Original Paper

Airborne *Pseudomonas* species in Healthcare Facilities in a Tropical Setting

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ABSTRACT: *Background and objectives: Pseudomonas* species are nosocomial pathogens that are capable of colonising moist surfaces. Little is known whether they get airborne. The study was undertaken to 1) characterise Gram-negative bacteria in indoor air of different hospitals; 2) characterise *Pseudomonas* sp. by phenotypic and genotypic methods; 3) determine homology of study environmental *Pseudomonas* isolates and correlate with established pathogenic strains' sequences. *Methods:* Samples were collected (duplicates) at the time of peak activity, by exposing media-containing plates (blood agar and MacConkey agar) for 30 minutes. Plates were incubated aerobically at 37°C for 24-48 h. Microorganisms were identified by standard microbiological procedures. Polymerase chain reaction targeting *Pseudomonas* specific 16S-rDNA was performed to obtain 618 bp amplicons. Representative strains were sequenced and compared with established sequences of pathogenic *Pseudomonas* strains from existing database for evolutionary details. *Results:* A total of six hospitals comprising 13 wards, 7 intensive care units (ICUs) and 8 operating rooms (ORs) were sampled over one-year period. A variety of Gram-negative bacilli were isolated, of which *Pseudomonas* sp. was predominant. Indoor air of 10 wards (77%), 5 ICUs (71%), 4 ORs (50%) harboured *Pseudomonas*. Similar strains of *Pseudomonas stutzeri* were isolated from indoor air of different hospitals. Phylogenetic analysis showed these environmental strains to be closely related to the pathogenic *Pseudomonas stutzeri* strain from the GenBank database. *Conclusions:* Isolation of airborne *Pseudomonas stutzeri* from different hospitals suggests a possible new reservoir in the hospital environment, indicating the need for appropriate engineering control measures to contain the spread of these nosocomial agents.

KEYWORDS: *Pseudomonas, airborne transmission, nosocomial infections, infection control*

Introduction

The incidence of Healthcare Associated Infections (HAI) in developed countries ranges between 5 – 15 % of hospitalized patients [1] . The incidence of HAI in developing countries is not well studied. It is estimated that HAI are higher in developing countries than in developed countries.[1] Limited financial resources, understaffing, poor hygiene and sanitation, lack or malfunctioning of basic equipments, inadequate infrastructures and overcrowding may be the contributing factors [1].

A one-day point analysis of the prevalence of infection was undertaken in 1417 intensive care units (ICUs) in 17 western European countries and it was found that *Staphylococcus aureus* was the most frequently isolated organism (30.1%) followed by *Pseudomonas aeruginosa* (28.7%), coagulase-negative *Staphylococci* (19.1%), yeasts (17.1%), and enterococci (11.7%) [2]. Studies conducted in India have shown that the HAI by Gram-negative organisms are higher than those by Gram-positive organisms. *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *E. coli*, *Acinetobacter baumanni*, *Enterobacter* species, *Citrobacter* species and other non-fermenters are the commonest Gram-negative organisms causing HAI; among Gram-positive organisms, *Staphylococcus aureus*, *Coagulase negative Staphylococci*, *Enterococcus* species and *Streptococcus* species were the predominant. [3,4].

Pseudomonas aeruginosa and other *Pseudomonas* species usually colonise moist surfaces[5], and have been implicated in airborne transmission of cystic fibrosis[6]. Few studies have documented their presence in indoor air of healthcare facilities [7,8,9]

The study was undertaken to 1) characterise Gram-negative bacteria in indoor air of different hospitals; 2) characterise *Pseudomonas* sp. by phenotypic and genotypic methods; 3) determine the homology of study environmental isolates of *Pseudomonas* sp. and correlate with sequences of established pathogenic strains.

