FIRST REPORT IN IRAQ : AMINO ACID SUBSTITUTION IN *PMRCAB* GENES AND THERE CORELLATION 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%),trimethoprimsulfamethoxazole(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.

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

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المستخلص

تم جمع 120 عينة من مختلف المصادر السريريه من مرضى دخلوا مستشفيات مختلفه في مدينه بغداد,تم الكشف عن 65 عينه فقط من نوع الراكده البومانيه. أظهرت نتائج اختبار الحساسية للمضادات الحيوية ان البكتريا كانت مقاومة لمضادات البيبراسيلين (92.31٪)، سيفوتاكسيم (87.69٪)، تريميثوبريم–سلفاميثوكسازول(73.85٪) . بينما الميروبينيم (70.60٪)، والجنتامايسين (69.67%). كانت نسبه المقاومه للايميبينيم والتتراسيكلين (46.58٪)،(43.08 ٪) على التوالي . وفي النهاية ، فقط (20٪) من بين جميع العزلات مقاومه لليفوفلوكساسين. بينما اظهرت نسبة مقاومة الكولستين بين ثلاثة وعشرين عزله ان جميع العزلات سجلت مقاومة بنسة 100% مع (16≥) مايكروغرام / 100 مايكروليتر قيم MIC. اظهرت نتائج الكشف عن جينات (*pmrCAB*) وتسلسلها بين سبعة عزلات بكتيريه والتي تم اختيارها بناءاًعلى مقاومتها لجميع المضادات الحيوية حتى الكولستين ,حيث ان جين (*pmrC*) يحتوي على اربعة عشر طفرة خطا . بينما يحتوي جين (*pmrA*) على طفرة خطأ واحده من بين أثني عشر طفرة .في حين ان نتائج جين (*pmrB*) اظهرت وجود ثلاثة طفرات خطأ.

الكلمات المفتاحية: الكولستين، مقاومه مضادات المايكروبات، الطفرة

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INTRODUCTION

A.baumannii is gram-negative, stricly 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 lipooligosaccharide (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 *lpxACD* 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 invesitigated 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 srRNA 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),Levofloxacine(5mg),Meropenem(10mg),piperacillin(100mg),Tetracycline(30mg),Trimethoprim-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).

PCR analysis

Five PCR fragments were selected for amplification,which respectively covered three different genetic loci in *A.baumannii* sequences, namely *pmrA*(one fragment), *pmrB* (two fragments), and *pmrC*(two fragments) (Table 1).

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

Set	Locus	Specific primer sequences (5′ to 3′)	Product size (bp)	Reference
1	*16srRNA*	5-CAGCTCGTGTCGTGAGATGT-3 5-CGTAAGGGCCATGATGACTT-3	150	17
2	*pmr*A	F: ATGACAAAAATCTTGATGATTGAAGAT R: TTATGATTGCCCCAAACGGTAG	675	This study
3	*pmr*B1	F: AAACGACTGATTTGGGGCACCTC R: GAGCAGCATCGGCAATAAATTGC	664	This study
4	*pmr*B2	F: ACTATTGACGAAATGAACCG R: ATTTCACTAATACAGAAAGACCG	697	This study
5	*pmr*C1	F: ATGTTTAATCTCATTATAGCCA R: ACCGCATGAGCTCACTT	792	This study
6	*pmr*C2	F: ACGAATCCGGAGCTTTCT R: TTAGTTTACATGGGCACAA	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 1× 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.

