Molecular Identification and Genotyping of *Pseudomonas aeruginosa* Isolates Using Double-Locus Sequence Typing (DLST) Analysis

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**ABSTRACT**

The opportunistic pathogen *Pseudomonas aeruginosa* is responsible for many life-threatening nosocomial and environmental acquired infections. In this study, the antibiotic susceptibility, molecular identification and genotyping of *P. aeruginosa* were performed. A total of 100 isolates of *P. aeruginosa* out of 523 specimens (19.12%) from clinical and environmental sources were analyzed. The results of antibiotic sensitivity profile grouped these isolates into non-multidrug resistant (non-MDR), multidrug resistant (MDR), and extensively drug resistant (XDR) by the ratios of 23.7%, 40.5% and 35.6%, respectively. The double-locus sequence typing (DLST) scheme was employed for genotyping a collection of 36 isolates of *P. aeruginosa* recovered from different clinical and environmental sources. Isolates were successfully typed into 19 different DLST genotypes with high discriminatory power (0.9206). In addition, three new alleles were recognized for the locus ms172 namely; ms172-128, ms172-129 and ms172-130. Thus, three novel DLST genotypes of this pathogen have been identified, which were exclusively belonged to the clinical sources, were exhibited XDR pattern. The phylogenetic analysis differentiated these genotypes into seven different genetic clusters supported by strong bootstrap values. However, there were indications of distinct evolutionary origins for some of the un-clustered genotypes (5/8). The DLST type 32-39 was the predominant cluster in this region with a majority of XDR pattern. Hereby, it can be concluded that DLST was capable of discriminating the phenotypically and genetically related isolates of *P. aeruginosa* and offered a reliable phylogenetic analysis.

1. INTRODUCTION

The bacterial pathogen *Pseudomonas aeruginosa* is well-known as a Gram-negative, extremely versatile and important opportunistic human pathogen that causes significant healthcare- associated infections, particularly in immunocompromised patients (Burstein *et al.*, 2015). Also, it has been recognized as a ubiquitous microorganism due to its extraordinary survival capability that can adapt to a wide range of ecological niches including soil, water, sewage, hospitals etc (Hare *et al.*, 2012). This pathogen is responsible for many serious and frequent hospital as well as environmental acquired illnesses including;
urinary tract infections (UTIs), folliculitis and skin rashes, pneumonia, bacteremia, osteomyelitis and ear infections (Burstein et al., 2015, Park et al., 2015 and Gba et al., 2018). The *P. aeruginosa* notorious fame is markedly contributes to its remarkable power to produce resistance against most commonly prescribed antibacterial drugs (Firouzi-Dalvand et al., 2016). This is either through innate resistance to many of the commonly used antibiotics by mutation in chromosomally encoded genes or through horizontally acquired resistance (Valot et al., 2015, Miranda et al., 2015 and Gba et al., 2018). In spite of many efficient infection control policies, outbreaks and infections in hospitals have been increasingly reported worldwide due to the prevalence of multi-drug resistant (MDR) strains of *P. aeruginosa* with significantly rising in patients’ morbidity and mortality (Janam et al., 2011 and Khosravi et al., 2016). Therefore, in order to understand the epidemiology of *P. aeruginosa*, it might be important to address the antibiotic resistance pattern, genotyping and phylogenetic relationship of its isolates from different environmental and clinical sources.

To date, many genotyping techniques have been employed to study and characterize *P. aeruginosa* isolates. Although the electrophoresis-based typing methods of bacterial strain typing such as pulsed field gel electrophoresis (PFGE), random amplification of polymorphic DNA (RAPD) and amplified fragment length polymorphism (AFLP) have many advantages and have been long used for genotyping of this pathogen, they are relatively time-consuming and require extensive inter-laboratory comparison of the results as well as coordination of protocols (Tang and Stratton, 2013). Therefore, the DNA sequencing-based genotyping approaches are becoming increasingly popular for bacterial typing. In contrast to current fragment based methods, DNA sequencing methods yield accurate, reproducible and highly-sensitive sequence results. Moreover, sequence data can be easily stored in online databases and shared between laboratories and/or compared to well-established libraries (Li et al., 2009 and Chen et al., 2015). Among these DNA sequence-based techniques is the recently developed scheme of double-locus sequence typing (DLST) based on the DNA sequencing and nucleotide analyses of two extremely variable loci (ms172 and ms217) of *P. aeruginosa* genome (Pappa et al., 2017). This technique allows obtaining unambiguous and uniformly systematized definition of types, since each isolate could be assigned with a definitive type and the data can be stored in databases for the purpose of surveillance investigations and/or comparing results with future studies (Cholley et al., 2015).

