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Full Length Research Paper

Prevalence of drug resistant and exotoxin A producing *Pseudomonas aeruginosa* in cutaneous infections in a tribal area in South India

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Pseudomonas aeruginosa* is one of the most important clinically significant and opportunistic pathogens, often causing nosocomial infections. *Pseudomonas* has been implicated to cause folliculitis and other papular or vesicular lesions in the skin of otherwise healthy individuals besides pyoderma gangrenosum in neutropenic patients. In this study, *Pseudomonas* species were isolated from patients with skin infections from a tribal area in South India. The isolates were confirmed as *P. aeruginosa* by biochemical tests and by a duplex polymerase chain reaction (PCR) targeting two loci from 16S rRNA. The isolates were examined for antibiotic susceptibility, production of exotoxin A, and fingerprinting pattern by enterobacterial repetitive intergenic consensus (ERIC) PCR. The isolates were found non-clonal in origin and all the isolates produced exotoxin A, a virulence trait of *P. aeruginosa*. All the isolates exhibited resistance towards several antibiotics including broad spectrum antibiotics, β-lactams, cephalosporin, macrolides, rifampicin and sulfonamides. However, the isolates were susceptible towards some commonly used antibiotics belonging to quinolones and aminoglycosides. A continuous monitoring of the *Pseudomonas* isolates for the drug resistance is important as it can help our clinicians in management of skin infections caused by *Pseudomonas.

Key words: Antibiotic resistance, skin lesions, exotoxin A, *Pseudomonas aeruginosa.*

INTRODUCTION

Pseudomonas aeruginosa is a Gram-negative versatile bacterium ubiquitous in nature. *P. aeruginosa* has become increasingly recognized as an emerging opportunistic pathogen of clinical relevance. This does not usually infect healthy tissues, yet there is hardly any tissue that it cannot infect if the tissue defenses are compromised in some manner. In most cases of infection, the integrity of a physical barrier to infection (for example, skin and mucous membrane) is lost or an underlying immune deficiency is present (von Graevenitz, 1976). It causes urinary tract infections, respiratory system infections, dermatitis, soft tissue infections, bacteremia, bone and joint infections, gastrointestinal infections and a variety of systemic infections. Adding to its pathogenicity, this bacterium has minimal nutritional requirements and can tolerate a wide variety of physical conditions. It may form a biofilm of cells in an extracellular polymeric matrix, on the mucous membranes of the lungs in cystic fibrosis patients and on many other surfaces (Guadarrama et al., 2005). In addition to causing serious and often life-threatening diseases, these organisms exhibit innate resistance towards many antibiotics and can develop new resistance after exposure to antimicrobial agents. Some *Pseudomonas* species are inherently resistant to many antibiotics and easily acquire resistance. To chalk out suitable antibiotic therapy, bacterial isolation and antibiotic sensitivity studies are always essential.

In this study, *Pseudomonas* species were isolated from the skin lesions of patients from a tribal area in South India. The isolates were subjected to biochemical and molecular characterization, DNA fingerprinting using

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enterobacterial repetitive intergenic consensus (ERIC)-PCR, antibiogram and production ability of extracellular exotoxin A.

MATERIALS AND METHODS

Sources of samples

A total of 7 *Pseudomonas* species were isolated from tissue fluid samples of patients having skin lesions. The tissue fluid samples were collected aseptically from the skin lesions during March to June 2010 from a tribal area of Paderu, South India (latitude 18°16′ N, longitude 82°40′ E). The samples were plated directly onto blood agar plates and incubated overnight at 37°C. The bacterial colonies were purified by streak plate method and used for further studies.

Phenotypic and biochemical characterization

All the isolates were examined for motility and Gram staining under a light microscope (Carl-Zeiss, Model no. HBO100). Standard biochemical tests like catalse and oxidase test, indole test, starch hydrolysis, nitrate reduction and gelatin liquefaction test were performed as described elsewhere (Harrigan and McCance, 1976).

