The 11th Arctic Ungulate Conference,
Saariselkä, Finland, 24-28 August, 2003.

The occurrence and prevalence of potentially zoonotic enteropathogens in semi-domesticated reindeer

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Abstract: The information about pathogens excreted by semi-domesticated reindeer (*Rangifer tarandus tarandus*) that might represent a health risk to humans and animals is insufficient. The objectives of this study are to find the occurrence and prevalence of important potentially enteropathogenic, zoonotic bacteria and parasites in reindeer. Faecal samples from clinically healthy, semi-domesticated reindeer (n=2243) from northern regions of Finland and Norway were examined for important potentially enteropathogenic bacteria (*Campylobacter* spp., *Enterococcus* spp., *Escherichia coli*, *Salmonella* spp. and *Yersinia* spp.) and parasites (*Cryptosporidium* spp.) following standard procedures. *Escherichia coli* were isolated in 2123 (94.7%), *Enterococcus* spp. in 2084 (92.9%), *Yersinia* spp. in 108 (4.8%) samples and *Campylobacter* sp., identified as *C. hyointestinalis*, in one sample only (0.04%). Neither *Salmonella* spp. nor *Cryptosporidium*-oocysts were detected. This study clearly shows that *E. coli* and *Enterococcus* spp. belong to the normal intestinal flora of healthy reindeer. However, only few of the isolated *E. coli*-strains possess genes encoding *stx1* (0.14%), *stx2* (0%), eae (0.52%) and hly_{EHEC} (0.99%), detected by PCR, that have the ability to cause health problems in humans and also animals. The isolated *Yersinia* spp. were further analysed for virulence factors, but examinations revealed no pathogenic strains. The public health risk due to excretion of important enteropathogenic microorganisms from reindeer has to be considered very low at present but a putative epidemiological threat to human health might arise when herding conditions are changed towards intensification and crowding. This study was performed as part of the EU-project RENMAN (www.urova.fi/home/renman/).

Key words: *Campylobacter*, *Enterococcus*, *Escherichia coli*, *Rangifer tarandus*, *Salmonella*, *Yersinia*, zoonosis.

Rangifer, 24 (1): 15-20

Introduction

Free-ranging animals may serve as sentinels or reservoirs for diseases in domestic livestock and man. Transmission of infectious agents to man may occur through direct contacts to free-ranging animals including cervids (Fanning & Edwards, 1991), by contamination of the environment through faecal shedding (Aavitsland & Hofshagen, 1999) or by consumption of venison (Keene *et al.*, 1997). In contrast, to domestic animals, however, the epidemiological situation in free-ranging animals and in their habitat is difficult to assess. Bacteria, such as *Campylobacter* spp., *Enterococcus* spp., *Escherichia coli*, *Salmonella* spp., *Yersinia* spp. and the parasites *Cryptosporidium* spp. are among the most important agents in causing zoonosis like enteric and other severe diseases and have been isolated from healthy and dieseased domestic ruminants (De Rycke *et al.*, 1986; Munoz *et*

al., 1996; Busato *et al.*, 1998 and 1999; Tham *et al.*, 1999). In *E. coli*, the ability to cause severe illness in humans and animals is associated to the occurrence of several virulence factors like the production of shigatoxins. Therefore the presence of shigatoxin genes can be an allusion for the virulence of certain strains, also known as shigatoxin producing *E. coli* (STEC). Besides STEC, *Campylobacter* spp., *Salmonella* spp. and *Yersinia* spp. are a problem for meat production with a high infection risk for humans consuming contaminated products. In addition, these bacteria are known to cause illness especially in young animals. Thus, a certain importance of these pathogens as well in reindeer production may not be excluded. *Salmonella* spp. (Kuronen *et al.*, 1998) have been found associated to mortality in reindeer in Finland and Sweden. *Campylobacter* spp., that have not been associated to mortality in reindeer, may also occur (Kobayashi *et al.*, 1999; Lahti *et al.*, 2001). A new genotype of *Cryptosporidium* closely related to *Cryptosporidium* (*Cr.*) *serpentis*, *Cr. muris* and *Cr. andersoni*, was isolated from 3 out of 49 examined caribous in Canada (Siefker *et al.*, 2002). Regarding *Cryptosporidium* spp. in northern European reindeer, no data is available. Ruminants, including reindeer, may be a reservoir for all these pathogens and more knowledge is required to better protect man and animals against outbreaks of diseases caused by these infectious organisms. This is of special importance as corralling of reindeer for winter feeding is increasing, eventually highering the prevalence of infectious mircroorganisms and the incidence of infectious diseases in reindeer.

