PCR Based Detection of *Pseudomonas aeruginosa* in Mastitic Cow Milk

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A B S T R A C T

Mastitis is the most economically significant disease of dairy animals. This condition is associated with a significant reduction in milk yield, increased costs of production and deteriorated milk quality. In the present study a total number of 40 bovine mastitis milk samples were collected to isolate and identify the *Pseudomonas aeruginosa*. All the samples were inoculated into nutrient broth which was further cultured on agar plates. Colonies showing typical Pseudomonas morphological characteristics were processed for biochemical analysis. Based on the morphological, cultural and biochemical characterization indicated that 6 isolates were identified as *Pseudomonas aeruginosa*. Investigations of the *Pseudomonas aeruginosa* proteolysis activity is the basal objective of this study. To achieve this objective 16S rRNA primer was used in the amplification of DNA extracted from bacterial isolates. In the present study six bacterial isolates were subjected to molecular identification. The 16SrRNA based PCR results, revealed 956bp amplicon sized product.

Introduction

Bovine mastitis is an inflammatory condition of udder (Bradley, 2002) of cattle and buffaloes which results in altered quality and decreased milk production (Sharma *et al.,* 2012). In severe cases, mastitis may results in partial or complete damage to udder tissues leading to decrease productive lifespan of the animal and less occasionally fatalities (Radostits *et al.,* 2007).

Mastitis is caused by many bacteria, which include the coliform group (Specifically *Escherichia coli*, *Enterobacter, Klebsiella species), Streptococci, Staphylococci, Corynebacteria, Pasteurella, Mycoplasma, Leptospira, Yersinia, Mycobacteria,*

Psuedomonas, Serratia and other organisms like fungi, yeasts and virus (Burns *et al.,* 1996).

Pseudomonas aeruginosa is a motile gram-negative rod from the family of Pseudomonadaceae. It is a frequently isolated from clinical specimens and identified as an accidental cause of bovine mastitis (Radostits, 2009). Most of the *P. aeruginosa* strains that have been isolated are strong biofilm producers that can reduce the efficacy of antibiotics and lead to chronic mastitis (Melchior *et al.,* 2006). The pathogen uses a wide range of virulence factors, including proteins capable of inducing toxicity, which effectively damage tissues (Lyczak *et al.,* 2000).

P. aeruginosa infections are generally detected by standard microbiological techniques such as phenotypic and biochemical profiles (Qin *et al.,* 2003), however these commercial tests tend to be lengthy and unreliable (Procop, 2007). Molecular techniques, such as polymerase chain reaction (PCR) are rapid and reliable for the identification of microbial pathogens (Procop, 2007); many PCR based diagnostic methods have been developed for *P. aeruginosa* (Motoshima *et al.,* 2007).

16S rRNA gene based identification method was also developed to identify P. *aeruginosa* (Spilker *et al.,* 2004). The aim of this study was PCR based molecular characterization of *Pseudomonas aeruginosa* from bovine affected with mastitis.

Materials and Methods

Samples

A total of forty milk samples from the cases of clinical mastitis in cattle were reported to Teaching Veterinary Clinical Complex (TVCC) with apparent clinical signs of mastitis and were referred for antimicrobial sensitivity test. All the samples were subjected to routine laboratory procedures for the bacterial growth in nutrient broth medium (Cruickshank *et al.,* 1975).

Isolation of *Pseudomonas aeruginosa*

Inoculation into broth

A total of 40 milk samples were collected from the mastits affected animals. The collected milk samples were brought to laboratory and transferred to the nutrient broth. After inoculation, the test tube was incubated at 37°C for overnight (Cruickshank *et al.,* 1975). Each of the mastitic milk samples were streaked on to nutrient agar, tryptic soy agar (TSA, Himedia - India), violet red bile glucose agar (VRBG, Liofilchem- Italy), MacConkey agar, EMB agar, blood agar and Cetrimide agar. The inoculated plates were incubated for 24-48 hours at 37°C. Suspected colonies were confirmed primarily as Gram-negative rods (Cruickshank *et al.,* 1975).

Identification of *Pseudomonas aeruginosa*

The pure colonies subjected for biochemical test using Indole test, Methyl red test Sugar fermentation reaction, Citrate utilization test and Voges-Proskauer test (Kavitha and Devasena, 2013).

