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Characterization of Antimicrobial Resistance and Enterotoxin Genes in Methicillin-Resistant *Staphylococcus aureus* Isolated from Mastitis Milk and Food Poisoning Cases

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Abstract: Enterotoxigenic *Staphylococcus aureus* is a main cause of Staphylococcal Food Poisoning (SFP). Here, researchers characterized the antimicrobial resistance, distribution and expression of enterotoxin genes of 41 Methicillin-Resistant *S. aureus* (MRSA) isolated from bovine mastitis milk and SFP cases. Apart from three SFP-acquired isolates which could produce extended-spectrum β-lactamases, all the others carried *mecA* gene and expressed the penicillin-binding protein 2a. The 92.7% of MRSA isolates tested showed twenty two multi-drug resistant patterns among which SFP-acquired isolates had higher resistant rate and the $MIC_{50/90}$ values of cefotaxime, amikacin, azithromycin, ciprofloxacin and gentamicin than those of milk-acquired isolates. The 95.1% of MRSA isolates carried at least one Staphylococcal Enterotoxins (*SEs*) gene and could produce the corresponding classical SEs, the differences in the prevalence of enterotoxigenic MRSA observed between milk-acquired and SFP-acquired isolates were not statistically significant. Six SEs genotypes were found, among which the genotypes sea-seg-sei-seln-selm and sea-seb-sec-seg-sei-seln-selm predominated, respectively in milk-acquired and SFP-acquired isolates.

Key words: Methicillin-resistant, *S. aureus*, antimicrobial resistance, enterotoxin gene, raw milk, food poisoning

INTRODUCTION

Staphylococcus aureus is the cause of infection in humans and animals and some strains are also responsible for Staphylococcal Food Poisoning (SFP), one of the most prevalent foodborne intoxication diseases worldwide by producing heat-stable enterotoxins in foods (Seo and Bohach, 2007; Le Loir *et al*., 2003). Methicillin-Resistant *S. aureus* (MRSA) were found primarily in humans, later they were also detected in animals (Enright *et al*., 2002). In recent years, the presence of MRSA in food animals and people who are in contact with these animals and livestock products has shown an upward trend and has become a serious clinical and food safety problem (Mulders *et al*., 2010; Gordoncillo *et al*., 2012; Deleo *et al*., 2010).

Staphylococcal Enterotoxins (SEs) belong to a large pyrogenic toxin family including classical SEs (SEA through SEE), newly SEs (seg, seh, sei, sej, ser, ses and set) and SE-like toxins (selk, sell, selm, seln, selo, selp, selq and selu) (Argudin *et al*., 2010; Omoe *et al*., 2013). Immunological methods were first developed for the detection of SEs, however those commercial immunoassay kits are limited to detect classical SEs only (Bennett, 2005). In an attempt to solve this problem, PCR assay has been developed to detect the prevalence of many *SEs* genes, especially newly *SEs* genes (McLauchlin *et al*., 2000).

Mastitic milk can serve as the main source of enterotoxigenic MRSA of animal origin (Mirzaei *et al*., 2012; Normanno *et al*., 2007). Therefore, continuous surveillance of enterotoxigenic MRSA in raw milk and raw milk products is essential. The objective of this study was to characterize the antimicrobial resistance, *SEs* genes distribution and enterotoxin production of MRSA isolates obtained from bovine mastitic milk and SFP cases.

MATERIALS AND METHODS

Bacterial isolates: A total of 41 MRSA isolates were analyzed in this study. The isolated strains were obtained from bovine mastitis milk (M1-M17) and SFP cases (SFP1-SFP24) and had been confirmed as *S. aureus* using conventional laboratory tests including the Gram stain, DNase test, coagulase test and 16S *rRNA* gene sequencing in the previous studies. *S. aureus* isolates were streaked onto selective CHROMagar MRSA (CHROMagar Microbiology, Paris, France) and the growth of colonies showing pink or mauve coloration was considered to be positive for MRSA.

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Preparation of bacterial DNA: Genomic DNA of MRSA isolates was prepared for *SEs* and *mecA* genes detection. A single colony was cultured in Luriae Bertani medium (LB medium, Oxoid, UK) for 18 h at 37°C. Overnight culture was pelleted by centrifugation then DNA was extracted using Bacterial Genomic DNA Mini-prep kit (Tiangen BioTech, Beijing) as described by the manufacturer.

Detection of the *mecA* gene and encoding protein PBP2a: The *mecA* gene which encodes the low-affinity Penicillin-Binding Protein 2a (PBP2a) is highly conserved in the methicillin-resistant species and is a useful molecular marker for determining MRSA. The distribution of the *mecA* gene among 41 MRSA isolates was surveyed by PCR using the following primers: 5'-GCGACTTCA CATCTATTAGG-3' and 5'-CTTCGTTACTCATGCCAT AC-3' which gave a PCR product of 394 bp. Reaction mixtures (25 μL) containing 2.5 μL of 10× PCR buffer, 1.5 μL of 25 mM $MgCl_2$, 0.5 μL of 10 mM dNTPs, 0.5 μL of 20 mM primers, 0.3 μL of Taq enzyme, 3 μL of DNA template and 16.2 μL ddH_2O were incubated for 5 min at initial denaturation temperature 94°C followed by 30 cycles of 30 sec at denaturation temperature 94°C, 30 sec at annealing temperature 53°C, 1 min at extension temperature 95°C and finally 10 min at final extension temperrature 72°C. The PCR products were checked on 1.5% agarose gels. Meanwhile, the *mecA* gene product, PBP2a was detected using the commercial Mastalex™ MRSA kit (Oxoid Ltd.). Briefly, a boiled, centrifuged extract of MRSA strain was detected by latex agglutination test with latex particles sensitised with monoclonal antibody directed against PBP2a. As a control, strains *S. aureus* ATCC25923 (MSSA) and *S. aureus* ATCC43300 (MRSA) were used respectively.

