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Molecular typing and cytotoxicity testing of *Staphylococcus aureus* isolated from milk, meat and clinical sources

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Staphylococcus aureus **is one of the main causes of foodborne diseases and a leading cause of nosocomial infections worldwide. Eleven (11) isolates from milk; 34 from meat and 23 of clinical source were subjected to phenotypic and biochemical characterizations for confirmation and further analysis for the presence of virulent genes, clonal relatedness and cytotoxicity profile. All isolates were positive for catalase production; 3, 11, and 7 isolates from milk, meat and clinical sources were coagulase positive. Polymerase chain reaction technique was used for the detection of 16S rRNA, clumping factor (*clfA*) and methicillin resistance (*mecA*) virulent genes. All isolates were positive for 16S rRNA; 100 and 45% of clinical; 73 and 64% of milk and none of the meat isolates were positive for *clfA* and *mecA* genes, respectively. Eight *Sma*I-based pulse-field gel electrophoresis (PFGE) clusters were identified at 80% similarity. Cytotoxic potential of the strains showed that 27.8, 24.1 and 22.2% of the strains tested had high, medium and low lactate dehydrogenase (LDH) release, respectively. These findings clearly demonstrate the presence of *S. aure*us in food and also in clinical infections, thus it should be a public health concern because even when virulent genes were not amplified in some strains, there may still be cytotoxicity effects.**

Key words: *Staphylococcus aureus*, pulse-field gel electrophoresis (PFGE), lactate dehydrogenase (LDH), cytotoxicity profile, molecular typing.

INTRODUCTION

Staphylococcus species are found normally in hair and skin of humans and animals, and according to many researchers, they are currently the leading cause of nosocomial infections worldwide (Lowy, 2003; Michelim et al., 2005; Bayles et al., 1998). Staphylococci, especially *Staphylococcus aureus*, are opportunistic pathogens if infection occurs in the susceptible individuals (Ghebremedhin et al., 2007).

Infections associated with *S. aureus* are extremely common, ranging from furuncles to foodborne intoxication

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and life threatening disease; therefore, the pathogen has potential to cause increased morbidity and mortality (Bayles et al., 1998). A recent study shows that almost 50% of meat in the grocery stores in the United States is contaminated with *S. aureus* (Waters et al., 2011); it is also the leading cause of intra-mammary infection in dairy animals. Furthermore, comprehensive epidemiological analysis has shown that *Staphylococcus* spp. was involved in several cases of outbreaks in United States (Ajayi et al., 2011).

According to reviews in Food Science and Food Safety (2006), there are antimicrobial usages at several stages in the food system, from the environment into the kitchen table, which creates an increasing spread of multi-drug resistant *S. aureus* in food worldwide and is a threat to food safety creating a public health concern (Xie et al., 2011). Because of the spread of multi-drug resistant pathogens in foods, the *Staphylococcus* spp. was subjected to resisto typing, applying 14 classes of antibiotics in order to establish the antimicrobial resistance profile of the isolates. It was found that majority of the isolates were resistant to 13 out of the 14 antibiotics tested (Ajayi et al., 2009).

Additionally the study on virulence genes characterization of *Staphylococcus* spp. isolated from food and clinical sources is important to estimate the prevalence of infections. Pulse-field gel electrophoresis (PFGE) is known to be a highly discriminatory technique, often used for characterizing genetic diversity of isolates and to research on outbreak sources (Xie et al., 2011). Furthermore, PFGE is particularly effective for molecular typing of methicillin resistant *S. aureus* (MRSA) (van Belkum et al., 1998).

The persistence of staphylococci in its hosts, despite the initiation of humoral and mucosal antibodies, continues to be an important issue for health of both humans and animals (Bayles et al., 1998). Furthermore, bacteria such as *Listeria monocytogenes, Salmonella* spp. and *Shigella* spp. are known to evade humoral immunity and then become internalized in host cells (Bayles et al., 1998). Although *S. aureus* is generally not considered to be an intracellular pathogen, it can be internalized, inducing apoptosis (cell death) in various cell types including epithelial cells, endothelial cells, keratinocytes, osteoblasts, lymphocytes and macrophages (Bayles et al., 1998; Wesson et al., 2000; Menzies and Kourteva, 1998; Nuzzo et al., 2000; Tucker et al., 2000; Jonas et al., 1994). The human INT-407 cell line is used in pathogenic enterobacterium studies, and to study cytotoxicity potential of foodborne pathogen *Escherichia coli, (Maldonado et al., 2005), Salmonella enterica* serovar Typhi (Santos et al., 2001*) and Klebsiella pneumoniae*.

Lactate dehydrogenase (LDH) is a low molecular weight enzyme, easily released from cell as a result of minor disturbances to the cell membrane integrity (Bhunia, 2008), and the release of LDH is one of the most widely used and accepted methods for measuring cellular death/lysis (Moran and Schnellmann, 1996). One of the advantages of this assay is that it can detect minimal membrane damage. Therefore, because of the prevalence in nature and the role of *S. aureus* in foodborne diseases and nosocomial infections, the focus of this study are to examine the virulence genes using multiplex PCR and the genetic relatedness of the strains using PFGE. Secondly, we aimed to determine the cytotoxic potential of strains isolated from milk, meat and clinical sources.

