Review Article

Molecular epidemiology and molecular typing methods of *Acinetobacter baumannii*

An updated review

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ABSTRACT

إن الهدف من هذه الدراسة هو الإطلاع على الطرق الجزيئية المستخدمة لعزلات CRAB لدراسة الوبائيات الجزيئية في جميع أنحاء العالم. هناك حاجة إلى تقنيات متعددة لفهم مصدر وطبيعة تفشي المرض الناجم عن *Acientobacter baumannii* والمقاومة المكتسبة لمضاد حيوي. في الوقت الحاضر، هناك تحول تدريجي من طرق التقليدية إلى الطرق الجزيئية الحديثة لدراسة علم الأوبئة الجزيئية ومكافحة العدوى. لقد أحدثت الجزيئية لسلالات *A. baumannii* ثورة كبيرة في العقدين الماضيين. وقد ثبت أن بعض التقنيات القائمة على التسلسل تمثل اختراقًا وفتحت آفاقًا جديدة، وهو ما لم يتم تحقيقه بالطرق التقليدية. في هذه المراجعة، تمت مناقشة طرق الكتابة المختلفة الموجودة مسبقًا والمستخدمة مؤخرًا لاستكشاف الوبائيات الجزيئية لـ *A. baumannii* المتعلقة بسياق العدوى البشرية.

The aim of this study was to go through the molecular methods used for typing of carbapenem-resistant *Acientobacter baumannii* (CRAB) isolates for investigating the molecular epidemiology all over the world. Multiple typing techniques are required to understand the source and nature of outbreaks caused by *Acientobacter baumannii* (*A. baumannii*) and acquired resistance to antimicrobials. Nowadays, there is gradual shift from traditional typing methods to modern molecular methods to study molecular epidemiology and infection control. Molecular typing of *A. baumannii* strains has been revolutionized significantly in the last 2 decades. A few sequencing-based techniques have been proven as a breakthrough and opened new prospects, which have not been achieved by the traditional methods. In this review, discussed different pre-existing and recently used typing methods to explore the molecular epidemiology of *A. baumannii* pertaining in context with human infections.

Keywords: *Acientobacter baumannii,* matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS), multilocus sequence typing (MLST), molecular typing, pulsed-field gel electrophoresis (PFGE)

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*A*cinetobacter baumannii (*A. baumannii*), recognized recently by the World Health Organization (WHO) as the top-priority bacterial pathogen, is infamous for its role in a variety of hospital-acquired infections.[1,2] Among the significant nosocomial infections caused by *A. baumannii* are urinary tract infection (UTI), meningitis, bacteremia, respiratory tract infections, wound infections, and surgical site infections.[3] The bacterium possesses numerous virulence factors and antimicrobial resistance mechanisms, rendering it sometimes an untreatable microorganism with a complex pathobiology.[1] Its exceptional capacity to cause hospital-wide outbreaks has encouraged microbiologists and research scientists to invent new technologies aimed at preventing infections and transmission. The accurate identification and molecular typing of the bacteria have become emerging areas for researchers and epidemiologists to initiate effective control measures. Over the past 2 decade, a number of molecular techniques have been developed for characterizing and typing of *A. baumannii* strains, including ribotyping, *A. baumannii* polymerase chain reaction (PCR)-based replicon typing/Rep-PCR (AB-PBRT), pulsed-field gel electrophoresis (PFGE), single locus sequence-based typing (SLST), trilocus sequence-based typing (3-LST), multilocus sequence typing (MLST),

matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS), whole genome sequencing (WGS), Fourier Transform Infrared (FTIR), Spectroscopy-sased IR Biotyping (IRB), random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP) analysis, and multiple-locus variable number of tandem repeat analysis (MLVA).[4-15] Amongst all, MLST and PFGE are considered as the gold standard technique for molecular typing of *A. baumannii*. Multilocus sequence typing relies on variations in the nucleotide sequence of 7 housekeeping genes, collectively generating a precise allele profile and finally assigning a sequence type (ST). Two schemes, namely the 'Oxford scheme'[16] and 'Pasteur Scheme'[17] are available for conducting MLST typing of *A. baumannii* (Figure 1 & Table 1). The aim of the present study is to gather and compile the available information on the diverse typing methods used, both currently and in the past, for characterizing *A. baumannii*.

