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ORIGINAL ARTICLE

Shiga-toxigenic *Escherichia coli* O157 and non-Shiga-toxigenic *E. coli* O157 respond differently to culture and isolation from naturally contaminated bovine faeces

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Keywords
E. coli O157, bovine, faeces, Shiga-toxin, immunomagnetic separation, non-STEC.

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Abstract

Aim: To quantify the effect of enrichment, immunomagnetic separation (IMS), and selective plating procedures on isolation of Shiga-toxigenic *Escherichia coli* O157 (STEC O157) and non-Shiga-toxigenic *Escherichia coli* O157 (non-STEC O157) from naturally contaminated bovine faeces.

Methods and Results: Two broth enrichment times, two IMS strategies, and two selective plating media were evaluated. STEC O157 and non-STEC O157 strains were often isolated from the same faecal specimen and responded differently to the isolation protocols. A large-volume IMS system was more sensitive than a conventional small-volume IMS method, but was also more expensive. STEC O157 was more frequently isolated from 6 h enriched broth and CHROMAgar plates containing 0·63 mg l^{-1} potassium tellurite (TCA). Non-STEC O157 was more frequently isolated from un-enriched broth and ChromAgar plates without tellurite (CA).

Conclusions: The combination of 6-h enrichment in Gram-negative broth containing vancomycin, cefixime and cefsuludin, large volume IMS and selective plating on TCA maximized STEC O157 recovery from naturally contaminated cattle faecal specimens.

Significance and Impact of the Study: The pairing of proper enrichment with a specific plating procedure is key for STEC O157 recovery from naturally contaminated bovine faeces. Incorporating tellurite into an *E. coli* O157 detection strategy may select for the subset of *E. coli* O157 that contains the Shiga-toxin genes.

Introduction

Shiga-toxigenic *Escherichia coli* O157:H7 (STEC O157) is an important cause of human gastrointestinal disease. Disease associated human isolates of *E. coli* O157:H7 usually carry a suite of virulence genes, including genes that code for shiga-toxin production (stx_1, stx_2), intimin (*eae*), and haemolysis (*hlyA*). Primary culture and isolation protocols for this pathogen from food, animal and environmental sources frequently result in the co-selection of *E. coli* that carry the O157 antigen (*E. coli* O157), but that may or may not carry the H7 antigen or the other virulence genes associated with human infection (Durso *et al.* 2005; Keen *et al.* 2006; Shelton *et al.* 2006). The potential of these isolates to cause human disease is unclear (Keen *et al.* 2006). Since the Shiga-toxins are important for disease symptoms in humans, it is important to distinguish between *E. coli* O157 isolates that carry the Shiga-toxin genes (STEC O157) and *E. coli* O157 isolates that do not carry the Shiga-toxin genes (non-STEC O157).

STEC O157 is ubiquitous in cattle and in cattle production environments. Control of this zoonotic pathogen is an important public health and food safety concern, and STEC O157 presence and prevalence in the preharvest cattle production environment is frequently monitored via bovine faecal shedding. One challenge in

isolating STEC O157 from faeces of naturally infected cattle is that this bacterium usually represents only a minor component of an animal's faecal microflora (Fegan *et al.* 2004; Pearce *et al.* 2004; LeJeune *et al.* 2006). Thus, immunomagnetic separation (IMS), with or without prior broth enrichment, is commonly used to concentrate STEC O157 and reduce the numerically dominant background flora. IMS has been shown to be effective for isolating *E. coli* O157 from complex sample matrices in laboratory-based experiments, including bovine faeces (LeJeune *et al.* 2006), soils (Hepburn *et al.* 2002), milk (Reinders *et al.* 2002) and ground beef (Wu *et al.* 2004; Arthur *et al.* 2005).

