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Research Article
Antibiotic Resistance and Methicillin Resistant *Staphylococcus aureus* Isolated from Bovine, Crossbred Etawa Goat and Human

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Abstract

Antimicrobial resistance patterns and gene encoding for methicillin/oxacillin resistance (*mec*A) were determined in 73 *Staphylococcus aureus*. The isolates of *S. aureus* originated from bovine (39 isolates) from Peranakan Etawa (PE) or crossbred Etawa goats (24 isolates) and from patients of Sarjito hospital (10 isolates) in Indonesia. The identification of *S. aureus* was based on cultural and biochemical tests and an amplification of a specific section of the 23S rRNA gene and thermonuclease (*nuc*) genes. *Staphylococcus aureus* originating from human and bovine were more resistant than those of goat origin. Seventeen *S. aureus* (23.29%) were resistant to single antibiotic and 15 isolates (20.55%) showed resistance to two antimicrobial agents. Multi resistances were found in 26 (35.62%) of *S. aureus* isolates. Resistance to ampicillin was the most common finding (80, 76.92 and 41.67%), followed by gentamicin (30, 51.28 and 25%), oxacillin (50, 38.46 and 58.33%), tetracyclin (40, 28.21 and 16.67%) and erythromycin (40, 23.08 and 20.83%) for human, bovine and goat, respectively. By PCR amplification could be observed in 5 (12.82%) methicillin/oxacillin resistant (*mec*A) genes for bovine isolates, 1 (4.17%) goat isolates and 9 (90%) human isolates. These isolates were identified as methicillin resistant *S. aureus* (MRSA). Most of MRSA were resistant to oxacillin (60%), ampicillin (66.7%), tetracyclin (40%), erythromycin (33.3%) and gentamicin (20%) The resistancy of *S. aureus* to methicillin/oxacillin and the other antibiotics in the present study might help to understand the distribution of methicillin resistant *S. aureus* (MRSA) among human bovine and goat isolates and might help to control *S. aureus* infections.

Key words: Antibiotic, bovine, crossbred Etawa goat, resistance, *Staphylococcus aureus*, human

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Staphylococcus aureus is the causative agent of clinical or subclinical mastitis in dairy ruminants. The intra mammary administration of antibiotics used on farms has increased, as it was proved to be effective for treating subclinical mastitis in dry small ruminants (Olechnowicz and Jaskowski, 2014). Antibiotics are used to treat diseases of cattle, sheep, goat, water buffalo and other animals (De Briyne *et al*., 2014). The indiscriminate use of antibiotics has led to the development of multiple antibiotic resistances thereby rendering the antibiotic treatment ineffective (Alian *et al*., 2012). The resistant bacteria present in environments are in contact with human beings and animals. Antimicrobial resistance is a major public health concern in many countries due to the persistent circulation of resistant strains of bacteria in the environment and the possible contamination of water and food (Gelbrand *et al*., 2015). *Staphylococcus aureus* has been reported to frequently show multiple antimicrobial resistance patterns (Nair *et al*., 2013).

Raw milk of dairy ruminants can be a potential source of antibiotic-resistant pathogens of animal, human and environmental origin. The microorganisms that contaminate raw milk may originate from the farm environment and include the etiological agents responsible for clinical and subclinical mastitis. The main reservoir of *S. aureus* is in the infected quarter of mammae, spreading in to the other animals during milking process (Akineden *et al*., 2001; Schroeder, 2012). In dairy cattle, *S. aureus* as well as coagulase negative staphylococci are usually considered to have the greater pathogenicity of subclinical mastitis agents (Hosseinzadeh and Saei, 2014). The subclinical mastitis in dairy cow and goat farms causing in significant economic losses due to reduction in milk production and poor milk quality (Sharma *et al*., 2012).

Staphylococcus aureus is still an important cause of foodborne intoxications worldwide (Cuny *et al*., 2015; Salasia *et al*., 2011). The ability of *S. aureus* to grow and produce staphylococcal enterotoxins under a wide range of conditions is evident from the variety of foods implicated in staphylococcal food poisoning (Johler *et al*., 2015). Several hospitals reported the increase of methicillin resistant *Staphylococcus aureus* (MRSA) frequency, because this strain was resistant to several antibiotics (Kaur and Chate, 2015). Methicillin is no longer manufactured because the more stable and similar penicillins such as oxacillin, flucloxacillin and dicloxacillin are used medically. Even though the methicillin is no longer the agent of choice for treatment, the acronym MRSA continues to be used (Batabyal *et al*., 2012).

