Pseudomonas aeruginosa

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8.1 Phenotyping Methods for *P. aeruginosa*

Serotyping is based on an agglutination reaction between bacterial lipopolysaccharide (LPS) and type-specific rabbit antisera. In 1983, an International Antigenic Typing System (IATS) was developed, establishing the existence of at least 17

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major heat-stable somatic antigens, and 3 new antigens have been recently added to the existing groups. This serotyping scheme characterizes most strains of P. aeruginosa possessing smooth LPS, although it shows poor discrimination against rough LPS isolates which are common in patients with cystic fibrosis.

Seventy-four isolates from 3 different hospitals and 18 reference strains were studied by Hernández et al. [1] Serotyping provided a good index of discrimination, although 11 isolates could not be serotyped. Eleven strains (12%) could not be assigned to any international serotype and were coded as autoagglutinable (one strain), non-agglutinable (three strains), or polyagglutinable (seven strains). The discrimination index of serotyping analysis referring to clinical isolates was 0.886. Overall, the predominant serotypes were O11 and O1, representing 24% and 19%, respectively, of serotypable strains. Three serotypes (O14, O15, and O17) were not found among the strains isolated from infected patients. The type strain of *P. aeruginosa* (NCTC 10332) was serotype O6. No association was observed between the strain serotype and the hospital.

Among the strains isolated in the hospital, O11 is the most common serotype. Serotyping of 88 non-repetitive clinical isolates is determined by the slide agglutination technique using specific antisera, polyvalent and monovalent. The results of serotyping showed that serotype O11 was the most common ($N = 14$, $P = 16\%$) followed by serotype O7 ($N = 11$, $P = 12.5\%$) and serotype O2 ($N = 10$, $P = 11.36\%$) [2]. In addition, During December 2013–December 2014, 229 *P. aeruginosa* were isolated from infected patients in the clinics of UCCK from a variety of clinical sites. Eighty isolates were studied as primary isolates from the group of infected patients. Serotyping resulted in the detection of eight serogroups. The most prevalent were O11 (65%) and O1 (17.5%). Other serotypes all with a prevalence of less than five were found in 17.5% of isolates: O4, O6, O9 were found in 3 (3.8%) samples each, O12 and O3 in 2 (2.57%) samples each, and O7 in 1(1.2%). No other serotypes were detected. O11 serotype was distributed in almost all clinics, but it was more common in the ICU. A greater diversity of serotypes was observed in the Pulmonology clinic where from six samples received during the study, 2 (O1), 1(O11), 1(O3), 1(O7), and 1(O9) were discovered [3].

Pyocin typing has been used extensively to trace the routes of infections within hospitals. Poor reproducibility has been of some, although lesser, concern in the case of pyocin typing. In the Gillies and Govan method of pyocin typing for *P. Aeruginosa*, a cross-streaking technique was used, and 105 main types and 25 subtypes were identified by the patterns of inhibition observed on 13 indicator strains [3]. The disadvantages of the technique included the need to remove the test strain growth before the application of the indicator strains, the 48-h period needed to obtain a result, and the inability to reliably type mucoid *P. aeruginosa*. The same 13 indicator strains which are already used internationally have been utilized in a revised technique by Janet et al. [4]. Test strains were rapidly applied to the surface of agar plates with a multiple inoculator. After incubation for 6 h and exposure to chloroform, the indicator strains were applied in agar overlays without prior removal of the test strain growth. After 18 h of incubation, the pyocin type was recognized by the inhibition of particular indicator strains. Additionally, the activity of

particulate (R and F) and nonparticulate (S) pyocins could be distinguished on the basis of inhibition zone size, which thus allowed further discrimination. The revised technique allows typing within 24 h, increases the number of identifiable types, and can be used to type mucoid strains.

One hundred and twelve clinical isolates of *P. aeruginosa* were determined by pyocin typing. Four pyocin types (1, 10, 3, and 5) dominated (respective frequencies: 56%, 15%, 12%, and 6%). The reproducibility of pyocin typing was distinctly inferior to both plasmid profiling and serotyping [5]. In addition, the typing method, which determined the pyocin activity of clinical isolates of *P. aeruginosa* on 27 indicator strains, was 43.7% reproducible, but the elimination of 9 indicator strains doubled the reproducibility and yielded more readable pyocin inhibition zones. Seventy-eight of 1084 isolates (7.2%) were untypable [6].

8.2 Restriction-Based Methods

8.2.1 Pulsed-Field Gel Electrophoresis (PFGE)

During the last decade, traditional phenotypic typing methods for epidemiologic and outbreak studies have been replaced by molecular methods. Pulsed-field gel electrophoresis (PFGE) typing is one of the most useful discriminating methods to type Pseudomonas spp. PFGE is considered as the "gold standard" typing method, due to its excellent discriminatory power and high epidemiological concordance [7]. PFGE is a technique used by scientists to generate a DNA fingerprint for a bacterial isolate. As the large DNA particles easily break, also due to their great viscosity, pipetting of them is difficult. For this reason, DNA and microrganisms are embedded in agarose gel and affected with restriction endonuclease enzymes.

Macrorestriction analysis by PFGE of DNA was performed according to USA Centers for Disease Control and Prevention's (CDC) highly standardized PFGE protocols for Gram-negative rods with some minor modifications [8]. Bacterial suspensions were prepared from individual bacterial colonies directly obtained from cultures incubated overnight on Mueller-Hinton agar. The suspensions were adjusted to a concentration of 10^9 CFU/mL, which is equal to 1:1.5 NTU in ethylenediaminetetraacetic acid (EDTA)-saline buffer (75 mmol/L NaCl and 25 mmol/L EDTA, pH 7.5). The cell suspension was mixed with an equal volume of 1% low-melting point seaKem Gold Agarose and was allowed to solidify in a 100 μL plug mold. The agarose plug was incubated for 24 h at 37 °C in 500 μL of lysis buffer (6 mmol/L Tris-HCl (pH 7.6), 0.1 mol/L EDTA, 1 mol/L NaCl, 0.5% Brij®58 (polyoxyethylene (20) cetyl ether; Sigma), 0.4% sodium deoxycholate, 0.5% sodium lauryl sarcosine, and 1 mg/mL lysozyme). Next, the lysis buffer was replaced with 500 μL of proteinase K buffer (1% sodium lauryl sarcosine, 0.5 mol/L EDTA (pH 9), and proteinase K (50 μg/mL; Sigma)), and this solution was incubated with gentle shaking at 50 °C for 20 h. The plugs were then washed four times for 30 min at 37 °C with 10 mL of Tris-EDTA buffer (10 mmol/L Tris-HCl (pH 8) and 1 mmol/L EDTA). One-third of a slice of each plug was cut and incubated for 18–20 h with

