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Biotypes and Enterotoxigenicity of Staphylococci Isolated from Camel's Meat in Jordan

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Research Article

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ABSTRACT

A total of 264 camel's meat and nasal swab samples were collected for isolation and typing of Staphylococci from Irbid Governorate in northern Jordan. About 97 % and 85% of meat and nasal swabs samples showed typical colonies of *Staphylococcus aureus* on Baird-Parker agar respectively. Out of 243 presumptively identified isolates, only 74 and 64 were confirmed as *S. aureus* by Microbact system and PCR technique respectively. About 67% of the isolates were typable by Devriese's scheme. Fifteen of those isolates (23%) were specifically allocated to human, bovine, ovine or abattoir where, 14% of these host specific isolates belonged to human biovar. The other 44% belonged to non-host specific biovars with majority of them were allocated to NHS1 biovar. When tested for the presence of toxin genes, 71.9% of *S. aureus* isolates had SE(s) genes with SEA being the most prominent at 91.3%. The study also showed that not only coagulase positive isolates contain toxin genes, coagulase negative isolates also possess toxin genes and thus are considered potential hazards in camel's meat.

Keywords: Staphylococcus aureus; biotyping; enterotoxins; Jordan.

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1. INTRODUCTION

Genus *Staphylococcus* contains 45 species and 24 subspecies as reported in the List of Prokaryotic Names with Standing in Nomenclature as of the full update in May 4, 2011 (http://www.bacterio.cict.fr/s/staphylococcus.html). Among those, only 19 species are of potential interest in food with *S. aureus* being one of the most pathogens incriminated in foodborne outbreaks (Le Loir et al., 2003).

Camel meat is a good source of food in areas where the climate adversely affects other animals (Knoess, 1977). The microbial surface contamination of carcasses has been repeatedly reported to have a significant effect on the meat shelf life. In a local study, 23 out of 25 camel's meat samples examined showed typical colonies of *S. aureus* on Baird-Parker agar (BPA), with a high mean count of 3×10^4 cfu/g which is much higher than that observed in beef, mutton or goats meat (Al-Tarazi et al., 2009).

S. aureus can cause food poisoning by ingesting preformed enterotoxin with abdominal pain, diarrhea, vomiting and nausea being the major symptoms (Le Loir et al., 2003). *S. aureus* produces a variety of extracellular protein toxins contributing to its virulence. Many of *S. aureus* strains produce pyrogenic exotoxins, such as staphylococcal enterotoxins (SE) and toxic shock syndrome toxin 1 (TSST-1). Staphylococcal enterotoxins (SEs) have been classified into many different types. Fifteen staphylococcal enterotoxins were identified; SEA-SEE-SEO and SEU (Atichou et al., 2004). The most common types of these enterotoxins are SEA, SEB, SECs, SED and SEE. Isolates carrying the aforementioned toxin genes are responsible for 95% of staphylococcal food poisoning outbreaks (Bergdoll 1989). Furthermore, not only coagulase positive *S. aureus* can produce toxins, coagulase negative *S. aureus* (CoNS) isolates were also reported to harbor enterotoxin genes (Cunha et al., 2006).

Isolation, identification and typing of *S. aureus* by conventional methods may take 5 to 6 days, even when rapid commercial systems are used for identification. Despite the lengthy procedures associated with conventional methods, they are still in use for *S. aureus* biotyping. Devriese (1984) developed a system that classifies *S. aureus* into human, bovine, ovine, poultry and five non-host specific biovars depending on unique biochemical characteristics. However, in the last two decades, many researchers have shifted to the use of polymerase chain reaction (PCR) for the detection and possible classification of food-borne pathogens to replace the time-consuming culture-based classical techniques (Candrian 1995; Hil 1996). For instance, one of the specific PCR primers used for PCR detection of *S. aureus* is directed to the thermonuclease (***nuc***) gene encoding thermo stable nuclease that gives 100% detection sensitivity for *S. aureus* (Hein et al., 2001; Brakstad et al., 1992; Palomares et al., 2003).

