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Genetic characterization of *Haemophilus ducreyi* from non-genital skin lesions in Cameroon

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SUMMARY

Background: *Haemophilus ducreyi*, traditionally recognized as the etiological agent of chancroid, a genital ulcer disease, is increasingly being identified as a significant cause of cutaneous ulcers in yaws-endemic regions across the South Pacific, Southeast Asia, and Sub-Saharan Africa. Despite its clinical relevance, this pathogen remains poorly characterized, and comprehensive genetic tools for analyzing isolate relationships are still lacking.

Methods: In this study, we present a follow-up of our previous research and developed a multilocus sequence typing (MLST) approach based on six of the seven loci from the *Haemophilus influenzae* MLST scheme and applied it to 82 primary clinical samples, previously confirmed to contain *H. ducreyi*, without culture. We also performed whole-genome sequencing (WGS) and antibiotic susceptibility testing on four cultured isolates obtained from cutaneous ulcers in yaws endemic health districts of Cameroon.

Results: Antibiotic susceptibility testing of *H. ducreyi* cultured isolates revealed sensitivity to all tested antibiotics, including ceftriaxone, azithromycin, and ciprofloxacin. MLST analysis, using data extracted from WGS and directly from clinical samples, identified 38 complete profiles across the six loci (34 from direct samples and four from cultured isolates), identifying 14 distinct sequence types (STs). BURST analysis of the six MLST genes grouped the STs into two distinct clonal complexes. An additional, polymorphism was observed in the *ftsI* gene, which encodes the penicillin-binding protein 3.

Conclusions: This study highlights the need for genetic typing of *H. ducreyi* strains circulating in the yaws-endemic regions of Cameroon. The developed MLST scheme offered effective strain discrimination and provided valuable insights into their genetic relationships in these areas.

Introduction

Haemophilus ducreyi is a fastidious Gram-negative coccobacillus of the polyphyletic family *Pasteurellaceae*. It is the etiological agent of chancroid, a sexually transmitted infection (STI) characterized by painful genital ulcers accompanied by inguinal lymphadenitis.[2] Chancroid was endemic in sub-Saharan Africa, gaining prominence in the early 1990s due to its association with increased HIV transmission risk.[5] Following the syndromic management introduced by the WHO to control bacterial genital ulcer diseases, prevalence has fallen drastically.[19,20] Historically, it was thought that transmission only occurred through sexual contact, via micro-abrasions, but evidence of skin to skin non-sexual transmission of *H. ducreyi* has been demonstrated in children in low-resource countries with chronic skin ulcerations.[21,22] Furthermore, while some *H. ducreyi* strains have been associated with leg ulcers, others have been found colonizing the healthy skin of asymptomatic people and can act as a continuous source of reinfection after mass drug treatments.[15] *H.*

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ducreyi is therefore considered a major cause of skin ulcers in tropical areas.[8,9] However, the genetic relationship between chancroid isolates and isolates from other skin lesions remains unknown. The ability of genetically distinct *H. ducreyi* isolates to cause non-sexually transmitted cutaneous lesions needs to be added to the differential diagnosis of skin ulcers in children in tropical areas.

Due to the fastidious growth of *H. ducreyi* and its relatively low growth rate on culture media, nucleic acid amplification tests (NAATs), which are essentially real-time PCRs, are employed to provide evidence of infection. Two principal targets are commonly used for the detection: the *hhdA* gene which encodes for the haemolysin A[1] and the more sensitive V8 region of the 16S ribosomal RNA gene.[11]

Various methods have been used to assess the diversity within *H. ducreyi*, but with suboptimal agreement. Ribotyping generated multiple restriction fragment length polymorphism (RFLP) profiles, but the designations derived from these variations have not gained widespread acceptance or use in epidemiological surveillance, partly due to the challenges in culturing this fastidious organism.[24] Outer membrane profiling was used to distinguish two clonal populations based on the autotransporter adhesion *dsrA* protein classifying the isolates into the so-called class 1 and class 2 strains. These correspond respectively to strains expressing *dsrA* type 1 (with the strain 35000 HP as reference) and type 2 (with the strain CIP 542 as reference).[27] A gene-by-gene approach based on the multilocus sequence analysis (MLSA) of 11 genes, including virulence and housekeeping genes, allowed for the identification of genetic differences between class 1 and class 2 strains. Out of the 11 genes examined, only three (*lspA2*, *ncaA*, and *dsrA*) provided meaningful differences. Subsequently, a multilocus sequence typing (MLST) method based on the analysis of three loci, notably *ncaA, hgbA* and *dsrA,* was developed and generated a tree similar to that obtained from whole genome phylogenetic analysis, supporting the reliability of the method.[6] A single-locus typing scheme was subsequently developed, focusing on the *dsrA* gene, which was sufficient to distinguish between different circulating strains of *H. ducreyi*.[10] To date, there is no standardized typing method that allows the exploration of the genomic diversity of *H. ducreyi* beyond class 1 and class 2.

