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Serosurvey for antibodies against *Salmonella* species in free-ranging moose (*Alces alces*) from Norway

Serologische Untersuchung auf Antikörper gegen *Salmonella* spezies in frei lebenden Elchen (*Alces alces*) in Norwegen

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Abstract: An indirect ELISA was developed as a tool for surveillance of antibodies against *Salmonella* sp. in free-ranging moose (*Alces alces*) in Norway. Serum samples from 303 clinically healthy moose sampled between 1993–2000 were examined. Anti-*Salmonella* antibodies were detected in samples from 6 individuals (1.98 %). This is the first evidence of *Salmonella*-seropositive free-ranging moose. Possible sources and transmission routes of *Salmonella* comprising environment, wildlife and man are discussed.

Keywords: *Salmonella* sp., moose, free-ranging, LPS-mix-ELISA

Zusammenfassung: Ein indirekter ELISA wurde entwickelt zur Bestimmung von Antikörpern gegen *Salmonella* spp. in frei lebenden Elchen (*Alces alces*) in Norwegen. Serumproben von 303 klinisch gesunden Elchen, entnommen in den Jahren 1993–2000, wurden untersucht und Anti-*Salmonella*-Antikörper wurden in 6 Individuen nachgewiesen (1,98 %). Dies ist der erste Nachweis von *Salmonella*-seropositiven frei lebenden Elchen. Mögliche Ursprünge und die Übertragungswege von Salmonellen werden diskutiert unter Berücksichtigung von Habitat, Wildtieren und dem Menschen.

Schlüsselwörter: *Salmonella* sp., Elch, frei lebend, LPS-mix-ELISA

Introduction

Salmonella sp. are opportunistic pathogens that can infect a wide range of hosts, including man (Morse and Duncan, 1974; Murray, 1991). Salmonellosis is one of the major health problems in many developing and industrialized countries (Bredal and Langeland, 1993). In the past decades there has been an increased number of *Salmonella* infections (Kapperud et al., 1998 a), implying a significant socio-economic impact. Food originating from domestic animals contaminated with *Salmonella* is the most important source for human infections (Hartung, 1993). Direct contact with animals is also a significant factor associated with enteric diseases in man (Fang et al., 1991), and has been documented in domestic animals (Bruner and Moran, 1949), pets (Fang et al., 1991; Torfoss and Abrahamsen, 2000; Gaulin et al., 2002), wild mammals (Thomas et al., 2001) and birds that may act as reservoirs (Coulson et al., 1983; Bredal and Langeland, 1993; Tauni and Österlund, 2000). However, as the occurrence of potential pathogenic bacteria in wildlife (Gates et al., 1995) and in the environment (Lindqvist et al., 2000), and its impact on human health, is difficult to asses, it is often underevaluated in public health risk assessments. There are several reports on potential transmission of *Salmonella* from wildlife to man (Aavitsland and Hofshagen, 1999; Thomas et al., 2001; Handeland et al., 2002); however information on the direct or indirect transmission of *Salmonella* from moose (*Alces alces*) or other wild ruminants to man seems to be absent, even though the occurrence of *Salmonella* has been reported quite frequently in diseased or clinically healthy, free-ranging or captive wild ruminants worldwide, such as elk (*Cervus elaphus nelsoni*) (Foreyt et al., 2001), red deer (*Cervus elaphus*) (Wetzel and Rieck, 1962; Nowotny and Hasitschka, 1976; McAllum et al., 1978), reindeer (*Rangifer tarandus tarandus*) (Kuronen et al., 1998), sika deer (*Cervus nippon*) (Sato et al., 2000), white-tailed deer (*Odocoileus virginianus*) (Cox, 1950; Debbie, 1968; Robinson et al., 1970) and blue wildebeest (*Counochaetes taurinus*) (Cameron et al., 1963). Therefore, a possible transmission of salmonellosis from moose to man cannot be excluded. For moose, only one report on the occurrence of *Salmonella* in an animal kept in a zoological garden from Germany (Selbitz et al., 1978) is known; however, no information is available from free-ranging moose.

The aim of this study was to contribute to the evaluation of the importance of *Salmonella* sp. in moose, consisting of development of methodology and investigation of antibodies against *Salmonella* sp. in moose in Norway. This study was part of a larger project to evaluate the importance of *Salmonella* sp. in northern wildlife and its impact on public health (Aschfalk et al., 2002 a, b).

