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Borrelia burgdorferi Sensu Lato Species in Europe Induce Diverse Immune Responses against C_6 Peptides in Infected Mice[▽]

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The diversity of Lyme-borreliosis-inducing *Borrelia* species in Europe set high standards for the use of serodiagnostic test systems in terms of specificity and sensitivity. In the United States, the one-step C_6 antibody test system based on the invariable domain IR6 of the VlsE molecule has been established as a successful diagnostic tool for testing canine samples. However, only a limited set of data are available regarding the antigenicity of the C_6 peptides in an experimental murine model and sensitivity of the test regarding European *Borrelia* species. In order to investigate antibody reactions induced by these spirochetes, a total of 142 C3H/HeN mice were inoculated with *Borrelia burgdorferi* sensu stricto N40, *B. garinii* PBi, two isolates of *B. afzelii*, *B. spielmanii* A14S, *B. valaisiana* Rio6, *B. valaisiana* VS116, or *B. lusitaniae*. Infection of the mice was documented utilizing tissue culture and PCR. The IR6 sequences of *B. burgdorferi* sensu stricto B31, *B. garinii* IP90, and two *B. afzelii* ACAI strains have been used to synthesize and test additional C_6 peptides. Compared to the well-established two-tiered test system, the results indicate that single C_6 peptides derived from *B. burgdorferi* sensu stricto and *B. garinii* can be used in an enzyme-linked immunosorbent assay-based technique to detect murine antibodies induced by either agent. Little is known about the prevalence or pathogenicity of the *B. afzelii* strains in mammalian hosts, but our experimental data indicate differences in the C_6 peptide test sensitivity for the detection of antibodies induced by different strains or isolates of *B. afzelii*.

Three members of the *Borrelia burgdorferi* sensu lato complex—*B. burgdorferi* sensu stricto, *B. garinii*, and *B. afzelii* (3)—are known to induce clinical symptoms associated with Lyme borreliosis (2, 3) in humans (43). Infections with *B. burgdorferi* sensu lato species are well documented in animals, especially infections in mice with *B. burgdorferi* sensu stricto, *B. afzelii*, and *B. garinii* have been proven (16, 31, 32) and in dogs *B. burgdorferi* sensu stricto has been demonstrated to cause infection and clinical disease (1). In Europe, *B. lusitaniae* (6, 21) and DNA from *B. valaisiana*, which can also be found in Asia (9, 39), have been detected in human patients with suspected Lyme borreliosis. *B. spielmanii* (36, 51) has been isolated from human patients. Regarding clinical symptoms, *B. garinii* and *B. afzelii* are the most prevalent pathogenic species in Europe and Asia, followed by *B. burgdorferi* sensu stricto, while in the United States, *B. burgdorferi* sensu stricto is the only species found to cause Lyme borreliosis in humans (42). The heterogeneity of *Borrelia* species in Europe led to the need of a species-dependent optimization of two-tiered serodiagnostic test systems (53) to determine specificity and sensitivity.

The variable surface protein VlsE (for variable major protein-like sequence, expressed) of *B. burgdorferi* sensu stricto is a highly specific serodiagnostic tool. The expression site *vlsE* undergoes a gene conversion mechanism (7, 58, 59) and, consequently, the VlsE protein itself is modified during infection in the mammalian host (58). The VlsE protein and especially its invariable region 6 (IR6) of *B. garinii* and *B. burgdorferi* sensu stricto were found to be highly immunogenic and specific for infection. Antibodies directed against these VlsE regions can be used in order to detect infections with *B. burgdorferi* sensu stricto strains in monkeys, mice, and humans (20, 27, 28). Based on these findings, a 26-mer synthetic peptide analogue of IR6, referred to as the C_6 peptide, has been successfully established as a serodiagnostic tool for human and canine Lyme borreliosis in the United States and in Europe (5, 22–24, 26, 34, 35, 48). The diversity of potential pathogenic *Borrelia* species in Europe and the unexplained state of infectivity or pathogenicity of some species raised the question of whether a single IR6-derived peptide can detect antibodies to all of these species. In a previous study, IR6 derived from *B. garinii* VlsE could successfully detect murine antibodies against *B. burgdorferi* sensu stricto, *B. garinii*, and *B. afzelii*, and antibodies from U.S. patients with different clinical manifestations of Lyme borreliosis (25). Investigations have been conducted, in which human sera collected during various stages of clinical Lyme borreliosis in Europe and North America have been tested with IR6/C_6 peptides derived from *B. burgdorferi* sensu stricto, *B. garinii*, and variants of *B. afzelii*. Several data indicate that IR6 sequences derived from *B. afzelii* are useful for the detection of C_6 antibodies in human sera from the United States and that *B. burgdorferi* sensu stricto-derived C_6 peptides can detect

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TABLE 1. Overview of *B. burgdorferi* sensu lato inoculation experiments[a]

Spirochete	Growth medium	Expt I		Expt II		Expt III		Total no. of mice (n = 142 [7])
		Dose (CFU)/ mouse	No. of mice (n = 48 [0])	Dose (CFU)/ mouse	No. of mice (n = 36 [2])	Dose (CFU)/ mouse	No. of mice (n = 58 [5])	
B. burgdorferi sensu stricto N40	BSK-II	10^6	6	10^6	10	10^6	5	21
B. garinii PBi	BSK-II	10^6	6	10^6	10	6.0×10^5	5	21
B. afzelii PKo	MKP	10^6	6	–	0	10^6	17	23
B. afzelii Slovakia	BSK-II	10^6	6	–	0	7.6×10^5	15	21
B. valaisiana VS116	BSK-II	10^6	6	–	0	–	0	6
B. valaisiana Rio6	BSK-II	–	0	10^6	8	–	0	8
B. spielmanii A14S	BSK-II	10^6	12	–	0	10^6	6	18
B. lusitaniae	BSK-II	10^6	6	10^6	8	10^6	10	24

[a] For experiments I and III, the collection of murine serum samples occurred on days 28, 56, and 63 postinoculation, and mice were sacrificed on day 63. For experiment II, the collection of murine serum samples occurred on days 28, 56, and 84 postinoculation, and mice were sacrificed on day 90. n, Total number of mice (values in brackets indicate the number of negative-control mice). –, No inoculation with this spirochete.

antibodies in sera from European patients. The *B. garinii*-derived C_6 peptide did not detect antibodies against *B. afzelii* or *B. burgdorferi* sensu stricto, as well as the just-mentioned *B. afzelii*- and *B. burgdorferi* sensu stricto-derived C_6 peptide variants (41). Regarding European patients' sera, it was proposed that the use of a C_6 peptide mixture might be more beneficial with regard of genospecies dependency and that early infections may not be detected by all C_6 test preparations (12, 49). Previously reported data showed differences in the amino acid sequences between IR6 within the *B. burgdorferi* sensu lato complex (13), which might result in different reactivities of patient sera. These different observations taken together lead to the need of further investigations regarding the use of C_6 peptides, especially for European applications. The antibody levels against different C_6 peptides should be measured separately in individuals who were known to be infected with only one of the pathogenic *Borrelia* species to exclude the influences of cross-reactive antibodies induced by different species. To our knowledge, no experimental studies exist using C_6 peptide sequences based on *B. burgdorferi* sensu stricto, *B. garinii*, and *B. afzelii* to detect immune responses at unique time points postinfection against different *Borrelia* genospecies, which also include *B. spielmanii*. This is important because *B. spielmanii* may be pathogenic, and serodiagnosis of specific antibodies against *B. spielmanii* might be recommended in the future. For this reason, we compared murine immune responses against *B. spielmanii* with *Borrelia* lysate antigen serology and C_6 peptides. In our study, mice were inoculated with *B. burgdorferi* sensu stricto, *B. garinii*, *B. afzelii*, *B. spielmanii*, *B. valaisiana*, and *B. lusitaniae*. The potential infectivity of spirochetes was characterized with plasmid lp25 and lp28-1 PCR.

Murine sera were collected and tested with whole-cell lysate-based two-tiered test method and enzyme-linked immunosorbent assay (ELISA) containing C_6 peptides originated from *B. burgdorferi* sensu stricto, *B. garinii*, or *B. afzelii*. Two-tiered testing was carried out to compare sensitivities with C_6 peptide test system. In addition, to confirm results obtained by serodiagnosis, tissue culture and PCR were carried out to demonstrate the successful infection of the mice. We focused on the experimental assay and not on the induction of clinical symptoms by *Borrelia* species and strains or their correlation with C_6 antibodies. For this reason, our results obtained for the sensitivity of C_6 peptides in the murine model do not allow the unrestricted transfer of the conclusions to infections occurring in other host species such as humans and dogs. However, they should offer a comprehensive contribution to the question of whether serological assays using a single C_6 peptide are sufficiently sensitive and cross-reactive to detect species-specific C_6 antibodies induced by defined European *Borrelia* species or strains.

MATERIALS AND METHODS

***Borrelia*.** Six species from the *B. burgdorferi* sensu lato group were used for the experiments. *B. burgdorferi* sensu stricto N40 (passage 3) is an isolate from a skin punch biopsy of an experimentally infected dog (1). *B. garinii* strain PBi (passage 16) and *B. afzelii* PKo (passage 32), representing European serodiagnostic reference strains (55, 56), have been originally isolated from humans. A second, uncharacterized *B. afzelii* isolate was named as *B. afzelii* Slovakia in this publication was an isolate from an ixodid tick. This isolate was shown to be infective in C3H mice (17). Two strains of *B. valaisiana*, strain VS116 (passage 46) from our institute stock and a tick isolate strain Rio6 (passage unknown) from Spain (10) were used for inoculation. *B. lusitaniae* (passage 14) from Portugal was an isolate originated from human skin (6). *B. spielmanii* strain A14S (passage 10) was originally isolated in The Netherlands.

Cultivation of spirochetes. To optimize bacterial growth in liquid media, BSK-H Medium (Sigma, Taufkirchen, Germany) and Barbour-Stoenner-Kelly (BSK-II) medium were prepared with different concentrations of heat-inactivated rabbit serum or gelatin for each *Borrelia* species and strain. All strains except *B. afzelii* PKo were grown at 33°C in BSK-II medium supplemented with 7.0 to 8.0% of rabbit serum and 8.5% bovine gelatin. *B. afzelii* PKo was grown in modified Kelly medium (MKP) (38). This protocol was kindly provided by Bettina Wilske, Max von Pettenkofer Institut, Munich, Germany.

Mice. Female C3H/HeN (C3H) mice were bred and purchased by Harlan Winkelmann GmbH, Borchen, Germany. During the time of experiment, mice were kept under specific-pathogen-free conditions in individually ventilated cages at the animal facility of the Max Planck Institute for Evolutionary Anthropology (Leipzig, Germany). The animal experiment was carried out in accordance with the guidelines approved by the Animal Care and Usage Committee of the Regierungspräsidium, Leipzig, Germany.

