Streptococcus agalactiae mastitis of bovine detection by Polymerase Chain Reaction (PCR) test in AL-Diwanyia province

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Keyword : *Streptococcus agalactiae* , mastitis , PCR.

Summary:

This study was conducted for exam 348 milk samples from (clinically mastitic and other healthy cows) in many areas in AL-Diwanyia province by using CMT and bacteriological testing , which appeared that (64.9%) as percentage of mastitis (clinically 15.9% , subclinically 84.0%) .

Streptococcus agalactiae mastitis 13.2% (26.6% clinically , 73.3 % subclinicaly) diagnose by PCR assay by using specific primer (*16SrRNA*). *Streptococcus agalactiae* (30 isolates) after classical methods applied for *Streptococcus agalactiae* identification (86 isolates)**.**

Introduction :

Mastitis is one of the most prevalent and most costly production diseases affecting the dairy cattle industry worldwide (1). In dairy cows *Streptococcus agalactiae* is a major cause of mastitis , which is currently considered the economically most important disease affecting the dairy industry (2 , 3) .

In Iraq many researches,(4) found that streptococcal mastitis (15.6%) , (5) was isolate *Streptococcus agalactiae* in Iraq from three cow stations for breeding cow as (39.75%) .

In the Dar es Salaam region of Tanzana, The *Streptococcus agalactiae* mastitis was 15.4% (6).

In Ahavaz ,Iran the most bacteria isolated from diary herd in this area were *Streptococcus agalactiae* (20 %) (7).

Prevalence of sub-clinical mastitis and bacterial etiology in the West Littoral Region of Uruguay were: *Staphylococcus aureus*, *Streptococcus agalactiae*, (62.8%, 11.3%) respectively (8) .

Materials and Methods:

Materials:

1.Blood agar base.

2. Nutrient agar.

3. Edward agar.

4. Nutrient Broth.

5- DNA Extraction kits .

All media were prepared according to company instructions .

Reagents :

1- Catalase reagent : Prepared according to (9) .

2- California Mastitis Test (CMT): Al-Syria for veterinary preparation (Syria) according to (10).

3- Coagulase reagent (rabbit plasma): Bacton , Dickinson Company (Spain) according to (11).

4- Gram Stain :Prepare according to (11) .

Commercial kits:

The commercial kits used in the present study are shown in Tables (1,2,3).

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 (forward and reverse)	BIONEER/Korea

Table (2) : DNA extraction Kit consist of :

1	Lyses cells solutions:- -Nuclei lyses solution
2	DNA precipitation alcohols:- -Isopropanol alcohol -Ethanol alcohol
3	DNA rehydration solution

Table (3) : 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)

primer : The obligonucluetide primers for detection of *Streptococcus agalactiae* (*16S rRNA* gene),the primers provided from (Bioneer, Korea) company as following table (4)**.**

Table (4) : Primers used in this study.

Gene name		Primer sequence	Primer size	Primer length
16sRNA	**F**	**TTTGGTGTTTACACTAGACTG**	**120**	**21**
	R	**TGTGTTAATTACTCTTATGCG**		**21**

Note : the primer designing according to NCBI site instructions.

Methods :

Clinical study :-

Three hindered forty eighty (348) milk samples collected from clinical mastitic cows (87) and (190) from cows appears healthy (without signs of mastitis) and (36) were taken from different areas of Al-Diwanyia province .Milk samples were collected in sterile tubes (2 tube)

for each sample (one for CMT and another for bacterial testes) and a septic technique used for milk sampling according to (12) .

California Mastitis Test (CMT) :

The normal milk samples were examined by CMT (California Mastitis Test) according to (13).

Bacterial Culture :

All milk samples which were collected from clinically mastitic cows and milk samples which positive for CMT were culture on blood agar by streaking method then incubated 37C for 24 hr. the bacterial colony on blood agar examined morphology (shape ; size ; colour) and examined by Gram stain ; then colony subcultured on Edward media as a selective media and incubated (37C) for (48) hr and then colonies examined at classical methods (morphology ; gram stain ; biochemical test) then streptococcal isolates finally give confirm diagnosis by PCR technique .

gram stain : by method of (14)

Biochemical tests :

Catalase test : by method of (15).

Coagulase test : by method of (15).

Oxidase Test : by method of (15).

Heamolysis Test : by method of (16).

The camp test : by method of (11**)** .

sodium hippurate test : by method of (11**).**

PCR technique :

Polymerase Chain Reaction (PCR):

Polymerase chain reaction assay was performed for confirmative detection of *Streptococcus agalactiae* by (*16SrRNA* gene) This assay was done according to method described by the company instructions.

