

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO	
RAD: 13-105202- -6	FECHA: 2015-05-16 22:54:25
DEP: 2020 DIRECCION DE NUEVAS CREACIONES	EVE: 378 FASE NACIONAL INCLUIDO CAPITULO I
TRA: 11 PCT-PATENTE DE INVENCION CAPITULOS I Y II	FOLIOS: 11
ACT: 430 REQUERSOLICITA	

DIRECCIÓN DE NUEVAS CREACIONES

2020
Bogotá, D.C.,

Abogado(a)/Señor(a)
LUZ CLEMENCIA DE PÁEZ

Asunto: Radicación: 13-105202- -6
 Trámite: 11
 Evento: 378
 Actuación: 430
 Oficio: 5132
 Folios: 11

Patente de Invención	X			Patente de Modelo de Utilidad	
Vía Nacional					
Vía PCT (Fase Nacional)	X	Capítulo I	X	Capítulo II	
No. Solicitud Internacional	PCT/CU2011/000005				
Fecha de Presentación Internacional	26/09/2011				

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:	Radicado No 13-105202-00000-0000	25/Abril/2013
Reivindicaciones:	Radicado No 13-105202-00000-0000	25/Abril/2013
	Radicado No 13-105202-00005-0000	24/Julio/2014
Secuencias:	Radicado No 13-105202-00000-0000	25/Abril/2013
	Radicado No 13-105202-00005-0000	24/Julio/2014
Prioridad:	CU 20100188	28/Septiembre/2010

2. Análisis de la Prioridad

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

3. Modificaciones no aceptadas (Art 34 D 486 y Art 19, 22 y 34 PCT)

Las reivindicaciones modificadas 2 y 9 folios 7 – 8 presentes en el Radicado No 13-105202-00005-0000 no son aceptadas porque mencionan secuencias nucleótidas SEQ ID NO 17, SEQ ID NO 18, SEQ ID NO 19, SEQ ID NO 20, SEQ ID NO 21, SEQ ID NO 22, SEQ ID NO 23, SEQ ID NO 24, SEQ ID NO 25, SEQ ID NO 26, SEQ ID NO 27 que no habían sido considerados inicialmente, por lo cual amplían el objeto de la solicitud.

El listado de secuencias anexo no se encuentra en el formato establecido, las secuencias se definen de manera incorrecta, se incluye las letras RWSY y no hay certeza de si corresponde a secuencias de aminoácidos o nucleótidos, por lo tanto no se acepta el listado de secuencias presentado en el Radicado No 13-105202-00005-0000.

Por lo anterior se estudiara el capítulo reivindicatorio Radicado No 13-105202-00000-0000.

4. Objeto de la invención

De acuerdo con lo establecido en el numeral 1.2.2.2 del capítulo primero del Título 10 de la Circular Única para efectos del examen de patentabilidad, se tendrá en cuenta la tasa pagada por el solicitante al momento de comenzar el examen para las reivindicaciones adicionales; esto es que cuando la solicitud contenga más de diez (10) reivindicaciones y no se haya pagado la tasa complementaria, sólo se estudiarán las cubiertas por la tasa pagada.

En el presente caso se estudiaran las reivindicaciones desde la 1 hasta la 14 (Radicado No 13-105202-00000-0000)

Título de la solicitud: “Composición vacunal para el control de las infestaciones por ectoparásitos”

El problema planteado en esta solicitud consiste en la necesidad de controlar las infestaciones por ectoparásitos en organismos hospedantes.

Para resolver este problema técnico la solicitud en estudio proporciona una composición vacunal que comprende un péptido de la proteína ribosomal P0.

El objeto de la presente solicitud se relaciona con la Clasificación Internacional de Patentes (CIP): A61K39/00; C07K14/435

5. Reivindicaciones relativas a un uso (Art 14 D 486)

El uso de las reivindicaciones 12-14 no es patentable, de acuerdo con el Art 14.

6. Excepción de la Patentabilidad (Art 20 D 486 literal (d))

El objeto de la reivindicación 9 no es patentable de acuerdo con el art. 20 D 486, toda vez que se refiere a la administración de la composición vacunal a un animal vacunado.

7. De la solicitud de patente

Reivindicaciones (Art 30 D 486, Art 22 PCT)

- 1- La reivindicación 1 no es clara porque menciona una composición vacunal definida por comprender un péptido de la proteína ribosomal P0 y la región aminoácida, lo cual no corresponde a una característica técnica esencial que permita tener certeza de cada uno de los ingredientes de la composición vacunal. Se sugiere definirla indicando los ingredientes y la proporción de los mismos.
- 2- La reivindicación 1 no es clara por cuánto se refiere a secuencias de ectoparásitos en sentido general. Se sugiere especificar la secuencia y el ectoparásito que la comprende.
- 3- El péptido de la reivindicación 2 y 11 definido a partir de fragmento de secuencias de aminoácidos y por un porcentaje de identidad, no porque no permite definir la secuencia específica a la que se refiere. Se sugiere definir el péptido indicando la secuencia aminoácida específica indicando el número de secuencia (SEQ ID NO).
- 4- La reivindicación 3 no es clara porque se refiere a un conjugado en términos generales indicando el origen de la estructura. Se sugiere definir la estructura que esté enteramente sustentada en la descripción, indicando la proteína KLH a la que se fusiona el péptido y el agente espaciador (anhídrido succínico).
- 5- En la reivindicación 3 y 4 no son claras porque mencionan una molécula con la que se conjuga el péptido de la composición, siendo inespecífica porque se refiere a toxinas y proteínas de diferentes virus sin indicar una denominación común o estructura. Se sugiere definirlas por las características estructurales.
- 6- En la reivindicación 5 se describe una composición vacunal por ser efectiva, lo cual corresponde a un resultado a alcanzar y no definen a la composición, puesto que la reivindicación incluiría no sólo la solución propuesta por el solicitante, sino todas las alternativas presentes o futuras que lleguen a ese resultado. Se sugiere reemplazarlo por las características esenciales, estructurales o funcionales, que contribuyen a la obtención de ese efecto.
- 7- En las reivindicaciones 6 y 7 la mención de la vía por la cual se obtiene el péptido que hace parte de la composición vacunal, no corresponde a una característica técnica esencial que defina el objeto reivindicado. Se sugiere definir la composición vacunal por sus ingredientes (Compuesto activo, excipientes, cualidades de los excipientes) y proporción de los mismos.
- 8- La reivindicación 8 menciona un adyuvante vacunal como parte de la composición, se sugiere indicar tipo de adyuvante y la proporción en la composición.
- 9- La reivindicación 10 no es clara porque menciona un ácido nucleico y está definido por codificar un péptido, lo cual no permite tener certeza de la estructura recombinante, se sugiere definirlo por la secuencia específica de nucleótidos, indicando el número de la secuencia (SEQ ID NO).
- 10- La reivindicación 11 no es clara porque define secuencias de aminoácidas en términos de porcentaje de homología de secuencias, lo cual no permite tener certeza de la estructura encargada del efecto al que aduce la solicitante. Se sugiere indicar la secuencia aminoácida específica e indicar el número de la misma (SEQ ID NO).

8. Unidad de invención (Art 25 D 486)

La solicitud no cumple con el requisito de Unidad de Invención, porque se han identificado 4

Grupos Inventivos diferentes que no guardan un concepto comun inventivp y son los siguientes:

1: seqid8	100.00	76.47	70.59	60.61	62.50
2: seqid9	76.47	100.00	68.57	57.58	60.61
3: seqid10	70.59	68.57	100.00	61.76	61.76
4: seqid4	60.61	57.58	61.76	100.00	93.94
5: seqid6	62.50	60.61	61.76	93.94	100.00

Por lo tanto, existen los siguientes grupos de invenciones:

1. Reivindicaciones 1, 2, 11 relativas a una composición vacunal para el control de infestaciones por ectoparásitos que comprende un péptido de la proteína ribosomal P0 correspondiente a la secuencia SEQ ID NO 4, SEQ ID NO 6.(tiene sustento)
2. Reivindicaciones 2, 11 relativas a una composición vacunal para el control de infestaciones por ectoparásitos que comprende un péptido de la secuencia SEQ ID NO 8.(No tiene sustento)
3. Reivindicaciones 2, 11 relativas a una composición vacunal para el control de infestaciones por ectoparásitos que comprende un péptido de la secuencia SEQ ID NO 9. (No tiene sustento)
4. Reivindicaciones 2, 11 relativas a una composición vacunal para el control de infestaciones por ectoparásitos que comprende un péptido de la secuencia SEQ ID NO 10.(no tiene sustento)

La búsqueda ha revelado que el concepto inventivo único es conocido, ya que el documento Genetic Analysis: Biomolecular Engineering 15 (1999) (Págs 143-148) divulga una vacuna contra infestación de garrapatas en animales, que comprende la proteína recombinante Bm86 (Pág.146-147).

Se le sugiere al solicitante restringir la materia a patentar en un solo concepto común inventivo especificando el avance tecnológico sobre el estado de la técnica. Esto se logra entre otras formas definiendo las características esenciales de la invención y mostrando las otras características particulares como dependientes, pero sin perjuicio de la concisión de las nuevas reivindicaciones tanto independientes como dependientes.

El solicitante también puede realizar una o más de las divisionales de acuerdo a los grupos de conceptos técnicos inventivos presentados en esta solicitud, esto claro, sin ampliar el objeto inicial de la invención

9. Determinación del Estado de la Técnica

El estado de la técnica se determinó con base en la fecha de presentación de la solicitud de prioridad: Septiembre 28 de 2010 y se encontraron los siguientes documentos que anticipan el objeto de esta solicitud:

Ítem	Documento	Título	Fecha de Publicación*
D2	<i>Genetic Analysis: Biomolecular</i>	"Vaccination against ticks (Boophilus spp.): the experience with the Bm86-based vaccine"	1999

	Engineering 15 (1999) Págs. 143-148	Gavac"	
D3	Journal of Biotechnology.. (Págs. 135-146 Vol. 33)	"High level expression of the B. microplus Bm86 antigen in the yeast Pichia Pastoris forming highly immunogenic particles for cattle"	1994

El documento D2¹ divulga una vacuna para el control de enfermedades transmitidas por garrapatas. La vacuna contiene la proteína recombinante Bm86 y su actividad biológica (resumen).

El documento D3² divulga la expresión del antígeno *B. microplus* Bm86 en *P. pastoris* (Pág. 137).