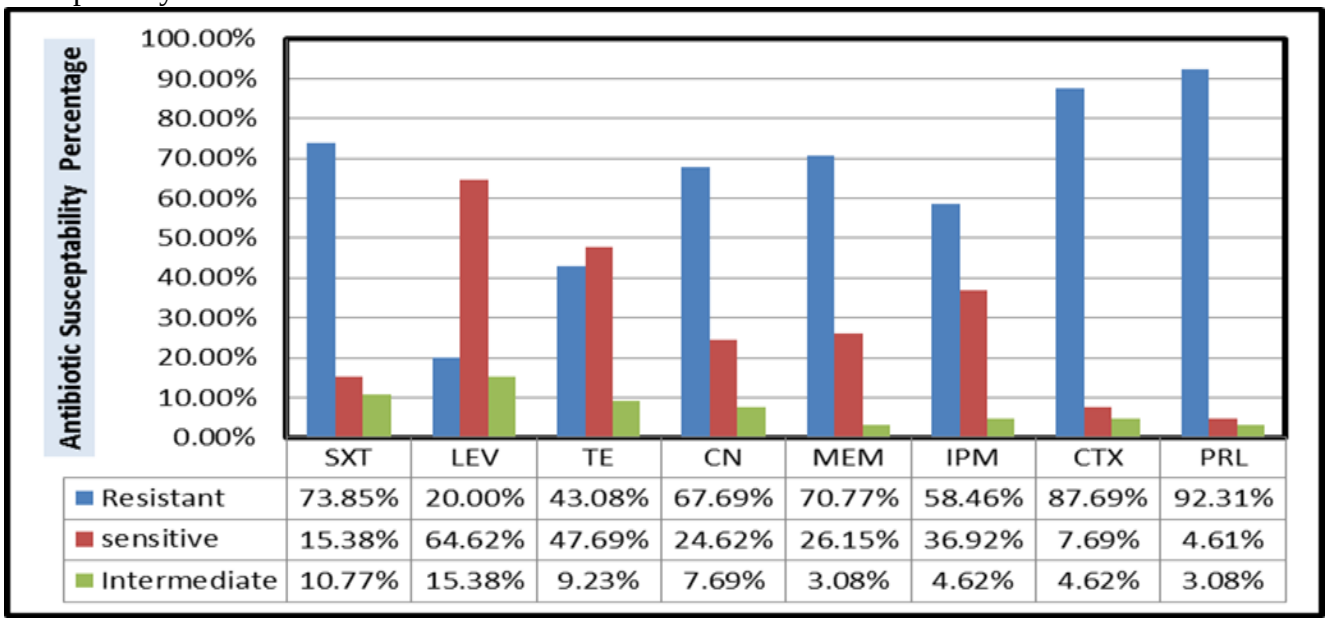
RESULTS AND DISCUSSION

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 percentag (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).



	SXT	LEV	TE	CN	MEM	IPM	CTX	PRL
Resistant	73.85%	20.00%	43.08%	67.69%	70.77%	58.46%	87.69%	92.31%
sensitive	15.38%	64.62%	47.69%	24.62%	26.15%	36.92%	7.69%	4.61%
Intermediate	10.77%	15.38%	9.23%	7.69%	3.08%	4.62%	4.62%	3.08%

Figure 1. Antibiotic susceptibility test percentage of *A.baumanniii* 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 hetero-resistance of these study varied from (7% to 100%) this may be due to multiple samples and varying standards to determine the hetero-resistance (21).

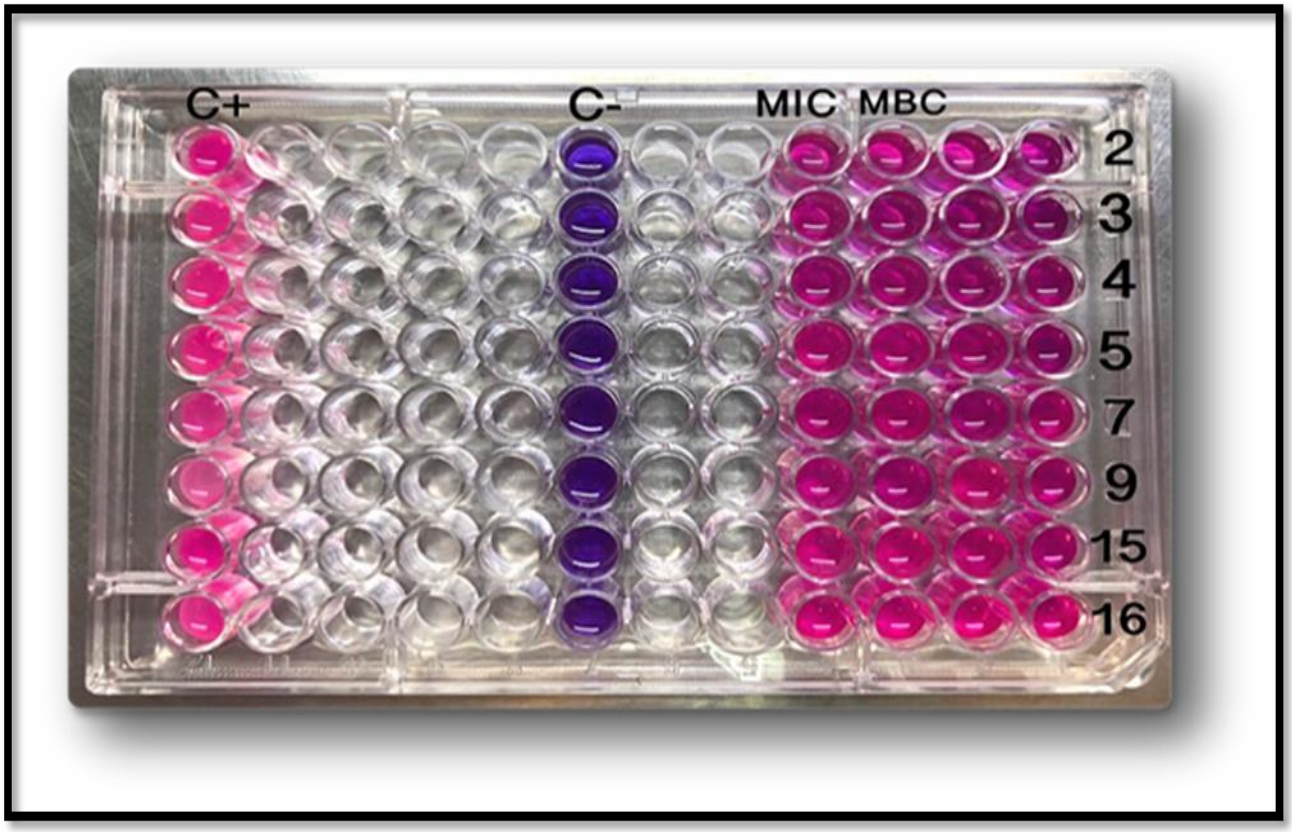


Figure 2. Minimum inhibitory concentration determination for *A.baumannii* isolates by using resazurin sodium stain, the change in color after incubation for 2-4 hours.Column (C-) (blue / purple) corresponds to the negative control, column (C+) (pink) to the positive control

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.