Accurate approaches are required to characterize *H. ducreyi* strains: 1) to understand the transmission dynamics of the pathogen, and 2) to track the spread of virulent or antibiotic-resistant strains. We aimed to follow up our previously conducted research to describe an MLST scheme for characterizing circulating strains of *H. ducreyi* based on the sequence diversity of seven housekeeping genes, adapted from the existing and widely used typing procedure for *H. influenzae*.

Methods

Patients and primary samples and bacterial isolates

The recruitment of participants and sample collection procedure for this study have been described elsewhere and was approved by the National Ethics Committee for Human Health Research (N°2020 / 12/ 1327/ CE/ CNERSH/ SP) and the Ministry of Public Health (approval N°631–021).[23] In brief, we conducted an active search of cutaneous ulcer cases in yaws endemic districts of Cameroon and collected ulcer swabs from 443 individuals including 271 individuals with ulcerative lesions clinically consistent with yaws and 172 healthy skin swabs from asymptomatic contacts. We collected swabs from individuals with yaws-like ulcers and asymptomatic contacts in yaws-endemic regions of Cameroon (A total of 271 subjects). In areas with limited electricity, a single swab per ulcer was collected in lysis buffer for molecular analysis (n=224). In areas with stable electricity, two swabs were collected (47×2samples): one for molecular analysis and the other for *H. ducreyi* culture. Asymptomatic contacts were sampled by swabbing both legs and placing the swab in lysis buffer.

Bacterial growth, DNA preparation

Bacteria were isolated by successive sub-culturing on polyvitex-chocolate agar plates (BioMérieux, Marcy-l'Etoile, France) and incubated at 33 °C and 5% CO_2 environment for 24 to 48 h. Although not typically used for *H. ducreyi*, this medium was chosen for its rich nutrient profile that supports the growth of fastidious organisms[26] (Fig. 1). The isolation process involved transferring colonies from initial growth to fresh plates to ensure purity. DNA extraction for polymerase chain reaction (PCR), Sanger, and next-generation sequencing was performed using two methods: thermal shock for pure bacterial culture DNA extraction and the QIAmp DNA mini kit (Qiagen, Germany) according to the manufacturer's recommendations for extracting DNA from primary ulcer swab samples.

Bacterial identification

Colonies that were morphologically suspected to be *H. ducreyi* (by the pushing test on the plate, and Gram staining (characteristics 'school of fish' or 'railroad track' appearance)) were further identified using Matrix-Assisted Laser Desorption Ionization-time of flight (MALDI- TOF) Biotyper, version 3.1 (BioMérieux, Marcy-l'Etoile, France) following the methodology previously described.[14] Whole genome sequencing on the cultured isolates (n=4) was performed with Illumina technology (NextSeq 500, Illumina) as earlier described.[3] The genomes of the four cultured isolates were uploaded to the PUBMLST database with the ID numbers (27495, 27497, 27498 and 27499). Molecular identification was performed using the "Species identification" tool on PubMLST https://pubmlst.org/species-id (Home > Species ID). Relatedness of the isolates were analyzed using the tools available on the Bacterial Isolates Genome Sequence database (BIGSdb) platform on PubMLST for *H. influenzae*[17] (https://pubmlst.org/organisms/haemophilus-influenzae) with the flow (Home > Organisms > *Haemophilus influenzae* > *Haemophilus influenzae* isolates > Search or browse database. Genetic characterization only used Ribosomal MLST (rMLST) analysis, due to the paucity of annotation for *H. ducreyi*, was employed to assess ribosomal gene diversity of the isolates. The neighbor-net network was visualized using SplitsTree4 (version 4.19.2).[16] For not-cultivated samples, *H. ducreyi* was detected by qPCR targeting the v8 portion of the 16S ribosomal RNA gene; probe, primers and qPCR conditions were published earlier.[23]