DNA Extraction and PCR

Total DNA was extracted from bacterial isolates by using commercially genomic DNA mini kit (Qiagen- Germany) following the mini spin protocol according to the manufacturer's instructions. To confirm the presence of *P. aeruginosa* DNA, a standard diagnostic PCR was carried using one 16SrRNA; Primer DG10-03F: 5'

GGGGGATCTTCGGACCTCA 3' and the primer DG10-04R: 5' TCCTTAGAGTGCCC ACCCG 3' as a primer sequence (5'-3').

The PCR amplification mixture (20µl) includes (10.0 µl) of master mix which contains bacterially derived Taq DNA polymerase, dNTPs, MgCl2 and reaction buffer at optimal concentration for efficient amplification of DNA templates by PCR (5 µl) of template DNA, (1 µl) of each forward and reverse primers and (3.0 µl) of nuclease free water to complete the amplification mixture to (20 µl). The PCR tubes containing amplification mixture were transferred to preheated thermocycler and started the program as follow; predenaturtion at 95°C for 3 min, then 35 cycles of denaturation at 95°C for 60 sec, annealing at 63°C for 30 Sec, extension at 72°C for 60 sec with one final extension of 10 min at 72°C and a subsequent hold temperature of 4°C using a thermocycler (Techne-UK).

The results of PCR were detected after the amplification process. (8µl) from amplification sample was directly loaded in a 1.2% agarose gel containing (3.0µl/30ml) ethidium bromide and DNA size marker as standard in electrophoresis and run at 50 V. The DNA was observed and photographed by using gel documentation system.

Results and Discussion

Identification of *Pseudomonas aeruginosa*

Forty milk samples were collected from mastitis affected cattle. The samples were inoculated in nutrient broth. After the overnight period of overnight incubation the samples were streaked on to different media namely MacConkey agar, Eosine Methylene blue agar, Cetrimide agar, Nutrient agar and Blood agar.

Eosin Methylene Blue agar (EMB)

Eosin Methylene Blue (EMB) Agar is used for identification of microorganism. *Pseudomonas aeruginosa produced* light purple colonies with spreading edges in Eosin Methylene Blue (EMB) Agar. In the present study 6 isolates which produced typical colonies indicating the *Pseudomonas aeruginosa*.

MacConkey agar

MacConkey agar is used to isolate *Pseudomonas aeruginosa*. In this media colourless colony with black tinge, typical fruity odour and metallic sheen was identified as *Pseudomonas aeruginosa*.

Nutrient agar

The samples were streaked on nutrient agar plates and the plates were incubated at 37 C° for 24 hours as described by (Pawel *et al.,* 2008). *Pseudomonas aeruginosa* isolated from milk samples were produces green tinged, circular, mucoid smooth colonies with emits sweat grape odour with metallic sheen on nutrient agar.

Blood agar

Blood agar is used to isolate Pseudomonas aeruginosa along with other media. In this media pseudomonas species produced gray coloured colony, β haemolytic with metallic sheen was identified as typical Pseudomonas species.

Cetrimide agar

Cetrimide agar is used as a selective media to isolate *Pseudomonas aeruginosa*. In this media pseudomonas species produced green tinged colony with metallic sheen was identified *Pseudomonas aeruginosa*.

Gram staining

Gram staining is used for identification of Gram positive and Gram negative organism. *Pseudomonas* is a gram negative rod Shaped bacterium. In the present study all the 6 isolates were subjected to gram staining and all the isolates were found to be gram negative typically thin rods.

