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Research article

Evaluation of in-house cefoxitin screening broth to determine methicillin-resistant staphylococci

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ABSTRACT

Methicillin-resistant staphylococci (MRS), including methicillin-resistant *Staphylococcus aureus* (MRSA) and methicillin-resistant coagulase-negative staphylococci (MRCoNS), have a global impact as a public health threat contributing significantly to morbidity, mortality, and socio-economic costs. Accurate and rapid detection of MRS results in effective antimicrobial therapy, immediate patient isolation, dissemination control, and appropriate disinfection measures. Herein, we developed an in-house cefoxitin screening broth and compared it to the cefoxitin disk diffusion method and polymerase chain reaction (PCR) for the detection of MRS. Verification of this screening broth on 52 MRSA, 37 MRCoNS, 44 methicillin-susceptible *S. aureus* (MSSA), and 11 MSCoNS revealed greater validity for MRSA/MSSA than for MRCoNS/MSCoNS. The kappa coefficient of 0.87 was superior for determination of MRSA and MSSA, whereas it was 0.54, which was considered poor, for determination of MRCoNS and MSCoNS. Application of this assay to screen MRSA should be useful in clinical laboratories and hospital infection-control units.

1. Introduction

Methicillin-resistant staphylococci (MRS), including methicillin-resistant *Staphylococcus aureus* (MRSA) and methicillin-resistant coagulase-negative staphylococci (MRCoNS) strains, have become an additional threat for human and animal health, contributing significantly to morbidity, mortality, and socio-economic costs [1]. Resistance in MRSA and MRCoNS is caused by the acquisition of the *mecA* gene that encodes a modified penicillin-binding protein 2a (PBP2a) which has a low binding affinity for all beta-lactam antibiotics [2]. The *mecA* gene is located within the *mec* operon carried by the staphylococcal cassette chromosome *mec* (SCC*mec*); to date, SCC*mec* I to XIII have been recorded [3].

MRSA is classified as a hospital-acquired (HA), community-acquired (CA), and livestock-associated (LA) infection [4]. MRSA strains carrying SCC*mec* I to III are commonly found in HA-MRSA, while SCC*mec* IV to XIII are usually detected in CA-MRSA and LA-MRSA [5]. In contrast to MRSA, MRCoNS carries variables of SCC*mec* types [6]. Although infections with MRSA have been well known, MRCoNS infections have also been frequently reported [7, 8, 9]. MRCoNS has been documented for high nasal prevalence in medical students, drug users, and in the university environment [2,10,11].

The Clinical and Laboratory Standards Institute (CLSI) recommends the use of cefoxitin as a surrogate marker to detect *mecA*-mediated oxacillin resistance in staphylococci because it is more reliable than oxacillin, a more potent inducer of the *mecA* regulatory system, and no special medium or incubation temperature is required for cefoxitin, as is required for oxacillin [12,13]. Accurate and rapid detection of MRS results support effective antimicrobial therapy, immediate patient isolation, and appropriate disinfection measures. Several methods for the detection of MRSA include latex agglutination assay, CHROM agar, VITEK, MALDI-TOF mass spectrometry, and PCR [14]. Although some of these methods are rapid, easy to perform, and are accurate in identification or detection, many of these methods are high in cost and have a high level of sophistication that requires operator experience and skill and special instrumentation. These constraints make such methods inappropriate in a laboratory with limited resources. The aim of this study was to compare an in-house cefoxitin screening broth with the cefoxitin disk diffusion method and PCR for the detection of MRS, specifically, to distinguish species or group of staphylococci using the

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screening broth. Our screening broth provides an alternative assay to detect MRS isolated from either human or non-human samples. This may be useful in laboratories where a large number of isolates must be screened at low cost and as such could reduce the costs associated with laboratory and infection control in hospitals with a high prevalence of MRS, especially MRSA.

