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Colistin Resistance Profiles, Molecular Investigation of *mcr*-1 and *mcr*-2 Plasmid Genes and Investigation of Carbapenemase Production in *Pseudomonas* and *Acinetobacter* Strains

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Abstract

Background and Purpose: The reintroduction of colistin as a last resort treatment against multi-resistant Gram-negative bacilli, is currently challenged by the emergence of colistin-resistant bacteria. The aim of this study was to assess the susceptibility of *Pseudomonas* and *Acinetobacter* strains to colistin, to identify carbapenemase production, and to investigate the plasmid genes involved in colistin resistance and carbapenemase production. **Methodology:** In order to establish the susceptibility profiles of 17 strains of *Pseudomonas* and *Acinetobacter* to colistin, their Minimum Inhibitory Concentrations (MICs) were determined using the liquid microdilution method. The possible production of carbapenemases was investigated with the modified Carbapenem Inactivation Method (mCIM). The search for genes encoding carbapenemases (bla_{OXA}, bla_{IMP}, bla_{Carba}) and those responsible for plasmid resistance to colistin (*mcr-1 and mcr-2*) was performed by conventional PCR. **Results and Conclusion:** Ninety-four percent (94%) (16/17) of the strains were resistant to colistin. Intraspecies distribution was 50% (8/16), 31%

(5/16), 13% (2/16) and 6% (1/16) for *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Pseudomonas luteola*, and *Pseudomonas fluorescens*, respectively. Twenty-nine percent (29%) (6/17) of the strains produced carbapenemases. No mcr-1 and mcr-2 plasmid genes were detected. On the other hand, 17.6% (3/17) of the strains possessed the carbapenemase genes distributed as follows: Carba type (60%), OXA type (40%) and IMP type (0%). The results of this study highlight a high resistance to colistin in strains belonging to the genera *Acinetobacter* and *Pseudomonas*, and some of these strains produce carbapenemases.

Keywords

Pseudomonas, *Acinetobacter*, Carbapenemase, Colistin, Suceptibility

1. Introduction

The efficiency of antibiotic molecules is currently compromised by the ability of bacteria to develop natural or acquired resistance mechanisms to bypass bactericidal or bacteriostatic activity [1]. This phenomenon is further aggravated by the expansion of multi-resistance or pan-resistant antibiotics in Gram-negative bacilli. It is particularly illustrated by the spread of resistance to carbapenems in *Enterobacteria*, *Pseudomonas* spp and *Acinetobacter* spp [2]. Carbapenems are the β-lactamines with the widest spectrum of activity due to their rapid penetration through the outer wall of Gram-negative bacilli and their stability to most natural or acquired β-lactamases [3]. Because of their broad-spectrum action, carbapenems are often used as last-resort antibiotics when infected patients are suspected to harbor multidrug resistant bacteria [4]. The increasing number of bacterial strains resistant to first-line antibiotics has resulted in an increase in the number of carbapenem prescriptions. This trend has contributed to the expansion of strains resistant to last-line molecules [1] [5]. The main bacterial species clinically involved are carbapenemase-producing enterobacteria (CPE), Meropenem-resistant *Pseudomonas aeruginosa* (MRPA), imipenem-resistant *Acinetobacter baumannii* (IRAB), all of which are currently referred to as highly resistant bacteria (HRB) [6]. In view of the spread of HRB, clinicians were forced to reintroduce a class of antibiotics which had been almost abandoned for decades because of its toxicity: colistin [2]. This molecule has a narrow spectrum of antibacterial activity, limited only to Gram-negative bacteria including enterobacteria, *Pseudomonas aeruginosa* and *Acinetobacter* spp [7]. Colistin is a cyclic polypeptide with detergent-like properties. It is naturally synthesized by *Bacillus polymyxa* subspecies *colistinus* and was discovered in the late 1940s in Japan. Colistin was reintroduced between 2015 and 2016 as a last resort treatment for severe nosocomial infections caused by bacteria resistant to other treatments, such as *Pseudomonas* and *Acinetobacter* [8]. It exerts its lethal action by interacting with the bacterium's outer membrane at the lipopolysaccharide (LPS)

level [1]. However, therapeutic alternative polymyxins are currently weakened by the emergence and sporadic spread of colistin resistance, the last line of defense against diseases due to highly resistant bacteria [7].

