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ORIGINAL ARTICLE

Molecular Characterisation of *Pseudomonas aeruginosa* Recovered in the Buea Health District of Cameroon: Implications for Nosocomial Spread.

La Caractérisation moléculaire de Pseudomonas aeruginosa A Retrouvé dans le Quartier de Santé de Buea de Cameroun : Les implications pour la Diffusion de Nosocomial.

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ABSTRACT

BACKGROUND: *Pseudomonas aeruginosa* is a ubiquitous gram-negative pathogen with a propensity to cause opportunistic infections in humans. Different strains of the organism could colonise patients heralding a wide spectrum of *P.aeruginosa* infections in the environment.

OBJECTIVE: To analyse isolates of *P. aeruginosa* from clinical and environmental samples using the Polymerase Chain Reaction (PCR) to establish strain relatedness.

METHODS: Fifty-two strains of the organism were isolated from wound swabs, urine, sputum of patients and environmental samples from the hospital environment using standard microbiological techniques and ethical consideration. Genomic DNA of the isolates was amplified with primers AF1 (5'-AGA GTT TGA TCC TGG CTCA-3') and 1541R (5'-AAG GAG GTG ATC CAG CC-3').

RESULTS: At least two bands were observed in all isolates typed and band sizes ranged from 0.07 – 1.5kb. The strains were genetically diverse, displaying profiles of 2 – 6 bands between 0.07 – 1.5kb.

CONCLUSION: The study demonstrates that strain diversity could be discerned between strains of *P. aeruginosa,* circulating in the environment of Buea, a finding which has important epidemiological and clinical significance bearing in mind that this pathogen is highly incriminated in nosocomial infections with attendant social implications. This therefore calls for more attention in the diagnosis and management of *P. aeruginosa* infections in the environment of Buea, Cameroon.

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Keywords: Pseudomonas aeruginosa, Nosocomial infection, Social implications, DNA, Polymerase Chain Reaction, Buea.

RESUMÉ

Contexte: *Le Pseudomonas aeruginosa est un pathogène à gramme-negative très répandu, ayant une tendance à causer les infections opportunistes chez les humains. Les différents souches de cette organisme peuvent coloniser les malades et représente ainsi un spectre large d'infection à P. aeruginosa dans l'environnement.*

Objectif: *Pour analyser les isolats de P. aeruginosa à partir des échantillons environnementaux et cliniques, la réaction en chaîne à base de polymérase a été utilisée à fin d'établir les relations entre les différentes souches.*

Methodes: *Après une clairance éthique, 52 souches de l'organisme ont été isoles des plaies, urines et crachats des malades par des méthodes et techniques standards usuels microbiennes. L'ADN des isolats étaient amplifiée avec les amorce AF1 (5'-AGA GTT TGA TCC TGG CTC A – 3') et 1541R (5'-AAG GAG GTG ATC CAG CC-3').*

Résultat : *Au moins deux bandes ont été observe dans les isolats type avec les tailles des fragments variant entre 0.07 et 1.5kb. Les souches étaient génétiquement diversifies montrant parfois 2 à 6 bandes.*

Conclusion: *l'Etude démontre que la diversitée peut être établir entre les souches en circulation à Buea faisant de cet étude une grande importance epidemiologiques et clinique. If faut se rappeler que cette pathogène est implique dans les infections nosocomiales amenant à des implications sociales et faire appel à une diagnostique et gestion sains dans l'environnement à Buea, au Cameroun.* ***WAJM 2007; 26(3): 191 – 195.***

Mots Cles: Pseudomonas aeruginosa, infections nosocomiales, implication social, ADN, PCR.

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Abbreviations:AIDS, Acquired Immunodeficiency Syndrome; DNA, deoxyribonulceni acid; PCR, Polymerase chain reaction.

