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Original Research Paper

DEVELOPMENT AND EVALUATION OF MONOCLONAL ANTIBODY-BASED ENZYME LINKED IMMUNOSORBENT ASSAY FOR BORRELIA GARINII ANTIGEN DETECTION IN URINE

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Summary: We developed antigen capture double sandwich ELISA (ACDS ELISA), based on borrelia flagellin-specific monoclonal antibody. Nested PCR analysis for borrelial DNA in the urine samples was used for comparison. Results obtained by ELISA and nested PCR analyses of urines from mice, experimentally infected with *B. garinii* by different routes of inoculation, confirmed the presence of borrelial antigens (antigenuria), while whole spirochetes or their DNA could not be detected or were not released into the urine (absence of spirocheturia). Such ELISA might become a good supplementary method in the diagnostics of Lyme disease, especially in seronegative subjects suspected to be infected with *B. burgdorferi.*

Key words: Borrelia antigens; urine; immunoenzime assay

Introduction

Borrelia burgdorferi sensu lato spirochete (B.b.) is the causative agent of Lyme borreliosis, a multisystemic illness and the most prevalent tick-borne disease. The diagnosis of this disease is achieved by evaluating clinical, epidemiological and laboratory findings (1, 2). Unfortunately, according to the protein electrophoresis patterns and specific monoclonal antibodies (mAbs') reactivity, B.b. is antigenically extremely heterogeneous microorganism which induces highly variable humoral immune response and is consequently difficult to confirm by serological methods (3-10). Serological diagnosis of Lyme disease is therefore unreliable and a direct method for detection of B.b. in presumably infected patients is needed to improve and to simplify borreliosis diagnostics. PCR is a method of choice (11-14) but unfortunately still too expensive, too elaborate and inaccessible for some laboratories. Since *B. burgdorferi* antigens have been detected in urine, blood and many tissues of infected hosts (15, 16) we tried to develop a simple but sufficiently sensitive and specific monoclonal antibody based ELISA for B.b. antigen detection in urine specimens. The method was evaluated by probing urine samples from experimentally infected BALB/c mice, analysing also the dynamics of.*B. burgdorferi* antigenuria, humoral immune response and the importance of inoculation route. The results were compared to those obtained with nested flagellin gene targeted polymerase chain reaction performed on the same samples.

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Materials and methods

B. burgdorferi antigen detection ELISA

The method we developed is an antigen capture double sandwich (ACDS) ELISA based on a monoclonal antibody (mAb) 1G6/G6/D12, directed against genus specific 41 kDa protein flagelin. MAb used in the test is of our own production (17) and can be substituted with adequatly purified mAbs specific for other borrelial proteins. In the first step 100µl per well of mAb (5 µg/ml, carbonate-bicarbonate buffer, pH 9'6) is adsorbed onto solid phase (96 microwell ELISA plate for antibody adsorbtion; Nunc Maxisorp, Copenhagen, Denmark) at 4oC overnight. After washing the wells with 3 x 350 µl of Phosphate

Buffered Saline (PBS) with 0,05% Tween 20 (washing solution, PBS -T; Tween 20: Sigma, St. Louis, USA), nonspecific antigen adsorption is limited by blocking unoccupied binding sites in the wells with 200 µl of 2 % Bovine Serum Albumine (Sigma, St. Louis, USA) in PBS, pH 7,2 (PBS-BSA) for one hour at 37oC. The wells are then washed as described above and borrelial antigens in the urine samples (if present), diluted 1:4 in PBS-BSA, are allowed to react with the mAb (100 µl per well, one hour at room temperature, shaking). The eventually bound flagellin is, after washing, recognized by previously standardized (crossboard titration in indirect ELISA) hyperimmune polyclonal anti B.b. rabbit serum at 1:800 dilution in PBS-BSA (100 µl, one hour at room temperature, shaking). Rabbit antibodies, specific for the *B. burgdorferi* flagellin (if present in the sample), react in the next step (after the washing of the wells) with pig anti-rabbit immunoglobulins conjugated with horse-radish peroxidase (HRP-conjugate; Dako, Copenhagen, Denmark; 1:1000 dilution in PBS-BSA, 100 µl per well, one hour at room temperature, shaking) and are revealed consequently, after washing the wells, by the Tetra Methyl Benzidine HRP substrate (TMB, Sigma, St. Louis, USA; 100µl, 10 minutes at room temperature) evaluating the results spectrophotometrically by ELISA test plate reader (Behring EF-200 ELISA reader, Germany; double OD at 450 and 630 nm) after the peroxidase activity in the wells was stopped by adding 50 µl per well of 0,5 M H_2SO_4. Positive (previously tested negative urine spiked with 30 ng/ml of sonicated *B. garinii*) and negative controls (negative urine) are included on every test plate, 4 wells each. Samples yielding optical densities higher than 3 SD of the negatives' average are considered positive.

