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In vitro reduction of antibacterial activity of tigecycline against multidrug-resistant *Acinetobacter baumannii* with host stress hormone norepinephrine

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ABSTRACT

The host stress hormone norepinephrine (NE), also called noradrenaline, is reported to augment bacterial growth and pathogenicity, but few studies have focused on the effect of NE on the activity of antimicrobials. The aim of this study was to clarify whether NE affects antimicrobial activity against multidrug-resistant *Acinetobacter baumannii* (MDR-AB). Time–kill studies of tigecycline (TIG) and colistin (COL) against MDR-AB as well as assays for factors contributing to antibiotic resistance were performed using MDR-AB clinical strains both in the presence and absence of 10 μM NE. In addition, expression of three efflux pump genes (*adeB*, *adeJ* and *adeG*) in the presence and absence of NE was analysed by quantitative reverse transcription PCR. Viable bacterial cell counts in TIG-supplemented medium containing NE were significantly increased compared with those in medium without NE. In contrast, NE had little influence on viable bacterial cell counts in the presence of COL. NE-supplemented medium resulted in an ca. 2 log increase in growth and in bacterial cell numbers adhering on polyurethane, silicone and polyvinylchloride surfaces. Amounts of biofilm in the presence of NE were ca. 3-fold higher than without NE. Expression of the *adeG* gene was upregulated 4–6-fold in the presence of NE. In conclusion, NE augmented factors contributing to antibiotic resistance and markedly reduced the in vitro antibacterial activity of TIG against MDR-AB. These findings suggest that NE treatment may contribute to the failure of TIG therapy in patients with MDR-AB infections.

1. Introduction

Norepinephrine (NE), also called noradrenaline, produced by the sympathetic nervous system and the adrenal medulla in response to an acute threat, acts as a hormone and neurotransmitter in the human body. In clinical settings, NE is commonly prescribed as a vasoactive agent for stabilisation of circulatory dynamics; indeed, NE and dopamine have been reported to be used for up to 50% of patients in intensive care units (ICUs) [1]. Recently, there has been increasing evidence to suggest that some micro-organisms sense and respond to the host stress hormones NE and epinephrine. Previous studies have shown that NE enhances the growth of different pathogenic bacteria, including bacteria inhabiting the gastrointestinal tract such as *Escherichia coli*, *Salmonella*, *Helicobacter*, *Listeria*, *Campylobacter* and *Yersinia* spp. [2–4]. In addition to stimulation of bacterial growth, NE is reported to enhance the production of virulence factors, such as motility, production of toxins and acquisition of iron [5–7]. Regarding the interaction between antimicrobials and the host stress hormones, NE is reported to increase viable bacterial cell counts in tobramycin-treated *Pseudomonas aeruginosa* [7]. However, it is still unclear whether NE has an influence on the pharmacodynamic activity of antibiotics against other bacteria, including multidrug-resistant bacteria [2–7].

In the last few decades, multidrug-resistant *Acinetobacter baumannii* (MDR-AB) has become an increasingly prevalent nosocomial pathogen among critically ill patients admitted to ICUs [8]. In these patients, MDR-AB causes diseases such as bacteraemia, pneumonia, and urinary tract and wound infections. Patients with infections caused by MDR-AB are very difficult to treat because MDR-AB is resistant to most of the available antimicrobial agents owing to a variety of intrinsic and acquired resistant determinants, including β-lactamases, aminoglycoside-modifying enzymes, outer membrane proteins and active efflux systems [9,10]. Tigecycline (TIG) and colistin (COL) have been shown to have good in vitro activity against MDR-AB. Some previous studies have reported that TIG and COL therapy is effective in the treatment of infections caused by

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MDR-AB [11,12]. Therefore, TIG and COL are indispensable antimicrobials against infections caused by this bacterium [13,14].

Increased plasma NE concentrations are common in critically ill patients in ICUs because they often receive high doses of NE and are in a state of sympathicotonia due to the severity of their illness [15]. Consequently, MDR-AB gaining entry into critically ill patients may well be exposed to elevated plasma NE concentrations; TIG and COL administered to these patients may well be similarly exposed to elevated plasma NE concentrations. Therefore, whether NE affects the antibacterial activity of TIG and COL against MDR-AB, and whether this may become a significant clinical problem when treating critically ill patients with infections caused by MDR-AB with TIG and COL, remains unclear. To clarify the interaction between NE and the antibacterial activities of TIG and COL against MDR-AB, time–kill studies of TIG and COL against MDR-AB as well as assays for some factors contributing to antibiotic resistance, bacterial growth, adhesion and biofilm formation were performed, both in the presence and absence of NE, using MDR-AB strains isolated from patients in ICUs in Japanese hospitals.

2. Materials and methods

2.1. Strains and medium used in this study

Strains used in this study comprised 25 MDR-AB strains isolated from ICU patients at the time of three independent outbreaks in Japanese hospitals (Table 1) as well as the reference strain *A. baumannii* ATCC 17978. To determine the minimum inhibitory concentrations (MICs) of antimicrobial agents, antimicrobial susceptibility testing was performed by agar plate dilution methods with Mueller–Hinton agar in accordance with the method recommended by the Clinical and Laboratory Standards Institute (CLSI) [16]. Pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST) were employed to determine the clonal relatedness of all MDR-AB strains according to the manufacturer's instructions (Supplementary material).

In all assays, a serum–SAPI medium was used, which contained ca. 6.25 mM NH_4NO_3, 1.84 mM KH_2PO_4, 3.35 mM KCl, 1.01 mM $MgSO_4$ and 2.77 mM glucose (pH 7.50) supplemented with 30% (v/v) adult bovine serum [2]. This is a host-like serum-supplemented minimal medium and has been used in almost all previous studies on the interaction between NE and bacteria [2–7].

Table 1
Profile of multidrug-resistant *Acinetobacter baumannii* strains used in this study.