Antibacterial susceptibility testing

Antibiotic susceptibility was assessed using the E-test method on polyvitex-chocolate agar medium through the determination of the minimal inhibitory concentrations (MIC). The antibiotics tested included ampicillin, amoxicillin + clavulanic acid, ceftriaxone, chloramphenicol, trimethoprim/ sulfamethoxazole, rifampicin, azithromycin, ciprofloxacin. The β-lactamase activity was screened for all isolates by the cefinase test (nitrocefin disks, bioMérieux, Marcy-l'Étoile, France). Interpretations were according to the guidelines for *H. influne*zae of the European Committee on Antimicrobial Susceptibility Testing v.13.0.[4]

MLST scheme of primary samples and analysis

We screened the 35 publicly available *H. ducreyi* genomes in the PubMLST database for the presence/absence of the seven genes used for MLST of *H. influenzae* in the available genome of *H. ducreyi.* Isolates that were identified as *H. ducreyi* in PubMLST databases were first selected using the "Search" tool and all the 35 isolates

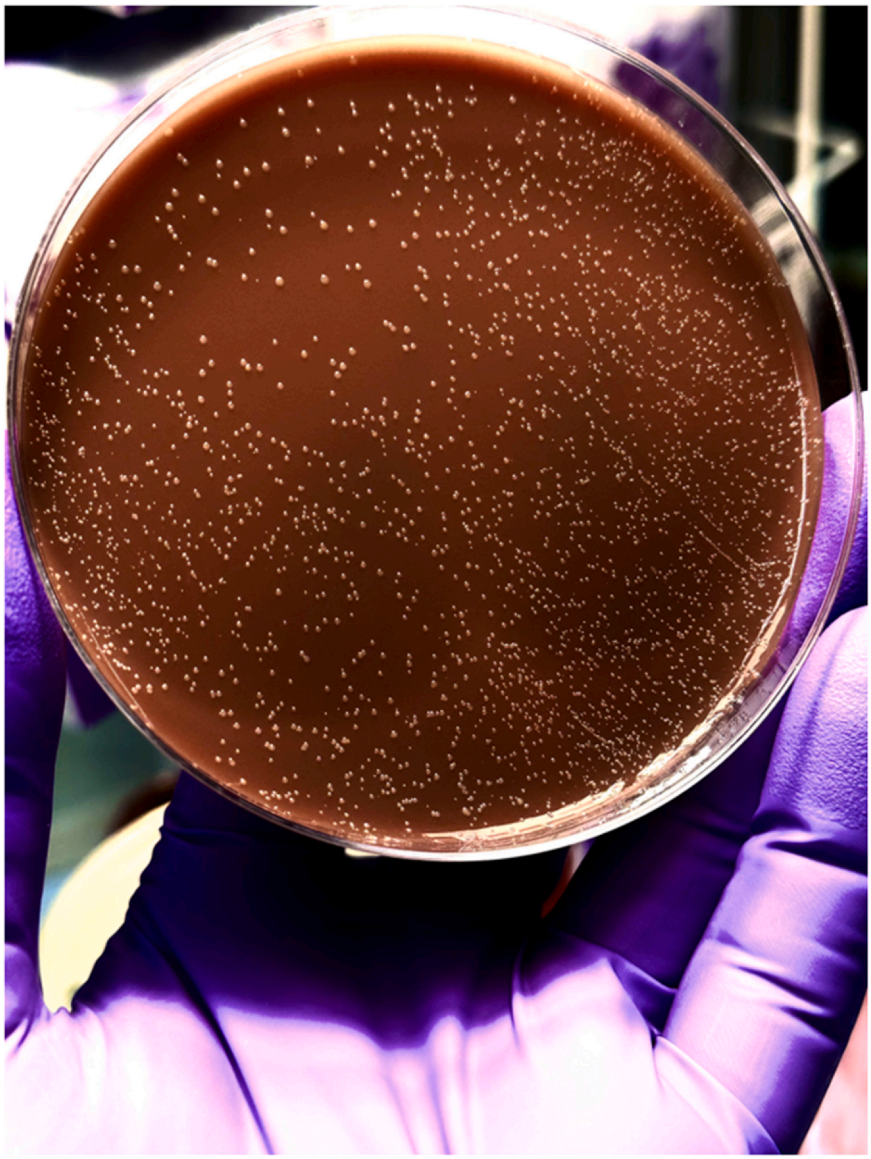

Fig. 1. Growth of small yellow-gray colonies identified as *H. ducreyi* as seen on the chocolate agar plate after an incubation period of 48 h.

