

Señor

Superintendente de Industria y Comercio

SUPERINTENDENCIA DE INDUSTRIA Y

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No. 09-144676- -00015-0000

Fecha: 2013-08-23 16:11:35 Dep. 2020 DIR.NUEVASCR
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Act. 412 REPOSPRESRECU Folios: 25

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Referencia: Expediente No. 09.144.676

Solicitud de Patente de Invención titulada: "Composiciones para vacuna en emulsión que comprende antígeno y un adyuvante en la fase acuosa y su método de elaboración"

Solicitante: Zoetis W LLC

RECURSO DE REPOSICIÓN

ÁLVARO CORREA ORDÓÑEZ, mayor de edad y vecino de Bogotá D.C., identificado según aparece al pie de mi firma, en mi condición de apoderado de la sociedad Zoetis W LLC, domiciliada en 100 Campus Drive, Florham Park, New Jersey 07932, Estados Unidos de América, presento oportunamente el siguiente recurso de reposición en contra de la Resolución 40517 de fecha 5 de Julio de 2013, notificado por listado único de notificación de propiedad industrial fijado el 16 de Julio de 2013, con el propósito de que se revoque la mencionada resolución.

1. OBJETO DEL RECURSO

Que se revoque la Resolución 40517 de fecha 5 de Julio de 2013, y en su lugar, se conceda el privilegio de patente de invención para la totalidad de las reivindicaciones 1 a 23.

2. ANTECEDENTES

2.1. La solicitud de patente tramitada en este expediente se presentó inicialmente con 36 reivindicaciones de las cuales 13 fueron desistidas, quedando finalmente 23 reivindicaciones (1 a 23). En la Resolución impugnada, el Despacho niega el derecho de patente para las reivindicaciones 1 a 23 con fundamento en falta de nivel inventivo.

2.2. Esta solicitud fue presentada ante ese Despacho el día 17 de Diciembre de 2009 y se le adjudicó el número de radicación 09.144.676. Mediante auto de fecha 12 de Febrero de 2010, la División de Nuevas Creaciones requirió al solicitante para que se pronunciara con respecto al primer examen de forma contenido en el Oficio No. 01837. Dicho requerimiento fue atendido mediante el memorial radicado el día 7 de Abril de 2010.

2.3. Habiendo cumplido con los requisitos formales, la solicitud fue publicada el día 21 de Febrero de 2011. No se presentaron oposiciones por parte de terceros. Se solicitó el examen de patentabilidad el día 12 de Agosto de 2011.

2.4. Siguiendo con el trámite normal, ese Despacho emitió un único examen de fondo el día 14 de Agosto de 2012, en el cual objetó la novedad de las reivindicaciones 24 a 26 con fundamento en las enseñanzas contenidas en el documento WO2004058142 (D1) y el nivel inventivo de las reivindicaciones 1 a 21, 27 a 36, con fundamento en las enseñanzas contenidas en los documentos US 5026546 (D2) y US 5109026 (D2). Este requerimiento fue oportunamente atendido el memorial radicado el 21 de Diciembre de 2012, en el cual se allegó un nuevo capítulo reivindicatorio conformado por 23 reivindicaciones.

2.5. El día 4 de Junio de 2013 se presentó modificación en el nombre del solicitante a Zoetis W LLC.

2.6. Por medio de la Resolución 40517 de fecha 5 de Julio de 2013, notificada por edicto desfijado el día 16 de Agosto de 2013, el Despacho niega las reivindicaciones 1 a 23 por cuanto considera que carecen de nivel inventivo a pesar de los argumentos presentados por la solicitante en la respuesta al examen de fondo.

2.7. Ese Despacho menciona que la falta de nivel inventivo se debe a las enseñanzas contenidas en los documentos US 5026546 (D2) y US 5109026 (D3). Particularmente, el Examinador establece que:

“El documento D3 se considera el estado de la técnica cercano a la invención definida en la reivindicación 1 porque divulga una emulsión agua en aceite que comprende una fase oleosa como aceite mineral y una fase acuosa que comprende un adyuvante polielectrolito policatiónico y el antígeno (...) el documento D2 divulga una suspensión acuosa como adyuvante que se encuentra estabilizada y comprende DDA y un polímero acrílico de ácido acrílico reticulado con polialilsacarosa. La diferencia entre la invención, definida en la reivindicación 1 y la composición divulgada en el documento D3 consiste en que el adyuvante se selecciona entre polímero acrílico, bromuro de dimetil dioctadecil amonio (DDA) o una combinación de éstos (...) Sin embargo, la utilización de un polímero de ácido acrílico reticulado con polialilsacarosa y bromuro de dimetil dioctadecil amonio (DDA) como adyuvantes para mejorar la respuesta inmune en una vacuna, se encuentra divulgada en el documento D2 que presenta una suspensión acuosa adyuvante estabilizada que comprende DDA y un polímero de ácido acrílico reticulado con polialilsacarosa. Adicionalmente, el documento D2 divulga la aplicación de DDA como adyuvante para varias vacunas y que su estabilidad en agua mejora cuando se mezcla con polímeros de ácido acrílico reticulado con polialilsacarosa, los cuales también tienen propiedades adyuvantes.

En consecuencia, la persona del oficio normalmente versada en la materia, incluiría como adyuvante bromuro de dimetil dioctadecil amonio (DDA), polímeros acrílicos reticulados con polialilsacarosa o una combinación de los mismos, según lo revelado en el documento D2, en la composición divulgada en el documento D3 para llegar así al objeto de la reivindicación 1 de la solicitud. Por esta razón la reivindicación 1 se considera obvia (...) El documento (D3), considerado el estado de la técnica más cercano a la invención definida en la reivindicación 22, divulga un método para la elaboración de una composición de vacuna que comprende preparar una solución acuosa de un antígeno y un adyuvante y posteriormente emulsificar con aceite mineral.

La diferencia entre el método reclamado y el método divulgado en el documento D3 consiste en que como adyuvante se utiliza DDA, un polímero acrílico o una combinación de los mismos (...)

Sin embargo, la utilización de bromuro de dimetil dioctadecil amonio (DDA) y un polímero acrílico como adyuvantes para preparar una fase acuosa de una vacuna ya está divulgada en el documento D2 que se refiere a la preparación de una suspensión acuosa adyuvante que comprende DDA y un polímero acrílico donde la solubilidad de DDA en agua se ha mejorado mediante la adición del polímero de ácido acrílico reticulado con polialilsacarosa.

En consecuencia, la persona del oficio normalmente versada en la materia, utilizaría los adyuvantes bromuro de dimetil dioctadecil amonio (DDA) y un polímero de ácido acrílico reticulado con polialilsacarosa tal como es divulgado en el documento D2 en el procedimiento del documento D3 para llegar así al objeto de la reivindicación 22 de la solicitud, por lo cual se considera obvia (...) El documento D3 se considera el estado de la técnica cercano a la invención definida en la reivindicación 23 porque divulga un método para elaborar una vacuna que consiste en preparar una fase acuosa que contiene un antígeno y un adyuvante y posteriormente adicionar lentamente la fase acuosa sobre una fase oleosa que contiene un emulsificador como monooleato de sorbitan, agitando al mezclar para producir una emulsión estable (...) Sin embargo, la utilización de bromuro de dimetil dioctadecil amonio (DDA) y un polímero de ácido acrílico reticulado con polialilsacarosa como adyuvantes para preparar una fase acuosa de una vacuna ya está divulgada en el documento D2 que se refiere a la preparación de una suspensión acuosa adyuvante que comprende DDA y un polímero acrílico, donde la solubilidad de DDA en agua se ha mejorado mediante la adición del polímero de ácido acrílico reticulado con polialilsacarosa. En consecuencia, la persona del oficio normalmente versada en la materia, según las enseñanzas del documento D2 utilizaría como adyuvantes bromuro de dimetil dioctadecil amonio (DDA) y un polímero de ácido acrílico divulgado en el método divulgado en el documento D3 para llegar así al objeto de la reivindicación 23 de la solicitud, por lo cual la reivindicación 23 se considera obvia (...) Es oportuno aclarar que, contrario a lo afirmado por la solicitante, los datos presentados en la solicitud no demuestran ningún efecto técnico inesperado. En la tabla 6 no se observa una diferencia significativa entre los datos correspondientes al grupo de tratamiento con emulsión estándar 1 y el grupo con emulsión que comprende carbopol y DDA 3, donde por ejemplo a las 9 semanas de la segunda dosis de vacuna los valores de hemaglutinación son 26 y 28 respectivamente para los grupos 1 y 3 (...)"

3. FUNDAMENTO DEL RECURSO

3.1. Objeto de la invención

Antes de entrar a discutir la patentabilidad de la presente invención, considero importante mencionar el objeto de la presente solicitud.

La presente solicitud se relaciona con composiciones para vacuna que comprenden emulsiones agua en aceite con un polímero acrílico en la fase acuosa que incrementa significativamente la respuesta inmune generada por la composición de vacuna, y se evita la reactividad adversa esperada del polímero acrílico en el animal y métodos para la elaboración de las formulaciones.

