Daignosis of *Staphylococcus aureus* mastitis in bovine in Al-Najaf province by using Polymerases chain reaction (PCR)

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Abstract

This study was conducted to collect 388 milk samples from cows at different villages and townships in Al-Najaf province to examine about *Staphylococcus aureus* mastitis .CMT was used for subclinical mastitis screening ,212(54.6%) milk samples were mastitic .The molecular method (PCR assay) was used to detected the presence (*glpF*) gene in classically diagnosed *S.aureus,* which appeared that 38(92.6%) *S.aureus* mastitis as 13(32.5%) clinical and 25(14.5%) subclinical mastitis .There was high significant incidence of *Staphylococcus aureus* mastitis in left posterior udder quarter rather than others quarters.

Key word: *S.aureus*, bovine mastitis, *glpf* gene

Introduction

Mastitis remains the most common disease of dairy cattle, causing the biggest economic losses to the dairy industry (1). *S.aureus* is among the most common etiologic agents of bovine mastitis (2) *Staphylococcus aureus* is a major pathogen in dairy cattle mastitis (3 , 4, 5) , it causes big financial/economic loss to the dairy industry worldwide, mainly due to reduced milk production and the need to discard contaminated milk (6,7).Iraq have many researches were done on mastitis in cows' herds for detection of causative agents, (8) were isolated just one isolate *S.aureus* from 29 cows suffered from acute mastitis in cows' herd in Al-Sulaymaniya governorate during two years (1978-1979),While (9) recorded the highest percentage to *S.aureus* isolates (36%). (10) had been revealed that *S.aureus* was isolated at 7.64% . (11) at Al-Nasir station of cows, She resulted that *S.aureus* mastitis was (28.73%) **.** (12) show the highest results of isolation of *S.aureus* from mastitic cows were (58%) , while (13) found the percentage of *Staphylococcus aureus* mastitis in cows in Ninevah governorate was 55% .

Materials and methods

Materials

Cultures media:-

1. Blood agar base :
2.Nutrient agar:
3. Mannitol Salt Agar :
4. Brain Heart Infusion agar:
5. Nutrient Broth:
6A. Urea Agar: All media were prepared according to information's of manufactured company .

Reagents :

1. Catalase reagent : According to (14)
2.Oxidase reagent:According to (15)
3.CaliforniaMastitisTest (CMT):It used for detection a subclinical mastitis (16)
4.Coagulase reagent (rabbit plasma): Bacton , Dickinson Company (Spain)
5. Gram Stain :It Prepared according to (15) .
6 . Urea solution (20 %) :

Commercial kits:The commercial kits used in the present study are shown in Table (1)and its appendices, as follow:-

Table (1): Commercial kits used in the present study

No.	Types of kits	Source
1	DNA extraction Kit(1)	Geneid/Korea
2	Green master mix 2X Kit(2)	BIONEER/Korea
3	Primers(3)	BIONEER/Korea

2. Green master mix consist of :-

1	DNA polymerase enzyme (Taq)
2	dNTPs
3	MgCl2
4	PCR loading buffer
5	PCR reaction buffer (pH 8.3)

3.Primers include from:

Target gene	Oligonucleotide	5´- 3´ Sequence	Product length	Reference
glpF	F	caatgggtgtgtttgctgtc	223 bp	(In this study)
	R	agccggtgctgtagagaaaa		

Methods :

1. Clinical study :-

Three hindered eighty eight (388) milk samples collected from clinical mastitic cows(40) and (174) from cows appears healthy (without signs of mastitis) were taken From different areas of Al-Najaf province. Milk samples were collected in sterile tubes (2 tubes) for each sample (one for CMT and physical exam and another for bacteriological test) and a septic technique used for milk samples collection. The procedure for milk sample collection according to (17) . The samples were transported to the laboratory in AL-Qadissiya University by cooling box.

2. Tests that used for examination of milk samples:

A. California Mastitis Test (CMT)

At laboratory of veterinary medicine collage Al- Qadissiya University , normal milk samples were examined by CMT(California Mastitis Test) according to (18) .

B. Bacterial Culture :

All milk samples from clinical mastitis and another samples which gave a positive reaction with (CMT) were submitted to centrifugation at 3000 rpm / 15 minutes, and the precipitate was cultured on Blood agar ,Nutrient agar by streaking method and then were incubated at 37 C° /48 hrs ,diagnosis depend on morphological character (shape ,color and size) of colony , then examined via gram stain, then after that the suspected colonies were subculture on selective and differentiate media then incubated at 37 C° f 48 hrs.

