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Capacity of multidrug-resistant clinical isolates of *Acinetobacter baumannii* to form biofilm and adhere to epithelial cell surfaces

H.-W. Lee[1], Y. M. Koh[1], J. Kim[2], J.-C. Lee[1], Y.-C. Lee[1], S.-Y. Seol[1], D.-T. Cho[1] and J. Kim[1]

[1]Department of Microbiology, Kyungpook National University, School of Medicine, Daegu and [2]Gangwan Province Minjok Leadership Academy, Korea

ABSTRACT

This study evaluated the capacity of 23 multidrug-resistant (MDR) clinical isolates of *Acinetobacter baumannii* to adhere to respiratory epithelial cell surfaces and to form biofilm on a polystyrene surface. All 23 *A. baumannii* isolates were capable of adhering efficiently to respiratory epithelial cells, and biofilm production was positively associated with epithelial cell adhesiveness (r 0.80, $p < 0.0001$). In the presence of the chelating agent EDTA, biofilm formation was markedly reduced. Cell adhesiveness and biofilm formation were significantly higher in isolates carrying the bla_{PER-1} gene as compared with isolates without this extended-spectrum β-lactamase gene ($p < 0.005$ and $p < 0.001$, respectively). Further examination by RT-PCR showed a positive correlation between the level of expression of the bla_{PER-1} gene and the level of biofilm formation (r 0.89, $p < 0.0001$) and cell adhesiveness (r 0.74, $p < 0.006$). Overall, the study demonstrated a high capacity of clinical isolates of MDR *A. baumannii* to form biofilm and to adhere to respiratory epithelial cells. This feature, combined with multidrug resistance, might contribute to the survival of these organisms and their dissemination in the hospital environment.

Keywords *Acinetobacter baumannii*, adherence, biofilm formation on epithelial cells, multidrug resistance, PER-1 β-lactamase

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INTRODUCTION

Acinetobacter baumannii is an important nosocomial pathogen that causes a range of infections, including respiratory and urinary tract infections, meningitis, endocarditis, wound infections and bacteraemia, especially in intensive care unit patients [1–3]. In the past, these organisms were frequently ignored when isolated from clinical samples, but significant improvements in resuscitation techniques have changed the types of infection caused by *Acinetobacter*, which is now a serious problem among patients in intensive care units [1–3]. In addition, interest in *A. baumannii* has been growing rapidly because of the emergence of multidrug-resistant (MDR) strains of this species, some of which are pan-resistant to antimicrobial agents [4–7].

MDR strains of *A. baumannii* are notorious for their ability to spread among hospitalised patients, and outbreaks caused by particular strains of *A. baumannii* have been reported with increasing frequency worldwide [4,5,7–11]. Risk-factors identified for the isolation of MDR *A. baumannii* include the severity of a patient's condition, the use of invasive devices, and the use of broad-spectrum antibiotics, all of which promote MDR *A. baumannii* colonisation, growth and invasiveness [1,3,12]. However, the ability of MDR *A. baumannii* strains to disseminate widely seems to depend on the expression of virulence factors that enable bacterial colonisation as well as on the expression of antibiotic resistance.

Adherence of bacteria to epithelial cells is considered to be an essential first step in colonisation and subsequent infection of a host [13]. In addition, biofilm formation is thought to be an important pathogenic feature, especially in relation to intravascular line infections and

Corresponding author and reprint requests: J. Kim, Department of Microbiology, School of Medicine, Kyungpook National University, 101 Dongin-2Ga, Junggu, Daegu, 700-422, Korea
E-mail: minkim@knu.ac.kr

ventilator-associated pneumonia. Biofilm is a microbially derived sessile community which is characterised by cells that are irreversibly attached to a substratum or interface with each other, and that are embedded in a matrix of self-produced extracellular polymeric substances [14]. Organisms that cause many device-related and other chronic infections grow in biofilms in or on these devices, and such infections are extremely difficult to eradicate because of the resistance of biofilm to numerous antimicrobial agents and products of the immune system [14,15].

