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Use of the 10-valent pneumococcal *Haemophilus influenzae* protein D conjugate vaccine (PHiD-CV10) in an Australian Indigenous paediatric population does not alter the prevalence of nontypeable *Haemophilus influenzae* without the protein D gene

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ABSTRACT

Background: Nontypeable *Haemophilus influenzae* (NTHi) is one of the main respiratory pathogens associated with otitis media and lung infections in Australian Indigenous children. PHiD-CV10, the 10-valent pneumococcal conjugate vaccine containing *H. influenzae* protein D was used in the Northern Territory infant vaccination schedule for two years from October 2009.
Methods: NTHi isolates from nasopharyngeal and ear discharge samples collected before, during and after the PHiD-CV10 era were screened for the *hpd* gene by PCR. Target amplicon sequence, extracted from available genomic sequence data, was analysed to identify variability in this region.
Results: There was no statistically significant difference in the proportion of *hpd*#3-PCR negative isolates from each era; overall 7% and 6% of nasopharyngeal and ear discharge isolates were negative, respectively. The nucleotide sequence data supported the *hpd*-PCR findings; truncations of the *hpd* gene precluding amplification and presumably expression of protein D were observed in approximately 7% of available genomes.
Conclusions: In the Northern Territory of Australia, a population at high risk of NTHi-associated infection, PHiD-CV10 use did not select for *hpd*-PCR negative isolates.

1. Introduction

Nontypeable *Haemophilus influenzae* (NTHi) is one of the main respiratory pathogens associated with otitis media, bronchiectasis, protracted bacterial bronchitis, and chronic suppurative lung disease among Australian Indigenous children [1].

Cross-sectional surveillance studies in remote communities of the Northern Territory (NT, Australia) during 2012–13 highlighted that 93% of Indigenous children (mean age 13 months) continue to experience some form of otitis media (OM), from OM with effusion (OME) through to chronic suppurative otitis media (CSOM) [2]. Of these children with OM, approximately 12% had a tympanic membrane perforation (TMP) and 9% had active ear discharge (ED), from which 63% cultured NTHi, 43% *Streptococcus pneumoniae* and 7% *Moraxella catarrhalis* [2]. In October 2009 the 10-valent pneumococcal *H. influenzae* protein D conjugate vaccine (Synflorix or PHiD-CV10) replaced the 7-valent pneumococcal conjugate vaccine (Prevenar or PCV7) on the NT childhood vaccine schedule. PHiD-CV10 utilises *H. influenzae* protein D (HiD) as a carrier for eight of the ten pneumococcal serotypes. In October 2011 Prevenar13 (or PCV13), which covers 3 additional pneumococcal serotypes but lacks HiD, replaced PHiD-CV10. The NT was the only Australian jurisdiction to use PHiD-CV10, with the rest of Australia moving directly from PCV7 to PCV13 in July 2011.

Early work showed HiD was antigenic [3,4] and constitutively expressed among all NTHi strains tested [5]; however, recent studies have found NTHi isolates lacking the *hpd* gene, which encodes HiD [6,7]. A randomised controlled trial of a pre-licensure HiD-conjugated vaccine (11Pn-PD) conferred a 35% reduction in NTHi-OM [8]. Since licensure of PHiD-CV10, most studies have had insufficient power to adequately describe the efficacy against NTHi carriage and middle ear infection [9]. In the NT, surveillance studies showed that compared to PCV7, PHiD-CV10 did not reduce

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NTHi carriage but did significantly reduce the prevalence of NTHi cultured from middle ear discharge specimens [10]. This is suggestive of a compartmental effect between middle ear and the nasopharynx (Np), whereby NTHi middle ear infection is prevented or NTHi is cleared from the middle ear during inflammation, yet remains unimpeded in the Np.

Since 2001, six studies of nasopharyngeal carriage and OM have been conducted in the NT during vaccine schedule transitions from PCV7 [11–13] to PHiD-CV10 [14] to PCV13 [2,10]. In addition, two RCTs are underway to compare PHiD-CV10, PCV13 and a combination schedule of both PCV vaccines [15]. These NT studies provide a unique opportunity to investigate vaccine selective pressures on the NTHi population. The aim of this study was to determine whether population-wide use of a vaccine containing HiD (PHiD-CV10; 2009–2011) positively selected for *hpd* negative NTHi in Np and/or middle ear discharge specimens of children in the NT.

