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Phenotypic and Genotypic Detection of Both *mecA*- and *blaZ*-Genes Mediated β-lactam Resistance in Staphylococcus Strains Isolated from Bovine Mastitis

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Abstract: In this study, 25 (52%) and 118 (67%) Staphylococcus strains were isolated from milk samples collected from cows suffering from clinical and subclinical mastitis, respectively. Identification of these isolates was performed using combined phenotypic and genotypic methods. A novel multiplex PCR assay was developed and used for both genotypic identification of the isolated Staphylococci and for detection of β-lactam-mediated resistance genes. Out of 25 Staphylococcal isolates recovered from clinical mastitic cases, one (4%) was *S.aureus*, 10 (40%) were coagulase positive staphylococci (CPS) other than *S.aureus* and 14 (56%) were coagulase negative staphylococci (CNS). Among the 118 isolates recovered from cases of subclinical mastitis, 32 (27%) were *S. aureus*, 30 (25%) were other CPS and 56 (48%) were CNS. Phenotypic prediction of *mecA* gene presence (historically referred to methicillin resistance, MR) was conducted by both cefoxitin and oxacillin disk diffusion tests, while prediction of *blaZ* gene presence was monitored by penicillin and amoxicillin/clavulinic acid (AMC) disk diffusion tests. Out of 25 Staphylococcal isolates recovered from clinical mastitic cases, 15, 19, 25 and 20 isolates were resistant to cefoxitin, oxacillin, penicillin and AMC, respectively. In subclinical mastitis, out of 118 isolates recovered, 43, 36, 43 and 22 isolates were resistant to cefoxitin, oxacillin, penicillin and AMC, respectively. According to PCR results, among the 25 Staphylococci isolated from clinical mastitis, 20 (80%) were methicillin resistant (MR) carrying the *mecA* gene, including 8 MRCPS and 12 MRCNS. Also, 17(68%) were β-lactamase producers harboring the *blaZ* gene, including 1 *S.aureus*, 8 CPS and 8 CNS. On the other hand, among the 118 Staphylococci isolated from cases of subclinical mastitis, 27 (22.9%) were MR including 5 MR *S. aureus* (MRSA), 7 MRCPS and 15 MRCNS. Also, 27 (22.9%) were β-lactamase producers including 12 *S.aureus*, 3 CPS and 12 CNS. The results showed higher incidence for MR and β-lactamase production in Staphylococci isolated from clinical mastitis versus those of subclinical mastitis (80 versus 22.9% and 68 versus 22.9%, respectively). Incidence of MRSA was found to be 15.6% of *S.aureus* caused subclinical mastitis. Close correspondence between phenotypic and genotypic tests was observed for the majority of isolates. Resistance to antimicrobials other than β-lactams was determined. In general, multi-drug resistance was more common among *blaZ* &/or *mecA* positive isolates ($P < 0.05$) in comparison to *blaZ* & *mecA* negative isolates. In conclusion, high incidence of staphylococci causing bovine mastitis was observed in this study. Most importantly, it is ascertained that CPS other than *S. aureus* and CNS are also deeply implicated in both clinical and subclinical bovine mastitis. Considering β-lactams resistance, it was found to be widely spreading among staphylococcal isolates. Additionally, association between blaZ &/or mecA mediated β-lactam resistance genes and resistance to other antibiotics was also declared.

Key words: *S. aureus* · CNS · CPS · Mastitis · Methicillin resistance · β-lactamase · PCR · Disk diffusion test

INTRODUCTION

Bovine mastitis continues to cause a huge economic burden to the dairy industry [1]. It is the most common frequent reason for antimicrobial drug use in dairy herds whereas antibiotic therapy is a major component and a primary tool for mastitis control in lactating and dry cows [2, 3]. As antimicrobial resistance is associated with the improper use of antimicrobial agents, it is important to monitor antimicrobial susceptibility of mastitis pathogens

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[4]. In recent years, antimicrobial resistance in bacteria of animal origin and its impact on human health have drawn much attention worldwide [5-9]. Among various antimicrobial drugs, varieties of β-lactams are currently licensed for use in veterinary medicine and thus provide opportunity for selection pressure in development of β-lactam resistance [4]. β-lactams are among the critically important antibiotics in the treatment of mastitis. They are a broad class of antibiotics, consisting of all antibiotic agents that contain a β-lactam nucleus in its molecular structure. β-lactam antibiotics work by inhibiting cell wall synthesis by the bacterial organism and are the most widely used group of antibiotics in the prevention and treatment of mastitis in dairy cows [1, 10]. Bacterial resistance to β-lactams is attributable to at least three mechanisms: inaccessibility of the drugs to their target (penicillin-binding proteins [PBPs]), the alterations of the drug target and/or inactivation of the drugs by β-lactamases [11-13].