Conventional methods for the detection of toxin-producing *S. aureus* strains are based on immunological procedures measuring the toxin in culture supernatants of suspected *S. aureus* strains or in contaminated food extracts or in patient specimens (Thompson et al., 1986). However, these methods are only useful in the presence of detectable amounts of toxins. Nucleotide sequences for toxin genes were obtained and subsequently, these sequences were used for the development of specific oligonucleotide primers for the detection of the presence of the toxin genes in *S. aureus* isolates and thus identifying potential enterotoxigenic isolates (Bayles and Iandolo, 1989; Blomster-Hautamaa et al., 1986; Johnson et al., 1991). The objectives of this study were to isolate *Staphylococcus aureus* from camel's meat and to biotype the isolates by the Devriese's scheme and to test

the isolates for the presence of toxin genes using the Polymerase Chain Reaction (PCR).

2. MATERIALS AND METHODS

2.1 Sample Collection, Transportation and Preparation

A total of 264 camel's nasal swabs and meat samples were collected from Irbid Governorate in northern Jordan and examined during the period between June 2007 and the end of July 2008. The 147 meat samples were collected from an abattoir or meat markets. Each meat sample represents one camel and about two hundred and fifty grams of meat collected from different locations of each carcass. Cut surfaces were taken from leg, flank and neck of camel carcasses. Thin wide meat slices were chosen under aseptic conditions using sterile blades and sterile containers (Gill, 2007). The 117 nose swabs were collected by inserting sterile swabs moistened in 0.1% buffer peptone (Hi-Media, India) in the external openings of the nose from the same camels that the meat was taken from, whenever it was possible. All samples were transported to laboratory under aseptic cooled condition as soon as possible and examined within 6 hours of collection time.

2.2 Isolation of *Staphylococcus aureus*

Isolation of *S. aureus* was performed by mixing 25g of each meat sample with 225 ml of 0.1% sterile buffered peptone water (Hi-Media, India), and homogenized in Stomacher (Seward, USA) at 300 cycles for 3 min. Serial decimal dilutions were prepared in buffered peptone and 0.1 ml of selected dilutions of each sample was spread onto Baird-Parker Agar (BPA) base (Oxoid, U.K.) supplemented with egg yolk-tellurite emulsion (Oxoid, U.K.) and plates were incubated at 37°C for 24 hours. If there was no growth, then plates were incubated for an additional 24 hours. Nasal swab samples were enriched by placing the cotton swabs in brain heart infusion broth (BHI) (Hi-Media, India) and incubated overnight at 37°C followed by subsequent streaking over BPA, and incubation at 37°C for 24-48 hours. Black to dark grey colonies with opaque zones, surrounded by clear halo was considered as presumptive *S. aureus*.

2.3 Identification of *Staphylococcus aureus*

2.3.1 Presumptive identification

Presumptive *S. aureus* isolates were further examined by Gram staining and tested for production of catalase and for coagulase production uses Latex Coagulase Kit (Plasmatec, Canada) according to the manufacturer's instructions (Koneman 1988). Presumptive isolates were then subjected to Microbact™ staphylococcal 12S system (Oxoid, U.K.) for species identification as per manufacturer's instructions.

