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Evaluation of Multi-antibiotic Resistance Index (MAR) and Molecular Characterization of *Pseudomonas aeruginosa* Isolated in Pathological Products from Chad

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Pseudomonas aeruginosa is a bacterium present in several ecological niches causing opportunistic infections in humans. In developing countries like Chad, this bacteria is responsible for various human pathologies. High-priority pathogens, such as *Salmonella* and *Shigella*, represent a particularly heavy burden in low- and middle-income countries, as do *Pseudomonas aeruginosa* and *Staphylococcus aureus*, which pose serious problems in health centers. The objective of this study was to evaluate the multi-antibiotic resistance index and to carry out a molecular characterization of *Pseudomonas aeruginosa* isolated in pathological products from Chad using by rep-PCR (GTG)5. Antibiotic resistance phenotypes were obtained by interpreting the results of our previous study on antimicrobial resistance of *Pseudomonas aeruginosa* isolated from human infections in N'Djamena, Chad. In this study, two phenotypes were obtained that is XDR and MDR. Thus, 24/36 of strains came from urine and 29/36 strains were MDR. The multi-antibiotic resistance (MAR) indexes varied from 0.33 to 0.86. The rep-PCR allowed good discrimination of *Pseudomonas aeruginosa* strains in this study. These isolates were grouped based on characteristic bands appearing in their genetic fingerprint profiles. In this study, Pseudomonas aeruginosa strains isolated from urinary tract infections and pus were classified as XDR and MDR type strains.

Keywords: Urinary tract infections; pus; Pseudomonas aeruginosa; molecular characterization; Chad.

1. INTRODUCTION

The discovery of antibiotics led to an increase in life expectancy during the 20th century and a significant improvement in patient care. However, antimicrobial resistance constitutes a major weakness in the treatment of infections leading to a sharp increase in morbidity and mortality rates [OMS, 2024]. Thus, Gram-negative bacilli (GNB) are the infectious agents most involved in human and animal pathologies [Kaboré et al. 2022a, Ouédraogo et al. 2022]. However, high-priority pathogens, such as *Salmonella and Shigella*, represent a particularly heavy burden in low- and middle-income countries, as do *Pseudomonas aeruginosa* and *Staphylococcus aureus*, which pose serious problems in health centers [OMS, 2024]. Among GNBs, *Pseudomonas aeruginosa* is responsible for 9.2% of nosocomial infections, placing it 4th on the list of high priority species for antimicrobial resistance (AMR), just after *Salmonella* Typhi, *Shigella* spp. and *Enterococcus faecium* [OMS, 2024, Amazian et al. 2010]. This microorganism may also cause community acquired pneumonia, bacteriemia, urinary tract infections, folliculitis, keratitis, endophthalmitis, otitis, enterocolitis, osteomyelitis or meningitis among other types of infections. However, GNB have a great capacity for acquisition and accumulation of antimicrobial resistance factors accompanied by expression of β-lactamases including carbapenemases having the most extensive substrate profiles of all β-lactamases and having a particular interest in a clinical setting [Nordmann 2010, Kaboré et al. Kaboré et al. 2022a]. This situation is a real threat to global health and a major challenge in search for new bioactive molecules against multi-antimicrobial resistant microbial strains [Ouango et al. 2024a]. Faced with these apparent problems, new strategies must be used to circumvent the scale of AMR in sub-Saharan countries [Ouango et al. 2024b]. However, over the last decade, the introduction of automated identification instruments has improved the reliability of medical diagnoses, but discrepancies in sensitivity and specificity exist between the different technological methods used [Jin et al. 2011]. However, there are molecular identification methods using universal primers, such as the repetitive element sequence-based PCR technique or rep-PCR [Cissé et al. 2019, Shin et al. 2023]. This technique is now widely used to study the diversity of microorganisms thanks to its discriminatory power based on the genetic fingerprints obtained by rep-PCR. It can also be applied to studies of microbial ecology and microbial evolution [Cissé et al. 2019, Shin et al. 2023, Ishii et al. 2008]. Thus, the objective of this study was to evaluate the multi-antibiotic resistance index and to carry out a molecular characterization of *Pseudomonas aeruginosa* isolated in pathological products from Chad using by rep-PCR (GTG)5.

2. MATERIALS AND METHODS

2.1 Strains Collection Site

Pseudomonas aeruginosa isolated were collected in three hospital centers in N'Djamena while respecting microbiological safety

instructions (Fig. 1). Strains were revived for performance of the antibiogram according to Lowbury and Collins [Lowbury and Collins 1955].

