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Original Research Paper

HUMORAL IMMUNE RESPONSE TO BACTERIA *BORRELIA BURGDORFERI* S.L. IN DAIRY CATTLE AFTER EXPERIMENTAL EXPOSURE

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Summary: To acquire insights into the humoral immunal response (HIR) of grazing diary cattle naturally infected with *B. burgdorferi* sensu lato (s.l.), an experimental group of 3 adult dairy cows was artificially infected with the bacteria. Three cows were intradermally (i.d.) infected with living *B. garinii*, and another was immunized with dead bacteria in Freund's incomplete adjuvant. The taxonomic identity and clonality of the isolates were confirmed by PCR amplification of the intergenic spacer between the 5S and 23S rRNA genes (1). The immune responses of the polyclonal, IgM and IgG class of antibodies were monitored both with whole-cell sonicated IgG and IgM and polyclonal ELISA and with indirect immunofluorescent assays (IIF). The immune response of the three i.d. infected animals remained low, but was detectable and characteristic in course, whereas in the vaccinated animal the immune response was strong.

Key words: *Borrelia burgdorferi*; dairy cattle; serological diagnosis.

Introduction

Lyme borreliosis (LB) – the most common arthropod-borne infection in Europe (2, 3) and in the United States (4) is a complex multisystem disorder in humans and animals, caused by *Borrelia burgdorferi* sensu lato, a genetically diverse species complex of spirochetes, comprising at least nine genospecies (5, 6, 7, 8). The principal vectors of these spirochetes are ticks belonging to the genus Ixodes (5). The different genospecies of *B. burgdorferi* s.l, appear to cause distinct clinical manifestations of Lyme borreliosis in humans (9). Serological tests and other diagnostic procedures are normally conducted to confirm *B. burgdorferi* s.l. infections.

Immune responses to *B. burgdorferi* s.l. have been analysed in rodents (10, 11, 12, 13), game birds (14), dogs (15, 16), horses (17, 18, 19), cattle (17, 20, 21, 24, 25) and rhesus monkeys (24). In our experiment, a fresh tick-borne isolate of *B. garinii* with a low spirochaete count was used and introduced by intradermal inoculation. In humans, serological confirmation of the diagnosis has been complicated by the delay in the development of the humoral immune response (HIR) to the spirochaete (27, 28) and it has been shown that up to 10 to 12 % of clinically ill patients have no HIR at all (29). The low sensitivity of serological tests in man early in the infection course has been a particular problem (29, 30, 32). However, in animals infected with the bacteria, the clinical aspects are often unclear and serological test results are relied upon to determine the cause of the illness. As established in previous investigations, levels of specific polyclonal antiborrelial antibodies rise in the grazing cattle according to the number of infections or the age of the animal (17, 33). In humans, however, a study of 15 patients in the first weeks of illness, when erythema migrans or meningitis was present, revealed that 14 of them had weak to strong IgM response, and of those 6 had weak to moderate IgG response (34). In the past, indirect-fluorescent antibody (IIF) procedures were used to detect antibodies to *B. burgdorferi* (34, 35); later ELISA (36, 37), which can quantify the level of immunoglobulins and specific polyclonal antibodies, was introduced and replaced the time consuming IF method.

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Assessment of IgM, IgG and specific polyclonal antibody responses may be helpful in determining the disease status or response to therapy (23, 38, 39, 40).

Consequently in our study we investigated whether the immunological response after experimental infection of cattle exists, what is it's dynamic during the first 20 weeks and whether it is high enough to be measured using ELISA and IIF.