Ribotyping. Ribotyping proves to be a valuable tool to study the epidemiology of various pathogenic bacteria, including *A. baumannii*.[4] The ribotyping procedure involves Southern blotting, a technique adept at accurately identifying several bacterial genera, followed by its genotyping.[18] In this method, specific restriction enzymes are employed to digest the genomic DNA at a fixed temperature. The resulting digested DNA is then sorted into smaller fragments and subsequently loaded onto electrophoresis units. These units run at a standard voltage to facilitate the separation of fragments, which are then shifted to a nitrocellulose membrane. Following this, a suitably labeled probe is utilized for hybridization with the ribosomal DNA. The ribotyping method becomes more accessible using non-radioactive labels within a well-established microbiology laboratory so that it is easier to perform. As a molecular taxonomy system, it has been found to be relatively stable and reliable. Ribotype profiles yield species- or strain-specific results. In 1992, Gerner-Smidt[4] started the detection of Acinetobacter species by employing 3 restriction enzymes—EcoRI, ClaI, and SalI—followed by labeling of cDNA with a digoxenin-11-UTP probe. Another restriction enzyme HindIII was used in accordance with AFLP analysis to generate the ribotype profiles for European clones.[19] Additionally, an automated ribotyper called Riboprinter

Table 1 - Different methods of molecular typing of *Acinetobacter baumannii*.

Molecular typing method	References
Ribotyping	Ahmed & Alp,[20] 2015
AB-PBRT/Rep-PCR	Chen et al, 2018[29]
Pulse field gel electrophoresis (PFGE)	Seifert & Gerner-Smidt,[33] 1995, Li et al[6]
Single locus sequence-based typing (SLST)	Hamouda et al,[35] 2010 Pournaras et al,[7] 2014
Trilocus sequence-based typing (3-LST)	Turton et al,[8] 2007 Tavares et al,[40] 2018
Multilocus sequence typing (MLST)	Bartual et al,[16] 2005 Kumar et al,[43] 2019
MALDI-TOF MS	Gautam et al,[10] 2017, Garcia-Salguero et al,[27] 2021
Whole genome sequencing (WGS)	Kumar et al,[11] 2015
FTIR-based IR biotyping	Daltonics, 2021,[12] Teng et al,[59] 2022

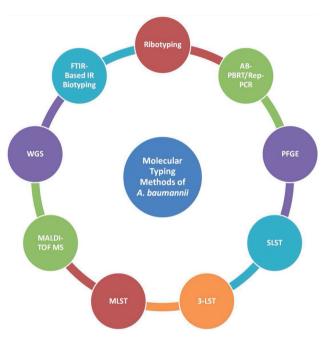AB-PBRT/Rep-PCR: *Acinetobacter baumannii* PCR-based replicon typing

Figure 1 - Various molecular typing methods of *Acinetobacter baumannii*.

(Dupont, USA) was introduced to the market and utilized in a number of epidemiological investigations, employing EcoRI enzymes.[20] In a separate study from Pakistan, the identification of *Acinetobacter junii* isolates from industrial wastewater was conducted using 16S ribosomal ribonucleic acid (rRNA) ribotyping.[21]

Acinetobacter baumannii PCR-based replicon typing (AB-PBRT/Rep-PCR). Plasmid typing emerged as the first molecular typing method for investigating the epidemiology of Acinetobacter species in 1990.[5]

However, due to various challenges and limitations associated with being the sole typing technique, its usage encountered obstacles, leading to its replacement by alternative methods. In 2010, a new AB-PBRT technique was proposed as a simple and cost-effective method to trace the resistance genes present on circulating plasmids in *A. baumannii*, facilitating investigations into its epidemiology.[22] A rapid molecular typing method is deemed essential for promptly tracing and investigating any probable bacterial outbreaks. The AB-PBRT method has been shown as an easy and quick technique for examining outbreak of nosocomial pathogens.[23] Repetitive extragenic palindromic (rep)-PCR is a polymerase chain reaction-based approach, utilizing the dispersion of repetitive sequences within the bacterial genome. It analyzes the length of DNA fragments situated between such repetitive sequences using outwardly designed oligonucleotides for annealing. *Acientobacter baumannii* PCR-based replicon typing method *A. baumannii* plasmids were classified into homogeneous groups based on nucleotide homology between the replicase genes of their respective replicates.[24] Repetitive extragenic palindromic-PCR has demonstrated success as a convenient method for analyzing the molecular epidemiology, clonality, and genomics of *A. baumannii*.[25] *Acientobacter baumannii* PCR-based replicon typing method has been applied to a collection of *A. baumannii* clinical isolates containing either blaOXA-58 or blaOXA-23 carbapenemase genes that have become multidrug-resistant.[26] Recently, a total of 21 strains of *A. baumannii* isolates underwent epidemiological and phenotypical investigations using rep-PCR and MALDI-TOF MS to assess their discriminatory power for *A. baumannii* clones.[27] In another study carried out in the southwest region of Iran, the Rep-PCR typing method was employed for detecting various pathogenic bacteria, including *A. baumannii*, in patients with hospital-acquired pneumonia.[28] Similarly, a study in Taiwan revealed the clonal spread of carbapenem-resistant *A. baumannii* (CRAB) across community healthcare centers and associated long-term care facilities using rep-PCR, which helped in identifying and tracing CRAB infections.[29]