Materials and methods

Sample collection

The study was conducted over a period of one year during January – December 2009 in different hospitals situated in and around the city of Chennai. The hospitals were sampled after obtaining the consent from the authorities. Sampling was carried out in six different hospitals (three ~ 100 bed hospitals, two < 10 bed hospitals and one > 100 bed hospital). Samples were collected only one time during the study period. Hospitals were coded in order to maintain confidentiality. Sampling locations included intensive care units (ICUs), operating rooms (ORs) and wards (general ward).

Hospitals with < 10 beds (6 and 8 beds respectively) were smaller hospitals and intensive care units were not available. Wards were naturally ventilated with multiple-beds. ORs were provided with conventional ventilation. In hospitals with ~100 beds (75, 50 and 40 beds respectively), intensive care units were provided with mechanical ventilation. Wards were provided with single-beds and natural ventilation with occasional mechanical ventilation through use of mechanical fans. ORs were provided with conventional ventilation, and were either fumigated or UV sterilised. In tertiary hospital (private) with > 500 beds, intensive care units were provided with A/C ventilation. Wards had multiple-beds, with natural ventilation, supported by mechanical fans. ORs were provided with ultra clean air.

Walk-through was conducted prior to every sampling to gather details on the existing local environmental conditions (such as water leakages and dampness in walls and ceilings that may favour microbial growth) and the extent of activity, to enable sampling to be carried out at the time of peak activity. Indoor air samples were collected (in duplicates) between 9.30 am and 12.30 pm of the day by exposed-plate gravitational (passive) method [10], by exposing media-containing plates (9 cm in diameter) for 30 minutes [11]. Petri plates were put at a height of 60 - 70 cm above the ground level during sampling. Media used for sampling included 5% sheep blood agar and MacConkey agar (Hi Media Company Limited, India). Plates were incubated aerobically at 37 °C for 24-48 h. Colony counts were taken and expressed in terms of colony forming units (CFU) per plate. Microorganisms were identified by standard microbiological procedures.

Phenotypic identification

All isolates of Gram-negative bacilli (GNB) were presumptively identified by conventional methods including haemolysis on sheep blood agar and growth on MacConkey agar for lactose fermentation. Lactose fermenting (LF) and non lactose fermenting (NLF) organisms were characterised to the genus level, based on the basic biochemical methods like catalase, oxidase, motility, indole, triple sugar iron test, urease, citrate and H_2S production [12].

The NLF motile and oxidase positive organisms, which were predominant, were further identified to the species level using the various standard sugar fermentation test like fermentation of 10 % lactose, starch, gelatin, oxidative fermentation of dextrose, maltose, mannitol, fructose, xylose, decarboxylation of lysine, arginine, ornithine and reduction of nitrate [13].

Antimicrobial susceptibility testing

Susceptibilities of the isolates were determined by disk diffusion method. The following antimicrobial agents (Hi-Media Company Limited, India) were tested: Amikacin (30 µg), Ampicillin (10 µg), Cefotaxime (30 µg), Cephalexin (30 µg), Ceftazidime (30 µg), Ciprofloxacin (5 µg), Imipenem (10 µg). Results were expressed as susceptible or resistant according to criteria recommended by the Clinical and Laboratory Standards Institute (2009) [14].

16S rDNA gene amplification and sequencing

The study isolates were subjected to conventional PCR to confirm phenotypic identification of the isolates as *Pseudomonas*. Bacterial DNA was extracted from pure cultures of the *Pseudomonas* isolates recovered from indoor air using commercially available kit (MEDOX-BIO^{TM} Gel Extraction Teaching Kit) as per the manufacturer's instructions. Amplification was carried out in a PTC-200 thermal cycler (MJ Research Inc., Watertown, Mass.), using commercially available kit (Red dye master mix kit – Bangalore Genei). Primer PA-GS-for (5-GAC GGG TGA GTA ATG CCT A-3) and PA-GS-rev (5-CAC TGG TGT TCC TTC CTA TA -3), which were found to amplify a 618 bp product from bacterial species, were used at 20 pmol concentrations [15]. The PCR mixtures (25 µL) were subjected to thermal cycling (initial denaturisation for 2 min at 95 °C and then 25 cycles were completed, each consisting of 20 s at 94 °C, 20 s at annealing

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temperature of 54 °C, and 40 s at 72 °C. A final extension of 1 min at 72 °C was applied. With this program, the total time for amplification of target DNA was approximately 1 h 24 min. Appropriate positive (*Pseudomonas aeruginosa* ATCC 28753) and negative (water) controls were included along with the test samples.

