Invasive *Haemophilus influenzae* disease: Changing epidemiology and host–parasite interactions in the 21st century

Marina Ulanova a, Raymond S.W. Tsang b, *

a Division of Medical Sciences, Northern Ontario School of Medicine, Lakehead University, Thunder Bay, Ontario, Canada
b Division of Vaccine Preventable Bacterial Diseases, National Microbiology Laboratory, Public Health Agency of Canada, Winnipeg, Manitoba, Canada

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**ABSTRACT**

Introduction of *Haemophilus influenzae* type b (Hib) conjugate vaccines in late 1980s–early 1990s made a dramatic effect on the incidence of invasive Hib disease among children in many industrialized countries with routine Hib immunization programs. However, cases of vaccine failure and an increased susceptibility to invasive Hib as well as non-type b *H. influenzae* disease have been consistently reported among individuals with various congenital and acquired immunodeficiencies. Remarkably, in the 21st century, diseases due to non-type b strains of *H. influenzae* are becoming relatively more frequent than before. Despite the overall successful immunization against Hib, some indigenous populations, i.e. Australian Aboriginal and North American Indian children still experience increased rates of invasive *H. influenzae* disease. In order to monitor the evolving nature of invasive *H. influenzae* disease, carefully documented surveillance data is required to capture the true magnitude of the problem. Developing new vaccines against non-type b *H. influenzae* is a potential solution to protect some vulnerable populations against the invasive disease.

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1. Introduction

*Haemophilus influenzae* is a small Gram-negative coccobacillus which may appear as pleiomorphic in its microscopic morphology. Besides human, there is no natural animal host. Most if not all *Haemophilus* species have fastidious growth requirements, and *H. influenzae* is dependent on X [hemin] and V [nicotinamide adenine dinucleotide] factors for growth. Many invasive virulent *H. influenzae* are encapsulated and on the antigenic basis of their capsular polysaccharides, six [a–f] capsular types or serotypes have been identified (Pittman, 1931). Most invasive *H. influenzae* infections are caused by serotype b strains, characterized by a polyribosyl ribitol phosphate [PRP] capsule (Crisel et al., 1975). Using animal infection model with genetically constructed isogenic mutants transformed with serotype-specific capsule-associated DNA from all six serotypes [a–f], serotype b was demonstrated to be the most virulent serotype, followed by serotype a, which is more virulent than serotypes c–f (Zwahlen et al., 1989). Invasive diseases caused by *H. influenzae* serotype b [Hib] include meningitis, epiglottitis, bacteremia without localized disease, septic arthritis, cellulitis, and pneumonia.

Strains without the capsules are termed non-typeable [NT], and they are most often associated with acute otitis media in children, sinusitis in adults and children, and community acquired pneumonia, especially in the elderly and in those with chronic obstructive pulmonary disease [COPD] (Murphy, 2005). Non-typeable *H. influenzae* has also been reported to cause invasive infection (Nizet et al., 1996). A list of invasive and mucosal diseases caused by Hib and NT *H. influenzae* is summarized in a book chapter by Ward and Zangwill (1999).

Since vaccination against Hib was introduced in the late 1980s, the epidemiology of invasive *H. influenzae* disease has changed. Also newer candidate vaccine(s) against all serotypes and NT *H. influenzae* are currently under development. As such, a review of our current understanding on various aspects of invasive *H. influenzae* disease in the post-Hib vaccination era appears to be appropriate. This review will cover the following topics: laboratory identification, typing, immunology, vaccination, disease susceptibility, epidemiology, and future outlook. The objective is to summarize our current understanding of this disease and the continued challenge that we may face with the control of this once common childhood infection.

2. Laboratory identification of *H. influenzae*

Accurate identification of pathogen is of utmost importance in the surveillance of notifiable infectious diseases. Without accurate
laboratory diagnosis and identification of pathogen, surveillance data may be compromised and erroneous control policies may be put in place. The traditional bacteriological approach for identification of *H. influenzae* involves microscopic morphology, hemolysis, specific requirements for X and V growth factors, and biochemical tests. However, some organisms are highly similar and may not be easily differentiated. For example, *H. aegypticus*, *H. influenzae* biogroup aegypticus [that causes the fulminating septicemic condition known as Brazilian purpuric fever, BPF] cannot be reliably differentiated from *H. influenzae* sensu stricto. Clinical history and additional tests are required to definitely identify *H. aegypticus* and *H. influenzae* biogroup aegypticus. Another example is the genetically distinct group of non-encapsulated or NT *H. influenzae*, mostly of biotype IV, associated with urogenital, neonatal, and mother–infant infections (Wallace et al., 1983). Traditional biochemical tests cannot distinguish between these two genetic populations of NT *H. influenzae*, and identification is based on differences in their 16S rRNA sequence (Quentin et al., 1993). Presence of an active sodC, copper- and zinc-containing superoxide dismutase, has also been described as a marker to differentiate between the cryptic genospecies that causes urogenital infections from the NT *H. influenzae* (Liangford et al., 2002).

Recent concerns of mis-identification of the strictly commensal *H. haemolyticus* as NT *H. influenzae* have been described because of increasing frequencies of finding non-hemolytic *H. haemolyticus* from respiratory sources (Murphy et al., 2007; McCrea et al., 2008). For example, in one study of 490 apparent *H. influenzae* isolates identified by conventional standard methods, 39.5% of sputum isolates (102 out of 258) and 27.3% of isolates identified by conventional standard methods, i.e. indole, urease, and ornithine decarboxylase (Kilian, 1976). However, biotyping or serotyping does not reveal the population structure or the genetic relationship between strains. The first genetic approach to examine the population biology of encapsulated *H. influenzae* was by multi-locus enzyme electrophoresis [MLEE] (Musser et al., 1988), and two phylogenetic groups, clonal divisions I and II, have been identified. Most isolates of *H. influenzae* involved in disease process belong to clonal division I (Musser et al., 1990). However, all serotype f strains and a minority of serotype a and serotype b strains are known to belong to clonal division II. Serotype e strains appear to form a distinct and may be intermediate group, only distantly related to the 2 clonal divisions I and II (Musser et al., 1988). The sodC gene, which encodes for the copper- and zinc-containing superoxide dismutase [CuZnSOD], has been found to be present in phylogenetic division II strains only and not in division I strains, with the exception of serotype e strains (Kroll et al., 1991b; Langford et al., 2002). However, a mutation in the enzyme active site in the sodC genes of encapsulated *H. influenzae* strains cause them unable to produce an active CuZnSOD protein (Kroll et al., 1991b). It has also been suggested that the organization of the capsule synthesis loci [cap] in clonal divisions I and II are different, partly due to its association with either the sodC gene or the insertion sequence IS1016 (Satola et al., 2003).

