Table 1
Minimum inhibitory concentrations (MICs) and percent resistance (%R) of *Aggregatibacter actinomycetemcomitans* to amoxicillin and amoxicillin/clavulanic acid (AMC) (n = 100 clinical isolates) and to metronidazole (n = 41 clinical isolates).

	Amoxicillin	AMC	Metronidazole	
			Aerobic growth	Anaerobic growth
MIC_{50} (μg/mL)	0.5	0.5	10	2.25
MIC_{90} (μg/mL)	1.5	1.0	56	8
MIC range (μg/mL)	0.38–2.0	0.25–2.0	0.75–256	0.38–256
%R[a]	0	0	[b]	

$MIC_{50/90}$, MIC required to inhibit 50% and 90% of the isolates, respectively.
[a] *Haemophilus influenzae* was used as the reference species: susceptible ≤2 μg/mL > resistant according to European Committee on Antimicrobial Susceptibility Testing (EUCAST) clinical breakpoint table v.3.1, valid from 2013-02-11.
[b] No reference species is available for metronidazole resistance breakpoints.

susceptibility of pathogens is regularly monitored. Significant differences between countries in antibiotic susceptibility profiles of periodontal bacteria have been noted in Europe [5] and South American countries [4]. In the present study, no β-lactamase-positive *A. actinomycetemcomitans* isolates were found and all strains appeared susceptible to amoxicillin according to the EUCAST breakpoint (≤2 μg/mL), which contradicts data from Colombia [3].

We also showed that in vitro metronidazole susceptibility is determined by growth conditions. In conclusion, although several reports show significant differences in susceptibility between amoxicillin and AMC, in this study we found no evidence for β-lactamase production in *A. actinomycetemcomitans*. Second, susceptibility testing of *A. actinomycetemcomitans* to metronidazole should be performed under anaerobic growth conditions.

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In vitro activities of tetracyclines against different clones of multidrug-resistant *Acinetobacter baumannii* isolates from two Iranian hospitals

Sir,

Acinetobacter baumannii has emerged as one of the most challenging healthcare-related pathogens and its occurrence has increased worldwide, especially in patients admitted to intensive care units [1]. *A. baumannii* isolates are frequently resistant to multiple antimicrobial agents and there are recent reports of isolates resistant to virtually all clinically relevant drugs [1].

Minocycline and tigecycline are both available for intravenous infusion and have been approved by the US Food and Drug Administration (FDA) for use in *A. baumannii* infections [2]. These two antibiotics have been shown to maintain encouraging activities against multidrug-resistant *A. baumannii* (MDR-AB) [2,3]. The Tigecycline Evaluation and Surveillance Trial TEST data reported minocycline and tigecycline as the most active agents by susceptibility rate and MIC_{90} value (minimum inhibitory concentration required to inhibit 90% of the isolates), respectively, against 483 carbapenem-resistant *A. baumannii* isolates [3].

In the present study, the in vitro activities of tigecycline, minocycline and doxycycline against 67 MDR-AB isolates recovered from 29 burn and 38 non-burn Iranian patients hospitalised in Tehran and Tabriz, respectively, were studied. MICs of tetracyclines, imipenem, meropenem, amikacin and gentamicin were determined by Etest (AB BIODISK, Solna, Sweden). The distribution and prevalence of the most relevant acquired resistance genes were assessed. Molecular epidemiological relationships were also investigated using DiversiLab repetitive sequence-based PCR (rep-PCR), multilocus sequence typing (MLST) and sequence group (SG) determination.

Amongst the 67 MDR-AB isolates, the prevalence rate of non-susceptibility to each antibiotic was >70%, with the exception of doxycycline (n = 30; 44.8%), tigecycline (n = 24; 35.8%) and minocycline (n = 7; 10.4%). The MIC_{50} and MIC_{90} values of tigecycline, minocycline and doxycycline, respectively, were as follows: 3 and 6 mg/L; 1 and 24 mg/L; and 8 and >256 mg/L. The MIC_{50} and MIC_{90} values of tigecycline in burn isolates were higher than those of non-burn isolates (3 and 6 mg/L vs. 2 and 4 mg/L, respectively).

All isolates were positive for *adeB* showing amplicons of the expected size (ca. 1 kb). The most common acquired resistance gene was *aph(3′)-VIa*, which was found in 64 isolates (95.5%), followed by $bla_{OXA\text{-}23\text{-}like}$ in 47 isolates (70.1%), *ant(2″)-Ia* in 38 isolates (56.7%), $bla_{OXA\text{-}40\text{-}like}$ in 24 isolates (35.8%), *aac(3′)-Ia* in 22 isolates (32.8%), *aph(3′)-Ia*/*ant(3″)-Ia* in 13 isolates (19.4%), *tetB* in 13 isolates (19.4%), *aac(6′)-Ib* in 11 isolates (16.4%) and *armA* in 6 isolates (9.0%). Thirteen *tetB*-carrying isolates, including seven non-burn

Table 1
Epidemiological, phenotypic and genotypic characteristics of 67 multidrug-resistant *Acinetobacter baumannii* isolates from Iran.

