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Detection of Sub Clinical Mastitis Pathogens by Multiplex PCR

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ABSTRACT

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A total of 200 quarter milk samples from 50 apparently healthy cows were examined by mPCR. In present study, the quarter wise prevalence of Sub clinical mastitis in cows 46.5 per cent (93/200) based on mPCR, respectively. Animal wise prevalence of sub clinical mastitis in cows was 52 per cent (26/50) based on mPCR. On examination *Staphylococcus spp.* (42 per cent) being major pathogen. Organism isolated in mixed infections was *Streptococcus spp.*, *Klebsiella pneumoniae*, *E. coli* and *Pseudomonas aeruginosa*.

Introduction

Mastitis is one of the most important disease of bovines and continues to have a major economic impact in the dairy industry through out the world (Bachaya *et al.*, 2012). Mastitis is classified into clinical and sub clinical form, according to degree of inflammation and severity of disease (Awale *et al.*, 2012). The sub clinical mastitis is asymptomatic, therefore milk appears to be normal. This form of mastitis is+ 30-40 times more common than clinical mastitis and causes the great loss in most of the dairy herds. Sub clinical mastitis is devastating disease in dairy industry through out

the world and in India it is enzootic and alarming. The sub clinical mastitis usually goes unnoticed because the milk and udder appear normal. The sub clinical mastitis is mainly caused by

Staphylococcus aureus, *Streptococcus agalactiae* and *Streptococcus* species and coliform (Mpatswenumugabo *et al.*, 2017). The *S. aureus* is the major pathogen of sub clinical mastitis (Piepers *et al.*, 2007). The Multiplex Polymerase Chain Reaction (mPCR) for mastitis diagnosis is helpful for rapid application of the preventive measures of the disease (Qing-Hil *et al.*, 2008).

Materials and Methods

A total of 200 quarter milk samples from 50 apparently healthy cows of different lacteal stage were collected aseptically from LRS, College of Veterinary and Animal Science, Bikaner and private dairies in surrounding areas of Bikaner. All the milk samples were screened by multiplex PCR.

Collection of milk samples

Milk samples were collected aseptically. Udder and teats were washed with water and air dried. Then each teat was wiped off by spirit swab. First Two three strippings of fore milk were discarded. Approximately 30 ml of fore milk from each teat was collected in sterilized test tube with caps. These were marked as right fore (RF), right hind (RH), left fore (LF), left hind (LH). Care was taken to avoid any type of contamination in the milk.

The milk samples from a total of 200 quarters of 50 cattle collected in the present study. All the samples of milk were brought to the laboratory and kept in refrigerator (4°C) until analysed. Milk samples were subjected to mPCR.

Multiplex PCR

Multiplex PCR helps in rapid detection and identification for more than one bovine mastitis pathogens at a time. The use of multiplex PCR resulted in identification of bacterial DNA of mastitis pathogens in culture negative milk samples also (Koskine *et al.*, 2010).

Procedure

Multiplex PCR helps in rapid detection and identification for more than one bovine mastitis pathogens at a time. The use of multiplex PCR resulted in identification of

bacterial DNA of mastitis pathogens in culture negative milk samples also (Koskine *et al.*, 2010)

Isolation of DNA from mastitic milk

DNA isolation from mastitic milk samples was done by using Thermo scientific DNA extraction kit, following protocol supplied with the kit.

Agarose gel electrophoresis

Agarose gel electrophoresis was carried out in horizontal submerged electrophoresis unit. It is used to check the integrity of DNA. For electrophoresis, 0.8 per cent agarose gel in TBE buffer containing ethidium bromide (0.5-1 µg) was used. After addition of bromophenol blue dye, each sample was loaded in the well of gel. 100 V at room temperature was required to carried out electrophoresis for about 1-2 hr depending upon the length of gel or till the dye migrated more than half of the length of the gel. At the end of electrophoresis, the gel was visualized under UV transilluminator and photographs were obtained.

Genotyping

Following sets of primers were used for species level confirmation of the isolates multiplex PCR.

Set No-1

Bacterial species Product size

Staphylococcus aureus 264bp

Pseudomonas aeruginosa 472bp

Klebsiella pneumoniae 555bp

Annealing temperature for following set of primers was 55°C.

Table.1 Reaction preparation for species multiplex PCR set no.1.

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

Table.2 Steps in species specific multiplex PCR reaction

Step		Temperature(°C)	Time
Step 1	Denaturation	96	5 min
Step 2 (35 cycle)	Denaturation-Annealing- Set	94	1min
	1	55	1 min
	Set 2	51	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	<i>Staphylococcus aureus</i>	39	41.93
2	<i>Streptococcus agalactiae</i>	11	11.8
3	<i>Staphylococcus hyicus</i>	8	8.69
4	<i>Streptococcus uberis</i>	4	4.30
5	<i>Klebsiella pneumoniae</i>	6	6.45
6	<i>Escherichia coli</i>	6	6.45
7	<i>Streptococcus dysgalactiae</i>	8	8.69
8	<i>Staphylococcus epidermidis</i>	2	2.15
9	<i>Pseudomonas aeruginosa</i>	1	1
10	<i>Streptococcus aureus</i> + <i>Streptococcus dysgalactiae</i>	3	3.22
11	<i>Staphylococcus aureus</i> + <i>Escherichia coli</i>	2	2.15
12	<i>Staphylococcus aureus</i> + <i>Streptococcus agalactiae</i>	2	2.15
13	<i>Staphylococcus aureus</i> + <i>Klebsiella pneumonia</i> + <i>Pseudomonas aeruginosa</i>	1	1
	Total	93	

