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Methicillin Resistant *Staphylococcus aureus* (MRSA) Isolated from Skin Infections in Poultry (Bumble Foot)

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Abstract
This study was aimed to isolate and identify of MRSA from infected skin of poultry, and testing the susceptibility of the isolates to different antibiotics especially methicillin, and confirmation of mecA gene encoded to methicillin resistance. Therefore, a total of 50 skin swabs were collected from the suspected poultry foot cases at different farm in Wasit province / Iraq, during November (2021) to January (2022). The swab samples were grown and purified, and then, the isolates were tested biochemically and phenotypically and subjected finally to antimicrobial sensitivity testing and molecular confirmation using conventional PCR assay. Examination of suspected S. aureus isolates revealed that the number of grown and fermented isolates on Mannitol salt agar (MSA) was 48%, and 12% for non-fermented MSA isolates; while, 40% of isolates were not grown on MSA. The colonies of suspected S. aureus were appeared on MSA as large, convex, shiny to opaque, circular, and golden-yellowish color; while on blood agar, colonies had signif, rounded, smooth, and grayish appearance. All grown and fermented isolates were showed positive reactivity to Catalase (100%) and Bound Coagulase (87.5%) test

Keywords: *mecA* gene, Polymerase chain reaction, Antimicrobial sensitivity testing, VITEK2 system, Biochemical testing, Iraq.

Introduction

Staphylococci are members of the family *Staphylococcaceae*, which appeared as a Gram positive and non-motile, non-spore-forming cocci, which can survive under many physiologic environmental conditions (1). The bacterium is characterized typically by smooth raised yellow to golden yellow color colonies, considers as one of the most opportunistic pathogens in human, as well as in veterinary medicine (2)(3). Interestingly, *S. aureus* has virulence factors that assist the bacterium to bind to host tissues, break down the host immune barrier, and invading the tissue causing sepsis and inducing the toxin-mediated syndromes (4)(5). Also, it has different mechanisms for generating resistance included the incorporation of compound structures such as the alteration of the target drug site, enzymatic inactivation and mutation at the target drug site, and horizontal gene transfer of resistance determinants (6)(7). At field, multiple antimicrobial resistances in *S. aureus* have raised a significant global veterinary and public problem since the organism results in mild infections such as abscesses of skin, impetigo and folliculitis; and severe infections such as the necrotizing fasciitis, pyomyositis and necrotic pneumonitis (7)(8). *Staphylococcus aureus* resistant to methicillin (MRSA) is the major pathogen responsible for infection in hospitals / communities, and livestock (9).

Skin is an important initial streak of protection against attacking bacterial pathogens like those found in the external environment and opportunistic microorganisms (10).

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Pododermatitis or bumble foot, general term refers to any inflammatory or degenerative condition, becomes a frequently seen disease in the avian foot in particular chicken and turkey (11)(12). In general, the condition ranged from subclinical and clinical mild or chronic infections (13). Additionally, there are several predisposing factors participated significantly in development of bumble foot including damage to the plantar surface of the foot, severe poxvirus lesions with secondary bacterial infections, hypothyroidism and hepatic dysfunction, stress, leg conformation or abnormalities, leg or foot injuries, overgrown toenails, fighting among flock members, poor husbandry, poor nutrition and vitamin A deficiency, coarse floor surfaces, inactivity, and obesity (11)(13).

This study was aimed to isolate and identify of MRSA from infected skin of poultry, and testing the susceptibility of the isolates to different antibiotics especially methicillin, and confirmation of *mecA* gene encoded to methicillin resistance.

Materials and methods

Ethical approval

This study was licensed by the Scientific Committee of the College of Veterinary Medicine, University of Wasit (Wasit, Iraq).

Preparation of reagents, solutions, and media

All study materials were either purchased [Gram stain, Normal saline and phosphate buffered saline (PBS)], prepared according to previously studies [Catalase reagent, and Human plasma (14) or following the manufacturer instructions [blood agar medium, Nutrient agar, Nutrient Broth, Manitol salt agar (MSA), Muller-Hinton agar, and Brain Heart infusion agar].

Samples collection and preparation

Totally, 50 skin swabs were collected from the suspected poultry foot cases at different farm in Wasit province / Iraq, during November (2021) to January (2022). All samples were transferred immediately to the laboratory on moistened sterile cotton-tipped applicator in 5 ml of freshly prepared nutrient broth in a test tube under the complete hygienic conditions and then incubated at 37°C for 24 hrs. Then, the inoculum was streaked on MSA plates, incubated for 24 hrs at 37°C, Gram stained, and examined microscopically (15) (16).

