Recent Techniques used for Isolation and Characterization of *Staphylococcus Aureus* from Mastitic Cows.

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Abstract: A total of 152 cows was examined in this study for Staphylococcus species, it was found that 44.3% of cows and were clinically mastitic whereas 14.5% were subclinically mastitic respectively. The identification of Staphylococcus species revealed that *S. aureus, S. epidermidis, S. intermedius* and *S. hyicus* for cows were (17.2%, 7.5%, 3.9% and 1.6%) respectively. *Staphylococcus aureus* isolates were confirmed after biochemical identification by API test. The study of virulence factors of total *S. aureus* isolates from mastitic cows revealed that lipase, fibrinolysin, DNase and protein A production were presented as percentage 67.3, 74.0, 85.6 and 84.6 respectively. The antibiotic sensitivity for *S. aureus* revealed that 96.2% of cow isolates were methicillin sensitive which considered the drug of choice for these isolates. The study also included the identification of *S. aureus* enterotoxins using set-RPLA and multiplex PCR. The incidence of enterotoxins C,A,B and D by set-RPLA were 36.5%, 14.4%, 10.6% and 2.9% respectively. Meanwhile the results of multiplex PCR were 7 isolates as enterotoxin C,4 isolates as enterotoxin E and one isolate for each A,B, and D respectively. The identification of MRSA of cow's isolates using PCR revealed that 3 isolates out of 5 isolates were positive.
[El-Seedy,F.R, El-Shabrawy, M; Hakim, A. S; Dorgham,S.M.m Ata, S. Nagwa; Bakry, M.A and Osman,N.M.N. Recent Techniques used for Isolation and Characterization of *Staphylococcus Aureus* from Mastitic Cows. Journal of American Science 2010;6(12):701-708]. (ISSN: 1545-1003). http://www.americanscience.org.

Key words: *Staphylococcus aureus*; mastitis; methicillin sensitive; set- RPLA, multiplex PCR.

1. Introduction:

Mastitis is the most common infectious disease affecting the dairy cows and remains the most economically important disease of dairy industries around the world. (Khan *et al.,* 1998).

Milk and its products can harbor a variety of microorganisms and can be important sources of food borne pathogens. The presence of food borne pathogens in milk is due to direct contact with contaminated sources in the dairy farm environment or with excretions from the udder of infected animals (Oliver *et al.,* 2005).

A wide variety of bacteria can be involved, but the most common mastitis pathogen is *Staphylococcus aureus*, *S. aureus* is a major pathogen of bovine mastitis worldwide. Despite implementing intensive control measures, it is difficult to eradicate the intramammary infections caused by this pathogen and it remains a substantial economic problem. (Salmon, 2002). *Staphylococcus aureus* produces a broad spectrum of surface components (proteins and capsular polysaccharide) and exotoxins, they are virulence factors involved in the pathogenesis of bovine mastitis as these toxins and products are injurious to milk producing cells of the mammary gland, impair glands and immune defense mechanisms, while they are capable to reside intracellular contributes the ability of *S. aureus* to establish a chronic infection that can persist for the life of the animal(Taverna *et al*., 2007). Enterotoxigenic *S. aureus* in raw milk posses a potential health hazard to consumers and the identification of such strains should be used as a part of analysis of milk and milk products (Zouharova and Rysanek, 2008).

Because of the organisms propensity to acquire antimicrobial resistance, whereas most infections can be treated or prophylacted with antibiotic; antimicrobial resistance of *S. aureus* especially methicillin resistant *S. aureus* (MRSA) continues to be a problem for clinicians worldwide justifies their recognition as a "New Emerging Pathogen" (Shittu and Lin, 2006).

So the present study was conducted to evaluate the recent techniques for isolation and characterization of antibiotic resistant staphylococci (*S. aureus*) from mastitic animals in correlation to its virulent factors.

2. Materials and methods

Samples

Six hundred and eight milk samples were collected from udder quarters of examined cows, 388 were collected from 97 clinically mastitic cows

which had clinical signs of abnormal secretions of mammary glands containing clots or flakes, with udders showing swelling and hardness and 220 from apparently healthy cows detected by palpation of udder and were subjected to California Mastitis Test (CMT) to detect subclinical mastitis.

