Turkish Journal of Veterinary & Animal Sciences

Volume 24 | Number 5 Article 7

1-1-2000

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Turk J Vet Anim Sci
24 (2000) 459-464

A Study on Selective Broths and Agar Media for the Isolation of *Escherichia coli* O157:H7 Serotype

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Received: 06.12.1999

Abstract: Modified tryptic soy broth, lauryl sulfate tryptose broth, modified EC broth, sorbitol MacConkey agar and Fluorocult E. coli O157 agar were tested for the recovery of 3 strains of *Escherichia coli* O157:H7 against *E. coli* type 1 and *Citrobacter freundii* as common competitive microflora of raw meat products. Two-stepped trials showed that modified EC broth and lauryl sulfate tryptose broth were superior to modified tryptic soy broth as selective enrichment media while sorbitol MacConkey agar was clearly better than Fluorocult E. coli O157 agar as a selective agar medium. Recovery of *E. coli* O157:H7 was difficult when selective enrichment broth-growing cohabitant microflora were present at more than 100 folds of *E. coli* O157. One percent *E. coli* O157:H7 in the cohabitant flora was the minimum level for recovery. This is probably more important than the minimum recovery rate of culture media and/or conventional culture methods when *E. coli* O157:H7 exists without competitive microorganisms.

Key Words: *E. coli* O157:H7, mTS Broth, mEC Broth, LST Broth, SMAC Agar.

Escherichia coli O157:H7 Serotipinin İzolasyonunda Selektif Broth ve Agar Besiyerleri Üzerine Bir Çalışma

Özet : Bu çalışmada, modifiye tryptic soy broth, lauryl sulphate broth, modifiye EC broth, sorbitol MacConkey agar ve Fluorocult E. coli O157 agar besiyerleri *E. coli* O157:H7 serotipinin üç şahit suşunun, çiğ et ürünlerinin tipik refakatçi florası olan *E. coli* tip 1 ve *Citrobacter freundii* 'nin ikişer suşuna karşı geri alınmasında denenmiştir. İki aşamalı denemeler modifiye EC broth ve lauryl sulphate brothun modifiye tryptic soy brotha ve sorbitol MacConkey agarın Fluorocult *E. coli* O157 agara göre daha iyi sonuç verdiğini göstermiştir. Selektif besiyerlerinde gelişebilen refakatçi flora sayısı *E. coli* O157 serotipi sayısından 100 mislinden fazla ise *E. coli* O157:H7' nin geri alınması zorlaşmaktadır. Bir diğer deyiş ile, selektif besiyerlerinde gelişebilen refakatçi flora içinde *E. coli* O157:H7 oranı en düşük olarak %1 olmalıdır. Bu oran, selektif besiyerleri ve/veya geleneksel izolasyon yöntemlerinde verilen minimum geri alınabilme sayısından muhtemelen daha önemlidir.

Anahtar Sözcükler : *E. coli* O157:H7, mTS broth, mEC broth, LST broth, SMAC agar.

Introduction

Since 1982, *Escherichia coli* O157:H7 has been reported to be responsible for several outbreaks of hemorrhagic colitis in the North American continent and other areas of the world. A more serious syndrome of hemolytic uremia has affected some of the cases, mostly children, and deaths have occurred particularly in nursing homes. This pathogen has been implicated in food-borne outbreaks of hemorrhagic colitis throughout North America and Europe (1). A significant outbreak was witnessed in Japan during the summer of 1996 (2).

It has been seen in sporadic cases that *E. coli* O157:H7 is a very serious pathogen (3, 4). Due to its importance, many investigations on its survival, growth characteristics, recovery, rapid detection, etc. have been undertaken. In spite of the fact that many improved methods based on genetics, immunology and enzymology have been developed for detection of enterohemorrhagic *E. coli* O157:H7 (5-8), conventional culture detection methods, particularly for routine screening studies, are still applied and are likely to be used in the future.

The conventional culture technique is based on inoculation of the sample in a selective broth, incubating and subculture on selective agar. Selective broths, namely, modified tryptic soy (mTS) broth, modified EC (mEC) broth and lauryl sulfate tryptose (LST) broth are the commonly used enrichment media. Both mTS and mEC broths contain novobiocin for inhibition of the

cohabitant flora (5, 9, 10). While other *E. coli* serotypes are sorbitol positive, *E. coli* O157:H7 serotype is sorbitol negative and sorbitol MacConkey (SMAC) agar contains sorbitol instead of lactose. Therefore, while sorbitol positives produce pink to red colonies, sorbitol negatives produce colorless colonies on SMAC agar (11).

