Sensitivity of *Escherichia coli* O157 Detection in Bovine Feces Assessed by Broth Enrichment followed by Immunomagnetic Separation and Direct Plating Methodologies

Jeffrey T. LeJeune,^{1*} Dale D. Hancock,² and Thomas E. Besser³

Food Animal Health Research Program, Ohio Agricultural Research and Development Center, The Ohio State University, 1680 Madison Ave., Wooster, Ohio 44691,¹ and Department of Veterinary Microbiology and Pathology² and Department of Veterinary Clinical Sciences,³ College of Veterinary Medicine, Washington State University, Pullman, Washington 99164

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In order to more precisely predict food safety risks, the fecal presence of food-borne pathogens among animals at slaughter must be correctly determined. Quantification of *Escherichia coli* O157 is also desirable. In two separate experiments, detection and enumeration of a nalidixic acid-resistant strain of *E. coli* O157 in bovine feces was assessed by culture on MacConkey agar supplemented with nalidixic acid (MACnal) and compared to overnight broth enrichment followed by immunomagnetic separation (IMS) and to direct plating of dilutions of bovine feces onto sorbitol MacConkey agar containing cefixime and tellurite (SMACct). The sensitivity of detection of *E. coli* O157 by both direct plating and IMS was highly dependent upon the initial concentration of the target organism in the sample. Sensitivity of detection by IMS was poor below 100 CFU/g but was better, and not affected by initial *E. coli* O157 numbers, above this concentration. Sensitivity of detection of *E. coli* O157 in bovine feces at low initial concentrations is very poor for both direct plating and IMS. Direct plating of dilutions of bovine feces on SMACct can be used to determine the magnitude of fecal *E. coli* excretion among cattle excreting greater than 100 CFU/g. Among positive samples identified by direct plating on SMACct, the direct counts of *E. coli* O157:H7 were highly correlated with the estimates obtained with the MACnal plates ($r = 0.88$, $P < 0.001$). Because the majority of cattle excrete less than 10^2 CFU *E. coli* O157/g feces, most studies, including those using IMS methods, probably grossly underestimate the prevalence of *E. coli* O157 in cattle.

Contamination of foods, water, and other environmental niches with bovine manure has emerged as an important public health concern, especially with regard to the epidemiology of *Escherichia coli* O157:H7 infections in both humans and animals. Since cattle harbor *E. coli* O157:H7 in their digestive tracts, albeit transiently, they have been considered the primary reservoir for this important food-borne pathogen. Extensive efforts to understand the epidemiology of *E. coli* O157:H7 in the bovine host have been made. Theoretical models and empirical data have demonstrated that control of the magnitude and prevalence of *E. coli* O157:H7 in live animals (preharvest) may have a great impact on reducing the rate of contamination of foods of bovine origin with this pathogen (4, 7). However, to date, limited data on the magnitude of fecal *E. coli* O157:H7 shedding among animals destined for slaughter are available. In order to quantify the risks associated with the slaughter of *E. coli* O157:H7-positive animals and the effectiveness of potential preharvest mitigation strategies, accurate estimates of the prevalence and the frequency distribution of *E. coli* O157:H7 concentrations in bovine feces are required.

To increase the diagnostic sensitivity, enrichment of large volumes (10 g or more) of bovine feces, followed by immunomagnetic separation (IMS), has been widely used for epidemi-

ological studies of *E. coli* O157 in cattle, despite the limited information on the sensitivity of this method with naturally contaminated bovine fecal samples (9, 11). Coupled with the reputed increase in assay sensitivity through the use of enrichment culture is the loss of the ability to directly quantify the concentration of *E. coli* O157:H7 present in a sample. The objectives of this study were to establish the analytical sensitivity of detection of *E. coli* O157:H7 in bovine feces by use of a direct plating method with the commonly used sorbitol MacConkey agar (containing 50 ng/ml cefixime and 2.5 μ g/ml potassium tellurite [SMACct]) and enrichment/IMS methodology. In addition, it was of particular interest to assess how well the concentration of *E. coli* O157:H7 estimated by direct plating correlated with the concentration estimates obtained by plating on a highly selective MacConkey agar supplemented with 20 μ g/ml nalidixic acid (MACnal).

