llII]1[I

SIIPERINDUSIRTh Y C0NEICIU
fillI Radicacioi

02053549 00000000 Folios: 43

11111 Fecha (fIND): 2002-06-20 16:41:53

rSUPEiINTENDENCIA

Ti’aiite

002 1’f4TENTES 0 1 RE6ISTRO/ 411 PRESthTAC1

udufriayCoinercio

Dependeniia: 2020 DIVISION DE NIJEVAS CRECIONES
02

05349FORMULAI
UNICO DE SOLICITUD DE PATENTEE
2000-3

O SOLICITUD DE:

I X I Patente de Invención I I Patente de Modelo de Utilidad

@ 1. AGRICULTURAL RESEARCH COUNCIL
2. INSTITUTE FOR ANIMAL SCIENCE

HEALTH
RESEARCH

Nombre: 3. BARTELING, SIMON JOHANNES

SOLICITANTE 1. Old Soutpan Road, Onderstepoort, Pretoria 0110, CC. NIT

(71) RepOblica de Sudâfrica

.. 2. 15 Edelhertweg, NL-8219 PH Lelystad, Holanda
Dlrecclon: 3. Nieuwe Keizersgracht 438, NL-1 018 VG,
Amsterdam, Holanda

TelØfono: 3123600 Fax: 3122410 C.E. Otro
E-Mail: genera lcardenasycardenas.com
NUmero
1. PRETORIA, SUDAFRICA
Domicilio: 2. LELYSTAD, HOLANDA
3. AMSTERDAM, HOLANDA

Nombre: LUZ HELENA ADARVE GOMEZ IDENTIFICACION

PREJTE DirecciOn: Carrera 7 NO 71-52 Torre B Piso 9 C.C. 117-71 NIT El
OAPODERADO TelØfono: 3123600 Fax: 3122410 C.E. El Otro El
(74) E-Mail: generaIcardenasycardenas.com NUmero 41.575.435 BTA
TP 14.884 C.S.J.

oNombre: BARTELING SIMON JOHANNES IDENTIFICACION

INVENTOR(ES) Dirección: Nieuwe Keizersgracht 438, NIL-11 018 VG, Amsterdam, Holanda C.C.NIT
(72)

TelØfono: 3123600 Fax: 3122410 CE. El Otro
E-Mail: genera Iccardenasycardenas.com
NCimero

Domicilio: AMSTERDAM, HOLANDA

Titulo(54):

"METODO PARA INACTIVAR

Clasificación Internacional (51): A Eb

K 3q /135

&Iz P4 Ioo 1 /06.

(33)Pals de Origen (32)Fecha (31)N’ de Solicitud
Prioridad

sifl N0RJ

0 Para publicar a partir de la fecha de la presente soilcitud a los:
6 meses 12 meses Fj 18 meses Otro

Comprobante de Pago No.: 02-38,978 Fecha junio 17, 2002

ANEXOS

11

Comprobante de pago de la tasa de presentación de la solicitud.
JComprobante de pago de la tasa por concepto de excedente de palabras en la put ElComprobante de pago de la tasa por concepto de reivindicación de prioridad. ElPoderes, si fuere el caso.
ElCertificado de existencia y representaciOn legal cuando el solicitante sea persona j ElCopia certificada de la primera solicitud, si se reivindica prioridad.
Traducción simple de la pnmera solicitud, Si se reivindica prioridad.
ElDocumento de cesión del inventor al solicitante o a su causante.

i: Descripción de la

ElUna o mÆs
ElDibujos y/o pianos necesarios.

El De ser ei caso, copia del contrato de acceso.

De ser el caso, documento que acredite ia licencia o autorización de uso de conoci tradicionales de la comunidad de las comunidades indIgenas.
ElDe ser el caso, certificado de depósito del material biológico.

El Arte final 12x12 y 6x6 cm.

Resumen y extracto para publicaciôn.

NOMBRE: LUZ HELENA ADARVE GOMEZ

FIRMA: L,&7t V I
C.C. 41.575435 BTA TIP 14.884
C.sJ.

BY JUAN DIEGO

fit 7
Industria y Comercio
SUP E RI NTEN DEN C IA

Radfracion: 02053549 00000 Feda (AND).- 202-0610 16:41:53 43 Dependemia.- 20 DIVISION DE CREACIONES

002 PATENTES D 1 RE TRO/ 411 P*EScjup.

NIT: 800176089-2

RECISO (IFICIAL DE CAJA: 02 - 38.978 FECHA : JUN10 17 DE 2002

***** CONSI6NACI

DEPOSITANTE TWO PASO BANCO

- -

Na. PASO FECHA P60 Yr. PASO --------------------------

ICARDENAS Y CARDENAS P.I. CONSISNACION BANCO

POPU7CONCEPTO

_00110_6 3348651 17/06/2002 1,020.000.Q0

*1*1* C P T 0 H***

CANT. RENTISTICO TOTAL CONCEPTO ---

1 50005-01-01 SOLICITUDES

SON SC!ENTOS SETENTA V (ICHO NIL PESOS

1 TRAMLIES DE SOL. DE PATENTE DE IN
TOTAL: $

IRESPONSABIE:

RECIBO DE CAJA APLICADO AL EXPEDIENTE Na.____________________________ c’r>pj 5 CTrV1

TA

378,000.00
378000.00

Carrera 13 No. 2 7-00 Pisos 5, 7 y 10 Conmutador 382 0840
Fax 350 5220

E-mail: info@sic.gov.co
www.sic.gov.co
Bogota, D.C., Colombia

Fax: 18 Jun 2002 13:55 P.03

WO 0/4390 . PCT/ZAOO/002S4

TITLE: METHOD OF INACTIVATING MICROORGANISMS

INTRODUCTION AND BACKGROUND TO THE INVENTION
This invention relates to methods of inactivating microorganisms and
5 manufacturing vaccines to vaccines manufactured by such methods, and to
kits incorporating such inactivated microorganisms. More particularly but not exclusively, this Invention relates to methods of inactivating viruses and manufacturing virus vaccines and to vaccines manufactured from such methods:
10

Virus inactivation and safety tests are the most critical steps in the production of inactivated vaccines. In the case of foot-and-mouth disease (FMD) vaccines in particular, guaranteed safety is essential because any occurrence of the disease will cause a blockade of all export trade of animals
15 and animal products.

A classical method of inactivating FMD virus and preparing FMD vaccine Is
with formaldehyde as described by Waldrnann at at.: Waldrnann 0. Pyl G, Hobohm KO, Mohimanri H: "Die Entwicklung des Riernser
20 Adsorbatimpfstoffes gegen Maul. und Klauenseuche und seine HersteIIung.
Zbl Bakt I Orig 148: 1, 1941.

In the past this method was used in the preparation of FMD vaccines and
eg.. applied in the preparation of vaccine based on Frenke! cultures; Frankel
25 S: "Modifications de la mØthode de culture du virus aphteuse salon Frankel.
Valeur des vacciris scion Jes donnØes du laboratoire". Bull OlE 61: 985, 1964.

