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Occurrence of shigatoxinogenic *Escherichia coli* O157 in Norwegian cattle herds

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SUMMARY

To investigate if there is a reservoir of *Escherichia coli* O157 in Norwegian cattle, faecal samples from 197 cattle herds were screened for *E. coli* O157 by the use of immunomagnetic separation (IMS) and PCR during the 1995 grazing season. Six *E. coli* O157:H-isolates were detected in two herds, one isolate in one and five in the other. The isolates carried the stx_1, stx_2, and *eae* genes, and a 90 MDa virulence plasmid. They were toxinogenic in a Vero cell assay. From 57 other herds, 137 faecal samples were positive for stx_1 and/or stx_2 genes detected by PCR run directly on IMS-isolated material. Among these samples, stx_2 were the most widely distributed toxin encoding genes. No difference was found among milking cows and heifers in the rate of stx_1 and/or stx_2 in positive samples.

INTRODUCTION

Enterohaemorrhagic *Escherichia coli* (EHEC) of serotype O157:H7 is an emerging human pathogen, causing haemorrhagic colitis and haemolytic uraemic syndrome [1–3]. The pathogenicity of EHEC is mainly associated with the ability to produce cytotoxins and their adherence capacity [4]. Calderwood and colleagues have recently proposed that the cytotoxins should collectively be referred to as members of a Shiga toxin family, due to their resemblance to the Shiga toxin produced by *Shigella dysenteriae*, type 1 [5]. The EHEC strains adhere to the intestinal epithelium by the attaching and effacing mechanism, in which the *eae*-genes play a major role [6].

Although more than 200 *E. coli* serotypes produce Shiga toxins, and consequently should be termed Shiga toxin-producing *E. coli* (STEC), only a limited, though increasing, number are considered as pathogenic to humans, and therefore classified as EHEC. Serotype O157:H7 is still the predominant serotype among EHEC, and the one most frequently associated with foodborne outbreaks. Whether this reflects the true distribution of EHEC serotypes, or is due to an increased awareness and thus overdiagnosing of O157:H7 compared to other EHEC serotypes, is under debate [7].

Dairy cattle, especially young animals, have been implicated as a principal reservoir of *E. coli* O157:H7 and foodborne infections caused by *E. coli* O157:H7 have most often been associated with consumption of bovine products, or products contaminated by bovine faeces [8–11]. Several epidemiological studies have detected STEC in cattle, but most of these isolates are not considered to be human pathogens [12, 13]. In the United States, the herd prevalence of *E. coli* O157:H7 has ranged from less than 1 to 8·3%, with animal prevalences ranging from 0·28 to 1·8% [14–16]. Similar animal prevalences have been found in two Nordic countries, Denmark and Finland, while a slightly higher prevalence (4%) has been reported

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from the United Kingdom (Jeppe Boel and Johanna Takkinen, personal communications) [8].

The aim of the present study was to investigate whether there is a reservoir of EHEC O157 in Norwegian dairy herds.

MATERIALS AND METHODS

Faecal samples

During the 1995 grazing season (June–October) a total of 1970 faecal samples from cattle from 197 herds were collected by local veterinarians and sent to the Norwegian College of Veterinary Medicine for analysis. The herds belonged to different veterinary districts in three high-density cattle regions in Norway (Fig. 1). Stool specimens were collected by rectalization from 10 animals on each farm, 5 samples from heifers and 5 from milking cows. Each faecal sample was put into a separate 50 ml Falcon tube and transported cooled with overnight express to the laboratory. On arrival, the samples were either analysed immediately or frozen at −70 °C until analysis.

Bacterial control strains

The stx_1- and stx_2 negative *E. coli* O157 ATCC 43888 was used as a negative control strain, and the stx_1- and stx_2 positive *E. coli* O157:H7 P1446 (Dynal A.S., Oslo, Norway) was used as a positive control strain.

Immunomagnetic separation (IMS)

From each faecal sample, approximately 1 g of faeces was added to 10 ml Gram Negative Broth (Difco Laboratories, Detroit, USA) and incubated with shaking at 37 °C for 5 h. IMS was performed using the Dynabeads® anti-*E. coli* O157 (Dynal A.S.), according to the manufacturer's recommendations.

Plating of IMS-isolated material

Fifty microlitres of the IMS-isolated material was spread onto a sorbitol–MacConkey agar plate (Oxoid, Unipath Ltd, England), containing 0·05 mg/l cefixime and 0·65 mg/l tellurite (CT-SMAC). The plates were incubated overnight at 37 °C. Presumptive *E. coli* O157 colonies were tested for agglutination with O157 antisera using the *E. coli* O157 Test Kit (Oxoid, Unipath Ltd, England).