En particular la reivindicación 1 de la solicitud en estudio reclama:

"Una composición de vacuna que comprende una emulsión agua en aceite, en donde dicha emulsión comprende un componente de aceite y un componente acuoso; y en donde dicho componente acuoso comprende:

- a) un antígeno y*
- b) un adyuvante seleccionado del grupo que consiste de un polímero acrílico, bromuro de dimetil dioctadecil amonio (DDA), o combinaciones de los mismos".*

3.2. El requisito de novedad

Su Despacho ha reconocido el requisito de novedad de la presente solicitud de patente, por lo tanto, el requisito a sustentar es el nivel inventivo.

3.3. Argumentos en favor del nivel inventivo

El nivel inventivo de una solicitud se encuentra definido en el Artículo 18 de la Decisión 486 y se establece cuando *"si para una persona del oficio normalmente versada en la materia técnica correspondiente, esa invención no hubiese resultado obvia ni se hubiese derivado de manera evidente del estado de la técnica"*.

A continuación se demostrará el nivel inventivo de la solicitud en estudio con relación a los documentos **D2** (que revela el bromuro de dimetil dioctadecil amonio (DDA) + Carbopol; documento que no contiene emulsiones en aceite) y el documento **D3** (que revela emulsiones agua en aceite pero no revela DDA + Carbopol).

Su Despacho afirma que debido a que las emulsiones agua en aceite ya se conocían y que debido a que las combinaciones de DDA y Carbopol ya se habían conocido, una persona medianamente versada en la materia técnica se vería motivada a combinar emulsiones agua en aceite con DDA+Carbopol.

Los argumentos de su Despacho se basan en una suposición de que si varios compuestos han sido conocidos como adyuvantes, habría sido obvio la combinación de estos exitosamente.

Respetuosamente, mi representada no está de acuerdo y presenta los siguientes argumentos:

En primer lugar, no es deseable cada combinación de adyuvantes. Por ejemplo, el uso de poliacrílicos en emulsiones agua en aceite habría sido desalentado en razón de que se esperaba que los polímeros acrílicos tales como Carbopol, serían altamente reactivos en asociación con emulsiones en aceite. Por favor, ver página 2 tercer párrafo de la solicitud en estudio.

En segundo lugar, una combinación de adyuvantes conocidos no produce necesariamente un mejor adyuvante

Mi representada presenta dos artículos de los autores Romera y Ramakrishnan, los cuales se adjuntan con este escrito.

En la divulgación del artículo de Romera's se demuestra que (agua en aceite, que es conocido como adyuvante) es tan buen adyuvante como la combinación agua en aceite + avridina (que también son conocidos por sus propiedades inmunomoduladores). De esta forma, la avridina, que es una amina cuaternaria como la DDA, no mejora las propiedades del adyuvante agua en aceite. Por lo tanto, una persona medianamente versada en la materia no se vería motivada a combinar el agua en aceite y una amina cuaternaria, tal como el DDA. Así mismo, con relación a Ramakrishnan et al., Veterinaria Italiana 2005 41(3): 149-

155, Se nota que al parecer el adjuvante saponina/ $\text{Al}(\text{OH})_3$ no fue mejor que la saponina sola o el $\text{Al}(\text{OH})_3$ solo.

En tercer lugar, es un principio bien aceptado en las leyes de patentes que resultados superiores demuestran el nivel inventivo de una composición. En el presente caso, la invención en estudio demostró resultados superiores de la emulsión agua en aceite + Carbopol y la emulsión agua en aceite + DDA + Carbopol con relación a las emulsiones agua en aceite sin DDA o Carbopol.

Mi representada se refiere a la tabla 4 de la solicitud en estudio, y hace notar respetuosamente que los títulos HI (media geométrica) en los grupos tratados con emulsión agua en aceite + Carbopol o emulsión agua en aceite + DDA + Carbopol (222.5 y 232.5, respectivamente) fueron casi dos veces más alto que en el grupo tratado solo con agua en aceite (133) y casi tres veces más alto que el grupo tratado con agua en aceite + Aluminio (82.5).

Además, el ejemplo 5 de la solicitud en estudio establece que " en este ejemplo, se observó una mejora general en la respuesta serológica de la influenza aviar en pavos a los que les fue administrada una emulsión de vacuna de agua en aceite inclusive de Carbopol o Carbopol + DDA en la fase acuosa.

Esta mejora ocurre con un aislado H5N9 de bajo patogénico convencional, similar a los resultados observados utilizando los aislados H5N3 de bajo patogénico de origen genético inverso (Ejemplo 4). Por lo tanto, la mejora en la respuesta inmune puede atribuirse al adjuvante y es por lo visto aplicable a una variedad de antígenos."

De lo anterior, se tiene que la presente solicitud de patente presenta resultados superiores a los del estado de la técnica y esto claramente es un indicio de nivel inventivo.

De conformidad con los argumentos arriba citados, se concluye que la presente invención es novedosa tal como lo ha reconocido su Despacho y también presenta altura inventiva con respecto a las enseñanzas de los documentos D2 y D3 considerados separadamente o en conjunto; y por lo tanto, la presente invención cumple con los requisitos de patentabilidad establecidos en el artículo 14 de la Decisión 486.

En virtud de los anteriores argumentos, solicito de manera respetuosa que se revoque la Resolución 40517 y se otorgue el privilegio de patente a la invención titulada:

"Composiciones para vacuna en emulsión que comprende antígeno y un adyuvante en la fase acuosa y su método de elaboración"

4. NOTIFICACIONES

Recibiré notificaciones en mis oficinas ubicadas en la Avenida 82 No. 10-62 Piso 6 de la ciudad de Bogotá, D.C.

Atentamente,



ÁLVARO CORREA ORDÓÑEZ
C.C. No. 79.143.366 de Usaquén
T.P. 20.281

Anexos: - Artículo 1¹
 - Artículo 2²

BOGMGT/BOGDMS-#1650898-V1-P2009_000289-RECURSO_DE_REPOSICIÓN.DOC

¹ *"Adjuvant effects of sulfolipo-cyclodextrin in a squalane-in-water and water-in-mineral oil emulsions for BHV-1 vaccines in cattle"*

² *"Immune response and protective efficacy in sheep immunized with hydroxylamine-inactivated bluetongue virus vaccine".*

Adjuvant effects of sulfolipo-cyclodextrin in a squalane-in-water and water-in-mineral oil emulsions for BHV-1 vaccines in cattle

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Received 22 September 1999; received in revised form 1 March 2000; accepted 1 March 2000

Abstract

The antibody and cell mediated immune responses induced by BHV-1 were analysed in cattle after vaccination and challenge exposure to the virulent strain LA of BHV-1. Animals were vaccinated intramuscularly (IM) with inactivated virus vaccines against BHV-1 containing either a water in mineral oil adjuvant (W/O), a water in mineral oil adjuvant plus Avridine (W/O+Avridine) or sulfolipo-cyclodextrin in squalane in-water emulsion (SL-CD/S/W). No significant differences were registered in the antibody response induced by the three evaluated vaccines. However, the BHV-1 specific cell-mediated immune response was stronger and appeared earlier when SL-CD/S/W was included in the formulation. The efficacy of the vaccines was also evaluated after intranasal challenge of the calves with a virulent BHV-1 LA strain. Animals vaccinated with SL-CD/S/W had reduced virus excretion and clinical symptoms compared with the mock-vaccinated animals. Comparison of levels of BHV-1 specific IgG2 and IgG1 with virus shedding revealed that, regardless of the adjuvant administered, animals showing BHV-1 specific IgG2/IgG1 ratios higher than 1 were those with a significant lower number of individuals shedding virus. Additionally, animals vaccinated with SL-CD/S/W presented no post-vaccinal reactions. These factors, combined with the higher efficacy and the ease of manipulation of the biodegradable oil, makes the vaccine formulated with this new adjuvant an important contribution for the veterinary vaccines industry. © 2000 Elsevier Science Ltd. All rights reserved.

Keywords: Bovine herpesvirus-1; Adjuvants; Sulfolipo-cyclodextrin; Avridine

1. Introduction

Infectious Bovine Rhinotracheitis (IBR) in cattle is caused by Bovine herpesvirus Type 1 (BHV-1) and is responsible for considerable economic losses due to decreased milk production, weight loss and abortion [1]. The wide spectrum of clinical symptoms includes rhinotracheitis, infectious pustular vulvovaginitis, balanopostitis, conjunctivitis, abortion, enteritis, and encephalitis [1].

Conventional BHV-1 vaccines including those based

on the use of modified live virus and also inactivated vaccines are available in many countries.