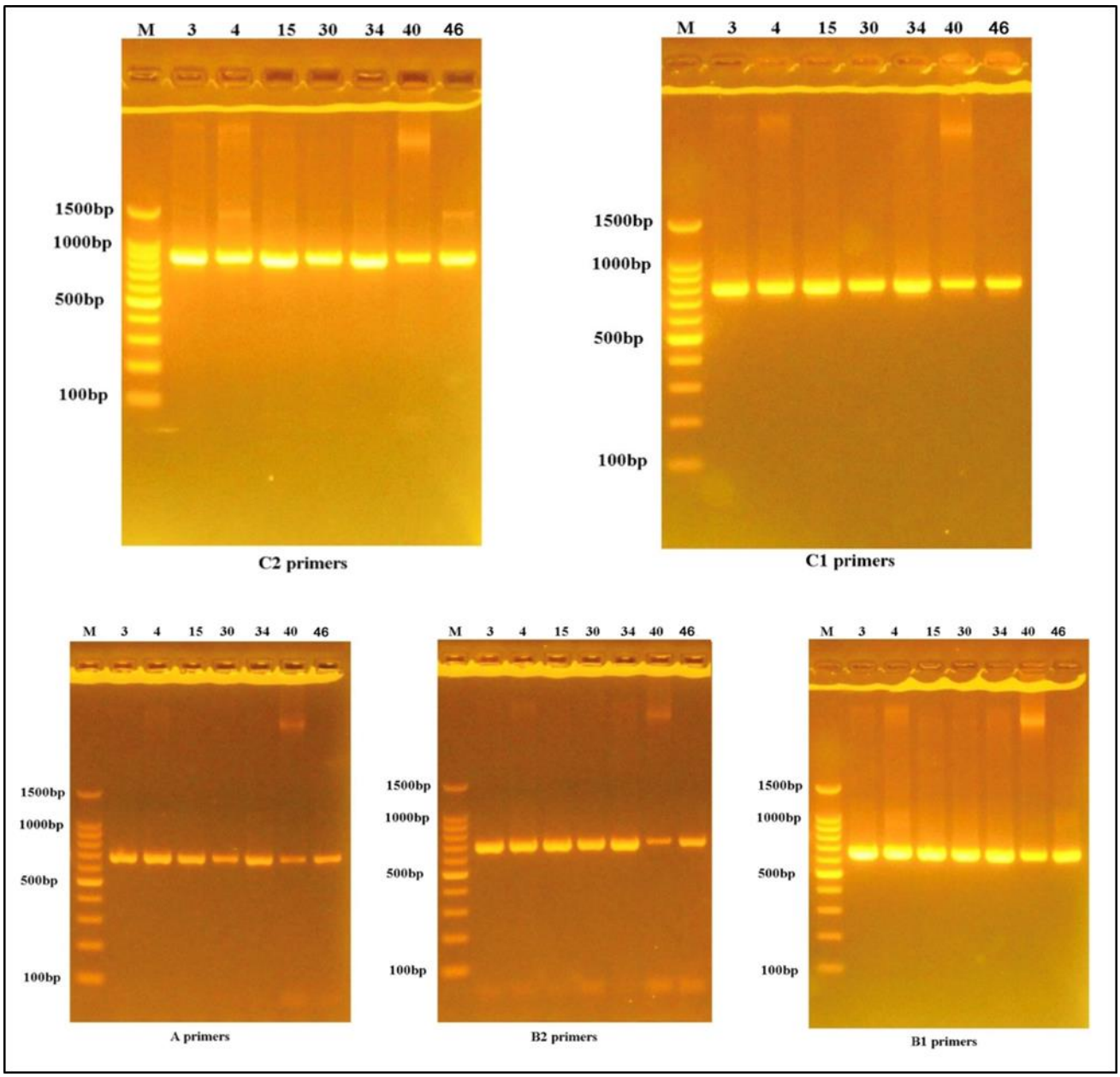


Figure 3. Gel electrophoresis image showing PCR amplification of the gene (*pmrA, pmr B, pmr C*)(2%Agarose. current 200 for 70 min at 90 voltage), Lane M: DNA marker (100-1500bp), Lane:(3,4,15,30,34,40,46) represents the positive results of A. baumannii isolates

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 *pmrB1* 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,MW315 723,MW315724,MW315725,MW315726,MW 315727,MW315728,MW315729,MW315730, MW315731,MW315732). Lastly, the *pmrC1* 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,MW315 737,MW315738,MW315739,MW315740,MW 315741,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: higherFe3+,elevatedAl3+,and lowpH) 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 conformative 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 phosphoethanol amine-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*.

