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Original Article

Detection and Molecular Characterization of Methicillin-Resistant *Staphylococcus aureus* from Table Eggs in Haripur, Pakistan

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Abstract

Table eggs are nutritionally important food consumed globally. Despite being protected inside the hard shell and a semipermeable membrane, the egg contents may be contaminated with microbes and thus become a possible carrier of infectious agents to humans. A number of medically significant bacterial species such as *Salmonella enterica, Listeria monocytogenes*, and *Yersinia enterocolitica* have already been reported from table eggs. More important is the presence of antimicrobial-resistant bacterial strains in this food source. The present study was aimed at detection and characterization of *Staphylococcus aureus* from table eggs collected from different retail shops in Haripur city of Pakistan. Staphylococci were isolated from 300 eggs collected from December 2015 to May 2016. *S. aureus* isolates were tested for antimicrobial susceptibility using broth microdilution and characterized using pulsed-field gel electrophoresis (PFGE), multilocus sequence typing (MLST), staphylococcal cassette chromosome *mec* (SCC*mec*) typing, and *spa* typing. The presence of Panton–Valentine leukocidin and antimicrobial resistance genes were detected using PCR. Staphylococci were isolated from 21.3% (64/300) of the table eggs tested. Of those, 59% (38/64) were identified as *S. aureus*, of which 33 (86.8%) were positive for *mec*A (MRSA, methicillin-resistant *S. aureus*). All MRSA were multidrug resistant (resistant to two or more antimicrobial classes), contained *aac-aph* (encoding aminoglycosides), and were *pvl*$^+$. Using MLST, *spa* typing, and SCC*mec* typing, three genotypic patterns were assigned: ST8-t8645-MRSA-IV, associated with USA300; and ST772-t657-MRSA-IV and ST772-t8645-MRSA-IV, both characteristic of the Bengal Bay community-associated MRSA clone. Molecular typing by PFGE revealed that the bacterial population was highly homogenous with only two patterns observed. This study is the first report of detection of human-associated *pvl*$^+$ MRSA from table eggs. The genetic similarities of MRSA present in the eggs to that of humans may suggest human to poultry transmission of MRSA via contamination.

Keywords: methicillin-resistant *Staphylococcus aureus*, antimicrobial resistant, Panton–Valentine leukocidin, table eggs

Introduction

Table eggs are one of the most wholesome and economical foods worldwide and are rich in proteins, fats, vitamins, and minerals (Kralik and Kralik, 2017). Eggs are usually considered safe and are naturally protected by the egg shell and a semipermeable membrane. Nonetheless, bacteria may enter the eggs by crossing both the egg shell and the membrane (Berrang *et al.*, 1999). Poor handling and storage under unhygienic conditions in the poultry farms or shops poses a risk to egg quality and may consequently affect human health (Pysniak, 2009; Pyzik and Marek, 2012). The diseases caused by the ingestion of contaminated eggs may range from mild symptoms to life-threatening situations requiring emergency

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treatment (Foley *et al.*, 2011; Safaei *et al.*, 2011). A number of bacterial species, including those causing foodborne illnesses such as *Listeria monocytogenes*, *Escherichia coli*, *Salmonella enterica*, and *Campylobacter jejuni*, have already been isolated from table eggs and reported in other studies (Pysniak, 2009; Sabarinath *et al.*, 2009; Abdullah, 2010). Bacteria may enter the eggs either through vertical transmission or by penetrating the egg shell (Messens *et al.*, 2006).

Staphylococcus aureus is a medically significant bacterial species responsible for diverse types of infections ranging from superficial skin and soft-tissue infections to fasciitis, otitis media, necrotizing pneumonia, and urinary infections (Tamarapu *et al.*, 2001; Harris *et al.*, 2002). In addition, *S. aureus* is one of the major causes of food poisoning due to their ability to produce heat-stable enterotoxins that may remain protected in the food environment and cause foodborne illness (Bergdoll *et al.*, 1967; Argudín *et al.*, 2010). There are over 20 types of staphylococcal enterotoxins (SE), two of them (SEA and SEB), that are best characterized and considered super antigens as they may bind MHC II molecules on the surface of antigen-presenting cells and may stimulate massive T cell proliferation and ultimately leading to toxic shock (McCormick *et al.*, 2001; Le Loir *et al.*, 2003; Rahimi *et al.*, 2013; Kadariya *et al.*, 2014).