Intradermal inoculation of *B. burgdorferi* sensu lato into mice. In total, 142 mice were inoculated with *B. burgdorferi* sensu stricto N40, *B. garinii* PBi, *B. afzelii* PKo, *B. afzelii* Slovakia, *B. valaisiana* VS116, *B. valaisiana* Rio6, *B. spielmanii* A14S, or *B. lusitaniae* (Table 1). Spirochetes were grown to late exponential phase until injection. The dose of spirochetes per mouse was between 6.0×10^5 and 1.0×10^6 in 100 μl of BSK-II or MKP medium. This volume was divided in two adjoining injections which were placed intradermally into shaven back of each mouse. Three C3H mice served as negative controls for tissue PCR.

Sera. Blood samples from all mice were obtained at days 28 and 56 after borrelia inoculation by retrobulbary bleeding under anesthesia using hematocrit glass capillaries (200 μl of blood) or by intracardial bleeding at day of sacrifice using a syringe. Serum was prepared in serum separators (BD Microtainer; Becton Dickinson) by a centrifugation step at 5,000 × g for 5 min at room temperature. Sera were stored frozen at −80°C. For the calculation of ELISA

TABLE 2. *B. burgdorferi* sensu lato C_6 peptide sequences used for ELISA

Species origin and recombination cassette	Peptide	Amino acid sequence (26-mer)[a]	Accession no.
B. burgdorferi B31 Vls15	*B. burgdorferi* C_6	CMKKDDQIAAAIALRGMAKDGKFAVK	AAC45189.1
B. garinii IP90 Vls7	*B. garinii* C_6	CMKKDDQIAAAMVLRGMAKDGQFALK	AAN87831.1
B. afzelii ACAI Vls1	*B. afzelii* $C_{6\text{-}1}$	CMKKSDKIAAAIVLRGVAKSGKFAVA	AAN87809.1
B. afzelii ACAI Vls3	*B. afzelii* $C_{6\text{-}2}$	CMKKRNDKIVAAIVLRGVAKDGKFAAA	AAN87811.1

[a] Including the N-terminal cysteine (C) conjugated.

cutoffs, 27 stored sera from uninfected C3H/HeN mice served as negative controls.

Tissue samples for cultivation and PCR. Mice were sacrificed at day 63 or 90 after borrelia inoculation. Tissues from the heart, skin from the right ear, right tarsal joint, bladder, and skin from inoculation area of the back were collected under sterile conditions. Tissue samples were washed in 70% of ethanol and in phosphate-buffered saline (PBS). Whole bladder and joint and parts of the ear, heart, and skin of back were squashed in 200 μl of BSK-II or MKP. These tissue mixtures were each individually transferred into 6 ml of BSK-II or MKP. Tissue cultures were kept at 33°C for 8 weeks and observed weekly for the presence of viable spirochetes. Except in experiment I, parts of the heart and skin from the ear and back were frozen at −80°C for later PCR. In the case of borrelia-positive tissue cultures, spirochetes were stored at −80°C in 100-μl aliquots containing added glycerol.

KELA using *Borrelia* lysate as antigen. A computerized kinetic ELISA (KELA) was performed as described previously (50). Sonicated whole cell-lysate of *B. burgdorferi* sensu stricto N40 served as the antigen. Murine sera were diluted 1:100 in PBS with 0.05% of Tween 20 and 2% milk powder (PBSTM). Borrelia-specific antibodies were detected with horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G (IgG; R&D Systems, Minneapolis, MN) in dilutions of 1:500 and 1:1,000 in PBSTM.

Western blotting. Western blot analysis of selected mice sera was performed with a test kit using *B. afzelii* lysate antigen and recombinant VlsE (*B. afzelii* + VlsE EcoBlot IgG Western Blot; Genzyme Virotech GmbH, Rüsselsheim, Germany). The test procedure was carried out according to the instructions supplied with the kit. Alkaline phosphatase conjugated affinity purified goat anti-mouse IgG (Rockland, Inc., Gilbertsville, PA) at a dilution of 1:4,000 was used as the secondary antibody. The substrate reaction was stopped after 10 min. The interpretation of test results was performed according to included kit-specific protein band templates and based on published criteria (14, 15).

C_6 peptide-specific ELISA commercial test system. The Lyme Quantitative C_6-antibody test kit (Lyme Quant C_6 Test; IDEXX Laboratories, Inc., Westbrook, ME) was used as a prescreening test for C_6-specific antibodies (24, 34). This test kit was originally developed for canine serum samples to measure the levels of C_6-specific antibody in the sera of positive dogs. Kit instructions were used but adapted with the following modifications: murine samples sera were diluted 1:100, and antibodies were detected with horseradish peroxidase-conjugated goat anti-mouse IgG (R&D Systems) using dilutions of 1:1,000 and 1:500 in PBSTM. The optical densities (OD) of the samples were measured spectrophotometrically at 650 nm and compared to sera from uninfected mice and sera from mice infected with *B. burgdorferi* sensu stricto N40.

ELISAs with *Borrelia* species-specific C_6 peptides serving as the antigen. Four synthetic C_6 peptides derived from the IR6 region of *B. burgdorferi* sensu stricto, *B. garinii*, or *B. afzelii* were synthesized with an N-terminal cysteine that was used for conjugation. The origin and amino acid sequences of the four synthetic C_6 peptides are shown in Table 2. Microtiter plates were coated with 100 μl of each C_6 peptide/well at a concentration of 0.5 μg/ml in a 0.05 M sodium carbonate coating buffer. After an incubation step at room temperature, the peptide solution was aspirated, and the plates were washed two times with PBS-Tween wash buffer. Plates were blocked by using 200 μl of detergent containing sucrose in 0.1 M Tris buffer/well for 2 h at room temperature. The blocking buffer was aspirated, and the plates were dried overnight at room temperature. Plates were stored in Mylar bags with desiccants at 4°C until use. ELISA was carried out and evaluated according to the protocol described previously for the commercial C_6 test system.

Statistical evaluation of ELISA data. For each plate, mean background values (wells incubated without serum but with anti-mouse IgG) were subtracted from mean samples values for each sample. For each infection group (mice infected with the same *Borrelia* species), the means (μ) and standard deviations (SD) (σ) of the obtained ELISA values were calculated. Cutoffs for each antigen tested in an ELISA were calculated by adding five SD to the mean values obtained by measuring 27 samples from uninfected mice. Samples were considered positive if the measured OD was equal or greater than the cutoff value.

Infectivity of *Borrelia* strains used for inoculation. To determine the infectivity of the *Borrelia* species used for inoculation, a PCR was performed that detected sequences of the linear plasmids lp25 and lp28-1. Lack of the lp25 plasmid results in a complete loss of infectivity and lack of the lp28-1 plasmid has been reported to result in diminished virulence (18, 19). *Borrelia* species and strains that were originally used for inoculation and those that could be isolated from murine tissues postinoculation were grown in BSK-II or MKP as previously described. Cultures were centrifuged at 10,000 × *g* for 15 min at 15°C. The supernatant was discharged, and the pellet was suspended in 200 μl of PBS. Centrifugation was repeated at 4°C. Supernatant was discarded again, and the pellet was suspended in 180 μl of PBS. Spirochetes and water used as a negative control were applied to a Qiagen Blood & Tissue kit (Qiagen GmbH, Hilden, Germany). DNA was eluted in 100 μl of distilled water. The yield of DNA and the purity were measured with a spectrophotometer (Eppendorf, Hamburg, Germany) at wavelengths λ = 230, 260, 280, and 320 nm. In the case of weak spirochetal growth, 100-μl portions of spirochete cultures were centrifuged and suspended in PBS and then boiled at 94°C for 5 min. A total of 10^5 spirochetes per μl were directly used for PCR. The primer sequences for plasmid lp25 (618 bp) and plasmid lp28-1 (291 bp) were previously described (16, 17). As DNA template, 5 μl of DNA were used. PCRs (50 μl) contained 4.0 mM $MgCl_2$, 1.0× PCR buffer, 1.25 U of *Taq* polymerase (AmpliTaq Gold DNA Polymerase with Gold Buffer and $MgCl_2$; Applied Biosystems Deutschland GmbH, Darmstadt, Germany), 0.2 mM concentrations of deoxynucleoside triphosphates (Fermentas GmbH, St. Leon-Rot, Germany), 1.0 μM concentrations of each primer (synthesized by Metabion International AG, Martinsried, Germany), and water. The PCR protocol was structured as follows: 94°C for 3 min; 40 cycles at 94°C for 1 min, 45°C for 1 min, and 72°C for 2 min; followed by a final extension step at 72°C for 6 min. A 1.6% agarose gel separation with ethidium bromide staining was used to resolve and visualize the PCR products.

Preparation and extraction of murine and borrelia DNA for positive control standard. Two mice, which were kept under specific-pathogen-free conditions as mentioned above, were sacrificed. Livers, spleens, and hearts were removed under sterile conditions. Tissue portions of 0.5 g were subjected to a phenol-chloroform-isoamyl alcohol extraction protocol as described previously (52). After extraction, DNA pellets were dried under vacuum and then dissolved in 200 μl of distilled water. *B. burgdorferi* sensu stricto N40, *B. garinii* PBi, *B. afzelii* PKo, and *B. spielmanii* A14S were grown as described previously. DNA extraction of 10^7 spirochetes per ml of medium was carried out as previously described. DNA was eluted in 55 μl of distilled water. Then, 55 μl of eluted DNA from 10^7 borrelia was used for the preparation of dilutions containing DNA from 10^7 to 10^{-2} borrelia in distilled water. Portions (200 μl) of DNA dilution from uninfected mice tissues were combined with 50 μl of each spirochete DNA dilution in a final DNA concentration of 240 ng/μl.

Extraction of DNA from mice tissues samples inoculated with *Borrelia* spp. Skin tissue samples from the ear or back of mice inoculated with *B. burgdorferi* sensu stricto, *B. garinii* PBi, *B. afzelii* PKo, *B. afzelii* Slovakia, or *B. spielmanii* were used for DNA extraction. Additional tissue samples from three uninfected C3H mice served as negative controls. DNA extraction was carried out using a Qiagen Blood & Tissue kit.