The method of MLEE which is based on gel electrophoresis of bacterial protein enzymes, has been superseded by a DNA sequence-based method of multi-locus sequence typing [MLST] (Meats et al., 2003). The typing method of MLST is based on assigning alleles to partial sequences of seven house keeping enzyme genes and sequence types were determined by the combinations of these seven gene alleles. Strains with at least four identical alleles are classified as a clonal complex. Strains belonging to the different serotypes have been found to reside within different clonal groups, sharing no or minimum housekeeping gene alleles (O'Brien et al., 2002; Sill et al., 2007; Tsang, 2008). In our analysis of invasive *H. influenzae* isolates (*n* = 122) from Manitoba, Canada, we found that strains that belonged to different serotypes did not have any common MLST housekeeping gene alleles, and hence they belonged to different clonal groups. This led us to believe that capsule switching did not occur among the 122 invasive isolates tested (Sill et al., 2007). Also of the 169 different sequence types (STs) in the *H. influenzae* MLST database that were found to be associated with encapsulated strains, most STs were linked to their serotypes and strains belonging to different serotypes appeared to have their own sets of STs (Tsang, 2008), again suggesting that capsule switching is not a common phenomenon among the different serotypes of *H. influenzae*.

In contrast to encapsulated *H. influenzae*, NT *H. influenzae* are a genetically diverse group of organisms. For example, in one study (Sill et al., 2007), we have found that 53 encapsulated *H. influenzae* were grouped into 11 different STs within 6 clonal clusters whereas 69 NT strains were divided into 45 different STs with most STs being unrelated to each other. Furthermore, this study of the population genetics of encapsulated and NT or non-encapsulated strains suggested that most non-capsular strains did not derive from encapsulated strains due to loss of

Serotyping of *H. influenzae* is usually accomplished by bacterial agglutination test using antibodies developed against the 6 different capsular polysaccharides. Molecular methods have also been developed to identify and differentiate the 6 different capsular types from either the non-encapsulated strains or from strains that have the capsule polysaccharide synthesis genes but have undergone a genetic rearrangement of their *cap* locus to become non-encapsulated (Kroll et al., 1991a; Falla et al., 1994).

Besides serotyping, another conventional approach to type *H. influenzae* is by biochemical reactions, and 8 biotypes have been described based on their differential reactivities in 3 biochemical tests, i.e. indole, urease, and ornithine decarboxylase (Kilian, 1976). However, biotyping or serotyping does not reveal the population structure or the genetic relationship between strains. The first genetic approach to examine the population biology of encapsulated *H. influenzae* was by multi-locus enzyme electrophoresis [MLEE] (Musser et al., 1988), and two phylogenetic groups, clonal divisions I and II, have been identified. Most isolates of *H. influenzae* involved in disease process belong to clonal division I (Musser et al., 1990). However, all serotype f strains and a minority of serotype a and serotype b strains are known to belong to clonal division II. Serotype e strains appear to form a distinct and may be intermediate group, only distantly related to the 2 clonal divisions I and II (Musser et al., 1988). The sodC gene, which encodes for the copper- and zinc-containing superoxide dismutase [CuZnSOD], has been found to be present in phylogenetic division II strains only and not in division I strains, with the exception of serotype e strains (Kroll et al., 1991b; Langford et al., 2002). However, a mutation in the enzyme active site in the sodC genes of encapsulated *H. influenzae* strains cause them unable to produce an active CuZnSOD protein (Kroll et al., 1991b). It has also been suggested that the organization of the capsule synthesis loci [cap] in clonal divisions I and II are different, partly due to its association with either the sodC gene or the insertion sequence IS1016 (Satola et al., 2003).

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### 3. Typing of *H. influenzae*

In order to understand the evolution of *H. influenzae* under immune selection from vaccine pressure, typing of strains, especially with molecular methods that can provide population biology and genetic structure information, is essential to track the changing nature of the organism. Traditional methods to type *H. influenzae* are based on biotypes and serotypes that reveal different phenotypic characteristics of the strains.
their capsule synthesis genes. Comparative genomic studies of NT H. influenzae strains suggested a “distributed genome hypothesis” whereby individual NT strain may each have their own sets of unique genes. In addition to a set of core genes (possessed by all strains) estimated to be about 1400, each strain may have up to 350 unique genes and 780 distributed genes (i.e. present in some but not all strains), and together they form a “supra-genome pool” that is available to different strains through continuous horizontal gene transfer (Hogg et al., 2007).

More recent studies based on MLST provided data that did not contradict this hypothesis but found that NT strains collected from different geographic origins and clinical backgrounds do separate into a number of well-defined phylogenetic groups (Erwin et al., 2008).

Other molecular methods proposed for typing of H. influenzae include 16S rRNA sequencing (Sacchi et al., 2005), pulsed-field gel electrophoresis (Saito et al., 1999), intergenic dyad sequence-PCR (IDS-PCR) (Bruant et al., 2003), ribotyping (Wang et al., 2001), enterobacterial repetitive intergenic consensus [ERIC] sequence-PCR (Van Belkum et al., 1994), and multiple-locus variable number tandem repeats analysis [MLVA] (Schouls et al., 2005).

4. Immunology of H. influenzae type b infection

The dynamics of invasive infections depend not only on the microbe, but also on the host. In the next few sections, current understanding on the immunology of H. influenzae infection, vaccination, and susceptibilities to infections will be discussed to form a basis to examine the changes in the epidemiology of invasive H. influenzae disease after introduction of the conjugate Hib vaccine.

Hib causes severe diseases, such as meningitis, epiglottitis, and acute lower respiratory tract infections in young children, acute pneumonia in adults, as well as septicaemia, pericarditis, pyelonephritis, arthritis in older or immunodeficient patients. However, Hib can also frequently colonize the nasopharynx of healthy individuals without causing the invasive disease. As the carriage rate is much greater than the incidence of invasive disease, natural defence mechanisms against this infection must exist. Protection against Hib is ensured by anti-capsular polysaccharide antibodies that mediate opsonophagocytosis of bacteria (Schreiber et al., 1986). Polysaccharide capsule of H. influenzae prevents both complement-mediated killing and phagocytosis and thus is the major virulence factor of these bacteria (Moxon and Kroll, 1988).

