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A simple modification in the DNA extraction process to extract good quality bacterial DNA from milk

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ABSTRACT

Mastitis is the inflammation of the udder characterized by pathological changes in the mammary gland tissue. The most common treatment regimen involves administration of antibiotics depending upon culture and antibiotic sensitivity test. Culture and antibiotic sensitivity testing requires a minimum of 2–3 days, thus search for alternative tests to quicken identification of causative agent has gained lot of focus. In mastitis, milk is the ideal sample for the identification of causative agents as well as for performing DNA based tests such as PCR. Milk though easy to collect, harbour certain inhibitors affecting isolation of DNA. Also, the DNA extracted might contain certain associated ions which interfere in PCR. In the present study, DNA was extracted from milk by initially treating it with SDS and triton and later DNA was extracted using standard phenol chloroform method (M1). The efficiency of extraction by this method (M1) was compared with that of a kit (Power food microbial DNA isolation kit) based method (M2). The DNA extracted from both the methods was evaluated and compared among each other using genus specific PCR for *E. coli*, *Klebsiella* spp., *Staphylococcus* spp. and *Streptococcus* spp. along with various antibiotic resistance genes present in these bacteria. From the study, it could be concluded that DNA could be extracted successfully using SDS and triton method directly from the milk more efficiently and is cost effective when compared with kit-based method.

Keywords: Antibiotic resistance genes, DNA isolation, Mastitis, Milk, SDS, Triton

Mastitis is one of the most important diseases in the dairy cattle because of its high incidence and production losses. It is the inflammation of the udder characterized by pathological changes in the mammary gland tissue, which may at times be accompanied by systemic disease, as well as several changes in the physical and chemical properties of milk (Radostitis *et al*. 2000). Mastitis could be categorized as acute or chronic based on duration and as clinical and sub clinical mastitis based on symptoms. Generally, the choice of treatment to be given in mastitis depends on the chronicity of infection, pathogen load, bacteriological culture and antimicrobial susceptibility testing. Since bacteriological culture and antimicrobial testing requires minimum 2–3 days, thus alternative diagnostic tests to hasten the process of diagnosis are currently being emphasized. Advanced molecular techniques are used for the diagnosis of the diseases in the animal health (D'Angelo *et al*. 2007) and polymerase chain reaction (PCR) is one such common technique that has been widely exploited.

In the case of mastitis, milk is the ideal sample used routinely for the diagnosis of infectious agent leading to mastitis. It is very easy to collect as it does not require any invasive technique. Milk though easy to collect, possesses certain inhibitors such as presence of high level of fats, proteins and calcium that might interfere in the extraction of DNA from milk. The extraction of DNA is the most critical step as for further downstream application we depend on the good quality DNA. Thus, the DNA extracted should be in sufficient quantity as well as should be amplifiable via PCR for it to be successful and repetitive test. The presence of inhibitors affecting efficacy of PCR assay has also been reported by various workers (Bickley *et al*. 1996, Cremonesi *et al*. 2006, Pirondini *et al*. 2010). There are several commercial kits as well as methods present for the isolation of DNA from the milk (Pirondini *et al*. 2010, Psifidi *et al*. 2010, Quigley *et al*. 2012) having their own advantages as well as disadvantages. Isolation of DNA from commercial kit is reported to be useful (Quigley *et al*. 2012) as well as time and labour saving (Psifidi *et al*. 2010) but it being expensive and thus cannot be widely used in the developing countries for routine diagnosis. Thus, the objective of the present study was to develop a simple, cheap and efficient procedure for isolating bacterial DNA directly from milk and evaluating its extraction efficacy with a commercial kit on the basis of PCR amplification.

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MATERIALS AND METHODS

Sampling: Milk samples (15–20 ml) suspected of mastitis

(clinical and subclinical) were collected aseptically (after discarding first few streaks of milk) from farms in and around Ludhiana, Punjab, India after taking due permission from Institutional Animal Ethics Committee (IAEC). The milk samples collected were tested immediately using Sodium Lauryl Sulphate test (SLS test) and those positive were selected and transported to the laboratory on ice for further processing.

