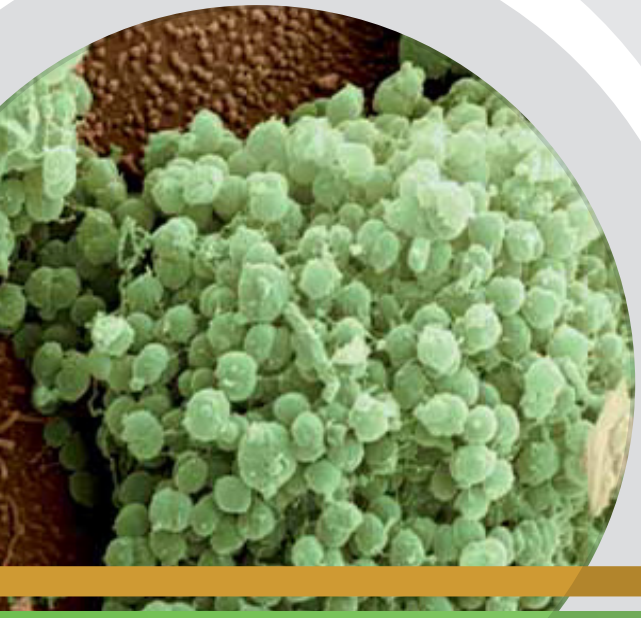

TECHNICAL REPORT

Molecular typing of *Neisseria gonorrhoeae* – a study of 2013 isolates

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ECDC TECHNICAL REPORT

Molecular typing of *Neisseria gonorrhoeae* – a study of 2013 isolates

This project was commissioned by the European Centre for Disease Prevention and Control and coordinated by Gianfranco Spiteri and Andrew Amato-Gauci (ECDC/2013/015).

The report was produced by Magnus Unemo, Susanne Jacobsson (Örebro University Hospital, Örebro, Sweden), Gianfranco Spiteri (ECDC), Michelle Cole, Franceso Tripodo (Public Health England, London, United Kingdom), Simon Harris, David Aanensen (The Centre for Genomic Pathogen Surveillance and The Wellcome Trust Sanger Institute, Cambridgeshire, United Kingdom). Antimicrobial resistance and NG-MAST testing were performed centrally by Vlad Grigorjev, Catherine Wiggins (Public Health England), Susanne Jacobsson (Örebro University Hospital). However, European STI Network members from Spain (Julio Vazquez), France (Agathe Goubard, Beatrice Bercout), Italy (Paola Stefanelli), Portugal (Maria José Borrego), Ireland (Brendan Crowley) also performed both antimicrobial resistance and NG-MAST testing decentrally. Whole genome sequencing was funded by The Centre for Genomic Pathogen Surveillance, and performed at the Wellcome Trust Sanger Institute. The European STI surveillance network was instrumental in the design of the study.

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Abbreviations

AMR	Antimicrobial resistance
bp	Base pairs
CI	Confidence intervals
DNA	Deoxyribonucleic acid
EEA	European Economic Area
EUCAST	European Committee on Antimicrobial Susceptibility Testing
Euro-GASP	European Gonococcal Antimicrobial Surveillance Programme
G	Genogroup
MIC	Minimum inhibitory concentration
MLST	Multilocus sequence typing
MSM	Men who have sex with men
NG-MAST	*Neisseria gonorrhoeae* multi-antigen sequence typing
OR	Odds ratio
PCR	Polymerase chain reaction
ST	Sequence type
STI	Sexually transmitted infection
TESSy	The European Surveillance System
WGS	Whole genome sequencing

Executive summary

In 2011, a pilot study was conducted to assess the public health value of molecular surveillance for gonorrhoea in 21 European Union/European Economic Area (EU/EEA) countries. A total of 1066 isolates collected as part of the 2010 European gonococcal antimicrobial surveillance programme (Euro-GASP) were typed using *Neisseria gonorrhoeae* Multi-Antigen Sequence typing (NG-MAST). The study highlighted many issues of importance for public health on national as well as EU/EEA level, including the identification of a highly heterogeneous gonococcal population with some predominant genogroups (Gs) and the establishment of a baseline for sequence types (STs) and Gs. The study showed that NG-MAST appeared to have a high discriminatory ability, provided enhanced surveillance data and improved the understanding of emergence and dissemination of gonococcal strains, including those with therapeutically relevant resistance profiles. Therefore NG-MAST (linked to antimicrobial resistance (AMR) profiles and epidemiological metadata) was considered useful for public health purposes at the EU/EEA level.

The present study represents a new molecular epidemiological survey using Euro-GASP isolates from 2013 to further assess the spread of AMR gonococcal strains in the EU/EEA, to investigate the stability of the relationships between AMR and STs/Gs and to address additional issues of importance for public health. In addition, whole genome sequencing (WGS) was performed on all available isolates to establish if multidrug resistant STs/Gs are clonal and to detail their distribution, to assess the public health relevance of WGS in the surveillance and control of *N. gonorrhoeae* at EU/EEA level and to form an evidence base for a molecular surveillance strategy for *N. gonorrhoeae* in the EU/EEA.

The 2013 study aimed to type 50 isolates from each country and 100 isolates from countries with higher rates of gonorrhoea by both NG-MAST and WGS. A total of 1189 isolates were typed by NG-MAST; 430 (STs) were identified, 146 of which were represented by two or more gonococcal isolates. Twenty-three types were represented by ten or more isolates. While considerable diversity was observed in different countries, four STs (ST1407, ST2992, ST2400 and ST4995) were predominant, albeit at different levels in individual countries. ST1407 and ST2992 also predominated in 2010. However, in 2013, ST1407 represented only 7.6% of all isolates, while in 2009-2010 it represented 15.6% of isolates. The continuing predominance of a single NG-MAST ST is interesting given that the gonococcus is highly heterogeneous and that NG-MAST measures variation within two highly variable genes.

Further analysis of the 23 most common STs showed that other STs within the study were strongly genetically related to these, enabling 18 major genogroups (49% of all typed isolates) of closely related STs to be defined. G1407 remained the predominant genogroup, but the overall prevalence of G1407 has decreased significantly (from 23.3% in 2009–2010 to 14.8% in 2013). Genogroup G1407 was observed in 19 out of 21 countries, although it was predominant in only six countries compared to 13 countries in 2010.

Statistical analyses demonstrated associations between patient characteristics and genogroups. For some genogroups (e.g. G51 and G2), patients were younger and a lower proportion of males were infected, which may indicate that some genotypes are more associated with heterosexual patients. In contrast, other genogroups were isolated more frequently in men who have sex with men (MSM), given the apparent associations with older patients and male gender (G2992, G4995 and G5624). However, given the limitations of the study population and the lower reporting rates of epidemiological variables such as sexual orientation, all associations should be interpreted with caution. This is a major limitation of the current study in terms of evaluating the public health benefits of linking genogroups or STs to epidemiological characteristics.

Significant associations were identified between ciprofloxacin resistance and the predominant genogroups G1407, G2400, G225, G4995, G5624, G5333, and G7232. A strong association was identified between G1407 and resistance to cefixime and ciprofloxacin, with also a relatively large proportion (34%) of the G1407 isolates showing intermediate susceptibility or resistance to azithromycin. Similar associations were also found in the previous Euro-GASP molecular typing study. Consequently, G1407 genogroup continues to predominate even though cefixime is no longer recommended as the first-line therapeutic agent to treat gonorrhoea. Six G1407 isolates were also resistant to the first-line treatment ceftriaxone. Accordingly, the continued predominance of G1407 isolates in many EU/EEA Member States is worrying, given their potential to be therapeutically challenging. While G1407 was more associated with MSM in the Euro-GASP molecular typing study in 2009–2010 (despite being present in the heterosexual population), G1407 was associated with heterosexual patients as well as older patients (>45 years of age) in 2013 at a higher level. Thus, this multidrug-resistant genogroup appears to have crossed over to the heterosexual population in 2013. Obviously, the G1407 genogroup is circulating both in the heterosexual population as well as among MSM and, consequently, the potential risks of future treatment failure are not restricted to any one patient group.

Quality assured WGS data linked to AMR profiles and epidemiological data was obtained for 1054 (89%) isolates and the phylogenomics based on the whole genome sequences revealed a significantly higher and more accurate resolution of the isolates. WGS further discriminated isolates with identical NG-MAST STs and Gs mostly reflecting specific evolutionary traits in the same country. The WGS data revealed that NG-MAST Gs (based on diversity in only two genes) do not always reflect the genomic similarities or differences of isolates. Additionally, 114 different multilocus sequence typing (MLST) STs (40 new STs), including 68 clusters (≥2 isolates with identical ST) and 46 STs represented by single isolates, were identified in silico from the WGS data. AMR determinants, mainly all known resistance determinants for sulphonamides, penicillins, tetracyclines, spectinomycin, rifampicin, fluoroquinolones, macrolides (e.g. azithromycin), cefixime and ceftriaxone were also identified in silico.

This report describes the second molecular typing survey of *N. gonorrhoeae* across the EU/EEA and provides recent information on NG-MAST STs/Gs and genotypes based on WGS circulating in 21 EU/EEA Member States. This is the first time WGS has been used in any international surveillance programme for sexually transmitted infections. This study provided an up-to-date baseline in regard to genetic heterogeneity of gonococcal isolates in the EU/EEA, including identification of many novel genotypes, which can inform outbreak investigations and identification of AMR gonococcal strains. The use of molecular typing to predict AMR profiles has clear public health benefits as it could aid understanding of the dissemination of resistance within a population and facilitate development of targeted mitigation strategies. If the prediction of AMR is sufficiently reliable this approach could have a direct impact on appropriate management of patients for whom culture and associated susceptibility testing has not been performed. WGS provided a significantly higher and more accurate resolution, and additional information can easily be evaluated such as additional markers (diagnostic, molecular epidemiologic (e.g. MLST), antimicrobial resistance and evolutionary traits). Using WGS it has been possible to appropriately assess whether G1407 and other multidrug resistant STs/Gs are clonal, stable and persist over time. In general, the use of WGS will provide enhanced and detailed understanding of the emergence and transmission of different gonococcal strains, AMR phenotypes, and gonorrhoea in general, both nationally and internationally.

There is a clear public health benefit to be gained from performing molecular surveillance of gonorrhoea in Europe, particularly by WGS. However there are a number of areas that require further development before the maximum benefit can be gained from such a scheme, such as improving representativeness, improving the reporting of epidemiological variables and the public availability of user-friendly external databases and analysis tools.

1 Introduction

Surveillance of the sexually transmitted infection (STI) gonorrhoea is implemented at the European Union/ European Economic Area (EU/EEA) level as part of enhanced STI surveillance, led by the European Centre for Disease Prevention and Control (ECDC). This includes monitoring of the number of reported cases of notifiable STI, associated epidemiological data, and antimicrobial susceptibility of gonococcal isolates through the European Gonococcal Antimicrobial Susceptibility Programme (Euro-GASP) [1,2].

Monitoring of molecular types and molecular epidemiology of the *Neisseria gonorrhoeae* strains circulating in the region has, however, only been previously performed in one sentinel study [3]. In that study, which was developed in accordance with the decisions taken at an expert group meeting in 2010, isolates collected in 2009 and 2010 through Euro-GASP were typed with the *N. gonorrhoeae* multi-antigen sequence typing (NG-MAST) [4]. This method was chosen because it was considered highly discriminative, reproducible, objective, providing easily transferable results and provided the international availability of a database (reviewed in [5]). In total, among the 1066 isolates investigated, 406 sequence types (STs) and seven major genogroups (Gs) were assigned. One of the key findings was that G1407 was the most common genogroup (23% of all isolates) and highly prevalent in the entire EU/EEA region (found in 20 of the 21 participating countries). G1407 is a multidrug-resistant gonococcal clone with decreased susceptibility and/or resistance to cefixime, ceftriaxone and azithromycin, and resistance to ciprofloxacin. Most importantly, G1407 accounted for most of the decreased susceptibility and resistance to the, at that time, recommended first-line antimicrobial for monotherapy (cefixime) in the whole EU/EEA region. The high prevalence of this multidrug-resistant gonococcal clone in the EU/EEA, and globally, is very worrying because most of the verified treatment failures with cefixime have also been caused by this clone [reviewed in 6–8]. Furthermore, many G1407 isolates only need a single additional mutation to develop high-level resistance to ceftriaxone [9], the last remaining option for empiric antimicrobial monotherapy of gonorrhoea [2,6,7].

The previous European typing study [3] highlighted many issues of importance for public health at national as well as EU/EEA level:

- the gonococcal population in the EU/EEA was highly heterogeneous but some main genogroups predominated;
- a baseline for STs and genogroups was established, which is of importance for outbreak investigations;
- NG-MAST appeared to have a sufficient discriminatory ability, at least for all the objectives of that study;
- NG-MAST provided enhanced surveillance data and improved the understanding of emergence and dissemination of gonococcal strains, including therapeutically relevant resistance profiles;
- NG-MAST (linked to antimicrobial resistance (AMR) profiles and epidemiological metadata) was considered useful for public health purposes at the EU/EEA level.

The usefulness of NG-MAST for public health purposes has also been emphasised through many national studies [reviewed in 5]. A recent large British study was of particular interest, showing a significant decline in the prevalence of isolates with decreased susceptibility/resistance to cefixime that was associated with the concomitant change in prescribing practice in 2010 from cefixime to ceftriaxone plus azithromycin. This decline was also associated with a decreased prevalence of the G1407 clone and, accordingly, a reservoir of this clone might have been effectively targeted for eradication with the changed treatment recommendations [10].

In general, the gonococcal antimicrobial susceptibility and resistance patterns appear to have changed over the years in the EU/EEA, such as decreasing cefixime resistance since 2010, and decreasing azithromycin resistance from 2010 to 2012 and subsequent increasing resistance from 2013 [1, 2]. It is crucial to understand if this is the result of changes in: treatment regimens (general or men who have sex with men (MSM)-specific), sexual behaviour in different sexual networks, the gonococcal strain distribution or due to other reasons. Molecular surveillance data linked to epidemiological data can provide evidence of changes to overall strain distribution and among risk groups and the link to antimicrobial susceptibility. A new Expert Group meeting (Euro-GASP co-ordination meeting) was therefore held in December 2013 in Stockholm, Sweden. At this meeting, it was decided to perform an up-to-date molecular epidemiological study to further assess the spread of AMR gonococcal strains in the EU/EEA, the stability of the relationships between AMR and STs/Gs, additional issues of importance for public health not addressed in the previous EU/EEA study, and to form the basis for a strategy for molecular typing of gonococcal isolates for surveillance in EU/EEA (see objectives below). The expert group agreed to implement a molecular epidemiological sentinel pilot study using similar methodology as in the 2010 study. The study would use NG-MAST and be based on Euro-GASP isolates obtained in 2013. In addition, considering developments in recent years in the field of whole genome sequencing (WGS), which has an ideal discriminatory ability and has become substantially less expensive, more effective and more frequently used for molecular typing, it was agreed that the 2013 European study would also include WGS of all available isolates, which became possible through external funding. In accordance with scientific practice, the expert group agreed that data would be publically available after publication. Following the expert group meeting, the protocol for the molecular typing survey was refined and agreed with the European STI surveillance network at their meeting in May 2014 in Dubrovnik, Croatia.

