j.Egypt.vet.med.Assoc 79, no 1. 155 – 167(2019)

# OCCURRENCE OF COMMON BACTEREAL ISOLATES FROM MASTITIS COWES

By

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## ABSTRACT

Mastitis, an inflammation of the mammary gland, is one of the most costly and complex diseases of the dairy cows. The present study was designed to evaluate the occurrence of etiological pathogens in 412 pooled milk samples from subclinical and clinical mastitic dairy cows at level before taking up antibiotic therapy. Also to study the molecular characterization of isolated bacteria. Samples of raw milk were taken from dairy cows in an around El-Behera, EI-Qalubia, EI-Sharkia, Beni suef and El-Menoufia Governorates of Egypt, for microbiological investigations. The predominant bacterial isolates from both clinical and subclinical mastitic milk samples were *Escherichia coli* (30.3 %) *Staphylococcus aureus* (29.9 %) followed by other streptococci. (28.6 %) meanwhile there was no growth in (11.2%) of samples. Subclinical mastitis (SCM) represented a major proportion of the burden of mastitis, 93.4 % .PCR protocol used for amplification and detection of (16-23srRNA gene (*S. aureus*), 16s rRNA gene (other streptococci*) and* tra T gene species specific (*Escherichia coli)*.

**Keywords:**

Mastitis; *Staphylococcus aureus; Escherichia coli*; *other* streptococci.

## INTRODUCTION

Bovine mastitis is an inflammation of udder tissue due to a microbial infection.

It is responsible for huge economic losses to the tune of billions of dollars every year to the dairy industry, **(Watts 1989)**. The losses are due to reduced milk quantity and quality contributes highest percentage of loss hence the major strategy to avoid this is by detecting the cause at early stage of infection. When infection is chronic, the udder loses its function permanently as the tissue becomes fibrosis. Hence, it is important to identify quickly the new clinical cases in order to control infection in the herd. The causative bacteria could be

classified as major or minor pathogens **Harmon (1994)**. Since subclinical mastitis does not show visible changes in the milk or udder, such affection is detected by reduction in milk yield, altered milk composition and the presence of inflammatory components and bacteria in milk. **Yuan *et al.*, (2011).** The merit of any detection method for routine diagnosis depends on factors, such as specificity, sensitivity, expense, time involved, and applicability to large numbers of milk samples. The most common but unspecific method to identify potential chronic infections is a SCC though it is not very specific. Early detection procedures have been shown to enhance cure rates and reduce the time required to return to normal milk when coupled with appropriate antimicrobial therapy **Milner *et al*., (1997).** Developments in molecular methods for the detection and characterization of microorganisms in a broad range of medical diagnostic fields, including Virology, Mycology, Parasitology, and Microbiology. Among these methods, Polymerase Chain Reaction (PCR) has generated great benefits and allowed scientific advancements. PCR is an excellent technique for the rapid detection of pathogens, including those difficult to culture **Marcela *et al., (*2009).** The aim of the present study designed to evaluate the occurrence of etiological pathogens from subclinical and clinical mastitic dairy cows, at level before taking up antibiotic therapy

## MATERIAL AND METHODS

**California Mastitis Test (CMT):**

Quarter milk samples were screened in the field using the California Mastitis Test (CMT) .Before sample collection the udders were thoroughly disinfected with 70% ethanol then dried. The first strips were discarded and a milk sample from each quarter was tested by CMT.CMT. Positive quarters' milk samples were collected under aseptic conditions in labeled sterile screw caped bottles and kept at 4ºC for further lab diagnosis **(Schalm *et al.*, 1971).**

**Animals and Sampling:**

In this study , a total of 412 pooled milk samples were collected out from 385 apparently healthy lactating cows, gave positive results of CMT, in and around El - Behera, EI- Qalubia, EI- Sharkia, Beni Suef and El - Menoufia Governorates of Egypt . Also 27 pooled milk samples were collected from mastitic cows showed all clinical signs of mastitis with clear changes in color and consistency of milk. Mastitic milk samples were collected during period from June 2015 until November 2016.

