

METHICILLIN-RESISTANT *STAPHYLOCOCCUS AUREUS* FROM READY-TO-EAT FOODS IN A HOSPITAL CANTEEN, SOUTHERN THAILAND: VIRULENCE CHARACTERIZATION AND GENETIC RELATIONSHIP

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Abstract. Due to the existence of sporadic cases caused by methicillin-resistant *Staphylococcus aureus* (MRSA) in Songklanagarind Hospital, Songkhla, southern Thailand where a canteen is located in close vicinity, this study investigated the presence of MRSA, including *mecA*-carrying *S. aureus* (MCSA), contamination in 105 ready-to-eat (RTE) food samples sold in this canteen. Coagulase-negative MRSA (MR-CoN) and coagulase-negative MCSA (MCSA-CoN) contaminations were observed in various RTE foods with unripened-papaya salad having significantly highest incidence of MCSA-CoN contamination ($p < 0.05$). The majority of MCSA-CoN isolates were resistant to clindamycin and fusidic acid. Two MCSA-CoN strains PSU172 and PSU180 were subsequently shown to be MR-CoN. Staphylococcal enterotoxins (SEs)-carrying MCSA-CoN strain PSU109 was isolated from seasoned rice. The SE-carrying MCSA-CoN strain PSU109, MR-CoN strain PSU172 and MCSA-CoN strain PSU173 were able to survive in the acidic unripened-papaya salad sauce (pH 4.0 - 4.5) for up to 6 hours. Typing by pulsed-field gel electrophoresis revealed 80% genetic relatedness among MCSA-CoN strains from RTE food and clinical strains. This study suggests the plausibility of RTE foods sold in a hospital canteen as vehicles of hospital-acquired MR-CoN and of MCSA-CoN.

Keywords: methicillin-resistant *Staphylococcus aureus* (MRSA), staphylococcal enterotoxin, pulsed-field gel electrophoresis, hospital canteen, ready-to-eat food

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INTRODUCTION

Incidence of infection caused by methicillin-resistant *Staphylococcus aureus* (MRSA) was first observed in late 1960 in south-eastern England (Jevons, 1961), and since then, a large number of MRSA

infections were subsequently reported in several areas of England, Germany and Canada (Johnson *et al*, 2005; Witte *et al*, 2007; Crago *et al*, 2012). MRSA plays a major role as a nosocomial pathogen, leading to high mortality rates worldwide. Methicillin resistance is due to the presence of *mecA*, coding for penicillin-binding protein2a (PBP2a), responsible for low affinity to β -lactam antibiotics (Utsui and Yokota, 1985).

Illness resulting from ingestion of MRSA occurs through the secretion of staphylococcal enterotoxins (SEs), which include toxic shock syndrome toxin 1 (TSST-1) belonging to the pyrogenic toxin superantigen (PTSAg) family (Hwang *et al*, 2007). Staphylococcal superantigens are capable of stimulating massive release of cytokines from T-cells and macrophages (Marrack and Kappler, 1990), resulting in excessive cellular immune response leading to toxic shock (Wang *et al*, 2008). In addition, they cause staphylococcal pneumonia and staphylococcal purpura fulminans (Fluer, 2007; Hussain *et al*, 2007). Several kinds of food samples can carry SE gene-carrying *S. aureus*, including coleslaw (Jones *et al*, 2002) and particularly raw meats (Hwang *et al*, 2007). The presence of SE gene-carrying *S. aureus* in ready-to eat (RTE) food items has recently been reported (Rizek *et al*, 2011; Hammad *et al*, 2012; Wang *et al*, 2013).

The first MRSA outbreak in Songklanagarind University Hospital, Songkhla, southern Thailand was reported in December 1986 (Jamulitrat *et al*, 1988) and infections by this bacterial species have been sporadically reported (Hortiwakul *et al*, 2004). Songklanagarind Hospital canteen is located in close vicinity to clinical buildings and high numbers of healthy people and patients attended this canteen. We suspected that *S. aureus*

and MRSA from clinical sources may be contaminating RTE foods in the hospital canteen. Thus, we investigated the prevalence of *S. aureus*, including MRSA, their antibiogram patterns, including their genetic relationships, in RTE foods sold in Songklanagarind Hospital canteen.

