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ORIGINAL ARTICLE

High prevalence of the PER-1 gene among carbapenem-resistant *Acinetobacter baumannii* in Riyadh, Saudi Arabia

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Abstract The prevalence of carbapenem-resistant *Acinetobacter baumannii* in Saudi Arabia and their resistance genetic mechanisms are yet to be identified. We studied the prevalence and genetic diversity of extended-spectrum beta-lactamase genes, particularly the PER-1 gene, among carbapenem-resistant *A. baumannii* strains from patients at a tertiary care hospital in Riyadh, Saudi Arabia between 2006 and 2014. Fresh subcultured samples were tested for antimicrobial susceptibility minimum inhibitory concentration (MIC). Total genomic DNA was extracted from each isolate and further used for polymerase chain reaction (PCR) genotyping, sequence-based typing (SBT) of PER-1 and OXA-51-like gene, and multilocus sequence typing (MLST) of positive isolates. Randomly selected clinical isolates (n = 100) were subjected to MLST. A total of 503 isolates were characterized as multidrug-resistant (MDR) using the MIC. Isolates were further PCR tested for $bla_{\text{-TEM}}$ and $bla_{\text{-PER-1}}$ resistance genes (n = 503). The genotyping results showed that 68/503 (14 %) isolates were positive to *bla* TEM. The genotyping results of PER-1-like genes showed that 384/503 (76.3 %) were positive among MDR *Acinetobacter* isolates. Based on SBT, the majority of these isolates were clustered into three main groups including isolates harboring PER-1: AB11 ($bla_{\text{-PER-1}}$), isolate AB16 ($bla_{\text{-PER-1}}$), and, finally, the plasmid pAB154 ($bla_{\text{-PER-7}}$). Remarkably, many isolates were concealing the PER-1 gene and harboring the TEM resistance genes as well. MLST results for selected isolates (n = 100) identified four main sequence types (STs: 2, 19, 20, and 25) and four novel isolates (ST 486–489). We report 76.3 % prevalence of the PER-1 resistance gene among *Acinetobacter* clinical isolates from Riyadh, Saudi Arabia. Further work is needed to explore the clinical risks and patient outcome with such resistance related to healthcare-associated infections and investigate the genetic and molecular mechanisms that confer the MDR phenotype.

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Introduction

Hospital-acquired infection due to *Acinetobacter baumannii* is a rising problem globally, including the Gulf region and Saudi Arabia [1–3]. *Acinetobacter baumannii* is a glucose non-fermentative Gram-negative bacilli which is widespread in hospitals and healthcare environments [4]. *Acinetobacter baumannii* plays an important role in hospital-acquired infections due to their extended survival time periods on surfaces and their high tendency to acquire resistance to multiple drugs. The resistance mechanism that *A. baumannii* uses is becoming a challenge for both physicians and microbiologists. One possible explanation of such a mechanism involves the selective permeability action in the outer membrane protein (porin)

which controls the efflux system and the presence of chromosomal beta-lactamase [5, 6].

PER is a term that refers to *Pseudomonas aeruginosa* extensive resistance, which is carried onto both chromosomes and plasmids [7–9]. Although the gene was first discovered in a *P. aeruginosa* strain, different hypotheses argue about its origin [8, 10]. The PER-1 gene has been detected in *A. baumannii* strains isolated from intensive care unit (ICU) patients in Riyadh, Saudi Arabia [3]. This might have triggered an outbreak case that was first found in the country. It is an extended-spectrum beta-lactamase enzyme and encoded by the $bla_{\text{-PER-1}}$ gene. This enzyme is known for its ability to hydrolyze the beta-lactam ring of antibiotics, such as penicillin, oxyimino-cephalosporins, and aztreonam [11]. PER resistance genes have been identified worldwide, including in Europe, North and South America, and the Far East, due to their chromosomal location [12]. PER-1 gene expression was first detected in 1993 in a Turkish patient in France who was infected with *P. aeruginosa* [13]. Different *P. aeruginosa* isolates harboring the enzyme were then reported in Turkey, and a widespread detection of the PER-1 enzymes has been reported in other European countries [14–16]. On the other hand, the prevalence and genetic diversity of PER-1 in the Middle East in general and Saudi Arabia in particular, are yet to be identified [2].