rep-PCR	SG	B/NB	Resistance genes									
			OXA-23	OXA-40	*armA*	*aac(3′)-Ia*	*aac(6′)-Ib*	*aph(3′)-Ia*	*aph(3′)-VIa*	*ant(2″)-Ia*	*ant(3″)-Ia*	*tetB*
A	1	0/6	1	0	1	5	1	1	5	3	2	1
A	–	0/2	0	0	0	1	0	0	2	1	0	0
B	1	1/2	3	1	0	3	1	0	3	1	0	0
C	–	2/0	0	2	0	0	0	2	2	2	0	0
D	–	6/0	0	6	1	0	0	1	6	2	1	0
E	3	7/0	4	3	0	1	1	1	7	7	1	0
E	–	2/0	0	2	0	0	1	0	2	2	0	0
F	2	7/0	7	7	0	1	2	1	6	6	1	0
G	–	2/0	2	0	0	0	0	0	2	0	0	0
H	–	2/2	4	0	0	0	1	0	4	4	2	3
I	1	3/8	11	1	2	6	2	3	10	1	1	2
J	1	1/5	6	0	1	4	0	2	6	2	1	1
Si 1,2	1	0/2	2	0	0	1	0	1	2	1	1	2
Si 3	–	1/0	1	0	1	0	0	0	1	1	0	0
Si 4	2	1/0	1	1	0	0	1	0	1	1	0	0
Si 5,8	–	1/1	2	0	0	0	1	1	2	2	2	2
Si 6	2	0/1	1	0	0	0	0	0	1	1	1	1
Si 7	–	1/0	1	0	0	0	0	0	1	1	0	1
Si 9	1	1/0	1	1	0	0	0	0	1	0	0	0

rep-PCR	SG	B/NB	MIC range (mg/L)						
			IPM	MEM	AMK	GEN	TIG	MIN	DOX
A	1	0/6	1.5–32	2–32	12–256	16–256	0.75–3	0.38–4	2–256
A	–	0/2	1.5–3	1.5–3	12–16	12–256	3	0.38	2–3
B	1	1/2	32	32	32–256	12–96	0.25–1.5	0.09–0.25	0.5–1
C	–	2/0	32	32	128–192	24–256	0.19–0.38	0.03	0.19–0.25
D	–	6/0	32	32	96–256	6–256	0.75–3	0.03–0.12	0.12–0.25
E	3	7/0	32	32	32–256	256	0.38–6	0.19–1	12–256
E	–	2/0	32	32	256	256	0.5–4	0.5–3	256
F	2	7/0	32	32	12–256	256	2–6	0.38–1	2–6
G	–	2/0	32	32	128–256	256	1–1.5	0.38	1–4
H	–	2/2	32	32	32–256	24–256	0.5–1.5	0.38–6	2–256
I	1	3/8	32	32	12–256	16–256	1.5–4	0.38–6	2–256
J	1	1/5	32	32	8–256	12–256	1–4	0.06–12	0.25–256
Si 1,2	1	0/2	32	32	48–256	48–256	2–4	12–24	256
Si 3	–	1/0	32	32	256	256	0.75	0.19	6
Si 4	2	1/0	32	32	64	256	3	0.25	2
Si 5,8	–	1/1	32	32	16–32	256	0.5–1.5	3–4	256
Si 6	2	0/1	32	32	96	256	3	6	256
Si 7	–	1/0	32	32	64	256	1	3	256
Si 9	1	1/0	32	32	256	48	0.5	0.02	0.19

rep-PCR, repetitive sequence-based PCR (DiversiLab); SG, sequence group; B, burn isolates; NB, non-burn isolates; IPM, imipenem; MEM, meropenem; AMK, amikacin; GEN, gentamicin; TIG, tigecycline; MIN, minocycline; DOX, doxycycline; Si, singleton; MIC, minimum inhibitory concentration.

and six burn isolates, were resistant to tetracycline and showed minocycline MICs ranging from 2 mg/L to 24 mg/L and doxycycline MICs > 256 mg/L.

A total of 10 DiversiLab rep-PCR subtype clusters including two or more isolates were defined (herein named A–J), whereas nine isolates were classified as singletons (Table 1). Ten sequence types (STs), including ST2, ST25, ST85, ST94, ST136, ST307 and four novel sequence types (ST323, ST324, ST325 and ST328), were detected. Multiplex PCR attributed 29, 9 and 7 isolates, respectively, to European Clones (ECs) II, I and III (SGs 1, 2 and 3).

Resistance to tigecycline was observed most frequently in isolates belonging to SG 2 and SG 1, with eight and six resistant isolates, respectively. Moreover, isolates attributed to SG 1 showed the highest prevalence of resistance to minocycline and doxycycline, with 5 and 13 resistant isolates, respectively. As summarised in Table 1, the resistance determinants were most frequently observed among isolates belonging to SG 1.

In this study, most of the MDR-AB isolates (87%) were classified as tetracycline-resistant. In contrast, except for one isolate, the tetracycline-resistant isolates were found to be susceptible or intermediately susceptible to minocycline. Tigecycline was also active with 64% of isolates displaying a MIC ≤ 2 mg/L. This prevalence is slightly lower than the rate recently reported in a survey in Iran where ca. 80% of the isolates proved to be susceptible [4]. This could be due to a higher prevalence of non-susceptible isolates in burn patients in whom tigecycline is being used for *A. baumannii* infections.

