INTERNATIONAL JOURNAL OF APPLIED AND EXPERIMENTAL BIOLOGY
(ISSN PRINT: 2790-6523; ISSN ONLINE: 2790-6531)
2026, Vol. 5, NO.1, 55-69
doi: https://doi.org/10.56612/ijaaeb.v1i1.164
ARK: https://n2t.net/ark:/71142/IJAaEB.v1i1.164
https://eminentscientists.com/

Insights into molecular characterization and post-vaccination dynamics of *Haemophilus influenzae*

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Abstract

Hemophilus influenzae is a Gram-negative bacterium exhibiting the characteristics of a facultative anaerobe. The bacterium requires capnophilic conditions for its growth. These bacteria are classified as fastidious organisms because they require X and V factors for their growth. They are present as commensals on the mucosa of the upper respiratory tract of humans. *Haemophilus influenzae* has no veterinary importance; it is exclusively adapted to its human host. The nontypeable strain (non-encapsulated) is mostly present in the normal flora of humans, but the typeable strain (encapsulated) can also exist as a commensal. To make a precise determination about infectious agents, methods of molecular characterization help distinguish among closely related isolates. Molecular characterization of *Haemophilus influenzae* is based on biochemical characteristics, capsular types, biotypes, lipopolysaccharide, and lipooligosaccharide, providing valuable insights into pathogenicity, resistance mechanisms, and epidemiology of this exclusive human pathogen. Laboratory diagnosis is done for the precise understanding of vaccine efficacy, disease outbreak monitoring, clinical management, and surveillance purposes. Protein-conjugated vaccine is used for its prevention. This has significantly reduced the incidence of Hib diseases. Still, simultaneously, the emergence of NTHi infections has also been reported, keeping in mind that NTHi is a prominent cause of otitis media, sinusitis, chronic obstructive pulmonary disease (COPD), and other invasive conditions in susceptible populations. However, utilizing surface proteins as a vaccine antigen can pave the way towards protective immunization against the NTHi strains. Additionally, the interdisciplinary relationship of molecular analysis with other areas of genomic research in determining pathogenicity, antibiotic resistance, vaccine efficacy, of pre- and post-vaccinal disease epidemiology is the main subject of this review; therefore, an effective approach towards molecular characterization can pave the way for developing protein-based vaccines for the control of NTHi infections in the post-Hib vaccine era.

ARTICLE TYPE
Original Review (OR)

SECTION
Human Biology (HB)

HANDLING EDITOR
Ashraf, M.A.B. (HB)

ARTICLE HISTORY
Received: 11 May, 2025
Accepted: 14 Jul, 2025
Online: 31 Jul, 2025
Published: 01 Jan, 2026

KEYWORDS
Gram-negative bacterium;
Hib;
Polyribosylribitol phosphate capsule;
Typeable and non-typeable strains;
Post-vaccination epidemiology

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CITATION (APA): Spaul, M.R., Khan, H., Zaheer, R., Raza, S., Faisal, M., Zahid, F. (2026). Insights into molecular characterisation and post-vaccination dynamics of *Haemophilus influenza*. *International Journal of Applied and Experimental Biology* 5(1):55-69.

Article No. 164; GP Template v202601

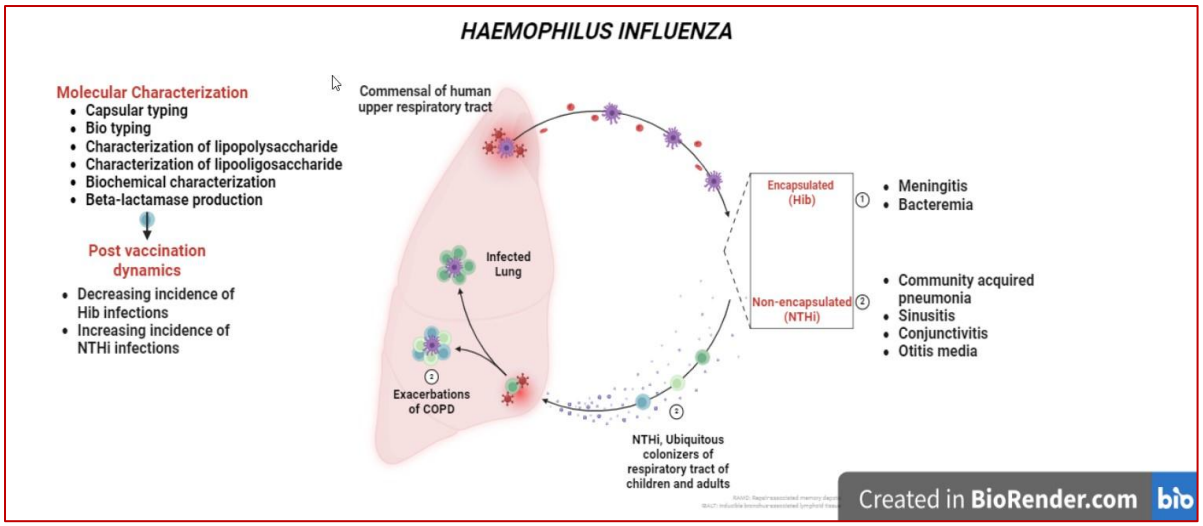

Figure 1: Graphical abstract created with BioRender.

