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Effects of erythromycin on *Pseudomonas aeruginosa* adherence to collagen and morphology *in vitro*

K.W. Tsang*, P. Ng*, P.L. Ho#, S. Chan*, G. Tipoe¶, R. Leung*, J. Sun*, J.C. Ho*, M.S. Ip*, W.K. Lam*

Effects of erythromycin on Pseudomonas aeruginosa *adherence to collagen and morphology* in vitro. *K.W. Tsang, P. Ng, P.L. Ho, S. Chan, G. Tipoe, R. Leung, J. Sun, J.C. Ho, M.S. Ip, W.K. Lam. ©ERS Journals Ltd 2003.*

ABSTRACT: The airways of patients with bronchiectasis and cystic fibrosis are often chronically colonised by *Pseudomonas aeruginosa* (PA), which is virtually impossible to eradicate. Low-dose erythromycin (EM), for unknown mechanisms, is efficacious in bronchiectasis and diffuse panbronchiolitis.

In this study, an *in vitro* model to investigate PA adherence to human type IV basement collagen was developed by using scanning electron microscopy (SEM). There were significantly less PA bacilli per 20 random SEM fields (4,000×) when PA was cultured in 0.05, 0.5 and 5 μg·mL^{-1} of EM compared with control (absence of EM). Adherence density (20 SEM fields·log^{-1} inocular size) for PA obtained from no EM (56.8±43.16) was significantly higher than that obtained from 0.05, 0.5, and 5 μg·mL^{-1} EM (21.5±17.56, 23.3±16.65, and 21.4±12.65 respectively). By using SEM it was found that PA, when incubated in EM (0.05, 0.5, 5 μg·mL^{-1}) had a significant reduction in its diagonal length, radius, height, volume and surface area.

It is possible, therefore, that these misshaped *Pseudomonas aeruginosa* bacilli are more susceptible to host defence mechanisms, while at the same time less adherent to the basement membrane of the airway *in vivo*. Therefore, this could help explain the clinical efficacy of low-dose erythromycin therapy on patients with *Pseudomonas aeruginosa* infection.

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*Depts of Medicine, #Microbiology and ¶Anatomy, The University of Hong Kong, Queen Mary Hospital, Pokfulam, Hong Kong SAR, China

Correspondence: K.W.T. Tsang, Division of Respiratory and Critical Care Medicine, University Department of Medicine, Queen Mary Hospital, The University of Hong Kong, Pokfulam, Hong Kong SAR, China.
Fax: 852 28725828
E-mail: kwttsang@hku.hk

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Pseudomonas aeruginosa (PA) is a versatile Gram-negative bacterium, which chronically infects the airways of patients with cystic fibrosis (CF) and severe bronchiectasis [1]. Basement membranes form the extracellular matrix and are complex and dynamic structures that are predominantly comprised of type IV collagen, laminin, fibronectin, and heparin sulphate proteoglycans [2]. Type IV collagen is the most abundant nonfibril-forming collagen within the lung and provides the scaffolding for the other components of the respiratory mucosal basement membrane [3]. *Post mortem* data have shown that a significant proportion of CF airways are denuded of mucosa, thereby exposing the basement membrane which PA can adhere to avidly [4]. A more recent *in vitro* study has revealed that PA appears to have a high affinity for basement membrane collagen fibrils in experimental infection of human respiratory mucosa [5]. Deoxyribonucleic acid finger-printing techniques suggest that most CF patients harbour genetically related PA strains in their respiratory tract over long periods of time [6]. The mechanism for PA persistence in the airway is probably complex and multiple. It is possible that adherence to basement membrane collagen fibrils is another mechanism, if not the most important, for PA to anchor to the bronchiectatic airways, and at the same time evade the sweeping actions of mucociliary clearance above it.

