

Neutrophil Extracellular Traps and Bacterial Biofilms in Middle Ear Effusion of Children with Recurrent Acute Otitis Media – A Potential Treatment Target

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Abstract

Background: Bacteria persist within biofilms on the middle ear mucosa of children with recurrent and chronic otitis media however the mechanisms by which these develop remain to be elucidated. Biopsies can be difficult to obtain from children and their small size limits analysis.

Methods: In this study we aimed to investigate biofilm presence in middle ear effusion (MEE) from children with recurrent acute otitis media (rAOM) and to determine if these may represent infectious reservoirs similarly to those on the mucosa. We examined this through culture, viability staining and fluorescent *in situ* hybridisation (FISH) to determine bacterial species present. Most MEEs had live bacteria present using viability staining (32/36) and all effusions had bacteria present using the universal FISH probe (26/26). Of these, 70% contained 2 or more otopathogenic species. Extensive DNA stranding was also present. This DNA was largely host derived, representing neutrophil extracellular traps (NETs) within which live bacteria in biofilm formations were present. When treated with the recombinant human deoxyribonuclease 1, Dornase alfa, these strands were observed to fragment.

Conclusions: Bacterial biofilms, composed of multiple live otopathogenic species can be demonstrated in the MEEs of children with rAOM and that these contain extensive DNA stranding from NETs. The NETs contribute to the viscosity of the effusion, potentially contributing to its failure to clear as well as biofilm development. Our data indicates that Dornase alfa can fragment these strands and may play a role in future chronic OM treatment.

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Introduction

Recurrent acute otitis media (rAOM) affects between 10–20% of children [1,2] and is a major reason for children requiring surgery, predominantly for ventilation tube insertion (VTI). Biofilm and intracellular infection have been demonstrated on the middle ear mucosa of children with chronic suppurative otitis media (CSOM), rAOM and chronic otitis media with effusion (OME) and are mechanisms of bacterial persistence in the middle ear causing recalcitrance to treatment and disease recurrence [3–6].

Biofilms on mucosal surfaces are a result of interactions between bacteria and the host and these differ physiologically from biofilms

formed on inert surfaces [7]. DNA, both host and bacterially derived, is important in biofilm formation, stabilisation and persistence of nontypeable *Haemophilus influenzae* (NTHi) and *Streptococcus pneumoniae in vitro* and in the chinchilla model of otitis media (OM) [8–12]. DNA may be derived from active immune mechanisms such as neutrophil extracellular traps (NETs) [13], necrotic neutrophil presence [14], or produced directly by the bacteria [8–11]. NETs consist of a DNA backbone embedded with antimicrobial peptides and enzymes [15] and are important in confining infections and killing bacteria [16]. Some pathogens, including *S. pneumoniae* and NTHi can resist NET killing [9,11,15–17] and NTHi has been shown to actively elicit NET formation, using the DNA as a protective niche [18]. High levels of NETs

may also lead to tissue damage as has been demonstrated in the liver and lung [19]. The presence of NETs and/or bacterial DNA in chronic OM may allow for novel treatments that breakdown DNA such as Dornase alfa used in the treatment of CF.

While middle ear mucosal biopsies have been used for demonstrating biofilm or intracellular presence of bacteria [3–6], biopsies are difficult to obtain and their small size limits analysis. The use of middle ear effusion (MEE) is an attractive alternative to examine the presence of biofilm-like formations in the middle ear and *Staphylococcus aureus* biofilm has been demonstrated in otorrhoea smears from children with CSOM [20]. Additional valuable data can be obtained from imaging the larger volume of MEE that cannot be acquired from single biopsies such as viability staining and fluorescent *in situ* hybridisation treatments, reducing bias in selecting probes and determining non-specific interactions between the probes and the sample. The presence of DNA in MEE from children with chronic and/or recurrent OM has not been previously demonstrated. In this study, we used FISH and viability staining to investigate whether bacterial biofilm, intracellular pathogens and extracellular DNA are present in MEE samples from children undergoing VTI for rAOM.

Materials and Methods

Patient population

MEE samples were obtained from children aged between 9 and 36 months with a history of at least 3 episodes of AOM and undergoing VTI as previously reported [21]. Children with diagnosed or suspected primary or secondary immunodeficiency, cystic fibrosis, immotile cilia syndrome, craniofacial abnormalities and chromosomal or genetic syndromes were excluded. Subjects were recruited from metropolitan hospitals in Perth, Western Australia, following written informed consent from parents or guardians. Approval for this study was obtained from the Princess Margaret Hospital for Children Ethics Committee (1295/EP) and from institutional review boards of hospitals where recruitment took place.

Specimen acquisition and preparation

Samples were collected as previously described [21]. In brief, MEEs were aspirated through a myringotomy incision using a sterile Leukotrap® (Pall Corporation, New York, USA) connected to the surgical suction system. The tubing system was rinsed with sterile saline to recover all the MEE present into the Leukotrap®. Nasopharyngeal samples were collected using a sterile flexible cotton-wool tip swab (Copan, Brescia, Italy) and stored in STGGB.

Bacterial culture

MEE (100 μ L) and nasopharyngeal samples (10 μ L) were assayed with standard culture techniques [22] on horse blood agar, chocolate agar containing bacitracin (300 mg/L), vancomycin (5 mg/L) and clindamycin (0.96 mg/L), and colistin nalidixic acid blood agar plates (Oxoid, Australia). Plates were incubated at 37°C in 5% CO₂ for 48 h and all predominant bacteria recorded.