DNA sequencing was carried out with an Applied Biosystems ABI model 3700 sequencer and the protocols of the manufacturer (PE Applied Biosystems, Foster City, California) by Sanger method, using the BigDye Terminator cycle sequencing ready reaction kit.

Table 1. Location wise distribution of Pseudomonas sp. in different hospitals

Hospital under study	**Sampling location**	**Sampling location code**	**Counts of *Pseudomonas* isolates (CFU/plate)**
H_1	ICU	H_1-ICU_1	7
		H_1-ICU_2	3
		H_1-ICU_3	0
		H_1-ICU_4	0
	OR	H_1-OR_1	0
		H_1-OR_2	0
		H_1-OR_3	3
	W	H_1-W_1	5
		H_1-W_2	0
		H_1-W_3	0
		H_1-W_4	2
		H_1-W_5	0
		H_1-W_6	2
		H_1-W_7	2
		H_1-W_8	2
H_2	ICU	H_2-ICU_1	2
	OR	H_2-OR_1	1
	W	H_2-W_1	12
H_3	ICU	H_3-ICU_1	10
	OR	H_3-OR_1	1
	W	H_3-W_1	4
H_4	ICU	H_4-ICU_1	4
	OR	H_4-OR_1	0
	W	H_4-W_1	3
H_5	OR	H_5-OR_1	1
	W	H_5-W_1	7
H_6	OR	H_6-OR_1	7
	W	H_6-W_1	55

OR – Operating room; ICU – Intensive Care Unit; W – Ward

Phylogenetic analysis

The partial nucleotide sequence of the 16S rDNA obtained from the study isolates were compared with other strains currently available in the National Centre for Biotechnology Information (NCBI) database and nucleotide homology percentages were calculated using the sequence similarity search tool, Basic Local Alignment Search Tool (BLAST), and the cDNA sequences were aligned using Clustal X 1.83 software [16]. Phylogenetic tree was constructed using Mega 4 kimura 2 parameter [17]. The interior branch test was carried out using 500 bootstrap replications to examine the statistical significance of the branching pattern. The pattern was then analysed for evolutionary details and homology. Sequences of Pseudomonas sp. of clinical origin available in the published literature were also included in the phylogenetic analysis to look for similarity.

Results

In all, 13 wards, 7 ICUs and 8 ORs from six different hospitals were sampled over one-year period. Of these, GNB were recovered from air samples collected from 6 ICUs, 5 ORs and 12 wards. A variety of GNB such as Pseudomonas sp., Klebsiella pneumoniae, Klebsiella oxytoca, Acinetobacter sp., Citrobacter diversus and Escherichia coli were isolated. Among the GNB isolated (n=155), Pseudomonas sp. was predominant (86 %). Indoor air of 10 wards (77%), 5 ICUs (71%) and 5 ORs (63%) were found to harbour Pseudomonas species.

When the loads of Pseudomonas in different locations were analysed (Table 1), it was found that Pseudomonas was isolated from indoor air of all the hospitals studied. Among the different areas sampled, the maximum load of Pseudomonas was obtained from indoor air of wards (0 – 55 CFU/plate) followed by ICUs (0 – 10 CFU/plate) and ORs (0 – 7 CFU/plate).In view of *Pseudomonas* being the commonest organism isolated irrespective of the hospital sampled, the strains from different hospitals were stocked and the study was undertaken to compare the strains obtained across all the hospitals sampled and determine the similarity.

A total of twenty five *Pseudomonas* isolates representative of different hospitals sampled were screened for similarity in phenotypic characteristics and antibiotic susceptibility pattern. Of these, seven *Pseudomonas* isolates were found to have similar phenotypic characteristics and antibiotic susceptibility pattern (Table 2). Four of the study strains were susceptible to all the antibiotics used; three strains were resistant to ampicillin and cephalexin.