Material and methods

In the RENMAN project, 2243 faeces samples from healthy reindeer, adults and calves, of both genders were examined for the occurrence of *Campylobacter* spp., *Enterococcus* spp., *E. coli*, *Salmonella* spp., *Yersinia* spp., and in addition, for the occurrence of the parasites *Cryptosporidium* spp. The samples were taken in the course of one year (June 2001 - April 2002) from Finnish and Norwegian free-ranging and corralled reindeer herds, considering parameters such as degree of intensification of husbandry, location and season. Samples were taken off the ground or per rectum from slaughter animals, sent to Kiel, Germany, directly after collection and were kept frozen (-4 °C) until further processed within max. one week.

The examination for *Campylobacter* spp. was done by inoculating 1 g faecal material into 9 ml Preston broth (Oxoid, Wesel, Germany). After 24 h incubation in a microaerophilic atmosphere (5% oxygen, 10% carbondioxide, 3% hydrogen and 82% nitrogen) at 37 °C, a loopful of the enriched suspension was plated on Preston agar (Oxoid) and incubated for 48 h under the above described conditions. *Campylobacter*-like colonies were analysed by Gram-staining, catalase and oxidase tests, and further biochemical reactions (ApiCampy, bioMérieux, Nürtingen, Germany).

To detect *Enterococcus* spp., 1 g faecal material was diluted in 9 ml glucose-azide broth (Merck, Darmstadt, Germany) and incubated for 48 h at 37 °C. A loopful broth was then spread both on kanamycin-aesculin-azide agar (Merck) and Slanetz and Bartley agar (Oxoid). After 48 h at 37 °C suspicious colonies were Gram-stained and their biochemical reactions were analysed further by catalase and oxidase tests.

Escherichia coli was isolated by adding 1 g faeces to 9 ml Gram-negative broth (Difco, Becton and Dickinson, Franklin Lakes, USA). After 24 h of incubation at 37 °C a loopful of broth was then plated onto Endo-c agar (Merck) and incubated under the above mentioned conditions for 24 h. Typical metallic shiny colonies were subcultured on blood agar (Oxoid), incubated for 24 h at 37 °C and tested for their biochemical reactions with API 20E (bioMérieux). PCR was used to detect the occurrence of shigatoxin1 and 2 genes (*stx1, stx2*), the intimin gene (*eae*) and EHEC-hemolysin gene (hly_{EHEC}). Primers were developed with help of the European Molecular Biological Library database and the oligo 6.0 software (Molecular Biology Insight, Cascade, USA) and manufactured commercially (Invitrogen, Karlsruhe,

Table 1. Primers used in PCR to amplify specific fragments from *E. coli stx1, stx2*, *eae* and hly_{EHEC} genes

Primer	Oligonucleotide sequence (5'- 3')	Size of amplified product
stx1 a stx1 b	TGT AAC TGG AAA GGT GGA GTA TAC A GCT ATT CTG AGT CAA CGA AAA ATA AC	210 bp
stx2 a stx2 b	GTT TTT CTT CGG TAT CCT ATT CC GAT GCA TCT CTG GTC ATT GTA TTA	484 bp
eae a eae b	ATT ACC ATC CAC ACA GAC GGT ACA GCG TGG TTG GAT CAA CCT	397 bp
hly a hly b	ACG ATG TGG TTT ATT CTG GA CTT CAC GTC ACC ATA CAT AT	166 bp

Germany). Base sequences and determined sizes of amplified products are shown in Table 1.