2. Materials and methods

2.1. Bacterial strains

In total, this study used 144 *Staphylococcus* spp., consisting of 96 *S. aureus* isolated from human specimens (n = 50) and non-human specimens (n = 46), and 48 coagulase-negative staphylococci (CoNS) isolated from human samples (n = 44) and non-human samples (n = 4). The human staphylococci were collected and sent from hospitals for further confirmation by the Public Health Microbiology Laboratory of the Faculty of Public Health, Kasetsart University Chalermphrakiat Sakon Nakhon Province Campus, Thailand. All isolates were kept in at -80 °C in a freezer.

Ethical review and approval were not required because no human specimens or data were used in the current study.

2.2. Microbiological analysis

Frozen isolates were cultured on sheep blood agar at 37 °C for 18 h. Identification of *S. aureus* and coagulase-negative staphylococci was performed using conventional phenotypic tests including Gram's stain, catalase testing, and tube coagulase testing [15]. *S. aureus* was presumptively identified based on Gram-positive cocci, a grape-like clustered arrangement, and being positive in catalase and coagulase. The Gram-positive cocci had grape-like clusters, were positive in catalase, but negative in coagulase; thus, they were identified as coagulase-negative staphylococci [15]. Confirmation of *S. aureus* was done using a multiplex PCR assay to simultaneously identify *S. aureus* based on *femA* and the methicillin-resistant genes *mecA* and *mecC*, respectively [16,17]. Genomic DNA from all isolates was extracted using a NucleoSpin® Tissue Kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions.

The PCR reaction mixtures contained 1X JumpStart™ REDTaq® ReadyMix™ Reaction Mix (Sigma) and 0.4 μM of each primer pair (Table 1). The following PCR thermocycling parameters were used: initial activation of DNA polymerase at 95 °C for 3 min; 30 cycles of denaturation at 95 °C for 30 s, primer annealing at 55 °C for 30 s, and extension at 72 °C for 1 min; with a final extension at 72 °C for 5 min. The PCR products were resolved using gel electrophoresis for 30 min on 2% agarose gels in 0.5× TBE buffer. The gels were stained with ethidium bromide and visualized under ultraviolet light using a GeneGenius Bio-imaging System (SynGene, Maryland, USA). The sizes of the PCR products were determined by comparison with a molecular size standard (GeneRuler™ 100 bp Plus DNA ladder, Thermo Fisher Scientific). *S. aureus* ATCC33591 (*mecA* positive), *S. aureus* ATCC (*mecC* positive), and *S. aureus* ATCC 25923 were used as positive control for PCR reaction.

Table 1. Primers used in multiplex PCR in current study.

Primer name	Sequence (5' - 3')	Target	PCR product size (bp)	Reference
femA-F	CGATCCATATTTACCATATCA	*S. aureus*	450	16
femA-R	ATCACGCTCTTCGTTTAGTT			
mecA-F	ACGAGTAGATGCTCAATATAA	*mecA*	293	
mecA-R	CTTAGTTCTTTAGCGATTGC			
mecC-F	GAA AAA AAG GCT TAG AAC GCC TC	*mecC*	138	17
mecC-R	GAA GAT CTT TTC CGT TTT CAG C			
d1	CCI TAY ICI TAY GAY GCI YTI GAR CC	*sodA*	438	18
d2	ARR TAR TAI GCR TGY TCC CAI ACR TC			

Where *S. aureus* was negative, based on the PCR results, *sodA* sequencing was conducted to identify the species of staphylococci [18]. PCR to amplify internal fragment of *sodA* was performed in a final volume of 50 μl containing 150 ng of DNA as the template, 0.5 μM each primer, and 1X JumpStart™ REDTaq® ReadyMix™ PCR Reaction Mix (Sigma-Aldrich, USA). The PCR mixtures were denatured (3 min at 95 °C) and then subjected to 30 cycles of amplification (30 s of denaturation at 95 °C, 60 s of annealing at 37 °C, and 45 s of elongation at 72 °C). The PCR products were purified using an E-Z 96® Cycle Pure Kit (Omega, USA) following the manufacturer's instructions. All *sodA*-PCR products were subjected to Sanger sequencing for confirmation by Apical Scientific Sdn Bhd, Selangor, Malaysia. Identification to the species level was based on sequence identity with a type strain sequence of ≥97% and a difference in sequence identity from the next closest species of ≥5% [18].