Materials and methods

Experimental design

An indirect Enzyme-linked Immunosorbent Assay (ELISA) was developed for detecting antibodies against *Salmonella* sp.

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in sera from moose. To cover a broad spectrum of *Salmonella (S.)* serogroups, a mixture of the lipopolysaccharides (LPS) from *S.* Typhimurium (somatic antigen O: 1, 4, 5, 12) and *S.* Choleraesuis (somatic antigen O: 6, 7) was used as antigen. Chicken anti-moose-Immunglobulin-horseradisch immunoconjugate (Ig-PO) served as the immunoconjugate. The ELISA was based on the procedure described by Carlsson et al. (1972). For further details on the solution and buffers used see Staak et al. (2000).

Screening of serum samples

Serum samples from 303 free-ranging moose, were examined in the described LPS-mix ELISA. The samples were collected from clinically healthy adult moose of both genders that were chemically immobilized for other scientific purposes. Sampling was carried out from 1993 to 2000 in six different areas of Norway: Vega (an island in Southern part of Nordland county), Nord-Østerdal (several communities in Northern part of Hedmark county), Beiarn (a community in Northern part of Nordland county), Bardu (a community in interior Troms county), Meldal (a community in southern part of Sør-Trøndelag county) and Stor-Elvdal (a community in central part of Hedmark county).

Negative and positive control sera

Sera from 19 adult moose, which were *Salmonella* culture-negative and had no clinical signs of salmonellosis, were used as negative controls. As no sera from naturally infected animals were available, positive control sera were obtained by immunization of 5 captive, *Salmonella* culture-negative adult moose with an inactivated *S.* Typhimurium and *S.* Dublin vaccine (Salmo Shield TD®, Novartis Animal Vaccines Inc., Overland Park, Kansas, USA). All animals used as positive controls were born in captivity at the Kenai Moose Research Center, Alaska, a facility with no documented occurrence of salmonellosis. All procedures followed guidelines for animal care and use adopted by the Alaska Department of Fish and Game, Division of Wildlife Conservation. During the trial, animals were kept in a 10-ha enclosure that prohibited contact with other moose and were offered a formulated ration (Schwartz et al., 1985) and water ad libitum. All five moose were clinically healthy during the experiment.

Animals were immobilized on days 0, 14 and 28 with a mixture of carfentanil citrate and xylazine HCl (Schmitt and Dalton, 1987) delivered by a dart rifle. Anesthesia was reversed with naltrexone and tolazoline HCl (Schwartz et al., 1997). Faecal samples were obtained from the rectum on days 0 and 14 and blood was sampled by jugular venipuncture during all immobilizations. Immunizations were administered on days 0 and 14 under guidelines provided by the manufacturer of the bacterin (Grand Laboratories, Freeman, SD, USA). The antibody titre against *Salmonella* sp. was measured and the sera with the highest titres were pooled for use (positive control serum). The appropriate dilutions of LPS, chicken anti-moose-Ig-PO and serum were determined by checkerboard titration.

Culture procedure

The standard procedures of the Norwegian Veterinary Institute were adopted for isolation of *Salmonella* sp. Faecal samples collected from the five moose just before immunization (day 0 and day 14) were cultured to confirm the absence of faecal shedding of *Salmonella*. A sample of 5 g of fecal material was cultured in buffered peptone water (1:9) (BPW) (Merck Industrikjemikalier, Oslo, Norway) and incubated at 37°C for 24 h. Selective enrichment was then done by inoculation of 0.1 ml of this pre-enrichment culture into 10 ml of *Salmonella* enrichment broth according to Rappaport and Vassiliadis (RVS-broth, Merck Industrikjemikalier) and of 1 ml of the pre-enrichment culture into 9 ml selenite-cystine enrichment broth (Merck Industrikjemikalier). After 24 h of incubation at 41.5° C, an incubation loop culture was plated both on bromthymol-blue-lactose agar and brilliantgreen-phenolred-lactose-sucrose agar (Merck Industrikjemikalier) and incubated 24 h at 37° C. The biochemical reactions of suspicious colonies were examined further.

