Effect of pH on the antimicrobial susceptibility of planktonic and biofilm-grown clinical *Pseudomonas aeruginosa* isolates

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ABSTRACT

The pH at the site of infection is one of a number of factors that may significantly influence the *in vivo* activity of an antibiotic prescribed for treatment of infection and it may be of particular importance in the treatment of cystic fibrosis (CF) pulmonary infection, as acidification of the airways in CF patients has been reported. As *Pseudomonas aeruginosa* is the most frequent causative pathogen of CF pulmonary infection, this study determines the effect that growth at a reduced pH, as may be experienced by *P. aeruginosa* during infection of the CF lung, has on the susceptibility of clinical *P. aeruginosa* isolates, grown planktonically and as biofilms, to tobramycin and ceftazidime. Time-kill assays revealed a clear loss of tobramycin bactericidal activity when the isolates were grown under acidic conditions. MIC and MBC determinations also showed decreased tobramycin activity under acidic conditions, but this effect was not observed for all isolates tested. In contrast, growth of the isolates at a reduced pH had no adverse effect on the bacteriostatic and bactericidal activity of ceftazidime. When the isolates were grown as biofilms, the pH at which the biofilms were formed did not affect the bactericidal activity of either tobramycin or ceftazidime, with neither antibiotic capable of eradicating biofilms formed by the isolates at each pH. This was in spite of the fact that the concentrations of both antibiotics used were much higher than the concentrations required to kill the isolates growing planktonically. These results show that growth in an acidic environment may reduce the susceptibility of clinical *P. aeruginosa* isolates to tobramycin.

KEY WORDS: Ceftazidime. Cystic fibrosis. pH. Pseudomonas aeruginosa. Tobramycin.

Introduction

The antibiotic regimen used to treat acute exacerbations of pulmonary infection in cystic fibrosis (CF) patients is primarily determined by the identity of the causative pathogen and its susceptibility to a range of antibiotics measured using *in vitro* susceptibility tests. However, the clinical value of standard susceptibility testing in the treatment of CF pulmonary infection has recently been called into question by data which show that the response of patients to intravenous antibiotic therapy did not appear to correlate with the antibiotic susceptibility pattern of the infecting bacteria.[1] There are many factors such as presence of high bacterial numbers[2] and growth in a biofilm[3] that influence the activity of an antibiotic *in vivo* and the fact that not all these are replicated in standard susceptibility tests may help to explain this finding.

A further important consideration in the susceptibility testing of isolates cultured from the lungs of CF patients is the pH at the site of infection in the lungs. It has been shown that the pH of both exhaled breath condensate and airway surface liquid is lower in patients with CF than in healthy controls.[4,5] Significantly, the activity of some antibiotics such as the aminoglycosides may be altered depending on the pH of the surrounding environment, with small changes in pH affecting the ratio of ionised and unionised antibiotic.

As *Pseudomonas aeruginosa* may be growing in the CF lung in an acidic environment, it is important to determine the effect that growth in such an environment has on antibiotic efficacy. Therefore, this study investigates the effect of growth at different pH on the susceptibility of clinical *P. aeruginosa* isolates grown planktonically and as biofilms to tobramycin and ceftazidime, the two antibiotics most frequently used to treat *P. aeruginosa* pulmonary infection in CF patients.

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Materials and methods

Bacterial isolates

Twelve clinical *P. aeruginosa* isolates cultured from the sputum of CF patients admitted to the Belfast City Hospital for treatment of an acute exacerbation of lung infection were tested. *P. aeruginosa* isolate NCTC 12934 was obtained from the National Culture Type Collection (London, UK). Isolates were stored at –70°C in glycerol and subcultured to Iso-Sensitest agar (ISA; Oxoid, Basingstoke, UK) slopes before testing.