MATERIALS AND METHODS

Isolation of *S. aureus* from clinical, rabbit meat and milk samples

The clinical samples were donated from a local hospital and the milk samples were collected weekly from a local diary (Huntsville, AL) and processed in the Food Microbiology Laboratory at Alabama A&M University. Isolation of *S. aureus* was conducted according to the procedure described by the FDA-Bacteriological Analytical Manual (FDA, 1998). For initial isolation of *Staphylococcus* in meat (Rabbit), one hind leg from each whole carcass was placed in a sterile Stomacher bag with 0.1% (w/v) peptone (Oxoid, Lenexa, KS) water and vigorously shaken for 2 min and further dilutions were prepared. Approximately 0.1 ml of each dilution was plated onto Baird-Parker agar (BPA) supplemented with egg yolk tellurite (Merck, Gibbstown, NJ) and incubated at 37°C for 24 h. For isolation of *S. aureus* from milk or clinical samples, a 0.1 ml of both undiluted and 1:10 serially diluted sample of each was plated and incubated as earlier described.

Plates containing colonies with typical growth morphology of *S. aureus* were selected, isolated and sub-cultured in trypticase soy broth (TSB). Staphylococcal isolates were identified by colony morphology, catalase, Gram-stain, Staphylococcal coagulase reaction and clumping factor (agglutination) (Food and Drug Administration, 1992). Identification was confirmed with API kits, either API STAPH or ID 32 STAPH (bioMerieux NA, Durham, NC).

Detection of virulence genes using multiplex polymerase chain reaction (PCR)

Isolation of bacterial DNA from overnight cultures was performed according to Karahan and Centinkaya (2006). PCR assays were performed in an Eppendorf Mastercycler (epGradient S model Thermal Cycler, NY, USA). PCR protocol and primers corresponding to *S. aureus* regions to 16S rRNA portion, clumping factor (*clfA)* and methicillin (*mecA)* genes of *S. aureus* as described by (Mason et al., 2001) were used. The primers used in this study were as follow: *Staphylococcus* specific 16S rRNA gene (791bp), 16S-F (5'-CCTATAAGACTGGGATAACTTCGGG 3') and 16S-R (5'-CTTTGAGTTTCAACCTTGCGGTCG 3'); clumping factor gene (638bp), *clfA*-F (5'-GCAAAATCCAGCACAACAGGAAACGA-3') and *clfA*-R (5'-CTTGATCTCCAGCCATAATTGGTGG-3'); and methicillin resistant gene (499bp), *mecA*-F (5'-TCCAGGAATGCAGAAAGACCAAAGC-3') and *mecA*-R(5'-GACACGATAGCCATCTTCATGTTGG-3')Invitrogen (Carlsbad, California).

S. aureus ATCC 700698, and *E. coli* which served as the positive and negative controls respectively were included in the PCR reactions. About 5 µL aliquot of PCR products was mixed with 3 µL loading dye and with molecular weight marker of 100-bp, were ana-

lyzed by electrophoresis on a 1% agarose gel at 70V for 1 h. Gels were stained in ethidium bromide, viewed by ultra-violet (UV) trans-illumination and photographed.

Pulsed PFGE

Only *Staphylococcus* isolates that tested positive for either *clfA* or *mecA* virulence genes were subjected to PFGE for research on clonal relatedness.

Preparation of DNA plugs

Chromosomal DNA was prepared as described by Reed et al. (2007). Briefly, 5 µL of lysostaphin enzyme was added to cell suspension equivalent to 3-4 x 10^9 CFU/mL, plus the addition of 250 µL of 60°C 1.8% SeaPlaque agarose (FMC Bio-product). Suspension was mixed well and 250 µL was immediately transferred into the plug molds. Plugs were allowed to solidify for 10 min at 4°C, washed and DNA plugs were cut and placed in an eppendorf tube containing 125 µL of 1X *Sma*I restriction buffer. Then, 30 U of *Sma*I restriction enzyme (Roche Diagnostics, Mannheim, Germany) was added to each tube, mixed gently and incubated for 2 h at 25°C. The digested DNA plugs were resolved on a 1% gel on a Chef Mapper (BioRad, Hercules, CA, USA), PFGE equipment with CHEF MAPPER parameters set as follows: 5 s initial switch time; 40 s final switch time; 20 h duration of run; voltage 200 V or 6 V/cm gradient; angle of 120° and chiller at 14°C. All gels were stained with ethidium bromide, documented and analyzed with a Foto/analyst Luminary FX Electrophoresis Documentation and Analysis System (Fotodyne Inc. Rochester, NY). Similarity indices were determined using the Dice coefficient (Dice, 1945) and the distances between clusters were calculated using the unweighted pair group method with arithmetic averages (UPGMA). Data analysis was performed using SPSS version 12.0.1. (SPSS Inc. Chicago, IL). Interpretations of PFGE patterns were performed according to Tenover et al. (1995).

