Journal of Food Protection, Vol. 71, No. 11, 2008, Pages 2233–2238

A Direct Plating Method for Estimating Populations of *Escherichia coli* O157 in Bovine Manure and Manure-Based Materials†

ELAINE D. BERRY* AND JAMES E. WELLS

U.S. Department of Agriculture, Agricultural Research Service, Roman L. Hruska U.S. Meat Animal Research Center, P.O. Box 166, Spur 18D, Clay Center, Nebraska 68933-0166, USA

MS 08-079: Received 13 February 2008/Accepted 3 July 2008

ABSTRACT

Escherichia coli O157:H7 outbreaks associated with produce consumption have brought attention to livestock manures and manure-based soil amendments as potential sources of pathogens for the contamination of these crops. Procedures for enumeration of *E. coli* O157:H7 are needed to assess the risks of transmission from these manures and their by-products. A direct plating method employing spiral plating onto CHROMagar O157 was investigated for enumeration of *E. coli* O157:H7 in feedlot surface material, aged bovine manure, bovine manure compost, and manure-amended soil. In studies utilizing samples spiked with a five-strain cocktail of *E. coli* O157:H7 at levels ranging from 10^2 to 10^5 CFU/g of sample, there were strong correlations between the observed and predicted levels of this pathogen. Although the addition of 2.5 mg/liter potassium tellurite and 5 mg/liter novobiocin made the medium more restrictive, these amendments enhanced the ability to identify and enumerate *E. coli* O157:H7 in feedlot surface material, which contained a higher proportion of fresh feces than did the other three sample types and therefore higher levels of interfering bacterial microflora. The spiral plating method was further assessed to determine its ability to enumerate *E. coli* O157:H7 in naturally contaminated feedlot surface material. Comparison of *E. coli* O157:H7 counts in feedlot surface material obtained by the spiral plating method and a most probable number technique were well correlated. We conclude that direct spiral plating onto CHROMagar O157 is effective for estimating *E. coli* O157:H7 levels in a variety of manures and manure-containing sample types to a lower detection limit of 200 CFU/g. The method has application for determining *E. coli* O157:H7 concentrations in manures and composts before their sale and use as soil amendments and for measuring the effectiveness of manure treatment processes to reduce or inactivate this pathogen.

Recent outbreaks of *Escherichia coli* O157:H7 infections linked to the consumption of spinach, lettuce, and other produce crops have focused attention on animal manures and manure-based soil amendments as potential sources of pathogens that can contaminate fruits and vegetables *(1, 10, 14, 30). E. coli* O157:H7 can be transmitted to food crops when manure containing these organisms is used as fertilizer or soil amendment or because of other inadvertent contact *(11, 25).* Runoff from animal feeding operations, manure storage, or manure-amended fields may contaminate fruit and vegetable crops or water that is used for human consumption or irrigation *(21, 27). E. coli* O157:H7 can contaminate and attach to external surfaces of plant tissues and may be internalized within the tissues when plants are grown in soil or water containing the pathogen *(33, 34, 36, 37, 39).*

With increases in produce-related outbreaks of *E. coli* O157:H7 infection *(30)* and the increased attention on manures and soils as sources of this microorganism, the occurrence and persistence of this pathogen in manure and soils has been a current research topic. As a result of this research, we now understand that *E. coli* O157:H7 can survive for long periods in manures after being shed by livestock *(8, 23, 38). E. coli* can persist up to several months and sometimes multiply in soils, including feedlot soils, pasture soils, and soils amended with contaminated manure *(3, 5, 7, 16, 24, 26).* An additional important component for assessing the risk of transmission of *E. coli* O157:H7 from manures, manure by-products, and manure-containing soils is the determination of the pathogen load in the materials in question. Methods for determining the levels of this organism in manures also are needed for establishing the effectiveness of treatment processes designed to reduce *E. coli* O157:H7 in manure and manure by-products. Brichta-Harhay et al. *(9)* recently described a spiral plate count method and media for enumeration of *E. coli* O157:H7 in bovine feces and on hides. Our objective was to evaluate the ability of this method to determine the levels of *E. coli* O157:H7 in aged bovine manure, bovine manure compost, feedlot surface material, and manure-amended soils.