Many molecular typing methods have been used to study the molecular epidemiology and genetic diversity among *A. baumannii* isolates. Multilocus sequence typing (MLST) is based on sequencing of the seven impartially selected genes, and the genes of each are allocated a number for each different allelic form [15, 17]. Based on the sequence types (STs) of isolates (*A. baumannii* isolates for example), the relatedness and phylogenetic analysis of these isolates will be determined by comparing their STs with the databases created by different laboratories worldwide (http://pasteur.fr/mlst). The purpose of the current study was to investigate the prevalence of PER-1 resistance gene among carbapenem-resistant *A. baumannii* isolates from Riyadh, Saudi Arabia.

Materials and methods

Bacterial identification

The clinical isolates used in this study were collected at the microbiology laboratory of the 800-bed tertiary care hospital, King Abdulaziz Medical City (KAMC), Riyadh, Saudi Arabia during 2006–2014. Isolates characterized by aerobic growth and were catalase-positive, oxidase-negative were identified using commercial the biochemical system MicroScan® WalkAway (Siemens, Germany). The isolates were initially identified at the species level using MicroScan and were then confirmed by polymerase chain reaction (PCR) of the RNA polymerase β-subunit gene (*rpoB*), followed by sequencing.

MIC and susceptibility testing

We collected 503 isolates for antimicrobial susceptibility testing. We used a randomly selected subset (n = 130) for the MLST to further study their minimum inhibitory concentrations (MICs). The susceptibility data were interpreted according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI). It was performed using the automated MicroScan® Walkaway system. All isolates that exhibited resistance were confirmed by the Etest® (bioMérieux, France) to detect the exact MIC value. Isolates were tested for their MIC using the MicroScan Neg MIC panel type 32 including the following antibiotics: amikacin, amoxicillin/clavulanate, aztreonam, cefepime, cefotaxime, ceftazidime, ceftriaxone, ciprofloxacin, colistin, gentamicin, imipenem, meropenem, tobramycin, and trimethoprim/sulfamethoxazole. Standardized suspensions of tested isolates were inoculated and incubated at 35 °C for a minimum of 16 h and the MIC was expressed as susceptible, intermediate, and resistant according to the 2011 European Committee on Antimicrobial Susceptibility Testing (EUCAST) report. Only one isolate per patient per year was included in the current analysis. Quality control was performed by testing these same antimicrobials against *Escherichia coli* ATCC 25922, *E. coli* ATCC 35218, and *P. aeruginosa* ATCC 27853. The isolates were defined as multidrug-resistant (MDR) [4, 18].

DNA extraction

DNA was extracted from 100 μL of fresh subcultured samples using the DNA extraction kit MagNA Pure Compact (Roche), according to the manufacturer's instructions. In brief, we used a combination of proteinase K enzyme and heat lysis, followed by magnetic bead capture and DNA wash. Finally, DNA was eluted with 200 μL of 10 mM Tris and 1 mM EDTA (pH 8.0) of elution buffer. The aliquots of extracted nucleic acids were stored at −20 °C until further analysis. The quantity and purity of the extracted nucleic acid samples were confirmed using a NanoDrop spectrophotometer (ND-1000, Fisher).