06/18/02 MAR 07:00 [# TX/RI 93941 tI003

Fax: 18 Jun 2002 13:56 PO4

we 01/46390 2 ’CTIZA00/00254

Formaldehyde is known for its cross-linking action (fixation) of proteins, which probably caused the high stability and Long shelf lives - of up to 5 years or longer - of FA-inactivated (Frankel) vaccines.
5

Several studies showed that at the FA concentration prescribed by Waldmann, inactivation plots were not linear and often showed ’tailing off"
which may cause incomplete inactivation. This was demonstrated in the following publications:
10 - Graves JH: "Formaldehyde inactivation of foot-and-mouth disease virus
as applied to vaccine preparation". Am J Vet Res 24, 1131;

C- Weslen, T. and Dinter, 1.: "The Inactivation of foot-and-mouth disease
virus by formalin". Arch. Ges. Vinisforsch. 1957,7.394;
- Barteling SJ, Woortmejer R, ’fisser N. "Innoculty testing of foot-and-
15 mouth disease vaccines. L Fon-naldehyde-inactivated aihydrogel
vaccines", J Blot. Stand. 1983, 11, 297..

Formaldehyde-inactivated vaccines induced good immunity and It was
shown that under well-defined conditions linear inactivation plots can be
20 obtained with FA (Barteling SJ, Woorimeijer R "Formaldehyde Inactivation of
foot-and-mouth disease virus. Conditions for the preparation of safe vaccine", Arch ViroL 1984, 80, 103). However, inactivation with FA remained suspicious and inactivation by aziridins (e.g. acetyl-ethylenirnine, AEI) which more rapidly inactivates, with linear inactivation plots, became the method of
25 choice..

- Inactivation with AM is described in the following publication:

06/18/02 MAR 07:00 j# TX/RX 93941 i]004

Fax: 18 JLIrI 2002 13:56 P.05

WO CL/46390 3 P1iiO1O(24

- Brown F, Hyslop NSG, Crick J, Morrow AW: "me use of acetyl-
ethyleneirnine in the production of Inactivated foot-and-mouth disease vaccines", J Hyg (Carnb) 1963b, 61: 337.

5 A widely used method for Inactivating FMD virus by an azirldln is described
by HG.Bahnemann: inactivation of viral antigens for vaccine preparation
with particular reference to the application of binary ethylenimine". Vaccine, 1999,, 299.

10 However, unlike PA, aziridins have no cross-linking reactivity and vaccines
prepared from some unstable FMD (vaccine) strains showed short shelf lives. Therefore, some labile vaccine strains are first fixed with PA before Inactivation is completed with an azisidin compound such as BEt. This method has been described in the following 3 publications:
15 - Rowlands et al. (1972) - Stabilizing the immunizing antigen of foot-and -
mouth disease virus by fixation with formaldehyde. Arch. Ges. Vlrusforsch. 39.274-283;
- Mowat et al. (1973) - Enhancement of immunizing potency of foot-and-
mouth disease vaccine for cattle by treatment of the antigen with
20 formaldehyde. Arch.ges.Virusforsch. 41, 365-370; and
- M M Rweyemarnu at at. (1989) - Effect of formaldehyde (FA) and binary
ethyleneirnine (BE1) on the integrity of foot-and-mouth disease virus
capsid. Rev. sci. tech. Off. tnt. EpIz. S. 747-767.

25 It is also known to inactivate the virus first with BEt and thereafter to cross-
link or fix the inactivated virus particles or antigens with FA e.g. FMD virus antigen of the SAT2 Zirn 7/83 strain.

0
0’

06/18/02 MAR 07:00 [# TX/RX 93941 Ij005

Fax: 18 Jun 2002 13:57 P.06

WO01146390 4 PCTM0/02S4 11

A disadvantage of the known methods is that it takes relatively long (-I- 2
days) to obtain satisfactory levels of inactivation, with the result that part of
the virus antigen may degrade. Vaccines manufactured from such
S inactivated virus antigens are therefore relatively less effective, In use.

Furthermore, BEI does not always give satisfying inactivation, in practice, the
extrapolated results sometimes indicate that there are still chances of the presence of surviving virus entities in a batch at the and of the inactivation
10 (at 48 hours) This means that such a batch is not acceptable and must be
destroyed.

OBJECTS OF THE INVENTION

15 Objects of the present invention are to provide methods of Inactivating
microorganisms and manufacturing vaccines, to provide vaccines manufactured by such methods and to provide kits incorporating such inactivated microorganisms, which are improvements of the known methods, vaccines and kits.
20

SUMMARY OF THE INVEN110N

According to a first aspect of the invention there is provided a method of inactivating a microorganism including the step of applying to said
25 microorganism a cross-linking agent simultaneously with a separate
inactivant.

06/18/02 MAR 07:00 [# TX/RX 93941 Q006

I-ax: 18 Jun 2002 13:57 P.O?

WO 01/46390 5 PCTtMIi0/054

The applicants have surprisingly found that by using the cross-linking agent simultaneously with the inactivant, a synergistic or augmenting effect is achieved whereby the microorganisms are inactivated, not only more thoroughly but, also, in a relatively much shorter period in comparison with
5 the prior art methods.

The Applicants have further found that vaccines manufactured according to
the invention are effective in use, probably due to the fact that the inactivatiOn period is relatively shorter and that the cross-linking agent fixes
10 the antigens of the microorganism, as they become available. The Applicants
believe that the antigens are thus preserved and not degraded and, therefore, vaccines manufactured according to the invention are expected to
be more stable with longer shelf lives than the known vaccines. Also, the Applicants further believe that vaccines will become less dependent on strict
15 cold-chain conditions during transport and use in the field.

20

25

The cross-linking agent may comprise an aldehyde.

The aldehyde may be a di-aldehyde.

Preferably the aldehyde is formaldehyde (FA).

The Applicants further believe that, at least in the case of FA, the cross linking agent prepares the virus for inactivation by the inactivant.

The inactivant may be an aziridine compound. The aziridine compound may be an ethyleneimine.

06/18/02 MAR 07:00 (# TX/RX 93941 IM 007

I-ax: 18 Jun 2002 13:58 P.08

WO 01/4390 TI1i/51254

Preferably the ethyleneimine is binary ethyleneimine (BEI).

The method may Include the further step of stopping the inactivation
5 process.

10

The inactivation process may be stopped by the addition of any one or n-iore
of sodium thiosuiphate, sodiumbisulfit, and trlshydroxymethyl-aminomethan (rris-buffer).

According to a second aspect of the invention there is provided.a vaccine including a microorganism inactivated by the above method according to the first aspect ofthe invention.

15 According to a third aspect of the invention there Is provided a method of
manufacturing a vaccine Including the steps of:
- providing a pathogen;
- inactivating the pathogen by applying to said pathogen a cross-linking
agent simultaneously with a separate inactivant
20 - the cross-linking agent may also inactivate the pathogen (by its cross-
linking activity).

The cross-linking agent may

aldehyde.

25 The aldehyde may be a dialdehyde.

Preferably the aldehyde Is formaldehyde (FA).

06/18/02 MAR 07:00 [# TX/RX 93941 I008

Fax: 18 Jun 2002 13:58 P.09

WO 01I46390 7 PAciOI435254 0

The inactivant may be an azlridlne compound. The aziridine compound may
be an ethyleneimirie.

5 Preferably the ethyteneimine is binary ethylenelmtne (BEI).

The method may Include the further step of stopping the inactivation

process.