MATERIALS AND METHODS

Compost, aged manure, manure-amended soils, and feedlot surface material. Mature compost was obtained from Dr. Daniel Miller (Agroecosystem Management Research Unit, U.S. Department of Agriculture, Agricultural Research Service, Lincoln, Nebr.). The compost was produced from bovine manure scraped from beef cattle feedlot pens, which was then windrowed and

* Author for correspondence. Tel: 402-762-4204; Fax: 402-762-4209; E-mail: elaine.berry@ars.usda.gov.

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turned approximately every 20 days over a 3-month period from July through early October 2006. Twenty separate compost samples were collected from different locations along the windrow and stored at −20°C until they were thawed and distributed among the experimental samples.

Aged bovine manure was collected from manure stockpiles located at the U.S. Meat Animal Research Center (USMARC). These stockpiles were composed of manure scraped from beef cattle feedlot pens at the USMARC feedlot during the summer of 2006 and represented manure from animals fed a variety of standard feedlot growing and finishing diets *(6).* The aged manure samples were collected from the stockpiles in February 2007, the week before they were used in experiments. Five separate manure samples were collected from different locations among the stockpiles, stored at 4°C, and distributed among the experimental samples.

Manure-amended soil (MAS) was collected 24 h after manure application to a USMARC research plot in early May 2007. The manure used in this application was aged manure from the same stockpiles described above; the manure was applied with a manure spreader at a rate of approximately 27,000 kg/ha (dry basis) and worked into the soil with a tandem offset disk to a depth of 10 to 15 cm. The soil at this site is a Crete silt loam (fine, smectitic, mesic Pachic Argiustolls). The MAS samples were collected to a depth of 10 cm at different locations throughout the plot, pooled, and stored at 4°C for up to 1 week before they were used in experiments.

Feedlot surface material (FSM) was collected from beef cattle feedlot pens at the USMARC feedlot on two different weeks in early March 2007 on the days before the experiments. At each time, separate samples were obtained from each of five different pens by collection and pooling of FSM taken from the pen surface just behind and along the length of the concrete feed bunk apron. Care was taken to avoid including freshly dropped feces in the FSM samples. The FSM samples were distributed among the experimental samples.

***E. coli* O157 inocula preparation.** For testing the direct plating method, a cocktail of five *E. coli* O157:H7 isolates was used to spike compost, aged manure, MAS, and FSM. The five isolates were reference strain *E. coli* O157:H7 ATCC 43895 and four bovine isolates *E. coli* O157:H7 96AC1, 258AC1, 294RC1, and 233AC1. The isolation of the bovine *E. coli* O157:H7 is described elsewhere *(12).* All five *E. coli* O157:H7 strains represent unique pulsed-field gel electrophoresis subtype groups *(4).* The cultures were inoculated from frozen glycerol stocks into separate 5-ml tubes of tryptic soy broth (TSB; Becton, Dickinson and Company, Sparks, Md.), which were incubated for 18 h at 37°C. One-milliliter volumes of each culture were collected by centrifugation (9,300 × *g* for 5 min), and the pellets were resuspended in 1 ml of buffered peptone water (BPW; Becton, Dickinson and Company). The separate 1-ml volumes were combined into a sterile tube to make the five-strain cocktail, which was diluted further in additional BPW to provide inocula with seven different concentrations of *E. coli* O157:H7 such that inoculation at the 1% level (0.5 ml of inoculum) into 50-g samples of the compost, manures, or soils provided seven different *E. coli* O157:H7 concentrations ranging from 10^2 to 10^5 CFU/g (wet weight). Levels of the pathogen in each of the seven inocula were determined by serial dilution in BPW and spiral plating onto tryptic soy agar (TSA; Becton, Dickinson and Company) plates using an Autoplate 4000 spiral plater (Spiral Biotech, Inc., Norwood, Mass.). The TSA plates were incubated at 37°C for 20 to 24 h, and colonies were enumerated. The inocula also were plated onto the three test media described below and incubated at 42°C, and the colonies were enumerated.

