RESEARCH ARTICLE

Molecular Characterization and Phylogenetic Profiling of Methicillin-Resistant *Staphylococcus aureus* from Poultry Meat in Nagpur

Sujata Gajanan Bhatkar, Shubhangi Rambhau Warke*, Mehak Ramesh Tikoo

Abstract

Antimicrobial resistance (AMR) represents a danger to public health, and a "One Health" strategy taking into account the reservoirs in humans, animals and the environment is need of the hour. The present study was designed to investigate the prevalence of Methicillin-resistant *Staphylococcus aureus* in poultry meat to establish genetic relationships among the MRSA isolates. In the present study, out of 50 samples of poultry meat, 19 (38%) were detected by conventional methods to be *S. aureus*, and 15 (30%) were confirmed by molecular detection of *nuc* gene. A prevalence of 9/19 (47.36%), 11/19 (57.89%), 16/19 (84.21%), 13/19 (68.42%), 9/19 (47.36%) and 6/19 (31.57%) resistance was observed in *S. aureus* isolates by phenotypic testing against Methicillin, Gentamicin, Penicillin-G, Tetracycline, Azithromycin and Vancomycin respectively, while as the *mecA* gene was detected in 56% (5/9) of the isolates that exhibited methicillin resistance. The sequencing and phylogenetic analysis of *mecA* gene of the representative positive samples revealed that the sequence of the isolate exhibited (98.52% and 98.88%) similarity with the human (*Homo sapiens*) sequences of Iraq and Egypt. The results of our study imply that the current management practices utilized in the poultry sector may be the cause of the increased prevalence and spread of antimicrobial-resistant bacteria in their environment.

Key words: Methicillin resistance, PCR, Phylogenetic analysis, Poultry meat, *Staphylococcus aureus*

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Introduction

Antimicrobial resistance (AMR) is a serious health problem brought on by the overuse and misuse of antibiotics. *Staphylococcus aureus* is a prevalent foodborne bacterium found in fresh and ready-to-eat foods that cause most illnesses worldwide (Diep *et al.,* 2006). *Staphylococcus aureus* is a common food-borne pathogenic bacteria found in raw and undercooked chicken and its consumption may lead to infection and/or toxicity (Herve and Kumar, 2017). The use of methicillin in food animal production facilities has led to a significant rise in the isolation of Methicillin-resistant *Staphylococcus aureus* (MRSA) strains (Oliveira *et al*., 2002). Conventional cultural, biochemical methods, PCR and DNA sequencing have been employed to validate and describe *Staphylococcus aureus* isolates and reveal their genetic variation and evolutionary links (Janda and Abbott, 2007). There is a lack of research concerning MRSA in Nagpur's poultry sector. The present study was designed to investigate the prevalence of Methicillin-resistant *Staphylococcus aureus* in poultry meat employing phenotypic and genotypic methods and further subjected for phylogenetic analysis of the to establish genetic relationships among the MRSA isolates.

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Materials and Methods

Collection and Processing of Samples

A total of 50 poultry meat samples were collected from the retail shops of Nagpur (India) using sterilized polyethylene bags and were transported to the laboratory on ice, followed by processing within the first 24 h. The collected samples were macerated before being subjected to inoculation in sterile nutrient broth at 37°C for 6-8 h before proceeding for primary bacterial isolation.

Isolation, Identification, and Biochemical Characterization

The collected samples were subjected to primary isolation of *Staphylococcus aureus* on Mannitol Salt Agar followed by further selective isolation on Baird Parker agar supplemented with egg yolk and potassium tellurite, and incubated overnight at 37°C (Ghabbour *et al*., 2022). The bacterial colonies were further subjected to characterization using Gram staining, growth on DNAse agar and coagulase testing (Cruickshank *et al.,* 1975).

Molecular Detection of Thermonuclease (*nuc*) Gene of *S. aureus*

DNA was extracted using a commercial kit (HiPurA ® Multi-sample DNA purification kit) from all the presumed positive isolates of *Staphylococcus aureus* obtained on Baird Parker agar. These presumptive positive isolates were confirmed using *nuc* gene targeting PCR (Ghabbour *et al*., 2022) using published primers, Forward primer: 5′ GCG ATT GAT GGT GAT ACG GTT 3′ and Reverse primer: 3′ AGC CAA GCC TTG ACG AAC TAA AG 3′ (Oliveira *et al.,* 2015).