INTRODUCTION

P. aeruginosa is a gram-negative bacterium widely distributed in nature with a propensity to cause opportunistic infections in humans.[1] The organism is an important nosocomial pathogen of hospitalised patients frequently associated with infections such as urinary and respiratory tract infections, surgical and wound infections, nosocomial pneumonia, bacteraemia, endocarditis and a variety of systemic diseases in patients with severe burns, cancer and AIDS who are immunosuppressed.[2,3] Colonised patients can be an important reservoir and the organism is maintained in the hospital environment by person-to-person transmission[4].

With the ever-increasing rate of nosocomial infection implicating *P. aeruginosa* and other organisms, there is need for a rapid and more discriminatory method of diagnosis.[5] There exist conventional systems to characterise strains of the organism, but these do not give satisfactory results as they rely on characteristics that are not stably expressed[6]. However, highly discriminative DNA-based methods have been used to genotype the organism. These include restriction endonuclease analysis, pulsed field gel electrophoresis and ribotyping.[7] However, the use of these methods is restricted to specialised centers due to their technical complexity and labour-intensive nature[8] constituting a hindrance for effective biotechnology transfer to Africa where infrastructural development is a limiting factor.

An alternative and simpler method readily available in our laboratory, the Polymerase Chain Reaction (PCR) has been used successfully for the typing of *P. aeruginosa* isolates.[9] In a similar study using PCR analysis, the genomic DNA was amplified using single oligonucleotide sequences and non-stringent amplification products with different sizes providing characteristic electrophoretic profiles.[10]

In the present study, in which we hypothesized strain unrelatedness between clinical and environmental isolates, we were able to report for the first time in the environment of Buea Cameroon, that genetic diversity exists between strains of the same *P. aeruginosa* species. This is important epidemiologically because a wide spectrum of *Pseudomonas* related infections could become common in our environment. Taking in to consideration the nosocomail nature of this pathogen, a situation which results in substantial morbidity and mortality leading to loss of productivity and invalidity, the social implications of this study is very important as the entire family of a patient(s) is bound to suffer in one way or the other. This becomes much more serious in the developing world, especially in Africa where affected families are ruined as little or no social care is provided.

SUBJECTS AND METHODS

Bacterial Isolates

A total of fifty-two strains of *P. aeruginosa* used in this study were isolated from health institutions in the Buea health district. Buea (4°9'N, 9°13'E) is a semi-rural town, and the provincial capital of the South West Province of Cameroon. The isolates were from 16 urine samples, 14 wound swabs and seven sputum samples[7] from infected patients while, 15 environmental isolates were recovered from hospital beds, tables, sinks and theatre equipment. The aim of the study was explained to the patients and their consent to participate in the study solicited. Ethical clearance to undertake the study was obtained from the local hospitals management board and the Provincial Delegation of Public Health for the South West Province. The bacteria were isolated and identified according to standard methods: gram stain, oxidase production, motility testing, pigment production and the API20E system (BioMérieux, France).[11, 12, 13]

DNA Extraction and Purification

Bacterial cells were cultured at 37°C for 24 hours on nutrient agar plates and suspended in 200μL of sterile distilled water. Each suspension was vortexed for 30 seconds, boiled for 10 minutes, centrifuged for 5 minutes at 14,000 rpm and supernatant collected.[14] The supernatant containing the crude DNA extract was mixed with 20μL of 3M sodium acetate and 500μL of ice-cold ethanol. After 24 hours of incubation at -20°C, tubes were centrifuged, washed and dried. They were finally resuspended in 30μL of TE (Tris-EDTA) and stored at –20°C until use.[15]

Polymerase Chain Reaction (PCR)

Following optimisation, fifty-two strains of *P. aeruginosa* were amplified using primers AF1 (5'-AGA GTT TGA TCC TGG CTCA-3') and 1541R (5'-AAG GAG GTG ATC CAG CC-3') (Cruachem Glasgow, UK).