Diffuse panbronchiolitis (DPB) is a recently recognised idiopathic chronic progressive suppurative and obstructive airway disease. DPB typically presents with wheezing, chronic bronchial sepsis, and often rapidly progresses to respiratory failure and death if untreated [7]. Long-term treatment with low-dose macrolides, particularly erythromycin (EM), improves respiratory symptoms, lung function parameters, arterial oxygen tension, and imaging findings in DPB [7–9]. The administration of low-dose EM to DPB patients with PA infection leads to an improvement of the 10-yr survival rate from 12.4% to >90% [10]. It was shown recently that the administration of low-dose EM, over an 8-week period, significantly reduces sputum volume and improves lung function in severe idiopathic bronchiectasis [11]. While the clinical efficacy of low-dose EM in DPB and bronchiectasis is undoubted, the precise mechanism of action for EM has not been determined.

Macrolides, despite their clinical efficacy in the treatment of lower respiratory tract infections in chronic obstructive pulmonary disease and pneumonia, penetrate poorly into the respiratory mucus and airway [12, 13]. For example, the ratio of sputum to serum macrolides is generally around 10% [14]. PA in the airways of patients with bronchiectasis and CF is, therefore, almost certainly exposed only to subminimal inhibitory concentrations (MIC) of EM. Although subMIC of EM reduces ciliotoxin production by PA [15], little is known of its effects on PA morphology and adherence to basement membrane collagen. Therefore, in this study, quantitative scanning electron microscopy (SEM) was performed to evaluate the effects of low-dosage EM on PA adherence. This was conducted using a newly developed model of basement membrane collagen and bacterial morphology.

Materials and methods

Inoculation of Pseudomonas aeruginosa

A clinical isolate of a nonmucoidal and piliated strain of PA (PACS001) was stored in brain heart infusion (BHI),

which contained 20% glycerol in liquid nitrogen. PA was retrieved on BHI agar plates (Oxoid, Basingstoke, UK) and incubated overnight at 37°C. Passage was limited to three times prior to the experiments. Following an overnight incubation, a colony of PA was agitated in 4 mL of BHI, which contained 0, 0.05, 0.5 or 5 $\mu g \cdot mL^{-1}$ of EM, in a 6 mL bijou mounted on a roller stage for 24 h at 37°C. The resultant bacterial suspension was then centrifuged for 10 min at 2,000×*g*. The supernate was discarded and replaced with 4 mL of phosphate buffered saline (PBS; Oxoid) containing 0, 0.05, 0.5 or 5 $\mu g \cdot mL^{-1}$of EM. This was repeated three times to wash the bacteria, which was finally resuspended in PBS containing 0, 0.05, 0.5 or 5 $\mu g \cdot mL^{-1}$ of EM. The final PA suspension was used for incubation in Eppendorf lids (Sorenson, Salt Lake City, UT, USA) to assess PA adherence to the collagen-coated surface, and it was also used for processing to assess bacterial morphology.

Collagen coating

Sterile human type IV collagen (Sigma, St. Louis, USA) solution (2 $mg \cdot mL^{-1}$ in 1% acetic acid) was prepared immediately before each experiment. The collagen had three major bands after sodium dodecyl-sulphate-polyacrylamide gel electropherisis under reducing conditions consistent with basement membrane collagen [16]. The lids of plastic Eppendorf tubes were carefully trimmed and removed from the body of the tubes and sterilised by autoclaving. A 50 μL aliquot of collagen solution was added to the inside of an inverted Eppendorf lid and allowed to air dry in an incubator maintained at 37°C for 24 h. Collagen coated lids were washed by immersing in sterile PBS three times, and air-dried for 0.5 h in a dehumidified incubator at 37°C.

Incubation of Pseudomonas aeruginosa *with collagen coated lids*

A 50 μL aliquot of PA suspension in PBS, which contained 0, 0.05, 0.5 or 5 $\mu g \cdot mL^{-1}$ EM, was added onto the collagen-coated lids by gentle pipetting. Viable counts of the inoculating PA suspension were performed to determine the bacterial concentration and purity. The lids were then incubated in the PA suspension for 45 min at 37°C in a dehumidified atmosphere. It had been determined previously that the optimum time for maximal adherence without any significant alteration in the PA viable count was 45 min. After incubation, the PA suspension was carefully decanted from the collagen-coated lids. The lids were rinsed in 5 mL sterile PBS solution three times to remove nonadherent bacteria. The lids were subsequently fixed in 2.5% glutaraldehyde and stored at 4°C until processing for electron microscopy.

