Infection and Drug Resistance

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REVIEW

Epidemic Trends and Biofilm Formation Mechanisms of Haemophilus influenzae: Insights into Clinical Implications and Prevention Strategies

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Abstract: *Haemophilus influenzae* (*H. influenzae*) is a significant pathogen responsible for causing respiratory tract infections and invasive diseases, leading to a considerable disease burden. The Haemophilus influenzae type b (Hib) conjugate vaccine has notably decreased the incidence of severe infections caused by Hib strains, and other non-typable *H. influenzae* (NTHi) serotypes have emerged as epidemic strains worldwide. As a result, the global epidemic trends and antibiotic resistance characteristics of *H. influenzae* have been altered. Researches on the virulence factors of *H. influenzae*, particularly the mechanisms underlying biofilm formation, and the development of anti-biofilm strategies hold significant clinical value. This article provides a summary of the epidemic trends, typing methods, virulence factors, biofilm formation mechanisms, and prevention strategies of *H. influenzae*. The increasing prevalence of NTHi strains and antibiotic resistance among *H. influenzae*, especially the high β-lactamase positivity and the emergence of BLNAR strains have increased clinical difficulties. Understanding its virulence factors, especially the formation mechanism of biofilm, and formulating effective anti-biofilm strategies may help to reduce the clinical impact. Therefore, future research efforts should focus on developing new approaches to prevent and control *H. influenzae* infections.

Keywords: *Haemophilus influenzae*, epidemiology, virulence factors, biofilms, prevention strategies

Introduction

H. influenzae is an opportunistic pathogen that commonly colonizes the human nasopharynx and causes a variety of infections, such as acute sinusitis, otitis media, conjunctivitis, community-acquired pneumonia, bacterial vaginitis, and invasive diseases, including septic meningitis, septicemia, peritonitis, and arthritis, among others.[1] Invasive diseases are becoming increasingly prevalent with each passing year. These conditions are marked by high mortality rates and have the potential to cause severe sequelae, posing a significant threat to the health of children.[2] Based on the presence or absence of cell wall capsular polysaccharide components, *H. influenzae* can be classified into six encapsulated types (Hia-Hif) and non-encapsulated NTHi. Of these, Hib is the most virulent and is associated with invasive diseases. The introduction of the Hib conjugate vaccine in some countries and regions has led to a change in the epidemiological characteristics of *H. influenzae*, resulting in a significant decrease in severe infections associated with Hib.[3] The public's waning attention to *H. influenzae* has resulted in the serious underestimation of other serotypes, particularly NTHi-related infections. The escalating rate of antibiotic resistance, as well as the evolution of virulence factors, specifically the formation of biofilms, have presented significant challenges to clinical treatment. As a result, anti-biofilm strategies and preventative methods for *H. influenzae* have become prominent areas of research.

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Epidemiology

The introduction of the Hib conjugate vaccine in Asia significantly trailed its implementation in developed Western countries. This lag, particularly in developing nations, led to considerable heterogeneity in *H. influenzae* epidemiology globally. Although Japan introduced the Hib vaccine in November 2008, the vaccination rate was initially low due to the voluntary nature of the program. Notably, it was not until December 2010, when the national policy to lessen the burden of self-pay vaccination was instituted, that the rate of vaccination increased significantly to over 90.0%.[4] In China, the overall coverage of the Hib conjugate vaccine was 54.9%. The vaccine coverage was found to be lower among foreign populations as compared to local residents. Furthermore, the eastern region of China had a higher coverage as compared to the central and western regions.[5] In Nepal, *H. influenzae* had been identified as the primary etiological agent of meningitis in pediatric patients.[6] In Vietnam, before the nationwide introduction of Hib conjugate vaccine, a cross-sectional Hib carriage study among 1000 children < 5 years of age found that the carriage of *H. influenzae* was detected in 37.0% of children and the Hib carriage rate was 3.0%.[7] The infection caused by *H. influenzae* remains a pertinent public health issue, particularly in emerging economies. As a result, it is advised to incorporate the Hib vaccine into national immunization schemes to address disparities in vaccination coverage.