Determination of levels of *S. aureus* and an evaluation of the antibiotic-resistant phenotypes of the isolates could serve as a tool for determining the hygiene standards implemented during milking. Data on antibiotic resistance could also be used to characterize these opportunistic pathogens, which may further limit the risks associated with the consumption of contaminated milk and its products (Alian *et al*., 2012). Few data on MRSA colonization rates in non-clinically affected animals are available. Although identification of colonized or infected animals is important in the prevention of the spread of MRSA, the routine screening of all animals is not yet practical, so there remains the possibility that a small percentage of colonized animals will remain undetected upon first admission to a veterinary clinic or hospital (Umaru *et al*., 2011).

The trend of resistance patterns to antibiotic use over time showed a long-term effect of over 3-7 years (Silva *et al*., 2013; Abo-Shama, 2014; Cuny *et al*., 2015). The prevalence of antibiotic resistance usually varies between isolates from different samples and even between herds in the same farm (Silva *et al*., 2013). Since antibiotics play an important role in the control of mastitis, a surveillance system for antibiotic resistance that will ensure optimal result and minimize the risk of development and spread of resistance in dairy farms is very crucial. The aim of this study was to determine the antimicrobial resistance and methicillin resistant *S. aureus* isolates from human, milk bovine and crossbred Etawa goat samples of selected dairy farms in Indonesia.

MATERIALS AND METHODS

Bacterial isolates: In this research we used *S. aureus* isolated from various places in dairy farms in Central Java and Riau and from human patients in Sardjito Hospital Yogyakarta Central Java, Indonesia. The isolates of *S. aureus* originated from bovine (39 isolates) from Peranakan Etawa (PE) or crossbred Etawa goats (24 isolates) and from patients of Sardjito hospital (10 isolates). *Staphylococcus aureus* strain BY7 isolated from bovine desribed in previous research (Salasia *et al*., 2011) was used as a control strain and non-staphylococcal isolate used as a negative control. Peranakan Etawa (PE) goat is descended originally from crossings between the Kacang goat (indigenous breed of goat in Indonesia) with Etawa (Jamnapari India) goat. All samples were identified based on Gram staining, fermentation on Mannitol Salt Agar (MSA), catalase and coagulase tests. The catalase test was done by placing a drop of hydrogen peroxide on a microscope slide. A small amount of bacterial isolate was added to hydrogen peroxide, bubbles of oxygen were observed for

catalase-positive. The coagulase test was performed by cultivation of the bacteria in the tube coagulase test (Bactident-Coagulase; Merck, Germany). The presence of coagulation was observed at 6 and 24 h.

Molecular identification: Molecular identification was done according to the amplification of 23S rRNA gene and *nuc* gene with the PCR technique with the program and primer design as previously decribed (Straub *et al*., 1999; Brakstad *et al*., 1992). The reaction mixture (25 µL) contained 1 µL primer 1 (20 pmol), 1 µL primer 2 (20 pmol; Invitrogen, USA), 12.5 µL PCR mix containing Taq DNA polymerase, $MgCl_2$ and dNTPs (KAPA Biosystem, USA), 2 µL of DNA template and 8.5 µL distilled water. The DNA of the isolates was prepared with the QIAamp DNA mini kit (Qiagen, Germany) as described by the manufacturer. The amplification of the genes was carried out with a thermal cycler (Mastercycler, Eppendorf, Germany). The PCR products were separated by gel electrophoresis in a 1.5% (w/v) agarose gel (Roth, Germany) in 0.5×TBE buffer (containing a mixture of tris base, boric acid and EDTA. A 100 bp DNA ladder (Geneaid, Taiwan) was used as a size marker. The resulting bands were visualized using FloroSafe (1st Base, Singapore) staining under UV transillumination.