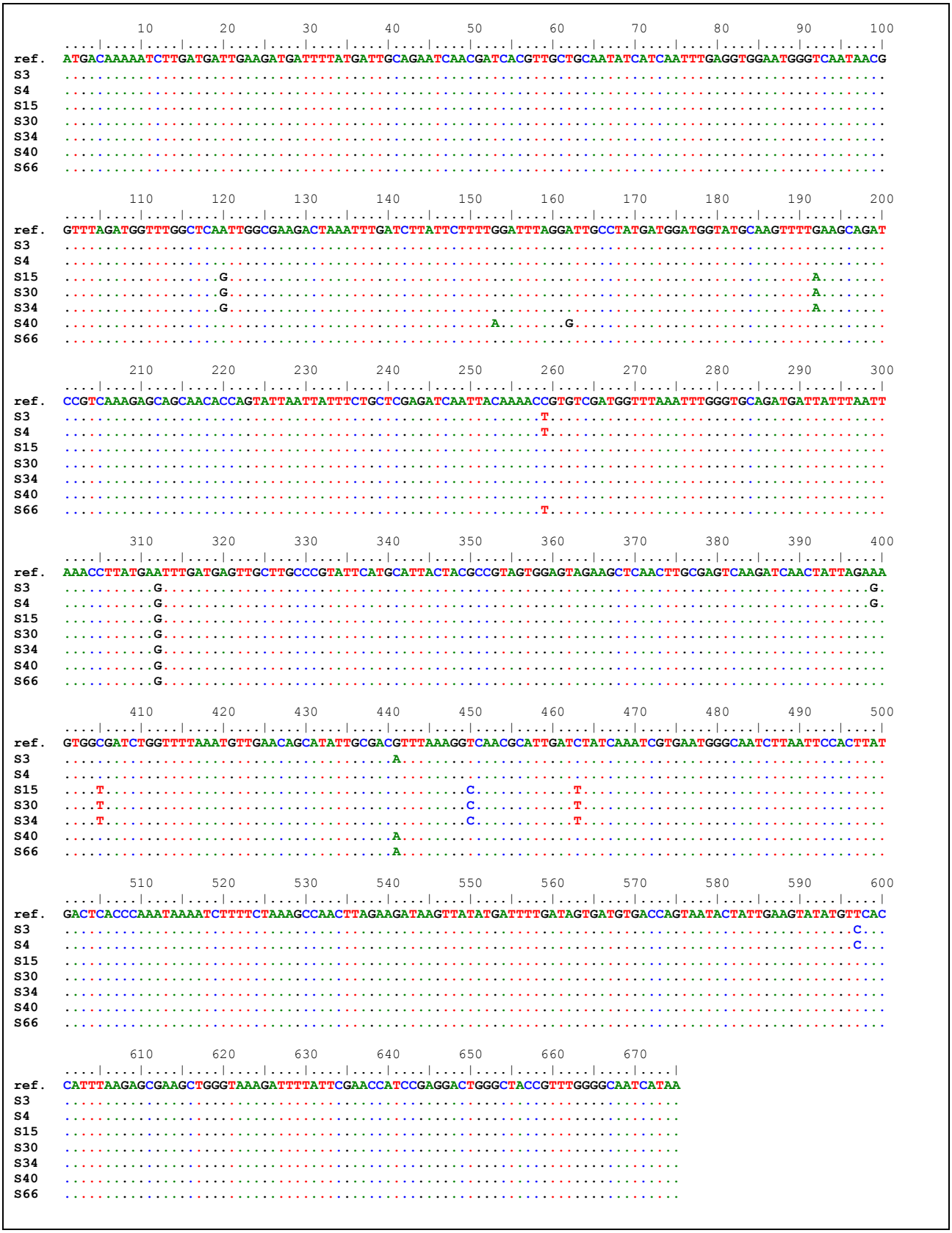


Figure 4. DNA sequences alignment of seven bacterial samples (forward sequence) compared with its reference sequences of the *pmrA* gene within the *Acinetobacter baumannii* sequences. The symbol "ref" refers to the NCBI reference sequences, while "S" refers to sample code

