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# Identification of Extended Spectrum Beta Lactamases Producing *Pseudomonas aeruginosa* from Subclinical Mastitis Milk Samples in an Organized Dairy Farm of SVVU, Andhra Pradesh

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## ABSTRACT

The present study was conducted during August 2021 at Buffalo Research Station, Venkatarammanagudem of West Godavari District, Andhra Pradesh, India and was aimed to detect the presence of extended-spectrum beta-lactamases (ESBL) producing *P. aeruginosa* from sub-clinical mastitis that are apparently healthy Murrah buffaloes. Inflammation of the udder by microbial infection is one of the leading economic disease in the dairy sector. Most of the pathogens especially *Pseudomonas aeruginosa* are refractory to the antibiotic theraphy. Nowadays, the antimicrobial resistance owed by the bacteria is at an alarming rate. *Pseudomonas aeruginosa*, a multi drug resistant pathogen carry the extended spectrum beta lactamase genes in its plasmids and thus become resistant to antibiotic therapy. A total of 276 milk samples were collected from 69 milch animals in the month of August, 2021 comprising from all the four quarters of each buffalo. Nine (n=9) (3.26%) *P. aeruginosa* isolates were identified from the sub-clinical mastitis milk samples from the total 276 samples collected in the study. The isolates on morphological analysis were typical to *P. aeruginosa*. Seven isolates were positive for phenotypical beta-lactamases production. A total of 1 and 2 isolates were found reactive of the $bla_{SHV}$ and $bla_{OXA}$ genes, and two isolates harboured both $bla_{SHV}$ and $bla_{OXA}$ genes, respectively on PCR assay. No $bla_{TEM}$ gene was found in the isolates. Antibiogram of the ESBL producing *P. aeruginosa* isolates (n=7) possessed 100% resistance against most of the commonly used antimicrobials like ampicillin, amoxycillin, clindamycin, co-trimoxazole oxytetracycline, streptomycin and ceftriaxone (85%). The *P. aeruginosa* isolates were sensitive to amikacin (100%), ciprofloxacin (87.4%), gentamicin (84%), norfloxacin (84.8%) and enrofloxacin (82%).

**KEYWORDS:** ESBL, Milk, *Pseudomonas aeruginosa*, sub-clinical mastitis

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***Data Availability Statement:*** Legal restrictions are imposed on the public sharing of raw data. However, authors have full right to transfer or share the data in raw form upon request subject to either meeting the conditions of the original consents and the original research study. Further, access of data needs to meet whether the user complies with the ethical and legal obligations as data controllers to allow for secondary use of the data outside of the original study.

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## 1. INTRODUCTION

India, a nation purely based on agriculture and livestock for its economy, and together they constitute nearly 25.6% of the total agriculture gross domestic product. Milk and milk products solely contribute for the GDP (Mouli et al., 2023) and India stands as a leading milk producer contributing nearly 23% of the total global production. The Dairy production is the backbone for most of the marginal and small farmers in India (Banerjee et al., 2017). Animals mainly act as the potential reservoirs and carriers for the resistance genes (Darwich et al., 2019). Many pathogens causes various diseases and ruin the health status of the animal. Among them, bovine mastitis is a serious concern in the dairy industry, as it is an irreparable loss if the treatment is not provided in time (Ali et al., 2021). The ability to recognize potentially harmful pathogens plays a vital role in the outcome of successful treatment. Mastitis includes clinical and sub-clinical stages, and *Pseudomonas aeruginosa* is one of the responsible pathogen for the infection (Schauer et al., 2021) and so far 131 bacterial microbes have been isolated from milk samples (Shoaib et al., 2021). Among the pathogens, *Pseudomonas aeruginosa* is very important as it is a multidrug resistant bacteria. (Park et al., 2014, Singh et al., 2005). In mastitis, the point source of infection was mainly from the contaminated soil and water (Petridou, 2021). In sub-clinical mastitis, clinically no changes could be seen externally except the increase in somatic cell count in the milk (Abbey et al., 2016). During the sub-clinical infection, the healthy milk samples also contribute nearly 20 to 50% of the microbial infection (Forsback et al., 2009) and in which *Pseudomonas aeruginosa* is one among them. The virulence and transmission of *P. aeruginosa* rely on various virulence factors such as flagella, pili, LPS, proteases, pyocyanin and those responsible for biofilm formation. *P. aeruginosa* can secrete extracellular polymeric substances such as exopolysaccharides, matrix proteins, extracellular DNA and extended beta spectrum lactamases. This enables the bacteria to cause persistent infections. Biofilm is refractory to several antibiotics due to change in phenotypic structure leading to multiple antibiotic resistance. Thus, it poses a significant threat to conventional antibiotic therapy.

