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Advanced Techniques Used for Isolation and Characterization of *Staphylococcus aureus* Isolated from Mastitic Buffaloes

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Abstract: A total of 107 buffaloes were examined in this study for Staphylococcus species, it was found that 32.9% were clinically mastitic where as 32.3% were subclinically mastitic respectively. The identification of *Staphylococcus aureus* revealed that in clinical mastitis was 25.5% while in subclinical mastitis was 10.9%. The *Staphylococcus aureus* isolates were confirmed after biochemical identification by API test. The study of virulence factors of *S. aureus* isolates from clinically and subclinically mastitic buffaloes revealed that lipase, fibrinolysin, DNase and protein A production were presented as percentage (63.9, 73.3), (69.4, 66.7), (86.1, 60.0) and (80.6, 83.3) respectively. The antibiotic sensitivity for *S. aureus* revealed that 98.0% of buffaloes isolates were methicillin sensitive which was 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 31.4, 15.7, 11.8 and zero respectively. Meanwhile the results of multiplex PCR were three isolates as enterotoxin C, two isolates as enterotoxin E, one isolate as enterotoxin B and no enterotoxin was produced by two isolates respectively. The identification of MRSA of buffaloes isolates using PCR revealed that one isolate out of two isolates was positive as the result of sensitivity test.

Key words: Buffaloes · *Staphylococcus aureus* · Mastitis · PCR

INTRODUCTION

Mastitis is the most common infectious disease affecting the dairy buffaloes and remains the most economically important disease of dairy industries around the world. The major cause of bovine mastitis is the infection of the udder by pathogenic bacteria. A wide variety of bacteria can be involved, but the most common mastitis pathogens are *S. aureus* [1, 2].

Bovine mastitis is one of the problems of animal health in dairy herds as from the economical point of view it causes decline in milk yield, affects milk quality and composition, shortens the productive life of affected animals and is recognized as a cause of serious economic losses to dairy farmers, also cost of drugs and therapy is one of the important problems since treatment of mastitis is costly to the dairy men [3, 4].

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 [5].

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 [6].

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 of the ability of *S. aureus* to establish a chronic infection that can persist for the life of the animal [7].

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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 [8].

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" [9].

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.

MATERIALS AND METHODS

Samples: Four hundred and seventy six milk samples were collected from udder quarters of examined buffaloes 192 were collected from 48 clinically mastitic buffaloes which had clinical signs of abnormal secretions of mammary glands containing clots or flakes, with udders showing swelling and hardness and 236 from 59 apparently healthy buffaloes detected by palpation of udder and were subjected to modified 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 Quinn *et al*. [10].

Staphylococci Latex Agglutination Test: Staphylococci were tested using dry spot kit. A fresh culture grown overnight 18-36 hours incubation was used. A positive result showed agglutination of the latex particles occurring 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 was inoculated into API staph medium to make a homogeneous bacterial suspension with a turbidity equivalent to McFarland tube No. 0.5 and this suspension was used immediately after preparation. Identification was 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 was obtained for the 20 tests of API strip.

Antimicrobial Sensitivity Test of *S. aureus* Isolates: It was carried out according to Finegold and Martin [11] using 15 antibiotics (Table 1).

Table 1: Antibiotics used for antimicrobial sensitivity test of *S. aureus* isolates.

			Zone diameter		
Antimicrobial agent	Code	Disc potency µg/disc	Resistant	Intermediate	Sensitive
Ampicillin	AMP	10	≤11	12-13	≥14
Amoxycillin	AMI	25	≤11	12-13	≥14
Amoxycillin +Clavulinic acid	AMC	20+10	≤13	14-17	≥18
Penicillin-G	P	10 unit	≤20	20-28	≥29
Ciprofloxacin	CF	10	≤20	21-27	≥29
Enrofloxacin	ENR	15	≤12	13-14	≥15
Gentamycin	CN	10	≤12	13-14	≥15
Clindamycin	DA	20	≤14	15-20	≥21
Neomycin	N	30	≤12	13-16	≥17
Streptomycin	S	10	≤11	12-14	≥15
Rifampicin	RD	30	≤12	13-21	≥22
Cloxacillin	OB	1	≤10	11-12	≥13
Methicilline	MET	5	≤11	12-15	≥16
Oxytetracycline	OT	30	≤14	15-18	≥19
Sulphamethoxazole-trimethoprim	SXT	23.75+1.25	≤10	11-15	≥16

Table 2: 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	

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] [12].