Among six serotypes of encapsulated H. influenzae (a–f), Hib that carries the PRP capsule is the most frequent cause of severe invasive disease. Non-capsulated, or NT H. influenzae are less protected against the host defence mechanisms and therefore cause invasive disease less frequently. Instead, non-capsulated H. influenzae are more commonly found in the upper respiratory tract.

In the pre-vaccination era, the development of protective immunity against Hib followed the natural history of exposure to this microorganism. Newborn babies and infants are protected by maternal IgG antibodies acquired through the transplacental transfer. The decline in maternal IgG usually occurs by the age of 6 months, and around this time infants become susceptible to invasive Hib disease. Carriage of Hib in the nasopharynx usually starts after 2 months of age and contributes to the development of natural immunity against this microorganism because it stimulates antibody production against PRP. Natural antibodies against Hib are also induced by the exposure to some non-pathogenic bacteria that are common in the environment and carry antigens cross-reacting with PRP, e.g. E. coli K100 (Insel and Anderson, 1982). The immunity of older children and adults against invasive Hib disease is mediated by naturally acquired antibodies against PRP that are highly efficient in opsonization of bacteria. With age, an increase in levels of anti-PRP antibodies in plasma coincides with decline in the incidence of the invasive Hib disease in the population [reviewed by Kelly et al. (2004)].

Hence, in unvaccinated children, generation of protective anti-PRP antibodies is caused by natural immunization resulting from Hib carriage as well as by immunization with cross-reactive antigens. The plasma levels of anti-PRP antibody in children follow a characteristic age-dependent pattern due to peculiar immune responses to bacterial polysaccharide antigens. As discussed below, maturation of a subpopulation of B cells responsible for production of anti-polysaccharide antibodies is delayed until the age >2 years. In general, children become fully competent to mount a protective immune response to PRP by the age of 5 years (Robbins et al., 1973). As the incidence of invasive Hib disease is inversely related to anti-PRP antibody levels, this explains why the disease rarely occurs in children above 5 years old.

Immune response to bacterial capsular polysaccharides is considerably different from the immune response to protein antigens. The capsular polysaccharides of H. influenzae, Streptococcus pneumoniae, Neisseria meningitidis, and some other encapsulated bacteria belong to the family of T-independent type II antigens [TI-2] that are characterized by poor or non-immunogenicity in infants [-2 years old] and failure to induce immunological memory and antibody affinity maturation at any age. The antibody response to capsular polysaccharides is a result of T-cell independent B-cell activation in contrast to the antibody response to protein antigens that requires certain signals from T-helper cells. Capsular polysaccharides are polyvalent antigens with repetitive epitopes and therefore are capable to cross-link multiple antigen receptors on the surface of a single B cell then initiating B-cell activation in the absence of T-cell derived co-stimulatory signals. Unlike proteins, capsular polysaccharides do not require MHC class II molecule-mediated antigen presentation [reviewed by Ves et al. (2000)].

One consequence of TI-2 responses is that pure capsular polysaccharide vaccines are inefficient in early childhood [when the disease mostly occurs], although can be protective in adults. In contrast, immunization with protein antigens is highly successful in small infants because T-helper function develops early in the ontogenesis.

The reasons why TI-2 antigens are not immunogenic until the age of 2 years are still incompletely understood. One suggested reason may be the delay in the development of the population of splenic marginal zone B cells that are primarily responsible for the anti-polysaccharide response (Timens et al., 1989; Ves et al., 2000) as well as of the major isotype of anti-polysaccharide antibody, IgG2 (Aucouturier et al., 1988). In addition, some co-stimulatory signals from accessory cells and the complement system that are essential for the response to capsular polysaccharides develop relatively late in the ontogenesis (Ves et al., 2000; Von Bulow et al., 2001; Kaur et al., 2007). As a result, infants respond poorly to immunization with Hib capsular polysaccharide (Smith et al., 1973).

Indeed, purified PRP polysaccharide vaccine induces an immature immune response in young children characterized by the prevalence of IgM antibody and a short duration of their production (Smith et al., 1973). Accordingly, the use of this vaccine for the immunization in the past demonstrated the lack of protection of children younger than 2 years against invasive Hib disease [reviewed by Ward (1991)]. Nevertheless, biological characteristics of anti-Hib capsular polysaccharide antibodies induced by immunization with PRP are identical to both natural and infection-induced antibodies (Schneerson et al., 1971). Anti-polysaccharide antibody responses to Hib invasive disease and to immunization with PRP also show remarkable similarities.
regarding their age-dependence. In contrast to responses of adults, infants and small children have a longer interval to reach the maximum antibody concentration post-immunization, their antibody levels are lower, and a decline in antibody levels is observed within several months (Robbins et al., 1973). Interestingly, children who had recovered from invasive Hib disease often had lower responses to the polysaccharide vaccine compared to the children of the same age without history of invasive disease (Norden et al., 1975). This indicates that the invasive Hib disease may suppress the immune response of small children to subsequent immunization with polysaccharide antigens of this microorganism, although the underlying immunological mechanisms are poorly understood.

5. Hib conjugate vaccines

To overcome poor immunogenicity of plain PRP, Hib conjugate vaccines have been developed. In these vaccines, PRP is covalently linked to a protein carrier, such as tetanus toxoid, diphtheria toxoid, or outer membrane protein complex of \( N. meningitidis \). The protein–polysaccharide conjugation ensures that the resulting immune response to PRP becomes T-cell dependent and hence is characterized by germinal centre formation, antibody class switching and affinity maturation as a result of co-stimulatory signals provided by T-helper cells. This response is also characterized by the formation of immunological memory: generation of memory B cells that induce the secondary (booster) immune response to the same antigen following subsequent exposure. These vaccines are highly immunogenic in young children and thus provide an excellent protection against invasive Hib disease in the most susceptible population [reviewed by Wenger and Ward (2004)].