These laboratory insights are an important first step in evaluating and optimizing methods for the detection and isolation of STEC O157 from field sources. Spiking complex sample matrixes with laboratory-reared or marker *E. coli* O157 or STEC O157 strains, however, may fail to adequately reproduce the bacterial growth characteristics found in naturally contaminated samples (Arthur *et al.* 2005). Typically, laboratory-based method evaluations overestimate their actual performance in the field. To avoid these issues, we used faecal specimens from naturally infected cattle to compare the effects of IMS, enrichment time, and selective plating on recovery rates of STEC O157 and non-STEC O157. Our primary study motivation was to test the hypothesis that STEC O157 IMS recovery rates can be improved by exposing the immunomagnetic beads to larger sample volumes, in this case, a 100-fold increase in sample volume exposed to anti-*E. coli* O157 paramagnetic beads (Ogden *et al.* 2000; Pearce *et al.* 2004; Echeverry *et al.* 2005).

In the present study, we assessed the effect of the large-volume semi-automated IMS Pathatrix™ system compared to conventional small-volume manual Dynal™ IMS system for STEC O157 and non-STEC O157 isolation from bovine faecal specimens originating from feedlot pens known to contain naturally STEC O157-infected cattle. In addition, we quantified the contribution of selective broth enrichment prior to IMS and the use of potassium tellurite in the final selective chromgenic plating media.

Materials and methods

Manual small-volume IMS

Bovine faecal broth enrichment and conventional manual small-volume IMS were performed as described previously (Durso *et al.* 2005; Keen *et al.* 2006). Briefly, 10 g of faeces was homogenized in 90 ml of Gram-negative (GN) broth containing vancomycin (8 mg l^{-1}), cefixime (0·05 mg l^{-1}) and cefsulodin (10 mg l^{-1}) (VCC). A 1-ml aliquot of the enrichment broth was removed for the time 0 IMS, and the remaining enrichment was incubated for 6 h at 37°C. IMS was performed using a 1-ml aliquot of the enrichment broth and 20 μl of Dynal anti-O157 immunomagnetic beads (Dynabeads; Invitrogen, Carlsbad, CA, USA). The 1-ml broth aliquot was shaker-incubated with the beads for 30 min at room temperature, the MPC was engaged for 5 min, samples were washed twice, and the washed beads were resuspended in 100 μl of bead wash (Fig. 1).

Semi-automated large-volume IMS

Faecal samples were enriched as described above. After enrichment was complete and a 1-ml aliquot was removed for manual IMS, sterile phosphate buffered saline (PBS) buffer was added to the GN-broth enrichment to bring the final volume of the sample from 99 to 250 ml. The entire 250 ml volume was then loaded onto the Pathatrix IMS machine (Pathatrix™; Matrix MicroScience Inc; Golden, CO, USA). The disposable filtering

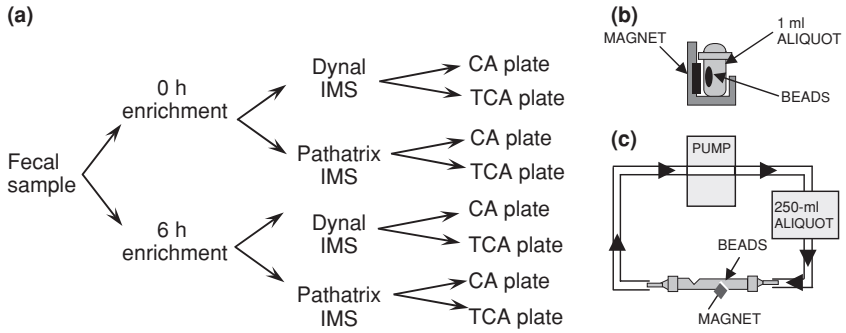

Figure 1 Experimental and immunomagnetic separation (IMS) design: (a) Each faecal specimen was cultured for *Escherichia coli* O157 under eight different conditions defined by enrichment time, IMS protocol and selective plate employed. (b) Conventional low volume Dynal IMS design: 1 ml aliquot of broth enrichment is exposed to 0·02 ml of magnetic beads. (c). Pathatrix IMS design: 250 ml of sample is exposed to 0·05 ml of magnetic beads.

apparatus was added and 50 μl of Pathatrix anti-O157 immunomagnetic beads were loaded into the filtering apparatus. Each sample was incubated for 30 min, during which a peristaltic pump circulated the entire volume of the sample over the magnetic beads several times per minute. IMS beads were held in place with a filtering apparatus in the Pathatrix machine. Upon completion of the incubation step, the disposable filter apparatus was removed, beads were washed using a disposable wash kit, and then resuspended in 100 μl of bead wash (Fig. 1).

