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Research Article

Evaluation of Bile Salt No. 3, Novobiocin, and Cefixime-Tellurite in Enrichment and/or Selective Plating Media for Detection of *Escherichia coli* O157:H7 from Ground Beef

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Abstract: Efficacy of commonly used selective supplements for detection of *E. coli* O157:H7 from ground beef was evaluated for suppression of sorbitol positive flora while supporting recovery of sorbitol negative flora. Selectivity of bile free modified *E. coli* broth (b-mEC) was not improved by the single addition of bile salts no. 3 (1.12 mg/ml), novobiocin (20 µg/ml), cefixime (0.05 µg/ml)-tellurite (2.5 µg/ml), or their combinations. The sorbitol positive flora was more resistant to the selective agents than sorbitol negatives. Sorbitol MacConkey agar and *E. coli* O157:H7 agar had almost equal selectivity. Adding cefixime (0.05 µg/ml)-tellurite (2.5 µg/ml) to the selective plating media highly enhanced the selectivity, and a general sorbitol based plating medium without cefixime-tellurite is not appropriate for this purpose due to its nonselectivity. As a result, a bile-free enrichment broth such as b-mEC and a sorbitol based selective plating medium containing cefixime-tellurite appeared to be reliable.

Key Words: *E. coli* O157:H7, ground beef, bile salt, novobiocin, cefixime-tellurite

Kıymalardan *Escherichia coli* O157:H7 İzolasyonu Amacıyla Zenginleştirme ve/veya Sorbitol Bazlı Katı Besi Yerlerinde Kullanılan Safra Tuzları No. 3, Novobiosin ve Sefiksim-Tellurit Saplementlerinin Değerlendirilmesi

Özet: Bu çalışmada sığır eti kıymalarından *E. coli* O157:H7 tayininde en sık kullanılan saplementlerin etkinliği sorbitol negatif floranın desteklenmesi ve sorbitol pozitif floranın (rekabetçi flora) en iyi şekilde baskılanması esasına göre araştırıldı. Safra tuzu ilave edilmemiş besi yeri (bile free modified *E. coli* broth, b-mEC)'ne safra tuzu (bile salts no. 3, 1,12 mg/ml), novobiosin (20 µg/ml), sefiksim (0,05 µg/ml)-tellurit (2,5 µg/ml) saplementlerinin ayrı ayrı veya kombine olarak ilave edilmesi besi yerinin etkinliğini artırmadı. Sorbitol pozitif floranın bu saplementlere sorbitol negatif floradan daha dirençli olduğu gözlendi. Sorbitol MacConkey agar ve *E. coli* O157:H7 agar yaklaşık olarak aynı düzeyde selektif özellik gösterdi. Bu besi yerlerine sefiksim (0,05 µg/ml)-tellurit (2,5 µg/ml) saplement ilave etmekle selektiflikleri oldukça arttı ve saplement kullanımının uygun olduğu görüldü. Sonuç olarak, b-mEC gibi bir zenginleştirme besi yeri ve sefiksim-tellurit saplement içeren sorbitol bazlı bir katı besi yerinin kullanımının güvenilir olduğu gözlendi.

Anahtar Sözcükler: *E. coli* O157:H7, sığır kıyması, safra tuzu, novobiosin, sefiksim-tellurit

Introduction

Escherichia coli O157:H7 is a foodborne pathogen, primarily associated with consumption of undercooked ground beef. The pathogen is an important food safety concern worldwide (1). In order to ensure the safety and quality of its products, the food industry needs to institute rapid, simple, and inexpensive pathogen screening procedures to monitor for the presence of this pathogen (2). However, it is an area which is continually developing and there is no universally accepted method for detection of this pathogen yet (3).

Since plating food specimens directly onto selective agar medium is not appropriate due to additional complication of natural flora, an initial enrichment procedure is generally required prior to introducing selective medium (4,5). A modified *E. coli* (mEC) broth with novobiocin (mEC+n) and Sorbitol MacConkey (SMAC) agar supplemented with Cefixime-tellurite

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supplement (CT-SMAC) are widely used media (6). It has also been stated that *E. coli* O157:H7 medium (EOH) has better potential than SMAC in terms of differentiating *E. coli* O157:H7 from other *E. coli* serotypes by their characteristic colony morphology (7).

