Comparative Studies between Traditional and Molecular Diagnosis of Mastitis in Goat

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Abstract

The primary causes of mastitis in cattle, goats and sheep are well-recognized groups of microorganisms as Staphylococcus spp.; Streptococcus spp.; Pasteurella spp.; *Escherichia coli*; Enterobacter spp. and Klebsiella spp. The objective of this study was to develop a Multiplex Polymerase Chain Reaction (MPCR) method for simultaneous detection of *Staphylococcus aureus, Streptococcus agalactiae,* and *Streptococcus uberis* directly from milk samples either positive or negative to California test, using specific primers for each microorganism. Two hundred and fifty milk samples represent 125 goats were tested by California test and submitted for isolation of different microorganism using traditional methods. *S.aureus, S.agalacteae, S.ubris, E.coli* and *C. pyogenes* were isolated from examined milk samples in different percentages. The results were discussed. Thirty two milk samples were chosen to represent milk samples analyzed by traditional methods were submitted to be examined by MPCR for diagnosis *S. aureus, S. agalactia,* and *S. uberis.* Results of conventional microbiological methods were used to determine the sensitivity and specificity of the multiplex PCR. Results of this study indicate that the multiplex PCR procedure has the potential to be a valuable diagnostic technique for simultaneous identification of *S. aureus, S. agalactiae,* and *S. uberis* directly from milk samples specially to diagnose subclinical methods.

Introduction

Ovine mastitis is an inflammation of the mammary gland, usually due to a microbial infection (**27**). It often becomes chronic, and it is important to identify quickly the new clinical cases in order to control infection. The suitability of a detection method for routine diagnosis depends on several factors, such as specificity, sensitivity, expense, amount of time and applicability to large numbers of milk samples. The most common but unspecific method (**4**) to identify potential subclinical mastitis

infections is a somatic cell count (SCM): The California mastitis test (CMT) in the field conditions.

Currently, the method of laboratory diagnosis is by in vitro culture which provides the "gold standard" however, this technique is labor-intestine and time consuming. Two other problems can be encountered when these methods of identification are used: first 2-3 days are required to grow, isolate and identify the pathogens, second, some bacteria, like *S. uberis* cannot be distinguished by biochemical assays (**17**).

It has been demonstrated that early detection procedures have been shown to enhance care rates and reduce the time required to return to normal milk when coupled with appropriate antimicrobial therapy (**19**). It is important to identify the pathogens not only for antimicrobial therapy purposes but also to monitor and control the rate of infection at the farm level (**2**).

During the last years, many tests have been developed for the diagnosis of mastitis. However, a rapid (less than 1 day), simple and specific test for each kind of bacterium involved has not been achieved. Many tests for the detection of pathogens already exist. Enzyme-linked immunosorbent assay methods exist for *S. aureus* detection in cases of mastitis, but the antibody titre does not correlate with the amount of infecting bacteria (**7**). Most PCR methods used for the detection of microorganisms in milk need a step of multiplying the bacteria in culture media (**23** and **30**). Rapid identification methods, in particular nucleic acid based tests, have the potential to be extremely specific and can also discriminate between closely related organisms, such as *S.paraubris* and *S.ubris*. It has been previously shown that milk samples could serve as substrate for the amplification of specific DNA sequence using PCR (**6**). The aim of this study was to identify pathogens causing ovine mastitis using molecular tools with rapidity, sensitivity, laboratory traditional methods and molecular diagnosis.

Material and Methods

Animals:

Egyptian Zaraibi goats are the most pronounced dairy goat amongst the local breeds (Baladi and Barki desert goat) in Egypt which considered being of high genetic potential as a dairy prolific breed (1). These animals were obtained from El-Serw Animal Production Research Station, Damietta governorate belonging to Animal production Research Institute (APRI).

Field diagnosis test:

It was applied using the California mastitis test (CMT). This test was applied according to (8).

Sampling:

A total of 250 milk samples were collected at midlactation from half udders of 125 Egyptian Zaraibi goats (40 mastitic goat; 55 from positive California test and 30 from apparently healthy goat). Before sample collection, teats of goats were dipped with a disinfectant, cleaned thoroughly and dried with individual disposable paper towels and ends were sanitized with swabs containing 70% isopropyl alcohol. Milk samples were transported on ice and maintained at -20C until analysis (21).