Materials and methods

Four Simmental cows, aged as follows: animal A 26 months, animal B 36 months, animal C 28 months and animal D 37 months, were selected for the trial. These were non-grazing animals which, after clinical, biochemical and haematological examination, were declared to be healthy; all were stabled from birth in the stall where only saw-dust was used for litter, and to the best of our knowledge had no contact with *B. burgdorferi* s.l. A negative serological reaction in the IIF test and ELISA confirmed this. Three animals (A, B and C) were intradermally inoculated with a pure culture of *B. garinii*, isolated and studied in Slovenia (1) in the subaxillar region. Animal D was immunized with dead *B. garinii* bacterias in Freund's incomplete adjuvant (Difco, Detroit, USA), and the procedure repeated 6 weeks later (booster). After taking blood samples for haematological, biochemical and serological investigations, and checking the general health of the animals, animals were infected as follows:

animal A, left subaxillar region, 5 times i.d., injections of 2 x 10^3 microorganisms in 100 µl of broth BSKII, right subaxillar region, single i.d. injection of 104 microorganisms in 100 µl of broth BSKII;

animal B, left subaxillar region, single i.d. injection of 104 microorganisms in 100 µl of broth BSKII, right subaxillar region, single i.d. injection of 104 microorganisms in 100 µl of broth BSKII;

animal C, left subaxillar region, 5 times i.d. injections of 2 x 10^4 microorganisms in 100 µl of broth BSKII, right subaxillar region, in doses of 5 times i.d. injections of 2 x 10^3 microorganisms in 100 µl of broth BSKII;

animal D, i.m., in the right *m. gluteus*, 10^9 microorganisms killed in 10 ml of Freund's incomplete adjuvant; the procedure was repeated after 6 weeks.

A tuberculin syringe set was used for injecting the bacteria. After infecting the animals, the remaining bacteria were monitored, and their viability evaluated. Blood was taken from *V. jugularis* and *V. epigastrica cranialis superficialis* with a 10 ml syringe, complete with siliconized wall (Sherwood-Monoject, Gosport, UK). After being centrifuged three times, 1 ml of each serum was frozen at -20 °C.

The blood samples were taken once a week over a twenty week period. During the experiment, a total of 84 samples were taken and analysed.

IIF

The antigen used in the test was a suspension of tick isolated *B. garinii*. After 20 minutes of centrifugation at 11,000 x g the culture was resuspended in PBS with 5 mM MgCl2 with a pH of 7.4. This suspension was centrifuged and the procedure repeated.

The sediment was resuspended in a PBS buffer with $MgCl_2$ and attenuated until 20 to 30 bacteria could be seen in the covered droplet at a magnification of 400 times. 12 µl of this suspension was poured onto object glasses (Bio-Merieux, Marcy l'Etoile, France), after being degreased for two hours in a suspension of equal parts of ethanol and acetone and then heated, three times in the flame of a gas burner. The object glasses were then dried for 12 hours overnight at 37° C, later put into methanol for 10 minutes, then wrapped in aluminium foil and stored at –70° C. The sera which were stored at -20° C were diluted 1 : 100 and 1 : 250. The positive control sera were taken from cattle immunized with the dead *B. burgdorferi* bacteria. The negative control sera were taken from a 3-month-old calf that was reared in a stable and which repeatedly tested negative in control tests. Labelling was performed with a rabbit anti-bovine gamma-globulin conjugated with FITC (DAKO, Glostrup, Denmark). The remaining procedures and methodologies have been preformed as described before (39).

ELISA

Bacteria *B. garinii* was used to prepare the antigen. After repeated centrifugation and washing of the culture in the PBS at 4° C, the microorganisms were disintegrated using ultrasonic disintegrator (8 x 15 seconds by 6000 Hz). After 30 minutes of centrifugation at 25,000 g, the protein content was checked in the supernatant from which proteins were concentrated to 1 µg/ml in an ultracentrifuge (Centriprep, Amicon, USA). The dilution of an antigen, 1:5000 in carbonated-bicarbonated buffered saline with a pH of 9.6 (Sigma, St. Louis, USA) and in a concentration of 5 µg/ml, was distributed across microtitre plates (NUNC, Roskfielde, Denmark) in quantities of 100 µg per well. After 18 to 20 hours of

