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Sensitivity of Bacteriologic Culture for Detection of *Escherichia coli* O157:H7 in Bovine Feces

M. W. SANDERSON,[1] J. M. GAY,[1]* D. D. HANCOCK,[1] C. C. GAY,[1]
L. K. FOX,[1] AND T. E. BESSER[2]

Department of Veterinary Clinical Sciences[1] and Department of Microbiology and Pathology,[2] Washington State University, Pullman, Washington 99164

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The sensitivities of several plating and broth enrichment methods for the detection of *Escherichia coli* O157:H7 in (i) bovine fecal samples directly inoculated with *E. coli* O157:H7, (ii) fecal samples from cattle in herds previously positive for *E. coli* O157:H7, and (iii) fecal samples from calves shedding *E. coli* O157:H7 after experimental oral inoculation were compared. Three enrichment protocols and three plating protocols were evaluated with directly inoculated fecal samples. All broth enrichment methods were superior to direct plating when they were combined with subsequent plating on sorbitol-MacConkey with cefixime and tellurite (SMACct). SMACct was the most sensitive plating medium, and the three alternative broth enrichment methods gave similar improvements in sensitivity. Of 351 fecal samples from known positive herds, 24 samples (6.8%) were positive by one or more methods. By the most sensitive plating method, cultures of 10-g samples were slightly more sensitive (19 of 351 [5.4%]) than cotton-tipped swab fecal samples (14 of 351 [4.0%]); however, this difference was not significant. For samples from calves orally inoculated with *E. coli* O157:H7, separation by immunomagnetic beads was slightly more sensitive (79%) than broth enrichment followed by plating at two dilutions (10^{-3} and 10^{-4}) (71%); however, this difference was not significant. The combination of overnight enrichment of swab fecal samples (0.1 g) and plating on SMACct at two dilutions (10^{-3} and 10^{-4}) appears to be a sensitive method for detection in large-scale studies involving hundreds of samples per week.

Escherichia coli O157:H7 was first associated with hemorrhagic colitis in humans in 1982 (15) and was later isolated from cattle (2, 4, 8, 20). Although the epidemiology of this organism is unclear, cattle appear to be a reservoir of *E. coli* O157:H7 through fecal shedding (7, 20).

To study the epidemiology of *E. coli* O157:H7 in bovines, the organism must be identified in the presence of large populations of other bacteria in cattle feces. Selective media for the isolation and identification of *E. coli* O157:H7 from feces were developed to increase detection sensitivity. Differences in sugar fermentation are used to differentiate *E. coli* O157:H7 from other coliforms (14). Sorbitol was initially added to MacConkey medium in place of lactose to differentiate *E. coli* O157:H7 strains, most of which do not ferment sorbitol, from other *E. coli* strains, which are predominantly sorbitol fermenters (10). Further sensitivity improvements resulted from the addition of rhamnose to sorbitol-MacConkey medium (SMAC) (3). Rhamnose is not fermented by *E. coli* O157:H7 as it is by most sorbitol-negative *E. coli* serotypes (3). Low concentrations of the antibiotic cefixime were added to SMAC media to inhibit the growth of *Proteus* species, which are often sorbitol negative and can be confused with *E. coli* O157:H7 on SMAC. Unlike most other *E. coli* serotypes, *E. coli* O157:H7 is β-glucuronidase negative (14). Media containing either 5-bromo-5-chloro-3-indoxyl-β-D-glucuronide (12) or 4-methylumbelliferyl-β-D-glucuronide (19) were developed to exploit this difference. More recently, adding tellurite to SMAC was shown to increase the sensitivity of detection of *E. coli* O157:H7 through differential inhibition of non-O157 *E. coli* and other organisms (22). Immunomagnetic separation (IMS) using O157-specific-antibody-coated beads followed by bacteriologic culture has been shown to improve the detection of *E. coli* O157:H7 in food (13, 21) and feces (5).