Biochemical characterization

The phenotypic characteristics of *P. aeruginosa* were determined using tests including Simmon's citrate utilization (Oxoid - UK), oxidase (1.0% tetramethyl), Urease test (Himedia - India) Gelatine liquefaction test, Oxidase test, Catalase test, Indole test, Methyl red test, Voges-Proskauer test, Sugar fermentation reaction, Phenyl alanine deaminase test, TSI and Carbohydrate fermentation tests (Smibert and Krieg, 1984; MacFadden, 2000).
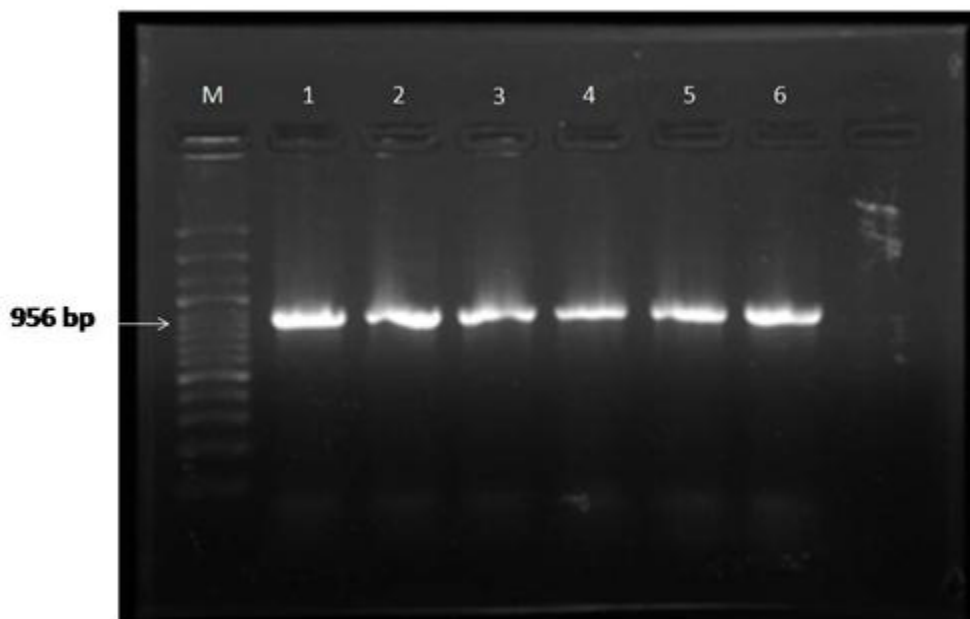
All suspected colonies of *Pseudomonas* *aeruginosa* on the basis of cultural and morphological properties were subjected to biochemical tests. All the 6 isolates were confirmed to be *Pseudomonas aeruginosa* after the biochemical results.

The isolates were sub-cultured on the same medium until pure cultures were achieved. The isolated bacteria were grown in nutrient broth (Oxoid - UK) and were then stored in 15% (v/v) glycerol at−20 °C and used as stock cultures in subsequent analysis (Dikbas, 2010).

PCR Analysis

To determine the presence of proteolytic *P. aeruginosa* in mastitis affected milk, 16S rRNA primer was used. Genomic DNA from a *Pseudomonas* positive isolate was used as the source of template DNA. The positive results for the prescence of 16S rRNA (956 bp) gene amplified products were displayed (Fig. 1).

Fig.1 PCR for *Pseudomonas aeruginosa* M: 1500bp DNA marker; Lane 1-6: Positive sample (956bp)

Pseudomonas aeruginosa was isolated by using nutrient agar which was promoted primarily based on characteristics colony morphology in nutrient agar, blood agar and MacConkey agar media and Gram's staining technique, Many strains of *P*. aeruginosa produce various species of pyocins and this pyocin producing strain of *P. aeruginosa* afford pigment on agar media (Cheesbrough, 1985). *Pseudomonas aeruginosa* produces circular mucoid smooth colonies with emits sweat grape odour on nutrient agar, these characteristics colonies were similar with finding of Haleem *et al.,* 2011.

In cultural isolation, colonies are selected based on phenotypic characteristics like size, colour, shape etc. Similarly, possible reasons for no growth in milk samples can include low concentration of bacteria in the milk samples, pathogens not growing in standard culture media, or presence of substances in the milk decreasing the viability of bacteria in culture (Taponen, 2009). All 6 isolates which had been biochemically identified as *P. aeruginosa* were subjected to genotyping using species specific primers reported earlier (Spilker*et al.,* 2004).

In conclusion, PCR assay can be used for rapid, sensitive, specific and reliable identification of the major mastitis pathogens from milk. As several bacterial species are involved in mastitis infection, inadequate pathogen detection or identification techniques often delay timely interventions in disease treatment and control.

Acknowledgement

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