As the incidence of Hib infection gradually decreased, the incidence of other serotypes and NTHi strains increased in relative terms. In North America, current epidemiological data suggested that invasive Hia disease predominantly affected indigenous communities, with Sequence Type (ST) 23 being the predominant clone described in most invasive diseases and MLST sites.[8] The incidence rate of invasive Hia cases in the United States increased by 11.1% annually on average from 2008 to 2017, and the total mortality rate was 7.8% (30/386), 42.7% of Hia cases occurred in children under 5 years old.[9] In Canada, *H. influenzae* diseases were predominantly caused by NTHi (74.2%, 950/1281), far exceeding serotype Hia (8.9%, 114/1281) and Hif (10.2%, 130/1281).[10] In Portugal, 79.2% (206/260) of the invasive *H. influenzae* disease isolates were NTHi, and ST103 and ST57 were the major STs.[11] The total carrying rate of *H. influenzae* among children aged 0–6 in daycare was 84.1% (1282/1524), of which 96.7% (1240/1282) were NTHi.[12] A study in Shanghai, China found that all 51 isolates collected were NTHi, of which 5.9% (3/51) caused invasive infections, with ST103 (7.84%, 4/51) being the most common.[13] A multicenter study in Zhejiang, China, found that *H. influenzae* infections accounted for 18.5% (280/1514) and NTHi accounted for 93.6% (262/280).[14] NTHi can lead to severe invasive diseases, especially in the elderly, pregnant/postpartum women, and newborns. In the United States from 2008 to 2019, 92.0% (207/225) of newborns with invasive NTHi infections occurred within the first week of birth, and 72.4% (163/225) were born prematurely. Compared to full-term newborns, the risk of NTHi in premature infants was 23 times higher.[15] In Belgium, the carrying rate of NTHi in children with acute otitis media and invasive diseases was 98.2% (214/218) and 68.1% (64/94) respectively.[16] 95.0% (190/200) of Japanese adults with invasive *H. influenzae* diseases were NTHi strains.[17] NTHi has become a major subtype of *H. influenzae* infections worldwide and has the ability to cause invasive disease. Due to the collective global morbidity and mortality, it has been recognized by the World Health Organization as one of the 12 priority pathogens.[18] With the introduction of Hib conjugate vaccine, the epidemiology of *H. influenzae* infection has changed, and NTHi has become a major subtype of lower respiratory tract infection and invasive disease, which requires great attention and focus. It is particularly important to carry out epidemiological investigations of *H. influenzae* in large samples and multicenter, which can provide an epidemiological basis for clinical diagnosis and treatment and national vaccine strategy.

H. influenzae Typing

There are currently multiple typing methods for *H. influenzae*, each with different focuses and advantages (Table 1). Choosing appropriate, convenient, and economical typing methods can provide scientific basis for clinical research and epidemiological investigations.

Table 1 Characteristics of Different *H. influenzae* Typing Methods

Typing Methods	Main Types	Characteristics and Significance
Capsule typing		
SAST	Hia-Hif and	Traditional, simple to operate, but low accuracy.
PCR	NTHi	High accuracy and specificity, worth clinical recommendation.
WGS		High accuracy and specificity, future development trend.
MALDI-TOF MS		Simple and fast, with potential in clinical microbiology laboratory.
Biotyping	I-VIII	Related with specific diseases, but low commonality and actual value.
MLST	ST and CC	Genetic information has storability and comparability, but significant differences in different regions and high cost.

Abbreviations: SAST, Slide agglutination serotyping; WGS, Whole-Genome Sequencing; MALDI-TOF-MS, Matrix-Associated Laser Desorption Ionization-Time Of Flight Mass Spectrometry; MLST, Multilocus sequence typing; ST, sequence type; CC, clonal complexes.

Capsule Typing

Slide Agglutination Serotyping (SAST)

Traditional serologic capsule typing was performed using a standard slide agglutination method. However, this traditional method's accuracy is limited and some encapsulated strains may end up being misclassified as NTHi due to inaccuracies in performing and interpreting the slide agglutination test, or altered ability to produce capsule due to partial deletion of the capsule gene *bexA*.[19]

PCR Typing

The capsular polysaccharide gene loci has three functional regions I~III. Regions I and III are functional genes, encoded by *bexDCBA* and *hcsAB* respectively. They are highly conserved in all capsular types and are related to cell transport and post-translational processing. Region II genes encode capsular type a to f specific proteins, and there are differences in genes between different serotypes.[20] The conventional typing method relies on the *bexA* gene and is not suitable for the study of a large number of isolates, which may lead to the misclassification of *bexA* deficient mutant strains. Some researchers described a molecular strategy that targeted the *bexB* gene, which provided a simple and reliable method for differentiating strains that lack the entire capsule locus from those containing a partial or complete capsule locus.[21]

Whole-Genome Sequencing (WGS) Typing

Due to the considerable accuracy provided by WGS, public health and clinical laboratories are currently beginning to incorporate WGS technology into diagnostic and disease surveillance projects.[22] Recently, two in silico approaches using WGS, "hinflfluenzae_capsule_characterization"[23] and "hicap"[24] have provided a more robust and reliable method for *H. influenzae* capsule typing. The consistency between the two typing methods and the actual phenotype serum typing method is 99–100%, respectively,[25] which are expected to replace the laborious traditional phenotype methods in clinical settings.

Matrix-Associated Laser Desorption Ionization-Time of Flight Mass Spectrometry (MALDI-TOF MS) Typing

Several studies have found that MALDI-TOF MS may be a simple and rapid alternative method for capsule serotyping of *H. influenzae*.[26] It is also the first report that MALDI-TOF MS can distinguish between Hie and Hif. MALDI-TOF MS can also be used as a surveillance tool in vaccinated populations to monitor the ongoing effectiveness of Hib conjugate vaccines and as a preliminary screening method for invasive *H. influenzae* strains.

