A THESIS FOR THE DEGREE OF MASTER OF SCIENCE

Development of a new selective medium for the detection of *Staphylococcus aureus* from food

황색포도상구균의 분리검출을 위한 새로운 선택배지 개발

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석사학위논문

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지도교수 강동현

이 논문을 석사학위 논문으로 제출함

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ABSTRACT

Baird-Parker agar (BP agar) was developed as a selective medium for detecting and differentiating *S. aureus* and has been used for several decades. BP agar relies on the characteristics of egg yolk hydrolysis and tellurite consumption for the differentiation of *S. aureus*. However, some other staphylococci (e.g. coagulase negative staphylococci) have these characteristics and can lead to false-positive results on BP agar. Also, numerous strains of *S. aureus* have low activity for the egg-yolk reaction. Therefore, a new selective medium (Cheon and Kang - CK medium) was formulated to more effectively isolate *Staphylococcus aureus* from food specimens. CK medium contains arbutin, D-cellobiose, raffinose pentahydrate and D-salicin as a differentiation marker. Acriflavine hydrochloride (12.5 μg/ml) and 5.0 μg/ml of polymycin B were included as selective antimicrobials. *S. aureus* produced evident yellow colonies on CK medium, whereas strains found to be false positive for *S. aureus* strains on BP agar as well as other staphylococci could not grow or appeared as

differentiable white to pink colonies on CK medium. A total of 127 *S. aureus* stock cultures were streaked onto CK medium and BP agar. The sensitivity of BP agar was 93.70% and that of CK medium was 100% after incubation for 48 hr. In the second phase of the study, *S. aureus*-suspected strains were isolated from a total of 72 naturally-contaminated food samples using CK medium and BP agar. When incubated for 48 hr, the specificity of CK medium (81.54 %) was superior to that of BP agar (72.31%). Compared with CK medium with TSAYE (supplemented 10% NaCl) and BP agar was no significant difference in recovering heat- or acid- injured *S. aureus*. Based on this study, CK medium can supplant BP agar as a selective and differential medium for isolation of *S. aureus* from foods.

***Keywords: Staphylococcus aureus*; selective and differential media; Food safety; Detection of foodborne pathogens**

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I. INTRODUCTION

Staphylococcus aureus is gram-positive coccus and a major human pathogen and causal agent of important nosocomial infections. Any part of the human body can be infected with *S. aureus* and it can cause a range of illnesses, from slight skin infections to lethal diseases such as acute pneumonia, bone and joint infection, and endocarditis (12). Approximately 500,000 patients in American hospitals acquire staphylococcal infections per year. *S. aureus* infections are regarded as especially hazardous because they can be extremely virulent, drug-resistant, and its toxin can cause widespread diseases like staphylococcal toxic shock syndrome (STSS). *S. aureus* is omnipresent in humans, livestock, and domestic animals (1, 5). The optimum conditions needed to growth of *S. aureus* are 35-38℃ temperature and 10% salinity of growth medium. This pathogen can be inactivated after heat treatment at 80℃ for 10 min (8). The food poisoning caused *S. aureus* is occurred by staphylococcal enterotoxin (SE) included in food (11). Though,

the major characteristics of this enterotoxin are it has strong resistant to proteolytic enzymes and heat treatment (13, 14). Therefore, it is crucially important to detect and control of contamination by *S. aurues* in food processing and handling plants (10). As mentioned earlier, *S. aurues* is important pathogen need to precise detect and control from various microorganisms in food, food contact surface and human body.

Coagulase-negative staphylococci (CNS) have been considered as human pathogenic bacteria in recent years. The precise reason of lesion by CNS isn't clearly established, however, it is already regarded that the attachment and/or persistence characteristics of bacterial polysaccharide components to foreign materials would be affected to its virulence (16). CNS stains are typical immanent pathogenic bacteria, and these strains are easily transmittable disease among hospitalized patients. The other important reasons of CNS strains are it could be infected native or prosthetic valve endocarditis, urinary tract, endophthalmitis and changed the route of central nervous system. Also, CNS strains have high and widespread resistant. Since, CNS strain can work as pathogen to human, should be accurately

differentiated with *S. aureus* (17, 18).

In 1962, Baird-Parker made of selective isolation medium of *S. aureus*. The principles are that *S. aureus* consumes tellurite using selective agent and form grey-black colonies and lipolytic activity of *S. aureus* can make hydrolyze the egg yolk and produce opaque zone surrounding grey-black colonies from this action (9, 19). Baird-Parker agar (BP agar) can distinguish *S. aurues* from other microorganism by including in the egg yolk tellurite before distributing sterilized medium. Overall, characteristics of colonies of *S. aureus* are smooth, rounded 2-3 mm in diameter on countable plates and grey to black with surrounded opaque zone (2).

During the fifty years since BP agar (20, 21) was developed, it has been used globally for detecting, isolating and enumerating *S. aureus* from food and clinical samples (2, 3). However, the BP agar method has critical limitations such as its high -price, a more complicated preparation due to the need to supplement with refrigerated stocks of egg-yolk tellurite, and 48 hr are required to obtain accurate colony counts of *S. aureus* on BP agar (6, 7). Also, BP agar has low selectivity and specificity. Under normal conditions, *S.*

aureus cannot be distinguished from other staphylococcal species because some of them produce colonies morphologically similar to *S. aureus*, or its multitude colonies may obfuscate those of *S. aureus*. For instance, some coagulase-negative staphylococci inhibit normal growth of *S. aureus* by causing them to form brown to black colonies when in juxtaposition, or will produce colonies similar to *S. aureus* on BPA medium (51). Some coagulase negative staphylococci inhibit normal growth of *S. aureus* by forming brown to black shade or showing similar colony with *S. aureus* on BP agar (20). These limitations of BP agar could lead to false-positive results, thus, developing a new medium for detection of *S. aureus* is an urgent a need (2, 4).

Also, it is difficult to obtain precise results due to the two aforementioned difficulties. Occasional false-negative results occur because numerous strains of *S. aureus* have low activity for the egg-yolk reaction. It is recommended that adding plasma or fibrinogen can help avoid these erroneous results. However, this alternative method is costly and requires a fastidious preparation process (22, 23, 24). Attempts have been made to

overcome these limitations of BP agar. Thus, various media have been developed and improved such as Lipovitellin Salt Mannitol agar (LSM), modified Vogel-Johnson (PCVJ) with phosphatidyl choline, Baird-Parker with phenol-phthalein (BP+PP, Ibrahim), Potassium Thiocyanate-Actidione-Sodium Azide-Egg Yolk-Pyruvate Agar (KRANEP). Lately, chromogenic media have been developed which utilize bacterial enzyme activity. These include specific chromogenic enzyme substrates which are degraded by certain bacteria and produce different colored colonies. Generally, chromogenic media have higher sensitivity and specificity than conventional selective media (48, 49). However, the cost of making chromogenic media is too inefficient to isolate and detect from a large number of food samples.

