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Coagulase Gene Typing with Emphasis on Methicillin-Resistance Staphylococci: Emergence to Public Health

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Abstract

Emerging antimicrobial resistance among CNS is a concern in veterinary and human medicine. Coagulase test is considered as the key test to differentiate staphylococci to two groups, coagulase positive staphylococci (CPS) and coagulase negative staphylococci (CNS). A total of 200 *Staphylococci* strains were isolated with percentage 66.7% (200/300) from quarter milk samples. The total of *S. aureus* strains are 70 with percentage 35% (70/200). Among 70 strains of *S. aureus*, 30 strains are coagulase positive *S. aureus* with percentage 43% (30/70) and coagulase negative *S. aureus* 57% (40/70). CNS other than *S. aureus* was detected with percentage 65% (130/200) from subclinical mastitic cows. We examine sixty isolates of staphylococci recovered from subclinical mastitis in dairy cattle which divided as ten isolates of coagulase positive *S. aureus* (CP *S. aureus*), ten isolates of coagulase negative *S. aureus* (CN *S. aureus*) and forty isolates of coagulase negative staphylococci (CNS) which identified using API-Staph Kits as *S. chromogenes*, *S. simulans*, *S. haemolyticus*, *S. epidermidis* and *S. cohnii.* The genotypic detection of *coa* gene and *mec*A gene was screened in CP *S. aureus*, CN *S. aureus* and CNS.

Keywords

Coagulase, *coa* Gene, *mec*A Gene, Staphylococci, Methicillin

1. Introduction

Staphylo-coagulase is an extracellular protein that has traditionally been used to differentiate *S. aureus* from the

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less virulent staphylococci. *S. aureus* secretes two clotting factors, coagulase (Coa) and von Willebrand factor binding protein (vWbp). The Coa and vWbp together are required for the formation of abscesses and promote the non-proteolytic activation of prothrombin and cleavage of fibrinogen, reactions that are inhibited with specific antibody against each of these molecules. Coa and vWbp specific antibodies confer protection against abscess formation and *S. aureus* lethal bacteraemia [1]. Although Coa enzyme is also produced by *Staphylococcus intermedius* and some *Staphylococcus hyicus* strains but coagulase production is one of the most reliable criteria for the identification of *S. aureus* [2].

Coagulase-negative staphylococci (CNS), have become the most commonly isolated microorganisms from bovine milk in many countries and are regarded as emerging mastitis pathogens [3]. In addition, CNS are abundantly present both in the cows' environment [4] and on their teat apices [5]. Also CNS showed high virulence by their ability to form biofilm which also get cells in biofilm more resist to antimicrobial [6]. So we are focusing in this study on subclinical mastitic cows and we examine all recovered coagulase positive and coagulase negative staphylococci.

The resistance to methicillin is caused by the presence of the *mec*A gene, which encodes the 78-kDa penicillin-binding protein (PBP) 2a (or PBP2') [7]. *Staphylococcus* strains with *mec*A are resistant to lactam antibiotics and frequently code for multi-drug resistance, which may represent a serious health and economic concern [8]. Consequently, it is highly important to detect *mec*A, especially in all *Staphylococcus* strains [9].

The resistance genes might in some instances transfer from staphylococci of animal origin to staphylococci that cause infections in humans, thereby compromising antimicrobial treatment [10]. CNS colonising the udder of buffaloes and cows may represent a reservoir of different antibiotic resistance genes and SCC*mec* elements. This raised the question of whether the genetic background could be a reservoir for interspecies gene transfer among CNS and *S. aureus* in the udder as it was previously suggested in the intestinal tract [11].

In recent years, increasing numbers of reports have shown that the *mec*A gene is present in CNS strains, including hospital-acquired infections, neighborhoods [12], animal epidermis [9] [13], beaches [14] and public transportation systems [15].

Therefore, the present work aimed to: 1) detect the presence of *coa* gene in *Staphylococci* field isolates from subclinical mastitic cows phenotypically and genotypically; 2) detect methicillin resistance gene in *S. aureus* and other CNS.

2. Materials and Methods

2.1. Sampling

The study took place in private dairy farms surrounding Giza, Egypt. A total of 300 quarter milk samples milk samples were aseptically collected from 85 mastitic dairy cattle. The animals had not been treated with an antibiotic for at least 30 days prior to collection. Milk sample collection was performed using the method of the National Mastitis Council, with some modifications, under aseptic conditions [16].

