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Antibiotics resistance and RAPD-PCR typing of multidrug resistant MRSA isolated from bovine mastitis cases in Thailand

Manakant Intrakamhaeng[a], **Tanaya Komutarin**[b,*]

[a] Department of Biology, Faculty of Science, Maha Sarakham University, Mahasarakham, Thailand
[b] Faculty of Medicine, Maha Sarakham University, Mahasarakham, Thailand

*Corresponding author, e-mail: tanaya_aor@yahoo.com

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ABSTRACT: Methicillin resistant *Staphylococcus aureus* (MRSA) strains are a global health concern in both human and veterinary medicine. In addition to β-lactam resistance, MRSA can carry resistance to other commonly used antibiotics. The present study sought to determine the antibiotic susceptibility profile and genetic relatedness of strains emerging in Thailand. A total of 229 isolates of *S. aureus* obtained from 598 mastitis cases were investigated for their susceptibility to several antibiotics. Among 229 isolates of *S. aureus*, 27 were found to be methicillin resistant and multidrug resistant. Multidrug resistant MRSA infection was detected in 4 provinces including Lopburi, Udon Thani, Khon Kaen and Saraburi. Multidrug resistant strains exhibited several antibiogram patterns (antibiotypes I to XII). The most frequent pattern was antibiotype VII, i.e., resistance to most commonly used antibiotics. Based on random amplification of polymorphic DNA (RAPD) technique and a combined amplicon-profile obtained with three primers, all isolates were grouped according to genetic similarity. Five different RAPD patterns (types I to V) were identified with 90% genetic similarity. The close genetic relatedness within RAPD type I was interesting because most RAPD type I isolates had antibiogram pattern III or VII. In addition, some isolates from different regions were identical in both antibiogram pattern and RAPD type. This revealed that some of the antibiotic-resistant isolates in our study were epidemic strains.

KEYWORDS: *Staphylococcus aureus*, methicillin resistance, genetic relatedness

INTRODUCTION

Bovine mastitis has been recognized as a costly disease with losses primarily affecting dairy farms. Among the various pathogens isolated as causative agents of bovine mastitis, *Staphylococcus aureus* is considered to be a frequent cause affecting both the yield and quality of milk[1–4]. In numerous locations worldwide, mastitis has been found to respond poorly to antibiotic treatment[5,6]. A possible reason for this is the spread of methicillin resistant *S. aureus* (MRSA) from humans to animals.

MRSA has traditionally been considered a nosocomial and community-acquired human pathogen. However, several recent studies have reported the incidence of MRSA in mastitis cases[5,7–9]. MRSA strains are considered to be resistant to many antibiotic agents[5,7,10]. MRSA isolates often possess a variety of other resistance genes[9], some conferring resistance to most available antimicrobials. MRSA isolates from animal origins are resistant to penicillin, and ampicillin, but less susceptible to erythromycin, gentamicin, or kanamycin[7]. The percentage of MRSA isolates resistant to several classes of antibiotics is significantly higher than methicillin susceptible isolates[5].

As many types of antibiotics are used in Thai dairy farms, increasing antibiotic resistance is a serious concern. Penicillin is the most widely used first-choice antibiotic for mastitis treatment, with various classes being used as alternatives including penicillinase-resistant penicillins, cephalosporins, macrolides, aminoglycosides, tetracyclines, lincosamides and folate pathway inhibitors. The present study aimed to investigate antibiotic resistance among *S. aureus* isolated from bovine mastitis cows in Thailand, with special attention paid to multidrug-resistant MRSA. MRSA strains isolated from different geographical regions were examined for genetic relatedness by random amplification of polymorphic DNA (RAPD-PCR).

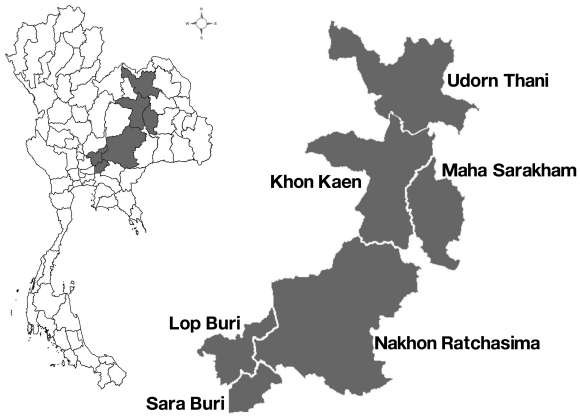

Fig. 1 Geographical location of the study area.

