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Verotoxinogenic *Escherichia coli* (VTEC) O157:H7 - A Nationwide Swedish Survey of Bovine Faeces

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Albihn A, Eriksson E, Wallen C, Aspán A: Verotoxinogenic *Escherichia coli* (VTEC) O157:H7 - A nationwide Swedish survey of bovine faeces. Acta vet. scand. 2003, 44, 43-52. – In the autumn of 1995 the first outbreaks of enterohemorrhagic *Escherichia coli* O157:H7 including ca 100 human cases were reported in Sweden. From outbreaks in other countries it is known that cattle may carry these bacteria and in many cases is the source of infection. Therefore, the present study was performed to survey the Swedish bovine population for the presence of verotoxin-producing *E. coli* (VTEC) of serotype O157:H7. Individual faecal samples were collected at the 16 main Swedish abattoirs from April 1996 to August 1997. Of 3071 faecal samples, VTEC O157 were found in 37 samples indicating a prevalence of 1.2% ($CI_{95\%}$ 0.8-1.6). All 37 isolates carried genes encoding for verotoxin (VT1 and/or VT2), intimin, EHEC-haemolysin and flagellin H7 as determined by PCR. Another 3 strains were of serotype O157:H7 but did not produce verotoxins. The 37 VTEC O157:H7 strains were further characterised by phage typing and pulsed-field gel electrophoresis. The results clearly show that VTEC O157:H7 is established in the Swedish bovine population and indicate that the prevalence of cattle carrying VTEC O157:H7 is correlated to the overall geographical distribution of cattle in Sweden. Results of this study have formed the basis for specific measures recommended to Swedish cattle farmers, and furthermore, a permanent monitoring programme was launched for VTEC O157:H7 in Swedish cattle at slaughter.

between phage typing; PFGE.

Introduction

The first case of enterohemorrhagic colitis due to infection with verotoxin-producing *Escherichia coli* (VTEC) O157:H7 in Sweden was reported in 1988 (*Sjöblad et al.* 1989). Since then, and until the second half of 1995, not more than 3 annual cases of the infection have been recorded. However, in 1995, 114 human cases of infection caused by VTEC O157:H7 were reported. In 1996 and 1997 more than 100 cases were reported each year, but since 1998 the number has declined to below 100 cases per year (www.smittskyddsinstitutet.se), during these years most infections being reported during the summer.

In Sweden, infections with VTEC O157:H7 in humans have been compulsorily notifiable since January 1, 1996 (www.sva.se/pdf/zoonosrapp2001.pdf). Other VTEC serotypes than O157:H7 are not notifiable, but may be an emerging problem also in Sweden as in other European countries. However, other VTEC serotypes have, so far, occurred only in sporadic cases, and not in community outbreaks.

The source of infection for verified human VTEC O157:H7 cases in Sweden has been determined only occasionally. When epidemiological investigations have indicated connections to farm animals or consumption of

non-pasteurised milk, investigations on farm level have been initiated. Between 1995 and 2002, VTEC O157:H7 was detected in animal samples on 19 farms in such investigations (unpublished data).

Experiences from outbreaks in other countries show that dairy and beef cattle in many cases carry *E. coli* O157:H7 (*Rasmussen et al.* 1993, *Wray et al.* 1993, *Chapman et al.* 1997). In 1995 it was not known whether VTEC O157:H7 was present in the Swedish bovine population. Hence, a nationwide monitoring programme was initiated in April 1996 to survey the Swedish bovine population for the presence of VTEC O157:H7, and if found, to what extent. Furthermore, isolates of VTEC O157:H7 were to be characterised by their virulence genes (vtx_1; vtx_2; *eaeA* and *EHEC hlyA*), phage typing and pulsed-field gel electrophoresis.