Extended-Spectrum β-Lactamases (ESBLs) testing: The bla_{TEM} gene which mediates the expression of β-lactamases. The distribution of the bla_{TEM} gene among 41 MRSA isolates was surveyed by PCR using the following primers: 5'-AGGAGGAGTATGATTGAACA-3' and 5'-CTCGTCGTTTGGTATGGC-3' which gave a PCR product of 535 bp. Reaction mixtures (25 μL) containing 2.5 μL of 10× PCR buffer, 1.5 μL of 25 mM $MgCl_2$, 0.5 μL of 10 mM dNTPs, 0.5 μL of 20 mM primers, 0.3 μL of Taq enzyme, 3 μL of DNA template and 16.2 μL ddH_2O were incubated for 5 min at initial denaturation temperature 95°C followed by 30 cycles of 30 sec at denaturation temperature 95°C, 30 sec at annealing temperature 54°C, 1 min at extension temperrature 72°C and finally 10 min at final extension temperature 72°C. The PCR products were checked on 1.5% agarose gels. Meanwhile, the 41 MRSA isolates were determined for ESBLs production with combined disk method as described by CLSI (2006). Briefly, the test inoculm (0.5 McFarland standard turbidity) was streaked onto Mueller-Hinton agar (Oxoid). One disk each of Ceftazidime-Clavulanate (CAZ/CA, 30/10 mg^{-1}) and cefotaxime-clavulanate (CTX/CA, 3010 mg^{-1}) was applied to the surface of the inoculated plate. A disk each of CAZ and CTX was also applied. A 5 mm increase in zone diameter for either antimicrobial agent tested in combination with Clavulanate (CA) versus its zone when tested alone was taken as ESBLs-producing strain. *E. coli* ATCC25922 and *K. pneumoniae* ATCC700603 were used as negative and positive controls for ESBLs protection, respectively.

Antimicrobial susceptibility testing: The Minimum Inhibitory Concentration (MIC) of antimicrobial in the MRSA strains was determined by E-test Method according to the manufacturer's instructions in which *S. aureus* ATCC29213 was used as quality control strain. The following 12 antimicrobial E-test strips (BioMerieux, France) were used: Oxacillin (OXA), Cefotaxime (CTX), Gentamicin (GEN), Amikacin (AMI), Clindamycin (CLI), Azithromycin (AZM), Minocyline (MNO), Chloramphenicol (CHL), Ciprofloxacin (CIP), Rifampicin (RIF), Teicoplanin (TEI) and Vancomycin (VAN). The MIC was recorded as the lowest concentration of the antimicrobials (in μg/mL) at which no more than two colonies were detected (CLSI, 2010) break-points were used for MIC interpretation. The concentrations of the antimicrobials inhibiting visible growth of 50 and 90% of bacteria were interpreted as the MIC_{50} and MIC_{90}, respectively. The MIC_{50} and MIC_{90} values were calculated using SAS for Windows, Version 9.0 (SAS Institute, Cary, NC).

Detection of *SEs* genes: All MRSA isolates were tested by PCR assay for ten *SEs* genes (*sea*, *seb*, *sec*, *sed*, *see*, *seg*, *seh*, *sei*, *selm*, *seln*) using primers and conditions as described in Table 1. Each PCR reaction mixture was listed in Table 2. The PCR products were checked on 1.5% agarose gels. Five reference strains of *S. aureus* harboring *SEs* genes, *FRI326* (*see*), *FRI569* (*seh*), *FRI361* (*sec*, *sed*, *selm*, *seln*), *ATCC25923* (*sea*, *seg*, *sei*), *CMCC26074* (*seb*) were included as positive control, respectively.

Detection of classical SEs: Classical *SEs* genes-positive MRSA isolates (*19sea*, *3sec*, *2sea-seb*, *11sea-seb-sec*, respectively) were assessed for corresponding enterotoxins production. Briefly, the tested bacteria were cultured in BHIB (Oxoid) for 24 h at 37°C. Culture filtrates were tested for enterotoxin protein using a Reversed Passive Latex Agglutination (RPLA) toxin detection kit (SET-RPLA; Oxoid) according to the manufacturer's instructions.

