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DIRECCIÓN DE NUEVAS CREACIONES**

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Abogado
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ASUNTO	Radicación	09-44585
	Trámite	011
	Evento	378
	Actuación	519
	Oficio	16482
	Folios	012

Patente de Invención	☒	Patente de Modelo de Utilidad	☐
Vía Nacional	☐		
Vía PCT (fase nacional)	☒	Cap. I	☒ Cap. II ☐
No. Sol. Internacional	PCT/US2007/023101		
Pub. Internacional	WO 2008/057396		

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 le comunica:

1. Documentos estudiados

<u>Descripción:</u>	Folios: 69 a 118	04/05/2009
<u>Reivindicaciones:</u>	Folios: 118 a 121	04/05/2009
<u>Figuras:</u>	Folios: 122 a 128	04/05/2009
<u>Prioridad:</u>	US 60/864,258	03/11/2006

2. Análisis de la Prioridad

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

3. Objeto de la Invención

Título de la solicitud: "Vacuna para la enfermedad de Lyme canina".

El objeto de la presente solicitud es suministrar una vacuna para la enfermedad de Lyme canina o borreliosis causada por la infección de especies de espiroquetas *Borrelia sp.*

El problema planteado en esta solicitud es la necesidad de vacunas alternativas que no utilicen la proteína OspA, como blanco para inducir anticuerpos borrelícos.

Para solucionar este problema técnico la solicitud en estudio suministra una vacuna para la enfermedad de lyme canina que utiliza organismos de una cepa individual de *Borrelia* sp., particularmente *B.burgdorferi*, que expresan el antígeno OspC7 conservado.

El objeto de la solicitud definida anteriormente se clasificó en la IPC: A61K 39/00.

4. No es invención (Artículo 15 D486 literal b)

La reivindicación 23 corresponde a un proceso biológico natural por lo que no es patentable de acuerdo con el Art citado.

5. Excepción a la patentabilidad (Artículo 20 D486 literal d)

El objeto de las reivindicaciones 17 a 22 consiste en un método de tratamiento aplicado a humanos o animales, por lo que no es patentable de acuerdo con el Art citado.

6. De la solicitud de Patente

Descripción (Artículo 28 D486)

La descripción no tiene información suficiente con relación a los anticuerpos reclamados en la solicitud. No hay información sobre la estructura de los anticuerpos (secuencias aminoácidas de regiones variables de ambas cadenas), pruebas de actividad, títulos de anticuerpos y demás que permitan a la persona del oficio reproducir la invención.

De esta manera, la descripción presentada incumple el artículo 28 de la D486.

Reivindicaciones (artículo 30 D 486)

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

- 1) La reivindicación principal se refiere a una composición de vacuna que comprende una cepa de *Borrelia*. Esta composición debe ser caracterizada a partir de todos los elementos que contiene, para este caso en particular debe indicar la presencia de adyuvantes y/o excipientes para diferenciarla del microorganismo como tal, e identificar la especie y cepa específica empleada, así como su estado (inactiva, atenuada, etc.);
- 2) La reivindicación 7 se refiere a un resultado a alcanzar con la vacuna, la obtención de anticuerpos en respuesta a la inmunización de un animal no puede considerarse un desarrollo alcanzado por la invención, sino un resultado del uso de la vacuna, donde este resultado corresponde además a un proceso biológico natural;
- 3) La reivindicación 13 define el Inmunógeno a partir del resultado a alcanzar con el mismo, este Inmunógeno debe definirse por su propia estructura;
- 4) El anticuerpo reclamado en las reivindicaciones 24 y 25 es definido por su actividad, sin embargo un anticuerpo debe definirse por su estructura indicando las secuencias de las regiones variables de cadena ligera y pesada. Esta información no esta presente en la descripción por lo que estas reivindicaciones se consideran inconsistentes con la materia divulgada en la solicitud.

De esta manera, el capítulo reivindicatorio presentado no cumple con los requisitos establecidos en el art. 30 de la Decisión 486.

6. Determinación del Estado de la Técnica

Para determinar el estado de la técnica se ha tenido en cuenta la fecha de presentación de la solicitud prioritaria reivindicada con la solicitud: 03/11/2006.

Teniendo en cuenta la fecha indicada se realizó búsqueda en el estado de la técnica de documentos relacionados con la solicitud, encontrándose los siguientes documentos relevantes a la misma:

Nº	Documento	Título	Fecha de publicación
D1	Lovrich S., et al. Clinical and Diagnostic Laboratory Immunology, vol 12 (6): 746-751	"Borrelia-specific OspC antibodies specific for a highly conserved epitope are immunodominant in human lyme disease and do not occur in mice or hamsters"	Jun, 2005
D2	Töpfer K., et al. Vaccine 25 (2007): 314-326	"Characterization of the humoral immune response in dogs after vaccination against the Lyme borreliosis agent. A study with five commercial vaccines using two different vaccination schedules"	Ago, 2006
D3	WO 2006/038115	"Multivalent canine vaccines against Leptospira Bratislava and other pathogens"	13/04/2006

Resumen de los documentos citados:

D1: Los humanos producen anticuerpos borrelicidas altamente específicos contra la proteína de superficie externa C (OspC) poco después de la infección con *Borrelia burgdorferi*. Se identifica el epítopo inmunodominante localizado en la región altamente conservada del extremo carboxi-terminal (resumen; folio 166).

D2: Se usaron cinco vacunas comercialmente disponibles para vacunar perros contra la enfermedad de Lyme, utilizando diferentes esquemas de vacunación y realizando mediciones de títulos de anticuerpos para establecer la protección por periodos extendidos (resumen, folio 169).

D3: La invención se relaciona con vacunas y métodos para proteger perros contra enfermedad causada por *Leptospira Bratislava*. La invención también se relaciona a la combinación de vacunas y métodos para proteger perros contra enfermedades o desordenes causados por patógenos caninos, incluidas especies de *Borrelia* sp (resumen).

<http://patentscope.wipo.int/search/en/search.jsf> ingresar WO2006038115

7. Análisis de patentabilidad (artículo 14 D486)

Novedad (artículo 16 D486)

La reivindicación 1 de la solicitud reclama una composición de vacuna la cual comprende una cantidad inmunológicamente efectiva de organismos provenientes de una primera o una única cepa de una genoespecie *Borrelia*; en donde cuando los organismos de dicha primera o dicha única cepa son cultivados bajo condiciones de crecimiento estándar de la genoespecie *Borrelia*, son erradicados en presencia de un anticuerpo OspC-específico educido en un animal inyectado con *B. burgdorferi* ss 50772 (ATCC N° PTA-439).

Adicionalmente, la reivindicación 23 reclama el suero que comprende anticuerpos borreliacídicos que se fijan al *B.burgdorferi* OspC obtenido a partir de un can inmunizado mediante el método de la reivindicación 21.

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

Características esenciales	D1	D2
Composición de vacuna	Composición de vacuna	Composición de vacuna
Organismos provenientes de una primera o una única cepa de una genoespecie <i>Borrelia</i>	<i>B. burgdorferi</i> 50772 aisladas originalmente de fluido cerebroespinal humano (Pág. 747 col izq, párr.1)	<i>B. burgdorferi</i> s. s. (de la vacuna comercial Merilym Tabla 1)
en donde cuando los organismos de dicha primera o dicha única cepa son cultivados bajo condiciones de crecimiento estándar de la genoespecie <i>Borrelia</i> , son erradicados en presencia de un anticuerpo OspC-específico educido en un animal inyectado con <i>B.burgdorferi</i> ss 50772	<i>B. burgdorferi</i> 50772 colocados en medio Barbour-Stoenner-Kelly (BSK) (Pág. 747 col izq) y se detectan anticuerpos borreliacidos (Pág. 747 col. der , párr. 1-2)	Se producen cantidades de anticuerpos específicos OspC en respuesta a la vacunación con Merilym (V1-Bbss-lys-adj lysate vaccination) (Pág. 323 col izq.)

El documento D1 divulga una composición de vacuna que contiene la cepa *Borrelia burgdorferi* 50772 aisladas de fluido cerebroespinal humano (Pág. 747 col izq, párr.1). La cepa es colocada en medio Barbour-Stoenner-Kelly (BSK) para su crecimiento (Pág. 747 col izq). Ratones o hamsters fueron infectados con la cepa *Borrelia burgdorferi* por inyección de dilución que contiene 100 µl de medio Barbour-Stoenner-Kelly (BSK). La composición contiene 10⁶ células 297 de *B. burgdorferi* (Pág. 747 col izq, párr. 3). Se detectaron anticuerpos anti- *B. burgdorferi* en las muestras de suero tomadas a los animales infectados (Pág. 747 col der).

De esta manera, el documento D1 contiene todas las características técnicas de la invención reclamada en la reivindicación 1 y 23 por lo que afecta su novedad.

Por su parte, el documento D2 también divulga varias opciones de vacunas comerciales empleadas para vacunar caninos contra la enfermedad de Lyme, con las cuales ensaya diferentes esquemas de vacunación y combinaciones de antígenos (resumen). De estas vacunas la Merilym, que contiene *B. burgdorferi* s. s. en lisado celular (Tabla 1), produce como respuesta inmunológica en los perros vacunados anticuerpos específicos contra OspC (Pág. 323 col izq).

De esta manera, el documento D2 contiene todas las características técnicas de la invención reclamada en la reivindicación 1 y 23 por lo que afecta su novedad.

Nivel inventivo (Artículo 18 D486)

En las reivindicaciones dependientes se reclama que la cepa utilizada expresa OspC y es la cepa *B.burgdorferi* ss 50772 (Reivindicaciones 3-5), que la composición tiene una segunda cepa que expresa OspA ó OspB (reivindicación 6), la composición también puede contener un Inmunógeno no proveniente de *Borrelia* (reivindicaciones 13-16). El documento D1 como se mencionó arriba divulga la composición vacunal que contiene la cepa *Borrelia burgdorferi* 50772 aisladas de fluido cerebroespinal humano, en una composición que contiene 10⁶ células 297 de *B. burgdorferi* (Pág. 747 col izq, párr. 3).

La diferencia entre la materia reclamada en la solicitud y D1 es que la solicitud realiza en forma preferente composiciones de vacuna combinadas que contienen además de

Borrelia burgdorferi 50772 otras cepas de *Borrelia*, mientras que en D1 solo se tiene esta cepa en la composición.

El efecto que produce esta diferencia es que la composición puede proteger contra otras cepas de *Borrelia*.

El problema resuelto por la invención es producir composiciones de vacuna contra la enfermedad de Lyme que tengan varias cepas de *Borrelia*, incluida *Borrelia burgdorferi* 50772.

Sin embargo, esta composición vacunal para proteger caninos contra la enfermedad de Lyme ya había sido divulgada por D2, donde se realiza una composición multivalente que contiene antígenos de lisados combinados de *B. garinii*, *B. afzelii* y *B. burgdorferi* s. s. (Pág. 316 col izq párr. 1). Este documento utiliza *B. burgdorferi* s. s. que induce anticuerpos específicos contra OspC (Pág. 323 col izq).

De este documento también se conoce los resultados de reactividad cruzada de los anticuerpos generados por las tres especies de *Borrelia* ensayadas (Fig. 4; Pág. 324 col der último párr.), mostrando que los anticuerpos contra *B. burgdorferi*, también pueden atacar antígenos de *B. garinii* y *B. afzelii*.

A partir de esta información, la persona del oficio normalmente versada en la materia estaba en capacidad de realizar una composición vacunal multivalente para proteger caninos contra la enfermedad de Lyme, con expectativa de tener éxito en la respuesta inmune y protección generada por esta vacuna. De esta manera, a partir de la información de D1 y D2 las reivindicaciones 1 a 12 carecen de nivel inventivo.

Respecto, a la composición multivalente con patógenos diferentes a *Borrelia* reclamada en las reivindicaciones 13 a 16. El documento D2 como se mencionó divulga varias opciones de vacunas comerciales empleadas para vacunar caninos contra la enfermedad de Lyme, con las cuales ensaya diferentes esquemas de vacunación y combinaciones de antígenos (resumen). De estas vacunas la Merilym, que contiene *B. burgdorferi* s. s. en lisado celular (Tabla 1), produce como respuesta inmunológica en los perros vacunados anticuerpos específicos contra OspC (Pág. 323 col izq). D2 también divulga una composición multivalente que contiene antígenos de lisados combinados de *B. garinii*, *B. afzelii* y *B. burgdorferi* s. s. (Pág. 316 col izq párr. 1).