Introduction

Haemophilus influenzae is a major community-acquired pathogen, being blamed for major health and life losses across the globe (Yamba Yamba et al., 2024; Seppanen et al., 2025). It is a small, Gram-negative, non-motile bacterium that is a non-spore-forming belonging to the family *Pasteurellaceae.* An ideal definition of *Haemophilus influenzae* can be attributed to its symbiotic nature in the human respiratory tract, causing upper and lower respiratory tract infections. So far, six capsular polysaccharide types of *H. influenzae* have been identified, namely: a, b, c, d, e, and f (Jin et al., 2007). Encapsulated (Virulent type b) strains of *H. influenzae* manifest the shape of coccobacilli, while nonencapsulated (non-virulent NTHi) exist in pleomorphic forms and exhibit as long threads and filaments (Su et al., 2023). They appear Gram-negative under Gram stain. However, smears from sputum may display bipolar staining, making its mistaken diagnosis with *Streptococcus pneumoniae* (Jin et al., 2007). It is highly acclimatized in humans, where it occurs as a commensal in the mucosa of the upper respiratory tract and causes disease during immunocompromised conditions (Jin et al., 2007)

Based on their requirement of hemin and nicotinamide adenine dinucleotide (NAD), *H. influenzae* has been placed into the group of fastidious organisms (Evans et al., 1974; Krisna et al., 2024), so chocolate agar is preferred for its growth, where the average size of the colonies has been measured between 1-2 mm. Studies have also depicted that the bacterium does not grow in the absence of either of the X or V factor (Mazloum et al., 1982). These factors have no endogenous origin; rather, they must be supplied to the bacterium exogenously in the form of various precursors (Windsor et al., 1993; Shalaginova et al., 2025). It grows in capnophilic conditions. Both its strains (Type b and NTHi) cause a variety of clinical infections that include otitis media, bacteremia, sinusitis, meningitis, conjunctivitis, pneumonia, etc.

In both industrialized and developing countries, respiratory tract infections linked to *H. influenzae* have a high morbidity and fatality rate (Foxwell et al., 1998; Yamba Yamba et al., 2024; Seppanen et al., 2025). However, NTHi has been accused of being a major culprit of respiratory tract infections (Murphy et al., 2009; Chatziparasidis et al., 2023). On the contrary, the invasive conditions such as bacteremia, pneumonia, epiglottitis, meningitis, and septic arthritis are mostly associated with typeable strains (Hib) (Mukundan et al., 2007).

The insights into pathogenicity, phylogeny, biology of bacterial cells, severity of disease, and control strategies, epidemiology, along with the knowledge of vaccinal antigens for enhanced protectivity can be achieved through molecular characterization of *H. influenzae* (Schiffer et al., 1974; Xiao et al., 2023b). The presence of polysaccharide capsular antigen serves as the basis for serotype classifications (Nørskov-Lauritsen et al., 2023). For this purpose, slide agglutination serotyping is a preferable method to classify capsular serotypes (Potts et al., 2019). The absence of capsular polysaccharide in non-typeable strains makes them exigent; however, the presence of certain adhesive factors such as lipooligosaccharides, IgA proteases, and glycolipids serves as a preliminary basis of their classification (Bajanca-Lavado et al., 2014). Characterization on the basis of ampicillin resistance provides a crucial molecular aspect. These strains are classified as β-lactamase-negative ampicillin-resistant (BLNAR) and β-lactamase-positive ampicillin-resistant (BLPAR). In the former, the

resistance is owned by alterations in penicillin-binding proteins, particularly PBP3, while in the latter strains, the production of β-lactamase is the key factor (Resman et al., 2012; Sethuvel et al., 2023).

Presenting the pre-vaccination scenario, 95% of all the invasive infections due to *H. influenzae* were attributed to Hib. The disease incidence has been documented in the US, Europe, Spain, France, etc. The rate was recorded as 12 to 21 cases per 100,000, 20 to 88 cases per 100,000 in Spain, France, and the US, respectively (High, 2002). The post-vaccination epidemiology depicts a remarkable success with a significant reduction in Hib cases due to the conjugate vaccine. Herd immunity played a significant role in this reduction (Berndsen et al., 2012; Bullen et al., 2023).

This study aims to analyze the importance of molecular research to tackle the emerging issues that are a threat to the sustainability of public health. Therefore, global efforts are currently in practice, particularly in the middle- and low-income countries where the introduction of Hib vaccination into national immunization programs is a key objective for the control of NTHi infections. Moreover, the insights into post-vaccination epidemiology and laboratory diagnosis of *H. influenzae* are crucial in suggesting improved diagnostic and therapeutic strategies.

