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Research Paper

PCR for detection of virulence and antibiotic resistance genes of coagulase positive *staphylococcus aureus* from clinical mastitis in Egypt

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Abstract

Coagulase positive *Staphylococcus aureus* is the most frequently bacterial pathogen causing clinical mastitis in cattle. In this work, 120 milk samples were collected from cattle suffering from clinical mastitis. The results revealed that S. aureus was 35 isolates (29.16%) and other staphylococci were 13 isolates (10.84%). The application of PCR was effectively in detection of Coa. SpA, mecA and blaZ genes of *S. aureus* by amplification at a single amplicon at 630 bp, 226 bp, 310 bp and 173bp, respectively. The results concluded that the detection of Coa and SpA genes can be used as a good method for typing of *S. aureus* strains as well as the detection of antibiotic resistance mecA and blaZ genes may be helpful for effective control of staphylococcal mastitis.

Keywords: *COA; SPA; MECA; BLAZ; PCR; Staphylococcus aureus; Mastitis*

1. Introduction

Mastitis is recognized as the most important dairy herd problem worldwide [1]. Economic losses of mastitis include decrease in milk quantity & quality and high costs of treatment [2]. S. aureus is one of the most common etiological pathogens, causing intramammary infection in dairy herds leading to severe economic losses in dairy industry [3] which colonize in the mammary gland [4]. S. aureus produce a large number of potential virulence factors which have an important role in the pathogenesis of mastitis [5].These include, Coagulase which is considered the most important virulence factor that clot plasma and coats the bacterial cell, so prevent the phagocytosis [6] via enables staphylococci to captured within a fibrin meshwork, disseminate and resist opsonophagocytic mechanism of host immune cells [7]. Staphylococcal protein A (spA) is a membrane-bound exoprotein of bacterial cell wall which considered an important virulence factor that impair opsonization by serum complement and phagocytosis of Polymorphonuclear leukocytes through binding to FC region of immunoglobulins [8]. *S.aureus* developed a high resistant against a wide variety of antibiotics which increase their virulence and difficulty in treatment [9]. The most important antibiotic resistant genes of *S.aureus* strains was blaZ gene which coded for β-lactamase. [10] and mecA gene which designated for methicillin resistance (MRSA) that coded for penicillin-binding protein 2a [11]. From epidemiological point of view the presence of virulent strains of *S .aureus* in mastitic milk give an importance to detect and characterize of these strains to control and reduction of the disease dissemination in dairy herds [12]. Therefore, this research was planned for characterization of *S. aureus* and detection of their virulence and antibiotic resistance genes from clinical mastitis cases.

2. Material and methods

1) Collection of milk samples according to [13]:
A total of 120 mastitic milk samples were collected from clinical cases of mastitis of lactating cattle at Menofia and Beheria Governorate from small-holders farmers.
2) Phenotypic characterization: through culturing onto blood agar, Baired parker agar medium and Gram's staining for demonstrating characteristic shape of staphylococci according to [14].
3) Biochemical and virulence activities:
All suspected strains of Staphylococci tested for catalase, tube coagulase using rabbit plasma [15] , haemolysis on blood agar and Deoxyribonuclease (DNase) activity onto DNase agar [16]:
4) PCR detection of Coa, spA, blaZ and mecA genes of *S. aureus*.
DNA extraction. DNA extraction from samples was performed using the QIAamp DNA Mini kit (Qiagen, Germany, GmbH) with modifications from the manufacturer's recommendations. Briefly, 200 µl of the sample suspension was incubated with 10 µl of proteinase K and 200 µl of lysis buffer at 56°C for 10 min. After incubation, 200 µl of 100% ethanol was added to the lysate. The sample was then washed and centrifuged following the manufacturer's recommendations. Nucleic acid was eluted with 100 µl of elution buffer provided in the kit.
Oligonucleotide Primer. Primers used were supplied from Metabion (Germany) are listed in table (1).
PCR amplification. Primers were utilized in a 25- µl reaction containing 12.5 µl of Emerald Amp Max PCR Master Mix (Takara, Japan), 1 µl of each primer of 20 pmol concentrations, 4.5 µl of water, and 6 µl of DNA template. The reaction was performed in an applied biosystem 2720 thermal cycler.

3. Analysis of the PCR products

The products of PCR were separated by electrophoresis on 1.5% agarose gel (Applichem, Germany, GmbH) in 1x TBE buffer at room temperature using gradients of 5V/cm. For gel analysis, 15 µl of the products was loaded in each gel slot. A 100 bp and 100DNA Ladders (Qiagen, Germany, GmbH) was used to determine the fragment sizes. The gel was photographed by a gel documentation system (Alpha Innotech, Biometra), and the data was analyzed through computer software.