All collected sample were transported directly, as soon as possible, in icebox to bacteriological laboratory of Animal Reproduction Research Institute in Giza Governorate.

**Isolation and identification of most common bacteria causing mastitis:**

**Cultural and biochemical identification.**

Ten milliliters of the milk samples were centrifuged at 3000 rpm for 20 min the cream supernatant fluid discarded. The sediment was suspended with equal volumes of sterile distilled water. A loop-full from each of the prepared milk samples was streaked on mannitol salt agar, Edwards medium, blood agar and Macconky agar respectively. Then it was incubated at 37 °C for 24 h. Suspected colonies were described for their morphological characteristic, appearance and hemolytic activity, followed by Gram staining before being transferred into slope agar to be subjected for further identification by biochemical test analysis , according to **Quinn *et al* ., (2011).**

**Molecular investigation by PCR:**

Chromosomal DNA was extracted using a rapid boiling procedure according to **Reischl *et al.,* (1994**) .Briefly; 1ml broth per each isolate was taken from the nutrient broth and centrifuged at 5000 rpm to sediment the bacterial pellet. The latter was washed twice-using Tris EDTA buffer and finally suspended in 200 µl of lysis buffer [1% Triton X-100, 0.5% Tween 20, 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA]. After boiling for 10 min, the suspension was centrifuged for 5 min to sediment bacterial debris. The supernatant was aspirated and from which five µl was used directly for PCR amplification PCR assays targeting 16-23srRNA gene (*S. aureus*), 16s rRNA gene (other Streptococci) and tra T gene species specific (*Escherichia coli)*. PCR primers were designed from highly divergent and species specific regions of the DNA coding for 16S–23S ISR rRNA, 16S r RNA and *traT* gene based on previously published sequence entries available in the NCBI - Gen Bank database for the detection of *Staphylococcus,* other Streptococci and *E. coli* respectively. All assays were performed using total volume of 25µl containing 5µl of template DNA, 20 pmol of each primer (Metabion international AG, Germany) and 1X of PCR mix (PCR Master Mix, Fermentas, Life Science). The PCR cycles were carried out in T100$^{TM}$ Thermal Cycler-Bio-Rad. Detailed sequences of primers and cycling protocols were depicted in (Table 1). PCR products were electrophoresed in 1.5% agarose gel containing 0.5X TBE at 100 volts for 40 min. Gels were stained by ethidium bromide and visualized by UV trans-illuminator.

**Table (1):** Primer sequences, target genes and cycling profiles of PCR assays used in this study.

| Primer name Specificity | Target gene | Sequence (5'–3') | cycles | PCR program | Size (bp) | Ref. |
|---|---|---|---|---|---|---|
| *Staphylococcus aureus* | 16s–23s ISR rRNA | F-TTC GTA CCA GCC AGA GGT GGA. R-TCT TCA GCG CAT CAC CAA TGC C. | 35 cycles | 95°C/45s 50°C/60s 72°C/30s | 229 bp | Pradhan, *et al* 2011 |
| Other Streptococci than agalactia | 16s rRNA | F-GAT ACA TAG CCG ACC TGA GA. R-AGG GCC TAA CAC CTA GCA CT. | 35 cycles | 95°C/45s 55°C/60s 70°C/45s | 561 bp | Pradhan. *et al* 2011 |
| *E. coli* | (tra T) gene | F-TCT GCG GGA GTC TCA GGG ATG GCT G. R-GTA TTT ATG CTG GTT ACC TGT TT | 35 cycles | 95°C/45s 58°C/60s 75°C/45s | 313 bp | Pradhan. *et al* 2011 |

