

## Humoral Immune Response in Dogs Naturally Infected with *Borrelia burgdorferi* Sensu Lato and in Dogs after Immunization with a *Borrelia* Vaccine<sup>∇</sup>

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**Lyme arthritis in dogs can be induced under experimental and natural conditions. However, the veterinary relevance of canine borreliosis is still under extensive investigation. The prevalence of symptoms is clearly low, although the risk of tick exposure is high. Current research focuses on case definitions, methods for diagnosing clinical disease in dogs, and discrimination between an immune response to a natural infection and an immune response to vaccination. In this experimental study, 23 dogs raised under tick-free conditions were allocated to two groups. The 11 dogs in the first group were vaccinated with a commercial borrelia vaccine and subsequently developed detectable antibody titers. The 12 dogs in the second group were walked on two consecutive days in an area where ticks were endemic. On day 5 after exposure, engorged ticks were removed from the 12 dogs and were analyzed for *Borrelia* DNA by a real-time PCR assay. Blood samples were taken before exposure/vaccination and at defined time points thereafter. Antibody responses were evaluated using an immunofluorescence antibody test (IFAT) and Western blotting. Seven dogs from which *Borrelia*-positive ticks were removed seroconverted and developed individual immune responses. Blood and urine samples taken from the tick-exposed group at weeks 1 and 3 for real-time PCR analysis and culture were always negative for bacterial DNA. In conclusion, despite serological evidence of infection/immunization, no clinical signs of disease were observed. The antibody patterns in a single Western blot did not permit differentiation between the different antigen sources (vaccine versus natural infection). However, repeated Western blot analyses may be useful for the confirmation of infection or vaccination status, since the time courses of the levels of specific antibodies seem to be different.**

After more than 20 years' research on canine borreliosis, diagnosis of the infection by interpreting laboratory results and correlating them with a dog's symptoms remains difficult and often unsatisfactory (27). When the first cases of canine borreliosis were published in the 1980s, it was assumed that the disease was similar to human Lyme disease. In recent years, these presumptions have had to be corrected, since studies have failed to correlate some clinical symptoms (neurological symptoms, renal failure, heart failure) and tissue analysis with definite confirmation of *Borrelia burgdorferi* sensu lato as the causative agent (7, 14, 16, 27). Thus far, Lyme arthritis is the only confirmed clinical outcome of infection with *Borrelia* spp. in dogs (12, 22).

Identification of *Borrelia* spp. in infected dogs has been documented with various degrees of success. Straubinger et al. (31) were able to regularly detect *Borrelia* spp. by PCR or culture from tissue specimens taken at the known site of tick attachment under experimental conditions, whereas with naturally infected dogs, this was impossible (27) or possible only for a low percentage (7, 14). *B. burgdorferi* DNA was rarely

detected in kidneys (14), the heart muscle, and joints (7) by PCR, immunohistochemistry (IHC) or modified silver staining, and fluorescence *in situ* hybridization. Most authors describe the isolation of spirochetes from blood samples from dogs as insensitive (28, 30), although others have reported the detection of *Borrelia* DNA in samples from one-third of dogs with suspected natural infections (26).

Canine immune responses to *B. burgdorferi* sensu lato have been tested by enzyme-based immunosorbent assays (ELISA) and Western blot assays based on recombinant or whole-cell antigens. Sensitivities of 43% to 74.3% and specificities of 60% to 85.1% were reported by Štefančíková et al. (29) for ELISA based on three different strains, demonstrating the importance of using local strains for serodiagnosis. Levy et al. (20) reported a sensitivity of 94.4% and a specificity of 99.6% for a C6 antigen ELISA, and Jacobson et al. (15) reported a sensitivity of 98.6% and a specificity of 91.9% for a kinetic ELISA. All values were calculated by comparing the test results to those of other ELISA or Western blot assays.

Cross-reactivity with other spirochetes (*Leptospira* spp., *Treponema* spp.) impairs the specificity of tests for Lyme borreliosis; Western blot bands at the levels of p33, p60 to p75, and p41 were detectable in canine sera containing antibodies to *Leptospira* spp. (4, 17).

Antibody responses to *B. burgdorferi* sensu lato are common in both symptomatic and asymptomatic animals in areas of

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endemicity, leading to the conclusion that only a very small percentage of dogs naturally infected by *B. burgdorferi* sensu lato become symptomatic after a typical incubation period of a few weeks (21). In previous studies, the rate of occurrence of clinical symptoms in experimentally infected dogs ranged from 0 to 77% (5, 6, 9). In Europe, the seroprevalence of antibodies for *B. burgdorferi* sensu lato in dogs (3.9% to 35.5%) has been documented in several studies (8, 24, 33, 35). Following the introduction of a commercially available vaccine for canine borreliosis (Merilyme; Merial, France), the percentage of dogs testing seropositive has increased considerably. In Austria, seropositivity increased from 38% to 59% of all dogs tested (18).

