RESEARCH ARTICLE

Isolation and Molecular Characterization of Methicillin-resistant *Staphylococcus aureus* (MRSA) from Swine following Spa Typing

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Abstract

The objective of this study was to isolate the Methicillin-resistant *Staphylococcus aureus* (MRSA) from the nasal swabs of swine and further characterize the isolates by spa typing. 116 (91.34%) Staphylococcal isolates were obtained from 127 nasal swabs of swine from organized and unorganized pig farms in and around the Puducherry region (India). The nuc gene specific for S. aureus was found in 97 (83.62%) isolates from swine. Methicillin resistant gene *mecA* was detected in 23 isolates (19.83%), and none of the staphylococcal isolates from swine carried the *mecC* gene. Ten representatives MRSA isolates from swine were subjected to further characterization using *spa* typing (*Staphylococcus aureus* protein A typing). Four different spa types were identified namely t657 (40.0%, 4/10), t5983 (40.0%, 4/10), t005 (10.0%, 1/10) and t127 (10.0%, 1/10) from Puducherry region.

Keywords: Methicillin-resistance, *mecA*, Public health, *Spa* typing, Swine.

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Introduction

Antimicrobial resistance (AMR) is an important public health issue being faced worldwide due to inappropriate antimicrobial usage in human and animal husbandry activities. It has contributed to an alarming increase in antimicrobial resistance (Aarestrup, 2005).

There has been increased concern in controlling these AMR pathogens that are resistant to commonly used antibiotics. Methicillin-resistant S. aureus is one of the most important super-bug present worldwide among different multi-drug resistant pathogenic bacteria. It is present in many animal species both as a cause of infection and in healthy carriers.

The *mecA* gene encodes an altered penicillin-binding protein (PBP, PBP2a, or PBP2), lowering the affinity for the β-lactam antibiotics (Kwon *et al*., 2006). So detecting the mecA gene's presence is considered a gold-standard approach in identifying the methicillin resistance from the Staphylococcal isolates (Anand *et al*. 2009). The recent finding of *mec*C which is a new *mecA* homologue *mecA*LGA251, with only 70 per cent nucleotide homology to the conventional *mecA* gene. This *mecC* gene is also used for identifying the methicillin resistance in the isolates from different host species (Stegger *et al*. 2011 and Worthing *et al*. 2016).

MRSA strains can be typed using various molecular approaches, in which DNA sequencing focused on a single locus sequence of the polymorphic region X of *S. aureus* protein A (spa) gene, paves an accurate method for *S. aureus* typing. This spa gene's high degree of polymorphism potentially suits for discriminating the isolates, especially in outbreak investigation (Moodley *et al*. 2006).

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Among food animals, swine have been implicated as one potential source of MRSA infections. Colonized animals may act as an MRSA reservoir for livestock and humans with close contact with animals (Wulf *et al.* 2008 and Rosenkranz *et al.,* 2014). The study on the prevalence of MRSA in swine is scanty in India; hence, the present study was undertaken to isolate *Staphylococci* from the nasal swabs of swine in Puducherry (Southern India) and molecular characterization of Methicillin-resistance in the isolated *Staphylococcus aureus* by *spa* typing.

Materials and Methods

Collection and Processing of Samples

A total of 127 nasal swabs of swine were obtained from both organized (68 samples) and unorganized (59 samples) pig farms in and around the Puducherry region (Southern India). Sterilized cotton swabs were used for nasal swabbing, and the samples were collected aseptically in sterile vials. All the samples were collected with prior consent from the swine handlers.

Isolation and Identification of Staphylococcus Species from Nasal Swabs of Pigs

The nasal swabs were subjected to enrichment in 5 mL of Luria Broth with 7.5% NaCl individually in sterile test tubes for 18 hours at 37°C. The enriched culture was loopful on Mannitol salt agar and incubated at 37°C for 24 hours. Growth on Mannitol salt agar in the form of yellow-colored colonies (mannitol positive) or red-colored colonies (mannitol negative) was observed. The isolated bacteria were subjected to Gram's staining, and isolated Gram-positive cocci were identified up to species level based on conventional cultural and biochemical characters as described in Bergey's Manual of Systematic Bacteriology (1984).