Scanning electron microscopy processing

The collagen-coated lids incubated with PA were fixed in 2.5% glutaraldehyde for 24 h prior to being rinsed in sodium cacodylate buffer, and postfixed in 1% osmium tetroxide for 1 h. Standard dehydration in graded ethanol then followed (three times in 50%, three in 70%, three in 90%, and three in 100% for 5 min each) before being stored in 100% acetone. Specimens were then critically dried in carbon dioxide and mounted on aluminum stubs before being sputter-coated with gold. These specimens were randomly coded and stored in individual desiccated tubes prior to SEM examination by an observer who was unaware of the treatment.

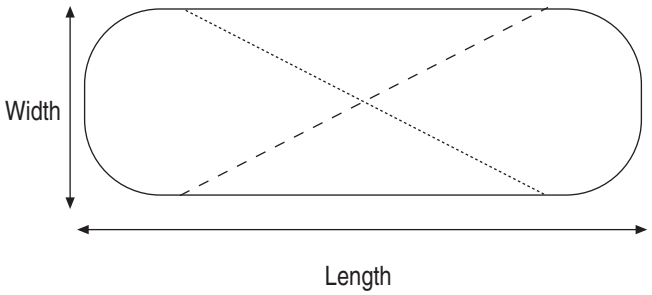

Fig. 1. – The mathematical model used to determine the morphology of a *Pseudomonas aeruginosa* bacterium under high power scanning electron microscopy. - - -: Diagonal length (DL)1; ·····: DL2.

Scanning electron microscopy assessment of Pseudomonas aeruginosa *adherence to collagen-coated Eppendorf lids*

Each lid was placed on the stage of an SEM and viewed at low magnification (×200) to confirm uniform collagen coating, or the specimen would be rejected. For each specimen, 20 random SEM fields, at ×4,000 magnification, were examined at the centre of the lid. The number of bacilli were counted manually for each of the SEM fields. The total number of PA bacilli was then calculated as PA density on collagen surface, which was a reflection of PA affinity towards collagen under the specific experimental condition. Adherence density was calculated as the total number of PA bacilli detected in 20 SEM fields divided by the logarithm of inocular size of PA, determined by viable counting as colony-forming units.

Scanning electron microscopy processing and assessment of bacterial morphology

Bacterial pellets obtained by centrifugation of each PA suspension were smeared on a coded sterile glass coverslip with a sterile plastic loop. The cells were then fixed in 2.5% cacodylate-buffered glutaraldehyde, post fixed in 1% osimum tetroxide, dehydrated in a series of graded ethanols, and critically dried using liquid carbon dioxide. Coverslips were mounted on aluminium stubs, sputter coated with gold and examined using SEM at ×10,000 with a Leica S440 scanning electron microscope (LEO Electron Microscopy Limited, Cambridge, England). The first 100 nondividing bacteria seen at random were electronically captured and assessed with an image analysis system (Improvision, London, UK).

After preliminary qualitative assessment of the nondividing bacteria under SEM a mathematical assumption of a bacterium shape as a uniform cylinder with two equal hemispheres on either end was made (fig. 1). Each bacterium was measured on SEM for width (W), diagonal length (DL)1, and DL2. The average DL was calculated for each bacterium. From the above measurements, calculations were performed using geometrical formulae of the following: the radius of the cylinder (and hemispheres) (R), the height of the cylinder (H), the surface area of each bacterium (A), and the volume of each bacterium (V).

$$R = 0.5W \tag{1}$$

$$H = \frac{1}{\sqrt{R^2 - W^2}} \tag{2}$$

$$A = (2\pi RH) + (4\pi R^2) \tag{3}$$

$$V = (\pi R^2 H) + (4/3\pi R^3) \tag{4}$$

Table 1. – Adherence of *Pseudomonas aeruginosa* to basement membrane collagen type IV in the presence of different concentrations of erythromycin (EM)