Antibiotic Sensitivity Test

The *Staphylococcus aureus* isolates were subjected to antibiotic sensitivity testing using a variety of antibiotics, *viz*., Penicillin-G (10 IU), Methicillin (10 µg), Tetracycline (30 µg), Gentamicin (30 µg), Azithromycin (15 µg) and Vancomycin (30 µg) as per the Clinical and Laboratory Standards (CLSI, 2018) criteria. The isolates were incubated overnight in nutrient broth at 37°C and compared to 0.5 Mc Garland standards followed by disk diffusion assay of the samples on Muller-Hinton agar incubated at 37°C for 24 h. The zone of inhibition of the samples was measured using a scale and the results were compared with the reference range (CLSI, 2018).

Molecular Detection of *mecA* Gene of *S. aureus*

The confirmed *S. aureus* isolates were subjected to conventional PCR for detection of the *mecA* gene and to describe isolates that demonstrated methicillin resistance (McClure *et al*., 2006). The PCR was carried out using published primers, Forward primer: 5′-GTA GAA ATG ACT GAA CGT CC GAT AA -3′ and Reverse primer: 3′-CCA ATT CCA CAT TGT TTC GGT C-5′ (McClure *et al*., 2006).

Sequencing and Phylogenetic Analysis

Sequencing of the one representative sample positive for the *mecA* gene was performed by Eurofins Genomics India Pvt Ltd, Bangalore. The sequence obtained was aligned using Bioedit software and subjected to NCBI BLAST to identify homologous global sequences similar to our sequence. The phylogenetic analysis of the sequencing data was carried out utilizing bioinformatic tools such as MEGA XI software and the phylogenetic tree was constructed using maximum likelihood methods. Sequences that belong to the same cluster are closely connected, and they display various clades indicating their evolutionary relationship within various isolates of *Staphylococcus aureus* (Kumar *et al*., 2020).

Results and Discussion

Out of 50 poultry meat samples processed, 20 (40%) samples were recovered on MSA as a result of mannitol fermentation manifesting yellow-colored colonies (Fig. 1)

Table. 1: Bacterial isolation, identification, and biochemical characterization of *S. aureus* from poultry meat

Samples	No. of samples examined	Colonies on MSA	Positive on Baired Parker agar	Coagulase positive	DNAse positive	Samples positive for *nuc* gene
Poultry meat	50	20 (40%)	19 38(%)	19 (38%)	19 (38%)	15 (30%)

Fig. 1: Mannitol fermenting and non-fermenting colonies of *Staphylococcus* spp. on Mannitol Salt Agar

Fig. 2: Typical jet-black colonies surrounded by a clear white halo zone showing lecithinase activity on Baird Parker Agar

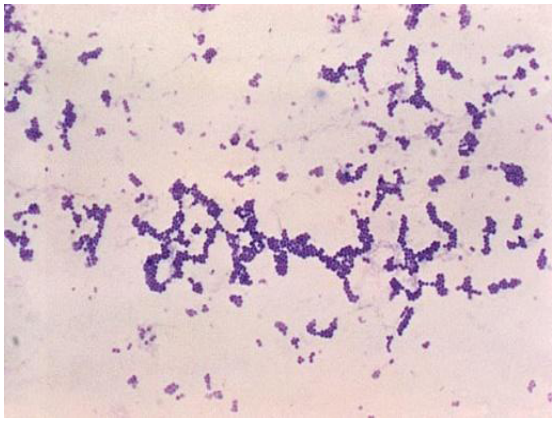

Fig. 3: Gram positive cocci of *Staphylococcus aureus* under 100X in oil immersion

and 19 (38%) of these samples produced jet-Black colonies surrounded by a clear white halo zone on Baird Parker agar, which is a characteristic of *S. aureus* (Table 1, Fig. 2). All of the positive *S. aureus* isolates exhibited typical morphology and staining property (Gram-positive cocci) characteristic of *Staphylococcus* spp. (Fig. 3). Colony morphology on MSA, Gram stain, catalase and coagulase tests have been used in previous studies for the primary identification of *S. aureus* in chicken samples (Nandy *et al*., 2009; Ameer, 2017).