Antimicrobial agents and buffers

Ceftazidime was obtained as Fortum from GSK (Uxbridge,

UK) and tobramycin was obtained from Sigma Chemical Co. (Dorset, UK). Morpholinoethanesulfonic acid (MES) and 4-morpholinepropanesulfonic acid (MOPS) were obtained from Sigma.

Planktonic antimicrobial susceptibility

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of tobramycin and ceftazidime were determined in triplicate by the broth microdilution method according to British Society for Antimicrobial Chemotherapy (BSAC) guidelines.[6] Serial two-fold dilutions (range: 0.25 to 128 µg/mL) of each antibiotic and the final inoculum ($2x10^5$ colony-forming units [CFU]/mL) for each isolate to be tested were prepared in Iso-Sensitest broth (ISB; Oxoid) and in ISB adjusted to pH 5.5, 6.5 and 7.0, with the final inoculum at each pH verified by total viable count. The pH of the ISB was adjusted to 5.5 and 6.5 by the addition of 50 mmol/L MES/NaOH buffer and to 7.0 by the addition of 50 mmol/L MOPS/NaOH buffer. Non-adjusted ISB had a pH of 7.4±0.2. The MICs and MBCs determined were used to calculate the antibiotic concentration required to inhibit (MIC) or kill (MBC) 50% (MIC_{50} and MBC_{50}, respectively) and 90% (MIC_{90} and MBC_{90}, respectively) of isolates tested. Quality assurance testing was performed in all experiments with *P. aeruginosa* isolate NCTC 12934.

Time-kill determinations

Time-kill assays were performed in sterile 250 mL flasks containing a final volume of 100 mL broth. The initial inoculum to be tested, $1x10^6$ CFU/mL, was prepared by growing isolates in ISB at each pH and in non-adjusted ISB. Flasks were incubated at 37°C on an orbital incubator with samples (1 mL) removed at 0, 2, 5, 8 and 24 h, serially 10-fold diluted, plated on ISA and the total viable count determined.

Biofilm susceptibility

Isolates to be tested were grown overnight in non-adjusted nutrient broth (NB; Oxoid) and NB adjusted as described previously to pH 5.5, 6.5 and 7.0. Bacterial biofilms were formed by adding 1 mL of each overnight bacterial culture to nine sterile vials containing 19 mL of the same NB preparation and a polyvinyl chloride (PVC) disc (1 cm^2), which were incubated on an orbital incubator at 37°C for 48 h. Following biofilm formation, the PVC discs were removed, washed with sterile phosphate-buffered saline (PBS) and transferred to vials containing the same preparation of fresh NB and either tobramycin or ceftazidime. Following incubation for a further 24 h, the PVC discs were removed, washed and adherent bacteria dislodged into 5 mL PBS by mild ultrasonication (5 min) and rapid vortex mixing (30 sec). Serial 10-fold dilutions were performed and the number of adherent bacteria on each disc was determined (CFU/cm^2). All biofilm experiments were performed in triplicate with antibiotic-free controls included for each isolate and pH tested.

Results

The results of planktonic antimicrobial susceptibility testing are summarised in Table 1. Total viable counts confirmed that the final inoculum density was similar for each isolate grown in ISB at each pH (data not shown). A reduction in the pH of the growth media from 7.4 (non-adjusted broth) to pH 7 and 6.5 decreased the ability of tobramycin to inhibit bacterial growth, with the MIC_{50} value increasing two-fold.

However, a further reduction in the pH of the growth medium to 5.5 had no effect on the MIC_{50} of tobramycin. Similarly, the bactericidal activity of tobramycin was decreased when the isolates were grown in media at a reduced pH, with the greatest increase in MBC_{50} value apparent at pH 6.5.

In contrast, altering the pH of the growth medium had no effect on either the bacteriostatic or bactericidal activity of ceftazidime, with no increases in either MIC_{50} or MBC_{50} values apparent when the isolates were grown in media with a reduced pH (Table 1).