This study produced CK medium, a new selective medium for *S. aureus* by incorporation of carbohydrates and antimicrobials. The effectiveness of CK medium was compared to BP agar using stock cultures and naturally contaminated food samples.

II. MATERIALS AND METHODS

2.1. Stock cultures

Test bacteria were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA) and the bacterial culture collection of Seoul National University (SNCC; Seoul, Korea) for this study and used for all experiments. Stock cultures were prepared by combining 700 μL of Tryptic Soy Broth (TSB; Difco, Becton Dickinson, Sparks, MD, USA) 24 h, 37℃ culture with 300 μL of 50% glycerol and stored frozen at –80°C.

2.2. Carbohydrates fermentation test

Characteristics of carbohydrate fermentation of 8 strains of *S.aureus*, 10 strains of coagulase negative staphylococci (CNS) isolated from clinical samples, and 26 strains of mesophilic non-target gram-positive bacteria such as *Kocuria kristinae, Kocuria rosea, Kocuria varians, Leuconostoc mesenteroides, Micrococcus spp., S. epidermidis, S. hominis, S. lentus, S.*

saprophyticus, S. sciuri, S. simulans, S. warneri, and *S. xylosus* were compared. The mesophilic bacteria were isolated from naturally contaminated chicken, ground beef and ground pork following the BAM (bacteriological analytical manual) method established by the U.S. Food and Drug Administration (Bennett, 1998) then colonies similar to *S. aureus* were selected. The API CH50 system (bioMérieux SA, Marcy 1'Etoile, France) was used according to manufacturer's instructions to obtain carbohydrate fermentation patterns.

2.3. Growth of test bacteria on CK medium including different concentrations of antimicrobials

One hundred µL of twofold serial dilutions of acriflavine hydrochloride (from 100 to 0.05 µg/ml) and polymyxin B (from 750 to 3.6 µg/ml) were added to 96-well plates. These wells were inoculated with 100 µL of tested bacteria to obtain a final concentration corresponding to approximately 10^4 to 10^5 CFU/ml per well. Absorbance was measured using a microtiter plate

(SpectraMax M2, Molecula Devices, USA) reader at 600 nm after incubating for 18-24 hr at 37℃.

2.4. Formulation of CK medium

The ingredients of CK medium are as follows: 5.0 g of arbutin (Sigma, China), 5.0 g of D-(+)-cellobiose (Fluka, United Kingdom), 5.0 g of D-(+)-raffinose pentahydrate (Fluka, China), 5.0 g of D-(-)-salicin (Sigma, China), 10.0 g of pyruvic acid, sodium salt (Acros organics, USA), 5.0 g of lithium chloride, anhydrous (Samchun Chemical, Korea), 0.01 g of nalidixic acid (Sigma-Aldrich, USA), 0.0125 g of acriflavine hydrochloride (Sigma-Aldrich, USA), 0.03 g of neutral red (Samchun Chemical, Korea), 15.0 g of pancreatic digest of casein (Difco), 5.0 g of enzymatic digest of soybean meal (Difco), 5.0 g of sodium chloride (Difco) and 15.0 g of agar per liter. The ingredients were added to 1 L distilled water, and sterilized at 121 ℃ for 15 min using an autoclave. After sterilization, the medium was cooled to 50 ℃ and 40,000 units of polymycin B (Oxoid, England) were added. Then the medium was poured into 9-cm-diameter petri dishes.

2.5. Comparison of CK medium with BP agar

Test bacteria (25 *Staphylococcus aureus* and non-*Staphylococcus aureus* strains) were incubated in 5 ml of tryptic soy broth (TSB; Difco) at 37℃ for 18 h. After incubation, one loopful of each culture was streaked onto BP agar and CK medium to obtain single, isolated colonies, and incubated at 37℃ for 24 h and 48 h. The morphology of colonies was compared and recorded. Colonies suspected of being *Staphylococcus aureus* was defined as black colonies surrounding opaque zone on BP agar and 1-2 mm diameter yellow colonies on CK medium.

2.6. Qualitative evaluation of the specificity of CK medium

Test bacteria (Table. 3.) were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA) and the bacterial culture collection of Seoul National University (SNCC; Seoul, Korea). *Bacillus cereus* (ATCC 10876, 13061, W-1, and 14579), *Listeria innocua* (ATCC 33090, 51742,

SNCC 1 and 2), *Listeria monocytogenes* (ATCC 19114, 19115, and 15315), *Proteus mirabilis*. (ATCC 12453, SNCC 1 to 21), *Escherichia coli* O157:H7 (7NE, ATCC 8624, 2026, 2027, 2029, 2257, 2264, 2321, 2324, and 2336), *Hafnia alvei* (ATCC 29926, 29926, and 29927), *Klebsiella pneumoniae* (ATCC 13883, Revco 41, Revco 55, and K1a), *Salmonella* Typhimurium (DT104 Killercow, ATCC 19586, and 43174), *Yersinia enterocolitica* (ATCC 55075, 9610, and 23715) were grown in 5 ml TSB at 37℃ for 18 h. After incubation, one loopful of each culture was streaked onto CK medium, then incubated at 37℃ for 24 hr and 48 hr. The growth of each bacterial strain was observed.

2.7. Assessment of the performance of CK medium using naturally contaminated food samples

Samples were analyzed according to the conventional culture method described by the U. S. Food and Drug Administration (Bennett, 1998) for the microbiological analysis of naturally contaminated foods (2). In summary, a total of 72 samples consisting of chicken (n = 45), ground beef (n = 12), and ground pork (n = 15) were purchased from local retail markets (Seoul, Korea). Each sample (50 g) was homogenized with a stomacher (EASY MIX,

AES Chemunex, Rennes, France) in sterile stomacher bags (Labplas Inc., Sainte-Julie, Quebec, Canada) containing 450 ml of Butter field's phosphate buffered dilution water for 2 min. After the sample homogenate was tenfold serially diluted, 1 ml sample suspensions were spread-plated onto 3 plates of BP agar and CK medium (e.g., 0.4 ml, 0.3 ml, and 0.3 ml), then inverted and incubated for 45-48 hr at 35℃. After incubation, each of 5 colonies suspected of being *S. aureus* and non-*S. aureus* on BPA and CK medium were selected for identification. Bacterial colonies were identified by using the VITEK system (bioMérieux SA).

2.8. Recovery of S. aureus on BP agar and CK medium

2.8.1. Bacterial strains and culture preparation

Three bacterial strains of *S. aureus* (ATCC 49444, ATCC 12692, ATCC 23235) were obtained from the bacterial culture collection of Seoul National University (Seoul, South 103 Korea) for this study and used for all experiments. Each strain of *S. aureus* (ATCC 49444, ATCC 12692, ATCC

23235) was cultured in tryptic soy broth (Difco, Becton Dickinson, Sparks, MD, USA) at 37˚C for 24 hr, harvested by centrifugation at 4000 × g for 20 min at 4℃, and washed three times with buffered peptone water (BPW, Difco). The final pellets were resuspended in sterile BPW, corresponding to approximately 10^7-10^8 log CFU/ml. Subsequently, suspended pellets of each strain of *S. aureus* were combined to produce culture cocktails which were used in subsequent experiments.