Polymerase Chain Reaction for *S. aureus* and Methicillin-resistance Genes

Preparation of template DNA from *Staphylococcus* strains was carried out as per Sowmya *et al*. (2012). The PCR was carried out with primers targeting 16S rRNA genes specific for *Staphylococci* with a 25 µL reaction mixture consisted of the following, Template DNA 5.0 µL, Primers 2.0 µL (20 pmol each primer), Mastermix (2X) 12.5 µL and Triple distilled water 5.5 µL. The PCR amplification was carried out in an automated thermal cycler (Eppendorf Mastercycler, Germany). The PCR products were analyzed by electrophoresis in 1.5 percent agarose gel in Tris-acetate EDTA (TAE) buffer (1x). The gel was visualized under a UV transilluminator, and the images were documented in a gel documentation system (Bio-Rad Laboratories, USA). All the isolates carrying genes specific for *Staphylococci* were subjected for a second round of PCR with primers targeting genes *nuc* genes specific for *Staphylococcus aureus*. Further all the Staphylococcal isolates were subjected to PCR with two sets of primers targeting *mec A* (162 bp) and *mec C* (136 bp) genes by uniplex PCR. The details of various primers used in this study are given in Table 1.

Characterization of MRSA by *spa* typing

Randomly selected ten MRSA isolates from swine were further characterized using *spa* typing (*Staphylococcus aureus* protein A gene) with primers targeting the *spa* gene of *Staphylococcus aureus* by PCR with variable product sizes (Mitra *et al*., 2013).

Sequence Analysis

The amplified PCR products targeting *spa* gene of 10 MRSA isolates from swine were custom sequenced. Custom sequencing was performed for both directions (5'-3' and 3'-5') to amplify the nucleotide sequence of *spa* gene, by sanger sequence method using the Automated sequencer, Applied Biosystems 3100. The obtained sequences were edited using MEGA 7 software, then the *spa* types were determined with the Ridom Spa server (www.spaserver.ridom.de) software. (Spa ID/ repeat ID/ repeat succession along with Kreiswirth IDs assigned for spa typing were mentioned.)

Reference strains used for Polymerase Chain Reaction

Staphylococcus aureus MTCC 87 as a positive control for genus and species-specific PCR assay and for *Spa* typing. *mec A* gene of Methicillin-resistant *Staphylococcus aureus* N-315 was also used as a positive control in this study (obtained from Dr. Teruyo Ito of Juntendo University from Tokyo, Japan).

Results and Discussion

In our study, 116 isolates were identified as *Staphylococci* out of 127 nasal swabs obtained from swine. Among these 116 isolates, 103 isolates showed the mannitol fermenting yellow-colored colonies, and 13 isolates were found to have

Table 1: Details of the primers used in the study

Primer name with Reference	*Target gene*	*Primer sequence (5'-3')*	*Size*
Staphylococci (Zhang *et al*., 2004)	Genus specific 16S rRNA	AACTCTGTTATTAGCGAAGAACA CCACCTTCCTCCGGTTTGTCACC	756 bp
Staphylococcus aureus (Braskstad *et al.*,1992)	*nuc* gene	GCGATTGATGGTGATACGGT AGCCAAGCCTTGACGAACTAAAGC	270 bp
Methicillin resistance in *Staphylococci* (Oliveira and de Lencastre, 2002)	*mec A*P4 *mec A*P7	TCCAGATTACAACTTCACCAGG CCACTTCATATCTTGTAACG	162 bp
ec C (mec A Variant) (Steggeret *al*., 2011)	*mec ALGA251*FP *mec ALGA251* RP	GAAAAAAAGGCTTAGAACGCCTC GAAGATCTTTTCCGTTTTCAGC	138 bp
Spa Typing (Mitra *et al*., 2013)	*Spa* gene 1067 F 1704 R	ACGTAACGGCTTCATCCA TCCACCAAATACAGTTGTACCG	Variable

