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Whole genome sequencing identifies opportunistic non-typeable *Haemophilus influenzae* rather than a hypervirulent clone

Sir,

Haemophilus influenzae (HI) is a strict human pathogen implicated in respiratory tract infections.¹ Invasive infections with capsulated HI type B (Hib) have all but disappeared due to Hib vaccination inclusion in the Australian immunisation

program since 1993.² Occasionally, non-typeable HI (NTHI) are isolated from sterile sites, e.g., infective endocarditis, meningitis.^{3,4} The growing cohort of immunocompromised patients from malignancy, its treatment and organ transplantation create a susceptible population.⁵ In the absence of an opsonising and phagocytosis evading polysaccharide capsule, IgA proteases, serum resistance and adhesins are the main virulence characteristics for NTHI.¹ We unexpectedly observed four cases of invasive NTHI over 7 months and investigated whether there was an unusually virulent clone circulating in our immunocompromised patient population.

Patients presented to our quaternary care metropolitan hospital from October 2018 to April 2019 with sterile site infections (Table 1). Bacterial identification was performed following isolation of pathogens using a matrix assisted laser desorption ionisation-time of flight mass spectrometry (MALDI-TOF MS; Bruker Daltonics, Germany) with HI identified by scores of >2. Susceptibility testing was performed according to Clinical and Laboratory Standards Institute (CLSI) M100 standards (28th ed)⁶ and β -lactamases detected by the nitrocefin disc test (Oxoid, UK).

Genomic DNA was extracted from the isolates using the Qiagen EZ1 Advanced Extractor (Qiagen, Germany) as per the manufacturer's instructions. Sequencing libraries were prepared using the Nextera DNA Flex Kit (Illumina, USA) as per the manufacturer's instructions and sequenced on an Illumina MiSeq. *De novo* assembly was performed on the read data using SKESA (v.2.3.0) and sequence typing performed using multilocus sequence typing (MLST). Virulence genes identified by Staples *et al.* were downloaded from NCBI and blasted against the assemblies.⁷ The sequencing reads were mapped to the complete genome *Haemophilus influenzae* strain NML-Hia-1 (GenBank accession: NZ_CP017811.1) using BWA and variants called using FreeBayes (v1.3.1-dirty). A maximum likelihood phylogenetic tree was constructed using FastTree (v2.1.10).

The clinical and microbiological characteristics of four male patients are shown in Table 1. Three patients were at risk for invasive NTHI infection from underlying defects in ear anatomy. The remaining patient was treated with rituximab for a lymphoproliferative disease. All were treated with ceftriaxone despite the availability of susceptibility testing. In the three bacteraemic patients, blood cultures were negative

Table 1 Clinical and microbiologic features in four patients with non-typeable *Haemophilus influenzae* invasive infection

Patient	Age	Risk	Site of infection	Site of isolation	Antibiotic treatment (weeks)	Phenotypic resistance	Molecular characteristics MLST type	Virulence genes
1	74	Lymphoproliferative disease	Bilateral pneumonia	Sputum	CTR (1)	β -lactamase negative	183	<i>hmw C</i> <i>pepN</i>
2	64	Eustachian tube anomaly, recurrent otitis media without bony erosion	Meningitis	Blood	CTR (4)	β -lactamase negative	103	<i>hmw C</i> <i>pepN</i>
3	59	Previous mastoidectomy with grommets, new mastoid fracture with CSF leak	Meningitis	Blood	CTR (2)	β -lactamase positive CTR/AUG sensitive	143	<i>hmw C</i> <i>pepN</i> <i>bla TEM-1</i>
4	53	Excision of acoustic neuroma 2 years prior complicated by CSF leak	Meningitis, subdural empyema	CSF	CTR (6)	β -lactamase negative CTR/ampicillin sensitive	136	<i>hmw C</i> <i>pepN</i>

AUG, amoxicillin clavulanic acid; CSF, cerebrospinal fluid; CTR, ceftriaxone; MLST, multilocus sequence typing.

2–3 days after commencement of ceftriaxone. One patient, without bacteraemia, had NTHI isolated from cerebrospinal fluid (CSF). This patient had a poor outcome (hydrocephalus, intellectual deficits) despite negative CSF cultures 48 h post-antibiotics. The four isolates were not identical based on *in silico* typing with more than 21,588 core SNPs between them (Fig. 1). Of the virulence genes investigated (*hmw C*, *pepN*, *hmw 1A*, *hmw2A*, *hap*, *igaI*), all four isolates harboured *hmwC* and *pepN*. Absence of capsular *BexA* and *Bex B* genes confirmed the phenotypic absence of a capsule in all isolates. *BlaTEM-1* was detected in the single isolate testing β -lactamase positive using phenotypic methods.

Globally, the average incidence of invasive NTHI is approximately 1/100 000 population.⁸ Reported reasons for the rise in NTHI infections include increases in nasopharyngeal colonisation in the healthy population from strain replacement post-Hib vaccination. This results in increased transmission of NTHI to vulnerable individuals. Typically, patients infected with NTHI have advancing age and underlying comorbidity, e.g., cardiopulmonary disease, HIV, alcoholism, malignancy, splenectomy, hypogammaglobulinaemia, etc.^{1,8} Our findings were consistent with the literature where one patient had lymphoma, while the remaining patients had meningitis related to anatomical defects and prior surgery. Although invasive infection is rare and our cases occurred over a short 7 month period, genome sequencing did not reveal clonal spread or novel virulence factors. Staples *et al.* also reported high genetic diversity by MLST and SNP typing without a dominant clone in 74 NTHI strains over a 15-year period in Queensland.⁷ However, Andersson *et al.* reported a respiratory outbreak in a Swedish long term care facility where all isolates clustered as MLST 14.⁹ Virulence factors associated with invasive NTHI are less well described. Biofilms prevent clearance in children with otitis media.¹⁰ Two Ig A proteases (*igaA*, *igaB* genes) are

implicated in chronic obstructive pulmonary disease associated pneumonia; serum resistance in infective endocarditis and adhesin proteins are described.^{5,11} Sequencing detected the presence of *hmw C* and *pepN* adhesins in our isolates. Adhesins mediate the attachment to human epithelial cells and promote colonisation. It is likely that our patients with meningitis were colonised with NTHI, and contiguous meningeal spread facilitated by disruption of anatomical planes was the pathogenesis.

In conclusion, the availability of whole genome sequencing in a clinical laboratory rapidly addressed our concerns of an unusually virulent clone or possible outbreak. NTHI have been implicated in clonal invasive disease, hence continuous surveillance and application of rapid genomic methods is relevant, particularly in our severely immunocompromised population.

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Fig. 1 Maximum likelihood phylogeny depicting the genetic relationship among isolates using *Haemophilus influenzae* strain NML-Hia-1 (GenBank accession: NZ_CP017811.1) as a reference. Scale bar indicates nucleotide substitutions per site.

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