



Targeted Screening of Membrane Proteins of *Haemophilus Ducreyi* with the Aim of Drug Targets Identification

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Abstract:

Chancroid is an STI characterized by the Gram-negative bacteria *Haemophilus ducreyi*. Controlling chancroid is challenging, and the primary treatment accessible is antimicrobial therapy. However, drug resistance has been seen in places where the disease is common. Due to recent global outbreaks of sexually transmitted infections (STIs), it is crucial to continue research on innovative treatment options and prevention measures. We used reverse vaccinology and subtraction genomic methods to determine potential vaccination and therapeutic targets against *H. ducreyi* in silico. We found 56 Secreted proteins, with 159 membrane molecules and 515 cytoplasmic proteins. We assessed their need, operation, and ability to cause disease. We identified 6 potential vaccination targets and three pharmacological targets inside pathogenicity islands. The discovered targets may be utilized in future initiatives to manage chancroid globally.

INTRODUCTION

The *Haemophilus ducreyi* bacteria bring on a sexually transmitted illness (STI) known as chancroid (1). Chancroid is prevalent in impoverished nations throughout Asia, Africa, and Latin America, suggesting a strong correlation between socioeconomic status and the occurrence of this disease among a specific community (1). In the 1990s, the World Health Organization predicted the global incidence of the condition to be approximately 7 million (2). Assessing the present distribution of chancroid is challenging due to the syndromic care of genital ulcer illnesses and the absence of monitoring and diagnostic methods (3).

Haemophilus ducreyi is a Gram-negative coccobacillus that is fastidious and non-motile (4). It harms catalase, ferments D-glucose, has fine pili and does not create endospores. It has no recognized animal or ecological reservoir and primarily infects the human mucosal epithelium (5). However, it can infect the keratinized stratified squamous epithelium, leading to ulcers, local discomfort, and inguinal

lymphadenopathy (5, 6). The bacteria stay outside cells containing white blood cells, collagen, and fibrin. It can avoid being engulfed by white blood cells, which seems to be its primary method of evading the body's immune system and is crucial for its ability to cause disease (5, 7). Ulcerative lesions are linked to mononuclear cell infiltrates in the dermis, mainly CD4⁺ lymphocytes, which might aid in co-infection with human immunodeficiency virus (HIV) (8).

Haemophilus ducreyi does not lead to general infection, even in individuals with HIV (9). Extragenital lesions may arise due to auto-inoculation. Recent research has revealed *H. ducreyi* as the cause of persistent epidermal limb ulcerations in both kids and adults that are not sexually transmitted (10). Genital bacteria are categorized into two classes (I and II) based on many phenotypic and genetic variants, particularly the membrane protein, despite the unclear association with non-genital isolates (11). Furthermore, *H. ducreyi* expresses many virulence factors like lipooligosaccharides, iron-regulated

proteins, outer membrane proteins, toxins, and other secreted products during disease (12). Most oligosaccharide compounds from *H. ducreyi* include a lactosamine terminal that interacts with sialic acid (11-13). Lipooligosaccharides aid in attaching *H. ducreyi* to keratinocytes in a laboratory setting. *H. ducreyi* expresses hemolysin that can break down keratinocytes, macrophages, and T and B cells (14).

Controlling chancroid is challenging because of the lack of a protective immunological response towards further *H. ducreyi* infections, while a delayed hypersensitivity reaction to *H. ducreyi* may develop (15). The lesions may last weeks or months and not fully heal without antimicrobial medication. This inadequate response could be because cell-mediated immunity successfully removes intracellular bacteria, but most of *H. ducreyi* stays extracellular (16). Although the specific reaction that could protect the organism from *H. ducreyi* infection is unknown, the reality that *H. ducreyi* is an extracellular bacterium indicates the possibility of a humoral immune response (17). Innate and acquired immune cells, including macrophages, dendritic cells, NK cells, polymorphonuclear leukocytes, memory CD4 β , and effector CD8 β T cells, are attracted to the lesions (18, 19). It remains uncertain whether Th1, Th2, Th17, Th9, Th22, and Treg immune system reaction profiles are effectively regulated in individuals with various disease characteristics. Additional *in vitro* and *in vivo* research is required to accurately determine the function of cellular and humoral immune defenses and establish the tolerance or susceptibility of people afflicted by the illness (18, 19).