Materials and methods

The study design

This study was designed as a nationwide monitoring study, with the aim to detect a prevalence in the population of at least 0.1% with a 90% confidence level (*Thrusfield* 1995). The total population of cattle in Sweden is about 1.8 million. Faecal samples were collected at the 16 main abattoirs; producing 90% of all Swedish beef, and 3000 cattle were aimed to be individually sampled. The collection of samples was performed from April 1996 to August 1997. All abattoirs sent samples each second week with a few exceptions when the premises were closed due to bank holidays, and the number of samples was proportional to the number of cattle passing through the abattoir. The local veterinary staff at each abattoir performed sampling, according to detailed instructions. Age of sampled animals was noted according to the abattoir classification system, i.e. older calves (mainly 5-8 months), young stock (mainly 12-24 months) or adult cattle (mainly 36 months or more). The breed was noted as dairy, beef or crossbreed. Surplus dairy calves may be raised as beef cattle, and in this study these animals are most often recorded as dairy breed. Due to legal aspects the supplier's identity was coded in order to protect identity. The sampling veterinarians were asked to, as far as possible, avoid multiple sampling from the same supplier. Individual samples were collected from ampulla recti after the digestive tract had been separated from the body. Samples were sent as refrigerated to the National Veterinary Institute and analysed within 24 h of sampling for the presence of *E. coli* serogroup O157 strains.

Isolation of VTEC O157

From each sample 10 grams of faeces were pre-enriched in 90 ml buffered peptone water (Oxoid CM 509; Oxoid, Basingstoke, England) for 6 ± 0.5 h in 37 °C. After pre-enrichment, 1 ml broth was processed by immunomagnetic separation (IMS) using paramagnetic beads coated with antibodies to *E. coli* O157 (Dynabeads anti *E. coli* O157; Dynal, Oslo, Norway). IMS was performed according to the manufacturer's instructions. The IMS step was performed either directly after 6 ± 0.5 h incubation or after storing the pre-enrichment broth in cold storage overnight, 16-20 h at 6-8 °C. After IMS, the beads were spread out on sorbitol McConkey agar supplemented with 0.05 mg/l cefixime and 2.5 mg /l of potassium tellurite (CT SMAC; Dynal, Oslo, Norway). After incubation at 37 °C for 18-24 h, the agar plates were screened for suspected sorbitole negative colonies of *E. coli* O157. From every sample up to 5 suspected colonies (if available) were picked for agglutination with a latex kit (Oxoid DR 622; Oxoid, Basingstoke, England). Colonies positive by agglutination were further confirmed with biochemistry using the API 20 E system (bioMérieux, Lyon, France).

Virulence typing
From each sample that yielded sorbitol negative E. coli O157 colonies, one colony was analysed by PCR to identify genes coding for verotoxin 1 and 2 (vtx_1 and vtx_2) according to *Woodward et al.* (1992), H7 *(fliC)* according to *Gannon et al.* (1997) and intimin *(eaeA)* and EHEC-haemolysin (EHEC-*hlyA*) according to *Paton & Paton* (1998).

Phage typing
The isolates of VTEC O157:H7 were phage typed using published methods at the Laboratory of Enteric Pathogens (Central Public Health Laboratory, London, UK) (*Ahmed et al.* 1987, *Khakhria et al.* 1990).

Genotyping of E. coli O157:H7 isolates by pulsed-field gel electrophoresis (PFGE)
DNA preparation. Chromosomal DNA for pulsed-field gel electrophoresis (PFGE) analysis was prepared essentially according to *Christensen et al.* (1994), but with the following modifications. Bacteria were grown overnight at 37 °C on Luria Broth-agar plates. Four colonies were suspended in 250 µl EC-buffer (1 M NaCl, 10 mM Tris, 200 mM EDTA, 0.5% sarcosyl, 0.2% sodium deoxycholate, pH 8.0) warmed to 37 °C and then mixed with 350 µl prewarmed Agarose Prep (Amersham Pharmacia Biotech, Uppsala, Sweden; 1.5% in EC-buffer) and 30 µl lysozyme-stock solution (20 mg/ml) (Roche Diagnostics Scandinavia AB, Bromma, Sweden). The mixture was distributed into disposable agarose plug moulds (Amersham Pharmacia Biotech, Uppsala, Sweden) and left to solidify for 15 min at 4 °C. The agarose plugs were transferred to 10 ml Falcon tubes containing 2.5 ml EC-buffer and 1mg proteinase K/ml buffer solution, and incubated at 56 °C for at least 20 h. After the proteinase K incubation the plugs were washed with TE-buffer (10 mM Tris, 1 mM EDTA, pH 8.0) and proteinase K was inactivated by adding 50 µl from a stock solution of 20 mM Pefablock (Roche Diagnostics Scandinavia AB, Bromma, Sweden) into 5 ml TE-buffer, leaving the plugs at 37 °C for 2 h. Finally, the plugs were washed with 5 ml TE-buffer at 37 °C (2×30 min), and then stored in TE-buffer at 4 °C until further use.