This resistance, observed up to 2015, involved mechanisms of resistance to colistin derived solely from the modification of chromosomal genes (vertical transfer) involved in the pathways of lipopolysaccharide (LPS) synthesis and modification [9]. Unfortunately, the first transferable colistin resistance mechanism capable of replication between bacterial strains (*Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*) was recently described in China in bacteria infecting animals, but also in bacterial strains isolated from hospitalized patients [10] [11]. This transferable mechanism, called mcr-1, was discovered by Liu *et al.* in 2015 [11]. This mechanism is not the only one that confers resistance to colistin. In 2016, a colistin resistance gene, detected in colistin-resistant *E. coli* isolates from pigs and cattle, mcr-2 was found on a plasmid. The latter was similar to mcr-1, with a high rate of transfer between bacterial strains. In addition, in 2017, the *mcr*-3 and *mcr*-4 genes were identified. To this date, nine other variants close to the mcr-1 gene (mcr-2 to mcr-10) have been described [12]. The current prevalence of the *mcr*-1 plasmid gene has been estimated to be about 20% in animals and 1% in humans. The work of Ahmed *et al.* on Gram-negative bacilli from birds in Egypt reported prevalence rates of 37.5% and 12.5% for *mcr*-1 and *mcr*-2 genes, respectively [13]. Similarly, the prevalence rates of *mcr*-3, *mcr*-4 and *mcr*-5 genes detected in vaginal swabs of women undergoing infertility testing in China were 1.5%, 12.7% and 0.7%, respectively [14].

In addition, the possibility or not of co-selection of resistance to colistin by the use of other antibiotics or even by the diversity of genetic carriers would be particularly conceivable in the current context. Indeed, Giani's 2015 study reveals that 43% of carbapenemase-producing strains are resistant to colistin [15]. Similarly, in the literature it has been reported that some strains of *E. coli* producing β-lactamase OXA and strains of *K. pneumoniae* producing carbapenemase type KPC are resistant to colistin [7].

Globally, the prevalence of resistance to colistin is low and varies according to country. Thus, in 2015, the European Antimicrobial Resistance Surveillance Network (EARSS) reported a prevalence of resistance to colistin of 1% and 4% for the genera *Pseudomonas* and *Acinetobacter*, respectively [7]. In the Africa area, in 2016, a study by Dortet *et al.* showed that in Tunisia between 2005 and 2010, the prevalence of resistance was 0% for *E. coli*, 1.2% for *K. pneumoniae* and 1.5% for *E. cloacae*. That same year in China, the prevalence rates of resistance for the same species were 5.4% for *E. coli*, 6.6% for *K. pneumoniae* and 26.5% for *E. cloacae* [16].

In Gabon, resistance to colistin was highlighted in 2019 by the work of Yala *et al.* on enterobacteria, specifically *E. coli*, whose prevalence of resistance to colistin was 40% [17]. Furthermore, in the *Salmonella* and *Shigella* genera, the overall resistance to colistin was showed to be 20% [18]. However, colistin prevalence

data for other members of the Gram-negative bacilli group such as *Pseudomonas* and *Acinetobacter* strains are lacking in this country. Furthermore, colistin is not prescribed in the neighboring provinces of the Haut-Ogooué and Ogooué-Lolo provinces where the strains have been isolated. In this context, this study aimed to phenotypically characterize their susceptibility, to investigate the plasmid genes *mcr*-1 and *mcr*-2 involved in colistin resistance, and to identify a possible production of carbapenemases.

2. Methodology

1) Biological material

Non-fermenting Gram-negative bacilli belonging to the *Pseudomonas* (9 strains) and *Acinetobacter* (8 strains) genera were tested. These were taken from the Molecular and Cellular Biology Laboratory (LABMC) of the Biology Department of the Masuku University of Science and Technology (USTM) (Table 1).