The method was tested by probing different types of samples (PBS, BSK medium, urine) spiked or not with borrelial antigens (sonicated or whole borrelia cells) in known concentrations (two-fold dilutions starting at 4µg/ml to 2 ng/ml and $7{,}2x10^6$ to 3,5x103 *B. burgdorferi* spirochetes/ml, respectively). Samples spiked with sonicated *Leptospira spp., E. coli* and *Salmonella* spp. at same concentrations were used to detect eventual cross reactions due to other bacteria flagellin.

Experimental infection of mice with B. garinii and sampling procedures

Fifteen 6-week old female BALB/c mice divided in 3 separately caged groups of.5 animals were infected by different routes of inoculation with the 3rd passage *B. garinii* M3/S (cultured from a tick infested mouse heart on our institute and determined as *B. garinii* by RFLP analysis after MluI digestion by dr. Eva Ruzic-Sabljic from Institute for microbiology, Medical Faculty, University of Ljubljana). The first group of mice (group A) was infected intraperitoneally, the second (group B) subcutaneously and the third (group C) intravenously with.0,1 ml BSK-H medium containing $1x10^7$ M3/S spirochetes. Additional 5 mice (group D) were inoculated with 0,1 ml sterile BSK-H medium and served as negative control. Blood and urine samples were taken at the beginning of the experiment (day 0), and on days 4, 8, 15 and 30 after initial inoculation of spirochetes. Serum and urine samples were stored at - 20oC immediately after sampling.

ELISA for the detection of B. garinii-specific antibodies in sera from experimentaly infected mice

B. garinii-specific antibodies in mouse sera were detected by indirect ELISA as described previously (17). Shortly, sonicated *B. garinii* was adsorbed onto the wells of ELISA plate overnight. Unoccupied binding sites in the wells were blocked by 2% BSA. Serum samples at 1/400 dilution reacted then with an adsorbed antigen and bound *B. garinii* specific antibodies were detected by anti mouse HRP conjugated rabbit immunoglobulins and TMB/stop substrate, measuring optical density at 450 nm in the test wells. Sera from uninfected group D mice served as negative controls. Sera yielding optical densities higher than calculated average + 3 SD of sera derived from uninfected controls were considered positive.

Detection of borrelial DNA in urine samples

A nested PCR, developed by Schmidt et al. (13), claimed to be capable of detecting less than 5 *B.burgdorferi* organisms was applied to detect *B. garinii* DNA in urine of experimentally infected mice. The target for this PCR was a specific region of the flagelin gene, common to all three pathogenic species of genus *Borrelia: B. burgdorferi* sensu stricto, *B. afzelli* and *B. garinii.* The primers and most of the PCR procedure were identical to the one described by Schmidt. Mouse urines collected during one month experimental infection were thawed and brought to room temperature. 50 µl of each sample was centrifuged at 14,000 g for 30 min. The pellet was resuspended in 600 µl PBS, pH 8. Half (300 µl)

of this suspension was used for PCR analysis. 200 *B. garinii* M3/S spirochetes in 50 µl of PBS, previously counted in Hauser - Petroff chamber by darkfield microscopy, were added to the other half to evaluate eventual inhibitory activity of each urine sample. Both suspensions were then centrifuged at 14,000 g for 20 min. The resulting pellets were dissolved in 100 µl of a 5% Chelex-100 suspension (Perkin-Elmer, USA) in PBS, heated at 100oC for 5 min and subsequently chilled on ice. After centrifugation (3000 g for 1 min), 10 µl of each supernatant was used as a template DNA in the first PCR.