Although precise immunological mechanisms behind the effects of polysaccharide–protein conjugate vaccines remain incompletely understood the widely accepted concept states the following. Upon administration of the vaccine, B cells take up the polysaccharide–protein conjugate. Protein is processed and presented as antigenic peptides to T cells in the context of MHC class II molecules. T cells recognize the antigenic peptides, become activated and differentiate into CD4+ T-helper cells. The latter then provide help to polysaccharide-specific B cells pushing them to proliferate, produce antibody against the polysaccharide, undergo isotype switch, affinity maturation and develop into B-memory cells. These cognate T- and B-cell interactions involve several co-stimulatory molecules, e.g. CD40-CD40L, as well as cytokines, such as IL-6 and TNF-\( \alpha \) (Breukels et al., 1999; Kamboj et al., 2001). Moreover, some components of conjugate vaccines, e.g. the outer membrane protein complex of \( N. meningitidis \) are able to stimulate accessory cells via pattern-recognition receptors, such as toll-like receptors [TLR]. It results in production of additional co-stimulatory signals further activating B cells and enhancing antibody production [adjuvant effect] (Latz et al., 2004). Conjugate vaccines may mimic natural responses to the whole microbial organism where T-cell epitopes are naturally combined with polysaccharides as well as with TLR ligands.

Hence, the major mechanism of action of conjugate vaccines is mediated by the recruitment of T cells to the immune response to polysaccharide antigens. Immunization with Hib conjugate vaccines introduced in 1990s made remarkable effects on invasive Hib disease for the following reasons. (1) T-cell dependence results in high immunogenicity of Hib capsular polysaccharide in infants. (2) Immunological memory develops and hence it becomes possible to booster the response with re-vaccination or re-exposure to the microorganism. (3) As a result of isotype switching, IgG_{1} antibodies are generated that are most efficient in opsonization of bacteria. (4) Elimination of the carriage of Hib in the population leads to removal of source of infection.

Several formulations of Hib conjugate vaccines have been developed that differ in carrier proteins and exhibit certain differences in immunogenicity and efficacy. Vaccines currently used include PRP conjugates with (1) tetanus toxoid [PRP-T], (2) diphtheria toxoid [PRP-D], (3) non-toxic variant of diphtheria toxin CRM197 [HbOC], and (4) \( N. meningitidis \) group B outer membrane protein [PRP-OMP]. Although all these vaccines use PRP, the size of the polysaccharide and the type of linkage with the protein carrier are different. Consequently, there are differences in the immune response that is induced by the immunization. Immunogenicity of these vaccines is routinely evaluated by the level of serum anti-PRP antibodies induced by the immunization: antibody level of 0.15 \( \mu \)g/ml is regarded as an indicator of positive vaccine response, and one of \( > 1 \) \( \mu \)g/ml as an indicator of clinical protection (Anderson, 1984; Peltola et al., 1984). The Hib conjugate vaccine formulations and their efficacy have been discussed in several recent review papers (Kelly et al., 2004; Trotter et al., 2008; Mond and Kokai-Kun, 2008). All of the vaccines are designed to be administered to infants and have demonstrated high efficacy in preventing invasive Hib disease.

Among Hib conjugate vaccines, PRP-D is the least immunogenic in infants and is not currently used. In contrast, PRP-T is highly immunogenic in infants. Based on measurement of antibody levels, PRP-T was found to be the most immunogenic vaccine following administration of three doses [in comparison to PRP-CRM, PRP-OMP and PRP-DJ (Decker and Edwards, 1998)]. HbOC is different from other conjugate vaccine since it contains short oligosaccharides. Despite the difference in the length of the polysaccharide part, HbOC shows similar immunogenicity and efficacy compared to PRP-T. PRP-OMP is a unique vaccine since it induces protective antibody response in 2-month–old infants after a single dose while other conjugate vaccines require at least two injections. The reason for this is that this vaccine contains outer membrane vesicles of \( N. meningitidis \) group B. Components of outer membrane of Gram-negative bacteria are capable of stimulating pattern-recognition receptors, i.e. TLR2 on innate immune cells and therefore elicit potent adjuvant effect (Latz et al., 2004). However, PRP-OMP demonstrates lower immunogenicity during boosting, compared to other conjugate vaccines (Decker and Edwards, 1998).

It is remarkable that 2-month–old infants are capable to develop protective anti-PRP antibody levels following immunization with Hib conjugate vaccines, especially considering that small children respond poorly to Hib invasive disease (Trollfors et al., 1992). This indicates that the conjugate vaccines do not simply mimic natural infection with whole bacteria but provide more immunogenic T-cell epitopes compared to bacterial cells, as well as induce more potent stimulation of the innate immune system due to the adjuvant effect. Such abilities of Hib conjugate vaccines are crucial because they overcome the low responsiveness of small children to polysaccharide antigens.

In most developed countries, Hib conjugate vaccines were licensed in the late 1980s–early 1990s. In the developing world, these vaccines were introduced more recently. Hib conjugate vaccines are commonly administered to infants with a 3-dose schedule starting at 2 months of age, followed with a booster at 11–18 months. The Hib conjugate vaccines are usually administered in combination with other pediatric vaccines, such as diphtheria and tetanus toxoids, pertussis, inactivated polio, and hepatitis B (Trotter et al., 2008).

Routine immunization against Hib with conjugate vaccines caused dramatic decrease in the incidence of the invasive disease in all countries where it has been implemented. For example, in
Canada, prior to the introduction of the conjugate Hib vaccine, i.e. in 1989, the incidence rate of invasive Hib disease was 1.89/100,000. After the introduction of the conjugate Hib vaccine, the incidence rate dramatically decreased to 0.3/100,000 in 2004 (data by Public Health Agency of Canada, 2006). Similar trends have been seen in other countries in North America and Europe where conjugate Hib vaccines are routinely used [reviewed by Morris et al. (2008)].

In particular, in USA, a series of population-based surveillance studies performed in 1970–1980s reported the rates of invasive Hib disease of between 40 and 100/100,000 children <5 years old (Wenger, 1998). Introduction of conjugate Hib vaccine in 1991 caused a decrease in invasive Hib disease of 85–99%; in 2006, the incidence rate was 0.21/100,000 children <5 years old (Morris et al., 2008). However, as discussed below, a significant difference in incidence rates exists between specific populations in USA, especially between North American Indians and the general population.