A total of 19 (38%) samples of poultry meat were found positive for coagulase production by tube method (Table 1). Citak and Duman (2011) reported 47.2% coagulase positive for *S. aureus from* poultry meat. Furthermore, the suspected isolates subjected to growth on DNAse agar showed a zone of clearance around the colonies in 19 (38%) samples (Table 1, Fig. 4).

The results of the present prevalence study in Nagpur and other studies conducted by researchers from various parts of the world differ vastly. The prevalence rates varied throughout different regions of India. However, results of our study showed a prevalence of 38 %, in poultry meat using conventional methods, which was closer to the reports of Karmi (2013) and Abdalrahman *et al.* (2015), who reported the prevalence of 40% and 41%, respectively. Das and Mazumder (2016) documented 48.57% and Herve and Kumar (2017) showed that 46.61% prevalence rate of *S. aureus* by conventional culture method in poultry meat. However, comparatively very low (13% and 15.2%) prevalence was documented by Bernier-Lachance *et al*. (2020) and Fawzy *et al*. (2017). The previous study has noted that the main cause of the low microbiological quality of chicken meat offered in common markets is high *S. aureus* contamination (Alvarez-Astorga *et al*., 2002).

Molecular Detection of *nuc* Gene

The oligonucleotide primer set amplifies the *nuc* gene, which encodes the thermonuclease produced by *S. aureus*. Identification of the *S. aureus* strain was based on 270 bp amplified products on 1% agarose gel. Prevalence of *S. aureus* in poultry meat by *nuc* gene was recorded to be 30% (15/50) in our study (Fig. 5), which was closer to the findings of Dutta *et al.* (2020), who reported 29% prevalence in poultry meat products across Ludhiana city. In comparison to our study, higher prevalence rates of 66.6% and 54.0 % were reported by Shylaja *et al*. (2018) and Mkize *et al*. (2017) in commercial broiler chickens slaughterhouses and retail outlets around the Durban metropolitan area in South Africa.

The findings of a different study demonstrated the variation in percentage between conventional and PCR methods for identifying *S. aureus* in chicken meat and concluded that the PCR assay is specific for diagnosing the strains in raw meat (Tamarapu *et al*., 2001). Our findings concurred with these observations. Worldwide, several studies suggest that *S. aureus* isolation rates in meat can vary from 28% to 80% in local chicken and imported frozen chicken (Abdulrahman *et al*., 2020).

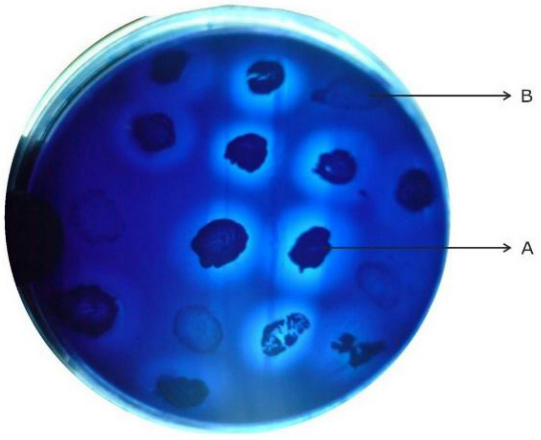

Fig. 4: Colonies of *S.aureus* positive isolates on DNAse agar. A: Positive, & B: Negative

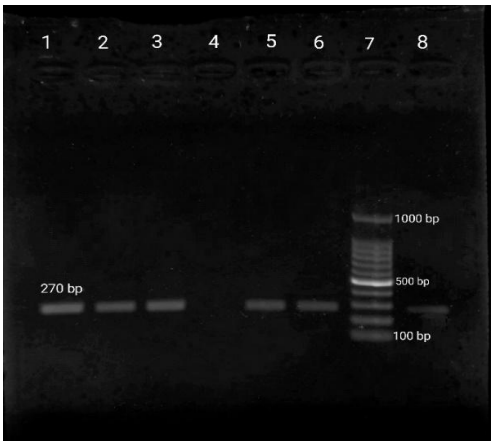

Fig. 5: Agarose gel electrophoresis showing amplification of *nuc* gene at 270 bp. Lane 1: Standard culture MTCC 96, Lane 2: P11, Lane 3: P5, Lane 4: Negative control (*E. coli*), Lane 5: P6, Lane 6: P4, Lane 7: 100 bp DNA ladder, Lane 8: P41