Extraction of DNA from the Staphylococcal Isolates According to Sritharan and Barker [13] for Amplification of Mec a Gene

Multiplex Polymerase Chain Reaction (Multiplex PCR) According to Becker *et al.* [14]: 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 for amplification of mec A gene of (SAEA F-SAEA R), (SAEB F-SAEB R), (GSECR.1-GSECR.2) and (GSEER.1-GSEER.2). All primers were used together and volume of the reaction mixture was completed to 50 µl using DDW. Forty 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.* [15]: Each reaction was performed in a final volume of 25 µl in PCR tubes (ependorff). Each reaction contained mixture consisting 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.

RESULTS AND DISCUSSION

From the results presented in Table (3) examination of 192 quarters milk samples collected from 48 clinically mastitic buffaloes by bacteriological examination revealed positive results in 141 (32.9 %) of them. On the other hand examination of 236 quarters milk samples collected from 59 subclinically mastitic buffaloes by CMT revealed positive results in 138 (32.3%) of them. These results are nearly similar to that reported by Verma [16] and Dhakal and Kapur [17]. On the other hand this finding is higher than that recorded by Hussian *et al*. [18] as they recorded an incidence of subclinical mastitis of 19% between buffaloes.

Table (4) demonstrates the distribution of affected quarters among mastitic buffaloes. It is clear that 18 buffaloes out of 48 clinically mastitic buffaloes (37.5%) showed 3 affected quarters while 16 buffaloes (33.3%) had all affected quarters. Buffaloes with two affected quarters constituted (18.8%) and those with one affected quarters

Table 3: Incidence of mastitis among the examined milk samples of buffaloes.

Healthy state of the udder	Examined Buffaloes	Examined quarters	Negative quarter milk samples		Positive quarter milk samples	
			No.	%	No.	%
Clinical mastitis	48	192	51	11.9	141	32.9
Subclinical mastitis	59	236	98	22.9	138	32.3
Total	107	428	149	34.8	279	65.2

Table 4: The distribution of infected quarters in the clinically and subclinically mastitic buffaloes.

Number of affected quarters	Clinically mastitic buffaloes		Subclinically mastitic buffaloes	
	No.	%	No.	%
One quarter	5	10.4	13	22.0
Two quarters	9	18.8	22	37.3
Three quarters	18	37.5	15	25.4
Four quarters	16	33.3	9	15.3
Total	48	100	59	100

Table 5: Prevalence of Staphylococcus species isolated from clinically and subclinically mastitic milk samples in buffaloes.

Source of milk samples	No. of examined milk samples	Staphylococcus species									
		S. aureus		*S. epidermidis*		*S. intermedius*		*S. hyicus*		Total number of isolates	
		No.	%	No.	%	No.	%	No.	%		%
Clinical mastitis	141	36	25.5	9	6.4	5	3.5	2	1.4	52	36.9
Subclinical mastitis	138	15	10.9	11	7.9	5	3.6	3	2.2	34	24.6
Total	279	51	18.3	20	7.2	10	3.6	5	1.8	86	30.8

Table 6: Incidence of virulent factors in *S. aureus* isolates from buffaloes.