Plating of samples

Suspensions of IMS bead-bacteria complexes from both the Dynal and Pathatrix systems were spread-plated onto selective chromogenic agar plates. The 100 ml IMS bead suspensions were dual plated (50 μl each) onto CHROMagar™ (CA) O157 plates (CHROMagar, Paris, France) and CA O157 plates containing 0·63 mg l^{-1} potassium tellurite (one quarter the manufacturers recommended amount) (TCA plates). All plates were incubated for 24 h at 37°C. Suspect STEC O157 colonies were picked and confirmed as described previously (Durso *et al.* 2005; Keen *et al.* 2006).

Confirmation of STEC and non-STEC O157 sample status

Up to five suspect *E. coli* O157 colonies displaying a mauve-pink colour on CA or TCA plates were serologically confirmed as *E. coli* O157 or H7 by enzyme-linked immuno-sorbent assay (ELISA) using anti-*E. coli* O157 monoclonal antibodies, (Laegreid *et al.* 1999) followed by PCR assays for stx_1, stx_2 (shiga-toxin), *eae* (intimin), *hly* (haemolyisn), $rfbE_{O157}$ (O157 O-antigen), and $fliC_{H7}$ (H7 flagellum) genes on all viable isolates (Gannon *et al.* 1997; Paton and Paton 1998a). Isolates were classified as STEC O157 or non-STEC O157 based solely on O157 antigen status and presence or absence of Shiga-toxin genes. Isolates were classified as STEC O157 if they were positive by ELISA or PCR for the O157 antigen, and they contained at least one Shiga-toxin gene, regardless of their *eae* or *hly* status. Likewise, isolates were classified as non-STEC O157 if they were positive by ELISA or PCR for the O157 antigen, but were PCR negative for both stx_1 and stx_2., again regardless of their *eae* or *hly* PCR results.

Comparison of IMS methods, enrichment time and plating media

Faecal specimens (n = 116) were collected from two Nebraska beef cattle feedlots and analysed in parallel in each of eight treatment combinations (Fig. 1), for a total of 928 samples processed. The STEC O157 and non-STEC O157 status of each sample was tabulated separately. A faecal specimen or method-defined sample was considered STEC O157 positive if an isolate was recovered which was *E. coli* O157 antigen positive by ELISA, $rfbE_{O157}$-positive by PCR, and *stx*1 and/or *stx*2 positive by PCR. A faecal specimen or method-defined sample was considered non-STEC O157 positive if an isolate was recovered that was *E. coli* O157 antigen positive by ELISA, $rfbE_{O157}$ positive by PCR and dual *stx*1 and *stx*2 negative by PCR.

Statistical analysis

Data were analysed by univariable and multivariable logistic regression for correlated data using SAS Proc Genmod (SAS, Cary, NC, USA). Statistical adjustment for correlated (clustered) data was used and necessary because of the split-sample nature of the experimental design (Fig. 1). For this analysis, a cluster was defined as the matrix of eight conditions (enrichment broth, IMS, and selective plating) applied to each of the 116 faecal specimens. Two dichotomous outcomes were modelled: isolation of STEC O157 (yes or no) and isolation of non-STEC O157 (yes or no). For logistic regression, odds ratios (OR) with 95% confidence intervals (CI) were generated as a measure of both magnitude and direction of association between an outcome (*E. coli* O157 isolation) and one or more potential explanatory variables. Explanatory variables with an OR >1·0 had an increased outcome likelihood relative to the reference condition while those with an OR <1·0 had a decreased outcome likelihood relative to the reference condition. The absolute diagnostic sensitivity was also calculated for specific enrichment, IMS, and selective plating method combinations. This was defined as the proportion of faecal samples positive for STEC O157 (or non-STEC O157) by a particular method divided by the number of 'true positive' faecal samples, where a 'true positive faecal' was one that was positive by any of the eight methods applied to a given sample.