2) Culture media and pre-culture preparation

The various culture media used were the Brain Heart Infusion (BHI) medium for bacterial revival and pre-culture preparation, and the Mueller-Hinton medium (MH) to demonstrate carbapenemase production and to determine the Minimum Inhibitory Concentrations (MICs) of colistin strains. Ten microlitres (10 µL) of the different bacterial suspensions were collected and deposited on BHI agar. Subsequently, streaked seeding was carried out and the boxes were incubated at 37˚C for 18 to 24 hours. The bacterial colonies obtained were used to prepare the various inocula.

3) Preparation of bacterial inocula

Inocula were prepared from the pre-cultures by taking one to two isolated colonies and then diluting them into a sterile tube containing physiological water (0.09% NaCl). The resulting suspensions were then adjusted by measuring optical densities (OD) spectrophotometer at a wavelength of 625 nm. The ODs between 0.08 and 0.132 corresponded to the standardized 0.5 McFarland inocula.

4) Determination of Minimum Inhibitory Concentrations

The different MICs of *Pseudomonas* and *Acinetobacter* strains to colistin were determined by microdilution in 96-well microplates. Thus, 100 µL of MH

Table 1. Distribution of the bacterial strains studied.

Names of strains	Urines	River waters	IMD	Total
Acinetobacter baumannii	7/14	1/2	0/1	8
Pseudomonas aeruginosa	4/14	1/2	1/1	6
Pseudomonas fluorescens	1/14	0/2	0/1	1
Pseudomonas luteola	2/14	0/2	0/1	2
Total	**14**	**2**	**1**	**17**

IMD: Implanted Medical Device.

broth was distributed to the different wells of the plate. Then, geometric dilution ranges of ratio 2 ranging from 16 µg/mL to 0.03 µg/mL were achieved, except in the control wells. Finally, 100 µL of inoculum was distributed to all wells except the negative control well, which was added to the culture medium the culture medium of 100 µL of ultra-pure water. The microplates were incubated at 37˚C for 18 to 24 hours and the results were interpreted according to the recommendations of the Comité de la Société Française de l'Antibiogramme (CASFM, 2020) and Clinical & Laboratory Standards Institute (CLSI).

5) Demonstration of carbapenemase production using the Carbapenem Inactivation Method

One to two young colonies of the strains were collected and diluted in 400 µL of BHI broth for testing. Subsequently, a disc of meropenem (10 µg) was incorporated into each tube and the preparations incubated at 37˚C for 4 hours 30 min. After incubation, the discs were removed and placed on pre-seeded MH agar of a merropenem-sensitive strain of *E. coli* (JFY 0117). Finally, the boxes were incubated at 37˚C for 18 to 24 hours. The diameters were read and interpreted with reference to the following conditions:

- ✓ If the diameter of inhibition is between 6 mm and 15 mm, the strain is considered carbapenemase-producing;
- ✓ If the diameter of inhibition is between 16 mm and 18 mm, the strain is considered indeterminate;
- ✓ Finally, if the inhibition diameter is greater than or equal to 19 mm, the strain is not considered carbapenemase-producing.

6) DNA extraction

Extraction of the total DNA was carried out with the heating technique. For each tested strain, 18- to 24-hour colonies were collected using a sterile Pasteur pipette, diluted in 300 µL of ultra-pure water, and the tubes were incubated at −20˚C for 20 min. The tubes were then transferred to a dry water bath at 100˚C for 10 min, then centrifuged at 12,000 rpm for 10 min and the resulting supernatant was transferred to a new tube and stored at −20˚C until use.

7) Search for resistance genes mcr-1 and mcr-2 and blaCarba, blaOXA, and blaIMP

The mcr-1 and mcr-2 genes and blacarba, blaIMP, blaOXA genes were tested on all 17 strains in this study. The sequences of the different primers and the sizes of the genes are shown in Table 2.

