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RESEARCH ARTICLE

SCREENING OF TOXIGENIC *S. AUREUS* ISOLATED FROM BOVINE AND HUMAN ORIGIN

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ABSTRACT

S. aureus produces extracellular protein toxins and virulence factors which contribute to the pathogenicity of the organism. The present work aimed to analysis the prevalence of enterotoxin producing *S. aureus* strains isolated from bovine and humans origin using reverse passive latex agglutination (RPLA) kit and PCR. A total of 25 *S. aureus* isolates out of 374 samples collected from cattle, buffaloes and human were identified, 20 from bovine and 5 from human. Using RPLA, 10 out of the 25 *S. aureus* isolates were found to be toxigenic with an incidence of 40%. They were distributed as enterotoxin C (50%), a (20%), A& B, A& C and enterotoxins A, B, C and D (10 % each). Using PCR 7 enterotoxigenic *S. aureus* isolates (28%) were detected. *sea* gene was detected in 2 isolates (28.6%), while *sec, seb, sea* & *seb, sec* & *see* and *sea, sec*, *sed* genes were detected in one isolate each (14.3% each). Analysis of the results obtained by RPLA and PCR for the productivity of classical enterotoxins A-D revealed approximately correlation between each other. It could be concluded that cows' and buffaloes' milk are of public health risk due to potent staphylococcal food poisoning strains.

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INTRODUCTION

Staphylococci often represent part of normal bacterial flora of the skin and mucosal surfaces of the respiratory, upper alimentary and urogenital tract of mammals and birds. Thus staphylococci are easily spread between animals and under certain conditions to humans. Moreover, staphylococci may be spread by animal products, such as milk (Werckenthin *et al*., 2001). *Staphylococcus aureus* is recognized to cause health care associated and community-acquired infections in every region of the world (Yilmaz *et al.*, 2007). It is recognized worldwide as an important food borne pathogen because of its ability to produce a wide range of toxins. Staphylococcal enterotoxins (SEs) are serologically grouped into five major classical types which are SEA, SEB, SEC, SED and SEE in addition to toxic shock syndrome toxin (TSST-1) which causes toxic shock syndrome in human (Rosengren *et al*., 2013). Enterotoxigenic *S. aureus* in milk posses a potential health hazard to consumers, the identification of such strains should be used as a part of a risk analysis of milk and milk products (Zouharova and Rysanek, 2008).

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SEA and SEB are usually more common in milk and milk products (Chiang *et al.,* 2006). Several methods have been developed for the detection of enterotoxigenic *S. aureus* strains. Based on immunological procedures staphylococcal enterotoxins are serologically grouped by reverse passive latex agglutination kit (RPLA) into four major classical types which are SEA, SEB, SEC and SED (Zouharova and Rysanek, 2008). Multiplex PCR assay for the detection of staphylococcal enterotoxins was developed and proved to be specific, sensitive and rapid method (Zschock *et al*., 2005). The present work aimed to analysis the prevalence of enterotoxin producing *S. aureus* strains isolated from bovine and humans origin using RPLA and PCR.

MATERIALS AND METHODS

Specimens

Milk samples

A total of 324 subclinical mastitic milk samples (cow n=172 and buffaloes n=152) were collected from dairy farms at Giza governorate, Egypt and Animal Health Research Institute (AHRI), Dokki, Giza, Egypt and kept at 4°C. All samples were

incubated for 18-24 hr. at 37ºC then centrifuged at 3000 rpm for 20 minutes. The cream and supernatant were discarded and the sediment was investigated bacteriologically to detect *S. aureus*.

Urine samples

Thirty samples were collected from diseased human under aseptic condition from clinics of Cairo University Hospitals (CUH) and human laboratories (Elborg and Alfa Lab.). Each specimen was centrifuged at 3000 r.p.m. for 10 minutes. The supernatant fluid was decanted and the sediment was used.

Septic wounds and abscesses

Twenty swabs were collected from human from clinics of Cairo University Hospitals (CUH) and human laboratories (Elborg and Alfa Lab.) after drawing off the pus under aseptic condition.