The decrease in the milk production caused by sub-clinical mastitis effects the farmers financially with a lower income than the cases of clinical mastitis cases (Romer et al., 2018, Cheng et al., 2020). In the recent years, there is an alarming increase in the sub-clinical mastitis cases as the herd size of the dairy increases (Ali et al., 2014). The reason may be due to the indiscriminate usage of the antibiotics irrespective of the need in animals and finally its effect was seen in the development of antibiotic resistance in humans too (ESBL's producing *P. aeruginosa* were considered as highly resilient bacteria as most of the antibiotics are not susceptible for the treatment (Falodun et al., 2020). Gram negative bacterium can easily acquire the antibiotic resistance through the horizontal transmission of plasmids, (ESBL's gene act as transposons i.e., transmissible genetic elements and leads to the development of antimicrobial resistance. ESBL's producing bacteria pose a serious public concern as they may cause risk to the humans in the food chain. The World Health Organization tagged *Pseudomonas aeruginosa* bacterium as the most critical antimicrobial resistant bacteria and there is an urge to develop newer antimicrobial agents against ESBL-producing *P. aeruginosa* or alternative methods like bacteriophages and use of phytochemicals have to be studied against the use of antibiotics.

Studies on ESBL's producing *P. aeruginosa* was extensively carried out in humans but less focussed in livestock. In concern, with the concept of one health with the increased antimicrobial resistance there is a much need to study of the occurrence of resistant antibiotic genes and there control measures. Therefore, the present study was aimed at the isolation and molecular characterization of ESBL's producing *P. aeruginosa* from subclinical mastitis cases of bovines.

## 2. MATERIALS AND METHODS

### *2.1. Collection of milk samples*

A total of 276 milk samples were collected from 69 milch animals comprising from all the four quarters of each lactating Murrah buffalo present in the Buffalo Research Station (BRS) maintained by Sri Venkateswara Veterinary University, Venkataramannagudem, West Godavari district of Andhra Pradesh during the period August 2021. The milk samples were collected under sterile conditions as described by Phuketes et al. (2001). Collected samples were transported on ice and immediately cultured or stored at 4°C for a minimum of 24 h until cultured/enriched.

### *2.2. Cultural and biochemical studies*

Milk samples were inoculated in Brain Heart Infusion broth and incubated at 37°C for 24 h. On observing the turbidity, the BHI broth culture was inoculated on PIA (Pseudomonas isolation agar). The plates were incubated at 37°C for 24 h to 48 h. The translucent colonies with green pigment presumed to be *Pseudomonas* spp. and were further employed to Gram's staining for morphological confirmation. Further the isolates were tested for IMViC test as per Quinn et al. (2011) for the biochemical characterization.

### *2.3. Molecular confirmation of P. aeruginosa isolates by PCR*

#### *2.3.1. Bacterial DNA extraction*

For genotypic detection of *P. aeruginosa*, DNA was extracted by simple boiling and snap chilling method from all the isolates .

*2.3.2. Detection of 16SrRNA gene specific for P. aeruginosa by PCR*

All the presumptively positive *P. aeruginosa* isolates were subjected to PCR for the detection of the 16S rRNA gene (956 bp) specific for *P. aeruginosa* as described by (Spilker et al., 2004). Standardized *Pseudomonas* spp. was used as a positive control in this study. The cyclical conditions were as follows with an initial denaturation at 95˚C for 5 min, denaturation at 95˚C for 45 sec, annealing 55˚C for 45 sec, extension at 72˚C for 60 sec and final extension at 72˚C for 5 min. The cycle was carried for 30 cycles. After completion of the cycles, the PCR product obtained was subjected to 1.7% agarose gel electrophoresis (Table 1).

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

| | Target gene | Nucleotide sequence (5'-3') | Amplicon size (bp) |
|---|---|---|---|
| *P. aeruginosa* | *16S rRNA* | Forward- GGGGGATCTTCG-GACCTCA Reverse- TCCT-TAGAGT-GCCCACCCG | 956 |

*2.4. Detection of ESBL production in Pseudomonas aeruginosa isolates*

*2.4.1. Phenotypical detection of ESBL production*

Phenotypical detection of ESBL production for the *P. aeruginosa* isolates were tested by phenotypic screening test (PST) for preliminary screening and double disk synergy test (DDST) for confirmation.