Strain	Sex	Patient age (years)	Specimen	Year of isolation	City in Japan where strain isolated
NUBL-1608	F	86	Pus	2009	Saitama
NUBL-1609	M	79	Pus	2009	Saitama
NUBL-1610	F	92	Sputum	2009	Saitama
NUBL-1611	F	86	Pus	2009	Saitama
NUBL-1612	F	93	Sputum	2009	Saitama
NUBL-1613	F	93	Sputum	2009	Saitama
NUBL-1614	M	54	Sputum	2009	Saitama
NUBL-1615	M	76	Sputum	2009	Saitama
NUBL-1616	M	61	Sputum	2009	Saitama
NUBL-1617	M	67	Sputum	2009	Saitama
NUBL-1618	F	86	Sputum	2009	Saitama
NUBL-1619	M	67	Sputum	2009	Saitama
NUBL-1620	M	85	Urine	2009	Saitama
NUBL-1621	F	86	Pus	2009	Saitama
NUBL-1622	M	67	Pus	2009	Saitama
NUBL-1623	F	91	Sputum	2009	Saitama
NUBL-1624	M	67	Sputum	2009	Saitama
NUBL-1625	M	58	Pus	2009	Saitama
NUBL-1626	M	87	Sputum	2010	Saitama
NUBL-1627	F	91	Urine	2010	Saitama
NUBL-1628	M	70	Sputum	2010	Tokyo
NUBL-11195	F	65	Sputum	2014	Nagoya
NUBL-11651	M	33	Sputum	2014	Mie
NUBL-11653	M	33	Sputum	2014	Mie
NUBL-13320	—	—	Environment	2014	Mie

2.2. Time–kill studies of tigecycline and colistin against multidrug-resistant A. baumannii

To examine whether NE can affect the pharmacodynamic activity of TIG and COL against MDR-AB, time–kill studies of TIG and COL were performed both in the presence and absence of NE as described previously with minor modifications [17]. To perform the time–kill studies in serum–SAPI medium, the MICs of TIG and COL were determined by the microdilution method with the serum–SAPI medium [16]. Flasks containing serum–SAPI medium and an antimicrobial drug were inoculated at a bacterial density of ca. 10^6 CFU/mL to a final volume of 10 mL and were incubated in a shaking incubator at 37 °C both in the presence and absence of 10 μM L-(−)-norepinephrine bitartrate (Sigma-Aldrich, St Louis, MO) (the concentration actually measured in patients following dopamine 3 μg/kg/min), using 1×, 2× and 4× MICs of TIG or COL measured by the microdilution method with serum–SAPI medium [15]. Bacterial growth was quantified at 1, 2, 4, 8 and 20 h post-incubation by plating 10-fold dilutions on Luria–Bertani (LB) agar [5 g/L Bacto™ yeast extract (Becton Dickinson & Co., Franklin Lakes, NJ), 10 g/L Bacto™ tryptone (Becton Dickinson & Co.), 5 g/L NaCl, supplemented with 15 g/L agar, adjusted to pH 7.0 with NaOH].

2.3. Growth assays in the presence and absence of norepinephrine

Bacterial growth was examined for 20 h both in the presence and absence of NE in the following manner. All strains were grown overnight at 37 °C in LB broth. Then, 1 mL samples were pelleted and were washed three times with phosphate-buffered saline (PBS) (pH 7.40) and were then re-suspended in PBS. These samples were inoculated at low cell density (10^2–10^3 CFU/mL) into serum–SAPI medium. The medium containing low bacterial density was incubated in the presence and absence of 10 μM L-(−)-norepinephrine bitartrate in a 5% CO_2 incubator at 37 °C. Bacterial cell numbers were enumerated by plating 10-fold dilutions on LB agar at every 2 h post-inoculation until reaching the 20-h growth period.

2.4. Bacterial adhesion to polyurethane, silicone and polyvinylchloride surfaces

To investigate whether NE affects MDR-AB adhesion to three abiotic surfaces (polyurethane, silicone and polyvinylchloride), bacterial cell numbers adhering to those materials both in the presence and absence of 10 μM NE at 24 h post-inoculation were examined as described previously with minor modifications [18,19]. Each strain was inoculated at a low cell density (10^2–10^3 CFU/mL) into sterile 10 mL flasks containing 5 mL of serum–SAPI medium and a 2-cm section of sterile non-coating polyurethane, silicone or polyvinylchloride tube (outside diameter 5 mm, inside diameter 4 mm). Each sample was incubated both in the presence and absence of 10 μM L-(−)-norepinephrine bitartrate for 24 h at 37 °C in a 5% CO_2 incubator. Following incubation, tube sections were washed five times in PBS (pH 7.40) at room temperature to remove planktonic and lightly adherent growth. Each tube section was transferred into a sterile 4.5 cm dish. Then, 1 mL of fresh PBS (pH 7.40) was added to a tube-containing dish and the inside and outside walls of the tube surfaces were vigorously scraped with a disposable cotton pestle. Dish contents were vortexed vigorously for 3 min and a uniform cell suspension was confirmed by microscopic observation. Bacterial cell numbers were enumerated by plating 10-fold dilutions on LB agar.

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2.5. Scanning electron microscopy (SEM)

Polyurethane, polyvinylchloride and silicone tube sections of 2 cm in length (outside diameter 5 mm, inside diameter 4 mm) incubated both in the presence and absence of 10 μM L-(−)-norepinephrine bitartrate with a low cell density (10^2–10^3 CFU/mL) of bacteria for 24 h were washed twice with PBS (pH 7.40) and were fixed with cold 1% glutaraldehyde in 0.1 M PBS (pH 7.00) for >3 h. Tubes were then fixed with 2% OsO_4 in 0.2 M PBS (pH 7.00) for 1 h and were dehydrated by increasing concentrations of ethanol. Samples were then sputter-coated with gold palladium and were observed by SEM (Model JSM-7610F; JEOL Ltd., Tokyo, Japan).

2.6. Biofilm formation assays

To investigate the ability of MDR-AB to form biofilm both in the presence and absence of NE, the relative amount of biofilm was measured using a biofilm formation assay in microtitre plates as described previously [20]. A 100 μL aliquot of serum–SAPI was added to each strain (10^4 CFU/mL) and was inoculated into each well of a 96-well flat-bottomed polystyrene plate both in the presence and absence of 10 μM L-(−)-norepinephrine bitartrate. The plate was incubated overnight at 37 °C in a 5% CO_2 incubator for 48 h to ensure similarity in growth rates between control and NE-added cultures. Following incubation, each well was washed and the biofilm was stained with 0.1% crystal violet solution. The biofilm-combined dye was solubilised with 200 μL of 95% ethanol and the optical density at 570 nm (OD_{570}) was measured using an iMark™ Microplate Reader (Bio-Rad Laboratories, Hercules, CA).