Peculiar characteristics

Physiological characteristics

Haemophilus influenzae ranges in shape from coccobacilli to pleomorphic, depending upon the virulence of the strain (Kumar and Sobhia, 2025).

Culturing methods for *Haemophilus influenzae*

One of its peculiar characteristics is its ability to grow in a complex medium like chocolate agar augmented with X (HEMIN) and V (NAD or NADPH) factors (Casino et al., 2023) that are released from the lysed RBCs. The chocolate agar is prepared by heating blood agar at about 80 °C, RBCs become lysed, causing the release of these factors and turning the medium to chocolate brown color, hence the name chocolate agar. The bacterium can also grow on Brain Heart Infusion (BHI) supplemented with hemin and NAD. Other *Haemophilus* species are devoid of such culturing requirements (Poje and Redfield, 2003; Costello et al., 2022).

Genomic map

The genome of *H. influenzae* is composed of 1.83 Mbp. The *bexA* and *bexB* genes in the cap locus of capsular polysaccharide in typeable *H. influenzae* strains can serve as a marker to differentiate between typeable and non-typeable strains (Davis et al., 2011).

Genetic transformation

Transformation in *Haemophilus* (Sharma et al., 2023) includes alterations in drug susceptibility patterns and integration of peculiar capsular antigens. Transformation in *Hemophilus influenzae* is coherent than in Enterobacteriaceae. It exhibits an enhanced competence mechanism. The organism creates membranous "blebs" in the cellular membrane as it gains competence in the presence of a certain DNA-binding protein in the outer membrane (Goodgal and Herriott, 1961; Müller, 2025).

Transmission

Through respiratory droplets, people can transmit *H. influenzae*, including Hib. When an infected person coughs or sneezes, the bacteria are released into the air in the form of tiny respiratory droplets. If other people breathe in such an environment, they are at risk of becoming ill. Even those who are healthy but have the bacteria in their throats and nostrils can spread the infection as they act as carriers of the disease. This is how this bacterium is transmitted. People who are in close or prolonged contact with a person who has *H. influenzae* disease are also at risk of contracting the infection (Petersen et al., 2020). Asymptomatic carriers are involved in the transmission of *H. influenzae* type b (Auranen et al., 2004). Studies have also reported the nosocomial transmission of non-typeable strains of *H. influenzae* (Goetz et al., 1994; Ulanova et al., 2024).

Direct close contact is a major contributing factor in the transmission of disease. A study was conducted in China during the COVID-19 pandemic, which has shown a decrease in transmission of disease among children owing to reduced direct contact among individuals (Zhou et al., 2023).

Pathogenesis and progression of disease

Colonization of the causative agent in the host's body is a stimulator of the process of infection and is mainly initiated by the adherence of the agent to the host's epithelial cells (Barber and Fitzgerald, 2024). This enables the bacterium to acclimatize to the host's internal physiological conditions and bypass all the barriers of immunity. This is indicated in the form of initial inflammatory reactions in the host, with the subsequent penetration of the bacterium into host tissues, aided by specific receptors, where fimbriae and high molecular weight outer membrane proteins play their paramount role. Capsule and LPS are also important antigenic determinants (Bhagwat et al., 2025).

Both localized and invasive infections are reported by *Haemophilus influenzae,* as bacteria are a normal resident of humans' commensal flora. Type b is of major public health concern as it is the culprit of grave infections in children, comprising meningitis and pneumonia (Moxon and Wilson, 1991; Khattak et al., 2023).

Different genetic variations of the NTHi strains have evolved the ability to enter the nasopharynx and infect mucosal tissues nearby (Van Eldere et al., 2014). NTHi are obtained through direct contact with respiratory secretions or through respiratory droplets that are shared between individuals (Murphy et al., 2009). Sinusitis, conjunctivitis, and otitis media are reported in children triggered by the colonization of NTHi in the nasopharynx (Collins et al., 2016). Because NTHi is an upper airway colonizer (Brown et al., 2022), it can also reach the lungs by inhalation, micro-aspiration events, and direct mucosal dispersion (Richardson et al., 2019). Pathogenesis is initiated by the disturbance of mucociliary clearance, adherence to ciliated airway epithelial cells, escape from immunological defense mechanisms, persistence and survival of NTHi in the lungs, followed by biofilm formation (Chatziparasidis et al., 2023).

