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Nontypeable *Streptococcus pneumoniae* as an Otopathogen

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Abstract

Among 34 *Spn* sequential isolates from middle ear fluid we found a case of a nontypeable *Streptococcus pneumoniae* (NT-*Spn*) in a child with AOM. The strain was pneumolysin PCR positive and capsule gene PCR negative. Virulence of the NT-*Spn* was confirmed in a chinchilla model of AOM.

Keywords

multi-locus sequence typing; nontypeable *Streptococcus pneumoniae*; acute otitis media

1. Introduction

Virtually all *Streptococcus pneumoniae* (*Spn*) causing acute otitis media (AOM) and sinusitis are encapsulated (Hanage *et al.*; 2006; Kadioglu *et al.*, 2008). Non-typeable *Streptococcus pneumoniae* (NT-*Spn*) have generally been viewed as an organism that does not cause suppurative infections; they have typically been described as pathogens in epidemic bacterial conjunctivitis (Barker *et al.*, 1999; Medeiros *et al.*, 1998; Carvalho *et al.*, 2003; Martin *et al.*, 2003; Berron *et al.*, 2005; Hanage *et al.*, 2006; Porat *et al.*, 2006; Williamson *et al.*, 2008) although one paper described NT-*Spn* as a cause of invasive pneumococcal disease (Lacapa *et al.*, 2006). Recently we isolated a NT-*Spn* from MEF of a child with suppurative AOM in Rochester, New York. This case report describes the suppurative AOM case and the characterization of the strain, including its genetic background and genetic relatedness to other *Spn* isolates, and its virulence in an experimental chinchilla model of AOM.

2. Case Report

A 7-month-old male developed an apparent upper respiratory viral infection followed 2 days later by fever of 39 Centigrade, and symptoms of an AOM. On examination the tympanic membranes were bulging and erythematous with a purulent effusion bilaterally. The remainder of the examination included coryza and an absence of conjunctival injection or

drainage. Since the child had enrolled in a prospective study sponsored by the NIH, NIDCD that called for a tympanocentesis whenever AOM occurred, the procedure was performed and produced copious purulent material from behind both tympanic membranes that later grew a NT-*Spn* (Pichichero et al., 2006). A culture from the nose and throat was also obtained per the study protocol and the nose culture grew the same NT-*Spn*. The child was treated with amoxicillin/clavulanate at a dose of 80 mg/Kg/day divided twice daily for 5 days and recovered uneventfully. He had two subsequent AOM infections where nontypeable *Haemophilus influenzae* was isolated. Thereafter he remained well with no further AOMs or other significant respiratory infections.

The bacterial isolates from MEF and nasopharyngeal were identified as *Spn* by tests were according to the 8th edition of Manual of Clinic Microbiology (Ruoff et al., 2003). Serotyping on both isolates was negative as determined by latex agglutination (Pneumotest-Latex, Statens Serum Institute, Copenhagen, Denmark). Both isolates were susceptible to optochin as tested using a Taxo P Disc (BD diagnostic Systems) and were bile soluble (Table 1).

To further confirm the strain was a *Spn*, bacterial genomic DNA was extracted to amplify the pneumolysin (*gly*) gene by PCR using forward primer CCCACTCTTCTTGCGGTTGA and reverse primer TGAGCCGTTATTTTTCATACTG, with a annealing temperature 55 °C (Murdoch et al., 2003). The strain from both the MEF and NP specimens expressed pneumolysin (Table 1). To determine that the strain did not have a capsule gene (*cps*) locus, the conserved capsular gene dexB (1714-3485 of GenBank Z47210) was amplified by PCR from genomic DNA using forward primer ATCCTTGTCAGCTCTGTGTC and reverse primer TCACTTGCAACTACATGAAC, with a with an annealing temperature of 50°C (Hanage et al 2006). An encapsulated strain with serotype 3 was used as a positive control. Both isolates from the MEF and NP specimens were PCR negative for capsule gene (Table 1), confirming that the isolate did not contain *cps* locus.

MICs of the NT-*Spn* isolates to 13 antibiotics were determined by broth microdilution (BMD) method according to CLSI (M100-S20). The isolates from MEF and NP were susceptible to all tested antibiotics including penicillin, ampicillin, amoxicillin, cefdinir, cefpodoxime, cefuroxime, ceftriaxone, faropenem, azithromycin, telithromycin, levofloxacin, moxifloxacin and trimethoprim.

To prove that the NT-*Spn* was the sole otopathogen, multiplex PCR (mPCR) was applied to detect DNA of 16S rRNA gene of *Haemophilus influenzae* (Hflue), *Moraxella catarrhalis* (Mcat) and *Streptococcus pyogenes* (Pyo) in the MEF as previously described (Hendolin et al., 1997) except reactions for each bacterial were separated in different tubes to avoid mutual interference. The mPCR was positive for *Spn*, but negative for the other bacteria in the MEF (Figure 1), indicating the NT-*Spn* was only pathogen causing AOM in this case.

Multilocus Sequence Typing (MLST) was performed to characterize the NT-*Spn* strain as described previously (Enright and Spratt, 1998) and in the *Spn* MLST database (<http://spneumoniae.mlst.net/>). The sequence type of the NT-*Spn* from both the MEF and NP specimens was the same, MLST 448 (Table 1). In order to illustrate nucleotide sequence divergence and explore the genetic relatedness of the NT-*Spn* isolate to other isolates, minimum evolution trees were constructed from the sequence of all seven alleles using the neighbor-joining method. The sequences of seven loci were concatenated to obtain a 3199-bps contigs and then a neighbor-joining tree was drawn using MEGA 4.0 (Tamura et al., 2007). The genetic linkage distance of the NT-*Spn* strain described in this report was close to three other, new strains that were sequence types 22, 35 and 9/34/35/42/47 (Figure 2).

