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MARCELA FORERO BARRIOS

ASUNTO	Radicación	08 025 146
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Patente de Invención	☒	Patente de Modelo de Utilidad	☐
Vía Nacional	☒		

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

1. Documentos estudiados

Descripción:	Folios 4 a 15	10/Marzo/2008
Reivindicaciones:	Folios 18 a 19	10/Marzo/2008
Dibujos:	Folios 16 a 17	10/Marzo/2008

2. Objeto de la invención

Título de la solicitud: “Compuesto Antigénico Multiprotéico Proveniente de Glándula Salival, Intestino y Saco Vitelino de Larva *Boophilus Microplus*, Procedimiento para su Fabricación y Vacuna Elaborada a Partir de ese Compuesto Antigénico”.

El problema planteado en esta solicitud consiste en la necesidad de un complejo de proteínas solubles purificadas de membrana que permiten la producción de un gran espectro de anticuerpos dirigidos contra varias estructuras vitales de las membranas celulares de la glándula salival, e intestino, así como del saco vitelino del parásito.

Para resolver este problema técnico la solicitud en estudio proporciona un compuesto antigénico purificado de multi-proteínas solubles derivado de glándula salival, intestino y saco vitelino de larvas de *Boophilus microplus*, que induce una respuesta inmune contra infestación de garrapatas *Boophilus microplus* en ganado bovino.

El objeto de la presente Solicitud se relaciona con la Clasificación Internacional de Patentes (CIP): A61K 39/00.

3. No es una invención (Art 15 D 486 literal (b))

El objeto de la reivindicación 1 no se considera una invención, de acuerdo con el Art citado.

4. De la Solicitud de Patente

Reivindicaciones (Art 30 D 486, Art 22 PCT)

Considerando que el objeto de la reivindicación 1 corresponde a material biológico aislado de la naturaleza el cual no es patentable, se sugiere al solicitante eliminar esta reivindicación y dejar como reivindicación independiente la reivindicación 3 incluyendo las características esenciales mencionadas en la reivindicación 1. Sin embargo el solicitante debe tener en cuenta que la reivindicación 1 no es clara porque se encabeza como compuesto antigénico, a pesar de que se trata de varias proteínas aisladas de la garrapata *Boophilus microplus* y no de una sola, por lo tanto se sugiere realizar las correcciones necesarias.

La reivindicación 1 hace referencia a un disclaimer que no tiene sustento en la descripción porque se dice que carece de tres proteínas de 35 kDa, 13 kDa y 6.5 kDa provenientes de glándula salival y una proteína de 27 kDa proveniente de intestino. Se sugiere incorporarla a la descripción para dar sustento. Esta modificación no supone ampliación puesto que las reivindicaciones iniciales se presentaron con la descripción (Art. 26 D 486).

La reivindicación 1 contiene la frase "inhibitorias del sistema inmune del huésped", lo cual no es una característica técnica que caracterice la invención. Se sugiere eliminarla del pliego reivindicatorio.

La reivindicación 2 se refiere a la ultrafiltración tangencial que si bien se nombra en el capítulo descriptivo no se encuentra soporte de su uso durante el proceso. Se sugiere restringir la solicitud a una razonable generalización según lo que se encuentra debidamente soportado en el capítulo descriptivo.

En la reivindicación 2, en el paso b se encuentra repetida la palabra "de". Se sugiere realizar la corrección respectiva.

La reivindicación 3 no es concisa porque se refiere a un inmunopotenciador, un preservativo y un adyuvante farmacéuticamente aceptables definiéndolos por su funcionalidad y no por un ingrediente como tal. Se recuerda al solicitante que esta manera de reivindicar no es adecuada porque no se definen los ingredientes como tal y por lo tanto no permiten distinguirlos del estado de la técnica.

La expresión "igual o mayor que" de la reivindicación 4 es imprecisa, por lo cual no permite distinguir la invención del estado de la técnica. Se sugiere sustituirlo por un término preciso o un rango concreto.

Las Reivindicaciones 4 y 6 - 8 no están sustentadas en la descripción porque las características como la bromo-etilenamina BEI como adyuvante, el adyuvante oleoso y el manitol como adyuvante oleoso están en las reivindicaciones pero no en la descripción. Se sugiere incorporarlas a la descripción para dar sustento. Esta modificación no supone ampliación puesto que las reivindicaciones iniciales se presentaron con la descripción (Art. 26 D 486).

Las reivindicaciones 4 – 6 y 8 si bien se nombran en el capítulo descriptivo para el escalamiento industrial, no se encuentra soporte descriptivo de la obtención o prueba de ninguna composición farmacéutica que contenga Listerio Lisina LLO inactivada derivada de *Listeria monocytogenes*, en una concentración entre 0,5 µg y 0,1 µg por mililitro, de composiciones con formaldehído o bromo-etilenimida BEI, ni de composiciones con aceite de manitol como adyuvante. Se sugiere restringir la

solicitud a una razonable generalización de lo que se encuentra debidamente soportado, es decir, que se fabricó o probó.

En la reivindicación 6 se hace referencia a formol y formaldehído de manera redundante puesto que estos dos son sinónimos. Se sugiere eliminar la palabra formol del capítulo reivindicatorio.

La reivindicación 9 no contiene ninguna característica esencial adicional a las divulgadas en las reivindicaciones previas por lo cual se sugiere eliminarla del pliego reivindicatorio.

Descripción (Art 28 D 486)

En los antecedentes de la invención se encuentran varios documentos de patentes, sin embargo algunos no se encuentran adecuadamente identificados con su número de publicación puesto que no tienen las dos letras que identifican el país en el que se realizó la publicación.

Se sugiere describir brevemente las graficas de los folios 16 y 17 en una sección dentro del capítulo descriptivo.

Las características del uso de bromo-etilenamina BEI como adyuvante, el adyuvante oleoso y el manitol como adyuvante oleoso, que se mencionan en las reivindicaciones, pero no se mencionan en la descripción, pueden ser incorporadas a la descripción para que estén sustentadas. Esta modificación no supone ampliación puesto que las reivindicaciones iniciales, que mencionan dichas características, fueron presentadas con la descripción.

Se requiere al Solicitante hacer las aclaraciones necesarias.

