*Article*

# Prevalence and Multidrug Resistance Pattern of Methicillin Resistant *S. aureus* Isolated from Frozen Chicken Meat in Bangladesh

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**Abstract:** Infections by methicillin-resistant *Staphylococcus aureus* (MRSA) are continuously expanding within the community. Chicken meat is usually contaminated by MRSA, and this contaminated chicken meat is an important source of foodborne infections in humans. In this study, a cross-sectional supershop survey was conducted to determine the prevalence and antimicrobial resistance pattern of MRSA in 113 domestic frozen chicken meat samples purchased from nine branded supershops available in five divisional megacities of Bangladesh. The study also focused on the determination of methicillin resistance gene in MRSA isolates. *S. aureus* was identified by standard culture-based and molecular methods, and subjected to antimicrobial susceptibility testing. MRSA was screened by cefoxitin disk diffusion test. Methicillin resistance gene was identified by PCR. Of samples, 54.9% were positive for *S. aureus*, and, of these, 37.1% isolates were identified as MRSA. All the isolates were multidrug resistant (MDR): 52.2% were resistant to 6−8 antimicrobial classes, and 47.8% isolates to 9−12 classes. Three (3.2%) isolates of *S. aureus* were possible extensively drug resistant. The highest rates of resistance were observed against cefoxitin (100%), followed by nalidixic acid, ampicillin and oxacillin (97.7%), colistin (91.3%), amoxicillin-clavulanic acid and amoxicillin (87%), penicillin-G and cloxacillin (82.6%), oxytetracycline (78.3%) and cefixime (73.9%). Screening of methicillin resistance gene revealed that 43.5% isolates of MRSA were positive for *mecA* gene. The high prevalence of MDR MRSA in frozen chicken meat samples in this study emphasizes the need for better sanitary education of food handlers in hygienic practices focusing on their potential role as reservoirs and spreaders of MRSA.

**Keywords:** Methicillin-resistant *Staphylococcus aureus*; Multidrug resistance; *mecA* gene; Frozen chicken meat; Bangladesh

## 1. Introduction

Foodborne diseases (FBDs) are a significant general well-being concern worldwide [1]. The World Health Organization (WHO) characterizes FBD as "disease of infectious or toxic nature caused by, or thought to be caused by, the consumption of food or water" [1]. *Staphylococcus aureus* (*S. aureus*) is one of the most important cause of FBD, causing an expected 241,000 illnesses each year in the United States [2]. The food poisoning caused by *S. aureus* ranks in 3rd position worldwide [3]. *S. aureus* could be a commensal and timeserving infective agent which will cause wide spectrum of

infections, from superficial skin infections to severe, and probably fatal, invasive illness [4]. This omnipresent microorganism is a vital infective agent because of combination of "toxin-mediated virulence, invasiveness, and antimicrobial resistance (AMR)." This organism has arisen as a significant microbe for each health facility and community-acquired infections [5]. *S. aureus* doesn't form spores however will cause contamination of food products throughout food preparation and handling. *S. aureus* can grow in a very wide selection of temperatures (7 to 48.5°C; optimum 30 to 37°C), pH (4.2 to 9.3; optimum 7 to 7.5), and common salt concentration up to 15% NaCl [6]. *S. aureus* could be a desiccation tolerant organism with the capacity to survive in probably dry and unpleasant environments, like the human nose and on skin and inanimate surfaces, for example, clothing and surfaces [7]. These attributes favor development of the organism in numerous food items [1]. *S. aureus* will stay viable on hands and environmental surfaces for extended durations following initial contact [8,9].

FBDs caused by *S. aureus* are mostly resisting toward antibiotic therapy, which represents one of the major challenges to human health [5]. Epidemiological surveys have recognized that the *S. aureus* isolated from diverse kinds of foodstuffs, particularly those with chicken origins, harbored considerable incidence of resistance toward frequently used antimicrobial agents [5]. Moreover, in step with the sensitivity to antibiotic medication, *S. aureus* was divided into methicillin-sensitive *Staphylococcus aureus* (MSSA) and methicillin-resistant *Staphylococcus aureus* (MRSA) [10]. MRSA constitutes a big public health threat worldwide. The threat is probably going equal or more in developing countries like Bangladesh. Thus, the WHO recently classified MRSA as "high priority 2 pathogens" that are a threat to the health of humans and animals [5]. Raw and frozen chicken meat may contain MRSA as a result of carcass contamination during slaughtering, processing and storage time [6,10]. MRSA, which is present in chicken meat and meat products, has become a serious concern in food safety, and represent a major health care problem, since it is the etiologic agent of various nosocomial and community-acquired infections related to FBDs [5]. The magnitude of the general public health burden because of AMR in MRSA is complicated, and is influenced by variety of factors like antimicrobial use practices in farming, control measure at slaughter, storage and distribution systems, the supply of fresh water, and correct preparation and residential hygiene etc [11]. In recent decades, the abuse of antibiotics for therapy and prophylaxis may prompt the development of multidrug-resistance (MDR) of MRSA, and later these resistant microorganisms will enter into the food chains to cause human infections [6]. In recent past, MRSA isolated from chicken meat samples were reported to be highly resistant to a number of multiple antimicrobials such as penicillin, methicillin, oxacillin, cefoxitin, chloramphenicol and erythromycin that pose a big threat to the consumers' health [5,6,10]. The *mecA* gene originally identified in MRSA, which encodes a low-affinity penicillin-binding protein which is responsible for β-lactam resistance [12]. The *mecA* gene is broadly dispersed among *S. aureus* and other staphylococcal species, and its appearance is fundamental for the methicillin-resistant phenotype [12].

Recently, microbial food safety has gotten a significant public wellbeing concern around the world including Bangladesh since of its huge effect on food chain. Chicken meat had been widely consumed for its high value in protein and nutrient substance including low cost and availability. However, chicken meat is usually contaminated by antibiotic resistant strains of *S. aureus*, especially MRSA, mostly due to improper handling by food-handlers and poor sanitation practices, and thus pose a great risk in the food safety [11]. Contaminated chicken meat is one of the significant causes of

FBDs in humans worldwide. Research has been conducted in Bangladesh on *S. aureus* contamination and AMR patterns in raw chicken meat from live bird markets [13-15]. Nowadays, city dwellers of Bangladeshi consumers prefer to buy their all essential daily commodities including frozen chicken meat and meat products from supershops because of easy availability, and this frozen chicken meat takes minimal processing for cooking, and also save consumers time. However, contamination of frozen chicken meat with *S. aureus*, especially MRSA has an important concern of food safety and public health hazards in Bangladesh. One inland study reported *S. aureus* contamination in preprocessed raw chicken meat collected from three supershops of Dhaka city [16], and another study reported the contamination of processed raw meat with MRSA [17]. However, prevalence and MDR pattern of MRSA as well as methicillin resistance gene isolated from frozen chicken meat samples has not been investigated yet in Bangladesh. Therefore, particular attention is required to be paid of studying the prevalence and AMR patterns of MRSA isolated from frozen chicken meat covering more outlets of available branded supershops to ensure taking effective measure. So, the objective of the present study was to determine the prevalence of MRSA and their AMR pattern isolated from frozen chicken meat of different supershops across different divisional megacities in Bangladesh. This study also focused on the determination of methicillin resistance gene in MRSA isolates. Such information is useful for better understanding of the risk of exposure to MRSA through food, particularly the chicken meat.

