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PREVALENCE AND CHARACTERIZATION OF METHICILLIN RESISTANT *STAPHYLOCOCCUS AUREUS* ISOLATED FROM RAW MILK IN EGYPT

By

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ABSTRACT

Methicillin-resistant *Staphylococcus aureus* (MRSA) has emerged globally as a potential pathogen, both in human and veterinary medicine. In recent years, the so-called livestock-associated MRSA has become an additional focus. The aim of the study was to isolation and identification of MRSA isolated from 56 samples raw bovine milk from different governerates in Egypt during the period of December 2016 through February 2017. This identification was carried out using conventional and molecular techniques. Out of 56 samples, 27 *S. aureus* strains were isolated (48%) and it was confirmed by PCR using *16S*rRNA gene. only 7 (25%) isolates were confirmed to be MRSA by molecular detection of *mec*A gene. The detection of MRSA in food of animal origin is a potential health hazard, thus it is necessary monitoring of food-producing animals and improving hygiene standards in food practices in order to reduce the microbiological risk to minimum.

Key words:

MRSA, Milk, Isolation, PCR.

INTRODUCTION

Staphylococcus aureus (S. aureus) is a common member of the natural microflora of human skin and nasal passage **(Hanson *et al.*, 2011).** In addition, as a potential pathogen, it may adversely affect human and animal health by causing sever necrotic lesions, abscesses **(Lowy, 1998) and bacteremia (Reacher *et al.*, 2000).** Moreover, besides these pathogenic symptoms, toxigenic foodborne strains of *S. aureus*, if they get multiplied in food to a certain level of about 105x10ˆ6 CFU/g, may secret potent, heat stable enterotoxins responsible for food-borne intoxication **(Tranter, 1990).** *S. aureus* intoxication ranked third of food

poisoning cases all over the world **(Asao *et al.,* 2003; Zhang *et al.,*1998)** and had been implicated with different categories of food including raw milk **(Jorgensen *et al.,* 2005),** dairy products **(Headrick *et al.,* 1998),** chicken, pork, beef and salad dishes **(Bryan, 1998).** Furthermore, *S. aureus* constitutes a primary cause of mastitis in dairy cattle **(Virgin *et al.,* 2009)**. Among *S. aureus*, Methicillin-resistant strains (MRSA), has emerged as a serious life-threaten infective agent, which does not respond to many antimicrobial treatments. Previous reports have shown an annual estimate of 94,000 MRSA infections in the United States, with nearly 20% mortality rate **(Klevens *et al.,* 2007)**. MRSA synthesizes a penicillin binding protein (PBP2a), encoded by the *mec*A gene on a mobile genetic element (Staphylococcal cassette chromosome mec SCCmec), which has a role of counteracting the inhibitory effect of Beta-lactam (b-lactam) antibiotics by preventing them from effectively binding to cell wall proteins. Moreover, MRSA may also resist vancomycin **(Cui *et al.,* 2000).** MRSA transmission has two main forms, hospital-acquired (HA) and community-acquired (CA). Although, HA MRSA infection was thoroughly investigated as the major form, CA-MRSA presently represents an imminent hazard and may have severe consequences **(Calfee *et al.,* 2003).** Whilst, HA MRSA is strictly linked to hospitals and health workers, CA-MRSA is more widespread and has no definite spreading vicinity **(Wannet *et al.,* 2004)** and its risk against industrialized nations have been increased **(Baggett *et al.,*2004)**. More recently livestock associated MRSA has been emerged in food production animals and farmers. **(Yu-yu Chaung, 2014).** *S. aureus* is highly prevalent in food and food environment, MRSA may have the same pattern of linkage. Many reports **(De Boe *et al.,* 2009; Kitai *et al.,* 2005; Kwon *et al.,* 2006; Lim *et al.,* 2010)** have identified presence of MRSA in different retailed meat products from different regions worldwide with varied prevalence. **(Normanno *et al.,* 2007)** isolated MRSA strains from bovine milk and some cheese varieties in Italy. Moreover, several food-borne acquired MRSA outbreaks have been also reported **(Jones *et al.,* 2002; Kluytmans *et al.,* 1995).** Recent reports revealed that MRSA was also associated with cases of bovine and caprine mastitis **(Aras, *et al.,* 2012 and Vanderhaeghen *et al.,* 2010).** MRSA strains have been found among the *S. aureus* strains isolated from bovine mastitis milk but they are not more **prevalent (Hendriksen*et al.,*2008;Juhàsz-Kaszanyitzky *et al.,* 2007;Kwon *et al.,*2005).** The Mediterranean region was considered a hyperactive endemic geographical area for MRSA, while Egypt was recorded as one of the Mediterranean countries