10 The inactivation process may be stopped by the addition of any one or more
of sodium thiosuiphate. sodiumbisulfit, and trishydroxymethyl-aminornethan (Tris-buffer).

The.Applicants have further found that the above methods according to the
15 Invention are particularly effective in the inactivation of viruses and the
preparation of vaccines for viral pathogens.

The virus may be of the famdy picorna viridae..

20 Preferably the virus 15 a foot-and-mouth disease virus, but It Will be
appreciated that the methods according to the invention will be effective in respect of most viruses and even other microorganisms.

T

According to another aspect of the invention there is provided e test kit
25 including a microorganism inactivated by the above method of the invention.
The inactivated microorganism may be In the form of an allergen or antigen
for use in an EUSA.

08/18/02 MAR 07:00 (# TX/RX 93941 11009

Fax: 18 Jun 2002 13:58 P.10

WO /4390-PCT1ZAOO1GW54

A preferred embodiment of the invention will now be described by means of
a non-limiting example.

5 In order to investigate the effect of BEI in combination with FA on FMD virus,
two groups of viruses namely a test group and a control group were inactivated as set out below.

EXAMPLE

10 A method according to the invention for the inactivation of foot-and-mouth
disease virus (FMDV) with a combination of formaldehyde (FA) and binary ethyleneimine (BEI) Is set out below. A standard operating procedure (SOP)
for the inactivation of FMDV for the purposes of FMD vaccine production Is also explained in detail hereinafter. Reference is made only to the procedure
15 followed in the test group. However, substantially the same procedures were
followed in the case of the test and control groups, with the exception that In
the test group, the 8E1 and FA was applied simultaneously, while in the control group. BEI was used on its own as the inactivating agent.

20 Definitions and abbreviations used below
BEI: Binary ethyleneimine
BEA: 2-Bromoethylarnine hydrobromide
NaOH: Sodium hydroxide
STS: Sodium thiosuiphate
25 FA:Formaldehyde
ITT 1; First Inactivation Tank
ITT 2: Second Inactivation Tank

06/18/02 MAR 07:00 [P TX/RX 93941 1@010

Fax: 18 Jun 2002 13:59 P.11

WO 0114390 g PCT!ZAOOI00254 N11-

Chemicals:
Sodium hydroxide pellets (NaOH) pa. Merck
2-Bromoethyiamrnonium bromide (SrCH2CH2NH3Br) - Merck
5 11.-Naphtol Violet solution
formalin p.a. solution containing approximately 37 % formaldehyde - Merck

Materials, and equipment
Measuring cylinder 1000 ml
10 5 liter sterile reagent bottle
Sterile stirring magnet
Sterile distilled water
10 ml sterile McCarthy bottles.
Fume hood

15 pH meter (combined electrode) Orion
Weighing scale
Magnetic stirrer
Autoclave

20 Method

A filtered chloroform-treated SAT 2 - ZIm 7183 virus culture is brought to a temperature of 30°C and then inactivated with a combination of BE[

FA

(BEI-FA). This combination inactivates FMD very rapidly (>2 loglO ID50 per hour).
25

The culture was contained in a tank.. Due to the fact that the BE[-FA may not enter some parts of the tank, such as dip-pipes, the virus might not be

06/18/02 MAR 07:00 [# TX/RX 93941 10011

Fax: 18 Jun 2002 13:59 P.12

WO 01/46390 0 PCT8100254

Inactivated completely and might, at the and of the process contaminate the inactivated virus antigen. Therefore, after approximately 4 hours the whole inactivation mixture is transferred into a second inactivationAsnk (with BEt
only after 24 hours)-
5

Samples are taken every 20 minutes to monitor the inactivation and verify
the correct shape of the regression line. In the present method, in batches of 150 liter, the extrapolated Inactivation plot must reach below - 6.3 loglO lD
at the end of the inactivation (at 24 h). The inactivation Is stopped by the
10 addition of 210/16 STS, which neutralizes BEt.

* Both, BEt and formaldehyde are toxic substances and should be handled with care. The BEt is prepared in a fume hood. After cyclisation (see below),
the BEt solution should be handled with extreme care because it is highly
15 toxic and probably carcinogenic. BEt remains must be neutralized with a
20% STS solution before it is discarded.

Note: The use of Tris or other quartemary ammonium salts e.g. for correction
of the pH, should be omitted because they react with FA. Tris can also be
20 used to stop further cross-hnkinglinactivation by FA.

Preparation of 8E1 and of BE[-FA (for 3001 virus harvest)
The preparation of BEL-FA solution must take place in a fume hood (at room temperature) as follows:
25 - Dispose 2970 ml ofsterile distilled water in a sterile 5-liter reagent bottle.
- Add 21g of NaOH to the 3 liter distilled water.

06/18/02 MAR 07:00 [# TX/RI 93941 I]012

Fax: 18 Jun 2002 14:00 P.13

WO 01146391 11

- Add a sterile magnet and stir until all the NaOH has been dissolved. (To
save time the NaOH can be prepared the day before the BE! is needed)
- Take the pH of the NaOH solution and write the measured pH value on
the bottle and on the record sheet.
5 - Add 61.5 g BE! to the 3 liter of NaOH solution.
- Add 1.5 ml of a 1% f-Naphtolviolet solution to the BE1.
- Allow cycllsation of BE! to take place for 1 hour 2t 370C on a magnetic

stirrer.

-. Measure the pH after cyclisatlon. (pH range from 10 to 11)
10 - Check the colour of the solution.
- After cyclisation the color of the F-Naphtol violet has changed from purple
into orange. -
For Inactivation with the BEI-FA combination, add 30 ml of formahn solution.
15

inactivation procedure with BE! only
- Add 10 ml of 0-IM BE) per liter virus harvest to reach a final concentration of 1 mM.

- Take during the first 6 hours of inactivation a sample (30 ml, see below)
20 every hour.
After 24 hours of inactivation, when the virus titer is expected to be below zero (less than 1 virus particle per ml), add the same quantity of DEl and transfer the inactivation mixture to the second inactivation tank (ITT2).
- Take a sample of 100 mi.
25 - Check the pH of the sample.
- At 43 hr add 10 % of a 20 % STS solution and stir (final STS
concentration: 2

06/18/02 MAR 07:00 [# TX/RI 93941 IJ013

Fax: 18 Jun 2002 14:00 P.14

WO 0114639 12 pcTJzAow06254

Take a. sample of 600 ml for checking the pH and for the’in vitro’ safety,
146 S. and sterility tests (see respective SOPS).
- Cool ITT 2 by flushing cold water through its jacket
- Now, the inactivated antigen can be transferred to the "Quarantine Zone"
5 for further processing.

Inactivation procedure with the BEI-FA combination
Add 10 ml of 0.IM BE[-FA per liter virus harvest to reach a Final concentration of 1 mM BE[and 0.04 % of FA (approx. I mM).
10 - Take during the first 3 hours a sample every 20 minutes.
- After 4 hours of inactivation, when the virus titer is expected to be below
zero (less than I virus particle per ml), transfer the Inactivation mixture to
the second inactivation tank (ITT2).
-. Take a sample of 100 ml. -
15 - After 20 hours of inactivation add 10 % of a 20 % STS solution and stir
(final ’TS concentration: 2
- Take a sample of 600 ml for checking the pt-I and for the tin vitro’ safety.
146S. and sterility tests (see respective SOPs).
Cool ITT 2 by flushing cold water through Its jacket.
20 Now, the inactivated antigen can be transferred to the "Buffer Zone" for
further processing.