MATERIALS AND METHODS

Source of *S. aureus* isolates

A total of 598 farms from the six milk-producing provinces in Thailand (Fig. 1), Khon Kaen, Maha Sarakham, Udon Thani, Nakhon Ratchasima, Lopburi, and Saraburi, were surveyed to investigate MRSA as the cause of bovine mastitis cases. One mastitic milk sample ($\sim$10 ml) was aseptically collected from each farm. An aliquot of each sample was spread onto plates containing Baird-Parker's medium (Oxoid Ltd, Thailand) which is a selective medium for growth of staphylococci. Samples were incubated in aerobic conditions at 37 °C for 24 h, to determine growth using *S. aureus* ATCC 25923 as a positive control. All grey-black shiny convex 1–1.5 mm diameter colonies growth on Baird-Parker's medium were considered to be *S. aureus*. Identification of these putative *S. aureus* colonies was based on standard biological tests, including Gram staining, colony morphology, catalase test, and coagulase test using human plasma.

Antibiotic susceptibility testing

All identified *S. aureus* isolates were tested for phenotypic methicillin resistance by antibiotic disc diffusion susceptibility with 1 µg oxacillin (Oxoid Ltd, Thailand) and 30 µg cefoxitin (Oxoid Ltd, Thailand) discs. Methicillin sensitive *S. aureus* ATCC 25923 and methicillin resistant *S. aureus* DMST 20625 were used as negative and positive controls, respectively. Both standard reference strains were from the Department of Medical Sciences, Ministry of Public Health, Thailand. The inhibition zone around each disc was measured after 24 h incubation at 37 °C and was interpreted according to CLSI recommendations in M100-S19 document[11]. Each isolate was tested in duplicate and the mean inhibition zone diameters were determined. Isolates with oxacillin resistance (inhibition zone diameter $\leqslant$ 10 mm) and cefoxitin resistance (inhibition zone diameter $\leqslant$ 21 mm) were identified as MRSA.

The disc diffusion technique was also used to test the effectiveness of other commonly used antibiotics. The following antibiotic discs (representative of different classes) were used: penicillin 10 units and oxacillin 1 µg (penicillins), cloxacillin 5 µg (penicillinase-resistant penicillins), cefoxitin 30 µg and cefquinome 30 µg (cephalosporins), erythromycin 15 µg (macrolides), gentamicin 10 µg (aminoglycosides), tetracycline 30 µl (tetracyclines), lincomycin 2 µg (lincosamides) and sulphamethoxazole/trimethoprim 23.75/1.25 µl (inhibitors of the folate pathway). All isolates were tested in duplicate to verify their antibiogram characteristics. Resistance of *S. aureus* isolates to three or more classes of antibiotics was considered multidrug-resistance[12].

Genomic DNA extraction

The multidrug resistant MRSA isolates were grown overnight in brain-heart infusion broth (Oxoid Ltd, Thailand) at 37 °C. DNA extraction was carried out using a Genomic DNA Extraction Kit (RBC Bioscience, Taiwan). Purity and concentration of the DNA were measured using a NanoDrop1000 spectrophotometer (Thermo Fisher Scientific, USA).

RAPD-PCR analysis

In accordance with the recommendations of previous studies, the oligonucleotide primers used were labelled GEN1-50-01 (5′-GTGCAATGAG-3′)[13], S (5′-TCACGATGCA-3′)[14–16] and C (5′-AGGGAACGAG-3′)[15–17]. The reliability of the technique was confirmed by performing the amplification twice for each isolate. PCR (25 µl) was performed using Bluemix DNA Polymerase Mastermix (RBC Bioscience, Taiwan). The amplification cycles were as follows: an initial cycle of 94 °C for 5 min; 35 °C for 5 min; 72 °C for 5 min, followed by 30 cycles of 94 °C for 30 s; 35 °C for 1 min; 72 °C for 1 min, and a final extension step of 72 °C for 5 min. All DNA primers were synthesized by Biodesign (Thailand). PCR products were checked using 1.5% agarose gel with 0.125 mg/l ethidium bromide. A 100 bp ladder (Invitrogen, USA) was run together with PCR products as a molecular weight marker. PCR banding sizes generated with each primer were measured by GelixOne G230 software (Biostep, Denmark). Only clear, unambiguous and reproducible bands were recorded.