MATERIALS AND METHODS

Source of bovine feces. Fecal specimens for the two experiments were obtained from multiple groups of 10-week-old male Holstein calves housed in a biosafety level 2 facility. All calves were first screened by use of overnight broth enrichment cultures followed by IMS (9) to ensure that no detectable fecal shedding of *E. coli* O157 was present. For each group, a single calf was orally challenged with 10 ml of an overnight broth culture (ca. 10^9 CFU) of a nalidixic acid-resistant strain of *E. coli* O157 (86-24nalR). Twenty-four to 48 h postinoculation, each inoculated calf was then introduced into a small pen containing three to five other uninoculated 10-week-old male calves, which subsequently began fecal shedding after transmission of the agent from the inoculated calf. Feces were collected from all calves in each pen at 3- to 4-day intervals for a period of up to 6 weeks.

* Corresponding author. Mailing address: Food Animal Health Research Program, Ohio Agricultural Research and Development Center, The Ohio State University, 1680 Madison Ave., Wooster, OH 44691. Phone: (330) 263-3739. Fax: (330) 263-3677. E-mail: lejeune.3@osu.edu.

FIG. 1. Sensitivity of detection of *E. coli* O157 in bovine feces, assessed using IMS. Results in column labeled "a" are statistically different ($P < 0.001$) from results in columns labeled "b."

Experiment 1: detection of *E. coli* O157 by IMS. For experiment 1, a total of 234 fecal samples were collected from 29 different calves on 12 different occasions. Each 10-g sample was combined with 90 ml tryptic soy broth (Becton Dickinson, Franklin Lakes, NJ) containing cefixime (50 ng/ml) and vancomycin (40 µg/ml). An aliquot of 1 ml of the fecal homogenate was spread plated onto 150-mm MACnals and incubated overnight at 37°C. The balance of the homogenate was also incubated overnight at 37°C, creating overnight enrichments. Following overnight incubation, the MACnals plates were screened for the presence of lactose-positive colonies. These colonies were enumerated, and then suspects were confirmed as *E. coli* O157 based on color of colonies on sorbitol MacConkey media and agglutination of latex beads coated with an anti-O157 antibody (Oxoid).

E. coli O157 colonies present in 1 ml of the overnight enrichments were concentrated using anti-O157 specific immunomagnetic beads by automated IMS (AIMS) according to the manufacturer's recommendations (Dynal, Oslo, Norway). Briefly, recovered beads were plated on SMACct and incubated overnight at 37°C. Up to five sorbitol-negative colonies on each SMACct plate were subsequently screened for growth on MACnals, growth and absence of fluorescence on EC medium (Becton Dickinson) with 100 µg/ml 4-methylumbelliferyl-β-D-glucuronide, agglutination with a latex agglutination test kit, and PCR amplification of *E. coli* O157-specific targets (6).

Experiment 2: enumeration of *E. coli* O157 colonies on SMACct. Two hundred eighty-one fecal samples collected from 27 different calves on 32 different sampling occasions were used in experiment 2. Although the calves used for feces collection for experiments 1 and 2 were exposed using the same challenge methodology, they were not the same animals. Each 1-g sample was suspended in 9 ml of tryptic soy broth and subsequently further diluted to 10⁻⁴. Three hundred microliters of the 10⁻³ dilution was spread onto 150-mm SMACct plates. One ml of each suspension was also spread plated onto a 150-mm MACnals plate, and suspect colonies were confirmed as *E. coli* O157 as described above. Remaining dilutions were stored at 4°C. Following incubation for 18 to 24 h at 37°C, sorbitol-negative colonies on SMACct plates were enumerated. Ten representative sorbitol-negative colonies were selected and tested for reaction with an anti-O157 latex agglutination kit. The number of sorbitol-negative colonies was multiplied by the fraction of latex-positive colonies and the fecal dilution to estimate the number of *E. coli* O157 colonies present in each gram of feces. Samples from which no isolated colonies could be obtained because of bacterial overgrowth were replated from higher dilutions of the stored aliquots required to obtain isolated colonies.

