Comparison of Virulence Determinants of Different Strains of *Haemophilus influenzae*.

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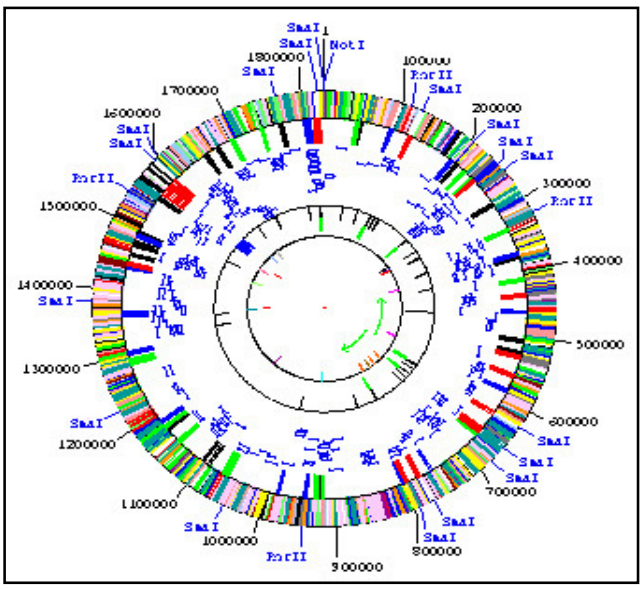
INTRODUCTION

Haemophilus influenzae is a small, nonmotile Gram-negative coccobacillus which was first described in 1982. It is generally aerobic but can grow as a facultative anaerobe too. *Haemophilus influenzae* was mistakenly considered to be the causative agent of the common flu, until the discovery of the influenza virus in 1933 as the causative agent; however, *Haemophilus influenzae* is still responsible for causing many other diseases. Encapsulated strains of *Haemophilus influenzae* isolated from cerebrospinal fluid are coccobacilli, 0.2 to 0.3 to 0.5 to 0.8 µm. Non-encapsulated organisms from sputum are pleomorphic and often exhibit long threads and filaments. The organism may appear Gram-positive unless the Gram stain procedure is very carefully carried out (16). Furthermore, as far as molecular analysis is concerned, *Haemophilus influenzae* was the first free living organism whose genome was completely sequenced by scientists, containing



1,830,137 base pairs of DNA in a circular chromosome which has 1740 protein-coding genes and 58 tRNA and 18 other RNA genes. (Figure 1)

FIG 1: The genome of *H. influenzae* (16).

Haemophilus influenzae is a part of normal bacterial flora of the upper respiratory tract, especially oropharynx and nosopharynx in almost 90% of the adult population, they are opportunistic pathogens that usually live in the host body without causing any harm or diseases and wait for an opportunity such as a viral infection or reduced immunity (8, 18, 21). Studies show that most of isolates sampled from the oropharynx of adults are non-encapsulated strains of *Haemophilus influenzae*, although encapsulated strains of *Haemophilus influenzae* can be isolated in the normal upper respiratory tract of both adults and children. There are 6 different encapsulated serotypes of *Haemophilus influenzae* (a, b, c, d, e and f), the most virulent form of which is *Haemophilus influenzae* type b (18, 20). In infants and small children *Haemophilus influenzae* causes bactraemia, epiglottitis, otitis media, acute pharyngitis bronchitis pneumonia, acute bacterial meningitis, endocarditis, and conjunctivitis. Generally, *Haemophilus influenzae* infections seem to occur just in human.

Currently, with the availability of the Hib conjugate vaccine, the incidence and subsequently the prevalence of the disease has dramatically dropped; but, according to scientists, Hib still remains a major causative agent of lower respiratory tract infection in young children, mostly in third world countries in which the vaccine is not routinely applied.

