Use of Salicin-Rhamnose-Cellobiose-4-Methylumbelliferyl-β-D Glucuronide–Sorbitol MacConkey (SRC-MUG-SMAC) Differential Agar for the Presumptive Identification of *Escherichia coli* O157 from the Primary Isolation Step

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Abstract: A differential agar for the further detection of presumptive *Escherichia coli* O157 strains among typical sorbitol negative and cefixime-tellurite resistant strains was investigated. For this purpose, in parallel to colonies of 8 reference strains, 1130 suspect colonies picked from the surface of cefixime-tellurite (CT)-sorbitol MacConkey (SMAC) (CT-SMAC) agar plates of food samples (white cheese, raw milk and ground beef) were transferred to differential agar plates. After a 6-h incubation period at 42 ºC, only 47 (4.15%) of the 1130 colonies appeared as typical *E. coli* O157 on the differential agar plates (100 x 15 mm) gridded into 25 numbered sections. Salicin (S), rhamnose (R) and cellobiose (C) were tested separately and together in combination with MUG. The best results were obtained from the combined use of the 3 in SMAC medium (SRC-MUG-SMAC). When violet red bile lactose agar (VL) was used in the same manner as SRC-MUG-SMAC, only 16 (1.41%) of the 1130 colonies remained typical. In this procedure, no biochemical test was required for further identification. The use of an SRC-MUG-SMAC plate immediately after the colony selection step appeared to be more discriminative than the addition of these sugars and MUG to the primary isolation media. As a result, the testing of presumptive *E. coli* O157 colonies using a differential agar plate including MUG and 3 (or more) sugars that are not fermented by this organism is a rapid, easy, precise and relatively inexpensive isolation procedure, and it is particularly ideal for use in routine screening laboratories.

Key Words: *E. coli* O157, salicin, rhamnose, cellobiose, MUG

İlk İzolasyon Aşamasında *Escherichia coli* O157'nin Şüpheli İdentifikasyonu Amacıyla Salisin-Ramnoz-Sellobiyoz-4-Metilumbelliferil-β-D Glukuronit–Sorbitol MacConkey (SRC-MUG-SMAC) Ayırıcı Katı Besiyerinin Kullanılması

Özet: Bu çalışmada, sorbitol negatif ve sefiksim-tellurite dirençli tipik suşlar arasından şüpheli *Escherichia coli* O157 suşlarının izolasyonu için bir ayırıcı katı besiyeri araştırıldı. Bu amaç için, 8 referans suş kolonisine paralel olarak gıda örneklerine (beyaz peynir, çiğ süt ve kıyma) ait sefiksim-tellurit (CT) içeren sorbitol MacConkey (CT-SMAC) besiyeri yüzeyinden seçilen 1130 şüpheli koloni ayırıcı katı besiyerine transfer edildi. Salisin (S), ramnoz (R), sellobiyoz (C) hem ayrı ayrı, hem de üçü birlikte MUG ile kombine edilerek test edildi. SRC ve MUG içeren ve dıştan çizimle 25 numaralı bölmeye ayrılan ayırıcı katı besiyeri (SRC-MUG-SMAC) plaklarının (100 x 15 mm) 42 ºC'de 6 saat inkübasyonundan sonra, 1130 suşun sadece 47 (% 4,15)'sinin tipik *E. coli* O157 özelliği gösterdiği gözlendi. Violet red bile lactose agar (VL) aynı tarzda kullanıldığında ise 1130 suşun sadece 16 (% 1,41)'sı tipik özellik gösterdi. Bu prosedürde ileri identifikasyon için başka bir biyokimyasal teste ihtiyaç duyulmadı. Koloni seçim aşamasında SRC-MUG-SMAC katı besiyerinin kullanılmasının ilk izolasyon katı besiyerlerine bu şekerlerin ve MUG'un katılmasına kıyasla daha ayırıcı olduğu görüldü. Sonuç olarak, şüpheli *E. coli* O157 identifikasyonunda MUG ile birlikte suşun fermente edemediği 3 veya daha fazla şeker içeren bir ayırıcı agar plağı kullanmanın hızlı, kolay, kesin ve göreceli olarak ucuz olması nedeniyle özellikle rutin tarama laboratuarları için ideal olduğu görüldü.

