirus. Existen 62 enterovirus

que no son poliovirus que pueden causar enfermedades en humanos: 23 virus Coxsackie A, 6 virus Coxsackie B, 28 echovirus, y otros 5 enterovirus. Los poliovirus, así como los virus Coxsackie y los echovirus, se propagan a través de la vía fecal-oral. La infección puede derivar en una amplia variedad de síntomas que van desde la enfermedad respiratoria leve (resfrío común), la enfermedad de las manos, los pies y la boca, la conjuntivitis hemorrágica, la meningitis aséptica, la miocarditis, la enfermedad similar a la sepsis neonatal y la parálisis fláccida aguda.

De los picornavirus, el *Enterovirus* representa un género de grupo grande y diverso de virus de ARN pequeños caracterizados por un ARN genómico de una sola cadena positiva. Todos los enterovirus contienen genoma de aproximadamente 7.500 bases y se sabe que tienen una alta velocidad de mutación debido a la replicación de baja fidelidad y a la recombinación frecuente. Después de la infección de la célula huésped, el genoma se traduce en una forma independiente del cierre en una sola poliproteína, que posteriormente se procesa por proteasas codificadas por virus en las proteínas de la cápside estructural y las proteínas no estructurales, que están involucradas principalmente en la replicación del virus.

Los enterovirus están asociados a varias enfermedades humanas y de los mamíferos. Los estudios serológicos han distinguido 68 serotipos humanos de *Enterovirus* sobre la base de ensayos de neutralización de anticuerpos. Otras variantes antigénicas se han definido dentro de varios de los serotipos sobre la base de la neutralización cruzada reducida o no recíproca entre variantes de cepas. Sobre la base de su patogénesis en humanos y animales, los *enterovirus* se clasificaron originalmente en cuatro grupos, los poliovirus, los virus Coxsackie A (CA), los virus Coxsackie B (CB) y los echovirus, pero se advirtió rápidamente que había superposiciones importantes en las propiedades biológicas de los virus en los diferentes grupos.

El género de los *Enterovirus* incluye las siguientes diez especies:

- *Enterovirus bovino*
- *Enterovirus humano A*
- *Enterovirus humano B*
- *Enterovirus humano C*
- *Enterovirus humano D*
- *Rinovirus humano A*
- *Rinovirus humano B*
- *Rinovirus humano C*
- *Enterovirus porcino B*

- *Enterovirus de simio A*

Dentro de estas diez especies existen diversos serotipos, por ejemplo:

Los serotipos de *Enterovirus* HEV71, EV-76, EV-89, EV-90, EV-91, EV-92 y virus Coxsackie A16 se hallan en la especie de *enterovirus humano A*.

Los serotipos de virus *Coxsackie* B1 (CV-B1), CV-B2, CV-B3, CV-B4, CV-B5 (que incluyen el virus de la enfermedad vesicular porcina [SVDV]), CV-B6, CV-A9, echovirus 1 (E-1; que incluye E-8), E-2, E-3, E-4, E-5, E-6, E-7, E-9 (que incluye CV-A23), E-11, E-12, E-13, E-14, E-15, E-16, E-17, E-18, E-19, E-20, E-21, E-24, E-25, E-26, E-27, E-29, E-30, E-31, E-32, E-33, enterovirus B69 (EV-B69), EV-B73, EV-B74, EV-B75, EV-B77, EV-B78, EV-B79, EV-B80, EV-B81, EV-B82, EV-B83, EV-B84, EV-B85, EV-B86, EV-B87, EV-B88, EV-B93, EV-B97, EV-B98, EV-B100, EV-B101, EV-B106, EV-B107, EV-B110 (de un chimpancé) y el enterovirus del simio SA5, se hallan en la especie *enterovirus humano B*.

Los serotipos EV-95, EV-96, EV-99, EV-102, EV-104, EV-105, y EV-109 se hallan en la especie *enterovirus humano C*.

Los serotipos EV-68, EV-70, y EV-94 se hallan en la especie *enterovirus humano D*.

Los serotipos de poliovirus PV-1, PV-2, y PV-3 se hallan en la especie *enterovirus humano C*.

Las enfermedades causadas por la infección por enterovirus incluyen la poliomielitis que es la enfermedad más notable causada por la infección por enterovirus. La enfermedad febril no específica es, sin embargo, la presentación más común de una infección por enterovirus.

Los enterovirus son las causas más comunes de la meningitis aséptica en los niños. En Estados Unidos de América, los enterovirus son los responsables de 30.000 a 50.000 casos meningitis. La encefalitis es una manifestación rara de una infección por enterovirus; cuando ocurre, el *Enterovirus* que se halla con más frecuencia que es el causante de la encefalitis es el echovirus 9.

La pleurodinia causada por enterovirus se caracteriza por un dolor paroxístico grave en el pecho y el abdomen, junto con fiebre, y algunas veces náuseas, dolor de cabeza y emesis.

La pericarditis y/o la miocarditis normalmente son causadas por enterovirus. También se han informado arritmias, la insuficiencia cardíaca y el infarto de miocardio.

La conjuntivitis hemorrágica aguda puede ser causada por enterovirus.

La enfermedad de las manos, de los pies y de la boca es la enfermedad infantil más comúnmente causada por la infección por el virus Coxsackie A o HEV71.

Un estudio de 2007 sugirió que las infecciones respiratorias o gastrointestinales agudos asociadas a los enterovirus pueden ser un factor en el síndrome de fatiga crónica.

Todos los miembros del género *Enterovirus,* que incluyen HEV71, poliovirus y virus *Coxsackie* A tienen un genoma de ARN de sentido positivo de una sola cadena que tiene un solo marco de lectura abierto que codifica una polipropteína, P1, que comprende las proteínas de la cápside VP4, VP2, VP3 y VP1 y varias proteínas no estructurales que incluyen las proteasas virales 3C y 3CD que son responsables para descomponer la poliproteína P1 en las proteínas de la cápside VP1, VP3 y VP0, cuyo VP0 finalmente se descompone en VP2 y VP4. Las proteínas

de la cápside pueden ensamblarse en partículas similares a un virus (VLP).

Los *enterovirus humanos 71* (HEV71) y el virus Coxsackie A son serotipos de *Enterovirus* notables como los agentes causantes principales de la enfermedad de las manos, de los pies y de la boca (HFMD) y HEV71 algunas veces se asocia a enfermedades graves del sistema nervioso central. HEV71 fue el primero que se aisló y se caracterizó a partir de casos de la enfermedad neurológica en California en 1989. Hasta la fecha, se sabe poco acerca de los mecanismos moleculares de la respuesta del huésped a la infección por HEV71, pero aumenta en el nivel de mARN que codifican quimioquinas, proteínas involucradas en la degradación de las proteínas, proteínas de complemento y proteínas apoptóticas han sido implicadas.

La Enfermedad de las Manos, de los Pies y de la Boca (HFMD) es una enfermedad autolimitante, común causada por un grupo de especies de enterovirus (familia Picornaviridae) tal como el virus *Coxsackie humano* A16 (CVA16), el virus *Coxsackie* A10 (CVA10) y el *enterovirus humano A* 71 (HEV71). El virus se segrega en las heces y también se halla en secreciones de la faringe. La transmisión está asociada al contacto cercano entre los niños y a través de la contaminación ambiental. La enfermedad se caracteriza por un inicio agudo de fiebre con

una erupción en las palmas de las manos, las plantas de los pies, las nalgas y las rodillas y las vesículas sobre las membranas bucales que habitualmente se resuelven en 7-10 días. Solamente una pequeña proporción de niños con HFMD desarrollan una enfermedad grave.

La enfermedad grave que involucra principalmente los sistemas neurológicos y cardiovasculares que se manifiestan como síndromes tales como la meningitis, la encefalitis, la parálisis fláccida aguda, el edema pulmonar y la insuficiencia cardíaca generalmente ocurren solamente con la infección por HEV71. En la Región del Asia-Pacífico, el síndrome neurológico más devastador es la encefalitis del tronco encefálico, que tiene un índice de mortalidad del 40-80 por ciento. Los niños con HFMD grave puede tomar meses para recuperarse y en algunos casos el daño neurológico puede ser permanente. Actualmente, no existe un tratamiento antiviral específico para HFMD y ninguna vacuna previene la infección por enterovirus diferente de la poliomielitis.

El HEV17 fue el primero que se aisló de un niño que murió de encefalitis en California en 1969 y el primero que informó en 1974. Si bien el virus se ha detectado en todo el mundo desde entonces, las epidemias regionales recientes de HFMD en Asia han creado una preocupación de que pueden estar surgiendo más

formas patogénicas de HEV71 en la región. El primer reconocimiento de un brote de HFMD con un alto número muertes fue en Sarawak, Malasia en 1997. El virus asociado al brote luego fue HEV71. Taiwán informó 129.106 casos de HFMD en una epidemia de 1998 con 405 que tienen una enfermedad grave con 78 muertes. Singapur informó una epidemia de 9000 casos con 7 muertes durante 2000-2001 y desde entonces ha experimentado epidemias recurrentes cada dos o tres años. Durante los primeros 8 meses de 2008, Singapur informó 19.530 casos y una muerte por HFMD. Desde entonces se han informado brotes de HEV71 regularmente en Singapur, Tailandia, Malasia, Taiwán, Japón, Corea y Vietnam.

China informó 83.344 casos con 17 muertes en 2007 y en 2008 experimentó un brote importante en la Ciudad de Fuyang en la Provincia de Anhui que se propaga en muchas partes de China. Estos importantes brotes fueron cubiertos ampliamente por la prensa, que destacó las preocupaciones de los padres por la salud de sus hijos y el trastorno social causado por el cierre de escuelas y de guarderías infantiles por los ministerios de salud pública en un intento de romper la cadena de transmisión. Desde entonces China ha informado grandes brotes anualmente.

Con respecto a la enfermedad causada por otros miembros de la familia *Picornaviridae,* la infección natural y el predominio de la poliomielitis han ocurrido exclusivamente en el ser humano desde la antigüedad como una enfermedad infecciosa. Un gran número de humanos aún se infectan con poliomielitis anualmente en países en vías de desarrollo. De ahí que la erradicación de la poliomielitis sea un proceso permanente.

Los poliovirus se clasificaban anteriormente como una especie que pertenece al género *Enterovirus* de la familia *Picornaviridae.* La especie Poliovirus se ha eliminado del género *Enterovirus.* Los poliovirus se clasifican como serotipos, poliovirus humano 1 (PV-1), poliovirus humano 2 (PV-2), y poliovirus humano 3 (PV-3), y se los considera subtipos de la especie *enterovirus humano C*, en género *Enterovirus* en la familia *Picornaviridae*. La especie de tipo del género *Enterovirus* se cambió de Poliovirus a *enterovirus humano C* en 2008.

Los tres subtipos de la especie *enterovirus humano C*, PV-1, PV-2 y PV-3, se caracterizan por una proteína de la cápside ligeramente diferente. Las proteínas de la cápside definen la especificidad del receptor celular y la antigenicidad del virus. PV-1 es la forma más común enfrentada en la naturaleza,

aunque las tres formas son muy infecciosas y pueden afectar a la médula espinal y causar poliomielitis.

La infección con el *enterovirus humano C* ha sido un problema ampliamente difundido y las vacunas de virus enteros inactivados se han usado para la inmunización masiva y están actualmente disponibles. Se han obtenido buenos resultados con vacunas para la poliomielitis inactivadas que se pueden preparar de acuerdo con un método que ha sido desarrollado por Salk y ha sido mejorado posteriormente en varios aspectos. Generalmente, estas vacunas contienen una mezcla del virus de la poliomielitis inactivada de las cepas Mahoney, MEF1 y Saukett.

Aunque se ha producido un *enterovirus humano C* atenuado y se lo ha usado como una vacuna para la poliomielitis oral atenuada, el *enterovirus humano C* atenuado puede ser peligroso debido a la posible reversión de la patogenicidad (neurovirulencia basada en la parálisis) en personas a las que se administra, o que están en contacto con virus enteros. Por lo tanto, existe una necesidad de una vacuna para la poliomielitis segura y efectiva que no tenga tal patogenicidad.

Al igual que todos los enterovirus, se han identificado cuatro polipéptidos de la cubierta/cápside del *enterovirus humano C* y se los ha designado VP1, VP2, VP3 y VP4, que se asocian para formar una cápside del virus icosahédrico. Normalmente, la vacunación con polipéptidos individuales del *enterovirus humano C* ha mostrado que los polipéptidos aislados no son capaces crear anticuerpos neutralizantes en humanos y animales (Meloen, et al., J. Gen. Virol. 45:761-763, 1979).

La Patente Estadounidense N° 4.508.708 enseña que los polipéptidos individuales del virus de la poliomielitis y el virus de la enfermedad de las manos, de los pies y de la boca, VP1, VP2, VP3 y VP4, no son capaces de crear anticuerpos neutralizantes en humanos y animales y que, entre los polipéptidos individuales del virus de las manos, de los pies y de la boca, solamente VP1 posee esta capacidad. La Patente Estadounidense N° 4.508.708 demuestra que entre los polipéptidos VP1, VP2 y VP3 del virión de MEF1 tipo 2 de *enterovirus humano C*, solamente el VP3 es capaz de inducir los anticuerpos neutralizantes, aunque el titulador del anticuerpo es bajo. Se ha descubierto, sin embargo, que los VP1, VP2 y VP3 son capaces de inducir anticuerpos neutralizantes solamente cuando la inmunización se lleva a cabo con una preparación que contiene arildona, un agente antiviral de amplio espectro que se ha mostrado que inhibe selectivamente

la replicación de los picornavirus (Langford, et al. Agentes Antimicrobianos y Química 28:578-580, 1985).

Por lo tanto, el problema que se debe resolver es la preparación de una vacuna efectiva que proporcione inmunidad protectora contra una infección por enterovirus y sin usar compuestos antivirales. Los enterovirus humanos para los cuales se desea la protección son, por ejemplo, el *enterovirus humano A*, que incluye el virus *Coxsackie* A16 y el *enterovirus humano* 71; el *enterovirus humano B*, que incluye los serotipos del virus Coxsackie B, los echovirus y serotipos de enterovirus; el *enterovirus humano C*, que incluye el *poliovirus humano* 1, el *poliovirus humano* 2 y el *poliovirus humano* 3; así como el *enterovirus humano D*, que incluye el EV 68.

Con los propósitos de la presente invención, los expertos en el arte entienden por una vacuna y la misma se define como un material profiláctico o terapéutico que contiene antígenos derivados de uno o más organismos patogénicos que, al ser administrados a un sujeto humano o a un animal, estimulan la inmunidad activa y protegen contra la infección con estos organismos u organismos relacionados (es decir, producen inmunidad protectora).

Además, los expertos en el arte pueden entender bien la inmunidad protectora. No obstante, la inmunidad protectora comprende, por lo menos, la inducción, o la producción de anticuerpos neutralizantes y/o la respuesta inmune a las células T que neutraliza el virus.

Se reconocerá bien en el arte de las vacunas, que no está claro si un antígeno derivado de un patógeno produce inmunidad protectora. Ellis (Capítulo 29 de Vacunas, Plotkin, et al. (eds) WB Saunders, Philadelphia, en la página 571, 1998) ejemplifica este problema en la cita “la clave para el problema (del desarrollo de vacunas) es la identificación de ese componente de proteína de un virus o patógeno microbiano que solo puede producir la producción de anticuerpos protectores…, y por lo tanto proteger al huésped del ataque del patógeno.”

Un enfoque para elaborar vacunas mejoradas contra picornavirus imitaría la estructura de la cápside del virus o sus componentes que pueden producir anticuerpos protectores tales como aquellos producidos con una vacuna de virus entero muerto. Este tipo de enfoque es más seguro que un enfoque de vacuna muerta, inactivada o atenuada porque no existe ninguna oportunidad para la reversión.

Todos los picornavirus comparten la misma estructura genómica, que incluye 4 genes estructurales dentro del gene P1: VP1, VP2, VP3, y VP4, el VP4 y el VP2 se expresan juntos como VP0, y las proteasas virales dentro de los genes de 3C y 3D. La proteasa viral descompone el gene P1, permitiendo de ese modo que el virus se ensamble en las partículas similares al virus (VLP), capsómeros del virus, complejos y/o antígenos de enterovirus.

Se han propuesto vacunas con éxito diferente. Se ha propuesto usar vacunas de subunidades que comprenden la proteína de la cápside principal, VP1, de enterovirus, como la base de vacunas para la prevención y el tratamiento de infecciones por enterovirus, que incluyen infecciones por HEV71 (Wu, et al., 2001).

Con respecto a la profilaxis contra una infección por enterovirus, es posible prever un enfoque de vacuna de virus muertos, que se ha mostrado que producen anticuerpos protectores. Los tituladores de virus bajos logrados en general hacen de la fabricación de tales vacunas de enterovirus un desafío.

La presente invención se refiere a vacunas que incluyen proteínas de la cubierta/cápside antigénicas de virus de la

familia de *Picornaviridae* y cuyas vacunas no tienen el ARN del virus que puede contribuir a la neurovirulencia. Las vacunas de la invención pueden comprender como antígenos: los polipéptidos P1 o VP0, o las proteínas de la cápside (VP), designados VP1, VP2, VP3 y/o VP4, o fragmentos inmunológica o biológicamente activos de ellos que producen anticuerpos neutralizantes contra enterovirus.

La presente invención se relaciona con vacunas en las cuales los antígenos de picornavirus están presentes en la forma de uno o más polipéptidos de picornavirus, especialmente los péptidos de *Enterovirus humanos* VP2, VP3 o VP0, sus fragmentos inmunogénicos y/o sus determinantes antigénicos. Los polipéptidos de picornavirus se pueden obtener mediante la síntesis química o por medio de técnicas de ADN recombinante usando secuencias de aminoácidos o de ácidos nucleicos de *Enterovirus humanos.*

Resumen de la invención

Una vacuna que comprende uno o más antígenos inmunológicamente activos que comprende: uno o más polipéptidos de Enterovirus humanos seleccionados de VP0, VP1, VP2, VP3, VP4 y sus fragmentos inmunológicamente activos, tal

vacuna produce una respuesta protectora y/o inmune neutralizante dirigida contra un Enterovirus humano, tal

vacuna, en donde el Enterovirus humano se selecciona del enterovirus humano A, el enterovirus humano B, el enterovirus humano C y el enterovirus humano D, tal

vacuna, en donde el enterovirus humano A se selecciona del enterovirus humano 71 (HEV71) y el virus Coxsackie A16, en donde el enterovirus humano B se selecciona del virus Coxsackie B y el echovirus, en donde el enterovirus humano C se selecciona del poliovirus humano 1, el poliovirus humano 2 y el poliovirus humano 3, y en donde el enterovirus humano D es EV 68, tal

vacuna, en donde la vacuna comprende un polipéptido de enterovirus activo VP0, tal

vacuna, en donde la vacuna comprende un polipéptido de enterovirus inmunológicamente activo VP2, tal

vacuna, en donde la vacuna comprende un polipéptido de enterovirus inmunológicamente activo VP3, tal

vacuna, en donde la vacuna comprende un polipéptido de enterovirus inmunológicamente activo VP1, tal

vacuna, en donde la vacuna comprende polipéptidos de una o más especies o serotipos de enterovirus, tal

vacuna, en donde la especie o el serotipo puede ser el enterovirus humano Ha seleccionado de HEV71 y el virus Cosxsackie A16, tal

vacuna, en donde la especie o el serotipo puede ser el enterovirus humano C seleccionado de PV-1, PV-2 y PV-3, tal

vacuna, en donde uno o más antígenos inmunológicamente activos que comprenden uno o más polipéptidos de Enterovirus humanos seleccionados de VP0, VP1, VP2, VP3, VP4 y sus fragmentos inmunológicamente activos, están en la forma de una partícula similar a un virus, capsómero, complejo y/o agregado, tal

vacuna, en donde la partícula similar a un virus comprende un polipéptido de enterovirus inmunológicamente activo VP0, tal

vacuna, en donde la partícula similar a un virus comprende un polipéptido de enterovirus inmunológicamente activo VP2, tal

vacuna, en donde la partícula similar a un virus comprende un polipéptido de enterovirus inmunológicamente activo VP3, tal

vacuna, en donde la partícula similar a un virus comprende un polipéptido de enterovirus inmunológicamente activo VP1, un

método para vacunar a un sujeto contra una infección por un Enterovirus, que comprende administrar al sujeto una vacuna que comprende uno o más antígenos inmunológicamente activos que comprenden uno o más polipéptidos de Enterovirus humanos seleccionados de VP0, VP1, VP2, VP3, VP4 y sus fragmentos inmunológicamente activos, en una cantidad efectiva para producir una respuesta protectora y/o inmune neutralizante cuando se administra al sujeto, tal

método, en donde la vacuna comprende un polipéptido de enterovirus inmunológicamente activo VP0, tal

método, en donde la vacuna comprende un polipéptido de enterovirus inmunológicamente activo VP1, tal

método, en donde la vacuna comprende un polipéptido de enterovirus inmunológicamente activo VP3, tal

método, en donde la vacuna comprende un polipéptido de enterovirus inmunológicamente activo VP2, tal como

método, en donde uno o más antígenos inmunológicamente activos comprenden uno o más polipéptidos de enterovirus humanos seleccionados de VP0, VP1, PV2, VP3, VP4 y sus fragmentos inmunológicamente activos, están en la forma de una partícula similar a un virus, capsómero, complejo y/o agregado, tal

método, en donde la partícula similar a un virus comprende un polipéptido de enterovirus inmunológicamente activo VP0, tal

método, en donde la partícula similar a un virus comprende un polipéptido de enterovirus inmunológicamente activo VP2, tal

método, en donde la partícula similar a un virus comprende un polipéptido de enterovirus inmunológicamente activo, tal

método, en donde la partícula similar a un virus comprende un polipéptido de enterovirus inmunológicamente activo VP1, tal

método, en donde la vacuna comprende polipéptidos de una o más especies o serotipos de enterovirus, tal

método, en donde la especie o serotipo de enterovirus puede ser el enterovirus humano Ha seleccionado de HEV71 y virus Coxsackie A16, tal

método, en donde la especie o el serotipo de enterovirus puede ser el enterovirus humano C seleccionado de PV-1, PV-2 y PV-3, un

cassette de expresión que comprende un promotor unido en forma operativa a un ácido nucleico que codifica un polipéptido de enterovirus humano P1, un Sitio de Entrada de Ribosoma Interno (IRES) y un ácido nucleico que codifica una proteasa de SCD de enterovirus humano, tal

cassette de expresión, en donde el polipéptido es un P1 de Enterovirus humano, tal

cassette de expresión, en donde la proteasa de SCD de enterovirus humano procesa el polipéptido P1 de enterovirus humano, tal

cassette de expresión, en donde los polipéptidos P1 de enterovirus humano procesados se asocian para formar partículas similares a un virus, capsómeros, complejos y/o agregados, tal

cassette de expresión, en donde el polipéptido P1 de enterovirus humano comprende combinaciones de polipéptidos seleccionados de VP0, VP1, VP2, VP3, VP4 y sus fragmentos inmunológicamente activos, tal

cassette de expresión, en donde el enterovirus humano se selecciona del enterovirus humano A y el enterovirus humano C, tal

cassette de expresión, en donde el enterovirus humano A se selecciona de HEV71 y el virus Coxsackie A16, tal

cassette de expresión, en donde el enterovirus humano C se selecciona del poliovirus humano 1 (PV-1), el poliovirus humano 2 (PV-2), el poliovirus humano 3 (PV-3), tal

cassette de expresión, en donde el IRES se obtiene del virus de la Encefalomiocarditis (EMCV), tal

cassette de expresión, en donde el IRES obtenido del virus de la Encefalomiocarditis (EMCV) se ha modificado genéticamente para reducir la actividad del IRES, tal

cassette de expresión, en donde el IRES obtenido del EMCV se ha modificado genéticamente agregando un nucleótido (A7) al bucle de bifurcación A6 en el segmento JK, tal

cassette de expresión, en donde el IRES se obtiene de un enterovirus humano, tal

cassette de expresión, en donde el promotor es un promotor eucariota, tal

cassette de expresión, en donde el promotor eucariota es un promotor de polihedrina, tal

cassette de expresión, en donde el promotor está unido en forma operativa a un ácido nucleico que codifica un polipéptido P1 de enterovirus A humano, un IRES de EMCV, y una proteasa 3CD de enterovirus humano A, tal

cassette de expresión, en donde el IRES de EMCV se ha mutado para reducir la actividad del IRES, tal

cassette de expresión, en donde el promotor está unido operativamente a un ácido nucleico que codifica un polipéptido P1 de enterovirus humano A, un IRES de HEV71 y una proteasa 3CD de enterovirus humano A, tal

cassette de expresión, en donde el promotor está unido operativamente a un ácido nucleico que codifica un polipéptido P1 de enterovirus humano A, un IRES de enterovirus humano C y una proteasa 3CD de enterovirus humano A, tal

cassette de expresión, en donde l promotor está unido operativamente a un ácido nucleico que codifica un polipéptido P1 de enterovirus humano C, un IRES de enterovirus humano C, y una proteasa 3CD de enterovirus humano C, tal

cassette de expresión, en donde el promotor está unido operativamente a un ácido nucleico de codifica un polipéptido P1 de enterovirus humano C, un IRES de HEV71 y una proteasa 3CD de enterovirus humano C, tal

cassette de expresión, en donde el promotor está unido operativamente a un ácido nucleico que codifica un polipéptido P1 de enterovirus humano C, un IRES de EMCV y una proteasa 3CD de enterovirus humano C, tal

método para elaborar una vacuna que comprende introducir un cassette de expresión de VLP en una célula huésped, cultivar la célula huésped durante un período de tiempo suficiente para producir los polipéptidos del cassette de expresión y

recuperar polipéptidos de enterovirus humanos de la célula huésped y/o del sobrenadante de cultivo, tal

método, en donde la célula huésped es una célula eucariota, tal

método, en donde la célula eucariota se selecciona de células de insectos, líneas de células de mamíferos, y células de levadura, tal

método, en donde las células de insectos se seleccionan de *Spodoptera frugiperda*, *Trichoplusia ni*, drosophila, y células de mosquitos derivadas de *Aedes albopictus*, tal

método, en donde las líneas de células de mosquitos se seleccionan de CHO, HEK 293, COS-1, HeLa, Vero y NIH3T3.

Breve Descripción de los Dibujos

Figura 1. Cassette de expresión de VLP de HEV71 [P1+IRES+3CD] y el plásmido pSN01.

Figura 2. Cassette de expresión de VLP de HEV71 [P1+IRES+3C] y el plásmido pSN03.

Figura 3. Expresión de VP1 en el sobrenadante células Sf9 infectadas por SN07.

Figura 4. VP1 expresado tantos en los sobrenadantes como en los lisados.

Figura 5. VP1 y VP0 están en el producto retenido después de la ultrafiltración sobre una membrana de corte de peso molecular de 100kDa (MWCO).

Figura 6. Programas de inmunización para producir anticuerpos neutralizantes.

Figura 7. Inmunotransferencias con sondas con antisueros policlonales de conejo contra VP0 (flecha) a tres tiempos diferentes de cosecha.

Figura 8. Sueros neutralizantes mezclados de ratones inmunizados con los antígenos oligoméricos en el sobrenadante de células Sf9 infectadas por SN07 tienen tituladores contra VP2 recombinante en ELISA.