Primers used in the first PCR were BBSCH31 (CAC ACC AGC ATC ACT TTC AGG GTC T) and BBSCH42 (CAA CCT CAT CTG TCA TTG TAG CAT CTT TTA TTT), targeting the chromosomal flagellin gene of *B. burgdorferi* (GenBank accession No X15661-2) and representing positions 477 to 501 and 913 to 881, respectively. The reaction mixture contained 50 mM KCl, 1,5mM MgCl2, 10mM Tris-HCl (pH 8,3), 0,05% glycerol, 0,1% TritonX-100, 0,1 mM EDTA, 00,1% BSA, 200µm (each) desoxynucleotide triphosphate (dAMP, dCTP, dTTP, dGTP), 50pM (each) outer primers, 2 U of *Taq* polymerase (Promega, USA) and 10µl of the DNA preparation (total volume 60 µl). The mixture was then overlaid with one drop of mineral oil. The amplification reactions were performed in 0,5 ml tubes with attached cap in a PTC-200 thermocycler (MJ Research, Watertown, USA). The first PCR with the outer primers was run for 25 cycles with denaturation at 94°C (30 s),.annealing at 55oC (30 s) and elongation at 72°C (30 s). Thermocycling was preceeded by 1 min at 95°C and followed by final 10 min extension at 72°C. After amplification, 3 µl of.the first PCR mixture was transferred to a second PCR mixture, consisting of the same master mixture as described abov; instead of outer primers, 25 pm (each) of inner primers FL-59 (TTT CAG GGT CTC AGG CGT CTT) and FL-7 (GCA TTT TCA ATT TTA GCA AGT GAT G), representing positions 491 to 511 and 767 to743, respectively, were used, yielding a 277-bp amplicon. The second PCR was done with 35 cycles of the same duration and same temperatures used for the first PCR for the denaturation and elongation, but the annealing temperature was increased to 58°C.

Blank controls, containing 10 µl of water instead of urine samples, as well as positive controls (10 fg of purified borrelial DNA) were run in parallel with each amplification assay.

DNA extraction, PCR mixture preparation, amplification, and analysis of amplicons were each done in separated areas. Filter barrier pipette tips and a dedicated set of pipetors were used for sample and PCR mixture preparation.

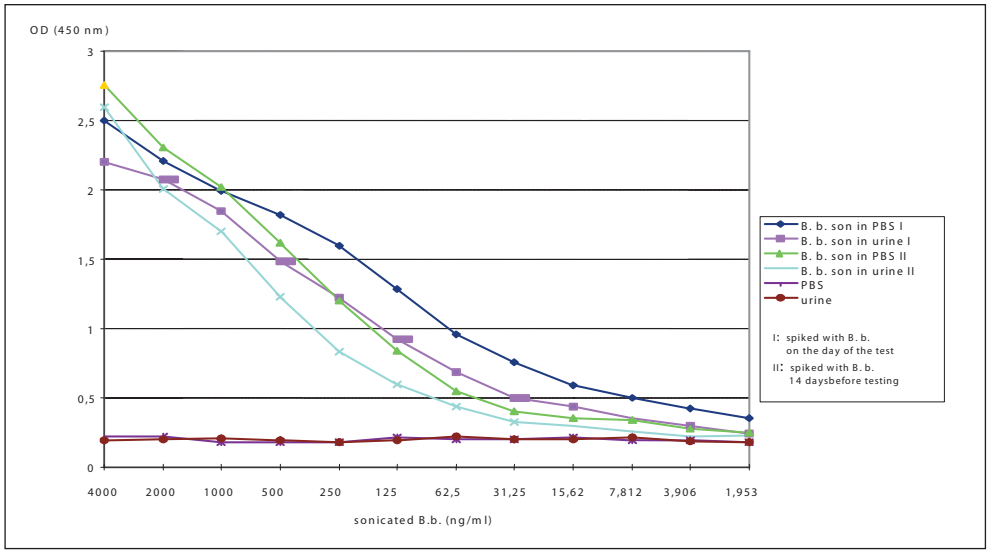
Amplified products were resolved by 3% agarose gel (NuSieve/SeaKem, 3:1; FMC Corporation, Rockland, Maine, USA) at 150 V for 25 min. The gel was then stained with ethidium bromide and the bands observed under UV illumination at 254 nm.