8) DNA amplification

The *mcr-1 and -2*, bla_{IMP}, bla_{OXA}, *and* bla_{Carba} genes were amplified using specific primers described in Table 2 from total DNA. PCR reactions were performed using a thermocycler (Bio-RAD, T100TM, USA) in a final reaction volume of 20 µL containing 3 µL of DNA and 17 µL of the PCR mix. PCR conditions and amplification programs are recorded in Table 3.

9) Migration and visualization on agarose gel

The size of the different amplified DNA fragments was determined by electrophoretic migration on a 1.5% agarose gel supplemented with Ethidium Bromide.

Table 2. Primers used for PCR amplification of mcr-1, mcr-2, bla_{carba}, bla_{IMP}, bla_{OXA} genes.

Primer	Sequence (5'-3')	Tailles (Kb)	Genes	References
IMP-F	GAAGGCGTTTATGTTCATAC	587	*bla*$_{IMP}$	[19]
IMP-R	GTACGTTTCAAGAGTGATGC			
Carba-F	AATGGCAATCAGCGCTTC	586	*bla*$_{Carba}$	[19]
Carba-R	GGGGCTTGATGCTCACT			
OXA-F	ACACAATACATATCAACTTCGC	813	*bla*$_{OXA}$	[19]
OXA-R	AGTGTGTTTAGAATGGTGATC			
mcr-1-F	AGTCCGTTTGTTCTTGTGGC	1497	*mcr1*	[20]
mcr-1-R	AGATCCTTGGTCTCGGCTTG			
mcr-2-F	CAAGTGTGTTGGTCGCAGTT	576	*mcr2*	[20]
mcr-2-R	TCTAGCCCGACAAGCATACC			

Table 3. PCR amplification program and conditions.

Genes	Condition/Duration	
	mcr-1/mcr-2	*Carbapenemases*
Initiale denaturation	95˚C/15min	95˚C/15min
Denaturation	93˚C/30sec	94˚C/1min
Hybridation	57˚C/1min	58˚C/1min
Elongation	72˚C/1min	72˚C/1min
Final elongation	72˚C/8min	72˚C/5min
Number of de cycles	**30**	**32**

Migration was performed at a voltage of 150 volts (V) and 400 milliampere (mA) for 25 min using Bio-RAD (Power Pac TM Basic, USA). Visualization was done under UV light using a transilluminator.

3. Results

This study first determined the susceptibility profiles to colistin of strains of the *Pseudomonas* and *Acinetobacter* genera and then demonstrated the possible production of carbapenemases by these strains.

1) Global prevalence of resistance of all strains tested

Strain susceptibility profiles were determined through their MICs for colistin. The results are presented in Table 4.

The results in Table 4 show that for the different MICs of the bacterial strains studied, a similar pattern of colistin resistance (94.1%) was recorded for both the CASFM and CLSI standards.

Table 4. Global prevalence of resistance of the tested strains.

Strains	CMI (µg/mL)	Phenotype/CASFM 2020	Phenotype/CLSI 2018
Pseudomonas aeruginosa 1	>16	R	R
Pseudomonas aeruginosa 2	>16	R	R
Pseudomonas aeruginosa 3	>16	R	R
Pseudomonas aeruginosa 4	>16	R	R
Pseudomonas aeruginosa 5	2	S	I
Pseudomonas aeruginosa 6	>16	R	R
Pseudomonas luteola 1	>16	R	R
Pseudomonas luteola 2	>16	R	R
Pseudomonas fluorescens	>16	R	R
Acinetobacter baumannii 1	>16	R	R
Acinetobacter baumannii 2	>16	R	R
Acinetobacter baumannii 3	>16	R	R
Acinetobacter baumannii 4	>16	R	R
Acinetobacter baumannii 5	>16	R	R
Acinetobacter baumannii 6	>16	R	R
Acinetobacter baumannii 7	>16	R	R
Acinetobacter baumannii 8	>16	R	R
Prévalence globale de résistance	-	**16/17 (94.1)**	**16/17 (94.1)**

R: Resistant; **I**: Intermediary; **S**: Susceptibility.

2) Resistance profile according to the bacterial species tested

The distribution of the resistance profile of the strains shows varying proportions of resistance according to the species (Table 5).