MATERIALS AND METHODS

Samples collection

A total of 105 RTE food samples were randomly collected approximately 3 hours after release for sale from Songklanagarind Hospital canteen, Hat Yai city, Songkhla Province, Thailand between August and December, 2013. The samples were brought immediately to the laboratory for analysis (Bunnoeng *et al*, 2014). In brief, 10 g of sample were aseptically mixed with 90 ml of normal saline solution (NSS) and homogenized for 1 minute. A 10 ml aliquot of mixture was added to 90 ml of tryptic soy broth (TSB) (Difco, Detroit, MI) and incubated at 37°C without shaking for 6 hours. A series of 10-fold dilutions of the solution in TSB was plated on mannitol salt agar (MSA) (Difco) and incubated at 37°C for 18 hours. Five yellow colonies per sample were selected randomly for further analysis.

S. aureus-specific nuclease gene detection

Identification of *S. aureus*-specific *nuc* (nuclease gene) was performed by PCR using primers described by Zhang *et al* (2004) (Table 1) and amplification conditions and analysis (279 bp) were described below.

Virulence genes detection

Each individual colony was inoculated into 3 ml of TSB and incubated at 37°C for 6 hours with aeration, then boiled for 10 minutes, immediately immersed on ice for 5 minutes and centrifuged at 11,000g for 10 minutes. The supernatant was di-

Table 1
Primers used in the study.

Target gene	Name	Sequence (5' to 3')	Annealing temperature (°C)	Amplicon size (bp)	Reference
<i>mecA</i>	mecA1	GTAGAAATGACTGAACGTCGGATAA	50	310	Geha <i>et al</i> , 1994
	mecA2	CCAAITCCACAITGTTTCGGTCTAA			
<i>luk-PV</i>	luk-PV1	ATCATAGGTAAATGCTGGACATGATCCA	57	433	Lina <i>et al</i> , 1999
	luk-PV2	GCAATCAASTGTATGGATAGCAAAAAGC			
<i>coa</i>	COA1	CGAGACCAAGAITCAACAAG	51	730	Wichelhaus <i>et al</i> , 2001
	COA2	AAAGAAAACCACTCACATCAGT			
<i>vWbp</i>	vWbp-F	GCTGGATTAAATGGTAAAAGTCAATG	50	320	Bunnoeng <i>et al</i> , 2014
	vWbp-R	GTTTATTA AAAACGTTTTGATGACC			
<i>femB</i>	FemB1	TTACAGAGTTAACTGTTACC	48	651	Kobayashi <i>et al</i> , 1994
	FemB2	ATACAAATCCAGCACGGTCT			
<i>spa</i>	SPA1	ATCTGTGGCGTAAACACCTG	55	1,500	Wichelhaus <i>et al</i> , 2001
	SPA2	CGCTGCACCTAACGGCTAATG			
<i>sea</i>	SEA-F	GCAGGGAACAAGCTTTAGGC	52	520	Monday and Bohach, 1999
	SEA-R	GTTCTGTAGAAAGTATGAAAACAG			
<i>seb</i>	SEB-SEC-F	ATGTAATTTGATATTCGCAGTG	50	643	Monday and Bohach, 1999
	SEC-R	TGCAGGCAICATAICATAACCA			
<i>sec</i>	SEC-F	CTTGTATGTATGGAGGAATAACAA	50	283	Monday and Bohach, 1999
	SEC-R	TGCAGGCATCATATCATAACCA			
<i>sed</i>	SED-F	GTGGTGAATATAGATAGGACTGC	50	384	Monday and Bohach, 1999
	SED-R	ATATGAAAGGIGCTCTGTGG			
<i>see</i>	SEE-F	TACCAATTAACITGTGGATAGAC	50	170	Monday and Bohach, 1999
	SEE-R	CTCTTTGCACCTTACCCG			
<i>seg</i>	SEG-F	CGTCTCCACCTGTTGAAGG	50	327	Monday and Bohach, 1999
	SEG-R	CCAAAGTATGTCIATITGTCG			
<i>seh</i>	SEH-F	CAACTGCTGATTTAGCTCAG	50	360	Monday and Bohach, 1999
	SEH-R	GTGGAATGAGTAATCTCTAGG			
<i>sei</i>	SEI-F	CAACTCGAATTTCAACACAGTAC	52	465	Monday and Bohach, 1999
	SEI-R	CAGGCAGTCCATCTCCTG			
<i>sej</i>	SEJ-F	CATCAGAACTGTTCCGCTAG	50	142	Monday and Bohach, 1999
	SEJ-R	CTGAAATTTTACCATCAAAGGTAC			
<i>tst</i>	tst-F	GCTTGGACAACCTGCTACAG	50	559	Monday and Bohach, 1999
	tst-R	TGGATCCGTCATTCATTTTAA			
<i>nuc</i>	nuc1	GTAGGTGGCAAGCGTTATCC	50	279	Zhang <i>et al</i> , 2004
	nuc2	CGCACATCAGCGGTACG			