Isolation and identification of *S. aureus* isolates

The collected samples were cultivated on the surfaces of mannitol salt agar (Oxoid, CM85), nutrient agar (Difco) and sheep blood agar. The inoculated plates were incubated at 37° C for 24 hours. The suspected colonies were picked up and examined microscopically and subjected for identification of staphylococci according to Quinn *et al.* (2002).

Determination of methicillin resistant *S. aureus* (MRSA**) agents**

The disk diffusion technique was adapted using cefoxitin sensitivity disk (30 ug, Oxoid) to detect MRSA according to CLSI (2013).

Reversed passive latex agglutination kits (RPLA)

S. aureus enterotoxins were extracted by sac cultural method according to Donnelly *et al*. (1967). The clear culture supernatant fluids were tested serologically by reversed passive latex agglutination technique using Oxoid SET-RPLA kits for detection of staphylococcal enterotoxins A, B, C and D according the manufacture.

Multiplex of PCR (Sambrook and Russell, 2001)

DNA was extracted according to Lina *et al*. (1999). DNA samples were tested in 50 µl reaction volume in a 0.5 ml eppindorf tube , containing PCR buffer (50 mM KCl , 10 mM tris – HCl ,1mM $MgCl_2$) each dNTPs (200 uM each), primer pairs (Table 1), each at 50 Pico mol / reaction and 0.5 of taq DNA polymerase. After layering with 40µl of mineral oil, thermal cycling in a programmable thermal cycler (PTC100 Mil Research, USA) was done and denaturized at 94°C for 30 sec. Annealing was done at 52°C for 30 sec., extension at 72°C for 1 minute and continued through 30 cycles. The cycling was preceded by 10 minutes incubation at 93°C and followed by a final 10 minutes extension period at 72°C. A negative control PCR reaction with no template also was included in this assay. The sample was electrophoresis at 96 volts for 45 minutes in an electrophoresis unit. The presence of specific amplified DNA bands was detected by visualization with UV light at wave length 421 nm and compared with 100 bp DNA marker (Axygen).

RESULTS

Identification of staphylococci isolated from the examined cases

A total of 374 samples were investigated bacteriologically to detect the occurrence of staphylococci among human, cow and buffalo samples, the isolation rates were 18%, 10.5% and 13.8% respectively (Table, 2). A total of 25 *S. aureus* isolates secured from bovine and humans origin were identified. It could be identified from septic wounds, and urine collected from diseased human with an incidence of 10% (each). Also it was isolated from mastitic cows (5.8%) and buffaloes (6.6%).

Characterization of *S. aureus* isolates

S. aureus isolates were characterized using colony pigment, tellurite reduction and hemolytic activity. *S. aureus* is coaggulase positive isolate produces endopigment when cultivated on nutrient agar. As shown in Table (3) 60% and 90% of *S. aureus* isolated from human and bovine samples respectively produced golden yellow pigment. While 10% of bovine isolated had creamy pigment colonies and 40% of human isolates produced white pigment colonies. It is clear that almost all *S. aureus* isolates were able to reduce tellurite to metallic tellurium produced a black coloration (96%) except one isolate from subclinical mastitic cow. 23 out of 25 *S. aureus* isolates were positive for hemolytic activity (92%).

Detection of toxigenic *S. aureus* strains

Using RPLA test, it is clear that 10 out of 25 *S. aureus* isolates were found to be toxigenic with an incidence of 40% (Table 4). Enterotoxin A was detected in 2 isolates with an incidence of 20%. Enterotoxin C was detected in 5 isolates (50%). Enterotoxins A & B, enterotoxins A & C and enterotoxins A, B, C & D were detected in one isolates each (10 % each). Using PCR 7 enterotoxigenic *S. aureus* isolates (28%) were detected as shown in Table (4) and Fig. (1). *sea* gene was detected in 2 isolates (28.5%), while *sec*, *seb*, *sea* & *seb*, *sec* & *see* and *sea*, *sec*, *sed* genes were detected in one isolate each (14.3% each).