Conventional bacteriological analysis:

Milk samples were examined following procedures recommended by (16) and (21). 10µl of fore milk was plated onto a trypticase soya agar plate supplemented with 5% defibrinated sheep blood and MacConkey agar. Plates were incubated at 37C and bacterial bacterial growth was observed at 24h intervals for 3 days. Bacteria on primary culture medium were identified tentatively according to colony morphologic features, hemolytic characteristics and biochemical characterization.

Isolates identified presumptively as staphylococci were tested for positive catalase, positive coagulase, mannitol salt and DNase agar. Isolates identified as streptococci were tested for negative catalase, evaluated for growth in Nacl, hydrolysis of esculin, streptococcal were identified to the species level using sugar fermentation tests and CAMP test. *S.agalacteae* was identified on the basis of positive CAMP test and negative esculin hydrolysis. *S. dysagalcteae* was identified on the basis of negative CAMP test and negative esculin hydrolysis, while *S. ubris* was identified on the basis of negative CAMP test and positive esculin hydrolysis.

Gram negative isolates were evaluated by their growthon MacConkey and identified biochemiclly on the following: oxidase test, triple sugar iron, urease test, motility, indol test, methyl red, Vogus Proskaur and citrate tests and other biochemical tests to identify the different species according to **(20).**

Isolation of bacterial DNA from milk: (22)

Fortynine milk samples (Table 6) were used as representative samples of milk for detection of bacterial DNA directly. 1ml milk sample was enriched with trypticase soya broth (1ml) and incubated overnight at 37C **(12).**

A 300µl of sample mixed with 300 µl of NTE buffer (0.1M NaCl. 20mM Tris-HCL [pH, 7.4], 1mMEDTA [pH, 7.5] containing 0.5%SDS and 100µg of proteanase K/ml.

The solution was then incubated at 37C for 4h., an equal volume of phenol – chloroform – isoamylalcohol (25:24:1) was added, and the solution was gently mixed for 3 min. and the upper phase collected. This process was repeated twice. The upper phase was collected and 60µl of 3M sodium acetate (pH 4.8) and 1.2ml of cold100% ethanol were added. Then mixed and held at 20C for 30min. to precipitate the DNA. The DNA was recovered by centrifugation at 10,000xg for 15 min. at 4C. The supernatant was discarded and the pelleted DNA was washed with ethanol 70% and centrifuged at 10,000xg for 5 min. at room temperature. The DNA pellet was used as template DNA.

Primers used in PCR to amplify specific fragments from genes for different microorganisms: (22)

Species	Oligonucleotide	Sequences (5`-3`)
S. aureus	STAA-AuI	TCT TCA GAA GAT GCG GAATA
	STAA-AuII	TAA GTC AAA CGT TAA TAA CAT ACG
S. agalacteae	STRA-AgI	AAG GAA ACC TGC CAT TTG
	STRA-AgII	TTA ACC TAG TTT CTT TAA AAC TCG AA
S. ubris	STRU-UBI	TAA GGA ACA CGT TGG TTA AG
	STRU-UBII	TTC CAG TCC TTA GAC CTT CT

Multiplex PCR assay: (22)

The reaction contained 0.4μM of *S.agalacteae* primers and 0.1 μM of *S. ubris* primers and 0.3 μM of *S.aureus* primers, 2mM $Mgcl_2$ and 2U of Taq DNA polymerase.

The reaction profile was 5 min. of denaturation at 95°C and 36 cycles of 95°C for 30 second and 72°C for 30 second, followed by final incubation of 7 min. at 72°C. PCR products were visualized by ultraviolet light transillumuation, where 7μl of amplified product was used for analysis by 20% polyacrylamide gel electrophoresis.

The gels were run at v/cm for 2h and then stained with silver **(15)**. The 50bp stepladder (sigma) was used as molecular weight marker.

Results and Discussion

An efficient vaccine against mastitis is not yet available and prevention as a measure of control needs sensitive, rapid and specific tests to identify the main bacteria that cause heavy losses in milk production.

CMT is only a screened test and must be followed by cultural or molecular identification of mastitis pathogens.

