RESEARCH ARTICLE

Identification of Antibiotic Resistance Genes in Multi-Drug Resistant *Acinetobacter Baumannii* Clinical Isolates of Iraqi Patients (Zq Strains), Using Whole-Genome Sequencing

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Received: 17th October, 19; Revised: 23th November, 19, Accepted: 15th December, 19; Available Online: 25th December, 2019

ABSTRACT

Background: The recent emergence of multidrug resistance (MDR) in *Acinetobacter baumannii* (*A. baumannii*) has raised concern in health care settings in Iraq. This is the first report of the whole genome sequence of *A. baumannii* ZQ isolated from Iraqi patients. To better comprehend the repertoire of MDR genetic elements and organization, we compared the genome sequences of eight extended drug-resistant (XDR) and two less drug-resistant *A. baumannii* ZQ strains with that of other completely sequenced *A. baumannii* from divergent worldwide distributed isolates.

Results: In consistence with their phenotypic antimicrobial resistance profiles, ZQ genomes harbors high to moderate numbers of genetic determinants, including β-lactamases, aminoglycoside-modifying enzymes, efflux pumps, modifications of target sites. Several strains showed nearly identical genome sequence, frequent structural variation was detected even between the closely related strains.

Conclusion: In general, the shorter the genetic distance among strains, the less insertion/deletion events proceed. However, frequent genomic changes was observed even inside the closely related strains of *A. baumannii*. Antimicrobial resistance genes are likely to be the target accumulating such variations, suggesting that the resistance elements respond actively to the selection pressure in the hospital setting. Besides the lateral acquisition of genetic material from resistant bacterial strains, the drastic issues is associated with continuous presence of intrinsic resistance genes in the genome of *A. baumannii,* which are ready to be boosted by exposure to sub-inhibitory levels of the antibiotics in the environment and might also play an important role in the evolution of resistance to the new derivatives of different antibiotic classes.

Keywords: *Acinetobacter baumannii*, Whole genomes sequences, phylogenetic tree analysis, Antibiotic-resistant determinants.

International Journal of Pharmaceutical Quality Assurance (2019); DOI: 10.25258/ijpqa.10.4.20

How to cite this article: Abood AL-Asadi, Z. H. (2019). Detection of *Aspergillus fumigatus* by Polymerase Chain Reaction (PCR). International Journal of Pharmaceutical Quality Assurance 10(4): 670-680.

Source of support: Nil

Conflict of interest: None

Consent for publication: Not applicable.

Funding: We are not a charity and not a funding agency; we are unfunded Ph.D. student researcher and seniors co-authors in the same paper

Competing interests: The authors of this manuscript certify that they have no financial or other competing interests concerning this article.

Authors' contributions: ZJQ performed different bioinformatics analyses, designed experimental procedures, collected the isolates that are included in this study, characterized the isolates and wrote the manuscript. HSK and ASA designed the study, performed the clinical study, and aided in the writing of the manuscript. All authors have read and approved the final manuscript.

BACKGROUND

Acinetobacter baumannii is a Gram-negative bacteria blamable for increasing numbers of the nosocomial infections, especially among immunocompromised patients,[1,2] community-acquired infections are also increasing in incidence.[3] Although *Acinetobacter spp.* primarily cause pneumonia; they are also frequent causes of the wound and burn infections, meningitis, urinary tract infections, and sepsis.[4] A well-recognized

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population at particular risk for *Acinetobacter* infections are military service members who have suffered combat-related injuries, who often acquire these infections in field hospitals.[5,6] In 2003-04, *A. baumannii* has become a major cause for concern in conflict regions, and has gained particular notoriety in the desert conflicts in Iraq, earning it the moniker "Iraqi-bacter." In particular, high incidences of multidrug-resistant MDR bacteremia (bloodstream infections) had been noted among U.S. Army service members in a military treatment facility.[7,8] While in the 1970s *Acinetobacter baumannii* is thought to have been sensitive to most antibiotics, today the pathogen appears to exhibit extensive resistance to most first-line antibiotics.[9] Gaining and spreading of antimicrobial resistance genes in *A. baumannii* are accomplished by combining resistance genes with an array of transposable elements that mediate the interchange of genetic determinants and reshuffle bacterial genomes, resulting in increased genetic combinations and providing a limitless source of genetic flexibility. Such an array comprises insertion sequences (IS), transposons, integrons, plasmids and eventually resistance islands (RI).[10] Inactivation of β-lactams by β-lactamases is a major antimicrobial drug resistance mechanism in *A. baumannii*. Based on sequence homology, β-lactamases are clustered into four molecular classes, A, B, C, and D.[11] All classes of β-lactamases were identified in *A. baumannii*. Many studies have shown that *A. baumannii* has natural competence to acquired exogenous DNA, and its genome has foreign DNA at high frequencies, inferring frequent horizontal gene transfer in this pathogen.[12] Therefore, the natural competence of *A. baumannii* may contribute to identification of a large number of β-lactamases in this threatening pathogen. Class D β-lactamases are called OXAs (oxacillinases), because they generally hydrolyze isoxazolylpenicillin oxacillin much faster than benzylpenicillin.[11] The existence of carbapenem-hydrolyzing class D β-lactamases is one of the major carbapenem resistance mechanisms in *A. baumannii*.[13] The subgroups of carbapenem-hydrolyzing OXAs, like the OXA-23, OXA-24, OXA-51, and OXA-58 subgroups, are prevalent in *A. baumannii.* Class D OXA-51-like carbapenemases are chromosomally encoded and appear to be intrinsic to *Acinetobacter baumannii*.[14] These enzymes are weak carbapenemases, and it has been proposed that they only confer carbapenem resistance if an additional promoter is given by the insertion of IS*Aba1* upstream of the structural gene.[15] The bla_{OXA-23} gene has been distributed worldwide, and the occurrence of OXA-23-producing *A. baumannii* strains is significantly high.[16] Insertion of IS*Aba1* in the bla_{OXA-23} promoter sequence has been described to be associated with overexpression of bla_{OXA-23}, bla_{OXA-51}, or bla_{OXA-58} in *A. baumannii*.[17]

MATERIALS AND METHODS

Bacterial strains and DNA sequencing

The *A. baumannii* ZQ strains were isolated from different clinical samples of Iraqi patients who were affiliated at Al-Imamain Al-khadhimain Hospital, Baghdad, Iraq as detailed in (Table 1). The species were identified by conventional methods and by using VITEK 32 GN system (bioMérieux). According to the Clinical and Laboratory Standards Institute (CLSI) guidelines,[18] antimicrobial susceptibility testing was performed by using VITEK II N211 system (bioMérieux), the minimum inhibitory concentrations (MIC)s of tigecycline, colistin, amikacin and meropenem were determined manually by the standard broth microdilution method using a two-fold dilution series following the recommendations given in document M100-S20 of the CLSI,[19] Disc Diffusion Susceptibility Testing were done for Chloramphenicol 30µg, Azithromycin 15 µg, and rifampin 30 µg (merseyside/u.k).