binding, the wells were washed with PBS containing 0.05 Tween 20; (PBST; Sigma, St. Louis, USA). Then over the course of one hour, uncoated parts of the wells were covered with 200 µl PBST containing 1 % powdered milk (Merck, Darmstadt, Germany), pH 7.2 at room temperature. After washing four times with PBS-T-BSA, the plates were placed on a shaker and incubated for one hour at room temperature with PBS-T-BSA and in several dilutions of cattle sera (1: 500; 1 : 1000; 1 : 2000). After washing the plates four times, both the protein G-HRP (DAKO) which was used to identify antibodies, and the conjugate – goat anti-bovine IgG conjugated-HRP or IgM conjugated-HRP (DAKO) were added to the microtitre wells in quantities of 100 µl per well in a dilution of 1:1000. The remaining procedures were performed following the manufacturer's instructions for goat anti-bovine IgG conjugated-HRP or IgM conjugated-HRP, DAKO). For negative control, the negative cattle sera were used in a dilution 1 : 500 and for positive control the immunised cattle sera with a titre of antibodies 1 : 6400, which was confirmed in an IFA in dilution of 1 : 5000 in the well.

The quotient of determination (R2), received by means of correlation analysis, was an illustration measure (connecting curve in figures) of the general tendency of dynamics, together with a cubic function with the common equation:

$$Yreg.= b_0 + b_1t + b_2t^2 + b_3t^3$$

Results

The humoral immune response in the treated animals in the class of polyclonal antibodies was low. An improvement was recorded at the end of the second week, post infection (animals A, B, C and D) and peaks were observed after eight weeks animals B and C), and after the eleventh, nineteenth and twentieth weeks animals A and C), and in an immunized animal, after the fifteenth week (animal D). The highest average post infection values can be expected at around fifteen weeks (Fig 1).