Automated identification by BD BBL Crystal

The BBL Crystal identification system for enteric/non-fermenter (Becton Dickinson and Co, Maryland, USA) was used for identification of *P. aeruginosa* isolates as per the manufacturer instructions.

Briefly, a single bacterial colony from overnight growth on blood agar plate was suspended in the BBL crystal enteric inoculum fluid. After vortexing for 10-15 s, the contents were poured into target area of the kit. Inoculum was allowed to fill in all the wells and the lid was closed. Inoculated kit panels were incubated with face down at 37°C for 18-20 h. The BBL Crystal Autoreader was used for reading of panels.

Preparation of genomic DNA

Genomic DNA was extracted by using the Fermentas genomic DNA extraction kit as per the manufacturer instructions (MBI Fermentas, Vilnius, Lithuania). The amount and purity of the DNA was measured by spectrophotometer (NanoDrop ND-1000, Australia).

PCR assay

Pseudomonas isolates were identified at the genera and species level by PCR as described elsewhere (Spilker et al., 2004). The primers were designed on the basis of genus- and species-specific 16S rDNA signature sequences. Primer pair PA-GS-F (GACGGGTGAGTAATGCCTA) and PA-GS-R (CACTGGTGTTCCTTCCTATA) was used to amplify all *Pseudomonas* species, while the pair PA-SS-F (GGGGGATCTTCGGACCTCA) and PA-SS-R (TCCTTAGAGTGCCCACCCG) was used only to amplify *P. aeruginosa* specifc DNA. PCR amplification of the target DNA was carried out using 200 µL PCR tube with a reaction mixture of 25 µL. Each of the reaction mixtures contained 1X reaction buffer, 200 µmol/L each of dATP, dCTP, dGTP and dTTP (Fermentas), 1.5 mmol/L $MgCl_2$, 1 U of Taq polymerase (Fermentas), 10X reaction buffer, 10 pM of each primers, 100 ng of bacterial DNA and milli-Q water up to 25 µl. The thermal cycler was programmed for 30 cycles starting with denaturation of the template DNA at 94°C for 1 min, annealing of primers with template DNA at 55°C for 1 min and extension of the primers at 72°C for 2 min. Before initiation of the first cycle, the reaction mixture was heated at 94°C for 10 min to allow complete denaturation of the template. After the last cycle, reaction mixture was subjected to 72°C for 10 min to ensure final extension. In control reaction, deionized water was added to reaction mixture instead of bacterial DNA. PCR products thus obtained were separated by electrophoresis on a 1.5% (*W/V*) agarose gel containing ethidium bromide (0.5 µg/ml) to resolve the amplified products. A 100 bp DNA ladder (Fermentas) was loaded on gel as a molecular size standard.

Genomic DNA fingerprinting analysis

Genetic relatedness among the *Pseudomonas* isolates was determined by using ERIC PCR. ERIC PCR was performed as described earlier with little modifications by using two oligonucleotides ERIC1R 5'-ATGTAAGCTCCTGGGGATTCAC-3' and ERIC2 5'-AAGTAAG TGACTGGGTGAGCG-3' (Rivera et al., 1995). The thermal cycler was programmed for 35 cycles of 1 min at 94°C, 1 min at 52°C, 10 min at 68°C followed by 20 min incubation at 70°C.

Antimicrobial susceptibility

The antimicrobial susceptibility of *P. aeruginosa* isolates was studied using disk diffusion method on Mueller–Hinton agar (CLSI, 2007). Antibiotic impregnated discs (Oxoid Ltd, Hants, UK) containing 43 different antibiotics were used (Table 1). The interpretation was made as per the zone size interpretation chart provided by the manufacturer of discs. Minimum inhibitory concentration (MIC) was determined on Mueller Hinton agar using HiComb discs (HiMedia, Mumbai, India) containing different concentrations of antibiotics. The value at which the zone convenes the comb-like projections of the strips was recorded as minimum inhibitory concentration.

