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Source: Journal of Wildlife Diseases, 40(1) : 32-41

Published By: Wildlife Disease Association

URL: https://doi.org/10.7589/0090-3558-40.1.32

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Journal of Wildlife Diseases, 40(1), 2004, pp. 32–41
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SEROLOGIC SURVEY FOR ANTIBODIES AGAINST *MYCOBACTERIUM AVIUM* SUBSP. *PARATUBERCULOSIS* IN FREE-RANGING CERVIDS FROM NORWAY

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ABSTRACT: Affinity between protein-G and immunoglobulins from red deer (*Cervus elaphus*), moose (*Alces alces*), roe deer (*Capreolus capreolus*), and reindeer (*Rangifer tarandus tarandus*) was tested in a competition binding assay. Sera from red deer, reindeer, and moose inhibited the assay less than sera from cattle (less affinity), whereas sera from roe deer showed a slightly higher affinity to protein-G than did sera from cattle. The conclusion was made that protein-G could be used instead of anti-species antibodies for these cervid species, where the aim of the screening was to look for exposure or lack of exposure to mycobacteria in the tested populations. Serologic screening of 1,373 free-ranging cervids for antibodies against *Mycobacterium avium* subsp. *paratuberculosis* was conducted. All sera were tested by a protein-G–based antigen-absorbed enzyme-linked immunosorbent assay (ELISA). Seropositive moose (10/537; 1.9%), red deer (14/371; 3.8%), roe deer (6/49; 12.2%), and semidomesticated reindeer (11/325; 3.4%) were found, whereas wild reindeer (n=91) were seronegative. In addition, the red deer sera were tested with a commercial ELISA, by which two animals tested positive and nine were suspicious of having *M. avium* subsp. *paratuberculosis* antibodies. Tissue samples and feces from 10 moose originating from a population with a clustering of seropositive animals were investigated by histology and bacteriology with negative results. Paratuberculosis has never been diagnosed in free-ranging or farmed cervid species in Norway. Thus, further studies are indicated to prove that the present findings reflect an infection with *M. avium* subsp. *paratuberculosis*.

Key words: Cervids, mycobacteria, paratuberculosis, protein-G, serology, wildlife.

INTRODUCTION

Paratuberculosis (Johne's disease) is caused by *Mycobacterium avium* subsp. *paratuberculosis*. The disease is characterized by chronic granulomatous enteritis, lymphadenitis, and emaciation in ruminants. All species of ruminants, including cervids, are assumed to be susceptible to infection (Manning and Collins, 2001). Fecal shedding from an infected animal and ingestion of the bacteria through contaminated food or water is the typical route of transmission. *Mycobacterium avium* subsp. *paratuberculosis* is relatively resistant against environmental factors (Lovell et al., 1944; Chiodini and Van Kruiningen, 1983) and the possible role of wild ruminants and other wildlife in the epidemiology of paratuberculosis has been addressed (Greig et al., 1997, 1999; Pavlik et al., 2000; Beard et al., 2001). In Norway, there are large populations of free-ranging cervids, including moose (*Alces alces*), red deer (*Cervus elaphus*), roe deer (*Capreolus capreolus*), and wild and semidomesticated reindeer (*Rangifer tarandus tarandus*), which often share pasture with cattle, sheep, and goats. Mycobacterial infections have not been diagnosed in Norwegian cervids, except for a few cases of *M. avium* subsp. *avium* infection in wild roe deer (Anonymous, 2002).

Paratuberculosis has been diagnosed in free-ranging red deer, roe deer, and fallow deer (*Dama dama*) in the Czech Republic (Pavlik et al., 2000), in fallow deer in Spain (Marco et al., 2002), and in red deer in the western Alps (Nebbia et al., 2000). Paratuberculosis also has been diagnosed in farmed red deer in Ireland (Power et al., 1993), Great Britain (Fawsett et al., 1995), Denmark (Jørgensen and Jørgensen, 1987), and Belgium (Godfroid

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et al., 2000). There is only one report indicating paratuberculosis in moose (Soltys et al., 1967), whereas no such report exists for wild reindeer. Several authors refer to this disease in semidomesticated reindeer in Yakutia in Russia, where it was first recognized in the 1950s (Strogov, 1973; Syroechkovskii, 1995).