Staphylococci are common causes of a wide variety of diseases in animals [14]. They are the most prevalent pathogens causing mastitis in ruminants [15]. Staphylococcal species associated with bovine mastitis have been classified as coagulase positive or coagulase-negative [16]. The most important Staphylococcal pathogens causing bovine mastitis include *S.aureus* and coagulase-negative staphylococci (CNS). *S.aureus* is increasingly recognized as etiological agent of bovine mastitis and a part from streptococci and coliform remain the most frequent bacterium isolated from clinical and subclinical forms of udder infections [17]. Coagulase-negative staphylococci (CNS) were isolated frequently from cows and primiparous heifers with clinical and subclinical mastitis worldwide [18-21]. Mechanisms of β-lactam resistance in Staphylococci include production of β-lactamases and/or production of low affinity penicillin-binding protein (PBP), termed PBP2' or PBP2a, designated as methicillin resistance (MR), preclude therapy with any of the currently available β-lactam antibiotics and may predict resistance to other classes of antibiotics beside β-lactams among all Staphylococci [22-24]. Production of β-lactamases was reported to be mainly encoded by the structural *blaZ* gene while production of an altered form of penicillin binding protein 2A (PBP2) is encoded by the *mecA* gene [25-27]. Staphylococci can produce β-lactamases, which belong to the Ambler class A and the Bush group 2a (as penicillinases) and are inhibited by clavulanic acid [11]. As indiscriminate use of antibiotics can lead to development of resistant strains and result in an increase in the cost of mastitis therapy. Therefore, detection of resistance is necessary for the selection of the most effective antimicrobial drug. So, the aim of this study was to find out the prevalence of β-lactam resistance among different Staphylococcal species isolated from bovine mastitic milk using antibiotic disk diffusion test. Also, a novel multiplex PCR assay that can detect both *blaZ* and *mecA* mediated β-lactam resistance genes with simultaneous identification of Staphylococci generally and *S.aureus* specially will be developed and used in comparison to phenotypic methods. Additionally, *In vitro* susceptibility for additional antibiotics will be determined to study the possible relationship between β-lactam resistance genes and resistance to other antimicrobial drugs.

MATERIALS AND METHODS

Collection of Milk Samples: A total number of 223 samples of bovine mammary secretions including 48 and 175 clinical and subclinical (according to California Mastitis Test; CMT) milk samples respectively were collected from mastitic cows in nine different dairy herds from different localities in Egypt.

Isolation and Identification of Staphylococci: Staphylococcal strains were isolated from milk samples in dairy herds by methods developed according to Harmon *et al.* [28]. An aliquot of 10 µL of each sample was spread over blood agar plates containing 5% defibrinated sheep blood and incubated at 37°C for 24 h. Colonies suspected of being staphylococci were subcultured on blood agar plates and tentatively identified according to morphologicl features, pigment production, type of hemolysis produced, gram staining, catalase test, coagulase test (in tubes) and characteristic growth on Mannitol salt agar which used as selective as well as differential medium for isolation and identification of Staphylococci according to the methods of Roberson *et al.* [29] and Sullia and Shantharan [30]. Reference strains used for quality control were as follows: *S.aureus* ATCC 25213 and S.*aureus* ATCC 25923.

Antibiotic Susceptibility Test: The isolated staphylococci were tested for their *In vitro* antimicrobial susceptibility using the disk diffusion technique on Mueller-Hinton agar (Difco, Sparks, MD). The results were recorded after 24 h of incubation at 37°C. The zone of inhibition of each antibiotic disc was recorded and interpreted referring to zone diameter interpretive Standards of CLSI [31]. Penicillin; P (10 units/disk), Amoxicillin/Clavulinic acid; AMC (30 µg/disk), Cefoxitin; Fox (30µg/disk) and

Table 1: Primer sequences, their specific targets and amplicon sizes

Primer name	Primer sequence 5'-3' (reference)	Product size	Specificity
16SrRNA f 16SrRNAr	5' GTA GGT GGC AAG CGTTAT CC 3' 5' CGC ACA TCA GCG TCA G 3' (Monday and Bohach,[33])	228 bp	Staphylococcus genus specific primers
nuc 1 *nuc* 2	5'-GCGATTGATGGT GATACGGTT-3' 5'-AGCCAAGCCTTGACGAACTAAAGC-3 ' (Brakstad *et al.*[34])	279 bp	S. aureus specific primers
blaZ f *blaZ* r	5' AAG AGA TTT GCC TAT GCT TC 3' 5' GCT TGA CCA CTT TTA TCA GC 3' (Vesterholm-Nielsen *et al.* [25])	517 p	β-lactamase producing Staphylococci
mecA f *mecA* r	5' GTG AAG ATA TAC CAA GTG ATT 3' 5' ATG CGC TAT AGA TTG AAA GGA T 3' (Zhang *et al.* [35])	147 bp	Methicilin resistant Staphylococci