Early studies revealed that all isolates of *H. influenzae* are different in terms of pathogenic potential. It is very important to mention that most systemic isolates express the type b capsule, whereas most respiratory tract isolates contain unencapsulated, referred to as non-typeable (12, 18). The fact that most of the *Haemophilus influenzae* diseases are caused by *Haemophilus influenzae* type b and non-typeable strains, makes it essential to know and compare the virulence factors of the *Haemophilus influenzae* strains in order to better understand which virulence factor or factors are most responsible for establishment of the disease. The purpose of this paper is to review and compare the virulence determinants of *Haemophilus influenzae* strains.

There are several main virulence factors in *Haemophilus influenzae* such as: capsule, fimbrial adhesin, HMW1 and HMW2, Hap Adhesin, Hia and Hsf Adhesins, Opacity-associated Protein A (OapA), Haemocin, IgA protease, Lipooligosaccharide, Outer Membrane Proteins and Protein D (18, 21). (***see table 1***). To better understand the characteristics of these virulence factors, each of them will be briefly explained below.

CAPSULE

All of the 6 encapsulated strains of *Haemophilus influenzae* have capsule as a virulence factor. The capsule is composed of polysaccharide containing two hexose sugars as subunit carbohyhdrates. But the type b polysaccharide is the only capsular type which has two pentose monosaccharide instead of the previously mentioned hexose sugars in other serotypes (18, 21). Also, the type b capsule is polyribosyl-ribitol-phosphate (PRP) which is composed of linear teichoic acid containing ribose, ribitol - an alcohol containing a five carbon sugar- and a phosphate linked by phosphodiester bonds and is a critical determinant of virulence. According to scientists, even though the virulence of type b *Haemophilus influenzae* is multifactorial, still the PRP capsule has a significant role in this

regard. This capsule helps *Haemophilus influenzae* to avoid phagocytosis and opsonization by neutrophils. Moreover, *Haemophilus influenzae* capsule does not induce alternative complement pathways; therefore, the bacterium invades the blood or CSF without aggravating an inflammatory response and attracting phagocytes (13, 16, 20, 21).

The genetic base of the production of the PRP capsule is multifaceted and engages both capsular and non-capsular genetic determinants. The genes involved in the expression of *Haemophilus influenzae* type b capsule are present as a duplication of an approximately 18-kb DNA segment (the Cap b locus). The only strain of *Haemophilus influenzae* that contains this duplicate-gene arrangement is *Haemophilus influenzae* type b, while other encapsulated serotypes only contain a single copy of the gene. Some recent evidence show that even capsule-deficient type b *Haemophilus influenzae* has also a single copy of the gene *capB* and is, therefore, less virulent than the normal type b (18, 21). Therefore, *capB* gene amplification is undertaken only in normal non-deficient type b *Haemophilus influenzae* stain and is one of the characteristics of this highly virulent strain. Further studies show that this cap b locus consists of three functionally distinct regions, named regions 1, 2 and 3. Region 3 contains the *hcsA* and *hcsB* genes. If *hcsA* is inactivated, it will lead in a partial decrease in surface-associated polysaccharide and an increase in the amount of polysaccharides in the periplasm, while the outcome of the inactivation of *hcsB* alone or of both *hcsA* and *hcsB* is a total loss of surface-associated polysaccharide and the gathering of polysaccharide in the periplasm. This implies that *hcsA* and *hcsB* genes are complementary in the transport of polysaccharide across the outer membrane and to the cell surface and are essential for virulence, (11, 13). It is worth noting that the process of encapsulation of type b organism is directly related to a decrease in the ability of the microorganism to adhere or to invade the host cells. In this regard, some mutations causing the loss of a copy of the duplication at the cap B locus result in capsule-deficiency that results in an increase in bacterial ability to adhere (50-fold) and invade (300-fold) the host epithelial cells. Meanwhile, the re-establishment of the encapsulation – in mutated strains – by transformation, results in a considerable reduction in both adherence and invasion of type b serotype. The capsule of *Haemophilus influenzae* strains, especially type b PRP, has been the basis of vaccine development for many years.