2.8.2. Recovery of heat-injured S. aureus

A 50 microliter of cell suspension diluted to about 7.0 log CFU/ml were added separately into 5 ml of BPW which had been preheated and maintained at 55℃. The screw cap tube turned a cap off tightly, and inoculated portion was immersed completely in shaking water bath at 55℃ for 10 min (25,26,27). After heating, test tube was removed from water bath and cooled immediately in a crushed ice/water mixture for maintain

temperature of cell suspension to about 25℃. Sample was ten-fold serially dilutions using 9 ml BPW, an 0.1 ml aliquots of diluted samples was spread plate onto nonselective medium and two selective medium such as Baird-Parker medium (BP agar, Difco) and CK medium as mentioned formulation. The nonselective medium used for this study was TSAYE which is supplemented with 10% NaCl to adjust the optimal salt level to growth of injured cells (28, 29). All plates were incubated at 37℃ for 24-48 h before counting.

2.8.3. Recovery for acid injured S. aureus

Cell suspension prepared as mentioned then 50 microliter of *S. aureus* into 5 ml of BPW adjusted to pH 2.44 with 2 % acetic acid and held for 2 min (30). Cell suspension treated acid exposure diluted serially 10 fold with BPW, then an 0.1 ml aliquots was spread plate onto nonselective medium

(TSAYE+10% NaCl) and selective medium (BP agar and CK medium). Also, all plates were incubated at 37℃ for 24-48 h before counting.

2.8.4. Statistical analysis

All experiments were repeated three times and converted to units of log CFU/g from each duplicate plate count. Data was analyzed using the ANOVA procedure of SAS (Version 9.2. SAS Institute Inc., Cary, NC, USA). The significant ($P \leq 0.05$) means were separated using Duncan's multiple range test.

III. RESULTS AND DISCUSSION

3.1. Characteristics of carbohydrates-fermenting of S. aureus and other bacteria

Fifty years ago, it is already revealed that lactic acid is produced as end-product of glucose fermentation by growth of *S. aureus* (54). Also, Schleifer and Kloos (55) demonstrated that *S. cohnii*, *S. haemolyticus*, and *S. xylosus* could be differentiated by comparing carbohydrate reaction pattern. Therefore, this study based on the hypothesis that if the selective medium were composed certain carbohydrates which are not fermented by *S. aureus*. Then colonies of other bacteria would be changed its color caused by below the pH by metabolites of carbohydrate. Since, acid is produced as one of metabolite of carbohydrate by bacteria.

In previously study, I was screening the background bacteria which isolated from diversity food following FDA's BAManual and have similar morphologic characteristic with *S. aureus* on BP agar (data not shown). The

carbohydrates fermenting abilities of 20 strains of *S. aureus*, 10 strains of staphylococci isolated from clinical samples, and 26 strains of background bacteria isolated from diversity food were compared by using the API 50 CH system. As shown in Table. 1., none of *S. aureus* strains fermented arbutin, D-cellobiose, D-raffinose, or salicin during 24 h and 48 h of incubation. When incubating for 24 h, among 10 strains of staphylococci, 1 strain fermented salicin. Of 26 strains of background bacteria, 8 strains fermented arbutin and D- cellobiose, 9 strains fermented D- raffinose, and 3 strains fermented salicin. Also, when incubating for 48 h, 1 strain of staphylococci strains fermented D-raffinose and salicin. Of background bacteria, 9 strains fermented arbutin and D-raffinose, and 12 strains fermented D-cellobiose, and 3 strains fermented salicin.

Table. 1. Fermentation of carbohydrates by S. aureus and other bacteria

Carbohydrate	Number of strains tested [a]					
	S. aureus (n = 20)		staphylococci (n= 11)		background mesophilic bacteria [b] (n = 20)	
	24 h	48 h	24 h	48 h	24 h	48 h
Arbutin	0 [c]	0	0	0	8	9
D-Cellobiose	0	0	0	0	8	12
D-Raffinose	0	0	0	1	9	9
Salicin	0	0	1	0	3	3

[a] n, number of strains examined

[b] Background mesophilic bacteria isolated from naturally contaminated foods such as ground beef, ground pork, and chicken. These have the characteristics of producing black colonies or black colonies surrounded by an opaque halo on BP agar (*Kocuria kristinae*, *Kocuria rosea*, *Kocuria varians*, *Leuconostoc mesenteroides*, *Micrococcus* spp., *S. epidermidis, S. hominis, S. lentus, S. saprophyticus, S. sciuri, S. simulans, S. warneri, S. xylosus*)

[c] Number of strains that fermented each carbohydrate.

3.2. Growth of test bacteria on CK medium including different concentrations of antimicrobials

As know from Table 1, it is hard to distinguish *S. aureus* from other bacteria by different carbohydrates-fermentation ability of bacteria. Generally known, *S. aureus* has the strong resistant against of antimicrobials. Thus, the antimicrobials added into the medium based on 4 carbohydrate source (arbutin, D-cellobiose, D-raffinose, salicin)

In this study, CK medium included in 5g/L of lithium chloride commonly used. Because, many selective medium such as Vogel Johnson agar (38), Barid-Parker Agar (39), Giolitti and Cantoni Broth (40), Liquid Baird-Parker (41) were used to it for discriminating *S. aureus* from other microorganism. Since, lithium chloride is a selective agents generally used for inhibiting the coagulase negative staphylococci and gram negative bacilli, although its mechanism hindering growth of bacteria is unknown clearly (42, 43).

Since introduced nalidixic acid, it has generally used the selective agent that inhibits most gram-negative pathogens including *Enterobacter species*, *Escherichia coli*, *Morganella* Morganii; *Proteus* Mirabilis, and *Proteus vulgaris* (44, 45). Hydroxynalidixic acid which is active metabolites of nalidixic acid could be bind to DNA. Subsequently, it interfere RNA synthesis and protein synthesis (46). Also, 0.01g/L of nalidixic acid which identified the effectiveness inhibiting gram negative bacteria incorporated in CK medium.

In addition, polymyxin B sulfate could inhibit a great part of gram negative bacilli exclude the Proteus group. In spite of remaining outside of target cell, it made change membrane permeability of target cell then causing cell death by interacting with lipopolysaccharide of the cytoplasmic outer membrane of gram negative bacteria (52). Sabath et al. (37) demonstrated that the MIC range of *S. aureus* against to polymycin B is 50 ~ >100 μg/ml while that of *S. epidermidis* is 3.1 ~ 100 μg/ml and staphylococci.