Detection of resistance genes

To identify the resistance genes, genotyping was performed by PCR amplification reactions with a list of primers representing the four different resistance mechanisms. Twenty pairs of primers were used for *A. baumannii* resistance genotyping (Eurofins, Germany) including TEM, VIM, and PER-1, as previously well established by Bonomo and others and explained elsewhere [3]. Final PCR reactions were carried out

in 20-μL volumes containing 20 ng DNA, 17 μL of MegaMix-Blue (Microzone, UK) containing 1.1× PCR reaction buffer, 220 μM of dNTP, 2.75 mM $MgCl_2$, 0.25 units of recombinant Taq DNA polymerase, and 0.40 μM each of the forward and reverse primers. PCRs were carried out using the PCR thermocycling program as recommended by the PCR mix supplier. In general, PCRs were done as follows: after the initial 10 min of incubation at 95 °C, a 30 cycle program comprising 94 °C for 30 s, 55 °C annealing temperature for 60 s, 72 °C for 60 s, and a final extension for 10 min at 72 °C was used. Some modifications in annealing temperature, number of cycles, etc. were made to optimize the amplification for certain genes. The PCR products were analyzed by electrophoresis on a 1.5 % (wt/vol) agarose gel containing 0.125 μg/mL ethidium bromide.

Sequence-based typing (SBT) and sequencing

The PCR amplicons were purified using the QIAquick PCR Extraction Kit (Qiagen, USA). Both strands of the amplicons were sequenced by using an ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit v1.1 (Applera, France) and an ABI 3100 automated sequencer (Biosystems, USA). PCR sequences were compared with all nucleotides sequences in the GenBank database using the PubMed National Center for Biotechnology Information BLAST program.

Multilocus sequence typing

MLST of 100 randomly selected isolates representing all clusters form the SBT-based phylogenies were carried out using the following seven housekeeping genes: *cpn60* (60-KDa chaperonin), *fusA* (elongation factor EF-G), *gltA* (citrate synthase), *pyrG* (CTP synthase), *recA* (homologous recombination factor), *rplB* (50S ribosomal protein L2), and, finally, *rpoB* (RNA polymerase subunit B), as described by Diancourt et al. [19]. The primers used for MLST amplification and sequencing are listed in Table 1 and the PCR conditions used were as previously described by others [19, 20]. We used the Pasteur MLST database for allelic profiles identification (http://pubmlst.org/perl/bigsdb/bigsdb.pl?db=pubmlst_abaumannii_pasteur_seqdef).

Data analysis

Data were collected and analyzed by Excel and SPSS version 20. A phylogenetic tree was generated from the MLST allelic profile using the START 2 software using default criteria and the unweighted pair group method with arithmetic mean [21]. The MLST genotyping alleles were used to construct the tree. Moreover, the identified sequence type, patients' demographic data, and their corresponding TEM and PER-1 genes were incorporated with the lineage tree. The isolates with new sequence types are shown with red arrows.

Results

MDR and carbapenem-resistant phenotypes

A total of 503 isolates were collected from KAMC during the period 2006–2014. The sources of the clinical isolates were as follows: respiratory 60 (46.2 %), wound and burn 31 (23.8 %), blood 18 (13.8 %), urine 5 (3.8 %), and others 16 (12.3 %). The demographic data showed that the isolates were collected from patients with the following age distribution: 2 to 24 years with 22.2 %, followed by aged 25 to 49 years (28.6 %), and, finally, 49.2 % of the patents were elderly, between 50 to 90 years. furthermore, we did not observe any significant differences in antibiotic resistance among *A. baumannii* isolated

Table 1 Scheme of *Acinetobacter* multilocus sequence typing (MLST) amplification and sequencing primers developed by Diancourt et al. [19]

Locus	Primer	Sequence	Amplicon size (bp)
cpn60	cpn60F	ACTGTACTTGCTCAAGC	405
	cpn60R	TTCAGCGATGATAAGAAGTGG	
fusA	fusA7	ATCGGTATTTCTGCKCACATYGAT	633
	fusA8	CCAACATACKYTGWACACCTTTGTT	
gltA	gltAF	AATTTACAGTGGCACATTAGGTCCC	483
	gltAR	GCAGAGATACCAGCAGAGATACACG	
pyrG	pyrG7	GGTGTTGTTTCATCACTAGGWAAAGG	297
	pyrG8	ATAAATGGTAAAGAYTCGATRTCACCMA	
recA	RA1	CCTGAATCTTCYGGTAAAAC	372
	RA2	GTTTCTGGGCTGCCAAACATTAC	
rplB	rplB7	GTAGAGCGTATTGAATACGATCCTAACC	330
	rplB8	CACCACCACCRTGYGGGTGATC	
rpoB	Vic4	GGCGAAATGGC(AGT)GA(AG)AACCA	456
	Vic6	GA(AG)TC(CT)TCGAAGTTGTAACC	

from males (49.2 %) versus females (50.8 %). Susceptibility testing showed that all isolates were resistant to four or more antimicrobial groups. Antibiotic susceptibility isolates against imipenem and meropenem are represented in Tables 2.

