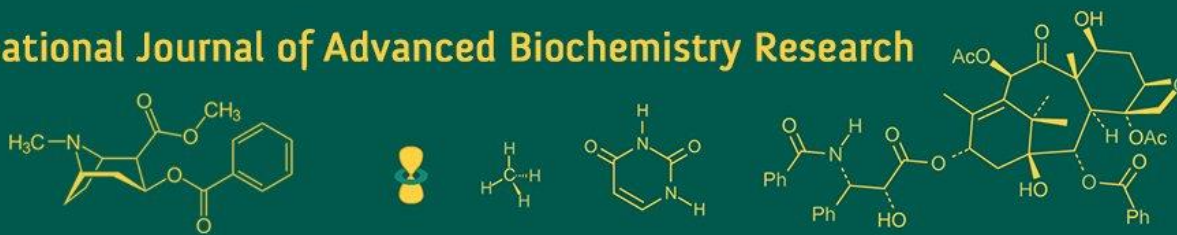


International Journal of Advanced Biochemistry Research



ISSN Print: 2617-4693
 ISSN Online: 2617-4707
 IJABR 2024; SP-8(4): 448-452
www.biochemjournal.com
 Received: 02-02-2024
 Accepted: 13-03-2024

Sonali Thakur
 Ph.D. Scholar, Department of
 Veterinary Public Health and
 Epidemiology, College of
 Veterinary Science and A.H.,
 Kamdhenu University, Anand,
 Gujarat, India

MN Brahmhatt
 Professor and HOD,
 Department of Veterinary
 Public Health and
 Epidemiology, College of
 Veterinary Science and A.H.,
 Kamdhenu University, Anand,
 Gujarat, India

JB Nayak
 Associate Professor,
 Department of Veterinary
 Public Health and
 Epidemiology, College of
 Veterinary Science and A.H.,
 Kamdhenu University, Anand,
 Gujarat, India

Nishant Patel
 RA, Department of Veterinary
 Public Health and
 Epidemiology, College of
 Veterinary Science and A.H.,
 Kamdhenu University, Anand,
 Gujarat, India

Corresponding Author:
Sonali Thakur
 Ph.D. Scholar, Department of
 Veterinary Public Health and
 Epidemiology, College of
 Veterinary Science and A.H.,
 Kamdhenu University, Anand,
 Gujarat, India

Biofilm-forming *Staphylococcus aureus* in retail meat: A study from Anand, Gujarat, India

Sonali Thakur, MN Brahmhatt, JB Nayak and Nishant Patel

DOI: <https://doi.org/10.33545/26174693.2024.v8.i4Sf.1005>

Abstract

Foodborne illnesses represent significant public health challenges worldwide. Developing countries face heightened risks due to insufficient food handling and sanitation practices. Although animal tissue begins as a sterile environment, contamination can occur during the slaughtering process from microorganisms present both externally and internally. *Staphylococcus aureus* stands as a major culprit in causing foodborne diseases. It has the capability to form biofilms on various surfaces and can thrive in the diverse environmental conditions commonly encountered in food processing facilities. The capacity of *Staphylococcus aureus* to form biofilms significantly contributes to its survival, persistence, antibiotic resistance, and virulence, making infections associated with biofilms challenging to manage and control. This study sought to evaluate the prevalence, molecular detection, and biofilm production of *Staphylococcus aureus* in raw meat sourced from retail shops in Anand, Gujarat. A total of 100 samples (50 chicken and 50 chevon) underwent cultural isolation and PCR confirmation for the presence of *Staphylococcus aureus* using the *nuc* gene. The identification of methicillin-resistant *Staphylococcus aureus* relied on the detection of the *mecA* gene. Biofilm formation was assessed using the Congo Red Agar method. Results revealed an overall isolation rate of *Staphylococcus aureus* at 21%, with a higher prevalence observed in chicken samples (71.42%) compared to chevon samples (28.57%). Methicillin-resistant *Staphylococcus aureus* was exclusively found in chicken samples, accounting for 9.52% of isolates. Among the confirmed isolates, 57.14% exhibited biofilm production. These findings underscore the critical importance of adhering to stringent hygiene practices during meat handling and highlight the potential health risks associated with consuming meat products.