1.1 Objectives

The objectives of the European typing study of isolates collected in 2013 were to:

- Update the baseline in regard to genetic heterogeneity of gonococcal isolates in the EU/EEA, including identification of novel genotypes, in order to inform outbreak investigations and the identification of AMR gonococcal isolates.
- Identify associations and stability over time of associations between AMR profiles for therapeutically relevant antimicrobials, risk groups and *N. gonorrhoeae* genotypes on a national as well as EU/EEA level in order to support the implementation and targeting of specific control measures.
- Compare current (2013 isolates) molecular epidemiology and associations with AMR with the previous study in 2009–2010 to further assess (temporally and geographically) the spread of antimicrobial-resistant gonococcal strains, with special focus on G1407, in the EU/EEA.
- Assess whether NG-MAST comprises sufficient discriminatory ability and adequately identifies different clones of *N. gonorrhoeae* in order to inform future molecular surveillance activities.
- Assess whether G1407 and other multidrug resistant STs/Gs are clonal and persisting using WGS in order to evaluate the stability over time of the associations between *N. gonorrhoeae* genotype and multidrug-resistant phenotype, detail the spread of multidrug resistant gonococcal clones and inform future molecular surveillance activities.
- Assess the public health relevance of WGS in the surveillance and control of *N. gonorrhoeae* at EU/EEA level.
- Form an evidence base for a molecular surveillance strategy for *N. gonorrhoeae* in the EU/EEA.

2 Methods

2.1 Isolate collection

Molecular typing was performed on gonococcal isolates mostly collected in the October/November 2013 Euro-GASP collection (see below). This strategy ensured that the isolates had already been collected and antimicrobial susceptibility testing performed and interpreted using breakpoints from the European Committee on Antimicrobial Susceptibility Testing (EUCAST). Permission to perform NG-MAST and WGS was obtained from all submitting laboratories. The protocol for this molecular typing survey was finalised in May 2014 and NG-MAST analysis of the isolates carried out from June 2014 to March 2015, with the NG-MAST report ready by April 2015. DNA extraction of the isolates for WGS was started in April 2015 and completed by July 2015. WGS was complete by December 2015 and analysis performed by May 2016.

2.1.1 Isolate numbers

Each country aimed to collect a minimum of 55 consecutive gonococcal isolates in the October/November collection period of 2013 (or from the whole year for countries with less than 50 isolates available), with the overall aim to retrieve and test 50 isolates. For countries where 55 isolates represent less than 10% of the total number of cases of gonorrhoea (Spain, United Kingdom and the Netherlands) in a six-month period, up to a maximum of 110 isolates were requested to be collected, with the aim to test 100 isolates.

2.1.2 Selection criteria

Isolates were included regardless of the antimicrobial susceptibility profile (i.e. include both antimicrobial susceptible and resistant isolates and obtain a baseline of homogeneity/heterogeneity). In addition, the following inclusion criteria were used:

- availability of a viable isolate;
- full antimicrobial susceptbility profile;
- permission from nominated representatives of the submitting country.

Details on the source of the isolates, country coverage, comprehensiveness and the samplying method can be found in 2013 Euro-GASP report [1].

2.2 NG-MAST

Centralised NG-MAST of the gonococcal isolates was performed at Public Health England, London, United Kingdom and Örebro University Hospital, Örebro, Sweden. Decentralised NG-MAST was performed by countries that preferred to type their isolates themselves (France, Ireland, Italy, Portugal and Spain). For all laboratories involved in decentralised NG-MAST, it was requested that an international reference strain (internal quality control) was included in each batch of testing. Bacterial DNA extracts were prepared for each isolate and either used promptly or stored at -20°C for subsequent use. NG-MAST was performed on the DNA extracts as previously described [4]. Briefly, internal regions of the *porB* and *tbpB* genes were amplified by polymerase chain reaction (PCR), and subsequently the PCR products were purified. Sequencing of both the forward and reverse strands was then performed. Allele numbers for the trimmed *porB* (490 base pairs (bp)) and *tbpB* (390 bp) sequences and sequence types were assigned via the online NG-MAST database[1].

A cluster was defined as two or more isolates with the same ST. Genogroups were defined for the most frequently observed STs (represented by 10 isolates or more) to demonstrate collections of closely related STs. To define genogroups, similarity of alleles was evaluated in BioNumerics v6.1 or BioEdit v7.0.9.0. For example, for ST1407 (*porB* 908, *tbpB* 110), all *porB* alleles from isolates with *tbpB* allele 110 were aligned and compared for similarity against *porB* allele 908, then all *tbpB* alleles from isolates containing *porB* allele 908 were compared for similarity against *tbpB* allele 110. A genogroup was therefore defined as all STs which shared one allele and showed >99% similarity in the other allele (≤5 bp difference for *porB* and ≤4 bp for *tbpB*) or an ST with two different alleles, but the concatenated sequence of both alleles (880 bp) displayed ≥99.4% (875 bp) similarity to the concatenated sequence of both alleles of the main ST in the genogroup. It should be noted that STs with two different alleles were not included in genogroups in the previous Euro-GASP typing study [3]. Genogroups were named after the most frequently occurring ST within the group (e.g. G1407 was named after ST1407).

NG-MAST and genogroups were linked to AMR and epidemiological information from each isolate/patient collected as part of Euro-GASP [1,2,11]. For the purposes of statistical analyses, patients were sub-divided into one of four age groups (0–24 years, 25–34 years, 35–44 years or ≥45 years).

[1] www.ng-mast.net

2.3 Whole genome sequencing

Centralised DNA extractions and whole genome sequencing (WGS) were performed in order to assure a very high quality of DNA preparations and sequencing, and to be able to substantially reduce the sequencing costs through a high-level of multiplexing. Highly concentrated and purified DNA extracts were prepared for each isolate using the Wizard Genomic Purification kit (Promega) or a custom protocol on QIAsymphony Robot (Qiagen). For all isolates, genomic DNA (approximately 1 µg) was fragmented to an average size of 500 bp and subjected to DNA library creation using the manufacturer's protocols. Adapter-ligated libraries were amplified and indexed via PCR. A portion of each library was used to create an equimolar pool comprising eight indexed libraries. Libraries were subjected to 125 base paired-end sequencing on an Illumina HiSeq 2500 following the manufacturer's instructions. Reads were aligned to the chromosome of *Neisseria gonorrhoeae* FA1090 (Accession number: NC_002946) using BWA MEM (version 0.7.12-r1039) [12] with options to output alignments for unpaired reads and to mark shorter split hits as secondary. Optical duplicates were removed and indels realigned using GATK [13] MarkDuplicates (version 1.127) and indelRealigner (version 3.4-46) respectively, under their default settings. Variant sites were identified from each isolate using SAMtools mpileup (version 1.2) [14]. Options used were: to report DP and DP4 statistics, count orphans, adjust the mapping quality to 50 and increase the maximum depth to 1000 (including for indel calling). This was followed by use of BCFtools (version 1.2) [14] call using a prior of 0.001, a ploidy of 1 and with the option to keep all alternate alleles at variant sites. All sites were further filtered, as described previously [15], to produce a multiple sequence alignment. Phylogenetic analysis was performed using RAxML (version 7.0.3) [16] using a GTRGAMMA evolutionary model and support was assessed via 100 bootstrap replicates.

NG-MAST alleles, multilocus sequence typing (MLST) alleles, and molecular AMR determinants (reviewed in 6) were identified in silico based on the de novo assemblies. The NG-MAST[2] and Neisseria MLST[3] websites were used to assign allele numbers and sequence types (STs). NG-MAST genogroups, comprising the main ST plus closely related STs, were assigned as detailed above.

2.4 Data collection

Isolate antimicrobial susceptibility/resistance data and patient epidemiological data (gender, age and sexual orientation) were collected through the routine Euro-GASP data collection in May 2014. Data were reported by participating laboratories to the European Surveillance System (TESSy). The antimicrobial susceptibility/resistance and epidemiological data were linked to NG-MAST STs generated by this study. The complete dataset obtained from the WGS are, as with the NG-MAST data, owned by ECDC, the Euro-GASP network and the Wellcome Trust Sanger Institute. For feasibility, the WGS data are stored in an external database at the Wellcome Trust Sanger Institute. In this database, WGS data were linked to the antimicrobial susceptibility/resistance data and epidemiological data from each isolate/patient in TESSy by the Euro-GASP project team and main collaborators at the Wellcome Trust Sanger Institute. No linked data for the whole dataset are publicly accessible.

2.5 Data analysis

The prevalence of NG-MAST sequence types and genogroups were assessed for 2013 and compared with the 2009–2010 survey. Potential associations between genogroups and antimicrobial susceptibilities and genogroups and patient characteristics (gender, age and sexual orientation) were explored, first by means of univariate analysis and then, where appropriate, by multivariable analysis using STATA v12.1. Statistical significance for all tests was assumed when $p<0.05$. Identified associations were compared with those observed in the 2009–2010 survey.

2.5.1 Univariate analysis

Where datasets contained sufficient numbers, crude odds ratios (OR) and 95% confidence intervals (CI) were calculated. A Pearson Chi^2-test (X^2) was used to test if these ORs were significantly different from 1 (i.e. testing the null hypothesis that there was no difference in odds of AMR between the group in question and the specified baseline group). In cases with small or zero cell numbers, Fisher's exact test was performed.

2.5.2 Multivariable analysis

The multivariable analysis used logistic regression to model the odds of associations between genogroup and AMR controlling for other variables. Using a forward stepwise approach, the most significant and strongest associations from the univariate analysis were added to a multivariable logistic regression model sequentially.

[2] www.ng-mast.net

[3] http://pubmlst.org/neisseria

3 Results

3.1 Frequency of NG-MAST STs

In total, 1189 isolates were successfully re-retrieved and tested with NG-MAST. Of these 1189 isolates, WGS was performed on all viable isolates, and quality assured WGS data linked to AMR profiles and epidemiological data were obtained for 1054 (89%) of the isolates (Table 1).

Table 1. Number of isolates available from Euro-GASP for molecular typing

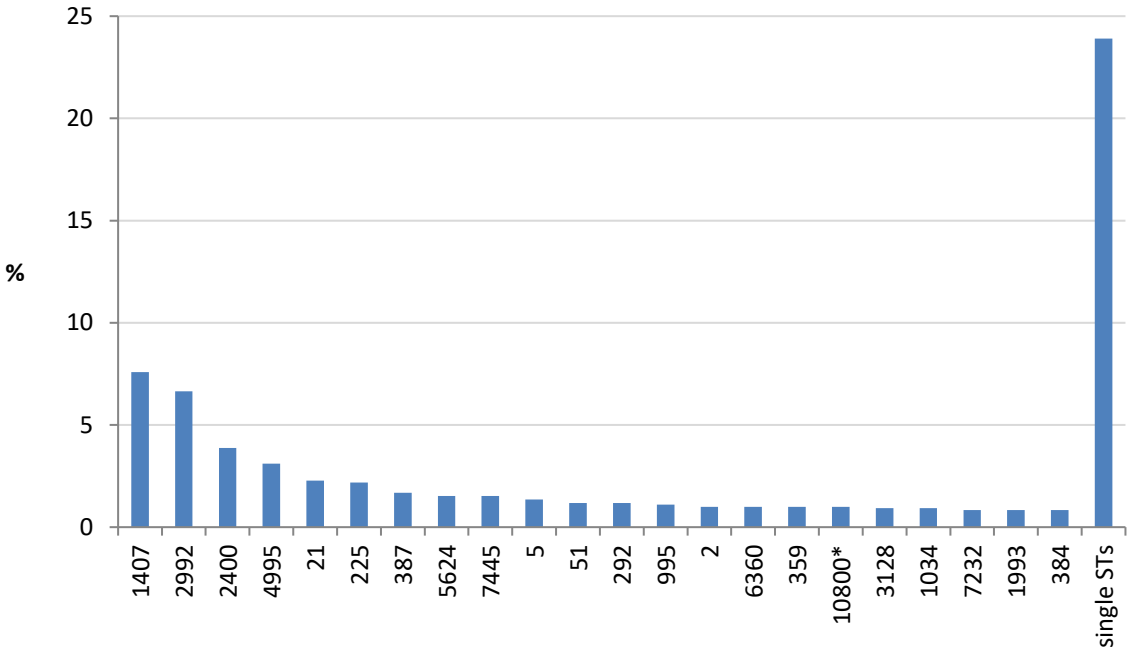
Country	Number of isolates available Oct/Nov 2013	Number of isolates tested using NG-MAST	Number of isolates tested using WGS
Austria	55	54	54
Belgium	55	55	55
Cyprus^	9	5	8
Denmark	56	56	55
France	58	58	57
Germany	50	48	47
Greece	50	48	48
Hungary	50	48	48
Iceland^	5	4	5
Ireland	48	45	-*
Italy	50	49	26
Latvia^	38	38	38
Malta^	31	21	20
Netherlands	88	89**	66
Norway^	112	55	55
Portugal	55	109**	108
Slovakia	56	56	38
Slovenia^	73	55	54
Spain^	119	119**	116
Sweden	50	50	50
United Kingdom	110	127**	106
Total	**1218**	**1189**	**1054**

** No viable isolates available for WGS. ** Additional isolates/data outside the October/November 2013 collection period submitted.*

^Isolates collected throughout 2013

Among these isolates, 430 different STs were identified, representing 333 different *porB* alleles and 106 different *tbpB* alleles. In addition, 146 clusters (two or more isolates with the same NG-MAST) were identified. There were 284 single STs and 225 new STs. The most frequently observed STs represented by ≥13 isolates were ST1407, ST2992, ST2400, ST4995, ST21, ST225, ST387, ST5624, ST7445, ST5, ST51, ST292 and ST995 (Figure 1). The *porB* and *tbpB* alleles defining these most frequently observed STs (represented by ≥10 isolates) are displayed in Table 2.