30 U of SpeI in the restriction buffer (Promega Buffer). DNA restriction fragments were separated by PFGE by using a CHEF DR III apparatus (Bio-Rad, Richmond, CA, USA) at 14 °C, 6 V/cm, for 20 h, with a time switch of 2–40 s. A Salmonella serotype Branderup strain (H9812) ladder (Bio-Rad Laboratories) restricted with XbaI was used as a universal size marker [9]. The gel was stained with ethidium bromide and visualized with the Gel-Doc system (Bio-Rad Laboratories). According to the criteria by Tenover et al. [10] isolates were considered to be genetically indistinguishable or identical if the restriction fragments had the same number of bands and the corresponding bands were with identical apparent size.

Currently, the SpeI enzyme usually produces 14–25 bands [11, 12] for each strain, and up to 37 [13, 14]. Reproducibility using the Spe I enzyme was 100%, and typeability ranged between 95 and 100% [13, 15]. In different studies, PFGE-Spe I was determined to have DIs between 0.98 and 0.998 [13, 15]. For some strains that cannot be typed, most of them are caused by DNA self-degradation. Studies have shown that this phenomenon can be eliminated by using HEPES buffer instead of Tris or adding 50 mM thiourea to the gel buffer to remove reactive Tris free radicals. An epidemiological typing study on the outbreak of *P. aeruginosa* using PFGE-Spe I showed that there are clones where the spatial and temporal distribution of isolates does not allow more than three band differences to be obtained in the PFGE files of different isolates. The correlation can therefore be suggested for transmission. Variations in the four to six bands seem to rule out direct transmission, but it is believed that clonal variations that may infect the same lineage [16, 17]. Studies have shown that DNA fingerprint variability in *P. aeruginosa* strains derived from macroscopic restriction analysis is usually the result of insertion/deletion rather than single nucleotide polymorphism (SNP). During evolution, large DNA fragments (gene islands) can be excised from the *P. aeruginosa* genome. The differences between the Spe I PFGE patterns were observed between different strains sharing the same SNP profile. This indicates that the core genome of *P. aeruginosa* is highly conserved, and the rate of change in the auxiliary DNA fragments is higher than that of the conserved core genome [18].

Between April 2011 and December 2016, samples of lung, liver, and spleen were collected from mink with this disease on 11 mink farms in 5 Chinese provinces. From these samples, 98 isolates of *P. aeruginosa* were obtained, which belonged to 5 serotypes: G ($n = 58$), I ($n = 15$), C ($n = 8$), M ($n = 5$), and B ($n = 2$); 10 isolates were not typable (10/98). More than 90% of the isolates formed biofilms, and 85% produced slime. All 98 isolates were resistant to 10 antibiotics (oxacillin, ampicillin, penicillin G, amoxicillin, ceftriaxone, cefazolin, cefaclor, tilmicosin, tildipirosin, and sulfonamide). However, almost all were susceptible to gentamicin, polymyxin B, and amikacin. Fifty-six unique genotypes by PFGE were identified. These findings have revealed genetic diversity and high antimicrobial resistance in *P. aeruginosa* isolated from mink with hemorrhagic pneumonia and will facilitate the prevention and control of this disease [19].

PFGE has been used as a molecular typing method in many studies to study multidrug resistance *P. aeruginosa* carrying multiple resistance determinants such as metal b-lactamases (MBL) or broad-spectrum b-lactamases (ESBLs) Clusters. A

molecular epidemiological examination in 2005 showed that hospitals in three different towns in northwestern Hungary were involved in an outbreak caused by multi-drug-resistant O11 VIM-4 MBL *P. aeruginosa* [20]. PFGE is performed according to the method described by Poh et al. After some modifications [21, 22], the genomic DNA insert was digested with 20 U Spe I enzyme at 37 °C for 2.5 h. Electrophoresis was performed in CHEF-DRII equipment (Bio-Rad). The DNA fingerprints were compared by Fingerprinting II Informatix software (Bio-Rad). The criticality of the PFGE genotype identified by using 1% band position tolerance and Dice coefficient was 80%. PFGE-Spe I analysis showed that isolates of the outbreak clones were obtained from three different intensive care units (ICU) in three different towns within 6 months, and showed ≥95% similarity by Dice coefficient. A VIM-4-producing *P. aeruginosa* carrier patient transferred between the two ICUs was also found to provide an epidemiological link between the two. The VIM-4 positive isolate from Huihui is an outbreak clone, indicating that the outbreak may play a depot role in the hospital environment in addition to spreading between patients.

The protocols have been established for clinical isolates, where high typeability and discriminatory power was achieved. The literature about PFGE-typing of environmental isolates is very limited and the method was applied in a very low number of isolates derived mainly from nosocomial environments, where the typeability and discriminatory power of the method could not be evaluated. The importance of typing *P. aeruginosa* isolates deriving from water and wastewater samples is crucial for public health reasons; *P. aeruginosa* in bottled water can be considered a risk to profoundly immunocompromised patients. According to European regulations (C. D. 98/83/EC), *P. aeruginosa* should be absent in potable water. Moreover, the choice of pool and spa waters for medical use is increasing. A number of recent studies emphasize the high prevalence of *P. aeruginosa* in hospital water facilities resulting in outbreaks. Epidemiological investigation to determine the source of an outbreak requires fast and reliable methods [23–25].

