

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO
DIRECCIÓN DE NUEVAS CREACIONES

2020
Bogotá, D.C.,

Abogado
EMILIO FERRERO

ASUNTO	Radicación	07 077 615
	Trámite	011
	Evento	379
	Actuación	519
	Oficio	
	Folios	19

1 2 4 5 3

Patente de Invención	☒	Patente de Modelo de Utilidad
Vía Nacional		
Vía PCT (Fase Nacional)	☒	Capítulo I ☐ Capítulo II ☒
No. Solicitud Internacional	PCT/US2005/047596	
Fecha de Presentación Internacional	29/Diciembre/2005	

Como resultado del examen de fondo realizado, con fundamento en el Artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se comunica:

1. Documentos estudiados

Descripción:	Folios 120 a 238	30/Julio/2007
Reivindicaciones:	Folios 239 a 240	30/Julio/2007
	Folios 258 a 270	30/Julio/2007
Prioridad:	US 60 640 510	30/Diciembre/2004
	US 11 034 797	13/Enero/2005
	US Sin número	29/Diciembre /2005

2. Análisis de la prioridad

Se acepta la reivindicación de prioridad porque esta solicitud fue presentada dentro del plazo establecido (Art. 9 D 486) y su contenido técnico corresponde con el de la prioridad reivindicada.

3. Objeto de la invención

Título de la solicitud: "Composiciones inmunogénicas PCV2 y métodos para producir tales composiciones"

El problema planteado en esta solicitud consiste en la necesidad de proporcionar un método para obtener proteína ORF2 que no requiera la extracción de la proteína

ORF2 desde dentro de las células infectadas y que además permitan obtener cantidades aceptables de la misma, así mismo proporcionar una composición inmunogénica.

Para resolver este problema técnico la solicitud en estudio proporciona métodos mejorados para producir y/o recuperar proteína ORF2 de PCV2 recombinante, donde el ORF2 es liberado en el sobrenadante en grandes cantidades.

El objeto de la presente Solicitud se relaciona con la Clasificación Internacional de Patentes (CIP): C07K14/08; C12P21/06.

4. Reivindicaciones relativas a un uso o a un segundo uso (Art 14 D 486)

El uso de las reivindicaciones 34-35 no es patentable, de acuerdo con el Art 14.

5. De la Solicitud de Patente

Reivindicaciones (Art 30 D 486, Art 22 PCT)

El capítulo reivindicatorio presentado carece de claridad y concisión por cuánto:

- 1) La reivindicación 1 caracteriza un método para recuperar una proteína que consiste en una serie de pasos A-F, en este caso es necesario que se defina la estructura de lectura abierta 2 a través de su respectiva secuencia; en el paso A, se debe definir la estructura de lectura y el vector de transferencia que se está utilizando. En el paso B, se debe definir el virus utilizado. El subproceso definido en el paso D, es un proceso esencialmente biológico por lo cual no se considera una invención. En el paso E se debe definir a que sobrenadante esta haciendo referencia, al igual que en el paso F.
Se sugiere al solicitante definir la invención completamente en la reivindicación independiente, incluyendo los pasos mencionados en las reivindicaciones 3-13 que contienen características esenciales de la invención, de esta manera el proceso será claro y se podrá observar una adecuada secuencia lógica de pasos completos. Se aplica el mismo concepto para los procedimientos de las reivindicaciones 14, 31, 36, 54 y 55 y sus respectivas reivindicaciones independientes.
- 2) La reivindicación 33 menciona "una composición de la materia obtenida" se solicita aclarar y especificar cual es la materia obtenida mediante sus características técnicas, es decir su respectiva secuencia en el caso de proteínas.
- 3) La reivindicación 38 define una composición inmunogénica, en donde se debe definir la secuencia de la proteína ORF2 de PCV2, cuál es la cadena de aminoácidos y además especificar los auxiliares de formulación y sus proporciones dentro de dicha composición.
- 4) El kit de la reivindicación 52 debe quedar definido mediante sus características técnicas, es decir especificar inequívocamente cada uno de los elementos que lo constituyen.
- 5) En general se observa una falta de concisión a lo largo de todo el capítulo reivindicatorio y un excesivo número de reivindicaciones independientes,

definiendo diferentes procedimientos que a la larga constan de los mismos pasos, se sugiere definir la invención de manera que una persona versada en la materia pueda reproducirla sin ambigüedad.

Se requiere al solicitante hacer las aclaraciones necesarias.

6. Unidad de invención (Art 25 D 486)

La solicitud no cumple con el requisito de unidad de invención porque se refiere a 2 grupos inventivos, estos están definidos por las siguientes características **esenciales**:

1° Un método para recuperar una proteína recombinante expresada por estructura de lectura abierta 2 (ORF2) del circovirus porcino tipo 2 (PCV2). (Reivindicaciones 1-13).

2° Un método para preparar una composición para invocar una respuesta inmune contra el circovirus porcino tipo 2 (PCV2). (Reivindicaciones 14-30).

3° Un método para recuperar una proteína recombinante expresada por estructura de lectura abierta 2 (ORF2) del circovirus porcino tipo 2 (PCV2). (Reivindicaciones 31-32).

4° Una composición de la materia obtenida mediante alguno de los métodos de los grupos anteriores (Reivindicación 33).

5° Un método para producir un kit de diagnóstico para la detección de una infección PCV2 (Reivindicaciones 36-37).

6° Una composición inmunogénica efectiva para reducir la severidad de los síntomas asociados con la infección por PCV2 (Reivindicaciones 38-48).

7° Un contenedor que comprende al menos una dosis de una composición inmunogénica efectiva que compuesta por la proteína estructura de lectura abierta 2 (ORF2) del circovirus porcino tipo 2 (PCV2). (Reivindicaciones 49-51).

8° Un kit que comprende un contenedor y un manual de instrucciones. (Reivindicaciones 52-53).

9° Un método para recuperar una proteína recombinante expresada al abrir la estructura de lectura abierta 2 (ORF2) del circovirus porcino tipo 2 (PCV2). (Reivindicación 54).

10° Un método para preparar una composición para invocar una respuesta inmune contra el circovirus porcino tipo 2 (PCV2). (Reivindicación 55).

Los anteriores grupos inventivos no se relacionan entre si mediante un único concepto inventivo, ya que no presentan las misma características técnicas. Se encuentra que la invención del grupo 1° ya estaba divulgada en el estado de la técnica mediante el documento D1, el cual divulga un método para insertar ORF2 de PCV2 dentro de un vector y la proteína que se recupera la cual es utilizada en un ensayo diagnóstico (Pág. 34, primera columna). Así mismo D2, divulga la clonación y

el uso de la estructura de lectura abierta 2 (ORF2) del circovirus porcino tipo 2 (PCV2) (Pág. 4566, 2.1 y 2.2). Es así que se evidencia que los diferentes grupos inventivos no comparten un único concepto inventivo y por ende no cumplen con la unidad de invención.

Se sugiere presentar las solicitudes divisionales que considere necesarias, ó limitar la solicitud inicial a una sola invención, eliminando de ella las otras invenciones.

El presente examen se llevará a cabo sobre los 3 primeros grupos inventivos.

7. Determinación del Estado de la Técnica

El Estado de la Técnica se determinó con base en la fecha de presentación de la solicitud de prioridad: Diciembre 30 de 2004, y se encontraron los siguientes documentos que anticipan el objeto de esta solicitud:

Ítem	Documento	Título	Fecha de Publicación*
D1	CLINICAL AND DIAGNOSTIC LABORATORY IMMUNOLOGY, vol 9, N°1, Enero 2002, páginas 33-40	Modified indirect porcine circovirus (PCV) type 2-based and recombinant capsid protein (ORF2)-based enzyme-linked immunosorbent assays for detection of antibodies to PCV.	Enero/2002
D2	VACCINE, vol. 21, Noviembre 2003, páginas 4565-4575.	Protection of swine against post-weaning multisystemic wasting síndrome (PMWS) by porcine circovirus type 2 (PCV2) proteins.	Noviembre/2003
D3	JOURNAL OF VETERINARY SCIENCE, vol3, n° 1. Marzo 1 de 2002, páginas 19-23.	Characterization of the recombinant proteins of porcine circovirus type 2 field isolate expressed in the baculovirus system.	01/Marzo/2002

D1
Divulga un método para insertar ORF2 de PCV2 dentro de un vector y la proteína que se recupera la cual es utilizada en un ensayo diagnóstico (Pág. 34, primera columna).