*2.4.2. Phenotypic screening test (PST)*

*Pseudomonas aeruginosa* was tested for susceptibility against four antimicrobial drugs, namely cefotaxime, ceftazidime, ceftriaxone, and aztreonam. The standardized inoculum was dispersed over MH agar with a sterile cotton swab. The antibiotic discs were placed on the agar surface and incubated at 37˚C for 18 hours. The zone of inhibition was measured and resistance to at least one of the four antibiotics used was regarded as a positive screening test for the development of ESBLs (CLSI, 2020).

*2.4.3. Phenotypic confirmatory test (PCT) for ESBL production by using double disk synergy test (DDST)*

*Pseudomonas aeruginosa* isolates positive in PST were further subjected to phenotypic confirmatory tests (PCT) as recommended by CLSI (2020) guidelines.

The standardized bacterial test suspension was inoculated on MH agar plates by uniformly swabbing the entire surface of the agar plates and were incubated at 37˚C for 18–24 h. Ceftazidime, cefotaxime, ceftriaxone and aztreonam discs were placed around augmentin (amoxycillin/clavulanic acid) 50µg disk at the distance of 15 mm from it (centre to centre). A clear visible zone of inhibition of any one of the antibiotic discs towards the zone of amoxyclav disc was regarded as phenotypic confirmation for the production of ESBLs. This enhanced zone of inhibition towards the clavulanate disc is called key hole phenomenon/ghost phenomenon/ champagne cork phenomenon, which indicates positive synergy test (Laudy et al., 2017).

*2.4.4. Detection of ESBL genes in P. aeruginosa isolates by m-PCR*

All phenotypically ESBL-producing *P. aeruginosa* isolates were screened for Multiplex PCR detection of the $bla_{\text{TEM}}$ (800 bp), $bla_{\text{SHV}}$ (713 bp), and $bla_{\text{OXA}}$ (564 bp) (table 2) as per the protocol of Spilker et al., 2004.

Table 2: Oligonucleotide primers used for the detection of ESBL genes in *P. aeruginosa* (Spilker et al., 2004)

| Primer | Target | Nucleotide sequence | Amplicon size (bp) |
|---|---|---|---|
| MultiTSO-T | $bla_{TEM}$ | CAT TTC CGT GTC GCC CTT ATT C | 800 |
| | | CGT TCA TCC ATA GTT GCC TGA C | |
| MultiTSO-S | $bla_{SHV}$ | AGC CGC TTG AGC AAA TTA AAC | 713 |
| | | ATC CCG CAG ATA AAA TCA CCA C | |
| MultiTSO-O | $bla_{OXA}$ | GGC ACC AGA TTC AAC TTT CAA G | 564 |
| | | GAC CCC AAG TTT CCT GTA AGT G | |

M-PCR assay was conducted with an optimized PCR mix of 10 µl under standardized thermal cycling conditions. The cyclical conditions were as follows with an initial denaturation at 94˚C for 10 min, denaturation at 94˚C for 40 sec, annealing 60˚C for 40 sec, extension at 72˚C for 60 sec and final extension at 72˚C for 7 min. The cycle was carried for 30 cycles. After completion of the cycles, the PCR product obtained was subjected to 1.7% agarose gel electrophoresis.

The PCR amplicons were analyzed 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.

## 3. 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). The total incidence of *P. aeruginosa* was found to be 3.26% in the present study. Association of *P. aeruginosa* in bovine subclinical mastitis cases were also reported earlier by Viswakarma (2008) and Singh et al. (2005) at 6.9% and 9.4%, respectively. Patel et al. (2012) and Heleili et al. (2012) reported a prevalence of *Pseudomonas* spp. in subclinical mastitis cases of cattle and buffaloes at 3.6% and 3.0%, respectively which are in correlation with the current study.

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) also detected 5.4% cases of bovine subclinical mastitis associated with *P. aeruginosa* in South Bengal, India.

### *3.1. Cultural isolation and biochemical characterization*

On proceeding for bacterial isolation on specific agars, out of 276 milk samples processed for isolation of the Gram negative bacillus, only nine samples were characteristic for *Pseudomonas*. All the 276 samples were inoculated in BHI medium preliminarily. Nine samples exhibited greenish pigmentation in the broth characteristic for *P. aeruginosa*. These nine samples were further streaked on to Pseudomonas isolation agar (PIA), they produced characteristic green pigmented and smooth mucoid colonies.

Banerjee et al. (2017) worked on sub-clinical mastitis, out of 422 samples on 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 were found to be Gram-negative bacilli on Gram's 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. (Figure 2). Similar blue green flourscence was also reported by Quinn et al. (2011) and Samanta (2013). Banerjee et al. (2017) 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 mainly South Bengal.