According to the results of previous study (50), *S. aureus* has more strong resistant (e.g. MIC is 12.5 μg/ml) against acriflavine as antimicrobial than staphylococci (*S. saprophyticus, S. sciuri* subsp. *sciuri, S. sciuri* subsp. *lentus, S. xylosus, S. capitis, S. warneri, S. hominis, S. haemolyticus, S. intermedius, S. epidermidis, S. simulans, S. cohnii, S. hyicus* subsp. *hyicus, S. hyicus* subsp. *chromogenes*). Besides, Kawai and Yamagishi (2009) demonstrated that acriflavine causes the thickened cell wall of *S. aureus* through transmission electron micrographs. However, it restores the thickened cell wall to its original form after acriflavine treatment removal. They interpreted *S. aureus* would be induced acriflavine-resistant genes after exposing to acriflavine.

In summary, CK medium was applied four selective antimicrobials, such as lithium chloride, nalidixic acid, polymyxin B, and acriflavine hydrochloride. CK medium was contained 5g/L of lithium chloride and 0.01g/L of nalidixic acid since lithium chloride and nalidixic acid have been generally used as inhibitor against gram negative bacteria. However, other

two agents should be conformed appropriate concentration which can inhibit only staphylococci not *S. aureus*.

Then making decision the concentration of antimicrobials, I compared to viability of tested bacteria such as 25 strains of *S. aureus*, 11 strains of staphylococci, and 20 strains of background bacteria under exposure conditions to these antimicrobials (Table. 2.). The tested bacteria incubated on broth medium included serially diluted each antimicrobial. When broth medium contained only 12.5 μg/ml of acriflavine hydrochloride, *S. aureus*, staphylococci, and background bacteria could grow respectively 21, 5, and 17 strains. In case of polymyxin B, when added 5.0 μg/ml of polymyxin B in broth medium, *S. aureus*, staphylococci, and background bacteria could growth respectively 21, 5, and 3 strains. However, *S. aureus* could grow 21 strains, though all staphylococci and background bacteria were completely inhibited under the combination condition which is 12.5 μg/ml of acriflavine hydrochloride and 5.0 μg/ml of polymyxin B.

There is one consideration that acriflavine hydrochloride could affect color of colonies because of its strong color. Therefore I did a screening for determination of compatible pH indicator. *S. aureus* released a quantity of acetic acid as its metabolite (53), also other bacteria produce acidic metabolites. One of the main principles is that carbohydrate which could not ferment by *S. aureus* added in order to differentiated from other bacteria could ferment the carbohydrate. When CK medium included acriflavine hydrochloride, it appeared yellowish brown color. Therefore CK medium should be added pH indicator with specific characteristics which its transition pH range is slightly acid to neutral and it did not produce yellow color in acidic environment. From this reason, I choose neutral red as pH indicator of CK medium.

Table. 2. Viability of *S. aureus* and other bacteria in broth medium supplemented with antimicrobials

	Number of strains [a]		
	S. aureus (n=25)	staphylococci (n=11)	background mesophilic bacteria [b] (n=20)
Acriflavine hydrochloride (12.5 μg/ml)	21 [c]	5	17
Polymycin B (5.0 μg/ml)	21	5	3
Combined Acriflavine hydrochloride and Polymycin B	21	0	0

[a] n, number of strains examined

b Background mesophilic bacteria isolated from naturally contaminated foods such as ground beef, ground pork, and chicken. Theses have the characteristics of producing black colonies or black colonies surrounding by an opaque halo on BP agar (*Kocuria kristinae*, *Kocuria rosea, Kocuria varians, Leuconostoc mesenteroides*, *Micrococcus* spp., *S. epidermidis, S. hominis, S. lentus, S. saprophyticus, S. sciuri, S. simulans, S. warneri, S. xylosus*)

[c] Number of strains showing viability in the presence of specific antibiotics

3.3. Comparison of CK medium with BP agar

The critical limit of BP agar is that having low sensitivity. That is BP agar frequently produced false-negative results. Also, to inhibit staphylococci strains was desirable since staphylococci often appear false-positive results or might make confuse distinction with *S. aureus* on BP agar.

On BP agar, 22 *S. aureus* strains appeared black colonies without halo (false-negative results) when incubated for 24 h, however, 11 strains of them produced opaque halo surrounding black colonies (true-positive results) after 48 h. When *S. aureus* incubated on CK medium, 8 *S. aureus* strains produced pink colonies after incubation for 24 h, all eight strains of them altered their color to yellow after 48 h. In other words, the sensitivity of BP agar was 82.68 % after incubated for 24 h and 91. 34 % after incubated for 48 h. While, that of CK medium was higher than results of BP agar as 93.70 % (24 h) and 100 % (48 h).

S. haemolyticus ATCC 29970 could not be distinguished through both on BP agar and CK medium. While *S. xylosis* ATCC 29971 produced *S. aureus*-

like colonies on BP agar, it was distinguished on CK medium by presenting pink colonies (data not shown). Also, *S. cohnii* ATCC 29974 represented *S. aureus*-like colonies on BP agar, it completely inhibits when incubated on CK medium. *S. capitis* ATCC 35661, *S. epidermidis* ATCC 12228, *S. intermedius* ATCC 29663, and *S. warneri* ATCC 10209 was grown and represented black colonies on BP agar. Whereas, they all completely were inhibited on CK medium even incubated for 48 h.

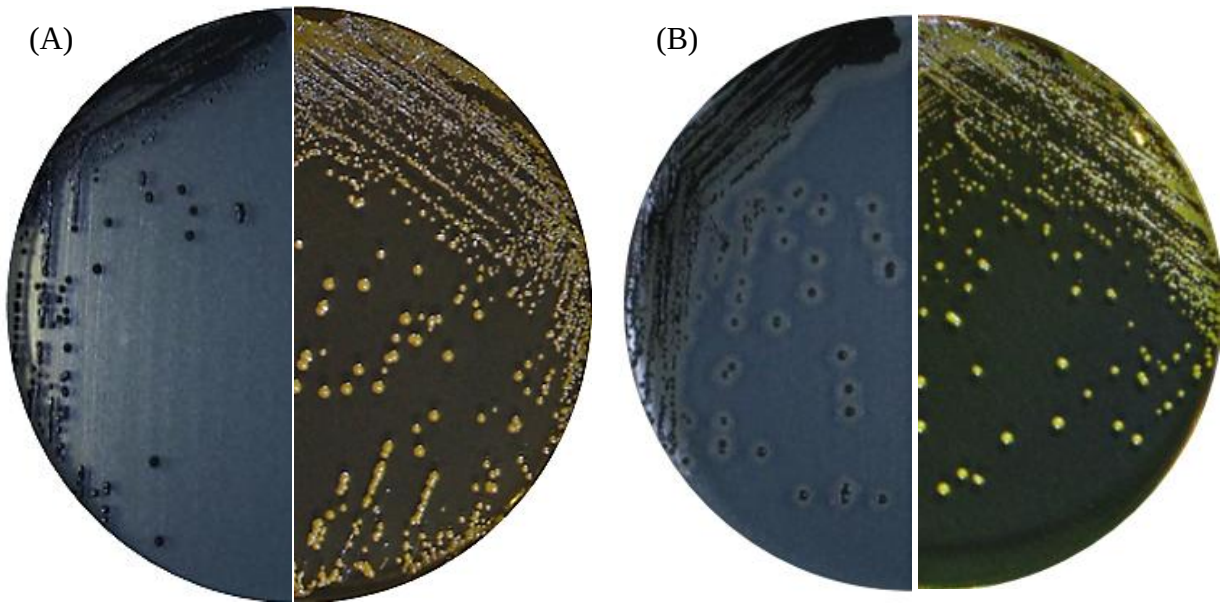

Fig. 1. Colonies produced by *S. aureus* ATCC 27664 (left) and ATCC 29213 (right) on BP agar (A, C) and CK medium (B, D). Colonies of *S. aureus* ATCC 27664 did not produce opaque haloes on BP agar. On CK medium, both strains produced yellow colonies