Oxacillin; Ox (1 µg/disk) disks are used to test phenotypic expression of *blaZ* and *mecA* genes. The following antibiotics were also tested in this study including Gentamicins, CN (10µg/disk), Neomycin; N (30 µg/disk), Tetracycline; T (30µg/disk), Streptomycin; S (10 µg/disk), Erythromycin; E (15µg/disk), Chloramphenicol; C (30µg/disk), Florphenicol, FFC (15 µg/disk) and Norfloxacin; NOR (30µg/disk).

DNA Extraction: A rapid boiling procedure was used to prepare template DNA from bacterial strains according to Reischl *et al.* [32]. Two to 5 loops of Staphylococci isolates taken from the brain heart infusion agar plate were collected and suspended in 200 µl of lysis buffer comprised of 1% Triton X-100, 0.5% Tween 20, 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA. After boiling for 10 min, the suspension was centrifuged for 2 min. to sediment bacterial debris. The supernatant was aspirated and from which 5 µl was used directly for PCR amplification.

Primers: Primers used for PCR amplification were synthesized in Bio Basic Inc. (Canada). Details of primer sequences, their specific targets and amplicon sizes are summarized in table 1.

Multiplex Polymerase Chain Reaction (PCR): A novel quadriplex PCR assay targeting *16S rRNA* gene (*Staphylococcus* genus specific), *nuc* (*S.aureus* species specific), *blaZ (*a determinant of β-lactamase production) and *mecA* (a determinant of methicillin resistance) was developed and used in our study. It was established using a total volume of 25 µl reaction mixtures contained 5µl of DNA as template, 20 pmol of each primer and 1X of PCR master mix (Dream Taq Green PCR Master Mix, Fermentas Life Science). The amplification cycles were carried out in a PT-100 Thermocycler (MJ Research, USA). Reaction conditions were optimized to be 94°C for 4 min. as initial denaturation, followed by 35 cycles of 94°C for 60 seconds, 55°C for 60 seconds and 72 °C for 60 seconds. A final extension step at 72°C for 10 min. was followed. DNA isolated from *S.aureus* ATCC 25923 & 29213 was used as positive controls while water was used as negative control. Both positive and negative controls were included in each PCR run to exclude both amplification failures due to presence of inhibitors and cross contamination. Amplification products were electrophorezed in 1.5% agarose gel containing 0.5X TBE at 70 volts for 60 min. and visualized under ultraviolet light. To assure that the amplification products were of the expected size, a 100 bp DNA ladder was run simultaneously as a DNA marker. Amplification of both 228 and 279 bp bands indicated the isolate to be *S. aureus* while amplification of 228 bp only indicated the strain to be Staphylococci other than *S. aureus*. Amplification of 517 bp fragment confirmed the strain to be β-lactamase producer while amplification of the 147 bp fragment confirmed it to be methicilin resistant. To ensure reproducibility of the results, nearly 30% of the isolates were tested twice.

Statistical Analysis: Differences in frequencies of *in vitro* resistance to antimicrobials other than β-lactams between *blaz* &/or *mecA* positive and *blaZ* & *mecA* negative isolates were determined by Pearson's chi-square test to study the possible relationship between β-lactam resistance genes and resistance to other antimicrobials [36]. Value of $P < 0.05$ was considered significant.

RESULTS

In this study, 25/48 (52%) Staphylococcal strains were isolated from milk samples collected from cows suffering from clinical mastitis. Also, 118/175 (67%) isolates were isolated from milk samples collected from cows with subclinical mastitis. Identification of these