Evaluation of bacterial viability and presence of DNA stranding in the MEE with BacLight staining kit

Viability of bacteria in the MEEs was assessed using the generic nucleic acid labelling of cells with the BacLight kit (Invitrogen Technologies). Briefly, 50 μ L of fresh MEE in saline was air dried for 30 min at room temperature on SuperFrost plus slides (Menzel-Glaser, Portsmouth, New Hampshire). Samples were incubated with 50 μ L of the BacLight working solution (consisting

of propidium iodide and SYTO9) (Invitrogen Technologies, Thornton, NSW) for 30 min at room temperature before three 5 min washes with PBS. Slides were mounted using an in-house low fade mounting media and stored at -20°C until CLSM imaging using the Leica TCS SP2 AOBs multiphoton confocal.

Evaluation of MEE specimens using 16S rRNA FISH with pathogen-specific probes

FISH was conducted as described by Hall-Stoodley *et al* and Thornton *et al* [5,6] for mucosal biopsies using 16S rRNA probes labelled with AlexaFluor 488, 546 or 633 dyes (Invitrogen Technologies, Thornton, NSW). A panel of probes was used to assess 5 slides for each MEE. Due to the availability of 4 lasers a maximum of 3 probes and one nucleic acid stain could be used on each sample. The probes included EUB338, a universal probe that hybridises all bacteria (with a 5'-3' sequence, GCT GCC TCC CGT AGG AGT) [23], Spn probe (GTG ATG CAA GTG CAC CTT) for *S. pneumoniae* [24], Haeinf (CCG CAC TTT CAT CTT CCG) for *H. influenzae* [25], Sau (GAA GCA AGC TTC TCG TCC G) for *S. aureus* [24], Mrc88 (CCG CCA CUA AGU AUC AGA) for *Moraxella catarrhalis* [5] and Psae (TCT CGG CCT TGA AAC CCC) for *Pseudomonas aeruginosa* [26] and NONEUB338 as the negative control for non-specific hybridisations [27]. Specificity of 16S rRNA probes was extensively tested and validated using ATCC and clinical isolates.

MEE samples stored in 50% PBS/Ethanol at -20°C were used for FISH. A 50 μ L aliquot from each MEE was air dried as above, incubated for 30 min at room temperature in 80% and then 100% ethanol. Samples were transferred to hybridisation chambers and incubated with lysozyme (10 mg/ml lysozyme in 0.1 M Tris (hydroxymethyl) aminomethane hydrochloride and 0.05 M Na₂ EDTA) for 2 h at 37°C. Slides were then washed with PBS. Samples were treated with 40 μ L of hybridisation buffer and 50 ng/ml of each probe at 46°C inside a hybridisation chamber for 2 h. Slides were then washed for 15 min at 48°C and incubated overnight with 1.2 μ g/ml Hoechst 33342 (Invitrogen Technologies, Thornton, NSW) to stain the nuclei of the host cells. Slides were mounted in low fade mounting media and imaged using four colour CLSM using the Leica TCS SP2 AOBs multiphoton confocal. Images were analysed for presence of bacteria, specific bacterial species, biofilm structure and composition.

Image analysis and interpretation

Samples were scored positive for biofilm presence when CLSM images showed bacterial aggregates resembling microcolonies or more extensive morphology, bacterial morphology based on size (0.5–2 μ m) and shape (cocci or coccobacilli) was correct, biofilm ultrastructure was present and fluorescent signal was only present in the appropriate fluorescent channel. Host cells were identified through the use of the Hoechst 33342 nucleic acid stain. Bacteria were marked as being intracellular when discrete clusters of bacteria were in close proximity to intact host cell nuclei as per Thornton *et al* [6].

Neutrophil elastase staining to determine whether the DNA in the samples was neutrophil derived

A subset of 10 MEEs was evaluated for neutrophil elastase by staining with anti-human neutrophil elastase antibodies (Abcam, UK). Fifty μ L of MEE in saline was air dried for 30 min at room temperature on SuperFrost plus slides (Menzel-Glaser, Portsmouth, New Hampshire). MEE was blocked with Zuk's blocking buffer and incubated at room temperature for 30 min. Block

Table 1. Summary of demographic data and the pathogens identified in MEE from 24 children undergoing VTI for rAOM. Pathogens were identified using standard culture and FISH, and viability was determined using Baclight staining.

Child No.	Sex (Age,m)	FISH 16S RNA Probe											NETs				
		NP culture	Abx	Ear	MEE culture	EUB	Spn	Hi	Mcat	Sau	PA	BaClight (Live/Dead/Both)		Biofilm present	Intracellular bacteria observed		
1	M (16.6)	Spn, Hi	Y	L	-	+	+	-	+	-	+	+	+	na	+	-	na
		Mcat		R	-	+	+	-	+	-	+	+	+	na	+	-	na
2	M (28.3)	Spn, Hi	N	L	-	+	+	-	-	-	-	-	-	Both	-	-	+
3	M (16.8)	Hi	Y	L	Sau	+	+	+	+	-	-	-	-	Live	+	-	+
		Sau		R	Sau	+	+	+	+	-	-	-	-	Live	+	+	+
4	M (20.0)	-	Y	R	-	+	+	-	-	-	-	-	-	Both	+	-	-
5	M (14.0)	-	Y	L	-	+	+	+	+	-	-	-	-	Both	+	+	-
				R	-	+	+	-	-	-	-	-	-	Live	+	-	+
6	M (23.3)	Hi	Y	L	Hi	+	+	+	+	-	-	-	+	Both	+	+	+
				R	Hi	+	+	+	+	-	-	-	-	Both	+	+	+
7	M (14.1)	Spn, Hi	N	R	Hi	+	+	+	+	-	-	-	+	Both	+	+	+
8	F (24.6)	Hi,	N	L	-	+	+	+	+	-	-	-	+	-	+	-	+
		Mcat		R	-	+	+	+	+	-	-	-	+	Both	+	+	+
9	F (18.0)	Spn	N	L	-	+	+	-	-	-	-	-	+	-	+	+	+
				R	-	+	+	-	-	-	-	-	+	Both	-	-	+
10	M (10.7)	Spn, Hi	N	R	Spn	+	+	+	+	-	-	-	-	Live	+	+	+
11	M (13.2)	Hi	N	L	Hi	+	+	+	+	-	-	-	-	Both	+	-	+
				R	Hi, Sau	+	+	+	+	-	-	-	-	Live	+	+	+
12	M (13.3)	Hi	N	R	-	+	+	-	-	-	-	-	-	Live	+	+	+
13	M (14.0)	Spn, Hi	Y	L	Spn, Hi	+	+	-	-	-	-	-	-	Live	+	-	+
				R	Spn, Hi	+	+	-	-	-	-	-	-	Live	-	-	+
14	F (26.6)	Hi,	U	L	-	+	+	+	+	-	-	-	+	Live	+	-	+
		Mcat		R	-	+	+	+	+	-	-	-	+	Live	+	-	+
15	M (17.9)	-	Y	L	-	+	+	+	+	-	-	-	-	Live	+	+	+
				R	AO	+	+	+	+	-	-	-	-	Live	+	+	+
16	M (18.5)	Sau	N	R	-	+	+	+	+	-	-	-	-	Live	+	+	-
17	F (16.9)	Spn, Hi	N	L	-	na	na	na	na	na	na	na	na	Live	+	+	+
		Mcat		R	-	na	na	na	na	na	na	na	na	Live	+	+	+
18	M (36.0)	-	Y	R	-	na	na	na	na	na	na	na	na	Both	+	-	+
19	M (9.7)	Mcat	N	R	-	na	na	na	na	na	na	na	na	Live	+	+	+
20	M (9.7)	Mcat, Hi	N	L	-	na	na	na	na	na	na	na	na	Live	+	-	+
				R	-	na	na	na	na	na	na	na	na	Live	+	-	+