Table 1: Primers Sequences, Target Genes, Amplicon Sizes and Cycling Conditions.

Target gene	Primers sequences	Amplified segment (bp)	Primary denaturation	Amplification (35 cycles)			Final extension	Reference
				Secondary denaturation	Annealing	Extension		
mecA	GTA GAA ATG ACT GAA CGT CCG ATA A CCA ATT CCA CAT TGT TTC GGT CTA A	310	94˚C 5 min.	94˚C 45 sec.	50˚C 45 sec.	72˚C 45 sec.	72˚C 10 min.	[17]
spa	TCA ACA AAG AAC AAC AAA ATG C GCT TTC GGT GCT TGA GAT TC	226	94˚C 5 min.	94˚C 30 sec.	55˚C 30 sec.	72˚C 30 sec.	72˚C 10 min.	[18]
Coagulase	ATA GAG ATG CTG GTA CAG G GCT TCC GAT TGT TCG ATG C	Four different types of bands may be detected 350 bp 430 bp 570 bp 630 bp	94˚C 10 min.	94˚C 1 min.	55˚C 1 min.	72˚C 1 min.	72˚C 10 min.	[19]
blaZ	ACTTCAACACCTGCTGCTTTC TGACCACTTTTATCAGCAACC	173 bp	94˚C 5 min.	94˚C 30 sec.	54˚C 30 sec.	72˚C 30 sec.	72˚C 7 min.	[20]

4. Results

Table 2: Prevalence of Staphylococci in Clinical Bovine Mastitic Cases.

Total number of clinical samples	No of positive cases on BPM		Phenotypic characterization Pathogenic staphylococci (*S. aureus*)		Other staphylococci	
	No	%	N	%	N	%
120	48	40%	35	29.16%	13	10.84%

% estimated according to number of samples (120).
BPM: Baired parker medium

Table 3: Enzymatic and Virulence Activities of Phenotypic *S. Aureus*

Total No of phenotypic S. aureus	Catalase		Lecithinase activity on BPM		B haemolysin on blood agar		Tube Coagulase test		DNase	
	N	%	N	%	N	%	N	%	N	%
35	35	100%	35	100%	28	80%	21	60%	7	20%

Results of Coa, SpA, mecA and blaZ genes of Staph.aureus by PCR.

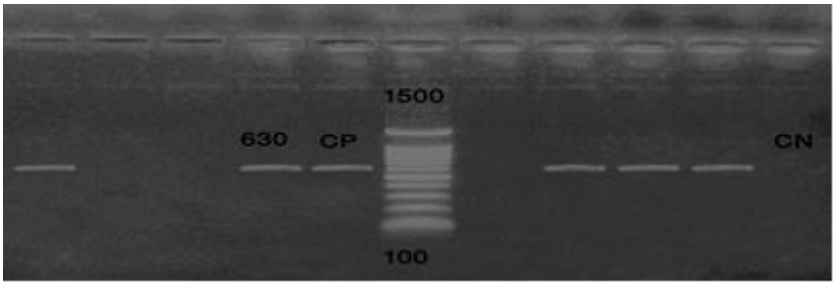

Fig. 1: 1.5% Agarose gel electrophoresis showed positive amplification of Coa gene of *S. aureus* isolates at 630 bp Fragment (S1, 2, 3, 5, 8). Cp (Control Positive), CN (Control Negative)).

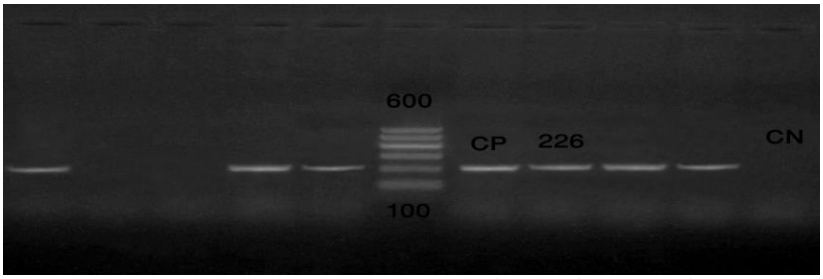

Fig. 2: 1.5% Agarose gel electrophoresis showed positive amplification of SpA gene of *S. aureus* isolates at 226 bp Fragment (S1, 2, 3, 4, 5, 8). Cp (Control Positive), CN (Control Negative).