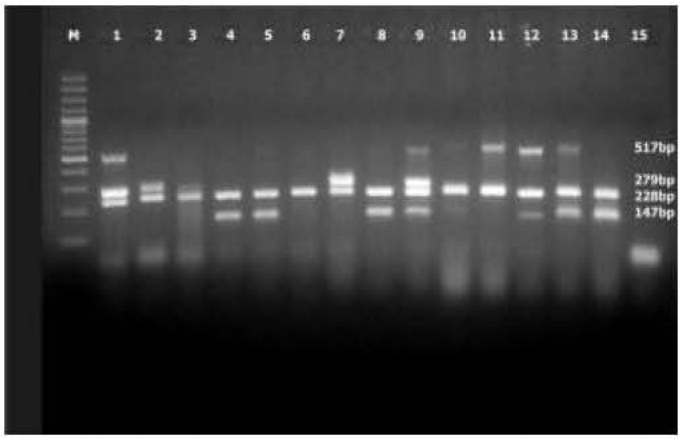

Fig. 1: Novel multiplex PCR assay detecting the *16S rRNA* (228bp), *nuc* (279bp), *mecA* (147bp) and *blaZ* (517bp) genes simultanously in *Staphylococcal strains*.
M: molecular size marker (100bp DNA ladder), lane 1: *S. aureus* ATCC29213, lane 2: *S. aureus* ATCC 25923, lanes 3-14: Representative Staphylococcal isolates, lane 15: negative control.

Table 2: Combined results of both phenotypic and genotypic identification of Staphylococci isolated from mastitic milk

Type of mastitis (no. of milk samples)	no of isolates (%)	Phenotypic identification	Genotypic identification			
			16s r RNA PCR result	*nuc* PCR result	Final result No (%)	
Clinical mastitis (48)	25 (52%)	CPS (11)	11 +ve	1 +ve	*S.aureus*	1 (4%)
				10 -ve	other CPS	10 (40%)
		CNS (14)	14 +ve	14 -ve	CNS	14 (56%)
Subclinical mastitis (175)	118 (67%)	CPS (62)	62 +ve	32 +ve	*S.aureus*	32 (27%)
				30 -ve	other CPS	30 (25%)
		CNS (56)	56 +ve	56 -ve	CNS	56 (48%)

isolates was performed using combined phenotypic and genotypic methods. Phenotypic identification classified the Staphylococcal isolates into coagulase positive (CPS) and coagulase negative Staphylococci (CNS). Moreover, the performed multiplex PCR assay confirmed all the isolates to be Staphylococci by successful amplification of the 228 bp PCR product of the Staphylococcal specific 16S rRNA gene. Using the same multiplex PCR, some isolates were confirmed to be *S.aureus* by successful amplification of 279 bp PCR product of the *S.aureus* specific thermonuclease gene (Fig 1). Table 2 showed that out of 25 isolates recovered from clinical mastitic milk, only one (4%) was *S.aureus*, 10 (40%) were identified as CPS other than *S.aureus* and 14 (56%) were CNS. Also it showed that out of the 118 isolates recovered from cases of subclinical mastitis 32 (27%) were *S.aureus*, 30 (25%) were other CPS and 56 (48%) were CNS.

Prevalence of both phenotypic and genotypic β-lactam resistance among the isolated Staphylococci was declared using disk diffusion tests (DD) and PCR assay. Table 3 shows the results of both phenotypic and genotypic methods used for detection of *blaZ* gene and *mecA* mediated β-lactam resistance. Both cefoxitin and oxicillin disk diffusion tests were used for detection of *mecA* mediated β-lactam resistance (methicillin resistance) while penicillin and AMC disk diffusion tests were used to monitor *blaZ* mediated resistance (indirect test for detection of β-lactamase production).

Out of 25 Staphylococcal isolates recovered from clinical mastitic cases, 15, 19, 25 and 20 isolates were resistant to cefoxitin, oxacillin, penicillin and AMC, respectively. In subclinical mastitis, out of 118 isolates recovered, 43, 36, 43 and 22 isolates were resistant to cefoxitin, oxacillin, penicillin and AMC, respectively.

Using the same multiplex PCR assay, detection of *blaZ* and/or *mecA* genes were performed through successful amplification of 517 bp and /or 147 bp specific products, respectively (Figure 1). According to PCR results, Out of 25 Staphylococci isolated from clinical mastitis, 20 (80%) were methicillin resistant (MR) carrying the *mecA* gene, including 8 MRCPS and 12 MRCNS.

Table 3: Comparison between phenotypic and genotypic detection of mecA and blaZ mediated β-lactam resistance among the isolated Staphylococci