Preparation of mammalian cell culture

The Human jejunal (INT-407) cell line was obtained from American Type Culture Collection (ATCC CCL-6), cultured and maintained in Dulbecco's modified Eagle's medium (DMEM) (Sigma Chemical Co., St. Louis, MO) with 10% fetal bovine serum ($D_{10}F$). Stock INT-407 cultures were maintained in a 37°C incubator with 7% CO_2 for 3 weeks to ensure a uniformed monolayer of culture. Confluent monolayer used for cytotoxicity assay was trypsinized, re-suspended in 24-well plates with fresh medium and incubated for 48 h.

Preparation of bacterial toxin

Bacterial cultures

Stock *Staphylococci* cultures isolated from food and clinical sources were maintained in Trypticase Soy Broth (TSB) with 20% glycerol and stored at -80°C. Prior to use, each strain was sub-cultured in 5 mL TSB and incubated overnight at 37°C.

Toxin preparation

Toxin was prepared as described by Maldonado et al. (2005). Briefly, a 1.5 mL aliquot of overnight bacterial culture was centrifuged at 10,000 rpm for 3 min and cell-free supernatants were stored in sterile tubes. Cell pellets were re-suspended in 75 µL of polymyxin B sulfate solution (2 mg/mL of PBS), incubated for 30 min and centrifuged. Following centrifugation, the supernatants were collected, combined with the original cell-free supernatants and filtered through 0.45 µm disc filters (Corning, NY). The filtrates were used immediately or stored at 4°C for up to two days.

Lactate dehydrogenase (LDH) assay

The cytotoxic effect of *Staphylococcus* on human cell lines was assessed, by measuring lactate dehydrogenase (LDH) released according to Roberts et al. (2001), with slight modification. Briefly, confluent INT-407 cells transferred to 24 well plates were used upon formation of monolayer. Each well was washed three times in 900 µL serum free (D0F) DMEM, then the low (PBS), high (1% Trition-X) controls and samples were prepared in triplicates in a 24 well plates as follows: (900 µL D_0F DMEM and 100 µL of controls or bacterial toxin filtrates), and incubated for 16 h at 37°C with 7% CO_2. After incubation, a 100 µL aliquot of supernatant from each bacterial isolate was dispersed into triplicate wells of a 96 well plates. Lactate dehydrogenase substrate was prepared according to the manufacturer's guidelines (Roche Applied Science, Indianapolis, IN) and 100 µL was dispensed into each well. Plates were incubated at room temperature and the absorbance was measured at a dual wavelength of 490 and 655 nm after 5 min and subsequently every 3 min for 15 min by a plate reader (Synergy HT, Vermont, USA). Percentage of cytotoxicity was calculated as follows:

$$\text{Cytotoxicity (\%)} = \frac{\text{Experimental value} - \text{Negative control}}{\text{Positive control} - \text{Negative control}} \times 100$$

Statistical analysis

Calculated percent cytotoxicity data were analyzed using Statistical Package for the Social Sciences (SPSS 16). Averages of the triplicates plus Standard deviation were reported. Tukey's studentized test was used for the separation of means and $P<0.05$ was taken as statistically significant association.

RESULTS

Multiplex PCR: 16S rRNA; (*clfA*) and (*mecA*) genes

Multiplex PCR analysis was performed on 61 samples of presumptive *Staphylococcus* isolates; 11 from milk; 27 from meat and 23 from human infections (clinical) and *S. aureus* ATCC 700698, and *E. coli* which served as the positive and negative controls respectively were tested. PCR amplifications yielded the fragments of the expected sizes. All *Staphylococcus* isolates were confirmed positive for 16S rRNA, 28 isolates for *clfA* and 16 strains were positive for *mecA* gene (Figure 1).

Clumping factor (*clfA)* gene was found in 20 clinical and eight milk isolates. None of the strains isolated from rabbit meat were positive for clumping factor gene. The *mecA* gene which resulted in a 499 bp fragment was found in 9 clinical and 7 milk isolates, while none of the meat isolates showed positive *mecA* gene (Figure 1). The