Table 1: Primers and PCR conditions for amplification *SEs* genes from MRSA isolates

Target gene	Primers sequence (5′→3′)	Amplicon size (bp)	PCR program[a]
sea	Forward: GCAGGGAACAGCTTTAGGC	521	1
	Reverse: GTTCTGTAGAAGTATGAAACACG		
seb	Forward: TAATCATGTATCAGCAATAAACG	599	2
	Reverse: TCTTCACATCTTTAGAATCAACC		
sec	Forward: GATGAAGTAGTTGATGTGTATGG	473	3
	Reverse: GTAAGGTGGACTTCTATCTTCAC		
sed	Forward: CTAGTTTGGTAATATCTCCT	317	1
	Reverse: TAATGCTATATCTTATAGGG		
see	Forward: ATGTGCTGGAGGCACACCAAAT	296	2
	Reverse: CGTGGACCCTTCAGAAGAATG		
seg	Forward: ATGTCTCCACCTGTTGAAGG	400	4
	Reverse: TGAGCCAGTGTCTTGCTTTG		
seh	Forward: CGAAAGCAGAAGATTTACACG	358	5
	Reverse: TCTACCCAAACATTAGCACC		
sei	Forward: CTCAAGGTGATATTGGTGTAGG	577	6
	Reverse: AAAAAACTTACAGGCAGTCCATCTC		
selm	Forward: ATACGGTGGAGTTACATTAGC	340	2
	Reverse: GAAACTTTCAGCTTGTCCTGTT		
seln	Forward: TTGGAAATAAATGTGTAGGCT	377	5
	Reverse: CCCACTGAACCTTTTACGTTA		

[a]1: 30 cycles 94°C×30 sec, 52°C×40 sec, 72°C×50 sec; 2: 30 cycles 94°C×30 sec, 48°C×40 sec, 72°C×50 sec; 3: 30 cycles 94°C×30 sec, 51°C×40 sec, 72°C×50 sec; 4: 30 cycles 94°C×30 sec, 55°C×40 sec, 72°C×50 sec; 5: 30 cycles 94°C×30 sec, 50°C×40 sec, 72°C×50 sec; 6: 30 cycles 94°C×30 sec, 54°C×40 sec, 72°C×50 sec; initial denaturation: 94°C×5 min; final extension: 72°C×10 min

Table 2: PCR reaction system to the different *SEs* genes (25 μL)

Genes	10×PCR buffer	25 mM $MgCl_2$	10 mM dNTPs	20 mM each primer	DNA template	5U Taq enzyme	ddH_2O
sea	2.5	1.5	0.5	1.0	1.5	0.3	17.7
seb	2.5	1.5	0.5	1.0	2.0	0.3	17.2
sec	2.5	1.5	0.5	1.0	2.0	0.3	17.2
sed	2.5	1.5	0.5	1.5	3.0	0.3	15.7
see	2.5	1.5	0.5	1.0	3.0	0.3	16.2
seg	2.5	1.5	0.5	0.5	3.0	0.3	16.7
seh	2.5	1.5	0.5	1.5	3.0	0.3	15.7
sei	2.5	1.5	0.5	1.0	3.0	0.3	16.2
selm	2.5	1.5	0.5	1.0	3.0	0.3	16.2
seln	2.5	1.5	0.5	0.5	1.5	0.3	18.2

Statistical analysis: The prevalence of *SEs* genes and antimicrobial resistance rate between milk-acquired and SFP-acquired isolates was compared by using the χ^2-test (SAS for Windows, Version 9.0; SAS Institute, Cary, NC). The $p<0.05$ was regarded as indicating statistical significance.

RESULTS AND DISCUSSION

Amplification with *mecA* gene and production of PBP2a and ESBLs: It is generally known that methicillin-resistance is primarily mediated by the overproduction of low-affinity PBP2a encoded by *mecA* gene (Arede and Oliveira, 2013). In this study, 92.7% (38/41) of MRSA strains analyzed carried *mecA* gene (Fig. 1) and produced the encoding protein PBP2a and only three SFP-acquired isolates (SFP1, SFP2 and SFP3) were lack of the *mecA* gene and failed to produce PBP2a in spite of their resistance to methicillin phenotypically. As shown in Fig. 2 and Table 3, the results are the same, 34.1% (14/41, 8 milk-acquired and 6 SFP-acquired strains) of MRSA isolates could produce ESBLs by an increase of 5 mm in the inhibition zone around the disc containing added clavulanic acid. It was interesting to note that three SFP-acquired MRSA isolates without carrying *mecA* gene were all ESBLs producers. The results confirm previous investigations and suggest that some MRSA strains can involve non-PBP2a-dependent mechanisms such as hyper-production of β-lactamases or production of a newly described methicillinase (Lee *et al*., 2004).

