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Genetic subtyping of *Escherichia coli* O157 isolates from 41 Pacific Northwest USA cattle farms

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SUMMARY

Escherichia coli O157 ($n = 376$) from 41 cattle farms were subtyped using pulsed field gel electrophoresis of endonuclease cleaved chromosomal DNA. Cleavage with *Xba*I resulted in 81 subtypes. Fifty-one isolates from subtypes found in more than one herd, or in herds on multiple sample collection dates were compared using the endonuclease *Not*I, resulting in 23 additional subtypes. Up to 11 *Xba*I subtypes were found per farm with up to 7 subtypes/farm identified from a single date. Indistinguishable subtypes (both *Xba*I and *Not*I) were found to persist on 4 farms for 6–24 months. Five subtypes were found on more than one farm separated by up to 640 km. Dairy farms where cattle had moved onto the farm had a similar number of subtypes as farms with no movement of cattle, and feedlots had more subtypes than dairy farms. These data indicate that there is a mechanism for multiple herd exposure to specific subtypes, there are multiple sources of exposure for cattle on farms, and on-farm reservoirs other than cattle may exist.

INTRODUCTION

Escherichia coli O157 is now recognized as an important agent of food borne human disease with worldwide distribution (1–3). Although some human infections of *E. coli* O157 have been acquired from direct contact with livestock [4, 5] most infections in humans are thought to be from food products of cattle origin, or indirectly linked to cattle [1, 2, 6, 7]. Both dairy and beef breeds of cattle are colonized with *E. coli* O157 at a low prevalence [8, 9–13] and the organism has been isolated from sheep, horses, dogs, deer and flies [14–16].

Pulsed field gel electrophoresis (PFGE) of endonuclease-cleaved chromosomal DNA can be used to classify *E. coli* O157 into subtypes that provide useful epidemiological information [17–19]. In the present study PFGE was used to subtype isolates of *E. coli* O157 collected over a 34 month period from 41 cattle herds in the USA states of Idaho, Oregon and Washington. This information was used to determine the level of diversity of *E. coli* O157 subtypes within and between cattle herds, the persistence of specific subtypes of *E. coli* O157 in cattle herds over time, and the geographical distribution of *E. coli* O157 subtypes in Pacific Northwest cattle herds. In addition, the diversity of *E. coli* O157 subtypes was compared between farms that did and did not receive cattle from outside sources during the period of study.

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MATERIALS AND METHODS

E. coli O157 isolates

Cattle isolates ($n = 376$) of *E. coli* O157 from 41 herds were obtained from previous investigations on *E. coli*

Table 1. *Sampling and number of subtypes based on PFGE of* Xba*I cleaved chromosomal DNA from culture positive farms*

Farm type	No. of farms	Average no. (range) of faecal samples/farm	Average duration (range) of sampling in months	No. of *E. coli* O157 isolates tested	Average no. (range) of subtypes/farm
Dairy	34	460·8 (179–1836)	8·5 (3–24)	310	3·4 (1–11)
Feedlot	6	182·8 (132–196)	3	40	5·5 (3–10)
Calf raiser	1	1515	14	26	5·0
Total	41			376	

O157 in Pacific Northwest cattle farms (Table 1). These were isolated using rectal or fecal swabs (approximately 0·1–0·3 g of faeces/swab) enriched in Tryptic Soy Broth and plated onto Sorbitol MacConkey agar. Identification of *E. coli* O157 isolates was based on the inability to ferment sorbitol and to produce beta-glucuronidase, and the possession of genes for Shiga toxin [1, 2 or both 1 and 2] and for the O157 antigen. None of the isolates in this study was assayed for the H flagellar antigen. Five isolates from 3 herds were obtained in a 1991 study [9], 129 isolates came from a study where 14 cattle herds were sampled monthly for approximately 1 year beginning in the summer of 1993 and ending in the summer of 1994 [11], 179 isolates came from a study where 12 dairy herds in each of Idaho, Oregon, and Washington were sampled monthly for 6 months [10] and 63 isolates came from a study where 6 feedlots and 6 dairies (3 each in ID, OR, and WA) were sampled monthly for 3 months in 1995 [14]. Some of the dairy herds were involved in more than one study, thus *E. coli* O157 isolates originated from a total of 41 herds, all herds were sampled monthly for a minimum of 3 months, and some herds were sampled 12 or more times over a 12–24 month period.