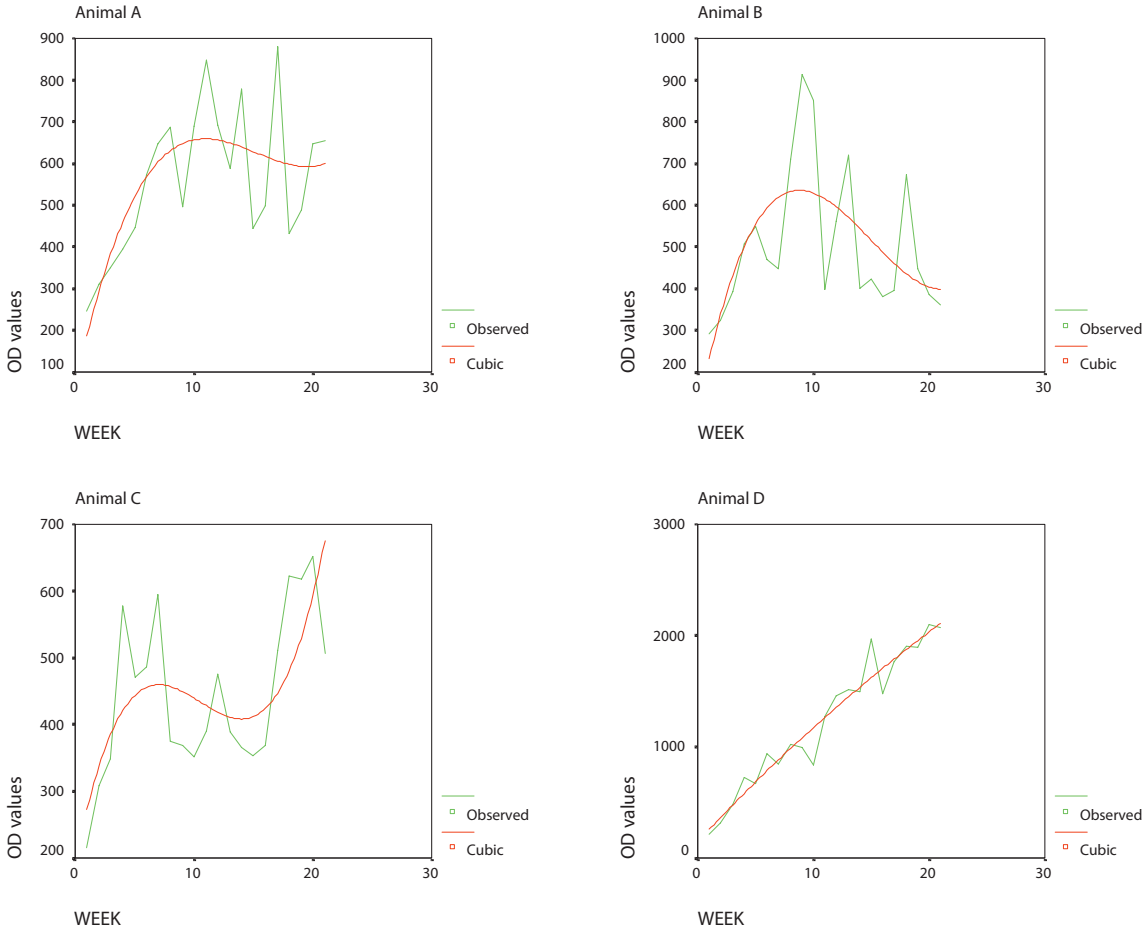

Figure 1: Levels of polyclonal antibodies response in treated animals measured by ELISA

Two weeks after infection, all animals showed a rise in the level of IgG. The highest values were recorded at the seventh and eighth weeks in animals A, B C and D and the fifteenth and eighteenth weeks (animals C and D). The highest values can be expected around 10.5 weeks (Fig. 2).

