Author manuscript, published in "Sexually Transmitted Infections 86, 6 (2010) 449"
DOI : 10.1136/sti.2010.044321

Genotypes and antimicrobial resistant phenotypes of *Neisseria gonorrhoeae* in Portugal (2004-2009)

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Contributions of each author

MJB was the PI; CF was the lead author for the paper, JPG contributed for the design of the study and study analysis; RP, MB, CF performed all the typing techniques, AP performed the bacterial detection and antimicrobial testing; BN performed the statistical analysis; and MJB, CF and JPG contributed to the write up.

Key messages box

1. STs formed two major groups separated by 159.8 [SE 8.9] nucleotide differences, yielding several subgroups.
2. Ciprofloxacin resistance seems to be associated with ST225 and genetically highly-related STs.
3. The multiplicity of STs (1 ST *per* 2.3 isolates) may reflect a high number of undiagnosed and not reported gonorrhoea cases.

Word count: 2271

ABSTRACT

Objectives: To determine the antibiotic phenotype and MAST-genotype distribution of *N. gonorrhoeae* isolates in Portugal between 2004 and 2009, and to evaluate specific associations between MAST-genotypes and sexual orientation, age, and antibiotic resistance.

Methods: A total of 236 *N. gonorrhoeae* isolates were typed through NG-MAST. The polymorphism degree and the phylogenetic relatedness among NG-MAST STs were evaluated by MEGA4 software on concatenated sequences of *por* and *tbpb* alleles. E-test was used to determine the susceptibility to ceftriaxone, ciprofloxacin, penicillin, and spectinomycin.

Results: No isolates displayed resistance to spectinomycin and ceftriaxone whereas 79.1% and 37.4% were resistant to penicillin and ciprofloxacin, respectively. We found 104 different STs (1 ST *per* 2.3 isolates), where the most frequent were ST210 (8.1%) and ST225 (7.6%). STs formed two major groups separated by 159.8 [SE 8.9] nucleotide differences, yielding several subgroups, one of them including the worldwide prevalent ST225. The probability of ciprofloxacin resistance among isolates within this subgroup was 73.5-fold higher than for the remaining isolates. Indeed, for the genetically closest subgroup, which includes the most prevalent ST210, only 8.0% of isolates were resistance to ciprofloxacin. There was a non-homogeneous distribution *per* year for ST225 ($p<0.001$), ST210 ($p=0.011$), and ST2 ($p=0.007$).

Conclusions: The heterogeneous STs scenario may represent the “tip of the iceberg”, reflecting a high number of undiagnosed and not reported gonorrhoea cases. A laboratory-based national surveillance of *N. gonorrhoeae* infections is mandatory to provide a broader spectrum of isolates that will allow the establishment of the scenario of the Portuguese sexual networks.

INTRODUCTION

Neisseria gonorrhoeae (*N. gonorrhoeae*), the etiologic agent of gonorrhoea, remains one of the most common bacterial sexually transmitted infections (STIs), as more than 60 millions of new cases are reported annually worldwide.[1, 2] Recent trends in gonorrhoea have shown a progressive increase in several countries of Western Europe (e.g. Sweden and Switzerland), resulting in the resurgence of an old problem for the public health.[2, 3] These alarming data may indicate a return to high risk sexual behaviours, contributing to onward transmission of STIs, as the HIV, which highlights the need for routine surveillance, prevention and control measures.[4]

Over the past decades, *N. gonorrhoeae* has repeatedly developed resistance to traditional antimicrobial agents, conditioning the choice of the first-line treatment.[5] Thus, third-generation cephalosporins are now recommended for European countries, despite emergence and spread of isolates displaying reduced susceptibility to cefixime and ceftriaxone has been recently reported.[6-9]

In Portugal, data resulting from the mandatory notification reveal that gonorrhoea incidence remained low and stable during the last decade (0.27 – 0.7 cases per 100 000 inhabitants).[2] However, current data on antimicrobial susceptibility profiles of Portuguese *N. gonorrhoeae* circulating isolates are not available, as well as information about the molecular epidemiology of pathogen. The only evaluation performed so far in Portugal regarded the determination of tetracycline resistance (which is no longer used for treatment) back in 1997.[10]

Typing strategies of *N. gonorrhoeae* isolates are valuable tools to define the sexual networks, to distinguish between relapse and re-infection episodes, and to monitor the emergence and spread of antibiotic resistant gonococci clones.[11-13] *N. gonorrhoeae* multiantigen sequence typing (NG-MAST) is a high-discriminatory tool to assess the genetic diversity of *N. gonorrhoeae*, through the evaluation of allelic combination between two highly polymorphic genes, *por* and *tbpB*. This can be especially important for short-term epidemiology enabling the identification of recent chains of transmission.[14]

In the present study we aimed to determine the antibiotic phenotype and MAST-genotype distribution of *N. gonorrhoeae* population in Portugal between 2004 and 2009, which constitutes the first molecular epidemiological study held in this country. Specific associations within genotypes and sexual orientation, age, and antibiotic resistance were also evaluated.