The main objectives of this study were molecular identification, investigation of the antibiotic resistance pattern, application of an efficient and recently developed DNA sequence-based genotyping technique, the DLST, to investigate the local epidemiology of *P. aeruginosa* and to detect the DLST genotypes of this pathogen in different districts in Kurdistan region-Iraq and eventually to assess the phylogenetic diversity among *P. aeruginosa* isolates obtained from different clinical and environmental sources.

2. MATERIALS AND METHODS

2.1. Bacterial isolates

The collection of *P. aeruginosa* isolates was comprised of 100 isolates out of 523 specimens investigated. Samples were obtained from both clinical (n=385) and environmental (n=138) sources, over a period from April to December 2014. This collection included: 86 clinical and 14 environmental isolates. Clinical specimens
were obtained from patients of both genders (various ages) at some of the major public hospitals in Kurdistan Region-Iraq including; the Emergency and Azadi (Teaching) Hospitals/Duhok, Rizgari (Teaching) Hospital/Erbil, Teaching, Emergency and General Medicine Hospitals/Sulaymaniyah, whereas the environmental samples were obtained from a number of animal farms (soils) at these three districts. The clinical specimens were taken from different wards at these hospitals and from various infection sources; such as infected burns and wounds, ear infections, urine samples and respiratory tract (bronchial-alveolar-lavage and sputum).

2.2. Bacterial identification

The classical identification of *P. aeruginosa* isolates was performed according to the methods described by Khan *et al.*, (2008), with minor modifications, using both selective and non-selective culture media, catalase and oxidase tests. Samples were first cultured into brain heart infusion broth and incubated at 37°C for 24 hours. Each sample was then streaked onto the MacConkey agar (Difco) plate and was kept incubated for 24 hours at 37°C. An isolated suspected colony of the organism was selected and streaked onto Pseudomonas Isolation Agar (Sigma-Aldrich-UK) plate and incubated overnight at 37°C. Isolates that showed growth and colony characteristics of this bacterium at 37°C on this medium, and showed positive reaction to catalase and oxidase tests with gram negative feature, were primarily identified as *P. aeruginosa*. A single pure bacterial colony was then cultured into 5ml of nutrient broth and kept incubated for 24 hours at 37°C. Bacterial growth was eventually stored in 50% glycerol and kept at -20°C before use.

2.3. Antimicrobial susceptibility test

The Kirby Bauer disc diffusion method on Mueller-Hinton agar was used for screening the antibiotic resistance pattern according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2014). All isolates were subjected to ten commonly used antibiotics (Oxoid, Basingstoke, UK); including imipenem 10µg, ciprofloxacine 5µg, amikacin 30µg, ceftazidime 30µg, erythromycin 15µg, gentamicin 10µg, cefotaxime 30µg, ticarcillin 75µg, amoxacillin/clavulanic acid 30/10µg, and cefixime 5µg discs.

Isolates that showed resistant to at least one agent in three or more antimicrobial categories were defined as multi-drug-resistant, while isolates showed susceptibility to only one or two in all antimicrobial categories were considered as extensively drug-resistant (Magiorakos *et al.*, 2012).

