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The use of selective and differential agars in the isolation of *Escherichia coli* O157 from dairy herds

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J.S. WALLACE AND K. JONES. 1996. The use of selective and differential agars for the isolation of *Escherichia coli* O157 from the faeces of dairy herds was investigated. Out of the 614 isolates which were positive for one or more of the selective criteria employed in the media only four proved to be *E. coli* O157. Ninety-nine per cent of the isolates were false positives. The procedure which resulted in the isolation of *E. coli* O157 from faecal samples was enrichment in modified Tryptone Soya Broth supplemented with novobiocin and subsequent growth on Chromagar® O157.

INTRODUCTION

Escherichia coli O157 is an emerging pathogen responsible for outbreaks of serious food-borne disease in North America and the UK (Morgan *et al.* 1988; Dorn 1993; Swerdlow *et al.* 1993). It has been established that cattle are the major reservoir for *E. coli* O157 (Chapman *et al.* 1994; Sanderson 1995; Zhao 1995) with outbreaks of disease directly associated with the consumption of foods of bovine origin (Paros *et al.* 1993; Synge *et al.* 1993; Willshaw *et al.* 1993). An increase in cases from two in 1993 and three in 1994 to 16 in 1995 in South Cumbria and the occurrence of sporadic cases in the area administered by the Morecambe Bay Health Authority (Griffiths, personal communication) have prompted this investigation into the extent to which *E. coli* O157 is found in local dairy herds.

Selective agars, based on the inability of *E. coli* O157 to ferment either sorbitol (March and Ratman 1986) or rhamnose (Chapman *et al.* 1991) and the absence of β-glucuronidase activity (Thompson *et al.* 1990), have been used to isolate *E. coli* O157 from a variety of samples by direct culture. Selectivity has been improved by the supplementation of Sorbitol MacConkey agar with cefixime in the presence of rhamnose (CR-SMAC) (Chapman *et al.* 1991) and by the inclusion of potassium tellurite (CT-SMAC) (Zadik *et al.* 1993). However, even with supplementation, the isolation of false positives still remains a major problem. Novel agars, such as SD-39 (QA Life Sciences, Inc.) and Chromagar® O157 (Chromagar), which distinguish *E. coli* O157 (pink) from other coliforms by colour, are designed to address this problem.

Direct culture may be suitable technique for the isolation of *E. coli* O157 from faecal samples of patients with acute illness but more sensitive methods are required for the isolation of *E. coli* O157 from food and environmental samples. In these circumstances, when target bacteria are present in low densities compared with the contaminating microflora, enrichment culture is superior to direct culture (Hindle *et al.* 1995; Sanderson *et al.* 1995; Zhao *et al.* 1995).

Other methods developed to improve the detectability of *E. coli* O157 in food and environmental samples include: the use of hydrophobic grid membranes filters (HGMF) to permit the screening of a large number of isolates (Doyle and Schoeni 1987; Todd *et al.* 1988) and immunomagnetic separation (IMS) techniques for separating *E. coli* O157 from the surrounding microflora (Chapman *et al.* 1994; Mortlock 1994).

This paper documents the problems encountered in the use of the above methods in the isolation of *E. coli* O157 from four dairy herds and describes a protocol which isolated the target bacterium and produced the lowest proportion of false positives.

MATERIALS AND METHODS

Farms

The faeces of four dairy herds were sampled. All of the herds were located within a 30 mile radius of Lancaster University.

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Faeces from the four dairy herds were collected immediately after milking on two different occasions.

Differential and selective agar

Four types of selective agar were used for the isolation of *E. coli* O157 by direct culture and following enrichment: Chromagar® O157 (Chromagar, France); SD-39 agar (QA Life Sciences, Inc., USA); CT-SMAC—Sorbitol MacConkey agar (SMAC) (Oxoid CM813) supplemented with 0·05 mg l^{-1} cefixime (Cyanamid, UK) and 2·5 mg l^{-1} potassium tellurite (Sigma); CR-SMAC—SMAC supplemented with 5 g l^{-1} rhamnose (Sigma) and 0·05 mg l^{-1} cefixime.