Results

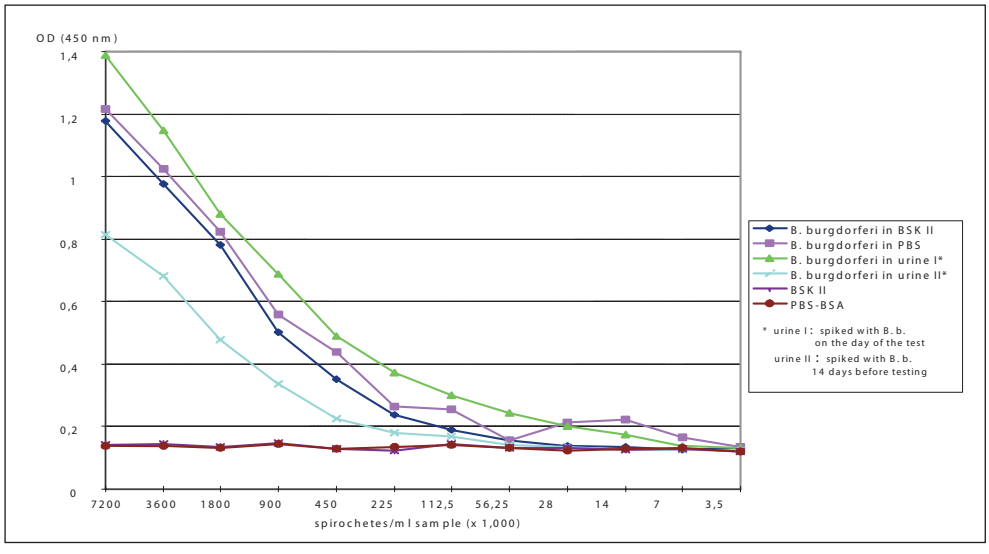
Results of our ACDS ELISA sensibility testing with serial dilutions of sonicated borrelial antigens and whole *B. garinii* cells in different types of samples are shown in Graphs No. 1 and 2, respectively. The detection limit was less than 0,5 ng/100 µl sample for sonicated borrelial antigens and cca 2,000 spirochetes/100 µl sample for whole borrelial cells. The type of the sample did not interfere with the results. Urine samples spiked with borrelial antigens reacted positively even after 14 days of storage at 4oC indicating that urine had no deterioration effect on B.b. flagelin. Cross reactions to the other bacterias' flagelin were not observed (Graph No. 3).

The detection limit of.the flagellin targeted nested PCR was in our hands slightly higher than described by the authors (13). The method was capable of detecting from 10 to 20 *B. garinii* borrelias per sample, as determined by assaying mouse urine samples spiked with known numbers of spirochetes, and thus prooved to be sufficiently sensible and specific (according to the authors) tecnique for *B. burgdorferi* DNA detection in urine. Yet, when urines obtained from mice from our experiment were examined, borrelial DNA could not be detected by this method. In view of the fact that the same samples, when spiked with 200 *B. garinii*, always.yielded the expected 277 bp amplicon, mouse urine tested were not found to be inhibitory.