Biotyping

Biotypic analysis used internal medium for urease reaction and ornithine decarboxylase reaction. *H. influenzae* can be classified into eight different biological types (I–VIII), depending on the biochemical reactions to urease fermentation, indole tests, and ornithine decarboxylase fermentation. There may be some correlation between the biotype and the source of the disease and strain. Different studies have found that among all biotypes, biotype II is usually the most common.[16,25] A survey found that biotype II was the most common in all groups, followed by type III found in the acute otitis media group and type I found in the invasive disease group.[16] Type I may be related to invasive diseases.[27]

Multilocus Sequence Typing (MLST)

Pathogenic strains were identified by sequencing the internal fragments of seven housekeeping genes (*adk, atpG, frdB, fucK, mdh, pgi*, and *recA*).[28] The typing results of isolates from different laboratories are comparable and can be stored, and the allele number, sequence type and corresponding clonal complexes (CC) of the strains are available in an Internet database. Through software, phylogenetic analysis can be conducted on different isolates to evaluate their phylogenetic relationships and diversity. Numerous studies have reported significant differences in MLST types among NTHi strains from different countries and regions. NTHi has multiple MLST types, indicating phylogenetic diversity among *H. influenzae* strains.[29,30] The differences in molecular epidemiology and genetic evolution and the correlation between MLST clustering types and antibiotic resistance, are still controversial and require further research.

H. influenzae Virulence Factors

The virulence factors of *H. influenzae* include capsule, pili, adhesins, IgA proteases and so on, which mediate bacterial adhesion, colonization, invasion, and biofilm formation (Table 2), and have important clinical significance. Some virulence factors have become potential targets for new drug development and vaccine research.

Capsule

The capsule is a polymer composed of ribose and polyribosylribol phosphate (PRP), the amount of which is related to complement resistance.[31] The capsule is encoded by the capsule gene in two identical copies of the chromosome. Amplification of the capsule gene can be seen in clinical isolates, which may be a mechanism for the bacteria to escape host defense. Bacteria containing 4 copies are significantly more resistant to antibody-dependent, classical complement pathway-directed phage cleavage compared to bacteria containing 2 copies.[32] Bacterial capsules are composed of negatively charged polysaccharides that limit interactions with similarly negatively charged host cells, such as leukocyte phagocytosis.[33]

Table 2 Genes and Functions of Common Virulence Factors in *H. influenzae*

Virulence Factor	Coding Genes	Characteristic	Function and Clinical Significance
Capsule	*bexABCD/hcsAB acs/bcs/ccs/dcs /ecs/fcs*	Expressing one of the six polysaccharide capsules (Hia-Hif) or none of them (NTHi)	Anti phagocytosis, escape host immunity.
Pili	*hifABCDE*; *pilABCD*; *comABCDEF*	The first fully studied adhesion factor	Mediate adhesion, colonization, and biofilm formation. PilA antibody becomes a vaccine target.
HMW1/HMW2	*hmw1ABC; hmw2ABC*	Present in 80% of clinical NTHi isolates but the expression varies.	Mediate bacterial adhesion and colonization. Highly homologous and immunogenic, with potential for candidate vaccines.
Hia/Hsf	*hia/hsf*	Hia exists in NTHi that does not express HMW1/HMW2, and its homologue Hsf is commonly present in capsule type strains.	Hia can mediate adhesion and colonization and is potential target for vaccine development and new drugs. Hsf can increase the virulence of Hib strain.
Hap	*hap*	Monomer self-binding autotransporter homologous to IgA1 protease	Mediate bacterial adhesion and colonization, but do not contribute to biofilm formation.
Omp5	*omp5*	A major outer membrane protein with a highly variable domain	Mediate adhesion and resist complement pathways.
IgA proteases	*igaA/igaB*	All *H. influenzae* strains have *igaA*, and 40% of the strains have *igaB*.	Mediate bacterial colonization and invasion, and related to clinical infection types.
msf	*SlrVA*	Belongs to the gene subfamily containing Sel1 like repeats	Mediate phagocytosis, persistence, and transportation.

Pili

The pili is a polymeric spiral protein structure on the surface of *H. influenzae*, another important virulence factor, and the first fully studied adhesion factor. Type IV pili (T4P) plays an important role in bacterial colonization. T4P is a filamentous polymer that spreads across the surface of bacterial cells. It is crucial for bacterial adhesion and colonization in respiratory epithelial cells, twitching movements, and biofilm formation.[34] The adhesion of NTHi expressing T4P is enhanced, and its receptor is intercellular adhesion molecule 1 (ICAM1) on the surface of epithelial cells[35]. The product of *PilA* gene in the coding gene *pilABCD* and *comABCDEF* gene cluster is the main fimbrial protein subunit.[36] In vitro experiments, antibodies targeting *pilA* could prevent the formation of NTHi biofilms and damage the formed biofilms.[37] Due to the importance of T4P in the colonization and pathogenesis, and the high conservation of the *pilA* subunit in different *H. influenzae* strains, *pilA* is being clinically studied as a candidate vaccine target for the prevention of NTHi disease.[38]