Direct isolation

Serial dilutions (1 : 10) of the amalgamated samples were prepared in 0·85% NaCl. Samples (0·1 ml) from each dilution were spread across the surface of CT-SMAC and CR-SMAC. Plates were incubated for 18 h at 37°C before examination.

Filtration method

Aliquots (1 ml) of appropriate serial dilutions were diluted in 10 ml of 0·85% NaCl and filtered individually through hydrophobic grid membrane filters (Iso-Grid®; QA Life Sciences, Inc.) (HGMF). Appropriate dilutions were determined on the basis of colony development on HGMF, with 10 to 500 colonies per filter taken as the acceptable range. Membranes were placed on to the surface of Chromagar® O157 and SD-39 agar and the plates incubated according to the manufacturer's instructions at 44·5°C (SD-39) and 37°C (Chromagar® O157) for 24 h.

Enrichment followed by direct culture

Twenty grams of each amalgamated faecal sample were added to 200 ml of modified Tryptone Soya Broth (TSB (Oxoid CM131), 30·0 g l^{-1}; bile salts No. 3 (Oxoid L56), 1·5 g l^{-1}; dipotassium hydrogen orthophosphate (Sigma), 1·5 g l^{-1}) *made selective by the addition of 20 mg* l^{-1} *novobiocin* (sodium salt) (Sigma) (mTSB-nov). Samples were incubated at 37°C without agitation for 18 h before removal of 1 ml aliquots. Log serial dilutions of each sample were prepared in 0·85% NaCl. In the case of SD-39 and Chromagar® O157 1 ml aliquots of the dilutions were filtered as above and the filters placed on SD-39 agar or Chromagar® O157 and incubated as above. To assess the efficacy of CT-SMAC and CR-SMAC 0·1 ml aliquots of each serial dilution were spread across the surface of each type of agar.

IMS procedure

Volumes (20 g) of each amalgamated faecal sample were added to 200 ml of buffered peptone water (Oxoid CM509) made selective by the addition of vancomycin (8 mg l^{-1}) (Sigma), cefixime (0·05 mg l^{-1}) (Sigma) and cefsulodin (10 mg l^{-1}) (Sigma) (BPW-VCC). Samples were incubated at 37°C without agitation for 6 h before removal of 1 ml aliquots. IMS was conducted according to the manufacturer's instructions using the Dynabead anti-*E. coli* O157 immunomagnetic separation (Dynal, UK) system according to the manufacturer's instructions. Beads were plated on to CT-SMAC.

Spiked controls

In order to be sure that the isolation methods were working, spiked controls were set up. On each sampling occasion duplicate 20 g of faeces from farm 1 were added to 200 ml of mTSB-nov and 200 ml of BPW-VCC and spiked with 250 cfu ml^{-1} of *E. coli* O157. Samples were examined for the presence of *E. coli* O157 using the direct and enrichment procedures as described above.

A strain of *E. coli* O157 of human origin was provided by Lancaster Royal Infirmary. It was cultured overnight in TSB at 37°C. The cell densities were determined spectrophotometrically at $O.D._{640}$, and numbers determined in triplicate by serial dilution and plate count methodology on Nutrient agar (Oxoid CM3). Cells were sedimented by centrifugation (1500 *g*, 10 min), re-suspended and washed three times in 0·85% NaCl. Half a ml of diluted inoculum, containing approximately 10^4 cfu ml^{-1}, was added to each of the 200 ml of faecal suspensions. Suspensions were mixed before incubation.

Verification and characterization of isolates

A maximum of 10 non-sorbitol fermenting (NSF), non-rhamnose fermenting (NRF) or pink colonies in the case of isolates from SD-39 and Chromagar® O157 were randomly selected from each agar. Selected colonies were purified on Nutrient agar and two to three 24-h-old colonies picked off and suspended in 2 ml of sterile water. A multipoint inoculator (Denley® A400) was used to inoculate the surface of six *types of agar to allow characterization of each isolate.*

The agars inoculated were those listed above together with Lactose Monensin Glucuronate (LMG) (QA Life Sciences, Inc.) and Buffered MUG agar (BMA) (QA Life Sciences, Inc.). LMG agar was used to presumptively identify isolates as coliforms whilst BMA agar was used to test for β-glucuronidase activity. LMG plates were incubated at 37°C for 24 h before examination for blue colonies (presumptive coliforms). BMA plates were incubated for 2 h at 35°C before examination under short-wave u.v. light (366 nm). Isolates

which lack β-glucuronidase activity do not fluoresce under these conditions.