where highest proportions of MRSA among invasive *S. aureus* isolated from blood cultures were reported **(Borg *et al.,* 2007).** In addition, specific MRSA strain CC398 has been linked with different food animals and people in contact, which arise a new MRSA form, livestock-associated (LA-MRSA). LA-MRSA has been isolated from both human and animal infections **(Krziwanek *et al.,* 2009)** and from bovine mastitis case **(Monecke *et al.,* 2007).** Aforementioned reports may elucidate a possible way of transmitting either CA-MRSA or LA-MRSA between food/animal handlers and foods. To the best of our knowledge,our study reveals the prevalence of MRSA strains in raw milk in Egypt. Molecular MRSA identification was accomplished by PCR detection of *mec*A gene. Antibiotic susceptibility of isolated MRSA strains was also tested.

MATERIAL AND METHODS

Samples collection:

Fifty-six raw milk samples were collected from the rural areas (local markets and/or villages) located in different governrates in Egypt during the period of December 2016 through February 2017. All samples were kept at 4˚C in insulated ice box before subjected to microbiological analysis. The collection of samples was carried out according to **(Hagon *et al.,* 1999).**

Bacteriological examination:

S. aureus was isolated by adding the aliquots of milk samples to trypticase soy broth then incubated overnight at 35°C. The broth was then streaked on Baird Parker agar (Becton and Dickinson (BD), Franklin lakes, NJ, USA) containing 30% eggyolk emulsion and 1% potassium tellurite and then on to mannitol salt agar plate (Oxoid Ltd, Hampshire, UK) Yellow colored colonies on the mannitol salt agar plate were presumed to be *S. aureus*. Pure cultures were further examined for morphological (convex elevation and smooth margin), staining, and cultural characteristics, and for biochemical reactions according to standard keys. Staphylococci were studied in particular for hemolysis and coagulase production. Coagulase test was carried out according to a tube method using oxalated rabbit plasma in a 1:10 dilution in brain heart infusion broth **(Haran *et al.,* 2012).** Also, samples were cultured on ORSAB media (Oxoid Ltd, Hampshire, UK) a chromogenic agar for isolation Methicillin Resistant *S. aureus* **(Simor *et al.,* 2001).**Only typical colonies identified as *S. aureus* were stored in cryogenic vials containing 1 mL of trypticase soy broth with 15% glycerin at-80˚C. Isolation and identification of *S. aureus* were performed according to **(Forbes *et al.,* 2007).**

Antimicrobial susceptibility testing:

Resistance to antibiotics was examined according to guidelines of the National Reference Centre for Antimicrobial Susceptibility and internationally recognized standards of the Clinical and Laboratory Standards Institute **(CLSI,2016).** Determinations were carried out using the diffusion disk method on Mueller-Hinton agar (Oxoid). Antibiotic discs (Oxoid, UK) were cefoxitin (FOX) (30 µg) vancomycin (VAN) (30 µg) teicoplanin (TEC) (30 µg), polymyxin B (PB) (1 µg), oxacillin (OX) (1 µg), fusidic acid (FD) (10 µg), erythromycin (E) (15 µg), clindamycin (DA) (2 µg). Zones of inhibition were measured after 18 and 24 h incubation at 35°C. Cefoxitin was used as an indicator of methicillin susceptibility disks and an inhibition zone diameter of ≤14 mm was reported as methicillin resistant and ≥ 18 mm was considered as methicillin sensitive. ATCC29213 (methicillin sensitive control strain) and ATCC 25923 (Methicillin resistant control strain) were used as a control for disc diffusion.

MRSA molecular identification:

S. aureus isolates were grown in BHI prior to DNA extraction and purification according to the DNA Purification Kit (Qiagen) procedure. *S. aureus* isolates were detected by multiplex PCR using the following primers set:

-16S rRNA FOR (5′CGCACATCAGCGTCAG3′) and 16S rRNA REV

(5′ GTAGGTGGCAAGCGTTAT 3′) **(Monday S. R. *et al.*, 1999)**. The primers of *16S* rRNA suspected PCR products are equal to (~ 228 bp).

-*MecA*For(5'GTAGAAATGACTGAACGTCCGAT3')and*mec*ARev

(5′ CCAATTCCACATTGTTTCGGTCTAA 3′) **(Hare and Malay, 2006).** The primers of *mec*A suspected PCR products are equal to (~ 310 bp).