2.2. Isolation and Identification

Each milk sample was plated on two plates the first plate containing Columbia Agar base with 5% defibrinated sheep blood (Oxoid) and the second plate was mannitol salt agar. Test plates were incubated for 24 - 48 hours at 37˚C ± 1˚C. All isolates were presumptively identified as staphylocooci based on colony morphology, Gram staining, catalase reaction, and oxidative-fermentative testing. After confirmation of the genus Staphylococcus, the enzyme coagulase was characterized among all isolates using both the slide and tube methods according to Quinn *et al.* [17]. Coagulase-negative isolates resistant to methicillin were subjected to identification to the species level using the API-Staph Kit (BioMerieux, P.O. Box 4328, Honeydew, 2040) as described by Petzer *et al.* [18].

2.3. Phenotypic Antimicrobial Resistance Tests

Antimicrobials were selected for testing based on the licensing for mastitis treatment in cattle, use in human medicine and potential resistant determinant phenotypes [19] [20]. Susceptibility of the isolates were determined against 10 antimicrobial agents as commercial discs (Oxoid) which commonly used for treatment of bovine mastitis in Egypt or considered as important agents for humans as follows: antimicrobials used for treatment of

bovine mastitis included in this study were ciprofloxacin (5 µg), erythromycin (15 µg), gentamicin (10 µg), penicillin (10 units) and tetracycline (30 µg). Antimicrobials not used for treatment of bovine mastitis but important for humans were clindamycin (2 µg), oxacillin (1 µg), rifampicin (5 µg) and vancomycin (30 µg). Isolates were inoculated into Mueller-Hinton broth (Oxoid) and incubated overnight at 37˚C. The turbidity of the suspensions were adjusted to a 0.5 McFarland standard and streaked onto Mueller-Hinton agar (Oxoid) plates. Antimicrobial disks were added on the plates and they were incubated aerobically at 35˚C for 16 - 18 h. The results were recorded as susceptible, intermediate, or resistant by measurement of the inhibition zone diameter. Resistance was determined by measurement of inhibition of growth around the antimicrobial disk according to the zone diameter interpretative standards of CLSI [21] or according to the antimicrobials manufacturers' instructions. The reference strain *S. aureus* ATCC 25923 was used as the quality control organism and included with each batch of isolates tested.

2.4. Molecular Detection of Coagulase (*coa*) Gene and Methicillin Resistance Genes (*mecA*) Gene in Staphylococcal Isolates

2.4.1. DNA Extraction

Prior to DNA extraction, the bacterial strains were cultivated on blood agar base (Oxoid, Germany) containing 5% defibrinated sheep blood for 24 h at 37˚C then genomic DNA of randomly selected 60 staphylococci strains were extracted by using an extraction kit (QIA amp mini kit, Qiagen). DNA was stored at −20˚C. To detect *coa* gene and *mecA* gene in examined staphylococcal isolates, specific oligonucleotide primers for *coa* gene and *mecA* gene were described in **Table 1**. All primers were supplied by Sigma Genosys (Sigma).

2.4.2. PCR for Detection of *coa* Gene Specific for Coagulase Production

The reaction with these primers were carried out using a total volume of 25 µl reaction mixtures contained 5 µl of DNA as template, 20 pmol of each primer and 1× of PCR master mix (Dream Taq Green PCR Master Mix, Fermentas Life Science). Amplification was conducted in Thermal cycler which was adjusted for detection of *coa* gene as follows, an initial denaturation at 94˚C for 45 sec. The cycling proceeded for 30 cycles of denaturation at 94˚C for 20 sec, annealing at 57˚C for 15 sec, and extension at 70˚C for 15 sec with a final step of final extension at 72˚C for 2 min.

2.4.3. PCR for Detection of *mecA* Gene Specific for Methicillin Resistance

The reaction with these primers were carried out using a total volume of 25 µl reaction mixtures contained 5 µl of DNA as template, 20 pmol of each primer and 1× of PCR master mix (Dream Taq Green PCR Master Mix, Fermentas Life Science). Amplification was conducted in Thermal cycler which was adjusted for detection of *mecA* gene as follows, an initial denaturation at 94˚C for 4 min. The cycling proceeded for 35 cycles of denaturation at 94˚C for 60 sec, annealing at 55˚C for 60 sec, and extension at 72˚C for 60 sec with a final step of final extension at 72˚C for 10 min.

3. Results

3.1. Isolates

All suspected colonies were examined under light microscope to detect Gram positive cocci occurring singly, in pairs, in short chain or in irregular clusters like bunch of grapes. The results of traditional biochemical tests and

Table 1. Primer sequences for c*oa* gene specific for coagulase production and *mecA* gene specific for methicillin resistance in staphylococci.