Table 1 Locations and prevalence of the multidrug resistant MRSA isolates.

Location	No. of samples	No. of *S. aureus* isolates	No. of multidrug resistant MRSA	Prevalence of multidrug resistant MRSA
Khon Kaen	197	92	6	6.5%
Maha Sarakham	58	16	0	0.0%
Udon Thani	149	63	11	17.5%
Nakhon Ratchasima	56	8	0	0.0%
Lopburi	72	26	9	34.6%
Saraburi	61	24	1	4.2%
Total	598	229	27	11.8%

Positions of scorable bands were transformed into a binary character matrix ("1" for the presence and "0" for the absence of a band at a particular position). A similarity matrix of distances based on the coefficient of simple matching was calculated. Based on simple matching coefficients, a dendrogram was constructed through the unweighed pair-group method with arithmetic mean using the sequential agglomerative hierarchical and nested cluster analysis (SAHN) program in NTSYS-PC 2.1 software.

RESULTS

S. aureus infections were found in 229 of 598 mastitis cases in all areas of study (Table 1). Regarding the agar diffusion test, a total of 27 isolates were shown to have a methicillin resistant phenotype (i.e., resistance to oxacillin and cefoxitin) and also exhibited multidrug resistance. Multidrug resistant MRSA infection was detected in 4 of 6 provinces, Khon Kaen, Udon Thani, Lopburi, and Saraburi (6.5%, 17.5%, 34.6%, and 4.2%, respectively).

Inhibition zone diameters obtained using the disc diffusion test are shown in Table 2. All of the multidrug resistant MRSA isolates were resistant to penicillin, oxacillin, cloxacillin, cefoxitin, and lincomycin. A high degree of susceptibility to sulphamethoxazole/trimethoprim, cefquinome, and gentamicin was found. Resistance to other antibiotics differed depending on the phenotypic characteristics of each isolate (Table 2).

Antibiogram results for the isolates were classified according to pattern of resistance (types I to XII). The most frequent pattern of resistance was type VII, which was found in 10 isolates from Lopburi, Udon Thani, and Khon Kaen. Antibiogram pattern VII represents resistance to eight of the drugs that are most commonly used for treatment (with the exception of cefquinome and sulphamethoxazole/trimethoprim).

RAPD fingerprints were generated to determine whether the 27 multidrug resistant MRSA isolates from the 6 locations were genetically clustered. RAPD reactions were performed in duplicate using the three random decamer oligonucleotide primers, and all amplification products were found to be reproducible. Based on a combined amplicon-profile obtained with the three primers, all isolates were found to have 86.5% genetic similarity. Isolates were grouped according to genetic similarity and intraspecies differentiation (Fig. 2).

Five different RAPD types (types I to V) were identified with 90% genetic similarity. Most isolates (23/27) were classified as RAPD type I. Within this RAPD type I group, antibiogram patterns III and VII occurred with highest frequency. The remaining RAPD types were presented by four isolates. One isolate from Khon Kaen exhibited RAPD type III, one isolate from Udon Thani was representative of type IV, and each of two isolates from Lopburi represented either type II or V (Table 2).

DISCUSSION

S. aureus was isolated from the milk of nearly 40% of mastitis cases, confirming it as an important aetiologic agent of this costly disease. Our finding that 27 of the isolates tested were multidrug resistant MRSA indicates that antibiotic resistance has emerged in dairy cattle in Thailand. This may explain recent reports of failure to treat bovine mastitis. The detection of multidrug resistant MRSA over different locations indicates this is a widespread problem. Using various antibiotics can create selection pressure [18, 19], ultimately resulting in the development of antibiotic resistance. We found the prevalence of multidrug resistant MRSA higher among isolates obtained in Lopburi, Udon Thani, and Khon Kaen. An important question to be answered was whether the isolates from each location displayed close linkage. Each antibiogram pattern obtained demonstrated similar resistance to commonly used antibiotics, such as penicillin, cloxacillin, and lincomycin. In the geographical areas investigated in this study, the penicillin class of antibiotics was the first choice with other types of antibiotic selected subsequently. While some antibiotics were found to have diminished efficacy, cefquinome, and sulphamethoxazole/trimethoprim remained very effective against bacterial strains isolated in the area of study. This may be because sulphamethoxazole/trimethoprim and cefquinome are less widely used. Our data suggests that these drugs should be used to treat cases of bovine mastitis that are unresponsive to other antibiotics. Increasing rates of resistance to sulphonamides and cephalosporins are being reported though [20], so continued antibiotic susceptibility monitoring is recommended.