Source of isolates	Clinical mastitic buffaloes				Sub Clinical mastitic buffaloes			
	36				15			
	positive		negative		positive		negative	
№ of samples	№	%	№	%	№	%	№	%
Lipase activity	23	63.9	13	36.1	11	73.3	4	26.7
Fibrinolysine activity	25	69.4	11	30.6	10	66.7	5	33.3
DNase	31	86.1	5	13.9	9	60	6	40
SPA	29	80.6	7	19.4	12	83.3	3	16.7

represent (10.4%). On the other hand, in subclinically mastitic buffaloes the incidence of infection in two quarters was comparatively high (37.3%) followed by three quarters infection (25.4%) then one and four quarters infection (22.0% and 15.3% respectively). The present results are in agreement with Bansal *et al.* [19] and Hasan [20]. The variation in the quarter involvement may be due to the differences in the defense reaction among quarters of the same animal [21].

Table (5) illustrates that the incidence of the *Staphylococcus* species among the examined quarters was 30.8 %. It is clear that, the majority of isolates recovered from clinical cases were *S. aureus* (25.5%) followed by *S. epidermidis* (6.4%), *S. intermedius* (3.5%) and *S. hyicus* (1.4%). Meanwhile the isolates recovered from subclinical cases showed an incidence of *S. aureus* 10.9%, S. *epidermidis* 7.9%, *S. intermedius* 3.6 % and the lowest incidence was *S. hyicus* as 2.2%. similar incidence of *S. aureus* was reported by Ulusoy *et al*. [22], Kapur *et al*. [23] and Mahbub *et al*. [24]. A lower incidence (22.5%) of *S. aureus* in clinical mastitis was reported by Silva [25]. In subclinical mastitis, a similar incidence of *S. aureus* was reported by Anderson *et al*. [26], Motie *et al*. [27],Malinowski *et al*. [28] and Aki [29].

In the present study, as shown in Table (6), 41 out of 51 *S. aureus* isolates (84.6%) showed positive SpA by agglutination test. This observation is in agreement with that mentioned by Rosenberg *et al*. [30] and Farage [31]. Moreover, the association of virulence genes and clinical mastitis proved the role of spa gene as risk factor [32]. Also the polymorphism of spa gene is confirmed to be scientifically associated with inflammatory response and growth rate [33, 34].

Table 7: Antibacterial sensitivity test of *S. aureus* isolates from milk samples of buffaloes with clinical and subclinical mastitis

		Sensitive		Intermediate		Resistant	
Antimicrobial agent	µg/disc	No.	%	No.	%	No.	%
Ampicillin	10	30	58.8	13	25.5	8	15.7
Amoxycillin	25	27	52.9	9	17.6	15	29.4
Amoxycillin +Clavulinic acid	20+10	43	84.3	3	5.9	5	9.8
Penicillin-G	10 unit	7	13.8	4	7.8	40	78.4
Ciprofloxacin	10	38	74.5	4	7.8	9	17.6
Enrofloxacin	15	44	86.3	2	3.9	5	9.8
Gentamycin	10	39	76.5	8	15.7	4	7.8
Clindamycin	20	34	66.7	4	7.8	13	25.5
Neomycin	30	40	78.4	5	9.8	6	11.8
Streptomycin	10	11	21.6	5	9.8	35	68.6
Rifampicin	30	42	82.4	3	5.9	6	11.8
Cloxacillin	1	31	60.8	7	13.8	13	25.5
Methicilline	5	50	98.0	-	-	1	1.9
Oxytetracycline	30	7	13.8	11	21.6	33	64.7
Sulphamethoxazole-trimethoprim	10+20	12	23.5	9	17.6	30	58.9

Table 8: Prevalence of toxigenic *S. aureus* isolates using RPLA test:

				Types of toxins							
		Toxigenic isolates		A		B		C		D	
Source of *S. aureus*	No. of *S. aureus* isolates	No.	%	No.	%	No.	%	No.	%	No.	%
Buffaloes	51	30	58.8	8	15.7	6	11.8	16	31.4	-	-

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).

Concerning lipase activity on egg yolk agar medium, only 17 *S. aureus* strains were negative to this test with an incidence of 33.3%. On the other hand 34 strains out of 51 *S. aureus* isolates had lipase activity with percentage of 66.7. These results goes parallel to that recorded by Leung *et al*. [35] and Annemuller and Zschock [36].