A loopful of *E. coli* colonies was diluted in 1 ml of treated saline solution (0.85 %) and heated in a thermoblock for 15 min at 100 °C. The samples were inserted in an ultrasonic bath for 3 min at 190 W and 50-60 Hz and centrifuged at 9875 g for 30 s. Five µl of the supernatant were added to one ready-to-go-bead (Amersham Pharmacia Biotech, Buckinghamshire, UK) dissolved in 18 µl sterile double distilled water. *EHEC EDL 933* (*stx1,2* positive) was used as a positive control and *E. coli ATCC 11 229* (*stx1,2* negative) was included as negative control. The conditions for the PCR were 95 °C for 12 min 30 s for initial denaturation, followed by 35 cycles of 95 °C for 20 s (denaturation), 57 °C for 30 s (primer annealing) and 72 °C for 40 s (DNA synthesis) and 5 min of final extension at 72 °C performed with a thermal cycler (Perkin-Elmer, Norwalk, USA). The amplified products were analysed by electrophoresis in a 2% agarose gel and were visualised following ethidium bromide staining (100 µl/100 ml gel) (Sigma-Aldrid, Steinheim, Germany) at UV-light and photographed (AlphaInnotech, Biozym, Hessisch Oldendorf, Germany) using the AlphaImager 1220 software (Biozym).

For the selective enrichment of *Salmonella* spp. 1 g faeces was inoculated into 14 ml of tetrathionate broth (Merck) and incubated for 24 h at 37 °C. One ml of this enriched broth was brought into tetrathionate broth the next day and incubated for another 24 h at 37 °C. This enrichment step was repeated a further time. On the fourth day one loopful of the cultured medium was plated both on *Salmonella-Shigella* agar (Difco) and Leifson agar (Merck). After 24 h of incubation at 37 °C presumptive *Salmonella* spp. colonies were Gram-stained and tested by API 20E (bioMérieux).

Cultural examination of *Yersinia* spp. was performed by adding 1 g faeces into 9 ml of Gram-negative broth and incubating for 48 h at 21 °C. One loopful of broth was then plated on *Yersinia*-selective agar (Difco) and incubated for another 48 h at 21 °C. Colonies with the typical bull's-eye-appearance were subcultured on blood agar and Gram-stained and biochemical tests were subsequently carried out with API 20E (bioMérieux) and Micronaut (Merlin, Bornheim-Hersel, Germany). To detect various *Yersinia*-genes, PCR was performed using the primers listed in Table 2. The PCR conditions consisted of an initial 94 °C denaturation step of 10 min followed by 30 cycles of 94 °C for 1 min, 57 °C for 1 min, and 72 °C for 1 min. The final cycle was followed by a 72 °C incubation for 10 min. Amplified DNA fragments were visualized as mentioned for the *E. coli* virulence genes.

For the detection of *Cryptosporidium* oocysts, immunomagnetic separation was applied using Dynabeads anti-*Cryptosporidium* (Dynal, Oslo, Norway). Twenty µl of the immunoconcentrate were used for a direct immunofluorescence test (*Cryptosporidium*-Antigen-IFT, medac, Wedel, Germany) (das Graças C. Pereira *et al.*, 1999).

Cryptosporidium parvum oocysts from a calf (Iowa isolate, USA) served as the positive control. Using a fluorescence microscope at x400–x1000 magnification *Cryptosporidium* oocysts appear as 6-10 µm in size, round or oval in shape with bright green fluorescence.

For statistical analyses, the data was evaluated with the Statistica 5.0 software (StatSoft GmbH, Hamburg, Germany), following the instructions of

Table 2. Primers used in the PCR to amplify *Yersinia*-genes.