2.7. Quantitative reverse transcription PCR (qRT-PCR)

To disclose whether NE can affect the expression of efflux pump systems in MDR-AB, transcription of three efflux pump genes (*adeB*, *adeJ* and *adeG*) was analysed both in the presence and absence of NE by qRT-PCR. Bacterial cells were inoculated at ca. 1×10^5 CFU/mL into serum–SAPI medium in the presence and absence of 10 μM NE and were grown in a 5% CO_2 incubator at 37 °C for 10 h. The RNA of bacterial cells was stabilised using RNA Protect Bacteria Reagent (QIAGEN, Mississauga, ON, Canada) and RNA extractions were performed using an RNeasy Kit (QIAGEN). cDNA was synthesised from 1 μg of total RNA using ReverTra Ace® qPCR RT Master Mix with gDNA Remover (TOYOBO Co., Ltd. Japan). Expression levels of *adeB*, *adeG* and *adeJ* were analysed by qRT-PCR using a StepOnePlus™ Real-Time PCR System (Applied Biosystems Inc., Foster City, CA) with THUNDERBIRD® SYBR® qPCR Mix (TOYOBO Co., Ltd.). The primer sequences are shown in Table 2. The *rpoB* gene, encoding the RNA polymerase B subunit, was used as a control housekeeping gene. Expression was quantified relative to that of *rpoB*, and normalised expression of *adeB*, *adeJ* and *adeG* was calculated relative to *rpoB* using the $2^{-\Delta\Delta C(T)}$ method [22]. qRT-PCR was performed in triplicate using freshly prepared cDNA and was repeated independently three times.

Table 2
Primers used in this study.

Gene	Orientation	Sequence (5′→3′)	Reference
adeB	Forward	CGAGTGGCACAACTAGCATC	[9]
	Reverse	CCTTGTCTTGGCTGCACTCT	
adeJ	Forward	CCTATTGCACAATATCCAACGA	[9]
	Reverse	AGGATAAGTCGCAGCAATCG	
adeG	Forward	GTCCTGAAATGGTCGTTCGT	[9]
	Reverse	AGCTTCTGCTTGGCTAGATGA	
rpoB	Forward	CGTGTATCTGCGCTTGG	[9]
	Reverse	CGTACTTCGAAGCCTGCAC	

2.8. Statistical analysis

Each experiment was performed in triplicate and was repeated independently three times unless otherwise stated. Numerical data shown are expressed as the mean ± standard deviation. Unpaired *t*-tests were used to compare pairs of conditions. Statistical significance was indicated by a *P*-value of <0.05. All statistical analyses were performed using IBM SPSS Statistics for Windows v.23.0 (IBM Corp., Armonk, NY).

3. Results

3.1. Characteristics of multidrug-resistant A. baumannii strains used in this study

All MDR-AB isolates used in this study were non-susceptible to carbapenems, aminoglycosides and quinolones but were susceptible to TIG and COL (Table 3). PFGE analysis classified the 25 strains into three pulsotypes, named A [sequence type (ST) 2, international clone (IC)-II], B (ST215, non-IC-I, II) and C (ST1, IC-I) (Fig. 1). Strains NUBL-1608, NUBL-11651 and NUBL-11195 were selected as representative of each pulsotype in this study.

3.2. Effect of norepinephrine on multidrug-resistant A. baumannii survival in the presence of tigecycline

MICs of TIG and COL measured by the microdilution method using serum–SAPI medium are shown in Table 4. MICs were the same as those measured by agar plate dilution methods with Mueller–Hinton agar in accordance with the method recommended by the CLSI. Results of the time–kill studies of TIG are shown in Fig. 2. Antimicrobials were considered bactericidal when a >3 $\log_{10}$ decrease in CFU/mL was achieved compared with the initial inoculum. Only 4× MIC of TIG resulted in a bactericidal effect during the 20-h test period when the time–kill studies were performed in medium

Table 3
Antimicrobial susceptibilities of *Acinetobacter baumannii* strains used in this study.

Clinical isolate	MIC (mg/L)											
	CAZ	PIP	TZP	IPM	MER	AMK	GEN	LFX	CIP	RIF	COL	TIG
NUBL-1608	>64	>256	128	16	>8	>64	>64	>4	>4	≤4	0.5	0.25
NUBL-1609	>64	>256	128	16	>8	>64	>64	>4	>4	≤4	0.5	0.25
NUBL-1610	>64	>256	128	16	>8	>64	>64	>4	>4	≤4	0.5	0.25
NUBL-1611	>64	>256	128	16	>8	>64	>64	>4	>4	≤4	0.5	0.25
NUBL-1612	>64	>256	128	>16	>8	>64	>64	>4	>4	≤4	0.5	0.25
NUBL-1613	>64	>256	64	16	>8	>64	>64	>4	>4	≤4	0.5	0.25
NUBL-1614	>64	>256	128	16	>8	>64	>64	>4	>4	≤4	0.5	0.25
NUBL-1615	>64	>256	64	16	>8	>64	>64	>4	>4	≤4	0.5	0.25
NUBL-1616	>64	>256	128	>16	>8	>64	>64	>4	>4	≤4	0.5	0.25
NUBL-1617	>64	>256	128	16	>8	>64	>64	>4	>4	≤4	0.5	0.25
NUBL-1618	>64	256	128	16	>8	>64	>64	>4	>4	≤4	0.5	0.25
NUBL-1619	>64	>256	128	16	>8	>64	>64	>4	>4	≤4	0.5	0.25
NUBL-1620	>64	>256	128	16	>8	>64	>64	>4	>4	≤4	0.5	0.25
NUBL-1621	>64	>256	64	16	>8	>64	>64	>4	>4	≤4	0.5	0.25
NUBL-1622	>64	>256	64	16	>8	>64	>64	>4	>4	≤4	0.5	0.25
NUBL-1623	>64	256	64	16	>8	>64	>64	>4	>4	≤4	0.5	0.25
NUBL-1624	>64	>256	64	16	>8	>64	>64	>4	>4	≤4	0.5	0.25
NUBL-1625	>64	>256	128	16	>8	>64	>64	>4	>4	≤4	0.5	0.25
NUBL-1626	>64	256	64	>16	>8	>64	>64	>4	>4	≤4	0.5	0.25
NUBL-1627	>64	>256	128	16	>8	>64	>64	>4	>4	≤4	0.5	0.25
NUBL-1628	>64	>256	128	32	>8	>64	>64	>4	>4	≤4	0.5	0.25
NUBL-11651	>64	>256	64	>16	>8	>64	>64	>4	>4	≤4	0.25	0.25
NUBL-11653	>64	>256	64	>16	>8	>64	>64	>4	>4	≤4	0.25	0.25
NUBL-13320	>64	>256	64	>16	>8	>64	>64	>4	>4	≤4	0.25	0.25
NUBL-11195	>64	>256	128	>16	>8	>64	>64	>4	>4	≤4	0.5	0.5

MIC, minimum inhibitory concentration; CAZ, ceftazidime; PIP, piperacillin; TZP, piperacillin/tazobactam; IPM, imipenem; MER, meropenem; AMK, amikacin; GEN, gentamicin; LFX, levofloxacin; CIP, ciprofloxacin; RIF, rifampicin; COL, colistin; TIG, tigecycline.