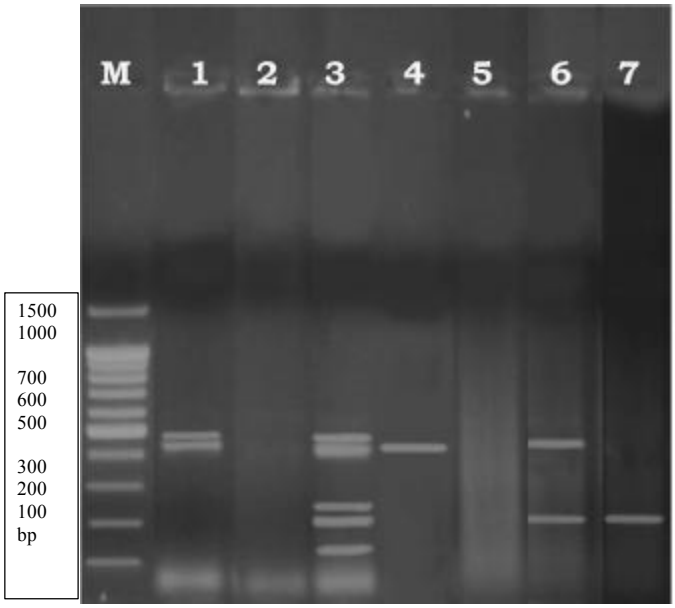
Analysis the data of fibrinolysis test showed that 35 (68.6%) of the examined *S. aureus* isolates had fibrinolysin activity while only 16 (31.4%) of these isolates failed to lyse fibrin. These results are in agreement with Wann *et al*. [37] and Elgabry [38].

In the present study 78.4% of *S. aureus* isolates had DNase activity. The highest DNase activity was recorded among *S. aureus* isolated from clinically mastitic buffaloes (86.1%) then subclinically mastitic buffaloes (60%) and these results nearly agree with the data obtained by Ata [39].

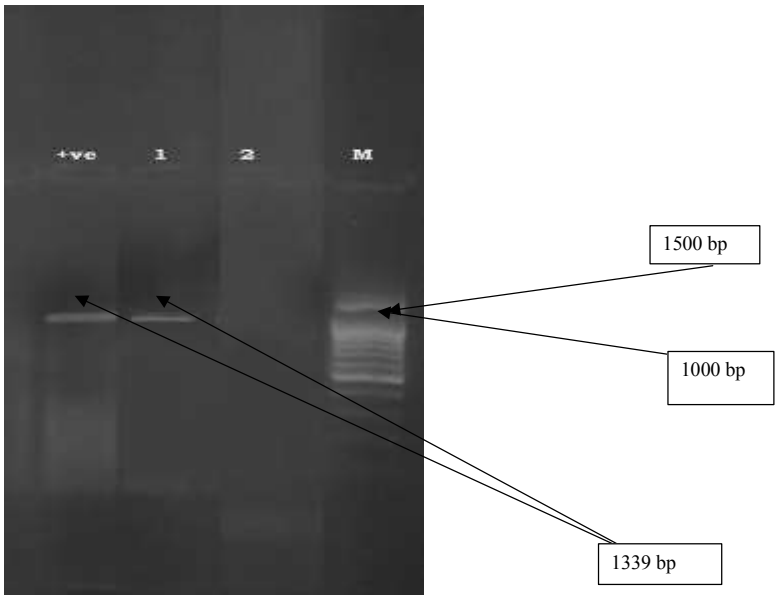
In the present investigation high sensitivity was recorded to methicillin (98%) among the examined *S. aureus* isolates in buffaloes followed by enrofloxacin (86.3%), amoxicillin + clavulinic acid (84.3%), rifampicin (82.4%), neomycin (78.4%), gentamycin (76.5%), ciprofloxacin (74.5%) and clindamycin (66.7%). Mean while moderate sensitivity was recorded to ampicillin (58.8%) and amoxycillin (52.9%) (Table 7). These results are nearly similar to those mentioned by Watts *et al*. [40], Ahlam and Khodry [41], Badia [42] and Mohamed [43].