#### *3.1.1. Biochemical characterization*

Biochemical profile revealed all the nine isolates of *P. aeruginosa* to be negative for indole and MR-VP and positive for citrate, catalase and oxidase. Banerjee et al. (2017) 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.

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

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

The 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 (Figure 1).

### *3.3. Detection of ESBL production in P. aeruginosa isolates detected from sub-clinical mastitis cases*

A total of 7 (2.53%) *E. coli* isolates were found to be positive for phenotypic detection of ESBL production in a combined disc diffusion assay. Falodun et al. (2020) carried out a study on Pseudomonas species isolated from cattle and reported that 71.5% of the isolates were *P. aeruginosa* and 37.5% of them were phenotypically positive for ESBL production by PST method. In another study performed by Laudy et al. (2017) out of 110 *P. aeruginosa* isolates 77 (70%) were positive for ESBL production by CDT method and 92 (83.6%) positive by DDST method. The results of our study differ slightly with those of Laudy et al. Isolates those suspected phenotypically for ESBL production were further subjected to molecular characterization. All the 7 positive isolates were tested for the detection of ESBL genes using m-PCR. Five isolates were found reactive of the ESBL genes $bla_{\text{SHV}}$ and $bla_{\text{OXA}}$ but not $bla_{TEM}$. Two isolates were positive for $bla_{\text{OXA}}$ and one isolate for $bla_{\text{SHV}}$ genes, and two isolates harboured both $bla_{\text{SHV}}$ and $bla_{\text{OXA}}$ genes, respectively on PCR assay. No $\text{bla}_{\text{TEM}}$ gene was found in the positive isolates. The total percentage of $bla_{\text{SHV}}$ was 42.38% and $bla_{\text{OXA}}$ was 57.14% in the present study. Shacharaghi et al. (2010) reported the frequency of $bla_{VEB}$, $bla_{SHV}$, $bla_{PER}$, $bla_{GES}$, and $bla_{TEM}$ among the ESBL positive *P. aeruginosa*

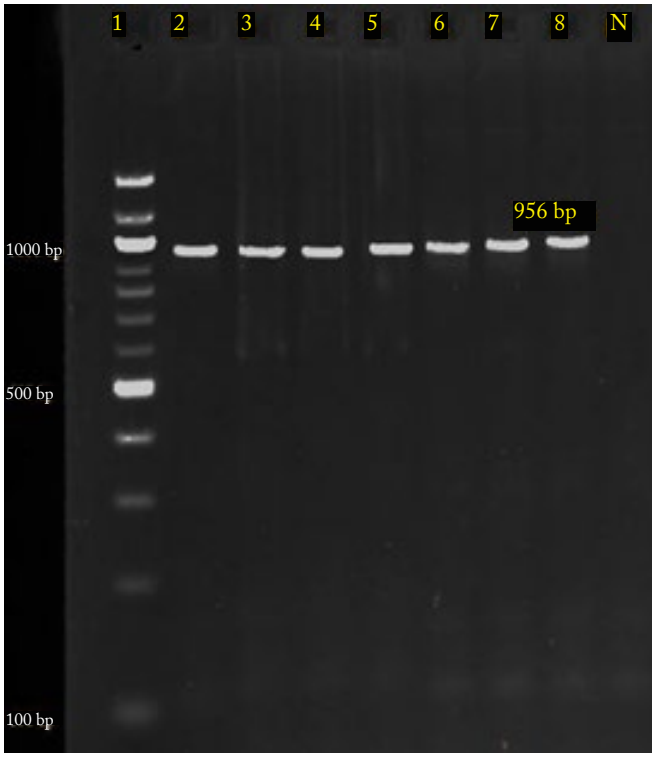


Figure 1: PCR test with species specific primers (16S rRNA) for detecting the *P. aeruginosa* isolates from sub-clinical mastitis cases

