VIRULENCE FACTORS AND MECHANISMS OF ANTIBIOTIC RESISTANCE OF
HAEMOPHILUS INFLUENZAE

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ABSTRACT
Haemophilus influenzae is a small gram-negative coccobacillus known as one of the major causes of meningitis, otitis media, sinusitis and epiglottitis, especially in childhood, as well as infections of the lower respiratory tract, eye infections and bacteremia. It has several virulence factors that play a crucial role in patient inflammatory response. Its capsule, the adhesion proteins, pili, the outer membrane proteins, the IgA protease and, last but not least, the lipooligosaccharide, increase the virulence of H. influenzae by participating actively in the host invasion the host by the microorganism. Some of these factors are used in vaccine preparations. In the post-vaccine era, an increase has been noticed in many European countries of invasive infections caused by non-encapsulated strains of H. influenzae which have a number of virulence factors, some of which are subject of serious research aiming at creating new vaccines. Numerous mechanisms of antibiotic resistance in H. influenzae are known which can compromise the empirical treatment of infections caused by this microorganism. The increasing incidence of resistance to aminopenicillins, induced not only by enzyme mechanisms but also by a change of their target is turning into a significant problem. Resistance to other antibiotics such as macrolides, tetracyclines, chloramphenicol, trimethoprim/sulfamethoxazole, and fluoroquinolones, commonly used to treat Haemophilus infections has also been described.

Key words: Haemophilus influenzae, virulence, antibiotic resistance

INTRODUCTION
Haemophilus influenzae is one of the major causes of meningitis, otitis media, sinusitis and epiglottitis, especially in childhood, as well as infections of the lower respiratory tract, eye infections and bacteremia. Before vaccines were introduced this microorganism was the most common causative agent of meningitis in children under 2 years of age with H. influenzae serotype b as the principal etiologic agent. The etiological profile of infections the microorganism causes has been modified after the conjugate vaccine containing parts of the capsule of this serotype was introduced; the other five serotypes of these bacteria along with their nontypable (non encapsulated) strains have been noticed to increase their incidence.

Another major H. influenzae-related problem is its sensitivity decline to some antibiotics, particularly to ampicillin and other beta-lactams, to tetracycline and to trimethoprim/sulfamethoxazole.

VIRULENCE FACTORS
The capsular polysaccharide of H. influenzae serotype b is a major virulence factor. It renders H. influenzae resistant to phagocytosis and helps the evadion of the intracellular killing in neutrophils. Six capsule serotypes have been described (a to f) of which type b is the most virulent. The native type b capsule is composed of linear teichoic acid containing ribose, ribitol, and phosphate, and called polyribosyl-ribitol-phosphate. The capsule synthesis is encoded by capB genes in two identical copies (17-18 kb) in the chromosome and are bound by a short region (1-1.3 kb). This region contains a gene called bexA which encodes a protein required for the export of the capsular material to the cell surface. The remaining 5 serotypes have the cap gene only in one copy. About 2% of H. influenzae type b also have only one copy of the gene. The loss of the other copy leads to lack of capsule synthesis and these strains are called capsule-deficient mutants.1,2
Unlike the encapsulated type b microorganisms that are associated with reduced adhesion and invasion of host cells, mutant strains have a significantly greater (50 times) ability to adhere and invade the macroorganism.

Nontypable invasive strains causing otitis media, sinusitis, bronchitis and pneumonia have also many adhesins which play a major role in causing infection.\(^3\),\(^4\)

The attachment of bacteria to the host epithelial cells is an important stage in the infection development and is a process that is mediated by bacterial adhesins and by specific receptors on the surface of cells of the macroorganism. In nontypable \textit{H. influenzae} adhesion is achieved by two main groups of adhesion proteins - high molecular weight proteinsHMW1/HMW2 and Hia adhesins. High molecular weight proteins are found in 75-80\% of all nontypable strains. The genes encoding them are arranged in clusters in the chromosome with the genes responsible for the expression of outer membrane proteins (OMP).\(^5\) HMW1 and HMW2 are encoded by two separate loci designated as \textit{hmw1} and \textit{hmw2}. Each locus consists of three genes encoding the adhesin (\textit{hmwA}), an integral outer membrane protein needed for the transport of HMW1 and HMW2 through the outer membrane (\textit{hmwB}) and cytoplasmic protein that stabilizes the adhesin prior to its export from the cytoplasm (\textit{hmwC}).

The level of expression of high molecular weight proteins is associated with the development of otitis media in children and bronchitis in patients with chronic obstructive pulmonary disease (COPD).

The so-called Hia adhesin is present in about 20\% of the non-encapsulated strains. Its analogue in encapsulated \textit{H. influenzae} serotypes is Hsf adhesin.