Due to the population's failure to embrace preventive measures and the absence of a reliable vaccination, the only current treatment options are azithromycin or ceftriaxone (for pregnant individuals) (20). Resistance to antibiotics was reported in endemic regions (21). Furthermore, research on non-genital ulcers indicates the presence of azithromycin-resistant non-genital strains, suggesting the possibility of an external reservoir or tolerance to the typical dosage of azithromycin (22). One goal of investigating *H. ducreyi* pathogenesis in this setting is to explore pathogenicity variables that might be considered for vaccine development. Due to the advancement of bioinformatics, the development of a vaccine for preventing *H. ducreyi* epidemics is nearing feasibility, as well as for other sexually transmitted infections (STIs) (23).

The horizontal gene transfer (HGT) process and bacterial evolution have led to the acquisition of novel antibiotic-resistance genes, necessitating the deployment of alternate infection control measures (24). Pathogenic and host genotypes increase the appeal of bioinformatics methods (25). Reverse

vaccinology utilizes computational methods to find virulence factors that are surface-exposed or secreted immunogenic proteins capable of binding MHC class I and II molecules for antigen presentation in the host's immune system (26). Successful vaccine potential molecules have established properties such as sub-cellular localization, including signal peptides, transmembrane domains, and antigenic epitopes. More research is needed on the genetic analysis of *H. ducreyi*. Most do not use reverse vaccinology methods for predicting vaccines or therapeutic targets. This presents new opportunities in the field of comparative genomics.

MATERIALS AND METHODS

Identification of the information

The genomic sequences of 10 *H. ducreyi* isolates were obtained from the GenBank dataset at the National Center for Biotechnology Information (NCBI). We used the Rapid Annotation utilizing Subsystem Technology (RAST) program to reannotate each of the 10 genotypes. This program standardizes the genome annotations to prevent unexpected outcomes and inaccurate gene interpretation, an important preparatory step for genome analysis.

Finding conserved non-host proteins that are homologous within a species

After applying RAST, we aligned all imported Coding Sequences (CDS) using global alignment techniques. We used the orthoMCL program for orthology definition, employing all-versus-all blast studies with an E-value threshold of 1×10^{-10} and the Markov Cluster (MCL) method. The core genome refers to the coding sequences (CDS) common to all 10 strains. In order to prevent autoimmunity, the medication and vaccination targets should not be similar to human proteins. To identify non-human homologous targets, we utilized orthoMCL (E-value 1×10^{-10}) to contrast the primary genome with the human genome.

Determination of islands with pathogenicity

Genome plasticity refers to the genome's dynamic nature, including DNA acquisition, loss, or reorganization. During this stage, the genomic plasticity studies concentrated on identifying genomic islands, portions of the genome that may have been acquired by horizontal gene transfer (HGT). Genomic islands are predicted based on variations in genome signature such as GC content and codon utilization, the existence of transposases and high levels of virulence, resistance, metabolic, and symbiotic factors, the existence of sequence insertions or flanking tRNA genes, dimensions ranging from 6 to 200 KB, and lack of them in non-pathogenic related

organisms. The Genomic Island Prediction Software (GIPSy) program was used for that.