Although they induce rapid and long-lasting immunity, live vaccines present considerable disadvantages such as the risk of induction of abortion [1–4] and the establishment of latent infections with the vaccine strain [5,6]. In addition, live vaccines may cause a temporary state of immunosuppression accompanied by impaired immunoresponsiveness and increased susceptibility to other infections [7]. Furthermore inactivated BHV-1 is, in general, poorly immunogenic and requires strong immunological adjuvants in order to obtain an effective immune response. Emulsions of mineral oil are often the adjuvant of choice for farm

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and laboratory animals since they induce high immune responses. However, the oil may persist at the site of injection for a long period of time [8], may cause excessive tissue reaction [8] and possess carcinogenicity [9,10]. Therefore, attempts to develop better and safer adjuvants lacking mineral oil have been undertaken. Emulsions of non-mineral oil such as squalene and squalane have been investigated extensively with variable results [11-13]. Squalene and squalane are normal constituents of the animal body and are considered to be biocompatible and biodegradable. Squalene is an unsaturated hydrocarbon and susceptible to oxidation, and is considered to be undesired in pharmaceutical applications. Squalane was therefore chosen as an alternative to the mineral oil in the formulation of Freund's complete adjuvant. Hilgers et al. [12-14] described a non-mineral oil-based adjuvant for veterinary vaccines. It was found that the incorporation of a hydrophobic sulfolipo-polysaccharide into a squalane-in-water emulsion strongly enhanced the humoral immune response to viral antigens (e.g. pseudorabies virus, influenza virus) in laboratory animals [12] and in target animals [13]. The concept was further optimised yielding a formulation comprising a hydrophobic sulfolipo-cyclodextrin, squalane, polysorbate 80 and aqueous phase [14]. This adjuvant combined high efficacy with low toxicity and good stability.

Another group of non-mineral oil adjuvants are lipid amines including dimethyldioctadecylammonium bromide [15] and a synthetic lipoid amine, Avridine, [16]. In addition to enhancing the humoral and cell-mediated immune responses, Avridine has been shown to have antitumor and immunomodulating properties [16,17]. Research with Avridine demonstrated that it induces interferon (IFN) secretion and production of interleukin-1 (IL-1), it enhances humoral immune response and promotes lymphoid cell proliferation [18].

In order to gain more insight into the mechanisms underlying protection against BHV-1 in cattle and to develop a vaccine with improved efficacy/toxicity-ratio, we investigated the effects of the sulfolipo-cyclodextrin/squalane adjuvant [14] and water-in-mineral oil emulsions (with and without Avridine) on the humoral and cellular immune responses as well as protection against virus challenge.

2. Material and methods

2.1. Virus and cells

BHV-1 strain LA (Los Angeles) was propagated in Madin Darby bovine kidney (MDBK) cells grown in Eagle Minimal Medium (MEM), supplemented with 10% foetal bovine serum (FBS).

Confluent monolayers in roller bottles were infected

at a multiplicity of infection of 0.1 and the virus was allowed to adsorb for 45 min at 37°C before the addition of fresh culture medium. When extensive cytopathogenic effect was observed, the supernatant was collected and cell debris was removed by centrifugation at 3000 g for 30 min. The titre of the resulting virus suspension was $10^{8.25}$ tissue-culture infective dose (TCID₅₀) per ml.

BHV-1 preparations were inactivated by treatment with 1% (v/v) of 0.1 M binary bromoethylenimine for 25 h at 37°C as described by Smitsaert et al. [19].

2.2. Vaccine formulations

One volume of inactivated BHV-1 suspension was mixed with 1 vol of mineral oil adjuvant (INTA, formulated with Arlacel C, Markol 52 and Tween 80) according to Smitsaert et al. [19] giving a water-in-mineral oil adjuvant (W/O). Avridine was incorporated to the vaccine dissolved in absolute ethanol and mixed with INTA oil adjuvant as described Berinstein et al. [20] giving a water-in-mineral oil adjuvant with Avridine (W/O + Avridine). The final dose of Avridine was 20 mg per animal. For the SL-CD/squalane vaccine 1 vol of inactivated BHV-1 suspension was mixed with 1 vol of adjuvant formulation sulfolipo-cyclodextrin in squalane in-water emulsion (SL-CD/squalane; Fort Dodge Animal Health Holland, Weesp, The Netherlands) as described by Hilgers et al. [14].

2.3. Experimental design

Forty-eight 8-12 month-old Holstein calves with no detectable BHV-1 serum antibodies were used in the experiment. They were randomly separated into three vaccine groups of 10 animals each and three control groups, two of four animals each received W/O + Avridine or SL-CD/squalane and the third contained 10 mock-vaccinated calves. The animals were injected intramuscularly with a 5 ml vaccine at days 0, 46 and 226. At day 400, five individuals for each vaccine group and the 10 mock-vaccinated calves were challenged. At different time intervals after vaccination and challenge blood samples were collected. Humoral responses were determined at days 6, 21, 46, 60, 76, 116, 176, 226, 234, 264, 294, 334, 375, 400, 406, 411, 414, 421, 440 and 470 and the antigen dependent lymphocyte proliferative response was tested at days 0, 6, 21, 76, 226, 234, 264, 400, 406 and 470.

2.4. Serology: Virus Neutralisation (VN) test

VN was performed in primary foetal bovine testis (FBT) cultures in 96 well microtitre plates using the constant virus-variable serum method [21]. Serial dilutions of serum were prepared, and 0.1 ml of diluted

samples were mixed with 0.1 ml of virus ($100 \text{ TCID}_{50}/\text{ml}$). Each dilution was tested in four wells containing FBT cell monolayers adsorbed for 1 h, the unadsorbed virus was removed and MEM with 5% FBS was added. Plates were then incubated for 3 days at 37°C and the cytopathogenic effect in the different wells was scored. $\log_{10}$ of the reciprocal value of the highest serum dilution in which a cytopathogenic effect was prevented was considered as the VN titre.

2.5. Serology: enzyme-linked immunosorbent assay (ELISA)

Partially purified antigen was prepared from MDBK cells infected with BHV-1. Infected cells and culture fluids were frozen/thawed three times and clarified by centrifugation for 30 min at 3000 g . Supernatants were pelleted by ultracentrifugation at $120,000 \text{ g}$ for 1 h at 4°C . Mock-infected MDBK cells were used as antigen control. The antigen preparations from infected cell cultures (MDBK + BHV) and mock-infected cell cultures (MDBK) were diluted 1/320 in carbonate-bicarbonate buffer (pH 9.6). This dilution was optimal as determined by checkerboard titrations using positive and negative reference sera.

Polystyrene microtitre plates (Immulon 1, Dynatech Laboratories) were incubated for 12 h at 4°C with $50 \mu\text{l}$ per well of either MDBK + BHV or MDBK in alternate wells. After incubating, the microtitre plates were washed three times and then incubated with serum samples diluted in phosphate buffered saline (PBS) containing 0.05% Tween 80 and 1% ovalbumin (PBST-OVA). Negative and positive sera (naturally infected bovine) were included in the test as controls. An anti-bovine rabbit antiserum labelled with peroxidase (Kirkegaard & Perry Laboratories, KPL) diluted in PBST-OVA was then added. After washing three times, the reaction was developed using 2,2'-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid) (ABTS) (SIGMA) with hydrogen peroxide as substrate in citrate buffer (pH 5). The reaction was allowed to continue for 20 min at room temperature and the optical density at 410 nm, was determined by a Dynatech microELISA-reader.

The titre was expressed as the $\log_{10}$ of the reciprocal dilution of the serum corresponding to an optical density (OD) of 0.1. The value of 0.1 was established because it is the mean OD of a negative serum plus two times the standard deviation.

2.6. Indirect ELISA for detecting anti BHV-1 isotype specific antibodies

Isotype detection was performed using an ELISA with anti-bovine IgG1 (DAS 17) and IgG2 (DAS 2) mouse monoclonal antibodies (mAbs) kindly provided

by Dr. S. Srikumaran (University of Nebraska, USA). Plates were coated with MDBK + BHV-1 for 12 h at 4°C . After washing, plates were blocked with PBST-OVA for 1 h at 37°C . Bovine sera were added in 4-fold dilutions and incubated for 1 h at 37°C . After washing, the isotype-specific anti-bovine mAbs were added and plates were incubated for 1 h at 37°C . After washing, plates were incubated with peroxidase labelled anti-mouse antibody (KPL) for 1 h at 37°C . After washing, H_2O_2 /ortho-phenyldiamine (Sigma) was added. The OD was determined at 490 nm. Titres were expressed as the $\log_{10}$ of the reciprocal value of the highest serum dilution giving an OD > 0.3 (the arithmetic mean of ODs of a negative serum plus two times the standard deviation).