Broth enrichment media have not been compared. Many procedures call for the direct streaking of feces onto SMAC plates (5, 17), while others employ buffered peptone water for enrichment before IMS (5).

The general thrust of this study was to identify methods applicable to studies involving large numbers of samples by specifically (i) comparing enrichment and plating media for the detection of *E. coli* O157:H7, (ii) evaluating the sensitivities of new methods, and (iii) determining the effects of increased sensitivity on the apparent prevalence of cattle shedding *E. coli* O157:H7 in known positive herds.

MATERIALS AND METHODS

***E. coli* O157:H7 preparation.** A nalidixic acid-resistant strain of *E. coli* O157:H7 (86-24 Nal-R) was used for experiment 1 (directly inoculated fecal samples) and experiment 3 (fecal samples from orally inoculated calves) (courtesy of P. Tarr, University of Washington). Prior to use, strain 86-24 Nal-R was incubated overnight in L broth at 37°C and diluted to 50% transmission at 540 nm with L broth. The resulting diluted broth contained approximately 2×10^8 CFU/ml and was further diluted as needed for the inoculation of fecal samples. The dilution accuracy was verified by plating each dilution and counting the colonies.

Experiment 1: detection of *E. coli* O157:H7 inoculated directly into bovine fecal samples. To compare bacteriologic plating media and enrichment broth formulations, strain 86-24 Nal-R was inoculated into fecal samples collected from 10 mature lactating dairy cows. Prior to inoculation with *E. coli* O157:H7 (strain 86-24 Nal-R), fecal samples were tested to confirm sample negativity. Each cow's fecal samples were then inoculated at the following three levels: 10, 100, and 1,000 CFU/g. Inoculated feces were mixed with a stomacher for 30 s before samples were taken. Cotton-tipped swabs were used to obtain samples of inoculated feces (0.12 ± 0.02 g per sample [mean ± standard deviation]) for comparison testing.

To compare plating media, swab samples of each inoculated fecal sample were placed in 3 ml of plain tryptic soy broth (TSB) (no. 0370-17-3; Difco, Detroit, Mich.) and incubated overnight at 37°C. The optimal dilutions for each plating medium were established by preliminary experiments. Because of increased bacterial inhibition by tellurite, a lower dilution was required to obtain accept-

* Corresponding author. Phone: (509) 335-0711. Fax: (509) 335-0880.

TABLE 1. Effects of plating media in experiment 1 on the percentages of *E. coli* O157:H7-positive samples detected and the 50% detection limit for each method[a]

Plating medium	% of positive samples detected	50% detection limit (CFU/g)
SMAC	3	2,511
SMACc	25	562
SMACct	70	25

[a] Data are averages for two replicates. TSB was the enrichment broth used with each plating medium.

TABLE 2. Effects of enrichment broths in experiment 1 on the percentages of *E. coli* O157:H7-positive samples detected and the 50% detection limit for each method[a]

Enrichment broth	% of positive samples detected	50% detection limit (CFU/g)
None (direct plating)	37[b]	251
TSBcv	80	13
TSBcvt[c]	77	16

[a] SMACct was the plating medium used with each enrichment broth.
[b] The direct plating result is significantly different than those for enrichment in TSBcv and TSBcvt ($P < 0.01$).
[c] TSBcvt, TSBcv with tellurite (2.5 μg/ml).

able plate growth. After overnight incubation, enriched broths were serially diluted to 10^{-6} with TSB for plating on plain SMAC and on SMAC with 50 ng of cefixime (Lederle Laboratories, Pearl River, N.Y.) per ml (SMACc) and to 10^{-4} with TSB for plating onto SMACc and 2.5 μg of tellurite (Sigma Chemical Co., St. Louis, Mo.) per ml (SMACct).

To compare broth enrichment media, swab samples from each inoculated fecal sample were (i) streaked directly onto SMACct, (ii) incubated in 3 ml of TSB with cefixime (50 ng/ml) and vancomycin (40 μg/ml) (Lyphocin; Lyphomed, Deerfield, Ill.) (TSBcv), and (iii) incubated in 3 ml of TSB with cefixime, vancomycin, and tellurite (2.5 μg/ml). After overnight incubation at 37°C, each enrichment was diluted to 10^{-4} with TSB and plated on SMACct for overnight incubation at 37°C.

