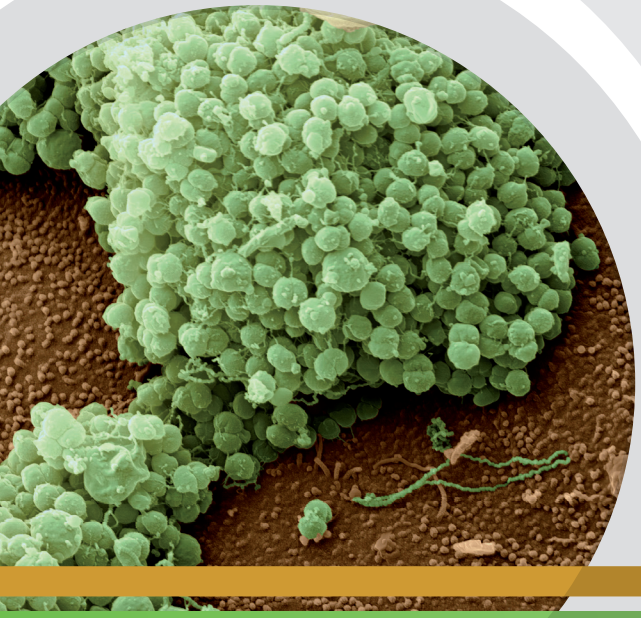

TECHNICAL REPORT

Molecular typing of *Neisseria gonorrhoeae*

Results from a pilot study 2010–2011

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ECDC TECHNICAL REPORT

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Results from a pilot study 2010–2011

This project was commissioned by the European Centre for Disease Prevention and Control and coordinated by Marita van de Laar (contract ECD.1699).

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Contents

Abbreviations

AMR	Antimicrobial Resistance
bp	Base pairs
CI	Confidence Intervals
DS	Decreased Susceptibility
ECDC	European Centre for Disease Prevention and Control
EEA	European Economic Area
EQA	External Quality Assurance
Euro-GASP	European Gonococcal Antimicrobial Surveillance Programme
LGV	Lymphogranuloma venereum
MIC	Minimum Inhibitory Concentration
MLST	Multi Locus Sequence Typing
MLVA	Multi Locus VNTR Analysis
MSM	Men who have sex with men
NG-MAST	*Neisseria gonorrhoeae* Multi-Antigen Sequence Typing
OR	Odds Ratio
PFGE	Pulsed Field Gel Electrophoresis
ST	Sequence Type
STI	Sexually Transmitted Infection
RFLP	Restriction Fragment Length Polymorphism
VNTR	Variable Number Tandem Repeat
WHO	World Health Organization

Executive summary

In 2011, a pilot study was conducted to assess the public health value of molecular surveillance for gonorrhoea in 21 EU/EEA countries. A total of 1 066 isolates collected as part of the 2010 European gonococcal antimicrobial surveillance programme were typed using *Neisseria gonorrhoeae* Multi Antigen Sequence typing (NG-MAST), which differentiates strains on the basis of sequence variation in two hypervariable loci, *porB* and *tbpB*.

The study aimed to type 50 isolates from each country and 100 isolates from countries with higher rates of gonorrhoea (e.g. the Netherlands, United Kingdom and Spain), using as recent a sampling period as possible (May/June 2010). For countries where only low numbers of isolates were available, additional gonococcal isolates collected in 2009 were also included.

A total of 406 sequence types (STs) were identified, 125 of which were represented by two or more gonococcal isolates, and 11 types were represented by ten or more isolates. While considerable diversity was observed in different countries, three types (STs 1407, 2992 and 225) were predominant, albeit at different levels in individual countries.

Further analysis of the 11 most common STs showed that other STs within the study collection were strongly related to these (differing by <1% in a single locus), enabling seven major genogroups of closely related STs to be defined. Genogroup G-1407 was observed in 20/21 countries and predominant in 13/21, while G-225 and G-2992 were observed in 19/21 and 14/21 countries respectively, but were predominant in just two.

Creation of genogroups allowed more robust statistical analysis and made it possible to explore associations between molecular type, patient characteristics and antimicrobial resistance. Associations between genogroups and younger age groups (G-2) or lower proportions of male patients (G-25, G-387) provided some indication of potential heterosexually associated types, which was substantiated by further analysis of the subset of 586 patients for whom sexual orientation was known. In contrast, potential indicators of association with men who have sex with men (MSM), such as older age group and male gender, were observed for genogroups G-1407 and G-2992, which were shown to be significantly associated with MSM in cases where sexual orientation was known.

Significant associations were identified between ciprofloxacin resistance and the predominant genogroups G-1407, G-225 and ST 5405. Fluoroquinolones are no longer recommended as first-line therapy, but the association between resistance and particular NG-MAST types could facilitate appropriate treatment of patients in the future, if antimicrobial resistance reaches levels requiring empirical therapy to be replaced by tailored treatment. Of greater concern was the strong association identified between G-1407 and decreased susceptibility to cefixime, one of the recommended first line empirical therapies. Isolates belonging to G-1407 also showed raised minimum inhibitory concentrations (MICs) to the other first-line treatment, ceftriaxone, as well as ciprofloxacin resistance and raised MICs to azithromycin. G-1407 therefore represents a collection of potential therapeutically challenging organisms which are currently predominant in many of the EU/EEA countries examined.

While the sample representativeness and quality of the epidemiological data were limitations of the current pilot study, performing molecular typing of gonorrhoea demonstrated a significant potential public health benefit. Identification of associations between molecular type and antimicrobial resistance profiles could aid understanding of the dissemination of resistance within a population and facilitate development of targeted intervention strategies. For maximum public health benefit, future work could aim to collate longitudinal, representative typing data linked to detailed epidemiological information and focus on achieving greater discrimination among the predominant types.

1 Introduction

Surveillance and monitoring of chlamydia, gonorrhoea and syphilis is implemented at the European Union level as part of enhanced surveillance of sexually transmitted infections (STIs), but does not presently include molecular typing methods. Since 2009, the European Centre for Disease Prevention and Control (ECDC) has been coordinating the enhanced surveillance of STIs in Europe. ECDC strives to ensure a high quality of standardised STI surveillance data from the countries of the European Union and the European Economic Area (referred to as EU/EEA). The STI microbiology project is part of the European STI surveillance scheme (launched in August 2009), contracted to an international team led by the Health Protection Agency (UK) and including the Statens Serum Institute [National Institute for Health Data and Disease Control] (Denmark) and Örebro University Hospital (Sweden).

The main objectives of the STI microbiology project are:

- To improve the quality of laboratory surveillance for gonorrhoea, syphilis, congenital syphilis and infection with *Chlamydia trachomatis* in EU/EEA Member States
- To strengthen the surveillance of *Neisseria gonorrhoeae* susceptibility in EU/EEA Member States, including an external quality assessment (EQA) scheme and training.

Background

Since 2007, all dedicated surveillance networks (funded by the EU Commission's Directorate-General for Health and Consumers) have been integrated into the work of ECDC, and molecular typing activities are included for several diseases. One of the aims of the STI microbiology project is to discuss and assess the added public health value of molecular typing with respect to bacterial STIs and if needed, to implement this molecular typing approach at the European level. It has the potential to provide insight into the dissemination of antimicrobial-resistant strains and to detect outbreaks not immediately apparent using current surveillance methods. Improving understanding of the molecular epidemiology of bacterial STIs may consequently help to inform intervention and control strategies.

An expert group meeting, consisting of both microbiologists and epidemiologists, was held in August 2010 to discuss and assess this approach. As detailed in the minutes of this meeting (Annex 1), it was agreed that typing of *N. gonorrhoeae* had the greatest potential to benefit public health, compared with typing of chlamydia or syphilis which would be of limited value at present, since the methods are insufficiently robust or discriminatory. A proposal was drawn up, with a potential model for applying molecular typing in EU/EEA Member States to explore associations between gonococcal molecular type and antimicrobial resistance (AMR) in Europe.

Selection of an appropriate typing method is critical in order to gain maximum public health benefit. The underlying population structure of the organism should also be considered in this decision. *N. gonorrhoeae* is naturally competent and so intra and inter-species transformation and recombinational events are a frequent occurrence, leading to a panmictic 'non-clonal' population structure. Therefore knowledge of the timescale of evolutionary change across the genome and within individual genes must be taken into consideration when interpreting data.

Key criteria for selection are that the method should be robust, reproducible, sufficiently discriminatory to distinguish between isolates from different sources but sufficiently stable to link isolates from sexual contacts or short transmission chains. The technology used should be transferrable and data generated should be unequivocal, to allow reliable inter-laboratory comparisons, and ideally have a high rapid throughput. Numerous methods are described for discriminating between strains of gonococci, but many do not fulfil these criteria. Methods for gonococcal typing have recently been reviewed in detail [32]. This report will consider the typing methods used most widely and therefore validated to the greatest extent.

Phenotypic methods for strain discrimination were used extensively in the pre-genomic era. Auxotyping differentiates strains on the basis of varying nutritional requirements but is insufficiently discriminatory, laborious and demanding in terms of technical and interpretative skills. Serotyping discriminates gonococci on the basis of variation in the outer membrane porin, encoded by porB, and is easier to perform and more discriminatory than auxotyping. However, the disadvantages of this method are the limited availability of serotyping reagents and the subjective interpretation and the problem of non-typeable strains. Even if a higher level of discrimination can be achieved by combining auxotyping and serotyping, these methods do not fulfil the criteria required for a typing method to be applied as part of a surveillance scheme.

Genotyping has largely superseded phenotyping as most methods can achieve 100% typeability. While some methods measure variability across the whole genome, others focus on variation within specific regions or genes. Genotypic methods can largely be subdivided into two categories: gel-based methods, which separate DNA bands in an agarose or polyacrylamide gel by electrophoresis, and sequence-based methods which determine the sequence of a PCR amplicon.

Gel-based typing methods

Many gel-based methods involve restriction fragment length polymorphism (RFLP) where genomic or amplified DNA is digested by restriction enzymes, and variation in restriction site sequences among strains results in DNA fragments of differing lengths. One of the challenges of RFLP methods is interpreting band patterns on gels to allow the type definition to be as objective as possible and to enable inter-laboratory comparison.

Pulsed Field Gel Electrophoresis (PFGE) uses rare-cutting restriction enzymes to limit the number of fragments generated, and has the advantage that it samples variation across the whole genome. PFGE is highly discriminatory and can differentiate within strains defined by other discriminatory molecular methods, such as porB sequencing and NG-MAST 29,38. It has been used in various studies to characterise local gonococcal populations and to characterise antibiotic resistance strains [7, 15, 29–31, 38]. While PFGE may have an application for further discrimination within dominant strains, it is not appropriate for the current application as it lacks standardisation, requires a high level of technical and interpretative expertise, is low-throughput, time-consuming and labour-intensive.