Table 2. Antibiotic susceptibility pattern of the study isolates

Hospital location	Date of Isolation	Organisms isolated	Antibiotic susceptibility						
			Ampicillin	Cephalexin	Cefotaxime	Ceftazidime	Ciprofloxacin	Amikacin	Imipenem
H_1 – OR	27-Aug-2009	*Pseudomonas stutzeri*	S	S	S	S	S	S	S
H_4 – ICU	17-Sep-2009	*Pseudomonas stutzeri*	S	S	S	S	S	S	S
H_6 – W	15-Sep-2009	*Pseudomonas* sp.	S	S	S	S	S	S	S
H_6 – OR	15-Sep-2009	*Pseudomonas* sp.	S	S	S	S	S	S	S
H_1 – OR	27-Aug-2009	*Pseudomonas* sp.	R	R	S	S	S	S	S
H_3 – W	12-Mar-2009	*Pseudomonas* sp.	R	R	S	S	S	S	S
H_3 – OR	12-Mar-2009	*Pseudomonas stutzeri*	R	R	S	S	S	S	S

H – Hospital; OR – Operating room; ICU – Intensive Care Unit; W – Ward

S – Susceptible; R – Resistant

□ – Strains sequenced

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The study strains that were environmental in origin were subjected to all possible tests as per standard microbiological procedures [12,13] They were non-pigmented, motile and oxidase-positive. Further, they were found to lack arginine dihydrolase activity and did not produce acid from lactose. In order to confirm the preliminary identification of the organisms recovered as *Pseudomonas stutzeri*, 16S rDNA sequencing was done and the sequences were blast analysed. The strains were first genotyped by performing polymerase chain reaction and confirmed as *Pseudomonas* (Fig.1). Four of these strains (three *Pseudomonas stutzeri* and one *Pseudomonas* sp.) with similar phenotypic characteristics, representative of different hospitals included in the study, were further subjected to sequencing (Table 2).

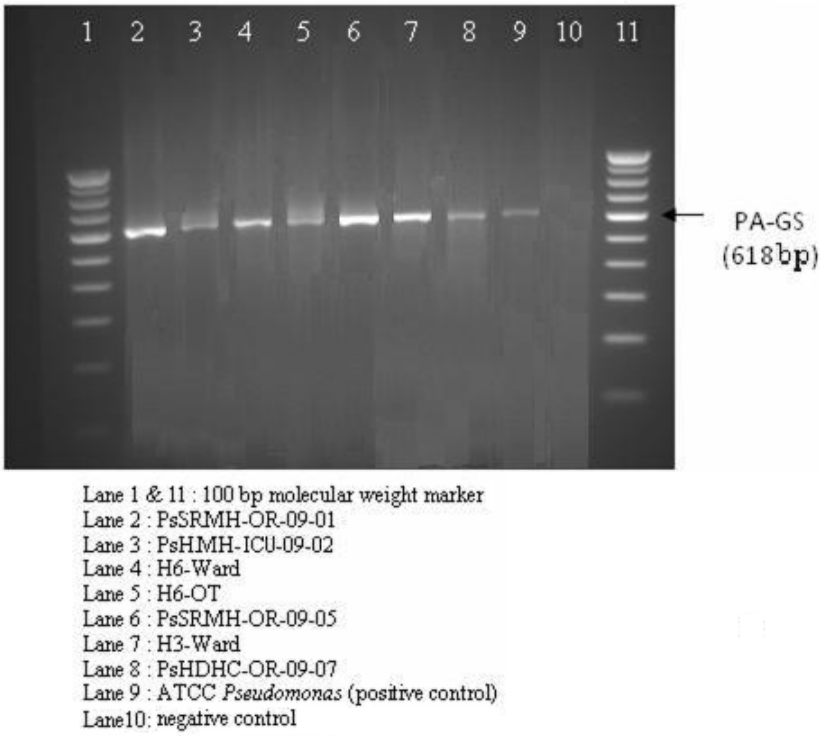

Fig.1. Gel electrophoresis of PCR products amplified from environmental strains of Pseudomonas stutzeri isolated from indoor air of different hospitals