Figure 1. NG-MAST STs represented by 10 or more isolates (%)



** New sequence type*

Table 2. The porB and tbpB alleles defining the most frequently observed NG-MAST STs (represented by ≥10 isolates)

ST (Number)	NG-MAST allele	
	porB	tbpB
ST1407 (90)	908	110
ST2992 (79)	1808	29
ST2400 (46)	1489	563
ST4995 (36)	3031	33
ST21 (28)	14	33
ST225 (26)	4	4
ST387 (20)	266	118
ST5624 (18)	90	953
ST7445 (18)	28	24
ST5 (16)	4	33
ST51 (14)	39	27
ST292 (14)	28	4
ST995 (13)	28	29
ST2 (12)	2	16
ST359 (12)	301	29
ST6360 (12)	3957	563
ST10800 (12)	6328*	27
ST1034 (11)	430	33
ST3128 (11)	1900	110
ST384 (10)	263	26
ST1993 (10)	1257	6
ST7232 (10)	1489	1388

** New allele*

Compared to 2009–2010, ST1407 and ST2992 have remained the two most common STs in EU/EEA. However, in 2013, ST1407 represented only 7.6% of all isolates, while this ST in 2009–2010 represented more than 15% (15.6%) of isolates [3]. Table 3 shows the most frequently occurring ST for each country (further details of all STs observed in each participating country are described in Annex 1). The prevalence of the three most common STs (ST1407, ST2992 and ST2400) varied considerably among countries (Table 3). ST1407 accounted for ≥10% of isolates in nine (43%) countries and was the predominant ST in seven (33%): Belgium, Cyprus, Hungary, Norway,

Portugal, Slovakia, and Spain. ST2992 was also common and ≥10% of isolates were assigned as this ST in six (29%) countries (Annex 1). For comparison, in 2009–2010, ST1407 accounted for ≥10% of isolates in 13 (62%) countries and was the most frequent ST in nine (43%) countries [3]. The predominant ST changed in all participating countries since the last survey, other than in Ireland (ST2992), Portugal (ST1407) and Spain (ST1407) (Table 3)

Table 3. Most frequently observed NG-MAST ST in each country

Country	Most frequent ST (in 2009/2010)	Total number of isolates typed	Total number of STs in each country	Number of ST 1407 (%)	Number of ST 2992 (%)	Number of ST 2400 (%)
Austria	ST3785 (ST1407)	54	26	1 (2)	0 (0)	2 (4)
Belgium	ST1407 (ST387)	55	24	9 (16)	8 (15)	4 (7)
Cyprus	ST1407 (ST3128)	5	2	4 (80)	0 (0)	0 (0)
Denmark	ST1993 (ST225/3158)	56	30	7 (12)	3 (5)	4 (7)
France	ST645 (ST2/2992)	58	45	0 (0)	2 (3)	2 (3)
Germany	ST4995 (ST25)	48	33	1 (2)	2 (4)	1 (2)
Greece	ST3128 (ST5405/5505)	48	20	0 (0)	1 (2)	1 (2)
Hungary	ST1407 (ST5332)	48	22	10 (21)	0 (0)	0 (0)
Iceland	ST1034/9541/ 10640/11080* (NA)	4	4	0 (0)	0 (0)	0 (0)
Ireland	ST2992 (ST2992)	45	27	0 (0)	7 (16)	2 (4)
Italy	ST2992 (ST1407)	49	19	5 (10)	9 (18)	6 (12)
Latvia	ST5 (ST3227)	38	15	0 (0)	0 (0)	2 (5)
Malta	ST2992 (ST225)	21	10	2 (10)	7 (33)	0 (0)
Netherlands	ST2400/2992 (ST1407)	89	48	1 (1)	9 (10)	9 (10)
Norway	ST1407 (ST2992)	55	41	5 (9)	2 (4)	3 (5)
Portugal	ST1407 (ST1407)	109	58	17 (16)	1 (1)	4 (4)
Slovakia	ST359/1407 (ST437)	56	21	8 (14)	5 (9)	1 (2)
Slovenia	ST21/10801* (ST1407)	55	27	2 (4)	1 (2)	0 (0)
Spain	ST1407 (ST1407)	119	66	13 (11)	8 (7)	0 (0)
Sweden	ST5445 (ST225)	50	34	0 (0)	1 (2)	2 (4)
UK	ST2992 (ST1407)	127	58	5 (4)	13 (10)	5 (4)

** New sequence type*

3.2 Definition and frequency of genogroups

Further analysis of the *porB* and *tbpB* alleles of the STs observed in ≥10 isolates showed that for many of the most common STs, several other STs within the total collection were highly related. STs were therefore clustered on the basis of this close relationship into 'genogroups' (see section 2.2 of Methods above), comprising the main ST plus all other closely related STs (Table 4). Eighteen genogroups were identified (Table 5) including the eight major genogroups G1407, G2992, G21, G2400, G51, G225, G4995 and G387 which were further defined (Table 4) and encompassed 585 (49%) of the 1189 isolates tested.

Table 4. Details of different NG-MAST STs within the eight major genogroups defined and level of variation in the porB and tbpB alleles for each ST

No. of bp difference from *porB* allele x*	Sequence Types within each Genogroup (G) (n=)							
	G1407	G2992	G21	G2400	G51	G225	G4995	G387
	1407 (90)	2992 (79)	21 (28)	2400 (46)	51 (14)	225 (26)	4995 (36)	387 (20)
1	2212 (7) 3149 (3) 3378 (5) 3431 (3) 4120 (3) 4275 (7) 5335 (2) 5622 (3) 8953 (2) 10026 (1) 11614 (1)	4397 (1) 4684 (1) 4751 (1) 5119 (3) 6335 (1) 7674 (2) 9193 (1) 9925 (1)	5 (16) 1034 (11) 5445 (7) 8329 (3) 10257 (1) 11075 (1)	6360 (12) 7345 (1) 8115 (4) 10789 (1)	25 (4) 239 (2) 273 (1) 3003 (1) 8148 (1)	346 (1) 437 (4) 891 (1) 1132 (2) 9164 (1) 11055 (5)	10846 (2)	5120 (3) 6232 (1) 11051 (1)
2	3158 (2) 4269 (4) 4706 (4) 5533 (1) 6827 (1) 8826 (2) 9815 (1) 10783 (1)	7994 (1) 11023 (1)	3485 (1) 9154 (1) 9155 (1)	11068 (1)	356 (2) 807 (5) 11067 (2) 11069 (1)	1103 (2) 11043 (2)	-	3227 (2)
3	3128 (11) 4947 (1) 6969 (1) 9158 (2) 9972 (3) 10006 (1) 10675 (2) 10778 (1) 10779 (1) 10782 (1) 10834 (1) 11050 (1)	-	9969 (1)	-	5341 (1) 10800 (12) 11029 (2) 11070 (2)	10843 (2)	-	5158 (1) 6982 (1) 11608 (1)
4	11048 (1) 11052 (1) 11053 (1) 11056 (2)	-	10993 (1)	-	4338 (2) 11042 (7)	8820 (1)	-	-
5	-	-	-	-	11065 (1)	-	-	-
1 bp difference from *tbpB* allele x†	-	-	11064 (2)	11098 (1)	-	9162 (1)	-	-
Differ in both *porB* and *tbpB* alleles	10004 (1)[a] 10005 (1)[b]	-	-	-	4330 (1)[c]	-	-	-
Total number (%)	176 (14.8%)	92 (7.7%)	74 (6.2%)	66 (5.6%)	61 (5.1%)	48 (4.0%)	38 (3.2%)	30 (2.5%)

** STs share an identical tbpB allele with the most frequent ST but vary at the porB allele*

† STs share an identical porB allele with the most frequent ST but vary at the tbpB allele

[a]STs with 2 bp difference in porB allele and 1 bp difference in tbpB allele

[b]STs with 3 bp difference in porB allele and 2 bp difference in tbpB allele

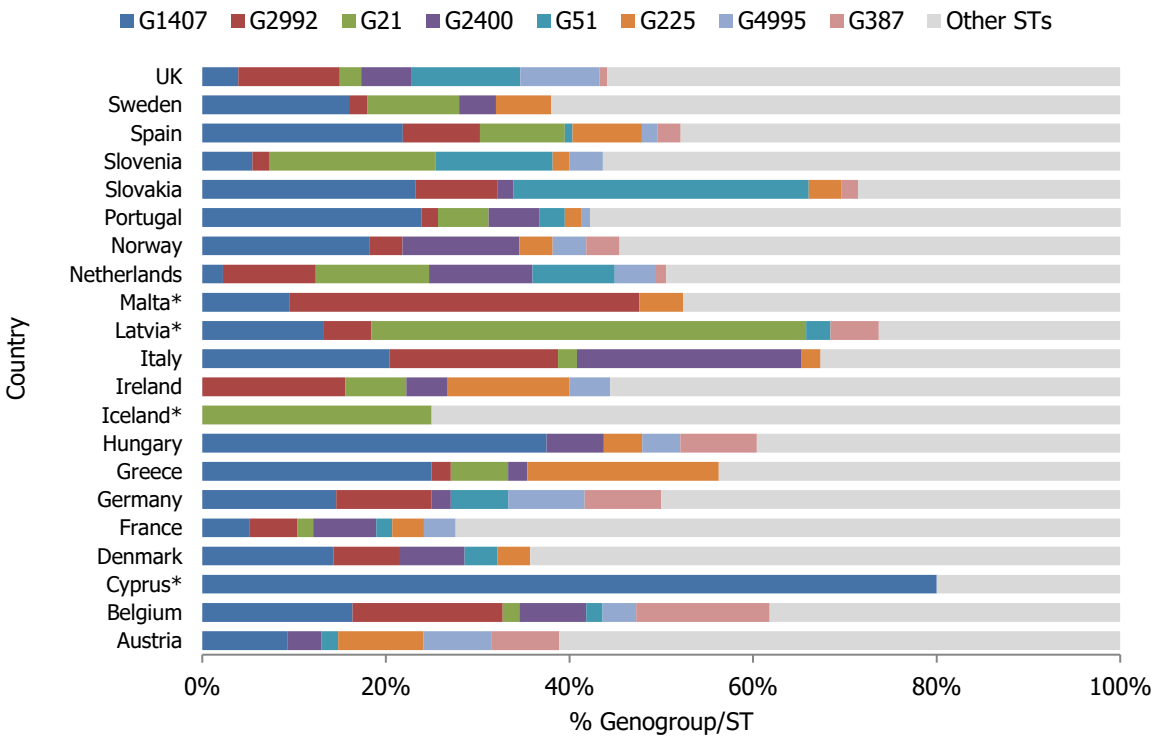
[c]STs with 1 bp difference in porB allele and 1 bp difference in tbpB allele

Table 5. Characteristics of patients infected with the most frequently observed NG-MAST genogroups

Genogroup (n=)	Mean patient age (range in years)	No. of patients with known gender	% male patients (n=)	No. of patients with known sexual orientation (no. of males)	% heterosexual (males and females) (n=)	% men who sleep with men (n=)
G1407 (176)	33.6 (15-72)	174	82.2 (143)	66 (55)	86.4 (57)	13.6 (9)
G2992 (92)	31.8 (17-63)	91	91.2 (83)	63 (58)	25.4 (16)	74.6 (47)
G21 (74)	32.4 (17-61)	73	89.0 (65)	46 (41)	50.0 (23)	50.0 (23)
G2400 (66)	31.4 (0-66)	66	95.5 (63)	39 (38)	20.5 (8)	79.5 (31)
G51 (61)	26.7 (17-50)	61	50.8 (31)	48 (23)	100 (48)	0
G225 (48)	33.4 (16-64)	48	83.3 (40)	24 (21)	58.3 (14)	41.7 (10)
G4995 (38)	34.4 (20-51)	38	100 (38)	17 (17)	5.9 (1)	94.1 (16)
G387 (30)	30.0 (18-47)	28	78.6 (22)	14 (10)	100 (14)	0
G2 (19)	25.3 (16-47)	19	52.6 (10)	9 (2)	100 (9)	0
G5624 (18)	38.0 (19-51)	17	94.1 (16)	12 (11)	8.3 (1)	91.7 (11)
G7445 (18)	34.7 (17-55)	18	100 (18)	6 (6)	33.3 (2)	66.7 (4)
G292 (17)	31.9 (19-42)	17	82.4 (14)	8 (7)	25 (2)	75 (6)
G384 (16)	29.4 (18-54)	16	93.8 (15)	3 (3)	66.7 (2)	33.3 (1)
G359 (15)	33.9 (17-54)	15	73.3 (11)	5 (4)	100 (5)	0
G995 (14)	31.8 (18-48)	14	92.9 (13)	7 (6)	42.9 (3)	57.1 (4)
G5333 (14)	35.4 (19-56)	14	78.6 (11)	8 (7)	75 (6)	25 (2)
G1993 (10)	31.7 (20-45)	10	30 (3)	10 (3)	100 (10)	0
G7232 (10)	33.8 (19-48)	10	90 (9)	0	0	0
All isolates (1189)	31.7 (0-73)	1179	84.6 (997)	508	55.3 (337)	44.7 (273)

The proportion of each genogroup observed in participating countries is presented in Figure 2. G1407 has widespread distribution, being found in all countries examined with the exception of Ireland and Iceland, and was predominant in Cyprus (80%), Denmark (14.3%), Hungary (37.5%), Norway (18.2%), Portugal (23.9%) and Spain (21.8%). In contrast, G1407 was comparatively uncommon (<10%) in Austria, France, Malta, the Netherlands, Slovenia and the United Kingdom. Compared to the previous study [3], the overall prevalence of G1407 has decreased significantly (from 23.3% in 2009–10 to 14.8% in 2013, Z-test $p<0.001$). In 2013, G1407 appeared to be most prevalent in southern Europe, Germany and the Scandinavian countries (Figure 3), whereas in 2009–2010 G1407 was prevalent in eastern and southern Europe (Figure 4). G2992 was observed in 17 (81%) countries, was predominant in Ireland and Malta and relatively common in Belgium, Italy, the Netherlands and the United Kingdom. G21 was observed in 13 (62%) countries and was predominant in Ireland, Latvia and Slovenia, while G51 was predominant in Slovakia (Figure 2). The number of isolates from some countries was rather small, in particular from Cyprus (5 isolates) and Iceland (4 isolates).

Figure 2. Distribution of NG-MAST genogroups within each participating country



** Countries with low isolate collections*

Figure 3. Prevalence of NG-MAST G1407 in participating EU/EEA countries, 2013

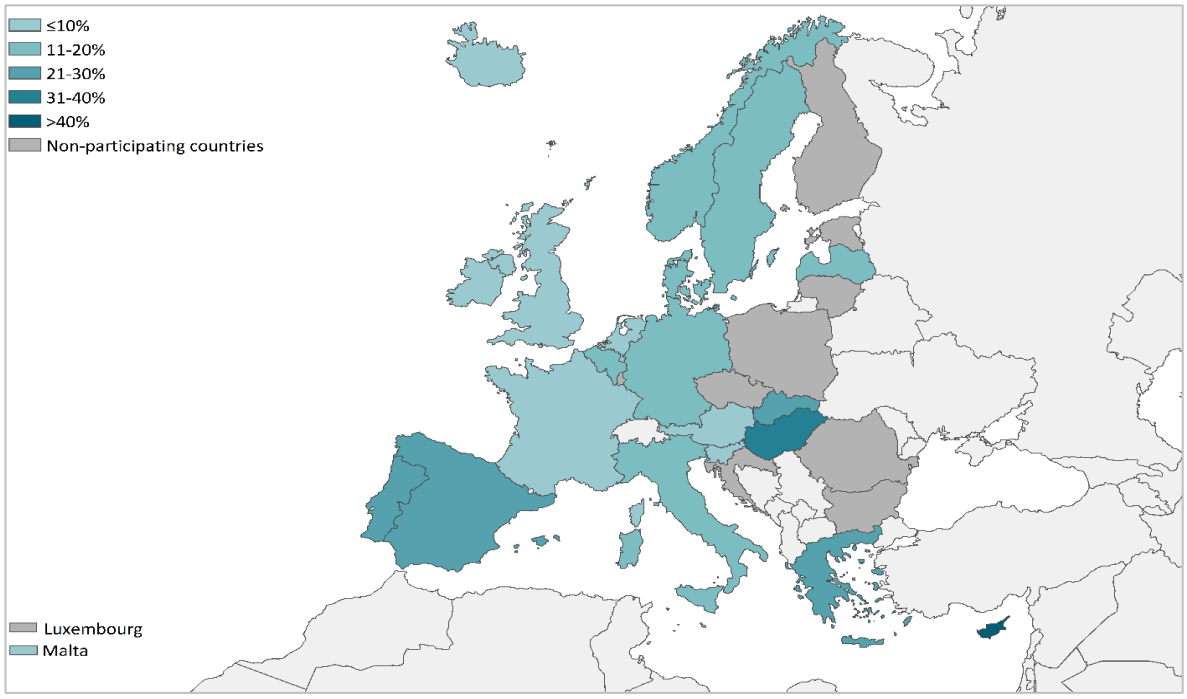
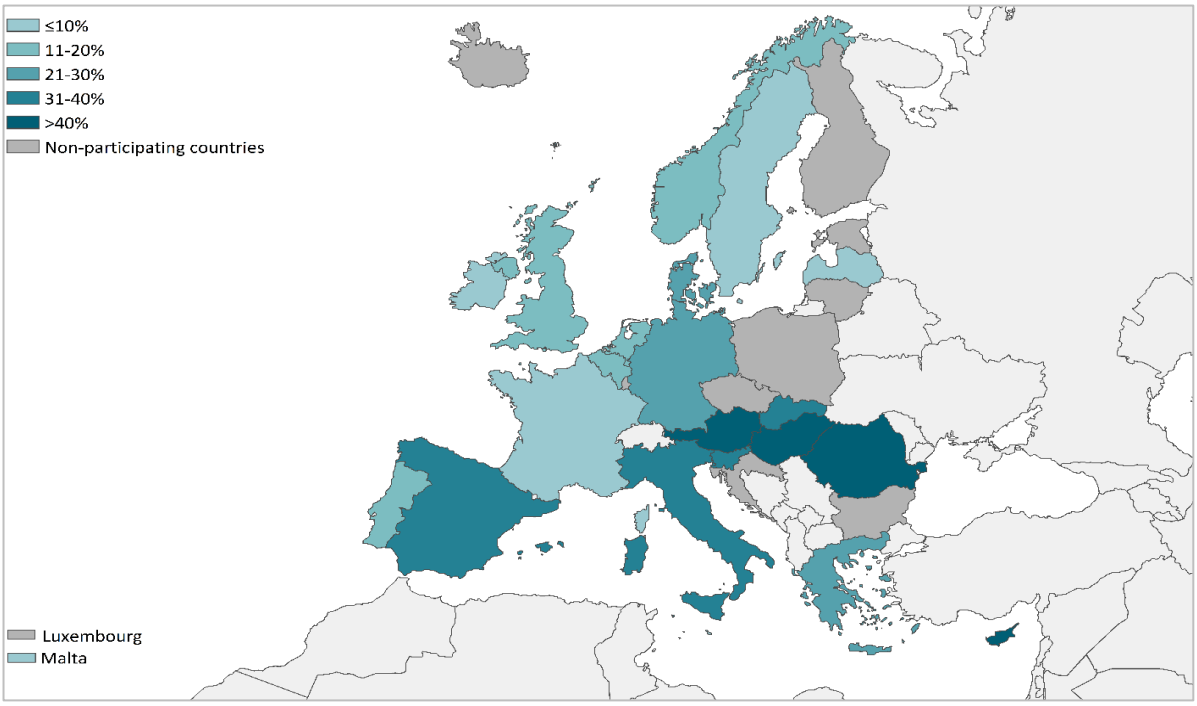


