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Identification of bacteria in sub clinical mastitis milk by using multiplex PCR

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Abstract
Two hundred milk samples taken from fifty cows which appears healthy. mPCR was used to test milk samples. In this study, the prevalence of subclinical mastitis was 46.5% (93/200) in the quarter and 52.5% (26/50) in the animals basis, according to mPCR. On investigation, Staphylococcus spp. (42%) were identified as the primary pathogen.

Keywords: Sub clinical mastitis, mPCR

Introduction
Mastitis is a prevalent disease among cattle. Mastitis is classified according to the degree of inflammation and severity of the ailment- subclinical mastitis and clinical mastitis (Awale *et al*. 2012). This form is 30-40 times more common than clinical mastitis and causes the greatest economic loss in most dairy animals. (Bachaya *et al*., 2012) [2]. Subclinical mastitis is mostly caused by Streptococcus, Staphylococcus and coliforms bacterial species. (Mpatiswenumugabo *et al*., 2017) [5]. S. aureus is the, major pathogen which responsible for subclinical mastitis. Piepers *et al*. (2007) [6]. Qing-Hil *et al*. (2008) [7] found that the Multiplex Polymerase Chain Reaction (mPCR) is useful for quick disease detection.

Material and Method
Aseptic collection of 200 quarter milk samples from 50 cows that appeared to be in good health was conducted at several government hospitals, the Veterinary Clinical Complex of the College of Veterinary and Animal Science in Bikaner, as well as some commercial dairy farms in the neighboring districts of Bikaner. Using multiplex PCR, all 200 milk samples were tested.

Collection of milk samples
Aseptic milk samples were taken from teat after properly cleaning. Drying them with air after cleaning. The teats were then cleansed with a spirit swab. The initial two or three strips of foremilk were removed. Then, 30 mL of foremilk from each teat was collected in a sterile test tube. These were identified as left fore (LF), left hind (LH), right fore (RF), and right hind (RH). Samples of 200 quarters from 50 cattle were taken. All samples were maintained in a refrigerator set at 40 degrees. Then the sample was submitted to mPCR.

Multiplex PCR
Multiplex PCR is useful for quick identification of diseases as well as identifying many bovine mastitis pathogens at once. Using multiplex PCR, bacterial DNA of mastitis pathogens was also identified in culture negative milk samples. (Koskine *et al*., 2010) [4].

Procedure
DNA extraction from mastitic milk
Genetic material was extracted from mastitic samples of milk using a Thermo Scientific Ltd. DNA extraction kit and its protocol.

Agarose gel electrophoresis

Agarose gel electrophoresis is used to assess the quality of DNA. It was performed in a horizontal submerged device using 0.8% agarose gel in Tris borate EDTA BE buffer with ethidium bromide. After adding the bromophenol blue dye, each sample was placed in the gel's well. To conduct electrophoresis, one hundred volts at room temperature was necessary for around 1-2 hours, depending on the length of the gel, or until the dye had moved over half the length of the gel. After electrophoresis, the gel was examined using an ultraviolet (UV) transilluminator and photographs were taken.

Genotyping

The following primer pairs were used to test the isolates at the species level using multiplex PCR.

Pair No-1: Bacterial species Product size

Staphylococcus aureus 264bp
Pseudomonas aeruginosa 472bp
Klebsiella pneumoniae 555bp

***Pseudomonas aeruginosa* identification based on the FecR gene**

Js PA F - 5` TGACCACGAAGAACACCTCG 3`
Js PA R- 5` TTCGCAGACGAAACCGAAGA 3`

Identification of *Klebsiella pneumoniae* based on Bar A gene

Js KN F- 5` GATGGGCGGGGATATTTCGT 3`
Js KNR- 5` TTCAGGTTAGCCGGGTTGTC 3`

Identification of *Staphylococcus aureus* based on VicK gene

Js SA F- 5`CAGACCGTCGTGGACGTATT 3`
Js SA R - 5`TCACGTCATGTAACACAGGGA 3`
For primer set no. 1, the annealing temperature was 55 °C.

