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CITROBACTER BRAAKII: A MAJOR CAUSE OF FALSE-POSITIVE RESULTS ON MACCONKEY AND LEVINE'S EOSIN METHYLENE BLUE SELECTIVE AGARS USED FOR THE ISOLATION OF *ESCHERICHIA COLI* FROM FRESH VEGETABLE SAMPLES

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ABSTRACT

Fresh vegetables are a frequent cause of foodborne disease outbreaks because they often do not undergo heat treatment prior to consumption. Pathogenic *Escherichia coli* strains are the major causative agents of produce-related outbreaks. In this study, we investigated the efficiency of conventional *E. coli* selective medium, MacConkey (MAC) agar, for the screening of fresh vegetable samples and evaluated the discrimination ability of Levine's eosin methylene blue (L-EMB) agar for *E. coli*-like colonies obtained from MAC agar. A total of 120 samples of lettuces ($n = 60$) and radish sprouts ($n = 60$) were analyzed. Among 17 (14.2%) MAC agar plates containing putative *E. coli* colonies, only one plate was confirmed *E. coli*-positive (positive predictive value = 5.9%). All 16 false-positive isolates were identified as *Citrobacter braakii* and formed *E. coli*-like colonies on L-EMB agar. These results indicate that in order to reduce false-positive results in screening fresh vegetable samples, both MAC and L-EMB selective agars should be modified with the aim of providing reliable differentiation between *E. coli* and *C. braakii*.

PRACTICAL APPLICATIONS

Although MacConkey (MAC) and Levine's eosin methylene blue (L-EMB) agars are not specific for the detection of pathogenic *Escherichia coli*, the media are recommended for the isolation of pathogenic *E. coli* (except *E. coli* O157:H7) in the Food and Drug Administration Bacteriological Analytical Manual. This is due to the fact that it is difficult to develop each pathogenic strains-specific selective media as they were not classified by their biochemical characteristics. However, the unexpected high prevalence of *Citrobacter braakii* in fresh vegetables observed in this study indicates that the screening potential of these *E. coli* selective media needs to be improved.

INTRODUCTION

In the Food and Drug Administration Bacteriological Analytical Manual, MacConkey (MAC) agar and Levine's eosin methylene blue (L-EMB) agar are listed as selective media for the isolation of pathogenic *Escherichia coli*, except enterohemorrhagic *E. coli* serotype O157:H7 (Bacteriological Analytical Manual 2011). Originally, MAC and L-EMB media were developed to detect not only *E. coli* but also other lactose-fermenting gram-negative bacteria (MacConkey 1905; Levine 1918). These bacteria produce acids by fermenting lactose; under acidic condition, the colonies are stained pink by neutral red in MAC agar and dark purple by eosin Y and methylene blue in L-EMB agar. *E. coli* is the most active lactose fermenter and is able to generate highly acidic conditions. This characteristic feature enables laboratory personnel to differentiate *E. coli* from other lactose-fermenting bacteria growing on these media

(Leininger *et al.* 2001). On MAC agar, *E. coli* forms brick red colonies with hazy surrounding zones due to precipitation of bile salts (Shuman and Silhavy 2003) and on L-EMB agar, *E. coli* colonies acquire a characteristic green metallic sheen due to the formation of an amide bond between eosin Y and methylene blue under strong acidic conditions (Horvath and Ropp 1974). However, previous studies have reported that some strains of coliform bacilli, including *Klebsiella*, *Serratia*, *Enterobacter* and *Citrobacter* spp. can produce typical *E. coli*-like colonies on both MAC and L-EMB agars (Venkateswaran *et al.* 1996; Leininger *et al.* 2001; Bacteriological Analytical Manual 2002). Thus, these bacteria at a high level may act as competing microflora and could interfere with the isolation of *E. coli* using the selective media.

Fresh vegetables are a frequent cause of foodborne disease outbreaks because they are often consumed raw without prior thermal treatment. Pathogenic *E. coli* strains are the major causative agents of produce-related outbreaks. A recent foodborne outbreak caused by a pathogenic *E. coli* variant O104:H4 in Germany was associated with sprouts (Buchholz *et al.* 2011) underscoring the need of screening for potentially pathogenic *E. coli* in fresh produce. In general, the levels of coliform bacteria in fresh lettuce and sprouts are high, reaching from 4 to 8 log cfu/g (Jo *et al.* 2011; Seow *et al.* 2012); such high prevalence may yield many false-positive results in screening tests for *E. coli* using MAC and L-EMB as selective media. However, there is very little information regarding the screening efficiency of these two *E. coli* selective agars in vegetable samples such as fresh lettuce and sprouts and the growth of major competing bacteria that can produce *E. coli*-like colonies.

