Shiga toxin genes (*stx*) in Norwegian sheep herds

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SUMMARY

The aim of this study was to describe the occurrence of shiga toxic genes (*stx*) in Norwegian sheep herds, and to identify herd management factors related to the occurrence of *stx* in herds. Faecal samples from 124 sheep-herds were collected at abattoirs in 1998. Pooled samples from lambs and from ewes were screened for *stx* by a PCR method directly on faeces. Of the 124 herds, 61 were positive for *stx*, giving an overall herd-prevalence of 49%. Twenty-one of the 61 positive herds were positive both in lamb and ewe samples, 24 only in lamb samples and 16 only in ewe samples. There was no difference in prevalence between regions. From the 21 herds positive both in lamb and ewe samples, stx encoding *E. coli* were detected in 18 herds using hydrophobic grid membrane filters and subsequent colony hybridization. Information about management factors was collected by telephone interviews. Having cattle at the same farm turned out to be a possible risk factor, with an Odds Ratio of 9·9 (CI 1·2 → ∞).

INTRODUCTION

Shiga toxin-producing *Escherichia coli* (STEC) are emerging potential human pathogens transmitted through the food chain from their animal hosts. The major virulence factors of STEC are the shiga toxins (Stx). The Stx family comprises Stx1 and many variants of Stx2. Other virulence factors include the ability to cause attaching-and-effacing lesions in the intestine and plasmid encoded haemolysins.

STEC have been isolated from a variety of domestic animals such as cattle, sheep, goats, pigs, dogs and cats, and also from wild animals and birds [1–6]. Domestic ruminants are, however, regarded as the major reservoir for STEC, and among these, cattle are considered as the most important vector in relation to human infection. Several studies have shown that cattle are a reservoir of a variety of STEC strains, of which some are indistinguishable from STEC strains causing human disease.

More recently, several studies have indicated a higher prevalence of STEC in sheep than in cattle [6–8]. STEC strains of serogroup O157, similar to those pathogenic to man, have also been isolated from sheep [3, 5], and Asakura et al. (1998) detected STEC from sheep belonging to the same non-O157 serogroups that had been isolated from STEC outbreaks in humans [9]. Although the understanding of STEC's epidemiology in sheep is still inadequate, these studies indicate that some of the STEC from sheep may pose a health hazard to humans. Limited data are available on the epidemiology of STEC in Norway. In screenings of faecal samples from dairy and beef cattle, the herd prevalence of *E. coli* O157:H7/H⁻ was 1% in dairy cattle and 7% in beef cattle [10, 11], while the herd prevalence of STEC in dairy cattle was 29% [10]. These results are consistent with results from similar studies undertaken in other countries. In a screening of faecal samples for *E. coli* O157:H7 from cattle,

sheep and pigs from the south-western region of Norway, Johnsen et al. found herd prevalences of 0·2%, < 0·5% and 0·1%, respectively [12]. These last results show a lower prevalence than those found by Vold et al. [10,11]. A number of studies have been performed in order to identify risk factors for STEC at the farm level, but most of these studies are limited by specifically focusing on *E. coli* O157:H7 in cattle [13–15]. Presumably, the results obtained are also valid as far as other zoonotic non-O157 STECs in ruminants are concerned [16], but the epidemiology of various *stx*'s may differ.

Increased faecal shedding of STEC by carrier animals, with a subsequent contamination of the environment, may be caused by stress and dietary changes [17]. To what degree specific management factors may influence, and possibly reduce, the carrier rate of STEC is, however, yet to be determined.

The aim of this study was to describe the occurrence of *stx* in Norwegian sheep herds and to identify herd management factors related to the occurrence of *stx* in herds.

MATERIALS AND METHODS

Study design

The cross-sectional study was designed so that 160 sheep herds (about 1% of all herds in Norway) were to be included in the study. Faecal samples were collected at 10 different abattoirs from the major sheep-producing regions of Norway during the slaughter season (September–November) of 1998. The number of herds selected at each abattoir was proportional to the number of herds in that area. Randomly selected herds, from which at least 3–5 ewes and 5 lambs were slaughtered that day, were sampled. In the south-western region several farmers had slaughtered their ewes early, so the number of herds sampled from that region was lower than planned. The final number of herds sampled was 134. Six of these did not have recognizable owner identity and four of them had only samples from either lambs or ewes, leaving 124 herds for further investigation. The number sampled from each region can be found in Table 1.