The present study used a bacterial adhesion assay and a biofilm formation assay to investigate the capacity of MDR clinical isolates of *A. baumannii* to adhere to respiratory epithelial cell surfaces and to form biofilm on a polystyrene surface. These properties were compared in isolates with and without the bla_{PER-1} gene, which encodes the PER-1 extended-spectrum β-lactamase and is prevalent among cefepime-resistant *A baumannii* [7,8,10,16]. In addition, the study investigated whether the biofilms formed could be inhibited by a chelating agent, i.e., EDTA, which has the ability to bind cations that may be required to stabilise the negatively charged molecules of the extracellular polymeric substances.

MATERIALS AND METHODS

Bacteria

Twenty-three clinical isolates of *A. baumannii* (12 isolates carrying the bla_{PER-1} gene and 11 isolates without bla_{PER-1}) were selected from among 40 MDR clinical isolates of *A. baumannii* that had been characterised previously in terms of their antimicrobial susceptibility, antimicrobial resistance genes and genotype [16]. These isolates were originally recovered from patients hospitalised in a university hospital in Korea between August 2004 and March 2005. The type strain of *A. baumannii* (ATCC 19606) was used for comparisons in cell adhesion and biofilm assays. *Escherichia coli* strain HB101 was used as a negative control in biofilm assays.

Bacterial adherence assay

Cells of the human bronchial epithelial cell line NCI-H292 (ATCC CRL-1848; American Tissue Culture Collection, Rockville, MD, USA) were cultured at 37°C in RPMI 1640 medium (Gibco BRL, Grand Island, NY, USA), supplemented with 25 mM HEPES, 2 mM L-glutamine, penicillin G 100 000 U/L, streptomycin 50 mg/L and fetal bovine serum (Gibco BRL) 10% v/v, in a humidified atmosphere containing CO_2 5% v/v.

NCI-H292 cells were prepared by seeding 2×10^5 cells on 13-mm-diameter plastic coverslips (Thermanox; Nunc, Rochester, NY, USA), which were then placed in four-well plates containing RPMI 1640 medium. The cells were incubated at 37°C for 16–24 h and were then washed with phosphate-buffered saline (PBS). Bacteria grown on blood agar plates at 35°C were suspended in RPMI 1640 medium at a density of *c.* 4×10^7 CFU/0.5 mL. Each cell monolayer was infected with 0.5 mL of bacterial suspension and incubated for 60 min at 37°C in a CO_2 5% v/v atmosphere. The non-adherent bacteria were then removed by washing three times with PBS. The bacteria that had adhered to NCI-H292 cells were fixed in methanol 100% for 20 min and stained for 30 min in a Giemsa staining solution. The coverslips were air-dried, mounted and viewed under a light microscope at a magnification of ×400. The number of bacteria adhering to 100 cells was determined. Three independent experiments were performed for each isolate.

Adherence to polystyrene surface and measurement of biofilm

Bacterial adherence to polystyrene was determined essentially as described by Rosenberg [17]. A polystyrene disk was pressed on the surface of a blood agar plate on which spots of bacterial isolates had been grown at 37°C for 24 h. The polystyrene disk was then washed extensively with tap water and stained with gentian violet. *E. coli* strain HB101 was used as a negative control.

The relative amount of biofilm formed by the *A. baumannii* isolates was measured using a biofilm formation assay in microtitre plates, based on procedures outlined by Heilman *et al.* [18]. In brief, overnight cultures were adjusted to an OD_{600} of 2.0 and diluted 200-fold in Luria–Bertoni medium. Aliquots (200 μL) of bacterial suspension were then inoculated into each well of a 96-well flat-bottomed polystyrene plate and incubated overnight at 37°C. The wells were then washed twice with 200 μL of PBS, air-dried, and stained with gentian violet 0.1% v/v for 15 min. The stained biofilms were then solubilised with ethanol 95% v/v for 5 min, and the biofilm cell-associated dye was measured at OD_{570} using an ELISA reader (VERSA max, Sunnyvale, CA, USA). Each experiment was performed in triplicate and repeated three times.

Inhibition of biofilm formation by EDTA

The inhibitory effect of EDTA on biofilm formation was evaluated using *A. baumannii* isolate 1656-2, which formed the highest amount of biofilm (see Results). A 200-μL aliquot of *A. baumannii* suspension was inoculated into each well of a flat-bottomed 96-well polystyrene plate and was incubated overnight at 37°C in the presence or absence of EDTA 125 mg/L, after which the amount of biofilm produced was measured as described above.