mannitol non-fermenting pink-colored colonies. All these 116 Staphylococcal isolates were subjected for genotypic confirmation by PCR in which 97 (83.62%) isolates were found to harbor the thermonuclease gene (*nuc*), which was specific for *S. aureus*. Comparing phenotypic and genotypic characterization, all the *S. aureus* isolates were obtained from mannitol fermenting yellow colonies only. Previous studies reported that the *nuc* gene-based PCR assay detected a total of 96 (94.1%) *S. aureus* from 102 nasal swabs of pigs in Andhra Pradesh, India (Reddy *et al.* 2015) and 139 (9.5%) *S. aureus* isolates from 1458 samples collected from pigs, in China (Guo *et al.* 2018).

In this study, among 116 *Staphylococci* isolates, 23 (19.82%) isolates were found to be positive for MRSA (*mecA*) gene with a product size of 162 bp (Fig. 1), but none of them were found to carry *mecC* gene for MRSA. A similar MRSA prevalence rate was also reported in studies from Ontario, Canada, in which 24.9% (71/285) colonization in pigs (Khanna *et al.* 2008) and 21.9% (78/355) in pork samples from Hong Kong (Boost *et al.* 2013). On the contrary, the presence of MRSA was lesser and varying about 3.2% (21/657) in pigs isolates were reported in Korea (Lim *et al.* 2012) and 4.6% (50/1085) isolates from 6 different states of United States (Smith *et al.* 2013). In a study conducted by Kalupahana *et al.* (2019) MRSA prevalence was 10 percent on 100 pig farms in Srilanka, with 1.2% (6/493) MRSA-positive samples in pigs.

In the present study, among the 10 MRSA isolates from pigs, four different *spa* types were identified to carry the *spa* gene-specific for *S. aureus* with PCR products of variable sizes (Fig. 2), they were t657 (40.0%, 4/10), t5983 (40.0%, 4/10), t005 (10.0%, 1/10) and t127 (10.0%, 1/10) from Puducherry region (Table 2). The most prevalent *spa*-type among pig isolates were *spa* type t5983 and t657. Similar *spa* types were reported in a study by Wu *et al.* (2018) from

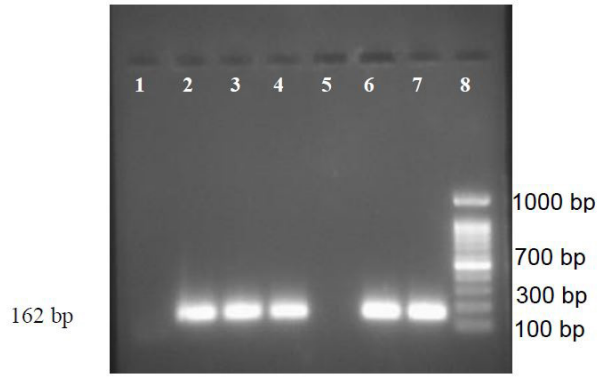

Fig. 1: Screening of field isolates for methicillin resistance (*mecA* gene). **Lane 1 & 5:** Negative control & Negative isolate; Lane 2 & 3,4,6,7: positive control & positive for *mecA* gene of *S. aureus* isolates respectively; Lane 8: 100 bp ladder.