The effect of reduced pH on the bactericidal activity of both antibiotics was further investigated with selected isolates by performing time-kill assays with the antibiotic concentrations for each isolate selected to be equal to or greater than the highest MIC observed for that particular isolate at all pH levels tested. For all three isolates examined, tobramycin exhibited decreased bactericidal activity when the isolates were grown in broth at a reduced pH (Fig. 1a). For example, when isolate 11F was grown in both non-adjusted broth and broth adjusted to pH 7.0 it exhibited an 8 log reduction in total viable count at 24 h when exposed to tobramycin. However, when this isolate was grown in a medium with a reduced pH, the bactericidal activity of tobramycin decreased with only a 3 log reduction (pH 6.5) and no change (pH 5.5) in total viable count apparent after exposure to tobramycin for 24 h.

In contrast, altering the pH of the growth medium had no effect on the killing kinetics of ceftazidime (Fig. 1b). Although ceftazidime exerted some initial bactericidal

Table 1. Effect of pH on the *in vitro* activity of tobramycin and ceftazidime against clinical *P. aeruginosa* isolates.

	Tobramycin				Ceftazidime			
pH of broth	5.5	6.5	7.0	Non-adjusted	5.5	6.5	7.0	Non-adjusted
MIC range (µg/mL)	<0.25–4	<0.25–32	<0.25–4	<0.25–16	<0.25–8	2–128	2–>128	2–>128
MIC_{50} (µg/mL)	1	2	2	1	2	4	4	4
MIC_{90} (µg/mL)	4	16	4	6	4	16	>128	128
MBC range (µg/mL)	<0.25–32	2–32	<0.25–16	<0.25–128	<0.25–4	2–128	2–>128	2–>128
MBC_{50} (µg/mL)	8	16	4	2	2	4	4	8
MBC_{90} (µg/mL)	16	32	8	64	4	32	>128	128

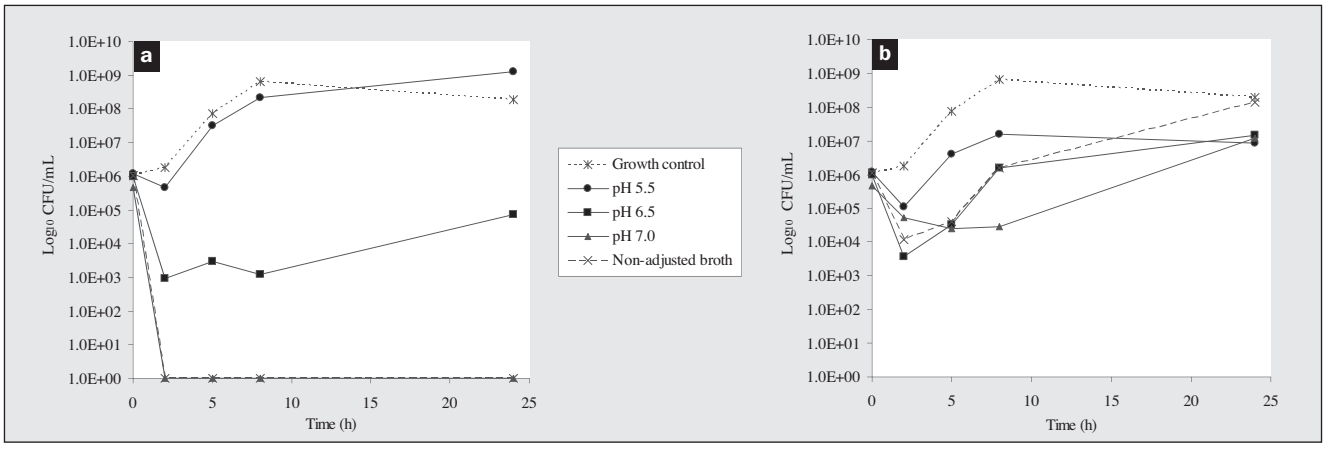

Fig. 1. Effect of pH on killing of representative *P. aeruginosa* isolate 11F exposed to **a)** 4 µg/mL tobramycin and **b)** 8 µg/mL ceftazidime.

activity against all three isolates grown at each pH, no pH-dependent differences in activity were apparent, with regrowth of all isolates occurring by 24 h, irrespective of the pH of the broth in which the isolate was grown.