2.4. DNA extraction and molecular confirmation of isolates

The High yield DNA Purification Kit was implemented to extract DNA from all 100 bacterial isolates as recommended by the supplier’s manual (Cinnagen-Iran). Molecular confirmation was performed for all isolates of *P. aeruginosa* by PCR using a primer pair PA-SS (Table 1) as previously described by (Spilker *et al.*, 20104) with minor PCR condition adjustments. The amplification reaction comprised of master mixture (12.5µl) (Promega/USA), primers (10pmol/µl) forward and reverse (1µl each), DNA sample (2µl) with a final concentration of 25-50ng/µl. The final reaction volume was completed to 25µl by adding 8.5µl of sterile nuclease free water. A Thermal Cycler (Applied Biosystems-2720) was used and the amplification conditions were; 1 cycle at 94°C for 5min as initial denaturation, 30 cycles starting with denaturation at 94°C for 1min, annealing at 58°C for 1min and amplification at 72°C for 1min with the final extension step at 72°C for 5min (Spilker *et al.*, 20104).
| Primer | Sequences (5′-3′) | Product size | Target | Reference |
|--------|------------------|--------------|--------|-----------|
| PA-SS  | F- GGGGGATCTTCGGACCTCA | 956bp | 16SrDNA | Spilker et al., 2004 |
|        | R- TCTTAGAGTGCCCCACCCG | | | |
| ms172  | F- GGATTCTCTCGACGAGGT | variable | ms172 locus | [http://www.dlst.org/CSS/i mages/LTM_PR_029.pdf](http://www.dlst.org/CSS/i mages/LTM_PR_029.pdf) |
|        | R- TACGTGAACCTGACGTGGTG | | | |
| ms217  | F- TTCTGGCTGTCGAGCTGAT | variable | ms217 locus | [http://www.dlst.org/CSS/i mages/LTM_PR_029.pdf](http://www.dlst.org/CSS/i mages/LTM_PR_029.pdf) |
|        | R- GAACAGCGTCTTTTTCCTGC | | | |

2.5. PCR amplification and sequencing of DLST loci

PCR amplification was performed for each of the two loci ms172 and ms217 using primers reported in (Table 1) as previously described by Basset and Blanc (2014) using a full DLST protocol available on the DLST website (http://www.dlst.org/Paeruginosa/) for genotyping of *P. aeruginosa*. Briefly, the PCR amplification reactions for each locus was carried out in 50μl reaction mixture composed of 25μl of master mix, 2μl of each of forward and reverse primers (10pmol/μl), 0.4μl of Taq DNA polymerase (1U/μl), 2μl of (1:7) diluted PWO DNA Polymerase (1U/μl), 16.6μl of dH₂O and 2μl of genomic DNA (25-50ng/μl). The PCR amplification conditions were as follow: initial denaturation at 94°C for 5 min; 35 cycles consisting of 30s at 94°C, 30s at 60°C (ms172) or 64°C (ms217), 45s at 72°C; with a final extension at 72°C for 5min (14). Gel electrophoresis was performed using 1.2% agarose gel. Gels were stained with ethidium bromide and evaluated for success and specificity of one clear band visible per PCR. The PCR products for each locus were purified with MinElute PCR Purification Kit (50) (Qiagen), according to the supplier’s protocol. Sequencing reactions were carried out with a reverse primer for each locus independently with a BigDye Terminator V3.1 (Applied Biosystems, USA). The purified PCR products were then sequenced using a 4 capillary ABI3130 Genetic Analyzer for Sanger sequencing at the DNA Sequencing Facility Unit of the Scientific Research Center, College of Science, University of Duhok.

2.6. Data analysis, Allele and DLST type assignment

The nucleotide sequences were imported, assembled, edited, trimmed and verified in Geneious, version R8.1 (Kearse et al., 2012) and then saved as Fasta format for sequence alignment and phylogenetic purposes. The *P. aeruginosa* DLST website at (http://www.dlst.org/Paeruginosa/) was used to assign numbers to each distinct allele within a locus after submitting good quality trace file sequences for each of ms172 and ms217 loci per each tested isolate. Thus, each individual isolate was given double numbers known as allelic profile that yielded to its DLST type. Any allele that did not match with the existing alleles in the database was designated and registered as “new” and a new allele number was given by the database.
The discriminatory power for DLST typing scheme, in the current study, was obtained through the index of diversity (ID) which was calculated by using an online tool at (http://www.comparingpartitions.info/).

3. RESULTS AND DISCUSSION

In the current study, out of 523 specimens investigated searching for the presence of *P. aeruginosa*, a total of 100 isolates of this pathogen have been identified from both hospitalized patients at Duhok, Erbil and Sulaymaniyah hospitals and from environmental samples, representing 19.12% of the total investigated specimens. This prevalence rate was found to be slightly lower than that previously reported in this region by Hassan *et al.*, (2012) in which *P. aeruginosa* accounted for 26.1% of the total nosocomial infections in Kurdistan. However, remarkably higher prevalence rates have been reported by studies elsewhere in Iraq, such as 46.6% in Najaf (Al-Dahhan, 2015) and 67.7% in Basrah (AbdulQader *et al.*, 2015). Probable variation and fluctuations in the prevalence rate of this bacterium over time and from study to another might be obvious due to the variation in study period, geographical location, sample type, hospital level, clinical department, economic condition, variation in the resistance pattern of this opportunistic pathogen in each hospital and/or province as well as type and quality of antibiotics used.