Data analysis. The concentration of nalidixic acid-resistant *E. coli* O157 in each fecal sample as determined with the MACnals plates was used as the gold standard. The probability of detecting *E. coli* O157 by direct plating and IMS as a function of the bacterial numbers on the MACnals plates was modeled using logistic regression (3). The Wald statistic was used to assess the statistical significance model coefficients. Goodness of fit was assessed using a Hosmer-Lemeshow goodness-of-fit test. In the case where logistic models failed to adequately fit the data, samples were stratified based on *E. coli* O157 concentrations, and then the distribution of positive tests among concentration groups was

FIG. 2. Likelihood model of detection of *E. coli* O157 from bovine fecal samples by use of direct plating methodology with SMACct.

compared using a chi-square goodness-of-fit test (17). For all samples in which the SMACct plates yielded positive results, Pearson correlation coefficients (r) were calculated for the relationships between the concentrations of *E. coli* O157 determined by use of SMACct and MACnals. All analyses were conducted using the SAS system for Windows (8.02; SAS, Cary, NC).

RESULTS

In experiment 1, 234 samples were plated on MACnals and tested by IMS. Of these 234 samples, 89 samples tested negative by both assays. Plating on MACnals detected 135 positive samples (57% prevalence), including 64 samples that were negative by AIMS. The AIMS procedure did, however, detect 10 positive samples that were negative by MACnals plating. Considering a positive result by either method as the gold standard, the overall sensitivities of MACnals and IMS were 93% and 56%, respectively. The increase in sensitivity of detection by IMS did not linearly increase with initial concentration of *E. coli* O157 in the sample, nor did the distribution of observed datum points closely fit a logistic model (P value by the Hosmer-Lemeshow goodness-of-fit test was 0.0375). Therefore, chi-square analyses were conducted. Samples containing between 10 and 99 CFU *E. coli* O157/g were poorly detected using IMS, while samples with any concentration at or greater than 100 CFU/g were identified at equal frequencies (Fig. 1).

In experiment 2, one or more *E. coli* O157 colonies were identified on 224 MACnals plates from the 281 fecal samples tested (80% prevalence). One hundred thirty of the 224 MACnals-positive samples had one or more confirmed colonies of *E. coli* O157 on the corresponding SMACct plate, resulting in a 58% sensitivity of detection by SMACct plating when MACnals was used as the gold standard. However, the probability of detection by direct plating increased to greater than 80% at high initial concentrations of *E. coli* O157 ($P < 0.001$) (Fig. 2). There was no evidence of a lack of fit of these data to a logistic model ($P < 0.444$). Among 130 samples having *E. coli* O157 colonies identified by SMACct culture, counts obtained with SMACct were strongly correlated with counts on MACnals ($r = 0.88$, $P < 0.001$) (Fig. 3).

FIG. 3. Correlation between *E. coli* O157 concentrations, as determined by plating on MACnal and positive SMACct samples ($r = 0.88$, $P < 0.001$).

DISCUSSION

Two conclusions can be drawn from the experiments described herein. First, the results of this study demonstrate the poor sensitivity of both IMS and direct plating for detecting *E. coli* O157 at low concentrations in bovine feces. Second, the value of direct plating of fecal dilutions to enumerate *E. coli* O157:H7 colonies in bovine feces at concentrations greater than 10^2 CFU/g was ascertained. Importantly, for these experiments, *E. coli* O157:H7 was present in the samples at concentrations, in the metabolic state, and admixed with background bacterial flora expected to be found under natural conditions. The use of a nalidixic acid-resistant strain of *E. coli* O157:H7 for calf challenge permitted the sensitive detection of the bacterium for use as the known standard from which comparisons with other culture methods could be made. Furthermore, the high prevalence of positive samples (80% and 57%) obtained from the "in-contact" calves provided a large number of fecal samples from which sensitivity analyses could be conducted.

The use of IMS has been reported to improve the sensitivity of *E. coli* O157 detection in human and bovine feces (1, 2). Theoretically, enrichment followed by IMS should be able to detect as little as a single organism present in the initial sample. In the laboratory, however, the type and number of background bacterial flora, the type of broth used, the temperature, and the incubation time impact the sensitivity of detection (10, 16, 18). The reliable detection limit of >100 CFU/g of feces which we report herein is comparable to the results that Omisakin et al. reported from a much smaller number of naturally colonized cattle (11).

