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Development of a multiplex PCR for the identification of *Pseudomonas aeruginosa*, *Escherichia coli*, *Klebsiella pneumoniae* and *Streptococcus agalactiae* in milk

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In the dairy industry financial losses mainly due to loss of milk production caused by mastitis and mastitis related problems is very common. In the mastitis, identification of the infectious agent/s could be of great help. In the present study, a multiplex polymerase chain reaction (PCR) to identify five common organisms implicated in the mastitis was tried. Primers for identification of *Staphylococcus aureus*, *Escherichia coli* (*E. coli*), *Klebsiella pneumoniae, Pseudomonas aeruginosa,* and *Streptococcus agalactiae* were selected/ designed. DNA was extracted from the affected milk using two different methods and was subjected to multiplex PCR. It was observed from the study that a multiplex PCR to amplify four organisms *E. coli*, *K. pneumoniae*, *S. agalactiae* and *P. aeruginosa* was developed successfully to quicken the detection of common mastitis causing organisms.

Keywords: Mastitis milk, microorganisms, multiplex PCR

Introduction

Dairy industry plays a significant role in the economy of the farmer as well as of the nation. Dairy industry faces financial losses due to various diseases such as brucellosis, infectious bovine rhinotracheitis (IBR) etc but among this major financial losses suffered by the industry are due to loss of milk production caused by mastitis and mastitis related problems. It is a multi-factorial disease and there are more than 150 bacterial species implicated in it and broadly they have been categorized into three major types; environmental, contagious and opportunists[1].

Preliminary diagnosis of mastitis is based upon the history and clinical signs like the inflammation of the udder or change in milk composition. But these symptoms are more apparent in clinical mastitis or chronic cases rather than in sub-clinical mastitis. Therefore, for an early diagnosis of mastitis in dairy animals various tests are performed, however, isolation and identification of bacteria is considered as gold standard for diagnosis. But the method is time consuming as it requires isolation of bacteria on selective and non-selective media and further confirmation by biochemical tests. Since remarkable progress has been made in molecular biology and techniques like polymerase chain reaction (PCR) have been developed which are highly sensitive, specific and less time consuming which could be used for the diagnosis of mastitis too. One modification of PCR is a multiplex PCR where we amplify multiple targets by adding more than one pair of primers in a single reaction thus saving considerable time without affecting its sensitivity[2]. It has been previously shown that milk samples could serve as template directly for the amplification using PCR[3-4] and thus this type of assay would be of great value in the dairy sector for diagnosis of mastitis. With the increase in the antimicrobial resistance in most of the bacterium it was also imperative to study those organisms which quickly adapt to antimicrobial genes and thus increase its spread in the environment. Bacterium *Pseudomonas aeruginosa* being one important bug which very quickly adapt to various conditions and develop antibiotic resistance[5]. As antimicrobial resistance is being observed in the dairy sector too[6-7] thus to understand the prevalence of *P. aeruginosa* would be of great help. Keeping in view the above facts the present study was designed with an objective of forming multiplex PCR for identification of *P. aeruginosa* along with other mastitis causing organisms.

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Materials and Methods

Sampling

A total of 101 milk samples (15 - 20 ml) from mastitic animals (sub-clinical and clinical cases) were collected aseptically (after discarding first few streaks of milk) from the Veterinary Clinical Complex, GADVASU, Ludhiana and also from nearby dairy farms in and around Ludhiana (Punjab). Sodium lauryl sulphate (SLS) test was performed and positive samples were transferred immediately to the laboratory on ice. All the samples were subjected to isolation of bacteria using blood agar, brain heart infusion agar, eosine methylene blue agar, Baird Parker agar, MacConkey's lactose agar, Edwards medium and cetrimide agar. All the bacterium isolated were identified using specific biochemical test as per[8].

