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CAPSULE AND FIMBRIAE EXPRESSED BY HAEMOPHILUS INFLUENZAE ISOLATES FROM NASOPHARYNGEAL CARRIAGE IN BULGARIAN CHILDREN

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Abstract: *H. influenzae* possesses an array of important virulence factors, which are found in certain strains and give them a selective advantage. We aimed to investigate the presence of fimbriae and encapsulated and non-capsulated variants from nasopharyngeal carriage in children at the onset of upper respiratory tract infections.

Methods. The capsule detection was performed by PCR for the *bexA* gene. All encapsulated *H.influenzae* were categorized into serotypes "a" to "f" by PCR-serotyping. The presence of haemagglutinating fimbriae was made by PCR for the hifA gene.

Results. A total of 163 nasopharyngeal *H.influenzae* isolates were studied. Most of them (96.3%) were non-capsulated variants, also known as non-typeable strains (NTHi). The presence of a capsule was detected in six strains. The PCR-serotyping disclosed three serotype "a" isolates, two isolates from serotype "f" and one from serotype "c". The PCR for *hifA* gene demonstrated 16.6% fimbriated isolates.

Conclusion. NTHi is predominant in nasopharyngeal carriage among children. The fimbriae are involved in the adherence to human epithelial cells, but there is no categorical evidence that the deficiency of fimbriae may influence significantly the pathogenesis of a disease. A better molecular understanding is essential to clarify the pathogenesis of the NTHi and non-fimbriated *H.influenzae* isolates.

Keywords: *H.influenzae*, fimbriae, capsule, PCR-serotyping.

INTRODUCTION

Haemophilus influenzae is a commensal pathogen and a part of the respiratory tract microbiota. The general habitat of *H. influenzae* is the mucous membranes of the respiratory tract in humans. The transmission from close contact with an infected individual with droplets from a sneeze and cough can be inhaled and may cause infection. *H. influenzae* may lead to otitis media, bronchitis, and even life-threatening diseases including epiglottitis, sepsis, and meningitis [1].

H. influenzae possesses an array of important virulence factors, including the capsule and fimbriae, which are found in certain strains and give them a selective advantage.

H. influenzae consists of various types based on the presence or absence of a capsule. The encapsulated or typable strains are six types (a through f) based on the antigenic structure of the capsular polysaccharide. The capsule protects the bacteria against phagocytosis and opsonization. Type b (Hib) is shown to be responsible for approximately 95% of systemic infections. The main clinical manifestations of invasive Hib strains are meningitis, pneumonia, epiglottitis, septicemia, cellulitis, and osteoarticular infections [1,2]. Capsule serotyping of *H. influenzae* is traditionally determined by serological methods—capsule swelling reaction and slide agglutination with specific antisera. Nowadays, PCR-serotyping has also been performed [3,4]. The *bex* locus in the encapsulated *Haemophilus influenzae* is a chromosomal region involved in the capsular polysaccharide export. The *bexA* gene is a common target in the PCR identification of encapsulated variants.

The strains that do not produce polysaccharide capsules are described as non-capsulated or non-typeable (NTHi) strains. NTHi can cause respiratory diseases such as otitis media, conjunctivitis, sinusitis, pneumonia, persistent bacterial bronchiolitis, and

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acute exacerbations in patients with chronic obstructive pulmonary disease [5-7].

Certain *H. influenzae* strains possess fimbriae structures, that assist the attachment to oropharyngeal epithelial cells [8, 9]. Four families of fimbriae have been distinguished morphologically but only LKP-type fimbriae are associated with the adherence of *H. influenzae* to human cells [8, 10]. LKP fimbriae also mediate binding to a variety of human mucosal epithelial cells during *H. influenzae* carriage and disease [8,11]. The fimbria gene cluster *hifA-hifE* is necessary for the formation of a complete fimbriae structure and fimbria-mediated adherence. The *hifA* encodes the fimbria subunit that mediates binding to a ganglioside receptor [11].

We aimed to investigate the presence of fimbriae and capsule types in nasopharyngeal specimens recovered from children at the onset of upper respiratory tract infections.

MATERIALS AND METHODS

Specimen collection

We studied 163 nasopharyngeal swabs recovered from patients with upper respiratory tract infections. All participating patients were up to 12 years of age. The specimens were collected in the Department of Medical Microbiology in Medical university of Sofia.

The nasopharyngeal swabs were cultured on Chocolate agar at 37°C in an atmosphere of 5% CO2 for 16-18h for growth. Identification of *H. influenzae* with standard diagnostic assays like oxidase test, Gram stain, satellite growth, and requirements on factors X and V was performed. Additionally, Remel Rapid NH biochemical identification tests (ThermoFisher Scientific, USA) were used for verification of the strains.

Capsule detection

The *H. influenzae* strains were differentiated into encapsulated and non-capsulated isolates by PCR based on the presence or absence of the *bexA* gene. The capsular variants "a" to "f" harbor the *bexA* gene, and it is not present in the NTHi isolates. The PCR reaction was done with oligonucleotide primers sets described by van Ketel [12] which results in an amplified product of 343 base pairs.