In addition, the 16S rRNA gene-PCR is an additional method based on DNA amplification and sequencing, widely used for identifying bacterial genera. This gene has a length of around 1550 base pairs (bp) and comprises both conserved and variable DNA segments. The length of the gene is sufficient to yield specific, consistent, and convincing results. It is a pan-bacterial gene that is used to generate universal primer sequences from the conserved regions for the bacterial identification up to species level.[30] The effectiveness of sequencing the 16S rRNA gene relies on accurately amplifying the target sequence of bacterial DNA and matching the resulting sequence with the nucleotide sequences already available in the National Centre for Biotechnology Information (NCBI) database for executing basic local alignment search tool (BLAST). Furthermore, the 16S rRNA gene sequencing method has been extensively performed for the identification of numerous bacterial strains. Consequently, an open access databank of NCBI called GenBank contains over 100,000 nucleotide sequences for 16S rRNA genes from different bacteria. This method can also be used to differentiate between bacteria belonging to different major phyla at the genus level, owing to its widespread use and popularity in the field. Nevertheless, this method has its limitations, specifically in cases where certain species of Acinetobacter share similar 16S sRNA sequences. The relatively slow rate of base substitution in the 16S sRNA gene contributes to its inadequacy as a technique for accurately identifying all Acinetobacter species.[31]

Conversely, the sequencing of the RNA polymerase β subunit (rpoB) gene has shown promise as a reliable technique with higher discriminatory power compared to 16S rRNA gene sequencing. A study conducted a comparison of rpoB and 16S rRNA gene sequencing results on a total of 99 well-characterized strains of the *Acinetobacter calcoaceticus-baumannii* (ACB) complex to evaluate intra- and inter-species inconsistency.[32] The findings indicated that rpoB sequencing provided more accurate results, displaying a higher precision in recognizing ACB complex strains than that of 16S rRNA gene sequencing.

Pulsed-field gel electrophoresis (PFGE). Public health laboratories have become increasingly reliant on molecular subtyping to categorize and classify organisms based on their genotypic characteristics. To carry out the epidemiological investigations and for tracing genetic association, PFGE continues to be acknowledged as the gold standard method for several bacterial genera.[6] In PFGE, restriction enzymes are used to digest the large fragments of genomic DNA, resulting in fragments of varying lengths, which are then separated by electric pulses at different angles. The counter-clamp homogeneous electric field (CHEF) electrophoresis unit is presently available and widely used as the primary PFGE instrument. In this system, fragmented DNA travels in a zigzag fashion within an agarose gel matrix under an alternating pulsed electric field maintained at a uniform temperature of 120°C. Despite the preference for some PCR-based methods

due to their faster protocols, PFGE remains the gold standard for genotyping bacterial strains. Generally, two restriction enzymes, SmaI and ApaI, are used for creating fragments of Acinetobacter genomic DNA for typing. It is displayed that PFGE exhibited higher discriminatory power when the ApaI restriction enzyme was applied to a set of Acinetobacter species, surpassing the results obtained from the ribotyping method.[33] In another study, an analysis of Acinetobacter strains was performed based on a meticulously standardized PFGE protocol for inter-laboratory comparative investigations.[34] Consequently, PFGE holds the potential to be a future method for establishing an internationally accessible database, promoting the monitoring of local and international clones. However, initial cost of equipment and reagents, and reproducibility issues in inter-laboratory results may pose challenges to its implementation soon. Although technique has been recognized as the "gold standard" for subtyping bacterial pathogens due to its high epidemiologic relevance as well as its sensitivity and discriminatory power. However, it takes approximately 5 days for PFGE to produce results and requires the use of costly software for result interpretation and analysis. Therefore, other PCR-based typing techniques are often favored, although a combination of both methods is occasionally employed.[33] There are few disadvantages associated with PFGE. The most significant is the labor-intensive nature of the protocol, which often takes several days to complete. Also, the results are often inconclusive when compared to other PCR-based techniques.[33]

