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Molecular Assessment of *MCR-1* Gene among Pandrug-Resistant *Acinetobacter baumannii*

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Abstract: Background Antimicrobial resistance has become one of the most widespread threats to humans. *Acinetobacter baumannii* is one of the pathogens responsible for healthcare-associated infections (nosocomial). Colistin is considered the last resort antibiotic against infections with pandrug-resistant (PDR) pathogens. Results: Eleven isolates were detected phenotypically as PDR *A. baumannii* and were confirmed molecularly using 16S rDNA. The *MCR*-1 gene was not detected within the chromosomal DNA of the selected isolates. Plasmid bearing the *MCR*-1 gene was identified in 10 selected isolates of *A. baumannii* that had not been previously observed to carry the *MCR*-1 gene. Moreover, the use of colistin in combination with anionic antibiotics or natural compound pterostilbene poses a viable therapeutic alternative for PDR and revives colistin's bactericidal effects on *MCR*-1-positive *A. baumannii*. Finally, the transmission electron microscopy studies proved the synergistic effect of these combinations and revealed the disruption of resistant *A. baumannii*'s outer membrane and alteration of the permeability properties that allowed overcoming the resistance of the isolates to colistin. Conclusions: Antimicrobial resistance of *A. baumannii* is related to the presence of the transferable plasmid-bearing *MCR*-1 gene. This study proved the ability of the combinations of colistin with anionic antibiotics and/or natural compound pterostilbene to restore the bactericidal effect of colistin. Overall, these combinations could be novel promising clinical alternatives against the increasing threat of the widespread multidrug-resistant *A. baumannii*.

Keywords: *Acinetobacter baumannii*; horizontal gene transfer; *MCR-1* gene; pandrug-resistant; overcoming colistin resistance

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1. Background

Acinetobacter baumannii is an opportunistic pathogen responsible for a widespread of healthcare-associated infections (nosocomial) [1]. *Acinetobacter baumannii* strains resist all known antibiotics and are classified as pandrug-resistant bacteria that should be characterized rapidly by the international healthcare community [2]. Colistin is considered the last resort for the treatment of severe infections caused by pandrug-resistant bacteria [3]. Recently resistance to colistin has been related to not only chromosomal mutations but also to the plasmid-mediated colistin resistance gene *(MCR-1)* that has been known in a number of Enterobacteriaceae species from different sources such as food, environment, animals, and humans [4,5]. The *MCR-1* gene is present in a bacterial plasmid that is able to move from one bacterium to another through horizontal gene transfer; therefore, the *MCR-1* gene has the potential to spread and has raised the resistance to colistin [6,7]. The first report of plasmid-mediated *MCR-1* gene was in *E. coli* as well as many *Klebsiella pneumoniae*, *Enterobacter aerogenes*, *Enterobacter cloacae*, *Salmonella species*, *Cronobacter sakazakaii*, *Moraxella* species, *Kluyvera* species, *Shigella sonnei*, and *Citrobacter* sp. [8,9]. The presence of plasmid-mediated *MCR-1* gene in MDR *A. baumannii* was proved first in Pakistan by Hameed et al. [10].

The phosphoethanolamine (PEtN) transferase encoded by *MCR-1* converts the electronegative charge of lipid A in the plasma membrane to electropositive by preventing colistin from binding to its bacterial target [8,11].

However, the restoration of the ability of colistin to treat severe clinical bacterial infections would be useful [12]. *MCR-1* changes lipid A with a positively charged PEtN residue, resulting in electrostatic repulsion of colistin, and then resistance [5,13]. Therefore, a change of charge by using a combination of anionic antibiotic and colistin could cause the electrostatic attraction of colistin to negatively charge lipopolysaccharide (LPS) that causes the self-promoted uptake of colistin and entering the cell, leading to cell death [14].

Previous studies had described the ability of pterostilbene (trans-3,5-dimethoxy-4′-hydroxystilbene) combined with colistin to reestablish the antibacterial activity of colistin against *MCR-1*-positive *Klebsiella pneumonia* [7]. Pterostilbene is a natural phytoalexin found in various plant species. In addition to studies on its effectiveness as antioxidant, anti-inflammatory and anti-cancer [15], Pterostilbene has a synergetic effect in vitro and in vivo on *E. coli* with colistin [7]. It has more favorable pharmacological properties with higher oral absorption efficacy, higher cellular uptake capability and extended half-life [7].

To the best of the authors' knowledge, there are very few studies on the detection of the *MCR-1* gene in *A. baumannii*; consequently, this study aims to identify PDR *A. baumannii* from different compartments, investigate their phenotypic and genotypic characteristics and confirm the presence of the plasmid-mediated *MCR-1* gene in comparison to previous studies. In addition, the study aims to find out whether combinations between colistin and anionic antibiotics and/or pterostilbene will have a synergetic effect in reviving colistin's bactericidal action against *MCR-1* positive *A. baumannii.*

2. Results

2.1. Phenotypic Identifications of Isolates and Susceptibility to Antibiotic

Out of 117 isolates, only 78 isolates (66.6%) were identified as suspected *A. baumannii* based on the morphological and biochemical characteristics; the colonies were Gram-negative coccobacilli, tiny, heavy and they resembled diplococci.

On blood agar, colonies were smooth, clear to matt without any hemolysis and non-pigmented, with smooth-to-pitted surfaces in a diameter of 1–2 mm.

On MacConkey agar, colonies of *A. baumannii* were non-lactose fermenters, pure purple and mucoid. All strains of *A. baumannii* were found to be catalase positive and oxidase/indole negative. The triple sugar iron agar (TSI) test had an alkaline bottom/alkaline slant, inadequate gas and H_2S produced. In the oxidative-fermentative medium (OF) test, glucose produced acid in aerobic conditions.

Sulphur, indole, and motility tests (SIM) showed immobility, and lack of both indole and H_2S production. Isolates grew at 37–44 °C and the optimum growth was at 42 °C. Isolates were confirmed as *A. baumannii* by API 20NE system (Biomerieux, Marcy-l'Etoile, France) code (0204042). The biochemical identification was illustrated in Supplementary Data S1.