10. Examen de Patentabilidad (Art 14 D486)

Nivel inventivo (Art 18 D486)

Grupo inventivo No 1

La reivindicación 1 consiste en “*Composición vacunal para el control de las infestaciones por ectoparásitos que comprende un péptido de la proteína ribosomal P0 de dichos ectoparásitos, correspondiente a la región entre los aminoácidos 267 y 301 de la proteína* (Folio 38 Radicado No 13-105202-00000-0000).

Comparación de las características técnicas esenciales, con las del estado de la técnica:

Características esenciales	D2	D3
Composición vacunal para el control de infestaciones por ectoparásitos que comprende:	Vacuna contra infestación de garrapatas que comprende (Pág. 145. Col. Der. Lit. 2.2)	Vacuna rBm86 que comprende (Pág. 144 Col. Der Lit. 3.3):
Péptido de la proteína ribosomal P0 de dichos ectoparásitos, correspondiente a la región de aminoácidos 267 y 301.	Proteína recombinante Bm86 (Pág.148. Col. Der.)	Antígeno Bm86 (Pág. 138)

El documento D2 se considera el estado de la técnica cercano a la invención definida en la reivindicación 1 porque divulga una vacuna contra infestación de garrapatas en animales, que comprende (Pág. 145. Col. Der. Lit. 2.2): la proteína recombinante Bm86 (Pág.148. Col. Der.)

La diferencia entre la invención, definida en la reivindicación 1 y la composición vacunal de D1 consiste en que la solicitud divulga un péptido de proteína ribosomal P0 correspondiente a la región de aminoácidos 267 y 301.

El efecto que logra el incluir la región de aminoácidos 267 y 301 en una vacuna contra la infestación por ectoparásitos es disminuir el número de parásitos por individuo afectado.

1
21http://worldwide.espacenet.com/nplReferenceDetails/biblio?CC=XP&NR=002957096&KC=&FT=E&DB=worldwide.espacenet.com&locale=en_EP&ND=4&II=3
2 <http://www.sciencedirect.com.ezproxy.unal.edu.co/science/article/pii/S0168165694901066>

Por lo tanto, el problema técnico objetivo que pretende resolver esta invención se puede formular así: cómo modificar la vacuna conocida en el documento D2 para disminuir el número de parásitos por individuo y hacerla más eficiente.

Sin embargo, una vacuna que comprende el antígeno rBm86 recombinante para aumentar el control de ectoparásitos en individuos afectados (Pág. 144 Col. Der Lit. 3.3), ya está divulgado en el documento D3 que se refiere a la expresión del antígeno rBm86 en *Pichia Pastoris* acoplado a otra proteína en la región terminal con un residuo de cisteína (Pág.140. Col. Der.). El documento también divulga ácidos nucleicos (Pág. 137. Col. Izq.).

Por lo tanto, una persona del oficio normalmente versada en la materia, incluiría un antígeno rBm86 expresado en *Pichia Pastoris* del documento D3 en la vacuna divulgada en el documento D2 para llegar así a la vacuna propuesta en la reivindicación 1 de la solicitud. Por lo cual se considera obvia.

En consecuencia, la reivindicación 1 de acuerdo con lo divulgado en los documentos D2 y D3 carece de nivel inventivo.

Las reivindicaciones dependientes 3 – 8 no mencionan características adicionales, por lo tanto tampoco tienen nivel inventivo.

Conclusión

La reivindicación 1 cumple el requisito de novedad (Art. 16), pero no cumple con el requisito de nivel inventivo (Art. 18).

Grupo inventivo No 2

La reivindicación 2 consiste en “*Composición vacunal que comprende un péptido que tiene la secuencia SEQ ID NO 8*” (Folio 38 Radicado No 13-105202-00000-0000).

Comparación de las características técnicas esenciales, con las del estado de la técnica:

Características esenciales	D2	D3
Composición vacunal para el control de infestaciones por ectoparásitos que comprende:	Vacuna contra infestación de garrapatas que comprende (Pág. 145. Col. Der. Lit. 2.2)	Vacuna rBm86 que comprende (Pág. 144 Col. Der Lit. 3.3):
Péptido de la proteína ribosomal P0 de dichos ectoparásitos, que comprende la secuencia SEQ ID NO 8.	Proteína recombinante Bm86 (Pág.148. Col. Der.)	Antígeno Bm86 (Pág. 138)

El documento D2 se considera el estado de la técnica cercano a la invención definida en la reivindicación 1 porque divulga una vacuna contra infestación de garrapatas en animales, que comprende (Pág. 145. Col. Der. Lit. 2.2): la proteína recombinante Bm86 (Pág.148. Col. Der.)

La diferencia entre la invención definida en la reivindicación 1 y la composición vacunal de D1 consiste en que la solicitud divulga un péptido de proteína ribosomal P0 que comprende la secuencia SEQ ID NO 8.

No se evidencia un efecto técnico asociado a una vacuna para el control de las infestaciones por ectoparásitos que comprenda la secuencia SEQ ID NO 8 porque la solicitud no define que la secuencia aminoácida SEQ ID NO 8 esté comprendida en la composición vacunal, solamente hace mención que las secuencias SEQ ID NO 4, SEQ ID NO 6 están comprendidas en la vacuna (Págs.30-32).

El problema técnico objetivo que pretende resolver esta invención se puede formular así: Cómo proporcionar una vacuna alternativa contra infestación por ectoparásitos.

Sin embargo, una vacuna que comprende el antígeno rBm86 recombinante para aumentar el control de ectoparásitos en individuos afectados (Pág. 144 Col. Der Lit. 3.3), ya está divulgado en el documento D3 que se refiere a la expresión del antígeno rBm86 en *Pichia Pastoris* acoplado a otra proteína en la región terminal con un residuo de cisteína (Pág.140. Col. Der.). El documento también divulga ácidos nucleicos (Pág. 137. Col. Izq.).

Por lo tanto, una persona del oficio normalmente versada en la materia, incluiría un antígeno rBm86 expresado en *Pichia Pastoris* del documento D3 en la vacuna divulgada en el documento D2 para llegar así a la vacuna propuesta en la reivindicación 1 de la solicitud. Por lo cual se considera obvia.

En consecuencia, la reivindicación 1 de acuerdo con lo divulgado en los documentos D2 y D3 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).

Grupo inventivo No 3

La reivindicación 2 consiste en “*Composición vacunal que comprende un péptido que tiene la secuencia SEQ ID NO 9*” (Folio 38 Radicado No 13-105202-00000-0000).

Comparación de las características técnicas esenciales, con las del estado de la técnica:

Características esenciales	D2	D3
Composición vacunal para el control de infestaciones por ectoparásitos que comprende:	Vacuna contra infestación de garrapatas que comprende (Pág. 145. Col. Der. Lit. 2.2).	Vacuna rBm86 que comprende (Pág. 144 Col. Der Lit. 3.3):
Péptido de la proteína ribosomal P0 de dichos ectoparásitos, que comprende la secuencia SEQ ID NO 8.	Proteína recombinante Bm86 (Pág.148. Col. Der.)	Antígeno Bm86 (Pág. 138)

El documento D2 se considera el estado de la técnica cercano a la invención definida en la reivindicación 1 porque divulga una vacuna contra infestación de garrapatas en animales, que comprende (Pág. 145. Col. Der. Lit. 2.2): la proteína recombinante Bm86 (Pág.148. Col. Der.)

La diferencia entre la invención definida en la reivindicación 1 y la composición vacunal de D1 consiste en que la solicitud divulga un péptido de proteína ribosomal P0 que comprende la secuencia SEQ ID NO 9.

No se evidencia un efecto técnico asociado a una vacuna para el control de las infestaciones por ectoparásitos que comprenda la secuencia SEQ ID NO 9 porque la solicitud no define que la secuencia aminoácida SEQ ID NO 9 esté comprendida en la composición vacunal, solamente hace mención que las secuencias SEQ ID NO 4, SEQ ID NO 6 están comprendidas en la vacuna (Págs.30-32).

El problema técnico objetivo que pretende resolver esta invención se puede formular así: Cómo proporcionar una vacuna alternativa contra infestación por ectoparásitos.

Sin embargo, una vacuna que comprende el antígeno rBm86 recombinante para aumentar el control de ectoparásitos en individuos afectados (Pág. 144 Col. Der Lit. 3.3), ya está divulgado en el documento D3 que se refiere a la expresión del antígeno rBm86 en *Pichia Pastoris* acoplado a otra proteína en la región terminal con un residuo de cisteína (Pág.140. Col. Der.). El documento también divulga ácidos nucleicos (Pág. 137. Col. Izq.).

Por lo tanto, una persona del oficio normalmente versada en la materia, incluiría un antígeno rBm86 expresado en *Pichia Pastoris* del documento D3 en la vacuna divulgada en el documento D2 para llegar así a la vacuna propuesta en la reivindicación 1 de la solicitud. Por lo cual se considera obvia.

En consecuencia, la reivindicación 1 de acuerdo con lo divulgado en los documentos D2 y D3 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).

Grupo inventivo No 4

La reivindicación 2 consiste en “*Composición vacunal que comprende un péptido que tiene la secuencia SEQ ID NO 10*” (Folio 38 Radicado No 13-105202-00000-0000).

Comparación de las características técnicas esenciales, con las del estado de la técnica:

Características esenciales	D2	D3
Composición vacunal para el control de infestaciones por ectoparásitos que comprende:	Vacuna contra infestación de garrapatas que comprende (Pág. 145. Col. Der. Lit. 2.2).	Vacuna rBm86 que comprende (Pág. 144 Col. Der Lit. 3.3):
Péptido de la proteína ribosomal P0 de dichos ectoparásitos, que comprende la secuencia SEQ ID NO 8.	Proteína recombinante Bm86 (Pág.148. Col. Der.)	Antígeno Bm86 (Pág. 138)

El documento D2 se considera el estado de la técnica cercano a la invención definida en la reivindicación 1 porque divulga una vacuna contra infestación de garrapatas en animales, que comprende (Pág. 145. Col. Der. Lit. 2.2): la proteína recombinante Bm86 (Pág.148. Col. Der.)

La diferencia entre la invención definida en la reivindicación 1 y la composición vacunal de D1 consiste en que la solicitud divulga un péptido de proteína ribosomal P0 que comprende

la secuencia SEQ ID NO 10.