Single locus sequence-based typing (SLST). Multilocus sequence typing, based on typically 7 housekeeping genes, offers superior resolution power; however, this procedure is time-consuming, labor-intensive, and costly. Meanwhile, SLST uses a genome mining strategy applicable to several bacteria with sufficient genome sequences available. After data mining, a fixed length of sequence (~500 bp) is selected and amplified in the test DNA of the bacterial isolate using accurately designed single-pair primers. This amplified single sequence is able to resolve the vast majority of the bacteria. For *A. baumannii*, SLST was evaluated using the blaOXA-51-like gene sequence, assessing its capability to correctly identify ST in clinical isolates.[7,35] Interestingly, blaOXA-51-like genes were specifically used as marker genes for the identification of *A. baumannii* up to the species level.[9,36] Single locus sequence-based typing demonstrated the ability to discriminate all epidemic and sporadic lineages, followed by the assignment of international

clones to the collection of isolates in this study. The results indicated that SLST can effectively identify the bulk of the main lineages of *A. baumannii*, supporting its potential implementation in clinical settings. A recent application of SLST involved investigating the detection of different clones of *A. baumannii* and their genetic relatedness in an ICU setting in South India.[37] Additionally, an SLST scheme was developed for *Propionibacterium acnes* with a resolving power comparable to MLST approaches.[38] However, one possible drawback of SLST in comparison with MLST, however, this method may have the disadvantage of decreasing resolution.

Trilocus sequence-based typing (3-LST). Three-LST or trilocus sequence-based typing-based multiplex-PCR method was first projected in 2007 to detect the international clones corresponding to the 'Pasteur scheme' of MLST.[8] The established 3-LST method utilizes multiplex-PCR of three genes: ompA, blaOXA-51-like, and csuE genes, for rapid identification of international clones, based on the sequences these alleles called, "3-LST". Using the 3-LST-based multiplex-PCR assay, isolates were rapidly assigned to predefined profiles (G) including G1, which corresponds to ICII; G2, which corresponds to ICI; and G3, to ICIII.[8] In 2016, Martin et al[39] introduced a new 3-LST multiplex-PCR to detect major clones of *A. baumannii* in Brazil. The aim of this study was to expand a substitute method for detecting clones known to be present on different continents. The new 3-LST multiplex-PCR, conducted in a single reaction, successfully detected five leading *A. baumannii* clones. A recent study identified a specific band profile for ST317 (Pasteur scheme) using Martin's 3-LST schemes, followed by a comparison with the Pasteur scheme of MLST.[40] During the outbreaks this method promising for rapid detection of *A. baumannii* and is facilitated by the new multiplex-PCR. Other techniques like MLST, usually are time consuming and initial cost of equipment are expensive; therefore, 3-LST could be achieved by overcoming these disadvantages.

Multilocus sequence typing. Multilocus sequence typing is a nucleotide sequence-based method, which is satisfactory for identifying genetic relatedness and molecular characterization of many bacterial genera.[41-43] Multilocus sequence typing has shown to be useful for the genotyping of pathogens in surveillance and epidemiological studies. It offers indisputable and conveniently transportable data, enabling the analysis of evolutionary relationships by numerous users through global databases.[9] This sequence-based high-resolution genotyping method has been effectively utilized for many clinically significant pathogenic bacteria along

with *A. baumannii*. Multilocus sequence typing serves as a substitute technique to PFGE and represents a relatively newer method using DNA sequence-based analysis. Initially used primarily for global surveillance and epidemiology of pathogenic bacteria, MLST is a technique that involves the amplification and comparison of multiple loci sequences of a bacterial genome. These loci, typically housekeeping genes, exist in all the bacterial isolates, and any variation within these loci is considered neutral. In MLST, diverse sequences are assigned allele numbers for each gene sequence. As mentioned earlier, 2 prevalent schemes for MLST are the Oxford and Pasteur schemes. The nucleotide sequences are around 500 bp in length, and each isolate is described by the combination of alleles of each housekeeping gene or ST used in both schemes.[16,17] In comparison to other types of typing, MLST is characterized by its superiority in terms of unambiguity and transferability of sequence information. However, a major disadvantage is the high cost of processing the DNA sequence.