Monitoring of the inactivation process
General

25 Both inactivation tanks are equipped with sampling devices that are steam-
sterilized in between the sampling.

06/18/02 MAR 07:00 [# TX/RX 93941 lJ014

Fax: 18 Jun 2002.14:01 P.15

WO O1/463 13

Sampling and titration
- Take samples of at least 30 ml (see below).
Split each sample immediately into 3 volumes of 8 ml and add them to sample bottles that contain I ml 20% STS and I ml fetal bovine serum
5 (FBS) free of anti-FMD virus activity, giving final concentrations of 10 %
of FBS and 2 % of STS. Store one sample at 40 C and 2 samples, for backing up, at- 70C.
Take a sample of 100 ml at 4 hour, before the inactivation mixture is transferred to the second Inactivation tank and measure the pH.
10 Take a final sample of 600 ml at the end of the inactivation.. -
- The reduction in virus titre is monitored by titration in microtitre plates with
MRS cells. The titers are expressed as 10 Jog units per ml (e.g. I million infectious doses per ml makes a titer of 6) and are plotted graphically.

15 Down stream processing
Inactivated virus antigen was concentrated by routine ultra-filtration to approximately 2 liter. Samples of the concentrated antigen were taken and send for routine checks and controls (sterility, 146 S antigen yields,, and ELISA), Other samples and the bulk of the concentrated antigen were stored

20 at -706C.

Experimental vaccine

An experimental AI(OH)3.-saponin vaccine was prepared from a mixture of all
2 ’5 FABEI-inactivated vaccine strains. The vaccines contained per 3 ml dose
0..8 pg 146 S antigen of each vaccine strain with the exception of SAT3 KNP90I3 of which 1.6 jig was added. 5 cattle were vaccinated with % of a

06/18/02 MAR 07:00 [# TX/RX 93941 Q015

Fax: 18 Jun 2002 14:01 P.16

WC) 01(46390 14

dose (0.75 ml). Blood-serum samples taken after the vaccination Were tasted
in a virus-neutralisatlon assay.

Results obtained with the Invention

5 A representative inactivation plot obtained with BE1 alone is given In fig Ia.
In our experience, with BEI alone, inactivation rates vary between 0.4 and
1.0 log per hour (see also Bahnemana, 1990) and sometimes, at low inactivation rates, the primary plots suggest some"tailing off’.

10 RoutInely, Inactivation is carried out for 48 hours. At 24 hours a second
portion of BEL Is added. Thus, even at the lowest Inactivation rates, sufficient safety can be expected, and final safety tests are passed.

In the enclosed figures Ia to f, the inactivation plots obtained with BEt alone
1 (a) for one of the SAT vaccine strains and with BEL-FA (b -t) for 5 vaccine
strains, are shown.

20

The Inactivation rates were varying from 2.0 (SATI-SAR9/81, fig 1 b) to more than 3 logs per hour (SAT2-ZIM 7183 and SAT2 KNP-19/89/2, fig id and le espectively, table I) ThUs, inactivation Is from more than 20 to over a 100-
fold faster if FA Is added.

06/18/02 MAR 07:00 [# TX/RI 93941 tJ016

Fax: 18 Jun 2002 14:02 P.17
10
10

5

WO 03/46390 15 PCT/1A0610854

Table 1: Inactivation rates (with BEI-FA) of five SAT vaccine strains,
Strain inactivation rate
SAT 1SAR 9/8T 2.0 log/hr
SAT 1- KNP 91/1 2.5 log/hr
SAT 2 ZIM 1183 3.1 log/hr
SAT 2 KNP 8912

331og/hr
SAT 3 KNP 10190-3 2.2 tog/hr

10 it is difficult to say what causes this synergistic effect. Under optimal
conditions, FA alone inactivates at a rate of approx. 0.3 log/hr only (Barteling, 1984). Thus hardly any addition is to be expected to the much faster inactivation of BEL

15 From these graphs it is clear that by the FA-BEI combination linear plots
were obtained and virus titres were found to be reduced by more than 2 logs
per hour. Thus already within 8 hours sufficient inactivation was reached for acceptable safety. In accordance, no surviving virus could be detected in a
large (200-rn!) sample taken, after 6 hours, as was the case for the final
20 samples (at 20 hr).

It should be noted that in the micro-titre system undiluted and 10-fold diluted
samples were causing a cyto-pathogenic effect but this was due to the ttoxicity of the formaldehyde (or the A-BEi combination) and no virus could
25 :be propagated from these cups.

I- ax: 18 Jun 2002 14:02 P.18

5

F,

15

WO 0/46390 16 PCTIZA00MG25A

Thus inactivation can be carried out within the time span of a working day or
Just overnight instead of the 48 hours required for the inactivation with Bl
alone. This gives greater flexibility in weekly production schedules.

With BEt atone, antigen yields (of SAT-strains) were often reduced from 10
to 30. percent during the 48 hours of inactivation. No reduction in 146 S antigen concentration was observed after the 24 hr of inactivation with FA-8EL Where conditions were otherwise identical, a reduction with approximately half that observed with BEt could be expected. The optimal yields. are probably due to fixation of the antigen by the cross-linking action
of FA (Barteling, 1984).

The results obtained after the vaccination of 5 cattle with the vaccine prepared from the S BEI-FA-inactivated antigens are presented in table 2.

20

25

06/18/02 MAR 07:00 (# TX/RI 93941 EI018

Fax: 18 Jun 2002 14:03 P.19

RE

5

10

15

WO 01.146.390 17 PCTIIAOOIU0254

Table 2 Mean virus neutralisation titres at 2. weeks post vaccinationand challenge results. The animals were vaccinated with Y4 of a dose containing
02pg of each of the SAT I and SAT 2 strains and 0.4pg of SAT 3.
Strain Mean-titre

AT I KNP 196/91-1 1.8

SAT I SAR 9181-1

2.4

SAT 2 KNP 191842 2.0

Sat 2 Zim 7183-2 2.3

SAT 3 KNP 10/90-3 2.3

Challenge (at 3 weeks post vaccination) was carried out with SAT I KNP 196/91-3. Out of 5 animals 2 were protected and I was partly protected (1 lesion).

20. Considering the tow quantities of antigen (and adjuvants) that were incorporated in the vaccine, the vaccine performed quite wall. The vaccines contained per 3 i-nI dose 0.8 pg per FMD vaccine strain and 14 of a dose (0.75 ml) was injected..

25 At 2 weeks post vaccination, the lowest neutralising antibody response was against SAT I KNP 9111 (with a mean titre of 1.8 log) and, therefore, this

06/18/02 MAR 07:00 (# TX/RX 93941 E019

Fax: 18 Jun 2002 14:03 P.20

/0
WO Il4390 15 pcTIzAeofo24

strain was selected as challenge strain (table 2). Two animals were protected
and one was parity protected, indicating a protection level of approximately
50 %0 PD 50). Because the injected vaccine dose contained approximately
0.4 p per FMD virus type (e.g. of SATI), these results indicate that (for the
5 weakest antigen) per pg a protection level of approximately 2.5 PD 50 can
be expected which is, in comparison with the industry standard, surprisingly good.

