Inhibition of quorum sensing in *Pseudomonas aeruginosa* by azithromycin and its effectiveness in urinary tract infections

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Pseudomonas aeruginosa, an opportunistic pathogen, is the third most common pathogen associated with nosocomial urinary tract infections (UTIs). The virulence of this organism is due to its ability to produce quorum-sensing (QS) signal molecules and form biofilms. These biofilms are usually resistant to conventional antibiotics and host immune responses. Recently, beneficial effects of macrolides, especially azithromycin (AZM), have been shown in patients suffering from chronic infections caused by *P. aeruginosa*. These were due to anti-inflammatory and modulatory effects of AZM on the expression of virulence factors of this pathogen. The present study was designed to evaluate the potential of AZM to inhibit QS signal molecules and its ability to attenuate the virulence of *P. aeruginosa* in an experimental UTI model. Sub-MIC concentrations of AZM significantly inhibited the production of QS signals, swimming, swarming and twitching motilities, and biofilm formation *in vitro*. The therapeutic evaluation of AZM in this experimental UTI model showed complete clearance of the organisms from the mouse kidneys. The results of this study highlight the potential effectiveness of AZM in attenuating the virulence of *P. aeruginosa* in a UTI model.

INTRODUCTION

Quorum sensing (QS) is an important global gene regulatory mechanism in bacteria that enables individual bacteria to coordinate their behaviour in populations. The QS system relies on self-generated signalling molecules, which coordinate gene expression in response to population density (Rumbaugh *et al.*, 2000). Many processes that assist in the survival, persistence and pathogenesis of *Pseudomonas aeruginosa*, such as the expression of virulence factors and biofilm formation, are under the control of QS. Two interrelated QS systems, *las* and *rhl*, have been reported in *P. aeruginosa* and act in a hierarchical manner (Davies *et al.*, 1998; Favre-Bonté *et al.*, 2003; de Kievit, 2009). Both systems consist of inducer and regulatory proteins and a cognate autoinducer signal molecule, *N*-(3-oxododecanoyl) homoserine lactone (OdDHL) and *N*-butanoyl homoserine lactone (BHL), respectively (Rumbaugh *et al.*, 2000; Venturi, 2006).

P. aeruginosa causes infections in a variety of situations, such as cystic fibrosis, burns, cancer and traumatic wounds, especially in immunocompromised hosts, and can be a problem in intensive care units (Van Delden & Iglewski, 1998; Singh *et al.*, 2000; Ehrlich *et al.*, 2002). It has a tendency to form biofilms on biotic and abiotic surfaces such as catheters and lead to persistent and chronic urinary

tract infections (UTIs) (Donlan & Costerton, 2002; Willcox *et al.*, 2008). These biofilms are usually resistant to antibiotics and host immune defence clearance, and hence are difficult to eradicate by antibiotic intervention (Ceri *et al.*, 1999; Costerton *et al.*, 1999; Donlan, 2001).

Although antibiotic therapy has great benefits in treatment, the emergence of multidrug resistance in *P. aeruginosa* has left clinicians with limited therapeutic options. Azithromycin (AZM), a member of the macrolide class of antibiotics, is used to cure certain bacterial infections, primarily caused by Gram-positive bacteria but also some Gram-negative pathogens. However, many clinical and experimental studies have shown beneficial effects of AZM in the treatment of patients with diffuse panbronchiolitis and cystic fibrosis, which are associated with *P. aeruginosa* infection (Pechère, 2001; Tateda *et al.*, 2001). Looking at the multiple actions of macrolides, such as their anti-inflammatory effect (Tsai *et al.*, 2004; Amsden, 2005) and inhibition of the synthesis of alginate (Skindersoe *et al.*, 2008), the present study aimed to evaluate the role of AZM in QS, swimming, swarming and twitching motilities, and biofilm formation of *P. aeruginosa*. Further therapeutic potential of AZM in a mouse model of UTI caused by *P. aeruginosa* was also analysed.

METHODS

Bacterial strains. Twenty-five clinical isolates of *P. aeruginosa* isolated from urine samples of patients having catheter-associated

Abbreviations: AZM, azithromycin; BHL, *N*-butanoyl homoserine lactone; MU, Miller unit; OdDHL, *N*-(3-oxododecanoyl) homoserine lactone; p.i., post-infection; QS, quorum sensing; UTI, urinary tract infection.