Studies have shown that PFGE is very effective in typing *P. aeruginosa* and has a high degree of discrimination. However, this method has the characteristics of high labor intensity, time consumption, and high cost. In addition, due to the lack of a widely accepted standard protocol for *P. aeruginosa* typing, results from different laboratories are not easy to compare [26, 27].

8.2.2 Restriction Fragment Length Polymorphism (RFLP)

This was the first methodto be widely used for typing strains of P. aeruginosa from patients with CF. Genomic DNA is extracted from the bacterium of interest, digested with one of several restriction enzymes, and the DNA fragments are then separated by electrophoresis. A radiolabelled probe, directed to a specific portion of the bacterial genome, is then added, and a hybridization reaction is carried out. The most discriminatory probes are those which react with a hypervariable portion of the bacterial genome. Generally, the genomic DNA of *P. aeruginosa* is digested with

two or three restriction enzymes. The obtained DNA digest is then separated by electrophoresis, transferred to a membrane and then hybridized with a radiolabeled probe (*exoA*). Obtain the size and number of different DNA genomic fragments, and then create fingerprints for different strains. Using Bgl II, Sal I and Xho I restriction enzymes to classify RFLP-*exoA* has a discrimination ability (DI) of 0.97, which can distinguish different strains. However, about 5% of the strains cannot achieve the purpose of typing because they do not contain *exoA*. Iisolates with different LPS serotypes and biotypes showed the same RFLP pattern, indicating that the phenotypic variation of *P. aeruginosa* is not necessarily the result of genetic heterogeneity. On the other hand, RFLP-*exoA* assigns serotypes, biotypes, and anti-bioGram indistinguishable isolates from unrelated patients as different types [26].

Although the type of *exoA* can also indicate the clonal relevance of the strain, the technique is proposed to distinguish infections caused by variants of the same clonal lineage, which persist in different geographic locations [17, 28]. At present, RFLP typing has been replaced by pulsed-field gel electrophoresis and random amplified of polymorphic DNA.

8.2.3 Ribotyping

Ribosomal RNA genes are the most conservative, and there are multiple copies on the genome. Using rRNA gene fragments as probes, the differences in the position and number of rRNA genes were detected, and the strains were typed. Generally, DNA restriction fragments are separated by gel electrophoresis, transferred to a membrane, and the conserved region of the rRNA gene is incubated with the probe. Ribotyping of *P. aeruginosa* uses Pvu II restriction endonucleases and rDNA gene probes with low discrimination. Pitt and his colleagues first studied the multi-resistant *P. aeruginosa* strain 012 from Europe using ribotyping. Their results are consistent with outer membrane protein electrophoresis, LPS analysis, and esterase typing results, indicating that the common origin of these strains implies the common origin of these strains [17, 29, 30].

In addition, when Pvu II enzyme and RiboPrinter are used together, DI values can reach 0.88 and 0.93 [31, 32]. The RiboPrinter™ microbial identification system (E.I. duPont de Nemours and Company) is an automated ribotyping system widely used in the past two decades. This method standardizes the technical and interpretive aspects of the program and also uses computer databases to compare products from a large number of isolates. Pvu II was selected for the analysis of *P. aeruginosa* isolates by automated ribotyping in a molecular surveillance study of European quinolone-resistant clinical isolates of *P. aeruginosa* using the RiboPrinter system [33]. The automated ribotyping method has excellent repeatability, Typeability, and high capacity. This method seems to be a convenient way to quickly identify and compare bacterial clones that are ubiquitous in distant geographic areas and time points.

Automated ribotyping was used to investigate the clonal diversity of the 56 *P. aeruginosa* isolates. The 56 clinical isolates of *P. aeruginosa* from 44 patients

warded in the intensive care unit were obtained from Aga Khan Hospital from March 1998 to March 2001. Automated ribotyping indicated that the clinical isolates were clonally related and could be clustered into four major ribogroups based on their similarity index, with ribogroup II being the dominant one. The *P. aeruginosa* isolates in ribogroup II were correlated with their antibiotic resistance pattern, and interestingly, there seemed to be a gradual acquisition of multiple antibiotic resistance associated with the isolates within this group over time [34].

The ribotyping method has low discrimination. Seventy-eight confirmed nosocomial Pseudomonus aeroginosa out of 1520 different sample (nosocomial infections & environmental samples) were collected during a period of 1 year. Six typing methods were evaluated, utilizing the confirmed 78 Pseudomonas strains, to assess their usefulness as tools to study the bacterial diversity. The methods used were antibiogram, pyocin typing, serotyping, extracellular enzyme typing, automated ribotyping, and PFGE. PFGE yielded 56 distinct types of *P. aeruginosa* with 100% distinction capacity (78/78) as all the strains were typable. Compared to PFGE, the distinctive capacities were 88.5% (69/78) for serotyping, 91% (71/78) for pyocin typing and 100% (78/78) for automated ribotyping analysis. The results obtained in PFGE were the easiest to read and interpret and most discriminating (0.99), followed by the pyocin typing (0.96), whereas ribotyping had (0.90) discriminatory power [35].

8.3 Amplified-Based Methods

8.3.1 Random Amplified of Polymorphic DNA (RAPD)

The random amplified polymorphic DNA genotyping (RAPD) technology generated by the random primer polymerase chain reaction was established in 1990 by Welsh et al. This technology uses short arbitrary sequence primers to perform PCR under non-strict conditions. As a result, many sites of genomic DNA were amplified simultaneously. Because the stringency of the annealing reaction is reduced, a single primer can be used for amplification simultaneously. The same primer can perform extension reactions at many sites on the template DNA. The length polymorphism of multiple sites can be detected by the presence or absence of bands at different migration positions in the gel. Using RAPD technology for nucleic acid analysis of *P. aeruginosa* can not only be used for genetics and epidemiology of *P. aeruginosa*.