Pair No. 2: Bacterial species product size

Escherichia coli 119bp
Streptococcus agalactiae 304bp

Identification of *Listeria monocytogenes* based on Atr gene

jsStrAg F- 5` CCCTTCTGGCTCTGGTAAGTC 3`
jsStrAg R- 5` TGCTGGATAAGCATTAGCCTTCT 3`

Identification of *Escherichia coli* based on Uid A gene

Js EC F -5` TACCGACGAAAACGGCAAGA 3`
Js EC R- 5` CGGTGATATCGTCCACCCAG 3`
For primer set no. 2, the annealing temperature was 51 °C.

Pair No- 3 Bacterial species Product size

Staphylococcus hyicus 173bp
Streptococcus uberis 338bp`

Identification of *Streptococcus uberis* based on pauA gene

jsStrU F- 5` AACTAGTCGACTTTGCGCCT3`
jsStrU R- 5` GTCAGGGTAGCGTTGCAAAA3`

Identification of *Staphylococcus hyicus* based on Sod A gene

Js SH F- 5` TAACAATGGTGGCGGTCACT 3`
Js SH R- 5`AAGCCCAGCCAGATCCAAAT 3
For primer set no. 3, the annealing temperature was 51 °C.

Multiplex Polymerase Chain Reaction: (Table no.- 1)

Result and Discussion

Out of two hundred milk samples, mPCR revealed that 93 of them contained pathogenic microbes. Eighty-six (8.60%) milk samples had a mixed infection, while ninety-five (91.3%) milk samples had a single bacterial infection. A total of 102 bacteria were identified from 93 quarters. (Table 3 and 4)

The results of the present investigation were similar to those of Choudhary (2018) [9], who isolated 97 isolates from 40 clinical mastitis samples using mPCR. Out of these, *Staphylococcus aureus* accounted for the greatest percentage (62.5%, 25/97), followed by *K. pneumonia* (32%), 13 S. hyicus (35%, 14/97), *Escherichia coli* (25%, 10/97), *Streptococcus uberis* (12.5%, 5/97), *Pseudomonas aeruginosa* (12.5%, 5/97), *M. bovis* (15%, 6/97), *L. monocytogenes* 5%, 2/97), and *S. agalactiae* (42.5%, 17/97), in that order.

Table 1: Set no.1 reaction preparation

S. No.	PCR components	Quantity
1	PCR assay buffer	5µl
2	Template DNA, Mgcl2	3µl
3	Primer- Frwd, dNTP, Primer- reverse	1µl
4	Taq DNA polymerase (5 U/µl)	0.25 µl
5	DEPC treated	6.75µl
	Total	25µl

Table 2: Steps in PCR reaction

Step		Temperature (°C)	Time
Step 1	Denaturation	96	5 min
Step 2	Denaturation-Annealing- Set 1	94	1min
	Set 2	55	1 min
	Set 3	51	1 min
	Extension	72	1 min
Step 3	Final extension	72	7 min
	Hold	4	infinite

Table 3: Relative frequency of different types of bacterial isolates in sub clinical mastitis infected quarters by m PCR

S. No	Bacterial isolates	No. of quarters	Percentage (%)
1	*Pseudomonas aeruginosa*	1	1
2	*Staphylococcus hyicus*	8	8.69
3	*Streptococcus agalactiae*	11	11.8
4	*Streptococcus uberis*	4	4.30
5	*Klebsiella pneumonia/ Escherichia coli*	6	6.45
6	*Streptococcus dysgalactiae*	8	8.69
7	*Staphylococcus epidermidis*	2	2.15
8	*Staphylococcus aureus*	39	41.93
9	*Streptococcus aureus+ Streptococcus dysgalactiae*	3	3.22
10	*Staphylococcus aureus + Escherichia coli*	2	2.15
11	*Staphylococcus aureus + Streptococcus agalactiae*	2	2.15
12	*Staphylococcus aureus + Klebsiella pneumonia + Pseudomonas aeruginosa*	1	1
	Total	93	