Primers target	Sequence	Anneali-ng	Amplified product size	References
(*coa*)	ATA GAG ATGCTG GTA CAG G GCT TCC GATTGT TCG ATG C	57˚C	750 bp	[22]
(*mecA*)	GTGAAGATATACCAAGTGATT3' ATGCGCTATAGATTGAAAGGAT3	55˚C	147 bp	[23]

API-Staph Kits indicated that all isolates are *Staphylococci* spp. A total of 200 *staphylococci* strains were isolated with percentage 66.7% (200/300).

3.2. Investigation of Coagulase Production by Coagulase Tube Test

The total of *S. aureus* strains are 70 with percentage 35% (70/200). Among 70 strains of *S. aureus*, 30 strains are coagulase positive *S. aureus* with percentage 43% (30/70) and coagulase negative *S. aureus* 57% (40/70). CNS other than *S. aureus* was detected with percentage 65% (130/200) from subclinical mastitic cows. The previous results were illustrated in **Table 2** and **Table 3**.

3.3. Detection of *coa* Gene Specific for Coagulase Enzyme Production

Sixty staphylococci strains were randomly selected for detection of *coa* gene, our results showed somewhat differences between phenotypic and genotypic detection of coagulase enzyme. It was noted that 10 strain of *S. aureus*, classified as coagulase negative by tube coagulase test were found to be positive with PCR. This result was illustrated in **Table 4**.

3.4. Detection of *mecA* Gene Specific for Methicillin Resistance

The results of PCR for amplification of 147 bp fragment for *mecA* gene performed with its specific primer were observed in **Table 5**.

Table 2. Number and percentage of CPS and CNS strains differentiated according to coagulase test and API kit.

No. of samples	*Staphylococci* strains	*S. aureus* strains	CP *S. aureus*	CN *S. aureus*	CNS other than *S. aureus*
300	200 (66.7%)	70 (35%)	30 (43%)	40 (57%)	130 (65%)

Table 3. Classification of CNS other than *S. aureus* according to API kit results.

No. of CNS other than *S. aureus*	CNS other than *S. aureus*	No %
130	*S. chromogenes*	40 (31%)
	S. simulans	10 (7.7%)
	S. haemolyticus	30 (23%)
	S. epidermidis	35 (27%)
	S. cohnii	15 (12%)

Table 4. Phenotypic and genotypic differences in detection of coagulase enzyme.

Strains	CP *S. aureus* (10 strains)		CN *S. aureus* (10 strains)		CNS other than *S. aureus* (40 strains)	
	Number of positive strains	%	Number of positive strains	%	Number of positive strains	%
Phenotypic (coagulase test)	10	100	0	0	0	0
Genotypic (*coa* gene)	10	100	10	100	0	0

Table 5. Results of PCR for amplification detection of 147 bp fragment for *mecA* gene performed with their specific primer.

Gene	CP *S. aureus* (10)		CN *S. aureus* (10)		CNS other than *S. aureus* (40)	
	positive amplification of *mecA* gene	%	positive amplification of *mecA* gene	%	positive amplification of *mecA* gene	%
mecA	2	20%	1	10%	9	22%

4. Discussion

Mastitis is one of the major causes in financial losses for dairy cattle farmers. Staphylococcal species associated with bovine mastitis have been classified as coagulase positive or coagulase negative. CNS has become the most common bovine mastitis isolate in many countries and could therefore be described as emerging mastitis pathogens. CNS is not as pathogenic as the other principal mastitis pathogens and infection mostly remains subclinical. However, CNS can cause persistent infections, which result in increased milk somatic cell count (SCC) and decreased milk quality [24]. The current results reported that CNS was the predominant isolate recovered from subclinical mastitis cows with percentage 65%, these result was in harmony with El-Jakee *et al.* [25] who reported that CNS is generally high in subclinical mastitic samples, but low in samples from animals with clinical mastitis.

In our study, *S. chromogenes*, *S. epidermidis* and *S. haemolyticus* were the most prevalent CNS with percentage 31%, 27% and 23% respectively.

Our results were nearly agreed with Waller *et al.* [26] who reported that *S. epidermidis* was the most common CNS in Subclinical mastitis and *S. haemolyticus* was also quite common, Piessens *et al.* [4] stated that S. *chromogenes* and *S. haemolyticus* to be the most common species in milk samples from cows with intramammary infections (IMI). Persistent IMI was common to quarters infected with *S. epidermidis* indicating a more udder-adapted origin [27].