In this investigation, vast majorities (86%) of the isolates were from clinical sources with a prevalence rate of 22.3% of the total investigated clinical specimens; meanwhile, 14% were from the environmental source with a prevalence rate of 10.1% of the total investigated environmental specimens. Isolates that have been identified as *P. aeruginosa* at the initial stages through means of selective agar cultures, biochemical and microscopic tests, were further confirmed at the molecular level by 16S rDNA-based PCR assay using species-specific primer pair (PA-SS). This primer pair yielded a DNA band of 956bp (Figure 1) corresponding with the specific band of *P. aeruginosa* across all tested isolates. Thus, all were identified and confirmed at the molecular level as *P. aeruginosa*.

Figure 1: Example of molecular identification of *P. aeruginosa* by PCR based assay using PA-SS primer pair. Lanes M represent DNA ladder (10kb). Numbers from 1-36 represent the tested samples. Electrophoresis was carry out using 1.2% agarose gel at 85V for 1hour.
The results of antibiotic sensitivity test of these isolates across 10 commonly used antibiotics were as follow: imipenem (18.8%), ceftazidime (26.7%), amikacin (39.6%), ciprofloxacin (42.5%), cefotaxime (44.5%), gentamicin (58.4%), cefixime (64.3%), ticarcillin (71.2%), amoxacillin/clavulanic acid (73.2%), and erythromycin (82.1%). These results were compatible with those obtained in the investigation of the antibiotic susceptibility profile of this pathogen from both clinical and hospital environmental samples in Nasiriyah, Iraq (Al-Zaidi, 2016). Also, variable results have been reported elsewhere in the antibiotic resistant pattern of 88 strains of P. aeruginosa against twelve commonly used antibiotics from 10 different wards of the university hospital centre of Abidjan- Côte d’Ivoire (Gba et al., 2018).

It has been reported that a proportion of the resistant P. aeruginosa as high as 34% is considered alarming and worrying (Pappa et al., 2017), therefore, based on the antibiotic sensitivity results obtained in the current study, surveillance investigations of such resistant pathogen in this region are recommended. The results of high drug resistance rate in the current investigation indicated that patients might be in high antibiotic resistance risk due to the circulation of highly drug-resistant strains of this pathogen in our hospitals as well as in the environment, which might also serve as a potential source of bacterial pathogens with multidrug resistant.

The prevalence of such high resistance rate in this region could be explained by the acquisition of highly resistant strains of this pathogen among clinical and environmental sources. The frequent and widely prescription and administration of antibiotics, the abuse and uncontrolled use of antibiotics by self-medication as well as flooding of the markets by antibiotics which might not always WHO pre-qualified that casts doubt on their true quality.

The results of PCR amplifications of the ms172 and ms217 loci revealed clear single amplified DNA bands with variable size which indicated that they are highly polymorphic loci (Figure: 2).

![Figure 2: Example of PCR amplification of ms172 and ms217 loci. Lanes M represent DNA ladder (10kb). Numbers from 1-18 represent the tested samples. Electrophoresis was carry out using 1.2% agarose gel at 85V for 1hour.](image-url)
The nucleotide sequences of both ms172 and ms217 loci have been determined for a collection of 36 isolates; represented different infection sources, environment and antibiotic resistant pattern. The nucleotide sequences obtained for each locus (allelic sequences) have been tested for their specificity implementing the BLAST algorithm of the GenBank database at the National Center for Biotechnology Information (NCBI) and then were submitted to the P. aeruginosa DLST database at (http://www.dlst.org/Paeruginosa/) to assign allelic numbers. The allelic numbers were assigned to each distinct allele within a locus. Data revealed that 13 different alleles for the locus ms172 and 15 different alleles for the locus ms217 have been determined. Furthermore, three new alleles for the locus ms172 study have been identified in the current namely; ms172-128, ms172-129 and ms172-130 which were previously not reported. These novel alleles were deposited at Allele definitions of ms172 section at the P. aeruginosa DLST database at (http://www.dlst.org/Paeruginosa/). Thus, three novel DLST genotypes have also been identified with the combinations of DLST128-60, DLST129-79, and DLST130-17 respectively, which were previously not described. The DLST technique has successfully typed all these isolates into 19 different DLST genotypes (Table 2).