## RESULTS AND DISCUSSION

The fact that bovine mastitis covers approximately 30% of all cows' diseases provides evidence for the potential of its economic significance in dairy cattle industry **(De Graves and Fetrow 1993)**.CMT not only provides a snap-shot in time of the udder health situation of a herd, but also provides a very effective means of plotting of identifying infection trends **(Pyorala, 2003).** In the present study, CMT showed that 385 (93.4%) positive polled samples with variable degrees. The degree was related to the CMT score (Table 2). It was found that 27/412 (6.6 %) represented clinical mastitis ,These cases of mastitis are termed sub-clinical, and can be up to 15 times more common than clinical cases of the illness. This is in agreement with **Bachaya *et al.*, (2011).** Based on CMT result and clinical examination, it is important to mention that, the percentage of prevalence of subclinical mastitis was (385 at 93.4%) which was greater than percentage of its prevalence in clinical mastitis cases (27 at 6.6%) in 412 isolates from cows. Some of the subclinical mastitis cases convert into clinical form causing more economic losses due to decreased milk production, cost of treatment, veterinarian's fee and culling. Therefore, the time of detection of subclinical mastitis is very important to initiate proper treatment, control and preventive measurements. This results correlated with **(Rahmeto *et al.*, 2016)** reported that, the very high prevalence sub-clinical f mastitis, in the herds examined revealed the huge potential economic loss the sector suffers. Perhaps all of the herd owners attributed this to lack of implementation of the routine mastitis prevention and control practices.

***Staphylococcus aureus.***

*Staphylococcus aureus* is one of the most important contagious mastitis pathogens in dairy cattle. Due to its zoonotic potential, control of *S. aureus* is not only of great economic importance in the dairy industry but also a significant public health concern **(Kümmel *et al.*, 2016)**. The presented data recorded that, the percentage of *S. aureus* in 412 collected samples was 29.9 %. The percent of single infection of *S. aureus* was 4.6 %, while the percent of mixed infection with *S. aureus* was 25.3% at a total percentage. The prevalence percentage of *S. aureus* was detected using conventional and PCR methods in DNA extracted from samples enriched cultures. The presence of *Staphylococcus aureus* in raw milk generally comes from cows with mastitis, Transmitted during milking via the teat cups or milkers hands.

When found in milk, high levels of contamination can be reached quickly under favorable conditions. Its presence in foods can be a risk to human health, causing a public health problem, as these bacteria produces toxins that can cause toxic food infections **(Quintana and Carneiro, 2006).**

***S. aureus Specific PCR:***

*S. aureus* specific primers targeting 16s–23s ISR rRNA were designed and employed for the specific confirmation of the *S. aureus* DNA. The isolates yielded a single DNA band of 229 bp amplicon of 16S-23S ISR rRNA Fig. (2). Molecular identification using *S. aureus* specific gene16S–23S ISR rRNA (229 bp) revealed 29.9% positive isolates .The benefit of handling 16S-23S rRNA gene was the high specificity for detecting *S. aureus* isolates as no other non-specific amplicon was seen. In consistence, amplification of the 16S-23S rRNA gene was used for quick genotyping numerous *S. aureus* isolates obtained from bovine herds in Switzerland **(Fournie*r et al.*, 2008).**

***Streptococcus* spp.**

The environmental streptococci are a significant cause of IMI and clinical mastitis in dairy herds and methods to control these pathogens are currently less than adequate) Smith **and Hogan, 1995**). Exposure of uninfected teats to the environmental streptococci can occur during the milking process, between milking, during the dry period and prior to parturition in first lactation heifers. This is in contrast to the contagious pathogens where exposure occurs primarily during the milking process **Hogan and Smith (2003).**

The presented data recorded that, the percentage of other streptococci. In 412 collected

samples was 28.6 %. The percent of single infection of other streptococci was 5.5 %, while the percent of mixed infection with other streptococci was 23.1% at a total percentage. The prevalence percentage of other streptococci was detected using conventional and PCR methods in DNA extracted from samples enriched cultures.

