

Original Article

Methicillin-resistant *S. aureus* colonization in intensive care unit patients: Early identification and molecular typingGul Durmaz¹, Ozlem Sancı², Yasemin Oz¹, Kiyimet Guven³, Abdurrahman Kiremitci¹, Filiz Aksit¹¹ Department of Microbiology, Eskisehir Osmangazi University Faculty of Medicine, Eskisehir, Turkey² Department of Microbiology, Bozuyuk State Hospital, Bilecik, Turkey³ Department of Biology, Anadolu University Faculty of Science, Eskisehir, Turkey**Abstract**

Introduction: Early detection of methicillin-resistant *Staphylococcus aureus* (MRSA) in colonized patients is very important for infection control procedures to prevent MRSA spread. We aimed to monitor MRSA carriage in intensive care unit (ICU) patients and to evaluate the speed and efficiency of conventional culture, immunological, chromogenic, and molecular methods together with genotyping.

Methodology: Nasal and axillar swab specimens were obtained from patients in the ICUs of the general surgery and neurosurgery wards in a tertiary hospital once a week over four weeks between December 2009 and July 2010. Oxacillin and cefoxitin disk diffusion tests, oxacillin agar screening test, latex agglutination test, chromogenic agar, and real-time polymerase chain reaction (PCR) tests were used for isolation and identification of MRSA. MRSA isolates were typed using ribotyping and pulsed-field gel electrophoresis (PFGE) typing.

Results: MRSA colonization was detected in 48 of 306 patients by real-time PCR. The MRSA colonization rate was 6.2%, 15.5%, and 38.5% at admission and in the first and second weeks, respectively. Sensitivity, specificity, positive and negative predictive values for all phenotypic tests were 98%, 99.6%, 98%, and 99.6%, respectively. The shortest handle time was observed in PCR. A total of three banding patterns were obtained from MRSA isolates by ribotyping, and PFGE analyses revealed 17 different pulsotypes varying from 11 to 18 distinct bands, showing high genetic diversity among the samples.

Conclusion: Phenotypic MRSA screening tests in our study exhibited similar performances. The superiority of real-time PCR is its short turnaround time.

Key words: MRSA; colonization; PFGE; real-time PCR.

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Introduction

Since methicillin-resistant *Staphylococcus aureus* (MRSA) is often resistant to multiple classes of antibiotics, it is an important agent of nosocomial infections. Nosocomial infections caused by MRSA have been associated with increased mortality and high healthcare costs [1]. In hospitals, transmission occurs from a colonized or infected individual to others mainly via the hands of transiently colonized healthcare workers [2,3]. Therefore, early detection of MRSA-colonized patients or healthcare workers is very important for infection control procedures to prevent the spread of MRSA. Clinical microbiology laboratories must choose an appropriate method to rapidly detect MRSA colonization in patients. Conventional microbiological culture methods have a diagnostic delay of three to five days for growth of organisms, while commercial selective agar-based methods yield the results within 18–24 hours. However,

the molecular methods can yield results in 2–3 hours [4]. The aims of this study were to monitor MRSA carriage in patients admitted to intensive care units (ICUs) and to evaluate the speed and efficiency of conventional microbiological culture, immunological, chromogenic, and molecular methods for identification together with genotyping of strains by ribotyping and pulsed-field gel electrophoresis (PFGE).

Methodology*Study design and samples*

This study was conducted in ICUs of general surgery and neurosurgery in a tertiary hospital between December 2009 and July 2010. Following the approval of the local ethics committee, two nasal and two axillar swab specimens were obtained from patients in the first 48 hours of admission. Later samplings were carried out once a week during the patients' four weeks in the ICU. The patients with MRSA or methicillin-susceptible *S.*

aureus (MSSA) colonization were treated with topical mupirocin twice daily for five days, and isolation precautions were added to standard infection control measures for these patients.

S. aureus ATCC 29312 and *S. aureus* ATCC 33593 were included in each run for quality control. In addition, *S. aureus* NRRL B 767 was used in each step of molecular studies as a control strain.

Isolation and identification of S. aureus

One of the swab specimens was inoculated onto 5% sheep blood agar. After incubation at 35°C for 18–24 hours, *S. aureus* was identified on the basis of colony morphology, Gram stain, catalase test, and tube coagulase test. Other specimens were used in MRSA polymerase chain reaction (PCR).