Identification of MRS was done by susceptibility to methicillin using cefoxitin (30 μg) disk diffusion according to the CLSI guideline [19]. A 0.5 McFarland standard suspension of the isolate was made and lawn cultures were made on Muller Hinton agar (MHA) plates (BBL, USA). The zone of inhibition was measured after incubation at 35 °C for 16–18 h. Zone size was interpreted according to the criteria in the CLSI guidelines [19]. Standard strains of MRSA (ATCC 43300) and MSSA (ATCC 25923) were used as quality control for the disk diffusion.

2.3. In-house cefoxitin screening broth

This broth was prepared using 2 tubes (A and B). Tube A (1 ml) contained 1.6% phenol red broth base (HiMedia, India), 1% D-mannitol (Univar, Australia), 7% NaCl (Daejung, Korea), and 0.2% 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal; GoldBio, USA). Tube B (1 ml) consisted of the same ingredients as tube A and also cefoxitin at 6 μg/ml. Interpretation is shown in Figure 1. A 0.1 ml amount of inoculum at a concentration of 0.5 McFarland was inoculated into the broth. Standard strains of MRSA (ATCC 43300) and MSSA (ATCC 25923) were used as quality control for the screening broth.

2.4. Statistical analysis

Diagnostic measures were calculated (sensitivity, specificity, positive and negative predictive values (PPV and NPV), and accuracy) for this in-house cefoxitin screening broth [20]. Kappa coefficients were calculated to evaluate the associations and levels of agreement of the assay in this study to the reference method (cefoxitin disk diffusion) [21].

Accession number. The GenBank accession numbers of *sodA* sequences of Staphylococci in this study were OL771262 - OL771286.

3. Results

3.1. Identification and methicillin resistance testing

In total, 144 isolates of staphylococci, 96 were presumptively identified to be *S. aureus* and 48 were CoNS, based on phenotypic testing. The 96 *S. aureus* suspected isolates were confirmed to be *S. aureus* using multiplex PCR and only 52 isolates (human isolates = 50 and non-human isolates = 2) carried the *mecA* gene. Cefoxitin susceptibility using disk diffusion revealed these 52 isolates resisted cefoxitin that were confirmed to be MRSA, whereas 37 out of the 48 CoNS were confirmed as MRCoNS based on cefoxitin disk diffusion testing and being positive for *mecA* (n = 36) and *mecC* (n = 1). Sequencing of the *sodA* gene demonstrated the 1 *mecC*-harboring MRCoNS isolate was *S. xylosus*, while the remaining 36 *mecA*-carrying MRCoNS were 16 *S. haemolyticus*, 9 *S. epidemidis*, 6