2.2 Determination of Antibiotic Resistance Phenotype

Antibiotic resistance phenotype was obtained by interpreting the results of our previous study on antimicrobial resistance of *Pseudomonas aeruginosa* isolated from human infections in N'Djamena, Chad [Ahmat et al. 2023]. The type of resistance was determined according to Cosentino et al. [Cosentino et al. 2023].

2.3 Evaluation of Multi-antibiotic Resistance Index

Multi-antibiotic resistance index (MAR) was determined according to Das et al. [Das S, et al. 2022]. MAR index was calculated using the following formula:

$$\text{MAR} = \frac{\textit{Number of antibiotics ineffective on the strain}}{\textit{Total number of antibiotics tested on the strain}}$$

The strains were classified according to their MAR value as Sandhu et al. [Sandhu et al. 2016].

2.4 Molecular Characterization of *Pseudomonas aeruginosa*

This part consists of studying the genetic fingerprint of *Pseudomonas aeruginosa* isolated using the universal primer (GTG)5.

2.4.1 Extraction of bacterial DNA

Bacterial DNA was extracted using the heat shock technique [Kaboré et al. 2022b]. To do this, a mass of each bacterial strain was introduced into an Eppendorf tube containing 350 µL of ultra-pure water. The whole was placed at -4 °C for 10 min for freezing. After freezing, the tubes were placed in water bath at 100°C for 10 min. The tubes were cooled to room temperature in the laboratory for 15 min. After cooling, the tubes were centrifuged at 12000 rpm for 15 mm. The supernatants containing the nucleic acids were collected in new Eppendorf tube then stored at 4°C for subsequent uses [Kaboré et al. 2022b].