Opa typing is an RFLP-based method which examines variation in 11 opa genes amplified by PCR. This method compares well with other highly discriminatory methods and can sub-divide types defined by porB sequencing or NG-MAST [21, 40]. Opa typing has been applied in studies to define gonococcal populations in a region [7, 13, 24], to monitor strain transmission within sexual networks [40] and to distinguish between cases of treatment failure, reinfection and mixed infections [19]. However, as with PFGE and other gel-based methods, interpretation of band patterns is subjective and the method is relatively labour intensive, limiting the use of opa typing to small-scale studies.

Multilocus variable-number tandem repeat (VNTR) analysis (MLVA) differentiates strains according to the number of repeat sequences within five loci across the gonococcal genome. DNA fragments vary according to the number of repeats and so strains are differentiated following accurate sizing of fragments by capillary electrophoresis. The advantage of MLVA over other gel-based methods that it is rapid and high-throughput however, standardisation is necessary to achieve comparable sizing of fragments between laboratories. Furthermore MLVA has not been extensively validated, having only been applied to large datasets within the Netherlands where it appeared to be less discriminatory than opa typing [9, 10]. MLVA was therefore not considered to be a suitable method for the current study.

Sequence-based typing methods

Sequence-based typing offers the advantage that it generates clear, unequivocal data which is easy to interpret and is transferrable between laboratories. In addition, as automated systems have evolved, this has become increasingly high-throughput and lower cost. With the advent of numerous competitively-priced commercial sequencing services, this technology is widely available to all potential users. The choice of gene(s) to sequence for typing is critical to the performance of the method and the downstream application of the typing. Sequencing of hypervariable genes may provide excellent differentiation for the purposes of short-term epidemiology but may be less appropriate for longitudinal studies over a number of years if types are insufficiently stable. In contrast, selection of highly conserved genes allows longer-term variation to be identified which may be more appropriate for studies examining strain evolution. Typing schemes based on multiple loci are preferable to those examining a single locus as recombination in *N. gonorrhoeae* is a relatively frequent event and therefore types defined by a single locus may not reflect the true genetic background of the strain. Several sequence-based typing methods are described for *N. gonorrhoeae* and the most widely used will be considered in this report.

PorB sequence analysis differentiates strains on the basis of variation in the whole porin (porB) gene or in a fragment encompassing the most variable regions. Both partial and full porB sequencing are highly discriminatory, being comparable to NG-MAST [9, 11, 14], although further differentiation can be achieved by combining these methods [39]. PorB sequencing has been used to define gonococcal types in a geographical region [11, 39] and to investigate sexual networks [37, 40] and treatment failures [33, 34]. However, comparison between laboratories remains difficult due to the lack of standardisation of the sequence length and the absence of any centralised database for type definition.

Multi Locus Sequence Typing (MLST) measures variation in the sequences of seven or more relatively conserved, slowly-evolving genes, usually encoding housekeeping enzymes, that are ideally distributed throughout the genome. The different alleles at each locus are assigned a specific number and each locus number is combined to define an MLST type which is unambiguous and transferrable between laboratories.

As MLST monitors slowly evolving loci, this method is most appropriate for studies investigating long-term and global epidemiology and gonococcal population dynamics. At present, there is no universally accepted MLST scheme for *N. gonorrhoeae*. Some studies have applied an MLST scheme using the same loci (abcZ, adk, aroE, fumC, gdh, pdhC and pgm) as for *N. meninigitidis* [2, 11, 18], whereas others have achieved greater discrimination by using more loci (abcZ, adk, aroE, fumC, gdh, glnA, gnd, pdhC, pgm, pilA, ppk, pyrD, and serC) [28]. While

MLST has been used to study long-term epidemiology and the gonococcal population structure, the suitability of the current schemes for these applications is yet to be extensively validated.

Neisseria-gonorrhoeae Multi Antigen Sequence Typing (NG-MAST) differentiates strains on the basis of sequence variation in fragments of two hypervariable genes, the porB gene and subunit B of the transferring-binding protein (tbpB) gene. NG-MAST is highly discriminatory, relatively easy to use and supported by an open-access, online database (www.ng-mast.net), enabling easy comparison of alleles and providing clear assignation of new alleles and types. For these reasons, NG-MAST has been accepted as the current method of choice and widely used to investigate specific gonococcal antimicrobial resistance phenotypes [3, 20, 25-27, 29, 41], to examine specific sexual networks [1, 6, 17, 23] and to investigate treatment failures [12, 33, 34]. There have been several studies examining consecutive gonococcal isolates within a geographical region [4, 8, 11, 22, 39] which have identified associations between ST and AMR phenotype and patient characteristics. Further differentiation within an NG-MAST has been possible using PFGE, opa typing or porB sequencing [11, 14, 21, 22, 29, 38].

The expert group concluded that NG-MAST is the appropriate method to use for molecular surveillance across Europe as it is highly discriminatory, relatively low cost, rapid, reproducible, robust, high-throughput and, unlike many of the other typing methods available, produces transferable data. NG-MAST and other similarly discriminatory methods (PFGE, opa typing and porB sequencing) are well suited to work on short term (months to a few years) epidemiological questions, whereas MLST which measures variation in less rapidly evolving genes is more appropriate for longer term studies of epidemiology and gonococcal population dynamics over many years or decades.

The aims of the current study, to assess the public health benefit of molecular surveillance for gonorrhoea in relation to antimicrobial resistance and outbreak investigation, would require a typing method compatible with these rapidly evolving events, which is why application of NG-MAST is appropriate in this context. Some NG-MASTs appear to be relatively stable, being observed in different studies spanning a time period of at least five years, which may indicate that this approach could have greater longitudinal application. However widespread longitudinal application has not been evaluated for NG-MAST or any other gonococcal molecular typing method. This could be an additional output of the current study if molecular surveillance is extended beyond the pilot period.

Objectives

A major aim of the European STI surveillance network is to assess the public health benefit of performing molecular surveillance of gonorrhoea in the EU/EEA Member States. The following objectives within the pilot study focus on achieving this aim:

- identify associations between NG-MAST and antimicrobial resistance profiles to determine the ability to predict AMR profile;
- provide a baseline for outbreak studies.

To maximise the value of this pilot, epidemiological variables should as far as possible be linked with the NG-MAST.

2 Methods

2.1 Isolate collection

NG-MAST was performed on the most recent isolates submitted to the 2010 European Gonococcal Antimicrobial Surveillance Programme (Euro-GASP) [5]. This was done to ensure that the isolates had already been collected, were ready for NG-MAST and were the most recently circulating isolates. Permission to perform NG-MAST on isolates submitted for AMR testing was obtained from the submitting laboratories.

2.1.1 Isolate numbers

Following the expert group meeting in 2010, it was agreed that the maximum number of isolates to be typed was 1 200. Each country aimed to collect a minimum of 55 gonococcal isolates in the May/June collection period of 2010, with the overall aim of retrieving and testing a minimum of 50 isolates. For countries where 55 isolates represented less than 10% of the total cases of gonorrhoea (Spain, United Kingdom and the Netherlands) in a six-month period, a request was made for up to a maximum of 110 isolates to be collected, with the aim of testing 100 isolates. The proposed aim was to type 50 isolates from each country other than Spain, UK and the Netherlands, where 100 isolates were typed. For those countries with less than 55 isolates, isolates were included from the 2010 second biannual AMR collection period (Nov/Dec 2010). If there were still less than 55, then isolates from 2009 were used (Table 1).

Table 1: Number of isolates available from Euro-GASP for NG-MAST

Country	Number of isolates available May/June 2010	Number of extra isolates taken from Nov/Dec 2010	Number of extra isolates taken from Oct/Dec 2009	Number of isolates to be tested using NG-MAST
Austria	55			**50**
Belgium	55			**50**
Cyprus		12		**12**
Denmark	55			**50**
France	54			**50**
Germany	55			**50**
Greece	36	14		**50**
Hungary		17		**17**
Ireland	24	26		**50**
Italy	55			**50**
Latvia	11	9	9	**29**
Malta	26	6	18	**50**
Netherlands	115			**100**
Norway	46		3	**49**
Portugal	31	0*	19	**50**
Romania		9		**9**
Slovakia	18	32		**50**
Slovenia	14	14	22	**50**
Spain	72	0*	29	**100**
Sweden	55			**50**
United Kingdom	111			**100**
Total	**888**	**139**	**100**	**1066**

**Not available as decentralised testing*

2.1.2 Selection criteria

The most recently circulating isolates were selected for typing. NG-MAST was performed on consecutive isolates submitted to the 2010 AMR sentinel surveillance, regardless of antimicrobial resistance profile, in order to include both sensitive and resistant isolates.

Inclusion criteria for this study were:

- Availability of a viable isolate
- Full antimicrobial resistance profile.

2.2 NG-MAST protocol

NG-MAST was performed on isolates in either London or Örebro. Bacterial DNA extracts were prepared for each isolate and stored at -20°C for subsequent use. NG-MAST was performed on the DNA extracts as previously described [16]. Internal regions of the porB and tbpB genes are amplified by PCR and the subsequent PCR products purified. Sequencing of both the leading and reverse strands was then performed and sequencing data analysed and edited in BioNumerics v6.1 (Applied Maths, St-Martens-Latem, Belgium) software. Allele numbers for the trimmed porB (490 bp) and tbpB (390 bp) sequences and sequence types were assigned via the online NG-MAST database (www.ng-mast.net). The chromatograms for all new alleles were carefully checked for any base call errors before sequences were uploaded to the online NG-MAST database for new allele number assignation. Similarly, both alleles for any novel STs were carefully checked before obtaining a novel ST number from the administrator section of the NG-MAST website.

2.3 Internal quality control and internal quality assurance

On each NG-MAST sequencing plate a WHO B strain was included for internal quality control, alongside a non template control. To ensure intra-laboratory consistency of results, internal quality assurance was conducted in the London laboratory by including one blinded DNA extract from an isolate of known ST on each NG-MAST plate.

2.4 Data analysis

All raw sequence data was analysed and edited in BioNumerics v6.1.

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 by means of neighbour joining multiple alignment, followed by individual pair-wise alignment, against the most frequent allele. This enables the number of base pair (bp) differences to be accurately determined. For example, for ST 1407 (*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 (≤5bp difference for *porB* and ≤4bp for *tbpB*). Genogroups were named after the most frequently-occurring ST within the group (e.g. in G-1407, 70% were ST 1407).

NG-MAST and genogroups were linked to AMR and epidemiological information from each isolate/patient collected as part of Euro-GASP, as described in the ECDC reporting protocol [5]. 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).

2.5 Statistical analysis

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 v11.2.

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 was used to test if these odds ratios were significantly different from 1 (i.e. testing the null hypothesis that there was no difference in odds of resistance/decreased susceptibility between the group in question and the specified baseline group). It should be noted that this analysis could not be performed for datasets where a cell equalled zero, and further multivariable analysis was not performed. In cases with small cell numbers, Fisher's exact test was performed.