Table 1. Cont.

Child No.	Sex (Age,m)	NP culture	Abx	Ear	MEE culture	FISH 16S RNA Probe							NETs		
						EUB	Spn	Hi	Mcat	Sau	PA	BacLight (Live/Dead/Both)		Biofilm present	Intracellular bacteria observed
21	M (16.5)	–	Y	L	–	na	na	na	na	na	na	Both	+	–	+
22	M (32.0)	–	N	L	–	na	na	na	na	na	na	Both	+	+	+
23	F (21.3)	Hi	U	L	–	na	na	na	na	na	na	Live	+	–	+
		–	–	R	–	na	na	na	na	na	na	Live	–	–	+
24	M (18.7)	–	Y	L	–	na	na	na	na	na	na	–	–	–	+
		–	–	L	–	na	na	na	na	na	na	–	–	–	+
Number positive						11/38 (29%)	19/26 (73%)	17/26 (65%)	7/26 (27%)	7/26 (27%)	1/26 (4%)	32/36 (89%)	32/38 (84%)	17/38 (45%)	33/36 (92%)

Biofilm and intracellular bacterial presence were also assessed.

Abbreviations: EUB – eubacterial probe, Spn – *S. pneumoniae* (organism and probe), Hi – *H. influenzae* (organism and probe), Mcat – *M. catarrhalis* (organism and probe), PA – *P. aeruginosa* (organism and probe), Sau – *S. aureus* (organism and probe), AO – *A. otitidis* (organism), na – Not available, Abx – taking antibiotics currently, U – Unknown, NP – nasopharyngeal culture. doi:10.1371/journal.pone.0053837.t001

buffer was removed and MEE was incubated with a 1/100 dilution of rabbit anti-neutrophil elastase (Abcam, UK) in Zuk's buffer with 1% saponin (Sigma, St Louis, MO) for 60 min at room temperature. Antibody was aspirated and the MEE was washed 3×10 min in PBS. MEE was then incubated with 1/100 AlexaFluor 647 labelled goat anti-rabbit antibody (Invitrogen Technologies, Thornton, NSW) in Zuk's buffer with 1% saponin for 60 min at room temperature in the dark. Antibody was aspirated and the MEE was washed 3×10 min in PBS at room temperature before being counterstained with propidium iodide (Invitrogen Technologies, Thornton, NSW), mounted in low fade mounting media and stored at –20°C until imaging using the Leica TCS SP2 AOBS multiphoton confocal.

Evaluation of actin presence using Phalloidin staining to determine whether neutrophil DNA was released via an active mechanism or necrosis

Seven MEEs were evaluated for actin presence by staining with Phalloidin. Briefly, 50µl of MEE in saline was air dried for 30 min at room temperature on SuperFrost plus slides. Samples were incubated with 50 µl of TRITC labeled Phalloidin (Invitrogen Technologies, Thornton, NSW) at room temperature for 60 min, washed 3 times with PBS, stained for 30 min at room temperature with 50 µl of SYTO9 and washed 3×5 min in PBS. Slides were mounted using a low fade mounting media and stored at –20°C until imaging using the Leica TCS SP2 AOBS multiphoton confocal.

Lectin staining to elucidate whether bacterial formations were surrounded by a matrix

A subset of 10 MEEs was stained for lectins by staining with Concanavalin A (ConA) and Wheat Germ Agglutinin (WGA). Fifty µl of MEE in saline was air dried for 30 min at room temperature on SuperFrost plus slides. MEE were fixed in 4% formaldehyde for 15 min at 37°C. Samples were then washed 3×10 min with PBS at room temperature. Samples were incubated at room temperature in the dark with 50 µl of a 5 µg/ml of WGA conjugated with Oregon Green 488 (Molecular Probes, Eugene, Oregon) in PBS for 10 min. Samples were washed twice in PBS. MEEs were then stained with 50 µl of 50 µg/ml FITC labelled ConA (Sigma, St Louis, MO) for 30 min at room temperature. Samples were then washed 3×5 min in PBS before being counterstained with propidium iodide and mounted in low fade mounting media and stored at –20°C until imaging.