FIMBRIAL ADHESION

Fimbriae are the colonization factors of the *Haemophilus influenzae,* meaning they mediate the bacterial adherence to human cells. Fimbriae are found on type b encapsulated and unencapsulated strains of *Haemophilus influenzae*. The adherence of *Haemophilus influenzae* to human mucosal and epithelial cells plays a significant role in infections and fimbriae play a considerable role in adhesion of the microorganism to host cells (9).

Some fimbriae facilitate the haemagglutination and adherence of the microorganism to human mucosal cells, especially to oropharyngeal epithelial cells, but on the other hand, they inhibit the mucosal cell invasion. The fimbriae of *Haemophilus influenzae* are composed of a 24

kDas major protein (HifA), two minor proteins of 20.6 kDa (HifD) and 45.5kDa (HifE) molecular weight. HifE has the ability to haemagglutinate and adhere to epithelial cells, and is located on the tip of the *Haemophilus influenzae* fimbriae. Any mutation in the *HifE* gene results in a reduced ability of the microorganism in haemagglutination and adherence. In fact, fimbriae facilitate adherence to human cells, by binding to glycoproteins and glycolipids present on the respiratory mucin proteins (21). The gene for these proteins (*HifA, HifD and HifE*) exist as a single-copy cluster together with two other genes *(HifC and HifB)* whose function is to code for proteins responsible in fimbrial assembly and protection of nascent fimbrial protein from degradation upon exporting from the cell. In a study by Van Ham and his collegues, it was shown that for *Haemophilus influenzae* fimbriae to be able to adhere to epithelial cells, major subunits are more important than minor subunits. Cloning of distinct *Haemophilus influenzae* fimbrial genes in an *E. coli* strain showed that minor *Haemophilus influenzae* subunits are not necessary for the adherence, because the adhesive domain is located on the major subunit gene-*HifA* (17, 21). The study also revealed that in *Haemophilus influenzae* itself, the minor subunits may impact the adherence of *Haemophilus influenzae* by increasing the number of fimbriae above the minimum level which is required to cause adherence. On the other hand, fimbriae composed of another protein called P5-fimbrin, have been indentified on non-typeable *Haemophilus influenzae* strains. P5-fimbrin is a 36.4 kDa protein which has a sequence homology with the P5 outer membrane protein (OMP) which will be discussed later. This protein also has the adherence capability to human cells. Although this protein shows a huge interstrain heterogeneity between different non-typeable strains, there are still some conserved regions in this protein that have been nominated as candidate vaccine antigens.

HMV1 AND HMV2 ADHESINS

HMW1 and HMW2 are the major virulence factors of non-typeable *Haemophilus influenzae* strains; they are high-molecular weight, non-pilus adhesins that were initially identified as the main targets of the human serum. The function of HMW1 (160 kDa) and HMW2 (155 kDa) is adherence to host epithelial cells. These adhesive proteins are present in almost 80% of non-typeable *Haemophilus influenzae,* (21, 22), but are absent from typeable strains (18). The genes encoding these high-molecular weight adhesins proteins are also arranged in clusters on the chromosomes of the microorganisms along with the other genes whose function is to code for proteins that are responsible for exporting, localization, and activation of these larger molecules. Non-typeable *Haemophilus influenzae* strains which cause otitis media and nasopharyngeal infection have a considerable amount of these two adhesive proteins (HMW1 and HMW2). Some of the non-typeable strains of *Haemophilus influenzae,* that do not express these two proteins, either express Hia protein, discussed below, or heamagglutinating fimbriae discussed above. Interestingly, the isolates of *Haemophilus influenzae* sampled from different anatomic sites of human body (e.g. ear in otitis media or lung tissue in chronic obstructive pulmonary diseases), show different levels of HMW genes expression.

Although HMW proteins are heterologous among different stains, there are still some conserved homologous regions present in these proteins. These large proteins express some surface-exposed epitopes that can be useful for establishment of recombinant peptide-based vaccines for non-typeable *Haemophilus influenzae* (18, 21, 22).