To determine if larger-volume samples of feces resulted in increased sensitivity, three 10-g fecal samples were collected from each of 10 mature lactating dairy cows. From each cow, one 10-g fecal sample was left uninoculated, one was inoculated with 4 CFU (0.4 CFU/g), and one was inoculated with 40 CFU (4 CFU/g) of *E. coli* O157:H7 (strain 86-24 Nal-R). Each 10-g fecal sample was then incubated overnight in 250 ml of TSBcv at 37°C, with subsequent dilution to 10^{-4} with TSB for plating on SMACct.

Non-sorbitol-fermenting colonies from SMAC plates from this experiment and subsequent ones were further identified as *E. coli* O157:H7 as previously described (8).

Experiment 2: detection of *E. coli* O157:H7 shedding in samples from naturally colonized dairy cattle. Fecal samples were collected from 234 2- to 4-month-old calves and 117 6- to 8-month-old calves from herds in which *E. coli* O157:H7 had been isolated during the previous year. Ten-gram fecal samples were collected in sterile plastic bags, and swab fecal samples were taken and placed in tubes containing 3 ml of TSBcv each. Samples were stored on ice for up to 24 h before being processed. Ten-gram fecal samples were incubated in 250 ml of TSBcv overnight at 37°C, diluted to 10^{-4} with TSB, and plated on SMACct. Swab fecal samples were incubated overnight in 3 ml of TSBcv at 37°C and then diluted to 10^{-6} for plating on SMACc and to 10^{-4} for plating on SMACct.

Experiment 3: detection of *E. coli* O157:H7 shedding in samples from calves orally inoculated with strain 86-24 Nal-R. Four 1-day-old calves were obtained from a commercial dairy and transported to an isolation facility. Calves were acclimated to the isolation facility and tested three times for fecal shedding of *E. coli* O157:H7 over a 1-week period by using rectal swab samples incubated overnight in 3 ml of TSBcv and subsequently diluted to 10^{-4} and plated on SMACct. Calves were then inoculated by a milk meal containing 5×10^8 CFU of *E. coli* O157:H7 (strain 86-24 Nal-R). Calf feces were sampled three times weekly, and fecal shedding of strain 86-24 Nal-R was quantified at each sampling as follows: feces were diluted to 10^{-1} and 10^{-2} with TSB containing 20 μg of nalidixic acid per ml. From the 10^{-1} dilution, 300 μl was spread on each of 10 MacConkey plates containing 20 μg of nalidixic acid per ml. In addition, 300 μl of the 10^{-2} dilution was spread on one MacConkey plate containing 20 μg of nalidixic acid per ml. Average colony counts were divided by the dilution factor to calculate shedding levels (in CFU per gram).

Five methods of detection were compared with all fecal samples. Samples of feces obtained with swabs were incubated in 3 ml of TSBcv and diluted to 10^{-6} with TSB for plating on SMACc (method 1) and to 10^{-4} with TSB for plating onto SMACct (method 2). On the basis of communication with another laboratory (18a), plating of a 10^{-3} dilution on SMACct (method 3) was added. Ten-gram fecal samples from each calf were incubated overnight in 250 ml of TSBcv and plated at 10^{-4} on SMACct (method 4). Swab fecal samples incubated in 3 ml of TSBcv were also used to perform IMS of samples incubated for 6 h with subsequent plating on SMACct (method 5). (Dynabeads [anti-*E. coli* O157; Dynal, Oslo, Norway] were used for IMS.)

Analytical methods. Methods were compared on the basis of discordant results from the same fecal sample. Differences between methods were compared by using an exact McNemar's test for paired comparisons (11). The 50% detection limit for experiment 1 was calculated by the method of Karber for 10-fold dilutions (6). The 50% detection limits for the methods used in experiment 3, in which colony counts per gram varied randomly, were calculated by logistic regression of detection on $\log_{10}$ of shedding (9).