2. Methods

2.1. Selection of isolates

Isolates were selected from OM and Np carriage surveillance studies conducted among children aged 0–6 years living in remote Indigenous communities across the NT between 2008 and 2013 [2,10]. We selected all available presumptive NTHi isolates (n = 509, 458 from Np and 51 from ED swabs) from 330 children aged <3 years, where individual parental consent had been given for future use of samples. ED samples were only collected when discharge was present upon examination. Presumptive NTHi isolates were identified by colony morphology (all morphotypes were selected), haemin and NAD dependence (Oxoid), and capsule absence using the Haemophilus Phadebact (Remel) test. Isolates were screened using the *hpd*#3 real-time PCR assay [16]. Those negative for *hpd*#3 were further scrutinised using a multiplex PCR targeting *siaT* and *hypD* to confirm the isolate as *H. influenzae* and not *H. haemolyticus* [17].

For this analysis children were allocated to one of three groups based on their vaccine history prior to the Np and/or ED swab collection.

PCV7 group: those vaccinated exclusively with PCV7 (primary course at 2, 4 and 6 months) prior to November 2009. Older children may have also received the scheduled 18 month pneumococcal polysaccharide vaccine (PPV23) booster.

PHiD-CV10 group: those vaccinated exclusively with of PHiD-CV10 (primary course 2, 4 and 6 months) between April 2010 and May 2012. Older children may have also received the scheduled 18 month PHiD-CV10 booster.

PCV13 group: those vaccinated exclusively with PCV13 (primary course 2, 4 and 6 months), who had swabs collected after June 2012. Older children may have received the scheduled 12 month PCV13 booster.

2.2. Genomic analysis

The *hpd*#3 amplicon represents 150 bp of the 1092 bp NTHi *hpd* gene [16]. To further clarify *hpd*#3 amplicon negative outcomes, we reviewed the *hpd* loci of 602 available *H. influenzae* genomes derived from isolates associated with lung, ear, invasive disease and Np carriage from varying geographic locations, both local and international. This included 41 isolates analysed with the *hpd#3* PCR in this study. The bioinformatic tool ARIBA [18] was used to generate local assemblies of the *hpd*#3 sequence from genomic short-read data to confirm the presence of an incomplete *hpd*#3 sequence, with reference to the *H. influenzae* PittEE genome (GenBank accession CP000671.1) [19] from which the *hpd*#3 assay was derived.

2.3. Statistical analysis

We compared the proportion of *hpd*#3-PCR negative swabs, *hpd*#3-PCR negative isolates and *hpd*#3-PCR negative children between vaccine groups using the Chi-square test. Children were considered *hpd* positive if any isolate from any swab (Np or ED) collected from that child was *hpd*#3-PCR positive. Swabs were considered positive if any isolate from the swab was *hpd*#3-PCR positive. Ages were compared using the Wilcoxon rank-sum test and logistic regression for association with *hpd*-negativity. Analysis was performed with Stata 14.

2.4. Ethics

Source studies had full ethical approval from the Human Research Ethics Committee of both Menzies School of Health and NT Department of Health (HREC 00153) and Central Australia (HREC 00155). All participants in this analysis had consented to future use of samples for related research.

3. Results

3.1. Isolate results

Of the 330 study children who contributed NTHi positive samples: 134 children from the PCV7 group contributed 130 Np swabs (219 isolates) and 18 ear discharge swabs (27 isolates); 132 children from the PHiD-CV10 group contributed 129 Np swabs (162 isolates) and 8 ED swabs (15 isolates); and 64 children from the PCV13 group contributed 64 Np swabs (77 isolates) and 8 ED swabs (9 isolates) (Table 1). Some swabs contributed more than one isolate. Of these children, 92% of the cohort had completed their primary PCV schedule at sampling (91% of PCV7 group, 98% of the PHiD-CV10 group and 83% of the PCV13 group). Most children with incomplete schedules were age appropriately immunised.