The antibiotic susceptibility character was studied using the simple disc diffusion technique as described by the Clinical and Laboratory Standards Institute (CLSI) guidelines 2022. Results showed that 20/78 (25.6%) *A. baumannii* isolates were detected as

MDRs showed the highest resistance for amikacin and aztreonam; whereas the lowest resistance rate was observed for cefepime and imipenem, while 16/78 (20.5%) *A. baumannii* isolates were detected as XDRs, the highest resistance was observed for aztreonam and piperacillin/tazobactam as well for imipenem and meropenem. Finally, 11/78 (14.1%) *A. baumannii* isolates (1,2,3,4,5,14,21,30,42,78,90) were detected as PDRs, the highest resistance was observed for aztreonam and piperacillin/tazobactam, imipenem and meropenem and also for colistin (Figure 1). Antibiotic resistance patterns of *A. baumannii* isolates as sensitive, MDR, XDR and PDR were illustrated in the Supplementary Data S2.

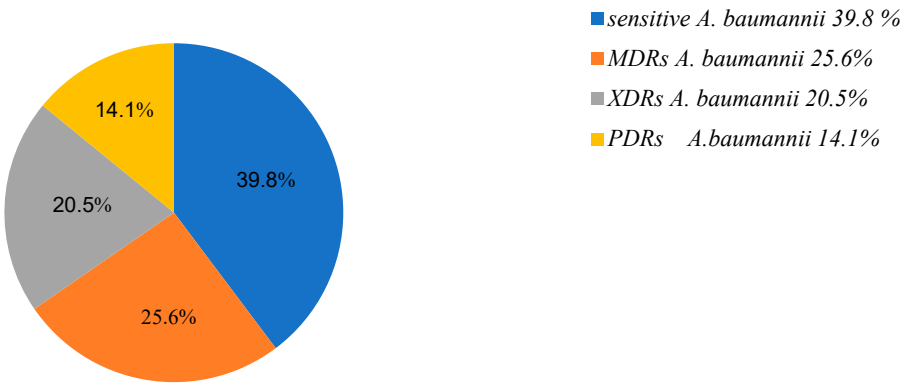

Figure 1. Categories of drug resistance *A. baumannii* isolates.

2.2. 16S rDNA Sequencing for the Selected Isolates

The 11 selected PDR *A. baumannii* strains (PDRs) (1,2,3,4,5,14,21,30,42,78,90) were molecularly identified using the 16S rDNA sequencing technique. The selected PDRs gave amplified bands at ~1500 bp (Figure 2).

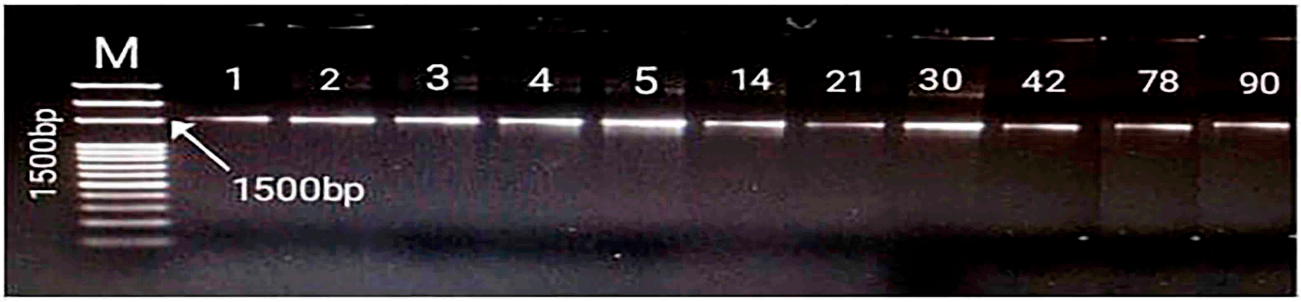

Figure 2. 16S rDNA of *A. baumannii* strains. M: 100 bp DNA ladder; 1,2,3,4,5,14,21,30,42,78,90 selected PDR *A. baumannii* isolates.

3. Data Analysis and Sequencing

The sequences of 16S rDNA for the selected PDRs were deposited to the National Center for Biotechnology Information (NCBI) and were listed and documented. They were recorded under accession numbers ON797630 to ON797640 (Table 1).

Table 1. Similarity percentage scores for 16S rDNA sequences of the identified strains.

Isolate Code	GenBank Accession Numbers	Similar Organisms	Query Cover	Similarity (%)	Accession Numbers of Published Strains
1	ON797630	*A. baumannii* ATCC 19,606	97%	97.65%	NR117620.1
3	ON797631	*A. baumannii* ATCC 19,606	97%	97.65%	NR117620.1
4	ON797632	*A. baumannii* ATCC 19,606	95%	98.95%	NR117620.1
5	ON797633	*A. baumannii* ATCC 19,606	95%	98.95%	NR117620.1
11	ON797634	*A. baumannii* ATCC 19,606	100%	99.49%	NR117620.1
14	ON797635	*A. baumannii* ATCC 19,606	98%	99.40%	NR117620.1
21	ON797636	*A. baumannii* ATCC 19,606	98%	99.40%	NR117620.1
30	ON797637	*A. baumannii* ATCC 19,606	100%	99.49%	NR117620.1
42	ON797638	*A. baumannii* ATCC 19,606	98%	99.40%	NR117620.1
78	ON797639	*A. baumannii* ATCC 19,606	100%	99.79%	NR117620.1
90	ON797640	*A. baumannii* ATCC 19,606		99.57%	

3.1. 16S rDNA Phylogenetic Tree Construction

The phylogenetic tree was constructed from the identified 16S rDNA *A. baumannii* sequences with their respective reference sequences from GenBank using the AlignStat Plot

tool (Figure 3). The similarity difference matrix heatmap showcases sequence similarity variations from alignment analysis. Significant nucleotide dissimilarities are represented by high values and reddish squares, while high sequence similarities are depicted in blue (Figure 4).

