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VIRULENCE MARKERS AND MOTILITY PATTERNS EXHIBITED BY *PSEUDOMONAS SPP.* ISOLATED FROM HIV AND NON-HIV POPULATION WITH LRTI

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***ABSTRACT:** Detection of biofilm, slime and motility patterns in Pseudomonas spp. can be valuable in empathetic the virulence of the creature. Here studies on the phenotypic methods of biofilm production, slime production and the motility patterns exhibited in 69 clinical isolates of Pseudomonas spp. and 2 environmental isolates were performed. Nineteen strains produced biofilm in which 6 were from HIV and 13 were from Non-HIV patients. All the 19 isolates positive for biofilm production were taken for detection of biofilm genes by PCR. The gene coding for alkaline protease aprA gene was detected in 13 (68.42%) isolates. The gene for phenazine biosynthesis phzA1 gene was detected in 14 isolates. The house keeping gene acpP was found to be positive for 18 (94.7%) isolates. 48 isolates were found positive for slime production. Pellicle formation was positive in 39 isolates. Swarming motility was positive in 42 isolates 29 were from HIV and 11 were from Non-HIV. Swimming motility was exhibited in 31 isolates. Twitching motility was exhibited in 35 isolates. The 2 environmental isolates were found to be positive only for slime production, swarming, and swimming motility. Among the clinical isolates, HIV isolates showed high production of slime, pellicle and positivity for the motility patterns seemed to be high in HIV compared to Non-HIV isolates except for the biofilm production which is high in Non-HIV isolates.*

***Keywords:** Pseudomonas, biofilm and slime production, motility pattern, virulence*

INTRODUCTION

Lower respiratory tract infections are public health problem due to their persistent and pervasive nature and place a considerable strain on the health budget. In HIV patients, lung infections are one of the foremost causes of illness and humanity. The most frequent respiratory complication in patients with HIV infection is caused by bacterial infections [1]. Virulence and inflammatory response of the lungs plays the vital role on the lower respiratory tract infections outcome. P. aeruginosa is an imperative pathogen in chronic breathing tract infections that induces chronic lung colonisation, ultimately causing respiratory disease and death. P. aeruginosa inflammation of the respiratory tract is a significant concern in sick patients and in individuals with cystic fibrosis (Mahenthiralingam et al 1996).

The change from an innocuous to a hyper virulent microbe under states of dysfunctional host protection frameworks is owing to a broad collection of specifically communicated destructiveness determinants including protease enzymes, mucoid exopolysaccharide, pili, exotoxin A, lipopolysaccharide, lipase, pigments, haemolysin, histamine, exoenzyme S, leukocidin in addition rhamnolipids (Tang et al 1996). These assist the microbes by damaging the host's defence response and framing an anti-toxin blockage to follow and attack their host. Without someone else, no single destructive force is serious, but the entire component cluster contributes to the pathogenicity of P. aeruginosa. Targeting bacterial virulence is one approach that has yet to be exploited. Our study aims to examine the prevalence of virulence factors like biofilm, slime, pellicle formation and their motility patterns (Biofilm genes) of isolated from HIV in addition non-HIV patients with infections of the lower respiratory tract, P. aeruginosa and to relate between the biofilm production and antimicrobial resistance among the isolates [2].

MATERIALS AND METHODS

Learning Population

Learning population comprised patients with symptoms of LRTI from HIV and Non-HIV population. Informed consent was taken from each patient after briefing them about the study. A total of 200 adult HIV patients of both sexes within the age group of 15-60 yrs admitted in Government Hospital of Thoracic medicine, Chennai, India were included in this study. Srifuengfung *et al.,* (2005) [3] reported 32.97% prevalence of *Pseudomonas* spp. among individuals with lower respiratory tract infection. Assuming a precision of 20% and α=5%, the required minimum sample size is 195.A total of 100 adult non-HIV patients of both sex within the age group of 15-60 yrs of age attending Dr. Kamashi Memorial Hospital, Pallikaranai, were also included in this study. The sample size for Non-HIV was calculated from the prevalence rate calculated for HIV. 50% of the sample size (100) was taken for the study.