Stability of the 146 S antigen Is an important parameter in the. selection of
10 new vaccine strain. By the cross-linking action of FA, which stabilises the
146 S antigen, this parameter becomes less critical and, thereforö, the BEI-
 ’ FA inactivation method will make the rapid introduction of new vaccine Strains relatively easier.

15 Because of the fixation of the antigen by the cross-linking action of FA the
- applicants expect the vaccines to be of superior stability. This is particularly
important for developing countries (e.g. in Africa), where maintenance of cold chain conditions Is not always possible.

20 1t will be appreciated that the method of inactivation according to the present
invention presents strongly augmented inactivation rates, where sufficient safety levels are reached within approximately 8 hours or teas, in comparison with the +1- 2 days with the known methods. Therefore, inactivation for. 20 :h0UJre with the method of the invention does not leave any possibility for a
25 dispute on the safety of a batch.

06/18/02 MAR 07:00 [# TX/RX 93941 11020

Fax: 18 Jun 2002 14:04 P.21

WO 1149O 19 PCTIZ,..400100254

5

The new inactivation method according to the invention shortens the process
of vaccine manufacture with at least one day. This makes a vaccine pioduction. system far more flexible and enables the process (including a chloroform treatment) to fit into a 4 days working week.

The Applicants further believe that during the method according to the invention, the FA fixes the virus antigens during the inactivation and,.. therefore, Will result in relatively more effective vaccines with a relatively long shelf live.
10

Where the inactivation with BEI alone gives, under favoiable conditions, inactivation rates of 0.5 - 1.0 logs per hour (Rweyemamu et al.), the 5 vaccine strains studiCdso farshowed that method according to the Invention presents inactivation rates from 2.0 to 3.3 logs (fig. 2b - 2f, table 1).
15 Therefore, the method of the Invention can give a guarantee for sufficient.
inactivation within 8 hours. This also means that the inactivation can be.

carried out within the time frame of a working day. This Is of great advantage
In a vaccine production process (virus production, harvest clarification. inactivation, and concentration of the antigen) which, according to the prior
20 art until now only just fits into a full working week.

The applicants foresee that a test kit inciudtng a microorganism inactivated according to the above method of the invention can be prepared. In the test
kit, the inactivated microorganism can be in the form of an allergen or
25 antigen for use in an EIJSA.

06/18/02 MAR 07:00 [# TX/RX 93941 t1021

Fax: 18 Jun 2002 14:04 P.23

WO 011390

CLAIMS

1.

5

b.2.

10 3.

4.

15 5.

j) 6.

20
7.

8.
25

21 . PCTJZAO4JfO4r-54

A method of inactivating a microorganism including the step of applying to said microorganism a cross-linking agent simultaneously
with a separate inactivant.

A method according to claim I wherein the cross-linking agent comprises an aldehyde.

A method according to claim 2 wherein the aldehyde is a di-aldehyde.

A method according to claim 3 wherein the aldehyde is formaldehyde (FA).

A method according to any one of the preceding claims wherein the Inactivant is an a2kidine compound.

A method according to claim 5 wherein the aziridine compound is an ethyleneimine.

A method according to claim 6 wherein the ethylenelmirte is binary ethyleneimine (BEI).

A method according to any one of the preceding claims which includes the further step of stopping the inactivation process.

06/18/02 MAR 07:00 (# TX/RI 93941 1j023

Fax: 18 Jun 2002 14:05 P.24

WO 01/460 22

. A method according to claim 8 wherein the inactivation process is:
stopped by the addition of any one or more of sodium thIosuiphate, sodiurnbisulfit, and tilshydroxymethyl..aminornethan ’(Tha-buffer).

5 1:0. A method of inactivating a microorganism substantially as herein
described and exemplified.

11. A vaccine including a microorganism inactivated by a method.
according to any one of the preceding claims.
10

12. A method of manufacturing a vaccine Including the steps of providing
a pathogen; and inactivating the pathogen by applying to said pathogen a cross-linking agent simultaneously with a separate mnactivarit.
15

13. A method according to claim 12 wherein the cross-linking agent
comprises an aldehyde.

14. A method according to claim 13 wherein the aldehyde is a dl-
20 aldehyde.

A method according to claim 14 wherein the aldehyde Is formaldehyde (FA).

A method according to any one of claims 12 to.15 wherein the lnacthrant is an aziridine compound.

06/18/02 MAR 07:00 1# TX/RX 93941 E1024

Fax: 18 Jun 2002 14:05 P.25

(0 WO 01/4639023 PCT/ZA(MWLJ0254 S &

17. A method according to claim 16 wherein the aziridiris compound. is an
ethyleneinilne.

1$.. A method according to claim 17 wherein the ethyleneimine Is binary
5 . ethyteneimine (BEL).

19. A method according to any one of claims 12 to 18 which includes the
further step of stopping the inactivation prOcess.

10 20. A method according to claim 19 wherein the inactivation process is
stopped by the addition of any one or more of sodium thiosuiphate, sodiumbisulfit, and tiishydroxymethyl-aminomethan (Tris-buffer).

21 A methodof manufacturing a vaccine substantially as herein described
15 and exemplified.

22. A vaccine manufactured by a method according to any. one of claims
12 to 21.

20 23. Use of a. method according to any one of claims 12 to: 21 in the
inactivation of viruses and the preparation of vaccines for viral pathogens.

24 Use according to claim 23 wherein the virus Is of the family picome
25 viridae.

06/18/02 MAR 07:00 [# TX/RX 93941 I1025

TITULO: METODO DE INACTIVAR MICROORGANISMOS INTRODUCCION Y ANTECEDENTES DEL INVENTO

Este invento se relaciona con mØtodos de inactivar microorganismos y fabricar vacunas; con vacunas fabricadas mediante dichos mØtodos, y con conjuntos que incorporan tales microorganismos inactivados. MÆs particularmente pero no exclusivamente, este invento se relaciona con mØtodos de inactivar virus y fabricar vacunas de virus y con vacunas fabricadas con esos mØtodos.

La inactivación de los virus y las pruebas de seguridad constituyen los pasos mÆs crIticos en la producciOn de vacunas inactivadas. En el caso de vacunas para enfermedades de fiebre aftosa (FMD) en particular, la seguridad garantizada es esencial porque cualquier suceso de la enfermedad causaria un bloqueo de todo el comercio de exportación de animales y de productos animales.

Un mØtodo clÆsico para inactivar elvirus FMD y preparar la vacuna FMD es con formaldehIdo tal como fue descrito por Waldmann y otros: Waldmann 0. Pyl G. Hobohm KO, Mohlmann H: "Die Entwicklung des Riemser Adsorbatimpfstoffes gegen Maul- und Klauenseuche und seine Herstellung". Zbl Bakt I Orig 148: 1, 1941.

En el pasado este mØtodo se utilizó en la preparaciOn de vacunas FMD y por ejemplo se aplicO en la preparación de vacunas basadas en cultivos Frenkel; Frenkel S: "Modifications de la mØthode de culture du virus aphteuse selon Frenkel. Valeur des vaccins selon les donnØes du laboratoire". Bull OlE 61: 985, 1964.

