Plasmid profile as fingerprinting of typing *Pseudomonas aeruginosa*

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Pyocine production typing and restriction fragment length polymorphism (RFLP) of plasmid DNA with BamH1 (BamH1 RFLP) were compared for intraspecies discrimination of 100 *Pseudomonas aeruginosa* isolates. Typeability of pyocine production method was 76% while that of BamH1 RFLP was 100%. BamH1 RFLP was highly discriminative so as to distinguish unrelated isolates of close lineage. However, it was not a good method to identify isolates of unrelated lineage because BamH1 RFLP appeared to be a subject to convergent evolution. On the other hand, conventional typing methods are based on the presence of specific bacterial surface structures. Therefore, conventional typing methods may sometimes lead to wrong classification of isolates especially when applied alone. A combination of typing methods decreased the reproducibility of the typing results. Combined use of pyocine production and BamH1 RFLP proved to be a very valuable tool for epidemiological identification of *Pseudomonas aeruginosa* isolates.

Introduction

Pseudomonas aeruginosa (*Ps. aeruginosa*) is a classic opportunist pathogen with innate resistance to many antibiotics and disinfectants. It is physiologically versatile and flourishes versatile and flourishes as a saprophyte in warm moist situations in the human environment, including sinks, drains respirators, humidifiers and disinfectant solutions. Isolation of *Ps. aeruginosa* from healthy carriers or environmental sites is only when there is a risk of transfer to compromised patients, e.g. by nurses' hands or via respirators. In patients with no clinical evidence of infection, isolation of *Ps. aeruginosa* particularly in association with other resistant organisms such as *Candida albicans*, is a consequence of selection by antibiotic therapy and of little direct clinical relevance. Infections due to Ps. aeruginosa are seldom encountered in healthy adults but in the last two decades the organism has become increasingly recognized as the etiological agent in a variety of serious infections in hospitalized patients with impaired immune defenses (Neu, 1983) including Human Immunodeficiency Virus (HIV) infections (Hickey & Shanson, 1993).

Colonization is often associated with prior instrumentation, e.g. catheterization, tracheostomy, etc. Susceptibility to infection with *Ps. aeruginosa* may also be occupational, e.g. ear infections in deep sea divers, or it may be recreational as in cases of whirlpool-associated (Jacuzzi) rash. *Ps. aeruginosa* is the most feared cause of corneal ulceration, hence great care must be taken in maintenance and cleaning of contact lenses (Dart & Seal, 1988). The organism is the major cause of malignant otitis media; otitis externa in saturation conditions necessary for deep water diving is painful and socially debilitating. Endocarditis and septicaemia caused by *Ps. aeruginosa* is relatively rare but carries a mortality rate exceeding 70% in patients compromised by severe burns, cancer or drug addiction. Arguably, the most significant pathogenic role for *Ps aeruginosa* at present is in the chronic debilitating pulmonary infections due to mucoid variants that are now the major cause of death in patients with cystic fibrosis (Govan & Nelson, 1992).

Ps. aeruginosa has high intrinsic resistance to many antibiotics at levels attainable in body tissues. Antibiotics likely to be most effective are the aminoglycosides, tobramycin and gentamicin in combination with an anti-pseudomonal penicillin such as ticarcillin, or the urieido-penicillins, azlocillin and piperacillin. Newer agents with good activity include the carbapenems, imipenem and meropenem and the monobactam aztreonam. Of the cephalosporins, ceftazidime has proved to be a useful non-toxic alternative to the aminoglycosides (Norrby *et al.*, 1993). Conventional biological typing methods for example biotyping, phage typing, serotyping and bacteriocin (Pyocin) are well established and have been applied to *Ps. aeruginosa* and a wide range of microorganisms as well (Hawkey, 1989). Plasmid profile, chromosomal analysis, restriction fragment length polymorphism (RFLP), protein fingerprinting, nucleic acid hybridization and amplification are the molecular methods applied to the diagnosis of nosocomial infections caused by *Ps.aeruginosa* (Relman *et al.*, 1990).

The present investigation aims to establish a molecular typing method for different strains of *Ps. aeruginosa* isolated from various clinical sources in Dakahlia province in Egypt, the attention was also directed towards the molecular methods of typing which include; restriction fragment length polymorphism (RFLP) of plasmid DNA of the tested strains.