	EM concentration $\mu g \cdot mL^{-1}$			
	0	0.05	0.5	5
Viable count $\times 10^9$ cfu·mL^{-1}	4.9±6.54	10.0±15.84	9.7±15.69	4.12±4.25
Log viable count	9.5±0.44	9.5±0.76	9.5±0.78	9.3±0.627
Bacilli in 20 SEM fields[#]	542.6±426.20*	202.2±163.34*	220.9±150.99*	200.1±127.77*
Adherence density	56.8±43.16*	21.5±17.56*	23.3±14.65*	21.4±12.65*

Data are shown as mean±SD. Cfu: colony-forming units. [#]: total number. Adherence density was calculated as the ratio of the total number of bacilli in 20 random scanning electron microscopy (SEM) fields to log viable count for each of the individual experiments. *: $p<0.05$ when compared with data obtained with no EM. n=12.

Statistical analysis

Data were presented as mean±SD. Paired data were compared using Wilcoxon signed-ranked test. Correlation was evaluated by Spearman's rank method. A $p<0.05$ was considered to be statistically significant.

Results

Bacterial adherence density to basement membrane collagen

A total of 12 experiments were performed for this series. The results are shown in table 1 and figure 2. There was no significant difference in the viable counts or log viable counts for the PA inocula used for 0, 0.05, 0.5 or 5 $\mu g \cdot mL^{-1}$ EM ($p>0.05$). However, there were more PA bacilli seen in 20 SEM fields in the control, when compared with 0.05, 0.5 or 5 $\mu g \cdot mL^{-1}$ EM (p=0.02, 0.02 and 0.01, respectively). Adherence density for PA to collagen in the absence of EM was significantly higher than those from 0.05, 0.5 or 5 $\mu g \cdot mL^{-1}$ EM (p=0.02, 0.02 and 0.01, respectively). However, there were no significant differences in the number of PA bacilli in 20 SEM fields or PA adherence densities among 0.05, 0.5 or 5 $\mu g \cdot mL^{-1}$ EM ($p>0.05$).

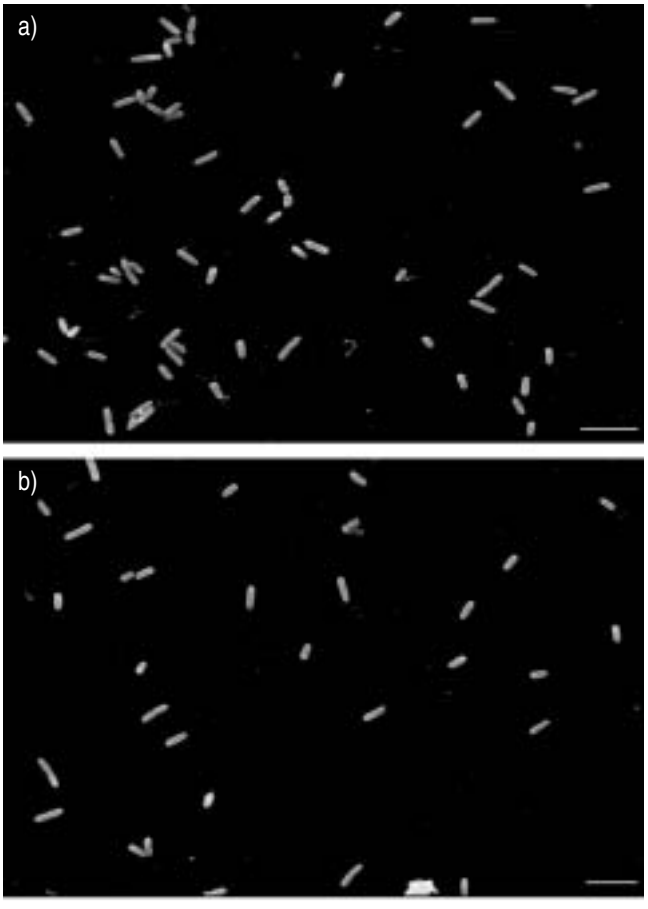

Fig. 2. – A scanning electron micrograph showing a) *Pseudomonas aeruginosa* (PA) bacilli adherence to human type IV basement membrane collagen, coated onto an inert surface and b) significantly less adherence by PA bacilli obtained from incubation with 0.05 $\mu g \cdot mL^{-1}$ erythromycin. Scale bars=4 μm.