The median age was statistically lower in each consecutive group (Table 1). Logistic regression showed no association between *hpd*#3-negative children, age, and vaccination group (data not shown). Gender was evenly balanced between groups. Each child contributed a swab or swabs from a single time point. Most children contributed a swab from only the Np, seven contributed only ED swabs and twenty contributed swabs from both sites (Table 1).

The median number of isolates per child was 2 (range 1–9). The median number of swabs per child was 1 (range 1–3). This gave a total of 458 Np isolates and 51 ED isolates for PCR scrutiny.

Overall, 7% (31/458) of NTHi isolates from the Np and 6% (3/51) of NTHi isolates from ED were *hpd*#3-PCR negative. By child, 6% (18/330) were *hpd*#3-PCR negative (both Np and ED swabs). Concurrent carriage of both a *hpd*#3-PCR negative and *hpd*#3-PCR positive NTHi isolate was observed for six PCV7 NP swabs, and one Np swab each in the PHiD-CV10 and PCV13 groups. Of the *hpd*#3-PCR negative isolates (n = 34; 6%), multiplex real-time PCR revealed that two Np isolates from children in the PCV7 group were *H. haemolyticus* [17]. Removal of these isolates from the analysis did not significantly affect p-values.

There were no statistically significant differences in the proportion of children with *hpd*#3-PCR negative NP swabs between vaccine groups (5% PCV7 vs 6% PHiD-CV10, p = 0.778; 3% PCV13 vs 6% PHiD-CV10, p = 0.364) (Table 2). Among the small number of ED swabs, no vaccine-group difference was apparent (6% PCV7 vs 0% PHiD-CV10, p = 0.497; 12% PCV13 vs 0% PHiD-CV10, p = 0.302).

Table 1
Demographics of children and swab sites, by group.

PCV era/years n = number of children	PCV7 <2009 n = 134	PHiD-CV10 2010–2012 n = 132	PCV13 >2012 n = 64	PCV7 vs PHiD-CV10 p-value	PHiD-CV10 vs PCV13 p-value
Age in years, median (range)	1.54 (0.43–2.58)	1.26 (0.5–2.76)	1.08 (0.42–2.42)	<0.001	0.014
Sex male (%)	51	46	50	0.388	0.550
No. children who contributed which type of swab					
Np only	118	126	59		
Np + 1 ED	10	2	2		
Np + 2 ED	2	1	3		
No Np, 1 ED swab	4	2	0		
No Np, 2 ED swabs	0	1	0	Totals	
Total Np isolates	219	162	77	458	
Total ED isolates	27	15	9	51	

Np: Nasopharyngeal; ED: ear discharge.

Table 2
hpd#3-PCR results by vaccine group.

	PCV7 <2009	PHiD-CV10 2010–12	PCV13 >2012	PCV7 vs PHiD-CV10 p-value	PHiD-CV10 vs PCV13 p-value
Np swabs (n)	130	129	64		
hpd#3 negative (%)	7 (5)	8 (6)	2 (3)	0.778	0.364
hpd#3 positive (%)	123 (95)	121 (94)	62 (97)		
Np isolates (n)	219	162	77		
hpd#3 negative (%)	16 (7)	11 (7)	4 (5)	0.846	0.635
hpd#3 positive (%)	203 (93)	151 (93)	73 (95)		
ED swabs (n)	18	8	8		
hpd#3 negative (%)	1 (6)	0	1 (12)	0.497	0.302
hpd#3 positive (%)	17 (94)	8 (100)	7 (88)		
ED isolates (n)	27	15	9		
hpd#3 negative (%)	1 (4)	0	2 (22)	0.451	0.057
hpd#3 positive (%)	26 (96)	15 (100)	7 (78)		