Amplifications were performed in 25μL reaction volumes containing nuclease-free water (14.78μL), 10X PCR buffer (100mM Tris Hcl pH 9; 500mM Kcl), 2.5mM $MgCl_2$, 5 units of AmpliTaq *Taq* polymerase, 200μM dNTP mix, 25pmol/L primers and 3μL template DNA. Thermal cycling proceeded under the following conditions: Initial denaturation at 95°C for 5 minutes, followed by 30 cycles of denaturation at 95°C for 1 minute, annealing at 57.5°C for 1 minute 30 seconds and extension at 72°C for 10 minutes (Applied Biosystems, GeneAmp PCR Systems 2700). All experiments included a negative control where the template DNA was substituted with sterile water. The amplified DNA products were separated by electrophoresis in a 2% agarose gel in 1X TBE buffer and detected by staining with 0.5μg/L ethidium bromide (Biometra T13, Germany). A 100bp DNA ladder (Promega, UK) was used as molecular weight marker.

RESULTS

Conventional Bacteriological Tests

After 24 hours of incubation, colonies appeared flat, round, mucoid with aerose margin and had a fried-egg shape. Microscopic examination revealed single, red, rod-like organisms' hence gram-negative rods. Further observation with oxidase test strip gave blue coloration after 10 seconds hence oxidase positive. Isolates were therefore found to be oxidase positive, gram negative, non-fermenters of carbohydrates and confirmed as *P. aeruginosa* by the API20E test (Biomerieux, France).

Table 1. Band distribution among isolates typed.

Origin	Number of isolates	Number of bands*				
		I	II	III	IV	V
Clinical samples	26	9	12	3	1	1
Environmental samples	6		3		3	

*Number of bands seen in the respective number of isolates typed.

Table 2: Band distribution and sizes in the environmental isolates typed.

Sample number*	Distance of migration from well (cm)	Band sizes (bp)
22E	0.2	1430
	4.3	100
16E	0.2	1430
	4.3	100
6E	1.2	740
	1.5	750
	4.3	100
29E	4.3	100
	5.4	70
36E	1.2	740
	1.5	750
	4.3	100
37E	1.8	500
	2.9	250
	4.3	100

*E, Environmental isolates

Typeability and Reproducibility

An initial screening with 2 primer sets was done to assess primer efficiency. As primers AF1 (5'-AGA GTT TGA TCC TGG CTCA-3') and 1541R (5'-AAG GAG GTG ATC CAG CC-3') gave clear and reproducible bands, they were selected for the study while primers PS1 (5'-GTT CGCCC-3') and PSII (5'AAGAGCCCGT-3'), which gave erratic results, were rejected. Fifty-two strains were subjected to PCR. No product was detected in distilled water, which served as the negative control.

PCR Profiles

The strain profiles presented between two and six fragments per lane with variable sizes and intensities (Figures 1 and 2). Figure 1 shows PCR profiles for environmental isolates typed while Figure 2 shows patterns for the clinical isolates. The number of bands obtained per strain typed is shown on Table 1 with fifteen of them having two bands.

Several DNA segments were amplified in each sample, and polymorphisms were apparent for primers AF1 and 1541R (e.g. the 0.1 Kb band seen in all the environmental samples but absent in the clinical isolates). Band sizes ranged from as small as 0.07kb to as large as 1.5 kb.

