SD Apo Lactoferrin-Tobramycin/Gentamicin Combinations are superior to monotherapy in the eradication of *Pseudomonas aeruginosa* Biofilm in the lungs

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KEYWORDS: lactoferrin, apo lactoferrin, spray drying, biofilm, cystic fibrosis

Introduction

Chronic lung infections from the opportunistic pathogeen *Pseudomonas aeruginosa* has been recognised as a major contributor to the incidences of high morbidity and mortality amongst cystic fibrosis (CF) patients (1,2). Currently, strategies for managing lung infections in CF patients involves the aggressive use of aerosolised antibiotics (3), however, increasing evidence suggests that the biofilm component of *P. aeruginosa* in the lower airway remains unperturbed and is associated with the development of antibiotic resistance. If this is so then, there is an urgent need to suitably adjust the current treatment strategy so that it includes compounds that prevent biofilm formation or disrupt established biofilms.

It is well understood that biofilm formation is strongly dependent on iron (Fe^{3+}) availability (4), therefore aerosolised anti-infective formulations which has the ability to chelate iron may essentially be a well suited therapy for eliminating P. aeruginosa biofilms on CF airway epithelial cells (5).

In this study, we report the use of combination therapy; an aminoglycosides (tobramycin and gentamicin) and an antimicrobial peptide (lactoferrin) to significantly deplete P. aeruginosa biofilms. We demonstrate that lactoferrin-tobramycin and lactoferrin-gentamicin combinations are superior to the single antibiotic regime currently being employed to combat *P. aeruginosa* biofilms.

MATERIALS AND METHOD

Antibiotics: The antibiotics used in this study included gentamicin and tobramycin supplied by Fagron, UK.

Bacterial strain and growth conditions: Pseudomonas aeruginosa strain PAO1 was provided by Prof. Peter Lambert of Aston University, Birmingham UK. The Strains were routinely grown from storage in a medium supplemented with magnesium chloride, glucose and casamino acids.

Dialysis of lactoferrin: Apo lactoferrin was prepared by dialyzing a suspension of lactoferrin for 24 hrs at 4 °C against 20 mmol/L sodium dihydrogen phosphate, 20 mmol/L sodium acetate and 40 mmol/L EDTA (pH 3.5). Ferric ion (Fe^{3+}) removal was verified by atomic absorption spectroscopy measurements.

Spray drying of combinations of lactoferrin and apo lactoferrin with the different aminoglycosides: Combinations of tobramycin and gentamicin with the different preparations of lactoferrin were spray dried (SD) as a 2% (w/v) aqueous suspension. The spray drying parameters utilized for the production of suitable micron-sized particles includes: Inlet temperature, 180°C, spray flow rate, 606 L/hr; pump setting, 10%; aspirator setting, 85% (34m3/hr) to produce various outlet temperatures ranging from 99 - 106°C.

Viability assay: To test the bactericidal activity of the various combinations, a viability assay was performed as previously described by Xu, Xiong et al. (6) with some

modifications. Briefly, 10µL of ~ c. 6.6 x 10^7 CFU mL^{-1} *P. aeruginosa* strain PAO1 suspension were incubated (37°C, 60 mins) with 90 µL of a 2 µg/mL concentration of the various combinations and sampled every 10 mins. After incubation, the cells were diluted in deionised water and plated in Mueller hinton agar plates. Following 24 h incubation of the plates at 37°C, the percentage of viable cells was determined relative to incubation without added antibiotics.