Table 2: location, Site, DLST types and antibiotic pattern of P. aeruginosa isolates.

| Isolate | Location | Site      | DLST Type ms172-ms217 | Antibiotic pattern |
|---------|----------|-----------|-----------------------|--------------------|
| Pa-1    | Duhok    | Soil      | 16-9                  | non-MDR            |
| Pa-2    | Erbil    | Burn      | 16-130                | MDR                |
| Pa-3    | Erbil    | Wound     | 16-9                  | non-MDR            |
| Pa-4    | Erbil    | Middle Ear| 4-101                 | MDR                |
| Pa-5    | Erbil    | Urine     | 28-130                | non-MDR            |
| Pa-6    | Erbil    | Respiratory| 8-78                 | XDR                |
| Pa-7    | Erbil    | Soil      | 16-9                  | XDR                |
| Pa-8    | Duhok    | Burn      | 16-11                 | MDR                |
| Pa-9    | Duhok    | Wound     | 16-11                 | XDR                |
| Pa-10   | Duhok    | Middle Ear| 28-9                 | non-MDR            |
| Pa-11   | Duhok    | Urine     | 32-39                 | non-MDR            |
| Pa-12   | Duhok    | Respiratory| 23-22                | XDR                |
| Pa-13   | Sulaymaniyah | Soil     | 16-9                  | XDR                |
| Pa-14   | Sulaymaniyah | Burn    | 32-22                 | XDR                |
| Pa-15   | Sulaymaniyah | Wound    | 6-54                  | MDR                |
| Pa-16   | Sulaymaniyah | Middle Ear| 4-101                | XDR                |
| Pa-17   | Sulaymaniyah | Urine    | 32-39                 | MDR                |
| Pa-18   | Sulaymaniyah | Respiratory| 32-39                | XDR                |
| Pa-19   | Duhok    | Wound     | 128*-60               | XDR                |
| Pa-20   | Sulaymaniyah | Burn    | 16-11                 | MDR                |
This sequence typing scheme showed a high discriminatory power in distinguishing each isolate from others which was 0.9206. This was evaluated according to the Simpson’s index of diversity (ID), in which an index of more than 0.90 is considered as an ideal indication that the typing method has the ability to distinguish each isolate from all others. In contrast, an index of zero is the indication that all isolates are of an identical type (Cholley et al., 2015). Furthermore, employing this sequence typing technique not only allowed the discrimination of the studied isolates from each other, but also was capable of identifying new DLST genotypes which were previously not reported. This genotyping scheme is available at the DLST website (www.dlst.org), which allows researchers to submit novel types directly and coordinately (Basset and Blanc, 2014).

To the best of our knowledge, this is the first report investigating P. aeruginosa DLST types and elucidating the predominant clones of this pathogen in this region. All novel DLST genotypes of P. aeruginosa were found to exhibit XDR pattern. Additionally, all new types were exclusively found to be from clinical sources; including wound, burn and urine. In contrast, none of the environmental isolates were presented new alleles. More so, the DLST scheme was successfully capable of discriminating the environmental isolates in different geographical locations (Duhok, Erbil and Sulaymaniyah), from clinical isolates by clustering them into a separate genetic cluster and all were typed as DLST16-9 genotype.

