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Can non-typeable *Haemophilus influenzae* carriage surveillance data infer antimicrobial resistance associated with otitis media?

Sophia Bowman-Derrick[1] | Tegan M. Harris[1] | Jemima Beissbarth[1] | Mariana Kleinecke[1] | Katrina Lawrence[1] | Teresa M. Wozniak[1,2] | Amy Bleakley[1] | Angela Rumaseb[1] | Michael J. Binks[1] | Robyn L. Marsh[1] | Peter S. Morris[1,3] | Amanda J. Leach[1] | Heidi Smith-Vaughan[1]

[1]Menzies School of Health Research, Charles Darwin University, Darwin, Australia
[2]Australian e-Health Research Centre CSIRO, Brisbane, Australia
[3]Royal Darwin Hospital, Darwin, Australia

Correspondence
Sophia Bowman-Derrick, Menzies School of Health Research, Charles Darwin University, Darwin, NT, Australia.
Email: Sophia.bowman-derrick@menzies.edu.au

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ABSTRACT

Importance: In remote communities of the Northern Territory, Australia, children experience high rates of otitis media (OM), commonly caused by non-typeable *Haemophilus influenzae* (NTHi). Few data exist on antibiotic susceptibility of NTHi from OM.

Objective: To determine whether population-level nasopharyngeal NTHi antibiotic susceptibility data could inform antibiotic treatment for OM.

Methods: NTHi isolates ($n = 92$) collected from ear discharge between 2003 and 2013 were selected to time- and age-match NTHi isolates from the nasopharyngeal carriage ($n = 95$). Antimicrobial susceptibility were tested. Phylogenomic trees and a genome-wide association study (GWAS) were performed to determine the similarity of nasopharyngeal and ear isolates at a population level.

Results: Among 174 NTHi isolates available for antimicrobial susceptibility testing, 10.3% (18/174) were resistant to ampicillin and 9.2% (16/174) were resistant to trimethoprim-sulfamethoxazole. Small numbers of isolates (≤3) were resistant to tetracycline, chloramphenicol, or amoxicillin-clavulanic acid. There was no statistical difference in the proportion of ampicillin-resistant ($P = 0.11$) or trimethoprim-sulfamethoxazole-resistant isolates ($P = 0.70$) between ear discharge and nasopharynx-derived NTHi isolates. Three multi-drug resistant NTHi isolates were identified. Phylogenomic trees showed no clustering of 187 *Haemophilus influenzae* isolates based on anatomical niche (nasopharynx or ear discharge), and no genetic variations that distinguished NTHi derived from ear discharge and nasopharyngeal carriage were evident in the GWAS.

Interpretation: In this population-level study, nasopharyngeal and ear discharge isolates did not represent distinct microbial populations. These results support tracking of population-level nasopharyngeal NTHi

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antibiotic resistance patterns to inform clinical management of OM in this population.

KEYWORDS
Haemophilus influenzae, Otitis media, Antimicrobial resistance

INTRODUCTION

Otitis media (OM) is a spectrum of diseases that includes OM with effusion (OME), acute OM with or without perforation, and chronic suppurative OM (CSOM).[1] Aboriginal children in remote communities of the Northern Territory (NT), Australia, develop OM within the first few weeks of life; 91% of children are affected by any OM, with 15% affected by CSOM.[2,3] OM has serious sequelae including hearing loss which may subsequently impact cognitive development and educational outcomes.[4]

Non-typeable *Haemophilus influenzae* (NTHi) is a common otopathogen, reported to be the cause of almost half of the cases of acute OM with a bacterial etiology, and is the main cause of chronic OM.[5] In the NT, previous polymerase chain reaction-based studies in Aboriginal children with acute OM or CSOM detected NTHi in 89% of ear discharge specimens.[6] Treatment failure of suppurative OM and CSOM is common among Aboriginal children who reside in remote areas, and there is currently no licensed vaccine for NTHi.[7,8]