The second part of the study was a semi-nested case-control study. Herds classified as *stx* positive both in lamb and ewe samples (lamb⁺, ewe⁺) (21 herds) were chosen as the case group. The control group was collected by random sampling among the

Table 1. *Frequency of STEC in sheep herds (lamb and ewe) in different regions in Norway (%)*

Region	Lamb⁻ Ewe⁻	Lamb⁻ Ewe⁺	Lamb⁺ Ewe⁻	Lamb⁺ Ewe⁺	Sampled
Eastern	20 (42)	9 (19)	9 (19)	9 (19)	47
South-western	7 (70)	0 (0)	1 (10)	2 (20)	10
Western	16 (70)	3 (13)	3 (13)	1 (4)	23
Middle	10 (53)	2 (11)	3 (16)	4 (21)	19
Northern	10 (40)	2 (8)	8 (32)	5 (25)	25
Total	63 (51)	16 (13)	24 (19)	21 (17)	124

stx negative herds (lamb⁻, ewe⁻), stratified by region to resemble the number of herds in the case group from the same area. Altogether, 46 farmers were contacted for follow-up interviews.

Faecal samples

Faecal samples were collected by cutting off the last part of rectum (about 20 cm) and then transported in coolers to the laboratory where they arrived the next day. Upon arrival, stool specimens of approximately 2 g were collected by sterile methods and frozen at −80 °C until analysed.

Screening for *stx* by PCR directly on faeces

DNA from faeces was prepared according to the boiling method described by Stewart et al. [18]. The lamb and ewe samples from each farm were pooled separately using 0·5 g from each animal and mixed 1:10 with brain heart infusion broth (BHI) (Difco Laboratories, USA). After 30 min at room temperature for sedimentation the supernatant was removed and incubated overnight at 37 °C. The following day, the supernatant was diluted 1:10 with phosphate-buffered saline (PBS) (Difco Laboratories, USA) and 1 ml was centrifuged at 13 000 *g* in an eppendorf centrifuge for 2 min. The pellet was washed in 1 ml PBS and centrifuged at 13 000 *g* for 2 min. Supernatant fluids were removed by aspiration. Washed pellets were re-suspended in 1 ml PBS and placed in a boiling water bath for 10 min. After cooling to room temperature, 4 μl 0·5 mg/ml RNAse was added and the sample was placed in 37 °C water bath for 30 min.

Ten μl of this preparation was added as template-DNA to a 40 μl PCR master-mix which contained 2·5 μl PCR 10× buffer, 2·5 μl Mg(OAc)₂, 1 μl dNTP

Mix (Finnzymes OY, Finland), 1 μl DNA Polymerase (Finnzymes OY, Finland), 1 μl of each stx primer [17] (MK1 and MK2) and sterile water, and run with a stx_1 and stx_2 positive control strain and negative control [10]. The MK1 and MK2 primers are degenerate primers, which are shown to detect the majority of known stx variants [19].

The Minicycler™ with Hot Bonnet™ (MJ research, MA, USA) was used with the following programme: 94 °C for 5 min, followed by 40 cycles of: denaturation at 94 °C for 1·5 min, annealing at 43 °C for 3 min and amplification at 72 °C for 4 min.

The amplified products were run on a 2% agarose gel (Sea Kem GTG, FMC BioProducts, Rockland, ME, USA) and visualized under uv-light with ethidium bromide staining.

Isolation and identification of stx-positive bacterial isolates

Herds positive both in lamb and ewe samples, were chosen for STEC isolation using the filter-hybridization protocol of Cobbold and Desmarchelier (2000) (20) with only a few minor changes. The probe was synthesized according to the protocol in the ECL direct nucleic acid labelling and detection systems Kit (Amersham International, Bucks, UK) using MK1/MK2 PCR products prepared from a stx_1 and stx_2 positive strain. Hybridization was performed as described in the same ECL protocol. Colonies that hybridized with the probe were picked from the replicate filter and streaked onto mHC agar. Isolates were confirmed as stx positive by PCR as described above.