Figura 9. Sueros neutralizantes mezclados de ratones inmunizados con antígenos oligoméricos en el sobrenadante de

células Sf9 infectadas por SN07 se unen más fuertemente a VP2 y VP0 que a VP1.

Figura 10. Región de IRES de EMCV (SEQ ID NO:1) l genoma de EMCV.

Figura 11. Fuera de marco del codón inicial de EMCV con secuencia de codificación de proteasa 3CD; secuencia de IRES nativa (SEQ ID NO:2) contra secuencia de IRES mutante (SEQ ID NO:3).

Figura 12. Plásmido pSN01-M1.

Figura 13. Plásmido pSN01-M2

Figura 14. Plásmido pSN01-M3.

Figura 15. Comparación de expresión de diferentes diseños de baculovirus recombinantes para la expresión de VLP de HEV71.

Figura 16. Plásmido pFastBac™ HT.

Figura 17. Constructo de expresión procariota para proteínas de fusión antigénicas del *enterovirus humano A* y del *enterovirus humano C*.

Figura 18. Anticuerpos de sueros neutralizantes mezclados ratones inmunizados con producto retenido de se une a todos los componentes de VLP de HEV71.

Figura 19. Caracterización de VLP de HEV71, tirar de VLP de HEV71 del sobrenadante del cultivo.

Figura 20. Análisis de la columna de afinidad (AFC) de VLP de HEV71 purificados.

Figura 21. Una micrografía electrónica de AFC de VLP de HEV71 purificados.

Figura 22. Expresión de VLP de *enterovirus humano C* (PV de poliovirus).

Figura 23. ELISA de VP3-VP1 de VLP de poliovirus.

Figura 24. Cassette de expresión de VLP de HEV71 con IRES de HEV71 y proteasa 3CD de HEV71.

Figura 25. Cassette de expresión de VLP de HEV71 con IRES de PV y proteasa 3CD de HEV71.

Descripción Detallada de la Invención

La invención proporciona partículas similares a virus (VLP), capsómeros de virus, agregados y complementos de antígenos de virus de la familia de *Picornaviridae* como una composición inmunogénica y/o una vacuna para la protección contra y/o el tratamiento de la infección por picornavirus. Ejemplos representativos pueden incluir un *Entero virus, un virus de Coxsackie* y un poliovirus.

La invención en otro aspecto proporciona proteínas de virus por ejemplo, una proteína P1 o una combinación de proteínas de VP0 de picornavirus, proteínas de VP1, proteínas de VP2, proteínas de VP3 y/o proteínas de VP4, o sus fragmentos inmunológica o biológicamente activos, que producen anticuerpos neutralizantes. La invención incluye proteínas de fusión que comprenden las proteínas de virus mencionadas y/o sus fragmentos, cuyas proteínas de fusión son inmunológicamente activas o biológicamente activas para producir la producción de anticuerpos neutralizantes que son protectores.

En una realización, un antígeno de *Enterovirus* puede ser una combinación de proteínas de la cubierta/cápside de *Enterovirus*, o sus fragmentos inmunológicamente activos. Las

proteínas de la cubierta/cápside de virus pueden ser cualquier combinación de proteínas de VP0, VP1, VP2, VP3 y/o VP4 y pueden tomar la forma de una partícula similar a un virus (VLP), un capsómero, complejo y/o agregado. La combinación puede estar en la forma de una proteína de fusión.

La invención en otro aspecto incluye un método para la producción de partículas similares a *Picornavirus* (VLP), capsómeros, complejos y/o agregados que puede incluir los pasos de: (i) construir un cassette de expresión unido operativamente a un promotor que comprende uno o más ácidos nucleicos cada uno de los cuales codifica una proteína de picornavirus, por ejemplo, una proteína P1 o una combinación de una proteína de VP0 de picornavirus, una proteína de VP1, una proteína de VP2, una proteína de VP3 y/o una proteína de VP4, que está/están unidas operativamente a un sitio de entrada de ribosoma interno (IRES), cuyo IRES está unido operativamente a una proteasa 3C o 3CD; (ii) transfectar o transformar una célula huésped adecuada con el cassette de expresión; (iii) cultivar las células huésped en condiciones en las cuales la célula produce las partículas similares a virus (VLP) y/o capsómeros y/o antígenos después de la expresión de los ácidos nucleicos comprendidos en el cassette.

Se puede obtener un ácido nucleico o una molécula de ADN recombinante mediante lo cual marcos de lectura abierta que codifican el virus Coxsackie A16, VEV71, enterovirus humano C (PV1, PV2 y/o PV3 de poliovirus humanos), EV 68, o cualquier otra proteína y proteasa de picornavirus se pueden amplificar mediante la amplificación de PCR usando iniciadores diseñados adecuadamente complementarios de las secuencias de ácidos nucleicos del virus Coxsackie A16, HEV71 o enterovirus humano C o cualquier otro picornavirus. Los iniciadores adecuados se pueden diseñar de acuerdo con técnicas estándar a partir de secuencias de ácidos nucleicos de enterovirus, que incluyen el virus Coxsackie A16, HEV71 y enterovirus humano C o cualquier otro picornavirus. Las secuencias de genomas completos están disponibles en GenBank y son accesibles en el Centro Nacional para la Información Biotecnológica (NCBI).

En una realización, una proteína P1 de picornavirus, o cualquier proteína P1 de enterovirus, se expresa como un polipéptido que posteriormente se descompone por la proteasa 3C o 3CD en la proteína de virus VP0, VP1 y VP3, o sus fragmentos inmunológica o biológicamente activos, cuyas proteínas de Enterovirus producen anticuerpos neutralizantes dirigidos contra enterovirus. La proteína de VP0 se puede descomponer nuevamente en las proteínas de VP2 y VP4, o sus fragmentos inmunológica o biológicamente activos que producen

anticuerpos neutralizantes dirigidos contra enterovirus. Las proteínas de virus se pueden autoensamblar en VLP, capsómeros y/o agregados de proteínas de enterovirus. Además se apreciará que los genes de proteasa pueden estar incluidos en la misma molécula recombinante de ADN del cassette de expresión de VLP o en diferentes moléculas de ADN y/o expresarse a partir de diferentes promotores o elementos de traducción.

Se pueden idear moléculas de ADN recombinante y los ácidos nucleicos de los cassettes de expresión de VLP mediante lo cual los marcos de lectura abierta que codifican proteínas o proteasas estructurales de picornavirus se pueden obtener mediante amplificación de PCR usando iniciadores diseñados adecuadamente para secuencias de ácidos nucleicos de picornavirus humanos.

En otra realización, la molécula de ADN recombinante puede codificar una proteína de fusión que tiene por lo menos dos proteínas estructurales de enterovirus, o sus proteínas, que se expresan como un antígeno de un solo polipéptido.

La presente invención comprende un cassette de expresión de VLP que aloja las secuencias de genes para proteínas estructurales de *Enterovirus* (región de P1) con una proteasa (3CD) que es necesaria para el procesamiento de las proteínas

P1 en las proteínas de la cápside del virus, permitiendo de ese modo el autoensamblaje de VLP de *Enterovirus*. El cassette de expresión es un vector bicistrónico que usa un promotor corriente arriba del ácido nucleico que codifica la secuencia para una proteína P1 de *Enterovirus*. Corriente abajo desde el cistrón que codifica la proteína P1 hay una secuencia del sitio de entrada de ribosoma interno (IRES) seguida por un cistrón que contiene la secuencia de nucleótidos que codifica la proteasa 3CD.

La expresión de la región de P1 y la proteasa 3CD avanza desde un solo mensaje bicistrónico en donde el gene de la proteasa 3CD se traduce en una forma independiente de la tapa bajo el control del IRES. Se observa que la expresión de la proteasa 3CD puede ser moderadamente tóxica lo cual deriva en la muerte de las células huésped, disminuyendo de ese modo el rendimiento de las proteínas y VLP de de la cápside de Enterovirus. La actividad de la proteasa se puede reducir mientras mantiene el alto nivel de expresión de la proteína P1 desde el cassette. Diferentes IRES y secuencias de IRES que comprenden mutaciones se insertaron en cassettes de expresión para controlar la expresión/actividad de la proteasa 3CD y para identificar IRES efectivos para procesar correctamente la P1 sin que fuera tóxico para la célula huésped. Para la producción eficiente de VLP, numerosos baculovirus

recombinantes que tienen la secuencia de codificación de P1 completa y la secuencia de codificación de la proteasa 3CD completa cuya expresión está bajo el control de IRES de diferentes especies o serotipos de virus, se ensayaron para la producción eficiente de VLP.

Por ejemplo, el cassette de expresión de la invención puede comprender un promotor que está unido operativamente a una secuencia de ácidos nucleicos que codifica un polipéptido P1 de enterovirus humano A, un IRES de EMCV y una proteasa 3CD de enterovirus humano A.

El cassette de expresión de la invención puede comprender un promotor que está unido operativamente a un ácido nucleico que codifica un polipéptido P1 de enterovirus humano A, un IRES del enterovirus humano C y una proteasa 3CD de enterovirus humano A.

El cassette de expresión de la invención puede comprender un promotor que está unido operativamente a un ácido nucleico que codifica un polipéptido P1 de enterovirus humano C, un IRES de HEV71 y una proteasa 3CD de enterovirus humano C.

Además, el cassette de expresión de la invención puede comprender un promotor que está unido operativamente a un

ácido nucleico que codifica un polipéptido P1 de enterovirus humano C, un IRES de EMCV y una proteasa 3CD de enterovirus humano C.

Además, hacer truncamientos y mutaciones de la proteasa 3CD en el cassette de expresión que comprende IRES eficientes puede alcanzar el máximo rendimiento de los VLP. Por ejemplo, la Glicina de la proteasa 3C de HEV71 que es un el aminoácido 1671 del GenBank acceso número DQ341362.1 se cambia a una Alanina usando la mutagénesis dirigida al sitio para la supresión de 3C de HEV71 mutante y el posterior procesamiento de un polipéptido P1 de HEV71.

Contrariamente al conocimiento convencional del arte con respecto a la meta alcanzar altos niveles de expresión y de actividad de una proteína a partir de un cassette de expresión, en una realización, la presente invención realmente intenta reducir la actividad de una proteína para alcanzar un rendimiento máximo de la proteína. La mutación del IRES o del ácido nucleico de la proteasa 3C para reducir la actividad inesperadamente deriva en un rendimiento aumentado de las proteínas de la cápside y los VLP de Enterovirus.

Los cassettes de expresión pueden clonarse en vectores adecuados y transformarse/transfectarse en células huésped

apropiadas para la expresión y la purificación de antígenos para vacunas y la protección contra infecciones de picornavirus, que incluyen enterovirus.

Los cassettes de expresión que codifican antígenos de picornavirus pueden estar comprendidos en plásmidos que pueden transfectarse en células huésped eucariotas y expresarse en condiciones de cultivo apropiadas. Los sistemas de expresión eucariotas adecuados son conocidos para los expertos en el arte e incluyen sistemas de expresión inducibles y células huésped eucariotas apropiadas.

Los vectores de expresión de células de mamíferos que comprenden un cassette de expresión de la invención incluyen aquellos que se pueden tranfectar transitoriamente en células huésped y líneas de células. Además, los vectores de expresión de células de mamíferos pueden ser vectores que se mantienen establemente dentro de la célula huésped después de la transfección.

Además, los vectores de expresión de células de mamíferos pueden incluir vectores que se transfectan estable o transitoriamente en células huésped de mamíferos o líneas de células en donde la expresión de la proteína de interés se induce agregando un agente inductor en el medio de cultivo.

Las células huésped de mamíferos y las líneas de células incluyen, por ejemplo, células CHO, HEK 293, COS-1, HeLa, Vero y NIH3T3. También se apreciará que otras células huésped eucariotas pueden incluir células de levadura u otras líneas de células de mamíferos.

El cassette de expresión puede estar contenido en virus recombinantes que pueden transfectar la célula huésped. Los virus adecuados que se pueden usar con este propósito incluyen baculovirus, vacunas, virus Sindbis, SV40, virus Sendai, retrovirus y adenovirus. Las células huésped adecuadas pueden incluir células huésped que son compatibles con los virus precedentes y éstos incluyen células de insectos tales como *Spodoptera frugiperda* (por ejemplo células Sf9), *Trichoplusia ni*, células CHO, fibroblastos embrionarios de pollo, células BHK, células SW13 humanas, drosophila, células de mosquitos derivadas de *Aedes albopictus.*

El cassette de expresión que comprende ácidos nucleicos de *Enterovirus* puede introducirse en una célula huésped apropiada por medios conocidos para los expertos en el arte. Las células huésped se propagan y se cultivan en condiciones que permiten la expresión de genes y proteínas de *Enterovirus*.

Un gene que codifica una proteína de VP2 de enterovirus, o sus fragmentos inmunológica o biológicamente activos que producen anticuerpos neutralizantes contra enterovirus, puede insertarse en un plásmido que contiene un promotor adecuado y expresarse en una célula huésped. Esta proteína de VP2 de enterovirus producida se aísla y se usa como la base de una composición inmunogénica para su uso como una vacuna o para el uso para diagnóstico.

Un gene que codifica una proteína de VP4 de enterovirus, o sus fragmentos inmunológica o biológicamente activos que producen anticuerpos neutralizantes contra enterovirus, puede insertarse en un plásmido que contiene un promotor adecuado y expresarse en una célula huésped. La proteína de VP4 de enterovirus producida se aísla y se usa como la base de una composición para su uso como una vacuna o para el uso para diagnóstico.

Un gene que codifica una proteína de VP0 de enterovirus, o sus fragmentos inmunológica o biológicamente activos que producen anticuerpos neutralizantes contra enterovirus, puede insertarse en un plásmido que contiene un promotor adecuado y expresarse en una célula huésped. La proteína de VP0 de enterovirus producida se aísla y se usa como la base de una

composición inmunogénica para su uso como una vacuna o para su uso para diagnóstico.

Un gene que codifica una proteína de VP0 de enterovirus puede unirse operativamente a un promotor adecuado e insertarse en un plásmido, cuyo plásmido presenta una proteasa de enterovirus unida a un promotor adecuado para proporcionar un plásmido recombinante doble, cuyo plásmido recombinante doble puede finalmente expresarse en un sistema de expresión de células eucariotas o procariotas.

Los vectores adecuados para la clonación de genes y la expresión de antígenos de polipéptidos de enterovirus incluyen cósmidos y plásmidos. Los sistemas de expresión adecuados incluyen sistemas de expresión procariotas conocidos para los expertos en el arte y células huésped procariotas, que incluyen *E. coli,* transformados con los cósmidos o plásmidos para la expresión de proteínas en células procariotas.

Los sistemas de expresión adecuados incluyen sistemas de expresión eucariotas conocidos para los expertos en el arte y células huésped eucariotas transformadas con plásmidos para la expresión de proteínas en diversas células huésped eucariotas y líneas de células.

Además, los antígenos de polipéptidos de *Enterovirus* pueden obtenerse de células huésped o sobrenadantes de cultivo por medios conocidos para los expertos en el arte.

Los VLP de *Enterovirus*, sus capsómeros, antígenos, componentes inmunológicamente activos y/o sus agregados pueden obtenerse de células huésped transfectadas y/o transformadas, un medio de cultivo de células huésped, sobrenadantes y lisados por cualquier medio adecuado de purificación conocido para los expertos en el arte. El aislamiento de proteínas liberadas en el medio de cultivo es un método sencillo para obtener VLP, capsómeros, antígenos y/o agregados de *Enterovirus*. Los VLP, capsómeros, antígenos de *Enterovirus,* sus componentes inmunológicamente activos y/o sus agregados, se pueden concentrar y purificar adicionalmente por medios conocidos para los expertos en el arte.

La invención en otro aspecto incluye una vacuna que contiene antígenos de picornavirus, tales como antígenos, VLP y/o capsómeros de *Enterovirus* en combinación con un coadyuvante adecuado. Los antígenos de picornavirus, sus fragmentos inmunológicamente activos, VLP y/o capsómeros se pueden combinar con cualquier coadyuvante tal como el Virus de Vacuna Modificado, ISCOMS, alumbre, hidróxido de aluminio, fosfato de aluminio, Coadyuvante Incompleto o Completo de Freund, Quil A

y otras saponinas o cualquier otro coadyuvante como se describió por ejemplo en Vanselow (1987) S. Vet. Bull. 57 881-896.

Las definiciones de los términos “fosfato de aluminio” e “hidróxido de aluminio” como se usan en la presente incluyen todas las formas de hidróxido de aluminio o fosfato de aluminio que son adecuadas para coadyuvar vacunas.

Además, los antígenos de picornavirus pueden prepararse mediante la síntesis química de polipéptidos basados en secuencias de ácidos nucleicos o de proteínas disponibles públicamente de poliovirus humanos o mediante síntesis química.

En un aspecto, la invención proporciona una vacuna que comprende uno o más antígeno(s) de *Enterovirus humano C*. Como se usa en la presente la expresión “antígeno de poliovirus” o “antígeno de *enterovirus humano C*” se refiere a todos los antígenos capaces de estimular anticuerpos neutralizantes a *enterovirus humanos C.* El antígeno de virus puede comprender una proteína de la cubierta/cápside, o sus fragmentos, determinantes antigénicos y/u otras proteínas de *enterovirus humano C.*

La invención en un aspecto incluye vacunas de la subunidad del virus *enterovirus humano C* comprenden una sola proteína de la cubierta/cápside del virus *enterovirus humano C* como un antígeno. Más específicamente la invención se refiere a vacunas que comprenden una proteína de la cubierta/cápside de VP2 de *enterovirus humano C*, o su fragmento inmunogénico, como antígeno.

La invención en otro aspecto incluye una vacuna que comprende partículas similares al *enterovirus humano C* (VLP), capsómeros, complejos y/o agregados que comprenden las proteínas VP1, VP2, VP3 y/o VP4 o VP0 del *enterovirus humano C*.

Se puede obtener una molécula de ADN recombinante mediante la cual se pueden obtener ácidos nucleicos que comprenden marcos de lectura abierta que codifican proteínas estructurales o proteasas del *enterovirus humano C* mediante amplificación de PCR usando iniciadores diseñados adecuadamente complementarios de secuencias ácidos nucleicos del *enterovirus humano C.* Los iniciadores adecuados se pueden diseñar de acuerdo con técnicas estándar a partir de secuencias de ácidos nucleicos del *enterovirus humano C*, tales como secuencias de genomas completos disponibles en GenBank y son accesibles en el Centro Nacional para la Información Biotecnológica (NCBI). Los

números de acceso para el genoma completo del genoma de poliovirus tipo I de *enterovirus humano C* incluyen V01149 y V01150.

Los cassettes de expresión de la invención pueden comprender ácidos nucleicos que codifican el polipéptido del *enterovirus humano C.* El polipéptido P1 se procesa (se descompone) por la proteasa 3CD traducida bajo el control del IRES del cassette de expresión para producir los polipéptidos VP1, VP3 y/o VP0. Las combinaciones de VP0, VP1 y VP3 pueden autoasociarse en partículas similares a virus.

El antígeno de polipéptido del *enterovirus humano C* puede comprender una proteína de VP2 de la cubierta/cápside del *enterovirus humano C*, un producto del nuevo procesamiento de VP0, en combinación con otras proteínas de la cubierta/cápside de VP0, VP1, VP3 y/o VP4 de poliovirus. La combinación de las proteínas de la cubierta/cápside de *enterovirus humano C* puede tomar la forma de una partícula similar a un virus (VLP), capsómero, complejo y/o agregado.

Un gene que codifica una proteína de VP2 de la cubierta/cápside de *enterovirus humano C* puede insertarse en un plásmido que contiene un promotor adecuado y expresarse en una célula huésped, la proteína se aísla y se usa como la base

de una composición inmunogénica para su uso como una vacuna. Además, el gene que codifica la proteína VP2 de poliovirus humano puede insertarse en un plásmido que contiene un promotor adecuado y expresarse en una célula huésped, la proteína se aísla y se usa como la base de una composición inmunogénica para su uso como una vacuna.

Un gene que codifica una proteína de VP4 de la cubierta/cápside de *enterovirus humano C* puede insertarse en un plásmido que contiene un promotor adecuado y expresarse en una célula huésped, la proteína se aísla y se usa como la base de una composición inmunogénica como una vacuna. Además, el gene que codifica la proteína de VP4 de poliovirus humano puede insertarse en un plásmido que contiene un promotor adecuado y expresarse en una célula huésped, la proteína se aísla y se usa como la base de una composición inmunogénica para su uso como una vacuna.

Un gene que codifica una proteína de VP0 de la cubierta/cápside de *enterovirus humano C* puede insertarse en un plásmido que contiene un promotor adecuado y expresarse en una célula huésped, la proteína se aísla y se usa como la base de una composición inmunogénica para su uso como una vacuna. Además, el gene que codifica la proteína de VP0 de poliovirus humano puede insertarse en un plásmido que contiene un

promotor adecuado y expresarse en una célula huésped, la proteína se aísla y se usa como la base de una composición inmunogénica para su uso como una vacuna.

La invención comprende una vacuna que comprende uno o más antígenos inmunológicamente activos que comprenden uno o más polipéptidos VP0, VP1, VP2, VP3, VP4 de enterovirus humano C y sus fragmentos inmunológicamente activos, cuya vacuna produce una respuesta protectora y/o inmune neutralizante dirigida contra un enterovirus humano.

En una realización, el cassette de expresión comprende esencialmente un ácido nucleico que codifica una poliproteína P1 de enterovirus humano C, un IRES y una proteasa 3CD de enterovirus bajo el control translacional del IRES, cuya proteasa procesa la poliproteína P1 de enterovirus humano C en proteínas de cápside estructurales.

En otro aspecto, la invención proporciona una vacuna que comprende un antígeno(s) de *enterovirus humano A*, derivado de una poliproteína P1 que incluye las proteínas VP2, VP4 y/o VP0 y/o sus fragmentos biológica o inmunológicamente activos. Los antígenos de *enterovirus humano A* se pueden obtener de HEV71 y/o del *virus Coxsackie* A16. El antígeno de HEV71 puede ser una proteína de la cubierta/cápside de virus de un solo

enterovirus humano. Específicamente, el antígeno de HEV71 puede ser una poliproteína P1 de HEV71, un polipéptido VP4, VP2 o VP0, o su fragmento, que produce una respuesta inmune cuando se administra a un humano.

En una realización, el antígeno de *enterovirus humano A* puede ser una combinación de proteínas de la cubierta/cápside de *enterovirus humano A,* o sus fragmentos inmunológicamente activos. Por ejemplo, el antígeno de *enterovirus humano A* puede comprender una proteína VP2 de poliovirus, en combinación con otras proteínas de la cubierta/cápside de poliovirus, seleccionadas de poliproteínas VP1, VP3 y/o VP4. La combinación de una poliproteína VP0 con otras proteínas de la cubierta/cápside de poliovirus puede tomar la forma de una partícula similar a un virus (VLP), capsómero, complejo y/o agregado. La combinación puede estar en la forma de una proteína de fusión.

Más específicamente, la invención se refiere a vacunas que comprenden una proteína de la cubierta/cápside de *enterovirus humano A*, o su fragmento inmunogénico, como un antígeno.

La invención en otro aspecto incluye una vacuna que comprende partículas similares a virus (VLP) del *enterovirus humano A*

y/o capsómeros que comprenden proteínas VP1, VP2, VP3 y/o VP4 de *enterovirus humano A.*

Se puede obtener una molécula de ADN recombinante mediante la cual marcos de lectura abierta que codifican proteínas estructurales y/o proteasas de *enterovirus humano A* se pueden amplificar mediante amplificación de PCR usando iniciadores diseñados adecuadamente complementarios de secuencias de ácidos nucleicos del *enterovirus humano A*. Los iniciadores diseñados adecuadamente se pueden diseñar de acuerdo con las técnicas estándar a partir de secuencias de ácidos nucleicos disponibles públicamente de HEV71. Los números de acceso para el genoma completo de HEV71 incluyen DQ341362, AB204852, AF302996 y AY465356.

Se puede obtener una molécula de ADN recombinante mediante la cual marcos de lectura abierta que codifican proteínas P1, VP1, VP2, VP3 y/o VP4 o VP0 de *enterovirus humano A* y sus fragmentos inmunológicamente activos y proteasas, también se pueden obtener mediante amplificación de PCR usando iniciadores diseñados adecuadamente complementarios de secuencias de ácidos nucleicos del *enterovirus humano A.*

En un aspecto de la invención, una proteína P1 de *enterovirus humano A* se expresa para formar una poliproteína o un

polipéptido que se descompone posteriormente por la proteasa 3C o 3CD en las proteínas VP0, VP1 y VP3. Las proteínas VP0 se pueden descomponer nuevamente en las proteínas VP2 y VP4. Las proteínas de enterovirus pueden autoensamblarse en VLP, capsómeros, complejos y/o agregados de proteínas de enterovirus. Además se apreciará que los genes no estructurales y los genes de proteasa pueden estar incluidos en la misma molécula de ADN recombinante o en moléculas recombinantes de ADN diferentes y/o expresarse desde promotores o elementos de traducción diferentes.

Los cassettes de expresión de la invención pueden comprender ácidos nucleicos que codifican un polipéptido P1 de *enterovirus humano A.* El polipéptido P1 se procesa (descompone) por la proteasa 3CD traducida bajo el control del IRES del cassette de expresión para producir las poliproteínas VP1, VP3 y VP0 y sus fragmentos inmunológicamente activos. Las combinaciones de polipéptidos VP0, VP1 y VP3 pueden autoasociarse en partículas similares a virus.

Un gene que codifica una proteína VP2 de *enterovirus humano A,* o sus fragmentos inmunológicamente activos, puede insertarse en un plásmido que contiene un promotor adecuado y expresarse en una célula huésped. El antígeno de *enterovirus humano A* aislado, por ejemplo, una proteína VP2 de HEV71, puede

aislarse y usarse como la base de una composición inmunogénica para su uso como una vacuna o para uso para diagnóstico.

Un gene que codifica una proteína VP4 de *enterovirus humano A*, o su fragmento inmunológicamente activo, puede insertarse en un plásmido que contiene un promotor adecuado y expresarse en una célula huésped. La proteína VP4 asociada puede aislarse y usarse como la base de una composición inmunogénica para su uso como una vacuna o para su uso para diagnóstico.

Un gene que codifica una proteína VP0 de *enterovirus humano A*, o su fragmento inmunológicamente activo, puede insertarse en un plásmido que contiene un promotor adecuado y expresarse en una célula huésped. La proteína VP0 aislada puede aislarse y usarse como la base de una composición inmunogénica para su uso como una vacuna o para uso para diagnóstico.

Un gene que codifica una proteína VP0 de *enterovirus humano A*, o su fragmento inmunológicamente activo, puede unirse operativamente a un promotor adecuado e insertarse en un plásmido, cuyo plásmido presenta una proteasa de *enterovirus humano A* unida a un promotor adecuado para proporcionar un plásmido recombinante doble, cuyo plásmido recombinante doble puede finalmente expresarse en un sistema de expresión de célula eucariota o procariota.

Los genes y ácidos nucleicos de *enterovirus humano A* comprendidos en el cassette de expresión pueden introducirse en una célula huésped apropiada por medios conocidos para los expertos en el arte. Las células huésped se propagan y se cultivan en condiciones que permiten la expresión de genes y proteínas de *enterovirus humano A.*

La invención comprende una vacuna que comprende uno o más antígenos inmunológicamente activos que comprenden una o más poliproteínas VP0, VP1, VP2, VP3, VP4 de *enterovirus humano A* y sus fragmentos inmunológicamente activos, cuya vacuna produce una respuesta protectora y/o inmune neutralizante dirigida contra un enterovirus humano.