A recent attempt to retain sensitivity and at the same time obtain quantitative results employed the most-probable-number analysis of IMS enriched samples (5). However, such methods are extremely labor intensive and consume large amounts of materials. Furthermore, because of the poor sensitivity of detection at low initial concentrations, these methods are likely to provide a high rate of false-negative test results at high dilutions of feces; therefore, the results should be interpreted with caution. An alternative to most-probable-number IMS for

quantitative measures of *E. coli* O157 in bovine feces is the direct plating of bovine fecal samples.

The detection of *E. coli* O157 by direct plating is limited by the total number of coliforms in each sample that can proliferate on the SMACct plates. The proportion of *E. coli* O157:H7 colonies among sorbitol-negative suspect colonies and dilution of feces required to obtain isolated colonies on the spread plates may reflect the microbial ecology of the gastrointestinal tract of the animal and be affected by the animal's age, diet, or environment. Supplements (cefexime and tellurite) to the SMAC successfully reduce growth of the non-*E. coli* O157 background flora present in enrichment cultures, thereby allowing for lower dilutions of sample to be plated (2). Likewise, the use of higher incubation temperatures during enrichment also affects the ratio of *E. coli* O157 to background flora (10). For most samples, plating of the 10^{-3} dilution was required to obtain isolated colonies on the SMACct plates. Nevertheless, despite these limitations, the direct plating method was highly correlated with the experimental (MACnal) plating method in terms of numbers of organisms detected and was successful in identifying greater than 80% of the positive samples which had over 100 CFU/g. Enumeration was possible with the direct plating method. The level of sensitivity of direct plating determined in this study is comparable to that determined by previous work (14, 15).

The consequences of low sensitivity for both the direct plating and the IMS procedure to studies on bovine epidemiology and food safety are unknown. Clearly, the diagnostic sensitivities of these assays are dependent upon the analytical sensitivity of the assay and the distribution of animals excreting numbers of organisms above the detection threshold. Most (61 to 85%) adult cattle excrete less than 100 CFU/g (8, 11, 12). Animals excreting low numbers of organisms contribute little to the overall environmental or food contamination relative to the contributions of animals excreting large numbers of organisms. As proposed by Omisakin et al., the magnitude of fecal *E. coli* O157 excretion may be more important than the prevalence (11). Thus, it may be more important to identify the high-shedding animals to be able to develop control measures targeted at these animals (13). The low sensitivity and variability due to Poisson variation of *E. coli* O157 cells at low initial bacterial concentrations may partially explain the apparent nonhomogeneous distribution of this organism in bovine fecal pats (12). Conversely, if numbers of bacteria vary greatly from gram to gram within the same fecal pat, some of the differences we observed between the MACnal and SMACct samples could be attributed to true differences in concentrations between samples. Because of this potential for heterogenic distribution of target bacteria in fecal pats, increasing the total weight of the sample analyzed may have impacted the sensitivity of the analysis. Using 10 g of feces mixed with 90 ml of diluent may further enhance the sensitivity of the direct plating method. Furthermore, the number of *E. coli* O157 organisms may have changed between the time the MACnal counts were determined and the time the IMS cultures were conducted. Wang et al. reported an increase in the concentration of *E. coli* O157 organisms in feces during the first 2 days of storage at 37°C and 22°C but not at 5°C (19). These two factors may account for the observation that 10 samples were positive by IMS (10 g of

sample used) but tested negative with the MACnals plates (1 g of sample used).

Quantitative data regarding the magnitude of fecal shedding available by direct plating of *E. coli* O157 can assist in refining risk assessment models for this important human pathogen. Although IMS appeared to be more sensitive than direct plating for detecting samples with small numbers of organisms, quantitative data were not available. The type of microbiological test used should be selected based on the desired outcome parameter of most interest: prevalence or quantitative results. One method to optimize sensitivity of detection and at the same time obtain quantitative data with the minimal amount of resources would be to first subject a portion of all samples to IMS, reserving the original fecal samples, and to subsequently directly plate for enumeration portions of the reserved samples that tested positive by IMS.

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