METHODS

N. gonorrhoeae isolates

Between 2006 and 2009, more than one hundred laboratories dispersed through the whole country were asked to send *N. gonorrhoeae* isolates to the national bacterial sexually transmitted infections laboratory located in the Portuguese NIH. The national collection was sent yearly to the “European Surveillance of Sexually Transmitted Infections” network. Twenty-five laboratories (including the NIH reference laboratory) from the Lisbon area (n=15), Porto area (n=6), Leiria (n=2) and Algarve (n=2) participated, leading to the collection of a total of 274 *N. gonorrhoeae* isolates. Participating laboratories provided the following data: date of isolation, specimen anatomic site, gender and age of the infected individual, as well as sexual orientation. All data were anonymous in order to avoid any ethical conflict.

A total of 236 from the whole 274 *N. gonorrhoeae* isolates collection were fully typed through NG-MAST. The infected individuals were aged from under one year to 58 years, median age 28. From these, 26 were isolated from women and 210 from men during the years 2004 (n=17), 2006 (n=29), 2007 (n=75), 2008 (n=50) and 2009 (n=65). Considering the sexual orientation, 74 were heterosexual (18 women and 56 men), 70 were MSM and for 92 infected individuals no information was provided.

Antimicrobial Susceptibility testing

The isolates were sub-cultured once in chocolate agar (BioMérieux, France) before susceptibility testing was performed. Suspensions of cultures aged 24 hours were prepared equivalent to McFarland's standard 0.5. The ceftriaxone, ciprofloxacin, penicillin, and spectinomycin MIC of isolates was determined in GC agar (Oxoid, Basingstoke, UK) with 1% Isovitalex (Becton, Dickinson and Company, Sparks, USA) using Etests (AB bioMérieux, Solna, Sweden) according to the manufacturer's instructions. Plates were allowed to incubate for 24 hours at 36ºC in 5% CO_2.

Isolates were categorised according to the following definitions: Ciprofloxacin resistant (MICs≥1 mg/L), spectinomycin resistant (MICs≥128 mg/L) ceftriaxone resistant (MIC≥0,5 mg/L), decreased-susceptibility to ceftriaxone (0.125≥MICs≤0,25 mg/L), and penicillin resistant (MIC≥2 mg/L). All penicillin intermediate or resistant isolates were tested for penicillinase production using the chromogenic reagent, Nitrocefin (Oxoid, Basingstoke, UK), according to manufacturer's instructions.

NG-MAST

The molecular genotyping of all *N. gonorrhoeae* isolates was performed using the NG-MAST method, as previously described.[14] Briefly, *N. gonorrhoeae* DNA was extracted with QIAamp DNA mini kit (Qiagen, Valencia, CA), according to manufacturer's instructions. Two highly polymorphic loci, *por* and *tbpB* were subjected to PCR amplification and both strands of internal fragments were sequenced (490 and

390 bp, respectively) by using Big Dye v.1.1 chemistry on an ABI3700 capillary sequencer (Applied BioSystems, Foster City, USA). Sequences were aligned by using Lasergene software (DNASTAR, Madison, USA). Alleles and sequence types (STs) were assigned in the international database of the NG-MAST website (www.ng-mast.net).

Genetic analysis

In order to evaluate the polymorphism degree and the phylogenetic relatedness among NG-MAST STs, concatenated sequences of *por* and *tbpb* alleles were created in a head-to-tail fashion, as previously described for other pathogens.[15] Subsequently, a Neighboor-Joining phylogenetic tree of all concatenated sequences was generated with MEGA4 software using Kimura two-parameter and p-distance models.[16-18] We have also generated matrices of pairwise comparisons to estimate the total number of variable sites, the overall mean genetic distance among all STs, and the genetic relatedness between putative ST clusters. The pairwise-deletion option was chosen to remove all sites containing missing data or alignment gaps for all genetic distance estimations. No analyses were done at the protein level because the *tbpb* allele becomes out-of-frame within the concatenated sequence.