Recovery of *E. coli* O157:H7 from foods is difficult by cultural methods, due to the outgrowth of other sorbitol negative, 1,4-Methylumbelliferyl glucuronide (MUG) negative and salicin negative colony populations besides the target organisms (1,4,8). Another problem is the overgrowth of sorbitol positive colonies (SPC) on selective agar plates resulting in masking the typical color of sorbitol negative colonies (SNC) (5,7,9,10). Thus, it is necessary to restreak the separated colonies onto another selective agar plate such as CT-SMAC medium supplemented with salicin (11). But, this procedure requires an additional 24 h incubation period. Therefore, the purpose of the present study was to evaluate four different enrichment broths (b-mEC, mEC, mEC+n, mEC+nct) and sorbitol based plating media (SMAC, EOH, CT-SMAC and CT-EOH) for maximum recovery of CT-resistant SNC population with the best suppression of population of sorbitol positive bacteria in ground beef.

Materials and Methods

Preparation of *E. coli* O157:H7 *inoculum*

One of the three strains of *E. coli* O157:H7 (strain no. 937) was kindly provided by Dr. Y. Özbaş (Univ. of Hacettepe, Ankara-Turkey) and other two strains (H71 and H72) were isolated from ground beef samples in our laboratory. Each strain was maintained on tryptic soy agar with 0.6% yeast extract (Oxoid) at 4 ^{o}C with monthly transfer. Strains were cultured at 37 ^{o}C for 20 h in tryptic soy broth with 0.6% yeast extract (BBL). Cells from broth culture were harvested by centrifugation at 9000 X g for 10 min at 4 ^{o}C. The cell pellets from individual strains were washed and resuspended in 5 ml of 0.85% saline solution. Equal volumes of the three strains were combined and serially diluted to obtain a cocktail of inoculum (11). The resulting inoculum was enumerated using sorbitol MacConkey medium plate for determination of colony forming unit (cfu) per ml. Each portion of enrichment media was inoculated with 6-12 cfu/ml *E. coli* O157:H7 mix culture.

Media

Bile salt-free modified *E. coli* broth (b-mEC) was prepared in the same formulation as the mEC broth (Oxoid). All ingredients incorporated in the medium were produced by Oxoid. Another three additional broths based upon modified *E. coli* broth including bile salts no. 3 (1.12 mg/ml) (mEC) were also used (12). These three were the mEC broth (Oxoid) with novobiocin (Oxoid, 20 µg/ml), (mEC+n); mEC with cefixime (0.05 µg/ml)-tellurite (2.5 µg/ml) (Oxoid) (mEC+nct) and mEC with novobiocin and cefixime-tellurite supplements (Oxoid) (mEC+nct). Four sorbitol based plating media were used: sorbitol MacConkey medium (SMAC) (Oxoid), *E.coli* O157:H7 medium (EOH; formulation of Kang and Fung) (7), Cefixime-tellurite (Oxoid) supplemented SMAC (CT-SMAC), and EOH (CT-EOH).

Sample preparation and analysis

Ground beef samples at four different times (five samples in every occasion, 1 kg each) between March and May 2001 were collected from local retailers in Kars, Turkey, and delivered to our laboratory in 1 h under cold storage. The samples in separate sterile bags were hand mixed and a 100 g portion from each of them was transferred to another separate sterile bag and mixed once again by hand massage. This 100 g portion was transferred to another sterile bag with 900 ml of b-mEC broth, hand massaged, filtered through sterile cheesecloth and divided into 12 of 50 ml portions. Some of these portions were inoculated with test strains, while some others were not. Sample distribution is shown in Table 1.

Table 1. Experimental design and treatment groups for enrichment.

Sample Treatment Enrichment Medium	A Natural Flora	B Inoculated	C* Heated, Inoculated
b-mEC: Bile free modified E. coli broth	A1	B1	C1
mEC+n: mEC broth with novobiocin	A2	B2	C2
mEC+ct: mEc broth with cefixime and tellurite	A3	B3	C3
mEC+nct: mEC broth with novobiocin, cefixime and tellurite	A4	B4	C4

* this group was heat treated at 85 ºC for 2 min to degrade natural vegetative flora of samples, and then inoculated with test culture at 20 ºC before incubation.