Results

The effects of IMS sample volume, enrichment time, and selective plating media were evaluated for the recovery of STEC O157 from naturally contaminated faecal samples (Fig 1). STEC O157 was isolated from 63 of 116 (54%) faecal specimens by at least one of the eight methods tested (Table 1). Non-STEC O157 was recovered from a different but overlapping subset of 63 of 116 (54%) faecal specimens by one or more of the eight methods evaluated (Fig. 2). Both STEC O157 and non-STEC O157 were isolated from 32 (28%) faecal specimens. Each faecal sample

Table 1 Method [enrichment time, immunomagnetic separation (IMS) strategy and selective plating] combination-specific isolation of STEC O157 and non-STEC O157 from bovine faeces originating from naturally infected cattle

Enrichment time (h)	IMS strategy	Selective plating	*n*	No. STEC O157 positive (%)	No. non-STEC O157 positive (%)
0	Dynal	CA	116	12 (10·3)	22 (19·0)
0	Pathatrix	CA	116	21 (18·1)	9 (7·8)
0	Dynal	TCA	116	17 (14·7)	4 (3·4)
0	Pathatrix	TCA	116	24 (20·7)	3 (2·6)
6	Dynal	CA	116	7 (6·0)	10 (8·6)
6	Pathatrix	CA	116	20 (17·2)	25 (21·6)
6	Dynal	TCA	116	35 (30·2)	10 (8·6)
6	Pathatrix	TCA	116	49 (42·2)	9 (7·8)
0 + 6	Dynal + Pathatrix	CA + TCA	116	63 (54·3)	63 (54·3)
0	Dynal + Pathatrix	CA + TCA	464	74 (15·9)	38 (8·2)
6	Dynal + Pathatrix	CA + TCA	464	111 (23·9)	54 (11·6)
0 + 6	Dynal + Pathatrix	CA	464	60 (12·9)	66 (14·2)
0 + 6	Dynal + Pathatrix	TCA	464	125 (26·9)	26 (5·6)
0 + 6	Dynal	CA + TCA	464	71 (15·3)	46 (9·9)
0 + 6	Pathatrix	CA + TCA	464	114 (24·6)	46 (9·9)
0 + 6	Dynal + Pathatrix	CA + TCA	928	185 (19·9)	92 (9·9)

STEC O157, Shiga-toxigenic *Escherichia coli* O157; non-STEC O157, non-Shiga-toxigenic *E. coli* O157.