On conventional cultural tests 61/125 (48.8%) were singly infected (Table, 2). The isolated microorganism was *E.coli* (17.5%); *S. aureus* (11.2%); *S.agalacteae* (4.8%); *S.disagalcteae* (9.4%) and *C. pyogenes* (5.6%). Table (3)

About 24% of 125 milk samples were considered as double infected milk (Table, 2). The isolated microorganism was *E.coli* plus *S.aureus* (5.7%); *E.coli* plus *S.disagalcteae* (7.2%); *S. aureus* plus *S. ubris* (6.4%) and *S. agalacteae* plus *S. aureus* (4.8%). Table (4)

On the other hand mixed infection where more than two microorganisms were observed in a percentage of 8% (Table, 2) and the isolated microorganisms were *E.coli* plus *S. aureus* and *S. ubris* (4.8%); *E.coli* plus Subris plus S.aureus and S.agalacteae (1.6%) and *S. aureus* plus *S. agalcteae* and *C. pyogenes* (1.6%). Table (5)

Thirty milk samples were considered as samples free from pathogens using conventional cultural methods. These results were relatively similar to **(22) and (13).**

Conventional procedures for the identify mastitis pathogens are labor-intensive **(17)** and most of the commercial identification systems are not designed to identify important veterinary pathogens **(29 and 17).**

However, there are several disadvantages associated with microbiological culture. It is limited by the dynamic nature of infection. Subclinical infected animals are intermittent shelders of organisms and may cycle through low and high shedding patterns during lactation. Milk culture may yield no bacteria from truly subclinically infected glands due to the submitted samples of residual therapeutic antibodies that may inhibit bacterial growth in vitro. The presence of leukocytes in milk samples from cases of clinical mastitis and in milk sample with high SCC may also potentially inhibit growth of bacteria due to the limitation of culture method. PCR has been developed to identify various mastitis pathogens **(9; 10; 18 and 11).**

Multiplex PCR, in a single assay, allows simultaneous screening for multiple pathogens that might be causing the disease. Primer used in this study for multiplex PCR are chosen to have similar length to avoid differential yields of amplification products and avoid amplification of shorter target fragments over the longer ones **(5).**

Species specific PCR based on detection of the 16S to 22SrRNA spacer region were developed for the four common bacterial causes of mastitis (*S. aureus; S.agalacteae; S.diagalacteae and S.ubris*) **(14 and 22).**

A preliminary assessment of multiplex PCR (MPCR) was performed on 46 milk samples (18 from single infected milk; 14 from double infected milk; 9 from mixed infected milk and 5 from free pathogen milk by conventional method). Table (6)

It was found that 2 samples positive for *E.coli* by culture were positive to *S.ubris* by MPCR and 2 samples positive to *C.pyogenes* were positive to *S.agalacteae* by MPCR (Table 7and Photo. 1). Moreover, one sample positive to *E.coli* plus *S.aureus* by culture was positive to *S.ubris* by MPCR and another sample were positive to *S.ubris* by MPCR while it was only positive to *S. agalacteae* and *S.aureus* (Table 8) and Photo. (2).

There is no difference between the mixed infection observed by culture method and MPCR (Table 9) and Photo. (3). Four samples free from pathogen by culture were positive to *S.aureus* and *S. ubris* (3 samples as single infection with *S.aureus* and 1 sample as double infection as *S.aureus* and *S.ubris* were detected by MPCR (Photo. 4).

The previous results revealed that by multiplex PCR, subclinical mastitis pathogens could be detected and proved the failure of conventional methods to diagnose subclinical mastitis in samples were positive to CMT.

In addition, MPCR test revealed the presence of *S.ubris* and *S.agalacteae* were negative to them by culture and positive to other pathogens detected by culture or MPCR techniques. These results were in agreed with that observed by **(9); (22) and (24).**

This study has shown that the multiplex PCR assay can be used as a rapid diagnostic tool to detect the presence of *S.aureus; S.agalacteae; S.ubris* in milk samples.

Conclusion:

Extra-mammary bacterial persistence is firstly due to hygienic or technical failures concerning the milking machine Secondly, high stocking density, particularly in intensively managed herds/flocks or during the suckling period, may result in large air concentrations of total microorganisms, mesophilic or coliform bacteria and staphylococci.

These effects are probably associated with incorrect ventilation and high relative humidity. The multiplication of various bacteria on the skin (and in the litter) can be subsequently enhanced **(25; 26 and 3).**

Conventional culture methods fail to diagnose subclinical mastitis in some milk samples and to diagnose or detect presence of *S.ubris* and *S.agalacteae* in single and double infected milk samples.

The diagnosis of subclinical mastitis is important to consider regarding the treatment strategies.

