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Protein A/G dependent ELISA a promising diagnostic tool in Lyme disease seroprevalence in game animals and hunting dogs

M.R. Bhide[a,*], J. Curlik[b], M. Travnicek[a], P. Lazar[b]

[a]*Department of Epizootology and Infectious Diseases, University of Veterinary Medicine, Komenskeho-73, Kosice, Slovakia Republic*

[b]*Department of Parasitology, Disease of Fish, Bees and Game, University of Veterinary Medicine, Komenskeho 73, Kosice, Slovakia Republic*

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Abstract

One of the major problems in serodiagnosis in wild animals is unavailability of specific antiglobulin conjugate. Our study focuses on validation of Protein A/G dependent ELISA in game animals like deer and mouflons as well as in hunting dogs. Binding ability of Protein A/G-conjugate to antibodies was the highest in dogs followed by fallow deer and mouflons. Three different whole cell *Borrelia* antigens were used to evaluate antigen dependent variation. In new Protein A/G-ELISA the highest sensitivities (90.50%, deer; 85.37%, mouflon & 94.29%, dog) were obtained by *B. garinii* antigen, with no statistically significant variation (χ^2, $P > 0.05$) among all other antigens used. Average seroprevalences observed in deer, mouflons and dogs were 44.90%, 29.41% and 30.43%, respectively. Marked influence of age on seroprevalence was noticed. Protein A/G – ELISA proved to be sensitive and promising diagnostic tool in serodiagnosis of Lyme disease in game ungulates and it can be used effectively for serosurvey in different wild mammals.

Keywords: Protein A/G; ELISA; Mouflon; Deer; Dog; Borrelia; Slovakia

Résumé

L'un des problémes majeur du sérodiagnostic chez les animaux sauvages est l'indisponibilité du conjugué anti-globuline spécifique.

Notre étude a été orientée sur la validation de la protéine A/G ELISA utilisée sur les gibiers comme le cerf et le mouflon et sur les chiens de chasse. La possibilité de combinaison de la proéine A/G avec l'anticorps a été la plus élevée chez le chien puis, sur les cerfs et les mouflons.

Trois différents antigénes Borrelia ont été utilisés pour l'évaluation de l'antigéne dans les nouvelles protéines A/G-ELISA. Les taux de sensibilité les plus élevés (90.5% chez le cerf, 85.35%

* Corresponding author. Fax: +421-55-632-36-33.
E-mail address: mangeshbhide@hotmail.com (M.R. Bhide).

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chez le mouflon, 94.9% chez le chien) ont été obtenues avec l'antigéne *B. Garinii* avec des variations statistiques non significatives (χ^2, $P > 0.05$).

Les séroprévalences moyennes ont été pour le cerf de 44.9, 29.41 et 30.43% pour le chien. Une influence significative de l'âge sur lar séroprévalence a été observée.

Mots-clé: Protéine A/G-ELISA; Mouflon; Cerf; Chien; Borrelia; Slovaquie

1. Introduction

Ungulates like deer and mouflons are the important hosts in epidemiology of Lyme borreliosis. The presence and density of game animals are key factors in Lyme disease eco-epidemiology. The sentinel status of deer for prediction of *Borrelia burgdorferi* (*Bb*) prevalence in habitat has been described [1]. Hunting dogs have close association with free-living wild animals as well as with humans. Comparison of the seroprevalences between hunting dogs and game animals can help to focus on possible circulation of *Borrelia* between these animals. In addition, dog has been identified as sentinel and competent reservoir for *B. burgdorferi* sensu stricto [2,3]. Some of the major obstacles in serological study in wild animals are unavailability of secondary antibodies, lack of sizable positive and negative controls, laborious capturing of wild animals for sample collection, etc. In Europe few studies have used ELISA or similar tests in deer [1,4,5]. On the other hand, reports on Lyme disease serosurvey and/or standardization of ELISA like assays in mouflons are very rare [6]. In our past studies we observed considerable levels of antibBorrelial antibodies in game animals [6,12].

ELISA is much preferable than various assays like heamagglutination test owing to its sensitivity and specificity. Some studies have effectively used Protein A or G conjugates in ELISA [7–9]. Recently, Protein A or G-ELISA has been used for detection of various infectious diseases including Lyme borreliosis in wild animals [10,11]. Binding capacity of Protein A and G to mammalian immunoglobulins differs from species to species [10].

