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Research Article

ASSESSMENT OF CORRELATION BETWEEN PHENOTYPIC AND GENOTYPIC RESISTANCE PROFILE OF *STAPHYLOCOCCUS AUREUS* ISOLATED FROM MEAT SAMPLES OF PUNJAB, INDIA

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ABSTRACT

The study intended to clarify the correlations between the genotypic and phenotypic antibiotic resistance pattern in *S. aureus* isolated from meat samples of Punjab. Six isolates were MRSA (*mec*A positive; oxacillin MIC 4 to 32 µg/ml). Seven other *S. aureus* (5.34%) isolates were *mec*A negative borderline oxacillin resistant *S. aureus* BORSA (*mec*A-BORSA). The *mec*A-BORSA isolates from chevon were ceftriaxone susceptible and β-lactamases hyper producers, whereas, isolates of pork origin were intermediate or completely resistant to ceftriaxone. All of the 27 *S. aureus* isolates that were phenotypically resistant to gentamicin with MIC >32 µg/ml carried *aac*A-*aph*D gene. However, 17.36% gentamicin susceptible isolates were positive for *aac*A-*aph*D gene. In this study, *tet*K, *tet*L and *tet*M genes were also present in *S. aureus* isolates. Only *erm*B and *erm*C genes were present in *S. aureus* isolates and 19.44% of the *S. aureus* isolates were genotypically negative yet demonstrated an intermediate phenotypic resistance with MIC 1-2 µg/ml. Two among the four MRSA erythromycin resistant isolates expressed inducible MLS_B phenotype ($ERY^+/CLI^-, D^+$) and 21.6% erythromycin-resistant and intermediate resistant MSSA (*erm*B/C positive) expressed inducible MLS_B phenotype ($ERY^+/CLI^-, D^+$).

Keywords: Antibiotic resistance genes, Correlation, Epsilometer test, Meat, *Staphylococcus aureus*

Penicillin resistance in *S. aureus* is mediated by the generation of a β-lactamases encoded by the *bla*Z gene (Olsen *et al.*, 2006) while as resistance to methicillin and other β-lactam antibiotics is regular because of *mec*A gene (Peacock and Paterson, 2015).

Resistance to tetracycline in *S. aureus* is because of by either active efflux (*tet*K and *tet*L) or ribosome-protective proteins (*tet*M or *tet*O). Contrary to *tet*K, the *tet*L gene is seldom found in *S. aureus* (Febler *et al.*, 2010).

Resistance to gentamicin and the related aminoglycosides is interceded by aromatic amino-acid decarboxylase [AAD(6')] and aminoglycoside phosphor-transferases [APH(2")] activities catalyzed by a solitary bifunctional protein encoded by *aac*A-*aph*D (Shannon and Phillips, 1982).

Additionally, Macrolides, lincosamides, and streptogramin B (MLS_B) resistance in *S. aureus* depends on the presence of atleast one *erm* genes of the classes A, B, C, F, T, Y, and 33 (Febler *et al.*, 2010). Lastly, vancomycin resistance is occasionally experienced in *S. aureus* and depends on the presence of the *van*A gene cluster.

MATERIALS AND METHODS

Isolates: A culture collection of four hundred thirty two *S. aureus* isolates from meat (chicken, chevon and pork) and swab samples (chopping block, butcher‘s hand and chopping knife) from SPH&Z, GADVASU were utilized in this study. One isolate for each sample including those isolates that demonstrated a unique biochemical profile were selected for Epsilometer test and genotypic test for antibiotic resistance genes.

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Coagulase Test: The *S. aureus* isolates were additionally described as coagulase positive and negative utilizing rabbit plasma (HiMedia lab, Mumbai) and the test was carried out according to the manufacturer‘s directions. Any level of coagulation within 4 hours was considered as positive outcome.