Adhesins

Adhesins are a large group of proteins including HWM1/HWM2, Hia/Hsf, Hap, and outer membrane proteins. The attachment of various adhesin proteins to host tissues is a critical step in the process of bacterial infection. In 80% of NTHi strains, bacterial attachment to the nasopharynx for colonization is mediated by two major groups of adhesion proteins, HMW1/HMW2.[39] They can bind to different specific receptors in host cells and act at different stages of *H. influenzae* invasion, and their expression correlates with *H. influenzae* aggressiveness and virulence. Some researchers have identified the promoting effects of HMW1 and HMW2 adhesins on respiratory tract colonization through a monkey model, which may be related to stimulating immune responses.[40] In patients with acute otitis media, HMW1 and HMW2 were the main targets of serum antibody response after infection, with high immunogenicity.[41] The high homology, high immunogenicity, and important roles of adhesion and colonization of HMW1 and HMW2 have made these adhesins promising as vaccine candidates.[41]

In approximately 25% of NTHi strains that do not express HMW1/HMW2, the Hia protein can be found, a key factor in the initial colonization of the nasopharynx. Hia is the main non fimbriae adhesin of NTHi. Recent studies suggest that Hia expression may vary in stages to evade immune responses to NTHi, and variants with lower Hia levels may help the bacteria avoid being killed by anti-Hia antisera.[42] Hia has dual binding activity, mimicking the combined activity of HMW1 and HMW2 adhesion proteins in function. Hia plays an important role in the biofilm formation and is expected to become a potential target for NTHi vaccine development and new drugs[43].

Hsf is commonly found in capsular *H. influenzae* strains and was originally reported in Hib strains.[44] Hsf is a unique twisted hairpin shaped trimer autologous transporter that is twice the size of Hia.[45] Hib strain mainly obtains multi-functional glycoprotein vitronectin (Vn) through Hsf and inhibits the formation of membrane attack complex (MAC) to protect bacteria from serum-mediated killing. The fine-tuned protein-protein interaction between Hsf and Vn can help inhibiting MAC formation and lung epithelial cell invasion, which may contribute to increasing the toxicity of Hib.[46]

Hap is a monomeric self-binding autotransporter homologous to serine-type IgA1 protease that promotes interaction with respiratory epithelial cells.[47] Hap is a widely expressed adhesion protein that binds to basement membrane components such as fibronectin, laminin and type IV collagen.[48] The C-terminal adhesion region and N-terminal serine protease structure region of Hap can promote adhesion and regulate Hap self-protein hydrolysis, respectively.[49]

The P5 protein, encoded by the *ompP5* gene, is a major outer membrane protein and is predicted to have a highly variable domain.[50] P5 can mediate the binding of bacteria to respiratory epithelial cells and mucin, leading to increased complement resistance.[49] P5 is a necessary condition for NTHi to resist classical and alternative complement pathways.[51] C4b binding protein (C4BP) plays an important role in complement escape of NTHi, and the P5 protein of NTHi is a new ligand of C4BP. The expression of P5 is positively correlated with the binding of C4BP.[52] However, the P5 protein does not seem to contribute to biofilm production.[49]

IgA Proteases

IgA proteases contribute to host mucosal colonization and tissue invasion and can cleave the heavy chain of the hinge region of human IgA1, disrupting IgA protection of the mucosal barrier. The *H. influenzae* genome has two alleles (*igaA*

and *igaB*) encoding IgA protease, with all strains having *igaA* and 40% of strains having *igaB*.[53] The expression of IgA protease varies among different NTHi strains. A study evaluated the relationship between IgA protease genotypes and clinical infection types and found that *igaB* was prevalent in 46% of respiratory tract infections, while it was relatively rare in invasive infections.[54] The activity level of IgA1 protease isolated from symptomatic NTHi infected individuals was significantly higher than that of asymptomatic individuals, which determined that IgA1 protease was a virulence factor.[55] Some researchers have discovered the first small molecule, non-peptide IgA1 protease inhibitor, which may be a potential treatment for resistant *H. influenzae* strains.[56]

Macrophage Survival Factor

Macrophage survival factor (msf) is a new virulence factor for phagocytosis, persistence, and trafficking to non-mucosal sites.[57] Statistical genetic analyses found a significant association between possession of the SlrVA subfamily (that is msf) and the disease isolates. Msf-containing isolates were significantly higher in disease isolates than in carrier isolates, and they may play a role in disease chronicity as well as in invasive disease by providing an adaptive advantage in biofilms and increasing the survival of macrophages.