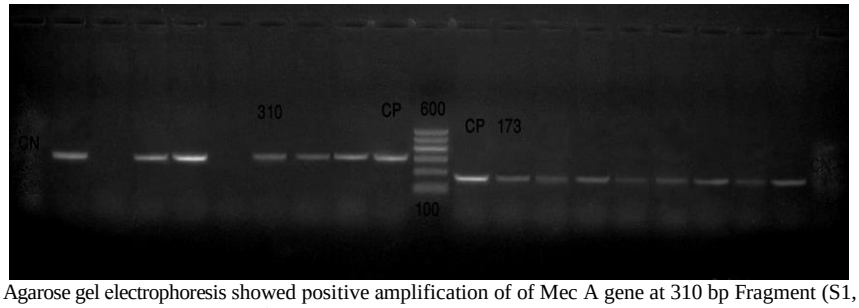

Fig. 3: 1.5% Agarose gel electrophoresis showed positive amplification of of Mec A gene at 310 bp Fragment (S1, 2, 3, 5, 6, 8) and blaz gene at 173bp Fragment of S. aureus isolates (S 1-8),Cp (Control Positive), CN (Control Negative)).

5. Discussion

Staph. aureus is the most important bacterial microorganism in bovines causing contagious mastitis and highly economic losses in dairy herds [21]. In the present study, phenotypic characterization of *S. aureus* was depended on Gram's stain, culturing on Baired parker medium, Catalase, tube coagulase and DNase tests. This finding was agree with [22] all *S.aureus* strains were positive for coagulase test and give a typical morphology on Baird Parker agar medium. In the present study, a prevalence rate of *S.aureus* was (29.16%). These results come in agreement with [23] isolated *S.aureus* (28.2%) from clinical cases in cattle. Moreover [24] revealed that *S.aureus* is responsible for about (30%) to (40%) of all mastitis cases. The high prevalence in the present study may be correlated to the transmission of infection by milker's hands, contaminated equipments and bad hygienic environment in small holder cows [25]. Several virulence factors were produced by S. aureus, including Coagulase protein, which is important in the pathogenicity [26] through turn fibrinogen to fibrin, then lead to abscess formation and persistence of microorganism in host tissue [27].Coagulase protein is considered to be a virulence factor in intrammmary infection and can be used as a simple and effective method for typing of *S. aureus* isolates from bovine mastitis. [28]. In the present study, all positive coagulase isolates subjected to Coa gene by PCR, and the result revealed that all isolates give a single amplicon at 630 bp. This nearly agree with [23] who reported a single amplicon of Coa gene at 600 bp of isolates from bovine mastitis. Moreover, the findings reported by [29] suggested that the amplicon of about 600 bp are predominant in bovine strains. As shown in fig (1) some isolates of CoPS didn't show any band at the set of this primer, which may be due to gene polymorphism. This finding agree with [30] achieved that S. aureus isolates give 5 different PCR products of Coa gene. Furthermore, [26] stated that coagulase gene had different PCR products indicating the polymorphism of the gene. Moreover [28]showed amplification of the coa gene of *S. aureus* isolates from mastitic cow produces 27 different PCR- products, which ranged from 579 to 1442 bp. The SpA gene is a major important surface protein of bacterial cell wall, which binds with FC region of immunoglobulin G, so the decreasing in SpA on cell surface of *S. aureus* resulted in increasing number of free receptor sites for complement and enhance phagocytosis [31]. The present study showed amplification of SpA gene of S.aureus at 229 bp. [32] reported that SpA gene can be used for typing the isolates of *S.aureus*. Furthermore [31] the detection of genetic polymorphisms in the X region of the SpA gene can be used for typing of *S. aureus*. Also [33] concluded that detection of SpA gene polymorphisms with Coa-PCR is proposed as a good diagnostic method for typing of *S. aureus* isolates which provided important results for effective control of staphylococcal mastitis. Recently, the prevalence of antibiotic resistance in *S. aureus* strains becomes a serious problem in dairy herds [34]. In the present study, mecA gene was detected at 310 bp. [35] mentioned that mecA gene of *S.aureus* strains was the main gene responsible for the resistance to methicillin through the production of Penicillin binding protein (PBP2a). The resistance to β-lactam antibiotics occur through hydrolyzing the β-lactam ring and convert to inactive form, so the application of PCR for detection of blaZ gene is recommended in veterinary laboratories for detection of the resistant strains of S. aureus [36]. In our result, all isolates of S. aureus subjected to PCR were positive for blaZ gene and gave a single amplicon at 173 bp. The same result obtained by [37] detected a single amplicon of 173 bp of blaZ gene. Our results showed that all tested strains for mecA gene were positive for blaZ gene and this similar to [38] who noticed that all positive strains to mecA were also positive for blaZ gene and the presence of both genes was correlated to phenotypic beta-lactam resistance of *S. aureus* strains.

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