HAP ADHESIN

Hap is a 155 kDa protein whose function is adherence to and invasion of epithelial cells. This non-fimbrial adhesin has both adhesive and protease activities and is associated with but different from IgA protease (discussed below) of *Haemophilus influenzae* (18, 21). Hap adhesin can be synthesized, secreted and can suffer an autoproteolytic cleavage when adhesive domains are released from the cell surface. Moreover, Hap autotransporter is a non-pilus adhesin whose function is adherence to epithelial cells and selected extracellular matrix proteins and mediating bacterial aggregation and microcolony formation. This adhesive protein has a 110 kDa extracellular internal passenger domain called Hap[S] with adhesive and protease activity and one 45 kDa outer membrane domain Hap[β] whose function is presentation of Hap[S] on the cell surface (2, 4). Because this molecule has a proteolytic activity, it can act better after cell invasion. The interaction of Hap adhesin molecule with collagen, fibronectin and laminen inhibit this proteolytic activity and facilitates the adherence of the microorganism to mentioned proteins (21). Interestingly, all genes encoding the adhesive activities of Hap adhesin, are located on the C-terminal of Hap[S], while the genes coding for protease activity of this molecule are located on a separate module of the protein. This, certainly, demonstrates the importance of this portion of Hap adhesin which definitely attracted the attention of vaccine scientists. In fact, it has been shown that nasopharyngeal colonization of non-typeable *Haemophilus influenzae* strain could be inhibited by intranasal immunization of mice with Hap adhesin molecule (4).

HIA AND HSF ADHESINS

These homologous proteins are high-molecular weight proteins that facilitate the adherence of the microorganism to host cells (18, 21). Hia (115 kDa) is another fimbrial protein found on those non-typeable strains of *Haemophilus influenzae* that do not express either HMW1 or HMW2. After synthesis and secretion, the Hia fimbrial protein tends to remain associated with the bacterial cell membrane. Hsf, on the other hand, is a homolog of Hia that is unanimously present among typeable *Haemophilus influenzae* strains including type b and non-type b strains (18). It is one of the major virulent factors of type b strain of *Haemophilus influenzae,* structurally similar to Hia protein and exists on the cell surface as short fiber-like structures and is different from fimbriae. The analyses of predicted amino acid sequence of Hsf revealed that there are three regions with high rate of homology to Hia binding domains (HiaBD1 and HiaBD2) in this protein. These analyses were based on the examination of glutathione *S*-transferase fusion proteins corresponding to these regions. Two of three of these regions had adhesive activity while the third was not adhesive when examined in an epithelial cell culture. These two adhesive regions showed an acidic binding pocket like the binding pocket previously recognized in the first binding domain of Hia (HiaBD1), interestingly while the acidic binding pocket was disrupted the adhesive activity was

also disrupted (3, 18). This shows that the acidic binding pocket is very important in the adhesive activity of Hsf in type b strain of *Haemophilus influenzae.*

OPACITY-ASSOCIATED PROTEIN A (OAPA)

This adhesin protein is the cause of the opaque colony morphology of *Haemophilus influenzae* on a transparent growth medium. This light-molecular weight protein (47 kDa) is found in all strains of *Haemophilus influenzae*. OapA facilitates the adherence of microorganism to epithelial cells in cell culture and is one of the key elements for pharyngeal colonization (19, 21). Opacity associated proteins are a type of membrane proteins found in *Haemophilus influenzae* that are useful as immunogens in designing vaccine candidates against infection by *Haemophilus influenzae*.

HAEMOCIN

One of the most important virulence factors of type b *Haemophilus influenzae* strain is haemocin (HMC). This small heat-stable protein is a type of bacteriocin produced by over 90% of type b *Haemophilus influenzae* strain and its function is to inhibit the growth of other bacteria belonging to the same or similar species in the site of the infection (21). In fact, haemocin is produced by most type b strains of *Haemophilus influenzae*, including strains determined to be genetically diverse, and is toxic to virtually all non-type b strains of *H. influenzae,* both encapsulated and non-encapsulated.