The sequences of the study isolates were submitted to GenBank and have been allotted the GenBank accession numbers HQ221549 – HQ221552. The nucleotide sequences of the partial 16S rDNA gene (618 nucleotides) of the four *Pseudomonas* isolates from indoor air of different hospitals were compared with other globally diverse *Pseudomonas* isolates of clinical and environmental origin, and they were found to be similar with *Pseudomonas* isolates from different ecological niches. Since clinical isolates of *Pseudomonas stutzeri* were not recovered from the study locations during the study period, established sequences of existing *Pseudomonas stutzeri* isolated from different sources deposited in GenBank were included in the study for phylogenetic analysis.

The phylogenetic tree was constructed using the study isolates and *Burkholderia* strain as an out group and forty five isolates from the GenBank as shown in Fig.2. The isolates included in the phylogram were isolated from all possible sources such as soil, water samples, plant surfaces, skin and gut, waste water activated sludge, industrial effluent and microbial biofilm. To the best of our knowledge, no documented sequences of *Pseudomonas* isolates from hospital air were available. Blast analysis showed that these environmental strains are closely related to other strains of *Pseudomonas* available in the NCBI database with 98−100 % sequence homology. Phylogenetic analysis showed that these environmental strains are closely related to pathogenic strains of *Pseudomonas*. When the study isolates of *Pseudomonas stutzeri* were compared with the clinical isolate of *Pseudomonas stutzeri* (CCUG 11256; GenBank Accession number U26262) from the GenBank database, the identity was found to be 99-100 % for three isolates, while the fourth study isolate (PsSRMH-OR-09-05) was found to be 97% identical.

Fig.2. Phylogenetic tree generated by using ClustalV-based alignment for 16S rDNA sequences of select Pseudomonas species of clinical and environmental origin

Discussion

Pseudomonas species are among the commonest etiologic agents of HAIs in India. Prevalence of *Pseudomonas* infections has been documented as 13.6 % in neonatal ICU and 12.7 % in general ICU [3,4]. They are known to survive in a variety of environmental niches. *Pseudomonas aeruginosa* can persist on inanimate objects for 6 h – 16 weeks, and upto 5 weeks on dry surfaces [18]. Principal reservoirs for *Pseudomonas* species are the hands of the personnel, sinks or other moist surfaces, and solutions used to rinse catheters [19], where transmission is known to occur primarily via hands. However, airborne dissemination has been implicated to play a significant role in patient-to-patient spread [20], This study was therefore carried out to determine whether air can be a reservoir of *Pseudomonas* species.

Different active and passive methods of air sampling are available; however, no single method is considered efficient for recovery of microorganisms, or is ideal in every sampling situation [10]. Exposed plate method is a well-known passive method that is reproducible and

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reliable [21]. Additionally, this method is cost-effective and finds its use in surveillance of air in healthcare facilities for isolation of GNB [7,8]. A sampling duration of 30 minutes was chosen for the study for better recovery of GNB that may require more duration of exposure when compared to Gram-positive cocci that are readily airborne [11].

Gram-positive cocci such as *Staphylococcus aureus* and Coagulase-negative *Staphylococci* were isolated along with GNB from indoor air of hospitals (data not shown). The focus of the current study was to determine the profile of indoor air with respect to GNB. Our study thus documented that all the hospitals that were sampled harboured *Pseudomonas* sp. in indoor air, including the ICUs and ORs, irrespective of the size of the hospital. When one-time sampling was conducted in duplicates in 28 different locations of the six hospitals, *Pseudomonas* sp. was present in indoor air of 20 (71 %) locations. Of the four isolates that were sequenced, three isolates were *Pseudomonas stutzeri* that were obtained from indoor air of three different hospitals. The recovery of these organisms from indoor air of different hospitals, including ICUs and ORs suggested the need to explore the possibility of *Ps. stutzeri* as a nosocomial pathogen.