Nucleic acid extraction: The HiPurATM bacterial genomic DNA purification kit (HiMedia Lab, Mumbai) was utilized for extraction of nucleic acid from *S. aureus* isolates as per manufacturer's directions and the extracted DNA was kept at -20 °C for further analysis.

Genotypic identification of *S. aureus*: The multiplex PCR for the detection of 16S *r*DNA (genus particular), *nuc* (species particular) and *mec*A genes was carried out according to Zehra *et al.*, 2017.

Antibiotic susceptibility testing of *S. aureus* isolates: The antibiotic susceptibility testing (AST) of *S. aureus* isolates was performed by the Epsilometer test (E-test) (Zehra *et al.*, 2017). All the selected *S. aureus* isolates were tested for their sensitivity to different antibiotics viz. oxacillin, penicillin, tetracycline, ceftriaxone, gentamicin, erythromycin and vancomycin utilizing Ezy MICTM strip (HiMedia Lab, Mumbai). The MIC value of the antibiotic was perused where zone of inhibition (ellipse) intersected with the MIC scale on the strip (Figure 1). AST for amoxicillin/clavulinic acid and D-test (Inducible clindamycin resistance test) was performed by disc diffusion method as per CLSI guidelines (M100-S21).

Identification of antibiotic resistant genes: To assess the

Table 1

Primers used for detection of antibiotic resistant genes in *S. aureus*

Target Gene	Oligonucleotide sequence (5'-3')	Amplicon size	Reference
*aac*A-*aph*D	TAA TCC AAG AGC AAT AAG GGCGCC ACA CTA TCA TAA CCA CTA	227	Strommenger *et al.*, 2003
*erm*A	AAG CGG TAA ACC CCT CTG ATTC GCA AAT CCC TTC TCA AC	190	Strommenger *et al.*, 2003
*erm*B	CTATCTGATTGTTGAAGAAGGATTGTTTACTCTTGGTTTAGGATGAAA	142	Martineau *et al.*, 2000
*erm*C	AAT CGT CAA TTC CTG CAT GTTAA TCG TGG AAT ACG GGT TTG	299	Strommenger *et al.*, 2003
*tet*K	GTA GCG ACA ATA GGT AAT AGTGTA GTG ACA ATA AAC CTC CTA	360	Strommenger *et al.*, 2003
*tet*M	AGT GGA GCG ATT ACA GAACAT ATG TCC TGG CGT GTC TA	158	Strommenger *et al.*, 2003
*tet*L	GTMGTTGCGCGCTATATTCCGTGAAMGRWAGCCCACCTAA	696	Huys *et al.*, 2005
*tet*O	AATGAAGATTCCGACAATTTCTCATGCGTTGTAGTATTCCA	781	Huys *et al.*, 2005
*mec*A	AAA ATC GAT GGT AAA GGT TGG CAGT TCT GCA GTA CCG GAT TTG C	532	Strommenger *et al.*, 2003
*bla*Z	ACT TCA ACA CCT GCT GCT TTCTGA CCA CTT TTA TCA GCA ACC	173	Martineau *et al.*,2000
*van*A	ATGAATAGAATAAAAGTTGCTCACCCCTTTAACGCTAATA	1032	Saha *et al.*, 2008
16S *r*DNA (*Staphylococcus genus* specific)	CAG CTC GTG TCG TGA GAT GTAAT CAT TTG TCC CAC CTT CG	420	Strommenger *et al.*, 2003
Coa	ATA GAG ATG CTG GTA CAG GGCT TCC GAT TGT TCG ATG C	547 (550-875)	Hookey *et al.*, 1998
Nuc	GCGATTGATGGTGATACGGTTAGCCAAGCCTTGACGAACTAAAGC	279	Brakstad *et al* ., 1992