Multiplex PCR, in a single assay, allows simultaneous screening for multiple pathogens that might be causing the disease.

The major disadvantage of using PCR as identification methods might be excessive sensitivity as minor contaminants in samples could lead to misdiagnosis. In addition, MPCR cannot provide the information on antimicrobial sensitivity that is necessary for choosing drugs for treatment of clinical mastitis.

Further studies should be applied to evaluate and detect molecular techniques to diagnose most of pathogens as gram negative bacteria with gram positive bacteria depending on primers with similar melting temperature and similar length and share in spacer regions.

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Table (1): Results of California mastitis test:

Total No. of animals	Mastitic milk		California mastitis test*									
			none		slight		mild		moderate		strong	
125			<10,000-200,000		150,000-500,000		400,000-1,500,00		800,000-5,000,000		Over 5,000,000	
	No.	%	No.	%	No.	%	No.	%	No.	%	No	%
	40	32	21	16.8	6	4.8	3	2.4	23	18.4	32	25.6

*None and slight considered as normal milk, mild, moderate and strong considered as subclinical mastitic milk.

Table (2): Rate and type of infection in milk samples (using conventional method):

Type of milk sample	Single infection		Double infection		Mixed infection	
	No.	%	No.	%	No.	%
Mastitic milk (40)	22	55	12	30	5	12.5
Subclinical mastitic milk (55)	34	61.8	15	27.2	4	7.2
Normal milk (30)	5	16.6	3	10	1	3.3
Total(125)	61	48.8	30	24	10	8

Table (3): Occurrence of different microorganisms as a single infection

Type of milk sample	*E. coli*		*S. aureus*		*S. agalacteae*		*S. diagalacteae*		*C. pyogenes*	
	No.	%	No.	%	No.	%	No.	%	No.	%
Mastitic milk (40)	7	17.5	5	12.5	2	9.09	5	12.5	3	7.5
Subclinical mastitic milk (55)	12	21.8	7	12.7	4	7.2	7	12.7	4	7.2
Normal milk (30)	3	10	2	6.6	0	0	0	0	0	0
Total (125)	22	17.6	14	11.2	6	4.8	12	9.6	7	5.6

Table (4): Occurrence of different microorganisms as a double infection

Type of milk sample	*E.coli*+ *S.aureus*		*E.coli*+ *S.disagalcteae*		*S.aureus*+ *S.ubris*		*S.aureus*+ *S.agalacteae*	
	No.	%	No.	%	No.	%	No.	%
Mastitic milk (40)	4	10	3	7.5	2	5	3	7.5
Subclinical mastitic milk (55)	4	5.45	4	7.2	5	9.09	3	4.45
Normal milk (30)	0	0	2	6.6	1	3.3	0	0
Total (125)	7	5.6	9	7.2	8	6.4	6	4.8

Table (5): Occurrence of different microorganisms as a mixed infection

Type of milk sample	*E.coli*+ *S.aureus*+ *S.ubris*		*E.coli*+ *S.disagalcteae*+ *S.ubris*+*S.aureus*		*S.aureus*+ *S.aga-lacteae*+ *C.pyogenes*	
	No.	%	No.	%	No.	%
Mastitic milk (40)	3	7.5	1	2.5	1	2.5
Subclinical mastitic milk (55)	2	3.6	1	1.8	1	1.8
Normal milk (30)	1	3.3	0	0	0	0
Total (125)	6	4.8	2	1.6	2	1.6

Table (6): Number and types of milk samples chosen for multiplex PCR

Type of infection	Type of microorganisms.	Type of milk samples			Total
		mastitic	Sub-clinical	normal	
Single infected	E.coli	2	2	1	5
	S.aureus	2	2	1	5
	S.agalactiae	1	1	0	2
	S.diagalacteae	0	2	0	4
	S.ubris	0	0	0	0
	C.pyogenes	1	1	0	2
Total		18			
Double infection	E.coli+S.aureus	2	2	0	4
	E.coli+S.disagalacteae	1	2	1	4
	S.agalacteae+S.aureus	1	0	0	1
	S.aureus+S.ubris	2	2	1	5
Total		14			
Mixed infection	S.agalacteae+ S.aureus+S.ubris	2	2	1	5
	E.coli+ S.agalacteae+C. puogenes	1	1	1	3
	S.aureus+ S.disagalacteae+ C.pyogenes	1	0	0	1
Total		9			
Pathogen free samples		5			