***Streptococcus* spp. S*pecific PCR:***

16S rRNA gene was targeted in the case of other streptococci. Upon amplification of the genus,specific 16S rRNA gene with designed primers using the DNA isolated.

As expected a single 561 bp DNA band was seen in the lane of 16S rRNA Fig (3) indicating that amplicon of 561 bp is specific for 16 S RNA genes of other streptococci revealed 28.6% positive isolates. This is in agreement with **Ramya *et al*., (2015)** as they shown that 16S gene based and clinical identities, a genus-level concordance rate of 96% and a species-level concordance rate of 87.5%.

***Escherichia coli*:**

Milk and dairy products can be contaminated with a variety of microorganisms from different sources. Raw milk and cheese made from raw milk can be a major source of potentially harmful bacteria to human, such as pathogenic *Escherichia coli* **(Oliver *et al.*, 2005).**

The presented data recorded that the percentage of *E. coli* in 412 collected samples was 30.3 %. The percent of single infection of *E. coli* was 11.3 %, while the percent of mixed infection with *E. coli* was 19.0 % at a total percentage. The prevalence percentage of *E. coli* was detected using conventional and PCR methods in DNA extracted from samples enriched cultures.

***E. coli specific PCR:***

The DNA from standard culture showed an amplified band of 313 bp Fig. (4) revealed 30.3% positive isolates indicating that, the gene is specific for *E.coli*, Similar size band was also seen in the reference cultures that were used as positive controls. This is in agreement with **Pradhan., *et al* 2011 ,**who reported that Primers were designed for *traT* gene of *E.coli* which is supposed to be conserved in >90% of all *E.coli* strains.

**No growth:**

Meanwhile about 11.2 % of clinical and subclinical mastitic samples recorded no growth on ordinary media of bacteriology. These results may be due to other causative agents as fungal infection, viral infection.

Nearly no clear differences between studded Governorates77.5%,78.3%,71.0%,80.2% and 79.4% from El-Behera, El-Qalubia, El-Sharkia, Beni suef and El-Menoufia respectively ),this due to equal chances of common bacterial infection ,that is because all Governorates have a big markets of animal, which enhance entrance of new breads of animal to the lactating farms or of different Governorates.

## CONCLUSION

Study of cattle clinical and subclinical mastitis is a critical aim to control economic losses of dairy farms, compared with culture method PCR is less time consuming, more accurate and highly specific. Therefore, PCR assay could be used as an alternative method in routine diagnosis of SCM for rapid, sensitive and specific simultaneous detection of most common bacterial isolates from subclinical and clinical cow mastitis.

**Table (2):** Number of examined subclinical and clinical mastitic milk samples collected from cattle cows in different governorates of Egypt.

| Governoate | Subclinical mastitis TOTAL Number 385 | | Clinical mastitis TOTAL Number 27 | |
|---|---|---|---|---|
| | No.(385) | % | No.(27) | % |
| El-behera | 84 | 21.8 | 6 | 22.2 |
| El-qalubia | 57 | 14.8 | 3 | 11.1 |
| El-Sharkia | 103 | 26..8 | 9 | 33.3 |
| Beni suef | 68 | 17.7 | 4 | 14.8 |
| El-menoufia | 73 | 19.0 | 5 | 18.5 |