Keywords: *Staphylococcus aureus*, methicillin-resistant *Staphylococcus aureus*, chevon, chicken, PCR, biofilms

Introduction

Foodborne illnesses, stemming from a myriad of agents such as bacteria, viruses, fungi, parasites, or chemicals, present formidable challenges to public health worldwide. With over 200 diseases linked to foodborne transmission, an estimated 600 million individuals, nearly 1 in 10 people globally, suffer from illness due to consuming contaminated food annually. This results in 420,000 deaths each year, accompanied by a loss of 33 million healthy life years, with diarrheal illnesses comprising most cases (World Health Organization, 2022) [24]. Common pathogens like *Salmonella* spp., *Campylobacter* spp., *Staphylococcus aureus*, *Escherichia coli*, and norovirus are often implicated in acute foodborne illnesses (Bintsis, 2017) [3]. Developing and underdeveloped nations face heightened risks of food contamination due to factors such as poor sanitation, inadequate infrastructure, and improper handling practices (Dandage *et al.*, 2017) [6]. In India, stagnant food safety standards are projected to lead to over 100 million cases of foodborne illnesses annually, a figure expected to rise to 150-177 million by 2030 (Smeets *et al.*, 2017) [21]. From 2009 to 2018, the Integrated Disease Surveillance Program documented 2,688 foodborne disease outbreaks in India, resulting in 153,745 illnesses and 572 deaths (Bisht *et al.*, 2021) [4]. The rapid globalization of food production and trade has further exacerbated the likelihood of food contamination, necessitating global collaboration among food safety authorities to effectively address these concerns (Jawad *et al.*, 2020) [11].

Staphylococcus aureus is characterized by irregular clusters resembling bunches of grapes, consists of spherical cells with diameters ranging from 0.5 to 1.0 μm , often occurring singly or in bunches.

These facultative anaerobic Gram-positive cocci are nonmotile and non-spore-forming, producing catalase when growing aerobically. On non-selective media, typical colonies of *S. aureus* appear yellow to golden-yellow, smooth, raised, glistening, circular, and translucent, reaching sizes of 6–8 mm in diameter. While *S. aureus* commonly colonizes the skin, anterior nares, and mouth of healthy individuals, nasal colonization is a significant source of infection and a risk factor for subsequent colonization, although most carriers remain asymptomatic (Gorwitz *et al.*, 2008) [9]. Nonetheless, *S. aureus* is a multifaceted pathogen accountable for both hospital and community-acquired infections, encompassing staphylococcal food poisoning (SFP) and toxic shock syndrome (TSS) (Song *et al.*, 2017) [22]. SFP, a prevalent cause of foodborne intoxication worldwide, manifests with violent vomiting shortly after ingestion and is typically self-limiting (Kerouanton *et al.*, 2007) [12]. Symptoms of acute gastroenteritis, such as nausea, abdominal cramps, diarrhoea, and fever, can appear within 2–6 hours of consuming food containing staphylococcal enterotoxins. Additionally, TSS, characterized by fever, erythema, hypotension, and multi-organ dysfunction, is associated with toxins produced by *S. aureus*, including TSST-1 and staphylococcal enterotoxins (Todd *et al.*, 1978) [23].