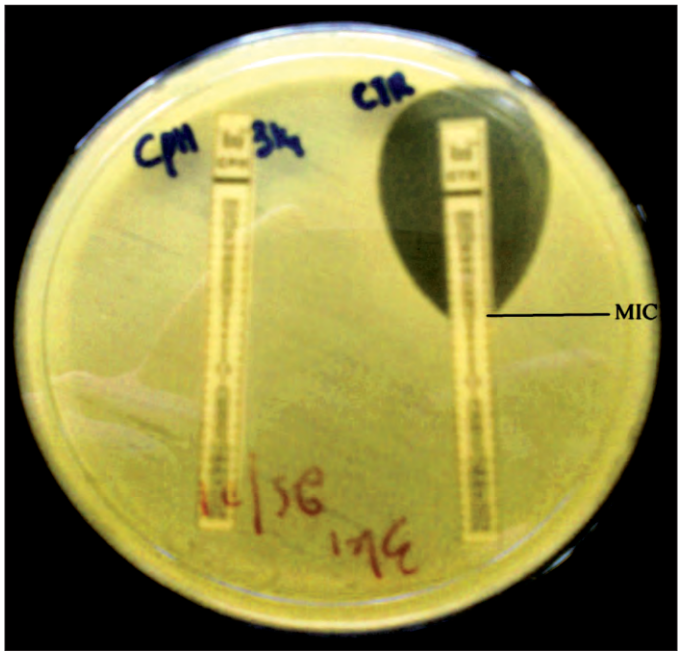

Fig. 1. The Epsilometer test showing the interaction of the inhibition zone with the strip of a *S. aureus* for Ceftriaxone (CTR)

presence of antibiotic resistance genes in isolates, multiplex PCR was utilized for the amplification of following genes: *bla*Z, *mec*A, *aac*A-*aph*D, *erm* (*erm*A, *erm*B, *erm*C), *tet* (efflux genes *tet*K and *tet*L, *tet*M and *tet*O of the ribosomal protection (RP) family) and *van*A encoding for penicillin, oxacillin, gentamicin, erythromycin, tetracycline and vancomycin resistance, respectively (Table 1).

S. aureus isolate ATCC 33591 and ATCC 25923 were used as MRSA (*mec*A +ve) and MSSA (*mec*A -ve) positive control, respectively. KU872013, KP834338/KP834339, KP658721, KP658723, KP886833, KT454736 and KT454737 S. aureus isolates were used as positive control for genes *bla*Z, *aac*A-*aph*D, *tet*K, *tet*L, *tet*M, *erm*B and *erm*C, respectively.

Each isolate was subjected to different multiplex PCR assays for recognition of each group (gp) of genes: gp1 (16S *r*DNA, *nuc*, *mec*A); gp2 (*tet*K, *tet*L, *tet*M and *tet*O); gp3 (*erm*A, *erm*B, *erm*C and *aac*A-*aph*D); gp4 (*coa* and *bla*Z). Separate PCR was run for vanA gene. The amplification was carried out in a total reaction volume of 25µl containing 0.4 mM dNTP, 4 mM $MgCl_2$ (Promega, U.S.A), 10 pmol/µl of each primer set containing forward and reverse primers, 1U *Taq*DNA polymerase (Promega, U.S.A), 0.01 µg- 0.2 µg template and sterile nuclease free water was added to make up the reaction volume of 25 µl.

The cycling conditions for multiplex PCR for gp1, gp2 and gp3 and of single PCR for *van*A gene were according to technique of Strommenger *et al.*, 2003 (with little adjustments as per Zehra *et al.*, 2017) and Saha *et al.*, 2008, respectively. However, cycling condition for *coa* and *bla*Z (gp 4) incorporated an initial denaturation of DNA at 94 °C for 45 s, followed by 30 cycles of denaturation at 94 °C for 20 s, annealing at 55 °C for 15 s and extension at 70 °C for 15 s, followed by a final extension of 2 min at 72 °C and hold at 4 °C. All the PCR amplicons were

envisioned utilizing a Gel documentation system (SynGene, USA) after electrophoresis on a 1.5% agarose gel containing 0.5 µg/ml ethidium bromide.