Dornase alfa treatment of middle ear fluid

Seven MEEs were treated with two concentrations of a highly purified solution of recombinant human deoxyribonuclease I (Dornase alfa, Pulmozyme, Roche) which is an enzyme that selectively cleaves DNA. Briefly, 250 µl MEE was incubated with 250 µl of 5 µg/ml or 1 mg/ml Dornase alfa for 15 min at room temperature. Samples were centrifuged using a serofuge and rinsed three times using PBS and stained as previously described using the BacLight kit (Invitrogen Technologies) followed by CLSM imaging.

Results

Patient characteristics

Thirty eight MEEs obtained from 24 children undergoing VTI for rAOM were assessed for the presence of extracellular DNA and live bacteria using generic nucleic acid staining and CLSM. Five children were female (20%) and the median age was

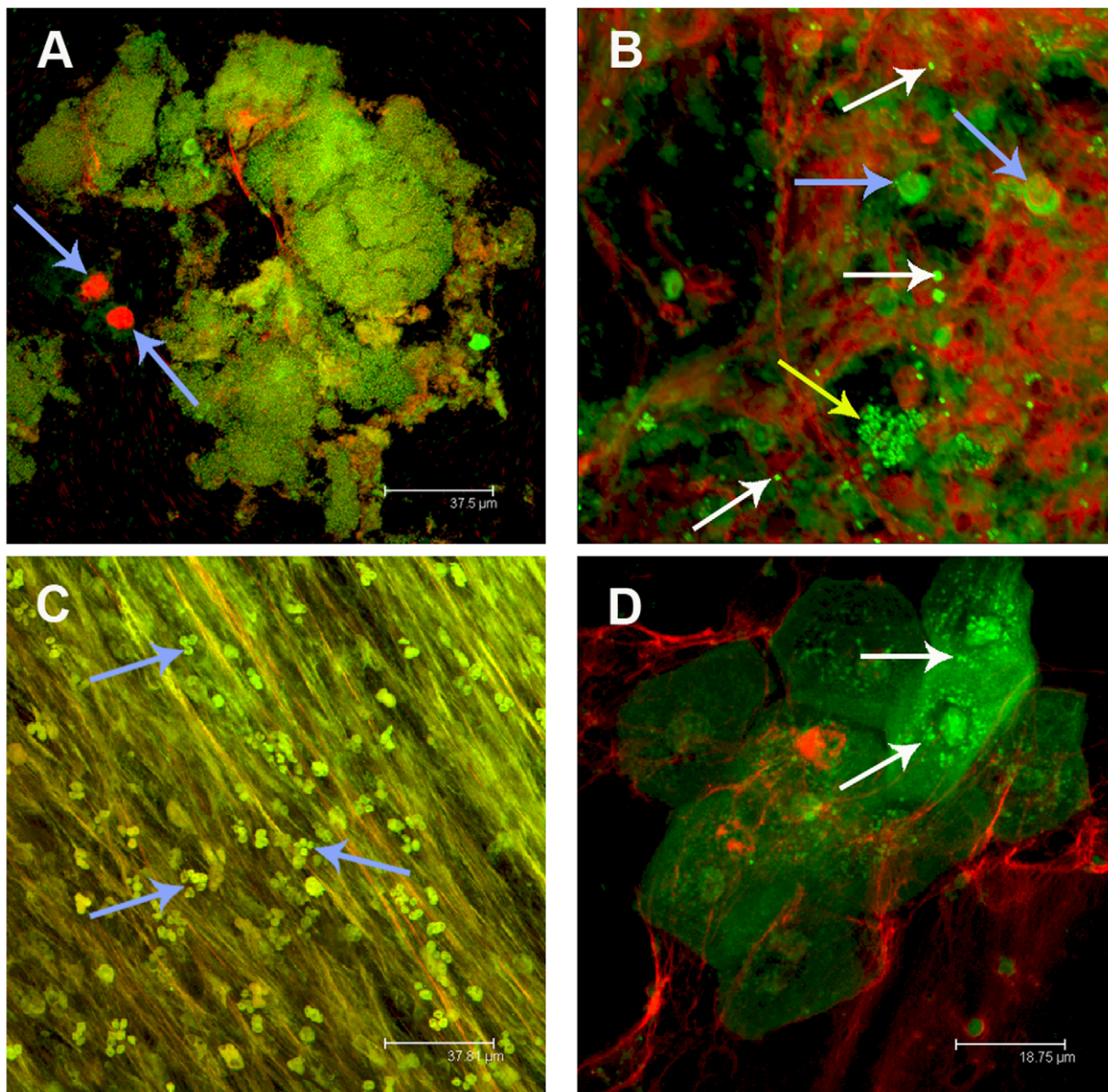


Figure 1. Biofilms (containing live, dead and intracellular bacteria) and DNA stranding were observed in MEE from children with rAOM. Representative images from MEEs stained with the BacLight kit. **A)** Large biofilm structures were observed. Both live (green) and dead bacteria (red) were present as were host cells (blue arrows). Bacteria with both dyes are evident (yellow). Host derived DNA strands were also present. **B)** Microcolonies of live bacteria (green; yellow arrow) within extensive nucleic acid matrices staining mainly with the propidium iodide component of the BacLight kit (red) were present throughout. Single bacteria (white arrows) and intact host nuclei were also observed (blue arrows). **C)** Extensive DNA stranding was present as were multi-lobed nuclei of intact neutrophils (blue arrows) apparent within the fibrotic DNA structure. **D)** Sloughed off epithelial cells containing live bacteria (arrows) were present in several MEEs.
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17.9 months (range 9.7–36.0 months; Table 1). All children enrolled in the study had received the 7-valent pneumococcal conjugate vaccine (Prevenar, Pfizer) at 2, 4 and 6 months of age as part of the Australian National Immunisation Program.