Non-type b encapsulated *Haemophilus influenzae* strains and non-typeable *Haemophilus influenzae* strains can not produce this lethal protein, but are highly susceptible to its lethal activity. This is why mostly type b *Haemophilus influenzae* can compete with non-typeable strains in nasopharyngeal colonization and infection. Analysis of the amino acid sequences of numerous genes of the previously identified HMC immunity gene (*hmcI)* indicate that there are several features common to class II bacteriocins of Gram-poistive bacteria. Mutation in the open reading frames immediately upstream of *hmcI* results in a complete failure in production of haemocin (7). In fact, HMC-producing strains of *Haemophilus influenzae* can invade the cells much earlier than the HMC-deficient isogenic mutants.

IGA PROTEASE

More than 97% of non-typeable strains of *Haemophilus influenzae* possess IgA protease (21). This molecule, as mentioned earlier, is associated with, but different from, Hap adhesin which share some sequence homology with this molecule. These two genes are highly similar and are of the major virulence factors and pathogenicity determinants of non-typeable strain of *Haemophilus influenzae*. There is a very considerable genetic polymorphism and mosaic-like pattern between these two genes, also it seems that *Hap* and *iga* are two paralogous genes that code for an adhesive and penetrative protein (Hap) and the protease activity of IgA protese. IgA protease produces IgA1 that inactivates human immunoglubolin A1. this immunoglubolin constitutes more than 90% of the IgA present in the nosopharynx. Recently, it was discovered that even though almost all non-typeable strains of *Haemophilus influenzae* have IgA protease, it seems that protease activity in strains isolated from throat swabs of an asymptomatic carrier is lesser than in clinical isolates from CSF, blood and sputum (2, 21).

LIPOOLIGOSACCHARIDE

All *Haemophilus influenzae* strains have an outer membrane-associated lipopolysaccharide (LPS) that contains lipid A connected via 2-keto-3-deoxyoctulosonic acid (KDO) to a core polysaccharide polymer consisting of neutral monosaccharides (21). But this LPS differs from that of enteric pathogens because it does not have the repeating terminal side chain which is the somatic antigen commonly known as antigen "O". Consequently, lipooligosaccharide is a better name for the LPS of *Haemophilus influenzae*. A number of surface molecules, such as adhesins including a structure found in LOS, contribute to the colonization of *Haemophilus influenzae* in the airway.

Furthermore, LOS lipid A has all of the endotoxic activities of LPS lipid A: mitogenicity, pyrogenicity in rabbits, aggregation of platelets, and the lethality in the mouse endotoximia model. Meanwhile, the important sugars in LOS – glucose, galactose, glucosamine, and heptose – are different in their composition and quantity between the different *Haemophilus influenzae* strains and among a specific strain (14, 21). Any changes in the amount of short genomic four-nucleotide sequences (5'-CAAT-3') causes variations in the LOS phase at the genetic level resulting in shifts in codon or anticodon reading frames, meaning small mutations cause variation in LOS phase in genetic level.

Moreover, some of the LOS found in most of *Haemophilus influenzae* strains are sialylated. Because the oligosaccharide of human glycolated sphingolipids are sialytated, those *Haemophilus influenzae* sialytated LOS are structurally and antigenically similar to these human glycolated sphingolipids. Therefore, any modification of microorganism's LOS allows the organism to evade opsonization and phagocytosis by being able to copy the molecular structures usually found in the hosts. Non-typeable Haemophilus influenzae attacks the host cell by binding to PAF (platelet-activating factor) receptor via their LOS glycoforms that contain phosphorylcholine (ChoP) (4, 5). For its ability to attract opsonization and phagocytosis, *Haemophilus influenzae* LOS has also been investigated as a potential antigen for non-typeable *Haemophilus influenzae* vaccine development. In fact, studies show that detoxified LOS that is conjugated to either *Haemophilus influenzae* outer membrance proteins or tetanus toxoid is immunogenic (17).