Identification of Western blot patterns specific for infection with *Borrelia* spp. and differentiation among dogs that are naturally infected, vaccinated, or vaccinated and subsequently infected are still major goals for diagnostic procedures for canine borreliosis. There are several indications of the need to test vaccinated dogs and to differentiate antibodies derived from vaccination from those induced after infection. (i) There is strong evidence for only minimal cross-protection by vaccinal antibodies against heterologous *Borrelia* spp., making infection and clinical symptoms possible (32). (ii) Dogs may be vaccinated during the incubation time, causing clinical symptoms weeks thereafter. (iii) Dogs may develop clinical signs similar to those of borreliosis after vaccination without natural infection (15).

Previous studies have identified specific Western blot bands as markers for infection or vaccination (1, 10, 15, 36) and have reported different patterns for symptomatic and asymptomatic dogs (11). Greene et al. (9) compared the Western blot patterns of experimentally infected dogs to the results for naturally exposed dogs, concluding that the lower number of bands for experimentally infected dogs represents an acute immune response after a single infection, whereas the higher number of bands for naturally infected dogs reflects a chronic response to repeated exposure.

However, because of differing experimental techniques (different strains, recombinant or whole-cell antigens for Western blotting), blot patterns differ greatly across the studies, making comparisons and consequently diagnostic conclusions in single cases difficult. Levy and Magnarelli (19) demonstrated that serological examinations of apparently healthy dogs had no predictive value for the subsequent development of limb or joint disorders. In the testing of immune responses in dogs, in many cases lack of information as to the exact time of natural infection, and consequently the current stage of infection, makes the interpretation even more difficult.

The aims of this study were to compare canine antibody responses to vaccination with those to natural infection with *B. burgdorferi* sensu lato under controlled conditions and to investigate possible hematogenous spread and urinary excretion in these dogs.

**MATERIALS AND METHODS**

**Animals.** Twenty-three adult beagles (ages, 12 to 18 months) were included in this study. The experiments were approved by the institutional ethics committee (Veterinary University Vienna) and the Austrian Ministry for Science and Research (GZ68.205/0148-C/GT/2007). All dogs were born and raised at the Veterinary University of Vienna under tick-free environmental conditions. The dogs were under continuing veterinary medical care and were examined for side

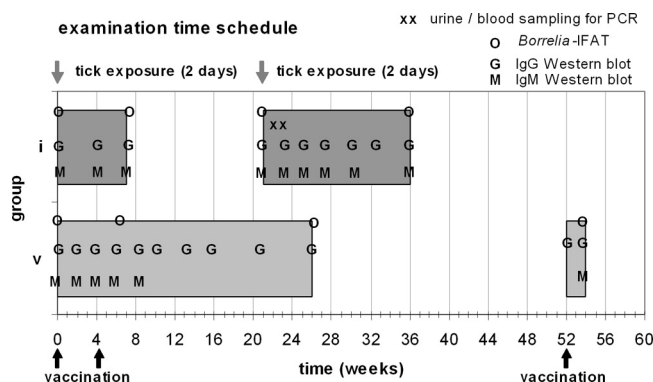


FIG. 1. Schedules for the vaccination and infection studies of 23 dogs. i, infection study group; v, vaccination study group.

effects of vaccination or symptomatic evidence of infectious disease. All dogs tested negative for *Borrelia* antibodies in an immunofluorescence antibody test (IFAT) prior to the study.

**Vaccination study.** Eleven healthy adult beagles were vaccinated with a commercially available whole-cell lysate *Borrelia* vaccine (*B. burgdorferi* sensu lato; Merilyme; Merial, France) according to the manufacturer's recommendations (2 vaccinations 4 weeks apart and 1 vaccination a year later). Blood was sampled 10 times for each dog at intervals of 2 to 5 weeks and twice more for six dogs 1 year after the first vaccination (Fig. 1).

*Borrelia* antibodies were evaluated by an IFAT (MegaScreen Fluorborrelia [MegaCor Diagnostik GmbH, Austria]; cutoff titer, 1:64; sensitivity, 90%; specificity, 98.6%) before the first and after the second vaccination (for 11 dogs), after 26 weeks (for 5 dogs), and after the third vaccination a year later (for 6 dogs). Serum samples from all dogs were subjected to two Western blot assays for detection of IgG and IgM antibodies (Fig. 1). The recomBlot assay (Mikrogen, Germany), using a recombinant antigen, provides specific bands for p100, VlsE, p41 (flagellin), p39, OspA (31 kDa), OspC (three bands at 22 kDa: *Borrelia garinii* strain T25, *B. garinii* strain 20047, and a combined band for *B. burgdorferi* sensu stricto and *Borrelia afzelii*), an internal part of the p41 antigen (two bands, one for *B. garinii* and one for *B. afzelii* [p41int.garinii and p41int.afzelii, respectively]), and p18 (decorin binding protein A). The second Western blot assay (MegaBlot; IgG and IgM; MegaCor Diagnostik GmbH, Austria), derived from *B. garinii* (strain VS102), was used for the detection of additional bands (75, 66, 58, and 37 kDa) and also served as an internal control. Band intensities were classified by a scoring system from zero to 7, where zero corresponds to no band and 7 corresponds to the highest-intensity band. The cutoff for positivity was intensity 2, which was the same strength as the weak positive control provided by the manufacturer.