Culture of nasopharyngeal swabs and MEE

Eighteen children had at least one pathogen isolated from the nasopharynx, with 14 children carrying NTHi (58%), 6 *M. catarrhalis* (25%), 7 *S. pneumoniae* (29%) and 1 *S. aureus*. Nine children had 2 or more otopathogens isolated from the nasopharynx. Eleven MEEs (29%) were culture positive for at least one

bacterial species, with 3 being positive for *S. pneumoniae* and 6 for NTHi (Table 1). Three MEEs were culture positive for *S. aureus* and 1 was culture positive for *Alloioococcus otitidis*. When bilateral effusions were collected, the same pathogen was isolated from both ears in 3/5 occasions (Table 1).

Bacteria in MEE are viable and present in biofilm morphologies

To investigate whether bacteria within the MEE were viable despite low culture rates we used BacLight viability staining. Live bacteria were identified in 32 of the 36 MEE samples tested. In 20

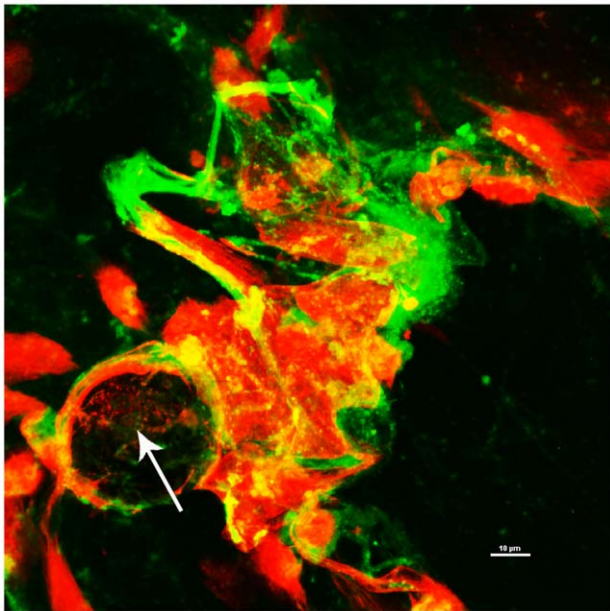


Figure 2. Bacterial biofilm matrices were demonstrated through staining with lectins. Concanavalin A (ConA) and Wheat Germ Agglutinin (WGA) (green) and propidium iodide (red) to stain for DNA. Co-localisation of the DNA and lectins are observed (yellow) suggesting that these are part of a biofilm structure. Bacterial biofilm can be observed (arrow) with bacteria being surrounded by a matrix that binds the lectin. Scale 10 μ m. doi:10.1371/journal.pone.0053837.g002

of these MEE only live bacteria were present, while in 12 both live and dead bacteria were identified. Bacteria were present in extensive biofilms, microcolonies and in the planktonic state (Figure 1). Nucleic acid staining revealed extensive extracellular DNA stranding in 33 of the 36 MEE samples (Figure 1B and 1C). These extensive DNA structures frequently had biofilms associated. To assess the association of the bacteria with an extracellular matrix, MEEs were stained with lectins. The 2 lectins bound the glycoproteins in the biofilm matrix, and these were associated with both the bacteria and the DNA strands (Figure 2). This association suggests the DNA may be a structural component of the biofilms within the MEE.

Polymicrobial biofilms containing known otopathogens were demonstrated in MEE

We conducted species specific FISH on 26 MEEs. All 26 MEE samples were positive for bacteria when tested with the universal EUB338 probe. Two or more otopathogenic species were observed in 70% of effusions (Table 1 and Figure 3) showing the presence of polymicrobial biofilms in the middle ear. Nineteen of 26 (73%) samples were positive for *S. pneumoniae*, 17 (65%) for *H. influenzae*, 7 (27%) for *M. catarrhalis*, 7 (27%) for *S. aureus* and 1 (4%) was positive for *P. aeruginosa*. Using the NONEUB338 probe non-specific hybridisation was not observed for any of the MEE samples (data not shown). Bacteria were evident within epithelial cells (based on morphology – Figure 1D), though specific staining to identify these cells was not performed.

Extensive DNA stranding evident within the MEE is largely NET derived

Given the extensive DNA stranding present and high proportion of neutrophil nuclei evident by morphology (Figure 1C) we

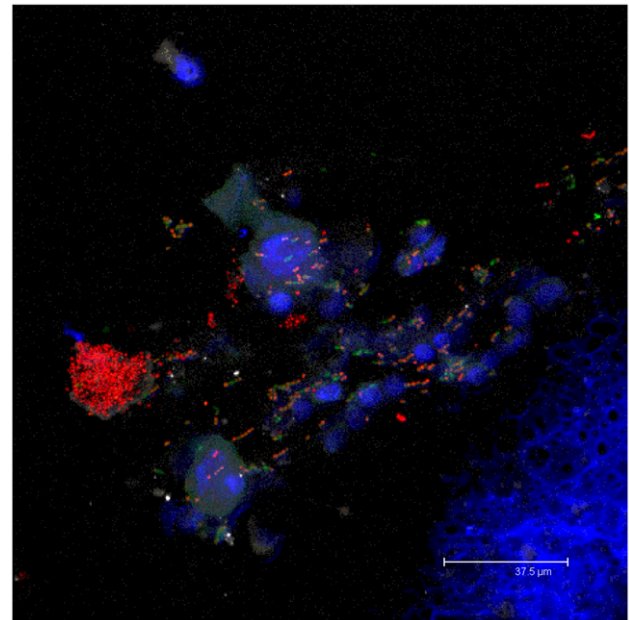


Figure 3. Multi-species bacterial biofilms containing known otopathogens, were demonstrated in the MEE of children with RAOM. Representative merged maximum intensity projection confocal image from FISH and Hoechst stained MEE (Child 3). MEE was culture positive for *S. aureus*. FISH: *S. pneumoniae* (AF488 green); universal EUB338 probe (AF546 red) *H. influenzae* (AF633 grey) Hoechst 33342 staining of host cell nuclei (blue). Chains of *S. pneumoniae* can be observed in this sample as well as those which are intracellular. Other unidentified bacteria (red) can be seen in chains and microcolony structures throughout this MEE. doi:10.1371/journal.pone.0053837.g003