D2
Divulga la clonación y el uso de la estructura de lectura abierta 2 (ORF2) del circovirus porcino tipo 2 (PCV2) (Pág. 4566, 2.1 y 2.2) y un método para preparar una composición para invocar una respuesta inmune contra PCV2 (Pág. 4566, segunda columna, primero y segundo párrafos)

D3
Divulga un método para recuperar VPLs de PCV2 del sobrenadante de células de insecto transducidas con un vector baculoviral que codifica ORF2 de PCV2 (Abstract; pág. 20, segunda columna párrafo 3).

8. Examen de Patentabilidad (Art 14 D486)

Novedad (Art 16 D486)

Las reivindicaciones 1 y 31 caracterizan en un método para recuperar una proteína recombinante, el cual consiste en las siguientes características técnicas:

Comparación de las características técnicas **esenciales**, con las del Estado de la Técnica:

Características esenciales	D3
Método para recuperar una proteína recombinante expresada por estructura de lectura abierta 2 de PCV2 que comprende:	Método para recuperar un VPLs de PCV2 presente en el sobrenadante del cultivo de células de insecto transducidas con un vector que codifica ORF2 de PCV2 que comprende:
Clonar dicha ORF2 de PCV2 dentro de un vector de transferencia.	Insertar el gen clonado dentro del vector de expresión de baculovirus (abstract).
Transfectar la porción de dicho vector de transferencia que contiene dicha ORF2 dentro de un virus.	
Infectar las células con dicho virus	Infectar células de insecto.
Causar que dicho virus exprese la proteína a partir de dicho ORF2	Causar que dicha proteína se expresen usando el sistema de expresión de baculovirus. (abstract)
Separar las células del dicho vector del sobrenadante	Separar las células de insecto congelándolas y descongelándolas con el fin de coleccionar el sobrenadante. (Pág. 20, segunda columna párrafo 3)
Recuperar dicha proteína de ORF2 en dicho sobrenadante.	Centrifugar el sobrenadante con el fin de obtener un precipitado el cual contiene la proteína obtenida. (Pág. 20, segunda columna párrafo 3)

El documento D3 divulga un método para recuperar VPLs de PCV2 del sobrenadante de células de insecto transducidas con un vector baculoviral que codifica ORF2 de PCV2 (Abstract; pág. 20, segunda columna párrafo 3).

Vemos que las características esenciales de las reivindicaciones 1 y 31 ya habían sido divulgadas previamente en D3, en consecuencia, dichas reivindicaciones carecen de novedad, según lo divulgado en el documento D3.

Las reivindicaciones dependientes 2-13 y 32 no mencionan alguna característica que haga nueva a la composición de la reivindicación 1, por lo cual se considera que no son nuevas.

La reivindicación 14 consiste en un método para preparar una composición para invocar una respuesta inmune contra PCV2, el cual presenta las siguientes características técnicas:

Comparación de las características técnicas **esenciales**, con las del Estado de la Técnica:

Características esenciales	D2
Método para preparar una composición para invocar una respuesta inmune contra PCV2 que comprende:	Método para preparar una composición para invocar una respuesta inmune contra PCV2 que comprende:
Transfectar una construcción dentro de un virus, dicha construcción comprende ADN recombinante de ORF2 de PCV2	Producir el baculovirus recombinante BacOrf2
Infectar células con dicho virus transfectado, dichas células están en un medio de crecimiento.	Infectar células Sf9 con el baculovirus recombinante BacOrf2. (Pág. 4566, segunda columna, primer párrafo)
Originar que dicho virus exprese la proteína recombinante de dicho ORF2.	Dichas células se cultivan por 72 h. (Pág. 4566, segunda columna, primer párrafo)

Recuperar dicha ORF2 expresada en el sobrenadante.	Llevar a cabo el lisado de las células con el fin de obtener la proteína. (Pág. 4566, segunda columna, primer párrafo)
Combinar dicha proteína recuperada con un adyuvante adecuado.	El lisado se combina con un buffer de fosfatos y con una mezcla adyuvante de agua/aceite. (Pág. 4566, segunda columna, segundo párrafo)

El documento D2 divulga un método para preparar una composición para invocar una respuesta inmune contra PCV2 (Pág. 4566, segunda columna, primero y segundo párrafos)

Vemos que las características esenciales de las reivindicaciones 1 y 31 ya habían sido divulgadas previamente en D2, en consecuencia, dicha reivindicación carece de novedad, según lo divulgado en el documento D2.

Las reivindicaciones dependientes 15-30 no mencionan alguna característica que haga nueva a la composición de la reivindicación 14, por lo cual se considera que no son nuevas.

9. Conclusión

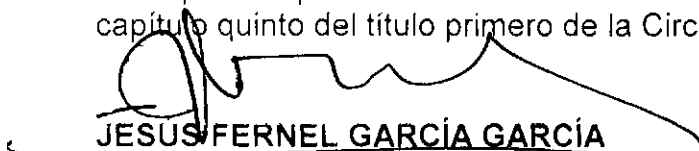
Los requisitos de patentabilidad de la presente solicitud están afectados así:

Ítem	Documento	Reivindicaciones afectadas	Requisito que afecta
D1	CLINICAL AND DIAGNOSTIC LABORATORY IMMUNOLOGY, vol 9, N°1, Enero 2002, páginas 33-40	1	Novedad
D2	VACCINE, vol. 21, Noviembre 2003, páginas 4565-4575.	14-30	Novedad
D3	JOURNAL OF VETERINARY SCIENCE, vol3, n° 1, Marzo 1 de 2002, páginas 19-23.	1 -13 y 32	Novedad

En cumplimiento del artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se le indica que debe dar respuesta dentro del término de sesenta (60) días contados a partir de la fecha de notificación de la presente comunicación. En caso de no dar respuesta al presente requerimiento, o si a pesar de la respuesta subsistieran los impedimentos para la concesión, se denegará la patente.

Se invita al solicitante se sirva indicar, si así lo considera, su renuncia al término faltante de sesenta (60) días para dar respuesta a este requerimiento y presentarla por escrito.

Notifíquese el presente oficio conforme a lo dispuesto en el numeral 5.2, literal e), del capítulo quinto del título primero de la Circular Única No. 10 de 2001.


JESÚS FERNEL GARCÍA GARCÍA
Director de Nuevas Creaciones (E)

El anterior requerimiento fue notificado por fijación en lista, de fecha

18 Jun. 2012
Realizado por: Ximena Andrade. **YA**
Revisado por: Consuelo Leguizamón.

Modified Indirect Fluorescent Virus (FCV) Type 2-Based and Recombinant Copied Protein (ORF2)-Based Enzyme-Linked Immunosorbent Assays for Detection of Antibodies to PCV

Department of
 Statistics
 University of
 California, Los Angeles
 Los Angeles, California 90024-1551
 U.S.A.
 E-mail: shirley@stat.ucla.edu
 Fax: +1 310 206 7131

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Figure 1. The effect of the concentration of the Fe^{2+} on the adsorption of Fe^{3+} on the surface of the Fe^{2+} -loaded zeolite. The concentration of the Fe^{3+} was 100 mg/L, and the pH was 5.0. The adsorption was carried out at 25°C for 24 h.

Presumptive multi-systemic wasting syndrome of some associated with parvovirus (PCV) is a recently reported and economically important disease. Simple and reliable diagnostic methods are needed for identifying antibodies to PCV type 2 (PCV-2) for monitoring of PCV infection. Here, we report the development of two modified indirect enzyme-linked immunosorbent assays (ELISAs): a PCV-2 ELISA based on cell culture propagated PCV-2 and an ORF2-ELISA based on recombinant major capsid protein, PCP2, and ORF2-ELISA detected antibodies to PCV-2 and the capsid protein, respectively, in sera from pigs experimentally infected with PCV-2 as early as 14 and 21 days post-inoculation (dpi). The kinetics of the antibody response to PCV-2 and the

serum capsid protein were similar. Reproducibility tests revealed that the coefficients of variation of positive EIA within and between runs for both assays were less than 10%. To validate the assays, EIA-2 and ORF2 EIA-3 assays were performed with 783 serum samples of young and adult pigs collected from different farms in Minnesota, United States, and compared with an indirect immunofluorescent assay (IF). Six out of 60 samples collected from nursery and growing pigs in 1987 were positive by EIA-2 and EIA-3, compared with IF; the remaining 54 samples were negative by all three assays. The specificity of EIA-2 and EIA-3 were similar to the tests performed with adult pigs, with specificity of 100% and 98.7%, respectively. The sensitivity of EIA-2 and EIA-3 were 100% and 98.7%, respectively, with specificity of 100% and 98.7%, respectively. The agreement between the EIA-2 and EIA-3 was 100%. The agreement between the two EIA-3s would accelerate our understanding of the host immune response to PCV-2 and facilitate the development of prevention and control strategies by characterizing the ecology of PCV-2 within some populations.