Biofilm Production – Microtitre Plate Method [4]

P. aeruginosa overnight culture was diluted 1:100 in a new Luria Bertani stock (LB) medium, administered (125 µl) to 96-well microtiter plate polyvinyl chloride (PVC) wells in addition grown for 15 h at 37 °C. Wells at room temperature were discoloured with 100 µl of 0.25 percent gem violet (CV) for 30 min. The stain was disposed of besides the plate was washed in standing water three to several times and allowed to dry. The stained biofilm was solubilized for 10 min with 200 µl of 95 percent ethanol in addition the optical concentration was determined by the micro ELISA auto reader at 570 nm (OD570).

Slime Production

Detection of slime production was done by standard tube method as described by Turkyilmaz and Esküüzmürlüler (2006). A loop of organism from pure culture that was inoculated on 5 ml of trypticase soy stock (TSB) was performed in the subjective examination for biofilm arrangement (Hi Media Laboratories ltd.). At 37°C, the tubes were incubated. The material was drained after 24 h. The tubes were then stained with 7 minutes of 1 percent safranin. The presence of an adherent layer of stained substance on the inner surface of the tube showed a favourable result. The appearance of stained material alone at the fluid air interface was not seen as a slime output trait.

Pellicle Formation Assay [5]
6ml LB-broth cultures were grown in 18-150 mm glass tubes at room infection (20 to 27 °C). Visual examination of the air-fluid boundary of the vertical society checked the pellicles. Total covering by an opaque coating of cells and structure content of the surface of the culture was regarded as a pellicle arrangement.

Motility Assay
Swarming Motility [6]: 0.5 percent (wt/vol) of bacto agar, 8 g/liter of nutrient broth besides 5 g/liter of glucose are in the swarming medium. Plates were allowed at room temperature to set for overnight. In LB stock, crops were aerobically grown overnight and calibrated to an visual density of 1.1 at 660 nm (OD660). Plates were seeded at the middle focal point of the agar surface with 1µl of cell suspension in addition incubated for 18 h at 37°C. The swarming area was assessed in addition a photograph was taken. In triplicates, the research was completed.

Swimming Motility **[6]:** 10 g/liter tryptone, 5 g/liter NaCl, in addition 0.3 percent (wt/vol) Bacto agar are found in the swimming medium. 3mm thickness On the day of use, swimming plates were made and dried for 3 h at room temperature. In LB broth, aerobically grown overnight cultures were grown aerobically overnight to an OD_{660} of 1.1. The plates were immunised in the centre of the agar using a disinfected needle and gestated for 24 h at 30 C, wrapped in plastic wrap. They weighed and photographed the swimming field. The test was carried out in triplicates.

Twitching Motility Assay [7]
Medium used a medium of 2 percent LB, 1 percent agar. Pseudomonas colonies grown overnight in LB medium were stabbed into the medium besides incubated for 48 h at 37 °C, extracting the agar after incubation. By staining with 0.25 percent (wt/vol) Coomassie brilliant blue for 30 min, the bacteria friendly to the surface of the Petri dish were visualized (Head & Yu, 2004). The diameter of the twitching region was measured.

Detection of Biofilm Genes by PCR [8]
Detection of biofilm genes (*aprA* and *phzA1)* were performed as per Lenz *et al.,* (2008) study. Genomic DNA was extracted as designated by the method of Porteous *et al.,* (2002) [9].The PCR was setup for 20µl reaction. The Quorum sensing regulating genes such as *phzA1, acpP* and *aprA* were detected by PCR.

Primer	Sequence	Temp (°C)	Product size (bp)
acpP-For *acpP*-Rev	ACTCGGCGTGAAGGAAGAAG CGACGGTGTCAAGGGAGT	60	80
aprA-For *aprA*-Rev	GCTTCAGCCAGAACCAGAAGAT TCGACACATTGCCCTTCAAC	60	78
phzA1-For *phzA1*-Rev	TAAAACGTAATCGCGAGTTCATG TTTTATTTGCGGAACGGCTATT	60	74

PCR cycling temperature

Denaturation at	-	95° C for 5 mins	
Denaturation at	-	95° C for 30 secs	
Annealing at	-	55° C for 30 sec	25 Cycles
Extension at	-	72° C for 30 sec	
Final Extension at	-	72° C for 10 mins	
Holding at	-	4° C for 5 mins	

After amplification, 2% agarose gel were used to visualize the amplicons. UV illumination was used for scanning gels. The bands were visualized and digitized using Gel Documentation system (Bio-Rad Laboratories).