C.Gram stain : According to (19).

D. Biochemical Tests:

1.Catalase test : (20) .

2.Coagulase test (21,22) .

3.Oxidase Test (19) .

4.Urease test : (23) .

5.Heamolysis Test: (24) .

E.Confirmative diagnosis of *staphylococcus aureus* by PCR by housekeeping gene (*glpF*)

The *Staphylococcus aureus* isolates which examine according classical methods may be submitted to Polymerase chain reaction assay was performed for confirmative detection of *staphylococcus aureus* by Housekeeping gene glycerol

kinase (*glpF gene*). All bacterial isolates were confirmated by PCR assay using (glycerol kinase) as conseveved gene in detection *Staphylococcus aureus* bacterium.This assay was done according to method described by (25,26,27,28) .

1. Primer

The oligonucleotide primers for detection of *Staphylococcus aureus* (*glpF*) gene were designed in this study. The primer provided from (Bioneer, Korea) company as following in table(2) .

Table (2): The Primers and their sequences and PCR product size.

Primer	Sequence		Product size
glpf	F	caatgggtgtgtttgctgtc	233bp
	R	agccggtgctgtagagaaaa	

2.Genomic DNA extraction

Genomic DNA of *staphylococcus aureus* isolate was extracted by using Genomic DNA Mini Kit, according to manufactured company , The extracted DNA was checked electrophoresis using 1.5% agarose gel.

3.Preparation of PCR master mix

The PCR master mix was prepared by using (AccuPower PCR PreMix Kit) and this master mix done according to company instructions.

4.PCR thermocycler conditions

The PCR thermocycler conditions of glpF primer . performed by using optimize PCR protocol writer online and done in convential PCR thermocycler system as following table

Table (3) :protocol steps for glpF primer in PCR assay

PCR cycle	Repeat cycle	Temp.	Time
Initial denaturation	1	94Cº	5min
Denaturation	30	72Cº	30sec
Annealing		55Cº	30sec
Extension		72Cº	30sec
Final extension	1	72Cº	5min
Hold	-	4 Cº	forever

5.PCR product analysis : The PCR products of for all genes was separation by electrophoresis using 1.5% agarose gel .

statistical analysis

The Chi-square test was applied to determine the statistical significance of the data. P value of <0.05 was considered significant

Results

Bacterial isolation

In this study, (44) suspected *S. aureus* isolates were detected their colonies morphologically on blood agar as smooth, yellow, white Colonies of 1 to 2 mm in diameter. All *S. aureus* colonies showed β-hemolysis ,and staining showed gram positive cocci arranged in clusters or spread of bacteria as spherical single cocci, diplococci, but the predominant shape was grape-like clusters of blue color under light microscope, and those mentioned features were characteristics features of staphylococci bacteria. Suspected *S.aureus* isolates were subcultured on mannitol salt agar for purification (selective agar containing 7.5% Nacl that inhibit all bacteria but not *S.aureus*), the colonies appeared as rounded, smooth convex colonies yellowish in color disseminated to the background of the agar indicated fermentation of mannitol sugar.

Biochemical Characteristics

Performing additional biochemical tests on suspected colonies for complete identification of staphylococci which revealed that 41 isolates out of 44 suspected isolate may be *S.aureus* as in table (4).

Table (4): Biochemical test of *S.aures* isolates

Suspected S.aureus becteria	Catalase +	Oxidase +	Coagulase +	Urease +
44	41	41	41	41

Polymerases chain reaction results

genomic DNA extraction

DNA from over night broth bacterial cultured was extracted by Geneiad Bacteria Genomic DNA Extraction Kit, Bioflux. The extracted DNA was checked by electrophoresis using 1.5% agarose gel**.** Genomic DNA (bands) were visualized by UV light as system showed in figure (1)**.**