While tympanocentesis is considered the gold standard for sterile collection of middle ear fluid for culture, this is not practicable for routine care, and not ethically approved for research in the NT.[1] The microbiology of OM is therefore only described in NT children where spontaneous perforation of the tympanic membrane has occurred.[6] Globally, the prevalence of antimicrobial resistance (AMR) among NTHi is increasing.[9,10] In Australia, NTHi is not included in ongoing AMR surveillance programs,[11] resulting in reliance on passive surveillance to track emerging resistance. Additionally, assessment of antimicrobial susceptibility among OM-associated NTHi is not routinely performed, therefore the level of resistance among ear strains in our setting is often unknown.[12] Addressing this gap is important to informing optimal treatments, as OM caused by NTHi can be more difficult to treat and is more likely to be chronic compared to OM caused by other bacterial species.[13]

First-line treatment of *H. influenzae* infections is β-lactam antibiotics; however, due to the prevalence of resistance, non-β lactams are increasingly used.[14] β-lactamase-positive ampicillin-resistant (BLPAR) and β-lactamase-negative ampicillin-resistant (BLNAR) *H. influenzae* strains have emerged.[14] BLPAR strains produce Class A β-lactamase (usually TEM-1 type) which confers aminopenicillin resistance, while BLNAR strains are resistant to multiple β-lactam antibiotics through mutations of the transpeptide domain of penicillin-binding protein 3 (PBP3), one of the targets of β-lactam antibiotics, encoded by the *ftsI* gene.[12–15] β-lactamase producing amoxicillin-clavulanic acid-resistant NTHi strains have also emerged but are less common than BLNAR strains.[14,16]

The current recommended OM treatments for Aboriginal and Torres Strait Islander children vary by condition, with amoxicillin and amoxicillin-clavulanic acid recommended for unresolved acute OM (AOM); azithromycin recommended where adherence to treatment is difficult or refrigeration unavailable; and trimethoprim-sulfamethoxazole recommended for unresolved CSOM.[17] Among NT children with OM, the prevalence of NTHi resistant to these antibiotics is currently unknown. Antibiotic treatment is prescribed empirically without confirmatory testing to determine whether the organism is susceptible. Improved understanding of NTHi resistance, at a population level, is therefore essential to inform the effective antimicrobial treatment of OM in our setting.

The nasopharynx is an important reservoir of NTHi, and nasopharyngeal colonization with NTHi is a precursor to otitis media.[5] Given the challenges inherent to sampling the middle ear among children without spontaneous perforations, the bacteriology of the nasopharynx is commonly used as a proxy for ear microbiology.

The primary aim of this study was therefore to determine the suitability of using population-level surveillance of key nasopharyngeal NTHi resistance profiles to inform future OM treatment guidelines. We also report the AMR profiles of nasopharyngeal and ear discharge NTHi from children with suppurative OM in a high-burden disease setting and describe the identified genetic determinants of resistance.

METHODS

Ethical approval

Analysis of *H. influenzae* isolates was approved by the Human Research Ethics Committee of the NT

TABLE 1 Non-typeable *Haemophilus influenzae* isolate source and study type

Study name and type	Years of data collected	Number of children enrolled	Number of isolates used in this study†
AATAAC, a randomized controlled trial[24]	2003–2005	320	Nasopharyngeal: 0 Ear discharge: 5
SHIMAR MARSi, cross-sectional[21]	2003, 2004	1420	Nasopharyngeal: 30 Ear discharge: 25
MARSiii, cross-sectional[22]	2010, 2011	1283	Nasopharyngeal: 10 Ear discharge: 14
MARSiv, cross-sectional[23]	2012	883	Nasopharyngeal: 32 Ear discharge: 1
MARSv, cross-sectional[23]	2013	371	Nasopharyngeal: 6 Ear discharge: 11
AAAOM (unpublished)	2008–2013	149	Nasopharyngeal: 17 Ear discharge: 36

†Only baseline swabs from clinical trials were used.