In addition to enterotoxin production, multidrug resistance (MDR) in *S. aureus* has emerged as a serious problem faced by healthcare professionals globally. Methicillin-resistant *S. aureus* (MRSA) shows resistance against almost all β-lactam antibiotics, including penicillin and cephalosporins. Resistance against β-lactam antibiotics is due to bacterial ability to produce an altered form of penicillin-binding proteins (PBP), that is, PBP2a that has lowered affinity for β-lactam drugs. The *mec*A gene located on the chromosomal DNA of MRSA strains encodes PBP2a. This *mec*A gene is a part of staphylococcal cassette chromosome *mec* (SCC*mec*), a mobile genetic element that may be horizontally transferred among strains of *S. aureus*, causing dissemination of antimicrobial resistance genes among the isolates. Strains devoid of SCC*mec* are sensitive to methicillin and are termed as methicillin-sensitive *S. aureus* (Stapleton and Taylor, 2002).

Panton–Valentine leukocidin (PVL) is also known as a synergohymenotropic toxin, acting by synergistic activity of two secretory proteins such as component S and F (Adler *et al.*, 2006). PVL enhances the pathogenicity of *S. aureus* and is the most virulent toxin of *S. aureus* that plays a role in wound and skin infections such as furuncles, cutaneous abscesses, and cellulitis and causes severe necrotic skin infections. This toxin has the ability to form pores in the membrane of host immune cells. PVL-positive *S. aureus* secretes two types of proteins such as LukS-PV and LukF-PV, which are encoded by two genes present on the *S. aureus* chromosome. The LukS-PV and LukF-PV have synergistic action. PVL is mostly associated with community-associated methicillin resistance in *S. aureus* infections (Adler *et al.*, 2006).

There is a very limited data available on the presence of *S. aureus* from table eggs (Abdullah, 2010; Pyzik *et al.*, 2014). These studies primarily focused on bacterial detection and antimicrobial susceptibility testing. A single study on molecular typing of *S. aureus* by Pyzik *et al.* (2014) utilized pulsed-field gel electrophoresis (PFGE) for phylogenetic analysis in addition to antibiotic resistance profiling. A more detailed study conducted in a different part of the world such as in Pakistan may further deepen our understanding of the problem. In the present study, *S. aureus* from table eggs were isolated and characterized using antimicrobial susceptibility testing, presence of antimicrobial resistance and *pvl* genes, PFGE, multilocus sequence typing (MLST), *spa* typing, and SSC*mec* typing. The presence of MRSA in table eggs will not only indicate a risk of foodborne illness, but also a source of dissemination of antimicrobial-resistant strains to humans and the environment (De Reu *et al.*, 2005; Woodridge, 2012).

Materials and Methods

Sample collection

Three hundred table egg samples were collected from different areas of Haripur, Pakistan district from December 2015 to May 2016 (Supplementary Table S1; Supplementary Data are available online at www.liebertpub.com/fpd). The samples were collected in sealed plastic bags from retail shops located in the Haripur city area, TIP colony and Sarai Saleh, and were transported to the microbiology laboratory at the University of Haripur.

Sample processing

The eggs were dipped in 70% ethanol for 3–5 s before analysis to disinfect their surface and then placed in the safety cabinet to air dry. Each egg was broken with the help of a sterile rod and the contents were poured in a sterile Petri plate. Microbial detection was carried out from three parts of the egg, that is, the inner membrane, egg white, and egg yolk. One milliliter of egg white and egg yolk was 10 times diluted with sterile phosphate-buffered saline (1X, PBS; Oxoid, Basingstoke, United Kingdom) and homogenized for 1–2 min. One hundred microliter of the mixture was spread evenly onto a mannitol salt agar plate (Oxoid) for detection of *S. aureus*. Similarly, the inner part of the egg membrane was washed with 1.0 mL sterile PBS, and 100 μL of the washing was inoculated onto mannitol salt agar. The plates were then incubated at 37°C for 24 h.