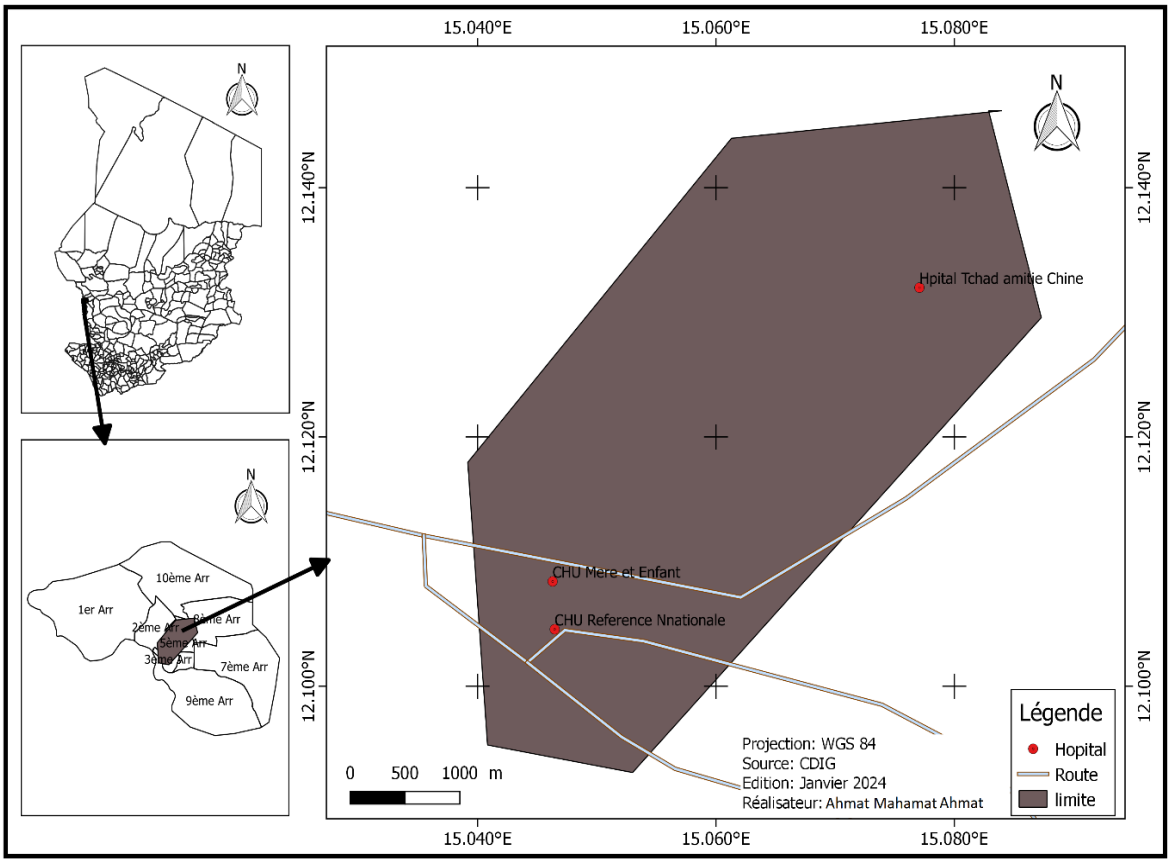

Fig. 1. Location of strain collection sites

2.4.2 Preparation of reaction mixture

The rep-PCR was carried out using the universal primer GTG5 (5'-GTGGTGGTGGTGGTG-3'). The total volume of reaction mixture was 25 µL composed of 4 µL of Master Mix (5X FIREPol® Master Mix), 2 µL of primer (GTG)5 with a concentration of 10 µmol per mL, 2 µL of MgCl2 (2.5 Mm), 2 µL of DNA and 15 µL of ultrapure water (Water free nuclease).

2.4.3 Amplification of DNA extracts

Amplification was carried out in three steps using the Mastercycler nexus gradient type thermal cycler (Eppendorf). PCR program described by Cissé et al. [2019] was used. This is a first initial denaturation step at 94°C for 4 min, followed by a second step of 30 cycles each consisting of denaturation at 95°C for 30s, hybridization at 45°C for 60s, an elongation at 65°C for 8 min and finally a third final elongation step at 65 °C for 16 min. PCR products were stored cool at 4°C.

2.4.4 Preparation of agarose gel

The agarose gel was prepared by dissolving 1 g of agarose in 100 mL of 0.5X Tris Acetate EDTA (TAE) buffer in the microwave (SHARP R65G10) until completely dissolved. After cooling the agarose solution, a volume of 10 µL of ethidium bromide (BET) at 10 mg/mL was added. The mixture obtained was poured into a horizontal tank containing a comb. After solidification the comb was removed. The gel was emerged in an ENDURO-GEL-XL type migration tank according to Kaboré et al. [2022b].

2.4.5 Electrophoresis of PCR products

The migration of amplicons was carried out under an electric field of 40 Volts and 30 mA for 2 hours. Thus, a volume of 10 µL of each amplicon was introduced into a well of gel. A 100 bp marker was used for band size comparison in each genetic profile of strains studied. After migration, gel was visualized and photographed by UVP PhotoDoc-It Imaging System Kaboré et al. [2022b].

2.4.6 Gel image processing

Visual observation with the naked eye of genetic fingerprint profile obtained from each strain by rep-PCR was made for strain grouping. Strains presenting the same genetic fingerprint profiles were affiliated to same group according to Cissé et al. [Cissé et al. 2019].

2.4.7 Construction of phylogenetic tree

Data obtained by genetic fingerprints of each strain were used. Data obtained were subjected to processing on DendroUPGEMA site using the Jaccard similarity index (Jaccard index, Tanimoto) according to Kaboré et al. [2022b]. *Pseudomonas aeruginosa* ATCC 27853 was used as a reference strain.

2.5 Statistical Analyzes

EXCEL 2021 software was used to calculate MAR index values and manage tables and figures. Descriptive statistics were used for data interpretation.

3. RESULTS AND DISCUSSION

3.1 Resistance Phenotype and Multi-Resistance Index of Strains to Antibiotics

The resistance phenotypes, MAR index values, origin and category of antibiotic resistance of the strains are recorded in Table 1. Fig. 2 presents the numbers of strains according to the origin and type of resistance to antibiotics. antibiotics. Of the strains studied, 24/36 came from urine and 29/36 were MDR. This result reveals a high prevalence of infections and the presence of multi-resistant strains in Chad. Thus, the MAR indexes varied from 0.33 to 0.86. Additionally, the multiple antibiotic resistance (MAR) index provides insight into the level of contamination and antibiotic use in the environment from which the isolates were obtained. It allows strains to be classified according to MAR indices into less resistant strains (MAR < 0.2) and highly resistant strains (MAR > 0.2). MAR index values are an important indicator of the potential risk of emergence of antibiotic resistance in the environment [Cosentino et al. 2023, Das S, et al. 2022]. On the other hand, in this study, all strains had MAR indices greater than 0.2, thus indicating a higher risk of exposure of patients to antibiotics or contamination of sources (water and food) frequently exposed to antibiotics [Fallah et al. 2024]. These results further highlight concern about the spread of antibiotic resistance in various environments in Chad, which could have adverse effects on public health and food security [Beshiru et al. 2022]. Thus, in Chad, if nothing is done there could be the appearance of