Disk diffusion test of oxacillin and cefoxitin

Bacterial suspension of each isolate was adjusted to the turbidity of 0.5 McFarland standards, spread onto Mueller-Hinton agar (MHA), and then 1 µg oxacillin and 30 µg cefoxitin disks were placed onto plates [5]. After incubation at 35°C for 18–24 hours, inhibition zone diameters around the disks were measured.

Oxacillin agar screening test

One milliliter of bacterial suspension adjusted to 0.5 McFarland was inoculated on MHA plates containing 4% NaCl and 6 mg/L oxacillin [5]. The plates were incubated at 35°C for 24 hours. In the presence of any colony on MHA plates, isolates were considered as MRSA.

Latex agglutination test

The *mecA* product (PBP2a) was detected using a commercial latex agglutination kit (Slidex MRSA Detection, bioMerieux, Marcy l'Etoile, France). The extract of an MRSA-suspected colony prepared by heating and centrifugation was mixed with latex particles sensitized with monoclonal antibody directed against PBP2a. Control included a suspension of unsensitized latex particles.

Chromogenic agar

At the same time, swabs were plated directly onto the selective chromogenic agar (Chrom ID MRSA Agar, bioMerieux, Marcy l'Etoile, France). After 18–24 hours of incubation at 35°C, the presence of green-pigmented colonies was considered as positive and no growth or colonies with other colors were considered as negative for MRSA.

MRSA PCR

The BD GeneOhm MRSA real-time PCR system (BD Diagnostics, Sparks, USA) was used. Other nasal and axillar swabs were transferred to the sample reagent buffer tubes and processed for cell lysis, and then DNA extraction was performed according to the manufacturer's recommendations. Three microliters of the lysed specimen were added to the PCR tubes containing 25 µL of the master mix. PCR was performed with a SmartCycler instrument (Cepheid, Sunnyvale, USA). Positive and negative controls were included in each run.

Ribotyping

The automated ribotyping was performed using a robotized instrument (Riboprinter Microbial Characterization System, Qualicon, Du Pont, Wilmington, USA) and the Riboprinter System Data Analysis Program. The procedure used for processing each sample is described in detail by the manufacturer. Briefly, the isolates were grown overnight at 35°C, suspended in buffer, heated at 80°C for 10 minutes, and lysed. The total DNA was restricted with *EcoRI*, electrophoretically separated, and transferred to a membrane, followed by hybridization. Ribotypes were recorded and numbered by the system.

PFGE typing

Isolates were compared by the DNA macrorestriction patterns obtained from PFGE following *SmaI* digestion. PFGE typing of strains was performed according to the methods of Bannerman *et al.* [6] and Hennekinne *et al.* [7], with some modifications. Briefly, 5 mL of brain-heart infusion broth (Merck, Darmstadt, Germany) was inoculated with a single colony of the strain and incubated at 35°C for 18–24 hours. The cells were pelleted, washed twice with Tris-EDTA (TE)-NaCl buffer, resuspended in lysis buffer (6 mmol/L Tris-HCl pH 7.6, 1 mol/L NaCl, 100 mmol/L EDTA, 0.5% Brij 58, 0.2% sodium deoxycholate, 0.5% sodium lauroyl sarcosine). Afterwards, 500 µL of cell suspension was mixed with 15 µL of lysostaphin (1 mg/mL; Sigma, Steinheim, Germany) and 500 µL of warm (55°C) 2% low melting-point agarose (Sea Plaque GTG, Cambrex BioScience Rockland, Rockland, USA). The mixture was poured into the slots of a plastic mold and cooled for 20–30 minutes at 4°C. Chromosomal DNA was prepared by lysing cells at 37°C for 4 hours in lysis buffer. This was followed by incubation at 55°C overnight in ESP buffer (0.5 M EDTA [pH 9–9.5], 1% sarkosyl, and 1 mg/mL proteinase K). The agarose plugs were washed four

times, each time for 60 minutes in TE buffer (10 mM Tris, 1 mM EDTA [pH 8]).

