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ISOLATION AND MOLECULAR CHARACTERIZATION OF *PSEUDOMONAS AERUGINOSA* FROM BOVINE SUB-CLINICAL MASTITIS IN WEST BENGAL

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ABSTRACT

Bovine sub-clinical mastitis is one of the significant problems in India which is affecting the milk producing capability of the affected cattlecausing huge loss of the rural and national economy. A total of 422 bovine milk samples with history of infection and drop in milk yield, were checked from different dairy farms of West Bengal and were tested by on-spot bromothymol blue (BTB) test, to finally collect 371 samples followed by secondary screening using somatic cell count (SCC) study. The finally tested milk samples showed significantly high SCC value of 3.26-4.88 lakh cells/ml of milk which was clearly indicative of infection. Approx. 23 samples (6.53%) were found to be positive for *Pseudomonas* sp. showing distinct bluish-green pigmentation on cetrimide agar and typical results during morphological and biochemical characterization. Among those 19 (5.39%)isolates were confirmed to be *Pseudomonas aeruginosa* by fluorescent technique for detection of characteristic blue-green fluorescence due to the presence of pigment pyoverdin. Molecular characterization of these isolates reportedthe presence of *tox*A gene in 11 isolates and *exo*S gene in 6 isolates whereas 2 isolates were having both the genes.

Keywords -*Pseudomonas aeruginosa,toxA, exoS*, sub-clinical mastitis, bovines

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Introduction

Pseudomonasaeruginosa is an opportunistic pathogenic bacterium causing sub-clinical mastitis in cattle though having slight occurrence, but is having great effect on dairy industry (Cheng *et al.*, 2010). These are short rods shaped bacteria, aerobic in natureand can produce nos. of pigments (pyocyanin, pyoverdin, pyurubin etc.), toxins and enzymes which help in their pathogenicity in different hosts (Pavlovskis and Wretlind,1982). Major toxins of this pathogen includes – exotoxin A (*tox*A) which inhibits protein synthesis by blocking activity of elongation factor 2, phospholipase C which is haemolytic in nature, exoenzyme S (*exo*S) andexoenzyme U (*exo*U) which are surface proteins causing pathogenicity in hosts, endotoxinor original endotoxic protein (OEP) having endotoxic effects, pyocinswhich can inhibits the growth of other bacteria and pigments like pyocyanin can generate superoxide damaging host cells (Quinn *et al.*, 2011). Among all these fatal factors, exotoxin A (*tox*A) and exoenzyme S (*exo*S) are two major toxins this pathogen is having which are associated with sub-clinical mastitis infection (Lanotte *et al.*, 2004; Narayanan, 2013). The infection by *P. aeruginosa* is a fatal septicemicwhich are very difficult to control due to the resistance power of this pathogen against varieties of common antibiotic drugs forming bio-films at the site of infection (Fatima*et al.*, 2012). This pathogen has become one of the major issues causing sub-clinical mastitis in bovine due to their pattern of pathogenicity and drug resistance capacity. So, aiming at the pathogenic effect of *Pseudomonas aeruginosa* in sub-clinical mastitis cases, this present study was targeted to detectand characterize *P. aeruginosa* isolates along with detection of *tox*A and *exo*S genes in those isolates screened from bovine sub-clinical mastitis cases.

Materials and Methods

Sample collection:Milk samples were screened by on-spot BTB test, from different dairy farms of West Bengal with history of previous or current infection and drop in milk yieldand positive samples were collectedto perform secondary screening by SCC study. The finally collected milk samples (n= 371) with

significantly high SCC values of more than 3-8 lakh cells/ml of milk were considered for further study.

Detection and confirmation of *P. aeruginosa*:All collected samples were tested following standard methods as per Carter and Wise (2004) and Narayanan (2013) to detect *Pseudomonas* sp. yielding cleargreenish / bluish-green pigmentation on cetrimide agar, typical results in morphological and biochemical characterization with the following tests –oxidase, catalase, citrate utilization, nitrate reduction, glucose fermentation, methyl red, Voges-Proskauer and indole test. Detection of *Pseudomonas aeruginosa*wasdone following fluorescent technique to detect of characteristic blue-green fluorescence by the pigment pyoverdin (Quinn*et al.*, 2011).