Restriction endonuclease digestion and pulsed-field gel electrophoresis. The restriction endonuclease *Xba*I was used as recommended by the manufacturer (Amersham Pharmacia Biotech, Uppsala, Sweden). A thin slice of the gel plug (1 mm) was loaded onto a 1.2% agarose gel (NA Agarose; Amersham Pharmacia Biotech, Uppsala, Sweden) and run in a Gene Navigator unit (Amersham Pharmacia Biotech, Uppsala, Sweden), at 175 V and 12 °C with buffer circulation. The programme was 10s, 3h / 15s, 6h / 20s, 6h / 40s, 5 h / 60s, 4h. After electrophoresis was completed the gels were stained with ethidium bromide for 20 min (0.1% in water), washed in water for 20 min and then photographed under UV-light. λ DNA (New England Biolabs, Hitchin, UK) was used as marker for molecular size.

Dendrograms. Polaroid photographs of macro restriction profiles were scanned with a UMAX Vista-S6E scanner (UMAX Technologies Inc, Dallas, Texas, USA) and digitalised using Adobe Photoshop 3.0.5 for Windows, and saved in TIFF format. These files were analysed using the GelCompar computer software version 4.0 (Applied Maths, Kortrijk, Belgium). Banding patterns were compared by the UPGMA (Unweighted Pair Group Method with Arithmetic Averages) clustering method, by using the Dice coefficient, according to the manufacturer's instruction.

Interpretation and nomenclature of PFGE patterns. Any difference in banding

Table 1. Number of faecal samples from which verotoxin-positive O157:H7 was isolated from a total of 3071 samples tested, and their corresponding virulence genes as determined by PCR.

Number of isolates	% of total number of sampled animals	vtx_1	vtx_2	*eaeA*	*EHEC hlyA*	*fliC*
1	0.03	+	-	+	+	+
21	0.68	-	+	+	+	+
15	0.49	+	+	+	+	+
37	1.2					

patterns (disregarding bands less than 50kB in size, i.e. below the lowest band of the molecular size marker used) between 2 genomic restriction profiles, was considered to be sufficient to regard the profiles as non-identical.

Statistical method

The differences in frequencies of positive isolates between age groups and breeds of animals, and between different seasons, respectively, were analysed by chi square analysis within the FREQ procedure in the SAS software package (*SAS Institute Inc.* 1989). Differences with *p*-values less than 0.05 were regarded as significant. The 95% confidence interval was also calculated in the SAS software package.

Results

A total of 3071 samples were analysed and 37 were positive for VTEC O157, indicating a prevalence of 1.2% ($CI_{95\%}$ 0.8-1.6). All 37 isolated strains carried the genes encoding for verotoxin (VT1 and/or VT2), intimin, EHEC-haemolysin and flagellin H7. According to the farm number given to each farm by the abattoir, the 3071 samples were collected from 2328 different premises. In 7 of the 3071 cases the farm number was not stated by the abattoir. Also, in some instances a cattle farm may have been given more than one farm number, and thus the exact number of premises sampled in this study is not known.