2.7. BHV-1 specific lymphocyte proliferation

Blood samples were collected by venipuncture in syringes containing preservative-free heparin. Lymphocyte-enriched cells were isolated from the buffy coats by centrifugation on Lymphoprep[®] (density 1.077 g/ml ; Nycomed Pharma A.S). Cells were then washed twice in RPMI 1640 medium supplemented with RPMI + 5% FBS. For the lymphocyte proliferation test (LPT), the virus suspension was diluted to 10^5 TCID_{50} and frozen at -70°C . Virus was inactivated by placing it for 1 min at a distance of 11 cm from two General Electric G875 ultraviolet (UV) bulbs. MDBK cell control cultures were also exposed to UV irradiation under similar conditions. The inactivated BHV-1 virus antigen and their respective MDBK control fluids, diluted 1:100 in RPMI 1640 culture medium, were added to the cultures which contained 0.1 ml of culture medium with $2 \times 10^6/\text{ml}$ lymphocytes. One microgram of the mitogen Concanavalin A (Con A; Sigma, St Louis, USA) was added to positive control wells. The lymphocytes were incubated for 4 days at 37°C in a humidified atmosphere with 5% CO_2 . Cell proliferation was measured by adding $0.4 \mu\text{Ci}$ [^3H] thymidine per well 20 h before harvesting. Each test was performed in triplicate. Thymidine uptake was measured in a liquid scintillation counter (LKB, Wallac, 1219 Rackbeta), and arithmetic means and standard deviations were calculated for the triplicate values. Results were obtained as mean counts per minute (CPM) and expressed as stimulation index ($\text{SI} = \text{mean CPM of culture with antigen} / \text{mean CPM with MDBK}$). The threshold value was established at 3 SI.

2.8. Challenge procedure

Five animals of each vaccine group randomly separated and 10 mock-vaccinated calves were infected with BHV-1 virus LA strain suspended in Eagle's medium

at a concentration of $10^{7.5}$ TCID₅₀/ml. Two millilitres of the inoculum were administered into each nostril of individual animals by aerosol exposition using an ultrasonic nebulizer (ELECTROLAB AP-300).

Prior to challenge, the calves were clinically examined and rectal temperature was recorded. Blood samples and nasal swabs were collected to establish baseline values. Nasal swabs were obtained by inserting tampons into the ventral meatus of the nasal passage and immediately dipping them in MEM containing 5000 IU penicillin/ml, 2500 µg streptomycin/ml and 10 µg amphotericin B/ml. Samples were collected 4, 6, 8, 11, 13, 15 and 18 days post-challenge (DPC). For virus detection all samples were inoculated immediately after collection: 0.1 ml of the nasal fluids were inoculated onto microcover glasses covered with MDBK cell monolayers in 24 well plates. Monolayers were inspected for three consecutive days for the appearance of cytopathological effect (CPE). To confirm BHV-1 infection, the microcover glasses were processed by direct immunofluorescence. Samples were considered negative when two additional passages over MDBK cells did not demonstrate CPE.

Serum samples of 0, 6, 11, 14, 21, 40 and 70 DPC were analysed in the ELISA and VN test and those of 0, 6 and 70 DPC in the LPT.

After challenge, the calves were clinically examined for 18 consecutive days by a veterinarian who was not aware of the treatments of individual animals. The parameters evaluated included the presence of anorexia, body temperature, rhinitis and vulvovaginitis.

2.9. Statistical analysis

The comparison among the vaccine profiles through the experimental period was performed using the analysis of variance (ANOVA) for repeated measures with the Greenhouse and Geisser correction of the significance levels (fixed at 5%). The post-ANOVA comparisons were performed using the Bonferroni test with the same level of significance. All the statistical calculations were performed using the SAS program (released 6.04), following the G.M.L. procedure [22].

In the proliferative test, the proportion of LPT positive animals with clinical symptoms were compared using the Fisher's exact test, significance levels are indicated for each case.

Correlation between the following parameters was evaluated: (1) VN antibodies titres and protection against virus challenge; (2) ELISA antibody titres and protection against virus challenge; (3) IgG2/IgG1 and protection against virus challenge. In this case also a non-parametric correlation (Spearman's rho) was used; (4) VN antibody and ELISA antibody titres.

3. Results

3.1. Anti BHV-1 antibody response

The titre of anti BHV-1 antibodies measured by ELISA, in animals immunised with the different vaccines did not differ significantly (Fig. 1a). Maximal responses were observed 60 and 234–264 days post-vaccination (DPV) while control animals did not demonstrate any antibody titres. The antibody titres before challenge ranged between 3.14 and 3.93 and 6 days after challenge, titres in all vaccinated calves started to increase reaching their maximal level at 11 DPC. In mock-vaccinated calves the antibodies appeared at 6 DPC and reached a maximal level at 40 DPC (Fig. 1b).

Although, as expected, there were statistically significant differences between vaccinated and mock-vaccinated cattle after challenge no differences were found between the different vaccine groups.

3.2. IgG isotype response in the vaccinated animals

At the time of the first booster vaccination, there was a significant increase in the titres of both IgG1 and IgG2 isotype antibodies in all vaccinated animals (Fig. 2a and c). However there were no significant differences between the different vaccine groups in the levels of IgG1 antibodies. Although the levels of IgG2 antibodies were significantly higher in the animals vaccinated with W/O (with or without Avridine) than in the group of animals vaccinated with SL-CD/S/W between 60–294 DPV, at the time of challenge, the levels of IgG2 were similar in all vaccinated animals. After challenge, titres of both Ig isotypes increased lasting for 3 weeks (Fig. 2b and d). No differences were observed between the different vaccine groups but there were statistically significant differences between the vaccinated and the mock-vaccinated animals.

3.3. Neutralising antibody responses in vaccinated animals

After the first and second booster vaccination, all vaccinated animals exhibited significant VN antibody titres whereas no titres were detected in mock-vaccinated animals (Fig. 3a). At the time of challenge, titres in vaccinated animals ranged from 0.5 to 1.16 and after challenge, titres increased from six DPC reaching the maximum 14 DPC. Mock-vaccinated animals demonstrated also significant VN titres, which were, on average, 10-fold lower than those of the vaccinated animals (Fig. 3b).

A relationship between VN titres and the ELISA titres and duration of virus shedding in the vaccinated groups could not be established (Table 1).

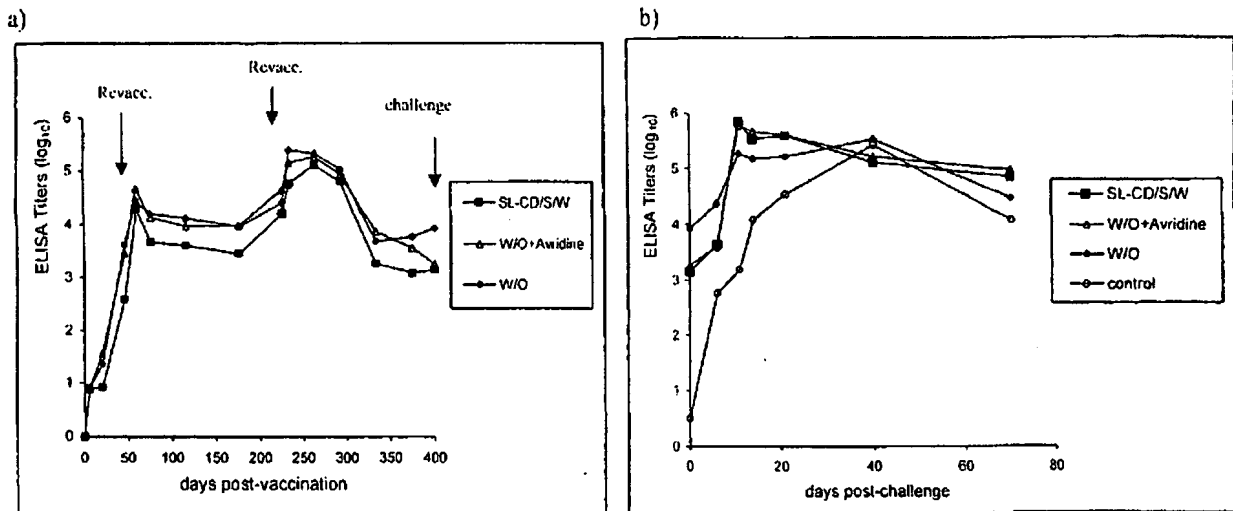


Fig. 1. Effect of different vaccine formulations on BHV-1 antibody titres measured by ELISA in cattle after vaccination and challenge. (a) ELISA antibody titres to inactivated BHV-1 plus SL-CD/Squalane/W (■), W/O plus Avridine (Δ) or W/O (◆) as adjuvant. Each point represents the mean titre of 10 animals expressed as log₁₀ of the reciprocal value of the serum dilution giving an O.D. above 0.1. SEM: 0.16. (b) Antibody titres after challenge of the animals. Each point represents the mean titre of five vaccinated or 10 mock-vaccinated animals. SEM: 0.21.