Analysis of the results in Table 5 shows that 94.1% (16/17) of the bacterial strains tested exhibit a colistin resistance profile. Of the 16 colistin-resistant BGN strains, 50.0% belong to *Acinetobacter baumannii*, followed by *Pseudomonas aeruginosa* (31.3%), *Pseudomonas luteola* (12.5%), and finally *Pseudomonas fluorescens* (6.2%) (Table 5). Regarding species variability, all strains belonging to *Acinetobacter baumannii* (100.0%) were resistant, as were strains of *Pseudomonas fluorescens* (100.0%) and *Pseudomonas luteola* (100.0%). In contrast, 83.3% of *Pseudomonas aeruginosa* strains were resistant. Therefore, the results in Table 5 also show different resistance rates depending on the origin of isolate strains. Resistance to colistin was 81.2% for strains from urine samples, 12.5% and 6.3% for strains from river samples and implanted medical devices (IMDs), respectively.

Table 5. Prevalence of resistance according to the different species and the origin of isolate strains.

Strains	Worforce (N)	Prevalence of resistance		Origins		
		Globale n/N (%)	Intra-espèces n/N	Urines n/N (%)	River water n/N (%)	IMD n/N (%)
Acinetobacter baumannii	8	8 (50.0)	8/8 (100.0)	7 (87.5)	1 (12.5)	-
Pseudomonas aeruginosa	6	5 (31.3)	5/6 (83.3)	3 (50.0)	1 (16.6)	1 (16.7)
Pseudomonas fluorescens	1	1 (6.2)	1/1 (100.0)	1 (100.0)	-	-
Pseudomonas luteola	2	2 (12.5)	2/2 (100.0)	2 (100.0)	-	-
Total	**17**	**16 (100.0)**	**16/17 (94.1)**	**13/16 (81.2)**	**2 (12.5)**	**1 (6.3)**

3) Search for plasmid genes involved in colistin resistance and Global prevalence of carbapenemase producing strains

The results in Table 6 show the absence or non-detection of *mcr*-1 and *mcr*-2 genes in the 17 bacterial strains studied.

In addition, the results in Table 6 show that 35.3% (6/17) of *Acinetobacter* and *Pseudomonas* strains are phenotypically carbapenemase-producing. They also highlight that 67% (4/6) of the carbapenemase producing strains belong to the *Acinetobacter baumannii* species and 33% (2/6) to the *Pseudomonas* genus, of which 16.67% (1/6) belong to *Pseudomonas aeruginosa* and *Pseudomonas luteola*. Their molecular screening revealed a detection rate of 17.6% (3/17). bla_{CARBA} was the most common gene (17.6%) compared to bla_{OXA} (11.8%) and bla_{IMP} (0.0%). In addition, the analysis of the results showed that *Acinetobacter baumannii* is predominant among the carbapenemase-producing strains, both phenotypically and genotypically.

4. Discussion

The objective of this study was to assess the susceptibility of hospital and environmental isolates of the *Pseudomonas* and *Acinetobacter* genera to colistin by determining their MICs and demonstrating the production of carbapenemases by these isolates.

The present study showed a high rate of resistance to colistin (94.1%) in the strains tested. Resistance to colistin has been reported in previous studies. Indeed, in the works of Mohanty *et al.* and Tilouch *et al.*, conducted in India and Tunisia, the rates of resistance to colistin were 14.4% and 0.0%, respectively [21] [22]. Similar studies in the United-States and Greece showed resistance rates of 1.6% and 18.6%, respectively [23]. This high level of resistance may be related to the size of the sample in this study, which may not be sufficiently representative. It has been clearly shown that the overall number of strains tested could potentially impact the prevalence of antimicrobial resistance, as highlighted by Frye and Jackson. This study reported gentamicin resistance rates of 7.38% (n = 1045/14,169) and 18.57% (n = 731/3941) for 14,169 and 3941 *Salmonella enterica* isolates, respectively [24]. Similarly, a study reported a prevalence of colistin

resistance of 11% in the United-States based on 56 strains out of 515 isolates of enterobacteria [2].