Preparation of LPS by phenol-water extraction

The coating antigens used were LPS from *S.* Typhimurium and *S.* Choleraesuis that had been grown in nutrient broth at 37° C for 24 h. Formalin (1 % final solution) was added to inactivate the bacteria. The medium was centrifuged at 10,000 g for 30 min and the sediment was washed and centrifuged three times in phosphate-buffered saline (PBS; ph 7.3; 0.15 mol/L) (at 10,000 g for 30 min). The sediment was then suspended in 30 ml sterile distilled water and kept at 4° C for 12 h before 170 ml of acetone was added and the mixture was kept at -22° C for 1 h. The upper aqueous layer was discarded and 100 ml acetone was added to the sediment. This was repeated five times. The acetone was evaporated at room temperature in the following 12 h and 8 ml sterile distilled water was added. This mixture was heated to 65° C for 5 min. An equivalent amount of 90 % phenol was preheated to the same temperature and then added to the mixture, which was kept at 65° C for 20 min with vigorous stirring. The mixture was then placed into an ice bath for 1 h and centrifuged for 40 min at 25,000 g. The aqueous layer was dialysed (15 KDa) against 5 L distilled water for 12 h and the dialysed material was centrifuged at 5,000 g for 20 min.

Preparation of chicken-anti-moose-Ig-PO immunoconjugate

Two laying hens were subcutaneously immunized with 0.5 ml moose Ig (4.5 mg/ml) emulsified with 0.5 ml Freund's complete adjuvant. The dose was divided equally and administered at both sides of the chest. Booster immunizations were carried out on days 21 and 100, respectively. The development of titres in the egg yolk was monitored by indirect ELISA. Egg yolk antibodies (IgY) of high titre phases were extracted from the yolk by dilution and ammonium sulphate precipitation (Wallmann et al., 1990). The specific IgY-anti-moose-Ig was purified by affinity chromatography using Sepharose 4B (Pharmacia, Uppsala, Sweden) as the matrix and moose-Ig as ligand. Subsequent conjugation with horseradish peroxidase was carried out as described by Wilson and Nakane (1978).

ELISA procedure

Salmonella-LPS-carbonate buffer (9 μg/ml; pH 9.6; 0.05 mol/L) was placed into each well (50 μl) of each second row of a 96-well polystyrene microtiter plate (Nunc Polysorb, Nunc A/S Roskilde, Denmark) and the plate was incubated at 37° C for 1 h, before being washed three times using distilled water. The wells were blocked with 200 μl of the carbonate buffer containing 1 % bovine serum albumin (BSA) and the plate was incubated for 15 min at 21° C, washed once with distilled water and subsequently washed three times with PBS with 0.05 % Tween 20 (PBS-T).

Serum samples were diluted 1:8 in PBS-T/BSA-buffer (0.5 % BSA) and 50 µl was added to one well with antigen and to one well lacking antigen. The plate was then incubated at 37° C for 30 min and washed once with distilled water and three times with PBS-T. Subsequently, 50 µl of conjugate was added to each well in a dilution of 1 : 1000 in PBS-T/BSA and incubated at 37° C for 30 min. The plate was washed again and this was followed by the addition of 50 µl of freshly prepared substrate indicator system (ABTS: 2,2'-Azino-bis(3-Ethylbenz-thiazoline-6-Sulfonic Acid) to all wells and incubation at 21° C for 15 min followed. After 15 min the optical density value (OD) was measured in a photometer (Labsystems Multiskan, Biochromatic type 348, LabSystems OY, Helsinki, Finland) at 405 nm.

Correction of sample OD and definition of cut-off

Due to interplate and day-to-day variation, a reference serum (pooled sera from the immunized moose after booster vaccination) was included in duplicate on each plate. The net ODs of all control and test sera were calculated to eliminate unspecific effects. In order to take account of a possible variation in the unspecific binding of different sera, individual blank values (antigen-free wells) were subtracted from the OD of the test well (with antigen) for each serum. Every net OD of the test sera was then corrected by using a plate specific factor calculated from the ratio of the mean of the control sera ODs (positive pool) of all plates used versus the mean of the positive control sera ODs on the actual plate. The cut-off was defined as the OD equal to the mean OD + 3 SD of the 19 moose of the negative sera pool tested each on 3 different plates.