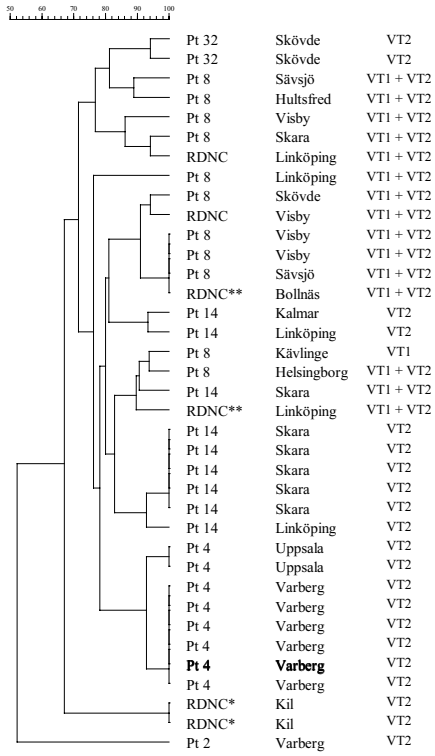

Figure 1. Dendrogram of *Xba*I macrorestriction electrophoretic patterns of the 37 isolates of VTEC O157:H7 described in the study; the phage type, abattoir of isolation and verotoxin type of each isolate.
RDNC = this culture reacts with the typing phages but does not conform to a recognised pattern
RDNC* = 2 strains with identical typing phage patterns, but without a given phage type name
RDNC** = 2 strains with identical typing phage patterns, but without a given phage type name

Table 2. Cattle with VTEC O157:H7-positive faecal samples, distributed according to age group.

Age group	No. positive	No. sampled	%
Older calves	7	181	3.9[a]
Young stock	23	1789	1.3
Adult cattle	7	1092	0.6[a]
Not recorded	0	9	0
Total	37	3071	1.2

[a] values significantly different at $p<0.001$

Table 3. Cattle with VTEC O157:H7-positive faecal samples, distributed according to breed.

Breed	No. positive	No. sampled	%
Dairy breed	28	1928	1.5[a]
Beef breed	3	631	0.5[a]
Crossbreed	0	43	0
Not recorded	6	469	1.3
Total	37	3071	1.2

[a] values significantly different at $p<0.1$

Of the 37 VTEC O157:H7 samples, 21 (57%) isolates carried the vtx_2 gene only, 15 (40%) both the vtx_1 and the vtx_2 genes and one isolate (3 %) the vtx_1 gene only (Table 1). All 37 isolates possessed the EHEC-*hlyA*, the *eaeA* and the *fliC* genes. In addition, 3 samples were found to contain *E. coli* of serotype O157 lacking verotoxins; these strains were not further characterised.

Phage typing of the 37 isolates divided them into 5 known phage-types (PT 2, PT 4, PT 8, PT 14 and PT 32). In addition, 6 strains had phage types not given numbers according to the phage-typing scheme used at the Laboratory of Enteric Pathogens, but which react with the typing phages (RDNC) (CPHL, London, UK). Of these, 2 were unique (RDNC in Fig. 1), and the other 4 grouped 2 and 2 (RDNC* and RDNC** in Fig. 1).

By pulsed-field gel electrophoresis 18 of the 37 isolates gave unique PFGE patterns after *Xba*I digestion (Fig. 1). Five groups of 2 or more isolates with identical PFGE patterns were found.

The distribution according to age of the animals showed that there was a higher ($p<0.001$) frequency of positive isolates from older calves (3.9%) than from adult cattle (0.6%) (Table 2).

The distribution according to breed of the animals showed that there was a tendency to higher ($p<0.1$) frequency of positive isolates from dairy cattle (1.5%) than from beef cattle (0.5%) (Table 3). However, fewer beef than dairy cattle were sampled (631 versus 1928).

During the grazing period (May to September), there was a higher ($p<0.05$) frequency of positive samples (1.6 %), than from the stabling period (0.8%) (Table 4). However, in February, a high frequency (3.1%) of animals was found to be positive, which could be explained by the fact that 5 of 6 young stock from the same farm were found to be positive when sampled, see below.

Table 4. Cattle with VTEC O157:H7-positive faecal samples, distributed according to sampling season.