No se evidencia un efecto técnico asociado a una vacuna para el control de las infestaciones por ectoparásitos que comprenda la secuencia SEQ ID NO 10 porque la solicitud no define que la secuencia aminoácida SEQ ID NO 10 esté comprendida en la composición vacunal, solamente hace mención que las secuencias SEQ ID NO 4, SEQ ID NO 6 están comprendidas en la vacuna (Págs.30-32).

El problema técnico objetivo que pretende resolver esta invención se puede formular así: Cómo proporcionar una vacuna alternativa contra infestación por ectoparásitos.

Sin embargo, una vacuna que comprende el antígeno rBm86 recombinante para aumentar el control de ectoparásitos en individuos afectados (Pág. 144 Col. Der Lit. 3.3), ya está divulgado en el documento D3 que se refiere a la expresión del antígeno rBm86 en *Pichia Pastoris* acoplado a otra proteína en la región terminal con un residuo de cisteína (Pág.140. Col. Der.). El documento también divulga ácidos nucleicos (Pág. 137. Col. Izq.).

Por lo tanto, una persona del oficio normalmente versada en la materia, incluiría un antígeno rBm86 expresado en *Pichia Pastoris* del documento D3 en la vacuna divulgada en el documento D2 para llegar así a la vacuna propuesta en la reivindicación 1 de la solicitud. Por lo cual se considera obvia.

En consecuencia, la reivindicación 1 de acuerdo con lo divulgado en los documentos D2 y D3 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).

11. Conclusión

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

Ítem	Documento	Reivindicaciones afectadas	Requisito que afecta
D1	<i>Genetic Analysis: Biomolecular Engineering 15 (1999) Páginas 143-148</i>	1-11	Nivel inventivo
D2	<i>Journal of Biotechnology.. (Págs. 135-146 Vol. 33)</i>	1-11	Nivel inventivo

12. Respuesta a los argumentos del señor solicitante

Argumentos con respecto a D1

1. El señor solicitante argumenta que “ el efecto de los anticuerpos generados contra el péptido de la proteína ribosomal P0 de ectoparásitos, ocurre por afectaciones de mecanismos biológicos diferentes a la respuesta inmune generada en D1, puesto que

en este caso la respuesta va dirigida contra una proteína con función biológica diferentes y se trata de vacunas cuyos principios activos son diferentes”

Se aclara que tiene razón en los argumentos en lo referente al documento D1 por lo cual se ha proyectado un segundo requerimiento de art 45 con documentos que anteceden el objeto reivindicado de la presente solicitud.

Argumentos con respecto al estudio de nivel inventivo

1. El señor solicitante reitera que “la persona del oficio normalmente versada en la materia, debe considerar que no es obvio, ni se deduce del estado de la técnica, ni del documento D1 que un péptido de la proteína ribosomal P0 descrito en las SEQ ID NO 4, 6, 8, 9 y 10 o los fragmentos de estas secuencias descritos en las SEQ ID NO 11, 12, 13, 14, 15 y 16 genere respuesta de anticuerpos capaz de afectar la viabilidad y fertilidad de garrapatas y piojos de mar”

Se aclara que el estudio de nivel inventivo se proyecta ahora a partir de la combinación de las enseñanzas de los documentos D2 y D3.

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 del día siguiente de la fecha de notificación de la presente comunicación. En caso de no dar respuesta al presente requerimiento, o si a pesar de la respuesta subsistieran los impedimentos para la concesión, se denegará la patente.

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

Finalmente, se le recuerda al solicitante que si como respuesta a este requerimiento llegara a modificar el capítulo reivindicatorio y éste contenga más de diez (10) reivindicaciones, no pagará la tasa de modificación voluntaria pero sí la correspondiente al pago por reivindicación adicional.

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 2015-05-19

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Vaccination against ticks (*Boophilus* spp.): the experience with the Bm86-based vaccine Gavac™[☆]

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Abstract

The control of tick infestations and the transmission of tick-borne diseases remain a challenge for the cattle industry in tropical and subtropical areas of the world. Traditional control methods have been only partially successful and the parasites continue to result in significant losses for the cattle industry. Recently, vaccines containing the recombinant *B. microplus* gut antigen Bm86 have been developed. Our vaccine formulation (Gavac™, Heber Biotec S.A., Havana, Cuba) has been registered and is commercially available in Cuba, Colombia, Dominican Republic, Brazil and Mexico. In controlled pen trials, Gavac™ has been effective for the control of artificial infestations of *B. annulatus*, *B. decoloratus* and chemical-sensitive and resistant *B. microplus* strains from Australia, Africa, America and Iran. In controlled field trials in Cuba, Brazil, Argentina and Mexico, Gavac has shown a 55–100% efficacy in the control of *B. microplus* infestations in grazing cattle 12–36 weeks after the first vaccination. Field trials under production conditions have been conducted in Cuba, Colombia, Brazil and Mexico in pure and cross-bred cattle herds. The application of Gavac™ has increased the time between acaricide treatments by an average of 32 ± 21 days ($P = 0.0005$) resulting in important savings for the cattle industry. In Cuba, a cost-effectiveness analysis was conducted in more than 260 000 animals. The cost-effectiveness analysis showed a 60% reduction in the number of acaricide treatments, together with the control of tick infestations and transmission of babesiosis, which resulted in savings of \$23.4 animal⁻¹ year⁻¹. These results clearly demonstrate the advantage of vaccination and support the application of Gavac for the control of *Boophilus* spp. infestations. © 1999 Published by Elsevier Science B.V. All rights reserved.

Keywords: Bm86; Babesia; Anaplasma; Boophilus; Cattle tick; Vaccine; Bovine; Gavac

1. Introduction

A major veterinary problem for cattle in tropical and subtropical regions is tick infestation and, particularly, infestation by *Boophilus* spp. Tick infestation produces weakness of the animals affecting milk production and transmits diseases like babesiosis and anaplasmosis. Conventional control methods include the use of chem-

ical acaricides with partially successful results, but this treatment has certain implicit drawbacks such as the presence of residues in the milk and meat and the selection of chemical-resistant tick strains [1–3].

Alternative approaches for tick control, such as the use of natural host resistance and development of vaccines to induce an immunological response against tick infestations, have been conducted [4,5].

The protective ‘concealed’ antigen Bm86 was identified from the gut of semi-engorged adult female ticks [1,6] and was obtained by recombinant DNA technology [5,7]. Two vaccines containing the recombinant Bm86 antigen have been registered and released to the market: one in Cuba in 1993 and in other Latin Amer-

[☆] Gavac is a trade mark of Heber Biotec S.A. (P.O. Box 6162, Havana, Cuba).

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ican countries during 1994–1997 [8] (Gavac, Heber Biotec S.A., Havana, Cuba) and the other in Australia in 1994 [9] (TickGARD, Hoechst Animal Health, Australia). Field trials performed in Cuba [10], Brazil [11], Argentina [12], Colombia [12] and Mexico [12] were successful in the control of tick populations by means of the recombinant vaccine Gavac against *B. microplus*. The Australian group was also successful in showing the efficacy of the vaccine in Australia [9]. These vaccines mainly reduce the average fertility of adult ticks instead of causing high mortality [5,7].

Babesiosis is transmitted by ticks in cattle and is mainly produced by species of protozoan parasites of the genus *Babesia* named *Babesia bovis* and *B. bigemina* [13]. These hemoparasites destroy bovine erythrocytes by haemolysis. Although substantial effort has been placed towards the development of vaccines against *Babesia* species [14,15], the main control method to prevent babesiosis in Africa and South America involves the use of chemical acaricides to control tick populations. In Australia, an attenuated live vaccine has been used successfully to control babesiosis [14,15].

This paper reviews the results obtained with the application of Gavac for the control of *Boophilus* spp. infestations in various countries of the world. The results clearly demonstrate the advantage of the vaccination and support the application of Gavac for the control of cattle tick infestations.

2. Materials and methods

2.1. Experimental designs

Three types of experiments are considered in the manuscript: (a) controlled pen trials; (b) controlled field trials; and (c) field trials under production conditions.

(a) In controlled pen trials, cattle were randomly assigned to vaccinated and control groups and artificially challenged with tick larvae employing cotton chambers or ear bags to assure tick collection (Table 1). Adult female ticks daily collected from vaccinated and control cattle were counted, weighted and assessed for egg laying capacity and fertility to determine the efficacy of the vaccine [8,12]:

Effect on the number of adult female ticks (%DT):

$\%DT = 100 [1 - (NTV/NTC)]$; NTV: number of adult female ticks in the vaccinated group; and NTC: Number of adult female ticks in the control group.

Effect on the egg laying capacity (%DO):

$\%DO = 100 [1 - (PATV/PATC)]$; PATV: average weight of the eggs per survived tick in the vaccinated group; and PATC: average weight of the eggs per survived tick in the control group.

Effect on fertility (%DF):

$\%DF = 100 [1 - (PPLOV/PPLOC)]$; PPLOV: average weight of the larvae per gram of eggs in the vaccinated group; and PPLOC: Average weight of the larvae per gram of eggs in the control group.

Table 1
Effect of vaccination with Gavac in controlled pen trials

Tick strain	% Reduction ^a			Efficacy (%) ^b	References
	DT	DO	DF		
<i>B. microplus</i>					
Camcord	74	60	12	91	[24]
Camcord	30	61	13	76	[5]
Camcord	46	43	10	72	[8,12]
Yeerongpilly	43	50	11	75	[24]
Cenapa	67	46	12	84	[24]
Tuxpan	9	38	13	51	[24]
Tuxpan	43	22	0 ^c	56	[25]
Mora	19	26	30 ^c	58	[25]
Colombian field	41	26	8.5	60	[8,12]
Brazilian field	40	8	10.2	51	[26]
<i>B. annulatus</i>					
Quemado	99.1	85	85.6	99.98	[20]
Rasht-Fouman	85	60	100	99.99	[20]

^a DT, adult female ticks; DO, egg laying capacity; DF, fertility.

^b Efficacy (%) = $100 \times [1 - (CRT \times CRO \times CRF)]$; CRT, reduction in the number of adult female ticks; CRO, reduction in the egg laying capacity; CRF, reduction in fertility. The reduction was measured with respect to the control-unvaccinated group.

^c The assay of effect on fertility was not completed.