**Infection study.** Dogs were walked on a long leash for a few hours in areas of high tick endemicity in Burgenland, Austria (autumn), and Vienna, Austria (spring), and thus were exposed to possibly infected ticks, on two consecutive days in October 2007 (6 dogs) and in April 2008 (11 dogs). One dog in the autumnal session had to be excluded from the study after 3 months because it developed behavioral incompatibility; therefore, only five remaining dogs and six new ones were walked in spring 2008. Five days after the end of tick exposure, the dogs were carefully examined for engorged ticks; these were subsequently removed and stored. Sites of tick bites were documented. All dogs received topical anti-tick permethrin treatment (Exspot; Essex, Germany) immediately after tick removal, and thereafter, no further ticks were found. Ticks were stored at -20°C until further examination. After being thawed, they were classified for species, sex, and developmental stage before dissection with sterile scalpel blades for DNA extraction with the DNeasy blood and tissue kit (Qiagen GmbH, Hilden, Germany).

Blood samples were collected three times at intervals of 3 to 4 weeks in autumn 2007 and seven times at intervals of 2 to 3 weeks in spring 2008 (Fig. 1). *Borrelia* antibodies were detected in IFATs, as described above, at two time points (before tick exposure and at the end of the observation period) in autumn 2007 and at two time points in spring 2008 (Fig. 1). Samples from all 11 dogs were tested in both Western blot assays as described for the vaccination study. In the spring, urine samples were collected from the dogs 1 and 2 weeks after tick exposure (Fig. 1). Urine and blood samples collected simultaneously were analyzed for *Borrelia* spp. by real-time PCR and culture.

TABLE 1. *Borrelia* IFAT titers in dogs before and after vaccination

Dog no.	Titer <sup>a</sup>			
	Before vaccination	2 wks after 2nd vaccination	22 wks after 2nd vaccination	2 wks after 3rd vaccination
1	N	1:128		1:128
2	N	1:128		1:64
3	N	1:64		1:128
4	N	1:64	N	
5	N	1:128	N	
6	N	1:64		1:128
7	N	1:128		1:128
8	N	1:128		1:128
9	N	1:64	N	
10	N	1:64	N	
11	N	1:256	N	

<sup>a</sup> N, negative.

**PCR and culture.** Ticks and EDTA-treated blood and urine specimens were analyzed by real-time PCR assays. A two-step procedure was used with two types of kit for each sample. First, the presence of *Borrelia* DNA was investigated by using part of the *Borrelia* flagellin gene (34) and part of the *OspA* gene from a commercially available real-time PCR assay (Ingenetix, Vienna, Austria). The primer and the TaqMan probe for fluorescent online detection were designed to react with all *Borrelia* species. In the second step, samples were typed using the ABI Prism sequence detector, model 7700 (Applied Biosystems, Foster City, CA) with three different probes for the spacer regions and with different kinds of dye. The reaction cycles were as follows: 2 min at 50°C and 15 min at 95°C, followed by 60 cycles of 15 s at 95°C and 1 min at 60°C. All preparations with positive signals were sequenced (Ingenetix, Vienna, Austria).

For the cultivation of *B. burgdorferi* sensu lato, 1-ml samples of each specimen (blood and urine) were placed in vials containing 7 ml BSK II medium (Sigma-Aldrich, St. Louis, MO) and were incubated at 33°C. The presence of spirochetes was checked by dark-field microscopy every week for 8 weeks.

**Statistics.** Concurrent band intensities for vaccinated and infected dogs, as well as the numbers of positive bands, were compared using an unpaired Student *t* test.

## RESULTS

Throughout the examination period of 60 weeks, none of the vaccinated dogs showed any symptoms suggesting a vaccination side effect. All seven dogs infested with *Borrelia* sp.-infested ticks remained healthy.

**Vaccination study.** All 11 vaccinated dogs seroconverted after the second vaccination. Titers of antibodies for dogs tested 22 weeks after the second vaccination returned to negative (Table 1). Six dogs were vaccinated a third time and tested positive by the IFAT again 2 weeks thereafter.

**Infection study.** A total of 104 ticks, attached and engorged on the 12 dogs, were collected and analyzed (Table 2). The possibility that a small number of ticks were missed when they were collected from the dogs cannot be excluded, because some ticks may have been removed by the dogs, and some, especially larvae and nymphs, may have fallen off earlier.

