APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Sept. 2003, p. 5243–5247 Vol. 69, No. 9
0099-2240/03/$08.00+0 DOI: 10.1128/AEM.69.9.5243–5247.2003

Prevalence of *Escherichia coli* O157 in Cattle Feeds in Midwestern Feedlots

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Received 15 January 2003/Accepted 23 June 2003

Comparisons of enrichment methods (with or without antibiotics and with or without a preenrichment step) using gram-negative (GN) broth or tryptic soy broth (TSB) were conducted with feeds inoculated with *Escherichia coli* O157:H7. TSB was more sensitive than GN broth, and TSB with a preenrichment step followed by TSB with antibiotics was more sensitive than plain TSB enrichment, in detecting *E. coli* O157 in inoculated feeds. Feed samples were collected from feed bunks from 54 feedlots to determine the prevalence of *E. coli* O157 in cattle feeds. TSB preenrichment followed by TSB with antibiotics and the standard GN broth enrichment were used for each feed sample. All samples underwent immunomagnetic separation and were plated onto sorbitol MacConkey agar with cefixime and potassium tellurite. Identification of *E. coli* O157 was based on indole production, positive latex agglutination for O157 antigen, API 20E test strip results, PCR for the *eaeA* gene, and the presence of at least one Shiga toxin. *E. coli* O157 was detected in 52 of 504 feed samples (10.3%) by using GN broth enrichment and in 46 of 504 feed samples (9.1%) by using TSB followed by TSB supplemented with cefixime and vancomycin. *E. coli* O157 was detected in 75 of 504 feed bunk samples (14.9%) by one or both methods. There was no correlation between *E. coli* O157 prevalence and generic coliform counts in feeds. The prevalence of *E. coli* O157 in cattle feed warrants further studies to increase our knowledge of the on-farm ecology of *E. coli* O157 in order to develop strategies to prevent food-borne disease in humans.

The source of *Escherichia coli* O157 that colonizes cattle is unknown. Feeds commonly contain coliforms (7) and generic *E. coli* (25), suggesting fecal contamination of feedstuffs. Individual cattle can be transiently colonized and shed *E. coli* O157 in their feces (15) for 30 to 60 days (3). The reported prevalence in cattle has generally been low in winter and high in summer (1, 15, 29), although some studies have found similar prevalences in winter and summer (11). On a herd basis, fecal shedding usually occurs in clusters, followed by relatively longer periods of low or no fecal shedding (13, 15). *E. coli* O157 can survive for an extended period in bovine feces (18, 33), providing opportunity for feed contamination.

E. coli O157 has rarely been detected in cattle feeds; in two previous studies, it was not detected (14, 25) and in a third it was detected in only 3 out of 32 (9.4%) feed samples taken from a farm over a 5-month period (36). Recently, 0.5% of feeds that were purchased and stored in certain farms were found to contain *E. coli* O157 (11). Certain conditions appear to support growth of generic *E. coli* and *E. coli* O157 in cattle feeds, such as increased concentrations of lactate and decreased concentrations of propionate (25) and high pH in poorly fermented silage (7). This ability of *E. coli* O157 to survive and grow in feed under appropriate conditions (7, 25), as well as the temporal clustering of fecal shedding in cattle, suggests that contaminated feed may be one source of *E. coli* O157 in cattle.

Little is known about the ecology of *E. coli* O157 in the farm environment (39). *E. coli* O157 can survive experimentally in cattle water troughs (23) and has been shown to cause fecal shedding in calves that ingest contaminated water under experimental conditions (37). The relative importance, associated risk factors, and specific role of cattle feeds in the colonization of cattle with and dissemination of *E. coli* O157 remain unclear. We hypothesized that *E. coli* O157 may be present in feed more commonly than recognized by previous culture techniques and that isolation methods need to be designed specifically for the feed environment. The purposes of this study were (i) to develop more-sensitive isolation techniques with feed samples inoculated with *E. coli* O157 and (ii) to assess the prevalence of *E. coli* O157 in cattle feeds from midwestern feedlots.

MATERIALS AND METHODS

Comparison of GN broth and TSB enrichment for detection of *E. coli* O157 in inoculated feeds. (i) Feces and feed inoculation. FRIK 2000, a strain of *E. coli* O157:H7 adapted to nalidixic acid (20 μg/ml; Nal^r), was used for experimental inoculations. The organism was grown overnight in gram-negative (GN) broth (BD, Franklin Lakes, N.J.) at 37°C and diluted with phosphate-buffered saline to obtain various concentrations of *E. coli* O157:H7. Colony counts of the dilutions were done by plating onto sorbitol MacConkey (SMAC) agar plates supplemented with nalidixic acid (20 μg/ml).