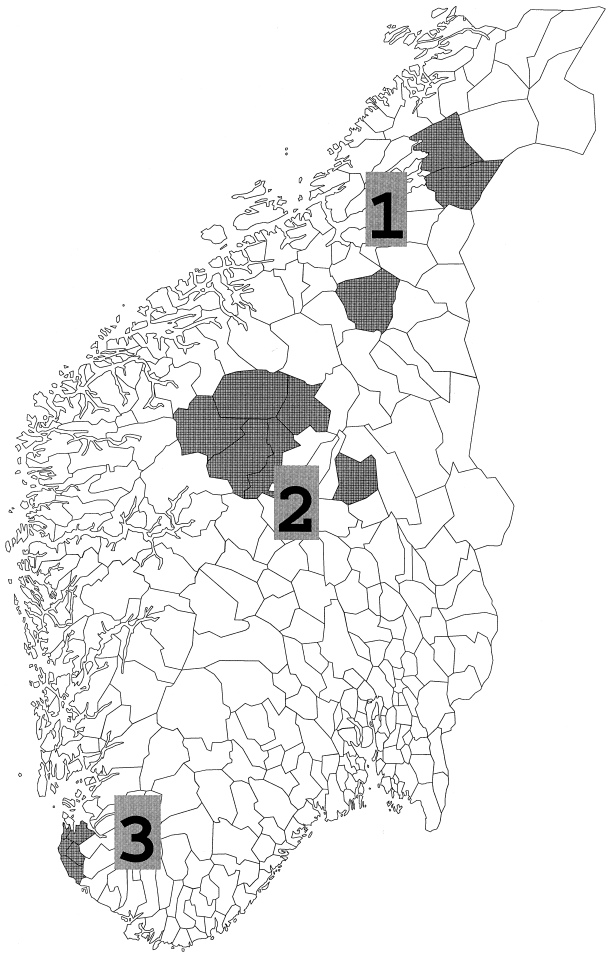

Fig. 1. Three high-density cattle regions in the southern part of Norway: Middle Norway (1), Eastern Norway (2), South-West Norway (3).

PCR for detection of stx_1 and stx_2 on IMS-isolated material

Ten microlitres of the IMS-isolated material was added directly as template DNA to a 40 μl mixture of PCR-reagents as follows: PCR buffer, 200 μM of each dNTP, 2 units DynaZyme DNA polymerase (Finnzymes OY, Finland), and 10 pmol of each primer (Genosys, Cambridge, England). The PCR was designed as a mixed PCR, simultaneously amplifying fragments from both the stx_1 and stx_2 genes. The primer sequences are listed in Table 1 (stx_1-1 + stx_1-2 + stx_2-1 + stx_2-2).

The Minicycler™ (MJ Research, MA, USA) was used for temperature cycling. The PCR sequences were: 94 °C for 5 min, followed by 40 cycles of:

Table 1. *Primer sequences*

Primer	Sequence 5′-> 3′	Reference
stx_1-1	CAG TTA ATG TGG TGG CGA AG	[42]
stx_1-2	CTG CTA ATA GTT CTG CGC ATC	[42]
stx_1-3	GAT AGT GGC TCA GGC GAT AA	[43]
stx_2-1	CTT CGG TAT CCT ATT CCC GG	[42]
stx_2-2	GGA TGC ATC TCT GGT CAT TG	[42]
stx_2-3	AAC CAC ACC CCA CCG GGC AGT TA	[43]
eae-1	CAG GTC GTC GTG TCT GCT AAA	[17]
eae-2	TCA GCG TGG TTG GAT CAA CCT	[17]

denaturation of 94 °C for 1 min, annealing at 50 °C for 1 min, and amplification at 72 °C for 1 min. The amplified products were run on a 2% agarose gel (SeaKem GTG, FMC BioProducts, Rockland, ME, USA) and visualized with ethidium bromide staining.

PCR for detection of stx_1 and stx_2 and *eae* on bacterial colonies

Bacterial colonies agglutinating O157 antisera and colonies from samples positive in the PCR on IMS-isolated material were run in a PCR as described above. All stx_1 and stx_2 positive isolates were also run in a PCR for detecting *eae*-genes specific for *E. coli* O157 [17]. *Eae*-primer sequences are listed in Table 1.

