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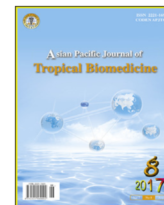

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Molecular characteristics, antibiogram and prevalence of multi-drug resistant *Staphylococcus aureus* (MDRSA) isolated from milk obtained from culled dairy cows and from cows with acute clinical mastitis

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ABSTRACT

Objectives: To study the molecular characteristics, antibiogram and prevalence of multi-drug resistant *Staphylococcus aureus* (*S. aureus*) (MDRSA) isolated from milk obtained from culled dairy cows and from cows with acute clinical mastitis.

Methods: Bacteria were cultured from 188 quarter milk samples obtained from cows before culling ($n = 139$) and from cows affected with acute mastitis ($n = 49$) belonging to 10 dairy farms. The bacteria were identified using colony morphology, Gram staining and biochemical characteristics. *S. aureus* isolates were then subjected to molecular characterization using PCR targeting *16S rRNA* and *mecA* gene to identify Methicillin resistant *S. aureus* (MRSA). The antibiogram of all isolates was performed using the Kirby–Bauer disk diffusion method against 10 commonly used antibiotics in dairy farms.

Results: *S. aureus* was isolated from 19 (13.7%) samples obtained from culled cows and 11 (22.4%) samples obtained from cows with acute mastitis. In both culled cows and cows with acute mastitis, *in vitro* antibiogram revealed that 100% of *S. aureus* isolates were resistant to erythromycin, penicillin G, streptomycin, doxycyclin, and trimethoprim/sulpha. The prevalence of MRSA in milk of culled cows and cows with acute mastitis was 26.3% and 18.2%, respectively, with an overall prevalence of 3.7% among all samples. All MRSA isolates were completely resistant to all tested antibiotics. All MRSA isolates were positive for the presence of the *mecA* gene.

Conclusions: MRSA carrying the *mecA* gene were isolated from mastitic milk from dairy cows in Jordan for the first time. MRSA may pose a potential health risk to the public, farm workers and veterinarians.

1. Introduction

Mastitis is one of the most clinically and economically important diseases in dairy cows [1–6]. The prevalence and causative agents of mastitis in dairy cows in Jordan have been reported previously [5–7]. In one study, the prevalence of clinical mastitis in pre-calving secretions, colostrum and milk in Jordan was reported at about 57% (89 of 156), 22% (40 of 180) and 19% (56 of 298), respectively [5]. The prevalence of subclinical mastitis was 94% (239 of 254 milk samples) [5]. In Jordan, *Staphylococcus aureus* (*S. aureus*) is considered the most common cause of clinical, subclinical and chronic mastitis in dairy cows with a prevalence rate ranging from 40% to 60% [5–7]. Other bacteria that have been isolated from clinical and subclinical mastitis cases include *Escherichia coli (E. coli)*, *Streptococcus* non-agalactiea and coagulase negative *Staphylococci* [8].

Systemic and intramammary antibiotic administration is a common practice used to treat mastitis worldwide [9]. Long history of using common antibacterial agents in dairy farms to combat infectious diseases including mastitis has resulted in the emergence of resistant bacterial strains. In one study in Jordan, 87.4% and 84.5% of isolates were resistant to sulfa/trimethoprim and penicillin G, respectively [8]. Similar results

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were reported against ciprofloxacin, gentamicin, lincomycin, streptomycin and penicillin G [5,6].

Resistant *S. aureus* strains to Methicillin or (MRSA) have been characterized by the presence of *mecA* gene that carries resistance to β-lactam antibiotics [10]. However, the resistance patterns of isolated *S. aureus* from cases of mastitis in recent years indicate the emergence of multi-drug resistance *S. aureus* (MDRSA) strains which may impose a significant threat to human health in Jordan [5–8]. Therefore, the aims of this research were to study the molecular characteristics, antibiogram and prevalence of multi-drug resistant *S. aureus* (MDRSA) isolated from milk obtained from culled dairy cows and from cows with acute clinical mastitis.

2. Materials and methods

2.1. Collection of milk samples

Quarter milk samples were collected aseptically from cows before culling from 10 dairy farms located in Northern Jordan. Milk samples were also collected from cases of acute mastitis in these farms during the period between January and December 2016. Samples were placed in sterile plain blood tubes and transported to the laboratory on ice within 2–4 h after collection for analysis.