The 376 isolates represent a smaller number of cattle, since some cattle were sampled multiple times during the individual studies. All isolates were demonstrated to possess genes for Shiga toxin using either PCR (20) or colony hybridization with digoxigenin labelled DNA probes [21, 22]. Representative isolates from each subtype were demonstrated to possess *eaeA* genes using PCR [23]. All isolates had been stored at −70 °C in brain heart infusion broth containing 25% (v/v) phosphate buffered glycerol prior to subtyping.

Subtyping *E. coli* O157 isolates

E. coli O157 isolates were initially subtyped using pulsed field gel electrophoretic (PFGE) patterns of *Xba*I cleaved chromosomal DNA. Only one isolate of *E. coli* O157 from each positive cattle faecal sample was PFGE typed. When identical PFGE patterns were observed for isolates originating from more than one herd, and for isolates detected in samples collected 6 or more months apart, additional analysis was performed using PFGE of *Not*I cleaved chromosomal DNA. *E. coli* O157 subtypes in this paper are defined as those isolates with unique PFGE patterns of *Xba*I and/or *Not*I cleaved chromosomal DNA.

Chromosomal DNA from each *E. coli* O157 isolate was prepared as follows (unpublished method obtained from D. Persing, Mayo Clinic Rochester, MN). Isolates were grown in LB-broth to an optical density of 50% transmittance at 540 nm. One millilitre of the bacterial culture was centrifuged at 14000 *g* for 3 min, the supernatant discarded, and the bacterial pellet suspended in 250 μl 100 mM EDTA, 10 mM EGTA, 10 mM Tris pH 8·0 (EET). The bacterial suspension was then mixed with 350 μl of 1·6% (w/v) chromosomal grade agarose (BioRad, Hercules, CA) in EET, placed into disposable agarose plug molds (BioRad, Hercules, CA) and cooled for 30 min at 4 °C. Agarose plugs for each isolate were then placed into sterile 50 ml centrifuge tubes containing 1 ml of EET with 200 μg/ml lysozyme and 0·05% *N*-laurlysarcosine sodium salt (EET–LS) and incubated for 4 h in a water bath at 30 °C. The EET-LS was removed and 1 ml of EET with 1 mg/ml of proteinase K and 1·0% (w/v) lauryl sulfate sodium salt (EET–SP) was added to each tube and incubated for 12–18 h in a water bath at 50 °C. The EET–SP was then removed, the plugs were rinsed by a 30 min soak in 40 ml of 10 mM Tris 1 mM EDTA disodium dihydrate pH 8·0 (TE) repeated four times, and then stored at 4 °C in TE.

Agarose embedded chromosomal DNA was cleaved with an endonuclease (either *Xba*I or *Not*I; Gibco-BRL, Gaithersburg, MD) following the manufacturer's directions. PFGE was performed on a CHEF–DRII PFGE apparatus (BioRad, Hercules,

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CA) using the following parameters; separation in 1% agarose-0·5 × TBE gels (PFGE agarose; BioRad, Hercules, CA), at 13 °C for 20 h at 6 volts/cm and a linear ramp of 5–50 sec Lambda concatameres (BioRad, Hercules, CA) were used as molecular weight markers in the first and last lane of each gel. Agarose gels were stained with ethidium bromide and photographed on an ultra violet transilluminator.

