Properties of enterotoxigenic *S. aureus* Isolated from mastitic cattle and buffaloes in Egypt

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Abstract: Enterotoxigenic *S. aureus* in milk posses a potential health hazard to consumers. In this paper 106 *S. aureus* isolated from cow and buffalo milk samples were investigated for production of enterotoxins. RPLA results showed high incidence of type C enterotoxin followed by type A and type B with incidence of 34 (32.1%), 19 (17.9%) and 15 (14.2%) respectively. Toxigenic *S. aureus* isolates produced golden yellow, creamy and white colonies on agar in percent of 69.11%, 27.94% and 2.94% respectively. Regarding to hemolytic activity on sheep blood agar, 92.65% of toxigenic *S. aureus* isolated from bovine milk samples were hemolytic. A correlation exists between toxigenic isolates and coagulase and DNase production. On crystal violet agar medium. 23.53% of the *S. aureus* isolates yielded yellow colonies, 64.71% yielded violet colonies, while 11.76% yielded white colonies from the toxigenic *S. aureus* isolates. It is clear that most of bovine isolates yielded violet colonies on the medium. Out of 68 isolates of toxigenic *S. aureus* isolates 51 (75%) showed SpA by agglutination test positive. Results obtained showed 100% agreement between RPLA and PCR techniques. [Journal of American Science. 2010;6(11):170-178]. (ISSN: 1545-1003).

Keywords: *S. aureus*, mastitis, enterotoxins, RPLA, PCR.

1. Introduction

Milk and its products can harbor a variety of microorganisms and can be important sources of food-borne pathogens. Livestock-associated *S. aureus* to be an underappreciated source of pathogenic strains (Bystron et al., 2010). Enterotoxigenic *S. aureus* in raw milk posses a potential health hazard to consumers, the identification of such strains should be used as part of a risk analysis of milk and milk products (Zouharova and Rysanek, 2008). Staphylococcal food poisoning is considered one of the leading food-borne illnesses in human worldwide and is associated with contaminated food of animal origin such as milk and dairy products (Tsegmed *et al.,* 2007). *S. aureus* is a major causative agent of mastitis which is the most economically important diseases for the dairy industry so more effective therapeutic treatment and prophylactic approaches are surely needed (Chiang *et al*., 2007; Oviedo-Boyso *et al*., 2008).

Regarding the public health, *S. aureus* is a commensal organism and versatile pathogen in animals and human. It produces a broad spectrum of surface components (proteins and capsular polysaccharides) and exotoxins. Staphylococcal enterotoxins (SEs) are serologically grouped into five major classical types which are SEA, SEB, SEC, SED and SEE. Also new SEs such as SEG through SEM has recently been identified and characterized (Chiang *et al*., 2006). In addition to toxic shock syndrome toxin (TSST-1) which is the causative agent in toxic shock syndrome in human (Kenny *et al.*, 1993). The direct detection of the pathogen in the raw milk and dairy products by PCR technique can provide rapid results and highlight the presence of loads of *S. aureus* potentially representing the risk of intoxication (Ercolini *et al.*, 2004). The analysis of the results obtained by SET-RPLA method for the productivity of classical enterotoxins A-D and the results obtained by PCR for the presence of *sea-sed* genes revealed the correlation between each other (Lawrynowicz-Paciorek *et al.*, 2007). The present work aimed to determine the role of *Staphylococcus* species in bovine mastitis and study the most virulence factors associated with isolated strains using recent techniques for the detection of gene sequence concerned with toxin production as RPLA and PCR techniques.

2. Material and Methods

Milk samples:

A total 0f 203 animals including 149 cows and 54 buffaloes from different farms in Egypt were examined for mastitis according to clinical observation (Schalm *et al.*, 1971). A total of 554 individual quarter milk samples were collected from 406 quarters of lactating cows and 148 quarters of lactating buffaloes, distributed as shown in Table (1). The examined udders were thoroughly washed, dried with a clean towel and the teats were sprayed with 70% ethanol. After that the first few jets of milk were discarded and 10 ml of milk samples from each quarter were collected in a sterile McCartney bottle. All samples were kept at 4°C and transported immediately to the laboratory.