Isolation of Staphylococci:

The mastitic milk samples were activated by incubation for 18-24 hours at 37°C, then milk samples were centrifuged at 3000 rpm for 20 minutes and the cream and supernatant fluids were discarded, the sediments were streaked onto the surface of the following media: Nutrient agar, Blood agar medium, Mannitol Salt agar, Baird Parker agar and Vogel Johnson agar. 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 identified according to Collee *et al*. (1996) and Quinn *et al*. (2002).

Staphylococci latex agglutination test:

Staphylococci were tested using dry spot kit and colonies from previous media Fresh culture grown overnight 18-36 hours incubation were used. A positive result showed agglutination of the latex particles occurs within 20 seconds. This indicates the presence of *S.aureus*.

Identification of *S. aureus* isolates using API system:

The organism was sub cultured onto Columbia blood agar at 37°C for 18-24 hours. Single well-isolated colony (young culture) from blood agar inoculated into API staph medium to make homogeneous bacterial suspension with a turbidity equivalent to McFarland tube No. 0.5 and this suspension used immediately after preparation. Identification is obtained with the numerical profile on the result sheet, the tests are separated into groups of 3 and a value 1, 2 or 4 is indicated for each. By adding together the values corresponding to positive reactions within each group, a 7-digit profile number is obtained for the 20 tests of API strip.

Antimicrobial sensitivity test of *S. aureus* isolates:

Using disk diffusion method which applied according to (Finegold and Martin, 1982).

Detection of staphylococcal enterotoxins by SET-RPLA kit:

The clear culture supernatant fluids were tested serologically by reversed passive latex agglutination technique using Oxoid SET-RPLA [A Kit for detection of Staphylococcal enterotoxins A, B, C and D] (Shingaki *et al*., 1981).

Extraction of DNA from the Staphylococcal isolates according to Sritharan and Barker (1991):

Extraction of DNA from the Staphylococcal isolates by Hexadechyl trimethyl ammonium bromide (CTAB) according to Sambrook *et al.* (1989):

Multiplex polymerase chain reaction (multiplex PCR) according to Becker *et al*. (1998):

All reactions were carried out in a final volume of 50 µl in micro application tubes (PCR tubes). The reaction mixture consists of 5 µl of the extracted DNA template from the bacterial cultures, 5 µl of 10x PCR buffer, (75 M Tris Hcl PH9.0, 2mM MgCl2, 50 mM Kcl , 20 mM$(NH4)_2So_4$),1 µl dNTPS (40µM),1µl(1U Ampli Taq DNA Polymerase) and 1µl from the forward and reverse primers of (SAEA F-SAEA R), (SAEB F-SAEB R), (GSECR.1-GSECR.2), (GSEDR.1-GSEDR.2) and (GSEER.1-GSEER.2). All primers were used together and volume of the reaction mixture was completed to 50 µl using DDW. 40 µl paraffin oil wax was added and the thermal cycler was adjusted as following program: initial denaturation at 92˚C for 5 minutes followed by 35 cycles of denaturation at 92˚C for 1 minute, annealing step at 52˚C for 1 minute and extension at 72˚C for 1 minute. A final extension step was done at 72˚C for 10 minutes. The PCR products were stored in the thermal cycler at 4˚C until they were collected.

Amplification of mec A gene from DNA of *Staphylococcus aureus* isolates according to Riffon *et al.* (2001):

Each reaction was performed in a final volume of 25 µl in PCR tubes (ependorff). Each reaction contained mixture consists of 3µl of the extracted DNA template from the bacterial cultures plus 20 µl of ready to used master mix and 1 µl from the forward and reverse primer of MecAR1-MecAR2. At the surface of the tube, 40 µl paraffin oil was added to avoid evaporation of the reaction mixture and the thermal cycle was adjusted as following program: initial denaturation at 94˚C for 4 minutes followed by 30 cycles of denaturation at 94˚C for 45 seconds, annealing step at 58˚C for 1 minute and extension at 72˚C for 2 minute. A final extension step was done at 72˚C for 10 minutes. The PCR products were stored in the thermal cycler at 4˚C until they were collected.