La diferencia entre la materia reclamada en la solicitud y D2 es que la composición de la solicitud puede contener inmunógenos diferentes a *Borrelia*, mientras que en D2 los inmunógenos ensayados provienen de especies de *Borrelia* únicamente.

El efecto que produce esta diferencia es que la vacuna multivalente puede proveer protección contra los otros inmunógenos de patógeno incluidos en la composición.

El problema técnico objetivo resuelto en la invención es proveer una vacuna multivalente que proteja a caninos contra la enfermedad de Lyme y contra otras enfermedades caninas.

Sin embargo, esta composición era conocida a partir de D3 el cual divulga una vacuna de *Leptospira bratislava* para uso canino, que puede ser aplicada a cachorros. Esta vacuna puede contener varios inmunógenos provenientes de virus o patógenos que infectan caninos, entre ellos *Borrelia* sp, preparación de células *Leptospira* de *Leptospira canicola*, *Leptospira grippotyphosa*, *Leptospira icterohaemorrhagiae* y *Leptospira pomona*, que comprende además una cepa atenuada de virus de moquillo canino (CD), una cepa atenuada de adenovirus canino de tipo 2 (CAV-2), una cepa

atenuada de virus de parainfluenza canina (CPI), una cepa atenuada de parvovirus canino (CPV) (Pág. 7 líneas 5-16).

De esta manera a partir de la información divulgada en D2 y D3, la persona del oficio normalmente versada en la materia podía obtener la invención tal como es reclamada en la presente solicitud. Por tanto, la materia reclamada carece de nivel inventivo e incumple el artículo 18 de la D486.

8. Conclusión

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

Ítem	Documento	Reivindicaciones afectadas	Requisito que afecta
D1	Lovrich S., et.al. Clinical and Diagnostic Laboratory Immunology, vol 12 (6): 746-751	1, 23 1-12	Novedad Nivel inventivo
D2	Töpfer K., et.al. Vaccine 25 (2007): 314-326	1, 23 1-25	Nivel inventivo
D3	WO 2006/038115	1-25	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 60 días contados a partir de la fecha de notificación de la presente comunicación. En caso de no dar respuesta al presente requerimiento, o si a pesar de la respuesta subsistieran los impedimentos para la concesión, se denegará la patente.

Se invita al solicitante se sirva indicar, si así lo considera, su renuncia al término faltante para dar respuesta a este requerimiento y presentarla por escrito

Notifíquese el presente oficio conforme a lo dispuesto en el numeral 5.2, literal e), del capítulo quinto del título primero de la Circular Única N° 10 de 2001.


JOSE LUIS SALAZAR LÓPEZ
Director de Nuevas Creaciones (C)

El anterior requerimiento fue notificado por fijación en lista, de fecha 18 SET. 2017

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Borrelia burgdorferi OspC Antibodies Specific for a Highly Conserved Epitope Are Immunodominant in Human Lyme Disease and Do Not Occur in Mice or Hamsters

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Humans produce highly specific borrelial antibodies against outer surface protein C (OspC) shortly after infection with *Borrelia burgdorferi sensu stricto*. We previously demonstrated the epitope recognized by immunoglobulin M (IgM) and IgG OspC borrelial antibodies was located within the 50 amino acids nearest the carboxy (C) terminus. In this study, we show the immunodominant epitope is located in the highly conserved region within the seven C-terminal amino acids. Six early Lyme disease sera that contained borrelial activity and IgM and/or IgG OspC antibodies were chosen randomly and adsorbed with truncated OspC containing the 16 or 7 amino acids nearest the C terminus. Adsorptions with each truncated protein abrogated the borrelial activity completely. In addition, only small concentrations of OspC antibodies remained detectable by enzyme-linked immunosorbent assay and Western blotting. Moreover, borrelial OspC antibodies were not induced in laboratory mice or hamsters despite heavy infections with *B. burgdorferi* spirochetes. These findings confirm that borrelial antibodies comprise the majority of the IgM and IgG OspC antibody response in human Lyme disease and that the epitope is located in the highly conserved C terminus. In addition, rodent animal models appear to be inappropriate subjects for assessing the effectiveness of the epitope for serodiagnosis or as a human Lyme disease vaccine.

Lyme disease caused by infection with *Borrelia burgdorferi sensu stricto* is the most prevalent arthropod-borne human illness in the United States. The bacteria thrive in a variety of diverse niches as they cycle between small mammals, *Ixodes scapularis* ticks, and other alternative hosts including humans. Their ability to survive appears dependent on the differential expression or repression of genes selected in response to a variety of environmental cues. For example, the spirochetes express outer surface protein A (OspA) in the midgut of resting nymphal and adult ticks but downregulate OspA and upregulate OspC in response to temperature after the tick begins feeding (27). More recently, researchers have demonstrated the ability of the spirochetes to regulate a multitude of genes in the tick (13), at the tick-host interface (14), and in the mammalian host (4) in response to additional environmental signals that likely include components in blood, biochemical reactions, and the host immune response.

Despite the propensity for antigenic variation, however, the complement-dependent borrelial (killing) antibody response detected in a human infection is remarkably predictable. Several *B. burgdorferi* proteins, including OspA, OspB, OspC, DhpA, Oms66, and the 39-kDa periplasmic protein, induce borrelial antibodies (7, 9, 10, 17, 19, 26, 28, 31), but the response in early human Lyme disease is specific primarily for OspC (5, 6, 16, 17, 25). In addition, borrelial antibodies specific for OspA and OspB may be produced, but the re-

sponse is almost exclusively seen in patients with Lyme arthritis (7, 10, 26). Moreover, traditional laboratory isolates that express OspA and OspB in vitro can be used to detect OspA and OspB borrelial antibodies, because the antibodies can attach to the outer surface proteins (Osps). However, OspC borrelial antibodies have not been detected by isolates that also express OspA and OspB, likely because the concurrent expression of these Osps hinders the attachment of OspC antibodies. Detecting OspC borrelial antibodies is therefore dependent on isolates such as *B. burgdorferi* 50772, because the spirochetes lack the plasmid containing *ospA* and *ospB* (2), and the absence of the Osps enables borrelial OspC antibodies to bind (6, 25). A borrelial antibody test that uses each type of isolate reliably provides sensitive and specific serodiagnostic confirmation of Lyme disease (5, 6, 8).

We previously exploited the susceptibility of *B. burgdorferi* 50772 to OspC borrelial antibodies (25) to demonstrate that the human antibody response was specific for the 50 amino acids (aa) nearest the carboxy (C) terminus of OspC (16). In this study, we examined smaller fragments of the OspC C terminus and again used borrelial activity specific for *B. burgdorferi* 50772 to more precisely identify the location of the borrelial epitope. We also determined whether the response was induced after the infection of laboratory mice or hamsters. The results confirm that the OspC borrelial antibody response is highly conserved in human Lyme disease and provide compelling evidence that the epitope responsible will be an extremely valuable serodiagnostic antigen and may provide the basis for an effective Lyme disease vaccine. Additionally, the results demonstrate that the production of OspC borrelial antibodies is unique to human infection.

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MATERIALS AND METHODS

Organisms. *B. burgdorferi* sensu stricto 50772 is a noninfectious isolate that lacks the plasmid containing *ospA* and *ospB* (36). *B. burgdorferi* sensu stricto 297 was isolated originally from human cerebrospinal fluid, and the spirochete expresses *OspA*, *OspB*, and *OspC*. *Escherichia coli* JM109 (Promega, Madison, Wis.) was used for cloning.

Animals. Ten-week-old female C3H/HeJ mice (Jackson Laboratories, Bar Harbor, Me.) or 6-week-old LVG hamsters (Charles River Laboratories, Kingston, N.Y.) were housed three or four per cage at ambient temperature. Food and water were available ad libitum. Animal experiments were reviewed and approved by the Gundersen Lutheran Animal Care and Use Committee (Institutional Animal Care and Use Committees).

Sera. Serum samples that contained significant concentrations of immunoglobulin M (IgM) and/or IgG *OspC* antibodies were obtained from patients with early Lyme disease characterized by single or multiple erythema migrans lesions. In addition, immune sera specific for host-adapted (3) Lyme disease spirochetes were obtained from hamsters and mice. Groups of donor animals were infected by injecting 100- μ l volumes of Barbour-Stoenner-Kelly (BSK) medium containing 10^6 *B. burgdorferi* 297 cells subcutaneously in the lower back. Five weeks postchallenge, 12 approximately 2- by 2-mm pieces of infected ear tissue were removed from each animal by punch biopsy and implanted subcutaneously into the lower back of a naive recipient animal. After 15 weeks, the recipient animals were bled to exsanguination and killed by inhalation of CO₂, and the bladder, kidney, spleen, heart, and ear tissues were cultured in BSK medium. Cultures were then examined weekly for 3 weeks by dark-field microscopy.

Cloning of *OspC* fragments. Recombinant *OspC* and *OspC*-Dra (50 C-terminal amino acids) were recovered from the previously described (16) *E. coli* JM109 containing either pX3-22 (*OspC*) or pX2-Dra (*OspC*-Dra). A recombinant protein containing the fragment with the final 16 *OspC* C-terminal amino acids (C16) was constructed using forward primer 5'-AGTTAAAGAACTTAC AAGTCTGTGTGG-3', reverse primer 5'-GGTTAAGGTTTTTTGGACTTCTG-3', and pX3-22 (15) as templates. The amplified C16 fragment was precipitated using Pellet paint (Novagen, Madison, Wis.) and ligated (pXT16) into a PinPoint Xa-1 T-vector (Promega, Madison, Wis.). A fragment containing the final seven C-terminal amino acids (C7) was similarly made by annealing primers 5'-GGCAGAAAGTCLAAAAAACCTTAACCA-3' and 5'-GGTTAAGGTTTTTTGGACTTCTTCTCTCTGCCAA-3' and also ligated (pXT7) into a PinPoint Xa-1 T-vector (Promega). Both pX16 and pX7 were then transformed into *E. coli* JM109, and proper orientations were confirmed by DNA sequencing (BigDye ABI, Foster City, Calif.).

Recovery of *OspC* or *OspC* fragments. The recombinant *OspC* or *OspC* fragments were recovered by culturing the *E. coli* in a 100-ml volume of 2 $\times$ tryptone-yeast extract (TY) broth containing 100 μ g/ml of ampicillin (Sigma Chemical Co., St. Louis, Mo.) for 12 h at 37°C, diluting tenfold with additional 2 $\times$ TY broth, and incubating for another hour. Isopropyl- β -D-thiogalactoside (IPTG) (Sigma) was then added to a final concentration of 0.1 μ M, and the cultures were reincubated for 4 h at 37°C. The suspensions were pelleted by centrifugation at 10,000 $\times$ g for 15 min at 4°C, resuspended in purification buffer containing 50 mM Tris (pH 8.0), 50 mM NaCl, 2 mM EDTA, and 0.1% Triton X-100, and lysed with a sonicator (model W350; Branson Sonic Power, Danbury, Conn.). Sonicated *E. coli* cells were pelleted by centrifugation at 10,000 $\times$ g for 20 min, and the supernatants were passed over columns containing SoftLink resin (Promega) at a rate of 0.5 ml/min at 4°C. The fusion proteins contained a 16-kDa biotinylated purification tag on the amino terminus of the protein that binds to SoftLink resin. Columns containing protein were then washed with 5 column volumes of purification buffer, and the bound proteins were eluted with purification buffer that also contained 5 mM biotin (Sigma). The proteins were then examined by using sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting with mouse monoclonal antibodies specific for *OspC* to confirm their purity.

Adsorption with *OspC* or *OspC* fragments. One-milliliter volumes of Tetralink tetrameric avidin resin (Promega) were washed and suspended in 20 ml of phosphate-buffered saline (PBS, pH 7.2) and loaded into separate 10- by 70-mm polypropylene columns. Amounts (0.5 μ g) of *OspC*, *OspC*-Dra (18), C16, or C7 were solubilized in 1-ml volumes of PBS and passed over separate columns. Complete binding was confirmed by protein assay (Bio-Rad). One-milliliter volumes of sera diluted 1:10 in PBS were then passed over the column four times, and the amounts of antibodies removed were confirmed by using *OspC*-Dra, C16, or C7 enzyme-linked immunosorbent assays (ELISAs).