Table 2
 Characteristics of SE gene-carrying MCSA-CoN strain PSU109 and MR-CoN strains PSU172 and PSU180.

Strain	Source	Virulence gene	^a Antimicrobial susceptibility pattern
PSU109 MCSA-CoN	^b Seasoned rice	<i>mecA, spa, sec, sed</i>	FOS, NV
PSU172 MR-CoN	Seasoned rice	<i>mecA</i>	DA, FD, FOX, NV
PSU180 MR-CoN	Chicken soup	<i>mecA</i>	DA, FD, FOX, NV

^aDA, clindamycin; FD, fusidic acid; FOS, fosfomycin; FOX, cefoxitin; NV, novobiocin. ^bSliced Hainan-style chicken with marinated rice.

luted 10-fold in sterile distilled water and used as DNA template for PCR.

Six distinct *S. aureus* virulence genes, namely, *mecA*, *coa*, *luk-PV* (panton valentine leukocidin), *spa* (staphylococcal protein A), *vWbp* (von Willebrand factor binding protein), and *femB* (Aminoacyl-transferase femB, a methicillin resistance regulator), together with 10 superantigens-coding genes (*sea*, *seb*, *sec*, *sed*, *see*, *seg*, *seh*, *sei*, *sej*, and *tst-1*) were identified by uniplex PCR. The primer sequences and annealing temperatures were shown in Table 1. Amplification, in a reaction volume of 25 µl, was carried out using GoTaq Flexi system (Promega, Madison, WI), consisting of 3.0 mM MgCl₂, 0.1 mM dNTPs, 0.4 µM each primer pair, 0.5 U GoTaq DNA polymerase, 1X GoTaq Flexi buffer and 2 µl of DNA template. Amplicons were analyzed by 1.0% agarose gel-electrophoresis, stained with ethidium bromide and recorded using WSE-5200 Printpraph 2M gel imaging system (ATTO, Tokyo, Japan). The absence of *coa* was confirmed by coagulase tube test using rabbit plasma (plasma dilution 1:4) (Sukhumungoon *et al*, 2014).

MRSA typing

Staphylococcal cassette chromosome *mec* (SCC*mec*) (type I to VI) was identified by PCR as described by Milheiriço *et al* (2007). In short, amplification was carried out in a 25-µl PCR reaction using GoTaq Flexi system (Promega), consisting of 3.0 mM MgCl₂, 0.1 mM dNTPs, 0.4 µM each primer pair, 0.5 unit GoTaq DNA polymerase, 1X GoTaq Flexi buffer and 2 µl of DNA template. Amplicons were analyzed by 1.2% agarose gel-electrophoresis and visualized as described above. PCR amplification of *mecA* was used as an internal control. In addition, pulsed-field gel electrophoresis (PFGE) was also carried out