Figure 4. Prevalence of NG-MAST G1407 in participating EU/EEA countries, 2009-2010



3.3 Sequence type and epidemiological characteristics of linked patients

For the 1189 patients included in this study, age and gender were the most frequently collected variables, known for 1159 and 1179 patients, respectively. Sexual orientation was known for 610 patients. Table 5 presents the epidemiological characteristics of the patients infected with a genogroup represented by ≥10 isolates and the univariate analysis results are presented in Table A2.1 (Annex 2).

Four patient age groups were assigned and were composed of the following patient numbers: ≥15 to <25 years (29%, n=335); ≥25 to <35 years (38%, n=440); ≥35 to <45 years (19.8%, n=229) and ≥45 years (13.2%, n=153). For 32 patients, age was unknown or patients were <15 years and therefore not included in the analysis. In total, five genogroups were composed of isolates from patients that differed significantly compared to the age group proportion of all patients; G1407, G4995, G2992, G51 and G2. For three genogroups there was a significant association with older patients (reference: patients aged 15-24 years): G1407 and patients ≥45 years (OR 1.8, 95% CI 1.08-2.98, p=0.0218, X^2 test); G4995 and patients 35-44 years (OR 2.77, 95% CI 1.01-7.63, p=0.0401, X^2 test); and G2992 and patients 25-34 years (OR 1.85, 95% CI 1.06-3.24, p=0.029, X^2 test). In contrast, G51 and G2 were significantly more common in the youngest age group (15-24 years) (G51: p<0.0001; G2: p=0.05, Fisher's exact test). A negative association of G1407 was observed with younger patients aged <25 years (reference: patients aged ≥45 years: OR 0.55, 95% CI 0.34-0.92, p=0.0218, X^2 test). The association of G51 and G2 and the youngest age group (15-24 years) was also observed in the 2009-2010 study [3].

The majority of gonococci in this study were collected from men (84.6%, n=997). A total of 182 isolates were from females (15.4%) and gender was reported as unknown for 10 cases. For most genogroups there was no clear link to gender, with only three genogroups composed of isolates from patients with a higher proportion of females when compared to the overall sample; G51, G2 and G1993 (G51: OR 6.15, 95% CI 3.57-10.59, p<0.0001, X^2 test; G2: OR 5.13, 95% CI 2.04-12.9, p=0.0001, X^2 test; G1993: p<0.0001, Fisher's exact test). In contrast, the proportion of males infected was significantly higher than the overall proportion for G2400 (p=0.008, Fisher's exact test) and G4995 (p=0.002, Fisher's exact test). The significant genogroup and gender associations observed in the previous Euro-GASP molecular typing study [3] and not observed in the current study were: G387 (lower proportion of males), G2992 and G1407 (higher proportion of males). The G2 association with females in this study was not observed in the previous study. Genogroups G51, G1993, G2400 and G4995 were not present in the previous study.

Sexual orientation information was available for 51.3% (610) of cases; 55.2% (337) were heterosexual and 44.8% (273) where MSM. Nine genogroups were composed of isolates from patients with a statistically different proportion of heterosexuals and MSM when compared to the overall mean: G1407, G51, G387, G2, G1993, G2992, G2400, G4995 and G5624. A strong association was observed between heterosexuals and infection with G1407 (OR 5.97, 95% CI 2.85-12.53, p<0.0001, X^2 test), along with infection in heterosexuals and G51, G387, G2 and

G1993 (p<0.0001, Fisher's exact test). In contrast, MSM were more frequently infected with G2992 (OR 4.17, 95% CI 2.28-7.64, p<0.0001; X^2 test), G2400 (OR 5.27, 95% CI 2.35-11.83, p<0.0001, X^2 test) and both G4995 and G5624 (p<0.0001, Fisher's exact test) when compared to heterosexuals. Notably, the 2009–2010 Euro-GASP molecular typing study already showed strong associations between MSM and G2992, in addition to links between heterosexuals and G387 and G2 being identified [3]. Compared to 2009-2010 [3], there was a change in the association of G1407 and MSM as previously described.

3.4 Sequence type and AMR associations

Table 6 shows the consensus antimicrobial susceptibility results for genogroups/STs represented by 10 or more isolates and the number of isolates that differ from the consensus. The majority of isolates that differed from the consensus for azithromycin were usually very close to the minimum inhibitory breakpoint (MIC) breakpoint by Etest.

Statistical analyses were applied to explore associations between genogroups and AMR as described in section 2.5 and the univariate analysis results are presented in Table A2.2 (Annex 2).

In Table 6, the consensus MIC for cefixime was the highest for G1407. The association between this genogroup and cefixime resistance (MIC >0.125 mg/L) was highly significant (OR 13.24, 95% CI 7.06-24.8, p<0.0001, X^2 test) by univariate analysis and also following multivariable analysis to control for patient age, gender and sexual orientation (OR 13.27, 95% CI 5.22-33.76, p<0.0001, X^2 test). Similar strong associations for G1407 were identified also in the Euro-GASP 2009-2010 molecular typing study [3], although it should be noted that the breakpoint of ≥0.125 mg/L was used in the previous study. This analysis was not performed for ceftriaxone as there were only six G1407 isolates showing resistance to ceftriaxone (MIC >0.125 mg/L), but these six isolates were the only ceftriaxone resistant ones in the entire material and the consensus MIC was raised compared with those for most other genogroups (Table 6). In addition, all but two isolates of G1407 were ciprofloxacin resistant (p<0.0001, Fisher's exact test), which was a strong association identified also in the Euro-GASP 2009-2010 molecular typing study [3]. While the consensus susceptibility for azithromycin was sensitive, the proportion of isolates showing MICs close to the breakpoint, and so in the resistant category, was significant in the univariate analysis (OR 3.78, 95% CI 2.26-6.31, p<0.0001, X2 test), as well as in the multivariable analysis after controlling for patient age, gender and sexual orientation (OR 3.06, 95% CI 1.32 – 7.09, p=0.009, X2 test). As MIC data for azithromycin were not available for all isolates, with susceptibility principally determined by a breakpoint method, it is only possible to speculate that these results indicate a significant association between G1407 and azithromycin MICs close to the breakpoint for resistance. None of the other genogroups included in Table 6 showed a modal MIC to cefixime as high as G1407, however the high-range MICs of G21, G2400, G292, and G7232, which included cefixime resistant isolates, are of concern. In contrast, G2992, the second most common genogroup, is mainly sensitive to all antimicrobials tested, with a significant association between G2992 and susceptibility to cefixime (p=0.03, Fisher's exact test) and ciprofloxacin (p<0.0001, Fisher's exact test) demonstrated. The modal MICs of the genogroups present in this study and the previous Euro-GASP molecular typing study (G1407, 2992, 225, 387, 359, and 2) were the same or differed by just one dilution [3].

For the remaining genogroups a significant association was detected between genogroups G21 and G7445, and azithromycin resistance (G21: OR 4.3, 95% CI 2.25-8.2, p<0.0001, X2 test. G7445: OR 36.1, 95% CI 12.43-105.04, p<0.0001, X2 test) and ciprofloxacin susceptibility (G21: OR 0.08, 95% CI 0.04-0.19, p<0.0001, X2 test. G7445: p<0.0001, Fisher's exact test). Isolates in genogroups G2400, G225, G4995, G5624, G5333 and G7232 were significantly associated with ciprofloxacin resistance (p<0.0001 for all genogroups other than G5333; p=0.002, Fisher's exact test). In contrast, isolates in genogroups G51, G387, G359, G2, G7445, G292, G384, G995 and G1993 were significantly associated with ciprofloxacin susceptibility (p=0.003 (G359), p=0.001 (G1993) and p<0.0001 (all other genogroups), Fisher's exact test), and G51 was additionally associated with azithromycin susceptibility (p=0.029, Fisher's exact test).

Table 6. Consensus antimicrobial susceptibility results for NG-MAST genogroups (G) represented by 10 or more isolates

Genogroup or ST	No. of isolates	Consensus resistance category (No. isolates differing from consensus) [No. of samples with associated data]			Modal MIC mg/L (range) [No. of samples with associated data]		
		Azithromycin	Ciprofloxacin	Spectinomycin	Gentamicin	Cefixime	Ceftriaxone
G1407	176	S (I=33, R=27)	R (S=2)	S	8 (2-16) [n=146]	0.125 (0.016-0.5)	0.047 (0.002-0.5)
G2992	92	S (I=17, R=3) [91]	S (R=3)	S	8 (3-16) [n=44]	0.016 (0.008-0.12)	0.004 (0.002-0.064)
G21	74	S (I=10, R=14) [72]	S (R=7)	S	8 (2-16) [n=52]	0.016 (0.004-0.25)	0.023 (0.002-0.125)
G2400	66	S (I=4, R=1)	R	S	8 (3-16) [n=42]	0.023 (0.016-0.25)	0.032 (0.008-0.064)
G51	61	S	S (R=2)	S	8 (1-16) [n=38]	0.016 (0.004-0.032)	0.004 (<0.002-0.016)
G225	48	S (I=8, R=4) [47]	R	S	8 (1-16) [n=31]	0.016 (0.016-0.125)	0.032 (0.002-0.094)
G4995	38	S (I=2)	R (S=1)	S	8 (4-16) [n=19]	0.016 (0.008-0.064)	0.008 (0.002-0.032)
G387	30	S	S (R=1)	S	8 (2-8) [n=20]	0.016 (0.002-0.064)	0.002 (<0.002-0.015)
G2	19	S	S (R=2)	S	4 (2-8) [n=12]	0.016 (0.004-0.125)	0.002 (0.002-0.064)
G5624	18	S (I=4)	R	S	4 (3-16) [n=10]	0.016 (0.008-0.032)	0.004 (0.002-0.015)
G7445	18	R (S=3, I=3)	S (R=1)	S	4 (3-12) [n=15]	0.016 (0.015-0.016)	0.003, 0.006 (0.002-0.125)
G292	17	S [16]	S (I=2)	S	8 (3-8) [n=9]	0.016 (0.008-0.25)	0.004 (0.002-0.064)
G384	16	S	S	S	4 (2-8) [n=4]	0.016 (0.004-0.032)	0.002 (<0.002-0.016)
G359	15	S	S (R=2)	S	8 (6-8)	0.032 (0.016-0.047)	0.016 (0.004-0.023)
G995	14	S	S	S	8 (3-8) [n=9]	0.016 (0.008-0.064)	0.008 (0.002-0.023)
G5333	14	S	R (S=1)	S	8 (3-8) [n=12]	0.016 (0.008-0.032)	0.023 (0.002-0.047)
G1993	10	S (I=3)	S	S	2, 3 (2-3)	0.016 (0.016)	0.002 (0.002)
G7232	10	S	R	S	8 (8)	0.064 (0.064-0.25)	0.064 (0.032-0.064)

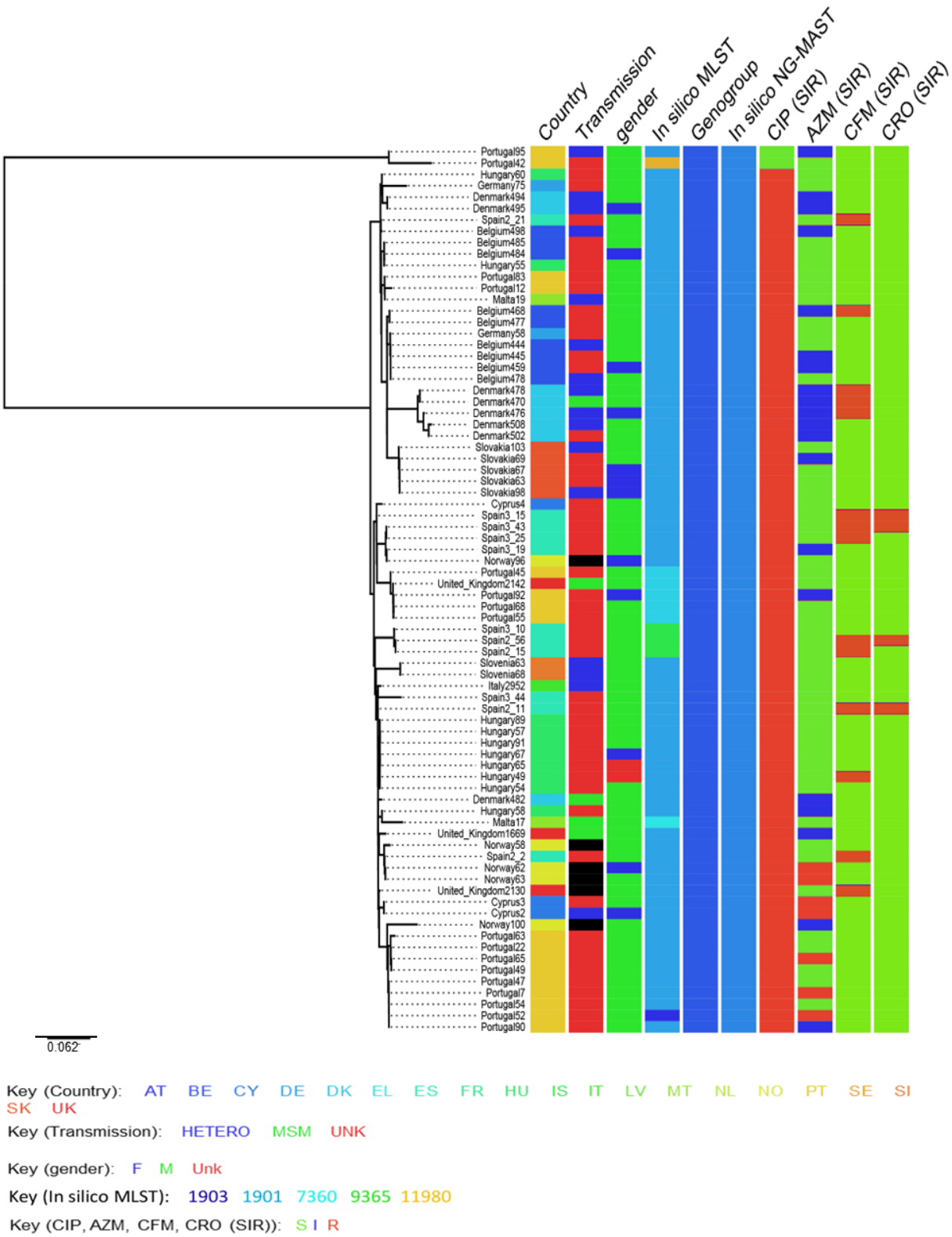
S – susceptible; I – intermediate susceptible; R – resistant

3.5 Whole genome sequencing (WGS)

WGS was performed on all viable isolates and quality assured WGS data that were linked to AMR profiles and epidemiological data were obtained for 1054 (89%) isolates (Table 1). Briefly, the phylogenomics based on the whole genome sequences (1054 isolates) showed a significantly higher and more accurate resolution of the isolates (Figures 5 and 6).