		Disk Diffusion tests										PCR results					
		Fox			Ox			P		AMC						(*mecA* & *blaZ*)	
Type of mastitis	Isolated M.O.(No)	R	M	S	R	M	S	R	S	R	S	Total *mecA*+	total *blaZ*+	*mecA*+ only	*blaZ*+ only	+	-
Clinical	*S. aureus* (1)	-	-	1	-	-	1	1	-		1		1		1		
(25)		-	-	(100)	-	-	(100)	(100)	-	-	(100)	-	(100)	-	(100)	-	-
	Other CPS (10)	6	1	3	5	4	1	10		8	2	8	8	2	2	6	-
		(60)	(10)	(30)	(50)	(40)	(10)	(100)	-	(80)	(20)	(80)	(80)	(20)	(20)	(60)	-
	CNS (14)	9	2	3	14	-	-	14	-	12	2	12	8	4	-	8	2
		(64.3)	(14.3)	(21.4)	(100)	-	-	(100)	-	(85.7)	(14.3)	(85.7)	(57.1)	(28.6)	-	(57.1)	(14.3)
Total clinical	25	15	3	7	19	4	2	25	-	20	5	20	17	6	3	14`	2
(%)		(60)	(12)	(28)	(76)	(16)	(8)	(100)	-	(80)	(20)	(80)	(68)	(24)	(12)	(56)	(8)
Subclinical	*S. aureus* (32)	13	2	17	8	4	20	13	19	5	27	5	12	2	9	3	18
(118)		(40.6)	(6.3)	(53.1)	(25)	(12.5)	(62.5)	(40.6)	(59.4)	(15.6)	(84.4)	(15.6)	(37.5)	(6.3)	(28.1)	(9.4)	(56.2)
	Other CPS (30)	8	-	22	8	1	21	9	21	5	25	7	3	5	1	2	22
		(26.7)	-	(73.3)	(26.7)	(3.3)	(70)	(30)	(70)	(16.7)	(83.3)	(23.3)	(10)	(16.7)	(3.3)	(6.6)	(73.4)
	CNS (56)	22	2	32	20	-	36	21	35	12	44	15	12	9	6	6	35
		(39.3)	(3.6)	(57.1)	(35.7)	-	(64.2)	(37.5)	(62.5)	(21.4)	(78.6)	(26.8)	(6.7)	(16.1)	(10.7)	(10.7)	(62.5)
Total subclinical	118	43	4	71	36	5	76	43	75	22	96	27	27	16	16	11	75
(%)		(36.4)	(3.4)	(60.2)	(30.5)	(4.2)	(64.4)	(36.4)	(63.6)	(18.6)	(81.4)	(22.9)	(22.9)	(13.5)	(13.6)	(9.3)	(63.6)

Table 4: Differences in resistance to antibiotics other than β-lactams between β-lactam resistance (mecA &/or blaZ mediated) and β-lactam sensitive staphylococci isolated from bovine mastitic milk

			No. of resistant isolates using disk diffusion tests							
species (no)	resistance genotype	no. of isolates	S	T	CN	N	E	C	FFC	NOR
S.aureus (33)	*mecA* +ve only	2	2*	2*	-	2*	-	-	-	-
	blaZ +ve only	10	7*	10*	3*	6*	4*	-	-	-
	mecA & *blaZ* +ve	3	2*	3*	1*	1*	2*	-	-	-
	mecA& *blaZ* -ve	18	2	4	-	-	1	1	-	-
other CPS (40)	*mecA* +ve only	7	6*	7*	2	4*	2	2	1*	1*
	blaZ +ve only	3	3*	3*	1	2*	2	1	-	-
	mecA& *blaZ* +ve	8	7*	7*	2	3*	5*	5*	3*	1*
	mecA& *blaZ* -ve	22	5	11	4	-	6	2	-	-
CNS (70)	*mecA* +ve only	13	13*	13*	6*	11*	9*	6*	6*	5*
	blaZ +ve only	6	4*	4*	1	4*	-	-	-	-
	mecA& *blaZ* +ve	14	7*	12*	3*	6*	6*	7*	7*	4*
	mecA& *blaZ* -ve	37	5	10	2	3	4	2	2	-
Total No.		143	63	86	25	42	37	26	19	11
(%)			(44.1)	(57.3)	(17.5)	(29.4)	(25.9)	(18.2)	(13.3)	(7.7)

Also, 17(68%) were β-lactamase producing (*blaZ* gene positive PCR) including 1 *S.aureus*, 8 CPS and 8 CNS. On the other hand, out of 118 Staphylococci isolated from cases of subclinical mastitis, 27 (22.9%) were MR including 5 MRSA, 7 MRCPS and 15 MRCNS. Also, 27 (22.9%) were β-lactamase producing (*blaZ* positive PCR) including 12 *S.aureus*, 3 CPS and 12 CNS. From the same Table it was noticed that 14(56%) and 11(9.3%) of the isolates carried both *mecA* and *blaZ* genes in clinical and subclinical milk samples, respectively.