In Slovakia mainly three members of the group *B. burgdorferi* sensu lato namely *B. burgdorferi* sensu stricto (*B. burgdorferi* s.s.), *B. garinii* and *B. afzelii* are present [13,14]. Because of the genospecies diversity in Slovakia we used all three Borrelial species for antigen preparation and different antigen based ELISAs were tested to compare effect of *Borrelia* genospecies on sensitivity of the assay. Here we report development and validation of Protein A/G-ELISA with three different Borrelial antigens in Lyme disease seroprevalence in deer, mouflons and hunting dogs.

2. Materials and methods

2.1. Serum samples

Blood samples from 49 fallow deer, 51 mouflons and 138 hunting dogs were collected during the year 2001 and 2002 from different wooded areas of Eastern Slovakia. Blood samples from hunting dogs were collected from villages located around

forests. Wild animals were trapped in wooden traps specially designed for large ungulates. Known positive and negative control sera for deer and mouflon were provided from our previous study (1998–2000) in Slovakia [6]. These sera were tested by commercial (BAG—Biologishe Analysensystem GmbH) indirect haemagglutination test (IHT). Positive dog sera were collected from those showing clinical signs and suspected for Lyme disease. Dog sera were tested by IHT as well as by common ELISA as a part of other study [15].

2.2. Antigens

B. burgdorferi s. s. and *B. garinii* isolated from Eastern Slovakia and characterized by PCR-Reverse line blot (data not published) were used for antigen preparation. For *Borrelia afzelii* antigen preparation, PKo (OspA serotype 2) strain was used. All the three strains were grown in BSK-H complete medium (Sigma) with 6% rabbit serum at 33 °C. Well-grown cultures were centrifuged at 20,000*g* for 30 min. at 10 °C. The pellets were washed three times with phosphate buffer saline (PBS, 7.4 pH) plus $MgCl_2$ (5 mM). Whole cell antigens were prepared by sonicating the pellets at 20 KHz for 3 min. on ice (Sonic Dysmembrator, Dynatech, UK). Sonicated antigens were diluted in phosphate buffer saline and stored at −70 °C. Protein concentration of the diluted antigens was measured by Genesys 5 (Spectronic Instruments, USA). Protein concentration for plate coating was fixed to 10 μg/ml. Microtiter plates were coated with different antigens (100 μl/well), incubated overnight at room temperature and then washed three times with PBS-Tween 20 (Tween 20–0.05%). Washed plates were air dried, packed and stored at 4 °C until use.

2.3. Standardization of binding ability—antibody and Protein A/G conjugate

To check the binding ability of new Protein AG horseradish peroxidase conjugate (Prozyme, USA) to deer, mouflon and dog antibodies, binding assay was carried out as described by Stobel et al. [10]. The details of antibody-Protein A/G conjugate binding assay are shown in Fig. 1. Based on the binding assay results, the optimum dilution combination was determined for each animal species. Serum dilutions were kept as 1:300 for dog and 1:100 for deer and mouflons. The optimum conjugate dilutions were 1: 2700 for dog and 1:900 for deer and mouflons.

2.4. Protein A/G-ELISA

Nonspecific binding sites in antigen coated plates were blocked by addition of 100 μl/well of 1% bovine serum albumin (Sigma). Diluted serum samples of deer, mouflons and dogs were added in duplicate (100 μl/well) in each microtiter plate. Positive and negative controls sera were diluted accordingly. After incubation for 1 h at 37 °C, plates were washed thrice with PBS–Tween20. Protein A/G conjugate was added (100 μl/well) with suitable dilutions (in PBS–Tween20) as determined in binding assay for each animal species. Plates were incubated for 1 h at 37 °C and washed thrice with PBS-Tween20. ABST

- Dilution of sera from each animal species (1:100 to 1:8100); 3 individuals each; in coating buffer (50mM Sodium carbonate buffer, pH 9.6).
- Coating of diluted sera in triplicate (100 μl/well) in microtiter plates – Overnight incubation at room temperature.
- Washing of plates, 3 times with PBS-Tween 20.
- Addition of 100 μl of diluted conjugate per well (1:300, 1:900, 1:2700 & 1:8100 in PBS-Tween20).
- Incubation for 1 h at 37ºC.
- Washing of plates, 3 times with PBS-Tween 20.
- Addition of freshly prepared ABTS substrate 100 μl/well (160mg dissolved in 400 ml citric phosphate buffer, pH 4.3); plus 1% of 1:40 diluted H_2O_2.
- Incubation at room temperature (10, 15, 25 & 30 min.).
- Measurement of absorption with ELISA reader.
- Comparison of results with negative controls, analysis and determination of the best suitable dilution combination.