Indirect ELISA. An *OspC* ELISA and ELISAs containing the C16 or C7 *OspC* fragments were prepared as described previously (16). Serum from a person not exposed to *B. burgdorferi* was used as a normal control.

Detection of borreliacidal antibodies. Borreliacidal antibodies were detected by a flow cytometric procedure (6, 8). Viable *B. burgdorferi* 297 or 50772 organisms in logarithmic growth phase were enumerated with a Petroff-Hausser counting chamber and diluted with fresh BSK medium to a concentration of approximately 5×10^5 organisms/ml. Concomitantly, serum samples were diluted 1:20 with BSK and filter sterilized by passage through a 0.2- μ m microfilter (Costar, Cambridge, Mass.). The filtered serum samples were then transferred to sterile 1.5-ml screw-cap microfuge tubes (Sarstedt, Newton, N.C.) and diluted serially (1:40 to 1:20,480) with BSK. Serum samples were heat inactivated at 56°C for 10 min, and a 100- μ l aliquot of the spirochetes and 10 μ l of sterile guinea pig serum (50% hemolytic component $\approx$ 200 units/ml) were added. The assay mixtures were mixed thoroughly and incubated for 16 to 24 h at 35°C.

Following incubation, 100 μ l of each assay suspension was transferred to a 12- by 75-mm polystyrene tube (Becton-Dickinson, Franklin Lakes, N.J.) containing 400 μ l of PBS and 1 μ g of acridine orange (Sigma) per ml. A FACSscan flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA) was then used to detect borreliacidal activity. Spirochetes were isolated by gating (CellQuest software; Becton Dickinson) and analyzed for 1 to 2 min with the flow rate set at low. Borreliacidal antibodies kill the spirochete by inducing a complement cascade that disrupts the outer membrane and causes the membrane to bleb. Borreliacidal antibodies were detected indirectly by monitoring the increased fluorescence intensity that occurs when the acridine orange intercalates into blebbed, nonviable spirochetes. A $\geq 3\%$ shift in the mean fluorescence intensity compared to that of a normal serum control was considered positive (5, 6). The presence of blebbed, nonviable *B. burgdorferi* was then confirmed by dark-field microscopy. The undiluted and adsorbed sera were assayed concurrently to eliminate the variability of titers caused by interassay variation in the concentrations of spirochetes.

Western blotting. Western blotting was performed using standard procedures. Briefly, 225 μ g of *B. burgdorferi* 50772 protein was loaded into the preparative wells of 0.1% sodium dodecyl sulfate-12% polyacrylamide gels, and the proteins were separated by running in an electrophoresis unit (PROTEAN IIx1, Bio-Rad Laboratories, Hercules, Calif.) at 24 mA for 4 h. The proteins were transferred from the gels to polyvinylidene difluoride membrane (Perkin-Elmer Life Sciences Inc., Boston, Mass.) by electrophoresing overnight at 10 V. The polyvinylidene difluoride was cut into strips and blocked with 1% bovine serum albumin in PBS (pH 7.2)-0.1% Tween 20 for 1 h at 22°C. Strips were incubated for 1 h at 22°C with serum diluted to a ratio of 1:100 and washed four times with PBS-0.1% Tween 20. Horseradish peroxidase-labeled anti-human IgM or IgG heavy and light chains (Kirkegaard and Perry Laboratories, Gaithersburg, Md.) were added, and the strips were incubated for 1 h at 22°C. Strips were washed and developed using the TMB membrane peroxidase substrate system (Kirkegaard & Perry).

RESULTS

Ability of adsorptions to remove *OspC* antibodies. The early Lyme disease sera contained high concentrations of IgM *OspC* antibodies, and three sera also contained significant amounts of IgG *OspC* antibodies (Table 1). In agreement with previous findings (16), the *OspC* antibodies were removed almost completely by adsorption with *OspC*-Dra. More notably, binding to the C16 and C7 *OspC* fragments also almost completely removed the *OspC* antibodies detected by ELISA (≥ 4 -fold reduction in titer) or by Western blotting (Fig. 1). In contrast, the reactivity on the Western blots for the other *B. burgdorferi* proteins appeared unaffected.

Effect of removing *OspC* fragment-specific antibodies on borreliacidal activity. We then determined whether removing the *OspC* antibodies by adsorption to the *OspC*-Dra, C16, or C7 *OspC* fragments also affected the borreliacidal activity. To confirm that the decreased activity was not due to nonspecific binding, we passed a Lyme disease serum that contained only *OspA* and *OspB* borreliacidal antibodies (7) over the columns prior to passing the Lyme disease sera that contained the *OspC* borreliacidal antibodies. In addition, we passed the Lyme disease sera over a column that contained just the fusion protein. As reported previously (16), adsorbing the early Lyme disease

TABLE 1. Removal of OspC antibodies from early Lyme disease sera by adsorption with OspC-Dra, C16, or C7

Serum sample	OspC ELISA reactivity ^a after adsorption with:									
	Untreated serum		OspC		OspC-Dra		C16		C7	
	IgM	IgG	IgM	IgG	IgM	IgG	IgM	IgG	IgM	IgG
1	40,960	20,480	ND	ND	160	640	640	320	640	640
2	81,920	2,560	ND	ND	ND	160	160	160	ND	320
3	20,480	640	ND	ND	ND	80	160	160	80	80
4	40,960	10,240	ND	ND	2,560	320	2,560	320	2,560	640
5	5,120	320	ND	ND	160	ND	160	ND	320	ND

^a Reciprocal of last dilution with absorbance value of ≥ 0.200 above that of normal serum control. ND, no ELISA reactivity detected.

sera with OspC-Dra also abrogated the borreliacidal activity completely (Table 2). In addition, the borreliacidal antibodies were removed completely by binding to either the C16 or the C7 OspC fragments. The collective results therefore confirmed that the IgM and IgG OspC borreliacidal antibodies were specific for an epitope contained within the C-terminal 16- and 7-aa fragments of OspC. Moreover, removing the borreliacidal

antibodies also removed almost the entire concentration (≥ 4 -fold reduction) of OspC antibodies.

OspC borreliacidal antibody response in mice and hamsters. We next determined whether the immune response in laboratory mice or hamsters would mimic the human antibody response after infection with Lyme disease spirochetes. To ensure that the antibodies were specific for proteins expressed in vivo (3), the animals were challenged with *B. burgdorferi* 297 spirochetes that were adapted to each animal by infecting donor animals with a needle challenge and harvesting spirochete-infected ear tissues to challenge additional naïve animals. The immune sera from the infected-tissue-challenged animals were then harvested, pooled ($n = 5$), and evaluated for borreliacidal activity and OspC antibodies. Both responses were detected, but the titers of all responses were significantly lower than the titers detected in human immune sera (Table 3), even though the animals were heavily infected (Table 4) and produced significant amounts of antibodies against many other proteins (Table 3). Furthermore, removing the OspC antibodies by adsorbing with the recombinant OspC had no effect on the borreliacidal activity (Table 3, Fig. 2).

DISCUSSION

Researchers showed previously (6, 16, 25) that the majority of IgM and IgG OspC antibodies produced after infection with *B. burgdorferi* sensu stricto were complement-dependent borreliacidal antibodies that killed the spirochetes without the necessity of scavenging by phagocytic cells. Moreover, the OspC borreliacidal antibodies were specific for an epitope(s) contained within the 50 C-terminal amino acids of the protein (16). In this study, we demonstrate that the OspC borreliacidal

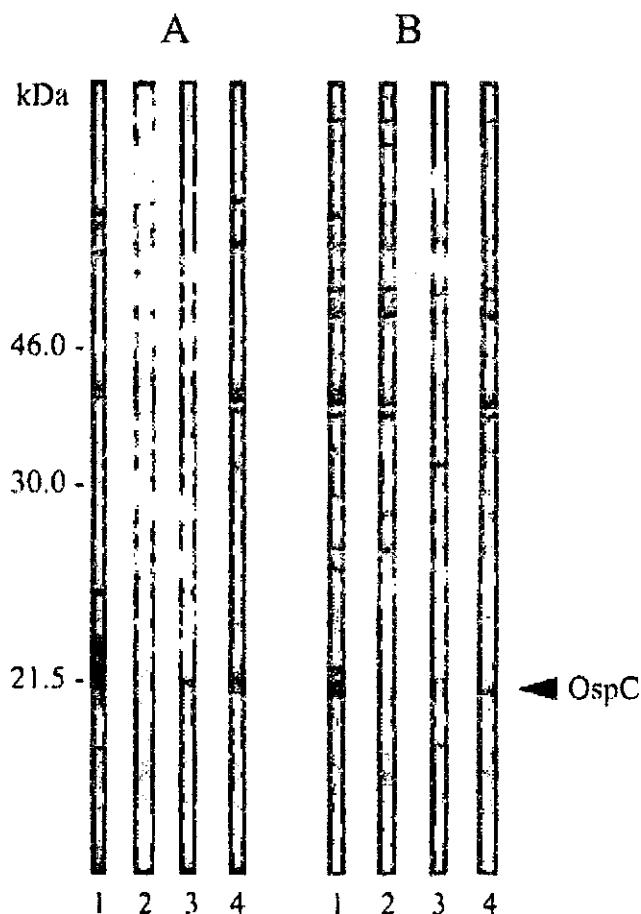


FIG. 1. Representative Western blots of antibodies in an early Lyme disease serum reactive against *B. burgdorferi* 50772 before adsorptions (lanes 1) and after adsorptions with OspC (lanes 2), C16 (lanes 3), or C7 (lanes 4). Panels A and B are IgM and IgG Western blots, respectively. Note that the effects of the adsorptions on the detection of OspC antibodies in the other four sera were identical.

TABLE 2. Removal of borreliacidal antibodies from Lyme disease sera after adsorption with OspC-Dra, C16, and C7

Serum sample	Borreliacidal activity ^a after adsorption with:			
	Untreated	OspC-Dra	C16	C7
Control ^b	5,120	5,120	5,120	5,120
1	10,240	ND	ND	ND
2	40,960	ND	ND	ND
3	10,240	ND	ND	ND
4	10,240	ND	ND	ND
5	2,560	ND	ND	ND

^a Reciprocal of last dilution with significant borreliacidal activity. ND, no borreliacidal activity detected.

^b Lyme disease serum containing OspA and OspB borreliacidal antibodies.

TABLE 3. Effect of removal of OspC antibodies on borreliacidal activity in mouse or hamster immune serum samples

Serum sample ^c	OspC IgG ELISA reactivity ^a		Borreliacidal activity ^b	
	Untreated	OspC adsorbed	Untreated	OspC adsorbed
Mouse	2,560	ND	160	160
Hamster	320	ND	320	320

^a Reciprocal of last dilution with absorbance value of ≥ 0.200 above that of normal serum control. ND, no ELISA reactivity detected.

^b Reciprocal of last dilution with significant borreliacidal activity

^c Pooled serum samples ($n = 5$).

antibodies are specific for a highly conserved epitope within the seven C-terminal amino acid residues.

This finding is remarkable. As an infected tick begins feeding on a human, OspC is expressed (27) and becomes the major outer membrane protein expressed in early infection, regardless of the *Borrelia* genospecies infecting the patient (11, 22, 24, 35). Correspondingly, OspC antibodies quickly become the dominant humoral immune response (22, 35); however, OspC ELISAs have lacked accuracy for confirming Lyme disease (12, 20, 24, 30, 33). The shortcomings were explained most often by the heterogeneity of ospC (15, 18, 29, 30), even among isolates within the same genospecies (32). The antibody responses induced by the highly variable regions would have widely variable specificities. Additionally, antibodies induced by other infections may also react to these regions. However, previous studies that characterized the ability of OspC antibodies to kill the spirochetes independent of scavenging by phagocytic cells have provided compelling evidence that the borreliacidal antibodies are specific for a conserved region of the protein. For example, a flow cytometric test that detects borreliacidal OspC antibodies by monitoring the ability of the antibodies to kill only *B. burgdorferi* sensu stricto 50772 detected the response in $>70\%$ of early Lyme disease sera collected from throughout the United States (5).