Among the 1054 isolates, 373 different NG-MAST STs, including 130 clusters (≥2 isolates with identical ST) and 243 STs represented by single isolates, were identified in silico from the whole genome sequences. No NG-MAST ST could be assigned for 16 isolates and multiple NG-MAST alleles were identified in 21 isolates. The most frequent STs were ST1407 (n=78), ST2992 (n=68), ST2400 (n=47), ST4995 (n=31), and ST21 (n=25). The phylogenomics based on the WGS data further discriminated isolates with identical ST. For example, among the NG-MAST ST1407 isolates (n=78) several smaller clades were observed, which mostly appeared to represent specific evolutionary traits in the same country. Furthermore, the whole genome sequences of two ST1407 isolates from Portugal were highly different from all the remaining whole genome sequences (Figure 5).

Figure 5. Phylogenomics of 78 *Neisseria gonorrhoeae* NG-MAST ST1407 isolates obtained in EU/EEA in 2013



Note: Scale represents genetic change measured as the number of nucleotide substitutions divided by length of the sequence.

Of the 1054 isolates, 646 (61%) could be assigned to an NG-MAST genogroup (in total 18 genogroups) and the eight predominant genogroups were G1407 (n=162), G2992 (n=81), G21 (n=68), G2400 (n=65), G51 (n=49), G225 (n=39), G4995 (n=30) and G387 (n=29). As observed for the NG-MAST STs, the WGS data further discriminated isolates with identical NG-MAST genogroups. For example, among the NG-MAST G1407 isolates

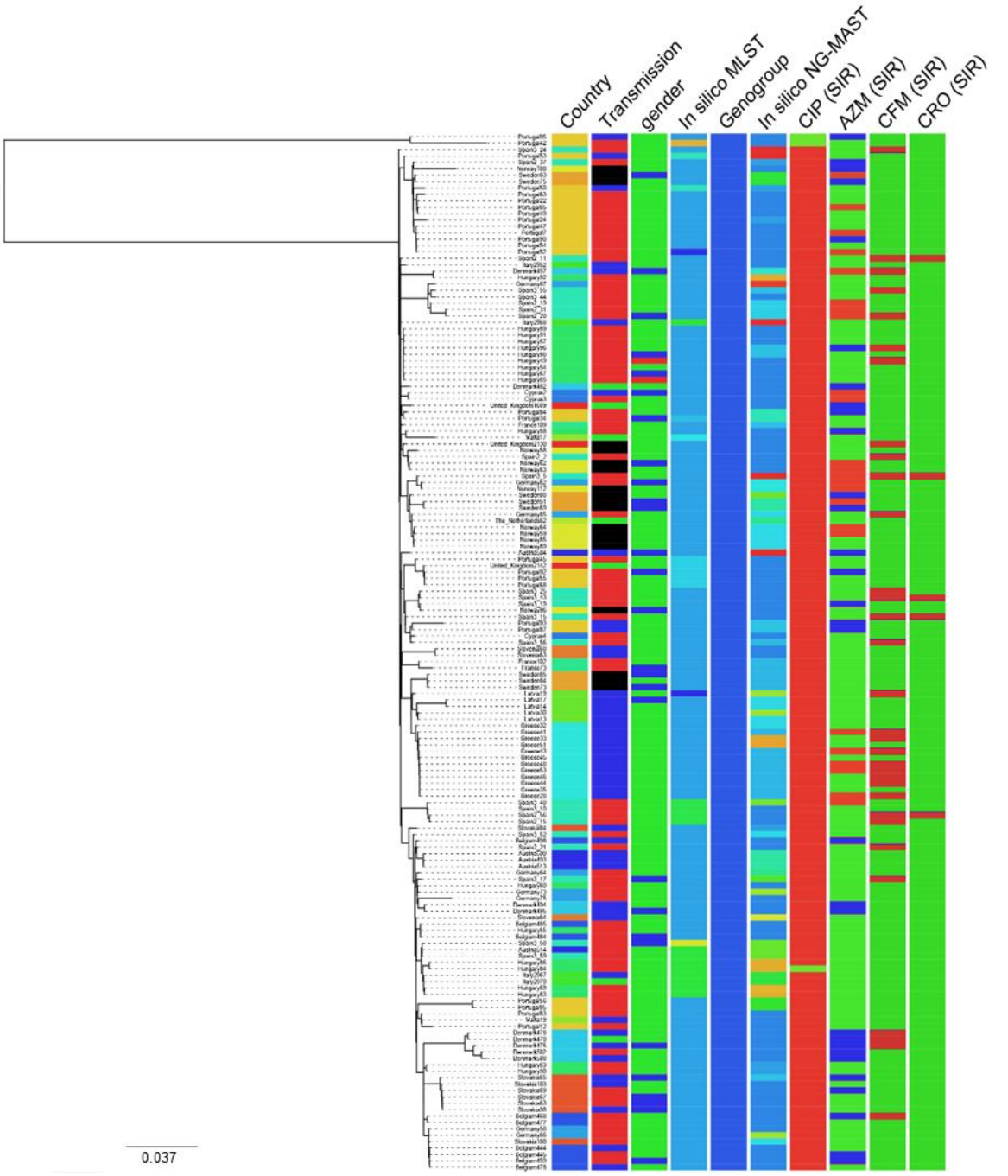
(n=162) many minor clades were identified, which appeared to reflect specific evolutionary traits in the same country (Figure 6).

Among the 1054 isolates, 114 different MLST STs (40 new STs), including 68 clusters (≥2 isolates with identical ST) and 46 STs represented by single isolates, were also identified in silico from the WGS data. All isolates could be assigned ST and the most frequent STs were ST1901 (n=220), ST9363 (n=87), ST7363 (n=78), ST1579 (n=68), and ST1588 (n=61). ST1901 was found in all countries except Iceland and was the predominant ST in 13 (65%) countries. ST1901 comprised most (83%) of the NG-MAST G1407 isolates, and also 95% of the NG-MAST ST225 isolates.

Finally, AMR determinants were also identified from the WGS data. These mostly included all known resistance determinants for sulphonamides, penicillins, tetracyclines, spectinomycin, rifampicin, fluoroquinolones, macrolides (e.g. azithromycin), cefixime and ceftriaxone.

The results of the WGS of all 1054 gonococcal isolates are detailed in a separate scientific publication.

Figure 6. Phylogenomics of 162 *Neisseria gonorrhoeae* NG-MAST genogroup 1407 isolates obtained in EU/EEA in 2013



Note: Scale represents genetic change measured as the number of nucleotide substitutions divided by length of the sequence; top five MLST and NG-MAST sequence types displayed in the legend.

4 Conclusions

4.1 Molecular typing of European gonococcal isolates from 2013

This report describes the second survey of *N. gonorrhoeae* genotypes across the EU/EEA and provides recent information on NG-MAST STs, NG-MAST genogroups, and genotypes based on WGS circulating in 21 EU/EEA Member States. In fact, this is the first time WGS has been used in any international surveillance programme for sexually transmitted infections. Although there is considerable diversity of gonococcal genotypes both within and among EU countries, some genotypes or clones are predominant. Nevertheless, given the variation in sampling strategy and number of isolates in different countries, the prevalence of genotypes (NG-MAST STs, genogroups, and genotypes based on WGS) should be interpreted with caution.

The multidrug-resistant NG-MAST G1407 remained the major genogroup and is widely distributed in the EU/EEA. It is found in all included countries except Ireland and Iceland, and was predominant in six (29%) countries. However, compared to the previous Euro-GASP molecular typing study [3] the overall prevalence of G1407 has decreased greatly, i.e. from 23.3% in 2009–2010 to 14.8% in 2013, with a consequent decrease in cefixime resistance from 8.9% in 2010 to 4.7% in 2013 [1]. In 2013, G1407 appeared to be most prevalent in southern Europe, Germany and the Scandinavian countries, whereas in 2009-2010 the G1407 was prevalent in eastern and southern Europe [3]. The continuing predominance of a single NG-MAST genogroup is interesting given that the gonococcus is highly heterogeneous and that NG-MAST measures variation within two highly variable genes.

Nevertheless, the WGS analysis further discriminated the NG-MAST G1407 isolates and identified many minor clades among them, which appeared to be associated with specific evolutionary traits in the same country. Two G1407 isolates were also found to be highly different from all other G1407 isolates using WGS. This, together with similar findings for other genogroups, e.g. G21, G2992, G2400, clearly showed that the NG-MAST genogroups (based on diversity in only two genes) do not always reflect the genomic similarities or differences of isolates. However, the multidrug-resistant profiles were retained in all G1407 isolates.

In 2013, a strong association was identified between G1407 and resistance to cefixime and ciprofloxacin, with a relatively large proportion (34%) of the G1407 isolates also showing intermediate susceptibility or resistance to azithromycin. Similar associations were also found in the previous Euro-GASP molecular typing study [3]. The G1407 genogroup continues to predominate even though cefixime is no longer recommended as the first-line therapeutic agent to treat gonorrhoea. Six G1407 isolates were also resistant to the first-line treatment ceftriaxone (recommended to be given together with azithromycin [17]), the last remaining option for empiric first-line monotherapy of gonorrhoea. Accordingly, the continued predominance of G1407 isolates in many EU/EEA Member States is worrying, given their potential to be therapeutically challenging. Interestingly, whereas G1407 was more associated with MSM in the 2009-2010 Euro-GASP molecular typing study, G1407 was more associated with heterosexual patients as well as older patients (>45 years of age) in 2013. Thus, in 2013 this multidrug-resistant genogroup appears to have crossed over to and spread in the heterosexual population. Obviously, G1407 is circulating both in the heterosexual population as well as among MSM and, accordingly, the potential risks of future treatment failure are not restricted to any one patient group.

The decrease in the proportion of G1407 isolates since the previous Euro-GASP molecular typing study in 2009-2010 and the change in the epidemiological features of patients in respect to sexual orientation could be due to many factors. These may take account that an increased use of more sensitive molecular diagnostics and increased testing of extra-genital sites (particularly the pharynx) among MSM might have contributed to reducing the reservoir of strains such as those of G1407. Effective diagnostics has subsequently allowed the administration of appropriate antimicrobial therapy, which now includes dual antimicrobial therapy which can target resistant strains more effectively. G1407 strain(s) might also have been replaced by cefixime-susceptible and possibly more biologically fit gonococcal genogroups/strains. Statistically significant associations were also identified between ciprofloxacin resistance and the predominant genogroups
G1407, G2400, G225, G4995, G5624, G5333, and G7232. Fluoroquinolones are no longer recommended as empirical first-line therapy for gonorrhoea, but any stable association between susceptibility or resistance and particular NG-MAST STs/Gs might facilitate targeted personalised treatment of patients in the future, i.e. if the AMR reaches levels requiring empirical therapy to be replaced or most likely supplemented by individually-tailored treatment.

Statistical analyses demonstrated associations between patient characteristics and genogroups. For some genogroups (e.g. G51 and G2), patients were younger and a lower proportion of males were infected, which may indicate that some genotypes are more associated with heterosexual patients. This was also supported by further analysis of the subset of patients for which sexual orientation was known. G51, G2, G1993, G387 and G359, for example, were not found in MSM. The association between G2 and G387 and heterosexual patients was also identified in the Euro-GASP 2009–2010 molecular typing study [3]. In contrast, other genogroups were isolated

more frequently in MSM, supported by the associations of some genogroups with older patients and male gender (G2992, G4995 and G5624). Once again, analysis of the patient subset for which sexual orientation was known confirmed potential associations between MSM and genogroups G2992 and G4995. This association between G2992 and MSM was also identified in the Euro-GASP 2009–2010 molecular typing study [3]. However, given the limitations of the sampled study population and the lower reporting rates of epidemiological variables such as sexual orientation, all associations should be interpreted with caution. This is a major limitation of the current study in terms of evaluating the public health benefits of linking genogroups or STs to epidemiological characteristics. It should also be noted that the frequency and distribution of the NG-MAST STs is dependent upon the sample selection. To help avoid selection bias, participating laboratories are requested to submit a representative sample set to Euro-GASP [11]. For example, isolates should be selected from consecutive patients and from patients representing different patient groups and geographical regions within the country to reflect the distribution of gonorrhoea cases in that country. This is not always possible and the Euro-GASP team are continually looking at ways to improve representativeness.

4.2 Public health benefits of molecular typing

The present molecular typing study provides a new baseline with regard to the genetic heterogeneity of gonococcal isolates in EU/EEA, including identification of many novel genotypes, which can help inform future outbreak investigations and identification of AMR gonococcal strains. The use of molecular typing to predict AMR profiles has clear public health benefits as it contributes to the understanding of the dissemination of resistance within a population, and facilitates development of targeted intervention strategies, particularly if the association between molecular type and epidemiological characteristics is well defined within the study period. However, as has been observed with G1407, the epidemiological profile of the strains can change over time. In addition, if the prediction of AMR is sufficiently reliable, this approach could have a direct impact on the appropriate management of patients for whom culture and associated susceptibility testing has not been performed.

The results of the present study were also compared to results from the previous Euro-GASP molecular typing study in 2009–2010 and, accordingly, the spread of AMR gonococcal strains, with special focus on G1407, in the EU/EEA could be assessed geographically, temporally and linked to different patient groups. Statistical analyses in the current study showed clear associations between the AMR profile and particular molecular type. Although the representativeness of the sample and particularly the low level of reporting of several epidemiological variables are major limitations, this study, as well as the Euro-GASP 2009–2010 molecular typing study, have successfully demonstrated that this molecular surveillance approach is effective and has clear public health value. The potential benefits are most apparent in the case of G1407, which shows a strong association with resistance to ciprofloxacin and cefixime and accounted for six isolates resistant to ceftriaxone. Treatment failures with ceftriaxone or cefixime in patients infected with G1407 isolates have been documented in many European countries, South Africa, Japan, Australia and Canada [reviewed in 6–8]. G1407 isolates have also shown their capacity to develop high level resistance to ceftriaxone, as shown in isolates cultured in France [9] and Spain [18]. G1407 clearly remains a potential major public health problem if it continues to be disseminated without control measures being taken. In the present molecular survey, enhanced understanding of emergence and transmission of gonococcal strains and their AMR in different risk groups nationally and internationally was obtained. It was encouraging that the prevalence of G1407 had decreased, despite its continued high rate of occurrence. This fact, together with the evolution of G1407 and other clones and the changing molecular epidemiology suggest that continuous monitoring using WGS, for ideal resolution, in Euro-GASP for public health purposes is crucial.