The second difference between the O157 serotype and other serotypes is the β-glucuronidase (β-GUR) activity. While others are β–GUR positive, *E. coli* O157:H7 is β–GUR negative, i.e., other serotypes split 4-methylumbelliferone glucuronide (MUG) and give fluorescence under 366 nm long-wave UV light whereas *E. coli* O157 cannot hydrolyze MUG. Some solid culture media have been developed on the basis of this difference, e.g., Fluorocult E. coli O157 agar and Fluorocult HC agar (5). In addition 5-bromo-4-chloro-3-indoxyl-ß-D-glucuronic acid cyclohexyl-ammonium salt (BCIG) plus SMAC agar, phenol red sorbitol agar plus MUG, HC agar, SMAC agar supplemented with rhamnose and cefixime (RC) or potassium tellurite and cefixime (TC) have been developed and used in some investigations (4, 5, 12-14).

The main disadvantage of conventional culture methods is the possibility of obtaining false negative results. Although *E. coli* O157:H7 may be differentiated mainly on the basis of its sorbitol and β–GUR negative properties, it is difficult to distinguish and isolate the suspected colonies among cohabitant colonies. A general laboratory practice is that 1 different colored colony (of the same size) can be easily separated among 100 colonies (150 colonies is the maximum level) on a 9 cm standard petri dish. In other words, 1% of well-grown colonies can be separated depending on their color and/or shape. The importance of the existence of a competitive flora in detection of *E. coli* O157 in screening studies has been described (11, 14). Hitchins et al. (15) reported difficulties due to high levels of contaminating coliforms which may mask the presence of *E. coli* O157:H7 strains when grown on SMAC agar.

When a food (or environmental) specimen contains *E. coli* O157:H7, it is most probable that a high level of contaminating microflora is present. Many of the species present are suppressed at the selective enrichment but others, for example, *E. coli* type 1, can grow well in many of the culture media selective for *E. coli* O157:H7. Some cultural practices such as alteration of the incubation temperature and/or higher levels of bile salts inhibit the growth of *E. coli* O157:H7, whereas *E. coli* type 1 grows well under these conditions also (16, 17). When isolating *E. coli* O157:H7 by conventional cultural methods, the level of *E. coli* O157:H7 in a competitive microflora grown in selective enrichment broth must be at least 1% and our study is based on this hypothesis.

The purpose of this study was the recovery of *E. coli* O157:H7 strain vs. *E. coli* type 1 and *C. freundii*, which are common cohabitants in animal-originated foods (18), by using different types of media.

Materials and Methods

Bacterial cultures: Three strains of *E. coli* O157:H7 serotype were collected from 3 different sources, i.e., Food Engineering Department of Hacettepe University (Turkey), Institute of Refik Saydam Public Health Center (Turkey) and Landbouw University (the Netherlands). For distinguishing the strains, their names were suffixed by (h1), (h2), and (h3) respectively. Two strains each of *E. coli* type 1 [suffixed by (c1) and (c2)] and *C. freundii* [suffixed by (f1) and (f2)] were isolated and identified by conventional biochemical tests (19). All 7 bacterial strains were maintained on nutrient agar slants and activated in nutrient broth at 37 °C for 18 h prior to analysis. None of the 7 bacterial strains was certified and/or categorized by national collections.

Culture media: Among many selective media, 3 selective enrichment broths and 2 selective agar media were tested for recovery of *E. coli* O157:H7 in 2-stepped trials. The compositions of the media are as follows.

mEC broth: Base medium was composed of tryptone (Oxoid) 2 %; lactose (Oxoid) 0.5 %; bile salts #3 (Difco) 0.112 %; K_2HPO_4 (Merck) 0.4 %; KH_2PO_4 (Merck) 0.15 %; and NaCl (Merck) 0.2 %. 20 mg/l filter sterilized novobiocin (Merck) was added to the autoclaved base medium after cooling to room temperature (10).

mTS broth: Base medium containing tryptic soy broth (Merck) 3.0 %; bile salts #3 (Difco) 0.15 % and K_2HPO_4 (Merck) 0.15 % was prepared and 20 mg/l filter sterilized novobiocin (Merck) was added to the autoclaved base medium after cooling to room temperature. Composition of the tryptic soy broth was peptone (casein) 0.17 %; peptone (soy meal) 0.3 %; D-glucose 0.25 %; K_2HPO_4 0.25 % (9).