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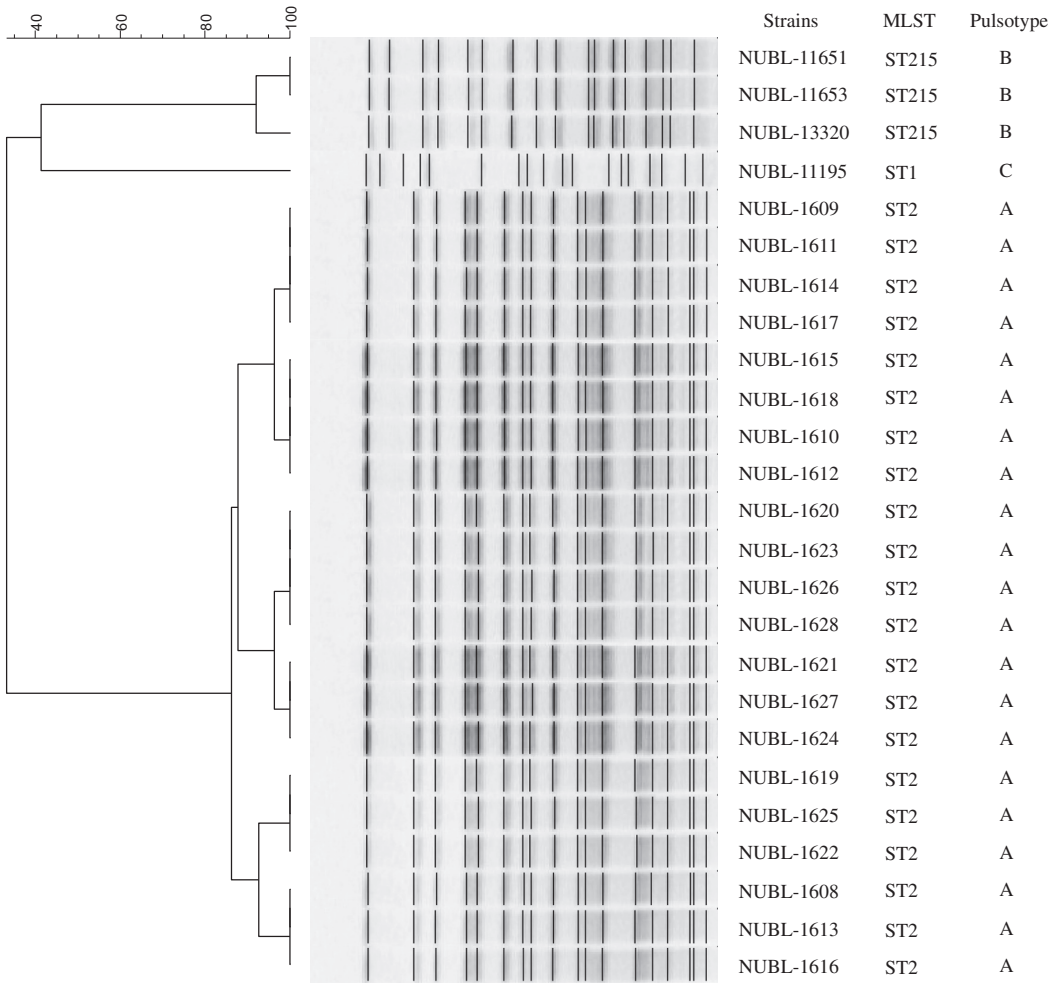

Fig. 1. Multilocus sequence typing (MLST) and pulsed-field gel electrophoresis (PFGE) pulsotypes in 25 multidrug-resistant *Acinetobacter baumannii* clinical strains used in this study.

without NE. At 1× and 2× MICs of TIG, viable bacterial cell counts of NUBL-1608 in the presence of NE were significantly higher than those in the absence of NE at 4 h post-inoculation, whilst those of NUBL-11651 and NUBL-11195 in the presence of NE were significantly increased at 8 h post-inoculation. Viable bacterial cell counts of *A. baumannii* ATCC 17978 in the presence of NE were significantly higher than those in the absence of NE only at 20 h post-inoculation. At 4× MIC of TIG, viable bacterial cell counts of all strains in the presence of NE were significantly higher than those in the absence of NE at 20 h post-inoculation. In particular, those of NUBL-1608 and NUBL-11195 in the presence of NE were already significantly increased at 8 h post-inoculation.

Table 4
Minimum inhibitory concentrations (MICs) of tigecycline (TIG) and colistin (COL) for three representative *Acinetobacter baumannii* strains as well as the reference strain *A. baumannii* ATCC 17978 measured by microdilution method using serum SAPI–medium.

Strain	MIC (mg/L)	
	TIG	COL
A. baumannii NUBL-1608	0.25	0.5
A. baumannii NUBL-11651	0.25	0.25
A. baumannii NUBL-11195	0.5	0.5
A. baumannii ATCC 17978	0.125	0.125
Escherichia coli ATCC 25922[a]	0.125	0.125

[a] Quality control strain.

3.3. *Effect of norepinephrine on multidrug-resistant* A. baumannii *survival in the presence of colistin*

Results of the time–kill studies with COL are shown in Fig. 3. In the medium without NE, 1× MIC of COL resulted in a bacteriostatic effect, whereas 2× and 4× MICs of COL resulted in a bactericidal effect. At 1× MIC of COL, viable bacterial cell counts of all strains in the presence of NE were slightly increased at 20 h post-inoculation compared with control cultures without NE, but with no statistical difference. On the other hand, at 2× and 4× MICs of COL, viable bacterial cell counts of all strains in the presence of NE were almost similar to those in the absence of NE at every time point.