Department of Health and Menzies School of Health Research (07/85) and conforms to the provisions of the Declaration of Helsinki. Isolates in this study were sourced from previous studies where parents had provided written informed consent for further laboratory analyses. Patient anonymity was preserved.

Study setting

The study was performed in Australia's NT. This region has a population of 246 000 residents dispersed over 1.4 million km.[2,18,19] The Aboriginal and Torres Strait Islander population of the NT is approximately 75 000; 80% of whom reside in remote or very remote areas.[18,20]

Nasopharyngeal carriage and ear discharge studies and isolates

Isolates were selected to represent: i) the nasopharyngeal NTHi population associated with no OM or non-suppurative OM, and ii) the ear discharge NTHi population associated with suppurative OM, which for this study included an NTHi isolate from a dry perforation. Nasopharyngeal NTHi isolates ($n = 95$) were matched to ear discharge isolates ($n = 92$) based on being collected in the same calendar year and from a child of the same age (in years). Among the 95 nasopharyngeal isolates, 81.1% ($n = 77$) were from children with healthy, aerated ears and 18.9% ($n = 18$) were from children with OME. Among the 92 ear discharge isolates, 65.2% ($n = 60$) were from cases of CSOM and 33.7% ($n = 31$) were from cases of acute OM with perforation; one case was from dry perforation.

The NTHi isolates used in this analysis were sourced from samples collected from five previously published[21–24] and one unpublished study set in the NT (Table 1). The "MARS"[22,23] and "SHIMAR"[21] studies were carriage and OM surveillance studies across the NT. The "AATAAC" study was a double-blind randomized controlled trial of azithromycin versus amoxicillin for the treatment of acute OM in 16 communities in the NT.[24] The "AAAOM" study was a double-blind placebo-controlled randomized clinical trial of the use of oral azithromycin in Aboriginal children presenting with asymptomatic AOM without perforation. Only baseline swabs from clinical trials were used. There were two pairs of isolates from the same child; the remaining isolates originated from different children.

Phenotypic characterization of NTHi isolates

NTHi were identified and serotyped for this analysis using methods previously described. Briefly, NTHi were identified from bacitracin-vancomycin-clindamycin chocolate agar plates based on colonial morphology (greyish, semi-opaque, smooth, flat or convex, 1–3 mm in size), X and V growth factor dependence, and lack of reaction with capsular antisera using the Phadebact® Haemophilus coagglutination test (Thermo Scientific, MA, USA). Where NTHi recovery was compromised by swarming Proteus species, a filtration step was included.[6] Capsular genotyping for *bexA* and *bexB* genes was performed to confirm NTHi identification.[25]

The European Committee on Antimicrobial Susceptibility Testing (EUCAST) method of antibiotic susceptibility testing[26] was performed on isolates for the following antibiotics: ampicillin, trimethoprim-sulfamethoxazole, meropenem, tetracycline, ciprofloxacin, chloramphenicol, ceftriaxone, and amoxicillin-clavulanic acid. Isolates with intermediate susceptibility were reported in line with the EUCAST updated definition (susceptible, increased exposure) and are categorized as susceptible.[27] Isolates with resistance to three or more antibiotics were classified as multi-drug resistant.

Genotypic characterization of NTHi isolates

Purified isolates were resuscitated by 16-streak culture onto chocolate agar for whole genome sequencing purposes. Agar plates were incubated at 37°C + 5% CO_2 for 18 h. Single colonies were subcultured on chocolate agar with the same culture conditions. DNA was extracted using growth from the lawn of the subculture plate. DNA extractions were performed on a QIAcube instrument (QIAGEN, Hilden, Germany) using the Gram-positive enzymatic lysis protocol of the QIAcube DNeasy Blood kit as per the manufacturer's instructions (QIAGEN). Enzymatic lysis buffer consisted of 20 mM Tris-Cl pH 8.0, 2 mM EDTA, 1.2% Triton, and 1 mg/ml lysozyme (Sigma, Castle Hill, Australia). Extractions were performed using the default QIAcube settings. Genome sequence data were obtained with paired-end libraries on the Illumina HiSeq 2500 (Illumina, USA) platform at the Australian Genome Research Facility (Parkville, VIC, Australia).