In domestic ruminants in Norway, paratuberculosis is only significant in goats. Approximately 50,000 goats produce approximately 20 million liters of milk annually, and paratuberculosis occurs endemically in goats in western Norway. The disease is controlled by vaccination, from 1967 to 2001 with a live vaccine (Saxegaard and Fodstad, 1985), and since 2001 with an inactivated vaccine (García Marín et al., 1999). In cattle, clinical paratuberculosis has occurred only sporadically, with no cases since 1979 (Djønne et al., 2001). In the period 1996–99, sera from 9,456 cattle from 754 herds were tested for antibodies against *M. avium* subsp. *paratuberculosis* as part of a national surveillance and control program for paratuberculosis. Antibodies were found in 728 animals (7.7%) originating from 40% of the herds (both milking cows and beef cattle). In an investigation of 4,038 fecal samples originating from 368 herds with seropositive animals, *M. avium* subsp. *paratuberculosis* was isolated from 0.1% of the animals. In an investigation of samples from small intestine, ileocecal valve, and mesenteric lymph nodes from 535 seropositive animals, 11 animals (2%) from five herds had histopathologic lesions compatible with paratuberculosis, and *M. avium* subsp. *paratuberculosis* was isolated from six of these animals. Cases that were confirmed by bacteriology originated from five different herds, of which four were beef cattle herds containing animals that had been imported from Denmark and Finland (Djønne et al., 2001). In a case-control study carried out in dairy cattle herds, one of the most important factors linked to high antibody titers was found to be red deer accessing the pasture (Fredriksen, pers. comm.).

The aim of the present study was to examine, by means of a protein-G–based enzyme-linked immunosorbent assay (ELISA), whether there are indications of *M. avium* subsp. *paratuberculosis* infection in Norwegian populations of wild cervids.

MATERIALS AND METHODS

Sampling

Serum samples from wild moose, red deer, roe deer, and wild and semidomesticated reindeer were obtained from various locations according to Table 1 and Figure 1. The sampling was carried out during hunting or chemical immobilization (wild animals) and at slaughter (semidomesticated reindeer). Of the moose sampled, 94 were calves, 12 were 1–2 yr old, and 414 were adults; age was not estimated for 17 animals. Of the red deer, 72 animals were calves, 89 were 1–2 yr old, and 195 were adults; age was not estimated for 16 animals. Eleven of the roe deer were calves, 10 were 1–2 yr old, and 26 were adults; age was unknown for two animals. All of the 325 semidomesticated reindeer tested were adults; age was not registered for the wild reindeer.

Protein-G affinity

Protein-G is described as a feasible conjugate for the detection of antibodies from a wide range of animal species, and is used in many serological assays. To address the affinity between protein-G and immunoglobulins from the different cervid species tested, a competition binding assay was used. Rabbit immunoglobulin G (IgG) was prepared as described previously (Tryland et al., 1998). Flat-bottomed microtiter plates (Nunc Immuno Polysorp, NUNC A/S, Roskilde, Denmark) were coated with 100 μl of coating buffer (0.06 M sodium carbonate buffer, pH 9.6) containing 1 μg/ml of rabbit IgG and incubated overnight at 4 C. The plates were washed five times with phosphate-buffered saline containing 0.1% Tween-20, pH 7.2 (PBS-T; repeated between steps), and blocked with 100 μl of PBS-T containing 1% bovine serum albumin (BSA; Sigma Chemical Co., St. Louis, Missouri, USA) and incubated at room temperature for 2 hr. Serum samples, obtained from 10 apparently healthy individuals each of moose, deer, roe deer, reindeer, polar bear (*Ursus maritimus*), and humans, were diluted twofold (1:10–1:655,360) in PBS-T with 0.02 μg/ml of recombinant biotinylated protein-G (Sigma-Aldrich AS, Oslo, Norway) and incubated at room temperature for 1 hr. The serum/protein-G mixture was add-

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TABLE 1. Number and origin of samples from moose, red deer, roe deer, and semidomesticated and wild reindeer and the seroprevalence of mycobacterial antibodies in Norway.