DNA preparation, library construction, and sequencing: The genomic DNA of *A. baumanii* ZQs strains were extracted using a bacterial genomic DNA purification kit (Promega, Wizard® Genomic DNA Purification Kit) according to the manufacturer's instructions. The quality of DNA was determined by gel electrophoresis and NanoDrop 2000 spectrophotometer (Nano-drop Technologies, Wilmington, DE).

Genome Sequencing

The DNA (whole genome) sequencing library type was paired-end libraries with 400-bps fragment size, and were constructed by using llumina - using Kapa DNA Library Covaris sheared, Kapa hyper preparation kit. Final Quality Control by Kapa qPCR and Agilent Tapestation included. Each library was deposited onto a MiSeq Flow Cell and sequenced using Next-Generation Sequencing Illumina - MiSeq v2 - PE 150 Cycle, Library preparation, and sequencing were performed by Oklahoma Medical Research Foundation NGS Core (Oklahoma City, OK. United States).

Table 1: *A. baumannii* ZQ strains distribution according to sample type

Isolates	*Specimen type/ infection*	*Date of isolation*
ZQ1	Wound swab/Wound infection	June 2016
ZQ2	Sputum/ pneumonia	September 2016
ZQ3	Blood/sepsis	June 2016
ZQ4	Blood/ bacteremia	July 2016
ZQ5	Wound swab/Wound infection	August 2016
ZQ6	Wound swab/Wound infection	August 2016
ZQ7	Sputum/ pneumonia	June 2016
ZQ8	Blood/ bacteremia	October 2016
ZQ9	Blood/sepsis	November 2016
ZQ10	CSF / meningitis	November 2016

Short reads processing and Genome assembly

Quality control. For the raw sequencing data, the reads were cleaned by removing the empty reads, adapter sequences and trim both ends reads with Error Probability Limit (0.05) using Geneious trimmer (Geneious 11.0.5 software, http://www.geneious.com, Kearse et al., 2012).[20] The BBDuk[21] plugin was used to trim further and filter the dataset. Reads shorter than 20 bp or with a minimum average quality score of less than 20 were removed, and paired read overlaps (where a read extends past the start of its mate) were trimmed to ensure complete adapter removal. (The length of overlapping sequences between the adaptor and read was at least >24 bp).[22]

Genome assembly

The clean reads were *de novo* assembled using Geneious *de novo* assembler with Basic *de novo* assembly options (11.0.5) with checking the circularize contigs with matching ends to construct A circular contig of plasmids.[20] Then the scaffolds and large contigs of each draft genome were moved and ordered using Mauve genome MCM algorithms 23 by using *A. baumannii* AYE (GenBank accession no. CU459141) as a reference genome.

Genome annotation and Bioinformatics analysis

Annotation was added by the NCBI Prokaryotic Genome Annotation Pipeline PGAP (released 2013). which uses best-placed reference protein set; GeneMarkS+, for identification of protein-coding genes (CDS); rRNA; tRNA; ncRNA; repeat_region; frameshifts, Mobile/fast-evolving genes like (insertion sequence and phage gene). Sequence alignment was also performed of the amino acid sequences of genes to the NCBI non-redundant (NR) database (E-value ≤ 10-10, identity score ≥35%, and coverage length ≥80%). If the amino acid sequence of a gene was aligned to multiple sequences in the databases, the optimal result was retained. Antimicrobial resistance genes were checked by using ResFinder-2.1 databases (https://cge.cbs.dtu.dk/services/ResFinder-2.1/).[24] Insertion sequence (IS) elements and transposons were identified using the ISfinder database (http://www-is.biotoul.fr/). The G+C contents in the genomic sequence were obtained by using Geneious sequence (11.0.5).

Comparative Genomics Analysis Data used in comparative analysis were downloaded from the NCBI database (ftp://ftp.ncbi.nlm.nih.gov/GenBank/genomes/ Bacteria/), including 25 whole genome sequences WGS and annotation of *A. baumannii* isolates including: MDR-ZJ06 (CP001937), MDR-TJ (CP003500), AB1656-2 (CP001921), AB0057 (CP001182), AB307-0294 (CP001172), ATCC 17978 (CP000521), ACICU (CP000863), AYE (CU459141), BAL062 (LT594095), BJAB07104 (CP003846), BJAB0715(CP003849), BJAB0868(CP003847), D1279779(CP003967),D36 (CP012952), DU202 (CP017152) LAC4 (CP007712), SDF (CU468230),TYTH-1 (CP003856), USA-2 (CP020592), XH386 (CP010779), ZW85-1 (CP006768) and ADP1 (CR543861). Draft genomes of Naval-17 (AFDO01000021), Naval-2 (AMSX00000000) and Naval-57 (AMFP00000000).

Multilocus sequence typing (MLST) was first performed for investigating the population structure of *A. baumannii* clinical isolates.[25] Sequence allele typing was performed with the multiple locus query tool at the publicly available *A. baumannii* MLST database at the Pasteur Institute's MLST website (www.pasteur.fr/recherche/genopole/PF8/mlst/Abaumannii.html) to analyze sequences of the seven housekeeping genes (*cpn60, fusA, gltA, pyrG, recA, rpIB, and rpoB*). Phylogenetic tree was built by concatenating the sequences at the seven housekeeping loci among the ten ZQs strains with the above mentioned *A. baumannii* strains, using the soil nonpathogenic *Acinetobacter baylyi* ADP1 as outgroup to root the tree, maintaining the correct reading frame, and construct a neighbor-joining tree based on these sequences using Geneious tree builder with default setting.

Nucleotide sequence accession number

The complete genome sequences of *Acinetobacter baumannii* ZQ strains have been deposited at DDBJ/ENA/GenBank under accession No.: ZQ1(PHHB00000000), ZQ2(PHKA00000000.2), ZQ3(PHJZ00000000), ZQ4(PHJY00000000), ZQ5(PHJX00000000), ZQ6(PHJW00000000), ZQ7(PHJV00000000), ZQ8(PHJU00000000), ZQ9(PHJT00000000) and ZQ10(PHJS00000000). The versions described in this study are version 2 for ZQ1(PHHB00000000.2), ZQ2(PHKA00000000.2), ZQ3(PHJZ00000000.2), ZQ4(PHJY00000000.2), ZQ5(PHJX00000000.2), ZQ6(PHJW00000000.2), ZQ7(PHJV00000000.2), ZQ8(PHJU00000000.2), ZQ10(PHJS00000000.2) and version 3 for ZQ9(PHJT00000000.3), The plasmids accessions appear in the WGS_SCFLD line at the bottom of the WGS master record.