Biochemical testing and phenotyping

Catalase, Coagulase, Tube and Slide tests were carried out as described by other studies (15)(17). Based on the zone of hemolysis produced by culturing on the Blood agar medium, the isolates were classified as α (wide complete zone with blurred edges), β (wide incomplete zone with sharp edges), δ (narrow incomplete zone with blurred edges) and γ (without zone), (18). Automated VITEK2 system (bioMérieux SA, France) was used for identification of *Staphylococcus* spp.

Antimicrobial susceptibility test

According to **Doulgeraki *et al.*** (19), antibiotic susceptibility test was performed using the disc diffusion method on Mueller–Hinton Agar.

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Molecular detection of S. aureus isolates

Conventional polymerase chain reaction (PCR) was selected to confirm of *S. aureus* isolates. Initially, the KAPA Express Extract (KAPA BIOSYSTEMS, South Africa) was applied to extraction the DNAs from the isolates. The extracted DNAs were checked for purification and concentration using the Nanodrop system (Thermo-Scientific, UK). Then, the mastermix tubes were prepared using the KAPA Taq Ready Mix (KAPA BIOSYSTEMS, South Africa) at a total volume of 20µL through targeting the *mecA* gene [(F: 5´- AAAATCGAT GGTAAAGGTTGGC -3´) and (R: 5´- AGTTCTGCAGTACCGGATTTGC -3´)]. The Thermal Cycler conditions were included 1 cycle initial denaturation (94ºC / 5 min); 40 cycles of denaturation (94ºC / 30 sec), annealing (55ºC / 30 sec) and extension (70ºC / 1 min); and 1 cycle final extension (72ºC / 5 min). The PCR products were analyzed by electrophoresis of the stained Agarose gel 2% with Ethidium Bromide at 100 volt and 80 Am for 1 hour. The positive samples were detected at an amplicon size of ~533.

Statistical analysis

All obtained data were analyzed using the Chi-square (x^2) test, *t-test*, and ANOVA of the SAS (2012) Software. Variation in large horizontal and small vertical letters refers to significant differences at $p<0.05$.

Results

Growth rate of S. aureus on MSA

The suspected *S. aureus* isolates revealed that the number of grown and fermented isolates on MSA was 24 (48%), and those grown and non-fermented MSA was 6 (12%); while, 20 (40 %) isolates were not grown on MSA (**Table 1**).

Table (1): percentages of suspect *S. aureus* isolate from skin infections of poultry

Total No.	Growth on ferment MSA		Growth on non-ferment MSA		No growth	
	No.	%	No.	%	No.	%
50	24	48 ^{A}	6	12 ^{C}	20	40 ^{B}

Characteristics of S. aureus colonies

The colonies of suspected *S. aureus* were appeared on MSA as large, convex, shiny to opaque, circular and golden-yellowish color that reflecting the mannitol fermentation; while on blood agar plates, colonies had the large, rounded, smooth and grayish appearance.

Microscopic examination

Staining of grown suspected colonies with Gram stain was demonstrated the Gram positive, pairs or grapelike clusters cocci.

Biochemical tests

In this study, all grown and fermented *S. aureus* isolates (Total No: 24) from skin of infected poultry were showed a positive reactivity to Catalase (100%) and Bound Coagulase (87.5%) tests but not for tube Coagulase test (0%), (**Table 2**).

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Table (2): Positive results of biochemical testing of grown and fermented *S. aureus* isolates on MSA

Total No.	Test [No. (%)]		
	Catalase	**Tube Coagulase**	**Bound Coagulase**
24	24 (100%) ^{A}	24 (100%) ^{A}	21 (87.5%) ^{B}

VITEK2 system

Among 30 *Staphylococcus*, fermented and non-fermented, isolates grown on MSA, the VITEK2 system demonstrated the species of these isolates (**Table 3**). Significantly, all fermented isolates were being positives as S. *aureus* (100%). While, the non-fermented isolates demonstrated that 2 (33.33%) isolates were *S. sciuri*, and 4 (66.67%) isolates were *S. saprophyticus*.