Statistical analysis: Statistical data analysis was performed using SPSS version 22. All statistical analyses were performed at a significant value of $p<0.05$. The $\chi 2$ test was performed to depict the associations between individual in silico recognition of procured antimicrobial resistant genes and OXA, PEN, GEN, ERY, and TET MIC levels at its particular non-susceptible threshold. The Fisher- Freeman-Halton exact test of independence was performed and the phi (ϕ) or Cramer's V (ϕc) coefficient was figured to portray the connections between's the genomic discovery designs and the MIC levels of *S. aureus* isolates as observed in OXA, PEN, GEN, ERY and TET. Cochran's-Mantel-Haenszel statistics were performed to assess the relationship between antimicrobial resistant genes and OXA, PEN, GEN, ERY, and TET phenotypic resistance after adjusting for type of sample.

In this study, resistant and intermediate resistant isolates were considered together as resistant isolates while as that had MIC between susceptible and intermediate range as per CLSI guidelines were considered as susceptible isolates for statistical analysis.

RESULTS AND DISCUSSION

Among the 144 selected isolates, 129 were coagulase positive, 15 were coagulase negative and 6 were MRSA. There was a statistically significant positive correlation ($p<0.05$) between phenotypic and genotypic resistance pattern in *S. aureus* isolates subjected to the antibiotic oxacillin, penicillin, tetracycline, gentamicin and erythromycin (Table 2). None of the isolates was phenotypically resistant to vancomycin (MIC 0.5-2 µg/ml) and none carried *van*A gene.

All the *S. aureus* isolates that had *bla*Z gene were likewise phenotypically resistant to penicillin ($\phi = 0.633$, $p = 0.001$, Fig. 2) aside from four of the isolates from chicken samples that were penicillin susceptible. These outcomes are in accordance with other studies (Ferreira *et al.*, 2017). However, in present study, five isolates (5/144, 3.5%)

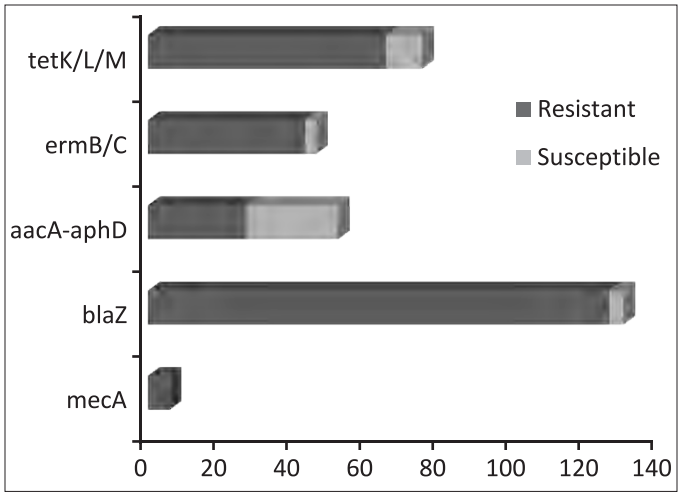

Fig. 2. Proportion of the resistant genes among the phenotypic *S. aureus*

demonstrated resistance to penicillin yet lacked *bla*Z gene. They were also coagulase positive *S. aureus*. These isolates indicated resistance with MIC value 0.25-1.5 µg/ml. This might be a direct result of *S. aureus* tolerance to the bacteriocidal effect of β-lactam antibiotics (Sabath, 1982). In addition to it, the presence of an extra protein having indistinguishable mobility as that of PBP in sensitive cells yet with decreased affinity for benzylpenicillin and other β-lactam antibiotics, has been reported as another reason for resistance to β-lactam antibiotics (Brown and Reynold, 1980).