Methicillin-resistant *Staphylococcus aureus* (MRSA), emerged in 1961 is a strain of the bacterium *S. aureus* known for its resistance to multiple antibiotics, presenting a significant challenge in healthcare settings. Methicillin resistance is mediated by the carriage of the staphylococcal cassette chromosome *mec* (SCC*mec*), a mobile genetic element containing the *mecA* gene, which encodes penicillin-binding protein 2a (PBP2a) with decreased affinity for β -lactam antibiotics (Doulgeraki *et al.*, 2017) [7]. Additionally, a new homologue of the *mecA* gene, designated as *mecC* gene, has also been identified. MRSA strains also produce β -lactamase encoded by *blaZ*, contributing to decreased activity of β -lactam antibiotics. Epidemiologically and molecularly, MRSA is categorized into hospital-associated MRSA (HA-MRSA), livestock-associated MRSA (LA-MRSA), and community-associated MRSA (CA-MRSA). Its ability to acquire multiantibiotic resistance poses challenges for future chemotherapy against multiresistant pathogens (Dahms *et al.*, 2014) [5]. LA-MRSA strains have been detected in pork and chicken products. These findings highlight the potential for meat products, to serve as a source of food poisoning.

Biofilm serves as a prevalent mechanism adopted by bacteria to endure diverse adverse environmental conditions, facilitating the formation of cellular communities encased within extracellular polymeric substances (EPS). Bacterial biofilms, pose challenges due to their resilience against antibiotic treatments and host immune defenses. These infections, often chronic and prone to relapse, account for a substantial portion of human bacterial infections and present significant challenges in both human and veterinary medicine (Hall-Stoodley *et al.*, 2004) [10]. *S. aureus* demonstrates notable biofilm formation abilities, particularly evident in various food-related settings such as raw materials, processing environments, equipment surfaces, and commodity circulation (Miao *et al.*, 2017) [15]. In the case of *S. aureus*, biofilm development progresses through attachment, microcolony formation, maturation, and detachment stages, with EPS playing a pivotal role in

cellular encasement and community function across these phases (Rosenthal *et al.*, 2014) [18].

Given the increasing importance of raising public awareness about food safety and quality, understanding the prevalence of *S. aureus* and methicillin-resistant strains (MRSA) in food animals is crucial. This study aimed to assess the prevalence of *S. aureus* and detect the presence of the methicillin resistance gene (*mecA*) in meat sold in retail outlets in Anand, using both conventional and molecular techniques.

Materials and Methods

Collection and processing of samples

Total 100 meat samples (50 chicken & 50 chevon) were collected from the retail meat shops in and around the Anand city (Gujarat, India). Samples were collected aseptically in pre-sterilized container. They were then immediately transferred on ice at 4°C to the laboratory of Department of Veterinary Public Health & Epidemiology, College of Veterinary Science and Animal Husbandry, Anand for further processing.

Isolation and identification of *Staphylococcus aureus*

All samples were enriched in Peptone Water broth at 37 °C for 24 hours. For the isolation of *S. aureus*, Mannitol Salt Agar (MSA) and Baird Parker Agar (BPA) were used as selective media. A loopful of inoculum from the enrichment broth was streaked onto both MSA and BPA supplemented with Egg Yolk Emulsion and 3.5% Potassium Tellurite solution. The inoculated plates were then incubated at 37 °C for 24-48 hours. Presumed *S. aureus* isolates were subsequently confirmed through Gram's staining using the HiMedia Gram Staining Kit.

Molecular detection of *Staphylococcus aureus* and Methicillin resistant *Staphylococcus aureus*

Culturally positive *S. aureus* isolates were subjected to molecular identification using PCR targeting the *nuc* and *mecA* gene, respectively. The snap chill process was used to obtain template DNA from isolates. The PCR reaction was set up in 200 μ L PCR tubes on ice, with a total volume of 25 μ L. The amplification aimed to screen the genes using a Thermocycler PCR machine (Eppendorf Mastercycler gradient). The reaction mixture comprised 12.50 μ L of PCR master mix (2X), 1 μ L each of forward and reverse primers (10 pmol each), 5.50 μ L of nuclease-free water, and 5 μ L of template. The specific oligonucleotide primer sequences for the *nuc* gene and *mecA* gene and the PCR thermal cycling conditions are outlined in Table 1 and Table 2, respectively. The resulting amplified product was subsequently analyzed via agarose gel electrophoresis using a 1% agarose gel, and the bands were visualized using a gel documentation system.