RESULTS

Phenotypic Detection Methods

***Biofilm Production*:** A total of 71 isolates were used in the study. 45 HIV besides 24 non-HIV and 2 environmental isolates and 1 ATCC standard strain were used in the study. Out of 71 *Pseudomonas spp.* studied, 19 (26.76%) biofilm poducers. 6/45 (13.33%) HIV detaches and 13/24 (54.17%) non-HIV isolates produced biofilm. None of the environmental isolates were biofilm producers.

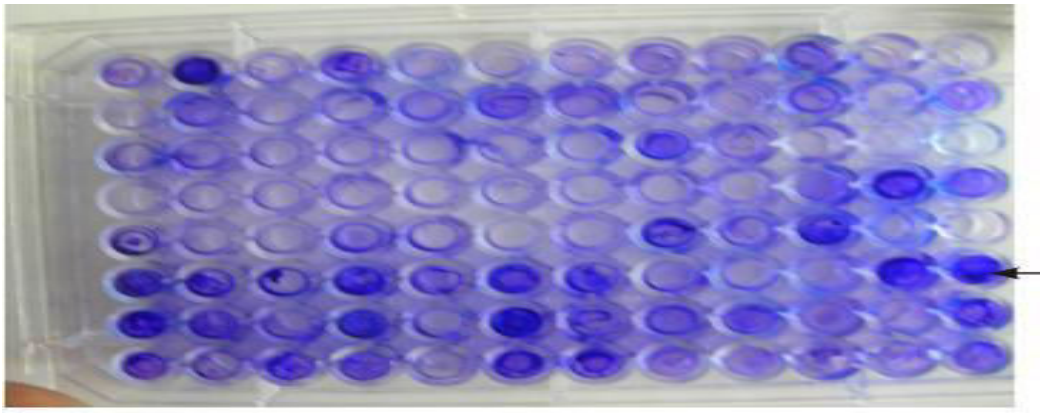

Table 18. Distribution of strong and moderate biofilm producers among HIV and Non-HIV

Source (Number of biofilm positive isolates)	No. of strong producers (%)	No. of moderate producers (%)
HIV (N=6)	5 (83.3)	1 (16.7)
Non-HIV (13)	5 (38.5)	8 (61.5)

Slime Production

Tube Method: A total of 71 isolates were used in the study. 45 HIV and 24 non-HIV and 2 environmental isolates and 1 ATCC standard strain were used in the study. Out of 71 *Pseudomonas spp.* studied, 48 (67.60%) were slime producers of which 29/45 (64.44%) HIV isolates and 17/24 (70.83%) non-HIV isolates produced slime. Both the environmental isolates were positive for slime production.

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Pellicle Formation Assay

A total of 71 isolates were used in the study. Forty-five HIV and 24 non-HIV and 2 environmental isolates and 1 ATCC standard strain were used in the study. Out of 71 *Pseudomonas spp.* studied, 39 (54.93%) formed pellicle. 21/ 45 (46.67%) HIV isolates and 18/24 (75%) non-HIV isolates formed pellicle. Both the environmental isolates were negative for pellicle formation.

Motility Assay:

Swimming Motility: A total of 71 isolates were used in the study. Forty-five HIV and 24 non-HIV and 2 environmental isolates and 1 ATCC standard strain were used in the study. Out of 71 *Pseudomonas spp.* studied, 31 (43.66%) showed swimming motility. 23/45 (51.11%) HIV isolates and 6/24 (25%) non-HIV isolates showed swimming motility. Both the environmental isolates were found to be positive for swimming motility.

***Swarming Motility*:** A total of 71 isolates were used in the study. Forty-five HIV and 24 non-HIV and 2 environmental isolates and 1 ATCC standard strain were used in the study. Out of 71 *Pseudomonas spp.* studied, 42(59.15%) showed swarming motility. 29/45 (64.44%) HIV isolates and 11/24 (45.83%) non-HIV isolates showed swarming motility. Both the environmental isolates were found to be positive for swarming motility.