Primer	Oligonucleotide sequence (5'-3')	Size of amplified product	Identified gen	Reference
SP1 SP3	GAA TAT TGC ACA ATG GGC GCA AAC AAA CCG CCT GCG TGC GC	233 bp	16S rRNA	(Neubauer *et al.*, 1999)
Adh2 Adh3	CAG GCG TTA ATT CTG TTG GTG TCC AAT GGC AAC AGA G	191 bp	yadA	(Blais & Phillipe, 1995)
V1 V2	CCT ACG AAC AAA ACC CAC AA GGA TTT ATC ATG GAT ATT TAT GG	524 bp	V-antigen	(Price *et al.*, 1989)
Ye1 Ye2	AAT ACC GCA TAA CGT CTT CG CTT CTT CTG CGA GTA ACG TC	330 bp	16S rRNA	(Neubauer *et al.*, 2000)

Trampisch & Windeler (1997). For all analyses, differences were considered significant at $P \leq 0.05$.

Results

In 2224 (99.2%) out of the total number of 2243 faecal samples one or more of the examined bacteria species were isolated. *Campylobacter* sp, identified as *C. hyointestinalis*, was detected in one sample only (0.04%). *Enterococcus* spp. were isolated in 2084 (92.9%) samples. *Escherichia coli* were isolated in 2123 (94.7%) samples. There was no evidence of the occurrence of *Salmonella* spp. nor *Cryptosporidium* spp.. Table 3 shows the results for the detection of the *E. coli* toxin genes by PCR.

Table 3. Occurrence of *E. coli* toxin genes in 2123 isolated strains (prevalences in parentheses)

	stx1	*stx2*	*eae*	hly_{EHEC}	*eae*+hly_{EHEC}
E. coli *n*=2123	3 (0.14%)	0	11 (0.52%)	21 (0.99%)	2 (0.09%)

One hundred and eight (4.8%) strains of *Yersinia* spp. were isolated, consisting of *Y. enterocolitica* biogroup 1A (29), *Y. intermedia* (2), *Y. kristensenii* (72), *Y. mollaretii* (3) and *Y. rhodei* (2). Fig. 1 illustrates the prevalences of *Yersinia* spp. and the other isolated bacteria in the examined reindeer faeces.

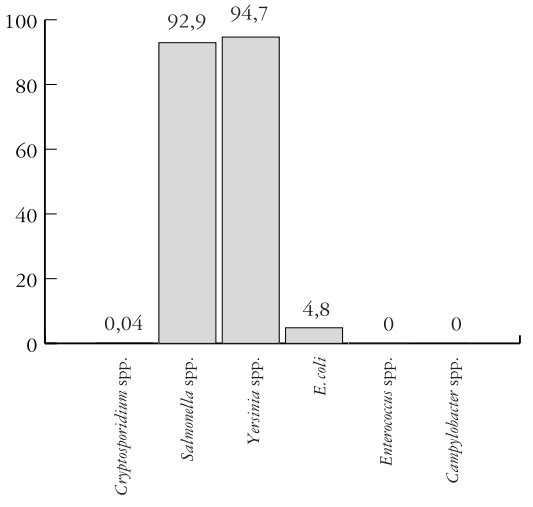

Fig. 1. Prevalences of analysed pathogens in faeces of reindeer (*n*= 2243)

Discussion

All bacteria investigated in this study may be found in northern Europe in the environment in aquatic, terrestrial and animal reservoirs (Kapperud, 1981) and were isolated from the intestinal tract of healthy or diseased ruminants worldwide (Adesiyun *et al.*, 1998; Busato *et al.*, 1998). Even though most of the isolated bacteria strains do not have the potential to cause severe human or animal health problems, certain strains might be a risk, especially for immunosupressed, old or very young persons and animals. Therefore one has to regard the epidemiological impact of transmission of these infectious agents from the environment to reindeer and man and vice versa.

In reindeer, *Enterococcus* spp. and *E. coli* occurred in very high prevalences, showing the affiliation of these two species to the normal intestinal flora of healthy reindeer. Concerning *E. coli*, there are only few reports on diseases caused by shigatoxin-producing bacteria in ruminants (Sherwood, 1985; Mainil, 1999), however these bacteria are of extreme importance in causing severe diseases in humans (Griffin & Tauxe, 1991). As the genes encoding *stx1*, *eae* and hly_{EHEC} were detected only in very low numbers of the isolated *E. coli*-strains, the human health risk due to *E. coli* excreted by reindeer can be considered very low. These results comply with another study detecting no *E. coli* O157:H7 in 1387 faecal and 421 meat samples from reindeer (Lahti *et al.*, 2001). It is however known that STEC virulence factors are mobile within bacterial populations (Yamamoto *et al.*, 1984; Pupo *et al.*, 1997).