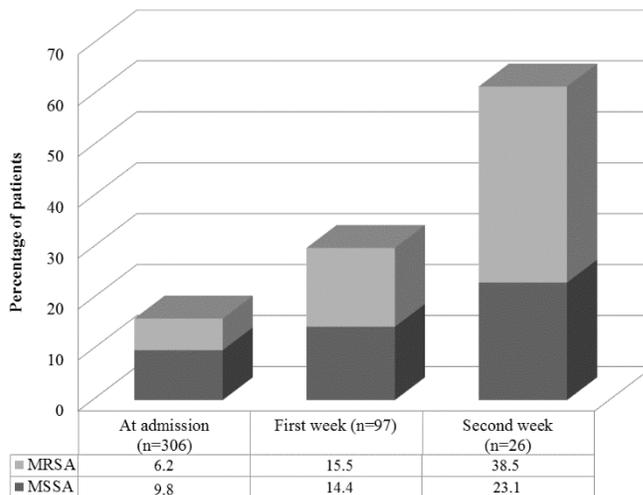
Approximately one-fourth of a plug was used for DNA digestion. Plugs were pre-incubated in restriction buffer for 30 minutes at room temperature, and then digested with 20 units of *SmaI* enzyme (New England Biolab, Ipswich, USA). DNA restriction fragments were separated in 1% agarose in 0.5X TBE using a Chef Mapper (Bio-Rad Laboratories, Hercules, USA) and pulse times were ramped from 5 seconds to 40 seconds for 19 hours. Gels were stained with gel red, visualized using an ultraviolet transilluminator, and photographed. Strains of *S. aureus* were placed in groups of identical or related strains by comparing the banding patterns produced, using a combination of photographic visual inspection and computer analysis (SPSS version 11.0, SPSS Inc., Chicago, USA) to create a similarity dendrogram.

A pulsotype (PT) was defined as a unique electrophoretic banding pattern. Strains with identical restriction profiles were assigned as the same type. The cluster cutoff was set at 78% similarity and all clusters were identified by Arabic numerals.

Results

A total of 306 patients (213 in ICU of general surgery and 93 in ICU of neurosurgery) were included in this study. The total numbers of *S. aureus*- and MRSA-colonized patients were 97 and 48, respectively. *S. aureus* colonization was detected in 49 patients at admission to ICUs, and the MRSA rate was 6.2% (n = 19); 5.2% (n = 11), and 8.6% (n = 8) in patients of general surgery and neurosurgery ICUs, respectively (Figure 1). During the follow-up period of the remaining non-colonized patients for four weeks, there were 97 patients at the end of the first week, 26 patients at the end of the second week, 6 patients at the end of the third week, and 2 patients at the end of the fourth week in ICUs. The numbers of *S. aureus*- and MRSA-colonized patients were 29 and 15 in the first week, and 16 and 10 in the second week, respectively. Although

Figure 1. The distribution and colonization rate with *S. aureus* isolates. The percentages in third and fourth weeks were not included in this figure because of the number of hospitalized patients was very low in this period.



half of the hospitalized patients were colonized with MRSA in the third and fourth weeks, the number of them was very low for justification of high prevalence of MRSA colonization. However, no new MRSA infections were detected in the general surgery and neurosurgery ICUs during the study.

When PCR was considered as the gold standard test for MRSA detection, all phenotypic tests exhibited similar performance results; discrepancies were observed for only three isolates (Table 1). Sensitivity, specificity, and positive and negative predictive values were found to be 98%, 99.6%, 98%, and 99.6%, respectively. The agreement between each phenotypic test and PCR was determined to be very high (about 98%) for both MRSA and MSSA (Table 2). However, the shortest handle time in laboratory was observed with PCR.

Automated ribotyping was applied to the 48 strains of *S. aureus* to assess the genetic similarity of the strains in the ICUs of general surgery and neurosurgery. Restriction of the total DNA with *EcoRI* yielded about 10–12 fragments of 2–13 kb in size

Table 1. Performance of phenotypic MRSA detection methods compared with PCR.

Isolates	OX DD	FOX DD	OX AT	CH agar	Latex agl
1	-	-	-	+	+
2	+	+	+	+	-
3	+	+	+	-	+
Sensitivity (%)	98	98	98	98	98
Specificity (%)	99.6	99.6	99.6	99.6	99.6
PPV (%)	98	98	98	98	98
NPV (%)	99.6	99.6	99.6	99.6	99.6

MRSA: methicillin-resistant *S. aureus*; PCR: polymerase chain reaction; OX DD: oxacillin disk diffusion; FOX DD: cefoxitin disk diffusion; OX AT: oxacillin agar screening test; CH agar: chromogenic agar; Latex agl: latex agglutination test; PPV: positive predictive value; NPV: negative predictive value.