2.5.2 Multivariable analysis

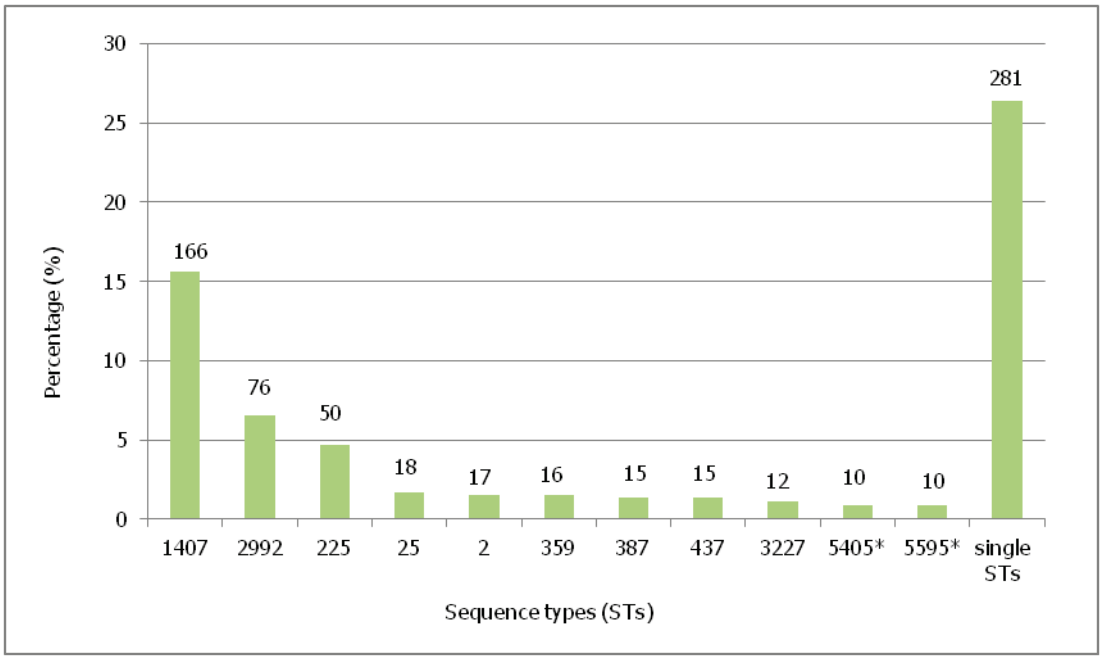
The multivariable analysis used logistic regression to model the odds of associations between genogroup and resistance controlling for other variables. The P value produced from the Wald Test was used to test the null hypothesis that the odds ratios were not significantly different to one.

3 Results

3.1 Frequency of STs

A total of 1 066 isolates were typed by NG-MAST. Within this collection, 406 different STs were identified, representing 313 different *porB* alleles and 113 different *tbpB* alleles. In addition, 125 clusters (two or more isolates with the same NG-MAST) were identified. There were 281 single STs and 216 new STs. The most frequently observed types represented by ≥10 isolates were STs 1407, 2992, 225, 25, 2, 359, 387, 437, 3227, 5405 and 5595, with 5405 and 5595 being new STs (Figure 1). Of these, all had unique *porB* alleles but some STs shared the same *tbpB* allele (STs 1407 and 5595, STs 225 and 437, STs 2292 and 359 and STs 387 and 3227) (Table 2).

Figure 1: Sequence types represented by 10 or more isolates (%)



** New sequence types*

Table 2: Details of the porB and tbpB alleles defining the most frequently observed STs (represented by ≥10 isolates)

ST (n=)	NG-MAST Allele	
	porB	*tbpB*
1407 (166)	908	110
2992 (76)	1808	29
225 (50)	4	4
25 (18)	18	27
2 (17)	2	16
359 (16)	301	29
437 (14)	14	4
387 (15)	266	118
3227 (12)	1954	118
5405 (10)	3279	1139
5595 (10)	3390	110

Table 3 shows the most frequently occurring ST for each country (further details of all STs observed in each participating country appear in Annex 2). The prevalence of the three most common STs (1407, 2992 and 225) varied considerably among countries (Table 3). ST 1407 accounted for more than 10% of isolates in 13/21

countries and was the predominant type observed in Austria, Belgium, Italy, the Netherlands, Portugal, Romania, Slovenia, Spain and United Kingdom (Annex 2).

Table 3: Most frequently observed ST in each country

Country	Most frequent ST	Total number of isolates typed	Total number of STs in each country	Number of ST 1407 (%)	Number of ST 2992 (%)	Number of ST 225 (%)
Austria	ST 1407	50	20	16 (32)	2 (4)	0 (0)
Belgium	ST 387	50	31	7 (14)	4 (8)	1 (2)
Cyprus	ST 3128	12	5	0 (0)	0 (0)	0(0)
Denmark	ST 225/3158	50	36	3 (6)	1 (2)	6 (12)
France	ST 2/2992	50	27	4 (8)	7 (14)	2 (4)
Germany	ST 25	50	18	14 (28)	0 (0)	0 (0)
Greece	ST 5405*/5505*	50	24	6 (12)	0 (0)	1 (2)
Hungary	ST 5332*	17	10	2 (12)	0 (0)	0 (0)
Ireland	ST 2992	50	25	3 (6)	16 (32)	0 (0)
Italy	ST 1407	50	25	17 (34)	5 (10)	0 (0)
Latvia	ST 3227	29	14	0 (0)	0 (0)	2 (7)
Malta	ST 225	50	16	3 (6)	1 (2)	23 (46)
Netherlands	ST 1407	100	52	16 (16)	7 (7)	3 (3)
Norway	ST 2992	49	27	7 (14)	11 (22)	0 (0)
Portugal	ST 1407	50	28	7 (14)	4 (8)	0 (0)
Romania	ST 1407/4120	9	7	2 (22)	0 (0)	0 (0)
Slovakia	ST 437	50	19	2 (4)	0 (0)	0 (0)
Slovenia	ST 1407	50	19	14 (28)	2 (4)	8 (16)
Spain	ST 1407	100	43	28 (28)	9 (9)	0 (0)
Sweden	ST 225	50	31	1 (2)	3 (6)	4 (8)
United Kingdom	ST 1407	100	62	14 (14)	4 (4)	0 (0)

**New ST*

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, differing by ≤1% at just one of the alleles (in most cases, the *porB* allele). STs were therefore clustered on the basis of this close relationship into 'genogroups', comprising the main ST plus all other closely related types (Table 4). For example, G-1407 comprising ST 1407 and a group of 27 other related STs, was defined by comparing *porB* alleles of all 52 STs containing *tbpB* allele 110 using the neighbour joining method and individual pair-wise alignment to establish how related these different STs were (Figure 2). This was repeated for the *tbpB* alleles of the eight STs sharing *porB* allele 908. Twenty-seven STs were identified that were >99% similar to ST 1407, with 25 STs differing by ≤5bp in *porB* allele and two STs differing by 1bp in *tbpB* (Table 4). Seven major genogroups, G-1407, G-225, G-2992, G-25, G-387, G-2 and G-25, were defined (Table 4) which encompassed 558 (52%) of the 1 066 isolates tested.

Table 4: Details of different STs within the seven 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=)						
	G-1407	**G-225**	**G-2992**	**G-25**	**G-387**	**G-2**	**G-359**
0	1407 (166)	225 (50)	2992 (76)	25 (18)	387 (15)	2 (17)	359 (16)
1	5595 (10) 3149 (9) 4120 (7) 5570 (5) 2212 (3) 5594 (3) 3431 (1) 3779 (1) 4275 (1) 4359 (1) 4951 (1) 5588 (1) 5619 (1) 5622 (1)	437 (14) 5463 (6) 1132 (5) 205 (1) 346 (1) 1342 (1) 1365 (1) 1399 (1) 2687 (1)	5049 (1) 5194 (1) 5237 (1)	51 (7)	0	226 (1)	1929 (2)
2	3158 (8) 5600 (4) 4974 (1) 5480 (1) 5625 (1) 5581 (1)	5017 (2) 5423 (2) 289 (1) 1340 (1) 2202 (1) 3141 (1) 3153 (1) 3952 (1) 4315 (1) 5703(1)	5515 (2) 5192 (1) 5385 (1)	3003 (4) 4589 (2) 273 (1) 881 (1) 5424 (1)	3227 (12)	0	5485 (1)
3	3128 (7) 5649 (1)	2616 (2) 3150 (2) 5180 (2) 2625 (1) 5655 (1)	0	356 (3) 807 (1)	5185 (4) 5191 (1) 5486 (1) 5503 (1)	0	0
4	5205 (1) 5599 (1)	3056 (1) 4473 (1) 5374 (1)	0	5384 (2) 5341 (1)	5186 (1) 5190 (1) 5498 (1)	0	0
5	5591 (1)	5330 (1)	0	4338 (1) 5172 (1)	0	0	1313 (1)
1 bp difference from *tbpB* allele x†	5332 (6) 4741 (4)	0	5227 (1)	0	0	0	0
Total number	248	107	84	43	37	18	20

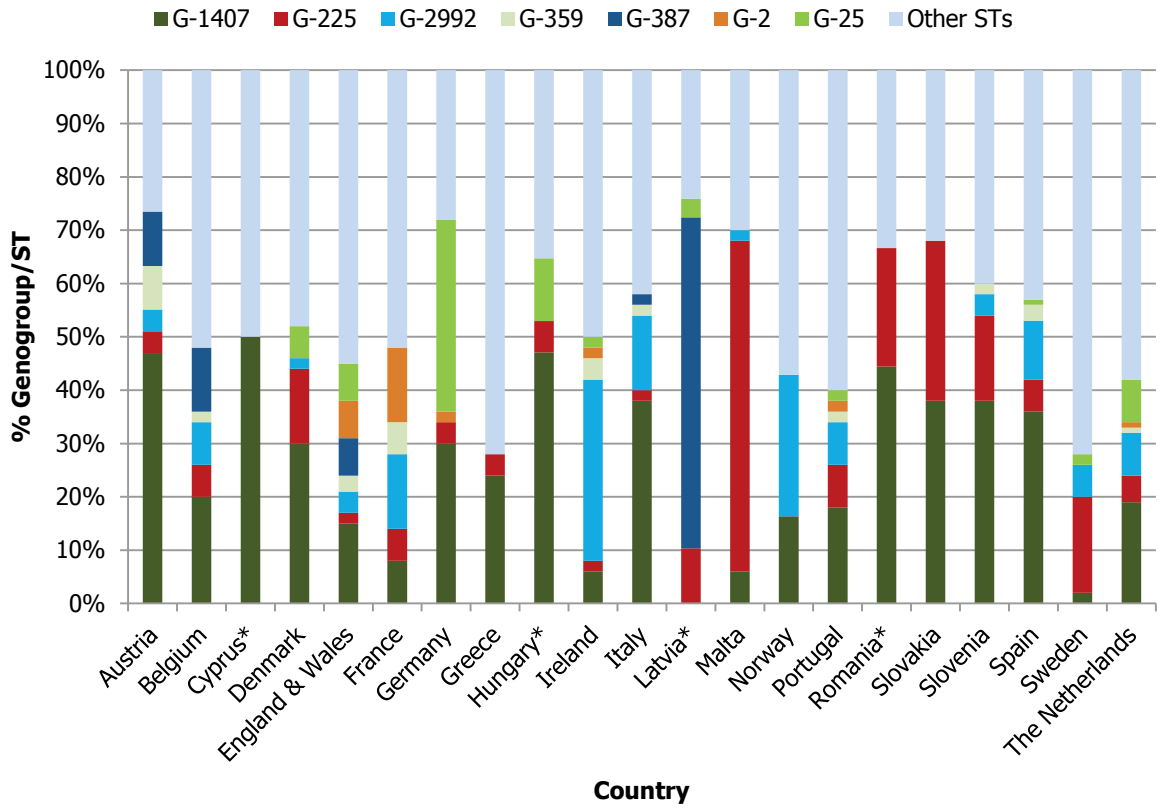
*STs share an identical *tbpB* allele with the most frequent ST but vary at the *porB* allele
†STs share an identical *porB* allele with the most frequent ST but vary at the *tbpB* allele