Molecular characterization of *Haemophilus influenzae*

Biochemical characterization

Common methods for characterizing *Haemophilus influenzae* include determining the organism's biotype and capsular serotypes. These techniques are not transparent about the strain's clonal origin and are prone to phenotypic changes (Gomez-De-Leon et al., 2000). Research on the epidemiology and pathophysiology of *H. influenzae* has made use of biochemical discrimination techniques such as multilocus enzyme electrophoresis, outer membrane protein analysis, and lipopolysaccharide profiling (Tsang, 2021). Numerous other biomolecular techniques have also been used, such as the investigation of DNA restriction fragment length polymorphisms (RFLP), random amplified polymorphic DNA (RAPD), and enzyme restriction amplification of the rDNA gene. The ribosomal region serves as a type and characterization target for both enzyme restriction and rDNA gene amplification. By using RFLP analysis, also known as ribotyping, it has proven feasible to differentiate between various isolates. The 16s RNA section is amplified via PCR-ribotyping, which limits the enzyme, and is widely used in genetic investigations on *H. Influenzae* (Mitsuda et al., 1999; Qurban and Ameen, 2020).

A study conducted in southeast Brazil reveals the higher incidence of type b capsule, followed by biotypes I, IV, VI, VIII, III, and V. By comparing the profiles of many strains, the above-mentioned ribotyping techniques revealed similarities that could indicate that pathogenic and non-pathogenic *H. influenzae* strains have comparable or same pathogenic mechanism genes (Lancellotti et al., 2008).

Capsular typing

So far, six capsular types (from a to f) of *H. influenzae* have been identified, which are chemically distinct from one another (Xiao et al., 2023a). Capsular type identification by the slide agglutination method is most commonly used, but due to its decreased authenticity (Shively et al., 1981; Månsson et al., 2018), an unequivocal method for capsular typing is the need of the hour. Therefore, targeting capsule-specific genes via polymerase chain reaction (PCR) is somewhat reliable (Falla et al., 1994).

A gene cluster associated with *H. influenzae* capsule expression is located on the "cap" region of the chromosome (Catlin et al., 1972). Regions 1-3 refer to the three areas that together comprise the same configuration of the cap loci for each of the six capsular types. All varieties share areas 1 and 3, although each variety has its own flank section 2 (Kroll et al., 1989). A DNA-based method can now be used to differentiate between the many capsular forms of *H. Influenzae* (Falla et al., 1994; Francesca et al., 2024).

Biotyping

Biotyping of isolated strains can be done based on the production of tryptophanase, urease and ornithine decarboxylase (ODC), but it has limited insights of differentiation (Kilian, 1976; Sunmonu et al., 2025), however, the criteria could function as a foundational structure for the initial targeted allocation of isolates that are not influenced by the X-factor (Nørskov-Lauritsen, 2014).

Characterization of lipopolysaccharide

Haemophilus influenzae LPS is composed of lipid A that is bound to a membrane; the oligosaccharide portion is connected to this moiety by a single 3-deoxy-D-manno-octulosonic acid (KDO) residue (Valvano, 2022). It is well established that the pathophysiology of *H. influenzae* infections involves the creation of specific oligosaccharide epitopes, and the carbohydrate portions of these LPS molecules act as targets for host immune response detection. Molecular structural studies of LPS from the mutant and wild-type strains of *H. influenzae* have led to the development of a structural model consisting of a conserved tripeptidyl inner-core moiety in which each heptose residue can serve as a point for elongation by hexose-containing oligosaccharide chains or for attachment of noncarbohydrate substituents (Risberg et al., 1999).

A study revealed identical biological, but distinct chemical lipopolysaccharide characteristics of *H. influenzae* as those of other members of Enterobacteriaceae. Thin-layer chromatography, gas-liquid chromatography, and calorimetric assays are commonly used methods for LPS characterization (Tønnessen et al., 2022).

Characterization of lipooligosaccharide in non-typeable strains

NTHi lacks an LPS with an O-antigen polysaccharide. NTHi exhibits diverse populations of LOSs that are distinct concerning the augmentation or deletion of glycose, phosphate (P), and phosphoethanolamine (PEA) substituents (Campagnari et al., 1987). The targeted expression is of paramount importance in regulating host-pathogen interactions, and it plays a role in determining the pathogenesis of non-typeable strains (Phillips et al., 1992).

Of many ways of characterizing LOS of non-typeable strains, the use of monoclonal antibodies against the antigenic determinants in the oligosaccharide portion of LOS remains dominant. SDS-PAGE and Western blot are being used to ensure that NTHI LOS-directed MAbs are bound to epitopes in the oligosaccharide region of NTHI LOS (Patrick et al., 1987). Other methods being implied for the sub-typing of NTHi strains include the use of SDS-PAGE to characterize the outer membrane protein content of the NTHi strains (Barenkamp et al., 1982; Su, 2023), by using polyclonal rabbit sera against the NTHi outer membrane proteins (Murphy and Apicella, 1985).