Antimicrobial susceptibility test: Antimicrobial susceptibility testing was performed by the Kirby-Bauer disc diffusion method using Müller-Hinton agar (Oxoid, England UK) supplemented with 5% defibrinated sheep blood, according to the Clinical Laboratory Standards Institute (CLSI). The following antimicrobial impregnated disks (Oxoid, England UK) were used: oxacillin (5 µg), tetracycline (30 µg), gentamicin (10 µg), ampicillin (10 µg) and erythromycin (15 µg). After incubation at 37°C for 48 h, the susceptibility of the *S. aureus* isolates to each antimicrobial agent was measured and the results were interpreted as shown in Table 1 in accordance with interpretive criteria provided by CLSI (Bedidi-Madani *et al*., 1992).

DNA isolation and purification: A QIAmp DNA mini kit (Qiagen, Germany) was used to purify the DNA from *S. aureus* according to the manufacturer's protocol. The bacterial strains were cultivated on blood agar base (Oxoid, Germany) containing 5% defibrinated sheep blood for 24 h at 37°C. A total of 5~10 *S. aureus* colonies were suspended with 180 µL TE buffer (10 mM Tris-HCl and 1 mM EDTA [pH 8]) containing 5 µL lysostaphin (1.8 U μL^{-1}; Sigma, USA) in 2 mL microfuge tubes. The suspension was incubated for 1 h at 37°C and 25 µL of proteinase K (14.8 mg mL^{-1}; Sigma, USA) and 200 µL of AL buffer (containing reagents AL1 and AL2; Qiagen, Germany) were then added. The suspensions were incubated for 30 min at 56°C and then for 10 min at 95°C before being spun at 6,000×g for a few seconds. A total of 420 µL ethanol was added to each sample and placed in a spin QIAmp column. After centrifugation at 6,000×g for 1 min, the spin columns were placed in a clean collection tube and the sample was washed twice with 500 µL of AW buffer (Qiagen, Germany). After the second wash and a centrifugation at 6,000×g for 3 min, the QIAamp spin columns were placed in a clean 2 mL microfuge tube and the DNA was eluted twice with 200 and 100 µL of AE buffer (Qiagen, Germany). DNA was stored at-20°C.

Detection of *mec*A gene: The *mec*A gene coding for methicillin resistance was detected by PCR as previously described (Al-Ruaily and Khalil, 2011). The primers used for the detection of the *mec*A gene were:

- AAAATCGATGGTAAAGGTTGGC (Forward)
- AGTTCTGCAGTACCGGATTTGC (Reverse)

RESULTS

According to cultural and biochemical properties, all 73 isolates used in the present study could be identified as *S. aureus*. All cultures investigated were Gram positive, positive for catalase, fermented mannitol in MSA and positive for coagulase. The identification of the isolates could be confirmed by PCR amplification of species specific of the gene encoding 23S rRNA and by amplification of thermonuclease gene *nuc*. The amplicons of these genes showed a uniform size of approximately 1250 and 279 bp, respectively. The amplicon of *nuc* genes of selected *S. aureus* is shown in Fig. 1.

Table 1: Interpretation of the inhibition zone diameter (mm) data

Antibiotics	Disc potency (µg)	Resistant (mm)	Sensitive (mm)
Oxacillin	5	<14	>15
Tetracycline	30	<19	>20
Gentamicin	10	<19	>20
Ampicillin	10	<25	>26
Erythromycin	15	<16	>20

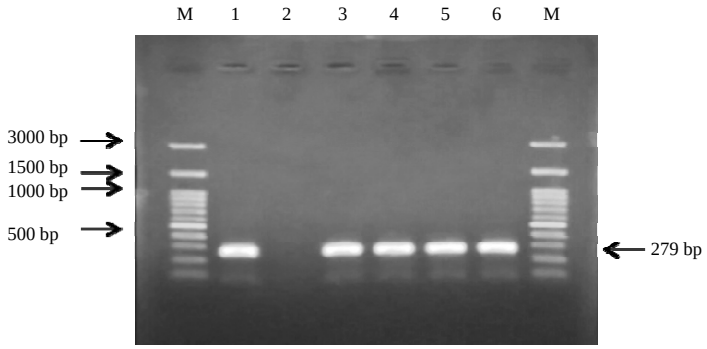

Fig. 1: Typical amplicons of the nuclease (*nuc*) gene of selected *S. aureus* strains, Lane M: 100 bp molecular-size DNA ladder, Lane 1: *Staphylococcus aureus* control strain, Lane 2: A negative isolate, Lane 3-6: Amplicon of 279 bp