**Table (3):** Prevalence of most common bacteria isolates from mastitic cows in different governorates of Egypt.

| Isolates | Governorates | | | | | | | | | | Total | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | El -Behera (90)* | | El -Qalubia (60 )* | | El - Sharkia (112 )* | | Beni Suef (72)* | | El -Menoufia (78)* | | (412)* | |
| | NO. | % | NO. | % | NO. | % | NO. | % | NO. | % | No. | % |
| *S. aureus* | 6 | 6.7 | 2 | 3.3 | 11 | 9.8 | 4 | 5.5 | 5 | 6.4 | 28 | 6.8 |
| other streptococci | 7 | 7.8 | 3 | 5.0 | 10 | 8.9 | 7 | 9.7 | 6 | 7.6 | 33 | 8.0 |
| *E. coli* | 12 | 13.3 | 13 | 21.6 | 19 | 16.9 | 11 | 15.2 | 13 | 16.6 | 68 | 16.5 |
| *S. aureus* + other *streptococci* | 17 | 18.9 | 14 | 23.3 | 16 | 14.2 | 12 | 16.6 | 13 | 16.6 | 72 | 17.5 |
| *Staph aureus* + *E. coli* | 13 | 14.4 | 9 | 15 | 10 | 8.9 | 8 | 11.1 | 7 | 8.9 | 47 | 11.4 |
| other streptococci +*E. coli* | 8 | 8.9 | 4 | 6.6 | 7.0 | 6.2 | 6 | 8.3 | 9 | 11.5 | 34 | 8.2 |
| *S. aureus* + other streptococci .+ *E. coli* | 6 | 6.7 | 2 | 3.3 | 8.0 | 7.1 | 9 | 12.5 | 9 | 11.5 | 34 | 8.2 |
| *Total bacterial isolates* | 69 | 76.6 | 47 | 78.3 | 81 | 72.3 | 57 | 79.1 | 62 | 79.4 | 316 | 76.6 |
| *No growth* | 13 | 14.4 | 10 | 16.6 | 24 | 21.4 | 9 | 12.5 | 12 | 15.3 | 68 | 16.5 |
| *Total* | 82 | 91.1 | 57 | 95 | 105 | 93.7 | 63 | 87.5 | 74 | 94.9 | 384 | 93.2 |

**Table (4):** Prevalence of bacterial strains isolated from cow subclinical and clinical mastitic milk samples.

| Isolates | SCM (N:316) | | CM (N:289) | | Total (N:605) | |
|---|---|---|---|---|---|---|
| | No. | % No/316 | No. | % No/289 | No. | % No/605 |
| **Single infection of *S. aureus*** | **20.0** | **6.3** | **8.0** | **2.8** | **28.0** | **4.6** |
| **Mixed infection of *S. aureus*** | **107.0** | **33.9** | **46.0** | **15.9** | **153.0** | **25.3** |
| **Single infection of other streptococci** | **15.0** | **4.8** | **18.0** | **6.2** | **33.0** | **5.5** |
| **Mixed infection of other streptococci** | **69.0** | **21.8** | **71.0** | **24.6** | **140.0** | **23.1** |
| **Single infection of *Escherichia coli*** | **22.0** | **7.0** | **46.0** | **15.9** | **68.0** | **11.3** |
| **Mixed infection of *Escherichia coli*** | **33.0** | **10.4** | **82.0** | **28.4** | **115.0** | **19.0** |
| **No bacterial infection** | **50.0** | **15.8** | **18.0** | **6.2** | **68.0** | **11.2** |
| **Total** | 316 | 100 | 289 | 100 | 605 | 100 |