3.4. *Effect of norepinephrine on multidrug-resistant* A. baumannii *growth*

The time course of growth both in the presence and absence of 10 μM NE is shown in Fig. 4. In strains NUBL-1608 and NUBL-11195, viable bacterial cell counts in the presence of NE were

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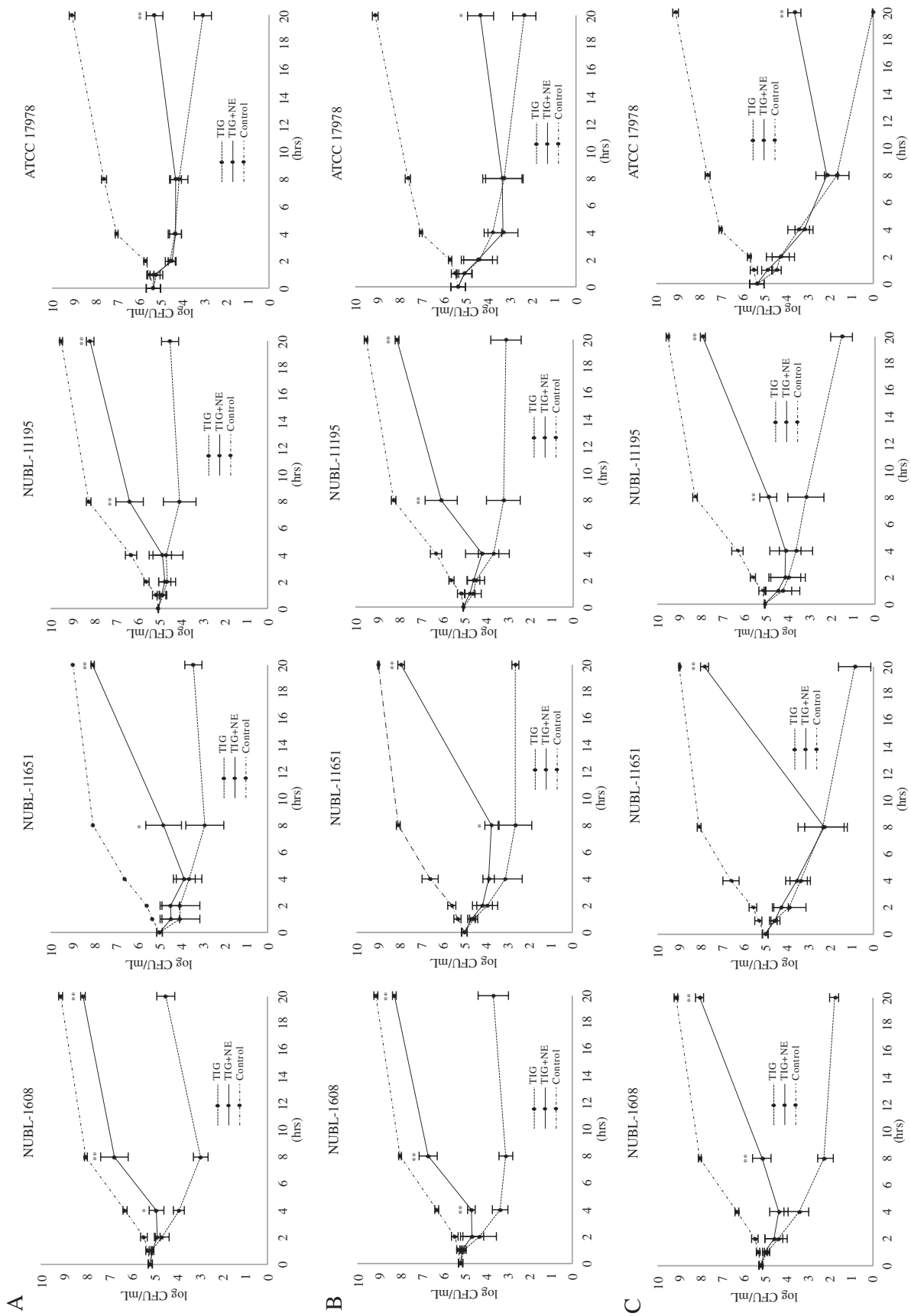

Fig. 2. Time–kill assay for tigecycline (TIG) at (A) 1× MIC, (B) 2× MIC and (C) 4× MIC with and without 10 μM norepinephrine (NE) against three representative *Acinetobacter baumannii* strains as well as the reference strain *A. baumannii* ATCC 17978. Data are the mean ± standard deviation of three separate analyses of triplicate assays. ***Statistically significant difference: $^{*}P < 0.05$; $^{**}P < 0.01$. MIC, minimum inhibitory concentration.

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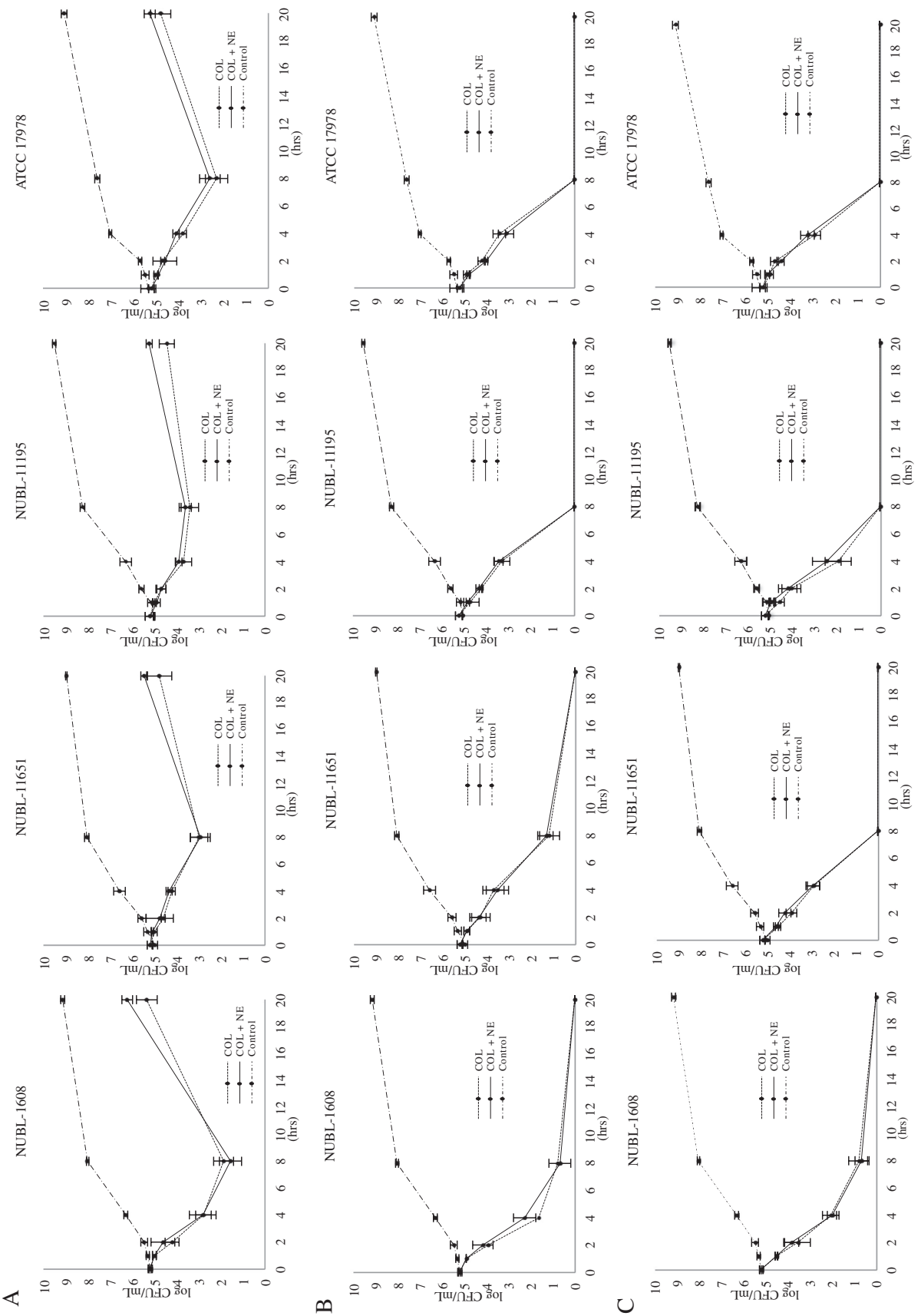