Table (1): Number of examined animals and quarters

Infected quarters	Cows		Buffaloes	
	No. of animals	No. of quarters	No. of animals	No. of quarters
One quarter	25	25	9	9
Two quarter	29	58	10	20
Three quarter	57	171	21	63
Four quarter	38	152	14	56
Total	149	406	54	148

Bacteriological examination:

The milk samples were activated by incubation for 18-24 hours at 37°C then the cream and supernatant fluids were discarded then milk samples were centrifuged at 3000 rpm for 20 minutes before bacteriological cultivation. The sediment was streaked on to the surface of nutrient agar (Difco) and Mannitol salt agar medium (Oxoid), then the inoculated plates were incubated for 24-48 hours at 37 °C after which they were examined for colony characters, cellular morphology and the purity of the culture. The suspected colonies were picked up and propagated on Baird-Parker agar (Oxoid) for further examination.

Identification and characterization of staphylococci isolates:

Pure cultures of the isolates were identified and characterized according to Cruickshank *et al*. (1975) and Mackie and MacCarteny (1996).-
Characteristics of coagulase positive staphylococci were identified according to Quinn *et al*. (2002) by: coagulase test using dry spot kit (staphy tect pIus), acetoin production, pigment production on nutrient agar" Difco", hemolysis activity on blood agar base (Oxoid) plus 5% sheep blood, deoxyribonuclease activity using DNase agar (Oxoid), growth on Baird-Parker medium (Oxoid) and crystal violet agar growth type (Rodgers *et al.*, 1999). Also SpA was detected using agglutination kits (welcome diagnostics) and latex slide agglutination test: Dry spot kit (staphy tect pIus) (Oxoid, DR100M) was used for the identification of staphylococci which possess clumping factor.

Detection of staphylococcal enterotoxins by SET RPLA kit (Oxoid):

Using reversed passive latex agglutination (RPLA) the *S. aureus* isolates were examined for production of enterotoxins A, B, C and D.

Detection of enterotoxin by PCR

The DNA was extracted from *S. aureus* isolates using enzymatic method and the PCR products were visualized according to Sambrook *et al*. (1989) using primers synthesized by Metabion Company, Germany as described in Table (2). DNA molecular weight marker was supplied by Amers Co. Cleveland, Ohio, USA and standard *S. aureus* and *S. epidermidis* donated from Department of bacteriology, Navy American research Unit (NAMRU 3).

3. Results

Tables (3) demonstrate the distribution of affected quarters among mastitic cows and buffaloes. It is clear that affection in 3 quarters is higher than the others quarters affection (42.12 - 42.57%), followed by affection in 4 quarters (37.43 - 37.84%), then in 2 quarters (14.29 – 13.51%) and in one quarter (6.16 – 6.08%) respectively.

The distribution of staphylococcal species among the examined mastitic quarters was 23.29% as shown in Table (4). It is clear that 106 isolates were identified as *S. aureus* with an incidence of 19.13%, followed by 16 isolates (2.89%) identified as *S. intermedius* and 7 isolates (1.26%) were identified as *S. hyicus.*

Results obtained in Table (5) showed that 68 out of 106 *S. aureus* isolates were found to be toxigenic with an incidence of 64.2% and distributed as follow: enterotoxin C were detected in 34 samples with an incidence of 32.1% , followed by enterotoxin A were isolated from 19 samples with an incidence of 17.9% and enterotoxin B were isolated from 15 samples with an incidence of 14.2% . It is clear from previous results that the enterotoxin C is the most predominant enterotoxin type than the others types.

S. aureus coagulase positive isolates produced endopigmets when cultivated on nutrient agar. As shown in Table (6) toxigenic *S. aureus* isolates produced golden yellow, creamy and white colonies on agar in percent of 69.1%, 27.94% and 2.94% respectively. Non toxigenic *S. aureus* isolates produced golden yellow and creamy colonies on agar in percent

of 71.05% and 28.95% respectively. It is clear that golden yellow colony was the most predominant pigment among bovine *S. aureus* isolates.

In the present investigation sheep blood agar was used to determine types of hemolysis among the *S. aureus* isolates and the results were illustrated in Table (7). It is clear that 92.65% of toxigenic *S. aureus* isolates were hemolytic and 92.1% of non toxigenic *S. aureus* isolates were hemolytic.

Out of 68 toxigenic *S. aureus* isolates 46 (67.65%) were DNase positive as shown in Table (8). While out of 38 non toxigenic *S. aureus* isolates 26 (68.42%) were DNase positive.