Month	No. positive	No. sampled	%
May	4	361	1.1
June	0	258	0
July	8	228	3.5
August	8	318	2.5
September	3	252	1.2
October	4	310	1.3
November	1	289	0.3
December	0	148	0.7
January	0	195	0
February	7	223	3.1
March	0	166	0
April	2	323	0.6
Total	37	3071	1.2

* For April, May, June and August the figures include samples from both 1996 and 1997.

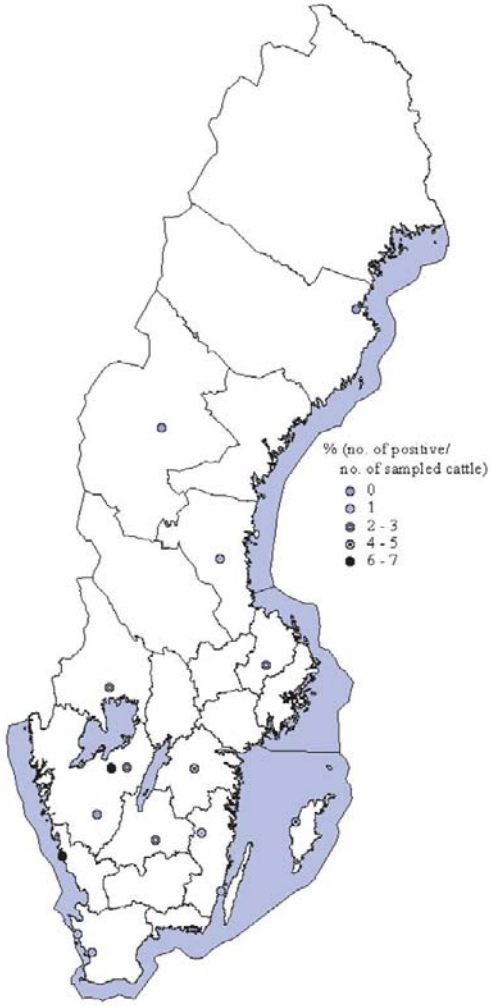

Figure 2. Map of the frequencies (% - no. positive / no. sampled cattle) of VTEC O157:H7 isolated and the localisation of the Swedish slaughterhouses.

A map was prepared (Fig. 2) depicting the different abattoirs and number of positive samples found by each abattoir. The geographic distribution of VTEC O157:H7 positive isolates shows a concentration in the south half of Sweden.

Discussion

Before 1996, verotoxin producing *E. coli* O157:H7 had never been diagnosed in Swedish cattle. However, only about 200 calves had been investigated in different research studies, and the methods used did not include *E. coli* O157:H7 antibody based immunomagnetic separation (IMS) (A. Franklin, National Veterinary Institute, Uppsala, Sweden, personal communication).

The results from the present study show that VTEC O157:H7 is present in the Swedish cattle population at a prevalence of ca 1.2% ($CI_{95\%}$ 0.8-1.6). The prevalence of VTEC O157:H7-positive healthy cattle is in the range of what has been reported (0.4%-15.7%) in other western countries where extended studies have been performed on faecal samples from animals at slaughter (*Chapman et al.* 1997, *Heuvelink et al.* 1998, *Bonardi et al.* 1999, *van Donkersgoed et al.* 1999, *Pradel et al.* 2000, *Johnsen et al.* 2001, *Lahti et al.* 2001, *Meyer-Broseta et al.* 2001, *Paiba et al.* 2002, *Tutenel et al.* 2002). However, it is precarious to compare the prevalence between different studies, as different methods have been used. It has been shown in several studies that IMS is superior in detecting VTEC O157:H7, as compared with direct plating on selective agar media or plating after incubation in enrichment broth (*Chapman et al.* 1994, *Heuvelink et al.* 1998, *Meyer-Broseta et al.* 2001).