Sequence data for the 187 isolates were mapped to the complete genome of *H. influenzae* 86-028 NP (GenBank accession no. CP000057.2) using SPANDx v3.2.1.[28] A midpoint-rooted maximum parsimony tree was constructed using 131 214 orthologous, biallelic single nucleotide polymorphisms (SNPs) using Paup v4.0a153[29] and visualized using iTOL[30] to determine whether isolates phylogenomically clustered based on anatomical niche.

Microbial Genome Assembler Pipeline v0.0.1 (https://github.com/dsarov/MGAP—Microbial-Genome-Assembler-Pipeline)[31] was used to generate reference-assisted (86-028 NP) genome assemblies of the 187 isolates. The multi-contig assemblies were merged into a single contig per NTHi isolate using kSNP, and the merged assemblies were annotated using Prokka v1.12-beta.[32] A pangenome was generated using the Prokka annotations as input into ROARY v3.12.0.[33]

A genome-wide association study (GWAS) was performed to determine whether any of the observed genetic variations in the dataset was associated with each anatomical niche (ear discharge or nasopharynx). The GWAS was performed using Pyseer v1.3.1,[34] comparing the ear discharge isolates to the nasopharyngeal isolates. To account for population structure in the dataset, multidimensional scaling components were used as fixed effects. Both clusters of orthologous groups and SNPs identified from the pangenome and SPANDx mapping, respectively, were subsequently analyzed for statistically significant genes/SNPs associated with the anatomical site. Supplementary to this, k-mers were identified from the 187 draft genome assemblies and analyzed using a linear mixed model, where a kinship matrix determined from the core genome phylogeny was used to account for population structure.

The presence of encoded AMR determinants in the 187 *H.influenzae* isolates was determined using ARIBA v2.14.5[35] and either the comprehensive AMR database (downloaded 4 January 2019) or a self-curated database containing AMR determinants specific to NTHi (*acrA*, *acrR*, $bla_{\text{ROB-1}}$, $bla_{\text{TEM-1}}$, *cat*, *dacA, dacB, folA, folP, ftsI, gyrA, gyrB*, *HIrrnF23S*, *parC, parE, rplD_L4, rplV_L22, and tetB*).

Isolates were categorized as β-lactamase positive ampicillin susceptible (BLPAS), BLPAR, or BLNAR based on the following criteria: BLPAS isolates were those with phenotypic susceptibility to ampicillin and the presence of $bla_{\text{TEM-1}}$; BLPAR isolates were those with phenotypic resistance to ampicillin and the presence of $bla_{\text{TEM-1}}$; BLNAR isolates were those with phenotypic ampicillin resistance and the absence of a $bla_{\text{TEM-1}}$. The term genetic BLNAR was used to describe ampicillin-susceptible strains with BLNAR substitutions N526K and/or R517H.[9,36]

Statistical analysis

Statistical analysis was conducted using STATA 15.1.[37] Differences in AMR between nasopharyngeal and ear discharge isolates were assessed using chi-squared tests. *P*-values of < 0.05 were considered statistically significant.

RESULTS

Characterization of *H. influenzae* isolates

Using the Phadebact Haemophilus coagglutination test, 181 of 187 isolates were identified as NTHi. Based on the results of *bexA* and *bexB* capsular genotyping, the six remaining isolates comprised two serotype b, and four types a, c–f. Based on the results of ARIBA analysis, six of 187 isolates were identified as capsular *H. influenzae*. These isolates included three serotype b, one serotype c, one serotype d, and one serotype e. A further isolate contained a truncated portion of the serotype e gene.

All capsular isolates originated from ear discharge. The phenotypic and genotypic tests were concordant on four of these isolates, while two genotypically capsular isolates were phenotypically identified as NTHi, and two isolates phenotypically identified as capsular serotypes did not possess capsular genes.