Species	Samples[a]	Location[b]	Number seropositive/number tested (%)[c]		
			Antigen-absorbed ELISA ≥0.15	HerdChek™ ≥0.15	HerdChek™ ≥0.30
Moose (*Alces alces*)	1992–99, chemical immobilization	1. Aust-Agder	1/51 (2.0)		
		2. Hedmark (central)	0/64 (0)		
		3. Hedmark (north)	0/65 (0)		
		4. Sør-Trøndelag (south)	0/45 (0)		
		5. Nordland (Vega)	8/99 (8)		
		6. Nordland (Beiarn)	0/80 (0)		
		7. Troms (central)	1/133 (0.8)		
		Total (moose)	10/537 (1.9)	NT	NT
		Total (moose >1 yr)	9/426 (2.1)		
Red deer (*Cervus elaphus*)	1998, hunters	8. Sogn og Fjordane (Flora)	9/119 (7.6)	4/119 (3.4)	1/119 (0.8)
		9. Sogn og Fjordane (Gloppen)	1/86 (1.2)	3/87 (3.5)	0/87 (0)
		10. Møre og Romsdal (Halsa)	0/48 (0)	1/48 (2.1)	0/48 (0)
		11. Møre og Romsdal (Aure)	1/51 (2)	3/51 (5.9)	1/51 (2)
		12. Sør-Trøndelag (Hitra)	3/67 (4.5)	0/67 (0)	0/67 (0)
		Total (red deer)	14/371 (3.8)	11/372 (3.0)	2/372 (0.5)
		Total (red deer >1 yr)	14/299 (4.7)	11/300 (3.7)	2/300 (0.7)
Roe deer (*Capreolus capreolus*)	1997, hunters	13. Møre og Romsdal (Aukra)	6/49 (12.2)	NT	NT
		Total (roe deer >1 yr)	6/38 (15.8)		
Reindeer, wild (*Rangifer tarandus tarandus*)	1996, hunters	14. Hedmark (Rendalen)	0/91 (0)	NT	NT
Reindeer, semidomesticated (*Rangifer tarandus tarandus*)	1994, slaughterhouse	15. Troms (north) and Finnmark (west)	1/25 (4)		
		16. Finnmark (east)	10/300 (3.3)		
		Total (semidomesticated reindeer)	11/325 (3.4)	NT	NT
Total (all individuals)			41/1.373 (3.0)		

[a] Date and source of blood samples.
[b] Location number corresponds with Figure 1.
[c] ELISA = enzyme-linked immunosorbent assay; NT = not tested.

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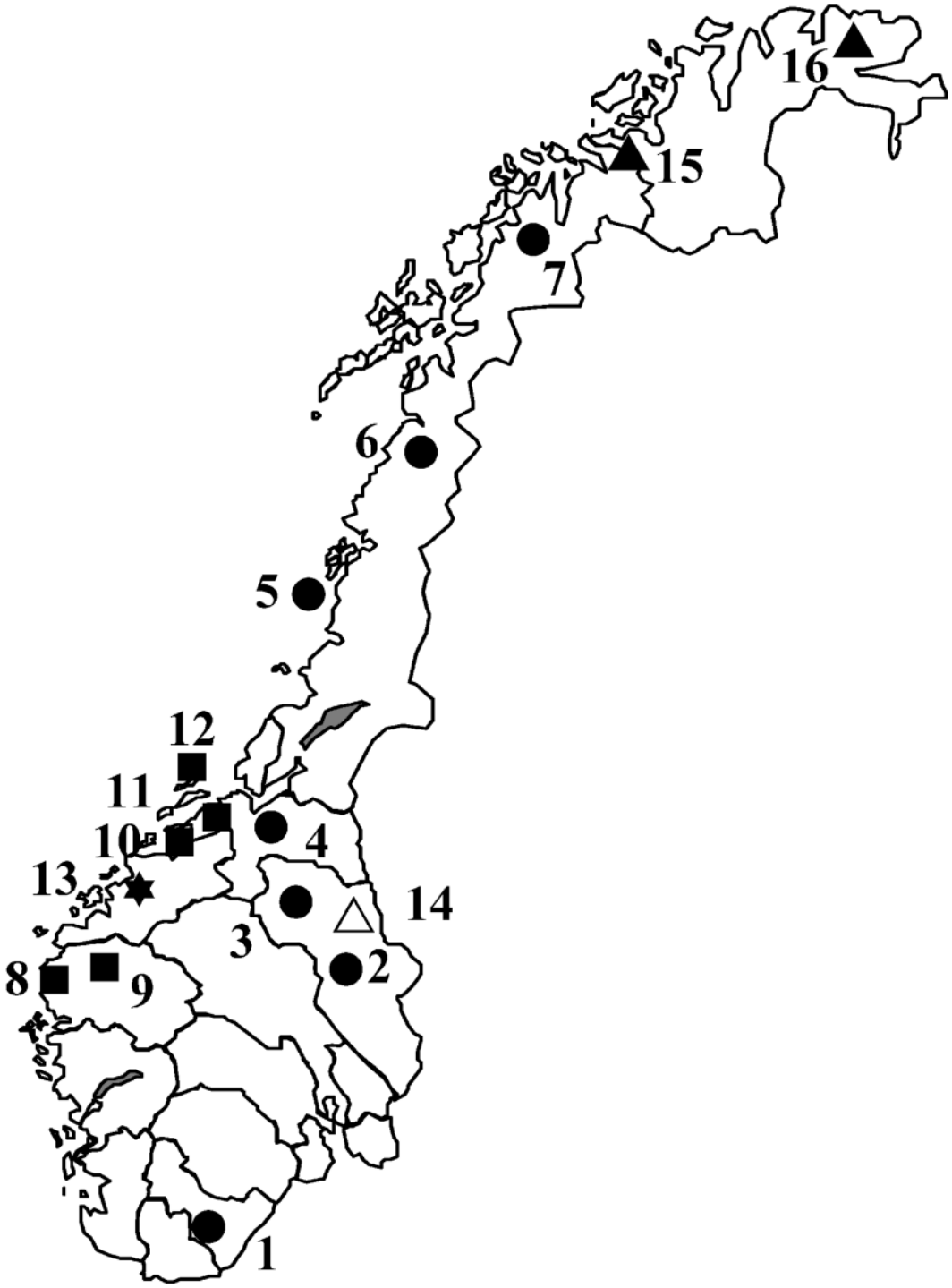