Table 6. Search for plasmid genes *mcr*-1 and *mcr*-2 and phenotypic and molecular frequency of carbapenemase producing strains.

Strains	Colistin's genes		Production phenotype/of carbapenemases	Carbapenemases genes		
	mcr-1	*mcr*-2		bla_{CARBA}	bla_{IMP}	bla_{OXA}
Pseudomonas aeruginosa 1	no	no	no	no	no	no
Pseudomonas aeruginosa 2	no	no	no	no	no	no
Pseudomonas aeruginosa 3	no	no	no	no	no	no
Pseudomonas aeruginosa 4	no	no	no	no	no	no
Pseudomonas aeruginosa 5	no	no	yes	no	no	no
Pseudomonas aeruginosa 6	no	no	no	no	no	no
Pseudomonas luteola 1	no	no	no	no	no	no
Pseudomonas luteola 2	no	no	yes	yes	no	yes
Pseudomonas fluorescens	no	no	no	no	no	no
Acinetobacter baumannii 1	no	no	no	no	no	no
Acinetobacter baumannii 2	no	no	yes	no	no	no
Acinetobacter baumannii 3	no	no	no	no	no	no
Acinetobacter baumannii 4	no	no	yes	yes	no	no
Acinetobacter baumannii 5	no	no	yes	no	no	no
Acinetobacter baumannii 6	no	no	yes	yes	no	oui
Acinetobacter baumannii 7	no	no	no	no	no	non
Acinetobacter baumannii 8	no	no	no	no	no	non
Global prevalence	**0/17 (0.00)**	**0/17 (0.00)**	**6/17 (35.3)**	**3/17 (17.6)**	**0/17 (0.0)**	**2/17 (11.8)**

In contrast, the colistin resistance observed in this study could probably be explained by the occurrence of chromosomal mutations in the genome of these strains, which led to the modification of lipopolysaccharide (LPS). Evidently, altering the LPS composition of Gram-negative bacilli by adding positively charged residues would induce a weakening of polymyxin binding at their site of action. This decrease in affinity would then result in preventing the binding of colistin to the LPS, inhibiting its action on the outer membrane of the strains [7] [25]. In addition to this phenomenon, the presence of mcr-type plasmid genes may also be implicated in the high-resistance rate observed. The multicenter study by Dortet *et al.*, published in 2016, showed the existence of different variants of the mcr gene (*mcr*-1, *mcr*-2) responsible for the colistin resistance phenotype in *E. coli* and *K. pneumoniae* species [2]. Therefore, other variants (*mcr* 1 - 10) have been identified in a variety of Gram-negative bacteria as a determinant of colistin resistance. These include the *mcr*-3 gene identified in China in the genus Aeromonas, as well as the *mcr*-9 and *mcr*-10 genes capable of conferring phenotypic resistance to colistin in Enterobacteriaceae [26] [27] [28]. Genes of the mcr family are responsible for polymyxin resistance by encoding a phosphoethanolamine transferase (PEtN). This enzyme is involved in the addition of PEtN to lipid A of the LPS, making it highly cationic, thus strengthening the bonds between the neighboring molecules of the LPS [25] [29].

Recent studies have pointed out that resistance to β-lactam antibiotics is not correlated with industrial production of β-lactamases, as countries with high prevalence rates of resistance have the lowest consumption of antibiotics [30]. It suggests that this resistance may be the result of other genetic determinants of antibiotic resistance.

Resistance to colistin in this study was dominated by strains belonging to *Acinetobacter baumannii* (50%). These results are similar to the work of in Algeria where the most resistant species was *Acinetobacter baumannii* as well (57.14%). This trait may be due to the strong upsurge of resistance genes to several families of antibiotics observed in this bacterial species [31]. Previous studies have reported the simultaneous presence of several genetic determinants of resistance in this species, including β-lactamases (e.g., $bla_{OXA\text{-}51}$, bla_{TEM}, $bla_{OXA\text{-}23}$, bla_{GES}, bla_{NDM}, bla_{VIM}), mobile genetic elements (ISAba1, Int II and ISII33), but also mutation points (gyrA, S83L, parC, S80L1, N283S) [32]. Furthermore, mutation and/or inactivation of the lipid A, lpxA, lpxC, and lpxD biosynthesis genes in *A. baumannii* may also be one of the causes of polymyxin resistance [32].