5. Determinación del Estado de la Técnica

El estado de la técnica se determinó con base en la fecha de presentación de la solicitud de patente nacional: 10/Marzo/2008 y se encontraron los siguientes documentos que anticipan el objeto de esta solicitud:

Ítem	Documento	Título	Fecha de Publicación
D1	US 5,587,311	DNA Encoding a Cell Membrane Glycoprotein of a Tuck Gut	24/Diciembre/1996
D2	da SILVA VAZ Jr, ET AL, Veterinary Immunology and Immunopathology	Immunization of Bovines with an Aspartic Proteinase Precursor Isolated from <i>Boophilus microplus</i> eggs	1998
D3	OLSEN, W. ET AL, Graduate Faculty of the University of North Dakota.	Induced Resistance to Ixodid Tick Infestation: Analysis and Isolation of Antigens	Diciembre/1987

D1<http://worldwide.espacenet.com/publicationDetails/originalDocument?CC=US&NR=5587311A&KC=A&FT=D&ND=3&date=19961224&DB=EPODOC&locale=en> EP divulga (Col. 1, líneas 11 - 22) un antígeno aislado de *Boophilus microplus* que administrado al ganado como una vacuna resulta en le producción de respuesta inmune. La vacuna comprende al menos un inmunógeno junto con un vehículo farmacéuticamente aceptable, adyuvante, inmunopotenciador o diluyente.

D2 divulga (Pág. 331, Abstract, Fl. 43) la capacidad de la pro-catepsina de la yema de *Boophilus* para inducir una respuesta inmune en el ganado en contra de

Boophilus microplus.

D3 divulga (Pág. 45, línea 6 – 26, Fl. 49; Pág. 51, líneas 22 – 24, Fl. 51; Pág. 52, líneas 19 – 20, Fl. 52; Pág. 53, líneas 21 – 25, Fl. 53; Pág. 59, líneas 15 – 20, Fl. 54) antígenos de *Boophilus microplus*, extractos de diferentes órganos, vacunas, antígenos y procesos para su aislamiento y purificación

6. Examen de Patentabilidad (Art 14 D486)

Nivel Inventivo (Art18 D486)

La reivindicación 3 consiste en una vacuna para el control de garrapatas *Boophilus microplus* en ganado bovino caracterizada por comprender como componente activo el compuesto antigénico de la reivindicación 1, en presencia de un inmunopotenciador, un preservativo y un adyuvante farmacéuticamente aceptables.

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

Características esenciales	D1	D2
Vacuna de multi-proteínas solubles de larvas de garrapata <i>Boophilus microplus</i> que comprende:	Vacuna de antígenos de <i>Boophilus microplus</i> de comprende:	Antígenos de <i>Boophilus microplus</i>
3 glicoproteínas de 147 kDa, 94 kDa y 73 kDa, y dos proteínas simples de 209 kDa y 68 kDa provenientes de intestino Dos glicoproteínas de 130 kDa y 66 kDa y una proteína simple de 110 kDa provenientes de glándula salival, 3 glicoproteínas de 160 kDa, 140 kDa y 85 kDa provenientes de saco vitelino; Carece de 3 proteínas de 35 kDa, 13 kDa y 6.5 kDa provenientes de glándula salival y una proteína de 27 kDa proveniente de intestino.	Antígenos derivados de glándulas salivales e intestino. Algunos antígenos son glicoproteínas. Antígenos entre 79 y 205 kDa	Antígeno de la yema.
Inmunopotenciador	Inmunopotenciador	No se menciona
Preservativo	Adyuvante	No se menciona
Adyuvante	Adyuvante	No se menciona

Los documentos D1 y D2 se consideran el estado de la técnica cercano a la invención definida en la reivindicación 1 porque divulgan vacunas que contienen antígenos aislados de *Boophilus microplus*. El documento D1 divulga una vacuna que contiene un antígeno aislado de intestino de *Boophilus microplus* (Col. 5, líneas 11 – 28) un vehículo farmacéuticamente aceptable, un adyuvante, un inmunopotenciador o un diluyente (Col. 6, líneas 28 – 31) y enseña que se han usado antígenos derivados de la glándula salival (Col. 2, líneas 2 – 4) e intestino (Col. 2, líneas 16 – 21), que se han encontrado antígenos de pesos moleculares entre 79 y 205 kDa con elevada eficacia como inmunogénos (Col. 3, líneas 22 – 28) y que algunos de los antígenos son glicoproteínas (Col. 12, líneas 48 y 49). El documento D2 da a conocer la capacidad de la pro-catepsina de la yema de *Boophilus* para inducir una respuesta inmune en el ganado en contra de *Boophilus microplus* (Pág. 331, Abstract, Fl. 43), la pro-catepsina fue identificada en extractos de intestino, embrión y larva de *Boophilus microplus* (Pág. 335, líneas 13 – 14, Fl. 45) y enseña que la protección se puede aumentar con una vacuna con múltiples sitios de acción y que la pro-catepsina puede ser usada además de los antígenos del intestino medio o glándula salival (Pág. 339, líneas 42 – 45, Fl. 47).

La diferencia entre la invención, definida en la reivindicación 3 y la vacuna del documento D1 son los antígenos aislados de saco vitelino y las proteínas con los pesos moleculares específicos de intestino, glándula salival y saco vitelino.

El efecto que logra el utilizar antígenos de saco vitelino y el uso de proteínas de peso molecular específico en la vacuna es la producción de anticuerpos luego de realizar la inoculación de los antígenos.

Por lo tanto, el problema técnico objetivo que pretende resolver esta invención se puede formular así: "cómo modificar la vacuna conocida en el documento D1 para obtener una vacuna alternativa que logre la formación de anticuerpos luego de su inoculación".

Sin embargo, la utilización de diferentes antígenos como estrategia para obtener protección por inmunización, así como el uso de otros antígenos como la pro-catepsina de la yema de *Boophilus microplus* además de los antígenos de intestino y glándula salival (Pág. 331, Abstract, Fl. 43; Pág. 339, líneas 42 – 45, Fl. 47), ya está divulgada en el documento D2. Adicionalmente, el documento D1 enseña que la vacuna puede contener más de un inmunógeno (Col. 6, líneas 28 y 29)

Como se mencionó anteriormente las reivindicaciones 4 – 6 no se encuentran soportadas y tampoco se demuestra ningún efecto técnico inesperado generado por el inmunopotenciador, el preservativo o el adyuvante. Adicionalmente, el aislamiento de antígenos del saco vitelino corresponde a una selección de las diferentes partes anatómicas de *Boophilus microplus* con el cual no se observa ningún efecto técnico inesperado.