Figure 2. Dendrogram showing similarity of porB alleles (using the neighbour joining method) for isolates sharing tbpB allele 110

**STs within this cluster which differed by >5bp and were not part of G-1407 were STs 5394, 5392, 5329, 5593, 5597, 5632.*

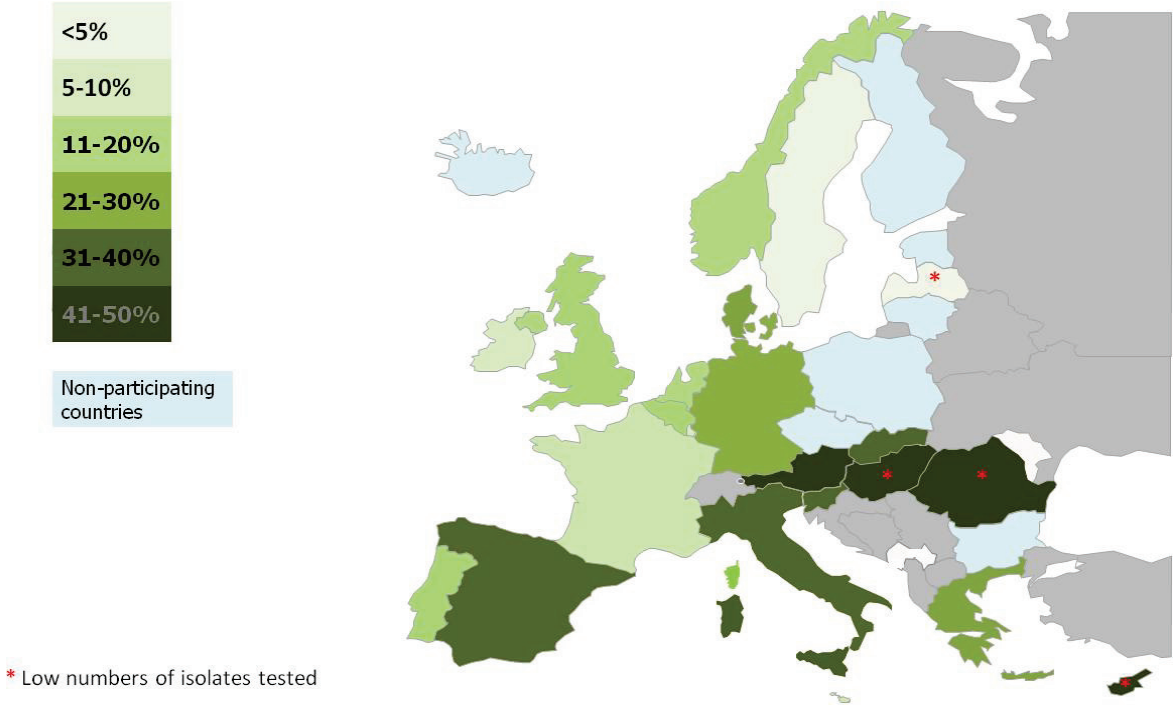
The proportion of each genogroup observed in each participating country is presented in Figure 3. G-1407 has widespread distribution, being found in all countries examined, with the exception of Latvia, and was predominant in Austria, Belgium, Cyprus, Denmark, England and Wales, Hungary, Italy, the Netherlands, Portugal, Romania, Slovakia, Slovenia and Spain. In contrast, G-1407 was comparatively uncommon (<10%) in France, Ireland, Malta and Sweden. G-1407 appeared to be most prevalent in eastern and southern Europe (Figure 4), which may suggest greater dissemination of this strain within specific sexual networks. It is not known whether ST 1407 or related types first emerged in any of these countries, which could also account for the higher proportions observed. The significance of this distribution should be interpreted with caution, given the low numbers of isolates submitted from some countries. The extreme predominance of a single clonal group is perhaps surprising given that the gonococcus is highly heterogeneous and that NG-MAST measures variation within two hypervariable genes. This may merit further work to determine the true extent to which this clonal group is related. G-225 was observed in 19/21 countries, was predominant in Malta and Sweden and relatively common in Denmark, Romania, Slovakia and Sweden. G-2992 was observed in 14/21 countries and was predominant in Ireland and Norway, while G-25 was predominant in Germany (Figure 3).

Figure 3. Distribution of genogroups within each participating country



*Countries with low isolate collections

Figure 4: Distribution of G-1407 in participating EU/EEA countries



3.3 Sequence type and epidemiological characteristics of linked patients

For the 1 066 patients included in this study, age and gender were the most frequently collected variables, known for 1 030 and 1 050 patients respectively. Sexual orientation was known for 586 patients. Table 5 below presents the epidemiological characteristics of the patients infected with a genogroup or ST (where a genogroup was not identified) represented by ≥10 isolates.

Statistical analysis was performed to explore associations between ST and patient characteristics. For most genogroups there was no clear link to an age category, with the exception of G-1407 which was significantly less common in younger patients aged <25 years (OR 0.43, 95% CI 0.29-0.63, p<0.0001, X2 test). In contrast, G-2 was significantly more common in this younger age group (OR 15.63, 95% CI 2.02-120.89, p=0.0004, X2 test).

Statistical analyses demonstrated that the proportion of males infected was significantly lower for G-25 (OR 0.13, 95% CI 0.07-0.25; p<0.0001, X2 test) and G-387 (OR 0.27, 95% CI 0.13-0.54; p=0.0001, X2 test). In contrast, the proportion of males infected was significantly higher for G-2992 in particular (OR 6.14, 95% CI 1.9-19.8; p=0.0005, X2 test), but also for G-1407 (OR 2.01, 95% CI 1.3-3.1; p=0.0017 X2 test). Where sexual orientation was known, a strong association was observed between MSM and infection with G-2992 (OR 15.86, 95% CI 6.4-77.8; p<0.0001, X2 test) and to a lesser extent G-1407 (OR 1.75, 95% CI 1.2-2.6; p=0.003, X2 test). However, it is important to note that G-1407 is distributed widely across different sexual networks, with 53.5% of G-1407 isolates recovered from heterosexual patients (Table 5). In contrast, MSM were less frequently infected with G-225 (OR 0.039, 95% CI 0.2-0.7; p=0.0015 X2 test), G-387 (p<0.0001, Fisher's exact test) and G-2 (p=0.01, Fisher's exact test), although it should be appreciated that statistical analysis of associations between sexual orientation and genogroup/ST is limited by the low completion rate for this variable in the current dataset.

Table 5: Characteristics of patients infected with the most frequently observed genogroups/STs

Genogroup (n=)	Mean patient age (range in years)	No. of patients where gender known	% male patients (n=)	No. of males where sexual orientation known	% heterosexual (n=)	% MSM (n=)
G-1407 (248)	33.3 (17 – 69)	244	89.3 (218)	129	53.5 (83)	46.5 (72)
G-225 (107)	29.1 (7 – 56)	104	80.9 (85)	59	83.4 (65)	16.6 (13)
G-2992 (84)	31.7 (18 — 66)	84	96.4 (81)	38	12.2 (5)	87.8 (36)
G-25 (43)	24.6 (16 – 40)	43	41.8 (18)	10	94 (33)	6.0 (2)
G-387 (37)	28.6 (18 – 69)	36	58.3 (21)	15	100 (30)	0
G-359 (20)	32.9 (18 – 69)	20	80 (16)	7	54.5 (6)	45.5 (5)
G-2 (18)	22.1 (15 – 38)	18	66.7 (12)	5	100 (11)	0
ST 5405* (10)	30.9 (6 – 51)	10	80 (8)	7	100 (9)	0
All isolates (1066)	31.4 (0 – 76)	1050	82.7 (868)	478	66.4 (438)	33.6 (222)

**ST 5405 was not found to be part of a larger genogroup*

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 MIC breakpoint by Etest.

Statistical analyses were applied to explore associations between genogroups and antimicrobial resistance/decreased susceptibility, as described in section 2.5.

In Table 6 the consensus MIC for cefixime was considerably increased for G-1407. The association between this genogroup and decreased susceptibility to cefixime (MIC ≥0.125 mg/L) was highly significant (OR 73, 95% CI 36.4-146.4, p<0.0001, X^2 test) by univariate analysis and also following multivariable analysis to control for patient age, gender and sexual orientation (OR 98.88, 95% CI 49.1 – 198.7, p<0.0001 X^2 test). This analysis was not performed for ceftriaxone as there were no examples of isolates showing decreased susceptibility (MIC ≥0.125 mg/L), but the consensus MIC was raised compared with those for other genogroups (Table 6). In addition, all examples of G-1407 were ciprofloxacin resistant (p<0.0001, Fisher's exact test). 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 after controlling for

patient age, gender and sexual orientation (OR 11.5, 95% CI 5.5 – 23.9, p<0.0001, X^2 test). As MIC data for azithromycin is 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 G-1407 and azithromycin MICs close to the breakpoint for resistance.

As cefixime is one of the recommended first-line therapeutic agents to treat gonorrhoea, the molecular epidemiology of isolates exhibiting decreased susceptibility to this agent, and the predominance of a major genogroup is of particular interest. None of the other isolates included in Table 6 showed decreased cefixime susceptibility but this was observed in a further 20 STs representing an additional 26 isolates in the total study population. Of these 26 isolates, 21 shared *tbpB* allele 110 but the 15 different *porB* alleles within this set differed from allele 908 by >5bp. One isolate possessed *porB* allele 908 but differed markedly at the *tbpB* allele from 110, and the STs of the remaining four isolates did not appear to be related to ST 1407.

All isolates except one belonging to G-225 were resistant to ciprofloxacin: this association was statistically significant according to Fisher's exact test (p<0.0001). All ten examples of ST 5405 were resistant to ciprofloxacin also (Table 6) which was significant by Fisher's exact test (p=0.0032), although this should be interpreted with caution given the small sample numbers.