Parameters of bacterial morphology

In total, six experiments were performed in this series. The results are shown in table 2 and figure 3. There was no significant difference in the viable count for PA among 0, 0.05, 0.5 or 5 $\mu g \cdot mL^{-1}$ EM ($p>0.05$). However, DL1 was significantly shorter for PA bacilli obtained from 0.05 and 0.5, but not 5 $\mu g \cdot mL^{-1}$ EM when compared with the control (p=0.006, 0.017 and 0.25, respectively). DL2 was also significantly shorter in the presence of 0.05, 0.5 and 5 $\mu g \cdot mL^{-1}$ EM, when compared with control (p=0.004, 0.02 and 0.02, respectively). In the presence of 0.05 and 0.5, but not 5, $\mu g \cdot mL^{-1}$ EM, R of PA bacilli was significantly shorter than in control (p=0.01, 0.02 and 0.31, respectively). Similarly, in the presence of 0.05 and 0.5, but not 5, $\mu g \cdot mL^{-1}$ EM, H of PA bacilli was significantly less than that obtained from the absence of EM (p=0.01, 0.03 and 0.11, respectively). In the presence

Table 2. – Parameters of bacterial morphology for *Pseudomonas aeruginosa* incubated in different concentration of erythromycin (EM)

	EM concentration $\mu g \cdot mL^{-1}$			
	0	0.05	0.5	5
Viable count $\times 10^9$ cfu·mL^{-1}	6.40±3.5	5.08±4.16	8.11±1.12	7.81±5.08
DL1	1.18±0.06	1.05±0.06*	1.08±0.06*	1.13±0.08
DL2	1.16±0.06	1.03±.01*	1.06±0.07*	1.08±0.05*
R	0.25±0.01	0.24±0.01*	0.24±0.01*	0.25±0.02
H	1.05±0.07	0.93±0.07*	0.95±0.07*	0.98±0.07
A μm^2	2.49±0.21	2.08±0.14*	2.13±0.16*	2.27±0.15*
V μm^3	0.29±0.04	0.22±0.02*	0.23±0.03*	0.25±0.03

Data are shown as mean±SD and presented as μm unless otherwise stated. cfu: colony-forming units; DL: diagonal length; R: radius of cylinder and hemisphere; H: height of cylinder; A: surface area of bacillus; V: volume of bacillus. *: $p<0.05$ when compared with data obtained from experiments with no erythromycin. n=6.