Scanning electron-microscopy

An *A. baumannii* suspension (500 μL) was inoculated into each well (containing a plastic coverslip) of four-well polystyrene plates and then incubated overnight at 37°C, with or without EDTA treatment (125 mg/L). Biofilms that formed on the plastic coverslips were then washed twice with 1 mL of PBS and fixed with paraformaldehyde 4% v/v in 0.1 M phosphate buffer (pH 7.4) for 30 min. The biofilms on the coverslips were sequentially dehydrated for 5 min in ethanol 50%, 70%, 90% and 100% v/v, and then fixed to aluminium stubs using carbon tape. The fixed biofilms were

then coated with a layer of gold (7 nm thick) and examined with an S-4300 scanning electron-microscope (Hitachi, Tokyo, Japan) at 15 kV.

RT-PCR analysis

Total RNA was isolated from each isolate using an Invisorb Spin Cell RNA Mini Kit (Invitek, Berlin, Germany). First-strand cDNA was obtained using a reverse transcription system (Gibco BRL) with 2 μg of total RNA, and the resulting cDNA was then used as a template for PCR using primers specific for the bla_{PER-1} gene (sense, 5′-ATGAATG-TCATTATAAAAGC; antisense, 5′-AATTTGGGCTTAGGG-CAAGAAA) and 16S rRNA (sense, 5′-TGGCTCAGATT-GAACGCTGGCGGC; antisense, 5′-TACCTTGTTACGA CTTCACCCCA). PCR amplification comprised 22 cycles of 94°C for 60 s, 50°C for 60 s and 72°C for 60 s, after which the amplified products were analysed by conventional agarose gel electrophoresis. Band intensities of the PCR products were measured using an image analysis program (MetaMorph; Universal Imaging Corp., Downingtown, PA, USA), with data expressed as ratios of each mRNA normalised to 16S rRNA amplified from the same cDNA sample.

Statistical methods

The software package SPSS 12.0K for Windows (SPSS Inc., Chicago, IL, USA) was used for statistical analyses. Student's *t*-test and Wilcoxon's rank sum test were used to compare clinical isolates with or without bla_{PER-1}. Correlations (cell adherence and biofilm formation; bla_{PER-1} mRNA expression level and cell adherence/biofilm formation) were evaluated using Pearson's correlation coefficient and Spearman's rank correlation. Data were expressed as mean values ± SD, with $p < 0.05$ considered to be statistically significant.

RESULTS

In the cell adherence assay, the total number of adhered bacteria/100 cells ranged between 29 ± 8 and 2077 ± 890 for the 23 clinical isolates of *A. baumannii*, and was 18 ± 5 for the type strain ATCC 19606 (Table 1). Among the isolates carrying bla_{PER-1}, two (1169 and 1656-2) showed *c.* 100-fold higher adherence than the ATCC 19606 type strain. The other ten isolates carrying bla_{PER-1} showed higher adherence (97.5 ± 59) than the 11 isolates without bla_{PER-1} (45.6 ± 10; Student's *t*-test, $p < 0.005$).

In a polystyrene adherence test, all 23 *A. baumannii* isolates and the *A. baumannii* type strain (ATCC 19606) were able to attach to a polystyrene disk, suggesting the presence of biofilm-forming ability. In a subsequent biofilm formation assay in microtitre plates, OD_{570} values (mean ± SD) for the 23 *A. baumannii* MDR isolates ranged between 1.14 ± 0.42 and 3.98 ± 0.13, as compared with 0.64 ± 0.11 for the type strain (Fig. 1). As with the cell adhesion assay, isolates carrying the bla_{PER-1} gene produced more biofilm than isolates without bla_{PER-1}. The mean OD_{570} value (2.4 ± 0.6) of the 12 isolates carrying bla_{PER-1} was significantly higher than that of the 11 isolates without bla_{PER-1} (1.6 ± 0.3; Student's *t*-test, $p < 0.001$; Wilcoxon's two-sample test, $p < 0.001$).

