FIRST REPORT IN IRAQ: AMINO ACID SUBSTITUTION IN PMR CAB GENES AND THERE CORRELATION WITH COLISTIN RESISTANCE AMONG A.BAUMANNII ISOLATES

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ABSTRACT
In the present study, out of 120 samples were collected from different clinical sites from patients who admitted to different hospitals in Baghdad city, only 65 samples were detected as A. baumannii. The antibiotics susceptibility test showed that the bacteria were resistant to piperacillin (92.31%), cefotaxime (87.69%), trimethoprim-sulfamethoxazole (73.85%). While Meropenem (70.77%) and gentamycin (67.69%). The resistance rate to imipenem and tetracycline were 58.46% and 43.08% respectively. Eventually, just (20%) among all isolates are resistant to levofloxacin. While the percentage of colistin resistance was performed among 23 isolates and the results showed that all isolates were reported resistance in a percentage 100% with MIC values ≥ 16 µg/100µl. The results of pmrCAB genes detection and sequencing among seven A.baumannii isolates, which were choices because they were resistant to all antibiotics even colistin, were showed that the entire region of the pmrC containing fourteen missense mutations. Out of twelve detected mutations in pmrA, only one of them was found to exhibit a missense mutations. While the results of pmrB gene showed three missenses mutations.

Key Words: colistin, antimicrobial resistant, mutation.

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التقرير الأول في العراق: استبدال الأحماض الأمينية في جينات pmr CAB ووجود ارتباط بمقاومة الكوليستين بين عزلات A.baumannii

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باحث
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المستخلص
تم جمع 120 عينة من مختلف المصادر السريري من مرضى دخلوا مستشفيات مختلفه في مدينة بغداد. تم الكشف عن 65 عينة فقط من نوع الراكد البومانيه. أظهرت نتائج اختبار الحساسية للمضادات الحيوية ان البكتريا كانت مقاومة لمضادات البيبيراسيلن (92.31%), سيفوتاكسيم (87.69%), تريميولوريم-سلفاميثوكساسول (73.85%). بينما الميروبيدين (70.60%), والجنتامايسين (69.67%). كانت نسبة المقاومة لللايميبينين والتراسكلين (46.58%). (43.08%) على التوالي. وفي النهاية ، فقط (20%) من بين جميع العزلات مقاومة لليفوفلوكساسين. بينما أظهرت نسبة مقاومة الكوليستين بين ثلاثة على ماضي. وعشرين علية ان جميع العزلات سجلت مقاومة بنسة 100% مع (MIC) ≥16 مايكروغرام / 100 مايكروليلتر. قيم اظهرت نتائج الكشف عن جينات pmrCAB وتسجلها بين سبعة عزلات بكتيريه والتي تم اختبارها بناءً على مقاومتها لجميع المضادات الحيوية حتى الكوليستين، حيث أن جين (pmrC) يحتوي على اربعة عشر طفرة خطاً. بينما يحتوي جين (pmrB) على فكلاً واحد من بين أثني عشر طفرة. في حين ان نتائج جين (pmrB) اظهرت وجود ثلاثة طفرات خطاً.