Production of *P. aeruginosa* specific exotoxin A

All the seven isolates were tested for the production of exotoxin A. The culture supernatant of overnight grown culture was concentrated using 50% (v/v) trichloroacetic acid (TCA). The pellet was washed twice with chilled acetone and re-dissolved in phosphate buffer saline (PBS). The proteins were separated by SDS-PAGE and transferred to PVDF membrane (Immobilon-P, Millipore). The membrane was blocked overnight in PBS containing 5% skimmed milk powder at 4°C before addition of Rabbit anti-exotoxin A anti-serum (Sigma Aldrich) diluted to 1:10,000 in 1% skimmed milk dilution buffer in PBS. Anti- Rabbit HRP conjugated Goat antibodies in 1: 4000 dilution were used as secondary antibodies. Bands were visualized with 3,3'-Diaminobenzidine (DAB) and hydrogen peroxide in PBS as substrate for HRP.

RESULTS AND DISCUSSION

Biochemical identification and characterization of *P. aeruginosa* isolates

All the 7 *Pseudomonas* isolates collected from skin lesions of patients from a tribal area in South India were

Table 1. Antibiotic susceptibility pattern of *P. aeruginosa* isolates.

S/N	Antibiotics	Conc. (mcg)	*P. aeruginosa* isolates						
			PVZ1	PVZ2	PVZ3	PVZ4	PVZ5	PVZ6	PVZ7
1	**Aminoglycosides**								
	Amikacin	30	S (8)	S (8)	S (8)	S (8)	S (8)	S (8)	S (8)
	Gentamicin	120	S (5)	S (5)	S (5)	S (5)	S (5)	S (5)	S (5)
	Kanamycin	30	R	R	R	R	R	R	R
	Neomycin	30	R	R	R	R	R	R	R
	Spectinomycin	100	R	R	R	R	R	R	R
	Streptomycin	10	R	R	R	R	R	R	R
	Tobramycin	10	S (ND)	S (ND)	S (ND)	S (ND)	S (ND)	S (ND)	S (ND)
2	**β- lactam**								
	Amoxicillin	25	R	R	R	R	R	R	R
	Amoxicillin/Clavulanate	30	R	R	R	R	R	R	R
	Ampicillin	10	R	R	R	R	R	R	R
	Aztreonam	30	R	R	R	R	R	R	R
	Cloxacillin	5	R	R	R	R	R	R	R
	Imipenem	10	S (<10)	S (<10)	S (<10)	S (<10)	S (<10)	S (<10)	S (<10)
	Meropenem	10	S (<10)	S (<10)	S (<10)	S (<10)	S (<10)	S (<10)	S (<10)
	Oxacillin	1	R	R	R	R	R	R	R
	Penicillin-G	10	R	R	R	R	R	R	R
	Piperacillin	100	S (ND)	S (ND)	S (ND)	S (ND)	S (ND)	S (ND)	S (ND)
3	**Cephalosporin**								
	Cefactor	30	R	R	R	R	R	R	R
	Cefixime	5	R	R	R	R	R	R	R
	Cefoxitin	30	R	R	R	R	R	R	R
	Cephalothin	30	R	R	R	R	R	R	R
4	**Macrolides**								
	Clarithromycin	15	R	R	R	R	R	R	R
	Erythromycin	15	R	R	R	R	R	R	R
	Roxithromycin	30	R	R	R	R	R	R	R
5	**Rifamycin**								
	Rifampicin	5	R	R	R	R	R	R	R
6	**Sulfonamide**								
	Sulfamethoxazole/Trimethoprim	25	R	R	R	R	R	R	R
	Sulfamethoxazole	100	R	R	R	R	R	R	R
	Trimethoprim	5	R	R	R	R	R	R	R
7	**Quinolone/Fluoroquinolones**								
	Ciprofloxacin	5	S (0.01)	S (0.01)	S (0.01)	S (0.01)	S (0.01)	S (0.01)	S (0.01)
	Gatifloxacin	5	S (ND)	S (ND)	S (ND)	S (ND)	S (ND)	S (ND)	S (ND)
	Levofloxacin	5	S(<0.005)	S(<0.005)	S(<0.005)	S(<0.005)	S(<0.005)	S(<0.005)	S(<0.005)
	Moxifloxacin	5	S(<0.005)	S(<0.005)	S(<0.005)	S(<0.005)	S(<0.005)	S(<0.005)	S(<0.005)
	Nalidixic Acid	30	R	R	R	R	R	R	R
	Norfloxacin	10	S (0.1)	S (0.1)	S (0.1)	S (0.1)	S (0.1)	S (0.1)	S (0.1)
	Ofloxacin	5	S (ND)	S (ND)	S (ND)	S (ND)	S (ND)	S (ND)	S (ND)