Table. 3. Colony colors of stock cultures on BP agar and CK medium

Strain	Strain [a]	Colony morphological shape on [b]			
		BP agar		CK medium	
		24 h	48 h	24 h	48 h
Staphylococcus aureus	ATCC 49444	B/H	B/H	Y	Y
Staphy. aureus	ATCC 12692	B/H	B/H	Y	Y
Staphy. aureus	ATCC 23235	B/H	B/H	Y	Y
Staphy. aureus	ATCC 27664	B/H	B/H	Y	Y
Staphy. aureus	ATCC 6538	B/H	B/H	Y	Y
Staphy. aureus	ATCC 29213	B/H	B/H	Y	Y
Staphy. aureus	ATCC 27213	B/H	B/H	Y	Y
Staphy. aureus	ATCC 25923	B	B/H	P	Y
Staphy. aureus	ATCC 27664	B	B/H	Y	Y
Staphy. aureus	ATCC 4012	B/H	B/H	Y	Y
Staphy. aureus	ATCC 10390	B/H	B/H	Y	Y
Staphy. aureus	ATCC 12598	B	B	P	Y
Staphy. aureus	ATCC 14458	B	B	P	Y
Staphy. aureus	ATCC 27154	B/H	B/H	Y	Y
Staphy. aureus	ATCC 27659	B/H	B/H	Y	Y
Staphy. aureus	ATCC 19095	B	B	Y	Y
Staphy. aureus	ATCC 8096	B/H	B/H	Y	Y
Staphy. aureus	ATCC 13301	B	B	Y	Y
Staphy. aureus	ATCC 13150	B/H	B/H	Y	Y
Staphy. aureus	ATCC 29737	B	B/H	P	Y
Staphy. aureus	ATCC 13565	B	B	Y	Y
Staphy. aureus	ATCC 12600	B/H	B/H	Y	Y
Staphy. aureus	ATCC 51650	B/H	B/H	Y	Y
Staphy. aureus	ATCC 51811	B/H	B/H	Y	Y
Staphy. aureus	ATCC 51651	B	B	Y	Y
Staphy. aureus	SNCC 1	B/H	B/H	Y	Y
Staphy. aureus	SNCC 2	B/H	B/H	Y	Y
Staphy. aureus	SNCC 3	B/H	B/H	Y	Y
Staphy. aureus	SNCC 4	B/H	B/H	Y	Y

(Continued)

Table. 3. (Continued)

Staphy. aureus	SNCC 5	B/H	B/H	Y	Y
Staphy. aureus	SNCC 6	B/H	B/H	Y	Y
Staphy. aureus	SNCC 7	B/H	B/H	Y	Y
Staphy. aureus	SNCC 8	B/H	B/H	Y	Y
Staphy. aureus	SNCC 9	B/H	B/H	Y	Y
Staphy. aureus	SNCC 10	B/H	B/H	Y	Y
Staphy. aureus	SNCC 11	B/H	B/H	Y	Y
Staphy. aureus	SNCC 12	B/H	B/H	Y	Y
Staphy. aureus	SNCC 13	B/H	B/H	Y	Y
Staphy. aureus	SNCC 14	B/H	B/H	Y	Y
Staphy. aureus	SNCC 15	B/H	B/H	Y	Y
Staphy. aureus	SNCC 16	B/H	B/H	Y	Y
Staphy. aureus	SNCC 17	B/H	B/H	Y	Y
Staphy. aureus	SNCC 18	B/H	B/H	Y	Y
Staphy. aureus	SNCC 19	B/H	B/H	Y	Y
Staphy. aureus	SNCC 20	B/H	B/H	Y	Y
Staphy. aureus	SNCC 21	B/H	B/H	Y	Y
Staphy. aureus	SNCC 22	B/H	B/H	Y	Y
Staphy. aureus	SNCC 23	B/H	B/H	Y	Y
Staphy. aureus	SNCC 24	B/H	B/H	Y	Y
Staphy. aureus	SNCC 25	B/H	B/H	Y	Y
Staphy. aureus	SNCC 26	B/H	B/H	Y	Y
Staphy. aureus	SNCC 27	B/H	B/H	Y	Y
Staphy. aureus	SNCC 28	B/H	B/H	Y	Y
Staphy. aureus	SNCC 29	B/H	B/H	Y	Y
Staphy. aureus	SNCC 30	B/H	B/H	Y	Y
Staphy. aureus	SNCC 31	B/H	B/H	Y	Y
Staphy. aureus	SNCC 32	B/H	B/H	Y	Y
Staphy. aureus	SNCC 33	B/H	B/H	Y	Y
Staphy. aureus	SNCC 34	B/H	B/H	Y	Y
Staphy. aureus	SNCC 35	B/H	B/H	Y	Y
Staphy. aureus	SNCC 36	B	B	Y	Y
Staphy. aureus	SNCC 37	B/H	B/H	Y	Y
Staphy. aureus	SNCC 38	B/H	B/H	Y	Y
Staphy. aureus	SNCC 39	B/H	B/H	Y	Y
Staphy. aureus	SNCC 40	B/H	B/H	Y	Y
Staphy. aureus	SNCC 41	B/H	B/H	Y	Y
Staphy. aureus	SNCC 42	B/H	B/H	Y	Y
Staphy. aureus	SNCC 43	B/H	B/H	Y	Y

(Continued)

Table. 3. (Continued)