Table 4: pathogens detected by using m PCR

S. No	Bacterial pathogens	Number of organism
1	*Staphylococcus aureus*	47(46.07%)
2	*Streptococcus agalactiae*	13 (12.74%)
3	*Staphylococcus hyicus*	8 (7.84%)
4	*Klebsiella pneumonia*	7(6.86%)
5	*E. coli*	8 (7.84%)
6	*Streptococcus uberis*	4 (3.92%)
7	*Streptococcus dysgalactiae*	11 (10.7%)
8	*Staphylococcus epidermidis*	2 (1.96%)
9	Pseudomonas aeruginosa	2 (1.96%)
	Total	102

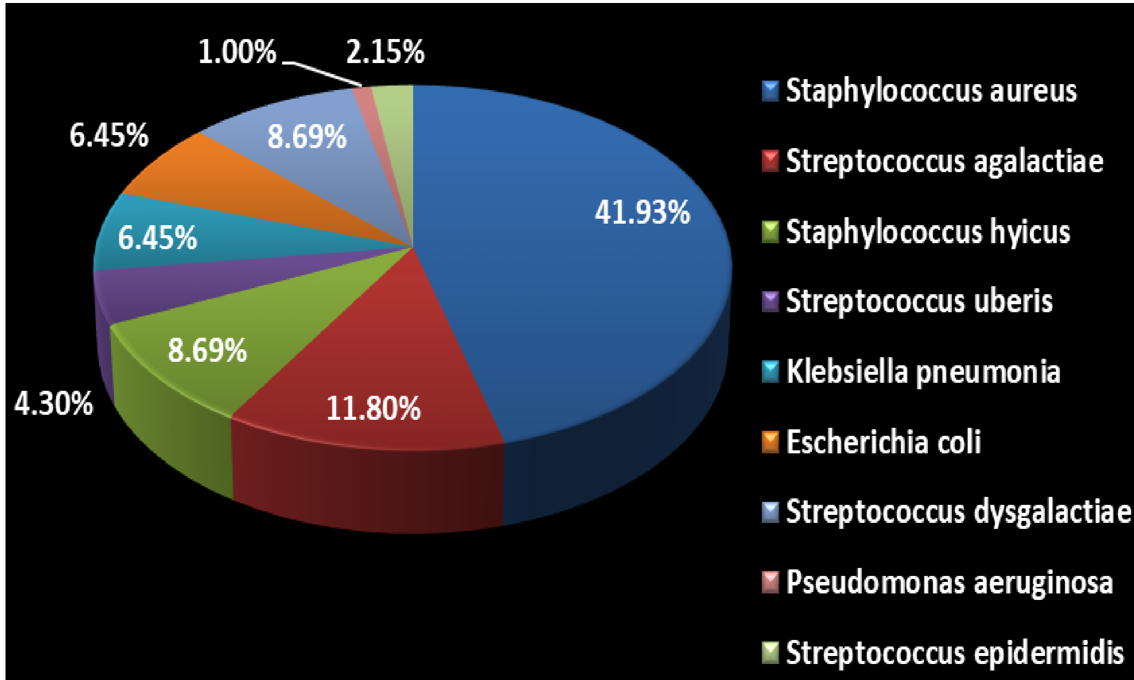

Fig 1: Relative frequency of bacteria isolate infected by mPCR (Sample having single infection)