Table 6: Consensus antimicrobial susceptibility results for genogroups (G)/sequence types (ST) represented by 10 or more isolates

Genogroup or ST	No. of isolates	Beta-lactamase	Consensus resistance category (No. isolates differing from consensus)				Modal MIC mg/L (range)	
			Azithro-mycin	Ciproflox-acin	Spectino-mycin	Genta-micin	Cefixime	Ceftriaxone
G-1407	248	Neg	S (44*)	R	S	8 (4-16)	0.125 (0.032-0.25)	0.047 (0.008-0.094)
G-2992	84	Neg	S (14†)	S (1)	S	8 (2-8)	0.023 (<0.016-0.064)	0.006 (0.002-0.016)
G-225	107	Neg	S (1‡)	R	S	8 (3-16)	0.023 (<0.016-0.064)	0.016 (0.003-0.047)
G-25	43	Neg	S	S (1)	S	8 (4-8)	<0.016 (0.016-0.064)	0.003 (<0.002-0.016)
G-387	37	Neg	S	S	S	8 (4-8)	<0.016 (<0.016-0.016)	<0.002 (<0.002-0.002)
G-359	20	Neg	S (1‡)	S	S	8 (4-16)	0.064 (<0.016-0064)	0.008/0.012 (0.004-0.023)
G-2	18	Neg	S	S	S	4 (2-8)	<0.016 (<0.016-0.032)	0.003 (<0.002-0.012)
ST 5405	10	Neg	S	R	S	8 (4-8)	<0.016 (<0.016-0.023)	0.004/0.006 (0.003-0.006)

**40 of the 44 with an R category were within one doubling dilution of the breakpoint*

†10 of the 14 with an R category were within one doubling dilution of the breakpoint

‡ Isolates that differ from the consensus were within one doubling dilution of the breakpoint.

4 Conclusions

4.1 Molecular typing of European isolates 2009/2010

This report describes the first survey of NG-MAST types across Europe and provides novel information on STs circulating in 21 EU/EEA Member States. Although there is considerable diversity of gonococcal STs both within and among EU countries, some types are predominant. This predominance is even more apparent if STs are grouped into genogroups to indicate highly related types. While one of the aims of the pilot study was to provide a baseline of STs circulating in Europe, given the variation in sampling strategy and number of cultures submitted for testing by different countries, the prevalence of different STs should be interpreted with caution.

Statistical analyses demonstrated associations between patient characteristics and genogroup/ST. For some genogroups, patients are younger (G-2) or a lower proportion of males are infected (G-25, G-387), which may be indicative of some types being heterosexually associated. This is supported by further analysis of the subset of patients for which sexual orientation was known: Genogroups G-2, G-25 and G-387 are rare in MSM. In contrast, other genogroups may occur more frequently in MSM, given the apparent associations with older patients (G-1407), and male gender (G-2992, G-1407). Once again, analysis of the patient subset for which sexual orientation was known confirmed potential associations between MSM and genogroups G-2992 and G-1407. However, given the limitations of the sampled study population and the lower completion rates of 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.

A strong association was identified between G-1407 and decreased susceptibility to cefixime. Raised MICs to the other first-line treatment, ceftriaxone, were also observed, as well as ciprofloxacin resistance and raised MICs to azithromycin, which also appears to be of statistical significance. The observed predominance of ST 1407 and related STs in many EU countries is worrying, given their potential to be therapeutically challenging. While G-1407 was shown to be associated with MSM, it is evident that this type is also circulating in the heterosexual population and so the potential risks of future treatment failure are not restricted to any one patient group. The observation that some other less closely related STs, sharing *tbpB* 110 but differing by >5bp at the *porB* locus, also showed raised MICs to cefixime suggests a need for additional typing to clarify the true relatedness of these strains to ST 1407. Statistically significant associations have been identified between ciprofloxacin resistance and the predominant genogroups G-1407 and G-225 and ST 5405. Fluoroquinolones are no longer recommended as first-line therapy, but the association between resistance and particular NG-MAST types could facilitate appropriate treatment of patients in the future, if AMR reaches levels requiring empirical therapy to be replaced by tailored treatment.

4.2 Public health benefits of NG-MAST

The use of molecular typing to predict AMR profile has clear public health benefits as it could aid understanding of the dissemination of resistance within a population and facilitate development of targeted intervention strategies, particularly if the association between molecular type and epidemiological characteristics is well defined. In addition, 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. Statistical analyses in the current pilot study have shown clear associations between the AMR profile and molecular type. Although the representativeness of the sample is a limitation, the pilot study has nevertheless successfully demonstrated a 'proof of principle' that this approach could be valid and have public health value if a sufficiently representative and longitudinal sample is used to generate baseline data. The potential benefits are most apparent in the case of G-1407, which shows a strong association with decreased susceptibility to cefixime, one of the first-line therapeutic options. Treatment failure in patients infected with gonococci exhibiting MICs of the levels in the current study are documented in the United Kingdom, Norway and Austria [12, 35, 36]. All of these failures belonged to G-1407, as did an isolate in France showing higher cefixime and ceftriaxone MICs of 4mg/L and 1-2mg/L respectively [34]. G-1407 therefore represents a potential major public health problem if it continues to be disseminated without measures being taken.

4.3 Molecular surveillance of gonorrhoea

This report indicates that there is a clear public health benefit to be gained from performing molecular surveillance 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 this 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 is a 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.
- A pilot study could be conducted to establish suitable additional loci to discriminate between predominant STs and clarify the relationships between STs. This would enable the optimisation of methods for future surveillance. Existing typing schemes (MLST, MVLA) have failed to discriminate within ST 1407 and novel approaches (e.g. whole genome sequencing) would therefore be required to establish whether this is a true clone.
- Further longitudinal typing of a representative sample would be required to monitor stability of associations among STs and AMR and/or epidemiological characteristics, and to identify temporal changes and emergence of novel STs. If this cannot be done on an annual basis 'snapshot' studies could be considered at regular intervals. For instance, as a minimum, future typing of isolates exhibiting decreased susceptibility to third generation cephalosporins could be performed to identify STs that have the potential to cause treatment failure and to monitor dissemination and evolution of this ongoing problem.

Retrospective typing of isolates exhibiting decreased susceptibility to cephalosporins and further characterisation of potential resistance determinants (e.g. penA, mtr, penB) in STs associated with cephalosporin decreased susceptibility would provide greater understanding of the emergence and dissemination of this phenotype.

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Annex 1. Minutes of the meeting of the expert group on molecular typing (August 2010)

Summary

The purpose of this expert group meeting was to discuss and provide an assessment of the added value to public health systems of introducing molecular typing for surveillance of gonorrhoea (including gonococcal antimicrobial resistance), chlamydia (including LGV, Lymphogranuloma venereum) and syphilis at the EU level.

The following items were suggested for broader consultation in the European STI surveillance network in order to move forward a pilot in molecular typing for STI providing the maximum public health benefit:

- Typing of *Neisseria gonorrhoeae* (NG) isolates from the antimicrobial resistance surveillance study will give the maximum public health benefit at this present time.
- *N. gonorrhoeae* multi-antigen sequence typing (NG-MAST) will be used for the typing of *N. gonorrhoeae* isolates, and the need for further discrimination will be explored.
- Typing will be performed to investigate associations between STs and antimicrobial resistance (AMR) profile, with a view to using NG-MASTs as a predictor of AMR directly on clinical samples collected for molecular testing.
- In addition, baseline data will be established which could be useful for future outbreak detection.
- Quality systems will be implemented for the use of NG-MAST.
- Typing methods for chlamydia and syphilis have no public health benefit at this time because the methods do not show sufficient robustness or discrimination.

1. Background

Since 2007, many disease specific networks (funded by the EU Commission's Directorate-General for Health and Consumers, DG SANCO) have been integrated into or outsourced by the European Centre for Disease Prevention and Control (ECDC), and molecular typing activities are included as part of these networks for several diseases. Some ECDC-funded outsourced laboratory networks are now operational in the European Union, performing typing for surveillance, with a wide variety of data flows and databases. Moreover, ECDC is currently investigating possible ways to implement integration of molecular typing data into EU-level surveillance.

Surveillance and monitoring of chlamydia (including LGV), gonorrhoea and syphilis is performed at EU level as part of enhanced STI surveillance, but does not currently include molecular typing methods. Since 2009, ECDC has coordinated the enhanced surveillance of STI in Europe. ECDC strives to ensure a high quality of standardised STI surveillance data from the countries of the EU and the European Economic Area (referred to as EU/EEA). The STI microbiology project is part of the European STI surveillance (launched in August 2009) and has been outsourced to an international team lead by the Health Protection Agency (UK) and including the Statens Serum Institute (Denmark) and Örebro University Hospital (Sweden).

In the former ESSTI (European Surveillance of Sexually Transmitted Infections) project, molecular typing of outbreaks was performed, however this was limited to gonococcal typing of selected isolates of decreased cephalosporin susceptibility and a study of the molecular epidemiology of syphilis in Scotland. Molecular typing of pathogens causing infectious diseases complements the traditional epidemiological surveillance. It provides appropriate discriminatory analyses to foster rapid, early detection of dispersed international clusters and outbreaks, for detection and investigation of transmission chains and relatedness of strains, emergence of antimicrobial resistance and new evolving pathogenic strains. It also supports studies to trace the source of an outbreak and identify new risk factors as the strains can be linked more accurately to epidemiological and clinical data.

The purpose of using typing techniques is to sequence the whole genome of a pathogen as this offers the greatest power of discrimination. However, for epidemiological purposes for many diseases, methods with lower discriminatory power are sufficient and molecular typing cannot completely override the need to isolate pathogens, which is still necessary for strain collection and phenotypic testing (e.g. for antimicrobial resistance).

Before adapting a typing method from a research setting to a broader context, a number of evaluation criteria must be considered. Among these are typeability, discriminatory power, epidemiological concordance and reproducibility. These characterise the 'technical' appropriateness of a method for the typing of a specific pathogen. Similarly, before molecular typing data can be included in surveillance at EU level or a typing method proposed for routine application, additional considerations are required.

The method should:

- be driven by practical needs for surveillance and provide essential information to achieve the surveillance objectives for the specific disease;
- produce comparable and valid data between laboratories across countries, including standardised typing protocols and nomenclature;
- be interpreted and assessed by molecular typing experts of the pathogen in order to guide public health response and decision making;
- give unambiguous typing data resulting in a single and uniform nomenclature for clones;
- provide typing data for 'eternity' and therefore be apparatus and company independent.

In addition, external quality control must be established and performed regularly, and all EU Member States should have access to the agreed molecular typing method/pathogen, either by building up capacity in their own country or by getting support from those Member States who have already developed the capacity.