PMWS is a disease of growing pig with low morbidity but high case mortality. The most common clinical signs are anorexia, depression, and jaundice (10). Histologic lesions include lymphohistiocytic to granulomatous infiltration in the liver, kidney, and lymphoid tissues as well as vasculopathy (11). This paper presents a case of PMWS in a 2-year-old pig with PCV-2 and porcine parvovirus (PPV) as well as the histologic lesions and features of PMWS (12), which is a solution with regard to the diagnosis of PMWS.

cases (13, 37, 38). Incubation with PMSV, PPMV, or a combination of both PVI and PPMV, failed to produce a clinical picture resembling PMSV (23). In a more recent study, the use of an immunosuppressant into PPMV2 infection, per examination of PPMV2 and confirmed clinical signs of SFTS, was associated with PPMV (25), and confirmed the role of PPMV as a potential cause of PMSV. The occurrence rate of PMSV is higher in the tropical world (47, 48) than in the temperate world (49).

The genomic organizations of PCV1 and PCV2 are similar. Both contain two major open reading frames (ORF1 and ORF2) (15, 32, 33). ORF1 of PCV1 encodes a polyprotein, and ORF2 of PCV1 encodes a capsid protein. The same is true for PCV2 (16, 34). The third protein we are interested in is PCV3. PCV3 has a unique structure, as we found ORF 2 on PCV1 and PCV2, but not on PCV3. Recently, we reported that ORF 2 of PCV3 encodes a major structural protein of approximately 100 kDa, containing structural proteins expressed in insect cells, as well as a component

Parine (*Parine*, 1971) was first identified as a PK-23 seed contaminant (Hay) and was subsequently classified in the family *Proteaceae* (S). The PK strains are species have a mean diameter of 17 mm, are almost single-stemmed, these creole and young PKs (PK-1, PK-2 and PK-3) are 10 cm, 15 cm and 20 cm, respectively, and they emerged in a field in South America. In contrast, the disease appeared again with high frequency and at late times was treated positively, as in the case of the washing syndrome (PMWS) (S, 1974).

The PK-15 cell contaminant PKV-15-1c has been shown to be immunogenic, not experimentally detectable (C. L. H. However, in recent studies PKV was consistently detected in PMWS faeces (2, 10, 11). Further porcine analyses revealed that in these animals two immunotypes of PKV (15, 32, 33, 36) PKV-15c and PKV-15d were designated for the nonpathogenic PKV-1 and PMWS-associated PKV-2, respectively. Different reactivity with non-specific antibodies to either PKV-1 or PKV-2 was observed, but they were antigenically identical (2, 4). However, in low levels of virus reactivity below the detection limit, antibodies to PKV-2 reacted to a low degree with PKV-1 (2).

* Corresponding author. Present address: University of Nottingham, Faculty of Chemical Engineering, Buxton Road, Nottingham NG5 7RD, U.K. Tel: 0115 9516411, fax: 0115 9516412, e-mail: p.palocz@open.ac.uk.

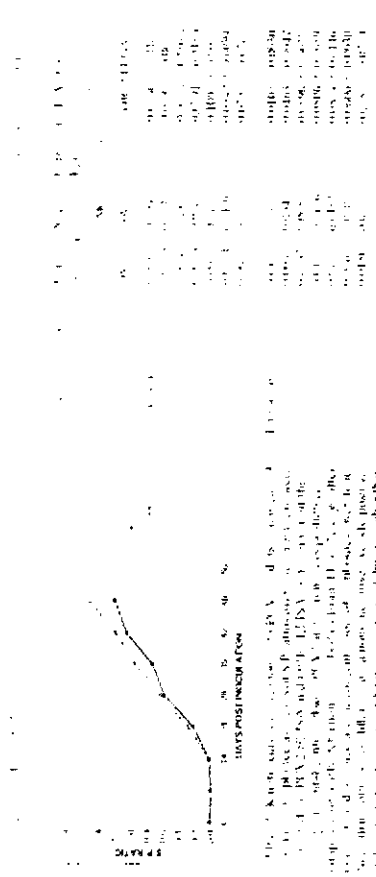


Fig. 5A results are field seen. We further divided these results into the 1-sec 1-A zone as within this consistently above

RESEARCH DESIGN

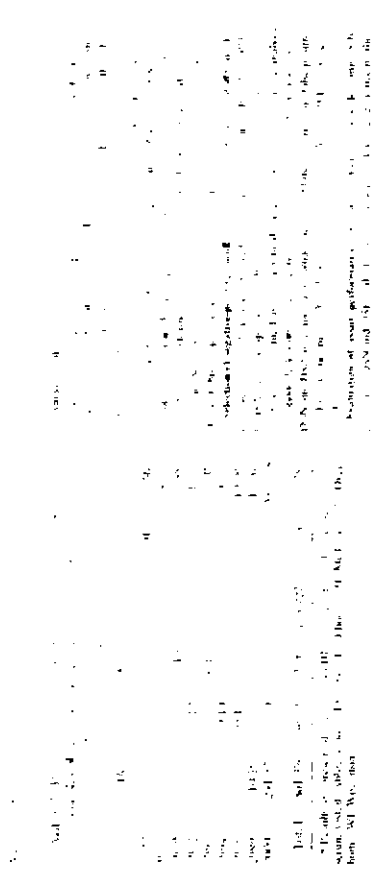
HF results. Results of HF of field serum samples are summarized in Table 1. In all 24 field sera tested by HF, 550 lipids were positive and 15 samples were negative for PCV. In addition, PCV positive results were detected as early as 1987 in 10 of 20 samples. This was followed by positive results from pigs in Michigan in 1989, nine from pigs in 1997, 10 of 28 samples, and 10 of 100 from the first herd in Iowa in 2006. Samples were positive for PCV antibodies.

ELISA results in experimental pigs. Sixty-one samples of serum pigs experimentally infected with PCV2 for culture media were used to evaluate the feasibility of PCV2 and ORP2 ELISA as diagnostic tools. Twenty-five negative sera were obtained from 20 pigs in the sham-inoculated group. An additional 36 HF-negative serum samples were collected from 43 pigs prior to 10 week postinfection with PCV2. Twenty HF-

(ii) PCV2 ELISA. In result is the PCV2 ELISA using field sera are presented in Table 3. Minimum and maximum values were classified by IF as negative, weakly positive and strongly positive for PCV2 antibodies. The distribution of IF-negative sera (5/17) (29.4%) was significantly different ($P < 0.05$) from the distribution of IF-positive sera (12/17) (70.6%) by chi-square test.

THE UNIVERSITY OF CHICAGO

Sample group	Protein	Protein	ORF2 ELISA
HP-negative (n = 217)	Aluminium Reaction	0.004	0.0052
	Mean	0.29 ±	0.276
	SD	0.084	0.084
	Mean	0.155	0.055
	Mean - SD	0.077	0.048
HP-positive, 0 - 480	Aluminium Reaction	0.034	0.33
	Mean	0.008	0.005
	SD	0.019	0.013

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the effect of 2,2,2-trifluoroethylamine on the growth of the cells. The results are shown in Table 1. The growth of the cells was significantly inhibited by the addition of 2,2,2-trifluoroethylamine to the culture medium. The inhibitory effect was dose-dependent, with the highest inhibition observed at a concentration of 10⁻⁴ M. The results also showed that the growth of the cells was not significantly affected by the addition of 2,2,2-trifluoroethylamine to the culture medium at a concentration of 10⁻⁵ M. The results suggest that 2,2,2-trifluoroethylamine may be a potential inhibitor of cell growth.

minimize the data, the χ^2 of a least-squares fit to the data, $\chi^2 = \sum_{i=1}^N (y_i - f(x_i))^2$, is used to assess the quality of the fit. The χ^2 is a measure of the goodness of fit, and is defined as the sum of the squares of the residuals, $\chi^2 = \sum_{i=1}^N (y_i - f(x_i))^2$, where y_i is the observed value, $f(x_i)$ is the predicted value, and N is the number of data points. The χ^2 is a measure of the goodness of fit, and is defined as the sum of the squares of the residuals, $\chi^2 = \sum_{i=1}^N (y_i - f(x_i))^2$, where y_i is the observed value, $f(x_i)$ is the predicted value, and N is the number of data points.

ELISA



Fig. 1. Frequency distribution of S/P ratios for ORF2 ELISA. The left histogram shows the frequency distribution of S/P ratios for ORF2 ELISA. The right histogram shows the frequency distribution of S/P ratios for ORF2 ELISA.

The results of the S/P ratio obtained from the ORF2 ELISA and the ORF2 ELISA are shown in Table 1. The results of the S/P ratio obtained from the ORF2 ELISA and the ORF2 ELISA are shown in Table 1.