Table (7): Evaluation of multiplex PCR (MPCR) for detection of different bacterial infection in milk samples (single infection observed by conventional methods)

Type of m.o isolated by conventional methods	No. of milk samples	No. of negative samples by MPCR methods	No. of positive samples by MPCR	Type of detected m.o
E.coli	5	2	3	*S.ubris*
S.aureus	5	0	5	*S.aureus*
S.agalacteae	2	0	2	*S.agalacteae*
S.diagalacteae	4	4	0	-
C.pyogenes	2	0	2	*S.agalacteae*
Total	18	6	12	

Table (8): Evaluation of multiplex PCR (MPCR) for detection of different bacterial infection in milk samples (double infection infected milk samples)

Type of m.o isolated by conventional methods	No. of milk samples	No. of negative samples by MPCR methods	No. of positive samples by MPCR	Type of detected m.o
E.coli +S.aureus	4	0	3	*S.dureus*
			1	*S.ubris*
E.coli+ S.diagalacteae	4	4	0	-
S.aureus+ S.ubris	1	0	1	*S.aureus+ S.ubris*
S.agalacteae+ S.aureus	5	0	4	*S.agalacteae+ S.aureus*
			1	*S.agalacteae+ S.aureus+ S.ubris*
Total	14	4	10	

Table (9): Evaluation of multiplex PCR (MPCR) for detection of different bacterial infection in milk samples (mixed infection infected milk samples)

Type of m.o isolated by conventional methods	No. of milk samples	No. of negative samples by MPCR methods	No. of positive samples by MPCR	Type of detected m.o
E.coli+ *S.aureus*+ *S.ubris*	5	0	5	*E.coli*+ *S.aureus*+ *S.ubris*
E.coli+ *S.ubris*+ *S.aureus*+ *S.agalacteae*	3	0	3	*E.coli*+*S.ubris* + *S.aureus*+ *S.agalacteae*
S.aureus+ *S.diagalacteae*+ *C.pyogenes*	1	0	1	*S.aureus*
Total	9	0	9	

Table (10): Evaluation of multiplex PCR (MPCR) for detection of different bacterial infection in milk samples (free from pathogen by conventional methods)

No. of milk samples	No. of negative samples by MPCR methods	No. of positive samples by MPCR	Type of detected m.o
5	1	3	*S.aureus*
		1	*S.aureus*+*S.ubris*

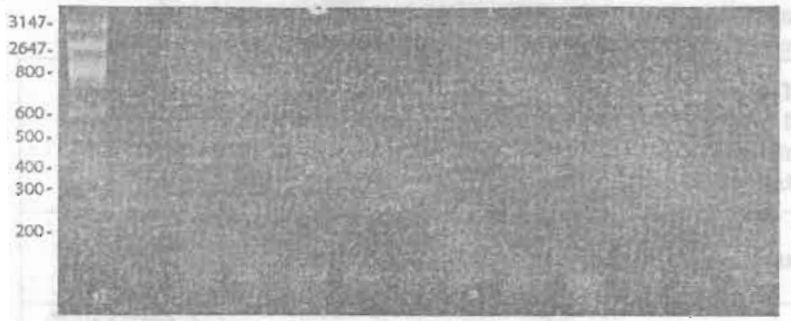

Photo. (1): Represent MPCR of milk samples singly infected on conventional methods. Lane 1 negative control; lane 2 control positive for *S. aureus* gene; lane 3 control positive for *S. agalacteae*; lane 4 positive control for *S. ubris*; lanes 5; 6 and 9 milk samples positive to *S. ubris*; lanes7 and 8 positive to *S. agalacteae*; lane 10 sample positive to *S. aureus* and lanes 11 and 12 negative milk samples for MPCR.