Testing of direct plating method and media for enumerating *E. coli* O157:H7 in spiked compost, aged manure, MAS, and FSM. The chromogenic medium CHROMagar O157 (CHROM; DRG International, Mountainside, N.J.) was the base medium tested. CHROMagar O157 containing 2.5 mg/liter potassium tellurite (T-CHROM) and CHROMagar O157 containing 2.5 mg/liter potassium tellurite and 5 mg/liter novobiocin (nt-CHROM) also were used.

Experiments for compost, aged manure, MAS, and FSM were performed at separate times. Samples (50 g) of the compost, aged manure, MAS, or FSM were measured into sterile Whirl-Pak bags (Nasco International, Inc., Ft. Atkinson, Wis.) and brought to room temperature before inoculation. Forty-two samples of each sample type were examined, 21 of each on two separate days. On each day, triplicate samples were inoculated at each of the seven different inoculation levels by dispensing 0.5 ml of the appropriate inoculum into the 50-g sample bags. Using a pipettor, the 0.5 ml of inoculum was spotted throughout the sample mass. The sample bags were then extensively massaged and shaken to distribute the inoculum throughout the sample. All inoculated samples were subsampled for *E. coli* O157:H7 enumeration within 1 h of inoculation. Ten grams of each sample was measured into a sterile filtered sample bag, and 90 ml of TSB was added. The contents of the bag were mixed by thorough massaging of the bag. The filtered sample was diluted further in BPW as necessary, left briefly to allow debris to settle, and spiral plated in duplicate onto CHROM, T-CHROM, and ntCHROM plates, except for the samples that were inoculated at the lowest *E. coli* O157:H7 level, which were plated in quadruplicate. All plates were incubated at 42°C for 20 to 24 h. *E. coli* O157 colonies on CHROMagar O157 are flat and mauve colored, typically without distinct centers. To confirm their identities, five or more colonies (as available or as needed) were tested for agglutination with *E. coli* O157 latex agglutination test reagents (Oxoid, Basingstoke, UK).

Enumeration of *E. coli* O157:H7 from naturally contaminated FSM. The direct plating method was further tested by examining its ability to enumerate *E. coli* O157:H7 in FSM that was naturally contaminated with this pathogen. For comparative purposes, a most-probable-number (MPN) procedure was used to estimate levels of *E. coli* O157:H7 in the same FSM samples.

To improve the probability of obtaining FSM with enumerable *E. coli* O157:H7, we collected FSM from USMARC feedlot pens during the summer months, when the reported incidence of fecal shedding of this pathogen is typically the highest *(2).* A total of 250 FSM samples were collected from 16 different pens from June through August 2007. Samples were returned to the laboratory for immediate processing. Ten grams of FSM was diluted and processed in 90 ml of TSB as described above, and this 10^{-1} dilution was spiral plated onto ntCHROM plates. The plates were incubated at 42°C for 20 to 24 h prior to examination for and enumeration of *E. coli* O157 colonies. Suspect *E. coli* O157 colonies were tested for agglutination with *E. coli* O157 latex agglutination test reagents, and identity was further confirmed by PCR for genes specific for enterohemorrhagic *E. coli* and *E. coli* O157 *(15).*

For enumeration of *E. coli* O157 with the MPN procedure, portions of the remaining 10^{-1} FSM sample dilution were used in a three-replicate tube MPN with immunomagnetic separation (IMS) and detection as described by Berry and Miller *(5).* Three 125-μl aliquots of each FSM sample dilution were transferred to

deep-well microtiter blocks in which each well contained 1,125 μl of TSB. After mixing the sample into the three wells, an additional three serial 10^{-1} dilutions were prepared from each of the three inoculated wells, for up to 12 wells per sample. The MPN dilutions were incubated at 37°C for 7 h and then held at 4°C until the next day, when the spiral-plated ntCHROM results were available. For reasons of economy and because of the lower threshold of detection of the MPN method (26.7 MPN/g for the MPN versus 200 CFU/g for the direct spiral plating method), only FSM samples for which an *E. coli* O157 count could be obtained via the direct plating method were processed further to obtain an *E. coli* O157 MPN count. One-milliliter volumes of each MPN tube were subjected to IMS using Dynabeads anti–*E. coli* O157 (Dynal Biotech ASA, Oslo, Norway) and plating onto ntCHROM plates, which were incubated at 37°C overnight and examined for *E. coli* O157 colonies. Suspect colonies were confirmed by latex agglutination and PCR as described above. The MPN count of each positive sample was calculated using the formula of Thomas *(35).*