In this study, we extend these findings by demonstrating that the dominant OspC borreliacidal antibody epitope is located within the surface-exposed (22) seven amino acid residues of the C-terminal end of the protein. More importantly, a BLAST search (1) confirmed the region is highly conserved among infectious *B. burgdorferi* sensu stricto, *B. garinii*, and *B. afzelii* isolates. The results therefore provide explanation for the increased accuracy (5, 6) of the flow cytometric borreliacidal antibody test. Moreover, the findings provide strong evidence that an ELISA comprised of the epitope would correlate directly with the highly specific borreliacidal antibody response. This should provide a sensitive and highly specific serodiagnostic confirmation of Lyme disease without the technical com-

TABLE 4. Recovery of *Borrelia burgdorferi* from mice and hamsters challenged with spirochete-infected ear tissues

Animal	No. of positive samples/no. tested in:					Total no. positive/no. tested
	Spleen	Kidney	Bladder	Heart	Ear	
Mice	0/5	2/5	5/5	4/5	1/5	5/5
Hamsters	2/5	0/5	3/5	4/5	4/5	5/5

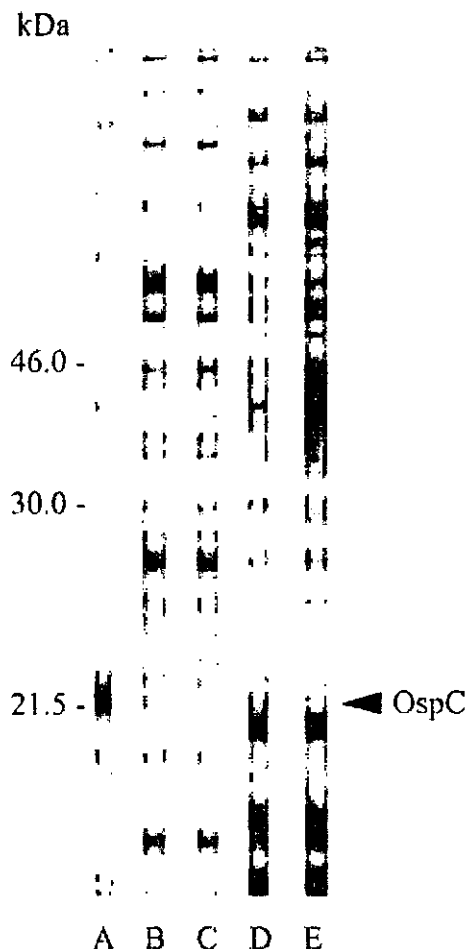


FIG. 2. Western blots of pooled ($n = 5$) hamster immune serum before (lane B) and after (lane C) adsorptions with OspC or pooled ($n = 5$) mouse immune serum before (lane D) and after (lane E) adsorptions with OspC. Lane A shows reactivity by polyclonal antibodies specific for OspC.

plexity and live organisms necessary to detect borreliacidal activity.

Mathiesen et al. (22) also reported that the OspC antibodies in sera from patients with neuroborreliosis caused by infection with *B. garinii* were specific predominantly for the identical sequence of 10 C-terminal amino acid residues of the protein. However, an ELISA with a synthetic peptide (21), while more reactive than the rOspC ELISA, detected OspC antibodies in European sera significantly less often (36%) than in sera from U.S. patients (11, 16, 20, 33). The authors noted the distinct absence of IgG OspC antibodies in sera from European patients (34) and suggested the failure of the response to reach maturity was because the epitope induced antibodies in a T-cell-independent fashion (22). However, this is clearly not the case after infection with *B. burgdorferi* sensu stricto. In this study, we detected high titers of borreliacidal IgG OspC antibodies, and numerous other investigators (5, 7, 11, 20, 25) have also confirmed that IgG OspC antibodies are commonly present in all stages of the illness. It seems unlikely the iden-

tical epitope would induce both a T-cell-independent and a T-cell-dependent response.

Rather, we suspect the failure of the OspC antibodies to switch classes in the European Lyme disease patients is more likely due to the predilection of the spirochetes to colonize the central nervous system, which may inhibit the maturation of the antibody response. In support, European patients are often infected with *B. garinii* or *B. afzelii*, which commonly cause neuroborreliosis (23). These genospecies are not found in the United States, where the patients are instead infected with *B. burgdorferi sensu stricto*, which most often colonizes the skin or joints. Additional studies to evaluate this hypothesis remain necessary, but the collective results still highlight the immunodominance of the OspC antibody response specific for the C terminus of the protein and provide support for the development of serodiagnostic procedures to exploit this finding.

In addition, the results provide compelling evidence that the C terminus of OspC is a good human vaccine candidate. However, we failed to detect borreliacidal OspC antibodies in sera from the laboratory mice or hamsters, despite vigorous antibody responses against many other *B. burgdorferi* proteins. The sera instead contained only small concentrations of OspC antibodies detected by ELISA or Western blotting. More significantly, the OspC antibodies were not borreliacidal antibodies. The findings therefore confirmed a significant difference in the antigenic reactivities of *B. burgdorferi sensu stricto* spirochetes that infect human or rodent hosts. More importantly, the results cast considerable doubt on the ability of rodent animal models to provide experimental data that accurately predict the ability of vaccination with OspC to provide protection against human infection.

In conclusion, the immunodominant IgM and IgG OspC antibody response in human Lyme disease is specific for the highly conserved seven C-terminal amino acid residues. This finding provides valuable information for future efforts to develop effective serodiagnostic tests and vaccines, but an accurate assessment of their effectiveness cannot be obtained by using laboratory mice or hamsters.

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Characterization of the humoral immune response in dogs after vaccination against the Lyme borreliosis agent

A study with five commercial vaccines using two different vaccination schedules

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Abstract

Using five commercially available vaccines, groups of dogs were vaccinated against Lyme borreliosis and followed for 13 months. A modified vaccination schedule was included in the study in an attempt to induce higher and therefore longer-lasting protective antibody levels during the first year of immunization. Following vaccination antibodies were monitored using ELISA and Western blotting. Serum samples were examined either with antigen preparations derived from either *Borrelia burgdorferi* s. s., *B. garinii* or *B. afzelii* lysates or separately with recombinant OspA derived from the three species. Regardless of the vaccine used the third vaccination induced significantly higher antibody levels. Further analyses of the sera with homologue and heterologue detection systems showed *in vitro* only a minor cross-reactivity of vaccinal antibodies towards antigens derived from heterologous *Borrelia* species *in vitro* and consequently only minimal cross-protection can be expected.

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1. Introduction

Borrelia burgdorferi, a bacterium transmitted by ticks, causes Lyme borreliosis, a common vector-borne disease in humans and animals [1,2]. After the successful generation of a lysate vaccine for dogs in the United States of America [3,4] research focused on additional vaccine-candidates with the introduction of formulations derived from recombinant OspA [5–7]. Studies have shown that antibodies directed against OspA are able to protect the host from the transmission of borrelia through the tick [8,9]. A recombinant OspA vaccine for the use in humans has been available for a few years [10,11] but was removed from the US American mar-

ket for economic reasons [12] even though studies showed a good immune response in humans [10,11,13–15]. This was also proven for dogs when whole cell preparations as well as lipidated recombinant OspA were administered [14,16–19].

The need for a protective vaccine is well-founded in the problematic nature of the disease. Lyme disease commonly causes severe lameness due to arthritis in one or more joints [20]. These early signs of Lyme disease in humans and dogs are sometimes not recognized and therefore remain untreated [21]. This allows the borrelia to disseminate in the host and to induce chronic disorders [22,23]. Treatment of human and animal Lyme borreliosis by antibiotic therapy is highly effective in the early state of disease [22], but the therapy of a chronic disease becomes more complicated [24]. In addition, research data suggest that a successful treatment of Lyme disease is not accompanied by the complete elimination of the spirochete from the host's tissues [25,26].

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Since treatment is difficult, the level of protection achieved with vaccines is important. Consequently an evaluation of recombinant OspA-vaccines was performed in two major studies in humans. In 1998 Sigal et al. were able to show that 68% of all vaccinees were protected after two immunizations within the first year, while the level of protection rose to 92% after the booster immunization the following year [10]. In a study carried out Steere et al. the percentage of protected individuals was even lower at 49% rising to 76% after the third vaccination in the second year [11]. A further study revealed that a third vaccination during the first year is able to raise the level of protection so that during the first 12 months 91–93% of the vaccinees were protected [27]. Since OspA antibodies are taken up by ticks when feeding on the immunized host, where they block Lyme borreliosis organisms from migrating within the tick, antibody levels have to be high at the time when ticks feed [9,28,29]. However, a specific value for a protective antibody level has not yet been defined for dogs as it has been for humans [15].

The goal of this study was to compare the current vaccination regime of two first-year immunizations with a modified three-time vaccination schedule in order to induce high antibody levels in dogs.

2. Materials and methods

2.1. Dogs and vaccines

Experiments were conducted in compliance with the Saxon Ministry for Social Affairs, Health, Youth and Family in Dresden, Germany. Dogs of both sexes, different breeds and age were chosen from the area of Saxony, Germany, and screened for specific antibodies against *B. burgdorferi* s. l. with serological assays (ELISA and Western blots) prior to immunization. Only animals negative for *B. burgdorferi* s. l. antibodies were included in the study for experimental immunization. Dogs were assigned randomly to one of the five vaccine groups as well as to one of the two vaccination groups. At the time of vaccination all dogs were healthy, based on pulse, temperature and clinical examination as well as information of the owners.

2.2. Vaccination and serology

Five commercial vaccines currently available for dogs were used for the study and each vaccinated dog was followed over a period of 13 months. Vaccines were administered subcutaneously either at the chest wall or between the shoulders. Each study group contained up to 10 dogs. Dogs received one of the five available vaccines and were immunized either according to schedules A or B. In total 49 animals received two vaccinations 3 weeks apart (schedule A, Table 2) and 50 dogs were vaccinated three times in 2- or 3-week intervals (schedule B, Table 2). Beginning with the second vaccination, blood samples of all dogs participating in the study were

collected after each immunization and in monthly intervals thereafter. The last sample was taken 1 month after the booster vaccination administered 376 ± 3 days after the first immunization. Blood was drawn either from the *Vena cephalica*, the *Vena saphena*, or the *Vena jugularis*.

Collected blood samples were allowed to coagulate for 30 min and then centrifuged at $3,500 \times g$ at 15°C for 15 min. The resulting serum was stored at -25°C .

2.3. Antigen preparation

Performing the study in Europe, where the causative agent of Lyme borreliosis is not only *B. burgdorferi* s. s. but also *B. garinii*, *B. afzelii* and probably other species [31,32], the need for heterogeneity in the immune response has to be considered. By using not only antigens derived from *B. burgdorferi* s. s. but also from *B. garinii* and *B. afzelii* as detection antigen vaccines were analyzed *in vitro* for their ability to induce cross-reactive and probably cross-protective antibodies.

Low-passage *B. burgdorferi* s. s. N40 (passage 2) were grown in 6 ml of Barbour–Stoenner–Kelly (BSK-II) medium [33], *B. afzelii* PKo and *B. garinii* PBi (passages 30 and 16, respectively) in 6 ml of Modified Kelly Pettenkofer (MKP) medium [34] (PKo and PBi borrelia strains and protocol were kindly provided by Dr. Bettina Wilske, Max von Pettenkofer Institut Munich, Germany). After reaching the desired density of 10^7 to 10^8 borrelia/ml, the organisms were placed in 1 l of the corresponding medium in air-tight screw cap glass bottles and grown to stationary phase (7–9 days). The preparation was concentrated by centrifuging the culture at $10,000 \times g$ at 20°C for 15 min. Supernatants were discarded and the pellets re-suspended in sterile phosphate buffered saline (PBS). The suspensions were centrifuged at $10,000 \times g$ at 4°C for 15 min. This step was repeated twice. After the last centrifugation borrelia were re-suspended in 5 ml PBS and placed on ice. Ultrasound disruption of borrelia was performed on ice at 35 W for 10 s repeating the procedure 10 times (Bandelin Sonoplus UW2070, Berlin, Germany). Using dark field microscopy (Zeiss, Jena, Germany) the solution was controlled for spirochete disintegration. Next, the solution of disrupted spirochetes was centrifuged at $10,000 \times g$ at 4°C for 10 min, supernatant was collected, and stored at -80°C until usage. Protein concentration of the cell lysate was measured using the BCA protocol (Pierce, Rockford, USA), the quality of the antigen preparation was evaluated after SDS PAGE electrophoresis [35] on gels stained with silver nitrate salt (SilverQuest Silver Staining Kit, Invitrogen, Karlsruhe, Germany) or Coomassie brilliant blue dye as described elsewhere [35].