The geographical distribution of animals harbouring VTEC O157:H7 showed a concentration in the south part of Sweden, where most cattle are kept. This may indicate that the occurrence of cattle carrying VTEC O157:H7 is correlated to the geographical distribution of the cattle population. It must be mentioned that this may not be an appropriate description of the geographic distribution of VTEC O157:H7-harbouring cattle, as some abattoirs drain a large geographical area, and the region covered

by each abattoir is not clearly defined. Also, the study was designed to answer the question if VTEC O157:H7-harbouring cattle were present in Sweden, not to show statistically relevant differences between the prevalence of this infection in different regions of Sweden.

The distribution according to age group of the animals showed that young animals (older calves) (3.9%) were more often found to be positive than adult cattle (0.6%). In other studies, it is also noted that young animals are more often found to be positive for VTEC O157:H7 than adult cattle (*Mechie et al.* 1997, *Hancock et al.* 1997, *van Donkersgoed et al.* 1999). In one study, *Cray & Moon* (1995) found that after experimental infection of calves and adult cattle with *E. coli* O157:H7, calves remained positive for longer periods.

The distribution according to breed of the animals showed that dairy cattle were more often positive than beef cattle, 1.4% versus 0.5%. However, fewer beef than dairy cattle were sampled. Also, surplus dairy calves may be raised as beef cattle, but were recorded as a dairy breed at slaughter. Other studies have shown a slight and probably not significantly higher rate of VTEC O157:H7 in beef than in dairy cattle, examples beeing *Hancock et al.* (1994) who found 0.71 and 0.28% respectively, and *Bonardi et al.* (1999) who found 16.6% and 16.1%, respectively. Conversely, *Chapman et al.* (1997) found 13.4% and 16.1% for beef and dairy cattle, respectively.

During summer and early autumn, i.e. the grazing period, more positive samples were found compared with other seasons. However, in February, many samples (3.1%) were found to be positive. Five of these 7 samples were collected at one slaughterhouse on the same day, and it was later found that 4 of these samples were from young stock reared on one farm (see below). However, a peak in incidence in cattle during summer and early autumn has been described by several authors studying VTEC O157:H7 epidemiology on cattle farms or prevalence of VTEC O157:H7 on carcasses at slaughter (*Hancock et al.* 1994, *Chapman et al.* 1997, *Hancock et al.* 1997, *Mechie et al.* 1997, *Bonardi et al.* 1999, *van Donkersgoed et al.* 1999, *Lahti et al.* 2001, *Paiba et al.* 2002, *Tutenel et al.* 2002).

Subtyping of *E. coli* O157:H7 has been performed in many studies to support epidemiological relationships between isolates within outbreaks or when transmission from cattle to humans has been suspected (*Willshaw et al.* 1997, *Allison et al.* 1998, *Watanabe et al.* 1999, *Louie et al.* 1999, *Crampin et al.* 1999). Among many methods suggested, phage typing and pulsed-field gel electrophoresis (PFGE) seem to be preferable, and although phage typing was found to be less discriminatory than PFGE in many studies, it was considered valuable to support PFGE, as PFGE may be more difficult to interpret. The authors found that isolates differing by more than one band with *Xba*I from the outbreak strain were clearly not outbreak related, but isolates differing by one band were difficult to classify (*Barrett et al.*1994, *Grif et al.* 1998). Authors investigating large numbers of strains from human outbreaks reported high clonal stability within outbreaks (*Allison et al.* 1998, *Watanabe et al.* 1999), whereas investigations on cattle farms suggest a fairly rapid mutation rate, with several, only slightly different, *Xba*I banding patterns found in cattle isolates from the same farm (*Crampin et al.* 1999, *Louie et al.* 1999).

In our study, the VTEC O157:H7 strains isolated from Swedish cattle were divided between 5 known phage types (PT 2, PT 4, PT 8, PT 14 and PT 32). In addition, 6 strains belonged to phage types not previously described. The 5 phage types identified in our study have all been found in cattle faeces in similar studies performed in Great Britain, Holland and Belgium

(*Chapman et al.* 1997, *Heuvelink et al.* 1998, *Richards et al.* 1998, *Paiba et al.* 2002, *Tutenel et al.* 2002). PFGE could further discriminate within phage types.