Statistical analysis. *E. coli* O157:H7 population numbers were converted to log CFU per gram (wet weight) or milliliter. Linear regressions were fit using the SigmaPlot data analysis and graphing software package (Systat Software, Inc., San Jose, Calif.), and *R*-square values are reported.

RESULTS AND DISCUSSION

There are numerous applications for *E. coli* O157:H7 enumeration procedures for manures and manure by-products. There is a need for methods to assess the safety of manures and composts prior to their use as soil amendments to fields that will be used to grow produce for human consumption *(40).* Likewise, such methods are needed to examine soils that will be planted to these crops. Determination of *E. coli* O157:H7 populations in manures is also required for measurement of the abilities of manure treatment processes to reduce or inactivate this pathogen. Furthermore, microbial risk assessments require data not only on the prevalence, but the concentration of *E. coli* O157:H7 in manures, for determination of the transmission risks associated with these products and their use.

Enumeration of *E. coli* O157:H7 populations in manures presents a special challenge because of high levels of generic *E. coli* and other closely related background microflora. Because of this difficulty, many researchers have relied upon the use of *E. coli* O157:H7 strains that have a specific antibiotic resistance or that contain marker genes for green fluorescent protein or other easily detectable traits to determine persistence of this organism in manures and soils and its survival during manure treatment processes *(19, 20, 22).* However, response of these manipulated strains or other laboratory strains may not always accurately represent the behavior of naturally occurring *E. coli* O157:H7. Real-time PCR quantification of *E. coli* O157:H7 in soils, manure, and feces is specific, rapid, and amenable to screening large numbers of samples, but detection limits for these samples typically are $>10^3$ CFU/g, and colonies are not available for further molecular characterization *(17, 18).* MPN procedures with IMS have been used effectively to determine levels of this pathogen in bovine feces and manures *(5, 13, 28).* However, MPN techniques have the disadvantage of being costly in terms of both labor and media and in combination with IMS are even more costly in terms of the expensive reagents that must be used for *E. coli* O157:H7 detection in each sample dilution. The requirement for preparing and testing multiple dilutions for each sample also makes use of MPN methods difficult for examining large numbers of samples.

Robinson et al. *(31)* and Brichta-Harhay et al. *(9)* have reported the effectiveness of spiral plating for enumeration of *E. coli* O157:H7 in bovine feces. As outlined in both of these studies, the spiral plating technique is advantageous for processing and plating large numbers of samples because fewer dilutions of each sample must be prepared and numerous samples can be plated rapidly. A drawback of the spiral plating technique is that low sample volumes typically are used, resulting in higher limits of detection. In this study, we plated 50-μl volumes of 10^{-1} dilutions, resulting in a lower limit of detection of 200 CFU/g of sample. Most current spiral plating models offer the options of increasing dispensed volumes to 100 or 250 μl, thereby lowering the detection level to the range of 50 to 100 CFU/g. We did not exercise this option to use higher sample volumes because we anticipated high levels of background bacteria that would interfere with identification of *E. coli* O157:H7 colonies in these samples.