Materials and methods

Materials

The following media were prepared: Nutrient broth (Oxoid) 8.0 gm/1, Nutrient agar (Oxoid) 15.0 gm/1, MacConkey's agar (Oxoid) 15.0 gm/1, Cetrimide broth and agar (Cruickshank *et al.*, 1975) Cetrimide 0.03 gm % in nutrient media (Oxoid), and Soy broth (Oxoid)(TSB) 8 gm/1.
The above mentioned media were sterilized by autoclaving at 121° C for 15 min.

Strains of *Ps. aeruginosa*

A total of 100 strains of *Ps. aeruginosa* from 5 hospitals located in Dakahleya governorate in Egypt. All patients were suffering from clinical lesions of these strains, 30 strains were isolated from urine, 28 strains from suppurative exudates (septic wounds) and 42 strains from burns.

Reagents and apparatus for plasmid DNA isolation (Sambrook et al., 1989)

Terrific broth, 1M D- glucose, Lysozyme Solution, Alkaline SDS (freshly prepared), 5 M Potassium Acetate (pH 7.5) and High Salt solution, horizontal gel electrophoresis apparatus, gel casting platform (Bio-Rad), Gel Combs and DC Power Supply (GIBCO-BRL) Model 2000/200, electrophoresis buffer (Tris-borate buffer) (TBE), agarose Gel (0.8-3%), gel loading buffer (Tracking Dye), DNA Molecular Weight Markers (Lambda Hind III digest marker & 1 Kb ladder marker GIBCO-BRL) ethidium Bromide solution (10 mg/ml), vortex (Fisher Scientific), eppendorf Geratebau Netheler & Hinz GmbH, Germany, horizontal gel electrophoresis apparatus and UV Transilluminator and Polaroid Camera (Fotodyne, Hartland, Wi, USA).

Methods

Collection, isolation and identification of *Ps. aeruginosa* strains

Clinical specimens were collected in sterile containers or by a sterile swab, according to the type of the material. All regulations concerning the collection of specimens (Blair *et al.*, 1970) have been thoroughly followed for a proper sampling technique. Two tubes of cetrimide broth were incouclated with a representative sample from each of the above mentioned collected specimens. After 24-48 hrs incubation at 37°C, subcultures were made onto nutrient agar and cetrimide agar plates. The formed colonies after 24 hrs incubation at 37°C were examined and identified. Identification of *Ps. aeruginosa* strains was carried out according to Topley and Wilson's principles of Bacteriology, Virology and Immunology and according to Sutter (1968). In this respect, the isolated strains were tested for their negative Gram stain, diffusible pigment production, gelation liquefaction, polar flagella, ready growth at 24°C on ordinary media and alkalization of litmus milk. Gluconate utilization was carried out by the method of Gaby and Free (1958), malonate utilization was performed according to Colwell (1964), oxidative utilization of glucose with acid production was done after Hugh and Liefson (1953), fluorescent pigment production was performed according to Colwell (1964). Oxidase production was tested for by the method of Gordon and Mcleod (1928).

Plasmid profile determination of *Ps. aeruginosa* isolates

Plasmid DNA was prepared using alkaline lysis method (Sambrook *et al.*, 1989), it includes; the inculation of a single bacterial colony into 2 ml of terrific broth (or containing ampicillin 100 ug/ml) in a loosely capped 15-ml tube then incubated overnight at 37°C with shaking Aliquots each of 1.5 ml of the culture were poured into a microfuge tube and centrifuged at 14,000 rpm for 30 seconds at 4°C in a microfuge. The medium was removed with a Pasteur pipette, leaving the bacterial pellets as dry as possible. The bacterial pellet was resuspended in 100 ml of ice-cooled lysozyme solution (in 50 mM glucose, 10 mM EDTA and 25 mM Tris-base) by vigorous vortexing, 20 µl of freshly prepared alkaline SDS (0.2 NaOH, 1% SDS) were added. The tube was closed tightly, and inverted rapidly 10 times to mix the contents. The tube was stored on ice for 5 min. Ice-cooled high salt solution (150 µl) was added, the tube was closed and mixed by inverting gently the capped tube 10 times without vortex, a visible precipitate should form and the tube was stored on ice for 5 min. The tube was centrifuged at 14,000 rpm for 5 min. 4°C in a microcentrifuge, the supernatant was transferred to a fresh tube. An equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) were added, mixed by vortexing, centrifuged at 14,000 rpm for 2 min. at 4°C in a microcentrifuge and the supernatant was transfered to a fresh tube. The double-stranded DNA was precipitated with 2 volumes of ethanol at room temperature, mixed and the mixture was allowed to stand for 2 min. at room temperature. The mixture was centrifuged at 14,000 rpm for 5 min. at 4°C in a microcentrifuge. The supernatant was removed by gentle aspiration. The pellet of double-stranded DNA was rinsed with 1 ml of 70% ethanol at 4°C, the supernatant was removed as described before and the pellet of nucleic acid was allowed to dry in the air for 10