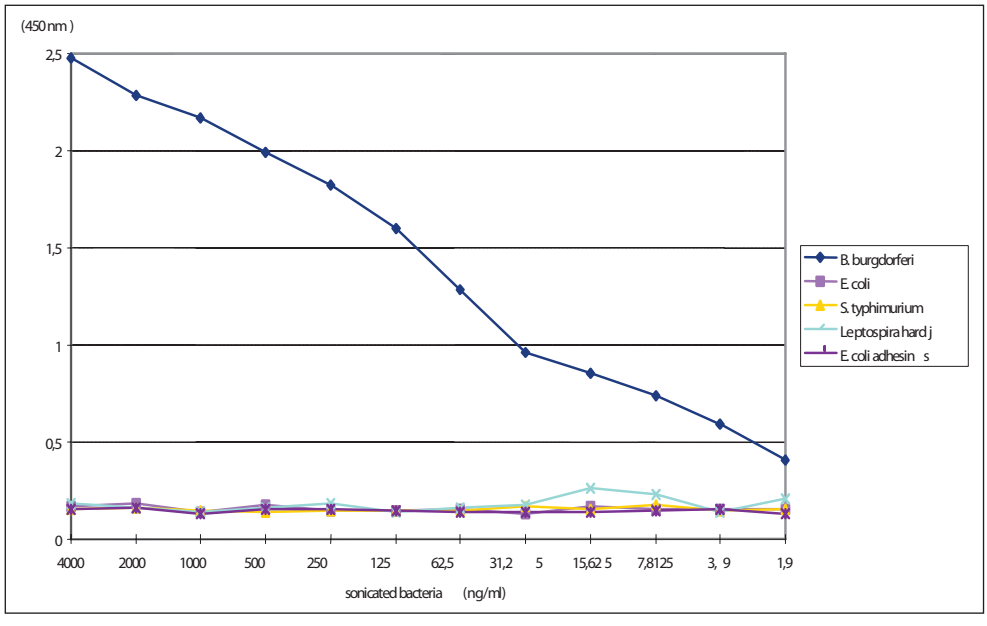
Contrariously, when the same samples were tested in ACDS ELISA, borrelial antigens could be detected in urine of virtually every *B. garinii* infected mouse at least in one of four samples taken (OD-450 nm > 0,192; cut off = average of negative controls + 3 SD = 0,145 + 3 x 0,015). The dynamics and the extent of *B. garinii* flagellin shedding in urine, together with humoral response of each animal in intraperitoneally, subcutaneously and intravenously infected groups of mice during the first month of.experimental infection, are shown in graphs 4, 5 and 6, respectively.



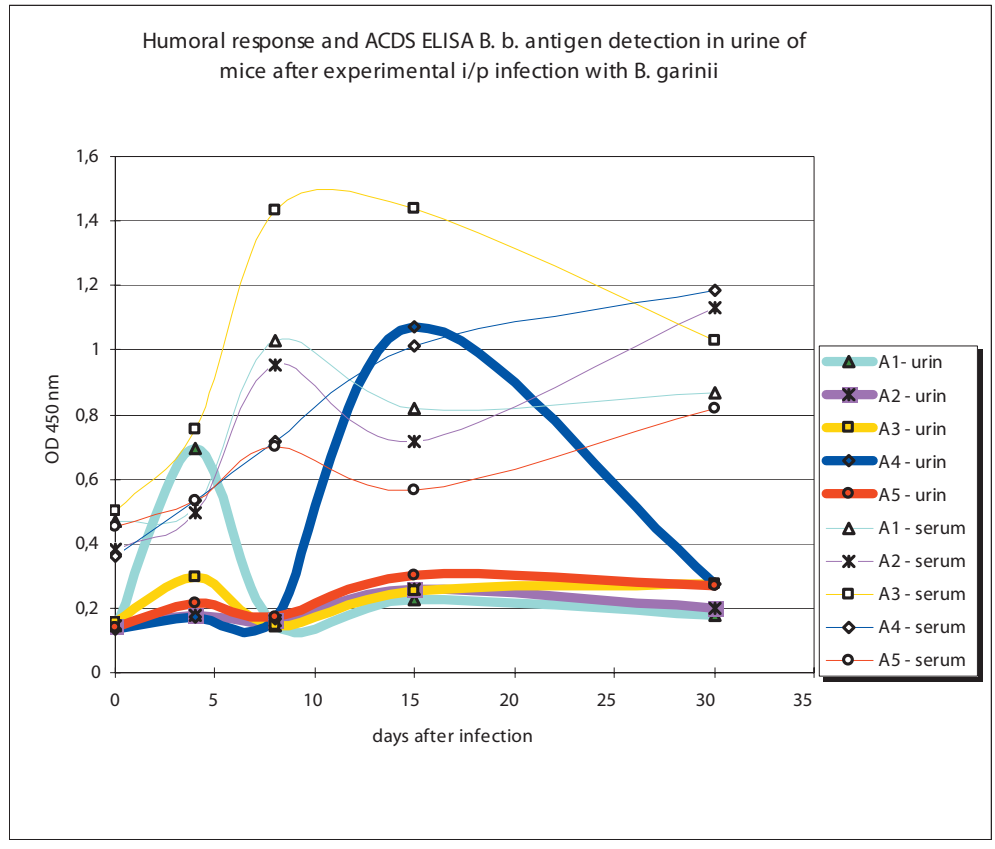
Graph 1: ACDS-ELISA *B. burgdorferi* flagelin detection in BSK II medium, PBS-BSA and urine spiked with sonicated *B. burgdorferi*