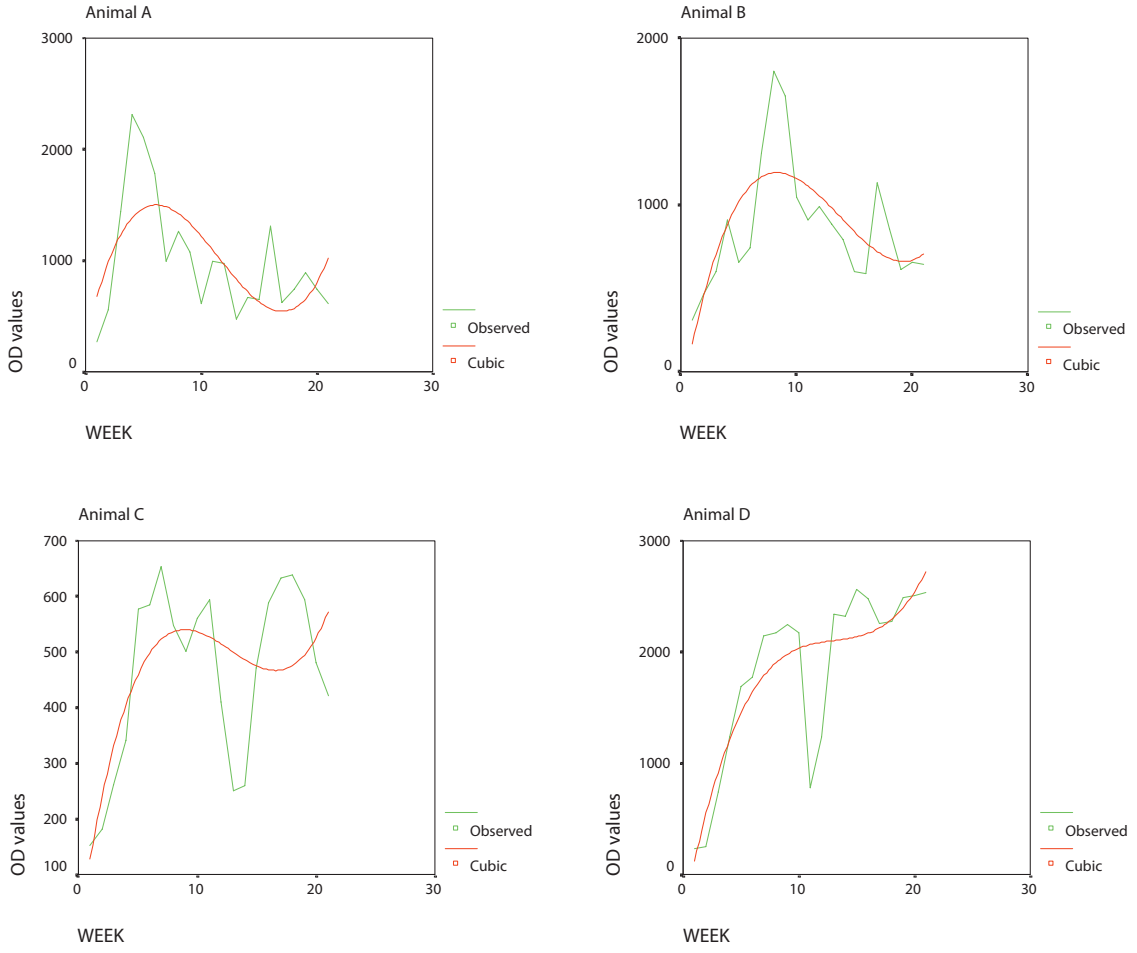

Figure 2: Levels of IgG antibodies response in treated animals measured by ELISA

The measurement of the IgM antibodies (Fig. 3) in animal A showed a rapid increase after the second week and reached the peak between the third and fourth week, in animals B and C between the eighth and ninth week, while in the vaccinated animal (animal D), the readings rose steadily until after the eighth week, and then declined, before again rising sharply between weeks twelve and fifteen. The highest values can be expected between the fourth and fifth weeks.

By comparing the values established by ELISA and IIF tests, it was concluded that there is a direct correlation between the two tests, with readings greater than 0.700 (OD values at 450/630) on the ELISA, comparable to readings of 1 : 256 and greater in the IIF test. This was considered highly reactive (+++ and ++). The range of 0.300 to 0.700 on the ELISA OD scale was selected as being definitely reactive (+). This was the equivalent to the range 1 : 64 to 1 : 256 in the IIF test. Optical readings, two standard deviations (SD) or more from the mean to 0.300 were interpreted as negative. The IIF test results matched (Fig. 4) the immune response values recorded by means of ELISA. Measurable, positive reactions were shown by the IIF test (dilution 1: 100) to start in weeks 2 (animal A, C and D) to 5 (animal B) in all animals.

The humoral immune responses resulting from experimental infection with *B. garinii* were weak, but detectable in animals A, B and C. However, a strong reaction was detected in the immunized animal (D), in which the value of the specific antibod-