Feces were collected per rectum from healthy, lactating dairy cows from a local dairy. Samples of a total mixed ration composed of corn silage, cracked corn, chopped brome hay, cottonseed, soybean meal, and a mineral and vitamin supplement were collected from feed bunks at the same dairy. Fresh feces were inoculated with 10^3, 10^4, and 10^5 CFU of Nal^r *E. coli* O157/g and stored over-

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night at room temperature (20°C). For each inoculation level, 5 g of inoculated feces was mixed with 15 ml of phosphate-buffered saline and vortexed to a uniform mixture. The suspension was added to 500 g of feed and thoroughly mixed. This resulted in feed inoculation concentrations of 10^1, 10^2, and 10^3 CFU/g, respectively. Mixed feeds were stored for 60 to 72 h at room temperature (20°C) before culturing by enrichment broths.

(ii) Experiment 1: GN broth enrichments. Ten-gram samples of inoculated feeds (10^3, 10^2, or 10^1 CFU/g) were placed in sterile plastic bags and mixed with 90 ml of GN broth with no antibiotics added; 90 ml of GN broth supplemented with cefixime (0.05 mg/liter), cefsulodin (10.0 mg/liter), and vancomycin (8.0 mg/liter) (GNccv); or 10 ml of GN broth with no antibiotics added that was held at room temperature (20°C) for 2 h as a preenrichment step, followed by the addition of 80 ml of GNccv broth (GN/GNccv). There were 10 replicates of feed samples at each inoculation level, for a total of thirty replicates for each enrichment method (GN broth only, GNccv, or GN/GNccv).

(iii) Experiment 2: comparison of GN versus TSB. Ten-gram samples of inoculated feeds (10^2 or 10^1 CFU/g) were placed in sterile plastic bags and mixed with 90 ml of GN broth or tryptic soy broth (TSB) with no antibiotics added. There were 20 replicates of feed samples at each inoculation level, for a total of 40 for each enrichment broth (GN broth and TSB).

(iv) Experiment 3: TSB comparison. Ten-gram samples of inoculated feeds (10^2, 10^1, or 10^0 CFU/g) were placed in sterile plastic bags and mixed with 90 ml of TSB with no antibiotics added, 90 ml of TSB supplemented with cefixime (0.05 mg/liter) and vancomycin (8.0 mg/liter) (TSBcv), or 10 ml of TSB with no antibiotics added that was held at room temperature (20°C) for 2 h as a preenrichment step, followed by the addition of 80 ml of TSBcv broth (TSB/TSBcv). There were 15 replicates of feed samples at each inoculation level, with a total of 45 replicates for each enrichment method (TSB only, TSBcv, or TSB/TSBcv).

All broths were incubated for 6 h at 37°C, followed by immunomagnetic separation (IMS) (Dynal; Biotech Inc., Lake Success, N.Y.) and spread plated onto SMAC agar supplemented with cefixime (50 ng/ml) and tellurite (2.5 μg/ml) (CT-SMAC). Plates were incubated overnight at 37°C, and up to six sorbitol-negative colonies with characteristic *E. coli* O157 morphology were streaked onto blood agar plates with sterile toothpicks and incubated overnight at 37°C. Growth on blood agar plates was tested for indole production. Indole-positive colonies were tested for nalidixic acid resistance by determining their growth on SMAC agar plates supplemented with nalidixic acid (20 μg/ml). Nalidixic acid-resistant isolates were tested for the O157 antigen by a latex agglutination assay (Oxoid Limited, Basingstoke, Hampshire, England).