Photo. (2): Represent MPCR of milk samples double infected on conventional methods. Lane 1 negative control; lane 2 control positive for *S. aureus* gene; lane 3 control positive for *S. agalacteae*; lane 4 positive control for *S. ubris*; lanes 5 and 8 milk samples positive to *S.aureus* and *S.ubris*; lane 6 milk samples positive to; *S. aureus, S. agalacteae and S. ubris*; lanes 7 and 11 samples positive to *S. aureus* and lanes 9 and 12 negative samples to MPCR

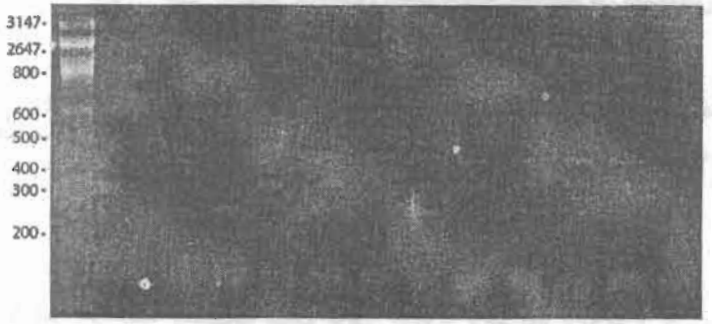

Photo. (3): Represent MPCR of milk samples mixed infected on conventional methods. Lane 1 negative control; lane 2 control positive for *S. aureus* gene; lane 3 control positive for *S. agalacteae*; lane 4 positive control for *S. ubris*; lanes 5; 8; 9 and 10 milk samples positive to *S. aureus* and *S. ubris*; lane 7 positive to *S. aureus*; *S. agalacteae* and *S. ubris*; lanes 6 and 11 positive to *S. aureus* and lane 12 negative sample for MPCR

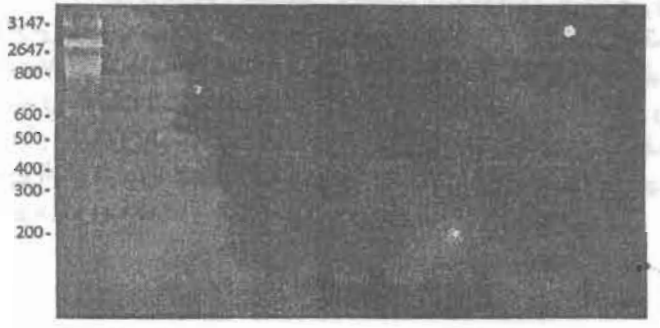

Photo. (4): Represent MPCR of milk samples free from pathogens on conventional methods. Lane 1 negative control; lane 2 control positive for *S. aureus* gene; lane 3 control positive for *S. agalacteae*; lane 4 positive control for *S. ubris*; lanes 5, 6 and 9 milk samples positive for *S. aureus* and lane 8 positive for *S. aureus* and *S. ubris* lanes 7 and 10 negative samples.

دراسات للمقارنة بين الطرق التقليدية والطرق التكنولوجية الحديثة فى تشخيص التهاب الضرع بالماعز

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الملخص العربى

السبب الاولى للاصابة بالتهاب الضرع فى الأبقار والماعز والأغنام تميز بمجموعة من الميكروبات مثل الميكروب المكور العنقودى والمكور السبحى وميكروب الباستيريلا والقولونى الايشريشى والانتيروباكتر والكلبسيلا.

الهدف من هذه الدراسة هو استخدام اختبار البلمرة المتعدد لتشخيص الاصابة بالميكروب المكور العنقودى والميكروب المكور السبحى اجلاكتى والميكروب المكور السبحى يوبريز مباشرة من عينات اللبن الايجابية والسلبية للاختبار الحقلى (اختباركاليفورنيا) باستخدام بادىء جينى لكل من هذه الميكروبات.

تم فحص ٢٥٠ عينة لبن تمثل ١٢٥ ماعزباختبار كاليفورنيا الحقلى وكذلك تم الكشف الميكروبات المسببة باستخدام الطرق التقليدية، وقد تم عزل الميكروب المكور العنقودى والميكروب المكور السبحى اجلاكتى والديس أجلاكتى والميكروب المكور السبحى يوبريزوالميكروب الايشريشى القولونى وميكروب الكورينى بيوجين وقد تم مناقشه هذه النتائج. تم اختيار ٣٢ عينة لبن تم فحصهم بالطرق التقليدية كعينات ممثلة لكل عينات اللبن لاجراء اختبار البلمرة المتعدد للكشف عن الميكروب المكور العنقودى والميكروب المكور السبحى اجلاكتى والميكروب المكور السبحى يوبريز وتم مناقشة النتائج. وقد وجد ان اختبار البلمرة المتعدد له الكفاءة لاستخدامه فى تشخيص كل من الميكروب المكور العنقودى والميكروب المكور السبحى اجلاكتى والميكروب المكور السبحى يوبريز مباشرة من عينات اللبن.