By real-time PCR analysis, 9 out of 104 ticks were positive for *Borrelia burgdorferi* sensu lato: 6 female *Ixodes ricinus* ticks, 1 *I. ricinus* nymph, and 2 female *I. canisuga* ticks. Of these borreliae, only four could be typed at the genospecies level: those from three female *I. ricinus* ticks were identified as *B. afzelii*, *B. garinii*, and a mixture of *B. afzelii* and *Borrelia spielmanii*, respectively, and one borrelia from an *I. ricinus* nymphal tick was identified as *B. afzelii*.

Among the 12 dogs in the infection study, 3 of 6 serocon-

TABLE 2. Numbers of engorged ticks collected from 12 dogs after natural exposure, numbers of ticks positive for *Borrelia* by PCR, and *Borrelia* IFAT titers before and after natural tick exposure

Dog no.	No. of ticks collected (no. PCR positive)		Titer <sup>a</sup>			
			Autumn		Spring	
	Autumn	Spring	Before infection	7 wks after 1st infection	Before 2nd infection	15 wks after 2nd infection
12	7 (0)	0 (0)	N	N	N	N
13	7 (1)	18 (1)	N	1:64	N	1:64
14	0 (0)	3 (1)	N	N	N	1:64
15	2 (1)	2 (0)	N	1:64	N	1:64
16	9 (1)	3 (1)	N	1:64	1:64	1:128
17	2 (0)	Excluded	N	N	Excluded	Excluded
18		5 (1)			N	1:128
19		4 (1)			N	1:64
20		23 (1)			N	1:64
21		4 (0)			N	N
22		4 (0)			N	N
23		11 (0)			N	N
Total	27 (3)	77 (6)				

<sup>a</sup> N, negative.

verted after autumnal exposure and 7 of 11 seroconverted or had increased titers after spring exposure (Table 2). Two of these 12 dogs had repeated infections (autumn and spring).

All dogs that were infested with infected ticks showed specific immune responses (rising titers by IFAT). The development of seropositivity in the IFAT and detectable *Borrelia* DNA in attached ticks identified dogs for further evaluation by Western blotting.

Blood and urine samples from all dogs in the infection study tested negative for *B. burgdorferi* sensu lato by PCR and culture.

**Comparison of Western blot results before/after vaccination and before/after tick exposure in dogs.** Baseline Western blot results were obtained from all dogs before the first vaccination or the first exposure to ticks. The results (recomBlot and MegaBlot, IgG and IgM) are displayed in Fig. 2; they indicate that several bands appear with variable frequencies even before *Borrelia* vaccination or infection of dogs. Major differences (>30%) between the results of the two IgM blots were detected when the frequencies of the p100, p41, and *OspC* bands were compared.

The frequencies of positive bands after vaccination or infection are displayed separately for Western blot assays performed early and late after vaccination/infection in Fig. 3a (IgG) and b (IgM). After vaccination, the frequencies of positive bands in the IgG Western blots tend to decrease (from early to late times); in particular, those of p100, *OspC*, and p41int.garinii decrease more than 40%. This is in contrast to the immune response after infection, when the frequencies of all bands except *VlsE* increase or stay stable.

In general, the IgM response after vaccination is of shorter duration than that after infection, and only 4 bands (p66, p41, p37, and *OspA*) are visible at late times after vaccination. Comparison of the IgM immune responses in the two study groups shows that the bands for p58, *OspA*, and p41int.garinii occur solely after vaccination, while that for p41int.afzelii occurs only after infection.

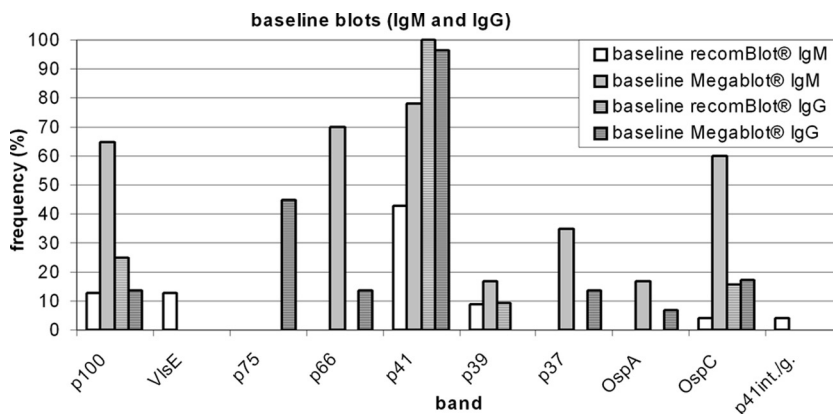


FIG. 2. Frequencies of *Borrelia* bands in baseline Western blots (recomBlot and MegaBlot, IgG and IgM) for 23 dogs.