2.4. Antibody detection using ELISA and Western blots

Sera were tested for *B. burgdorferi*-specific antibodies with a computerized kinetic enzyme-linked immunosorbent assay (KELA) and by Western blotting as described elsewhere [36].

Positive control serum samples were included in the study: sera of dogs experimentally infected with *B. burgdorferi* s. s. N40 by tick infestation (dog S98-4/1, dog S98-4/12, dog A96-4/11), serum from a dog immunized experimentally with OspA derived from *B. burgdorferi* s. s., *B. garinii* and *B. afzelii* (triple OspA, dog S98-5/8) as well as serum of a dog experimentally immunized with rOspA of *B. burgdorferi* s. s. without adjuvant (dog S98-4/2). All sera were collected in earlier studies [19]. Additionally a mixture of monoclonal antibodies directed against OspA, OspC, p39 and p60 of *B. burgdorferi* s. s. ZS7 were included in Western blot experiments (antibodies kindly provided by Dr. M. Simon, Max-Planck-Institut für Immunbiologie, Freiburg, Germany).

ELISA plates (C96 Maxisorb, Nunc, Wiesbaden, Germany) were coated as previously described [36] with 100 µl per well carbonate buffer containing 0.34 µg per well cell lysate of either *B. burgdorferi* s. s. or a mixture of *B. garinii* and *B. afzelii* mixed with 0.04 µg per well rOspA of the corresponding species. The spiking with rOspA was performed to ensure maximal OspA-antibody detection (rOspA antigens were kindly provided by Dr. Vincent Weynants, GlaxoSmithKline Biologicals, Rixensart, Belgium). A goat-anti-dog IgG HRP (ICN/Cappel, Durham, USA) diluted 1:4000 was used as secondary antibody. Tetramethylbenzidine (TMB 2-C microwell substrate, KPL, Gaithersburg, USA) served as substrate. The extinction was read three times in 45-s intervals (kinetic ELISA, SpectraMAX340PC, Molecular Devices, Munich, Germany). Knowing that after vaccination protection from the infection with *B. burgdorferi* sensu lato organisms is correlated with high antibody levels of anti-OspA-antibodies [18,30], the level of protecting antibodies of canine vaccines was additionally measured with ELISA using plates coated with recombinant OspA. A coating with 1.0 µg rOspA per well of either *B. burgdorferi* s. s. ZS7, *B. afzelii* ACA1 or *B. garinii* ZQ1 allowed the detection of vaccine-induced OspA antibodies.

To correct for inter-day variations canine control sera were tested repeatedly and correlated back to ELISA values from previous experiments [19]. This correlation was applied to subsequent ELISA analyses.

In addition, a set of samples including the first serum sample of each dog (207 samples) as well as samples with high titers from each vaccinated dog (days 42 or 72) were analyzed in a quantitative C6 ELISA test (IDEXX Laboratories, Maine, USA). Four serum samples of each vaccinated dog were tested with a semi-quantitative C6-SNAP test (IDEXX Laboratories): base line at day 0, samples with the highest antibody levels (days 42 or 72), samples with low antibody

levels (day 376) and samples after booster vaccination (day 407).

SDS PAGE gels (12%, 115 mm × 130 mm) were prepared for Western blotting as described previously [35,36]. Gels were loaded with 300 µg of protein diluted in sample buffer (single 55-mm lane) and with two molecular weight markers in separate lanes (prestained Precision Protein Standards and biotinylated broad range SDS-PAGE Standard, Biorad, Munich, Germany). Separation was performed at 200 V for 3.5 h followed by a transfer onto a nitrocellulose membrane (membraPure CN-Membran 33 cm × 3 mm, 0.45 µm, Bodenheim, Germany) at 30 V over night. After washing and blocking in 3% gelatin solution with TBST, the nitrocellulose membrane was placed in a 24-lane miniblottor chamber (Immunetics, Boston, USA). Serum samples were applied on the membrane in a 1% gelatin solution with TBST and incubated for 1 h at 37 °C on a slowly rotating platform. After washing, membranes were incubated with goat-anti-dog IgG-HRP (1:1000 dilution; ICN/Cappel, Durham, USA) or with goat-anti-mouse IgG-HRP (1:1000 dilution; R&D, Minneapolis, USA) for 1 h at room temperature. Signals were visualized with Opti4CN (Sigma-Aldrich, Munich, Germany).

2.5. Statistical analyses

Mean KELEA-values were calculated for each single time point. Only experiments carried out with the sera of all 92 dogs were statistically analyzed. Sera from dogs vaccinated according to schedule A were compared to those drawn from dogs immunized according to schedule B. Since effects of an additional antigen application can only be documented after basic immunization has been completed, statistical analyses were performed after this time point. Using the restricted maximum likelihood method, (REML) the antibody levels between days 42 (maximum antibody response) and 376 (minimum antibody level) were compared in each vaccination group in order to find significant differences between the two vaccination schedules. In the beginning data were analyzed for interactions between vaccine and regimen but no interactions were detectable. Therefore the REML was calculated without the consideration of interactions between vaccine and regimen. This resulted in the comparison of all dogs vaccinated according to schedule A with all dogs vaccinated according to schedule B independent of the vaccine used. The differences of the least square means were adjusted using Tukey–Kramer. All statistical analyses were performed at the Department for Biometry, Epidemiology and Information Processing (IBEI), University of Veterinary Medicine Hannover, Germany.

Fig. 1. Antibody levels induced by five commercial vaccines detected with *B. burgdorferi* s. s.-derived (A) or with a mixture of *B. garinii*- and *B. afzelii*-derived antigens (B). Dogs in group A (vaccinated twice) are shown with open symbol, dogs in group B (vaccinated three times) are shown with closed symbol. IgG antibody detection was accomplished with bacterial lysates produced with *B. burgdorferi* s. s. N40 organisms or with a mixture of *B. garinii* PBI and *B. afzelii* PKo organism enriched with rOspA of the corresponding species. Graphs with grey-shaded backgrounds show measurements conducted with homologous antigens.

Total antibody levels measured with bacterial lysates derived from

(A) *B. burgdorferi* s. s.

(B) *B. garinii* + *B. afzelii*

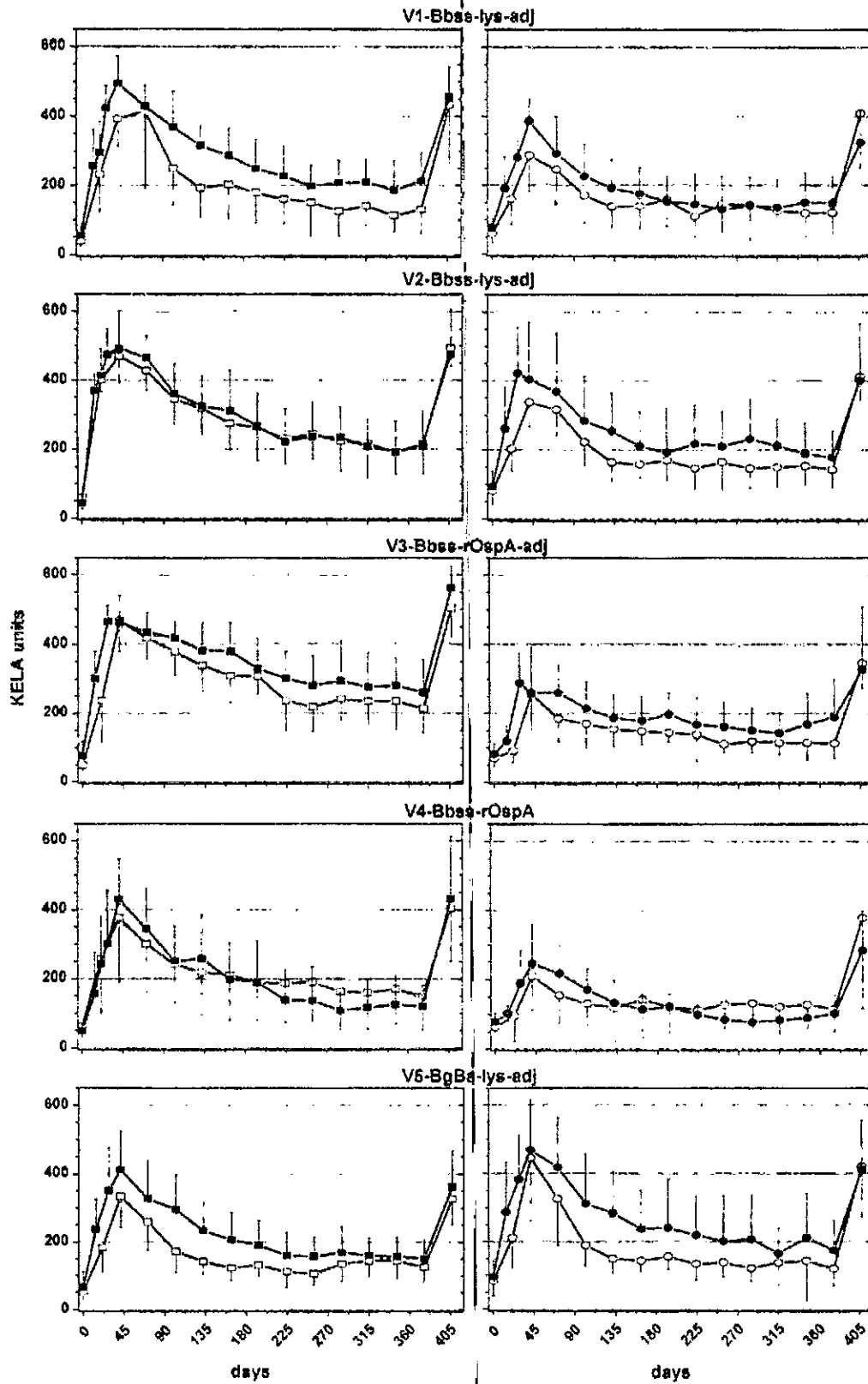


Table 1
Vaccines

Vaccine	Company, country	Species	Antigens	Adjuvant	Abbreviation
Menlym	Maral, Germany	<i>B. burgdorferi</i> s. s.	Lysate	Yes	V1-Bbss-lys-adj
LymeVax	Fort Dodge, USA	<i>B. burgdorferi</i> s. s.	Lysate	Yes	V2-Bbss-lys-adj
ProLyme	Intervet, USA	<i>B. burgdorferi</i> s. s.	rOspA	Yes	V3-Bbss-rOspA-adj
Recombitek Lyme	Meriel, USA	<i>B. burgdorferi</i> s. s.	rOspA	No	V4-Bbss-rOspA
Biocan B	Biovera, Czech Republic	<i>B. afzelii</i> , <i>B. garinii</i>	Lysate	Yes	V5-BgBa-lys-adj

Table 2
Number of dogs per study group and vaccine

	Vaccine				
	V1-Bbss-lys-adj	V2-Bbss-lys-adj	V3-Bbss-rOspA-adj	V4-Bbss-rOspA	V5-BgBa-lys-adj
Group A	9	10(1)	10(2)	10	10(2)
Group B	10(1)	10	10(1)	10	10

Parentheses indicate the number of animals which left the study prematurely.

3. Results

3.1. Screening of 207 base line serum samples

Of 207 dogs analyzed 46 (22.2%) reacted positive for *B. burgdorferi* antibodies using an in-house two-tier-test-system (ELISA combined with a confirmatory Western blot). The infection rate was 20.3%; the remaining four animals (1.9%) had vaccine-induced antibodies. Seropositivity was not accompanied by any clinical signs of illness. Analyzing the sera with the quantitative C6-ELISA lowered the number of positive reacting dog sera to 11, an infection rate of 5.3%.

3.2. Total antibody levels after immunization

Ninety-nine of 207 dogs tested were chosen to be vaccinated with one of the five vaccines listed in Table 1. During the course of the experiment five dogs left the study due to their owners' moving (two dogs) or sale to new owners (three dogs). In addition, one dog died of a generalized infection, another one of cancer. Thus the study consisted of 92 dogs over the complete survey period as shown in Table 2.