DTR type strains (difficult-to-treat resistance) referring to non-sensitivity to all first-line drugs [Cosentino et al. 2023]. This emergence of new strains of *Pseudomonas aeruginosa* type (DTR) is a crucial challenge and must be considered in the burden of clinical care [Cosentino et al. 2023]. *Pseudomonas aeruginosa* is a gram-negative opportunistic pathogenic bacterium and a frequent inducer of urinary tract infections in human [Wu and Li 2015]. The LPS of *Pseudomonas aeruginosa* is less toxic than other gram-negative rods, which facilitates the establishment of chronic infections by eliciting a low inflammatory response [Wu and Li 2015, Hassuna et al. 2024]. The increasing prevalence of chronic and hospital-acquired infections produced by multidrug-resistant (MDR) or extensively drug-resistant (XDR) *Pseudomonas aeruginosa* strains is associated with significant morbidity and mortality [Sastre-Femenia et al. 2024, Osundiya et al. 2013]. This growing threat results from the extraordinary capacity of this pathogen for developing resistance through chromosomal mutations and from the increasing prevalence of transferable resistance determinants, particularly those encoding carbapenemases or extended-spectrum β-lactamases (ESBLs) [Shank et al. 2019, Magiorakos et al. 2012]. *Pseudomonas* aeruginosa has a nonclonal epidemic population structure, composed of a limited number of widespread clones which are selected from a background of a large quantity of rare and unrelated genotypes that are recombining at high frequency. Noteworthy, the vast majority of infections by MDR, and specially XDR, strains are produced by these and few other clones worldwide [Sastre-Femenia et al. 2024, Osundiya et al. 2013, Shank et al. 2019]. *Pseudomonas aeruginosa* is one of the most frequent and severe causes of acute nosocomial infections, particularly affecting immunocompromised. Indeed, *Pseudomonas aeruginosa* is the number one pathogen causing ventilator associated pneumonia and burn wound infections, both associated with a very high (>30%) mortality rate [Kreusch et al. 2003]. Likewise, *Pseudomonas* aeruginosa is the most frequent and severe driver of chronic respiratory infections in patients suffering from cystic fibrosis or other chronic underlying diseases such as bronchiectasis and chronic obstructive pulmonary disease [Oliver et al. 2003].

3.2 Grouping Isolates Based on Genetic Fingerprint Profile

The rep-PCR allowed good discrimination of *Pseudomonas aeruginosa* strains in this study. These isolates were grouped based on characteristic bands appearing in their genetic fingerprint profiles following visual observation (Fig. 3). This observation was confirmed by the phylogenetic tree (Fig. 4). Groups 16,17 and 33 were made up of 2 representatives while all other groups were made up of a single representative. The primer (GTG)5 was used for discrimination of subspecies of *Bacillus cereuis* and *Lactoballus lactis* respectively by Cissé et al. [Cissé et al. 2019] and Alegria et al. [Alegria et al. 2010]. Phylogenetic tree obtained presents the relationship between the different strains studied thanks to their genetic fingerprint profile in comparison with the reference *Pseudomonas aeruginosa* ATCC27853. The relationship is determined by the Cophenetic Correlation Coefficient (CP = 66%). Thus, from the dendrogram the reference of isolate *Pseudomonas aeruginosa* ATCC27853 is positioned at the same level as the P29 strain.

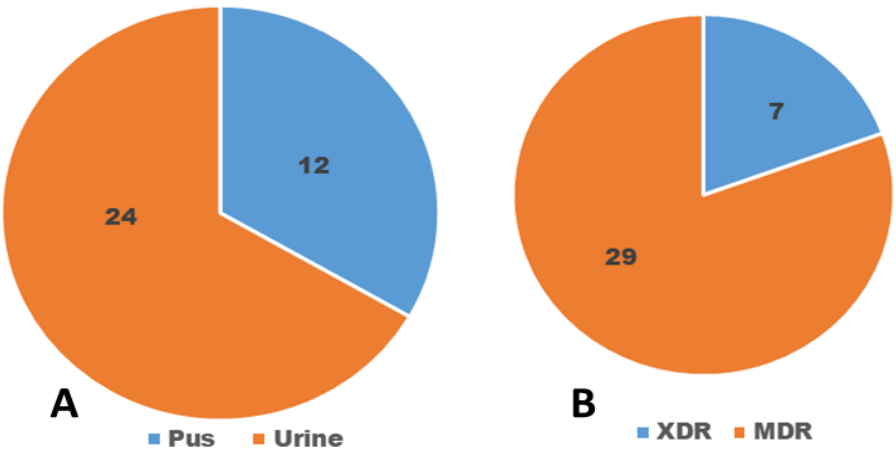

Fig. 2. Origin (A) and type of antibiotic resistance of strains (B)

