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Research article

Detection of methicillin resistance of *Staphylococcus aureus* gene isolates from contaminated cow's milk

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Abstract

One hundred samples of raw cow milk was collected from different market centers in Basrah city during a period from 16 June to 21 August 2017 and analyzed for the presence of Staphylococcus aureus. The biochemical test revealed that 26 isolates on mannitol salt agar were (S. epidermidis), and 32 isolates were (S. aureus) and give a positive result to coagulase, catalase urease and citrate utilization tests, whereas negative results to indole test. The high rate of S. aureus was observed in Abu Skair 55% followed by Al-Dair 40%, Al-Zubair 35%, Al Karma 25%, and Al-Hartha 5%. The presence of the mecA gene by using pairs of primers revealed that 6.25% of S. aureus strain contain that gene. By using the disc diffusion method, all tested isolates revealed high sensitivity toward chloramphenicol and erythromycin, intermediate sensitivity to tetracycline and novobiocin, and 100% resistance toward methicillin and amoxicillin.

Keywords: *Staphylococcus aureus, mecA gene*, Cow, Milk.

Introduction

Milk and dairy products of a cow are crucial foods for human beings. They are universally recognized as a complete diet due to their essential components (1). It available to our mass is lower in food value due to high prevalence of mastitis in dairy animals (2). It is a major consumption in human diet around the world. It is a good medium for growth of many microorganisms. The quality of milk is considered necessary to consumer's health. It was found that all cases of dairy illness belong to bacterial origin. Consumption of milk is involved in infectious diseases such as Salmonella, Listeria monocytogenes, Staphylococcus aureus, Campylobacter, and Yersinia (3). Milk normally has a varied microflora collecting from interior and exterior surfaces of the animal (4). It is susceptible to contamination by different microorganisms which result in infection and risk to consumer's health. There are different disease can be transferred from milk to human such as tuberculosis, brucellosis, typhoid, and listeriosis (5). Although microbial load in fresh milk is very low (less than 10-3 CFU/L), but this level may increase up to 100 fold if milk is stored at room temperature (6). Staphylococcus aureus is an important bacterium due to toxin-mediated virulence, invasiveness, and antibiotic resistance (7). The spectrum of S. aureus infection ranges from pimple and furuncles to toxic shock syndrome and sepsis (8) S. aureus also is important mastitis pathogens in animals (9). Most of which depend on numerous virulence factors. On the other hand, Staphylococcal food poisoning rely on one single type of virulence staphylococcal enterotoxins factors (7).

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Identification

Isolates were identified by sub culturing on Mannitol salt agar, colony morphology, staining, and submitted to different biochemical tests (coagulase test, catalase test and urease test, indole test) as described previously by (10, 11, 12); respectively. Citrate utilization test using Simon citrate agar. Then molecular detection of gene.

Antimicrobials susceptibility testing

Disc dilution method used as antimicrobial susceptibility testing as described by (13).

Extraction of bacterial DNA:

Bacterial DNA was extracted as described by manufacturer's bacterial extraction kit. Isolates were grown in 5 ml of brain heart infusion broth overnight at 37°C.

Primers

One primer pair was used. PCR technique performed using this primer pair to identify (*mecA*) gene (14) as show in Table (1)

Materials and Methods

Ethical approval

The Animal Ethical Committee of Veterinary Medicine College, University of Al-Qadisiyah, Iraq, has approved the present study under permission No: 328

A total of 100 cow's samples were collected in the period extending from 16 June to 21 August 2017 from different sites of Basrah province markets. Milk was collected randomly under sterile conditions. Milk was transported to laboratory in a cooling box.

Culturing

After enrichment of samples with peptone water for 18h, a loop-full of culture was streaked into plates of Mannitol salt agar. The plates were then incubated for 18h at 37°C and checked for the growth of typical colonies of S. *aureus.*

Table (1): Oligonucleotide primers for PCR amplification

Primer	Sequence (5´→3´)	S	P(bp)	Reference
F	GTAGAAATGACTGAACGTCCGATGA	*mecA* gene	310	14
R	CCAATTCCACATTGTTTCGGTCTAA			

S: Specify; P: Product size

PCR design and amplification conditions

The reaction mixture was prepared in a total volume of 25µl for *mecA* gene Table (2). Amplification of the *mecA* gene was achieved on the thermocycler using the reagent Table (3).