In Fig. 4, the mean intensities of specific IgG bands over time are displayed, and the results for vaccinated dogs are contrasted with those for naturally infected dogs.

All mean band intensities increased after each consecutive vaccination or infection; only the IgG p58 and p18 bands were seen solely after vaccination.

Intensities for OspA remained high between the second and third vaccinations, whereas all other bands responsive to vaccination decreased in intensity to negative levels within 4

months. Mean band intensities after the second and/or third vaccination became positive or were classified as rising within 2 weeks for p100, VlsE, p66, p41, p39, p37, OspA, OspC, and p41int.garini. The intensities of most bands increased even more after the third vaccination than after the second vaccination (Fig. 4).

For subclinically infected dogs, mean band intensities became positive or were classified as rising for p100 (after 7 to 9 weeks), p41 (after 2 to 7 weeks), p37 (after 9 weeks), p39 (after

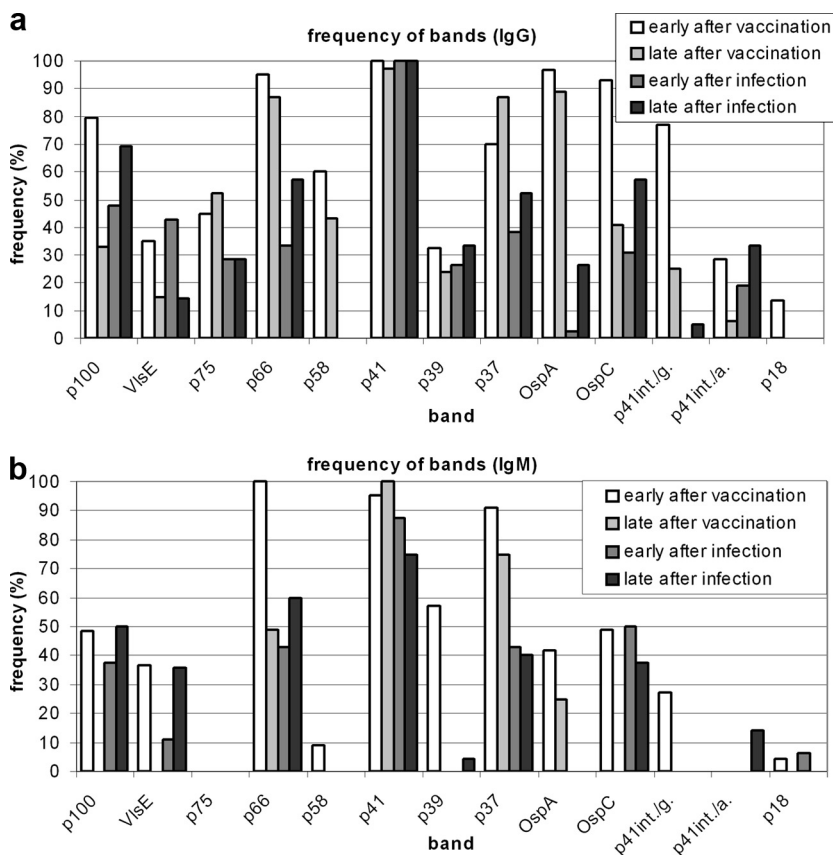


FIG. 3. Frequencies of *Borrelia* Western blot bands (recomBlot and MegaBlot) for IgG (a) and IgM (b) in dogs after vaccination (early, 2 to 6 weeks after the 2nd or 3rd vaccination; late, 9 to 48 weeks after the 2nd vaccination) and after infection (early, 2 to 7 weeks after infection; late, 9 to 15 weeks after infection). p41int./g., p41int.garini; p41int./a., p41int.afzelii.