OUTER MEMBRANE PROTEINS (OMPS)

All strains of *Haemophilus influenzae* have outer membrane proteins on their cell outer membrane. OMPs are composed of many types of proteins, from which P2, or the major protein (38-40 kDa), comprises more than 50% of the OMPs. This protein exists on the outer membrane as a trimer and acts as a prion, so it is mostly located on the outer membrane of non-typeable *Haemophilus influenzae* strains. Also, P2 and P6 OMPs have been investigated and proven as good potential vaccine antigens. In fact, antibodies produced against P2 have a considerable bactericidal activity and are, therefore, protective against *Haemophilus influenzae* infection in animal models and in human when infected by a non-typeable *Haemophilus influenzae* strain, (21). The bioinformatics and biological sequence-analysis of purified P2 protein shows that the portions of this protein which are inside the outer membrane have conserved amino acids in their sequences; in contrast, amino acid analysis of the surface-exposed portions of this protein shows that this portion is highly variable and lacks conserved regions between different strains. Moreover, the exposed P2 proteins isolated from respiratory samples have the capability to change over time because of sequential base changes in P2 structural genes.

The genetic loci coding for P2 protein actually do not experience many mutations and genetic variations in coding sequences, but rather areas where repetitive sequences are located might show some variations (6). Therefore, to consider the P2 protein as a candidate of vaccine development, attempt should be made to consider the conserved regions of this OMP protein instead of the variable portion, and it is necessary to study and analyze P2 OMPs in different *Haemophilus influenzae* strains to identify epitopes that are immunologically and structurally conserved. Interestingly, studies focused on animal models, have revealed that some antibodies produced against P2 protein target a single surface-exposed loop in this protein, any changes in this region would eliminate the immunological property of the microorganism that has this recognized-P-phenotype (6). This phenotype is displaced as the time passes altering the P2 phenotype of the microorganism. This can also partially explain why sometime the infection with non-typeable *Haemophilus influenzae* stain remains chronic.

On the other hand, another important OMP, P6 protein, can be found in both typeable and non-typeable *Haemophilus influenzae* strains. P6, a peptidoglycan-associated lipoprotein, constitutes 1-5% of all OMPs and is a light-weighted protein of 16.6 kDa. Outer membrane protein P6 of non-typeable *Haemophilus influenzae* is a potent immunomodulator and selective inducer of human macrophage proinflammatory cytokines (1). Antibodies produced against this protein have bactericidal activity in animal models and can induce both P6-specific humoral and cell mediated immunity. Unlike P2 protein, P6 protein show a very high homology (97%) in the amino acid analyses of type b and non-typeable *Haemophilus influenzae* strains, which shows that this protein is stable and conserved (21). Because P6 have a conserved nature, the bactericidal antibodies produced against this protein have a long-term efficacy and can remain in human serum for long time; therefore, efforts are focused on inclusion of P6 OMP as a potential candidate vaccine against non-typeable *Haemophilus influenzae* strains.

PROTEIN D (PD)

This light-weighted (42 kDa) and highly conserved surface lipoprotein can be found in all types of *Haemophilus influenzae* strains, especially in non-typeable strains of *Haemophilus influenzae*. The main function of this protein is damaging the ciliary function in human nosopharyngeal tissue and is, therefore, involved in the pathogenesis of upper respiratory tract infection (5). The most probable mechanism of its virulent characteristics is its glycerophos-phodiesterase activity, which in turn pilots the release of phosphorylcholine from human epithelial cells. The scientists discovered that this protein has shown to be one of the most capable vaccine candidates against non-typeable *Haemophilus influenzae* strain. In fact, rats that have been vaccinated by PD-based vaccine have cleared non-typeable *Haemophilus influenzae* upon challenge. Moreover, the cases of *Haemophilus influenzae*-dependent acute otitis media in human have dropped significantly after PD vaccine application. In a clinical trial involving children, protein D was utilized as an antigenically-active-carrier protein in an 11-valent pneumococcal conjugate experimental vaccine; considerable protection was achieved against acute otitis media not only caused by pneumococci but also caused by non-typeable *Haemophilus influenzae* (10). Therefore, protein D is one of the most important virulence factors of *Haemophilus influenzae* that can successfully be used to design a vaccine against *Haemophilus influenzae* and prevent at least one of the most significant diseases caused by this microorganism in human, otitis media.