## 2. Materials and Methods

### 2.1. Sample collection

From April to December 2019, a cross-sectional supershop survey was conducted in 40 outlets of nine different supershops in five divisional megacities (Dhaka, Chattogram, Sylhet, Rajshahi, and Mymensingh) of Bangladesh. A total of 113 domestic frozen chicken meat samples (82 broilers, 31 cockerels) including whole chicken or meat cuts (breast, drumstick, leg, and wing muscle) were purchased from these outlets. Each supershop had its own branded frozen chicken meat. On purchase, each sample was placed in a separate sterile tightly sealed plastic bag and kept in a cold box at a temperature lower than 4°C for transportation. On arrival at the laboratory, frozen chicken meat in the sealed plastic bag was placed in a cool area until it defrosted. In addition, data on brand name, source of chicken, processing and packaging of meat, and special labels (e.g., green or organic chickens) were also collected.

### 2.2. Isolation and identification of S. aureus

Isolation and identification of *S. aureus* were performed according to the EN ISO 6888-1 standard procedure of the International Organization for Standardization [18]. For pre-enrichment of bacteria, 25 g portion from each piece of meat sample was chopped into very small fine pieces, homogenized with 225 mL of buffered peptone water and incubated at 37°C for 18—24 h. After pre-enrichment in buffered peptone water, 1 mL of the culture was mixed with 5 mL of nutrient broth and incubated for 24 h at 37°C. Then, a loopful of culture broth was streaked onto Mannitol Salt Agar in duplicate and incubated at 37°C for 24 h. Three presumptive *S. aureus* yellow colour colonies with yellow zones from each selective agar plate were picked, and then subcultured to obtain a pure culture. Gram staining, catalase, and coagulase tests were performed with the pure

culture. All presumptive *S. aureus* isolates were subjected to DNA extraction using the "boiling" method as described earlier [19]. A duplex PCR was carried out for the confirmation of *S. aureus* with two sets of genus- and species-specific primers. Primers used were Staph756F (5'-AACTCTGTTATTAGGGAAGAACA-3') and Staph750R (5'-CCACCTTCCTCCGGTTTGTCACC-3' for the amplification of 756 bp from *16S rRNA* gene, and Nuc450-F (5'-AGTATATAGTGCAACTTCAACTAAA-3') and Nuc450-R (5'-ATCAGCGTTGTCTTCGCTCCAAATA-5') for the amplification of 450 bp from *nuc* (thermonuclease) gene [20,21]. The PCR reaction mixture (25 μL) consisted of 12.5 μL of OneTaq Quick-Load PCR master mix with standard buffer (New England BioLabs Inc., Ipswich, MA), 1.5 μL (15 pmol) each of forward and reverse primers, 2 μL of DNA template, and 4.5 μL of nuclease-free water. The thermal profile consisted of initial denaturation at 94°C for 5 min, 30 cycles of separation at 94°C for 1 min, primer annealing at 55°C for 1 min, and extension at 72°C for 2 min with a final elongation at 72°C for 10 min. After amplification, PCR products were analyzed by gel electrophoresis on 1.5% UltraPure™ Agarose gel stained with ethidium bromide (5 μg/mL) including a 100-bp DNA ladder (New England, BioLabs Inc.) which served as a molecular weight marker. The resulting band of PCR product was visualized under UV translluminator and photographed. All PCR-confirmed *S. aureus* isolates were stored on nutrient broth containing 50% (v/v) glycerol at –20°C for further study.

*2.3. Antimicrobial susceptibility testing*

The AMR profile of all *S. aureus* isolates was determined using the Kirby-Bauer disk diffusion method as described by the Clinical and Laboratory Standards Institute (CLSI) [22] with a panel of 38 antimicrobials representing 14 different antimicrobial classes. The antimicrobials commonly used for antimicrobial susceptibility testing include, the fluoroquinolones [nalidixic acid (NA, 30 μg), ciprofloxacin (CIP, 5 μg), levofloxacin (LEV, 5 μg), norfloxacin (NX, 10 μg), ofloxacin (OFX, 5 μg), gatifloxacin (GAT, 5 μg), pefloxacin (PEF, 5 μg)], non-extended spectrum cephalosporins [first-generation cephalosporins: cephalexin (CL, 30 μg), cephradine (CE, 30 μg); second-generation cephalosporins: cefuroxime (CXM, 30 μg), cefaclor (CEC, 30 μg)], extended-spectrum cephalosporins [third-generation cephalosporins: cefotaxime (CTX, 30 μg), ceftriaxone (CRO, 30 μg), ceftazidime (CAZ, 30 μg), cefixime (CFM, 5 μg); fourth-generation cephalosporins: cefepime (FEP, 30 μg)], cephamycins [cefoxitin (FOX, 30 μg)], carbapenems [imipenem (IPM, 10 μg), meropenem (MEM, 10 μg)], tetracyclines [tetracycline (TE, 30 μg), oxytetracycline (OT, 30 μg), doxycycline (DO, 10 μg)], penicillins [ampicillin (AM, 10 μg), amoxycillin (AX, 10 μg), penicillin-G (P, 10 units), methicillin (MET, 5 μg), oxacillin (OX, 1 μg), cloxacillin (CX, 5 μg)], penicillins + β-lactamase inhibitors [amoxicillin-clavulanic acid (AMC, 30 μg)], aminoglycosides [gentamicin (CN, 10 μg), neomycin (N, 30 μg), tobramycin (TOB, 10 μg)], folate pathway inhibitors [trimethoprim-sulfamethoxazole (SXT, 25 μg), lincosamide [clindamycin (DA, 2 μg)], glycopeptides and lipoglycopeptides [vancomycin (VA, 30 μg)], macrolides [azithromycin (AZM, 15 μg), erythromycin (E, 15 μg)], and polymyxins [colistin (CT)]. For colistin, minimum inhibitory concentrations (MICs) were determined by broth microdilution method, according to the CLSI guidelines [22]. The interpretive category (susceptible, intermediate, and resistant) of each isolate was determined according to the CLSI guidelines [22], and in some cases, of European Committee on Antimicrobial Susceptibility Testing (EUCAST) [23]. Isolates resistant to at least one agent in

three or more antimicrobial classes were defined as multidrug resistant (MDR) while isolates resistant to at least one agent in all but two or fewer antimicrobial classes were defined as possible extensively drug resistant (pXDR) [24].