RESULTS AND DISCUSSION

The antimicrobial susceptibility results of 18 antimicrobial drugs indicated that eight of ZQ strains including ZQ (1, 2, 3, 5, 6, 7, 9, and 10) were resistant to 16 out of the 18 tested antibiotics including piperacillin, cephalosporin analogs, aminoglycosides, fluoroquinolones, trimethoprim, sulfonamides, rifampin, and chloramphenicol but sensitive to tigecycline and colistin, so they were designated to be extensive drug-resistant (XDR) strains.[26] Compared to the less antimicrobial resistance profile of ZQ (4 and 8) strains which were susceptible to almost all tested antibiotics, but resistant to cephalosporin analogs and rifampin, while ZQ4 was sensitive to piperacillin, ZQ8 showed intermediate resistance for it (Table 2).

Genomic characteristics

The number obtained of pass filter reads per sample was about 3.2 million paired-end reads (2 x 150 pb), produced (1.6 M reads in each direction). The raw sequencing data were uploaded to the public database, the National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov/) under the BioProject PRJNA419147. General features of the *A. baumannii* ZQ strains are listed in Table 3 Including whole genome size,

Table 2: Susceptibility profiles of ZQ strains

	Isolates											
AB	*ZQ1*	*ZQ2*	*ZQ3*	*ZQ4*	*ZQ5*	*ZQ6*	*ZQ7*	*ZQ8*	*ZQ9*	*ZQ10*		
AK	R									= 128 S	R = 128	R = 128
										R = 128 S	R = 128	R = 128
										R = 128	R = 128	
ATH	R	R	R	S	R	R	R	S	R	R		
C	R	R	R	S	R	R	R	S	R	R		
CAZ	R ≥ 64	R > 32	R ≥ 64	S	R > 32	R > 32	R > 32	R = 32	R > 64	R > 32		
CIP	R ≥ 4	R > 2	R ≥ 4	S	R > 2	R > 2	R ≥ 4	S	R > 2	R > 2		
CO	S	S	S	S	S	S	S	S	S	S		
CTR	R ≥ 64	R > 32	R ≥ 64	R=16	R > 32	R > 32	R > 32	R = 32	R > 32	R > 32		
CTX	R > 128	R > 128	R > 128	R = 32	R > 128	R > 128	R > 128	R = 32	R > 128	R > 128		
FEP	R = 32	R > 32	R > 32	I = 4	R > 32	R > 32	R > 32	4 = I	R > 32	R > 32		
GEN	R≥ 16	R > 8	R ≥ 16	S	R > 8	R > 8	R > 8	S	I = 8	I = 8		
IMP	R > 8	R > 8	R > 8	S	R > 8	R > 8	R > 8	S	R > 8	R > 8		
LEV	R≥ 8	R > 4	R ≥ 8	S	I = 4	I = 4	R ≥ 8	S	R > 4	R > 4		
MEM	R≥ 8	R > 16	R > 16	S	R > 16	R > 16	R = 16	S	R > 16	R > 16		
PRL	R ≥ 128	R > 64	R ≥ 128	S	R > 64	R > 64	R ≥ 128	I = 4	R > 64	R > 32		
RIP	R	R	R	R	R	R	R	R	R	R		
TCG	S	S	S	S	S	S	S	S	S	S		
TMX	R ≥ 320	R > 160	R > 160	S	R>160	R>160	R>160	S	R > 160	R > 160		
TOB	R ≥ 16	R > 8	R > 8	S	R> 8	R>8	R> 8	S	R > 8	R > 8		

AB, antibiotics; **Ak,** Amikacin**; ATH,** Azithromycin**; C,** Chloramphenicol; **CTX**, Cefotaxime; **CRT**, Ceftriaxone; **CAZ**, Ceftazidime; **CIP**, Ciprofloxacin; **LEV**, Levofloxacin; **TGC**, Tigecycline ; **PRL**, Piperacillin ; **CO**, Colistin; **GEN**, Gentamicin; **FEP**, Cefepime ; **IMP**, Imipenem; **MEM**, Meropenem; **RIP**, rifampin; **TOB**, Tobramycin; **TMX,** Trimethoprim/sulfamethoxazole. **R***: resistance; **S***: sensitive; **I***: intermediate; the numbers associated with them are the MIC values (µg/ml).

Table 3: General Features of ZQ genomes

Features	*Isolates*									
	ZQ1	*ZQ2*	*ZQ3*	*ZQ4*	*ZQ5*	*ZQ6*	*ZQ7*	*ZQ8*	*ZQ9*	*ZQ10*
Assembly level	scaffolds	scaffolds	scaffolds	scaffolds	scaffolds	scaffolds	scaffolds	scaffolds	scaffolds	scaffolds
whole genome size	3,994,097	4,283,539	4,107,093	3,927,904	4,002,057	4,105,592	3,987,480	4,123,841	4,123,732	4,066,290
Chromosome size	3,951,859	4,148,030	4,005,327	3,877,698	3,975,389	4,044,253	3,956,147	4,094,093	4,003,752	4,022,741
No. of plasmids	2	4	5	4	4	6	2	3	4	3
G+C content	38.90%	39%	38.90%	39%	39.20%	39.20%	38.90%	38.90%	38.80%	38.90%
No. of genes	3,894	4,345	4,095	4,122	4,028	4,130	3,849	4,098	4,210	4,139
No. of protein-encoding genes	3,755	4,261	4,007	4,033	3,946	4,050	3,933	4,019	4,210	4,054
No. of tRNAs	56	66	67	67	65	63	63	63	67	66
No. of rRNAs	1, 1, 1 (5S, 16S, 23S)	6, 4, 4 (5S, 16S, 23S)	6, 5, 6 (5S, 16S, 23S)	1, 1, 1 (5S, 16S, 23S)	6, 5, 3 (5S, 16S, 23S)	6, 4, 2 (5S, 16S, 23S)	1, 1, 1 (5S, 16S, 23S)	8, 2, 2 (5S, 16S, 23S)	6, 5, 4 (5S, 16S, 23S)	6, 4, 4 (5S, 16S, 23S)

These features were obtained based on annotations using the NCBI Prokaryotic Genome Annotation Pipeline PGAP (released 2013). Best-placed reference protein set; GeneMarkS+,

chromosome size, number of plasmids associated with each isolate, number of RNAs, % GC content, and number of transposase. Genome features of ZQ strains were comparable to previously published whole-genome A. *baumanii*.[2,9,27-31]

Sequencing analysis also identified 35 complete plasmids and 2 partial sequence plasmids from these ten ZQ strains, 12 of them were directly associated with antibiotic resistance genes, interestingly it is found that the phylogenetically closely related strains harbor plasmids that are almost identical in their size and protein coding genes including antibiotic resistance genes. The general features of plasmid sequences associated with antibiotic resistance in ZQ strains are demonstrated in Table 4.