Characterization based on β-lactamase production

Penicillin and third-generation cephalosporins have been the drug of choice for the respiratory infections caused by *Haemophilus influenzae*. For a very long time, ampicillin was an effective treatment for *H. influenzae* infections. Regretfully, during the past few decades, resistance to this antibiotic and other β-lactams has grown. Penicillin-binding protein (PBP) and changes in the β-lactamases' enzymatic hydrolysis have been identified as the two main resistance mechanisms. The former ones are termed as β-lactamase-negative ampicillin-resistant (BLNAR), while the latter ones have been designated as β-lactamase-positive ampicillin-resistant (BLPAR) (Kiedrowska et al., 2017). BLNAR is the result of the expression of β-lactamase (Yamba Yamba et al., 2024; Seppanen et al., 2025) and the mutations in penicillin-binding proteins, particularly PBP3. These are called β-lactamase-negative ampicillin-resistant (BLNAR) strains, and these strains have high prevalence in Japan (Yokota et al., 2009).

Putative virulence factors

Hemagglutinating pili, sometimes called fimbriae, are a major adherence factor leading to the attachment of bacteria in the upper respiratory tract, followed by colonization (Gilsdorf et al., 1997). Phase variation controls the expression of the pilus, which switches spontaneously between the piliated and nonpiliated states at a frequency of roughly 10-4 to 10-3/generation. In general, nasopharyngeal colonizing strains express pili more frequently than those isolated from invasive infections (Geluk et al., 1998; Belayhun et al., 2023). Not all *H. influenzae* contain pili-producing genes (Marrs et al., 2001).

High molecular weight proteins (HMW1, HMW2) trigger significant antibody responses in

people with acute otitis media, which led to their first identification as *H. influenzae*-associated antigens (Barenkamp and Leininger, 1992). Studies have revealed that these high molecular weight proteins act as attachment factors and may aid in the expansion towards human epithelial cells (St Geme et al., 1993; Kalograiaki et al., 2018).

Polyribosylribitol phosphate capsule: Six serotypes of the capsule, the primary virulence factor characterizing pathogenic *H. influenzae* strains, have been described. The most virulent encapsulated strains are those with type b capsules (Hib strains), which are made of polyribosylribitol phosphate **(PRP)** (Slack et al., 2021) and can infect normal, non-immune hosts with prolonged bacteremia and localized infection. *Haemophilus influenzae* capsules, like those of other Gram-negative organisms, increase the virulence of the bacteria by resistance to phagocytosis (Marrs et al., 2001). The strong negative charge of the polysaccharide capsules may provide an electrostatic force of repulsion to phagocytic immune cells, and the capsular material itself may sterically interfere with the binding of opsonizing antibodies and complement to the bacterial surface.

IgA proteases on the mucosal surface are involved in the early defense against microbes, such as neutralizing poisons, preventing pathogens from adhering to the epithelium, and agglutinating organisms in mucus (Brandtzaeg, 1992). *Haemophilus influenzae* secretes IgA protease. These proteins serve as enzymes to cleave the antibodies at the heavy chain region, releasing two fragments (Fab and Fc). This saves the bacteria from the process of neutralization by the antibody (Reinholdt and Kilian, 1997).

Lipooligosaccharide: *Haemophilus influenzae* not only has distinct adhesin proteins on the cell surface and complicated sticky organelles, but it may also modify intrinsic structural elements and components of the cell membrane that act as attachment determinants (Theodorakis et al., 2024). One noteworthy example of this is a study showing that specific glyco-modifications of *H. influenzae* lipooligosaccharide (LOS) confer an ability to adhere to human epithelial cells. Prior research has indicated that the LOS core contains a considerable amount of complex carbohydrates, and their inclusion is reliant on one or more biosynthetic enzymes expressing phase variables. Nontypeable strains, which lack the LOS oligosaccharide side-chain, were demonstrated to be less efficient than wild-type cells to adhere to and penetrate respiratory epithelial cells (Swords et al., 2000).

Laboratory evaluation and sequential testing

Isolation of the bacterium from the clinical samples is required for the diagnosis and identification of the presence of *H. influenzae* in the samples. Samples vary concerning each clinical manifestation. For example, in the case of meningitis, the sample should be of CSF (Cerebrospinal fluid). Other samples include throat swabs, sputum, joint aspirates, and bronchial aspirates, etc. (Ishiwada et al., 1998). Standard optimum medium used for the growth of *H. influenzae* in the laboratory includes chocolate agar, prepared by heating BA at 80 °C, which will cause the release of X factor (hemin) and V factor (NAD) from the red blood cells, causing the bacterium to grow. *Haemophilus influenzae* cannot grow on an unsupplemented blood agar. Before carrying out diagnostic and identification procedures, a pure culture of the bacterium should be obtained, and diagnosis should be performed on 18–24-hour growth from chocolate agar under 5% carbon dioxide.

Gram staining: The bacteria appear as small, pleomorphic, Gram-negative bacilli or coccobacilli (Wu et al., 2013).