Genomic DNA extraction :

Genomic DNA of *Streptococcus agalactiae* isolates was extracted by using Genomic DNA Mini Kit.

DNA profile :

The extracted DNA was checked electrophoresis using 1.5% agarose gel .

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 as showing in Table:(5).

Table (5): Components of PCR master mix reaction.

PCR Master mix		Volume
DNA template		5µl
Primers	F. primer	1.5µl
	R. primer	1.5µl
PCR water		12µl
Total volume		20µl

The DNA template and primers was added in to standard PCR master mix tube which is PCR Premix it is lyophilized materials that containing all other components needed to PCR reaction such as (Taq DNA polymerase, dNTPs, Tris-HCl pH: 9.0, KCl, $MgCl_2$,stabilizer, and tracking dye). And the tubes completed to final volume 20ul by PCR water, then the tube mixed briefly by vortex.

Statistical analysis :

The data was analysis by using Chi – square test , $p< (0.05)$ as a criterion for significant (17).

Results :

Out of 348 milk samples were collected from dairy cows in AL-Diwaniyia province ; The results showed that (226) milk samples were mastitic (64.9%) which appeared 36 (15.9%) as clinical mastitis and 190 (84 %) subclinical mastitis, table(6).

Table(6): Clinical and subclinical mastitis of examined milk samples.

Number of examined sample	clinical mastitis		subclinical mastitis		Total mastitis	
	NO.	%	No.	%	NO.	%
348	36	15.9%A	190	84%B	226	64.9%

Different letters refers to the significant differences (P < 0.05).

The results of CMT for detecting subclinical mastitic samples was showed that the degree with trace result (T) were 42 samples in a percentage 22.1%, while the degree (1+) were 42 samples in a percentage 22.1 %,the degree (2+) were 47 sample in a percentage 24.7 % and the degree (3+) were 47 samples as 31.7 % .Table(7).

Table (7): Degree of CMT of sub clinical mastitic samples.

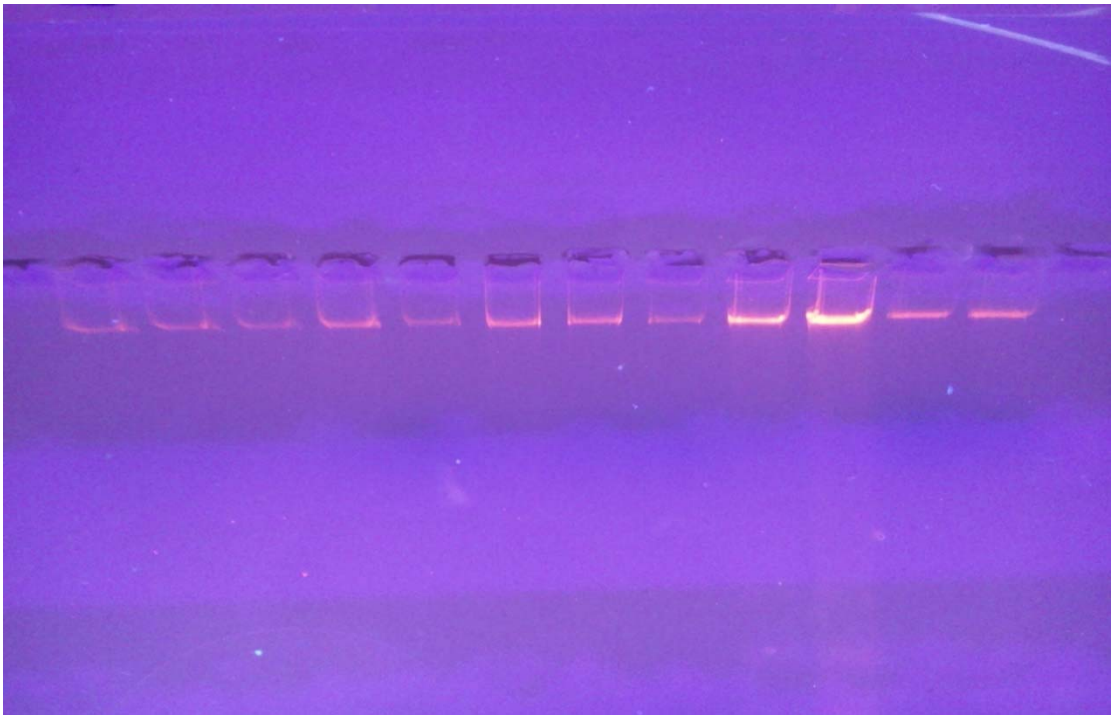
CMT degrees	Subclinical mastitic samples	%
(T)	42	22.1
+1	42	22.1
+2	47	24.7
+3	59	31.1