Anahtar Sözcükler: *E. coli* O157, salisin, ramnoz, sellobiyoz, MUG

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Introduction

Escherichia coli O157:H7 is a foodborne pathogen, primarily associated with the consumption of contaminated ground beef and is an important food safety concern worldwide (1). In order to ensure the safety and quality of its products, the food industry needs rapid, simple and inexpensive screening procedures to monitor this pathogen on a routine basis (2). However, it has been stated that routine analysis procedures for *E. coli* O157 are continually developing and that we are still some way from a universally accepted method for this purpose (3).

Recovering low but significant levels of *E. coli* O157 strains from foods by culture methods is difficult, due to the outgrowth of colonies of sorbitol-positive and non-O157 sorbitol-negative indigenous flora on sorbitol-based primary isolation media (1,4,5). It has been reported that several carbohydrates, such as sorbitol, salicin (S), adonitol, inositol, cellobiose (C) and sorbose, are not fermented by *E. coli* O157:H7 strains (6-9). Separate incorporation of S, R or C, with or without 4-methylumbelliferyl-β-D glucuronide (MUG), into the selective plating media has been recommended to improve differentiation (7,10,11). Commercial sorbitol-based selective plating media including MUG and/or rhamnose are also available.

It has been reported that, when many sugars are incorporated into a selective plating medium, colony coloration patterns and the MUG reaction are not discriminitive because of high acidity (11,12). Salicin, R, C and MUG are expensive substances and their incorporation into primary isolation media increases the cost of analysis per sample. We, therefore, decided to search for an accurate, rapid, simple and inexpensive differential agar for use after selective plating. This agar was used to test sugar fermentation and MUG activity of sorbitol-negative colonies picked from the sorbitol-based primary isolation media. For this purpose, S, R, C and MUG were added to SMAC agar. The lactose fermentation phenotype was also evaluated in parallel with these tests.

Materials and Methods

Reference strains. One strain of *E. coli* O157:H7 (strain no. 937), provided by Dr. Y. Özbaş (Univ. of Hacettepe, Ankara, Turkey), and 7 other *E. coli* O157:H7 strains, isolated from ground beef samples in our laboratory, were used as reference strains in the experiments. Each strain was separately maintained on tryptic soy agar (Difco) with 0.6% yeast extract (Difco) at 4 °C with monthly transfer. Each strain was transferred to tryptic soy broth (TSB, Difco) with 0.6% yeast extract (Difco) (mTSB) and grown at 37 °C for 20 h before use.

Media. Double strength mTSB with novobiocin (Oxoid) (mTSBn) was used for the enrichment of raw milk samples, and mTSBn was used for cheese samples, according to Reinders et al. (13). Modified *E. coli* broth (mEC, Oxoid) was used for the enrichment of ground beef samples.

SMAC agar (Oxoid) supplemented with cefixime-tellurite (CT, Oxoid) (CT-SMAC) was used as the primary isolation medium.

The differential agar formulations are listed below:

1. MUG-SMAC: 4-methylumbelliferyl-β-D glucuronide (MUG, 100 mg/l, Difco)-SMAC agar.

2. S-MUG-SMAC: Salicin (S, 5 g/l, Difco)- MUG (100 mg/l, Difco)-SMAC agar.

3. R-MUG-SMAC: Rhamnose (R, 5 g/l, Sigma)-MUG-SMAC agar.

4. C-MUG-SMAC: Cellobiose (C, 5 g/l, Sigma)-MUG-SMAC agar.

5. SRC-MUG-SMAC: Salicin rhamnose cellobiose-MUG-SMAC agar.

6. VL: Violet red bile lactose agar (Difco).

The indicated sugars and MUG were added to the media before, and novobiocin and CT supplements were added after autoclaving. All media, except VL, were autoclaved at 121 °C for 15 min.