Statistical analysis

Chi-square, Fisher Exact and Linear-by-Linear tests were used to evaluate the associations between ST types and antibiotic resistance, year of isolation and population characteristics (sexual orientation, age, and gender). Observed differences were considered statistically significant for $p<0.05$. All statistical results were obtained with the package of statistical programs SPSS 15.0.

RESULTS

Antimicrobial susceptibility phenotypes

From the 236 *N. gonorrhoeae* isolates, 187 were subjected to antimicrobial susceptibility testing. Antibiotic resistance to spectinomycin and ceftriaxone was not observed in the current studied population. Furthermore, only four isolates presented reduced susceptibility to ceftriaxone, which occurred in 2007. Contrarily, 148 (79.1%) and 70 (37.4%) isolates displayed resistance to penicillin and ciprofloxacin, respectively, where 15.5% of the former were penicillinase producers. Multiresistance was detected in 35.8% of isolates considering that 95.7% of ciprofloxacin resistant isolates were also resistant to penicillin.

Genetic diversity of *N. gonorrhoeae* isolates

According to the NG-MAST method, 236 *N. gonorrhoeae* isolates were distributed into 104 different STs, 60 (57.7%) of which had not been reported before to the international database. The majority of STs (66.3%, 69 of 104) were represented by a single isolate, whereas the remaining STs included two to 19 isolates. This high genetic diversity arose from the allelic combination of 86 *por* and 39 *tbpB* alleles, which is supported by the fully-branched phylogenetic tree based on concatenated sequences of these alleles (fig 1). The overall mean genetic distance was 97.3 [SE 5.1] nucleotide differences, where a maximum distance of 205 [SE 12.5] nucleotides was observed between ST4324 and ST4334. Interestingly, the 104 STs formed two major groups (fig 1) separated by 159.8 [SE 8.9] nucleotide differences, yielding several subgroups of genetically close STs, as the one that includes the worldwide prevalent ST225 (subgroup A2 in fig 1), that shows a genetic distance within the same-group strains solely of 5.3 [SE 1.1].

The most frequent STs were ST210 (n=19, 8.1%) and ST225 (n=18, 7.6%), which, together with other eight STs (table 1) represent 42% (100/236) of all isolates. There was a statistically significant non-homogeneous distribution over the five years for ST225 (p<0.001), ST210 (p=0.011), and ST2 (p=0.007), with a decreasing tendency for the first two (p=0.016 and p=0.002, respectively) and an increasing tendency for the latter (p=0.005).

The evaluation of STs from nine pairs of isolates recovered from known sexual partners has shown 88.9% of concordance. These are represented by a diverse set of STs and are dispersed throughout the study period. The single pair of isolates that presented non-identical STs, involved two novel STs (ST3628 and ST3630), which share the *por* 2171 allele.

Table 1 – Characterisation of the most frequent STS

ST	n	Sexual Orientation			Age			Antibiotic susceptibility[a]: Penicillin		Antibiotic susceptibility[a]: Ciprofloxacin		
		MSM	HS[b]		<25	≥25		S	I/R	S	I/R	
		n (%)[c]	n (%)[c]	p	n (%)	n (%)	p	n (%)[c]	n (%)[c]	n (%)[c]	n (%)[c]	p
210	19	5 (35.7)	9 (64.3)		5 (26.3)	14 (73.7)		6 (40.0)	9 (60.0)	14 (93.3)	1 (6.7)	0.02
225	18	9 (100)	0 (0.0)	0.002	2 (11.1)	16 (88.9)	0.028	0 (0.0)	18 (100)	0 (0.0)	18 (100)	<0.001
783	9	0 (0.0)	7 (100)	0.014	7 (77.8)	2 (22.2)	0.009	1 (11.1)	8 (88.9)	9 (100)	0 (0.0)	0.02
1318	9	0 (0.0)	7 (100)	0.014	5 (55.6)	4 (44.4)		2 (22.2)	7 (77.8)	9 (100)	0 (0.0)	0.02
1407	9	5 (71.4)	2 (28.6)		3 (33.3)	6 (66.7)		0 (0.0)	9 (100)	1 (11.1)	8 (88.9)	0.002
1479	9	1 (25.0)	3 (75.0)		4 (44.4)	5 (55.6)		2 (22.2)	7 (77.8)	0 (0.0)	9 (100)	<0.001
1780	9	5 (71.4)	2 (28.6)		3 (33.3)	6 (66.7)		5 (55.6)	4 (44.4)	9 (100)	0 (0.0)	0.02
2	6	0 (0.0)	4 (100)		5 (83.3)	1 (16.7)	0.02	4 (66.7)	2 (33.3)	6 (100)	0 (0.0)	
1034	6	1 (33.3)	2 (66.7)		3 (50.0)	3 (50.0)		0 (0.0)	5 (100)	5 (100)	0 (0.0)	

