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APPLICATION OF PHENOTYPIC AND GENOTYPIC STRATEGIES TO IDENTIFY PSEUDOMONAS AERUGINOSA

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Abstract:-
A case study of patient presented with developed wound infection with Pseudomonas aeruginosa (leukocytosis). The strains of P. aeruginosa isolated from Sheffield General Northern Hospital (UK) and studied for phenotypic and genotypic identifications. This present study were conducted in Sheffield Hallam University (UK) and was aimed to evaluate the application of Phenotypic and Genotypic techniques to identify P. aeruginosa. Generally, the results of Real time PCR (RTPCR) revealed that the diagnosis viability was confirmed for P. aeruginosa. However, identification on the basis of phenotype by differences in types of growth on cetrimide and acetamide agar medium presents that both agars were conducive to the growth of P. aeruginosa. The strains of P. aeruginosa were also tested for antibiotic susceptibility to six different antibiotics, imipenem showed the greatest inhibition effect on the bacteria. It was observed that genetic techniques in accordance with phenotypic tests have facilitated to conduct a comprehensive characterization of P. aeruginosa strains obtained from a particular environment at a particular time.

Keywords: - *Pseudomonas aeruginosa, phenotype, genotype, RT-PCR, antibiotic resistance.*

INTRODUCTION

The discovery of *Pseudomonas aeruginosa* is attributed to the French chemist and bacteriologist Carle Gessard, whose experimental work in 1882 led to the isolation of this bacterium based on the fact that exposure to ultra-violet light colouredits watersoluble pigments blue-green (1).Gessard documented this work in his article entitled "On the Blue and Green Colouration that Appears on Bandages." Based on the empirical results, he not only gave the bacterium its name, but also identified its pigment derivative and formulated assumptions regarding its pathogenic quality and how this compared to that of other similar bacteria(2). A highly opportunistic pathogen, the Gram-negative bacterium *P. aeruginosa* causes infections that may require hospital admission or may result in death. The infectiousness of this bacterium is worrying enough, but its behavior is even more (3). The bacterium has low susceptibility to the action of a wide range of antibacterial agents and frequently the only outcome of exposure to such agents is augmentation of bacterial resistance. Consequently, *P. aeruginosa* can proliferate even as it is actively treated. The high rate of endurance of this bacterium has prompted several measures to be taken, including close monitoring in clinical institutions and continuous updating of its genome in a global database to keep track of any possible attempts to use the bacterium as a biological weapon (2).

Pseudomonas aeruginosa Having the shape of a rod with dimensions of 0.5-0.8μm by 1.5-3.0 μm, *P. aeruginosa* is usually contracted in a hospital setting and can induce both acute and chronic infections which are difficult to treat as the bacterium is resistant to numerous antibiotics (4). Individuals with burn injuries, cystic fibrosis, acute leukaemia, organ transplants, and intravenous-drug addiction are particularly susceptible to infection with this bacterium. Causing nosocomial infection, *P. aeruginosa* has been found to thrive on a broad range of items and surfaces in a hospital environment (5). Therefore, the risk of patient infection increases the longer the stay in hospital .the most severe infections caused by *P. aeruginosa* aremalignant external otitis, endophthalmitis, endocarditis, meningitis, pneumonia, and septicaemia. The chances of patients recovering from such infection depend on how severe their underpinning disease conditions are (6).*P. aeruginosa* can be discriminated into subdivisions byroutinely methodsincluding: serotyping, biotyping, pyocintyping, phage typing and susceptibility to antibiotic of tested strains. Nevertheless, these methods of discrimination are much lower than that obtained by molecular typing methods. DNA typing methods have been mainly used to examine the variety of collections of *P. aeruginosa* (7).

MATERIALS AND METHODS

Bacterial strains

Astrain of *P. aeruginosa*, was originally isolated from a clinical specimens thick puscontaining discharge from the wound has a distinctive odor and is the result of tissue decay that is associated with *P. aeruginosa* infection. The strain was identified as *P. aeruginosa* on the basis of typical morphology by gram-negative staining, a positive oxidase reaction, growth at 42^{o}C and conventional biochemical tests using the API 20NE system (Bio-Mérieux, France). Also *P. aeruginosa* was identified by PCR amplification of 16 S ribosomal RNA (8).