Prevalence of toxigenic *S. aureus* isolates using RPLA test is illustrated in Table (8). Only 30 out of 51 *S. aureus* isolates from buffaloes were recorded as toxigenic strains with perecentage of 58.8% and enterotoxins were distributed as follow: enterotoxin C was the highest among the other types of enterotoxins 16 (31.4%) followed by enterotoxin A, enterotoxin B and enterotoxin D whose numbers of isolates were 8 (15.7%), 6 (11.8%) and zero respectively.

These results are in agreement with that mentioned by Jorgensen *et al*. [44] who found SEC was the most common enterotoxin detected in *S. aureus* isolates from bovine mastitis. In addition to that mentioned by Abd-El-Rashid *et al*. [45] who found that obtained results showed high incidence of type C (7 - 50%) followed by enterotoxin A, enterotoxin B and enterotoxin D whose numbers of isolates were 4 (28.57%), 3 (21.4%) and zero (0%) 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 [46]. From our point of view the distribution of infection in the udder tissues may be related to the role played by toxins, this observation is in accordance to that mentioned by Hillerton and Walton [47].



M : The DNA molecular weight marker (100bp ladder). (100-200-300-400-500-600-700-800-900-1000-1500 bp)
Lane (1): positive amplification of 451 bp for enterotoxin C and 482 bp for enterotoxin B in mastitic buffaloes
Lane (2 & 5): No amplification in mastitic buffaloes
Lane (3): Positive control
Lane (4): Positive amplification of 451 bp for enterotoxin C in mastitic buffaloes
Lane (6): Positive amplification of 209 bp for enterotoxin E and 451 bp for enterotoxin C in mastitic buffaloes
Lane (7): Positive amplification of 209 bp for enterotoxin E in mastitic buffalo
Photo 1: Agarose gel electrophoresis showing the result of multiplex PCR for detection of enterotoxin genes from *S. aureus* DNA molecular weight Marker was supplied by Amers Co. Cleveland, Ohio, USA.



M : Marker: (100 bp) (100-200-300-400-500-600-700-800-900-1000-1500 bp) +ve: positive control
Lane (1): Methicillin resistant mastitic buffaloes
Lane (2): Methicillin sensitive mastitic buffaloes
Photo 2: Agarose gel electrophoresis showing amplification of the 1339 bp fragment of mecAgene The DNA molecular weight marker (100bp ladder

Detection of toxigenic strains in *S. aureus* isolates was done using multiplex polymerase chain reaction technique (multiplex PCR). Total number of 6 isolates previously tested by using RPLA and the results were confirmed using multiplex PCR. Results obtained showed 100% agreement between the two tests RPLA and multiplex PCR. Our findings also agree with that of Zouharova and Rysanek [8] 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 [48, 49].

Results presented in photo (2) revealed positive amplification of the 1339 bp fragment of mecA gene from the extracted DNA of one *S. aureus* isolate out of 2 examined samples. This 5 isolate was methicillin-resistant which indicated that PCR technique could detect the mecA gene in the mecA resistant. This finding is supported by Riffon *et al*. [17] and Keith *et al*. [50].

CONCLUSIONS

It is concluded from present study that:

- Incidence of subclinical mastitis in buffaloes is relatively high from our point of view were attributed to the immune status system of the animals.
- *Staphylococcus aureus* is a major pathogen that causing mastitis among Staphylococcus species.
- Results obtained showed that 100% agreement between the 12 selected strains examined by RPLA and multiplex PCR test.
- There is no clear relationship between multi drug resistant, â-lactamase production and mecA gene.