In an attempt to better simulate conditions *in vivo* in the lungs of CF patients, where *P. aeruginosa* has been shown to grow as a biofilm within mucus,[3,7] we determined the effect of reduced pH on the activity of both antibiotics against selected isolates growing in biofilms. Biofilms were formed and challenged at each pH, with the concentration of both antibiotics used (80 µg/mL) selected to be greater than the highest MBC observed for the isolates growing planktonically at all pH levels tested.

Reducing the pH at which the isolates were grown had no effect on biofilm formation, with biofilms formed for each of the isolates in similar numbers at each pH (Fig. 2). Similarly, the pH at which the biofilms were formed had no effect on antibiotic activity as tobramycin and ceftazidime, at the concentrations tested, were unable to eradicate biofilms formed by the isolates at each pH (Fig. 2).

Discussion

pH at the site of infection may be particularly important in the antibiotic treatment of CF pulmonary infection because acidification of the CF airways has been reported by measurements of both exhaled breath condensate[5] and lung airway surface liquid in CF patients.[4] Despite this fact, *in vitro* susceptibility tests are performed in broth at pH values similar to human serum (pH 7.4).[8] However, a small change in pH at the site of infection may impact on the effectiveness of antibiotic therapy, as the pH of the surrounding environment affects the ratio of ionised and un-ionised antibiotic. This change in pH has the most significant effect on antibiotics such as the aminoglycosides and macrolides, which are most active when they are un-ionised and become less active when they become ionised under acidic conditions. Therefore, this *in vitro* study looked at the effect that growth at a reduced pH, as may be experienced by *P. aeruginosa* during infection of the CF lung, had on the susceptibility of *P. aeruginosa* to tobramycin and ceftazidime.

Bactericidal killing curves revealed a clear loss of tobramycin bactericidal activity when the isolates were grown under acidic conditions. Determination of MIC and MBC also showed decreased tobramycin activity under acidic conditions, but this effect was not observed for all isolates tested. This change in susceptibility with changing pH cannot be accounted for by differences in the growth rate of the isolates at different pH levels, as growth rates were not affected by pH. This finding is similar to the results of previous studies that also examined the antimicrobial susceptibility of bacterial isolates under acidic conditions.

Gudmundsson *et al*.[8] reported that an acidic pH had a detrimental effect on the activity of aminoglycosides and ciprofloxacin, with the MIC of tobramycin for *P. aeruginosa* increasing four- to eight-fold when the isolate was grown at an acidic pH, in comparison to when it was grown at an alkaline pH. The rate of bactericidal killing for aminoglycosides and ciprofloxacin measured at 1 h and 4 h was also lower when the isolates were grown at an acidic pH.

Similarly, Garrison *et al*.[9] found, using an *in vitro* pharmacodynamic model, that at an acidic pH (6.4) similar to that found in lung tissue in patients with pneumonia, the bactericidal activity of clarithromycin against *Haemophilus*