Table 1. Primers used in the study and the PCR conditions for each primer

Gene	Primers 5'…….3'	Amplification conditions			Product size (bp)	Reference
		Temp	Time	No. of Cycles		
Thermonuclease (nuc)	**Pri-1** GCGATTGATGGTGATACGGTT **Pri-2** AGCCAAGCCTTGACGAACTAAAGC	94° C 94° C 55° C 72° C 72° C	5 min 30 s 45 s 45 s 10 min	1 35 1	270	Pinto et al., (2005)
Coagulase (coa)	**Coa F** ATA GAG ATG CTG GTA CAG G **Coa R** GCT TCC GAT TGT TCG ATG C	94° C 94° C 57° C 72 ° 72° C	45 s 20 s 15 s 15 s 2 min	1 35 1	Variable [a]	Hookey et al., (1998)
Toxin (SeA)	**SEA1** TTGGAAACGGTTAAAACGAA **SEA2** GAACCTTCCCATCAAAAACA	94° C 94° C 55° C 72 ° 72° C	2 min 2 min 2 min 1 min 7 min	1 40 1	120	Johnson et al., (1991)
Toxin (SeB)	**SEB1** TCGCATCAAACTGACAAACG SEB2 GCAGGTACTCTATAAGTGCC	Same as SeA			478	Johnson et al., (1991)
Toxin (SeC)	**SEC1** GACATAAAAGCTAGGAATTT SEC2 AAATCGGATTAACATTATCC	Same as SeA			257	Johnson et al., (1991)
Toxin (SeD)	**SED1** CTAGTTTGGTAATATCTCCT SED2 TAATGCTATATCTTATAGGG	Same as SeA			317	Johnson et al., (1991)
Toxin (SeE)	**SEE1** TAGATAAAGTTAAAACAAGC **SEE2** TAACTTACCGTGGACCCTTC	Same as SeA			170	Johnson et al., (1991)

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

2.3.2 Coagulase test

Tube method was used to detect both free and bound coagulase enzyme. Half ml of sterile rabbit plasma was placed in a clean test tube, mixed with loop full or colony from nutrient agar culture, incubated at 35-37 in water bath and result were taken every 30 minutes for 4 hours. Positive results were manifested by solidifying the plasma. If the result remains negative, the tubes were re-incubated overnight and tested again (Baird 1996).

2.3.3 DNase test

DNase activity was tested on DNase test agar following the manufacturer's recommendations (Difco). Only strong DNase activities indicated by clearing zone around growth similar to that of the *S. aureus* control strain were recorded as positive. Weak DNase activities with clearing zones noticeably smaller than that of the positive control were recorded as negative (Boerlin et al., 2003).

2.3.4 Molecular methods

The DNA which was used in the molecular methods was extracted from 15 ml Brain Heart Infusion (BHI) overnight culture using Promega wizard® genomic DNA purification kit according to manufacturer's instructions. Purity of DNA was evaluated by agarose gel electrophoresis.

2.3.5 Identification, confirmation and toxin genes detection of *S. aureus* isolates using PCR

All the isolates were confirmed as *S. aureus* using PCR primers for *nuc* and *Coa* gene. In addition, the isolates were tested for the presence of coagulase and SE toxin genes (SEA, SEB, SEC, SED and SEE) using PCR specific primers listed in table 1. All PCRs were performed in 50 µl volumes, with each reaction mixture containing the following: 1X PCR buffer (20 mM Tris-HCl, pH 8.0, 100 mM KCl, 0.1 mM EDTA), 200 mM of each deoxynucleoside triphosphate, 1.5 mM of $MgCl_2$, 2.0 U of *Taq* DNA polymerase (All from Promega Inc.), 0.2 pmole from each primer and 5 µl of DNA template. Thermal cycling for each PCR amplification was performed as described in Table 1. PCR products were separated by electrophoresis on 2% agarose gel and visualized under U.V light. All DNA of SE(s) positive controls were kindly provided by Dr Karsten Becker (Institute of Medical Microbiology, University Hospital of Munster, Germany).

2.4 Biotyping of *Staphylococcus aureus*

Biotyping of *S. aureus* strains were performed according to Devriese's scheme (Devriese 1984). This method depends on 4 different biochemical reactions; detection of staphylokinase, β-haemolysin, coagulase activity on bovine plasma, and the type of growth on crystal violet agar media.

3. RESULTS

3.1 Isolation of *Staphylococcus aureus*

One hundred and forty three out of 147 (97.27%) camel meat samples examined showed typical colonies of *S. aureus* on BPA, with a mean count of $2.58 \times 10^4 \pm 1.8 \times 10^4$cfu/g.

Whereas, 100 of 117 (85.4%) nasal swabs revealed typical colonies of *S. aureus* on BPA. From each positive meat or swab sample, one characteristic colony was chosen and subjected for further confirmation.