It is the aim of ECDC to discuss and assess the added public health value of these molecular typing activities with respect to bacterial STIs and if needed, to implement this molecular typing approach at European level. It has the potential to provide insight into the dissemination of antimicrobial resistant strains and to detect outbreaks not immediately apparent using current surveillance methods. Improving understanding of the molecular epidemiology of bacterial STIs may help to inform intervention and control strategies. For this reason, one of the key overall aims of the STI microbiology project is to assess the benefits of molecular typing in investigating antimicrobial resistance in *N. gonorrhoeae* and in detecting and investigating outbreaks of bacterial STI.

To achieve this aim, the following will be required:

- Establishment of an expert group as a panel for discussion and expert opinions; the expert group should consist of both microbiologists and epidemiologists.
- Assessment of the public health added value of introducing EU-level molecular typing surveillance for gonorrhoea (including gonococcal antimicrobial resistance), chlamydia (including LGV) and syphilis.
  - Discussion should focus on the potential public health benefits of molecular typing of STIs taking into consideration the logistical and technical challenges of introducing molecular typing at the EU level.
  - If considered of public health benefit, the purpose – i.e. outbreak investigation or routine surveillance – needs to be discussed.
- Discussion of the available methods for STI molecular typing;
  - Appropriate methods for typing bacterial STIs that are discriminatory, reproducible and transferable;
  - Strategies for implementing molecular typing in Europe.
- Decision on the scope and nature of a possible pilot study to evaluate the feasibility and benefits of proposed strategies.
  - In the event of a negative assessment, a description should be provided of the main reasons and/or obstacles identified.
- Provision of training in the proposed typing methods can be considered after the pilot has been successfully evaluated and implementation is planned to take place across EU.

2. Scope and purpose of the meeting

The purpose of this expert group meeting was to discuss and assess the public health added value of introducing EU level molecular typing surveillance for gonorrhoea (including gonococcal antimicrobial resistance), chlamydia (including LGV) and syphilis.

The scope of the meeting is detailed in the agenda (Appendix 1) and included the following topics :

- Public health added value of molecular surveillance
- Molecular surveillance of antimicrobial resistance (AMR) and outbreaks in *Neisseria gonorrhoeae* (NG) including typing methods and types of isolates to be tested
- Molecular surveillance of other bacterial STIs; syphilis and chlamydia
- Quality systems for typing
- Any other business.

The meeting also aimed to include the expected outcome of a proposal being discussed at the annual meeting of the network of microbiologists and epidemiologists.

3. Public health added value of molecular surveillance

Discussions focused on assessing the public health benefit of molecular typing activities with respect to bacterial STI and included the following points:

3.1 Outbreaks: gonorrhoea

- Typing of resistant isolates from an outbreak offers the potential of improved information when planning public health interventions and intensive control efforts; however there is insufficient evidence to indicate public health benefit.
- Molecular typing can identify whether a collection of isolates of *N. gonorrhoeae* are from a common source or part of a transmission chain if typing is performed in a timely manner. However the use of typing for outbreaks requires knowledge of a background level of different types and there was a general agreement that typing in outbreaks would not lead to any real time public health benefits.
- For linked cases in an outbreak the source would need to be identified, which is not currently the case for STIs unless it is something new like the new chlamydia variant. This was the public health approach to NG resistance when contact tracing for gonorrhoea was more widespread. However, partner notification has been reduced and MSM contact tracing does not work well, making it necessary to investigate outbreaks in different ways. For outbreak detection it would be necessary to focus on certain patient groups, such as MSM with LGV and/or syphilis, where typing could discriminate sub-epidemics to become a public health advantage.
- Typing data could be given back to clinics and any identified clusters would allow targeted public health interventions. However there are confidentiality issues over this and concerns that health advisors lack understanding and may make assumptions regarding the direction of STI transmission.

Conclusion: Typing of isolates of N. gonorrhoeae from outbreaks does not have sufficient public health value at this time**.**

3.2 Monitoring antimicrobial resistant gonorrhoea

- The movement towards more timely and comprehensive coverage for surveillance of gonococcal AMR will need support from Member States. The additional information that could be used for public health purposes, such as monitoring and investigation of outbreaks, could be an incentive for additional EU countries to participate in the surveillance of gonococcal AMR.
- The public health benefit of molecular typing of isolates from AMR surveillance would depend upon sample representativeness.
- The advantage of molecular typing of gonococcal isolates is the provision of enhanced surveillance data. It would also be useful to perform molecular typing directly on residual NAAT specimens to provide additional data. This is one of the reasons for increased support and funding by Health Protection in Scotland for the typing of gonococcal isolates, where Scotland has complete coverage.
- For European added value a reference population could be created with both typing data and culture-based AMR data. It was suggested that only resistant isolates should be typed, however it was felt that a more representative data set would be needed. Resistant and non-resistant isolates could be typed over a time period to detect changes. Countries that do not have culture could potentially use the typing data from NAAT specimens to establish resistance profiles as there is a real possibility that there will be a shift away from culture, and a benefit of typing would be the use of NAAT specimens to monitor for AMR.
- One idea would be to have sentinel sites for the collection of isolates or NAAT specimens. This is the model Euro-GASP uses for isolate collection which could be extended to collect NAAT specimens.
- Regular repeated studies would be required to monitor and check the correlation between phenotypic AMR data and *Neisseria gonorrhoeae* Multi-Antigen Sequence Typing (NG-MAST).

Conclusion: Molecular typing of gonococcal isolates is of public health value but would be most powerful when combined with the epidemiological data collected through TESSy.

3.3 Chlamydial infection

- A number of typing methods have been described for *Chlamydia trachomatis* but none of the typing methods shows sufficient discrimination to give useful information, such as whether a certain type is linked to PID (pelvic inflammatory disease), which would be a real public health benefit.

Conclusion: Molecular typing for *C. trachomatis* is not sufficiently advanced to be used at the EU level at this time.

3.4 Syphilis

- Europe has a need to perform molecular epidemiological studies for syphilis but the typing methods available are technically tedious, have low throughput and lack discriminatory power. Pilots would be useful although they would only answer research questions and not tackle public health issues at the present time.

Conclusion: Molecular typing for *Treponema pallidum* is not sufficiently advanced to warrant a pilot across the EU.

4. Appropriate methods for molecular surveillance

4.1 AMR: *Neisseria gonorrhoeae* and outbreaks

Typing methods

The typing method needs to be discriminatory, have transferable technology and data for laboratory comparisons and have a rapid throughput. The most valuable method would be one which could produce an association between ST and AMR profile. The use of NG-MAST was discussed and it was agreed by the expert group that this would be the most appropriate typing method. NG-MAST can distinguish between isolates from different sources but is sufficiently stable to identify isolates from sexual contacts or short transmission chains (such as those found in clusters or outbreaks). It is reproducible, robust, high-throughput and produces transferable data.

NG-MAST is now accepted as the current method of choice and has replaced the phenotypic methods, auxotyping and serotyping, and molecular methods based either on restriction fragment length polymorphisms (RFLP) or sequence-based methods. Phenotypic methods, which were widely used in the past, did show high discrimination when used in combination. However, they were technically demanding, had subjective endpoints and displayed variable reproducibility. All molecular methods showed improved reproducibility but the sequence-based methods produced more robust data than RFLP methods. RFLP patterns produced by pulse field gel electrophoresis (PFGE) and opa typing are both discriminatory methods but the sequence data produced by NG-MAST has proved more robust, particularly for use with large numbers of strains where comparison of patterns is difficult.

NG-MAST is adequate but it does not discriminate between resistant organisms of the same ST, suggesting that an alternative method may be useful for further discrimination. For example, the most prevalent ST in Scotland is 1 407 in about 15–20% of isolates, but we do not know if these are clonal. Similarly, clusters of the penA mosaic gene are found but this does not demonstrate that they are directly linked to one another.

NG-MAST currently examines diversity in two very polymorphic genes (por and tbpB), and it would be possible to add an additional typing method or a third locus to add discrimination. One such additional method could be sequence-based typing of one of the opa genes or the penA mosaic gene. However, it was felt that penA mosaic detection is important for characterisation and not typing.

Conclusion: NG-MAST is an appropriate method to use for molecular surveillance although additional alleles should be investigated to provide more discrimination.

AMR: typing

As described in the draft proposal there were three suggested strategies:

- Type all isolates identified in EURO-GASP exhibiting decreased susceptibility to third generation cephalosporin to provide greater understanding of the epidemiology. The possibility of characterising the *penA* gene could be introduced.
- Type all high-level azithromycin resistant isolates (e.g. 128 mg/L and above), up to a maximum of 100 per year in the pilot. The value of this would be to monitor and control dissemination of high-level azithromycin resistance which could limit future therapeutic options, since this agent has been proposed as a component of combination therapies.
- Identify countries where typing of isolates resistant to other antimicrobials may be of public health benefit, for example if resistance levels are low enough (<5%) for these agents to have some therapeutic benefit.

Conclusions:

- All isolates from the AMR surveillance study should be typed, including susceptible isolates, as opposed to a case control study.
- Typing all isolates will allow investigation of the association between STs and resistance/susceptible profiles. Although it was suggested that both the 2009 and 2010 isolates should be typed, the sampling frame and strategy needs to be discussed further, including the budget and logistics.
- Typing data should be linked to the AMR and epidemiological data and therefore the extra variables for typing should be added to the Euro-GASP proposal.

- A smaller study consisting of only a few isolates may be conducted to compare NG-MAST detection from NAAT samples. This would help to establish whether typing can be performed in the absence of a viable isolate, as NAATs are increasingly being used as the sole method of detection for *N. gonorrhoeae*.

Outbreak typing

The typing strategy for outbreaks would require typing any isolate thought to be involved in an outbreak. A percentage of the population would need to be typed to gain a representative baseline in each country.

Conclusion: the molecular surveillance pilot should concentrate on typing strains from the AMR study and not include outbreak strains.

5. Syphilis typing

Potential methods for typing syphilis were raised and discussed. The choice of typing methods for syphilis is limited. At present, there is only been one appropriate, published, molecular typing method (developed by CDC) which has also been evaluated in other studies. Other typing methods are still in research and there is currently no typing method that meets ECDC criteria. Typing of syphilis is important as there is very little known about the types circulating in Europe and typing could therefore provide an insight into the epidemiology of syphilis. However, it may be best to put this forward as a research proposal to DG-SANCO (or the Directorate-General for Research and Innovation, DG Research).

The current method works best on ulcer specimens and is less sensitive with blood specimens; however this typing method could be a starting point for collating data on types prevalent in Europe. The use of specimens directly from patients may have ethical and safety issues which would need to be taken into consideration.

Conclusion: Due to the lack of a suitable typing method, syphilis typing will not be included in the pilot. A research proposal on typing for *T. pallidum* in molecular epidemiological studies should be considered.

6. Chlamydia typing

At present there is no method for typing chlamydia that meets ECDC criteria, and the large number of cases is prohibitive. At this stage typing of specimens positive for LGV may be the most beneficial, as this is a restricted group, is more severe and has a lower prevalence. However a suitably discriminatory method would need to be identified as current methods have indicated that the European outbreak is clonal.