Prevalence of *E. coli* O157 in feedlot feed samples. (i) Feed sample collection. Feed samples were collected as a part of a concurrent *E. coli* O157 prevalence study in cattle feedlots in Nebraska, Kansas, Oklahoma, and Texas during June, July, and August 2001 (J. M. Sargeant, D. D. Griffin, R. A. Smith, M. W. Sanderson, and X. Shi, Abstr. 82nd Conf. Res. Workers Anim. Dis., abstr. 94, 2001). Feed samples were collected from two to four feedlots each week. At each feedlot, approximately 1.0-kg samples of total mixed feed, consisting of 10 smaller grab samples from different areas of the feed surface, were collected from feed bunks of 10 pens of cattle that were within 1 month of market. All feeds were corn-based finishing diets typical of feedlots in the region, including corn, corn silage or hay, and a protein supplement such as soybean meal or urea. Feed samples were shipped overnight to the Food Animal Health and Management Center laboratory at Kansas State University. A total of 504 feed bunks from 54 feedlots were sampled. Data on the number of animals in each pen, square feet of pen space per animal, and available feed bunk length were collected on the day of visit.

(ii) Culture methods. Based on the results obtained with inoculated feeds, the most sensitive method (TSB/TSBcv) was selected and applied in parallel with a standard method (GN broth) to feed samples. Ten grams of feed samples, collected from feedlots, was placed in a sterile plastic bag containing 90 ml of GN broth with no antibiotics added or 10 ml of TSB with no antibiotics added and held at room temperature (20°C) for 2 h as a preenrichment step, followed by the addition of 80 ml of TSBcv.

All samples were incubated for 6 h at 37°C followed by IMS and spread plated onto CT-SMAC. Plates were incubated overnight at 37°C, and up to six sorbitol-negative colonies with characteristic *E. coli* O157 morphology were streaked onto blood agar plates with sterile toothpicks. Following incubation overnight at 37°C, growth on blood agar was tested for indole production. Indole-positive colonies were further tested for the O157 antigen by a latex agglutination assay (Oxoid Limited). Isolates that were positive by the latex agglutination assay were confirmed to be *E. coli* by API (Rapid 20E; BioMerieux, Inc., Hazelwood, Mo.). *E. coli* O157 presumptive isolates were then transferred onto storage beads (Protect beads; KEY Scientific Products, Round Rock, Tex.) for storage at −80°C.

TABLE 1. Comparison of enrichment methods with GN broth or TSB to detect *E. coli* O157 in inoculated cattle feeds

Expt	Enrichment procedure	Total no. of samples	No. of positive samples	Sensitivity[a] (%)
1	GN broth with no antibiotics added	30	21	70.0*
	GNccv[b]	30	26	86.7*
	GN/GNccv[b]	30	24	80.0*
2	GN broth with no antibiotics added	40	10	25.0*
	TSB with no antibiotics added	40	20	50.0†
3	TSB with no antibiotics added	45	16	35.6*
	TSBcv[c]	5	20	44.4*†
	TSB/TSBcv[c]	45	24	53.3†

[a] Sensitivity values within each experiment with different symbols are significantly different ($P \leq 0.05$).
[b] Cefixime, cefsulodin, and vancomycin were included in the medium at 0.05, 10.0, and 8.0 mg/liter, respectively.
[c] Cefixime and vancomycin were included in the medium at 0.05 and 8.0 mg/liter, respectively.

(iii) PCR for detection of virulence genes. For each isolate, PCR was used for the detection of the *eaeA*, *stx1*, and *stx2* genes. A Protect bead was placed into 5 ml of GNccv broth and incubated for 12 h at 37°C. Twenty-five microliters of the culture was removed, and DNA was extracted by the guanidinium thiocyanate extraction method (30). The *eaeA* gene was determined with the TaqMan *E. coli* O157:H7 detection kit (PE Applied Biosystems, Foster City, Calif.). The presence of *stx1* and/or *stx2* genes was determined by 5′ nuclease assays with the TaqMan *E. coli* STX1 and STX2 detection kits (PE Applied Biosystems). Samples and corresponding data were analyzed with the ABI Prism 7700 sequence detection system (PE Applied Biosystems). Isolates possessing the *eaeA* gene and one or both Shiga toxins were considered to be *E. coli* O157.

(iv) Standard coliform counts. Standard coliform counts were performed on each feed sample collected from feedlots. Ten grams of feed was placed in a sterile plastic bag containing 90 ml of distilled water. The sample was mixed for 30 s and serially diluted (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5}), and 100 μl from each dilution was spread plated onto MacConkey agar and incubated at 37°C for 18 h.

Statistical analysis. Analyses were performed with a commercially available software package (Stata statistical software, release 7.0; StataCorp). Experimental isolation methods were compared by the exact McNemar's chi-square test, controlling for multiple-comparison error rate by the Bonferroni method. The agreement of isolation methods used for prevalence data was determined by using the kappa test of agreement.