Table (3): Results of *Staphylococcus* isolates grown on MSA by VITEK2 system

Isolate	Total No.	Species [No. (%)]		
		S. aureus	***S. sciuri***	***S. saprophyticus***
Fermented	24	24 (100%) ^{A}	0 (0%) ^{B}	0 (0%) ^{B}
Non-fermented	6	0 (0%) ^{C}	2 (33.33%) ^{B}	4 (66.67%) ^{A}
Total No.	30	24 (80%) ^{A}	2 (6.67%) ^{B}	4 (13.33%) ^{B}

Antimicrobial susceptibility

The findings of antimicrobial susceptibility for the confirmed S. *aureus* isolates by VITEK2 revealed that all isolates were sensitive to Azithromycin (100%), Nitrofurantoin (58.33%), Clindamycin (41.67%) and streptomycin (20.83%), (**Table 4**).

Table (4): Results of antimicrobial susceptibility for *S. aureus* isolates (Total No: 24)

Antibiotics	Concentration	Sensitivity	Resistance	*p-value*
Azithromycin	15 mcg	24 (100%) ^{Aa}	0 (0%) ^{Be}	<0.0029
Ampicillin	25 mcg	0 (0%) ^{Bf}	24 (100%) ^{Aa}	
Nitrofurantoin	100 mcg	14 (58.33%) ^{Ab}	10 (41.67%) ^{Bd}	
Trimethoprim	10 mcg	0 (0%) ^{Bf}	24 (100%) ^{Aa}	
Amoxicillin / Cloxacillin	35/5 mcg	0 (0%) ^{Bf}	24 (100%) ^{Aa}	
Carbenicillin	25 mcg	0 (0%) ^{Bf}	24 (100%) ^{Aa}	
Doxycycline	10 mcg	0 (0%) ^{Bf}	24 (100%) ^{Aa}	
Bacitracin	10 mcg	0 (0%) ^{Bf}	24 (100%) ^{Aa}	
Chloramphenicol	30 mcg	3 (12.5%) ^{Be}	21 (87.5%) ^{Ab}	
Clindamycin	10 mcg	10 (41.67%) ^{Bc}	14 (58.33%) ^{Ac}	
Cloxacillin	10 mcg	0 (0%) ^{Bf}	24 (100%) ^{Aa}	
Streptomycin	30 mcg	5 (20.83%) ^{Bd}	19 (79.17%) ^{Ab}	
Methicillin	10 µg	0 (0%) ^{Bf}	24 (100%) ^{Aa}	
p-value		<0.0016		-

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Cefoxitin sensitivity testing

As described by **Kirby and Bauer** (20), the disc-diffusion method was carried out on *S. aureus* isolates to estimate their sensitivity to Cefoxitin at a concentration of 30 mg. Among all study samples, the findings revealed that only 14 (58.33%) isolates were resistance to Cefoxitin (30 mg) and considered to be MRSA with a diameter inhibition zone of ≤ 21 mm.

Molecular PCR assay

Targeting the *mecA* gene, the positive findings for testing a totally 14 MRSA *S. aureus* isolates by PCR assay was 5 (28.57%), (**Figure 1**).

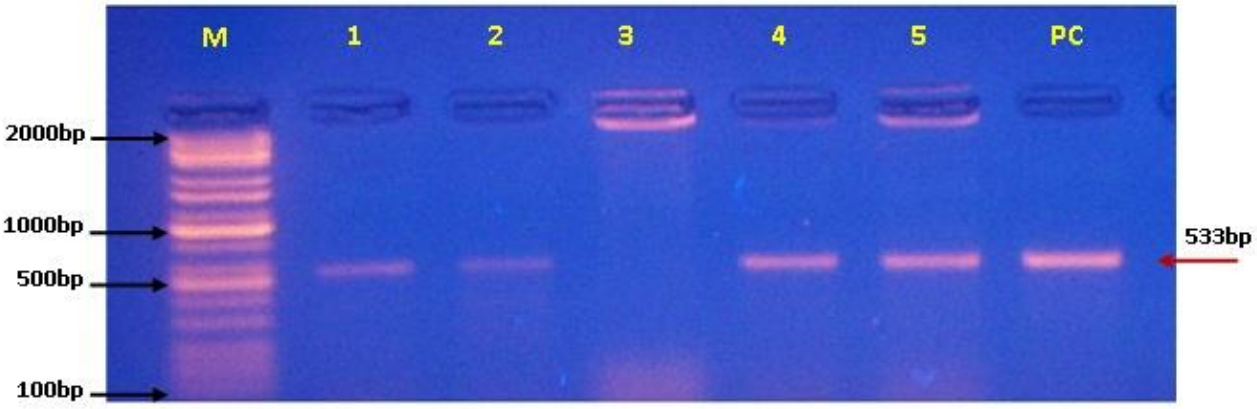

Figure (1): Agarose-gel electrophoresis (2%) in targeting the *mecA* gene at ~533bp product size; M: Ladder marker (100-2000bp); Lanes (1, 2, 4 and 5): Representative positive samples; Lane (3): Negative sample; PC: Positive control