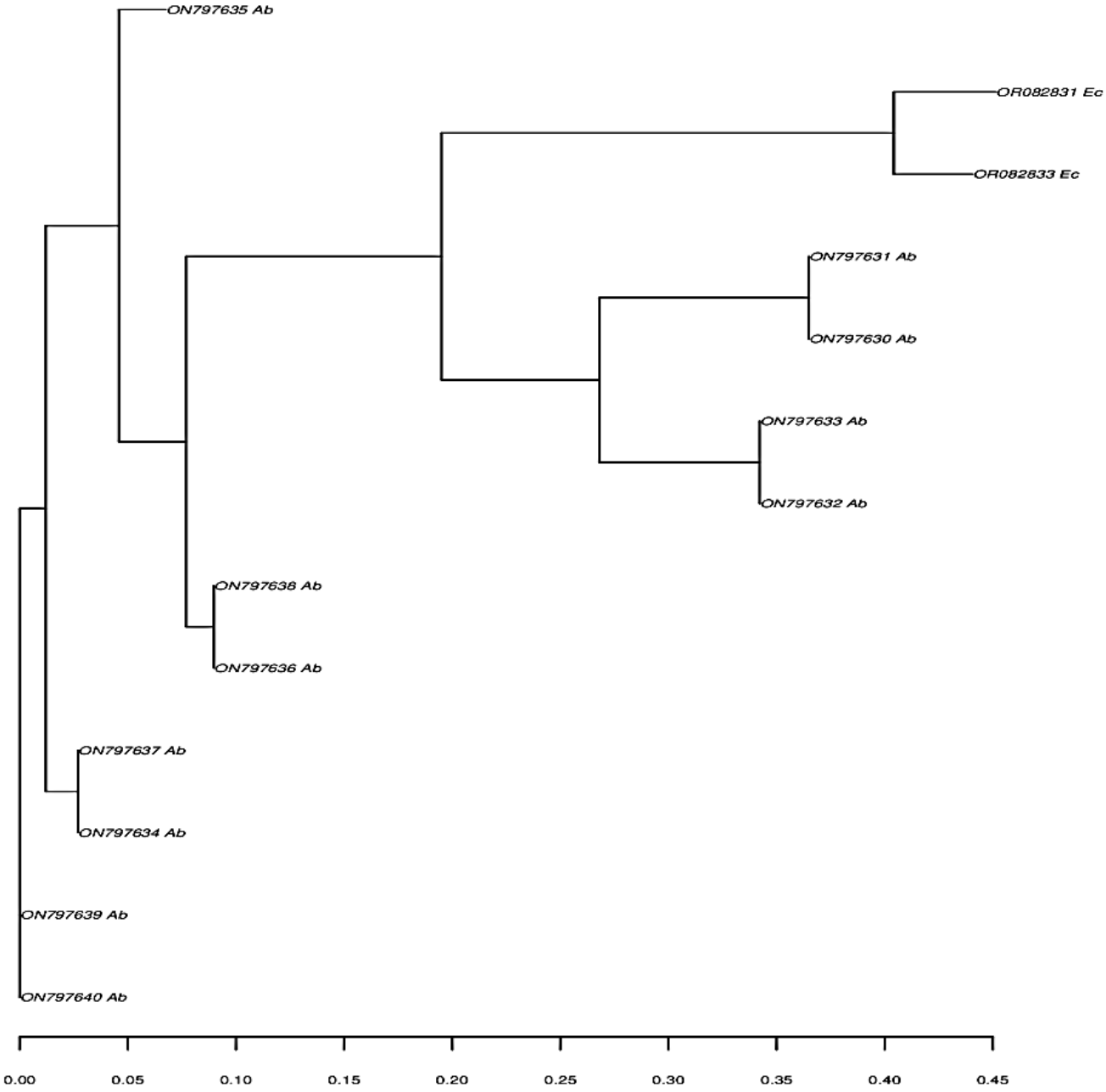

Figure 3. The phylogenetic tree of eleven PDRs *A. baumannii*.