Multilocus sequence typing and phylogenetic analysis

ZQ5 and ZQ6 isolates were found to have the same allelic profile (cpn60-1, fusA-1, gltA-1, pyrG-1, recA-5, rpIB- 1 and rpoB-1) under the Institute Pasteur Multi Locus Sequence Typing (MLST) scheme, which belong to sequence type 1 (ST-1), while ZQ3 showed single allelic mismatch (cpn60-1, fusA-1, gltA-1, pyrG-1, recA-5, rpIB- 1 and rpoB-30) and were designated to be ST-717. ST1 and ST717 were recommended to be designated by CC1 (where CC stands for clonal complex) for uniform nomenclature. We also found that the isolates of ZQ1, ZQ2 and ZQ7 showed the same allelic profile (cpn60-2, fusA-2, gltA-2, pyrG-2, recA-2, rpIB- 2 and rpoB-2), and were recommended to be designated by ST2 or CC2. For ZQ9 and ZQ10 strains, they exhibited similar allelic profile (cpn60-1, fusA-3, gltA-2, pyrG-1, recA-4, rpIB-4 and rpoB-92), and were recommended to be designated by ST575 or CC8. ZQ4 and ZQ8 strains showed different allelic profiles (cpn60-3, fusA-4, gltA-2, pyrG-2, recA-7, rpIB-1 and rpoB-2) (cpn60-56, fusA-3, gltA-55 pyrG-2, recA-9, rpIB-4 and rpoB-14), and were recommended to be designated by ST-203 and ST-513 respectively.

Phylogenetic tree analysis

The phylogeny of *A. baumannii* of ZQs, with respect to other sequenced *A. baumannii* isolates from geographically worldwide divergent origins, with varied degrees of antimicrobial resistance profile, was inferred using a Pasteur MLST approach.[32] These included 18 highly -resistant completely sequenced *A. baumannii* strains (str.AYE, AB0057, ACICU, 1656-2, TYTH-1, XH386, USA-2, BAL062, BJAB07104, BJAB0715, BJAB0868, D36, ZW85-1, MDR-TJ, MDR-ZJ06, Naval-2, and Naval 57), moderately resistance strains (LAC-4, DU202, Naval-17), two susceptible clinical strains (ATCC 17978 and AB307-0294), one community-acquired strain (D1279779), and 1 nonclinical strain (SDF) isolated from a human body louse . ADP1, a soil living *Acinetobacter baylyi* strain, was used as outgroup for comparison. On the basis of the phylogenetic data, three of the ten ZQ strains (ZQ1, ZQ2 and ZQ7) along with nine previously reported Asian strains (including MDR-ZJ06, MDR-TJ, XH386, BJAB07104 and BJAB0868 from China; BAL062 and TYTH-1 from Taiwan and 1656-2, USA-2, DU202 from South Korea) and two strains (Naval-2, Naval 17) which were isolated form wounded American soldiers returning from Iraq about 1 decade ago and treated at the National Naval Medical Center(NNMC) were grouped together with ACICU (from Italy), a strain previously assigned of the global clone II (GC II) group. Also, This MLST based analysis suggested that the closest phylogenetic relatives of ZQ4 strain were D1279779 strain from Australia, which were previously assigned to international clonal (IIC) lineage. ZQ5, ZQ6, and ZQ3 were grouped together with the strains (Str. AYE from France, AB0057 (USA) strain, AB307-0294 from the USA, and D36 from Australia) which belong to global clone I (GC I) (27). Interestingly, ZQ8 (ST14), Naval-57 from USA (ST155) and ZW85-1 from china (ST 639) were grouped with the earliest isolated strain, ATCC17978 (ST437) which belong to international clonal group III,[33] the closest neighbors for ZQ9 and ZQ10 were (LAC-4 from USA and BJAB0715 from china) that have unusual sequence type of ST-10 and ST-23 respectively, indicative of possible divergent evolution of these strains from their original clones.[2,34] Interestingly, the full susceptible nonpathogenic strain SDF is separated with all other strains, which may suggest SDF has a different origin comparing with other drug-resistant strains. The Pasteur sequence typing of all isolate are listed in (TableS1).

Antibiotic resistance of ZQ strains and resistance genetic determinants

The present article focuses on the genes expected to be involved in antimicrobial resistance; these genes, together with the type of antibacterial resistance they show, are listed in Table 5 and

Table 4: General Features of ZQ strains' plasmids associated with antibiotic resistance

plasmid	*GenBank Accession No.*	*size (pbs)*	*Antibiotics resistant genes*
P1ZQ1	CM008887.2	18253	*Cat8, Sul1, MphE, msrE,aacA4, Arma, AadA1* associated with *Int1*
p2ZQ2	CM009647.1	12769	*MphE, msrE*
p3ZQ2	CM009648.1	6078	*aadB*
p1ZQ3	CM009028.2	6078	*aadB*
p1ZQ5	PHJX02000072.1	20429	*IntI, Arr2, CmlA, blaPER-7,qacE,sul1, MphE, msrE, Arma,*
P6ZQ6	PHJW02000090.1	20429	*IntI, Arr2, CmlA, blaPER-7,qacE,sul1, MphE, msrE, Arma,*
p2ZQ7	PHJV02000053.1	20142	*Cat8, Sul1, MphE, msrE,aacA4, Arma, AadA1* associated with *Int1* and *Apha1*
p2ZQ8	CM009034.2	6078	*aadB*
p1ZQ9	CM009083.3	35194	*Mph(E) , msrE, Tet(39)*
p3ZQ9	CM009085.3	6078	*aadB*
p1ZQ10	CM009030.2	35194	*Mph(E) , msrE, Tet(39)*
p2ZQ10	CM009031.2	6078	aadB