The results of the initial examination of the method to enumerate *E. coli* O157:H7 in spiked manure, compost, MAS, and FSM are shown in Figure 1. For all experiments, the predicted levels were calculated from *E. coli* O157:H7 concentrations in the inocula as obtained by plating onto TSA. In each of the four sample types, levels of *E. coli* O157:H7 determined on all three test media were strongly correlated with the predicted levels. As expected with restrictive selective media, the *E. coli* O157:H7 populations on the test media were in general slightly lower than the predicted values. Additional restriction of *E. coli* O157:H7 can be noted for some sample types with the addition of potassium tellurite (in the T-CHROM) or the combination of potassium tellurite and novobiocin (in the ntCHROM). For example, average percent recoveries of *E. coli* O157:H7 from FSM on CHROM, T-CHROM, and ntCHROM were 78.2, 22.0, and 26.8% of the predicted values, respectively (Fig. 1). The addition of potassium tellurite to the base medium CHROMagar O157 is suggested by the manufacturer for inhibiting various non–*E. coli* O157 bacteria that may produce colonies of similar color. Brichta-Harhay et al. *(9)* found that the addition of novobiocin to CHROMagar O157 reduced interfering background microflora in fecal, hide, and carcass samples. The effects of population underestimation in the increasingly selective media were not sample specific but also were observed in the *E. coli* O157:H7 inocula (Fig. 2). In comparison to values obtained on TSA, recoveries of *E. coli* O157:H7 in the inocula were 102.1, 50.5, and 52.4% on CHROM, T-CHROM, and ntCHROM, respectively.

As can be expected for foods or other sample types, underestimation of *E. coli* O157:H7 populations in the manure-based samples also may be due in part to attachment or interaction of the inoculated cells to components of the sample matrices, such as manure particulates and soil par-

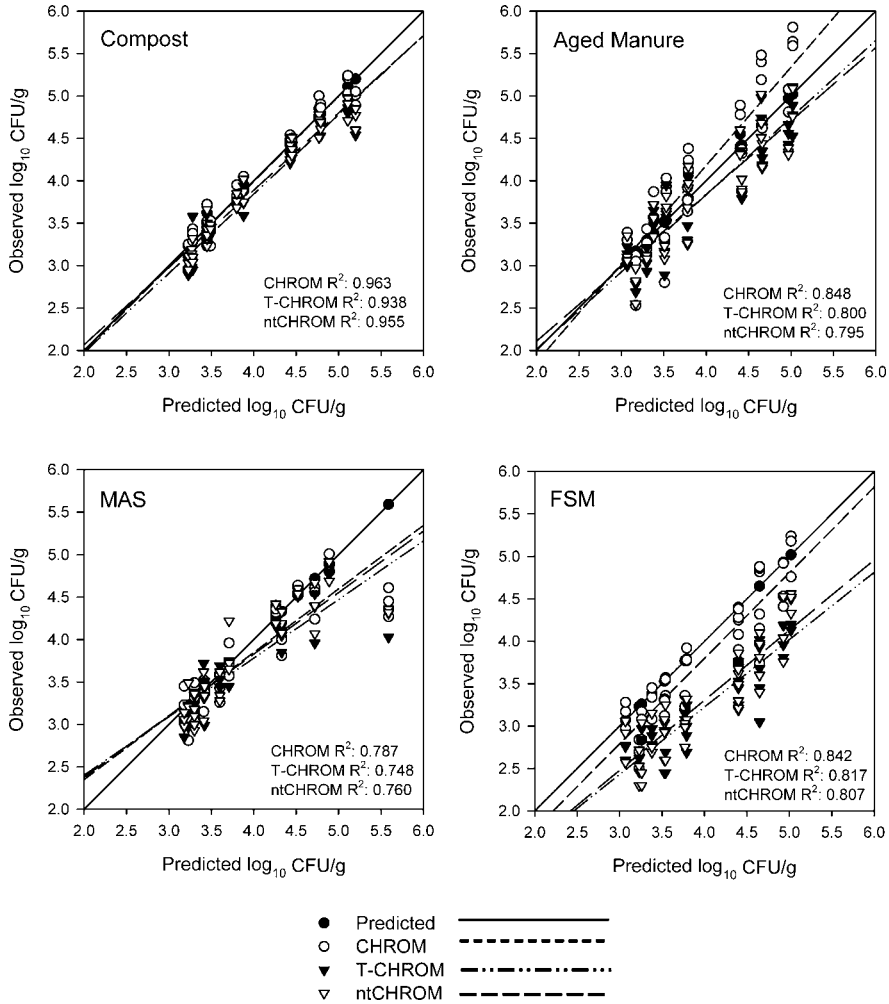

FIGURE 1. *Comparison of* E. coli *O157:H7 counts in compost, aged manure, manure-amended soil (MAS), and feedlot surface material (FSM) obtained on CHROM, T-CHROM, and ntCHROM agars with those levels predicted by counts in the inocula determined on TSA.*

ticles. All spiked samples were subsampled for determination of *E. coli* O157:H7 concentrations within 1 h of inoculation with the pathogen. Although the time necessary for cells to attach to sample particles is uncertain, attachment can affect recovery rates.