Table 1. Resistance phenotype of *Pseudomonas aeruginosa*

Isolates	Antibiotic susceptibility phenotype	MAR	Category	Pathological product
P01	$S^{PIP, IMI, TZP, GMN, CIP, LEV, FOS, COL}$ $R^{AUG, CRO, CPM, CAZ, AZT, AK, TOB}$	0.47	MDR	Urine
P02	$S^{IMI, TZP, GMN, TOB, LEV, FOS, COL}$ $R^{AUG, CRO, CPM, CAZ, PIP, AZT, AK, CIP}$	0.53	MDR	Pus
P03	$S^{IMI, GMN, FOS, COL}$ $R^{AUG, CRO, CPM, CAZ, PIP, AZT, TZP, AK, TOB, CIP, LEV}$	0.73	MDR	Urine
P04	$S^{IMI, LEV, FOS, COL}$ $R^{AUG, CRO, CAZ, AZT, AK, PIP, TOB, CIP, TZP, CPM, GMN}$	0.73	MDR	Pus
P05	$S^{IMI, TZP, LEV, FOS, COL}$ $R^{AUG, CRO, CAZ, AZT, AK, PIP, TOB, CIP, GMN, CPM}$	0.66	MDR	Urine
P06	$S^{IMI, GMN, TZP, FOS, COL}$ $R^{AUG, CRO, CAZ, AZT, AK, PIP, TOB, CIP, CPM, LEV}$	0.60	MDR	Urine
P07	$S^{IMI, GMN, FOS, COL}$ $R^{AUG, CRO, CAZ, AZT, AK, PIP, TOB, CIP, CPM, LEV, TZP}$	0.60	MDR	Urine
P08	$S^{GMN, TOB, CIP, LEV, TZP, FOS, COL}$ $R^{AUG, CRO, CAZ, AZT, AK, PIP, CPM, IMI}$	0.53	MDR	Urine
P09	$S^{GMN, FOS, COL}$ $R^{AUG, CRO, CAZ, AZT, AK, PIP, TOB, CIP, CPM, LEV, TZP, IMI}$	0.80	MDR	Urine
P10	$S^{GMN, TOB, PIP, CRO, LEV, FOS, COL}$ $R^{AUG, CAZ, AZT, AK, CIP, CPM, IMI, TZP}$	0.53	MDR	Urine
P11	$S^{IMI, AZT, CPM, LEV, TZP FOS, COL}$ $R^{AUG, CRO, CAZ, AK, PIP, GMN, TOB CIP}$	0.53	MDR	Urine
P12	$S^{GMN, IMI, CIP, TZP LEV, FOS, COL}$ $R^{AUG, CRO, CAZ, AZT, AK, PIP TOB CPM}$	0.53	MDR	Pus
P13	$S^{GMN, IMI, CIP, LEV, FOS, COL}R^{AUG, CRO, CAZ, AZT, AK, PIP, TOB, CPM, TZP}$	0.60	MDR	Pus
P14	$S^{PIP, TOB, LEV, FOS COL}$ $R^{AUG, CRO, CAZ, AZT, AK, CPM, TZP GMN, IMI, CIP}$	0.66	MDR	Pus
P15	$S^{LEV, TZP, FOS, COL}$ $R^{AUG, CRO, CAZ, AZT, AK, PIP, TOB, CPM, GMN, IMI, CIP}$	0.73	MDR	Pus
P16	$S^{AZT, PIP, CAZ, GMN, TZP, AK, FOS, COL}$ $R^{AUG, CRO, CPM, IMI, CIP, TOB, LEV}$	0.46	MDR	Pus
P17	$S^{IMI, GMN, AK, LEV, CIP, TOB, FOS, COL}$ $R^{AUG, CRO, CPM, AZT, PIP, CAZ, TZP}$	0.46	XDR	Pus
P18	$S^{CIP, PIP, FOS, COL}$ $R^{AUG, CRO, CPM, IMI, TOB, LEV, CAZ, GMN TZP AK, AZT}$	0.73	MDR	Pus
P19	$S^{IMI, LEV, FOS, COL}$ $R^{AUG, CRO, CPM, AZT, PIP, CAZ, TZP, GMN, AK, CIP, TOB}$	0.73	MDR	Urine
P20	$S^{IMI, GMN, AK, LEV, CIP, TOB, FOS, COL}$ $R^{AUG, CRO, CPM, AZT, PIP CAZ, TZP}$	0.46	MDR	Urine
P21	$S^{FOS, COL}$ $R^{AUG, CRO, CPM, AZT, PIP, CAZ, TZP, IMI, GMN, AK, LEV, CIP, TOB}$	0.86	XDR	Urine
P22	$S^{IMI, AK, FOS, COL}$ $R^{AUG, CRO, CPM, TOB, LEV, CAZ, GMN, TZP, AZT, CIP, PIP}$	0.73	MDR	Urine
P23	$S^{IMI, COL}$ $R^{AUG, CRO, CPM, TOB, LEV, CAZ, GMN, TZP, AZT, CIP, PIP, FOS, AK}$	0.86	XDR	Urine
P24	$S^{FOS, COL, PIP}R^{AUG, CRO, CPM, TOB, LEV, CAZ, GMN, TZP, AZT, CIP, IMI, AK}$	0.80	MDR	Urine
P25	$S^{IMI, TZP, AK, FOS, COL}$ $R^{AUG, CRO, CPM, TOB, LEV, CAZ, GMN, AZT, CIP, PIP}$	0.66	MDR	Urine
P26	$S^{IMI, FOS, COL}$ $R^{AUG, CRO, CPM, TOB, LEV, CAZ, GMN, TZP, AZT, CIP, AK, PIP}$	0.80	MDR	Pus
P27	$S^{IMI, PIP, FOS, COL}$ $R^{AUG, CRO, CPM, TOB, LEV, CAZ, GMN, AZT, CIP, TZP, AK}$	0.73	MDR	Urine