En una realización, el cassette de expresión consiste esencialmente en un ácido nucleico que codifica una poliproteína P1 de *enterovirus humano A*, un IRES y una proteasa 3CD de enterovirus bajo el control translacional del IRES, cuya proteasa procesa la poliproteína P1 de *Enterovirus* en proteínas estructurales de la cápside de enterovirus. Las proteínas estructurales pueden tomar la forma de VLP, capsómeros, complejos y/o agregados.

De hecho, la expresión de una o más proteínas de *Enterovirus*, como se describe en la presente, proporciona antígenos que producen anticuerpos, cuyos anticuerpos son funcionales y capaces de neutralizar enterovirus seleccionados de HEV71, virus Coxsackie A16, *enterovirus humano C* o cualquier otro picornavirus a un alto titulador.

La expresión de una o más de las proteínas de *Enterovirus* sugiere que los polipéptidos VP2 y/o VP0 contienen epítopos reconocidos por antisueros neutralizantes.

Estos anticuerpos funcionales sorpresivamente se unen más fuertemente a polipéptidos VP2 y VP0 de *Enterovirus* que los polipéptidos VP1, cuyo polipéptido VP1 se entiende en el arte que es la proteína de la cápside principal necesaria para la generación de anticuerpos neutralizantes. Es inesperado que los polipéptidos VP2 sean, de hecho, importantes para generar anticuerpos neutralizantes contra la infección por HEV71.

Por lo tanto, se sorpresivo que un polipéptido VP2 de *Enterovirus* sea el epítopo dominante, o determinante antigénico, de las proteínas de la cápside para la generación de anticuerpos neutralizantes contra la infección por enterovirus. Los polipéptidos VP2 de HEV71, solos, o en combinación con otras proteínas de la cápside HEV71, por

ejemplo, polipéptidos VP0, es el antígeno dominante que produce anticuerpos neutralizantes dirigidos contra HEV71.

En un aspecto de la invención, están contempladas vacunaciones profilácticas para la prevención de la infección por enterovirus cuyas vacunas incorporan las proteínas estructurales VP0 y/o VP2 de enterovirus humanos en una composición inmunogénica. La composición inmunogénica o la vacuna pueden comprender las proteínas estructurales VP0 o VP2 del *enterovirus humano A*, que incluyen HEV71 y el virus Coxsackie A16, o una combinación de ellos. La composición inmunogénica se puede administrar a un sujeto para producir anticuerpos neutralizantes dirigidos contra enterovirus humanos. La composición inmunogénica puede estar comprendida en una vacuna que se administra a un sujeto para la prevención de la infección por la enfermedad de las manos, los pies y la boca causada por el *enterovirus humano A,* tales como a partir de los virus HEV71 y/o del virus Coxsackie A16.

En otro aspecto de la invención, las vacunaciones terapéuticas se proveen para prevenir y/o aliviar complicaciones de la infección por HEV71 y/o virus Coxsackie A16, por ejemplo, las complicaciones neurológicas y cardiovasculares que se manifiestan como síndromes tales como meningitis, encefalitis,

parálisis fláccida aguda, edema pulmonar e insuficiencia cardíaca.

En un aspecto de la invención, están contempladas vacunaciones profilácticas para la prevención de la infección por enterovirus, cuyas vacunas incorporan proteínas estructurales VP0 o VP2 del *enterovirus humano C,* por ejemplo, las proteínas estructurales PV1, PV2, PV3, o sus combinaciones, o sus fragmentos biológica o inmunológicamente activos. La composición inmunogénica puede estar comprendida en una vacuna que se administra a un sujeto para la prevención de la poliomielitis causada por el *enterovirus humano C*, que incluye PV1, PV2 y PV3.

Además, la composición inmunogénica o la vacuna pueden comprender una combinación de antígenos derivada del *enterovirus humano C* así como del *enterovirus humano A.*

Se puede hacer referencia ahora a diversas realizaciones de la invención como se ilustra en las figuras adjuntas. En estas realizaciones se debe señalar que los VLP, capsómeros, antígenos y agregados de *Enterovirus* y constructos específicos de moléculas recombinantes de ADN se dan a modo de ejemplo.

Se puede llegar a la conclusión de que los polipéptidos VP2 de *Enterovirus* son importantes para alcanzar anticuerpos neutralizantes. Los polipéptidos VP2 pueden ser suficientes para formular una vacuna contra una infección con picornavirus, tales como *enterovirus humano A,* tipos de HEV71 y virus Coxsackie A16, *enterovirus humano C* tipos 1, 2 y 3 y *enterovirus humanos D* tipo EV68.

En un aspecto de la invención, están contempladas vacunaciones profilácticas para la prevención de la infección por picornavirus, cuyas vacunaciones incorporan por lo menos proteínas estructurales VP0 y/o VP2 y VP4 del virus en una composición inmunogénica. La composición inmunogénica se puede administrar a un sujeto para producir anticuerpos neutralizantes dirigidos contra un picornavirus. La composición inmunogénica puede estar comprendida en una vacuna que se administra a un sujeto para la prevención de la infección por picornavirus.

En otro aspecto de la invención, se proporcionan vacunaciones terapéuticas para prevenir y/o aliviar complicaciones de la infección por picornavirus, por ejemplo, las complicaciones neurológicas y cardiovasculares que se manifiestan como síndromes tales como meningitis, encefalitis, parálisis fláccida aguda, edema pulmonar e insuficiencia cardíaca.

También en otro aspecto de la invención se proporciona una composición de vacuna de acuerdo con la invención para su uso en medicina.

En aún otro aspecto, la invención proporciona una vacuna bivalente o multivalente que comprende antígenos de VP0 y/o VP2 y/o VP4. Por ejemplo, se pueden combinar antígenos de VP0 y/o de VP2 y/o de VP4 de *enterovirus humano A.* Además, los antígenos mencionados anteriormente de diferentes serotipos del *enterovirus humano A*, tales como antígenos del *virus Coxsackie A* y de HEV71, se pueden combinar en una vacuna, por ejemplo, dirigida contra una enfermedad humana de los pies y de la boca.

Los antígenos de enterovirus de vacunas bivalentes o multivalentes se pueden producir a partir de los cassettes de expresión descritos en la presente. Los antígenos de enterovirus pueden estar en la forma de partículas similares a virus, capsómeros, complejos y/o agregados.

En aún otro aspecto, la invención proporciona una vacuna bivalente o multivalente que comprende antígeno(s) de enterovirus y un antígeno que proporciona inmunidad contra uno o más de los siguientes patógenos: difteria (D); tétanos (T);

tos ferina (P); *Haemophilus influenzae* b (Hib); Hepatitis A (HA) Hepatitis B (HB), y Enterovirus humano 71.

En una vacuna pediátrica, también se pueden incluir otros antígenos compatibles, por ejemplo, antígenos que se sabe que son efectivos contra la meningitis B, la meningitis A y C y otitis media.

La cantidad del antígeno de picornavirus en cada una de las dosis de vacuna se selecciona como una cantidad que induce una respuesta inmunoprotectora sin efectos colaterales adversos importantes en vacunas típicas. Tal cantidad varía según qué inmunógenos es empleen. Una cantidad óptima para una vacuna particular se puede determinar mediante estudios estándar que involucran la observación de tituladores de anticuerpos y otras respuestas en sujetos. Un curso de vacunación primaria puede incluir 2 o 3 dosis de una vacuna, administradas en intervalos óptimos para proporcionar una respuesta inmunoprotectora.

La invención entonces proporciona un método para prevenir infecciones por picornavirus en humanos, cuyo método comprende tratar a un sujeto humano que lo necesita, con una dosis inmunológicamente efectiva de una vacuna de acuerdo con

cualquier aspecto de la invención como se describió anteriormente.

Como se usa en la presente y en las reivindicaciones, los términos y las frases expuestas a continuación tienen las definiciones siguientes:

“Anticuerpo” se refiere a una molécula de inmunoglobulina producida por células linfoides B con una secuencia de aminoácidos específica evocada en humanos u otros animales por un antígeno (inmunógeno). Estas moléculas se caracterizan por la reacción específica con el antígeno.

“Respuesta al anticuerpo” o “respuesta humoral” se refieren a un tipo de respuesta inmune en la cual se producen anticuerpos por células linfoides B y se segregan en la sangre y/o en la linfa en respuesta a un estímulo antigénico. En una respuesta inmune que funciona correctamente, el anticuerpo se une específicamente a antígenos sobre la superficie de células (por ejemplo, un patógeno), que marca la célula para la destrucción por células fagocíticas y/o mecanismos mediados por complementos.

“Antígeno” se refiere a toda sustancia que, como resultado de la entrada en contacto con células apropiadas, induce un

estado de sensibilidad y/o sensibilidad inmune y que reacciona en una forma demostrable con anticuerpos y/o células inmunes del sujeto sensibilizado in vivo o in vitro.

“Epítopo” se refiere a la forma más simple de un determinante antigénico, sobre una molécula de antígeno complejo. Ésta es una parte específica de un antígeno reconocido por un receptor de inmunoglobulina o de células T.

“Proteína de fusión” se refiere a un antígeno de proteína formado mediante la expresión de un polipéptido hecho combinando dos o más secuencias de genes derivadas de diferentes proteínas estructurales de enterovirus.

“Fragmentos inmunológicamente activos” o “fragmentos biológicamente activos” son fragmentos de proteínas estructurales de *Enterovirus* que producen anticuerpos neutralizantes dirigidos contra enterovirus. Por consiguiente, en el contexto de la invención, tales fragmentos inmunológicamente activos se presentan al sistema inmune de un organismo para afectar, o más preferentemente para inducir, una respuesta inmune específica y, de ese modo, vacunar o proteger profilácticamente al organismo contra una infección con un *Enterovirus.*

La respuesta inmune al anticuerpo neutralizante es donde las células especializadas del sistema inmune reconocen la presentación de tales proteínas heterólogas, péptidos o epítopos y lanzan una respuesta inmune específica.

En una realización, el antígeno de vacuna de acuerdo con la invención puede inducir una respuesta inmune protectora. Por el término “respuesta inmune protectora” y/o “respuesta inmune neutralizante” como se usa en la presente se entiende que el sujeto vacunado puede resistir o protegerse contra una infección con el agente patógeno contra el cual se hizo la vacunación.

“Respuesta celular” o “respuesta del huésped celular” se refieren a un tipo de respuesta inmune mediada por células T helper y killer capaces de eliminar directamente células infectadas por virus o cancerosas.

“Célula que presenta antígenos” se refiere a las células accesorias de eventos inductivos de antígenos que funcionan principalmente manipulando y presentando el antígeno a linfocitos. La interacción de las células que presentan antígenos (APC) con antígenos es un paso esencial en la inducción inmune porque permite que los linfocitos enfrenten y reconozcan moléculas antigénicas y se activen. Ejemplos de APC

incluyen macrófagos, células dendríticas de Langerhans, células dendríticas foliculares y células B.

“Célula B” se refiere a un tipo de linfocito que produce inmunoglobulinas o anticuerpos que interactúan con antígenos.

“Linfocito T citotóxico” es un tipo especializado de linfocito capaz de destruir células extrañas y células huésped infectadas con los agentes infecciosos que producen antígenos virales.

La expresión “que consiste esencialmente en” significa que además de aquellos componentes que son obligatorios, otros componentes también pueden estar presentes en las composiciones, siempre que las características esenciales, básicas y/o novedosas de las composiciones no se vean afectadas sustancialmente por su presencia.

Por la expresión “unido operativamente” se entiende que los componentes descritos están en una relación que les permite funcionar en su forma deseada. Por lo tanto, por ejemplo, por un promotor “unido operativamente” a un ácido nucleico se entiende que el promotor y los ácidos nucleicos de un cistrón, o más de un cistrón, se combinan de manera tal que se pueda producir un solo ARN mensajero (mARN) cistrónico, bicistrónio

o multicistrónico. La expresión de proteína del ARN mensajero se puede regular de acuerdo con los elementos de transcripción/traducción de la secuencia de ácidos nucleicos. Una secuencia de IRES que se inserta en un cassette de expresión en una orientación que está corriente arriba (5') a un cistrón significa que la secuencia de IRES y los ácidos nucleicos del cistrón están ligados de manera tal que la traducción del mARN cistrónico se regule bajo el control del IRES.

Ahora se puede hacer referencia a diversas realizaciones de la invención como se ilustra en las figuras adjuntas a la presente. En estas realizaciones, se debe señalar que los VLP de picornavirus, capsómeros, antígenos y agregados y constructos específicos de moléculas recombinantes de ADN se dan a modo de ejemplo.

EJEMPLO 1. Descripción de Cassettes de Expresión y Vectores de VLP de HEV71

Se pueden construir cassettes de expresión por medios entendidos en el arte.

1.1. Cassette de expresión de VLP de HEV71

Características

Tamaño del cassette: 5172 bp

prPs: promotor sintético temprano/tardío fuerte del virus de la viruela, 43 bp

P1: secuencia de codificación de la proteína P1 de EV71-SB12736-SAR-03 (Acceso de GenBank: DQ341362) con el agregado de un codón de parada, 2588 bp

IRES: Sitio de Unión de Ribosoma Interno, 585 bp

3CD: Secuencia de codificación de proteínas C y D de P3 de EV71-SB12736-SAR-03 (Acceso de GenBank: DQ341362) con el agregado de un codón de inicio y un codón de parada de ATG, 1940 bp

Pac I: Cortadores raros, permite que el cassette se clone en pSNX01 (MVA del vector de integración 3).

El cassette se clonó en el vector de entrada de Gateway pDONR221 (Invitrogen) para producir pSN01.

Véase la Figura 1 para un diagrama del cassette de expresión y el plásmido pSN01.

1.2. Cassette de expresión de VLP de HEV71 [P1+IRES+3C]

Características:

Tamaño del cassette: 3773 bp

prPS: Promotor sintético temprano/tardío fuerte del virus de la viruela, 43 bp

P1: Secuencia de codificación de la proteína P1 de EV71-SB12736-SAR-03 (Acceso de GenBank: DQ341362) con el agregado de un codón de parada, 2588 bp

IRES: Sitio de Unión de Ribosoma Interno, 585 bp

3CD: Secuencia de codificación de la proteína C de P3 de EV71-SB12736-SAR-03 (Acceso de GenBank: DQ341362) con el agregado de un codón de inicio y un codón de parada de ATG, 551 bp

Pac I: Cortadores raros, permite que el cassette se clone en pSNX01 (MVA del vector de integración 3)

El cassette se clonó en el vector de entrada de Gateway pDONR221 (Invitrogen) para producir pSN03.

Véase la Figura 2 para un diagrama del cassette de expresión y el plásmido pSN03.

3.1. Métodos para obtener Baculovirus Recombinantes que contienen el inserto HEV71[P1+IRES+3CD] o [P1+IRES+3C]

El material de la fuente para P1 de HEV71 más 3CD fue pSN01 y para P1 más 3C fue pSN03. El objetivo fue introducir el cassette de HEV71-VLP desde pSN01 y pSN03 (vectores de Entrada) en el plásmido de expresión del baculovirus pDEST8

(vector de Destino) mediante recombinación attL/aaR *in vitro* usando LR CLONASE®, siguiendo las instrucciones del manual Invitrogen BAC-TO-BAC® (2009).

Se expusieron dos reacciones de recombinasa:

1. pSN01 (EV71-P1+3CD) x pDEST8 para producir pSN07;
2. pSN03 (EV71-P1+3C) x pDEST8 para producir pSN08

pSN07 y pSN08 se usaron para producir los bácmidos recombinantes bacSN07 y bacSN08 transformando DH10bac como se describió en el manual Invitrogen BAC-TO-BAC® manual. Los bácmidos recombinantes se transfectaron en células Sf9 para rescatar los baculovirus recombinantes SN07 y SN08.

Los baculovirus recombinantes SN07 y SN08 se usaron para infectar adicionalmente a células Sf9 en placas de 6 receptáculos para evaluar la expresión de las proteínas de la cápside procesadas. Se usó un antisuero de conejo policlonal específico para VP1 para identificar la proteína VP1 en Western blots de lisados y sobrenadantes de células Sf9 infectadas por baculovirus.

1.4. Expresión de VP1 en el sobrenadante de células Sf9 infectadas por SN07

Los sobrenadantes de células Sf9 infectadas se cosecharon diariamente desde el día 3 hasta el día 7 después de la infección y las proteínas se resolvieron sobre un 12% SDS-PAGE y se transfirieron a membranas de nitrocelulosa que luego se

sondearon con antisuero anti-VP1 de conejo policlonal (dilución 1:4000) toda la noche y luego se conjugaron anti-conejo con HRP (dilución 1:1000) durante 1 hora a temperatura ambiente. Los Western blots se desarrollaron posteriormente usando TMB. Los resultados se muestran en la Figura 3. Se observó que VP1 de HEV71 se procesó y que la expresión de la proteína se observó en el sobrenadante el día 3 y el día 4 después de la infección y que la cantidad de la expresión de VP1 disminuyó después de ello. El baculovirus recombinante SN07 al infectar las células Sf9 genera antígenos que se hallan en el sobrenadante.

Ejemplo 2. VP1 procesado en los sobrenadantes y en los lisados

Se infectaron células Sf9 a una Multiplicidad de Infección (MOI) de 10 con diferentes productos aislados de baculovirus, que incluyen SN07, SN08, un baculovirus control bacGUS y una simulación infectada. Los sobrenadantes y productos lisados se cosecharon los días 3 y 4 después de la infección y la expresión de las proteínas se evaluó mediante Western blots usando antisueros anti-VP1 de conejo (dilución 1:4000) para comparar los rendimientos de proteínas producidas por SN07 y SN08. Como se muestra en la Figura 4, el constructo de expresión SN07 produjo VP1 más descompuesta que el constructo de expresión SN08 tanto en el sobrenadante como en el producto lisado en los días 3 y 4 después de la infección.

Ejemplo 3. VP1 y VP0 están en el producto retenido después de la ultrafiltración sobre una membrana de corte de peso molecular de 100 kD (MWCO)

Los sobrenadantes de células Sf9 infectadas por SN07 el día 3 después de la infección se aclararon y se pasaron a través de filtros AMICON® (Millipore Corp.) con un MWCO de 100 kD. El producto retenido se ensayó por la presencia de VP1 y VP0 procesadas. Dado que el peso molecular de VP1 es aproximadamente 33 kDa y que el peso molecular de VP0 es 36 kDa, no se esperaría que estas proteínas permanezcan en el producto retenido a menos que estuvieran en una forma oligomérica. Como se muestra en la Figura 5, estos antígenos permanecen en el producto retenido o pasando los sobrenadantes a través de un ultrafiltro de MWCO de 100 kDa. Esto sugiere que los antígenos están asociados a una forma oligomérica. Por lo tanto, se puede llegar a la conclusión de que VP1 y VP0 se procesan y están en una asociación oligomérica con otras proteínas de la cápside.

EJEMPLO 4. El producto retenido, cuando se usa para inmunizar a ratones, produce anticuerpos neutralizantes fuertes contra HEV71.

Se inmunizó a dos grupos de ratones blancos multiplicados por mezcla de razas con el concentrado de sobrenadante de Sf9 infectadas con SN07 preparado en el Ejemplo 3. Los programas

de inmunización usados se muestran en el diagrama de la Figura 6.

Todos los ratones del grupo inmunizado produjeron anticuerpos que neutralizaron HEV71 como se muestra en la Tabla 1. El producto retenido, cuando se usa para inmunizar a ratones, produce anticuerpos neutralizantes fuertes contra HEV71.

Tabla 1. Anticuerpos neutralizantes dirigidos contra HEV71

Identificación de ratón	**Titulador recíproco neut**
m1-1	160
m1-2	640
m1-3	>=2560
m1-4	640
m2-1	80
m2-2	640
m2-3	640
m2-4	160
control1	<10
control2	<10

Por lo tanto, se puede llegar a la conclusión de que las proteínas oligoméricas pueden producir anticuerpos neutralizantes en ratones.

Las proteínas oligoméricas se pueden utilizar en composiciones inmunogénicas y/o estar comprendidas en vacunas para la administración y la profilaxis de la infección por enterovirus.

EJEMPLO 5. VP0 se expresa en los lisados de células Sf9 infectadas por SN07

Células Sf9 se infectaron con SN07 y los lisados se cosecharon a las 72, 96 y 120 horas. Las inmunotransferencias se sondearon con antisueros policlonales de conejo dirigidos contra VP0 de EV71 y se mostró que VP0 se expresa a las 72 horas después de la infección. Como se muestra en la Figura 7, el VP0 se descompuso parcialmente a VP2 comenzando a las 96 horas después de la infección. Por lo tanto, VP0 así como VP2 están presentes en los lisados de células infectadas por SN07.

EJEMPLO 6. Sueros neutralizantes mezclados de ratones inmunizados con los antígenos inmunogénicos en el sobrenadante de células Sf9 infectadas por SN07 tienen altos tituladores contra VP2 recombinantes en ELISA

Las placas de ELISA se recubrió con cantidades iguales de VP1 y VP2 recombinantes y se usó para ensayar antisueros de ratón neutralizantes mezclados. La Figura 8 muestra que los ratones

que recibieron la preparación de antígenos de producto retenido de sobrenadante pudieron unirse a VP0 mejor que VP1. Por lo tanto, se puede llegar a la conclusión de que los anticuerpos son funcionales y pueden neutralizar HEV71 a altos tituladores.

EJEMPLO 7. Sueros neutralizantes mezclados de ratones inmunizados con antígenos oligoméricos en el sobrenadante de células Sf9 infectadas por SN07 se unen más fuertemente a VP2 y VP0 que a VP1.

Los Western blots de la Figura 9 muestran que los antisueros de ratón mezclados neutralizantes se unieron más fuertemente VP2 y a VP0 que a VP1 aún cuando se agregó la misma cantidad de proteína total a cada receptáculo. Esto sugiere que VP2 y VP0 contienen epítopos reconocidos por antisueros neutralizantes.

Estos anticuerpos funcionales sorpresivamente se unen más fuertemente a VP2 y a VP0 que a VP1 que se considera que es la principal proteína de la cápside necesaria para la generación de anticuerpos neutralizantes. Por lo tanto es importante que VP0 es de hecho importante para generar anticuerpos neutralizantes.

Se puede llegar a la conclusión de que la presencia de VP2 en una formulación de vacuna es importante para obtener anticuerpos neutralizantes. VP2 puede ser suficiente para formular una vacuna contra HEV71 y otras especies de enterovirus A, B y C que incluyen el virus Coxsackie A16, Echovirus 30 y poliovirus tipos 1, 2 y 3.

También se apreciará que la invención incluye dentro de su alcance un método para generar una respuesta dirigida contra poliovirus que incluye el paso de administrar una cantidad efectiva de una vacuna que comprende un antígeno de poliovirus.

EJEMPLO 8. Comparación de los niveles de anticuerpos neutralizantes

Se usan composiciones inmunogénicas que comprenden VP1 de HEV71 para inmunizar a ratones. En forma similar, se usan composiciones inmunogénicas que comprenden VP2 y/o VP0 de HEV71 para inmunizar a ratones. Los niveles de anticuerpos neutralizantes en los ratones inmunizados con VP2 y/o VP0 de HEV71 se comparan con los niveles de anticuerpos neutralizantes en los ratones inmunizados con VP1 de HEV71. Los niveles de anticuerpos neutralizantes en los ratones inmunizados con VP2 y/o VP0 de HEV71 son mucho más altos que

los niveles de anticuerpos neutralizantes en los ratones inmunizados con VP1 de HEV71.

EJEMPLO 9. Construcción del vector de baculovirus recombinante para la expresión de la región de P1 de *enterovirus humano C* y la proteasa 3CD de un solo mensaje bicistrónico

Este ejemplo proporciona un método que deriva en la producción eficiente de VLP de *enterovirus humano C* (poliovirus) reduciendo la proteasa 3CD mediada eliminando células infectadas por baculovirus.

La construcción de un vector de baculovirus recombinante para la expresión de la región de P1 y la proteasa 3CD de un solo mensaje bicistrónico se muestra, por ejemplo, en la Figura 1. El gene de proteasa 4CD se traduce en una forma independiente de la tapa bajo el control del IRES de EMCV. Este sistema proporciona la palanca para regular la expresión de la proteasa 3CD, es decir, evaluar las secuencias de IRES mutante para hallar el IRES más débil de manera tal que se produzca una menor cantidad de proteasa comparada con las proteínas P1.

Se construye un vector bicistrónico en el cual el plásmido contiene un promotor de polihedrina corriente arriba desde la secuencia de codificación para la P1. Corriente abajo desde

los cistrones que codifican P1 hay una secuencia de un sitio de entrada de ribosoma interno del virus de la encefalomiocarditis (EMCV) (GenBank acceso número AF113968.2; nucleótidos 1666 a 2251) seguidos por los cistrones que contienen la secuencia de nucleótidos que codifica la proteasa 3CD. Se puede accede al material de la fuente para P1 de *enterovirus humano C* con 3CD en la Colección de Cultivos de Tipo Americano (ATCC) y se lo sintetiza a partir de secuencias de *enterovirus humano C* (GenBank disponible a través del Centro Nacional para la Información Biotecnológica (NCBI)).

Se generan baculovirus recombinantes usando el sistema BAC-TO-BAC® siguiendo las instrucciones del fabricante (Invitrogen). En resumen, se usa LR CLONASE® para introducir el cassette de VLP de *enterovirus humano C* en el plásmido de expresión de baculovirus pDEST8 (vector de uso de Destino) mediante recombinación attL/aaR in vitro. La reacción de LR CLONASE® se lleva a cabo a 25°C durante 1 hora mediante la incubación con proteinasa K. la mezcla de la reacción de LR CLONASE® se transforma en células competentes DH5α de Eficiencia de Biblioteca para obtener clones de expresión. Se aísla el ADN de las colonias resultantes y se confirma la presencia del cassette de *enterovirus humano C* mediante el análisis de enzima de restricción. Se construyen bácmidos recombinantes introduciendo el cassette de expresión, en el genoma de

baculovirus alojado en las células DH10bac por recombinasa de transposición T7. Los bácmidos recombinantes se verifican por su fenotipo blanco sobre placas de agar de LB complementadas con 50 µg/ml de kanamicina, 7 µg/ml de gentamicina, 10 µg/ml de tetraciclina, 100 µg/ml de X-gal, y 40 µg/ml de IPTG. El Estuche de Minipreparación de Plásmidos de Alta Pureza de Unión Pura (Invitrogen) se usa para purificar el ADN del bácmido de alta calidad desde DH10Bac *E. coli*. M13 de ida, M13 de vuelta e iniciadores internos de la inserción se usan para confirmar la existencia del cassette de *enterovirus humano C*. Se usa el reactive de transfección EFFECTENE® (Qiagen) para rescatar baculovirus recombinantes transfectando los ADN en células de insectos Sf9. En resumen, las células Sf9 se siembran a 2 millones por cada matraz T25 y se incuban para adherirse durante 6 horas a 28°C. Un microgramo de ADN de bácmido recombinante se resuspende en 150 µl de tampón de condensación de ADN y 8 µl de solución de mejorador se mezclan y se incuban a temperatura ambiente durante 5 minutos. Luego se agregan 25 µl de reactivo EFFECTENE® en la mezcla de ADN y se incuba durante 10 minutos a temperatura ambiente. Un ml de medio de cultivo se agrega a los tubos que contienen los complejos de transfección y se transfieren a matraces de cultivo de células y se distribuyen uniformemente. El día 3, el sobrenadantes se cosecha mediante la centrifugación a 500 g durante 5 minutos. Después de la transfección, se prepara el

material viral de título alto. Una vez que se obtiene un material viral alto, se lo emplea para determinar los tiempos óptimos para la expresión de la proteína blanco.