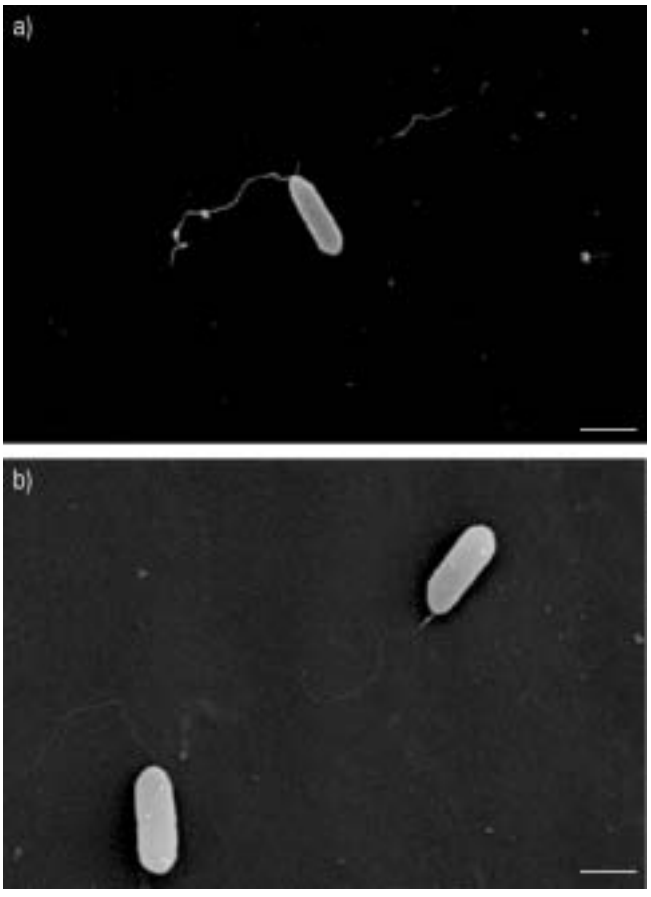

Fig. 3. – A scanning electron micrograph showing the reduction in the diagonal lengths 1 and 2, and radius of *Pseudomonas aeruginosa* bacilli obtained after 24 h incubation in a) 0.05 μg·mL^{-1} erythromycin, and b) no erythromycin. Scale bars=1μm.

of 0.05, 0.5 and 5 μg·mL^{-1} EM, the A of PA bacilli was significantly smaller than control (p=0.003, 0.008 and 0.05, respectively), although the V of bacilli was only different from control for 0.05 and 0.5, but not for 5, μg·mL^{-1} EM (p=0.004, 0.01 and 0.10, respectively). There was no dose-dependent effect, *i.e.* significant differences between 0.05, 0.5 or 5 μg·mL^{-1} EM, on the aforementioned parameters (p>0.05, table 2).

Table 3. – Results of correlation analysis between collagen adherence densities for *Pseudomonas aeruginosa* and parameters of bacterial morphology determined by scanning electron microscopy

	EM concentration μg·mL^{-1}			
	0	0.05	0.5	5
Viable count	-0.25, 0.63	-0.75, 0.15	0.48, 0.33	-0.76, 0.08
DL1	0.37, 0.48	0.35, 0.57	-0.54, 0.27	0.37, 0.47
DL2	0.32, 0.54	0.34, 0.58	-0.63, 0.18	0.25, 0.63
R	0.56, 0.25	0.40, 0.50	0.60, 0.21	-0.54, 0.27
H	0.29, 0.58	0.34, 0.58	-0.71, 0.12	0.43, 0.39
A	0.53, 0.29	0.45, 0.45	0.02, 0.98	-0.30, 0.57
V	0.52, 0.29	0.46, 0.44	0.17, 0.75	-0.41, 0.42

Data are presented as r- and p-values, respectively, when correlated with adherence density. DL: diagonal length; R: radius of cylinder and hemisphere; H: height of cylinder; A: surface area of bacillus; V: volume of bacillus. n=6.

Correlation between bacterial morphology and adherence density

Correlation analysis revealed no significant correlation between adherence density with viable count, DL1, DL2, R, H, A or V of the PA bacilli (p>0.05) (table 3).

Discussion

By using direct counting of the number of PA bacilli adherent to human type IV collagen, the key constituent of airway basement membrane, the data from this study showed that the presence of low EM significantly reduced bacterial adherence to human basement membrane collagen *in vitro*. In addition, PA bacilli incubated in low levels of EM were significantly shorter, thinner, and lower in total V and A than the control. As the concentrations of EM studied, 0.05, 0.5 and 5 μg·mL^{-1}, correspond to the sputum levels of EM in patients with chronic bronchitis (0.10–0.60 μg·mL^{-1}) treated with 500 mg of EM *t.i.d.* [14], the findings from this study should, therefore, reflect the *in vivo* situation. There were no obvious dose-dependent effects and the highest concentration of EM used (5 μg·mL^{-1}) had the least effect on bacterial morphology. This strongly suggests that a low concentration of EM affects PA morphology *via* a mechanism other than the traditional antibiotic mechanism *i.e.* through the disruption of bacterial ribosomal action, leading to inhibition of protein synthesis. Although low-dose EM had significant effects on the parameters of PA bacterial morphology, these parameters did not correlate with adherence densities. This suggests that the inhibitory effects of subMIC EM on PA adherence to human basement membrane collagen, could occur independently of the simple alteration in bacterial morphology. At the same time, PA metabolism is obviously grossly altered by subMIC EM, which led to the morphological changes observed. By using the same adherence model and SEM assessment for bacterial adherence density, it was shown recently by the authors that PA specifically adhere to human basement membrane collagen *via* recognition of the β-D-galactose-1-4-β-D-*N*-acetylglucosamine sequence on type IV collagen and this process could be inhibited by heparin and Ca^{2+} [17].