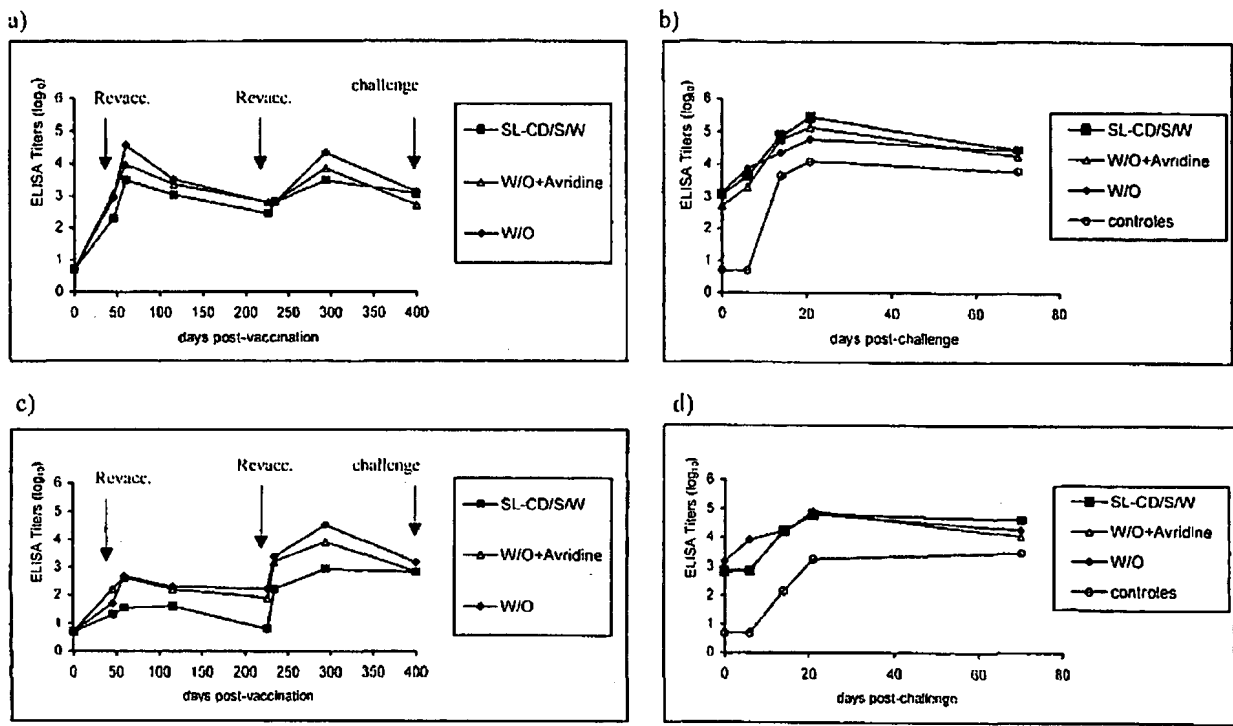


Fig. 2. Isotype profiles of anti BHV-1 antibody titres, measured by ELISA, after vaccination and subsequent challenge. (a) IgG 1 titres in vaccinated animals with inactivated BHV-1. Symbols in all cases were the same with animals vaccinated with inactivated BHV-1 plus SL-CD/Squalane/W (■), W/O plus Avridine (Δ) or W/O (◆) as adjuvant as shown in the graphs. SEM: 0.19. (b) IgG 1 titres after challenge of vaccinated and control animals. SEM: 0.17. (c) IgG 2 titres in vaccinated animals with inactivated BHV-1. SEM: 0.25. (d) IgG 2 titres after challenge of vaccinated and control animals. SEM: 0.21.

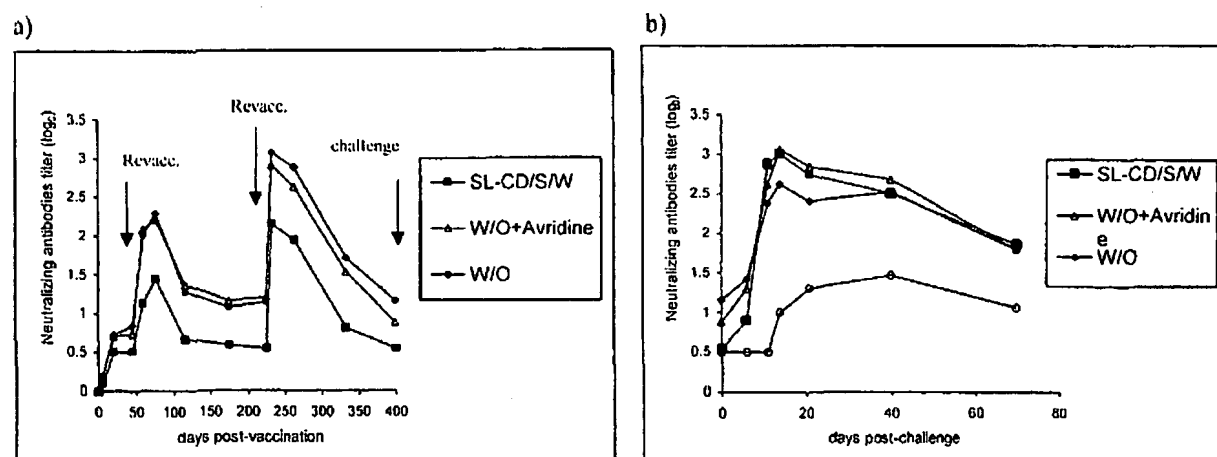


Fig. 3. Neutralising antibody titres after vaccination and challenge. (a) Neutralising antibody titres following vaccination with different formulations of inactivated BHV-1 vaccines. SEM: 0.13. (b) Neutralising antibody titres after challenge of the vaccinated and control animals. SEM: 0.17.

3.4. Lymphocyte proliferation test (LPT)

The percentage of individuals showing a positive LPT response in the group of animals vaccinated with SL-CD/S/W were notably higher than in those vaccinated with W/O (with or without Avridine) (Fig. 4). The highest value was obtained with SL-CD/squalane/W at six DPV where nine out of ten vaccinated animals showed positive LPT responses when compared with the other two groups (where five out of ten vaccinated animals showed positive responses — Fisher's test revealed $p < 0.07$). At 76 DPV (corresponding to 30 days after the booster), significant LPT responses were detected in 50% of the SL-CD/squalane/W vaccine group and in 20% of the W/O and W/O plus Avridine group. At the time of the second revaccination, responses were equally low in the three vaccine groups.

Seven days after the second booster, the three vaccinated groups demonstrated significantly increased LPT responses. Among the animals immunised with vaccine plus SL-CD/squalane/W, 50% gave positive responses

while within the groups vaccinated with W/O or W/O plus Avridine, only 20% of the animals demonstrated significant LPT results. No significant LPT responses were observed in the mock-vaccinated animals during the tested period.

At the moment of challenge, each group of vaccinated animals showed one animal positive for LPT responses. At six DPC, this percentage increased to 100% in the vaccinated animals and to 80% in the mock-vaccinated controls.

3.5. Clinical observation and virus shedding

After challenge, the temperature response of all vaccinated animals maintained a mean value of 38°C while three out of ten control animals showed temperatures higher than 40°C at four to six DPC.

The first symptoms, characterised by bilateral serous rhinitis, were observed four DPC which increased in severity as manifested by severe sero-mucosa rhinitis by eight DPC and returned to normal at 14 DPC. At eight DPC, when symptoms were most severe, the vac-

Table 1
ELISA antibody titres and VN titres at the day of challenge (day 400) vs viral excretion in calves after intranasal challenge^a

Adjuvant	ELISA titre (400 DPV)	VN titre (400 DPV)	Mean (range) of maximal titre of virus excreted (10 log TCID ₅₀ per ml nasal liquid)	Average number of days (range) of virus shedding
SL-CD/squalane/W	3.1	0.54	1.9 (0–4)	4.0 (0–7)
W/O + Avridine	3.3	0.88	3.4 (0–4.5)	4.0 (0–7)
W/O	3.9	1.16	2.6 (0–4.5)	4.2 (0–7)
No antigen/no adjuvant	0.5	0.50	5.2 (2–6.5)	9.3 (0–12)
SEM	0.11	0.14	0.79	1.5

^a Data were expressed as geometric means and standard error means (SEM).