**SCM: subclinical mastitis**

**CM: clinical mastitis**

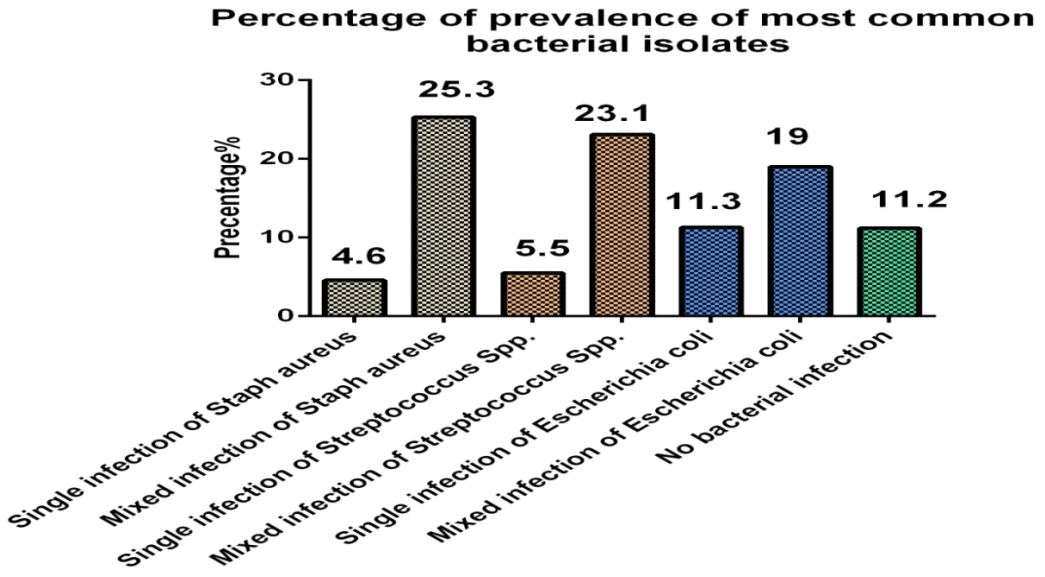


**Fig. (1):** Prevalence of most common bacterial isolates from cows subclinical and clinical mastitis identified by PCR technique.

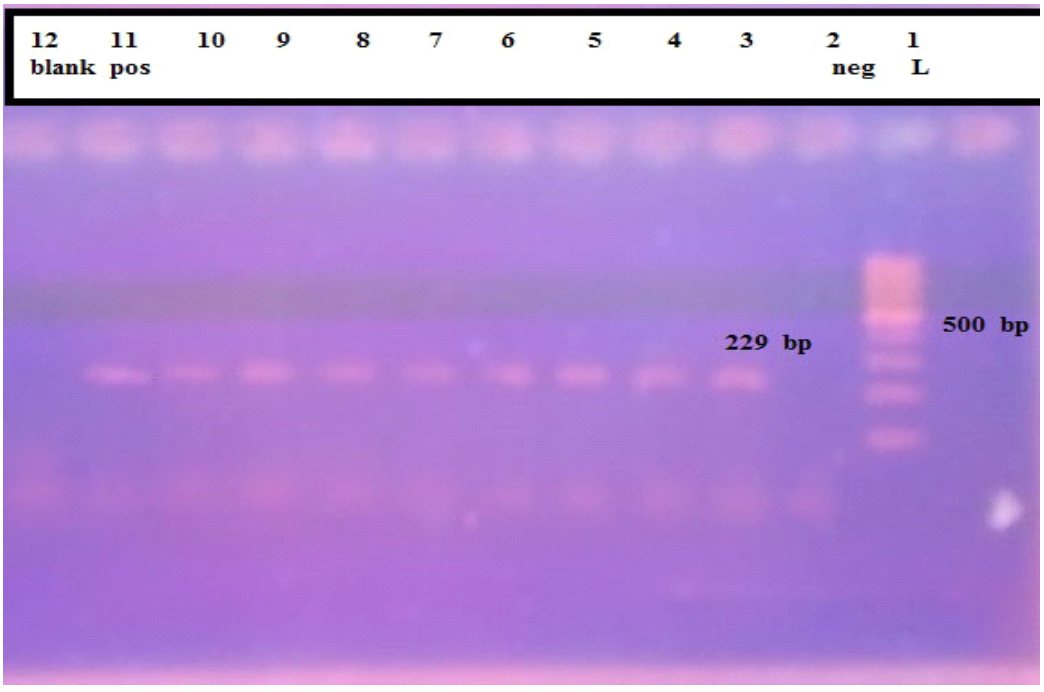


**Fig. (2):** PCR product of 229 bp using *staphylococcus aureus* 16s–23s ISR rRNA species specific PCR assay. Lane 1:100 bp ladder DNA marker, lane 2: control negative (*Escherichia coliATCC:9637*),lane3-10:*staphylococcus aureus* positive isolate,lane 11: *staphylococcus aureus* control positive (*Staphylococcus aureus*ATCC: 6538), and Lane 12: blank).