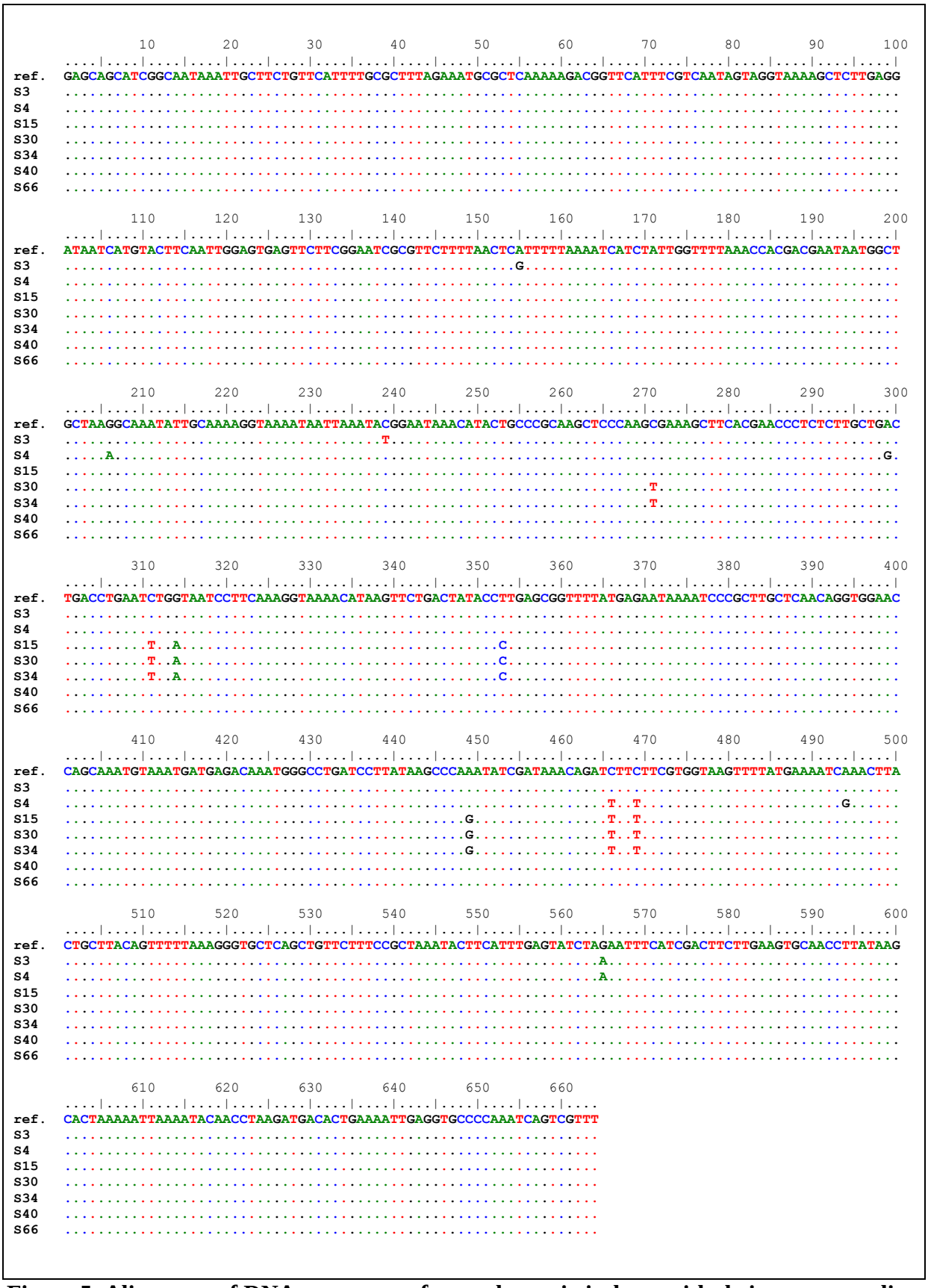


Figure 5. Alignment of DNA sequences of seven bacteria isolates with their corresponding *pmrB1* locus reference sequences inside the genomic sequences of *Acinetobacter baumannii*. The symbol "ref" refers to the NCBI reference sequences, while "S" refers to sample code