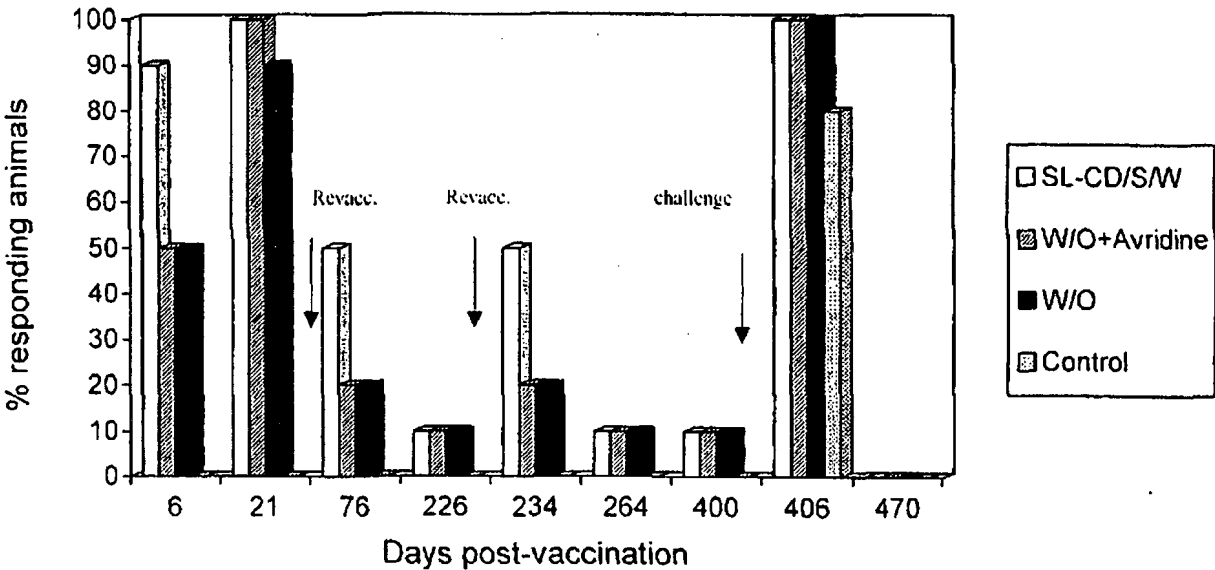


Fig. 4. The percentage of animals exhibiting significant lymphocyte proliferation after vaccination and subsequent challenge with BHV-1.

inated with SL-CD/squalane had significantly less sero-mucosa rhinitis than the unvaccinated group (Table 2) $p \leq 0.07$.

Virus shedding, monitored at time intervals after challenge, was maximal by eight DPC and was detected in 10 out of 10 mock-vaccinated animals, in three out of five animals receiving either SL-CD/squalane or W/O and in four out of five calves receiving W/O + Avridine (Fig. 5a-d). The three groups of vaccinated animals showed significant shorter period of virus shedding than the control group ($p \leq 0.08$; Table 1). As compared to mock-vaccinated animals, animals vaccinated with BHV-1 + SL-CD/squalane but not with W/O + Avridine or W/O presented significantly lower average of maximal titres of virus excreted ($p \leq 0.05$; Table 1).

Interestingly, there was a reverse relationship between virus shedding and ELISA antibody titres ($r = -0.59$; $p \leq 0.01$; Table 1).

Comparison of levels of IgG2 and IgG1 with virus shedding revealed that regardless of the vaccine adjuvant administered, animals showing IgG2/IgG1 ratios

≥ 1 were those with significant less individuals presenting virus shedding (29%), when compared with the individuals showing ratios < 1 (100%) ($p \leq 0.01$) (Table 3).

The exact Fisher's test allowed us to determine the existence of association between high values of the IgG2/IgG1 ratio and protection ($p \leq 0.01$). Additionally, a non-parametric correlation (Spearman's rho) between the same determinations also gave a significant value, $\rho = 0.625^*$ ($p \leq 0.01$).

4. Discussion

In this report we presented data regarding the effects of a new adjuvant formulation, SL-CD/squalane/W, on the immune response elicited by inactivated BHV-1 when compared with two previously characterised adjuvants: a water-in-mineral oil emulsions with or without the addition of Avridine. The efficacy of these three inactivated BHV-1 vaccines was determined by analysing the humoral and cell-mediated immune re-

Table 2
Clinical symptoms in vaccinated and non-vaccinated animals after challenge with BHV-1 determined by nasal secretion 8 DPC

Adjuvant	Number of animals with nasal secretion per group		
	No secretion	Serous or slight	Severe serous or sero-mucous
SL-CD/squalane/W	0/5	4/5	1/5
W/O + Avridine	0/5	3/5	2/5
W/O	0/5	3/5	2/5
No antigen/no adjuvant	0/10	3/10	7/10

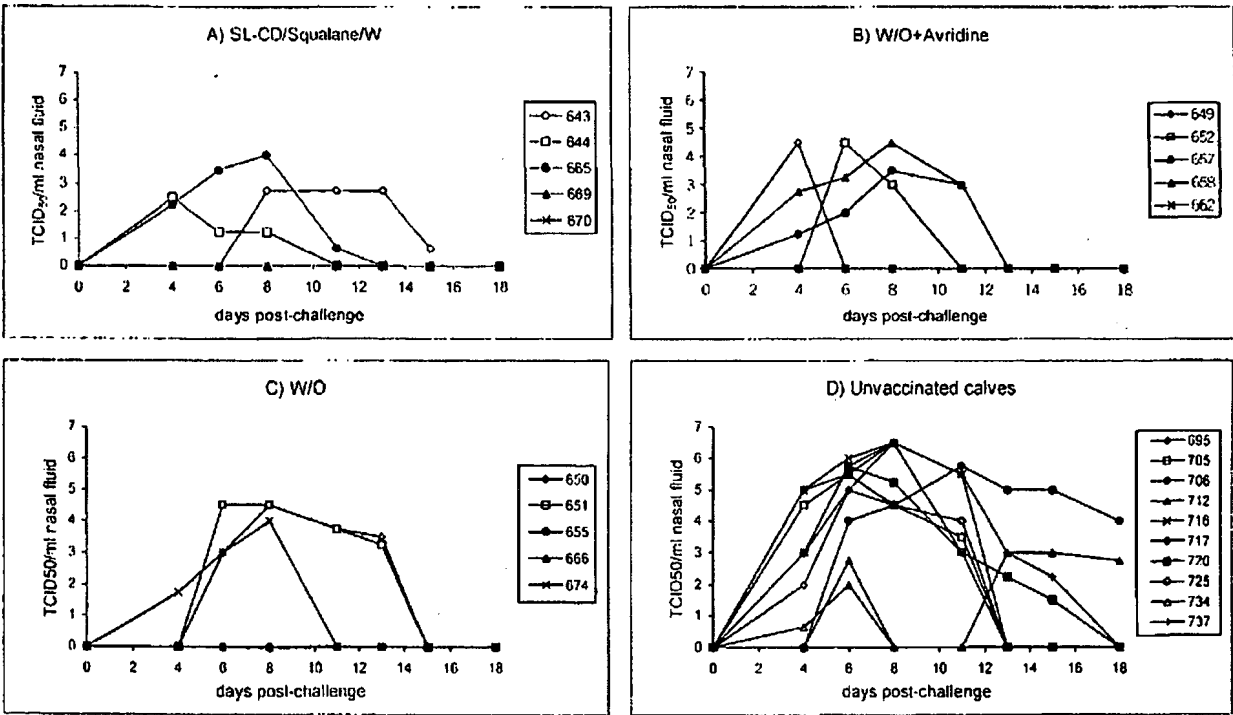


Fig. 5. Virus shedding in vaccinated and mock-vaccinated calves after challenge. The quantity of infectious BHV-1 virus in nasal secretions after challenge was expressed as the TCID₅₀ per ml nasal fluid.

sponses induced by vaccination and by evaluating the secondary immune responses, virus excretion and disease symptoms after intranasal challenge of vaccinated calves with the virulent BHV-1 LA strain.

The results presented here show that the three vaccines tested significantly reduced both the amount of virus excreted and the length of the time of virus shedding when compared with unvaccinated animals. In addition, the SL-CD/squalane/W vaccine was more efficient in reducing the amount of virus shedding (Table 1) as well as the disease symptoms (Table 2) than the W/O-based vaccines.

Regarding the induced anti-BHV-1 antibody response, the three adjuvants elicited significant ELISA and VN antibody titres although, at the time of challenge, no differences were seen between the antibody titres among the three vaccines tested. Additionally, it

was not possible to statistically establish a relationship between VN antibody titres and the degree of protection in vaccinated animals, which is in agreement with results previously reported by others [23,24]. Nevertheless, VN antibodies are thought to play a limited role in the protective immunity against BHV-1 infection as these antibodies appear late after the infection and, as it has been shown, BHV-1 can spread from cell to cell through intercellular bridges without being exposed to intercellular fluids [2,25].

There was a statistically significant inverse correlation between the ELISA antibody titres and the degree of virus shedding. This fact would indicate a contribution of non-VN antibodies to the protection against BHV-1. Interestingly, the lowest titres in virus shedding were observed in those animals presenting relatively high IgG2 and low IgG1 antibody

Table 3
Virus shedding in vaccinated calves divided into two groups with either high (>1) or low (<1) IgG2/IgG1-ratio

IgG2/IgG1-ratio	Number (%) of bovine shedding virus	Mean (range) of maximal titre of virus excreted (10 log TCID ₅₀ per ml nasal liquid)	Average number of days (range) of virus shedding
> 1	2/7 (29%)	1.2 (0.0–4.5)	1.57 (0–2)
< 1	8/8 (100%)	3.84 (2.5–4.5)	5.25 (1–7)

titres. The putative importance of IgG2 antibodies could be explained by the fact that antibody-dependent cellular cytotoxicity (ADCC) appears to be one of the protective immunological mechanisms in BHV-1 infections [26] and that the ADCC effector cells in cattle (e.g. neutrophils and macrophages) specifically possess IgG2-receptors [27].