FIGURE 1. Sampling locations (county) for free-ranging cervids from Norway: 1, Aust-Agder; 2, Hedmark (central); 3, Hedmark (north); 4, Sør-Trøndelag (south); 5, Nordland (Vega); 6, Nordland (Beiarn); 7, Troms (central); 8, Sogn og Fjordane (Flora); 9, Sogn og Fjordane (Gloppen); 10, Møre og Romsdal (Halsa); 11, Møre og Romsdal (Aure); 12, Sør-Trøndelag (Hitra); 13, Møre og Romsdal (Aukra); 14, Hedmark (Rendalen); 15, Troms (north) and Finnmark (west); 16, Finnmark (east). Symbols: ● = moose, ■ = red deer, ★ = roe deer, △ = wild reindeer, ▲ = semidomesticated reindeer.

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ed and the plates were incubated at room temperature for 1 hr, followed by streptavidin-peroxidase (Boehringer Mannheim, GmbH, Mannheim, Germany) diluted 1:10,000 in PBS-T and incubation at room temperature for 30 min. As substrate, 50 μl of orthophenylene-diamine (OPD; DAKO, Glostrup, Denmark), 2 mg diluted in 0.05 M citric acid phosphate buffer (pH 5.0) with 0.6 μl of 30% H_2O_2 per milligram of OPD, was added and incubated in the dark at room temperature for 10 min. The enzyme reaction was stopped by adding 50 μl of 2 M H_2SO_4, and the plates were subsequently read in a spectrophotometer (Multiskan EX version 1.0, Labsystems Oy, Helsinki, Finland) at 492 nm. Sera were tested separately (intraspecies variation) and subsequently pooled by species (interspecies variation).

Serum from cattle and humans was included to have a reference to the use of the ELISA kit designed for cattle and as a reference to previously reported investigations on protein-G, respectively (Åkerström and Björck, 1986). Polar bear serum was included as a species of which immunoglobulins have restricted affinity to protein-G (Tryland et al., 2001).