Table 1. Adherence of clinical isolates of *Acinetobacter baumannii* to respiratory epithelial cells[a]

Isolates without bla_{PER-1}			Isolates with bla_{PER-1}		
Strains	Specimen	No. of bacteria/100 cells	Strains	Specimen	No. of bacteria/100 cells
ATCC 19606	Type strain	18 ± 5	335N6	Open pus	181 ± 64
5075	Blood	40 ± 5	667	Sputum	177 ± 55
1322	Sputum	29 ± 8	870	Pleural fluid	90 ± 20
375	Sputum	66 ± 15	1169	Sputum	1767 ± 550
502	Urine	44 ± 17	1656-2	Sputum	2077 ± 890
450	Urine	41 ± 12	1007	Sputum	99 ± 21
439	Urine	51 ± 18	489	Open pus	60 ± 15
671	Sputum	41 ± 10	690	Sputum	45 ± 11
263	Sputum	54 ± 19	287	Sputum	55 ± 14
298	Urine	55 ± 21	872	Open pus	178 ± 38
831	Urine	39 ± 14	726	Sputum	40 ± 15
913	Sputum	42 ± 16	609N6	Open pus	50 ± 17

[a]The number of bacteria adherent to 100 NCI-H292 cells was determined. Each experiment was performed in duplicate and repeated on three occasions. Data are expressed as means ± SD.

Fig. 2 shows the level of expression of bla_{PER-1} in the isolates that carried this gene. The levels of PER-1 mRNA were expressed as ratios of PER-1 mRNA normalised to 16S rRNA, with the relationships between the levels of PER-1 expression and biofilm formation/cellular adhesiveness being evaluated using Pearson's correlation coefficient. There were significant correlations between the level of PER-1 expression and biofilm formation ($n = 12$, r 0.89, $p < 0.001$), and between the level of PER-1 expression and cellular adhesiveness ($n = 12$, r 0.74, p 0.006).

The two isolates that showed the greatest epithelial cell adhesiveness (isolates 1169 and 1656-2) also produced large amounts of biofilm, suggesting a positive association between biofilm-forming capacity and cell adhesiveness. When the correlation coefficient between these two variables was calculated for all 24 isolates of *A. baumannii*, there was a highly significant correlation between biofilm-forming capacity and cell adhesiveness ($n = 24$, r 0.77, $p < 0.0001$ with Pearson's correlation coefficient; $n = 24$, r 0.80, $p < 0.0001$ with Spearman's rank correlation).

Finally, biofilm formation by *A. baumannii* isolate 1656-2, which formed the highest amount of biofilm, was markedly reduced (55–65%) in the

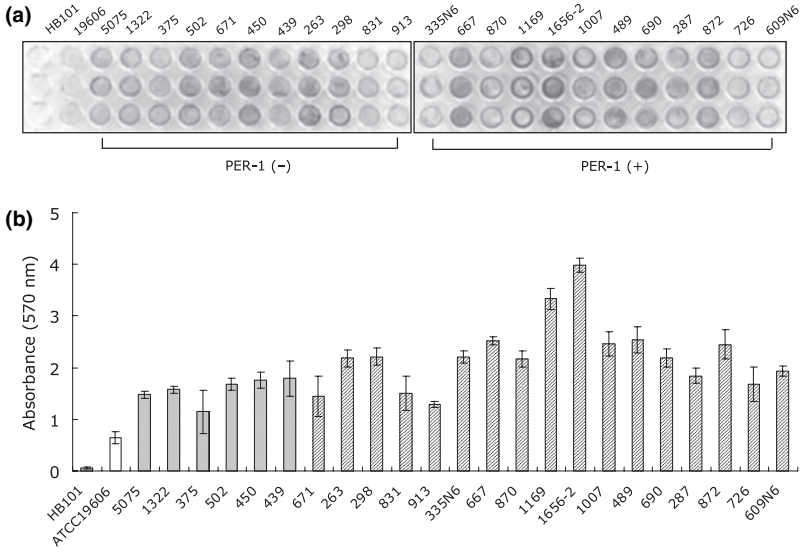

Fig. 1. Biofilm formation assay results for clinical isolates of *Acinetobacter baumannii*. (a) Biofilms stained with gentian violet 0.1% v/v for 15 min. (b) To measure the relative amount of biofilm, the gentian violet-stained biofilms were solubilised with ethanol 95% v/v for 5 min, and biofilm cell-associated dye was measured at OD_{570} using an ELISA reader. Each experiment was performed in triplicate and repeated on three occasions. HB101 is the *Escherichia coli* control strain; ATCC 19606 is the *A. baumannii* type strain.