Thus, in this study, we identified *E. coli*-competing microflora on MAC agar during screening and isolation of *E. coli* from fresh lettuce and sprouts, and evaluated the discrimination ability of L-EMB agar for *E. coli*-like colonies on MAC agar.

MATERIALS AND METHODS

A total of 120 fresh vegetable samples, including 60 lettuce and 60 radish sprout items, were purchased from a supermarket in Seoul, South Korea, over 5-month period from May to September 2014. All samples were delivered to the laboratory within 30 min of purchase and immediately prepared for analysis.

Each sample (25 g) was mixed with 225 mL buffered peptone water (Oxoid, Hampshire, UK) and homogenized for 1 min using a blender (Bagmixer, Interscience, St Nom, France). The homogenates were incubated for 3 h at 37C to revive bacteria and then left for another 21 h at 44C. After the enrichment, a loopful of broth culture was streaked onto MAC agar (Oxoid), followed by incubation at 37C for 24 h. Suspected colonies (brick red, nonmucoid, round-shaped, surrounded with a hazy zone of precipitated bile) were subcultured on tryptic soy agar (Oxoid) at 37C for 24 h, followed by final identification of the colonies using the VITEK 2 GN kit (bioMérieux, Marcy 1'Etoile, France). The predictive positive value (PPV) was calculated as the ratio of VITEK2-positive *E. coli* plates to the total number of MAC staining-positive plates.

To evaluate the discrimination ability of L-EMB agar, the *E. coli*-like colonies from MAC agar were subcultured on L-EMB agar (Oxoid) for 24 h at 37C and selected based on the appearance of the green metallic sheen.

RESULTS AND DISCUSSION

Table 1 shows the efficiency of *E. coli* screening using MAC agar as a selective medium. Among the 120 samples of fresh vegetables analyzed in this study, the presence of typical *E. coli*-like colonies on MAC agar was observed in 14.2% (17 of 120 samples). Among the 17 plates of positively stained colonies, one plate was confirmed as *E. coli*-positive by the VITEK 2 test, whereas the colonies on the remaining 16 plates were identified as *Citrobacter braakii*. When streaked onto L-EMB agar, all *C. braakii* isolates produced typical *E. coli*-like colonies with the metallic green sheen.

Until recently, MAC and L-EMB agar have been widely used as selective media for the screening of *E. coli*, including non-O157:H7 pathogenic strains (Karim *et al.* 2013; Pinaka *et al.* 2013; Selim *et al.* 2014). However, in this study, MAC agar presented poor screening efficiency in *E. coli* selective isolation from vegetable samples, as indicated by the low PPV of 5.9%, which may have resulted from low prevalence of *E. coli* in fresh vegetables (Choe *et al.* 2013; Ma *et al.*

TABLE 1. PREVALENCE OF FALSE-POSITIVE *ESCHERICHIA COLI* ON MACCONKEY AGAR USED FOR THE ISOLATION OF *E. COLI* FROM FRESH VEGETABLES

Number of putative *E. coli*-positive plates/number of tested vegetable samples (%)	Number of samples confirmed as†		
	True-positive	False-positive	PPV‡
17/120 (14.2)	1	16	5.9%

† The putative *E. coli* isolates were confirmed as *E. coli* (true-positive) or *Citrobacter braakii* (false-positive) by the VITEK2 test.

‡ PPV (positive predictive value) was calculated as the percentage of true *E. coli*-positive plates by the VITEK2 test to the number of putative *E. coli*-positive MacConkey agar plates.