PFGE patterns of *Xba*I cleaved DNA for each isolate were compared using scanned images of photographs of each agarose gel. A restriction fragment length polymorphism (RFLP) analysis software programme (DNA ProScan PRO-RFLP, Nashville, TN) was used to generate an estimated size in kb for the largest nine bands of DNA for each isolate. The nine largest bands were chosen because these were consistently resolved on each PFGE gel and because they included most of the observed diversity in PFGE patterns. A SAS clustering procedure (SAS Institute, Cary, NC) was then used to designate the possible indistinguishable subtypes, and comparison of individual isolates within clusters was performed in a spread sheet that utilized the data on DNA band size. This comparison allowed for a 5% margin of error in DNA bands < 150 kb and a 10% margin of error in DNA bands $\geqslant 150$ kb.

The isolates belonging to indistinguishable subtypes that were identified as occurring in more than one herd, and over time within herds were further analysed using PFGE patterns of *Not*I cleaved chromosomal DNA. These isolates were compared directly by running together on agarose gels as previously described.

Statistical analysis

The average number of subtypes identified per farm was compared for 11 dairy farms into which animal movements occurred during a 6-month study period to that of 25 dairy farms into which animal movements did not occur. These herds were sampled on 6 consecutive monthly visits to the farm (July–December, 1995) where approximately 60 faecal samples were collected per visit. Significance of the difference was tested using the Mann–Whitney Rank Sum test (24). A similar comparison was done on the number of subtypes per farm for 6 dairies and 6 feedlots that were visited on 3 consecutive monthly visits (during the period July–November 1996) where approximately 60 faecal samples were collected per visit.

Regression analysis was used to determine if the number of samples collected and frequency of sampling on farms were correlated with the number of subtypes detected in cattle herds (25).

RESULTS

All 376 isolates of *E. coli* O157 possessed Shiga toxin genes. Two isolates (0·5%) possessed genes for ST1 only, 124 (33%) possessed genes for ST2 only, and 250 (66·5%) possessed genes for ST1 and ST2. There was no clear association of ST type with PFGE subtypes, although most members of a subtype also had the same ST type. All isolates possessed genes for *eaeA* genes, thus, all of the *E. coli* O157 isolates used in this study were members of EHEC.

Eighty-one subtypes of *E. coli* O157 were identified using PFGE of *Xba*I cleaved chromosomal DNA. Up to 11 subtypes were isolated per herd with as many as 7 subtypes found on one sample data (Table 2). Subtypes that occurred in more than one herd, or that persisted in herds were further analysed by PFGE using *Not*I cleaved chromosomal DNA. This analysis of 51 isolates yielded 23 additional subtypes. Subtypes with indistinguishable PFGE patterns of chromosomal DNA cleaved with *Xba*I and with *Not*I were shown to persist on 4 farms for 6–24 months. In most cases cattle herds had subtypes that were unique to the herd. However, 5 subtypes were found in more than one herd separated by as many as 640 km. One of these subtypes was found in 3 herds, one was found in 4 herds and another was found in 6 herds (Fig. 1). Four of these subtypes were found in cattle from both dairy herds and feedlots.

No relationship between the number of subtypes and animal movement into farms was observed in the 36 dairy herd study where duration of sampling and number of samples collected were similar for each herd. Farms which experienced movement of animals onto the farm had an average of 2·6 subtypes compared to 2·0 for herds which were closed to animal movements ($P = 0{\cdot}46$) (Table 3). However, in the study that compared 6 feedlots (which experienced frequent movement of animals on to the facility) to 6 dairy herds (which experienced few animal movements) a weak association was observed ($P = 0{\cdot}08$). The mean number of *E. coli* O157 subtypes in these feedlots was 5·5 compared to 3 for these dairies (Table 4). A dairy calf raising facility, which received approximately 200 new calves/month from many

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Table 2. *Number of PFGE subtypes based on* Xba*I cleaved chromosomal DNA found in Pacific Northwest cattle farms*

No. of subtypes	No. of farms	Average no. (range) of isolates/farm	Maximum no. of subtypes/sample date	Average no. (range) of months sampled
1	10	4·5 (1–28)	1	8·4 (6–13)
2	4	10·0 (5–20)	1	6·0 (6)
3	11	9·9 (3–25)	3	5·7 (3–12)
4	3	6·3 (4–11)	3	6·0 (3–9)
5	5	16·4 (5–27)	5	8·0 (3–13)
6	3	11·0 (8–15)	4	8·0 (6–9)
7	1	25·0	4	24·0
8	2	12·5 (9–16)	5	6·0 (3–9)
10	1	15·0	7	3·0
11	1	13·0	3	9·0