LST broth: Commercially available dehydrated medium (Merck) was used (35.5 g/l) (20).

SMAC agar: Commercially available dehydrated medium (Oxoid) was used (51.5 g/l) (11).

Fluorocult O157 agar: Commercially available dehydrated medium (Merck) was used (55.0 g/l) (20). Fluorocult O157 agar is abbreviated as F-O157 agar in this study.

The broth media were used on the day of preparation whereas the agar media were dried for 1 day at room temperature before use. Although addition of novobiocin was not needed in our study, as it is generally used for suppressing the cohabitant flora in screening tests, mTS and mEC broths were prepared by adding novobiocin according to the original formulae (9, 10).

First recovery studies

The 7 bacterial strains were separately inoculated to selective enrichment broths and incubated for up to 28 h at 37 °C. Decimal dilutions were made from 0, 16, 20, 24, 28 h cultures and then 0.1 ml of diluted cultures were spread on both SMAC agar and F-O157 agar. After 24 h incubation at 37 °C, plates containing 20-200 colonies were selected for colony counting. Log_{10} values of the counts (cfu/ml) for each culture were statistically analyzed.

Second recovery studies

E. coli O157:H7 strains were mixed with competitive bacterial strains in the following ratios:

a) *E. coli* O157:H7 (h1):*E. coli* type 1 (c1):*E. coli* type 1 (c2) = 1:50:50 and 5:50:50 (v/v)

b) *E. coli* O157:H7 (h1):*C. freundii* (f1):*C. freundii* (f2) = 1:50:50 and 5:50:50 (v/v)

c) *E. coli* O157:H7 (h1):*E. coli* type 1 (c1):*E. coli* type 1 (c2):*C. freundii* (f1):*C. freundii* (f2) = 1:25:25:25:25 and 5:25:25:25:25 (v/v).

The same mixing procedure was also applied to *E. coli* O157:H7 (h2) and (h3) strains, resulting in 18 mixed cultures. Twenty-four hour nutrient broth cultures of all the 7 bacterial strains were considered to be 10^8 cfu/ml on the basis of previous analyses. These 18 mixed cultures were inoculated to selective enrichment broths (2% v/v) and incubated for 24 h at 37 °C. 0.1 ml of decimal dilutions of the enrichment cultures were spread on both SMAC agar and F-O157 agar. After 24 h incubation at 37 °C the plates were analyzed for recovery of *E. coli* O157:H7 vs. cohabitants' colonies. The plates were evaluated and scored as 0 (no visible *E. coli* O157:H7 colony), 1 (small numbers of *E. coli* O157:H7 colonies; 1 to 10), 2 (adequate isolation possibility; 11 to 20 *E. coli* O157:H7 colonies) and 3 (well growth; >20 colonies of *E. coli* O157:H7).

Statistical analyses

Data from independent triplicate trials at each step were statistically evaluated by applying analysis of variance and Duncan's Multiple Range (DMR) Test by Minitab program.

Results

In the first recovery studies, results obtained by analysis of variance indicate that interactions between agar media x incubation time and the difference between the 2 agar media were significant ($p<0.05$). The means of colony counts (log_{10} cfu/ml; n = 315) were 9.5315 ± 0.0261 for SMAC agar and 9.3438 ± 0.0269 for F-O157 agar. The results reveal that SMAC agar gave better results than F-O157 agar for recovery of *E. coli* O157:H7 (Table 1).

The results of the second recovery trials are given in Table 2. Each number in the table indicates a sum of scores of 6 plates (3 independent replicates and 2

Table 1. Analyses of Variance of First Recovery Trial.