En consecuencia, la persona del oficio normalmente versada en la materia, aislaría antígenos de partes de *Boophilus microplus* diferentes al intestino y a la glándula salival como se encuentra divulgado en el documento D2 y los utilizaría junto con antígenos de intestino y glándula salival divulgada en el documento D1 para llegar así al objeto de la reivindicación 3 de la solicitud. Por lo cual se considera obvia.

Puesto que las reivindicaciones 4 - 9 no mencionan características inventivas adicionales tampoco se pueden considerar inventivas.

Conclusión:
Las reivindicaciones 3 - 9 cumplen el requisito de novedad (Art. 16), pero no cumplen con el requisito de nivel inventivo (Art. 18).

La reivindicación 2 consiste en: Un procedimiento para obtener un compuesto antigénico como en la reivindicación 1 como se encuentra en los folios 18 y 19.

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

Características esenciales	D1	D5
Procedimiento para obtener un compuesto antigénico que comprende:	Procedimiento de aislamiento y purificación de antígeno de <i>Boophilus microplus</i>	Procedimiento de aislamiento y purificación de antígenos de garrapata
Congelación gradual al vacío de larvas de <i>Boophilus microplus</i> , remoción de cutícula y tejidos de envoltura a través de la acción subsecuente de hidróxido de sodio, ácido nítrico y la adición de enzima quitinaza, con la posterior separación de saco vitelino, intestino y glándulas salivales por micro succión.	Remoción de cutícula.	Obtención de extractos de glándula salival, huevos e intestino.

Características esenciales	D1	D5
Extracción de proteínas solubles de saco vitelino y membranas de células de glándula salival e intestino de <i>Boophilus microplus</i> y eliminación de tres proteínas de glándula salival con pesos moleculares de 35 kDa, 13 kDa y 6.5 kDa y 1 proteína de intestino con peso molecular de 27 kDa, por ultrafiltración tangencial y electroforesis preparativa en SDS-PAGE, para obtener una fracción de proteínas de peso molecular de 250 kDa 60 kDa.	Extracción, centrifugación y congelamiento de precipitados. Electroforesis de gel de poliacrilamida SDS.	Extractos de glándula salival e intestino. Cromatografía de membrana. Electroforesis SDS-PAGE.
Purificación del eluido electroforético por cromatografía de inmunoafinidad donde al sustrato se anclan anticuerpos policlonales IgY, obtenidos de yema de huevo de gallina previamente inmunizada con los antígenos de larva de <i>Boophilus microplus</i> , específicos para capturar un complejo único de tres glicoproteínas de 147 kDa, 94 kDa y 73 kDa y dos proteínas simples de 209 kDa y 68 kDa provenientes de intestino, dos glicoproteínas de 130 kDa y 66 kDa y una proteína simple de 110 kDa provenientes de glándula salival y tres glicoproteínas de 160 kDa, 140 kDa y 85 kDa provenientes de saco vitelino que constituye el material antigénico multiproteico purificado.	No se menciona	Técnica Inmunoblot. Purificación de anticuerpos por técnicas de inmunoafinidad.

Los documentos D1 y D3 constituyen el estado de la técnica cercano a la invención porque dan a conocer procedimientos para el aislamiento y purificación de antígenos de *Boophilus microplus*. El documento D1 da a conocer un procedimiento de aislamiento de antígenos de *Boophilus microplus* en el que adultas femeninas de *Boophilus microplus* son homogenizadas, se retira la cutícula (Col. 3, líneas 12 y 13; Col. 16, líneas 30 – 31) y se realiza electroforesis en gel con SDS (Col. 17, líneas 29 – 32). El documento D3 enseña la obtención de los órganos como el intestino, la glándula salival y los huevos de la garrapata (Pág. 59, líneas 15 – 20, Fl. 54), el uso de extractos de glándula salival e intestino (Pág. 45, línea 6 – 26, Fl. 49), el aislamiento de antígenos por cromatografía de membrana (Pág. 50, líneas 18 y 19, Fl. 50), electroforesis SDS-PAGE (Pág. 51, líneas 22 – 24, Fl. 51), el uso de la técnica inmunoblot (Pág. 52, líneas 19 – 20, Fl. 52) y el uso de anticuerpos para obtener antígenos purificados por técnicas de inmunoafinidad (Pág. 53, líneas 21 – 25, Fl. 53).

Las diferencias entre la invención, definida en la reivindicación 2 y el procedimiento del documento D1 es el congelamiento inicial de las larvas, la remoción de la cutícula y envoltura con hidróxido de sodio, ácido nítrico y quitinaza, la obtención de extractos de glándula salival, intestino y saco vitelino y el uso de cromatografía de inmunoafinidad para la purificación de los antígenos.

El efecto que logra separar los órganos en extractos diferentes y la purificación de los antígenos es la obtención de un antígenos purificados de glándula salival, intestino y saco vitelino de *Boophilus microplus*.

Por tanto, el problema técnico objetivo consiste en “cómo modificar el procedimiento del documento D1 para obtener antígenos purificados de glándula salival, intestino y saco vitelino de *Boophilus microplus*”.

El documento D3 describe una manera de solucionar el problema técnico, en el que se separan los órganos de la garrapata como el intestino, la glándula salival y los huevos y se preparan extractos de cada uno de éstos (Pág. 45, línea 6 – 26, Fl. 49; Pág. 59, líneas 15 – 20, Fl. 54), y el uso de técnicas de inmunoafinidad para obtener antígenos purificados (Pág. 53, líneas 21 – 25, Fl. 53).

En cuanto al congelamiento inicial de las larvas y el uso de hidróxido de sodio, ácido nítrico y quitinaza para la remoción de la cutícula y envoltura, no se observa ningún efecto técnico inesperado generado, por lo cual no le otorgan nivel inventivo al procedimiento.

La reivindicación es obvia, porque la persona del oficio normalmente versada en la materia enfrentada al problema de "obtener antígenos purificados de glándula salival, intestino y saco vitelino de *Boophilus microplus*", obtendría "extractos de los órganos de la garrapata y purificaría los antígenos con técnicas de inmunoafinidad como se revela en el documento D2 logrando de esta forma el procedimiento objeto de esta solicitud.