Amplification was done with the following profile: Initial denaturation at 94˚C for 5 min, 40 cycles as follow: denaturation at 94˚C for 30 s, annealing at 55˚C for 30 s and extension at 72˚C for 30 s, followed by final extension step at 72˚C for 5 min. The PCR products were electrophoreted on 0.5% agarose gel and visualized by ethidium bromide staining. Gene Ruler 50 bp DNA ladder (Fermentas) was used as a molecular weight standard. PCR was performed on *S. aureus* ATCC 33591 (positive control) and *S. epidermidis* (locally isolate, negative control).

RESULTS

56 bovine milk samples were cultured for *S. aureus* was isolated from 27(48%) of the 56 samples and total MRSA isolates which was isolated from *S. aureus* were 7 (25%).

Isolation and biochemical characterization of *S. aureus*:

From all the 56 samples, 33 samples had growth on Baird parker agar. When those colonies were subcultured , 27samples had growth on the mannitol salt agar which is a selective media for *S. aureus* .When those colonies were subjected to biochemical characterization which involves Gram staining, hemolysis on blood agar, coagulase and catalase test, all 27samples showed positive result for all the three tests.

Selective isolation of MRSA on ORSAB media:

Only 18(67%) samples that give result on ORSAB (Oxacillin Resistant *S. aureus* Base) media, which is a chromogenic agar for isolation of methicillin resistant *S. aureus.*

Susceptibility of *S. aureus* isolates:

Overall, 19 (70%) of the *S. aureus* isolates were methicillin resistant according to break points of CLSI 2016.

PCR amplification:

Based on multiplex PCR amplification results only seven (25%) samples out of 27 samples, which was identified as *S. aureus* by 16SrRNA, were positive for *mec*A gene. (Table 1) PCR amplification of *16S* rRNA and *mec*A gene demonstrating amplicons of 228 and 310 bp products respectively Fig. (1).

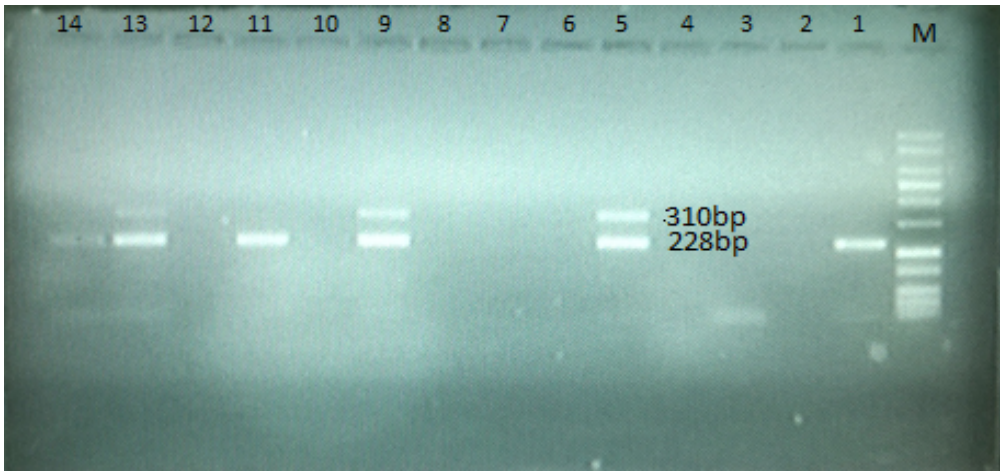

Fig. (1): Gel electrophoresis shows multiplex PCR for *16S* rRNA gene (228bp), *mec*A gene fragments of *S. aureus* (310bp), M: 50bpDNA marker, lane :1, 5,9,11,13,14:*16S*rRNApositive, lane:5,9,13:*mec*A positive.

(Table1): Sources and characteristics of MRSA isolates (n=7) in this study. *Mec* A: gene encoding methicillin resistant *staphylococcus aureus* type A."FOX: Cefoxitin, OX: Oxacillin, PB: Polymyxin B, FD: Fusidic acid, DA: Clindamycin, VA: Vancomycin", ORSAB: Oxacillin Resistant Staphylococcal Base.

Isolate ID	Specimen Source	Phenotypic resistance profile	ORSAB	Genotypic characteristics (16SrRNA,mecA)
1M	**Bovine milk**	**FOX,OX,PB**	**positive**	**Positive**
2M	**Bovine milk**	**FOX,OX,PB,FD,DA**	**positive**	**Positive**
12M	**Bovine milk**	**FOX,OX,PB,FD,DA.VA**	**positive**	**Positive**
13M	**Bovine milk**	**OX,FD**	**positive**	**Positive**
17M	**Bovine milk**	**FOX,OX,PB,FD**	**positive**	**Positive**
24M	**Bovine milk**	**FOX,OX**	**positive**	**Positive**
48M	**Bovine milk**	**OX,FD**	**positive**	**Positive**