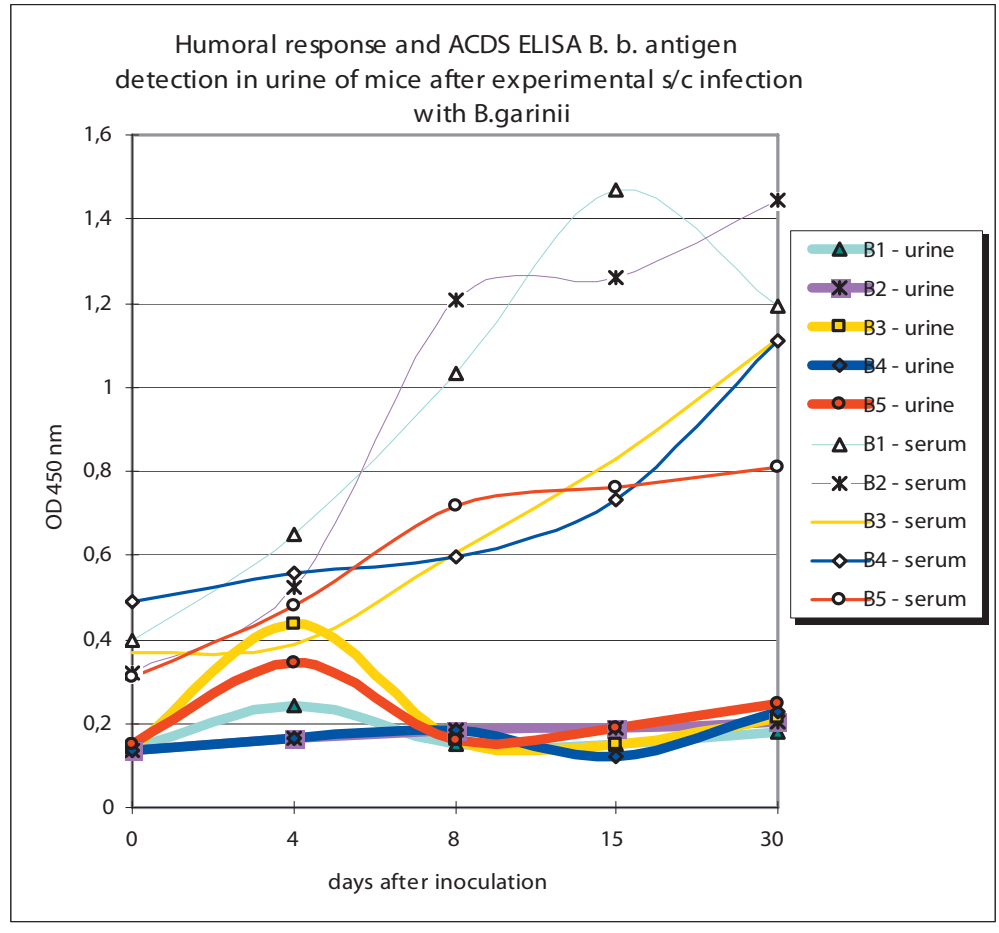
Graph 2: ACDS-ELISA *B. burgdorferi* flagelin detection in BSK II medium, PBS-BSA and urine spiked with whole *B. burgdorferi* cells