DISCUSSION

We have successfully developed and validated two versions of ELISA for the detection of antibodies to PCV2 and ORF2. The results of the S/P ratio obtained from the ORF2 ELISA and the ORF2 ELISA are shown in Table 1. The results of the S/P ratio obtained from the ORF2 ELISA and the ORF2 ELISA are shown in Table 1.

This is the first published report demonstrating the existence of PCV2 infection in U.S. swine herds as early as 1987. Six of the 30 serum samples obtained from a herd in Wisconsin in 1987 were positive by all three assays with titers of more than 100 and S/P ratios ranging from 0.50 to 0.65 for PCV2 ELISA and 0.27 to 0.50 for ORF2 ELISA. This finding was in accordance with several previous reports of PCV2 infection as detected by immunofluorescence in pigs reared in 1989 in Japan (35). In addition, serum samples from 15 herds in Belgium collected in 1985 (34) and serum samples

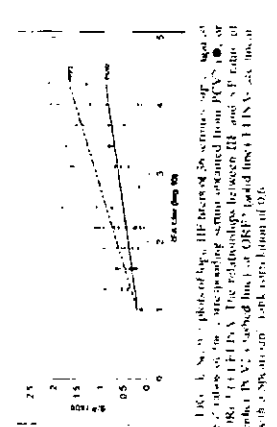


Fig. 2. Relationship between the S/P ratio of PCV2 ELISA and the S/P ratio of ORF2 ELISA. The relationship between the S/P ratio of PCV2 ELISA and the S/P ratio of ORF2 ELISA is shown in this graph.

The results of the S/P ratio obtained from the ORF2 ELISA and the ORF2 ELISA are shown in Table 1. The results of the S/P ratio obtained from the ORF2 ELISA and the ORF2 ELISA are shown in Table 1.

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Table 1. Results of the S/P ratio obtained from the ORF2 ELISA and the ORF2 ELISA.

Sample	PCV2 ELISA	ORF2 ELISA
1	0.50	0.27
2	0.55	0.30
3	0.60	0.33
4	0.65	0.36
5	0.70	0.39
6	0.75	0.42
7	0.80	0.45
8	0.85	0.48
9	0.90	0.51
10	0.95	0.54

The results of the S/P ratio obtained from the ORF2 ELISA and the ORF2 ELISA are shown in Table 1. The results of the S/P ratio obtained from the ORF2 ELISA and the ORF2 ELISA are shown in Table 1.

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306



Protection of swine against post-weaning multisystemic wasting syndrome (PMWS) by porcine circovirus type 2 (PCV2) proteins

P. Blacchard^{a,*}, D. Mahé^a, R. Canole^a, A. Keramiec'h^a,
M.A. Buechard^b, P. Cerdillo^c, E. Albini^d, A. Jestin^a

^a Centre de Recherche en Santé Animale, INRA, UR1139, 37075 Le Mans, France

^b Laboratoire de Pathologie Animale, Université de Bourgogne, 21078 Dijon, France

^c Istituto Nazionale per lo Studio e la Cura delle Leucemie, 20133 Milano, Italy

^d Centre de Recherche en Santé Animale, INRA, UR1139, 37075 Le Mans, France

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Abstract

Porcine circovirus type 2 (PCV2) is known to be associated with post-weaning multisystemic wasting syndrome (PMWS), a common described disease of young pigs. Since no PCV2 vaccine was available so far, we have developed a specific PCV2 vaccine candidate. The ORF1-encoded replication protein and ORF2-encoded capsid protein of PCV2 were expressed and detected in either mammalian or insect expression systems. In a first trial, ORF2 protein was found to be a major immunogen, inducing protection in a mouse model. The piglets received a first injection with plasmid expressing ORF2 protein and gradually immunized with a vaccine containing ORF1-ORF2 protein followed by a second injection. A fortnight later, associated with bacterial-mycoplasmal colitis, stimulating factor (GM-CSF) expression elicited signs (fever, anorexia, etc.), the pigs were protected against a PCV2 challenge after vaccination. In a second trial, protection induced by a subunit vaccine was even better than the one induced by DNA vaccine, since PCV2 replication was completely inhibited.

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1. Introduction

Post-weaning multisystemic wasting syndrome (PMWS) is a new disease affecting 8–13-week-old piglets, that was first observed in Britain in 1996 [1]. PMWS first emerged in western Canada in 1991 [2], and similar disorders were subsequently observed in herds in European countries and in the United States and Asia [3–8]. Clinically, the disease is characterized by pallor, fever and progressive wasting, together with respiratory and digestive disorders. Morbidity rates of 5–30% have been reported in affected pigs [9]. Isolation of virus from tissues of affected pigs led to the identification of a porcine circovirus type 2 (PCV2), considered to be of etiological importance in PMWS [10–14]. Many studies have confirmed the pivotal role of PCV2 in experimental reproduction of the disease [15–19]. Moreover, coinfections with PCV2 and other viral agents, such as

* Corresponding author. Tel.: +33 243 764 42 42.

E-mail address: p.blacchard@le Mans.inra.fr (P. Blacchard).

DNA encoding a replication protein (pORF1) subcloned into two expression vectors for injection in cells against viral infection. A 1.5 kb fragment of DNA encoding porcine circovirus type 2 (PCV2) was subcloned into a pORF1 vector. The recombinant plasmid was transfected into cells. The cells were harvested and the supernatant was collected. The cells were washed twice with PBS, counted and resuspended in PBS at a concentration of 10⁶ cells per ml. The cells were lysed by freeze-thaw. 70 µl of nuclear lysis buffer (1.5 × 10⁶ cells were separated on a 1% SDS-PAGE) and transferred onto a nitrocellulose membrane (Bio-Rad). Immunoreactivity of recombinant pORF1 and pORF2 was checked by Western blot analysis with a swine anti-PCV2 antiserum.

2.1. Vaccination protocol
The DNA vaccine preparation was composed of 200 µg of each plasmid in a total volume of 1 ml in PBS pH 7.2. The protein vaccine was prepared immediately before use, as follows. For 2 ml of emulsion to be prepared, 500 µl of crude lysate from each recombinant baculoviruses (Baculovirus), pORF1, alone or in association, were completed to 1 ml of PBS pH 7.2 and mixed with 1 ml of a water-in-oil adjuvant (Montanide IMS 131) (PR provided by Seppac). For the control, 1 ml of crude lysate from wild-type ACNPV was mixed under the same conditions. The preparations were emulsified and stored on ice until inoculation of the pigs.

2.2. Immunization of pigs according to a prime-boost protocol
In this first trial, 35 25-day-old SPF piglets were divided into five groups of seven piglets randomized according to sex and weight in our facilities under strictly controlled conditions (level 1 biosecurity) [45]. Piglets from four groups received a first intramuscular injection of DNA plasmid preparation on one side of the neck, followed by a second injection, 2 weeks later on the same side, completed by a third injection of recombinant protein emulsion on the opposite side. Pigs were challenged according to the experimental model described by Albini et al. [17], namely intratracheally with 10^{5.2} TCID50 in a dose volume of 5 ml and intramuscularly with 10^{4.5} TCID50 in 1 ml of a tissue preparation-based inoculum, 10 days after the second vaccine injection. In the fifth group, piglets were not vaccinated and not challenged. The composition of the vaccines for the various groups of piglets is presented in Table 1. Clinical observations and rectal temperatures were recorded daily and the piglets were bled and weighed weekly. Duration of the pyrexia phase and relative daily weight gains (RDWG) kg bodyweight were determined for 5 weeks after challenge. The clinical protection conferred in the vaccinated groups was evaluated with reference to the challenge control (CC) group according to the

2. Materials and methods

2.1. Cloning of PCV2 genes

The ORF1 and ORF2 genes encoding PCV2 proteins (replication and capsid) were cloned into the pCDNA3.1 mammalian expression vector (Invitrogen) or the pVL1393 baculovirus transfer vector (PharMingen), as described elsewhere [30]. Recombinant clones were sequenced to ensure that no mutation occurred.

2.2. Production and *in vitro* analysis of plasmids from mammalian expression vectors

Plasmids from pCDNA3.1 encoding ORF1, PCV2 and ORF2, PCV2 were designated pORF1 and pORF2. A pCDNA3 plasmid expressing porcine granulocyte-macrophage colony-stimulating factor (designated pGM-CSF) was supplied by INRA (Jouy-en-Josas, France). The three plasmids were purified by Endo-free plasmid Giga kit columns (Qiagen, Hilden, Germany) and resuspended in PBS. The DNA concentration was estimated by spectrophotometry.

pORF1, pORF2 and pGM-CSF expression were confirmed in transfected PK15 cells, either by IPMA for coronavirus proteins [30], or by a bone marrow proliferation assay for porcine GM-CSF [25].