Table 1. Cont.

8	**Broad Spectrum**								
	Chloramphenicol	30	R	R	R	R	R	R	R
	Tetracyclin	30	R	R	R	R	R	R	R
	Doxycyclin hydrochloride	30	R	R	R	R	R	R	R
9	**Others**								
	Amphotericin	100	R	R	R	R	R	R	R
	Clindamycin	10	R	R	R	R	R	R	R
	Colistin (Polypeptide)	10	R	R	R	R	R	R	R
	Linezolid (Oxazotidione)	30	R	R	R	R	R	R	R
	Polymyxin B	300	R	R	R	R	R	R	R

Values in parenthesis indicate MIC of antibiotics; R, resistant; S, susceptible and ND, not done.

found Gram negative, motile, catalase and oxidase test positive, exhibited nutrient gelatin liquefaction and starch hydrolysis but were negative for indole, and nitrate reduction test. These are the characteristics features of all pseudomonads as described in Bergey's Manual for Systematic Bacteriology (Palleroni, 1984). BD Crystal automatic identification system also confirmed the isolates as *P. aeruginosa*. The isolates utilized only galactose and did not ferment arabinose, mannose, sucrose, melibiose, rhamnose, sorbitol, mannitol, adonitol and inositol. The isolates hydrolyzed urea, glycine and utilized citrate, malonic acid, triphenyl tetrazolium chloride, arginine and lysine.

PCR identification of *P. aeruginosa* isolates

A duplex PCR was performed for molecular identification of *P. aeruginosa.* Among the two sets of primers used, first *Pseudomonas* genera specific set of primers (PA-GS) yielded amplicon of 618 bp and another *Pseudomonas aeruginosa* specific set of primers (PA-SS) gave amplicon of 956 bp in all the isolates (Figure 1). Thus, all the isolates were confirmed as *P. aeruginosa* by PCR.

Antibiogram of *P. aeruginosa* isolates

In this study, *P. aeruginosa* from cutaneous infections in tribal areas exhibited resistance towards several antibiotics including broad spectrum antibiotics, β-lactams, cephalosporin, macrolides, rifampicin and sulfonamides (Table 1). However, the isolates were susceptible towards many commonly used antibiotics belonging to quinolones and aminoglycosides. The MIC values for all the isolates were found less than 10 mcg for all the groups of antibiotics (Table 1).

Emergence of resistance in *Pseudomonas species* for most of the antibiotics is a challenge for our clinicians. A total of 43 antibiotics were tested and only few antibiotics like levofloxacin, norfloxacin, ciprofloxacin, gatifloxacin, ofloxacin, moxifloxacin, meropenem, imipenem, piperacillin, tobramycin, gentamicin and amikacin were proved to be effective for the treatment. Globally, most of the *P. aeruginosa* isolates from hospitals have shown resistance towards quinolones, β-lactams and aminoglycosides (Philippe et al., 1999; Gad et al., 2007; Rodriguez-Morales et al., 2007; Rodriguez et al., 2005). Multiple antimicrobial resistances of the bacterial pathogens are of great concern both in veterinary and human medicine worldwide (Masaadeh and Jaran, 2009; Ranjan et al., 2010). Hence, a continuous monitoring of the *Pseudomonas* isolates for drug resistance is important from the clinical standpoint.