Using reverse vaccination to identify potential targets for the Haemophilus ducreyi vaccine

The subtractive genome technique was used to determine vaccination targets. We first used the core genome, which comprises the critical genes of the virus, and then conducted BLASTp analysis to forecast the non-host homologous domains. Using the SutfGI software, which categorizes proteins into cytoplasmic, secreted, putative surface exposed (PSE), and membrane proteins based on the existence or absence of signal peptides, retention signals, and transmembrane helices, we predicted the subcellular localization of all the proteins from this non-host homologous conserved proteome. We analyzed the secreted proteins for adhesion probability (above 0.51) and MHC I and MHC II binding characteristics using the Vaxijn tool after identification. We identified cleavage locations and transmembrane helices utilizing SignalP and TMHMM, respectively, and determined functional domains using InterProScan. We analyzed the potential targets for their existence in pathogenicity islands (PAIs).

RESULTS

The process in Figure 1 summarizes the essential phases for target identification, the approaches used, and the total number of proteins reported in each step.

Discovering conserved non-host homologous proteins within a species and pathogenicity islands

Ten *H. ducreyi* strains' sequences were compared

(table 1), with *H. ducreyi* 35000HP serving as the standard strain for the orthoMCL study. The core genome, or 1257 CDSs, consists of coding DNA fragments common to all strains. Taking the human genome as the host genetic code, we discovered that 847 CDSs are non-host homologous molecules.

We also conducted genomic islands identification. *H. ducreyi*'s closest relatives are either harmful to humans or animals or lack a fully sequenced genome, making them unsuitable for predicting genomic islands. To prevent inaccurate adverse outcomes, we utilized two closely similar non-pathogenic organisms, *Haemophilus somnus*129Pt strain and *Manheimia haemolytica* USMARC_2286 strain, which are not harmful to humans. We used the GIPSy program for this method. MEGA analysis indicates that *H. ducreyi* is more closely linked to species within the Pasteurallaceae family rather than other species in the *Haemophilus* genus, which comprises pathogenic microbes. Additional research conducted phylogenomic research on *H. commas* 129Pt and *M. haemolytica* USMARC_2286, which are commensal organisms in animals belonging to the family Pasteurellaceae, revealing a close connection to *H. ducreyi*. We used GIPSy research to predict three pathogenicity islands and then visualized the findings using the BRIG program. Pathogenicity island prediction (PAI) is crucial to comprehending bacterial evolution, the virulence elements encoded, their mobility and construction, and the relationships between the pathogen and eukaryotic host cell populations (28). As a result, these PAI host infectiousness factors that may be attractive candidates for vaccines that elicit a reaction from the immune system.