Crude bacterial lysates were prepared by suspending a 1 µl loopful of bacteria in 20 µl of 50 mM NaOH-0.25% sodium dodecyl sulfate (SDS) and heating for 15 min at 95 °C. Lysates were diluted with 980 µl of water, and 2.5 µl was used for amplification in a 25 µL PCR mixture. PCR tubes further contained 0.5 U of DNA polymerase, a 400 µM concentration of each deoxynucleoside triphosphate, primer, reaction buffer, and 2.5 mM $MgCl_2$. Amplification was performed with annealing at 52 °C, extension at 72 °C, and melting at 97 °C. The PCR products were mixed with 2.5 µl of gel loading buffer and electrophoresed on 2% agarose gel 1.0XTAE buffer

(TrisAcetate-EDTA) at 100 V for 2 h. Molecular size markers used were a 100 bp ladder. Isolates from each patient typed by RAPD analysis in a single PCR run were analyzed by photographing the gels and marking the position of the bands in order to facilitate the comparison of strains between patients. Strain differentiation was done by observing readily discernible band patterns. In most protocols, at least in the first few cycles of PCR, a lower annealing temperature is used, which allows imperfect hybridization at multiple random locations on the chromosome to amplify its random fragments. The PCR products were separated by agarose gel electrophoresis, stained, and analyzed by visual inspection or by calculation methods. Compared with PFGE and several other molecular typing technologies in many laboratories, this method is simpler, faster, lower cost, and more labor intensive.

RAPD typing was performed on 200 strains of *P. aeruginosa* by using 10 nucleotide primers. The results showed that the typeability was 100% and the intralaboratory reproducibility was 98.5% [36]. However, due to most aspects of the PCR program, including the temperature profile and source, and the small differences in different batches of Taq polymerase, it is possible to affect the repeatability of the band diagram. In different laboratories, the band patterns observed for the same *P. aeruginosa* isolates typed by the same RAPD method may show large differences. Under the same conditions, equipment and the same operator, the measurement is repeated, and the measurement is performed regularly rather than sporadically. The RAPD band pattern has the highest repeatability. Therefore, RAPD can only be used for laboratory comparison of *P. aeruginosa* strain collection [37].

Ten to twenty-five bands of 200–3000 bp for the *P. aeruginosa* isolates were obtained when applying the method evaluated by Campbell and colleagues by the use of the 10-nucleotide primer 208, an Invitrogen Taq polymerase and 40 ng purified genomic DNA. The concentration of genomic DNA will not interfere with the experimental results, but the use of different Taq polymerases has a great influence on the quantity and intensity of PCR products. Among the types of Taq enzymes used, Invitrogen Taq polymerase obtains the best results (i.e., the maximum number and intensity of bands) [36, 38].

The RAPD classification can be assisted by using software. In a typing study on MDR *P. aeruginosa* clinical isolates in Hungary and other European countries, it was found that the epidemiologically related *P. aeruginosa* isolates showed genetic similarity>90%. MLST results show that gifts with more than 80% similarity belong to the same clonal complex. Although MLST type and RAPD type of *P. aeruginosa* are in correspondence, the genetic similarity is less than 80% [39, 40].

In the molecular epidemiology and colonization of *P. aeruginosa* in the burn department of Shahid Motahari Hospital in Tehran, Iran, RFLP and RAPD analysess were used to study 127 clinical and two environmental collected from January 2008 to June 2008. In RFLP, the PCR product of the 16S rRNA gene was restriction enzymes Alu I, Hae III, and Rsa I, and the resulting fragments were analyzed by agarose electrophoresis. Molecular typing by RFLP did not show discrimination against P. aeruginosa isolates, but RAPD-PCR showed eight different genotypes designated RAPD1 to RAPD8 in clinical and environmental isolates. RAPD1

is the main genotype of clinical (n = 64, 50.4%) and environmental isolates (n = 1, 50%). The results show that RAPD may have better typing and discriminating ability than RFLP in studying P. aeruginosa [41].

Due to the simplicity and high speed of RAPD, it can be used as the first screening for *P. aeruginosa* epidemic typing. Through RAPD screening, clonal relevance can be determined at a relatively low cost within 24 h.

8.3.2 Amplified Fragment Length Polymorphisms (AFLP)

AFLP is known as the third-generation molecular labeling technology after RFLP, RAPD, etc. The principle is to cut genomic DNA with two or more restriction enzymes to produce sticky ends to form restriction fragments of different sizes, and then connect the artificial short double junction to the stickiness of these fragments. At the end, a pre-amplification reaction and a selective amplification reaction are performed using the specific fragment with a linker as a template. The linker and several adjacent base sequences serve as the binding sites of the primer. Finally, only the digested fragments paired with the selected base can be amplified to achieve specific amplification [44]. AFLP analysis is a selective restriction fragment amplification technique in which adaptors are ligated to genomic restriction fragments, and then these fragments are PCR amplified using adaptor-specific primers. For AFLP analysis, only a limited amount of purified genomic DNA (50–100 ng) is required. Digested with two restriction enzymes, one has an average cutting frequency (such as Eco RI) and the other has a higher cutting frequency (such as MseI). After adaptor ligation and PCR amplification, a polyacrylamide gel electrophoresis of the PCR product can obtain a pattern of usually 40–200 bands [42].

The genotypic relatedness was assessed by using AFLP fingerprinting. Sixty-six *P. aeruginosa* isolates were obtained from sputa/deep-pharyngeal swabs from 27 CF patients belonging to 17 families. Twenty-three distinct genotypes of *P. aeruginosa* were identified. Eleven families each had one distinct genotype. In the other six families more than one genotype was observed; three families each showed two genotypes, two families each had three genotypes, and one family had four genotypes of *P. aeruginosa*. In several cases, siblings with CF from the same family harbored the same strain of *P. aeruginosa*, which were different from the genotypes in other families. On the other hand, there was an overlap in *P. aeruginosa* between closely related families. Some patients show persistent colonization with the same genotype of *P. aeruginosa* over the longitudinal period. The presence of the same genotypes in the siblings of the same family and closely related families suggests cross-transmission of *P. aeruginosa* or acquisition from common environmental exposure [43].