Antigen-absorbed ELISA

An antigen-absorbed ELISA, based on antigens from *M. avium* subsp. *paratuberculosis* absorbed on antiserum against *M. avium* subsp. *avium* (Olsen et al., 2000, 2001) was carried out on all serum samples. Briefly, flat-bottomed 96-well microtiter plates (Nunc Immuno Polysorp, NUNC A/S) were coated with 100 μl of 0.06 M sodium carbonate buffer (pH 9.6) containing 0.5 μg of antigen per milliliter at 4 C. Plates were washed five times (repeated between all steps in the ELISA) with PBS-T and blocked with 150 μl of PBS-T containing 1% BSA (Sigma Chemical) at 20 C for 1 hr. A serum dilution of 1:600 was chosen, based on the affinity titration curve for protein-G. Serum samples were diluted in PBS-T and 100 μl was added and incubated at 20 C for 1 hr. Biotinylated protein-G (Sigma-Aldrich AS) diluted 1:15,000 in PBS-T was added (100 μl/well) and incubated at 20 C for 1 hr. Streptavidin-peroxidase (Boehringer Mannheim) diluted 1:10,000 in PBS-T was added and incubated at 20 C for 30 min. As substrate, 50 μl of OPD (DAKO) diluted in citric acid phosphate buffer (pH 5.0; 0.66 mg/ml) with 0.6 μl of 30% H_2O_2 per milligram of OPD was added and incubated in the dark at 20 C for 10 min. The enzyme reaction was stopped by adding 50 μl of 2 M H_2SO_4. The plates were read in a spectrophotometer (Multiskan EX version 1.0) at 492 nm.

As positive control sera, one sample obtained from a Norwegian cow and one from a farmed red deer from Belgium, both with clinical paratuberculosis, were used. The cow and the deer were culture positive on feces and organs, respectively, and sera from both animals tested positive in a commercial paratuberculosis ELISA kit (HerdChek™, IDEXX Laboratories, Inc., Scandinavia AB, Öserbybruk, Sweden) (Godfroid et al., 2000). As negative control serum, one serum sample from a Norwegian cow and one from a farmed red deer from Belgium were used. Both animals tested negative in the commercial ELISA kit, and the deer also was culture negative on feces and tested negative in a skin test and in a lymphoproliferation test (Godfroid et al., 2000). All serum samples were tested in duplicate. Blank wells were added serum dilution buffer only and were treated as the test wells. By using the optical density (OD) ratio (mean OD of sample/mean OD of positive control), the OD value for each test serum was adjusted relative to the positive control on the respective plate, compensating for variation between plates. To assess the variability of the method, inter- and intraassay coefficients of variation (coefficient of variation = standard deviation×100/mean) were calculated based on the OD values for the positive control serum on each plate (Kemeny and Chantler, 1988). Samples with an OD ratio ≥0.15 were considered as seropositive.

Commercial ELISA kit

The serum samples from red deer also were tested by a commercial test kit designed for screening cattle for paratuberculosis (HerdChek, IDEXX), by using protein-G with horseradish peroxidase as conjugate. As recommended for cattle, an OD ratio (OD sample − mean OD negative control/mean OD positive control − mean OD negative control) ≥0.3 was defined as seropositive, whereas a ratio between ≥0.15 and <0.3 was regarded as suspicious. Each sample was tested once, whereas samples with OD ratio ≥0.15 were retested in duplicate, with the final OD ratio being based on the mean of the two retest values.

Histopathology and bacteriology

Ten moose, five calves, two 1–2 yr old, and three animals >10 yr old shot in 1999 during ordinary hunting at Vega, a 119-km^2 island in Nordland County (65°40′N, 11°55′E; location 5) , were sampled for pathologic and bacteriologic examination. Sampling included fresh and formalin-fixed tissues from medial and distal jeunum, ileum, ileocecal junction, jejunal and cecal lymph nodes, and feces. Histologic sections were prepared from the formalin-fixed tis-

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TABLE 2. Comparison of affinity between protein-G and immunoglobulins from cervids, humans, and polar bears in a competition binding assay (sera diluted 1:640).

Species		Mean OD[a]	Mean OD/mean OD cattle	Percent inhibition of protein-G[b]
Roe deer	(*Capreolus capreolus*)	0.34[c]	0.89	112
Cattle		0.38	1	100
Human		0.39	1.02	98
Moose	(*Alces alces*)	0.51	1.34	75
Reindeer	(*Rangifer tarandus tarandus*)	0.55	1.45	69
Red deer	(*Cervus elaphus*)	0.65	1.71	58
Polar bear	(*Ursus maritimus*)	1.24	3.26	30

[a] OD = optical density.
[b] Percent inhibition compared to cattle.
[c] Highest affinity (strongest inhibition).

sues by following standard procedures and were stained with hematoxylin-eosin and Ziehl-Neelsen (ZN) stain, and examined by light microscopy for pathologic lesions and presence of acid-fast bacteria. Fresh tissue samples were homogenized and decontaminated with 4% sodium hydroxide and 5% oxalic acid with 0.1% malachite green (Jørgensen, 1982), and, like the fecal samples, were inoculated onto selective and nonselective Dubos medium with mycobactin (2 μg/ml) and pyruvate (4 mg/ml) (Saxegaard, 1985). The samples were incubated at 37 C for 16 wk and examined for growth of mycobacteria by ZN staining.