DNA quantification. To detect the *ospA* gene from *B. burgdorferi* sensu stricto N40 primer, probe sequences and an amplification protocol were used as previously described (44). Tests showed that the probe specific for *B. burgdorferi* sensu stricto *ospA* could detect *B. garinii* and *B. afzelii ospA* but not the corresponding *B. spielmanii ospA* sequence. The following primer and probe sequences were synthesized by Metabion: for *B. garinii* PBi *ospA*, BgPBi-ospA-16F (5′-AAATG TTAGCAGCCTTGATGAAA-3′) and BgPBi-ospA-119R (5′-GACTGTAATT ACCATCTTTGTCTTT-3′); for *B. afzelii* PKo *ospA*, BaPKo-ospA-15F (5′-AA

TABLE 3. Recultivation of spirochetes from murine tissues

Borrelia inoculated	No. of mice tested/ no. of mice inoculated[a]	No. of tissue samples positive for spirochetes/no. of cultivated tissue samples					Total mice with *Borrelia*-positive tissue samples	
		Heart	Bladder	Joint	Ear	Back	No. of mice/total no. of mice examined	Rate (%)
B. burgdorferi sensu stricto N40	12/20	12/12	12/12	12/12	12/12	6/6	12/12	100.0
B. garinii PBi	18/21	0/18	0/18	0/18	0/18	0/15	0/18	0.0
B. afzelii PKo	20/23	9/20	6/20	1/20	5/20	0/17	14/20	70.0
B. afzelii Slovakia	20/21	6/20	7/20	4/20	0/20	2/14	11/20	55.0
B. valaisiana VS116	0/6	–[b]	–	–	–	–	–	–
B. valaisiana Rio6	8/8	0/8	0/8	0/8	0/8	0/8	0/8	0.0
B. spielmanii A14S	18/18	0/18	0/18	0/18	0/18	0/12	0/18	0.0
B. lusitaniae	24/24	0/24	0/24	0/24	0/24	0/18	0/24	0.0

[a] That is, the number of mice tested in tissue culture/the number of mice inoculated with *Borrelia*.
[b] –, not tested.

AATGTTAGCAGCCTTGATGAA-3′) and BaPKo-ospA-119R (5′-GACTGTA CTTACCGTCTTTGTCTT-3′); and for *B. spielmanii ospA*, Bsp-ospA-F (5′-AA TGTTAGCGGCCTTGACGAGAA-3′), Bsp-ospA-R (5′-AGGCTGTATTTAC CGTCTTTGTCCT-3′), and Bsp-ospA-P (FAM-5′-AACAGCACTTCAGTAG ATGTACCTGG-3′-TAMRA).

A volume of 2.5 μl of DNA per well in triplicate was used with 25 μl of Mastermix for testing. Species-specific DNA from borrelia served as the positive control in each specific quantitative PCR (qPCR) assay. Measurements were carried out with an iCycler iQ Multi-Color real-time PCR detection system (Bio-Rad Laboratories GmbH, Munich, Germany). Optimal results regarding slope, efficacy, and sensitivity could be achieved with a total DNA concentration of 120 ng/μl (300 ng/well). For every sample, threshold cycle mean values and SD were calculated and correlated to the values of the positive-standard dilution series to define the number of spirochetes per 300 ng of extracted murine DNA.

Image processing. Images taken from Western blots, and PCR gels were processed (size, contrast, brightness, and labeling) using CorelDRAW 9 computer software.

RESULTS

Re-cultivation of spirochetes. To determine whether the inoculated *Borrelia* species and strains were able to disseminate through the host's tissue, samples of murine tissues were cultivated in liquid media for eight weeks. The results are shown in Table 3. From 100% of the murine tissues and mice inoculated with *B. burgdorferi* sensu stricto N40 spirochetes could be reisolated, while 14 of 20 mice (70.0%) inoculated with *B. afzelii* PKo and 11 of 20 mice (55.0%) inoculated with *B. afzelii* Slovakia were culture positive. The majority of these cultures were found to be positive for heart tissue and bladder tissue samples. No tissues from mice inoculated with *B. garinii* PBi, *B. spielmanii*, *B. lusitaniae*, and *B. valaisiana* Rio6 were positive. Tissues from mice inoculated with *B. valaisiana* VS116 were not assayed.

IgG antibody response to *B. burgdorferi* sensu stricto lysate antigen KELA. As a first serological screening test for immunological responses to borrelia, murine sera were tested at days 28 and 56 postinoculation for rising antibody reactions with a KELA based on whole-cell lysate antigen (Fig. 1). All sera from mice inoculated with *B. burgdorferi* sensu stricto N40 (Fig. 1A), *B. afzelii* PKo (Fig. 1C), and *B. afzelii* Slovakia (Fig. 1D) showed strong and rising total antibody levels during the first 56 days postinoculation. The group of mice which had been inoculated with *B. garinii* PBi reacted heterogeneously (Fig. 1B). Seven murine sera (G02, G04, G15 to G17, G19, and G20) remained antibody negative, while three mice sera (G01, G07, and G10) showed high and eleven mice sera (G03, G05, G06, G08, G09, G11 to G14, G18, and G21) showed moderate antibody levels. These variations were also found in mice inoculated with *B. spielmanii* A14S (Fig. 1G). Six sera (S07, S09 to S12, and S17) showed low antibody levels, while twelve sera showed a clear rising of IgG levels over time. Of 24 sera from mice inoculated with *B. lusitaniae*, two (L01 and L04) responded positively (Fig. 1F). The rest of the serum samples did not exceed the cutoff line, including four sera (L02, L05, L07, and L17) that showed a weak increase in antibodies over time. None of the mice which had been inoculated with *B. valaisiana* VS116 or Rio6 showed a detectable antibody reaction (Fig. 1E).

Confirmation of previous KELA results with Western blots based on *B. afzelii* lysate antigen. Following the two-tiered testing method for serodiagnosis, immune responses detected with ELISA were confirmed by using a lysate antigen-based Western blot with sera obtained day 56 postinoculation. When samples that had exceeded the KELA cutoff line were tested, mice were considered overall positive for a specific contact with borrelial antigen if protein bands for recombinant VlsE and two of the following bands were detectable: p83/100, p58, p43, p39, p30, p23 (OspC), p21, and p17. The number of sera that reacted against the different protein bands and the percentages are shown in Table 4. A representative selection of murine serum samples is shown in the Western blots in Fig. 2. The majority of mice (Table 4) inoculated with *B. burgdorferi* sensu stricto N40 (represented by number 1 and the sera B02, B12, and B19 on stripes a, b, and c) showed serum reactions against the VlsE protein, p41, p39, OspC, and p21. All sera were considered seropositive. A total of 100% of mice that were inoculated with *B. afzelii* PKo and 85.0% of *B. afzelii* Slovakia-inoculated mice showed antibody reactions against recombinant VlsE (Table 4). All sera responded against p83/p100, p41, OspC, and p21, as shown with serum samples A08, A12, and A17 for *B. afzelii* PKo (Fig. 2, number 3, stripes a to c) and AS07, AS14, and AS15 for *B. afzelii* Slovakia (Fig. 2, number 4, stripes a to c). In summary, all mice inoculated with *B. afzelii* were considered seropositive. Seventeen mice inoculated with *B. garinii* PBi showed no or weak antibody responses on Western blots (data not shown). Despite the fact that 18 sera reacted against OspC (Table 4), 17 were considered seronegative because of missing reactions against VlsE and com-

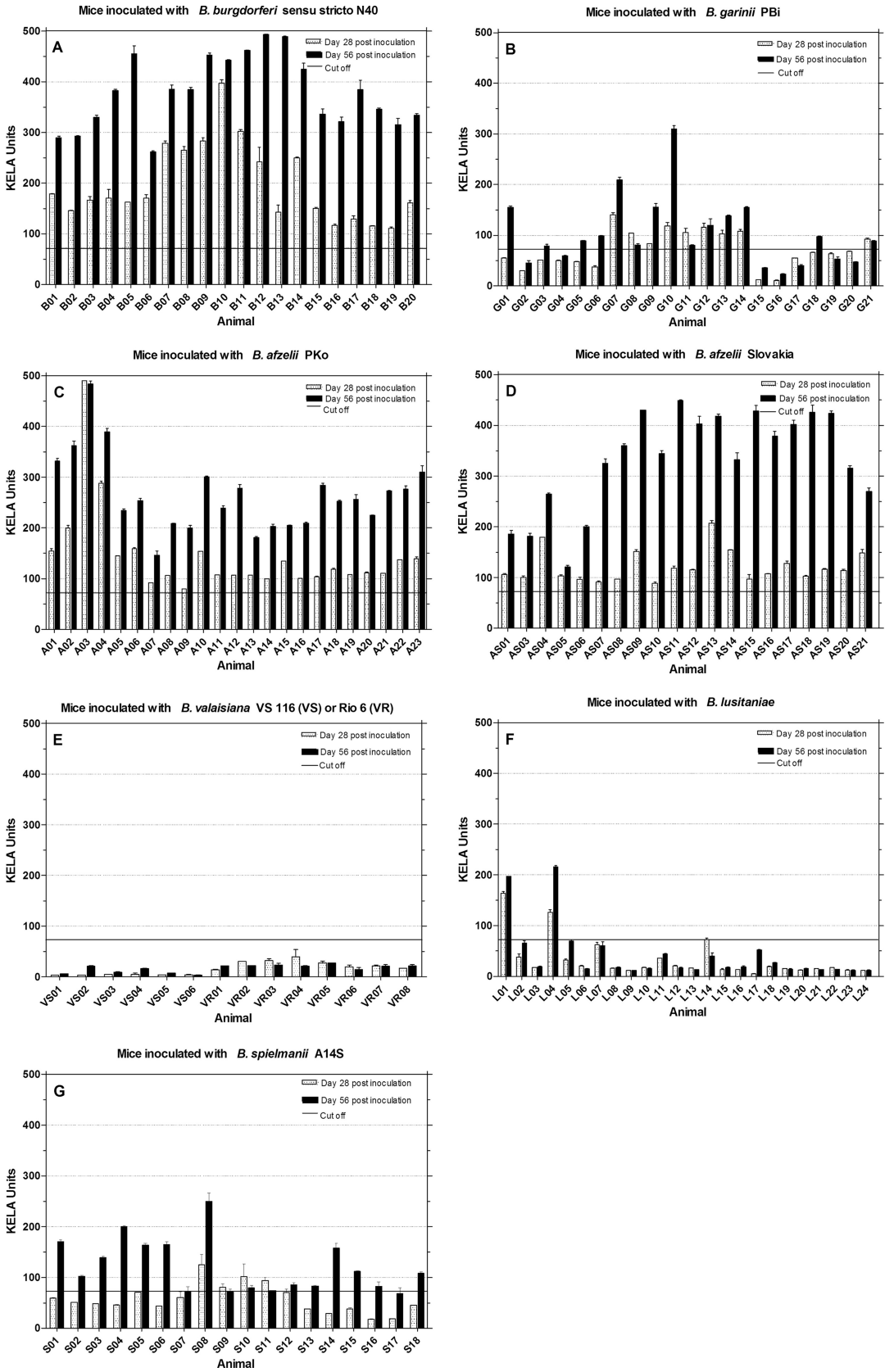

FIG. 1. IgG antibody response to *B. burgdorferi* sensu stricto lysate antigen. Mice were inoculated with different *Borrelia* species (A to G), and sera were tested at days 28 and 56 postinoculation for the presence of a rising overall *Borrelia* IgG response. The cutoff (73.03) was set at mean KELA units value of sera from 27 uninfected mice plus five times the SD of the mean.