The isolates were identified by their biochemical reactions in the Roscozym 4-hour ent kit (A/S Rosco, Taastrup, Denmark).

Herd classification

One pooled faecal sample was classified as stx positive by the presence of a DNA product of the expected size (224/227 bp), determined by comparison with the molecular weight marker (123 bp ladder; Life Technologies) and the positive control. In the prevalence study the herd was classified as stx positive if at least one of the pooled samples (lamb or ewe) from the herd was positive in the PCR. In the case control study the herds selected to the case group were positive both in lamb and ewe samples.

Epidemiological information

Six of the farmers in the case-control study decided not to participate in the study. Two of these were positive herds; 4 were negative herds, leaving 19 positive herds and 21 negative herds for further statistical analysis. A standard questionnaire on herd characteristics and management factors was mailed to the farmers and telephone interviews were conducted a few days later.

Statistical analysis

Tabular analysis with different herd outcomes (ewe$^+$/lamb$^+$, ewe$^+$/lamb$^-$, ewe$^-$/lamb$^+$, ewe$^-$/lamb$^-$) was performed using JMP (version 3.2.5, SAS Institute, NJ).

In the nested case-control study, the herd status on stx (ewe$^+$, lamb$^+$) was the outcome variable. Descriptive analysis of herd characteristics and management factors for case and control herds was done by the Kruskal–Wallis test for continuous variables and Fisher's Exact test for categorical variables using JMP. Variables showing a P-value < 0.20 in univariate analyses were tested further in a multiple logistic regression model, using LogXact (Cytel Software Corp., Cambridge, MA). Linearity of continuous variables was tested using graphical plots and by categorization into quartiles. As the case-control groups were stratified on regions, this variable was kept in the model as a stratifying variable.

RESULTS

Sixty-one of the 124 herds were positive for stx in the PCR, giving an overall herd prevalence of 49%. Twenty-one of the 61 positive herds were positive both in the lamb and the ewe samples, 24 only in the lamb samples and 16 only in the ewe samples. Thus, no effect of age on the distribution of stx was observed. There was no difference of prevalence between regions. The results for different regions in Norway and frequency of STEC (in lamb and ewe) are shown in Table 1.

stx-positive isolates were detected from 18 of the 21 herds initially positive both in lamb and ewe samples. From nine herds STEC were isolated from both samples, from five herds only from lamb samples and from four herds only from ewe samples. All these isolates were identified as $E.\ coli$ in the Roscozym test.

Results from the descriptive statistical analyses are

Table 2. *Descriptive analyses of continuous herd characteristic factors from herds in the final case-control study. Results for case herds* (n = 19) *and control herds* (n = 21). *The* P-*values given are from the Kruskal–Wallis test*

Variable	Case herds median (range)	Control herds median (range)	P
No. ewes during winter	65 (22–250)	72 (15–185)	0·48
Cultured meadow (m²/ewe)*	130 (84–633)	100 (28–186)	0·02
No. contact farms at pasture	4 (0–25)	4 (0–60)	0·20
No. sheep purchased the last 3 years	2 (0–34)	3 (0–38)	0·36
Days indoor with lamb	17·5 (10–21)	17·5 (7–45)	0·30
Months fed hay (winter)	7 (0–8)	6 (0–8)	0·37
Months fed grass silage (winter)	5 (0–9)	6·5 (0–9)	0·29

* *P*-value < 0·20, candidate variable in multiple logistic regression.

Table 3. *Descriptive analyses of categorical herd characteristic factors for herds in the final case-control study. Results for case herds* (n = 19) *and control herds* (n = 21). *The* P-*values given are from Fisher's exact test*

Variable	Case herds	Control herds	P
Participating in ram circle	5/18	5/21	1·0
Using their own ram	14/19	18/21	0·44
Sharing ram with other farms	5/19	6/21	1·0
Having cattle at the same farm*	6/19	0/21	0·01
Water-supply from well	14/19	13/21	0·51
Water-supply from the surface	2/19	2/21	1·0
Water-supply from water works	4/19	7/21	0·49
Sharing transportation to/from pasture	3/19	5/21	0·70
Using the abattoirs transportation truck	17/19	20/21	0·60

* *P*-value < 0·20, candidate variable in multiple logistic regression.

shown in Tables 2 and 3. There was no difference between case and control herds for herd size, feeding practices or contact with other herds. However, a larger area of cultured meadow/ewe was found in case herds. More noteworthy, there were six farms that had cattle as well as sheep, and all these sheep herds were in the case group. Detailed examination of the data showed that the farms with cattle had a considerably larger area of meadow/ewe than the other farms. Thus the variable meadow/ewe was dropped due to co-linearity, and only the cattle factor remained as a potential risk factor. An Odds Ratio of 9·9 (95% CI 1·2 → ∞) was found in exact stratified logistic regression.