TABLE 1: The summary table comparing virulence factors of Haemophilus influenzae in different strains.

Virulence factors	Functions	Molecular Properties	Type a	Type b	Type c	Type d	Type e	Type f	Non-typeable
Capsule	The most potent virulent factor, resist phagocytosis and opsonization, encapsulation decrease the adherence and invasion capbility of the microorganism	Non-type b: polysaccharide sugar contaning hexose as subunits. Type b: two pentose sugar instad of hexose.polyribosyl-ribitol-phosphate (PRP)	+	+	+	+	+	+	–
Fimbrial Adhesin	Mediates haemagglutination and adherence to human mucosal cells, however it inhibits mucosal cell invasion.	One major protein (HifA) 24 kDa, and two minor proteins of 20.6 kDa (HifD) and 45.5kDa (HifE)		+					30-40%
HMW1 and HMW2	Mediate adherence to epithelial cells.	HMW1 (160 kDa) protein HMW2 (155 kDa) protein	–	–	–	–	–	–	70-80%
Hap Adhesin	Adhesion to epithelial cells and extra cellular matrix proteins, mediates bacterial aggregation and microcolony formation.	155 kDa protein, a110 kDa Hap[S], 45 kDa Hap[β], C-terminal of Hap[S] is the most important part of the molecule	+	+	+	+	+	+	+
Hia and Hsf Adhesins	Mediate adherence to epithelial cells.	Hia (115 kDa), Hsf has a high rate sequence homology with Hia Binding domains HiaBDs	Hsf	Hsf	Hsf	Hsf	Hsf	Hsf	Hif
Opacity-associated Protein A (OapA)	Mediates adherence to epithelial cells in culture and is required for pharyngeal colonization.	(47 kDa) membrane proteins	+	+	+	+	+	+	+
Haemocin	Inhibits the growth of other encapsulated types and non-typeable strains of *Haemophilus influenzae*.	Small heat-stable proteins	–	90 %	–	–	–	–	–
IgA Protease	Inactivates the human immunoglubolin A1 that accounts for 90% of the Ig A present in the oropharynx.	Similar to Hap, share homology and are paralogous.	+	+	+	+	+	+	97%
Lipooligosaccharide	Posses endotoxic activities of LPS: mitogenicity, pyrogenicity platelet aggregation. Attracts opsonization and phagocytosis.	lipid A connected via 2-keto-3-deoxyoctulosonic acid (KDO) to a core polysaccharide polymer consisting of neutral monosaccharides, lacks "O" somatic antigen, therefore named LOS	+	+	+	+	+	+	+
Outer Membrane Proteins	P 2: Functions as prions, can induce bactericidal antibody production P6: Can induce production of bactericidal antibodies, induce P6-specific humoral and cell-mediated immunity. Both P2 and P6 potential for candidate vaccine for Non-typeable *Haemophilus influenzae.*	P2: (38-40 kDa), The portion of the molecule inside the outer membrane has conserved amino acid sequences. But surface-exposed portions are highly variable. P6: (16.6 kDa), Peptidoglycan-associated lipoprotein show 97% homology between different strains.	P6	P6	P6	P6	P6	P6	P2 & P6
Protein D (PD)	Damages the ciliary function in human nasopharyngeal tissue	(42 kDa) surface lipoprotein, has glycerophosphodiesterase activity	+	+	+	+	+	+	+

Upon looking to *Haemophilus influenzae* virulence determinants, it can be seen that various strains of *Haemophilus influenzae* are different in possessing these virulence factors. Type b strain and non-typeable *Haemophilus influenzae* strain have the most significant virulence factors that have significant pathogenicity and cause the major diseases. For a better comparison, table 1 summarizes the virulence factors of different *Haemophilus influenzae* strains.