Evaluation of biofilm production by *Staphylococcus aureus* isolates

The isolates identified morphologically and molecularly were streaked on Congo Red Agar as described by Freeman *et al.* (1989) [8]. The plates were incubated at 37°C for 24 h.

Results and Discussions

Out of a total of 100 samples, 24% were isolated as *S. aureus*, with 66.66% (16/24) originating from chicken samples and 33.33% (8/24) from chevon samples. These

isolates exhibited distinctive characteristics: jet-black colonies on BPA and golden-yellow colonies on MSA, along with a gram-positive cocci arrangement in clusters (Fig 1, 2, 3). In a study conducted by Owuna *et al.* (2015) [17], it was found that out of 40 poultry meat samples, 29 samples (72.50%) tested positive for the presence of *S. aureus*. Sangeetha *et al.* (2020) [19] reported, 55% prevalence rate of *S. aureus* in chevon whereas chicken samples had 27% prevalence rate.



Fig 1: Jet black colonies of *S. aureus* on Baird Parker Agar



Fig 2: Yellow colonies of *S. aureus* on Mannitol Salt Agar

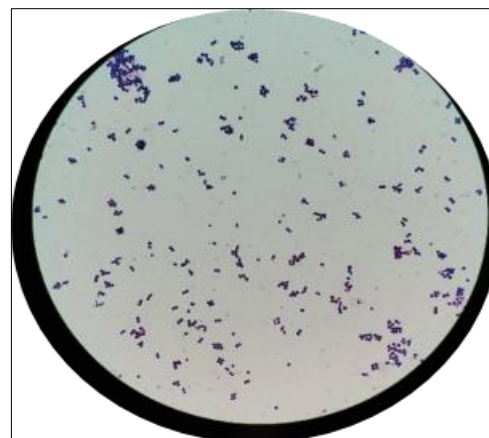


Fig 3: Gram positive cocci in bunches

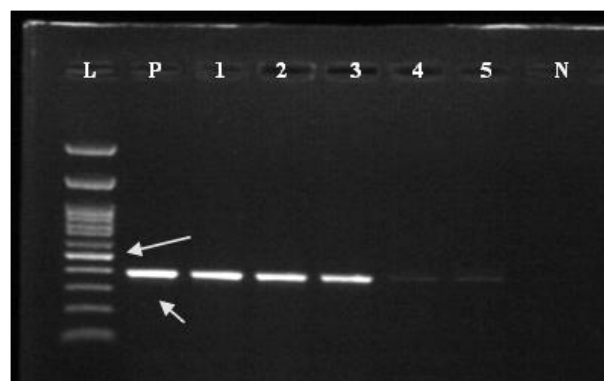


Fig 4: Agarose gel Electrophoresis of PCR products for detection of nuc gene (L: 100 bp DNA ladder; P: Positive control; Lane 1-5: Positive samples; N: Negative control)

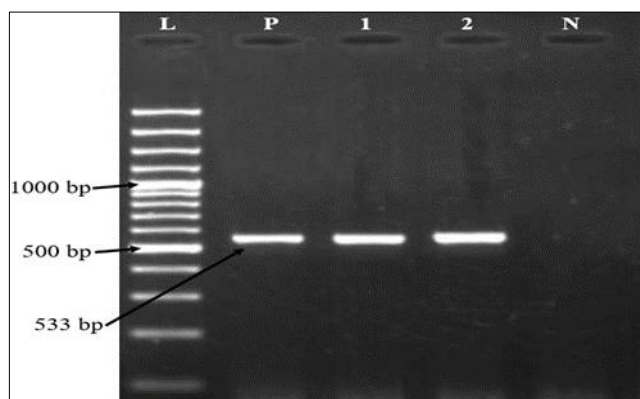


Fig 5: Agarose gel Electrophoresis of PCR products for detection of *mecA* gene (L: 100 bp DNA ladder; P: Positive control; Lane 1&2: Positive samples; N: Negative control)