H. influenzae Biofilm

Biofilm is a microbial community with a certain spatial configuration that is attached to the surface of an object and consists of coated bacteria, lipopolysaccharide, matrix proteins, nucleic acids, and other components. Its formation is influenced by the factors of bacterial pathogens themselves, such as pili, liposaccharides, proteins, nucleic acids, quorum sensing system (QS) and two-component signaling system.[58,59] Biofilms protect bacteria from environmental, host, and chemical stressors, and can enhance bacterial virulence and resistance. Biofilm formation is associated with recurrent respiratory diseases and asymptomatic colonization.[60] More than 80% of human infectious diseases may be mediated by biofilms.[61] *H. influenzae* can form biofilms in vivo and in vitro. A variety of clinical diseases and *H. influenzae* biofilms are closely related, such as chronic rhinosinusitis, secretory otitis media, adenoid hypertrophy, chronic obstructive pulmonary disease, cystic pulmonary fibrosis, and prolonged bacterial bronchitis. The in-depth study of *H. influenzae* biofilm can provide new treatment ideas for recurrent, chronic, and refractory infections.

Biofilm Classification

The formation of *H. influenzae* biofilms can be evaluated by modified microtitration plate method stained with crystal violet.[62] However, there is currently no recognized classification standard for determining the ability of biofilm formation. Referring to some relevant literatures,[63,64] classification is based on the calculated cut-off optical density (OD_c). OD_c is defined as the average optical density value of the blank control group plus three times the standard deviation. $OD \leq OD_c$ indicates no biofilm formation, $OD_c < OD \leq 4OD_c$ indicates weak biofilm formation, and $OD > 4OD_c$ indicates strong biofilm formation.

Structure and Components

The process of biofilm formation can be divided into five stages: initial adhesion, proliferation, maturation, depolymerization and recolonization.[65] In the adhesion stage, *H. influenzae* is irreversibly attached to respiratory epithelial cells through pili and adhesins, including binding ICAM-1[35] receptor and carcinoembryonic antigen-related cell adhesion molecules (CEACAMs).[66] During the proliferation and maturation stages, bacteria can produce extracellular polymeric substances (EPS) and it mediates signal transmission, metabolic changes, gene expression changes, nutrition and oxygen gradient changes through the QS system, and finally forms a mature biofilm with mushroom like or tower like structure.[67] When the internal environment of mature biofilm reaches saturation, bacteria can be depolymerized to become planktonic again and colonize other positions.

Biofilm formation is influenced by numerous autochthonous factors, such as T4P and adhesin proteins, as previously described. During NTHi biofilm formation in vitro, the relatively low average temperature of the human nasopharynx (34°C) significantly enhances T4P expression and motility compared to in vivo temperature (37°C).[68] NTHI biofilms contain variants expressing lipooligosaccharide (LOS) that contain phosphorylcholine (PCho) and sialic acid. The

expression of PCho is significantly correlated with an increase in biofilm thickness, surface coverage, total biomass, and a decrease in biofilm roughness.[69] LOS containing sialic acid can promote NTHi to form biofilm in vitro and promote the persistence of bacteria in the middle ear or lung in vivo.[70] However, some studies have found incorporation of phosphorylcholine into the LOS of NTHi does not correlate with the level of biofilm formation in vitro,[71] so further in-depth research is needed.

QS is a density-dependent coordination system of gene expression among bacteria in a community, which is essential for the formation, development, and diffusion of biofilms. Most biofilms achieve QS through the Auto-Inducer 2 (AI-2) pathway controlled by the luxS manipulator.[72] These signals are detected by the ABC translocator protein RbsB, leading to a downstream transcriptional cascade response that drives biofilm formation.[73] The expression of AI-2 can promote the formation of NTHi biofilm and the persistence of bacteria in vivo.[74,75] A study used NTHi mutant experiments to confirm that activation and interruption of the luxS operon in the AI-2 pathway can affect the maturation and diffusion of biofilms, respectively.[76] Induction of luxS synthesis can lead to increased transcription of glycosyltransferase. Glycosyltransferase is a key enzyme involved in the production of extracellular matrix in bacterial biofilms formed by streptococci found in the lungs of COPD patients.[77] NTHi also expresses a QSeB/C two-component secondary system independent of the AI-2 system, which may support the coordination of biofilm gene expression when the luxS-RbsB system is damaged.[58]

Extracellular DNA (eDNA) and DNABII proteins are important components of NTHi biofilm EPS. Immunofluorescence techniques have shown that eDNA provides structural stability, maintenance, and extension of the biofilm to secondary sites.[78] In the chinchilla otitis media model, eDNA can reduce the biological activity of an important effector of innate immunity, namely Human β-Defensin-3 (hBD-3).[79] The degradation of eDNA can destabilize the biofilm of NTHi and also reduce surface adhesion and biofilm formation of bacteria in the planktonic state.[80] The integration host factor (IHF) and histone-like protein HU in the DNABII protein family, located in different regions of the extracellular matrix of biofilm, have independent functions and play a central role in the structural integrity of NTHi biofilms.[81] In the chinchilla model, targeted immunization with these proteins can lead to rapid removal of biofilms.[81] NTHi T4P can transport eDNA and DNABII from bacterial cytoplasm to periplasm through the expression of ComE pore, which suggests a novel form of DNA release from viable NTHi.[82] Monoclonal antibodies targeting DNABII protein can significantly damage NTHi biofilms.[83]