Table 2
Effect of vaccination with Gavac in the control of tick infestations in the field

Location	Breed ^a	Maximal control ^b		References
		Time (in weeks) ^c	% ^d	
Limonar (Matanzas, Cuba)	Holstein	28–31	81	[10]
Fazenda Resgate (S. Paulo, Brazil)	Cross-bred (dairy)	36	79	[11]
	Cross-bred (beef)	24	96	[11]
	<i>B. indicus</i>	24	97	[11]
	Cross-bred (beef)	20	55	[27]
Corrientes (Argentina)	Cross-bred (beef)	12	100	[28]
Peregrino (Tamaulipas, Mexico)	Cross-bred (beef)	7	97	unpublished
Kikapu (Chiapas, Mexico)	Cross-bred			

^a Cross-bred cattle were *B. taurus* × *B. indicus* cattle.

^b Measured by counting the number of adult female ticks on the animals.

^c Time after the first injection with Gavac.

^d Measured with respect to a control-unvaccinated group.

Table 3
Effect of vaccination with Gavac on the frequency of acaricide treatments

Location	Breed ^a	Time between treatments (days) ^b			References
		Before vaccination	After vaccination	Increase in time between treatments	
Cuba (several provinces with >100 000 animals)	Holstein	14	32	18	[16]
Los Naranjos (La Habana, Cuba)	Holstein	7	22	15	[21]
Colombia (several regions)	Crossbred	14	40	26	[23]
	Holstein	15	27	12	[23]
	<i>B. indicus</i>	15	75	60	[23]
Doima (Colombia)	Crossbred	21	76	55	Unpublished
Santa Cruz (Ibague, Colombia)	Crossbred	21	90	69	Unpublished
Barra Mansa (Rio do Janeiro, Brazil)	Holstein	12	27	15	[29]
Peregrino (Tamaulipas, Mexico)	Crossbred	14	47	33	[28]
Kikapu (Chiapas, Mexico)	Crossbred	16	34	18	Unpublished
Average ± SD	–	15 ± 4	47 ± 24	32 ± 21*	–

^a Cross-bred cattle were *B. taurus* × *B. indicus* cattle.

^b Represents the average values of several experiments including cattle with one to four injections with Gavac. **P* = 0.0005 (Student *t*-test).

Efficacy:

Efficacy (%) = 100 [1-(CRT × CRO × CRF)];
CRT = NTV/NTC: reduction in the number of adult female ticks as compared to the control group; CRO = PATV/PATC: reduction in the egg laying capacity of the survived ticks as compared to the control group; and CRF = PPLOV/PPLOC: reduction in fertility as compared to the control group.

(b) Controlled field trials were conducted to assay the effect of the vaccination with Gavac in the control of cattle tick infestations under field conditions (Table 2). Cattle were distributed into vaccinated and control unvaccinated animals. Adult female ticks (≥ 4.5 mm) were counted in vaccinated and control cattle to follow the tick infestation rate and to determine the maximal control of tick infestation obtained after vaccination (Table 2).

(c) Field trials under production conditions gave the information necessary to evaluate the effect of the vaccine on the frequency of acaricide treatments and on the transmission of tick-borne diseases (Tables 3 and 4). Also, in a cost-effectiveness analysis conducted in Cuba in more than 260 000 animals, production parameters were evaluated and total savings calculated for the application of the vaccine in comparison with the use of traditional acaricide treatments [16].

2.2. Vaccination with Gavac

Vaccination was conducted employing three intramuscular injections of 2 ml (50 µg *Pichia pastoris*-derived Bm86 per ml) in the neck in weeks 0, 4 and 7 as a prime immunization, followed in field trials by a maintenance dose approximately every 6 months [11,12,16].

2.3. Antibody response to vaccination

To monitor the serum antibody response against Bm86, sera were collected from animals randomly selected from the vaccinated and control groups, before the start of vaccination (preimmune serum), at the moment of each injection dose and at least 15 days after the completion of the vaccination schedule. The antibody titres were determined by ELISA [5]. Correlation studies were conducted between the *anti*-Bm86 antibody titers and the weight of ticks and eggs laid per tick engorged on vaccinated cattle and between the antibody response to vaccination and the physiological status of the animals [16].

3. Results and discussion

The recent development of vaccine formulations against cattle tick infestations has become an alternative way for the control of this ectoparasite which causes important economic losses in tropical cattle production.

The vaccine formulation Gavac contains a *P. pastoris*-derived antigen obtained after purification in a particulated and glycosylated form [17]. These antigenic particles are highly immunogenic and immunostimulatory [18,19].

Controlled pen trials showed that the vaccine is able to control artificial *Boophilus* infestations in vaccinated cattle (Table 1). The protection covered tick strains isolated from different regions of the world and including chemical-sensitive and -resistant strains (Table 1). This result is not unexpected because the mechanism of action of the vaccine is different from that used by chemical acaricides.

The vaccine was efficacious in protecting against different *Boophilus* species (Table 1). In fact, although not included in the table, the vaccine has been able to control *B. decoloratus* infestations also [20]. This wide range of protection permits the application of the vaccine in regions where various *Boophilus* species coexist, a situation that is found in regions of America, Africa and Asia.

Although controlled pen trials can serve as an indicator for vaccine efficacy, field trials are necessary to evaluate the vaccine under field conditions. The results compiled in the Table 2 indicate that the vaccine controls tick infestations in the field. Vaccination with Gavac has been shown to protect cattle infected with different tick isolates, in a range of cattle breeds, in a range of geographical locations, in cattle of both sexes and of different ages, in pregnant and lactating cows and in cattle receiving other treatments with vaccination [8,9,11,12,16]. However, herd to herd variations have been found [8,9,16]. The degree of protection in our trials varied between different experiments. This result could be attributed to differences in the tick strains, experimental conditions and/or physiological status of the animals. Nevertheless, protection in the field is usually higher than in controlled pen trials (Tables 1 and 2).

In a field trial conducted in Cuba in cattle vaccinated under production conditions, the correlation between the antibody response to vaccination and the effect on tick fertility was established [16]. This result permits the evaluation of vaccine efficacy through the measurement of *anti*-Bm86 antibody titers in vaccinated animals. The antibody response to Gavac has been studied in different herds of cattle and the response is usually homogeneous (Table 5). However, as previously noted, the physiological status of the animals has been found to affect the primary response to vaccination but not the antibody titers after revaccination [16].

To evaluate the effect of the vaccine on cattle production two indicators were considered: variations in the frequency of the acaricide treatments and on the transmission of tick-borne diseases and particularly of babesiosis.

The application of Gavac™ in different geographical regions has increased the time between acaricide treatments by an average of 32 ± 21 days ($P = 0.0005$; Student-*t* test; Table 3) resulting in important savings for the cattle industry. In Cuba, the cost-effectiveness analysis showed a 60% reduction in the number of acaricide treatments, together with the control of tick infestation [16].

Table 4
Transmission of tick-borne diseases in cattle vaccinated with Gavac^a

Location	Cases per 1000 animals			
	Babesiosis		Anaplasmosis	
	Before vaccination	After vaccination	Before vaccination	After vaccination
Cuba (several provinces with >260 000 animals)	18.8	18.0	19.5	19.8
Cienfuegos and S. Spiritus (Cuba)	25.2	3.8	18.8	3.1

^a Data were obtained from Ref. [16]. All cases were recorded after a confirmative diagnosis.

Table 5
Antibody response to vaccination with Gavac in cattle under field conditions^a

Location	Antibody titers (mean $\pm$ SE)	Number of animals tested	References
Los Naranjos (Havana, Cuba)	8194 $\pm$ 402	480	[8,16,21]
ICA (Havana, Cuba)	8968 $\pm$ 962	80	[16]
Triangulo 1 (Camaguey, Cuba)	5022 $\pm$ 341	150	[16]
Triangulo 5 (Camaguey, Cuba)	5620 $\pm$ 689	50	[16]
Villena Revolucion (Havana, Cuba)	8188 $\pm$ 783	141	[30]
Holguin (Cuba)	6835 $\pm$ 113	1370	Unpublished
Sancti Spiritus (Cuba)	3093 $\pm$ 183	477	[16]
Tamaulipas (Mexico)	3555 $\pm$ 1167	20	[28]
Bucaramanga (Colombia)	7682 $\pm$ 565	300	Unpublished
Total	6351 $\pm$ 578	3068	–

^a Anti-Bm86 geometric mean titers were determined by ELISA 2 weeks after the third injection with Gavac, coincidentally with the peak in the antibody response.

An important impact of the control of tick infestations is the effect on the transmission of tick-borne diseases. Early experiments with Gavac showed an effect on the transmission of babesiosis in vaccinated herds [21,22]. In our experiments, the effect of the vaccine has been shown mainly in the control of babesiosis (Table 4; Ref. [16]). However, in regions such as Cienfuegos and Sancti Spiritus where ticks are probably the main vectors for the transmission of anaplasmosis, the vaccination with Gavac has also controlled the transmission of this parasite (Table 4; Ref. [16,23]).

These results have an ultimate effect on the economy of cattle industry through the reduction in the number of dead cattle and spending for acaricides and treatment of diseased cattle and an elevation in milk, meat and leather production. In a study conducted in Cuba, savings of \$23.4 animal⁻¹ year⁻¹ have been estimated for the application of the vaccination with Gavac in combination with acaricide treatments [16]. In Colombia, only considering the costs and labor for vaccination and acaricide treatments, savings equivalent to \$3 animal⁻¹ year⁻¹ were estimated [23]. In a recent evaluation in the dairy farms 'Colombia' and 'Santa Cruz' in Tolima (Ibagué, Colombia) after 3 years of vaccination with Gavac, acaricide treatments were reduced by over 70% resulting in average savings of \$4 animal⁻¹ year⁻¹ (Dr Norma C. Vargas Espinosa, personal communication).

4. Conclusion

The results summarized in this report demonstrate the feasibility of employing the vaccination with Gavac for the control of *Boophilus* infestations, resulting in the reduction of the acaricide-associated drawbacks with important savings for the cattle industry.

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High level expression of the *B. microplus* Bm86 antigen in the yeast *Pichia pastoris* forming highly immunogenic particles for cattle

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Abstract

Recently, a gene coding for the Bm86 tick gut glycoprotein was cloned, expressed in *Escherichia coli* and shown to induce an immunological response in cattle to damage ticks engorging on these animals (Rand et al., 1989). We report here the increased expression of the Bm86 antigen from the cattle tick *Boophilus microplus* in the methylotrophic yeast *Pichia pastoris*. The recombinant protein was obtained with a purity higher than 95% by a procedure with a high yield. The conducted biochemical studies demonstrated the antigen to be glycosylated and found to form particles of around 17 to 45 nm in diameter with enhanced immunogenic properties. Ticks engorging on vaccinated cattle were significantly damaged as a result of the immune response against the recombinant antigen. This system permits the obtainment in a high yield of the tick Bm86 antigen, in a glycosylated and particulated form.