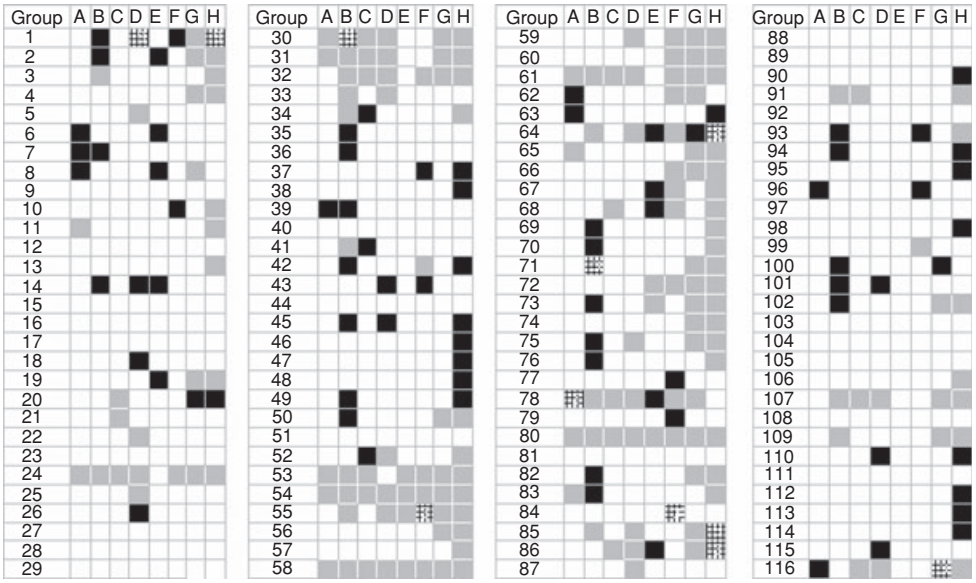

Figure 2 Distribution of STEC O157 and non-STEC O157 positive isolates. Column A: Pathatrix, TCA, 6 h; column B: Pathatrix, CA, 6 h; column C: Pathatrix, TCA, 0 h; column D: Pathatrix, CA, 0 h; column E: Dynal, TCA, 6 h; column F: Dynal, CA, 6 h; column G: Dynal, TCA, 0 h; column H: Dynal, CA, 0 h. Grey boxes are samples that were positive for STEC O157 only. Black boxes are samples that were positive for non-STEC O157 only. Hatched boxes are samples that were positive for both STEC and non-STEC O157.

was evaluated by all eight methods, which generated a total of 185 STEC O157 and 92 non-STEC O157 isolates. Method-specific summaries of isolation rates for both STEC O157 and non-STEC O157 are shown in Table 1. The two most sensitive method combinations for detecting STEC O157 were Pathatrix IMS after 6 h enrichment plated onto TCA, which found 49 of 116 (42%) faecal samples positive, followed by the Dynal IMS after 6 h enrichment plated onto TCA, which found 35 of 116 (30%) samples positive. The least sensitive method combination for STEC O157 detection was Dynal IMS with 6 h enrichment on CA plates, with seven of 116 (6%)

samples positive (Table 1). In contrast, for non-STEC O157 detection, Pathatrix IMS with 6 h enrichment plated onto CA was the most sensitive method combination, with 25 (22%) of 116 samples positive. The next most sensitive method was Dynal IMS without (0 h) broth enrichment plated onto CA, with 22 (19%) positive samples (Table 1). Although a small number of positive plates contained suspect mauve colonies exclusively, over 54% of the plates from which STEC O157 was isolated contained fewer than 30 suspect mauve colonies, and most positive plates were composed of a mixture of relatively rare suspects among frequent background colonies. Among the 185 STEC O157 isolates, 39% were PCR positive for both stx_1 and stx_2, 60% were positive for stx_2 only, and 1% were positive for stx_1 only; 88·3% of the STEC O157 isolates were PCR positive for *eae*, compared to only 11·9% of the non-STEC O157 isolates. Additionally, 94·6% and 82·1% of the STEC O157 isolates were positive for *hly* and $fliC_{H7}$, respectively, compared to 39% and 15·3% among the non-STEC O157 isolates.

Univariable and multivariable logistic regression analysis of the isolation data demonstrated significant effects of IMS method, enrichment time, and selective plating on STEC O157 isolation rates (Table 2). For example, in the multivariable model that simultaneously examined enrichment, IMS and plate effects (Table 2, Model IV), 6 h enrichment was better than 0 h (OR = 1·84, 1·24–2·74 95% CI), Pathatrix was more sensitive than Dynal (OR = 1·78, 1·34–2·37 95% CI), and TCA was better than CA (OR = 2·00, 1·41–2·86 95% CI) (Table 2 and Fig. 3). In contrast, multivariable logistic regression analysis of non-STEC O157 isolation (Table 2, Model VIII) showed only plate having a significant effect, with CA plates better than TCA plates (OR = 0·318 0·56–0·18 95% CI). There were no significant effects of IMS or enrichment time on non-STEC O157 isolation (Table 2 and Fig. 3).

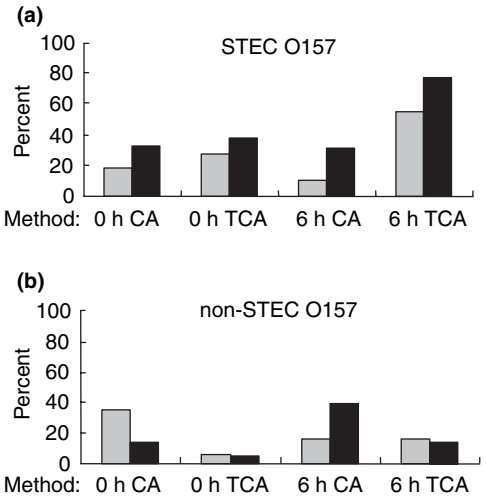

Figure 3 Effect of immunomagnetic separation (IMS) method, enrichment time, and plating media on recovery of *Escherichia coli* O157 strains from naturally contaminated bovine faecal pats. (a) Per cent of STEC O157-positive samples recovered, expressed as absolute sensitivity. (b) Per cent of non-STEC O157-positive samples recovered, expressed as absolute sensitivity. □, Dynal; ■, Pathatrix.