3.2 Species Identification

3.2.1 Biochemical

To identify the 243 presumptive *S. aureus* isolates, Microbact™ staphylococcal 12S (Oxoid, UK) biochemical identification system was used. *S. aureus* was identified in 58 and 16 of meat and swab isolates respectively. The distribution of the isolated Staphylococcal species based on Microbact™ staphylococcal 12S identification system is presented in Table 2.

3.2.2 Molecular identification

Isolates identified as *S. aureus* by the biochemical profiling system were confirmed by amplifying the thermonuclease gene specific for *S. aureus* (***nuc***) using PCR. The identities of only 64 (out of 74 biochemically identified *S. aureus*) isolates were confirmed as *S. aureus,* of which 48 out of them revealed the presence of coagulase genes. The distribution of swabs and meat isolates are presented in Table 3.

3.3 The Enterotoxigenicity of *S. aureus* isolates

When all the isolates (coagulase +ve and coagulase –ve) were tested for toxin production, 46 out of the 64 confirmed *S. aureus* isolates (71.9%) found to have SE gene(s). Five of these isolates were recovered from nasal swabs while the other 41 isolates were of meat origin. The most prominent type of SE gene was SEA with 42 strains (65.6%) harboring this toxin gen followed by 3 isolates containing SEA+SEB genes (4.6%) and 1 strain containing SEC gene (1.5%). However, none of the isolates had SEE or SED genes.

3.4 Biotyping

When typed with the Diverse Scheme, 43 out of the 64 *S. aureus* isolates (67.2%) were typable, while the other 21 (32.8%) were untypable. Of the untypable strains, 20 isolates were of meat origin while 1 isolate was from swabs samples. The number and prevalence of each biovar and their distribution are presented in (Table 4).

Table 2. The distribution of the *Staphylococcus* species depending on Microbact™ staphylococcal 12S system

Items	*S. aureus*	*S. hemolyticus*	*S. simulans*	*S. captis*	*S. saprophiticus*	*S. hominis*	*S. warneri*	*S. xylosus*	*S. cohni*	*S. schleifri*	*S. avrecaris*	*S. lygnesi*
Meat	58	0	24	13	14	11	10	4	5	2	1	1
Swab	16	3	12	12	44	3	5	5	0	0	0	0
Total	74	3	36	25	58	14	15	9	5	2	1	1

Table 3. Distribution of *S. aureus* isolates depending on Microbact 12S and PCR confirmation system

Sample items	No.of isolates confirmed by Microbact 12S	No. of isolates confirmed by *nuc* gen (%)	No. of isolates having both *nuc* and coagulase genes (%)
Meat	58	54 (93.1)	42(72.4)
Swabs	16	10 (62.5)	6 (37.5)
Total	74	64 (86.4)	48(64.9)

Table 4. The distribution of the 43 typable *S. aureus* isolates obtained from the 64 camel's meat and nasal swabs isolates

Biovars	No. of isolates (%)	Meat	Swab
Human	9 (14)	7	2
Bovine	3 (4.6)	2	1
Ovine	2 (3.1)	1	1
Abattoir	1 (1.5)	0	1
NHS1	18 (28.1)	17	1
NHS2	3 (4.6)	2	1
NHS3	1 (1.5)	1	0
NHS4	3 (4.6)	2	1
NHS5	3 (4.6)	2	1
Total	43 (67.1)	34 (53.1)	9 (14)

NHS: Non host specific.

Table 5. The distribution of the 21 untypable *S. aureus* isolates obtained from 64 camel's meat and swabs isolates

(H, S, B, CV)	No. of isolates (%)	Meat	Nose swabs
(-, +, +, c)	10 (15.6 %)	9	1
(+, +, +, c)	6 (9.4%)	6	0
(-, -, +, a)	3 (4.7%)	3	0
(-, -, +, c)	2 (3.12%)	2	0
Total (%)	21 (32.8%)	20 (31.3%)	1 (1.6%)

H: β-hemolysin; positive (+), negative (-)
S: staphylokinase; positive (+), negative (-)
B: Bovine plasma coagulation; positive (+), negative (-)
CV: Crystal violet types of growth, c: type c, a: type a

Among the isolates, 14 (32.5%) were specifically allocated to human and different animals biotypes, whereas 28 (65.1%) belonged to non-host specific biovars and only one isolate (2.4%) belonged to abattoir biovar. The untypable isolates are presented in (Table 5).