Five groups of isolates with identical *Xba*I banding patterns were found in our study. To elucidate whether isolates were from the same source, we asked for further information from the abattoir, where the suppliers were coded with unique but anonymous numbers. In the case of the 2 isolates from Kil slaughterhouse, they were isolated on the same day and had the same supplier number. As for the 2 isolates from Uppsala slaughterhouse, they were collected on different dates (in 1996 and 1997, respectively) and were marked with different supplier numbers. Four isolates that grouped together by PFGE, came from 3 different abattoirs situated in different parts of Sweden. However, of these 4, 2 isolates from Visby slaughterhouse were collected on the same day and were marked with the same supplier number. From the slaughterhouse in Skara, 5 positive samples were collected on one day, in February 1996 (see above), with 4 different supplier numbers, indicating that they were from 4 different farms. However, as they all shared the same PFGE pattern, further investigations were made with the abattoir. It was thus revealed that the calves had been bought by the same cattle trader and kept on one farm before slaughter. Finally, of the isolates from Varberg slaughterhouse, 6 out of 7 showed identical PFGE patterns (Fig. 1). All but one of the 6 identical isolates shared supplier number, indicating that these 5 isolates originated from the same farm. Furthermore, the samples found positive from this particular supplier had been taken on 4 different occasions. The seventh positive sample from Varberg slaughterhouse had a different supplier number, and a different phage type as well as PFGE profile. Thus, PFGE analysis of the material has provided further information valuable to the evaluation of this monitoring study.

To reduce the risk of spreading the infection to other farms and to humans, the national authorities have issued a number of recommended restrictions for farms harbouring animals carrying the VTEC O157:H7 bacteria. These include restrictions concerning visits to farms, and guidelines on personal hygienic behaviour, cleaning of stables, no consumption of non-pasteurised milk, animals should be clean when sent to the abattoir, no on-farm slaughter of cattle for the farmer's own consumption, slurry should not be spread on grassland, slurry should not drain off to running water or lakes, and grazing cattle should be fenced-off from recreational areas, etc. Based on the results of the present study, the Swedish Board of Agriculture has introduced permanent monitoring at slaughter to continually screen for the presence of VTEC O157:H7 in the Swedish bovine population. The results of this permanent monitoring will be reported elsewhere.

To conclude, the results clearly show that VTEC O157:H7 is present in the Swedish bovine population and indicate that the distribution of cattle testing positive for VTEC O157:H7 is correlated to the overall geographical distribution of cattle in Sweden.

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Sammanfattning

Verotoxinproducerande Escherichia coli (VTEC) O157:H7 i Sverige - en landstäckande undersökning av nötträck efter slakt.

Under hösten 1995 drabbades Sverige för första gången av ett utbrott av enterohemoragisk *Escherichia coli* O157:H7, då ett 100-tal humanfall rapporterades kliniskt. Från studier i andra länder har det visats att idisslare kan vara bärare av bakterien, och anses ofta vara direkt eller indirekt källa till human smitta. För att undersöka om även svensk nötboskap bär verotoxinproducerande *E. coli* (VTEC) av serotypen O157:H7, gjordes en studie genom provtagning av nötträck på slakterier. Mellan april 1996 och augusti 1997 samlades 3071 träckprover in vid de 16 största slakterierna i Sverige. Av dessa prov var 37 (1,2%) positiva för verotoxin-producerande (VT1 och/eller VT2) *E. coli* av serotypen O157:H7. Alla dessa isolat bar på generna *eaeA,* EHEC *hlyA* och H7. Ytterligare 3 isolat var av serotypen O157, men producerade inte verotoxin. De 37 verotoxinproducerande isolaten karakteriserades vidare genom fagtypning och makrorestriktionsenzymanalys. Undersökningen visade att VTEC O157:H7 finns etablerad hos svensk nötboskap och prevalensen är väl korrelerad med den geografiska förekomsten av nötboskap i Sverige. Resultaten från denna studie har legat till grund för specifika rekommendationer till svenska djurägare. Studien övergick direkt efter avslutande till ett permanent övervakningsprogram för VTEC O157:H7 vid slakt.

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