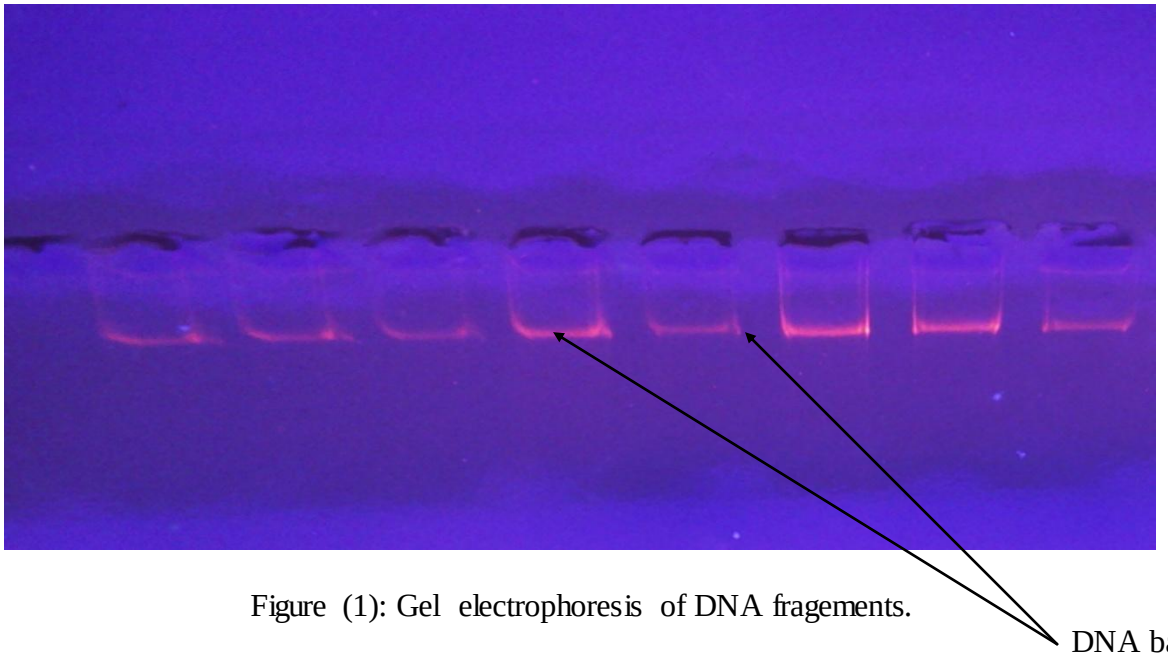


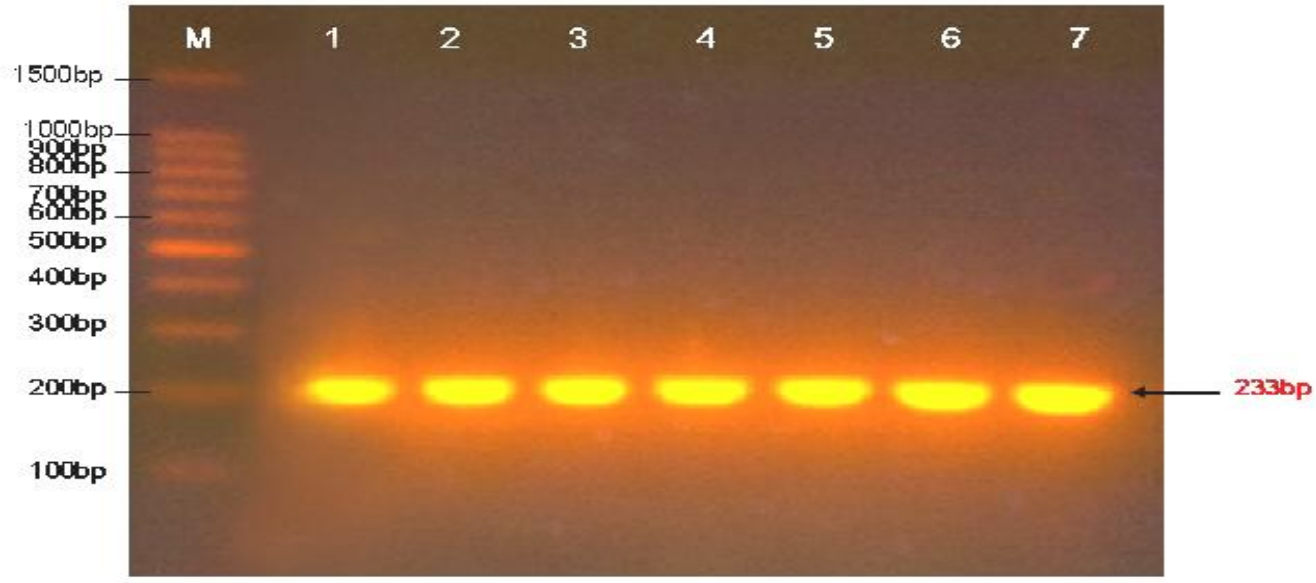
Figure (1): Gel electrophoresis of DNA fragements.