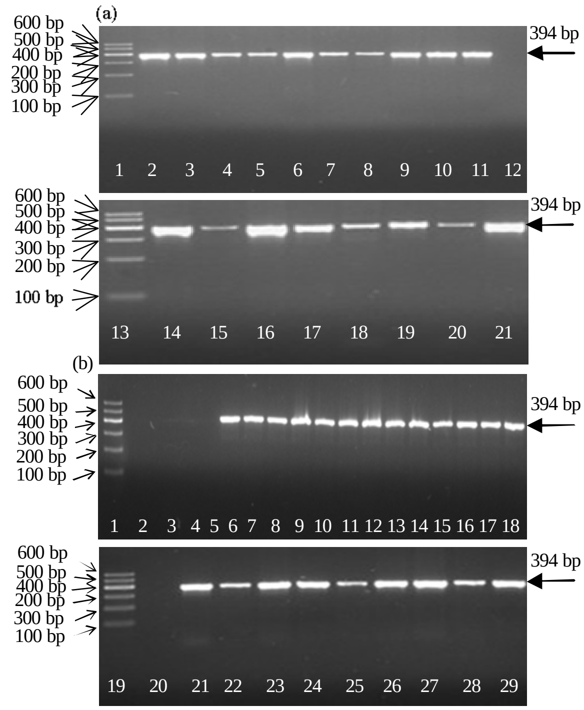

Fig. 1: Agarose gel electrophoresis analysis for the *mecA* gene in MRSA isolates; a) PCR products obtained from 17 milk-acquired isolates; Lane 1 and 13: DNA molecular size marker of 100-600 bp; Lane 2-10: M1-M9 isolates; Lane 11: MRSA ATCC43300 positive control; Lane 12: *S. aureus* ATCC29213 negative control; Lane 14-21: M10-M17 isolates; b) PCR products obtained from 24 SFP-acquired isolates; Lane 1 and 19: DNA molecular size marker of 100-600 bp; Lane 2, negative control; Lane 3-18: SFP1-SFP16 isolates; Lane 20: negative control; Lane 21: positive control; Lane 22-29: SFP17-SFP24 isolates

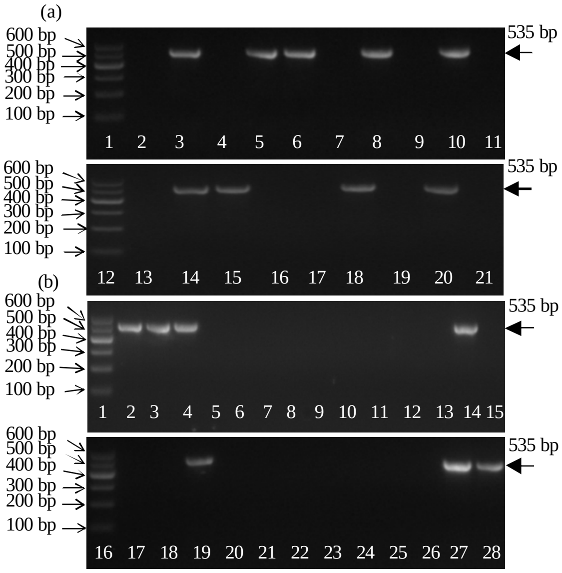

Fig. 2: Agarose gel electrophoresis analysis for the *blaTEM* gene in MRSA isolates; a) PCR products obtained from 17 milk-acquired isolates; Lane 1 and 12: DNA molecular size marker of 100-600 bp; Lane 2-9: M1-M8 isolates; Lane 10: *K. pneumoniae* ATCC700603 positive control; Lane 11: *E. coli* ATCC25922 negative control; Lane 13-21: M9-M17 isolates; b) PCR products obtained from 24 SFP-acquired isolates; Lane 1 and 16: DNA molecular size marker of 100-600 bp; Lane 2-13, SFP1-SFP12 isolates; Lane 14: positive control; Lane 15: negative control; Lane 17-28: SFP13-SFP24 isolates

Antimicrobial resistance: The results concerning antimicrobial resistance were shown in Table 4. Except for three milk-acquired isolates (M1, M8, M12), 92.7% of isolates (38/41) showed multiple resistant phenotype (resistance to three or more antimicrobials). The most common resistance observed was oxacillin (100%) followed by clindamycin (78.1%), rifampicin (65.9%), azithromycin and ciprofloxacin (63.4% for each one), cefotaxime and gentamicin (41.5% for each one), amikacin (39%) and chloramphenicol (12.2%). None of the isolates were resistant to minocyline, teicoplanin and vancomycin. This is valuable information because these three drugs have been usual choice for the treatment of MRSA infection.

Compared with milk-acquired isolates, SFP-acquired isolates had a higher antimicrobial resistance rate for cefotaxime ($p<0.01$), amikacin ($p<0.01$), azithromycin ($p<0.01$), ciprofloxacin ($p<0.01$), gentamicin ($p<0.05$) and the MIC_{50} and MIC_{90} values of these antimicrobials were 3>128 and 2.9>64 fold higher than those of the milk-acquired isolates, respectively. For other antimicrobials, i.e., oxacillin, clindamycin, chlortetracycline and rifampicin, the resistance rates were not significantly different between milk origin and SFP origin isolates ($p>0.05$). In addition, 22 different resistance patterns were found in MRSA strains analyzed, among them the resistance pattern OXA-CLI-CIP-RIF was shared both in milk-acquired and SFP-acquired isolates (Table 5). These findings are interesting, however due to the small number of isolates examined in this study, further large scale studies would be necessary to confirm this observation.