The typical development of vaccine-induced antibodies was seen in both groups (A and B) shortly after the completion of the first year's immunization as shown in Fig. 1. Both *B. burgdorferi* s. s.-derived lysate vaccines (V1-Bbss-lys-adj and V2-Bbss-lys-adj) produced antibody levels of nearly 500 KELA units that declined to 200 KELA units within the first 6 months when tested on homologous *Borrelia* antigens (Fig. 1A). The use of heterologous antigens derived from *B. garinii* and *B. afzelii* organisms, however, allowed the detection of cross-reactive antibodies and consequently lower antibody-levels were observed during the same time periods (Fig. 1B). Dogs vaccinated according to schedule A or B with rOspA combined with adjuvant (V3-Bbss-rOspA-adj) produced the highest antibody levels of 500 KELA units which did not drop below 200 KELA units during the first year. The use of heterologous detection antigens however resulted in lower peak antibody-levels that disappeared quickly during the first 6 months. Dogs having received rOspA only (V4-

Bbss-rOspA) showed a maximum of 430 KELA units and the antibody levels declined rapidly to 150 KELA units within the first 12 months when tested with *B. burgdorferi* s. s.-derived antigens. Again, the heterologous detection system was much less efficient in detecting antibodies. In contrast, the lysate produced from both *B. garinii* and *B. afzelii* organisms (V5-BgBa-lys-adj) induced a peak antibody level of 430 KELA units that dropped off swiftly to 150 KELA units within the first 12 months when tested on heterologous *B. burgdorferi* s. s.-derived antigens, while elevated and longer lasting antibody levels were observed when the same sera were tested with the homologous detection system.

All vaccine groups combined showed a statistically significant difference between regimen A and B of $p = 0.0331$ when tested with *B. burgdorferi* s. s. antigens. Concerning the *B. garinii*, *B. afzelii* antigen preparation the p -value was even lower at 0.0115.

3.3. OspA-specific antibodies induced by immunization

Samples from three individual dogs representative for each single study group (30 dogs in total) were analyzed in species-specific OspA-ELISA systems. Plates were coated with rOspA derived from either *B. burgdorferi* s. s. (Fig. 2A), or *B. garinii* (Fig. 2B), or *B. afzelii* (Fig. 2C).

When a homologous detection system was used, OspA antibody levels between 400 and 500 KELA units were measured after completion of the basic immunization with V1–V4. Afterwards peak antibody levels dropped off rapidly and reached pre-vaccination levels within the following 10 months. V5-BgBa-lys-adj produced low peak OspA antibody levels after a regular immunization consisting of two vaccine applications. Three immunizations in 3-week-intervals, however, raised peak OspA antibody levels by approximately 200 KELA units when measured in the homologous detection system.

When heterologous detection systems were used, only cross-reactive antibodies were detected and a loss of detection sensitivity was observed in all study groups (Fig. 2).

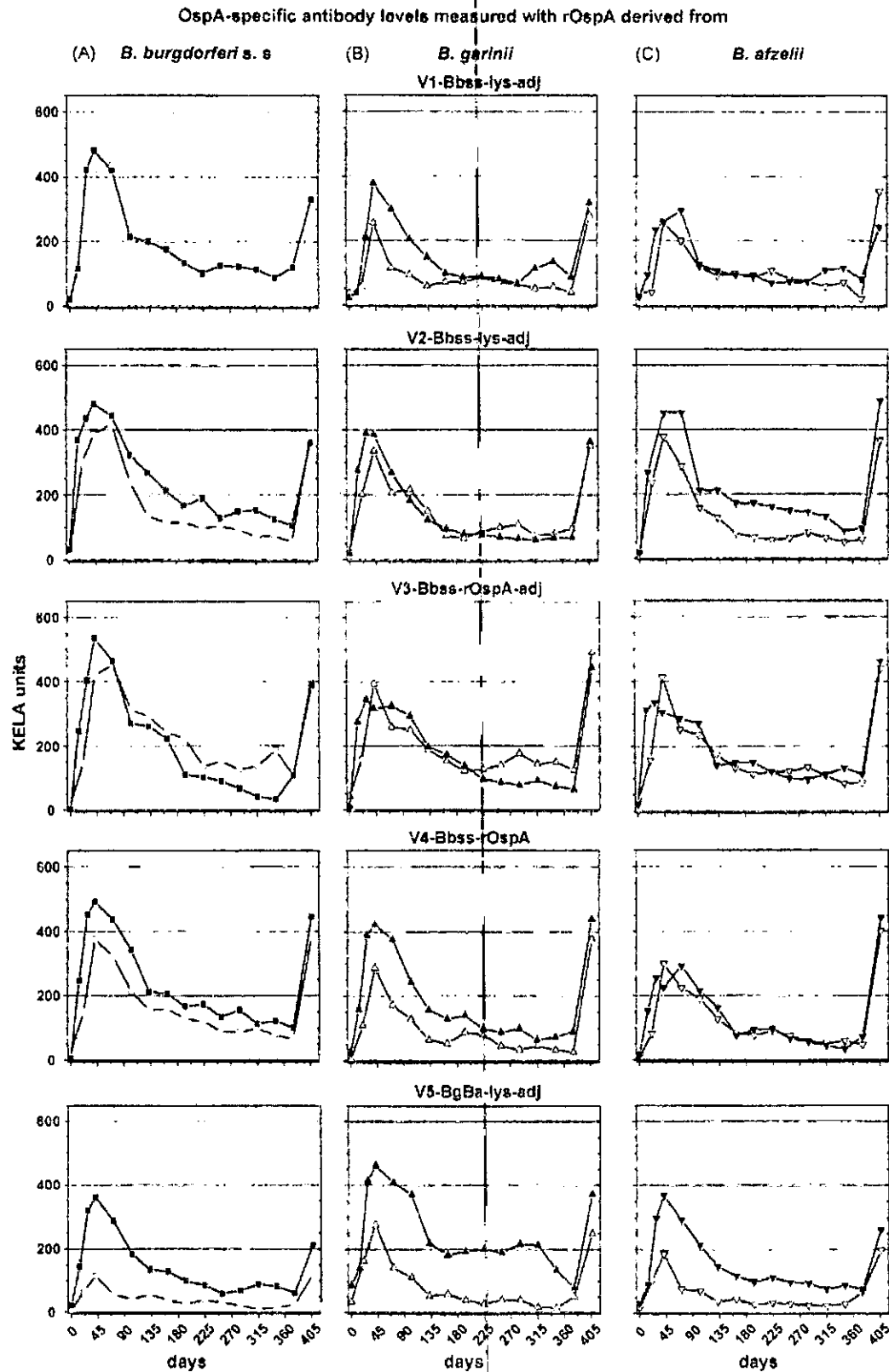


Fig. 2. Antibody levels induced by five commercial vaccines detected with species-specific rOspA proteins. Dogs in group A (vaccinated twice) are shown with open symbol, dogs in group B (vaccinated three times) are shown with closed symbol. The rOspA derived from either *B. burgdorferi* s. s. ZS7 (A), or *B. garinii* ZQ1 (B) or *B. afzelii* ACA1 (C) served as detection antigens for IgG antibodies. Graphs with grey-shaded backgrounds show measurements conducted with homologous antigens.

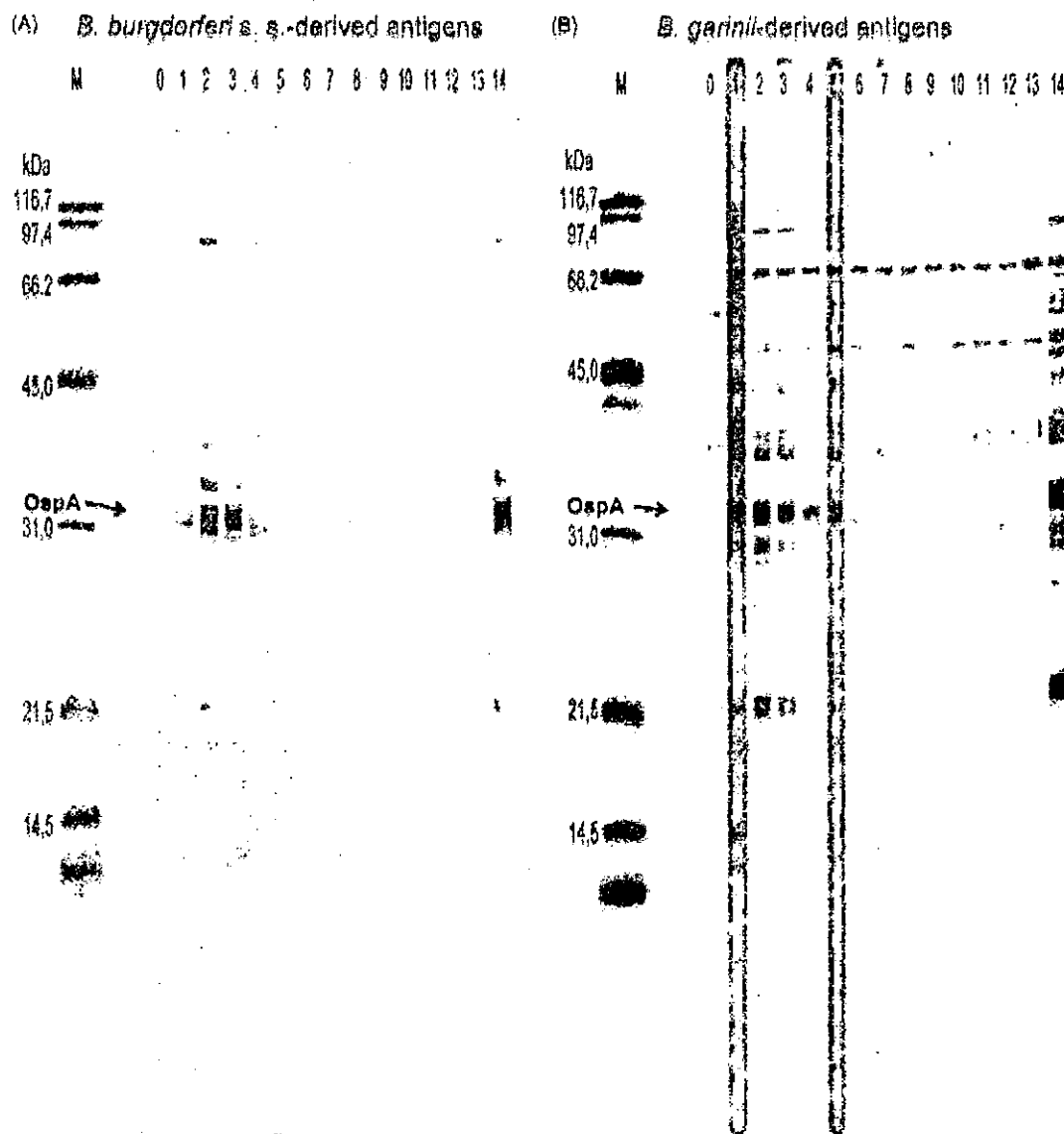


Fig. 3. Western blots showing vaccine-induced antibodies from a dog immunized twice with V1-Bbsa-lys-adj detected with antigens derived from *B. burgdorferi* s. s. N40 (A) or *B. garinii* PBj (B). The first sample was drawn at day 0 (pre-vaccination); lane 1 represents the time of the second vaccination; samples shown in lanes 2–14 (days 42–407) were taken in monthly intervals; the sample in lane 13 (day 376) was collected before the booster vaccination was applied. Vaccine-induced antibodies are seen beginning with lane 1. Mainly OspA antibodies develop, accompanied by various others as expected after vaccination with a lysate-derived vaccine. Signals fade quickly until booster immunization is administered 1 year later.

3.4. Characterization of vaccine-induced antibody responses by Western blot analysis

Fig. 3A shows the vaccine-induced antibody response of a dog immunized with a lysate-based vaccine derived from *B. burgdorferi* s. s. tested with *B. burgdorferi* s. s.-derived antigens. Prior to vaccination the serum showed no specific antibodies for infection (lane 0). After vaccination (lane 1 and following) antibodies mainly directed against OspA (31 kDa), OspB (34–36 kDa) and flagellin (41 kDa) were visible. Starting at day 72 (lane 3) signal intensity decreased continuously until day 376 (lane 13) and then increased again after the booster vaccination at day 407 (lane 14).