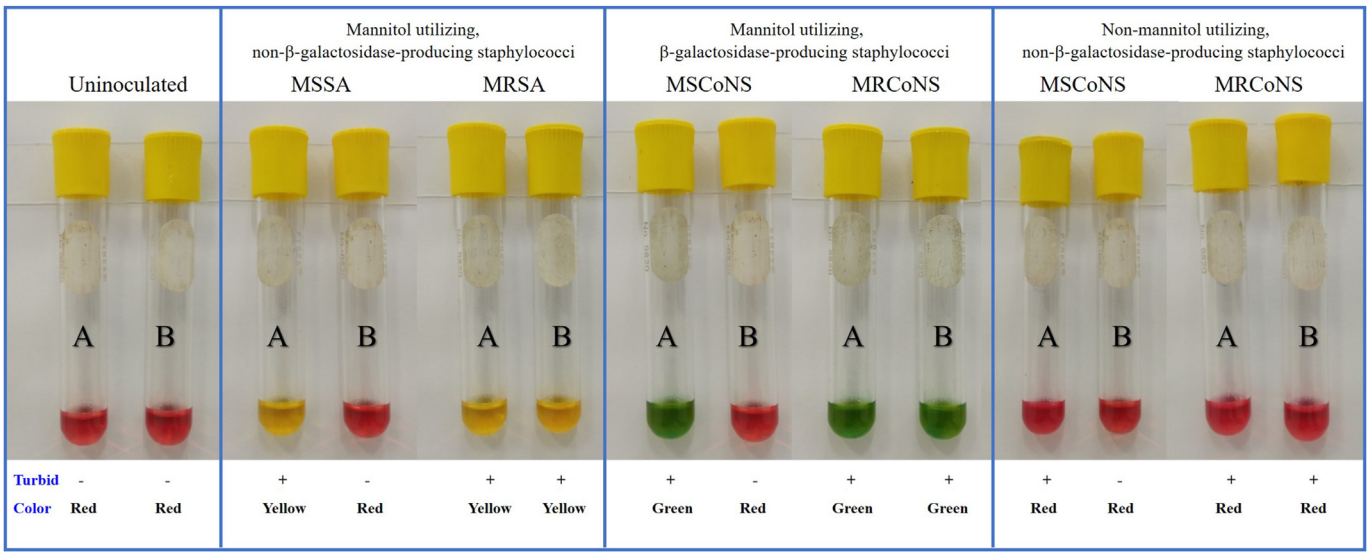

Figure 1. Interpretation of an in-house cefoxitin screening broth for methicillin-resistant staphylococci (MRS) and methicillin-susceptible staphylococci (MSS).

S. sciuri, and a single isolate each of *S. cohnii*, *S. saprophyticus*, *S. capitis*, *S. hominis*, and *S. arlettae*.

In contrast with the methicillin-susceptible staphylococci (MSS), cefoxitin disk diffusion assay confirmed 55 isolates consisting of 44 MSSA (methicillin-susceptible *S. aureus*) and 11 MSCoNS. The *sodA* sequencing identified 11 MSCoNS to be 4 *S. xylosus*, 2 *S. haemolyticus*, 2 *S. saprophyticus*, 2 *S. sciuri*, and a single *S. epidemidis*. In addition, multiplex PCR detected *mecC* in 4 isolates of *S. xylosus*.

3.2. Evaluation of in-house cefoxitin screening broth

The principle of the in-house cefoxitin screening broth determined resistance to cefoxitin based on turbidity and distinguished species or group of staphylococci based on color changes. The broth was used in 2 tubes (A and B). Tube A did not contain cefoxitin, whereas tube B did. Basically, all staphylococci including those being methicillin resistant or susceptible could grow in tube A as was confirmed by turbidity, while tube B depended on their resistance to the antibiotic. If these bacteria could utilize mannitol, they produced acid to affect the pH of the medium resulting in the phenol red indicator changing from red to yellow. However, if the organism could produce β-galactosidase, the enzyme degraded to X-Gal that produced a blue product. Therefore, staphylococci utilizing mannitol and producing β-galactosidase produced a green color due to the mixing of the blue and yellow products. Distinguishing between these staphylococci to the group or species levels was based on the color appearance. A yellow color was presumably due to mannitol utilization but not β-galactosidase, suggesting staphylococci including *S. aureus* and *S. sciuri* (Figure 1). A green color indicated mannitol utilization and β-galactosidase production, suggesting several species of *Staphylococcus*, such as *S. xylosus*, *S. arlettae*, and *S. intermedius* (Figure 1). Red or orange colors with turbidity indicated non mannitol utilization, suggesting staphylococci, such as *S. epidemicus*, *S. capitis*, and *S. saprophyticus* (Figure 1).