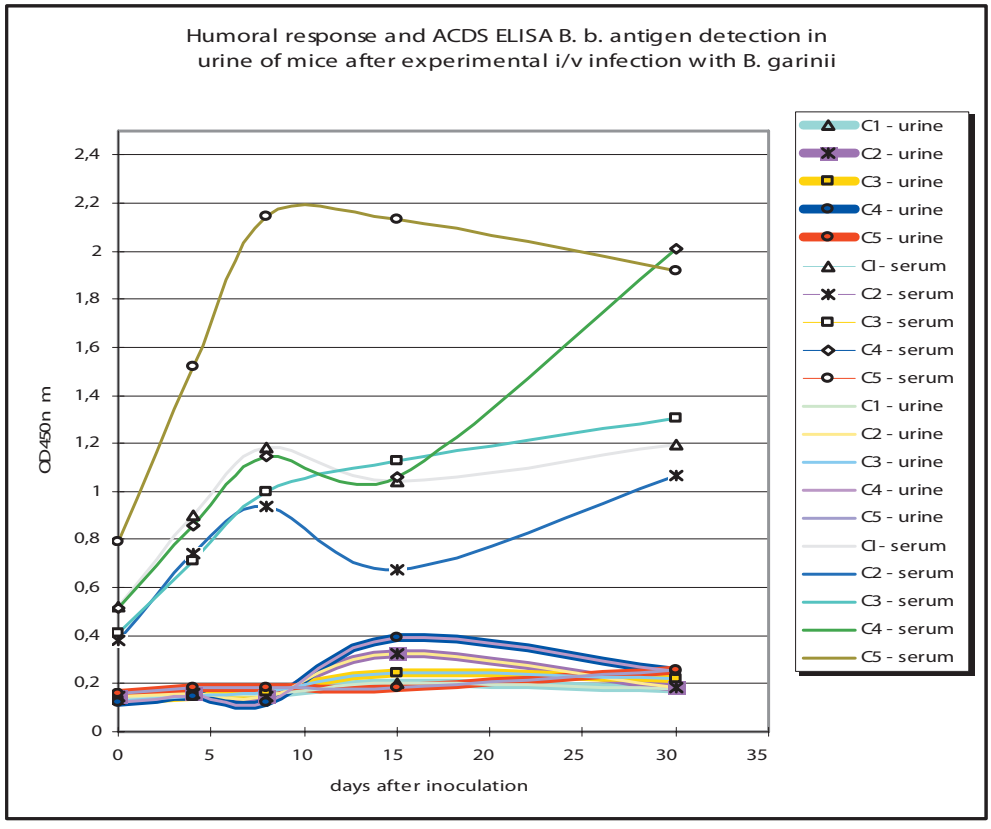
Graph 3: Reactivity of sonicated heterologous bacteria in ACDS-ELISA for *Borrelia burgdorferi* detection in urine samples



Graph 4: Humoral response (indirect ELISA) and borrelial antigen detection in urine samples (ACDS ELISA) of mice after experimental intraperitoneal infection with *B. garinii*



Graph 5: Humoral response (indirect ELISA) and borrelial antigen detection in urine samples (ACDS ELISA) of mice after experimental subcutaneous infection with *B. garinii*



Graph 6: Humoral response (indirect ELISA) and borrelial antigen detection in urine samples (ACDS ELISA) of mice after experimental intravenous infection with *B. garinii*

Discussion

Our developed antigen capture ELISA, grounded on the flagellin-specific monoclonal antibody might be, at least in our view, a tool for improving the complex Lyme disease diagnostics. Being capable of detecting less than 0,5 ng of sonicated borrelial antigens, the method offers substantially better sensibility than equivalent inhibition ELISA (18). The detection limit was higher when whole spirochetes were used as an antigen in sensitivity testing; this fact can be explained by periplasmic and not surface localization of *B. burgdorferi* flagella in intact spirochetes. The catching mAb can consequently not react with the flagellin molecules at the same extent as when sonicated borreliae were used. Although not tested with comparable *B. burgdorferi* s. s. and *B. afzelli* antigens, the assay should be, due to flagellin protein invariability.among B. burgdorferi sensu lato genomospecies, able to detect all the causative agents of Lyme borreliosis, too. This is not the case with the OspA antigen capture ELISAs, which can recognize only a limited number of *B. burgdorferi* Osp A serotypes (19), even if the detection limit of the method is superior in comparison to our method. In addition to this, OspA detection in urine and other biological fluids might be questionable since this protein is generally down-regulated in infected hosts (20). Cross reactions to the other bacterias' flagellin were not observed (Graph No. 3), but further investigations in this field are necessary. Reports on cross-reactivity of antibodies raised against other bacteria to this protein exist (21); other authors however claim that these antibodies do not react with 41 kDa flagellin but with another borrelial protein of 40 kDa (22). The type of the sample (urine or PBS) did not interfere essentially with the results. Urine samples spiked with borrelial antigens reacted positively even after 14 days of storage at 4°C indicating that urine had no serious deterioration effect on *B.burgdorferi*.flagellin.