Por lo cual el procedimiento de la reivindicación 2 carece de Nivel Inventivo.

Conclusión:
La reivindicación 2 cumple el requisito de novedad (Art. 16), pero no cumple con el requisito de nivel inventivo (Art. 18).

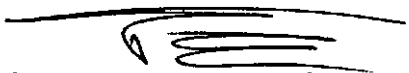
7. Conclusión

Los requisitos de Patentabilidad de la presente Solicitud están afectados así:

Ítem	Documento	Reivindicaciones afectadas	Requisito que afecta
D1	US 5,587,311	2 - 9	Nivel Inventivo
D2	da SILVA VAZ Jr, ET AL, Veterinary Immunology and Immunopathology	3 - 9	Nivel Inventivo
D3	OLSEN, W. ET AL, Graduate Faculty of the University of North Dakota.	2	Nivel Inventivo

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

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


JOSÉ LUIS SALAZAR LÓPEZ
Director de Nuevas Creaciones (C)

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

Elaborado por: Jeny Paola Villate Becerra *P. Villate*
Revisado por: Consuelo Leguizamón *CL*

1. Introduction

The methods for the control of the tick *Boophilus microplus* are based mainly on the use of acaricides in spite of the high costs of these products and resistance induced on tick populations (Nalau, 1985). Due to these facts, the possibility of protecting bovines by means of an artificially induced immune response against the tick has been intensively pursued by different research groups as an additional method of control (Wikel, 1982; Willadsen et al., 1989; Riding et al., 1994; Rodriguez et al., 1994).

The capacity of animals to produce an immune response to crude tick extracts and purified proteins has been described (Wikel, 1996). It was observed that the fecundity rates of ticks fed on bovines immunized with egg, larva or adult tissues from *B. microplus* were lower than those fed on unimmunized bovines (Opdebeeck et al., 1989). Two antigens (Bm86 and Bm91) were isolated, characterized, cloned and both showed to be able to induce a protective immunity against *B. microplus* (Rand et al., 1989; Willadsen et al., 1989; Riding et al., 1994). Recombinant Bm86 has been used to produce the first commercial vaccine against the cattle tick and the results obtained with this protein have demonstrated the possibility of controlling tick populations by an immunological approach. However, it will be necessary to search for other protective antigens, since chemicals still have to be used together with Bm86 in order to achieve an efficient control of ticks (Labarta et al., 1996).

In this paper we show that BYC, a protein isolated from *B. microplus* eggs (Logullo et al., 1998), is able to induce a partially protective immune response when inoculated into cattle. We also use the inoculation of monoclonal antibodies (MAbs) against BYC into fully engorged *B. microplus* females to evaluate their effect on tick physiology.

2. Material and methods

2.1. Antigen preparation

BYC was isolated from 1-day tick egg homogenate by anion-exchange chromatography on DEAE-Toyopearl, followed by gel filtration in a Sephacryl S-200 column (Logullo et al., 1998).

One-day eggs were homogenized in a tissue grinder in PBS with 0.1% of pepstatin A, 0.1% leupeptin and 0.1 mM (TPCK) and centrifuged at 11 000 × g for 10 min at 4°C. The supernatant was used for SDS-PAGE.

The tissues (salivary gland, gut, embryo and larva) were sonicated, solubilized with 0.5% sodium dodecylsulfate and centrifuged at 32 000 × g for 40 min (da Silva Vaz Jr. et al., 1994).

2.2. Production of monoclonal antibodies (MAbs)

BA1.B/c mice were inoculated three times at intervals of 10 days by the intraperitoneal route with 100 µg BYC plus 0.2 mg of saponin in phosphate-buffered saline pH 7.2 (PBS). For the intrasplenic booster with 50 µg of BYC in PBS the mice were

Immunization of bovines with an aspartic proteinase precursor isolated from *Boophilus microplus* eggs

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Abstract

The capacity of the *Boophilus* Yolk pro-Cathepsin (BYC) to induce a protective immune response in cattle against *Boophilus microplus* infestation was tested by vaccination experiments and by inoculation of monoclonal antibody (MAb) against BYC into fully engorged tick females. In immunization experiments the measurement of various biological parameters demonstrated a partial protection against *B. microplus*. A continuous decrease in the levels of specific antibodies was observed over 11 months when six bovines were maintained in field conditions. The inoculation of the MAb into tick females produced a dose-dependent decrease in oviposition and survival of the ectoparasite compared to the control. © 1998 Elsevier Science B.V. All rights reserved.

Keywords: *Boophilus microplus*; Monoclonal antibodies; Vaccine; Tick; Bovine; Aspartic proteinase

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anesthetized. After 3 days the animals were sacrificed and the spleen cells were fused to SP2.0 myeloma cells with polyethylene glycol according to the method of Köhler and Milstein (1975). Hybridoma culture supernatants were screened for antibodies to BYC by ELISA (Harlow and Lane, 1988). Cloned hybridoma cells secreting MAbs were inoculated into BALB/c mice previously injected with pristane[®] to induce ascites formation (Harlow and Lane, 1988). The ascites were dialyzed with 20 mM sodium phosphate buffer, pH 8.4 and applied to a protein G-Sepharose column (Pharmacia Biotech, Uppsala, Sweden) equilibrated with the same buffer and eluted with 100 mM glycine HCl buffer, pH 2.7. The fractions containing IgG were dialyzed against PBS.

2.3. Inoculation of MAbs into the ticks

Fully engorged female ticks were inoculated on the ventral surface with a microsyringe containing 10–60 µg of MAb against BYC (BrBm5). As control groups, ticks non-inoculated or inoculated with 40 µg of a non-related MAb (MAb OC3 against Foot and Mouth Disease Virus, supplied by Dr Sandra F. Farias, Centro de Biotecnologia do Estado do Rio Grande do Sul, Brazil) or 20 µl of PBS alone were used. Each group of ticks (5 g each) was maintained at 28°C and 85% relative humidity until egg hatching. The effects of these inoculations were evaluated from alterations in biological parameters such as weight of the eggs produced, number of ticks that completed oviposition and weight of fertile eggs.