We verified this screening broth on 52 MRSA, 37 MRCoNS, 44 MSSA, and 11 MSCoNS. The results are shown in Table 2. All *S. aureus* and *S. sciuri* samples were turbid with a yellow color in tube A (no cefoxitin); however, turbidity in tube B (containing cefoxitin) depended on resistance to cefoxitin. Where isolates were susceptible to cefoxitin, they

Table 2. Results using an in-house screening broth on 144 staphylococcal isolates in this study.

Type	Species	N	Tube A (no cefoxitin)			Tube B (containing cefoxitin)			% Concordance
			Turbidity	Color	N	Turbidity	Color	N	
Methicillin-resistant	*S. aureus*	52	+	Yellow	52	+	Yellow	46	88.5
	S. sciuri	6	+	Yellow	6	+	Yellow	4	66.7
	S. xylosus	1	+	Green	1	+	Green	1	100
	S. arlettae	1	+	Green	1	+	Green	1	100
	S. haemolyticus	16	+	Red/Orange	16	+	Red/Orange	12	75
	S. epidermidis	9	+	Red	9	+	Red	8	88.9
	S. cohnii	1	+	Red	1	+	Red	1	100
	S. saprophyticus	1	+	Red	1	+	Red	1	100
	S. capitis	1	+	Red/Orange	1	+	Red/Orange	1	100
	S. hominis	1	+	Red	1	+	Red	1	100
Methicillin-susceptible	*S. aureus*	44	+	Yellow	44	-	No change (Red)	44	100
	S. sciuri	2	+	Yellow	2	+	Yellow	1	50
	S. xylosus	4	+	Green	4	-	No change (Red)	4	100
	S. haemolyticus	2	+	Red/Orange	2	-	No change (Red)	2	100
	S. epidemidis	1	+	Red	1	-	No change (Red)	1	100
	S. saprophyticus	2	+	Red	2	+	Red	1	50
Total		**144**			**144**			**129**	**89.6**

would not grow and tube B would remain a red color but if they could resist cefoxitin, then a yellow color with turbidity was observed. Mannitol-utilizing and β-galactosidase-producing staphylococci and non-mannitol-utilizing staphylococci were identified by color differences (Table 2). The levels of accuracy, specificity, sensitivity, PPV, and NPV of this screening broth for these MRS isolates are presented in Tables 3 and 4. The results indicated that our in-house cefoxitin screening broth produced greater validity in MRSA/MSSA than for MRCoNS/MSCoNS, with kappa coefficients of 0.87 for determination of MRSA and MSSA and 0.54 for determination of MRCoNS and MSCoNS. Based on the kappa coefficient criteria, our screening broth revealed strong agreement (values in the range 0.80–0.90) for MRSA/MSSA determination, but weak agreement (values in the range 0.40–0.59) for MRCoNS/MSCoNS. In addition, our in-house screening broth showed rapid positivity after incubation for at least 8 h and the cost per test (2 tubes) was USD 1.02 or EUR 0.9.

4. Discussion

MRS, especially MRSA, has a global impact as a threat to public health by contributing significantly to morbidity, mortality, and socio-economic costs. The World Health Organization has prioritized MRSA in group 2 (high) of the global priority list of antibiotic-resistant bacteria (https://www.who.int/medicines/publications/WHO-PPL-Short_ Summary_ 25Feb-ET_NM_ WHO.pdf; accessed August, 2021). Global epidemiology of MRSA revealed 19.1%, 31.9%, 17.4%, 67.9%, 27.3%, and 43.2% in Africa, the Americas, the Mediterranean, Europe, Southeast-Asia, and the Western Pacific, respectively [22]. A SENTRY study in Europe revealed that MRSA isolates constituted 25% of all *S. aureus* isolates, with the highest prevalence in hospitals in Portugal (54%) and Italy (43–58%) [9], with some areas in Europe having even higher levels (60–70%) of MRCoNS [9].

