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### Permalink

<https://escholarship.org/uc/item/2ms3f3bz>

### Journal

BMC Infectious Diseases, 4(1)

### ISSN

1471-2334

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### Publication Date

2004-12-01

### DOI

10.1186/1471-2334-4-12

Peer reviewed

Research article

Open Access

## Antimicrobial activity of innate immune molecules against *Streptococcus pneumoniae*, *Moraxella catarrhalis* and nontypeable *Haemophilus influenzae*

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Published: 05 May 2004

Received: 14 November 2003

BMC Infectious Diseases 2004, 4:12

Accepted: 05 May 2004

This article is available from: <http://www.biomedcentral.com/1471-2334/4/12>

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### Abstract

**Background:** Despite its direct connection to the nasopharynx which harbors otitis media pathogens as part of its normal flora, the middle ear cavity is kept free of these bacteria by as yet unknown mechanisms. Respiratory mucosal epithelia, including those of the middle ear and eustachian tube, secrete antimicrobial effectors including lysozyme, lactoferrin and  $\beta$  defensins-1 and -2. To elucidate the role of these innate immune molecules in the normal defense and maintenance of sterility of respiratory mucosa such as that of the middle ear, we assessed their effect on the respiratory pathogens nontypeable *Haemophilus influenzae* (NTHi) 12, *Moraxella catarrhalis* 035E, and *Streptococcus pneumoniae* 3, and 6B.

**Methods:** Two assay methods, the radial assay and the liquid broth assay, were employed for testing the antimicrobial activity of the molecules. This was done in order to minimize the possibility that the observed effects were artifacts of any single assay system employed. Also, transmission electron microscopy (TEM) was employed to evaluate the effect of antimicrobial innate immune molecules on OM pathogens. For the statistical analysis of the data, Student's t-test was performed.

**Results:** Results of the radial diffusion assay showed that  $\beta$  defensin-2 was active against all four OM pathogens tested, while treatment with  $\beta$  defensin-1 appeared to only affect *M. catarrhalis*. The radial assay results also showed that lysozyme was quite effective against *S. pneumoniae* 3 and 6B and was partially bacteriostatic/bactericidal against *M. catarrhalis*. Lysozyme however, appeared not to affect the growth of NTHi. Thus, lysozyme seems to have a more pronounced impact on the growth of the Gram-positive *S. pneumoniae* as compared to that of Gram-negative pathogens. Lactoferrin on the other hand, enhanced the growth of the bacteria tested. The results of the radial

assays were confirmed using liquid broth assays for antimicrobial activity, and showed that lysozyme and  $\beta$  defensin-2 could act synergistically against *S. pneumoniae* 6B. Moreover, in the liquid broth assay,  $\beta$  defensin-1 showed a modest inhibitory effect on the growth of *S. pneumoniae* 6B. As assessed by ultrastructural analysis, lysozyme and  $\beta$  defensin-2, and to a much lesser extent,  $\beta$  defensin-1, appeared to be able to cause damage to the bacterial membranes.

**Conclusions:** Here we report that lysozyme and the  $\beta$  defensins can inhibit the growth of clinical isolates of otitis media pathogens – namely NTHi strain 12, *S. pneumoniae* strains 3 and 6B and *M. catarrhalis* strain 035E – and cause ultrastructural damage to these pathogens. Moreover, we demonstrate that lysozyme and  $\beta$  defensin-2 can act synergistically against *S. pneumoniae*. These findings are consistent with the concept that secreted antimicrobial peptides and other components of innate immunity constitute the first line of defense protecting host mucosal surfaces, including the tubotympanal (eustachian tube and middle ear cavity) mucosa, against pathogens.

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## Background

Otitis media (OM) is the most prevalent mucosal infectious disease affecting young children, second only to the common cold [1]. OM is also the major cause of conductive hearing loss among this group and the leading indication for antibiotic therapy [1]. OM results in 31 million annual visits to physicians' offices and is estimated to have a yearly cost exceeding \$5 billion.

Most cases of OM are caused by three major pathogens: *S. pneumoniae* (30–40%), nontypeable *Haemophilus influenzae* (NTHi, 30%) and *M. catarrhalis* (20%) [1,2]. These organisms are also pathogens for sinusitis and chronic obstructive pulmonary disease (COPD) [3]. In the past three decades, there has been a dramatic worldwide increase in antibiotic resistance in respiratory pathogens [4,5]. This has resulted in a reduction of the number of effective antibiotics for OM and has begun to pose a major public health threat. There is, thus, an urgent need to develop new and innovative non-antibiotic approaches to prevent and manage this disease. To this end, it is imperative to understand the mechanisms that defend the tubotympanum (the middle ear and eustachian tube) against invading pathogens and determine if the defense mechanisms can be augmented to prevent or treat OM.

The pathogenesis of OM is multi-factorial with risk factors such as delayed development of the adaptive immune system, complement system abnormality, as well as other environmental and genetic factors playing an important role [6-10]. Although anatomic immaturity of the eustachian tube and delayed development of systemic immunity are considered to be the most important factors underlying OM susceptibility among young children, other factors may be of greater importance before the adaptive immune system is fully matured [1,8]. For example, in a study of children with recurrent acute otitis media (rAOM), Prellner and colleagues showed that only 1/3 of two-year-old children with rAOM had significantly lower

levels of antibodies against the pneumococcal capsular type 19F. This suggests that factors other than circulating antibody titers affect susceptibility to rAOM [8]. One group of factors that may be of particular relevance to OM susceptibility consists of the innate immune molecules, including the defensins, lysozyme and lactoferrin [11-16], as well as the pathogen recognition receptors such as the toll-like receptors (TLR) [3,17]. Many studies have demonstrated the presence of lysozyme and lactoferrin in chronic middle ear effusion [18-21] and recent work has shown that TLR-2, TLR-4, the defensins, lysozyme and lactoferrin are expressed, albeit at different levels, in normal middle ear epithelial cells, as well as in those cells from patients with chronic otitis media with effusion (OME) [3,17,22-24]. The exact role however, that these molecules play in normal mucosal defense and maintenance of tympanic cavity sterility as well as in the OM susceptibility has not yet been clearly elucidated.