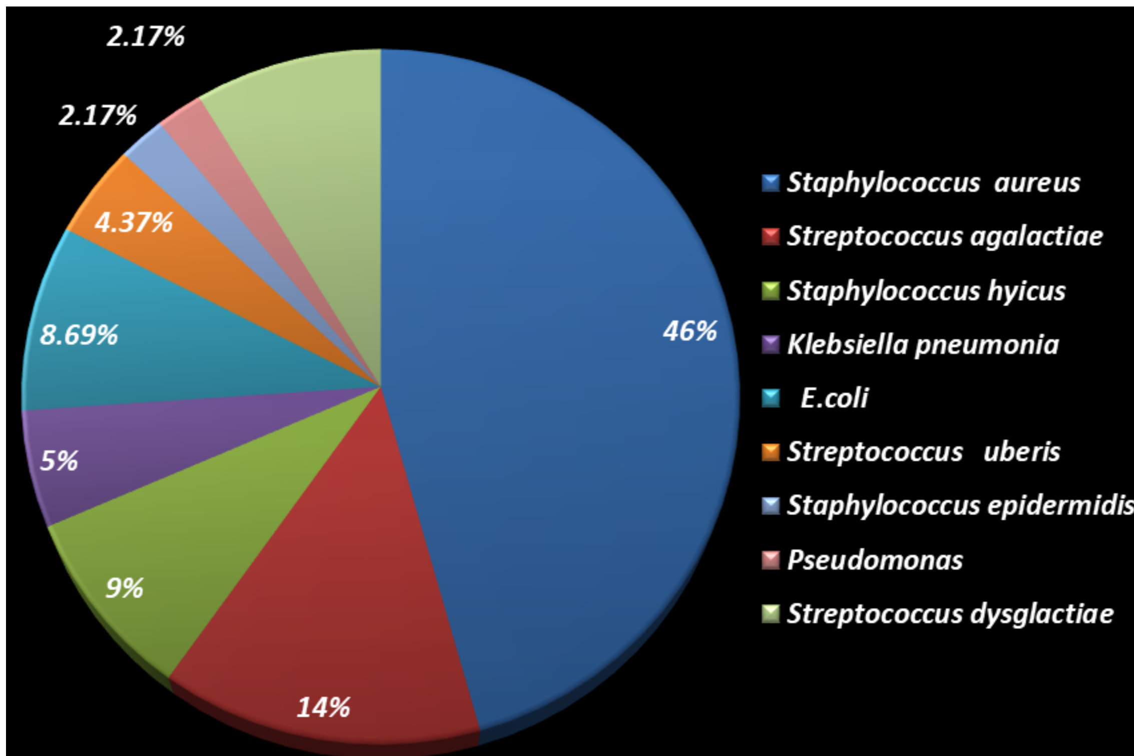

Fig 2: Relative Frequency 93 isolates from 82 quarters by mPCR

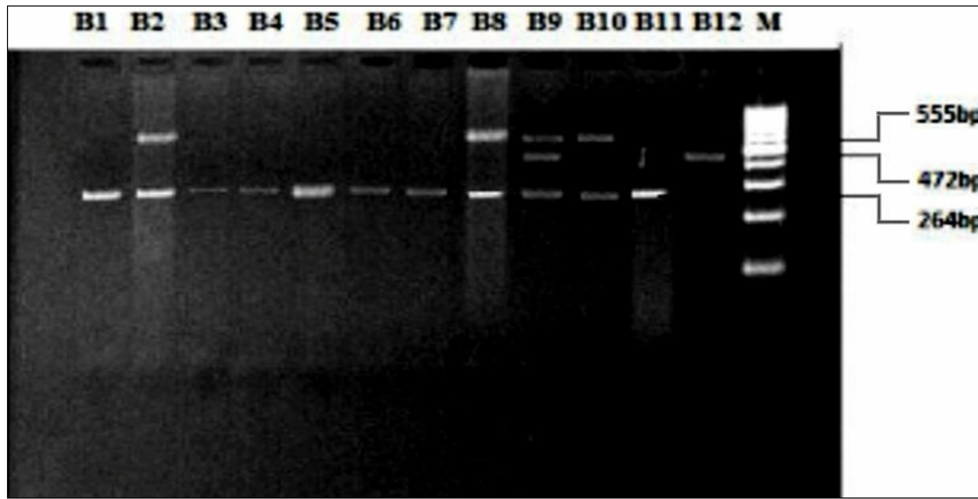

Fig 3: Pathogen identification in Set no. 1

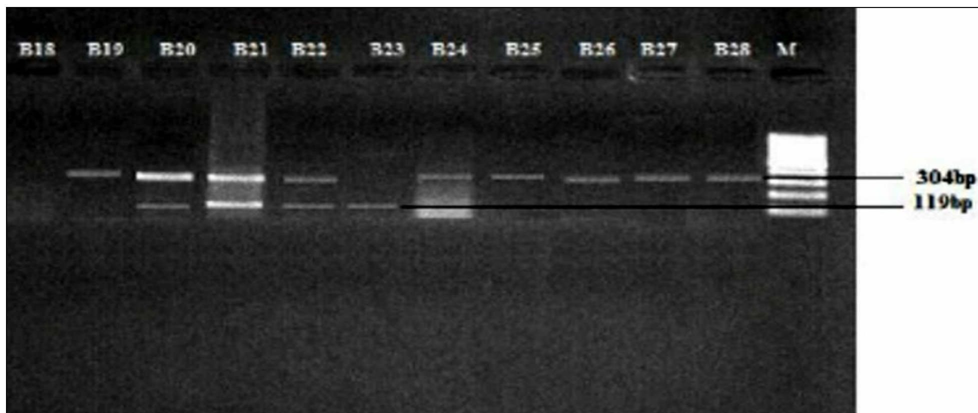

Fig 4: Identification of pathogens in set no.2

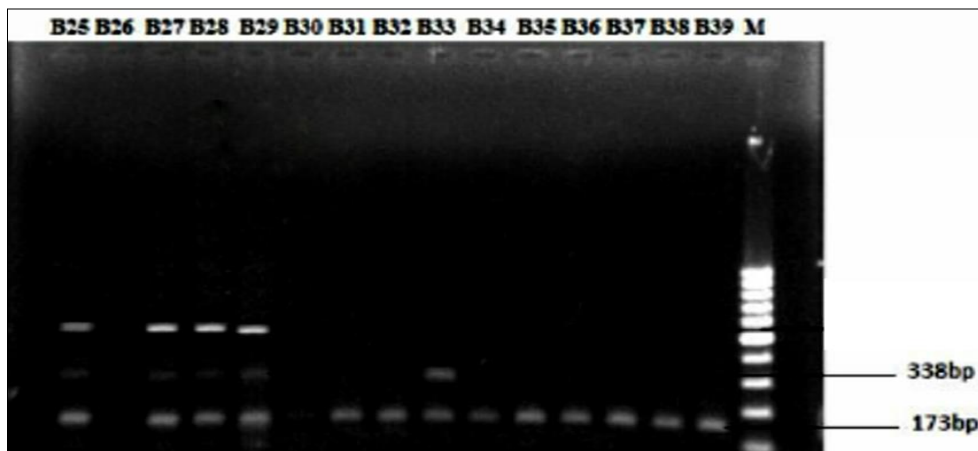

Fig 6: Identification of pathogens in set no 3

Summary and Conclusion

The objective of the current study, "Studies on some aspects of subclinical mastitis in cattle," was to use mPCR to determine the prevalence of subclinical mastitis in cattle. Using mPCR, ninety three of the two hundred quarter milk samples tested positive for pathogenic bacteria. 102 bacterial isolates were found in 82 samples; 85 milk samples (91.39%) had a single bacterial infection, and 8 (8.60%) had a mixed infection. It was discovered that mixed infections included Pseudomonas aeruginosa, E. coli, Klebsiella spp., and Staphylococcus spp. The cultural examination takes time to identify an organism. Therefore, multiplex PCR analysis should be employed. It is useful for quickly diagnosing and identifying more than one pathogen in mastitic milk.

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