EJEMPLO 10. Producción de VLP de HEV71, así como *enterovirus humano C,* mediante la reducción de la eliminación mediada por proteasa 3CD de células infectadas por baculovirus

Este experimento describe la construcción del vector de baculovirus recombinante para la expresión de la región de P1 y la proteasa 3Cd a partir de un solo mensaje bicistrónico. El gene de proteasa 3CD se traduce en una forma independiente de la tapa bajo el control del IRES de EMCV, que se muestra en la Figura 10. Este sistema proporciona la palanca para regular la expresión de la proteasa 3CD, es decir evaluar las secuencias de IRES mutante para hallar el IRES más débil de manera tal que se produzca una cantidad menor de la proteasa comparada con las proteínas P1.

Se construyó un vector bicistrónico en el cual un plásmido contiene un promotor de polihedrina corriente arriba desde la secuencia de codificación para la P1. Corriente abajo desde los cistrones que codifican P1 es una secuencia del sitio de entrada de ribosoma interno (IRES) del *virus de la encefalomiocarditis* (EMCV) seguidos por los cistrones que

contienen la secuencia de nucleótidos que codifica la proteasa 3CD, véase la Figura 1. El IRES usado en el Ejemplo 1 contiene la secuencia de IRES de EMCV nativo ya que existen formas alteradas. La secuencia del IRES del EMCV nativo de la Figura 10 muestra el bucle de bifurcación A6 en el segmento JK. El agregado de un nucleótido, por ejemplo, una adenina (A7), reduce la expresión. Además, en el constructo usado en el Ejemplo 1, la proteasa 3CD se fusiona con el IRES del *virus de la encefalomiocarditis* en el extremo de amino. Encuadrar mejor que el codón de inicio de EMCV con la secuencia de codificación de proteasa 3CD debe reducir considerablemente la expresión de los genes corriente abajo, véase la Figura 11. Ambas modificaciones se incorporan en la secuencia del IRES de EMCV de pSN01 y se denomina pSN01-M1, como se muestra en la Figura 12 y se sintetiza mediante DNA2.0.

Se diseñaron experimentos para caracterizar los VLP expresados desde el IRES de EMCV mutante de pSN01-M1. Se generaron baculovirus recombinantes usando el sistema BAC-TO-BAC® siguiendo las instrucciones del fabricante (Invitrogen). En resumen, se usó LR CLONASE® para introducir el cassette de VLP de HEV71 en el plásmido de expresión de baculovirus pDEST8 (vector de uso de Destino) mediante recombinación attL/aar in vitro. La reacción de LR CLONASE® se llevó a cabo a 25°C durante 1 hora seguida por la incubación con proteinasa K. La

mezcla de la reacción de LR CLONASE® se transformó en células competentes DH5α de Eficiencia de Biblioteca para obtener clones de expresión. Se aisló el ADN de las colonias resultantes y se confirmó para la presencia del cassette de HEV71/poliovirus mediante análisis de enzima de restricción. Los bácmidos recombinantes se construyen introduciendo el cassette de expresión de pSN07-M1, en el genoma de baculovirus alojado en células DH10bac por recombinasa de transposición T7 para dar bacSN07-M1. Los bácmidos recombinantes se verifican por su fenotipo blanco sobre placas de agar de LB complementadas con 50 µg/ml de kanamicina, 7 µg/ml de gentamicina, 10 µg/ml de tetraciclina, 100 µg/ml de X-gal, y 40 µg/ml de IPTG. El Estuche de Minipreparación de AND de Plásmido de Alta Pureza de Unión Pura (Invitrogen) se usó para purificar el ADN de bácmido de alta calidad desde DH10Bac *E. coli*. M13 de ida, M13 de vuelta y los iniciadores internos desde la inserción se usaron para confirmar la existencia del cassette de HEV71/poliovirus. Se usó el reactivo de transfección EFFECTENE® (Qiagen) para rescatar el baculovirus recombinante transfectando los ADN en células de insectos Sf9. En resumen, las células Sf9 se sembraron a 2 millones por cada matraz T25 y se incubaron para adherirse durante 6 horas a 28°C. Un microgramo del ADN de bácmido recombinante se resuspendió en 150 µl de tampón de condensación de ADN y 8 µl de mejorador se mezclan y se incuban a temperatura ambiente

durante 5 minutos. Luego 25 µl de reactivo EFFECTENE® se agregaron a una mezcla de ADN y se incubaron durante 10 minutos a temperatura ambiente. Un ml del medio de cultivo se agregó a los tubos que contienen los complejos de transfección y se transfirieron a los matraces de cultivo de células y se distribuyeron uniformemente. El día 3 el sobrenadante se cosechó mediante centrifugación a 500 g durante 5 minutos. Después de la transfección, se prepara un material viral de alto título. Una vez que se obtiene un material viral alto, se lo emplea para determinar los tiempos óptimos para la expresión de proteína blanco. Para el análisis de la proteína de interés, células Sf9 cultivadas en 10% de medio de células de insectos de Grace (Invitrogen) se resuspendió en 1% de medio de FBS Sf900-II SFM (Invitrogen) para obtener una sola célula y se sembraron a un millón por ml de densidad en los matraces y se incubaron durante 4 horas a 28°C. Los materiales virales se agregaron a células lavadas con PBS a un MOI de 10 y se agitaron suavemente durante 1 hora. Las células infectadas se lavaron tres veces con PBS y las células se cultivaron en SF900II-SFM durante diferentes puntos de tiempo. Las células se lisaron con tampón hipotónico /1% TRITON® X-100 (TX-100) (1,5 mM $MgCl_2$, 50 mM KCl, 20 mM HEPES, 1% TX-100) agitando el matraz durante 30 minutos a temperatura ambiente y los lisados de células se prepararon recolectando las células lisadas desde el matraz y centrifugando a 4°C durante 30

minutos a 7000 rpm. Los componentes de los lisados de células y los sobrenadantes se analizaron mediante inmonotransferencia y ELISA usando anticuerpos específicos.

EJEMPLO 11. Producción eficiente de VLP de HEV71, así como de *enterovirus humano C* (poliovirus) mediante la reducción de la eliminación mediada por la proteasa 3CD de células infectadas por baculovirus

La construcción de un vector de baculovirus recombinante para la expresión de la región de P1 y la proteasa 3CD desde un solo mensaje bicistrónico se muestra en la Figura 1. El gene de proteasa 3CD se traduce en una forma independiente de la tapa bajo el control del IRES de EMCV, que se muestra en la Figura 10. Este sistema proporciona la palanca para regular la expresión de proteasa 3CD, es decir, evaluar las secuencias de IRES mutante para hallar el IRES más débil de manera tal que se produzca una cantidad menor de proteasa comparada con las proteínas P1.

Se construyó un vector bicistrónico y el plásmido contiene un promotor de polihedrina corriente arriba desde la secuencia de codificación para la P1. Corriente abajo desde los cistrones que codifican P1 hay una secuencia del sitio de entrada de ribosoma interno (IRES) del *virus de la encefalomiocarditis*

(EMCV) seguido por los cistrones que contienen una secuencia de nucleótidos que codifica la proteasa 3CD, véase la Figura 1. El IRES usado en el Ejemplo 1 contiene la secuencia de IRES de EMCV nativo ya que existen formas alteradas. La secuencia de IRES de EMCV nativo tiene el bucle de bifurcación A6 en el segmento JK, en realidad agregando un nucleótido (A7) que se sabe que reduce la expresión, véase la Figura 10. El bucle de bifurcación A6 se modificó a A7 en la secuencia de IRES de EMCV de pSN01 y se lo denominó pSN01-M2, véase la Figura 14, sintetizado por DNA2.0.

Se caracterizaron los VLP expresados por el IRES de EMCV mutante de pSN01-M2. Los baculovirus recombinantes se generaron usando el sistema BAC-TO-BAC® siguiendo las instrucciones del fabricante (Invitrogen). En resumen, se usó LR CLONASE® para introducir el cassette de VLP de HEV71 en el plásmido de expresión de baculovirus pDEST8 (vector de uso de Destino) mediante recombinación attL/aar in vitro. La reacción de LR CLONASE® se llevó a cabo a 25°C durante 1 hora seguida por la incubación con proteinasa K. La mezcla de la reacción de LR CLONASE® se transformó en células competentes DH5α de Eficiencia de Biblioteca para obtener clones de expresión. Se aisló el ADN de las colonias resultantes y se confirmó para la presencia del cassette de HEV71/poliovirus mediante análisis de enzima de restricción. Los bácmidos recombinantes se

construyen introduciendo el cassette de expresión de pSN07-M2, en el genoma de baculovirus alojado en células DH10bac por recombinasa de transposición T7 para dar bacSN07-M2. Los bácmidos recombinantes se verificaron por su fenotipo blanco sobre placas de agar de LB complementadas con 50 µg/ml de kanamicina, 7 µg/ml de gentamicina, 10 µg/ml de tetraciclina, 100 µg/ml de X-gal, y 40 µg/ml de IPTG. El Estuche de Minipreparación de AND de Plásmido de Alta Pureza de Unión Pura (Invitrogen) se usó para purificar el ADN de bácmido de alta calidad desde DH10Bac *E. coli*. M13 de ida, M13 de vuelta y los iniciadores internos desde la inserción se usaron para confirmar la existencia del cassette de HEV71/poliovirus. Se usó el reactivo de transfección EFFECTENE® (Qiagen) para rescatar el baculovirus recombinante transfectando los ADN en células de insectos Sf9. En resumen, las células Sf9 se sembraron a 2 millones por cada matraz T25 y se incubaron para adherirse durante 6 horas a 28°C. Un microgramo del ADN de bácmido recombinante se resuspendió en 150 µl de tampón de condensación de ADN y 8 µl de mejorador se mezclan y se incuban a temperatura ambiente durante 5 minutos. Luego 25 µl de reactivo EFFECTENE® se agregaron a una mezcla de ADN y se incubaron durante 10 minutos a temperatura ambiente. Un ml del medio de cultivo se agregó a los tubos que contienen los complejos de transfección y se transfirieron a los matraces de cultivo de células y se distribuyeron uniformemente. El día 3

el sobrenadante se cosechó mediante centrifugación a 500 g durante 5 minutos. Después de la transfección, se prepara un material viral de alto título. Una vez que se obtiene un material viral alto, se lo emplea para determinar los tiempos óptimos para la expresión de proteína blanco. Para el análisis de la proteína de interés, células Sf9 cultivadas en 10% de medio de células de insectos de Grace (Invitrogen) se resuspendió en 1% de medio de FBS Sf900-II SFM (Invitrogen) para obtener una sola célula y se sembraron a un millón por ml de densidad en los matraces y se incubaron durante 4 horas a 28°C. Los materiales virales se agregaron a células lavadas con PBS a un MOI de 10 y se agitaron suavemente durante 1 hora. Las células infectadas se lavaron tres veces con PBS y las células se cultivaron en SF900II-SFM durante diferentes puntos de tiempo. Las células se lisaron con tampón hipotónico /1% TRITON® X-100 (TX-100) (1,5 mM $MgCl_2$, 50 mM KCl, 20 mM HEPES, 1% TX-100) agitando el matraz durante 30 minutos a temperatura ambiente y los lisados de células se prepararon recolectando las células lisadas desde el matraz y centrifugando a 4°C durante 30 minutos a 7000 rpm. Los componentes de los lisados de células y los sobrenadantes se analizaron mediante inmonotransferencia y ELISA usando anticuerpos específicos.

EJEMPLO 12. Producción eficiente de VLP de HEV71, así como de *enterovirus humano C* (poliovirus) mediante la reducción de la eliminación mediada por la proteasa 3CD de células infectadas por baculovirus

La construcción de un vector de baculovirus recombinante para la expresión de la región de P1 y la proteasa 3CD desde un solo mensaje bicistrónico se muestra en la Figura 1. El gene de proteasa 3CD se traduce en una forma independiente de la tapa bajo el control del IRES de EMCV, que se muestra en la Figura 10. Este sistema proporciona la palanca para regular la expresión de proteasa 3CD, es decir, evaluar las secuencias de IRES mutante para hallar el IRES más débil de manera tal que se produzca una cantidad menor de proteasa comparada con las proteínas P1.

Se construyó un vector bicistrónico y el plásmido contiene un promotor de polihedrina corriente arriba desde la secuencia de codificación para la P1. Corriente abajo desde los cistrones que codifican P1 hay una secuencia del sitio de entrada de ribosoma interno (IRES) del *virus de la encefalomiocarditis* (EMCV) seguido por los cistrones que contienen una secuencia de nucleótidos que codifica la proteasa 3CD (Figura 1). El IRES usado en el Ejemplo 1 contiene la secuencia de IRES de EMCV nativo ya que existen formas alteradas. En el constructo

de pSN01 la proteasa 3CD se fusiona con la poliproteína del virus de la encefalomiocarditis en el extremo de amino. Encuadrar mejor el codón de inicio de EMCV con la secuencia de codificación de proteasa 3CD debe reducir considerablemente la expresión de los genes corriente abajo, véase la Figura 11. Esta modificación se incorporó en la secuencia de IRES de EMCV de pSN01 y se denominó pSN01-M3, véase la Figura 14 y se sintetiza mediante DNA2.0.

Se analizaron los VLP expresados por el IRES de EMCV mutante de pSN01-M3. Los baculovirus recombinantes se generaron usando el sistema BAC-TO-BAC® siguiendo las instrucciones del fabricante (Invitrogen). En resumen, se usó LR CLONASE® para introducir el cassette de VLP de HEV71 en el plásmido de expresión de baculovirus pDEST8 (vector de uso de Destino) mediante recombinación attL/aar in vitro. La reacción de LR CLONASE® se llevó a cabo a 25°C durante 1 hora seguida por la incubación con proteinasa K. La mezcla de la reacción de LR CLONASE® se transformó en células competentes DH5α de Eficiencia de Biblioteca para obtener clones de expresión. Se aisló el ADN de las colonias resultantes y se confirmó para la presencia del cassette de HEV71/poliovirus mediante análisis de enzima de restricción. Los bácmidos recombinantes se construyen introduciendo el cassette de expresión de pSN07-M3, en el genoma de baculovirus alojado en células DH10bac por

recombinasa de transposición T7 para dar bacSN07-M3. Los bácmidos recombinantes se verificaron por su fenotipo blanco sobre placas de agar de LB complementadas con 50 µg/ml de kanamicina, 7 µg/ml de gentamicina, 10 µg/ml de tetraciclina, 100 µg/ml de X-gal, y 40 µg/ml de IPTG. El Estuche de Minipreparación de AND de Plásmido de Alta Pureza de Unión Pura (Invitrogen) se usó para purificar el ADN de bácmido de alta calidad desde DH10Bac *E. coli*. M13 de ida, M13 de vuelta y los iniciadores internos desde la inserción se usaron para confirmar la existencia del cassette de HEV71/poliovirus. Se usó el reactivo de transfección EFFECTENE® (Qiagen) para rescatar el baculovirus recombinante transfectando los ADN en células de insectos Sf9. En resumen, las células Sf9 se sembraron a 2 millones por cada matraz T25 y se incubaron para adherirse durante 6 horas a 28°C. Un microgramo del ADN de bácmido recombinante se resuspendió en 150 µl de tampón de condensación de ADN y 8 µl de mejorador se mezclan y se incuban a temperatura ambiente durante 5 minutos. Luego 25 µl de reactivo EFFECTENE® se agregaron a una mezcla de ADN y se incubaron durante 10 minutos a temperatura ambiente. Un ml del medio de cultivo se agregó a los tubos que contienen los complejos de transfección y se transfirieron a los matraces de cultivo de células y se distribuyeron uniformemente. Después del día 3, el sobrenadante se cosechó mediante centrifugación a 500 g durante 5 minutos. Después de la transfección, se

prepara un material viral de alto título. Una vez que se obtiene un material viral alto, se lo emplea para determinar los tiempos óptimos para la expresión de proteína blanco.

Para el análisis de la proteína de interés, células Sf9 cultivadas en 10% de medio de células de insectos de Grace (Invitrogen) se resuspendieron en 1% de medio de FBS Sf900-II SFM (Invitrogen) para obtener una sola célula y se sembraron a un millón por ml de densidad en los matraces y se incubaron durante 4 horas a 28°C. Los materiales virales se agregaron a células lavadas con PBS a un MOI de 10 y se agitaron suavemente durante 1 hora. Las células infectadas se lavaron tres veces con PBS y las células se cultivaron en SF900II-SFM durante diferentes puntos de tiempo. Las células se lisaron con tampón hipotónico /1% TRITON® X-100 (TX-100) (1,5 mM $MgCl_2$, 50 mM KCl, 20 mM HEPES, 1% TX-100) agitando el matraz durante 30 minutos a temperatura ambiente y los lisados de células se prepararon recolectando las células lisadas desde el matraz y centrifugando a 4°C durante 30 minutos a 7000 rpm. Los componentes de los lisados de células y los sobrenadantes se analizaron mediante inmonotransferencia y ELISA usando anticuerpos específicos.

EJEMPLO 13. El constructo de IRES mutante M2 expresa niveles más altos de VP1 en el sobrenadante

Los baculovirus recombinantes que expresan proteínas de la cápside de HEV71 bajo el control de IRES de EMCV de tipo silvestre o mutantes se evaluaron con respecto al nivel de expresión de las proteínas de la cápside desde los IRES de ECMV. Los baculovirus produjeron VLP que se expresan bajo el control del IRES de EMCV de tipo silvestre desde SN07 del Ejemplo 1 y los 3 IRES mutantes, M1, M2 y M3 de los Ejemplos 10, 11 y 12, respectivamente, se analizaron con respecto al nivel de expresión de baculovirus de las proteínas de la cápside de HEV71. También se incluyeron en el estudio un baculovirus recombinante que expresa P1 y 3CD bajo diferentes promotores (F) y un baculovirus recombinante control que expresa bacGUS (G). Las células Sf9 se infectaron a un MOI de 5 y tanto los lisados como los sobrenadantes se cosecharon el día 3 como se describió en los Ejemplos 10–12 precedentes. Los lisados y los sobrenadantes se sondearon con un anticuerpo anti–VP1 para detectar la expresión de las proteínas de la cápside de HEV71.

Las inmunotransferencias de la Figura 15 muestran que cuando los lisados se sondearon con anticuerpos a VP1, los mutantes M1 y M2 expresan niveles más altos de VP1 que el mutante M3 o el constructo impulsado por 2 promotores. Sin embargo, el mutante M2 produjo más VP1 en el sobrenadante. Estos datos se muestran en el panel superior.

El panel inferior de la Figura 15 muestra las inmunotransferencias que se sondearon con un anticuerpo anti-gp64 control que se dirige a la proteína de la cubierta del baculovirus. La inmunotransferencia muestra que se produjeron cantidades equivalentes de baculovirus en cada muestra.

EJEMPLO 14. Clonación, expresión y purificación de vacunas de subunidades usando el sistema de expresión de baculovirus

La presente invención está destinada a la generación y el uso de proteínas estructurales de HEV71 y poliovirus recombinante que se fusionan como un solo inmunógeno para producir una respuesta inmune protectora en los individuos vacunados. La presente invención se relaciona en general con la preparación de composiciones de vacunas de proteínas de fusión de HEV71 y/o poliovirus recombinante que comprenden proteína de subunidad de HEV71 y/o poliovirus, o sus fragmentos inunogénicos y un coadyuvante en combinación con la proteína de fusión de subunidad de HEV71 y/o poliovirus. Las proteínas de fusión de HEV71 y poliovirus pueden comprender proteínas de la cápside seleccionadas de VP1, VP2, PVP3 y VP4, sus combinaciones y combinaciones de sus fragmentos inmunogénicos. En un aspecto de la presente realización, la proteína de fusión de HEV71 y/o poliovirus recombinante comprende la proteína de subunidad de HEV71 o poliovirus y una proteína

pareja en asociación genética con la proteína de subunidad de HEV71 o poliovirus. La presente invención contempla métodos para generar los constructos para expresar las siguientes vacunas de subunidad en *E. coli* así como baculovirus: VP0, fusión de VP4-VP2-VP3, fusión de VP2-VP3-VP1.

Para generar cADN a partir de VEV71 y/o poliovirus que codifica VP0, fusión de VP4-VP2-VP3, o VP2-VP3-VP1, se llevó a cabo la reacción en cadena de polimerasa con transcripción inversa (El Estuche de Ácido Nucleico de Alta Pureza, Roche) usando el ARN viral genómico purificado. Un iniciador de ida y de vuelta se hizo desde el extremo de 5' y 3' de los genes y cuyos iniciadores incorporan un codón de inicio y de parada. Los productos de PCR amplificados sometieron a digestión con las enzimas de restricción EcoRI y NotI y se clonaron en el vector pFastBac HT (Invitrogen) como se muestra en la Figura 16. El sistema de expresión BAC-TO-BAC® de Invitrogen está comercialmente disponible y se usaron métodos siguiendo las instrucciones del fabricante. Los genes de fusión se clonan en el plásmido donante pFastBac HT y la producción de proteínas recombinantes se basó en el sistema de expresión de baculovirus BAC-TO-BAC® (Invitrogen). El plásmido donante PFastBac HT que transporta los genes de fusión se transfirió a un vector de lanzamiento de baculovirus (bácmido) mediante la recombinación específica del sitio por recombinasa de

transposición T7. Esto se logró en DH10Bac de la cepa de E. coli. Las células DH10Bac contienen el bácmido, lo cual confirió resistencia a kanamicina y un plásmido helper, que codificó la transposasa y confirió resistencia a tetraciclina. Los plásmidos pFastBac HT recombinantes con el gene de interés se transfirieron en células DH10Bac para la transposición para generar bácmidos recombinantes. Las células transformadas se diluyeron en forma serial y cada dilución se colocó sobre placas de Agar LB complementadas con 50µg/ml de kanamicina, 7 µg/ml de gentamicina, 10 µg/ml de tetraciclina, 100 µg/ml de X-gal, y 40 µg/ml de IPTG y se incubó durante por lo menos 48 horas a 37°C. Las colonias blancas se recogieron y se rayaron nuevamente para confirmar un fenotipo blanco. Los bácmidos recombinantes se aislaron mediante el Estuche de Minipreparación de ADN de Plásmido de Alta Pureza de Unión Pura (Invitrogen) y las muestras de ADN se disolvieron en 40 µl de TE (10 mM Tris-HCl pH 8, 1 mM EDTA) y se usaron para transfecciones.

El ADN del bácmido aislado se detectó por el gene de interés insertado mediante PCR. Se usaron reactivos de transfección EFFECTENE® (Qiagen) para rescatar baculovirus recombinantes transfectando los ADN en células de insectos Sf9. En resumen, se sembraron células Sf9 a 2 millones por cada matraz T25 y se incubaron para adherir durante 6 horas a 28°C. Un microgramo

del ADN de bácmido recombinante se resuspendió en 150 µl de tampón de condensación de ADN y 8 µl de mejorador se mezclan y se incuban a temperatura ambiente durante 5 minutos. Luego 25 µl de reactivo EFFECTENE® se agregaron a una mezcla de ADN y se incubaron durante 10 minutos a temperatura ambiente. Un ml del medio de cultivo se agregó a los tubos que contienen los complejos de transfección y se transfirieron a los matraces de cultivo de células y se distribuyeron uniformemente. El día 3 el sobrenadante se cosechó mediante centrifugación a 500 g durante 5 minutos. Después de la transfección, se prepara un material viral de alto título. Una vez que se obtiene un material viral alto, se lo emplea para determinar los tiempos óptimos para la expresión de proteína blanco. Para el análisis de la proteína de interés, células Sf9 cultivadas en 10% de medio de células de insectos de Grace (Invitrogen) se resuspendió en 1% de medio de FBS Sf900-II SFM (Invitrogen) para obtener una sola célula y se sembraron a un millón por ml de densidad en los matraces y se incubaron durante 4 horas a 28°C. Los materiales virales se agregaron a células lavadas con PBS a un MOI de 10 y se agitaron suavemente durante 1 hora. Las células infectadas se lavaron tres veces con PBS y las células se cultivaron en SF900II-SFM durante diferentes puntos de tiempo. Las células se lisaron con tampón hipotónico /1% TRITON® X-100 (TX-100) (1,5 mM $MgCl_2$, 50 mM KCl, 20 mM HEPES, 1% TX-100) agitando el matraz durante 30 minutos a

temperatura ambiente y los lisados de células se prepararon recolectando las células lisadas desde el matraz y centrifugando a 4°C durante 30 minutos a 7000 rpm. La expresión de la proteína de baculovirus se verificó mediante electroforesis de gel de poliacrilamida de SDS (SDS-PAGE) y Western blots usando el anticuerpo His Probe-HRP (Thermo Scientific) como la sonda. Una vez que la producción de baculovirus y la expresión de la proteína se confirmaron, el material de virus se amplificó para producir un material concentrado de los baculovirus que transportan el gene de interés. También se establecieron la concentración más apropiada del virus para infectar células de insectos y el punto de tiempo óptimo para la producción de la proteína deseada. Para la purificación en condiciones de desnaturalización, las células se lisaron en un tampón de lisis que contiene 6 M HCl de guanidinio en 100 mM NaH_2PO_4, 10 mM Tris, 300mM NaCl, 10 mM imidazol, pH 8,0 (tampón de lisis). La suspensión se sometió a ultrasonido sobre hielo con 5 pulsaciones de 1 minuto por pulsación a un ajuste de potencia de 60 vatios, y se mezcló a temperatura ambiente durante 1 hora. El producto lisado se centrifugó a 27K g durante 30 minutos para eliminar restos de células. El sobrenadante se cargó sobre una columna de HisTrap (GE healthcare life sciences) previamente equilibrada con el tampón de lisis. Después de la carga, la columna se lavó con 20 volúmenes de

columna de 6 M HCl de guanidinio en 100 mM NaH_2PO_4, 10 mM Tris, 300 mM NaCl, 40 mM Imidazol, pH 8,0 (tampón de lavado 1), seguido por lavados con 20 volúmenes de columna de 8 M urea en 100 mM NaH_2PO_4, 10 mM Tris, 300 mM NaCl, 40 mM imidazol, pH 8,0 (tampón de lavado 2). La proteína unida se eluyó con un tampón que contiene 8 M urea, 100 mM NaH_2PO_4, 10 mM Tris, 300 mM NaCl, 250 mM imidazol, pH 8 (Elution Buffer). Las fracciones que contienen la proteína se mezclaron y se dializaron contra PBS, toda la noche a 4°C. Se usó proteasa TEV para la eliminación del marcador de histidina después de la purificación de la proteína siguiendo las instrucciones del fabricante.