Here we report that lysozyme and the  $\beta$  defensins can inhibit the growth of clinical isolates of otitis media pathogens, namely, NTHi 12, *M. catarrhalis* 035E, and *S. pneumoniae* 3, and 6B and cause ultrastructural damage to these pathogens. Moreover, we demonstrate that lysozyme and  $\beta$  defensin-2 can act synergistically against *S. pneumoniae*.

## Methods

### Bacteria

All bacteria used in this study were clinical isolates.

### NTHi and *M. catarrhalis*

Stocks of NTHi 12 (obtained from Dr. Steve Barenkamp, St. Louis University, School of Medicine) and *M. catarrhalis* 035E (obtained from Dr. Eric Hansen, University of Texas, Southwestern Medical Center) were maintained at -80°C. The bacteria were plated on chocolate agar and incubated overnight at 37°C in 5% CO<sub>2</sub>. A single colony was used to inoculate 10 ml of brain heart infusion (BHI,

Becton Dickinson, Cockeysville, MD), supplemented with hemin (10 µg/ml, Sigma, St. Louis, MO) and nicotinamide adenine dinucleotide (NAD, 10 µg/ml, Sigma, St. Louis, MO), and allowed to grow overnight. In the morning, 1/10 volume of the overnight culture was transferred to fresh medium and incubated for 3 hours. The subculture was then washed twice with 10 mM sodium phosphate and the optical density (O.D.) at 620 nm was determined.

#### *S. pneumoniae*

For the *S. pneumoniae* 3 and 6B (obtained from American Type Culture Collection, ATCC), a 50 µl aliquot of the frozen stock was thawed on ice, added to 10 ml of Todd-Hewitt broth (THB, Becton Dickinson, Cockeysville, MD) and cultured overnight. One tenth of the volume of the overnight culture was then transferred to fresh medium and incubated for 3 hours. The subculture was then washed twice with 10 mM sodium phosphate and the O.D. at 620 nm was determined.

### **Testing of antimicrobial activity**

#### *Radial assay*

The radial assay method of Lehrer and coworkers was used, with minor modifications [39]. The subcultured bacteria ( $4 \times 10^6$  CFU/10 ml underlay gel for NTHi and *M. catarrhalis* and  $10^6$  CFU/10 ml underlay gel for *S. pneumoniae*) were mixed with melted underlay agarose medium at 42°C (0.1X culture broth – BHI for NTHi and *M. catarrhalis* and THB for *S. pneumoniae*- 10 mM sodium phosphate, 0.8% low electroendosmosis (EEO)-type agarose) and poured into 8 cm × 8 cm square petri plates. The gel was allowed to solidify in the petri plates and wells were punched out with a cork borer (4 mm, VWR Scientific, West Chester, PA). The antimicrobial peptides and proteins, human milk lysozyme (Sigma, St. Louis, MO), human milk apo-lactoferrin (0.03% iron content, Sigma, St. Louis, MO), human β defensin-1 and human β defensin-2 (Peptides International, Louisville, KY), were dissolved in 0.01% acetic acid/0.1% bovine serum albumin (Sigma, St. Louis, MO) and 4 µl was added to each well. The plates were then incubated for 3 hours at 37°C in the 5% CO<sub>2</sub> incubator. They were next overlaid with the overlay agarose medium (0.5X culture broth, 10 mM sodium phosphate, 0.8% low EEO-type agarose) and covered. The plates were then allowed to incubate overnight at 37°C in a 5% CO<sub>2</sub>. The diameter of the zones of inhibition produced by each of the antimicrobial molecules against the OM pathogens (NTHi, *M. catarrhalis* and *S. pneumoniae*) was then measured in three separate experiments and the mean and standard deviation were calculated. To analyze the salt dependency of the activity of antimicrobial molecules against OM pathogens, NaCl was added to the underlay gel solution to obtain a final NaCl concentration of 100 mM.

#### *Antibacterial liquid broth assay*

For the antibacterial liquid broth assays, 1/10 of the volume of each overnight culture was transferred to fresh medium and allowed to grow to mid-log phase. The bacteria were then exposed to various concentrations of lysozyme, β defensin-1, β defensin-2 or a combination of the lysozyme and β defensin-2 molecules for 3 h at 37°C. Cell viability was measured by plating several dilutions and counting the number of colonies after and overnight incubation. The assay was done in triplicate for each concentration of test molecules.

#### **Statistical analysis**

Student's *t*-test was used to perform statistical analysis of the data.

#### **Electron microscopy**

Transmission electron microscopy (TEM) was employed to evaluate the effect of antimicrobial innate immune molecules on OM pathogens. The bacterial cultures treated with antimicrobial proteins or peptides for 30 minutes (β defensin-1 treatment was for 3 hours) were mixed with an equal volume of 5% buffered glutaraldehyde (pH 7.4) and centrifuged (5000 × g, for 20 minutes). The bacterial pellets were left in the fixative at 4°C for 2 hr, post-fixed in 2% osmium tetroxide, dehydrated in graded ethanol and embedded in Eponate 12 (Ted Pella, Inc. Redding, CA). Thin sections (70 µm) were mounted on coated specimen grids, contrasted with uranyl acetate and lead citrate and examined in a transmission electron microscope (CM120 BioTwin, FEI-Philips, Hillsboro, OR) operating at 80 kV.