After static incubation at 42 °C for 22 h, all of the 12 enrichment cultures were held in ice water for 2 minutes, serially diluted using 0.85% saline, and then the dilutions were kept at 4 ± 2 °C to prevent growth of microorganisms in broth culture during the selective plating process. The SMAC and EOH were spread plated from fourth and sixth, and CT-SMAC and CT-EOH were spread plated from original enrichment and the first four dilutions (25 µL) of each enrichment culture. Plates were incubated aerobically at 42 °C for 18-24 h, and then SPCs and SNCs were counted separately. Colony counts were transformed to cfu/ml and mean values of the replica represented in the figures with standard deviations.

For the determination of presence of *E. coli* O157 in uninoculated samples (Group A), a total of 100 SNCs selected from agar plates after colony counting were analysed by using the standard FDA, Bacteriological Analytical Manual (BAM) methods (6). Briefly, target organism was detected by colony morphology on Eosin Methylene Blue (EMB) agar (BBL), fermentation reactions in Triple Sugar Iron (TSI) agar (Merck). Indol test reaction, and *E. coli* O157 latex agglutination test results.

Statistical analysis

Enumerated SNC and SPC populations (CFU/ml) grown on four different selective agar media were transformed to log_{10} values. Experiments were repeated four times and means and standard errors were determined and demonstrated in figures. Analysis of variance (ANOVA) was applied to detect the differences between bacterial counts in different enrichment and selective plating combinations, and significance was based on a levels of 5% and 1% ($P < 0.05$ and $P < 0.01$).

Results

Escherichia coli O157:H7 was not detected in any of the 20 samples used in the current study. The SNCs on SMAC and CT-SMAC media were whitish gray, while the SPCs were pink to red; also the SNCs were pink and SPCs were yellow on EOH and CT-EOH media.

Only SNCs arose on selective agar plates of heat treated and inoculated group (Group C). Effectiveness of heat treatment on flora of samples was confirmed by the enrichment of heat treated but not inoculated portions of Group C samples in b-mEC broth and plating of enrichments using SMAC and EOH media.

Although *E. coli* O157 was not detected in any of the 20 uninoculated samples used in the current study (Group A), there were large numbers of SPCs on selective plating media compared to SNCs (Figure 1a, 1b). The same aspect was observed in Group B samples (inoculated) (Figure 2a, 2b). None of the enrichment broth and selective plate combination produced more SNC population than SPC population (Figures 1a, 1b, 2a, 2b). It was also observed that the outgrowth of SPCs on selective media affected the typical color of media and SNC.

There was no statistical difference in terms of SNC and SPC populations (Figures 1a, 1b, 2a, 2b) among SMAC and EOH media. We also demonstrated that b-mEC broth yielded SNC population almost equal to mEC+n, mEC+ct, and mEC+nct broths. When all of the four different enrichment broths (b-mEC, mEC+n, mEC+ct, mEC+nct) were combined with SMAC or EOH, it gave at least equal amounts of SPC and SNC populatons in both uninoculated and inoculated groups (Group A and B) (Figures 1a, 1b, 2a, 2b). Contrary to these findings, both SNC and SPC populations were suppressed dramatically ($P < 0.01$) when CT-SMAC and CT-EOH were used for selective plating (Figures 1a, 1b, 2a, 2b). This suppressive effect was more pronounced on SNC population than on SPC population in uninoculated samples (Group A) (Figure 1a, 1b) ($P < 0.05$). But in inoculated Group B samples (Figure 2a, 2b), the same difference did not appear either. Also, a very limited suppression of CT-SMAC and CT-EOH media on SNC population was noticed in the heat treated and inoculated (Group C) samples compared to others (Group A and B) (Figures 1a, 2a, 3).

In the present study, the SNCs grew well on CT supplemented agar plates when inoculated from all enrichment cultures, except for mEC+nct (Figures 1a, 2a,). However, those findings were not observed clearly when the ground beef flora was destroyed by heat treatment and then inoculated with culture mix (Group C) (Figure 3).