Administration of low-dose EM, probably by the inhibition of glycoconjugate release [18], reduces sputum production in patients with bronchorrhoea [19] and DPB [7,9]. Low-dose EM, with unclear mechanisms, also reduces airway responsiveness in patients with bronchiectasis [20]. A recent study by the authors has shown that an 8-week administration period of low-dose EM (500 mg *b.i.d*) significantly reduced sputum volume and improved lung function in steady state severe idiopathic bronchiectasis. The underlying mechanism was not considered to be due to bacteriostatic or bactericidal actions, or to be anti-inflammatory. This conclusion was surmised as there were no significant changes in: 1) sputum densities of leukocytes; 2) pro-inflammatory mediators including interleukin-1 and -8; 3) tumour necrosis factor-α and leukotriene B_4; or 4) sputum bacterial densities, respectively [11].

SubMIC antibiotics are known to influence bacterial interactions with host cells in many ways. These include bacterial synthesis of the cell wall and extracellular products, bacterial adherence to cells, and changes in bacterial morphology [21, 22]. SubMIC EM reduces PA adherence to acid-injured murine tracheal mucosa [23], PA ciliotoxin, haemagglutinin and protease production [24, 25], neutrophil migration [26], and superoxide generation [26]. SubMIC macrolides suppress biofilm formation by PA, which also appears to be an important factor for PA to persist in the lungs of patients with

bronchiectasis and CF [27]. Although subMIC EM has been shown to alter the morphology of *Legionella pneumophilia* [28], its effects on the bacterial morphology of PA has not been investigated previously.

Although the mechanism underlying the reduced PA adherence to collagen and alteration of bacterial morphology is unclear, it has been known for some time that subMIC antibiotics could alter bacterial morphology [29, 30]. The disorganisation of the bacterial surface architecture could occur, which, in turn, could change the surface electrical charges, thereby affecting the bacterial interaction with environmental surfaces [31]. Exposure to subMIC β-lactams causes clustering of Gram-positive cocci (which are linked by thick cross walls) and filamentous formation by Gram-negative bacilli [29]. These altered forms of bacteria appear to exhibit lower pathogenicity than their respective normal counterparts. For example they have decreased adherence to epithelial cells, higher susceptibility to phagocytosis and decreased output of bacterial enzymes [29]. It is possible, therefore, that the misshaped PA bacilli are more susceptible to host defence mechanisms, whilst being less adherent to the basement membrane of the airway *in vivo*. These could obviously severely hinder their persistence in the bronchiectatic airways, and could be an important mechanism for the clinical efficacy of EM in bronchiectasis and DPB patients with PA infection.

Although significant morbidity and mortality have been attributed to *Pseudomonas aeruginosa* infections over the past two decades [1, 32], current available treatment modalities for these infections, *i.e*. the use of intensive use and prolonged treatment with antibiotics, fails to eradicate *Pseudomonas aeruginosa* from the airways of bronchiectasis and cystic fibrosis patients. Consequently due to the poor understanding of the pathogenecity of *Pseudomonas aeruginosa* infection, there is little prospect for the development of novel and effective treatment for *Pseudomonas aeruginosa* infection in bronchiectasis and cystic fibrosis. The findings from this study on the effects of low-dose erythromycin on *Pseudomonas aeruginosa* could have important implications on future development of novel therapies against such infection for patients with bronchiectasis and cystic fibrosis.

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