REFERENCES

1. Bakr, M.A., 1986. Comparative studies on different methods used for detection of subclinical mastitis in dairy animals.Thesis Ph.D. Fac. Vet. Med. Cairo Univ.
2. Chiang, Y.C., W.W. Liao, C.M. Fan, W.Y. Pai, C.S. Chiou and H.Y. Tsen, 2008. PCR detection of staphylococcal enterotoxins (SEs), N, O, P, Q, R and U and survey of SE types in *S. aureus* isolates from food poisoning cases in Taiwan.Int. J. Food Microbiol., 121(1): 66-73.
3. EL-Rasheedy, A.A., A. Amin, A.S. Mostafa and W.S. Tawfik, 1988. Evaluation of mass screening tests in diagnosis of subclinical cases compared with the conventional bacteriological method. Vet. Med. J. Giza., 36: 91-100.
4. Ebrahim, S.A.H., 1994. Incidence and public health importance of sub clinical mastitis in dairy cattle in Suez-canal area.M.V.Sc.Thesis,milk-hygiene-Dept. Fac.Vet. Med., Suez-Canal University.
5. Oliver, S.P., B.M. Jayarao and R.A. Almeida, 2005. Food borne pathogens in milk and the dairy farm environment: Food Safety and Public Health Implications. Food borne Path. Dis., 2: 115-129.
6. Salmon, S.A., 2002. Use of antimicrobial susceptibility data to assist in determining the best therapy for clinical mastitis. Proceedings of the NMC 41st Annu Mtg., Orlando, FL. National Mastitis Council Madison, W.I., pp: 36.
7. Taverna, F., A. Negri, R. Piccinini, A. Zecconi, S. Nonnis, S. Ronchi and G. Tedeschi, 2007. Characterization of cell wall associated proteins of *S. aureus* isolated from bovine mastitis. Vet. Microbiol., 119(2-4): 240-247.
8. Zouharova, M. and D. Rysanek, 2008. Multiplex PCR and RPLA identification of *S. aureus* enterotoxigenic strains from Bulk Tank milk. Zoonoses Public Health., 55(6): 313-9.
9. Shittu, A.O. and J. Lin, 2006. Antimicrobial susceptibility patterns and characterization of clinical isolates of *S. aureus* in KwaZulu-Natal Province, South Africa. BMC Infect. Dis., 6(1): 125.
10. Quinn, P.J., B.K Markey, M.E. Carter, W.J.C. Donnelly, F. C. Leonard and D. Maguire, 2002. Veterinary Microbiology and Microbial Diseases. 1st published Blackwell Science 1td.
11. Finegold, S.M. and W.J. Martin, 1982. Bailey and scott's Diagnostic Microbiology. 6th Ed., The C.V. Mosby Company, St. Louis, Torento, London.
12. Shingaki, M., H. Igarashi, H. Fujikawa, H. Ushioda, T. Terayama and S. Sakai, 1981. Study on reverse passive latex agglutination test for detection of staphylococcal enterotoxins A, B and C. Annu. Rep. Tokyo, Metrop. Res. Lab. Public Health., 32(1): 128-131.
13. Sritharan, V. and R.H. Barker, 1991. A simple method for diagnosis of mycobacterium tuberculosis infections in clinical samples using PCR. Mol. Cell Probes, 5: 385-395.