Discussion

Kang and Fung (7) demonstrated that, in spite of SMAC, SPCs on EOH did not mask the typical colors of SNC. However, in the current study, we did not detect any statistical difference in terms of SNC and SPC

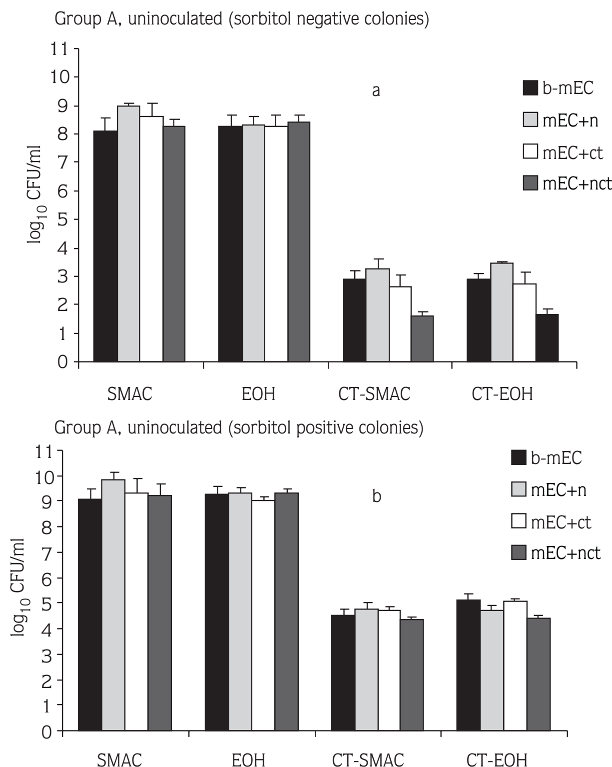

Figure 1. Sorbitol negative (1a) and positive (1b) colony populations in enrichment cultures of uninoculated ground beef samples (Group A), shown as mean±S:E of four repetition. (b-mEC: bile free modified *E. coli* broth; n, novobiocin; ct or CT: cefixime-tellurite supplement; SMAC: sorbitol MacConkey agar; EOH; *E. coli* O157:H7 agar).

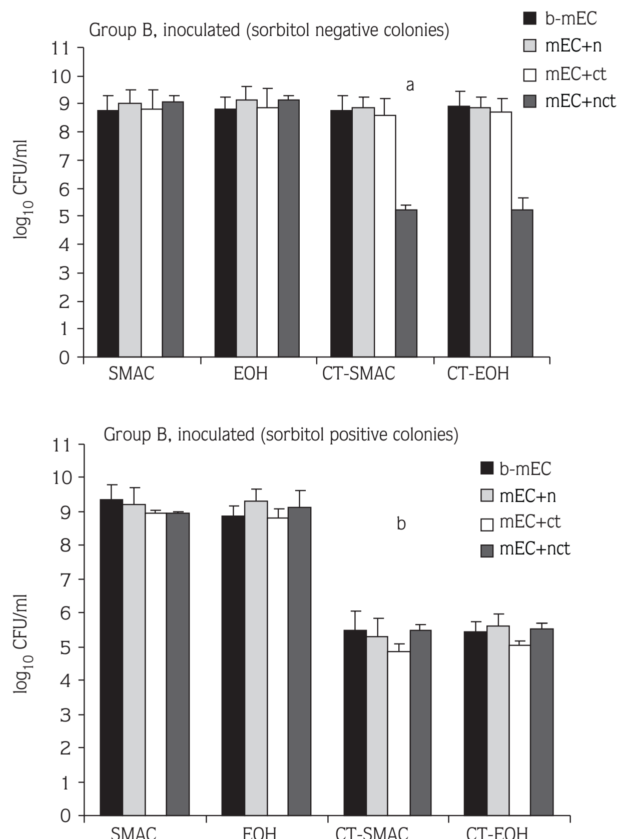

Figure 2. Sorbitol negative (2a) and positive (2b) colony populations in enrichment cultures of inoculated ground beef samples (Group B). Key: the same as in Fig. 1 legend.

populations (Figures 1a, 1b, 2a,2b) among these media. For that reason, it is possible to speculate that EOH may not have advantages over SMAC when used as selective plating medium for selection of SNC from enriched ground beef samples.