3.2. Genomic analysis of hpd#3 amplicon variation

For this analysis, 602 *H. influenzae* genome sequences were investigated, of which 41 genomes corresponded to isolates included in this study. The *hpd* gene sequence was assembled for each genome, revealing twenty-four unique *hpd*#3 amplicon variants (AVs) (Fig. 1). Four of the AVs likely result in disrupted *hpd*#3 PCR amplification due to the absence of a primer binding site due to 5′ or 3′ *hpd* gene truncations (AV 21–24). Multiple partial *hpd* assemblies were observed for four genomes. A full *hpd*#3 amplicon was observed in 3 of the 4 genomes (AVs 2, 9, 10), one of which corresponded to a study isolate and amplified in the *hpd*#3-PCR as expected (AV 10). The remaining genome had two partial *hpd* assemblies, neither containing a full *hpd*#3 amplicon (AV 23, 24) and this genome did not overlap with the study cohort.

Of the 602 genomes reviewed, 43 (7%) had the 5′ truncation of the *hpd* gene that included the *hpd*#3 forward primer binding site (AV 21, 22) (Fig. 1). We expect that this truncation would abrogate Protein D expression. As mentioned, four genomes (0.7%) had partial *hpd* genome assemblies that would also likely preclude Protein D expression. An additional four genomes (0.7%) had small 3′ truncations (86–118 bp) which did not affect the *hpd*#3 amplicon (data

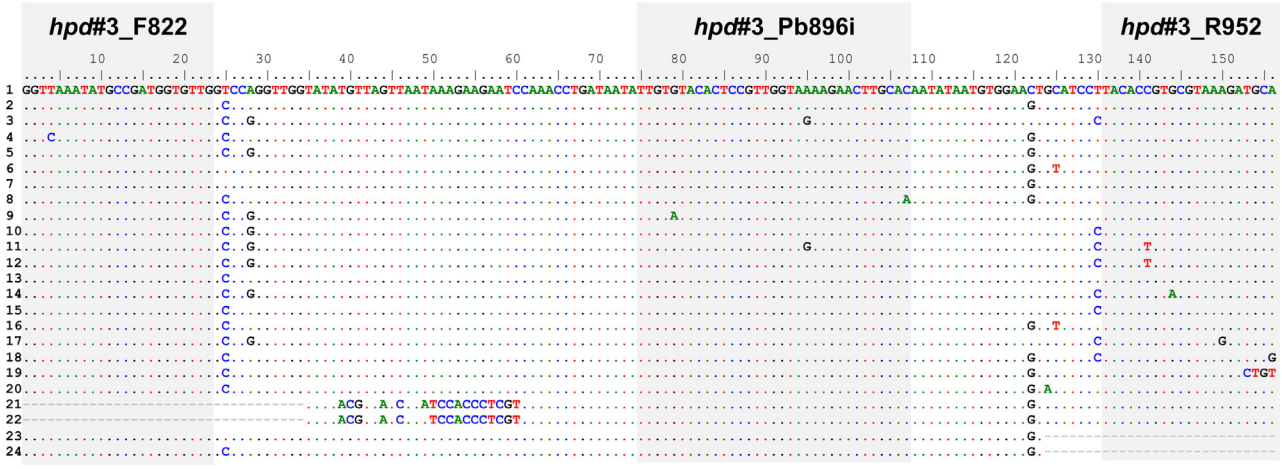

Fig. 1. Nucleotide sequences of the unique hpd#3 amplicon variants (AVs). The primer (*hpd*#3_F822 and *hpd*#3_Pb896i) positions are indicated by grey boxes. AV nucleotide sequences are presented relative to AV1, where letters denote observed variation at the relevant nucleotide position, and dots signify identical sequence. Missing sequence is denoted by a dash.

not shown). The impact of this truncation on Protein D expression is not yet known.

Among the 41 genomes corresponding to isolates used in this study, 41 harboured full *hpd*#3 amplicons (AVs 2, 4, 7, 8, 10, 15, and 18) and were *hpd*#3-PCR positive. The remaining isolate was *hpd*#3-PCR negative, which correlated with the presence of a truncated *hpd*#3 (AV 21) in its genome.