In our study, MRSA had lower prevalence in non-human isolates (3.8%) than human isolates (96.2%). Several studies reported high prevalence of MRSA among raw foods, environmental surfaces, and sand and water samples [23, 24, 25]. However, some studies indicated low prevalence of MRSA in non-human samples, for example, 2.2% from retail food and food handlers' gloves, 1.7% in beef, 1.2–1.9% in pork, 0.3% in chicken, 3.5% in turkey, 1.86% in secondary school environments, and 1.58% from environmental contamination in railway stations and coach stations [26, 27, 28, 29, 30]. Several reasons could account for this low prevalence of MRSA in non-human samples. For example, differences in the sampling period, sample size, sampling site, sampling techniques, isolation method, single enrichment step, frequency of MRSA in different samples, or geographical locations could partially explain the variation of prevalence [27,29,31]. Also of interest was the high prevalence of methicillin-resistant *S. haemolyticus* carrying *mecA* (MRSH; n = 16; 44.4%) among the MRCoNS-carrying *mecA* (n = 36). A study in Brazil revealed 88% MRSH (n = 64) as defined by the cefoxitin disc diffusion assay but 56 of these isolates were *mecA*-positive [32]. Another study reported 87% and 66% MRSH on the hands of healthcare workers and in a hospital environment, respectively [33]. The increase in the frequency of MRSH as the causal agent of hospital infections, the possibility of the emergence of resistance to other antibiotics, and clonal spreading within hospitals should be closely monitored.

Table 3. Validity of the in-house cefoxitin screening broth for determining *S. aureus* compared to cefoxitin disk-diffusion.

In-house screening broth	Cefoxitin disk-diffusion		Validity
	Positive (MRSA)	Negative (MSSA)	
Positive (MRSA)	46	0	PPV = 100%
Negative (MSSA)	6	44	NPV = 88%
Validity	Sensitivity = 88.5%	Specificity = 100%	Accuracy = 93.7%

PPV = positive-predictive value; NPV = negative-predictive value.

Table 4. Validity of the in-house cefoxitin screening broth for determining coagulase-negative staphylococci compared to cefoxitin disk-diffusion.

In-house screening broth	Cefoxitin disk-diffusion		Validity
	Positive (MRCoNS)	Negative (MSCoNS)	
Positive (MRCoNS)	30	2	PPV = 93.7%
Negative (MSCoNS)	7	9	NPV = 56.3%
Validity	Sensitivity = 81.1%	Specificity = 81.8%	Accuracy = 81.3%

PPV = positive-predictive value; NPV = negative-predictive value.

Bassetti et al., (2017) discussed potential determinants influencing the future dissemination and control of antibiotic resistance and nominated the rapidity and accuracy of laboratory techniques that allowed for rapid identification of the infecting pathogen and antibiotic susceptibility testing [34]. The current study developed an in-house cefoxitin screening broth to distinguish *Staphylococcus* species or groups and their resistance to cefoxitin in a single reaction. Our assay could differentiate *S. aureus* from other staphylococci, except for *S. sciuri*. In addition to *S. sciuri*, the staphylococci *S. capitis*, *S. lentus*, *S. simiae, S. chromogenes, S. warneri, S. pisciferm*entas, *S. pasteuri, S. caprae,* and *S. cohnii* subsp. *cohnii* may not be differentiated from *S. aureus* by the screening broth because these species can utilize mannitol but do not produce β-galactosidase [15]. However, further testing based on coagulase production can distinguish *S. aureus* from these species [15]. Furthermore, these bacteria do not frequently occur in human specimens. However, further validation and modification of this screening broth should be studied so it is effective for the mentioned bacteria.