El formaldehIdo se conoce por su acciOn de uniOn intermolecular (fijaciOn) de las proteinas, Ia que probablemente causO la alta estabilidad y larga vida de conservaciOn - hasta de 5 aæos amÆs- de las vacunas inactivadas FA (Frenkel). Varios estudios demostraron que a la concentraciOn FA prescrita por Waldmann, los trazados de la inactivaciOn no eran lineales y con frecuencia mostraban apartarse de las seæales ("tailing off’), Ia que puede causar inactivación incompleta. Esto se demostrO en las siguientes publicaciones:

Graves JH: "Formaldehyde inactivation of foot-and-mouth disease virus as applied to vaccine preparation", Am J Vet Res 24, 1131;
Weslen, T. And Dinter, Z.: "The inactivation of foot-and-mouth disease virus by formalin". Arch. Ges. Virusforsch. 1957, 7, 394;

Barteling SJ, Woortmeijer R, Visser N: "Innocuity testing of foot-and-mouth disease vaccines. I. Formaldehyde-inactivated alhydrogel vaccines", J Biol. Stand. 1983, 11,297.

F’

Las vacunas inactivadas con formaldehido inducian buena inmunidad y se demostrO que bajo condiciones bien definidas se podlan obtener trazados de inactivaciOn lineal con FA (Barteling SJ, Woortmeijer R: "Formaldehyde inactivation of foot-and mouth disease virus. Conditions for the preparation of safe vaccine", Arch Virol. 1984, 80, 103). Sin embargo, la inactivaciOn con FA continuó siendo sospechosa y la inactivaciOn por medio de las aziridinas (como por ejemplo acetil-etilenimina, AEI) que son las que mÆs rÆpidamente inactivan, con trazados de inactivación lineal, se convirtiô en el mØtodo seleccionado.

La inactivaciôn con AEI se describe en la siguiente publicacion:
Brown F, Hyslop NSG, Crick J, Morrow AW: "The use of acetylethyleneimine in the production of inactivated foot-and-mouth disease vaccines", J Hyg (Camb) 1963b,61:337.

Un mØtodo ampliamente utilizado para inactivar el virus FMD mediante una aziridina fue descrito por H.G. Bahnemann: "Inactivation of viral antigens for vaccine preparation with particular reference to the application of binary ethylenimine". Vaccine, 1999, 8 299.

Sin embargo, a diferencia del FA, las aziridinas no tienen reactividad de uniOn intermolecular y las vacunas preparadas a partir de algunas cepas inestables de FMD (vacuna) mostraron un corto tiempo de conservaciOn. Por consiguiente, algunas cepas de vacunas lÆbiles primero se fijan con FA antes de que se complete la inactivaciOn con un compuesto de aziridina tal como el BEI. Este mØtodo ha sido descrito en las siguientes tres publicaciones:

Rowlands et al. (1972) - Stabilizing the immnunizing antigen of foot-and-mouth disease virus by fixation with formaldehyde. (EstabilizaciOn del antIgeno inmunizante del virus de fiebre aftosa mediante fijaciOn con formaldehido). Arch. Ges. Virusforsch. 39, 274-283;
Mowat et al. (1973) - Enhancement of immunizing potency of foot-and-mouth disease vaccine for cattle by treatment of the antigen with formaldehyde. (Mejora de la potencia inmunizante de la vacuna de la fiebre aftosa para el ganando mediante el tratamiento del antIgeno con formaldehido). Arch. Ges. Virusforsch. 41, 365-370; y
M M Rweyemamu et al. (1989) - Effect of formaldehyde (FA) and binary ethyleneimine (BEI) on the integrity of foot-and-mouth disease virus capsid. (Efecto del formaldehido (FA) y de la etilenimina binaria (BEI) sobre la integridad de cÆpsido del virus de fiebre aftosa). Rev. sd. tech. Off. mt. Epiz. 8, 747-767.

TambiØn se sabe que inactiva el virus primero con BEI y posteriormente une intermolecularmente o fija las partIculas o antIgenos de virus inactivado con el FA, como por ejemplo el antigeno del virus FMD de la cepa SAT2 Zim 7/83.

Una desventaja de los mØtodos conocidos es que toma relativamente largo tiempo (+1- 2 dias) obtener niveles satisfactorios de inactivaciOn, con el resultado de que parte del antIgeno del virus puede degradarse. Las vacunas fabricadas a partir de tales antIgenos de virus inactivado son por consiguiente relativamente menos eficaces en su uso.

AdemÆs, Ia BEI no siempre da una inactivación satisfactoria. En la prÆctica, los resultados extrapolados algunas veces indican que existe todavia la probabilidad de presencia de entidades de virus sobrevivientes en un tote at final de la inactivación. (A las 48 horas). Esto significa que ese lote no es aceptable y debe ser destruido.

OBJETIVOS DEL INVENTO

El objeto del presente invento es el de suministrar mØtodos para inactivar microorganismos y para fabricar vacunas, asi como proporcionar vacunas fabricadas con esos mØtodos at igual que conjuntos que incorporen dichos microorganismos inactivados, que constituyen mejoras a los mØtodos, vacunas y conjuntos conocidos.

RESUMEN DEL INVENTO

De acuerdo con un primer aspecto del invento se proporciona un mØtodo para inactivar un microorganismo, inctuido el paso de aplicar a dicho microorganismo un agente de union intermolecular simuttÆneamente con un inactivante separado.

Los solicitantes han encontrado sorpresivamente que con Ia utilizaciOn del agente para uniOn intermolecular simultÆneamente con el inactivante, se logra un efecto sinergIstico o de aumento por medio del cual los microorganismos se inactivan, no solamente en forma mÆs completa, sino tambiØnen un perlodo relativamente mÆs corto en comparacion con los mØtodos de la tØcnica anterior.

Los Solicitantes han encontrado ademØs que las vacunas fabricadas de acuerdo con el invento son eficaces en su uso, probabtemente debido al hecho de que el periodo de inactivaciOn es relativamente mÆscorto y at hecho de que el agente de la uniOn intermolecular fija los antfgenos del microorganismo a medida que se dispone de ellos. Los Solicitantes consideran que los antIgenos se preservan en esta forma y no se degradan y, por lo tanto, se espera que las vacunas fabricadas de acuerdo con el invento sean mÆs estables y tengan vidas de conservaciOn mayores que aquellas de las vacunas conocidas. Igualmente, los Solicitantes consideran ademÆs que las vacunas serÆnmenos dependientes de las estrictas condiciones de cadena fria durante el transporte y utilizaciOn en el campo.

El agente de uniOn intermolecular podria comprender un aldehido.
El atdehido podrIa ser un di-atdehido.
Preferiblemente el atdehido es formaldehIdo (FA).

/1?

La preparación de la solución BEI-FA debe tener lugar en una campana de humos (a temperatura ambiente) de la siguiente manera:
Disponga de 2970 ml de agua destilada estØril en una botella estØril de 5 litros
de reactor.