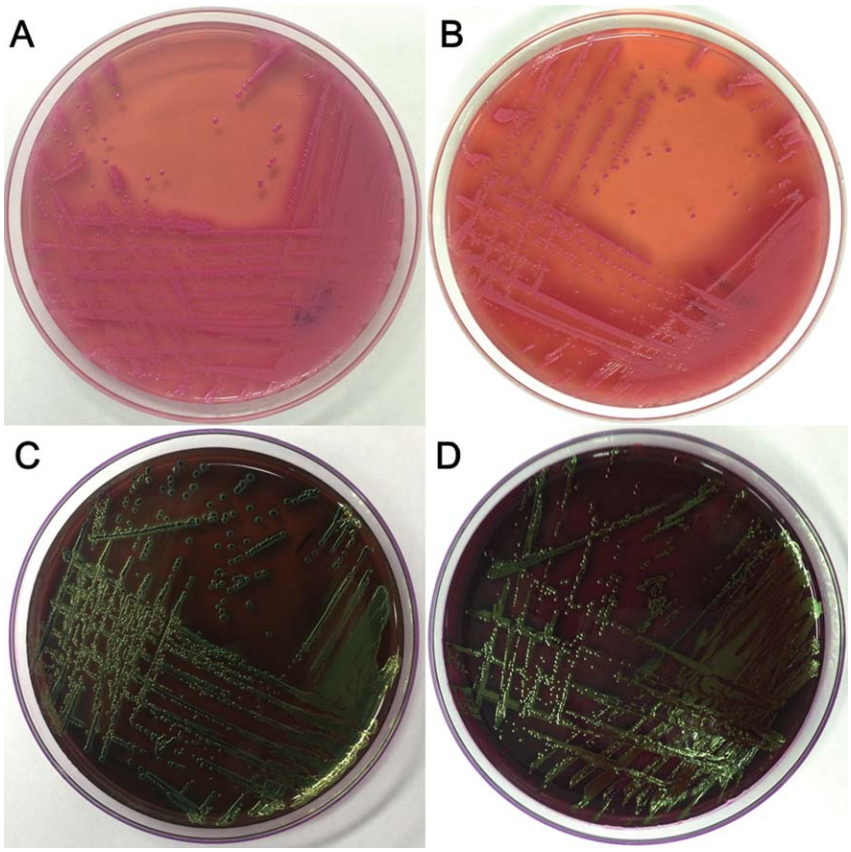

FIG. 1. IMAGES OF *ESCHERICHIA COLI* AND *CITROBACTER BRAAKII* COLONIES ON MACCONKEY AGAR (A AND B) AND LEVINE'S EOSIN METHYLENE BLUE AGAR (C AND D) AFTER 24-H INCUBATION AT 37C. (A AND C) *E. COLI* ISOLATED FROM VEGETABLE SAMPLES IN THIS STUDY; (B AND D) *C. BRAAKII* ISOLATED FROM VEGETABLE SAMPLES IN THIS STUDY. THE COLONIES FORMED BY *E. COLI* AND *C. BRAAKII* ON BOTH SELECTIVE MEDIA LOOK ALMOST IDENTICAL

2014). The presence of *E. coli* in foods indicates fecal contamination and the possibility of foodborne infection (Santos *et al.* 2012). In addition, as fresh produce does not undergo heat treatment prior to consumption (Rodríguez-Caturla *et al.* 2012), i.e., contaminating microflora is not inactivated, the level of *E. coli* in these foods is subject to strict control: it should not exceed 10^2 cfu/g in the European Union and 10 cfu/g in Korea (Santos *et al.* 2012; Korea Food and Drug Administration 2013). These strict policies for fresh produce may result in low prevalence of *E. coli* in fresh vegetables, evident through recent studies; *E. coli* isolation rates in fresh vegetables were reported at 6.3%, 4.0% and 2.3% in Spain, Portugal and South Korea, respectively (Rodríguez *et al.* 2011; Campos *et al.* 2013; Kim *et al.* 2014). L-EMB agar would have similar *E. coli* screening performance to MAC agar in testing fresh vegetables because all false MAC-positive isolates formed typical *E. coli*-like colonies on L-EMB agar. These results indicate that the use of these media for *E. coli* presumptive screening in fresh vegetables would require additional cost for further following confirmation steps.

Interestingly, all false-positive isolates in this study were identified as *C. braakii* which is known as an unusual gram-negative bacterium (Carlini *et al.* 2005). *E. coli* and *C. braakii* colonies formed on MAC and L-EMB agars were almost identical, indicating that lactose-fermenting ability of *C. braakii* isolates is similar to that of *E. coli* (Fig. 1). To our knowledge, this is the first report on *C. braakii* strains having the ability to produce typical *E. coli*-like colonies on MAC and L-EMB agars, suggesting that this species could complicate accurate *E. coli* detection in vegetables using these two selective media.

Citrobacter spp., including *C. braakii*, are found in food products such as meats, seafood and vegetables, as well as in water and soil (Arens and Verbist 1997; Ananchaipattana *et al.* 2012). Although some studies showed that *C. braakii* could cause opportunistic infections, such as peritonitis and sepsis (Gupta *et al.* 2003; Carlini *et al.* 2005); in general, *Citrobacter* spp. are considered to have low virulence in the gastrointestinal tract except for enterotoxigenic *Citrobacter freundii* (Schmidt *et al.* 1993; Samonis *et al.* 2009). To the best of our knowledge, no cases of food poisoning by *C. braakii* have been reported, suggesting that this species does not pose a significant danger regarding food safety and should be distinguished from a potentially pathogenic *E. coli* using efficient and reliable detection methodology.

