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RESEARCH ARTICLE

Molecular Characterization of *Staphylococcus aureus* Isolated from Meat and Their Antibiotic Resistance Profiles

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ABSTRACT

The aims of this study were to characterize *S. aureus* isolates from different meat sources in Jordan and study their genetic relationship using PCR-RFLP in addition to their antibiotic resistance profiles. Thirty *S. aureus* isolates were identified and confirmed by PCR techniques. The isolates from goat and camel meats were sensitive to the majority of the tested antibiotics. Plasmid profiling revealed that 26 isolates contained at least one plasmid with no correlation between the number of plasmids and the resistance profiles. PCR-RFLP of the coagulase gene (coa) classified the isolates to several clusters upon digestion with *Alu* I or *Cfo* I restriction enzymes. This study concluded that the 30 *S. aureus* isolates were genetically diverse and comprised heterogeneous population with 7 genotypes at both 33.1 and 51.2 similarity levels.

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INTRODUCTION

Staphylococcus aureus has been implicated in a variety of infections in human and animals (Bartlett and Hulten**,** 2010; Gu *et al*., 2013; Khan *et al*., 2013). The treatments of such infections become difficult due the emergence of multidrug resistant strains such as methicillin resistant *S. aureus* (MRSA) MRSA and *S. aureus* is also considered as a major food borne pathogen (Hennekinne *et al*., 2010; Kenar *et al*., 2012). In Jordan, Disease Control Directorate/Ministry of Health reported that staphylococcal food poisoning during seven years (1999-2005), was implicated in 176, 83, 172, 133, 103, 278, and 90 cases, respectively. Relatively high numbers of staphylococcal food poisoning in Jordan stressed the need to study and characterize *S. aureus* isolated from locally produced meat. Therefore, the detection and typing of *S. aureus* isolates and understanding their antibiotic susceptibility are essential to maintain rigor in quality control within the food processing environment. Traditional methods of *S. aureus* typing have been replaced by molecular typing (Sabat *et al*., 2006). In this study, RFLP of the coagulase gene was performed, as it is cheap and easy to proceed, and has high level of reproducibility (Hookey *et al*., 1998; Morandi *et al*., 2010). Typing the *S. aureus* isolates by RFLP enables our understanding of their genetic structure and improve our capability of tracing the origin of *S. aureus* outbreaks. Therefore, the aim of this study was to characterize *S. aureus* isolates from meat of domestic animals used in Jordan; camels, beef, sheep and goats by PCR-RFLP of the coagulase gene, the isolates were tested for the presence of methicillin resistance genes and the antimicrobial susceptibility patterns.

MATERIALS AND METHODS

Sampling, processing and isolation of *S. aureus*: Two hundred raw meat samples were collected from camels, beef, sheep and goats during 2009 from abattoirs of four governorates in Jordan. Samples were processed as previously reported (Al-Tarazi *et al.,* 2009). All samples were cultured on Baird-Parker agar base (BPA) supplemented with egg yolk-tellurite emulsion (Oxoid UK). Presumptive *S. aureus* isolates were further identified using Microbact Staph 12S system (Oxoid, UK).

Antibiotic test: Agar disk diffusion test of Penicillin (P; 10U), Oxacillin (OX; 1µg), Cefoxitin (FOX; 10µg), Gentamicin (GN; 10µg), Tobramycin (TOB; 10µg), Amikacin (AK; 30µg), Teicoplanin (TEC; 30 µg), Ciprofloxacin (CIP; 5µg), Chloramphenicol (C; 30µg),

Erythromycin (E; 15µg), Tetracycline (TE; 15µg), Trimethoprim/Sulphamethoxazole (SXT; 1.25/23.75µg), Amoxicillin-clavulanic acid (AMC.20/10µg), Azithromycin (AZD; 15µg), Moxifloxacin (MXF; 5µg), Clindamycin (DA; 2µg), Rifampicin (RA; 5µg), Quinupristin/ Dalfopristin (QA; 15µg), Linezolid (LZD; 30µg) and Vancomycin (VA; 30µg) was carried out using the Clinical Laboratory Standard Institute guidelines, supplement M2-A9 (CLSI, 2008).

***S. aureus* identification and confirmation of *Mec A* and *Coa* genes by PCR:** DNA from the isolates was extracted using the Wizard Genomic DNA purification kit (Promega, USA) and the purity of the extracted DNA was tested by running samples on agarose gel electrophoresis and pure DNA stored in -80°C. Thermal cycling for PCR amplification was performed as described in Table 2.