The allelic nucleotide sequences of the two loci (ms172 and ms217) were concatenated and used to construct phylogenetic tree among these strains, in order to allow better discrimination and greater tree robustness. Thus, the genotypes were differentiated into seven different clusters in the phylogenetic tree, supported by strong bootstrap values (Figure 3). However, eight genotypes were not grouped with any of these genetic clusters. This might indicate distinct evolutionary origins for some of these un-clustered genotypes (5/8; 62.5%) in Kurdistan, as they were found to be

| Pa-21 | Sulaymaniyah | Burn | 129*-79 | XDR |
| Pa-22 | Sulaymaniyah | Wound | 10-76 | XDR |
| Pa-23 | Sulaymaniyah | Burn | 32-39 | XDR |
| Pa-24 | Sulaymaniyah | Burn | 9-124 | XDR |
| Pa-25 | Sulaymaniyah | Burn | 32-39 | MDR |
| Pa-26 | Duhok | Respiratory | 28-130 | XDR |
| Pa-27 | Sulaymaniyah | Burn | 32-39 | non-MDR |
| Pa-28 | Duhok | Middle Ear | 23-22 | XDR |
| Pa-29 | Erbil | Respiratory | 4-101 | MDR |
| Pa-30 | Sulaymaniyah | Burn | 32-39 | non-MDR |
| Pa-31 | Sulaymaniyah | Wound | 32-39 | XDR |
| Pa-32 | Duhok | Respiratory | 23-39 | XDR |
| Pa-33 | Sulaymaniyah | Middle Ear | 32-39 | MDR |
| Pa-34 | Duhok | Respiratory | 10-25 | XDR |
| Pa-35 | Duhok | Urine | 21-96 | non-MDR |
| Pa-36 | Sulaymaniyah | Urine | 130*-17 | XDR |

* New alleles identified in this study.
unrelated and shared no alleles with other genetic clusters. Furthermore, one of these un-clustered genotypes was assigned as new (DLST129-79).

![Figure 3: Phylogenetic relationships of P. aeruginosa isolates.](image)

Also, the phylogenetic analysis revealed that the DLST-32-39 was the predominant cluster (9/36; 25%) in this region. In addition, the vast majority of the predominant cluster isolates (7 out of 9; 77.7%) were found to exhibit an XDR pattern and identified from different infection sources. This may be due to their adaptation and fitness to a variety of environmental niches that make them squeeze away from the immune as well as the antibiotic barriers. Identification of the new types as well as the predominant cluster of this pathogen with extensively drug-resistant patterns might further confirm that patients are in high antibiotic resistance risk. Therefore, applying efficient infection control policies and measures might be required in order to minimize hospital as well as environmental related infections due to particular persistent MDR and/or XDR clones of *P. aeruginosa*. In the present study, the results of DLST typing indicated that different DLST genetic profiles might serve as a driving force and a potential source for the distribution of the resistant strains in our hospitals and environments as well. However, further investigations might be
required to provide important information in terms of the possible correlations between a particular DLST clusters and the production as well as the transmission of the resistant mechanisms.

The major goal of investigating the local epidemiology is to identify the transmission of isolates. The markers used for this purpose are required to be stable through the period of investigations. The new, cost effective and promising DLST typing scheme has been proved to produce a stable results for several months or even years for comparison of isolates recovered throughout the duration of investigations (Pappa et al., 2017). Therefore, it can be implemented to study the epidemiology of isolates with low cost and in a short of time as well. In the present study, the DLST technique was implemented to assess the diversity of *P. aeruginosa* isolates through a period of one year and it was proved to be reliable and powerful molecular tool to distinguish and discriminate clusters of isolates belonging to the same infection sources and environments. Also, it does not require a special search algorithm as well as no assembly procedure is needed. Additionally, a web-based database (http://www.dlst.org), which has recently been developed, can be used to identify allele profiles and DLST types as well as to identify new alleles and types in unambiguous way. Also, in terms of the *P. aeruginosa* population, this database could be used as a reference and implemented to assess the local and/or international diversity of this pathogen.

5. CONCLUSIONS

Lastly, it can be concluded that *P. aeruginosa* strains, which have been isolated from different geographical and infection sources, have showed variations in their antibiotic resistant pattern, allelic profiles and DLST types. This may reflect their diversity as well as their adaptive ability to different environmental niches and diseases caused. The exact identifications and characterization of this pathogen in clinical settings and assessing the phylogenetic relationship among its strains are crucial for epidemiological investigations. Thus, this study may consequently have significant role in diagnosis, antibiotic treatment, and infection control policies as well. The DLST typing scheme was successfully used for genotyping and to investigate the local epidemiology of a collection of *P. aeruginosa* isolates in Kurdistan. This technique was also found to be highly reproducible, typable as well as to have high discriminatory power to distinguish between these isolates.

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Conflict of Interest

The author has no conflicts of interest to disclose.

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