investigated whether the DNA was derived from neutrophils. Cells were confirmed to be neutrophils in a selection of samples using haematoxylin and eosin staining, as well as CD15 staining (data not shown). Cells positive for the histiocytic lineage marker (CD68), which is indicative of macrophages were also observed (data not shown). Samples were stained with Phalloidin to determine if the cytoplasmic marker actin was present thereby implying that host derived DNA strands were due to degradation of necrotic neutrophils rather than produced as an active response by the neutrophils. Of the 7 samples tested only one was positive for actin (Figure 4) but this was not associated with DNA. Another marker of neutrophil DNA release is the association of the DNA with the enzyme neutrophil elastase. When labelled with anti-neutrophil elastase antibodies granular staining colocalised with DNA (Figure 5A) and bacteria were observed to be associated with DNA and neutrophil elastase (Figure 5B).

Treatment with Dornase alfa effectively breaks down the DNA structures and associated biofilms in the MEE

When MEEs were treated with Dornase alfa at a concentration used in CF therapy (1 mg/ml) and at 5 μ g/ml, complete fragmentation of the DNA matrix was observed in all samples within 15 minutes (Figure 6).

Discussion

Bacterial biofilm is increasingly recognised to play a role in the recurrence and persistence of infections such as OM. Demonstrating biofilm in very small tissue biopsies from the middle ear of children is technically difficult and may be biased by sampling and

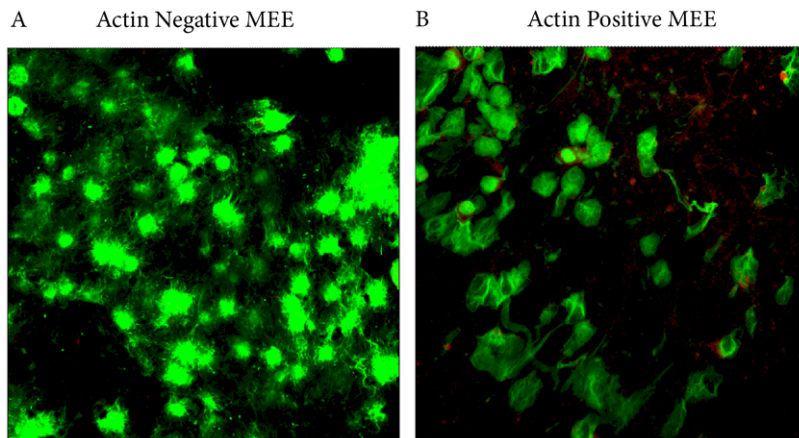


Figure 4. Most DNA present in the MEE was negative for actin, indicating it was actively produced and not released due to necrosis. MEE were stained with phalloidin to detect actin presence. MEEs were treated with the nucleic acid stain SYTO9 (green) and TRITC labelled Phalloidin (red). A) No red fluorescence was observed in most samples indicating actin was not present. This compared to B) where some actin is present (red). Even when the samples were positive for actin, this was not extensive, suggesting a combination of active DNA release and the presence of necrotic neutrophils is likely. doi:10.1371/journal.pone.0053837.g004

probe selection [5,6]. This is the first study to identify multi-species biofilms consisting of live otopathogens in MEE samples from children with rAOM. Furthermore this is the first study to demonstrate the association of these biofilms with a DNA matrix produced by NETs which may represent a treatment target through the use of the therapeutic agent Dornase alfa which was able to fragment the DNA matrix.

In this study, bacterial biofilms were demonstrated in 84% of MEE from children undergoing VTI for rAOM. This is similar to rates of biofilm presence on the middle ear mucosa of children with chronic OME and rAOM (64–92%) [5,6] and in CSOM discharge samples (83%) [20]. MEE biofilms appear similar to

those described in bronchoalveolar lavage fluids from children with CF, where bacteria adhere indirectly to the mucosa [28] resulting in small bacterial aggregates in highly viscous fluid [29]. Biofilms in the MEE were not homogenous, suggesting discrete microcolonies, similar to patchy biofilm formations on the middle ear mucosa in the chinchilla model of OM [30].

Using FISH, biofilms within MEE were shown to be polymicrobial, consistent with those observed in the environment [31] and previously identified on the middle ear mucosa [5,6]. This is clinically relevant as in animal models, polymicrobial biofilms provide a protective advantage for bacteria compared to a single species biofilm [32]. Host inflammatory responses are