PER-1 and other carbapenems resistance genes

Carbapenems resistance phenotypes among the isolates were confirmed by MIC ≥8 μg/ml for both meropenem (96.4 %) and imipenem (88.3 %). PCR typing showed that 76.3 % of the isolates (*n* = 384/503) contained the PER-1 gene. On the other hand, the genotyping results showed that *n* = 132/503 (26.2 %) isolates were positive to $bla_{\text{-TEM}}$ and 457/503 (90.8 %) were positive for the OXA-51-like gene. Nevertheless, all isolates were negative to the OXA-24-like gene.

Sequence types of *A. baumannii* isolates and identification of regional clones

The sequence types for 100 randomly selected isolates typed by MLST were gathered in four clusters. The MLSTs of some isolates (*n* = 34) were not available because they were untypable or missing alleles. However, the most common sequence type (ST) was ST2 (*n* = 26), which is part of clonal complex 2 (CC2) and represents the European clone II (EUII) or Worldwide clone 2 (WW2). The second most common type was ST20 (CC1) (*n* = 19), followed by other STs. Moreover, MLST enabled the identification of four new isolates (486–489) with novel allelic profiles (Table 3). The MLST allelic profiles of the four novel STs isolates have been entered into the Institut Pasteur MLST databases. Full data of the new isolates ST-486, ST-487, ST-488, and ST-489 can be accessed at (http://pubmlst.org/perl/bigsdb/bigsdb.pl?page=info&db=pubmlst_abaumannii_isolates&id=1602).

Diversity of PER among *Acinetobacter* isolates

The PCR primers used for sequencing $bla_{\text{-PER-1}}$ genes in *A. baumannii* identified two genotypic forms, PER-1-1 and PER-1-7. In addition, based on hit definition, the PER-1-positive clones were sub-divided into five groups (Table 4). The majority of PER-1 isolates are similar to isolate AB11 ($\text{bla-}_{\text{PER-1–1}}$) (Table 4) and the remaining four groups represents one isolate of PER-1-1 in each, as shown in Table 4. On the other hand, the sequences of 29 isolates were submitted to the BLAST server and found to be similar to PER-7 (Table 4). This group was sub-divided into two groups: 17 isolates belong to isolate AB16 and the remaining 12 belong to pAB154.

Table 2 Antibiotic susceptibility of multidrug-resistant (MDR) *Acinetobacter* isolates represented as number of isolates (percentage)

Antibiotic	Amikacin	Cefepime	Cefotaxime	Ceftazidime	Ceftriaxone	Ciprofloxacin	Colistin	Gentamicin	Imipenem	Meropenem	Tobramycin	Trimethoprim/sulfamethoxazole
Resistant	74 (56.9)	126 (96.9)	124 (95.4)	125 (96.2)	125 (96.2)	125 (96.2)	2 (1.5)	122 (93.8)	100 (76.9)	115 (88.5)	52 (40)	129 (99.2)
Intermediate	17 (13.1)	0 (0)	0 (0)	1 (0.8)	0 (0)	1 (0.8)	0 (0)	1 (0.8)	15 (11.5)	8 (6.2)	7 (5.4)	0 (0)
Susceptible	39 (30)	4 (3.1)	6 (4.6)	4 (3.1)	5 (3.8)	4 (3.1)	128 (98.5)	7 (5.4)	15 (11.5)	7 (5.4)	70 (53.8)	1 (0.8)