Biofilm assay: To test the susceptibility of the *P. aeruginosa* strain to various antibiotics in the biofilms mode of growth, overnight cultures of *P. aeruginosa* were diluted 1:100 into fresh medium supplemented with magnesium chloride, glucose and casamino acids. Aliquots of the dilution were dispensed into a 96 well dish and incubated (37°C, 24 h). Excess broth was removed and the number of colony forming units per milliliter (CFU/mL) of the planktonic bacteria was quantified. The biofilms were then washed and stained with 0.1% (w/v) crystal violet for 15 mins at room temperature. Following vigorous washing with water, the stained biofilms were solubilized in 30% acetic acid and the absorbance at 550nm of a 125 µL aliquot was determined in a microplate reader (Multiskan spectrum, Thermo Scientific) using 30% acetic acid in water as the blank. Aliquots of the broth prior to staining were used as an indicator of the level of planktonic growth.

RESULTS AND DISCUSSION

Following spray drying, the mean yield, volume weighted mean diameter and moisture content of lactoferrin powder were measured and were as follows (Table 1 and table 2);

Table 1: Spray drying parameters

Formulation	Inlet temp (°C)	Outlet temp (°C)	Airflow rate (L/hr)	Mean yield (%)	Moisture content (%)
SD Lactoferrin	180	99 - 100	606	45.2 ±2.7	5.9 ±0.4
SD Apo Lactoferrin	180	100 - 102	606	57.8 ±1.8	5.7 ±0.2
Tobramycin	180	102 - 104	606	82.1 ±2.2	3.2 ±0.4
Lactoferrin + Tobramycin	180	104 - 106	606	87.5 ±1.4	3.7 ±0.2
Apo Lactoferrin + Tobramycin	180	103 - 104	606	76.3 ±2.4	3.3 ±0.5
Gentamicin	180	99 - 102	606	85.4 ±1.3	4.0 ±0.2
Lactoferrin + Gentamicin	180	102 - 104	606	87.3 ±2.1	3.9 ±0.3
Apo Lactoferrin + Gentamicin	180	99 -103	606	80.1±1.9	3.4 ±0.4

Table 2: Particle size distribution

	d10	d50	d90
SD Lactoferrin	1.38	4.91	11.08
SD Apo Lactoferrin	1.28	4.79	11.04
SD Tobramycin	1.25	4.90	11.29
SD Lactoferrin + Tobramycin	1.17	5.27	15.23
SD Apo Lactoferrin + Tobramycin	1.11	5.06	14.31
SD Gentamicin	1.40	6.06	14.38
SD Lactoferrin + Gentamicin	1.47	6.23	14.41
SD Apo Lactoferrin + Gentamicin	1.46	5.15	11.53

The bactericidal activity of the various combinations were tested against *P. aeruginosa* PAO1 following a 60 minute incubation period (Figure 1 and Figure 2). While 2 µg/mL of a 1:1 combination of spray dried apo lactoferrin and Gentamicin was able to completely kill all bacterial cells within 40 mins, the same concentration was not as effective for the other antibiotic combinations. However, there was an overall reduction of bacterial cells by over 3 log units by the other combinations within 60 mins.