3.5 Distribution of Enterotoxigenic *S. aureus* Biotypes

When the relationship between biotypes and toxin production was analyzed, there was no relationship observed between any biotype and the type of enterotoxin gene. However, the SEA type was detected in the different biotypes while SEC was detected in one bovine biotype and the combination of SEA + SEB genes was observed in either two NHS1 or one untypable isolates.

4. DISCUSSION

The mean count of presumptive *S. aureus* in camel's meat examined was $2.5 \times 10^4 \pm 1.8 \times 10^4$ cfu/g. Nearly similar findings of 3×10^4 were obtained in a local study on camel meat (Al-Tarazi et al., 2009). There are no specific standards for permissible number of *S. aureus* in raw meat items. Traditionally *S. aureus* count of 10^3 cfu/g is the highest permissible number usually recommended as a specification by the international agencies (Sally and Mark, 2003). The lowest number of *S. aureus* cells required to produce the minimum level of

enterotoxin that is considered necessary to cause gastroenteritis in humans differs for substrates and for types of enterotoxins, however, it is ranged from ~10^4 cfu/g to 10^7 cfu/g, with 10^6 cells/g being the average number required to cause *Staphylococcus* food poisoning (Jay, et al., 2005). Some of the leading factors responsible for Staphylococcal food poisoning outbreaks include improper holding temperatures, poor personal hygiene and contaminated equipments (Bean and Griffin, 1990). This indicates that, high *S. aureus* counts and potential food poisoning usually results from food abuse, particularly in cooked or ready to serve food. Therefore, presence of low number of *S. aureus* in fresh food doesn't necessarily guarantee that such meat is safe for consumption (ICMSF, 1986). The majority of the nasal swab isolates were identified as *S. saprophyticus* in contrast to *S. aureus* in meat isolates. When typed using Devriese scheme, the percentages of the untypable *S. aureus* were 31.3 % and 1.6% in meat and nasal swabs respectively. The above findings may suggest a limited correlation between meat isolates and that obtained from the camel nasal cavity. This also may imply that the camel carcass gets contaminated during slaughtering and processing and such contamination had increased the percentage of the enterotoxigenic *S. aureus* isolates from 45% (5 out of 11 isolates) in camel nose flora up to 76% (41 out of 54 isolates) in camel meats.

Knowing that about half of the staphylococcal species are host adapted to human and animals, while the other half including coagulase negative species are adapted solely to nonhuman hosts, therefore the entry of these species into human foods is not precluded (Jay et al., 2005). In this study, out of presumptive 243 *S. aureus* isolates, only 30%, 74 isolates were identified using Microbact 12S as *S. aureus*, while, the other 70% were identified as following; *S. hemolyticus, S. simulans, S. captis, S. saprophityicus, S. hominis, S. warneri, S, xylosus, S. cohni, S. schleifri, S. auricularis* and *S. lugdenesis.*

Eighty six percent of biochemically confirmed as *S. aureus* by the Microbact system (64 isolates) were confirmed as *S. aureus* using *S. aureus* specific ***nuc*** gene. These confirmed isolates were subjected to biotyping using Devriese biotyping scheme. Only 43 (67.2%) of isolates were typable, while the other 21 (32.8%) isolates did not belong to any known biovar. Among the typable isolates, 15 (34.9%) of isolates were specifically allocated to either human, bovine, ovine or abattoir biovars. The other 28 (65.1%) of the isolates belonged to non-host specific biovars. This may indicates the broad diversity of contamination sources. It's hard to draw a comparison with other food items simply because each food has its own environment and sources of contamination. However in a local study on different meat items including camel's meat, it was reported that the majority of host specific biovars were of bovine (24.33%) and human (22.16%) (Al-Tarazi et al., 2009). Other two separate studies on *S. aureus* food isolates showed that 51.3% and 41.3% of the specific typable biovars were of human biovar (Devriese, 1984; Rosec et al., 1997).