Extraction of DNA from the Standard Cultures

Standard cultures of *S. aureus* (MTCC 902), *E. coli* (MTCC 901), *K. pneumoniae* (MTCC 432) and *S. agalactiae* and *P. aeruginosa* (available in the Department of Veterinary Microbiology) were revived. These cultures were stored in trypticase soy agar (slant) at 4°C throughout the period of study. The extraction of bacterial genomic DNA was done using phenol: chloroform: isoamyl alcohol (25:24:1) (PCI)[9]. In brief, 1.8 ml of overnight grown bacterium in Luria Bertani (LB) broth was centrifuged and to the pellet, 50 µl each of 10% SDS (sodium dodecyl sulphate) and proteinase K (200 µg/ml) were added and incubated at 60°C for 1 h. Next, 500 µl of phenol: chloroform: isoamyl alcohol (PCI; 25:24:1) was added to it, mixed gently and centrifuged (10,000 x g) for 10 minutes to collect the supernatant and the step was repeated. To the supernatant equal volume of isopropanol and to the one-tenth volume of supernatant, 3M sodium acetate (pH 5.2) was added and kept at -20°C for overnight. Next day, the precipitated DNA was collected after centrifuging (10,000 x g) for 20 minutes. DNA was washed twice with 500 µl of 70% ethanol and after removal of residual ethanol it was reconstituted to 50 µl by adding nuclease free water (NFW) and stored at -20°C for further use.

Extraction of the DNA from the Milk

The DNA was extracted from the milk using two different methods viz., SDS-triton method[10] and power food microbial DNA isolation kit (Mo Bio Laboratories, Inc. USA).

Extraction of Bacterial DNA Directly from Milk Using SDS-Triton

One millilitre (ml) each of 10% SDS and 20% Triton-X-100 was added to eight ml of milk and incubated at 60°C for 1 h to remove or lessen the fat/cream layer. Later, 1.8 ml milk was transferred and centrifuged and to the pellet, 50 µl each of 10% SDS and proteinase K (200 µg/ml) were added and incubated at 60°C for 1 h. Five hundred µl of phenol: chloroform: isoamyl alcohol (25 : 24 : 1) was added to it, mixed gently and centrifuged (10,000 g) for 10 minutes to collect the supernatant and the step was repeated and equal volume of isopropanol and one-tenth volume of supernatant, 3M sodium acetate (pH 5.2) was added and kept at -20°C for overnight. Next day it was centrifuged (10,000 g) for 20 minutes to collect the pellet and the pellet was washed twice with 500 µl of 70% ethanol and centrifuged (10,000 g) for 10 minutes and residual ethanol was removed and the pellet was reconstituted into 50 µl of nuclease free water (NFW).

Extraction of Bacterial DNA Directly from Milk

The DNA from the milk was extracted using power food microbial DNA isolation kit (Mo Bio Laboratories, Inc. USA).

Estimation of the Quality of the DNA extracted

The DNA extracted from all the three methods was tested for its optical density (OD) at 260/280 nm using Nanodrop spectrophotometer (Thermo Scientific, USA). A ratio of 1.7 to 1.9 (260/280 nm) was considered satisfactory. The DNA was then stored at -20°C for further use in PCR and multiplex PCR.

Polymerase Chain Reaction (PCR)

For performing PCR specific primers for *S. aureus*, *E. coli*, *K. pneumoniae, P. aeruginosa,* and *S. agalactiae* were selected/ designed (Table 1). The primers were designed using Primer3 Software (https://www.ncbi.nlm.nih.gov/tools/primer-blast) and were synthesized commercially (Flarebio Biotech Inc., China). A 25 µl PCR reaction mixture was formulated using 12.5 µl of master mix (1X one *Taq* master mix) (New England Bio Labs, USA), 0.5 µl of 20 pmol/µl of each forward and reverse primers (Cusabio, China), 1.0 µl of template DNA and finally the reaction volume was made up to 25 µl using nuclease free water.