min. The nucleic acids were redissolved in 50 µl of deionized water containing DNAase-free pancreatic RNAase and the plasmids were stored at -20°C.

Plasmids were subjected to electrophoresis at 80 V in horizontal gels containing 0.8% agarose with Tris-borate buffer. Lambda Hind III digest (Sigma) was used as marker. The gel was stained with ethidium bromide, exposed to UV light to visualize the fragments and photographed.

Restriction fragment length polymorphism (RFLP) analysis with Bam H1

The restriction enzyme digest was usually performed in a volume of 20 µl on 0.2-1 µg of substrate DNA, using a 2 to10 fold excess of enzyme over DNA. The following is an example of a typical RE (Restriction Enzyme) digest. 16.3 µl of sterile, deionized water, 2 µl of RE 10x buffer, 0.2 µl of acetylated BSA, 10 µg/µl, 1 µl of DNA, 1 µg/µl and 0.5 µl of restriction enzyme, 10 units/µl were mixed in a sterile tube. The final volume was 20 µl, mixed gently by pipetting, the tube was closed, centrifuged for a few seconds in a microcentrifuge and incubated at the optimum temperature for 1- 4 hrs.

Results

Collection, isolation and identification of strains of *Ps. aeruginosa*

In the present study, 100 strains of *Ps. aeruginosa* were isolated from Mansoura University Hospitals. In this respect, 30 strains with serial from N° 1 to N° 30 were isolated from urinary tracti (urine samples), 28 strains from strain N° 31 to N° 58 were isolated from septic wounds and the remaining 42 strains from strain N° 59 to N° 100 were isolated from burns. All strains were Gram-negative actively motile bacilli and show different colonies especially large and small ones; with irregular translucent edges and dark center. Most strains produced colored pigments after incubation at 37°C for 24 hrs. All strains were oxidase positive, fermented glucose with acid production, liquefied gelatin and formed slime and surface pellicle when grown in nutrient broth. All strains grow rapidly at 42°C and optimally at 37°C.

Restriction fragment length polymorphism (RFLP) of plasmid DNA of *Ps. aeruginosa*

The isolated plasmid DNAs of *Ps. aeruginosa* strains were purified and digested by different restriction endonucleases such as EcoR1, EcoRV, Hind III, Spe 1 and Bam H1. By digestion of Plasmid DNA of 78 strains by Bam H1, we obtained forteen different restriction patterns ranged from 20 kbp to 2kbp. Restriction pattern 1 (one strain) pattern 2 (twenty eight strains), pattern 3 (one strain), pattern 4 (two strains), patterns 5 (eight strains), pattern 6 (eight strains), pattern 7 (one strain), pattern 8 (two strains), pattern 9 (four strains), pattern 10 (four strains), pattern 11 (four strains), pattern 12 (six strains), pattern 13 (five strains) and pattern 14 (four strains).