RESULTS

Comparison of GN broth and TSB enrichment for detection of *E. coli* O157 in inoculated feeds. There were no significant differences ($P > 0.05$) in the sensitivity of detection of *E. coli* O157 in inoculated feeds among GN broth, GNccv, and GN/GNccv enrichment methods (Table 1, experiment 1). The 10^3 concentration in experiment 1 was excluded in experiments 2 and 3, as it allowed overgrowth of *E. coli* O157. Comparison of the two enrichment broths indicated that TSB with no antibiotics added was more sensitive for detection ($P < 0.05$) than GN broth with no antibiotics (Table 1). In experiment 3, the TSB/TSBcv enrichment method was more sensitive for detection ($P < 0.05$) than the TSB enrichment method. This was the only significant difference between the three TSB methods (Table 1).

Prevalence of *E. coli* O157 in feedlot feed samples. The numbers of feedlots sampled in Nebraska, Kansas, Oklahoma, and Texas were 23, 12, 12, and 7, respectively. The average number of animals in each pen, square feet of pen space per animal, and available feed bunk length were 124.8, 313 ft^2, and

TABLE 2. Comparison of generic coliform concentrations and prevalences of *E. coli* O157 in cattle feeds

Generic coliform concn (CFU/g)	Total no. of feed samples	No. of positive samples	% Positive
10^1	131	25	19.1
10^2	76	17	22.4
10^3	117	16	13.7
10^4	117	9	7.7
10^5	53	6	11.3
10^6	8	1	12.5
10^7	2	1	50.0

136.3 ft, respectively. Animals in all feedlots sampled were provided feed ad lib.

E. coli O157 was detected by one or both culture methods in 75 of 504 (14.9%) of feed bunk samples from 54 feedlots. At least one positive feed bunk sample was found in 44.4%, or 24 of 54, of the feedlots. There was no significant difference in the prevalence of *E. coli* O157 detected by the two methods ($P > 0.05$), but there was only moderate agreement between the two, with a kappa statistic of 0.41. The GN broth enrichment method detected *E. coli* O157 in 52 feed bunk samples (10.3%), and the TSB/TSBcv method detected *E. coli* O157 in 46 feed bunk samples (9.1%). There were 29 pens for which only the GN broth method detected *E. coli* O157. There were 23 pens for which only the TSB/TSBcv method detected *E. coli* O157. Both methods detected *E. coli* O157 in 23 out of 75 (30.7%) positive samples.

PCR analysis revealed that 75 out of 85 (88.2%) latex agglutination-positive isolates possessed the *eaeA* gene. Of the 75 *eaeA*-positive isolates, 65 isolates (86.7%) possessed both *stx1* and *stx2* genes, 8 isolates (10.7%) possessed only the *stx1* gene, and 2 isolates (2.6%) possessed only the *stx2* gene.

Generic coliform counts in feed samples ranged from 0 to 1.2×10^7 CFU/g, with a mean count of 1.6×10^5 CFU/g and a median count of 5.9×10^3 CFU/g. There was no correlation between *E. coli* O157 prevalence and generic coliform counts in feed samples ($P > 0.05$; Table 2).

DISCUSSION

Previous studies have addressed the growth and survivability of *E. coli* O157 in cattle feeds (7, 25). Detection methods with various media have generally balanced selectivity with enrichment of *E. coli* O157 (4). *E. coli* O157 organisms can be injured or stressed or can enter a viable-but-nonculturable state in certain environments (4, 26). Lynn et al. (25) found that moistening feeds with sterile, distilled water increased recovery of generic *E. coli* from cattle feeds. Additional studies have found that use of a nonselective preenrichment step increased the recovery of potentially stressed *E. coli* O157 (4, 16, 38). Isolation of *E. coli* O157 in water was also improved by using a nonselective preenrichment step (24). In our experiments, we chose to vary the selectivity of the enrichment broths while maintaining the rest of the isolation procedures (IMS, CT-SMAC, indole, latex agglutination, API, PCR) constant. We compared TSB and GN broth because they have been previously used to detect *E. coli* O157 in feces, feed, and water (17, 24, 25). GN broth is a selective medium that supports coliform growth while inhibiting the growth of most competitive flora; TSB is not a selective medium. After determining that TSB was significantly more sensitive in detecting *E. coli* O157 in inoculated feeds than GN broth, we compared three enrichment procedures with TSB. We found that the TSB/TSBcv enrichment method was more sensitive than the TSB enrichment method in inoculated feeds. Because this was the only significant difference among the three TSB methods, we chose to use TSB/TSBcv in our feed prevalence study.