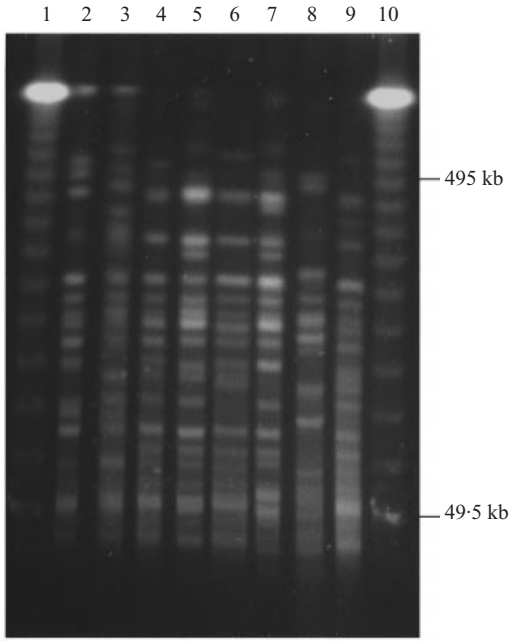

Fig. 1. The most common pulsed field gel electrophoresis patterns of *Xba*I cleaved chromosomal DNA from *E. coli* O157 isolated from cattle. Lanes 1 and 10 are λ-DNA concatemer molecular weight standards (Bio-Rad laboratories, Hercules, CA). The subtypes shown in lanes 2–6 occurred in at least 2 and up to 6 cattle farms. Those shown in lanes 6–9 persisted in herds for at least 6 and up to 24 months.

Table 3. *Comparison of the number of* E. coli *O157 subtypes based on* Xba*I cleaved chromosomal DNA from 36 dairy farms classified by movement of cattle onto the farm during the study period*

Animal movement	No. of farms	No. pos. for *E. coli* O157	Average no. (range) of subtypes/farm
Yes	11	9	2·6 (0–11)
No	25	18	2·0 (0–8)

Table 4. *Comparison of the number of* E. coli *O157 subtypes, based on* Xba*I cleaved chromosomal DNA, in 6 feedlots and 6 dairy farms*

Type of farm	No. of farms	No. pos. for *E. coli* O157	Average no. (range) of subtypes/farm
Dairy	6	6	3·0 (2–5)
Feedlot	6	6	5·5 (3–11)

different dairies, had 5 subtypes over a 14 month period of sampling (Table 1).

There was no correlation between the number of subtypes detected in cattle farms with the number of samples collected ($P = 0{\cdot}76$) and with the duration of sampling ($P = 0{\cdot}85$). The average number of subtypes detected in farms where < 200 samples were collected was 5·1 compared to 2·5 for farms where 200–400 samples were collected, 5·9 for farms where 400–800 samples were collected, and 3·5 for farms where > 800 samples were collected. The number of subtypes detected in farms sampled for 6 or less months averaged 3·3 compared to 4·8 for farms sampled 6–12 months and 4·6 for farms sampled for more that 12 months.

DISCUSSION

This study necessitated the characterization, by PFGE, of a large number of *E. coli* O157 isolates. This was accomplished through the combination of a software programme that utilized scanned images of PFGE gel photographs, a clustering procedure using a statistical analysis programme to reduce the number of isolates for direct comparison of clustered isolates, and a spreadsheet program.