In recent years, WGS has become less expensive and is more frequently used for molecular typing. WGS provided a number of advantages compared to NG-MAST: significantly higher and more accurate resolution; increased discrimination (including of isolates with identical NG-MAST ST); identification of NG-MAST STs and Gs that did not appropriately reflect the genomic backbone of the isolates; typing of all isolates; identification of samples with multiple sequences/isolates (which would not be possible using NG-MAST); evaluation of additional information such as diagnostic, molecular epidemiologic (e.g. MLST) and antimicrobial resistance markers (also for antimicrobials not tested in Euro-GASP) etc.), evolutionary traits etc. Due to the ideal resolution offered by WGS, it is possible for the first time to appropriately assess whether G1407 and other multidrug-resistant STs/Gs are clonal, stable and persist over time. In general, the use of WGS will provide enhanced and detailed understanding of the emergence and transmission of different gonococcal strains, AMR phenotypes, and gonorrhoea at a national and international level.

4.3 Molecular typing of gonorrhoea on European level

This report illustrates that there is a clear public health benefit to be gained from performing molecular typing, particularly using WGS, of gonorrhoea in Europe. However there are a number of areas that require further development before the maximum benefit can be gained from such a scheme:

- The variation in sampling strategy and sample size (affecting representativeness) among countries is a limitation of using this survey for surveillance, as is also the case for AMR surveillance. Further training and continued support to individual countries will be required to improve this situation.
- The quality of the epidemiological information collected (particularly the level of reporting of many variables) is a major limitation of the current study which hinders full evaluation of the public health benefit of molecular surveillance. This should be addressed as part of the general development of Euro-GASP.
- Further longitudinal typing of a representative sample would be required to monitor stability of associations among STs, genogroups, genomic clades and AMR and/or epidemiological characteristics, and to identify temporal changes and emergence of novel genotypes. This could be done on Euro-GASP isolates at least every third year using WGS (if funding is available).
- A number of challenges remain to practically consider WGS-based European *N. gonorrhoeae* molecular typing for surveillance. These include:
  - the development of an appropriate publicly available and user-friendly external database (including analysis tools). This type of database has also been developed during the present molecular typing study in collaboration with colleagues at the Wellcome Trust Sanger Institute and will be launched shortly;
  - the lack of unified and practical nomenclature for the results of WGS makes communication difficult. Therefore, it is recommended that in silico NG-MAST analysis (from the whole genome sequences) is performed, and NG-MAST STs and NG-MAST genogroups are continuously reported in TESSy. In TESSy, a unique identifier for each gonococcal isolate should be able to link to its whole genome sequence stored in the external database;
  - it is crucial to find simplified mechanisms for sharing epidemiological data on gonococcal isolates (stored in TESSy) with an external database. This is essential to allow for the full public health benefits through appropriate analysis of the whole genome sequences together with all relevant epidemiological data;
  - appropriate quality assurance measures for WGS need to be developed. This would include, as a priority, an adequate external quality assessment scheme before any decentralised WGS can be performed as part of the Euro-GASP molecular typing programme. It is crucial that WGS and subsequent analysis is performed centrally in the near future until appropriate expertise, capacity, quality assurance and cost-effectiveness issues are addressed. This should be supplemented by appropriate training and the development of quality assurance measures. Decentralising WGS would be aimed for once countries have fulfilled strict quality critera and external quality assessments.

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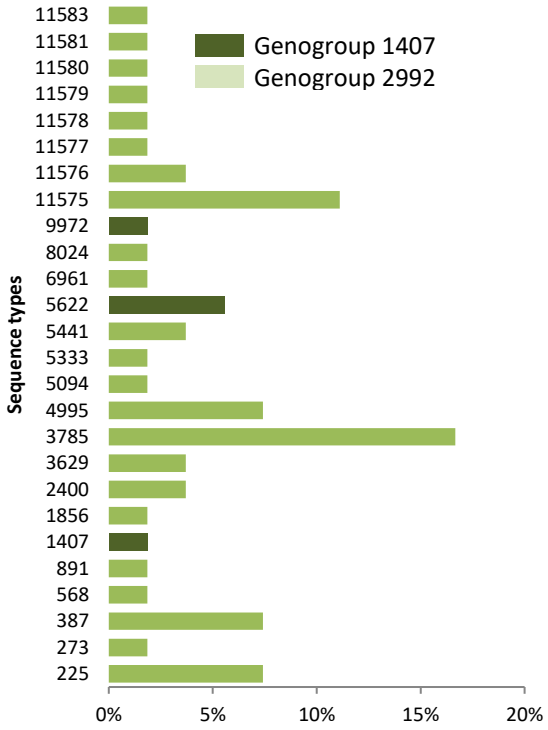
Annex 1. Sequence types for each country

Figure A1.1. Sequence types in Austria



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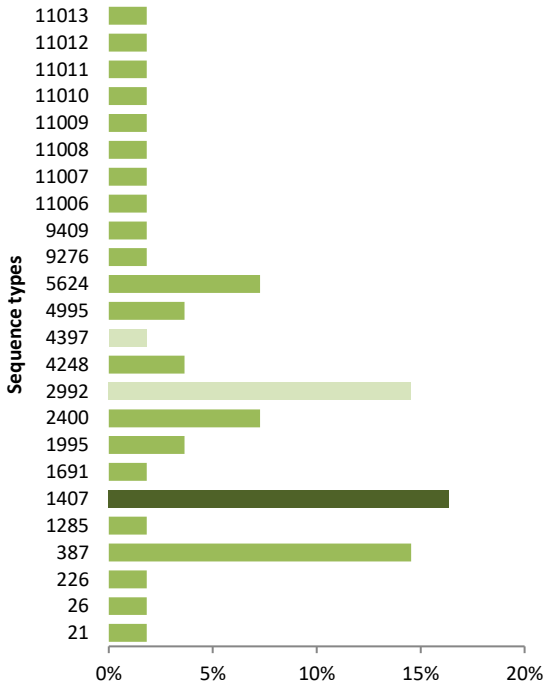
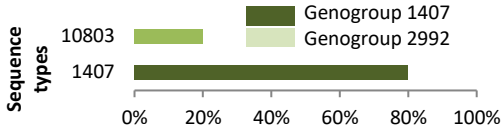


Figure A1.3. Sequence types in Cyprus



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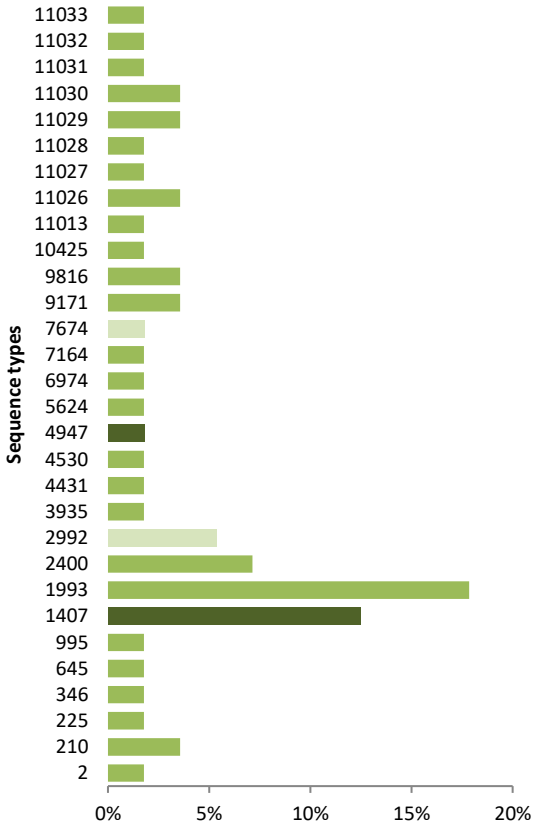

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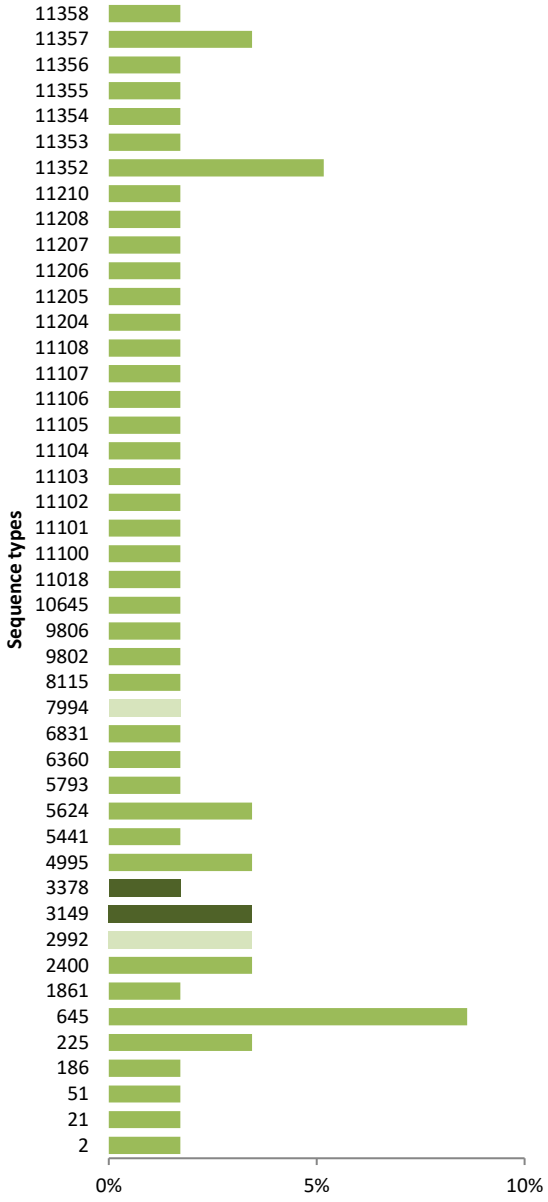
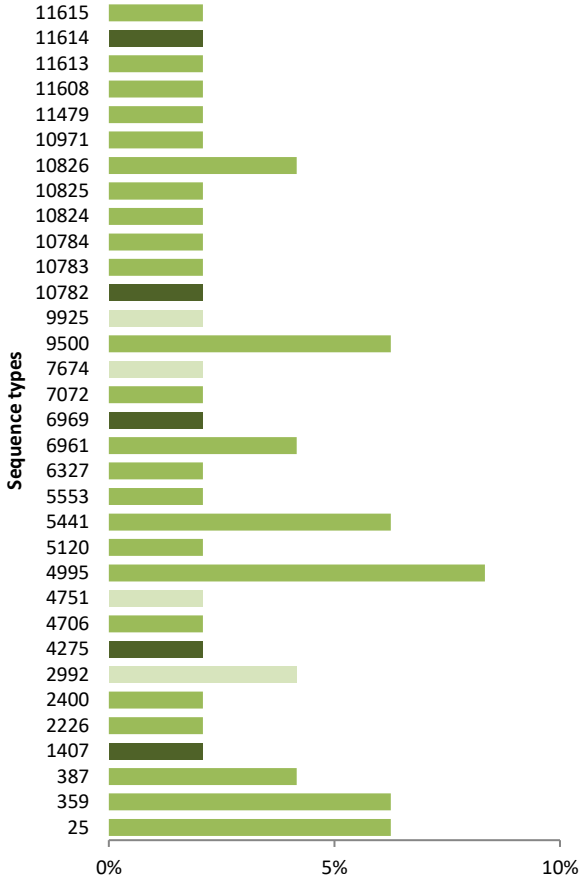


Figure A1.6. Sequence types in Germany



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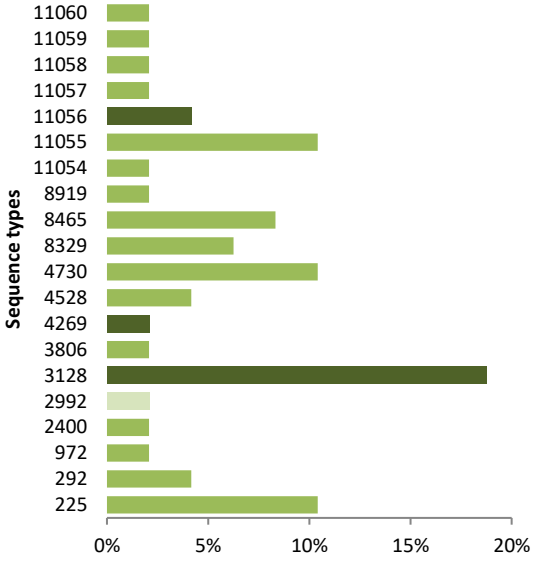

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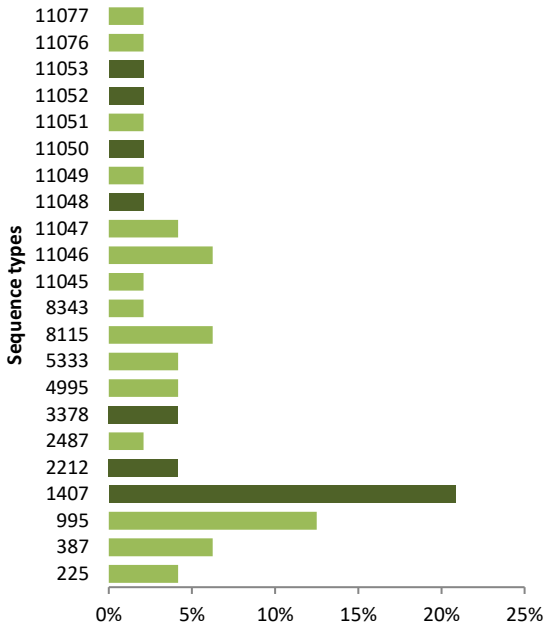

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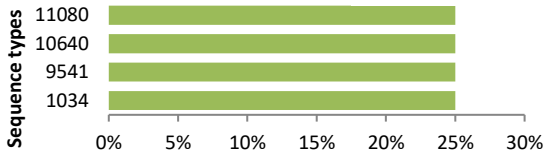

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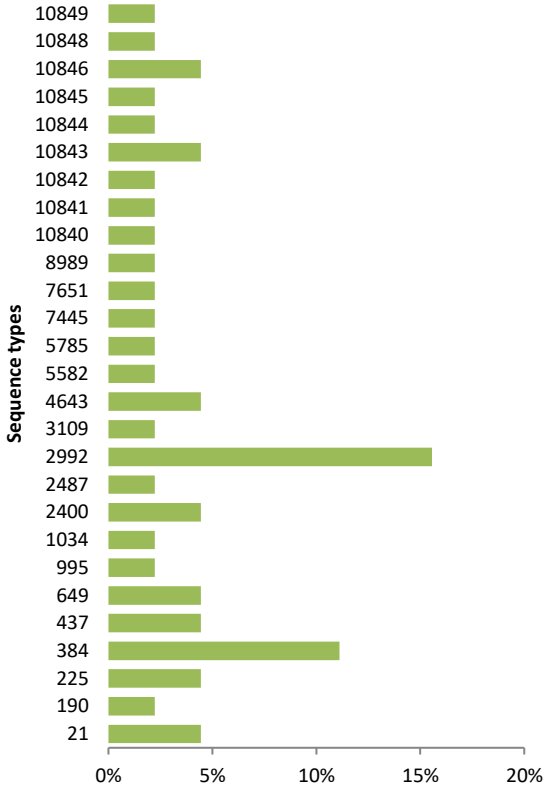