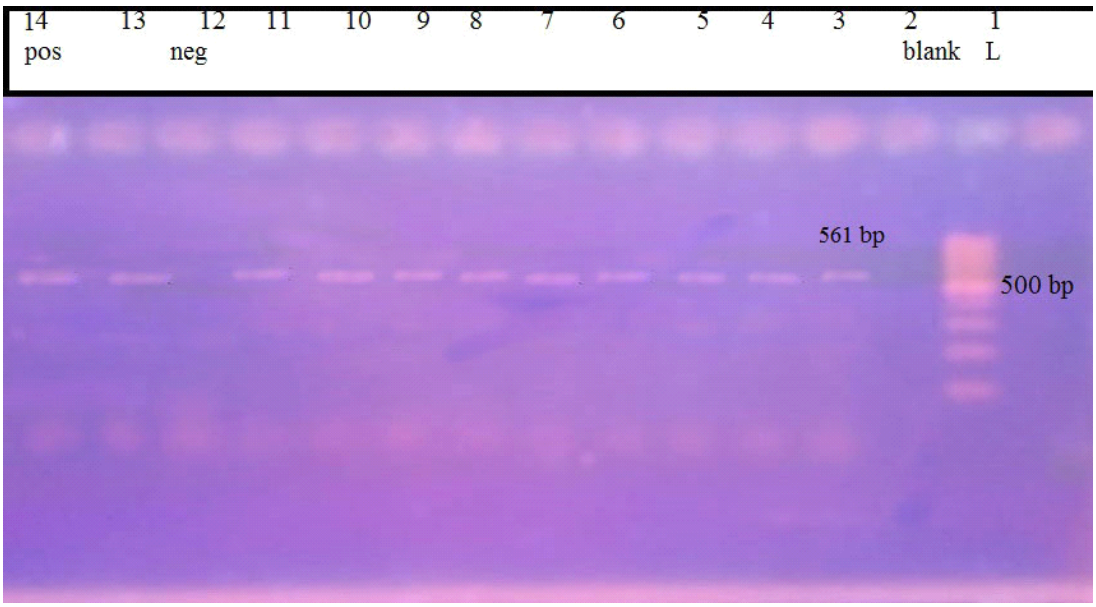


**Fig. (3):** PCR product of 561 bp using 16s rRNA (Streptococcus species*) species*-specific PCR assay. Lane 1:100 bp ladder DNA marker, lane 2: blank lane 3-11 and 13: streptococcus species positive isolate, lane 12: control negative (*Escherichia coliATCC: 9637*), and lane 14 Streptococcus species control positive (Streptococcus species *ATCC: 25175*).