Discussion

Livestock faeces may contain a variety of *E. coli* O157 strains (Keen *et al.* 2006), and this study confirmed this observation. We isolated both STEC O157 and non-STEC

Table 2 Logistic regression analysis of the effects of broth enrichment, immunomagnetic separation strategy and selective plating on the isolation of STEC O157 and non-STEC O157 from bovine faeces originating from naturally-infected cattle

Model outcome	Model no.	Model type	Explanatory variable(s)	Odds ratio	OR 95% CI	*P*-value
STEC O157	I	Univariable*	Enrich 6 h (*vs* 0 h)	1·66	1·21–2·27	<0·0001
	II	Univariable	Pathatrix (*vs* Dynal)	1·80	1·44–2·26	<0·0001
	III	Univariable	Plate on TCA (*vs* CA)	2·48	1·86–3·32	<0·0001
	IV	Multivariable†	Enrich 6 h (*vs* 0 h)	1·84	1·24–2·74	0·0027
			Pathatrix (*vs* Dynal)	1·78	1·34–2·37	<0·0001
			Plate on TCA (*vs* CA)	2·00	1·41–2·83	0·0001
Non-STEC O157	V	Univariable	Enrich 6 h (*vs* 0 h)	0·44	0·24–0·82	0·0100
	VI	Univariable	Pathatrix (*vs* Dynal)	0·46	0·21–1·01	0·0528
	VII	Univariable	Plate on TCA (*vs* CA)	0·36	0·21–0·61	0·0002
	VIII	Multivariable	Enrich 6 h (*vs* 0 h)	1·53	0·99–2·38	0·0579
			Pathatrix (*vs* Dynal)	1·01	0·66–1·55	0·9487
			Plate on TCA (*vs* CA)	0·32	0·18–0·56	<0·0001

STEC O157, Shiga-toxigenic *Escherichia coli* O157; non-STEC O157, non-Shiga-toxigenic *E. coli* O157.

*A single explanatory variable is examined for association with the model outcome.

†Multiple explanatory variables are examined simultaneously for their association with the model outcome.

O157 from 36 of the 116 cattle faecal samples tested for this investigation. *E. coli* O157:H7 strains that do not carry either of the Shiga-toxin genes have been identified from both humans (Schmidt *et al.* 1999) and animals (Heuvelink *et al.* 1998), and some STEC O157 strains appear to readily lose the Shiga-toxin genes upon *in vitro* cultivation or *in vivo* passage (Karch *et al.* 1992; Murase *et al.* 1999). Surface water (Shelton *et al.* 2006), agricultural soils (Durso *et al.* 2005) and livestock faeces (Keen *et al.* 2006) can also contain a variety of *E. coli* O157 strains with different virulence gene profiles, including both STEC and non-STEC O157. In other words, detection of the *E. coli* O157 antigen or gene is necessary but insufficient for isolate characterization. In this study, for example, 94 of the original 116 faecal samples were positive for *E. coli* O157, but only 63 of those were positive for STEC O157.