Table (2): PCR conditions

Stage	Step	Temperature	Time	No. of cycle
I	Initial denaturation	95ºC	5 min.	1
II	Denaturation	95ºC	35 sec.	35
	Annealing	65ºC	30 sec.	
	Extension	72ºC	35 sec.	
III	Final extension	72ºC	10 min.	1

Table (3): PCR design

Reagent	Volume
Green master mix	12.5 µl
Primer forward	2.25µl (0.75µl for each primer)
Primer reverse	2.25µl (0.75µl for each primer)
DNA template	3µl
Nuclease free water	5µl
Total	25µl

Analysis of PCR product for *S. aureus*

The electrophoresis of amplified product was carried out in 1.5 % agarose gel using 7µl DNA ladder as molecular weight marker and 7µl of mPCR reactions at 70 V for 30 min and at 80 V for 20 min. The gel was visualized using UV trans illuminator and photographed.

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Results

Isolation and characterization of colonies on mannitol salt agar

On mannitol salt agar, the growing colonies were clear pink to red colonies with no yellow color change in the medium (mannitol non-fermenter) or yellow to golden colonies and changed mannitol salt agar from pink to yellow color (mannitol fermenter).

Biochemical tests

Specific biochemical tests were used for the detection of isolates on mannitol salt agar. These tests were coagulase, catalase, citrate utilization, and indole and urease activity. The result gives coagulase positive, catalase positive, citrate positive, urease positive and indole negative considered as *Staphylococcus aureus*. The other colonies considered as *S. epidermidis* according to their characteristics as shown in Table (4). The high rate of *S. aureus* was observed in Abu-Skair 55% followed by Al-Dair 40% and Al-Zubair 35%, Al-Karma 25%, and Al Hartha 5% respectively Table (5).

Table (4): Comparison of the isolates according to the biochemical tests

Biochemical tests	Results	*S. aureus*	*S. epidermis*
Urease test	Positive	32	26
Simon's citrate	Positive	32	26
Indole test	Negative	32	26
Coagulase test	Positive	32	
	Negative		26
Catalase test	Positive	32	26

Table (5): Number and percentage of *S. aureus*, *S. epidermidis* isolated from milk during this study.

Name of Region	No of sample	*S. aureus* No. (%)	*S. epidermidis* No. (%)
Abu Skair	20	11(55)	3(15)
Al Dair	20	8(40)	10(50)
Al Karma	20	5(25)	2(10)
Al Hartha	20	1(5)	7(35)
Al Zubair	20	7(35)	4(20)
Total	100	32	26

X^2 =0.0002 (p<0.05)
X^2 =0.0001 (p<0.05)

Extraction of bacterial genomic DNA

Extraction of bacterial genomic DNA was done on 32 isolates S. aureus Figure (1)

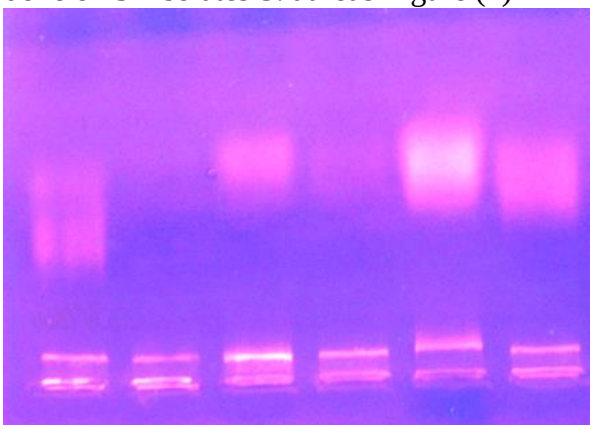

Figure (1): Electrophotogram of total DNA extracted from bacteria, which were identified as *S. aureus* by biochemical tests.

PCR assay

The presence of *mecA* gene (310bp) in *S. aureus* isolates were tested by PCR, only 2/32 (6.25%) were *mecA* positive. However, the remaining 30/32 (93.75%) were negative to create the band of 310 bp specific for *mecA* gene Figure (2)

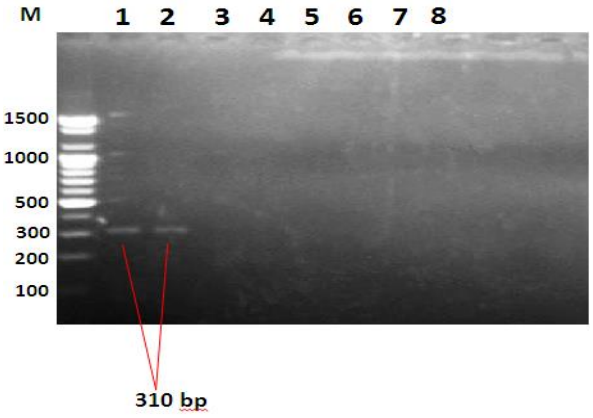

Figure (2): Representative gel showing the presence of *mecA* gene (310bp) separated on 1.5% agarose gel stained with ethidium bromide.