As shown in Table (9), out of 68 toxigenic *S. aureus* isolates 66 were positive for tellurite reduction with an incidence of 97.06%, while all the 38 non toxigenic *S. aureus* isolates (100%) were positive.

Crystal violet agar medium was used as a selective medium for characterization of *S. aureus*. 3 characteristic appearances were recorded as shown in Table (10). Among toxigenic *S. aureus* isolates type A growth (yellow colonies) was detected in 23.53% of the isolates, and type C growth (violet colonies) was detected in 64.71% of the isolates, while type E (white colonies) was detected only in 11.76%. In non toxigenic *S. aureus* isolates Type A growth (yellow colonies) was detected in 23.68% of the isolates, and type C growth (violet colonies) was detected in 65.79% of the isolates, while type E (white colonies) was detected only in 10.53%. It is clear that most of bovine isolates had violet colonies on the medium.

Out of 68 isolates of toxigenic *S. aureus* isolates, 51 (75%) showed SpA agglutination test positive as shown in Table (11). Also out of 38 isolates of non toxigenic *S. aureus* isolates 27 (71.05%) were SpA positive.

Table (2): shows the primers used for PCR

Genes	Primer sequence (5'- 3')	PCR Program*				Size (bp)	Reference
		No. of cycles	Temperature(°C) / time(minutes) of				
			Denaturation	Annealing	Extension		
16 S rRNA F	GTAGGTGGCAAGCGTTATCC	35	92 ^{O}C / 1 min	52^{0}C / 1 min	72^{0}C / 1 min	228	**Løvseth *et al.* (2004)**
16 S rRNA R	CGCACATCAGCGTCAG						
sea F	CCTTTGGAAACGGTTAAAACG	35	92 ^{0}C / 1 min	58^{0}C / 1 min	72^{0}C / 1 min	127	**Becker *et al.* (1998)**
sea R	TCTGAACCTTCCCATCAAAAAC						
seb F	TCGCATCAAACTGACAAACG					477	
seb R	GCAGGTACTCTATAAGTGCC						

Photo (1) showed that the *S. aureus* isolates previously proved to be toxigenic strains by using RPLA were confirmed to be toxigenic by using PCR. Results obtained showed that 100% agreement between RPLA & PCR.

Table (3): Distribution of quarters showing clinical signs of mastitis in 149 cows and 54 buffaloes.

Quarter	Cows		Buffaloes	
	No.	%	No.	%
1 Quarter	25	6.16	9	6.08
2 Quarter	58	14.29	20	13.51
3 Quarter	171	42.12	63	42.57
4 Quarter	152	37.43	56	37.84
Total	406	100	148	100

No. Positive number. % was calculated according to the total number of quarters.

Table (4): Distribution of *Staphylococcus* species isolated from the examined milk samples.

Sources of the isolates	No. of examined milk samples	*Staphylococcus* species						Total No. of isolates	%
		S. aureus		*S. intermedius*		*S. hyicus*			
		No.	%	No.	%	No.	%		
Cows	406	85	20.94	11	2.71	5	1.23	101	24.88
Buffaloes	148	21	14.19	5	3.38	2	1.35	28	18.92
Total	554	106	19.13	16	2.89	7	1.26	129	23.29

Table (5): Prevalence of toxigenic *S. aureus* isolates using RPLA test.

	No. of *S. aureus* isolates	Toxigenic isolates		Types of toxins					
				A		B		C	
		No.	%	No.	%	No.	%	No.	%
Cows	85	56	65.9	16	18.8	12	14.1	28	32.9
Buffaloes	21	12	57.1	3	14.3	3	14.3	6	28.6
Total	106	68	64.2	19	17.9	15	14.2	34	32.1

Table (6) : Percentage of pigment production among *S. aureus* isolates.

Sources of the isolates	Toxigenic isolates							Non toxigenic isolates *							Total isolates						
	No. of examined *S. aureus*	Golden yellow		Creamy		White		No. of examined *S. aureus*	Golden yellow		Creamy		White		No. of examined *S. aureus*	Golden yellow		Creamy		White	
		No.	%	No.	%	No.	%		No.	%	No.	%	No.	%		No.	%	No.	%	No.	%
Cows	56	38	67.9	16	28.6	2	3.6	29	20	68.97	9	31.03	-	0	85	58	68.2	25	29.4	2	2.4
Buffaloes	12	9	75	3	25	-	0	9	7	77.8	2	22.2	-	0	21	16	76.2	5	23.8	-	0
Total	68	47	69.1	19	27.94	2	2.94	38	27	71.05	11	28.95	-	0	106	74	69.81	30	28.3	2	1.89

Table (7): Percentage of hemolytic activity of *S. aureus* isolates on sheep blood agar.