PFGE of *Xba*I cleaved chromosomal DNA and

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allowed the discrimination of numerous subtypes of *E. coli* O157. The identification of 81 subtypes among 376 isolates (one isolate from each positive sample) suggests that hundreds or possibly even thousands of subtypes must exist in the cattle population. PFGE of *Not*I cleaved chromosomal DNA was performed on 51 isolates that either occurred on more than one farm or that persisted on farms. This additional endonuclease cleavage of the chromosome identified an additional 23 subtypes indicating that the use of a single endonuclease for epidemiological analysis is not sufficient to determine the relatedness of *E. coli* O157 isolates. The diversity of subtypes found in the current study is supported by another study that found 20 PFGE subtypes in 160 isolates from 29 dairy cattle and 3 water troughs [26]. Given this diversity, the probability of isolating indistinguishable subtypes from two separate farms by chance alone would seem to be low, suggesting the possibility that there exist common reservoirs of colonization for some cattle farms.

On four farms indistiguishable subtypes were collected at points in time separated by 6–24 months, indicating that some mechanism exists that allows *E. coli* O157 to be maintained in an individual farm ecosystem. The finding that the number of *E. coli* O157 subtypes on cattle farms was not correlated with the number of samples collected from cattle farms nor with the duration of sampling on cattle farms further supports the existence of a mechanism for the perpetuation of specific subtypes on cattle farms. In one herd, which was sampled monthly for a year, a single subtype was found at a relatively high prevalence (5–25%) of cattle in the herd. No other subtypes were found in this herd during the 12 months of sampling, indicating a lack of observable mutation events, which is thought to be a possible explanation for the diversity of *E. coli* O157 subtypes observed in cattle herds. This finding could indicate that there are certain unidentified management variables that serve to perpetuate colonized cattle in the herd, or that this herd occurs in a unique micro-environment with a very specific reservoir for this strain. This apparent contradiction to the general observation that farms have several subtypes of *E. coli* O157 in cattle at any one time offers intriguing evidence for further speculation of on-farm epidemiology and ecology of this organism.

In contrast to those subtypes that were maintained on farms for long periods, most appeared within a particular farm only briefly, and most farms had a succession of unique subtypes. Seven subtypes of *E. coli* O157 were found on one farm on a single sample collection date. These subtypes differed by three or more bands indicating that they were quite different. Although this diversity could be explained by mutation of resident subtypes to new subtypes, this phenomena alone does not seem adequate to explain all of the diversity. It is likely that at least some of this diversity is a result of new subtypes that are introduced to the farm, colonize cattle, and then fail to maintain in the herd. This suggests a source of *E. coli* O157 for colonizing cattle that is external to individual farms. The finding of five subtypes that were shared among more than one farm, several of which were separated by over 600 km, indicates some mode of intra-regional transmission. Cattle movement among farms is a logical suspect, but the data are conflicting in this regard. The failure to find a significant difference in the number of subtypes between dairy farms into which outside cattle moved and dairy farms which remained closed does not support this hypothesis. However, the finding of a greater number of subtypes per farm in feedlots than found in dairies is compatible with the introduction of new subtypes via cattle movement onto farms. It is also possible that the greater diversity observed in feedlot cattle *vs*. dairy cattle was due to differences in the way feedlot cattle are managed. It seems likely that other means of intra-regional transmission, in addition to cattle movement, must exist. Marketed cattle feed and the movement of wildlife and humans are other possible modes of dispersion. Cattle feeds sold in the USA feeds are often contaminated with *Salmonella enterica* (9·8% of feeds tested) and *E. coli* (30·1% of feeds tested) [27, 28]. This supports the possibility that *E. coli* O157 contamination also occurs in cattle feeds, however, *E. coli* O157 has not been reported to have been found in marketed cattle feeds.

Of the 376 isolates of *E. coli* O157 evaluated in this study 336 came from dairy cattle and 40 came from beef cattle in feedlots. Thirty-one of the 40 beef cattle isolates (78%) belonged to *E. coli* O157 subtypes that also occurred in dairy cattle. In fact most of these isolates belonged to the subtypes that were most frequently isolated from all cattle. Since cattle feed is one of the few resources shared by these two types of cattle the finding of shared subtypes in both dairy cattle and beef cattle adds further evidence that cattle feed may be an important consideration for potential vehicles of colonization of cattle farms with *E. coli* O157.

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