Source	DF	F	p
Ba	6	83.58	0.000
Se	2	33.81	0.000
Am	1	90.79	0.000**
It	4	8.75	0.000
Ba x Se	12	26.17	0.000
Ba x Am	6	58.98	0.000
Ba x It	24	16.51	0.000
Se x Am	2	1.23	0.293
Se x It	8	2.84	0.004
Am x It	4	0.64	0.637
Ba x Se x Am	12	0.85	0.603
Ba x Se x It	48	2.42	0.000**
Ba x Am x It	24	0.90	0.602
Se x Am x It	8	0.27	0.976
Ba x Se x Am x It	48	0.22	1.000

**: $p<0.05$; Ba: Bacteria; Se: Selective enrichment media; Am: Agar media; It: Incubation time; DF: Degree of Freedom; F: F value; p: probability.

Table 2. Recovery of *E. coli* O157:H7 Against Competitive Flora

Competitive Flora =		*E. coli* type 1			*C. freundii*			*E. coli* + *C. freundii*		
E. coli O157 strain =		H7 1	H7 2	H7 3	H7 1	H7 2	H7 3	H7 1	H7 2	H7 3
mTS	SMAC	5	4	5	2	3	7	3	3	4
	F-O157	2	1	2	0	0	0	0	0	0
mEC	SMAC	3	4	4	18	17	18	3	6	5
	F-O157	2	1	2	12	9	12	0	1	0
LST	SMAC	4	2	5	17	18	18	2	3	6
	F-O157	2	1	4	15	18	17	1	1	2

* See Materials and Methods; Second Recovery Trials section for explanation of numerals

different *E. coli* O157:H7 inoculum rates). The scoring of plates as 0, 1, 2 or 3 is explained above. In other words, in the table, higher figures show higher opportunity for isolation of *E. coli* O157:H7 vs. cohabitants.

Discussion

First Recovery Trials

It is expected that 1 agar medium will give higher counts for *E. coli* O157:H7 and lower counts for competitive flora and that medium will be assumed to be better than the other agar media for *E. coli* O157:H7 isolation in the presence of competitive flora. In this experiment SMAC agar gave higher plate counts than F-O157 agar for all the 7 bacterial strains. But from this point of view it is hard to say that SMAC agar is clearly better than F-O157 agar because the growth of cohabitant flora was also good in the case of SMAC agar. On the other hand, false negative results might be obtained with F-O157 agar.

The DMR test was applied to the groups of bacteria, selective enrichment media and incubation time. A summary of these statistical analyses is given below.

- 2 strains of *E. coli* O157:H7 and 1 strain of *C. freundii* grew well in the mTS broth at 16, 20, 24 and 28 h of incubation while the growth of others was suppressed.
- 2 strains of *E. coli* O157:H7 and 2 strains of *C. freundii* grew well in the mEC broth at 16, 20, 24 and 28 h of incubation while the growth of others was suppressed.
- 2 strains of *E. coli* O157:H7 grew well in the LST broth while others grew differently in different incubation periods.

These results indicate clearly that strain difference is important. *E. coli* O157:H7 (h3) grew weakly in every 1 of the 3 selective enrichment mediums. There are a number of studies emphasizing strain difference directly. Karch et al. (8) and Palumbo et al. (17) showed that strain difference was important in *E. coli* O157:H7 analysis.

mTS broth seems to have some advantages due to the weak growth of 2 strains of *E. coli* type 1 and 1 strain of *C. freundii.* The performance of LST broth was not clear since different growth levels were found at different incubation times. This is undoubtedly critical for routine analysis.

In order to eliminate strain difference, the 3 strains of *E. coli* O157:H7 were assumed to be 1 strain, and similarly 2 strains each of *E. coli* type 1 and *C. freundii* were assumed to be 1 strain. Statistical analysis was applied to these 3 theoretically created bacteria which indicated that interactions among bacteria x selective enrichment media, bacteria x agar media and bacteria x incubation time were statistically significant ($p<0.05$). For *E. coli* O157:H7 and *C. freundii* all 3 selective enrichment media were similar while LST broth was found to be better for *E. coli* type 1 than the other strains. As expected, the results of statistical analysis of the 3 theoretically created bacteria were similar to those of the independent analysis of 7 bacteria.

Two important results obtained in the first step were that the SMAC agar was superior to the F-O157 agar and

strain differences were significant ($p<0.05$). Selection of the best selective enrichment broth was difficult at this stage.