UTI attending the Government Medical College and Hospital, Chandigarh, India, along with the *P. aeruginosa* standard strain PAO1 were used in this study. The study protocol was approved by the Panjab University Ethical Committee. Two reporter strains, *Escherichia coli* MG4(λ 1.4) and PAO-JP2(pECP61.5), were also used. These reporter strains were grown under antibiotic pressure from ampicillin ($100 \mu\text{g ml}^{-1}$) and carbenicillin ($250 \mu\text{g ml}^{-1}$) for the detection of OdDHL and BHL, respectively. All strains were maintained as 10 % glycerol stocks and stored at -80°C . Fresh subcultures were prepared for each new experiment from glycerol stocks.

MIC determination. The MIC of AZM (HiMedia Laboratories) was determined according to NCCLS (2002) guidelines against the standard *P. aeruginosa* strain PAO1 and clinical strains PA1 and PA2. Briefly, from stock solutions of AZM (10 mg ml^{-1}), different dilutions of $5\text{--}200 \mu\text{g ml}^{-1}$ were prepared. A 6 h culture was used as the inoculum. The concentration of antibiotic resulting in no visible growth was taken as the MIC. For all further experiments, a sub-MIC of AZM was used for all three strains.

Estimation of QS signals (N-acyl homoserine lactones). Supernatant was extracted from overnight cultures grown in the presence or absence of AZM and β -galactosidase activity was determined. The reporter culture was diluted 1:1 in Z buffer ($\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ 0.06 M, $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ 0.04 M, KCl 0.01 M, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.001 M, β -ME 0.05 M, pH 7.0) and assayed for β -galactosidase activity by using ONPG as substrate, as described by Miller (1972).

Motility assays. Swimming, swarming and twitching motilities were assayed on agar plates containing specialized medium with or without a sub-MIC of AZM (Rashid & Kornberg, 2000). For the motility assays, organisms were grown overnight in the absence of AZM and then inoculated onto motility plates with AZM.

Swimming. Plates containing 1 % tryptone, 0.5 % NaCl and 0.3 % (w/v) Bacto agar were prepared for the assay. Swimming plates were point-inoculated from an overnight culture with a sterile toothpick and incubated at 30°C for 24 h. The zone diameter was measured to assess swimming motility.

Swarming. Nutrient broth (8 g l^{-1}) supplemented with 5 g glucose l^{-1} was prepared. The medium was solidified by the addition of 0.5 % (w/v) Bacto agar. The plates were point-inoculated from an overnight culture with a sterile toothpick and incubated at 37°C for 24 h. Swarming motility was assessed by measuring the circular turbid zones formed by the bacterial cells migrating away from the point of inoculation.

Twitching. Freshly prepared Luria–Bertani agar plates (1 % Bacto agar) were used for the assay. Overnight cultures were stabbed with a sterile toothpick through the agar layer to the bottom of the Petri dish. The plates were then incubated at 37°C for 48 h. The ability of bacteria to adhere on the polystyrene surface was examined by removing the agar and staining the attached cells with crystal violet (0.1 %, w/v). The plates were washed gently with tap water to remove any unattached cells before staining. The diameter of the stained zone was measured to assess the twitching motility.

Biofilm-forming capacity. Biofilm-forming capacity was determined by a microtitre plate assay, as described by Wagner *et al.* (2007). Biofilms were allowed to develop in microtitre plates with and without AZM. The plate was incubated at 37°C for 24 h under static conditions. After 24 h, the wells were drained and washed three times with sterile PBS to remove free cells. The wells were stained with crystal violet (0.1 %, w/v) for 15 min at room temperature. Excess dye was removed by washing the wells with sterile PBS. Dye taken up by

the biofilm cells was extracted with 95 % (v/v) ethanol. Absorbance was measured at 570 nm and biofilm-forming capacity was calculated as A_{570} of the strain in the presence of AZM/ A_{570} of the strain in the absence of AZM. A control without antibiotic was run simultaneously.

Biofilm generation. Sterile Foley catheter pieces (Rusch) of 1 cm were cut and a biofilm was allowed to develop under static conditions for 7 days as described previously in the presence or absence of a sub-MIC of AZM (Mittal *et al.*, 2006). The catheter pieces were transferred to fresh medium every 24 h. Each day, the catheter pieces in duplicate were removed, rinsed three times with PBS and cells were removed from the surface by scraping the surface with a sterile scalpel blade. The cells were sonicated using a low-level sonication cycle and vortexed for 30 s. Dispersed samples were then centrifuged and the biofilm cells were suspended in 1 ml PBS. Serial dilutions were prepared and plated on MacConkey agar plates.