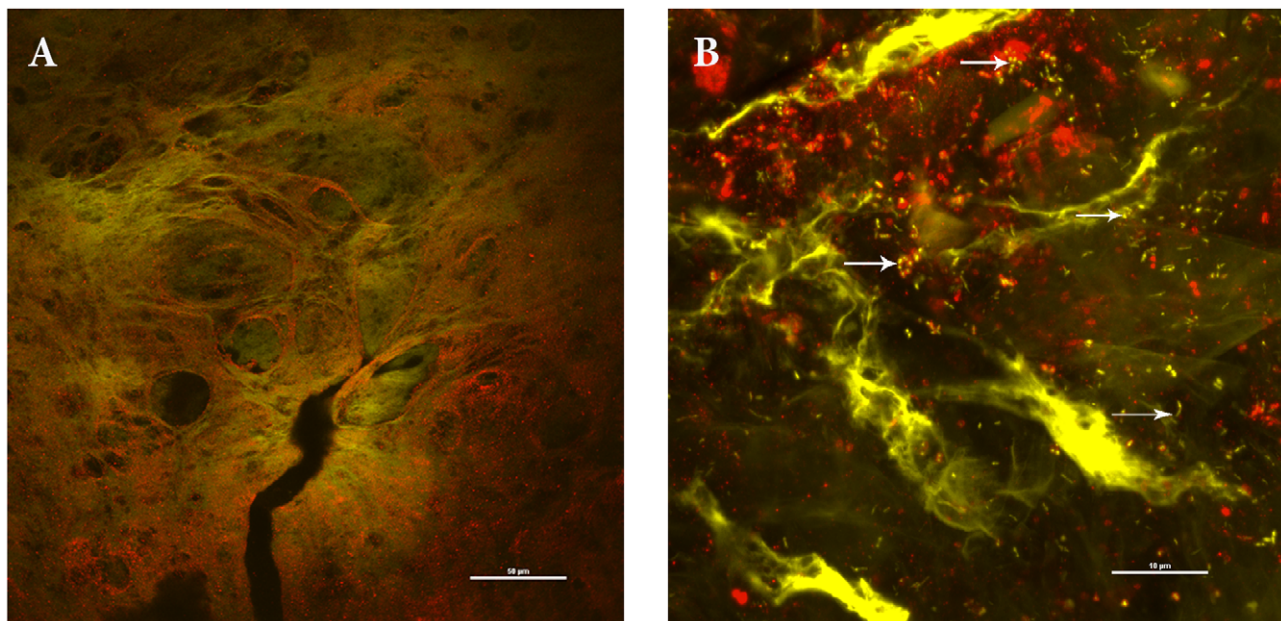


Figure 5. Most DNA present in the MEE was neutrophil-derived. MEE stained with neutrophil elastase AlexaFluor647 (Red) and propidium iodide (Yellow) and. A) Co-localisation of DNA and neutrophil elastase is present throughout the sample indicating the DNA that is present is neutrophil derived. Scale 50 µm. B) Co-localisation of DNA and neutrophil elastase is present throughout the sample as well as being bacterially associated throughout the sample (arrows). Scale 10 µm. doi:10.1371/journal.pone.0053837.g005

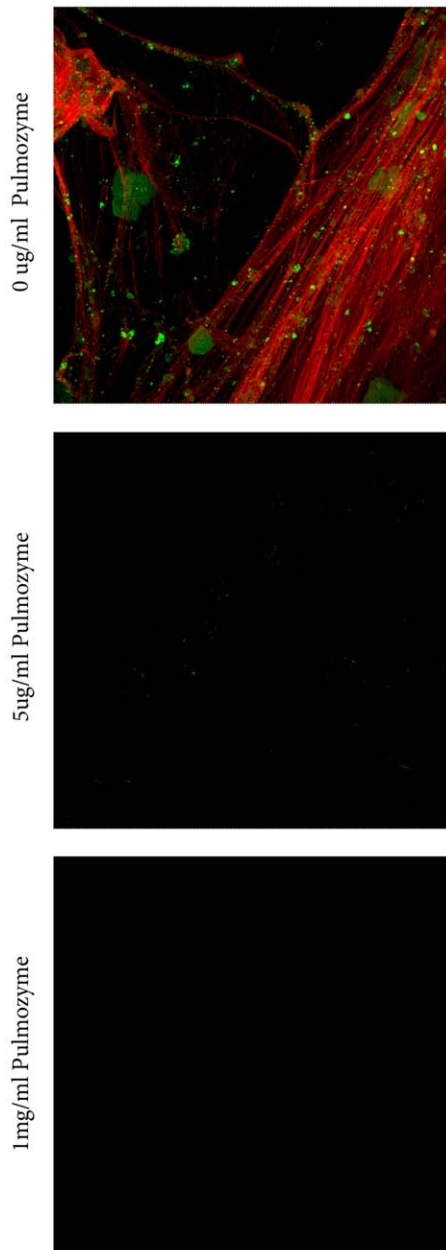


Figure 6. Dornase alfa (Pulmozyme®) treatment of MEE results in disintegration of the NET structures. DNA staining of MEEs from children with rAOM treated with Dornase alfa or left untreated. In the top panel, a control MEE untreated with Dornase alfa where extensive DNA stranding, epithelial cells and bacteria are evident. When treated with Dornase alfa even at concentrations as low as 5ug/ml complete fragmentation of the strands in the MEE are seen and nothing remains on the slide at 1 mg/ml.

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increased when multiple bacterial species are present regardless of whether they are able to be cultured [33,34]. While 92% of MEE's were positive for at least one of the known otopathogens, none bound the NONEUB338 (the nonsense or negative control) probe indicating the specificity of this technique. Large numbers of unidentified species were also present within the MEE (as identified by binding the EUB338 (universal) probe) and the high rates of bacterial detection using FISH suggest that this technique

represents a sensitive and specific tool to assess bacteria present in MEE.

We demonstrated extensive extracellular DNA stranding in MEE, predominantly neutrophil derived through NET formation, and live bacteria within these. Both animal [10] and *in vitro* studies [8] suggest that the presence of such DNA, both host and bacterially derived, play a role in biofilm development and bacterial persistence in OM. NETs also play roles in interspecies competition for colonising the nasopharynx [35,36] and may also do so in the middle ear. Similarly to previous studies [21,37] we found *S. pneumoniae* and *H. influenzae* the most commonly identified pathogens in the MEE of children with rAOM. These species can resist NET killing despite high levels of antimicrobial proteins [9,16,18] due to production of DNases and through alteration of the bacterial surface [18]. The lipooligosaccharide modifications promoting NTHi survival in NETs are similar to those that promote biofilm formation [18]. In the chinchilla model of NTHi OM, a bacterial histone-like protein (IHF) is involved in structuring the extracellular DNA that plays a major role in stabilising biofilms [38]. The inability of NETs to clear otopathogens may contribute to establishing stable biofilm communities in the MEE [9,11,18]. This combined with the viscosity of the DNA may impede clearance of the MEE and the associated bacteria. Mast cells and eosinophils are also able to form extracellular traps [39] and their role in rAOM requires further investigation.