Antimicrobial susceptibility testing for *Staphylococcus aureus* isolates

Antimicrobial susceptibility against six antimicrobial agents was determined by disc diffusion Table (6). Most isolates showed that 100% resistance to Amoxil line and methicillin, while found to

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be 100% sensitive to chloramphenicol and erythromycin. Whereas 100% found to be intermediate to novobiocin and tetracycline.

Table (6): Susceptibility test for *Staphylococcus aureus* isolates (n=2)

Antimicrobial	Percentage		
	Sensitive	Intermediate	Resistance
Amoxicillin	0	0	100
Methicillin	0	0	100
Chloramphenicol	100	0	0
Erythromycin	100	0	0
Tetracycline	0	100	0
Novobiocin	0	100	0

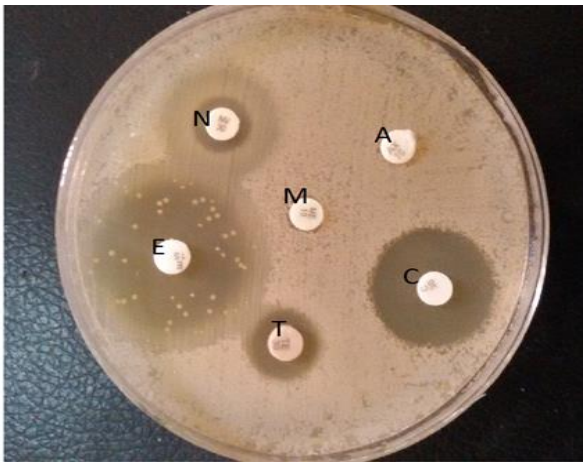

Figure (3): Antimicrobial susceptibility testing for *Staphylococcus aureus* isolates

A=Amoxilline.M=Mcthicillin.C=Chloramphenicol.E=Erythromycin.T=Tetracycline.N=Novobiocin.

Discussion

The methods used for detection depend on enrichment of the samples in peptone water, followed by spreading on selective media, then submitting to biochemical confirmation. These colonies were considered as *S. aureus* and constituted 32% (32/100). On the other hand, the isolates of mannitol non-fermenting were clear pink to red colonies with no yellow color change in the medium and these colonies were considered as *S. epidermidis* and constituted 26% (26/100) (15). In the current study the Isolation rate of S. aureus was similar to that of (16) whose isolates of *S. aureus* from cow milk sample were in percentages of 34.7% ; 38% and 40% respectively .However, lower results were detected by (17); (18) whom isolates of *S. aureus* from cow milk samples were in the percentage of 14.38% and 23% respectively. While the isolation rate of *S. epidermidis* from samples was 26%. This finding is in agreement with previous study (19). Previous studies revealed that *mecA* gene is present in all MRSA strains (20). The primer pair used in the PCR of the present study designed according to the nucleotide sequences of the *mecA* gene, amplified and produced a double-stranded fragment of 310bp. The amplification of *mecA* gene was tested in all suspected methicillin resistant *S. aureus* (MRSA) isolates; 32/100, only 2/32 (6.25%) were *mecA* positive. However, the remaining 30/32 (93.75%) failed to create the band of 310 bp specific for *mecA* gene. This finding is in agreement with a previous study, in which the complete absence *mecA* genes in isolates were phenotypically MRSA suggesting a probability of hyperproduction of lactamase (21). Previously, the absence of *mecA* gene within resistant staphylococcal isolates was also listed (22). The low percentage (6.25%) of methicillin resistant *S. aureus* (MRSA) isolates may be attributed to the fact that there are mechanisms responsible for beta-lactam resistance of MRSA. The use of antimicrobial drugs in food animals may result in transferring of resistance to human. It has been found that antimicrobial drug will be restricted to use in human medicine (23). By using disc diffusion method, two isolates of *S. aureus* submitted for their antimicrobial susceptibility to six antimicrobial agents. The isolates showed that 100% of the isolates resistance to Amoxicillin and methicillin, 100% sensitive to chloramphenicol and

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erythromycin and 100% intermediate to novobiocin and tetracycline .This finding is in agreement with previous study(24) , in which the *S. aureus* was resistant to methicillin, intermediate to tetracycline, and sensitive to chloramphenicol. However, the current study is in disagreement with previous studies (25) in which 75% of *S. aureus* was resistant to Erythromycin and only 4% of *S. aureus* resistant to Novobiocin. In addition, the results of Amoxil line is in agreement with (26).

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