Table 2. MRSA screening test results.

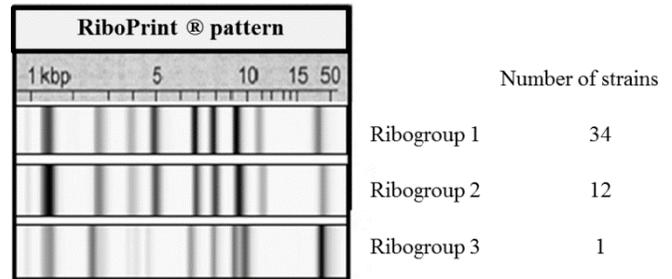
	PCR	Chromogenic agar	Latex agglutination	Oxacillin disk test	Cefoxitin disk test	Oxacillin agar screening
MSSA (n)	49	48	48	48	48	48
MRSA (n)	48	47	47	47	47	47
Test time (h)*	2	18–48	18–24	36–48	36–48	36–48

MRSA: methicillin-resistant *S. aureus*; MSSA: methicillin-susceptible *S. aureus*; PCR: polymerase chain reaction; *Elapsed time for identification after the sample reached to the laboratory

(Figure 2). A total of three banding patterns (ribogroups) was obtained among the isolates. The 47 MRSA isolates were confirmed as *S. aureus*, but one of the isolates was identified as *S. haemolyticus* by the ribotyping system. The latter isolate was excluded from typing studies. The differences in the ribopatterns were mostly located in bands between 3 and 11 kb in size (Figure 2). The number of ribotypes determined and the number of isolates representing them are shown in Figure 2. Ribogroup 3 contained only one isolate obtained from a male in the general surgery unit. However, ribogroups 1 and 2 contained isolates from both general surgery and neurosurgery units, indicating no relation between the sources of the isolates and ribotype patterns.

A total of 47 *S. aureus* strains were typed using PFGE. All of the strains tested were typeable. The genetic analyses revealed 17 different PTs varying from 11 to 18 distinct bands in the range from 679 kb to 48.5 kb, showing high genetic diversity among the strains (Figure 3). A dendrogram that included all patterns was constructed on the basis of the similarity levels (Figure 4). A cut-off point of 78% of similarity was considered to define two main clusters. The dominant cluster

Figure 2. Ribotypes from *S. aureus* strains isolated from ICUs of general surgery and neurosurgery (ribogroup numbers were given by an automatic ribotyping system).



(cluster 1) included PTs 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 13, 14, 15, 16, and 17, representing strains isolated from both general surgery and neurosurgery units. Cluster 2 included only PT 12, representing a strain obtained from the general surgery unit.

Discussion

In our study, while 6.2% of patients had MRSA colonization at ICU admission, 15.5% of remaining patients in the first week and 34.6% in the second week

Figure 3. Restriction endonuclease digestion of total genomic DNA of strains representing some of the groups (pulsed-field gel electrophoresis [PFGE] profiles) of *S. aureus* restriction digestion with *Sma*I and separation by PFGE. Lanes show some of the pulsotypes. M, 48.5–1,000-kB concatamer ladder. The PFGE conditions were 1% (w/v) agarose gel in 0.5X TBE, switching pulses of 5 to 40 seconds at a period of 19 hours 6 V/cm.