The effectiveness of immunization is not equal in different populations, and certain groups of children show a reduced vaccine response or even vaccine failure [occurrence of invasive Hib disease despite the vaccination]. In several countries, i.e. UK, Ireland, and The Netherlands, a rise in the incidence of invasive Hib disease was reported several years after the introduction of Hib conjugate vaccines (Rijkers et al., 2003). The reasons for this phenomenon are not fully understood. A recent study found that immunologically competent children who experienced Hib conjugate vaccine failure produced low-avidity antibodies that lacked a protective effect against the disease (Lee et al., 2008). Production of low-avidity antibodies may result from some defect in immunological priming (Lee et al., 2008). It was suggested that a combination of Hib conjugate with acellular pertussis vaccine can reduce the immunogenicity of the Hib component (McVernon et al., 2003). As the vaccination caused a decrease in carriage rate among children, it is also possible that the lack of natural boosting due to reduced circulation of Hib in the community affects the vaccine efficacy (McVernon et al., 2004).

In the UK and Ireland, the omission of a booster dose has been implicated as the major cause of an increased number of vaccine failures in 2000–2002 [reviewed by Kelly et al. (2004)]. At that time, the UK and Ireland were the only countries in Western Europe that did not routinely provide a booster dose of Hib conjugate vaccine for children in their second year of life. Therefore, the lack of natural boosting due to a decreased circulation of Hib in the population or the omission of a booster dose of the vaccine could have caused the resurgence of Hib invasive disease in these countries. Indeed, implementation of a booster campaign in 2003 quickly resulted in a reduction of Hib invasive disease in the UK (Ladhani et al., 2008b).

However, in The Netherlands, the rise in vaccine failures from 2002 onwards cannot be explained by the same factors as in the UK. Indeed, the Dutch vaccination program includes a booster at 11 months of age. In addition, no acellular pertussis vaccine has been used before 2005 (Schouls et al., 2005). Instead, a sharp increase in genetic diversity of Hib strains circulating among small children has been reported after introduction of routine Hib immunization (Schouls et al., 2005). These observations suggest that changes in genetic structure of bacteria may be responsible for the appearance of Hib strains that are successful in evading vaccine-induced immunity, especially in certain groups of children that may have an increased susceptibility to Hib infection (Schouls et al., 2005). Although there was some decrease in the incidence rate of invasive Hib infections in The Netherlands in 2006–2007 compared to 2005, the rates were still higher than during the first years following introduction of the vaccine in 1993 (The Netherlands Reference Laboratory for Bacterial Meningitis, 2008).

Despite the great complexity and lack of complete understanding of mechanisms underlying vaccine failures, it is important to distinguish between failure rates and their causes on a population basis. Indeed, the vaccine failures can be caused by some environmental or associated factors, such as a decrease in natural boosting or interference between different vaccines. Alternatively, a subset of children may have lower responsiveness to the vaccine because of some underlying conditions causing a delay in the maturation of anti-PRP antibody production. Among cases of Hib vaccine failure recorded in Great Britain in 1992–1998, 44% of children had such risk factors as prematurity, malignancy, or immunoglobulin subclass deficiency (Heath et al., 2000). The significance of impaired immune responses is discussed in detail in the next section.

### 6. Increased susceptibility to invasive Hib disease and Hib conjugate vaccine failure in immunocompromised individuals

Hib conjugate vaccine failure can be caused by any defect in the development of protective immunity against Hib that is normally induced by the immunization, such as various congenital [primary] and acquired [secondary] immunodeficiencies. The latter can result from infectious damage to the critical components of immunity [HIV infection], severe underlying conditions affecting the immune system [e.g. lymphoblastic leukemia], or immunosuppressive therapy. Because of the age-dependence of anti-polysaccharide immunity children can be more affected by underlying conditions than adults. Data from a recent study suggest that premature infants may have an increased risk of clinical vaccine failure (Heath et al., 2003). However, severe invasive Hib disease, as well as Hib conjugate vaccine failure may also occur in apparently normal immunocompetent adults as a manifestation of a selective antibody deficiency (Pollin et al., 1997).

Because of the major importance of anti-capsular polysaccharide antibody and opsonophagocytosis in the defence against Hib, any clinical conditions that cause impaired humoral immunity or deficient function of spleen greatly enhance susceptibility to infections caused by this organism.

#### 6.1. Primary immunodeficiencies

Patients with primary immunodeficiencies affecting antibody production, such as Bruton agammaglobulinemia, X-linked hyper IgM syndrome, Wiscott-Aldrich syndrome, X-linked agammaglobulinemia, or common variable immunodeficiency have an increased risk of invasive Hib disease and vaccine failure (Van der Hilst et al., 2002; Overturf, 2003). Because of importance of complement system in killing Hib by lysis and opsonophagocytosis, various genetic defects in the complement system, such as early-phase components in the classical pathway C1–C3 or the alternative pathway [i.e. factor I deficiency] are associated with an increased susceptibility to Hib (Colten, 2002).

#### 6.2. Asplenia

Individuals with complete or partial lack of splenic tissue, most often caused by congenital asplenia or surgical splenectomy, are highly susceptible to systemic infections with encapsulated bacteria including Hib. The spleen has the unique role in clearance of bacteria from the blood by the phagocytic cells in the red pulp. In addition, as discussed above, the marginal zone B cells are critically important in anti-polysaccharide antibody production (Zandvoort and Timens, 2002). Small children with congenital asplenia represent the most vulnerable group for invasive Hib disease
although adults with anatomical or functional [e.g. caused by sickle-cell disease] asplenia also have a greatly increased risk of such infections. However, adults who have anti-PRP antibody prior to splenectomy are less susceptible to the disease than children. In many countries, administration of vaccines against encapsulated bacteria is recommended for asplenic individuals, but adherence to such recommendations varies, and impact of vaccination on Hib invasive disease has not been systematically studied [reviewed by Mourtzoukou et al. (2008)]. Some studies have reported good response of asplenic individuals including children, to Hib conjugate vaccine (Kristensen, 1992; Webber et al., 1993). However, asplenic patients’ IgM responses are impaired even though they may develop normal IgG responses to polysaccharide antigens (Molrine et al., 1999). The response to vaccine following splenectomy also depends on the underlying condition. In adult patients with splenectomy and hematological malignancies, the response to Hib conjugate vaccine is significantly lower compared to patients with posttraumatic splenectomy (Eigenberger et al., 2007).