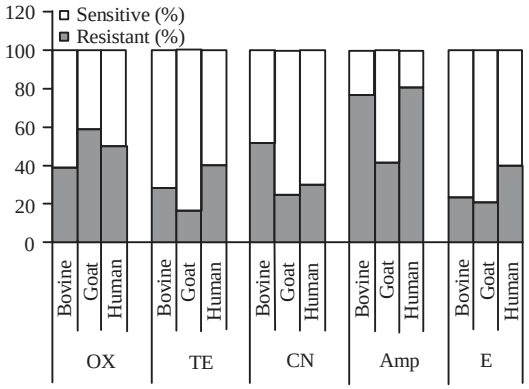

Fig. 2: Comparison of sensitivity to antibiotics in *S. aureus* isolated from bovine, goat and human, OX: Oxacillin, TE: Tetracycline, CN: Gentamicin, Amp: Ampicillin, E: Erythromycin

Table 2: Antibiotic susceptibility profiles of *Staphylococcus aureus* isolated from bovine, goat and human originating from Central Java and Riau, Indonesia

	Resistant (%)			Sensitive (%)		
Antibiotics	Bovine (n = 39)	Goat (n = 24)	Human (n = 10)	Bovine (n = 39)	Goat (n = 24)	Human (n = 10)
Oxacillin (5 µg)	15 (38.46)	7 (58.33)	5 (50)	24 (61.54)	5 (41.67)	5 (50)
Tetracycline (30 µg)	11 (28.21)	4 (16.67)	4 (40)	28 (71.79)	20 (83.33)	6 (60)
Gentamicin (10 µg)	20 (51.28)	6 (25.00)	3 (30)	19 (48.72)	18 (75.00)	7 (70)
Ampicillin (10 µg)	30 (76.92)	10 (41.67)	8 (80)	9 (23.08)	14 (58.33)	2 (20)
Erythromycin (15 µg)	9 (23.08)	5 (20.83)	4 (40)	30 (76.92)	19 (79.19)	6 (60)

The antibiotic susceptibility tests for *S. aureus* isolates were subjected to five antimicrobial agents from different antibiotic classes were used. Antibiotics of veterinary and human health relevance were also considered. The percentage of resistance of *S. aureus* to several antibiotics is summarized in Table 2. Results of antimicrobial susceptibility are shown in Fig. 2. In total *S. aureus* originating from human and bovine were more resistant than those of goat origin. Seventeen *S. aureus* (23.29%) were resistant to single antibiotic and 15 isolates (20.55%) showed resistance to 2 antimicrobial agents. Multiresistance was found in 26 (35.62%) of *S. aureus* isolates. Resistance to ampicillin was the most common finding (80, 76.92 and 41.67%), followed by gentamicin (51.28, 30 and 25%), oxacillin (50, 38.46 and 58.33%), tetracyclin (40, 28.21 and 16.67%) and erythromycin (40, 23.08 and 20.83%) for human, bovine and goat, respectively.

By PCR amplification could be observed that 15 isolates expressed methicillin resistant (*mec*A) genes for 5 (12.82%) bovine isolates, 1 (4.17%) goat isolate and 9 (90%) human isolates. These isolates were identified as methicillin resistant *S. aureus* (MRSA). Most of MRSA were resistant to oxacillin

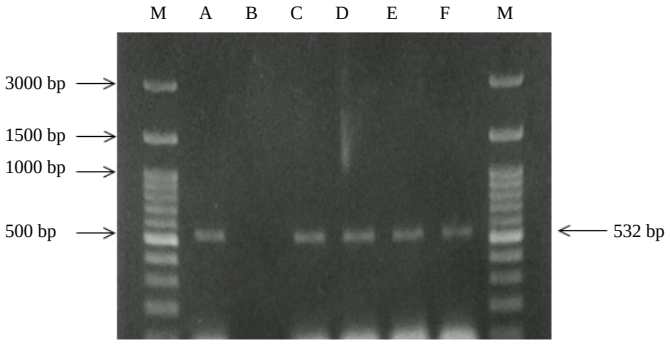

Fig. 3: A positive control (MRSA) showed a PCR product of *S. aureus* specific *mec*A gene 532 bp (Lane A). A negative control (Lane B, no band), B: No band, Lane C-F: PCR product of *mec*A gene 532 bp for the selected isolates was applied which showed clear bands confirmed that, all the isolates were MRSA, Lane M: 100 bp molecular weight marker