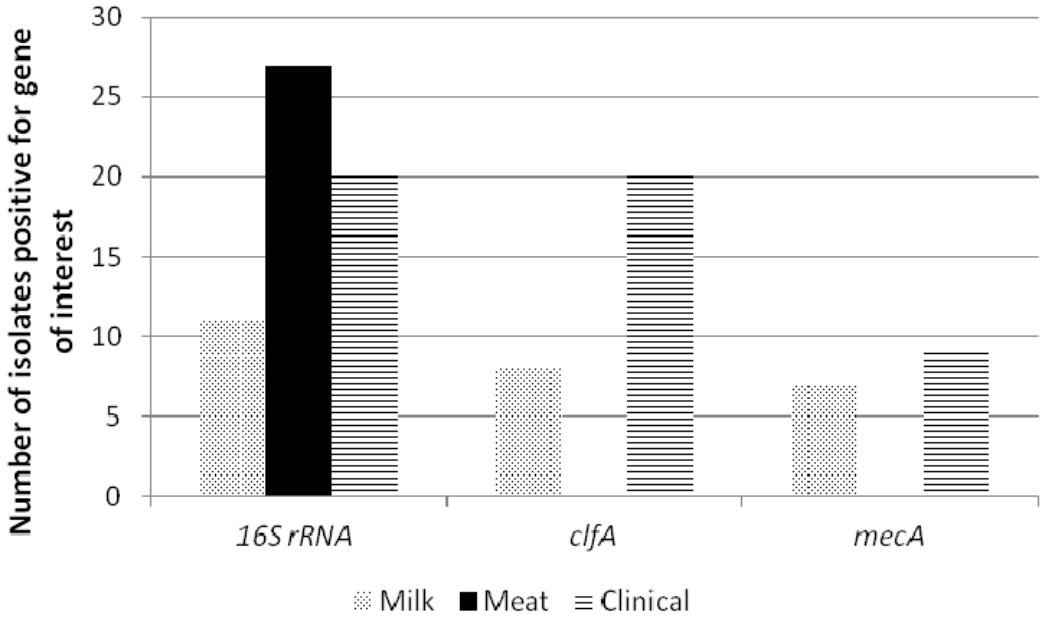

Figure 1. Detection of 16Sr RNA, clumping factor (*clfA*) and methicillin resistant (*mecA*) genes from *S. aureus* in milk, meat and clinical isolates.

complete results of the presence of 16S rRNA, *clfA* and *mecA* virulent genes for each strain are summarized in Table 1.

Clonal relatedness in *Staphylococcus*

To investigate the clonal relationship, a total of 33 *S. aureus* strains (9 isolates from milk and 24 from clinical sources) were subjected to *Sma*I macro restriction fragment analysis of their chromosomal DNA by PFGE, including 2 ATCC (8325-4 and 700698) strains of *S. aureus* isolates. The PFGE profiles of the clinical isolates were compared with the PFGE profile of the milk isolates.

As reported by Tenover et al. (1995), isolates are designated genetically indistinguishable if the restriction patterns have the same number of bands and the bands appear to be of similar size. Figure 2A is representative of the PFGE gels for clinical strains, two strains in lanes 3 and 4 had PFGE banding patterns that were indistinguishable.

The other clinical strains were closely related, since there were two to three differences in bands between the observed patterns, possibly due to point mutations (insertions or deletions). The *S. aureus* strains from milk are shown in Figure 2B. Lanes 1, 7 and 15 correspond to ATCC 700698 and lanes 6, 8 and 13 to one milk sample that appeared to be genetically indistinguishable.

Of the 33 isolates typed by *Sma*I-based PFGE, each of the two ATCC strains had distinct PFGE patterns different from the strains isolated from milk or clinical sources. The horizontal bar above the dendrogram indicates the percentage of genetic relatedness among the different strains. Each cluster had varying number of strains.

Cytotoxicity profile

A total of 57 *Staphylococcus* isolates were evaluated for their cytotoxicity potential, based on the release of lactate dehydrogenase from jejunal (INT-407) cells, and were compared with the positive control, Triton-X 100 (1%) and PBS and the negative control. Cytotoxicity assay were performed in triplicate. The LDH release values for Triton-X 100 ranged from 1.459 to 1.47 and the negative control ranged from 0.334 to 0.338. The results of LDH values (averages of absorbance ± standard deviations) and calculated percent cytotoxicity for each isolate tested are listed in Table 1.

The percent of cytotoxicity were grouped as negative (<1%); low (1 to 20%); medium (21 to 49%) and high (>50%) categories arbitrarily according to Maldonado et al. (2005). Four (36%) from milk, 9 (33%) from meat and 1 (5.5%) from clinical isolates were in the negative category (Figure 4).

Three (27%), 4 (14%) and 5 (31%) from milk, meat and clinical respectively were in the low category; 2 (18%) 5 (18.5%) and 6 (33%) isolates from milk, meat and clinical were in the medium category while 1 (9%) from milk, 9 (32%) from meat and 4 (22%) clinical isolates were in the high category.

The strains isolated from milk had LDH had range from -24 to 80%; the strains from meat ranged from -27 to 194%; while the clinical strains LDH ranged from -17 to 113%. There was a statistically significant difference (p<0.05) in LDH release between the clinical and milk

Table 1. Cytotoxicity Profile of *Staphylococcus aureus* isolated from milk, meat and clinical sources.