In this study, (226) mastitic milk samples were cultured on blood agar and their Colonies morphologically appeared as pinpoint , white to milky in color , Colonies of 1 to 2 mm in diameter and colonies showed β-hemolysis and then transferred suspected colony for culture on Edward media (selective media) that grow up (199) isolate (37 ċ/ 48 h) and then submitted to classical methods of diagnosis the *Streptococcus agalactiae* bacteria (Gram stain ,Camp test ,Sodium Hippurate hydrolysis ,Coagulase , and Catalase test), and these isolates on Edward agar appeared as rounded, white yellowish in color and convex in shape. Gram positive cocci arranged in chains (shot chains) or spread bacteria as spherical single cocci, diplococci, but the predominant shape was strep-like clusters of blue color under light microscope, and those mentioned biochemical features (table8) were characteristics of *Streptococcus agalactiae* as(86) isolates.

Table (8) : Biochemical tests of *Streptococcus agalactiae* .

Character	Results
Gram stain	**Cocci pairs or short chian blue in colour**
Camp test	**+**
Sodium hippurate hydrolysis	**+**
Catalase	**–**
Coagulase	**–**

Eighty six *Streptococcus agalactiae* isolates were testing by molecular methods (PCR technique by using *16SrRNA* primer) to confirm the identification of these isolates after the step of genomic DNA extraction figure (1).



Figure(1) : Bands of extracted DNA on gel Electrophoresis.

The PCR assay Figure (2) was revealed that only 30 out of 86 isolates table(9)that may be *Streptococcus agalactiae* , and this percentage (15 %) was represented the percent of *Streptococcus agalactiae* as the causative agent of mastitic cases in our study.

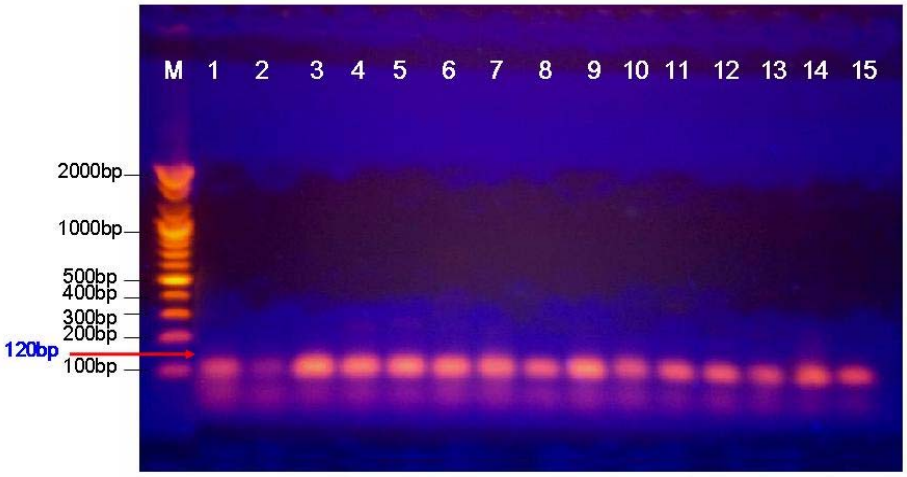


Figure (2): Bands of *16SrRNA* gene on agarose gel 120 bp that represent *Streptococcus agalactiae* isolates.

Table(9): Percentage of *Streptococcus agalactiae* as causative of mastitic cases according to classical and molecular tests.

Suspected streptococal isolates	Classical methods		Molecular methods	
NO.	NO.	%	NO.	%
199	86	43.2	30	15

Discussion :

The results of the present study was showed that bovine mastitis the percentage was (64.9%) which was agreement with the result by (Miltenburg J. *et a1*.1996) as (65 %) and disagreement with mastitis percentage in cattle by (18) , (8) As (77%) in AL- Esehaky station , (52 %) Respectively.

The present study also showed subclinical mastitis as (89%) that was closely with finding by (19) in Al-Mosul (80.8 %) , but disagree with reports by (20) as (93.3 %) and (77 %) by (21).

Clinical mastitis (15.9%) in this study is supported by resultant of (22) as (16.1 %) , while it less than percentage by (23)as (33.01 %).