Table 3: Susceptibility of MRSA to different antibiotics

Code	Source	*mec*A gene	Oxacillin	Tetracycline	Gentamicin	Ampicillin	Erythromycin
SU 2	Bovine	+	R	R	R	R	R
SU 10	Bovine	+	S	S	S	S	S
SU 16	Bovine	+	R	S	S	S	S
SU 24	Bovine	+	R	S	S	R	S
SU 25	Bovine	+	R	R	S	R	S
SK01	Goat	+	S	S	S	S	S
169	Human	+	R	S	S	R	S
179	Human	+	S	S	S	R	S
198	Human	+	S	R	S	R	S
199	Human	+	R	R	R	S	R
255	Human	+	S	S	S	R	S
262	Human	+	R	S	S	R	R
870	Human	+	S	R	S	S	R
979	Human	+	R	S	S	R	S
1091	Human	+	R	R	R	R	R
			R = 9 (60%)	R = 6 (40%)	R = 3 (20%)	R = 10 (66.7%)	R = 5 (33.3%)

+: Positive *mec*A gene (532 bp), R: Resistant, S: Sensitive

(60%), ampicillin (66.7%), tetracyclin (40%), erythromycin (33.3%) and gentamicin (20%) (Table 3). In Fig. 3, a positive control (MRSA) showed a PCR product of *S. aureus* specific *mec*A gene 532 bp (lane A). A negative control, PCR product was applied on lane B showed no band on the figure. The PCR product of *mec*A gene 532 bp for the selected isolates was applied on lane C-F which showed clear bands confirmed that all the isolates were MRSA.

DISCUSSION

In this study all 73 isolates originated from bovine (39 isolates) from Peranakan Etawa (PE) or crossbred Etawa goats (24 isolates) and from human patients of Sardjito Hospital (10 isolates) could be identified as *S. aureus*. The identification of the isolates based on the cultural and biochemical and confirmed by PCR amplification of species specific parts of the gene encoding 23S rRNA and by amplification of thermonuclease gene *nuc* with the size of approximately 1250 and 279 bp, respectively. Amplification of gene species specific sequences by PCR such as 23S rRNA and *nuc* with a size of approximately 1250 bp and 279 bp is used for identification of *S. aureus* (Akineden *et al*., 2001; Nashev *et al*., 2004; Salasia *et al*., 2011; Szweda *et al*., 2014).

The prevalence of *S. aureus* has been reported to vary with the size and geographic region in the world. The improper hygiene and poor farm management practices contributed to the presence of *S. aureus* in the milk (Abo-Shama, 2014). Lee *et al*. (2015) reported that MRSA infection rate decreased from 3.58 (baseline) to 0.42‰ (intervention period), re-surged to 2.21‰ (interruption period) and decreased to 0.18‰ (re-introduction of intervention period). Patients admitted to the surgical ICU during the intervention periods had a lower in-hospital mortality (13.5% (155 out of 1,147) versus 16.6% (203 out of 1,226), p = 0.038). After adjusting for effects of confounding variables, the active

screening and decolonization program was independently associated with a decrease in hospital MRSA infections (adjusted odds ratio: 0.3, 95% CI: 0.1-0.8) and 90-day mortality (adjusted hazard ratio: 0.8, 95% CI: 0.7-0.99). Cost analysis showed that $22 medical costs can be saved for every $1 spent on the intervention.

Staphylococcus aureus is normally resident in humans, therefore, the *S. aureus* present in cow's and goat's milk may have resulted from transmission from humans. Improving the hygienic conditions of the milking environment and/or utensils may reduce the prevalence of *S. aureus* in milk and prevent its transmission to humans (Abo-Shama, 2014). *Staphylococcus aureus* is the most pathogenic among the mastitis causing agents in cattle and small ruminants (Salasia *et al*., 2004; Arga *et al*., 2012). Dorgham *et al*. (2013) detected that *S. aureus* represented 60% of the isolates from subclinical mastitis in goats. *Staphylococcus aureus* is one of the most common agents in bacterial food poisoning outbreaks (Kadariya *et al*., 2014). It is also a major causative pathogen of clinical or subclinical mastitis of dairy domestic ruminants (Ahmady and Kazemi, 2013).