Isolate	Source	Coagulase	Virulence genes present[a]			Cytotoxicity[b]	
			16S rRNA	*clfA*	*mecA*	LDH ($A_{490/655}$)	%
Low control	PBS					0.347	
High control	Triton X					1.464	
M 11	Milk	NT[c]				0.302 ± 0.02^{xy}	- 4
M 3-5	Milk	+	+	+	+	0.413 ± 0.021 ^{wx}	6
M 9	Milk	-	+	+	+	0.144 ± 0.004^{y}	- 18
M 8	Milk	-	+	+	+	0.481 ± 0.046^{p}	12
M 4-3	Milk	-	+	+	+	0.155 ± 0.004^{y}	- 17
M 4-1	Milk	-	+	+	+	0.872 ± 0.044^{h}	47
M 3-1	Milk	NT	+	+	-	NT	
M 3-2	Milk	+	+	-	-	0.077 ± 0.004^{z}	- 24
M 3-3	Milk	-	+	+	+	0.374 ± 0.033^{xy}	2
M 3-4	Milk	+	+	+	+	0.719 ± 0.007^{lm}	33
M 4-4	Milk	NT				1.238 ± 0.074^{e}	80
PC8	Meat	-	+	-	-	2.509 ± 0.107^{a}	194
PC5-12	Meat	-	+	-	-	0.749 ± 0.030^{kl}	36
PC5	Meat	-	+	-	-	0.717 ± 0.338^{lm}	33
PC7-14	Meat	-	+	-	-	1.729 ± 0.051^{b}	123
PC7	Meat	+	+	-	-	0.423 ± 0.009^{wx}	7
PV1-16	Meat	NT	+	-	-	0.919 ± 0.190^{fg}	51
PV 2-17	Meat	-	+	-	-	1.659 ± 0.228^{bc}	117
PV 4-18	Meat	+	+	-	-	0.217 ± 0.008^{xy}	-12
PV 5-2	Meat	+	+	-	-	0.080 ± 0.001^{z}	-23
PV 5	Meat	+	+	-	-	0.717 ± 0.338^{lm}	33
PV 6	Meat	-	+	-	-	0.164 ± 0.017^{yz}	-16
PV 7-21	Meat	NT				0.072 ± 0.002^{z}	-24
PV 8-22	Meat	-	+	-	-	0.230 ± 0.011^{xy}	-10
PR10	Meat	+	+	-	-	0.181 ± 0.025^{yz}	-14
PR11	Meat	+	+	-	-	0.441 ± 0.043^{uv}	8
PR 2	Meat	+	+	-	-	0.761 ± 0.107^{jk}	37
PR 3R3	Meat	-	+	-	-	0.159 ± 0.007^{yz}	-17
PR 5-3	Meat	+	+	-	-	0.069 ± 0.005 ^{z}	-24
PR 5	Meat	-	+	-	-	0.410 ± 0.008 ^{wx}	6
PR 7-4	Meat	+	+	-	-	0.912 ± 0.016^{fg}	51
PR 9-6	Meat	+	+	-	-	1.488 ± 0.042d	102
PC10	Meat	NT	+	-	-	0.383 ± 0.063x	3
PC13	Meat	NT	+	-	-	0.907 ± 0.053fg	50
PC3-16	Meat	NT	NT			0.582 ± 0.225qr	21
PC4-11	Meat	-	+	-	-	1.303 ± 0.101de	86
PR 9 (6-1)	Meat	+	+	-	-	1.805 ± 0.228b	130
PR9	Meat	-	+	-	-	0.413 ± 0.003q	-27
C10-12	Clinical	-	+	+	+	0.599 ± 0.086q	23
C1	Clinical	-	+	+	-	0.687 ± 0.061mn	30
C2	Clinical	-	+	+	-	0.632 ± 0.029op	26
C13	Clinical	-	+	+	-	0.457 ± 0.058uv	10
C17	Clinical	+	+	+	+	0.987 ± 0.128f	57
C14	Clinical	-	+	+	+	0.289 ± 0.008xy	-5
C15	Clinical	+	+	+	-	0.583 ± 0.016qr	21
C16	Clinical		+	+	-	1.238 ± 0.088e	80
C18	Clinical	-	+	+	-	1.318 ± 0.021de	87
C19	Clinical	+	+	+	-	0.433 ± 0.004w	8

Table 1. Contd.

Isolate	Source	Coagulase	Virulence genes present			Cytotoxicity	
			16S rRNA	*clfA*	*mecA*	LDH ($A_{490/655}$)	%
C28	Clinical	-	+	+	+	0.547 ± 0.017^{op}	18
C22	Clinical	+	+	+	-	0.154 ± 0.006yz	-17
C24	Clinical	+	+	+	-	0.501 ± 0.069^{t}	14
C10-3	Clinical	-	+	+	-	1.614 ± 0.018^{bc}	113
C4	Clinical	+				0.531 ± 0.006^{rs}	16
C6	Clinical	NT	+	+	+	0.776 ± 0.036^{ij}	38
C7	Clinical	+	+	+	+	0.663 ± 0.008^{no}	28
C9	Clinical	-	+	+	-	0.378 ± 0.011^{y}	3
C29	Clinical	-	+	+	+	NT	
C5	Clinical	-	+	+	+	NT	
C12	Clinical	NT	+	+	+	NT	
C27	Clinical	NT	NT			NT	
C26	Clinical	NT	NT			NT	
ATCC 8325-4		NT	+	+	+	0.840 ± 0.050	44

a = Virulence genes detected by PCR; b=The LDH release values are averages ± standard deviations for three replicates. Means with same alphabets are not different (p<0.05); c NT=Not tested