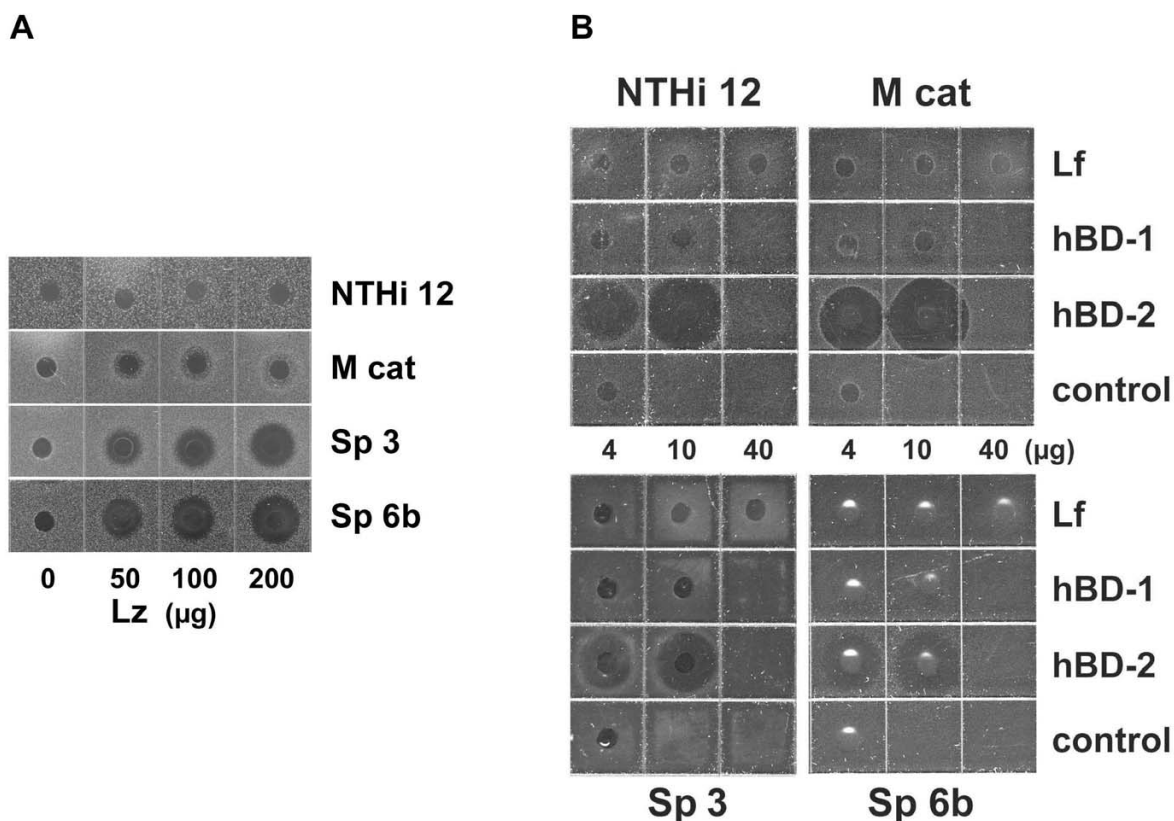
### **Results**

#### **Antimicrobial activity of innate immune molecules against OM pathogens**

We tested the bacteriostatic/bactericidal effects of the innate immune molecules on four clinically relevant otitis media pathogens: NTHi 12, *M. catarrhalis* 035E and *S. pneumoniae* 3 and 6B. Two assay methods, the radial assay and the liquid broth assay, were employed for testing the antimicrobial activity of the molecules. This was done in order to minimize the possibility that the observed effects were artifacts of any single assay system employed.

#### *Radial assay*

The results of the radial assay indicated that the treatment of the bacteria with lysozyme resulted in a visible inhibition of the growth of *M. catarrhalis* and *S. pneumoniae* 3 and 6B (Figure 1A and Table 1). This effect was seen at concentrations of 12.5 mg/ml (50 µg of the protein per well) and higher. There appeared to be a dose-dependent increase in the effect of lysozyme on the growth of *S. pneumoniae* and *M. catarrhalis*, although the dose dependence of the effect was not as apparent with the latter pathogen.



**Figure 1**  
**Radial inhibition assay results demonstrating the effect of innate immune molecules on otitis media pathogens.** Representative radial inhibition assay for the measurement of the activity of innate immune molecules against NTHi 12, *M. catarrhalis* 035E (M cat), *S. pneumoniae* 3 (Sp 3) and *S. pneumoniae* 6B (Sp 6B). Panel A shows the results of testing three doses (50 µg, 100 µg and 200 µg) of human lysozyme (Lz) against the bacteria. Panel B shows the results of testing the lactoferrin (Lf) and the defensins against the bacteria. Three doses (4 µg, 10 µg and 40 µg) of human lactoferrin (Lf) were tested, as were two doses (4 µg and 10 µg) each of human β defensin-1 (hBD-1) and β defensin-2 (hBD-2). Each dose was delivered in a total volume of 4 µl. The control well received only solvent (0.01% acetic acid). The diameter of the inhibition zone was measured and averaged in three separate experiments.

Lysozyme appeared not to affect the growth of NTHi (Figure 1A and Table 1). Thus, lysozyme appears to have a more pronounced impact on the growth of the Gram-positive *S. pneumoniae* compared to the Gram-negative pathogens (Figure 1A and Table 1).

It should be noted that the concentration of lysozyme decreases as it diffuses radially into the three-dimensional gel matrix. Moreover, the concentration of lysozyme ranges from 1.1 mg/ml in serous middle ear effusions to 3.71 mg/ml in mucoid in middle ear effusions [20]. Thus, once dilution as a result of diffusion is taken into account the amounts used in our assays are likely to give near physiologic levels of this protein in the gel matrix.

Apo-lactoferrin treatment did not inhibit bacterial growth, and appeared to result in an enhancement of the growth of all bacteria tested at 40 µg dosage (Figure 1B and Table 1).

Treatment with β defensin-1 was effective only against *M. catarrhalis* and only at the high dose (10 µg). In contrast, β defensin-2, at 4 µg and 10 µg, significantly inhibited the growth of all four bacterial types (Figure 1B and Table 1). The 40 µg dose of β defensin-2 was not tested because the effects were already observable at the lower doses. Likewise, because β defensin-1 showed an effect against *M. catarrhalis* at 10 µg, a higher dose was not tested. *M. catarrhalis* 035E was most susceptible to β defensin-2, followed by NTHi 12, *S. pneumoniae* 3 and *S. pneumoniae* 6B.