Key words: *Boophilus microplus*; Tick; Antigen; *P. pastoris*; Gene expression

1. Introduction

The ectoparasite *Boophilus microplus* is a major veterinary problem affecting cattle health, as a debilitating agent itself and as a vector of severe diseases. The traditional control methods include the use of chemicals with partially successful results, but this approach has certain drawbacks

such as environmental and residue problems, the high incidence of acaricide resistance within tick populations on the field, and its high cost (Willadsen and Kemp, 1988; Wikel, 1988). Cattle acquire a partial immunity to the ectoparasite after extensive natural exposure, due largely to an immediate hypersensitivity reaction to the tick which is, nevertheless, unable to prevent serious losses in cattle production. There have been different approaches to develop a method for tick control by the artificial induction of host-resistance, using

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extracts from whole ticks, salivary glands and antigens associated with the saliva, so far unsuccessful (Wikel, 1981, 1988; Wikel and Allen, 1976; Geraint, 1991; Allen, 1979). The vaccination with 'concealed' gut antigens for tick control to provoke efficient tick gut damage was recently achieved using the *B. microplus* Bm86 glycoprotein (Willadsen et al., 1989).

Employing the Bm86 antigen purified from the plasmatic membrane of tick gut epithelial cells, an induction of resistance to the ixodid infestation was obtained by immunization. As a result, the blood digestion in these ticks was strongly and rapidly inhibited and the antibodies, together with other components of the immune system such as the complement, caused lysis of the tick gut epithelia (Willadsen et al., 1989; Johnston et al., 1986; Opdebeeck et al., 1988; Kemp et al., 1989; Willadsen et al., 1988). Partial protection against tick infestation was also obtained in cattle vaccinated with the recombinant Bm86 antigen derived from either *Escherichia coli* (Rand et al., 1989) or *Aspergillus nidulans* or *niger* (Turnbull et al., 1990) transformed cells, but the expression levels or the antigen recovery were apparently very low.

The methylotrophic yeast *Pichia pastoris* has been shown to be a suitable host for the industrial production of heterologous glycoproteins (Tschopp et al., 1987; Digan et al., 1989; Clase et al., 1991; Margolles et al., 1992). In this paper we report a high level expression of the *B. microplus* Bm86 antigen in *P. pastoris*. The described process is simple, easy to scale up and cost effective for industrial production, and allows the obtainment of a glycosylated and particulated antigen preparation. Ticks engorging on vaccinated cattle were significantly damaged as a result of the immune response against this recombinant antigen.

2. Materials and methods

2.1. Cloning of the Bm86 gene and construction of the yeast expression vector pPBm

The cloning strategy is depicted in Fig. 1. The 912 bp and 941 bp fragments corresponding to

the nucleotides 90–1001 and 975–1916 of the published sequence for Bm86 cDNA (Rand et al., 1989), respectively, were amplified using the primers 5'-GTCTAGAGGAATCATTTGCTCTGACTTC (bmt-1; upstream primer including an *Xba*I site), 5'-CCAGATCTTTAAGCACTTGAC-TTTCAGGATC (bmt-2; downstream primer including a *Bgl*II site) and 5'-GCTGCTATTGTC-CATGGAAATCAAGG and 5'-CCTTGATTTC-CATGGACAATAGCAGC (bmt-4 and bmt-3; internal primers including a unique *Nco*I site).

Boophilus microplus RNA was isolated from neoginas by the procedure of Chomczynski (Chomczynski and Sacchi, 1987). The poly A⁺ mRNA was purified by oligo (dT)-cellulose (Chirgwin et al., 1979) and diluted in RNase free water. The cDNA reaction was done employing 4 µg of poly A⁺ RNA in 100 µl of a reaction buffer containing 50 mM Tris-HCl, 50 mM KCl, 6 mM MgCl₂, 1 mM dXTPs, 50 U of RNasin, 4 µg oligo dT primers, 10 mM DTT, pH 8.8 and 100 U of avian myeloblastosis virus reverse transcriptase (Promega, USA). After incubation at 42°C for 50 min, the reaction was stopped by freezing to -20°C. It was then thawed and 10 µl were mixed in a total volume of 100 µl containing 10 mM Tris-HCl, 50 mM KCl, 2 mM MgCl₂, pH 8.3, 200 mM dXTPs, 0.1 nM of each oligonucleotide primer (bmt-1 and bmt-3 in the first reaction tube and bmt-2 and bmt-4 in the second reaction tube), and 3 U of *Thermus aquaticus* DNA polymerase (Heber Biotec, Cuba). Polymerase chain reaction (PCR) was conducted in 30 cycles of 20 s at 94°C (1 min for the first cycle), of 20 s at 55°C, and of 1 min at 72°C (3 min for the last cycle).

After phenol/chloroform extraction and ethanol precipitation, the amplified products were subjected to agarose gel electrophoresis and extracted from the gel (Maniatis et al., 1989). The 912 bp (Bm 5') and 941 bp (Bm 3') fragments (Fig. 1) were resuspended in TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.1), containing 200 µM ATP and 2 U of T4 polynucleotide kinase (PNK, Heber Biotec, Cuba). The phosphorylation reaction was incubated for 1 h at 37°C and after phenol/chloroform extraction and ethanol precipitation, phosphorylated fragments were cloned into a Bluescript plasmid digested with *Eco*RV to gen-

erate the plasmids pSBm5' and pSBm3' where both inserts were sequenced by the dideoxy chain termination method (Sanger et al., 1977).

Plasmids pSBm5' and pSBm3' were then digested with *Xba*I-*Nco*I and *Nco*I-*Bgl*II, respectively, and the obtained fragments were inserted into an *Xba*I-*Bam*HI pTrp digested vector (Estrada et al., 1992) to assemble the Bm86 cDNA lacking the 3' terminal region in the resulting plasmid pTBm.

Employing the plasmid pTBm, a second PCR reaction was performed as previously described, but using the oligonucleotides 5'-GAATCATC-CATTGCTCT (bmt-5) and bmt-2 as 5' and 3' amplification primers, respectively, and 50 ng of plasmid DNA. The 1.83 kb amplified fragment was then extracted and phosphorylated as previously described and inserted into the S1-nuclease-blunted *Nco*I site of the vector pPS 7 for expression of the Bm86 cDNA in the yeast *P. pastoris*. The plasmid pPS 7 is an integrative vector for the expression of heterologous proteins in *P. pastoris*, which contains the 1.15 kb fragment from the methanol regulated alcohol oxidase (AOX1) promoter, followed by the signal peptide of the sucrose invertase (spSUC2) gene from *Saccharomyces cerevisiae* (Kaiser et al., 1987), the 960 bp fragment of the glyceraldehyde 3-phosphate dehydrogenase transcription terminator (GAPt) from *S. cerevisiae*, besides the histidine 3 (HIS 3) gene from *S. cerevisiae* as a selection marker in histidine requiring auxotrophs *P. pastoris* strains. Furthermore, this plasmid has a 3' sequence of 2.1 kb of the AOX1 gene, inserted in the pUC18 vector (Fig. 1). The resulting plasmid was designed as pPBm (Fig. 1).

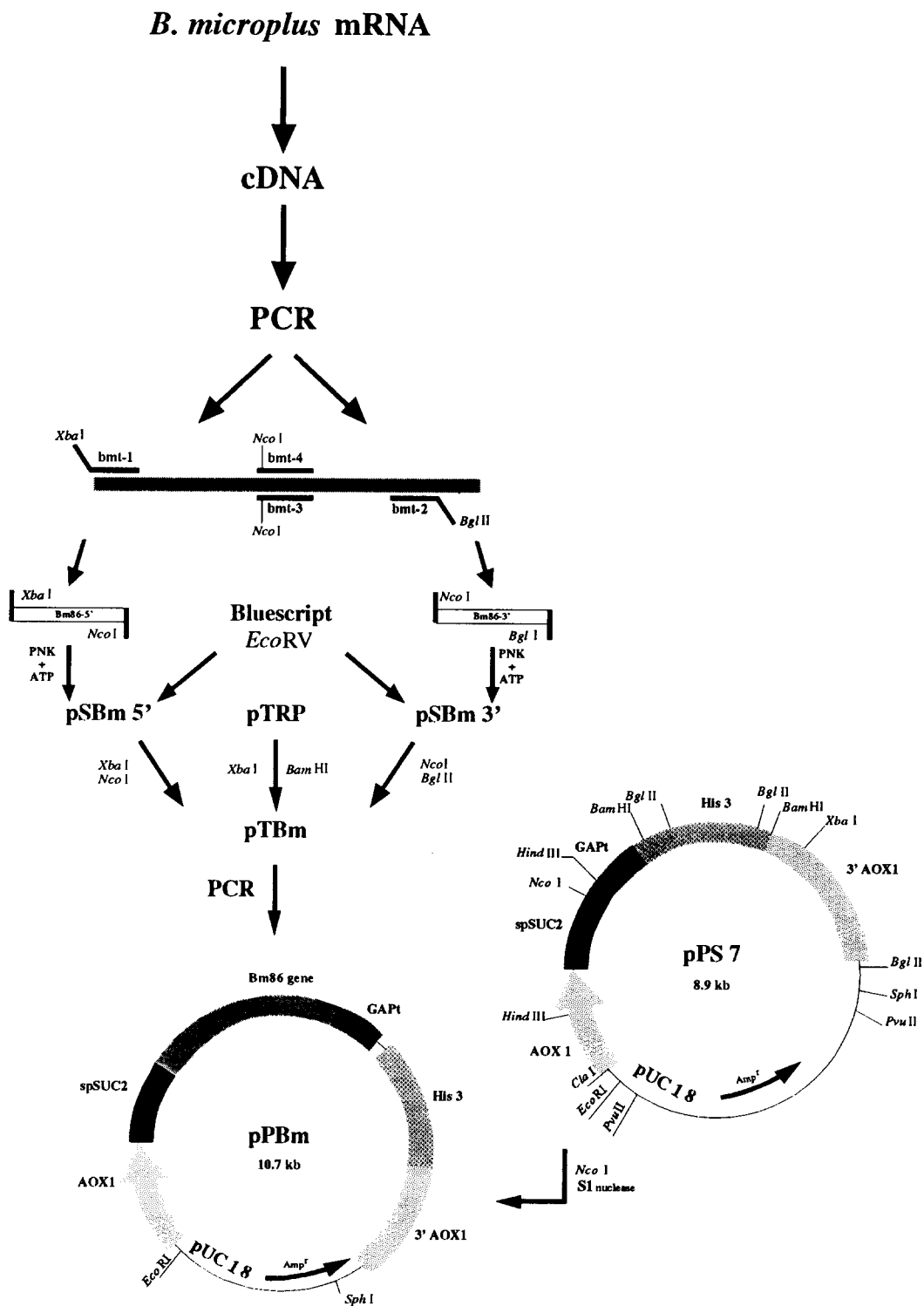
2.2. Expression of the recombinant Bm86 (rBm86) antigen in yeast

The expression plasmid (pPBm) was *Eco*RI-*Sph*I digested and the expression cassette transformed into histidine-requiring auxotrophs (HIS 3⁻), MP 36 *P. pastoris* strain (Yong et al., 1992) which acquired a His⁺ phenotype following transformation. The transformants were analyzed by Southern blot to check for correct integration by replacement of the *P. pastoris* host AOX1

gene by the expression cassette of the pPBm to assure stable expression of a single copy integrant.