TABLE 4. Western blot immune responses of murine sera inoculated with different *Borrelia* species against lysate antigen proteins

Protein	No. of sera with specific present protein bands and expressed as % of tested sera															
	B. burgdorferi sensu stricto N40		*B. garinii* PBi		*B. afzelii* PKo		*B. afzelii* Slovakia		*B. spielmanii* A14S		*B. lusitaniae*		*B. valaisiana* VS116		*B. valaisiana* Rio6	
	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
VlsE	20	100.0	3	15.0	23	100.0	17	85.0	17	94.4	0	0.0	0	0.0	0	0.0
p83/p100	15	75.0	1	5.0	23	100.0	20	100.0	14	77.8	0	0.0	0	0.0	0	0.0
p58	7	35.0	1	5.0	18	78.3	11	55.0	3	16.7	0	0.0	0	0.0	0	0.0
p43	0	0.0	0	0.0	1	4.3	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0
p41	19	95.0	13	65.0	23	100.0	20	100.0	16	88.9	1	4.2	0	0.0	0	0.0
p39	14	70.0	4	20.0	23	100.0	17	85.0	12	66.7	0	0.0	0	0.0	0	0.0
OspA	1	5.0	4	20.0	0	0.0	4	20.0	8	44.4	7	29.2	0	0.0	0	0.0
p30	6	30.0	0	0.0	2	8.7	3	15.0	3	16.7	0	0.0	0	0.0	0	0.0
OspC	16	80.0	18	90.0	23	100.0	20	100.0	3	16.7	0	0.0	0	0.0	0	0.0
p21	14	70.0	13	65.0	23	100.0	20	100.0	13	72.2	0	0.0	0	0.0	0	0.0
p17	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0
Total sera	20	100.0	20	100.0	23	100.0	20	100.0	18	100.0	24	100.0	6	100.0	8	100.0

binations of infection-specific proteins. Three mice reacted moderately seropositive with recombinant VlsE (Fig. 2, number 2, G07, G13, and G18 on stripes a to c). In addition, these three sera produced antibody reactions against OspC and p21 and were considered seropositive. Mice inoculated with *B. spielmanii* A14S are represented by sera S09, S22, and S25 (Fig. 2, number 5, stripes a to c). As shown in Table 4, 94.4% of sera reacted positively against VlsE. A total of 77.8% showed antibody responses to p83/p100, and 72.2% showed antibody responses to p21. Eight sera responded to OspA (44.4%) and 16 to p41 (88.9%). The 17 VlsE-positive sera were considered seropositive. None of the mice inoculated with *B. lusitaniae* showed antibodies to VlsE or other proteins specific for *Borrelia* infections. As represented by the sera L04, L01, and L12 (Fig. 2, number 6, stripes a to c), 29.2% of the mice inoculated with *B. lusitaniae* (Table 4) reacted against OspA. None of the mice were considered seropositive, which is also true for mice inoculated with *B. valaisiana* VS116 and *B. valaisiana* Rio6.

C_6 peptide-specific quantitative ELISA results obtained with a commercial test system. All murine sera were tested with the Lyme Quant C_6 Test to document the relative level of C_6-specific antibodies over time. The data are shown in Fig. 3. Mice inoculated with *B. burgdorferi* sensu stricto N40 developed strong and mostly rising antibody responses over time from days 28 to 56 postinoculation ($\mu = 1.7$; $\sigma = 0.56$ at day 56) (Fig. 3A). In comparison, sera from mice inoculated with *B. afzelii* PKo (Fig. 3C) produced weaker C_6 signals ($\mu = 0.65$; $\sigma = 0.36$ at day 56). Thirteen mice (A01 to A04, A07, A10, A11, A15 to A18, A20, and A22) showed a decline in antibody levels from days 28 to 56. Of the mice inoculated with *B. afzelii* Slovakia, seven (AS04, AS05, AS08, AS09, AS12, AS14, and AS20) (Fig. 3D) showed a decline in C_6 antibody levels and five (AS06, AS07, AS16, AS19, and AS21) had higher antibody reactions (OD > 0.5; $\mu = 0.35$; $\sigma = 0.33$) at day 56. Only three mice inoculated with *B. garinii* PBi showed rising antibody reactions (G07, G13, and G18) with OD values of $\mu = 0.49$ and $\sigma = 0.40$ at day 56. Six mice inoculated with *B. spielmanii* A14S (Fig. 3G) developed rising antibody levels over time (S01, S04, S06, S07, S17, and S18). Two sera (S02 and S03) showed a decrease in C_6 antibodies from days 28 to 56 (in total $\mu = 0.13$, $\sigma = 0.18$ at day 56). Mice inoculated with *B. valaisiana* or *B. lusitaniae* strains, with the results depicted in Fig. 3E ($\mu = 0.04$, $\sigma = 0.0$) or Fig. 3F ($\mu = 0.02$ and $\sigma = 0.02$), respectively, had no detectable C_6 antibody response.

Antibody responses against *Borrelia* species-specific C_6 peptides at day 56 postinoculation. Figure 4 to 7 show IgG antibody reactions of murine sera against *B. burgdorferi* sensu stricto C_6 (Fig. 4), *B. garinii* C_6 (Fig. 5), and *B. afzelii* C_6 peptides with sequence variable 1 (*B. afzelii* $C_{6\text{-}1}$) (Fig. 6) and variable 2 (*B. afzelii* $C_{6\text{-}2}$) (Fig. 7). Considerable overall antibody levels were found in mice inoculated with *B. burgdorferi* sensu stricto N40 (Fig. 4 to 7A). The highest overall responses were detected to *B. garinii* C_6 with ($\mu = 1.50$; $\sigma = 0.43$) in Fig. 5A, followed by *B. burgdorferi* C_6 ($\mu = 1.33$; $\sigma = 0.17$) in Fig. 4A. Lower and individually different immune responses were detected using *B. afzelii* $C_{6\text{-}1}$ ($\mu = 0.59$; $\sigma = 0.70$) and *B. afzelii* $C_{6\text{-}2}$ ($\mu = 0.43$; $\sigma = 0.46$). Twelve (Fig. 6A) and seven sera (Fig. 7A), respectively, did not exceed the cutoff, while specific sera showed higher antibody levels (for example, B02, B07, B12, or B19 in Fig. 6A and B04 to B07 and B17 to B19 in Fig. 7A). Mice inoculated with *B. afzelii* PKo (Fig. 4 to Fig. 7C) built peak antibody responses against *B. afzelii* $C_{6\text{-}1}$ ($\mu = 1.40$; $\sigma = 0.55$) and *B. afzelii* $C_{6\text{-}2}$ ($\mu = 1.60$; $\sigma = 0.57$). Some sera reacted more strongly against *B. afzelii* $C_{6\text{-}1}$ (A08); others reacted more strongly against *B. afzelii* $C_{6\text{-}2}$ (A05). Responses to *B. burgdorferi* C_6 ($\mu = 1.00$; $\sigma = 0.65$; Fig. 4C) varied. The lowest overall antibody responses were detected against *B. garinii* C_6 peptide ($\mu = 0.71$; $\sigma = 0.60$). Here, eight mice developed reactions of <0.25 OD (Fig. 5C).

Mice inoculated with *B. afzelii* Slovakia (Fig. 4 to Fig. 7D) responded most strongly to *B. afzelii* $C_{6\text{-}1}$ ($\mu = 0.50$; $\sigma = 0.52$), as shown in Fig. 6D, followed by peptide *B. afzelii* $C_{6\text{-}2}$ ($\mu = 0.27$; $\sigma = 0.36$), as shown in Fig. 7D. The highest levels were reached by the sera AS01, AS05 to AS07, AS16, AS17, AS19, and AS21. Twelve sera did not reach cutoff line (Fig. 7D). Four sera reached moderate antibody levels against *B. garinii* C_6 in Fig. 5D (AS03, AS06, AS07, and AS19), while the overall titer was $\mu = 0.16$ with $\sigma = 0.20$. Three mice inoculated with *B. garinii* PBi (G07, G13, and G18 in Fig. 5B) had detectable antibody levels against *B. garinii* C_6 ($\mu = 0.10$; $\sigma = 0.36$).