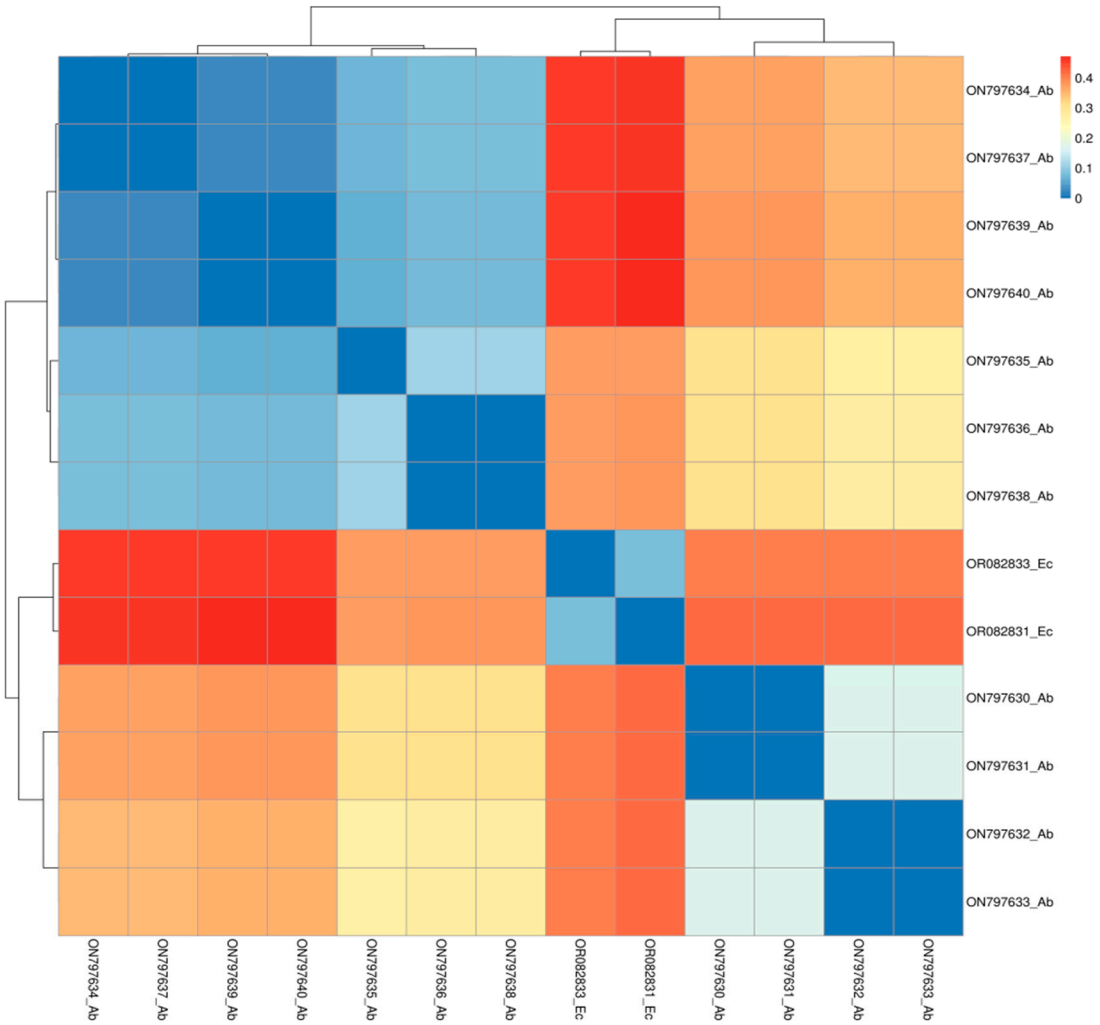

Figure 4. Distance matrix methods of phylogenetic analysis.

3.2. Detection of Phosphoethanolamine-Lipid a Transferase MCR-1 Gene in PDRs

Phosphoethanolamine-lipid A transferase *MCR-1* gene was not detected in the chromosomal genes of *A. baumannii.* However, the plasmid-bearing phosphoethanolamine-lipid A transferase *MCR-1* gene was extracted and amplified. Ten out of 11 PDR *A. baumannii* gave the expected fragment size of ~700 bp of phosphoethanolamine-lipid A transferase *MCR*-1 gene and only one strain (strain 4) was negative (Figure 5).

Figure 5. PCR amplification of *MCR-1* gene in *A. baumannii*, M: 100-bp DNA marker; 1,2,3,4,5,14,21,30,42,78,90 selected PDRs.

3.3. Data Analysis of Phosphoethanolamine-Lipid a Transferase MCR-1 and Phylogenetic Construction

The *MCR-1* gene sequence for both strains 78 and 90 was compared to their respective reference sequences from GenBank using the AlignStat Plot tool. The phylogenetic

tree based on the *MCR-1* gene sequence for both strains 78 and 90 of *A. baumannii* was constructed alongside the sequences of *MCR-1* for *Escherichia coli* strain L36 (accession no. MT070408). The phylogenetic tree showed that the *MCR-1* gene sequence was nearly the same and it was presented in the same clade (Figure 6a).

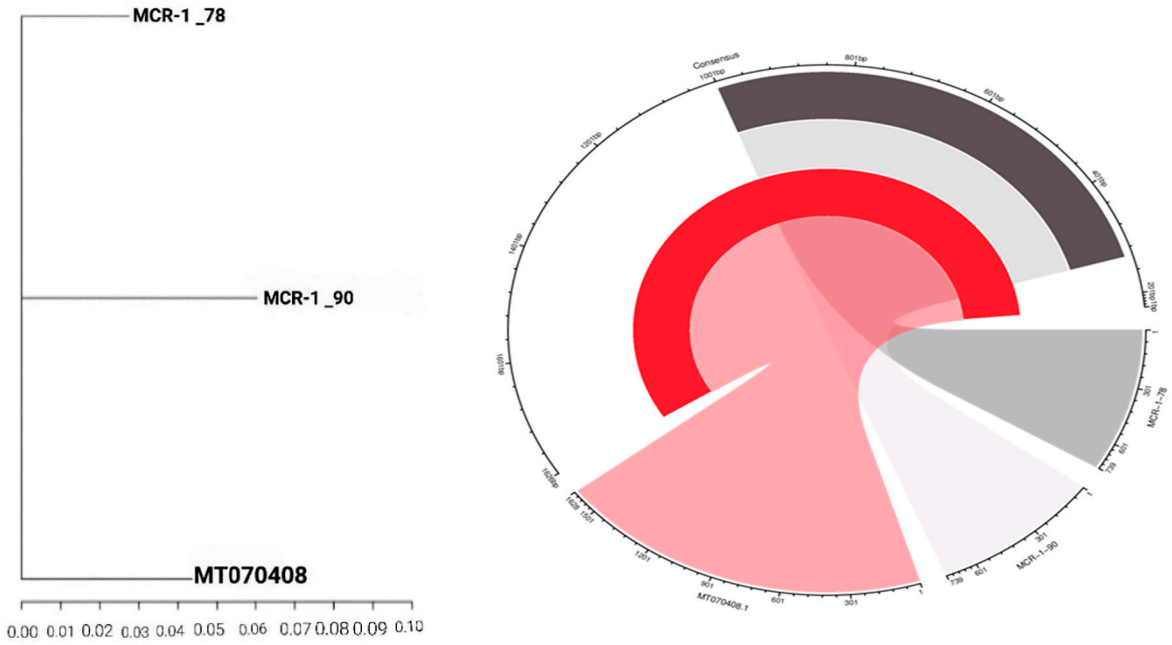

Figure 6. (**a**) phylogenetic tree of *MCR-1* gene; (**b**) Seq alignment circle with links of *MCR-1* gene. Consensus region between the three strains, *MCR-1* sequence of strain 78, *MCR-1* sequence of strain 90, *MCR-1* sequence of *Escherichia coli* strain L36 (accession no. MT070408).

Figure 6b shows similar nucleotide regions of *the MCR-1* gene sequence for both strains 78 and 90 of *A. baumannii* was constructed with the sequences of *MCR-1* for *Escherichia coli* strain L36 (accession no. MT070408).