2.4. Cattle vaccination and challenge trials

Hereford cattle 6 months old were purchased from a tick-free area and housed in individual tick-proof pens on slatted floors. In the Experiment 1, the test group (T5, T6, T7 and T8) received four intramuscular inoculations of 100 µg of BYC plus 1 mg of Quil A[®] in PBS at 2 week intervals and the control group (T1, T2, T3 and T4) received Quil A[®] inoculations alone. Cattle were challenged 2 weeks after the last inoculation with 30 000 larvae 10 days old. In the Experiment 2, the same immunization protocol was used for the test group (T13, T14, T15 and T16) and for the control group (T9, T10, T11 and T12) with the difference that they were challenged with 17-day old larvae. Tick larvae used in both experiments originated from the same pool of eggs. After the infestation period the cattle were maintained in field conditions for 8 months.

2.5. Analysis of the ticks

From the 20 post-infestation day until the end of the infestation, the ticks that detached spontaneously were collected once a day. Then the ticks were classified as fully or partly engorged, counted and weighed. Every day, up to 5 g of fully engorged ticks were maintained at 28°C and 85% relative humidity for 20 days, when the eggs were collected, classified as fertile or infertile, weighed and incubated again until hatching to ascertain their viability.

The protection was calculated by variation in biological parameters such as: number of fully engorged tick, egg laying capacity and egg fertility. The overall protection was

calculated by the interaction of these parameters with the formula:

$$\text{Overall protection ratio} = 100 \times [1 - (\text{NFE} \times \text{WE} \times \text{WL})]$$

where NFE = number of fully engorged ticks from vaccinated bovine/control bovine, WE = egg laying capacity of ticks from vaccinated bovine/control bovine, WL = egg fertility of ticks from vaccinated bovine/control bovine.

The statistical significance of the results shown in Table 2 was tested in two ways. Differences between egg-laying capacities and egg fertilities of ticks on control and vaccinated cattle were evaluated with a standard *t*-test and the variation in the number of engorged ticks in both the groups was examined as a two-way analysis of variance (ANOVA) with replication (Sokal and Rohlf, 1969).

2.6. ELISA

To test the immune response of the bovine against BYC, bovine sera were collected at various intervals during vaccination, infestation and in the field. The humoral immune response was determined by ELISA as previously described, with minor modifications (da Silva Vaz, Jr. et al., 1994). Microtitration plates were coated with 0.5 µg of BYC per well and the test sera diluted 1:50 up to 1:6400. The serum was considered positive when the reaction of post-inoculation samples showed an optical density (OD) difference higher than the mean plus 2 standard deviation of the OD from pre-inoculation serum (control).

For the detection of BYC in tick tissues (salivary gland, gut, embryo and larva), the microtitration plates were coated with 3 µg well⁻¹ of tissue antigens or with 50 ng well⁻¹ of BYC and tested with MAb (2 µg well⁻¹). The result was considered positive when OD was two times the OD obtained with the non-related MAb (negative control).

2.7. SDS-PAGE

Sodium dodecyl sulfate polyacrylamide gel electrophoresis with 3% acrylamide in stacking gel and 10% in running gel was used to run the tissue extracts (20 µg protein lane⁻¹) and BYC (10 µg protein lane⁻¹) in sample buffer containing 1% SDS, 1% Tris pH 6.8, 0.04% bromophenol blue and 10% glycerol. Electrophoresis was performed at 70 mA in stacking gel and at 150 mA in running gel and then stained with Coomassie blue.

2.8. Western blot

For Western blot analysis, 1 day egg samples (in a concentration of 50 µg of proteins cm⁻¹ of gel) were separated by SDS-PAGE and transferred to nitrocellulose at 70 V for 1 h at 4°C in 12 mM carbonate buffer pH 9.9 (Dunn, 1986). The nitrocellulose sheet was blocked with 5% blotto for 1 h at room temperature. The MAbs (4 µg ml⁻¹), the bovine serum obtained after the fourth inoculation of BYC inoculation (1:400) or rabbit serum anti-vitellogenin (1:100) were incubated in 5% blotto for 2 h at room temperature. Then goat anti-IgG-peroxidase conjugate in 5% blotto was incubated for 1 h

at room temperature. After three washes with 1% blotto and once with PBS, the chromogen and the substrate were added (5 mg 3,3'-diaminobenzidine in 30 ml PBS plus 150 µl H₂O₂ and (400 µl CoCl₂) and incubated until the bands were suitably dark (Towbin et al., 1979; Burnette, 1981).

3. Results

To test the feasibility of using BYC as a protective antigen, ticks were injected with MAb against BYC. Five IgG1 MAbs with specificity for BYC were produced (BrBm5, BrBm6, BrBm7, BrBm8 and BrBm9). The BrBm5 was used for subsequent experiments since it had a higher reactivity than the other MAbs and by ELISA competitive assay all MAbs seem to react with the same epitope of the antigen. By means of ELISA and Western blot performed with this MAb, we identified BYC in gut, embryo and larva extracts from *B. microplus* (data not shown).

Several ticks inoculated with BrBm5 did not complete oviposition and the females that successfully oviposited in the groups injected with BrBm5 showed survival rates ranging from 5.4% to 60% less than the control group and laid 2.5–52.6% less eggs (Table 1). The dose of MAb inoculated and the quantity of eggs laid ($r = -0.97$, $p < 0.003$) and the survival rates of ovipositing females ($r = -0.97$, $p < 0.003$) has significant correlation. The eclosion index was not altered (data not shown).

From the data of inhibition of tick reproductive capacity obtained with the BrBm5 inoculation we decided to conduct vaccination experiments with bovines. BYC used in the immunizations was isolated from 1 day tick eggs by anion-exchange and gel filtration chromatography. The degree of purification was verified by SDS-PAGE stained with Coomassie blue as shown in Fig. 1 and the serum antibody titers to BYC were followed

Table 1
Effect of MAb inoculation into fully engorged female tick (5 g each group), results expressed as mean (±SEM) of six independent experiments

	Inoculation group (Mean ± SEM)				
	Control ^a	10 µg	20 µg	40 µg	60 µg
Total egg weight (g)	2.42 (±0.15)	2.30 (±0.10)	2.17 (±0.14)	1.36 (±0.30)**	1.12 (±0.31)**
Egg weight reduction (%) ^b		2.5	7.7	42.2	52.6
Survival index ^c	0.95 (±0.04)	0.84 (±0.04)	0.82 (±0.09)	0.49 (±0.14)*	0.35 (±0.11)**
Survival reduction to oviposition (%) ^d		5.4	7.1	44.8	60.0

^aControl group without any treatment is not statistically different from control group inoculated with 40 µg of non-related MAb OC3 ($p = 0.31$) and from control group inoculated with PBS ($p = 0.27$).