Table 5: Distribution of resistance gene determinants among ZQ strains

Antibiotic Resistance Genes	*Resistance type*	*ZQ1*	*ZQ2*	*ZQ3*	*ZQ4*	*ZQ5*	*ZQ6*	*ZQ7*	*ZQ8*	*ZQ9*	*ZQ10*
Aminoglycoside acetyltransferases AAC3 (1,2)	Aminoglycosides modifying enzymes										
Aminoglycoside acetyltransferases AAC6 (4)	Aminoglycosides modifying enzymes	1						1			
Aminoglycoside adenyltransferases ANT(2″) (aadB)	Aminoglycosides modifying enzymes		1	**1**					1	1	1
Aminoglycoside adenyltransferases ANT(3″)-I (aadA1)	Aminoglycosides modifying enzymes	1	1	1		2	2	1		1	
Aminoglycoside nucleotidyltransferase ANT(3'')-II	Aminoglycosides modifying enzymes	1	1	1	1	1	1	1	1	**1**	1
Aminoglycoside phosphotransferases APH(3′)	Aminoglycosides modifying enzymes (Streptomycin)	1	4	**1**		2	2	2	2	**1**	1
Aminoglycoside phosphotransferases APH(6)	Aminoglycosides modifying enzymes (Streptomycin)	1	1			1	1	1	1		
Streptothricin N-acetyltransferase Sat2	Aminoglycosides modifying enzymes (Streptothricin)										
Arma 16S rRNA *methylase*	Alteration of target sites, aminoglycosides	1				1	1	1			
β-lactamase class PER-7	Expanded spectrum cephalosporins inhibited by clavulanic acid and tazobactam.					1	1			1	1
β-lactamase class PER-10	Cephalosporins								1		
β-lactamase class CARB2	Carbenicillinase (Carbencillin resistance genes)									1	1
β-lactamase class TEM-1	Penicillins and first-generation cephalosporins		1								
β-lactamase class C ADC	Extended-spectrum cephalosporins but not cefepime	1	1	1	2	1	1	1	1	1	1
β-lactamase class D 51 like	Narrow-spectrum hydrolysis profile including, at a low level, imipenem and meropenem	1	1	1	1	1	1	1	1	1	1
β-lactamase class D 23 like	Carbapenems	1	1	1		1	1	1		1	1
Two-component sensor histidine kinase (adeS)	AdeABC efflux pump expression control	1	1	1		1	1	1		1	1
Efflux system DNA-binding response regulator (AdeR)	AdeABC efflux pump expression control	1	1	1		1	1	1			
RND efflux AdeABC	MDR efflux pump *	1	1	1		1	1	1		1	1
RND efflux AdeIJK	MDR efflux pump ǂ	1	1	1	1	1	1	1	1	1	1
ABC multidrug resistant transporter	MDR efflux pump									1	1
Mph(E) family macrolide 2'-phosphotransferase	Macrolides	**1**	1			1	1	1		1	1
ABC-F type ribosomal protection protein Msr (E) efflux pump	Macrolide, Lincosamide and Streptogramin B	**1**	1			1	1	1		1	1
GyrA (R: Ser-Leu mutation at position 83	Fluoroquinolones	**1**	1	1		1	1	1		1	1
ParC (R: Ser-Leu mutation at position 84	Fluoroquinolones	**1**	1	1				1		1	1
tet A efflux pump	Tetracycline efflux pump			1	1	1	1		1		
tet (39)-tetR efflux pump	Tetracycline efflux pump									1	1
tet (B)-tetR efflux pump	Tetracycline efflux pump	1	1			1	1	1			
NAD(+)rifampin ADP ribosyltransferase Arr-2	Rifampin					1	1				
Dihydropteroate synthase Sul1	Sulfonamide	1	1			1	1	1		1	1
Dihydropteroate synthase Sul2 CDS (folP1)	Sulfonamide			1		1	1				
Trimethoprim resistant (dihyrofolate reductase (DHFR)	Trimethoprim			1		1	1				
Chloramphenicol resistance efflux pump (CmlA)	Chloramphenicol efflux pump					1	1				
Chloramphenicol acetyltransferase (CatB)	Chloramphenicol	1						1			

TableS2. A deep analysis of these ten genome sequences will be published elsewhere. In consistence with their phenotypic antimicrobial resistance profiles, ZQ genomes harbor high to moderate numbers of genetic determinants, including β-lactamases, aminoglycoside-modifying enzymes, efflux pumps, modifications of target sites, and permeability defects. Some of these are linked to mobile genetic elements (IS, Tn, integron, plasmids), with the potential to encode resistance functions detected in these bacterial strains. Among the 34 drug-resistance-related genes and mutations identified from ZQ genomes **(Table 5)**. The highest number of antimicrobial resistance genes were associated with ZQ5 and ZQ5 with 27 genes; however 23, 21, 20, 20, 19, and 17 drugs resistance-related genes were identified in ZQ2, ZQ7, ZQ1, ZQ9, ZQ10, and ZQ3 respectively. The least numbers of the detected antimicrobial resistance genes were for ZQ8 and ZQ4 with only 10 and 6 genes, respectively. Among those drug-resistance-related genes from ZQ genomes, 4 of them were shared by all ZQ strains include aminoglycoside nucleotidyltransferase ANT (3'')-II (confer resistance to aminoglycosides), β-lactamase class D 51 like (resistance to carbapenems); bla$_{ADC}$ (resistance to cephalosporins), as well as *ade* genes (adeIJK) encoding for multi-drug resistant efflux pumps.

The aminoglycoside phosphotransferases APH (3″) which give resistance to streptomycin was plentifully detected in all but not in ZQ4 strain, four copies of this gene were identified in ZQ2 while two copies of the same gene were detected in ZQ (5, 6, 7 and 8) and one copy was identified ZQ (3, 9 and 10). The common drug-resistance genes shared by all XDR ZQ strains (all except ZQ (4 and 8)) include β-lactamase class D 23 like (Carbapenems resistance) and *ade* genes (adeABC) encoding for multi-drug resistant efflux pumps, A mutation (Ser83Leu) in (*gyrA*) gene which encodes for DNA gyrase and is responsible for resistance to fluoroquinolones was also identified in all XDR ZQ strains. They were also found that the closely related strains (ZQ1 and ZQ7) ; (ZQ5 and ZQ6) shared the same drug-resistant genes, interestingly, ZQ9 and ZQ10 strains shared all drug-resistant genes except aminoglycoside adenyltransferases ANT(3″)-I (aadA1) (aminoglycosides modifying enzymes) which was unique in ZQ9.