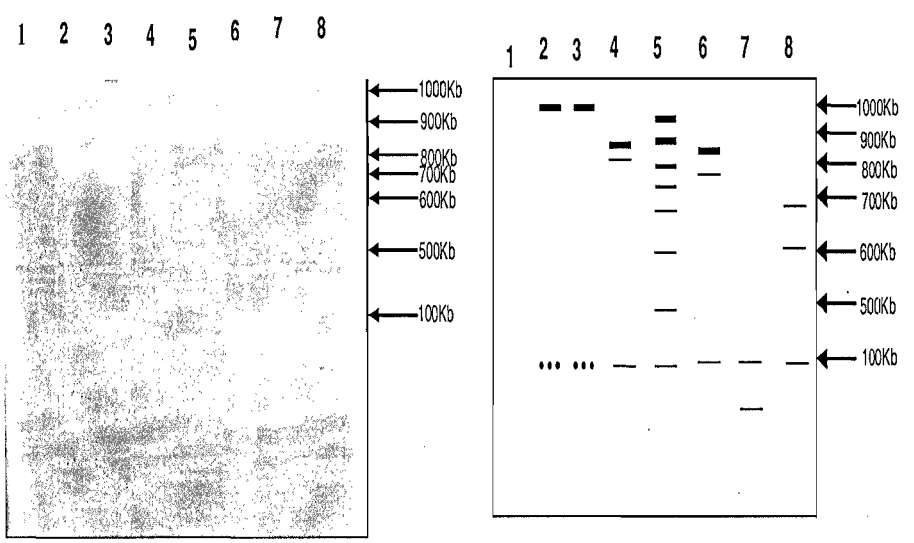

Figure 1. Left panel: Agarose gels showing amplification of genomic DNA of *P. aeruginosa* from environmental isolates. 7μL of amplified products were electrophoresed on 2 % agarose gel in 1X TBE buffer. The gel was stained in 0.5μg/mL ethidium bromide solution and photographed. Lane 1, negative control. Lanes 2-4, Environmental samples. Lane

Table 3: Band distribution and sizes of clinical isolates typed.

Sample numbers	Distance of migration from well (cm)	Band sizes (bp)
18W	3.1	220
36W	1.9	470
	2.7	280
37W	1.9	470
	2.7	280
50W	1.8	500
	2.5	330
	3.0	230
2U	3.0	230
3U	2.7	280
5U	2.9	260
25W	1.9	470
	2.7	280
35W	1.9	470
	2.7	280
41U	2.9	260
	3.1	220
	3.2	200
47U	2.9	260
	3.1	220
	3.2	200
16U	1.9	470
	2.7	280
25U	3.0	230
26U	2.9	260
	3.1	220
34S	1.9	470
	3.0	230
42S	1.9	470
46S	2.0	440
47S	2.1	470
5S	1.9	470
	3.0	230
12W	2.0	440
	2.5	330
11S	0.1	1500
	0.3	1300
	1.1	780
	1.5	600
	2.1	470
	2.6	300
34S	1.0	890
	1.1	780
	1.2	750
	1.9	470
	2.8	260
35S	1.2	750
	1.9	470
10W	1.9	470
	2.8	260
11W	1.9	470
49S	2.9	260
	3.1	220

*S, Sputum; U, Urine,; W, wound swab.

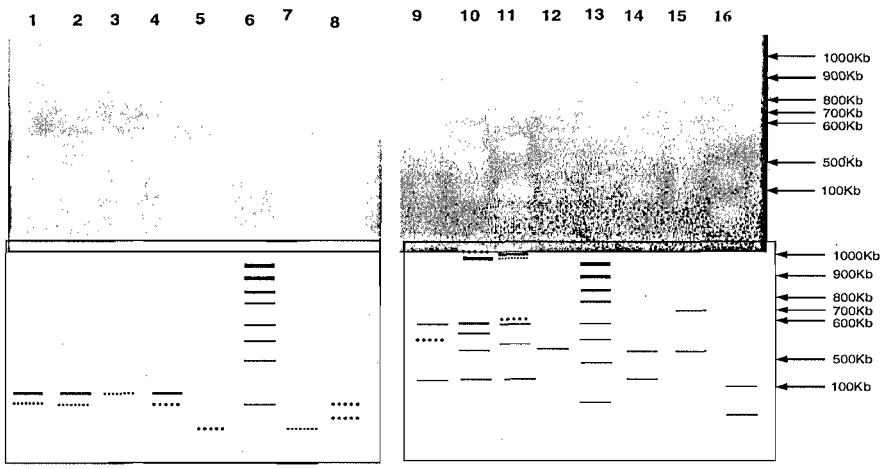

Figure 2: Upper panel: agarose gel showing amplification of genomic DNA of *P. aeruginosa* from some clinical isolates using primers AF1 and 1541R. 7μL of amplified products were electrophoresed on 2% agarose gel in 1X TBE buffer. The gel was stained in 0.5μg/ mL ethidium bromide and photographed. In each run, a 100 bp DNA ladder was used to determine band sizes. A negative control was used to show that the bands seen were not artefacts. Lanes 1and 2, Wound swabs. Lanes 3-5, 7 and 8, Urine samples. Lanes 6 and 13, 100bp DNA ladder. Lanes 9, 14 and 15, Wound swabs. Lanes 10-12 and 16, Sputum samples. Lower panel: schematic representation of the PCR profiles of the upper panel.