In vitro resistance of the isolated Staphylococci to antimicrobial drugs other than β-lactams was detected using disk diffusion test. Difference in resistance between β-lactam resistant and β-lactam sensitive isolates was shown in Table (4). In general, the results revealed that multidrug resistance was more common among *blaZ* &/or *mecA* positive isolates. On the contrary, resistance was less frequent among *blaZ* & *mecA* negative isolates. Staphylococcal isolates containing *blaZ* &/or *mecA* genes showed significant *in vitro* resistance to Tetracycline,

Streptomycin and Neomycin ($p< 0.05$) and for less extent to Erythromycin and Gentamycin and Chloramphenicol. It was found that multidrug resistance was very aggressive in *mecA* positive CNS. Five out of 13 *mecA* positive CNS showed multidrug resistance to all the tested antibiotic disks. It was also concluded from the same table that NOR followed by FFC were the most effective antibiotics *In vitro* where only 11/143 isolates (7.7%) were resistant to NOR and 19/143 (13.3%) were resistant to FFC.

DISCUSSION

Worldwide, mastitis is the most common infectious disease affecting dairy cows and the most economically important disease of the dairy industry [37]. Successful managing, preventing and treatment of bovine mastitis are great inevitable task for dairy producers. Because of the importance of Staphylococci specifically *S.aureus* as major mastitis pathogen which is very difficult to be treated, this work focused on studying Staphylococci causing clinical and subclinical mastitis in bovine and their antibiotic resistance.

In this study, 25/48 (52%) Staphylococcal isolates were isolated from milk samples collected from cows suffering from clinical mastitis. Also, 118/175 (67%) isolates were isolated from milk samples collected from cows with subclinical mastitis. Identification of these isolates was performed using combined phenotypic and genotypic methods. Phenotypic identification classified the Staphylococcal isolates into CPS and CNS. A novel multiplex PCR, utilizing four pair of primers simultaneously, was developed and used for both genotypic identification of the isolated Staphylococci and detection of β-lactam-mediated resistance genes. The assay was optimized and validated using *S.aureus* ATCC (25213 & 25923) reference strains as positive control. At first, the DNA was isolated from both the reference strains and the isolated strains using the reported crude DNA extraction method of Reischl *et al.* [32]. This method was very simple and takes only few minutes to obtain amplifiable crude DNA. It also saves the expensive lysostaphin which usually used in ordinary methods of extracting pure DNA from Staphylococci. The performed multiplex PCR assay confirmed all the isolates to be Staphylococci through successful amplification of the 228 bp fragment of Staphylococcal specific 16S rRNA gene (Fig 1). Using the same multiplex PCR, some isolates were confirmed to be *S.aureus* through successful amplification of 279 bp fragment of *S.aureus* specific thermonuclease gene (Fig 1). As shown in table 2, out of 25 isolates recovered from clinical mastitic milk, only one (4%) was *S.aureus*, 10 (40%) were identified as coagulase positive Staphylococci (CPS) other than *S.aureus* and 14 (56%) were coagulase negative Staphylococci (CNS). Also it showed that out of the 118 Staphylococcal isolates recovered from cases of subclinical mastitis 32 (27%) were *S.aureus*, 30 (25%) were other CPS and 56 (48%) were CNS. Our results presented an increased incidence of CPS and CNS. The incidences of CPS among isolates recovered from clinical and subclinical mastitis were 40 and 25%, respectively. These relatively high incidences came in agreement to those reported by Arslan *et al.* [16] who reported the isolation of 35 *S. intermedius* out of 77 Staphylococcal isolates recovered from subclinical mastitic milk. Moreover, the increased incidence of CNS among our isolates (56% & 48%) ascertained the growing importance of CNS as mastitis pathogens as previously reported by Smith [38]. Coagulase negative Staphylococci were reported as significant causes of subclinical mastitis and elevated somatic cell counts in infected quarters [19-21, 39, 40]. They were also reported to cause clinical mastitis and persistent IMI that can last for several months during lactation in the absence of intervention [41-43]. Additionally, Haltia *et al.* [44] included the CNS within contagious bacteria that caused high mastitis prevalence. They also reported the CNS infections to be usually spread from cow to cow when bad hygiene was found. Considering the CPS other than *S.aureus*, Park *et al.* [45] classified them with *S.aureus* as contagious pathogens infecting milk samples of their study.

Because of the frequent use of β-lactams in intramammary infusions used for mastitis treatment in the Egyptian dairy farms, therefore, β-lactam resistance of Staphylococci as udder pathogens is of greatest concern from a clinical perspective. So, this study aimed to detect the prevalence of β-lactam resistance among our Staphylococcal isolates. As β-lactam resistance in Staphylococci was reported to be mainly mediated by *mecA* gene which is the determinant of methicillin resistance in all Staphylococci [27] and/or *blaZ* gene, the determinant of β-lactamase production [25] so, phenotypic and genotypic methods were directed toward detection of both mechanisms.