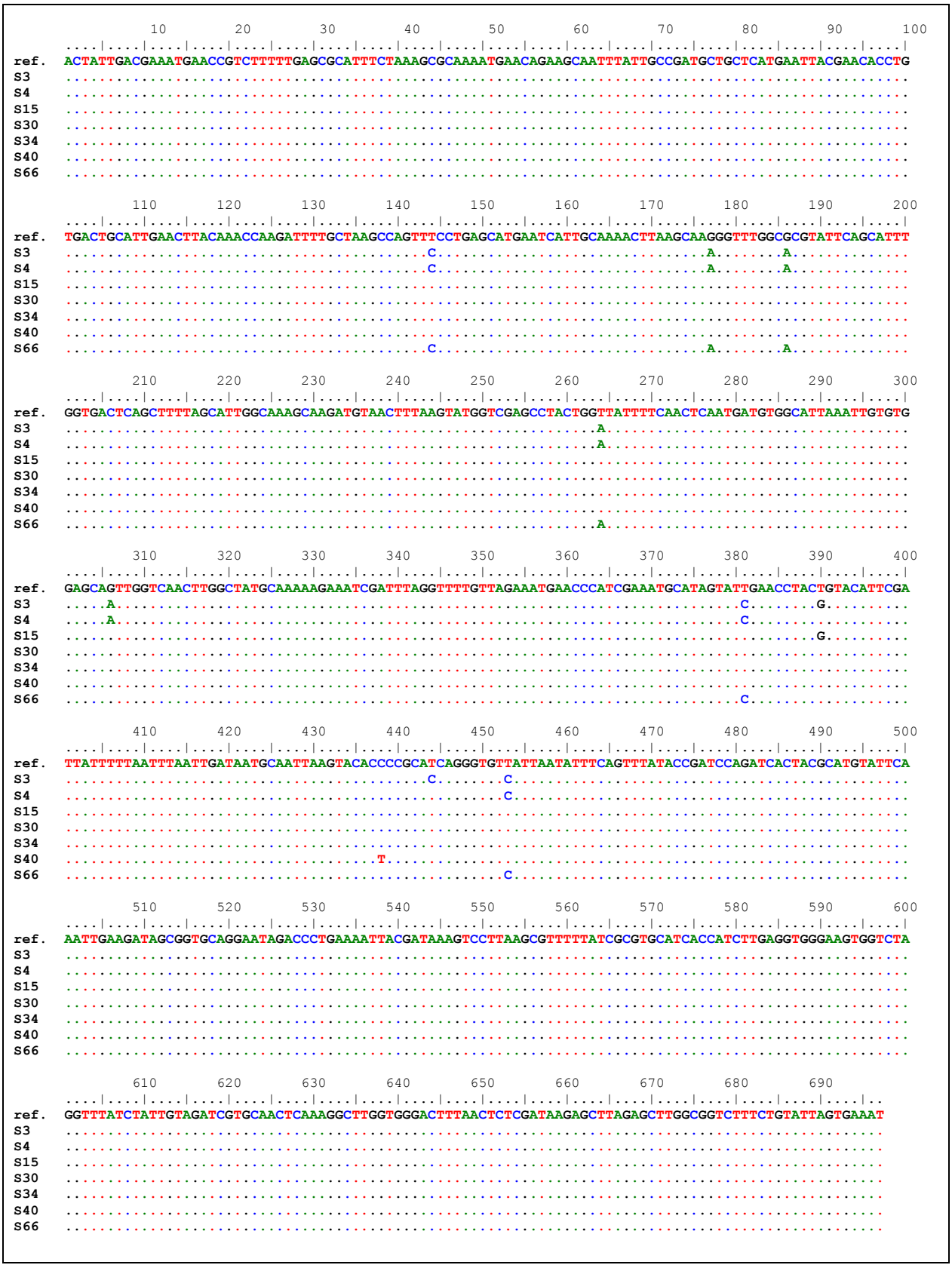


Figure 6. Alignment of the DNA sequences of seven bacterial specimens with their corresponding *PmrB2* locus reference sequences inside the genomic sequences of *Acinetobacter baumannii*. The symbol "ref" refers to the NCBI reference sequences, while "S" refers to sample code

