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# Cultural characterization and molecular identification of *Pseudomonas aeruginosa* from milk samples

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## Abstract
Inflammation of udder by microbial infection is one of the leading economic disease in dairy sector. Most of the pathogens especially *Pseudomonas aeruginosa* are refractory to the antibiotic theraphy. In the present research, a total of 87 milk samples (Mastitis, M=32 and healthy milk, N=55) were collected from surrounding areas of Krishna District and Buffalo Research Station, Venkataramannagudem of West Godavari District, Andhra Pradesh. The samples were analyzed for microbial isolation and molecular characterization of *Pseudomonas aeruginosa*. On cultural isolation and molecular analysis, mastitis infected milk detected *Pseudomonas aeruginosa* in four samples and the pathogen was also detected in three healthy milk samples suggesting that *Pseudomonas aeruginosa* is responsible for sub-clinical mastitis infection in the herd.

**Keywords:** Milk, molecular detection, *Pseudomonas aeruginosa,* sub-clinical mastitis

## Introduction
India is the leading milk producer contributing nearly 23% of the total global production. Dairy production is the backbone for most of the marginal and small farmers in India (Banerjee *et al* 2017) [1]. Many diseases and pathogens ruin the health status of the animal. Among them, bovine mastitis is a serious concern in the dairy industry, as it is a irreparable loss if the treatment is not provided in time. The ability to recognize potentially harmful pathogens plays a vital role in the outcome of successful treatment. Among the pathogens, *Pseudomonas aeruginosa* is very important as it is a multi drug resistance pathogen. (Park *et al* 2014, Singh *et al* 2005) [7, 10]. Sub clinical mastitis infection is generally seen in dry or non lactating period. During the sub clinical infection the healthy milk samples also contribute nearly 20 to 50% of the microbial infection (Forsback *et al*., 2009) [2] and in which *Pseudomonas aeruginosa* is one among them. Therefore, the present study was focused on the isolation and molecular characterization of *P. aeruginosa* from mastitis affected and subclinical mastitis cases of bovines from surrounding areas of Krishna District and from Buffalo Research station, of Andhra Pradesh for having an idea about the incidence of *P. aeruginosa* in bovines.

## Material & Methods
### Collection of samples
Eighty seven milk samples, mastitis milk from suspected mastitis cases and healthy milk from buffaloes (Mastitis, M=32 and healthy milk, N=55) were collected from Veterinary Clinical Complex of NTR College of Veterinary Science, Gannavaram, Veterinary Dispensary (VD) / Veterinary Hospital (VH) / Veterinary Polyclinics (VPC) in Krishna district and Buffalo Research Station (BRS), Sri Venkateswara Veterinary University (SVVU), Venkataramannagudem, West Godavari district of Andhra Pradesh during the period April 2021 to 1st week of May 2021 and from July 2021 to December 2021. The milk samples were collected under sterile conditions as described. Collected milk samples were shipped on ice and stored at 4 °C for a minimum of 24 h until cultured/enriched.

**Cultural and biochemical studies**

Milk samples of about 10 µl were inoculated in BHI broth and incubated at 37 °C for 24 h. After observing the turbidity, the BHI broth culture was inoculated on Pseudomonas isolation agar. The inoculated plates were further placed at 37 °C for 24 h to 48 h. The plates that produced translucent colonies with green pigment were examined for their morphology and staining characters by employing Gram's method. Positive cultures were further processed for biochemical identification.

**Catalase test**

This test was performed with the help of slide on to which a small amount of colony growth was added to a drop of 3% hydrogen peroxide. Immediate production of gas bubbles was considered as a positive test, where as in negative test no gas bubbles was observed.

**Oxidase Test**

Standard oxidase discs were used to perform the test. The single colony was placed on the disc. Immediate development of blue colour was considered as positive test whereas in negative test there is no colour change.