Genetic analysis

RT-PCR (Polymerase chain reaction): PCR was performed in order to investigate the expression of genes in current bacteria. Endotoxin gene and gyrase gene was the target gene. 3 of PCR reaction tubes were labeled as positive control, negative control and PCR product. Reaction mixture was prepared by adding 4μl of forward primer, 4μl of reverse primer, 100μl Syber green master mix and 52μl water. Then, 10 μl of sterile water was added to negative control, 10μl of control DNA was added to positive control and 10 μl of DNA relevant to our case study was added to PCR product tube and after that 40 μl of master mix was added to each PCR tube and finally placed in thermocycler for 2.5 hours. PCR amplification of 16S rRNA, PCR 16s was carried out by using primer 16S.Three of PCR reaction tubes were labeled as positive control, negative control and PCR product. Reaction mixture was prepared by adding 4μl of forward primer, 4μl of reverse primer, 100μl Taq-containing PCR master mix and 52μl water. Then, 10 μl of sterile water was added to negative control, 10μl of control DNA was added to positive control and 10 μl of DNA relevant to our case study was added to PCR product tube and after that 40 μl of master mix was added to each PCR tube and finally placed in thermocycler for 2.5 hours. The result was detected by using agarose gel electrophoresis.

Phenotypic study

Pyocin and fluorescein production was tested on both selective Cetrimide and acetamide Agars (Merc, Germany). Susceptibility to antibacterial drugs was studied by the disk diffusion method according to CLSI (Clinical and Laboratory Standards Institute) for 6 following antimicrobial agents (Bio- Mérieux, France): Ampicillin (AP, 10μg), colistin (COL, 25 μg), ceftazidime (CAZ, 30 μg), imipenem (IMP, 10 μg), gentamicin (GM, 30μg) and ciprofloxacin (CIP, 5 μg) (9).

RESULTS

In the context of the Gram stain procedure, the bacteria was established to be *P. aeruginosa* as they were revealed to be Gram negative and were rod-shaped and pink in colour under the microscope. Meanwhile, the diagnosis viability was confirmed by the culture result. It was shown that both cetrimide and acetamide agars were conducive to the growth of *P. aeruginosa*. The pigments of *P. aeruginosa* which display a blue-green and yellow-green colour, respectively. In addition, the yellow-green culture was circular mucoid in shape and emanated a fruity smell, which is characteristic of *P. aeruginosa*. The positive result of the oxidase test indicated by the fact that the filter paper turned purple in colour. The *P. aeruginosa* species and genus were determined on the basis of the API sequence number (Fig. 1).

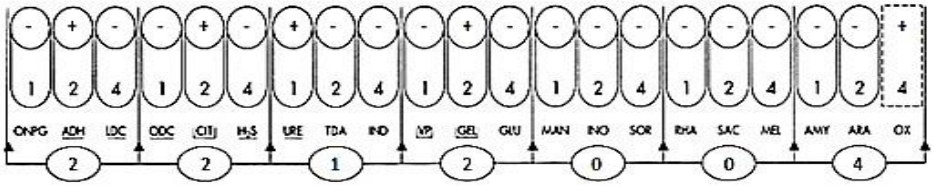

Figure 1. The *P. aeruginosa* species and genus were determined on the basis of the API sequence number (standard results)

The strain of *P. aeruginosa* isolate showed much differentiated resistance to antimicrobial agents tested. Different resistance patterns in various arrangements were observed from sensitivity to all tested antibiotics, through resistance to only two or three antibiotics, to multidrug resistance for almost all tested drugs: *P. aeruginosa* susceptibility and resistance to the antibiotics gentamicin 30µg (21 mmradius), ceftazidime 30µg (19mm radius), colistin25µg (11mm radius), ciprofloxacin5µg (20 mmradius), imipenem10µg (25 mm radius) and ampicillin10µg (0mm radius). The results of the RT-PCR, alongside the peaks for gyrase gene and endotoxin gene displayed by the positive and negative controls as well as by the case study (Fig. 2). The 16s rRNA band in negative and positive controls as well as in the case sample (Fig.3).The blast result revealed that the strains were *P. aeruginosa* (Fig. 4).