الكلمات المفتاحية: الكوليستين، مقاومة مضادات الماكبرويات، الطفرة
INTRODUCTION

*A. baumannii* is gram-negative, strictly aerobic, catalase-positive, non-motile, non-fermenting, non-fastidious. It can expand over a range of temperatures, pHs and nutrient content making pathogens highly adapted to existence in both human and ecological conditions (16,34). *A. baumannii* is typically a pathogen associated with health care and has been identified as the cause of outbreaks and nosocomial infections in several studies (35). Rising the levels of *A. baumannii* infection related to the difficulties encountered in administering efficient antimicrobial therapy has been a great concern in recent decades. Intrinsic properties, such as the powerful permeability barrier, efflux pump and chromosomally coded lactamases have greatly decreased the number of successful antibiotics that could be used in few cases to zero against some isolates. In the last few years, the largest proportion of resistance mechanisms in several antibiotic classes have resulted in the removal of it such as cephalosporin, quinolone, penicillin, tetracycline and aminoglycoside as effective treatment choices for antimicrobial agents. Carbapenem, due to its high efficiency and little toxicity, remains the only major sustainable class of antibiotic therapy for *A. baumannii* contagion. Nevertheless, The growing emergence of carbapenem resistance also threatened to compromise this therapeutic alternative in recent years (1,4,6). After the accumulation of many processes of resistance in *A. baumannii* and reduced the number of available groups of antibiotics to treat it, as the final treatment choice for infections caused by carbapenem-resistant bacteria, colistin is used by clinicians (19). Later unfortunately, the resistance of colistin between the strains of *A. baumannii* have risen over time (29). Mostly due to the acquisition of plasmid-mediated resistance genes, also *A. baumannii* quickly develops colistin resistance from in vitro and in vivo drug exposure to selective pressure by genetic mutations (12,22). Two major mechanisms for colistin-resistant induction: modulation of lipoooligosaccharide (LOS) by the acquisition of single nucleotide polymorphisms in *pmrAB*; or total depletion of LOS due to SNPs in genes encoding the *lpxA*, *lpxC* and *lpxD* genes of lipid A biosynthesis. The LOS modification or loss led to a decrease in the net negative charge of the LOS, hence the connection between colistin and the cell membrane decreases (9). Mutations in genes other than *pmrAB* and *lpxA* can cause resistance to colistin (25). In particular, the role of the *mla* genes in *A. baumannii* membranes composition. The Mla pathway stops phospholipids from gathering in the outer membrane. MlaA is a protein associated with the outer membrane, and MlaC has been recognized as the protein that returns stray phospholipids back to the inner membrane through the periplasmic region. The roles of *MlaB*, -D, and -F are less evident, but resistance to colistin has been correlated with mutated versions of these proteins (27). The aim of study: colistin is one of promising option for treatment of *Acinetobacter baumannii*. The current study investigated the emergence of colistin resistance among carbapenem resistant strains of the *A. baumannii*.

MATERIALS AND METHODS

Samples collection and identification

Approximately 120 samples were collected randomly from various clinical sources including (burns, blood, sputum, urine, wounds) from patients who admitted to different hospitals in period between September 2019 to December 2019. The bacterial isolates were identified by using specific *A. baumannii* biochemical tests. Then the previous bacterial identification findings were verified by the use of the API 20E and molecular identification using the 16 s rRNA gene.

Antibiotic susceptibility test

Disk diffusion method was used to confirm the susceptibility of all known isolates using 8 types of antibiotics including cefotaxim (30mg), gentamycin (10mg), imipenem (10mg), L. evofloxacine (5mg), Meropenem (10mg), piperacillin (100mg), Tetracycline (30mg), Trimethoprim-m-sulphamethoxazol (1.25/23.75 mg) on MH agar in compliance with the guidelines of the Clinical and Laboratory Standards Institute CLSI 2018 (11).

Minimum inhibitory concentration (MIC)

Micro-titer dilution method was used to determine the MIC of colistin antibiotic for 23 isolates which choose depends on their ability to resist most antibiotics. After diluted the
antibiotic in ranges of 2,4,8,16 μg/100 μl, the result must be interpreted after 16-18 hours of incubation at 37°C according to the Clinical Laboratories Standards Institute CLSI (2018).

Molecular study
The genomic DNA of all isolates was extracted using the Genaid Kit according to the manufacturers instructions (Geneaid Biotech, Taiwan).

Table 1. The specific primer pairs that selected to amplify 16s rRNA, pmrA, pmrB, and pmrC genetic loci within the Acinetobacter baumannii genomic sequences

<table>
<thead>
<tr>
<th>Set</th>
<th>Locus</th>
<th>Specific primer sequences (5’ to 3’)</th>
<th>Product size (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>16s rRNA</td>
<td>5-CAGCTCGTGTGCTGATGATGT-3 5-CGTAAGGGCCATGATGACTT-3</td>
<td>150</td>
<td>17</td>
</tr>
</tbody>
</table>
| 2   | pmrA  | F: ATGACAAAAATCTTGTGATGTAAGAT  
R: TTATGATTGCCCAAACCGTAG | 675 | This study |
| 3   | pmrB1 | F: AACAGACGGATTGGGACACCTC  
R: GAGCAGCTCGCAAAATACCC | 664 | This study |
| 4   | pmrB2 | R: ATTTTCAATACAGAAAAGCCG | 697 | This study |
| 5   | pmrC1 | F: ATGTTTAATCTCTATTATAGCCA  
R: ACCGCATGAGCTCATT | 792 | This study |
| 6   | pmrC2 | R: TTAGTTTACATGGGCAACAA | 870 | This study |