EJEMPLO 15. Expresión y purificación de vacunas de subunidad de *enterovirus humano A y enterovirus humano C* (poliovirus) en *E. coli*

El Sistema de Expresión Champion® pET SUMO (Invitrogen) produce los niveles más altos de proteína soluble en *E. coli*. Utiliza una fusión de modificador relacionado con ubiquitina pequeño (SUMO) para mejorar la solubilidad de la proteína de fusión expresada. Después de la expresión, el grupo SUMO 11 kD se puede descomponer con la proteasa de SUMO altamente específica y activa (ULP-1) en el extremo de carbonilo, produciendo una proteína nativa. También contiene el marcador

6xHis del extremo de N para la detección y purificación de proteína.

La construcción del vector de expresión de pET SUMO-VP0, pET SUMO-VP4-VP2-VP3 y pET SUMO-VP2-VP3-VP1 para las proteínas de fusión antigénicas de HEV71 y poliovirus, como se muestra en la Figura 17, es la siguiente. Los fragmentos de VP0, fusión de VP4-VP2-VP3 y fusión de VP2-VP3-VP1 se usaron como los antígenos para las vacunas de subunidades de HEV71 y de poliovirus. Un motive de SUMO y el marcador de 6XHis se conjugaron al extreme de N de las fusiones para contribuir en la solubilización de la proteína y la purificación de la proteína, respectivamente. Las proteínas de fusión antigénicas se crearon mediante una tecnología de clonación de genes que comprende clonar secuencias de cADN que codifican proteínas respectivas en un vector de expresión para formar vectores de expresión de pET SUMO-VP0, pET SUMO-VP0, pET SUMO- VP4-VP2-VP3 y pET SUMO-VP2-VP3-VP1. Los fragmentos de ADN que codifican parejas de fusión se amplificaron por PCR usando iniciadores específicos que comprenden un codón de inicio y un codón de parada en los iniciadores de ida y de vuelta, respectivamente. La unión del producto de la PCR se llevó a cabo de la siguiente manera: producto de PCR nuevo, 10X tampón de unión, vector de pET SUMO (25 ng/µl) 2 µl, agua estéril agregada a un volumen total de 9 µl, y se agregó 1 µl de T4 ADN ligasa (4,0

unidades de Weiss) y la reacción de unión se incubó a 15°C durante toda la noche luego se procedió a transformar células competentes One Shot® Mach1™-T1R (Invitrogen). Se seleccionaron diez (10) colonias y el ADN de plásmido se aisló de ellas cuando el Estuche de Purificación de Mini Plásmido PureLink™ HQ (Invitrogen). Los plásmidos se analizaron mediante análisis de restricción para confirmar la presencia y la orientación correcta del inserto. Desde los recombinantes, se aisló el ADN de plásmido como el anterior y los plásmidos se transformaron en células BL21 (DE3) One Shot® (Invitrogen). Los transformantes se cultivaron y la inducción de la expresión con IPTG en varios puntos de tiempo se llevó a cabo para determinar el tiempo óptimo de expresión. Para cada punto de tiempo, se retiraron 500 µl de los cultivos inducidos y no inducidos y cada una de las pelotillas de células se resuspendió en 80 µl de tampón de muestra de SDS-PAGE. Después de centrifugar las muestras en ebullición, 10 µl de cada una de las muestras se cargaron en un gel de SDS-PAGE y se sometieron a electroforesis.

La escala ascendente de la purificación de la proteína de fusión recombinante usando una columna de níquel HisTrap (GE Healthcare Life Sciences), se adaptó el siguiente procedimiento. Un cultivo de toda la noche (5%) se inoculó en 100-300 ml de LB más 50 µg/ml de kanamicina y se indujo

después de 2 horas con 1 mM IPTG. Después de 2 horas, las células se cosecharon centrifugando a 3000 g durante 10 minutos. La pelotilla se resuspendió en 10% (volumen total del cultivo) del tampón de unión (20 mM fosfato de sodio, 0,5 M NaCl y 20 mM imidazol a pH 7,4). Las células se sometieron a ultrasonido con el procesador de Líquidos Misonic UltraSonicate cinco veces durante un minuto con un espacio de un minuto en un balde de hielo. Las muestras sometidas a ultrasonido se separaron en la forma soluble e insoluble centrifugando a 4000 rpm durante 1 hora a 4°C. La fracción insoluble se resuspendió con tampón de unión que contiene 6M urea. Las fracciones soluble e insoluble se centrifugaron a 4000 rpm durante 1 hora a 4°C y luego se filtraron a través de la unidad de filtro de 0,22 µm. Una columna de HisTrap se equilibró con el tampón de unión y las muestras filtradas se cargaron en la columna. Luego, la columna se lavó con el tampón de unión con 40 mM imidazol y la proteína recombinante se eluyó con el tampón de unión que contiene 0,5 M imidazol (6M urea para la fracción insoluble). Todas las muestras recolectadas se ensayaron usando el protocolo de azul de Coomassie para las proteínas. La proteína recombinante pura que contiene la fracción eluida se dializó usando la tubería de Merck en el tampón de Tris-HCl. Después de la diálisis, se estimaron las concentraciones de las proteínas usando un reactivo de Bradford. La proteína nativa se generó usando

proteasa SUMO para descomponer el péptido del extremo de N que contiene el marcador 6xHis y SUMO siguiendo las instrucciones del fabricante.

EJEMPLO 16. Anticuerpos de sueros neutralizantes mezclados de ratones inmunizados con producto retenido SN07 se unen a todos los componentes de las VLP de EV71.

Las secuencias de codificación de VP1, VP2 y VP3 se clonaron por separado en el vector de PET SUMO como se describió en el Ejemplo 15. Cada proteína de la cápside se expresó en E. coli y se purificó como se describió en el Ejemplo 15. Las proteínas purificadas VP0, VP1, VP2 y VP3 se sometieron a Western blot y se sondearon con los sueros neutralizantes mezclados usados en el Ejemplo 4 a la dilución de 1:1000. Los sueros de ratones control usados en el Ejemplo 4 también se incluyen en los estudios.

Los resultados de los Western blot de la Figura 18 muestran que los sueros neutralizantes mezclados de ratones inmunizados con antígenos oligoméricos en el sobrenadante de células Sf9 infectadas por SN07 se unen a la totalidad de los componentes de VLP, VP0, VP1, VP2 y VP3.

EJEMPLO 17. Caracterización de VLP de HEV71: Extracción de VLP de HEV71 de sobrenadantes de cultivo

20 ml del sobrenadante de células infectadas con baculovirus recombinante SN07 se mezclaron con 10 ml del anticuerpo monoclonal neutralizante (EV18/4/D6-1/F1/G9) y se las dejó a temperatura ambiente durante 1 hora. La mezcla se cargo lentamente a través de una columna de 1 ml de MabSelect SuRe™ (GE Health Care) de Proteína recombinante A, 85 µm de tamaño de perla de agarosa, que se equilibró previamente con PBS, luego se lavó con 20 mls de PBS y luego se eluyó con 0,5 ml de 0,1M HCl de glicina, a pH 3,0. Las fracciones se neutralizaron con 30 µl de Tris-HCl, pH 8,8. Las fracciones de elución se corrieron sobre SDS-PAGE seguido por el manchado con azul de Coomassie y desmanchado.

La Figura 19 muestra que todos los componentes de VLP (VP0, VP1 y VP3) están a vista en el gel de SDS-PAGE manchado con azul de Coomassie.

EJEMPLO 18. Análisis de VLP de HEV71 purificadas por columna de afinidad (AFC)

Se preparó una columna de afinidad (AFC) con un anticuerpo monoclonal neutralizante (EV18/4/D6-1/F1/G9), que se

desarrolló usando un producto retenido de sobrenadante de SN07 de baculovirus. La fracción eluida se analizó mediante Western blot y se muestra en la Figura 20. La fracción eluida en los Carriles 1 y 2, se sondeó usando un anticuerpo anti-VP1, muestra que VP1 está presente en las VLP purificadas por AFC La fracción eluida en los Carriles 3 y 4, sondeada usando un anticuerpo anti-VP2, muestra que VP2 también está presente en las VLP purificadas por AFC. La fracción eluida en los Carriles 5 y 6, sondeada usando un anticuerpo monoclonal anti-VP2, muestra que VP2 está presente en las VLP purificadas por AFC.

EJEMPLO 19. Micrografía electrónica de VLP de HEV71 purificadas por AFC

La fracción eluida desde la columna de afinidad (AFC) del Ejemplo 18 se evaluó mediante un microscopio electrónico. Una imagen de la micrografía electrónica de las VLP purificadas por AFC se muestra en la Figura 21. Los resultados del microscopio electrónico confirman que la proteínas estructurales de HEV71 se ensamblan en VLP.

EJEMPLO 20. Estudios de protección de ratones usando producto retenido

El antígeno usado fue el producto retenido crudo concentrado 20x de SN07 de baculovirus recombinante preparado como se describió anteriormente. Se determinaron tapones vaginales indicadores de preñez la mañana después del apareamiento, se lo designó como Día Embrionario E0.5. Dos dosis de 100 µL del producto retenido mezclado con IMJECT® (alumbre) administrado por vía intraperitoneal a madres preñadas a E3.5 y, al confirmarse el embarazo mediante medición de peso, se administró una segunda dosis en E17.5. La muestra de sangre inicial se extrajo de cada ratón antes de la administración de la primera dosis de vacuna, se extrajeron muestras semanalmente después de ello hasta 14 días después del desafío viral de las crías. Se inmunizó a tres grupos de 20 ratones Balb/c y luego se los desafió (20 ratones), se simuló la inmunización de 10 ratones y se los desafió (20 ratones), 5 ratones no inmunizados, se infectó a crías de cinco días no desafiados con 50 µl de virus MP-26M que contienen 100 x HD_{50} de MP-26M. Se observó a todos los animales dos veces por día por los signos clínicos de enfermedad hasta 14 días después de la inoculación. Los signos anormales incluyeron no poder medrar; bajar de peso; falta de desarrollo; estómago sin leche; letargo; inclinación de la cabeza; postura encorvada; pelaje erizado; deshidratación; hipotermia; parálisis de extremidades. La parálisis se calificó de acuerdo con el siguiente sistema de puntaje:

0 – normal;

1 – debilidad de extremidades pero aún puede mover las extremidades;

2 incapacidad para mover las extremidades afectadas

3 cuadriplejia, es decir incapacidad para mover las extremidades

Se sacrificó a los animales que padecen la parálisis de puntaje 3 por dislocación cervical bajo anestesia (HD_{50}). La histopatología se realizó en cortes de una variedad de órganos blanco.

Los resultados del estudio de protección se muestran en la Tabla 2 siguiente.

Tabla 2

83% DE PROTECCIÓN EN PROTECCIÓN PASIVA

VACUNA	NÚMERO DE RATONES APAREADOS	NÚMERO DE RATONES PREÑADOS	NÚMERO DE CRÍAS / CAMADA	NÚMERO TOTAL DE CRÍAS	NÚMERO DE CRÍAS SOBREVIVIENTES	ÍNDICE DE SUPERVIENCIA	SUPERVIVENCIA MEDIANA (DÍAS)
PRODUCTO RETENIDO	20	2	1, 5	6	5*	83%	14
ALUMBRE /PBS	20	2	5, 7	12	0	0%	5
NINGUNA VACUNA / NINGÚN DESAFÍO	5+5	2+1	5, 7, 6	18	18	100%	14

Dos camadas de ratones nacieron de madres vacunadas con VLP. Una madre tuvo cinco crías y mostró buen cuidado maternal. La segunda madre tuvo una cría que fue muy débil y no mostró buen cuidado maternal.

Se puede llegar a la conclusión de que el 83% de los ratones que nacieron de madres inmunizadas quedaron protegidos del desafío de los virus.

En la Tabla 3 se muestra un resumen de las observaciones histopatológicas.

Tabla 3. RESUMEN DE OBSERVACIONES HISTOPATOLÓGICAS (N = no se observó ninguna anormalidad)

VACUNA	EDAD	DÍAS P1	RATÓN	SÍNTOMAS	BAZO	HÍGADO	CORAZON	MÚSCULO ESQUELÉT	MÉDULA ESPINAL	CEREBRO
VLP	19	14	V87P4	NGINGÚN SIGNO CLÍNICO DE INFECCIÓN	N	N(1)	N	0,5	N	N
	19	14	V87P5	NINGÚN SIGNO CLÍNICO DE INFECCIÓN	N	N(1)	N	0,5	N	N
	19	14	V87P6	NGINGÚN SIGNO CLÍNICO DE INFECCIÓN	N	N(1)	N	0,5	N	N
ALUMBRE / PBS	10	5	DC4	PARÁLISIS PUNTAJE 3	N	N(2)	N	3	N	N
			DC5	PARÁLISIS PUNTAJE 3	N	N(2)	N	3	N	N

			DC6	PARÁLISIS PUNTAJE 3	N	N(2)	N	03	N	N
NINGUNA VACUNA	19	NI	V116P4	NINGÚN SIGNO CLÍNICO DE INFECCIÓN	N	N(0, 5)	N	N	N	N
			V116P5	NINGÚN SIGNO CLÍNICO DE INFECCIÓN	N	N(0, 5)	N	N	N	N

Cinco ratones dieron a luz a camadas y se los incluyó en el estudio de protección de la vacuna (11% de índice de preñez). El valor del punto final humano (HD_{50}), calculado mediante el método de Reed y Muench, 1938, se determinó que fue $2,0 \times 10^2$ $TCID_{50}$ de MP-26M. Un 83% de los ratones que nacieron de madres inmunizadas quedaron protegidos del desafío.

Los resultados de la histopatología mostraron que 3 ratones de madres del grupo inmunizado por VLP de HEV71 a los que se desafió con el virus HEV71 no mostraron ningún signo de infección.

Sin embargo, 3 ratones de madres de la simulación de inmunización (alumbre/PBS) y a los que luego se desafió con HEV71 sucumbieron a la infección. Dos ratones de madres del grupo no inmunizado, no desafiado no mostraron ningún signo clínico de infección.

La vacuna de VLP protegió a una camada de ratones pequeños (5/6 crías de madres inmunizadas contra 0/12 crías de madres sometidas a simulación de inmunización) contra el desafío letal con una cepa adaptada de ratones del genotipo B3 de HEV71.

EJEMPLO 21. Expresión de VLP de *enterovirus humano C* (PV de poliovirus)

Se crearon variantes de cassettes de expresión poliovirus para generar VLP de poliovirus. Todos los cassettes de expresión comprendieron un polipéptido P1 de poliovirus y difirieron con respecto a al IRES, cuyo IRES dirige la expresión de una proteasa 3CD de poliovirus: (PV-P1+HEV71-IRES+PV-3CD); (PV-P1+EMCV-IRES+PV-3CD); (PV-P1+PV-IRES+PV-3CD). Se ensayaron los baculovirus recombinantes que alojaban a los cassettes de expresión de VLP de poliovirus. Los lisados de las células infectadas por baculovirus se cosecharon el día 3 después de la infección y el VP3 de poliovirus se evaluó usando anticuerpos anti-PVP3 de conejo (1:2000).

Solamente el constructo que contiene IRES de PV produjo una proteína VP-3 del tamaño esperado. A diferencia del constructo de IRES de PV, la proteína VP3 de poliovirus no se procesa

correctamente cuando la proteasa 3CD está bajo el control del IRES de HEV71 o el IRES de EMCV y en consecuencia, afecta negativamente la producción de las VLP de poliovirus. Por lo tanto, el cassette de expresión de VLP de PV de poliovirus que aloja el IRES de PV es muy eficiente para la producción de VLP de poliovirus.

EJEMPLO 22. ELISA para demostrar el ensamblaje de VLP de PV de poliovirus

Para demostrar la generación de VLP de poliovirus del cassette de expresión de VLP de PV, se realizó un ELISA de dos sitios usando lisados y sobrenadantes de baculovirus recombinantes que transportan el cassette de expresión de VLP de PV en donde la proteasa 3CD de PV está bajo el control de un IRES de poliovirus. Se infectaron células Sf9 con los baculovirus recombinantes que transportan un cassette de expresión de las VLP de PV que incluye el IRES de PV. Se cosecharon lisados y sobrenadantes el día 3 después de la infección. La formación de las VLP de poliovirus se evaluó usando un ELISA de dos sitios.

El procedimiento de ELISA de dos sitios se realizó preparando anticuerpos VP3 anti-poliovirus de conejo purificados por proteína A como los anticuerpos de captura y diluyendo en el

tampón de recubrimiento, 0,05M tampón de Carbonato-bicarbonato, pH 9,6, a 50µg/mL. 100 µl/receptáculo de los anticuerpos se distribuyó en inmunoplacas NUNC y se almacenó a 4°C toda la noche. Después de lavar con PBST (0,05%), las inmunoplacas se bloquearon con 200 µl/receptáculo de tampón de bloqueo, 1% de caseína en PBS, a temperatura ambiente durante 2 horas. Las muestras de lisados y sobrenadantes de baculovirus recombinantes que transportan un cassette de expresión de VLP de PV en donde la proteasa 3CD de PV está bajo el control de un IRES DE poliovirus se diluyeron en diluyentes, 0,2% caseína en PBS, se distribuyeron a 100 µl/receptáculo, y se incubaron a temperatura ambiente durante 1 hora y luego se lavaron con PBST (0,05%).

Para la detección de VLP, los anticuerpos monoclonales de ratón, anti-VP1 (Clon 5-D8/1; Dako), se prepararon en 0,2% de caseína en diluyentes de PBS a una dilución de 1:250. Los anticuerpos monoclonales de detección diluida se distribuyeron a 100 µl/receptáculo y luego se incubaron a temperatura ambiente durante 1 hora. Se prepararon anticuerpos 2° conjugados por HRP anti-ratón en 0,2% de caseína en diluyentes de PBS a una dilución de 1:1000. Después de lavar la placa con PBST (0,05%), 100µl/receptáculo de los anticuerpos 2° diluidos se distribuyeron y la placa se incubó a temperatura ambiente durante 1 hora. Las places se lavaron con tampón de lavado,

PBST (0,05%). El sustrato de peroxidasa de microreceptáculo de componente SureBlue™ TMB 1(KPL) se distribuyó a 100 µl/receptáculo y se incubó a temperatura ambiente durante 10 minutos para el desarrollo. 100 µl/receptáculo de solución de parada, 5 mM NaOH, se agregó para detener la reacción. La absorbencia de cada uno de los receptáculos se leyó a un diámetro exterior de 650 nm.

Los resultados de ELISA de dos sitios que se muestran en la Figura 23 demuestran que las proteínas VP1 y VP3 están en asociación una con otra lo cual indica que las VLP en realidad se forman desde el cassette de expresión de VLP de PV.

EJEMPLO 23. Construcción de Cassettes de Expresión de VLP de HEV71 con IRES de HEV71 (P1+IRES de HEV71+3CD)

La estructura esquemática de un cassette de VLP de HEV71 con IRES de HEV71 se muestra en la Figura 23. El cassette de expresión es similar al constructo que se muestra en el Ejemplo 1 (pSN01) excepto que la expresión de la proteasa 3CD se impulsa por el IRES de HEV71 en lugar del IRES de EMCV. La secuencia de IRES de HEV71 se halla en GenBank, Acceso Número DQ341362,1; nucleótidos 1 a 747. Un vector que contiene el cassette de expresión se introduce en el plásmido de expresión de baculovirus pDEST8 (vector de Destino) mediante

recombinación de attL/aaR in vitro usando LR CLONASE®, siguiendo las instrucciones en el manual BAC-TO-BAC® Invitrogen (2009). La recombinación entre el vector de entrada y pDEST8 produce un clon de expresión. Los clones de expresión da origen al bácmido recombinante transformando DH10bac como se describió en el manual BAC-TO-BAC® Invitrogen. La transfección del bácmido recombinante en células Sf9 rescata el baculovirus recombinante que transporta el cassette de expresión que aloja P1, IRES de HEV71 y 3CD.

Se puede usar otra infección de células Sf9 para evaluar la expresión de proteínas de la cápside procesadas con baculovirus recombinantes rescatados en placas de 6 receptáculos. Un antisuero de conejo policlonal específico para VP1, VP0 y VP3 identifica las VLP ensambladas mediante Western blot de lisados y sobrenadantes de células Sf9 infectadas por baculovirus recombinantes.

EJEMPLO 24. Construcción de Cassettes de Expresión de VLP de HEV71 con IRES de PV (P1+IRES de PV+3CD)

El cassette de expresión es similar al cassette de expresión del Ejemplo 24 excepto que la expresión de la proteasa 3CD se impulsa por un IRES de poliovirus (IRES de PV) en lugar de un

IRES de HEV71. La secuencia de IRES de poliovirus se halla en GenBank, Acceso Número V01150.12; nucleótidos 1 a 628.

Un vector que contiene el cassette de expresión de HEV71 se introduce en el plásmido de expresión de baculovirus pDEST8 (vector de Destino) mediante recombinación attL/aaR in vitro usando LR CLONASE®, siguiendo las instrucciones del manual BAC-TO-BAC® Invitrogen (2009). La reacción de recombinación entre el vector de entrada y pDEST8 se instala para producir un clon de expresión. Un clon de expresión da origen al bácmido recombinante transformando DH10bac como se describe en el manual BAC-TO-BAC® Invitrogen. La transfección del bácmido recombinante en células Sf9 rescata baculovirus recombinante que transporta el cassette de expresión que aloja P1, IRES de PV y 3CD.

Se puede usar otra infección de células Sf9 para evaluar la expresión de proteínas de la cápside procesadas con baculovirus recombinantes rescatados en placas de 6 receptáculos. Un antisuero de conejo policlonal específico para VP1, VP0 y VP3 identifica las VLP ensambladas por Western blot de lisados y sobrenadantes de células Sf9 infectadas por baculovirus recombinantes.

TRATADO DE COOPERACIÓN EN MATERIA DE PATENTES

PCT

INFORME DE BÚSQUEDA INTERNACIONAL

(Artículo 18 y Normas 43 y 44 del PCT)

<table>
<tr><td>Referencia de Archivo del Solicitante o agente:
SENTINEXT 2 PCT</td><td colspan="2">PARA REALIZAR OTROS TRAMITES
Véase Notificación de Transmisión de Informe de Búsqueda Internacional (Formulario PCT/ISA/220) y, si fuere aplicable, del punto 5 más abajo.</td></tr>
<tr><td>Solicitud de Patente Internacional No:
PCT/IB2012/003114</td><td>Fecha de Presentación Internacional (día/mes/año):
1-11-2012</td><td>Fecha de Prioridad (Más antigua) (día/mes/año):
3-11-2011</td></tr>
<tr><td colspan="3">Solicitante:
SENTINEXT THERAPEUTICS SDN BHD</td></tr>
<tr><td colspan="3">Este informe de búsqueda internacional ha sido preparado por esta Autoridad de Búsqueda Internacional y es transmitido al solicitante conforme al Artículo 18. Se transmite una copia del mismo a la Oficina Internacional.
Este informe de búsqueda internacional consta de _7_ páginas.
[] Está acompañado asimismo de una copia de cada documento de arte previo citado en el informe.</td></tr>
<tr><td colspan="3">1. Fundamento de Informe</td></tr>
</table>

a. Con respecto al **idioma,** la búsqueda internacional se llevó a cabo basada en:

[x] la solicitud de patente internacional en el idioma en que ésta se presentó.

[ ] una traducción de la solicitud de patente internacional al _____, que es el idioma de la traducción provista para los fines de la búsqueda internacional (Normas 12.3(a) y 23.1(b)).

b. [] Este informe de búsqueda internacional se ha establecido tomando en cuenta la **rectificación de un error evidente** autorizada o notificada por a esta Autoridad conforme a la Norma 91 (Norma 43.6bis(a)).

c. [X] Con respecto a cualquier **secuencia de nucleótidos y/o de aminoácidos** revelada en la solicitud de patente internacional, véase el Casillero Nº I

2. [] **Ciertas reivindicaciones se consideraron no buscables** (Véase el Casillero Nº II)

3. [X] **Falta Unidad de Invención** (Véase el Casillero Nº III).

4. Con respecto al **título**:

[] el texto se aprueba tal como lo presentó el solicitante

[X] el texto ha sido establecido por esta Autoridad como sigue:

VACUNAS DIRIGIDAS CONTRA ENTEROVIRUS HUMANOS

5. Con respecto al **resumen:**

[x] el texto es aprobado como lo presentó el solicitante

[] el texto ha sido establecido, conforme a la Norma 38.2(b), por esta Autoridad como aparece en el Casillero N° IV. El Solicitante puede, dentro de un mes desde la fecha de remisión de este informe de búsqueda internacional, presentar comentarios ante esta Autoridad.

6. Con respecto a los **dibujos**,

a. La Lámina de **dibujos** que se publicará con el resumen es: Figura No. __.

[] sugerida por el Solicitante

[] seleccionada por esta Autoridad, debido a que el Solicitante no sugirió una Figura.

[] seleccionada por esta Autoridad, debido a que ésta es la figura que mejor caracteriza a la invención

b. [X] No se debe publicar ninguna de las figuras con el resumen.

INFORME DE BÚSQUEDA INTERNACIONAL	Solicitud de Patente Internacional Nº PCT/IB2012/003114

Casillero Nº II. Secuencia(s) de nucleótidos y/o de aminoácidos (Continuación del punto 1.c de la página 1)

1. Con respecto a todas las secuencias de nucleótidos y/o aminoácidos reveladas en la solicitud de patente internacional y necesarias para la invención reivindicada, la búsqueda internacional se llevó a cabo sobre la base de:

a. (medio)

[] en papel

[x] en forma electrónica

b. (tiempo)

[x] en la solicitud de patente internacional como se presentó

[] junto con la solicitud de patente internacional en forma electrónica

[] con posterioridad a esta Autoridad con el propósito de la búsqueda

2. [] Además, en el caso de que se haya presentado o provisto más de una versión o copia de un listado de secuencias y/o tabla relacionada con ella, se presentaron las declaraciones necesarias de que la información de las copias posteriores o

adicionales es idéntica a aquella de la solicitud de patente presentada o que no vaya más allá de la solicitud de patente presentada, según corresponda.

3. Comentarios adicionales:

INFORME DE BÚSQUEDA INTERNACIONAL	Solicitud de Patente Internacional N° PCT/IB2012/003114

Casillero Nº II. Observaciones cuando ciertas reivindicaciones se consideraron no buscables (Continuación del punto 2 de la página 1).

El presente informe de búsqueda internacional no se ha establecido con respecto a ciertas reivindicaciones conforme a al Artículo 17(2)(a) por los siguientes motivos:

1. [] Reivindicaciones N°:

porque se relacionan con un objeto que no se requiere que busque esta Autoridad, a saber:

2. [x] Reivindicaciones N°:

porque se relacionan con partes de la solicitud internacional que no cumplen con los requisitos establecidos en tal magnitud que no se puede realizar una búsqueda significativa, específicamente:

3. [] Reivindicaciones N°:

porque son reivindicaciones dependientes y no están redactadas de acuerdo con la segunda y tercera oraciones de la Norma 6.4(a).

Casillero Nº III. Observaciones cuando falta unidad de invención (Continuación del punto 3 de la página 1)

Esta Autoridad de Búsqueda Internacional encontró varias invenciones en esta solicitud de patente internacional, a saber:

Véase la página adicional

1. [] Como todos los aranceles de búsqueda adicionales se pagaron oportunamente por el solicitante, este informe de búsqueda internacional abarca todas las reivindicaciones buscables.