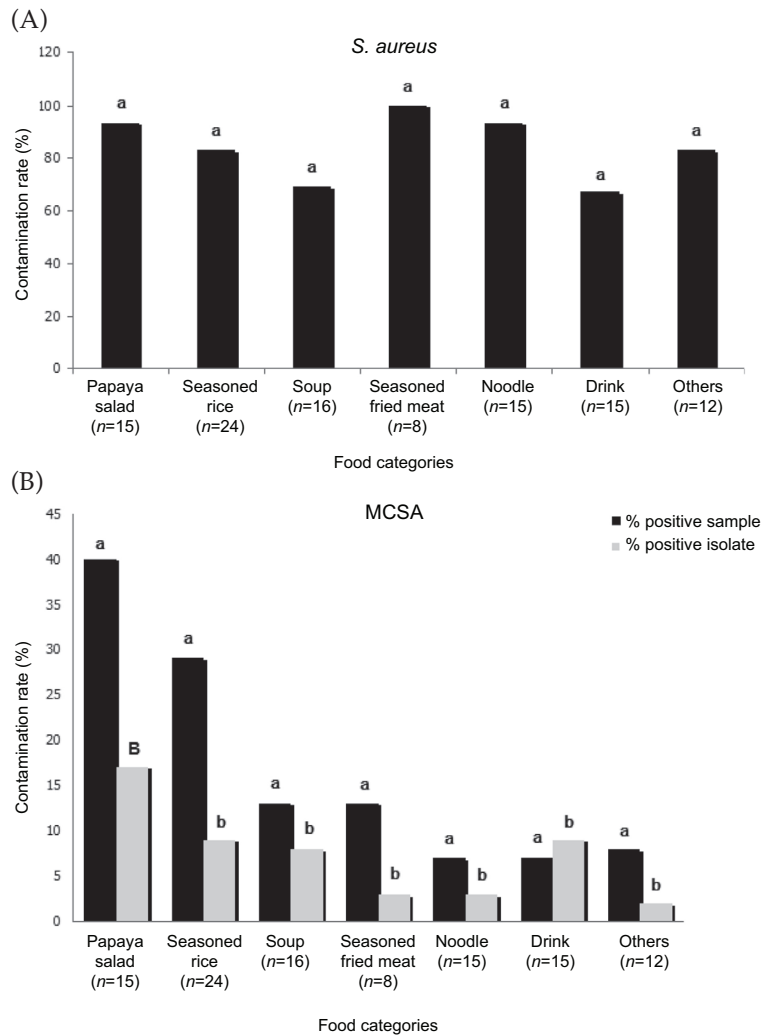


Fig 1—*S. aureus* (A) and MCSA (B) contamination in different RTE food categories sold in Songklanagarind Hospital canteen. *S. aureus* and MCSA were identified by the presence of *nuc* and both *nuc* and *mecA*, respectively. There were 34 MCSA isolates obtained from 105 RTE food samples. Uppercase letter, B, indicates significant difference ($p < 0.05$) and lowercase letter, a and b, indicate non-significant differences among the food categories.

as described by Bunnoeng *et al* (2014). In brief, bacterial spheroplast formation was generated by treating with 50 $\mu\text{g}/\text{ml}$ of lysothaphin (Sigma-Aldrich, St Louis, MO) for 30 minutes. After plug preparation, genome of *S. aureus* was digested with

50 U FastDigest *Sma* I (Thermo Scientific, Rockford, IL) at 37°C for 1 hour and separated by 1.0% agarose gel (Invitrogen, Carlsbad, CA) at 14°C in 0.5X TBE buffer using a CHEF DRIII system (Bio-rad, Hercules, CA) at 6V/cm, field angle 120°, initial and final switch times of 5.0 and 40.0 seconds, respectively, and an overall electrophoresis time of 21 hours. Then, the gel was stained with ethidium bromide and images recorded as described above. The 48.5 kb Lambda DNA was used as a molecular size standard. Dendrogram was constructed using unweighted pair-group method of arithmetic average (UPGMA) (BioNumerics software version 7.0, Applied maths, Sint-Martens-Latem, Belgium). Clinical MRSA strains, 4846-12 SPT, 988-12 SPT, and 4898-12 SPT isolated from patients in Songklanagarind Hospital during approximate time as RTE food collection, were included for comparison.