Biofilms and Antibiotic Resistance

NTHi biofilms exhibit a complex multidrug resistance strategy to a variety of widely used antibiotics. Studies have shown that ciprofloxacin, azithromycin and amoxicillin are 100% effective against NTHi planktonic strains, while killing only 68%, 57% and 4% of NTHi strains in biofilms.[84] Sub-inhibitory concentrations of β-lactam antibiotic stimulated the biofilm-forming ability of NTHi strains, genes involved in glycogen production and transporter function were up-regulated and down-regulated genes were linked to multiple metabolic processes but not those involved in stress response.[85] The present proteomic study indicates that the NTHi biofilm exists in a semi-dormant state with decreased energy metabolism and protein synthesis yet is still capable of managing oxidative stress.[86] The eDNA-rich extracellular matrix can impede the penetration of a range of antimicrobials, including ampicillin and ciprofloxacin.[80] Fluctuating gene expression and cellular metabolism within biofilms result in a drug-resistant bacterial phenotype. Planktonic *H. influenzae* has been shown to exhibit a heterogeneous drug-resistant phenotype to imipenem,[87] and this resistance can be enhanced in biofilm communities.[88] Differences in biofilm formation depend on the type of diseases, and there is a high degree of heterogeneity among biofilms produced by *H. influenzae* clinical isolates,[89] but there does not appear to be a significant correlation between biofilm thickness and resistance to antibiotics.[90]

Biofilms and the Immune System

Biofilms have important host components, including neutrophil extracellular traps (NETs). However, the formation of NETs is not an important determinant of NTHi clearance, but NTHi communities with biofilm phenotypes can survive in NETs.[91] The study found that peroxidase and peroxiredoxin-glutaredoxin (pdgx) are the decisive factors to promote the

survival and persistence of bacteria in NETs.[92] Biofilms also induce metabolically inactive persistent cells and provide physical and chemical barriers to immune system effectors and antimicrobials. Biofilms can produce IgA proteases that integrate into the extracellular matrix and block the host immune response by cleaving human IgA. IgA proteases are differentially expressed in vivo but play an important role for bacterial survival in respiratory epithelial cells.[93] The eDNA of *H. influenzae* biofilms can also bind to hBD-3, reducing its antimicrobial properties.[79] HBD3 is a member of the innate immune system and is essential in the protection of respiratory epithelial cells from bacterial invasion.[94]

Polymicrobial Biofilms

Biofilms are rarely formed by a single species, and the clinical significance of polymicrobial biofilms has been increasingly recognized. In polymicrobial biofilms, pathogens continuously communicate with each other through direct contact or the release and uptake of QS molecules.[95] NTHi and Streptococcus pneumoniae often colonize the respiratory tract of COPD patients together and interact synergistically to promote initial attachment, biofilm formation and survival.[96] The mechanism of interaction between these two bacteria has been previously studied. NTHi can provide passive protection for Streptococcus pneumoniae in vivo through two distinct mechanisms: production of β-lactamase and formation of biofilm communities.[97] NTHi and Streptococcus pneumoniae can mutually regulate the expression of virulence genes. The *lytA* and *cbpD* gene expression, which regulate autolysis and self-cleavage in Streptococcus pneumoniae, were significantly downregulated[98] and the expression of T4P structural protein in NTHi was significantly upregulated.[99] However, another study claimed that NTHi could coexist with Streptococcus pneumoniae during the first 24 hours, but after 48 hours, the viability of NTHi rapidly decreased to undetectable levels, when moraxella catarrhalis was present in the polymicrobial biofilm NTHi could survive for 48 hours.[100]

Biofilms and Clinical Correlation

The study on the correlation between biofilm formation ability and clinical characteristics has clinical value. A study of NTHi of lower respiratory tract origin found biofilm formation in 10.2% (10/98) of isolates, with no significant differences in antimicrobial susceptibility patterns and severity of clinical symptoms between the biofilm-forming and non-forming groups, but correlated with ICU length of stay, demonstrating for the first time the clinical impact of NTHi biofilm production.[101] Another study found that the biofilm index was higher in the invasive disease group compared to the non-invasive group, but the difference was not statistically significant.[102] NTHi isolated from children with fever or fever with earache could produce biofilms more frequently than children without these symptoms.[103] The production of biofilms was not significantly associated with treatment failure or recurrence of acute otitis media, but the production of biofilms was significantly lower in conjunctivitis-otitis media syndrome and *H. influenzae* strains modified with penicillin-binding proteins.[62]