The conditions for growth of *E. coli* O157:H7 in different enrichment media vary considerably due to the inhibitor substances used for selective recovery of this pathogen from ground beef (2,13). Also, antimicrobial supplements may lead to the reporting of false negative samples (1). Restaino et al. (5) demonstrated the superiority of modified buffered peptone water (mBPW) to mEC+n. We also determined in this study that addition of bile salts and/or novobiocin, cefixime and tellurite into enrichment broths is not necessary for successive isolation of *E. coli* O157:H7 from ground beef. But, to supplement the primary isolation medium (SMAC) with cefixime-tellurite (CT-SMAC) may increase the selectivity. Our findings may also demonstrate that the sensitivity of other SNCs higher than *E. coli* O157:H7 strains used in this study. MacRae et al. (1) have demonstrated the sensitivity of *E. coli* O157 to CT supplement and this sensitivity has been overcome by an extended lag time of strains for 10 to 16 h, but enrichment for a 24 h has tolerated that sensitivity at an important range.

In the present study, the SNCs grew well on CT supplemented agar plates when inoculated from all enrichment cultures, except for mEC+nct (Figure 1a, 2a,). However, those findings were not observed clearly when the ground beef flora was destroyed by heat treatment and then inoculated with pure culture mix (Group C) (Figure 3). It is, thus, possible to speculate that predictive modeling in pure cultures shows a significantly different growth profile from that observed in the

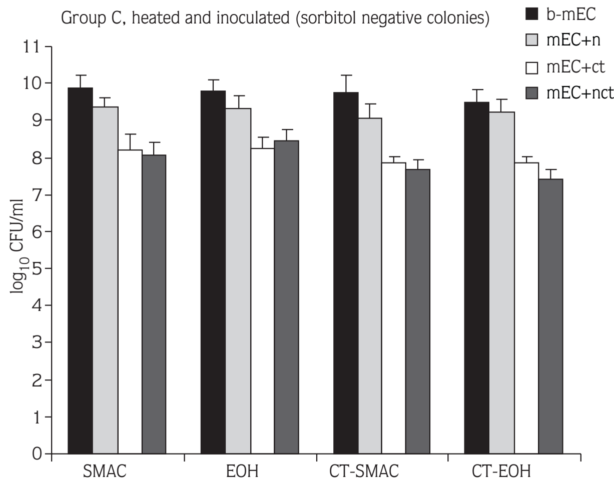

Figure 3. *E. coli* O157:H7 populations (sorbitol negative colonies) in enrichment cultures of heated treated, inoculated ground beef samples (Group C). Key: the same as in Fig. 1 legend.

presence of a competitive microflora. Duffy et al. (14) has also stated previously that there is an inherent danger in applying predictive models developed for pathogen growth in pure cultures directly to foods.

Methods for the detection and isolation of *E. coli* O157:H7 with a summary of comparative enrichment media and plating media studies have been well documented by De Boer and Heuvelink (15) and Harrison et al. (16). Although the superiority of other selective media over SMAC medium in recovery of stressed *E. coli* O157:H7 has been stated (17,18), CT-SMAC medium has been demonstrated to be superior to other agar media (19). Using two sorbitol based selective media, one with CT supplement, for spread plating from enrichment of the sample, may be acceptable for minimizing the false negative sample results. In this study, acceptable growth pattern and countable numbers of colonies were detected on SMAC and EOH media when these media spread plated from 5th or 6th ten-fold serial dilution tubes. The original enrichment culture and also first three dilution tubes of the same enrichment were the choice for making a good inoculation onto CT-SMAC and CT-EOH media. When CT supplemented selective medium gives adequate SNCs for further isolations, selection of suspect colonies among these CT resistant colonies may not require an additional CT resistance test. Thus, the analysis time can be shortened by at least a day with economical benefit for isolation of *E. coli* O157:H7 in ground beef samples. Also, when adequate numbers of SNC do not grow on the plate, selecting the target colonies on other CT free selective plating medium inoculated from the fifth and sixth serial ten-fold dilution of the same sample can help to prevent false negative sample results. Supplementation of selective media with some chromogenic and/or flourogenic substances can also enhance selectivity (6).

We believe that b-mEC broth may be an alternative of other formulas tested in the present study, and also selection of one sorbitol based agar medium over another depends on researcher's choice, but using a combination of either SMAC and CT-SMAC media or EOH and CT-EOH media for selective plating from b-mEC broth may be suitable for detection of *E. coli* O157:H7 from ground beef on a routine basis. Nevertheless, our results should be evaluated by comparing with other enrichment broths and selective agar media on retail ground beef samples for recommendation as an improved routine isolation method for *E. coli* O157:H7.

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