Therapeutic effect of AZM *in vivo*

Establishment of experimental UTI. The method of Harjai *et al.* (2005) was used for induction of a UTI. Ethical approval for the use of animals was granted by the Panjab University Ethical Committee. Female LACA (Swiss Webster) mice, 6–8 weeks old and free of bacteraemia, were used for the experiment. Four groups consisting of eight animals each were used. Control group 1 was infected without any further treatment. In control group 2, 1 ml sterile normal saline was given orally and intravenously to infected animals. In groups 3 and 4, infected animals were treated orally ($400 \mu\text{l}$) or intravenously ($100 \mu\text{l}$) with AZM [$500 \text{ mg (kg body wt)}^{-1}$] from the day of infection to the day of sacrifice. On day 0, $50 \mu\text{l}$ adjusted inoculum ($10^8 \text{ c.f.u. ml}^{-1}$) was introduced into the bladder of the mice using a soft intramediac, non-radio-opaque polyethylene tubing (outer diameter 0.61 mm; Clay Adams).

Bacteriological examination. Animals were sacrificed on days 3 and 5 post-infection (p.i.). Aseptically removed kidneys were weighed and homogenized in 1 ml sterile saline. Quantitative bacterial counts (c.f.u.) were calculated ($\text{g kidney tissue}^{-1}$), as reported by Kakar *et al.* (1986).

Histopathological examination. Kidney tissue from infected animals from all groups of mice was fixed in 10 % normal buffered saline and dehydrated in 30–100 % ethanol. Paraffin wax blocks were prepared and $5 \mu\text{m}$ thin sections were stained with haematoxylin and eosin. The medulla, cortex, calyx and subcalyx of each kidney were evaluated for histopathological changes with the help of an expert pathologist.

Statistical analysis. All experiments were carried out in triplicate to validate the reproducibility of the experiments. The results were analysed statistically using Student's *t*-test with GraphPad Prism software to calculate *P* values. $P < 0.05$ was taken as statistically significant.

RESULTS AND DISCUSSION

Effect of AZM on QS signals

The pathogenicity of *P. aeruginosa* is controlled by QS signals (Van Delden & Iglewski, 1998). Therefore, in the present study, the effect of AZM on production of the QS signal molecules OdDHL and BHL was evaluated. Two clinical isolates, PA1 and PA2, producing comparable levels of QS signal molecules compared with the standard strain PAO1 and having the maximum biofilm-forming capacity

of all the isolates, were used for further study, along with strain PAO1. The MIC for AZM for *P. aeruginosa* PAO1 was determined as $177 \mu\text{g ml}^{-1}$. A sub-MIC ($88.5 \mu\text{g ml}^{-1}$) was used for all further experiments. Strain PAO1 produced 818 Miller units (MU) BHL and 137 MU OdDHL in absence of AZM (Fig. 1). The levels of BHL and OdDHL decreased to 372 MU ($P<0.001$) and 120 MU ($P<0.01$), respectively, in the presence of AZM. The clinical isolates PA1 and PA2 also showed a reduction in the production of BHL and OdDHL in the presence of AZM (Fig. 1), indicating that macrolides interfere with the production of QS signals in *P. aeruginosa*. It has been observed by other workers that a sub-MIC of erythromycin, another macrolide, also reduces the production of QS signal molecules (Sofer *et al.*, 1999). AZM is neither bactericidal nor bacteriostatic in *P. aeruginosa*, but it inhibits QS signal production and may lead to attenuation of the virulence of *P. aeruginosa*. This approach could prove beneficial when exploited in different *P. aeruginosa*-induced infections in various animal models.

Effect of AZM on motility

P. aeruginosa exhibits three types of motility – swimming, swarming and twitching – which are mediated by flagella and type IV pili (Köhler *et al.*, 2000; Rashid & Kornberg, 2000). Whilst flagellum-mediated motility has been implicated in bringing *P. aeruginosa* within proximity of the surface, type IV pili enable the organism to migrate across biotic surfaces, recruit cells from adjacent monolayers and form cell aggregates (O'Toole *et al.*, 1999). To see the effect of AZM on the swimming, swarming and twitching motilities of *P. aeruginosa*, strains PAO1, PA1 and PA2 were grown overnight in the absence of AZM and then inoculated onto motility plates with AZM. It was observed that the presence of AZM significantly reduced all three types of motility (Table 1, Fig. 2). Molinari *et al.* (1992) also observed a reduction in motility of *P. aeruginosa* on exposure to a sub-MIC of AZM, hence

confirming the ability of AZM to reduce the expression of virulence factors. Nalca *et al.* (2006) also demonstrated an inhibitory effect of AZM on swimming and twitching motilities, with delayed and impaired alginate production.