Typing methods for *C. trachomatis* have relied on differentiation based on the outer membrane protein (OmpA) resulting in a small number of types of which D-K are found in oculogenital disease and L in lymphogranuloma. OmpA typing is recognised as insufficiently discriminatory. Newer approaches that have been developed to increase discriminatory power, include multilocus sequence typing (MLST) and sequencing of three variable number tandem repeats (VNTR). However, although they show promise they still require further validation to explore the degree of enhanced discrimination achievable.

Conclusion: Typing of chlamydia needs more research and it was agreed not to include chlamydia typing in the pilot**.**

7. Quality

Molecular surveillance quality systems were discussed, such as IQC, IQA and EQA. In addition, because sequencing is used more in public health it is important to define and interpret quality.

7.1 Internal quality control (IQC)

It was agreed to use WHO strains for internal quality control (IQC). The appropriate strains for NG-MAST will be established and recommendations will be made in the proposal for all laboratories conducting molecular typing.

7.2 Internal quality assurance (IQA)

To ensure intra-laboratory testing and consistency of results it was recommended that IQA should be conducted in all laboratories performing molecular typing. The long term involvement in IQA would be down to the individual laboratories and recommendations would be provided. The individual laboratories would be able to set up the most practical system for themselves, provided that they ensure the results of previously typed samples are blinded to the laboratory staff performing re-testing and defining of types. All repeat testing should generate the same type as was defined previously in the sample.

7.3 External quality assurance (EQA)

An EQA system has been implemented for antimicrobial susceptibility testing of NG. All EQA strains have been typed using NG-MAST. Any countries performing NG-MAST typing can submit NG-MAST results for the EQA strains to ensure accuracy.

7.4 Assignation of novel types

It is important to ensure that data is of sufficient quality to enable true type assignation as there is concern that some sequences submitted are not of sufficient quality and therefore not genuine new sequence types. It was suggested that the checking of sequence quality and confirmation of novel types could be centralised, but this will require further investigation. Automated quality checking for NG-MAST could also be developed.

8. Any other business

- The expert group report will be written up and distributed to group members for comment.
- The proposal for molecular surveillance will be amended and disseminated to the expert group. When the proposal has been agreed it will be distributed at the annual meeting in September.
- The addition of an extra variable will be explored to accommodate the typing result in the TESSy database which will receive AMR data.
- The creation of an NG-MAST database to collate existing and new typing data from across Europe will also be considered.

Meeting agenda

European Network of STI Microbiology – Molecular surveillance expert group
6 August 2010, London, United Kingdom.

1. Public health added value (09:00–12:30)

09:00 – 09:10	Scope and purpose – ECDC working group	Marita van de Laar
09:10 – 09:20	Public health benefits of routine typing of *N. gonorrhoeae*	Kirstine Eastwick
09:20 – 09:30	Overview of methods for typing of *N. gonorrhoeae*	Cathy Ison
09:30 – 09.40	*Chlamydia trachomatis* typing	Magnus Unemo
09:40 – 09.50	Overview of methods for typing of *Treponerna palladium*	Michelle Cole
09:50 – 10:00	Detailed typing of *Treponerna palladium* for molecular epidemiological links in syphilis transmission	Sylvia Bruisten
10:00 – 10:10	Developing molecular typing systems for surveillance	Jon Green
Discussion on public health added value of molecular surveillance		
Draft protocol for molecular surveillance of bacterial STIs		Stephanie Chisholm

2. Review appropriate methods and public health benefits (13:00–16:00)

AMR Neisseria gonorrhoeae – outbreaks of gonorrhoea

- Molecular surveillance of gonorrhoea – public health benefits
- Which method is most appropriate for these objectives?
- Strategies for setting up a pilot study
  - Criteria for typing (which resistance profiles?)
  - Creation of representative baseline data for each STI
- Data analysis – how to identify a potential outbreak within the pilot.

Chlamydia and syphilis – to detect and investigate potential outbreaks

- Public health benefits of molecular surveillance
- Which typing method is most appropriate?
- Strategies for setting up a pilot study
  - Creation of representative baseline data for each STI
- Data analysis – how to identify a potential outbreak within the pilot.

3. Any other business

List of participants

Name	Organisation, country
Maria José Borrego	Portugal
Sylvia Bruisten	The Netherlands
Stephanie Chisholm (Co-chair)	United Kingdom
Michelle Cole	United Kingdom
Kirstine Eastwick	United Kingdom (Scotland)
Jonathan Green	United Kingdom
Catherine Ison (Co-chair)	United Kingdom
Dzintars Ozolins	Latvia
Paola Stefanelli	Italy
Eberhard Straube	Germany
Magnus Unemo	Sweden
Marita van der Laar	ECDC
Helen Ward	UK
Nerteley Quaye (minutes)	UK