Discussion

Ps. aeruginosa is an opportunistic pathogen that affects a wide range of patients with a predisposing condition. Earlier epidemiological studies were based on evaluation of one or more phenotypic characteristics (Pitt, 1988). Several typing methods have been applied to distinguish between isolates which reinfect the individual patient following antibiotic therapy (Ojeniyi *et al.*, 1993). In the present study, a total of 100 strains of *Ps. aeruginosa* were isolated from patients at Mansoura University Hospitals (Egypt). Molecular methods including plasmid profiles, and plasmid BamH1/RFLP of the isolated strains were adopted. It was also of interest to find out the differences, similarities or relationships between the conventional and molecular methods of typing. Recently, emphasis in clinical microbiology is on rapid diagnostic techniques that will reduce the time required to identify the presence of pathogenic microorganisms in clinical. Rapid techniques can be divided into three major categories; microscopic methods, immunologic methods and molecular methods. Whereas each has a role to play in the laboratory diagnosis of infectious diseases, nucleic acid-based methods have the greatest potential for increasing the sensitivity of detecting infectious agents directly in clinical samples while maintaining high specificity. Molecular techniques also have the broadest diagnostic applications, including the ability to detect and identify infectious agents that cannot be cultured *in vitro*. Although the major strength of molecular methods is for organism identification, molecular techniques can also be adapted for strain typing and for decoction of antimicrobial resistance genes to guide therapy early in the course of disease. Although many of the newer nucleic acid amplification methods appear complex, they are based on simple DNA-to-DNA or DNA-to-RNA hybridization reactions. The basic principles of nucleic acid hybridization and amplification are the molecular methods applied to the diagnosis of infectious diseases. These methods showed low ability, reproducibility and/or discriminatory power (Ogle and Vasil, 1993). To overcome these difficulties, methods based on chromosomal DNA polymorphisms have been developed. Genetic markers on chromosomal DNA are more stable and typing results do not depend on variable henotypic expression .

The problems associated with many of the phenotypic techniques have stimulated interest in DNA-based typing methods. The techniques of molecular biology were first applied to the epidemiologic study of bacterial infections to trace the spread of gram-negative bacilli causing nosocomial outbreaks of sepsis and urinary tract

infection (Schaberg *et al.*, 1981). Recently, molecular genetic methods, including plasmid profile, RFLP, ribotyping, and repetitive element sequence-based polymerase chain resection, have been useful in validating endemic diseases (Robert & Macelis, 1991).

Plasmid profiles were relatively simple and reliable typing technique which doesn't require any sophisticated equipements. The potential usefulness of plasmid profiles to bacterial epidemiology depends upon the degree to which three requirements are satisfied, the bacterial strains under study should contain plasmids, plasmids must be sufficiently diverse so that independent isolates, not recently derived from a common progenitor, are likely to carry distinctly different plasmid DNA and the methods for plasmid profiling must be rapid, reproducible, inexpensive and available in clinical laboratories. Plasmid analysis was done using gel electrophoresis to obtain profiles of plasmid sizes (Mayer, 1988). Most strains of *Ps. aeruginosa* contained plasmid (96% of the tested strains) indicating that this method will be useful for typing strains of *Ps. aeruginosa*. Plasmid analysis cannot, of course, be used for typing purposes if the strains being examined lack plasmids. It is worth noting at the outset that it is not always true that identical plasmid profiles are indicative of an epidemiological relationship, since it is quite possible that evolutionary-related organisms will have acquired a similar basic collection of plasmids. Problems of interpretation may arise if different molecular forms of a single plasmid are visualized on the same gel, thereby giving three bands where only one plasmid is present. In addition, problems may arise when using plasmid analysis for typing purposes, particularly if preparations contain large plasmids, because agarose gel electrophoresis is not a sensitive method of detecting small differences between plasmid of similar size. Similarly, simple analysis of plasmid profiles will not detect the difference between two dissimilar plasmids of identical size. Such problems can be overcome by using restriction endonucleases to generate "plasmid fingerprints". Cutting plasmid DNA with restriction endonucleases adds only about 1 hr to the procedure and provides much additional information. Plasmid DNA molecules can, therefore, be compared by examining the number and size of fragments generated by digestion of the DNA with restriction endonucleases. The pattern of fragments produced is termed the plasmid fingerprint, while variations observed between related molecules are termed restriction fragment length polymorphisms (RFLPs). Determination of DNA fragment sizes is not required when plasmid fingerprints are compared side-by-side, but it is difficult to arrange this when large numbers of strains are being examined. In addition, if the precise sizes of the DNA fragments can be recorded, it is then possible to compare fingerprints obtained across time from many different laboratories. Unfortunately, most published data have been obtained from comparisons of migration distances on agarose gels with only a few fragment size standards (Grundmann *et al.*, 1995).