Results obtained by ACDS ELISA and nested PCR analysis of urines from mice, expeimentally infected with *B. garinii* by different routes of inoculation confirm the presence of.borrelial antigens (including flagellin) in urine from infected mice (antigenuria), while whole spirochetes or their DNA could not be detected or were not released into the urine (absence of spirocheturia). Our data correlate well with available articles on borrelial antigenuria in mice (15,16, 18), inasmuch as our negative PCR results combine well with reports on failed attempts

of *B. burgdorferi* cultivation from mouse urine during experimental infection, despite the fact that the spirochetes were easily recovered from the walls of obviously infected urinary bladders (23, 24, 25)..As far as we know, with an exception of one work where *B. burgdorferi* has been succesfully isolated from mouse urine, but only from mice coinfected with *Babesia microti* (26), this spirochete has not been cultivated from, nor has its DNA been detected in mouse urine yet.

B. burgdorferi flagellin has been found in at least one of four urine samples taken from each of experimentally infected mice. The dynamics and the extent of flagellin shedding in urine appeared to be dependent on inoculation mode, which apprently influenced the humoral response development. Flagellin concentration in urine seemed to be inversely proportional to the.amount of.*B. burgdorferi* specific antibodies present in serum: flagellin could be detected in higher levels when the humoral response was still weak (in the first 4 days of infection) and a slight rise-ups in flagellin concentration were observed when specific antibody levels fell, usually around day 15. This antibody level - flagellin urine shedding relationship was most transparent in animals inoculated intravenously, which.responded immediately and with higher levels of specific immunoglobulins than those infected intraperitoneously or subcutaneously, and shed evidently less flagellin in urine than mice belonging to the other two groups (graphs 4-6). One month after initial inoculation antibody levels were elevated in all of the infected mice and flagellin could not be detected in urine any of the animals in the experiment. Urine and sera derived from the negative control group of mice reacted negatively throughout the study in the ACDS and antibody ELISA, respectively. These results suggest that the infection with *B. garinii* is being controlled by the specific humoral response level. Tiny rise-ups in flagellin in urine present in parallel with temporary regress in antibody titers might be a sign of multiplication of *borreliae*, possibly escape variants with new antigens expressed, and are then followed by a new rise in antibody titer..

The assay might become a good supplementary method in the Lyme disease diagnostics, especially in seronegative subjects suspected to be infected with *B. burgdorferi.* Further cross reactivity testing with broader range of bacteria, particularly other spirochetes, is necessary in order to calculate the exact specificity of the test, and the test needs to be evaluated also by probing urine samples from humans and other animal species. Although not so perfectly sensitive and specific as diverse PCR technique, the method demonstrates by these preliminary results an acceptable sensitivity for the *B. burgdorferi* antigen detection in the urine specimens with the possibility of improving it by concentrating the urine samples. In contrast to PCR, it does not suffer from unknown inhibition substances present in the samples and can detect borrelial antigens even if (or when) the spirochetes are not released into urine. Besides this, it is cheap, simple and fast and thus easily adaptable to every laboratory.

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RAZVOJ IN PREIZKUŠANJE METODE ELISA Z MONOKLONSKIMI PROTITELESI ZA UGOTAVLJANJE ANTIGENOV SPIROHETE BORRELIA GARINII V URINU

I. Gruntar, T. Malovrh

Povzetek: V prispevku je opisan razvoj antigenske lovilne metode ELISA, ki temelji na načelu dvojnega sendviča z monoklonskimi protitelesi, specifičnimi za borelijski flagelin. Za primerjavo metod smo uporabljali vgnezdeni PCR specifičen za borelijsko DNK v vzorcih urina. Rezultati pridobljeni z ELISA in z vgnezdenim PCR v vzorcih urina eksperimentalno okuženih miši z različnimi načini inokulacije *B. garinii*, potrjujejo prisotnost borelijskih antigenov (antigenurija), medtem ko celotnih spirohet ali njihove DNK ni bilo možno dokazati ali pa celih spirohet ni bilo prisotnih v urinu (ni spiroheturije). Takšno metodo ELISA bi torej lahko uporabljali kot dopolnilno metodo v diagnostiki lajmske borelioze, še posebej pri seronegativnih primerih, pri katerih se domneva, dag re za okužbo z *B. burgdorferi.*

Ključne besede: borelijski antigeni; urin; imunoencimske analize