Molecular typing of microorganisms is very essential for infection control programs. These molecular techniques are rapid and accurate. On the other side, traditional phenotypic methods have several drawbacks.

Recent studies recommended that phenotypic and genotypic identification of CNS do not necessarily agree [28].

According to our results, we found that10 strains, classified as coagulase negative by tube coagulase test were found to be positive with PCR, this result was completely agreed with Gharib *et al.* [29] who mentioned that 2 strains, classified as coagulase negative by tube coagulase test were found to be positive with PCR amplification of the gene which clearly emphasizes the use of molecular methods in detecting *S. aureus*. The *coa* gene amplification has been considered a simple and accurate method for typing of *S. aureus* isolated from distinct sources, the coagulase protein is an important virulence factor of *S. aureus*. Like *spa*, *coa* has a polymorphic repeat region that can be used for differentiating *S. aureus* isolates. The variable region of *coa* gene is comprised of 81 bp tandem short sequence repeats (SSRs) [30].

In our study, all PCR products for *coa* gene were detected at 750 bp, this result agreed with Schlegelova *et al.* [31] who reported the size of *coa* gene PCR product of *S. aureus* isolates from dairy cow and human are 650 - 1050 bp.

Emerging antimicrobial resistance among CNS is a concern in veterinary and human medicine. Archer and Climo [32] reported an escalation of resistance for almost all antimicrobial classes excluding glycopeptides: *β*-lactams aminoglycosides, trimethoprim, rifampin, fluoroquinolones, macrolides, and tetracyclines. Humans and dairy cattle may share CNS strains, implying that bovine multidrug resistant staphylococci might be zoonotic pathogens [33].

The widespread use of antibiotics on dairy farms and other food-producing animals could lead to the selection and emergence of antibiotic resistant bacterial strains [34] to become a serious public health problem because of the possibility of dissemination of the antimicrobial resistant bacteria to humans via food. In Egypt, very little is known about the use of antibiotics on small dairy farms as is the case in lower/middle-income countries. Redding *et al.* [35] found that the farmers' knowledge of antibiotics was significantly associated with the use of antibiotics for preventative reasons, the purchase of antibiotics from feed-stores, the experience of complications in animals after having administered antibiotics, the number of workers on the farm, the educational level of the farmer and its infrequent use, because therapeutic interventions were sought only when the animal had reached an advanced stage of clinical disease. Also, because of their inability to define an antibiotic and in contrast to Redding *et al.* [35], many farmers do not understand that the use of antibiotics carried inherent risks to their animals and potentially to the consumers of dairy products from treated animals. In the recent study to Osman *et al.* [9], the highest resistance rate was observed against penicillin, ampicillin and oxacillin in CNS isolates. The emergence of high levels of penicillin resistance followed by the development and spread of strains resistant to the semisynthetic penicillin (oxacillin), macrolides, tetracyclines, and aminoglycosides has made the therapy of staphylococcal disease a global challenge [33]. In the present work, *mecA* gene was detected with high incidence

in CNS other than *S. aureus* followed by CP *S. aureus* and CN *S. aureus* with percentage 22%, 20% and 10% respectively. Our result supported by Bochniarz *et al.* [36] who reported that, recently a significant increase has been observed in the number of isolated methicillin resistance CNS (*mec*A-gene-positive) which are resistant to all groups of *β*-lactam antibiotics. Taponen and Pyorala [37] stated that, mastitis-causing coagulase-negative staphylococci (CNS) tend to be more resistant to antimicrobials than *S. aureus* and may be a source of *β*-lactam resistance genes.

The importance of CNS has increased and they have become the predominant pathogens isolated from subclinical mastitis in several countries which leading to economic losses resulting in decreased milk production. In addition to that, studying antimicrobial susceptibility of CNS at the species level can provide valuable information about species-specific differences that can be vital data for effective mastitis therapy and control [33]. The significance of our study in the prevention of staphylococci contamination in milk is to avoid spread of resistant strains of staphylococci from animal to anther and to human.

5. Conclusion

Because of the possibility of misidentifying *S. aureus* as CNS depending on coagulase test only so we recommend that genotypic detection to *coa* gene is very important for identification as well as the possibility of dissemination of the antimicrobial resistant bacteria to humans via the food processing chains. Screening the dairy industry for antimicrobial resistant bacteria should be performed as the resistance genes might in some instances transfer from staphylococci of animal origin to staphylococci that cause infections in humans, thereby compromising antimicrobial treatment.

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