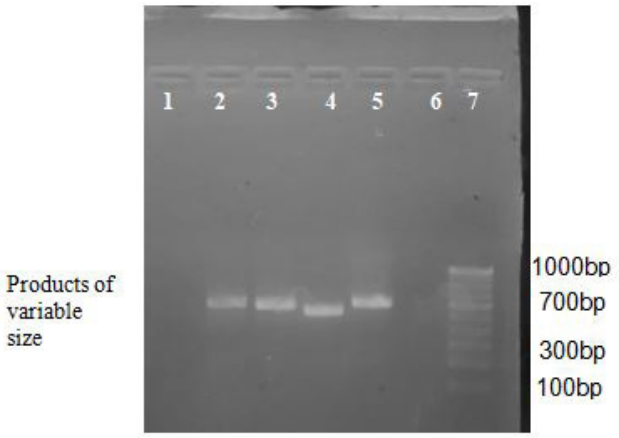

Fig. 2: Amplification of the Spa gene
Lane 1 & 6: Negative control & Negative isolate; Lane 5 & 2,3,4: positive control & positive for *spa* gene of *S. aureus* isolates respectively; Lane 7: 100 bp ladder.

Table 2: *Spa* Types of MRSA isolates obtained from swine

S.No.	*Isolate name*	*No: of Repeat units*	*Length of VNTR in base pairs*	*Repeat sequence (ridom nomenclature)*	*Spa type*
1.	PYP-44	8	192	T1:J1:E1:F1:M1:B1:P1:B1 r26:r23:r13:r21:r17:r34:r33:r34	t657
2.	PYP-10	11	264	U1:J1:E1:N1:C1:M1:O1:M1:O1:K1:R1 r07:r23:r13:r31:r05:r17:r25:r17:r25:r16:r28	t5983
3.	PYP-20	11	264	U1:J1:E1:N1:C1:M1:O1:M1:O1:K1:R1 r07:r23:r13:r31:r05:r17:r25:r17:r25:r16:r28	t5983
4.	PYP-87	8	192	T1:J1:E1:F1:M1:B1:P1:B1 r26:r23:r13:r21:r17:r34:r33:r34	t657
5.	PYP-31	8	192	T1:J1:E1:F1:M1:B1:P1:B1 r26:r23:r13:r21:r17:r34:r33:r34	t657
6.	PYP-54	11	264	U1:J1:E1:N1:C1:M1:O1:M1:O1:K1:R1 r07:r23:r13:r31:r05:r17:r25:r17:r25:r16:r28	t5983
7.	PYP-65	11	264	U1:J1:E1:N1:C1:M1:O1:M1:O1:K1:R1 r07:r23:r13:r31:r05:r17:r25:r17:r25:r16:r28	t5983
8.	PYP-62	8	192	T1:J1:E1:F1:M1:B1:P1:B1 r26:r23:r13:r21:r17:r34:r33:r34	t657
9.	PYP-91	7	168	U1:J1:F1:K1:B1:P1:E1 r07:r23:r21:r16:r34:r33:r13	t127
10.	PYP-102	12	288	T1:J1:E1:J1:N1:C1:M1:O1:M1:O1:K1:R1 r26:r23:r13:r23:r31:r05:r17:r25:r17:r25:r16:r28	t005

meat and meat products in China. Agerso *et al.* (2012) has reported MRSA associated with *spa* type t127 in pigs from Danish slaughterhouse. Merialdi *et al.* (2019) reported that the spa type t127 associated with ST1 SCCmec V LA-MRSA is a commonly found clone of MRSA reported in Italian pig holdings. Ivbule *et al.* (2017) has reported *Spa* type t127 and several other *spa* types in MRSA from pigs and the environment. Kalupahana *et al.* (2019) obtained the MRSA strains from pigs, and the *spa* types identified in pig farms were t127, t002, t304, t10744 from Sri Lanka. The Spa types found in our study can be used in understanding the genetic relationship among *S. aureus* strains in swine, which in turn helps for the epidemiological tracking of the source of infection to other hosts species, especially about the inter-species transmission. Therefore the prevention of MRSA infection between pigs and community settings will be possible in the future.

Conclusions

This study indicates the prevalence of MRSA infection in pigs (19.82%) and four different *spa* types in pigs from the Puducherry region. Among them, the most prevalent *spa* types were t5983 and t657. Further studies on swine and the swine handlers may helps us to understand the transmission of MRSA between the species, which will help formulate the strategies for containment of antimicrobial resistance in humans and animals.

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