Table 3: Production of ESBLs among 41 MRSA isolates

No. of milk-acquired	CTX (CAZ)[a]	CTX/CA[b] (CAZ/CA)	ESBLs producer	No. of SFP-acquired	CTX (CAZ)	CTX/CA (CAZ/CA)	ESBLs producer
M1	24 (15)	22 (13)	−(<5)	SFP1	22 (20)	30 (25)	+(≥5)
M2	11 (9)	20 (14)	+(≥5)	SFP2	15 (11)	20 (17)	+(≥5)
M3	16 (10)	15 (10)	−(<5)	SFP3	26 (20)	31 (26)	+(≥5)
M4	7 (11)	19 (16)	+(≥5)	SFP4	6 (6)	6 (8)	−(<5)
M5	16 (10)	22 (15)	+(≥5)	SFP5	6 (6)	6 (6)	−(<5)
M6	30 (20)	29 (18)	−(<5)	SFP6	23 (17)	22 (19)	−(<5)
M7	24 (13)	29 (19)	+(≥5)	SFP7	6 (6)	6 (6)	−(<5)
M8	23 (15)	22 (16)	−(<5)	SFP8	6 (6)	6 (6)	−(<5)
M9	24 (16)	26 (16)	−(<5)	SFP9	6 (6)	6 (6)	−(<5)
M10	24 (13)	29 (19)	+(≥5)	SFP10	6 (6)	6 (6)	−(<5)
M11	20 (17)	26 (22)	+(≥5)	SFP11	19 (16)	21 (14)	−(<5)
M12	34 (24)	30 (22)	−(<5)	SFP12	6 (6)	6 (8)	−(<5)
M13	26 (10)	26 (12)	−(<5)	SFP13	6 (6)	6 (10)	−(<5)
M14	28 (16)	33 (32)	+(≥5)	SFP14	19 (12)	18 (16)	−(<5)
M15	29 (20)	28 (19)	−(<5)	SFP15	17 (10)	22 (19)	+(≥5)
M16	27 (12)	33 (24)	+(>5)	SFP16	18 (10)	16 (8)	−(<5)
M17	20 (11)	21 (12)	−(<5)	SFP17	6 (6)	6 (6)	−(<5)
-	-	-	-	SFP18	6 (6)	6 (6)	−(<5)
-	-	-	-	SFP19	6 (6)	6 (6)	−(<5)
-	-	-	-	SFP20	24 (17)	20 (20)	−(<5)
-	-	-	-	SFP21	6 (6)	6 (6)	−(<5)
-	-	-	-	SFP22	6 (6)	6 (6)	−(<5)
-	-	-	-	SFP23	21 (10)	26 (16)	+(≥5)
-	-	-	-	SFP24	19 (9)	24 (20)	+(≥5)

[a]CTX: Cefotaxime; CAZ: Ceftazidime; CAZ/CA: Ceftazidime-Clavulanate; [b]CTX/CA: Cefotaxime-Clavulanate

Table 4: Antimicrobial resistance of the 17 milk-acquired and 24 SFP-acquired isolates against 12 antimicrobial agents

		MIC_{50} ($\mu g\ mL^{-1}$)[d]		MIC_{90} ($\mu g\ mL^{-1}$)[e]		MIC ranges		Resistance rate		
Antibiotic[a]	Break point ($\mu g\ mL^{-1}$)	Milk-acquired	SFP-acquired	Milk-acquired	SFP-acquired	Milk-acquired	SFP-acquired	Milk-acquired	SFP-acquired	Total
OXA	4	34	56	118	212	64-128	48-192	17 (100%)	24 (100%)	100 (%)
CTX[b]	64	2	>256	4	>256	0.75-256	2->256	1 (5.9%)	16 (66.7%)	41.5 (%)
GEN[c]	16	4	19	16	47	0.064-24	0.25-128	4 (23.5%)	13 (54.2%)	41.5 (%)
AMI[b]	64	9	28	94	403	2-128	0.5-128	2 (11.8%)	14 (58.3%)	39.0 (%)
CLI	4	10	20	176	478	0.5-128	0.125-128	12 (70.6%)	20 (83.3%)	78.1 (%)
AZM[b]	8	2	13	2	55	1.5-32	0.25-128	5 (29.4%)	21 (87.5%)	63.4 (%)
MNO	16	1	1	1	2	0.023-8	0.032-8	0 (0)	0 (0)	0
CHL	32	3	24	34	86	1.5-48	1.5-32	4 (23.5%)	1 (4.2%)	12.2 (%)
CIP[b]	4	2	64	16	>256	0.023-192	0.064-128	5 (29.4%)	21 (87.5%)	63.4 (%)
RIF	4	4	5	69	99	0.064-128	0.032-128	11 (64.7%)	16 (66.7%)	65.9 (%)
TEI	32	1	2	1	4	0.50-2	1.0-6	0 (0)	0 (0)	0
VAN	32	1	1	1	1	0.5-4	0.5-4	0 (0)	0 (0)	0

[a]OXA = Oxacillin; CTX = Cefotaxime; GEN = Gentamicin; AMI = Amikacin; CLI = Clindamycin; AZM = Azithromycin; MNO = Minocyline; CHL = Chloramphenicol; CIP = Ciprofloxacin; RIF = Rifampicin; TEI = Teicoplanin; VAN = Vancomycin; [b]Indicates a very significant difference of resistance rate between Milk-acquired and SFP-acquired at p<0.01; [c]Indicates a significant difference of resistance rate between Milk-acquired and SFP-acquired at p<0.05; [d]MIC_{50} indicates minimal inhibitory concentration value of the agents inhiiting 50% of the number of isolates; [e]MIC_{90} indicates minimal inhibitory concentration value of the agents inhiiting 90% of the number of isolates