Confirmation diagnosis *staphylococcus aureus* by detection Housekeeping (*glpF) gene* by PCR

All bacterial isolates (44) which isolated from blood agar according type of hemolysis and gram stain these isolates cultured on selective media (manitol salt agar) and diagnose according classical method as(41) *S.aureus* isolates were identified by PCR that revealed 38(92.6%) *S.aureus* isolates were detected which have (*glpF*) gene with product size 223bp. Table (5) , Figure (2).

Table(5) :Detection *S.aureus* by classical methods and PCR technique

Isolates numbers Suspected *S.aureus*	Classical diagnostic methods of *S.aureus*		Confirm detection by PCR technique of *S.aureus*	
44	41	88.1	38	92.6



Figuer (2): Gel electrophoresis of DNA fragements 223 bp amplified fragment of *glfa* gene among examined *S.aureus* isolates.

Out of 388milk samples which collected from cows in some villages and townships of the AL-Najaf province ,there were 212 milk samples (54.6%) are infected (mastitic) as 40(18.8%) clinical mastitis and 172(81.1%) subclincal mastitis .Table (6) .

Table (6): Percentage of clinical and subclinical of examined milk samples.

Numbers of examined milk samples	Mastitis milk samples		Clinical Mastitis		Subclinical Mastitis	
388	No	%	No.	%	No.	%
	212	54.6	40	18.8a	172	81.1b

*The different letters refers to significant differences at ($p< 0.05$)

Percentage of *S.aureus* and CoNS in clinical and subclinical mastitis :-

But staph mastitis was classified into *S.aureus* mastitis 38(17.9%) as 13(32.5%) clinical and 25(14.5%) subclinical form ,and *coagulase negative staphylococci* (CoNS) mastitis 93(43.8%) as 2(5%) clinical and 91(52.9%) sub clinical form .Table (7)

Table (7): Percentage of *S.aureus* and *CoNS* mastitis

Mastitis form	No.	*S.aureus*		CoNS	
		No.	%	No.	%
Clinical mastitis	40	13	32.5 Aa	2	5 bA
Subclinical Mastitis	172	25	14.5 Ba	91	52.9 bB
Total	212	38	17.9a	93	43.8b

*CoNS :Coagulase Negative *Staphylococci*

*The different letters refers to significant differences at ($p< 0.05$)

Relationship between the isolation of *S.aureus* and Udder quarters

S.aureus was isolated from different udder quarters ,the posterior udder quarters were recorded the higher percentage of *S.aureus* isolate as showed in table (4-4) RA 5 (13.1%) ,RP 7 (18.4%),LA 10 (26.3%)and LP 16 (42.1%) respectively .Table (8)

Table(8) **:**Relationship between the isolation of *S.aureus* and Udder quarters of examined cows

Udder quarters	*S.aureus*	
	No.	%
Right anterior RA	5	13.1a
Right posterior RP	7	18.4a b
Left anterior LA	10	26.3b
Left posterior LP	16	42.1c
Total	38	

*The different letters refers to significant differences at ($p< 0.05$)

Discussion

prevalence and occurrence of bovine mastitis

Mastitis is the most important worldwide disease in dairy milk production (29), and it is notoriously difficult to estimate the losses associated with clinical and subclinical mastitis ,which arise from the costs of treatment, culling ,death and decreased milk production and constituent quality (30) . Bovine mastitis continues to cause a huge economic burden to the dairy industry (31) .Results from our study showed that the percentage of bovine mastitis was 54.6% which similar to percentage of bovine mastitis were found by (32) was 52.4%, while our results contradict all (33) and (34) in Iraq were found percentage of bovine mastitis 77.5% and 77.7% respectively .This different of mastitis percentage due to several factors as season of study , type of housing , breed, age of animals (17).According our knowledge may be due to laboratory technique and degree of contamination found and sanitary measurement that applied or not indifferent herds were effective.The present study were showed that the percentage of clinical mastitis 18.8 % which accordance with (35) who examined 223 mastitic milk samples in Egypt and found 21.5% were clinical mastitis ,while (36) were founded 22.50% clinical mastitis in Assiut, Egypt. Our results lower than percentage by (9) 33.01% in Iraq .The variation in incidences of clinical mastitis may be due to many causes as the type and severity of the causative agent,size of herd and sampling collectionary (randomly or selectivity) also that high milk producing cows are more susceptible and the nutritionary status of the herd more effective (17).Results from accurate study ,the subclinical mastitis percentage was 81.1% which was like the result of (37) in Mosul as 80.85% and also nearest with the result of (38) in a percentage 77% , (33) 77.6% in Iraq and 87.2% in Egypt reported by (35) while unlike with the result the study by (39) with a percentage of 92.3% ,the discrepancy depend on environment factor as contamination ,we thought due to milkier hygiene and may be external parasites as ticks which cause mini wounds result from tick bite as well as that coagulase negative *Staphylococci* (CoNS) found as normal flora on teat skin ,all that stimulate mastitis occurrence. The prevalence of mastitis effected by extensive investigation and research of mastitis etiology may be capable of helping to provide an important and optimistic approach to control this disease (40) .