2. [] Como las reivindicaciones buscables pudieron buscarse sin esfuerzo que justifique un arancel adicional, esta Autoridad no intima al pago de ningún arancel adicional.

3. [] Como ninguno de los aranceles de búsqueda adicionales requeridos se pagaron oportunamente por el solicitante, este informe de búsqueda internacional abarca solamente aquellas reivindicaciones para las cuales se pagaron aranceles, específicamente las reivindicaciones Nº:

4. [x] El solicitante no pagó ninguno de los aranceles de búsqueda adicionales. En consecuencia, este informe de búsqueda internacional se limita a la invención mencionada en

primer lugar en las reivindicaciones; está abarcada por las reivindicaciones Nº: 1-55 (parcialmente)

Observación Bajo Protesta:

[] Los aranceles de búsqueda adicionales estuvieron acompañados por la protesta del solicitante y cuando correspondía por el arancel de protesta

[] Los aranceles de búsqueda adicionales estuvieron acompañados por la protesta del solicitante pero el arancel de protesta aplicable no se pagó dentro del plazo especificado en la invención.

[] Ninguna protesta acompañó el pago de los aranceles de búsqueda adicionales.

INFORME DE BÚSQUEDA INTERNACIONAL	Solicitud de Patente Internacional N° PCT/IB2012/003114

A. Clasificación de la Materia:

INV. A61K39/125 A61K39/13 A61K39/135

ADD.

De acuerdo con la Clasificación Internacional de Patentes (IPC) o con la clasificación nacional e IPC.

B. CAMPOS DE BÚSQUEDA

Documentación mínima buscada (sistema de clasificación seguido por símbolos de clasificación)

A61K

Documentación buscada además de la documentación mínima en la medida en que tales documentos están incluidos en los campos de búsqueda:

Base de datos electrónica consultada durante la búsqueda internacional (nombre de la base de datos y, si es posible, términos de búsqueda usados):

EPO-Internal, BIOSIS, CHEM ABS Data, WPI Data

C. Documentos Considerados Relevantes		
Categoría*	Cita de documento, indicando, si corresponde, los pasajes relevantes	Relevante para la reivindicación N°
X	CHUNG YAO-CHI ET AL: "Inmunización con partículas similares a virus de enterovirus 71 produce respuestas inmunes potentes y protege a ratones contra un desafío letal". VACUNA, volumen 26, N° 15, marzo de 2008 (03-2008), páginas 1855-1862, XP022534445, ISSN: 0264-410X página 1856, columna de la izquierda, ultimo párrafo - columna derecha, párrafo 2 página 1861, último párrafo	1-55
[x] Se mencionan otros documentos en la continuación del Casillero C. [x] Véase el anexo de familia de patentes		

* Categorías especiales de documentos citados:	"T": documento posterior publicado con posterioridad a
"A": documento que define el estado general del arte que no se considera de particular relevancia	la fecha de presentación internacional o a la fecha de prioridad y que no está en conflicto con la solicitud
"E": documento anterior pero publicado en la fecha de presentación internacional o con posterioridad a ella.	pero citado para comprender un principio o teoría que sustenta la invención.
"L": documento que puede arrojar dudas sobre la reivindicación de prioridad o que se cita para establecer la fecha de publicación de otra cita o por otra razón especial (especificada).	"X": documento de particular relevancia: la invención reivindicada no puede considerarse nueva o no puede considerarse que implica una actividad inventiva cuando el documento se toma solo.
"O": documento que refiere a una revelación oral, uso, exhibición u otro medio.	"Y": documento de particular relevancia: la invención reivindicada no puede considerarse que implica una actividad inventiva cuando el documento se combina con otro
"P": documento publicado antes de la fecha de presentación internacional pero con posterioridad a la fecha de	u otros documentos, siendo dicha combinación obvia para

prioridad reivindicada.	un experto en el arte. "&": documento miembro de la misma familia de patentes.
Fecha de la finalización efectiva de la búsqueda internacional 21 de junio de 2013	Fecha de envío del informe de búsqueda internacional 13/09/2013
Nombre y domicilio para correspondencia de la ISA/ Oficina de Patentes Europea, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040 Fax: (+31–70) 340–3016	Funcionario Autorizado Mata Vicente, Teresa

Formulario PCT/ISA/210 (página 2) (abril de 2005)

INFORME DE BÚSQUEDA INTERNACIONAL	Solicitud de Patente Internacional N° PCT/IB2012/003114

C (Continuación). Documentos Considerados Relevantes

Categoría*	Cita de documento, indicando, si corresponde, los pasajes relevantes	Relevante para la reivindicación N°
X	CHUNG CHENG-YU ET AL: "Vacuna de partículas similares a virus de Enterovirus 71: condición de producción mejorada para el rendimiento mejorado", VACUNA, volumen 28, N° 43, octbre de 2010 (10-2010), páginas 6951-6957, XP027392042, ISSN: 0264-410X resumen	1-55
X	MATRAHIM N A ET AL: “Constructo de Vacuna de ADN en la Presencia del IRES de EV71 produjo un Título de Anticuerpo Neutralizante Más Alto”, REVISTA INTERNACIONAL DE	1-4, 8-11

	ENFERMEDADES INFECCIOSAS, volumen 12, N° Suplemento 1, diciembre de 2008 (12-2008), página E254, XP002693933, y 13° C0NGRESO INTERNACIONAL DE ENFERMEDADES INFECCIOSAS; KUALA LUMPUR, MALASIA; 19-22 DE JUNIO DE 2008 ISSN: 1201-9712 Véanse las últimas cuatro líneas	
X	US 4.508.708 A (VAN WEZEL ANT0NIUS L 2 de abril de 1985 (2-04-1985) resumen; reivindicación 8	1-4, 6-11

INFORME DE BÚSQUEDA INTERNACIONAL Información sobre miembros de familia de patentes		Solicitud de Patente Internacional Nº PCT/US2012/003114	
Documento de patente citado en informe de búsqueda	Fecha de publicación	Miembro(s) de familia de patentes	Fecha de publicación
US 4508708 A	2-04-1985	CA 1193545 Al	17-09-1985
		DE 3273145 D1	16-10-1986
		EP 0066932 Al	15-12-1982
		NL 8102740 A	3-01-1983
		US 4508708 A	2-04-1985

Formulario PCT/ISA/210 (anexo de familia de patentes) (abril de 2005)

	Solicitud de Patente Internacional N° PCT/IB2012/003114

OTRA INFORMACIÓN CONTINUACIÓN DE PCT/ISA/210

Esta Autoridad de Búsqueda Internacional halló varias (grupos de) invenciones en la presente solicitud de patente internacional, de la siguiente manera:

1. Reivindicaciones: 1-55 (parcialmente)

   Una vacuna que comprende uno o más antígenos inmunológicamente activos que comprenden uno o más polipéptidos de HEV71 de VP0, PV1, VP2, VP3, VP4 y sus fragmentos inmunológicamente activos. Dicha vacuna, en donde está en la forma de una VLP, un capsómero, un complejo y/o un agregado. Un cassette de expresión que comprende un promotor unido operativamente a un ácido nucleico que codifica un polipéptido P1 de HEV71, un IRES y un ácido nucleico que codifica una proteasa 3CD de HEV71; el uso del cassette para elaborar una vacuna.

2-8. reivindicaciones: 1-55 (parcialmente)

   Ídem 1, pero limitado a cada uno de los virus Virus Coxsackie A16, virus Coxsackie 8, echovirus, HP1, HP2, HP3 y EV 68.

(12) SOLICITUD DE PATENTE INTERNACIONAL PUBLICADA CONFORME AL TRATADO DE COOPRACIÓN EN MATERIA DE PATENTES (PCT)

(19) Organización Mundial de la Propiedad Intelectual
Oficina Internacional
(43) Fecha de Publicación Internacional:
4 de julio de 2013 (4-07-2013)

WIPO | PCT

(10) Publicación Internacional Número:
WO 2013/098655 A2

(51) Clasificación Internacional de Patentes:
A61K 39/125 *(2006.01)*

(21) Solicitud de Patente Internacional Número:
PCT/US2012/003114
(22) Fecha de Presentación Internacional:
1 de noviembre de 2012 (1-11-2012)
(25) Idioma de presentación: Inglés
(26) Idioma de publicación: Inglés
(30) Datos de la Prioridad:
PI2011005318 3 de noviembre de 2011 (3/11/2011) US

(71) Solicitante*:* **SENTINEXT THERAPEUTICS SDN BHD [MY/MY];** Suite 19-h, Level 19, Menara Northam, 55 Jalan Sultan Ahmad Shah, Penang, 10050 (MY).

(72) **Inventores***:* **CARDOSA, Mary, Jane;** No. 8, Richmond Hill, Lorong Stampin Tengan 5g2, Kuching Sarawak, 93250 (MY). **JAMILUDDIN, Mohamad, Fakruddin;** 10-13-p2 Sri Saujana Apts., Lorong Sungai Dua, Gelugor Penang, 11700 (MY). **HAMID, Sharifah, Binti;** No. 39 Jalan Anggerik Tainia, 31/120 Kota Kemuning, Seksyen 31, Shah Alam Selangor, 40460 (MY).

(81) Estados Designados *(a menos que se indique de otro modo, para todos los tipos de protección nacional existentes):* AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PE, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW**.**

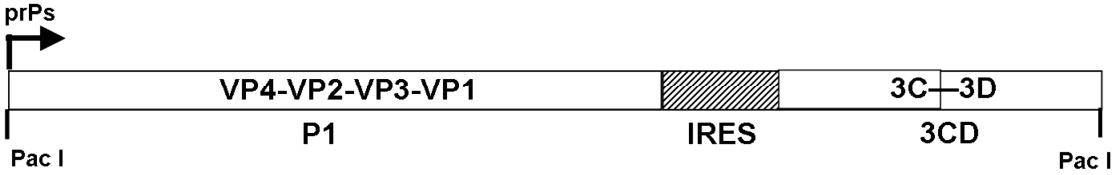
(84) Estados Designados *(a menos que se indique de otro modo, para todos los tipos de protección regional existentes):* ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasia (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), Europeo (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Publicada:
-- sin informe de búsqueda internacional y se publicará nuevamente cuando se reciba ese informe (Norma 48.2(g)).
-- con la parte del listado de secuencias de la memoria descriptiva (Norma 5.2(a)).

(56) Título: ANTÍGENOS Y VACUNAS DIRIGIDOS CONTRA ENTEROVIRUS HUMANOS

(57) Resumen de la invención: La presente invención proporciona materiales y métodos para producir en forma antígenos inmunológicamente activos derivados de miembros de la familia del virus *Picornaviridae*. Los antígenos del picornavirus de la invención pueden estar en una forma para su uso como una vacuna administrada a un sujeto en un tratamiento terapéutico o para la prevención de una infección por el picornavirus. Los antígenos del picornavirus de la invención pueden estar en la forma de una composición inmunogénica para su uso en vacunas que se administran para la prevención de una infección por un *Enterovirus*. La presente invención además comprende composiciones inmunogénicas que comprenden antígenos de *enterovirus A Humano, enterovirus B Humano*, *enterovirus C Humano*, *enterovirus D Humano* y su uso en vacunas para la prevención de una infección por un *Enterovirus*.

Figura 1. Cassette de expression de VLP de HEV71 [P1+IRES+3CD] y el plásmido pSN01



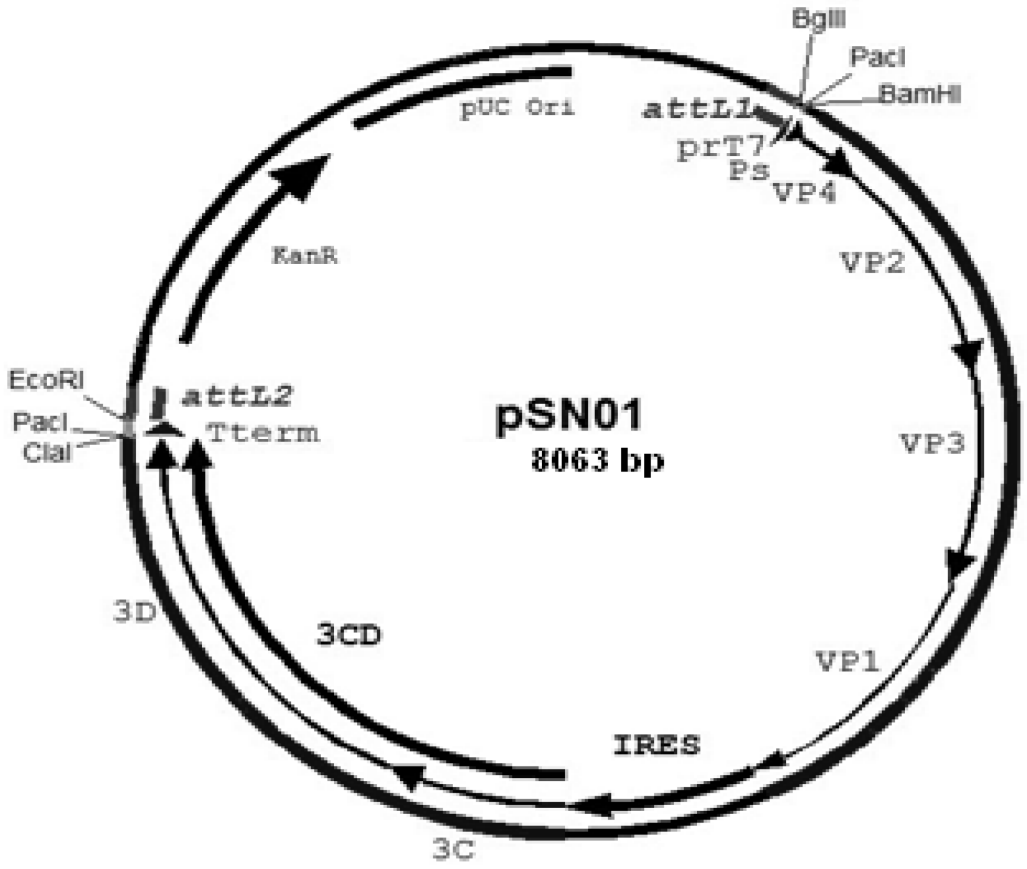
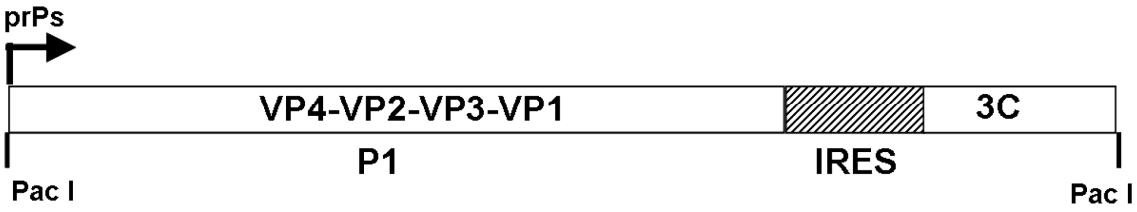


Figura 2. Cassette de expression de VLP de HEV71 [P1+IRES+3C] y el plásmido pSN03.



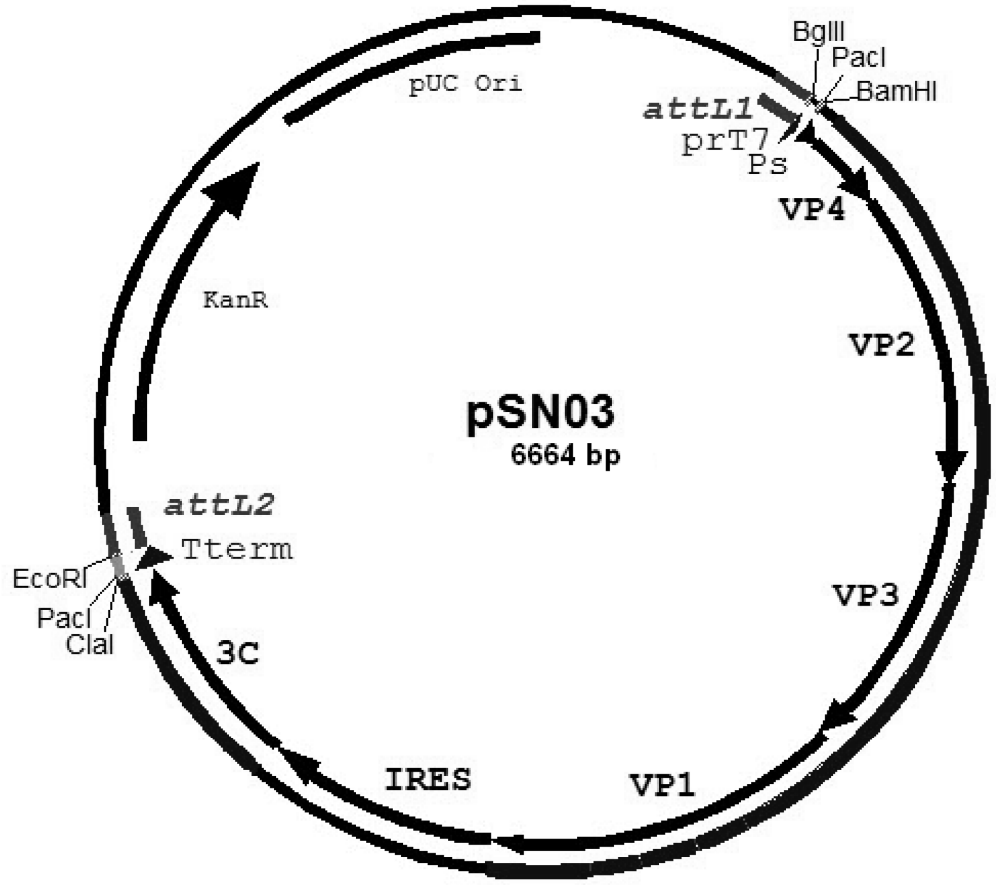


Figura 3. Expressión de VP1 en sobrenadante de células Sf9 infectadas por SN07

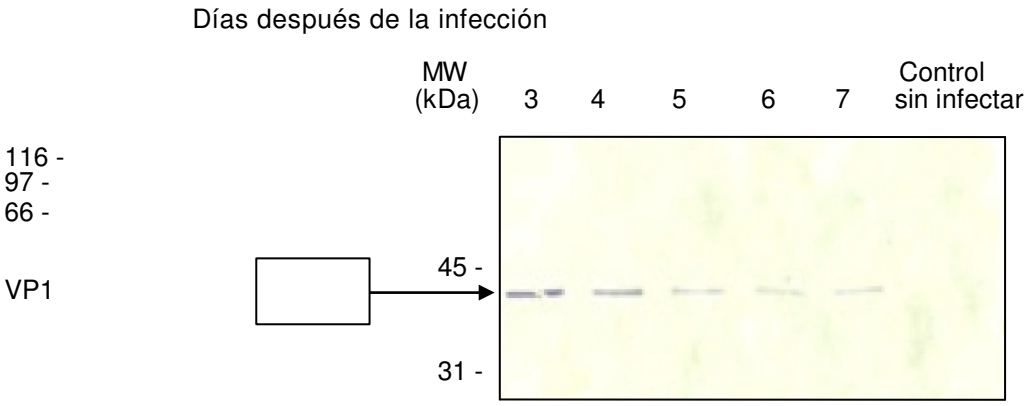


Figura 4. VP1 procesado tanto en los sobrenadantes como en los lisados

Carriles 1 y 5:bacSN07p3

Carriles 2 y 6:bacSN08p3

Carriles 3 y 7:bacGUSd2

Carriles 4 y 8: Sf9 control

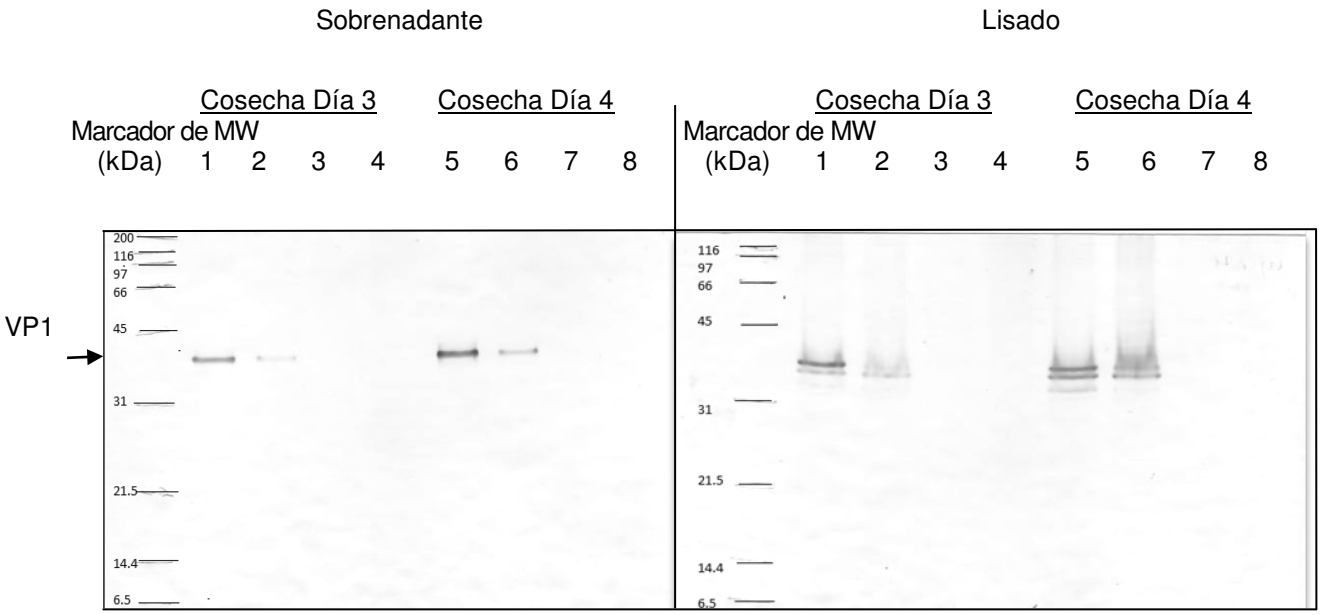


Figura 5. VP1 y VP0 están en el producto retenido después de la ultrafiltración sobre una membrana de corte de peso molecular (MWCO) de 100 kDa

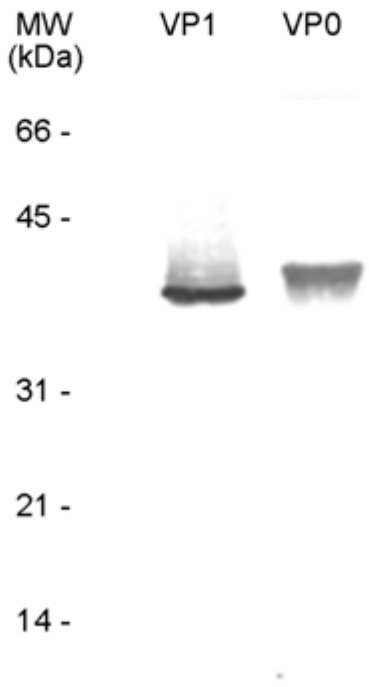
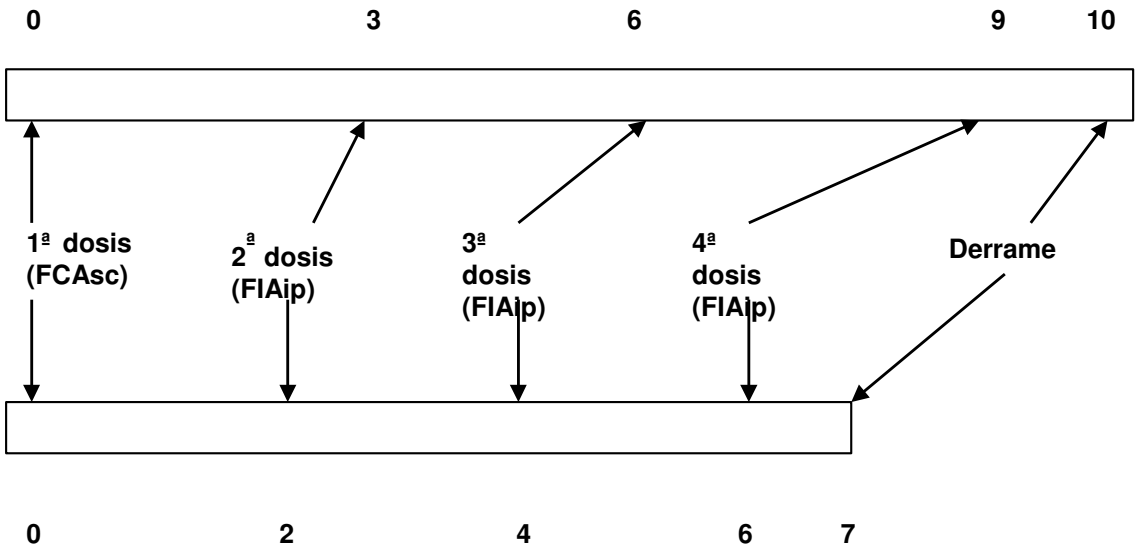


Figura 6.

PRIMERA PRUEBA DEL CONCEPTO DE NUESTRA ESTRATEGIA: DOS PROGRAMAS DE INMUNIZACIÓN (grupo de 4 ratones).

El inmunógeno se preparó en Coadyuvante Completo de Freund (FCA) o Coadyuvante Incompleto de Freund (FIA) y se administró mediante inyección por vía subcutánea (sc) o inyección intraperitoneal (ip).



SEMANAS

Figura 7. Inmunotransferencias sondeadas con antisueros policlonales de conejo contra VP0 (flecha) en 3 tiempos diferentes de cosecha

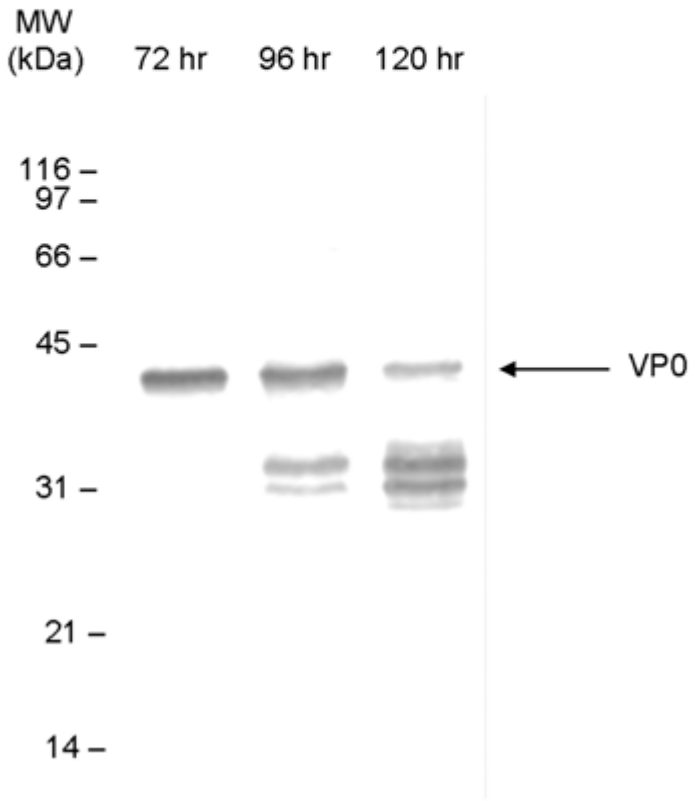


Figura 8. Sueros neutralizantes mezclados de ratones inmunizados con los antígenos oligoméricos en el sobrenadante de células Sf9 infectadas por SN07 tienen altos títulos contra VP2 recombinante en ELISA.