In conclusion, MAC and L-EMB agars as *E. coli* selective media should be improved to reduce false-positive results in

screening food products, especially fresh vegetables that contain high levels of different coliform bacilli with high lactose fermentation activity. In this study, *C. braakii* was the only identified species to form typical *E. coli*-like colonies on MAC agar and also could not be differentiated in L-EMB agar, indicating the need for further studies aimed to improve the level of differentiation between *E. coli* and *C. braakii* using conventional selective media to prevent the increase of testing time and cost.

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REFERENCES

ANANCHAIPATTANA, C., HOSOTANI, Y., KAWASAKI, S., PONGSAWAT, S., BARI, M.D.L., ISOBE, S. and INATSU, Y. 2012. Bacterial contamination in retail foods purchased in Thailand. Food Sci. Technol. Res. *18*, 705–712.

ARENS, S. and VERBIST, L. 1997. Differentiation and susceptibility of *Citrobacter* isolates from patients in a university hospital. Clin. Microbiol. Infect. *3*, 53–57.

BACTERIOLOGICAL ANALYTICAL MANUAL. 2002. Chapter 4, Enumeration of *Escherichia coli* and the Coliform Bacteria. http://www.fda.gov/Food/FoodScienceResearch/LaboratoryMethods/ucm064948.htm (accessed February 9, 2015).

BACTERIOLOGICAL ANALYTICAL MANUAL. 2011. Chapter 4A, Diarrheagenic *Escherichia coli.* http://www.fda.gov/Food/FoodScienceResearch/LaboratoryMethods/ucm070080.htm (accessed February 9, 2015).

BUCHHOLZ, U., BERNARD, H., WERBER, D., BÖHMER, M.M., REMSCHMIDT, C., WILKING, H., DELERÉ, Y., AN DER HEIDEN, M., ADLHOCH, C., DREESMAN, J., *ET AL*. 2011. German outbreak of *Escherichia coli* O104:H4 associated with sprouts. N. Engl. J. Med. *365*, 1763–1770.

CAMPOS, J., MOURÃO, J., PESTANA, N., PEIXE, L., NOVAIS, C. and ANTUNES, P. 2013. Microbiological quality of ready-to-eat salads: An underestimated vehicle of bacteria and clinically relevant antibiotic resistance genes. Int. J. Food Microbiol. *166*, 464–470.

CARLINI, A., MATTEI, R., MAZZOTTA, L., LUCAROTTI, I., PIOLI, R., BARTELLONI, A. and ANTONELLI, A. 2005. *Citrobacter braakii*, an unusual organism as cause of acute peritonitis in PD patients. Perit. Dial. Int. *25*, 405–406.

CHOE, H.S., LEE, D.S., LEE, S.J., HONG, S.H., PARK, D.C., LEE, M.K., KIM, T.H. and CHO, Y.H. 2013. Performance of Anyplex™ II multiplex real-time PCR for the diagnosis of seven sexually transmitted infections: Comparison with currently available methods. Int. J. Infect. Dis. *17*, e1134–e1140.

GUPTA, R., RAUF, S.J., SINGH, S., SMITH, J. and AGRAHARKAR, M.L. 2003. Sepsis in a renal transplant recipient due to *Citrobacter braakii*. South. Med. J. *96*, 796–798.

HORVATH, R.S. and ROPP, M.E. 1974. Mechanism of action of eosin-methylene blue agar in the differentiation of *Escherichia coli* and *Enterobacter aerogenes*. Int. J. Syst. Bacteriol. *24*, 221–224.

JO, M.J., JEONG, A.R., KIM, H.J., LEE, N., OH, S.W., KIM, Y.J., CHUN, H.S. and KOO, M. 2011. Microbiological quality of fresh-cut produce and organic vegetables. Korean J. Food Sci. Technol. *43*, 91–97.

KARIM, M., AKHTER, N. and HOQUE, S. 2013. Bacterial status and pollution level in the sediment of Gulshan Lake, Dhaka. J. Asiat. Soc. Bangladesh Sci. *38*, 155–161.