The ZQ Iraqi strains were compared with eighteen of the most highly resistant completely sequenced *A. baumannii* strains from Genbank (str.AYE, AB0057, ACICU, 1656-2, TYTH-1, XH386, USA-2, BAL062, BJAB07104, BJAB0715, BJAB0868, D36, ZW85-1, MDR-TJ, MDR-ZJ06, Naval-2, and Naval 57), moderately resistance strains (LAC-4, DU202, Naval-17), two susceptible clinical strains (ATCC 17978 and AB307-0294), one community-acquired strain (D1279779), and 1 nonclinical strain (SDF) isolated from a human body louse (Figure 1). Nevertheless, the availability of complete genome sequences of *A. baumannii* strains with various degrees of antibacterial resistance is exceedingly useful for scientific research, especially from the perspectives of emergence and spreading of antibacterial resistance genes.

Table 5 The cell is grey indicates the presence of the gene, the cells in black color indicate the presence of more than one copy of the gene while the white cells indicate the absence of the gene. The details of the resistance genetic determinants with their locus tag in the GenBank are listed in **(TableS2).**

The first type of beta-lactamase detected in ZQ strains is a class D-type oxacillinase named *bla$_{OXA-51-like}$*, which was demonstrated to have a narrow-spectrum hydrolysis profile including, at low level, imipenem and meropenem.[35] All of ZQ strains and the other analyzed *A. baumanii* strains including sensitive *A. baumanii* strains SDF and ATCC17978 carried *bla* like OXA-51, which is thought to be intrinsic to *A. baumanii* and normally does not confer carbapenem resistance.[14] The bla$_{OXA-23,}$ which could be the main contributor to carbapenem resistance in ZQ strains owing to the absence of *ISAba1* or other insertion sequences upstream of the intrinsic bla$_{OXA-51-}$ like gene in all ZQ strains that could drive its overexpression leading to carbapenem resistance. As expected, the absence of *bla*$_{OXA-23-like}$, *bla*$_{OXA-40-like}$ and *bla*$_{OXA-58-like}$ genes in the ZQ4 and ZQ8 genomes could explain their susceptible phenotypes to imipenem and meropenem. In MDR strains like AB0057 (from USA), MDR-TJ, MDR-ZJ06, BJAB07104, BJAB0868, and BJAB0715 strains (from china), the presence of *bla*$_{OXA-23}$ could account for their carbapenem resistance.[2,27,31,36]

The *amp*C β-lactamase was detected in all ZQ and other examined *A. baumanii* strains listed in (Figure 1), but was absent in the susceptible SDF strain (9), The class C cephalosporinase *AmpC* is a class of enzymes that target extended-spectrum cephalosporins, and novel AmpC-type enzymes from *Acinetobacter* were designated ADC (for Acinetobacter-derived cephalosporinase).[37] Consistent with all ZQ strains being resistant to piperacillin, older versions of the penicillins, and most of cephalosporin antibiotics, bla$_{ADC}$ genes were detected ZQ1, ZQ2, ZQ7 ZQ5 and ZQ6 strains, all of them were upstream by *ISAba1* elements. On contrary, *bla$_{ADC}$* in closely related ZQ9 and ZQ10 strains along with bla$_{ADC}$ of ZQ8 were not flanked by insertion sequence, although the presence of an upstream IS element (known as IS*AbaI*) promotes increased expression of *bla*$_{ADC}$ and resistance to extended-spectrum cephalosporins but not to cefepime and carbapenems.[38] IS*Aba1* has also been found in the genomes of other *A. baumanii* strains associated with antibiotic resistance genes (including *ampC*), most likely driving robust expression of the resistance phenotypes.[2,36] It is worthy of mentioning that ZQ4 strain which was showed resistance to cephalosporins but intermediate resistance to cefepime, has two copy of class C beta-lactamase, the first one was annotated according to PAGP pipeline, as incomplete, partial *bla$_{ADC}$* and was downstream by *ISAba*19 (IS3 family), while the second class C beta-lactamase found to be flanked by two insertion sequences (IS3 upstream and IS5/IS1182 family transposase downstream)**.** To our knowledge, this is the first report of a gene cluster encoding class C beta-lactamase being flanked by IS3 and IS5/IS1182 insertion elements, which indicate the horizontal gene transfer and the probable modification gene expression, thus increase resistance to a number of cephalosporins analogs including cefepime.

The presence of extended-spectrum β-lactamase (ESBL) PER-7 (*blaPER-7*) gene cluster was found in ZQ (5, 6, 9