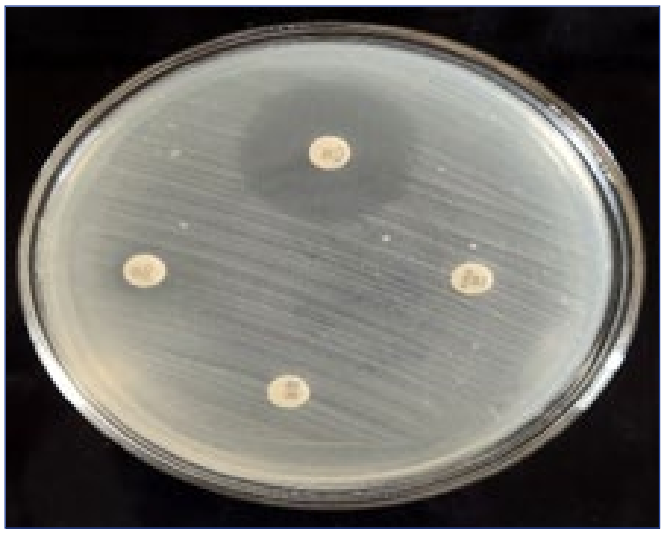

Figure 2: Phenotypic screening test

isolates to be 24%, 22%, 17%, 0%, and 9%, respectively. Hosu et al. (2021) analysed 82 *P. aeruginosa* isolates which were phenotypically positive for ESBL production. The most significant finding of this investigation was that, the most prevalent genotype for ESBL production was $bla_{TEM}$ (79.9%), followed by $bla_{SHV}$ (69.5%) and $bla_{CTXM}$ (31.5%). It has been reported that ESBL genes show variation depending on the geographical location. The findings of Ehlers et al. (2009), Chen et al. (2015) and Miranda et al.

Figure 3: Double disk synergy test

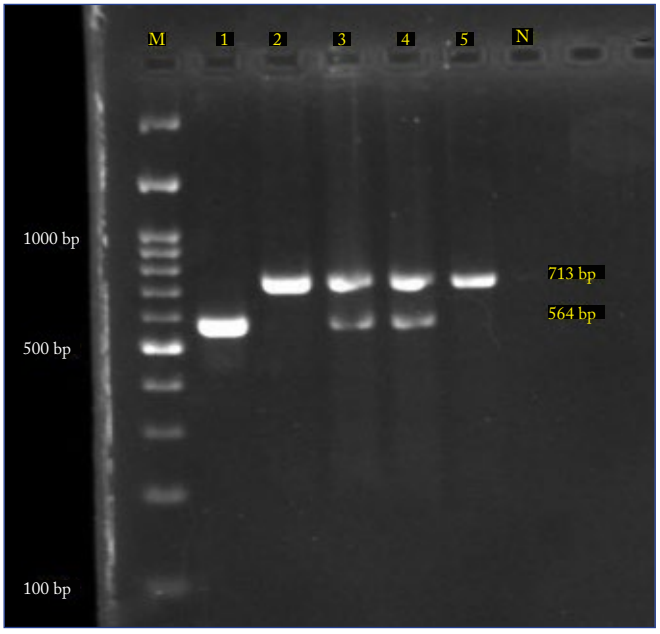


Figure 4: Multiplex PCR test with $bla_{OXA}$ (564 bp), $bla_{SHV}$ (713 bp) and $bla_{TEM}$ (800 bp) primers for detecting ESBL genes in *P. aeruginosa* isolates of sub-clinical mastitis

(2015) from South Africa, China and Brazil, respectively found the prevalent genotype as $bla_{TEM}$ while, Jamali et al. (2017) reported the prevalent gene to be $bla_{SHV}$.

Variation was exhibited in the presence of ESBL-producing genes in the isolates and in their phenotypical expression. The discrepancies may be due to varied geographic location, different levels of healthcare facilities involved, varied levels

of exposure to healthcare settings, choice of antibiotic use and antibiotic stewardship practices, environmental factors and host immune responses play a vital role (Becerio et al., 2013)

*3.4. Antimicrobial sensitivity test of ESBL positive P. aeruginosa isolates*

The antibiotic sensitive patterns of *P. aeruginosa* vary depending on the dosage and prolific usage of antimicrobials, the inherent resistance capacity and biofilm forming ability of the bacteria. All the ESBL-producing *P. aeruginosa* isolates were subjected for antibiogram. Antimicrobials like ampicillin, amoxycillin, clindamycin, co-trimoxazole oxytetracycline, streptomycin attained 100% resistance and ceftriaxone (85%) which may be due to the presence of ESBL's. most of the isolates showed multidrug resistance which were similar to the reports of Turkyilmaz et al., 2023. The *P. aeruginosa* isolates were sensitive to amikacin (100%), ciprofloxacin (87.4%), gentamicin (84%), norfloxacin (84.8%) and enrofloxacin (82%), respectively.

## 4. CONCLUSION

3.26% of the milk samples were positive for sub-clinical mastitis caused by *P. aeruginosa*. High number of antimicrobial resistances was conferred to most of the commonly used antimicrobials. *P. aeruginosa* acted as a causative bacterial pathogen for Bovine sub-clinical mastitis.

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