Kovac's oxidase test: A negative oxidase test indicates the absence of *H. influenzae,* while a positive oxidase test is an indication of the presence of *H. influenzae.*

Growth factor requirement testing: In case of a +ve oxidase reaction, testing is done to ascertain which growth factor is required, either X or V.

Latex agglutination test: If the growth factor requirement testing gives an indication that the isolate may be *H. influenzae*, then serological tests are performed with the blood sample that includes the latex agglutination test, which uses the monoclonal antibodies to detect the capsular antigen from the patient's sample.

Isolation: Isolation of the bacterium from the clinical samples is performed on the chocolate agar. For encapsulated strains, the colonies on the chocolate agar are mucoid, convex, colorless, and opaque. On the other hand, the colonies for non-encapsulated strains are non-mucoid, convex, opaque, and of a grey color.

Molecular analysis: One-step multiplex PCR is a preferable approach for the confirmation of *H. influenzae* in clinical samples. The main goal is to distinguish typeable strains from the non-typeable ones. To achieve this goal, two genes are targeted, i.e., the *ompP6* gene, for outer membrane protein 6, and present in all the strains, and the *bexA* gene encoding the capsule transport protein, which

persists only in the encapsulated ones (Carrera-Salinas et al., 2021).

Satellitism: It is a very interesting phenomenon for isolation of a fastidious organism like *H. influenzae*. In it, an organism is grown with *H. influenzae* on the same agar plate, other than the chocolate agar, which is known to produce X and V factors required for growing *H. influenzae*. The factors are released into the agar, which are then used by *H. influenzae* in order to grow.

Streptococcus pneumoniae is known to produce these growth factors, which are then used by *H. influenzae* to grow (Evans et al., 1975).

Discrimination between *H. influenzae* and *H. haemolyticus*

Haemophilus influenzae is an important colonizer of the upper respiratory tract of humans, where it is associated with recurrent tonsillitis and retropharyngeal abscesses (Brook, 2004), however, the incidence is particularly high in children and lessens with increasing age (Kilian et al., 2002). *Haemophilus parainfluenzae, H. haemolyticus*, and *H. parahaemolyticus* (Kilian et al., 2002) are comparatively low pathogenic, but have phylogenetic relationships with *H. influenzae* (Frickmann et al., 2014). Therefore, the probability of their isolation from clinical URT infections is occasional. Even some studies abandon any clinical association of *H. haemolyticus*. In order to check this hypothesis, a study was conducted where amplification of the gene that codes for the P6 protein of *Haemophilus* spp. was carried out (Murphy et al., 2007). The sequence was identical in all strains of *H. influenzae,* but *H. haemolyticus* strains differed by 4 out of 156 amino acids from *H. influenzae* strains. Immunoblot assays with monoclonal antibodies are also an effective approach to distinguish between *H. influenzae* and *H. haemolyticus* (Murphy et al., 1992). Apart from this, various sequence-based approaches, PCR and hybridization-based approaches, MALDI-TOF-MS etc., provide confirmatory diagnosis (Hinz et al., 2015).

Vaccines

Infections caused by encapsulated Hib strains of *H. influenzae* have shown a substantial decrease owing to the administration of vaccines composed of polysaccharide capsule conjugated to protein carriers. But these vaccines are not effective in the control of infections caused by non-typeable strains. The principal cause of otitis media in children and lower airways infection in adults could be due to the absence of polysaccharide capsules (Murphy, 2015).

Conjugated vaccines against Hib infections

Early vaccines against typeable strains of *H. influenzae* containing only a polysaccharide capsule elicit weak and short-term immune responses in infants and adults. To overcome this, conjugate vaccines were developed in which the polysaccharide capsule is conjugated with an immunogenic carrier protein to develop protective immunity in individuals. These vaccines trigger T-cell-dependent immune responses (Goldblatt, 2000). Another aspect of this reaction is immunological memory, which is the generation of memory B cells that, upon re-exposure, initiate a secondary (booster) immune response to the same antigen. These immunizations provide the most vulnerable populations with significant protection against invasive Hib disease, because they elicit robust immune responses in newborn infants (Ulanova and Tsang, 2009). For the formulation of such vaccines, various carrier proteins are currently in use, which include tetanus toxoid, mutated diphtheria toxin CRM197, and outer membrane proteins of *Neisseria meningitidis* (OMP) (Trotter et al., 2008). The following conjugated vaccines against Hib infections have been licensed for use: PRP-D (diphtheria), PRP-OMP (outer membrane protein of *Neisseria meningitidis*), HbOC (CRM_{197}), and PRP-T (tetanus). PRP-OMP induces higher antibody titers when administered during primary infection compared to other conjugate vaccines (Decker and Edwards, 1998).

There has been limited comprehension about the enhanced immunogenicity of the conjugated vaccines. The covalent linkage between the capsular polysaccharide and the conjugated carrier protein may be the contributing factor (Anderson et al., 1985).