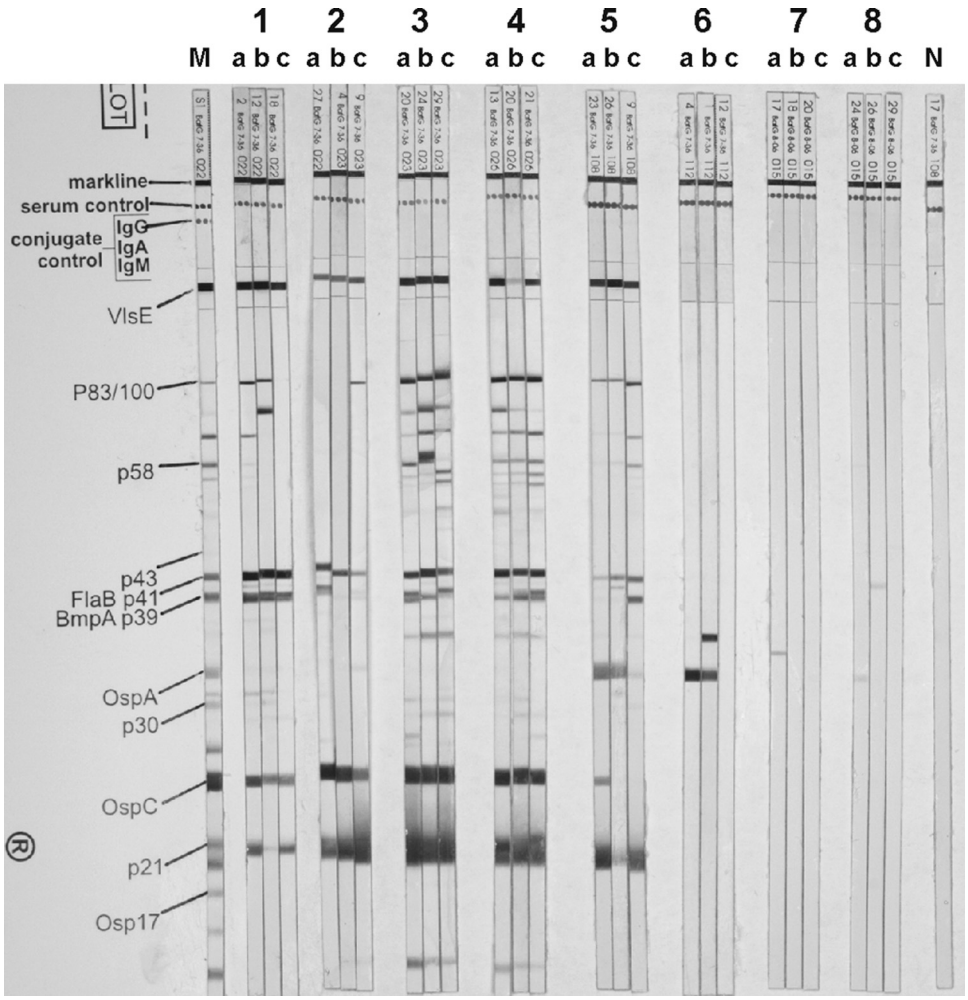

FIG. 2. Antibody response to recombinant VlsE and *Borrelia* lysate antigen on day 56 postinoculation in a Western blot. M, positive control marker for protein bands; N, negative murine serum. Number 1, stripes a to c: mice inoculated with *B. burgdorferi* sensu stricto N40 (B02, B12, and B19). Number 2, stripes a to c: mice inoculated with *B. garinii* PBi (G07, G13, and G18). Number 3, stripes a to c: mice inoculated with *B. afzelii* PKo (A08, A12, and A17). Number 4, stripes a to c: mice inoculated with *B. afzelii* Slovakia (AS07, AS14, and AS15). Number 5, stripes a to c: mice inoculated with *B. spielmanii* A14S (S09, S22, and S25). Number 6, stripes a to c: mice inoculated with *B. lusitaniae* (L04, L01, and L12). Number 7, stripes a to c: mice inoculated with *B. valaisiana* VS116 (VS02, VS03, and VS05). Number 8, stripes a to c: mice inoculated with *B. valaisiana* Rio6 (VR03, VR05, and VR08).

Mouse G07 reacted the most strongly with an OD of 1.68. This distribution of antibody reactions can also be seen in Fig. 4B for *B. burgdorferi* C_6, but the overall titers are clearly lower ($\mu = 0.06$; $\sigma = 0.12$). Only one mouse (G13) exceeded the cutoff if *B. afzelii* $C_{6\text{-}2}$ ($\mu = 0.04$; $\sigma = 0.05$) antibodies were detected (Fig. 7B). No marked antibody responses could be detected against *B. afzelii* $C_{6\text{-}1}$ ($\mu = 0.02$; $\sigma = 0.04$), as shown in Fig. 6B. In mice inoculated with *B. spielmanii* (Fig. 4 to Fig. 7G), only weak overall responses to the different C_6 peptides were detected. The highest levels were reached using *B. burgdorferi* C_6 (Fig. 4G) with $\mu = 0.09$ and $\sigma = 0.07$. Nine sera showed moderate titers with OD values of <0.25 (S01, S03 to S06, S08, S14, S15, and S17). Five sera (S01, S03, S04, S13, and S18) showed weak responses to *B. garinii* C_6 (Fig. 5G) and overall ODs with $\mu = 0.03$ and $\sigma = 0.03$. Two sera (S02 and S06) exceeded the cutoff with responses against *B. afzelii* $C_{6\text{-}2}$ (Fig. 7G), while S07 and S18 showed weak reactions to *B. afzelii* $C_{6\text{-}1}$ (Fig. 6G). No immune responses against *B. burgdorferi* C_6, *B. garinii* C_6, or *B. afzelii* $C_{6\text{-}1}$ and *B. afzelii* $C_{6\text{-}2}$ peptides were detected in sera from *B. valaisiana*- or *B. lusitaniae*-inoculated mice (Fig. 4 to Fig. 7E and F.)

Sensitivity of different C_6 peptides compared to two-tiered testing method. Table 5 demonstrates the sensitivity of different C_6 peptides compared to the two-tiered testing method whose results were set as the standard. Calculations show that sensitivities of 100.0% were reached by homologous systems with C_6 peptides and C_6 antibodies originated from the same *Borrelia* species such as *B. burgdorferi* sensu stricto and *B. garinii*. In the case of C_6 antibody detection against *B. afzelii* $C_{6\text{-}1}$, *B. afzelii* $C_{6\text{-}2}$, and *B. burgdorferi* C_6, 100.0% sensitivity was obtained for *B. afzelii* PKo antibodies; however, clearly lower sensitivities of 70.6% (*B. afzelii* $C_{6\text{-}1}$) and 47.1% (*B. afzelii* $C_{6\text{-}2}$) were obtained when the sera of mice inoculated with *B. afzelii* Slovakia were tested. According to this, *B. burgdorferi* C_6 (58.8%) and *B. garinii* C_6 (64.7%) detected *B. afzelii* Slovakia antibodies with less sensitivity than *B. afzelii* PKo

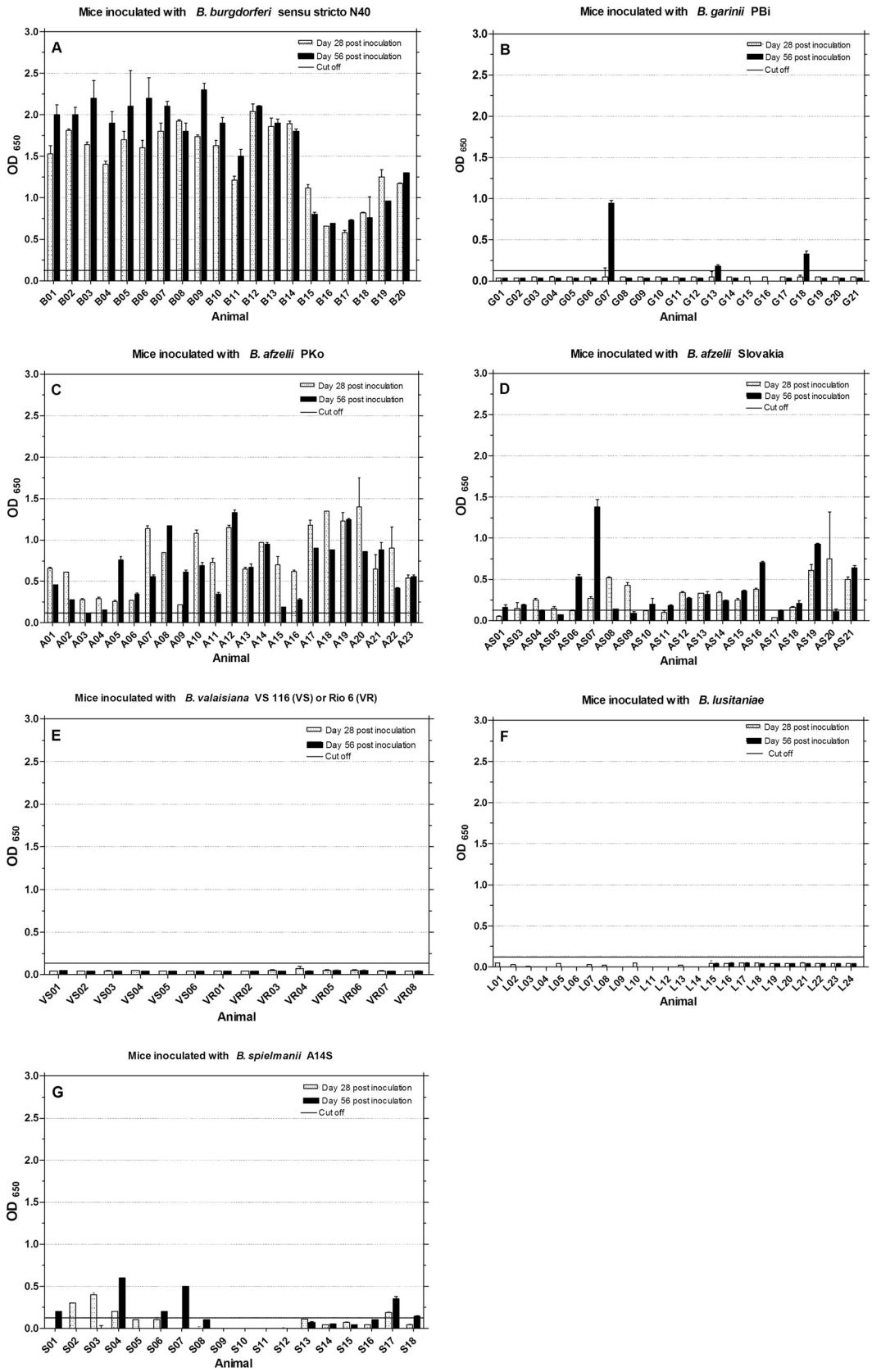

FIG. 3. IgG antibody response to Lyme Quant C_6 Test ELISA. Mice were inoculated with different *Borrelia* species (A to G), and sera were tested at days 28 and 56 postinoculation for the presence of a rising anti-C_6 peptide IgG response. The cutoff (OD = 0.13) was set at mean OD value of sera from 27 uninfected mice plus five times the SD of the mean.