Infectious agent isolation and identification: Milk samples brought to the laboratory were mixed thoroughly and inoculated onto Brain Heart Infusion (BHI) egar, Eosin Methylene Blue (EMB) agar, MacConkey's Lactose (MLA) agar, Baird Parker agar (BPA) supplemented with egg-yolk tellurite emulsion, Edward's medium and Blood Agar (BA) for 18–24 h at 37ºC. Isolated bacterial colonies were examined culturally, microscopically and biochemically (catalase, oxidase, indole, methyl red, voges proskauer's, citrate, triple sugar iron, esculin, arginine and fermentation of sugars) for the confirmation of the organisms.

Antibiotic sensitivity test: The test was performed as per the protocol of NCCLS (2002). Bacteria having zone of inhibition more than 15 mm were classified as sensitive and those equal to or less than 15 mm were classified as resistant.

Extraction of DNA: The DNA was extracted from the from milk using SDS and triton method indicated as M1 and Power food microbial DNA isolation kit (MO BIO Laboratories, Inc. USA) indicated as M2.

Extraction of bacterial DNA directly from milk using SDS-Triton (*M1*): One ml each of 10% SDS and 20% Triton-X-100 was added to 8 ml of milk and incubated at 60°C for 1 h to remove or lessen the fat/cream layer. This step was added so as to make DNA isolation from the milk easy. 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. Phenol: Chloroform: Isoamyl alcohol (PCI, 25: 24: 1) (500 µl) was added to it, mixed gently and centrifuged (10,000 ×g) for 10 min to collect the supernatant and the step was repeated. Equal volume of isopropanol and one-tenth volume of supernatant, 3 M sodium acetate (*p*H 5.2) was added and kept at -20°C for overnight. Next day it was centrifuged (10,000×g) for 20 min to collect the pellet and the pellet was washed twice with 500 µl of 70% ethanol and centrifuged (10,000×g) for 10 min and residual ethanol was removed and the pellet was reconstituted into 50 µl of nuclease free water (NFW). Later optical density (OD) at 260 and 280 nm of individual sample was measured using Nano drop (Thermo Scientific, USA). A ratio of 1.7 to 1.9 (260/280) was considered satisfactory. The DNA was then stored at –20°C for further use (Sambrook and Russels 2001).

Extraction of bacterial DNA directly from milk using power food microbial DNA isolation kit (*M2*): The DNA from the milk was extracted directly using kit as per the manufacturer's instructions. Briefly, 1.8 ml milk was centrifuged at 13,000 ×g for 1 min at room temperature (RT). The supernatant was decanted and the tubes were spun again to re-suspend the pellet in 450 µl of pre-warmed solution PF1 and transferred to micro Bead tube and vortexed for 10 min. The tubes were centrifuged at 13000 ×g for 1 min at RT and the supernatant was transferred to a new microcentrifuge tube. To this, 100 µl of solution PF2 was added, vortexed and incubated at 4°C for 5 min followed by centrifugation at 13,000 ×g for 1 min at RT and the supernatant was carefully transferred to another microcentrifuge tube after avoiding pellet. To the supernatant, 900 µl of solution PF3 was added and vortexed. After this, 650 µl of the supernatant was loaded onto a spin filter and centrifuged at 13,000 ×g for 1 min to discard the flow through and it was repeated until all the supernatant was loaded on the spin filter. Spin filter basket was placed to a new microcentrifuge tube and 650 µl of PF4 solution was added and centrifuged at 13,000 × g for 1 min at RT. The flow through was discarded and 650 µl of solution PF5 was added to the spin filter and centrifuged at 13,000 × g for 1 min at RT. The flow through was discarded and centrifuged at 13,000 ×g for 2 min to remove residual wash. The spin filter basket was placed in a new microcentrifuge tube and 100 µl of solution PF 6 was added and centrifuged at 13,000 × g for 1 min to collect the DNA. The DNA was stored at –20°C for further use.