Molecular characterization of *P. aeruginosa*: All positive isolates were considered for molecular characterization following standard protocol as per Lanotte*et al.* (2004). Fresh culture of each strain was harvested in 15ml TE buffer (40mM Tris/HCl, 2mM EDTA, pH 8.0) and lysed in 220µl of a 25% (w/v) aqueous solution of SDS and 30µl Pronase. Then the mixture was incubated overnight at 37°C to allow cellular lysis. Bacterial DNA was extracted as per the method of Brenner*et al.* (1982) and was resuspended in 1ml 1X TE buffer.

Specific primers were used for amplification and detection of *tox*Aand *exo*S genes(**Table 1**)in thermal cycler under specific PCR conditions as per method described by Lanotte*et al.* (2004):the PCR mixture was having PCR buffer (10mM Tris/HCl, 50mM KCl, 1.5mM $MgCl_2$, pH 8.3), 200µM of each dNTP, 12.5 pmol of each primer, DMSO at the final concentration of 4%, 1U Ampli*Taq*DNA polymerase and 25ng DNA template. The previously extracted bacterial DNA samples were amplified in Thermal cycler (Eppendrof, Germany) using the following condition: 94°C for 3min, 30cycles of 94°C for 30s, 55°C for 1min and 72°C for 1min 30s and 72°C for 5min. Each gene was amplified separately. A known strain of *P. aeruginosa* (ATCC no. 27853) and one *E. coli* strain (departmental isolate) are used as positive and negative controls in this study. PCR products were separated in a 1% agarose gel for 1h at 100V, stained with ethidium bromide and detected by UV transillumination.

Table 1: Primers used in molecular characterization of *P. aeruginosa* isolates

Primers	Sequences	Gene product bp	Reference
ETA1 ETA2	5' GGT AAC CAG CTC AGC CAC AT 3' 5' TGA TGT CCA GGT CAT GCT TC 3'	*tox*A, 352	Lanotte *et al.* (2004)
exoS F exoS R	5' CTT GAA GGG ACT CGA CAA GG 3' 5' TTC AGG TCC GCG TAG TGA AT 3'	*exo*S, 504	

Results and Discussion

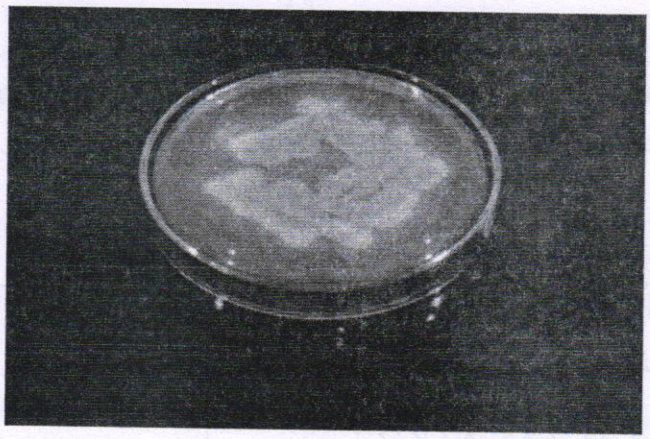
Upon screening of 422 bovine milk samples obtained from different dairy farms of West Bengal with history of previous infection and drop in milk yield,a total of 371 samples were considered for collection and further testing as found positive in BTB test and showed significantly high SCC values *i.e.* 3.26-4.88 lakh cells/ml of milk in secondary screening by SCC study. When screened for bacterial infection or for

the presence of *Pseudomonas* sp., approx. 23 (6.53%) samples were detected to be positive showing characteristicgreenish or bluish-green colour colonies on cetrimide agar (Carter and Wise, 2004). This much presence of *Pseudomonas* sp. @3.6% causing sub-clinical mastitis in bovines in J&K were also reported bySingh *et al.* (2005) which arealmost in accordance with this finding. All 23 isolates weredetected to be *Pseudomonas* sp. as those were typical Gram negative bacillimorphologicallyand showed positive results to oxidase, catalase, citrate utilization, nitrate reduction and glucose fermentation whereas negative to MR, VP and indole tests (Carter and Wise, 2004; Quinn *et al.*, 2011 and Samanta, 2013) which were typical with this pathogen.