Anti-Biofilm Strategy

Biofilms have strong drug resistance and immune defense effects and are closely related to clinical severe and chronic infections. The urgent need to develop effective anti-biological strategies will be an important direction for future research. Physical clearance methods such as surgical debridement, ultrasound, and electrical current clearance are not suitable for respiratory mucosa. Biological clearance methods such as bacteriophages have demonstrated anti biofilm activity against various bacteria such as Pseudomonas aeruginosa and Staphylococcus aureus in vitro and in vivo experiments.[104,105] However, there are few studies on *H. influenzae*. Bacteriophages are highly heterogeneous and have potential biosafety risks, so strict in vivo research is still needed. Chemical clearance methods such as antibacterial drugs, antibodies, enzymes, surfactants, and some QS inhibitors are currently the mainstream direction of applied research. In vitro models and animal experiments, relevant research on antibodies has achieved phased results, which will be described in detail in the subsequent chapter on vaccine prevention. Some natural compounds, such as plant essential oils, Chinese herbal extracts, and synthetic chemicals can inhibit and destroy biofilms by inhibiting the QS system, like Oldenlandia diffusa extract,[106] plant essential oils,[107–109] 3-hydroxychalcone (chalcone 8)[110] and so on. However, due to the possible cytotoxicity and complex synthesis and extraction processes, in vivo research and even clinical trials still require further in-depth research. In addition, serum proteases have proteolytic activity against bacterial

adhesions involved in biofilm formation.[111] Antibacterial photodynamic therapy (aPDT) of photosensitizer Chlorin e6 (Ce6) has significant bactericidal activity against NTHi in planktonic and biofilm states, and is an effective and promising adjuvant treatment for acute and recurrent otitis media.[112,113]

In clinical practice, rational selection of antibiotics or combined use of certain drugs is more clinically valuable. Studies have found that high concentrations of amoxicillin cannot remove NTHi from biofilms, while respiratory quinolones can inhibit biofilm formation and eradicate mature biofilms and can kill NTHi in biofilms even under sub-MIC conditions.[114] Simultaneous administration of carbapenem, neoquinolone and macrolide has the effect of anti NTHi mature biofilm.[115] The combination of metal chelating agent EDTA and antibiotics can inhibit biofilm formation and damage mature biofilms, and increase the sensitivity of NTHi biofilms to ampicillin and ciprofloxacin.[80]

Antibiotic Resistance

The resistance rate of *H. influenzae* to common antibiotics, particularly β-lactam antibiotics, has been continuously increasing. The resistance mechanism of *H. influenzae* includes the production of β lactamase (encoded by *TEM−1* and *ROB−1* resistance genes)[116] and the decrease of PBP3 affinity caused by mutations in *ftsI* genes (encoded by *PBP3S* and *PBP3BLN* resistance genes).[117] The mechanism of the β-lactamase-negative ampicillin resistant (BLNAR) strain is the alteration of the PBP3 protein encoded by the *ftsI* gene. In addition, strains with both mechanisms are referred to as β-lactamase-positive amoxicillin-clavulanate resistant (BLPACR).[118] The β-lactamase positivity and BLNAR strains varied in different countries and regions. In Shanghai, China, 33.3% (17/51) of the strains produced TEM-1-type β-lactamase, and BLNAR strains accounted for 11.8% (6/51),[119] while the proportion of BLNAR strains in Guangzhou, China in 2016–2017 was 22.1% (89/402).[120] In Portugal, the β-lactamase positivity rate was 7.5% (96/1282).[12] In contrast, in France, the rate of β-lactamase positivity was as high as 27.1% (157/580) and 35.0% (208/595) for the BLNAR strains.[121] In Belgium, β-lactamase positivity was 23.8% (132/554) and 16.0% (15/94) in the carrier and invasive groups, respectively.[16]

In addition to β-lactam antibiotics, fluoroquinolones are also commonly used for respiratory infections. Fluoroquinolone-resistant *H. influenzae* was first reported in 1993, and child fluoroquinolone-resistant *H. influenzae* was first reported in Hong Kong in 2004.[122] Subsequently, fluoroquinolone-resistant *H. influenzae* increased gradually, with different prevalence trends worldwide. No fluoroquinolone-resistant strains were found in invasive *H. influenzae* isolates collected in Canada between 2007 and 2014.[123] In Spain, 0.4% (28/7267) strains were resistant to fluoroquinolone.[124] 2.7% (11/402) ciprofloxacin-resistant *H. influenzae* were detected in Guangzhou, China.[120] A study in Taiwan found that 41.7% (20/48) of *H. influenzae* isolated from nursing home residents were not sensitive to fluoroquinolones, and the reduced sensitivity to fluoroquinolones was associated with a *gyrA* gene mutation at site 84.[125] The drug resistance situation of *H. influenzae* is very serious. The in-depth investigation of large samples and multi-centers to grasp the mechanism of drug resistance and the changing situation is of great significance for the scientific and rational selection of antibiotics in the clinic.

H. influenzae Vaccine Prevention

Prevention against *H. influenzae* is mainly based on vaccines. In China, the main vaccine is Hib polysaccharide conjugate vaccine, including single Hib vaccine, DTaP-Hib (quadruple vaccine) and DTap-Hib-IPV (pentavalent vaccine). In addition, in Western countries, there is the DTap-Hepatitis B-Inactivated Polio-Hib (hexavalent vaccine). But there is no specific vaccine for NTHi. Several studies on potential vaccine targets for NTHi have achieved certain results.