6.3. HIV infection

HIV infection is associated with invasive *H. influenzae* infections [although not as often as with infections caused by another encapsulated bacteria, *S. pneumoniae*] (Berger et al., 1994; Munoz et al., 1997). Response to Hib conjugate vaccines in HIV-infected children is lower and the risk of vaccine failure is 35 times greater than in uninfected children (Madhi et al., 2002; Madhi et al., 2005). In HIV-infected children, immunological memory is impaired and cannot be restored by repeated immunizations (Spoullou et al., 2003). Nevertheless, high effectiveness of the vaccines in protection against Hib meningitis was reported in a population with high HIV prevalence (Daza et al., 2006). Children treated with highly active antiretroviral therapy [HAART] developed protective antibody after repeat immunizations against Hib, and the antibody persisted in the majority for at least 1 year (Melvin and Mohan, 2003). Some studies reported reduced response to Hib conjugate vaccines in HIV-infected adults, especially in those with low CD4+ T cells counts \( \leq 100 \times 10^6/l \) (Kroon et al., 1997; De Sousa dos Santos et al., 2004).

6.4. Secondary immunodeficiencies

Patients with various clinical conditions that cause impaired humoral immunity are highly susceptible to invasive Hib disease as well as to infections caused by other encapsulated bacteria.

An increased risk for Hib infections associated with failure to produce and maintain protective antibody responses to vaccines was reported in children with acute lymphoblastic leukemia (Feldman et al., 1990; Ek et al., 2004; Brodtman et al., 2005). Such humoral immunological defect can be inherent in the disease itself or due to depletion of lymphocytes as a result of chemotherapy (Brodtman et al., 2005). Reduced response to vaccination was reported in children with solid tumors receiving chemotherapy (Shenep et al., 1994). Nevertheless, a recent study showed that vaccination of children with acute lymphoblastic and myeloid leukemia following completion of standard chemotherapy induced anti-PRP antibody levels >1 µg/ml in 93% of patients (Patel et al., 2007). These data indicate that protection against invasive Hib disease can be achieved in the majority of these children via re-vaccination.

Multiple myeloma and chronic lymphocytic leukemia are also associated with impaired humoral immunity and therefore such patients are highly susceptible to infections caused by encapsulated bacteria including Hib [reviewed by Rolston (2001)]. Reduced response to Hib conjugate vaccine was reported in adult patients with chronic lymphocytic leukemia (Hartkamp et al., 2001; Sinsalo et al., 2002). However, in adult patients with multiple myeloma, response to Hib vaccination was comparable with the healthy controls despite the chemotherapy (Robertson et al., 2000).

Patients experience immunodeficiency, i.e. decreased levels of circulating immunoglobulins, impaired immunoglobulin class switching, and a loss of complexity in immunoglobulin rearrangement patterns, for at least 1 year following allogeneic bone marrow transplantation [BMT]. As a result, BMT recipients have a high risk of infections with encapsulated bacteria, i.e. Hib, and therefore re-vaccination is recommended after BMT (Avigan et al., 2001; Machado, 2004). However, patients surviving 20–30 years after BMT are able to recover both total immunoglobulin levels and anti-PRP antibody without vaccination, likely as a result of natural exposure to these bacteria (Storek et al., 2001). Interestingly, PRP-specific B memory cells from recently immunized donors are capable of persisting in BMT recipients for several months and contribute to the antibody response against Hib (Lausen et al., 2004).

Secondary immunodeficiency associated with an increased risk of invasive Hib disease also occurs in solid-organ recipients, both because of the immunosuppressive therapy and the end-stage organ dysfunction which was the reason for organ transplantation (Avery and Ljungman, 2001).

Patients with chronic renal failure have impaired cell-mediated and humoral immunity due to both the metabolic consequences of the uraemia and the effect of therapeutic interventions such as dialysis and immunosuppression. In addition, the underlying diseases that caused renal failure, i.e. diabetes mellitus and systemic lupus erythematosus, affect immune defence mechanisms [reviewed by Pesanti (2001)].

The resulting secondary immunodeficiency manifests as poor response to vaccination and increased incidence of various infections, including septicaemia [reviewed by Janus et al. (2008)]. Hib conjugate vaccine failures in children with chronic kidney disease, particularly in those receiving peritoneal dialysis have been reported (Fivush et al., 1993; Neu et al., 1996; Laube et al., 2002), as well as cases of *H. influenzae* peritonitis in both pediatric and adult patients on peritoneal dialysis (Neuhaus et al., 1996; Chew et al., 1997).

7. Increased susceptibility of Aboriginal people to invasive *H. influenzae* disease

Some indigenous populations have an increased susceptibility to invasive disease caused by encapsulated bacteria, including *H. influenzae* as well as lower protective effect of immunization. Although the reasons for this are poorly understood, both genetic and environmental factors have been implicated by some studies.

It has been well known since the pre-vaccination era, that some Aboriginal populations have an increased susceptibility to invasive Hib disease. Before the introduction of Hib conjugate vaccines, rates of invasive Hib disease among Navajo and White Mountain Apache children in the south-west United States were 3–5-fold higher than the rates in the general US population and were among the highest reported worldwide (Losonsky et al., 1984; Coulehan et al., 1984). Before 1991, Alaska Native children also experienced one of the highest rates of invasive Hib disease (Ward et al., 1990). In Canadian Arctic, in 1972–1977, an annual incidence of Hib meningitis was significantly higher in Aboriginal populations than in non-Aboriginal children (Wotton et al., 1981). Similarly, in Australia, a significantly higher incidence of invasive Hib disease, as well as earlier onset of Hib meningitis, was reported among Aboriginal children compared to non-Aboriginal children (Hanna and Wild, 1991).
Immunization with Hib conjugate vaccines has resulted in a dramatic decrease in invasive Hib disease in Alaska; however, despite high rates of immunization coverage, Hib disease rates among rural Alaska Native children <5 years of age remain higher than the rates among non-Native Alaskan and other US children (Singleton et al., 2006). Among Navajo and White Mountain Apache children, Hib conjugate vaccines have led to a sustained reduction in both invasive Hib disease and oropharyngeal carriage of this microorganism. However, the incidence of invasive Hib disease among children <2 years of age remained 20 times higher than in the general US population (Millar et al., 2000). In the post-vaccination era, the epidemiology of Hib disease is still markedly different between rural Aboriginal and Caucasian children who live in urban areas. A study by Guthridge et al. (2000) showed that Aboriginal children contract invasive Hib disease earlier than non-Aboriginal urban children [at the age of 6 months versus 12 months]. The Aboriginal children had lower pre-immunization levels of maternal anti-PRP antibody which was consistent with early onset of invasive Hib disease. Caucasian children had significantly higher mean antibody levels in response to booster vaccination and a significantly higher percentage of good responders than Aboriginal children. This study suggests that the difference in epidemiology of invasive Hib disease between Aboriginal and non-Aboriginal children may be due to a difference in both the environment and the genetics (Guthridge et al., 2000). A recent study reported that higher rates of invasive Hib disease persisted in Australian Aboriginal and Torres Strait Islander children in 2003–2006 despite the successful vaccination program delivery (Menzies et al., 2008).