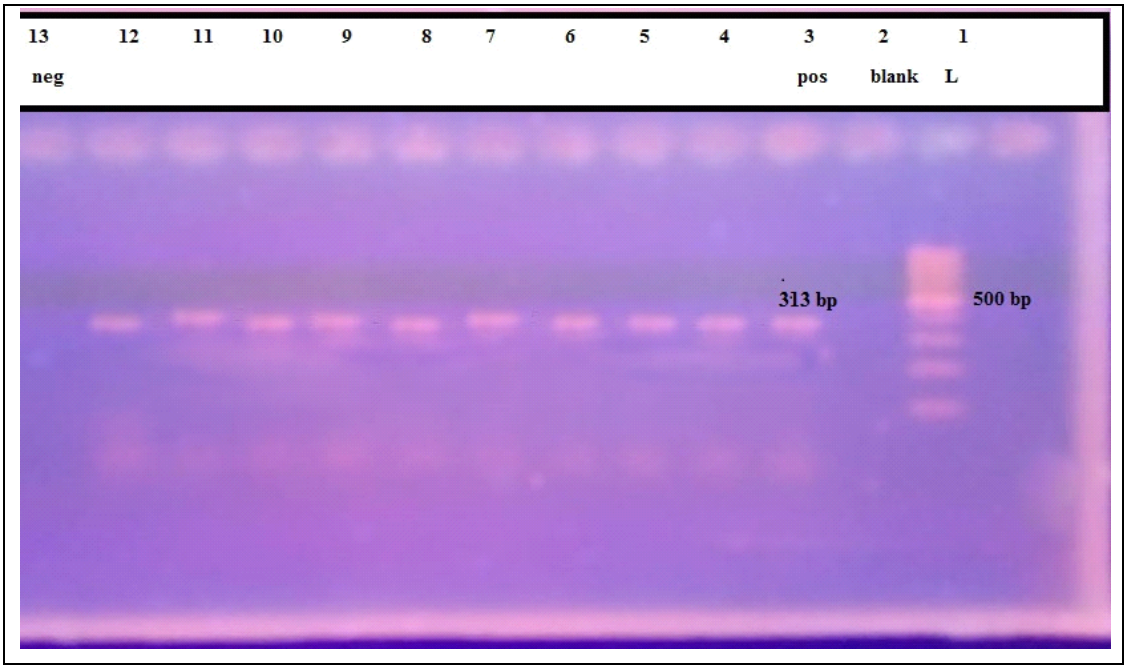


**Fig. (4):** PCR product of 313 bp using *Escherichia coli*. (Tra T) gene species-specific PCR assay. Lane 1:100 bp ladder DNA marker, lane 2: blank, lane 4-12: *Escherichia coli* positive isolate, lane 3:control positive (*Escherichia coliATCC: 9637*) and lane13: *staphylococcus aureus* control negative (*Staphylococcus aureus*ATCC: 6538).

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**الانتشار والتوصيف الجزيئي لمعظم العزلات البكتيرية الشائعة من التهاب الضرع البقرى**

**دون الإكلينيكي والإكلينيكي**

**الملخص العربى**

**ارجوا كتابه الاسامى عربى وجهه العمل**

يعد التهاب الضرع ، وهو التهاب في الغدة الثديية ، أحد أكثر الأمراض المكلفة والمعقدة في الأبقار الحلوب. تم تصميم هذه الدراسة لتقييم حدوث مسببات الأمراض المسببة في 412 من عينات الالبان المجمعة من الأبقار الحلوب السريرية والسريرية ، على مستوى قبل تناول العلاج بالمضادات الحيوية. أيضا لدراسة اشتملت التوصيف الجزيئي للبكتيريا المعزولة. تم أخذ عينات من اللبن الخام من أبقار الألبان في منطقة البحيرة ، محافظة القليوبية ، الشرقية ، بني سويف ومحافظ المنوفية في مصر ، للتحقيقات الميكروبيولوجية. كما تم عزل البكتيريا السائدة فى عينات الحليب للا بقار المصابه بالتهاب الضرع الإكلينيكي وتحت الإكلينيكي وكانت الإشريكية القولونية (30.3٪) المكورات العنقودية الذهبية (29.9٪) و البكتريا العقدية (28.6٪) ولم يكن هناك نمو في (11.2٪) من العينات. التهاب الضرع تحت الإكلينيكي يمثل نسبة كبيرة من عبء التهاب الضرع، وتشير النتائج ان 93.4٪ التهاب الضرع تحت الإكلينيكي .ولذلك يشترط الكشف على مسببات الأمراض الرئيسية في الضرع أثناء التهاب شأنه أن يساعد في إنقاذ الحيوان من التهاب الضرع هناك عن طريق تجنب تلف دائم في الضرع. وبالتالي هناك حاجة للكشف السريع والحساس والمحددة لمسببات الأمراض المسببة لالتهاب الضرع في مرحلة مبكرة من العدوى. مع أخذ ذلك في الاعتبار لدينا PCR محدد لنوع جنس مسببات الأمراض الرئيسية.