were analyzed using the tool "Gene presence"(Home > Organisms > *Haemophilus influenzae* > *Haemophilus influenzae* isolates > Plugins > Gene presence). Six of the seven genes were always present (*adk- atpG- frdB- mdh- pgI- recA*), the *fucK* gene was absent in all the 35 isolates (Supplementary file 1). We also added the *ftsI* gene to the MLST scheme, this gene is used to characterize resistance to beta-lactam in *H. influnezae*.[3] Primers were designed and used with adaptors corresponding to universal forward and reverse oligonucleotides added to the 5′ ends of the upstream and the downstream oligonucleotides, respectively (Supplementary Table 1). After amplification, the universal forward and reverse oligonucleotides were used for sequencing as previously described.[25] The lengths and positions of sequences used for each target gene are summarized in Table 1. All sequences produced in the present study were submitted to the NCBI GenBank database (accession numbers PQ310525 to PQ285371).

As several alleles of the sequenced genes differed in length from those of *H. influenzae* on the PubMLST database it was not possible to use the alleles numbers from PubMLST database. We, therefore, used a specific designation for the alleles from our collection (two alleles

Table 1
Summary of nucleotide sequence information after trimming.

Locus	Gene product	Length of fragment (bp)	Number of alleles	Number of variable sites	Position (min)[a]
adk	Adenylate kinase	372	4	13	656350- 656721
atpG	ATP synthase	498	7	36	7531- 8028
frdB	Fumarate reductase	456	2	1	31086- 31112
ftsI	Penicilin binding protein 3	579	6	27	181092- 181670
mdh	Malate dehydrogenase	348	6	5	200001- 200348
pgi	Glucose-6-phosphate isomerase	528	12	18	330805- 331332
recA	RecA protein	495	7	7	325933- 326427

[a] The positions in the table correspond to those obtained from the *H. ducreyi* 35000 HP reference strain.