The ability to respond to Hib conjugate vaccine with a robust antibody production has a genetic component and has been found to be lower in some Native American populations compared to Caucasian populations (Siber et al., 1990; Ward et al., 1990; Santosham et al., 1992). A polymorphism in the VKa2 gene encoding the variable part of the k light chain of antibody to PRP was detected in Navajos (Feeney et al., 1996). Since 60% of total anti-PRP antibody repertoire is encoded by this gene, and these antibodies exhibit a high avidity, such allelic polymorphism may play a role in an increased susceptibility to invasive Hib disease as well as in less effective antibody response to the vaccine in some Aboriginal populations.

Hence, in certain populations, both immunogenetic factors and acquired immunodeficiencies may have a significant impact on the results of immunization programs as well as on the epidemiology of invasive Hib disease in the post-vaccine era. Therefore, in the process of developing and testing new vaccines, it is important to consider such non-responding or low responding groups. Presence of non-responders in the population can also have an effect on the estimation of the percentage of vaccinated individuals required to achieve herd immunity.

8. Epidemiology of H. influenzae infections

To understand the changing epidemiology of invasive H. influenzae disease, a brief review on the carriage, followed by a review of invasive diseases caused by all H. influenzae isolates regardless of their encapsulation status before and after introduction of Hib vaccination, will be described below.

8.1. Transmission and respiratory tract colonization

In unvaccinated population, 3–5% of infants and 8–12% of preschool children are colonized in the nasopharynx by type b strains with higher rates in day-care centres (Peerbooms et al., 2002). Higher carriage rates have also been found among household members of a case of invasive Hib disease (Campbell et al., 1980). By 5 years of age, most children would have been colonized at some point by Hib (Mpairwe, 1970; Michaels et al., 1975, 1976). Pharyngeal carriage of Hib in preschool children attending day-care centres and school-aged children serve as a source of infection.

Non-typeable H. influenzae frequently colonizes the nasopharynx of healthy children, and essentially every child is colonized at some point. Non-typeable H. influenzae also colonizes the upper respiratory tract of healthy adults and adults with COPD (Murphy et al., 1999). Colonization of the upper respiratory tract of adults with COPD by new strains of NT H. influenzae is associated with the occurrence of an exacerbation (Sethi et al., 2002).

8.2. Invasive H. influenzae disease before introduction of the Hib conjugate vaccine

Before vaccination against H. influenzae was introduced, most invasive diseases were caused by Hib. In both the US and UK, 93–96% of all invasive H. influenzae recovered from children were typed as Hib (Mason et al., 1982; Falla et al., 1993). In both countries, Hib was the most common cause of pyogenic meningitis in children, especially those 3–18 months of age; and most invasive diseases occurred in children less than 5 years old (Shapiro and Ward, 1991). In the US, incidence rate of invasive Hib disease prior to introduction of Hib vaccination was as high as >300 cases per 100,000 children and the rates among the Native Alaskans were even higher (Ward et al., 1986). In the Oxford region in the UK, the incidence of Hib disease in children <5 and <10 years of ages were 35.5 and 17 cases per 100,000 per year, respectively (Falla et al., 1993). In contrast to the disease incidence in children, the annual incidence of invasive Hib disease in adults >18 years of age was only 0.85 cases per 100,000, and many had chronic lung disease, HIV infection, and other immunocompromising conditions (Farley et al., 1992). Hence in the pre-Hib vaccine era, invasive H. influenzae disease was predominantly caused by Hib and affected mainly children under the age of 10.

Practically all of the non-Hib strains recovered from invasive disease cases in children involved NT strains, 6% in one study (Mason et al., 1982) and 7% in another (Falla et al., 1993). In each of the above studies, only one strain of H. influenzae serotype f was identified among the 346–413 H. influenzae strains typed; and there were no other serotypes identified. In a small study involving adults (Farley et al., 1992), 2.5% of all invasive H. influenzae isolates were serotype f strains, 47.5% were NT strains, and the remainder were Hib strains. The incidence of NT H. influenzae invasive disease in children <10 years of age (and excluding neonates) was only 0.5 per 100,000 (Falla et al., 1993), while in adults, the incidence was estimated to be 0.81 per 100,000 (Farley et al., 1992).

8.3. Invasive H. influenzae disease in the post-Hib conjugate vaccine era

With the introduction of conjugate vaccines, incidence of Hib disease has declined rapidly and significantly in those countries which have incorporated conjugate Hib vaccine into their routine immunization programs. In both the US and Canada, even in children <5 years of age, Hib now accounts for only a very small proportion of all invasive disease cases. For example, among 522 invasive isolates of H. influenzae submitted to the Illinois Department of Public Health between 1996 and 2004, only 78 (14.9%) belonged to Hib (Dworkin et al., 2007). Also of the 111 cases of invasive H. influenzae diseases in children <5 years old, only 28 cases (or 25.2%) were due to Hib. In Manitoba, Canada, of the 122 isolates of H. influenzae from invasive disease...
cases, only 5 or 4.1% were Hib; and among the 51 cases of invasive *H. influenzae* diseases that occurred in children <5 years old, only 4 or 7.8% were due to Hib (National Microbiology Laboratory's unpublished data). As a result of the dramatic reduction of Hib disease in children, potential elimination of Hib disease among infants and children appears to be possible (Bisgard et al., 1998; Bath et al., 2002).