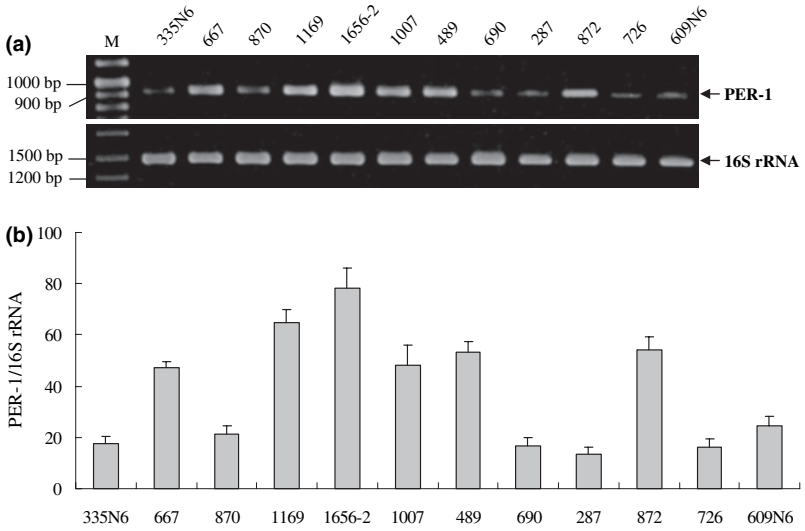

Fig. 2. Comparative RT-PCR analysis of bla_{PER-1} gene expression by clinical isolates of *Acinetobacter baumannii*. (a) Agarose gel showing representative RT-PCR results for the bla_{PER-1} gene and 16S rRNA. (b) The density of each band in each lane was quantified by scanning densitometry and then expressed as means ± SD. Data are expressed as ratios of PER-1 mRNA normalised to 16S rRNA. M, DNA size marker.

presence of EDTA. Subsequent analysis by scanning electron-microscopy revealed a reduction in the number of colonising bacterial cells and in the amount of biofilm produced in the presence of EDTA (Fig. 3).

DISCUSSION

A few previous reports have described the ability of clinical isolates of *A. baumannii* to attach to and form biofilms on glass surfaces [9,19,20]. Vidal *et al*. [9] found that an *A. baumannii* isolate from a respiratory tract infection formed biofilm on the surface of coverslips, and that this comprised an amorphous material similar to exopolysaccharide. Tomaras *et al*. [19] demonstrated that *A. baumannii* ATCC 19606 adhered to and formed biofilm on plastic and glass surfaces, and that disruption of the *csuC* and *csuE* open reading frames resulted in non-piliated cells, and the abolishment of cell attachment and biofilm formation. More recently, Siroy *et al*. [20] demonstrated that a clinical MDR isolate formed significant amounts of biofilm, and that this correlated with the accumulation of certain outer-membrane proteins, e.g., NlpE and CsuD, that have been described previously as being involved in bacterial adhesion. In *E. coli*, NlpE is a sensing molecule that generates an adhesion-specific signal to a two-component system that is involved in surface sensing and biofilm formation [21,22]. Overall, these previous observations agree with the main finding in the

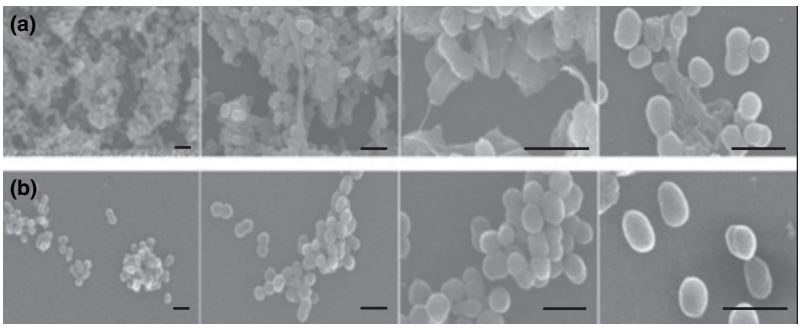

Fig. 3. Inhibition of biofilm formation by EDTA in a clinical strain of *Acinetobacter baumannii*. The morphology of biofilms formed by *A. baumannii* strain 1656-2 in (a) the absence or (b) the presence of EDTA was viewed under a scanning electron-microscope at ×5000, ×10 000, ×20 000 and ×25 000 magnification. The scale magnification bar indicates 2 μm.