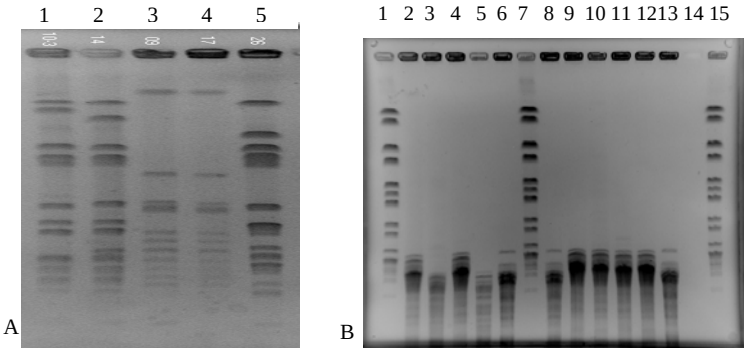

Figure 2. PFGE patterns of *Sma*1 restriction enzyme digested DNA of *S. aureus.* Lanes 1 to 5 are representative of PFGE pattern from clinical (A) and milk (B) strains; lanes 1, 7 and 15 correspond to ATCC 700698; lanes 2 to 6 and 8 to 13 correspond to milk strains.

isolates, and also between meat and milk isolates as shown in Figure 5. However, there was no significant difference (p<0.05) in LDH release between the clinical and meat isolates. The meat isolates displayed the widest range of LDH release, followed by the clinical isolates. However, the clinical isolates had the smallest number of isolates with negative LDH release. This assay is used to assess the pathogenic capabilities of the strains isolated from milk, meat and clinical sources.

DISCUSSION

In this study, 61 *S. aureus* strains isolated from milk, rabbit meat and human infections were investigated for 16S rRNA gene and *cflA* and *mecA* virulence genes by PCR. PCR techniques provide increased sensitivity, allow for more rapid laboratory processing times and enhance the likelihood of detecting bacterial pathogens (Riyaz-Ul-Hassan et al., 2008). It has also been proposed for detection of foodborne pathogens to replace the time consuming classical techniques (Olsen, 2000).

The strains were also genotyped using PFGE and the cytotoxicity profile was determined. All 61 isolates from this study were positive for 16S rRNA gene, as well as in previous studies (Mason et al., 2001; Zhang et al., 2004; Fan et al., 2008). The 16S rRNA gene is specific for *Staphylococcus* spp. Clumping factor (*clfA)* gene, was amplified for 20 of the clinical, 8 milk isolates and none of the strains isolated from rabbit meat. The isolation of