There have been several lines of evidence indicating the putative role of the cell-mediated immunity in protection against BHV-1 infection [28,29]. In this report, we have assessed cell-mediated immunity by analysing LPT responses of white blood cells to BHV-1. Our results show that inactivated BHV-1 plus SL-CD/squalane/W generated significantly higher lymphocyte proliferation responses than vaccines containing W/O, either with or without Avridine (Fig. 4), which is manifested by an earlier onset of the response and a higher percentage of responding animals.

Studies performed by us, as well as by others [28], show that specifically sensitised lymphocytes appear by the 5th DPC. Thus, the animals vaccinated with vaccine containing SL-CD/squalane/W presented, throughout the experimental period, the highest percentage of animals showing a positive lymphoproliferation and, coincidentally, these animals were the most protected from virus challenge.

In addition to the immunological efficacy, we were interested in comparing the toxicity of the three vaccines in terms of production of local reactions at the site of vaccine administration. Results demonstrated that the vaccine containing SL-CD/squalane/W did not present any reactions in the studied animals in contrast with the reaction observed in bovines vaccinated with W/O adjuvanted vaccines (data not shown).

Taking all these factors into consideration, the results presented here led us to conclude that SL-CD/squalane/W could be an attractive alternative to W/O adjuvants since it combines a higher protective efficacy with milder local inflammatory reactions. An additional technical advantage of SL-CD/squalane/W is that it produces very stable emulsions by simply mixing the aqueous antigen suspension with a ready-to-use adjuvant formulation [14].

Acknowledgements

The authors are grateful to A. Varone for technical laboratory assistance, and F. Marchovcechio for the assistance in the formulations of the vaccines, and F. Cristo for the dedication and care of the calves used in the experiment. This work was sponsored by Solvay Animal Health now Fort Dodge Animal Health Holland.

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Immune response and protective efficacy in sheep immunised with hydroxylamine-inactivated bluetongue virus vaccine

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Summary

Vero cell adapted bluetongue virus (BTV), serotype 18 (BTV-18) was inactivated with hydroxylamine and adjuvanted with aluminium hydroxide (Al(OH)₃) gel, saponin, or Al(OH)₃ gel/saponin prior to immunisation of sheep. Group-specific non-neutralising antibodies to BTV were detected in all vaccinated sheep as soon as seven days after vaccination. However, before challenge, serotype-specific neutralising antibodies were not detected in vaccinated sheep. Upon challenge with homologous live BTV, there was a reduction in delayed type of hypersensitivity response in control sheep but not in the vaccinated animals. All vaccinated sheep had significant ($P < 0.01$) reductions in their clinical reaction index (CRI) and duration of viraemia.

Keywords

Bluetongue virus - Clinical reaction index - Delayed type of hypersensitivity, Hydroxylamine - Immune response - Immunosuppression - Vaccines.

Introduction

Bluetongue (BT) is an infectious, non-contagious, insect-borne viral disease of sheep and other ruminants. The disease has major implications for international trade of livestock. Bluetongue is caused by the bluetongue virus (BTV), genus *Orbivirus*, family *Reoviridae*. The virion is a non-enveloped, triple-layered, icosahedral particle containing 10 linear dsRNA segments. The outer layer contains two major proteins, VP2 and VP5, that are involved in the generation and specificity of neutralising antibodies (1, 3). The inner bi-layered icosahedral core is composed of two major proteins, VP7 and VP3, three minor proteins, VP1, VP4 and VP6, which surround the genomic RNA. The VP7 protein is the major BTV group reactive antigen (13). In addition to these structural proteins, the BTV genome encodes four non-structural proteins, NS1, NS2, NS3 and NS3A (14).

The BTV is transmitted between its vertebrate hosts by the bites of vector species of *Culicoides* and has a global distribution that extends approximately between latitudes 35°S and 50°N (7). It has been estimated that BTV causes losses of US\$3 billion each year throughout the world (16). There are at least 24 serotypes of BTV of which 21 (except 19, 22 and 24) are reported to be circulating in India based on sero-surveillance and/or virus isolation studies (12).

Clinical signs of BT are usually observed in certain breeds of sheep and certain species of deer. Cattle and goats usually experience sub-clinical infections but can serve as important reservoirs of the virus

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(6). The severity of the disease in sheep is dependent on the virus serotype and strain, certain environmental factors such as solar radiation, and the breed of sheep (2). Mortality can be as high as 70% in individual flocks but generally is much lower (10-20%). However, the main consequences of BTV infection are a result of indirect losses due to abortion, loss of condition and prolonged convalescence. Clinical signs of disease in sheep include fever, oedema, congestion, lameness and depression (9).

Vaccination of susceptible species is the most efficient way of controlling the disease. Live attenuated BTV vaccines have been developed and are used in many countries. However, attenuated BTV vaccines have potential safety problems, such as abortion in pregnant sheep, vector-borne transmission, genetic recombination, reversion to virulence (8) and immunosuppression (5). It is important, therefore, to develop inactivated vaccines against BT. This study was undertaken to determine the efficacy of a hydroxylamine-inactivated BTV vaccine that incorporates three different adjuvants.

Materials and methods

Virus and cells

A strain of serotype 18 of BTV (Bhopal isolate) was isolated from sheep in 1988 during a clinical outbreak of BT disease in Khandwa village, Madhyapradesh, India. The virus was passaged three times in baby hamster kidney (BHK-21) cells and freeze dried. For the present study, the virus was reconstituted and passaged three times in African green monkey kidney (Vero) cells. The cells were grown in Glasgow minimum essential medium supplemented with 10% foetal calf serum, 2 mM L-glutamine, 100 IU/ml benzyl penicillin and 100 µg/ml streptomycin sulphate (growth medium). This medium without serum served as

the maintenance medium.

Animals

All sheep (local nondescript breed) used in this study were provided by the Division of Pharmacology and Toxicology, Livestock Production and Management Section, Indian Veterinary Research Institute (IVRI), Izatnagar, India. The sheep included both male and female animals and were between the ages of two and four years. Before the commencement of the experiment, the animals were shorn, dewormed with albendazole at 7.5 mg/kg body weight and dipped in cypermethrin High Cis. They were held in insect-proof housing and blood samples were obtained two weeks before the first vaccination to ensure that they were free of BTV antibodies. Animal experimentation was performed in accordance with the guidelines of the Committee for the Purpose of Control and Supervision on Experiments on Animals (CPCSEA), India.

Inactivation of virus

Hydroxylamine was used as hydrochloride, $\text{NH}_2\text{OH}\cdot\text{HCl}$. A solution of 2 M hydroxylamine was freshly prepared in 5 N NaOH. The pH of the solution was adjusted to 8.8 with 12 M HCl and this solution was added to the virus suspension to achieve a final concentration of 0.25 M. The BTV was allowed to react with hydroxylamine for a total of 48 h at 37°C. Inactivation was first performed for 36 h in one vessel, and then transferred to another vessel for a further 12 h to inactivate any residual live virus. After 48 h incubation, inactivation was stopped by the addition of ice cold phosphate buffered saline (PBS) in a ratio of 1:5 (for each ml sample 4 ml of PBS) and dialysed for 24 h against PBS to remove any excess or unbound hydroxylamine. The inactivated virus suspensions were inoculated onto blood agar and Sabourad dextrose agar to confirm the absence of bacterial and mycotic contamination, respectively.

The inactivated virus suspensions were inoculated onto Vero cell monolayers to confirm complete virus inactivation.

Formulation of vaccines

Al(OH)₃ gel-adjuvanted BTV vaccine

Equal parts of Al(OH)₃ gel (kindly provided by the late S.M. Lal, IVRI, Bangalore, India) and inactivated virus suspension were stirred for 30 min on ice. The mixture was kept at 4°C for 7 days for further maturation of the gel.

Saponin-adjuvanted BTV vaccine

Saponin was prepared as a stock solution of 100 mg/ml in PBS and added to a final concentration of 1 mg/ml. The mixture was stirred for 30 min and kept at 4°C for 7 days.

Al(OH)₃ gel/saponin-adjuvanted BTV vaccine

In addition to equal parts of Al(OH)₃ gel and virus suspension, saponin was added to a final concentration of 1 mg/ml. The mixture was stirred and kept at 4°C for 7 days.

Experimental design

Twelve seronegative sheep were randomly assigned to four groups of three animals each. Sheep in groups 1, 2 and 3 each received 4 ml of Al(OH)₃ gel-adjuvanted vaccine, 2 ml of saponin-adjuvanted vaccine and 4 ml of Al(OH)₃ gel/saponin adjuvanted vaccine, respectively. The sheep in group 4 served as unvaccinated controls. An equal quantity of vaccine was inoculated intramuscularly at two sites (neck and posterior thigh) and all vaccinated animals were revaccinated on day 21 with the same dose, route and vaccine type. All animals were challenged with virulent BTV-18 six weeks after the first vaccination. A total of 4 ml (1.2×10^5 PFU/ml) of virus per animal was injected simultaneously at multiple sites by the intradermal and subcutaneous routes.