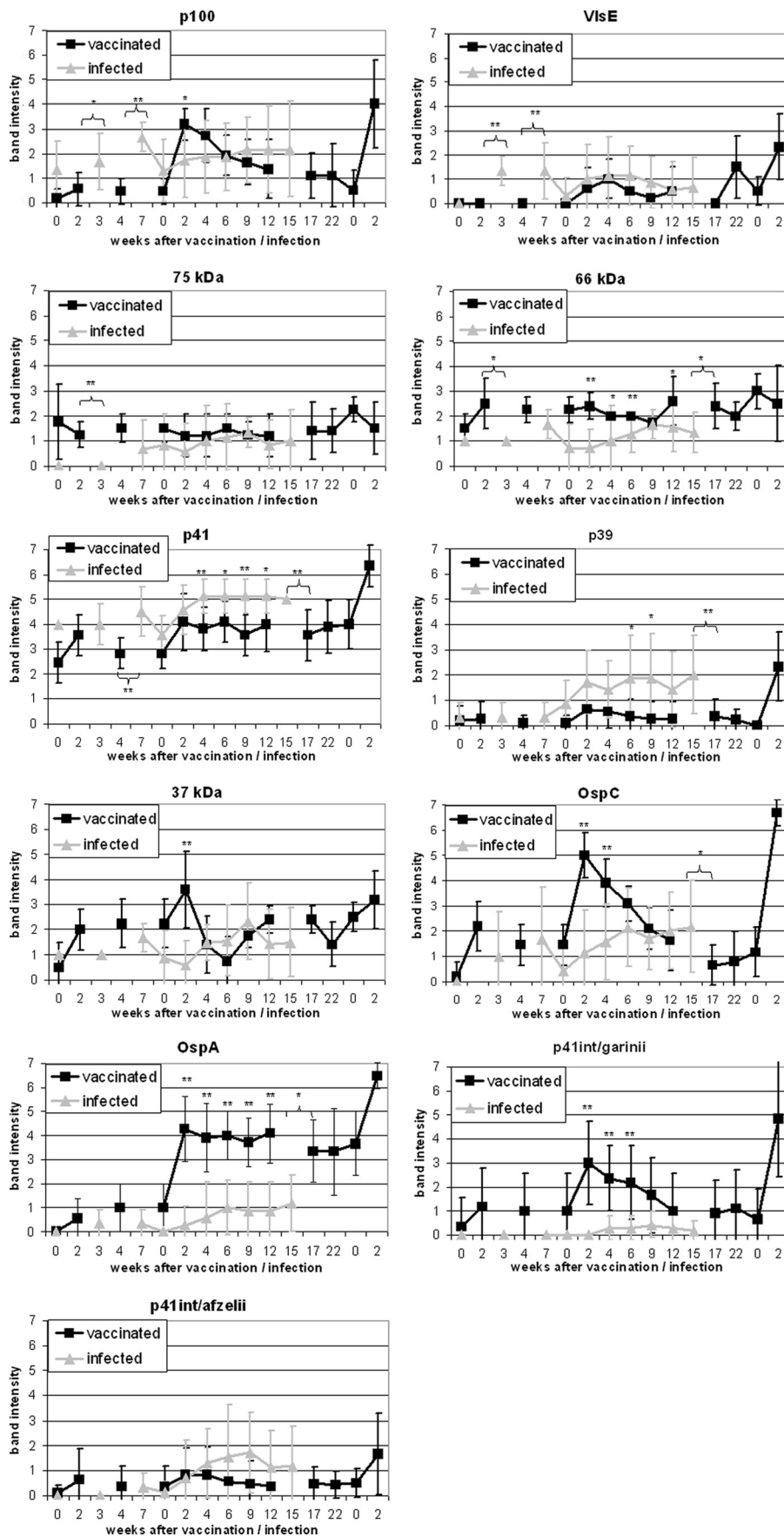


FIG. 4. Mean intensities ( $\pm$  standard deviations) of IgG Western blot bands for dogs after *B. burgdorferi* vaccination or natural infection (at week 0). Asterisks indicate significant differences (\*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ).

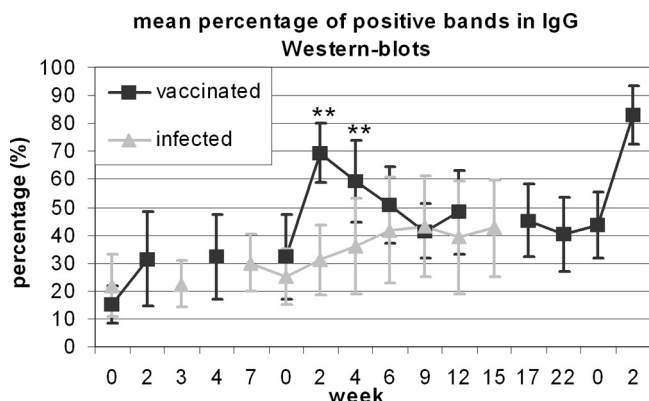


FIG. 5. Mean percentages of positive bands in IgG Western blots for dogs after *B. burgdorferi* vaccination or natural infection (at week 0). Asterisks indicate significant differences (\*\*,  $P < 0.01$ ).

15 weeks), and OspC (after 6 weeks). In some individual dogs' Western blots, band intensities for p100, p41, p37, p39, OspC, and p41int.afzelii even increased within 2 weeks after infection.

When consecutive band intensities for the two groups were compared, vaccinated dogs had significantly higher scores for p66 and p75 after the first vaccination/infection and for p100, p66, p37, OspA, OspC (within 2 to 4 weeks), and p41int.garinii after the second vaccination/infection. Infected dogs showed significantly higher scores for p100, VlsE, and p41 after the first vaccination/infection and for p41, p39, and OspC (only after 15 weeks) after the second vaccination/infection.