4317	6	0 (0.0)	2 (100)		4 (66.7)	2 (33.3)		2 (40.0)	3 (60.0)	5 (100)	0 (0.0)

[a] S, Susceptible; I, Intermediate; R, Resistant
[b] HS, Heterosexual
[c] Percentage according to known information

STs *versus* socio-demographic data

There were substantial differences in ST distribution by age and sexual orientation. Indeed, ST2 and ST783 were predominantly found in patients under 25 years of age (p=0.02 and p=0.009, respectively) (table 1). On the other hand, one of the most common STs, ST225, was identified predominantly in patients above 25 years (p=0.028). Regarding sexual orientation, there was a statistically significant association between ST225 and the MSM (p=0.001) whereas ST783 and ST1318 were associated with heterosexual individuals (p=0.014). No statistical association regarding geographic origin of isolates and ST was found in the present study.

STs *versus* antibiotic resistance

There was a statistical significant association between resistance to ciprofloxacin and isolates with ST1479 (100%, p<0.001), ST3615 (100%, p=0.007), ST225 (94.4%, p<0.001) and ST1407 (88.9%, p=0.002). For all the other antibiotics no association was found.

DISCUSSION

In Portugal it is assumed that only a small fraction of the *N. gonorrhoeae* infections are notified to health authorities, which may be in the basis of the apparently stable incidence rates, contradicting reports from other countries that evidence increasing rates.[2] It is expected that the implementation of laboratory and clinical notification (according to ECDC guidelines) will provide more accurate epidemiological data on this STI. Considering the lack of data in Portugal and the recent development of the NG-MAST technique, we aimed to determine the antibiotic phenotype and MAST-genotype distribution of *N. gonorrhoeae* population in Portugal since 2004.

Globally, the results from the antibiotic susceptibility profiles matched the ones reported for other countries.[7, 8, 19-21] In fact, no isolates displayed resistance to spectinomycin and ceftriaxone whereas 79.1% and 37.4% were resistant to penicillin and ciprofloxacin, respectively, which supports the current recommend therapy strategy.[6]

However, contrarily to what has been described in some countries, we did not observed an increase tendency for reduced susceptibility to ceftriaxone.[7-9] Nevertheless, the continuous monitorization of susceptibility profiles to this antibiotic is advisable. Considering the association between antibiotic susceptibility phenotypes and STs, our results also supported previous reports. Indeed, ST225, which was the second most frequent ST in our study, and the only disseminated worldwide, was mostly found in ciprofloxacin resistant *N. gonorrhoeae* isolates ($p<0.001$).[8, 13, 21, 22] We also found a similar association for ST1459 ($p<0.001$), so far only described in France, and that had comprised solely isolates resistant to this antibiotic. Curiously, the rate of ciprofloxacin resistance within a subgroup of isolates that includes ST225 was 96.4%. Indeed, the probability of ciprofloxacin resistance among these isolates was 73.5-fold higher than for the remaining isolates with other STs. Interestingly, the genetically and phylogenetically closest subgroup (subgroup A1 in fig 1), which includes the most prevalent ST210, presented only 8.0% resistance to ciprofloxacin. Thus, based on our results and on the literature we can speculate that ciprofloxacin resistance is associated with ST225 and genetically highly-related STs.[8] However, the opposite cannot be stated as isolates resistant to ciprofloxacin can be found phylogenetic distant from ST225, as for ST1479 (within group B in fig 1), which reveals 148 [SE 11] nucleotide differences to ST225. Further studies comprehending more isolates and STs are essential to understand this phenomenon.