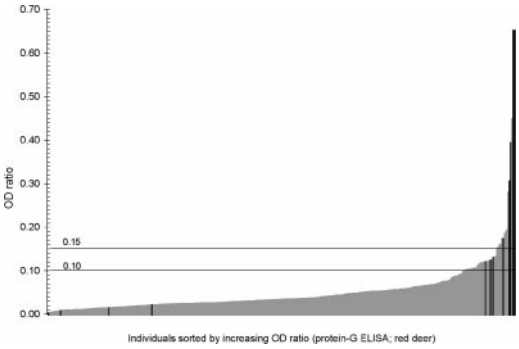

FIGURE 2. Distribution of optical density (OD) ratio values for red deer (n=271) in the antigen-absorbed enzyme-linked immunosorbent assay (ELISA), compared with the results from the commercial ELISA kit (HerdChek, IDEXX). Each black bar represents a serum sample defined to be positive or suspicious in the commercial ELISA kit (OD ratio≥0.15). The horizontal lines represent two different OD ratio cutoff values for the antigen-absorbed ELISA.

Statistical methods

Agreement between the commercial ELISA kit (HerdChek, IDEXX) and the antigen-absorbed ELISA (red deer) was tested by calculating kappa (Martin et al., 1987).

RESULTS

The intraspecies variation in affinity between immunoglobulins and protein-G was low (data not shown), whereas the interspecies variation (pooled sera) was moderate and affinity generally were more comparable between species, including cattle and humans (Table 2). Roe deer sera demonstrated a slightly higher affinity to protein-G compared to cattle, whereas sera from moose, reindeer, and red deer showed lower affinity to protein-G than did cattle sera.

The results of the antigen-absorbed ELISA are shown in Table 1. Roe deer had the highest proportion of seropositive animals (6/49; 12.2%), with two of the seropositive animals being 1.5 yr old and four being adults. The arrays of OD ratios for red deer tested with the antigen-absorbed ELISA are shown in Figure 2. Red deer had a relatively large variation in seroprevalence, which was highest at location 8 (7.6%). Of the 14 seropositive red deer, 12 were adults and two were 1.5 yr old. Moose had a seroprevalence of 1.9%, with samples from most areas being negative, with a notable exception of Vega (lo-

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cation 5). On the island Vega, eight (8%) of 99 individuals were seropositive, including six adults, one 1.5-yr-old animal, and one calf. Of the 325 semidomesticated reindeer, 11 animals (3.4%) were seropositive, of which 10 were from the western region of the pasture area (location 15). All the wild reindeer were seronegative.

Of red deer tested with the commercial ELISA, nine were classified as suspicious (OD ratio≥0.15), whereas two were classified as seropositive (OD ratio≥0.3). Seven of these 11 animals were also seropositive in the antigen-absorbed ELISA with a cutoff value of 0.10 (as used for cattle), whereas four were seropositive with a cutoff value of 0.15 (Fig. 2). The calculated agreement (kappa) between the commercial test kit (sample/positive [S/P] ratio cutoff≥0.15) and the antigen-absorbed ELISA (OD ratio cutoff 0.15) was 0.5. Based on the positive control on each plate, the coefficient of variation between the different plates was found to be 9.9%, whereas the mean coefficient of intraplate variation was 2.9%.

Histologic examination of the intestine and intestinal lymph nodes from the 10 moose from Vega revealed no lesions consistent with paratuberculosis, and results of cultivation for mycobacteria also were negative.