### 2.4. Screening of methicillin-resistant S. aureus (MRSA)

Phenotypically, MRSA were detected by Cefoxitin Disk Diffusion method as per CLSI guidelines [22]. Briefly, for each isolate, a minimum of four to five *S. aureus* colonies isolated from an overnight growth were transferred to nutrient broth. Bacterial suspensions in nutrient broth at a density equivalent to a 0.5 McFarland standard was inoculated onto Mueller–Hinton agar plate in duplicate with cefoxitin (30 μg), oxacillin (1 μg), cloxacillin (5 μg) and  methicillin (5 μg) disk. The plates were incubated at 37°C for 24 h. The isolates of *S. aureus* that showed resistance to cefoxitin (≤ 21 mm zone diameter) were considered MRSA.

### 2.5. Detection of methicillin resistance gene

A uniplex PCR targeting methicillin resistance gene (*mecA*) in *S. aureus* was standardized, and used in this study with specific primer as described earlier [21]. The sequence of the forward primer was MecA1 (5'-GTAGAAATGACTGAACGTCCGATAA-3) and of the reverse primer was MecA2 (5'-CCAATTCCACATTGTTTCGGTCTAA-3') for the amplification of 585 bp. Each PCR reaction mixture was constituted in a final reaction mixture of 25 μL made up of 12.5 μL PCR master mix (New England, BioLabs Inc.), 1.5 μL (15 pmol) each of forward and reverse primers, 7.5 μL of nuclease-free water, and 2 μL of DNA template. Amplification was performed by using a Veriti 96-well thermal cycler (Thermo Fisher Scientific) with this thermal profile: heating at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 1 min, primer annealing at 55°C for 1 min, extension at 72°C for 2 min, and a final extension step for 10 min at 72°C. Positive (*mecA* gene) and negative (sterile phosphate buffer saline) controls were included in each run of PCR. After amplification, PCR product was subjected to electrophoresis on 1.5% UltraPure™ agarose gel containing ethidium bromide (0.5 μg/mL). The resulting band of PCR product was examined under UV-transilluminator and photographed. A 100-bp molecular weight standard ladder was included on each run.

### 2.6. Data analyses

Data were extracted, and entered into spread sheet (Microsoft Excel) and transferred into SPSS software v22.0 (IBM Corp., Armonk, N.Y., USA) for statistical analysis. Descriptive statistics were used to compute the prevalence of *S. aureus* and resistance percentage. The significant differences in prevalence of *S. aureus* and resistance percentage among different brands, sampling area, chicken types, production types, and meat types were determined using chi-square test (Z-test for proportions) and Fisher's exact test (wherever appropriate). The association between resistance phenotypes (outcome) and their corresponding methicillin resistance genes was analysed by binary logistic regression. The level of significance was set at $p < 0.05$. An UpSet plot was constructed to show the antimicrobial resistance pattern of methicillin- resistant *S. aureus* and methicillin-susceptible *S. aureus* by using online tools (https://asntech.shinyapps.io/intervene/).

## 3. Results

*3.1. Prevalence and distribution of methicillin-resistant S. aureus and methicillin-susceptible S. aureus*

Among 113 frozen chicken meat samples, the overall prevalence of *S. aureus* was 54.9% (n = 62) (Table 1). All isolates of *S. aureus* produced expected product size of 756 bp by PCR (Figure 1). Of the 62 *S. aureus* isolates, 23 (37.1%) were phenotypically identified as methicillin-resistant *S. aureus* (MRSA) based on cefoxitin disc diffusion test, and the remaining 39 (62.9%) isolates were methicillin-susceptible *S. aureus* (MSSA) (Table 1).
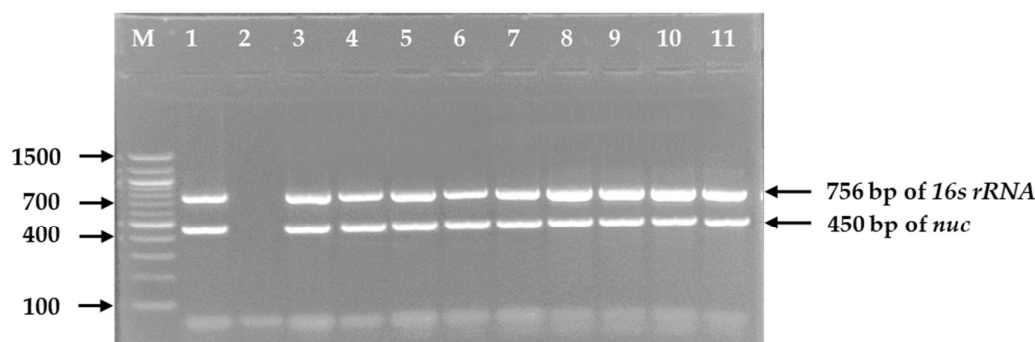


**Figure 1.** PCR amplified products of 756 bp of *16s rRNA* gene of *Staphylococcus* spp. and 450 bp of *nuc* gene of *S. aureus* in 1.5% agarose gel electrophoresis and ethidium bromide staining.
**Legends:** M = DNA marker (100 bp), Lane 1 = Positive control of *S. aureus*, Lane 2 = Negative control, Lane 3-11 = Tested *S. aureus* isolates.

The distribution of MRSA and MSSA related to brands, divisions, chicken types, production types, and meat types is also summarized in Table 1. Among the 9 brands, the prevalence of *S. aureus* from brand 7 (100%) was highest than those from brand 4 (11.3%). *S. aureus* was not recovered from frozen chicken meat of brand no. 8 and brand no. 9. The prevalence of MRSA among different brands varied from 36.8% to 50%, whereas the prevalence of MSSA varied from 50% to 100%. A significant difference was observed in the prevalence of MSSA among brands. Regarding division-wise distribution, the prevalence of MRSA was significantly higher in Chattogram division (66.7%) than Sylhet (33.3%) and Dhaka divisions (38.3%), while the highest prevalence of MSSA was found in Mymensingh and Rajshahi divisions (100%) than Chattogram division (33.3%). Chicken type-wise distribution revealed that there was significant difference in the prevalence of both MRSA and MSSA between broiler and cockerel chickens. MRSA prevalences in broiler and cockerel chickens were 45.2% and 20%, respectively, whereas the MSSA prevalences were 54.8% and 80%, respectively. When looking at the production type-wise distribution of MRSA and MSSA, there was no significant difference between production types. Meat type-wise distribution revealed that 100% of leg muscles were contaminated with MRSA, however, 73.9% of whole chicken pool sample were contaminated with MSSA.