The same serum samples were also applied to membranes coated with *B. garinii*-derived antigens as shown in Fig. 3B. Bands developed at 31, 41, 66 and 83 kDa as well as a variety of signals for larger and smaller antigens. The intensity and number of signals increased after the booster vaccination. This analysis was also performed with blots coated with a lysate derived from *B. afzelii* organisms (data not shown). Here the serum samples showed fewer bands in the high and low molecular range and a strongly reduced intensity of the 31, 41 and 83 kDa signals.

Fig. 4A is a Western blot of serum collected from a dog vaccinated with the combined lysates derived from *B. garinii* and *B. afzelii* and assessed with *B. burgdorferi* s. s.-derived

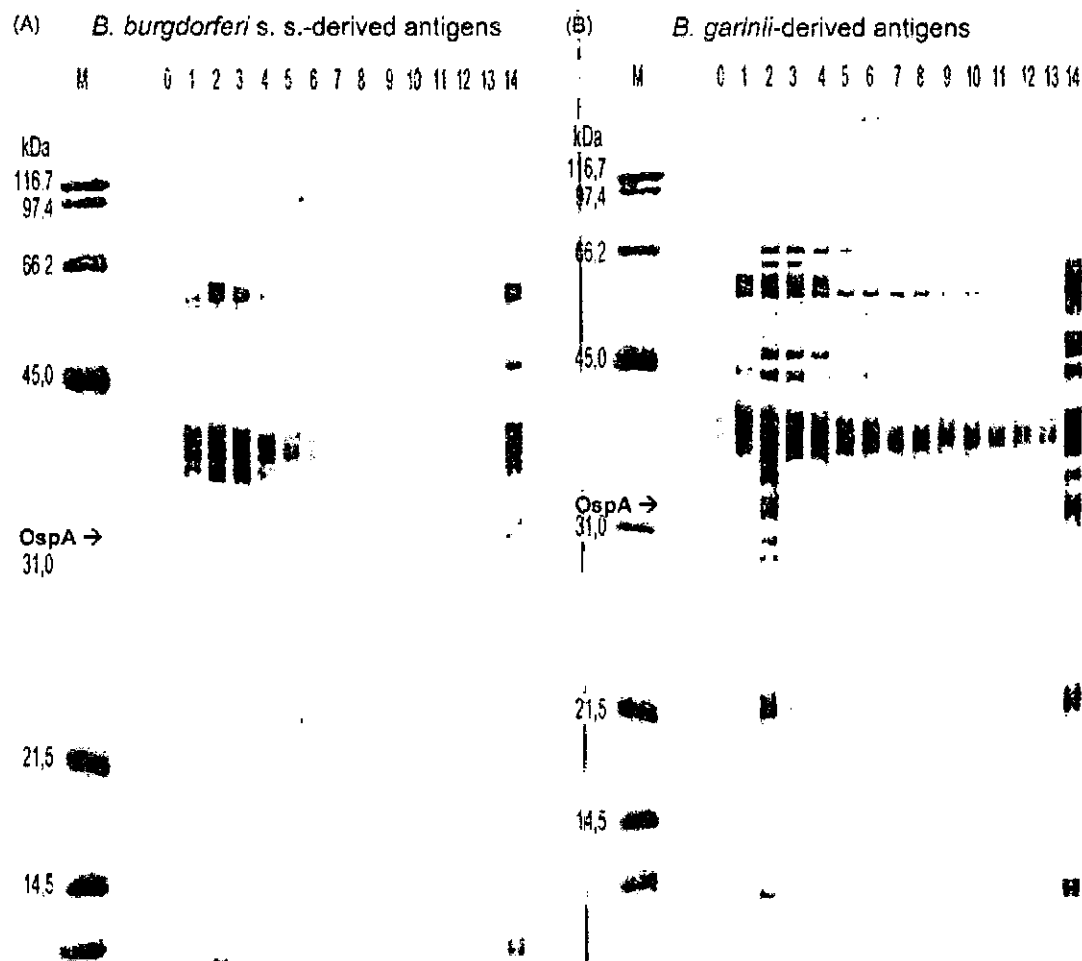


Fig. 4. Western blots showing vaccine-induced antibodies from a dog immunized twice with V5-BgBa-lys-adj detected with antigens derived from *B. burgdorferi* s. s. N40 (A) or *B. garinii* PBi (B). The first sample was drawn at day 0 (pre-vaccination); lane 1 represents the time of the second vaccination; samples shown in lanes 2–14 (days 42–407) were taken in monthly intervals; the sample in lane 13 (day 376) was collected before the booster vaccination was applied. Vaccine-induced antibodies are seen beginning with lane 1. Mainly antibodies directed against 41–43 kDa develop, accompanied by various others as expected after vaccination with a lysate-derived vaccine. Signals fade quickly apart from the strong reaction against the 41–43 kDa-sized antigens until booster immunization is administered 1 year later.

antigens. Again, no signals were visible before vaccination. After the first immunization, on day 21 (lane 1) one strong signal developed at approximately 41–43 kDa, which increased in intensity by the second vaccination and lasted throughout the year. After day 42, bands faded within the first 6 months, including p39, p60 and others. With the booster immunization signals appeared again, but their intensity was reduced compared to the reaction in the previous year. Up to day 376, no signal was detected at 31 kDa, but after the booster a weak band became visible. The same serum was applied to a membrane coated with the lysate produced from a *B. garinii* culture (Fig. 4B). The number of bands and their intensity was stronger compared to the Western blot coated with *B.*

burgdorferi s. s.-derived antigens. A prominent signal was observed at 41–43 kDa and a weak signal visible at 31 kDa. A similar Western blot with *B. afzelii*-derived antigens (data not shown) showed a reduced number of bands in comparison to the Western blot using *B. garinii*-derived antigens and only one prominent signal at 41–43 kDa.

3.5. Cross reactivity of vaccine-induced antibodies

A Western blot was prepared carrying separately the antigens of all three species, that allowed the comparison of three representative vaccinees as well as the reactivity of a serum sample from a dog experimentally infected with *B. burgdor-*

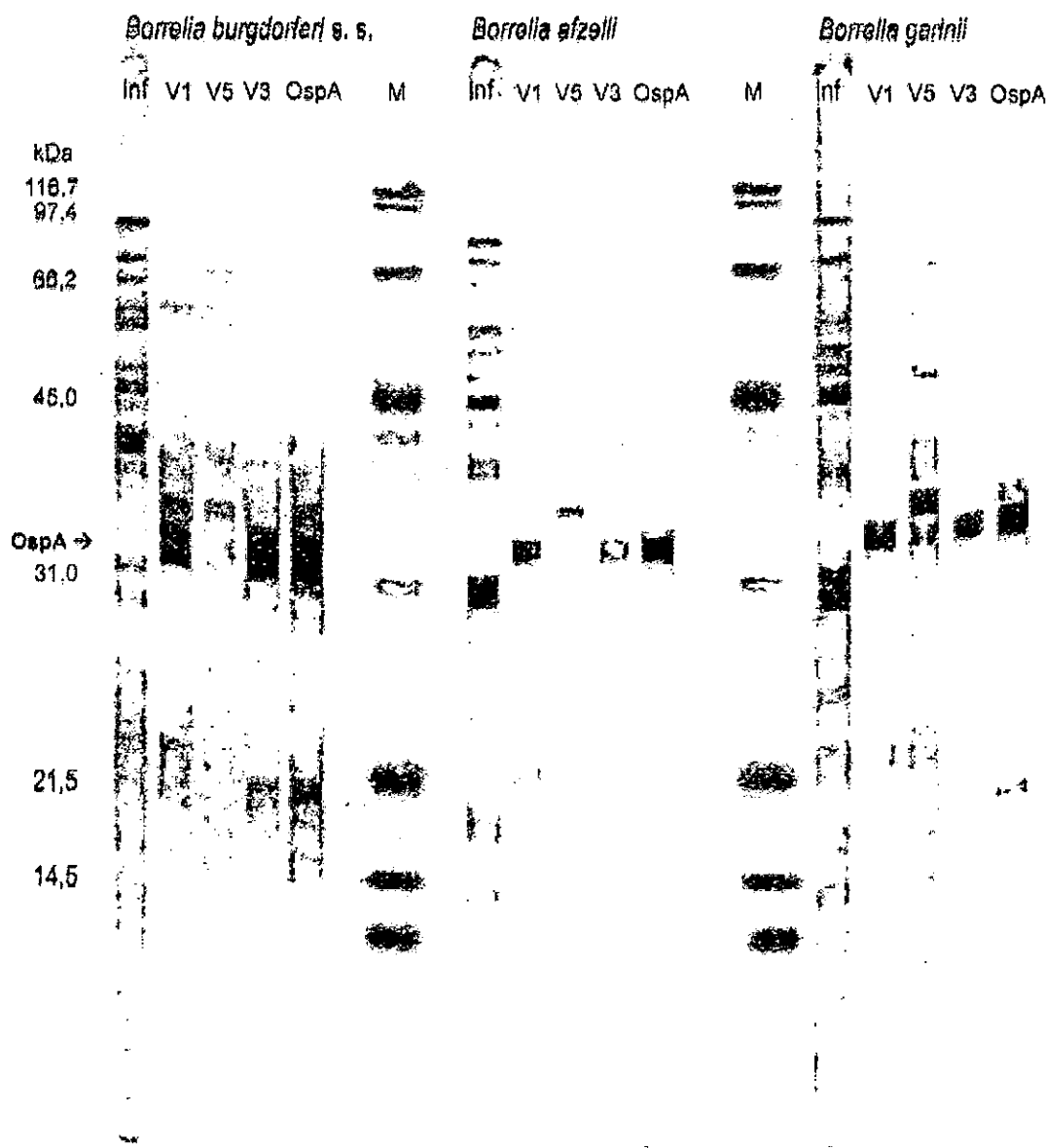


Fig. 5. Western blot showing serum samples from an infected and from vaccinated dogs analyzed with antigens produced with *B. burgdorferi* s. s. (N40), *B. garinii* (PB1), or *B. afzelii* (PKo) organisms. Inf.: serum from a dog experimentally infected with *B. burgdorferi* s. s. by tick exposure; V1: serum from a dog vaccinated three times with V1-Bbss-lys-adj; V5: serum from a dog vaccinated three times with V5-BgBa-lys-adj; V3: serum from a dog vaccinated three times with V3-Bbss-rOspA-adj; OspA: serum from a dog vaccinated experimentally with rOspA of all three species (*B. burgdorferi* s. s., *B. garinii* and *B. afzelii*). The comparison of infected and vaccinated dog sera shows the variations in detectable antibodies when tested with different species. OspA antibodies induced with vaccines V1–V4 (derived from *B. burgdorferi* s. s.) bind weakly to *B. garinii* and *B. afzelii* antigens and vice versa.

feri s. s. N40 (Fig. 5). This experiment was also conducted with monoclonal antibodies described in materials and methods to ensure a correct interpretation of detected signals (data not shown).

Antibodies induced by infection showed similar but not identical patterns when tested with antigens derived from the three different borrelia species. The pattern visualized with *B. burgdorferi* s. s.-derived antigens resembled a typical reaction after an infection with *B. burgdorferi* s. s. as it was reported earlier [37–39]. In contrast, reactions at 14 kDa as well as

17 kDa were seen with *B. garinii*- and *B. afzelii*-derived antigens but were missing on blots coated with *B. burgdorferi* s. s. antigen. A band at 30 kDa varied from a double signal on the *B. burgdorferi* s. s.-specific blot to a single broad signal on the *B. afzelii*-specific blot to a triple signal when tested with *B. garinii*-derived antigens. A missing signal at 80 kDa on the *B. afzelii*-specific blot represented another difference.