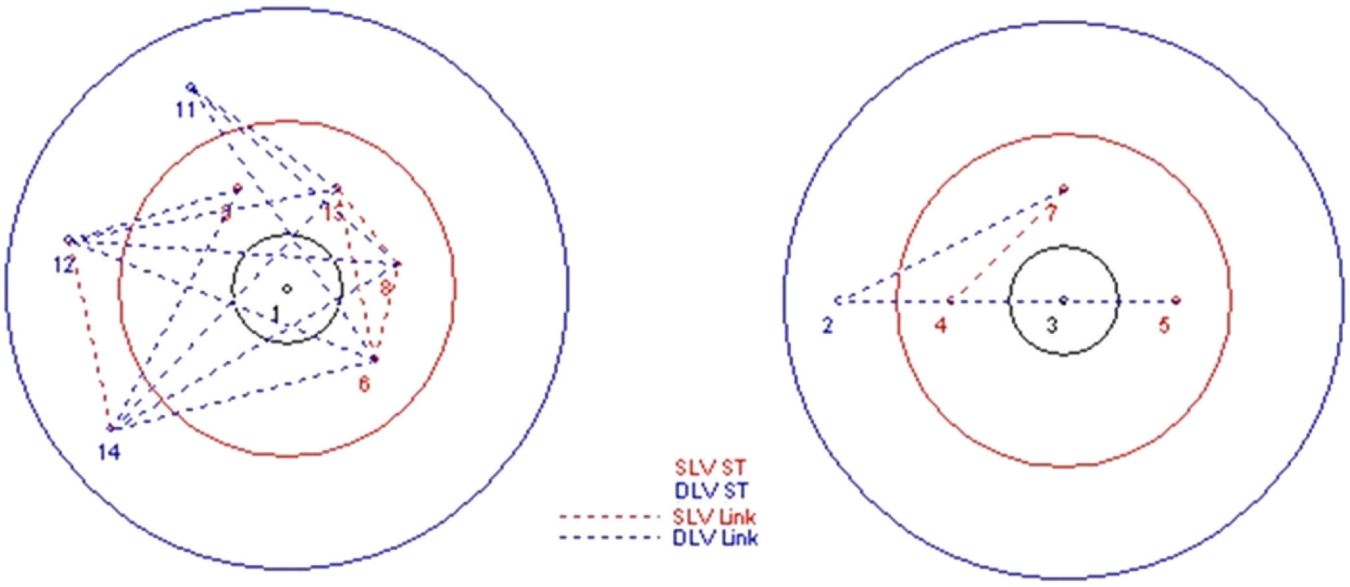

Fig. 2. BURST-based clustering of the isolates with full profiles of the 6 MLST loci. As shown, this analysis clustered STs into groups according to their allelic profiles. BURST analysis defines the number of Single Locus Variants (SLV), Double Locus Variants (DLV)) for each sequence type (ST) they are located within concentric circles. The analysis also identifies a founder ST in each group that corresponds to the ST that displays the highest number of SLV and located in the center of the circles.

differ by at least one nucleotide) and sequence types were named for isolates sharing the same six alleles. A BURST analysis was also used to analyze the relationships between isolates showing complete allelic profiles for the six analyzed genes. The results clustered STs into groups according to their allelic profiles, defining the number of Single Locus Variants (SLV), Double Locus Variants (DLV), and Satellites (SAT) for each sequence type (ST). Additionally, a founder ST was identified for each group, corresponding to the ST with the highest number of SLV.[18]

Results

Description of cases

We initially screened 271 ulcer samples and 172 healthy skin swabs screened.[23] In this study, 106 suspected cases were included of which 97 samples were positive for *H. ducreyi* by qPCR.[23] Of these, 82 were from individuals with skin ulcers and 15 from asymptomatic individuals. Of these 82 samples, a total of 71 ulcer samples and 7 asymptomatic samples had a cycle threshold < 30 by qPCR and were included in the typing analysis.

We attempted culture from 47 samples from the skin ulcer cases enrolled in the study, among which 13 were positives to *H. ducreyi* PCR. From these samples, we were able to isolate nine potential isolates that were identified as *H. ducreyi* on the basis of colony morphology and characteristic growth requirements.

Identification and MLST analysis for non-cultured samples

As described in the Method section, we focused on 71 ulcer samples and 7 samples from healthy individuals that met the inclusion criteria of strong positive qPCR amplification of *H. ducreyi* DNA (cycle threshold < 30). These samples were included in the MLST analysis, which allowed amplification and sequencing of these genes. Complete profiles for the seven genes (*adk*, *atpG*, *frdb*, *mdh*, pgi, *recA*, and *ftsI*) were obtained for 34 out of 78 samples. For the remaining samples partial profiles were obtained (1 to six genes). The number of alleles identified among these seven genes ranged from two (*frdb*) to twelve (*pgi*) (Supplementary file 2). The 38 complete profiles (34 samples and 4 cultured isolates) revealed 14 distinct combinations or sequence types (STs) that are depicted in (Supplementary file 3). BURST analysis (Fig. 2) using the six MLST genes allowed clustering of the STs of the isolates/samples into two groups (clonal complexes sharing at least four of the six MLST loci), The first group contained 8 STs corresponding to 26 samples. The second group contained five STs corresponding to 11 isolates/samples. This second group included the four cultured isolates sharing ST-2. Only one profile (ST-10) persisted as a singleton, representing a single sample (Supplementary file 3). Adding *ftsI* alleles to the analysis allowed further discrimination within ST-4 of the second group (Supplementary file 3). Sequences of *ftsI* identified 7 alleles corresponding to 6 amino acid sequences that differed by one to 11 residues. The four cultured isolates harboring the *ftsI* allele 2 were susceptible to all tested beta-lactam antibiotics (Table 3).