**Table 1.** Prevalence and distribution of MRSA and MSSA isolated from frozen chicken meat.

| Variables | Total no. of samples | No. (%) of *S. aureus*-positive isolates | MRSA, no. (%) | MSSA, no. (%) |
|---|---|---|---|---|
| **Brands** | | | | |
| Brand 1 | 23 | 14 (60.9) | 7 (50.0)[a] | 7 (50.0)[a] |
| Brand 2 | 40 | 19 (47.5) | 7 (36.8)[a] | 12 (63.2)[a] |
| Brand 3 | 28 | 15 (53.6) | 6 (40.0)[a] | 9 (60.0)[a] |
| Brand 4 | 8 | 7 (11.3) | 3 (42.9)[a] | 4 (57.1)[a] |
| Brand 5 | 2 | 1 (50.0) | 0 | 1 (100.0)[b] |
| Brand 6 | 2 | 1 (50.0) | 0 | 1 (100.0)[b] |
| Brand 7 | 5 | 5 (100.0) | 0 | 5 (100.0)[b] |
| Brand 8 | 3 | 0 | 0 | 0 |
| Brand 9 | 2 | 0 | 0 | 0 |
| **Divisions** | | | | |
| Dhaka | 82 | 47 (57.3) | 18 (38.3)[a] | 29 (61.7)[a] |
| Chattogram | 10 | 6 (60.0) | 4 (66.7)[b] | 2 (33.3)[c] |
| Sylhet | 11 | 3 (27.3) | 1 (33.3)[a] | 2 (66.7)[a] |
| Mymensingh | 5 | 5 (100.0) | 0 | 5 (100.0)[b] |
| Rajshahi | 5 | 1 (20.0) | 0 | 1 (100.0)[b] |
| **Chicken types** | | | | |
| Broiler | 82 | 42 (51.2) | 19 (45.2)[a] | 23 (54.8)[b] |
| Cockerel | 31 | 20 (64.5) | 4 (20.0)[b] | 16 (80.0)[a] |
| **Production types** | | | | |
| Organic | 10 | 8 (80.0) | 4 (50.0)[a] | 4 (50.0)[a] |
| Non organic | 103 | 54 (52.4) | 19 (35.2)[a] | 35 (64.8)[a] |
| **Meat types** | | | | |
| Breast | 27 | 13 (48.1) | 5 (38.5)[ab] | 8 (61.5)[a] |
| Drumstick | 30 | 14 (46.7) | 5 (35.7)[ab] | 9 (64.3)[a] |
| Leg | 3 | 3 (100.0) | 3 (100.0)[c] | 0 |
| Wing | 19 | 9 (47.4) | 4 (44.4)[a] | 5 (55.6)[a] |
| Whole chicken pool sample | 34 | 23 (67.6) | 6 (26.1)[b] | 17 (73.9)[a] |
| Total | 113 | 62 (54.9) | 23 (37.1) | 39 (62.9) |

[a,b,c]Values in the same column with different superscripts differ significantly ($p \leq 0.05$).

*3.2. Antimicrobial resistance pattern*

Of the 62 *S. aureus* isolates, two isolates (3.2%) were possible extensively drug resistant (pXDR); it showed resistance to 11 to 12 of 14 antimicrobial classes. Regarding brand-wise distribution, it was observed that the pXDR *S. aureus* isolates were mostly observed in brand 1 and brand 3.

The overall MDR patterns of MRSA and MSSA have shown in Table 2.   Among 62 *S. aureus* (both MRSA and MSSA) tested, all the isolates were MDR. It was revealed that more than half (52.2%

for MRSA, and 53.8% for MSSA) of the isolates were resistant to 6–8 antimicrobial classes while 35.9% of MSSA isolates were resistant to 3–5 classes. Notably, 47.8% of MRSA and 10.3% of MSSA isolates were resistant to 9–12 classes. The distribution of MRSA and MSSA resistant to multiple antimicrobial classes showed that the cent percent isolates, recovered from brand 4 in case of MRSA, and brand 5 in case of MSSA, were resistant to 6–8 antimicrobial classes. Furthermore, 85.7% MRSA isolates from brand 1, and 100% MSSA isolates from brand 6 showed resistance to 9–12 and 3–5 antimicrobial classes, respectively. All the MRSA isolates from Sylhet division and 75% from Chattogram division were resistant to 9–12 classes of antimicrobials. On the other hand, 100% MSSA isolates, resistant to 3–5 and 6–8 antimicrobial classes, were observed in Sylhet and Rajshahi, and Chattogram divisions, respectively. Regarding chicken types, it was observed that 57.9% of MRSA isolates from broiler chicken meat, and 75% isolates from cockerel chicken meat were resistant to 6–8 and 9–12 antimicrobial classes, respectively. In case of MSSA, ≥ 50% of the isolates from broiler and cockerel chickens expressed resistance to 6–8 classes of antimicrobials. As for production types, 75% of MRSA and MSSA isolates from organically produced chickens were resistant to 6–8 and 3–5 antimicrobial classes, respectively, while more than 50% isolates of MRSA and MSSA from non-organically produced chickens were resistant to 9–12 and 6–8 classes, respectively. The meat type-wise analysis revealed that the highest resistance to 9–12 antimicrobial classes was observed in leg muscles (100%) in case of MRSA while the resistance to 6–8 classes was higher in wing muscles (80%) in case of MSSA.
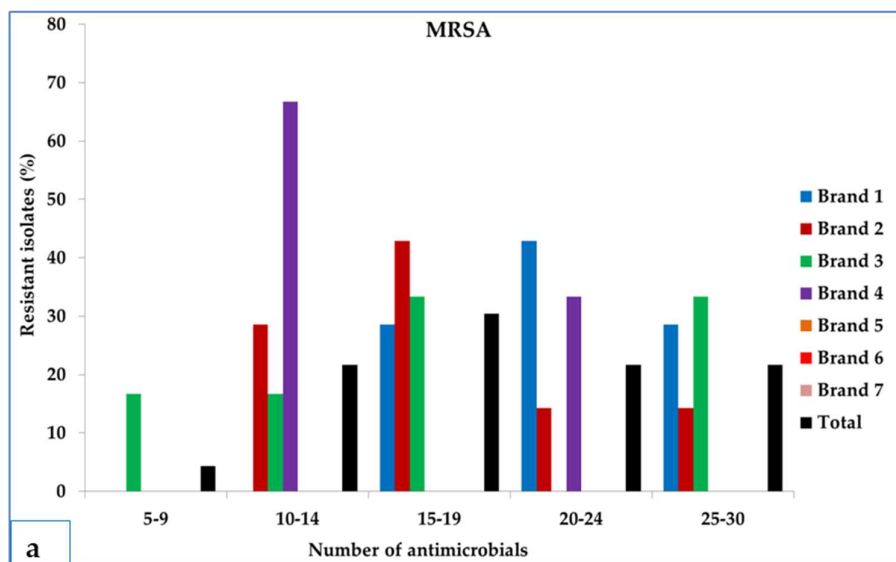
**Table 2.** Distribution of MRSA and MSSA isolates resistant to multiple classes of antimicrobials.