***Twitching Motility*:** A total of 71 isolates were used in the study. Forty-five HIV and 24 non-HIV and 2 environmental isolates and 1 ATCC standard strain were used in the study. Out of 71 *Pseudomonas spp.* studied, 35(49.29%) showed Twitching motility. 21/45 (46.67%) HIV isolates and 14/24 (58.33%) non-HIV isolates showed Twitching motility. Both the environmental isolates were also found to be negative for twitching motility.

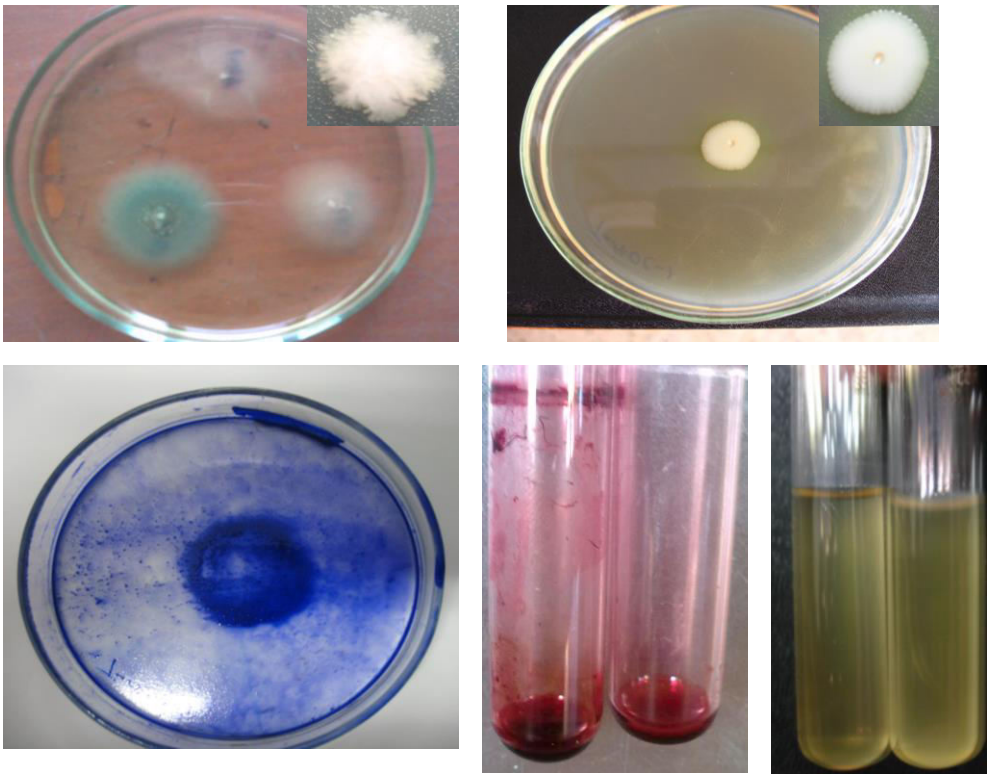

Table.23 Percentage of positivity for Motility patterns exhibited by Pseudomonas spp.

Source	Swimming (%)	Swarming (%)	Twitching (%)
HIV (n=45)	23 (51.1)	29 (64.4)	21 (46.7)
Non-HIV (n=24)	6 (25)	11 (45.8)	14 (58.3)
Environmental isolates (n=2)	2 (100)	2 (100)	- (0)

Table.26 showing the production of different virulence factors among HIV and Non-HIV population.