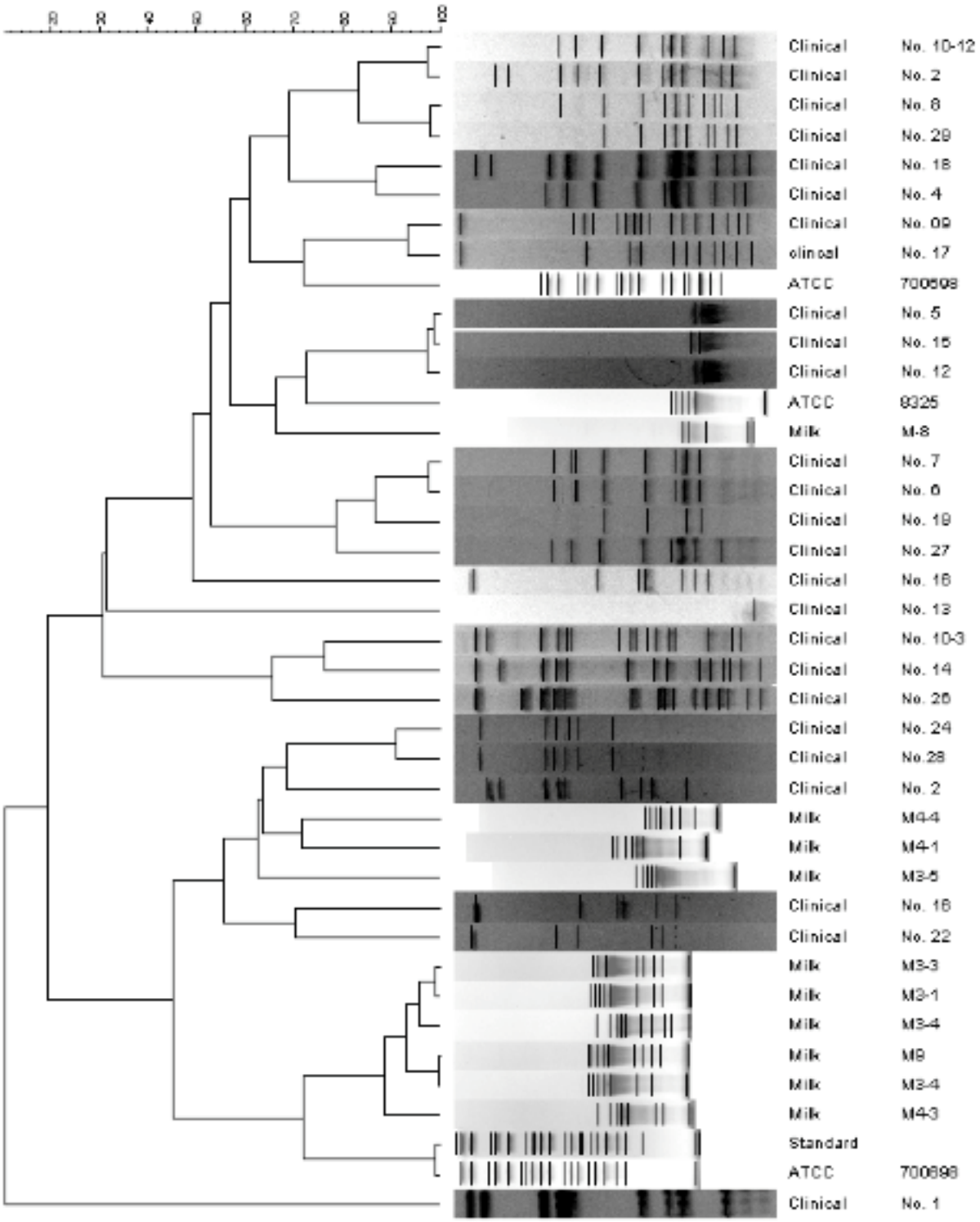

Figure 3. PFGE macro restriction profiles (SmaI) of *S. aureus* strains from clinical and milk sources. Pair comparison and cluster analysis performed with the Dice correlation coefficient and UPGMA.

clumping factor A is specific to *S. aureus.* Furthermore, *clfA* gene codes for a multifunctional protein, in addition to binding fibrinogen and it can also contribute to *S. aureus* capabilities of binding to platelet (Siboo et al., 2001). *S. aureus* have also been documented to produce other fibrinogen binding proteins such as *clfB*, although the primary fibrinogen-binding protein is *clfA* (Ni Eidhin et al., 1998).

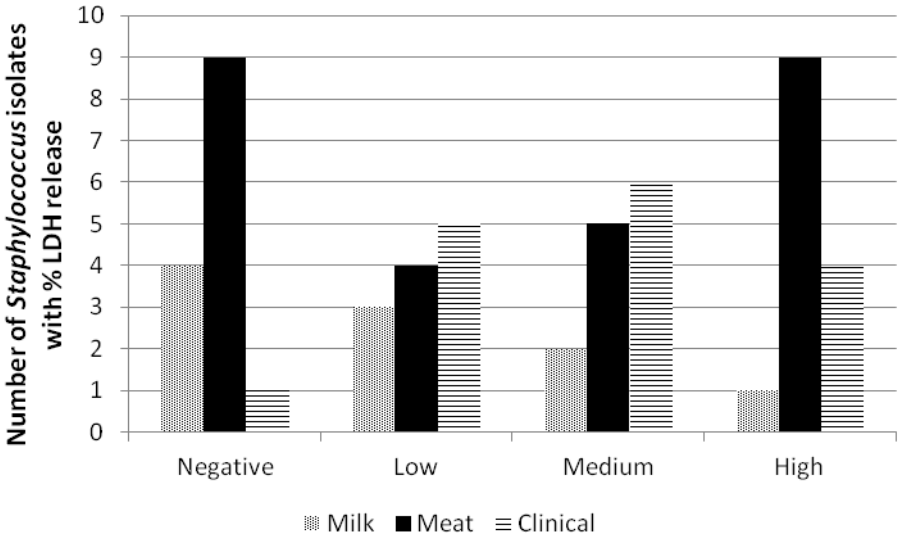

Figure 4. Percentages of cytotoxic *Staphylococcus* strains from milk, meat and clinical sources. Cytotoxicity values were grouped into negative (<1%); low (1 to 20%); medium (21 to 49%) and high (>50%) categories.

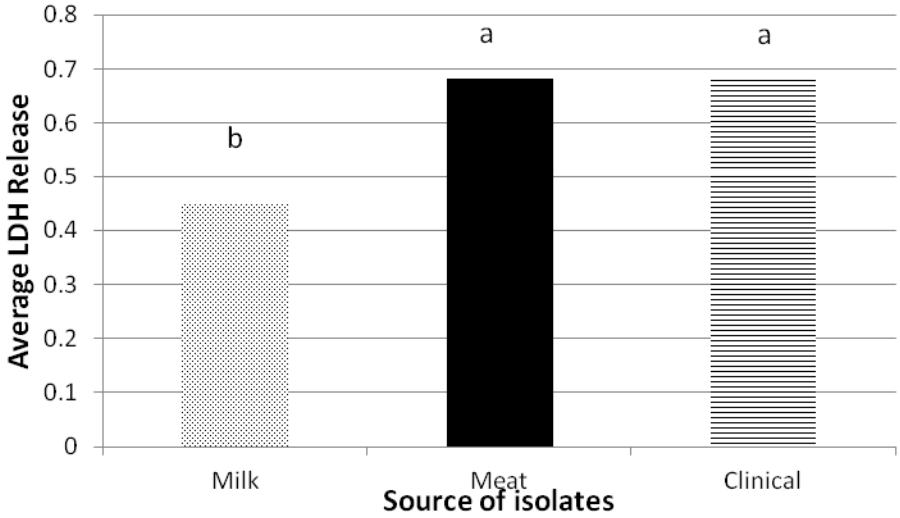

Figure 5. LDH release of *Staphylococcus* strains isolated from milk, meat and clinical sources. Mean separation is by Tukey's studentized analysis, means with same letters are not statistically different ($p<0.05$).