Strains with four or more of the diagnostic characteristics of *E. coli* O157, i.e. they did not ferment sorbitol or rhamnose, lacked β-glucuronidase activity, produced pink growth on SD-39 and Chromagar® O157 and blue colonies on LMG agar, were presumptively identified as *E. coli* O157 by testing for agglutination with an *E. coli* O157 latex kit (Oxoid DR620). Presumptive isolates were confirmed biochemically as *E. coli* using API 20E (BioMérieux).

RESULTS

Using the criteria of growth on selective media or colour change on differential media, 614 isolates were selected as presumptive *E. coli* O157 from the faecal samples of the four herds. They were purified and tested for characteristics typical of *E. coli* O157. The results (Fig. 1) show that 85% of the isolates were β-glucuronidase negative; 53% were non-sorbitol and non-rhamnose fermentors; 37% were non-sorbitol fermentors but capable of growth in the presence of cefixime and potassium tellurite; 40% produced blue colonies on LMG agar (confirmatory for coliforms); 34% produced pink colonies on SD-39 agar; 20% produced pink colonies on Chromagar® O157 and 7% produced pale blue colonies with pink peripheries on Chromagar® O157. The latter were easily mistaken as pink during the initial isolation (not shown in Fig. 1).

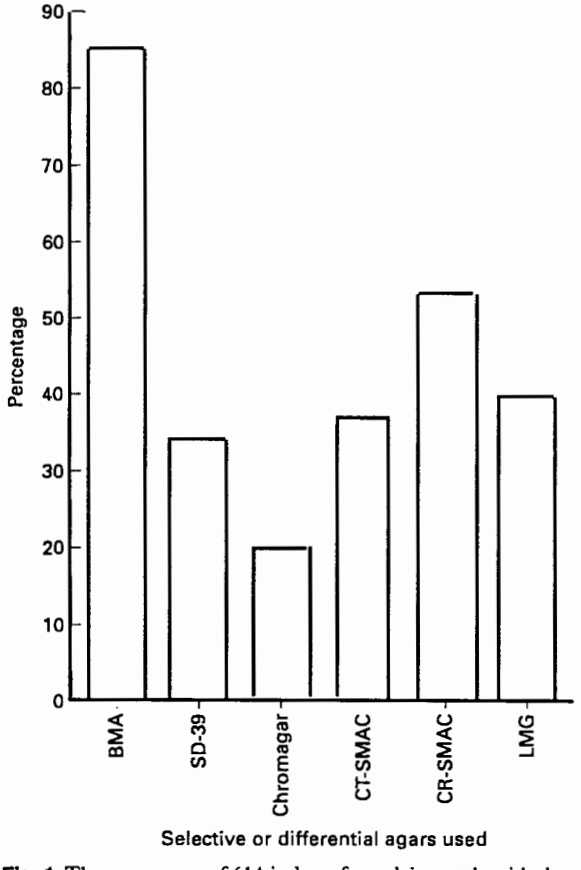

Fig. 1 The percentage of 614 isolates from dairy cattle with the same phenotypic characteristics as *Escherichia coli* O157 when grown on selective or differential agar

The number of characteristics similar to those of *E. coli* O157 possessed by the isolates following direct and enrichment culture are shown in Table 1. Forty-seven isolates (7·7% of the total) had four or more of the *E. coli* O157 characteristics and were tested for latex agglutination. Only four isolates were positive. These were the same isolates which were positive for all six of the characteristics tested for.

The four confirmed *E. coli* O157 isolates from the unspiked faecal samples were from the same farm. Two were isolated on the first sampling date and two on the second. They were isolated only using the following method, namely, enrichment in mTSB-nov for 18 h at 37°C, followed by filtration using the HGMF technique and growth on Chromagar® O157. Of the six agars used to distinguish *E. coli* O157 from false positives, following enrichment and isolation on Chromagar® O157, CT-SMAC and CR-SMAC were more discriminatory than SD-39, LMG, BMA and Chromagar® O157 (Fig. 2). Only 11% of the isolates presumptively identified as *E. coli* O157 by characteristic growth on Chromagar® O157 were NSF and NRF.