When each plated sample of aged manure, compost, MAS, and FSM was analyzed, background microflora on CHROM, T-CHROM, and ntCHROM were compared, different colony colors were noted, and populations were estimated and scored. For all four sample types, background microflora was the highest on CHROM, followed by T-CHROM, and then ntCHROM (data not shown). For all three test media, FSM samples had the highest background microflora, followed by aged manure and MAS samples (which had similar backgrounds); mature compost had the lowest level of background microflora. These results were anticipated because effective bovine manure composting processes substantially inactivate the fecal bacteria initially present in the manure, thus eliminating many of the organisms that may be able to form colonies on selective media designed for detection of *E. coli* O157:H7 *(22, 32).* In comparison, FSM collected from feedlot pens containing cattle is expected to have a higher content of fresh fecal material and therefore a higher population of interfering flora. In spite of media and sample differences with regard to background microflora, CHROM appeared to be adequate for easy identification and enumeration of *E. coli* O157 colonies in compost, aged manure, and MAS. However, many FSM samples contained non–*E. coli* O157 bacteria that were capable of growth on CHROM and T-CHROM that closely resembled *E. coli* O157:H7 colonies in color and morphology, and these colonies were sometimes difficult to discern from the actual target colonies. These nontarget bacterial colonies were not observed on FSM ntCHROM plates. Further work in a larger variety of composts, aged manures, and soils from different sources is needed to extend these findings.

Our previous work demonstrated that *E. coli* O157:H7 can persist and sometimes multiply in feedlot surface soils *(5).* Furthermore, its survival in FSM may play a role in its persistence in the cattle production environment. Several factors likely impact the incidence of the pathogen on the feedlot surface, the most important of which is the prevalence and load of *E. coli* O157:H7 shed by cattle in the pen. Our current work indicates that the incidence of *E. coli* O157 in FSM, as determined by enrichment and IMS, can sometimes approach 100% (unpublished data). FSM was the most challenging of the manure-containing samples examined because it contained fresher fecal material and concomitantly higher levels of interfering background flora. For these reasons, FSM was used to examine the ability of the direct plating method to enumerate naturally occurring

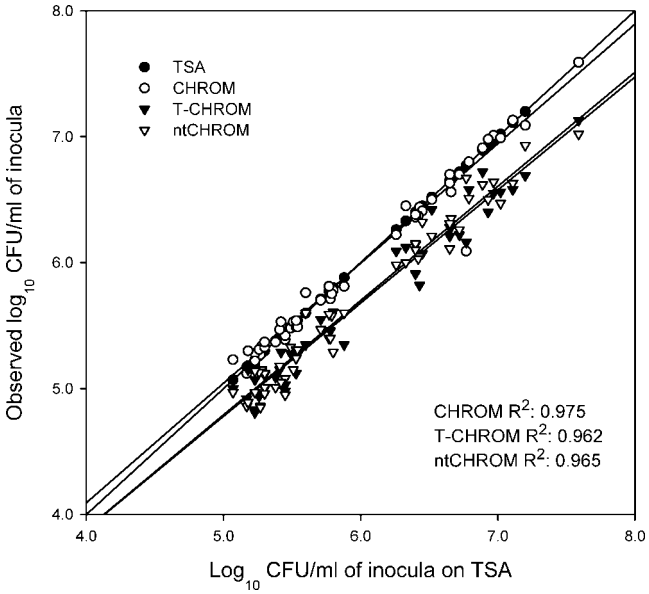

FIGURE 2. *Comparison of* E. coli *O157:H7 levels in the five-strain composite inocula obtained on CHROM, T-CHROM, and ntCHROM agars with those levels in the inocula determined on TSA.*