According to this study , the *Streptococcus agalactiae* mastitis is (13.2 %) which confirm the fact that *Streptococcus agalactiae* (Group B streptococcus) is an important mastitic bovine pathogen in both clinical and subclinical mastitis according to (24) , (12).

Also this result is closely related to reports of many researchers as (15.1%) by (25),(15.4%) by (6) in Tanzania , (26) as (10%).

While this result is disagreement with higher percentage as (2008) (27) (26%) in Iran and the resultant as (44.7%) in Massachusevs between 1976-1982 (28).

And incontrast the decline infection rates that recorded as (7.9%) by (29) and (1-8%) as *Streptococcus agalactiae* mastitis (12) .

This huge variation in mastitis and strep. mastitis (clinically and subclinicaly) rates in our study and another resultant of many researchers in different courtiers are attributed to many factors as season of study , type of animals housing . breed , age , and general conditions as well as the type of natural animal feeding also milking hygiene and different degree of sanitary measurement that applied in these dairy cattle during calving and lactation status .

The result of CMT was raveled the most subclinical mastitis as degree (+3) which was very closely to reports by (29) and (30) were show (25-30%) of subclinical mastitis take degree (3+) by CMT due to that infection induce leukocytes especially neutrophil and lead to increase somatic cells counts in mammary tissue and milks within short time as acute infection.

While (31) show this degree of CMT not stable but changeable and depend on time of sampling regarding to type of pathogen invasive process and severity as well as lactation period .

Molecular detection of *Streptococcus agalactiae* by PCR in this study is support by (32) whom show that exact identification of pathogens is important for mastitis control and epidemiological studies , and development of molecular biological techniques such PCR may be useful significantly .

Our study revealed that percentage of identification of *streptococcus agalactiae* by PCR (15%) in compared by (43%) by classical methods was agreements with (33) and (34) as that PCR identification is more accurate and in percentage lower than by ordinary methods for bacterial isolation and they illustrated that by PCR technique highly specify into specific genome which impossibility represent as sero-var from *Streptococcus agalactiae* as species that may be detected by classical methods .

But , (35) was found incontrast of our result by that PCR isolation percentage was higher that by classical methods because the bacteriological culturing was negative in milk sample could be due to presence antibacterial substance in milk lead to decrease the viability of bacteria in culture (36) , or failure in conventional culture compare with identification of bacteria using PCR (37).

Refreance :

(5) علي ، رقية مصطفى(٢٠٠١) ، عزل وتشخيص جراثيم ***Strept- agalactiae*** من التهاب الضرع والمهبل في الابقار والتهاب المهبل في النساء الحوامل ودراسة العلاقة بينهما . **رسالة ماجستير ، كلية الطب البيطري ، جامعة بغداد.**

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تشخيص التهاب الضرع في الأبقار المتسبب عن المكورات السبحية الحليبية باستخدام تقنية تفاعل السلسلة المتعدد التبلمرة في محافظة الديوانية

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الخلاصة

صممت الدراسة لفحص ٣٤٨ عينة حليب من (أبقار مصابة بالتهاب الضرع سريريا وأخرى سليمة ظاهريا) في العديد من مناطق محافظة الديوانية للتحري عن نسبة الإصابة بالتهاب الضرع بشكل عام ونسبة الإصابة بالتهاب الضرع الذي تسببه جراثيم السبحيات الحليبية بشكل خاص باستخدام اختبار كاليفورنيا والفحص الجرثومي والذي ظهر إن نسبة التهاب الضرع بلغت ٦٤,٩ % (١٥,٩ % التهاب الضرع ألسريري و ٨٤,٠ % التهاب الضرع التحت ألسريري) .

في حين كانت نسبه التهاب الضرع الذي تسببه جراثيم السبحيات الحليبية *Streptococcus agalactiae* هي ١٣,٢ % (٢٦,٦% سريري و ٧٣,٣% تحت ألسريري) وتم تأكيد التشخيص باستخدام تقنية تفاعل السلسلة المتعدد من خلال استخدام بادئ خاص يدعى (16SrRNA) الخاص بالشريط المفرد للمادة الوراثية لجرثومة السبحيات الحليبية ٣٠ عزلة,وبنسبة ١٥ % من مجموع العزلات المشكوك بكونها سبحيات حليبية تم ذلك التشخيص بعد إجراء الفحوصات الكيمحيوية الكلاسيكية التي أفرزت (٨٦) عزلة على إنها جرثومة السبحيات الحليبية .