Table 2 Antibiogram and RAPD types of the 27 multidrug resistant MRSA isolates.

Isolate	Location		Antibiogram	Inhibition zone of each antibiotic disc (mm)										RAPD type			
	Province	District	pattern	P10	OX1	OB5	FOX30	CEQ30	E15	CN10	TE30	MY2	SXT25	C	S	GEN1	All[†]
MRSA01	Khon Kaen	Phangthui	III	24[a]	⩽ 6[a]	⩽ 6[a]	⩽ 6[a]	25[c]	⩽ 6[a]	13[c]	9[a]	⩽ 6[a]	13.5[b]	I	I	I	I
MRSA02	Khon Kaen	Phangthui	VII	25.5[a]	⩽ 6[a]	⩽ 6[a]	9[a]	26.5[c]	⩽ 6[a]	12[a]	⩽ 6	⩽ 6[a]	24.5[c]	VI	II	I	III
MRSA03	Khon Kaen	Phangthui	VII	24.5[a]	⩽ 6[a]	⩽ 6[a]	⩽ 6[a]	25.5[c]	⩽ 6[a]	11[a]	8	⩽ 6[a]	26.5[c]	II	I	I	I
MRSA04	Khon Kaen	Phangthui	VII	26[a]	⩽ 6[a]	⩽ 6[a]	⩽ 6[a]	25[c]	⩽ 6[a]	12[a]	9[a]	⩽ 6[a]	25[c]	I	I	I	I
MRSA05	Khon Kaen	Phangthui	VII	24[a]	⩽ 6[a]	⩽ 6[a]	⩽ 6[a]	23.5[c]	⩽ 6[a]	11[a]	9[a]	⩽ 6[a]	26[c]	II	I	I	I
MRSA06	Khon Kaen	Kranuan	IX	20[a]	⩽ 6[a]	⩽ 6[a]	⩽ 6[a]	18[a]	24[c]	11[a]	12[a]	⩽ 6[a]	30[c]	I	I	I	I
MRSA07	Udon Thani	Srithaat	I	⩽ 6[a]	⩽ 6[a]	⩽ 6[a]	21[a]	27[c]	⩽ 6[a]	16[c]	20[c]	8[a]	21.5[c]	IV	I	I	I
MRSA08	Udon Thani	Srithaat	II	12[a]	⩽ 6[a]	7[a]	7[a]	17.5[a]	25.5[c]	21[c]	28[c]	7[a]	26[c]	I	I	IV	I
MRSA09	Udon Thani	Srithaat	VI	6[a]	⩽ 6[a]	⩽ 6[a]	12[a]	23.5[c]	⩽ 6[a]	11[a]	24[c]	⩽ 6[a]	23.5[c]	VII	I	I	I
MRSA10	Udon Thani	Thungfon	VII	21.5[a]	⩽ 6[a]	⩽ 6[a]	⩽ 6[a]	20.5[b]	⩽ 6[a]	11[a]	7[a]	⩽ 6[a]	28[c]	II	I	I	I
MRSA11	Udon Thani	Nongwuaso	VII	25[a]	⩽ 6[a]	⩽ 6[a]	⩽ 6[a]	20.5[b]	⩽ 6[a]	11[a]	10[a]	⩽ 6[a]	27.5[c]	I	I	II	I
MRSA12	Udon Thani	Srithaat	VII	21[a]	⩽ 6[a]	⩽ 6[a]	⩽ 6[a]	19[b]	⩽ 6[a]	12[a]	9[a]	⩽ 6[a]	28[c]	I	I	I	I
MRSA13	Udon Thani	Nongwuaso	VII	23.5[a]	⩽ 6[a]	⩽ 6[a]	⩽ 6[a]	24[c]	⩽ 6[a]	11[a]	9[a]	⩽ 6[a]	27[c]	VIII	I	I	I