Among the selected 144 isolates, six isolates from the chicken samples were MRSA i.e., *mec*A positive (Fig. 3). The oxacillin MIC values of those isolates extend from 4 to 32 µg/ml ($\phi = 0.662$, $p=0.001$). According to literature, isolates that are *mec*A positive, borderline oxacillin resistant with MIC ranging from 1 to 8 µg/ml were designated as *mec*A+BORSA (Huang *et al.*, 2018). In this study, two isolate indicated MIC of 4 and 6 µg/ml and conveyed *mec*A gene. In spite of that, seven *S. aureus* (5.34%) isolates that were phenotypically resistant to oxacillin yet genotypically without *mec*A indicated borderline resistance with MIC ranging from 4-8 µg/ml and in this manner assigned as *mec*A-BORSA. Martineau *et al.* (2000) likewise detailed the presence of isolates that

Table 2

Genomic detection patterns revealed to be correlated with respective antibiotics

Antibiotic_gene	Chicken (ϕ, p)*	Chevon (ϕ, p)	Pork (ϕ, p)	Swab (ϕ, p)
PEN_*bla*Z	0.191, 0.196	1.000, 0.000	0.746, 0.002	0.633, 0.045
OXA_*mec*A	0.662, 0.000	**	**	**
GEN_ *aac*A-*aph*D	0.716, 0.000	0.550, 0.003	0.650, 0.000	0.490, 0.003
TET_*tet*K/L/M	0.875, 0.000	0.809, 0.000	0.769, 0.000	1.000, 0.000
ERY_*erm*B/C	0.532, 0.000	0.672, 0.000	0.586, 0.000	0.576, 0.000

*ϕ = phi (chi square based measure of nominal association) and p = level of significance; ** as none other sample was *mec*A positive

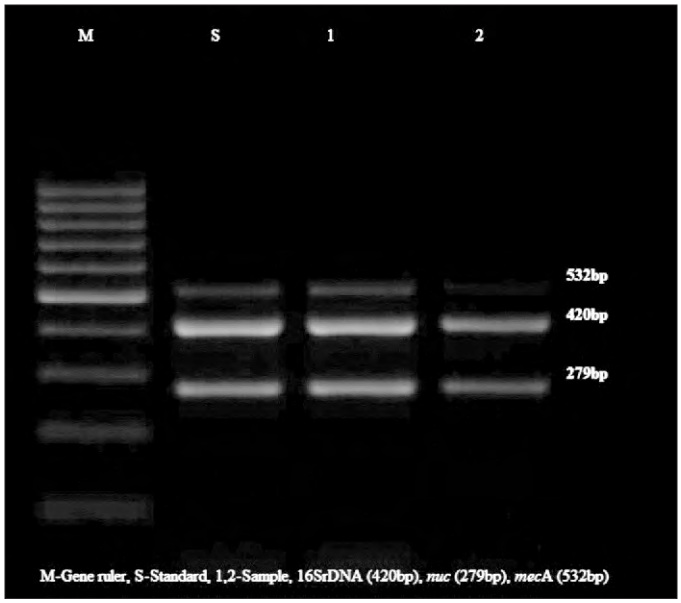

Fig. 3. Agarose gel electrophoresis of PCR-amplified products using genus (16S *r*DNA- 420bp)/species-specific (*nuc*-279bp) and *mec*A (532bp) primer sets. Lane M-100-bp Plus DNA size marker; lane S-*S. aureus* ATCC 33591 reference strain; lane 1-2-examined *S. aureus* isolates