Aæada 21 g de NaOH a los 3 litros de agua destilada.
Aæadaun imÆnestØril y agite hasta que todo el NaOH se haya disuelto. (Para ahorrar tiempo el NaOH puede prepararse el dIa anterior al que se necesite la BEI).
Tome el pH de la solución de NaOH y escriba el valor del pH medido en la botella y en la hoja de registro.
Aæada61.5 g de BEI a la soluciOn de NaOH de 3 litros.
Aæadaa la BEI 1.5 ml de una soluciOn de p--Naftol violeta al 1%,
Permita que la ciclaciOn de la BEI tenga lugar por I horas a 37°C en un agitador magnØtico.
Mida el pH despuØs de la ciclaciOn (el rango del pH debe estar entre 10 y 11) Verifique el color de la solución.
DespuØs de la ciclaciOn el color del p--Naftol violeta ha cambiado de morado a anaranjado.
Para inactivación con la combinaciOn de BEI-FA, aæada 30 ml de soluciôn de formalin.

Procedimiento de inactivación con BEI solamente
Aæada 10 ml de 0.1 M BEI por litro de cosecha de virus para lograr una concentración final de 1 mM.

Tome una muestra durante las primeras 6 horas de inactivaciôn (30 ml, ver a continuación) cada hora.
DespuØs de 24 horas de inactivación, cuando se espera que el titulo del virus estØ por debajo de cero (menos de I particula de virus por ml), aæadala misma cantidad de BEI y transfiera Ia mezcla de inactivaciOn al segundo tanque de inactivación (ITT2).
Tome una muestra de 100 ml.
Verifique el pH de la muestra.
A las 48 horas aæada el 10% de una soluciOn de STS al 20% y agite (la concentraciOn final del STS debe ser 2%).
Tome una muestra de 600 ml para verificar el pH y para la seguridad "in vitro", 146 S, y pruebas de esterilidad (vØanse los respectivos SOPs).
EnfrIe el ITT 2 haciendo fluir agua frIa a travØs de la camisa del tanque.
Ahora, el antIgeno inactivàdo puede transferirse a la "Zona de Cuarentena" para el procesamiento posterior.

Procedimiento de inactivación con la combinación de BEI-FA.
Aæada 10 ml de 0.IM de BEI-FA por litro de cosecha de virus para lograr una concentraciôn final de 1 mM BEI y un 0.04% de FA (aprox. 1 mM).

Tome durante las primeras 3 horas una muestra cada 20 minutos.
DespuØs de 4 horas de inactivaciOn, cuando se espera que el tItulo del virus estØ por debajo de cero (menos de una partIcula de virus por ml), transfiera la mezcla de la inactivaciOn a un segundo tanque de inactivación (ITT2).
Tome una muestra de 100 ml.
DespuØs de 20 horas de inactivación aæada 10% de una soluciOn de STS al 20% y agite (concentración final de STS: 2%).
Tome una muestra de 600 ml para verificar el pH y para la seguridad "in vitro", 146S, y las pruebas de esterilidad (vØanse los respectivos SOPs).
EnfrIe el ITT2 haciendo fluir agua fria a travØs de la camisa del tanque.
Ahora, el antigeno inactivado se puede transferir a la "Zona de Tampon" para procesamiento posterior.

Monitoreo del proceso de inactivaciOn
Generalidades

Ambos tanques de inactivaciOn estÆn dotados con implementos de muestreo esterilizados al vapor entre un muestreo y otro.

Muestreo y TitulaciOn
Tomar muestras de por lo menos 30 ml (vØase mÆs abajo).
Divida cada muestra inmediatamente en 3 volümenes de 8 ml y aæÆdalosa botellas de muestra que contengan I ml de STS al 20% y I ml de suero fetal bovino (FBS) libre de actividad viral anti-FMD, obteniendo concentraciones finales de 10% de FBS y de 2% de STS. Almacene una muestra a 4°C y 2 muestras para respaldo a -70°C.
Tome una muestra de 100 ml a las 4 horas, antes de que la mezcla de. inactivaciOn se transfiera al segundo tanque de inactivación y mida el pH.
Tome una muestra final de 600 ml a la finalizaciOn de la inactivaciOn.
La reducciOn en la titulaciOn del virus se monitorea mediante titulaciôn en las placas de microtItulo con cØlulas IBRS. Los tItulos se expresan como 10 unidades logarItmicas por ml (por ejemplo I millOn de dosis infecciosas por ml hacen un titulo de 6) y se trazan graficamente.

Procesamiento corriente abajo
El antigeno de virus inactivado se concentró mediante ultra-filtraciOn de rutina a aproximadamente 2 litros. Las muestras del antigeno concentrado se tomaron y enviaron para chequeos y controles rutinarios (esterilidad, rendimientos de antigeno 146 S y ELISA). Otras muestras junto con el grueso del antigeno concentrado se almacenaron a -70°C.

Vacuna experimental
Se preparo una vacuna experimental de Al(OH)3-saponina a partir de una mezcla de todas las cepas de vacunas inactivadas 5 FA-BEI. Las vacunas contenlan por dosis de 3 ml 0.8 jzg de antigeno 146 S de cada cepa de vacuna con la excepciOn

del SAT3 KNP90/3 del cual se aæadieron 1.6 /zg. Se vacunaron 5 cabezas de ganado con 1/4 de una dosis (0.75 ml). Se probaron las muestras de suero sangumneo tomadas despuØs de la vacunaciOn en un ensayo de neutralizaciOn del virus.

Resultados obtenidos con el invento
Un trazo de inactivación representativa obtenido con la BE! sola se ilustra en Ia Figura Ia. En nuestra experiencia, con la BEI sola, las tasa de inactivación varlan entre 0.4 y 1.0 log. por hora (ver tambiØn a Bahnemann, 1990) y algunas veces, a bajas tasas de inactivaciôn, los trazos primarios sugirieron apartarse de las senates ("tailing off’).

Rutinariamente, la inactivaciOn se Ileva a cabo por 48 horas. A las 24 horas se aæade unasegunda porcion de BEI. Por consiguiente, inclusive a las tasas mÆs bajas de inactivaciOn, se puede esperar una seguridad suficiente y so aprueban las pruebas finales de seguridad.

En las figuras adjuntas I a a f se muestran los trazos de inactivación obtenidos con la BEI sola (a) para una de las cepas de vacuna SAT y con el BEI-FA (b - f) para 5 cepas de vacunas.

Las tasas de inactivaciOn estuvieron variando entre 2.0 (SATI-SAR9/81, fig Ib) a mÆs de3 logaritmos por hora (SAT2-ZIM 7/83 y SAT2 KNP-19/89/2, figuras Id y le respectivamente, tabla 1). Por consiguiente, la inactivación es mÆs de 20 por encima de 100 veces mÆsrÆpidasi se adiciona FA.

Tabla 1: Tasas de inactivación (con BEI-FA) de cinco cepas de vacunas SAT. Cepas Tasa de inactivaciOn
SAT 1-SAR 9/81 2.0 log/hr
SAT 1-KNP 91/1 2.5 log/hr
SAT 2-ZIM 7/83 3.1 log/hr
SAT 2-KNP 89/2 3.3 log/hr
SAT 3-KNP 10/90-3 2.2 log/hr

Es difIcil decir quØ causa este efecto sinergistico. Bajo condiciones Optimas el FA solo inactiva a una tasa de aproximadamente 0.3 log/hora solamente (Barteling, 1984). Por consiguiente, casi no debe esperarse ninguna adición a la inactivaciOn mÆs rÆpidade la BEI.