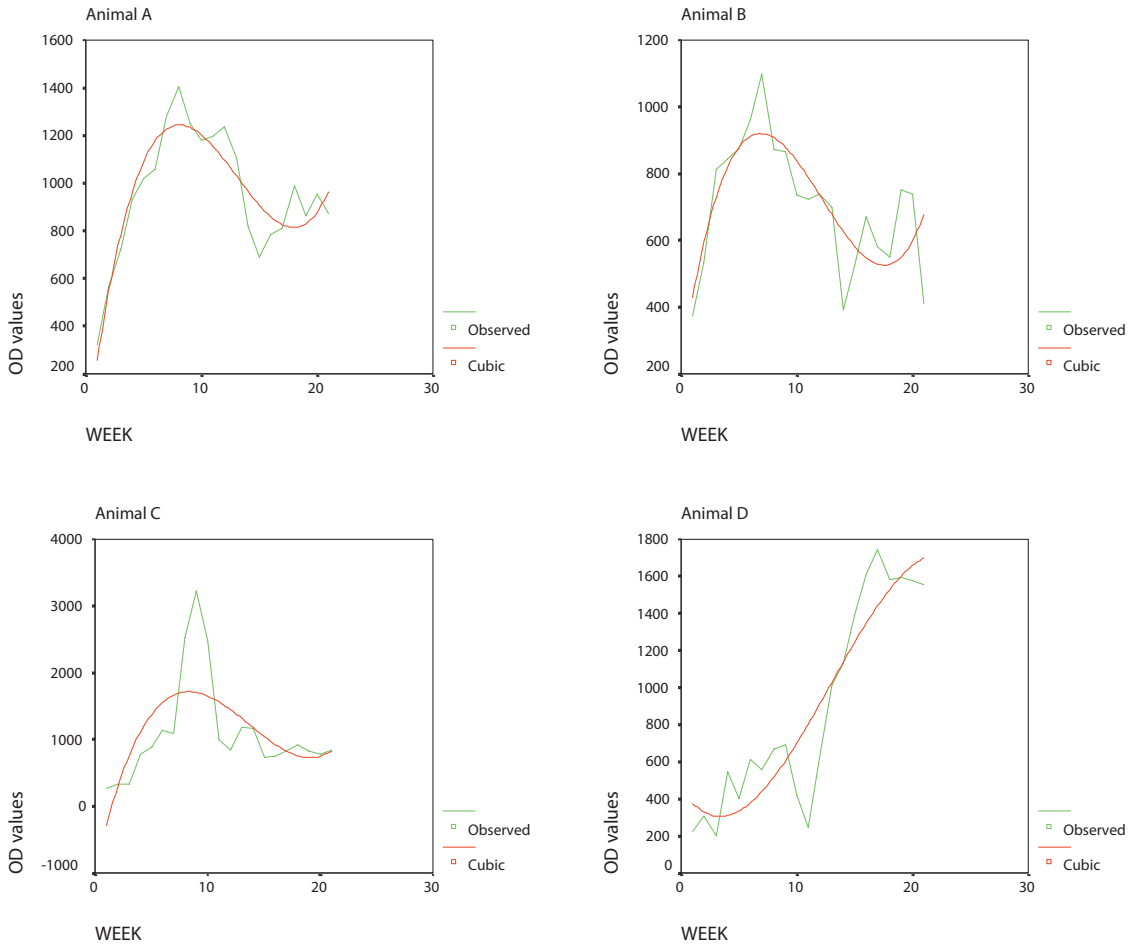

Figure 3: Values of IgM antibodies response in treated animals measured by ELISA

ies after 20 weeks showed a positive reaction in the IIF tests (dilution 1:12800) and exceeded 2680 units (by OD values at 450/630) or titer 1 : 38400 in the ELISA tests.

Discussion

The knowledge about the serological responses to Lyme borreliosis in animals, and especially in cattle, is limited and we are as yet unable to explain the numerous clinical events that could be connected to *B. burgdorferi* s.l. infection (swelling of the joints, lameness, interdigital dermatitis, emaciation).

In Slovenia, Lyme borreliosis is endemic, and is the most frequently diagnosed tick-borne disease in humans (40). Investigations carried out by the Veterinary Faculty in Ljubljana show that titres greater than 1:100 in IF seropositivity were detected in 18 % dogs, 24 % in wild animals (41) and 64 % in grazing cattle (32).

While the HIR in cases of Lyme disease generally follows a basic immunological pattern, it is common to see a delayed response, a quick elevation of IgG antibodies and in humans a complete absence of HIR (42), although less frequent (up to 12 %).

A rapid elevation of IgG was noted in all experimental animals and this is characteristic of secondary immune response. The same was observed in mice (43).

Specific and quite strong IgM response was detected in all four experimental animals.