Fig. 1. Steps in antibody-Protein A/G peroxidase conjugate binding assay.

substrate (2,2′Azino-di-3-ethyl-benzothiazolin-sulfonate; Sigma) was added (100 μl/well) and plates were incubated for 25 min. Absorbance was measured at 405 nm.

2.5. Statistical analysis

Cutoff value for each animal species was measured with the help of Win-Episcope 2.0 software. Mean absorbance of each test samples was compared with corresponding cutoff value for determining positivity or negativity. Paired *t*-test (STATGRAPHICS plus 5.1) was used to assess the age and sex dependent variations. Effect of different antigens on Protein A/G-ELISA was studied with Chi square (χ^2) test.

3. Results

3.1. Antibody–protein A/G conjugate binding ability

Protein A/G conjugate diluted at 1:900 showed the best binding results with 1:100 serum dilution in deer and mouflon. In dog, the best binding conjugate—serum combination was 1:2700 and 1:300, respectively. Though the higher dilutions of conjugate as well as sera showed comparable binding ability (Table 1), optimum dilution combinations were set to obtain maximum sensitivity in actual anti-*Borrelia* antibody testing.

Table 1
Binding ability of combined Protein A/G horseradish peroxide conjugate and antibodies of different animal species under study

Animal and serum dilutions	Conjugate dilutions			
	1:300	1:900	1:2700	1:8100
Deer				
1:100	++++	++++	+++	+++
1:300	++++	+++	+++	++
1:900	+++	++	++	++
1:2700	+	+	−	−
1:8100	−	−	−	−
Mouflon				
1:100	++++	++++	+++	+++
1:300	+++	+++	++	++
1:900	+++	+ +	+	+
1:2700	+	+	−	−
1:8100	−	−	−	−
Dog				
1:100	++++	++++	++++	+++
1:300	++++	++++	++++	+++
1:900	+++	+++	+++	++
1:2700	+++	++	++	++
1:8100	+	+	−	−

Binding ability of conjugate and antibody is rated as shown above ranging from the best (++++), good (+++), poor (++) to very poor (+) and negative (−).

3.2. Antigen dependent variation in Protein A/G-ELISA

B. garinii antigen showed maximum sensitivity (90.5%) followed by *B. burgdorferi* s.s. antigen (88.25%) in deer, whereas the sensitivity obtained by PKo antigen was the least (Table 2). In contrast, PKo antigen showed better sensitivity (94.00%) in case of dogs. In dogs, Receiver operating characteristic (ROC) areas were the largest for PKo and *B. garinii*(97.32 and 97.67%, respectively), giving maximum efficacy of the test. In case of mouflons, sensitivity pattern was similar to dog showing *B. garinii* antigen most sensitive

Table 2
Antigen dependent sensitivities of Protein A/G-ELISA

	Animal species		
	Deer	Mouflon	Dog
B. garinii	90.50% (96.51%)	85.37% (92.22%)	94.29% (97.67%)
B. burgdorferi s.s.	88.25% (93.87%)	83.12% (90.32%)	92.31% (96.99%)
PKo *(B. afzelii)*	88.0% (93.03%)	84.08% (92.15%)	94.00% (97.32%)

Figures in parenthesis indicate ROC area for specific antigen based ELISA for particular animal species.

Table 3
Age and sex dependent seropositivity in deer, mouflons and dogs

Animals	Adult	Young	Male	Female	Total
Deer	56.76% (37)	8.33% (12)	47.37% (19)	43.33% (30)	44.90% (49)
Mouflon	36.59% (41)	0% (10)	26.09% (23)	32.14% (28)	29.41% (51)
Dog	35.90% (117)	0% (21)	31.31% (67)	23.94% (71)	30.43% (138)

Figures in the parenthesis indicate number of individuals (n).

followed by PKo and *B. burgdorferi* s.s. (Table 2). Overall sensitivities differed depending on specific antigen used in ELISA for different animal species, but no significant variations were noticed.