A partir de estos graticos queda claro que con la combinaciOn FA-BEI se obtuvieron trazos lineates y se encontraron titulaciones de virus reducidas en mÆs de 2 logaritmos por hora. As!, ya dentrode las 8 horas se habla logrado una inactivaciOn suficiente para una seguridad aceptable. Por consiguiente, no se

pudo detectar ningün virus sobreviviente en una gran muestra (200-ml) tomada despuØs de 6 horas, tal como fue el caso para las muestras finales (a 20 hr.).

Debe anotarse que en el sistema de micro-titulación las muestras del sistema no diluido y las muestras diluidas 10 veces estaban causando un efecto citopatogØnico, pero esto se debiO a la toxicidad del formaldehIdo (0 a la combinaciOn de FA-BEI) y ningün virus podia ser propagado de estas tazas.

De esta manera la inactivación puede Ilevarse a cabo dentro del lapso de tiempo de un dia hÆbilojusto de un dia para otro en vez de las 48 horas requeridas para la inactivaciôn con la BEI sola. Esto brinda una mayor flexibilidad en los programas de producciOn semanales.

Con la BEI sola, los rendimientos del antIgeno (cepas de SAT) con frecuencia se redujeron del 10 al 30% durante las 48 horas de inactivación. No se observO ninguna reducciOn en la concentración del antIgeno 146 S despuØs de las 24 horas de inactivaciôn con FA-BEI. Cuando las condiciones fueron por 10 demÆs idØnticas, podria esperarse una reducciOn con aproximadamente la mitad de 10 que se observO con el BEI. Los rendimientos Optimos probablemente se deben a la fijaciOn del antIgeno mediante la acciôn de la union intermolecular del FA (Barteling, 1984).

Los resultados obtenidos despuØs de la vacunaciOn de 5 cabezas de ganado con la vacuna preparada a partir de los antigenos inactivados 5 BEI-FA se presentan en la tabla 2.

Tabla 2: TitulaciOn de la neutralizaciOn del virus medio a las 2 semanas posteriores a la vacunaciOn y resultados del reto. Los animales se vacunaron con …de una dosis que contenia 0.2 jig de cada una de las cepas SAT I y SAT 2 y
fl t in HPRATA

Cepa TitulaciOn media
SAT KNPI96/91-1 1.8
SAT I SAR 9/81-1 2.4
SAT 2 KNP 19/842 2.0
Sat 2 Zim 7/83-2 2.3
SAT 3 KNP 10/90-3 2.3

El reto (a las 3 sernanas siguientes a la vacunaciOn) se llevó a cabo con el SAT I KNP 196/91-1. 5 de cada 2 animales estaban protegidos y I estaba parcialmente protegido (1 lesiOn).

Considerando las bajas cantidades de antigeno (y de adyuvantes) que se incorporaron a la vacuna, Østa se desempeæO bastante bien. Las vacunas

contenian por cada dosis de 3 ml 0.8 ILg por cepa do vacuna FMD y se inyectó 14 de una dosis (0.75 ml).

A las 2 semanas siguientes a la vacunación la respuesta mÆsbaja del anticuerpo neutralizador fue contra 01 SAT I KNP 91/1 (con una titulación media de 1.8 logaritmos) y, por consiguiente, esta cepa se seleccionó como la cepa reto (tabla 2). Dos animales estaban protegidos y uno estaba parcialmente protegido, indicando un nivel de protección de aproximadamente 50% (1 PD 50). Como la dosis de la vacuna inyectada contenia aproximadamente 0.4 jig por tipo de virus FMD (v.g. ver SAIl), estos resultados indican que (para el antIgeno mÆs dØbil) por gg se puede esperar que un nivel de protección do aproximadamente 2.5 PD 50 la cual, en comparación con la norma de la industria, es sorprendentemente buena.

La estabilidad del antIgeno 146 S constituye un parÆmetro importante en la selecciOn de la cepa de la nueva vacuna. Por la acción do uniOn intermolecular del FA, la cual estabiliza el antIgeno 146 S, este parÆmetrose toma menos critico, y por consiguiente, el mØtodo do inactivaciOn de BEI-FA harÆ relativamente mÆs fÆcilintroducir rapidamente las nuevas cepas do vacunas.

Debido a la fijaciOn del antigeno mediante la acción de union intermolecular del FA, los solicitantes esperan quo las vacunas tengan una estabilidad superior. Esto es particularmente importante para los palses en via de desarrollo (coma par ejemplo en Africa), donde el mantenimiento de las condiciones do cadena fria aün no es posible.

Se apreciarÆ quo elmØtodo de inactivaciOn conforme al prosente invento presenta unas tasas do inactivaciOn fuertemente aumentadas, en donde los niveles do seguridad suficiente se logran dontro de aproximadamente las 8 horas siguientes o menos, en comparacion con los +1- 2 dias con los mØtodos conocidos. Por consiguiente, la inactivaciOn para las 20 horas con el mØtodo del invento no deja ninguna posibilidad para una disputa sobre la seguridad de un lote.

El nuevo mØtodo de inactivaciOn do acuerdo con el invento acorta el proceso do la fabricaciOn do la vacuna por Ia menos en un dIa. Esto hace el sistema de producciOn do la vacuna mucho mÆs flexibley permite quo el proceso (incluyendo un tratamiento con cloroformo) encaje dentro do una semana de 4 dIas do trabajo.

Los Solicitantes consideran ademÆs quo durante el mØtodo segün 01 invento, 01 FA fija los antigenos del virusdurante la inactivaciOn y, par Ia tanto, esto traerÆ coma resultado vacunas relativamente mÆs efectivas con una vida do consorvaciôn relativamente larga.

Donde la inactivación con la BE[sola proporciona, bajo condiciones favorables, tasas de inactivación de 0.5 - 1.0 logaritmos par hora (Rweyemamu y otros), las 5 cepas de vacuna estudiadas hasta ahora muestran que el mØtodo de acuerdo con el invento presenta tasas de inactivación entre 2.0 a 3.3 logaritmos (Figura 2b - 2f, tabla 1). Por consiguiente, el mØtodo del invento puede proporcionar una garantia para suficiente inactivación dentro de las 8 horas siguientes. Esto tambiØn significa que la inactivación se puede Ilevar a cabo dentro del marco de tiempo de un dIa de trabajo. Esta constituye una gran ventaja en el proceso de producciOn de vacunas (producción, cosecha, clarificaciOn, inactivaciOn, y concentración del antIgeno en el virus) que, de conformidad con la tØcnica anterior encaja solo hasta
ahora en un dia total de trabajo.

Los Solicitantes prevØn que por Ia menos un conjunto de prueba, incluido un microorganismo inactivado de acuerdo con el mØtodo precedente del invento se
puede preparar. En el conjunto de pruebas, el microorganismo inactivado puede
estar en la forma de un alØrgeno a de un antIgeno para utilización en un ELISA.

(Se apreciarÆ queson posibles las variaciones en detalle con los mØtodos, vacunas y conjuntos de acuerdo con el invento sin apartarse del alcance de las reivindicaciones adjuntas.

V