3.4. Sequencing of MCR-1 Gene

The PCR products containing amplicons of 700 bp for 90 strains were sequenced. After annotation, the partial nucleotide sequences of the *MCR-1* gene were found to be nearly identical to the sequences of published *MCR-1* genes in NCBI.

The annotated sequence of the *MCR-1* gene was submitted to NCBI to be listed in the gene bank. The partial sequence of the *MCR-1* gene has been deposited in Gene Bank under accession number LC754315.

3.5. Overcoming MCR-1 Mediated Colistin Resistance A. baumannii

The minimum inhibitory concentration (MIC) and the antimicrobial activity of the anionic antibiotics and colistin against colistin-resistant strain *A. baumannii* (strain 90) are listed in Table 2. The results showed that the antimicrobial activity of linezolid against the colistin-resistant strain was 0.1 mg/mL when used separately, while in combination with 10 mg/mL colistin, the MIC became 0.001 mg/mL. On the other hand, clindamycin has the lowest antimicrobial activity with MIC 10 mg/mL when used separately and enhanced to 1 mg/mL in combination with 10 mg/mL colistin. However, the MIC of vancomycin was 1 mg/mL when used separately and reached 0.001 mg/mL in combination with 10 mg/mL colistin. The MIC of both rifampicin and azithromycin separately have 1 mg/mL and 10 mg/mL, respectively and enhanced to 0.1 mg/mL in combination with 10 mg/mL colistin.

The MIC and the antimicrobial activity of pterostilbene and colistin against colistin-resistant strain *A. baumannii* are presented in Table 3. None of the concentrations of pterostilbene from 10 mg/mL to 0.0001 mg/mL had antimicrobial activity against colistin-resistant strain 90. The present findings proved the synergetic effect of pterostilbene with colistin against *A. baumannii* strains that are colistin-resistant and its MIC in combination with 10 mg/mL colistin reached 0.01 mg/mL. On the other hand, the best dilution of pterostilbene was 0.01 mg/mL with serial dilution of colistin to enhance the MIC of colistin. The results indicated that the MIC of colistin can be enhanced to 0.001 mg/mL and accordingly enhance its antimicrobial activity against colistin-resistant *A. baumannii* isolates.

3.6. Transmission Electron Microscope (TEM) Analysis of A. baumannii

The transmission electron microscope images illustrated the change in the outer membrane of *A. baumannii.* Plate A showed a ruptured outer membrane that confirmed the sensitivity of the *A. baumannii* strain. Plate B showed the resistant strain of *A. baumannii* with an intact, regular, and clear outer membrane. In Plate C the outer membrane of *A. baumannii* was affected and deformed due to the synergistic effect of the combination of colistin with the anionic antibiotics. Finally, plate d showed that the outer membrane of *A. baumannii* was also deformed and affected due to the combination of pterostilbene with colistin (Figure 7).

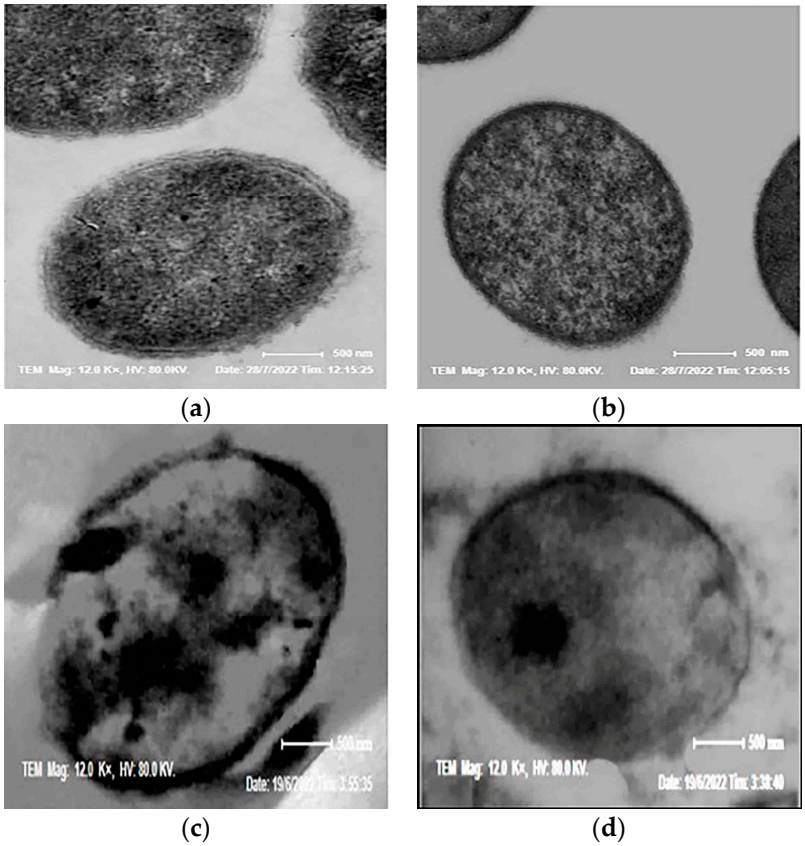

Figure 7. Transmission electron micrograph of *A. baumannii* outer membrane. (**a**) colistin-sensitive *A. baumannii* strain, (**b**) colistin-resistant *A. baumannii* strain (positive for *MCR-1* gene), (**c**) *A. baumannii* strains (positive for *MCR-1* gene) after combination with anionic antibiotic and colistin, (**d**) *A. baumannii* strains (positive for *MCR-1* gene) after combination with pterostilbene and colistin.

Table 2. Antimicrobial activity and MIC of the anionic antibiotics and colistin against colistin-resistant strain 90.

Tested Strain	Antibiotic	MIC of Different Antibiotic Separately	MIC of Combination of Different Antibiotics with 10 mg/mL Colistin
Strain 90	linezolid	0.1 mg/mL	0.001 mg/mL
	Vancomycin	1 mg/mL	0.001 mg/mL
	Rifampicin	1 mg/mL	0.1 mg/mL
	Azithromycin	10 mg/mL	0.1 mg/mL
	Clindamycin	10 mg/mL	1 mg/mL

Table 3. Antimicrobial activity and MIC of pterostilbene and colistin against colistin-resistant strain 90.