DNA restriction endonuclease analysis of the PCR-amplified coagulase gene: Restriction analysis was conducted as described by Hookey *et al*. (1998) and Plasmid profiling of the isolates was performed using the Wizard Plus SV minipreps DNA purification system (Promega, USA).

RFLP cluster analysis of the isolates: RFLP banding patterns of the restriction *coa* PCR-products for the 30 *S. aureus* isolates were examined by estimating the size of each fragment on the electrophoresis gel and bands were scored, with the data coded as a factor of 1 or 0, representing the presence or absence of a restriction band, respectively. A similarity matrix among *S. aureus* isolates was produced using the Jaccard coefficient. A dendrogram showing the genetic relatedness among the isolates was constructed from the resulting data using SPSS version 15 (SPSS Inc. 1989-2007). The cutoff for the dendrogram was selected based on the average of mean similarity matrix.

RESULTS AND DISCUSSION

Isolation and enumeration of *S. aureus*: Thirty of 200 meat samples revealed presence of PCR-confirmed *S. aureus* (Table 1). Those isolates were then tested for antibiotic sensitivity, plasmid profile and cluster analysis to understand their genetic relatedness using *Alu* I and *Cfo* I restriction enzyme analysis. The combination of phenotypic and genotypic characterization is often effective in tracing a particular isolate incriminated in an outbreak. Genomic fingerprinting is necessary for classifying multiple isolates into groups with similar or close similarity indices (Sabour *et al*., 2004). In this work, phenotypic and genotypic properties and relatedness of the 30 *S. aureus* isolates were investigated.

Testing of antibiotic resistance by disk diffusion and PCR methods: Thirty *S. aureus* isolates were tested for sensitivity to commonly used antibiotics. All the tested isolates were completely sensitive to six antibiotics (OX, GN, AK, TOB, AZM and C) (Table 1). Also the isolates showed high susceptibility (>90%) to Qd, RA, MET, LZD and DA antibiotics. This indicates the lower frequency of using these antibiotics for therapy or as animal feed additive. In contrast, the majority of the isolates (87 and 80%) were resistant to penicillin and amoxicillin-clavulanic acid, respectively, highlighting the frequent use of these antibiotics for animals. This appears to be in agreement with other report (Guven *et al*., 2010).

Among the 30 isolates, one isolate was sensitive to all the antibiotics tested and five other isolates exhibited resistance to two, while the rest exhibited resistance to more than two antibiotics. These results are similar to those published by Andrew *et al*. (2011). In this study, when analyzing multidrug resistance patterns of antibiotics to each animal species, it appeared that isolates of beef meats exhibited the highest resistance; one isolates resistance to 11 and another to 8 antibiotics, followed by two isolates from camel and goat's meat which was resistant to 8 and 7 antibiotics, respectively (Table 1).

Table 1: Source of the PCR *S. aureus* confirmed isolates, number of plasmids, number of antibiotics each isolate resistant to and types of these antibiotics.

Isolate ID	Source	No. of Plasmids	No. of Anti-biotics[@]	Types of antibiotics[$]
55	Goat's meat	0	6	P,VA,TEC,AMC, CIP, MXF
56	Goat's meat	0	3	P, AMC, CIP
62	Goat's meat	2	7	P,FOX,VA, E, TE, SXT, LZD
66	Goat's meat	2	6	P, MET, VA, AMC, TE, SXT
76	Goat's meat	2	3	P, AMC, SXT
79	Goat's meat	0	4	AMC, TE, CIP, SXT
83	Goat's meat	2	2	P, SXT
84	Goat's meat	1	4	P, AMC, TE, SXT
92	Goat's meat	1	3	P, AMC, SXT
94	Goat's meat	2	7	P, FOX, MET, TE, CIP, SXT
96	Goat's meat	3	2	P, AMC
99	Goat's meat	1	5	P, FOX, AMC, TE, SXT
251	Camel's meat	4	6	P, VA, TEC, AMC, TE, CIP
252	Camel's meat	1	0	All tested antibiotics are sensitive
254	Camel's meat	1	6	P, TEC, AMC, TE, CIP, SXT
258	Camel's meat	3	2	VA, SXT
259	Camel's meat	1	5	P, AMC, TE, CIP, DA
263	Camel's meat	0	2	CIP, SXT
266	Camel's meat	1	4	P, AMC, CIP, SXT
267	Camel's meat	1	8	P,AMC, E,TE, CIP, DA, SXT, LZD
270	Camel's meat	1	3	P, TEC, AMC,
272	Camel's meat	1	6	P, AMC, TE, CIP, MXF, SXT
4cd	Beef's meat	1	3	P, AMC, CIP,
6cd	Beef's meat	7	6	P, VA, AMC, E, CIP, MXF
12cd	Beef's meat	1	8	P,VA,TEC, AMC, E,TE, CIP, MXF
22cd	Beef's meat	1	11	P, VA, TEC, AMC, E, TE, CIP, MXF, DA, SXT, QD
28cd	Beef's meat	1	5	P, AMC, TE, CIP, SXT
33cd	Beef's meat	2	3	P, AMC, RA
38cd	Beef's meat	0	2	P, AMC
512	Sheep meat	4	3	P, TEC, AMC