Sample analysis. A total of 298 samples consisting of 150 ground beef and 90 white cheese, and 58 raw milk (1 l each) were obtained during September-November 2003 from local retailers in Kars, Turkey, and delivered to our laboratory in 1 h under cold storage. After thorough mixing, 100 ml of each milk sample was added to 100 ml of double strength mTSBn broth. Each cheese and ground beef sample was mixed separately in sterile bags by hand massage applied to the outside of the bag, and 25 g of this sample was transferred to another separate sterile bag. Then 225 ml of enrichment broth was added to each sample, followed by hand massaging

for 2 min applied to the outside of the bag. The further analysis protocol is listed below:

a. Resuscitation: Static incubation at 25 °C for 2 h.

b. Enrichment: Static incubation at 42 °C for 22 h.

c. Primary isolation: After enrichment, samples were serially diluted using 0.85% saline, and CT-SMAC was spread-plated from the original and 6 consecutive 10-fold serial dilutions of each enrichment culture (50 µl from each). The plates were incubated aerobically at 42 °C for 16 h.

Differential agar tests: For the determination of presumptive *E. coli* O157 isolates, 5 to 10 typical colonies (sorbitol negative, colorless), if present, were picked from the primary isolation plates (CT-SMAC) of each sample and spread onto the center of a section on the differential agar plates (MUG-SMAC, S-MUG-SMAC, R-MUG-SMAC, C-MUG-SMAC, SRC-MUG-SMAC and VL). Inoculations were made onto a 2-mm diameter surface area of the plate, and the plates were incubated at 42 °C for 6 to 24 h. A 6-h incubation period seemed to be sufficient for the development of typical colonies. By this method, approximately 25 colonies were tested on each 100 x 15 mm plate gridded into 25 numbered sections. Typical colonies appeared colorless and MUG negative (no blue fluorescence occurred under a 366 nm UV lamp (Merck) in a dark room) on the differential agar plates.

Atypical colonies fermented any of the 3 sugars and developed a red color.

Results

The 8 reference strains did not ferment S, R or C, but fermented lactose, and none of these strains cleaved MUG. As seen in the Table, by using separate rhamnose, cellobiose, MUG, salicin and lactose tests, 29.64%, 42.56%, 51.32%, 53.89% and 61.15% of 1130 sorbitol-negative colonies demonstrated the properties of *E. coli* O157, respectively. Rhamnose appeared to be the most selective substance, particularly in terms of eliminating false positive colonies picked from the primary isolation medium (CT-SMAC). When S, R, C and MUG were incorporated into a single sorbitol-based plating medium (SRC-MUG-SMAC), only 47 (4.15%) of the 1130 sorbitol-negative strains developed typical (colorless and MUG negative) colonies. When the lactose test plate (VL) was used in parallel to this differential agar (SRC-MUG-SMAC), only 16 (1.41%) of the 1130 strains developed typical colonies.

A total of 25 sorbitol-negative colonies were transferred onto one differential agar medium. Inoculations made onto 1-2 mm diameter surface areas of the plates enabled the rapid development of colonies. A 6-h incubation period seemed to be sufficient to

Table. Differential test results of sorbitol-negative presumptive *E. coli* O157 colonies picked from the primary isolation medium (CT-SMAC).

Tests/Samples	Medium	Ground beef n:150	White cheese n:90	Raw milk n:58	TOTAL n:298
Samples (% in total) including sorbitol-negative organisms	CT-SMAC	99 (66.00)	76 (84.44)	37 (63.79)	242 (81.20)
Total sorbitol-negative colonies picked from primary isolation medium	CT-SMAC	600	368	162	1130
Lactose positive (%)	VL	313(52.16)	293 (79.61)	85 (52.46)	691 (61.15)
MUG* negative (%)	MUG-SMAC	370 (61.66)	111 (30.16)	99 (61.11)	580 (51.32)
Salicin and MUG negative (%)	S-MUG-SMAC	405 (67.50)	144 (39.13)	60 (37.03)	609 (53.89)
Rhamnose and MUG negative (%)	R-MUG-SMAC	217 (36.16)	34 (9.24)	84 (51.85)	335 (29.64)
Cellobiose and MUG negative (%)	C-MUG-SMAC	298 (49.66)	128 (34.78)	55 (33.95)	481 (42.56)
SRC** and MUG negative (%)	SRC-MUG-SMAC	26 (4.33)	9 (2.44)	12 (7.40)	47 (4.15)
SRC and MUG negative and lactose positive (%)	SRC-MUG-SMAC and VL	7 (1.16)	3 (0.81)	6 (3.70)	16 (1.41)

*MUG; 4-methylumbelliferyl-β-D glucuronide, **SRC; salicin (S), rhamnose (R), cellobiose (C).

differentiate typical colonies from atypical ones using this procedure. The red coloring and blue fluorescence of all 25 colonies on each plate were easily monitored.