Table 1 — Primers for PCR and multiplex PCR

Organisms		Primer 5' to 3'	Size (bp)	Position		Reference
				From	To	
Staphylococcus aureus	F	CTGTACGCTAGGTGGAGCG	532			Kour *et al* (2018)
	R	TTTTGCAGGATGTCCGCCTT				
Pseudomonas aeruginosa / NC_002516.2	F	CTGGCCTTGACATGCTGAGA	183	6043208	6044743	This Study
	R	TCACCGGCAGTCTCCTTAGA		6043584	6043766	
Klebsiella pneumoniae / CP024838.1	F	ATGGCCGGGCATGGTACTTC	156	3416904	3416923	This Study
	R	ACCGGAGGTGATGTTTTCGGT		3417059	3417039	
E. coli	F	ATCAACCGAGATTCCCCCA	232			Riffon *et al* 2001
	R	TCACTATCGGTCAGTCAGGAG				
Streptococcus agalactiae	F	CTGTGAGATGGACCTGCGTT	352			Singh *et al* (2019)
	R	ACGCCCAATAAATCCGGACA				

Multiplex PCR on Bacterial DNA

A 25 µl multiplex PCR reaction mixture was formulated using 12.5 µl of master mix (1X one *Taq* master mix) (New England Bio Labs, USA), 0.5 µl of 20 pmol/µl of each forward and reverse primers of each bacteria viz., *S. aureus*, *E. coli*, *K. pneumoniae, P. aeruginosa,* and *S. agalactiae* (Cusabio, China), 1 µl of genomic DNA (100 ng) from each organism and the reaction volume was made up to 25 µl using nuclease free water.

Multiplex PCR on DNA from Milk

A 50 µl reaction mixture was formulated for multiplex PCR consisting of 25 µl of master mix (1X one *Taq* master mix) (New England Bio Labs, USA), 0.25 µl of 20 pmol/µl of each forward and reverse primers (Cusabio, China), 10 µl of template DNA and rest of the volume was made upto 50 µl using nuclease free water.

PCR and multiplex PCR was performed on a thermocycler (Veriti, ABI, USA) with the following conditions; an initial denaturation at 94°C for 5 minutes and 35 cycles each of denaturation at 94°C for 45 seconds, annealing at 60°C for 1 minute and extension at 72°C for 1 minute. This was followed by a final extension at 72°C for 10 minutes. The products were run on 1.5% agarose gel along with 100 bp DNA molecular weight marker (New England Biolabs, USA) at 5 V/cm and visualized using gel documentation system (AlphaImager, Alpha Innotech, USA).

Results & Discussion

Prevalence of Microorganisms

In the present study out of a total of one hundred and one milk samples collected 92 samples were positive via SLS test. From ninety two milk samples, eighty samples yielded bacterial growth. All the isolates were identified using appropriate biochemical tests and PCR and a total of 33 (41.25%) *S. aureus*, 24 (30.0%) *E. coli*, 16 (20.0%) *K. pneumoniae*, 5 (6.25%) *P. aeruginosa* and 2 (2.5%) *S. agalactiae* were isolated.

Evaluation of Two Different Methods for Extracting DNA from Milk

The DNA was extracted by two different methods from milk viz., using SDS-triton method[10] and power food microbial kit. The DNA extracted using both the methods was used as template DNA for PCR. On the basis of the PCR for *S. aureus*, *K. pneumoniae*, *E. coli, P. aeruginosa* and *S. agalactiae* it was observed that via SDS triton method we could amplify 63 culture positive samples out of a total of 80 culture positive samples whereas *via* kit method we could amplify 58 culture positive samples out of 80 culture positive samples. The application of PCR depends on the critical step of extraction of DNA from the heterogeneous food material as food constitutes a complex mixture containing PCR inhibitors that may compromise the amplifiability of DNA. The presence of high levels of fats, proteins, and calcium in milk can adversely affect the DNA amplification efficiency[11-16]. Since there are several methods to extract DNA directly from food (experimental protocols and commercial kits). Thus the objective of this study was to validate an earlier developed method of DNA extraction from milk which proved to be reliable, fast and economical. Bacterial DNA was extracted using two methods directly from milk sample using SDS triton and power food microbial

DNA isolation kit respectively. The bacterial DNA extracted from both the methods was subjected to PCR using genus specific primers revealing that the detection of individual microorganism was best using SDS triton method.