The virulence of the NT-*Spn* strain was evaluated in an experimental chinchilla model of AOM using two different previously described routes for middle ear challenge (Babl *et al.*, 2002; Sabharwal *et al.*, 2009). In an experiment of NP challenge followed by barotrauma, 2 chinchilla (4 ears) were challenged intranasally with 10E7 cfu of our NT-*Spn* strain in 100ul per nares. One day following challenge, NP colonization was present in both animals at 2×10^4 cfu/ml. None of 4 ears subsequently developed signs of experimental AOM and all four ears were culture negative when cultured 3 days following barotrauma. In an experiment of direct inoculation of the middle ear, 2 animals (four ears) were challenged directly with 52 cfu in 100 μ l of HBSS of our NT-*Spn* strain. Two days following inoculation the right ear of one animal was culture positive (100cfu/ml); five days following inoculation the left ear of the same animal was culture positive (10E5 cfu / ml). Two additional chinchilla (4 additional ears) were challenged directly with 1000 cfu in 100 μ l of HBSS of the NT-*Spn* strain. All four had culture positive middle ear disease present at 3 days following challenge (1,000 cfu per ml).

3. Discussion

This case report of suppurative AOM in a child caused by NT-*Spn* is the first, to our knowledge, to be confirmed in the US as a sole otopathogen.

Up to now, there have been 320 nontypeable *Spn* isolates with 205 sequence types registered into MLST database. Of them, 27 isolates with 10 sequence types were isolated from AOM cases, but most sequence types were from the nasopharynx. Only ST448(ours) and ST13 (Lipsitch M 2007) were from MEF (Lipsitch *et al.*, 2007). Lipsitch *et al* (2007) did not provide any clinical information about the ST13 strain nor did they exhaustively characterize the strain as we have. In 2005 and 2006, Hanage WP *et al* found three NT-*Spn* strains with STs 448, 449 and 344 from the MEF of Finnish children with AOM (Hanage *et al.*, 2005; Hanage *et al.*, 2006). Their strains were not included in the MLST database. Neither Lipsitch *et al* (2007) nor Hanage *et al.* (2005, 2006). mentioned if any other otopathogens were involved in the AOM.

The ST448 seems to be an international strain that broadly causes conjunctivitis (Porat *et al.*, 2006), and has begun to associate with AOM (Hanage *et al.*, 2006). There are 17 isolates reported to MLST database that are nonserotypeable *Spn* from 8 countries (Australia, China, Germany, Portugal, Switzerland, Thailand, UK, USA) (Hathaway *et al.*, 2004; Porat *et al.*, 2006; Lipsitch *et al.*, 2007; Sá-Leão *et al.*, 2006). Nine of 17 ST448 isolates in the MLST database are resistant to at least one of five antibiotics (penicillin, erythromycin, tetracycline, chloramphenicol, cefotaxime). In genetic relatedness, ST448 is most close to other five STs (1229, 1290, 3064, 3065 and 4141) that were non nonserotypeable and two STs (3278, 3283) that were serotype 6A and 7C. ST448 differs from these strains in only one of seven loci. Among three major nonserotypeable strains (STs 344,448,449) from middle ear fluid, ST 448 does not cluster with STs 344 and 449 (Hanage *et al.*, 2006), while ST 344 and ST449 differ only in one of seven loci (*ddl*). ST448 has only one same locus as STs 499 and 344.

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Figure 1.

Only *Spn* but *Hflu*, *Mcat* and *Spyo* was detected in MEF by multiplex PCR. DNA was extracted from MEF and nasal wash samples, NT-*Spn* isolates and positive control strain to amplify *Spn* (panel A), *Hflu* (panel B), *C Mcat* (panel C) and *Spyo* (panel D). PCR products size was evaluated on 2% agarose gel. Panel A, PCR for *Spn*, lane 1, DNA ladder; Lane 2, positive control; Lane 3, NT-*Spn* isolate from NP (0702064AOM1NP); Lane 4, NT-*Spn* isolate from MEF (0702064AOM1MEF); lane 5, MEF samples; lane 6, negative control. Panel B-D, PCR for *Hflu*, *Mcat* and *Spyo*, lane 1, DNA ladder; Lane 2, positive control; Lane 3, DNA from MEF; lane 4, negative control.

Figure 2. Genetic relatedness (neighbor-joining tree) of the non-encapsulated strain of *Spn* described here with other isolates. Scale bar indicates number of nucleotide substitutions per site.

Table 1

Nontypeable *S. pneumoniae* strain ST448

Strain	MLST Type	Sera type	Optochin (mm)	Bile test	CpsA PCR	Ply PCR	Allele number of house keep genes					
							<i>aroE</i>	<i>gdh</i>	<i>gki</i>	<i>recP</i>	<i>spi</i>	<i>ddl</i>
0702064AOM1MEF	ST448	NT	+(22)	+	-	+	8	5	2	27	2	11
0702064AOM1NP	ST448	NT	+(24)	+	-	+	8	5	2	27	2	11