Clinical signs

Sheep were monitored daily for body temperature

and other clinical signs for 14 days following challenge. Clinical signs were quantified by a clinical reaction index (CRI) as described by Huismans *et al.* (3). A maximum score of 12 was given to the cumulative total of body temperature of more than 40°C from days 3 to 14 post inoculation (dpi), a score from 0 to 4 each for mouth, nose and foot lesions, and a score of 4 for death. These scores were added to a maximum possible score of 28.

Post challenge viraemia

Heparinised blood samples were collected and centrifuged at $1\,500 \times g$ for 10 min. After removing the plasma, red blood cells (RBCs) were washed three times with PBS and lysed with chilled sterile distilled water to the original volume. Three tubes of confluent monolayers of BHK-21 cells were inoculated with 0.1 ml of lysed RBCs and kept at 37°C for 1 h for virus adsorption. After washing the cells twice with maintenance medium, 1 ml of maintenance medium per tube was added and incubated at 37°C for 7 days. The cells were observed microscopically for BTV-specific cytopathic effects (CPE). Three blind passages were performed for each sample.

Humoral immune response

The presence of group-specific, precipitating, non-neutralising antibody was measured by the agar gel immunodiffusion (AGID) test (4). Serotype specific neutralising antibody was determined by the standard beta-microtitre serum neutralisation test (10).

Delayed type hypersensitivity test

Phytohaemagglutinin (PHA) was used as a non-specific mitogen to evaluate cellular immunity (11). An area of approximately 6 × 6 cm was clipped on both sides of the neck of the sheep and approximately 2 × 2 cm was delineated with an indelible marker. The initial skin-fold thickness at each site was measured using Vernier calipers. The amount of intradermal mitogen had previously

been titrated in normal sheep to determine the optimal concentration and response time. Sheep were intradermally inoculated on one side of the neck with 100 µl of PHA diluted to 1 mg/ml in distilled water. Distilled water (100 µl) was injected into the opposite side of the neck to serve as a negative control. Skin fold thickness was measured again 24 h after injection. The response to the mitogen was determined by subtraction of initial skin thickness measurement from 24 h post-injection measurements.

Statistical analysis

Statistical analysis was performed using the Student *t* test.

Results

Clinical signs

No untoward effects or clinical signs were observed in vaccinated sheep before challenge. After challenge, clinical signs were scored as CRI as shown in Table I.

The sheep in all three vaccine groups presented a significant CRI reduction ($P<0.01$). With the exception of a single animal (ID No. 6; group 2; fever on 5 dpi), none of the vaccinated sheep exhibited fever or clinical signs of BT. In contrast, the control sheep had a mean CRI of 4.43 ± 0.23 and fever occurred in all three animals for 3 to 4 days, lameness (ID No. 11), and mouth lesions (ID No.12).

Post challenge viraemia

The duration of post challenge viraemia amongst sheep in the different groups is shown in Table I. The virus was isolated for up to 7 dpi in vaccinated and for up to 13 dpi in control sheep.

Humoral immune response

Group-specific, non-neutralising antibodies to BTV were detected in vaccinated animals as early as 7 days after first vaccination. No neutralising antibodies were detected in any of the vaccinated sheep before challenge. Neutralising antibodies

Table I
Clinical reaction index and duration of post challenge viraemia

Group	Sheep ID No.	Clinical reaction index	Duration of viraemia (days)
1. (Al[OH] ₃) gel adjuvant	1	0	7
	2	0	5
	3	0	7
	Mean ± SE	0.00 ± 0.00*	6.33 ± 0.66*
2. Saponin adjuvant	4	0	7
	5	0	7
	6	1	5
	Mean ± SE	0.33 ± 0.33*	6.33 ± 0.66*
3. (Al[OH] ₃) gel + saponin adjuvants	7	0	7
	8	0	7
	9	0	7
	Mean ± SE	0.00 ± 0.00*	7.00 ± 0.00*
4. Control (unvaccinated group)	10	4	11
	11	4.5	13
	12	4.8	11
	Mean ± SE	4.43 ± 0.23	11.66 ± 0.66

* $P<0.01$

were detected one to two weeks after challenge in all vaccinated and unvaccinated animals (titres 1:4 to 1:512).

Delayed type hypersensitivity response

Prior to challenge, there was no significant difference in mean skin-fold thickness following administration of PHA to either the vaccinated or unvaccinated sheep. After challenge, the control group had reduced mean skin-fold thickness compared to thickness prior to challenge; specifically, the mean skin-fold thickness of control sheep prior to challenge was 4.33 ± 1.2 compared to 0.33 ± 0.33 , 0.67 ± 0.17 , and 1.83 ± 0.17 at 7, 14, and 21 days post challenge, respectively. There was no such reduction in skin thickness following PHA administration to vaccinated sheep after challenge.

Discussion

The aim of this study was to develop and evaluate an inactivated BTV vaccine. Vaccination of sheep with hydroxylamine-inactivated BTV vaccine (with all three adjuvants) resulted in a significant reduction ($P < 0.01$) in clinical signs and duration of viraemia. Viraemia of limited duration in vaccinated sheep would potentially reduce the spread of vector-borne BTV in field conditions, leading to effective disease control.

All vaccinated sheep developed group-specific non-neutralising antibodies as early as 7 days after first vaccination. However, before challenge, none of the vaccinated animals developed neutralising antibodies. Potential explanations for the failure of the hydroxylamine-inactivated BTV vaccine to induce neutralising antibodies in vaccinated sheep include the following:

- a) hydroxylamine may have altered some antigenic properties of proteins that invoke neutralising antibodies (VP2 and/or VP5)
- b) insufficient antigen was used for immunisation

c) the adjuvants used were not effective.

In our previous study, sheep vaccinated with a binary ethylenimine (BEI)-inactivated, saponin adjuvanted BTV vaccine that incorporated the same strain and concentration of BTV, route of inoculation, and challenge dose etc. developed neutralising antibodies whereas similar vaccines adjuvanted with $Al(OH)_3$ or $Al(OH)_3$ /saponin did not (12). Therefore, antigen concentration and adjuvant might not be responsible for the lack of immunogenicity observed in this study. Analysis of dsRNA extracted from hydroxylamine-inactivated BTV in RNA-PAGE did not show degradation of viral RNA (M.A. Ramakrishnan, unpublished observation), therefore we assume that hydroxylamine may have altered some antigenic properties of the proteins involved in the induction of neutralising antibodies. Specifically, epitopes on outer capsid proteins VP2 and VP5 may be altered because they are involved in the generation and the specificity of neutralising antibodies (1, 3).

Vaccination with VP2 isolated from purified virus particles or expressed from recombinant baculoviruses, can protect sheep against challenge with virulent virus of the same serotype (3, 14). Co-expression of VP2 with VP5 alone, or in combination with core proteins to form double-shelled virus-like particles (VLPs) enhances the neutralising antibody response of inoculated sheep (14). However, virus neutralising antibody may not be the only immune response capable of affording protective immunity, since sheep can resist challenge with live virus in the absence of neutralising antibody (15). This implies that although neutralising antibodies may play a role in protection, other factors or mechanisms are also involved. Vaccination with recombinant capripox virus expressed VP7 protein (group reactive antigen) partially protected sheep against virulent virus of heterologous serotype (17). Stott

et al. (15) showed that an inactivated virus conferred protection through a cellular immune response in the absence of neutralising antibodies.

Phytohaemagglutinin was used as a non-specific mitogen to evaluate the cellular immune response in vaccinated and unvaccinated sheep. Upon challenge with live BTV, control animals had reduced delayed type hypersensitivity (DTH) response compared to pre-challenge, as also observed by Quist *et al.* (11) and Lacetera and Ronchi (5). In a study in deer, Quist *et al.* (11) observed that both epizootic haemorrhagic disease virus- and BTV-infected deer had reduced *in vitro* and occasionally *in vivo* proliferation of lymphocytes in response to mitogen (PHA) during acute infection. Recently, Lacetera and Ronchi (5) showed that inoculation of goats with a live attenuated BTV serotype 2 vaccine caused discernible and profound depression of non-specific lymphocyte blastogenesis as measured by DNA synthesis in peripheral blood mononuclear cells stimulated with PHA, concanavalin-A, and pokeweed mitogen.

Vaccines that incorporated each of the three adjuvants elicited similar humoral and cell-mediated immune (CMI) responses. The reduced duration of viraemia and reduction in CRI that occurred following challenge in vaccinated sheep were associated with the presence of only non-neutralising antibodies prior to challenge, however CMI could also have a role in protecting the vaccinated sheep. The authors also conclude that BTV causes immunosuppression (reduced DTH response) in sheep, and that inactivated vaccines can prevent the immunosuppressive effects of virulent BTV.

Acknowledgements

This work was supported by All India Network Programme on Bluetongue Disease, Indian Council of Agricultural Research, New Delhi, India.

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