**Table 1: Measurements of the effect of human lysozyme (hLz), lactoferrin (hLf),  $\beta$  defensin-1 (HBD-1) and  $\beta$  defensin-2 (HBD-2) on the growth of otitis media pathogens, using the radial inhibition assay.**

Molecule	Total amount of protein/peptide added (in a total volume of 4 $\mu$ l)	Bacteria			
		NTHi	<i>M. catarrhalis</i>	<i>S. pneumoniae</i> 3	<i>S. pneumoniae</i> 6B
hLz	50 $\mu$ g	-	4.6 $\pm$ 0.4 (P)	6.2 $\pm$ 0.7 (C)	6.7 $\pm$ 0.9 (C)
	100 $\mu$ g	-	5.6 $\pm$ 0.7 (P)	7.2 $\pm$ 0.5 (C)	7.4 $\pm$ 0.6 (C)
	200 $\mu$ g	-	6.2 $\pm$ 0.5 (P)	7.7 $\pm$ 0.6 (C)	8.2 $\pm$ 0.7 (C)
hLf	4 $\mu$ g	-	-	-	-
	10 $\mu$ g	E	-	E	-
	40 $\mu$ g	E	E	E	E
HBD-1	4 $\mu$ g	-	-	-	-
	10 $\mu$ g	-	7.7 $\pm$ 0.6 (P)	-	-
	40 $\mu$ g	ND	ND	ND	ND
HBD-2	4 $\mu$ g	9.7 $\pm$ 2.5 (C)	11.7 $\pm$ 0.6 (C)	8.7 $\pm$ 1.5 (C)	8.7 $\pm$ 1.2 (C)
	10 $\mu$ g	12.3 $\pm$ 2.9 (C)	14.7 $\pm$ 0.6 (C)	10 $\pm$ 1.3 (C)	10.7 $\pm$ 1.5 (C)
	40 $\mu$ g	ND	ND	ND	ND

Diameter (mm) of the inhibition zone (average of 3 separate experiments  $\pm$  SD) caused by the different concentrations of the antimicrobial molecules is given in the column below each pathogen. -: No effect; E: Enhanced growth; C: Complete inhibition; P: Partial inhibition; ND: Not done

**Table 2: Measurements of the effect of 100 mM NaCl on the inhibition of the growth of otitis media pathogens by human lysozyme (hLz), lactoferrin (hLf),  $\beta$  defensin-1 (HBD-1) and  $\beta$  defensin-2 (HBD-2), using the radial inhibition assay.**

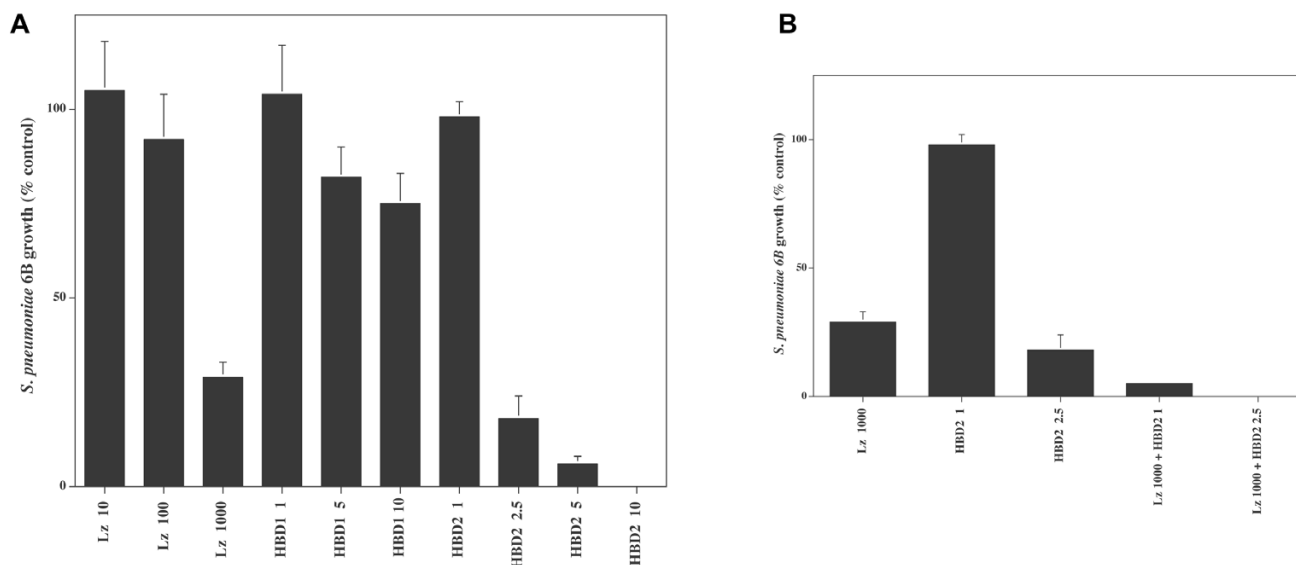
Molecule	Total amount of protein/peptide added (in a total volume of 4 $\mu$ l)	Bacteria			
		NTHi	<i>M. catarrhalis</i>	<i>S. pneumoniae</i> 3	<i>S. pneumoniae</i> 6B
hLz	4 $\mu$ g	-	-	-	-
	10 $\mu$ g	-	-	-	-
	40 $\mu$ g	-	-	-	-
hLf	4 $\mu$ g	-	-	-	-
	10 $\mu$ g	-	-	-	-
	40 $\mu$ g	-	-	-	-
HBD-1	4 $\mu$ g	-	-	-	-
	10 $\mu$ g	-	-	-	-
	40 $\mu$ g	ND	ND	ND	ND
HBD-2	4 $\mu$ g	8.8 $\pm$ 1.0 (C)	9.0 $\pm$ 0.7 (C)	-	-
	10 $\mu$ g	11.3 $\pm$ 0.3 (C)	11.0 $\pm$ 0.7 (C)	-	-
	40 $\mu$ g	ND	ND	ND	ND

Diameter (mm) of the inhibition zone (average of 3 separate experiments  $\pm$  SD) caused by the different concentrations of the antimicrobial molecules is given in the column below each pathogen. -: No effect; ND: Not done

In order to test the effect of high salt concentration on the effectiveness of the antimicrobial molecules, NaCl was added to the gel to obtain a final concentration of 100 mM. As shown in Table 2, 100 mM salt completely blocked the growth inhibitory effect of lysozyme and  $\beta$  defensin-1 against all bacteria tested. It also blocked the effect of  $\beta$  defensin-2 against *S. pneumoniae* 3 and *S. pneumoniae* 6B. High salt concentration however, did not inhibit the activity of  $\beta$  defensin-2 against NTHi and *M. catarrhalis*, although it did result in a reduction of this effect.