Table 5: Resistance pattern of the 17 milk-acquired and 24 SFP-acquired isolates

		No. (MRSA isolates (%))		
No. of antimicrobial	Antimicrobial resistance pattern[a]	Milk-acquired	SFP-acquired	Total
2	OXA-CHL	3 (17.6%)	0 (0)	3 (7.3%)
3	OXA-CLI-CIP	1 (5.9%)	0 (0)	1 (2.4%)
	OXA-CLI-RIF	4 (23.5%)	0 (0)	4 (9.8%)
	OXA-AZM-CIP	0 (0)	2 (8.3%)	2 (4.9%)
4	OXA-AMI-CLI-AZM	1 (5.9%)	0 (0)	1 (2.4%)
	OXA-AZM-CIP-RIF	1 (5.9%)	0 (0)	1 (2.4%)
	OXA-GEN-CLI-RIF	2 (11.8%)	0 (0)	2 (4.9%)
	OXA-CLI-CIP-RIF	2 (11.8%)	1 (4.2%)	3 (7.3%)
	OXA-CLI-AZM-CIP	0 (0)	2 (8.3%)	2 (4.9%)
	OXA-AMI-AZM-RIF	0 (0)	1 (4.2%)	1 (2.4%)
	OXA-CLI-AZM-RIF	0 (0)	1 (4.2%)	1 (2.4%)
5	OXA-GEN-AMI-AZM-RIF	1 (5.9%)	0 (0)	1 (2.4%)
	OXA-CLI-AZM-CHL-RIF	1 (5.9%)	0 (0)	1 (2.4%)
	OXA-AMI-CLI-CIP-RIF	0 (0)	1 (4.2%)	1 (2.4%)
6	OXA-CTX-GEN-CLI-AZM-CHL	1 (5.9%)	0 (0)	1 (2.4%)
	OXA-CTX-CLI-AZM-CIP-RIF	0 (0)	2 (8.3%)	2 (4.9%)
	OXA-CTX-GEN-AZM-CIP-RIF	0 (0)	1 (4.2%)	1 (2.4%)
	OXA-CTX-AMI-CLI-CIP-RIF	0 (0)	4 (16.7%)	4 (9.8%)
7	OXA-CTX-GEN-AMI-CLI-AZM-CIP	0 (0)	1 (4.2%)	1 (2.4%)
	OXA-CTX-GEN-CLI-AZM-CIP-RIF	0 (0)	1 (4.2%)	1 (2.4%)
8	OXA-CTX-GEN-AMI-CLI-AZM-CIP-RIF	0 (0)	7 (29.2%)	7 (17.1%)

[a]OXA = Oxacillin; CTX = Cefotaxime; GEN = Gentamicin; AMI = Amikacin; CLI = Clindamycin; AZM = Azithromycin; MNO = Minocyline; CHL = Chloramphenicol; CIP = Ciprofloxacin; RIF = Rifampicin; TEI = Teicoplanin; VAN = Vancomycin

Distribution of *SEs* genes: Analyzing *S. aureus* isolates from SFP cases indicated that SEA and SED were the two predominant SEs followed by SEB (Cha *et al*., 2006; Kerouanton *et al*., 2007). On the other hand, the SEC producers were often linked to dairy product-borne intoxications (Pelisser *et al*., 2009; Balaban and Rasooly, 2000). In a French study, sea was the most prevalent *SE* gene followed by sed, seg, sei and seh (Jarraud *et al*., 2001). In the present study, 39 (95.1%) of 41 MRSA isolates examined were found to carry at least one of ten *SEs* genes identified, the differences in the prevalence of enterotoxigenic MRSA isolates observed between milk-acquired and SFP-acquired isolates were not statistically significant (p>0.05). The most frequently found *SEs* gene was *sea* (36/41, 87.8%) followed by *seg*, *sei*, *selm* and *seln* (25/41, 60.9% for each one), *sec* (20/41, 48.8%) and *seb* (19/41, 46.3%), respectively but neither SFP-acquired nor milk-acquired MRSA isolates harbored *sed*, *see* and *seh* genes (Table 6). These differences might account for the differences in isolates origin and geographical locations. Furthermore, six SEs genotypes were observed (Table 6), among which the genotypes sea-seg-sei-seln-selm predominated at the rate of 47.1% in milk origin isolates whereas the *sea-seb-sec-seg-sei-seln-selm* gene combination predominated at the rate of 54.1% in SFP origin isolates (Fig. 3).