Strains of *Pseudomonas aeruginosa* were the second species with high levels of resistance to colistin (31.3%). Various mechanisms are clearly involved in colistin resistance in this species, including the regulation of a two-component system: the ParRS system [5]. The ParRS operon codes for two proteins, a ParR regulatory protein and a ParS membrane sensor protein kinase. In the presence of sub-inhibitory concentrations of colistin, the ParS protein is activated by autophosphorylation and in turn activates the ParR protein. The latter activates the pmrHFIJKLM operon, which leads to the synthesis of 4 amino-4-deoxy-L-arabinose

on lipid A. The latter, being positively charged, will cause a loss of affinity for colistin [5] [33].

Thus, our work indicates that the resistance pattern recorded applies to both hospital strains (81%) and environmental strains (13%). This result suggests the spread of resistant strains due to contamination of aquatic ecosystems by hospital or human discharges [34].

Colistin, a last resort antibiotic used to treat multi-resistant Gram-negative Bacilli (GNB), is compromised by the presence of mcr plasmid genes which are prevalent in most Asian and European countries. The search for the most common variants of this family in this study showed the non-amplification (0%) of *mcr*-1 and *mcr*-2 genes in *Pseudomonas* and *Acinetobacter* strains. These results are similar to those found in the literature where very low rates or non-detection of these plasmid genes in BGN strains are reported. Indeed, in the work Zhang *et al* conducted in China, the prevalence of mcr-1 and mcr-2 genes in multi-drug resistant BGNs isolated from human samples was 0.7% and 1.5%, respectively [14]. In contrast, among the 146 clinical isolates of *Acinetobacter baumannii* and *Pseudomonas aeruginosa* from hospitals in Pakistan, approximately 3% of strains harbored the mcr-1 gene [35]. In addition, in a study by Furong *et al*., no mcr-carrying strains were found in 90 colistin-resistant clinical isolates of *Acinetobacter baumannii* [36].

Furthermore, this non-detection of the *mcr*-1 and 2 genes could probably be related to the origin of the strains screened, since many studies detect lower rates of mcr variants in bacterial isolates of human origin. These make up most of the strains in this study (n = 15/17). Indeed, the work of Ahmed *et al.* (2019) conducted in Egypt found prevalence rates of 37.5% and 12.5%, respectively, for the *mcr*-1 and *mcr*-2 genes in *Pseudomonas aeruginosa* strains from birds, while it was 0% for *Pseudomonas aeruginosa* strains from human isolates [13]. Similarly, Caselli's work in Italy detected the *mcr*-1 gene in 10% of animal isolates and 0.1% of human isolates [37]. Furthermore, results obtained in a Chinese study by Chang *et al.* also recorded a fairly high prevalence of the *mcr*-1 gene in fecal swine samples (14.02%), compared to those observed among farmers, which was 3.45% [26]. The high use of colistin in animals, particularly in pig production, may be the main cause of the spread of bacterial resistance to colistin in humans [38].

Although these variants are the most common, their absence in this study suggests the involvement of another variant of mcr. The variants *mcr*-3, *mcr*-4, and *mcr*-5, responsible for resistance to colistin in the enterobacteria, *Pseudomonas aeruginosa* and *Acinetobacter baumannii*, isolated from vaginal swabs of women undergoing infertility testing in China, have already been reported. Their prevalence was 1.5% for *mcr*-3, 12.7% for *mcr*-4 and 0.7% for *mcr*-5 [14].