The phylogenetic tree evidenced a huge strain diversity which would be expected due to the highly polymorphism of the two alleles involved in the NG-MAST method, and grouped STs in two major groups (fig 1) that present a genetic distance of 159.8 [SE 8.9] nucleotide differences. However, there is a high number of STs represented by a single isolate, and no predominant ST exists, which may be due to the local emergence of new STs, recent introduction of foreign STs or incomplete/insufficient epidemiological surveillance. In Portugal, although our data do not represent the entire country, this heterogeneous STs scenario may represent the "tip of the iceberg", reflecting a high number of undiagnosed and not reported gonorrhoea cases. Thus, such diversity of STs within the studied population did not allow the identification of sexual networks that spread this infection, contrarily to what has been successfully achieved in the UK.[23] However, eight of the nine sexual partners included in this study were infected by isolates with the same ST, which reinforces the interest of NG-MAST as a tool for the construction of sexual networks. Nonetheless, the high rate of polymorphism of the two genes could lead to slight differences in the resulting combined genotype. In fact, the discordant couple (ST3628 and ST3630) evidenced the same *por* allele but a *tbpB* allele diverging by a single nucleotide mutation. Overall, the couple concordance rate (88.9%) was similar to the described in the literature.[24, 25] Considering the inexistence of sexual networks in the present study and the dynamic fluctuation on the predominance of *N. gonorrhoeae* types per year, these apparently prevailing types likely represent ecological succeeded STs during a short period of time, although *N. gonorrhoeae* is typically considered as 'nonclonal' where alleles are at linkage equilibrium.[26, 27] This can explain the short-term emergence of STs in all the NG-MAST studies performed to date. As an example, ST210 and ST225 frequency has been declining in Portugal since 2004 and are eventually being replaced by other STs.

In the future, we expect that the laboratory-based national surveillance of *N. gonorrhoeae* infections will provide a broader spectrum of isolates that will allow the establishment of the scenario of the Portuguese sexual networks. Furthermore, it would be desirable that molecular epidemiology studies could be extended to a large number of countries, in order to evaluate the accuracy of the associations reported so far, involving STs, age, sexual orientation, and antibiotic resistance, and to perform further research to investigate such associations.

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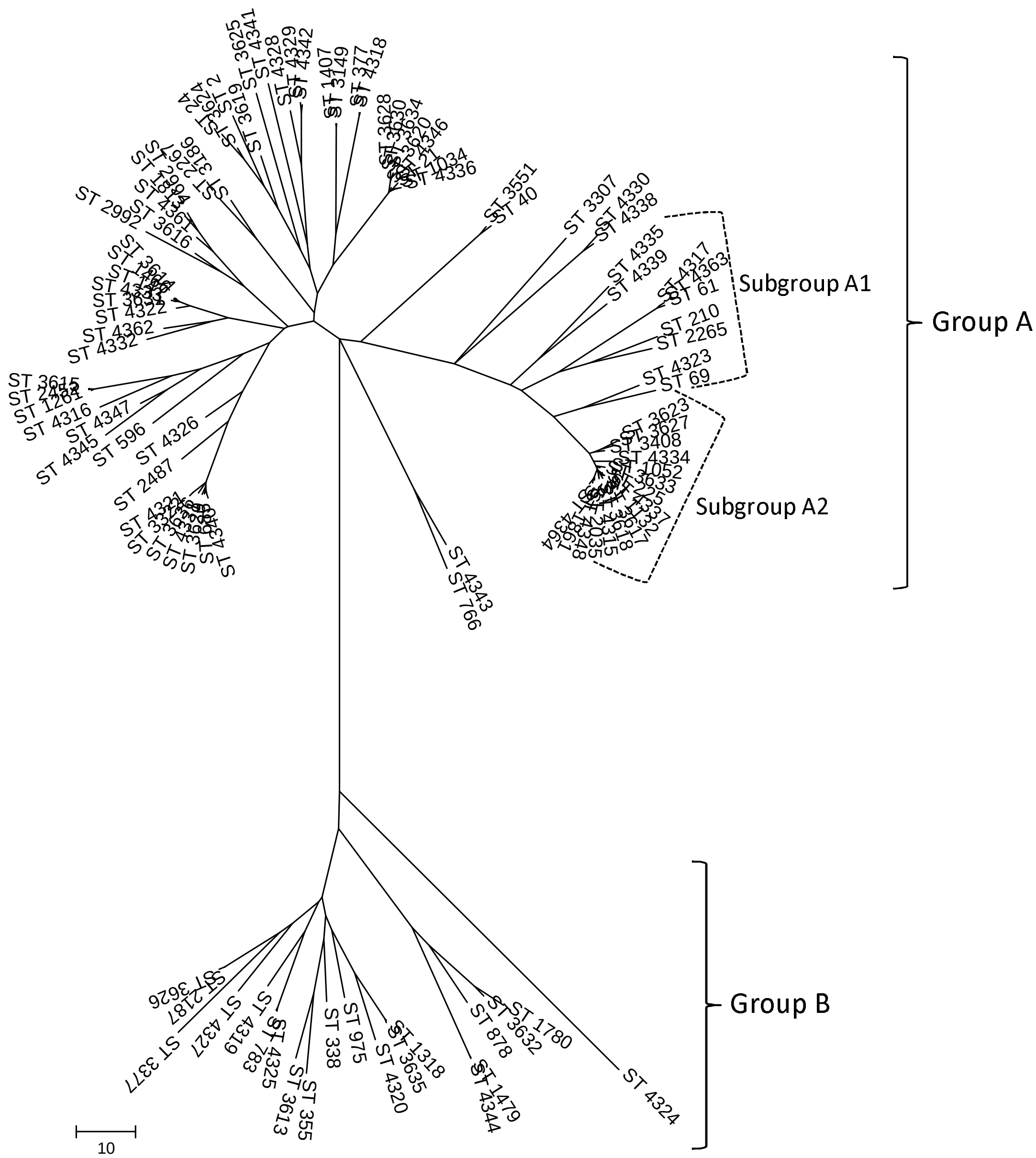
Competing interests: None.