Phylogenetic analysis showed that the environmental strains of *Ps. stutzeri* isolated from indoor air of different hospitals were similar and their clustering in the same group in the phylogenetic tree along with the clinical strain of *Ps. stutzeri* (CCUG 11256; GenBank Accession number U26262) available in the existing database.

16s rDNA sequence analysis was performed as it helps to identify organisms that are difficult to be identified phenotypically [22]. The study strains were thus confirmed as *Ps. stutzeri*. Since 16S rRNA gene sequencing provides accurately grouped organisms for analysis [23], an attempt was made for preliminary assessment of the similarity between study environmental isolates and established sequences from NCBI database.

Ps. stutzeri are capable of causing opportunistic infections in humans [24] and have been associated with bacteraemia/septicaemia, bone infections, endocarditis, eye infections (endophthalmitis and panophthalmitis), meningitis, pneumonia and/or empyema, skin infection, urinary tract infection and ventriculitis, resulting in increased morbidity [25,26], and to cause pseudobacteremia [27].

Ps. stutzeri are commonly found in the hospital environment. *Ps. stutzeri* has been isolated from deionised water used for hemo-dialysis [28]. Procedures or activities that cause aerosolisation may be the source of these organisms especially from moist surfaces [27.29]. Studies have shown that *Ps. stutzeri* are capable of causing nosocomial infections [25,30]. An 8-year prospective study on the incidence of postoperative infectious endophthalmitis in a tertiary care ophthalmic hospital in Chennai, India showed infections by *Ps. stutzeri* (n=12) to be more in numbers when compared to that of *Pseudomonas aeruginosa* (n=7) [31].

The study strains were susceptible to majority of the commonly used antibiotics. However, a longitudinal follow-up study was not undertaken to determine if these strains acquired resistance to antibiotics. Susceptible strains of *Ps. stutzeri* have been recovered from hospital environment [26]. However, the ability of these organisms to gradually develop resistance to chlorhexidine with increasing concentrations of chlorhexidine diacetate has been documented [32].Further, these strains were found to acquire resistance subsequently following exposure to antibiotics in the hospital environment resulting in survival of resistant mutant strains [33].

The similarity documented between the study isolates of *Ps. stutzeri* strains that are environmental in origin and the clinical isolate of *Ps. stutzeri* in the NCBI database suggests that a pathway may exist, allowing for the introduction of these organisms from the hospital environment into patients during healthcare delivery. Further studies may be undertaken to perform simultaneous sampling of environmental and clinical samples to obtain a correlation of the isolates.

The presence of *Ps. stutzeri* in indoor air of special environments such as ICUs and ORs is worrisome; their presence indicates the need for clean practices since settled dust in these environments can be a source. *Pseudomonas* species are present in hospital environment on surfaces and instruments, from which they may get temporarily airborne during activity.

The repeated isolation of *Ps. stutzeri* from different hospitals which showed a homology with the clinical isolate of *Ps. stutzeri* in the NCBI database suggests a possible new reservoir for *Ps. stutzeri* in the hospital environment. Airborne transmission is a possible route for acquisition of surgical site infection

and wound infection. Since *Pseudomonas aeruginosa* and other *Pseudomonas* species have predilection for the hospital environment, it is not possible to rid the hospital environment of these nosocomial pathogens. There is a need to heighten awareness on possible sources of such nosocomial agents in order to plan for appropriate engineering control measures to contain the spread of these nosocomial agents, especially in areas where immuno-compromised patients are admitted. *Pseudomonas* may get aerosolised during procedures, indicating the need for use of personal protective equipments such as masks. The possibility of settling of these airborne organisms on surfaces indicates the need for cleaning to maintain dust-free environment. Newer technologies such as ultrasonic cleaning may be adopted to reduce the use of disinfectants.

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