The pili are another virulence factor of \textit{H. influenzae}. They are found in the encapsulated strains of serotype b and in almost half of the non-encapsulated strains. They consist of one large protein (HifA) and two small ones (HifD and HifE). Pili mediate the attachment of the bacterial cell to the eukaryotic cell by binding to glycoproteins and glycolipids on its surface. \textit{H. influenzae} has one copy of each of the genes encoding fimbrial proteins (hifA, hifD and hifE). In the cluster containing these genes, there are two more genes (\textit{hifV} and \textit{hifS}) that carry information about the synthesis of proteins involved in the assembling of fimbrial proteins and preventing them from destruction in cell export. Nontypable \textit{H. influenzae} has another fimbrial protein called P5-fimbria, similar to one of the outer membrane proteins (P5).\(^6\)

Outer membrane \textit{H. influenzae} proteins are six to eight in number. Some of them such as P2 and P6 are being extensively studied as antigens that may be included in vaccine preparations against nontypable strains. Antibodies against P2 have bactericidal and protective effect. P2 are the most common outer membrane proteins. They are porins whose outer part is highly variable and differs between strains. Their inner part, located in the outer membrane, has conservative amino acid sequence. The outer part of these proteins may vary with time as a result of single changes in structural genes for P2. This leads to a chronic form of some \textit{H. influenzae} caused infections.

P6 is an outer membrane protein expressed on the surface of the encapsulated and nontypable strains. The gene that encodes it is highly preserved, which determines the high degree of similarity in terms of its nucleotide sequence in different strains.\(^7\)

An important factor of \textit{H. influenzae} virulence is the IgA1 protease that inactivates human immunoglobulin A1 and facilitates the colonization of mucosae. About 95\% of nontypable strains possess the gene (\textit{iga}) for this enzyme. Protease activity is preserved in a greater percentage in invasive isolates (blood and cerebrospinal fluid) and isolates from sputum. This virulence factor occurs more frequently in strains from the upper respiratory tract (primarily nontypable strains). The presence of a second IgA protease has been detected, more common in isolates from patients with COPD.\(^8\)

Like all Gram-negative microorganisms, \textit{H. influenzae} has a lipopolysaccharide, but it is with a shorter polysaccharide chain and is termed lipooligosaccharide. Except possessing the properties inherent to endotoxins of Gram-negative bacteria, the lipooligosaccharide helps to evade opsonisation and phagocytosis by imitating molecular structures present in the macroorganism. This is due to the sialated ends of the lipooligosaccharide which are structurally and antigenically similar to the sialated oligosaccharides in the sphingolipids of the human body.

\textbf{MECHANISMS OF ANTIBIOTIC RESISTANCE}

Some of the main mechanisms of resistance of \textit{H. influenzae} are those to \textit{β}-lactam antibiotics. More frequent (in about 30\%) is the plasmid-mediated production of \textit{β}-lactamase of types TEM-1 and ROB-1, called \textit{β}-lactamase positive ampicillin-resistant strains (BLPAR). The TEM-1 type \textit{β}-lactamase, which is more common, is associated with different (at least 5) promoter regions: P3, partial
overlapping of Pa/Pb; Pdel; Prpt and duplicate repeatability of Prpt.\textsuperscript{9} Promoters have different affinity to RNA polymerase, altering the expression of the β-lactamase, thus leading to different levels of resistance to β-lactams.\textsuperscript{10} In a small percentage of the ampicillin-non-susceptible strains a change in penicillin-binding protein (PBP3) is detected, which reduces its affinity to penicillins and cephalosporins. PBP3 is a transpeptidase, responsible for the synthesis of peptidoglycan in the septum region of dividing bacteria, which is encoded by the ftsI gene.\textsuperscript{11} Mutations in this gene lead to amino acid substitution in the transpeptidase domain of PBP3. These strains are called β-lactamase negative ampicillin resistant (BLNAR). Ubukata et al. distinguish three groups of isolates depending on the amino acid substitutions: Arg-517 → His or Asn-526 → Lys, close to Lys-Thr-Gly (KTG) motive are groups I and II, and substitutions Met-377 → Ile, Ser-385 → Thr and Leu-389 → Phe, close to Ser-Ser-Asn (SSN) motive, as well as Asn-526 → Lys are group III.\textsuperscript{12} A total of 24 different amino acid changes in the transpeptidase domain of PBP3 have been described, which is probably a result of the selective pressures of different β-lactams. Strains with mutations in the ftsI gene showed values of minimal inhibitory concentrations (MIC) of ampicillin from 0.5 to 16 μg/ml under which they are divided into low-level BLNAR (0.5-4 μg / ml) and high-level BLNAR (8-16 μg / ml).\textsuperscript{13,14} BLNAR have reduced susceptibility except to aminopenicillins and to their β-lactamase inhibitors, and also to second generation cephalosporins. Resistance to Cefaclor is an indicator for the presence of this mechanism.