Tested Strain	MIC of Pterostilbene only (Group 3)	MIC of Pterostilbene in Combination with 10 mg/mL Colistin
Strain no. 90	No activity	0.01 mg/mL

4. Discussion

4.1. Pandrug Resistant A. baumannii

Acinetobacter is one of the main causes of healthcare-associated infections in recent years [16]. It is very complicated to be controlled due to the prevalence of multi-drug-resistant microorganisms in hospitals. Recently, the emergence of PDR *A. baumannii* isolates worldwide was observed, which is associated with the rapid spread of a few colistin-resistant epidemic families producing the acquired plasmid-mediated *MCR-1* gene that resists colistin [8]. There are only a few studies concerning the epidemiology and colistin susceptibility of *A. baumannii* [10].

4.2. Molecular Features

The molecular identification of *A. baumannii* isolates that were collected, and phenotypically and biochemically identified, was performed by 16S rDNA using universal primers. Recently, 16S rDNA sequencing has played a vital role in the precise detection and identification of bacterial isolates [17]. The current study compared the molecular findings with phenotypic findings to explore the genetic relationship between *A. baumannii* isolates. Nowadays, microbial identification by molecular techniques is considered the best tool due to its accuracy and speed [17].

However, little is known regarding genetic traits and clonal diversity patterns because most investigations have focused on evaluating the antibiotic sensitivity patterns in clinical strains of *A. baumannii* [18]. In the present study *A. baumannii* isolates were detected as PDRs that are resistant to all examined antibiotics.

4.3. Plasmid-Mediated MCR-1 Gene Transfer

The resistance mechanism was due to phosphoethanolamine lipid A transferase plasmid-mediated *MCR-1* gene activity [7]. The first work on the plasmid-mediated *MCR-1* gene was discovered in *E. coli* [8]. In this study, the unusual plasmid-mediated phosphoethanolamine transferase gene *MCR-1* was identified in the selected *A. baumannii* isolates and the sequence of the *MCR-1* gene was identical to the sequences previously described in *E. coli* (*MCR-1*: NG_052663.1). Phylogenetic analysis demonstrates the similarities between *MCR-1* nucleotide sequences of *A. baumannii* samples and those of *E. coli.* This suggests the probability of horizontal gene transfer of *MCR*-positive *A. baumannii* to another. However, these isolates were collected from different hospital compartments (inanimate objects) and for this reason this study is considered the first one that focused on and ensured the probability of the occurrence of horizontal gene transfer.

Moreover, the *MCR-1* is common and widespread in the selective isolates of *A. baumannii* and was considerably higher than those previously reported in *E. coli*. These results indicate that more precautions should be maintained to the spread of *MCR-1* in different ranges of *A. baumannii*. The high level of the *MCR-1* found in *A. baumannii* is likely associated with the widespread use of colistin as a last-line antibiotic used to treat XDR-*A. baumannii* [19].

4.4. Mechanisms of MCR-1 Gene Transfer

The risk of horizontally transferable colistin resistance via the *MCR-1* gene has increased due to the expanding clinical dependence on colistin [14]. The bactericidal effect of colistin occurred in 3 major events: first of all disruption of the outer membrane by interaction with surface LPS, then self-promoted uptake, and finally the lysis of the cytoplasmic membrane due to the formation of instability region in the cytoplasmic membrane [14]. This is supported by the addition of cationic phosphoethanolamine to phosphate groups on the lipid A component of LPS, which reduces the network of anionic charge of the cell surface. Colistin resistance happened through a reduction of the electrostatic attraction between colistin and the *A. baumannii* outer membrane.

4.5. Overcoming Colistin Resistance by Combination with Anionic Antibiotics and/or Pterostilbene

Previous studies declared colistin was unable to traverse an intact bacterial outer membrane, while colistin in combination with antibiotics could cause disruptive effects on the outer membrane [20]. The *MCR-1* gene represents the expected cause of colistin resistance. The traditional mechanism for the *MCR-1* gene and other colistin resistance is the addition of a cationic part to the phosphates of lipid A, which diminishes the electrostatic interaction between colistin and lipid A and prevents disruption of the outer membrane and, consequently, self-promoted uptake and lysis [20].

The present study shows that re-sensitization of *MCR-1*-expressing *A. baumannii* can be attained by using combinations of colistin with anionic antibiotics that are generally effective against Gram-positive bacteria.

A natural compound used in traditional Chinese medicine Pterostilbene (trans-3,5-dimethoxy-4′-hydroxystilbene) shows synergistic activity with colistin against *E. coli* in vitro and in vivo [7]. This proves the effectiveness of pterostilbene in combination with colistin to restore the bactericidal activity of colistin against *MCR*-1-positive *A. baumannii*. The microdilution method confirmed that pterostilbene lowers colistin MIC in *MCR*-1-positive *A. baumannii* strains. Furthermore, by reducing the amount of colistin used in clinical therapy, we could reduce the possibility of mutations forming in the LPS modification pathways in *A. baumannii*, which can occur with long-term use of colistin.

4.6. Transmission Electron Microscopy

Moreover, transmission electron microscopy studies proved the synergistic effect of the combination of colistin with anionic antibiotic and the combination with pterostilbene on cell membranes. The results showed that treated *A. baumannii* strains can disrupt the membrane of resistant cells, indicating that the combination could alter the permeability properties to mediate the disruption of the *A. baumannii* membrane, resulting in the efflux of essential cytoplasmic components that allow them to overcome the resistance of the isolates to colistin which then leads to the death of bacterial cells. Overall, the combination of colistin with anionic antibiotic and the combination with pterostilbene could be a novel and promising clinical alternative against the increasing threat of a widespread dissemination of multidrug-resistant *A. baumannii*.

5. Conclusions

The study focused on the identification of *A. baumannii* isolates exhibiting PDR, as they displayed resistance to all tested antibiotics except for colistin. The underlying resistance mechanism was attributed to the presence of the plasmid-mediated phosphoethanolamine transferase *MCR-1* gene in these isolates. The *MCR-1* gene, responsible for conferring

resistance, encodes a plasmid-mediated phosphoethanolamine lipid A transferase. This gene's activity leads to the observed resistance phenotype in the *A. baumannii* isolates.