As was mentioned earlier, the most potent and virulent strain of *Haemophilus influenzae* is the type b strain. This is due mainly to having a different version of capsular polysaccharide called PRP or polyribosyl-ribotol-phosphate (figure 2).

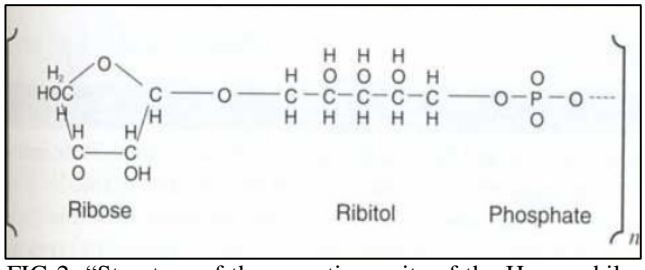


FIG 2: "Structure of the repeating units of the Haemophilus influenzae type B capsular polysaccharide polyribosyl-ribitol-phosphate (PRP). This model consists of the five-carbon monosaccharide ribose, linked by an ester bond to ribitol, a five-carbon sugar alcohol which, in turn, is linked to a phosphate group."
(Image and description has been taken from winn et al. (21).

Even though there are many other virulence factors for type b *Haemophilus influenzae* strain that makes it the most virulent strain, this PRP capsule has great significance (21). This capsule allows *Haemophilus influenzae* to avoid phagocytosis and opsonization processes. Other virulence factors found in type b *Haemophilus influenzae* strain (such as: fimbriae, Hap and Hsf adhesins) are mainly functioning as adhesins, invasins and colonization factors in respiratory tract epithelial cells in human hosts. Another very important virulent factor of type b *Haemophilus influenzae* is haemocin which inhibits other non-type b encapsulated and non-encapsulated strains of *Haemophilus influenzae* to grow on the same site. The remaining virulence factors are common between all the strains of *Haemophilus influenzae*. Comparing all strains of *Haemophilus influenzae*, it is obvious that the most virulent strains of *Haemophilus influenzae* are type b and non-typeable strains. On the other hand, non-typeable or unencapsulated strains of *Haemophilus influenzae* do not possess capsule in their structure. Their main virulence factors are the adhesin proteins, such as: Hia, Hap, OapA and a hemagglutinating pili, that facilitate or mediate the adherence of the microorganisms to the cells and also pave the way for invasion of those cells. It might be of importance to mention that most of virulence factors of non-typeable strains of *Haemophilus influenzae* are immunogenic, in that, they can induce both humoral and cell-mediated systems to induce strain-specific antibody and T-cells to protect the host from the subsequent challenge and infection by that strain.

To sum up, it can be said that *Haemophilus influenzae* is a clinically important microorganism, there are 6 encapsulated strains of *Haemophilus influenzae*, the most clinically important and virulent type of which is type b, having a different and exceptional version of capsule and possessing some specific and unique virulence factors, such as haemocin. The other types of encapsulated *Haemophilus influenzae* strains, which are mainly called as non-type b encapsulated strains, also possess some virulence factors, (summarized in table 1), but are not as virulent as type b and, therefore, not as clinically important. Moreover,

non-typeable strains of *Haemophilus influenzae*, which are also called as unencapsulated strains or non-encapsulated strains of *Haemophilus influenzae*, are in the second degree in pathogenicity after type b strain. Their main virulence factors are the adhesin proteins which mediate the adherence of microorganism to epithelial cell surfaces paving the way for invasion, colonization and other subsequent pathogenesis of the microorganism. Fortunately, some of the main virulence factors present in *Haemophilus influenzae* are immunogenic and can be used in designing vaccine candidates against infection by *Haemophilus influenzae*. Hopefully, understanding the structure and virulence factors of this microorganism can help scientists overcome the infection by this bacterium.

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