In humans, the IgG response at the early stage of the disease is generally considered to be of little diagnostic value (45). Most patients have a good IgG response in the early stages of the disease and some reinfected patients may only have an IgG response (28). Our results suggest that the IgG-ELISA response can be of value in the serodiagnosis of early Lyme disease in the cattle. It is interesting to note that almost immediately after the second im-

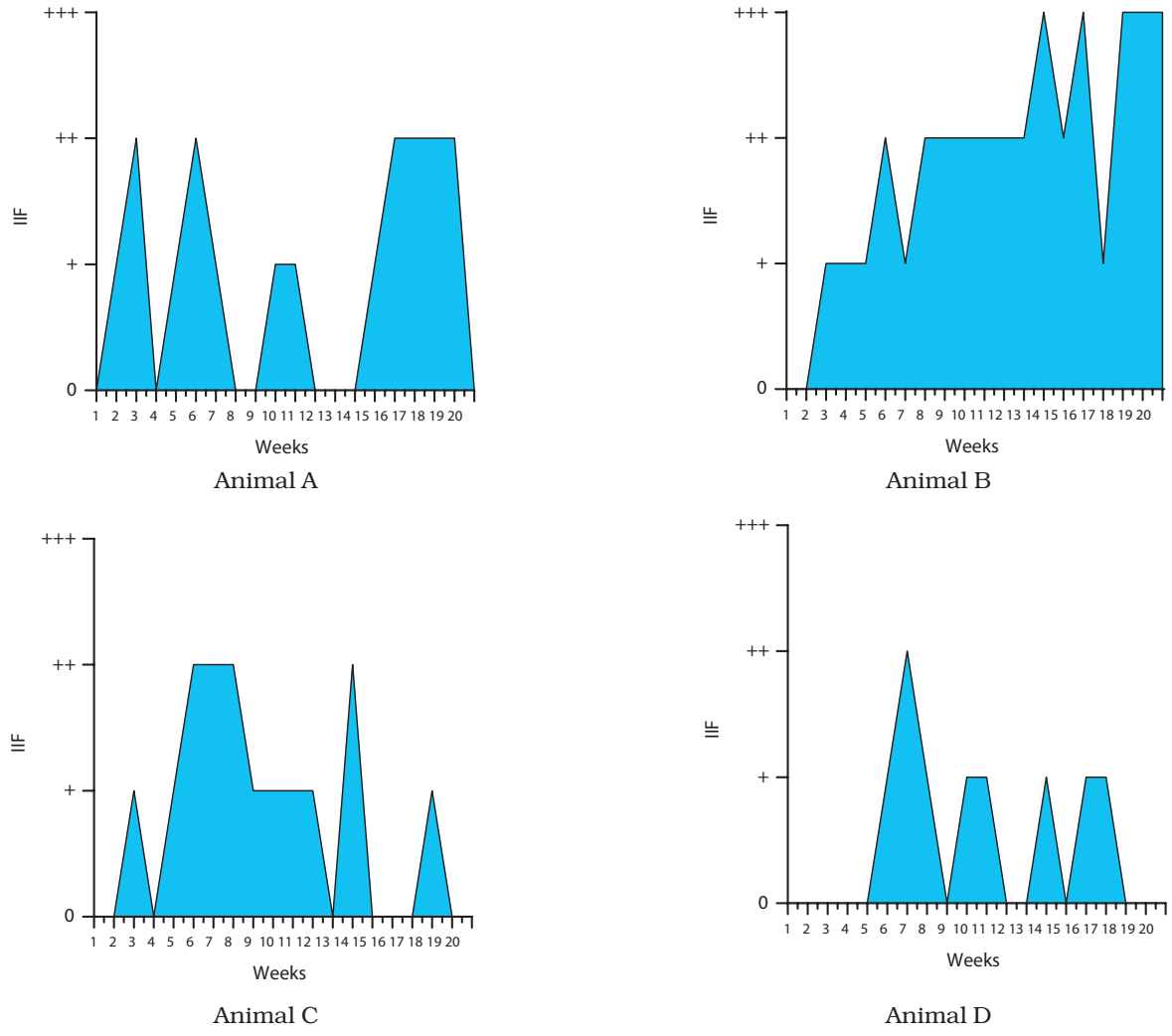

Figure 4: Specific polyclonal antibodies response in treated animals measured by IIF

munization (booster) in week 6, animal D displayed a quick fall in the levels of IgG and IgM antibodies. This continued until approximately the tenth week, before rising again. Although the predominant human isolate in Slovenia is the *B. afzelii* species (45), for our experiment we selected the tick-borne isolate of *B. garinii*, the first most frequently isolated species in animals and the second most frequently isolated species in humans in Slovenia(1, 46).