Identification of S. aureus

Preliminary identification of the strains was made using standard microbiological and biochemical tests, including microscopy, catalase, coagulase, and DNase tests as well as mannitol fermentation on mannitol salt agar. Confirmation of *S. aureus* was made on the basis of amplification of the *nuc* gene by PCR as described (Poulsen *et al.*, 2003). All resulting clones were verified and identified to the genus and species level using the Vitek 2 system (bioMérieux, Durham, NC) and the Vitek 2 Gram-positive identification cards according to the manufacturer's directions.

Antimicrobial susceptibility testing

Minimum inhibitory concentrations (MIC, μg/mL) for *S. aureus* were determined by broth microdilution with the Sensititre™ semiautomated antimicrobial susceptibility system (Trek Diagnostic Systems, Inc., Cleveland, OH) and the Sensititre MRSA plate CMV1MRSA according to the manufacturer's directions. Antimicrobials and breakpoints were as follows: cefoxitin (≥8 μg/mL), clindamycin (≥4 μg/mL),

daptomycin (>1 μg/mL), erythromycin (≥8 μg/mL), gentamicin (≥16 μg/mL), levofloxacin (≥4 μg/mL), linezolid (≥8 μg/mL), moxifloxacin (≥2 g/mL), mupirocin (256 μg/mL), nitrofurantoin (≥128 μg/mL), oxacillin (≥4 μg/mL), penicillin G (≥0.25 μg/mL), Quinupristin/Dalfopristin (Q/D) (≥4 μg/mL), tetracycline (≥16 μg/mL), tigecycline (>0.25 μg/mL), trimethoprim/sulfamethoxazole (≥4/76 μg/mL), and vancomycin (≥16 μg/mL). MIC values were manually recorded by using the Sensitouch system, and Clinical and Laboratory Standards Institute (CLSI) standards were used to determine resistance (CLSI, 2016). Only susceptible breakpoints for daptomycin (≤1 μg/mL) and tigecycline (≤0.25 μg/mL) have been established by CLSI; resistance for these two drugs was defined as MICs greater than those values. *S. aureus* ATCC 29213 was used as a quality control strain.

Molecular characterization

Presumptive MRSA were confirmed using multiplex PCR used to detect the presence of staphylococcal 16S rDNA, an internal *S. aureus* control, and *mec*A (Strommenger *et al.*, 2003). Resistance to macrolides [*erm*(A), *erm*(C)], aminoglycosides (*aacA-aphD*), tetracycline [(*tet*(K), *tet*(M)], and streptogramins [*vat*(A), *vat*(B), *vat*(C)] was also tested using the multiplex PCR. Positive and negative controls were included with all PCR runs performed. Positive controls were as follows: *aacA-aphD-E. faecalis* ATCC 49532, *erm*(A)-*S. aureus* RN1389, *erm*(C)-*S. aureus* RN4220, *mec*A-*S. aureus* ATCC 33591, *tet*(K)-*S. aureus* RB 36-1 (Jackson *et al.*, 2013), *tet*(M)-*E. faecalis* OG1-SSp, *vat*(A)-*S. aureus* CIP 107907, *vat*(B)-*S. aureus* CIP 108540, and *vat*(C)-*S. aureus* CIP 107908. SCC*mec* type (Oliveira and de Lencastre, 2002), *spa* type (Harmsen *et al.*, 2003; Strommenger *et al.*, 2008a), MLST (Enright *et al.*, 2000), and PVL gene presence (Strommenger *et al.*, 2008b) were performed as previously described. PFGE was used to generate macrorestriction patterns as previously described using 30 U of *Sma*I (Roche, Indianapolis, IN) (McDougal *et al.*, 2003). Cluster analysis was performed with BioNumerics software v6 (Applied Maths, Belgium) using Dice coefficient and the unweighted pair-group method with arithmetic mean. Optimization settings for dendrograms were 2% with a band tolerance of 2%.

Results

Bacterial isolation and identification

Of 300 table egg samples collected, 21.3% (64/300) of samples were confirmed as *Staphylococcus* on the basis of all microbiological and biochemical tests as well as PCR. *S. aureus* were isolated from the three parts of table egg, which included egg shell, white, and yolk. The maximum number of staphylococci were isolated from egg yolk that represented 37.5% (24/64), followed by 34.4% (22/64) from egg white, and 28.1% (18/64) from the inner membrane of egg shells. Further identification of the 64 staphylococci on Vitek 2 revealed that 38/64 (59.4%) of staphylococci strains were *S. aureus*; 33 of those were resistant to cefoxitin and *mec*A positive using PCR. Thus, overall prevalence of MRSA in table eggs was found to be 11% (33/300). The distribution of the remaining 27 isolates of staphylococci were as follows; *S. haemolyticus* (1/64; 1.6%), *S. simulans* (7/64; 10.9%), *S. simulans/S. haemoliticus* (13/64; 20.3%), *S. vitulinus* (2/64; 3.1%), *S. sciuri* (1/64; 1.6%), and *S. lentus* (2/64; 3.1%). The overall prevalence of all *Staphylococcus* species is presented in Figure 1.