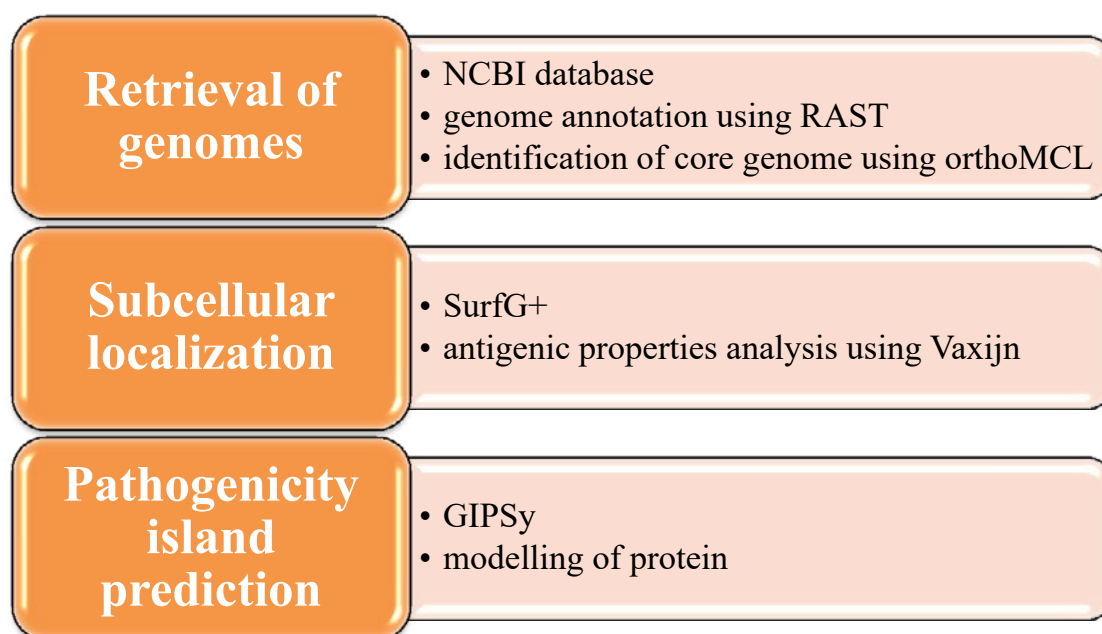


Fig 1. Created a process detailing the approaches used and the total proteins detected at each stage.

Table 1. Details regarding the 10 *H. ducreyi* strains that were employed in this study.

Strain	GenBank number	Size (MB)	Gene number	GC (%)	Protein number
Hd_GU1	CP011228	1.62	1566	38.10	1407
Hd_GU2	CP011229	1.55	1504	38.00	1340
Hd_GU3	CP011230	1.59	1539	38.10	1385
Hd_GU4	CP011231	1.57	1522	38.10	1363
Hd_GHA1	CP015429	1.62	1551	37.90	1389
Hd_AUSPNG1	CM004377	1.72	1695	38.01	1541
Hd_CLU1	CP011220	1.65	1611	38.20	1450
Hd_CLU2	CP011227	1.59	1557	38.40	1382
Hd_VAN1	CP015424	1.66	1629	38.10	1468
Hd_35000HP	AE017143	1.69	1668	38.20	1509

The lack of certain pathogenicity islands in particular strains may be linked to the variability in virulence genes, various phenotypes, and genetic diversity that categorize *H. ducreyi* strains into three categories: non-genital, genital class I, and genital class II. The absence of a portion of PAI 2 in genital class II strains (Hd_GU1, Hd_GU2, Hd_GU3, and Hd_GU4) indicates this. PAIs have precarious locations that may be gained or lost over time. Their absence in certain situations may be linked to the inclusion of draft genotypes in the dataset utilized for the research. PAIs are significant because they are a category of GEIs with virulence genes. High densities of two groups of genes are likely to be found inside PAIs: shared genes present in two or more, but not all, strains and singletons (strain-specific genes).

Estimating the potential vaccination target for Haemophilus ducreyi

We analyzed all genetic sequences from *H. ducreyi* using the reverse vaccinology technique. We examined the genes conserved across many genomes and found they are crucial for pathogenic and non-host homologous organisms. We anticipated the subcellular distribution of protein molecules that are likely to be antigenic by focusing on secreted proteins, surface-exposed proteins, and membrane proteins. We analyzed their MHC I and MHC II binding properties and adhesion probability greater than 0.51, ensuring they did not resemble mammalian proteins. We examined the correlation of virulence factors with pathogenicity islands (PAIs) since they are considered more effective targets due to their encoded nature. Molecules encoded by shared PAIs are suitable candidates, but this selection does not exclude targets from the previous phase. PAIs presence suggests that the proteins might be crucial virulence agents and should be given priority.

The protein's subcellular distribution was forecasted utilizing the SurfGp program. 847 gene products were discovered, with 332 categorized as probable surface-exposed molecules, secreted proteins, or membrane proteins (Fig 2). We next analyzed 332 proteins in Vaxijn to identify proteins with adhesion probability above 0.51. We discovered 31 proteins from them. We analyzed 31 proteins versus DEG and identified 6 as strictly necessary to the pathogen based on an E-value cut-off of 1×10^{-4} .

The biological data for these 6 proteins was obtained by analyzing cleavage locations and transmembrane helices utilizing SignalP and TMHMM and subsequently forecasting functional domains utilizing InterProScan. Proteins with a molecular weight of 110 kDa or less are preferred candidates for vaccine development due to their ease of purification. The molecular weights of specific proteins were determined utilizing UniProt. All projected proteins fall within this range and may serve as viable vaccine targets (Table 2).