MRSA14	Udon Thani	Nongwuaso	VII	25[a]	⩽ 6[a]	⩽ 6[a]	⩽ 6[a]	25.5[c]	⩽ 6[a]	11[a]	9[a]	⩽ 6[a]	28[c]	VIII	I	I	I
MRSA15	Udon Thani	Srithaat	VIII	6[a]	⩽ 6[a]	⩽ 6[a]	21[a]	14.5[a]	⩽ 6[a]	11[a]	21[c]	⩽ 6[a]	16.5[c]	I	I	I	I
MRSA16	Udon Thani	Srithaat	VIII	8[a]	7.5[a]	⩽ 6[a]	19[a]	10.5[a]	13[a]	12[a]	27[c]	9[a]	19.5[c]	III	I	VII	IV
MRSA17	Udon Thani	Srithaat	XII	⩽ 6[a]	⩽ 6[a]	⩽ 6[a]	⩽ 6[a]	12[a]	⩽ 6[a]	10[a]	10[a]	⩽ 6[a]	20.5[c]	I	I	I	I
MRSA18	Lopburi	Suanmaduea	III	21[a]	⩽ 6[a]	⩽ 6[a]	⩽ 6[a]	21.5[b]	⩽ 6[a]	13[c]	12[a]	⩽ 6[a]	22.5[c]	I	I	I	I
MRSA19	Lopburi	Suanmaduea	III	20.5[a]	⩽ 6[a]	⩽ 6[a]	⩽ 6[a]	20[b]	⩽ 6[a]	13[c]	7[a]	⩽ 6[a]	25.5[c]	I	I	I	I
MRSA20	Lopburi	Nong ri	III	25[a]	⩽ 6[a]	⩽ 6[a]	⩽ 6[a]	26.5[c]	⩽ 6[a]	15[c]	10[a]	⩽ 6[a]	31.5[c]	I	I	I	I
MRSA21	Lopburi	Nong muang	III	22[a]	⩽ 6[a]	7[a]	⩽ 6[a]	22.5[c]	⩽ 6[a]	15[c]	⩽ 6[a]	⩽ 6[a]	27.5[c]	V	I	I	I
MRSA22	Lopburi	Nong ri	III	26[a]	⩽ 6[a]	⩽ 6[a]	9[a]	23[c]	13[a]	17[c]	9[a]	⩽ 6[a]	31[c]	I	I	V	II
MRSA23	Lopburi	Suanmaduea	IV	⩽ 6[a]	⩽ 6[a]	⩽ 6[a]	19[a]	⩽ 6[a]	⩽ 6[a]	17[c]	22[c]	⩽ 6[a]	21[c]	I	I	VI	V
MRSA24	Lopburi	Suanmaduea	V	15[a]	⩽ 6[a]	⩽ 6[a]	9[a]	22[c]	⩽ 6[a]	16[c]	25[c]	⩽ 6[a]	⩽ 6[a]	I	I	III	I
MRSA25	Lopburi	Suanmaduea	VII	24.5[a]	⩽ 6[a]	7[a]	⩽ 6[a]	25.5[c]	⩽ 6[a]	12[a]	⩽ 6[a]	⩽ 6[a]	29.5[c]	IV	I	I	I
MRSA26	Lopburi	Suanmaduea	X	24[a]	⩽ 6[a]	⩽ 6[a]	⩽ 6[a]	22.5[c]	⩽ 6[a]	13[c]	13[a]	⩽ 6[a]	⩽ 6[a]	I	I	I	I
MRSA27	Saraburi	Sapkradan	XI	22[a]	⩽ 6[a]	⩽ 6[a]	⩽ 6[a]	22[c]	⩽ 6[a]	⩽ 6[a]	⩽ 6[a]	⩽ 6[a]	⩽ 6[a]	I	I	I	I

P10, Penicillin 10 IU; OX1, Oxacillin 1 µg; OB5, Cloxacillin 5 µg; FOX30, Cefoxitin 30 µg; CN10, CEQ30, Cefquinome 30 µg; E15, Erythromycin 15 µg; CN10, Gentamicin 10 µg; TE30, Tetracycline 30 µg; MY2, Lincomycin 2 µg; SXT25, Sulphamethoxazole/trimethoprim 23.75/1.25 µg.