Other groups of staphylococci that could be differentiated using the screening broth were those that were mannitol-utilizing and β-galactosidase-producing and also staphylococci that were not mannitol-utilizing. The Manual of Clinical Microbiology lists clinical mannitol-utilizing and β-galactosidase-producing staphylococci including *S. xylosus*, *S. saprophyticus*, *S. succinus*, *S. arlettae*, *S. simulans*, *S. pseudintermedius*, *S. nepalensis*, *S. felis*, *S. intermedius*, *S. condiment*, *S. carnosus* subsp. *carnosus*, *S. kloosii*, *S. gallinarum*, *S. lutrae*, *S. equorum* subsp. *equorum*, and *S. cohnii* subsp. *urealyticus*, and clinically non mannitol-utilizing staphylococci including *S. epidermidis, S. hyicus, S. lugdunensis, S. muscae, S. pettenkoferi, S. saccharolyticus*, and *S. carnosus* subsp. *utilis* [15].

Another study demonstrated that ChromID MRSA showed higher sensitivity, but its specificity was lower than Brilliance agar II due to the false-positive growth of Enterococcus spp. and methicillin-resistant coagulase-negative Staphylococci (MRCoNS) [35]. High levels of false positives (~50%) obtained with all chromogenic media when tested with *mec*-negative borderline oxacillin-resistant *S. aureus* (BORSA) isolates have been reported [36]. In our in-house assay, no false-positives were detected in the MRSA isolates; in contrast, two MRCoNS isolates were false-positive. However, false-positives due to BORSA should be noted as possibilities associated with our assay.

Although the CLSI guidelines recommend oxacillin salt agar (OSA; Mueller-Hinton agar +4% NaCl +6 μg/ml oxacillin) to detect MRSA [19], the OSA screen test is recommended only for *S. aureus* and also can be hard to interpret, especially heterogeneous *mecA* harboring staphylococci are not detected due to low expression of resistance [37,38]. One study revealed the specificity of OSA was only 75% as it falsely recognized 7 isolates as MSSA [38]. In addition, it has been reported that cefoxitin is more reliable than oxacillin to detect MRSA harboring either *mecA* or *mecC* [39,40]. Fang and Hedin (2006) demonstrated that cefoxitin-based selective broth was more sensitive at the rapid detection of MRSA strains, especially heterogeneously resistant strains, than the oxacillin-based broth [41]. Our assay included cefoxitin in the broth to facilitate detection of hetero-resistant MRSA in addition to homogeneous MRSA or MRCoNs.

Validity testing based on the kappa coefficient demonstrated that our screening broth had superior determination of MRSA and MSSA but limited determination of cefoxitin resistance for CoNS. In addition, this screening broth could provide rapid detection after at least 8 h of incubation. Therefore, application of this assay to screen MRSA is useful for clinical laboratories and hospital infection-control units to provide prompt information to facilitate the rapid control of MRSA dissemination in hospitals and a faster implementation of isolation precautions regarding MRSA carriage or infection from other patients, as well as reducing the cost of surveillance or investigation. A previous study demonstrated that rapid diagnostic testing procedures, such as PCR, could safely reduce the number of unnecessary isolation days in ICUs by 44%, at a cost of EUR 121.76–136.04 per isolation day in a low endemic setting for MRSA [42]. Another study revealed the benefits of chromogenic agar, with a reduction of 47% in isolation days at a cost saving of EUR 6.74 per isolation day. Chromogenic screening can be considered as a cost-saving procedure [43]. However, the unit cost of our screening broth was about EUR 0.9 (USD 1.02) per test (2 tubes), whereas the cost per test using chromogenic agar was EUR 2.08 [43]. Thus, the proposed in-house cefoxitin screening broth method could result in substantial reductions in the cost. Therefore, a clinical laboratory could apply our screening broth to screen a large number of suspected staphylococci with a substantial reduction in costs, making this useful for laboratories, especially in resource-limited countries.

Declarations

Author contribution statement

Anusak Kerdsin: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Peechanika Chopjitt: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

Rada Kansan: Performed the experiments.

Parichart Boueroy and Rujirat Hatrongjit: Contributed reagents, materials, analysis tools or data.

Natkamon Saenhom: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

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Data availability statement

Data included in article/supplementary material/referenced in article.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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