Noticeably, all 25 seg-positive isolates were positive for sei, selm and seln (Table 6). The coexistence of seg, sei, selm and seln was not surprising

Table 6: Prevalence of *SEs* genes profiles among 17 milk-acquired and 24 SFP-acquired MRSA

	MRSA isolates possessing a specific profile (%)		
Ses genes profile	Milk-acquired (N = 17)	SFP-acquired (N = 24)	Total (N = 41)
sea	2/17 (11.8)	3/24 (12.5)	5/41 (12.2)
sec	2/17 (11.8)	1/24 (4.2)	3/41 (7.2)
sea-seb	1/17 (5.9)	1/24 (4,2)	2/41 (4.8)
sea-seb-sec	2/17 (11.8)	2/24 (8.3)	4/41 (9.7)
sea-seg-sei-seln-selm	8/17 (47.1)	4/24 (16.6)	12/41 (29.2)
sea-seb-sec-seg-sei-seln-selm	0	13/24 (54.1)	13/41 (31.7)
Any *SE* gene	15/17 (88.2)	24/24 (100)	39/41 (95.1)
None *SE* gene	2/17 (11.8)	0	2/41 (4.9)

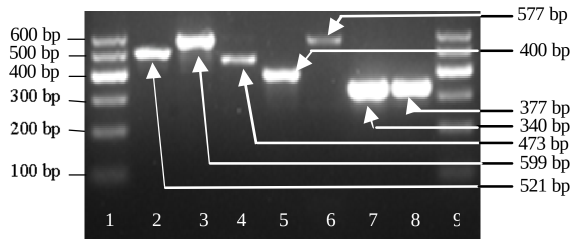

Fig. 3: Agarose gel electrophoresis analysis for the *SEs* genes in reference strains of *S. aureus*; Lane 1 and 9: DNA molecular size marker of 100-600 bp; Lane 2: sea (521 bp); Lane 3: seb (599 bp); Lane 4: sec (473 bp); Lane 5: seg (400 bp); Lane 6: sei (577 bp); Lane 7: selm (340 bp); Lane 8: seln (377 bp)

because together with selo and sometimes selu they belong to an enterotoxin gene cluster (egc) and the detection of one usually indicates the presence of others (Kerouanton *et al*., 2007). Consistent with the findings, a high prevalence of *egc* genes in *S. aureus* was also reported by other researchers (Bania *et al*., 2006; Becker *et al*., 2004; Lawrynowicz-Paciorek *et al*., 2007; Omoe *et al*., 2002).

Production of classical SEs proteins: A high frequency of *SEs* genes does not necessarily produce enterotoxins at a level sufficient to cause SFP. SEs production begins when MRSA strain populations exceeds 10^5 cfu mL^{-1} which is influenced by culture conditions such as temperature, pH and water activity (Valero *et al*., 2009). Therefore, it is important to evaluate whether the MRSA strain can produce SEs. Boynukara *et al*. (2008) reported that 25.5% of *S. aureus* isolates produced classical SEs enterotoxins detected by RPLA test. The findings were that 95.1% of MRSA isolates analyzed were enterotoxigenic, among which17 isolates were positive for sea, 3 for sec, 2 for sea and seb, 17 for sea, seb and sec together detected by RPLA test, respectively. Nonetheless, since commercial immunoassay kits are only available to classical SEs detection, PCR assay used in the present study was an efficient method for the detection of *SEs* genes, especially newly *SEs* genes. In addition, RT-PCR has been shown to be a rapid and useful method to demonstrate the expression level of SEs mRNA.

CONCLUSION

The present study clearly demonstrated that 82.4% of the milk-acquired MRSA isolates and 100% of the SFP-acquired MRSA isolates were enterotoxigenic and multidrug resist-ant. Six SEs genotypes and twenty-two resistance patterns were found among them. In order to obtain sufficient data for the correct risk assessment, more studies are needed concerning the pre-alence of enterotoxigenic MRSA in food processing environment, food handlers and the products.

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REFERENCES

Arede, P. and D.C. Oliveira, 2013. Proteolysis of mecA repressor is essential for expression of methicillin resistance by *Staphylococcus aureus*. Antimicrob. Agents Chemother., 57: 2001-2002.

Argudin, M.A., M.C. Mendoza and M.R. Rodicio, 2010. Food poisoning and *Staphylococcus aureus* enterotoxins. Toxin, 2: 1751-1773.

Balaban, N. and A. Rasooly, 2000. Staphylococcal enterotoxins. Int. J. Food Microbiol., 61: 1-10.

Bania, J., A. Dabrowska, J. Bystron, K. Korzekwa, J. Chrzanowska and J. Molenda, 2006. Distribution of newly described enterotoxin-like genes in *Staphylococcus aureus* from food. Int. J. Food Microbiol., 108: 36-41.

Becker, K., A.W. Friedrich, G. Peters and C. von Eiff, 2004. Systematic survey on the prevalence of genes coding for staphylococcal enterotoxins SElM, SElO and SElN. Mol. Nutr. Food Res., 48: 488-495.

Bennett, R.W., 2005. Staphylococcal enterotoxin and its rapid identification in foods by enzyme-linked immunosorbent assay-based methodology. J. Food Prot., 68: 1264-1270.

Boynukara, B., T. Gulhan, M. Alisarli, K. Gurturk and H. Solmaz, 2008. Classical enterotoxigenic characteristics of *Staphylococcus aureus* strains isolated from bovine subclinical mastitis in Van, Turkey. Int. J. Food Microbiol., 125: 209-211.

CLSI, 2006. Performance standards for antimicrobial susceptibility testing; M100-S16. 16th International Supplement (M100-S16), National Committee for Clinical Laboratory Standards, Wayne, PA., USA.

CLSI, 2010. Methods for antimicrobial susceptibility tests for bacteria that grow aerobically (M07-A8). National Committee for Clinical and Laboratory Standards. Wayne, PA., USA.