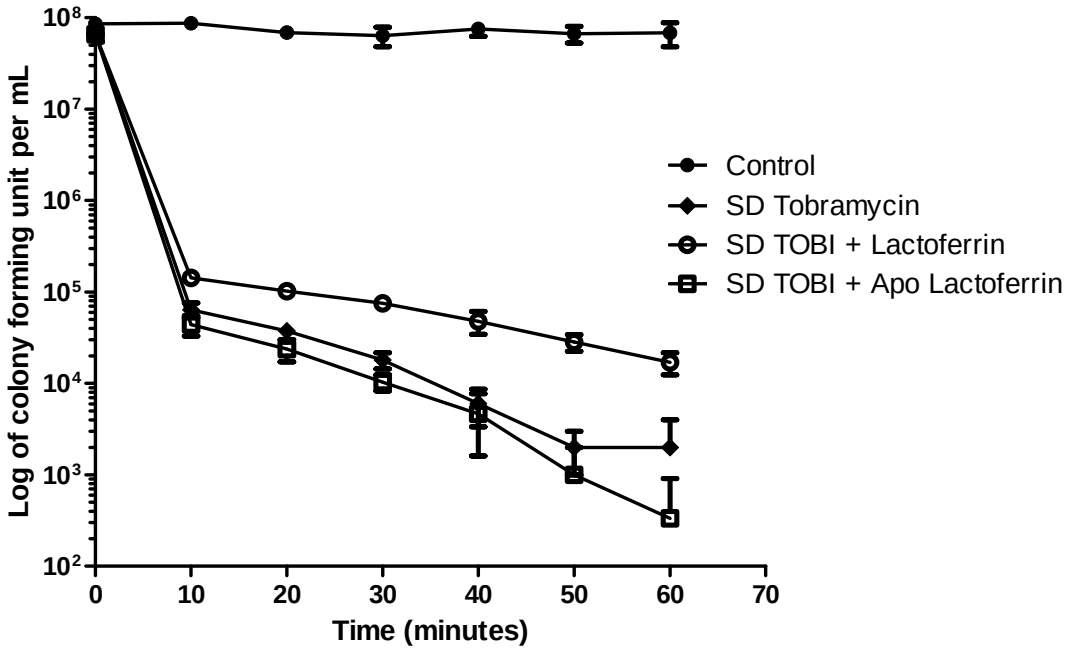

Figure 1: Logarithmic plot of bacterial cell viability of various combinations of tobramycin and lactoferrin preparations at 2µg/mL (n = 3).

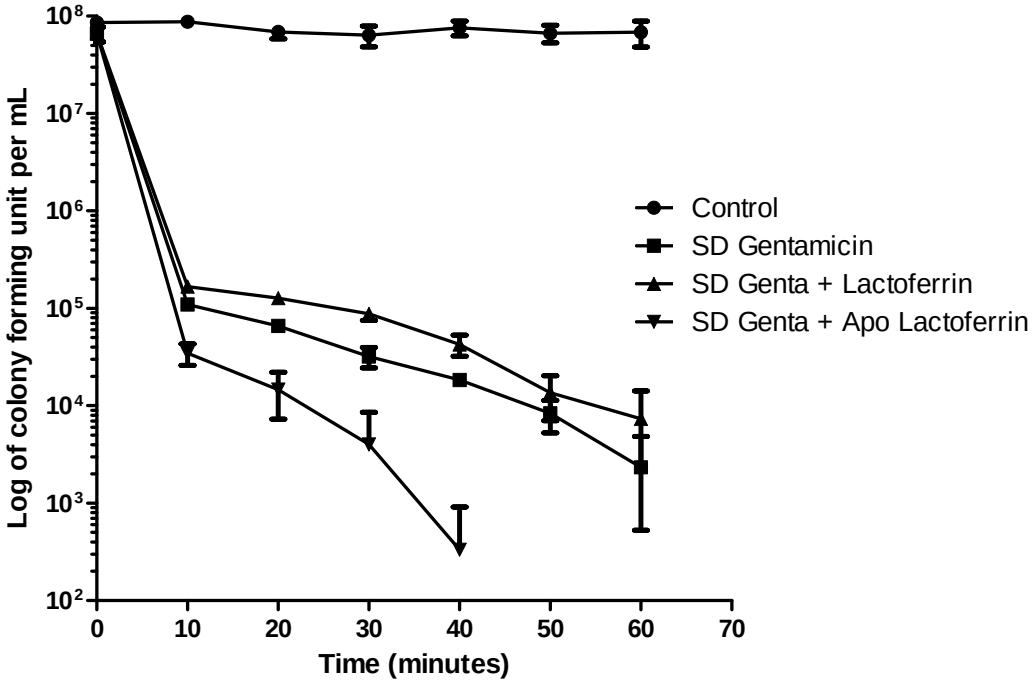

Figure 2: Logarithmic plot of bacterial cell viability of various combinations of gentamicin and lactoferrin preparations at 2µg/mL (n = 3).