Polymerase chain reaction (*PCR*): The PCR assay was carried out using genus specific primers for *E. coli*, *Klebsiella* spp., *Staphylococcus* spp. and *Streptococcus* spp. (Table 1). For *Staphylococcus* spp. genus, specific primers targeting 16SrRNA from *Staphylococcus aureus* subsp. *aureus* NCTC 8325, Accession No. CP000253.1 was designed using Primer 3 Software (Untergasser *et al*. 2012) and tested *in silico* initially. A PCR reaction mixture consisting 2.5 µl of 10× PCR buffer (New England Biolabs Inc, USA), 1 µl of 20 pmol/µl of each forward and reverse primers (Flarebio Biotech Inc, China), 0.6 µl for *Klebsiella* spp., 0.75 µl for *E. coli* and *Staphylococcus* spp. and 1 µl for *Streptococcus* spp., $MgCl_2$ (50 mM), 1 µl of 10 mM dNTPs mix, 0.2 of µl 5U/µl of Taq DNA polymerase, 2 µl of template DNA containing approximately 100 ng of DNA was made up to 25 µl with NFW. PCR was performed on a thermocycler (Veriti, Applied Biosystems, USA) with an initial denaturation at 94°C for 5 min and later 30 cycles of denaturation at 94°C for 45 sec, annealing at 60°C for *E. coli*, 58°C for *Klebsiella* spp. and 55°C for *Staphylococcus* spp. and *Streptococcus* spp. for 45 sec and extension at 72°C for 1 min. This was followed by a final extension at 72°C for 7 min. The products were run on 1.5% agarose along with 100 bp DNA molecular weight marker (New England Biolabs, USA) at 5V/cm and visualized using gel documentation system (AlphaImager, Alpha Innotech).

Identification of antibiotic resistance genes: Further, in order to check the efficacy of the method, the antibiotic resistance genes of the identified organisms were targeted. *E. coli*, *Klebsiella* spp., *Staphylococcus* spp. and *Streptococcus* spp. isolates isolated were subjected to PCR for detection of antibiotic resistance genes (Table 2). A PCR reaction mixture comprising 1× PCR buffer, 1.5 mM of

Table 1. Primers used for detection of *E. coli*, *Klebsiella* spp., *Streptococcus* spp. and *Staphylococcus* spp.

Organism	Oligonucleotide sequences (5′ to 3′)	Amplicon size (bp)	Annealing temp (°C)	Reference
E. coli	F: ATC AAC CGA GAT TCC CCCA R: TCACTATCGGTCAGTCAG GAG CAG GAG	232	60	Riffon *et al.* 2001
Klebsiella spp.	F: ATTTGAAGAGGTTGCAAACGAT R: TTCACTCTGAAGTTTTCTTGTGTTC	130	58	Turton *et al.* 2010
Staphylococcus spp.	F: CTGTACGCTAGGTGGAGCG R: TTTTGCAGGATGTCCGCCTT	532	55	Present study
Streptococcus spp.	F: CAGGAAGTGCTGTTACGTTAAAC R: CGTCCCATTTAGGGTTCTTCC	369	55	Jain *et al.* 2012.

$MgCl_2$, 200 µM of each deoxynucleoside triphosphate and 1U of Taq DNA polymerase, 20 pM each of the respective forward and reverse primer and 2 µl of template DNA was made up 25 µl by adding nuclease free water. PCR was performed on a thermocycler (Veriti, ABI, USA) with an initial denaturation at 94°C for 5 min followed 30 cycles each of denaturation at 94°C for 45 sec, annealing for 45 sec and extension at 72°C for 1 min followed by a final extension at 72°C for 10 min. The products were run on 1.5% agarose 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).

RESULTS AND DISCUSSION

Isolation and identification of the causative agents: Out of a total of 50 samples yielding bacterial growth, organisms belonging to 4 genus, viz. *Staphylococcus* spp. (43), *E. coli* (26), *Klebsiella* spp. (19) and *Streptococcus* spp. (15) were isolated and confirmed using gram's staining, cultural characters, biochemical tests and PCR using genus specific primers (Supplementary Fig. 1)