Antimicrobial susceptibility testing of S. aureus

Percent resistance of *S. aureus* isolates ($n=38$) to the tested antimicrobials is shown in Figure 2. High levels of resistance to the β-lactam antibiotics (cefoxitin [94.7%], oxacillin [92.1], and penicillin [97.4%]), aminoglycoside (gentamicin [86.8%]), and fluoroquinolones (levofloxacin [97.4%] and moxifloxacin [63.2%]) were exhibited.

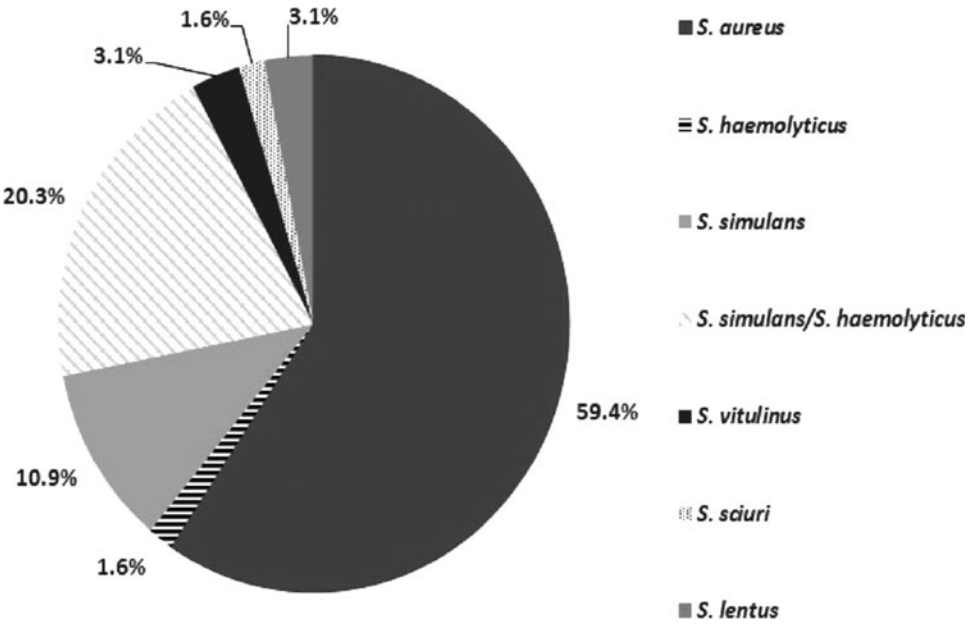


FIG. 1. Distribution of staphylococcal species from table eggs. Values are given in percentage ($n=64$).