2.3. Production and characterization of baculovirus-expressed proteins

Two recombinant baculoviruses were obtained after co-transfection with transfer vectors and linearized bacu-

lating virus (Baculovirus) as described previously [31]. Briefly, we used the ORF1-PCV2 protein and Baculovirus expressed the ORF1-PCV2 protein.

SP2 cells were infected with wild-type *Adenovirus* (Adenovirus) nuclear protein (AdNP) or recombinant baculoviruses (BacV1 or BacV2) in a multiplicity of infection (moi) of 10–100 (multiplicity of infection per cell). The cells were harvested 72 h post-infection (pi) and washed twice with PBS, counted and resuspended in PBS at a concentration of 10⁶ cells per ml. The cells were lysed by freeze-thaw. 70 µl of nuclear lysis buffer (1.5 × 10⁶ cells were separated on a 1% SDS-PAGE) and transferred onto a nitrocellulose membrane (Bio-Rad). Immunoreactivity of recombinant pORF1 and pORF2 was checked by Western blot analysis with a swine anti-PCV2 antiserum.

2.4. Inoculation protocol

The DNA vaccine preparation was composed of 200 µg of each plasmid in a total volume of 1 ml in PBS pH 7.2. The protein vaccine was prepared immediately before use, as follows. For 2 ml of emulsion to be prepared, 500 µl of crude lysate from each recombinant baculoviruses (Baculovirus), pORF1, alone or in association, were completed to 1 ml of PBS pH 7.2 and mixed with 1 ml of a water-in-oil adjuvant (Montanide IMS 131) (PR provided by Seppac). For the control, 1 ml of crude lysate from wild-type ACNPV was mixed under the same conditions. The preparations were emulsified and stored on ice until inoculation of the pigs.

2.4.1. Immunization of pigs according to a prime-boost protocol

In this first trial, 35 25-day-old SPF piglets were divided into five groups of seven piglets randomized according to sex and weight in our facilities under strictly controlled conditions (level 1 biosecurity) [45]. Piglets from four groups received a first intramuscular injection of DNA plasmid preparation on one side of the neck, followed by a second injection, 2 weeks later on the same side, completed by a third injection of recombinant protein emulsion on the opposite side. Pigs were challenged according to the experimental model described by Albini et al. [17], namely intratracheally with 10^{5.2} TCID50 in a dose volume of 5 ml and intramuscularly with 10^{4.5} TCID50 in 1 ml of a tissue preparation-based inoculum, 10 days after the second vaccine injection. In the fifth group, piglets were not vaccinated and not challenged. The composition of the vaccines for the various groups of piglets is presented in Table 1. Clinical observations and rectal temperatures were recorded daily and the piglets were bled and weighed weekly. Duration of the pyrexia phase and relative daily weight gains (RDWG) kg bodyweight were determined for 5 weeks after challenge. The clinical protection conferred in the vaccinated groups was evaluated with reference to the challenge control (CC) group according to the

weeks later, vaccinated by a second intramuscular injection. Before the PCV2 challenge, 10 days later (Day 1), all animals were observed in the five groups during the vaccination period. Comparisons of the relative daily weight gain (DWS) of vaccinated pigs with that of control pigs (value = 1) did not reveal any statistically significant differences between the groups.

After an incubation period of 19–21 days after challenge, all piglets from the challenge control (CC) group developed pyrexia and growth retardation. The number of days during which the rectal temperature exceeded 40.5 °C and the relative daily weight gain compared to the control group were recorded (Fig. 1a and b). In this group, the pyrexia phase and growth retardation were essentially observed during the second week post-infection, whereas in the previous phase — 1–1.55 days and RDWG = 0.33 ± 0.23, $P < 0.05$ — and the third-week post-infection duration of pyrexia phase — 2.43 ± 1.72 days and RDWG = 0.38 ± 0.29, $P < 0.01$ — compared to the CC group (RDWG = 1), in which pyrexia and growth retardation were not observed. These results were in agreement with those reported by Alhina et al. [17] during development of the experimental model of PMWS in SPF piglets. Several animals presented clinical symptoms (dyspnea, hemorrhagic, rough hair-coat), and one animal developed infection and was killed for ethical reasons, 4 weeks post-infection.

As shown in Fig. 1a, the duration of the pyrexia phase observed in the vaccinated groups (Ort1, Ort2 and Ort1&Ort2 groups) was shorter than in the CC group. In the Ort1 group, the difference was significant ($P < 0.05$) only during the third-week post-infection compared to the CC group, whereas the Ort2 and Ort1&Ort2 groups showed a more significant difference during the second post-infection, while only the Ort2 and Ort1&Ort2 groups presented a significant difference during the third-week post-infection ($P < 0.01$ and ≤ 0.05 , respectively). To evaluate clinical protection, we determined a ΔGW3 index, corresponding to the difference in mean daily weight gain expressed as a percentage of weight in kg per day) during the third-week post-challenge period between vaccinated pigs and challenge control pigs (Table 1). These results confirmed a significant enhancement of protection after vaccination with an Ort2-based preparation (DNA and recombinant protein), confirming Ort2 as a major protective immunogen. One piglet in the Ort1 group also showed severe symptoms of PMWS (dyspnea, ataxia, rough hair-coat, tremor, and wasting).

The relative daily weight gain was also better in vaccinated groups compared to the CC group (Fig. 1b). A significant difference was observed during the second-week post-infection, while only the Ort2 and Ort1&Ort2 groups presented a significant difference during the third-week post-infection ($P < 0.01$ and ≤ 0.05 , respectively).

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same expression system. Serological analysis was then performed by a square phase blotting (ELISA) (Fig. 2). In this assay, the reaction was performed using two PCV2-specific monoclonal antibodies (MAbs) as a substrate and a second and a second peroxidase-conjugated MAbs was used as a detection antibody. Not purified PCV2, at an optimal concentration and were incubated and then transferred onto ELISA plates. Results were expressed as the percentage of absorbance produced by the serum compared to the non-inhibited reaction. Sera giving an inhibition higher than 50% at the initial screening dilution, set at 1:6, were outside of the positive sera. The titers were expressed as the log₂ of the highest dilution of antisera indicating 50% inhibition. A mean titer was calculated for the sera from each group.

2.5.2. PCR detection of PCV2 in tissue samples

All tissue samples from the eight groups of piglets were kept frozen at -72 °C prior to DNA extraction. DNA was extracted using the Qiagen DNeasy tissue kit according to the manufacturer's recommendations. A previously described PCR technique for the detection of PCV2 was carried out [30]. Amplification reactions were performed in a 50 µl mixture containing 0.4 µM of each primer (forward: 5'-CTCTTCCTACTGCTGAGTACCTTGTTC-3' and reverse: 5'-CCAGGAGACAGCTCCTCCGTGTC-3') 0.1 mM of each dNTP, 5 U Taq DNA polymerase (Boehringer) with the following cycling parameters: denaturation at 95 °C, 5 min; 35 cycles of 91 °C, 30 s, annealing at 60 °C, 30 s; 72 °C, 2 min; final elongation at 79 °C, 10 min. Amplification generated 177-bp DNA fragments, corresponding to the PCV2 genome. An internal control was added to each PCR reaction to reveal any false negative samples. This internal control was produced and cloned from the PCV2 genome, and revealed a 428-bp fragment with the same primers.

2.6. Statistical analysis

Parametric statistical tests were applied using the SYSTAT 9 computer software package (SPSS Inc.). Relative daily weight gains were analyzed by ANOVA procedure during the vaccination period and by the Kruskal-Wallis non-parametric test for the 5 weeks post-infection. The number of days when the rectal temperature was higher than 40.5 °C was analyzed by the Yates χ^2 test.