Pathogenesis of *S. aureus* results from the combined action of a diversity of factors, and infection begins with bacterial adhesion to host tissues (Jarraud et al., 2002). The *clfA* gene amplification results (87%) for the clinical strains in this study are comparable to previous studies by Tristan et al. (2003), who reported that *clfA* gene was present in 100% of the clinical samples tested while Peacock et al. (2002) reported 98% of *clfA* gene. Raw unpasteurized milk has been shown to be natural reservoir for *S. aureus* and other pathogenic microorganisms. In addition, studies have further demonstrated that *S. aureus* can be isolated from raw milk, 43-62% of the times (Foschino et al., 2002; Chye et al., 2004). From the raw and unpasteurized milk procured for this study, 73% of the *S. aureus* isolates were positive for *clfA* gene. The emergence and increasing number of methicillin resistant *S. aureus* in foods and the environment are alarming. Furthermore, methicillin resistance gene in *S. aureus* is conserved and its amplification can be beneficial during outbreak investigation. Only 45% of clinical and 64% of milk strains was positive for *mecA* gene. Previously, it was reported by Suzuki et al. (1993) that in some strains

of *Staphylococcus* the loss of their regulator gene by deletion occurs and we speculate that in this research some clinical strains may have lost the regulator gene also. However, more of the strains isolated from milk had *mecA* gene. This poses a considerable public health risk, in particular because of the increase in number of individuals who consume raw milk (Riyaz-Ul-Hassan et al., 2008). It is known that the food handlers are most often exposed to *S. aureus* contamination (Jablonski and Bohach, 2011; Jørgensen et al., 2005) and that raw milk and raw milk products from dairy animals, un-clean tanker and food handlers are probably equally involved as source of the contamination.

All the *S. aureus* strains from rabbit from this study were negative for the clumping factor A and methicillin resistance genes. According to Vancraeynest et al. (2006), a high virulence (HV) rabbit *S. aureus* clone causes transmission of chronic staphylococcosis, whereas low virulence (LV) strains only infect a limited number of animals. It is therefore speculated that the *Staphylococcus* strains from the rabbit isolates could be low virulent strains and that probably explains the inability of PCR to detect either clumping factor A or methicillin resistant gene.

All the strains were positive for 16S rRNA gene in *Staphylococcus* isolates. The PFGE was used to further discriminate the isolates. As reported by Revazishvili et al., (2006), PFGE is more useful for short-term studying of *S. aureus;* and is considered the gold standard technique for MRSA typing because of its high discriminatory power, its reproducibility, and its good correlation with epidemiologically linked data (Tenover et al., 1994). The results of the PFGE typing showed some degree of genetic heterogeneity among the *S. aureus* strains from clinical and milk sources. At 80% similarity level, 8 clusters were identified (Figure 3). The isolates from similar clinical or milk sources were grouped into the same PFGE cluster in this study in contrast to Xie et al. (2011) who reported same PFGE cluster organization from strains of *Staphylococcus* isolated from different sources. Five MRSA strains from clinical sources were not grouped into PFGE cluster while six strains isolated from milk were in one cluster.

Lactate dehydrogenase is a low molecular weight enzyme carried in the cytoplasm of the majority of cells, which is then released from the cells after a minor disturbance of the integrity of the cell membrane. The LDH results were comparable with LDH release in *E. coli,* using Vero cell assay (Maldonado et al., 2005), although in this study jejunal INT-407 cell culture was used to access the cytotoxicity of the *Staphylococcus* strains. The LDH release cytotoxicity assay from human intestinal cell line was applicable to determining the virulence capabilities of *Staphylococcus* strains isolated from food and clinical sources. Lactate dehydrogenase release also signified the pathogenic effect of *Staphylococcus* by causing cell damage and cell death of the INT-407. However, the results of this study contradict Buxser and Bonventre (1981) study, which found that *Staphylococcus* enterotoxins A and B did not initiate LDH release in INT-407 cells.

Lactate dehydrogenase assay was performed to show the virulence potential of *Staphylococcus* isolates from milk, meat and clinical sources. According to Bhunia (2008) although *S. aureus* is generally not considered to be an intracellular pathogen associated with classical facultative intracellular pathogens such as *Listeria, Salmonella*, and *Shigella* spp., it has been documented that some *S. aureus* strains isolated from bovine mastitis can adhere and invade bovine mammary epithelial cell line MAC-T and primary bovine mammary secretory epithelial cells (Almeida et al., 1996; Bayles et al., 1998).

Conclusion

These findings show the prevalence of *S. aure*us in food and clinical specimen, and we provide evidence that even if virulent genes such as *clfA* or *mecA* genes were not amplified in some strains, there may still be cytotoxicity effects presenting a public health concern particularly in individuals that consume game animals or raw milk and might have compromised immunity.

Conflict of interest

The authors declare that they have no conflict of interest.

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