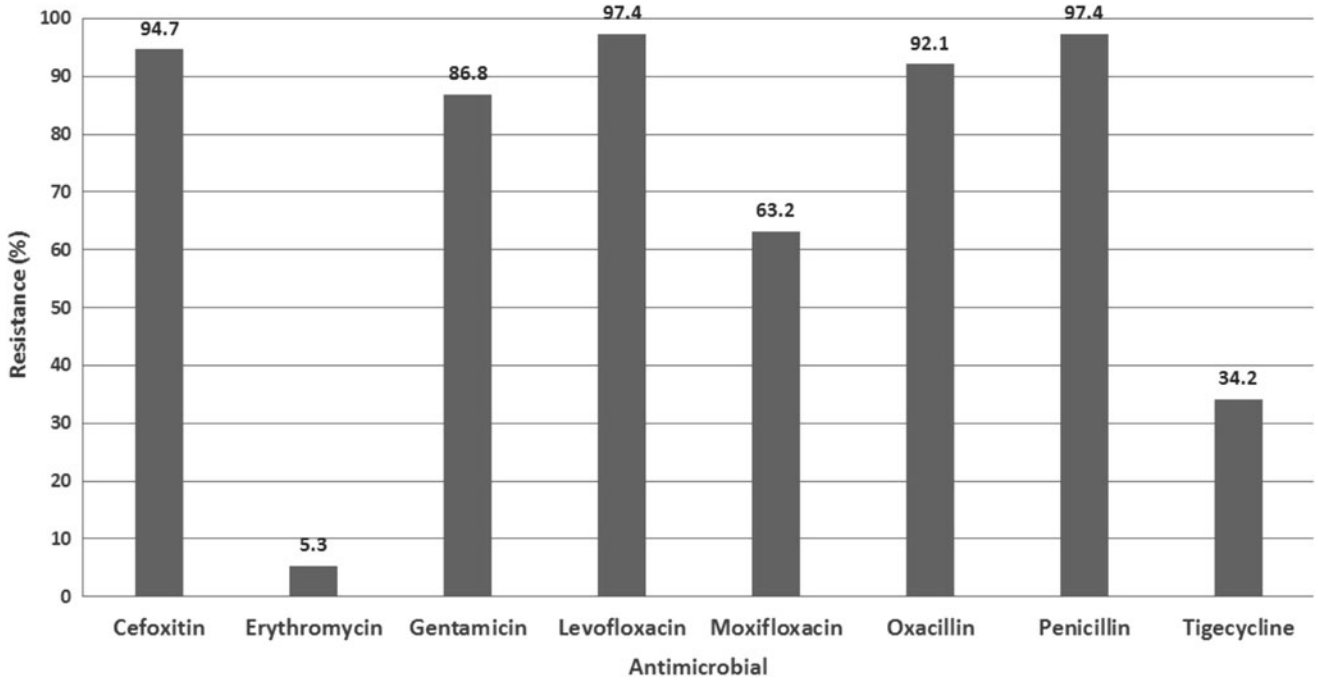


FIG. 2. Percent resistance of *Staphylococcus aureus* ($n=38$) to tested antimicrobials using broth microdilution. No resistance to clindamycin, daptomycin, linezolid, nitrofurantoin, Quinupristin/Dalfopristin, tetracycline, trimethoprim/sulfamethoxazole, or vancomycin was detected.

Resistance to the glycylcycline, tigecycline, was lower (34.2%), while the lowest level of resistance (5.3%) was observed to the macrolide, erythromycin. None of the *S. aureus* strains was resistant to clindamycin, daptomycin, linezolid, nitrofurantoin, quinupristin/dalfopristin, tetracycline, trimethoprim/sulfamethoxazole, or vancomycin. For the MRSA strains ($n=33$), all were resistant to cefoxitin, levofloxacin, oxacillin, and penicillin (Fig. 3). MRSA isolates were also resistant to gentamicin (30/33; 90.9%), moxifloxacin (21/33; 63.6%), and erythromycin (3/33; 9%); 10 isolates (30.3%) were resistant to tigecycline. Multidrug resistance (MDR; resistance to two or more classes of antimicrobial classes) was observed with most common combinations of β-lactams and fluoroquinolones (Fig. 3). Other MDR class combinations included aminoglycoside-β-lactam-fluoroquinolone, aminoglycoside-β-lactam-fluoroquinolone-glycylcycline, and β-lactam-fluoroquinolone-macrolide. One isolate, 15.C, was resistant to an aminoglycoside, β-lactam, fluoroquinolone, macrolide, and glycylcycline.