Phenotypic testing identified 7.7% (14/181) NTHi isolates as β-lactamase positive. Genotypically, 7.7% (14/181) of NTHi isolates carried β-lactamase genes; 13 isolates carried a $bla_{\text{TEM-1}}$ gene and one isolate carried a $bla_{\text{ROB-1}}$ gene. One isolate was phenotypically β-lactamase positive, for which no β-lactamase genes were identified. In addition, there was one isolate that was phenotypically β-lactamase negative, which carried $bla_{\text{TEM-1}}$.

Antimicrobial resistance profiles were similar among nasopharyngeal and ear discharge *H. influenzae* isolates

All nasopharyngeal isolates ($n = 95$) had antimicrobial susceptibility results; 90.2% (83/92) of the ear discharge isolates had available antimicrobial susceptibility results as nine isolates had inadequate growth. Of the isolates with available antimicrobial susceptibility, all nasopharyngeal isolates were NTHi, while the ear discharge isolates included four capsular *H. influenzae*. All capsular *H. influenzae* were susceptible to all antibiotics tested; these isolates were excluded from the analysis of phenotypic resistance to enable comparison of only non-typeable isolates.

In total, 174 NTHi isolates were included in the analysis of phenotypic resistance, and of these 10.3% ($n = 18$) were resistant to ampicillin, 9.2% ($n = 16$) were resistant to trimethoprim-sulfamethoxazole, 1.7% ($n = 3$) were resistant to chloramphenicol, 1.1% ($n = 2$) were resistant to amoxicillin-clavulanic acid, and 1.1% ($n = 2$) were resistant to tetracycline. All isolates were susceptible to meropenem, ciprofloxacin, and ceftriaxone. Of the two isolates resistant to amoxicillin-clavulanic acid, one was also resistant to ampicillin, while one was susceptible to ampicillin. There was no statistically significant difference in the proportion of AMR among nasopharyngeal and ear discharge isolates (Table 2). There were three nasopharyngeal isolates displaying multi-drug resistance, of which one was a BLNAR isolate (Isolate 1, Table 3).

Of 18 NTHi isolates with ampicillin resistance, 14 were BLPAR (zone diameter range 0–8 mm) and four were BLNAR isolates (zone diameter range 0–8 mm). Of the BLPAR isolates, two were multi-drug resistant; one of these tested phenotypically as a β-lactamase isolate however no β-lactamase gene was identified on ARIBA analysis (Isolate 2, Table 3).

Genetic determinants of AMR

None of the four BLNAR isolates contained known PBP3 substitutions associated with ampicillin resistance;[9,38] however, 26 NTHi isolates, and one serotype d *H. influenzae* isolate, that were susceptible to ampicillin and/or amoxicillin-clavulanic acid were identified as genetic BLNAR containing an N526K PBP3 substitution.

Tetracycline resistance was conferred by *tet* genes A, B, D, and R. Chloramphenicol resistance was conferred by *catII* genes in two of three isolates. There were no known trimethoprim-sulfamethoxazole resistance determinants identified.

Genetic determinants associated with ear disease

The phylogenomic tree generated via mapping of the 187 *H. influenzae* genomes to the 86-028 NP reference genome demonstrated an absence of isolate clustering based on the anatomical niche of the specimen (nasopharyngeal or ear discharge; Figure 1), indicating that NTHi from the middle ear and nasopharynx do not represent distinct genomic populations. Of the capsular isolates, five were dispersed throughout the phylogenetic tree, and one isolate which was phenotypically NTHi but genetically serotype e was part of the Clade I described by de Chiara et al.[39]

Factoring in multiple multidimensional scaling dimensions (6–9), no genes (clusters of orthologous groups) nor SNPs (140 555/186 690 statistically tested) were statistically significantly associated with ear discharge isolates compared to the nasopharyngeal isolates. Of the 18 380 416 k-mers identified, 17 077 574 were statistically tested. There were 3 666 443 unique k-mers observed; however, no k-mer was statistically significantly associated with ear discharge isolates compared to the nasopharyngeal isolates.