present study that MDR clinical isolates of *A. baumannii* have the ability to form large amounts of biofilm, and that this ability shows a significant correlation with epithelial cell adherence.

Acquisition of the ability to form biofilm could be a good strategy to enhance a microorganism's survival under stressed conditions, e.g., during host invasion or following antibiotic treatment. This is because cells growing in biofilms are highly resistant to the components of the human immune system and to numerous types of antimicrobial agent. In addition, the ability of bacterial cells to transfer genes horizontally is enhanced within biofilm communities, thereby facilitating the spread of antibiotic resistance [1,15]. Thus, *A. baumannii* strains capable of forming biofilm might be selected under antibiotic pressure, or conversely, *A. baumannii* might acquire resistance to multiple drugs within biofilm communities. In either event, the high colonising capacity of *A. baumannii*, combined with its resistance to multiple drugs, will contribute to the organism's survival and further dissemination in the hospital setting.

An interesting finding of the current study was that *A. baumannii* isolates carrying $bla_{\text{PER-1}}$ showed a significantly higher capacity for epithelial cell adherence and biofilm formation when compared with *A. baumannii* isolates without $bla_{\text{PER-1}}$. Although the 12 isolates carrying the $bla_{\text{PER-1}}$ gene were clonally related [16], the levels of expression of $bla_{\text{PER-1}}$ varied among these isolates, and showed a significant correlation with the capacity of each isolate to form biofilm and to adhere to epithelial cells. Similarly, Sechi *et al.* [23] showed that PER-1 production in *A. baumannii* was related to cell adhesion. Vahaboglu *et al.* [7] found that PER-1 production was an independent indicator of poor prognosis in a cohort study that compared the clinical outcomes of patients in an intensive care unit who were infected with *Acinetobacter* strains resistant to third-generation cephalosporins. Cells growing in biofilms are known to be highly resistant to components of the human immune system and to numerous types of antimicrobial agents. The high capacity for biofilm formation shown by *A. baumannii* isolates carrying $bla_{\text{PER-1}}$ could therefore be a possible explanation for the findings of Vahaboglu *et al.* [7]. Further studies should focus on the clinical outcomes of patients infected with PER-1-producing *A. baumannii* strains with a high capacity for biofilm formation.

The metal chelator EDTA is known to have activity against biofilms associated with bacteria such as *Staphylococcus* spp., *Candida* spp. and *Pseudomonas aeruginosa* [24–26]. Raad *et al.* [25] showed that a flush solution consisting of minocycline–EDTA was highly efficacious in preventing catheter-related colonisation, bacteraemia and endocarditis in rabbits, and that this flush solution was also highly effective in reducing colonisation with *Staphylococcus epidermidis*, *Staphylococcus aureus* and *Candida albicans* cells embedded in fresh and mature biofilm adhering to catheter surfaces [26]. In the present study, treatment with EDTA caused a 55–65% reduction in biofilm formation. This inhibitory effect was not observed following treatment with trypsin (data not shown), and it was therefore concluded that the major constituent of biofilms formed by *A. baumannii* is polysaccharide. A reduction in the number of colonising bacterial cells, as well as biofilm formation, in the presence of EDTA was revealed by scanning electron-microscopy. EDTA had no effect on the planktonic growth of bacteria (data not shown), so the reduction in the number of colonising bacterial cells in the presence of EDTA seems to be a result of the detachment of biofilm cells, as opposed to the inhibition of

bacterial growth. The efficient reduction in biofilm formation by *A. baumannii* in the presence of EDTA suggested that the flushing strategy employed in previous studies [24–26] could also be applicable for *A. baumannii*.

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