**Methyl Red (MR) Test**

The test was performed using methyl red broth in which pure culture of *Pseudomonas aeruginosa* was inoculated. The tubes were incubated at 37 ºC for 48 h. After incubation, 5 drops of methyl red indicator solution were added to the test tube. Positive results show bright red colour however yellow colour indicates negative results.

**Voges-Proskauer (VP) Test**

The test was performed using Voges Proskauer (VP) broth in which pure culture was inoculated. The tubes were incubated for 48h at 37 ºC. After incubation, 3 mL of Barrit A reagent (alpha naphthol) and 1 ml of Barrit B (40% KOH) reagent were added. The tube was exposed to atmospheric oxygen for 10-15 minutes. Positive results show pink colour in 2-5 minutes and negative result shows yellow or copper colour at the surface.

**Indole Test**

The test was performed by using 5 ml indole broth in which pure culture was inoculated and incubated at 37ºC for 48h. After incubation, 0.5 mL of Kovac's reagent was added slowly by slightly tilting the test tube. Formation of pink ring was considered as positive reaction and a yellow colour ring at the surface indicates the test as negative.

**Citrate Test**

Simmon's Citrate agar slants were inoculated from 18-24 h old culture colony and incubated at 37ºC and observed upto 7days. A positive reaction was observed by colour change from green to blue in the slant and negative showing no colour change of the slant.

**Molecular characterization of *P. aeruginosa* by polymerase chain reaction (PCR)**

**Template preparation by boiling and snap chilling method**

About two ml of overnight grown *P. aeruginosa* culture was taken in a micro centrifuge tube and centrifuged at 10,000 rpm for five minutes. The pellet was suspended in 400 µl of nuclease free water and heated for 10 min in a boiling water bath. The microcentrifuge tube was transferred immediately on to ice. After 20 min, the tube was centrifuged at 8000 rpm for five minutes at 4 ºC and the supernatant was used as a template for PCR reactions.

**Measurement of DNA concentration and purity**

The concentration of DNA was measured with Nanodrop 200C and adjusted to 50 ng for further molecular studies. Pure DNA samples with an optical density ratio of 1.8 to 2 at 260/280 nm were used.

**Molecular confirmation of *P. aeruginosa***

In PCR test, the *P. aeruginosa* isolates were confirmed using species specific oligonucleotide primers under thermal cycler conditions as described in Table 1 & 2. All the reactions were put in a volume of 10 µl in 0.2 ml PCR tubes (Table 3).

The PCR amplicons were analysed by electrophoresis on a 1.7% agarose gel stained with 0.5 µg of ethidium bromide / mL in Tris-Borate EDTA (TBE) buffer. Electrophoresis was carried out at 90V for 60 min in submarine gel electrophoresis unit and the PCR products were visualized in Gel documentation system. The sizes of PCR products were verified by comparison with quantitative DNA ladder. Negative control i.e., distilled water was used in PCR tests.

**Table 1:** Nucleotide sequences and amplicon sizes of *P. aeruginosa* (Spilker *et al.,* 2004) [11]

| | Target gene | Nucleotide sequence (5'-3') | Amplion size(bp) |
|---|---|---|---|
| *P. aeruginosa* | *16S rRNA* | Forward- GGGGGATCTTCGGACCTCA<br>Reverse- TCCTTAGAGTGCCCACCCG | 956 |

**Table 2:** Standardized thermal cycling conditions for *P. aeruginosa* (Spilker *et al.,* 2004) [11]

| | Standardized conditions | | |
|---|---|---|---|
| **Steps** | **Temperature** | **Duration** | **No. of cycles** |
| Initial denaturation | 95 °C | 5 min | 1 |
| Denaturation | 95 °C | 45 sec | 30 |
| Annealing | 55 °C | 45 sec | |
| Extension | 72 °C | 60 sec | |
| Final extension | 72 °C | 5 min | 1 |

**Table 3:** Composition of PCR mix for *P. aeruginosa* species-specific PCR

| S. No | Reagents | Quantity (µL) |
|---|---|---|
| 1 | 2x Master Mix (Appendix) | 5 |
| 2 | Forward primer (20 pmol/ µL) | 0.5 |
| 3 | Reverse primer (20 pmol/ µL) | 0.5 |
| 4 | DNA template | 1 |
| 5 | Distilled water | 3 |
| | Total | 10 |

**Results and Discussion**

Mastitis has been a problem in the dairy industry, though steady efforts were followed to control the infection. Recurrent infections of the udder is a persistent problem that leads to high prevalence of mastitis and sub clinical mastitis (Park *et al* 2014, Hillerton and Kliem, 2002) [7, 3].