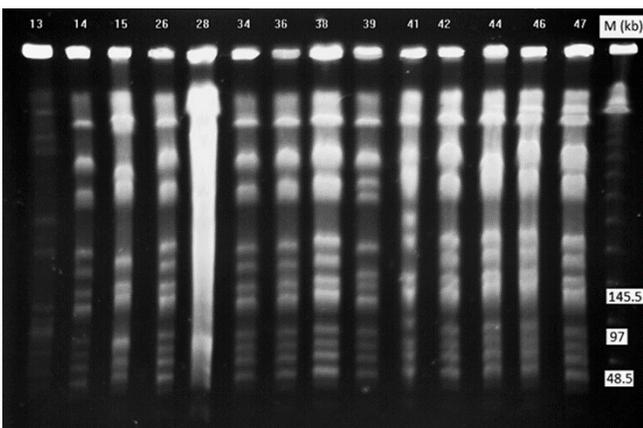
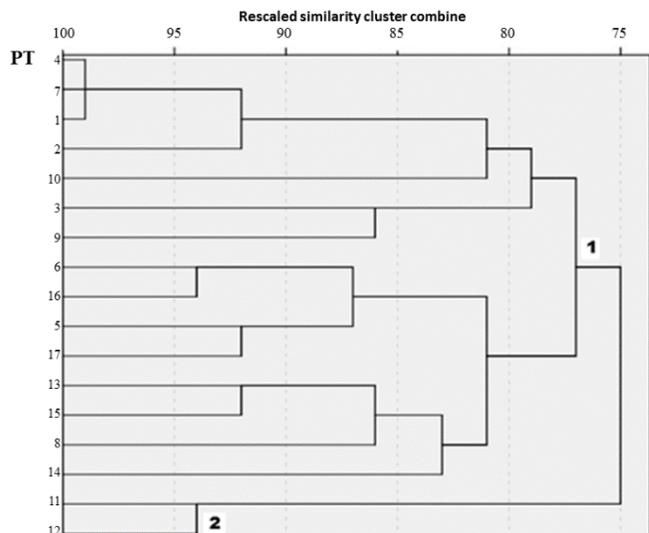


Figure 4. Clonal relationships of 17 *S. aureus* isolates established with *Sma*I PFGE analysis. Clusters (CL) are labeled with numbers. PT: pulsotype.



acquired MRSA in ICUs; since the number of isolates in the third and fourth weeks was very few, it was not included in this discussion. Previously reported prevalence of MRSA colonization at ICU admission ranged between 6.7% and 11.0% [8-11]. However, in countries where MRSA is endemic such as in India, MRSA colonization rate is higher [12,13]. Marshall *et al.* [11] and Mathanraj *et al.* [13] reported that the strongest risk factor for acquisition of MRSA was length of stay in the ICU. These studies confirmed our results that the prevalence of unknown MRSA colonization at admission to ICU is high in settings with endemic MRSA transmission. Prolonged hospitalization times in ICUs have been associated with increased colonization rates [8,11]. In highly endemic countries, routine surveillance for MRSA carriage in ICUs, with subsequent isolation of documented carriers, has been associated with reductions in MRSA infections in ICUs and across the hospital [14-17]. Therefore, rapid and accurate detection and eradication of colonization will be extremely beneficial in preventing the nosocomial spread of MRSA. Rapid diagnostic tests may allow early identification of previously unknown MRSA carriers at ICU admission.

There are several phenotypic tests for detection of MRSA from colonies isolated on routine media preferred by the Clinical and Laboratory Standards Institute (CLSI) [5]. The cefoxitin disk screen test for detection of oxacillin resistance in staphylococci is preferred by the CLSI. For *S. aureus* and *S. lugdunensis*, the cefoxitin disk test is comparable to the oxacillin disk test for prediction of *mecA*-mediated resistance to oxacillin; however, the cefoxitin disk test is easier to read, and therefore, it is preferred method of the CLSI [18]. The oxacillin agar screen method has also been recommended for confirmation of suspected strains by the CLSI. The studies evaluating these tests have showed that they have acceptable performance; in general, sensitivity (94.1%–100%) and specificity (87.4%–100%) ranges of the oxacillin disk diffusion method, cefoxitin disk diffusion method, and oxacillin agar screen test in these studies were found to be similar to those found in our study [19-21]. In Baddour *et al.*'s study [22], the oxacillin agar screen and PBP2a latex agglutination methods were reported to be more sensitive than the oxacillin and cefoxitin disk diffusion methods, and cefoxitin disk diffusion was found to be the most specific method. Also, Valesco *et al.* [21] reported that cefoxitin disk diffusion and PBP2a detection were the most sensitive methods and that the cefoxitin disk was the best predictor of methicillin resistance in *S. aureus* strains among oxacillin,

cefazolin, cefoxitin, cefotaxime, and imipenem disk tests, oxacillin Etest, oxacillin agar screening, and latex agglutination. However, these phenotypic methods require an additional 18–24 hours on standardized culture methods for results.