Effect of AZM on biofilm formation

The biofilm-forming capacity of strains PAO1, PA1 and PA2 was calculated in the presence and absence of AZM. The results demonstrated that AZM significantly reduced biofilm-forming capacity in all three strains ($P<0.001$; Fig. 3). The effect of AZM was also determined on different stages of biofilm formation by quantifying log (c.f.u. ml^{-1}) values. Biofilm was generated on Foley catheters for 7 successive days in the absence or presence of AZM. In the absence of AZM, strain PAO1 showed an increase in the log count of biofilm formation from day 1 with a maximum peak value (11.0 ± 0.32) on day 4, after which a decline was observed up to day 7 (Fig. 4). The peak on day 4 was indicative of maximum accumulation of bacterial cells on the surface of the catheter. In the presence of AZM, a significant reduction in the log c.f.u. of biofilms was observed (5.6 ± 0.08) for PAO1 on the peak day of biofilm formation, i.e. day 4. The clinical isolates PA1 and PA2 also showed a similar trend with a reduction in the peak on day 4 and a significant reduction in biofilm formation over 7 days in the presence of AZM (Fig. 4). The reduction in log count was indicative of thinner and reduced biofilm formation. These kinds of biofilms show increased permeability and penetration of antibiotics through the extracellular polymeric substance (Mikuniya *et al.*, 2005). Ichimiya *et al.* (1996) have also shown that AZM can inhibit biofilm formation significantly by inhibiting the production of alginate acid by mucoid strains and the production of exopolysaccharides by non-mucoid strains of *P. aeruginosa*. Impairment of the ability of *P. aeruginosa* to form fully polymerized alginate biofilms may help in attenuating the virulence of *P. aeruginosa* (Hoffmann *et al.*, 2007).

Fig. 1. Production of the QS signal molecules BHL and OdDHL as estimated by a β -galactosidase assay in culture supernatants of standard strain PAO1 and clinical isolates PA1 and PA2 in the absence or presence of a sub-MIC of AZM. The results are presented as means $\pm$ SD obtained from three independent experiments. **, $P<0.01$; ***, $P<0.001$.

Table 1. Comparison of the swimming, swarming and twitching motilities shown by standard strain PAO1 and clinical isolates PA1 and PA2 in the absence or presence of a sub-MIC of AZM

Results are given as zone diameters (cm).

Motility	AZM	PAO1	PA1	PA2
Swimming	+	0.73 ± 0.058	0.53 ± 0.058	0.73 ± 0.06
	–	$0.1 \pm 0.01^{**}$	$0.1 \pm 0.01^{**}$	$0.27 \pm 0.056^{**}$
Swarming	+	0.53 ± 0.057	1.7 ± 0.012	1.03 ± 0.058
	–	$0.12 \pm 0.029^*$	$0.4 \pm 0.01^{***}$	$0.4 \pm 0.029^{**}$
Twitching	+	0.23 ± 0.058	1.15 ± 0.05	0.45 ± 0.05
	–	0.1 ± 0.01	$0.2 \pm 0.029^{***}$	0.2 ± 0.06

*, $P < 0.05$ (significant); **, $P < 0.01$ (very significant); ***, $P < 0.001$ (extremely significant).

Potential efficacy of AZM in treatment of experimental UTIs

A significant reduction in QS signal molecules and a reduction in the formation of biofilm by *P. aeruginosa* was observed *in vitro*. The therapeutic potential of AZM in a mouse model of ascending UTI, where the motility of the organism provides an additional benefit in ascending to the renal tissue, was then evaluated. Standard strain PAO1 was used to infect the animals. Animals in all groups were sacrificed on day 3 or 5 p.i. On day 3 p.i., control group animals (untreated and saline-treated mice) showed

comparable renal bacterial counts of 5.75 ± 0.11 and 5.38 ± 0.06 [$\log$ (c.f.u. g^{-1})], respectively. A significant reduction in log count of 3.18 ± 0.11 and 3.36 ± 0.31 was observed in the groups of mice treated intravenously and orally with AZM, respectively, on day 3 p.i. ($P < 0.05$). On day 5 p.i., untreated and saline-treated mice showed renal bacterial counts of 3.67 ± 0.07 and 3.50 ± 0.05 , whereas no bacteria could be recovered from the renal tissue of mice on day 5 p.i. from the treatment groups (Fig. 5), indicating clearance of infection by day 5 p.i. following AZM treatment. Inhibition of the production of QS signal molecules and the inability to form a thick and mature biofilm in the presence of AZM could have led to complete clearance of the organisms from the kidney tissue of infected mice.