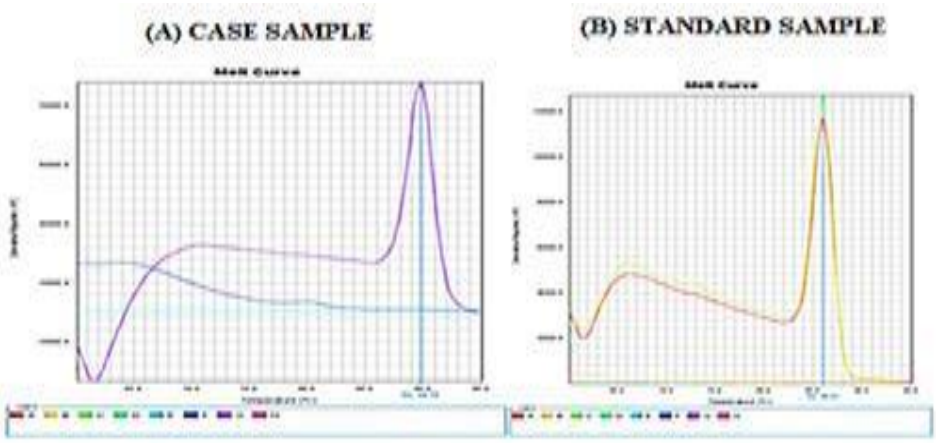

Figure 2. The results of the RT-PCR, alongside the peaks for gyrase gene and endotoxin gene displayed by the positive and negative controls as well as by the case study.

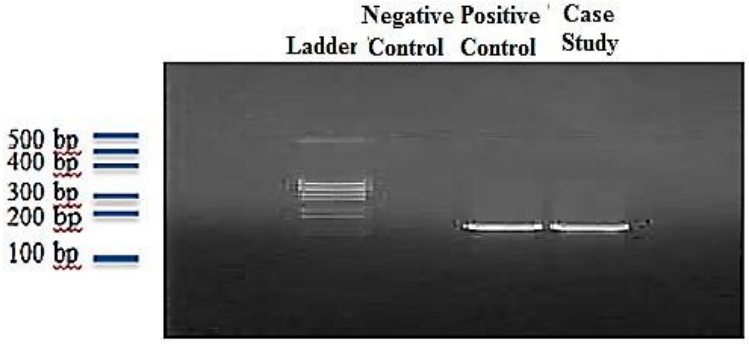

Figure 3. The 16s rRNA band in negative and positive controls as well as in the case sample.

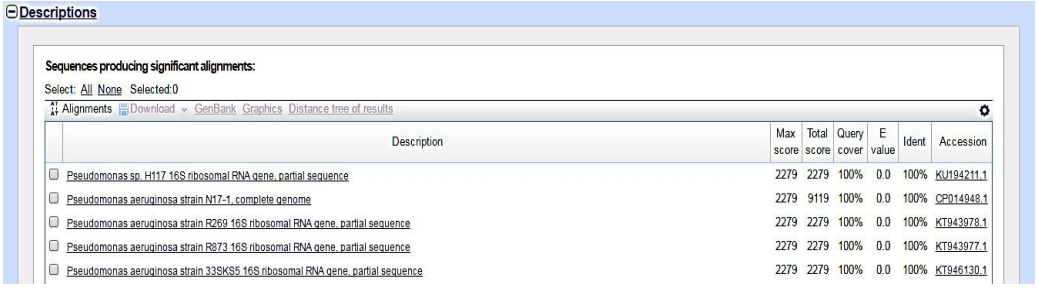

Figure 4. The blast result revealed that the strains were *P. aeruginosa* in a proportion of 100%

DISCUSSION

Infections acquired in a hospital setting, particularly wound infections, are overwhelmingly caused by *P. aeruginosa*. In the context of the Gram stain procedure, the bacteria were established to be *P. aeruginosa* as they were revealed to be