Commercial chromogenic media and latex agglutination tests detecting PBP2a are alternative and cost-effective approaches to screening clinical specimens for MRSA carriage. Other advantages of these tests are shorter time for detection of MRSA, enhanced recovery, minimal labor, and no additional antimicrobial susceptibility or screening tests [4,23]. In a study evaluating three different commercial chromogenic media, sensitivity and specificity values were found to be 83.8%–89% and 92.1%–98.6%, respectively [23]. A commercial latex agglutination test was reported to show good correlation with PCR as a gold standard test and was an alternative method that could be used in most laboratories [24]. However, Denys *et al.* [23] observed that experience is needed for the recognition of suspected colonies on chromogenic media, and they recommend follow-up confirmation of questionable colonies by a coagulase test or latex agglutination test and Gram stain to increase the specificity of MRSA interpretation [23]. The use of a latex agglutination test along with a chromogenic medium has been shown to rule out false-positive results and increase specificity up to 99% [25].

Although it is very expensive and not practical for most routine clinical laboratories, a molecular test based on the detection of the *mecA* gene is considered as the gold standard test for methicillin resistance. [19-21,24]. Compared with the chromogenic agar MRSA assay, PCR had sensitivity, specificity, and positive and negative predictive values of 100%, 98.6%, 95.8%, and 100%, respectively, and the mean PCR turnaround time was 14.5 hours [4]. In a systematic review, Polisená *et al.* [3] found small differences in the MRSA colonization, infection, and transmission rates between screening using PCR and chromogenic agar, but the turnaround time and number of isolation days were lower for screening by PCR versus chromogenic agar. Thus, not only high performance but also short turnaround time has important advantages. Although culture-based MRSA screening tests have proven to be cheaper and more sensitive methods, the long time required to report the results remains a major problem; isolation and identification results are usually available at least 24 to 72 hours after sample collection. This time delay could allow MRSA cross-transmission. Therefore, a molecular MRSA detection test permits early identification of MRSA carriage in critically ill

patients. It could help to improve MRSA control strategies, especially if it is linked to systematic on-admission screening and preemptive isolation of newly admitted patients [10]. During this study, new cases of MRSA infection did not occur, probably due to early detection and eradication of the MRSA-colonized patients in the general surgery and neurosurgery ICUs. Wassenberg *et al.* [26] compared two different real-time PCR assays with conventional culture and showed that the number of isolation days was reduced by 44.3% with PCR-based screening at the additional costs of 327.84€ and 252.14€ per patient screened, and costs per isolation day avoided were 136.04€ and 121.76€.

Controlling the spread of MRSA by screening patients, personnel, and the environment remains a high priority in infection control programs. Tracing the source and transmission routes of MRSA relies on typing methods as tools for the genetic characterization of isolates. PFGE has been accepted as the reference method for molecular strain typing of MRSA. PFGE is known to be highly discriminatory, and therefore it is frequently used for outbreak analysis [27]. However, this strategy is labor intensive, time consuming, and technical instability has an adverse effect on reproducibility. Therefore, automated ribotyping may be used for genetic characterization of a high number of clinical isolates. The discriminatory power of ribotyping as an automatable technique for differentiation of bacteria for systematic, epidemiological, ecological, and population studies has been well reviewed previously [28]. In this study, automated ribotyping was applied to the 47 strains of *S. aureus* to assess the genetic similarity of the strains isolated from different patients of the ICUs of general surgery and neurosurgery. All the strains tested were found to be typeable, and ribogroup 1 was dominant among the strains tested.

It is known that PFGE-nontypeable isolates are found in samples from humans; however, in this study, all of the strains tested were typeable. The genetic analyses of 47 isolates revealed 17 different PTs, indicating high genetic diversity among the samples. A dendrogram that included all patterns was constructed on the basis of the similarity levels defined two main clusters without any epidemiological indication among the strains.

Conclusions

MRSA colonization rate at admission to ICUs is high, and prolonged hospitalization times in ICUs have increased the colonization rate. Therefore, early, rapid, and accurate detection and eradication of colonized

patients at admission to ICU may help to prevent the nosocomial spread of MRSA. Although commercial and conventional MRSA screening tests evaluated in our study exhibited similar performance results, the superiority of real-time PCR is that it has a short turnaround time compared with the required time of about 48–72 hours by agar-based tests. In this study, ribotyping was shown to be a fast and reliable method for identification, but the discriminatory power of PFGE compared to ribotyping for molecular strain typing of *S. aureus* remains the highest.

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