2.2. Bacterial culture and identification

Blood and MacConkey agar plates were used to initially culture the bacteria from milk samples as described previously [11]. After inoculation, plates were incubated aerobically at 37 °C for 24 h [11]. Initial identification of bacteria was based on colony morphology and Gram's staining characteristics. Further classification of bacteria was carried out using catalase and coagulase reactions. Bacteria with large hemolytic zone and catalase and coagulase positive were considered *S. aureus*. *Staphylococcus* colonies with negative coagulase activity were considered coagulase-negative staphylococci (CNS). All staphylococci isolates were then confirmed using the API ID 32 Staph (Bio Merieux, France). Gram-negative, lactose fermenter on MacConkey agar and oxidase negative bacteria were identified as *E. coli*.

2.3. Antibiogram

To determine the antibiogram of isolated bacteria, the Kirby–Bauer disk diffusion method was used with modification [12]. Ten commonly used antibiotics in dairy farms were used namely: ampicillin (10 μg), penicillin G (10 IU), streptomycin (10 μg), gentamycin (10 μg), erythromycin (15 μg), ciprofloxacin (5 μg), oxytetracycline (30 μg), trimethoprim/sulphamethoxazole (25 μg), enrofloxacin (5 μg), and doxycyclin (30 μg). Sensitivity results were classified as susceptible or resistant. In this study, we modified the classification of the sensitivity results from susceptible, intermediate, or resistant to only susceptible or resistant. We considered the result ‘intermediate’ as resistant because of our field clinical observation of the widespread resistance of bacteria against antibacterial therapy.

2.4. Determination of methicillin-resistant S. aureus

Isolated strains of *S. aureus* were further examined to identify methicillin-resistant *S. aureus* (MRSA) [13]. Bacteria were inoculated on Tryptone soya broth (TSB) with 10% sodium chloride salt (TSB-S) (Hi-media) and incubated at 37 °C for 24–48 h. Samples with turbid appearance were subcultured on mannitol salt agar (Hi-media) with 6 mg/L oxacillin (O-MS agar) and incubated for 24–48 h at 37 °C. Bacterial growth on mannitol salt agar with round-shaped, golden-yellow, or pale-colored colonies were considered oxacillin-resistant staphylococci. Grown bacteria on mannitol salt agar were further confirmed using Gram's staining and catalase test. Gram-positive bacteria that was oxacillin resistant and mannitol fermenter were considered as MRSA isolates.

2.5. Identification of S. aureus using PCR targeting 16S rRNA

Isolated strains that were classified as MRSA were further confirmed by *S. aureus*-specific polymerase chain reaction (PCR) targeting 16S rRNA using the primer sequences: F-5′ GTAGGTGGCCAAGCGTTATCC 3′ and R-5′ CGCACATCAGCGTCAG 3′ [13]. The PCR reaction was performed with 1× PCR buffer (pH-8.3; 15 mM $MgCl_2$), 5 mM of deoxynucleotide triphosphates (dNTPs), 20 pmol of forward and reverse primers, 1 U Taq DNA polymerase, 2 μL of DNA template per reaction in a final volume of 25 μL. The PCR cycling conditions were optimized with initial denaturation at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 50 °C for 1 min, and extension at 72 °C for 1 min with the final extension at 72 °C for 10 min.

2.6. Detection of mecA gene in isolated S. aureus

The presence of *mecA* gene in *S. aureus* isolates was also evaluated as previously described [13]. Briefly, a 25.0 μL volume of PCR reaction mixture contained 1.0 μL of genomic DNA, 12.5 μL of PCR master mix, 7.5 μL PCR H_2O and 2 μL each of *mecA* primers. The following primers were used: F-5′ TCCAGATTACAACTTCACCAGG 3′ and R-5′ CCACTTC ATATCTTGTAACG 3′ with the amplicon size of 162 bp. The PCR cycling conditions were: denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, extension at 72 °C for 1 min, final extension at 72 °C for 5 min, and cooling to 4 °C.

The products of PCR amplification were analyzed using agarose gel electrophoresis (Hi media, India) on tris acetate-ethylene diamine tetra acetic acid buffer containing ethidium bromide (0.5 μg/mL). Gels were visualized and photographed under gel documentation system (Biorad).

3. Results

In total, 188 quarter milk samples were collected from culled dairy cows (139) and from clinical mastitis cases (49). Culled cows were removed from the herd because of different reasons but not necessarily because of mastitis. *S. aureus* was revealed from 19 (13.7%) milk samples obtained from culled cows and 11 (22.4%) milk samples obtained from acute mastitis cases. Coagulase-negative staphylococci (CNS) were revealed from 6 samples obtained from culled cows and 5 samples obtained from cows with acute mastitis. *E. coli* was revealed from 1 sample obtained from culled cows and 18 samples obtained from cows with mastitis. No growth was reported in 113 samples obtained from culled cows and 15 samples obtained from cows with acute mastitis.

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