4. Discussion

We found no difference in proportions of children with *hpd*#3-PCR negative NTHi Np carriage between the PCV7, PHiD-CV10 and PCV13 groups investigated. This suggests that the use of PHiD-CV10 did not select for NTHi Np carriage isolates with a truncated or absent *hpd* gene. Additionally, among the small numbers of ED swabs tested, we found no difference in the proportion of *hpd*#3-PCR-negative isolates between vaccine groups. Due to the small sample size we are not able to definitively conclude there is no difference in *hpd*#3-PCR-negative NTHi isolated from ear discharge. A larger study of ear isolates may clarify the role of *hpd* in observed compartmental effect [10]. There was a statistically significant difference in age between the vaccine groups and hence potential group differences in vaccine-induced immunity at the time of Np and ED sampling, however logistic regression showed no association of age with the odds of a child having *hpd*#3-PCR negative NTHi.

Overall, 92% of the analysed cohort had received their primary schedule vaccines (2, 4, and 6 months of age) at sampling. The majority of the incomplete schedules were age appropriate. The overall NT paediatric population has high vaccination rates, with PCV vaccination completed by age 12 months in approximately 90% of children from 2007 to 2012 [20–23]. While we did not observe a NTHi *hpd*#3-PCR-negative population difference, nor a change in overall NTHi Np carriage, PHiD-CV10 was only in use in the NT for two years, and longer-term use could potentially drive subtle bacterial population changes beyond those measured here.

Antibiotic use by the child in the weeks prior to sampling was not taken into consideration for this analysis. All available NTHi were tested for *hpd*#3 without review of antibiotic susceptibility. In the original collection studies there was a significant increase in antibiotic prescriptions in the PHiD-CV10 era compared to PCV7, (28% to 42%, p=<0.0001) [10], however this did not impact on the NTHi carriage, which remained between 63% and 71% across the three eras [2].

Our data suggest that the *hpd*#3-PCR negative NTHi isolates in this study could have a truncated or absent *hpd* gene, resulting in *hpd*#3-PCR non-amplification. The ramifications of the non-amplification of *hpd*#3-PCR negative NTHi isolates where *hpd*-based PCR methods are used in isolation to identify or quantify *H. influenzae*, include species misidentification, and/or underestimation of prevalence and density. More robust *H. influenzae* PCR targets have since been published that complement (*fucP* [24,25]), or replace entirely (*siaT* [17]) the *hpd*#3 PCR, providing the opportunity to identify NTHi with and without the *hpd* gene.

The impact of observed *hpd* gene truncations on Protein D formation and expression will be the focus of further investigation. However, we speculate that a significant *hpd* truncation (AV 21–24) or complete gene absence would directly negatively affect protein D expression. This may allow NTHi to evade PHiD-CV10 vaccine-mediated antibody response. However, the associated fitness costs and impact to NTHi virulence is not currently known. Stability at approximately 7% of the NTHi population suggests *hpd*#3-PCR negative NTHi does not noticeably affect bacterial fitness. Ahren *et al* reported that protein D promoted adherence and internalisation on NTHi into human monocytic cells [26], therefore from a clinical perspective these *hpd*#3-PCR negative isolates may be less infective.

The genome collection used is not a true NTHi population representation, as disease-related and non-standard NTHi were deliberately selected for whole genome sequencing as part of other studies. However, the prevalence of truncated *hpd* conferring a non-amplifying *hpd*#3 sequence (AV 21 – 24, Fig. 1) in the NTHi genomes was similar to the prevalence of *hpd*#3-PCR negative Np isolates from the study population, suggesting the NTHi genome dataset provided a reasonable approximation.

In conclusion, the use of PHiD-CV10 does not appear to select for NTHi Np carriage isolates with a truncated or absent *hpd* gene. However, our previous finding of a potential compartmental impact of PHiD-CV10 on NTHi culture from the Np versus middle ear suggests that further investigation of *hpd* gene variation in NTHi isolates from ear discharge is warranted [9,10]. Further investigation of *hpd* truncations and their effect on Protein D formation and expression is also warranted to help elucidate implications for vaccine efficacy.

Declaration of Competing Interest

The authors declare they have no competing interests.

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