Sources of the isolates	Toxigenic isolates			Non Toxigenic isolates *			Total isolates		
	No. of examined *S. aureus*	Hemolytic activity		No. of examined *S. aureus*	Hemolytic activity		No. of examined *S. aureus*	Hemolytic activity	
		No.	%		No.	%		No.	%
Cows	56	52	92.86	29	27	93.1	85	79	92.94
Buffaloes	12	11	91.67	9	8	88.89	21	19	90.48
Total	68	63	92.65	38	35	92.1	106	98	92.45

*non toxigenic *S. aureus* using RPLA

Table (8) : Percentage of deoxyribonuclease activity of *S. aureus* isolates.

Sources of isolates	Toxigenic isolates			Non Toxigenic isolates *			Total isolates		
	No. of examined *S. aureus*	DNase activity		No. of examined *S. aureus*	DNase activity		No. of examined *S. aureus*	DNase activity	
		No.	%		No.	%		No.	%
Cows	56	38	67.86	29	21	72.41	85	59	69.41
Buffaloes	12	8	66.67	9	5	55.56	21	13	61.9
Total	68	46	67.65	38	26	68.42	106	72	67.92

*non toxigenic *S. aureus* using RPLA

Table (10): Percentage of growth types of *S. aureus* isolates on crystal violet agar medium.

Sources of the isolates	Toxigenic isolates							Non toxigenic isolates *							Total isolates						
	No. of examined *S. aureus*	violet		yellow		White		No. of examined *S.aureus*	violet		yellow		White		No. of examined *S.aureus*	violet		yellow		White	
		No.	%	No.	%	No.	%		No.	%	No.	%	No.	%		No.	%	No.	%	No.	%
Cows	56	36	64.29	13	23.21	7	12.5	29	19	65.52	7	24.14	3	10.34	85	55	64.71	20	23.53	10	11.76
Buffaloes	12	8	66.67	3	25	1	8.33	9	6	66.67	2	22.22	1	11.11	21	14	66.67	5	23.81	2	9.52
Total	68	44	64.71	16	23.53	8	11.76	38	25	65.79	9	23.68	4	10.53	106	69	65.1	25	23.58	12	11.32

*non toxigenic *S. aureus* using RPLA

Table (11): Incidence of protein A in *S. aureus* isolates using agglutination test.

Sources of isolates	Toxigenic isolates			Non Toxigenic isolates *			Total isolates		
	No. of examined *S. aureus*	Staphylococcal protein A (SpA)		No. of examined *S. aureus*	Staphylococcal protein A (SpA)		No. of examined *S. aureus*	Staphylococcal protein A (SpA)	
		No.	%		No.	%		No.	%
Cows	56	42	75	29	22	75.86	85	64	75.29
Buffaloes	12	9	75	9	5	55.56	21	14	66.67
Total	68	51	75	38	27	71.05	106	78	73.58