The enterotoxigenic character was much greatly related to *S. aureus* in comparison with other staphylococci and was highly correlated with coagulase positive rather than CoNS isolates. In this study, out of the 16 CoNS identified isolates, only one isolate (6.2%) appears to be potentially enterotoxigenic by exhibiting the presence of SEA gene. The observed low prevalence of enterotoxigenic isolates among CoNS is similar to results obtained in a local study conducted by Al-Tarazi et al. (2009) in different meat items including camel's meat where only 2.4% of the coagulase negative strains were enterotoxigenic. Our results also are in accordance with two other studies on CoNS isolates from goat and sheep milk reported that the percentage of enterotoxigenic strains among the CoNS were 10.5% and 6.2% respectively (Bautista et al.,1988; Vernozy-Rozand et al., 1996).

Staphylococcus enterotoxin gene(s) were detected in 71.9% of *S. aureus* isolates. The presence of SE(s) genes does not necessarily mean that the isolate can produce intact and biologically active toxin, and that could be due to possible mutations in the coding regulatory regions leading to impairment in translation (Sharma et al., 2000). This explanation was demonstrated in two studies (Sharma et al., 2000; Mornadi et al., 2007) where toxin genes were detected in some non-toxin producing *S. aureus* isolates. However, these results contrasts other study conducted by Fueyo et al. (2001) in which the detection of the classical enterotoxin genes in human *S. aureus* isolates was concomitant with the production of toxin. These observations may needs further verification, but in all cases, isolates carrying SE(s) genes are considered potential enterotoxigenic strains. The 71.9% enterotoxigenic strains reported here is closely similar to those reported in Brazil for *S. aureus* isolates from raw and pasteurized milk (68.4%) (Rall et al., 2008) and in Italy for isolates from milk and dairy products (67%) (Mornadi et al., 2007). Nevertheless, other studies reported lower percentage of enterotoxigenic strains for isolates from milk (37%) or much higher than the 13.5% for isolates from different local meat items (Adwan et al., 2005; Al-Tarazi et al., 2009). In the later study for instance none of the *S. aureus* isolated from camel's meat were enterotoxigenic. The variability of food examined in the former studies could explain the diversity of enterotoxigenic prevalence among *S. aureus*, indicating that substrate type and processing conditions exert an effect on the production of toxin by *S. aureus* pathogens. This was evident from study by Wieneke (1974) who reported a higher production of enterotoxin in cooked rather than raw food. Other factor may affect the prevalence of enterotoxigenic strains and should be taken in consideration is the animal species.

As for the different types of toxins, *S. aureus* isolates in this study mainly produced SEA (91.3%) while, 2.1% isolates produce SEC and isolates produced both SEA+SEB toxins represent 6.5%. Similarly, the highest percentage of SEA and SEC producers was noticed for isolates from raw pork in Germany (Atanassova et al., 2001). Also in Italy, isolates from meat, milk pastry, delicatessen, fish and eggs showed that SEA produced alone or in combination with other enterotoxins was the most prevalent (49.1%) followed by SEC (34.9%), and very low percentage of the isolates produce SED or SEB (Normanno et al., 2005). The dominance of SEA was also reported in Spanish dry-cured hams at 54.3% while the combinations of more than two enterotoxins were detected in 32.7% of enterotoxigenic strains, with SEA and SEB combinations were the most frequent reported ones (Marin et al., 1992). Although it is commonly known that most of human biotypes are enterotoxigenic strains producing SEA type (Bergdoll, 1989), but in this study none of the human biotypes identified were enterotoxigenic, whereas, other identified biovars were enterotoxin producers. Therefore, there is no correlation exist between the type of enterotoxin and any specific biovar.

5. CONCLUSION

This study showed that camel's meat is heavily exposed to Staphylococcal cross contaminants particularly of human and NHS1 biotypes. Noticeable differences in *S. aureus* identification were observed between conventional and molecular techniques and SEA was the most predominant enterotoxin among camel isolates.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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