When the mean standard deviations of band intensities, used as a parameter for the heterogeneity of Western blot results in each group, were compared, we calculated mean standard deviations of band intensities of  $>1$  for OspA and p41int.garinii (1.07 and 1.56) in the vaccination group and for p100, VlsE, p39, OspC, and p41int.afzelii (range, 1.01 to 1.43) in the infection group.

In Fig. 5, the mean percentages of positive IgG bands in each Western blot (recomBlot and MegaBlot) assay over time are displayed, and the results for vaccinated dogs and naturally infected dogs are contrasted. Values were significantly higher for the vaccinated group than for the infected group 2 to 4 weeks after the second vaccination/infection.

### DISCUSSION

Natural infections with *B. burgdorferi* sensu lato or vaccination with a *Borrelia* whole-cell lysate vaccine resulted in an individual and specific humoral immune response in each of the vaccinated or infected dogs. The IFAT titers of seropositive dogs were similar irrespective of the groups to which the animals were allocated. Although there were differences between the Western blot patterns of vaccinated versus infected dogs, individual variations within each group were considerable, and a single Western blot analysis may be unsuitable for differentiation between infection and vaccination. Even the baseline patterns prior to vaccination or tick exposure were highly individual and displayed positive results for individual dogs with no history of tick infestation. Thus, for proper interpretation of test results and a definitive diagnosis, it is neces-

sary that the clinical history and physical examination be taken into account.

In the present study, the infection pressure exerted by infected ticks on dogs was low. Despite the short exposure time, 7 of 12 dogs became infected. This is a strong indication of a high infection risk for dogs that are walked in areas of high tick endemicity. The infection risk is also based on the prevalence of *B. burgdorferi* sensu lato in ticks, which is approximately 10% in both areas (3). Although multiple transmissions increase infection stress for the host and may consequently increase the probability of clinical signs, this could not be observed in our study.

In dogs, the incidence of clinical symptoms after the bite of a *Borrelia*-infected tick is estimated as being very low ( $<5\%$ ) (13, 21). In an experimental setting, ticks were attached to the thoracic walls of dogs and were prevented from moving to another part of the dog's body. The joints next to the site of the tick bite developed more-severe signs of arthritis than other joints (6, 31). In our natural setting of exposure, ticks were attached at different sites. Most ticks were collected from the head, neck, and ventral abdomen, which might influence bacterial distribution and give the host's immune system more time to eliminate the bacteria.

Identification of the infectious agent *in vivo* is a major goal in the diagnosis of infectious diseases. In canine borreliosis, it is particularly difficult and unproductive to attempt isolation of the bacteria or amplification of DNA from blood and urine samples (15, 27, 28), although some research groups have described successful identification of *Borrelia* DNA in canine blood and urine (2, 26). Even under controlled conditions and with precise knowledge of the time of infection, indicating the most promising time for the identification of bacteria in blood and urine, we were not able to detect *Borrelia* spp. by culture or by PCR assay. No correlation between the absence of clinical signs and the lack of detectable *Borrelia* DNA can be drawn from the results given. Detection of *B. burgdorferi* DNA in tissue samples from infected dogs has been successful mostly after experimental infection (30) but was much less effective when paraffin-embedded tissue sections from dogs with presumptive and naturally acquired Lyme borreliosis were examined (7). Thus, for dogs with clinical evidence of arthritis, it will be more promising to test synovia or synovial membranes than blood or urine samples for *B. burgdorferi* DNA.

Serology has been considered a major diagnostic criterion for canine borreliosis, in addition to tick exposure, clinical symptoms, and response to antibiotics (15).

Quantitative detection of specific antibodies in dogs in our study was highly sensitive; all vaccinated dogs and all dogs infested by infected ticks seroconverted.

Commercial Western blot test systems for dogs utilize interpretation patterns for humans regarding the occurrence of specific bands in the time course of disease. This practice must be reconsidered in light of the present study, which shows that the time course of pattern development for dogs is different from that for humans, a finding also documented by Lovrich et al. (23) for OspC.

The specificity of single bands will have to be demonstrated for canine samples, since many bands were detected in some dogs even before vaccination or infection (Fig. 2). This phenomenon might be explained by the cross-reactivity of spe-

cific components of the bacterial flora in the gastrointestinal tracts of dogs. In previous studies, antibodies directed against bands at the level of p33, the 60- to 75-kDa range, p41, OspC, and p41int.garinii showed detectable levels of cross-reactivity (4, 17).

In addition to the choice of bands, the combination and number of positive bands is another challenge in the diagnostic workup of canine borreliosis. A European multicenter study of immunoblotting for the serodiagnosis of Lyme borreliosis in humans showed remarkable variations in results from different test systems (25). It was reported that when more bands defining positivity were included, the specificity increased and the sensitivity decreased.