Fig. 3. Time–kill assay for colistin (COL) at (A) 1× MIC, (B) 2× MIC and (C) 4× MIC with and without 10 μM norepinephrine (NE) against three representative *Acinetobacter baumannii* strains as well as the reference strain *A. baumannii* ATCC 17978. Data are the mean ± standard deviation of three separate analyses of triplicate assays. MIC, minimum inhibitory concentration.

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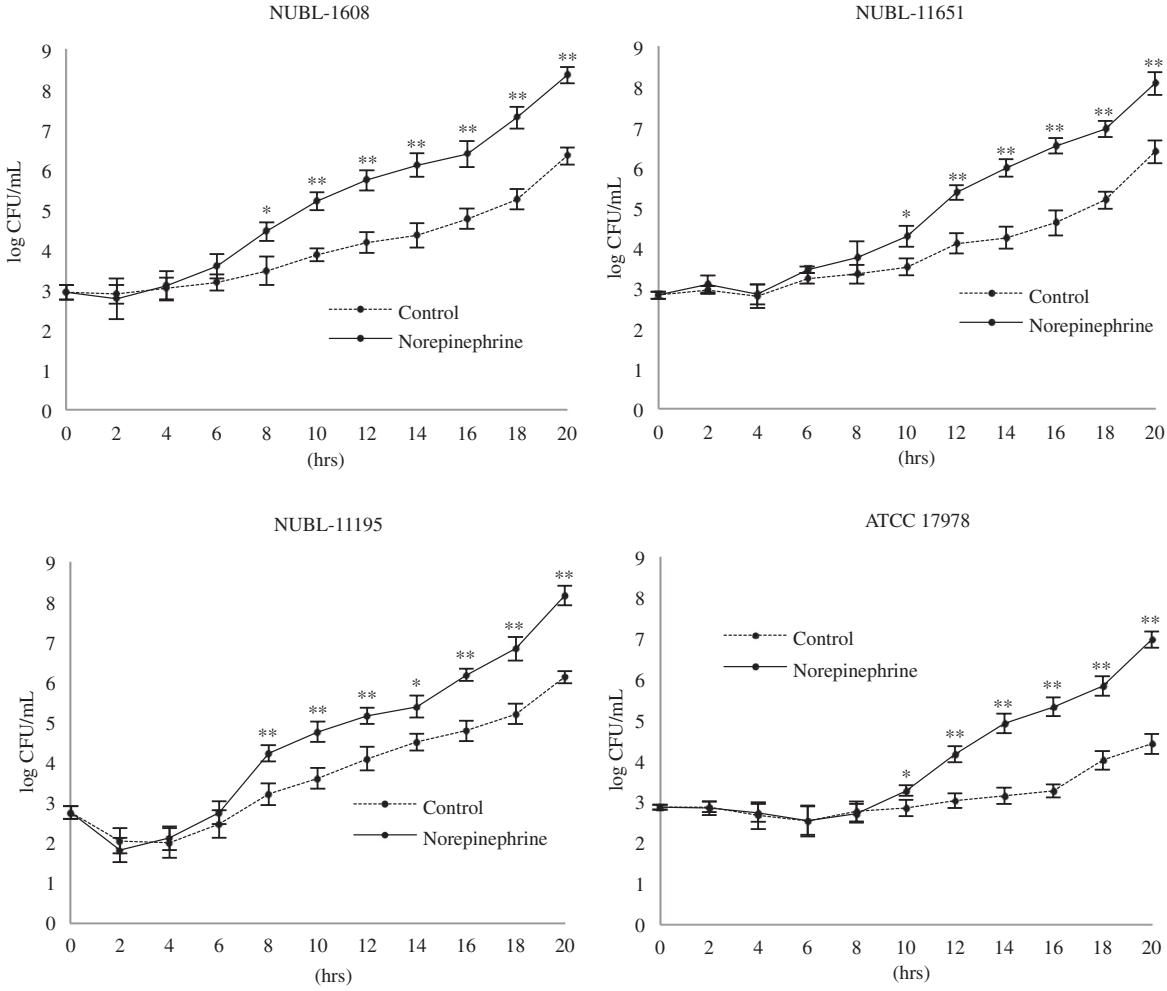

Fig. 4. Time course of growth of three representative *Acinetobacter baumannii* strains as well as the reference strain *A. baumannii* ATCC 17978 in the presence and absence of 10 μM norepinephrine. Viable bacterial cell count was performed in triplicate every 2 h post-inoculation. Data are the mean ± standard deviation log CFU/mL of three separate analyses of triplicate assays. *,**Statistically significant difference: $^{*}P < 0.05$; $^{**}P < 0.01$.

significantly higher than those in the absence of NE at 8 h post-inoculation. On the other hand, in strains NUBL-11651 and ATCC 17978, viable bacterial cell counts in the presence of NE were significantly higher than those in the absence of NE at 10 h post-inoculation. This result showed that NE took ≥8–10 h to exhibit a significant effect on the enhancement of bacterial growth at a low initial inoculum of MDR-AB.

3.5. *Effect of norepinephrine on multidrug-resistant* A. baumannii *adhesion on polyurethane, silicone and polyvinylchloride surfaces*

MDR-AB adhesion on polyurethane, silicone and polyvinylchloride surfaces was significantly increased by almost 2 log orders in NE-supplemented medium compared with control cultures without NE (Fig. 5). This effect of NE was not affected by differences in the chemical quality of tubes in all strains. In medium without NE, all strains exceedingly adhered to the silicone tube. Fig. 6 shows SEM images of NUBL-1608 seeded onto polyurethane, silicone and polyvinylchloride tubes and incubated in the presence or absence of NE. NE induced extensive biofilm production with large amounts of exopolysaccharide clearly visible (Fig. 6a–c), whereas control cultures without NE (Fig. 6d–f) showed little production of biofilm. A very similar NE-induced enhancement of biofilm production on each tube was observed in strains NUBL-11651, NUBL-11195 and ATCC 17978.