Virulence factors	HIV (N=45)		Non-HIV (N=24)		Total (N=69)	
	N	%	N	%	N	%
Biofilm production*	6	13.3	13	54.2	19	27.5
Slime production	29	64.4	17	70.8	46	66.7
Pellicle formation*	21	46.7	18	75	39	56.5
Swarming motility	29	64.4	11	45.8	40	57.9
Swimming motility	23	51.1	6	25	29	42
Twitching motility*	20	44.4	17	70.8	37	53.6

Genotypic Detection: Detection of Biofilm genes by PCR

The existence of the gene biofilm was screened in 19 Biofilm producing isolates. The studies for gene coding were done for alkaline protease *aprA* gene which was detected in 13 (68.42%) isolates. 5/6 (83.3%) HIV isolates and 8/13(61.5%) Non-HIV isolates were found to show the presence of the gene (Plate 43). The gene for phenazine biosynthesis *phzA1* gene was detected in 14 isolates. 4/6 (66.7%) HIV isolates and 10/13 (76.9) of the Non-HIV isolates showed the presence of the gene (Plate 44). The house keeping gene *acpP* was found to be positive for 18 (94.7%) isolates which included 5 (83.3%) HIV and 13 (100%) of the Non-HIV isolates (Fig 1, 2,3) (Table-38).

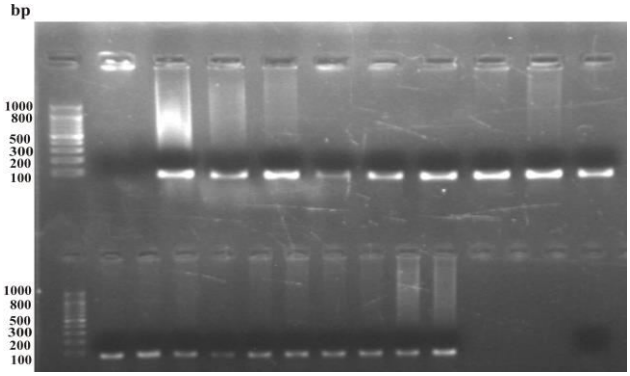

Fig 1. Gel showing amplification of the House keeping gene - *acpP* gene by PCR

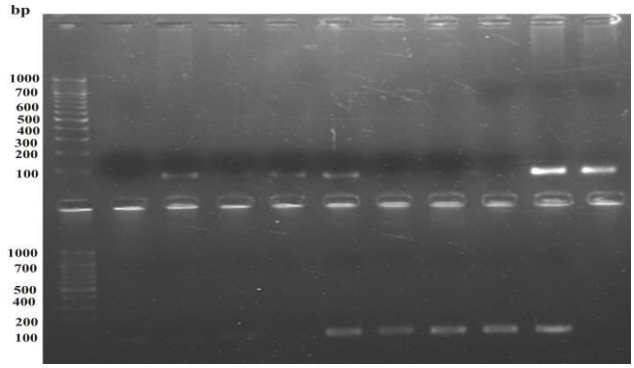

Fig 2. Gel picture showing the amplification of the *aprA* gene by PCR

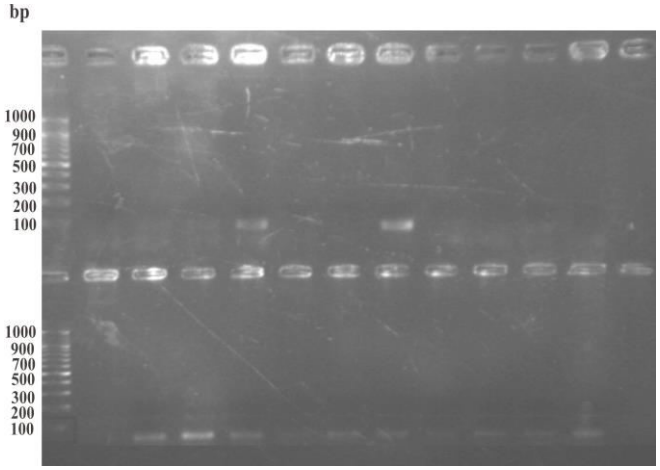

Fig 3. Gel picture showing the amplification of the *PhzA1* gene by PCR

Positivity for biofilm genes among *Pseudomonas* spp. (N=19)

Biofilm genes	HIV (N=6)		Non-HIV (N=13)		Total (N=19)	
	N	%	N	%	N	%
*aprA**	5	83.3	8	61.5	13	68.42
*phzA1**	3	50	10	76.9	13	68.42
*acpP**	5	83.3	13	100	18	94.7

Statistical Analysis

Statistical analyses were performed using 2-tailed Fisher's exact test were p-values > 0.05 considered significant. When association of HIV and Non-HIV isolates with biofilm production was compared statistically significant difference p-value < 0.001 was obtained. Only twitching motility was substantially correlated with biofilm when the combination of total resistance to biofilm, virulence and motility was contrasted. Twitching motility was statistically significant with a p value = 0.045.HIV status and twitching are significantly associated with biofilm. A multiple logistic regression was done with biofilm as dependent variable and HIV status and

twitching as independent variables. Odds ratio for twitching was 8.1 (95% CI: 1.8,35.7) and for HIV status 9.7 (95% C.I.: 2.6 , 35.8). Also the pellicle the formation with a p value = 0.002 was statistically important.