Table 3 For selected isolates, demographic data and sequence type alleles and beta-lactamases and metallo-beta-lactamases resistance genotyping among MDR *Acinetobacter* isolates (full dataset in Supplementary Table 1)

Isolate ID	Age/sex	Source	Beta-lactamase								MLST alleles								
			bla		*bla*-OXA				metallo										
			TEM	PER	24	23	51	58	IMP	VIM	cpn60	fusA	gltA	pyrG	recA	rplB	rpoB	ST	Antibiotic
ACI-R-161	03M	Tracheal	−	+	−	+	+	−	+	+	1	1	1	1	5	1	1	**1**	10
ACI-R-130	22M	Tracheal	−	+	−	+	+	−	−	−	1	2	1	1	5	1	1	**19**	11
ACI-R-002	20M	Unk	+	+	−	+	+	−	+	+	2	2	2	2	2	2	2	**2**	10
ACI-R-015	66F	Bronchial	−	+	−	+	+	−	−	+	2	2	2	2	2	2	2	**2**	8
ACI-R-033	35M	Blood	−	+	−	+	+	−	−	−	2	2	2	2	2	2	2	**2**	9
ACI-R-036	77F	Tissue	+	+	−	+	+	−	+	+	2	2	2	2	2	2	2	**2**	7
ACI-R-051	64F	Bronchial	+	+	−	+	+	−	+	+	2	2	2	2	2	1	2	**195**	11
ACI-R-057	27M	Sputum	−	+	−	+	+	+	+	+	2	2	13	2	2	1	2	**196**	11
ACI-R-068	30M	Unk	+	+	−	+	+	−	+	+	2	2	1	2	2	2	2	**487**	11
ACI-R-074	47M	Sputum	+	+	−	+	+	−	+	+	2	2	2	2	2	8	2	**489**	9
ACI-R-082	50F	Tracheal	−	+	−	+	+	−	+	−	2	4	7	1	7	−	4	**Unk**	11
ACI-R-097	64F	Unk	−	+	−	+	+	−	+	+	3	1	1	1	5	1	1	**20**	9
ACI-R-099	43F	Sputum	−	+	−	+	+	−	−	−	3	1	1	1	5	1	1	**20**	11
ACI-R-173*	24M	Tracheal	+	+	−	+	+	−	−	−	3	3	2	4	7	2	4	**25**	9

*Some isolates ($n = 34$) were not available, due to being untypable or missing an allele

Table 4 Results of BLAST of the isolated clones

Hit definition	PER genotype	PubMed hit_id	Isolate	Length, bp	Isolate	Length, bp
Isolate AB11 (bla_{PER-1})	1	107123296	AC_002	757	AC_121	849
			AC_009	845	AC_127	845
			AC_013	802	AC_134	847
			AC_016	843	AC_138	844
			AC_035	845	AC_140	846
			AC_037	847	AC_142	849
			AC_040	848	AC_144	845
			AC_043	845	AC_146	844
			AC_069	847	AC_148	844
			AC_076	853	AC_162	847
			AC_083	843	AC_183	843
			AC_084	848	AC_188	850
			AC_095	794	AC_191	843
			AC_098	844	AC_197	844
			AC_104	765	AC_206	845
			AC_107	844	AC_224	796
			AC_110	850	AC_232	765
			AC_111	844	AC_236	843
			AC_115	847	AC_249	794
			AC_118	848	AC_256	785
Isolate AB16 (bla_{PER-1})	1	107123317	AC_176	731	AC_106	546
			AC_002	879	AC_109	871
			AC_004	558	AC_123	875
			AC_006	871	AC_133	886
			AC_011	884	AC_148	671
			AC_013	868	AC_204	876
			AC_047	870	AC_232	879
			AC_055	878	AC_254	573
			AC_104	878		
pAB154 (bla_{PER-7})	7	406507747	AC_006	759	AC_142	778
			AC_035	764	AC_162	775
			AC_076	789	AC_183	748
			AC_089	684	AC_197	761
			AC_110	765	AC_206	761
			AC_127	759	AC_236	761
Isolate AB31 (bla_{PER-1})	1	107123341	AC_111	758		
PER-1 gene, complete cds	1	146216707	AC_016	752		
Strain AP2 mosaic class 1 integron	1	336441522	AC_224	880		
Strain AB-15094 class 1 integron Int1	1	406507747	AC_065	850		