The PCR products were electrophoresed in 1.5% agarose gel using Tris-acetate EDTA buffer. The gel containing separated DNA was stained with ethidium bromide. Standard marker containing known fragments of DNA either 100 bp or 250 bp ladders was used.

Table (1): The primers used for PCR

Primer	Sequence(5¯- 3¯)	Product size(bp)
SAEA-F	CCTTTGGAAACGGTTAAAACG	127
SAEA-R	TCTGAACCTTCCCATCAAAAC	
SAEB-F	TCGCATCAAACTGACAAACG	477
SAEB-R	GCAGGTACTCTATATAGTGCC	
GSECR-1	AGATGAAGTAGTTGATGTGTATGG	451
GSECR-2	CACACTTTTAGAATCAACCG	
GSEDR-1	CCAATAATAGGAGAAAATAAAAG	278
GSEDR-2	ATTGGTATTTTTTTTCGTTC	
GSEER-1	AGGTTTTTTCACAGGTCATCC	209
GSEER-2	CTTTTTTTCTTCGGTCAATC	
Mec AR1	GTGGAATTGGCCAATACAGG	1339
Mec AR2	TGAGTTCTGCAGTACCGGAT	

3. Results and Discussion:

From the results presented in table (2) examination of 388 quarter milk samples collected from 97 clinically mastitic cows by bacteriological examination revealed positive results in 232 (44.3%) of them while the examination of 136 quarter milk samples collected from 34 subclinically mastitic cows by CMT revealed positive results in 76 (14.5%) of them as shown in table ().These results are nearly similar to those mentioned by Bakken (1981) and Kossaibat *et al.*, (1998). El –Rashidy *et al.*, (1990) recorded that the incidence of subclinical mastitis was 26.08% and Seddek *et al.*, (1999) 7.1% to 29% among cows.

It is clear from table (3) The affection in two quarters is higher than the other quarter's affection in clinically and subclinically mastitic cows with an incidence of 34.0% and 38.2% respectively. In clinically mastitic cows three quarters affection are 27.8% followed by one quarter affection 21.6% then four quarter affection 16.5% meanwhile in subclinically mastitic cows one quarter affection is 26.5% followed by three quarter affection 20.6 % then four quarter affection 14.7 % . Concerning quarter involvement in mastitic cows, the rate of involvement in one and two quarters were relatively higher in subclinical mastitis (26.5% and 38.2%) than in clinical mastitis (21.6% and 34%) while the affection in the three and four quarters were higher in clinical mastitis (27.8% and 16.5%) than in subclinical mastitis (20.6% and 14.7%), whereas Bansal *et al.*,(1990) found that 64% of the lactating cows were infected in one quarter, 25% in two quarters, 5% in three quarters and 0% in all four quarters respectively, the variation in the quarter involvement maybe due to the differences in the defense reaction among quarters of the same animal (Dopfer *et al.*, 1999).

Table (2): Incidence of mastitis among the examined milk samples of cows.

Healthy state of the udder	Examined cows	Examined quarter	Negative quarter milk samples		Positive quartermilk samples	
			No.	%	No.	%
Clinical mastitis	97	388	156	29.8	232	44.3
Subclinical mastitis	34	136	60	11.5	76	14.5
Total	131	524	216	41.2	308	58.8

Table (3): The distribution of infected quarters in clinically and subclinical mastitic cows:

Number of affected quarters	clinically mastitic cows		subclinically mastitic cows	
	No.	**%**	**No.**	**%**
One quarter	21	21.6	9	26.5
Two quarters	33	34	13	38.2
Three quarters	27	27.8	7	20.6
Four quarters	16	16.5	5	14.7
Total	97	100	34	100