The PCR reaction was performed using 20μl of PCR premix was contained 1U of Top DNA polymerase, 250μM of dNTPs, 10mM of Tris-HCl (pH 9.0), 30mM of KCl, 1.5mM of MgCl2. The reaction mixture was completed with 10 pmol of each primers utilized in this study and 50ng of genomic DNA. The amplification was begun by initial denaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C, annealing 55,56.6,61,54°C for 16Sr RNA , pmr C,A,B respectively and elongation at 72°C, and was finalized with a final extension at 72°C for 10min for all the primers .Amplification was verified by electrophoresis on an ethidium bromide(0.5mg/ml) pre-stained 1.5%(w/v) agarose gel in 1x TBE buffer (2mM of EDTA, 90 mM of Tris-Borate,pH 8.3), using a 100-bp ladder(Cat#D-1010,Bioneer,Daejeon,South Korea) as a molecular weight marker. It was made sure that all PCR resolved bands are specific and consisted of only one clean and sharp band to be submitted into sequencing reactions.

DNA sequencing of PCR amplicons
The solved PCR amplicons were sequenced from forward, according to the instructions of the sequencing company(Macrogen Inc.Seoul, South Korea). The sequencing results of the PCR products have been edited, aligned, and analyzed in the reference database with the corresponding sequences by using BioEdit suit. The variations observed were counted in PCR amplicons in each sequenced sample and also in their specified location within the referred genome. The amino acid sequence of the target proteins was collected from the protein data bank online.

RESULTS AND DISCUSSION
The results showed that out of 120 samples, only 90 samples had Acinetobacter spp positive growth. Based on the results of diagnosis by biochemical tests, the API 20E system, and using a molecular method (16SrRNA), 65 out of 90 isolates were recognized as A. baumannii isolates. While the other 55 isolates belonging to another pathogenic bacterium.

Antibiotic susceptibility
The arising of A. baumannii strains that resist broad-spectrum antimicrobial agents in clinical settings have become a major health problem, due to the restriction treatment options for the infections caused by this pathogen(7,13,32). It
is important to use precise tests for resistance in order to reduce treatment times and to ensure that antibiotics are successful such as utilizing the Disk diffusion method. The results showed a high resistance rate to piperacillin (92.31%), cefotaxime (87.69%), trimethoprim-sulfamethoxazole (73.85%) among *Acinetobacter baumannii* isolates. In addition, all isolates have shown a resistance rate to gentamycin in percentage (67.69%) also the resistance rate to imipenem and tetracycline (58.46%, 43.08%) respectively. Finally, only (20%) of all isolates are resistant to levofloxacin Figure 1. In our study, high resistance noted among *A. baumannii* isolates to piperacillin 92.31% and cefotaxime 87.69%. This result was accepted with study by Ghaima (14) which found that the resistance rate for PRL was 90% in addition to CTX 95%. Also, resistance to trimethoprim-sulphamethoxazole was high 73.85%. This resistance rate for the same antibiotic was 100% that confirmed by Abdallah et al. (2). Its worthy notice that from all tested isolates 52.3% were resistant to carbapenems. The susceptibility rate of the isolate toward the meropenem and imipenem was 70.77% and 58.46% respectively. This result was agreed with the study conducted by Mshachal et al. (28) who recorded that the resistance rates for meropenem and imipenem were 80.64% and 50% respectively. In this study, 67.69% of isolates resistant to gentamicin. Analysis of antibiotic resistance by Aliakbarzade et al. (5) showed high resistance to aminoglycoside in 86%. Moreover, all the isolates were showed moderate resistance to tetracycline in 43.08%. This results in accordance with the rate of resistance observed by Beheshti et al. (8). On other hand, the isolates were mostly sensitive to levofloxacin in percentage 64.62% with a low resistant rate of 20%. This result disagreed with the study of Mera et al. (26) who found all isolates were resistant to levofloxacin in 52.3%. Complex factors such as the presence of the mobile component, misuse of antimicrobial drugs, poor infection control practices, and increased international travel facilitate the patterns of the spread of resistance among *Acinetobacter baumannii* isolates (3,36).