Yersinia spp. was isolated in 108 samples. The identified species *Y. intermedia*, *Y. kristensenii*, *Y. mollaretii* and *Y. rhodei* have been isolated before from various environmental samples (fresh water, soil, *etc.*), food, healthy animals and healthy and diseased humans (Baier & Puppel, 1981; Sulakvelidze, 2000). Even though these species are widely distributed in nature, their actual impact on human health is a matter of controversy. But as they are isolated from persons with gastrointestinal disorders, the role of these species should not be disregarded (Sulakvelidze, 2000). The isolated *Y. enterocolitica* strains belonged to Biogroup 1A, which embraces the nonpathogenic European *Y. enterocolitica* strains, often isolated from environmental samples, foods, animal and human faeces (Bottone, 1997).

Campylobacter hyointestinalis was isolated from one sample only. As the cultivation of *Campylobacter* spp. is exceedingly difficult, the real prevalence might be higher. Hitherto *Campylobacter hyointestinalis* has been associated only sporadically with human gastrointestinal disorders (Edmonds *et al.*, 1987; Gorkiewicz *et*

al., 2002). Even though the prevalence for *Campylobacter* spp. in this study was very low, it shows that reindeer can be carriers. This is approved by another study detecting *Campylobacter hyointestinalis* in a prevalence of 6% in Finnish reindeer faeces (Hänninen *et al.*, 2002). It is surprising that neither *Salmonella* spp. nor *Cryptosporidium* oocysts were detected in reindeer in this study as both pathogens have been isolated from the environment, farm animals and man in Fennoscandia (Refsum *et al.*, 2002; Horman *et al.*, 2004), as was *Salmonella* spp. from reindeer in Finland as well (Kuronen *et al.*, 1998).

Conclusion: The examined enteropathogens were either not detected at all (*Salmonella* spp. and *Cryptosporidium* spp.), in very small numbers (*Campylobacter* spp.) or if detected, their virulence and pathogenicity was very low (*E. coli* and *Yersinia* spp.). The potential human and animal health risk by reindeer, excreting various important enteropathogenic bacteria and *Cryptosporidium* spp., should be considered very low.

Acknowledgements

This examination was performed in the context of the RENMAN-Project, funded by the EC's 5th framework programme.

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Manuscript received 25 November, 2003
revision received 29 April, 2004
accepted 19 May, 2004

Abstract in Norwegian / Sammendrag:

Det er mangelfull kunnskap om hvorvidt det i reinmøkk kan finnes mikroorganismer som kan representere en helserisiko for dyr og mennesker. Hensikten med denne studien var å undersøke forekomsten av mulige sykdomsfremkallende mikroorganismer i reinmøkk. Prøver av reinmøkk ble samlet fra 2243 klinisk friske tamrein i nordre deler av Finland og Norge. Prøvene ble undersøkt for bakteriene *Campylobacter* spp., *Enterococcus* spp., *Escherichia coli*, *Salmonella* spp., *Yersinia* spp. og parasitten *Cryptosporidium* spp. ved bruk av standardiserte laboratoriemetoder. *E. coli* ble funnet i 2123 prøver (94,7%), *Enterococcus* spp. i 2084 prøver (92,9%) og *Yersinia* spp. i 108 prøver (4,8%). *Campylobacter* spp., identifisert som *Campylobacter hyointestinalis*, ble bare funnet i én prøve (0,04%). *Salmonella* spp. og *Cryptosporidium* spp. ble ikke påvist. Videre undersøkelser av *E. coli* viste at bare svært få (<1%) av isolatene hadde gener som kodet for mulige sykdomsfremkallende toksiner. Videre undersøkelser av *Yersinia* spp. viste at ingen av disse isolatene var sykdomsfremkallende. Studien viser at helserisikoen knyttet til de undersøkte mikroorganismene i reinmøkk må betraktes som svært liten.