#### Antimicrobial liquid broth assay

The results of the radial assays were confirmed by performing liquid broth antimicrobial assays using lysozyme and  $\beta$  defensin-2. Because of the robust antimicrobial activity of the two molecules against *S. pneumoniae* 3 and 6B, we chose one of these, 6B, as a test organism to validate the result of the radial assay. As shown in Figure 2A, 1  $\mu$ g/ml of  $\beta$  defensin-2 did not demonstrate any significant antimicrobial activity against *S. pneumoniae* 6B, while at concentrations of 2.5  $\mu$ g/ml and 5  $\mu$ g/ml, it inhibited growth by 80% and 95%, respectively. At a concentration

**Figure 2****Liquid broth assay results demonstrating the susceptibility of *S. pneumoniae* to lysozyme and the  $\beta$  defensins.**

Antibacterial liquid broth assays for the measurement of the activity of lysozyme (Lz) and  $\beta$  defensins-1 and -2 (HBD1 and HBD2), alone (A) and in combination (B) (Lz+HBD2), against *S. pneumoniae* 6B (Sp 6B). The combination of lysozyme (1000  $\mu$ g/ml) and  $\beta$  defensin-2 (1  $\mu$ g/ml or 2.5  $\mu$ g/ml) was significantly more effective than lysozyme (1000  $\mu$ g/ml), or  $\beta$  defensin-2 (1  $\mu$ g/ml or 2.5  $\mu$ g/ml) alone. ( $P = 0.008$  and  $0.005$ , respectively, for the difference in the effect of the Lz-HBD2 combinations versus Lz (1 mg/ml) alone.  $P = 0.0007$  and  $0.03$  for the difference in the effect of the Lz-HBD2 combinations versus 2.5  $\mu$ g/ml of  $\beta$  defensin-2 alone). Four concentrations (1  $\mu$ g/ml, 2.5  $\mu$ g/ml, 5  $\mu$ g/ml and 10  $\mu$ g) of human  $\beta$  defensin-2 (HBD-2) and one concentration (1 mg/ml) of lysozyme (Lz) were tested against the bacteria – either alone, or in combination.

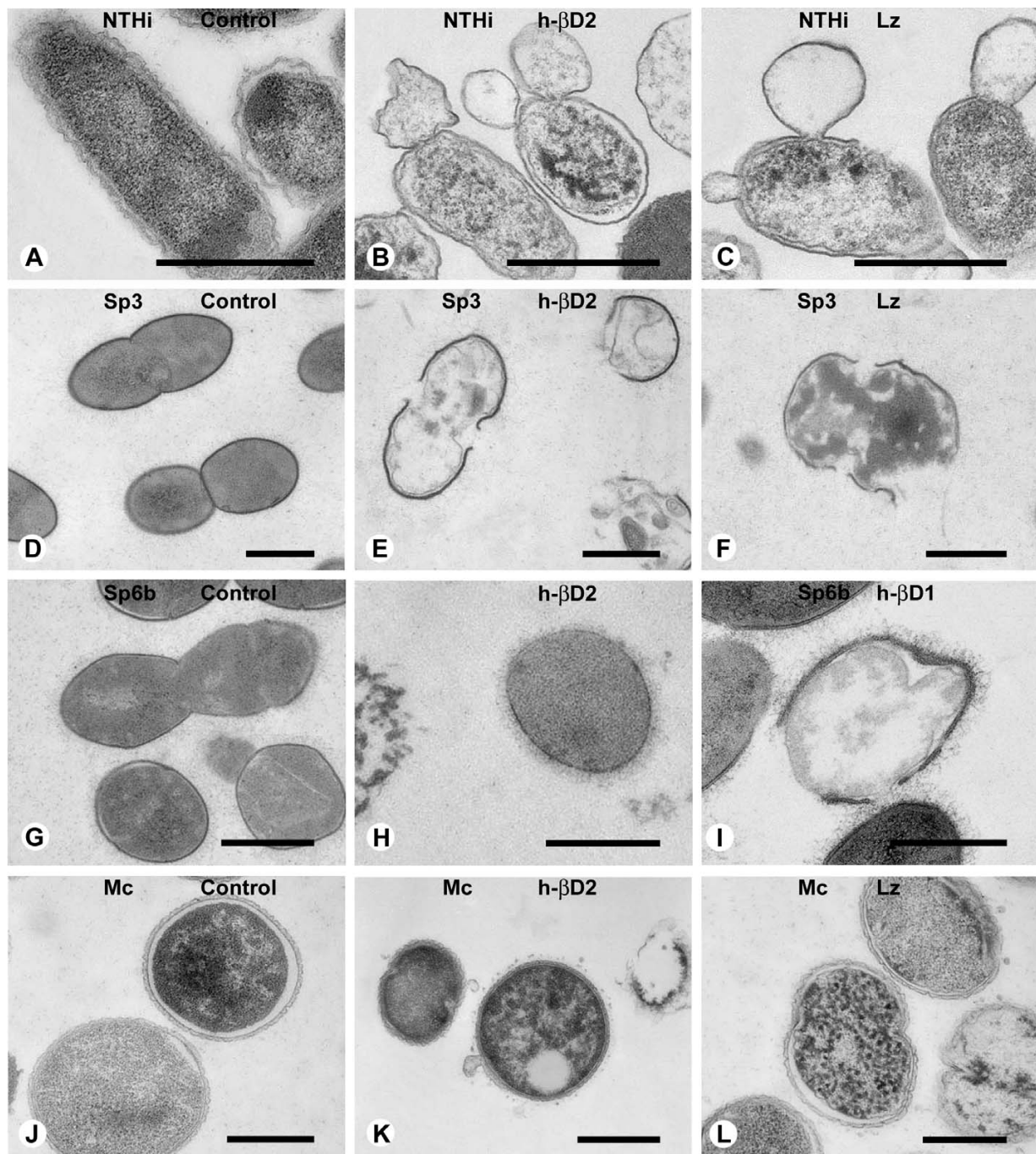
of 10  $\mu$ g/ml,  $\beta$  defensin-2 completely inhibited the growth of the bacteria. Beta defensin-1, on the other hand, did not have a significant growth inhibitory effect at concentrations of 1 and 5  $\mu$ g/ml, but inhibited the growth of *S. pneumoniae* 6B by 25% at a concentration of 10  $\mu$ g/ml. Lysozyme also had a significant impact on bacterial growth and at a concentration of 1 mg/ml resulted in a 70% inhibition of growth (Figure 2A). As shown in Figure 2B, a combination of  $\beta$  defensin-2 (1  $\mu$ g/ml or 2.5  $\mu$ g/ml) and lysozyme (1 mg/ml) appeared to have a significant synergistic effect and resulted in 95–100% inhibition of bacterial growth.