[@]: the number of antibiotics the isolate is resistant to
[$]: the types of the antibiotics the isolates are resistant to

When the isolates were tested for the resistance to methicillin, only two isolates from goat meat samples exhibit intermediate resistance (Table 1). However, when tested for presence of *mec* A gene, only two isolates no. 55 and no. 33 cd did not contain the *mec* A gene. Those two isolates were sensitive to methicillin, cefoxitin and oxacillin confirming the absence of the resistance genes. Although it was reported that the presence of *mec* A gene is related to the resistance to cefoxitin and oxacillin as *mec* A gene encodes for penicillin binding proteins that leads to the oxacillin resistance (Anand *et al*., 2009). In

Table 2: Oligonucleotide primer pairs and PCR running conditions used for *S. aureus*

Gene	Primers 5'…….3'	Amplification conditions			Product size (bp)	Reference
		Temp	Time	No. of Cycles		
Thermonuclease (nuc)	Pri-1	94°C	5 min	1	270	Pinto *et al.* (2005)
	5'GCGATTGATGGTGATACGGTT 3'	94°C	30 s	35		
	Pri-2	55°C	45 s			
	5' AGCCAAGCCTTGACGAACTAAAGC 3'	72°C	45 s			
		72°C	10 min	1		
Methicillin resistance (mecA)	*mec*A F	94°C	4 min	1	310	Geha *et al.* (1994)
	5-'GTAGAAATGACTGAACGTCCGATGA 3'	94°C	45 s	35		
	*mec*A R 5'CCAATTCCACATTGTTTCGGTCTAA 3'	50°C	45 s			
		72°C	60 s			
		72°C	2 min	1		
Coagulase (coa)	Coa F	94°C	45 s	1	Variable[a]	Hookey *et al.* (1998)
	5'-ATA GAG ATG CTG GTA CAG G-3'	94°C	20 s	35		
	Coa R	57°C	15 s			
	5'-GCT TCC GAT TGT TCG ATG C-3'	72°C	15 s			
		72°C	2 min	1		

[a] 875, 660, 603, or 547 bp

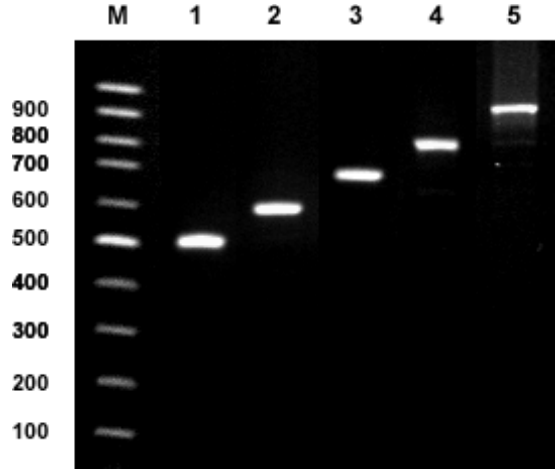

Fig. 1: Agarose gel electrophoresis of amplified PCR fragments of coagulase gene for *S. aureus* sub species aureus isolates. Lane 1: three isolates of 500 bp, lane 2: fifteen isolates of 600 bp, lane 3: nine isolates of 660 bp, lane 4: two isolates of 800 bp, lane 5: one isolate of 850 bp.

this study, there was no relationship between these two facts which appears to be in agreement with results reported by Yamazumi *et al*. (2001), where the difference in phenotypic and genotypic methicillin resistance might be due to heterogeneous expression of *mec* A gene by Staphylococcus strains.