RESULTS

Experiment 1: detection of *E. coli* O157:H7 inoculated directly into bovine fecal samples. The average effects of plating media on the percentages of positive samples detected for two replicates and the 50% detection limits of *E. coli* O157:H7 are summarized in Table 1. For both replicates, plating on SMACct was more sensitive than plating on SMAC or SMACc ($P < 0.01$), and plating on SMACc tended to be more sensitive than plating on SMAC.

Table 2 summarizes the effects of broth enrichment on the percentages of positive samples detected and the 50% detection limits. Broth enrichment improved the sensitivity of direct plating ($P < 0.01$), and no differences between the broth enrichment media were found. TSBcv was chosen as the broth enrichment medium for further comparisons.

Three of 10 samples inoculated at 4 CFU/g and 3 of 10 samples inoculated at 0.4 CFU/g were detected among large-volume (10-g) fecal samples incubated in TSBcv.

Experiment 2: detection of *E. coli* O157:H7 shedding in samples from naturally colonized dairy cattle. As summarized in Table 3, the incubation of 10-g fecal samples in TSBcv and subsequent plating on SMACct was more sensitive for detecting *E. coli* O157:H7 in samples from naturally colonized cattle than was the incubation of swab fecal samples in TSBcv and subsequent plating on SMACc ($P < 0.01$). More positive samples were detected by using large-volume (10-g) fecal samples than swab fecal samples for plating on SMACct; however, the difference was not significant ($P = 0.15$).

Experiment 3: detection of *E. coli* O157:H7 shedding in samples from calves orally inoculated with strain 86-24 Nal-R. For analysis of experiment 3, fecal samples were categorized into four groups by the following strain 86-24 Nal-R concentrations: <1,000 CFU/g, 1,000 to 10,000 CFU/g, 10,000 to 100,000 CFU/g, and >100,000 CFU/g. Positive samples were

TABLE 3. Comparison of detection methods with samples from naturally colonized cattle in experiment 2[a]

Method	No. of samples positive/no. tested	Prevalence (%)[b]	Relative sensitivity (%) vs:	
			All methods	Large-volume (10-g) samples
SMACc	8/351	2.3*	33	42
SMACct	14/351	4.0*,**	58	74
Large-volume (10-g) samples	19/351	5.4**	79	
All methods	24/351	6.8		

[a] All samples were incubated in TSBcv.
[b] Prevalence pairs without the same pair of asterisks are significantly different ($P < 0.01$).

TABLE 4. Percentages of positive samples detected and the 50% detection limit for each method in experiment 3[a]

Method [dilution(s)]	% of positive samples detected (*n*)	50% detection limit (CFU/g)
1, SMACc (10^{-6})	42**** (52)	10^4
2, SMACct (10^{-4})	50*** (52)	10^3
3, SMACct (10^{-4}, 10^{-3})	71*,**,*** (31)	10^2
4, Large-volume (10-g) samples (10^{-4})	58**,*** (52)	10^3
5, 6-h IMS	79* (52)	10^2

[a] All samples were incubated in TSBcv.
[b] Percentage pairs without the same pairs of asterisks are significantly different ($P < 0.01$).

distributed approximately equally among the four shedding levels. Calves shed for an average of 32 days after inoculation, with a range of 24 to 43 days.

Table 4 summarizes the percentages of positive samples detected and the 50% detection limit for each test. The 6-h IMS method detected the most positive samples but was not significantly more sensitive than broth enrichments of swab fecal samples plated on SMACct at 10^{-3} and 10^{-4} ($P = 0.25$).