Notably, the study found that combining colistin with a variety of anionic antibiotics, typically effective against Gram-positive bacteria, exhibited an impact on *MCR*-1-expressing *A. baumannii* strains. This suggests a potential synergy between colistin and these anionic antibiotics in addressing the antibiotic resistance mediated by the *MCR-1* gene.

Furthermore, the administration of pterostilbene, in conjunction with colistin, demonstrated the ability to restore the bactericidal activity of colistin against *MCR-1*-positive *A. baumannii*. This combination was observed to alter the permeability properties of the *A. baumannii* membrane, as evidenced by transmission electron microscopic assays, ultimately leading to the disruption of the bacterial membrane.

These findings provide valuable insights for future studies aiming to overcome antibiotic resistance mediated by the *MCR-1* gene in *A. baumannii.* Building upon the observed synergy between colistin and anionic antibiotics, as well as the potential of pterostilbene to restore colistin's efficacy, further investigations can explore optimized combination therapies and delve into the underlying mechanisms involved. Such research endeavors hold the potential to enhance our understanding and contribute to the development of innovative approaches in combating multidrug-resistant *A. baumannii* infections.

6. Materials and Methods

6.1. Sample Collection

The samples were collected by swabbing technique from public hospitals in Cairo (Egypt) of different compartments (ICU, wards, surgery rooms) from inanimate sources including (bed rails, ventilators, monitors, etc.) during the period of six months from April to October 2020. A total of 117 isolates were confirmed as *Acinetobacter* spp.

6.2. Phenotypic Identifications of Isolate

All isolates were confirmed to be *A. baumannii* based on phenotypical tests (colony morphology and Gram stain results) and biochemical tests including catalase [21], urease [22], oxidative-fermentative medium (OF) [23], oxidase [24], triple sugar iron agar (TSI) [25], citrate [26], SIM tests [27], and finally confirmed by API NE20 multi-test system (Biomerieux, Marcy-l'Etoile, France), according to Risan [28]. Isolates were stored in the MacConkey broth medium supplemented with 40% glycerol at stored at −80 °C for further analysis.

6.3. Antibiotic Susceptibility Test

The pattern of antimicrobial resistance was studied using the simple disk diffusion technique [29]. All isolates were tested for antibiotic susceptibility on Muller Hinton Agar (MHA) plates by diffusion with Kirby-Bauer discs according to 2021 Clinical and Laboratory Standards Institute (CLSI) guidelines. Nine Antibiotics including Aztreonam (Monobactam) (30 µg/disc); amikacin (aminoglycoside) (30 µg/disc); ciprofloxacin and levofloxacin (quinolone) (5 µg/disk); cefepime (cephalosporin) (30 µg/disc); cefotaxime (cephalosporin) (30 µg/disc); Imipenem (carbapenem) (10 µg/disc; piperacillin/tosabactam (penicillins) (10 µg/disk); colistin (polymyxin) (10 µg/disk) were used to determine the multidrug-resistant (MDR), extensively drug-resistant (XDR) and pan-drug-resistant (PDR) status of *A. baumannii.* All inoculated plates were incubated aerobically at 42 °C for 18–24 h in an aerobic atmosphere. The results were interpreted based on the information provided by CLSI [30].

6.4. Molecular Identification of Bacterial Strains Using 16S rDNA

6.4.1. Extraction of Genomic DNA from *Acinetobacter baumannii* Isolates

A single colony of *A. baumannii* PDR isolates was inoculated into 5 mL MacConkey broth and incubated at 42 °C overnight. Genomic DNA was extracted from bacterial colonies using the GeneJET™ PCR purification Kit (Thermo Scientific, Los Angeles, CA,

USA) according to the manufacturer's instructions. The quality and quantity of DNA were decided upon by determining the absorbance of the sample at 260/280 nm with a spectrophotometer (Spectrum lab 721S, Guangzhou, China). DNA samples were stored at −80 °C for further investigation.

6.4.2. 16S rDNA PCR Amplification and Sequencing

16S rDNA was amplified in a programmable thermal cycler (NYXTECHNIK, San Diego, CA, USA). Universal primers of 16S rDNA obtained from Macrogen company were used in PCR amplification.

Forward primer (27 F) 5′-AGAGTTTGATCATGGCTCAG-3′
Reverse primer (1492 R) 5′-GGTTACCTTGTTACGACTT-3′ [31]

Polymerase chain reaction (PCR) cycles were programmed for the denaturation step for 5 min at 95 °C, followed by 35 cycles of 95 °C for 2 min, 48 °C for 1 min and 72 °C for 4 min, plus one additional cycle of a final chain elongation at 72 °C for 20 min. A volume of 15 µL of the PCR products was separated on a 1.0% agarose gel stained with TBE-ethidium bromide at 75 Volts for 1 h. With ladder DNA marker (100 bp–3000 bp) (AXYGEN). The gel was photographed using Gel Doc™ XR+ Gel Documentation System. Successful PCR products were sent to Macrogen company to be sequenced by the Dideoxy-chain termination method using 3500 genetic analyzers. The nucleotide sequences of the 16S rDNA of *A. baumannii* have been deposited to NCBI to be listed and documented in the GenBank database and were analyzed for similarities to other known sequences found in the GenBank using the Basic Local Alignment Search Tool. The (BLASTn) tool indicates the best matches based on percent sequence identification. (https://blast.ncbi.nlm.nih.gov/Blast.cgi, accessed on 20 June 2022)

6.5. Phylogenetic Construction

AlignStatPlot (Molecular Evolutionary Genetics Analysis software tool, version no. 1.0) (https://bioinformatics.um6p.ma/AlignStatPlot, accessed on 6 November 2022) was used for sequence alignment and phylogenetic tree construction [32].