This study indicates that the prevalence of *E. coli* O157 (14.9%) in cattle feed may be significantly higher than previously reported (11, 14, 25, 36). The prevalence may reflect the use of more-sensitive detection methods (29, 34, 41). This study provides evidence that feed may be a vehicle for dissemination and colonization, yet the source of *E. coli* O157 contamination in cattle feed remains unidentified. The cattle in the pens that we sampled had access to the feed bunks prior to sampling; therefore, it is plausible that the feed could have been contaminated by *E. coli* O157 from cattle saliva (17). Another obvious source is shedding cattle that defecate in the feed. Other possible sources include fecal contamination by other livestock species (19) or by wildlife (8, 31), including birds (14, 39, 40), rodents (14), and insects (14, 29). Another possible source is contaminated feed components that are mixed into the feed (11).

If *E. coli* O157 fecal shedding is clustered temporally (1, 11, 13), we would expect that our collection of samples at a single point in time would underestimate the feed bunk prevalence (12, 25, 35), assuming that there is a direct relationship between these two factors. We sampled during the summer, which could increase our chance of detecting *E. coli* O157 if there is a seasonal effect or unknown risk factors associated with season (27). Lack of correlation between feed generic coliform counts and the feed bunk prevalence of *E. coli* O157 was surprising. If coliform counts are a measure of fecal contamination, feed *E. coli* O157 prevalence should generally parallel coliform counts (25); alternately, a high number of coliforms could competitively inhibit *E. coli* O157 recovery. We found no association between feed coliform count and *E. coli* O157 presence. Ten feed bunk samples that were positive for *E. coli* O157 had coliform counts that were less than 10. This suggests that fecal contamination may not be the source of *E. coli* O157 in the feed in all cases.

The lack of agreement between the two methods that we used to detect prevalence suggests that there were interactions that allowed one method to detect when the other didn't within the same sample. This lack of agreement could be due to sampling variance from within each composite feed sample, or the enrichment methods we used could have interacted with the feed additives or components present in the feed, environmental conditions at the feed bunk, or other factors unknown to us at this time. Additionally, while the TSB/TSBcv method was the most sensitive in the inoculation study, it did not detect more positives in the field samples. The reasons for this are not clear but may include feed interactions with the method as well. The inoculation study used a total mixed ration for lactating cows from a local dairy that was different from the typical feedlot ration and that did not include ionophores. It is plausible that the TSB/TSBcv method may have been more sensitive for this ration but not for general feedlot rations. Clarification of these possible interactions will require more

investigations of isolation methods with various feed types and additives.

A majority (88.2%) of our isolates possessed a portion of *eaeA*, which has been identified as highly specific for *E. coli* O157:H7 (28), and at least one *stx* gene. The lack of virulence genes in some latex agglutination-positive isolates indicates that the prevalence of *E. coli* O157 could be overreported if PCR was not used. The presence of both Shiga toxin genes in a majority (86.7%) of the confirmed isolates was similar to findings from other studies of *E. coli* O157 isolates from cattle (2, 22, 32).

Further studies are needed to determine sources and associated risk factors for feed contamination before we can establish appropriate safety programs for cattle feed. Possible risk factors for feed contamination are numerous, including components of the feed (5, 6, 20, 29), *E. coli* O157 shedding in calves before they enter the feedlot (9, 21), and manure handling practices (12). To assess this potential risk of colonization in cattle, we need to know the role of feed in the colonization of cattle and persistence of *E. coli* O157 within the feedlot environment. The use of typing methods and geographical mapping of specific *E. coli* O157 isolates (2, 10, 28, 32) will also aid in determining feed contamination sources and links. Further investigations of *E. coli* O157 detection methods and feed-related factors are needed so that we can learn more of the on-farm ecology and develop strategies to prevent food-borne diseases in humans.

ACKNOWLEDGMENTS

This work was supported by U.S. Department of Agriculture CSREES grants 00-35212-9393 and 01-34359-7474.

We thank the feedlot producers in Nebraska, Kansas, Oklahoma, and Texas who participated in this study. We appreciate the technical assistance provided by Ryan Church, Amy Hanson, Mike Hays, and Xiaorong Shi in the laboratory.

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