Discussion

Based on data of present study, we confirm that some MRSA isolates found in skin infections of poultry lacking the *mecA* gene, and this may be attributed to different factors such as types of strains if MRSA or MSSA, specificity and sensitivity of methodology, number of samples, type of clinical samples, and geographical difference (21)(22)(23). However, this study determined that MRSA is associated significantly with the skin infections of poultry. This study revealed different percentage of susceptibility to different antibiotics. The history of *S. aureus* to antibiotic resistance could be related to 3 greatly used antibiotics are vancomycin, methicillin, and penicillin (24)(25). Penicillin resistance had been recorded for first, shortly, post introducing of penicillin for clinical practices in 1940 (26). This resistance to penicillin is achieved by producing of β-lactamase enzyme that hydrolyzes the β-lactam ring of the penicillin molecule, effectively interrupting penicillin's cell wall disruption (19). Penicillin-resistant *S. aureus* (PRSA) is emerged in the 1950s and caused pandemic infections in hospitals, and are considered as the first wave of antibiotic resistance in *S. aureus* (27). With the usage of methicillin, the pandemic PRSA is controlled (28). Methicillin is a semisynthetic β-lactamase resistant β-lactam derivative, which introduced to treat of PRSA infections in 1959 (29)(30). Only one year later, the first case of MRSA and confirmed to be mediated by the *mec*A gene that encodes the penicillin-binding protein 2A (PBP2A) that has a low-affinity for β-lactam antibiotics (31). The *mec*A gene located within the Staphylococcal Cassette Chromosome *mec* (*SCCmec*), allows a bacterium to be resistant to antibiotics such as

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methicillin, penicillin and other penicillin-like antibiotics; suggesting that this gene is be able to transfer through horizontal gene transfer (HGT) among various strains (31)(32). However, an improper use of antibiotics creates several problems such as the emergence of bacterial resistance to antibiotics, and an occurrence of methicillin resistance as observed frequently in recent (33)(34)(35).

In most routine microbiological settings, the detection of methicillin resistance among staphylococcal isolates is based on phenotypic assays such as the disk diffusion test and identification of the minimum inhibitory concentration (MIC), (36)(37). Genetic confirmation of positive findings based on detection of the *mecA* gene has also been (38). MRSA strains were multi-drug resistant which might be due to production of betalactamase and PBP2a (penicillin binding protein), (39)(40). The disk diffusion and broth dilution method require at least 24 h for evaluation of the results (36)(41). However, the detection of antibiotic resistance genes such as *mecA* gene by PCR techniques is considered the gold standard method since infections by MRSA require rapid and accurate in most routine microbiological settings (42).

Significant correlation between phenotypic antibiotic susceptibility patterns and genotypic analysis by PCR was also reported (1)(43). The critical parameters for success of PCR to detect the multidrug resistant bacteria as MRSA are multiple and depended on its cost, reliability, accuracy and sensitivity (44)(45). In veterinary microbiology, several applicable techniques can be applied to diagnosis and characterizing of *S. aureus* strains that having a particular importance to be detected, prevented and controlled, since these strains express many potential virulence related-factors like surface proteins that promote colonization of host tissue surface factors and inhibit phagocytic engulfment, capsule protein A (46); in addition to biochemical properties that enhance their survival in phagocyte, catalase production (47), immunological disguises, protein A and clotting factor (48); inherent and acquired resistance to antimicrobial agent (49); and membrane damaging toxins like hemolysis that lyseeukaryotic cell membranes (50).

Conclusion

This study confirms the usefulness of PCR for the detection of antibiotic resistance genes associated with *S. aureus* infections since it offers a rapid, simple, and accurate identification of antibiotic resistance profiles. Combination of high sensitive and specific laboratory methods must be aimed for detection the multidrug resistant bacteria.

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