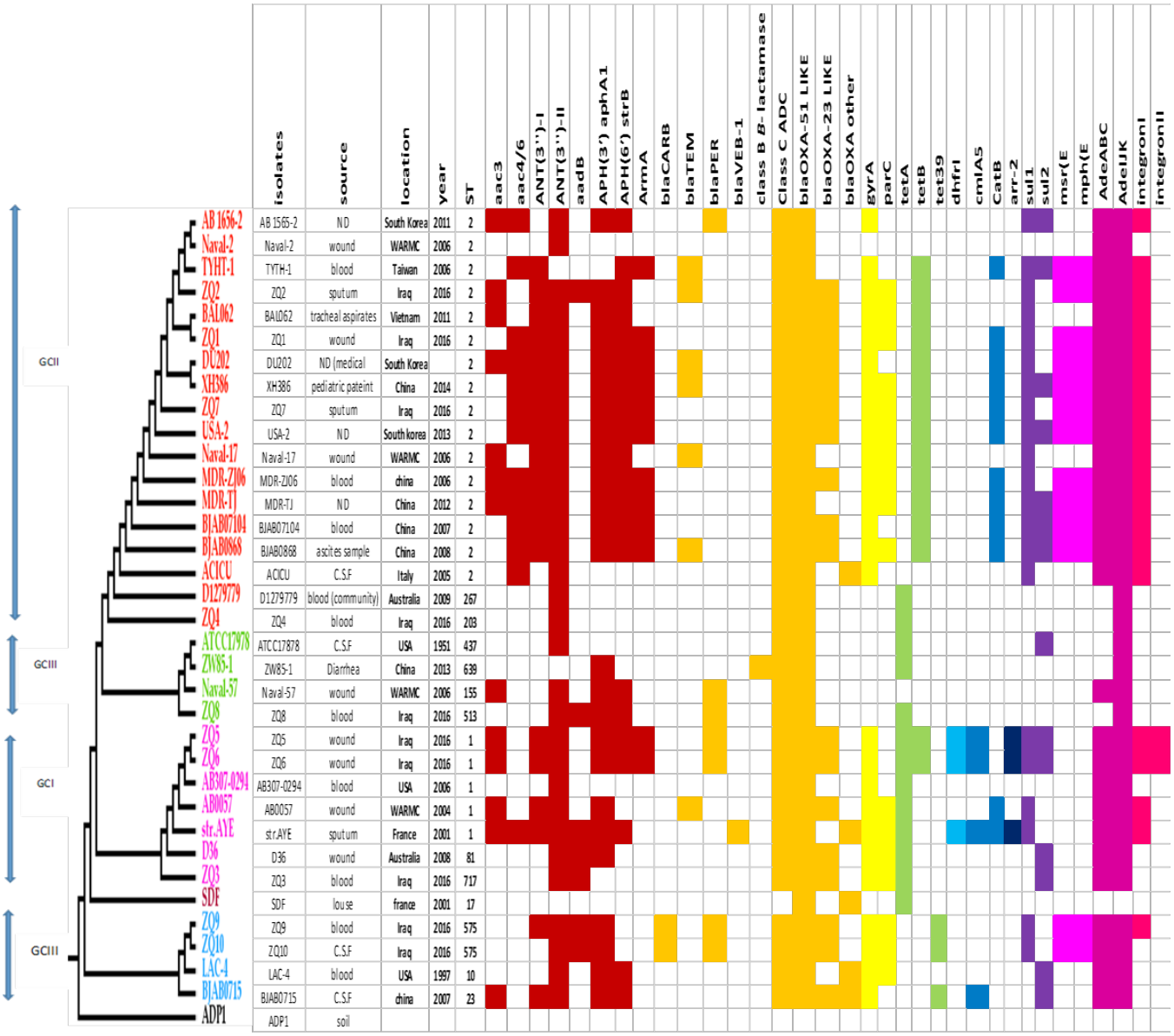

Figure 1. Phylogenetic analysis of 35 A. *baumannii* strains with soil nonpathogenic *Acinetobacter baylyi* ADP1 as outgroup. Phylogentic tree were built by concatenating the sequences at the seven loci under the Institute Pasteur Multi Locus Sequence Typing (MLST) scheme among the ten ZQs strains and 25 other *A. baumannii* strains with whole genomes in GenBank. (AYE, AB0057, ACICU, 1656-2, TYTH-1, XH386, USA-2, LAC-2, BAL062, BJAB07104, BJAB0715, BJAB0868, DU202, D36, ZW85-1, MDR-TJ, MDR-ZJ06, Naval-2, Naval-17, and Naval 57), 2 susceptible strains (ATCC 17978 and AB307-0294), 1 community-acquired strain (D1279779), and 1nonclinical strain (SDF) isolated from a human body louse, maintaining the correct reading frame, and construct a neighbor-joining tree based on these sequences using Geneious tree builder with the default setting. (Geneious version 11.0.5 created by Biomatters. Available from https://www.geneious.com). The center of the figure showed the source of infection, year isolation and the Pasteur MLST of these *A. baumannii* strains. The left of the figures showed the abundance of the resistance genes among these *A. baumannii* strains.

and 10); it is worthy of mentioning that ZQ8 strain harbor β-lactamase PER-10, which differs from PER-7 by 4 amino acid substitutions. PER-7 possessed higher-level of hydrolytic activities against cephalosporins and aztreonam,[39] and was associated with a mosaic class 1 integron structure. While *the* $bla_{\text{PER-10}}$ gene was found to be part of a composite transposon named Tn*1213*.[40] Inconsistent with ZQ8 antimicrobial resistance profile that showed antimicrobial resistance to cephalosporins and intermediate resistance to piperacillin was awing to the presence of $bla_{PER-10,}$ because bla_{ADC} (AmpC) of ZQ8 strain were not flanked by insertion sequence, Being non-inducible, the basal expression levels of bla_{ADC} enzymes from *A. baumannii* do not significantly alter the susceptibility profile to β-lactams. The bla_{CARB-2}, a carbenicillinase gene, which was also designated bla_{PSE-1}. The genetic context of bla_{CARB-2} was also located in class 1 integron in ZQ (9 and 10), TEM-1 is another type of class A beta-lactamase, which was characterized only in ZQ2 strain, TEM-1 is capable of hydrolyzing penicillins and first-generation cephalosporins but is unable to attack the oxyimino cephalosporin.

Resistance to the fluoroquinolones can be accounted by mutations in both housekeeping genes *gyrA* and *parC* in AYE strain,[9] AB0057 (27), and a mutation in *gyrA* for ACICU strain (30) these point mutations were not present in fluoroquinolones sensitive strains ATCC 17978 or D1279779 (29), in consistence with fluoroquinolones resistance profile among ZQ stains, all fluoroquinolone resistant ZQ strains shared 100% identity to *gyrA* and *parC* genes of *A. baumannii* AYE , and thus the Ser83Leu mutation in *gyrA* and Ser84Leu mutation in *parC* were might be the cause of their resistance to fluoroquinolones, while the intermediate resistant ZQ5 and ZQ6 strains, showed only Ser83Leu mutation in *gyrA* but no Ser84Leu mutation in *parC*, and neither mutations were present in ZQ4 nor in ZQ8. Additional ORFs encoding a putative aminoglycoside adenylyltransferase and a tetracycline efflux pump *tet*A were also found in all ZQ strain and other A. *baumannii* strains including SDF and ATCC17978. As these predicted resistance genes did not confer a resistant phenotype to the SDF strain, their exact function is not known (9). Outer membrane proteins (OMPs), also known as porins, play a significant role in the mechanisms of drug resistance (9). However, the OMPs CarO and OprD were complete and not interrupted in ZQ strains. OMP changes seem to make little influence to antibacterial resistance in ZQ strains since loss or mutation of OMPs has not been observed.

An important group of drug-resistance genes recognized in ZQ strains are the genes related to efflux pump function, Including resistance-nodulation-cell division (RND) family, major facilitator superfamily (MFS) and multidrug and toxic efflux (MATE) family (Table S2). Among the RND gene family, all ZQ strain, but not the ZQ4 and ZQ8 strain, exhibited the *Ade*ABC efflux pump-encoding genes (*ade*A, *ade*B, and *ade*C) , the overexpression of *ade*ABC efflux pump may confer high-level resistance to carbapenems (2), it is not surprisingly *Ade*ABC were not found in sensitive strains like SDF (9), D1279779 (29). Another efflux pump system identified from all ZQ strain is the *adeIJK* efflux pump (encoded by *ade*I, *ade*J, and *ade*K) It has been suggested that the *Ade*IJK proteins contribute to intrinsic but not acquired antibiotic resistance in *A. baumanii*.[41] Finally, the efflux pump system identified from the ZQ strains is *tetA* (B), which drives the efflux of tetracycline.