Cha, J.O., J.K. Lee, Y.H. Jung, J.I. Yoo, Y.K. Park, B.S. Kim and Y.S. Lee, 2006. Molecular analysis of *Staphylococcus aureus* isolates associated with staphylococcal food poisoning in South Korea. J. Applied Microbiol., 101: 864-871.

DeLeo, F.R., M. Otto, B.N. Kreiswirth and H.F. Chambers, 2010. Community-associated meticillin-resistant *Staphylococcus aureus*. Lancet, 375: 1557-1568.

Enright, M.C., D.A. Robinson, G. Randle, E.J. Feil, H. Grundmann and B.G. Spratt, 2002. The evolutionary history of Methicillin-Resistant *Staphylococcus aureus* (MRSA). Proc. Natl. Acad. Sci. USA., 99: 7687-7692.

Gordoncillo, M.J., N. Abdujamilova, M. Perri, S. Donabedian, M. Zervos and P. Bartlett, 2012. Detection of Methicillin-Resistant *Staphylococcus aureus* (MRSA) in backyard pigs and their owners, Michigan, USA. Zoonoses Public Health, 59: 212-216.

Jarraud, S., M.A. Peyrat, A. Lim, A. Tristan and M. Bes *et al*., 2001. *egc*, a highly prevalent operon of enterotoxin gene, forms a putative nursery of superantigens in *Staphylococcus aureus*. J. Immunol., 166: 669-677.

Kerouanton, A., J.A. Hennekinne, C. Letertre, L. Petit, O. Chesneau, A. Brisabois and M.L. de Buyser, 2007. Characterization of *Staphylococcus aureus* strains associated with food poisoning outbreaks in France. Int. J. Food Microbiol., 115: 369-375.

Lawrynowicz-Paciorek, M., M. Kochman, K. Piekarska, A. Grochowska and B. Windyga, 2007. The distribution of enterotoxin and enterotoxin-like genes in *Staphylococcus aureus* strains isolated from nasal carriers and food samples. Int. J. Food Microbiol., 117: 319-323.

Le Loir, Y., F. Baron and M. Gautier, 2003. *Staphylococcus aureus* and food poisoning. Genet. Mol. Res., 2: 63-76.

Lee, J.H., J.M. Jeong, Y.H. Park, S.S. Choi and Y.H. Kim *et al*., 2004. Evaluation of the Methicillin-Resistant *Staphylococcus aureus* (MRSA)-Screen latex agglutination test for detection of MRSA of animal origin. J. Clin. Microbiol., 42: 2780-2782.

McLauchlin, J., G.L. Narayanan, V. Mithani and G. O'neill, 2000. The detection of enterotoxins and toxic shock syndrome toxin genes in *Staphylococcus aureus* by polymerase chain reaction. J. Food Prot., 63: 479-488.

Mirzaei, H., H. Farhoudi, H. Tavassoli, M. Farajli and A. Monadi, 2012. Presence and antimicrobial susceptibility of methicillin-resistant *Staphylococcus aureus* in raw and pasteurized milk and ice cream in Tabriz by culture and PCR techniques. Afr. J. Microbiol. Res., 6: 6224-6229.

Mulders, M.N., A.P.J. Haenen, P.L. Geenen, P.C. Vesseur and E.S. Poldervaart *et al*., 2010. Prevalence of livestock-associated MRSA in broiler flocks and risk factors for slaughterhouse personnel in The Netherlands. Epidemiol. Infect., 138: 743-755.

Normanno, G., G. La Salandra, A. Dambrosio, N.C. Quaglia and M. Corrente *et al*., 2007. Occurrence, characterization and antimicrobial resistance of enterotoxigenic *Staphylococcus aureus* isolated from meat and dairy products. Int. J. Food Microbiol., 115: 290-296.

Omoe, K., D.L. Hu, H.K. Ono, S. Shimizu and H. Takahashi-Omoe *et al*., 2013. Emetic potentials of newly identified staphylococcal enterotoxin-like toxins. Infect. Immun., 81: 3627-3631.

Omoe, K., M. Ishikawa, Y. Shimoda, D.L. Hu, S. Ueda and K. Shinagawa, 2002. Detection of seg, seh and sei genes in *Staphylococcus aureus* isolates and determination of the enterotoxin productivities of *S. aureus* isolates harboring seg, seh, or sei genes. J. Clin. Microbiol., 40: 857-862.

Pelisser, M.R., C.S. Klein, K.R. Ascoli, T.R. Zotti and A.C.M. Arisi, 2009. Ocurrence of *Staphylococcus aureus* and multiplex pcr detection of classic enterotoxin genes in cheese and meat products. Braz. J. Microbiol., 40: 145-148.

Seo, K.S. and G.A. Bohach, 2007. *Staphylococcus aureus*. In: Food Microbiology: Fundamentals and Frontiers, Doyle, M.P. and L.R. Beuchat (Eds.). ASM Press, Washington, DC., USA., pp: 493-518.

Valero, A., F. Perez-Rodriguez, E. Carrasco, J.M. Fuentes-Alventosa, R.M. Garcia-Gimeno and G. Zurera, 2009. Modelling the growth boundaries of *Staphylococcus aureus*: Effect of temperature, pH and water activity. Int. J. Food. Microbiol., 133: 186-194.