A comparative study of PFGE and AFLP methods on 22 *P. aeruginosa* isolates shows that AFLP has 100% typability, DI value is 0.97, and PFGE is 0.96. In this study, Eco RI and Mse I restriction enzymes were used in AFLP, and clusters of *P. aeruginosa* isolates related to epidemiology have a similar PFGE pattern, showing>90% identity source. On the other hand, using GelCompar software to assist in

fluorescence-labeled AFLP fingerprint analysis, PFGE with more than six epidemiologically unrelated strains with differences showed less than 90% homology [44].

8.3.3 Multilocus Variable-Number Tandem Repeat (VNTR) Analysis (MLVA)

The MLVA technique has been developed to typing bacteria such as Salmonella, Listeria monocytogenes, and Escherichia coli [45]. The basis of the MLVA technique is the identification of the variable number tandem repeat (VNTRs) in specific locuses on the genome of microorganisms [46]. In the MLVA technique after selecting the desired locus and designing the primer for them and extracting the desired strains DNA, the proliferation of the sequences containing the VNTR is performed by PCR. The product obtained from PCR is sequenced and the number of replicates is calculated. Vergnaud and colleagues developed the MLVA protocol for *P. aeruginosa*, which was subsequently improved by adding new epidemiological information markers. The MLVA scheme involves 15 loci with repeated tandem sequences (VNTR). The MLVA genotype of the *P. aeruginosa* isolate (MLVA15) with 15 VNTRs is expressed as an allele, showing the number of repeats for each analyzed VNTR. When a difference is observed on any of the 15 VNTRs, a new genotype number will be given. Pedigree is defined as a group of isolates with up to two different genotypes (VNTR). The total diversity index of 15 markers calculated from 190 isolates was 0.97. For a simpler and more reliable MLVA scheme, when only microsatellites ms142, ms211, ms212, ms213, ms214, ms215, ms216, ms217, ms222, and ms223 participate in the analysis, the number of characteristic VNTRs can be reduced to 10 [47, 48].

In the genetic typing for the number of bacterial strains isolated from patients using MLVA technique [49]. Seventy samples from different strains of *P. aeruginosa* were isolated from a wide range of patients, including those admitted to the ICU and CCU units to outpatients. Although these strains were similar in nature to the biochemical characteristics and analyzes, 39 strains were classified in the MLVA analysis. This number of strains obtained from *P. aeruginosa* strains indicates the high accuracy of this method in differentiating the differences in these strains. These differences, which led to the creation of different types in the MLVA method, showed that they were not detectable by conventional biochemical methods. Urinary tract infections (UTIs) are one of the three most common causes in the community, especially at the hospital level. Urinary tract infection caused by pathogenic bacteria after respiratory infection is the most common type of infection in patients admitted to different parts of hospitals [50]. Patients with UTI are very diverse, and a wide range of bacterial strains have been identified and reported for different characteristics and pathogens [51, 52]. Due to the high variation in the pathogenic strains that come from patients from different parts of the world, which often carry antibiotic resistance genes, researchers are seeking more practical and precise solutions to differentiate these close to each other strains. As most diagnostic methods require

high volume of clinical specimens, and are time consuming and not cost effective, techniques such as MLVA, have become more common to therapists. In recent years, MLVA-based typing, in which isolates are evaluated by the number of replicates in several genetic regions, for a number of important bacteria such as Bacillus anthraise, Staphylococcus aureus, Enterococcus faecium, Haemophilus influenzae, Bordetella pertosis, and many others has been used [53].

MLVA was used to investigate the source of *P. aeruginosa* infection in a pediatric CF center in Paris, France. Between January 2004 and December 2006, *P. aeruginosa* was detected in 46 children, 17 of whom had primary colonization. A total of 163 strains were recovered. After genotyping 15 VNTRs, a total of 39 lineages were observed, consisting of indistinguishable or closely related isolates. One of them corresponds to "Clone C," which is widely distributed in Europe. This shows that the MLVA genotype of *P. aeruginosa* strains recovered from individual patients proved to be stable over time, except for the occasional insertion of IS elements and the addition or deletion of repeated sequences in a single VNTR [47].

During 2004–2008, researchers collected 81 non-repetitive *P. aeruginosa* in two universities and two hospitals in Bulgaria. Determine the 100% repeatability and 97.5% typability of the MLVA scheme. In order to study the difference between MLVA, MLST, and PFGE in the classification of *P. aeruginosa*, 32 strains from sputum samples from CF patients in the Netherlands were typed using three methods. Only 9 of the first 15 VNTRs were analyzed. The DI of PFGE, MLVA, and MLST are 0.988, 0.980, and 0.952, respectively, with overlapping confidence intervals of 95%. There is a high degree of consistency between the three methods at the cloning cluster level. The authors underscored the advantages of MLVA and MLST in their portability and ease of interpretation, and a further advantage of MLVA over MLST was also highlighted in being more cost effective as it does not require sequencing [54]. However, Johansson et al. analyzed 232 isolates of *P. aeruginosa* isolated from cystic fibrosis patients by both methods. In this comparative study, 91% of the results were similar to each other. However, they emphasized that, despite the expensive and time-consuming PFGE, its accuracy is higher than that of MLVA [55].

Seventy *P. aeruginosa* isolates were collected from different hospitals located in Tehran city in 2018. After the amplification of the genes by PCR, electrophoresis on the agarose gel was performed for the products of each of the eight genes. The bonds produced on the gels representing the size of each of the sequences were analyzed by Gene Tools software by comparison with the 100 bp size marker (ZR 100 bp DNA Marker ™). After sequencing and examining the frequency of repetitions in 70 strains, 39 types were obtained. In the phylogeny tree, the relationship between different strains was based on the similarity in their repetitions in a branch. The length of each branch also shows the difference in the number of repetitions in different branches [56]. The MST (minimum spanning tree) pattern derived from the MLVA analysis for the desired strains. Seventy strains classified into 39 types are presented as a clone based on the number of strains that were categorized. In total, the MST pattern obtained in this study consists of 11 clonal complexes (CC). This concept is based on the relationship between the numbers of repetitions that

have been considered as comparisons in each category. The distance between each CC represents markers that are common in each clone. And, if the similarity between the indices was more, the distance between the clones is closer to each other. The MST pattern usually gives us a better understanding of the typing of strains that cause the creation of a common attribute, because it indicates the differences and common indicators as clonal complex simultaneously [57].