Multiple species of *B. burgdorferi* sensu lato differ in their molecular and antigenic properties, and therefore it could be expected that the recognition of a *B. burgdorferi* s.l. infection is difficult and dependent on the validity and specificity of the diagnostic assays and heterologous antigens used (47, 48).

It is important to note that the established specific early immune responses in our experiment may appeared due to homologous antigens of the same genospecies that were used in the tests. The same results were reported in the Finnish experiment, where only the homologous antigen (an antigen of the same genospecies) was sufficiently sensitive to give a positive result in the serological test (22). However, it is still not clear, what is the meaning of genospecies-binding differences of B. burgdorferi s.l. in pathogenecity and clinical manifestation of the disease in animals.

As established in this and other studies, the levels of specific anti-borrelial antibodies in grazing cattle depend on the number of infections or the age of animal (32, 35). The same pattern can be observed in humans, where HIR develops gradually over a period of months to years (28, 29, 30).

The weak immunological response established in our experiment probably mirrors the existing data, although the 20 - week period is not long enough to confirm this.

The results of serological investigations of clinically and subclinically ill animals are still not comparable to those in humans. In our previous study, it was established that basic HIR in grazing cattle in Slovenia is high, but the differences between the reactions of infected, though healthy animals, and those suspected of having Lyme disease, are not significant enough to be used as confirmation of the disease (32).

The enzyme-linked immunosorbent assay (ELISA) has been widely used for detecting antibodies to *B. burgdorferi* s.l. in human and veterinary medicine, and its efficiency and reliability is comparable with other available tests for Lyme disease (21, 44). These assays are still not standardised, thus requiring tests with various levels of sensitivity and specificity. As our results have shown, IIF and ELISA tests can be sufficiently sensitive to detect an infection in the cattle during the early phases of Lyme disease, but due to their weak humoral immunological response it is not easy to evaluate or use them. Generally, by comparing the values established by means of ELISA and IIF in our study, it was concluded that the IIF test results matched those of the immunological response values recorded by means of ELISA. Reactions in IIF tests in dilutions 1 : 100 were observed from 2.5 to 3 weeks (17 to 21 days) after infection.

It has been shown in several previous studies that humoral immunological response definitely indicates a reaction to *Borrelia burgdorferi* infected organisms; however since the strength of the HIR frequently depends on the phase of the illness, the clinical importance of HIR as a means of diagnosis is not yet clear.

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HUMORALNI IMUNSKI ODZIV GOVEDA PO POSKUSNEM STIKU Z BAKTERIJO *BORRELIA BURGDORFERI* S.L

Č. Borko

Povzetek: Da bi ugotovili značilnosti humoralnega imunskega odgovora (HIR) pašnega goveda, naravno okuženega z baketrijo *B. burgdorferi* sensu lato (s.l.), smo skupino treh odraslih goved umetno okužili z bakterijo. Tri goveda smo intradermalno (i.d.) okužili z živimi bakterijami *B. garinii*, četrto pa smo imunizirali z mrtvimi bakterijami v Freundovem nepopolnem adjuvansu. Taksonomsko identiteto in klonsko pripadnost izolata smo potrdili s PCR pomnoževanjem 5S in 23S r RNA (1). Ugotavljali smo imunski odgovor poliklonalnih, IgM in IgG protiteles z ELISO in z indirektno imunofluorescenčno analizo (IIF). Imunski odgovor treh intradermalno okuženih živali je bil nizek, vendar ugotovljiv in značilen v toku, medtem ko je bil pri vakcinirani živali visok.

Ključne besede: *Borrelia burgdorferi*; govedo; serološka diagnostika