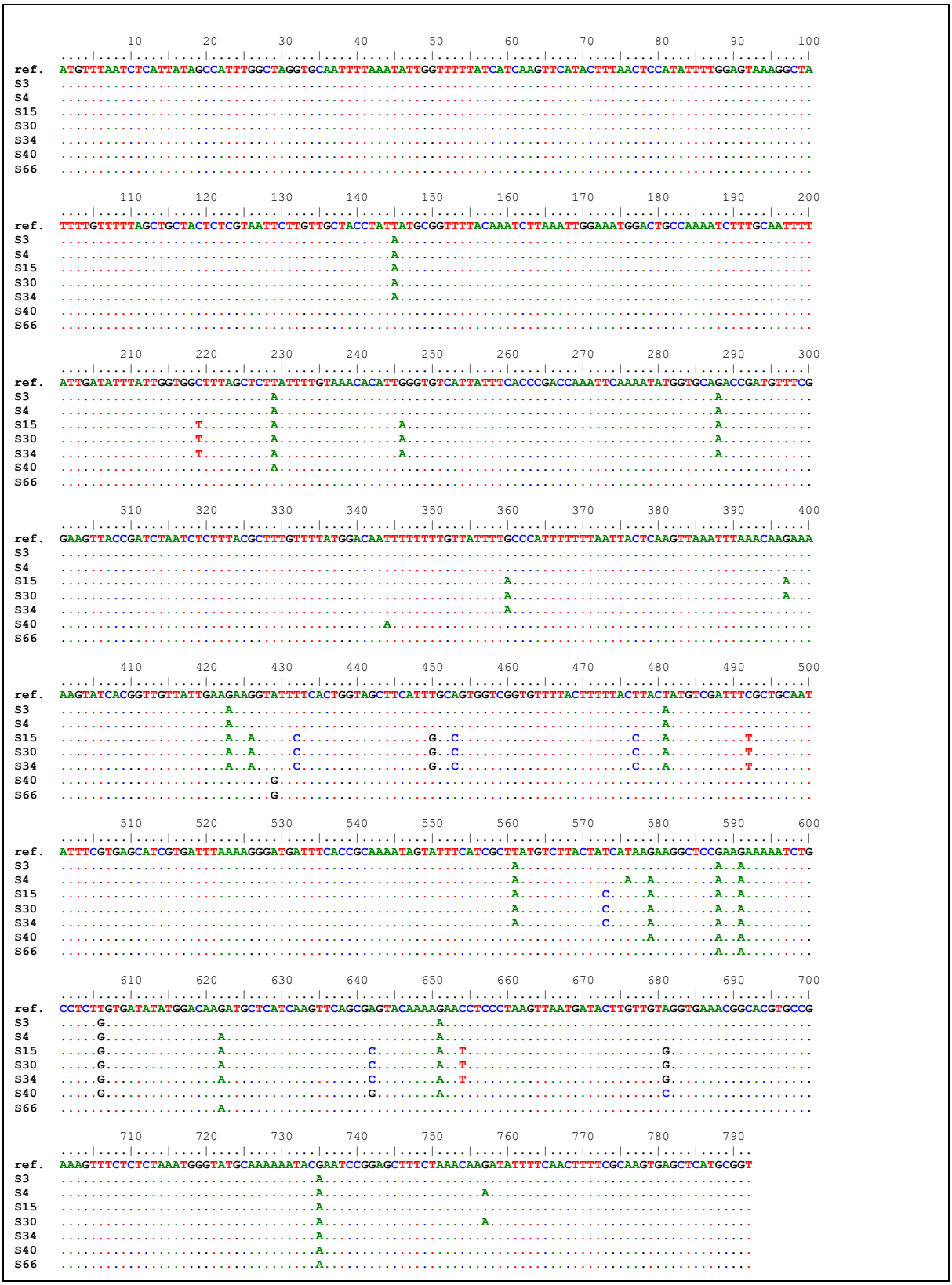
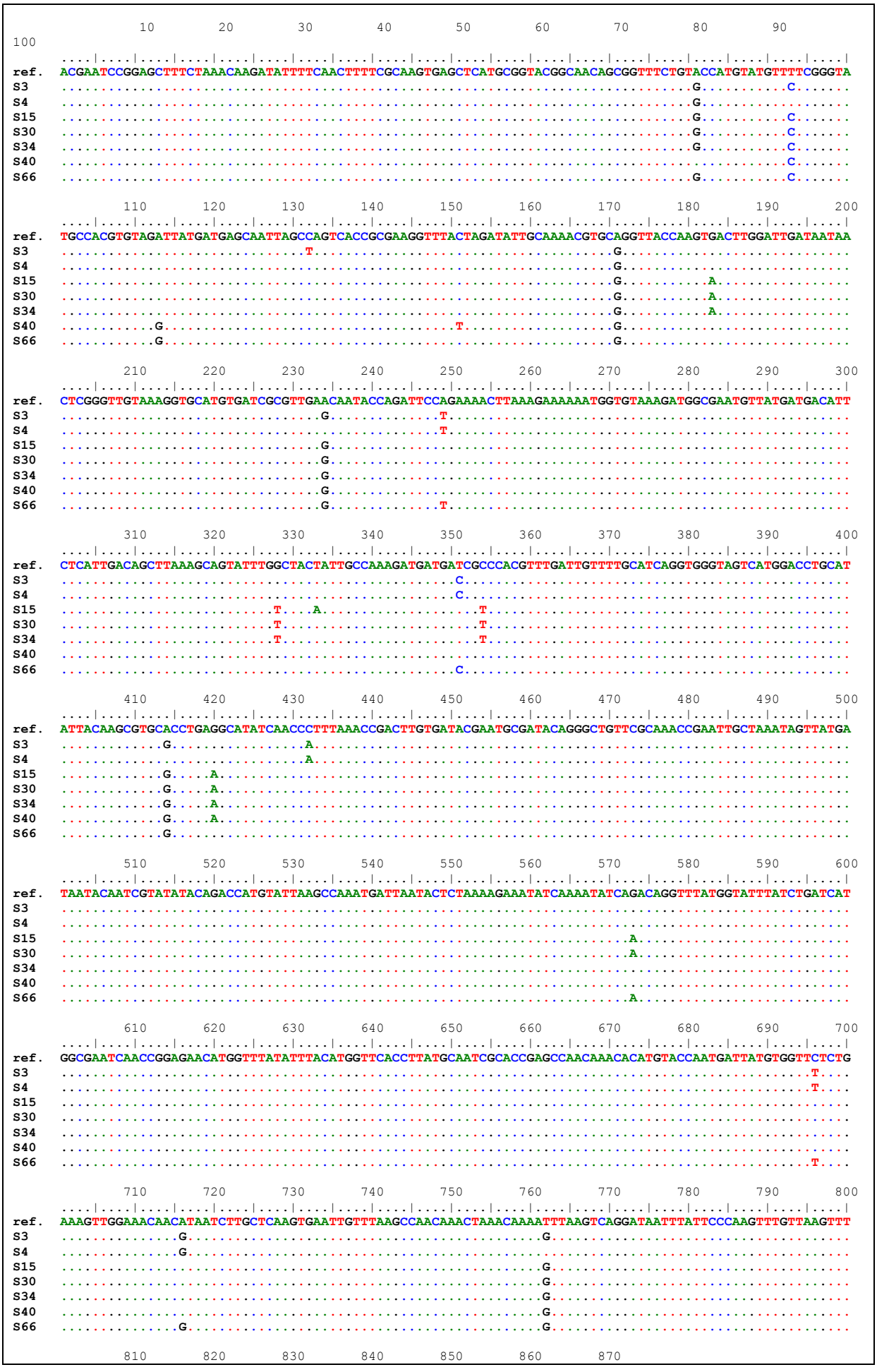


Figure 7. Alignment of seven bacterial samples with their corresponding *pmrC1* locus reference sequences inside the genomic sequences of *Acinetobacter baumannii* with DNA sequences.The symbol "ref" refers to the NCBI reference sequences, while "S" refers to sample code