Antimicrobial susceptibility testing

All MCSA-CoN (coagulase-negative, *mecA*-carrying *S. aureus*) samples were determined for their antimicrobial susceptibilities by the disk diffusion method (CLSI, 2014). Ten antimicrobial agents

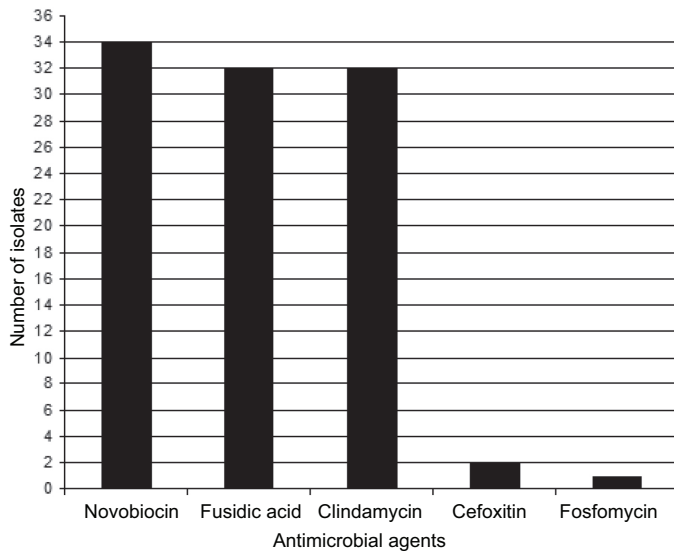


Fig 2—Antimicrobial resistance of 34 MCSA isolates from various RTE foods from Songklanagarind Hospital canteen. Antimicrobial susceptibility test was performed by disk diffusion method using cefoxitin (30 µg), clindamycin (2 µg), fosfomycin (50 µg), fusidic acid (10 µg) and novobiocin disk (5 µg).

were used, namely, cefoxitin (30 µg), ciprofloxacin (5 µg), clindamycin (2 µg), cotrimoxazole (23.75/1.25 µg), erythromycin (15 µg), fosfomycin (50 µg), fusidic acid (10 µg), gentamicin (10 µg), norfloxacin (10 µg), and vancomycin (256-0.015 µg). Oxacillin resistance was interpreted using cefoxitin disk (30 µg) and novobiocin disk (5 µg) was used to discriminate between *S. saprophyticus* and coagulase-negative Staphylococci. Clear zone was measured using a Vernier caliper. Antimicrobial agents were from Oxoid (Hamshire, UK). Vancomycin susceptibility was performed by the E-test method, with susceptibility breakpoint for vancomycin of coagulase-negative *S. aureus* of ≤ 4 µg/ml (CLSI, 2014).

Evaluation of bacterial survival in acidic food

Bacteria were grown in TSB and col-

lected as described above. Bacterial cells were washed with 0.85% NaCl solution (NSS) and adjusted to 0.5 McFarland turbidity standard unit with a McFarland densitometer (Biosan, Riga, Latvia). Ten-fold dilution was performed to obtain a working solution of 1.5×10^7 CFU/ml. A 1 ml aliquot of the working suspension was added to 99 ml of a sterile unripened-papaya salad sauce (from Songklanagarind Hospital canteen) at a final concentration of 1.5×10^5 CFU/ml. Survival of bacteria was monitored at 7 time points (0-6 hours) by surface plate count on MSA. The experiment was performed in triplicate. The pH of unripened-papaya

salad sauce was measured using a pH meter (Sartorius, Goettingen, Germany).

Statistical analysis

Data were analyzed using SPSS for Windows software, version 11.0 (SPSS, Chicago, IL). Pearson chi-square (X^2) was used to compare the relationship between the presence of *S. aureus* or MCSA and type of food. Kruskal-Wallis test was employed to determine the difference in *S. aureus* or MCSA existence between groups. Level of significance was set at a $p < 0.05$.

RESULTS

Bacteria isolates and the presence of *mecA*

In order to obtain data on the possible contamination of *S. aureus* and hospital acquired MRSA methicillin in RTE food