DISCUSSION

Many identified vaccine targets consist of surface-exposed or secreted proteins more likely to be pathogenic or pathogenic, making them good candidates for vaccines (27). We emphasize anticipated proteins in PAIs, but other PSE and secreted proteins, such as the lipoprotein NlpD amino acids and the secreted arginine ABC transporter, are also possible vaccine targets. A01_0584 is a member of the LysM domain, a standard protein module that binds peptidoglycan in bacterium and chitin in eukaryotes (28). The domain was first discovered in enzymes that break down bacterial cell walls and serve as a signal for precise plant-bacteria identification in bacterial pathogenesis (29). Protein A01_0636 is a member of the solute-

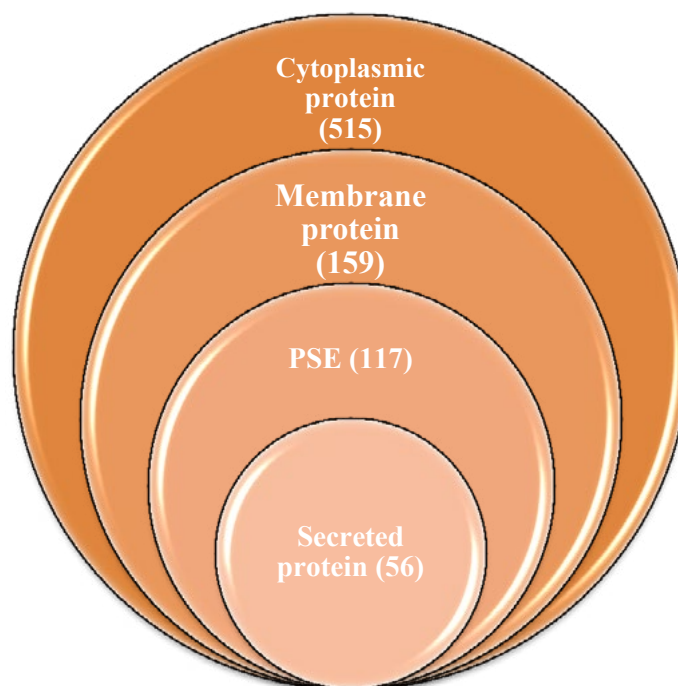


Fig2. Subcellular localization of conserved non-host homologous proteins from *H. ducreyi*. PSE stands for presumed surface exposed.

binding polypeptide family 3/N-terminal domain of MltF, which plays a role in actively transporting solutes across the cytoplasm membrane (30). In addition, it is crucial to note the discovery of another secreted amino acid, a DNA uptake protein, and a related DNA-binding protein derived from the competence protein molecule ComEA (31). This protein contains a helix-hairpin-helix domain, which enables a cell to absorb external DNA, leading to a process known as transformation. This activity is controlled in reaction to cell-to-cell communication and nutritional circumstances and is common among bacteria, likely serving as a significant route for gene transfer (32).

Most of the outer layer of the protein's composition is situated inside the membrane. It may exhibit sequence diversity in the protecting epitopes found in the exterior loops, enabling evasion of immunity. Research (33) shows that the first stage in most illnesses involves pathogenic bacteria attaching to eukaryotic cells or host proteins in the extracellular matrix. Preventing this connection effectively stops the illness. To avoid issues, we added the recognition of proteins related to adhesion, namely released outer membrane proteins. The first two amino acids are members of the BamA protein family responsible for assembling and integrating beta-barrel molecules into the outer membrane. The third protein is associated with the bacterial surface protein D15 domain. A protein is necessary for maintaining the integrity of the pilus and for its activities, such as adhering to human cells. DsrA was not anticipated to form part of the core genome due to its absence in some strains or the large

number of draft genomes included in the analysis. Nevertheless, we must be rigorous in identifying effective targets against all strains (33).