DISCUSSION

The overall STEC herd prevalence of 49% found in this study cannot be directly compared to the herd prevalence study in dairy cattle [10], due to the difference in study design and methods. Results from other countries indicate that the prevalence of STEC in sheep is higher than in cattle [6–8]. In Australia, Fegan & Desmarchelier detected *stx* in 88% of faecal samples from sheep grazing on pasture and on all the 13 farms investigated [8]. The within-herd prevalence was in the range 56–100% [8]. Beutin et al. isolated STEC from 66·6% of the sheep investigated [7], and in Spain, STEC was isolated from 68% of 93 herds (1300 lambs) [21]. In general, the occurrence of STEC seems to be higher in cattle and sheep than in non-ruminant animals, and the results of this study support the view of STEC as an organism that can be frequently detected among healthy sheep. Recently, indications that *E. coli* O157:H7 might be better adapted to persist in the alimentary tract of ruminants than other pathogenic *E. coli* was presented [22]. These findings can partly explain the widespread occurrence of STEC in sheep.

In this study there was no difference in herd

prevalence between regions. Fewer herds than planned were sampled from the south-western region of the country, and the estimated prevalence is less precise for this area. The sensitivity of the boiling method for DNA preparation combined with PCR as described by Stewart et al. was estimated to approximately 3 cfu/g [18]. This is concordant with the results obtained in initial spiking experiments in this study (data not shown). Because of some expected loss of sensitivity analysing pooled and frozen faecal samples, the overall herd prevalence is probably even higher than the 49% found in this study.

The hybridization-hydrophobic-grid membrane filter technique is useful for isolating all STEC, and is applicable to faeces, food and environmental samples. Other studies have shown that this technique gives a very good recovery of the isolates [20]. Follow-up analysis of the *E. coli* isolates indicates that the Stx-encoding genes are unstable (data not shown), and this might explain why there were three herds, initially positive in the MK1/MK2 PCR, from which it was not possible to recover any STEC.

The results from the case-control study indicate that having cattle at the same farm is a risk factor for the occurrence of *stx*. Further investigation will be performed to determine whether or not cattle and sheep on the same farms share the same STEC pool. The herds investigated in this study were also analysed for prevalence and risk factors for *Salmonella enterica* serovar 61:k:1,5,(7) (unpublished observations). Results from these analyses showed clear regional differences in the prevalence of this specific variety of salmonella. Breed and number of animals kept during the winter were identified as the dominant risk factors. Those results are different from the ones found in this study, and although *Salmonella* spp. and *E. coli* share the faecal–oral route for transmission there are some differences in their epidemiology.

The effect of diet has previously been shown to influence on the colonization of *E. coli* O157:H7 in sheep [17]. In this study there was no effect of diet. This might be due to the fact that most of the sampling was done before start of the housing period, and during grazing most sheep in Norway are fed on a similar regime.

The use of exact inference methods was necessary in this study. Standard asymptomatic statistical methods could not produce any estimate of odds ratio because of empty strata. Further, any estimate based upon the low number of cases as in this study may be severely biased if based upon approximate asymptotic methods. With a specificity of 100%, the lack of sensitivity will be a conservative error, causing a bias of the odds ratio estimate towards one [23].

Only 6–13 human clinical cases of STEC-infections in Norway have been notified to the National Institute of Public Health since 1996, and about half of these cases are associated with travelling abroad. In 1999, a small domestic outbreak including four cases was identified, and probably associated with the consumption of contaminated lettuce [24–26]. With these data in mind, there is no strong indication that the reservoir of *stx*-genes in sheep represents any human health hazard in Norway, but whether sheep isolates represent a reservoir of mobile genes, which can be transferred to more virulent *E. coli* is a question for future research.

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