Besides, the major benefit of the MLST technique is its capacity for data transfer among different laboratories worldwide and over the internet, distinguishing it from other genotyping methods. In the Oxford scheme of MLST, internal fragments from seven housekeeping genes—gltA, gyrB, gdhB, recA, cpn60, gpi, and rpoD—are selected for an MLST platform.[16] While in the Pasteur scheme, the cpn60, fusA, gltA, pyrG, recA, rplB and rpoB genes are amplified and sequenced to generate phylogenetic interpretations.[17] Notably, we performed the MLST typing of *A. baumannii* previously in 2019 and revealed the presence of multiple international clones circulating in a tertiary care hospital in North India.[9] Recently, the molecular epidemiology of imipenem-resistant MLST bacteremia was studied using MLST in a pediatric intensive care unit (PICU) of a tertiary care hospital from 2000 to 2016 in Seoul, Korea.[44] Another study highlighted the differences in both phenotypic and genotypic characteristics among multilocus sequence typing isolates obtained from environmental and clinical settings using MLST.[25] A recent study compared the discriminatory abilities of PFGE, MLST, and core genome MLST (cgMLST) techniques in China.[6] The findings of the study concluded that cgMLST exhibited more effectiveness compared to MLST and PFGE, suggesting its potential as an open method for molecular typing *A. baumannii* isolates in epidemiological research and surveillance.

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). Presently, MLST stands as the predominant technique for epidemiological analysis and molecular characterization, although it is both expensive and requires significant time and labor.[10,45,46] The swift detection of Acinetobacter species is crucial due to distinct susceptibility patterns within the *A. baumannii* group. As an alternative and substitute typing scheme, MALDI-TOF MS is drawing global attention as it is nowadays the preferred and cost-effective method for routine bacterial identification.[10,47] Matrix-assisted laser desorption ionization-TOF MS is widely used for the identification and typing of microorganisms to the subspecies level.[48] Despite its escalating importance in detecting bacteria up to the species level, a study confirmed the incompatibility of this method in showing clonal relatedness among *A. baumannii* isolates.[49] However, another study demonstrated that MALDI-TOF MS accurately identified Acinetobacter species by generating reference signatures through the processing of species-specific peaks using dedicated data software.[50] The investigators derived the mean spectra from two strains of *A. dijkshoorniae*, 3 of *A. seifertii*, and 2 of *A. nosocomialis*. These spectra were clubbed with the Bruker taxonomy database, allowing the accurate detection of all strains, whether from direct colonies or bacterial extracts, using the BioTyper software.[50] Recently, a constitutive phenotypic modification of lipid A by phosphoethanolamine (PEtN) incorporation was noticed in the multidrug-resistant (MDR) strains of carbapenem-resistant *A. baumannii* using MALDI-TOF MS, particularly in the polymyxin B resistant strains.[51] There are several advantages of using this method, including the fact that it is very precise and sensitive as well as being capable of identifying whole bacteria cells, as well as bacteria isolates. There is a great deal of success being achieved with MALDI-TOF MS for the identification and typing of microorganisms down to the subspecies level, Further, bacteria cultures that are difficult to grow can also be used to identify them, and as a result, the method is not sensitive to modifications made in microorganism growth protocols prior to analysis.

Whole genome sequence (WGS). Whole genome sequence is gradually becoming a routine method in microbiology laboratories, facilitated by the advent of high-throughput methods.[52] Regarded as an 'all-in-one test,' WGS delves into the full genetic details of a bacterial isolate, providing both antimicrobial susceptibility patterns alongside the identification of bacterial species.[53] Its extensive precision in strain information makes WGS valuable for epidemiological investigations and typing on both local and global