Approach to vaccine development for non-typeable strains

The development of protective immunity in the host is the key indicator of the effectiveness of the vaccine antigen (Murphy, 2015). Non-typeable strains lack polysaccharide capsules, so PRP is not a favorable target. However, the use of surface proteins as a vaccine antigen is under consideration for protective immune response in the host (Murphy, 2009). The high genetic variability among non-

typeable *H. influenzae* strains poses a barrier to this strategy since it causes sequence heterogeneity in numerous surface antigens (Eutsey et al., 2013; Chatziparasidis et al., 2023).

Lack of a PRP capsule makes vaccine development against NTHi a challenging game. Antibodies against LPSs and OMPs are present in human serum. Finding OMPs with immunogenic and antigenic properties required for invasion, colonization, and survival in the human host has been the main focus of research on human immunity against NTHi infections (Latz et al., 2004; Zaman et al., 2025). Low-molecular-weight proteins from *H. influenzae* can be divided into two categories: major OMPs (P1, P2, and P4–P6) and minor OMPs (protein D and the transferrin binding proteins 1, 2 (Tbp1/Tbp2). OMP P2's acceptance as a vaccine candidate has been hampered by its high variability and the antigenic drift that follows recurring infections in patients with chronic bronchitis (Poolman et al., 2000).

Impact of immunization on *H. influenzae* infections

A chain of studies was carried out during the 1970s and 1980s to ascertain the burden of disease due to *H. influenzae* in America. A population-based survey of between 40 and 100 cases per 100,000 children <5 years old was carried out in different geographical locations within the United States to ascertain the rate of invasive disease which revealed that every one out of 200 children is the sufferer of disease by 5 years of age and the disease was recorded in highest frequency in children of 6 to 11 months of age (Wenger, 1998).

Typeable strains of *H. influenzae* (Hib) are primarily associated with meningitis (Slack et al., 2021), accounting for 60% of all cases. Epiglottitis (comprising 15 to 30% of all cases), and the rest of the infections are associated with arthritis, facial cellulitis, otitis media, and bacteremia (Slack, 2021). The mortality rate in children owing to invasive infections is 3-6%, while prolonged sequelae like mental retardation and hearing loss are linked to 25% of the meningitis cases, which are fortunate enough to survive (Wenger, 1998).

In the United Kingdom, the Hib conjugate immunization program was initiated in October 1992. Vaccinations were administered to 2, 3, and 4-month-old infants without the use of a "booster" dose (Heath et al., 2000). With the advent of a less immunogenic Hib conjugate vaccine (DTaP-Hib) in 2000 and 2001, the relatively small post-immunization antibody concentrations that followed the combination of acellular pertussis, tetanus, and diphtheria vaccinations were drastically lowered (McVernon et al., 2003). Although there was a noticeable drop in incidence at first, eight years after the vaccine was introduced, infections started to resurface, requiring a nationwide immunization drive to make up for lost time. The United Kingdom's first experience with a highly successful vaccination program boosted confidence in the immunological memory's capacity to protect against low Hib antibody levels (Heath et al., 2000). Memory immunological responses are demonstrated by the rapid production of high-avidity antibodies upon re-exposure to an antigenic challenge. Direct protection, however, was shown to be less effective than anticipated following the UK infant primary course, at just 61% for the first two years and 27% for the years thereafter (Ramsay et al., 2003). It is probable that the early implementation of the catch-up plan covered this up (Slack et al., 2021).

Various studies conducted in Finland, the United States, and the United Kingdom have revealed a prominent reduction in the incidence of Hib infections (Coen, 1999). A meta-analysis study revealed the reduced incidence of Hib diseases by 84% involving the use of Hib conjugate vaccine with a confidence interval range of 69-92%. A significant improvement in the efficacy of this vaccine was even observed after the second administration, stressing the need for double doses to achieve the required protection (Obonyo and Lau, 2006; Slack et al., 2021).

Antibacterial immunizations lower the morbidity and mortality from bacterial infections and may also make antibiotic-resistant organisms that colonize and perhaps infect vaccine recipients less resistant. It has been demonstrated that the Hib vaccination dramatically lowers the incidence of Hib infections in Finland, to the point that Hib resistance to β-lactam antibiotics does not need to be taken into account when diagnosing septic arthritis in young, immunized children. Consequently, narrow-spectrum antibiotics may be the initial medicines used in treatment (Gilsdorf, 2021).