Polymerase Chain Reaction and Mulitplex PCR

For PCR all the primers selected/ designed in the study were tested on the genomic DNA of the known individual organisms. PCR was able to amplify the desired organisms. When these selected primers were able to amplify desired product for the organisms targeted they were subjected for simultaneous identification of five most common organisms causing mastitis (*S. aureus*, *K. pneumoniae*, *E. coli*, *P. aeruginosa* and *S. agalactiae*). Various combinations of reagents viz., DNA template 1-10 µl, concentrations of $MgCl_2$, annealing time (45-60 sec) temperature (55-60^{o}C) combinations were tried to standardize a single multiplex PCR to identify all the etiological agents. After conducting various experiments a DNA template volume of 10 µl, 1X one *Taq* master mix 25 µl and annealing time of 60 seconds at 60^{o}C for 35 cycles were finalized.

After the individual amplification we tested our multiplex and could multiplex four organisms *S. agalactiae*, *K. pneumoniae, E. coli* and *P. aeruginosa* in a single multiplex PCR. However, *S. aureus* could not be combined in the multiplex PCR. Though, we tried to vary initial template concentration for *S. aureus* in both the direction i.e. increasing and decreasing concentration but we could not make them to amplify all five etiological agents in single mPCR (Fig. 1).

In the present study the DNA was extracted by two different methods from milk viz., using SDS-triton method and power food microbial kit. There are several other methods exist to extract DNA directly from food experimental protocols and commercial kits. Thus the objective of this study was to validate an earlier developed method of DNA extraction from milk which proved to be reliable, fast and economical. The results suggested that isolation of organisms to extract DNA and using this DNA as template in PCR was much superior to the methods in which DNA was extracted from milk and used for PCR. PCR was used to identify the quality of the isolated bacterial DNA taking clue again from the study of Rossen *et al*. (1992) and Kour *et al*. (2020) where they found PCR to be sensitive to large volume of complex food samples containing high amount of fat and proteins.

For developing a multiplex PCR it was essential that the amplicons besides being specific should differ in length by at least 50 bp from each other so that they could be clearly distinguishable on agarose gel electrophoresis. Primers were designed keeping all the

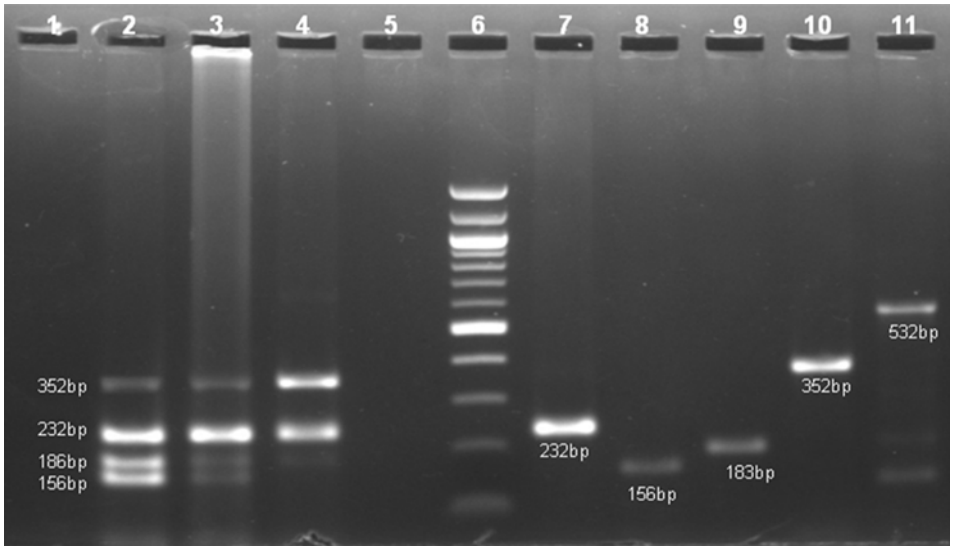

Fig. 1 — Single and multiplex PCR on DNA isolated from organisms and from milk using SDS triton method and kit