The compatibility of the results obtained by the two assay methods suggests that the radial assay, which is simpler to perform than the liquid broth assay, is indeed a suitable methodology for studying the antimicrobial activity of innate immune molecules.

**Ultrastructural changes in OM pathogens after treatment with lysozyme and  $\beta$  defensin-2**

Ultrastructural analysis of NTHi 12, *S. pneumoniae* 3, *S. pneumoniae* 6B and *M. catarrhalis* treated in solution with

the antimicrobial molecules revealed that changes occurred in the bacteria following exposure to  $\beta$  defensin-2 or lysozyme. The morphology of the untreated NTHi is shown in Figure 3A. Figures 3B and 3C show the morphology of NTHi treated with 10  $\mu$ g/ml  $\beta$  defensin-2 for 30 minute, and 1 mg/ml of human milk lysozyme for 30 minute, respectively. It should be noted that lysozyme, at concentrations lower than 1 mg/ml, had no observable effect on the ultrastructure of the bacteria (data not shown). In the presence of  $\beta$  defensin-2 or lysozyme, NTHi show blebbing of the membranes with extrusion of the cytoplasmic contents into the blebs (Figures 3B and 3C). The percentage of profiles showing membrane "blebbing" was estimated by examination of approximately 100 bacterial profiles using systematic random sampling methods [25]. With  $\beta$  defensin-2 treatment, at least 30% of the bacteria showed signs of membrane damage, while with lysozyme only 5% of the bacteria showed blebbing. NTHi, incubated in liquid broth alone for 30 min, showed blebbing in approximately 5% of the cases. This low incidence of blebbing in the untreated bacteria suggests that the observed changes in treated bacteria were not fixation

**Figure 3**

**Ultrastructural changes in OM pathogens treated with innate immune molecules.** Panels A, B and C are electron micrographs showing the experimental results with NTHi. Untreated NTHi are shown in panel A. Bacteria were treated for 30 minutes with  $\beta$  defensin-2 (10  $\mu$ g/ml) (panel B), or with 1 mg/ml human milk lysozyme (panel C). Bar = 0.5 micron. Panels D, E and F show results of experiments with *S. pneumoniae* 3. Untreated bacteria are shown in panel D. Bacteria were treated for 30 minutes with human  $\beta$  defensin-2 (10  $\mu$ g/ml) (panel E), or with 1 mg/ml human milk lysozyme (panel F). Bar = 0.5 micron. Panels G, H and I show the results of experiments *S. pneumoniae* 6B. Untreated bacteria are shown in panel G. Bacteria treated for 30 minutes with human  $\beta$  defensin-2 (10  $\mu$ g/ml) are shown in panel H and those treated for 3 hours with human  $\beta$  defensin-1 (10  $\mu$ g/ml) are shown in panel I. Bar = 0.5 micron. Panels J, K and L show experiments results with *M. catarrhalis*. Untreated bacteria are shown in panel J. Results of a 30-minute incubation with  $\beta$  defensin-2 are shown in panel K and those of a 30 minute treatment of the bacteria with lysozyme are shown in panel L. Bar = 0.5 micron.



artifacts. Also, the difference in the percentage of damaged bacteria could account for the absence of lysozyme's growth inhibitory effect on NTHi in the radial assay.

Treatment of *S. pneumoniae* 3 with  $\beta$  defensin-2 or lysozyme also resulted in damage to the bacteria, although the former molecule appeared to be much more potent. The untreated bacteria are shown in Figure 3D, while Figures 3E and 3F show the effect of a 30 minute treatment with  $\beta$  defensin-2 and lysozyme, respectively. As seen in Figure 3E, treatment with  $\beta$  defensin-2 results in the disruption of the cell membrane and the lysis of the bacteria. Although lysozyme had a similar effect, fewer bacteria appear to be lysed, consistent with the results of the radial assay.

Human  $\beta$  defensin-2 showed activity against *S. pneumoniae* 6B as well. A 30 minute treatment with  $\beta$  defensin-2, resulted in damage to the cell membrane and condensation of the cytoplasmic material (Figure 3H). Attempts to evaluate the ultrastructural effect of lysozyme on *S. pneumoniae* 6B were unsuccessful because the bacteria appeared to have been fully lysed by lysozyme. Treatment of *S. pneumoniae* 6B with 10  $\mu$ g of human  $\beta$  defensin-1 for 3 hours had a relatively small effect on the ultrastructure of the bacteria (Figure 3I). Based on the enumeration of lysed *S. pneumoniae* 6B in a random sampling of multiple electron micrographs, less than 6% of the bacteria appeared to have been lysed by this treatment, consistent with the modest reduction in growth observed in the solution assay (data not shown).

The effect of  $\beta$  defensin-2 or lysozyme on *M. catarrhalis* was also tested. Untreated *M. catarrhalis* are shown in Figure 3J. As shown in Figure 3K, a 30 minute incubation of *M. catarrhalis* with  $\beta$  defensin-2 resulted in substantial damage to the cells. Results of a 30 minute treatment of the bacteria with lysozyme are shown in Figure 3L and suggest that this treatment can also result in the formation of blebs. Samples from later time points, however, showed no evidence of blebs and, as expected, contained more lysed bacteria (data not shown).