The estimated size of each band obtained is shown on Tables 2 and 3. A 0.1kb fragment was found in all environmental isolates typed, but absent in the clinical isolates indicating the genetic diversity between the clinical and environmental samples.

Strain comparison

There was a common 0.1kb fragment observed in all the environmental isolates typed (Figure 1). In addition to this, two of these isolates also had a very large and intense 1.4kb fragment. Overall therefore, the clinical and environmental isolates differed genetically since differences were observed between the two groups. This genetic disconcordance is in conformity with the stated hypothesis. The results show that primers of arbitrary sequence can be used to amplify genomic DNA segments, and that polymorphisms can be detected between the amplification products of different isolates. Within the clinical and environmental isolates, there exists no concordance.

DISCUSSION

Typing to evaluate the existence of genetically different strains is commonly advised in epidemiological investigations, and the use of very high discriminatory typing methods is recommended for differentiating between bacterial species. [16] Complete knowledge of an organism's genetic make up allows for exhaustive identification of virulence genes, vaccine and antimicrobial targets.[17] Compliations due to *P. aeruginosa* are usually associated with immunocompromised states and with the opportunistic nature of the pathogen; rapid diagnosis of an infection in a reliable way becomes necessary. It is widely accepted that traditional phenotypic methods for typing *P. aeruginosa* do not permit accurate investigation of infections because phenotypic markers are relatively unstable[5].

In this study, a high degree of reproducibility was achieved with PCR and thus confers its usefulness for epidemiological investigations. The PCR fingerprinting results show a degree of genomic diversity among *P. aeruginosa* isolated from clinical and environmental samples in conformity with our hypothesis that strain diversity exists between clinical and environmental isolates of *P.aeruginosa*. The difference in banding pattern of the environmental and clinical isolates could be attributed to genetic divergence arising from point mutations, insertions and deletions.[18]

Kerr *et al*[19].used a 26-mer primer to investigate a nosocomial outbreak of *P. aeruginosa* in an intensive care unit and showed identical profiles of all isolates indicating no diversity. However, in another study by Ruimy *et al*[20], *P. aeruginosa* strains isolated from ventilated, cancer, bacteraemic patients and environmental water, showed that the organism was genetically diverse. Sawaril *et al*[21], using ribotyping for characterisation of *P. aeruginosa* from patients in an intensive care unit in Singapore, provided evidence for different ribotypes. Our study therefore corroborates previous findings.

The results show that single oligonucleotide primers can be used to amplify genomic DNA segments and that polymorphisms can be detected between the amplification products of different isolates. The present investigation therefore demonstrates the relevance of molecular typing techniques such as PCR in the analysis of evolutionary relationship in an organism as well as providing reliable epidemiological data. The subject of our future investigations will be to design more specific primers based on resistance genes and sequences in the 6.3 mbp genome of *P. aeruginosa* since the organism accounts for a significant proportion of nosocomial infections and a high tendency to develop or acquire new antibiotic resistance traits.[22, 23]

Also observed in this study were common strain types detected in the six hospitals surveyed but the majority of the strains were associated with one hospital indicating that there could be patient- to- patient spread in this hospital. Because of the relatively small sample size in each hospital, it is difficult to draw any general conclusions about the significance of the strain type distributions among hospitals. The common strain was isolated from urine and wound samples predictive of the fact that the same strain may have been etiologic in urinary tract and wound infections. Most of the wound isolates were obtained from theatre equipment and this may explain the high prevalence of the organism in wound infections. In

conclusion therefore, the existence of different genetic types of *P. aeruginosa* observed in patients and the hospital environments merits special attention considering that this may lead to a spread of the organism in the environment with a consequent emergence of resistant strains. The far reaching social and financial implications of this can not be over emphasised.

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