Table 1. Distribution of encapsulated and non-capsulated variants of fimbriated *H.influenzae* isolates recovered from nasopharyngeal carriage in children up to 12 years of age

	Strains	Clinical source	Capsular type	Gender	Age
1	15n	nasopharynx	NTHi	m	1yr
2	12t	throat	NTHi	m	3yr,4mo
3	32n	nasopharynx	NTHi	m	2yr,7mo
4	33n	nasopharynx	NTHi	m	10yr
5	45n	nasopharynx	Serotype "a"	m	8yr
6	48n	nasopharynx	NTHi	m	6yr
7	50n	nasopharynx	NTHi	f	11yr
8	51n	nasopharynx	Serotype "a"	m	4yr,2mo
9	54n	nasopharynx	NTHi	m	4yr,6mo
10	58n	nasopharynx	NTHi	f	7yr
11	62n	nasopharynx	NTHi	m	5yr
12	68n	nasopharynx	NTHi	f	2yr
13	28t	throat	NTHi	f	1yr
14	36t	throat	NTHi	f	1yr,7mo
15	71n	nasopharynx	NTHi	m	2yr
16	75t	throat	NTHi	m	3yr
17	48t	throat	NTHi	f	12yr
18	80n	nasopharynx	NTHi	m	9yr
19	83n	nasopharynx	NTHi	f	12yr
20	52t	throat	NTHi	f	4yr
21	66t	throat	NTHi	m	8yr
22	85n	nasopharynx	NTHi	m	2yr
23	90n	nasopharynx	Serotype "f"	m	2yr
24	101n	nasopharynx	NTHi	f	3yr,5mo
25	112n	nasopharynx	NTHi	m	6yr
26	120n	nasopharynx	NTHi	f	11yr
27	131n	nasopharynx	NTHi	f	5yr

Notes: NTHi- non-typeable *H.influenzae*; m-male, f-female.

PCR-Serotyping

All encapsulated strains were subjected to a PCR-serotyping with six primer pairs in separate PCR reactions to prove the specific capsule types "a" to "f". The primers were designed by Falla *et al.* [4].

PCR detection of fimbriated H. influenzae isolates

A PCR method was performed for the detection of the presence of haemagglutinating fimbriae. The target gene was the hifA gene that encodes for the major subunit of haemagglutinating fimbriae of *H. influenzae*. The PCR was carried out with an oligonucleotide primer set: TGCTGTTTATTAAGGCTTTAG and TTGTAGGGTGGGCGTAAGCC, described previously in the report of Geluk *et al.* [8].

RESULTS

We studied 163 nasopharyngeal *H. influenzae* specimens recovered from children aged 6 months to12 years. Most of the colonized children were up to 6 years of age (52.2%).

The PCR results for the *bexA* gene revealed 96.3% non-capsulated variants, also known as non-typeable strains (NTHi). The presence of a capsule was detected in six strains (3.7%). PCR serotyping was conducted for the encapsulated isolates.

PCR-serotyping

PCR-serotyping of the encapsulated variants disclosed capsular serotype "a" in three isolates, two isolates were from serotype "f" and one from serotype "c".

PCR detection of fimbriated H. influenzae isolates

The fimbrial typing was carried out for all encapsulated and non-capsulated variants. The majority of the nasopharyngeal *H. influenzae* isolates did not show a presence of haemagglutinating fimbriae. Twenty-seven isolates (16.6%) contain the fimbrial gene *hifA*. Among the fimbriated *H. influenzae* isolates, two were from type "a", one belong to serotype "f" and 24 were non-typeable *H. influenzae*. Most of the fimbriated isolates were recovered from males up to 5 years of age (59.3%).

DISCUSSION

Our study investigated the distribution of encapsulated and non-capsulated variants, and the presence of fimbriae among *H. influenzae* isolates from nasopharyngeal carriage in children. Following the implementation of Hib in the routine immunization programs in many countries, including Bulgaria, the most virulent capsule type b of *H. influenzae* is decreased significantly. Hib is a part of the hexavalent vaccine (DTPa-HBV-IPV/Hib) that covers six main diseases: diphtheria, tetanus, pertussis, hepatitis B, polio and *Haemophilus influenzae* type b. Protein D, a highly conserved surface lipoprotein found in all *Haemophilus influenzae*, including NTHi was used as a conjugate in the pneumococcal vaccine PHiD-CV [13]. Reports discussed that the postvaccine effect may lead to a replacement of type b with a novel virulent type [14, 15].

We detected three capsular types: "a", "c", and "f" in a small number of isolates in our study. All others were non-typeable strains. NTHi is considered a common respiratory tract pathogen in many investigations [6, 7, 16, 17]. NTHi is involved in the pathogenesis mainly of local respiratory tract infections, but also in invasive infections in the last years [17,18].

The presence of fimbriae was not significant in our study (~17%). We do not observe a correlation between the distribution of fimbriae and the gender and age of the patients, or the capsulated or encapsulated variants of *H. influenzae*. It has been demonstrated previously that fimbriae of Hib are important for the adherence to respiratory epithelial cells and can modulate the invasion of *H. influenzae*, but non-fimbriated strains were shown to attach to mucosa cells as well [19]. *In vitro* study on mice revealed that the fimbriated Hib correlated with lower mortality and higher susceptibility to complement-mediated bacteriolysis compared to non-fimbriated variants, although fimbriae accelerated the adherence to the mucosal surface [20]. The *in vitro* resistance of non-fimbriate bacteria to the bactericidal effects of normal human serum was reported at least 400 times greater than that of fimbriate bacteria [20].

There are known more than 14 LKP fimbriae for hemagglutination and adherence to human epithelial cells [11] and this variety may contribute to the pathogenesis of the infections. The expression of LKP fimbriae is subject to phase variation, and non-fimbriated and fimbriated organisms can occur in a given population at any time.

In conclusion, continuous surveillance is essential to clarify the mechanisms for adherence and invasion in non-fimbriated and non-capsulated variants of *H. influenzae*.

Conflict of interest

The authors declare that they have no conflict of interest.

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