Lane 1 and 5 NTC, Lane 2 multiplex PCR for *Streptococcus agalactiae* (352 bp), *E. coli* (232 bp), *Pseudomonas aeruginosa* (183 bp) and *Klebsiella pneumoniae* (156 bp) on known DNA; Lane 3 multiplex PCR for *S. agalactiae*, *E. coli*, *P. aeruginosa* and *K. pneumoniae* (SDS triton method); Lane 4 multiplex PCR for *S. agalactiae*, *E. coli* and *P. aeruginosa* using kit; Lane 6 100 bp Ladder; Lane 7 *E. coli* (232 bp); Lane 8 *K. pneumoniae* (156 bp); Lane 9 *P. aeruginosa* (183 bp); Lane 10 *S. agalactiae* (352 bp); Lane 11 *Staphylococcus aureus* (532 bp)

above parameters in mind and checked *in silico* to observe their characters of specificity as well as G+C percentage and the primers once fulfilling most of the characters were synthesized and used in the multiplex PCR. Multiplex PCR has been developed for use on milk samples by various earlier workers. Troncarelli[17] developed a multiplex PCR for *S. aureus*, *S. agalactiae* and *E. coli* and multiplex PCR protocol was efficient for identification of *S. agalactiae* and *E. coli* but failed to detect *S. aureus* in evaluated bulk tank milk samples which is similar to our study where *Staphylococcus* could not be identified in multiplex PCR. In another study[18] a multiplex PCR was developed to identify bacterial pathogens of mastitis viz., *S. aureus, S. epidermidis, S. chromogens, S. agalactiae, S. dysgalactiae, S. uberis* and *E. coli* simultaneously in milk and tissue samples of mastitis affected buffaloes. The results based on multiplex PCR revealed that *S. aureus* was predominant bacteria in milk and tissue samples followed by *E. coli, S. agalactiae, S. epidermidis, S. chromogens, S. dysgalactiae* and *S. uberis* similar to our findings but we could not get *S. aureus* in our multiplex PCR. Similarly Singh *et al* (2019) conducted a study and developed a multiplex PCR for identification of *E. coli, K. pneumoniae, S. aureus* and *S. agalactiae* but their assay was able to amplify only three organisms' viz., *S. agalactiae, K. pneumoniae* and *E. coli* only but could not amplify *S. aureus* where *Staphylococcus* was positively isolated from the microbiological cultures but failed to amplify with the other organisms in the multiplex PCR reaction. However, the exact reason for this could not be identified. In this study multiplex PCR for identification of *P. aeruginosa* along with three other common mastitis causing organisms was achieved. Identification of *P. aeruginosa* is very important as this is the organism which can take up antibiotic resistant genes at a relatively faster rate[19]. In the present scenario various strategies are being formulated to prevent development of antibiotic resistance. In the dairy sector use of antibiotics is rampant[20] so with this PCR we would additionally be aware of the presence of an important antibiotic resistant genes transmitting organism. However, further studies are required to understand failure of *Staphylococcus* in getting amplified in this multiplex PCR.

Conclusions

Dairy industry plays a significant role in the economy of the farmer as well as of the nation. Control measures of mastitis in dairy cattle needs sensitive, rapid and specific tests to identify the main bacteria that cause heavy losses in the dairy industry. Hence, from the study it could be concluded that with the help of multiplex PCR we could identify *S. agalactiae, E. coli, P. aeruginosa* and *K. pneumoniae* and in a single PCR reaction directly on the DNA extracted from the milk.

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