Test-specific scoring systems for individual bands, resulting in a summed cutoff score for distinguishing positive from negative results, are commonly used in commercial test systems. Our results show the problem with these scoring systems: 30% of baseline IgG Western blots and 39% of baseline IgM Western blots would have been interpreted as positive on the basis of a single specific band (p100, p39, or OspC). The mean percentage of total positive bands for dogs is significantly higher at 2 to 4 weeks after vaccination than for dogs at the same time point after infection. Later on, the number of positive bands in the Western blot does not allow one to distinguish between vaccinated and naturally infected dogs (Fig. 5).

In general, band patterns change in the time course after vaccination and infection. The intensities of the OspA and p41 bands remain high after vaccination over a much longer period than those of bands for the p100, OspC, and p41int antigens. This is in contrast to the vaccination study by Töpfer and Straubinger (32), who found that levels of specific OspA antibodies decreased similarly to total antibody levels a few months after vaccination.

After experimental infection, the first episode of lameness occurred after an incubation time of 50 to 123 days after tick exposure (30), which might be similar the time to testing for canine borreliosis in veterinary practice. The corresponding Western blots in our study (Fig. 4, 6 to 15 weeks after infection) show that infected dogs had significantly lower mean band intensities for OspA and higher intensities for p39 and to some extent OspC, which is in concordance with the findings of Barthold et al. (1).

Nevertheless, it was nearly impossible to assign individual immunoblots to the vaccination group or the infection group by the occurrence of a single band, as demonstrated by high standard deviations (>1) even in the most promising bands (p100, VlsE, p39, OspA, OspC, and both p41/int antigens) (Fig. 4). Repeating the Western blot assay within this critical period after the onset of clinical signs may assist in the interpretation. Decreases in the intensities of the p100, OspC, and p41int.garinii bands and stable high OspA bands are indicative of a postvaccinal immune response. Stable or rising intensities of the p100, p39, OspC, and p41int.afzelii bands are a strong indication of an ongoing immune response after natural infection (Fig. 3a and 4).

The VlsE protein and its synthetic C6 peptide supposedly are not expressed in ticks, cell culture, or Lyme vaccines and are therefore recommended as specific markers for infection in humans and dogs (22). In the present study, however, VlsE was not helpful in distinguishing humoral responses to vaccination

from humoral responses to infection. The IgM recombinant blot assay detected the VlsE band in some baseline blots as well as in dogs presumed to be uninfected; the IgG recombinant blot assay showed the VlsE band to be a target of a strong immune response in both groups. These results highlight the difficulty of reproducing findings with differing test procedures and under different field conditions.

In general, the IgM responses of the dogs were less intense than their IgG responses and appeared to develop within the same period. The differing results for the IgM recomBlot and IgM MegaBlot assays show insufficient reproducibility for the p100, p41, and OspC bands and low specificity for several bands, as evident in the baseline blots (Fig. 2). The p100, VlsE, p39, OspC, and p41int.garinii bands never occurred in the "late blots" after vaccination, and the p58, OspA, and p41int.garinii bands were seen only after vaccination (Fig. 3a and b). Due to the generally low frequency of specific bands, testing for IgM in the diagnosis of canine Lyme disease is not recommended, a view also shared by Jacobson et al. (15).

In conclusion, the present study has highlighted some of the difficulties in the diagnosis of canine borreliosis and the need to validate existing assays in dogs as well as to develop more-accurate criteria for defining canine borreliosis.

Veterinarians should follow strict guidelines in the diagnostic workup of canine borreliosis. The detection of a specific canine antibody response is one major diagnostic criterion for canine borreliosis, but first arthritis should be confirmed by clinical findings and laboratory results. Because *Borrelia* spp. are of minor importance in the etiology of canine polyarthritis, differentials have to be ruled out. Serology is easy to perform, but it should not replace the attempt to detect antigens (by culture and/or PCR) in synovial specimens from symptomatic dogs, although sensitivity is known to be low.

Serology alone can be misleading in the workup of a lame dog without any further investigation.

A single Western blot test is insufficient for differentiation between vaccinated and infected dogs. However, repeated Western blot assays may be useful for the confirmation of infection or vaccination status, since the time courses of the levels of specific antibodies seem to be different.

Western blot analysis is recommended for the confirmation of screening tests, but individual immunoblot assays have several limitations. The interpretation aids supplied with commercial test kits reflect the situation in human and not canine borreliosis. The latter is certainly overdiagnosed, because Western blot assays seem to be nonspecific in several cases, as demonstrated in the present work, where baseline blots appeared to be positive even though tick infestation, and thus previous exposure to *Borrelia* spp., was extremely unlikely. The exact time point of infection is unknown in the majority of canine cases, and therefore, differentiation of single Western blot patterns as early or late infection does not seem appropriate, particularly since dogs have a high incidence of tick infestation, making multiple transmissions of pathogens likely. Assays for detection of canine immune responses should be validated and adapted to the knowledge of canine borreliosis.

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