DISCUSSION

Food-borne diseases are an important public health problem as it not only affects human health, but also has a significant impact on economic and trade issues. The global changes affecting population growth,lifestyle, international food trade, food production and processing, agricultural, animal husbandry practices, and antimicrobial resistance, have posed a threat to the emergence of food borne diseases. Food-borne diseases, especially dairy products infections, are not limited to the third world countries. Even in developed countries, it has been reported that around 2%-6% of the bacterial outbreaks, in which the food vehicle is known, were related to milk and dairy products **(De Buyser *et al.,* 2001).** Improper food handling and unhygienic practices among food handlers during production, processing and distribution, have contributed to food poisoning episodes **(Angelillo IF *et al.,* 2000).** The incidence of notifiable food-borne diseases, namely, cholera, typhoid, food poisoning, hepatitis A and dysentery, is less than 5/100 000 population sporadic in nature, and outbreaks

are confined to certain areas only **(Morteza, 2010).** Methicillin-resistant *Staphylococcus aureus (MRSA)* can cause a number of human diseases, ranging in severity from minor to life-threatening infections. Handling/consumption of food contaminated with MRSA are potential vehicles of colonization or infections for humans **(EFSA, 2009),** thus the monitoring of food for the presence of MRSA is mandatory in order to better assess the foodborne risk. Hence, this study was conducted to isolate *S. aureus* and determine the prevalence of antimicrobial resistance among the isolates from the raw bovine milk in Egypt. In the present study, the rate of *S. aureus* isolation in raw bovine milk was investigated and the results out of 56 samples were 27(48%) samples were positive *S. aureus* by phenotypic and genotypic detection. While disc diffusion results show that 19 (70%) samples of isolated *S. aureus* were MRSA and only 7 (25%) samples were MRSA by genotypic detection by *mec*A gene. In this study, the prevalence of MRSA in raw milk was 25%, which considered high compared the low prevalence reported by other authors in Europe and the USA. Studies from Hungary, UK, Germany and Belgium have revealed a prevalence of 0%, 0.3%, 4.4% and 7.4% respectively **(Peles *et al.,* 2007; Kreausukon *et al.,* 2012; Paterson *et al.,* 2012; Vanderhaeghen *et al.,* 2010).** A study on Minnesota (USA) reported two MRSA-positive samples out of 150 raw milk samples **(Haran *et al.,* 2012).** Other authors investigating the presence of MRSA in raw cow's milk in other US States **(Erskine *et al.,* 2002; Virgin *et al.,* 2009)** reported similar findings**.** A low prevalence (1.4%) of MRSA in raw cow's milk was also reported in China **(Wang *et al.,* 2014).** Researchers from Africa reported a prevalence of MRSA in raw milk ranging from 4.8% in Nigeria to 8.6% in Egypt **(Karmal *et al.,* 2013, Umaru *et al.,*2013).** With regard to the methods used in our study, the phenotypic assays used for the isolation and the identification of MRSA from the raw milk samples revealed a high level of specificity. The chromogenic medium (ORSAB®OXOID) used for the detection and the presumptive identification of MRSA was able to reveal correctly all the MRSA strains isolated from the milk samples analysed. Similar results were obtained with the cefoxitin disc diffusion test, considered the preferred phenotypic method to predict the presence of *mec*A-mediated oxacillin resistance in *Staphylococcus aureus* **(CLSI, 2016).** The further genotypic characterization confirmed the identity of the strains as MRSA, thus the use of these methods is very useful as a first step in the isolation and presumptive identification of MRSA from raw milk sample. **(Alipour *et al.,* 2014).** Thus in our study we combined both genotypic and phenotypic methods for more accurate MRSA detection.

It is well known that, the antimicrobial susceptibility of MRSA isolated from human and environmental sources is very variable, and this is of concern in human and veterinary therapy. Moreover, MRSA strains are frequently multidrug-resistant. In this study, all MRSA isolates were resistant to between three or more antimicrobial groups, revealing a variable rate of resistance to vancomycin, teicoplanin, polymxin B, oxacillin, cefoxitin, erythromycin and clindamycin (Table 1). Our findings are comparable with those of other authors, revealing the widespread diffusion of multidrug-resistant MRSA strains of bovine origin worldwide **(Haran *et al.,* 2012; Mi Nam H. *et al.,* 2011; Wang *et al.,* 2014).** In conclusion, the presence of MRSA in food of animal origin poses a potential risk of infection and/or colonization to humans, both through direct contact and through food consumption. Thus, additional research is required to better understanding the ecology and evolution of bacterial resistance to antimicrobial agents in the environment as a whole.

Conflict of interest statement:

We declare that we have no conflict of interest.

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