Reliable detection of antibiotic resistance may therefore be important for treatment of acute exacerbations in patients with skin lesions. One important reason for treatment failure is assumed to be indiscriminate use of antibacterials without testing *in vitro* sensitivity of causal organisms (Lister et al., 2009; Zhang et al., 2005). This practice, at one hand increases the economic losses and on the other hand results in development of resistance to commonly used antimicrobials.

Genetic relatedness of *P. aeruginosa* isolates

The *P. aeruginosa* isolates were characterized by ERIC PCR to reveal their clonal relationships. ERIC PCR with genomic DNA of various *P. aeruginosa* isolates resulted in amplification of multiple fragments of DNA with sizes ranging from 0.3 to 1.4 kb. PCR-based methods of fingerprinting take advantage of the presence of repetitive sequences that are interspersed throughout the genome of diverse bacterial species (Ghosh et al., 2011). The fingerprinting method used in this study is well established and have been applied to both clinical and environmental strains for their identification (Kumar et al., 2009). The fingerprinting analyses revealed different patterns among the isolates, suggesting the non-clonal

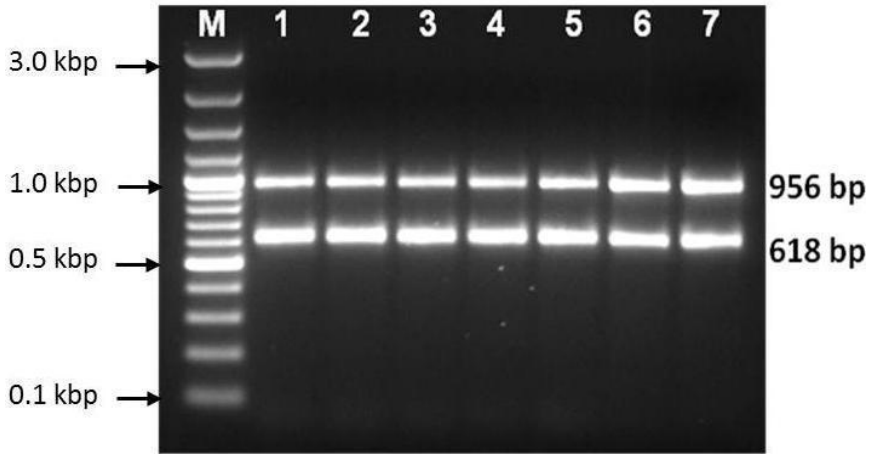

Figure 1. Duplex PCR for detection of *P. aeruginosa*. Lane M: 100 bp (Fermentas); Lane 1-7: Clinical isolates PVZ1-PVZ7.

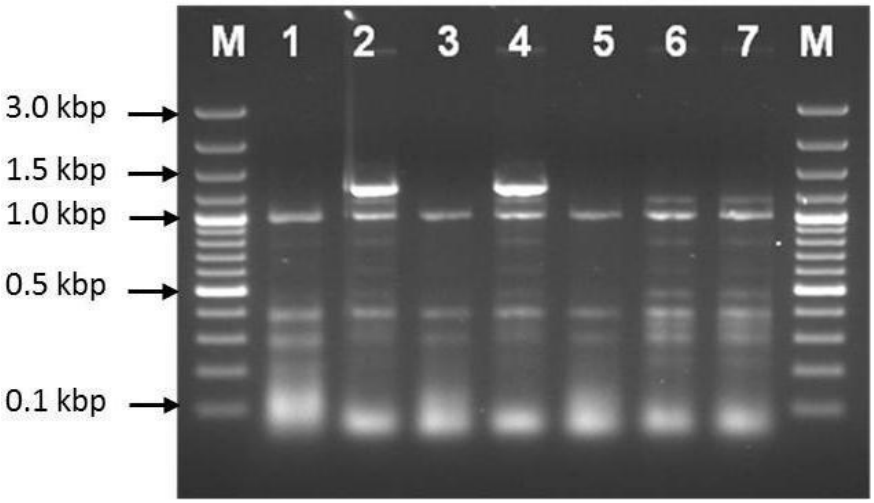

Figure 2. ERIC PCR of *P. aeruginosa* isolates. Lane M: 100 bp plus DNA ladder (Fermentas); Lane 1-7: Clinical isolates PVZ1-PVZ7.

origin of the isolates (Figure 2).