Figure 1. Antibiotic susceptibility test percentage of *A. baumannii* isolates. Cefotaxime (CTX), Gentamycin (CN), Imipenem (IPM), Levofloxacin (LEV), Meropenem (MEM), Piperacillin (PRL), Tetracycline (TE), Trimethoprim-sulfamethoxazole (SXT).

Minimal inhibitory concentration (MIC) of colistin
In our study, the percentage of colistin resistance among *A. baumannii* isolates (n=23) that resist to most of the tested antibiotics including carbapenem, was reported as 100% with MIC values ≥ 16µg/100 µl Figure 2. These results disagreed with the findings by Hameed et al. (15) who found that 9.6% of *A. baumannii* isolates with MIC ranging from 8 to 16mg/ml. 7% of bacterial isolates were documented to be colistin-resistant with a MIC range of 16 to 64 mg/ml as reported by Oikonomou et al. (30). Another study by
Lescat et al. recorded that 57 of *A. baumannii* isolates were resisting with MIC ranging from 4 to 128 mg/ml (20). However, the heteroresistance of these study varied from (7% to 100%) this may be due to multiple samples and varying standards to determine the heteroresistance (21).

**Figure 2. Minimum inhibitory concentration determination for *A. baumannii* isolates by using resazurin sodium stain, the change in color after incubation for 2-4 hours.** Column (C-) (blue/purple) corresponds to the negative control, column (C+) (pink) to the positive control.

**Molecular detection of pmrCAB genes**

The three types of genetic fragments (pmrCAB), covering three loci were detected among 7 isolates of 23 isolates which showed a high resistance rate to most antibiotics even carbapenems and colistin namely S3, S4, S15, S30, S34, S40, S46. Five selected fragments of pmrA, pmrB1, pmrB2, pmrC1, and pmrC2 were utilized to amplify genomic DNA sequences of 675 bp, 664 bp, 697 bp, 792 bp, and 870 bp respectively which represent the entire pmrCAB. The results showed the presence of (pmrA, pmr B, pmrC-like genes) in all 7(100%) of *A. baumannii* clinical isolates.
Figure 3. Gel electrophoresis image showing PCR amplification of the gene (pmrA, pmrB, pmrC) (2% Agarose. current 200 for 70 min at 90 voltage), Lane M: DNA marker (100-1500bp), Lane: (3, 4, 15, 30, 34, 40, 46) represents the positive results of A. baumannii isolates.