ref. ACGAATCCGGAGCTTTCTAAACAAGATATTTTCAACTTTTCGCAAGTGAGCTCATGCGGTACGGCAACAGCGGTTTCTGTACCATGTATGTTTTCGGGTA
S3 .....G.....C.....
S4 .....G.....
S15 .....G.....C.....
S30 .....G.....C.....
S34 .....G.....C.....
S40 .....C.....
S66 .....G.....C.....
ref. TGCCACGTGTAGATTATGATGAGCAATTAGCCAGTCACCGCGAAGGTTTACTAGATATTGCAAAACGTGCAGGTTACCAAGTGACTTGGATTGATAATAA
S3 .....T.....G.....
S4 .....G.....
S15 .....G.....A.....
S30 .....G.....A.....
S34 .....G.....A.....
S40 .....G.....T.....G.....
S66 .....G.....G.....
ref. CTCGGGTTGTAAAGGTGCATGTGATCGCGTTGAACAATACCAGATTCCAGAAAACTTAAAGAAAAAATGGTGTAAAGATGGCGAATGTTATGATGACATT
S3 .....G.....T.....
S4 .....T.....
S15 .....G.....
S30 .....G.....
S34 .....G.....
S40 .....G.....
S66 .....G.....T.....
ref. CTCATTGACAGCTTAAAGCAGTATTTGGCTACTATTGCCAAAGATGATGATCGCCCACGTTTGATTGTTTTGCATCAGGTGGGTAGTCATGGACCTGCAT
S3 .....C.....
S4 .....C.....
S15 .....T...A.....T.....
S30 .....T.....T.....
S34 .....T.....T.....
S40 .....
S66 .....C.....
ref. ATTACAAGCGTGCACCTGAGGCATATCAACCCTTTAAACCGACTTGTGATACGAATGCGATACAGGGCTGTTCGCAAACCGAATTGCTAAATAGTTATGA
S3 .....G.....A.....
S4 .....A.....
S15 .....G.....A.....
S30 .....G.....A.....
S34 .....G.....A.....
S40 .....G.....A.....
S66 .....G.....
ref. TAATACAATCGTATATACAGACCATGTATTAAGCCAAATGATTAATACTCTAAAAGAAATATCAAAATATCAGACAGGTTTATGGTATTTATCTGATCAT
S3 .....
S4 .....
S15 .....A.....
S30 .....A.....
S34 .....
S40 .....
S66 .....A.....
ref. GGCGAATCAACCGGAGAACATGGTTTATATTTACATGGTTCACCTTATGCAATCGCACCGAGCCAACAAACACATGTACCAATGATTATGTGGTTCTCTG
S3 .....T....
S4 .....T....
S15 .....
S30 .....
S34 .....
S40 .....
S66 .....T....
ref. AAAGTTGGAAACAACATAATCTTGCTCAAGTGAATTGTTTAAGCCAACAAACTAAACAAAATTTAAGTCAGGATAATTTATTCCCAAGTTTGTTAAGTTT
S3 .....G.....G.....
S4 .....G.....
S15 .....G.....
S30 .....G.....
S34 .....G.....
S40 .....G.....
S66 .....G.....G.....

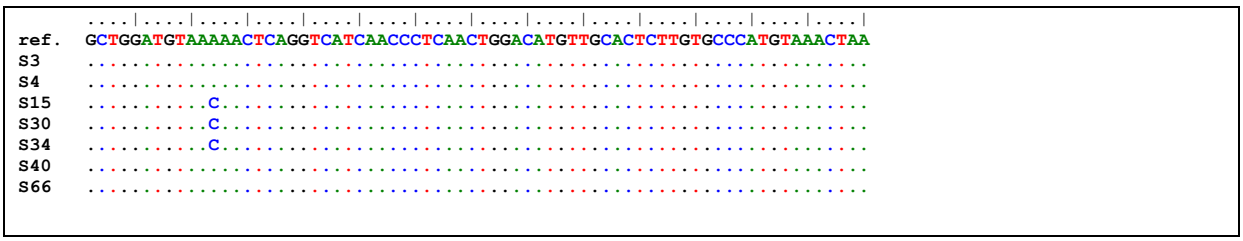


Figure 8. Within the *Acinetobacter baumannii* genomic sequences, DNA sequences align seven bacterial samples with their corresponding *pmrC2* locus reference sequences. The symbol "ref" refers to the NCBI reference sequences, while "S" refers to sample code

Table 2. Amino acid substitutions in the *pmrCAB* genes

Strain	PXEЬMIC (µg/ml)	*pmr*C (549 aa) aa (1-236)	Sulfatase (aa237-532)	*Pmr*A (224 aa) Rec (aa 5-116)	aa (117-131)	*pmr*B (444 aa) aa (1-215)	aa (216-276) HisK	aa (277-330)	aa (331-419) HATPaseC
S3	≥ 16	Y65N,Y93N, D224N	H499R, N514K	R87C					
S4	≥ 16	Y65N,Y93N, H208Q,D224N	D269N,D298G ,H499R,N514 K	R87C		E72K,D73N ,A138T			
S15	≥ 16	Y65N,Y93N, E149K,F166L, Y177N,D224N	A370S,N514K, K531T			E72K,D73N ,A138T			
S30	≥ 16	Y65N,Y93N, E149K,F166L, Y177N,D224N	D269N,A370S, N514K, K531T			E72K, D73N, A138T			
S34	≥ 16	Y65N,Y93N, F166L,Y177N, D224N	A370S,N514K K531T			E72K, D73N, A138T			
S40	≥ 16	Y93N,I131N D224N	N514K						
S46	≥ 16	D224N	D298G,H499R N514K	R87C					

The predicted domains according to the NCBI domain predictor (www.ncbi.nlm.nhi.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 process

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