Staphy. aureus	SNCC 44	B/H	B/H	Y	Y
Staphy. aureus	SNCC 45	B/H	B/H	Y	Y
Staphy. aureus	SNCC 46	B/H	B/H	Y	Y
Staphy. aureus	SNCC 47	B/H	B/H	Y	Y
Staphy. aureus	SNCC 48	B/H	B/H	Y	Y
Staphy. aureus	SNCC 49	B/H	B/H	Y	Y
Staphy. aureus	SNCC 50	B/H	B/H	Y	Y
Staphy. aureus	SNCC 51	B/H	B/H	Y	Y
Staphy. aureus	SNCC 52	B/H	B/H	Y	Y
Staphy. aureus	SNCC 53	B/H	B/H	Y	Y
Staphy. aureus	SNCC 54	B/H	B/H	Y	Y
Staphy. aureus	SNCC 55	B/H	B/H	Y	Y
Staphy. aureus	SNCC 56	B/H	B/H	Y	Y
Staphy. aureus	SNCC 57	B/H	B/H	Y	Y
Staphy. aureus	SNCC 58	B/H	B/H	Y	Y
Staphy. aureus	SNCC 59	B	B	Y	Y
Staphy. aureus	SNCC 60	B/H	B/H	Y	Y
Staphy. aureus	SNCC 61	B/H	B/H	Y	Y
Staphy. aureus	SNCC 62	B/H	B/H	Y	Y
Staphy. aureus	SNCC 63	B/H	B/H	Y	Y
Staphy. aureus	SNCC 64	B/H	B/H	Y	Y
Staphy. aureus	SNCC 65	B/H	B/H	Y	Y
Staphy. aureus	SNCC 66	B/H	B/H	Y	Y
Staphy. aureus	SNCC 67	B/H	B/H	Y	Y
Staphy. aureus	SNCC 68	B	B/H	P	Y
Staphy. aureus	SNCC 69	B/H	B/H	Y	Y
Staphy. aureus	SNCC 70	B	B/H	P	Y
Staphy. aureus	SNCC 71	B/H	B/H	Y	Y
Staphy. aureus	SNCC 72	B/H	B/H	Y	Y
Staphy. aureus	SNCC 73	B/H	B/H	Y	Y
Staphy. aureus	SNCC 74	B/H	B/H	Y	Y
Staphy. aureus	SNCC 75	B/H	B/H	Y	Y
Staphy. aureus	SNCC 76	B/H	B/H	Y	Y
Staphy. aureus	SNCC 77	B/H	B/H	Y	Y
Staphy. aureus	SNCC 78	B/H	B/H	Y	Y
Staphy. aureus	SNCC 79	B/H	B/H	Y	Y
Staphy. aureus	SNCC 80	B/H	B/H	Y	Y
Staphy. aureus	SNCC 81	B/H	B/H	Y	Y
Staphy. aureus	SNCC 82	B/H	B/H	Y	Y

(Continued)

Table. 3. (Continued)

Staphy. aureus	SNCC 83	B/H	B/H	Y	Y
Staphy. aureus	SNCC 84	B/H	B/H	Y	Y
Staphy. aureus	SNCC 85	B/H	B/H	Y	Y
Staphy. aureus	SNCC 86	B/H	B/H	Y	Y
Staphy. aureus	SNCC 87	B/H	B/H	Y	Y
Staphy. aureus	SNCC 88	B/H	B/H	Y	Y
Staphy. aureus	SNCC 89	B/H	B/H	Y	Y
Staphy. aureus	SNCC 90	B	B	Y	Y
Staphy. aureus	SNCC 91	B/H	B/H	Y	Y
Staphy. aureus	SNCC 92	B/H	B/H	Y	Y
Staphy. aureus	SNCC 93	B/H	B/H	Y	Y
Staphy. aureus	SNCC 94	B	B	Y	Y
Staphy. aureus	SNCC 95	B	B	Y	Y
Staphy. aureus	SNCC 96	B/H	B/H	Y	Y
Staphy. aureus	SNCC 97	B	B/H	Y	Y
Staphy. aureus	SNCC 98	B	B/H	Y	Y
Staphy. aureus	SNCC 99	B	B/H	Y	Y
Staphy. aureus	SNCC 100	B	B/H	Y	Y
Staphy. aureus	SNCC 101	B	B/H	P	Y
Staphy. aureus	SNCC 102	B	B/H	P	Y
Staphylococci					
Staphy. epidermidis	ATCC 14990	-	-	-	-
Staphy. hominis	ATCC 27844	-	-	-	-
Staphy. saprophyticus	ATCC 15305	-	-	-	-
Staphy. haemolyticus	ATCC 29970	-	-	-	-
Staphy. xylosis	ATCC 29971	-	-	-	-
Staphy. capitis	ATCC 27840	-	-	-	-
Staphy. cohnii	ATCC 29974	B/H	B/H	-	-
Staphy.capitis	ATCC 35661	-	B	-	-
Staphy.epidermidis	ATCC 12228	B	B	-	-
Staphy. intermedius	ATCC 29663	B	B	-	-
Staphy. warneri	ATCC 10209	B	B	-	-

[a] ATCC, American Type Culture Collection; SNCC, the bacterial culture collection of Seoul National University.

[b] B/H, black colonies surrounded with opaque haloes; B, black colonies without haloes; Y, yellow colonies; P, pink colonies; -, not growth.

3.4. Qualitative evaluation of the specificity of CK medium

To evaluate qualitative specificity of CK medium, non *S. aureus* strains such as *B. cereus, L. innocua, L. monocytogenes, Proteus mirabilis, E. coli* O157:H7, *Hafnia. alvei, Salmonella* Typhimurium, *Klebsiella pneumoniae*, and *Yersinia enterocolitica* were incubated on CK medium and colony color and morphology was compared (Table. 4.).

All three *B. cereus* strains and three *P. mirabilis* strains were completely inhibited on CK medium. Most gram -positive strains grew on CK medium, however, *P.mirabilis* produced turbid yellow colonies and *L. innocua* and *L. monocytogenes* produced white colonies on CK medium. They could be readily distinguished from colonies of *S. aureus*, which produced yellow colonies.

Among *S. aureus* - like strains isolated from food, 6 strains grew on CK medium. *Kocuria rosea*, *Kocuria varians*, and *S. sciuri* produced yellow colonies after 24 h incubation time, however, it turned over turbid yellow colonies. *Kocuria rosea* produced pink colonies and *S. warneri* produced yellow colonies with changing the medium to dark brown color.

When *Proteus* spp. coexist with *S. aureus* in the same sample, it is more difficult to isolate *S. aureus* (19). The ease of distinguishing *S. aureus* from *Proteus* mirabilis was compared in simultaneously inoculated BP agar and CK medium plates (Fig. 2.). *P. mirabilis* appeared as black colonies without opaque haloes on BP agar (data not shown). However, it was difficult to differentiate between *S. aureus* and *P. mirabilis* on BP agar when they were present in similar concentrations (Fig. 2A). Conversely, *P. mirabilis* produced turbid and wide-spreading colonies on CK medium (Fig. 2B). Of gram-negative bacteria tested, ten *E. coli* O157:H7 strains, 3 *H. alvei* strains, 3 *S.* Typhimurium strains, 4 *K. pneumoniae* strains, and 3 *Yersinia enterocolitica* strains, were completely inhibited on CK medium.