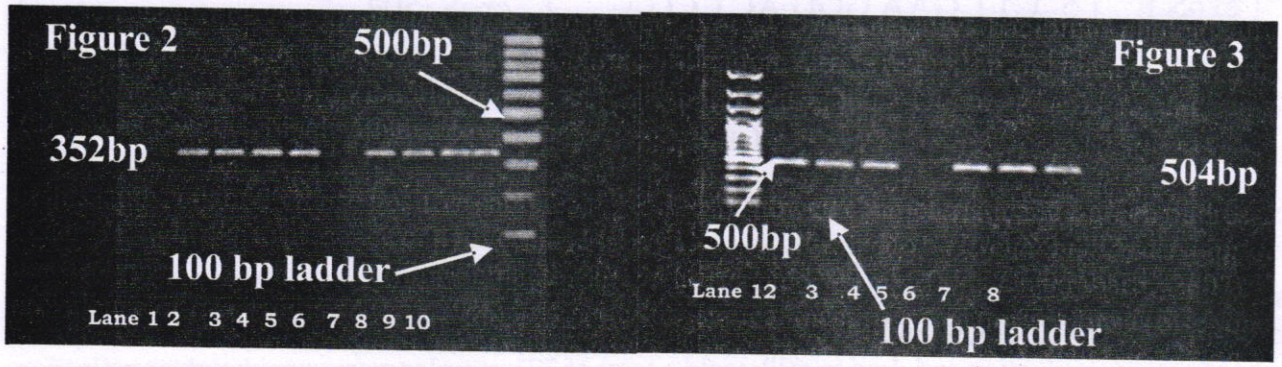
In this study, out of 23 those positive samples, 19 (5.39%) showed characteristic blue-green fluorescence under UV light due to presence of pyoverdin **(Figure 1)**thus confirmed to be *Pseudomonas aeruginosa*, as this is typical for this bacterium(Samanta, 2013; Carter and Wise, 2004; Chakrabarti, 2007). This prevalence of *P. aeruginosa* (@5.39%) in bovine sub-clinical mastitis cases (@3.6%) was also reported by Singh *et al.* (2005)from Jammu and Kashmir. Again, Heleili*et al.* (2012), Patel *et al.*(2012)and Vishwakarma (2008) also reported prevalence of *P. aeruginosa*in sub-clinical mastitis cases of bovines and buffaloes with a range of 3.2% - 9.4% which are almost matching with this report.

Molecular characterization of these isolates confirmed the presence of *tox*A genes **(Figure 2)** in 12(63.16%) isolates and *exo*S genes**(Figure 3)**in 7(36.84%) isolates (**Table 2**)in which 2 isolates were found to possess both the genes. Lanotte *et al.* (2004) also reported *tox*A (approx. 100%) to be more frequently available gene than *exo*S (84.5%) in *P. aeruginosa* strains isolated from different sources which are in agreement with the present study. Again, detection of these genes from *P. aeruginosa* isolates were also reported by Dacheux*et al.* (2000), Feltman*et al.* (2001),Badr*et al.* (2008) and Nikbin*et al.* (2012) in their studies.

Figure 1: Showing characteristic blue-green fluorescence by *Pseudomonas aeruginosa* isolates due to pigment pyoverdin under UV light



Figures 2 & 3: PCR detection of *tox*A (352bp) and *exo*S (504 bp) genes in pathogenic *P. Aeruginosa*isolates



Lane 2-4, 6-9: test samples, 1: +ve control 5: -ve control

Lane 3-4, 6-8: test samples, 2: +ve control, 5: -ve control

Table 2: Showing distribution pattern of toxA and exoS genes in *P. aeruginosa* isolates

Toxin genes Place of sampling	*tox*A	*exo*S	*tox*A*and* *exo*S*both*	Total (%)
Haringhata Dairy Farm	1	0	0	1 (5.26)
Kalyani SLF	1	1	0	2 (10.53)
Borsul Block	2	2	0	4 (21.06)
Memari Area	3	3	1	7 (36.84)
Mogra Area	4	0	1	5 (26.31)
Total (%)	11 (57.89)	6 (31.58)	2 (10.53)	19 (100)

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