Experiments performed by Ogden *et al.* (2000) with spiked samples demonstrated that increasing the volume of enrichment broth exposed to IMS beads (from 1 to 10 ml or 50 ml) greatly improves (up to eightfold) *E. coli* O157 recovery, presumably by increasing the number of target organisms exposed to the magnetic beads. The Pathatrix™ IMS system is an extreme example of this concept, as it is designed to potentially expose a census of every target organism present in a 250-ml volume of enrichment broth to the magnetic beads. Our 6 h enrichment plated on TCA was only 1·4-fold more sensitive for 250 ml (49/63 = 0·78 absolute diagnostic sensitivity) *vs* 1 ml (35/63 = 0·56 absolute diagnostic sensitivity). The average fold-increase in sensitivity for all conditions was only 1·8-fold for 250 *vs* 1 ml. These differences highlight the importance of evaluating primary culture and isolation procedures using naturally contaminated samples. While most field studies looking for STEC O157 include a broth enrichment step prior to IMS, we included a 0 h enrichment (i.e. nonenriched) sample primarily to see if the large volume Pathatrix system might be able to detect *E. coli* O157 without an enrichment step, thereby decreasing the time required for isolation. Unfortunately, the Pathatrix system on TCA without enrichment was only about 50% as sensitive compared to 6 h enriched samples (Table 1).

Diagnostic sensitivity is only one factor used in choosing the most appropriate method for any particular application. Although the Pathatrix method was 1·4-fold more sensitive than our standard method for STEC O157 detection from naturally contaminated bovine faecal specimens, the daily throughput in our laboratory was fourfold lower, the generation of biohazardous waste was fourfold greater by volume, and the cost per sample for IMS supplies and consumables was 4·7-fold greater (Table 3).

Table 3 Comparison of immunomagnetic separation (IMS) methods

Measurement	Dynal	Pathatrix
Cluster-level diagnostic sensitivity*	71/464	114/464
Maximum throughput†	120 samples per day per person	30 samples per day per person
Amount of biohazardous waste generated	0·5 bag per day	2 bags per day
Cost per sample for IMS supplies‡	$1·99	$9·40

*Number of IMS-method specific STEC O157 positive samples.
†In our laboratory, per 8 h working day by one trained person.
‡At time experiment was performed in August 2004.

In addition to IMS methods, we also quantified the effects of a 6 h enrichment and selective plating on the recovery of STEC O157 from our samples. Unlike STEC O157 culture from human faecal samples, which can be accomplished by direct plating of faecal swabs onto a sorbitol MacConkey (SMAC) plate (Paton and Paton 1998b), the low number of target organisms compared to background flora in cattle faeces requires selective enrichment to improve STEC O157 recovery from these samples (Chapman *et al.* 1997; Heuvelink *et al.* 1998). Our data show that a 6 h enrichment significantly improved STEC O157 recovery from cattle faecal specimens but only when used in conjunction with selective plating on TCA. Suspect STEC O157 are easy to distinguish from background flora on TCA plates, because they display a mauve-pink morphology, while most of the background flora has blue or white colonies. These colony morphologies remain stable over many days, unlike the transient stability displayed by sorbitol-negative STEC O157 colony morphology on SMAC agar. The 6 h enrichment did not improve the recovery of STEC O157 from CA plates, which tended to be heavily overgrown with background flora. Thus, the pairing of enrichment procedure with a specific plating procedure is key for STEC O157 recovery from bovine faeces.

One difficulty in using naturally contaminated samples compared to laboratory spiked samples is that it is impossible to know which samples represent 'true' positives. We defined a true positive faecal specimen as one that was positive by any of the eight treatments. No single method could account for all positive samples. Samples from only three faecal specimens were positive by all eight of the method combinations tested, 19 were positive by one method only, and 22 were negative across all eight methods (Fig. 2). In addition to method-specific effects such as enrichment time or selective plate used, some of

these differences across methods may also be attributable to natural variation in *E. coli* O157 density (i.e. nonuniform bacterial distribution) within a single positive faecal specimen (Pearce *et al.* 2004; Echeverry *et al.* 2005).

In conclusion, large volume Pathatrix IMS has definite diagnostic sensitivity advantages over small volume Dynal IMS for STEC O157 detection from naturally contaminated bovine faeces. However, this increased sensitivity is countered by the increased labour and laboratory costs of using this large volume protocol. In addition, our findings suggest that incorporating tellurite into an *E. coli* O157 detection strategy may select for the subset of *E. coli* O157 that contain the Shiga-toxin genes.

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Names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standards of the product, and the use of the name by USDA implies no approval of the product to the exclusion of others that may also be suitable.

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