Virulence profile of *P. aeruginosa* isolates

All the isolates of *P. aeruginosa* were found positive for the production of exotoxin A in the secretome. Immunoblot with anti-exotoxin A rabbit antibodies showed intense band of 66 KDa confirming virulence of all the isolates (Figure 3). Exotoxin A appears to be involved in local *P. aeruginosa* infections and systemic disease. Studies that compare the virulence of exotoxin A producing strains of *P. aeruginosa* to mutant strains that do not produce exotoxin A suggest that it is an important virulence factor (Blackwood et al., 1983; El-Zaim et al., 1998).

Exotoxin A is produced by most *P. aeruginosa* strains that cause clinical infections. Like diphtheria toxin, *P. aeruginosa* exotoxin A catalyzes ADP-ribosylation and inactivation of elongation factor 2, leading to inhibition of protein biosynthesis and cell death. Exotoxin A is responsible for local tissue damage, bacterial invasion, and (possibly) immunosuppression (Woods and Iglewski, 1983).

Pseudomonas species has become increasingly prevalent worldwide. Strengthening surveillance and screening of high-risk patients appears as an important component of effective infection control programs to limit the spread of *Pseudomonas species* in hospitals. Tribal people are generally custodians of medicinal plants and avoid antibiotics and other modern medicines. Hence, the *P. aeruginosa* isolates were found susceptible to commonly

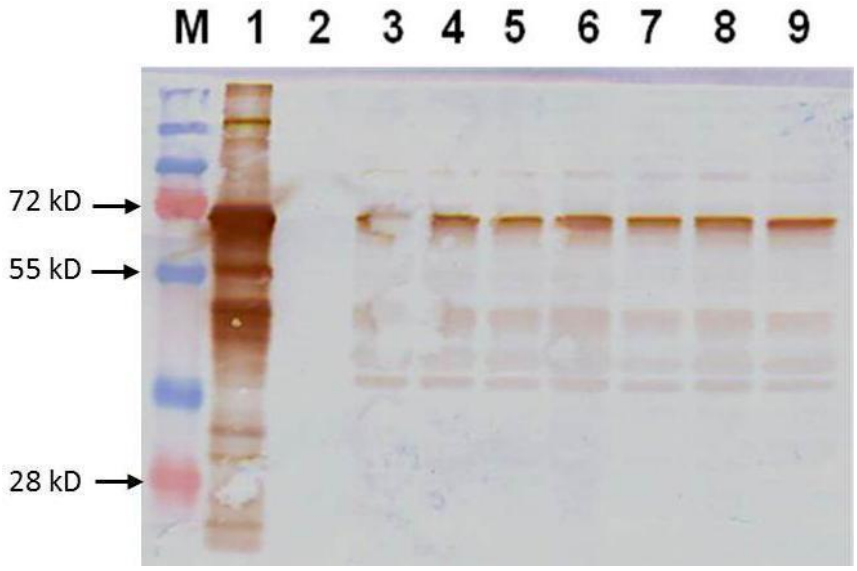

Figure 3. Western Blot of *P. aeruginosa* exotoxin A with rabbit anti-exotoxin A antibodies. Lane M: Prestained protein page ruler (Fermentas); Lane 2: Negative control; Lane 3-9: Clinical isolates PVZ1-PVZ7.

used quinolones and aminoglycosides. Hence, judicious use of antibiotics is imperative for controlling spread of antibiotics resistance among bacteria.

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