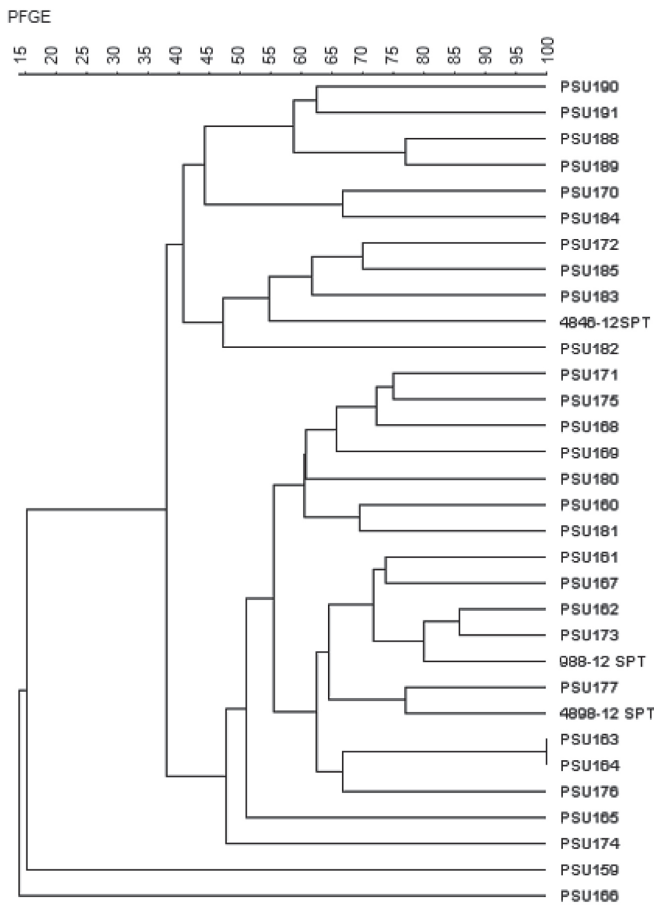


Fig 3—PFGE-based dendrogram of 29 MCSA isolates (PSU strains) from various RTE foods, Songklanagarind Hospital canteen and 3 clinical MRSA strains (988-12 SPT, 484612-SPT and 4898-12 SPT) from Songklanagarind Hospital in-patients isolated during the same period.

sold in Songklanagarind Hospital canteen, 105 RTE food samples were analyzed. *S. aureus* was found in 87 samples (83%), with seasoned fried meat having the highest percent *S. aureus* contamination (8/8) while drinks the lowest (10/15, 67%), but these values among the food categories were not significantly different ($p > 0.05$) (Fig 1A). On the other hand, when considering the numbers of MCSA contamination in each type of food, the presence of MCSA was significantly related to the type of food ($p < 0.05$), with unripped-pa-

paya salad being the most important MCSA vehicle (17%) (Fig 1B).

Antimicrobial susceptibility test and virulence genes investigation

Among 452 bacterial isolates obtained from all RTE food types, 34 (7.5%) were confirmed as coagulase-negative *S. aureus* by PCR, which showed the absence of *coa* and *femB*, and also were resistant to novobiocin. Coagulase tube tests verified the lack of *coa* phenotype. Of these 34 isolates, ceftiofur resistance was present in two MCSA-CoNs, PSU172 and PSU180, derived from seasoned rice and soup, respectively, and so were classified as coagulase-negative MRSA (MR-CoN) (Table 2). Clindamycin and fusidic acid resistance were observed in 32/34 (94%) isolates (Fig 2). One isolate (PSU109) carrying SE genes, *sec* and *sed*, including *spa* gene, was

resistant to fosfomicin (Table 2, Fig 2). All isolates were susceptible to vancomycin (minimum inhibitory concentration of 0.5-1.5 $\mu\text{g/ml}$).

MRSA genotyping

Because most of MCSA-CoNs from various food types exhibited the same genotype and antibiogram pattern, bacterial genotyping was performed to seek for their genetic relationships. SCC_{mec} typing could not be performed due to absence of specific amplicons (data not shown), and

PFGE was carried out. Profiles of DNA fragments from 29 isolates from each type of RTE food sample and 3 isolates collected from patients during the same period revealed 29 distinguishable patterns (80% similarity), with PSU166 being the most distantly related (Fig 3). MCSA-CoN strain PSU162 isolated from sliced Hainan-style chicken with marinated rice and MCSA-CoN strain PSU173 from unripened-papaya salad demonstrated 85% genetic similarity and 80% relatedness to a clinical MRSA strain 988-12 SPT (Fig 3). A similar result (77.5% similarity) was obtained with PSU177, from unripened-papaya salad, and clinical MRSA strain 4898-12 SPT.