3.6. *Effect of norepinephrine on multidrug-resistant* A. baumannii *biofilm formation*

Results of the biofilm formation assay are shown in Fig. 7. In the absence of NE, all strains were weak biofilm producers. Each strain grown in the presence of NE produced an ca. 3–4-fold higher amount of biofilm than strains grown in medium without NE at 48 h post-inoculation. When NE was absent the OD_{570} value of NUBL-1608 was the highest, but biofilm formation of NUBL-11195 was most promoted by exposure to NE.

3.7. *Effect of norepinephrine on the transcription of efflux pump genes*

Levels of expression of the efflux pump genes in strains exposed to NE relative to those in strains not exposed to NE are shown in Fig. 8. For all strains, relative expression of the *adeG* gene in the presence of NE was significantly increased compared with that in the

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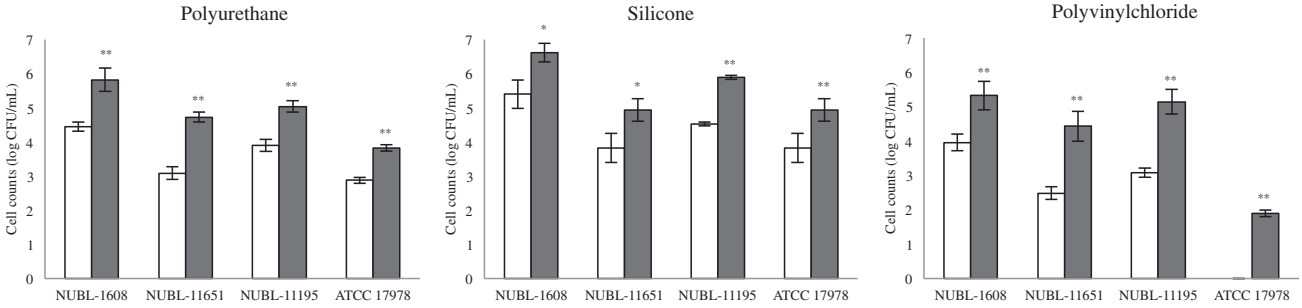

Fig. 5. Adhesion assays for three representative *Acinetobacter baumannii* strains as well as the reference strain *A. baumannii* ATCC 17978 in the presence (grey bars) and absence (white bars) of 10 μM norepinephrine. Results are presented on a log scale representing the mean calculated from raw CFU counts obtained from three separate analyses of triplicate assays. Error bars indicate the standard deviation. *,**Statistically significant difference: *$P < 0.05$; **$P < 0.01$.

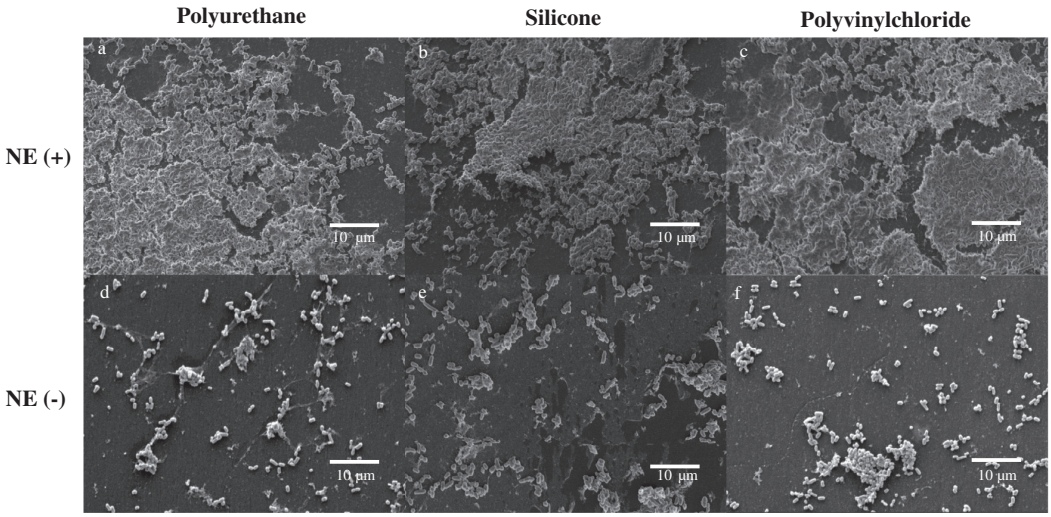

Fig. 6. Scanning electron microscopy images of multidrug-resistant *Acinetobacter baumannii* strain NUBL-1608 biofilm growth in (a–c) the presence and (d–f) the absence of norepinephrine (NE).

absence of NE, resulting in 6.08-fold (NUBL-1608), 4.51-fold (NUBL-11651), 5.32-fold (NUBL-11195) and 4.47-fold (ATCC 17978) changes in expression, respectively. On the other hand, the relative expressions of *adeB* and *adeJ* genes in the presence of NE were almost similar to those in the absence of NE.

4. Discussion

In the present study, NE markedly reduced the in vitro antibacterial activity of TIG against MDR-AB but had little influence on the activity of COL. In recent years, some studies have shown that patients undergoing TIG therapy for MDR-AB infections had higher mortality and treatment failure rates and lower microbial eradication rates than those treated with other appropriate antimicrobial agents such as COL [21,23–26]. In a matched cohort analysis by Chuang et al, patients treated with TIG-based therapy had an excess mortality of 16.7% compared with those who received COL-based therapy for the treatment of MDR-AB pneumonia [24]. Furthermore, in a systematic review and meta-analysis, Ni et al reported that there was no significant difference between TIG and other regimens in terms of all-cause mortality when treating MDR-AB infections, but subgroup analysis indicated that treatment with TIG was associated with higher in-hospital mortality [odds ratio (OR) = 1.57, 95% confidence interval (CI) 1.04–2.35; $P = 0.03$] and a

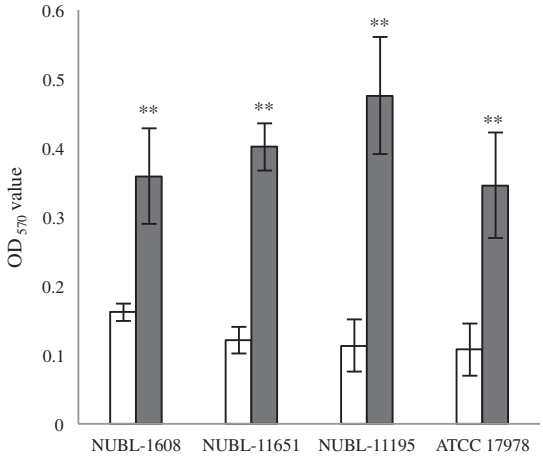

Fig. 7. Biofilm formation assay in the presence (grey bars) and absence (white bars) of 10 μM norepinephrine. Data are expressed as the mean ± standard deviation. **Statistically significant difference ($P < 0.01$). OD_{570}, optical density at 570 nm.