In the MEE, it is important to use both the nucleic acid staining and the specific FISH methodology to assess biofilms. Generic staining protocols such as the BacLight kit do not involve enzyme treatments or stringent wash steps, which are necessary to conduct FISH staining, which means that the biofilm morphology within the MEE is retained more reliably. Indeed biofilm presence was more often scored using this technique compared with using FISH. FISH however allows identification of specific species and their relationship within the sample. Intracellular pathogens were detected in 45% of effusions, and 36% of children. These rates are lower than those detected in biopsies from children in previous studies (71%) [6]. The lower detection rate likely reflects a higher proportion of cells containing pathogenic bacteria being sequestered within the tissue rather than shed into the MEE. While MEE may be useful to assess biofilm presence in the middle ear, it may not be as representative in determining intracellular sequestration.

The presence of non-cultureable but metabolically active bacteria in the MEE provides further evidence of biofilm involvement in OM. In this study 21% of children were culture positive for any of the main OM pathogens which is comparable with other published studies [5,40]. However, using viability staining, 89% of MEEs tested were positive for live bacteria, predominantly *H. influenzae* followed by *S. pneumoniae*. Discrepancies were present comparing FISH and culture, with bacteria observed more often using direct microscopic examination. These differences have been observed previously when comparing standard culture and a direct detection method such as FISH [41,42]. Detection of bacterial species in one ear of a single subject did not always correlate with the opposite ear using either culture or FISH. Findings of different pathogens in the ears of individual children are not isolated to our study [5] and may suggest the presence of different microbial reservoirs in each ear.

FISH represents a specific and sensitive tool for assessing bacteria in the MEEs and in addition provides spatial and morphological data. This could be important when considering larger scale studies, particularly monitoring clinical interventions to treat biofilm, where it is easier to collect and examine MEEs than biopsies. Using MEE to monitor intracellular persistence may not be as reliable as much lower rates of intracellular infection

were observed in the MEE when compared to mucosal biopsies. Direct comparisons of MEE and middle ear biopsies from the same child are required to confirm this. Data from animal models have also demonstrated a difference in the niches occupied by bacterial species in polymicrobial infections. Specifically, in the chinchilla model of *H. influenzae* and *M. catarrhalis* OM, where *M. catarrhalis* is seen to be exclusively associated with the epithelial surface while *H. influenzae* is detected in both the epithelium and the MEE [32].

Studies often use nasopharyngeal carriage of otopathogens as a surrogate for the infecting pathogens of the middle ear. This may be indicative with acute infection but less reliable in children with recurrent AOM. In this small study, we did not see a direct correlation between the pathogens in the middle ear with any of the detection methodologies with those cultured from the nasopharynx. This suggests that different bacteria may be found only in the middle ear space reflecting the presence of biofilm as a separate source of microbes causing the recurrent acute infections. Investigation of MEE and NPS samples using molecular detection techniques rather than relying solely on culture methodology is required as previous studies have demonstrated that non-cultureable organisms can be present in up to 36% of NPS using molecular methods [43].

Dornase alfa is the only licensed DNase with efficacy in the treatment of CF [44] and has been demonstrated to improve lung function, reduce DNA levels, and to reduce the viscosity of airway secretions [45]. When we treated MEEs with Dornase alfa in the current study, complete and rapid degradation of the matrix was observed even at concentrations lower than those used clinically in CF. While the number of MEEs tested was small, this data suggests that Dornase alfa may be a useful adjunct treatment in recurrent or chronic OM. Directly degrading the DNA may improve bacterial clearance from the middle ear by reducing biofilm stability and causing bacteria to return to their planktonic form which are susceptible to locally (or systemically) administered antimicrobials and host immune mechanisms. In *in vitro* systems of neutrophil induced biofilms by *P. aeruginosa*, DNases had decreased activity in treating thicker, more mature biofilms or those containing mucoid strains of bacteria, possibly due to increased polysaccharide masking DNA cleavage sites [29]. This effect of DNase appears to be species specific, [8,12,38] and needs to be better characterized for otopathogens and for polymicrobial biofilms. Other biofilm components in the MEE also need to be

better assessed as they may affect the efficacy of a DNase treatment.

This study includes a relatively small number of children with a specific clinical presentation of OM, being persistent effusion following rAOM episodes. Investigation of the role of biofilm in MEE and the middle ear mucosa in a population of children with a broader OM spectrum, including children with chronic OME and CSOM, and the potential of Dornase alfa are required.

These data provide an insight into the role of biofilm in the middle ear which is not only surface associated but present in the entire middle ear cavity. Live bacteria in microcolonies are present in extensive DNA stranding from NETs in the MEE of children with rAOM, contributing to effusion viscosity and may affect its failure to clear. The involvement of these biofilms with NET associated DNA indicates a potential for the novel use of Dornase alfa combined with currently used topical antibiotics at the time of surgery. We are currently conducting a clinical trial to assess the use of Dornase alfa as an adjunct therapy to VTI to prevent recurrent episodes of OM and possibly the need for repeat surgery. The potential use of Dornase alfa as a treatment for recurrent or chronic OM prior to surgery requires development of suitable methods of delivery to the middle ear and this warrants further investigation.

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Author Contributions

Conceived and designed the experiments: RT SW LK PR SV HC PCR. Performed the experiments: RT SW. Analyzed the data: RT SW LK PR PCR. Contributed reagents/materials/analysis tools: RT SW LK PR SV HC PCR. Wrote the paper: RT SW LK PR SV HC PCR.

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