Because of the highest sensitivities as well as maximum ROC areas obtained by *B. garinii* antigen in all three animal species, we considered the results from this test for calculating seroprevalences.

3.3. Seroprevalence

Average prevalence of anti-*Borrelia* antibodies in deer during the years 2001–2002 was 44.90% whereas in mouflon it was 29.41%. In the study, only one young deer ($n = 12$) and none of the young mouflon ($n = 10$) were found positive (Table 3). No significant ($P > 0.05$) difference between male and female seroprevalences was noticed in deer and mouflon. In dogs average prevalence was 30.43%. Age dependent variation was also observed in dogs. In the study, dogs less than 1 year age had very few visits to forest resulting no positivity in this age group ($n = 21$). Breed and sex dependent variation in seroprevalence was not found in dogs.

4. Discussion

Antibody–Protein A or G binding affinity changes from species to species. Recent study [10] on different wild animal species clearly indicated the inter-species difference in binding capacity. Further, significant variation in binding affinity between domestic and wild dogs has been reported. Scanty work has been done to standardize binding capacity for mouflon and fallow deer. We used recombinant combined Protein A/G peroxidase conjugate instead of using separate proteins. Proteins A and G possess a specific affinity towards Fc region of mammalian IgG [16] which provides more sensitivity of ELISA. Nevertheless, some studies [17,18] documented the increased specificity of ELISA with these protein conjugates than commercial polyclonal anti-IgG conjugates. In our study we obtained maximum 90.50, 85.37 and 94.29% sensitivities of Protein A/G-ELISA in deer, mouflons and dogs, respectively (Table 2). In other study Protein G based ELISA [19] exhibited sensitivity of 97%. So far, Protein A or G-ELISA has been used successfully in some wild and domestic animals [20–24]. Various studies have reported the significant correlation of Protein A or G-ELISA with other routine tests like latex agglutination test

[20], Passive hemagglutination test [21], polyclonal conjugate ELISA [17], Gel diffusion test [22], etc.

The deer is described as noncompetent reservoir host [27], but its importance in eco-epidemiology of Lyme disease is immense. In the present study average seroprevalence in deer reported is consistent with our previous seroprevalence in the years 1998–2000 [6]. In Denmark specific anti-*Borrelia* antibodies in fallow deer were detected in 30% of total serum samples [26]. In Czech Republic antibodies against *B. burgdorferi* were detected in 50.70% of fallow deer by IHA [28]. In case of mouflon, Lyme disease seroprevalence in the Czech Republic was 76.50% [4]. Seropositivity detected by us in this study was 29.41%. Interestingly we observed age dependent significant ($P < 0.05$) difference in seropositivity which disagrees with findings of Juricova et al. [28]. Like other authors [26,28,29], sex dependent variation in seroprevalence was not observed in this study.

The seropositivity observed during the years 2001–2002 in dogs was lower than the positivity (40%) observed by Stefancikova et al. [30]. On the other hand, our results are in accordance with other seroepidemiological studies [31–33]. Dogs stay seropositive for much shorter period after Borrelial infection. The seropositivity lasts for approximately 1 year [34]. On the other side the seropositivity in other animals as well as in humans persists for several years. As a result, screening of dog sera for seropositivity can be good indicator of present Lyme disease risk in particular area.

In humans and dogs, Borrelial antigen dependent variation in ELISA is well documented [35,36]. According to Hauser et al. [35] variation was mainly between *B. afzelii* based test on one side and *B. burgdorferi* s.s. or *B. garinii* based test on the other side. In our study, all antigens were reacted well with antibodies of three animal species. No significant variation ($\chi^2, P > 0.05$) was observed between *B. afzelii*, *B. burgdorferi* s.s. and *B. garinii* antigens. It might be due to the sharing of common antigens by three genospecies. Antigenic variation and its effect on reactivity with mammalian antibody and host immune response is discussed elsewhere [25,35,37–39]. Furthermore, Borrelial antigen–antibody affinity can be influenced by passage of the particular strain. Low passaged isolates showed greater reactivity than high passaged isolates [39]. Nevertheless, environmental factors can influence the protein composition of *Borrelia* strains. It is better to use local and low passaged strains in seroprevalences and epidemiological studies to avoid antigenic variations and reactivity differences.

Protein A/G-ELISA proved its promising use in disease diagnosis in wild ungulates. It can be used effectively in other wild animals for Lyme disease serodiagnosis.

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