Phenotypic prediction of *mecA* gene presence which is historically referred to methicillin resistance (MR) was conducted by both cefoxitin and oxacillin disk diffusion tests. On the other hand, prediction of *blaZ* gene presence was conducted by penicillin and AMC disk diffusion tests [46, 47].

As shown in table 3, out of 25 Staphylococcal isolates recovered from clinical mastitic cases, 15, 19, 25 and 20 isolates were resistant to cefoxitin, oxacillin, penicillin and AMC, respectively. In subclinical mastitis, out of 118 isolates recovered, 43, 36, 43 and 22 isolates were resistant to cefoxitin, oxacillin, penicillin and AMC, respectively.

Interpretation of the results revealed difference between results of both cefoxitin and oxacillin disk diffusion tests. These differences can be attributed to variation in inoculum size, growth conditions (e.g., the temperature or osmolarity of the medium), making susceptibility testing of methicillin resistant Staphylococci (MRS) by standard microbiological methods potentially difficult [11, 48]. Conflicting recommendation regarding the most reliable method of identification of MR can be another cause for these discrepancies [49]. These discrepancies were previously reported by many authors when comparing different phenotypic tests [14, 50-53].

Additionally, phenotypic methods were found to be time consuming and labour intensive [54]. Moreover, performing disk diffusion tests was also reported to lead to false positive and false negative results [55]. On the hand, several advantages were reported for genotypic methods in resistance detection compared to conventional susceptibility methods. Genotypic tests provide resistance profiles rapidly, diminish the biohazard risk associated with the propagation of the microorganisms by culturing and it can be used as a gold standard for evaluating new, improved susceptibility methods for testing clinical isolates with difficult-to-detect resistance profiles where CLSI guidelines now accepted that checking for presence of *mecA* and *blaZ* genes by PCR is the most reliable method for detection of MR [56, 57]. Because of the above mentioned disadvantages of phenotypic tests and the advantages of genotypic methods, two pairs of primers were included in our novel multiplex PCR. The first pair targeted the *mecA* gene, the determinant of methicillin resistance [35] while the second pair targeted the *blaZ* gene, the determinant of β-lactamase production [25, 50]. Using our novel multiplex PCR assay, detection of *blaZ* and/or *mecA* genes were performed through successful amplification of 517 bp and /or 147 bp specific products, respectively (Figure 1). According to PCR results, out of 25 Staphylococcal strains isolated from clinical mastitis, 20 (80%) were methicillin resistant (MR) carrying the *mecA* gene, including 8 MRCPS and 12 MRCNS. Also, 17/25 (68%) were β-lactamase producers (*blaZ* gene positive PCR) including 1 *S.aureus*, 8 CPS and 8 CNS. Additionally, these 25 isolates can be also classified into four groups: isolates having *mecA* gene only (n=6, 24%), isolates having *blaZ* gene only (n=3, 12%), isolates having both *mecA* & *blaZ* genes (n=14, 24%) and isolates had neither *mecA* nor *blaZ* genes (n=2, 8%).

On the other hand, out of 118 Staphylococci isolated from cases of subclinical mastitis, 27 (22.9%) were MR including 5 MRSA, 7 MRCPS and 15 MRCNS. Also, 27/118 (22.9%) were β-lactamase producers (*blaZ* positive PCR) including 12 *S.aureus*, 3 CPS and 12 CNS. Furthermore, these 118 isolates can be classified into 4 groups: isolates having *mecA* gene only (n=6, 13.5%), isolates having *blaZ* gene only (n=16, 13.6%), isolates having both *mecA* & *blaZ* genes (n=11, 9.3%) and isolates had neither *mecA* nor *blaZ* genes (n=75, 63.7%). Our results showed higher incidence for MR in isolates of clinical mastitis versus those of subclinical mastitis (80% versus 22.9%). Also, incidence of Staphylococci producing β-lactamase was higher in clinical mastitis than subclinical mastitis (68% versus 22.9%). Incidence of MRSA was found to be 15.6% of *S.aureus* caused subclinical mastitis. According to our knowledge, this is the first record for genotypic detection of MRSA in Staphylococci recovered from bovine mastitis in Egypt. This high incidence was previously reported by Vanderhaeghen *et al.* [58] who found 10% of isolated *S.aureus* to be MRSA. The increase in resistance incidence can be attributed to the selective pressure enforced by the improper use of β-lactams in mastitis treatment [59-61]. Our results also cleared that the incidence of MR among CPS and CNS were higher than that among *S.aureus* isolates whether in cases of clinical or subclinical mastitis. These high incidences declared the importance of both CPS and CNS as mastitis pathogens. Resistance to β-lactams has been reported in *S.aureus*, CPS and CNS isolated from cow's milk with clinical and subclinical mastitis [20, 23, 62-65]. It has also been hypothesized that MRCNS of the agricultural animals may serve as important reservoirs for the transfer of antimicrobial resistance genes to *S.aureus* [66, 67]. Some authors even recommended that animals that carry MR-CNS should be culled [20].