Table 1: Details of primers used in PCR

Name of primers	Primer Sequence (5'-3')	Amplicon Size (bp)	Reference
<i>nuc</i>	F: GCTGGCATATGTATGGCAATT R: GCTTCAGGACCATATTTCTCTAC	385	Shylaja <i>et al.</i> (2010) [20]
<i>mecA</i>	F: AAA ATC GAT GGT AAA GGT TGG C R: AGT TCT GCA GTA CCG GAT TTG C	533	Lee (2003) [14]

Table 2: Thermal cycling conditions for primer pairs in PCR

Primers (Forward and Reverse)	Cycling Conditions				
	Initial Denaturation	Denaturation	Annealing	Extension	Final Extension
<i>nuc</i>	94 °C 5 min	94 °C 30 sec	55 °C 30 sec	72 °C 30 sec	72 °C 5 min
<i>mecA</i>	94 °C 4 min	94 °C 30 sec	55 °C 30 sec	72 °C 1 min	72 °C 5 min
35 cycles					

Of the 24 *S. aureus* isolates identified via culture technique, PCR confirmed 21 samples out of the total isolates. Specifically, 15 chicken samples tested positive, accounting for 71.42%, while 6 chevon samples were positive, constituting 28.57% (Fig 4). Additionally, 2 isolates of *S. aureus* from chicken samples were confirmed as MRSA (Fig 5). Zehra *et al.* (2019) [25] and Latha *et al.* (2017) [13] reported 17.70% and 40% prevalence of *S. aureus* from chevon samples. Regarding chicken samples, Naas *et al.* (2019) [16] and Zehra *et al.* (2019) [25] reported a prevalence of 40% and 26.50%. Zehra *et al.* (2019) [25] reported 2.72% prevalence of MRSA from chicken samples. These variations in findings underscore potential differences in prevalence rates across diverse geographic regions or sample populations.

Isolates confirmed with PCR were subjected to streaking on

CRA, which revealed 57.14% (12/21) biofilm producers as they produced black colour colonies on CRA, whereas no change in colouration was observed in non-biofilm producing isolates (Fig 6). According to Ballah *et al.* (2022) [2], among 100 *S. aureus* isolates, 89% exhibited characteristic colonies on Congo Red Agar (CRA) and were classified as biofilm producers. In contrast, Achek *et al.* (2020) [1] reported that all *S. aureus* isolates (8) from food samples were biofilm producers, totalling 100%. The overall results of this study are compiled in Table 3.

The significance of *S. aureus* and MRSA and their role in human infection through consumption of contaminated food are emphasised in this work. We hope that this study will serve as a guide for other scientists working in related domains.

Table 3: Overall Prevalence (%) of *S. aureus* observed by Biochemical and Molecular methods

Sample type	No. of samples examined	No. of positive samples for <i>S. aureus</i> (culturally)	No. of positive samples for <i>S. aureus</i> (PCR)	No. of positive samples for MRSA (PCR)	Biofilm producers (CRA)
Chicken	50	16	15	2	9
Chevon	50	8	6	0	3
Overall	100	24	21	2	12



Fig 6: Biofilm forming *S. aureus* producing black colonies on CRA

Conclusions

The current research reveals a moderate occurrence of *S. aureus* and methicillin-resistant *S. aureus* in meat samples, suggesting a need for enhanced sanitary and hygienic measures in meat handling practices. Moreover, the formation of biofilms in settings associated with food processing is notably significant, as they can serve as a continuous reservoir of microbial contamination, leading to food spoilage or the transmission of diseases.

Acknowledgements

Authors gratefully acknowledge to Dean Veterinary Faculty for their technical assistance with instruments and lab work. Authors are thankful to the College of Veterinary Science and Animal Husbandry, Anand for providing necessary instrumentations and consumables to carry out the study.

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