Previous studies, both in laboratory settings and in living organisms, have found *H. ducreyi* transcripts that are active during human infection, and several of these amino acids were detailed in our findings (33, 34). A study utilized selective capture of transcribed motifs using RNA obtained from pustules of three volunteers infected with *H. ducreyi* and RNA obtained from bacteria cultured in broth. They discovered many genes in the bacterium that have been recognized as possible factors contributing to virulence, such as the anticipated outer membrane protein (34). A different transcriptomic investigation (35) analyzed the *H. ducreyi* transcriptome in biopsy samples from human lesions. It contrasted it to the transcriptome of bacteria cultured in mid-log, transition, and stationary phases. *H. ducreyi* harvested from pustules exhibited upregulated genes related to nutrient transport, anaerobiosis, and fermentation compared to the inoculum in the mid-log phase. These included the anticipated formate efflux transporter *focA* and the virulence determinant *hgbA*, which is responsible for haemoglobin uptake. The research only analyzed the transcriptome of the whole lesion at a single point in time. Other virulence indicators may be expressed in varying ways throughout time or by bacteria in various microenvironments within the lesions.

CONCLUSION

Throughout the last century, multiple traditional

Table 2. VaxiJn found a potential candidate vaccination target for *Haemophilus ducreyi*. TMHMM is a server that predicts transmembrane helices. PSE refers to probable surface-exposed regions, SEC indicates secreted proteins, and MEM stands for membrane proteins.

protein ID	gene	localization	signal-P	TMHMM	InterProScan	molecular weight (Da)	adhesin probability	length (AA)
WP_010944348.1	—	PSE	no	1	etratricopeptide-like helicaldomain	20238	0.550	181
WP_010944572.1	lptC	PSE	no	1	LptC related	21798	0.673	193
WP_010944666.1	—	PSE	no	1	thioredoxin-like fold/ thioredoxin domain/ alkyl hydroperoxide reductase subunit C /thiol-specific antioxidant	19907	0.563	172
WP_010944716.1	—	PSE	yes (between 20 and 21)	0	LysM domain/peptidase M23/ duplicated hybrid motif	40279	0.541	372
WP_010944817.1	—	PSE	no	2	Rossmann-like alpha/beta/ alphas and wich fold/ domain of unknown function DUF218	27983	0.533	248
WP_010945328.1	nlpc	PSE	yes (between 31 and 32)	0	endopeptidase, NLPC/P60 domain	19295	0.519	71

methods have been effectively used in vaccine creation, including the culture of the pathogen and the utilization of biochemical, immunological, and microbiological techniques [74]. This approach is time-consuming, only detects numerous antigens which may not provide protection, and often proves unsuccessful when the infectious agent cannot be grown in a laboratory setting. The genetic revolution has enabled computational vaccinology to achieve optimal outcomes in vaccine formulation by predicting all antigens using in silico methods. Creating a worldwide vaccination for *Neisseria meningitidis* strains marked the first use of immune informatics in vaccinology. Subsequently, other effective vaccines were developed using reverse vaccinology, including vaccines targeting *Listeria monocytogenes*, Malarian protozoans, *Streptococcus pneumoniae*, *Porphyromonas gingivalis*, *Chlamydia*

pneumoniae, and *Staphylococcus aureus*. Comparative genomics, subtractive genome research, and reverse vaccinology have been effectively used in vaccine development. Due to the failure to prevent global sexually transmitted illnesses and the development of antimicrobial resistance, some infections like syphilis have resurfaced, necessitating the implementation of new techniques for STI management. Preventive strategies such as developing vaccines and medicines against *H. ducreyi* are necessary to guard against potential chancroid epidemics. The work used in silico reverse vaccinology and subtraction genomic methods to identify potential vaccine and therapeutic targets from the genome of *H. ducreyi* strains.

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Conflicts of Interest

The authors declare no conflict of interest.

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