P. pastoris cells expressing Bm86 were grown in shake flasks with 50 ml of YPG medium (10 g yeast extract, 20 g peptone, and 2% w/v of glycerol for 1 l) at 30°C until obtaining an OD_{600nm} of 5–10. Then they were harvested, washed once with YP (glycerol free YPG medium), resuspended in YP at approx. OD_{600nm} 5 and incubated at 30°C for 3–4 d with methanol to induce expression. The culture media from methanol induced transformants were tested to assess the percentage of Bm86 expression by densitometric scanning of the Coomassie brilliant blue stained protein bands fractioned by SDS-PAGE using a LKB laser densitometer and also determined by ELISA. The production of Bm86 was examined in two batch-type runs, each one performed in a 5 l Marubishi reactor equipped with monitor and controls for pH, dissolved oxygen, agitation speed, temperature and air flow measurements. Temperature was maintained at 30°C. Cell yields were estimated by the wet cell pellet weight. Inocula for the bioreactor runs were grown in a 1 l Erlenmeyer flask containing 500 ml of YPG medium. Bioreactor cultures grown in the batch mode were propagated in YPG medium until the available glycerol was exhausted, then methanol was added to the culture for 96 h at a rate of 2.5 ml l⁻¹ h⁻¹ for batch mode bioprocess.

Yeast cells were washed once in disruption buffer (50 mM sodium phosphate, 5 mM EDTA, 10% sucrose, 0.3 M NaCl, 1 mM 2-mercaptoethanol, pH 7) and resuspended in the same buffer at a concentration of 350 g l⁻¹. The cells were disrupted for 14 min on a bead mill (Dynomill) equipped with a cold water cooling jacket at a flow rate of 20 ml min⁻¹. The clarification of 1 l of sample was conducted by centrifugation at 9000 × *g* for 20 min in RP 12-2 (HITACHI) rotor. The pellet was washed with 1 l of washing buffer (50 mM sodium phosphate, 5 mM EDTA, 0.5% Triton X-100, 0.3 M NaCl, 1 mM 2-mercaptoethanol, pH 7), centrifuged at 9000 × *g* for 20 min and subjected to another three washings with a buffer containing 50 mM sodium phosphate, 5 mM EDTA, 0.3 mM NaCl, 1 mM



2-mercaptoethanol, pH 7. The pellet resulting from the last centrifugation at $9000 \times g$ for 20 min at room temperature was extracted by homogenization with a Polytron (IKA T-50) in 2 l of extraction buffer (8 M urea, 50 mM sodium phosphate, 5 mM EDTA, 1 mM 2-mercaptoethanol, pH 7). All washings and subsequent steps were performed at room temperature. Combined centrifugation supernatants of the extraction were pumped into a Sephadex G-25 column previously equilibrated with a 50 mM sodium phosphate, 5 mM EDTA, pH 7 buffer. The total protein peak eluted from the column was sedimented by differential precipitation in 35% NH_4SO_4 to eliminate the excess of contaminants and the antigen was concentrated at 65% of NH_4SO_4 . The concentrated antigen pellet was resuspended in a 50 mM sodium phosphate, pH 7 buffer for desalting in a Sephadex G-25 column equilibrated with 50 mM sodium phosphate, pH 7.

2.3. Preparation of a rabbit antisera against Bm86 peptides

For tracing the expression of the rBm86, the reported amino acid sequence of the natural protein (Rand et al., 1989) was analyzed in the Hoops and Woods (1981) computer program, to select the possible antigenic determinants. Five immunogenic peptides were designed and synthesized by the method of simultaneous multiple peptide synthesis (Hughten, 1985). They were cleaved with liquid hydrogen fluoride, their composition controlled by amino acid analysis and their purity assayed by a RP-C 18 4.6×100 mm HPLC column (J.T. Baker) at 34°C . The synthesized peptides had the following sequences:

- (1) E M T R G R L R R S V C K A G V S C N E N E Q S E C A K G Q (amino acids 474 to 505)
- (2) T T T K A K D K D P D P G K S S A (amino acids 612 to 628)
- (3) L S K H V L R K L Q A C E H (amino acids 397 to 410)

(4) S S I C S D F G N E F C R N A (amino acids 21 to 35)

(5) C D C G E W G A M N M T T R (amino acids 132 to 145)

Peptides were coupled to bovine serum albumin (BSA) as carrier protein via the terminal cystein residue, using *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester (Liu et al., 1979), and were used to immunize New Zealand white rabbits. After obtaining preimmune sera, groups of two rabbits were immunized as follows: on day 0, each rabbit was injected intradermally (i.d.) with 200 μg of the peptide-albumin complex (PAC) in 1 ml of complete Freund's adjuvant (CFA); on day 14, they were injected i.d. with the same dose of the PAC emulsified with incomplete Freund's adjuvant (IFA) and on day 21, the animals were injected intraperitoneally with 200 μg of PAC in 1 ml of IFA. Rabbits were bled 15 d after the last immunization, serum was obtained, divided into aliquots, and stored at -20°C for later use.

The presence and specificity of anti-peptide antibodies were tested by dot-blot. Briefly, in different pieces of Hybond nitrocellulose sheets (Amersham) 5, 2.5, 0.62, 0.31, 0.15 and 0.08 μg of the PAC were spotted and 1 μg of BSA and 20 μg of an unrelated coupled peptide were used as negative controls. Unbound reactive sites were blocked by incubation with 2% skim milk in PBS. The sheets of nitrocellulose were incubated with various rabbit sera diluted in 2% skim milk in PBS and the unbound material was removed by washing three times with 0.2% Tween 20 in PBS. Peroxidase-labeled affinity-purified goat anti-rabbit IgG was added and the Hybond sheets were washed five times before substrate was added. Finally, the anti-peptides sera were pooled and used in Western blot analysis of cells extract from methanol induced MB 9 transformant.

2.4. Biochemical characterization of the rBm86 antigen

The molecular weight of rBm86 was estimated by polyacrylamide gel electrophoresis in the pres-

Fig. 1. Handling of mRNA from *B. microplus* to obtain the Bm86 gene and cloning into the *P. pastoris* expression vector pPS7 to construct the pPBm vector which directed the synthesis of rBm86. Details are described in Materials and Methods.

ence of sodium dodecyl sulfate (SDS-PAGE) and by molecular exclusion high performance liquid chromatography (HPLC) with a TSK-G 5000 PW (TOSO-HAAS Corp.) column in PBS with 0.25 M NaCl, as compared to 2400 kDa HBsAg particles of the hepatitis B virus, and with a TSK-3000 (TOSO-HAAS Corp.) column in denaturalizing conditions (50 mM Tris-HCl, 8 mM urea, 150 mM 2-mercaptoethanol, pH 7.2). The protein in 50 mM Tris-HCl was centrifuged and mixed with 2% of phosphotungstic acid, to check it by electron microscopy in the JEOL-JEM 2000 EX microscope. For Western blot analysis, the protein was fractionated by SDS-PAGE at room temperature (Maniatis et al., 1989). Blots were developed with an anti-peptide antibody (1:5 dilution) and with a goat anti-rabbit IgG-colloidal gold complex. The N-terminal sequence analysis was performed on a KNAUER protein sequencer. The amino acid sequence was corroborated by mass spectrometry. The peroxidase-lectin staining of glycoproteins on membranes (Hsi et al., 1991; García et al., 1992) was used to determine the glycosylation of rBm86. Briefly, 100 μ l of the sample were spotted on the membrane and the unbound reactive sites were blocked with 5% skim milk in TBS (50 mM Tris-HCl, 150 mM NaCl, pH 8.0), 1 h at 37°C. The membrane was then incubated for 1 h with concanavalin A-peroxidase complex, 1:1000 diluted in PBS, and washed twice with TBS and once in TBS with 0.05% Tween 20. The development was performed with a 4-chloronaphtol solution (2 mg in 600 μ l of methanol, 10 ml TBS and 10 μ l H₂O₂). The purified rBm86 was carboxymethylated and the sulphhydryl bonds reduced (Crestfield et al., 1963) before deglycosylation. For this reaction, 1 mg of rBm86 in 0.02% SDS was heated 10 min at 100°C, 10 mU of EndoH were added and incubated during 16 h at 37°C in 0.1 mM citrate buffer.

2.5. Cattle vaccination and challenge trials

Cattle were primarily grade Holstein, about 12 months old. They were purchased from cattle tick-free areas. Three groups of five heads were immunized with 100 μ g and 400 μ g of rBm86

antigen, and the third group was inoculated with 50 mM sodium phosphate, pH 7. All vaccination trials were done by intramuscular route, in Freund's complete adjuvant the first time and the second and third shots were at the 4th and 7th weeks in Freund's incomplete adjuvant. The serum antibodies to rBm86 were monitored by immunoassay (ELISA) and the antigens were applied to the microtitre plate wells in a concentration of 5 μ g ml⁻¹. The serum from each animal was tested for anti-Bm86 antibodies prior to vaccination and several times later, by serial dilution of sera in the microtitre plate. After washing the plates, the antibody titres were determined with rabbit anti-bovine IgG immunoglobulins conjugated with peroxidase. Cattle were infested 21 d after the last shot with 1000 *B. microplus* larvae applied individually to each animal per day during 3 d. After approx. 24 d, engorged adult female ticks dropped from the cattle and each day ticks were collected, counted and weighed to calculate the mean weight per tick. The ticks were examined for obvious signs of damage, to determine the number of ticks that were visibly damaged on each day. All the female ticks that dropped from each animal were weighed, and assessed for egg laying capacity.