Confirmative stx_1 and stx_2 PCR

Positive results from the stx_1 and stx_2 PCR on bacterial colonies were confirmed by running a semi-nested PCR, utilizing PCR products from the first PCR diluted 1:10 as target DNA. The semi-nested PCR reactions were run separately for stx_1 and stx_2, under conditions as described for the first PCR. The inner primer sequences are listed in Table 1 (stx_1-3 and stx_2-3).

Biochemical identification of *E. coli*

All bacterial isolates positive in the stx_1 and stx_2 PCR were verified as *E. coli* by standard biochemical tests [18].

Serology

All bacterial isolates positive in the stx_1 and stx_2 PCR were sent to the Reference Laboratory for Enteric Pathogens, National Institute of Public Health, Oslo, Norway for serotyping. The isolates were tested against the following antisera: O26, O78, O86, O112, O119, O125, O126, O128, O157, and H7.

Vero cell test

The isolates identified as stx_1 and stx_2 positive *E. coli* were tested for toxicity in a Vero cell assay [19].

Plasmid profiling

Plasmid profile analyses were performed on STEC O157 isolates according to the methods described by Kado & Liu and Birnboim & Doly [20, 21]. The plasmids were separated on a 1% agarose gel and visualized by ethidium bromide staining.

Faecal flora

Faecal samples, from which *E. coli* O157 was detected, were thawed and pre-enriched in Gram Negative Broth. After pre-enrichment, 0·1 ml was spread onto SMAC agar plates to approximate the relative amount of other enterobacteria constituted relative to typical sorbitol–negative *E. coli* O157.

Spiked samples

Before the collection of the 1970 field samples, stool samples from five cattle were spiked with the *E. coli* strain P1446 in the following concentrations; 10^6 cfu/g faeces, 10^4 cfu/g faeces, 10^2 cfu/g faeces, and 10^1 cfu/g faeces. These samples, in addition to a non-spiked stool sample, were analysed by IMS with subsequent plating on CT-SMAC, and stx_1 and stx_2 PCR on IMS-isolated material, in order to estimate

the sensitivity of the test procedure. To evaluate a possible reductive effect of transport of samples, an identical set of spiked stool samples were analysed after 2 days storage at 4 °C. In order to evaluate the effect of IMS, direct plating of spiked, pre-enriched faeces onto CT-SMAC was performed. An stx_1 and stx_2 PCR after extraction of DNA from crude samples was also included [22].

RESULTS

Sensitivity of procedure as estimated on spiked samples

IMS-enriched samples gave positive PCR and CT-SMAC results on spiking levels of 10^6, 10^4 and 10^2 cfu/g faeces. Sporadic positive PCR results without growth on CT-SMAC were obtained from samples spiked with 10^1 cfu/g faeces. No effect on the results from storing the stool samples at 4 °C for 2 days could be demonstrated. The negative control samples were always negative.

When using direct plating of spiked, pre-enriched faeces onto CT-SMAC, a high number of colonies resembling *E. coli* O157 was obtained, thus making the plates difficult to interpret. Direct stx_1 and stx_2 PCR on spiked samples after DNA extraction always gave negative results.

Plating of IMS-isolated material

Sorbitol-negative colonies confirmed as *E. coli* O157:H− were isolated on the CT-SMAC agar plates from six faecal samples from two herds. PCR on these isolates revealed the presence of both stx_1 and stx_2 genes. They were positive in the O157-specific *eae*-PCR, and harboured a 90 MDa plasmid. One of the six isolates produced high levels of toxicity in the Vero cell-test, whereas the others produced low levels of toxicity. The low-level toxicity producers originated from five animals belonging to the same herd. Based on these results and the plasmid profiles, we assume that these five isolates are progenies of the same *E. coli* strain.

Stx$_1$ and *stx*$_2$ PCR on IMS-isolated material

A total of 137 (7%) samples representing 57 herds (29%) were positive for stx_1 and/or stx_2 in the PCR run on IMS separated material. The distribution of stx_1 and stx_2 in the three geographical regions, on a herd basis and on an animal basis are presented in Figures 2 and 3. The difference between the three geographical regions was most notable for stx_2 ($\chi^2 = 12{\cdot}7$, $P = 0{\cdot}0017$), while no difference was found for stx_1 ($\chi^2 = 0{\cdot}27$, $P = 0{\cdot}87$), or $stx_1 + stx_2$ ($\chi^2 = 2{\cdot}02$, $P = 0{\cdot}36$). A clear trend towards clustering of *stx*-positive animals into specific herds was observed. No age difference was found for stx_1 ($\chi^2 = 0{\cdot}42$, $P = 0{\cdot}52$) or stx_2 ($\chi^2 = 1{\cdot}03$, $P = 0{\cdot}31$).