Fig. 2. Demonstration of the swimming (a, b), swarming (c, d) and twitching (e, f) motilities of standard strain PAO1 and clinical isolates PA1 and PA2 in the absence (controls: a, c, e) and presence (test plates: b, d, f) of AZM. **Fig. 3.** Demonstration of biofilm-forming capacity estimated by a crystal violet assay of *P. aeruginosa* standard strain PAO1 and clinical isolates PA1 and PA2 in the absence or presence of a sub-MIC of AZM. A standard reference value of 1 was taken as the control (no AZM) for each strain. The results are presented as means $\pm$ SD obtained from three independent experiments. ***, $P < 0.001$.

Fig. 4. Generation of biofilms on Foley catheters as log (c.f.u. ml⁻¹) of *P. aeruginosa* standard strain PAO1 and clinical isolates PA1 and PA2 from day 1 to day 7 in the absence or presence of a sub-MIC of AZM. The results are presented as means $\pm$ SD obtained from three independent experiments.

On histopathological examination, the renal tissue of untreated but infected mice and saline-treated mice showed severe inflammation with infiltration of polymorphonuclear leukocytes and plasma cells in the glomeruli and subepithelium of the renal pelvis (Fig. 6). In contrast, infected mice treated with AZM showed mild inflammation with normal glomeruli and tubules in the medulla and cortex regions of the renal tissue. The renal tissue of uninfected mice without treatment or treated with AZM did not show any histopathological changes, suggesting no effect of AZM on renal tissue. The histopathological results were in accordance with the bacteriological findings, showing significant differences between the test and control groups. In the treated group, renal tissue destruction was reduced in comparison with the control groups. The role of *N*-acyl homoserine lactones in tissue inflammation has been observed previously by a number of workers (Rumbaugh *et al.*, 1999; Smith *et al.*, 2002; Kumar *et al.*, 2009). Reduced tissue inflammation in the treatment

groups indicated that AZM, besides inhibiting QS, also plays an anti-inflammatory role during UTIs and hence protects tissue from inflammation. The ability of AZM to reduce tissue inflammation further confirmed the effectiveness of AZM for treatment of experimental UTIs.

Although AZM has been shown to exert a therapeutic effect in cystic fibrosis and diffuse panbronchiolitis in mouse lung infection models (Wolter & McCormack, 1998; Saiman *et al.*, 2002; Hansen *et al.*, 2005), to the best of our knowledge, the present report is the first to show the potential efficacy of AZM in the treatment of UTIs caused by *P. aeruginosa* in a mouse model. Increased clearance of *P. aeruginosa* biofilms, reduced lung pathology and favourable modulation of the immune response in the lung infection model has also been observed by a number of workers (Nicolau *et al.*, 1999; Moser *et al.*, 2002; Hoffmann *et al.*, 2007). In the present study, treatment with AZM improved the outcome of UTIs caused by *P.*

Fig. 5. Renal bacterial load (c.f.u. g⁻¹) of infected and treated mice sacrificed on days 3 and day 5 p.i. after intraurethral challenge with *P. aeruginosa* strain PAO1. Mice groups were given oral or intravenous (i.v.) treatment with AZM. Control group 1 comprised infected mice without treatment and control group 2 comprised infected mice with saline treatment. *, $P < 0.05$; ***, $P < 0.001$.

Fig. 6. (a) Photomicrograph of renal tissue of normal uninfected mice. Normal glomeruli are indicated by arrows. (b) Infiltration of polymorphonuclear leukocytes along with plasma cells in the interstitium and severe inflammation in tubules in the medulla region in the kidney tissue of control untreated mice infected with *P. aeruginosa*. (c) Normal to mild inflammation with reduced infiltration of polymorphonuclear leukocytes in the interstitium, with normal tubules of the medulla region in kidney tissue of infected and AZM-treated mice. Sections were stained with haematoxylin and eosin. Original magnification, $\times 100$. The arrows in (b) and (c) indicate histopathological changes.

aeruginosa in a mouse model. It was concluded that AZM can be used as a QS inhibitor or antagonist to potentially

reduce the virulence of *P. aeruginosa*. The use of AZM could also be explored further as an adjunctive therapy with other antibiotics for the treatment of UTIs caused by *P. aeruginosa*. More studies on the use of AZM in a UTI model in the context of modulation of the immune response will throw more light on the therapeutic potential of AZM in kidney infection of mice.

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