Instead of caused by Hib, most of the invasive *H. influenzae* diseases are now caused by NT *H. influenzae* strains (Dworkin et al., 2007; Tsang et al., 2007). This finding in North America appears to be confirmed by data from the EU where incidence rates of invasive *H. influenzae* disease due to NT *H. influenzae* strains exceeded that of Hib or all of the encapsulated strains combined for the period of 2000–2004. According to the data from the EU, incidence rates of Hib during the period of 2000–2004 were 1.11, 1.34, 2.27, 2.14, and 1.50 per million population. The incidence of non-typeable *H. influenzae* for the same period of time was reported to be 2.33, 2.52, 1.99, 2.65, and 2.33, respectively (Ladhani et al., 2008a,b). Although invasive diseases due to non-Hib encapsulated strains as well as NT *H. influenzae* have not reached the prevalence of Hib disease in the pre-Hib vaccine era, nevertheless their numbers are disturbing and seem to have increased in the past years. Furthermore, the age of the patients suffering from invasive *H. influenzae* disease has also shifted from children (under 12) to adults, neonates and those over 65 years of age (Dworkin et al., 2007; authors’ unpublished data). In the study by Dworkin et al. (2007), the incidence rate of invasive *H. influenzae* disease was highest in those <5 and in those ≥65 years old, giving incidence rates of about 2.75 per 100,000 and about 3.75 per 100,000, respectively in 2004.

A recent publication confirmed that in post-vaccination era, there is an increased incidence of *H. influenzae* invasive disease among Aboriginal populations in Northern Canada (Degani et al., 2008). According to this study, annual incidence rates for *H. influenzae* disease during 2003–2005 were 4-fold higher among Aboriginal compared to non-Aboriginal persons in the Canadian Arctic. Some recent papers have also indicated that there is a trend of an increasing incidence of invasive non-type b *H. influenzae* disease, particularly type a, in the post-vaccination era in Canada and United States, with the highest prevalence among Aboriginal populations (Jin et al., 2007; Millar et al., 2005; Tsang et al., 2006, 2007; McConnell et al., 2007; Degani et al., 2008; Bruce et al., 2008).

### 8.4. Diseases caused by *H. influenzae* serotype a [Hia] and serotype f [Hif]

When Margaret Pittman described the 6 serotypes of *H. influenzae*, she noted that a small percentage of diseases were caused by Hia and Hif (Pittman, 1931). Both Hia and Hif had subsequently been shown to resist antibody-free complement-mediated bacteriolysis more than Hic, Hid, and Hie strains (Sutton et al., 1992). However, most of the invasive Hia disease described in the literature occurred in the Aboriginal population (Hammitt et al., 2005; Millar et al., 2005; Bruce et al., 2008). This might be related to an increased susceptibility of the Aboriginal populations in North America to invasive Hib disease compared to the general American population (Losonsky et al., 1984; Singleton et al., 2000).

Although invasive diseases due to Hif have been reported with increasing frequency (Slater et al., 1990; Urwin et al., 1996) and the spectrum of disease caused by Hif is similar to that of Hib (Frayha et al., 1996; Zacharisen et al., 2003), the patients involved are different from those affected by Hib. Unlike Hib which affects mainly children, many patients with invasive Hif disease are adults, and with underlying conditions (Urwin et al., 1996; Bruun et al., 2004; Taube et al., 2006). Also, despite a relative increase in the number of invasive Hif isolates, the incidence of disease has only increased slightly and occurs only as a small fraction of the magnitude of Hib disease in the pre-Hib vaccine era.

### 9. Molecular typing and evolution of invasive *H. influenzae* disease

Pulsed-field gel electrophoresis provided evidence to support the hypothesis that the source of invasive *H. influenzae* originates from the nasopharynx of carriers since paired isolates obtained from the nasopharynx and blood or CSF of patients gave identical DNA fingerprints (Saito et al., 1999). With the decline in Hib disease in those countries with a routine Hib vaccination program, a relative increase in non-Hib disease is observed. Using PFGE or MLST, encapsulated non-Hib strains collected in the post-Hib vaccine era have been found to be mainly clonal and of limited genetic diversity (Omikunle et al., 2002; Sill et al., 2007). In contrast, based on an analysis of 520 Hib strains isolated from patients with invasive disease in The Netherlands 10 years before and 9 years after the introduction of the Hib conjugate vaccine, a sharp increase in the genetic diversity of Hib strains was observed among the Hib strains isolated in the post-Hib vaccine era from neonates to those <4 years old (Schouls et al., 2005). However, a more sensitive method of MLVA was used in the later study. Finally, unlike the encapsulated serotypeable strains, NT *H. influenzae* are very heterogeneous in their genetic background and no particular clone(s) predominant among the strains analysed suggesting that the increase in disease caused by NT *H. influenzae* in the post-Hib vaccination era is not due to proliferation of one or a small number of virulent clones (Bruit et al., 2003; Sacchi et al., 2005; Erwin et al., 2008). These pathogenic strains of NT *H. influenzae* probably did not arise from their serotypeable encapsulated counterparts. Their increase in invasive disease association in the post-Hib vaccine era is probably a result of replacement of Hib strains by NT isolates due to unmasking as described by Marc Lipstich (1999). The emergence of NT *H. influenzae* as a major pathogen in the post-Hib vaccine era may be enhanced by the supragenome known to exist among NT strains.

### 10. Future outlook on invasive *H. influenzae* disease

The Hib conjugate vaccines can be regarded as one of many success stories in the history of vaccinology, with near elimination of Hib carriage and disease among children in many industrialized countries which have routine Hib vaccination programs, even among the high risk American Indian children (Millar et al., 2000). However, diseases due to non-type b strains are becoming relatively more frequent than before. Although the magnitude of disease caused by non-type b *H. influenzae* is still far from the burden of disease caused by Hib in the pre-Hib vaccine era, reports of outbreaks of infections due to NT *H. influenzae* have appeared in recent literature (Hershkowitz et al., 2004; Van Dort et al., 2007; Gransden et al., 2007). In order to monitor the evolving nature of invasive *H. influenzae* disease, carefully documented surveillance data is required to capture the true magnitude of the problem, including who is now vulnerable to the non-type b *H. influenzae*. Newer vaccines for control of all *H. influenzae* strains, including the NT strains, are now under clinical trials (Prymula et al., 2007; Forsgren et al., 2008). Complete surveillance data is required in order to define the patient population and the potential cost-benefits of using these newer vaccines.

In summary, in the post-Hib conjugate vaccine era, non-Hib *H. influenzae* strains are the major cause of invasive *H. influenzae*
In certain patient populations, Hia and Hif are becoming invasive disease. In certain patient populations, Hia and Hif are becoming invasive disease. In certain patient populations, Hia and Hif are becoming invasive disease. In certain patient populations, Hia and Hif are becoming invasive disease. In certain patient populations, Hia and Hif are becoming invasive disease.