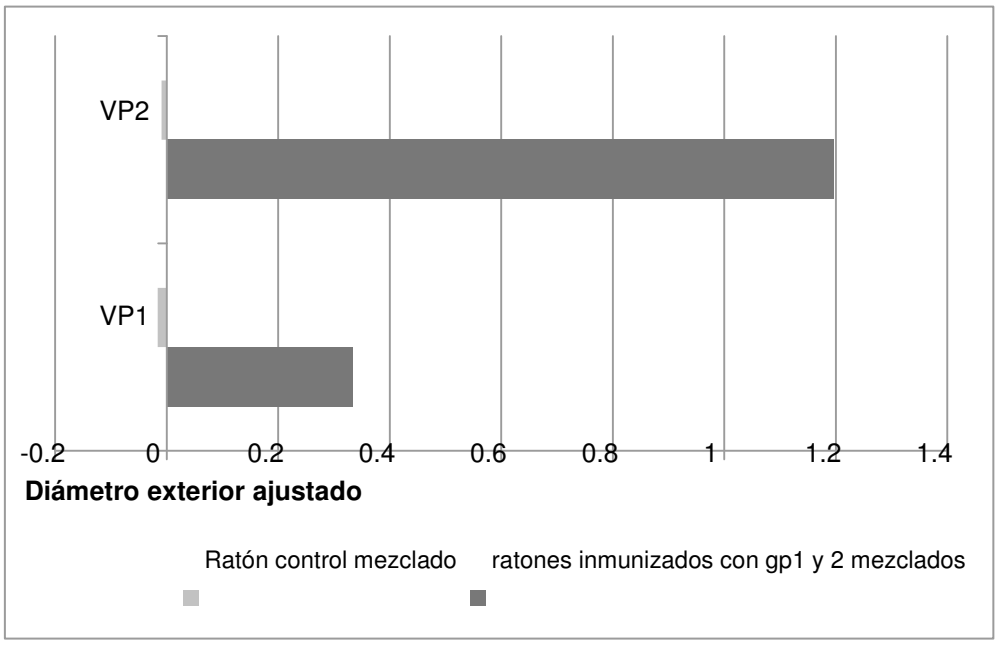
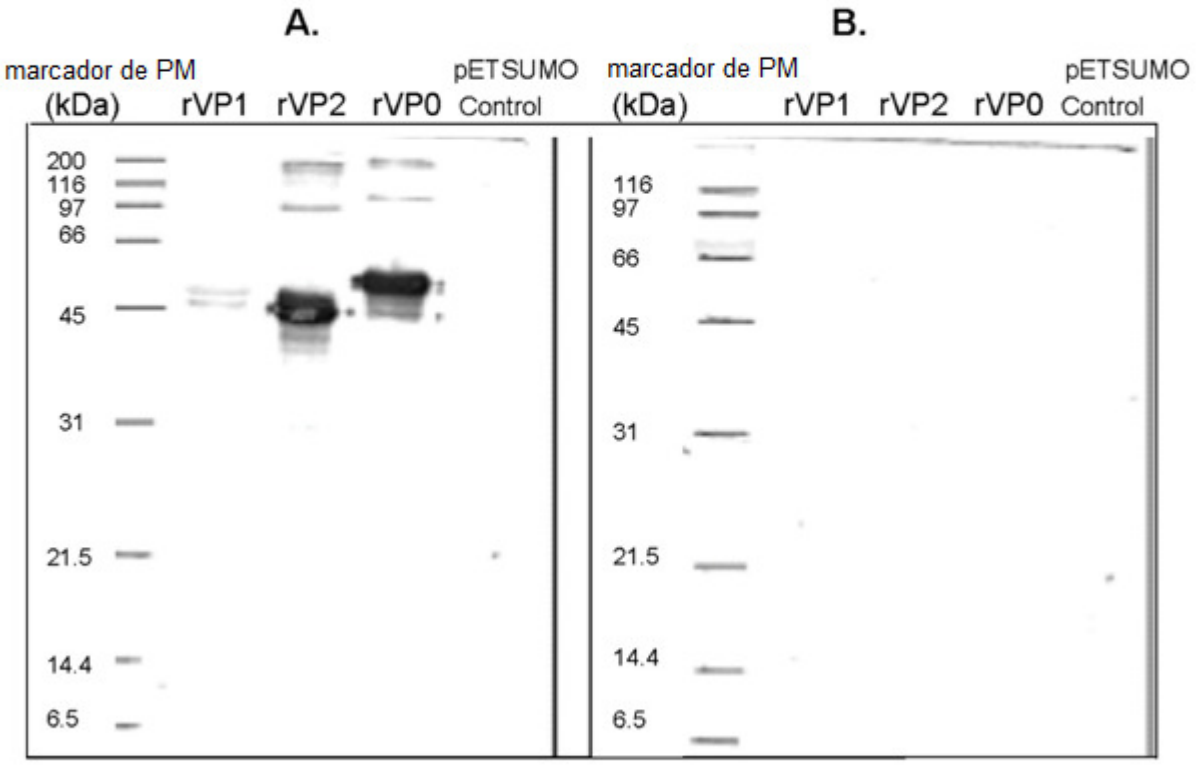


Figura 9. Sueros neutralizantes mezclados desde ratones inmunizados con antígenos oligoméricos en el sobrenadante de células Sf9 infectados por SN07 se unen a más fuertemente a VP2 y VP0 que a VP1



A. Sondeado con VLP anti-bacSN07p3 de suero de ratones a dilución 1/2000
B. Sondeado con células control anti-SF9 de seuero de ratones a dilución 1/500

Figura 10. Región de IRES del genoma de EMCV (SEQID NO:1). La región de IRES representa la secuencia genómica de EMCV en GenBank acceso número AF113968.2; nucleótidos1666 a 2251.

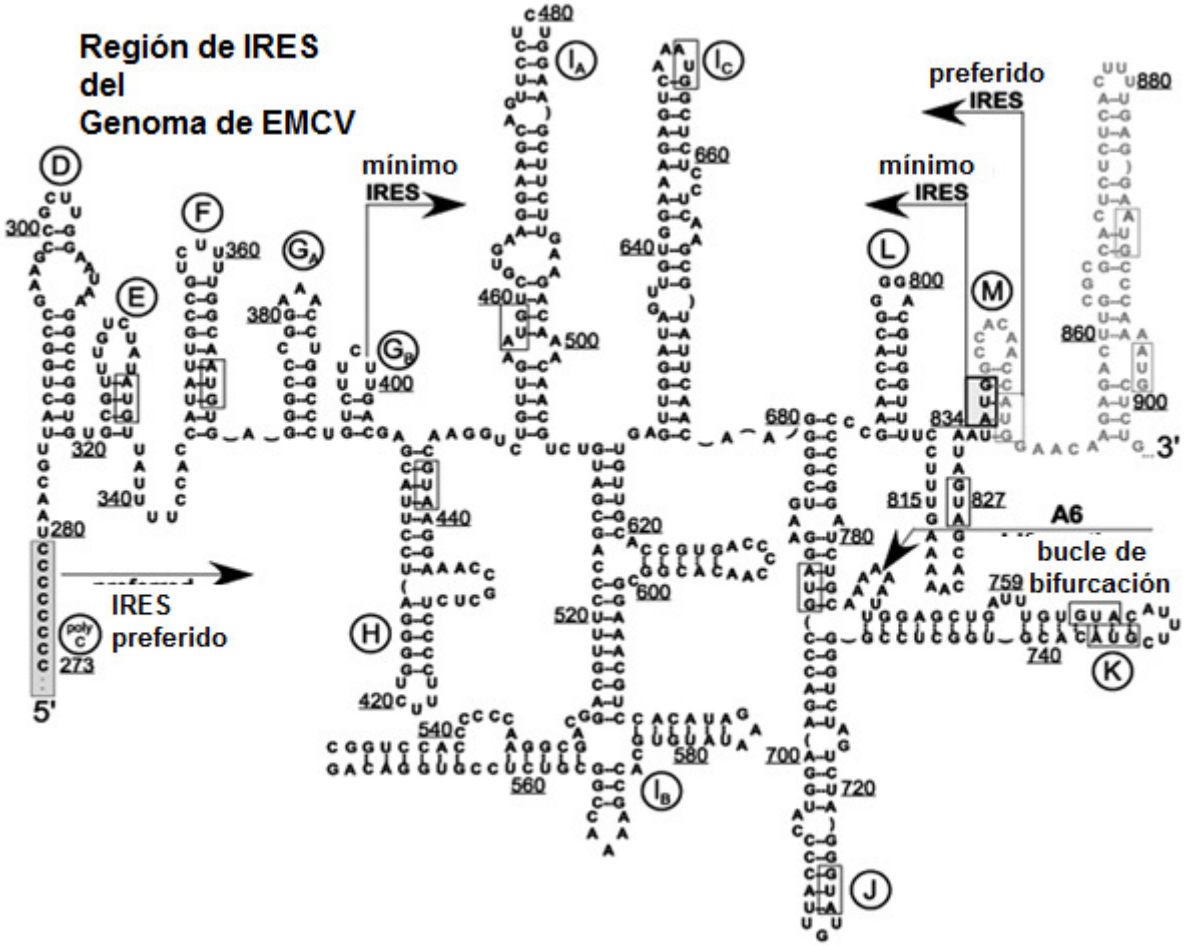


Figura 11. Mejor encuadre del codón de inicio de EMCV con la secuencia de codificación de proteasa 3CD; Secuencia de codificación de IRES de EMCV (SEQIDNO:2) se compara con una secuencia de codificación de IRES de EMCV mutante (SEQIDNO:3).

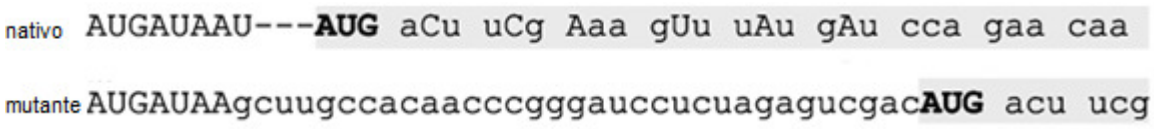


Figura 12. Plásmido pSN01-M1.

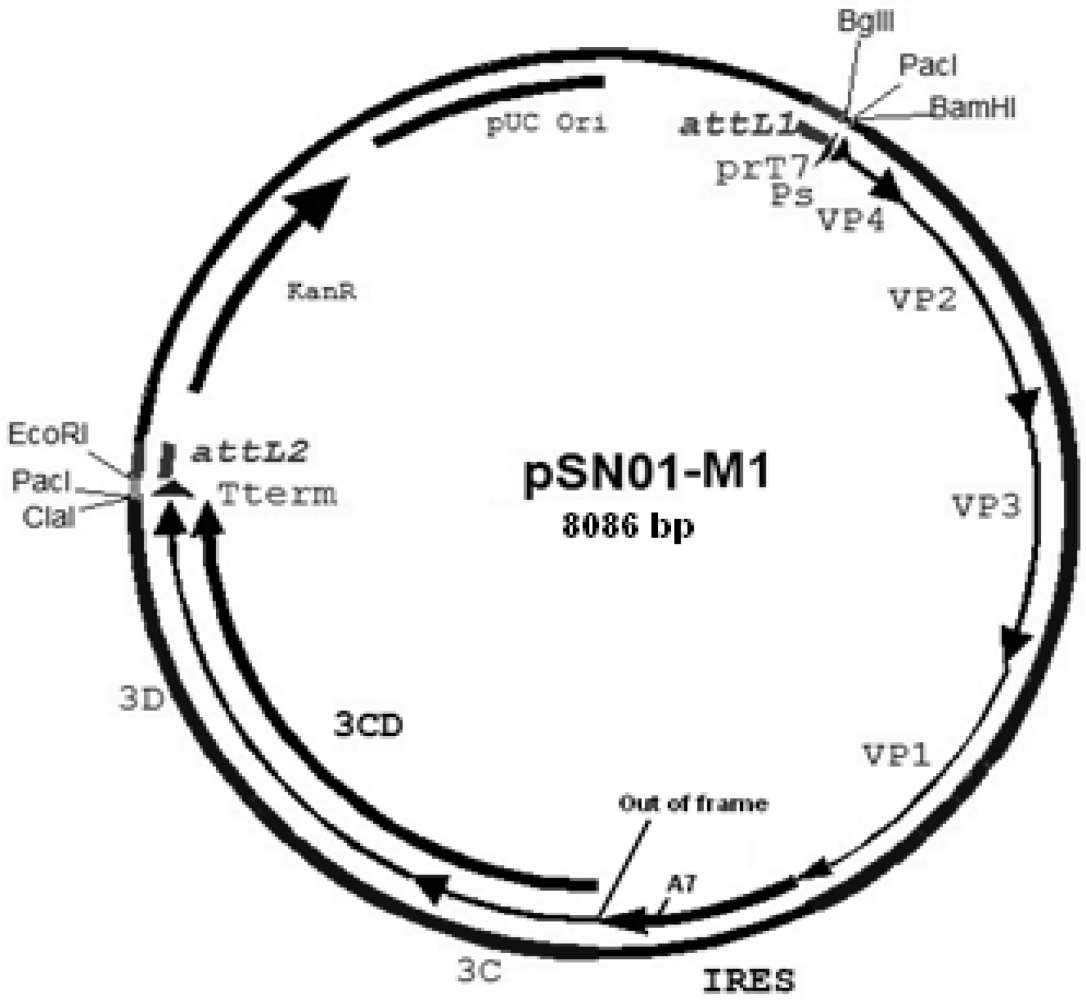


Figura 13. Plásmido pSN01-M2

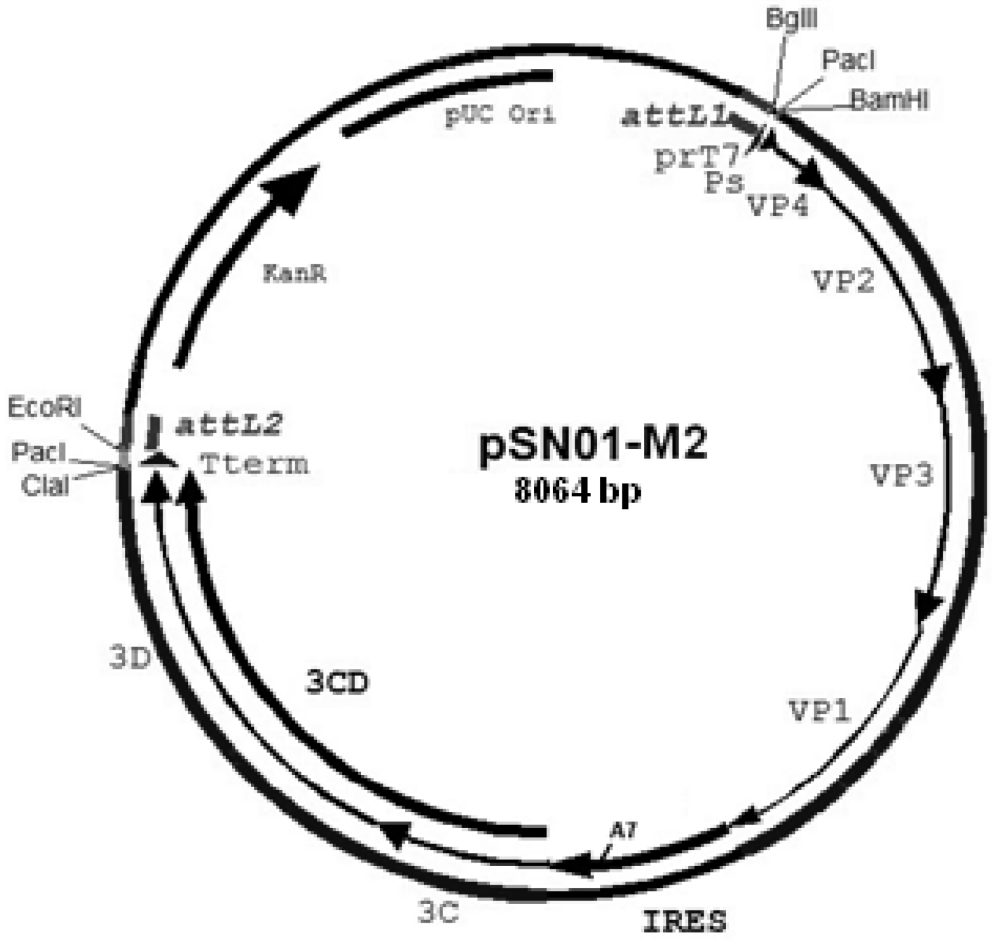


Figura 14. Plásmido pSN01-M3.

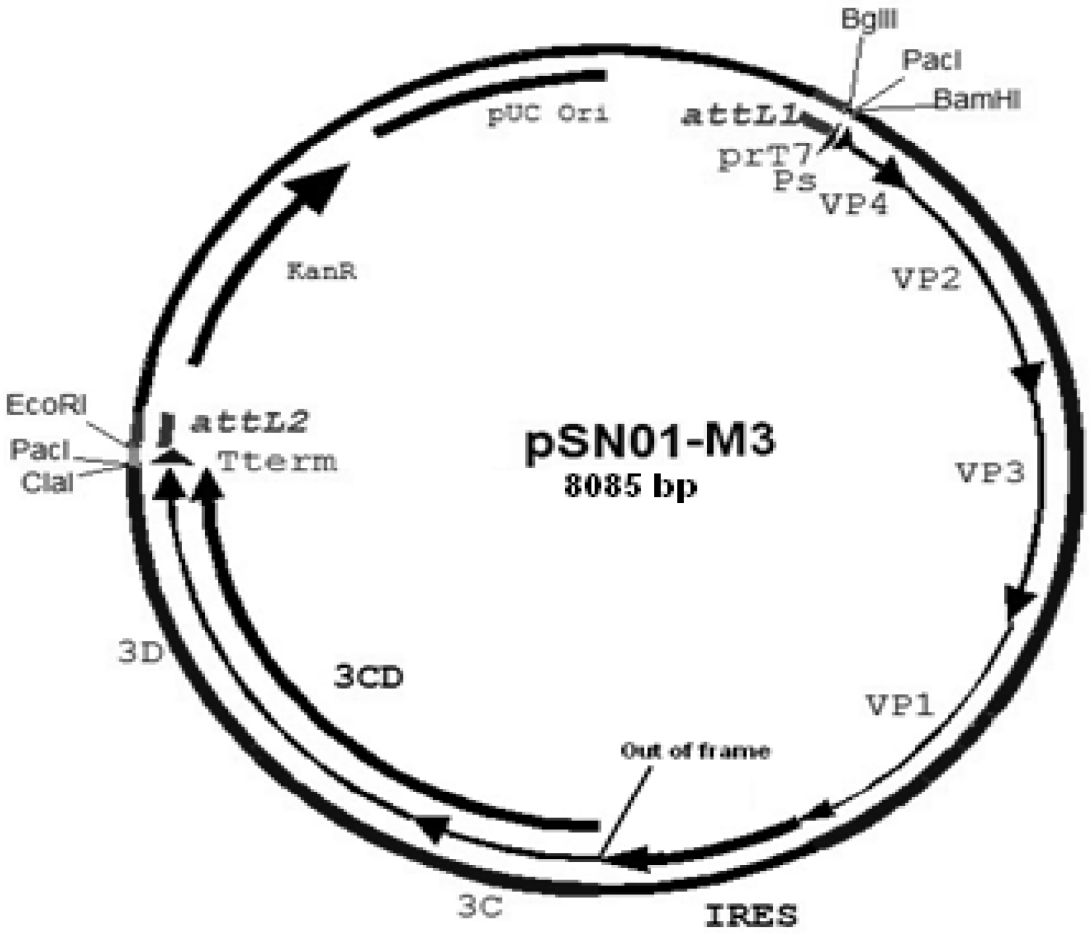


Figura 15. Comparación de expresión de diferentes diseños de baculovirus recombinantes para la expresión de VLP de HEV71.

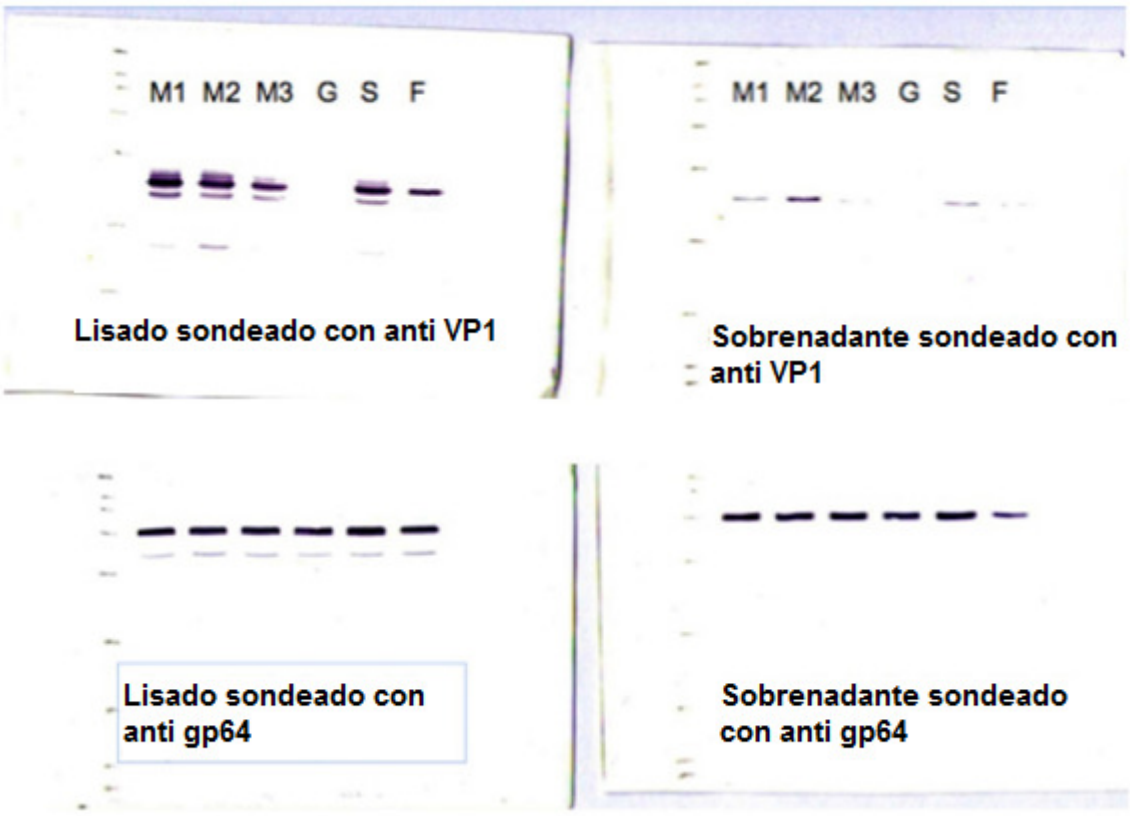


Figura16. Plásmido pFastBac^{TM}HT

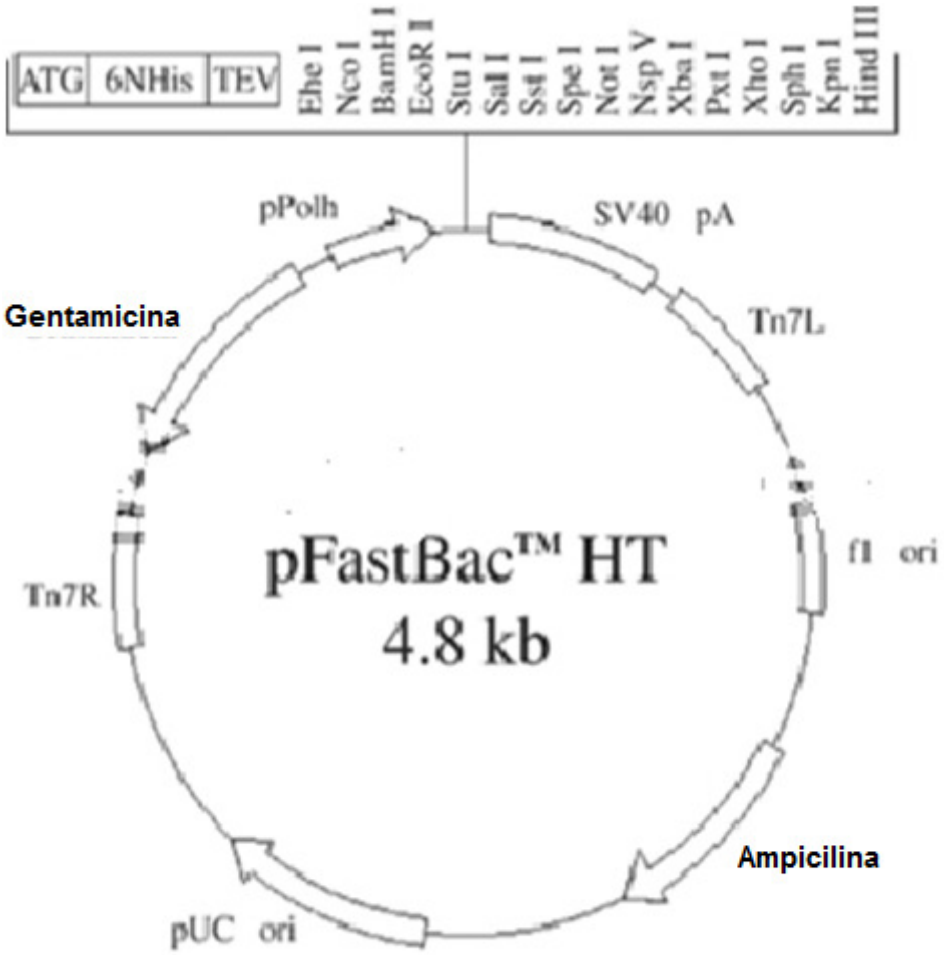


Figura 17. Contructo de expresión procariota para proteínas de fusión antigénica de *Enterovirus humano A* y *enterovirus humano C*.

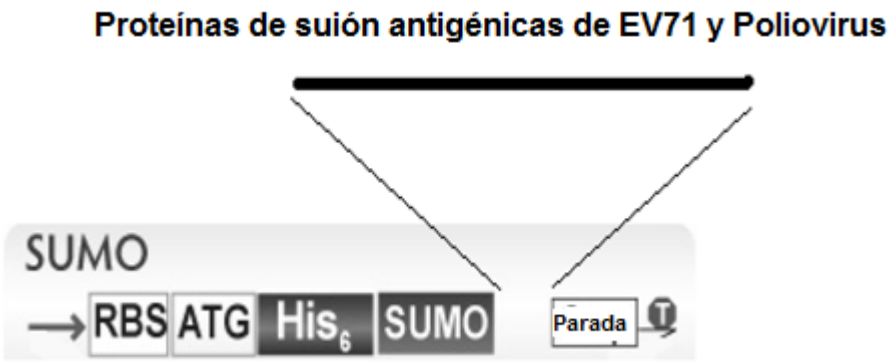


Figura 18. Anticuerpos de sueros neutralizantes mezclados de ratones inmunizados con producto retenido SN07 se unen a todos los componentes de VLP de HEV71 VLPs.