DISCUSSION

Mastitis is one of the economically important diseases of dairy animals**.** Subclinical mastitis cases are those in which no visible appearance of changes in the milk or udder, but milk production decreases, bacteria are present in the secretion and composition is altered (Zeryehun *et al*., 2013). *S. aureus* is the leading cause of nosocomial infections and is responsible for a wide range of human diseases, including endocarditis, food poisoning, toxic shock syndrome, septicemia, skin infections, soft tissue infections and bone infections, as well as bovine and ovine mastitis (Koreen *et al*., 2004).

The purpose of the present study was to investigate enterotoxigenic properties of *S. aureus* strains isolated from bovine and human sources using RPLA and PCR.

Table 1. The primer pair of *S. aureus* toxin used in multiplex PCR

Gene	Primer	Oligonucleotide sequence (5'__4')	Size (bp) of PCR product	Reference
Sea	SEA-1	cctttggaaacggttaaaacg	127	Becker *et al.* (1998)
	SEA-2	tctgaaccttcc cat caaaaa c		
Seb	SEB-1	tcg cat caa act gacaaa cg	478	Jarraud *et al.* (1999)
	SEB-2	gcaggt act ctataagtg cc		
Sec	SEC-1	cttgtatgtatg gag gaataacaa	283	Monday and Bohach (1999)
	SEC-2	tgcagg cat catatcatacca		
ed	SED-1	ctagtttggtaa tat ctcct	317	Jarraud *et al.*(1999)
	SED-2	taatgc tat atctta tag gg		
See	SEE-1	tag ataaggttaaaacaagc	482	Jarraud *et al.* (1999)
	SEE-2	taacttaccgtggacccttc		

Table 2. Incidence of *S. aureus* from the collected samples

Type of the Samples	No. of the examined sample	*Staphylococcus* species		*S. aureus*	
		No.	%	No.	%
Milk from subclinical mastitic cows	172	18	10.5	10	5.8
Milk from subclinical mastitic buffaloes	152	21	13.8	10	6.6
Total bovine	324	39	12	20	6.2
Urine samples from human	30	6	20	3	10
Abscesses from human	20	3	15	2	10
Total human	50	9	18	5	10
Total	374	48	12.8	25	6.7

No.: Number of Positive, %: was calculated according to the examined samples.

Table 3. characteristic features of the examined *S. aureus* isolates

Type of samples	No. of *S. aureus*	Colony pigment						Tellurite reduction		Hemolytic activity	
		White		Creamy		Golden yellow					
		No.	%	No.	%	No.	%	No.	%	No.	%
Milk from subclinical mastitic cows	10	0	0	2	20	8	80	9	90	10	100
Milk from subclinical mastitic buffaloes	10	0	0	0	0	10	100	10	100	8	80
Total bovine	20	0	0	2	10	18	90	19	95	18	90
Urine samples from human	3	1	33.3	0	0	2	66.7	3	100	3	100
Abscesses from human	2	1	50	0	0	1	50	2	100	2	100
Total human	5	2	40	0	0	3	60	5	100	5	100
Total	25	2	8	2	8	21	84	24	96	23	92

Staphylococcus species were characterized and identified using the most important conventional biochemical tests (Quinn *et al*., 2002). *S. aureus* could be identified from subclinical mastitic cows (5.8%) and buffaloes (6.6%). Akram *et al*. (2013) reported higher prevalence rates of *Staphylococcus aureus* in mastitic milk. *S. aureus* was isolated by El-Jakee *et al*. (2008) from mastitic cow (22.7%) and buffaloes (16%), as well as from cattle septic wounds (22%). Staphylococci are one of the most common pathogens that cause mastitis worldwide (El Behiry *et al*., 2012).