Unlike the conventional methods of typing, *Ps. aeruginosa* strains were found to be typeable using molecular typing methods. In this study (Figure 1 and 2), the value of RFLP analysis of plasmid DNA for differentiation *Ps. aeruginosa* strains was investigated. Digestion with restriction enzyme BamHI produced fourteen RFLPs band patterns which result in successful differentiation of tested strains of *Ps. aeruginosa*. The RFLP analysis is the typing method of choice for strains from patients with *Ps. aeruginosa* infections, however, this method is technically complex and currently can be done at only a few research laboratories. Eighteen strains out of the 24 non typable strains (by pyocin typing) were successfully typed by BamH1/RFLP as follows, digestion pattern 2 (8 strains), digestion pattern 12 (4 strains), digestion pattern 6 (2 strains), and digestion patterns 5, 9, 11 and 14 (on strains for each).

In conclusion, BamH1/RFLP analysis was a valuable tool not only for characterizing Ps. aeruginosa isolate colonality but also to evaluate the degree of genetic relatedness between lineages. This approach may present a general interest because it is very highly discriminative, it does not require sophisticated equipment and can be applied to many other bacterial species. This study may contribute to a better understanding of which extend in conclusions regarding the epidemiology of a particular setting, deduced from application of each one of the molecular methods tested, may be influenced by intrinsic limitations of each technique.

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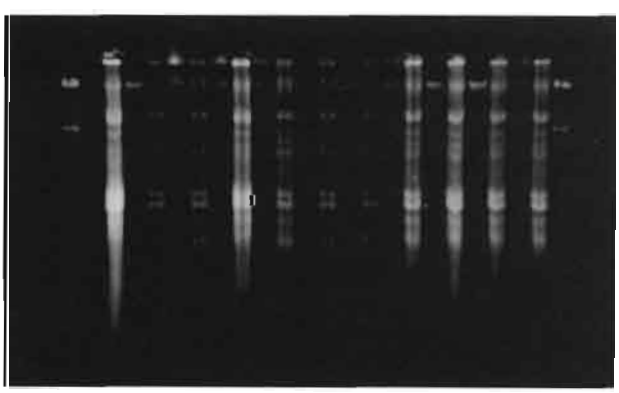

Figure 1. Agarose gel electrophoresis of BamH1 RFLP profile of plasmid DNA of *Ps. aeruginosa* isolates. Lanes 1 and 18, Lambda Hind III digest marker; Lanes 2, 4,6,8,10,12,14 and 16 are plasmid of strains No 64, 65, 66, 68, 69, 81, 82 and 84 respectivly, lanes 3, 5,7,9,11,13,15 and 17 are the corresponding Bam H1 RFLP profiles

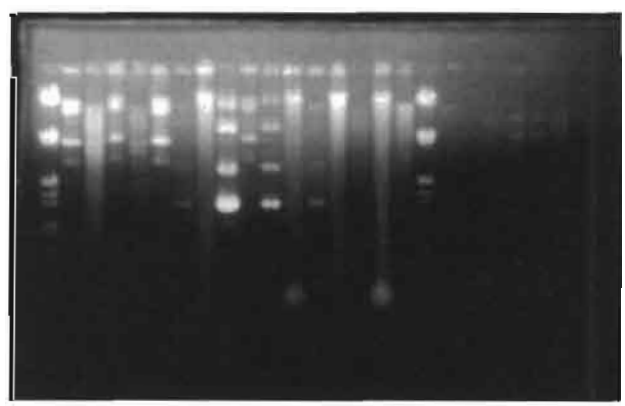

Figure 2. Agarose gel electrophoresis of BamH1 RFLP profile of plasmid DNA of Ps. aeruginosa isolates. Lanes 1 and 22, Lambda Hind III digest marker; Lanes 2,4,6,8,10,12,14,16,18 and 20 are plasmids of strains No. 2, 5, 7, 16, 17, 33, 38, 73, 77 and 83 respectively, lanes 3, 5, 7, 9, 11, 13, 15, 17, 19 and 21 are the corresponding Bam H1 RFLP profiles.