The total incidence of *P. aeruginosa* was found to be 8.04%. In mastitis infected milk it was 12.5% (4/32) and 5.45% (3/55) from healthy milk samples. The incidence of *P. aeruginosa* in the mastitis milk was 12.5% and similar prevalence of 9.6% was reported in Brazil by Vasquez *et al.* (2017) [12] from bovine mastitis infections and 5.6% prevalence in Korea from bovine mastitis between 2003 and

2008 by Nam *et al*. (2009) [6]. Similarly, prevalence of *Pseudomonas* spp. in sub clinical mastitis was reported by other workers like Heleili *et al*. and Vishwakarma at the incidence of 3.0% and 6.9%, respectively from bovines and buffaloes. Banerjee *et al*. (2017) [1] also detected 5.4% cases of bovine subclinical mastitis associated with *P. aeruginosa* in South Bengal, India.

**Cultural isolation and biochemical characterization**

On proceeding for bacterial isolation on specific agars, out of 87 milk samples processed for isolation of the Gram negative bacillus, only seven samples (4 from mastitis milk and 3 from healthy milk) were characteristic for *Pseudomonas.* All the 87 samples were inoculated in BHI medium preliminarily. Seven samples exhibited greenish pigmentation characteristic for *P. aeruginosa* (Fig. 1). These seven samples were further streaked on to Pseudomonas isolation agar (PIA), they produced characteristic green pigmented and smooth mucoid colonies (Fig. 1).

Banerjee *et al.* (2017) [1] worked on sub clinical mastitis, out of 422 samples tested for bacterial isolation, only 23 (6.5%) samples were found to be positive for Pseudomonas sp. as they showed characteristic bluish-green pigmentation on cetrimide agar and found to be Gram-negative bacilli on Grams staining.

To further confirm and visualize the pyoverdin pigment, the colonies on PIA were focused on fluorescent microscope and characteristic blue-green fluorescence was exhibited under UV light. (Fig.2). Similar blue green flourscence was also reported by Samanta (2013) [9]. Banerjee *et al.* (2017) [1] collected a total of 23 mastitis samples out of which 19 (5.4%) isolates were confirmed to be *P. aeruginosa* showing characteristic pyoverdin associated blue-green fluorescence causing subclinical mastitis cases of bovines in different districts of South Bengal.