Sequencing results of pmrCAB operon
A direct sequencing strategy was conducted in the currently investigated samples to resolve the pattern of genetic diversity in each analyzed isolate (S3, S4, S15, S30, S34, S40, S46). The sequencing reactions revealed the accurate locations after the analysis of NCBI blastn. This engine showed about 99% sequences of similarity between the sequenced samples and these targets. NCBI BLASTn engine indicated the presence of remarkable homology with the expected targets (pmrCAB) within the Acinetobacter baumannii sequences. The results of pmrCAB operon sequences of seven colistin resistance A. baumannii isolates showed that in pmrA amplicons according to the alignment results by Bio Edit software Figure 4, about twelve nucleic acid substitutions were observed with only one missense effect (R87C) that was observed in three investigated isolates (S3, S4, and S46). The novel pmrA mutations were deposited under accession numbers (MW315712, MW315713, MW315714, MW315715, MW315716, MW315717, MW315718) in the NCBI database. Concerning pmrBI amplicons that covered the upstream portion of the pmrB locus, thirteen nucleic acid substitutions were observed with three missense effect (E72K, D73N, and A138T).
that were observed in two to four investigated isolates (S4, S15, S30, S34), respectively. Though ten nucleic acid substitutions were also observed in pmrB2 amplicons (that covered the downstream portion of the pmrB locus) based on the results of alignment by using bio edit software Figure5 and Figure 6, no missense effects were observed and all detected variations were just silent variations within the pmrB2 region. Novel of entire pmrB mutations were stored under accession numbers in NCBI database (MW315719, MW315720, MW315721, MW315722, MW315723, MW315724, MW315725, MW315726, MW315727, MW315728, MW315729, MW315730, MW315731, MW315732). Lastly, the pmrCl amplicons that covered the upstream portion of the pmrC locus, thirty-three nucleic acid substitutions were observed with nine missense effects (Y65N, Y93N, I131N, E149K, F166L, Y177N, H208Q, D224N and D269N) that were observed in the investigated isolates in variable density. Likewise, twenty-one nucleic acid substitutions were observed in the pmrC2 that covered the downstream portion of the pmrC locus, according to the consequence of alignment Figure 7 and Figure 8. Within these observed variations, five missense effects (D298G, A370S, H499R, N514K and K531T) were observed with variable distributions on the same analyzed pmrC-encoded protein. The novel mutation of entire pmrC have been deposited in NCBI database under accession numbers (MW315733, MW315734, MW315735, MW315736, MW315737, MW315738, MW315739, MW315740, MW315741, MW315742, MW315743, MW315744, MW315745, MW315746). The microevolution of a bacterium is strongly affected by single nucleotide polymorphisms (SNPs). These mutations also have a significant impact on antibiotic resistance and pathogenicity. Colistin resistance has been found in recent years to be due to SNPs in the genes that encode the pmrAB two-component system responsible for the synthesis and control of lipopolysaccharide in the bacterial cell wall (31). In particular, external stimuli (example: higher Fe3+, elevated Al3+, and low pH) cause PmrB autophosphorylation at the preserved residue of histidine in its cytoplasmic domain accompanied by phosphoryl group transfer to the preserved residue of PmrA aspartate (18). That activating PmrA, therefore to encourage the expression of PmrA activated genes, active PmrA binds to DNA. One of the genes that are positively regulated by PmrA, is eptA (also known as pmrC) which is encoded in the pmrCAB operon (33). The expression of pmrC leads to the addition of phosphoethanolamine (pEtN) to one or two lipids A phosphate positions decreases the negative LPS charge and hence the association with colistin. The most popular resistance mechanism found in clinical A. baumannii isolates is pEtN mediated resistance (23). In the present study, only one amino acid substitution was observed represented by substitution of Arg I with Cysteine I (R87C) in the receiver domain residues of the entire PmrA encoded protein. The receiver domain is important for sensing the activation of PmrB and facilitating the DNA binding domain residues to identify the DNA binding site, thus binding and activating the transcription of the targeted genes (24). Conformational changes in the receiver domain caused by mutations are likely to increase PmrA phosphorylation and lead to improved DNA binding ability and up-regulation of the targeted genes. Three amino acid substitutions were observed as represented by the substitution of Glu I with Lys (K) (E72K), Asp (D) with Asn (N) (D73N), and Ala (A) with Thr (T) (A138T) in the transmembrane domain of the entire pmrB-encoded two-component system sensor histidine kinase PmrB. Relevant physiological signals (e.g. high Fe3+ and high Al3+) are detected by the transmembrane domain and the phosphorylation of PmrB by conformation changes is subsequently enhanced. Even in the absence of these environmental signals, mutations in the transmembrane domain may trigger these conformational changes and thus constitutively promote the phosphorylation of PmrB (10). Also, nine amino acid substitutions (Y65N, Y93N, I131N, E149K, F166L, Y177N, H208Q, D224N, and D269N) were observed in the entire pmrC1-encoded phosphoethanolamine-lipid A transferase, while five amino acid substitutions were observed as represented by the substitution of five amino acids (D298G, A370S, H499R, N514K, and K531T) in the entire pmrC2-encoded
phosphoethanol amine-lipid A transferase. Some mutations located in the transmembrane domain and the other in the sulfatase domain in PmrC (table 2) which is responsible for increasing the MIC of colistin and is correlated with overexpression of pmrC and resistance to colistin antibiotic in our A.baumannii.