AKNOWLEDGEMENTS

We thank the Portuguese collaborators that contributed with their isolates and data for the Portuguese national collection of *Neisseria gonorrhoeae* for the years 2006 to 2009: J. Azevedo and I. Santo (CS Lapa), F. Alegria (Aqualab), A. Morais (CS Oeiras), M. Azevedo (Clin. Fernando Teixeira), J. M. Amorim (Clin Lab. Edgar Botelho Moniz), F. Pereira (H. Cruz Vermelha), P Paixão (H. Luz), M. D. Pinheiro (H. S. João), J. Melo-Cristino (H. Sta Maria), M. A. Faustino (H. S. Marcos), J. Diogo (H. Garcia Orta), F. Martins (H. S. F. Xavier), Teresa Paramês (Imunolab), M. A. Santos (INSA Porto), M. Pereira (Lababa), M. Menezes (Labdiagnóstica), M. B. Santos (Labeto), M. C. Guimarães (Labmed Center), C. Marques (Labomarques), L. Ferreira (Lab. David Santos Pinto), M. Charneca (Lab. Ascensão Afonso), S. Sá (Lab. Isabel Vicente), F. Lencastre (Lab. V. M. Roldão), J. L. Viana (Lab. Nova Era), A. Gomes (Med. Lab. Carlos Silva Torres), and A. Raimundo (Previlabor).

FIGURE LEGEND

Figure 1 - Phylogenetic reconstruction of concatenated sequences of *por* and *tbpB* alleles for all STs identified in Portugal by Neighbor-joining tree topology based on distance estimates using the "number of nucleotide differences" model. Two major groups of STs are marked (group A and B), which are genetically separated by 159.8 [SE8.9] nucleotides. For group A, two subgroups are identified, A1 and A2, including ST210 and ST225, respectively. Branch lengths are proportional to nucleotide distances between STs. Bootstrap analysis with 500 replications was applied. Phylogenetic reconstruction based on the Kimura two-parameter model for substitutions events yielded a similar topology.


Group A
Subgroup A1
Subgroup A2
Group B
10
ST 3551
ST 40
ST 3307
ST 4330
ST 4338
ST 4335
ST 4339
ST 4317
ST 4363
ST 61
ST 210
ST 2265
ST 4323
ST 69
ST 3623
ST 3627
ST 3408
ST 4334
ST 4343
ST 766
ST 2992
ST 3616
ST 4322
ST 4362
ST 4332
ST 3615
ST 1261
ST 4316
ST 4347
ST 4345
ST 596
ST 4326
ST 2487
ST 3625
ST 4341
ST 4328
ST 4329
ST 4342
ST 1407
ST 3149
ST 377
ST 4318
ST 3628
ST 1034
ST 4336
ST 3626
ST 2187
ST 3377
ST 4327
ST 4319
ST 783
ST 4325
ST 3613
ST 355
ST 338
ST 975
ST 1318
ST 3635
ST 4320
ST 878
ST 3632
ST 1780
ST 1479
ST 4344
ST 4324