The potential disadvantage of the MLVA method is that even with a fluorescence detection system, it is difficult to accurately determine the size of the fragment because it depends on the mobility and the sequence composition. In addition, the evolution of repeated DNA sequences may be too fast, compromising epidemiological consistency. Although this method has rarely been used for epidemiological studies so far, MLVA may become a more widely used typing technology for *P. aeruginosa* in the future, especially if portable typing data is required between laboratories or intercontinental in regional or international studies [54].

8.4 Sequencing-Based Method

8.4.1 Multilocus Sequence Typing (MLST)

Multilocus sequence typing (MLST) is a strain-typing system that focuses exclusively on conserved housekeeping genes [58]. Though pulsed-field gel electrophoresis (PFGE) possesses higher discriminatory power, the lack of a universal standard and portability makes MLST more ideal for comparative analysis of strain types regardless of region or source. Moreover, in a comparative study of molecular techniques for typing *P. aeruginosa*, MLST had the greatest predictive value (100%) in labeling strains as unique [59]. The standardization of MLST has given rise to databases that enable comparative analysis of allele sequences and identification of unique sequence types.

The seven genes acsA, aroE, guaA, mutL, nuoD, ppsA, and trpE were selected according to the MLST scheme for *P. aeruginosa* created by Curran et al. [60]. The loci were originally chosen based upon biological role (e.g., a range of differing central housekeeping roles including mismatch repair, DNA replication, and amino acid biosynthesis), size (>600 bp), location (i.e., a minimum of 6 kbp upstream or downstream from known virulence factors, lysogenic phage, or insertion sequence elements), and suitability for nested primer design and sequence diversity.

During the PCR amplification of the target sequence, some *P. aeruginosa* isolates may require some modifications to the PCR program. These modifications include the addition of pure dimethyl sulfoxide at a concentration of 5 ml per 100 ml of the PCR master mix and increasing the annealing temperature from 55 °C to 58 °C [37]. In addition, in some cases, in order to generate clear and unambiguous DNA sequence data to search in the MLST database, it is also possible to perform additional sequencing reactions on the purified PCR fragments using amplification primers. The polymerase chain reaction products were sequenced and submitted to the *P. aeruginosa* MLST database (https://pubmlst.org/paeruginosa/) for the

assignment of allelic numbers. Each isolate was then assigned a sequence type (ST) based on the combination of seven allelic numbers. Isolates with the same ST are considered as members of the same clone. Up to now, there are 3391 ST types in the database.

A comparative typing study of 90 *P. aeruginosa* isolates obtained from swab cultures around the rectum in patients in the intensive care unit showed that SpeI digestion of the 90 *P. aeruginosa* isolates from different patients identified 85 unique patterns and MLST revealed 60 different STs. Among the 60 STs, 36 were not previously submitted to the *P. aeruginosa* database. Although both PFGE and MLST have a high discriminating ability (DI values of 0.999 and 0.975, respectively), PFGE has greater discriminating power than MLST [61]. Because the results of MLST are highly reproducible and easy to compare between different laboratories, the interpretation of the data is clear, and it is used to determine the cloning relationship between bacterial strains that differ greatly in time and geography and genetic diversity is particularly useful. Therefore, MLST is a key epidemiological tool for studying regional and global epidemiology of multidrug-resistant *P. aeruginosa*. Studies have shown that *P. aeruginosa*, which is popular in Europe when carrying bla_{VIM} MBL and bla_{PER-1} ESBL, mainly contains two clonal complexes of serotype O11 and O12 isolates [39, 62–64].

In order to identify the major multidrug-resistant hospital clones of *P. aeruginosa*, many molecular typing studies were conducted at the National Epidemiology Center in Budapest, Hungary. The research object was a total of 1500 strains of *P. aeruginosa* between 2003 and 2008. The clinical isolates were screened for MBL and ESBL production. The selected isolates were also used for the overexpression of AmpC b-lactamase and the presence of aminoglycoside resistance determinants carried by integrins. A variety of typing methods have been performed on the representative MDR *P. aeruginosa*, including serotyping, RAPD and MLST. The results of the study indicate the key role of four different *P. aeruginosa* clonal complexes (as determined by MLST) in the emergence of MDR isolates. These four clonal complexes also seem to have a wide geographical distribution outside Hungary, but the acquired resistance determinants may show a high degree of variability among isolates from different geographic sources [39, 40, 64, 65]. The first complex (CC4) is characterized by a serotype of O12 and the founder sequence type ST111, which corresponds to the major multi-resistant P12 clone in Europe. In addition, some isolates of this clonal complex in Hungary are associated with the spread of VIM-4 MBL. The second complex (CC11) is characterized by serotype O11 (the founder sequence type ST235) and contributes to the spread of VIM-4 MBL and PER-1 ESBL. The remaining two clonal complexes distributed throughout the country are characterized by serotypes of O4 and O6, sequence types of ST175 and ST395, and contain isolates that overproduce the chromosomal AmpC b-lactamase and carry Integra aadB (aminoglycoside 2′-O-adenylate transferase) gene. In addition, observing the changes based on the resistance gene complexes CC4 and CC11 carried in different countries and hospitals, multiple independent concepts have proposed the acquisition of these two resistance factors of these two universal clones of *P.*

aeruginosa, which seem to be particularly adept at acquiring resistance determinants that result in an MDR phenotype [37, 39, 66, 67].