Table (4) illustrated the bacteriological examination of 232 milk samples and 76 milk samples collected from clinical and subclinical mastitis in cows respectively. It was found that only 71 and 22 were positive milk samples for staphylococcal species with an incidence of 30.6% and 28.9% respectively. In clinical mastitis the percentage of *S. aureus* isolates were (17.7%) as major pathogen followed by *S. epidermidis* (6.9%), *S. intermedius* (4.3%) and the lowest incidence was *S. hyicus* (1.7%). On other hand, in subclinical mastitis the incidence of *S. aureus* was (15.8%), *S. epidermidis* was (9.2%), *S. intermedius* was (2.6 %) and *S. hyicus* was (1.3%). These results showed that *Staphylococcus aureus* was the most microorganisms incriminated as cause of clinical and subclinical mastitis, as it represented 17.7% and 15.8% of the total bacterial isolates from examined quarter milk of cows respectively. In agreement with this result Esmat and Bader (1996) and Dego *et al.,* (2002) recorded that *S .aureus* was the most prevalent bacterial agent associated with mastitis in cows.

Table (5) illustrated the tested virulence factors of the *S. aureus* isolates in the present study, 88 out of 104 *S. aureus* isolates (84.6%) showed positive SpA by agglutination test. This observation was in agreement with that mentioned by Rosenberg *et al*, (2000) and Farage (2008). Moreover, the association of virulence genes and clinical mastitis proved the role of spa gene as risk factor (Zecconi *et al.* ,2005). Also the polymorphism of spa gene was confirmed to be scientifically associated with inflammatory response and growth rate (Zecconi *et al.* ,2006). (Kalorey *et al*., 2007).

In the present work all isolates of *S.aureus* were subjected for detection of clumping factor and capsular polysaccharide using dry spot kit (staphtect plus) (Oxoid).It is a latex slide agglutination test for differentiation of *S.aureus* than other staphylococci.
Concerning lipase activity on egg yolk agar medium, only 34 *S. aureus* strains were negative to this test with an incidence of 32.7%. On the other hand 70 strains out of 104 *S. aureus* isolates had lipase activity with percentage of 67.3. These results goes parallel to that recorded by Leung *et al*.,(1993) and Annemuller and Zschock

Table (4): Prevalence of Staphylococcus species isolated from clinically and subclinically mastitic milk samples in cows (1999).

Source of milk samples	No. of examined milk samples	Staphylococcus species								Total number of isolates	%
		S. aureus		*S. epidermidis*		*S. intermedius*		*S. hyicus*			
		No.	%	No.	%	No.	%	No.	%		
Clinical mastitis	232	41	17.7	16	6.9	10	4.3	4	1.7	71	30.6
Subclinical mastitis	76	12	15.8	7	9.2	2	2.6	1	1.3	22	28.9
Total	308	53	17.2	23	7.5	12	3.9	5	1.6	93	30.2

(100%) then subclinically mastitic cows (66.7%).

Table (5): Incidence of virulent factors in *S. aureus* isolates from cows.

Source of isolates	Clinical mastitic cows				Sub Clinical mastitic cows			
No of samples	41				12			
	positive		negative		positive		negative	
	No	%	No	%	No	%	No	%
Lipase activity	29	70.7	12	29.3	7	58.3	5	41.7
Fibrinolysine activity	33	80.5	8	19.5	9	75	3	25
DNase	41	100	-	-	8	66.7	4	33.3
SPA	37	90.2	4	9.8	10	83.3	2	16.7

In the present investigation high sensitivity was recorded to methicillin (96.2%) among the examined *S. aureus* isolates in cows followed by gentamycin (90.6%) and amoxicillin ,clavulinic acid and enrofloxacin (84.9% each) then ciprofloxacin (83.0%) and rifampicin (79.2%). Meanwhile 71.7% of the examined *S. aureus* isolates were resistant to streptomycin, 64.2% to penicillin and 54.7% to oxytetracycline. These results agreed to large extent with the finding of Pengov (1996) and Bhalerao *et al*., (2000).

Table (6): Antibacterial sensitivity test of *S. aureus* isolates from milk samples of cows with clinical and subclinical mastitis.