Discussion

There are several alternatives to traditional techniques for the isolation and identification of *E. coli* O157. However, although sensitive and specific, these techniques are expensive and likely to be beyond to scope of most routine diagnostic laboratories (7). In contrast to these alternative methods, selective culture is simple, quick and relatively inexpensive, and isolates may be readily and reliably identified using any rapid commercial test kit available. Furthermore, it has been stated that many routine analysis laboratories lack the capability of carrying out DNA probe and verocytotoxin assay techniques (3,14). The results in the Table show that in the examination of 298 food samples 242 samples required further investigation. Of 1130 colonies picked from the selective plates (CT-SMAC) of the 242 samples that required presumptive identification, only 47 did so from SRC-MUG-SMAC differential medium. This demonstrates that if this procedure is used, isolation and further identification tests such as O157 and H7 latex agglutination, micro-identification systems (API test kits), ELISA and PCR will be applied to considerably fewer colonies than in the case when routine valid procedures are performed (8). Thus, there will be savings in the terms of cost, labor and time.

Sorbitol fermentation and rapid MUG hydrolysis are characteristic of 95% and 92% of *E. coli* strains, respectively (6,8,10). While isolates of *E. coli* O157 fail to ferment sorbitol, S, R and C, or to cleave MUG, many sorbitol-based primary isolation media supplemented with MUG and/or R are commercially available. The addition of S, R or C to sorbitol-based primary isolation media, separately or in combination with MUG, has been recommended (3,5,7,10,11,15-17). However, undesirable outcomes have been reported under these conditions such as an outgrowth of indigenous flora, resulting in coloration problems on the plates, due to the occurrence of high acidity in the medium caused by the fermentation of sugars by non-O157 *E. coli* colonies (4,5,18,19). It has also been stated that the reaction of chromogenic substances is not discriminitive when many colonies grow on a single agar plate and make the environment of the plate acidic (11,12). Another problem is that the fluorescent product of MUG hydrolysis diffuses out of the colony and obscures the actual colonial source of the glucuronidase enzyme in selective plating medium (12). However, in this study, SRC-MUG-SMAC differential agar was successfully used to select the presumptive *E. coli* O157 colonies among other sorbitol negative colonies. Neither coloration nor fluorescence problems occurred at this step, owing to adequate distancing of the 25 colonies grown on each plate.

It is necessary to pick suspect colonies from selective plating media and transfer them to another test medium for further isolation and identification of pathogenic bacteria (8,14,20). It is also known that *E. coli* strains can produce identical colonies on selective agar media after 16-18 h incubation, particularly when inoculated from enrichment cultures. In this study, a 16-h incubation period for the primary isolation medium (CT-SMAC) and 6 h for the differential media (SRC-MUG-SMAC and VL) were found to be sufficient for typical colony development. In this way, the selective plating and differential agar procedure could be completed in 22 to 24 h after the enrichment of food samples. Typical colorless colonies on SRC-MUG-SMAC agar could be appropriate sources for further latex tests and other spot tests.

Some rhamnose and/or MUG positive strains of *E. coli* O157 have been reported (9,16,21-24). In another study, a good correlation between pathogenicity and rhamnose negativity of *E. coli* O157 has been demonstrated (25). In this study, we did not test these properties; however, we think that we used the most useful sugar combination in the differential agar medium for the easy selection of pathogenic *E. coli* O157 strains.

In conclusion, the use of SRC-MUG-SMAC, in combination with a lactose-based medium such as VL, is a good candidate for the effective selection of presumptive *E. coli* O157 strains grown on a primary isolation medium.

Acknowledgment

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