^bPercent of reduction in egg weight was calculated by comparison with the weight of eggs produced by ticks that did not receive any treatment.

^cSurvival index was calculated from the number of ticks that completed oviposition divided by the total number of ticks incubated for oviposition.

^dPercent of reduction in survival to oviposition was calculated by comparison with the number of ticks that did not receive any treatment that completed oviposition.

* $p < 0.05$.
[†] $p < 0.01$ compared to control (Student's t -test).

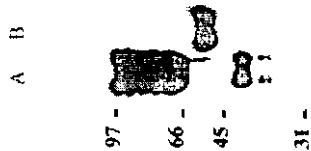


Fig. 1. SDS-PAGE (10% gel performed under reducing conditions) of (A) 1 day egg homogenate; (B) BYC from *B. microplus*. Molecular weight markers $\times 10^3$.

by ELISA and Western blot. After the fourth inoculation, bovines showed titers of antibodies between 1600 and 6400 against BYC (data not shown). All sera recognized BYC in the egg extract; however, other bands were also recognized (Fig. 2). No reaction was observed with the sera obtained from control cattle (data not shown).

Fifteen days after the last immunization the bovines were challenged with *B. microplus* larvae. The efficacy of vaccination was 14% in Experiment 1 and 36% in Experiment 2, considering the reduction in the number of engorged tick, the egg-laying capacity and egg fertility. The principal change in ticks engorged in immunized bovines when compared to

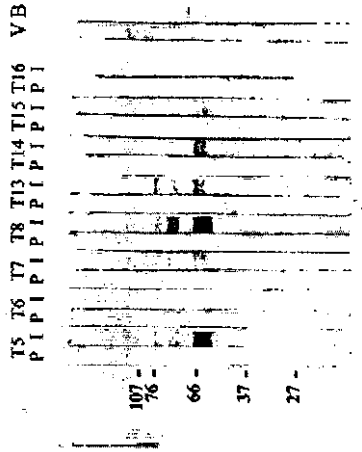


Fig. 2. Western blot analysis of *B. microplus* egg homogenate (SDS-PAGE, 10% gel performed under reducing conditions). Probed with pre-immunized bovine sera (P); immunized bovine sera (P); rabbit serum anti-vitellogenin (V); MAb against BYC (B). Molecular weight markers $\times 10^3$.

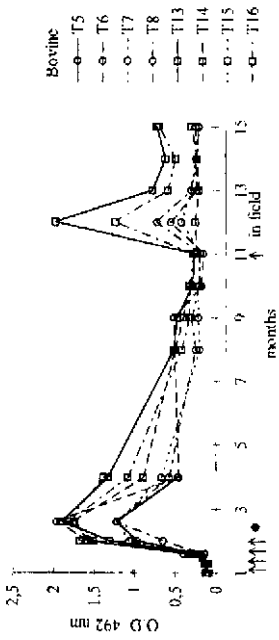


Fig. 3. Dynamics of the humoral immune response of bovines immunized with BYC from *B. microplus*, determined by ELISA (sera diluted 1:100). Arrows indicate the days of immunization and the day of infestation.

anti-BYC antibody levels, three had an intermediary response and only one did not respond (Fig. 3).

4. Discussion

BYC is the precursor of an acidic aspartic proteinase that is synthesized in the fat body and in the gut of the tick, it is secreted to the hemolymph and stored in the oocyte. BYC is processed by limited proteolysis during embryogenesis (Logullo et al., 1998), and it is suggested to play a role in the degradation of yolk proteins, a process in which acid proteinases of the lysosomal type have been described in other invertebrates, including ticks (Fagotto, 1990). In a previous report, we demonstrated the presence of functional anti-BYC antibodies in the hemolymph of ticks feeding on vaccinated cattle (da Silva Vaz Jr. et al., 1996). Here we show that immunization of cattle with BYC can interfere with the reproductive process of the tick. In the case of vaccination with Bm86 the deleterious effects of antibodies on the parasite have been attributed to lesions on the tick digestive tract, which are in direct contact with antibodies from bovine plasma (Willadsen et al., 1989), and the reduced oviposition rates are probably secondary to a general impairment of the parasite physiology. In contrast, analysis of several tick biologic parameters after vaccination with BYC showed a great contribution of effects on the reproductive tract to the overall protection (Table 2). Ticks fed on BYC-injected bovines showed no increase in the number of hemorrhagic females (data not shown) indicating that lesions in the gut are not produced, which is the most striking effect of Bm86 vaccination. On the other hand, the number of larvae hatched from laid eggs was affected in both experiments, this effect being consistent with the role in yolk degradation during embryogenesis proposed for BYC (Logullo et al., 1998).

When analyzed by Western blot, the sera of bovines inoculated with purified BYC (judged by SDS-PAGE) showed reaction with other bands that appeared to be vitellogenin since they have a similar pattern of bands as the ones recognized by 1-day egg extract

Table 2
Cattle vaccination and challenge trial

	Tick number		Weight (gm)		Egg fertility ^a
	Animal	Fully engorged	Egg laying capacity ^b	Egg fertility ^a	
Experiment 1 Control	T1	1,164	0.13		0.84
	T2	2,230	0.45		0.90
	T3	1,211	0.46		0.90
	T4	1,617	0.49		0.89
	T5	1,556 ± 247	0.46 ± 0.01		0.88 ± 0.01
	T6	1,396	0.34		0.88
	T7	1,597	0.39		0.80
	T8	1,209	0.43		0.82
		1,776	0.40		0.83
	Mean ± SE	1,595 ± 151	0.41 ± 0.01		0.81 ± 0.01
Experiment 2 Control	T9	505	0.36		0.77
	T10	659	0.40		0.89
	T11	1,352	0.38		0.69
	T12	1,280	0.45		0.81
	T13	949 ± 215	0.40 ± 0.02		0.80 ± 0.04
	T14	915	0.36		0.68
	T15	665	0.40		0.70
	T16	922	0.40		0.69
		459	0.35		0.68
	Mean ± SE	740 ± 111	0.38 ± 0.01		0.69 ± 0.01
Vaccinated		22	4.2		13.9 ^c
	Difference (%) ^a				

^aDifference (%) = 100 × (1 - mean value of vaccinated group/control group).
^bThe weight of layed eggs by samples of fully engorged ticks, daily collected during 11 days in the course of infestation period, was used to calculate the proportion of the weight of ticks that was converted into eggs.
^cProportion between weight of fertile eggs and weight of layed eggs.
^dp < 0.005.
^ep < 0.01 compared to control.

the control group was in egg fertility, resulting in a reduction of 7.7% and 13.9% in Experiment 1 and 2, respectively. In addition, the egg-laying capacity was significantly reduced (9.2%) in the immunized group of Experiment 1 when compared to the control group. The absolute number of fully engorged ticks produced in Experiment 2 was lower than in Experiment 1. Difference was not observed in the weight of individual fully engorged ticks between vaccinated and control groups indicating that anti-BYC antibodies did not affect feeding (Table 2).