The pneumococcal NTHi protein D conjugate vaccine (PHiD-CV) had a vaccine protective effect of 35.3% against NTHi-associated otitis media, suggesting for the first time that a vaccine strategy targeting NTHi is possible.[126] The vaccine efficacy of PHiD-CV in treating clinical acute otitis media and bacterial otitis media in children<24 months was 24.0% and 48.0%, respectively, and the efficacy in treating moderate and severe clinical otitis media was 17.7% and 32.7%, respectively.[127] Researches into the development of vaccine targets in vitro and vivo primarily focused on *H. influenzae* adhesion proteins, particularly those that are critical in biofilm formation and stabilization. The anti-recombinant soluble form of PilA (rsPilA) antibody could destroy and prevent the formation of NTHi biofilms in vitro, and could also prevent experimental otitis media caused by NTHi in animal models.[37] A chimeric immunogen,

"chimV4", against both the P5 and T4P, could provide significant protection against otitis media caused by NTHi in chinchilla models by percutaneous immunization.[128] Recombinantly expressed protein D and outer membrane protein 26 could induce a robust antibody response after vaccination as a separate vaccine in a mouse model.[129] The fusion protein of E protein and PilA also expressed certain vaccine potential against NTHi biofilm in vitro mouse models.[130] The cationic cholesterol pullulan based (cCHP) - P6 nasal vaccine in the mouse model could also induce effective protective effect in the airway mucosa.[131] The mixture of anti IHF and amoxicillin clavulanate completely inhibited the formation of NTHi biofilms.[132] Australian Aboriginal otitis-prone children had lower serum IgG titres to major NTHi vaccine candidate antigens (rsPilA, ChimV4 and outer membrane protein 26), suggesting that Aboriginal children may benefit from immunisation with vaccines containing these antigens to increase titres of protective antibodies.[133] There are also other NTHi surface dominant antigens that can be used as candidate vaccine molecules. However, unlike capsular type *H. influenzae*, NTHi lacks a single surface antigen and may require a multi component binding targeted vaccine. How to combine candidate vaccine antigen molecules will be a research hotspot in the future.

Discussion and Future Work

H. influenzae is an important pathogen that causes respiratory tract infections and invasive diseases, posing a serious threat to children's health and remaining a public health issue that requires attention at present. With the introduction of the Hib conjugate vaccine, there has been a significant reduction in severe Hib-related infections. However, the infection situation in developing countries with low conjugate vaccine coverage is still severe, leading to a serious disease burden. It is recommended to incorporate the Hib vaccine into national immunization planning as soon as possible.

With the decline in the Hib incidence rate, other serotypes, especially the NTHi subtype, have gradually become epidemic strains and have led to severe infections. Due to the global incidence rate and mortality, NTHi has been recognized as one of the 12 priority pathogens by the WHO. Additionally, the drug resistance situation of *H. influenzae* is severe, and the high β-lactamase positivity and the emergence of BLNAR strains have made clinical treatment more difficult. There are significant regional differences in the detection rate of β-lactamase, drug resistance genes, and drug resistance patterns. Timely regional and multicenter epidemiological surveys are necessary to understand the epidemic trend of *H. influenzae* strains and provide epidemiological evidence for clinical treatment and national vaccine strategies.

Virulence factors, such as the capsule, fimbriae, adhesion proteins, and IgA protease, play an important role in the adhesion, colonization, invasion, and biofilm formation of *H. influenzae*. In-depth research on these factors provides ideas for clinical treatment and prevention strategies. Some antibodies like PilA antibody,[37] HMW1 and HMW2 protein antibodies,[41] Outer membrane protein P5[128], P6[131], IgA1 protease inhibitor[56] have shown promising results in vitro experiments and are expected to become candidate vaccine targets. Bacterial biofilm is a complex spatial structure that can protect bacteria from environmental, host, and chemical stress sources, and can enhance bacterial virulence and drug resistance. Its complex regulatory mechanisms, strong drug resistance, and correlation with clinical chronic infections have been research hotspots in recent years. The ultimate goal is to actively develop effective and clinically translatable anti-biofilm strategies. Drugs targeting bacterial biofilm constituent proteins and QS systems may be new research directions and approaches. Fortunately, some protein antibodies targeting biofilm formation and stability have achieved certain results in vitro and in vivo experiments. We hope to see more scientific clinical research results, ultimately benefiting the public.

In summary, the increasing prevalence of NTHi strains and antibiotic resistance among *H. influenzae* has increased clinical difficulties. *H. influenzae* infection is still a public health problem that can cause serious disease burden, although it has been underestimated by the public. Future studies should concentrate on the epidemic trends and drug resistance characteristics of *H. influenzae* in different regions, the mechanisms underlying biofilm formation, and the development of anti-biofilm strategies. These efforts will provide a scientific basis for the clinical diagnosis, treatment, and prevention of *H. influenzae* infections.

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically

reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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