Antibiotic Sensitivity Test

The *in vitro* antibiogram testing revealed that 9/19 (47.36%) *S. aureus* isolates obtained in our study were resistant to Methicillin, 11/19 (57.89%) to Gentamicin, 16/18 (84.21 %) to Penicillin-G, 13/19 (68.42%) to Tetracycline, 9/19 (47.36%) to Azithromycin and 6/19 (31.57%) to Vancomycin. These results were in agreement with Herve and Kumar (2017) and Amoako *et al*. (2020) for the respective antibiotics. At the same time contradictory results to our study were reported by Can *et al*. (2017).

Molecular Detection of *mecA* Gene

PCR-based molecular methods are preferred for the determination of antibiotic-resistant genes. In the present study, the detection of methicillin-resistant genes was accomplished by PCR method directed at the *mec A* gene (Kumar *et al*., 2010). The findings of our study revealed that 5 out of 9 (56%) phenotypic methicillin-resistant *S. aureus* isolates were found to possess *mecA* gene, which generated an amplicon size of 310 bp (Fig. 6). Significant differences in the detection of antimicrobial resistance to methicillin by phenotypic and genotypic testing noted in our study could be due to varying degrees of methicillin resistance that appear every 10^4 or 10^6 cells or it could be due to lack of the penicillinase plasmid which is crucial for the stability and phenotypic expression of the *mec*A gene (Hiramatsu *et al.,* 1990). Our results showed relevance to a study by Mkize *et al*. (2017) who reported a 56% presence of the *mec* A gene in poultry meat. However, a lower percentage of genes (34.11% and 21.4%) were found in the studies carried out by Ghabbour *et al*. (2022) and Rao *et al*. (2022).

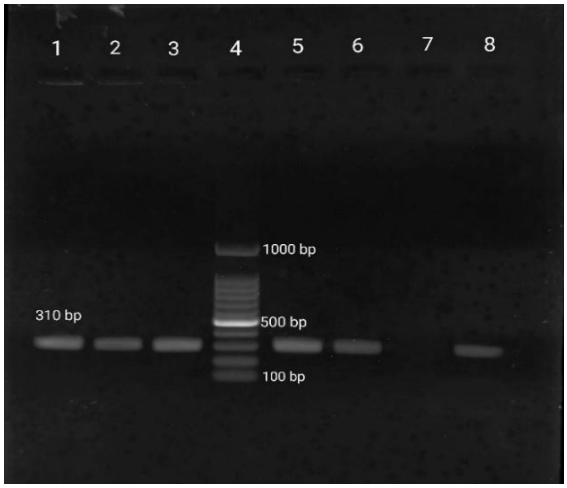

Fig. 6: Agarose gel electrophoresis showing amplification of *mecA* gene at 310 bp. Lane 1: P5, Lane 2: P42, Lane 3: P4, Lane 4: 100 bp DNA ladder, Lane 5: P10, Lane 6: P24, Lane 7: Negative control (*Psedomonas auruginosa*), Lane 8: P31