KOREA FOOD AND DRUG ADMINISTRATION. 2013. Korea Food Code, Chapter 5–29, Ready-to-Eat and Convenience Foods. http://fse.foodnara.go.kr/residue/RS/jsp/menu_02_01_03.jsp?idx=99 (accessed February 9, 2015).

KIM, J.W., CHOI, I.W., NA, W.S., BALJII, E., YU, Y.M., YOUN, Y.N. and LEE, Y.H. 2014. Assessment of the microbiological quality of vegetable from urban community gardens in Korea. J. Food Hyg. Saf. *29*, 1–5.

LEININGER, D.J., ROBERSON, J.R. and ELVINGER, F. 2001. Use of eosin methylene blue agar to differentiate *Escherichia coli* from other gram-negative mastitis pathogens. J. Vet. Diagnostic Investig. *13*, 273–275.

LEVINE, M. 1918. Differentiation of *B. coli* and *B. aerogenes* on a simplified eosin-methylene blue agar. J. Infect. Dis. *23*, 43–47.

MA, H.M., IP, M., WOO, J. and HUI, D.S.C. 2014. Development and validation of a clinical risk score for predicting drug-resistant bacterial pneumonia in older Chinese patients. Respirology *19*, 549–555.

MACCONKEY, A. 1905. Lactose-fermenting bacteria in feces. J. Hyg. *5*, 333–379.

PINAKA, O., POURNARAS, S., MOUCHTOURI, V., PLAKOKEFALOS, E., KATSIAFLAKA, A., KOLOKYTHOPOULOU, F., BARBOUTSI, E., BITSOLAS, N. and HADJICHRISTODOULOU, C. 2013. Shiga toxin-producing *Escherichia coli* in Central Greece: Prevalence and virulence genes of O157:H7 and non-O157 in animal feces, vegetables, and humans. Eur. J. Clin. Microbiol. Infect. Dis. *32*, 1401–1408.

RODRÍGUEZ, M., VALERO, A., POSADA-IZQUIERDO, G.D., CARRASCO, E. and ZURERA, G. 2011. Evaluation of food handler practices and microbiological status of ready-to-eat foods in long-term care facilities in the Andalusia region of Spain. J. Food Prot. *74*, 1504–1512.

RODRÍGUEZ-CATURLA, M.Y., VALERO, A., CARRASCO, E., POSADA, G.D., GARCÍA-GIMENO, R.M. and ZURERA, G. 2012. Evaluation of hygiene practices and microbiological status of ready-to-eat vegetable salads in Spanish school canteens. J. Sci. Food Agric. *92*, 2332–2340.

SAMONIS, G., KARAGEORGOPOULOS, D.E., KOFTERIDIS, D.P., MATTHAIOU, D.K., SIDIROPOULOU, V., MARAKI, S. and FALAGAS, M.E. 2009. *Citrobacter* infections in a general hospital: Characteristics and outcomes. Eur. J. Clin. Microbiol. Infect. Dis. *28*, 61–68.

SANTOS, M.I., CAVACO, A., GOUVEIA, J., NOVAIS, M.R., NOGUEIRA, P.J., PEDROSO, L. and FERREIRA, M.A.S.S. 2012. Evaluation of minimally processed salads commercialized in Portugal. Food Control *23*, 275–281.

SCHMIDT, H., MONTAG, M., BOCKEMÜHL, J., HEESEMANN, J. and KARCH, H. 1993. Shiga-like toxin II-related cytotoxins in *Citrobacter freundii* strains from humans and beef samples. Infect. Immun. *61*, 534–543.

SELIM, S.A., AHMED, S.F., AZIZ, M.H.A., ZAKARIA, A.M., KLENA, J.D. and PANGALLO, D. 2014. Prevalence and characterization of Shiga-Toxin O157:H7 and Non-O157:H7 enterohemorrhagic *Escherichia coli* isolated from different sources. Biotechnol. Biotechnol. Equip. *27*, 3834–3842.

SEOW, J., ÁGOSTON, R., PHUA, L. and YUK, H.-G. 2012. Microbiological quality of fresh vegetables and fruits sold in Singapore. Food Control *25*, 39–44.

SHUMAN, H.A. and SILHAVY, T.J. 2003. The art and design of genetic screens: *Escherichia coli*. Nat. Rev. Genet. *4*, 419–431.

VENKATESWARAN, K., MURAKOSHI, A. and SATAKE, M. 1996. Comparison of commercially available kits with standard methods for the detection of coliforms and *Escherichia coli* in foods. Appl. Environ. Microbiol. *62*, 2236–2243.