Comparing the results of both phenotypic and genotypic tests revealed close correspondence between them for the majority of the isolates. On the contrary, this correlation was not found for 2 groups of isolates. The first group of isolates didn't carry the *mecA* gene while showing phenotypic resistance to cefoxitine and /or oxacillin. Non-*mecA* methicillin resistance can be

attributed to many reasons: the first is the production of modified intrinsic PBPs with altered affinity for methicillin [68], the second reason can be the inactivation of oxacillin or methicillin by increased production of β-lactamase which can be declared by detection of *blaZ* gene by PCR. Another explanation of this phenomenon could be the disadvantages previously reported for disk diffusion tests to call *mecA* negative strains resistant [69]. The other group of strains carried the *mecA* gene but was sensitive by disk diffusion tests. This can be explained by the possible involvement of other genes in the process of β-lactam resistance which can affect the expression of *mecA* gene [48, 70-72]. Another cause for such problem was reported to be the heteroresistance phenomenon which is difficult to be classically detected and was reported mainly to occur in CNS of agricultural origin [67, 73]. In this case some strains produce low levels of PBP2a and so escape classic detection and phenotypically misidentified as methicillin sensitive despite having the *mecA* gene [74-76]. In comparison to *mecA* PCR, Zhang *et al.* [53] reported the broth microdilution test to have the best sensitivity in detecting MR phenotypes among CNS of animal origin. Additionally, some strains of CNS are slow growing, so they may be misidentified as sensitive while they possess the *mecA* gene [77]. This minor miscorrelation between phenotypic and genotypic tests was also mentioned by many authors [1, 50, 51, 69, 77, 78].

Appropriate therapy of β-lactam resistant staphylococcal infection requires the knowledge of antimicrobial resistance profile. Therefore, resistance to antimicrobials other than β-lactams was also determined.

The isolates were classified according the PCR results into four groups: isolates having *mecA* gene only, isolates having *blaZ* gene only, isolates having both *mecA* & *blaZ* genes and isolates had neither *mecA* nor *blaZ* genes. *In vitro* resistance of the isolated *Staphylococcus* strains to antimicrobial drugs other than β-lactams was detected using disk diffusion test. Difference in resistance between these four groups was shown in table (4). In general, the results revealed that multidrug resistance was more common among *blaZ* &/or *mecA* positive isolates ($P<0.05$) in comparison to *blaZ* & *mecA* negative isolates. Staphylococcal isolates contained positive *blaZ* &/or *mecA* genes showed more *In vitro* resistance to Tetracycline, Streptomycin and Neomycin and for less extent to Erythromycin, Chloramphenicol and Gentamycin. It was found that multidrug resistance was very aggressive in *mecA* positive CNS where 5/13 showed multidrug resistance to all the tested antibiotic disks. This multidrug resistance of Staphylococci carrying the β-lactams resistance genes can be attributed to that both *mecA* and *blaZ* genes often encode resistance to other antimicrobials [22, 79, 80]. The majority of the tetracycline resistant isolates were also resistant to penicillin. This combination of resistance has been previously reported for *S.aureus* isolated from intramammary infections [50, 81, 82]. It was also concluded from the same Table that Norfloxacin followed by Florphenicol were the most effective antibiotics *In vitro* where only 11/143 isolates (7.7%) were resistant to Norfloxacin and 19/143 (13.3%) were resistant to Florphenicol. Nearly similar result was reported by Imran *et al.* [83] who found that enrofloxacin was the most effective antibiotic against MRS followed by norfloxacin and chloramphenicol.

In conclusion, this study showed high incidence of staphylococci causing bovine mastitis. Most importantly, it is ascertained that CPS other than *S.aureus* and CNS are also deeply implicated in both clinical and subclinical bovine mastitis. Accordingly, attention must be paid toward the identification of these strains to the species level. Considering β-lactam resistance, it was found to be widely spreading among staphylococcal isolates. Additionally, association between *blaZ* and/or *mecA* mediated β-lactam resistance genes and resistance to other antibiotics was also declared. Therefore, to guide therapy and measures to counteract spread of resistant clones, regular monitoring for β-lactam resistance is recommended in herds with udder health problems.

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