were phenotypically oxacillin resistant but negative for *mec*A gene (Martineau *et al.*, 2000). In similar manner, Pereira *et al.* (2009) discovered 38% of *S. aureus* isolates resistant to oxacillin yet just 0.68% of the isolates conveyed *mec*A gene. BORSA has been frequently observed phenotype among *S. aureus* isolates (Pereira *et al.*, 2009). These isolates are cefoxitin/ceftriaxine susceptible and do not carry the *mec*A or *mec*C genes, however demonstrated oxacillin resistance with MIC between 1-8 µg/ml (Shore and Coleman, 2013). Such example might be a direct result of hyperproduction of β-lactamases or production of normal PBP with altered binding capacity (Martineau *et al.*, 2000). To exclude the likelihood of hyperproduction of β-lactamase, amoxyclave antibiotic disk diffusion test was performed (Martineau *et al.*, 2000). The isolates from chevon (oxacillin resistant, *mec*A negative) were ceftriaxone susceptible and β-lactamase hyperproducers with inhibition zone more than 20 mm however uneven outcomes were found in case of isolates from pork samples (Table 3). These isolates were intermediate or completely resistant to ceftriaxone and one was not β-lactamase hyperproducer. This can be clarified by another reason for being BORSA and that is modified *S. aureus* (MODSA) with mutations in pbp genes and/or PBP4-overexpression (Argudin *et al.*, 2018).

All the 27 *S. aureus* isolates that were phenotypically resistant to gentamicin (18.75%, 27/144) with MIC>32 µg/ml were additionally positive for *aac*A-*aph*D gene (ϕ = 0.639, p = 0.001; Fig. 4). This noteworthy relationship between the gentamicin resistance and presence of *aac*A-*aph*D gene stays strong after adjusting for type of sample (Table 2). However, 17.36% (25/144) of the *S. aureus* isolates had *aac*A-*aph*D gene yet were phenotypically sensitive to gentamicin (Fig. 2). This might be because of the lack of expression or incomplete expression of *aac*(6')/*aph*(2") gene (Martineau *et al.*, 2000). In the present study, *aac*A-*aph*D gene was found among MRSA isolates. This gentamicin-resistant MRSA is regularly experienced in community isolates and is less frequently found among clinical isolates (Ida *et al.*, 2001). This study, therefore, reports the presence of *aac*A-*aph*D gene among isolates without phenotypic gentamicin resistance.

In this study, *tet*K, *tet*Land *tet*M genes were present in *S. aureus* isolates (Fig. 5). None of the CNSA could be confirmed for *tet*L. Of the total *S. aureus* isolates, 43.05% (62/144) were certain for *tet*K, 18.05% (26/144) were positive for both *tet*K and *tet*M genes, 2.08% (3/144) for *tet*L and *tet*M genes and 0.69% (1/144) isolates for *tet*K and *tet*L. Based on the results of this study, tetracycline resistance to presence of *tet*K/L/M gene are significantly

Table 3

Characteristics of isolates resistant to oxacillin but negative for *mec*A gene by PCR

Source	Isolate	Oxacillin MIC (µg/ml)	Ceftriaxone MIC (µg/ml)	Amoxyclave Inhibition zone* (mm)	blaZ (PCR detection)	β-lactamase hyper producers
Pork	P12a	6	16	50	+	+
Pork	P72	6	16	46	+	+
Pork	P44(CNSA)	4	64	18	+	-
Pork	P59	4	64	30	+	+
Chevon	Che1	4	8	28	+	+
Chevon	Che2	4	8	30	+	+
Chevon	Che72(CNSA)	6	8	45	+	+

*The diameter of the inhibition zone with amoxyclave (amoxicillin-clavulinic acid: 20 and 10 µg, respectively) if exceed 20mm then strains were considered β-lactamases hyperproducers

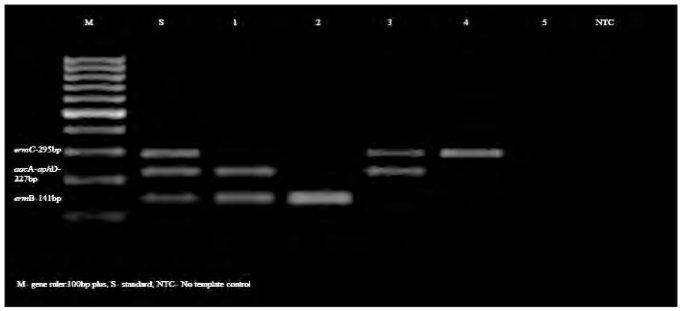

Fig. 4. Agarose gel electrophoresis of PCR-amplified products using erythromycin and gentamicin resistance genes primer sets. Lanes 1-5- examined *S. aureus* isolates; lane M-100-bp Plus DNA size marker; lane S-Standard; lane NTC-no template control