Antimicrobial agent	µg/disc	Sensitive		intermediate		resistant	
		No.	%	No.	%	No.	%
Ampicillin	10	26	49.0	16	30.2	11	20.8
Amoxycillin	25	32	60.4	9	16.9	12	22.6
Amoxycillin +Clavulinic acid	20+10	45	84.9	2	3.8	6	11.3
Penicillin-G	10 unit	15	28.3	4	7.5	34	64.2
Ciprofloxacin	10	44	83.0	5	9.4	4	7.5
Enrofloxacin	15	45	84.9	3	5.7	5	9.4
Gentamycin	10	48	90.6	3	5.7	2	3.8
Clindamycin	20	35	66.0	5	9.4	13	24.5
Neomycin	30	31	58.5	7	13.2	15	28.3
Streptomycin	10	11	20.8	4	7.5	38	71.7
Rifampicin	30	42	79.2	3	5.7	8	15.1
Cloxacillin	1	36	67.9	5	9.4	12	22.6
Methicilline	5	51	96.2	-	-	2	3.8
Oxytetracycline	30	14	26.4	10	18.9	29	54.7
Sulphamethoxazole-trimethoprim	23.75+ 1.25	20	37.7	12	22.6	21	39.6

The present study detected toxigenic strains in *S. aureus* isolates using commercial available kits, reverse passive latex agglutination test (RPLA). Results obtained showed high incidence of type C enterotoxin followed by type A then type B and type D. These results are in agreement with that mentioned by Jorgensen *et al.*, (2005) who found SEC was the most common enterotoxin detected in *S. aureus* isolates from bovine mastitis. In addition to that mentioned by Soriano *et al.*,(2002) and Badia (2004) who found that obtained results showed high incidence of type C (22 - 41.5%) followed by enterotoxin A, enterotoxin B and enterotoxin D whose numbers of isolates were 7 (13.2%), 5(9.4%) and 3 (5.7%) respectively. 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 number of serological methods for detection of enterotoxins in food and culture media has been proposed (Dacunha *et al.*, 2007). From our point of view the distribution of infection in the udder tissues may be related to the role played by toxins, this observation was in accordance to that mentioned by Hillerton and Walton (1991).

Table (7): Prevalence of toxigenic *S. aureus* isolates using RPLA test:

Source of *S. aureus*	No. of *S. aureus* isolates	Toxigenic isolates		Types of toxins							
				A		B		C		D	
		No.	%	No.	%	No.	%	No.	%	No.	%
Cows	53	37	69.8	7	13.2	5	9.4	22	41.5	3	5.7
Buffaloes	51	30	58.8	8	15.7	6	11.8	16	31.4	-	-
Total	104	67	64.4	15	14.4	11	10.6	38	36.5	3	2.9

Detection of toxigenic strains in *S. aureus* isolates using multiplex polymerase chain reaction technique (multiplex PCR). Total number of 12 isolates previously tested by using RPLA and the results were confirmed using multiplex PCR as recent technique. Results obtained showed that 100% agreement between the two tests RPLA and multiplex PCR. Our findings also agree with that of Zouharova and Rysanek(2008) who found that the results of both methods were identical concerning SEB and SED. It was concluded that detection of SEs by multiplex PCR was a useful additional tool to support identification of enterotoxigenic strains. Photo (1) showed 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).

To amplify the mecA gene from the extracted DNA of the previously selected *S. aureus* isolates with MR1-MR2 primers which amplify 1339

bp fragment of mecA gene were used. Results presented in photo (2) revealed that positive amplification of the 1339 bp fragment of mecA gene from the extracted DNA of 3 *S. aureus* isolates out of 5 examined samples. These 5 results of antibiogram of such five isolates were "3 strains methicillin-resistant while 2 strains were sensitive" which indicated that PCR technique could detect the mecA gene in the mecA resistant. This finding was supported by Riffon *et al*., (2001)