S. aureus could be identified from human septic wound, and urine collected from diseased human (10% each). Humans are known to carry *S. aureus* in their anterior nares, with a mean carriage rate of 37.2% in the general population (Kluytmans *et al*., 1997). *S. aureus* causes a wide variety of human illnesses, including scalded skin syndrome, toxic shock syndrome (TSS), necrotizing pneumonia and colonizes the anterior nares and, from there, may colonize other body surfaces, including other mucous membranes and damaged skin (Nada *et al.*, 2012). 92% of *S. aureus* isolates were hemolytic and 96% were able to reduce tellurite to metallic tellurium producing a black coloration. *S. aureus* isolates produced golden yellow, creamy and white colonies on nutrient agar, in percent of 84%, 8% and 8% respectively. It is clear that golden yellow colony was the predominant pigment among *S. aureus* isolates (El-Jakee *et al*., 2010b).

Leone *et al*. (2015) concluded that methicillin-resistant *Staphylococcus aureus* (MRSA) associated infection has become a worrisome issue worldwide. All of *S. aureus* isolates were methicillin sensitive**.** Staphylococcal abscesses have been described with both methicillin resistant *S. aureus* and methicillin sensitive *S. aureus* (Barnett *et al*., 2012). Various methods have been developed for the detection of enterotoxin production (Jassim *et al*., 2013).

Reverse passive latex agglutination test (RPLA) test was used in this study as a recent technique for detection of the presence of staphylococcal enterotoxins and this fact was in accordance with that mentioned by Schumacher-Perdreau *et al.* (1994) and El-Jakee *et al*. (2010a) who confirmed the accuracy of commercial available RPLA for detection of enterotoxins.

Exactly 10 out of 25 *S. aureus* isolates were found to be toxigenic using RPLA (40%). Enterotoxin C was detected with an incidence of 50% followed by enterotoxin A (20%). Jorgensen *et al.* (2005) found that *sec* was most common toxin detected in *S. aureus* isolates from bovine mastitis.

Polymerase chain reaction techniques have been developed for detection of staphylococci enterotoxins (Jassim *et al.*, 2013). Using PCR, 7 out of 25 *S. aureus* isolates were found to be toxigenic (28%).

Table 4. Prevalence of toxigenic *S. aureus* isolates using RPLA and PCR

Type of the samples	No. of S. aureus isolates	RPLA test							PCR							
		Types of toxins					Toxigenic *S. aureus* isolates		sea	sea & seb	sec	seb	sec & see	Sea, sec & sed	Toxigenic *S. aureus* isolates	
		A	A&B	A&C	C	A,B,C& D	No.	%							No.	%
Milk from subclinical mastitic cows	10	1	0	1	2	0	4	40	2	0	1	0	0	0	3	30
Milk from subclinical mastitic buffaloes	10	1	1	0	1	0	3	30	0	1	0	0	0	0	1	10
Total bovine	20	2	1	1	3	0	7	35	2	1	1	0	0	0	4	40
Urine samples from human	3	0	0	0	0	1	1	33.3	0	0	0	0	0	1	1	33.3
Abscesses from human	2	0	0	0	2	0	2	100	0	0	0	1	1	0	2	100
Total human	5	0	0	0	2	1	3	60	0	0	0	1	1	1	3	60
Total	25	2	1	1	5	1	10	40	2	1	1	1	1	1	7	28

It is clear that, enterotoxin A, B and C could be detected among subclinically mastitic bovine. Many strains of *S. aureus* produce one or more exotoxins, which may contribute to the pathogenesis of mastitis in ruminants (Ünal and Çinar, 2012).

Enterotoxins A, B, C, D and E could be identified from *S. aureus* isolated from the collected human sample. The ability of *S. aureus* to cause human disease depends on the production of cell-surface adhesin, antiphagocytic factors, and secreted exotoxins (Nada *et al.,* 2012). The present study concluded that *S. aureus* isolated from bovine and human could serve as a potential reservoir of staphylococci enterotoxin genes. And detection of SEs by RPLA and PCR was a useful additional tool to support identification of enterotoxigenic strains.

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