Figure 4. DNA sequences alignment of seven bacterial samples (forward sequence) compared with its reference sequences of the pmrA gene within the Acinetobacter baumannii sequences. The symbol “ref” refers to the NCBI reference sequences, while “S” refers to sample code
Figure 5. Alignment of DNA sequences of seven bacteria isolates with their corresponding pmrB1 locus reference sequences inside the genomic sequences of Acinetobacter baumannii. The symbol “ref” refers to the NCBI reference sequences, while “S” refers to sample code.
Figure 6. Alignment of the DNA sequences of seven bacterial specimens with their corresponding *PmrB2* locus reference sequences inside the genomic sequences of *Acinetobacter baumannii*. The symbol “ref” refers to the NCBI reference sequences, while “S” refers to sample code.
Figure 7. Alignment of seven bacterial samples with their corresponding pmrC1 locus reference sequences inside the genomic sequences of Acinetobacter baumannii with DNA sequences. The symbol “ref” refers to the NCBI reference sequences, while “S” refers to sample code.
Figure 8. Within the Acinetobacter baumannii genomic sequences, DNA sequences align seven bacterial samples with their corresponding pmrC2 locus reference sequences. The symbol “ref” refers to the NCBI reference sequences, while “S” refers to sample code.

Table 2. Amino acid substitutions in the pmrCAB genes

<table>
<thead>
<tr>
<th>Strain</th>
<th>PXEβ/MIC (µg/ml)</th>
<th>pmrC (549 aa)</th>
<th>PmrA (224 aa)</th>
<th>pmrB (444 aa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S3</td>
<td>≥ 16</td>
<td>Y65N,Y93N,</td>
<td>H499R,</td>
<td>R87C</td>
</tr>
<tr>
<td></td>
<td>D224N</td>
<td></td>
<td>N514K</td>
<td></td>
</tr>
<tr>
<td>S4</td>
<td>≥ 16</td>
<td>Y65N,Y93N,</td>
<td>D269N,D298G</td>
<td>E72K,D73N</td>
</tr>
<tr>
<td></td>
<td>H208Q,D224N</td>
<td>H499R,N514K</td>
<td></td>
<td>A138T</td>
</tr>
<tr>
<td>S15</td>
<td>≥ 16</td>
<td>Y65N,Y93N,</td>
<td>A370S,N514K,</td>
<td>E72K,D73N</td>
</tr>
<tr>
<td></td>
<td>E149K,F166L,</td>
<td></td>
<td>K531T</td>
<td>A138T</td>
</tr>
<tr>
<td></td>
<td>Y177N,D224N</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S30</td>
<td>≥ 16</td>
<td>Y65N,Y93N,</td>
<td>D269N,A370S,</td>
<td>E72K,</td>
</tr>
<tr>
<td></td>
<td>E149K,F166L,</td>
<td></td>
<td>N514K</td>
<td>D73N,</td>
</tr>
<tr>
<td></td>
<td>Y177N,D224N</td>
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<td>K531T</td>
<td>A138T</td>
</tr>
<tr>
<td>S34</td>
<td>≥ 16</td>
<td>Y65N,Y93N,</td>
<td>A370S,N514K,</td>
<td>E72K,</td>
</tr>
<tr>
<td></td>
<td>F166L,Y177N,</td>
<td></td>
<td>K531T</td>
<td>D73N,</td>
</tr>
<tr>
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<td>D224N</td>
<td></td>
<td></td>
<td>A138T</td>
</tr>
<tr>
<td>S40</td>
<td>≥ 16</td>
<td>Y93N,N123N,</td>
<td>N514K</td>
<td></td>
</tr>
<tr>
<td></td>
<td>D224N</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S46</td>
<td>≥ 16</td>
<td>D224N</td>
<td>D298G,H499R,</td>
<td>R87C</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>N514K</td>
<td></td>
</tr>
</tbody>
</table>

The predicted domains according to the NCBI domain predictor (www.ncbi.nlm.nih.gov/protein) are indicated as follows: sulfatase, sulfatase domain; aa, amino acid; Rec, signal receiver domain; HisK, histidine kinase (dimerization/phosphor acceptor) domain; and HATPaseC, histidine kinase-like ATPase. Polymyxin E (PXE) MICs were evaluated according to the CLSI broth microdilution method.

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