Comparison of the DNA extraction methods: DNA was extracted from all the milk samples that yielded bacterial growth. The DNA extracted from both methods (M1 and M2) was subjected to PCR using genus specific primers for *E. coli*, *Klebsiella* spp., *Staphylococcus* spp. and *Streptococcus* spp. Samples found positive using M1 and M2 method were 88.46% and 73.07% (genus *E. coli*); 84.21% and 73.68% (*Klebsiella* spp.); 86.04% and 74.41% (*Staphylococcus* spp.); 80% and 66.66% (*Streptococcus* spp.). Thus on the basis of number counts the results

Table 2. Primers for the detection of antibiotic resistance genes

Organism	Antibiotic gene	Oligonucleotide sequences (5' to 3')	Amplicon size (bp)	Annealing temp (°C)	Reference
E. coli and *Klebsiella* spp.	*aac*(*3*)-*iv*	F: CTTCAGGATGGCAAGTTGGT R: TCATCTCGTTCTCCGCTCAT	285	60	Momtaz *et al*. 2012
E. coli and *Klebsiella* spp.	*aad*A	F: GTGGATGGCGGCCTGAAGCC R: AATGCCCAGTCGGCAGCG	527	60	Boerlin *et al*. 2005
E. coli and *Klebsiella* spp.	*tet*B	F: CCTCAGCTTCTCAACGCGTG R: GCACCTTGCTGATGACTCTT	634	60	Momtaz *et al.* 2012
E. coli	*bla*SHV	F: TCA GCG AAA AAC ACC TTG R: TCC CGC AGA TAA ATC ACCA	472	60	Karczmarczyk *et al.* 2011
Klebsiella spp.	*bla*SHV	F: TCGCCTGTGTATTATCTCCC R: CGCAGATAAATCACCACAATG	768	58	Momtaz *et al.* 2012
Staphylococcus spp. and *Streptococcus* spp.	*tet*M	F: AGT GGA GCG ATT ACA GAA R: CAT ATG TCC TGG CGT GTC TA	158	55	Duran *et al*. 2012
Staphylococcus spp. and *Streptococcus* spp.	*blaZ*	F: ACTTCAACACCTGCTGCTTTC R: TGACCACTTTTATCAGCAACC	173	55	Duran *et al*. 2012
Staphylococcus spp.	*erm*A	F: TCTAAAAAGCATGTAAAAGAA R: CTTCGATAGTTTATTAATATTAG	645	55	Arana *et al*. 2014
Staphylococcus spp.	*mec*A	F: CCTAGTAAAGCTCCGGAA R: CTAGTCCATTCGGTCCA	331	55	Duran *et al*. 2012
Streptococcus spp.	(*erm*B)	F: ATTGGAACAGGTAAAGGGC R: GAACATCTGTGGTATGGCG	442	55	Marimon *et al*. 2005
Streptococcus spp.	*mef*A	F: AGTATCATTAATCACTAGTGC R: TTCTTCTGGTACTAAAAGTGG	346	55	Arana *et al*. 2014

aac(3)-*iv* (gentamicin), *aad*A (streptomycin), *tet*B and *tet*M (tetracycline), *bla*SHV and *blaZ* (β-lactams), *erm*A, *erm*B (erythromycin), *mec*A (methicillin), *mef*A (macrolides).

Table 3. Amplification using PCR and its comparison with the methods of isolation of the bacteria, and antibiotic resistance genes and its comparison with the antibiotic sensitivity test

Organism	No. of isolates	M1	M2	Antibiotic	AST	M1	M2
E. coli	26	23/26	19/26	Gentamicin	7	6/7	2/7
				Penicillin	25	23/25	19/25
				Streptomycin	21	18/21	14/21
				Tetracycline	11	10/11	9/11
Klebsiella spp.	19	16/19	14/19	Gentamicin	5	4/5	3/5
				Penicillin	19	16/19	14/19
				Streptomycin	13	9/13	8/13
				Tetracycline	9	7/9	7/9
Staphylococcus spp.	43	37/43	32/43	Erythromycin	24	21/24	18/24
				Penicillin	41	33/41	29/41
				Methicillin	33	29/33	24/33
				Tetracycline	22	18/22	16/22
Streptococcus spp.	15	12/15	10/15	Erythromycin	10	9/10	7/10
				Penicillin	15	10/15	8/15
				Macrolide	12	10/12	8/12
				Tetracycline	8	7/8	5/8

indicated that the identification of individual microorganisms from milk was best using method M1 as compared to method M2.