## Discussion

Respiratory mucosal epithelia, including the epithelium of the middle ear, paranasal sinuses and lung, serve as an effective barrier against pathogen invasion and help to maintain the sterility of these sites. Innate immune molecules produced by the epithelial cells provide the host with a constitutive or immediately-inducible defense system that is capable of effectively dealing with the continuous attacks of a variety of pathogens at the mucosal epithelial surfaces. Under normal conditions, the middle ear of humans and laboratory animals remains sterile, although OM pathogens, which are part of the normal

nasopharyngeal flora, can reach the middle ear via the eustachian tube [26]. Furthermore, non-inflamed tubal and middle ear mucosa have been shown to contain relatively few immunocytes [7,27]. These findings suggest that the components of the innate immune system may be important in the defense of the tubotympanum (middle ear and eustachian tube). Furthermore, as the development of adaptive immunity occurs in late childhood, innate immune molecules are likely to play an especially important role in protecting infants and young children against OM pathogens. Consistent with the hypothesis that innate immune molecules protect the tubotympanum during the neonatal and early postnatal periods, Park and Lim [15] showed that the number of secretory cells expressing lysozyme was similar in the epithelium and glandular structures of the eustachian tube of both adult and early post-natal animals. Innate immune molecules may, however, also be important for keeping the tubotympanum sterile later in life, by serving as a first line of defense even after the adaptive immune system has fully matured.

As little is known about the antimicrobial activity of innate immune molecules against respiratory pathogens, in the present study we analyzed the effect of lysozyme, lactoferrin,  $\beta$  defensin-1 and  $\beta$  defensin-2 on the growth of known OM pathogens. The bacteria used in the present study were chosen on the basis of their clinical relevance in OM and other respiratory infections. NTHi 12 and *M. catarrhalis* 035E are middle ear effusion clinical isolates that have been used extensively in vaccine studies [28], while *S. pneumoniae* 6B and 3 are prevalent OM pathogens and have been isolated from middle ear effusions in as many as 18.7% and 8.7%, respectively, of children in the US [29].

The results of our studies suggest that lysozyme,  $\beta$  defensin-1 and  $\beta$  defensin-2 have activity against the above pathogens. Moreover, lysozyme and  $\beta$  defensin-2 appeared to have a profound synergistic effect against *S. pneumoniae* 6B. These results are consistent with our previous observations and suggest that alone, or in combination, innate immune molecules can be effective against several clinically relevant OM pathogens [13].

Of the four molecules tested in the radial inhibition assay,  $\beta$  defensin-2 displayed the highest potency and was effective against all pathogens tested. These results were confirmed using the liquid broth assay and additionally showed that  $\beta$  defensin-2 had a potent, dose-dependent effect on the growth of *S. pneumoniae*, even at concentrations as low as 2.5  $\mu$ g/ml. Previous studies have shown the LD<sub>90</sub> of  $\beta$  defensin-2 (the dose that achieves 90% reduction of colony-forming units) to be between 4 and 10  $\mu$ g/ml for Gram negative bacteria and as high as 100  $\mu$ g/ml

for Gram positive bacteria [30,31]. Other studies have shown the minimal inhibitory concentrations of  $\beta$  defensin-2 for *E. coli* to be between 4 and 62  $\mu\text{g/ml}$  [32].  $\beta$  defensin-2 levels in the saliva and gingival crevicular fluid have been shown to be approximately 150 ng/ml and 75–175 ng/ml, respectively [33]. The normal plasma concentration of  $\beta$  defensin-2 has been shown to be in the 30 pg/ml range and its epidermal concentration is estimated to be between 3.5–16  $\mu\text{M}$  [31,32]. It should be noted, however, that local production of  $\beta$  defensin-2 and its secretion into the periciliary fluid underneath the mucous blanket is likely to result in higher concentrations of this molecule on mucosal surfaces protected by the mucociliary system.

$\beta$  defensin-2 is produced by epithelial cells following contact with microorganisms, or exposure to cytokines such as TNF- $\alpha$  and IL-1 [34,35]. It has been shown to exhibit potent antimicrobial activity against Gram-negative bacteria and *Candida*, but was less effective against Gram-positive *Staphylococcus aureus* [36,37]. We recently showed that  $\beta$  defensin-2 could inhibit the growth of NTHi [13]. Our observations were later confirmed by Starner and colleagues [38] who showed that  $\beta$  defensins-2 and 3 could kill NTHi.  $\beta$  defensin-2 functions as a NF- $\kappa\text{B}$  target gene in the middle ear epithelium and blocking NF- $\kappa\text{B}$  activation inhibits the up-regulation of human  $\beta$  defensin-2 in response to IL-1 $\alpha$  stimulation or bacterial infection [24]. Although the effect of  $\beta$  defensin-2 on NTHi was consistent with our previous results [13] and those obtained by Starner and coworkers [38], to our knowledge, its effect on *M. catarrhalis* and *S. pneumoniae* has not been previously reported.

In contrast to  $\beta$  defensin-2,  $\beta$  defensin-1 had a relatively minor impact on the growth of the respiratory pathogens.  $\beta$  defensin-1 is not upregulated by inflammatory stimuli, is predominantly expressed in the distal tubules of the kidney and in the digestive and reproductive tracts and serves as a defense molecule in the absence of inflammation [34,39-41]. Consistent with previous observations of the antimicrobial activities of this molecule against Gram-negative bacteria [36],  $\beta$  defensin-1 did show some activity against *M. catarrhalis* in the radial diffusion assay. In the liquid broth assay however, it also showed activity against *S. pneumoniae* and caused a 25% inhibition of the growth of this bacterium. When the damage to the  $\beta$  defensin-1 treated *S. pneumoniae* was assessed by electron microscopy, it was seen that only a small fraction of the bacteria (less than 6%) were affected by the treatment. Such a modest effect is unlikely to be observable in the radial diffusion assay, although, as our results demonstrate, it is detectable in the liquid broth assay. Regardless, the results of both assays indicate that relatively high concentrations of  $\beta$  defensin-1 may be required for its anti-

microbial effects and are consistent with the idea that this molecule may be acting in concert with other components of the innate immune system in order to be most effective. As expected, defensin molecules showed salt sensitivity and were inhibited by 100 mM salt, as previously reported [42]. But, as the defensins have been shown to display a concentration-dependent resistance to salt inactivation [43], we believe that they will be active *in vivo* where their high local concentration in the periciliary fluid space will be sufficient to overcome the salt effect despite the presence of physiological levels of NaCl.