Detection of antimicrobial resistance genes

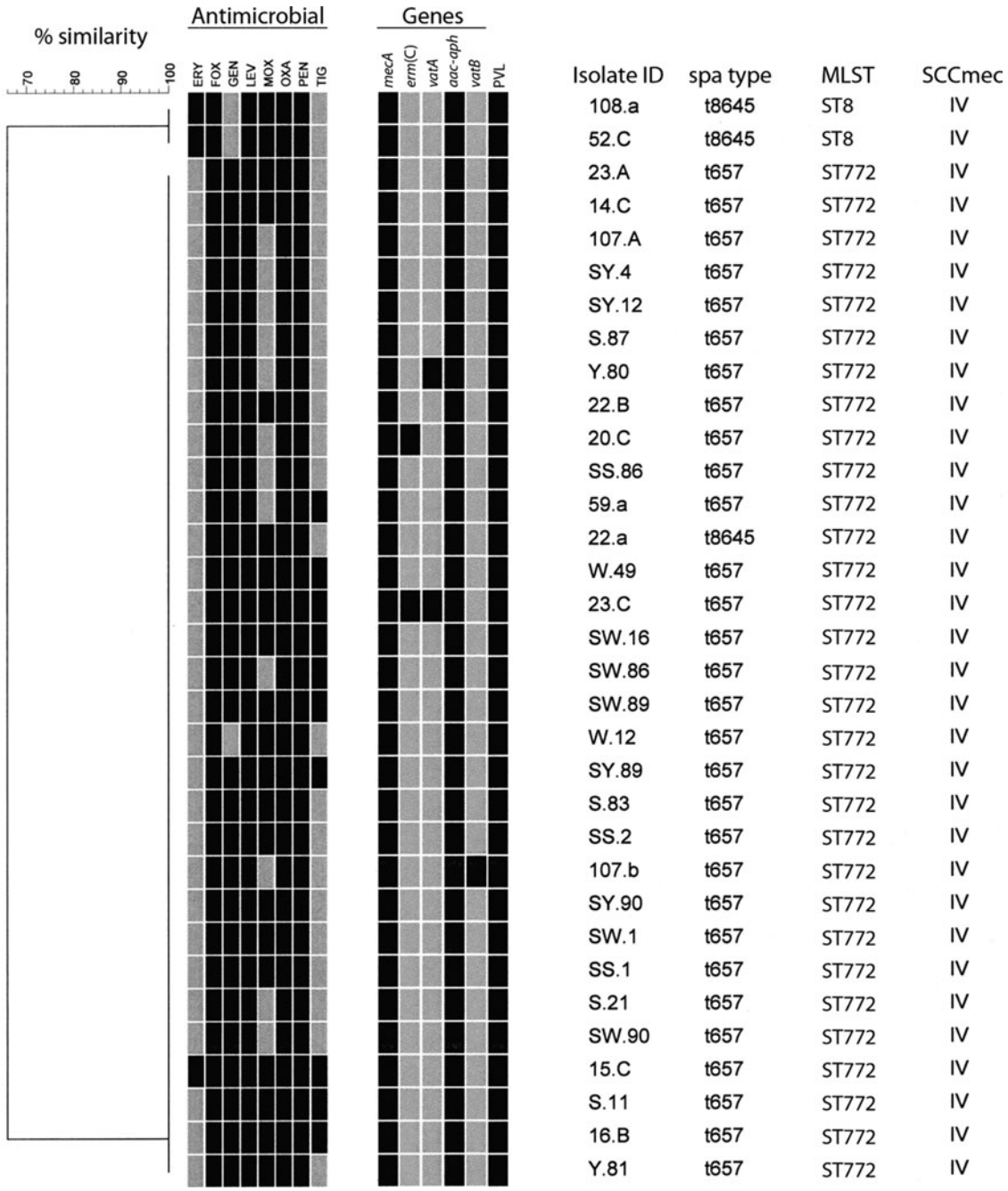
In addition to *mec*A, all MRSA isolates were positive for *aac-aph* (Fig. 3). The presence of *mec*A corresponded 100% with the oxacillin phenotype. However, three isolates that were positive for *aac-aph* were not resistant to gentamicin. Conversely, three isolates were resistant to erythromycin without a corresponding resistance gene and the two isolates that contained *erm*(C) were not resistant to erythromycin. Other resistance genes rarely detected in the isolates were *vat*A, present in two isolates and *vat*B found in one MRSA isolate. Other genes such as *erm*(A), *tetM*, *tetK*, and *vatC* could not be detected in any of the strains.

Molecular typing

All MRSA strains were positive for PVL and were SCC*mec* type IV (Fig. 3). MLST analysis revealed that the MRSA population was largely homogenous; 31 (93.9%) of the MRSA strains belonged to ST772, while only two (6%) belonged to ST8. *Spa* typing of the isolated MRSA strains identified two major types, that is, t657 and t8645. With the exception of one isolate which was *spa* type t8645, all ST772 types identified by MLST corresponded to t657, whereas all ST8 corresponded to t8645. As with MLST and *spa* typing, PFGE analysis of all MRSA strains showed two major groups. The majority of the strains ($n=31$) belonged to a single cluster, which were 100% identical, while the remaining two isolates were grouped together because they were 100% identical to each other (Fig. 3).