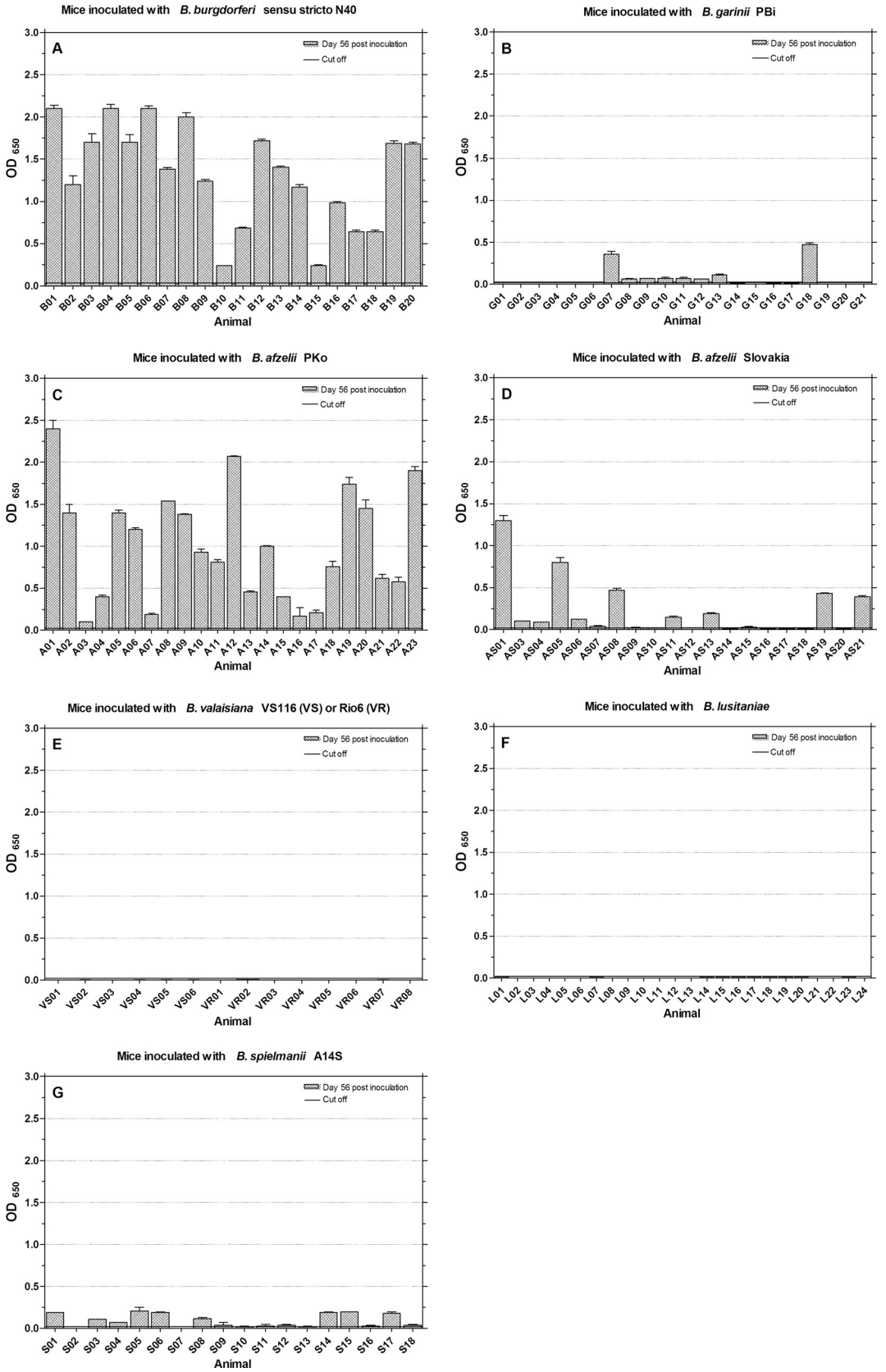

FIG. 4. IgG antibody response to *B. burgdorferi* sensu stricto C_6 peptide antigen. Mice were inoculated with different *Borrelia* species (A to G), and sera were tested at day 56 postinoculation. The cutoff (0.02) was set at the mean OD value of sera from 27 uninfected mice plus five times the SD of the mean.

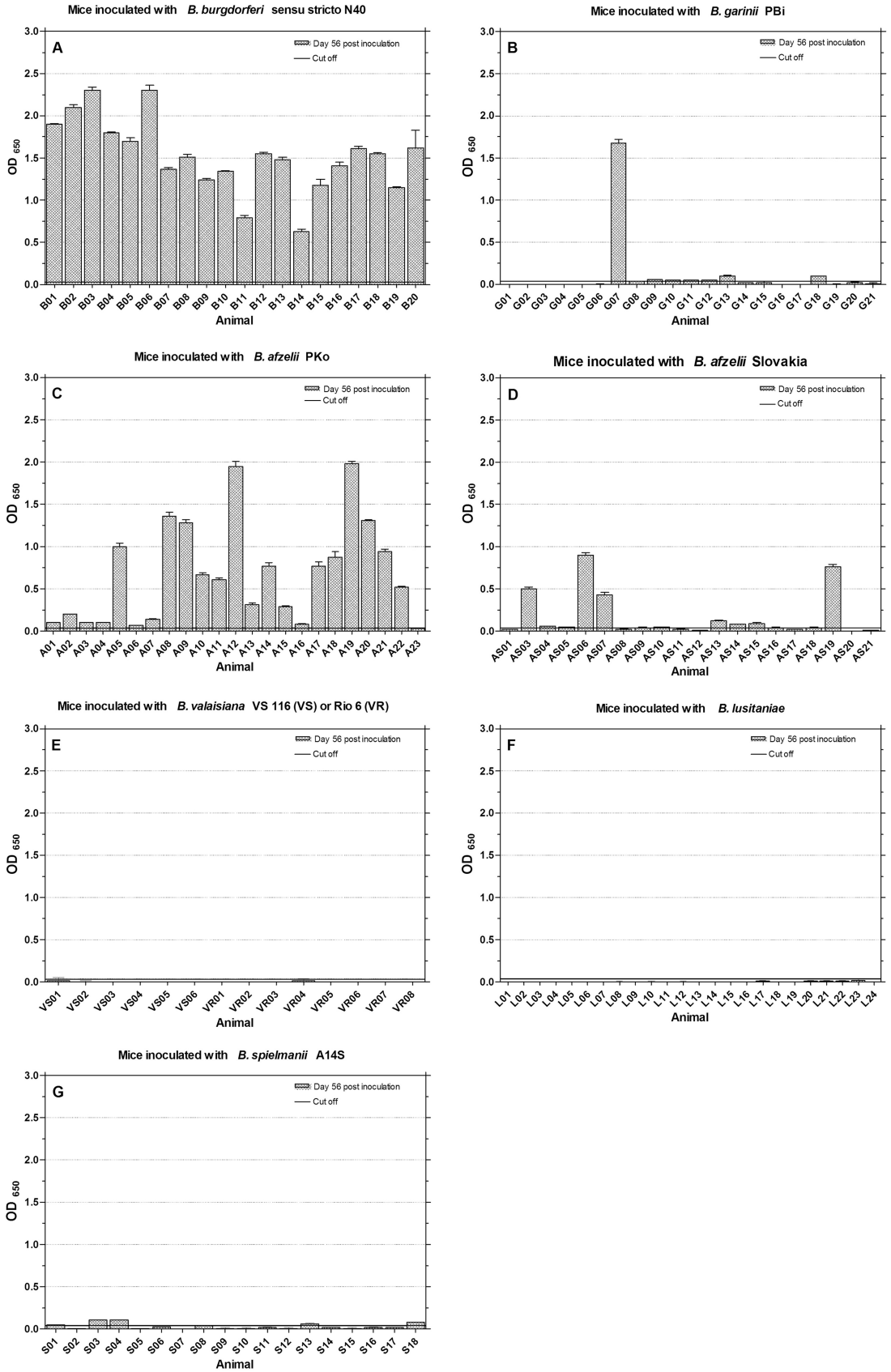

FIG. 5. IgG antibody response to *B. garinii* C_6 peptide antigen. Mice were inoculated with different *Borrelia* species (A to G), and sera were tested at day 56 postinoculation. The cutoff (0.04) was set at mean the OD value of sera from 27 uninfected mice plus five times the SD of the mean.

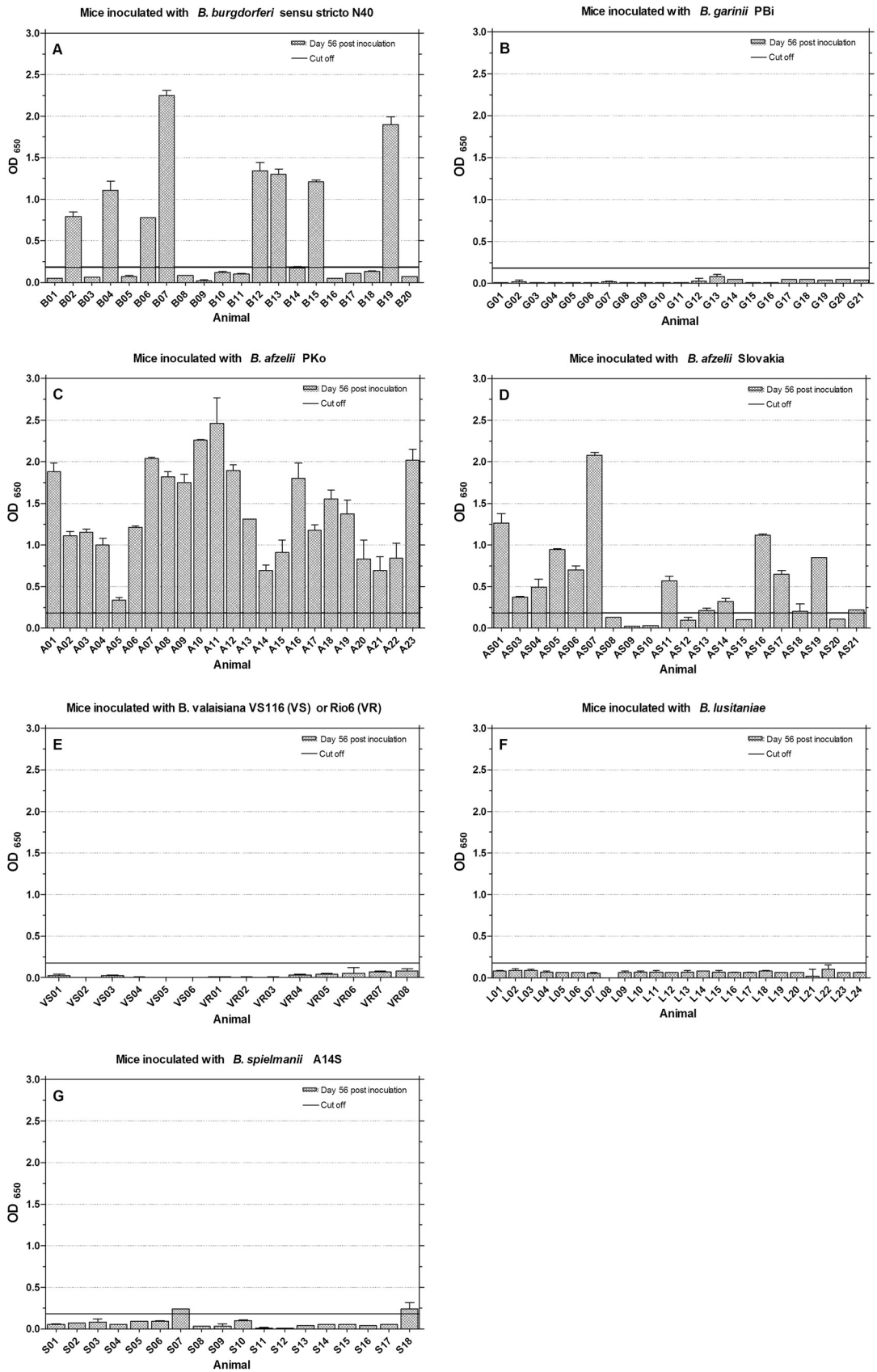

FIG. 6. IgG antibody response to *B. afzelii* $C_{6\text{-}1}$ peptide antigen. Mice were inoculated with different *Borrelia* species (A to G), and sera were tested at day 56 postinoculation. The cutoff (0.18) was set at the mean OD value of sera from 27 uninfected mice plus five times the SD of the mean.