| Variables | No. (%) of isolates resistant to multiple antimicrobial classes | | | | | |
|---|---|---|---|---|---|---|
| | MRSA | | | MSSA | | |
| | 3–5 | 6–8 | 9–12 | 3–5 | 6–8 | 9–12 |
| **Brands** | | | | | | |
| Brand 1 | 0 | 1 (14.3)[b] | 6 (85.7)[a] | 1 (14.3)[b] | 5 (71.4)[a] | 1 (14.3)[b] |
| Brand 2 | 0 | 4 (57.1)[b] | 3 (42.9)[b] | 5 (41.7)[c] | 7 (58.3)[ab] | 0 |
| Brand 3 | 0 | 4 (66.7)[b] | 2 (33.3)[b] | 4 (44.4)[c] | 5 (55.6)[ab] | 0 |
| Brand 4 | 0 | 3 (100.0)[a] | 0 | 3 (75.0)[a] | 1 (25.0)[bc] | 0 |
| Brand 5 | 0 | 0 | 0 | 0 | 1 (100.0)[d] | 0 |
| Brand 6 | 0 | 0 | 0 | 1 (100.0)[a] | 0 | 0 |
| Brand 7 | 0 | 0 | 0 | 0 | 2 (40.0)[b] | 3 (60.0)[a] |
| **Divisions** | | | | | | |
| Dhaka | 0 | 11 (61.1)[a] | 7 (38.9)[b] | 11 (37.9)[b] | 17 (58.6)[b] | 1 (3.4)[b] |
| Chattogram | 0 | 1 (25.0)[b] | 3 (75.0)[a] | 0 | 2 (100.0)[a] | 0 |
| Sylhet | 0 | 0 | 1 (100.0)[a] | 2 (100.0)[a] | 0 | 0 |
| Mymensingh | 0 | 0 | 0 | 0 | 2 (40.0)[b] | 3 (60.0)[a] |
| Rajshahi | 0 | 0 | 0 | 1 (100.0)[a] | 0 | 0 |
| **Chicken types** | | | | | | |
| Broiler | 0 | 11 (57.9)[a] | 8 (42.1)[b] | 10 (43.5)[a] | 13 (56.5)[a] | 0 |
| Cockerel | 0 | 1 (25.0)[b] | 3 (75.0)[a] | 4 (25.0)[b] | 8 (50.0)[a] | 4 (25.0)[a] |
| **Production types** | | | | | | |
| Organic | 0 | 3 (75.0)[a] | 1 (25.0)[b] | 3 (75.0)[a] | 1 (25.0)[b] | 0 |

| Variables | No. (%) of isolates resistant to multiple antimicrobial classes | | | | | |
|---|---|---|---|---|---|---|
| | MRSA | | | MSSA | | |
| | 3–5 | 6–8 | 9–12 | 3–5 | 6–8 | 9–12 |
| Non-organic | 0 | 9 (47.4)[b] | 10 (52.6)[a] | 11 (31.4)[b] | 20 (57.1)[a] | 4 (11.4)[a] |
| Meat types | | | | | | |
| Breast | 0 | 3 (60.0)[ab] | 2 (40.0)[ab] | 4 (50.0)[a] | 4 (50.0)[b] | 0 |
| Drumstick | 0 | 3 (60.0)[ab] | 2 (40.0)[ab] | 4 (44.4)[a] | 5 (55.6)[b] | 0 |
| Leg | 0 | 0 | 3 (100.0)[c] | 0 | 0 | 0 |
| Wing | 0 | 3 (75.0)[a] | 1 (25.0)[a] | 1 (20.0)[b] | 4 (80.0)[a] | 0 |
| Whole chicken | 0 | 3 (50.0)[b] | 3 (50.0)[b] | 5 (29.4)[ab] | 8 (47.1)[b] | 4 (23.5)[a] |
| Total | 0 | 12 (52.2) | 11 (47.8) | 14 (35.9) | 21 (53.8) | 4 (10.3) |

[a,b,c,d]Values in the same column with different superscripts differ significantly ($p \leq 0.05$); Samples from brand 8 and brand 9 were negative for *S. aureus*.

Among 62 *S. aureus* isolates (both MRSA and MSSA), all the isolates were resistant to at least five, and up to 30 antimicrobial agents tested (Figure 2a-b). Regarding MRSA, 30.4% isolates were resistant to 15–19 antimicrobials, 21.7% isolates were resistant to 10–14 antimicrobials, and 4.3% isolates were resistant to 5–9 antimicrobials (Figure 2a). Notably, a noticeable number of MRSA isolates (10, 43.4%) demonstrated resistance to 20–30 antimicrobial agents (Figure 2a). For MSSA, resistance to 5–9 and 15–19 antimicrobials were observed in 30.8%, and 28.2% isolates, respectively (Figure 2b). Of note, 7 (18%) isolates showed resistance to 20–30 antimicrobial agents (Figure 2b). Analysis by brands revealed that comparatively higher percentages of MRSA isolates demonstrated resistance to 10–14 antimicrobials in brand 4, followed by 15–19 antimicrobials in brand 2 and 20–24 antimicrobials in brand 1 than other brands (Figure 2a). On the contrary, the highest resistance to 5–9 and 15–19 antimicrobial agents was observed in brand 6 and brand 5, respectively, whereas resistance to 20–24 antimicrobial agents was higher in brand 7 (60%) in case of MSSA (Figure 2b).
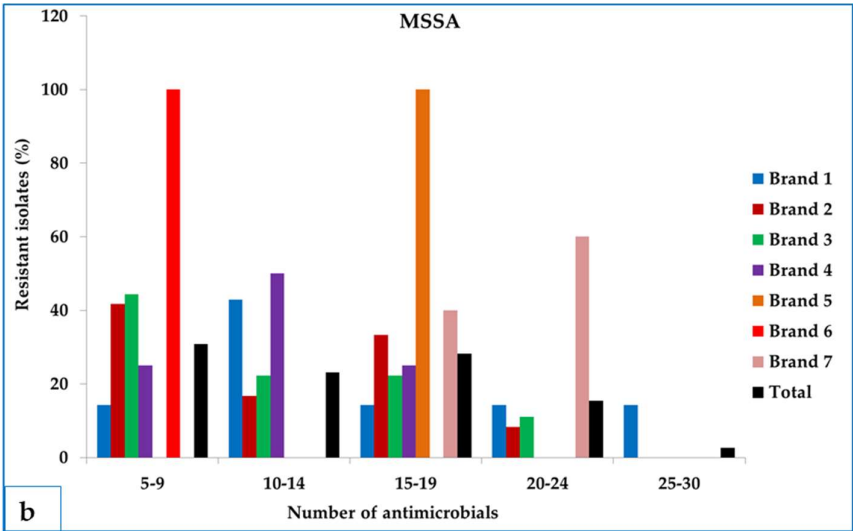
**Figure 2a-b.** Brand-wise distribution of (a) MRSA and (b) MSSA isolates resistant to multiple antimicrobial agents isolated from frozen chicken meat.