Figure A1.11. Sequence types in Italy

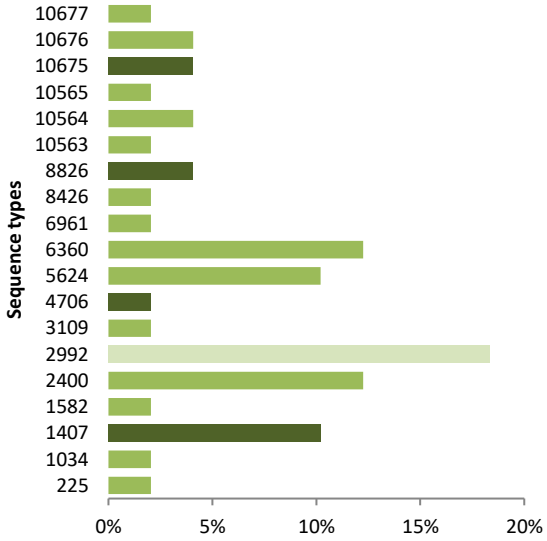


Figure A1.12. Sequence types in Latvia

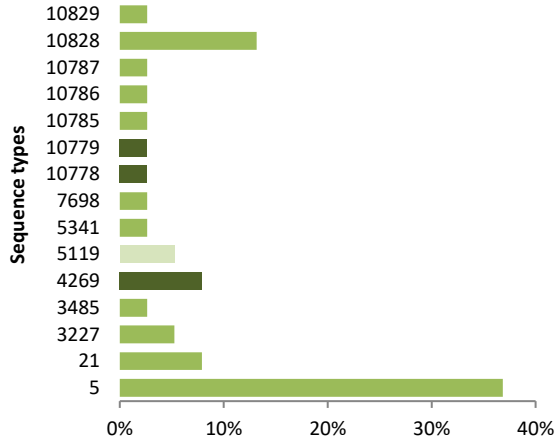
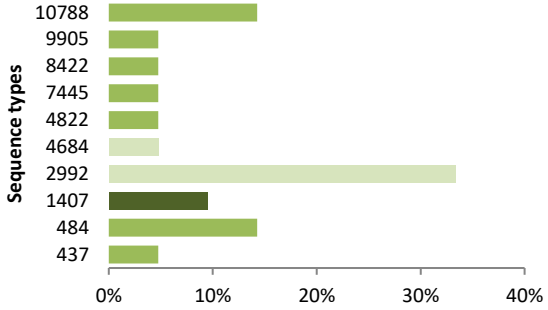


Figure A1.13. Sequence types in Malta



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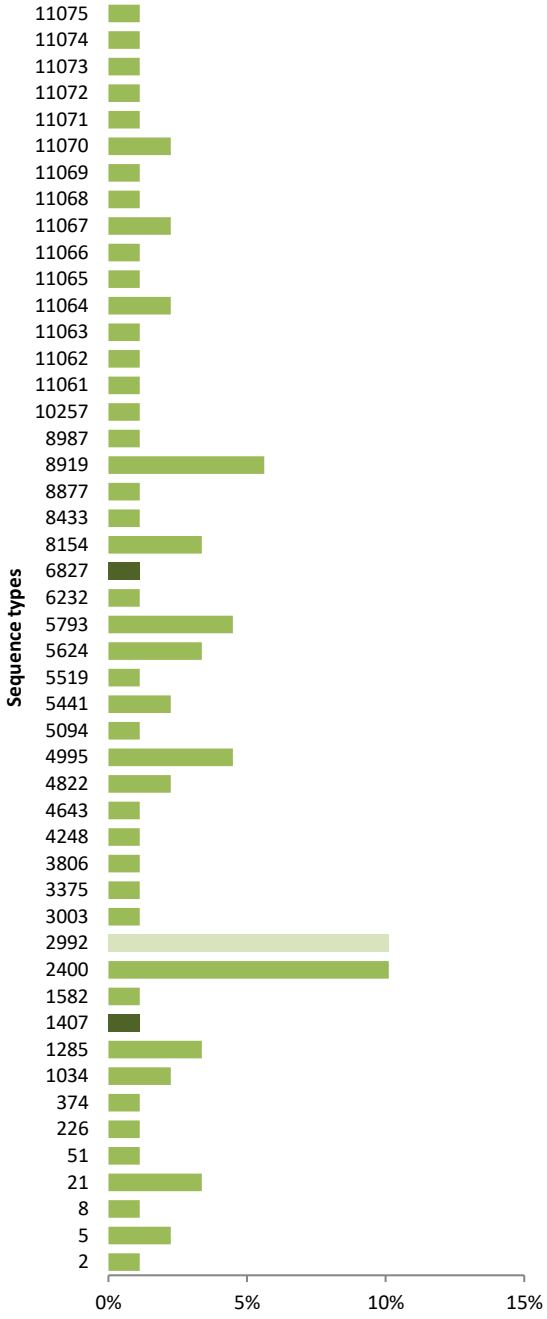


Figure A1.15. Sequence types in Norway

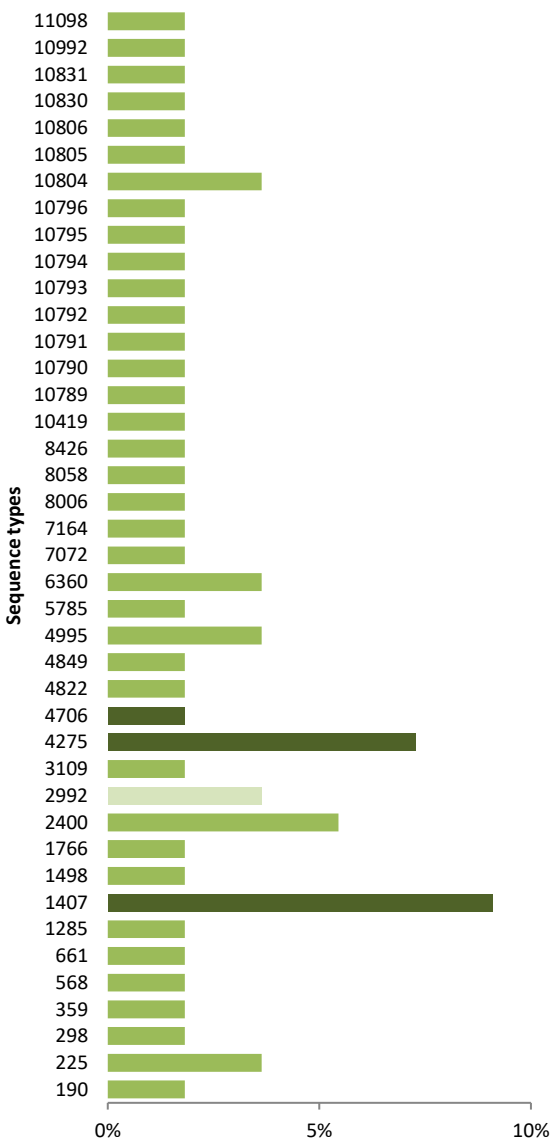
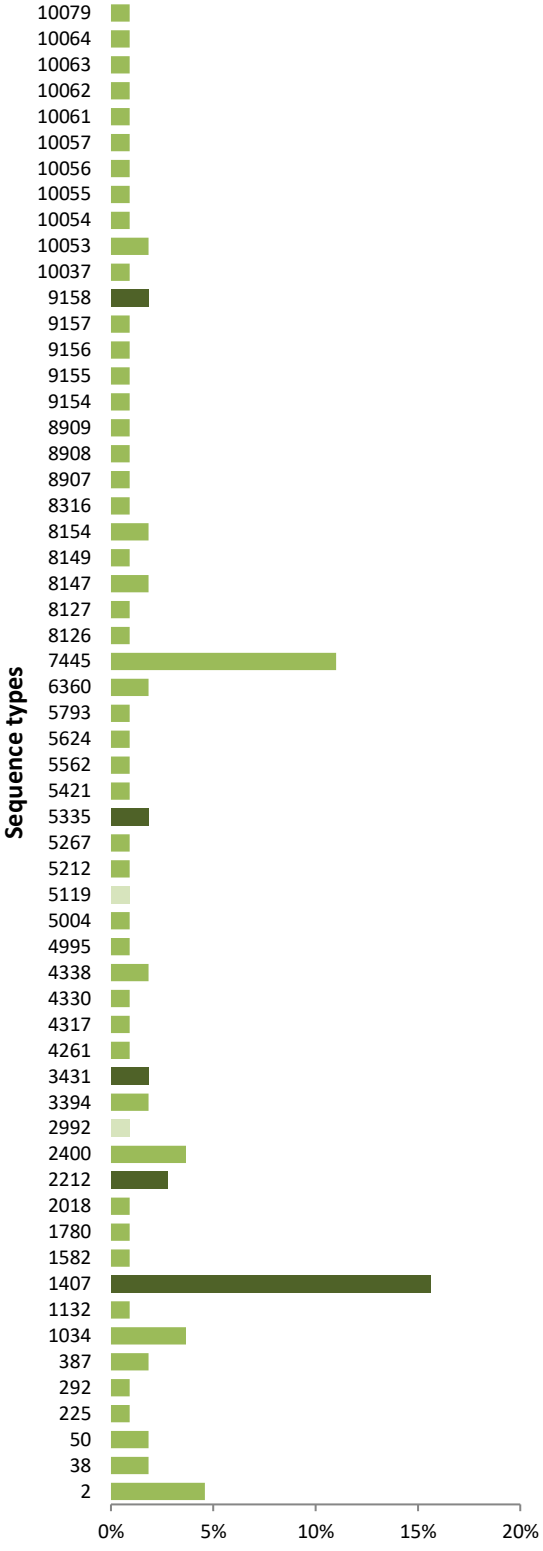


Figure A1.16. Sequence types in Portugal



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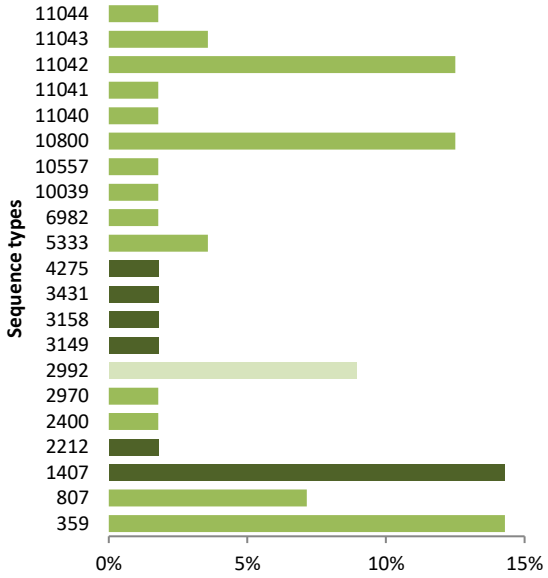
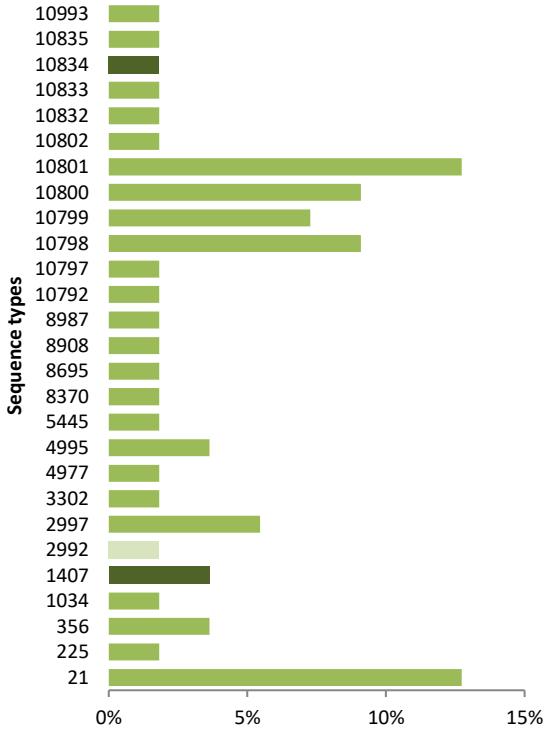


Figure A1.18. Sequence types in Slovenia



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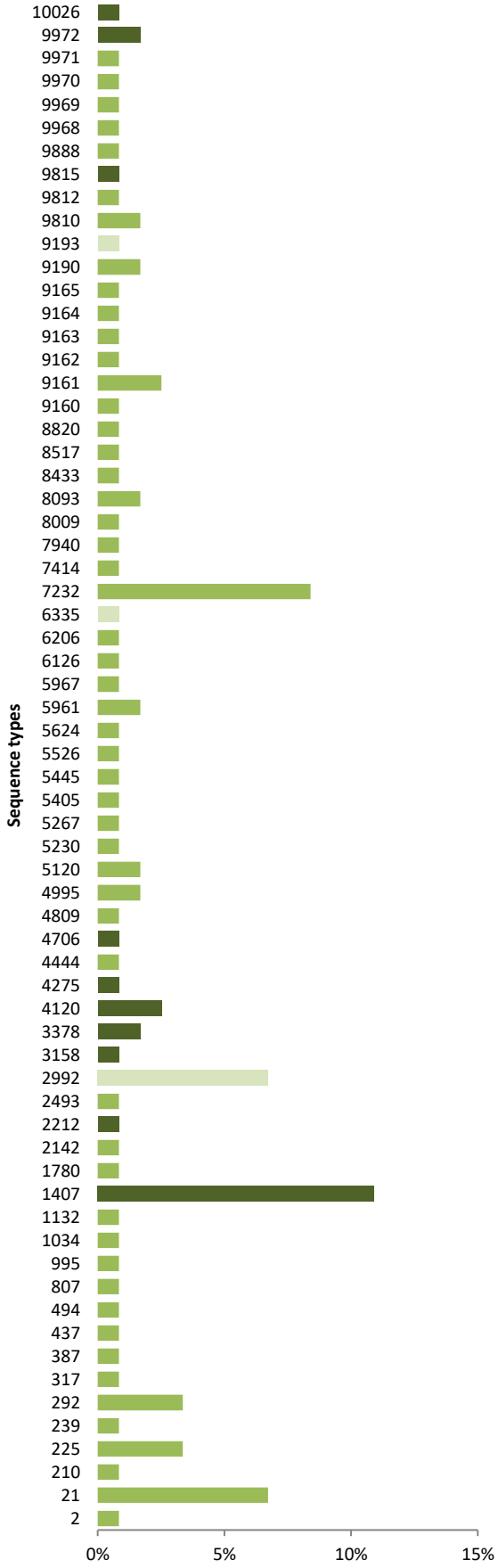