DISCUSSION

The affinity between protein-G and immunoglobulins from roe deer and red deer has been reported (Deruaz et al., 1996). A recent report stated that immunoglobulins from white-tailed deer and elk have approximately the same affinity to protein-G as cattle, and that protein-G does not necessarily bind immunoglobulins uniformly in closely related species (Kramsky et al., 2003). This necessitates affinity investigations on the actual species to be screened. Results from our investigation indicated that it was feasible to use protein-G instead of anti-species antibodies for red deer and roe deer as well as moose and reindeer, although some interspecies variation was observed (Table 2). To establish serologic tests for determination of seroprevalences in a population, this interspecies variation must be taken into consideration and the tests may have to be validated for each species separately. In the paratuberculosis screening reported here, where the aim of the study was to look for indications of exposure of deer species to mycobacteria, the species-related affinity variation was regarded to be of minor importance. Roe deer showed almost the same affinity as cattle, whereas red deer, moose, and reindeer demonstrated less affinity to protein-G than did cattle, which indicates that the difference in affinity between immunoglobulins and protein-G may lead to a generally lower detection of bound immunoglobulins to the antigen as compared to that in cattle, which may lower the sensitivity of the serological test when cutoff values established for cattle are used. To adjust for the fact that roe deer sera showed a higher affinity to protein-G than cattle, we have used an OD ratio cutoff of ≥15 for the antigen-absorbed ELISA instead of a cutoff of ≥10 as previously used for this test for cattle sera (Olsen et al., 2001), which is expected to raise the specificity of the multispecies screening.

Discrepancy between the antigen-absorbed ELISA and the commercial ELISA for the red deer sera (Fig. 2) may be explained by the fact that in the commercial ELISA, the test serum was preabsorbed on antigen from *M. phlei,* whereas in the antigen-absorbed ELISA the antigen was absorbed against antibodies against *M. avium* subsp. *avium* in a column (Olsen et al., 2001). These are two different approaches to deal with the serologic cross-reactivity between species of mycobacteria, of which several are regarded as environmental bacteria, being associated with birds, water, feed, and soil and only sporadically infecting mammals (Thorel et al., 2001). However, *M. avium* subsp. *avium* has been isolated from lymph nodes and lesions in farmed deer (Wards et al.,

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1991; de Lisle et al., 1996) and has caused high mortality in these animals (Mackintosh et al., 1999). A focus on *M. avium* subsp. *avium* is thus assumed to be relevant when dealing with wildlife investigations, which supports the method chosen in the antigen-absorbed ELISA, where antigens shared between *M. avium* subsp. *paratuberculosis* and *M. avium* subsp. *avium* are removed from the coat. The fact that seven of the 11 animals that were classified as suspicious or seropositive on the commercial ELISA kit also were seropositive in the antigen-absorbed ELISA supports the idea that the measured antibody titer may be a result from exposure to *M. avium* subsp. *paratuberculosis.*

The highest seroprevalences among cervids in Norway were found in roe deer and red deer from western Norway, where paratuberculosis is endemic in goats. These results may indicate a connection between mycobacterial infections in wildlife and domestic animals. This also was recently suggested in a case-control study, in which access of red deer to pastures was found to be one of the most important factors connected with high mycobacterial antibody titers in diary cattle (Fredriksen, pers. comm.). No seropositive wild reindeer were found, whereas 3.4% of the semidomesticated reindeer in Troms and Finnmark were seropositive. Semidomestic reindeer are subjected to seasonal relocations between summer and winter pastures, and they also may have contact with other reindeer herds as well as domestic animals. However, no contact occurs between reindeer across the border between Russia and Norway, and paratuberculosis is to our knowledge not diagnosed in reindeer in northwestern Russia. In the moose population at the island Vega (location 5), consisting of 20–30 animals during the sampling period (1992–99), antibodies were found in eight (8%) of 99 animals sampled during this period, whereas the 10 animals shot in 1999 were all negative (by serology, bacteriology, and histopathology).

With the serologic findings reported here, free-ranging cervids in Norway, with the possible exception of wild reindeer, likely are exposed to mycobacteria. Whether these mycobacterial infections provoke pathologic conditions is not known, except for a few diagnosed cases of *M. avium* subsp. *avium* infection in roe deer (Anonymous, 2002). The possibility exists that an exchange of mycobacterial agents occurs between wild cervids and livestock, and that the results achieved in the surveillance program for paratuberculosis in domestic ruminants in Norway are influenced by exposure from a wildlife reservoir. Further efforts should be made to isolate and characterize the agent(s) causing mycobacterial seroconversion in Norwegian cervids, and, because of the broad host range of *M. avium* subsp. *paratuberculosis*, other wildlife species also should be taken into consideration.

ACKNOWLEDGMENTS

We thank I. A. Heffernan and K. B. Soleim for excellent laboratory work. J. Godfroid and T. Tollersrud are acknowledged for providing positive and negative control sera from farmed red deer and from cattle, respectively. We also thank R. Müller and the moose hunters at Vega for their cooperation. The help of M. Heim during field work at Vega is acknowledged.

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Received for publication 4 April 2003.

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