Lysozyme is an important component of innate immunity against pathogens at mucosal surfaces, including the middle ear, where it is produced by the secretory cells of the epithelium, as well as by neutrophils and macrophages [12,19,44-46]. In studies by Liu and coworkers [20] they showed that chronic mucoid middle ear effusions contained on average 3.71 mg/ml of lysozyme, while chronic serous middle ear effusions contained 1.1 mg/ml of this protein. Moreover, their results suggested that the majority of the lysozyme produced did not come from recruited macrophages and neutrophils, but from local sources such as epithelial cells. The presence of lysozyme in serous cells of the tubal glands and the secretory epithelial cells [18] also suggests an important role for this molecule in the defense of the tubotympanum against pathogens [21].

Lysozyme is a muramidase that cleaves the glycan backbone by catalyzing the hydrolysis of 1–4-glycosidic bonds between N-acetylmuraminic acid and N-acetyl-D-glucosamine, which are constituents of the cell walls of most bacteria. The amount of lysozyme used in the liquid broth (1 mg/ml) was not outside the physiological range as evidenced by the fact that concentration of lysozyme in human milk is nearly 0.9 mg/ml [47]. In the radial diffusion assay however, higher concentrations of lysozyme (12.5, 25 and 50 mg/ml) were required in order to see an effect. This is because, the radial assay, in contrast to the liquid broth assay, is based on the diffusion of molecules, thus translating into a lower concentration of lysozyme per unit volume in the gel.

The results of the radial diffusion assay showed that lysozyme had activity against both *S. pneumoniae* serotypes, as well as against *M. catarrhalis*. Lysozyme however, was not shown to cause any detectable inhibition in the growth of NTHi, although lysozyme-treated NTHi did show changes at the ultrastructural level. This apparent disparity may be due to the fact that only a small fraction (5%) of lysozyme-treated NTHi showed any sign of damage. Thus, with 95% of the cells still undamaged, it is unlikely that any inhibition of growth would have been detectable in the radial assay. Consistent with the observations of Chun and Hancock [48], lysozyme also appeared

to display salt sensitivity and its effect was inhibited by 100 mM salt. As with the defensins, we believe that high local concentrations of lysozyme in the periciliary fluid space may be able to overcome the inhibitory effect of physiological levels of NaCl.

The results of the solution assay corroborated our radial diffusion assay data and demonstrated that physiological concentrations of lysozyme do indeed inhibit the growth of *S. pneumoniae* 6B. Moreover, our results showed that lysozyme and  $\beta$  defensin-2 act synergistically against this pathogen.

Unlike the results obtained with lysozyme and the  $\beta$  defensins, treatment of the four respiratory pathogens with lactoferrin appeared to have a growth enhancing effect using the current culture conditions. All four bacteria tested had a positive growth response to lactoferrin, with NTHi 12 and *S. pneumoniae* 3 showing a higher sensitivity than the other two. This result, however, is not surprising, as *M. catarrhalis* has been shown to be able to use lactoferrin as an iron source for growth *in vitro* and *S. pneumoniae* has been shown to specifically recognize and bind human lactoferrin [49]. Moreover, other bacteria have also been shown to be able to use lactoferrin for growth. For example, when grown under iron starvation, *Neisseria meningitidis* expresses receptors for transferrin and lactoferrin in the outer membrane [50].

Lactoferrin is a proteolytic, iron-binding antimicrobial glycoprotein found in the milk and exocrine secretions of mammals and is released from neutrophilic granules during inflammation [51,52]. It has been suggested that the presence of lactoferrin (and lysozyme) in human milk may be at least one reason why babies raised on breast milk are more resistant to OM than their formula-fed counterparts [10,53]. Although in the present study we did not observe any antibacterial activity by lactoferrin when tested alone, in our previous studies we showed that in the solution assay this molecule could enhance the effect of lysozyme and defensins against respiratory pathogens [13]. Consistent with our observations, Plaut and colleagues have shown that although the proteolytic activity of lactoferrin causes Hap-positive NTHi strains to lose their ability to adhere to Chang epithelial cells, it does not by itself affect the viability of the bacteria [54]. Moreover, by using apo-lactoferrin with a 0.03% iron content, we ruled out the possibility that the growth-enhancing effect of lactoferrin may be due to its saturation with iron and thus its activity as an iron source for the bacteria.

Although it is currently not known if an *in vivo* deficiency of innate immune molecules is a risk factor for OM, our preliminary results with mice deficient in surfactant protein D (SP-D), suggest that it is (Lee et al., unpublished

data). Surfactant proteins A (SP-A) and D are members of the collectin family of innate immune molecules and have been reported to bind lipopolysaccharide (LPS), opsonize microorganisms, enhance the clearance of lung pathogens and inhibit the growth of Gram-negative bacteria [55]. As part of our future studies, we plan to expand our *in vivo* studies by studying OM susceptibility in SP-A knockouts as well in knockouts of other innate immune molecules.

## Conclusions

Our results demonstrate that innate immune molecules such as lysozyme,  $\beta$  defensin-1 and  $\beta$  defensin-2 can reduce the viability of major OM pathogens, perhaps by disrupting their membrane integrity. Our results also show that  $\beta$  defensin-2 and lysozyme can act synergistically against *S. pneumoniae* 6B, an important OM pathogen. The current findings appear to be consistent with earlier observations of the effect of these molecules on bacterial integrity [48,56,57]. We, therefore, suggest that the innate immune molecules such as lysozyme and  $\beta$  defensins-1 and -2 provide the first line of defense against respiratory pathogens in the tubotympanum and may be useful for treating otitis media as an adjunct to antibiotics.

## Competing interests

None declared.

## Authors' contributions

HYL performed all antimicrobial assays and performed data analysis

AA oversaw study design and data analysis, drafted the manuscript and performed statistical analysis

PW performed EM studies

SKM did Q-PCR on middle ear specimens

KT collected middle ear specimens and helped with Q-PCR

SHK helped to perform antimicrobial assays

JDL provided expert advice with regards to the pathogens used in the study

MN collected middle ear specimens

TG provided expert advice regarding the defensins and antimicrobial assays

DJL is recipient of NIDCD R01 DC05025, which supported this work and the studies were conducted in his laboratory, with him as the principal investigator

## Acknowledgments

We would like to thank Drs. Xing Xing Gu (NIH, NIDCD) and Howard Faden (SUNY, Buffalo) for helpful discussions. This work was supported in part by a grant from the NIDCD (R01 DC05025) to DJL.

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