Figure A1.20. Sequence types in Sweden

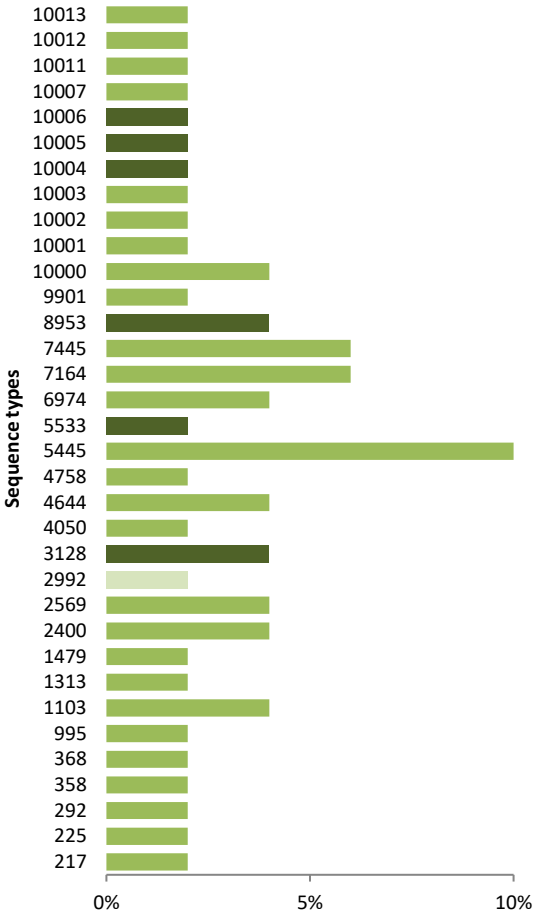
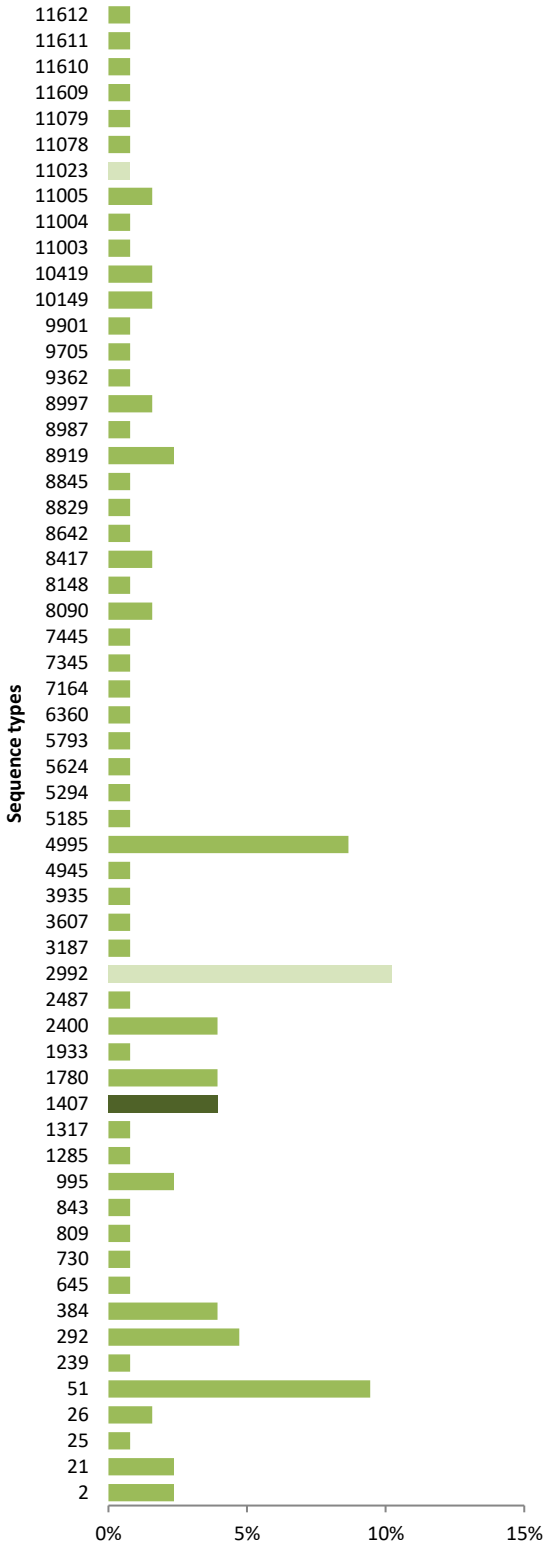


Figure A1.21. Sequence types in the UK



Annex 2. Statistical tables

Table A2.1. Univariate association of genogroups and patient characteristics

Genogroup	Sexual orientation (MSM=273, Heterosexual=337)					Gender (males=997, females=182)					Age group (≥15 to <25 years=335, ≥25 to <35 years=440, ≥35 to <45 years=229, ≥45 years=153)				
	Cat	No. (%)	OR	95% CI	P value	Cat	No. (%)	OR	95% CI	P value	Cat	No. (%)	OR	95% CI	P value
1407	MSM	9 (3.3)	1			M	143 (14.3)	1			≥15 to <25	43 (12.8)	1		
	Het	57 (16.9)	5.97	2.85-12.53	**<0.0001**	F	31 (17.0)	1.22	0.8-1.88	0.347	≥25 to <35	56 (12.7)	0.99	0.65-1.52	0.9643
											≥35 to <45	40 (17.5)	1.43	0.9-2.3	0.1277
											≥45 (153)	32 (20.9)	1.8	1.08-2.98	**0.0218**
2992	MSM	47 (17.2)	4.17	2.28-7.64	**<0.0001**	M	83 (8.3)	1			≥15 to <25	19 (5.7)	1		
	Het	16 (4.8)	1			F	8 (4.4)	0.51	0.24-1.07	0.0679	≥25 to <35	44 (10)	1.85	1.06-3.24	**0.029**
											≥35 to <45	20 (8.7)	1.59	0.83-3.06	0.1596
											≥45 (153)	8 (5.2)	0.92	0.39-2.15	0.8428
21	MSM	23 (8.4)	1			M	65 (6.5)	1			≥15 to <25	15 (4.5)	1		
	Het	23 (6.8)	0.8	0.44-1.45	0.4572	F	8 (4.4)	0.66	0.31-1.4	0.2745	≥25 to <35	14 (3.2)	0.7	0.33-1.48	0.3467
											≥35 to <45	10 (4.4)	0.97	0.43-2.21	0.95
											≥45 (153)	9 (5.9)	1.33	0.57-3.12	0.506
2400	MSM	31 (11.4)	5.27	2.35-11.83	**<0.0001**	M	63 (6.3)			**0.008***	≥15 to <25	17 (5.1)	1		
	Het	8 (2.4)	1			F	3 (1.7)				≥25 to <35	26 (5.9)	1.17	0.62-2.2	0.6154
											≥35 to <45	15 (6.6)	1.31	0.64-2.69	0.4573
											≥45 (153)	5 (3.3)	0.63	0.23-1.75	0.3727
51	MSM	0 (0.0)				M	31 (3.1)	1			≥15 to <25	31 (9.3)			**<0.0001***
	Het	48 (14.2)			**<0.0001***	F	30 (16.5)	6.15	3.57-10.59	**<0.0001**	≥25 to <35	17 (3.9)			
											≥35 to <45	12 (5.2)			
											≥45 (153)	1 (0.7)			
225	MSM	10 (3.7)	1			M	40 (4.0)	1			≥15 to <25	15 (4.5)	1		
	Het	14 (4.2)	1.14	0.5-2.61	0.7565	F	8 (4.4)	1.1	0.51-2.39	0.8098	≥25 to <35	14 (3.2)	0.7	0.33-1.47	0.3467
											≥35 to <45	10 (4.4)	0.97	0.43-2.21	0.95
											≥45 (153)	9 (5.9)	1.33	0.57-3.12	0.506
4995	MSM	16 (5.9)				M	38 (3.8)			**0.002***	≥15 to <25	6 (1.7)	1		
	Het	1 (0.3)			**<0.0001***	F	0 (0.0)				≥25 to <35	14 (3.2)	1.8	0.68-4.74	0.2267
											≥35 to <45	11 (4.8)	2.77	1.01-7.63	**0.0401**
											≥45 (153)	6 (3.9)	2.24	0.71-7.08	0.159
387	MSM	0 (0.0)				M	22 (2.2)	1			≥15 to <25	8 (2.4)			0.476*
	Het	14 (4.2)			**<0.0001***	F	6 (3.3)	1.51	0.6-3.78	0.3747	≥25 to <35	10 (2.3)			
											≥35 to <45	7 (3.1)			
											≥45 (153)	1 (0.7)			
359	MSM	0 (0.0)				M	11 (1.1)			0.269*	≥15 to <25	3 (0.9)			0.614*
	Het	5 (1.5)			0.068*	F	4 (2.2)				≥25 to <35	5 (1.1)			
											≥35 to <45	5 (2.2)			
											≥45 (153)	2 (1.3)			

Genogroup	Sexual orientation (MSM=273, Heterosexual=337)					Gender (males=997, females=182)					Age group (≥15 to <25 years=335, ≥25 to <35 years=440, ≥35 to <45 years=229, ≥45 years=153)				
	Cat	No. (%)	OR	95% CI	P value	Cat	No. (%)	OR	95% CI	P value	Cat	No. (%)	OR	95% CI	P value
2	MSM	0 (0.0)				M	10 (1.0)	1			≥15 to <25	11 (3.3)			**0.05***
	Het	9 (2.7)			**0.005***	F	9 (5.0)	5.13	2.04-12.9	**0.0001**	≥25 to <35	4 (0.9)			
											≥35 to <45	2 (0.9)			
											≥45 (153)	1 (0.7)			
5624	MSM	11 (4.0)				M	16 (1.6)			0.496	≥15 to <25	2 (0.6)			0.118*
	Het	1 (0.3)			**0.002***	F	1 (0.6)				≥25 to <35	8 (1.8)			
											≥35 to <45	2 (0.9)			
											≥45 (153)	5 (3.3)			
7445	MSM	4 (1.5)				M	18 (1.8)			0.094	≥15 to <25	6 (1.8)			0.13*
	Het	2 (0.6)			0.416*	F	0 (0.0)				≥25 to <35	3 (0.7)			
											≥35 to <45	3 (1.3)			
											≥45 (153)	5 (3.3)			
292	MSM	6 (2.2)				M	14 (1.4)			0.737	≥15 to <25	5 (1.5)			0.103*
	Het	2 (0.6)			0.148*	F	3 (1.7)				≥25 to <35	5 (1.1)			
											≥35 to <45	7 (3.1)			
											≥45 (153)	0 (0.0)			
384	MSM	1 (0.4)				M	15 (1.5)			0.491	≥15 to <25	6 (1.8)			0.572*
	Het	2 (0.6)			1*	F	1 (0.6)				≥25 to <35	7 (1.6)			
											≥35 to <45	1 (0.4)			
											≥45 (153)	2 (1.3)			
995	MSM	4 (1.5)				M	13 (1.3)			0.708	≥15 to <25	3 (0.9)			0.976*
	Het	3 (0.9)			0.706*	F	1 (0.6)				≥25 to <35	5 (1.1)			
											≥35 to <45	2 (0.9)			
											≥45 (153)	2 (1.3)			
5333	MSM	2 (0.7)				M	11 (1.1)			0.464	≥15 to <25	2 (0.6)			0.174*
	Het	6 (1.8)			0.308*	F	3 (1.7)				≥25 to <35	4 (0.9)			
											≥35 to <45	6 (2.6)			
											≥45 (153)	2 (1.3)			
1993	MSM	0 (0.0)				M	3 (0.3)			**<0.0001***	≥15 to <25	3 (0.9)			0.841*
	Het	10 (3.0)			**0.003***	F	7 (3.9)				≥25 to <35	3 (0.7)			
											≥35 to <45	3 (1.3)			
											≥45 (153)	1 (0.7)			
7232	MSM	No sexual orientation data for G7232				F	1 (0.6)			1	≥15 to <25	3 (0.9)			0.074*
	Het										≥25 to <35	1 (0.2)			
											≥35 to <45	5 (2.2)			
											≥45 (153)	1 (0.7)			

Cat – Category *Het – Heterosexuals* *F – Females*

No. – Number *M – Males*

Table A2.2. Univariate association of genogroups and antimicrobial susceptibility/resistance

Genogroup	Cefixime (sensitive=1137, resistant=52)					Azithromycin (sensitive=1108, resistant=73)					Ciprofloxacin (sensitive=563, resistant=626)				
	Cat	No. (%)	OR	95% CI	P value	Cat	No. (%)	OR	95% CI	P value	Cat	No. (%)	OR	95% CI	P value
1407	S	142 (12.5)	1			S	149 (13.5)	1			S	2 (0.4)			**p<0.0001***
	R	34 (65.4)	13.24	7.06-24.8	**<0.0001**	R	27 (37.0)	3.78	2.26-6.31	**<0.0001**	R	174 (27.8)			
2992	S	92 (8.1)			**0.029***	S	88 (7.9)			0.361*	S	89 (15.8)			**<0.0001***
	R	0 (0.0)				R	3 (4.1)				R	3 (0.5)			
21	S	72 (6.33)			0.767*	S	58 (5.2)	1			S	67 (11.9)	1		
	R	2 (3.9)				R	14 (19.2)	4.3	2.25-8.2	**<0.0001**	R	7 (1.1)	0.08	0.04-0.19	**<0.0001**
2400	S	63 (5.5)			0.763*	S	65 (5.9)			0.119*	S	0 (0.0)			**p<0.0001***
	R	3 (5.8)				R	1 (1.4)				R	66 (10.5)			
51	S	61 (5.4)			0.106*	S	61 (5.5)			**0.029***	S	59 (10.5)			**<0.0001***
	R	0 (0.0)				R	0				R	2 (0.3)			
225	S	48 (4.2)			0.265*	S	43 (3.9)			0.528*	S	0 (0.0)			**p<0.0001***
	R	0 (0.0)				R	4 (5.5)				R	48 (7.7)			
4995	S	38 (3.3)			0.407*	S	38 (3.4)			0.165*	S	1 (0.2)			**p<0.0001***
	R	0 (0.0)				R	0				R	37 (5.9)			
387	S	30 (2.6)			0.638*	S	30 (2.7)			0.253*	S	29 (5.2)			**<0.0001***
	R	0 (0.0)				R	0				R	1 (0.2)			
359	S	15 (1.3)			1*	S	15 (1.4)			0.618*	S	13 (2.3)			**0.003***
	R	0 (0.0)				R	0				R	2 (0.3)			
2	S	19 (1.7)			1*	S	19 (1.7)			0.625*	S	17 (3.0)			**<0.0001***
	R	0 (0.0)				R	0				R	2 (0.3)			
5624	S	18 (1.6)			1*	S	18 (1.6)			0.621*	S	0 (0.0)			**p<0.0001***
	R	0 (0.0)				R	0				R	18 (2.9)			
7445	S	18 (1.6)			1*	S	6 (0.5)	36.13	12.43-105.04	**p<0.0001**	S	17 (3.0)			**<0.0001***
	R	0 (0.0)				R	12 (16.4)				R	1 (0.2)			
292	S	17 (1.5)			1*	S	16 (1.4)			0.618*	S	15 (2.7)			**0.001***
	R	0 (0.0)				R	0				R	2 (0.3)			
384	S	16 (1.4)			1*	S	16 (1.4)			0.618*	S	16 (2.8)			**<0.0001***
	R	0 (0.0)				R	0				R	0 (0.0)			
995	S	14 (1.2)			1*	S	14 (1.3)			1*	S	14 (2.5)			**<0.0001***
	R	0 (0.0)				R	0				R	0 (0.0)			
5333	S	14 (1.2)			1*	S	14 (1.3)			1*	S	1 (0.2)			**p=0.002***
	R	0 (0.0)				R	0				R	13 (2.1)			
1993	S	10 (0.9)			1*	S	10 (0.9)			1*	S	10 (1.8)			**0.001***
	R	0 (0.0)				R	0				R	0 (0.0)			
7232	S	9 (0.8			0.362	S	10 (0.9)			1*	S	0 (0.0)			**p=0.002***
	R	1 (1.9)				R	0				R	10 (1.6)			

*Note: * Expected value for one cell < 5, so Fisher's exact test performed*

Cat – Category *S – Sensitive*

No. – Number *R – Resistant*

European Centre for Disease Prevention and Control (ECDC)

Postal address:
Granits väg 8, SE-171 65 Solna, Sweden

Visiting address:
Tomtebodavägen 11A, SE-171 65 Solna, Sweden

Tel. +46 858601000
Fax +46 858601001
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