Discussion

Staphylococcal food poisoning is a major health problem caused by ingestion of food contaminated with *S. aureus* (Kadariya *et al.*, 2014). Foods such as eggs may easily be contaminated with *S. aureus*, and the cooking process may not denature heat-stable enterotoxins. The few studies on *S. aureus* and eggs have shown that the bacterium can be present in the egg yolk, egg white, and on the shell of eggs and that some of the isolates may be resistant to antimicrobials (Pysniak *et al.*, 2009; Pyzik *et al.*, 2012, 2014). In the present study of *S. aureus* isolated from table eggs collected from different shops of Haripur city of Pakistan, prevalence, antimicrobial resistance, and molecular genetic characteristics were determined, which may aid in defining how the bacterium is transmitted to the egg (e.g., via a human or animal source).



Isolate ID	spa type	MLST	SCCmec
108.a	t8645	ST8	IV
52.C	t8645	ST8	IV
23.A	t657	ST772	IV
14.C	t657	ST772	IV
107.A	t657	ST772	IV
SY.4	t657	ST772	IV
SY.12	t657	ST772	IV
S.87	t657	ST772	IV
Y.80	t657	ST772	IV
22.B	t657	ST772	IV
20.C	t657	ST772	IV
SS.86	t657	ST772	IV
59.a	t657	ST772	IV
22.a	t8645	ST772	IV
W.49	t657	ST772	IV
23.C	t657	ST772	IV
SW.16	t657	ST772	IV
SW.86	t657	ST772	IV
SW.89	t657	ST772	IV
W.12	t657	ST772	IV
SY.89	t657	ST772	IV
S.83	t657	ST772	IV
SS.2	t657	ST772	IV
107.b	t657	ST772	IV
SY.90	t657	ST772	IV
SW.1	t657	ST772	IV
SS.1	t657	ST772	IV
S.21	t657	ST772	IV
SW.90	t657	ST772	IV
15.C	t657	ST772	IV
S.11	t657	ST772	IV
16.B	t657	ST772	IV
Y.81	t657	ST772	IV

FIG. 3. PFGE analysis, antimicrobial resistance pattern, and genetic profiles of MRSA from table eggs. DNA for PFGE was digested with *Sma*I. Levels of similarity were determined using Dice coefficient and the unweighted pair-group method with arithmetic mean. Black boxes represent positive results; gray boxes represent negative results. Antimicrobials are erythromycin (ERY), cefoxitin (FOX), gentamicin (GEN), levofloxacin (LEV), moxifloxacin (MOX), oxacillin (OXA), penicillin (PEN), and tigecycline (TIG). MLST, multilocus sequence typing; MRSA, methicillin-resistant *S. aureus*; PFGE, pulsed-field gel electrophoresis; PVL, Panton–Valentine leukocidin; SCC*mec*, staphylococcal cassette chromosome *mec*.

Similar to other studies, *S. aureus* and other staphylococcal species were isolated from the eggs (Pysniak *et al.*, 2009; Pyzik *et al.*, 2012). Although the number of different species in this study was slightly lower than that of those studies, this may be a reflection of the type of eggs sampled, the location of the study, or the identification method used. It is noteworthy that *S. aureus* strains isolated from table eggs in this study exhibited MDR against a number of commonly used antibiotics in humans. There is concern about the high level of antimicrobial resistance among clinical isolates (Ullah *et al.*, 2016); however, strains of similar antimicrobial resistance profiles have been and continue to be isolated from food samples such as eggs (Pyzik *et al.*, 2014). The prevalence of MRSA among all *S. aureus* in this study is of concern as chicken eggs have become a significant source of dissemination of antimicrobial-resistant bacteria. The scope of

the present study was restricted to eggs, but their feces and waste material may also be an important source of antimicrobial-resistant bacteria. Some *S. aureus* strains showed the presence of antimicrobial resistance genes, but phenotypically they were susceptible, which indicates nonfunctional or inactive antimicrobial resistance genes (e.g., erythromycin). Alternatively, certain strains were resistant, but a corresponding resistance gene was not detected. This could be due to the limited number of resistance genes tested and the absence of the gene conferring resistance from that group. The bacteria may also have developed an alternative mechanism of drug resistance.