Identification of cultured isolates

Using MALD-TOF and real-time PCR, four of the nine isolates were confirmed as *H. ducreyi*. WGS sequencing of the isolates were submitted to the "species identification tool" on PUBMLST.org[17] that confirmed the identification of *H. ducreyi* while the five other isolates were identified as *Arcanobacterium haemolyticum*, a bacterium also reported in tropical cutaneous ulcer disease.[12] rMLST analysis showed that the four isolates were highly linked. Indeed, the rMLST analysis identified two main clusters among the 35 isolates available on the PUBMLST.org (including our 4 isolates) (Fig. 3). Several isolates from class I and II genital ulcers or from cutaneous ulcers were previously published.[7] The four isolates in our study clustered with the isolates of class I genital ulcers and with isolates from cutaneous ulcers, and were distantly separated from isolates of class II genital ulcers (Fig. 3).

Antibiotic susceptibility

All four *H. ducreyi* isolates tested were susceptible to all the antibiotics assessed. Table 3 summarizes the values of the minimal inhibitory concentrations of each antibiotic tested per sample. Ceftriaxone was the most active antibiotic with a MIC < 0.016 mg/L for most of the isolates (Table 3). None of the four isolates was positive for beta-lactamase by cefinase test and a BLAST search for beta-

Table 2
Characteristics of study population.

Cases n=106[a]	
Sex	
Male	76
Female	30
Age group	
< 5	6
5–15	99
> 15	1
Median age	6 [6–11]
Antibiotic treatment	
yes	10
no	77
don't know	19
Site	
Bankim	50[b]
Doume	37
Maroua	8
Mbang	4
Messamena	1
Ndelele	3
Yokadouma	3

[a] 97 cases were qPCR-positive samples, and 9 cases were culture-positive.
[b] 5 cases from this site for which culture was positive were not confirmed positive to *H. ducreyi*.

Table 3
Minimal inhibitory concentration of the antibiotic tested per sample (mg/L).

Sample	AMP	AML	CRO	AZM	CIP	CHL	RIM	SXT
JPN-2	0.125	0.047	< 0.016	0.016	0.032	0.38	0.004	0.047
JPN-4	0.125	0.064	< 0.016	0.023	0.032	0.38	0.004	0.047
JPN-5	0.064	0.047	< 0.016	0.023	0.016	0.38	0.004	0.032
JPN-6	0.125	0.047	< 0.016	0.023	0.032	1	0.004	0.047

AMP: ampicillin, AML: Amoxicillin/clavulanic acid, CRO: Ceftriaxone, AZM: azithromycin, CIP: ciprofloxacin, CHL: chloramphenicol, RIM: rifampicin, SXT: cotrimoxazole.

lactamase did not detect any homology in the genomes of the four sequenced isolates.

Discussion

In this study, we present an integrated work of multiple molecular approaches to analyze the *H. ducreyi* isolates. We developed a non-culture method for detecting cases directly from clinical samples, which can then be followed by a targeted MLST scheme for genetic analysis and clustering. These approaches in addition to the PCR-based detection of *H. ducreyi* will help establish a standardized protocol for the identification and typing of this difficult-to-culture bacterium. Moreover, sequence of genes involved in antimicrobial resistance can also the prediction of resistance to these antibiotics as already established for *H. influnezae*.[3] Our data confirmed the role of *H. ducreyi* in non-genital skin lesions by data obtained from both culture and non-culture samples. However, caution is needed when using culture methods, as other bacterial species, such as *A. haemolyticum*, can be co-cultured from clinical samples. However, the presence of this bacterium in skin lesions highlights the need for pathophysiological studies to better understand its role in the pathogenesis of these lesions.

The use of qPCR targeting the v8 portion of the 16S ribosomal RNA gene seems to be reliable and allowed confirmation of isolates in most of the clinical samples. We also used a stringent qPCR criterion for diagnosis (i.e., a PCR cycle threshold < 30). This stringency was further corroborated by the MLST analysis that yielded DNA sequences (partial or complete profiles for the 6 MLST genes in all the 78 tested samples).

Our data also showed that the current MLST schemes of *H. influenzae* as it stands cannot be applied to *H. ducreyi*. The gene *fucK* is absent in *H. ducreyi* and the other six genes are quite distant from their homolog in *H. influnezae*. We therefore presented here a specific set of primers to allow a specific MLST for *H. ducreyi* that succeeded in clustering all the samples we tested in this work including the four cultured isolates. Of interest the BURST analysis suggested that at least two genotypes, based on our MLST approach, were identified among the non-culture samples. These data suggest that isolates from cutaneous ulcers may show a high diversity. Our MLST approach can be a reliable tool in the genetic analysis of *H. ducreyi* when cultured isolates are not available.