DISCUSSION

Our genomic comparisons found that NTHi from age- and time-matched nasopharyngeal and ear discharge samples did not represent distinct microbial populations. Thus, at the population level, our results support the use of AMR data from nasopharyngeal carriage screening to predict antimicrobial susceptibility among middle ear isolates in our setting. Consistent with this interpretation, we did not identify statistically significant differences in the antimicrobial susceptibility of nasopharyngeal and ear discharge isolates at the population level.

The phenotypic AMR levels identified among NTHi isolates in this population between 2002–2013 were relatively low compared to many countries where resistance is increasing.[12] For example, the 10% ampicillin resistance we report here for NTHi is comparable to NTHi isolated from children with bronchiectasis in the NT (2007–2010, 8%–15%),[40] whereas in China and Korea, the proportion of ampicillin resistance among NTHi in 2005–2014 has been reported as up to 59%.[41,42] In addition, we found that the proportion of NTHi isolates with β-lactamase production was low compared with other studies reported between the years 2005–2007 that found a prevalence of 13%–52%.[16,42,43] Consistent with global studies, most β-lactamase positive *H. influenzae* isolates contained $bla_{\text{TEM-1}}$, with a single isolate identified as containing $bla_{\text{ROB-1}}$.[42] One isolate was phenotypically β-lactamase negative but carried the $bla_{\text{TEM-1}}$ gene; this may be a result of a mutation in the promoter region or the presence of an inactive mutant TEM enzyme as described previously by Tristram.[44] Another isolate tested phenotypically

TABLE 2 Antimicrobial susceptibility results and comparison between ear discharge and nasopharyngeal non-typeable *Haemophilus influenzae* isolates

Antibiotics	Ear discharge ($n = 79$)	Nasopharyngeal ($n = 95$)	*P*
Ampicillin			
Resistant	5 (6.3)	13 (13.7)	0.11
Zone diameter range (mm)	0–35	0–31	
Trimethoprim-sulfamethoxazole			
Resistant	8 (10.1)	8 (8.4)	0.70
Zone diameter range (mm)	0–44	6–43	
Meropenem			
Resistant	0	0	–
Zone diameter range (mm)	26–52	25–44	
Tetracycline			
Resistant	0	2 (2.1)	0.64
Zone diameter range (mm)	23–44	20–41	
Ciprofloxacin			
Resistant	0	0	–
Zone diameter range (mm)	34–54	33–48	
Chloramphenicol			
Resistant	0	3 (3.2)	0.11
Zone diameter range (mm)	31–45	6–45	
Ceftriaxone			
Resistant	0	0	–
Zone diameter range (mm)	32–62	35–50	
Amoxicillin-clavulanic acid			
Resistant	0	2 (2.1)	0.20
Zone diameter range (mm)	19–34	14–34	

–, not applicable.

TABLE 3 Non-typeable *Haemophilus influenzae* nasopharyngeal isolates displaying multi-drug resistance

		Zone diameter (mm)				
Isolate number	Resistance genes	TET	AMP	SXT	CHLOR	AMC
1	None identified	35	15	16	38	14
2	$bla_{\text{TEM-1}}$, *catII*, *tetA*, *D*, *R*	20	6	35	18	15
3	$bla_{\text{TEM-1}}$, *catII*, *tetA*, *B*, *D*, *R*	21	6	35	20	18

Abbreviations: AMC, amoxicillin-clavulanic acid; AMP, ampicillin; CHLOR, chloramphenicol; SXT, trimethoprim-sulfamethoxazole; TET, tetracycline.