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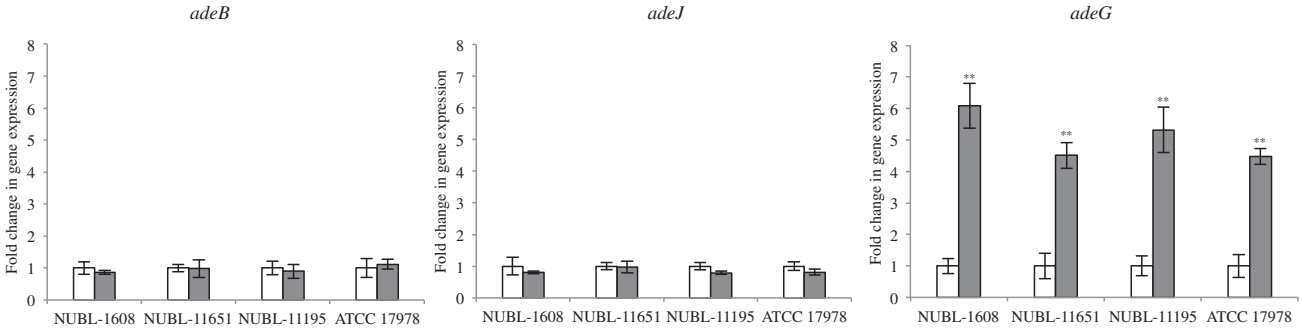

Fig. 8. Relative expression of the *adeB*, *adeJ* and *adeG* genes in the presence (grey bars) and absence (white bars) of 10 μM norepinephrine. Data shown are the mean ± standard deviation of three separate analyses of triplicate assays. **Statistically significant difference ($P < 0.01$).

lower microbial eradication rate (OR = 0.20, 95% CI 0.07–0.59; $P = 0.003$) [21]. This was reported to be due to increased superinfections, more adverse events and recent decreasing susceptibility in TIG-based therapy. Almost all of these studies were conducted using severely ill patients with high Acute Physiology and Chronic Health Evaluation (APACHE) II scores who received a catecholamine, including NE. Therefore, it is possible the NE-induced reduction of TIG activity against MDR-AB could contribute to poor outcomes in patients undergoing TIG therapy for MDR-AB infections.

Previous studies have shown that *A. baumannii* strains with high biofilm-forming capacity are selected under antibiotic pressure and acquire resistance to multiple antibiotics within biofilm communities [27–30]. In the current study, the microtitre plate assay showed that MDR-AB strains with low biofilm-forming capacity transformed to strong biofilm-producers in the presence of NE. Even so, whilst the time–kill assays demonstrated that NE significantly increased viable bacterial cell counts in the presence of TIG, there was only a slight effect in the presence of COL. One probable reason for this is that COL had a much stronger initial and short-term bactericidal activity against MDR-AB than TIG; therefore, most strains in the presence of COL were killed within 8 h of inoculation before NE fully showed an effect on MDR-AB. In fact, the growth assays demonstrated that NE required ≥8–10 h to enhance bacterial growth. Because NE induced high TIG resistance in MDR-AB, we additionally investigated whether NE affects expression of the efflux pump genes *adeB*, *adeJ* and *adeG*, which are reported to be associated with decreased susceptibility of MDR-AB to TIG [31–34]. NE upregulated expression of the *adeG* efflux pump gene. Therefore, in addition to enhanced bacterial growth and biofilm formation, overexpression of AdeFGH induced by NE may play a role in the induction of TIG resistance in MDR-AB.

Recently, multidrug efflux pump systems in some bacteria, such as AcrAB–TolC in *E. coli* and MexAB–OprM and MexCD–OprJ in *P. aeruginosa*, have been shown to have a role not only in drug excretion but also in biofilm formation [35,36]. In fact, some efflux pump inhibitors were shown to reduce bacterial biofilm formation [37]. For *A. baumannii*, He et al reported that biofilm formation was associated with overexpression of the AdeFGH efflux pump [38]. In the present study, we found that NE could upregulate expression of the *adeG* gene and increase MDR-AB biofilm formation. Therefore, upregulation of expression of the AdeFGH efflux pump induced by NE stimulation may play a role not only in the decreased antibacterial activity of TIG against MDR-AB but also in increasing biofilm production, which may increase the in vivo TIG resistance level of MDR-AB.

Two well-known pathogenic mechanisms in device-related hospital infections are bacterial adhesion and biofilm formation [39,40]. In the present study, we found that NE had the ability to augment bacterial adhesion and biofilm formation of MDR-AB on polyurethane, silicone and polyvinylchloride surfaces. Intravascular indwelling catheters are currently made of polyurethane, silicone or polyvinylchloride and are routinely used in critically ill patients. Long-term use of these catheters makes patients vulnerable to microbial colonisation. In clinical settings, we use 4 mg of NE, which is added to 1000 mL of solvent (ca. 23.6 μM) as per the recommendations of the US Food and Drug Administration (FDA), and a higher level of NE is administered through the catheters as needed. Consequently, MDR-AB migrating to and inhabiting inside or very close to a catheter used in patients receiving NE are directly exposed to >10 μM NE. The first step of catheter-related bloodstream infection (CR-BSI) is considered to be bacterial adhesion and biofilm formation on the catheter, which is a significant source of bacterial inoculation leading to CR-BSI. Thus, patients receiving a high level of NE are likely in a condition that allows MDR-AB-related CR-BSIs to develop more easily when MDR-AB colonises their catheters.

This study has several limitations. First, it is not clear whether the responsiveness to NE is generalised among different types of MDR-AB clones because only two epidemic clones and one non-epidemic clone of MDR-AB were investigated. Second, we did not demonstrate our findings in vivo using animal models. This limitation will be the subject of future research in our laboratory.

5. Conclusions

NE augmented the factors relevant to antibiotic resistance and markedly reduced the in vitro antibacterial activity of TIG against MDR-AB, but had only a slight influence on the activity of COL.

These findings suggest that NE treatment may contribute to the failure of TIG therapy in patients with infections caused by MDR-AB.

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Competing interests: None declared.

Ethical approval: Not required.

Appendix. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ijantimicag.2016.09.022.

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