Analysis of the genetic relatedness of all genomes available in the PUBMLST showed the clustering of the corresponding isolates into two groups. Class II genital ulcer isolates corresponded to one group, while class I genital ulcer isolates and cutaneous ulcer isolates corresponded to another group that included our four cultured isolates. This observation supports previous findings, suggesting that cutaneous ulcer isolates are closely related to, and may be derivatives of class I genital ulcer isolates.[7] Several non-culture samples were obtained from the healthy skin of asymptomatic individuals, indicating that isolates genetically linked to those from cutaneous ulcers may colonize human skin asymptomatically in endemic areas.

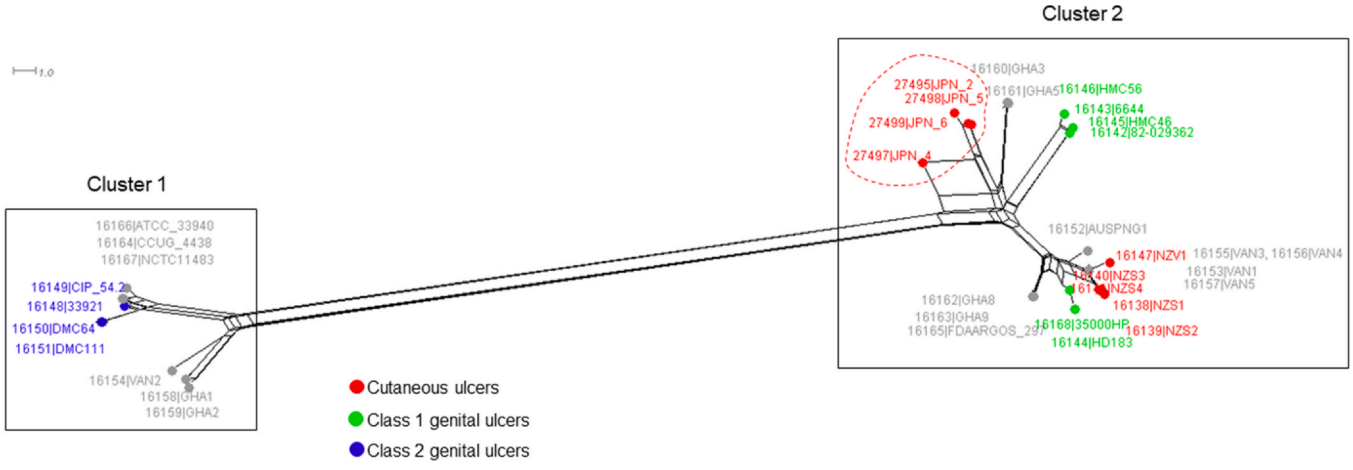

Fig. 3. Phylogenetic Tree Based on rMLST allelic profiles using Genome comparator analysis of 35 *H. ducreyi* Genomes: 31 from the pubMLST Database and 4 from This Study (surrounded by dashed red line). Two genetic clusters are recognized and indicated. The ID on PUBMLST data base and the name of isolates are indicated. Isolates from cutaneous ulcers are indicated in red. Isolates that were described as class I genital ulcers are indicated in green and those with class II in blue. Isolates with unknown sites are indicated in gray. Each Node Represents a Genome ID.

Indeed, isolates from genital ulcers have also been detected in asymptomatic sex workers during chancroid outbreak The Gambia.[13]

Additionally, analysis of the *ftsI* gene could allow the prediction of susceptibility to beta-lactams similar to its application in *H. influenzae*.[3] Antibiotic resistance predictions can also be extended to other antibiotics used in mass drug administration. However, sequencing isolates with known susceptibility profiles is essential to establish correlations between phenotypic and genotypic data. One limitation of our study is the low number of isolates, nevertheless, the MLST approach represents significant progress by providing a standardized molecular scheme to enhance the diagnosis/ typing and analysis of infections by this difficult-to-culture bacterium.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jinf.2025.106448.

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