Table. 4. Qualitative evaluation of the specificity of CK medium with various genera of gram-positive and negative bacteria

Bacterial species	No. of strains tested	No. of strains that grew on CK medium	No. of strains on CK medium: that produced	
			Yellow colonies	White to pink colonies
Bacillus cereus	4	0	0	0
Listeria innocua	4	4	0	4
Listeria monocytogenes	3	3	0	3
Proteus mirabilis	22	19	0	19
Background bacteria isolated from food	20	6	3	3
E. coli O157:H7	10	0	0	0
Hafnia. alvei	3	0	0	0
Salmonella Typhimurium	3	0	0	0
Klebsiella pneumonia	4	0	0	0
Yersinia enterocolitica	3	0	0	0

b Background bacteria isolated from naturally contaminated food such as ground beef, ground pork, and chicken. Also, it has the characteristics presenting black colonies or black colonies surrounding opaque film on BP agar (*Kocuria kristinae, Kocuria rosea, Kocuria varians, Leuconostoc mesenteroides, Micrococcus* spp., *S. epidermidis, S. hominis, S. lentus, S. saprophyticus, S. sciuri, S. simulans, S. warneri, S. xylosus*)

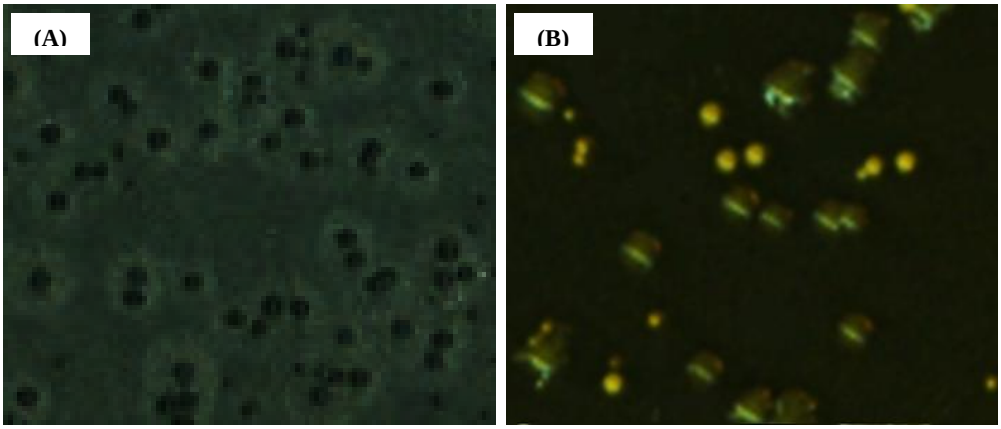

Fig. 2. Colonies produced by *P. mirabilis* and *S. aureus* on BP agar (left) and CK medium (right) when both species were present in similar concentrations. The two species could not be differentiated on BP agar because both produced black colonies surrounded by opaque haloes (A). *S. aureus* produced yellow colonies while *P. mirabilis* produced turbid and wide-spread colonies on CK medium (B)

3.5. Assessment of the performance of CK medium and BP agar using naturally contaminated food samples

Table. 5. shows the bacteriological analysis of naturally contaminated food samples. By monitoring after 48 hr incubation, sensitivity and specificity of each medium was calculated as detailed. From 72 food samples, 7 *S. aureus* strains were isolated on BP agar and CK medium.

The 2 strains of them represent black colony without opaque halo on BP agar medium after incubation for 24 h, while show yellow colony on CK medium regardless of incubation time. The sensitivity of CK medium (100 %) was higher than that of BP agar medium (71.43 %). After 48 h incubation, both medium show 100% sensitivity.

It takes a 48 h for incubation after spreading diluted food sample according to the microbiological analysis of naturally contaminated foods (recommended by FDA's BAM manual). I found that *Kocuria krisinae* (*n*=1) and *Lactococcus garvieae* (*n*=15) change its morphologic characteristic on BP agar medium relied on incubation time. They represent the black colony

without opaque halo after incubation for 24 h, however, it could not differentiate true-positive strain from false-positive strains. On the other hand, total of 6 and 12 false-positive results, including *S. sciuri* (n=2), *Kocuria* spp. (n=2), *Lactococcus garvieae* (n=15). While, *Kocuria* spp. (n=2), *S. eidermidis* (n=1), *S. sciuri* (n=2), *S. xylosus* (n=1), *S. warneri* (n=4), *S. saprophyticus* (n=1), *Micrococcus* spp. (n=1), found on CK medium after incubation for 24 h and 48 h. The specificity of CK medium (81.54 %) is superior to that of BP agar (71.21%) after 48 h incubation, albeit the specificity of CK medium (90.77 %) is lower than that of BP agar (96.92 %) after 24 h incubation.

Table. 5. The specificity of CK medium compared with that of BP agar on the microbiological analysis of naturally contaminated foods

	48 hr incubation	
	% Sensitivity	% Specificity
BP agar	100.00	71.21
CI	100.00	81.54

3.6. Comparing recovery capacities of S. aureus on BP agar and CK medium

Thermal treatment has been widely using for pasteurization and sterilization in food processing (34). Also, organic acids are recommended to sterilize during slaughter process (35, 36). However, it can increase the risk of outbreak that the existence of injured pathogens in food. Since they have potential ability for resuscitate and can normally function given the appropriate environment. Most food can furnish many nutrients, water, and other elements for growth of injured bacteria. It is used selective medium in order to isolate and detect pathogens from food. Though, injured bacteria may not grow well on these selective conditions (31, 32, 33).

Fig. 3. shows the viable counts of injured *S. aureus* after heat treatment at 55℃ for 10 min. Compared to TSAYE (supplemented 10% NaCl), BP agar, and CK medium, there are no significant differences ($P > 0.05$). Likewise, Fig. 4. shows that enumerating acid injured *S. aureus* (2%

acid) for 2 min using TSAYE (supplemented 10% NaCl), BP agar, and CK medium is no significant difference.

Therefore, it can be demonstrated that the new selective medium (CK medium) is applicable for isolation and detection to heat- and acid-injured *S. aureus*.