β-lactamase positive but did not contain a known β-lactamase gene. This suggests the presence of an undescribed enzyme that is responsible for β-lactamase activity as proposed previously by Farrell et al.[45]

We identified a small number of BLNAR isolates. Global surveillance studies have reported less than 0.2% of *H. influenzae* isolates as BLNAR; however, prevalence is increasing in Europe and Japan.[13,46–48] In 2006, the Australian Group on Antimicrobial Resistance reported that 8.5% of Australian *H. influenzae* isolates were BLNAR.[49] While the clinical significance of BLNAR isolates is debated,[16] these isolates tend to demonstrate reduced susceptibility to amoxicillin-clavulanic acid and other β-lactam antibiotics, including cephalosporins.[9] In addition, Japanese BLNAR *H. influenzae* isolates have recently been associated with multi-drug resistance.[14] As BLNAR isolates are associated with an increased risk of

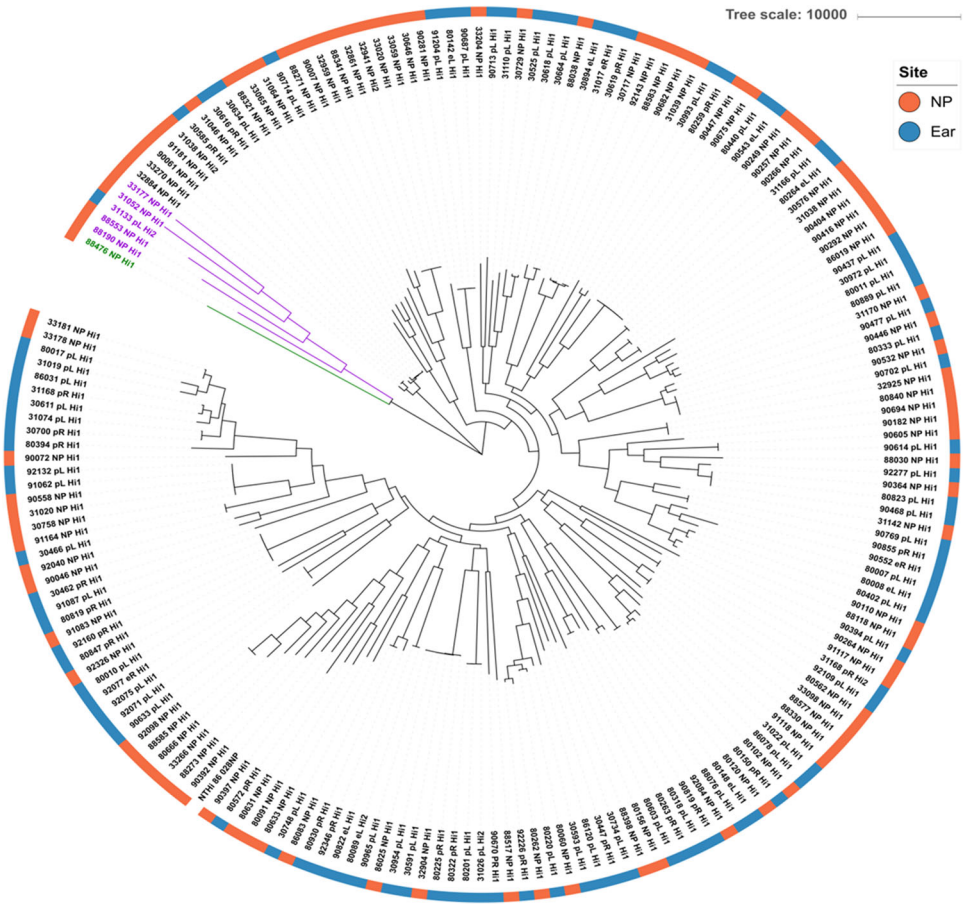

FIGURE 1 Phylogenetic tree of 187 *Haemophilus influenzae* isolates. A midpoint-rooted maximum parsimony tree was constructed using 131 214 orthologous, biallelic single nucleotide polymorphisms (95% confidence interval, 0.1410). The site of the isolate is indicated by the outer colored ring. Purple coloring indicates clade I;[39] green indicates *fucP* negative clade.[57] NP, nasopharyngeal.