These results were confirmed using two faecal suspensions spiked with *E. coli* O157 at a concentration of 250 cfu ml^{-1}. *Escherichia coli* O157 was not isolated from either of the spiked suspensions following direct culture or enrichment and subsequent plating on to SD-39, CT-SMAC or CR-SMAC. It was, however, isolated from the suspensions after enrichment for 18 h and subsequent plating following filtration onto Chromagar® O157, and by the IMS procedure. Even with the spiked samples the number of isolates recovered and confirmed as *E. coli* O157 was low. Ninety per cent of those isolated on Chromagar® O157 and 95% of those isolated using IMS were false positives for *E. coli* O157.

Of the original, presumptive, isolates 99·4% were false positives, that is, they possessed one or more of the phenotypic characteristics of *E. coli* O157. Representatives of these isolates were identified using the API 20E and API 20NE system (Table 2). Most were members of the Enterobacteriaceae except for *Pseudomonas* spp. and *Actinetobacter* spp.

DISCUSSION

Dairy cattle have been implicated as a major reservoir of *E. coli* O157 in the UK (Chapman *et al.* 1993). In the present

		Number of characteristics similar to those of *E. coli* O157					
		1	2	3	4	5	6
Direct	Chromagar® O157	72	68	54	0	0	0
	SD-39	68	68	48	12	0	0
	CT-SMAC	72	58	48	0	0	0
	CR-SMAC	70	68	28	2	0	0
	Total	282	262	178	14	0	0
Enrichment	Chromagar® O157	70	70	60	10	4	4
	SD-39	72	72	38	8	0	0
	CT-SMAC	66	56	52	12	0	0
	CR-SMAC	64	62	48	0	0	0
	IMS	60	38	33	3	0	0
	Total	332	298	231	33	4	4

Table 1 The number of bacteria isolated using different cultural methods which possessed one or more of the phenotypic characteristics of *Escherichia coli* O157

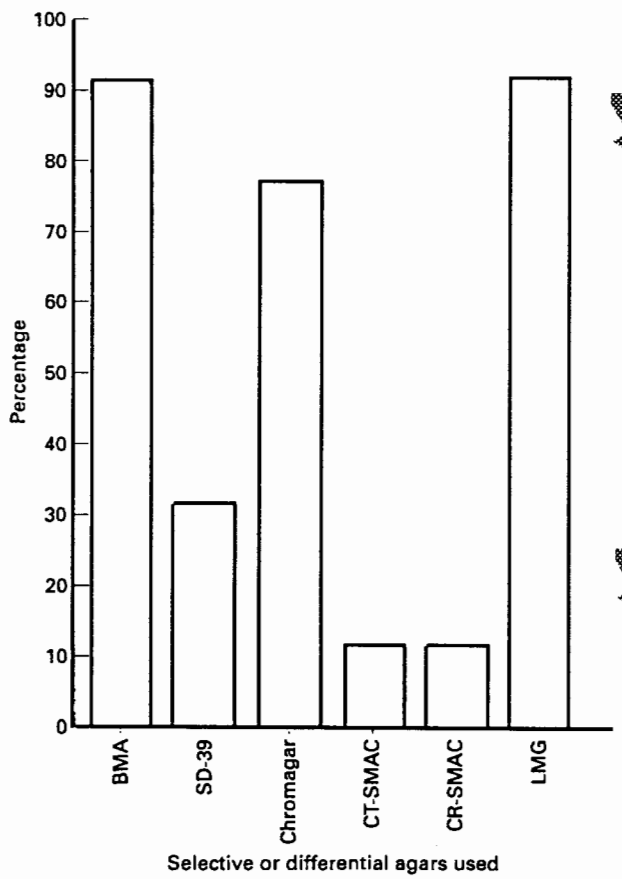

Fig. 2 The percentage of isolates taken from Chromagar® O157 following enrichment with the same phenotypic characteristics as *Escherichia coli* O157 determined by plating onto six types of selective or differential agars