Our finding resulted that a large number of *S. aureus* were resistant to ampicillin, gentamicin, tetracycline and oxacillin. These drugs are usually used in veterinary medicine in Indonesia. As reported by other investigators, the resistance of *S. aureus* isolates to β-lactams such as ampicillin, penicillin, tetracycline and oxacillin was evident (Szweda *et al*., 2014). Alian *et al*. (2012) reported a heightened multiresistance of *S. aureus* was found in 34.8% of *S. aureus* isolates. Resistance (resistance and intermediate resistance) to ampicillin was the most common finding (54.3%), followed by resistance to oxacillin (28.3%), tetracycline (26.1%), penicillin G (23.9%), erythromycin (23.9%).

Since antibiotic-resistant isolates might be transmitted to humans by the consumption of food products containing such resistant bacteria, the use of antibiotics as growth promoters in animal husbandry, especially of those commonly used for both human and animal care should be avoided (Jassim and Limoges, 2014). The present study demonstrated that the resistant strains may have been transferred to cow and goat then to milk, which can be the reason of infection in human beings if they take raw milk. The hygiene conditions and careful handling of cow during milking should be improved to prevent the transmission. Antibiotics are used to treat diseases of cattle, sheep, goat, buffalo and other animals and as well as used as preservatives for milk (De Briyne *et al*., 2014). *Staphylococcus aureus* has been reported to frequently show multiple antimicrobial resistance patterns (Nair *et al*., 2013). This may be due to the indiscriminate use of antibiotics has led to the development of multiple antibiotic resistances thereby rendering the antibiotic treatment ineffective. The resistant bacteria present in environments are in contact with human beings and animals. Antimicrobial resistance is a major public health concern in many countries due to the persistent circulation of resistant strains of bacteria in the environment and the possible contamination of water and food (Wooldridge, 2012).

Staphylococcus aureus strains were once nearly uniformly susceptible to semi-synthetic penicillinase-resistant β-lactams (e.g., methicillin, oxacillin) the most commonly used class of antibiotics for skin infection. These strains were termed methicillin resistant *Staphylococcus aureus* (MRSA) a term that implied cross-resistance to all β-lactams including all penicillins and cephalosporins. Among antibiotic resistant isolates in this study, could be observed 15 isolates expressed methicillin resistant (*mec*A) genes for 5 (12.82%) bovine isolates, 1 (4.17%) goat isolate and 9 (90%) human isolates by PCR amplification. These isolates were identified as methicillin resistant *S. aureus* (MRSA). Most of MRSA were also resistant to oxacillin (60%), ampicillin (66.7%), tetracyclin (40%), erythromycin (33.3%) and gentamicin (20%). The results of the present study confirm that methicillin-resistant staphylococci prevalence is still low in ruminants as observed in previous research (Luini *et al*., 2015) in contrast to human. The finding of some *mec*A-negative isolates which were phenotypically resistant to β-lactam antimicrobial agents could be related to a less common type of resistance due to either over production of β-lactamase or the presence of altered Penicillin Binding Protein (PBP) not related to 2a or 2' (Grema *et al*., 2015). For many years, MRSA was considered only a human pathogen, until a report of a MRSA mastitis (udder infection) in a dairy cow surfaced in 1972 (Mohammed and Nigatu, 2015). It has now become an increasingly urgent problem in veterinary medicine with MRSA infections reported in small animals and cattle (Rahimi *et al*., 2015).

CONCLUSION

This study confirms that over 35.0% of the tested *S. aureus* were multiple antibiotic resistances. *Staphylococcus aureus* originating from human and bovine were more resistant than those of goat origin. Most of methicillin resistant *S. aureus* (MRSA) was found in human isolates. The occurrence of *mec*A gene in bovine and goat isolates indicated the important transmission of MRSA among human and animals. The resistance of *S. aureus* to methicillin/oxacillin and the other antibiotics in the present study might help to understand the distribution of MRSA among human bovine

and goat isolates and might help to control *S. aureus* infections. The MRSA infections should be prevented and controled based on a multidisciplinary task, involving surveillance, patient screening, decolonisation, isolation and cohorting of patients both human and animals, environmental cleaning and antimicrobial management. The prevention and control of MRSA are the responsibility of all those who work in the healthcare sector and not just those professionally involved in infection prevention and control. While, MRSA is known to be resistant to various antibiotics, the future studies are needed to develop better therapeutic treatments by using herbal medicine or new vaccines.

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