Table.4 Pathogens detected by Multiplex PCR

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

Fig.1 Relative frequency of different types of bacterial isolate in sub clinical mastitis infected quarters by mPCR (Sample having single infection)

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

Fig.3 Set-1 Identification of *Staphylococcus aureus*-264bp *Klebsiella pneumoniae*-555 bp and *Pseudomonas aeruginosa* 472 bp

Fig.4 Set 2 - Identification of *E. coli*-119bp *Streptococcus agalactiae*-304bp amplicon size

Fig.5 Set 3 Identification of *Staphylococcus hyicus*- 173bp, *Streptococcus uberis*-338bp

VicK gene based identification of *Staphylococcus aureus*

Js SA F- 5`CAGACCGTCGTGGACGTATT
3`

Js SA R -
5`TCACGTCATGTAACACAGGGA 3`

FecR gene based identification of *Pseudomonas aeruginosa*

Js PA F - 5`
TGACCACGAAGAACACCTCG 3`

Js PA R- 5`
TTCGCAGACGAAACCGAAGA 3`

Bar A gene based identification of *Klebsiella pneumoniae*

Js KN F- 5` GATGGGCGGGGATATTTCGT
3`

Js KNR- 5` TTCAGGTTAGCCGGGTTGTC
3`

Set No. 2

Bacterial species product size

Escherichia coli 119bp

Streptococcus agalactiae 304bp

Annealing temperature for following set of primers was 51°C

Uid A gene based identification of *Echerichia coli*

Js EC F -5`
TACCGACGAAAACGGCAAGA 3`

Js EC R- 5` CGGTGATATCGTCCACCCAG
3`

Atr gene based identification of *Listeria monocytogenes*

jsStrAg F- 5`
CCCTTCTGGCTCTGGTAAGTC 3`

jsStrAg R- 5`
TGCTGGATAAGCATTAGCCTTCT 3`

Set No- 3

Bacterial species product size

Staphylococcus hyicus 173bp

Streptococcus uberis 338bp

Annealing temperature for following set primers was 51°C.

Sod A gene based identification of *Staphylococcus hyicus*

Js SH F- 5` TAACAATGGTGGCGGTCCT
3`

Js SH R- 5`AAGCCCAGCCAGATCCAAAT
3`

pauA gene based identification of *Streptococcus uberis*

jsStrU F- 5`
AACTAGTCGACTTTGCGCCT3`

jsStrU R- 5`
GTCAGGGTAGCGTTGCAAAA3`

Multiplex Polymerase Chain Reaction (Table no.- 1)

Results and Discussion

Out of 200 quarters milk samples, 93 quarters were found positive for pathogenic bacteria on mPCR. Out of 93 samples, 102 bacterial

isolates were identified in which 85 (91.3 %) milk samples were having single bacterial infection where as 8 (8.60 %) milk samples were having mixed infection (Fig. 1–5 and Table 2). Organisms isolated in mixed infections were *Staphylococcus* spp., *Streptococcus* spp., *Klebsiella* spp., *E. coli* and *Pseudomonas*. (Table-3 and 4)

The findings of present study are similar with Choudhary (2018) who isolated 97 isolates from 40 clinical mastitis samples by mPCR, out of that *S. aureus* was maximum (62.5%, 25/97) followed by *S. agalactiae* (42.5%, 17/97), *S. hyicus* (35 %, 14/97), *K. pneumonia* (32%, 13/97), *E. coli* (25%, 10/97), *S. uberis* (12.5%, 5/97), *P. aeruginosa* (12.5%, 5/97), *L. monocytogenes* (5 %, 2/97) and *M. bovis* (15 %, 6/97), respectively. The multiplex PCR in present study provided a convenient and accurate identification of 9 important mastitis pathogens simultaneously.

The multiplex PCR successfully detected all those bacteria isolated in culture studies. It was helpful in detecting the organisms even in culture negative samples.

Taponen *et al.*, (2009) reported a large proportion of positive samples via molecular methods from culture-negative milk samples in conventional bacteriology.

The present study entitled “Studies on some aspects of sub clinical mastitis in cattle” was undertaken to determine the prevalence of subclinical mastitis in cattle on the basis of California mastitis test, cultural examination and mPCR, changes in composition of milk and oxidative stress in animals having sub clinical mastitis.

Out of 200 quarters milk samples, 93 were found positive for pathogenic bacteria on mPCR. Out of 82 samples, 102 bacterial isolates were identified in which 85 milk

samples were having single bacterial infection (91.39%), where as 8 (8.60%) milk samples were having mixed infection. Organisms isolated in mixed infections were *Staphylococcus* spp., *Klebsiella* spp., *E. coli* and *Pseudomonas aeruginosa*.

For identification of organism cultural method is time consuming. Therefore, multiplex PCR analysis should be used which helps in rapid detection and identification for more than one bovine mastitis pathogens at a time that could produce results in a single day with the help of species specific primers.

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