Overall individual antimicrobial resistance pattern of MRSA and MSSA has shown in Figures 3-4. Among MRSA isolates, the resistance to cefoxitin (100%, n = 23), nalidixic acid, ampicillin and oxacillin (97.7%, n = 22), colistin (91.3%, n = 21),   amoxicillin-clavulanic acid and amoxicillin (87%, n = 20), penicillin-G and cloxacillin (82.6%, n = 19), oxytetracycline (78.3%, n = 18) and cefixime (73.9%, n = 17) were found highest, whereas, resistance to vancomycin (4.3%, n = 1), and neomycin and gentamicin (8.7%, n = 2) were observed lowest (Figure 3).
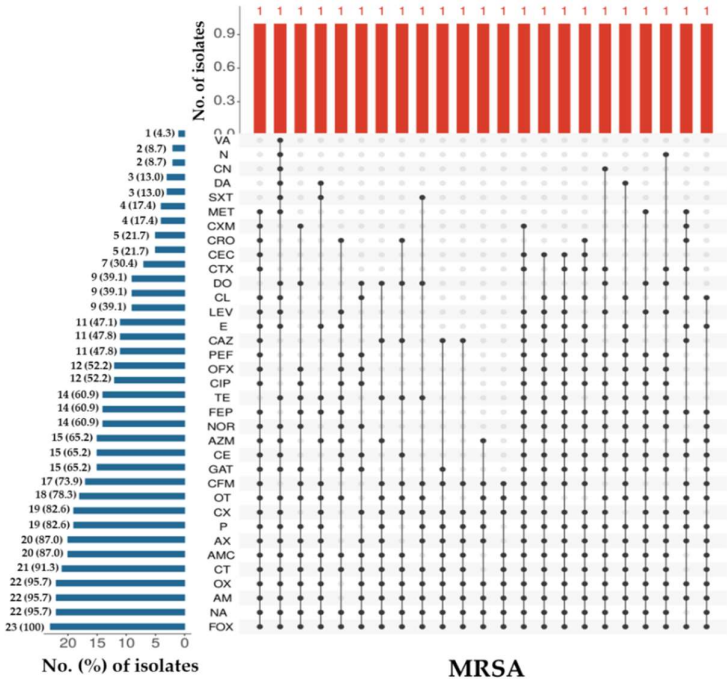


**Figure 3.** Individual antimicrobial resistance pattern of methicillin-resistant *S. aureus* (MRSA) isolated from frozen chicken meat.

For MSSA, the highest individual AMR was observed against amoxicillin (97.4%, n = 38), oxacillin (92.3%, n = 36), ampicillin (89.7%, n = 35), colistin (87.2%, n = 34), penicillin-G (79.5%, n = 31), and nalidixic acid and oxytetracycline (76.9%, n = 30). Low resistance was observed to meropenem and cefaclor (2.6%, n = 1), and trimethoprim-sulfamethoxazole (5.1%, n = 2) (Figure 4). When looking at the AMR patterns, 23 different resistance patterns were observed for MRSA isolates, and 37 different resistance patterns were observed for MSSA isolates (Figure 3-4). The most frequent AMR patterns observed among the MSSA isolates were AX-OX-AM-CT-P-NA-OT-CFM-CX (n = 2) and AX-OX-AM-CT-P-OT-TE-CFM-CX (n = 2) (Figure 4).
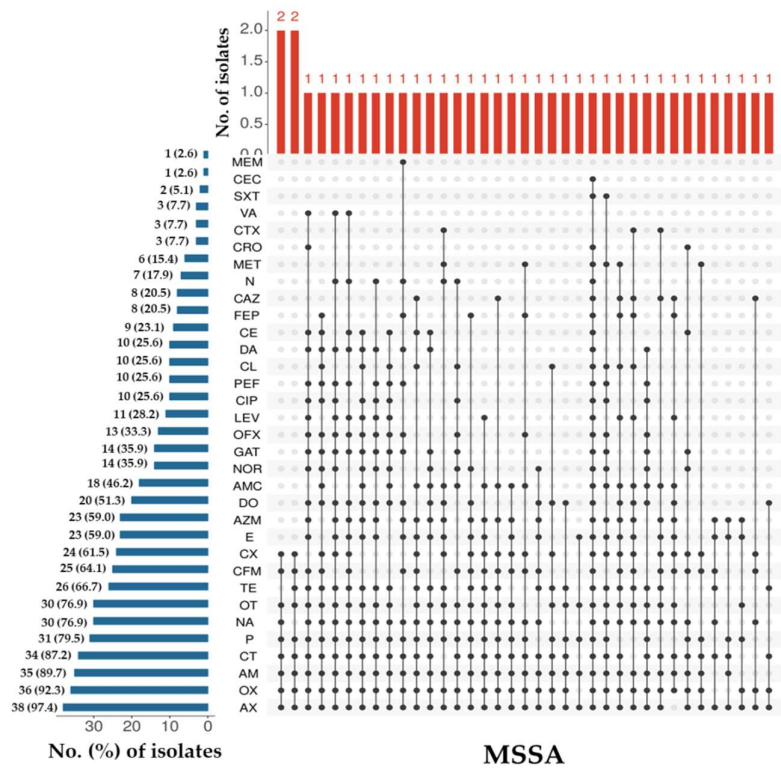


**Figure 4.** Individual antimicrobial resistance pattern of methicillin-susceptible *S. aureus* (MSSA) isolated from frozen chicken meat.

*3.3. Prevalence of mecA gene*

Among 23 MRSA and 39 MSSA, 10 (43.5%) and 17 (43.6%) isolates, respectively were positive for *mecA* gene as they generated expected size of 310 bp on amplification (Figure 4).
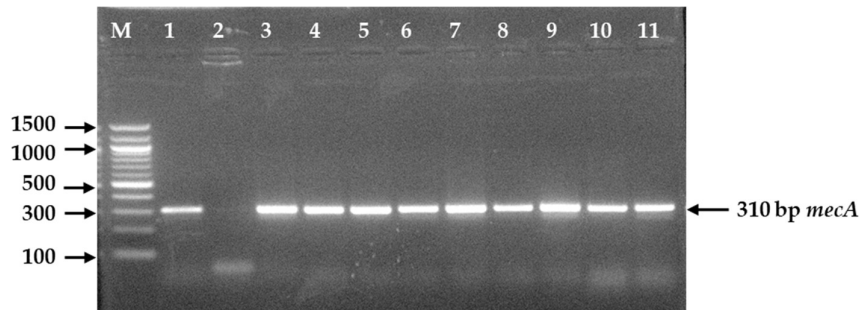


**Figure 4.** PCR amplified product of 310 bp from *mecA* gene of MRSA and MSSA following 1.5% agarose gel electrophoresis and ethidium bromide staining.