Plasmid Profiling: When the 30 *S. aureus* isolates were tested for the presence of plasmids, 5 isolates did not contain any plasmid although they exhibit resistance to some antibiotics indicating that the resistance genes might be located on the chromosomal genome of these isolates. Also, the majority (14 isolates) contained only 1 plasmid. Moreover, the isolate that contained the highest number of plasmids (7) exhibited resistance to only 6 antibiotics while an isolate showed resistance to 11 different antibiotics contained only 1 plasmid highlighting the lack of correlation between the number of plasmids and the antibiotic resistance profiles ($r^2 = 0.003$) (Table 1). These results are supported by a study of Dharmalingam *et al*. (2003) who reported no correlation between the presence of the plasmids in *Helicobacter pylori* isolates and their antibiotic resistance. These results indicated the inadequacy of plasmid analysis to obtain any results on the antibiotic resistance profiles of *S. aureus* isolates.

PCR-RFLP analysis of coagulase gene: Production of coagulase by *S. aureus* isolates is considered an important phenotypic feature and is used to type this pathogen. The variability of the 3' end region of the *coa* gene is the basis for typing which is used to trace pathogens among animals or humans (da Silva and da Silva, 2005). Upon testing the isolates for the presence of *coa* gene by PCR, the size of the amplified genes were 500, 600, 660, 800 and 850 bp in 3, 15, 9, 2 and 1 isolate, respectively, with one amplicon for each isolate (Fig. 1). This indicates the presence of the same allelic forms of *coa* gene (da Silva and da Silva, 2005). In this study, PCR products of the *coa* gene were digested with *Cfo*I and *Alu* I enzymes in an attempt to confirm the resulting RFLP patterns for these isolates. Both enzymes generated the same number of clusters but differ in the distribution of the isolates among the clusters in that, clusters appeared to be identical after digestion with one enzyme are different when digested with the other enzyme (Fig. 2 and 3).

Among the clusters, cluster A for both enzymes shared 6 isolates and cluster B for *Alu* I enzyme shared 12 isolates with cluster C of the *Cfo* I enzymes. These results differ from a study conducted by Hookey *et al*., (1998) who reported very similar clusters obtained for the *S. aureus* isolates digested with *Alu* I and *Cfo* I enzymes.

Dendrogram was constructed on the basis of similarity index among *S. aureus* isolates using *Alu*-generated RFLP (Fig. 2). A 33.1% cutoff value gave 3 major clusters with 26 isolates while 4 isolates formed single clusters. Cluster C contains 15 isolates (9 from goats and 6 from camel meat) with similarity range of 0.36-1.0. The genotype C cluster was predominant with three sub clusters, sub cluster I for isolates from camel's meat, sub-clusters II and III form goat meat collected from two different locations suggesting the existence of the same clones in different geographical regions.

Cluster B contained only 3 isolates (similarity level 1.0) from camel meat suggesting that these isolates are indistinguishable or have been derived from similar clones or represent the same strain. Whereas, all meat samples 7 beef's and 3 sheep's formed one cluster (A) with a similarity ranged from 0.5-1.0. Genotypes D, E, F and G contain one isolate in each with minimal similarity.

It is noteworthy that the goats were slaughtered in different slaughterhouses than where the camels were slaughtered. This could be explained by the fact that the

contaminating strains are predominant in slaughterhouses in Jordan and animals are moved easily before being slaughtered. In addition, there were some isolates belonging to the same cluster although isolated from animals in different places. This substantiates the existence of certain strains of *S. aureus* that might have spread among the animals in these places (Aires-de-Sousa *et al*., 2007).