Results and discussion

Six out of the 303 sera (1.98 %) from free-ranging moose examined showed a higher OD than the cut-off (0.311) in this ELISA. Table 1 shows the OD of the control sera from culture-negative moose, from the pooled sera of the immunized moose and from the test-positive sera from the wild test population. In all regions except Vega ≥ 1 *Salmonella*-seropositive moose were documented.

This is the first ELISA developed for the indirect detection of antibodies against *Salmonella* sp. in moose. LPS from two different *Salmonella* serovars were used to cover the O-antigens found in the most common *Salmonella* serogroups isolated from animals, including all the serovars, known to the authors, which have been isolated from wild ruminants.

Salmonella has been isolated from other wild ruminants before (Foreyt et al., 2001), but this is the first report of *Salmonella*-seropositive, free-ranging moose. This finding is of some practical interest, since this species is hunted for human consumption (approximately 38,000 capita/year, a third of the total estimated Norwegian moose population) and thus a transmission to man following the food chain or by direct contact with moose cannot be excluded, as it was assumed for other bacteria (*Streptococcus* sp.) having caused skin infections in moose hunters (Herva, 1979).

In free-ranging reindeer from Norway, a seroprevalence to *Salmonella* sp. of 0.6 % was detected (Aschfalk et al., 2002 a). Even though it is difficult to compare the seroprevalences between different species determined by different assays, the elevated seroprevalence found in moose may be explained by the fact that, compared to reindeer, moose are mostly found in more southern Norwegian regions. These areas are more densely populated and having more intensified agricultural systems, presumably increasing the risk of transmission of bacteria to wildlife through agriculture, household and industry waste and sewage, faecal and manure disposal. Indeed, occurrence of bacteria such as *Salmonella* in wildlife must be seen in the context of the epidemiological situation in an area, considering public health as well as livestock health situation. Compared to the situation in central Europe, salmonellosis is of less importance in northern European countries (Hopp et al., 1999), both in animals (Bredal and Langland, 1993) and consequently in humans (Kapperud et al. 1998a). The favourable epidemiological situation in Norway may be due to (1) a relatively isolated geographical location, (2) to the cold climate, (3) strict import-regulations for animals and products of animal origin, (4) strict control and preventive measures (Bredal and Langland, 1993), and (5) the structure of animal husbandry characterized by small, scattered production units with a relatively low degree of industrialization (Sandvik and Næss, 1994).

Several outbreaks of human salmonellosis in Norway were related to occurrence of *Salmonella* in free-living animals, such as hedgehogs or several wild bird species, and in the environment, such as untreated drinking-water (Brendal and Langeland, 1993), snow and soil (Kapperud et al., 1998 b; Handeland et al., 2002). Especially free-living birds may function as effective spreaders of infectious agents through faecal contamination of the environment, including surface water (Kapperud et al., 1998b). Stagnant drinking water is considered the most commonly reported source of infection (Blood and Henderson, 1968) and ingestion of contaminated surface waters may lead to gastroenteritis from various potential human pathogens that are part oft the normal flora of wild animals (Fang et al., 1991; Murray, 1991; Polo et al., 1999). The epidemiology of a *Salmonella* infection comprising man, wildlife and the environment was shown by Aavitsland and Hofshagen (1999) who traced outbreaks of salmonellosis in man to untreated drinking water and a bird cadaver found in the water reservoir. Moose, which are regularly using water areas as their natural food resources may easily encounter contaminated water. Infections in this species may be acquired by contact with livestock (Fayer et al., 1982) as well as other free-ranging ruminants such as reindeer (Honour and Hickling, 1993). Infected, moose may serve as a potential reservoir transmitting infectious agents in a wide geographical range to other wild animals, to livestock and consequently also to man. However, it may be assumed that man and his pollution of the environment may cause transmission vice versa, posing a potential health risk to wildlife (Foster et al., 1998).

Conclusions

This is the fist study which documents the exposure to *Salmonella* sp. in free-ranging moose from Norway. Investigations on human cases of salmonellosis should include simultaneous stu-

Table 1: OD (corrected) of control sera from culture-negative moose, from the pooled sera of immunised moose and from the test-positive sera from the wild moose population

Moose samples	n sample	mean x n tested	SD
Culture-negative moose	19 x 3	0.104	0.069
Immunized moose	1 x 10	0.813	0.094
Test-positive moose	5 x 2	0.503	0.102

SD = Standard deviation

dies on wild and domestic animals in contact with the case (Thomas et al., 2001).

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