[†] All primers combined.

[a] resistant to the antibiotic; [b] intermediate resistant to the antibiotic; [c] susceptible to the antibiotic.

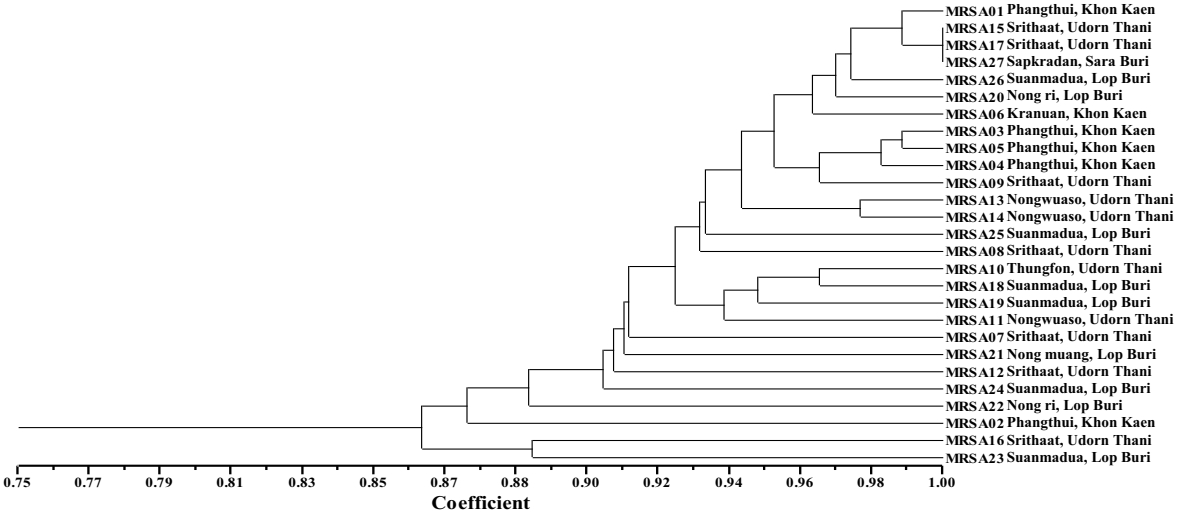

Fig. 2 Dendrogram of the 27 multidrug resistant MRSA from four regions of Thailand, based on simple matching coefficients using the sequential agglomerative hierarchical and nested cluster analysis program in NTSYS-PC 2.1 software.

Antibiotic resistance data obtained for MRSA isolates in the present study are consistent with those obtained in previous reports. In a 2003 study, Lee found that the all MRSA isolates from dairy cattle were resistant to penicillin, less susceptible to erythromycin and gentamicin, and very susceptible to sulphamethoxazole/trimethoprim[7]. In another study, Juhász-Kaszanyitzky et al[8] reported that all MRSA isolated from subclinical mastitis cases were resistant to tetracycline and erythromycin, and susceptible to gentamicin and sulphamethoxazole/trimethoprim. Minor differences in antibiotic susceptibility may be due to different antibiotic usage in different geographic locations.

The use of multiple primer sets in RAPD analysis can be used as a rapid method for preliminary biotyping of multidrug resistant MRSA strains. In a previous study using primer GEN1-50-01[13], the discriminatory power of RAPD and its ability to characterize strains was demonstrated. The primers S and C have also been used in several previous studies[14–17], and demonstrated to powerfully discriminate epidemiologically related isolates. Based on antibiotic susceptibility and a combined amplicon-profile obtained using the three primers, our study found considerable diversity among the multidrug resistant MRSA isolates obtained in the different geographical locations. For Lopburi, Udon Thani, and Khon Kaen provinces, various antibiogram patterns were obtained but patterns VII and III occurred with highest frequency. The close genetic relatedness within RAPD type I was interesting because most RAPD type I isolates exhibited antibiogram patterns III and VII. In some cases, isolates from different areas were identical in both antibiogram pattern and RAPD type. This result indicates that some of the antibiotic-resistant isolates were epidemic strains. Work to further characterize the genomic relationships and multiple antibiotic resistance of isolated strains is currently underway in our laboratory. This will be achieved using various recently developed methods of analysis including the Xpert MRSA assay[21] and PFGE analysis[22,23].

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