The dendrogram was constructed on the basis of the similarity index among *S. aureus* isolates using *Cfo* I RFLP Fig. 3. A 51.2% cutoff value gave 7 distinct clusters. Cluster A contained 6 indistinguishable isolates (similarity of 1.0), 5 of them from beef meat, 1 isolate from sheep meat. The high rate of similarity within a particular group (same abattoir) indicates cross contamination among the isolates at some point during processing (van Loo *et al*., 2007). Cluster B contained 14 isolates (similarity 0.5-1.0) of which 6 isolates from goat meat that was obtained from 3 different abattoirs while the other 8 isolates from camels meat that was obtained from the same abattoir. It is noteworthy that all the isolates but one (isolate no. 55) were indistinguishable with 100% similarity when digested with *Cfo* I while they were not identical when digested with *Alu* I enzyme. The similarity in the genotypes of some of the isolates necessitates the use of two enzymes to resolve differences among isolates. Genotypes C and D contained mixtures of the isolates from both camels and goats meat with cluster D contains 3 isolates (similarity 0.7-0.8) one from camels and two from goats meat while cluster C containing two isolates sharing 0.7% similarity. Cluster F contained 3 isolates (similarity 1.0) all from goat's meat. Clusters E and G contained one isolate each (no. 99 and no. 28 cd, respectively). Only isolate (no. 28 cd) was not digested by the *Cfo* I enzyme. Typing these isolates by RFLP using two restriction enzymes revealed that the isolates were genetically diverse and comprise a heterogeneous population with 7 genotypes at 33.1% and 51.2% similarity levels after restriction with *Alu* I and *Cfo* I enzymes, respectively.

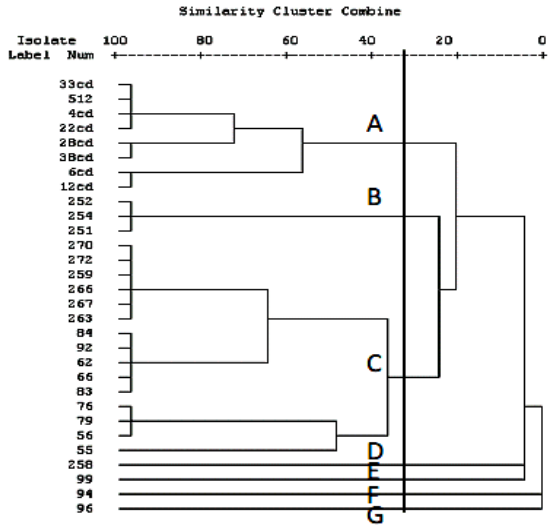

Fig. 2: Dendrogram derived from Jaccard Coefficient Cluster analysis based on combined similarity matrix obtained from Coagulase RFLP-using *Alu* I restriction enzyme showing genetic relatedness among *S. aureus* isolates from meat in Jordan. The scale at the top shows the similarity index. The cutoff was set at 33.1% similarity. Clusters are numbered by capital letter A-G. Numbers at the left side of the dendrogram denote the isolate identification numbers.

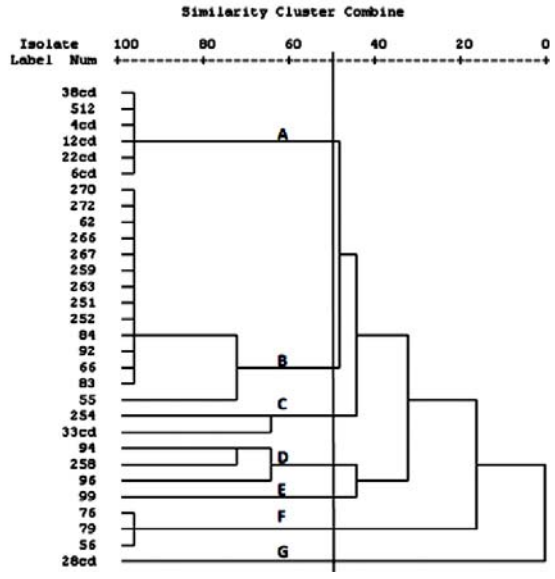

Fig. 3: Dendrogram derived from Jaccard Coefficient Cluster analysis based on combined similarity matrix obtained from Coagulase RFLP-using *Cfo* I restriction enzyme showing genetic relatedness among *S. aureus* isolates from meat in Jordan. The scale at the top shows the similarity index. The cutoff was set at 51.2% similarity. Clusters are numbered by capital letter A-G. Numbers at the left side of the dendrogram denote the isolate identification numbers.

Conclusion: The presence of multidrug resistant *S. aureus* isolated mainly from beef's meat indicating the misuse of the antibiotics, and that aminoglycopeptides are the antibiotics of choice for treatment of a multi-resistant *S. aureus* infection in animals. RFLP of the *coa* gene offers a good discriminatory power in typing *S. aureus* isolates collected from different geographical regions in Jordan. The data presented would help food and animal health workers to enhance safety precautions before slaughtering and while handling meat originated from different animals.

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