*non toxigenic *S. aureus* using RPLA

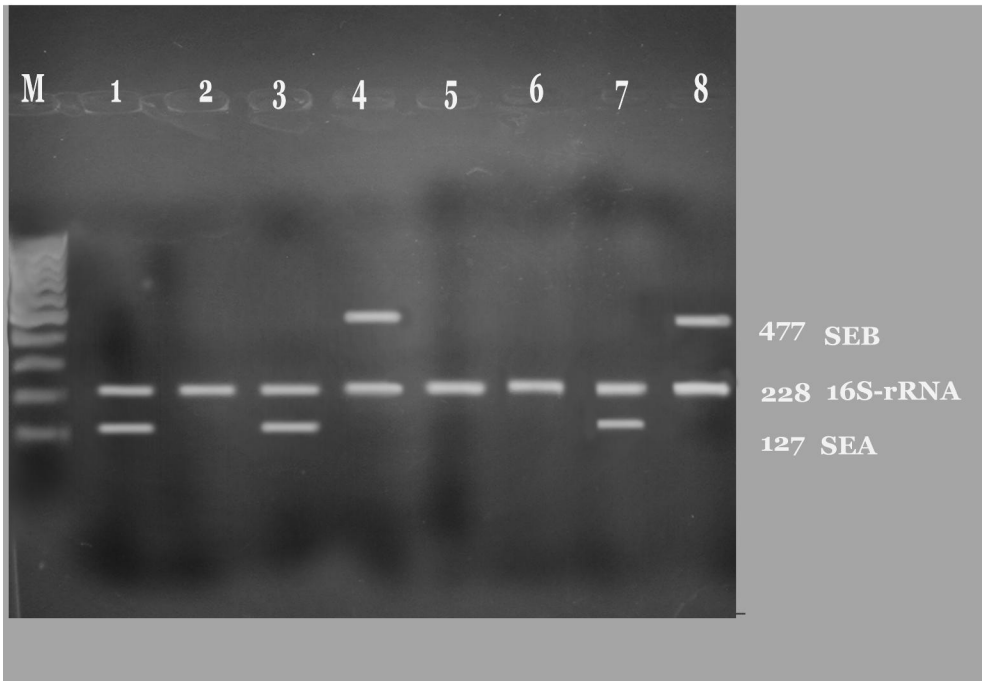

Photo (1): Shows SDS profile analysis of amplified PCR products among the examined *S. aureus*. 3 isolates produced type a toxin (lanes 1, 3 and 7). 2 isolates produced type B toxin (lanes 4 and 8) by using polymerase chain reaction technique (PCR). Lane 2: standard *S. aureus* strain. Lane 5: *S. aureus* isolate produce c toxin as detected by RPLA. Lane 6: *S. aureus* negative for production of toxins as detected by RPLA.

4. Discussion

S. aureus is involved in intramammary infections in bovine causing economic losses and milk-safety problems (Taverna *et al.*, 2007). Mastitis control is complex problem for which there are no simple solutions.

Bacteriological study of mastitic milk samples was carried out and results obtained revealed that staphylococcal species were isolated from 129 samples with the percentage of 23.29 % this percentage was calculated according to the total number of quarters (554) as cleared from Table (4). These results were nearly similar to those mentioned by Pankey *et al.* (1991) (25.4 %); Mahbub *et al.* (1996); Badia (2004) (27.21%) and Elgabry (2006) (21.2%). Among coagulase–positive *Staphylococcus* species: *S. aureus, S. hyicus and S. intermedius. S. aureus* is a major agent of bovine mastitis as mentioned by Schleifer (1986). The results obtained in Table (3) showed that 106 isolates were identified as *S. aureus* with an incidence of 19.13%, followed by 2.89% were identified as *S. intermedius* and 1.26% were identified as *S .hyicus.* These results goes in the direction which indicated that high incidence of staphylococcal mastitis was mainly due to *S. aureus*. The present results are in agreement with Badia (2004); Ekman *et al*. (2004) and Elgabry (2006) who found that *S. aureus* isolates were of high incidence than the other types of *Staphylococcus*. High incidence of *S. aureus* may be attributed to that *S. aureus* has a wide spread during the different seasons of the year. Nickerson *et al.* (1995) recorded that *S. aureus* was known to be easily spread between animals so that one *S. aureus* case may lead to more cases. The invasion of *S. aureus* in the interstitial tissue of the mammary gland and the nature of capsular polysaccharide type 5 (CP5) probably help bacteria to withstand the host defense mechanism (Hensen *et al.* 2000).

A number of different phenotypic and genotypic techniques are available to classified *S. aureus* strains for epidemiological investigation (Wildemauwe et al., 2010). One of the goals of this study was to explore the phenotypic characters including different virulence factors of *S. aureus* isolates. *S. aureus* is a major food borne pathogen due to its capability to produce a wide range of heat-stable enterotoxins (Peles *et al.*, 2007). Detection of staphylococcal enterotoxins is decisive for confirmation of an outbreak and determination of the enterotoxigenicity of the strains. Since the recognition

of their antigenicity, large numbers of serological methods for the detection of enterotoxins in food and culture media have been proposed (Da Cunha *et al*., 2007). Major virulence factors of *S. aureus* organism include enterotoxins (SEs) that cause both food poisoning and toxic shock syndrome. Recently, a novel SE tentatively designated SEL was identified in a bovine mastitis isolates, the toxin lacked emetic activity (Orwin *et al*., 2003). A little as 0.1 µg of enterotoxins can be sufficient to produce food poisoning after incubation period which can be as short as 1 hour out of usually 4 - 6 hours (IASR, 2001).