E. coli giving positive stx_1 and/or stx_2 PCR results could be isolated from CT-SMAC plates only from 20 of the 137 stx_1 and/or stx_2 positive samples. These 20 isolates did not react with any of the available antisera for enteropathogenic *E. coli*. All, except three isolates, were toxigenic in the Vero cell-test. Two of the non-toxigenic isolates had apparently lost their toxin genes during subcultivation, as shown by negative results in a control PCR. All isolates were negative in the *eae*-PCR.

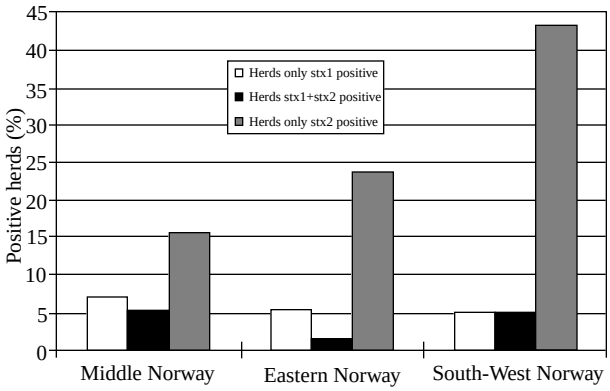

Fig. 2. The distribution of stx_1 and stx_2 on a herd basis in the three high-density cattle regions in Norway.

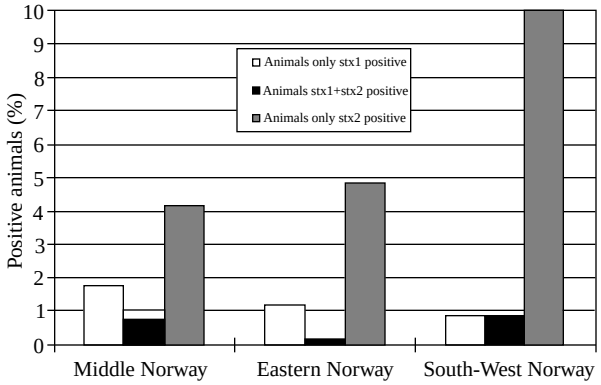

Fig. 3. The distribution of stx_1 and stx_2 on an animal basis in the three high-density cattle regions in Norway.

Semi-nested stx_1 and stx_2 PCR

All positive results from the stx_1 and stx_2 PCR on bacterial colonies were confirmed in the semi-nested PCR.

Faecal flora

After thawing, pre-enrichment and plating on SMAC, faecal samples from which *E. coli* O157:H− had been detected gave rise to a flora dominated by typical *E. coli* O157 colonies.

E. coli O157:H− carrying animals

The two herds from which *E. coli* O157:H− carrying animals were detected were located in separate geographical locations, Eastern Norway and South-West Norway (Fig. 1). *E. coli* O157:H− was detected from five animals in the Eastern Norway herd, three of which were heifers. The *E. coli* O157:H− carrying animal in the other herd was a lactating cow.

DISCUSSION

The *E. coli* O157 herd prevalence of 1% is similar to or lower than results presented in studies of cattle faeces in other countries. However, due to differences of study design and methods, different studies cannot be directly compared. The epidemiological significance of this finding of a small reservoir of *E. coli* O157:H− in Norway is unclear. From 1992–6, only 12 human cases of EHEC infections have been notified to the National Institute of Health. Ten of these cases have been associated with travelling abroad before the onset of disease. For the other two cases, the origins of infection are unknown. None of the *E. coli* isolates from the 12 cases has belonged to the O157:H− phenotype. Data indicate that each individual animal only transiently or sporadically sheds *E. coli* O157 [23, 24]. As demonstrated in sheep, diet may have an effect on *E. coli* O157 shedding, and stressful situations such as transport to the slaughter facilities and hold-off from feed can increase shedding rate prior to slaughter [25]. Thus, on-farm one-time faecal sampling of individual animals may not accurately reflect the number of animals which shed *E. coli* O157 at slaughter.

In humans, *E. coli* O157-associated illness occurs in a seasonal pattern, with a peak during the summer months [26]. Conflicting results are presented whether this phenomenon is reflected in a similar season variation in shedding of *E. coli* O157 from cattle [14, 16]. The faecal samples analysed in this study were collected during the grazing period or shortly after housing.