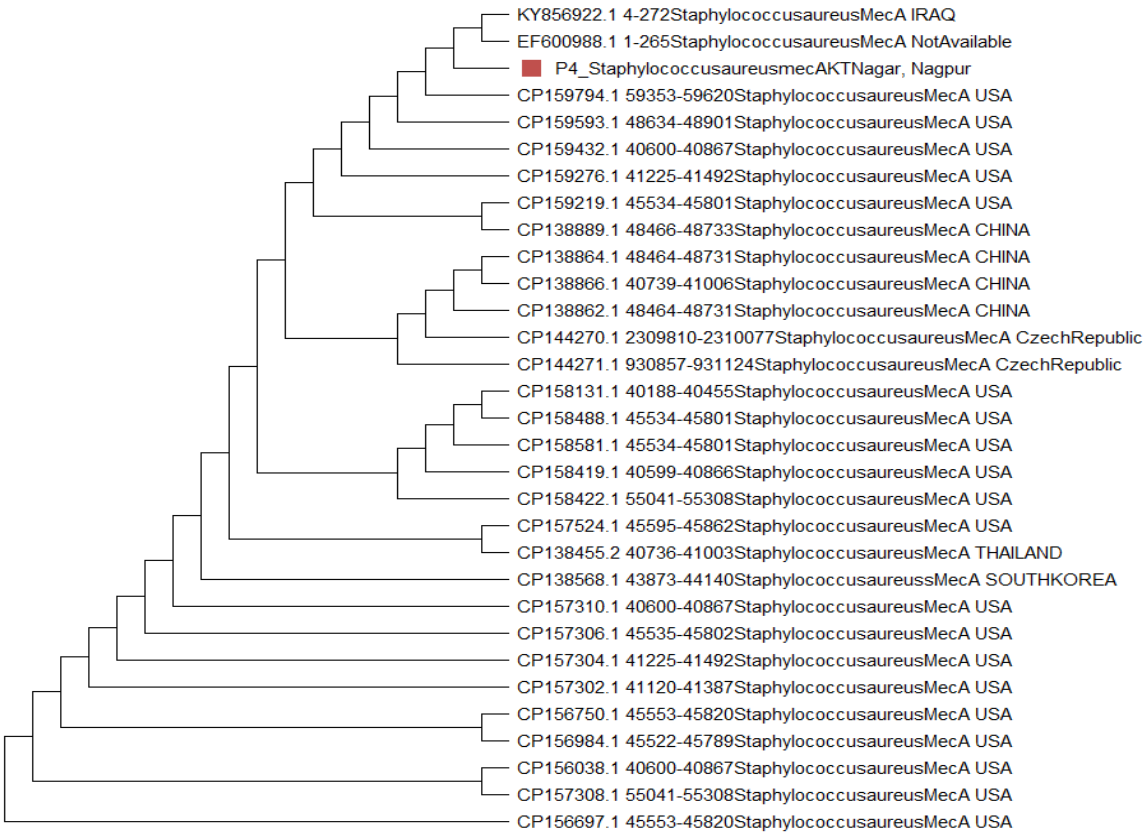

Fig. 7: Phylogenetic analysis of study isolates of methicillin-resistant*S.aureus* with already reported sequences

Sequencing and Phylogenetic Analysis

The sequences of *mec* A gene amplified poultry meat (P4) subjected to phylogenetic analysis and BLAST search with BLASTN program against non-redundant database showed 98.52% homology with the sequence obtained from human (*Homo sapiens*) of Iraq (Accession no. KY856922) and 98.88% homology with Egypt (Accession no. EF600988) and showed high diversity and out group from other isolates from China, Czech Republic, USA, Thailand, and South Korea (Fig. 7). This study reported first genetic relationship of methicillin-resistant *S. aureus* isolates obtained from poultry meat of Nagpur. In earlier studies, it has been reported that MRSA isolates are closely related to the human source and are categorized as community-acquired owing to the accumulation of methicillin resistant strains in foods of animal origin and their environment which is then transferred to humans though consumption of contaminated animal foods. This study further revealed that the *S. aureus* strain positive for *mecA* gene showed homology to the human sequence from USA and was grouped in a different cluster from human sequence of Pakistan (Yaiphathoi and Sharma, 2020). The present findings highlight the significance of teamwork within the One Health framework in tackling this complex problem and guaranteeing the security of our food supply and public health.

Conclusion

Our data of 31.75% Methicillin-resistant *S. aureus* confirms the alarming increase in the prevalence of community-acquired MRSA in poultry meat from local meat shops of Nagpur region and its emerging multiple antibiotic resistances in foods as a serious problem for public health. Additionally, this study highlighted the serious risks to public health associated with eating those foods contaminated with *S. aureus*. AMR gene presence in the poultry meat production environment was investigated and this research yielded invaluable information for estimating the risk to public health posed by antibiotic use in the poultry sector.

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