Furthermore, this lack of detection would suggest that in addition to the low dissemination of plasmid resistance, colistin resistance is currently dominated by a chromosomal origin. Indeed, many studies have reported that the most recurrent genetic determinism of colistin resistance is due to chromosomal mutations

in various genes leading to changes in the structure of lipopolysaccharide (LPS) and to the abolition of colistin binding on the bacterial wall [7]. Similarly, a study showed that several genes and operons are involved in LPS modification, such as the PmrC and naxD genes in *Acinetobacter baumannii*, which allow the addition of phosphoethanolamine and galactosamine [39]. The eptA gene and the PmrHFIJKLM operon in *Pseudomonas aeruginosa* add L-amine4-arabinose (Lara4N). The addition of these cationic groups to the LPS decreases the affinity of colistin and thus leads to the acquisition of resistance. Indeed, in *Pseudomonas aeruginosa* and *Acinetobacter baumannii*, the acquisition of resistance to colistin is due to mutations in the PmrA genes. These are activated in *Pseudomonas aeruginosa* by phoP and PmrB. They lead to constitutive activation of the PmrAB regulon and thus to the production of cationic groups and the amendment of the LPS. In addition, the acquisition of colistin resistance in *Pseudomonas aeruginosa* is also a consequence of mutation in the phoP and phoQ genes. It results in constitutive activation of the phoPQ regulon and thus the production of cationic groups and modification of the LPS [39].

In this study, 29% of strains produced carbapenemases. This result, similar to that reported by Tahmasebi in 2020 shows the spread of carbapenemase-producing strains (25.74%) worldwide but also in our locality. The presence of these enzymes may potentially contribute to resistance to colistin as reported in previous work, suggesting an association between the presence of carbapenemase and increased resistance to colistin [23] [40]. Moreover, in a study conducted in Italy, 43% of carbapenemase-producing strains had resistance to colistin. Carbapenemase typing in this work also revealed that the strains producing KPC and OXA-48 types had the highest levels of resistance to colistin [15] [40]. As such, a new genetic mechanism responsible for the emergence of resistance to colistin has been reported. It is believed to be related to the presence of KPC carbapenemases following exposure to colistin. This mechanism involves the inactivation of the mgrB gene, which codes for a transmembrane regulator that exercises feedback on the PhoQP signalling system. Once regulated upwards, the PhoQP system activates the LPS modifying system, which is ultimately responsible for polymyxin resistance [41].

Among the strains studied, 17.6% produced carbapenemase types OXA-48 and Carba. These results are corroborated by studies by Vala *et al.* in which in a total of 75 non-fermenting isolates (47 *Pseudomonas aeruginosa* and 28 *Acinetobacter baumannii*), the prevalence of the carbapenemase gene was 10.6% [42]. This low level is explained by the small size of the type of carbapenemase genes sought (OXA, Carba, IMP). In fact, Nordmanns studies have shown that the most frequent and threatening carbapenemases are KPC-type carbapenemases (KPC-2 to KPC-8) and that in *Pseudomonas aeruginosa*, the most frequently described carbapenemases are KPC, GES, and metallo β-lactamases [5] [6]. These include β-lactamase KPC-2 which has been identified in South America and the Caribbean in *Pseudomonas aeruginosa* and in Puerto Rico in *Acinetobacter baumannii*. In addition, another carbapenemase type VIM-1 was identified in Italy

in *Pseudomonas*, followed by VIM-2 in France [43].

The presence of carbapenemase-producing strains in our study may indicate an alarming resistance to colistin. Indeed, some work has shown that in Italy, resistance to colistin reaches alarming proportions in carbapenemase-producing strains, particularly in carbapenemase-producing *K pneumoniae*. In 2011, in Italy, 22.4% of KPC carbapenemase-producing strains were resistant to colistin, and in 2014, 43% were resistant to colistin [43].

5. Conclusion

In this study, the *Pseudomonas* and *Acinetobacter* strains show a high resistance to colistin, a last-line therapeutic molecule, marked by a predominance of the species A*cinetobacter baumannii*. This resistance pattern could be linked to carbapenemase production in these bacterial strains or to other unexplained mechanisms. In view of these results, it seems necessary to systematically determine the susceptibility profiles of the germs to colistin and to characterize the resistance mechanisms underlying this potential resistance. Further studies would make it possible to better identify the risks and possibilities of resistance gene transfer.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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