**Fig 1:** Green pigmented smooth mucoid colonies of *Pseudomonas* on Pseudomonas Isolation Agar

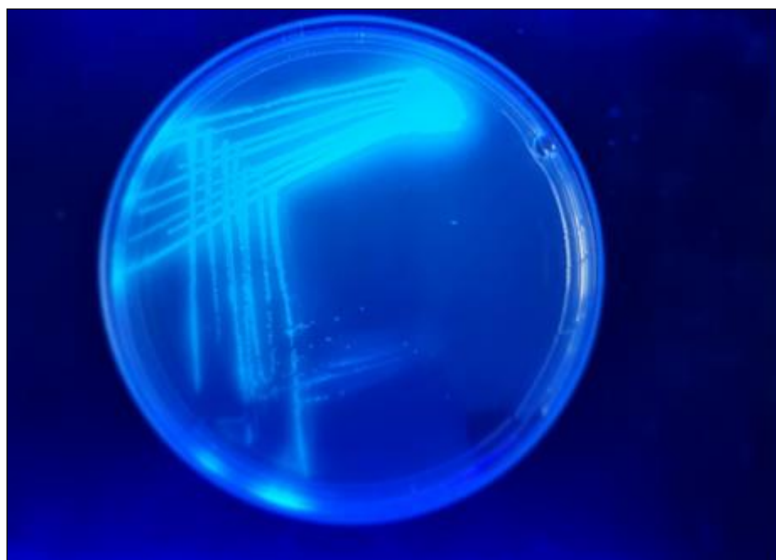

**Fig 2:** Blue green fluorescence of *Pseudomonas aeruginosa* under UV light

**Biochemical Characterization**

Biochemical profile revealed al the seven isolates of *P. aeruginosa* to be negative for indole and MR-VP and positive for catalase, citrate and oxidase (Table 4)

**Table 4:** Biochemical sensitivity profile of *P. aeruginosa* isolates

| S No. | Name of the biochemical test | No. of isolates tested | No. of isolates (+) | No. of isolates (-) |
|---|---|---|---|---|
| 1 | Indole | 7 | 0 | 7 |
| 2 | MR-VP | 7 | 0 | 7 |
| 3 | Citrate | 7 | 7 | 0 |
| 4 | Catalase | 7 | 7 | 0 |
| 5 | Oxidase | 7 | 7 | 0 |

Banerjee *et al.* (2017) [1] studied on biochemical characterization of *Pseudomonas* isolates and revealed that 21 samples i.e., positive for oxidase, catalase, citrate utilization, nitrate reduction, and glucose fermentation whereas were negative for methyl red, Voges-Proskauer, and indole tests whereas two *Pseudomonas* samples showed variable result in glucose fermentation test as revealed in this study.

**PCR test for detecting *P. aeruginosa***

In the recent years, 16s ribosomal RNA (rRNA) gene has become a convenient way for developing newer molecular biological techniques and aiding in identifying and detecting the species of bacteria. Kohne *et al*. (1986) [4] were the first to describe the use of the rRNA gene as a target for DNA probes to detect and classify the microorganisms.

All the 7 isolates that were culturally confirmed as *P. aeruginosa* were subjected to species specific (16S *rRNA*) and yielded a PCR product of 956 bp. The results were presented in (Fig. 3).

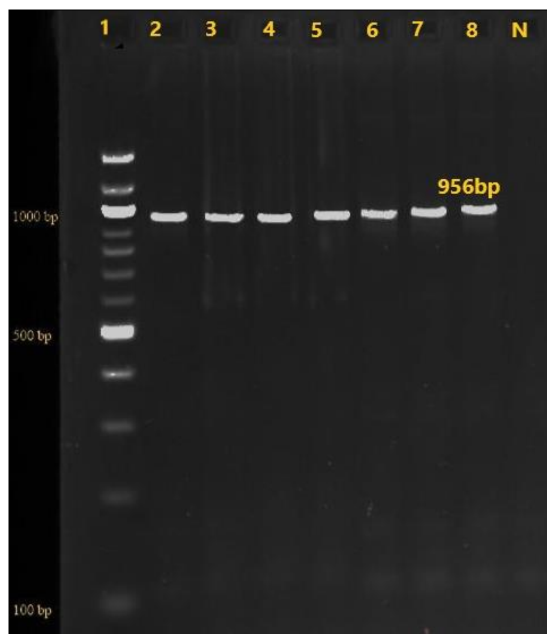


**Fig 3:** PCR test with species specific primers (16S rRNA) for detecting the *P. aeruginosa* isolates from mastitis and sub clinical mastitis cases

## Conclusion

It can be concluded that *P. aeruginosa* acts as one of the causative bacterial pathogens for Bovine mastitis. Timely detection and confirmation of the pathogen is of plays a vital role in decreasing the antimicrobial resistance for the microbe. Hence, suitable control strategies both microbiological and molecular tests for clinical mastitis and sub clinical mastitis cases must be performed at an early stage in prevention of the disease.

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