Clearly, prevalence data vary due to the use of diagnostic tests with different specificities and sensitivities. The use of IMS for separating and concentrating the *E. coli* O157, together with plating on selective media, has been demonstrated to be a sensitive and specific method for detecting *E. coli* O157 from faecal samples, food samples and environmental samples [27–30]. For other toxinogenic *E. coli*, the combination of IMS with toxin-genes-detecting PCR has been successful [31]. According to our spiking experiments, the sensitivity of IMS/plating and PCR on IMS-isolated material were similar. We have no explanation as to why the *E. coli* O157 isolates in this study only were isolated by plating and not by the PCR on IMS separated material.

Dynal A.S. recommend the use of SMAC agar plates containing 0·05 mg/l cefixime and 2·5 mg/l tellurite for selective growth of *E. coli* O157. Cefixime at this concentration inhibits *Proteus* spp. but not *E. coli*, while tellurite is suggested to have a selective activity for *E. coli* O157 [29]. Some strains of *E. coli* O157 are apparently sensitive to tellurite in the recommended concentration (Vigfrid Næss, personal communication), and Dynal A.S. therefore recommend the parallel use of SMAC agar plates. Our experience is that distinguishing *E. coli* O157 colonies from the rich background flora on the SMAC agar plates can be difficult. Thus, we chose to reduce the amount of tellurite in the CT-SMAC agar medium to 0·65 mg/l. By the use of this tellurite concentration, growth of background flora was still inhibited, while growth of *E. coli* O157 strains that did not grow on the original CT-SMAC agar plates was stimulated (data not shown).

The *E. coli* O157 isolates from the present study did not agglutinate with anti-H7 sera. Non-motile variants, designated NM or H−, have been recorded among many of the EHEC serotypes, and *E. coli* O157:H− strains have frequently been isolated in Europe [32–34]. Recently, Feng and colleagues have discussed the possibility that many *E. coli* O157:NM isolates have been mistyped and actually could be isolates of *E. coli* O157:H7 that are non-motile [35]. Their assumption is based on the fact that a large portion of their investigated *E. coli* O157:NM isolates harbour many other genotypic and phenotypic charac-

teristics for *E. coli* O157:H7, and seem to belong to the *E. coli* O157:H7 clonal group. To which extent our O157:H− isolates have carried the H7 flagellae, but then lost the antigens during isolation and subcultivation, is unknown. The isolates also carried other pathogenicity factors enabling the bacterium to cause disease; they all carried functional stx_1 and stx_2 genes and the *eae* genes encoding proteins necessary for adhesion. They also harboured a 90 MDa plasmid considered crucial for pathogenicity [36].

In spite of the use of the Dynabeads® anti-*E. coli* O157, 137 (7%) of the faecal samples gave a positive stx_1 and/or stx_2 PCR result without a corresponding *E. coli* O157 isolate. Since we only succeeded in isolating stx_1 and/or stx_2 positive *E. coli* strains from 20 of these samples, it is not known whether the remaining 117 PCR positive samples harboured *E. coli* O157 or other non-specifically attached STEC/EHEC strains. However, the finding of a relative high proportion of STEC of serotypes that have not yet been associated with human disease is consistent with other studies [12, 37–40], as is the dominance of the stx_2 in the toxin gene profile of these samples.

Half the animals sampled in the present study were heifers (< 24 months), and half were milking cows. No significant differences in shedding of STEC were observed. This finding contrasts with other studies claiming that weaned calves and heifers more frequently carry STEC than adult cattle [12, 14]; a phenomenon attributed to a relative lack of immunity against STEC, age-related physiological and/or morphological characteristics of the gastrointestinal tractus promoting STEC colonization, and aspects of calf management increasing exposure to these agents [12].

Although the non-EHEC strains do not represent a direct reservoir of human-pathogenic bacteria, these STEC may represent a reservoir of toxin genes, which can be transferred to *E. coli* serotypes able to colonize the human intestinal tract. The phage-mediated stx_1 and stx_2 genes are located on mobile genetic elements, and the transferable nature of Shiga toxin production in *E. coli* O26 and O128 has been demonstrated in a laboratory setting [41]. The frequent occurrence of stx_2 compared to stx_1 in STEC isolates, and the difference between stx_1/stx_2 profile in different areas, may indicate a difference in the mobility pattern between the two stx_1- and stx_2-carrying phages. Whether, and to what extent, such transfer of stx_1 and stx_2 can occur in stool- or food samples, is currently under investigation in our laboratory.

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