The results of the application of PCR depends on the critical step, i.e. extraction of bacterial DNA from the milk as milk harbours various PCR inhibitors that might interfere with the amplificability of DNA (D'Angelo *et al*. 2007). Also, the presence of high levels of fats, proteins, and calcium in milk could adversely affect the DNA amplification efficiency (Rossen *et al*. 1992, Bickley *et al*. 1996). Power food microbial DNA isolation kit (M2) was selected to be used in the present study for reference as well as for the comparison. This was done after taking clue from an earlier study (Quigley *et al*. 2012) where it was revealed that the extraction of pure and highly concentrated DNA from milk using this kit was found to be consistent. In the present study, SDS, triton along with heat was used for isolation of DNA from milk as SDS, triton along with heat helped in the removal of the fatty layer, which was the major impediment in the extraction of good quality DNA. Later the DNA was extracted using standard phenol chloroform method as per Sambrook and Russels (2001). PCR was used to identify the quality of the isolated bacterial DNA taking clue again from the study of Rossen *et al*. (1992) where they found PCR to be sensitive to large volume of complex food samples containing high amount of fat and proteins.

The results of the study indicate that it is possible to extract DNA from both methods but with a bit of difference in the yield which was similar to the findings of Pirondini *et al*. (2010) who compared seven DNA extraction procedures and evaluated the DNA quality on the ability to successfully amplify specific products using PCR and Real-time PCR. In an study, Psifidi *et al*. (2010) optimized six genomic DNA extraction methods from ovine milk samples and concluded that SDS triton method protocol developed by them was robust in extracting DNA which is similar to the findings of the present study where DNA extracted using SDS triton too yielded good quality of amplifiable DNA.

In another study, Usman *et al*. (2014) optimized and evaluated three methods for genomic DNA extraction from bovine milk and found that modified nucleospin blood kit method was significantly better than the Phenol-Chloroform method in terms of quantity as well as the quality of the DNA. In line with the result of Usman *et al*. (2014) we too failed to extract good quality DNA using just phenol chloroform method directly from milk. Thus, to get good quality DNA we modified the protocol by adding SDS, triton and heat in the phenol chloroform method which yielded good quality amplifiable DNA.

Since we wanted to further study the quality of the DNA extracted through the method developed in the study, DNA extracted using both the methods was used to amplify antibiotic resistance genes. The selection of antibiotic resistant genes was done depending on the antibiotic sensitivity (AST) results. The primers for some of the genes which were found resistant in the isolated organisms based on AST results were selected from the available literature and used in the present study. On the basis of amplification of the antibiotic resistant genes it was observed that using method M1, we could get more positive PCR amplification of the antibiotic resistance genes than with method M2 (Table 3, Supplementary Figs 2, 3). Antibiotic resistant genes amplification results were consistent for the entire genus tested in the present study (Table 3). Thus, the results of the present study revealed that the method M1 which is a modification of phenol chloroform method used in the study was cost effective in extracting bacterial DNA directly from milk without compromising on the quality of the extracted DNA. However, more studies with additional samples are needed to establish its robustness.

Mastitis is an important disease in the dairy animals and

for its diagnosis using molecular tests good quality DNA from the organisms implicated in mastitis should be extracted. Though, DNA method directly from the bacterial growth has been standardized but due to presence of many inhibitors in milk, extracting quality that could be used in downstream applications DNA is a challenge. In the present study, DNA was extracted from milk using two methods. In the first method, milk was treated initially with SDS and triton and later subjected to standard phenol chloroform method (M1) and secondly with a kit (Power food microbial DNA isolation kit) (M2). DNA extracted from both the methods was evaluated using genus specific PCR along with various antibiotic resistance genes and it was observed that DNA extracted using M1 method was efficient and useful for downstream applications such as PCR. Thus, it could be concluded from the study that M1 method incorporating SDS-triton developed in the study is a cheaper and efficient method to extract bacterial DNA from milk that could be used for downstream applications.

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