In contrast to the results published by Pyzik *et al.* (2014), our data from PFGE revealed that most of the strains were genetically homogenous and further testing using *spa* typing, MLST, and SCC*mec* typing confirmed those results. This may be due to the number of farms from which the eggs were collected, suggesting clonal spread of bacterial strains. MLST analysis showed that the majority of strains belong to ST772, which agrees with our previous study on MRSA in human clinical isolates from Pakistan (Madzgalla *et al.*, 2016). The majority of MRSA strains from skin and wound infection cases from that study belonged to the same sequence type. Two of the *S. aureus* from eggs were ST8 that has been primarily linked with community-associated MRSA (CA-MRSA) (Gonzalez *et al.*, 2006). Studies by groups from other locations in Pakistan reported ST8 and ST239 as the most prevalent MLST type in human clinical or community MRSA (Shabir *et al.*, 2010; Zafar *et al.*, 2011).

SCC*mec* type IV was the only *mec* type found in eggs from this study. Two SCC*mec* types, that is, type III and type IV, have already been reported from Pakistan from healthcare-associated MRSA (HA-MRSA) and CA-MRSA infections, respectively. In the study conducted by Zafar *et al.* (2011), association of ST8 with SCC*mec*-IV (ST8-MRSA-SCC*mec*IV) was reported. This ST-SCC*mec* combination was also found in the present study and is most prevalent among CA-MRSA infection cases characteristic of USA300 clones (Monecke *et al.*, 2011). Of the two *spa* types detected, t657 is associated with Bengal Bay CA-MRSA clones (Rajan *et al.*, 2015; Ho *et al.*, 2017). Although the Bengal Bay clone was first reported in Bangladesh, it has spread to other locations due to travel to India or contact with travelers to that region (Brennan *et al.*, 2012; Rajan *et al.*, 2015). It has recently been reported in seafood and associated water samples from India (Murugadas *et al.*, 2017). Presently, *spa* typing data are unavailable from Pakistan to compare with our results. Interestingly, all MRSA strains detected from egg samples in this study were *pvl* positive, which is the first report to the best of our knowledge. PVL is an important virulence factor of *S. aureus* strains capable of causing skin and soft-tissue infections as well as necrotizing pneumonia (Adler *et al.*, 2006). The presence of *pvl* may also indicate transmission of MRSA from humans to poultry.

Humans have been fighting the battle against antimicrobial resistance among bacteria; the disease caused by resistant *S. aureus* strains among those on prolonged antibiotic therapy may be difficult to be controlled. Of further concern is dissemination of these resistant bacteria to food, the environment, and animals. Antimicrobial resistance profiles of the *S. aureus* strains isolated from egg samples were comparable to those reports published in other parts of Pakistan in humans, which is an alarming situation (Taj *et al.*, 2010). Injudicious and uncontrolled use of antimicrobials while treating farm animals and antimicrobial use in poultry feed may be possible explanations for MRSA in eggs in this study, since previous experimental studies on table eggs have already proved bacterial penetration of egg shells (Berrang *et al.*, 1999). Therefore, resistant bacterial strains may easily enter the egg and contaminate them. An additional explanation for the results of this study may be transfer of human antimicrobial-resistant strains to poultry since both the USA300 and Bengal Bay lineages are common among humans in different areas of the world and were found in the eggs. This scenario is plausible because chickens are short-lived, having maximum contact with humans who feed or care for them. There may not be direct transmission of these bacteria to eggs from humans, but there may be transmission from humans to poultry to eggs.

Conclusion

In the present study, we report for the first time the detection of human-associated, *pvl*-positive *S. aureus* strains with multiple antimicrobial resistance patterns from table eggs. The presence of MDR MRSA in eggs is a matter of food safety and alternatively a source of dissemination of resistant strains to humans through the food chain. Antimicrobial resistance of these strains against commonly used antibiotics to cure human diseases is of public health concern. Proper sanitation in the poultry farms, improved storage conditions, as well as judicious and controlled use of antibiotics in the poultry industry are all important to control bacterial carriage and health risks by contaminated table eggs.

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Disclosure Statement

No competing financial interests exist.

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