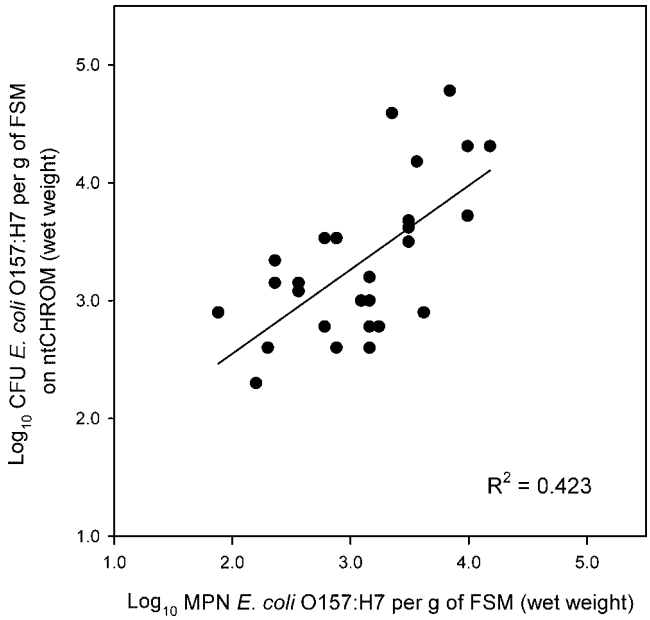

FIGURE 3. *Comparison of* E. coli *O157:H7 populations in naturally contaminated feedlot surface material (FSM) obtained by spiral plating onto ntCHROM and by the most-probable-number technique.*

E. coli O157:H7, with ntCHROM as the plating medium. Of the 250 FSM samples that were screened, 32 samples had levels of the pathogen that were at least 200 CFU/g and therefore were enumerable by spiral plating. *E. coli* O157:H7 concentrations in these samples ranged from 2.30 to 6.19 log CFU/g, similar to concentrations that have been reported elsewhere for bovine feces *(9, 13, 31).* The MPN procedure was completed for these 32 FSM samples. Four of these 32 FSM samples had *E. coli* O157:H7 numbers greater than 4.48 log MPN/g (the upper limit of the MPN estimate for the dilution series used), and 1 sample had less than 1.42 log MPN/g (the lower limit). The comparison of *E. coli* O157:H7 levels obtained by the two enumeration methods in the remaining 27 FSM samples is shown in Figure 3. Comparison of *E. coli* O157:H7 levels obtained by the two methods indicate that they are well correlated. The results of the comparison may be affected by the use of the more restrictive ntCHROM in the spiral plating method, which can result in underestimations of the pathogen populations. In addition, when using spiral plating to enumerate *E. coli* O157:H7 in bovine feces, lower precision for values at lower cell concentrations has been reported *(9, 31).* Also, as discussed previously, attachment of target cells to soil or manure particulates may reduce recovery rates obtained by spiral plating; in contrast, the MPN technique, with the use of target cell enrichment as performed here, can be assumed to be more robust with regard to the effects of cell attachment to sample particulates. Finally, the MPN technique is more variable than direct plating procedures and is more likely to produce higher population estimates *(29).*

We concluded that the direct spiral plating method is useful for estimating *E. coli* O157:H7 concentrations in a variety of manure-containing sample types, including FSM, aged bovine manure, bovine manure compost, and MAS. The addition of potassium tellurite and novobiocin to CHROMagar O157 medium can improve enumeration of *E. coli* O157:H7 in manure-based materials containing fresh feces and high levels of interfering bacteria. Data regarding the levels of this pathogen in manures, composts, and soils should enhance the assessment of transmission risks associated with these products, thereby reducing the risk of human foodborne illness.

ACKNOWLEDGMENTS

The authors thank Jane Long, Sandra Cummins, and Dee Kucera for technical support and Debbie Kummer for secretarial assistance. We are grateful to Daniel Miller for the gift of the compost samples.

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