Some strains of \textit{H. influenzae} can produce β-lactamase, while showing resistance to amoxicillin/clavulanic acid, and are called β-lactamase positive amoxicillin/clavulanic acid-resistant (BLPACR).\textsuperscript{15,16} These isolates probably combine the two resistance mechanisms described above.

Resistance to tetracyclines is due to an efflux mechanism encoded by the tetB gene flanked by the conjugate transposons Tn416 and Tn916. Plasmids carrying genes for resistance to ampicillin-chloramphenicol-tetracycline-kanamycin are described in \textit{H. influenzae} type b.\textsuperscript{17}

Resistance of \textit{H. influenzae} to macrolides may be due to congenital or acquired efflux pump, ribosomal methylation and mutations in ribosomal protein and RNA.\textsuperscript{18} The presence of some of the genes \textit{erm}(A), \textit{erm}(B), \textit{erm}(C), \textit{erm} F and \textit{mef}(A) determines the resistance to macrolides.\textsuperscript{19}

Resistance to chloramphenicol is associated with the production of chloramphenicol acetyltransferase (\textit{cat}), encoded by the \textit{cat} gene. The gene is part of a conjugate plasmid. These plasmids can be integrated in the chromosome.\textsuperscript{20} The loss of an outer membrane protein of \textit{H. influenzae} can be a cause for a permeability barrier for the antibiotic. Resistance to trimethoprim/sulfamethoxazole is quite common in \textit{H. influenzae} and is due to increased production of dihydrofolate reductase with reduced affinity for trimethoprim.\textsuperscript{21}

Resistance to fluoroquinolones among isolates of \textit{H. influenzae} is rare. It occurs after changes of the region, determining the quinolone resistance of the genes encoding DNA gyrase and topoisomerase IV.\textsuperscript{22} It should be borne in mind that in the presence of quinolone \textit{in vitro} spontaneous quinolone-resistant mutants are easily selected.

**CONCLUSIONS**

As a result of the widespread use of vaccines against \textit{H. influenzae} type b, a shift of the etiological spectrum of Haemophilus infections is being observed. More and more often, cases of invasive infections caused by non-type b or nontypable \textit{H. influenzae} are being reported\textsuperscript{23-25}, among which a higher frequency of aminopenicillin resistance occurs due to non-enzymatic mechanisms, namely changes in the penicillin-binding proteins. Therefore, study of virulence factors and mechanisms of antibiotic resistance of \textit{H. influenzae} is essential for the introduction of more effective specific prevention and treatment of infections caused by this microorganism.

**REFERENCES**


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ФАКТОРЫ ПАТОГЕННОСТИ И МЕХАНИЗМЫ АНТИБИОТИЧЕСКОЙ РЕЗИСТЕНТНОСТИ ПРИ HAEMOPHILUS INFLUENZAE

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РЕЗЮМЕ

Haemophilus influenzae является одним из основных возбудителей менингита, синуита, эпиглотита (преимущественно в детском возрасте), а также и возбудителем инфекций нижних дыхательных путей, глазных инфекций и бактериемии. Этот возбудитель обладает рядом факторов патогенности, играющих основную роль для возникновения инфекций. Капсула, адгезивные протеины, пили, внешнемембранные протеины, IgA1 протеаза и не на последнем месте липоолигосахарид повышают вирулентность Haemophilus influenzae, участвую активно в инвазии макроорганизма. Некоторые из вышеуказанных факторов патогенности находят применение в составе вакцин. В поствакцинальной эре в ряде европейских стран все больше нарастает часть инвазивных инфекций, вызванных бес kapsулями штаммами H. influenzae, обладающими рядом факторов патогенности. Некоторые из этих штаммов изучаются активно с целью создания новых вакцин. Известно множество механизмов антибиотической резистентности при H. influenzae, которые могут компрометировать эмпирическую теорию инфекций, вызванных этим микроорганизмом. Нарастающая частота резистентности к аминопеницилинам в мировом масштабе, объясняющаяся не только энзимными механизмами, но и изменением мишени их действия, представляет все более существенную проблему. Наблюдается резистентность и к другим антибиотикам (макролиды, тетрациклины, хлорамфеникол, триметоприм/сульфаметоксазол, флуорхинолоны), часто применяемые при лечении гемофилусных инфекций.