Gram negative and were rod-shaped and pink in colour under the microscope. Meanwhile, the diagnosis viability was confirmed by the culture result. It was shown that both cetrimide and acetamide agars were conducive to the growth of *P. aeruginosa*. The reason why these two types of agar were employed was that not only do they possess selectivity and therefore make it possible to isolate Gram-negative bacteria, but they also incorporate a selective agent targeting microbial flora and intensify pyocin and fluorescein, the pigments of *P. aeruginosa* which display a blue-green and yellow-green colour, respectively. In addition, the yellow-green culture was circular mucoid in shape and emanated a fruity smell, which is characteristic of *P. aeruginosa* (10).The tests related to antibiotic susceptibility produced different results. All of the six types of antibiotics that were employed had an effect on *P. aeruginosa*, but they were each associated with a distinct inhibition area. Thus, the largest inhibition area (25 mm) pertained to imipenem, meaning that this antibiotic had the greatest effect on the bacteria; meanwhile, gentamicin, ceftazidime and ciprofloxacin had an intermediary effect, with inhibition areas of 21 mm, 19 mm and 20 mm, respectively. Colistin had the smallest inhibition area (11 mm), meaning that it was least effective against *P. aeruginosa*. By contrast, no inhibition area was noted for ampicillin, indicating bacterial resistance to this antibiotic. The ability of the bacteria to secrete an enzyme associated with the bacterial electron transport chain, cytochrome C oxidase, was confirmed by the positive result of the oxidase test indicated by the fact that the filter paper turned purple in colour. The *P. aeruginosa* species and genus were determined based on the API reaction which distinguished enterobactericaea from other Gram-negative bacteria (11). The bacteria were certified to be *P. aeruginosa* based on the 100% proportion obtained from the blast gene sequencing.The results of the RT-PCR had a high level of reliability. As anticipated, there was a difference between the negative control peak, and the positive control and case sample (A) peaks, on the other hand, in the context of amplification of gene coding for endotoxin. By contrast, the peak of the negative sample was the same with that of the positive control and the standard sample (B) in the context of gyrase gene amplification. One reason for this result might have been the contamination of the DNA genome (12). As regards the 16s rRNA amplification and sequencing, only the positive control and the case sample exhibited a band within the range 200-300 bp of molecular weight, whereas the negative control did not exhibit any band. Since the positive control and the case study displayed identical band and molecular weight, the diagnosis viability was confirmed for *P. aeruginosa* (13). Compared to phenotypic methods, PCR-based genotypic methods are more advantageous as they are characterised by time-effectiveness, ease of use, and straightforward interpretation, while the equipment necessary to conduct them is accessible in an increasing number of laboratories (14). The sensitivity, specificity and depth of the quantitative real-time PCR (qPCR) method exceed those of the culture method and therefore, qPCR is less time-consuming and can detect *P. aeruginosa* more accurately. On the downside, qPCR is expensive and can be undertaken only by suitably qualified and experienced individuals (15). For the purposes of *P. aeruginosa* identification and differentiation between bacteria of interest and other bacteria of similar genus or species, the PCR amplification form of 16s rRNA was employed. Conversely, identification on the basis of phenotype presents greater difficulties, takes longer to perform and has reduced accuracy (16). Moreover, excessive growth on the culture plate might lead to misinterpretation of the results of phenotypic analysis. On the other hand, if RT-PCR and genotypic techniques are unavailable or too costly, phenotypic methods (e.g. culture and API techniques) may be more convenient to use for identification purposes. Infections caused by Pseudomonas require antibiotic treatment. In the case of patients suffering from neutropenia, bacteraemia, sepsis, severe upper respiratory infections (URIs), or abscesses, Pseudomonas infections are initially treated with a mixture of two antibiotics, such as beta-lactam antibiotic and aminoglycoside (17).Several factors determine which antibiotic is most suitable, including infection site and severity, and level of bacterial resistance. There is growing alarm that existing antibiotics have an unsuccessful effect against an increasing number of Pseudomonas strains. Based on the results obtained in the present study, *P. aeruginosa* was most susceptible to the action of imipenem, which targets and suppresses cell wall synthesis in both Gram-negative and Gram-positive bacteria. Furthermore, β-lactamase (both penicillinase and cephalosporinase) secreted by certain bacteria does not affect the stability of this antibiotic which, unlike other β-lactam antibiotics, can strongly suppress β-lactamases produced by some Gram-negative bacteria (18).In conclusion, amongst all used procedures in this study RT-PCR turned out to be a powerful tool for the study of clinical *P.aeruginosa* isolates diversity. Nevertheless, the suggestion that maximum discrimination can be best achieved by a combination of phenotypic and genotypic methods.