3. Results and discussion

3.1. Expression of the Bm86 gene in the yeast *Pichia pastoris*

The Bm86 gene fragment was obtained from *B. microplus* neogina by cDNA-PCR, using the bmt-1, bmt-3 primers in the first reaction and bmt-2, bmt-4 primers in the second. The two fragments Bm3' (912 kb) and Bm5' (941 kb) from the cDNA-PCR reactions, were cloned into the Bluescript vector to obtain the plasmids pSBm3' and pSBm5'. These plasmids were digested with *Xba*I-*Nco*I and *Nco*I-*Bgl*II, respectively, to assemble the Bm86 gene into the *Xba*I-*Bam*HI site of the pTrp vector (Estrada et al., 1992), originating the pTBm plasmid. Another PCR reaction was carried out with the pTBm as template, using bmt-5 and bmt-2 primers, to obtain a DNA frag-

ment of 1.83 kb corresponding to the Bm86 gene, which was cloned into the pPS7 vector to obtain the pPBm (Fig. 1).

The Bm86 gene resulting from this PCR and sequenced by us, had three nucleotide changes at DNA level, in the positions 239 (C to T), 364 (C

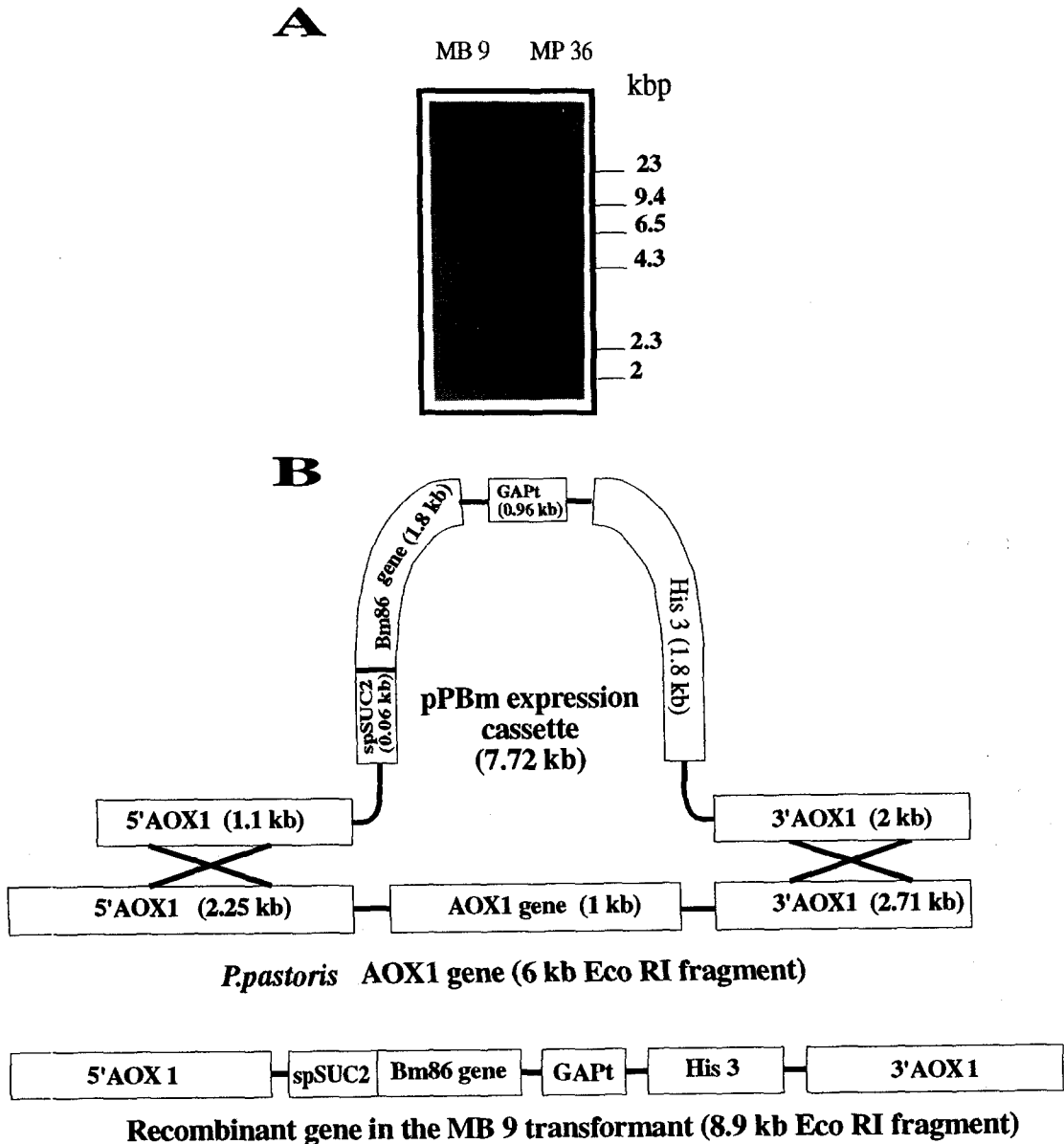


Fig. 2. (A) Southern blot analysis of AOX1 loci in MB 9 and MP 36 *P. pastoris* strains. The filter was hybridized with a ^{32}P -labeled AOX1 promoter probe from the plasmid pPS 7(*Eco*RI-*Hind*III). The lanes contain 10 μg of *Eco*RI digested DNA from the MB 9 and MP 36 strains, size markers correspond to *Hind*III digested λ DNA. (B) is a diagram of the double crossover leading to the replacement of the (6 kb) AOX1 gene with the 7.72 kb *Eco*RI-*Sph*I fragment containing the Bm86 expression cassette derived from pPBm.

to T) and 377 (T to C), as compared to the reported sequence of the Bm86 gene (Rand et al., 1989), but only one of these changes in the position 364 implied a substitution of the amino acid Thr for Ile. This was confirmed by mass spectrometry analysis. This result could be a product of some heterogeneity of the tick population, although we can not discard the possibility of a misreading from the PCR reaction.

The *P. pastoris* expression vector pPBm contains the 608 amino acid coding sequence of the Bm86 gene, but lacking 23 amino acids of the carboxyterminal transmembrane region and the 19 amino acids fragment of the signal peptide sequence of the natural Bm86 glycoprotein. The expression cassette (pPBm) had 7.8 kb after *SphI-EcoRI* cleavage with free ends homologous to the AOX1 promoter and 3' sequences of the AOX1 gene from *P. pastoris*, to help in directing the integration of this expression cassette into the AOX1 locus of the *P. pastoris* genome, by homologous recombination resulting in the substitution of the endogenous AOX1 structural gene. Southern blot hybridization analysis of the DNA isolated from the MB9 transformant showed that the AOX1 structural gene was replaced by a single copy *SphI-EcoRI* fragment from pPBm expression vector (Fig. 2), showing a Mut^S phenotype (Cregg and Malden, 1987).

The MB 9 transformant was grown until OD_{600nm} 250 in a 5 l culture medium bioreactor. After exhausting the glycerol from the culture medium, the cells were induced with methanol for 96 h. The cells were collected by centrifugation and the rBm86 antigen was purified by a simple washed pellet procedure from the MB 9 disrupted pellet, with a very high percentage of recovery (80%) and purity. The antigen obtained by this method with a purity higher than 95%, allowed the determination of a single N-terminal sequence, Glu-Ser-Ser-Ile-Cys-Ser-Asp-Phe-Gly-Asn-Glu-Phe-Cys-Arg-Asn-Ala-Glu-Cys-Glu-Val-Val, which was in agreement with the reported Bm86 sequence (Rand et al., 1989). This also indicated the appropriate cleavage of the sucrose invertase signal peptide, used for secretion of the antigen.

The level of rBm86 secreted in *P. pastoris*

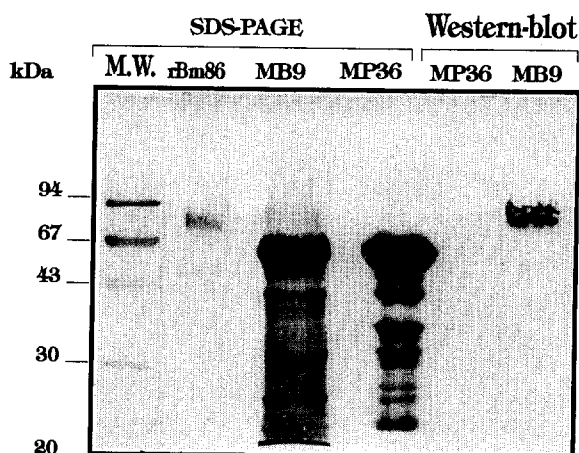


Fig. 3. Expression analysis of rBm86. Coomassie blue-stained SDS-10.5% polyacrylamide gel (SDS-PAGE) showing total cell extracts from the MB 9 transformant and MP36 *P. pastoris* strains induced with methanol, in each lane were loaded 200 μ g of total protein. In lane 1, 5 μ g of purified rBm86 were loaded. Western blot analysis of cell extracts from methanol induced MB 9 and MP36 strains. For development, a rabbit anti-Bm 86 peptides polyclonal antiserum was used. Molecular weight markers (M.W.) correspond to phosphorylase B (94 kDa), albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa) and β -lactalbumin (18 kDa).

during continuous biosynthesis on methanol was determined to be about 1.5 g l⁻¹ of bioreactor medium at a cell density of 33 g l⁻¹ (dry weight). This expression level was higher than those obtained in *A. nidulans* or *A. niger* (Turnbull et al., 1990). This high expression level in *P. pastoris* has also been reported for other recombinant proteins such as invertase (Tschopp et al., 1987), lysozyme (Digan et al., 1989), tetanus toxin (Clase et al., 1991) and α -amylase (Margolles et al., 1992).