study *E. coli* O157 was isolated from only one of the four dairy herds examined. Enrichment was found to be an essential step in the isolation of *E. coli* O157 from cattle faeces. Other researchers have also highlighted this (Sanderson *et al.* 1995; Bolton *et al.* 1995; Zhao *et al.* 1995). Extremely low recovery rates for *E. coli* O157 were found, even after spiking faecal samples with control suspensions. We found that *E. coli* O157 could only be isolated from unspiked dairy faeces using the following procedure, namely, enrichment in mTSB-nov for 18 h followed by plating onto Chromagar® O157 in conjunction with the HGMF. This method was also slightly more sensitive than the IMS procedure for the isolation *E. coli* O157 from the spiked samples. None of the direct methods or enrichment followed by inoculation onto CR-SMAC or CT-SMAC resulted in the isolation of *E. coli* O157 from either the spiked or unspiked samples. Similarly, *E. coli* O157 was not isolated using the HGMF in conjunction with SD-39 agar prior to or following enrichment. The absence of *E. coli* O157 may be a direct consequence of using the elevated temperature (44·5°C) recommended by the manufacturer for the incubation of SD-39 agar, since the organism grows poorly at temperatures above 41°C (Raghubeer and Matches 1990). To the authors' knowledge this is the first report on the use of Chromagar® O157, in conjunction with the HGMF system, for the detection of *E. coli* O157 in bovine faecal samples.

The increased sensitivity of the IMS procedure in comparison to the direct methods or enrichment followed by inoculation onto the supplemented SMAC agars or SD-39 concurs with the results of others. Chapman *et al.* (1994), Wright *et al.* (1994) and Mortlock (1994) have reported increased sensitivity of between 100- and 1000-fold when examining for the presence of *E. coli* O157 using the IMS procedure as opposed to direct plating.

Table 2 The phenotypic characteristics of *Escherichia coli* O157 and of selected strains identified as false positives isolated using different agars

	Chromagar Pink	SD-39 Pink	LMG Blue	BMA Negative	CT-SMAC NSF	CR-SMAC NSRF
E. coli O157	+	+	+	+	+	+
E. coli	+		+	+		
E. coli		+	+	+		
E. coli	±	+	+	+		+
E. coli		+	+		+	+
Hafnia alvei		+		+	±	+
Pseudomonas spp.		±		+	+	±
Acinetobacter spp.		±		+	+	±
Citrobacter spp.	+		+			
Morganella morganii				+	+	+
Escherichia fergusonii		+		±		+

+, Gave growth characteristic of *E. coli* O157; NSF, non-sorbitol fermenting; NSRF, non-sorbitol or rhamnose fermenting.

A major problem seen with all of the solid media employed in this study was the growth of false positives, i.e. exhibiting the same colour changes on selective and differential agars as *E. coli* O157. This problem has also been reported by other workers when using both the IMS procedure (Mortlock 1994) and direct culture techniques (Chapman *et al.* 1991, 1993).

Several other methods have been described for the detection of *E. coli* O157. These include: immunoblot techniques (Szabo *et al.* 1990; Doyle and Schoeni 1987); immunoassay (Padhye and Doyle 1992; Kim and Doyle 1992; Johnson *et al.* 1995; Dylla *et al.* 1995); DNA probes specific to *E. coli* O157 (Gunzer *et al.* 1992; Olsen and Oshiro 1996). Although these techniques are more sensitive in the detection of *E. coli* O157 they are technically more demanding, expensive and tend to give results that cannot be confirmed by culture. In contrast selective culture is technically simple, relatively inexpensive and within the scope of most routine laboratories.

The use of Chromagar® O157 in conjunction with the HGMF system following enrichment culture was the most sensitive technique of those used in the present study. The use of a variety of agars to characterize the presumptive strains isolated considerably reduced the number of isolates which needed to be confirmed using latex bead agglutination. Combinations of methods have also been used by other workers to aid in the identification of *E. coli* O157. For example, Scotland *et al.* (1993) showed that testing for β-glucuronidase activity was a useful secondary test in the identification of *E. coli* O157. The results of the present study indicate, however, that plating of pure cultures isolated from Chromagar® O157 following enrichment on to either CT-SMAC or CR-SMAC is a more discriminatory second test than any of the other solid media tested. This cost effective method reduced the number of false positives which required further confirmation by latex agglutination by approximately 90%.

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