treatment failure, ongoing surveillance of the prevalence of BLNAR isolates among NTHi-causing OM should be conducted.[13]

We did not identify known PBP3 mutations in the BLNAR isolates in this population. We identified 14% of isolates as genetic BLNAR, which is consistent with a previous Australian study.[36] While amino acid substitutions have been described in BLNAR isolates for 24 amino acids in the transpeptide domain of PBP3, there is no substitution that is found in all BLNAR isolates.[9]

The recommended treatment for OM among Aboriginal and/or Torres Strait Islander children includes oral ampicillin (first-line), oral amoxicillin-clavulanic acid (second-line), and oral trimethoprim-sulfamethoxazole where topical treatment does not resolve CSOM. Oral treatment is used with or without topical ear medication (typically ciprofloxacin).[17] Trimethoprim-sulfamethoxazole resistance was identified among 9% (n = 16) of NTHi isolates in this study; this compares with a previous Australian *H. influenzae* survey in 2006 that reported 20.1% non-susceptibility.[49] In comparison, trimethoprim-sulfamethoxazole resistance is reported in England among 12% of invasive (cerebrospinal fluid and bloodstream) NTHi isolates[50] and in China among 57% of *H. influenzae* isolates from the nasopharyngeal carriage.[41] There are no known mutations conferring trimethoprim-sulfamethoxazole resistance in NTHi, therefore further analysis to assess the associated genetic determinants would be beneficial.

We report resistance to amoxicillin-clavulanic acid (1%), chloramphenicol (2%), and tetracycline (1%). Concerningly, we identified three multi-drug resistant isolates. Of these, one isolate was resistant to the recommended oral treatments of ampicillin, amoxicillin-clavulanic acid, and trimethoprim-sulfamethoxazole.

Our genomic comparisons found that ear discharge and nasopharyngeal NTHi populations were not significantly different, suggesting that the middle ear environment is

not selected for a genomically distinct population. Previous studies have found that nasopharyngeal samples cannot be used to predict the middle ear fluid culture microbiology of individual patients.[51–53] A 2013 systematic review found that nasopharyngeal samples collected at the onset of acute OM vary compared to middle ear fluid isolates, and therefore at an individual level, cannot be used to predict the antimicrobial susceptibility of the middle ear for antibiotic prescribing.[53,54] A 2014 review found that the AMR of microorganisms in the middle ear fluid correlated poorly with nasopharyngeal swabs when taken from healthy patients; however, the authors identified that AMR of microorganisms (including *H. influenzae*) could be predicted by nasopharyngeal samples collected at the onset of acute OM.[52] Our results indicate that for the purposes of population-level surveillance, in our setting, the AMR of NTHi middle ear isolates is likely predicted using the antimicrobial susceptibility results of nasopharyngeal samples.

Strengths of this study include the use of whole genome sequencing and GWAS to demonstrate that NTHi from the nasopharynx and ear discharge do not represent distinct populations and that the middle ear niche is not selecting for NTHi with specific genetic features. The main limitation related to the difficulty in accessing NTHi isolates from the middle ear; isolates from six past studies were opportunistically accessed meaning that the isolates and AMR data were dated.

In summary, this study identified several key points relevant to the management of NTHi and OM in this population. We additionally identified the emergence of BLNAR isolates, trimethoprim-sulfamethoxazole resistance, and multidrug resistance among local NTHi strains. These findings emphasize the need for ongoing monitoring to track the further emergence of resistance to these antibiotics that are currently recommended for OM treatment. Our findings support efforts to improve AMR surveillance in high disease-burden settings in Australia that are outside of current surveillance reach.[55] We recommend the inclusion of NTHi AMR data from nasopharyngeal isolates in the HOTspots geospatial surveillance tool,[56] to improve monitoring of changes in NTHi resistance patterns over time, to inform local treatment guidelines, and to provide local epidemiological data for clinical management of patients with otitis media.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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