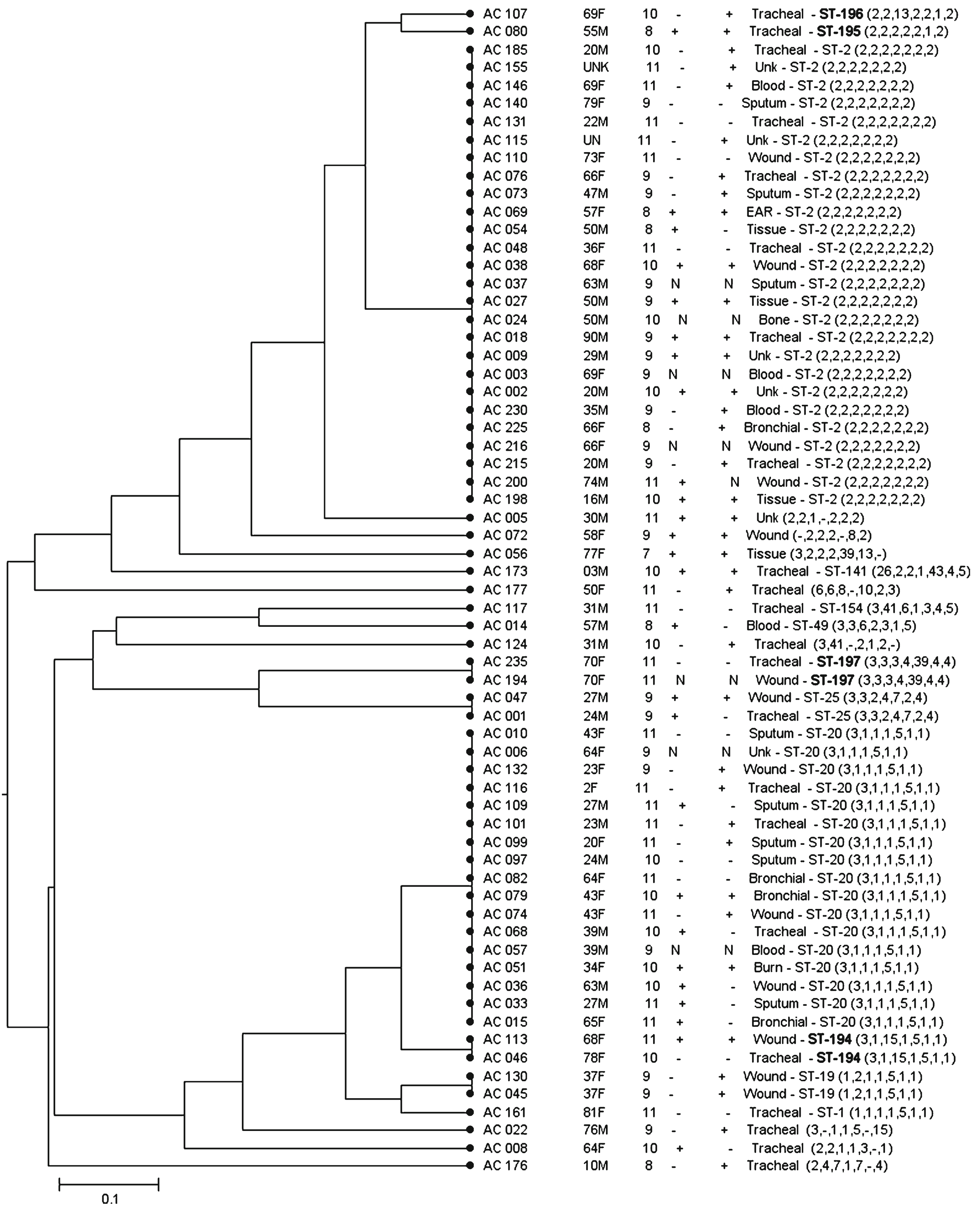

Fig. 1 Lineage tree generated from the multilocus sequence typing (MLST) allelic profile and showing the MLST a sequence type, patients' demographic data, and their corresponding TEM and PER-1 gene occurrence in *Acinetobacter* clinical isolates from Saudi Arabia, with new sequence type isolates shown with bold font