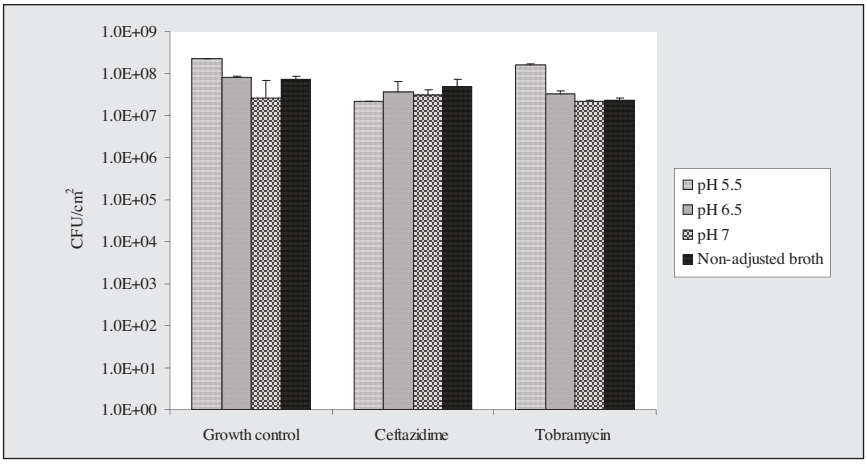

Fig. 2. Effect of pH on susceptibility of 48-hour-old biofilms of representative *P. aeruginosa* isolate 11A challenged for 24 hours with tobramycin (80 µg/mL) and ceftazidime (80 µg/mL). Error bars represent the standard deviation of the mean for each value.

influenzae was significantly reduced when compared to its activity at an alkaline pH.

In the present study, growth of the isolates at a reduced pH had no adverse effect on the bacteriostatic and bactericidal activity of ceftazidime as measured by MIC/MBC and killing kinetics. This finding is similar to that reported previously by Gudmundsson *et al*.,[8] who also found that initial bactericidal activity of ampicillin against bacterial isolates was not dependent on pH.

The differences in the effect of an acidic pH on the activity of tobramycin and ceftazidime may be related to the effect of pH on drug ionisation. As stated previously, aminoglycosides are most active when they are un-ionised and become less active when they become ionised under acidic conditions. Increased ionisation under acidic conditions has been proposed to decrease both the permeability and binding to bacterial surface and intracellular receptors of aminoglycosides, resulting in decreased bactericidal activity.[8] In contrast, ceftazidime remains primarily un-ionised at an acidic pH and therefore its bactericidal activity is not adversely affected by a reduction in pH.

Furthermore, the electrical potential across the bacterial membrane is reduced when the surrounding environment is anaerobic, highly osmolar or acidic.[10] As aminoglycosides are transported across the bacterial membrane using energy-dependent oxidative phosphorylation, their uptake is reduced when the electrical potential is reduced as a result of an acidic external pH such as that present in the CF lung.

When the isolates were grown as biofilms, the pH at which the biofilms were formed did not affect the bactericidal activity of either tobramycin or ceftazidime, with neither antibiotic capable of eradicating biofilms formed by the isolates at each pH. This was despite the fact that the concentrations of both antibiotics used were much higher than the concentrations required to kill the isolates growing planktonically, and also in excess of the maximum achievable sputum concentration of each antibiotic following intravenous administration.[11] These results are similar to those reported by previous studies which have also shown that antibiotic eradication of *P. aeruginosa* biofilms is extremely difficult.[12–14]

P. aeruginosa biofilms are protected from antibiotic attack through the reduced diffusion of antibiotics through alginate, low metabolic activity and the presence of persister cells.[15] The acidic conditions in the CF lung may also play a role in the protection of biofilms from antibiotic challenge in the CF lung through aminoglycoside inactivation.

Furthermore, *P. aeruginosa* biofilms can generate an acidic microenvironment throughout the biofilm and, although this acidic environment may not be a natural defence mechanism, it is likely to further protect *P. aeruginosa* biofilms from aminoglycoside activity.[16]

In conclusion, this study has shown that growth in an acidic environment such as that which may be found in the CF lung reduces the activity of tobramycin but not ceftazidime against planktonically grown *P. aeruginosa*. Growth in an acidic environment was not shown to have an effect on the bactericidal activity of either antibiotic against the isolates growing in biofilm, with neither antibiotic, at concentrations greater than those achievable at the site of infection in the lungs, capable of eradicating biofilms formed at each pH. □

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