DISCUSSION

The goal of this study was to determine the relative sensitivities of several methods of detecting *E. coli* O157:H7 in bovine fecal samples. The results reported here are consistent with those of other reports that have compared different plating media for detection in feces (3, 5, 22). On the basis of this study, enrichment in TSBcv followed by plating on SMACct is a sensitive and practical method for the detection of *E. coli* O157:H7 in bovine fecal samples. In our study, the differences in sensitivity between swab fecal-sample enrichments plated at 10^{-3} and 10^{-4} on SMACct and IMS of 6-h enrichments were small and statistically insignificant. The 10^{-3} plating, however, was not included until the calf inoculation study was already under way and was applied to only 31 positive samples, limiting the power to detect a difference. Even with this power limitation, the differences in sensitivity between swab fecal-sample enrichments plated on SMACct at 10^{-3} and 10^{-4} and 6-h IMS do not appear to be large.

On the basis of the results of experiment 2 (detection in samples from naturally colonized herds), the addition of tellurite to the plating medium results in an approximately twofold increase in apparent prevalence. The prevalence of all methods applied in parallel was three times higher than that of the least sensitive method (TSBcv enrichment plated on SMACc). Additionally, in inoculated fecal samples, the use of TSBcv enrichments was significantly more sensitive than direct plating, suggesting that application of the more sensitive methods reported in this study may increase apparent prevalence by several times over those stated in previous reports that used direct plating (1, 2, 4, 5, 8). This is supported by the fourfold increase in prevalence found in one study when the use of buffered peptone water enrichment followed by IMS was compared with direct plating (5).

An important question regarding the prevalence of *E. coli* O157:H7 in cattle is whether there is a large population of colonized cattle that are not detected by current methods because of low-level shedding. The calves in experiment 3 shed for an average of 32 days after inoculation, with a range of 24 to 43 days, and positive samples were approximately equally divided among the four shedding levels. These calves did not shed for an extended period at low but detectable levels nor did they resume shedding during a 3-week follow-up period after the last detected positive sample. The prevalence of *E. coli* O157:H7 fecal shedding must be interpreted with caution. The minimum detection threshold for experiment 3 (orally inoculated calves) suggests that if cattle shed *E. coli* O157:H7 at less than 100 CFU/g of feces, they would not be consistently detected. Prevalence could be higher. In balance, the data from this study suggest that the prevalence of fecal shedding in herds in which *E. coli* O157:H7 is endemic is seldom more than 10%, even with the most sensitive tests and multiple tests in series.

The minimum detection threshold was markedly different between experiment 1 (directly inoculated fecal samples) and experiment 3 (fecal samples from orally inoculated calves) (Tables 1 and 3). This difference in detection level may be due to differences in the physiologic state of strains used in direct feces inoculation studies compared with that of feces-adapted organisms. Environmentally inducible enzyme changes have been described for *E. coli* (16). *E. coli* O157:H7 isolates grown in medium for direct inoculation of fecal samples followed by enrichment in growth medium may have physiologic advantages over competing fecal species sufficient to allow them to overgrow competing species and be detected more easily. Alternatively, this difference may be due to the effects of competing flora in bovine feces on the detection of *E. coli* O157:H7. Fecal samples for inoculation were taken from mature lactating dairy cows on a typical milking ration, while inoculated calves were 1 to 7 weeks old and on a diet of milk and concentrate. The differences in intestinal flora between milk-fed and mature cattle have previously been described (18). Competing floral differences may have an impact on the sensitivity of detection of *E. coli* O157:H7, making it more difficult to detect in samples from young or milk-fed animals. However, previous studies have detected the lowest prevalence of *E. coli* O157:H7 in samples from preweaned calves and mature cows (8). Despite the differences in detection limits, the order of tests from least sensitive to most sensitive remained unchanged for all three experiments.

In conclusion, the culture of 0.1-g fecal samples on cotton-tipped swabs with TSBcv for enrichment followed by the plating of 10^{-3} and 10^{-4} dilutions on SMACct appears to be a cost-effective and sensitive method for large-scale studies involving hundreds of samples per week. The culture of large-volume (10-g) fecal samples and the use of IMS provided relatively small additional increases in sensitivity.

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