One hundred and sixty *P. aeruginosa* strains were isolated from a hospital in China. Multilocus sequence typing analysis demonstrated that these isolates were highly diverse; 68 sequence types were identified, of which 28 were novel sequence types. Polygenic and eBURST analysis demonstrated genetically similar clones with dissimilar resistance profiles. The results showed that 68 STs were segregated into 11 CCs because of the sequence identity among five or more alleles. The largest CC consisted of eight STs (ST2378, ST2372, ST274, ST2383, ST2370, ST2373, ST2405, and ST209, with ST209 being the primary founder). The second CC consisted of four STs (ST244, ST2374, ST2371, and ST597, with ST597 being the primary founder). The nine other groups contained two or three STs. Twenty-six STs were classified as singletons. The MLST tree revealed a high genetic diversity in those isolates. The analysis revealed a weak bootstrapping value, especially in major branches. The phylogenetic tree shows that ST2378, ST2372, ST274, ST2383, ST2370, ST2373, ST2405, and ST209 were clustered together. Several close clusters were identified; these were also previously obtained by the eBURST algorithm. In addition, the results showed that the MLST STs and antimicrobial resistance profiles were not correlated in this study. Isolates with the same ST did not show a unique resistance profile pattern. This demonstrated that there is no definitive link between the ST of isolates and their resistance to these 14 antimicrobial agents. Mapping of resistance profile data onto the eBURST analysis data and the phylogenetic tree revealed the following: (1) the most similar resistance profiles did not cluster together and (2) isolates with the same STs did not share similar resistance phenotypes. Taken together, this shows that these strains displayed a relatively high degree of genetic variability, demonstrating that antibiotic resistance was most likely determined by individual genetic combinations.

A total of 2818 *P. aeruginosa* isolates were collected in 2010 from 65 hospitals in 22 regions of China. Susceptibilities to 16 antimicrobial agents were evaluated by the disk diffusion method. Since carbapenems and ceftazidime are currently the most widely used effective anti-Pseudomonas drugs, we selected 896 imipenem-, meropenem-, or ceftazidime-nonsusceptible isolates for a further MLST study to investigate the clonal relationships among drug-nonsusceptible isolates. Of the 896 isolates, 632 belonged to 116 known STs, 201 demonstrated 104 new STs (new combinations of known alleles), and 63 others belonged to 34 new STs (STs contain novel alleles of certain genes). The regional distribution of the top 10 STs revealed the geographic dispersion of STs. The five of the top 10 STs were distributed in more than 10 regions. ST274, ST244, ST235, ST277, and ST357 were found in 16, 13, 11, 10, and 10 regions, respectively. This suggested that they were more common in China. To investigate the clonal relationships of 896 isolates, BioNumerics was used to create a minimum spanning tree and cluster STs into CCs. Although the whole population was nonclonal, there were several large CCs, which meant that the population was partially clonal, and some of those CCs contained globally spread STs and were related to local outbreaks of *P. aeruginosa* infections, such as ST235,

ST244, ST357, etc. Spain reported two outbreaks of *P. aeruginosa* in 2007 and 2008, one of which was caused by ST235 in a hematology department. South Korea also reported the dissemination of multidrug-resistant *P. aeruginosa* belonging to ST235 [68]. ST244 and ST235 were responsible for an outbreak of infections with *P. aeruginosa* that carried the PER-1 β-lactamase in Poland [69]. ST357 producing the IMP-7 metallo-β-lactamase has been reported in Singapore, and it also spread in the Czech-Polish border region [70]. In addition, the relatedness between STs and numbers of isolates was evaluated by generating a correlation curve. A marked linear relationship between ST categories and numbers of isolates was observed in the correlation curve, which meant that the number of ST categories increased with the addition of isolates.

To investigate the drug resistance and genetic background of *P. aeruginosa* at Shaanxi Provincial People's Hospital between July 2016 and January 2017. Sixty-eight STs were segregated into 11 CCs because of the sequence identity among five or more alleles. The largest CC consisted of eight STs (ST2378, ST2372, ST274, ST2383, ST2370, ST2373, ST2405, and ST209, with ST209 being the primary founder). The second CC consisted of four STs (ST244, ST2374, ST2371, and ST597, with ST597 being the primary founder). The nine other groups contained two or three STs. Twenty-six STs were classified as singletons. The MLST tree revealed a high genetic diversity in those isolates. The analysis revealed a weak bootstrapping value, especially in major branches. The phylogenetic tree shows that ST2378, ST2372, ST274, ST2383, ST2370, ST2373, ST2405, and ST209 were clustered together. Several close clusters were identified; these were also previously obtained by the eBURST algorithm. In addition, the results showed that the MLST STs and antimicrobial resistance profiles were not correlated in this study. Isolates with the same ST did not show a unique resistance profile pattern. This demonstrated that there is no definitive link between the ST of isolates and their resistance to these 14 antimicrobial agents. Mapping of resistance profile data onto the eBURST analysis data and the phylogenetic tree revealed that the most similar resistance profiles did not cluster together and isolates with the same STs did not share similar resistance phenotypes. In conclusion, this shows that these strains displayed a relatively high degree of genetic variability, demonstrating that antibiotic resistance was most likely determined by individual genetic combinations [63, 71].

8.4.2 Double-Locus Sequence Typing (DLST)

It has recently been shown in Staphylococcus aureus that, by sequencing small regions (ca. 500 bp) of only two highly variable loci (double-locus sequence typing, DLST), it is possible to investigate the epidemiology of this pathogen [72–74]. Similar to other sequence-based methods, it gave unambiguous definition of types, allowing inter-laboratory comparisons and high reproducibility. Moreover, the possibility to work with batches of 96 isolates allowed a reduction of costs and working time. Consequently, this method can be easily incorporated into long-term routine

surveillance programs. An efficient sequence-based typing scheme similar to the DLST scheme of *S. aureus* to investigate the local epidemiology of *P. aeruginosa* was set up by Basset et al. in 2013.

To identify potentially highly variable loci in the *P. aeruginosa* genome, eleven loci (ms142, ms172, ms173, ms194, ms207, ms214, ms215, ms217, ms222, ms223, oprD) were tested on a subset of isolates. Among these, only three loci (i.e., ms172, ms217, oprD) showed a product size that was larger than 300 bp for all isolates. Therefore, only these loci were selected for further analyses.