3.2. Characterization of the rBm86 antigen

The rBm86 antigen produced in *P. pastoris* was analyzed by SDS-PAGE. This protein appeared in SDS-PAGE as a wide band, similar to a heterogeneous molecular species with size from 90 to 100 kDa (Fig. 3), but treatment with EndoH resulted in a single band of 70 kDa, corresponding to the molecular weight of the rBm86 protein

without the presence of the N-linked oligosaccharides.

Three peptides numbered 3, 4 and 5 in Materials and Methods section were able to raise antibodies in rabbits. These anti-peptide antisera were pooled and used in the immuno-identification of the rBm86 protein by Western blot analysis. This assay showed that the 90–100 kDa band in the sample of medium from methanol induced cells was linked to rBm86 (Fig. 3).

The glycosylation of rBm86 was corroborated in a blotting assay with a concanavalin A-colloidal gold complex, because this complex has a very high affinity to the *N*-acetyl-D-glucosamine. This kind of N-linked oligosaccharides are the principal type of glycosylation by *P. pastoris* yeast, in the same way that the high mannose oligosaccharide glycosylation is found in higher eukaryotic organisms (Ratner, 1989).

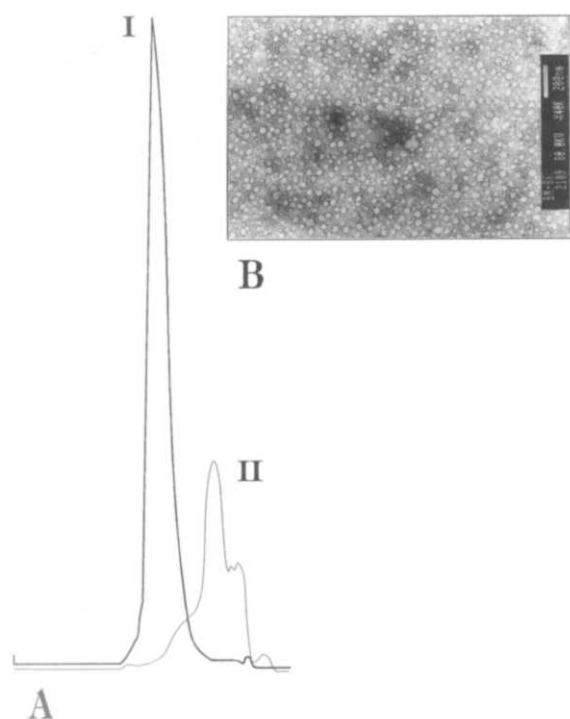


Fig. 4. (A) Comparison between the HPLC exclusion patterns of recombinant HBsAg (peak I) and rBm86 particles (peak II). Running conditions: Column-TSK G 5000 PW, buffer-PBS and 0.25 M NaCl, 0.2 ml min⁻¹ flow rate. (B) Analysis of rBm86 particles by electron microscopy ($\times 40\,000$).

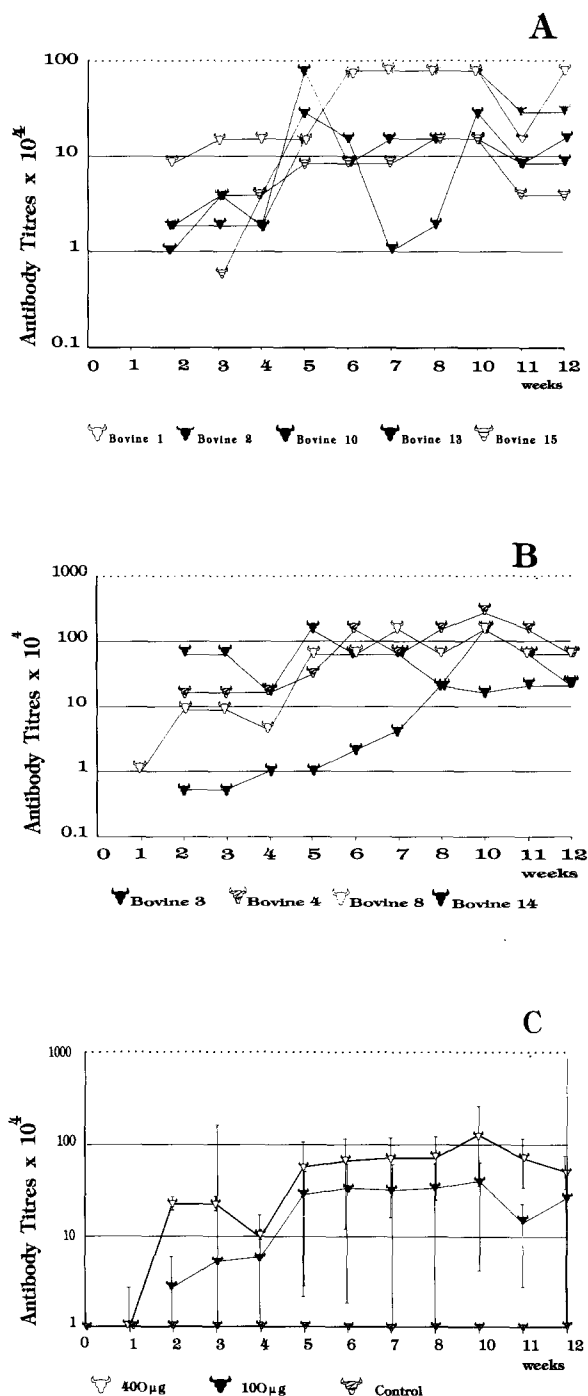


Fig. 5. Bovine serum antibody titres to rBm86 antigen determined by ELISA in cattle vaccinated with 100 µg (panel A) and 400 µg (panel B). The panel (C) represents the weekly mean titres of the anti-Bm86 antibodies in both vaccinated and control groups.

In the TSK-G 5000 PW HPLC pattern without denaturalization, the retention time of rBm86 was 90 min (peak II, Fig. 4A), very similar to protein complexes such as the hepatitis B surface antigen (HBsAg), which was used as control. The latter has a retention time of 66 min (peak I, Fig. 4A) in the same running buffer as rBm86 (Fig. 4A). A sample of this protein was checked by electron microscopy to verify that the rBm86 antigen has a particulated structure, with particle size between 17–43 nm (Fig. 4B). Due to this reason the peak II did not have a symmetric shape like the peak I corresponding to HBsAg. This particle is larger than hepatitis B virus particles. How-

ever, in HPLC our protein ran slower than the hepatitis B surface antigen, perhaps because the rBm86 particle has a lower density than the HBsAg particle. Finally, the particulation and glycosylation characteristics are very important in several proteins because they could enhance the immunogenic performance of antigens in the immunized animal.

3.3. Protection of cattle vaccinated with the rBm86 antigen

The particulated Bm86 antigen purified from *P. pastoris* was used in two different concentra-

Table 1

Results of the protection trial against *B. microplus* in cattle immunized with different doses of rBm86 antigen

Animal		Cumulative tick number ^a	Cumulative tick weight ^b (g)	Mean tick weight ^c (mg)	Damaged ticks ^d (%)	Cumulative egg weight ^e (g)	Egg/tick weight ^f (g)
Control	5	886	167	188	4	79	0.47
	6	997	171	171	4	58	0.34
	7	526	88	167	3	41	0.46
	9	906	141	155	16	48	0.34
	12	667	118	176	3	54	0.47
Mean $\pm$ SD		796 $\pm$ 194	137 $\pm$ 35	171 $\pm$ 12	6 $\pm$ 6	56 $\pm$ 14	0.42 $\pm$ 0.07
Total		3982	685	857		280	
Vaccinates (100 μ g)	1	668	79	118	87	18	0.23
	2	918	130	141	85	35	0.27
	10	397	26	65	90	6	0.23
	13	420	55	131	82	13	0.24
	15	382	24	63	95	4	0.15
Mean $\pm$ SD		557 $\pm$ 233	63 $\pm$ 44	103 $\pm$ 37	88 $\pm$ 5	15 $\pm$ 12	0.22 $\pm$ 0.04
Total		2785	314			76	
Reduction		30%	40%	40%		73%	48%
Vaccinates (400 μ g)	3	764	75	98	95	17	0.22
	4	1006	102	101	85	25	0.25
	8	566	62	109	82	10	0.16
	14	647	57	88	83	11	0.19
Mean $\pm$ SD		746 $\pm$ 191	74 $\pm$ 20	99 $\pm$ 9	86 $\pm$ 9	16 $\pm$ 7	0.22 $\pm$ 0.04
Total		2983	296			63	
Reduction		25%	42%	42%		71%	50%

^a Total number of ticks (all females) with approx. 0.8 cm of size were collected from each animal over the day 21 after the challenge.

^b Total weight of ticks collected from each animal.

^c (a) and (b) were used to calculate the mean weight per tick.

^d The percentage of tick damage is the sum of all ticks visibly damaged from the total number of ticks collected per animal.

^e The total weight of eggs produced by the surviving ticks under ideal conditions. All the female ticks collected from each animal, were incubated at 28°C and 80% of humidity, until eggs production had ceased.

^f The eggs weight was used to calculate the proportion of the tick weight that was converted into eggs.

tions to immunize five animals per group, which were subsequently challenged with ticks. In the group immunized with 400 μg rBm86, one animal did not complete the experiment and only four animals were challenged. The immunologic response was tested in each animal and the antibody titres were followed in these experiments by ELISA. The animals immunized with 100 μg and 400 μg had the same tick protection and immunological response (Fig. 5). Engorged adult female ticks were collected on completion of the parasitic part of the life cycle, approx. 23 d after the infection. Almost all ticks collected from both groups of vaccinated animals changed their normal gray color to a red color, as a signal of damage, due to the leakage of bovine erythrocytes through the damaged tick gut wall into the hemolymph, and the average weight of ticks dropping after engorgement from vaccinated animals was significantly reduced (50%). The animals vaccinated with 100 μg doses presented a modest decrease in the number of ticks (31%), and a dramatic effect of vaccination was the reduction by 70% of the reproductive ability of ticks on vaccinated animals as compared to the control group (Table 1). The differences in the mean weight, percentage of damage and total egg production between both vaccinated groups and the control group, were statistically significant ($P < 0.01$). These results were essentially similar to those reported using the rBm86 expressed in *E. coli* (Rand et al., 1989). We have shown that the MB 9 transformant of *P. pastoris* yeast constitutes a highly productive source for obtaining large quantities of the rBm86 antigen, which have the right protective epitopes for damaging and decreasing *B. microplus* tick populations.

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