1. 1 µg of rEV71-VP0
2. 1 µg de rEV71-VP1
3. 1 µg de rEV71-VP2
4. 1 µg de rEV71-VP3

kDa kDa 1 2 3 4 1 2 3 4

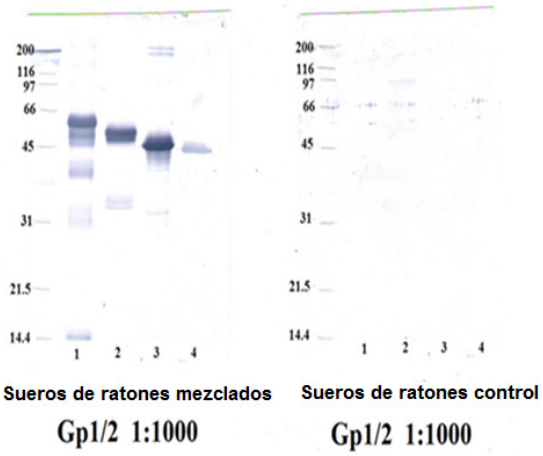


Figura 19. Caracterización de HEV71VLPs, extracción de VLP de HEV71 del sobrenadante de cultivo. Los componentes de las VLP (VP0, VP1 y VP3) son visibles en el gel de SDS-PAGE manchado con Coomassie. (MW: marcador de peso molecular; CB: manchado con azul de Coomassie)

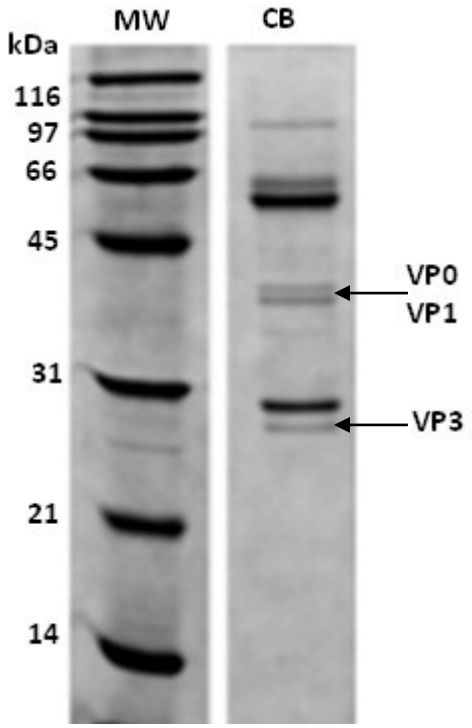


Figura 20. Análisis de columna de afinidad (AFC) de VLP de HEV71 purificadas.

Se preparó un AFC con un anticuerpo monoclonal neutralizante (EV18/4/D6- 1/F1/G9), cuyo anticuerpo monoclonal se desarrolló usando un producto retenido de SN07. La fracción eluida se analizó mediante Western blot. La fracción eluida de los Carriles 1 y 2, sondeada usando un anticuerpo anti-VP1, muestra que VP1 está presente en el AFC de de VLP purificadas. La fracción eluida de los Carriles 3 y 4, sondeada usando un anticuerpo anti-VP2, muestra que VP2 también está presente en el AFC de VLP purificadas. La fracción eluida de los Carriles 5 y 6, sondeada usando un anticuerpo monoclonal anti-VP2, muestra que VP2 está presente en el AFC de VLP purificadas.

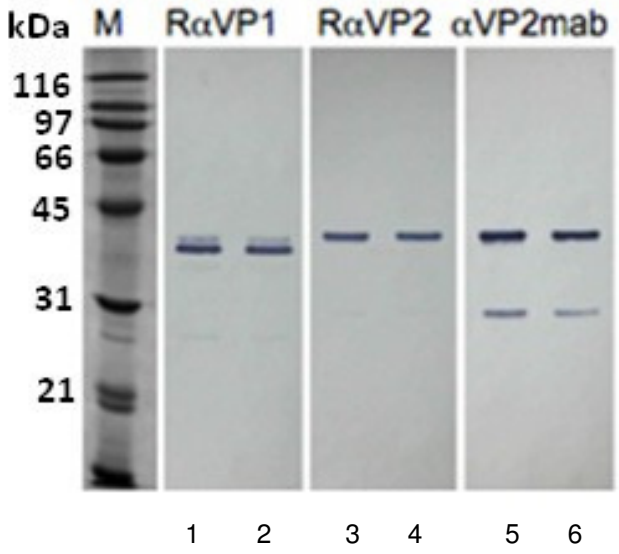


Figura 21. Una imagen de micrografía de AFC de VLP de HEV71 purificadas. Se preparó un AFC con un anticuerpo monoclonal neutralizante (EV18/4/D6-1/F1/G9), cuyo anticuerpo monoclonal se desarrolló usando un producto retenido de SN07. La fracción eluida se analizó mediante Western blot (Figura 20) microscopio electrónico. Las flechas apuntan a las partículas similares a virus.

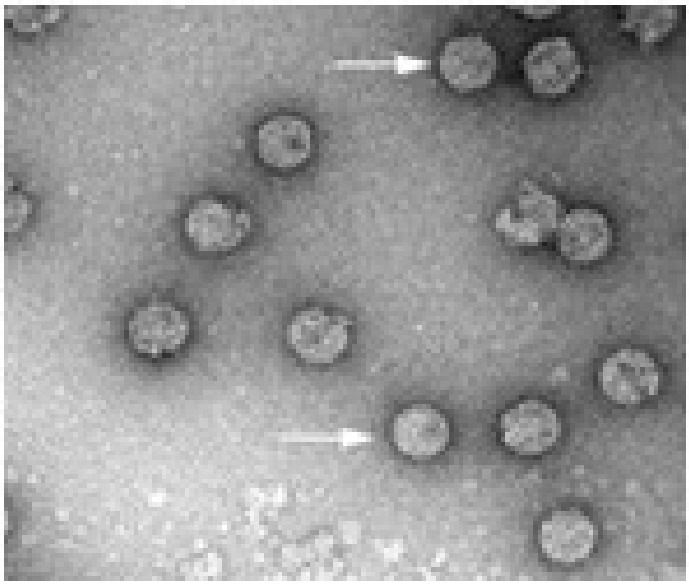
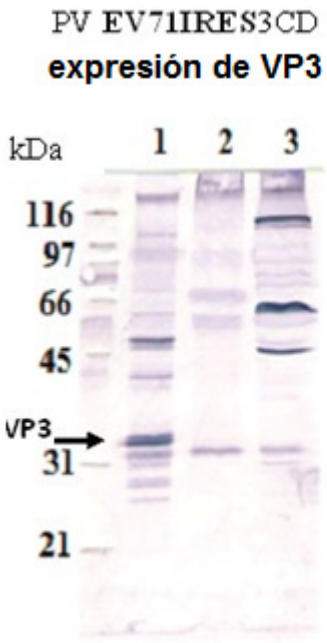
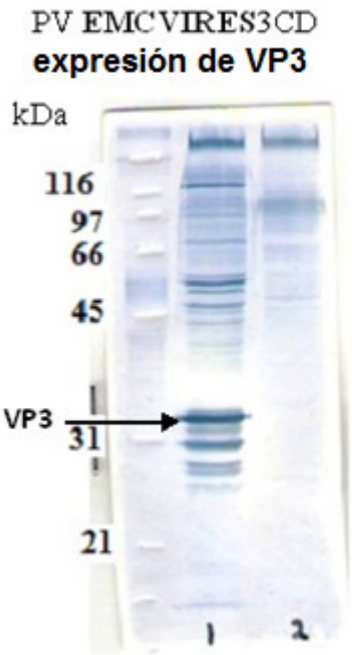


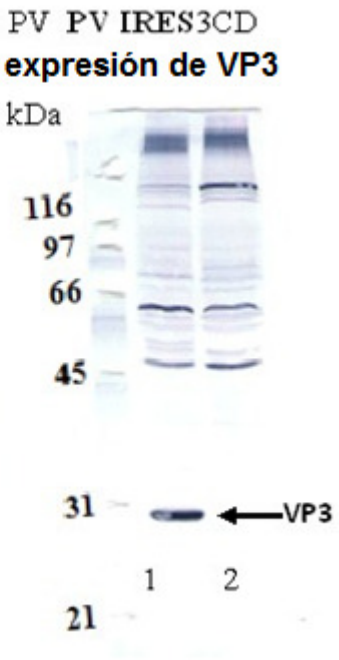
Figura 22. Expresión de VL de *enterovirus humano C* (poliovirus-PV). Se construyeron tres cassettes de expresión diferentes para generar VLP de poliovirus. Todos los cassettes de expresión comprenden un polipéptido P1 de poliovirus y difieren con respect al IRES, cuyo IRES dirige la expresión de una proteasa 3CD de poliovirus. Se ensayaron baculovirus recombinantes que alojan los cassettes de expresión de VLP de poliovirus. Los lisados de las células infectadas por baculovirus se cosecharon el día 3 después de la infección y la expresión de VP3 de poliovirus se evaluó usando anticuerpos anti-PVP3 de conejo (1:2000).



1.Lisado de IRES de HEV71
2.Sobrenadante de IRES de HEV71
3-Lisado de bacGus



1. Lisado de IRES de EMCV
2.Lisado de bacGus



1.Lisado de IRES de PV
2.Lisado de bacGus

Figura 23. ELISA de VP3 de VLP de Poliovirus. Para demostrar la generación de VLP de poliovirus, se realizó ELISA de dos sitios usando lisados y sobrenadantes de baculovirus recombinantes que transportan un cassette de expresión de VLP de poliovirus en donde la proteasa 3CD de poliovirus está bajo el control de un IRES de poliovirus. Se usaron anticuerpos anti-VP3 de poliovirus de conejo purificado como los anticuerpos de captura. Las VLP de poliovirus se detectaron usando anticuerpos monoclonales anti-VP1 de ratón.

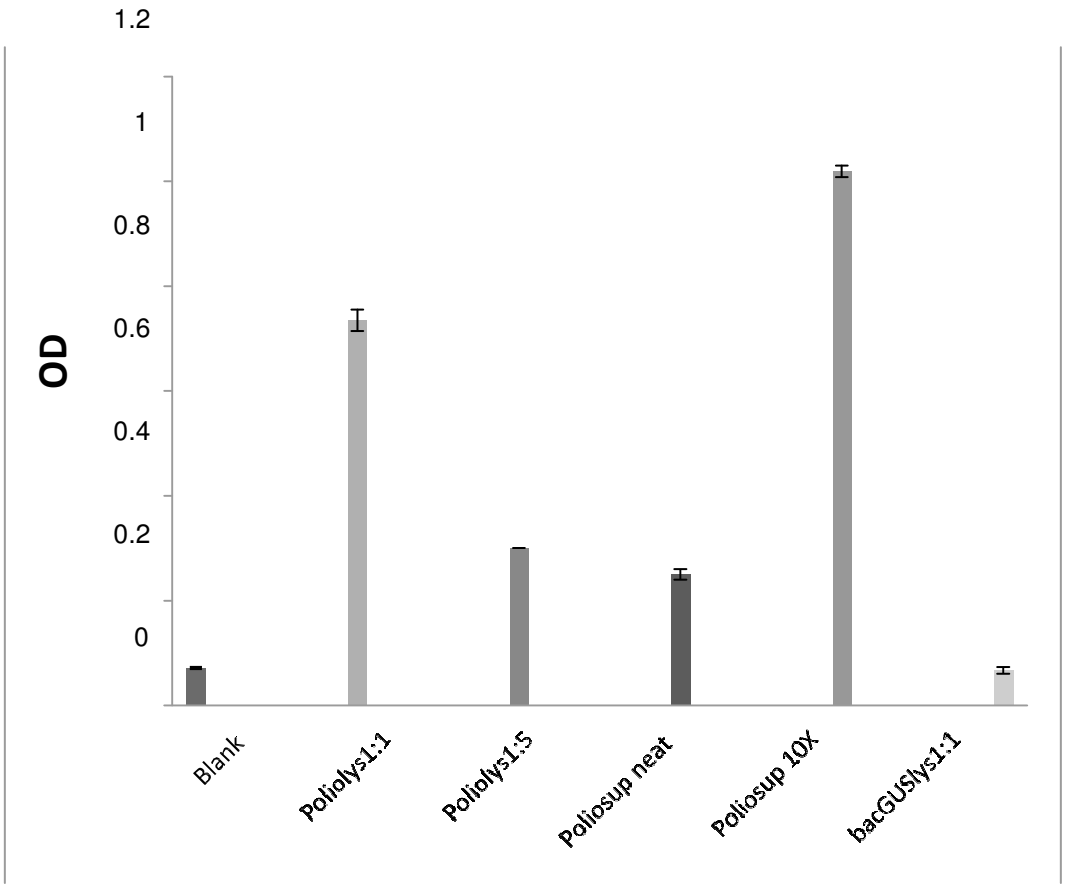


Figura 24. Cassette de expresión de VLP de HEV71 con IRES de HEV71 (P1+IRES de HEV71+3CD).

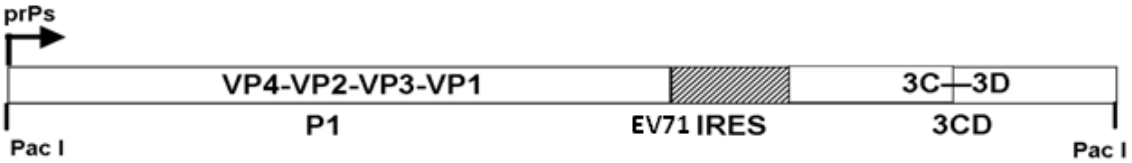
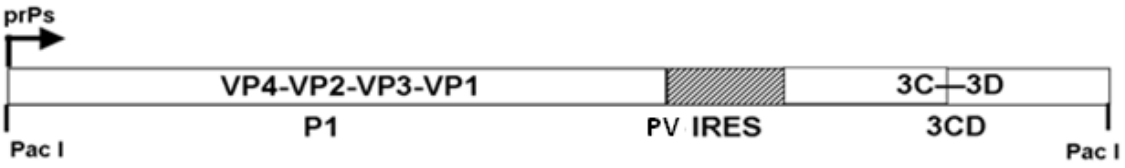


Figura 25. Cassette de expresión de VLP de HEV71 con IRES (PV) de Poliovirus (P1+IRES de PV+3CD).



LISTADO DE SECUENCIAS

<110> Cardosa, Mary Jane
Jamiludden, Mohamad
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REIVINDICACIONES

1. Una vacuna **CARACTERIZADA** porque comprende uno o más antígenos inmunológicamente activos que comprenden uno o más polipéptidos de *enterovirus humano* seleccionados de VP0, PV1, VP2, VP3, VP4 y sus fragmentos inmunológicamente activos.

2. La vacuna de acuerdo con la reivindicación 1, **CARACTERIZADA** porque produce una respuesta inmune protectora y/o neutralizante dirigida contra un *Enterovirus* humano.

3. La vacuna de acuerdo con la reivindicación 1, **CARACTERIZADO** porque el *Enterovirus humano* se selecciona de *enterovirus humano A, enterovirus humano B, enterovirus humano C y enterovirus humano D.*

4. La vacuna de acuerdo con la reivindicación 3, **CARACTERIZADA** porque el *enterovirus humano A* se selecciona de *enterovirus humano 71* (HEV71), *virus Coxsackie* A16, en donde el *enterovirus humano B* se selecciona de *virus Coxsackie* B y echovirus, en donde el *enterovirus humano C* se selecciona de *poliovirus humano 1, poliovirus humano 2 y poliovirus humano 3* y en donde el *enterovirus humano D* es EV 68.

5. La vacuna de acuerdo con la reivindicación 1, **CARACTERIZADA** porque la vacuna comprende un polipéptido VP0 de *Enterovirus* inmunológicamente activo.

6. La vacuna de acuerdo con la reivindicación 1, **CARACTERIZADA** porque la vacuna comprende el polipéptido VP2 de *Enterovirus* inmunológicamente activo.

7. La vacuna de acuerdo con la reivindicación 1, **CARACTERIZADA** porque la vacuna comprende el polipéptido VP3 de *enterovirus* inmunológicamente activo.

8. La vacuna de acuerdo con la reivindicación 1, **CARACTERIZADA** porque la vacuna comprende un polipéptido VP1 de *Enterovirus* inmunológicamente activo.

9. La vacuna de acuerdo con la reivindicación 1, **CARACTERIZADA** porque la vacuna comprende polipéptidos de una o más especies o serotipos de *Enterovirus.*

10. La vacuna de acuerdo con la reivindicación 9, **CARACTERIZADA** porque la especie o el serotipo pueden ser *enterovirus humano A seleccionado* de HEV71 y virus Coxsackie A16.

11. La vacuna de acuerdo con la reivindicación 9, **CARACTERIZADA** porque la especie o el serotipo pueden ser *enterovirus humano C* seleccionado de PV-1, PV-2 y PV-3.

12. La vacuna de acuerdo con la reivindicación 1, **CARACTERIZADA** porque uno o más antígenos inmunológicamente activos que comprenden uno o más polipéptidos de *enterovirus humano* seleccionados de VP0, VP1, VP2, VP3, VP4 y sus fragmentos inmunológicamente activos, están en la forma de una partícula similar a un virus, un capsómero, un complejo y/o un agregado.

13. La vacuna de acuerdo con la reivindicación 12, **CARACTERIZADA** porque la partícula similar a un virus comprende un polipéptido VP0 de *enterovirus* inmunológicamente activo.

14. La vacuna de acuerdo con la reivindicación 12, **CARACTERIZADA** porque la partícula similar a un virus comprende un polipéptido VP2 de *enterovirus* inmunológicamente activo.

15. La vacuna de acuerdo con la reivindicación 12, **CARACTERIZADA** porque la partícula similar a un virus comprende un polipéptido VP3 de *enterovirus* inmunológicamente activo

16. La vacuna de acuerdo con la reivindicación 12, **CARACTERIZADA** porque la partícula similar a un virus comprende un polipéptido VP1 de *enterovirus* inmunológicamente activo.

17. Un método para vacunar a un sujeto contra una infección por un *Enterovirus*, **CARACTERIZADO** porque comprende administrar al sujeto una vacuna que comprende uno o más antígenos inmunológicamente activos que comprenden uno o más polipéptidos de *Enterovirus* seleccionados de VP0, VP1, VP2, VP3, VP4 y sus fragmentos inmunológicamente activos, en una cantidad efectiva para producir una respuesta inmune protectora y/o neutralizante cuando se administra al sujeto.

18. El método de acuerdo con la reivindicación 17, **CARACTERIZADO** porque la vacuna comprende un polipéptido VP0 de *Enterovirus* inmunológicamente activo.

19. El método de acuerdo con la reivindicación 17, **CARACTERIZADO** porque la vacuna comprende un polipéptido VP1 de *enterovirus* inmunológicamente activo.

20. El método de acuerdo con la reivindicación 17, **CARACTERUZADO** porque la vacuna comprende un polipéptido VP3 de *enterovirus* inmunológicamente activo.

21. El método de acuerdo con la reivindicación 17, **CARACTERIZADO** porque la vacuna comprende un polipéptido VP2 de *enterovirus* inmunológicamente activo.

22. El método de acuerdo con la reivindicación 17, **CARACTERIZADO** porque uno o más antígenos inmunológicamente activos que comprenden uno más polipéptidos de *enterovirus humano* seleccionados de VP0, VP1, VP2, VP3, VP4 y sus fragmentos inmunológicamente activos están en la forma de una partícula similar a un virus, un capsómero, un complejo y/o un agregado.

23. El método de acuerdo con la reivindicación 22, **CARACTERIZADO** porque la partícula similar a un virus comprende un polipéptido VP0 de *enterovirus* inmunológicamente activo.

24. El método de acuerdo con la reivindicación 22, **CARACTERIZADO** porque la partícula similar a un virus comprende un polipéptido VP2 de *enterovirus* inmunológicamente activo.

25. El método de acuerdo con la reivindicación 22, **CARACTERIZADO** porque la partícula similar a un virus comprende un polipéptido VP3 de *enterovirus* inmunológicamente activo.

26. El método de acuerdo con la reivindicación 22, **CARACTERIZADO** porque la partícula similar a un virus comprende un polipéptido VP1 de *enterovirus* inmunológicamente activo.

27. El método de acuerdo con la reivindicación 17, **CARACTERIZADO** porque la vacuna comprende polipéptidos de una o más especies o serotipos de *enterovirus.*

28. El método de acuerdo con la reivindicación 27, **CARACTERIZADO** porque la especie o serotipo de *enterovirus* puede ser *enterovirus humano Ha seleccionado* de HEV71 o *virus Coxsackie* A16.

29. El método de acuerdo con la reivindicación 27, **CARACTERIZADO** porque la especie o serotipo de *enterovirus* puede ser el *enterovirus humano C* seleccionado de PV-1, PV-2 y PV-3.

30. Un cassette de expresión **CARACTERIZADO** porque comprende un promotor unido operativamente a un ácido nucleico que codifica un polipéptido P1 de *enterovirus humano,* un Sitio de Entrada de Ribosoma Interno (IRES) y un ácido nucleico que codifica una proteasa 3CD de *enterovirus humano.*

31. El cassette de expresión de acuerdo con la reivindicación 30, **CARACTERIZADO** porque el polipéptido es P1 de *enterovirus humano.*

32. El cassette de expresión de acuerdo con la reivindicación 30, **CARACTERIZADO** porque la proteasa 3CD de *enterovirus humano* procesa el polipéptido P1 de *enterovirus humano.*

33. El cassette de expresión de acuerdo con la reivindicación 32, **CARACTERIZADO** porque los polipéptidos P1 de *enterovirus humano* procesados se asocian para formar partículas similares a virus, capsómeros, complejos y/o agregados.

34. El cassette de expresión de acuerdo con la reivindicación 30, **CARACTERIZADO** porque el polipéptido P1 de *enterovirus humano* comprende combinaciones de polipéptidos seleccionados de VP0, VP1, KVP2, VP3, VP4 y sus fragmentos inmunológicamente activos.

35. El cassette de expresión de acuerdo con la reivindicación 30, **CARACTERIZADO** porque el *enterovirus* humano se selecciona de *enterovirus humano A y enterovirus humano C.*

36. El cassette de expresión de acuerdo con la reivindicación 35, **CARACTERIZADO** porque el *enterovirus humano A* se selecciona de HEV71 y *virus Coxsackie* A16.

37. El cassette de expresión de acuerdo con la reivindicación 35, **CARACTERIZADO** porque el *enterovirus humano C* se selecciona de poliovirus humano 1 (PV-1), poliovirus humano 2 (PV-2) y poliovirus humano 3 (PV-3).

38. El cassette de expresión de acuerdo con la reivindicación 30, **CARACTERIZADO** porque el IRES se obtiene del virus de la *encefalomiocarditis* (EMCV).

39. El cassette de expresión de acuerdo con la reivindicación 30, **CARACTERIZADO** porque el IRES se obtiene del *virus de la encefalomiocarditis* (EMCV) se ha modificado genéticamente para reducir la actividad de IRES.

40. El cassette de expresión de acuerdo con la reivindicación 39, **CARACTERIZADO** porque el IRES derivado de EMCV se ha modificado genéticamente agregando un nucleótido (A7) al bucle de bifurcación A6 en el segmento JK.

41. El cassette de expresión de acuerdo con la reivindicación 30, **CARACTERIZADO** porque el IRES se obtiene de un *enterovirus* humano.

42. El cassette de expresión de acuerdo con la reivindicación 30, **CARACTERIZADO** porque el promotor es un promotor eucariota.

43. El cassette de expresión de acuerdo con la reivindicación 42, **CARACTERIZADO** porque el promotor eucariota es un promotor de polihedrina.

44. El cassette de expresión de acuerdo con la reivindicación 30, **CARACTERIZADO** porque el promotor está unido operativamente a un ácido nucleico que codifica un polipéptido P1 de *enterovirus humano A*, un IRES de EMCV y una proteasa 3CD de *enterovirus humano A.*

45. El cassette de expresión de acuerdo con la reivindicación 44, **CARACTERIZADO** porque el IRES de EMCV se ha mutado para reducir la actividad del IRES.

46. El cassette de expresión de acuerdo con la reivindicación 30, **CARACTERIZADO** porque el promotor está unido operativamente a un ácido nucleico que codifica un polipéptido P1 de

enterovirus humano A, un IRES de HEV71 y una proteasa 3CD de *enterovirus humano A.*

47. El cassette de expresión de acuerdo con la reivindicación 30, **CARACTERIZADO** porque el promotor está unido operativamente a un ácido nucleico que codifica un polipéptido P1 de *enterovirus humano A*, un IRES de *enterovirus humano C* y una proteasa 3CD de *enterovirus humano A.*

48. El cassette de expresión de acuerdo con la reivindicación 30, **CARACTERIZADO** porque el promotor está unido operativamente a un ácido nucleico que codifica un polipéptido P1 de *enterovirus humano C,* un IRES de *enterovirus humano C* y una proteasa 3CD de *enterovirus humano C*.

49. El cassette de expresión de acuerdo con la reivindicación 30, **CARACTERIZADO** porque el promotor está unido operativamente a un ácido nucleico que codifica un polipéptido P1 de *enterovirus humano C,* un IRES de HEV71 y una proteasa 3CD de *enterovirus humano C.*

50. El cassette de expresión de acuerdo con la reivindicación 30, **CARACTERIZADO** porque el promotor está unido operativamente a un ácido nucleico que codifica un polipéptido P1 de

enterovirus humano C, un IRES de EMCV y una proteasa 3CD de *enterovirus humano C*.

51. Un método para elaborar una vacuna **CARACTERIZADO** porque comprende introducir el cassette de expresión de acuerdo con la reivindicación 30 en una célula huésped, cultivar la célula huésped durante un período de tiempo suficiente para producir los polipéptidos del cassette de expresión y recuperar polipéptidos de *enterovirus humano* de la célula huésped y/o el sobrenadante de cultivo.

52. El método de acuerdo con la reivindicación 51, **CARACTERIZADO** porque la célula huésped es una célula eucariota.

53. El método de acuerdo con la reivindicación 52, **CARACTERIZADO** porque la célula eucariota se selecciona de células de insectos, células de mamíferos y células de levadura.

54. El método de acuerdo con la reivindicación 53, **CARACTERIZADO** porque las células de insectos se seleccionan de *Spodoptera frugiperda*, *Trichoplusia ni*, drosophila, y células de mosquitos derivadas de *Aedes albopictus.*

55. El método de acuerdo con la reivindicación 53, **CARACTERIZADO** porque las líneas de células de mamíferos se seleccionan de CHO, HEK 293, COS-1, HeLa, Vero y NIH3T3.

RESUMEN DE LA INVENCIÓN

La presente invención proporciona materiales y métodos para producir en forma antígenos inmunológicamente activos derivados de miembros de la familia del virus *Picornaviridae*. Los antígenos del picornavirus de la invención pueden estar en una forma para su uso como una vacuna administrada a un sujeto en un tratamiento terapéutico o para la prevención de una infección por el picornavirus. Los antígenos del picornavirus de la invención pueden estar en la forma de una composición inmunogénica para su uso en vacunas que se administran para la prevención de una infección por un *Enterovirus*. La presente invención además comprende composiciones inmunogénicas que comprenden antígenos de *enterovirus A Humano*, *enterovirus B Humano*, *enterovirus C Humano*, *enterovirus D Humano* y su uso en vacunas para la prevención de una infección por un *Enterovirus*.