3. Results

3.1. Identification of PCV2 major immunogen in a prime-boost pig immunization approach trial (n = 1)

In trial no. 1, 25-day-old pigs were vaccinated with a DNA plasmid preparation, followed by a second injection

Composition of the vaccines for the various groups of piglets in trial no. 2 and vaccination protocol		Injection 1 (28-day-old piglets)		Injection 2 (42-day-old piglets)		PCV2 challenge (53-day-old piglets)	
Challenge control groups (CC1 and CC2)	Without	Without	Without	Without	Without	Without	Without
PCV2 vaccine groups (Ort1, Ort2, Ort1&Ort2)	Ort1/Ort2: 20 µg Ort2/Ort2: 20 µg Ort1/Ort2: 20 µg	Ort1/Ort2: 20 µg Ort2/Ort2: 20 µg Ort1/Ort2: 20 µg	Ort1/Ort2: 20 µg Ort2/Ort2: 20 µg Ort1/Ort2: 20 µg	Ort1/Ort2: 20 µg Ort2/Ort2: 20 µg Ort1/Ort2: 20 µg	Ort1/Ort2: 20 µg Ort2/Ort2: 20 µg Ort1/Ort2: 20 µg	Ort1/Ort2: 20 µg Ort2/Ort2: 20 µg Ort1/Ort2: 20 µg	Ort1/Ort2: 20 µg Ort2/Ort2: 20 µg Ort1/Ort2: 20 µg
Subunit vaccine groups (SU1 and SU2)	SU1/PCV2: 5 × 10 ⁶ cells SU2/PCV2: 5 × 10 ⁶ cells SU1/SU2: 5 × 10 ⁶ cells	SU1/PCV2: 5 × 10 ⁶ cells SU2/PCV2: 5 × 10 ⁶ cells SU1/SU2: 5 × 10 ⁶ cells	SU1/PCV2: 5 × 10 ⁶ cells SU2/PCV2: 5 × 10 ⁶ cells SU1/SU2: 5 × 10 ⁶ cells	SU1/PCV2: 5 × 10 ⁶ cells SU2/PCV2: 5 × 10 ⁶ cells SU1/SU2: 5 × 10 ⁶ cells	SU1/PCV2: 5 × 10 ⁶ cells SU2/PCV2: 5 × 10 ⁶ cells SU1/SU2: 5 × 10 ⁶ cells	SU1/PCV2: 5 × 10 ⁶ cells SU2/PCV2: 5 × 10 ⁶ cells SU1/SU2: 5 × 10 ⁶ cells	SU1/PCV2: 5 × 10 ⁶ cells SU2/PCV2: 5 × 10 ⁶ cells SU1/SU2: 5 × 10 ⁶ cells
Control groups (C1 and C2)	Without	Without	Without	Without	Without	Without	Without

Note: DNA vaccine was prepared in a total volume of 1 ml in PBS (pH 7.2), and subunit vaccine was prepared in a total volume of 1 ml in PBS (pH 7.2) mixed with 1 ml of adjuvant (Mycobacterium). The PCV2 vaccine used for the challenge consisted of a mixture of 10⁶ cells in 1 ml of PBS (pH 7.2) mixed with 1 ml of adjuvant (Mycobacterium).

experimental model previously described by Alhina et al. [11].

2.4.2. Comparison between DNA vaccine and subunit vaccine

In the second trial, 63-day-old SPF piglets were divided into eight groups of eight piglets, randomized as described in the first trial. In the DNA vaccine protocol, piglets received a first intramuscular injection of DNA plasmid preparation, followed by a second injection, 2 weeks later. In the subunit vaccine protocol, piglets received a first intramuscular injection of baculovirus-expressed protein emulsion, followed by a second injection, 2 weeks later. Piglets were challenged 11 days after the second injection, as described in the first trial. The second trial was conducted in duplicate, a first series of four groups was monitored for 5 weeks after challenge and a second series of four groups was subjected to early killing between 4 and 17 days after challenge. The composition of the vaccines of the various groups are presented in Table 2. In the first series (groups CC1, DNA1, SU1 and C1), clinical observations and re-

lative daily weight gain were recorded during the pyrexia phase and relative daily weight gain/lymph node weight were determined for 5 weeks after challenge. At necropsy, tissue samples (thoracic lymph nodes, tonsil) were collected for laboratory investigation. In the second series (groups CC2, DNA2, SU2, C2), two piglets from each group were killed and necropsied at days 4, 8, 11 and 17 post-infection (dpi) and tissue samples (thoracic, inguinal, mesenteric lymph nodes, tonsil, lung and ileum) were collected for laboratory investigations.

2.5. Laboratory investigations in the second trial

2.5.1. Injection of PCV2-antibodies by a liquid phase blocking ELISA (LPEB)

The sera collected from the CC1, DNA1, SU1 and C1 groups just before vaccination and up to 4 weeks after challenge were analyzed for the presence of PCV2-specific antibodies. The ELISA protein test using the baculovirus-expressed (b12) protein developed in our laboratory [11] could not be used, because of the cross-reaction with the



Fig. 1. Number of days with rectal temperature higher than 40.5 °C (a) and relative daily weight gain (RDWG) (b) for each vaccinated and challenged group in trial no. 1. Columns represent the average values for each group: challenge control group (CC1), DN1 vaccine group / DN2 challenge group (DN1), CC2 vaccine group (CC2) and DN2 vaccine group (DN2). The RDWG of challenged pigs is compared with that of the control pigs (value = 1). Bars represent standard deviations.

This piglet was finally killed, for ethical reasons, at 35 dpi.

3.2. Comparison between a DN1 vaccine and a subunit vaccine (trial no. 2)

In the second trial, the pigs were vaccinated at the age of 4 and 6 weeks with either an Ortlé/DN2-based plasmid preparation (groups DN1 and DN2), or an Ortlé/DN2-based plasmid preparation (groups SU1 and SU2). They were then PCV2 challenged 14 days after the second vaccine injection. Pigs in groups CC1 and CC2 (challenge control) were not vaccinated, but were challenged; and pigs in groups C1 and C2 (control) were not vaccinated and not challenged. No clinical signs were observed in any of the groups before the challenge. Only pigs vaccinated with the plasmid preparation presented hyperthermia just after vaccination. However, comparison of relative daily weight gain (RDWG) (28-34) of vaccinated pigs with that of control pigs (value = 1)

Table 3. Clinical protection of vaccinated groups after PCV2 challenge in trial no. 1.

Groups of pigs	RDWG, third-week post-infection	ADWG, third-week post-infection	ADWG, clinical signs
Challenge control group	0.91 ± 0.09	44/40	Yes (1)
Ortlé vaccine group	1.29 ± 0.11	44/40	Yes (1)
DN2 vaccine group	1.99 ± 0.13**	11/19	No
Ortlé/DN2 vaccine group	1.5 ± 0.07*	10/6	No

Note: ADWG index was determined after PCV2 challenge as the following: in mean daily weight gain (RDWG), expressed as % kg per day (S.D.). during the third-week post-challenge period between vaccinated pigs and challenge control pigs.

* Several animals showed clinical symptoms, such as diarrhoea, inappetence, weight loss, and were killed for ethical reasons. (1) One animal died, not wasting, and was killed for ethical reasons. (2) Two of the group of 5 weeks of C1 group post-infection.

** Statistical analysis was performed as comparison with the challenge control group (P < 0.05).

did not reveal any significant difference between the groups.

3.2.1. Follow-up of the first series of pigs (groups CC1, DN1, SU1 and C1) for 5 weeks post-infection

As in the first trial, all piglets of the CC1 group developed preveia and growth retardation after challenge. The number of days during which the rectal temperature exceeded 40.5 °C and the relative daily weight gain compared to the C group were recorded (Fig. 2a and b). In this group, the preveia phase and growth retardation were essentially increased during the third-week post-infection, respectively, compared to the C1 group, in which preveia and growth retardation were not observed (RDWG = 1). A significant growth retardation was also observed during the first- and second-week post-infection. However, clinical symptoms were less pronounced than during the first trial (digestive disorders only). The difference of expression of PMWS in CC pigs in these two trials cannot be ascribed to

placebo vaccination with GM-CSF and Montanide applied to CC pigs in the first trial. Indeed, clinical symptoms were also observed in other trials without placebo vaccination [17].

Table 1. Clinical protection of vaccinated groups after PCV2 challenge in trial no. 2.

Groups of pigs	ADWG, third-week post-infection	ADWG, clinical signs
Challenge control group	0.79 ± 0.18	Yes*
DN1 vaccine group	1.49 ± 0.07**	11/20
Subunit vaccine group	1.45 ± 0.05**	11/51

Note: ADWG index was determined after PCV2 challenge as the difference in mean daily weight gain (RDWG), expressed as % kg per day (S.D.), during the third-week post-challenge period between vaccinated pigs and challenge control pigs.

* Several animals showed clinical symptoms, such as diarrhoea, inappetence, weight loss, and were killed for ethical reasons. (1) One animal died, not wasting, and was killed for ethical reasons. (2) Two of the group of 5 weeks of C1 group post-infection.