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 *et al.* (1995) who confirmed the accuracy of commercial available RPLA for detection of enterotoxins. Results obtained in Table (5) showed that 68 out of 106 *S. aureus* isolates were found to be toxigenic with an incidence of 64.2% and distributed as follow: enterotoxin C were detected from 34 samples with an incidence of 32.1% followed by enterotoxin A from 19 samples with an incidence of 17.9% and enterotoxin B from 15 samples with an incidence of 14.2%. It is clear from previous results that the enterotoxin C is the predominant one, this observation were in agreement with that mentioned by Jorgensen et *al.* (2005) who found that SEC and *sec* were most common toxin detected in *S. aureus* isolates from bovine mastitis. Samah (2003) recorded that 16.6% isolates of 106 *S. aureus* isolates obtained from milk were enterotoxigenic type SEC producing isolates. In addition to that mentioned by Soriano *et al*. (2002) who found that obtained results showed the high incidence of the type C followed by type B and then type A.

S. aureus isolates were characterized as coagulase positive isolates produce endopigmets when cultivated on nutrient agar. As shown in Table (6) toxigenic *S. aureus* isolates produced golden yellow, creamy and white colonies on agar, in percent of 69.11%, 27.94% and 2.94% respectively. Non toxigenic *S. aureus* isolates produced golden yellow and creamy colonies on agar in percent of 71.05% and 28.95% respectively. It is clear that golden yellow colony was the predominant pigment among *S. aureus* isolates. These results are in agreement with Elgabry (2006) who found that toxigenic *S. aureus* isolates produced golden yellow, creamy and white colonies on agar in percent of 64.1%, 29.5% and 6.4% respectively. 92.65% of toxigenic *S. aureus* isolates had hemolytic activity on sheep blood agar as shown in Table (7) and 92.1% of non toxigenic *S. aureus* isolates were hemolytic. Lam *et al.* (1995) and Aarestrup *et al*. (1999) showed that approximately 1/5 to 1/4 of the *S. aureus* isolates of bovine mastitis do not present any detectable beta-hemolytic activity in primary cultures.

Out of 68 toxigenic *S. aureus* isolates 46 (67.65%) were DNase positive, while out of 38 non toxigenic *S. aureus* isolates 26 (68.42%) were DNase positive as shown in Table (8). Abd El-Salam (2003) recorded that all toxigenic strains of *Staphylococcus* were coagulase positive and DNase producers. Boerlin *et al*. (2003) illustrated that 71.8% of *S. aureus* isolates had DNase activity. It is clear from Table (9) that out of 68 isolates of toxigenic *S. aureus* isolates 66 (97.06%), were able to reduce tellurite to metallic tellurium producing a black coloration, and all non toxigenic *S. aureus* isolates (100%) were positive. *S. aureus* isolates was able to reduce tellurite to metallic tellurium with an incidence of 96.2% (Elgabry, 2006). Selective agars like modified Baird-Parker agar have been used successfully for the detection and identification of *S. aureus* and other coagulase-positive staphylococci (Roberson *et al.*, 1992). Three characteristic appearances were recorded among *S. aureus* isolates after having been grown on crystal violet agar medium, as shown in Table (10). Yellow colonies were detected in 23.53% of the isolates, and violet colonies were detected in 64.71% of the isolates, while white colonies were detected only in 11.76% from the toxigenic *S. aureus* isolates. It is clear that most of bovine isolates had violet colonies on the medium. The present results are in agreement with Wan *et al*. (1999).

Several rapid identification tests for *S. aureus* are commercially available and have been extensively in use. For instance, the slidex staph plus kit from Bio-Merieux is an agglutination test used for the simultaneous demonstration of protein A, clumbing factor and other surface antigens specific for *S. aureus* (Boerlin *et al*., 2003). In the present study 51 out of 68 isolates of toxigenic *S. aureus* isolates (75%) showed SpA by agglutination test positive as shown in Table (11). Detection of toxigenic strains in *S. aureus* isolates using polymerase chain reaction technique (PCR) was illustrated in photo (1). The isolates proved to be toxigenic using RPLA were confirmed using PCR (detection of toxin C was not available) as recent technique. Results obtained showed that 100% agreement between the 2 tests RPLA & PCR. Zouharova and Rysanek (2008) found that the results of both methods were identical concerning SEB and SED. It was concluded that detection of SEs by PCR was a useful additional tool to support identification of Enterotoxigenic strains.

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