Evaluation of bacterial survival in acidic food

Among the large numbers of MCSA-CoN, certain strains of MR-CoN were found in unripened-papaya salad, a popular acidic food consumed throughout Thailand, this food type was used as a surrogate for evaluating the survival of MR-CoN strain PSU172, MCSA-CoN strain PSU173 and SE-carrying MCSA-CoN strain PSU109. The pH of the unripened-papaya salad sauce was 4.0 - 4.5. These strains survived in unripened-papaya salad throughout 6 hours of incubation with a bacterial population numbers maintained between 10^4 and 10^5 CFU/ml (data not shown).

DISCUSSION

MRSA has been recognized as being responsible for infections in hospital settings for over a decade (de Sousa *et al*, 2003; Johnson *et al*, 2005). In addition there is the existence of community-acquired MRSA, which poses a problem in the health care system (Gordon and Lowy, 2008). Recently, *S. aureus* and MRSA have been detected in various food products,

including RTE food, in many areas of the world (Pu *et al*, 2009; Feßler *et al*, 2011; Hammad *et al*, 2012; Wang *et al*, 2013). These bacteria display several potential virulence factors, including SEs, members of PTSAg family responsible for food poisoning in humans.

In this study, MCSA-CoN strain PSU109, isolated from sliced Hainan-style chicken with marinated rice, carried *sec*, *sed*, and *spa*, encoding toxins proposed to cause illness in humans (Chang *et al*, 2005). Crago *et al* (2012) found contamination in food samples of several bacterial species, including *S. aureus* [73/693 (10.5%) of the samples] but not MRSA, in Alberta, Canada. Hammad *et al* (2012) also demonstrated the presence of *S. aureus* and MRSA in Sashimi, an RTE raw fish. In addition to MR-CoN, we found MCSA-CoN strains which were susceptible to cefoxitin, exhibited the growth on MSA containing 4µg/ml cefoxitin, suggesting the sign of heterogeneous MRSA (data not shown). This phenomenon is believed possibly to affect the bacterial pleiotropic regulatory mechanisms (Rohrer *et al*, 2003). The presence of heterogeneous MRSA also poses health risks to humans.

There is a significant degree of MCSA-CoN contamination in unripened-papaya salad compared with other kinds of RTE food. This food item is naturally not subjected to high temperatures during its preparation, and is often produced under poor hygienic practices. The high detection rate of MCSA-CoN in this study is associated with the large numbers of isolates obtained per sample ($p < 0.05$). Therefore, we suggest that to successfully detect MCSA from food samples, low number of selected isolates should be avoided to increase the detection threshold.

PFGE, a reliable tool extensively used

for investigations of bacterial genetic relatedness (Tenover *et al*, 1995; Weese *et al*, 2010), demonstrated that there were at least two MCSA-CoN strains contaminating the food samples, and which were genetically distantly related. This phenomenon has been shown previously in MCSA-CoN strains isolated from raw meats sold at retail shops in this area (Bunnoeng *et al*, 2014). More importantly, MCSA-CoN strain PSU162 collected from sliced Hainan-style chicken with marinated rice and MCSA-CoN strain PSU173 from unripened-papaya salad had a high degree (80%) of relatedness with the clinical MRSA strain 988-12 SPT (Fig 3), supporting the notion that these MCSA-CoN strains may have been acquired from clinical sources.

Unripened-papaya salad is a popular food consumed throughout Thailand and neighboring countries. However, unripened-papaya salad is an acidic food where bacterial growth should be suppressed. Although the favorable pH required for the growth of *S. aureus* is in the range of 4.5-9.3 (Lawley *et al*, 2008), the tested PSU172, PSU173 and PSU109 strains were able to survive in the unripened-papaya salad (pH of 4.0-4.5). Thus, only good hygienic practice in food preparation, not only for food items at high risk but for all RTE food items, can reduce bacterial contamination of such pathogenic microbes as MCSA-CoN and MR-CoN, especially in settings geographically located near to clinical institutions.

ACKNOWLEDGEMENTS

Financial support for this study was from a Research Assistant Scholarship, Faculty of Science, Prince of Songkla University, fiscal year 2014 (grant no. 2-2557-02-011).

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