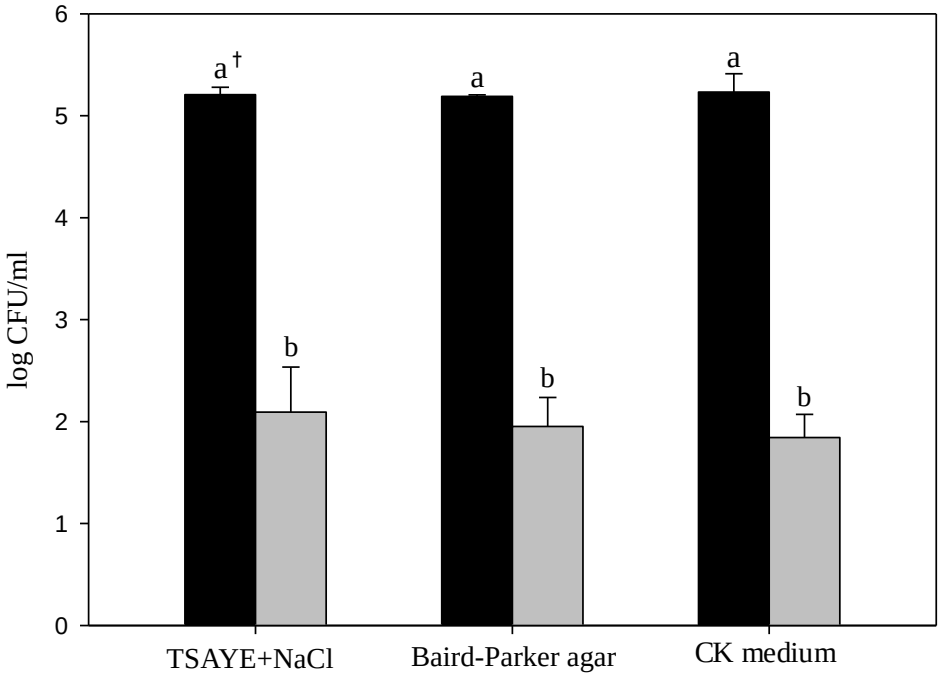

Fig. 3. Comparison of Tryptic soy agar-Yeast extract supplemented with 10% NaCl, BP agar, and CK media for recovery of injured *Staphylococcus aureus*. Heat treatment; in 0.1% peptone water at 55℃ for 10 min. ■ control samples, ■ after heat treatment

† Different letters within a treatment indicate significant differences ($p<0.05$)

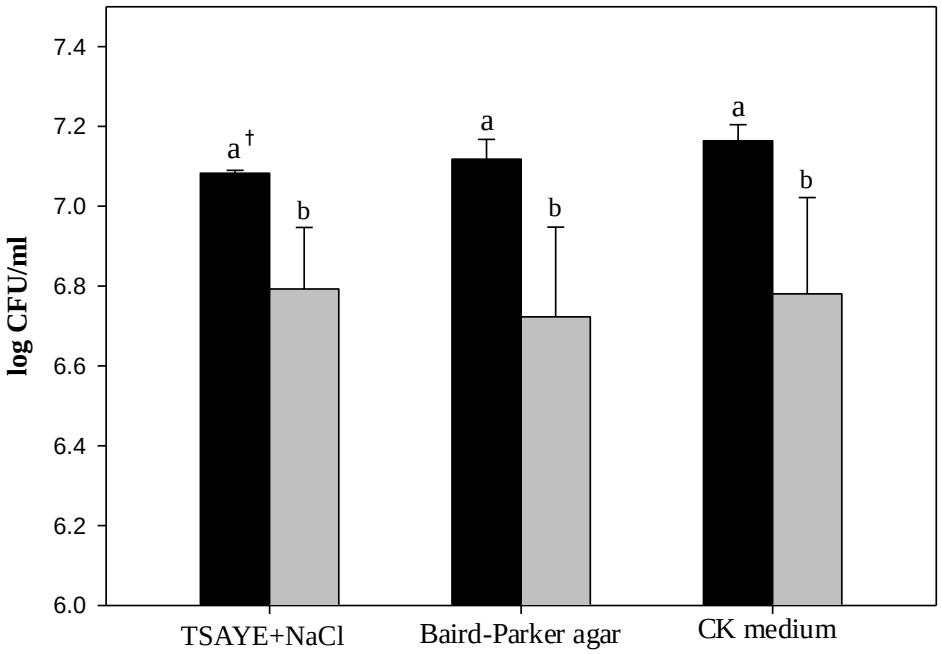

Fig. 4. Comparison of Tryptic soy agar-Yeast extract supplemented with 10% NaCl, BP agar, and CK media for recovery of injured *Staphylococcus aureus*. Acid treatment; in 0.1% peptone water at 2% lactic acid for 2 min. ■ control samples, ■ after acid treatment

† Different letters within a treatment indicate significant differences ($p<0.05$)

IV. CONCLUSIONS

In conclusion, the aim of this study was to develop a new selective medium for isolation of *S. aureus*. CK medium utilizes a basic growth principle of microorganisms, that is, producing acid as a result of carbohydrate fermentation. CK medium yielded an improved sensitivity, specificity and good recovery capacity, namely, it produces less false-positive and false-negative colonies than does BP agar. It can supplement established selective and differential media for the isolation of *S. aureus*. Therefore, CK medium may provide a valuable addition to the array of selective media available for the detection and isolation of *S. aureus* from foods.

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Ⅵ. 국문초록

Baird-Parker agar (BP agar)는 수십 년 간 황색포도상구균의 대표적인 선택배지로 이용되어 왔다. BP agar 는 황색포도상구균이 난황을 가수분해하고 tellurite 를 분해하는 특성을 이용하여 균을 선택적으로 분리해 낼 수 있다. 하지만 몇몇의 다른 staphylococci (e.g. Coagulase negative staphylococci)도 이러한 특성을 가지고 있기 때문에 BP agar 에서는 위 양성 결과를 가져올 수 있다는 단점이 있다. 따라서 본 연구에서는 새로운 황색포도상구균 선택배지 (Carbohydrate and inhibitor; CK medium)을 개발하였다. CK medium 은 분별마커로 arbutin, D-cellobios, raffinose pentahydrate 그리고 D-salicin 을 첨가하고, 선택적인 항생물질로 12.5 ㎍/ml acriflavine hydrochloride 와 5.0 ㎍/ml polymyxin B 를 첨가하여 *S. aureus* 이외의 균의 성장을

저해시켰다. CK medium 상에서 *S. aureus* 는 분명한 노란색 콜로니를 형성하지만, BP agar 를 이용하여 분리한 *S. aureus* 로 의심되는 strains 이나 다른 staphylococci strains 은 성장하지 않거나 노란색 콜로니와 분별 가능한 분홍색 또는 흰색 콜로니를 형성하였다. 총 127 개의 *S. aureus* 를 CK medium 과 BP agar 에 streaking 하여 48 시간 배양하였을 때, 각 배지의 선별성은 BP agar 가 91.34%, CK medium 이 93.70%로 나타났다. 또한 총 72 개의 식품 샘플을 이용하여 각 배지의 선택성을 테스트 하였다. 24 시간을 배양한 경우에는 CK medium 의 선택성이 90.77%로 BP agar (96.92%)보다 낮게 나타났지만, 48 시간을 배양한 경우에는 CK medium 은 81.54%로 BP agar (72.31%)보다 높게 나타났다. 분리한 strains 중에서 BP agar 에서는 19 개, CK medium 에서는 12 개의 위 양성 결과가 나타났다. 손상된 *S. aureus* 의 회복력을 테스트하기 위하여 heat- 그리고 acid-injured *S. aureus* 를 CK medium, 10% NaCl 을 첨가한 TSAYE,

그리고 BP agar 에 접종하여 배양하였다. 그 결과 세 종류의 배지에서 손상된 *S. aureus* 의 회복력은 유의적인 차이가 없는 것으로 나타났다. 이상의 결과로 볼 때, CK medium 은 BP agar 를 대체하여 식품으로부터 황색포도상구균을 선택적으로 검출해 내는데 유용하게 사용될 수 있을 것이다.

주요어: 황색포도상구균, 선택분별배지, 식품안전성, 식중독균 검출

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