Isolates	Antibiotic susceptibility phenotype	MAR	Category	Pathological product
P28	$S^{IMI, CIP, GMN, TOB, FOS, COL}$ $R^{AUG, CRO, CPM, LEV, CAZ, AZT, PIP, TZP, AK}$	0.60	MDR	Urine
P29	$S^{IMI, CIP, GMN, TOB, LEV, AK, TZP, FOS, COL}$ $R^{AUG, CRO, CPM, CAZ, AZT, PIP}$	0.40	XDR	Urine
P30	$S^{IMI, GMN, TOB, LEV, FOS, COL}$ $R^{AUG, CRO, CPM, CAZ, AZT, PIP, TZP, AK, CIP}$	0.60	MDR	Urine
P31	$S^{IMI, GMN, AK, TZP, COL}$ $R^{AUG, CRO, CPM, CAZ, AZT, PIP, CIP, TOB, LEV, FOS}$	0.66	MDR	Pus
P32	$S^{IMI, GMN, AK, AZT, TZP, PIP, TOB, LEV, FOS, COL}$ $R^{AUG, CRO, CPM, CAZ, CIP}$	0.33	XDR	Urine
P33	$S^{FOS, COL}$ $R^{AUG, CRO, CPM, CAZ, IMI, GMN, AK, AZT, TZP, PIP, CIP, TOB, LEV}$	0.86	XDR	Urine
P34	$S^{IMI, GMN, AK, TOB, PIP, TZP, LEV, FOS}$ $R^{AUG, CRO, CPM, CAZ, AZT, CIP, COL}$	0.47	MDR	Pus
P35	$S^{IMI, GMN, AK, FOS}$ $R^{AUG, CRO, CPM, CAZ, AZT, PIP, CIP, TOB, LEV, TZP, COL}$	0.73	MDR	Urine
P36	$S^{IMI, GMN, AK, TOB, PIP, TZP, CIP, LEV, FOS, COL}$ $R^{AUG, CRO, CPM, CAZ, AZT}$	0.33	XDR	Urine

Legend: MDR : Multi-Drug Resistance ; XDR : eXtensive Drug Resistance, AUG : Augmentin ; PIP = Piperacillin; TPZ = Piperacillin + Tazobactam; CRO = Ceftriaxon; CAZ = Ceftazidim; CPM = Cefepim; AZT = Aztreonam; IMI = Imipenem; GMN: Gentamicin; AK: Amikacin; TOB: Tobramycin; CIP = Ciprofloxacin; LEV = Levofloxacin; FOS = Fosfomycin; COL = Colistin