**Legends:** M = DNA marker (100 bp), Lane 1 = Positive control of *mecA* gene, Lane 2 = Negative control, Lane 3-11 = Positive for *mecA* gene.

### 3.4. Phenotypic and genotypic association of antimicrobial resistance

In case of MRSA, among the 7 isolates resistant to cefotaxime, 6 isolates (85.7%) carried *mecA* gene (Table 3). For the 15 isolates resistant to gatifloxacin and azithromycin, *mecA* gene was detected in 9 isolates (60%). Eight (8/12, 66.7%) of the ciprofloxacin and ofloxacin resistant isolates also carried *mecA* gene. However, these phenotypes were positively associated (OR > 1) with the presence of *mecA* gene. For MSSA, a certain percentage of isolates (10/31, 32.3%) resistant to penicillin-G harbored the *mecA* gene (Table 3). Penicillin-G resistance was negatively associated with the presence of *mecA* gene (OR = 0.07, p = 0.02).

**Table 3.** Association between antimicrobial resistance phenotypes and methicillin resistance *mecA* gene in MRSA and MSSA isolated from frozen chicken meat.

| Antimicrobials | $N_P$ | ARG | P+/G+ | P+/G- | P-/G+ | P-/G- | $N_G$ | OR | 95% CI | p-value[a] |
|---|---|---|---|---|---|---|---|---|---|---|
| **MRSA** | | | | | | | | | | |
| Cefotaxime | 7 | *mecA* | 6 | 1 | 4 | 12 | 10 | 18.0 | 1.6-198.5 | 0.02 |
| Norfloxacin | 14 | *mecA* | 9 | 5 | 1 | 8 | 10 | 14.4 | 1.4-150.8 | 0.03 |
| Ciprofloxacin | 12 | *mecA* | 8 | 4 | 2 | 9 | 10 | 9.0 | 1.3-63.0 | 0.03 |
| Gatifloxacin | 15 | *mecA* | 9 | 6 | 1 | 7 | 10 | 10.5 | 1.0-108.6 | 0.05 |
| Pefloxacin | 11 | *mecA* | 8 | 3 | 2 | 10 | 10 | 13.3 | 1.8-100.1 | 0.01 |
| Ofloxacin | 12 | *mecA* | 8 | 4 | 2 | 9 | 10 | 9.0 | 1.3-63.0 | 0.03 |
| Azithromycin | 15 | *mecA* | 9 | 6 | 1 | 7 | 10 | 10.5 | 1.0-108.6 | 0.05 |
| **MSSA** | | | | | | | | | | |
| Penicillin-G | 31 | *mecA* | 10 | 21 | 7 | 1 | 17 | 0.07 | 0.01-0.63 | 0.02 |

$N_P$: No. of isolates expressing phenotypic resistance to the indicated antimicrobials.

P+/G+: No. of phenotypically resistance isolates (P+) with resistance genes (G+) for antimicrobials identified.

P+/G-: No. of phenotypically resistance isolates (P+) with no resistance genes (G-) for antimicrobials identified.

P-/G+: No. of phenotypically susceptible isolates (P-) with resistance genes (G+) for antimicrobials identified.

P-/G-: No. of phenotypically susceptible isolates (P-) with no resistance genes (G-) for antimicrobials identified.

$N_G$: No. of isolates carrying the indicated resistance gene.

[a]Only statistically significant (p ≤ 0.05) associations are shown.

ARGs = Antimicrobial resistance genes; OR = Odds ratio; CI = Confidence interval.

## 4. Discussion

The present study provides the first comprehensive evidence on the extent and distribution of methicillin-resistant *S. aureus* (MRSA) and their AMR profile including methicillin resistance gene (*mecA*) isolated from frozen chicken meat in Bangladesh. Around 55% of frozen chicken meat collected from almost all branded supershops located in five divisional megacities of Bangladesh was found positive for *S. aureus*, and of them 37% were identified as MRSA. The findings of the present study demonstrated that the prevalence of MRSA was relatively higher than that of 5.6% prevalence in processed raw meat samples as reported earlier in Bangladesh [17]. Compared with studies from other countries, a variable prevalence of MRSA (5.6% to 8.1%) in frozen chicken meat was reported in Egypt, China and Hong Kong [6,10,25]. Attribute to the sample sizes, sample types, slaughterhouse sanitation during handling, meat handler's hygiene, and geographic locations of investigation may be the reason for these differences [10]. The present study demonstrated that the prevalence of MRSA as well as methicillin-susceptible *S. aureus* (MSSA) varied among different brands of frozen chicken meat. The differences in the prevalence among brands could be because of differences in the personal hygienic practices and sanitation levels of each brand. The highest distribution of MRSA was observed in the Chattogram division than Sylhet and Dhaka divisions of Bangladesh, which is in contrast with a previous report, where a comparatively lower percentage of MRSA contamination was observed in processed raw meat samples in Dhaka division of Bangladesh [17]. On the contrary, the prevalence of MSSA was higher in Mymensingh and Rajshahi divisions than Chattogram division. In previous investigation on frozen chicken rinse samples, the prevalence of MSSA was observed to be higher in Chattogram division of Bangladesh [26]. The chicken types (broiler vs cockerel) had significant effect on the prevalence of MRSA and MSSA, however, production types (organic vs non-organic) had no significant effects on the occurrence of MRSA and MSSA. Within the present study, a variable degree of contamination with MRSA was found among different types of meat samples. Contamination of frozen chicken meat with MRSA can easily occur due to poor hygienic practices of meat handlers during slaughtering, or processing and packaging of chicken meat. In spite of the fact that the nasal area is viewed as the primary site of colonization with *S. aureus*, these organisms are also present in the gastrointestinal tract of chickens [27]. During slaughtering, carcasses may get contaminated by the contents of the intestinal tract, from the slaughtering environment or even by MRSA-infected handlers who have direct contact with carcasses or meat [28].