14. Becker, K., R. Roth and G. Peter, 1998. Rapid and specific detection of toxigenic *S. aureus* (use of two multiplex PCR enzyme immunoassay for amplification and hybridization of *Staphylococcal enterotoxin* genes, exfoliative toxin genes and toxic shock syndrome1 gene. J. Clin. Microbiol., 36: 2548-2553.
15. Riffon, R., K. Sayasisth, H. Khalil, P. Dubereuil, M. Droletr and J. Lagace, 2001. Development of a rapid and sensitive test for identification of major pathogens in bovine mastitis by PCR. J. Clin. Microbiol., 39: 2584-2586.
16. Verma, R., 1988. Studies on clinical and subclinical bovine mastitis. Indian J. Comp. Microbiol. Immunol. Infect. Dis., 9(1): 28-33.
17. Dhakal, I.P. and M.P. Kapur, 1992. Prevalence of subclinical and latent mastitis in buffaloes as detected by somatic cell count(SCC), California Mastitis Test (CMT) and cultural examination using fractioned milk Ind. J. Dairy Sci., 44: 585-586.
18. Hussian M.K. Naeem and N. Jabal, 1984. Subclinical mastitis in cows and buffaloes identification and drug susceptibility of causative organisms. Pakist. Vet. J., 4(3): 161-164.
19. Bansal, B.K., K.B. Singh, S.K. Jand, D.C. Nauriyal and S.S. Randhawa, 1990. Incidence of clinical mastitis in dairy animals. Indian J. Dairy Sci., 43(3): 355-358.
20. Hasan, N.M.K., 1992. Studies on mastitis causative organisms in dairy animals.M. V.SC. Thesis, Hygiene and control of milk and its products, Fac. Vet. Med., Cairo University.
21. Dopfer, D., H.W. Barkema, T.J.G.M. Lam, Y.H. Schukken and W. Gaastra, 1999. Recurrent clinical mastitis caused by Escherichia coli in dairy cows.. Dairy Sci., 82(1): 80-85.
22. Ulusoy, E., M. Izgur, O. Akay, K.S. Diker, N. Aydin and M. Arda, 1985. Identification and antibiotic sensitivity of microorganisms isolated from mastitic dairy cows. Vet. Fakult. Dergisis Ankava Univ., 32(2): 358-372.
23. Kapur, M.P., A. Sharma and R.M. Bhardwaj, 1988. Bacteriology of clinical mastitis in buffaloes.Indian Society of Buffaloe development and Indian Council of Agricul. Res., India, 12: 12-16(1988). Cited after Dairy Sci. Abst., 53(1): 55(1991).
24. Mahbub, E., A.T. Elahi, M.M. Rahman and M.A.M. Prodhan, 1996. Isolation and identification of bacterial from different quarters of mastitis affected dairy cows in Bangladesh Bang. Vet. J., 30(1-2): 63-65.
25. Silva, J.C., 1996. On the microbiology of bovine mastitis in Pourtugal. Veterinaria Techica., 6(5) : 22-26.
26. Anderson, K.L., A.R. Smith, B.K. Gustafsson, S.L. Spahr and H.L. Whitmore, 1982. Diagnosis and treatment of acute mastitis in a large herd. JAVMA, 181(7): 690-693.
27. Motie, A., S. Ramudit and R. Mohabir, 1985. Subclinical mastitis in dairy cattle in Guyana. Trop. Anim. HIth. Prod., 17(4): 245-246.
28. Malinowski, E., A. Klossowska and M. Szalbierz, 1992. Pathogenicity to the mammary gland of microorganisms isolated from clinical and subclinical bovine mastitis. Med. Vet., 48(10): 467-469.
29. Aki, S., 2000. Bacterial agents causing contagious and environmental bovine mastitis in Trakya district and their susceptibility to antibiotics. Veteriner Fakiiltesi Dergisi, 26(2): 353-365.
30. Rosenberg, J.B., B. Love and D.L. Patterson, 2000. Bacterial cure and somatic cell count response of dairy cows with a positive CMT at calving to therapy with cephapirin Sodium. Veterinary therapeutics, 3(4): 381-386.
31. Farage S. Randa, 2008. Properties of enterotoxigenic *S. aureus* isolated from mastitic cattle and buffaloes. Thesis M.V. Sc. (Microbiology), Fac. of Vet. Med. Cairo University.
32. Zecconi, A., E. Binda, V. Borromeo and R. Piccinini, 2005. Relationship between some S. aureus pathogenic factors and growth rates and somatic cell counts. J. Dairy Res., 72(2): 203-208.
33. Zecconi, A., L. Cesaris, E. Liandris, V. Dapra and R. Piccinini, 2006. Role of several *S. aureus* virulence factors on the inflammatory response in bovine mammary gland. Microbiol. Path., 40(4): 177-183.
34. Kalorey, D.R., Y. Shanmugam, N.V. Kurkure, K.K. Chousalkar and S.B. Barbuddhe, 2007. PCR based detection of genes encoding virulence determinants in *S. aureus* from bovine clinical mastitis cases. J. Vet. Sci., 8(2): 151-154.
35. Leung, D.Y.M., H.C. Meissnen, D.R. Fulton, D.L. Murray, B.L. Kotzin and P.M. Schlievert, 1993. Toxic shock syndrome toxin-secreting *S. aureus* in Kawasaki syndrome. Lancet, 342: 1385-1388.
36. Annemulller, T. and M. Zschock, 1999. Genotyping of *S. aureus* isolated from bovine mastitis. Vet. Microbiol., 69: 217-224.
37. Wann, E.R., B. Dassay, J.M. Fourier and T.J. Foster, 1999. Genetic analysis of the cap 5 locus of *S. aureus*. FEMS Microbiol. lett., 170: 97-103.
38. Elgabry, A.E., 2006. Characterization of *S. aureus* from different sources with special reference to protein A.Thesis M. V. Sc. (Microbiology), Fac. Vet. Med. Cairo University.