Detection of phosphoethanolamine-lipid A transferase *MCR-1* gene in PDR *A. baumannii*
Detection of phosphoethanolamine-lipid A transferase *MCR-1* gene in chromosomal DNA

The previously extracted genomic DNA of the eleven selected PDR *A. baumannii* was used to detect the presence of the *MCR-1* gene using specific primers for the gene:

Forward primer (F)5′-Fa ATGACAAAAATCTTGATGATTG-3′
Reverse primer (R)5′-Fa TTATGATTGCCCCAAACGGTAG-3′

The specific primers for the *MCR-1* gene were designed on the primer 3 program. Primer relied on the conserved region of *Escherichia coli* strain L36 phosphoethanolamine-lipid A transferase *MCR-1* with accession numbers MT070408, it was obtained from Macrogen company.

6.6. Detection of Phosphoethanolamine-Lipid a Transferase MCR-1 Gene in Plasmid

Extracted plasmid from eleven PDR *A. baumannii* were subjected to genotyping using PCR according to Schwarz and Johnson [33].

A single colony of PDR *A. baumannii* isolates was inoculated into 5 mL MacConkey broth and incubated overnight at 42 °C. Plasmid DNA was extracted from the bacterial colony using a plasmid DNA extraction kit GeneJET™ PCR Purification Kit (Presto™ Mini plasmid kit) according to the manufacturer's instructions.

6.7. PCR Amplification and Sequencing of Phosphoethanolamine-Lipid a Transferase MCR-1 Gene

The amplification reactions of PCR cycling conditions were cycled 35 times after an initial denaturation step for 5 min at 94 °C. The thermocycling profile consisted of 30 s at 94 °C (denaturation), 1 min was used for annealing using different temperatures (52 °C),

and 1 min at 72 °C (extension) with a final extension step for 10 min at 72 °C. The results of the amplified region of a given gene were visualized on a 1.5% agarose gel stained with TBE-ethidium bromide at 75 volts and compared with 100 bP Ladder DNA marker (100 bp–3000 bp) (GeneRuler 100 bp DNA Ladder, Thermo Scientific, Los Angeles, CA, USA). The gel was photographed using Gel Doc™ XR+ Gel Documentation System. The PCR products containing amplicons of 700 bp for 78 and 90 strains were sequenced by the Sanger sequencing method (ABI 3730XL DNA Analyzer) in (Colore Company).

6.8. Data Analysis of Phosphoethanolamine-Lipid a Transferase MCR-1, Phylogenetic Construction and Sequencing of MCR-1 Gene

The sequence comparison was performed using the BLAST available at the NCBI (http://www.ncbi.nlm.nih.gov/blast/, accessed on 15 February 2023). For the sequence annotation of the gene, https://www.expasy.org/tools/dna.html was used.

The partial nucleotide sequences of the *MCR-1* genes detected in this study were found to be like the published sequences of different entries of *MCR-1* genes. AlignStatPlot (https://bioinformatics.um6p.ma/AlignStatPlot) was used for sequence alignment of the *MCR-1* gene and phylogenetic tree construction [32].

The annotated sequence of the *MCR-1* genes for the two isolates 78 and 90 were submitted to the National Center for Biotechnology Information (GenBank) to be listed in the gene bank.

6.9. Overcoming MCR-1 Mediated Colistin Resistance in A. baumannii

The Minimum inhibitory concentration (MIC) assays were used to identify synergies between anionic antibiotics such as Rifampicin (RA), Clindamycin (CLI), Vancomycin (VA), Azithromycin (AZ), linezolid (LIZ) and colistin against colistin-resistant strain 90. Additionally, it was used to identify the synergetic effect between the natural compound pterostilbene and colistin against colistin-resistant strains.

Different anionic antibiotics and a natural compound pterostilbene were used in serial dilutions from 10 mg/mL to 0.0001 mg/mL in combination with a fixed concentration of colistin 10 mg/mL to determine MIC of antimicrobial activity and the synergistic effect between anionic antibiotics and colistin against colistin-resistant strains. In the meanwhile, the synergistic effect between pterostilbene and colistin against colistin-resistant strains was also examined. *A. baumannii* isolates were cultured on MacConkey broth medium at 42 °C with shaking at 180 rpm to obtain an OD 600 value of ~0.3. The MIC test was grouped as illustrated in Table 4.

Table 4. Groups of the tested antibiotics and pterostilbene for MIC test.

Group No 1	Anionic antibiotics against colistin-resistant strain no 90.
Group No 2	Anionic antibiotics and 10 mg/mL of colistin against colistin-resistant strain no 90.
Group No 3	Pterostilbene (10 mg/mL to 0.0001 mg/mL) and 10 mg/mL of colistin against colistin-resistant strain no 90.
Group No 4	Pterostilbene 0.01 mg/mL (the best dilution) and colistin (10 mg/mL to 0.0001 mg/mL) against colistin-resistant strain no 90.

6.10. Transmission Electron Microscope (TEM) Analysis of A. baumannii

Four groups were prepared to examine the outer membrane of *A. baumannii* (strain no. 90) by TEM. The first group is the colistin-sensitive *A. baumannii*, the second group is the colistin-resistant *A. baumannii* (positive for *MCR-1* gene), the third group is the colistin-resistant *A. baumannii* after combination with the best anionic antibiotic and colistin, and the fourth group is the colistin-resistant *A. baumannii* after combination with pterostilbene and colistin. The four samples were fixed in glutraldhyde and then fixed in 4% osminum tetroxide at 4 °C for 1 h. The samples were dehydrated in graded ethanol/water series, followed

by infiltration by resin (Epoxy resin). The specimen was placed in a mold filled with liquid resin and cured into a hard block using heat. A section of 1 µm thin was cut and mounted on copper grids and stained by uranyl acetate and lead citrate [34,35]. *A. baumannii* slides were observed using JEOL JSM 1400, (Tokyo, Japan) TEM at the Electronic Microscope Unit, Research Laboratories Complex, Faculty of Agriculture, Cairo University, Giza, Egypt.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/microbiolres14030083/s1, Supplementary Data S1. Table S1. Characteristics of *Acinetobacter* isolates. Supplementary Data S2. Table S2. Antibiotic resistance patterns of *Acinetobacter baumanni*.

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