DISCUSSION

The main reason for establishment of infection is biofilms with increased chemo resistance than bacteria in suspensions [10]. Even without movement against sessile microscopic species, antimicrobial managers potent contrary to planktonic microbes can be many periods less active [10,11]. Our study screens for the ability of the organisms to produce biofilm. The assay selected, a notable technique for discoloration biofilms formed by many gram-positive microbial strains, gram-negative strains in addition fungi, founded on crystal violet staining, is (Stepanovic *et al*., 2000; Djordjevic, 2002; Pratt & Kolter, 1998; Li *et al*., 2003; Matz *et al*., 2005; O'Toole & Kolter, 1998) [12-17].

Our research examined the effect of motility, twitching, swimming and swarming on the development of biofilms on 69 Pseudomonas spp. isolates. In order to survey the biofilm-forming ability of these strains, a typical technique based on bacteriological growth inside 96-well microtiter plates [17] was used. A high amount and accurate analysis of the development of biofilm on an abiotic shallow is considered in this specific technique.

It appeared that the actions of flagella besides type IV pili were involved in the production of biofilms [18]. Since all strains scrutinized in this study had flagella, our findings on the relation between motility in addition biofilm arrangement recommend that the flagellar movement of the isolates, as seen by the spinning area observed, is not the transcendent force, including the in vitro reformist advancement of biofilms. Besides, in all the examined isolates, shuddering, as interceded by type IV pili, was not seen. Nevertheless, without this motion, a large amount of isolates developed more biofilm.

Slime polysaccharide has been isolated from patients experiencing CF, respiratory tract sickness and urinary tract disease [19]. It is important to determine the occurrence of slime development amongst P. aeruginosa straining confined to experimental models as it helps to select pathogenicity in addition to evaluate its diagnostic importance as a virulent pathogenic indicator. Such kind of examination is as of now detailed in gram negative microorganisms by staining techniques [20]. To recognize the slime-producing strains of P. aeruginosa from clinical samples, straightforward and strong methods are needed, as anti-microbial resistance is typically found in these strains. The development of slime may be one reason why P.aeruginosa will thrive in clinical settings for longer periods, going around as a potent wellspring of nosocomial diseases. In our investigation, we used tube method for detection of slime production. We observed 66.7% showing slime production which was high compared to the report of Vishnu Prasad, *et al.,* 2009 who reported only 56.20% [21].

A pellicle is a biofilm that collects at a standing fluid culture's air-fluid interface. Investigations of the pellicle development of numerous strains of P. aeruginosa indicated a wide scope of auto aggregative aggregates, proposing that there is hereditary changeability in the loci controlling these aggregates [22-24]. The capacity of *P. aeruginosa* to form pellicles causes us to discover whether the pellicles are one of the variables associated with biofilm forming capacity of the living beings. The gene engaged with pellicle development is *pel* gene. The identifiable evidence of the pel genes and the disclosure of a matrix content rich in glucose in P. aeruginosa should be seen to satisfy potential examinations aimed at determining the role of the structure of a biofilm. In addition, the pel genes are probable targets for the regulation and conceivable annihilation of

P. aeruginosa biofilms and associated microorganisms. In our study, we found that among 69 isolates, 39 (56.5%) were found to produce pellicle.

CONCLUSION

Targeting the virulence factors may prove to be fruitful avenue to pursue. It is possible that interfering with the virulence will put selective pressure on survival and resistance of the pathogens and their antibiotics resistance can be relieved. Potentially antibacterial agents can be targeted against bacterial virulence factors to combat with the pathogens causing infection.

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