correlated (ϕ=0.875, p=0.001, Table 2) and this significance (p=0.001) demonstrated that the association between the tetracycline resistance and tetK/L/M gene stays strong after adjusting for type of sample. All MRSA isolates were positive for *tet*K/*tet*M gene. This demonstrated a high prevalence of *tet*K/*tet*M gene among MRSA isolates. Among the *S. aureus* isolates that carried *tet*K/L/M gene, 79% demonstrated resistance to tetracycline with MIC values ≥32µg/ml (Fig. 2). The isolates that carriedonly *tet*M gene were phenotypically sensitive to tetracycline. This might be on the grounds that *tet*M is less expressive gene (Huys *et al.*, 2005).

Among the erythromycin resistant genes, *erm*B and *erm*C genes were present in *S. aureus* isolates (Fig. 4). The greater proportion of the *S. aureus* isolates that were carrying any of the *erm* genes (18.05%, 26/144 *erm*B$^+$; 15.97%, 23/144 *erm*C$^+$; 2.08%, 3/144 *erm*B$^+$*erm*C$^+$) indicated complete or intermediate resistance to erythromycin (ϕ = 0.576, p = 0.001, Table 2). In present study, 19.44% (28/144) of the *S. aureus* isolates were genotypically negative, however demonstrated an intermediate resistance with MIC 1-2 µg/ml phenotypically. This intermediate resistance to erythromycin could be because of different genes like *msr*A or novel gene *erm*TR (Martineau *et al.*, 2000). Likewise, Martineau *et al.* (2000) additionally revealed two *S. aureus* isolates resistant to erythromycin phenotypically yet not carrying any of the *erm* resistance genes. These isolates that were resistant or intermediate to erythromycin *in vitro* were tried for inducible clindamycin resistance (D-test). Among the 17 erythromycin-resistant *S. aureus*, four were MRSA while 2 of the 4 erythromycin-resistant MRSA expressed inducible MLSB phenotype (ERY$^+$/CLI$^-$, D$^+$). Eight (21.6%) of 37 erythromycin-resistant and intermediate resistant MSSA (*erm*B/C positive) expressed inducible MLS_B phenotype (ERY$^+$/CLI$^-$, D$^+$). Four isolates demonstrated a constitutive MLS_B phenotype (ERM$^+$/CLI$^+$, D$^-$). There was no significant difference in MLS_B identification rate between Erythromycin-resistant MRSA and Erythromycin- resistant MSSA (p>.05).

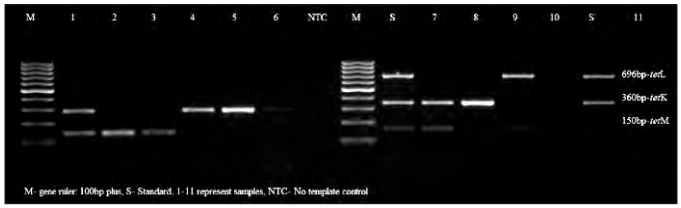

Fig. 5. Agarose gel electrophoresis of PCR-amplified products using tetracyline resistance genes primer sets. Lanes 7-11-examined *S. aureus* isolates; lane M-100-bp Plus DNA size marker; lane S-Standard; lane NTC-no template control

CONCLUSIONS

The present study uncovered the pervasiveness of BORSA in the food of animal origin and is a public health concern as there are odds of it getting transmitted to people prompting genuine outcomes like treatment failure as that by MRSA. It was additionally seen that many isolates carried the genes of resistance, however, lacked the phenotypic expression, in this way exhibiting the future potential of these isolates to end up resistant to the assessed antimicrobial agents.

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