Isolation *S.aureues* from clinical and subclinical mastitis

S.aureus isolated from clinical mastitis in this study (32.5%) was higher than that was earlier reported by (41) (20.59%) from Abu-Ghraib zone from cows suffering acute mastitis in Baghdad government and (42) in Estonia found 20% from clinical mastitis samples were positive to *S.aureus* .This result comparable with Swedish Study (28.3%) *S.aureus* isolated from clinical mastitis by (43) but our result was lower from another studies as (44) who found that 54.4% from clinical bovine mastitis were +ve to *S.aureus* infection and (12) which conducted to the highest results in isolation of *S.aureus* from mastitic cows; she found that out of 48 milk samples from acute cases there were 28 (58%) *S.aureus* +ve isolates.Subclinical *Staphylococcus aureus* mastitis was 14.5 %. Which was in similar to the result (16.6%) as obtained by (45) and closed to the range of *S.aureus* isolated from subclinical mastitis (12-37%) in England, Spain and USA(46,47) and in Sweden(19%) reported by (48). Substantial differences were found in result obtained from another studies (44.44%) ,(44.03%) , (41%) ,(6%) by (49), (50), (40) and (51) respectively. We observed there clear differences in percentages of isolate *S.*

aureus in clinical and sub clinical mastitis of our study compare with another studies ,there may be for more than reason as possible reasons for bacteriologically negative findings in milk samples could be the presence of antibacterial substances in the milk that lead to a decrease in the viability of bacteria in the culture (52), or failures in conventional culture compared with identification of bacteria using the real-time polymerase chain reaction (53), growth of staphylococci was inhibited to a lower extent by lactoferrin which found in bovine milk(54,55) which effect on percentage of bacterial isolation. We thought a mount, type and right selection of antibacterial which used also available professional veterinarian service and culture and knowledge of owner, all these factors effect on prevalence. In this study, the number of isolates from left hindquarter higher than other quarters , which is similar to previous reports by (10,33) which was attributed to normal laying down of cow and that caused attachment of the posterior quarters with bed and also posterior quarters can contaminated by feces on hindlegs , tail of cow and uterus secretion (56,57,17).

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تشخيص التهاب الضرع البقريالذي تسببه المكورات العنقوديه الذهبيه البقري في محافظة النجف الاشرف باستخدام تقنية تفاعل سلسلة البلمره المتعدد

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الخلاصة

تضمنت الدراسه جمع (388) عينة حليب ابقار من مناطق (نواحي وقرى) مختلفه تابعه لمحافظة النجف الاشرف للتحري عن التهاب الضرع التي تسببه جراثيم المكورات العنقوديه الذهبيه وقد استخدم اختبار كاليفورنيا CMT لتحديد حالات التهاب الضرع تحت السريري وبلغت نسبة التهاب الضرع العام 212 (54,6%) .وتم استخدام تفاعل سلسلة البلمره المتعدد لتاكيد تشخيص المكورات العنقوديه الذهبيه باستخدام البادىء (glpF) لعزلات المشخصه بالطرق الكلاسكيه والتي بلغت نسبه عزلها 13 (32,5%) واعتبرت هي نسبة التهاب الضرع المتسبب بواسطة المكورات العنقوديه الذهبيه والتي كانت سريريا15,3 % و20%تحت السريري وكانت نسبة التهاب الضرع المتسبب بواسطة المكورات العنقوديه الذهبيه مرتفعة معنويا في الربع الايسر الخلفي مقارنة مع بقية الارباع من الضرع.

بحث مستل من رسالة الماجستير للباحث الاول