REFERENCES

[1].BOTZENHART, Konrad and DÖRING, Gerd (1993). Ecology and epidemiology of Pseudomonas aeruginosa. In: Pseudomonas aeruginosa as an Opportunistic Pathogen. Springer, 1-18.

[2].PIRNAY, Jean-Paul, et al. (2009). Pseudomonas aeruginosa population structure revisited. PLoS one, 4 (11), e7740.

[3].TILLOTSON, JAMES R. and LERNER, A. MARTIN (1968). Characteristics of nonbacteremic Pseudomonas pneumonia. Annals of internal medicine, 68 (2), 295-307.

[4].MOORE, Nicholas M. and FLAWS, Maribeth L. (2011). Introduction: Pseudomonas aeruginosa. Clinical laboratory science, 24 (1), 41.

[5].KANG, Cheol-In, et al. (2003). Pseudomonas aeruginosa bacteremia: risk factors for mortality and influence of delayed receipt of effective antimicrobial therapy on clinical outcome. Clinical infectious diseases, 37 (6), 745-751.

[6].MURPHY, Timothy F., et al. (2008). Pseudomonas aeruginosa in chronic obstructive pulmonary disease. American journal of respiratory and critical care medicine, 177 (8), 853-860.

[7].Speert, D.P. (2002). Molecular epidemiology of Pseudomonas aeruginosa. Front. Biosci. 1 (7), e354-361.

[8].Kingsford, N.M.; Raadsma, H.W. (1995). Detection of Pseudomonas aeruginosa from ovine fleece washings by PCR amplication of 16S ribosomal RNA. Vet. Microbiol. 47, 61-70.

[9].Clinical and Laboratory Standards Institute. Performance standards for antimicrobial testing (2006). 16th informational supplement M100-S16. Wayne, Pa: CLSI.
[10]. HOIBY, Niels, et al. (1977). Pseudomonas aeruginosa infection in cystic fibrosis. Diagnostic and prognostic significance of Pseudomonas aeruginosaprecipitins determined by means of crossed immunoelectrophoresis. Scandinavian journal of respiratory diseases, 58 (2), 65-79.
[11]. WELLINGHAUSEN, N., et al. (2005). Superiority of molecular techniques for identification of gram-negative, oxidase-positive rods, including morphologically nontypical Pseudomonas aeruginosa, from patients with cystic fibrosis. Journal of clinical microbiology, 43 (8), 4070-4075.
[12]. ANUJ, Snehal N., et al. (2009). Identification of Pseudomonas aeruginosa by a duplex real-time polymerase chain reaction assay targeting the ecfX and the gyrB genes. Diagnostic microbiology and infectious disease, 63 (2), 127-131.
[13]. YOKOYAMA, Keiko, et al. (2003). Acquisition of 16S rRNAmethylase gene in Pseudomonas aeruginosa. The lancet, 362 (9399), 1888-1893.
[14]. QIN, X., et al. (2003). Use of real-time PCR with multiple targets to identify Pseudomonas aeruginosa and other nonfermenting gram-negative bacilli from patients with cystic fibrosis. Journal of clinical microbiology, 41 (9), 4312-4317.
[15]. MOTOSHIMA, Maiko, et al. (2007). Rapid and accurate detection of Pseudomonas aeruginosa by real-time polymerase chain reaction with melting curve analysis targeting gyrB gene. Diagnostic microbiology and infectious disease, 58 (1), 53-58.
[16]. MELLMANN, A., et al. (2008). Evaluation of matrix-assisted laser desorption ionization-time-of-flight mass spectrometry in comparison to 16S rRNA gene sequencing for species identification of nonfermenting bacteria. Journal of clinical microbiology, 46 (6), 1946-1954.
[17]. MICEK, S. T., et al. (2005). Pseudomonas aeruginosa bloodstream infection: importance of appropriate initial antimicrobial treatment. Antimicrobial agents and chemotherapy, 49 (4), 1306-1311.
[18]. BERTRAND, X., et al. (2001). Pseudomonas aeruginosa: antibiotic susceptibility and genotypic characterization of strains isolated in the intensive care unit. Clinical microbiology and infection, 7 (12), 706-708.