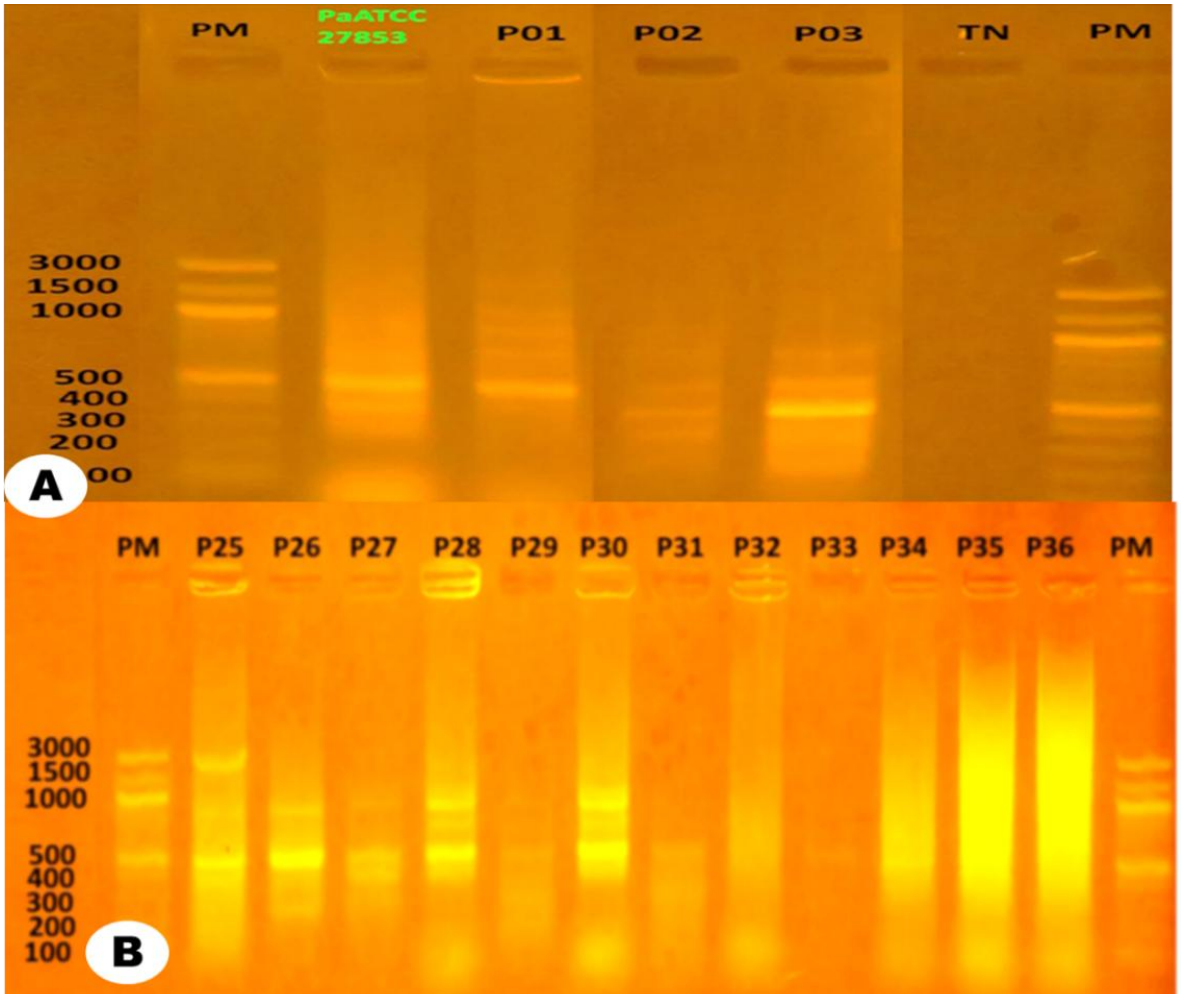

Fig. 3. Genetic fingerprint profile of *Pseudomonas aeruginosa* by rep-PCR

Legend: MP: Molecular weight marker, Pa ATCC27853: Pseudomonas aeruginosa ATCC27853, TN: negative control; P01 to P36: Strains studied