Crystal violet staining showed that biofilm formation by *P. aeruginosa* PAO1 was significantly (ANOVA, $p < 0.05$) inhibited in the presence of the different lactoferrin preparations. Interestingly, apo lactoferrin and spray dried lactoferrin exhibited greater inhibition of both biofilm formation and biofilm persistence (Figure 2).

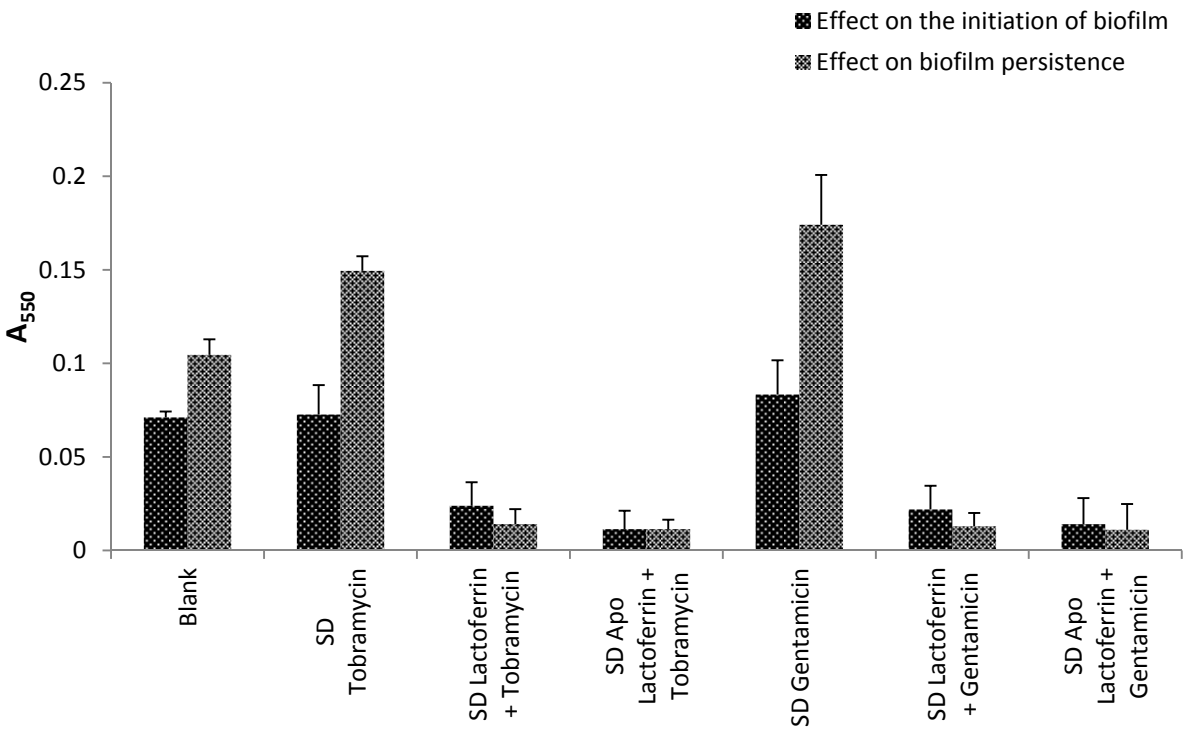

Figure 2: Crystal violet staining of residual biofilms of *P. aeruginosa* following a 24hr incubation with the various combinations of antibiotics and an exposure to 48 hr formed biofilms.

CONCLUSION

In conclusion, combination therapy comprising of an antimicrobial peptide (lactoferrin) and an aminoglycosides (tobramycin or gentamicin) provides a feasible and alternative approach to monotherapy since the various combinations are more efficient than the respective monotherapy in the eradication of both planktonic and biofilms of *P. aeruginosa.*

ACKNOWLEDGEMENT

The authors would like to thank Mr. John Swarbrick and Friesland Campina for their generous donation of the Lactoferrin.

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