Association of PER-1 diversity and MLST

A lineage tree was generated from MLST allelic profiles and SBT-PER using the unweighted pair group method with arithmetic mean (Fig. 1). MDR *A. baumannii* isolates were grouped into three clusters, where the first cluster consisted of isolates that harbored the PER-1 gene (n = 28) and belonged to ST2. Distinctively, many isolates typed as ST20 and possessing the PER-1 gene (n = 19) were the main contributors to the second cluster. However, the third cluster was a mix of STs that confer positive TEM and PER resistance genes together (Fig. 1).

Discussion

Genetically, PER-1 assumed to be part of the class A beta-lactamase enzyme but different studies contradict the idea by separating it into independent groups within classes [7–9]. This is due to the fact that the PER-1 gene shares only 26 % sequence homology with other class A beta enzymes such as TEM and SHV. Also, there are two essential conserved domains in the class A beta-lactamase structure: the omega loop fold and the cis confirmation peptide bond between 166 and 167 residues were not present in the PER-1 protein [22]. On the other hand, PER-1 was found to form a new fold in the omega loop which possessed an aspartic acid residue 138, conferring trans confirmation between 166 and 167 residues [23]. Recently, *A. baumannii* isolates with antibiotic resistance have been on the increase in the Gulf countries [22–26]. The characterization of its prevalence will help contain this problem. Studies dissecting the genetic structure and mode of acquisition of certain specific elements revealed that two transposons elements, ISPa12 in the upstream and ISPa13 in the downstream, surround the PER-1 gene to form the TN1213 transposon in both *A. baumannii* and *P. aeruginosa*. Also, there is a promoter in the ISPa12 element that activates the expression of the PER-1 gene [23]. In this study, 73 isolates were characterized by PCR and sequencing of the bla_{PER} gene. The sequenced products were then analyzed for homology with equivalent sequences available at NCBI BLASTN (http://blast.ncbi.nlm.nih.gov/Blast.cgi). All of the isolates were found to be positive for this gene. Among the 73 isolates, 55 % of samples were related to the Chinese origin isolate AB11 ($bla_{\text{PER-1}}$), 23 % related to the Chinese origin isolate AB16 ($bla_{\text{PER-1}}$), 16 % related to the Egyptian origin plasmid pAB154 (blaPER-7), and 6 % of the isolates were related to Chinese, French, and British origin of *A. baumannii*. Wang et al. [27] reported a similar high prevalence of PER-1 *A. baumannii* in China as well. In their study, about 78 % of the Chinese imipenem-resistant *Acinetobacter* isolates were found to harbor the PER-1 gene. These results indicated that the prevalence of PER-1 genes is similar to that China (77.8 %) and higher than in Italy (34.61 %) and Turkey (46 %) [13, 27, 28]. Thus, the isolated strains in Riyadh, Saudi Arabia are either transferred by nationals of these countries or by Saudis who visited these countries. It is noteworthy that PER-7 represents a slight challenge, as it can hydrolyze cephalosporins more rapidly than PER-1 [29].

Conclusion

Here, we report for the first time the high prevalence of PER-1 resistance gene among *Acinetobacter baumannii* in Saudi Arabia. The PER-1 resistance gene was previously reported in some European countries (Turkey, France, and Italy) and North America. Further studies are needed to explore the origin and molecular mechanisms that confer the resistance phenotype

Compliance with ethical standards

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Conflict of interest All authors confirm that there is no conflict of interest or financial relationship with the organization that funded the research.

Ethics statement Ethical approval and consent were not required for this study because no human or animal subjects were used.

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