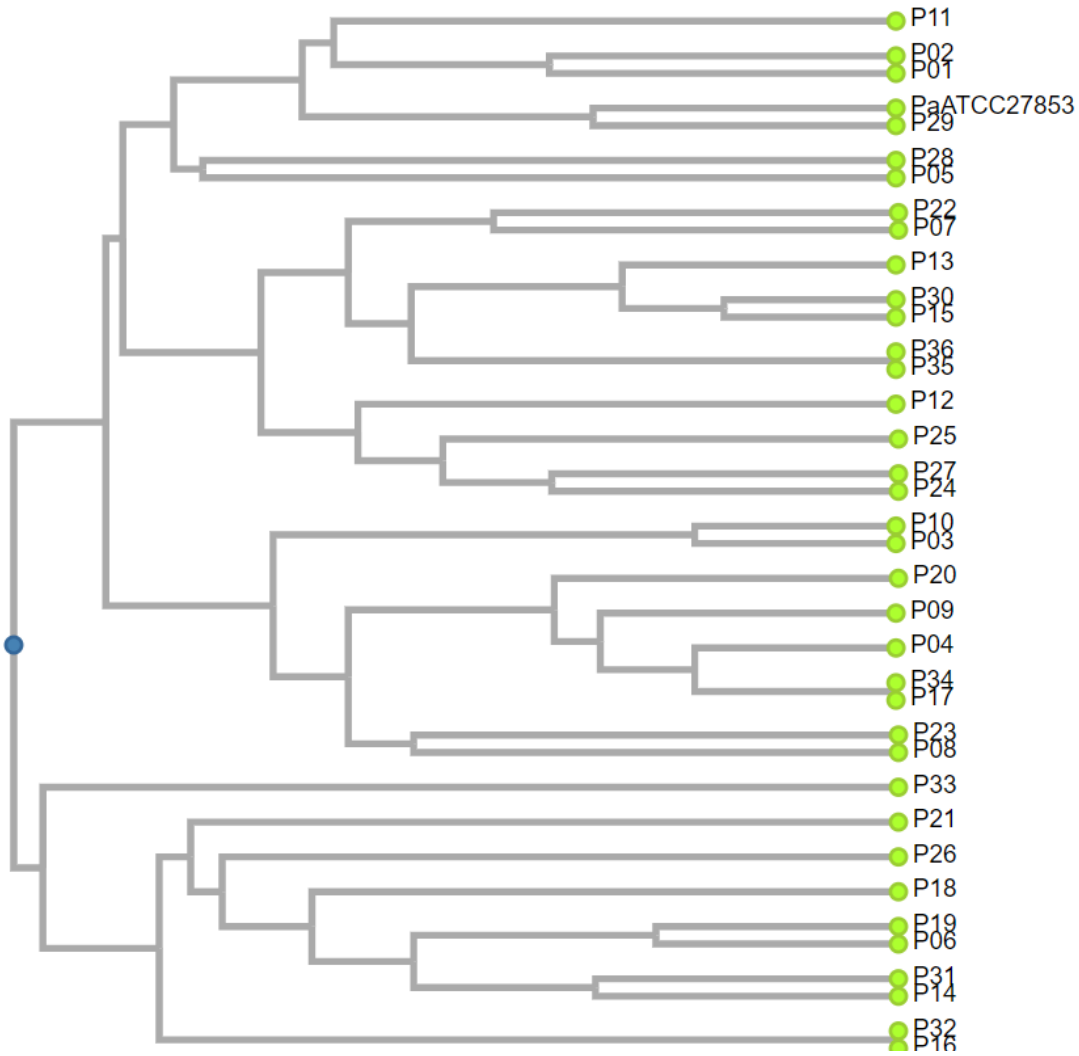

Fig. 4. Dendrogram based on genetic fingerprints by GTG5-PCR of Pseudomonas aeruginosa clinical strains according to UPGMA with Cophenetic Correlation Coefficient (CP) = 0.66

Analysis of the phylogenetic tree shows that there is genetic diversity between the strains studied. Thus, 33 subgroups were obtained. Cophenetic Correlation Coefficient (CP = 66%) reveals phylogenetic diversity between the strains studied (CP less than 88%). Ouédraogo et al. [Ouédraogo et al. 2022], Kaboré et al. [Kaboré et al. 2022b] and Khare et al. [Khare et al. 2020] used the same method to group of *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *E. coli*, respectively. According to genetic fingerprint profile of the strains based on phylogeny tree, we find that the strains of groups G16 (P16 and P32), G17 (P17 and P34) and G33 (P35 and P36) isolated from pus could also be at the origin of urinary tract infections due to their affiliation with strains responsible for urinary pathologies according to Fig. 4. However, the G6 group (P13) isolated from pus is unique compared to other urinary tract infection, so it might be specific only to suppurative infections. However, isolates from groups G1, G2, G3, G5, G6, G7, G8, G9, G11, G12, G14, G15, G16, G17, G18, G20, G21 and G22 are uropathogenic depending on their affiliation (Fig. 4). Indeed, in certain previous studies, primer (GTG)5 was used to discriminate of *E. coli*, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* responsible for pathologies in humans and animals [Ouédraogo et al. 2022, Kaboré et al. 2022a, Khare et al. 2020]. Thus, the molecular profiles of *P. aeruginosa* isolates responsible for infections, as well as that of *P. aeruginosa* ATCC27853 strain used as reference in this study, reveal remarkable clonal diversity, with most of isolates represented having unique genetic fingerprint profiles.

4. CONCLUSION

This study made it possible to know the antibiotic resistance phenotype and the molecular identification of Pseudomonas aeruginosa clinical strains circulating in Chad. Additionally, the study compared the reported MAR indices against the allowable limit. In this study, Pseudomonas aeruginosa strains isolated from urinary tract infections and pus were classified as XDR and MDR type strains.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc.) and text-to-image generators have been used during the writing or editing of this manuscript.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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