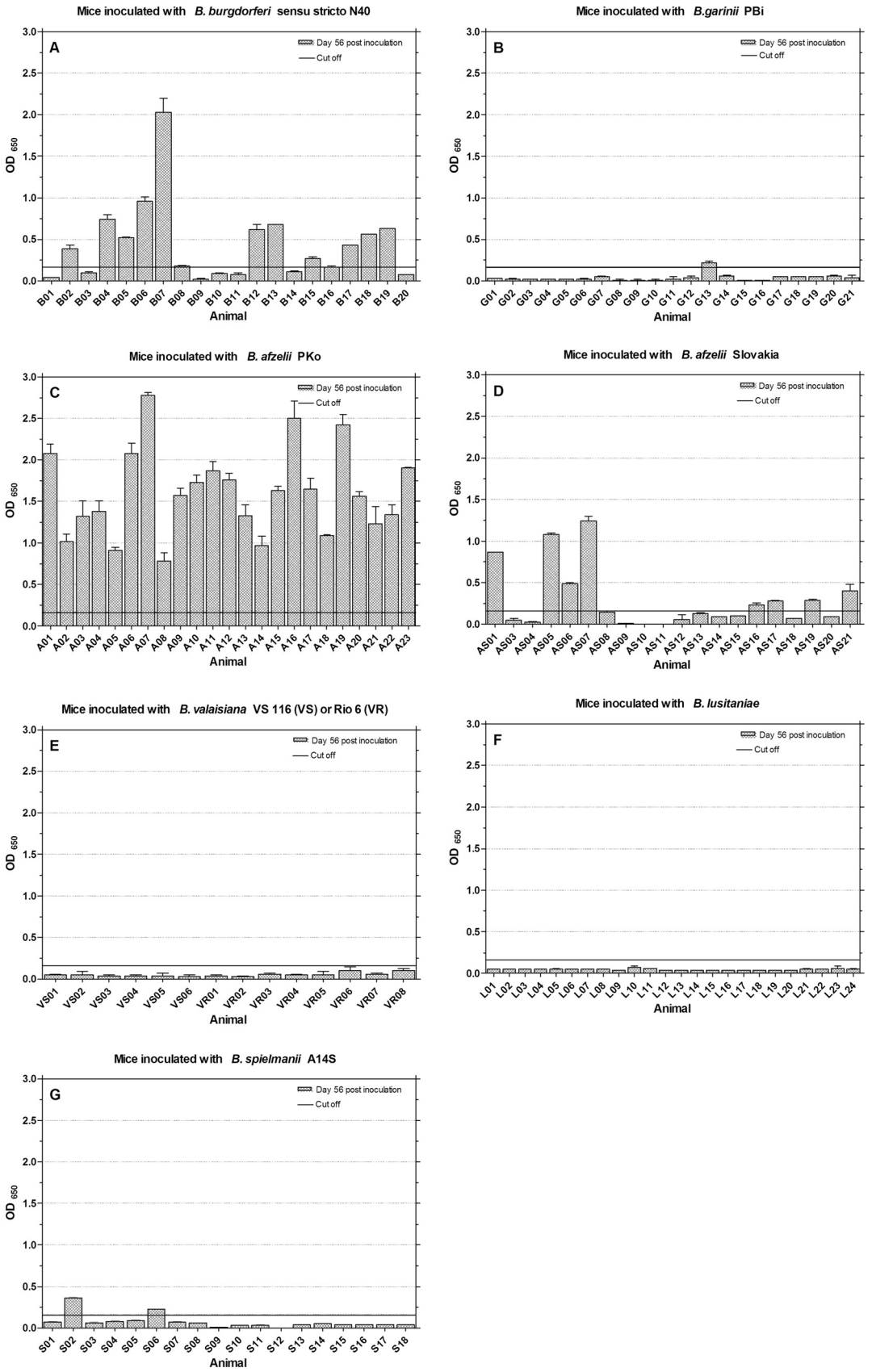

FIG. 7. IgG antibody response to *B. afzelii* $C_{6\text{-}2}$ peptide antigen. Mice were inoculated with different *Borrelia* species (A to G), and sera were tested at day 56 postinoculation. The cutoff (= 0.16) was set at mean OD value of sera from 27 uninfected mice plus five times the SD of the mean.

TABLE 5. Sensitivity of different C_6 peptides used as ELISA antigens compared to two-tiered testing[a]

Borrelia strain	C_6 peptide sensitivity (%)[b]			
	B. burgdorferi C_6	*B. garinii* C_6	*B. afzelii* C_{6-1}	*B. afzelii* C_{6-2}
B. burgdorferi sensu stricto N40	100.0	100.0	40.0	65.0
B. garinii PBi	100.0	100.0	0.0	33.3
B. afzelii PKo	100.0	95.7	100.0	100.0
B. afzelii Slovakia	58.8	64.7	70.6	47.1
B. spielmanii A14S	52.9	23.5	11.8	11.8

[a] True positive was defined as KELA positive and Western blot positive in two-tiered testing.
[b] C_6 peptides from *B. burgdorferi* sensu stricto, *B. garinii*, and *B. afzelii*.

(100.0 and 95.7%, respectively). C_6 antibodies against *B. garinii* PBi were not detected with *B. afzelii* C_{6-1}, while 33.3% of the same sera reacted with *B. afzelii* C_{6-2}. The highest sensitivity for *B. spielmanii* A14S C_6 antibodies was achieved using *B. burgdorferi* C_6 with 52.9%; it was less with *B. garinii* C_6 (23.5%) and weakest with the two *B. afzelii* peptide preparations (11.8% each).

Plasmid content of *Borrelia* used for inoculation. Figure 8 shows stained electrophoresis gels with PCR products from lp25 (Fig. 8A, 618 bp) and lp28-1 (Fig. 8B, 291 bp). All *Borrelia* strains and species with the exception of *B. valaisiana* VS116 (number 5a) and *B. valaisiana* Rio6 (number 6a) contained lp25. DNA from lp28-1 (Fig. 8B) was found in all *Borrelia* species and strains except *B. lusitaniae* (number 8a), *B. valaisiana* VS116 (number 5a), and *B. valaisiana* Rio6 (number 6a). In the case of these *B. valaisiana* strains, DNA products with lower sizes (~280 bp) were found.

Quantitative detection of spirochetes in selected murine tissues. Table 6 shows the results from qPCR analyses of DNA from the tissues of selected mice. Tissues from the ears or backs of five mice inoculated with *B. burgdorferi* sensu stricto N40 were tested for the presence of the *ospA* gene. Except for two mice (B08 and B12), specific borrelia DNA was found in all of the animals. The spirochete content per 300 ng of murine DNA was between 146,000 and 20.9 organisms. Reisolation from these mice with culture could be confirmed by qPCR. No DNA from *B. garinii ospA* was detected in tissues from four tested mice inoculated with *B. garinii* PBi, and recultivation of the spirochetes also failed. In all four tested ears from mice that were inoculated with *B. afzelii* PKo, DNA from *B. afzelii ospA* was detected with a spirochete content ranging from 19 to 222 organisms per 300 ng of murine DNA. The culture results were confirmed for three of the four mice. DNA from *B. afzelii ospA* was detected in the ears of two of three mice inoculated with *B. afzelii* Slovakia with contents ranging from 16.8 and 30.2 organisms per 300 ng of murine DNA. Three mice inoculated with *B. spielmanii* A14S were tested with DNA from ears and backs. *ospA* was present in all three samples obtained from the backs.

DISCUSSION

The focus of this investigation was to verify the applicability of single C_6 peptides originated from defined *Borrelia* species for antibody testing in terms of cross-reactivity and sensitivity to species-specific C_6 antibodies in the mouse. In this context, the sensitivity of these C_6 peptide-based test systems was compared to the two-tiered test method using lysate antigen-based ELISA and Western blotting. Because of the experimental design of the present study, specificity as defined by an epidemiological approach was not determined. The data we obtained show clearly, that in a mouse model, antibodies against *B. burgdorferi* C_6 or *B. garinii* C_6 can be detected with sensitivities of 100% for each of the two corresponding *Borrelia* species compared to two-tiered approach (Table 5). This confirms similar results from previous studies (25, 26, 29), which used a single C_6 peptide derived from *B. burgdorferi* or *B. garinii* and concluded that IR6 is antigenetically conserved among strains of *B. garinii* and *B. burgdorferi* sensu stricto. We observed that the antibody levels against *B. burgdorferi* sensu stricto and *B. garinii* were generally higher when *B. garinii* C_6 was used as detection antigen; however, individual antibody levels varied clearly among the experimentally infected mice. For example,

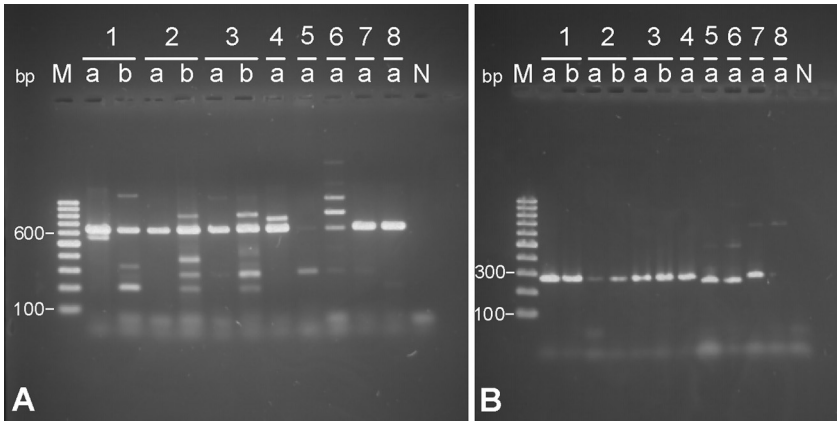

FIG. 8. Detection of linear plasmids lp25 (A) and lp28-1 (B) in *B. burgdorferi* sensu lato (numbers 1 to 8). Number 1, *B. burgdorferi* sensu stricto N40; number 2, *B. afzelii* PKo; number 3, *B. afzelii* Slovakia; number 4, *B. garinii* PBi; number 5, *B. valaisiana* VS116; number 6, *B. valaisiana* Rio6; number 7, *B. spielmanii* A14S; number 8, *B. lusitaniae*. a, Used for inoculation; b, reisolated from murine tissue. M, marker for DNA band size in base pair length. N, negative control (water).