** Statistical analysis was performed in comparison with the challenge control group (P < 0.05).

significantly reduced virulence, and this protective phase more efficiently in the Orf2 and Orf1&Orf2 vaccine groups than in the Orf1 vaccine group. Moreover, in the Orf2 and Orf1&Orf2 post-infection, the Orf2 and Orf1&Orf2 groups showed a significantly reduced viral titra (log10 TCID50) compared to the CC group, which was twice presented in the Orf1 group. No difference was observed between the Orf2 group and the CC group (mean of viral load group) while the Orf1&Orf2 group showed a significant difference with the CC group ($P < 0.05$) during the third week. These results were confirmed by a higher AGW3 index in the Orf2 group than in the Orf1&Orf2 group (1.09 versus 0.76), in the third week post-challenge. Although no significant difference was observed between these two groups, the lower AGW3 index in the Orf1&Orf2 group suggests the existence of a reduction effect of Orf1 addition on Orf2 vaccine efficacy. However, no clinical symptoms were observed in these two groups, while pigs in the Orf1 group presented typical PMWS symptoms (ataxia, rough hair coat, wasting). We therefore showed that the Orf2 encoded capsid protein, used in a preparation-based DNA and subunit vaccine, neutralizes the major immunogen to induce protection of piglets against a PCV2 challenge. In contrast, the Orf1-encoded replication protein appears to be only weakly immunogenic. These results differ from those reported for Chicken anemia virus (CAV), for which co-synthesis of VP1 (capsid protein) and VP2 are necessary to mount a protective immune response [33,42].

In our trial no. 2, we focused on the vaccine composition to compare the efficacy of the protection conferred by either a DNA vaccine or a subunit vaccine. Independently of the previous results, we decided to use the two associated major PCV2 proteins (Rep and Cap1), in two injections. As in the first trial, on the basis of growth retardation and fever parameters presented by piglets in the challenge control group, we observed that the subunit vaccine significantly reduced the duration of the pyrexia phase ($P < 0.05$) during the third-week post-infection, while the DNA vaccine did not have any effect on the duration of the pyrexia phase. All piglets of the DNA group presented hyperthermia higher than 40.5 °C for one to 3 days versus three piglets of the subunit group for 1–3 days. In contrast, the two vaccinated groups (DNA and SU) showed a better growth, compared to the CC group. These results were confirmed by a high Δ GW3 index, 1.20 and 1.54 respectively. The AGW3 index obtained in trial no. 2 was higher than that obtained in trial no. 1. However, we cannot consider so significant the difference in AGW3 obtained in the various groups of animals (Orf2, Orf1&Orf2, DNA, subunit groups). In the case of conventional commercial vaccines against Aujeszky's disease virus (ADV), the official requirement is a minimum Δ GW7 index of 1.5. Although these two diseases are not comparable, the AGW3 index obtained in the present study indicates a useful protection performance for the production of vaccines against a PCV2 challenge.

To complete the results of trial no. 2, obtained by evaluation of rectal temperature and weight gain, we analyzed the

the immune response presented by the vaccine B-Vax, as influence the expression of PMWS.

The objective of this study was to evaluate the efficacy of protection induced by vaccination of piglets against a PCV2 challenge. This is the first report to relate the use of vaccines in PCV2 infection. In 2 vaccine groups (in a prime boost protocol) and the subunit-vaccine group appeared to provide a significant protection against PCV2 infection. However, the subunit group did not present a significant growth retardation compared to the control group at the third- and fourth-week post-infection ($P < 0.05$). We therefore demonstrated the essential role of the recombinant capsid protein produced by the baculovirus system in a vaccine concept against PCV2 infection. Further trials must be conducted in order to define a better vaccine protocol. Prime-boost vaccination with DNA and recombinant baculovirus expressed protein successfully enhances the immune response to other viruses, resulting in both humoral and cell-mediated immune responses [45, 46]. In the case of a vaccine against PCV2 infection, more detailed studies are necessary to characterize the immune response. However, previous studies have demonstrated induction of immune priming T and B cells with DNA vaccination in the pig model [28] and on the basis of the results obtained with our subunit vaccine in terms of seroconversion and clearance of virus following PCV2 challenge, we can predict a good efficacy of our subunit vaccine in a prime-boost approach that would induce both antibodies and cell-mediated immunity.

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Characterization of the Recombinant Proteins of Porcine Circovirus Type2 Field Isolate Expressed in the Baculovirus System

Yoon Kim, Jibmyun Kim, Kyungwon Kang¹ and Young S. Lyoo

¹Immunopathology Lab, College of Veterinary Medicine, Konkuk University, Seoul, 143-701, Korea
²Dongbaekcheon River Vet. Hospital, Dongbaekcheon, Kyunggi-do, Korea

ABSTRACT

Porcine circovirus (PCV) type2 was isolated using primary porcine kidney cells from lymph node of piglets with typical PMWS. The presence of the virus was identified by PCR using primers specific to PCV type2. The ORFs 1 and 2 were amplified by RT-PCR using primers corresponding to the target genes of the PCV type-2. Cloned genes were inserted into the baculovirus expression vector and PCV recombinant proteins were expressed using baculovirus expression system. Recombinant protein expression was determined by indirect immunofluorescent assay (IFA) and immunoblotting using polyclonal antiserum to PCV. ORF1 gene expressed two proteins with approximately 17 kDa and 31 kDa proteins in the baculovirus system. Recombinant protein of the ORF2 was similar to that of the native virus except minor bands with different molecular weight were detected. Recombinant protein expressed in the baculovirus system showed at least two glycosylation sites based on the tunicamycin treatment. Recombinant protein of the ORF2 assembled virus-like particle in recombinant virus infected insect cells.

Key words : PCV type2, recombinant protein, baculovirus system

Introduction

Post-weaning multisystemic syndrome (PMWS) has been hot issue in swine industry with controversy of causative agent that may produce this poorly recognizable pig disease. Among several pathogens detected PCV type2 has been consistently identified from animals with typical PMWS (1,2,10,11). However non-pathogenic porcine circovirus (PCV

^{*} Corresponding author: Dr. Young S. Lyoo, Assistant professor, Immunopathology Lab, College of Veterinary Medicine, Konkuk University, Seoul Korea 143-701
Phone: +82-2-450-3719, e-mail: lyoo@ Konkuk.ac.kr

type1) was first detected as a contaminant of the cell culture pig kidney cell line PK-15 (13,14). This small non-enveloped single-stranded circular DNA virus has been classified in the circoviridae family (6), along with other viruses including chicken anemia virus, psittacine beak and feather disease virus (1, 17). The presence of antibodies reactive with PCV has also been detected in humans, mice and cattle (10), but challenge experiment to pigs of PK-15, contaminant PCV didn't induce disease (3,15). The viral genome DNA is very short (approximately 1.7kb) compared to that of other viruses but it possesses tentatively 11 (ORFs) by computer analysis (5,9), but major two (ORFs of ORF1 and ORF2) have been elucidated their role in the virus (7,8,12). The genome size of ORF1, encoded replicase protein, is 945bp and ORF2, encoded capsid protein, is 1846bp, respectively (5). Recently recognized unique PCV is known to associate with pig's new disease, post-weaning multisystemic syndrome (PMWS) worldwide. This PCV named to PCV type2 and 70% of the sequence is homologous compared to PCV type1. In two major ORFs, 89% of ORF1 sequence and 67% of ORF2 is homologous between two strains (11).

In this research, baculovirus protein expression system was used for the expression of the major two ORFs of PCV type-2 known to associate with PMWS in swine. The expressed proteins were characterized with molecular techniques described elsewhere.

Materials and Methods

PCR and cloning

Lymph nodes and tissues were collected from pigs with PMWS, and DNA was extracted using phenolchloroform/isoamylalcohol solution. Extracted viral genome DNA was used as a template to amplify ORFs 1 and 2 with primers specific to corresponding genes. This amplified PCR product contained open reading frame of the gene, encoding the protein associated with viral replication. A primer sequence for the upstream 5' end of the ORF1 designated as P1 was 5'-ATC⁺ ATCC⁺ CACA⁺ GTT⁺ CTA⁺ CAG⁺ A and 3' reverse primer R1 sequence was 5'-TAA⁺ TAC⁺ TCC⁺ GAT⁺ ATG⁺ ATG⁺ C-3', respectively. PCR conditions for the amplification was

Dr. Yoon, Kim, Jibmyun Kim, Kyungwon Kang¹ and Young S. Lyoo²

as follows. The first one cycle was performed with denaturing for 2 min at 94°C, annealing for 30 sec at 52°C and extension for 3 min at 72°C. Additional 25 cycles of 30 sec at 94°C, 1 min at 52°C and 90 sec at 72°C and final step comprised of 9 min at 72°C. Initial at 42°C and 10 min at 72°C was carried out. Amplified gene was cloned into TA vector (Pharmacia Lincotec vector system, USA). TA vector containing PCV type2 ORF1 was digested with Bst48 and subcloned into CMV⁺ based baculovirus transfer vector pVLCB3 (Invitrogen, USA).