There are numerous commercial selective plating media for various microorganisms. Although all of them are applicable for routine analysis as well as for research work, selectivity and sensitivity are 2 most important factors for choosing among them. Especially if the bacterium studied is very low in number compared to the cohabitant flora, selection of the best medium is important. Generally, "the best medium" is variable mainly according to time and other factors. For *E. coli* O157:H7 isolation, many selective media have been compared over time by researchers. While SMAC agar is used very commonly for traditional cultural techniques, SMAC agar plus BCIG (16), SMAC agar plus CR (12), and SMAC agar plus an antiserum for *E. coli* serogroup O157 (21) were found to be superior to SMAC agar. In this research we found that SMAC agar is superior to F-O157 agar.

Second Recovery Trials

The effect of the competitive microflora was very clear in this step. Similar to the first recovery trials, *E. coli* O157:H7 strains showed different growth patterns. Interactions among selective enrichment broth x agar media x cohabitant concentrations and selective enrichment broth x type of cohabitant x cohabitant concentration were statistically significant ($p<0.05$). Better results were obtained with SMAC agar than F-O157 agar in this step also. However, in contrast to the first recovery studies, SMAC agar combined with mEC broth and LST broth gave higher isolation possibilities than that of the SMAC agar and mTS broth combination. A correct combination of the selective enrichment broth and agar media is important. For *Salmonella* analysis, there are some examples such as RVS broth and MLCB agar (22), and Salmosyst broth and Rambach agar (20).

SMAC agar has clearly shown better results than F-O157 agar. In the presence of cohabitant flora, *E. coli* O157:H7 was recovered from every combination of SMAC agar and the selective enrichment broth while F-O157 agar gave 8 false negative results; 6 of those were obtained from mTS broth while the other 2 were from mEC broth combinations.

There was no significant difference between selective enrichment media in the first step, but in the second step lower plate counts were obtained with mTS broth than the others. Blais et al. (6) found that higher *E. coli* O157:H7 cell densities were achieved after enrichment with mEC broth plus novobiocin than in mTS broth for the inoculated meat samples.

The recovery of *E. coli* O157:H7 is easy when only *C. freundii* is present as a cohabitant microflora but the possibility of the presence of *C. freundii* as the only competitive flora is very unlikely. The presence of *E. coli* type 1 plus *C. freundii* as cohabitant flora gave similar results to *E. coli* type 1. It indicates that *C. freundii* is not an important cohabitant for *E. coli* O157:H7 isolation while *E. coli* type 1 is clearly an important cohabitant.

As expected, the 5 % *E. coli* O157:H7 concentration in cohabitant flora gave better recovery than that of 1 %. According to Smith and Scotland (23), it might be because of the low proportion of VTEC in the fecal flora (often less then 1%) that picking and testing of individual colonies did not always detect the presence of VTEC. Okrend et al. (16) reported that the ability to isolate *E. coli* O157:H7 from mixed cultures was dependent upon the number of the background microflora. This was particularly true when testing raw meat samples which might contain 10^6 microorganisms per gram.

In a conclusion, if a study on selective media preparation and/or selection of media is based on target bacteria alone, the results may not reflect application in practice. The existence of selective media-growing cohabitant bacteria is more important than the target bacteria's recovery rate or recovery limits. Also, it must be kept in mind that a correct combination of selective enrichment and selective agar media is important, depending on the composition of the microflora of the sample.

According to the results, SMAC agar is clearly superior to F-O157 agar, whereas mEC broth and LST broth must be used as selective enrichment media instead of mTS broth when *E. coli* type 1 and *C. freundii* are part of the competitive microflora of the sample. The ratio of selective enrichment media-growing bacteria is very important for the detection of *E. coli* O157:H7 from food and environmental specimens. If the concentration of *E. coli* O157:H7 is less than 1 % of the cohabitant flora in the sample, the recovery is almost impossible by the use of conventional culture methods and it is independent of the number of this bacteria in the sample. One must also

take into account the cohabitant species and strain differences.

Acknowledgments

This study was supported by the Veterinary and Husbandry Research Group (Grant No. TUBITAK-VHAG-1192), Scientific and Technical Council of Turkey. The authors thank Dr. K. Ayhan, Dr. A. Aytaç and S. Es for supplying the *E. coli* O157:H7 strains and also Dr. E. Başpınar for statistical analyses.

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