Nontypeable *H. influenzae* (NTHi): nonencapsulated strains are a critical culprit of respiratory tract infections comprising acute otitis media, cystic fibrosis, and community-acquired pneumonia among children, particularly in developing countries (Weeks et al., 2021). In adults, bacteria are the colonizers of the lower respiratory tract, causing chronic obstructive pulmonary disease (COPD) (Adderson et al., 2001). COPD (Short et al., 2021) is the fourth leading cause of death in adults (Bruun et al., 2004). Similar to *Pseudomonas aeruginosa* in cystic fibrosis patients, NTHi bacteria have been linked to the development of biofilms in the respiratory tracts in people with COPD (Wu

et al., 2014). An uncommon bacterial colony known as a biofilm adheres to solid surfaces because it is encased in a polymer matrix. Ninety-nine percent of bacteria on various surfaces form biofilms on their own, in contrast to planktonic germs that can become resistant to drugs and immune clearance systems. Therefore, creating efficient management strategies for illnesses brought on by bacteria in biofilms, including otitis media with effusion, requires an awareness of the function that biofilm growth plays in bacterial pathogens (Behrouzi et al., 2017; Principi and Esposito, 2024).

Even with the wide availability of medications, NTHi infections are still thought to be major contributors to morbidity and death. This highlights how crucial it is to develop vaccine candidates that offer protection against NTHi infections (Atto et al., 2025).

Owing to the inherent "heterogeneity" of microorganisms, research and production of effective NTHi vaccines are hindered. Putative conserved NTHi antigens have been the subject of numerous searches in recent years, and a number of candidates are presently being studied. Great strides are expected in the next years towards the creation, manufacturing, and testing of potent NTHi vaccines (Buckland et al., 2024).

In the United Kingdom (UK), the incidence of invasive non-thermal hydrolysis infection (NTHi) in pregnant women was 17 times greater than that in non-pregnant women between 2009 and 2012 (Oliver et al., 2023). It's interesting to note that the infection resulted in the pregnancy's termination in all but two of these cases—either by miscarriage, stillbirth, or childbirth at the time of infection. Compared to term neonates, infants born at less than 28 weeks of gestation had a 365-fold higher risk of developing invasive NTHi illness. There was a noticeably increased incidence of invasive NTHi illness in preterm newborns. NTHi was the etiology of 97% of instances of invasive Hi illness in neonates in the United Kingdom (Oliver et al., 2023). Therefore, considering the antigenic variability of NTHi, the development of a protective vaccine against NTHi infections is the need of the hour. It has been suggested that a number of the NTHi outer-membrane constituents could serve as vaccine antigens. Protein diversity has led to the elimination of many antigens. The many antigens being studied for potential inclusion in vaccines have been chosen in part due to their immunogenicity, sequence conservation, and/or ability to produce notable protection in animal models after immunization (Kyd and Cripps, 1999; Gloanec et al., 2025).

NTHi was responsible for around 70% of all invasive Hi diseases in the US between 2008 and 2019. This is consistent with other studies that concluded that NTHi is primarily responsible for invasive Hi disease following the introduction of the Hib immunization. The study conducted in 2023 in the US also reveals that the incidence of NTHi has been increasing in the US, with the most dramatic rises taking place in recent years, much like in other countries. The largest annual rate increases were seen by teenagers and young adults (15–44 years old). Additionally, the prevalence of invasive NTHi varies with race, particularly in neonates and women who are pregnant (Oliver et al., 2023).

A retrospective study of NTHi diseases in Central Asia conducted revealed that out of 34 adults with invasive *H. influenzae* infections, NTHi contributed to 79% cases of pneumonia, followed by Hif (11%), Hia (5%), and Hib (5%) (Slack et al., 2021).

Conclusion

Despite the global success of *Haemophilus influenzae* type b (Hib) vaccination, the increasing clinical relevance of non-typeable strains is still a major concern. Epidemiological trends following immunisation indicate an increase in the prevalence of NTHi-associated infections. Therefore, this review underscores the need to comprehend NTHi at the molecular level for developing novel, protein-based, next-generation vaccines for NTHi in the post-Hib vaccine era.

Author(s), Editor(s) and Publisher's declarations

Source of funding
None declared.

Contribution of authors
Research superior (s): MRS; HK. Member(s) of advisory group: SR. Concetualization and designing the study: MRS and RZ. Intellectual Contribution: RZ. Proof reading and approval of the final version: MF, FZ.

Permissions and ethical compliance
This study does not involve human/animal subjects, and thus no ethical approval is required.

Handling of bio-hazardous materials
Since this is a review article, it does not involve any experimentation or use of any types of materials/chemicals.

Supplementary material
No supplementary material is included with this manuscript.

Conflict of interest
The authors declare no conflict of interest.

Availability of primary data and materials
As per editorial policy, experimental materials, primary data, or software codes are not submitted to the publisher/Journal management. These are available with the corresponding author (s) and/or with other author(s) as declared by the corresponding author (s) of this manuscript.

Authors' consent
All authors have critically read this manuscript and agreed to publish in IJAaEB.

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Declaration of generative AI and AI-assisted technologies in the writing process
It is declared that the authors did not use any AI tools or AI-assisted services in the preparation, analysis, or creation of this manuscript submitted for publication in the International Journal of Applied and Experimental Biology (IJAaEB).

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