Concluding markers

In this study, we present for the first time the draft whole-genome sequences of clinical A. *baumanii* ZQ strains which were isolated from Iraqi patients, and demonstrated that these strains belong to divergent global clones, *in silico* analysis was also performed to elucidate the antibiotic resistance mechanisms of *A. baumanii* ZQ, identifying a large number of resistance genes encoding b-lactamases, aminoglycosides, macrolides, sulfonamides, tetracyclines, efflux pumps and which are important in antibiotic resistance mechanisms. Resistance to antimicrobial agents are likely to be the target accumulating these different mechanisms, suggesting that the resistance elements respond actively to the selection pressure in the hospital setting. Besides the direct lateral acquisition of genetic material from resistant bacterial strains, the drastic issues that might also play a part in their rapid adaptation to new derivatives of the main antibiotic classes, is associated with continuous presence of intrinsic resistance genes in the genome of *A. baumanii,* which are ready to be boosted by exposure to sub inhibitory levels of the antibiotics in the environment.

Finally, some discrepancies were noted by the identification of a number of putative resistance genes in some susceptible ZQ strains, which suggests that it is potentially only be a step away from acquiring this new resistance through understated changes in expression level or a specific mutation or series of mutations that alter the substrate profile and enhance catalytic activity.

Abbreviations

AAC: Aminoglycoside acetyltransferases; **aadA1:** aminoglycoside adenyltransferases ANT(3″)-I; **aadB**: aminoglycoside nucleotidyltransferase ANT (2'')-Ia; **AbaRI**: Acinetobacter baumannii antibiotic resistance islands; **ABC:** ATP-binding (ABC) transporters; **ADC:** Acinetobacter-derived cephalosporinases; **Ade:** A. baumannii multidrug-resistant efflux pump; **AmpC:** Ampicillin class C β-lactamase; **ANT:** Aminoglycoside adenyltransferases; **APH:** Aminoglycoside phosphotransferases; **ARR-2**: rifampin ADP-ribosylating transferase; **Arma:** *a*minoglycoside *r*esistance *m*ethyltransferase**; CarO:** Carbapenem-associated outer membrane protein; **Cat8:** Chloramphenicol acetyltransferase; **CDS:** protein coding genes; **CLSI**: Clinical Laboratory Standards Institute; **CmlA:** Chloramphenicol resistance Acinetobacter; **comM:** ATPase encoding gene; **cpn60:** 60-KDa chaperonin; **Dhfr:** Dihydrofolate reductase; **FolP1:** Folate reductase; **fusA:** elongation factor EF-G; **GC** content: guanine-cytosine content; **gltA:** citrate synthase; **glms:** glutamine--fructose-6-phosphate transaminase; **gyrB:** DNA topoisomerase (ATP-hydrolyzing) subunit B; **indels:** insertions/ deletions; **IntI1**: class I integron; **IS:** insertion sequences; **ISAba and ISPa**: predefined common nomenclature forIS of A. baumannii and Pseudomonas aeruginosa respectively; **ISCR1:** Insertion sequence common region or IS91-like elements; **Leu:** leucine amino acid; **MATE:** multidrug and toxic compound extrusion family; **MCM:** mauve Contigs mover; **MdfA:** Multidrug facilitator; **MDR:** Multidrug resistant; **MFS:** major facilitator superfamily; **Mph(E):** family macrolide 2'-phosphotransferase; **Msr(E):** ABC-F type ribosomal protection protein; **ncRNA:** non-coding ribonucleic acid; **NGS:** next-generation sequencing; **Omp:** Outer membrane protein; **ORFs**: open reading frames; **OXA:** oxacillinase; **parC:** subunit A of topoisomerase IV; **PBP:** Penicillin binding protein; **PE:** paired-end sequencing; **PER:** Pseudomonas extended resistant; **PGAP**: Prokaryotic Genome Annotation Pipeline; **pyrG:** Cytidine triphosphate synthase; **qacE:** ethidium bromide resistance locus; **recA:** homologous recombination factor; **RepB:** plasmid replication initiator; **RI:** resistance islands; **RND: r**esistance-nodulation-

cell division; **rplB:** 50S ribosomal protein L2; **rpoB:** DNA-directed RNA polymerase subunit beta; **Sat2:** Streptothricin N-acetyltransferase; **Ser:** Serine an α-amino acid; **SMR:** small MDR family; **sul1:** Sulfonamide-resistant dihydropteroate synthase; **TCS**: two-component system; **TEM:** Temoneira; **Tet:** Tetracycline resistant Acinetobacter; **tn:** transposase; **XDR:** extensively drug resistance.

DECLARATIONS

Ethical approval and patients consent

The current study was approved by the institution review board of College of Medicine/ Al-Nahrain University in accordance with the World Medical Association's Declaration of Helsinki guidelines. Patient's consents are not applicable

Availability of data and materials

The raw sequencing data were uploaded to the public database of the National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov/) under the BioProject PRJNA419147. The complete genome sequences of *Acinetobacter baumannii* ZQ strains have been deposited at DDBJ/ENA/GenBank under accession No.: ZQ1(PHHB00000000), ZQ2(PHKA00000000.2), ZQ3(PHJZ00000000), ZQ4(PHJY00000000), ZQ5(PHJX00000000), ZQ6(PHJW00000000), ZQ7(PHJV00000000), ZQ8(PHJU00000000), ZQ9(PHJT00000000) and ZQ10(PHJS00000000). The versions described in this study are version 2 for ZQ1(PHHB00000000.2), ZQ2(PHKA00000000.2), ZQ3(PHJZ00000000.2), ZQ4(PHJY00000000.2), ZQ5(PHJX00000000.2), ZQ6(PHJW00000000.2), ZQ7(PHJV00000000.2), ZQ8(PHJU00000000.2), ZQ10(PHJS00000000.2) and version 3 for ZQ9(PHJT00000000.3), The plasmids accessions appear in the WGS_SCFLD line at the bottom of the WGS master record.

ACKNOWLEDGMENTS

We sincerely thank Dr. Mervet Basim and the Bacteriology laboratory department staff at Al-Imamain Al-Khadhimain Hospital, Baghdad, Iraq, for their help with bacterial samples collection and characterization.

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Additional file1

42. Table S1: Details of A. baumannii strains multilocus sequence type according to Pasteur institute.
43. Table S2: The details of the resistance genetic determinants with their locus tag in the GenBank. (XLS 14 KB)