Single-strand sequencing of three highly variable loci (ms172, ms217, and oprD) was performed on a collection of 282 isolates recovered between 1994 and 2007 (from patients and the environment). As expected, the resolution of each locus alone (number of types (NT) = 35–64; index of discrimination (ID) = 0.816–0.964) was lower than the combination of two loci (NT = 78–97; ID = 0.966–0.971). As each pairwise combination of loci gave similar results, the most robust combination with ms172 [reverse; R] and ms217 [R] to constitute the double-locus sequence typing (DLST) scheme for *P. aeruginosa* was selected. This combination gave: (i) a complete genotype for 276/282 isolates (typability of 98%), (ii) 86 different types, and (iii) an ID of 0.968. Analysis of multiple isolates from the same patients or taps showed that DLST genotypes are generally stable over a period of several months. The high typability, discriminatory power, and ease of use of the proposed DLST scheme make it a method of choice for local epidemiological analyses of *P. aeruginosa*. Moreover, an Internet database (http://www.dlst.org) was developed to give an unambiguous definition of DLST types.

Reliable molecular typing methods are necessary to investigate the epidemiology of bacterial pathogens. Reference methods such as MLST and PFGE are costly and time-consuming. Cholley et al. compared the DLST method for *P. aeruginosa* to MLST and PFGE on a collection of 281 isolates. DLST was as discriminatory as MLST and was able to recognize “high-risk” epidemic clones. Both methods were highly congruent [75].

An increase in *P. aeruginosa* incidence was observed in the ICUs of the Lausanne University Hospital between 2010 and 2014 [76]. One hundred fifty three isolates retrieved during this period were typed with double locus sequence typing (DLST), which detected the presence of three major genotypes: DLST 1-18, DLST 1-21, and DLST 6-7. DLST 1-18 (ST1076) isolates were previously associated with an epidemiologically well-described outbreak in the burn unit. Nevertheless, DLST 1-21 (ST253) and DLST 6-7 (ST17) showed sporadic occurrence with only few cases of possible transmission between patients. In addition, the comparison of DLST and MLST showed that all DLST 1-18 isolates belonged to ST1076, DLST 1-21 to ST253, and DLST 6-7 to ST17, except for one DLST 6-7 isolate, which was found to be of ST845, a single-locus variant from ST17 at the nuoD locus. This confirms the previously documented congruence between both methods [77].

8.4.3 Whole Genome Sequencing (WGS)

In order to understand the colonization and infection pathways, a strong typing method is needed to study the correlation between strains. Although the PGGE method currently commonly used is the gold standard, it has low reproducibility between different laboratories and is not suitable for large-scale research. At present, methods based on sequence analysis such as MLST and DLST have shown advantages. MLST has shown high efficiency in studying the overall population structure of *P. aeruginosa*. DLST has been successfully used to study the epidemiology of Staphylococcus aureus and *P. aeruginosa*. At present, with the continuous development of new technologies, in the hospital environment, the latest research in *P. aeruginosa* evolution research and epidemiological research has used whole genome sequencing (WGS) [73, 76].

The emergence of high-throughput methods has promoted WGS, bringing hope for the separation of single base pairs between isolates, making it the ultimate molecular typing method for bacteria. Analysis of single nucleotide polymorphisms (SNPs) in the bacterial genome provides a method to determine the correlation between isolates that are epidemiologically linked and to track the evolution of bacteria over months to years. High-throughput sequencing analysis was performed on the five isolates that broke out in UH-NHST-CC hospital. Although these five strains of *P. aeruginosa* belong to the same PFGE and RAPD lineage, the genomic sequence data obtained by high-throughput sequencing technology shows that the isolate PANOTK11 is an outlier compared to the other four isolates and does not belong to the same outbreak pedigree. PANOTK11 has a 48 kb sequence, which is not present in the other four strains. This area is assembled into a contig and annotated as containing 24 CDS. Furthermore, the single nucleotide polymorphism (SNP) between the genomic sequence data showed the key single-base differences accumulated during the outbreak process, thus providing an in-depth understanding of the evolution of the outbreak strain. Differential SNPs have been found in various genes, including lasR, nrdG, tadZ, and algB. The rate of these generations is estimated to be one SNP every 4–5 months. In conclusion, this study proves that single-base resolution of whole-genome sequencing is one of the powerful tools for the analysis of outbreak isolates. It shows the similarity of strains, and evolve over time passing through the gene sequence adapt to changes [77].

After the incidence of P. aeruginosa in the ICU of Lausanne University Hospital increased, clinical and environmental isolates were typed using DLST. Three main types of DLST were identified (DLST 1-18, DLST 1-21, and DLST 6-7), and the identification capabilities of whole genome sequencing (WGS) were used to further study these three main types of DLST. It is one of the advantages of WGS to identify the ST of isolates by MLST inspection. DLST is both cheap and efficient. It is a routine monitoring method for *P. aeruginosa* in ICU wards and classifies all patients and environmental isolates quarterly. Only when several patients have similar genotypes, on-site epidemiological investigation and WGS are performed. Although the cost of WGS is decreasing, as a routine monitoring method for *P. aeruginosa*, its implementation cost is still higher than the currently used DLST. In addition, the

analysis of WGS data requires a certain level of bioinformatics expertise, but not all laboratories have it [77]. Therefore, using DLST as a first-line molecular typing tool for monitoring and WGS to solve problematic clusters will ultimately be an accurate and cost-effective typing strategy.

8.5 Summary

Pseudomonas aeruginosa is characterized by excellent biochemical and ecological versatility, and the genome size in different strains may vary by as much as 30%. Phenotyping methods for *P. aeruginosa* have been reported, including serotyping, pyocin, and antibacterial susceptibility typing and serotyping. Phenotypic characterization continues to play an important role in the management of *P. aeruginosa* infection. The distinguishing ability of molecular typing technology must be high enough to distinguish unrelated strains, but not high enough to assign isolates of a common lineage (such as epidemic clones) to different genotypes. Currently, a variety of molecular typing methods have been developed. This chapter introduces the application of several molecular typing methods such as restriction-based methods, amplified-based methods, sequencing-based methods in the typing of *P. aeruginosa*.

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