The SG 1/EC II isolates showed the most heterogeneous pattern of genetic determinants and the highest resistance rates to tigecycline, minocycline and doxycycline. Clonality of non-susceptible isolates was further investigated using rep-PCR. Although minocycline-non-susceptible isolates were equally distributed between clusters and singletons, tigecycline-non-susceptible isolates mainly clustered into rep-PCR subtypes E and F, the two prominent clusters in burn isolates. This finding agrees with previous reports and supports the view that multiple MDR-AB epidemic strains can be selected by antibacterial use pressure in healthcare settings [5].

This study confirms the major role of the highly successful ECs I and II in the spread of MDR-AB strains in Iran. Striking genetic

versatility may allow EC II strains to develop resistance to nearly all clinically relevant agents. Tigecycline and minocycline may be still considered effective therapeutic options for MDR-AB infections. However, ongoing monitoring of *A. baumannii* susceptibility to these antibiotics is required.

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A novel GyrB mutation in meticillin-resistant *Staphylococcus aureus* (MRSA) confers a high level of resistance to third-generation quinolones

Sir,

Fluoroquinolones are widely used to treat respiratory and urinary tract infections. The development of first-generation quinolones began with nalidixic acid, and the new quinolone (fluoroquinolone) antimicrobial agents were developed [1].

Second-generation quinolones, such as ciprofloxacin, nadifloxacin, sparfloxacin and levofloxacin, unequally inhibit topoisomerase IV and DNA gyrase, and the primary target enzymes differ with each drug type [2]. The third-generation quinolones, such as pazufloxacin, moxifloxacin and sitafloxacin, have strong antimicrobial activity, especially against Gram-positive bacteria such as *Streptococcus pneumoniae*, because they target both topoisomerase IV and DNA gyrase [3]. Treatment with systemic-acting fluoroquinolones induces resistance in meticillin-resistant *Staphylococcus aureus* (MRSA), which are generally off-target bacteria [4]. High-level fluoroquinolone-resistant *S. aureus* emerge through processive mutations in the quinolone resistance-determining region (QRDR) of GrlA and GyrA. To investigate the mechanism of high-level resistance to third-generation quinolones, the amino acid mutations present in GrlA, GrlB, GyrA and GyrB were analysed.

A total of 189 MRSA isolates were collected from five Japanese hospitals between 1999 and 2005. All strains were isolated from non-respiratory departments. The minimum inhibitory concentration (MIC) was determined using the agar doubling dilution method according to Clinical and Laboratory Standards Institute (CLSI) guidelines. Pazufloxacin (Toyama Chemical Co., Ltd., Tokyo, Japan), moxifloxacin (Bayer Yakuhin, Ltd., Osaka, Japan) and sitafloxacin (Daiichi Sankyo Co., Ltd., Tokyo, Japan) were kindly provided by their manufacturers. Ciprofloxacin, sparfloxacin and levofloxacin were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). The amino acid sequences of GrlA, GrlB, GyrA and GyrB were determined by sequencing the relevant genes. Nadifloxacin (Otsuka Pharmaceutical Co., Ltd., Tokyo, Japan) was used to select for mutations in GyrA and/or GyrB because it primarily targets and binds to DNA gyrase. Mutant strains were constructed by the following method. Strains were grown for 16 h in tryptone soya broth (Oxoid Ltd., Basingstoke, UK). Cells (ca. 10^{10} CFU) were then spread on tryptone soya agar (Oxoid Ltd.) containing 128 μg/mL nadifloxacin. Strains that grew on the selective medium were considered high-level third-generation quinolone-resistant mutants.

Approximately one-third of the strains (34.4%) were moxifloxacin-resistant (MIC > 2 μg/mL). High-level moxifloxacin resistance (MIC ≥ 128 μg/mL) was found in 10 strains (5.3%). These data show that fluoroquinolone-resistant MRSA are disseminated on the non-targeting sites of third-generation quinolones. On the other hand, no high-level sitafloxacin-resistant strain (MIC ≥ 128 μg/mL) was found in the present study.

When the amino acid sequences of GrlA, GrlB, GyrA and GyrB were determined, two high-level moxifloxacin-resistant strains carrying the mutations S80Y + E84K in GrlA and S84L + E88R in GyrA were found (group D) (Table 1). This is the first time the E88R mutation in GyrA has been observed in *S. aureus*. Despite identical mutation locations, the MICs of pazufloxacin, moxifloxacin and sitafloxacin for the mutant group D strains were two- to fourfold higher than those of the mutant group C strains. Furthermore, eight high-level moxifloxacin-resistant strains carrying the mutations S80Y + E84K in GrlA, S84L + E88G in GyrA and E477D in GyrB were found (group E). The mutation E477D in GyrB is a novel amino acid substitution for *S. aureus*. The MICs of moxifloxacin and sitafloxacin for mutant group E were higher than those for mutant group C. The