39. Ata S.S. Nagwa, 1990. Characterization of *S. aureus* isolated from different sources with particular reference to protein A and its significance. Ph.D., Thesis, Microbiology, Fac. Vet. Med., Cairo University.
40. Watts, J.L., S.A. Salmon, R.J. Yancy, S.C. Nickerseon, L.J. Weaver, C. Halmberg, J.W. Pankey and L.K. Fox, 1995. Antimicrobial susceptibility of micro organisms isolated from mammary glands of dairy heifers. J. Dairy Sc., 78(7): 1637-1648.
41. Ahlam, E. and R.M. Khodry, 1996. Sensitivity of some bacteria isolated from mastitic milk of Egyptian buffalo-cows to some recent antimicrobial.3rd Vet. Med. Cong. Zagazig 8-10 October 1996.
42. Badia, S.E., 2004. Studies on microbial causes of mastitis in buffaloes. Thesis Ph.D. Fac. Vet. Med. Cairo University.
43. Mohamed B. Heba, 2008. Microbiological studies on *S. aureus* with regard to antibiotic resistance. M.V. Sc. Thesis, Microbiology, Fac. Vet. Med., Cairo University.
44. Jorgensen, H., T. Mork, H.R. Hogasen and L.M. Rorvik, 2005. Enterotoxigenic *S. aureus* strains in bulk milk in Norway. J. Appl. Microbiol., 99(1): 158-166.
45. Abd EL–Rashid, F.A., E.R. Zaki and E.M. Riad, 2003. Occurrence and detection of *Staphylococcus aureus* toxins in bovine mastitic milk.7th Sci. Cong. Egyptian Society for cattle Disease, 7-9 Dec. Assiut, Egypt.
46. Dacuncha Mde, L., R.A. Calsolari and J.P. Junior, 2007. Detection of enterotoxins and toxic syndrome toxin-1 genes in Staphylococcus by PCR and RPLA assay. Microbiol. Immun., 51(4): 381-390.
47. Hillerton, J.E. and A.W. Walton, 1991. Identification of subclinical mastitis with a hand –held electrical conductivity meter. Vet. Rec., 128: 513-515.
48. Lawrynowicz-Paciorek, M., M. Kochman, K. Piekarska, A. Grochowska and B. Windyga, 2007. The distribution of enterotoxin and enterotoxin-like genes in *S. aureus* strains. Int. J. Food Microbiol., 117(3): 319-323.
49. Morandi, S., M. Brasca, R. Lodi, P. Cremonesi and B. Castiglioni, 2007 Detection of classical enterotoxins and identification of enterotoxin genes in *S. aureus* from milk and dairy products. Vet. Microbiol., 124(1-2): 66-72.
50. Keith, E., W. Kerry, J. Nicola and D. Peter, 2006. Methicilline – resistant Staphylococci in animals. Vet. Rec., 144: 60-64.