Serum antibody reactivity to BYC gradually declined after the infestation period, and after 11 months, titers close to pre-immune levels were observed. Therefore, to test if a memory response had been generated by immunization with BYC, the cattle were boosted with a single injection of 100 µg of BYC plus 1 mg Quil A⁴⁰. Five out of six animals showed an increase in the serum antibody levels again. Two animals showed high

probed with a rabbit serum anti-vitellogenin. These results could be due to the presence of low quantities of vitelin in the BYC preparation used for immunization or to the cross-reactivity caused by phosphorylated and glycosylated amino acid residues in both proteins.

Protection rates of different cattle breeds immunized with Bm86 antigen challenged with different tick strains show remarkable variability (Canales et al., 1997). Moreover, differences were also observed when animals of the same breed and in the same herd were used. The immunization of bovines with BYC induced a mean overall protection between 14% and 36%, when the various biological parameters from Table 2 were considered together. There was a decrease in the capacity of ticks to complete the parasite life cycle in vaccinated cattle, although the effect of the vaccine was not so remarkable as Bm86 immunization (Canales et al., 1997). The lower protection index observed in our experiment can be explained by the low capacity of BYC to induce a protective immune response or can be related to tick strain variation or to different experimental conditions.

The variation of the protection rate observed between Experiment 1 and 2 could be explained by the difference in the viability of larvae used to challenge the bovines. This may be due to the age of larvae employed in the experiments. The larvae of Experiment 1 were 1 week younger than those of Experiment 2, although they were obtained from the same pool of eggs. In fact, the absolute number of fully engorged ticks in Experiment 1 was higher than the number obtained in Experiment 2. A variation observed in the number of fully engorged ticks in different vaccination experiments has been associated with different conditions of tick larva and climate conditions during the experiment (Opdebeeck et al., 1989; Ridling et al., 1994). Another hypothesis is that an immunosuppressive effect, acting in the feeding process, caused by a higher number of viable ticks in Experiment 1 could be masking the effect of the anti-BYC immune response (Barriga et al., 1993; Schorderet and Brossard, 1993).

The protection rate induced by BYC was insufficient to permit a complete control of the *B. microplus*. One possible reason could be the fact that BYC is abundant in the eggs, therefore a high concentration of antibodies is necessary to induce a significant injury to the tick. Data that corroborate this assumption are the observation that the inoculation of Mab anti-BYC in fully engorged ticks induces a dose-dependent reduction in tick fertility (Table 1). Based in these observations it is possible to infer that the modulation of the anti-BYC immune response can improve the protective capacity of the BYC. This modulation can be achieved by altering the adjuvant or the inoculation protocol (dose and inoculum site) (Brown et al., 1984; Barriga, 1994).

As antibody titers showed a marked decrease some months after the first vaccination (Fig. 3), changes in the immunization protocol to obtain a more sustained antibody response should be examined. However, it is important to note that the immunized cattle showed a memory response after 11 months of primary immunization, essential for long-term protection. Another strategy to increase the protection rate might be the combination of the BYC with other antigens, such as Bm86 and Bm91, generating a multitarget vaccine. Moreover, as has already been discussed, the protection obtained by immunization with BYC seems to be derived from the effects on the reproductive tract which opens the way to search for other protective antigens that do not need to be restricted to the midgut or salivary gland.

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INDUCED RESISTANCE TO IXODID TICK INFESTATION:
ANALYSIS AND ISOLATION OF ANTIGENS

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ineffective while those prepared from organs of ticks which had fed for five days induced resistance.

Organ Extracts

Trager (1939a) speculated that since only the ticks mouth parts contact host tissue, it was probable that some antigenic material in the saliva was injected into the host. Thus guinea pigs were immunized with crude extracts of salivary gland and digestive tract from Dermacentor variabilis (Trager, 1939b). The greatest degree of resistance was obtained using salivary gland extract (Trager, 1939b), but, unfortunately, statistical analysis of results was not presented. Immunization with salivary gland extracts induced resistance to challenge infestations with Hyalomma anatolicum excavatum (Kohler et al., 1967) and Rhipicephalus sanguineus (Garin and Grabarev, 1972). Brossard (1976) immunized calves with Boophilus microplus salivary gland extract prepared from partially engorged female ticks. This resulted in resistance which reduced the number of ticks able to engorge successfully. Wikel (1981) induced a high degree of resistance in guinea pigs using salivary gland antigens (SGA) from partially engorged, adult, female Dermacentor andersoni. Various routes of administration and use of two different adjuvants were evaluated. Intradermal administration of either one or ten micrograms of SGA induced resistance which was not significantly different statistically than that found in guinea pigs made resistant by repeated infestation (Wikel, 1981).

Research Methods

Multiple immunogenic molecules, which can be found in various tick extracts, have been shown to be involved in the complex mechanism of resistance to tick infestation (Whelen et al., 1984; Shapiro et al., 1986; Gill et al., 1986). The goal of this research was to separate immunogenic components of tick extracts and determine if they have a role in inducing resistance to tick infestation. A long range objective of research in this laboratory is to develop an immunization capable of artificially inducing resistance to tick infestation. Development of immunological and biochemical techniques with increased sensitivity and specificity have resulted in tools which can be used to analyze the significance of specific immunogens on the development of resistance to tick infestation. Specific tools used in this endeavor include: membrane ultrafiltration, gel filtration chromatography, electrophoretic analysis, immunoblotting, production of monoclonal antibodies, and Dot-ELISA.