levels. Whole genome sequence has been proven to be a highly powerful method for exploring the molecular epidemiology of *A. baumannii*, offering a comprehensive overview of different bacterial species in a single assay. It helps the immediate detection of various species, lineages, resistance mechanisms, and virulence factors across all types of bacteria.[11] Through WGS analysis conducted at 4 United States centers, it was revealed that CRAB poses a potential menace to admitted patients in healthcare centers, accounting for 24% of 30-day mortality.[54] Their findings further highlighted a reallocation within the CRAB isolates in the United States over the last decade and underlined the significance of molecular epidemiology and immediate surveillance for tracing CRAB transmission. The WGS technique is gaining popularity as the method of choice for executing epidemiological and surveillance studies of *A. baumannii* due to its ability to reveal genetic relatedness and complexity. Another WGS study has recently provided insights into the distribution of antibiotic resistance genes and population genetics of CRAB isolates causing infections in six hospitals in 3 Mediterranean countries: Israel, Greece, and Italy.[55] The study highlighted that almost 97% of isolates harbored acquired carbapenems and further suggested that the geographical variations in CRAB isolates and their clonal dispersion resulted in endemic transmission in hospitals and regions. Whole genome sequence analysis of 221 CRAB isolates from Thailand has also resulted in extensive detection of mobile genetic elements (MGE) and antimicrobial resistance genes and virulence genes.[28,56] For routine diagnosis in microbiology laboratories, WGS is now complementing traditional methods, providing rapid and accurate antibiotic susceptibility testing (AST) results. As a consequence, EUCAST recommends the adoption of WGS for reporting AST. In a recent study from India, a web-based automated platform called 'Galaxy ASIST' was developed using whole genome data and antimicrobial resistance patterns from 6500 *A. baumannii* strains, offering the detection of determinants of antimicrobial resistance.[57] This platform provides a centralized storehouse along with metadata to offer a globally accepted framework for determining AST profiles of *A. baumannii* clinical isolates in accordance with global standards. Whole genome sequence is rampantly swapping other typing techniques for studying outbreaks of infectious diseases. Moreover, WGS-based cgMLST has been investigated recently for *A. baumannii* using Ridom SeqSphere+ and AST prediction in ARESdb.[58]

Fourier transform infrared-based IR biotyping. Detecting the presence of probable spread before the onset of an outbreak is crucial for the timely implementation of preventive measures to control infection. Fourier transform infrared is an impending typing technique that utilizes infrared spectroscopy for biotyping. Developed by Bruker Daltonics GmbH, Germany, FTIR spectroscopy is an effective system that serves as an early warning for any outbreaks or transmission of infectious agents.[12] This cost-effective and rapid spectra-based typing method produces whole biochemical fingerprinting information of a bacteria. Recently, in Amsterdam, the Netherlands, a range of bacterial strains, including 25 *A. baumannii* isolates, were screened in real-time to differentiate between resistant and susceptible bacteria at the strain level using the FTIR spectroscopy typing technique.[59] FTIR proves to be a promising method for hospital hygiene management by establishing cutoff values and calibrating culture techniques. Importantly, it offers a more cost-effective alternative to generally used typing techniques such as PFGE, MLST, and WGS.[60,61] The clustering outcomes of the IR biotyping were closely aligned with those obtained from WGS and PFGE, enabling a prompt and reliable typing process. In addition to its advantages, such as reduced costs and time savings, FTIR spectroscopy proves to be an efficient technique for typing bacterial isolates, making it a reliable choice for real-time studies of healthcare outbreaks.[60,61]

Random amplified polymorphic DNA-PRC. Deoxyribonucleic acid-based typing techniques generate specific DNA banding patterns through the digestion of PCR products with restriction endonuclease enzymes or solely by multiplex PCR. These methods are prevalently used globally for epidemiological investigations of *A. baumannii*. In this direction, RAPD has gained huge achievement in typing *A. baumannii* strains locally.[13] In recent times, the molecular epidemiology of a total of 119 *A. baumannii* isolates was determined in Iran using RAPD-PCR, revealing substantial diversity among the isolates.[62] In a groundbreaking investigation in Saudi Arabia, plasmid-borne sulfonamide and aminoglycoside resistance genes were observed among clinical isolates of *A. baumannii* utilizing the RAPD-PCR-based genotyping method. Additionally, this study detected a new mutation in the sul1 gene and a novel allele variation in the aac (6')-Ib gene.[63] Random amplified polymorphic-PCR-based genotyping of 23 MDR *A. baumannii* strains were executed, clustering them into 11 different genotypes, which revealed unseen community-acquired infections (CAI) in Egypt.[64]