The PCV type2 ORF2 was amplified with 700bp full sequence. Primer set was 5'-T⁺ TTT⁺ AGC⁺ TTT⁺ GAT⁺ GAT⁺ GAT⁺ CTT⁺ C-3' and 5'-T⁺ TTT⁺ AGC⁺ TTT⁺ GAT⁺ GAT⁺ GAT⁺ CTT⁺ C-3'. This primer set had minor modification from the one designed by Naeve et al. and reverse primer was included BamHI restriction site for cloning (12). PCR conditions for the amplification consisted of denaturation step at 95°C for 5 min, and additional 30 cycles of denaturation at 94°C for 30 sec, annealing at 53°C for 1 min and extension at 72°C for 1 min. PCR was carried out using thermocycler (Mettler Blocker, USA). PCR product was cloned into baculovirus expression vector pVLCB3 as same manner as ORF1.

Sequencing

To confirm proper start and stop regions cloned DNAs of ORF1 and ORF2 of the PCV type2 were sequenced by Sanger's method using automated sequence analyzer (Bioer Co. Ltd., Korea).

Transfection

Heffie cells were maintained with EX-cell 400 (Gibco scientific, USA) with 1% of antibiotics and antimycotic solution (Wako-Pure, USA). Insect cells were seeded into 6 well plate and placed 25°C for 2 hrs. Transfection mixture containing linearized baculovirus DNA (BioLabs, Beverly, USA) and pVLCB3 vector with insertion of PCV ORF1 or 2 genes were mixed with Lipofectin (Gibco-BRL, USA) and was placed in room temperature for 10 min. And cells were transfected with transfection mixture drop by drop and placed at room temperature. After 6 hrs of incubation, the medium using transfection was removed and replaced with 5ml fresh insect cell culture medium (Ex-cell 400).

After 72 hrs of incubation at 25°C, supernatant was collected and the cell plate was air dried and fixed with methanol at -20°C for 15 min. To determine expression of the recombinant protein from transfected cells by IFA using polyclonal antiserum was carried out.

Immunoblotting

Heffie cells (5X10⁶) were seeded into 75cm² plastic cell flask and 2 hrs later, recombinant baculovirus was inoculated and placed at 25°C incubator for 90min. After 72 hrs of infection, when obvious CPE was observed, cells and supernatant were harvested. Control cells and supernatant

were mixed with 2X trans-blot buffer, boiled at 100°C for 5 min, placed on ice and then re-precipitated at 12,000 rpm for 1min. Samples were electrophoresed in 10% SDS/PAGE gel and proteins were transferred onto PVDF membrane using electrotransfer system. Membrane was blocked with 5% skim milk, and reacted with 1:100 dilute of polyclonal anti PCV antiserum for two hours, and washed three times with PBS-Tween 140 (BioLabs) gel. Anti-swine secondary antibody (HRP, USA) was reacted for one hour to capture the recombinant protein bound swine IgG. The membrane was washed and agitated into diaminobenzidine-tetrahydrochloride for 1 min. Then the membrane was exposed to X-ray film and developed.

Tunicamycin treatment

Insect cells were seeded into 75cm² cell flask and 2 hrs later, recombinant baculovirus was inoculated and placed at 25°C for 90 min. The inoculums were replaced with fresh insect cell culture medium Ex-cell 400 including Lipofectin tunicamycin. After 72 hrs of incubation, cells were harvested and immunoprecipitated by same manner with previous protein analysis using SDS PAGE.

Virus-like particle formation

VLP formation was determined by electron microscopy (JEM-100A, Japan) with negative staining using 2% PTA. Recombinant virus infected insect cells was frozen and thawed three times and supernatant was collected. Insect cell culture supernatant was centrifuged and pellet was resuspended with distilled water for the electron microscopy.

Results

ORFs 1 and 2 of the PCV type2 have been amplified by PCR using primer set corresponding to target gene with expected genomic size. The PCR products was purified using GeneClean II kit (Bio101, USA) for the expression vector construction. Each of the ORF was appropriately ligated into the baculovirus expression vector pVLCB3 and confirmed with restriction endonuclease digestion followed by agarose gel electrophoresis with ethidium bromide staining. Cloned DNA sizes were similar to that of the expected in agarose gel electrophoresis. Recombinant expression vector (lane 2) and ORFs (lane 3) and 2 (lane 4) were released using appropriate restriction endonucleases (Fig. 1). Recombinant baculoviruses containing PCV type2 ORF1 or ORF2 were generated from linearized baculovirus DNA and baculovirus construction vectors transfected insect cells. PCV type2 recombinant proteins were successfully expressed from recombinant virus infected insect cells. Expression of the recombinant proteins from recombinant virus infected insect cells were confirmed by IFA test using polyclonal antiserum to PCV type 2. Intracytoplasmic fluorescence was detected from recombinant DNA transfected insect cells but from vector alone transfected non control cells (Fig. 2).

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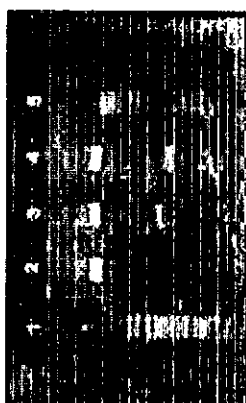


Fig. 1. Electron micrograph of the PTA stained PCV ORP2 recombinant protein expressed in the baculovirus system showing virulent particles. Majority of VP1 was similar to that of the native PCV particles but size of particles was heterogeneous. Bar represents 100 nm in length.

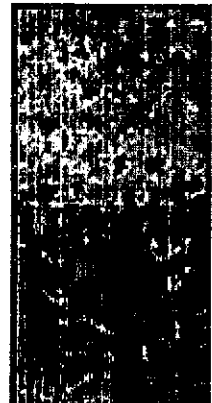


Fig. 2. Western blot analysis of the porcine circovirus recombinant proteins. Lane 1 represents insect cell lysate and recombinant PCV type2 ORP2 recombinant proteins with high approximately 20kDa and low molecular weight (7kDa), respectively. Lane 2 is a recombinant protein of the ORP2 and lane 3 represents recombinant ORP2 with tunicamycin treatment.



Fig. 3. Electron micrograph showing recombinant baculovirus containing ORP2 of the PCV2 infected insect cells were released virus like particles. Irregular form of virus like particles were detected from ORP2 recombinant virus infected insect cells but ORP1 infected cells (Fig. 3).

VP1 formation
Electron microscopy revealed recombinant baculovirus containing ORP2 of the PCV2 infected insect cells were released virus like particles. Irregular form of virus like particles were detected from ORP2 recombinant virus infected insect cells but ORP1 infected cells (Fig. 3).

Discussion
Among new emerging swine diseases PMWS became one of a most controversial diseases. PCV type2 has been speculated as a major causative agent of the PMWS in pigs. However there is no clear evidence that the virus causes PMWS except PCV was detected from majority of the pigs showing clinical signs. Since PCV replicated in the secondary immune system it has been raised great concern on the xenotransplantation using pig as an animal model. Immunosuppressed individual may cause life threatening serious problem when organs transplanted into the human recipient from genetically modified pig donors infected with PCV. So far known molecular characteristics of the PCV



Fig. 1. Electron micrograph of the PTA stained PCV ORP2 recombinant protein expressed in the baculovirus system showing virulent particles. Majority of VP1 was similar to that of the native PCV particles but size of particles was heterogeneous. Bar represents 100 nm in length.

type 1 and 2 are similar to each other with genetic organization and size of the viral genome. But the finding of the fairly small viral genome size and function of the each ORP of the PCV is not fully understood in the current. The ORP2 of the PCV type2 showed heterogeneity compare to that of the PCV type1 (5).

VP1 protein sequence of the PCV ORP2 possessed at least a glycosylation site but native recombinant protein expressed in the baculovirus system did not show any noticeable changes in the molecular mass by tunicamycin treatment. This may indicate that the ORP2 of the PCV has minor glycosylation modification or insect cell expression system does not mimic porcine cell translation system. The post translational modification of the recombinant ORP2 need to be further characterized to understand nature of the ORP2 in the PCV type 2. At the moment we do not understand two minor bands appeared to be shifted from major band after tunicamycin treatment.

The recombinant proteins expressed in the eukaryotic expression system could be utilized as a diagnostic antigen, immunogen for the recombinant vaccine and clue to understand molecular characteristics of the viral protein. Further research of the recombinant protein such as immunogenicity comparison with native viral protein function of the each recombinant protein in the pathogenicity and viral replication would be beneficial to solve the PMWS in pigs. Currently immunogenicity of the recombinant protein is under testing in the host animals to understand role of the recombinant protein in swine and piglets received maternal antibody from vaccinated sows.

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