In comparison, the sera from dogs immunized with V1-Bbss-lys-adj and V5-BgBa-lys-adj showed some signals similar to those detected after infection, but the vaccines induced

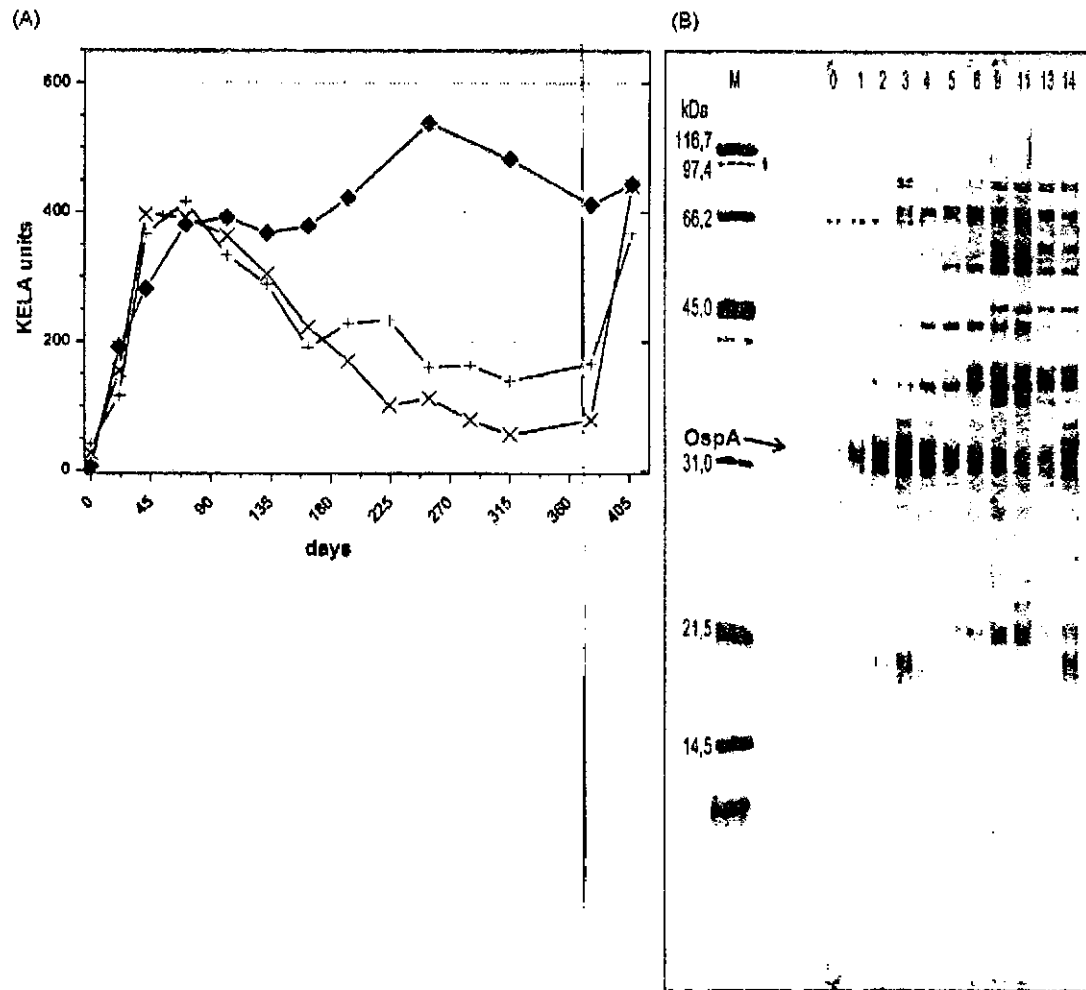


Fig. 6. (A) Antibody levels of serum samples collected from dog KHT-156 (closed rhombus). Two additional vaccinated dogs are shown with a characteristic antibody response after immunization with V4-Bbss-rOspA. (B) Western blot of the serum samples collected from dog KHT-156. The first sample was drawn at day 0 (pre-vaccination); lane 1 represents time of the second vaccination; samples shown in lanes 2–14 (days 42–407) were taken in monthly intervals; sample in lane 13 (day 376) was collected before the booster vaccination was applied 1 year later. Beginning with day 72, development of signals indicative for an infection with *B. burgdorferi* can be observed.

fewer bands. In addition, V1-Bbss-lys-adj induced a strong response against OspA, OspB, and p60 when tested with *B. burgdorferi* s. s.-derived antigens. Small amounts of OspC-specific antibodies appear in response to V1-Bbss-lys-adj lysate vaccination, but are absent in response to V2-Bbss-lys-adj (data not shown). When the same serum samples were applied to antigens from the other two species, all signals were less intense. The recombinant vaccine V3-Bbss-rOspA-adj induced two signals, one against OspA, the other appearing in the lower molecular range, showing breakdown products of the OspA antigen preparation as seen in earlier experiments [19]. On the heterologous detection systems the previously strong OspA signal changed to a very weak response directed against the OspA molecules from *B. garinii* and *B. afzelii*.

Dogs vaccinated with V5-BgBa-lys-adj showed a small reaction against homologous OspA detection antigens and almost no binding of the same antibodies was observed

against heterologous OspA proteins, while the main reaction was directed against an antigen at 41–43 kDa.

3.6. Infection after vaccination

The analysis of total antibody levels of all dogs made one sample appear conspicuous. While two other dogs in Fig. 6A developed normal peak antibody levels of 400 KELA units after being vaccinated twice with rOspA, antibody levels in the conspicuous dog reached 550 KELA units by day 235. As shown in Fig. 6B Western blotting illustrated an increase of signal number and also intensity for most signals beginning with day 72. Due to OspA vaccination the dog developed antibodies directed against OspA, but later (lane 11) also against OspC (23 kDa), flagellin (41 kDa), p39 (39 kDa), and antigens at 45 kDa as well as 55 and 90 kDa, signals that are characteristic for an infection. When the same serum samples were tested with the semi-quantitative C6-SNAP test

provided by IDEXX Laboratories, Inc., the first sample prior to vaccination was negative. Samples collected at later times were highly positive for C6-specific antibodies. In contrast, samples from all other vaccinated dogs were negative before and at all times after vaccination.

4. Discussion

By vaccinating 800 human participants van Hoeske et al. showed that the addition of a third dose of rOspA within 6 months of starting the basic immunization protocol could increase the proportion of protected individuals from 53–55% to 91–93% [27]. Applying this approach to veterinary medicine led to the outline of the study presented here. The expansion of the vaccination schedule by a third vaccine application was intended to achieve long-lasting, protective antibody levels that exceed those induced by a regular two-application-schedule used for killed vaccines. In addition, the vaccine schedule was simplified by reducing the application interval between the second and third immunization from 5 months to 2–3 weeks.

Prior to vaccination, the evaluation of 207 serum samples of possible study participants showed an infection rate with *B. burgdorferi* s. l. organism of 20.3%. Consequently, in the geographic area where these dogs reside, an infection with *B. burgdorferi* s. l. occurs frequently, underlining the need for a good protective vaccine. However, it is important to point out that seropositivity does not necessarily equate with the expression of clinical manifestations as it was reported by Levy and Magnarelli [40]. But more importantly, a significant discrepancy in the specificity for antibody detection was observed using different test systems: the commercial ELISA and Western blot system using only recombinant proteins found 60.9% positive, mainly infected and a few vaccinated dogs in a group of 169 individuals. This rate was probably due to a very high reactivity of the test kit resulting in the development of many weak signals difficult to interpret. In addition, by using the read out scheme and evaluation points for each signal, questionable results were found. In contrast, a detection system using the synthetic peptide C6 in an ELISA-format lowered seropositivity to 5.3%. Most likely this low-level detection rate has its origin in the different nature of this test being designed to monitor C6-antibody levels specific for *B. burgdorferi* s. l. after antibiotic treatment is applied in infected individuals [41]. Considering the effect of antibiotic therapy on C6 antibodies and the inherent and purposely established lower sensitivity of the test, it is conceivable that a smaller number of infected animals was detected compared to the standard two-tier-test-system. In summary, an infection rate of approximately 20% as detected with the conventional in-house two-tier-test-system was considered a trustworthy approximation of the true infection rate since no questionable cases were observed and this seropositivity is in accordance with results published by other groups using well-established tests [42,43].

As a consequence all subsequent analyses involving vaccinated dogs were conducted with the in-house test system. All vaccinated dogs (with one exception) showed antibody levels as expected after an immunization with a killed vaccine. For V1-Bhss-lys-adj a positive impact of a third antigen dose is apparent with higher antibody levels during the year and after booster immunization in group B. The fact that the major fraction of bacterin-induced antibodies is directed against OspA was already shown by Barthold et al. as well as Gauthier et al. They achieved similar results when analyzing sera of vaccinated dogs in comparison to infected animals [37,44]. The reaction seen on the Western blot resembles a typical pattern expected after vaccination with a lysate vaccine [19] and was also observed for V2-Bhss-lys-adj [37]. This vaccine, however, does not show such a strong antibody-rising effect as a result of a third antigen dose as seen in V1-Bhss-lys-adj. In this solitary case a regular first-year immunization based on only two vaccinations already produced high antibody levels almost undistinguishable from those induced with the modified extended immunization schedule. The third lysate vaccine, V3-BgBa-lys-adj again illustrates the impact of an additional antigen application. But the OspA signal appears weak, raising the question of the underlying protection mechanism.

In contrast to the lysate-derived vaccines their counterparts containing only single recombinant proteins induced only a limited array of OspA-specific antibodies. Unexpectedly, V3-Bhss-rOspA-adj showed antibody kinetics that surpassed the other vaccine formulations and probably provides longer-lasting protection. The differences in antibody levels observed with *Borrelia*-lysate-coated and rOspA-coated ELISA are possibly the consequence of the reduction of tested animals from 10 to 3 per group and therefore individual variations influenced the analysis much stronger. In contrast, dogs vaccinated with V4-Bhss-rOspA produced lower antibodies levels that declined rapidly. This vaccine induced the weakest antibody response probably due to the lack of adjuvant.

In conclusion the majority of the antibody kinetics described above as well as their combined statistical analyses showed a positive impact of a third vaccination on the antibody levels. It was possible to induce higher, longer-lasting antibodies in dogs by applying additional antigens within the basic immunization schedule.

In addition to the kinetics of vaccine-induced antibody levels the detection of cross-reactive antibodies was a special aspect of this study. Modifying the detection system by exchanging *B. burgdorferi* s. s.- against *B. garinii*- or *B. afzelii*-derived antigens and vice versa in ELISA and Western blot systems made it possible to gain detailed information about the antibodies' cross-reactivity *in vitro*. As seen in Fig. 5, antibodies of a dog experimentally infected with *B. burgdorferi* s. s. attach to many *B. garinii*- and *B. afzelii*-derived antigens. Signals appear to a certain extent at different molecular weights. The reaction against OspC is variable and a clear gap at 31 kDa in all three species demonstrates a lack of OspA-specific antibodies after natural infection as it was published previously [37,38,44–47]. Staining with

monoclonal antibodies and with a canine serum containing OspA-specific antibodies against all three species confirmed that sufficient OspA antigens were present in the *B. garinii*- and *B. afzelii*-derived lysates and therefore in the detection systems. The variations of serum reactions to the different species are in accordance with those seen on Western blots prepared with sera of infected humans described by Hauser et al. [48]. In addition, van Dam showed the different behavior of sera from individuals infected with different European strains and related the results to the difficulties of generating a protective vaccine [49]. Therefore, weaker signals were expected when vaccine-induced antibodies were allowed to bind to heterologous antigens. This low binding affinity of vaccine-induced antibodies against related borrelia species was even more evident in quantitative assays using either ELISA coated with lysate or single rOspA (Figs. 1 and 2). This probably is indicative for a low-level or even the lack of cross-protection as it was demonstrated with the murine model for Lyme borreliosis when species-specific rOspA proteins were used to induce antibodies [50]. The serologically confirmed infection of one dog in this study may point in the same direction (Fig. 6). However, we cannot rule out the possibility that the dog contracted its infection during the early phase of the study when vaccinal antibodies were low and rising and thus were not protective.

In summary, this work illustrates that (i) a third antigen application within 2–3 weeks after the second immunization induces an elevated antibody response. The significant increase leads to the recommendation for it to be put into practice. However, we suggest applying the third vaccination to dogs preferably at a later interval, 4–6 months following the second immunization. This allows a booster of antibody levels at times when in our study animals showed very low antibody levels. (ii) Our data also indicate, that the protection achieved by an immunization might be species-specific, as it was also shown with recombinant OspA by Gern et al. [50]. Therefore, protection from infection by immunization only is difficult to achieve within Europe and tick control seems to be an additional adequate measure to prevent infection. Daily tick checks and substances, which prevent tick attachment, are recommended. Acaricide substances alone or in combination with repellents are advisable to reduce the risk of infection with *B. burgdorferi* s. l. and with other tick-borne organisms. (iii) A substantial variability of outer surface protein patterns among Lyme borrelia species exists. This has to be considered when diagnostic assays are established and interpreted to avoid a large number of false-negative and especially false-positive test results.

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