Considering the obvious importance of *S. aureus* as foodborne pathogen, and the worldwide emergence of MDR in this foodborne bacterium, we screened the AMR profiles of *S. aureus* isolated from frozen chicken meat. An important finding of concern in this study is that all the isolates of MRSA and MSSA were MDR (resistant to 3 or more classes of antimicrobials), of which a significant proportion of the isolates were resistant to 6–8 and 9–12 antimicrobial classes, which is in contrast with previous observation in Bangladesh, where 37% MRSA isolates recovered from processed raw meat showed MDR [17]. The high prevalence of MDR MRSA and MSSA in frozen chicken meat was related with the type of brands, which might be due to brand level differences in production, and handling including usage of antimicrobials. The majority of MRSA isolates from Sylhet and Chattogram divisions showed MDR, which is inconsistent with the previous report in Bangladesh [17]. Of note, the current study additionally found that 3.2% of *S. aureus* isolates were possible extensively drug-resistant (pXDR). A report from India depicts the development of an extensively

drug-resistant *S. aureus* isolates in humans [29]. The high percentage of MDR and existence of pXDR may be due to the result from random chromosomal mutations and transfer of resistance genes via conjugation and transformation of the resistance transfer factor and resistance determinants or could be considered a preliminary evidence suggesting the extensive use of antimicrobial agents in veterinary and medical practices for the control of bacterial diseases [30]. Another conceivable clarification is that the high prevalence of MDR might be credited to the cross-contamination during slaughtering [28].

Clinical management of Staphylococcal disease is depended on antimicrobial treatment which frequently fails due to forceful resistance of organisms to antimicrobials. We found that all the isolates in this study were resistant to at least five antimicrobial agents, and 43.4% isolates of MRSA, and 18% isolates of MSSA were resistant to 20–30 antimicrobial agents. A high percentage of antimicrobial-resistance among MRSA and MSSA, which is in disagreement with the previous report in China, in which 2.3% of *S. aureus* isolates in frozen chicken meat was resistant to 16–24 antimicrobials [6]. This may be due to the fact that the indiscriminate uses of antimicrobial agents in poultry production for therapy, prophylaxis and growth promotion, along with poor biosecurity and waste management systems accelerate the emergence of antimicrobial resistant pathogens, which may be implicated in foodborne antimicrobial resistant bacterial infection in humans.

Although antimicrobial use is a fundamental technique for control of *S. aureus* infection, especially for MRSA, attributable to its formidable ability to adapt to variable environmental conditions, this organism has an extraordinary capability to quickly get resistant to essentially all antimicrobials [31]. In the current study, we observed that all the MRSA isolates were resistant to cefoxitin. This prevalence was higher than the finding of earlier study in Bangladesh, where the author reported 33.3% isolates showed resistance to cefoxitin [17]. Resistance to penicillin-G, amoxycillin, ampicillin and oxacillin was the most common among MRSA and MSSA, which is consistent with previous report conducted in China [6]. These prevalences were higher than the finding of earlier study in Central Africa [32]. *S. aureus* is well known to express the highest resistance to penicillins antimicrobial class, and penicillins resistance by Gram-positive bacteria has been reported since 1940 [31]. Nalidixic acid and oxytetracycline resistance were also commonly observed among MRSA and MSSA isolates. These prevalences were higher than the findings of earlier studies on frozen chicken meat in China and Central Africa [6,32]. This is not surprising, because oxytetracycline is one of the most commonly used antibiotics for treatment of infections in poultry and humans without basic programs and restrictive policies on the use of this antibiotics, therefore, very frequent occurrence of resistance in MRSA and MSSA are probably a consequence of this. On the other hand, quinolone resistance among *S. aureus* emerged quickly, a lot of conspicuously among the methicillin-resistant strains. As a result, the capacity to use fluoroquinolones as antistaphylococcal agents was dramatically diminished. The explanations for the difference in rates of quinolone resistance between MRSA and MSSA strains are unsure. One causative issue is probably going antibiotic selective pressure, particularly within the hospital setting, leading to the choice and spread of the more antibiotic resistant MRSA strains [33]. Of note, in the present study, resistance to colistin, last-resort antimicrobials used for human therapy, was detected in 91% MRSA and 87% MSSA isolates, might be due to the chromosomal mutations through amino acid substitution [34]. Furthermore, environmental, meat processing, and human hygiene related factors may have an effect on AMR of *S. aureus* isolated from frozen chicken meat

samples. On the other hand, MRSA isolates in this study showed relatively low resistance to vancomycin, and MSSA isolates to meropenem and cefaclor, which may be due to the fact that these antimicrobials do not have veterinary preparations, and are not available for veterinary use, and also are not routinely used in clinical setting in Bangladesh.

MRSA has become a serious concern in food safety, and constitutes a major health care problem [5]. *S. aureus* isolates were designated as MRSA based on the presence of the methicillin resistance gene *mecA*. Interestingly, the frequency of *mecA* gene in both MRSA and MSSA recovered from frozen chicken meat reached around 44% in the current study. Our result contrast sharply with data published in Egypt and China, reported that 5.6% and 8.6%, respectively of the *S. aureus* isolates from frozen chicken meat were *mecA* gene positive, suggesting that the presence of *mecA* gene is the principal evidence for the detection of MRSA isolate [6,10]. However, harboring *mecA* gene is not sufficient for methicillin resistance, because some *S. aureus* isolates that contain the *mecA* gene are still shown to be susceptible to methicillin [35].

This study also described the association between AMR phenotypes and presence of *mecA* gene in MRSA and MSSA isolated from frozen chicken meat samples. Among the 23 MRSA and 39 MSSA isolates, we observed that the occurrence of methicillin resistance gene (*mecA*) was found not only in phenotypically resistant isolates but also in phenotypically non-resistant isolates. The association between the AMR and the presence of *mecA* gene in MRSA and MSSA might be because of colocalization of resistance gene on the same genetic elements, and the possible co-selection of many resistance genes by a single antimicrobial [36]. However, resistance genes can be linked to genetic elements, and the use of a particular antimicrobial can select for resistance not only to its own, but also potentially to a variety of other antimicrobials [36]. Since methicillin resistance gene alone is insufficient to confer resistance, further mechanisms are likely associated with the resistance phenotype of MRSA and MSSA strains.

It would be worthy if samples were taken from more outlets of various supershops, however, frozen chicken meat were purchased from most of the renowned supershops situated in five divisional megacities of Bangladesh; in this manner, the information are illustrative of the entire of Bangladesh.

## 5. Conclusions

To our knowledge, this is the first thorough study on the prevalence of MRSA in frozen chicken meat samples from different supershops of Bangladesh, and is the only study describing the presence of MDR, pXDR and *mecA* gene in meat samples. This study reported a relatively high prevalence of MRSA and high rates of MDR amongst the isolates from frozen chicken meat samples, thus indicating the potential role of chicken meat in the dissemination of MDR MRSA strains in Bangladesh and highlighting the health risks for consumers. The high prevalence of MDR MRSA in frozen chicken meat samples emphasizes the need for better sanitary education of food handlers in hygienic practices focusing on their potential role as reservoirs and spreaders of MRSA. Hygienic measures should be taken to ensure the safety of food products, and a proper risk assessment should be conducted to further clarify the possible health hazard for consumers.

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