TABLE 6. Quantitative detection of species-specific *ospA* DNA in selected murine tissues and comparison with reisolation results from skin tissue

Borrelia strain and mouse no.	Skin type	Test result[a]	
		qPCR	Culture
B. burgdorferi sensu stricto N40			
B07	Ear	146,000	+
	Back	4,940	+
B08	Ear	0.0	+
B12	Ear	0.0	+
	Back	199	+
B11	Ear	568	+
B15	Ear	20.9	+
B20	Ear	531	+
B. garinii PBi			
G07	Ear	0.0	–
	Back	0.0	–
G09	Ear	0.0	–
	Back	0.0	–
G13	Ear	0.0	–
	Back	0.0	–
G18	Ear	0.0	–
	Back	0.0	–
B. afzelii PKo			
A08	Ear	34.3	–
A13	Ear	208	+
A15	Ear	222	+
A17	Ear	19	+
B. afzelii Slovakia			
AS07	Ear	30.2	+
AS14	Ear	16.8	–[b]
AS15	Ear	0.0	–[c]
B. spielmanii A14S			
S13	Ear	0.0	–
	Back	13.8	–
S14	Ear	0.0	–
	Back	13.6	–
S17	Ear	0.0	–
	Back	32.59	–

[a] qPCR results are indicated as the number of spirochetes per 300 ng of extracted murine DNA. Culture results are indicated as positive (+) or negative (–).

[b] Due to contamination of the culture medium, *Borrelia* could be reisolated from the joint.

[c] Due to contamination of the culture medium, *Borrelia* could be reisolated from the bladder.

in mice B10 and B15 a less intense reaction to *B. burgdorferi* C_6 than to *B. garinii* C_6 (Fig. 4 and 5A) was observed. When we focused on the detection of C_6 antibodies against the *B. afzelii* PKo strain and the *B. afzelii* Slovakia isolate, it became apparent that immune responses against C_6 peptides were clearly varied. To assure genospecies identity, *B. afzelii* PKo and *B. afzelii* Slovakia were characterized by amplification and sequencing based on a 600-nucleotide fragment of their gene encoding the 16S rRNA as described previously (37). The results confirmed that both are *B. afzelii* (data not shown).

The overall C_6 antibody levels were noticeably lower in mice inoculated with *B. afzelii* Slovakia compared to mice inoculated with *B. afzelii* PKo. Furthermore, compared to *B. afzelii* PKo-inoculated mice, more *B. afzelii* Slovakia inoculated-animals were nonreactive against the different C_6 peptides (Fig. 4 to 7D). In contrast, antibody detection with *Borrelia* lysate antigens (KELA and Western blot) did not show broad differences between the *B. afzelii* isolate and the strain PKo. However, a smaller fraction of *B. afzelii* Slovakia-inoculated animals was responsive to recombinant VlsE in Western blot analyses (85.0%; 17 of 20 mice; Table 4), which might be a sign for a less immune effective VlsE presentation of this borrelia isolate, which probably results in lower C_6 responses of sera obtained from these mice. In addition, antibody populations in the *B. afzelii* PKo-infected mice might bind more efficiently to C_6 peptides based on *B. afzelii* ACAI than antibodies of *B. afzelii* Slovakia-infected mice. We assume that this observation may be dependent on the *B. afzelii* strain or isolate we used for this experiment and that there appear to be general differences in the sensitivity to detect specific antibodies against *B. afzelii* genospecies members. The choice of C_6 peptides for European applications might be reviewed focusing on this aspect. For example, in another study, there was little difference in sensitivity using a C_6 ELISA to detect antibodies against diverse *ospC* genotypes or strains of *B. burgdorferi* sensu stricto in U.S. patients (57).

In our study, the measured C_6 responses against *B. burgdorferi* sensu stricto N40, *B. garinii* PBi, and *B. afzelii* PKo support the experimental data, which show that *B. burgdorferi* sensu stricto N40 successfully induces infection and clinical symptoms in dogs (45, 46) and, furthermore, *B. garinii* PBi and *B. afzelii* PKo in mice (4, 8). In addition, *B. afzelii* PKo and *B. garinii* PBi have been isolated from humans and detected in patients (11, 54) and are therefore recommended for human serodiagnosis (14).

No broad data are available regarding the prevalence of these defined *Borrelia* strains in naturally infected hosts such as humans and dogs or the clinical impact of the isolate *B. afzelii* Slovakia. For this isolate, the clinical relevance cannot be estimated. Further studies should focus especially on the impact of defined single-species or single-strain borrelia infections on the detection of C_6 antibodies, including their potential inducing clinical signs in different susceptible hosts.

Standardized experimental conditions in our mouse model allowed a careful characterization of the C_6 peptide antibody detection capabilities in animals with defined monoinfections. The results of the present study show that the use of C_6 peptides derived from *B. garinii* and *B. burgdorferi* works best for the detection of immune responses against *B. burgdorferi*, *B. garinii*, and *B. afzelii* PKo. Looking at the quantities of *B. afzelii* antibodies detected with *B. afzelii* $C_{6\text{-}1}$ or *B. afzelii* $C_{6\text{-}2}$, it can be concluded that both peptides are sufficiently reactive to detect *B. afzelii* PKo (100.0% each). *B. afzelii* $C_{6\text{-}1}$ is more sensitive for the detection of antibodies against *B. afzelii* Slovakia than *B. afzelii* $C_{6\text{-}2}$.

A quantitative C_6 ELISA test, which was originally developed to monitor changes in C_6 antibody levels in dogs, was used to demonstrate changes in C_6 antibody levels over time (day 28 to day 56). This test kit was adequate in terms of sensitivity for the detection of C_6 antibodies against *B. burgdorferi* sensu stricto N40, *B. garinii* PBi (100.0%), *B. afzelii* PKo (95.7%), and *B. afzelii* Slovakia (88.2%) compared to two-tiered testing (data not shown). Antibodies against *B. spielmanii* were detected with a sensitivity of 35.3%. Unexpectedly,

detectable C_6 antibody levels to *B. afzelii* declined from days 28 to 56, which could be observed in sera from mice inoculated with *B. afzelii* PKo and in sera from mice inoculated with *B. afzelii* Slovakia (Fig. 3C and D). This decline may be part of individual variations in mice over time and a reflection of the specific characteristics of the C_6 immune responses induced by *B. afzelii*. Another explanation may be that some strains of *B. afzelii* are less C_6 immunoreactive in mice, resulting in a corresponding decline of the C_6 antibody level. However, viable spirochetes could be isolated from *B. afzelii*-inoculated mice via tissue culture, which confirms the ongoing process of infection.

Regarding characteristics of inoculated species used in the murine model, *B. spielmanii* A14S was not reisolated from mice, which may be a result of the demands of this particular strain for in-vitro growth conditions (data not shown). lp25 and lp28-1 were present in the organisms used for inoculation and DNA from *B. spielmanii ospA* was detected in all mice that were tested. Interestingly, *B. spielmanii*-specific DNA was not detected in skin tissues from ear but was detected in skin from the back near the injection site. This may be a result of *B. spielmanii*-specific dissemination behavior and tissue presence in murine hosts. In summary, low C_6 antibody levels were detected in mice inoculated with *B. spielmanii* A14S. The assay using the C_6 peptide derived from *B. burgdorferi* sensu stricto showed the highest sensitivity (52.9%). The *B. garinii* C_6, *B. afzelii* $C_{6\text{-}1}$, and *B. afzelii* $C_{6\text{-}2}$ peptides were clearly less capable of detecting specific antibodies. To our knowledge, this is the first investigation using C_6 peptides to document *B. spielmanii* immune responses. No data are available for the *vlsE* gene sequences of *B. spielmanii*. Western blots show that antibodies against VlsE are produced as a result of the host's *B. spielmanii*-specific immune response. Phenomena such as antigenic variation including the invariable regions of VlsE, recombination, and segmental gene conversion occurrence similar to that described in *B. burgdorferi* sensu stricto B31 (58–60) might be expected. Specific IR6 sequences and C_6 peptides derived from *B. spielmanii* VlsE should be investigated for future applications.

Serial in vitro passages of *Borrelia* can result in the loss of infectivity (33, 40, 47). This may have been one reason why only three mice inoculated with *B. garinii* PBi reacted clearly seropositive and none of the 21 mice was culture positive or PCR positive, although lp25 and lp28-1 were present in the inoculated spirochetes. *B. lusitaniae* contained lp25, but the reduced infection rate may have be due to the lack of lp28-1, as previously shown for *B. burgdorferi* sensu stricto B31 (19). This would explain the absence of the immune responses against VlsE and the C_6 peptides. Regarding the results for *B. valaisiana* VS116 and Rio6, no positive results could be obtained for culture, serology, or plasmid PCR. For this reason, we concluded that these passages of strains most likely are not infective in mice.

Our data verify that the use of *Borrelia* lysate antigen as a two-tiered approach with ELISA, followed by Western blotting, is a sensitive and specific method for the detection of antibodies against *B. burgdorferi* sensu stricto, *B. garinii*, and *B. afzelii*. We can furthermore show that this test system detects antibodies directed against *B. spielmanii* and *B. lusitaniae*.

The addition of VlsE to the diagnostic test systems (30, 35) could specify the decision for the presence of acute Lyme borreliosis. With the use of recombinant VlsE in our study, the immune responses to successful infections with *B. burgdorferi* sensu lato was confirmed and was related to the lp28-1 contents of spirochetes used for infection. Despite the time-consuming method and difficult interpretation criteria, the two-tiered test-systems is still recommended primarily because of its increased sensitivity for European strains of *B. burgdorferi* sensu lato.

In summary, we conclude that the use of the present single-step C_6 peptide test system to detect murine immune responses against *B. burgdorferi* sensu lato occurring in Europe is excellent in terms of sensitivity, when sera are tested only for antibodies against *B. burgdorferi* sensu stricto, *B. garinii*, and *B. afzelii* PKo. In our study we found that antibodies against the isolate *B. afzelii* Slovakia were less reactive with the C_6 antigen compared to *B. afzelii* PKo. To understand the impact of this finding on the C_6 test performance in Europe, it would be important to analyze *B. afzelii* Slovakia's prevalence and pathogenicity versus the other recognized strains of *B. burgdorferi* sensu lato. A combination of C_6 antigens originally derived from different strains of *B. burgdorferi*, *B. garinii*, or *B. afzelii* may provide improved sensitivity. Further experimental investigations in natural hosts such as dogs should be performed to clarify the transferability of the results obtained from our experimental murine infection model.

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