Defined molecular weight range fractions of the tick extracts used in this study were prepared using membrane ultrafiltration. Blatt et al. (1965) demonstrated that ultrafiltration using membranes formed from the complex interaction product of polyanions and polycations (Dialplex or Diaflo membranes, Amicon, Blatt et al., 1965; 1967) concentrated protein solutions rapidly with no signs of denaturation. Membranes are available to fit specific ultrafiltration tasks and capable of screening solutes of varying molecular weights (Blatt et al., 1965). The chief advantage of this ultrafiltration technique is

that it rapidly and simply resolves complex mixtures into size graded classes, but one achieves limited fractionation rather than total resolution (Blatt et al., 1967).

Gel filtration chromatography using a Sephadex G-200 column was also used to fractionate antigens. Sephadex (Gel filtration, 1972) is a bead-formed gel prepared by cross-linking dextran with epichlorohydrin. Containing a large number of hydroxyl groups, the gel is very hydrophilic and swells readily in water and electrolyte solutions. The gel beads contain pores which function as the stationary phase. Very large molecules, above the exclusion limits, never enter the stationary phase, but move through the column with the liquid phase. Molecules which can enter the gel pores, spend time in the stationary phase, and move more slowly through the column. Thus, molecules are eluted in order of decreasing molecular size. This is basically a sieving action. Sephadex G-200 has a molecular weight fractionation range of five to 800 Kdal for polypeptides and globular proteins. In addition to fractionation of solutes, these columns can be calibrated and used to determine molecular weights of proteins (Ackers, 1964; Gel filtration, 1972; 1984). By pooling specific aliquots, molecular weight range fractions of tick extracts similar to those obtained by ultrafiltration were prepared.

Fraction separations obtained by ultrafiltration and gel filtration chromatography were analyzed by SDS-PAGE gels stained with the Morrissey modified silver stain (Morrissey, 1981). This staining method is 100 times more sensitive than Coomassie blue staining (Morrissey, 1981). Polyacrylamide gels are the medium of choice for

zone electrophoresis of most proteins because it can be reproducibly prepared with a wide range of pore sizes (Hames, 1981). The principle of polyacrylamide electrophoresis is that molecules are separated based on molecular charge and size. The sieving action of polyacrylamide results in high resolution (Hames, 1981). Superior resolution of complex protein mixtures is obtained using the Laemmli SDS-discontinuous buffer system (Hames, 1981; Laemmli and Favre, 1973). In this system, the protein mixture is denatured at 100 C in the presence of excess SDS and a thiol reagent which cleaves disulphide bonds generating polypeptide subunits. This results in most polypeptides binding SDS in a constant ratio of 1.4 g SDS/g of polypeptide. The very high negative charge of SDS negates the normal charge of the polypeptide and separation is then strictly according to polypeptide size. In addition to determining composition, molecular weight can be established by comparing the relative mobility to that of molecular weight standard markers on the same gel (Hames, 1981). This method was used to determine the composition and molecular weight of components and/or subunits of tick extracts.

Involvement of multiple immunogenic molecules has been demonstrated by immunoblotting. Immunoblotting is an advanced immunological procedure developed by Towbin et al. (1979) in which complex mixtures of proteins are first fractionated into their polypeptide components by polyacrylamide gel electrophoresis, usually in the presence of SDS, and are then electroeluted to a nitrocellulose sheet. The polypeptide bands are immobilized on the nitrocellulose in exactly the same pattern found in the gel. This nitrocellulose sheet can then be

stained for protein pattern or reacted with antibodies (first antibody) after blocking unoccupied protein binding sites. Visualization or immunodetection of bands is accomplished by reacting the nitrocellulose with a second antibody (antispecies immunoglobulin) which has been tagged with radioisotopes, fluorochromes, or one of the very sensitive enzyme tags such as horseradish peroxidase and visualized by autoradiography, illumination with ultraviolet light, or addition of a precipitable substrate respectively. Use of horseradish peroxidase allows detection of as little as 100 pg protein. This was an important advance in that smaller amounts of antibody, or ligand, can be used to determine reactivity and the problem of gel pore size limiting diffusion is avoided (Towbin et al., 1979; Gershoni and Palade, 1983). Although a faithful replica of SDS-PAGE gel patterns could be transferred, they were not quantitative (Towbin et al., 1979). The modifications of Burnette (1981) resulted in quantitative transfer of SDS-PAGE gel patterns to nitrocellulose. Greater than 90% of all proteins with molecular weights up to 100 Kdal were transferred in 20 hours. The sensitivity of immunodetection is a function of specific antibody titer; but Burnette (1981) was able to detect as little as one to two nanograms of protein using this system.

In a continuing effort to accurately define and acquire antigens, which are involved in resistance to tick infestation, monoclonal antibodies are being produced to immunogenic tick polypeptides. These monoclonal antibodies will eventually be used in immunoaffinity techniques to obtain purified tick antigens. Cell fusion techniques were used to develop monoclonal antibody producing hybridoma cell

Serum was aliquoted into five milliliter screw top glass vials, frozen and maintained at -20 C.

Small amounts of blood were taken from rabbits by nicking the marginal ear vein and collecting the blood in heparinized capillary tubes (Sherwood Medical Industries, ST. Louis, MO). The capillary tubes were spun in a hematocrit centrifuge (International Equipment Company, Boston, MA) to separate the plasma. Larger volumes of blood were taken, using 18 gauge 1 1/2 inch needles and 20 ml syringes, by blind cardiac puncture or the animal was anesthetized and exanguinated by open chest cardiac puncture. Blood was processed in the same manner described for guinea pig blood.

Antigen Preparation

Tick extracts used in this investigation were prepared from both Amblyomma americanum and Dermacentor andersoni ova and salivary glands. Amblyomma americanum gut extract, brush border fragments, and 27000 x g supernatant were also prepared and used in immunization regimens.

Ticks were dissected to obtain the desired organs for extract preparation. This was accomplished by embedding adult ticks in a paraffin filled petri plate such that the extended legs, head, and ventral side were secured in the paraffin leaving the dorsal side exposed. The dish was then flooded with 0.15 M phosphate buffered saline pH 7.2 (PBS, Appendix II) and placed on the stage of a dissecting microscope. Using a Bard-Parker # 11 disposable scalpel blade (Becton Dickinson and Co., Rutherford, NJ) the scutum and exoskeleton