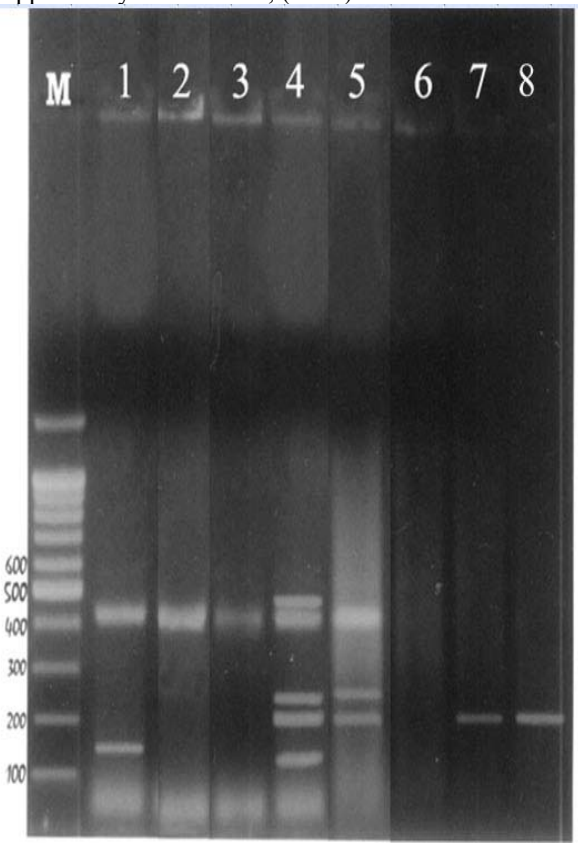

Photo (1): Agarose gel electrophoresis showing the result of multiplex PCR for detection of enterotoxin genes from *S. aureus*

M: The DNA molecular weight marker (100bp ladder)
Lane (1): positive amplification of 127 bp for enterotoxin A and 451bp for enterotoxin C in mastitic cows
Lane (2): positive amplification of 451 bp for enterotoxin C in mastitic cows
Lane (3): positive amplification of 451 bp for enterotoxin C in mastitic cows
Lane (4): positive control
Lane (5): positive amplification of 209 bp for enterotoxin E and 278 bp for enterotoxin D and 451bp for enterotoxin C in mastitic cows
Lane (6): no amplification in mastitic cows
Lane (7): positive amplification of 209 bp for enterotoxin E in mastitic cows.
Lane (8): positive amplification of 209 bp for enterotoxin E in mastitic cows.

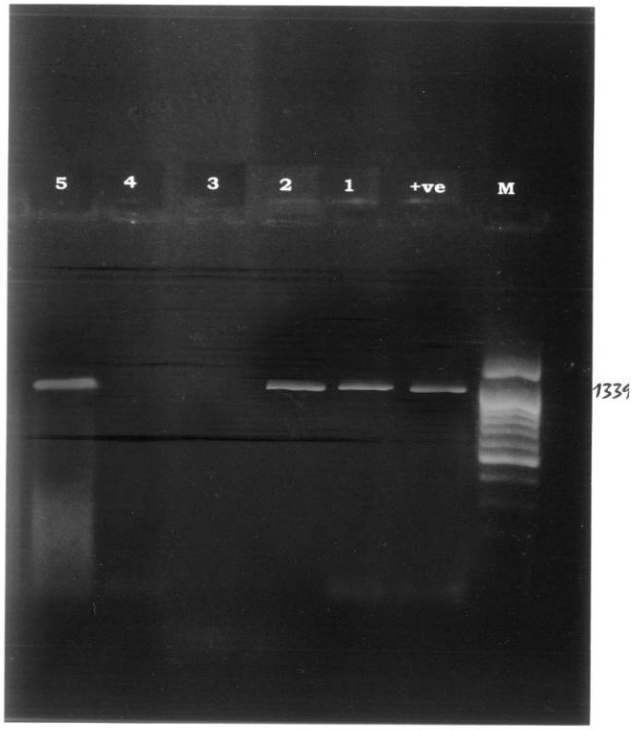

Photo (2): Agarose gel electrophoresis showing amplification of the 1339 bp fragment of mecAgene

M: The DNA molecular weight marker (100bp ladder)
+ve: positive control
Lane (1 & 2): methicillin resistant mastitic cows
Lane (3): methicillin sensitive mastitic cows.

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10/15/2010