This also marked the first report regarding CAI in Egypt caused by *A. baumannii*. In 2018, the molecular epidemiology of 32 ventilator-associated pneumonia cases and 47 burn wound cases showed the colonization of extensively drug-resistant isolates of *A. baumannii* in 2 teaching hospitals in Tehran, Iran, indicating genetic similarity among all the isolates.[65] Furthermore, the genome analysis of MDR *A. baumannii* has identified the presence of transposons, integrons, and other MGEs like AbaR (*A. baumannii* antibiotic resistance) islands. In a separate study from South India, the phylogenetic relationship of 14 biofilm-producing *A. baumannii* strains was investigated using RAPD-PCR. This study also correlated the findings with antimicrobial resistance patterns caused by beta-lactamases, MGEs, and virulence factors.[66]

As stated earlier, this approach could be expanded to multidrug-resistant bacteria. Random amplified polymorphic-PCR typing methods considered as a cost-effective surveillance system to evaluate the possibility of multiple drug resistance *A. baumannii* outbreaks in especially in local hospital settings, and those hospitals which has limited testing facility for microbial-associated disease.

Amplified fragment length polymorphism (AFLP). It was initially considered a novel genomic fingerprinting method for *A. baumannii* in 1997, relying on the amplification of specific PCR fragments.[14] This typing technique involves the use of a single set of restriction enzymes (HindIII and TaqI) and a single set of specific PCR oligonucleotides as primers. In contemporary times, other more cost-effective and reliable techniques are favored over AFLP, rendering this technique obsolete for molecular typing of *A. baumannii*. As per the current PubMed search, the latest AFLP-based typing study was performed in 2013 in Arizona, USA, aiming for strain-level resolution and species characterization of Acinetobacter species.[67] The outcomes of the investigation presented high congruency between PCR/ESI-MS and AFLP genotyping methods. Due to its wide range of applications and high reproducibility as sequence data for primer construction are not required. AFLP has proven to be a useful tool for *Acinetobacter spp.* outbreak exploration and subspecies analysis. Although this method is labor-intensive and challenging in results analysis due to difficulties in transporting data between laboratories, due to different sequencing platforms are used.

Multilocus variable-number tandem-repeat (MLVA). Multilocus variable-number tandem-repeat technique employs the number of repetitions at various loci within a set of repetitive DNA. It is a PCR-based technique known as variable number of tandem repeats (VNTR).[15] Multilocus variable-number tandem-repeat genotyping offers information about outbreaks, such as clonal diversity, genetic relatedness, spatial and temporal spread, and their effect on epidemic or endemic situations. Some distinctive features that make MLVA a promising method for typing *A. baumannii* strains include high resolution, portability, elevated resolution power, and adaptability to automation.[68] Similar to many other typing techniques, MLVA also faces numerous limitations. Factors such as high mutation rates and swift evolution render MLVA unsuitable for being a globally accepted typing method. Due to the necessity of designing primers that target the pathogen being investigated, MLVA is not a universal method. Additionally, the results of MLVA cannot be directly compared with those of other laboratories because of the amplicon banding pattern.[68] The broad array of small markers in MLVA does not allow clustering. In a study conducted in Southwestern Iran, a sum of 70 clinical isolates of *A. baumannii* underwent MLVA to study their molecular characterization and genotyping.[69] The study displayed 12 distinct MLVA types among *A. baumannii* isolates, which were further grouped into five clusters and four singleton genotypes. Another study identified MLVA-8Orsay L as a potent marker for drawing evolutionary relationships due to its enhanced stability, yielding epidemiological results comparable to the MLST method.[70,71] Lately, a study in Lithuania characterized 194 MDR *A. baumannii* strains using MLVA genotyping methods. This study further highlighted the circulation of several AMR genes and the genetic relatedness among various *A. baumannii* isolates.[72] Notably, according to a PubMed search, there is no available study from India on MLVA-based molecular typing of *A. baumannii*. However, the molecular epidemiology of 73 CRAB isolates from tertiary care hospitals in Java and Sulawesi islands, Indonesia, was recently determined using MLVA.[73]

The typing methods for *A. baumannii* strains have undergone significant transformation over the last two decades. Emerging tools such as spectroscopic technologies and CRISPR-based typing show promise but necessitate in-depth validation. WGS, MLST, SLST, and 3-LST represent sequencing-based methods, which have witnessed an evident revolution in this horizon, introducing new perspectives not previously attainable with traditional methods. Currently, many countries have embraced WGS or are in the process of integrating it for regular surveillance of several pathogenic bacteria. Thus, a comparable course of action is imperative for the surveillance of *A. baumannii* in the near future.

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