Annex 2. Sequence types for each country

Figure A2.1: Sequence types in Austria

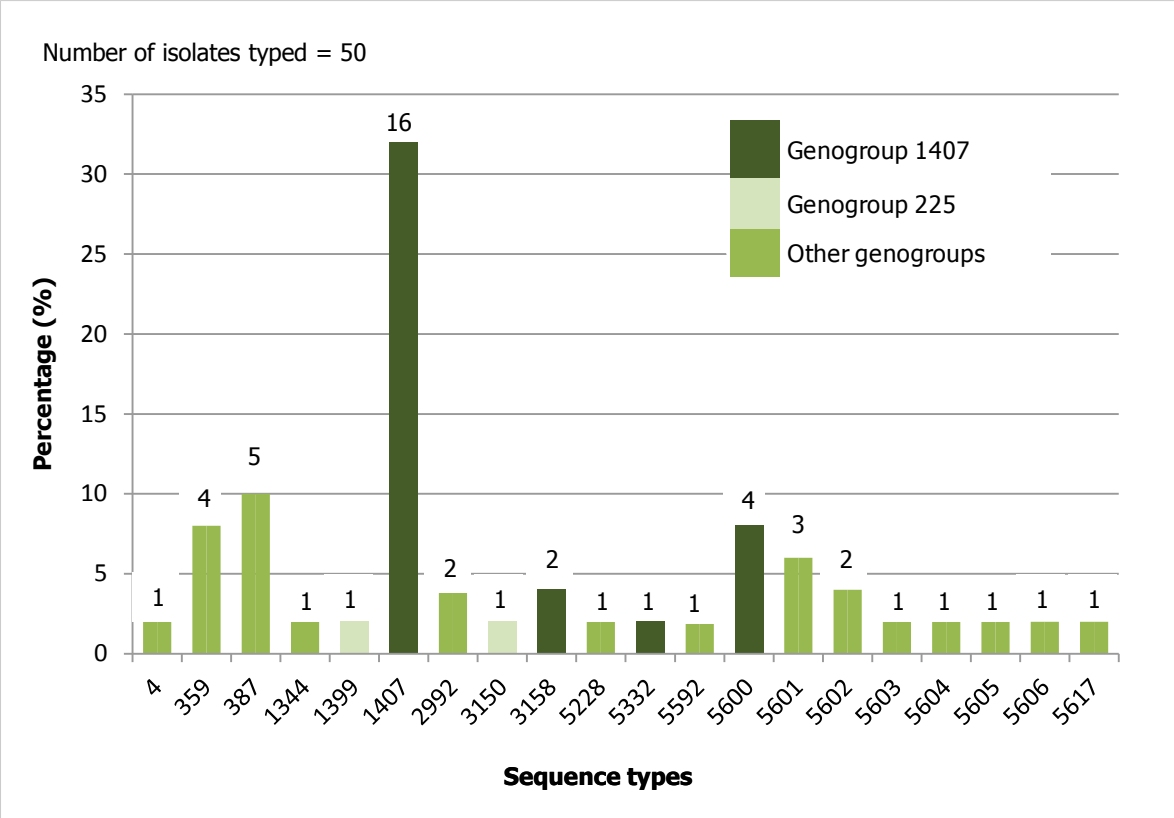

Figure A2.2: Sequence types in Belgium

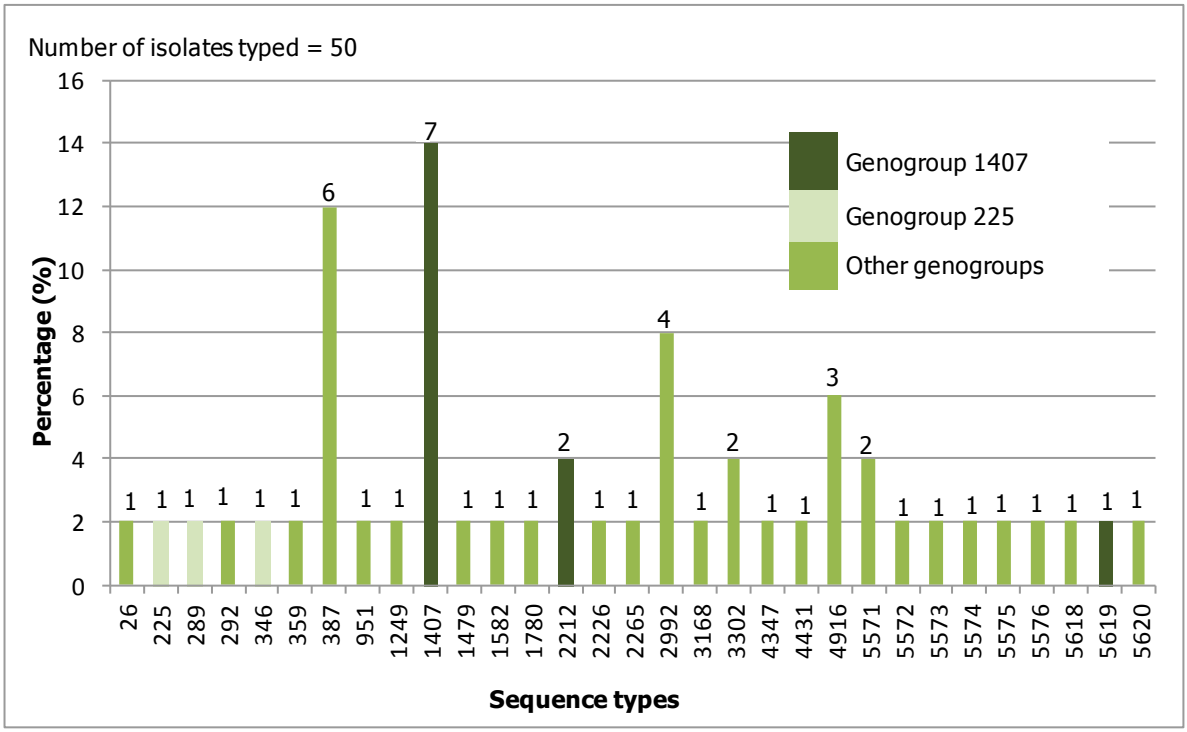

Figure A2.3: Sequence types in Cyprus

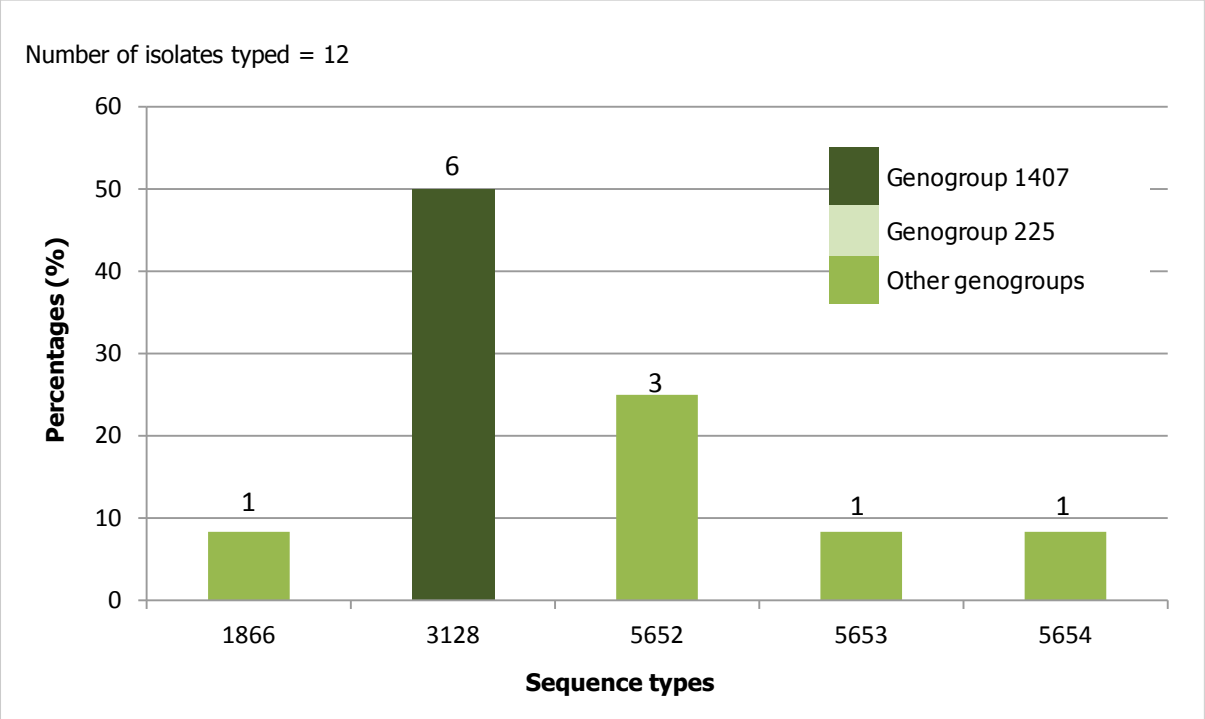

Figure A2.4: Sequence types in Denmark

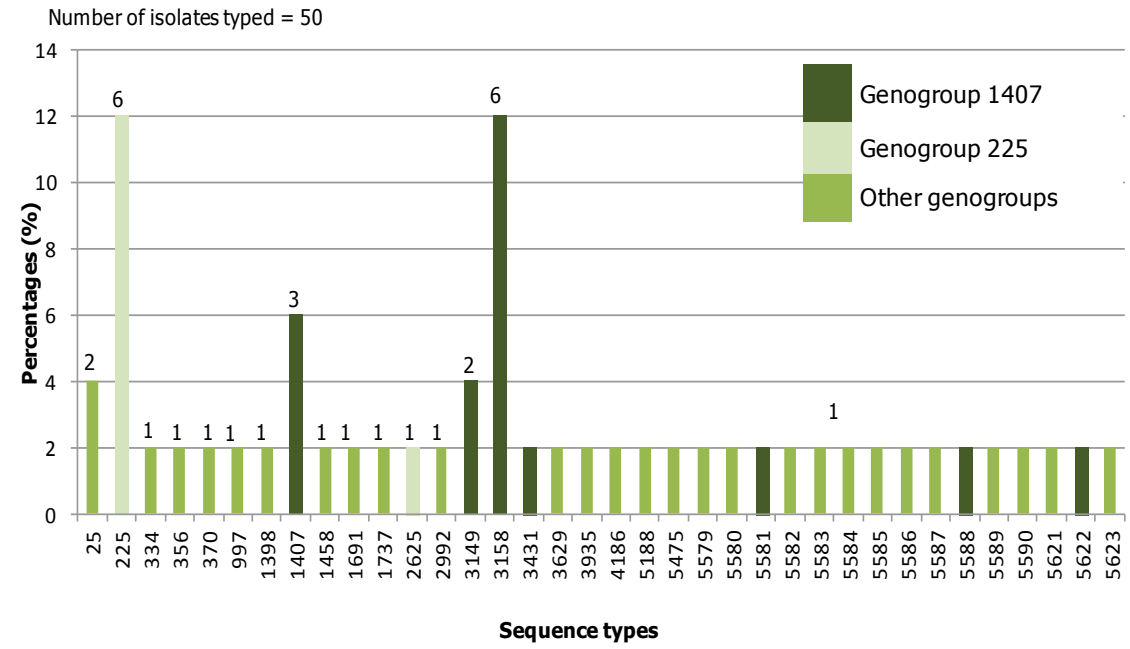

Figure A2.5: Sequence types in France

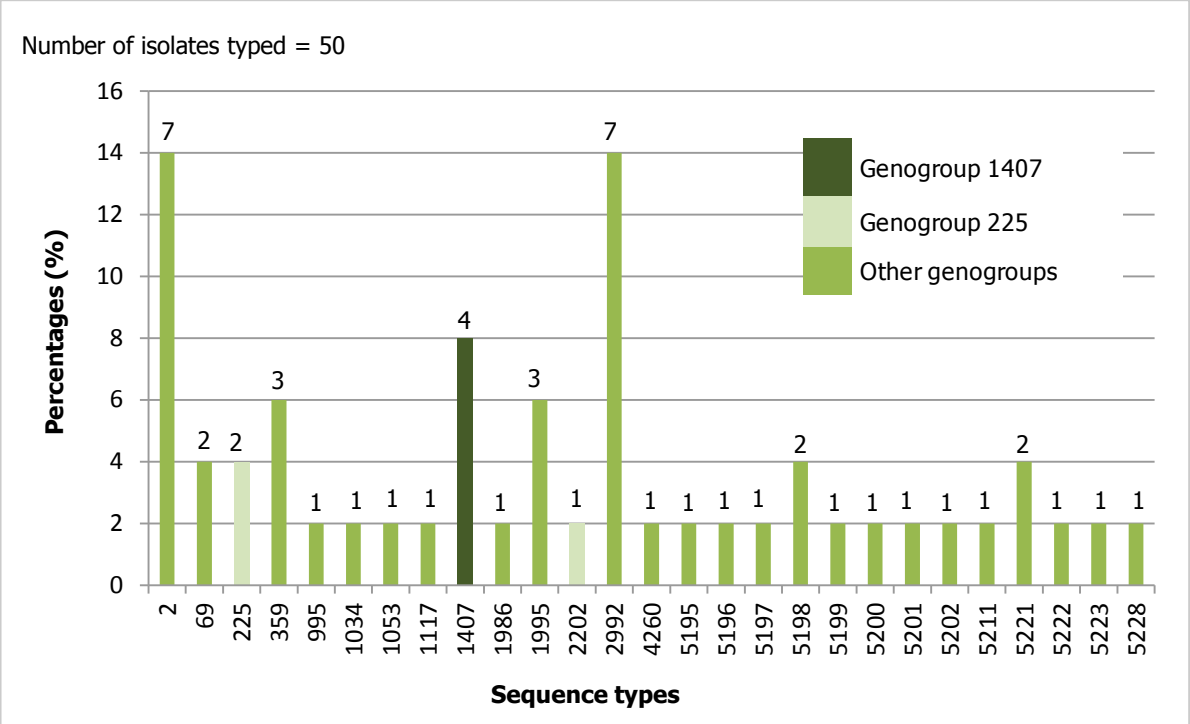

Figure A2.6: Sequence types in Germany

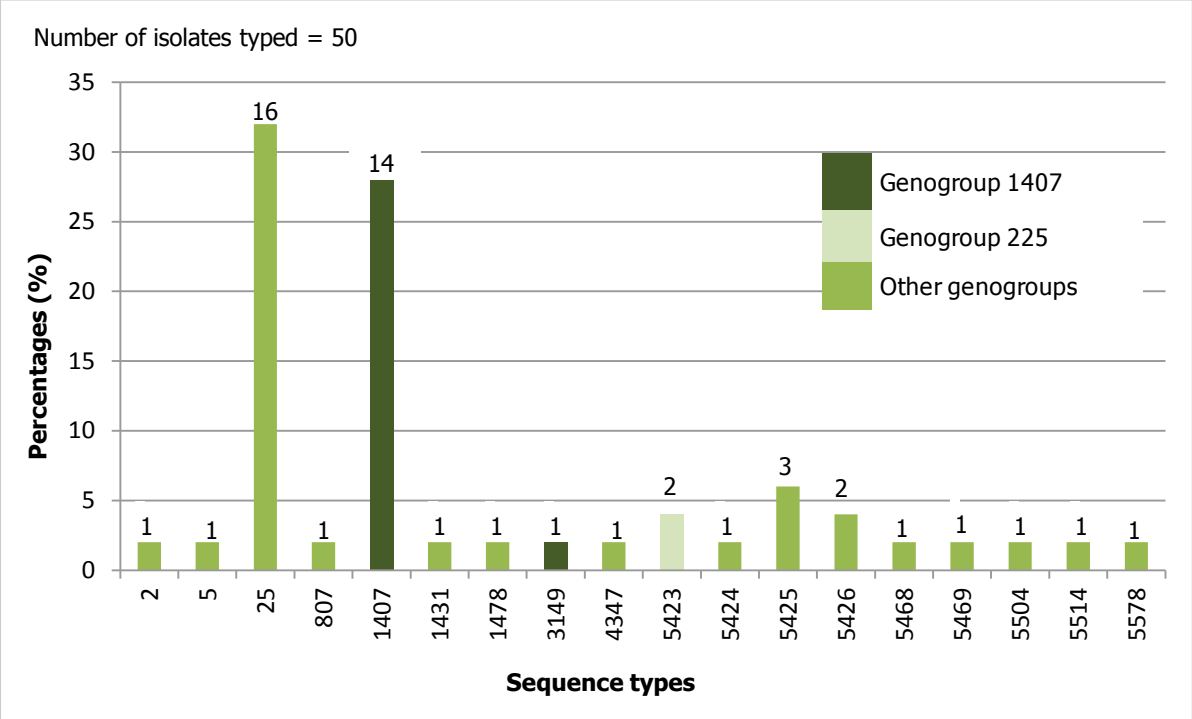

Figure A2.7: Sequence types in Greece

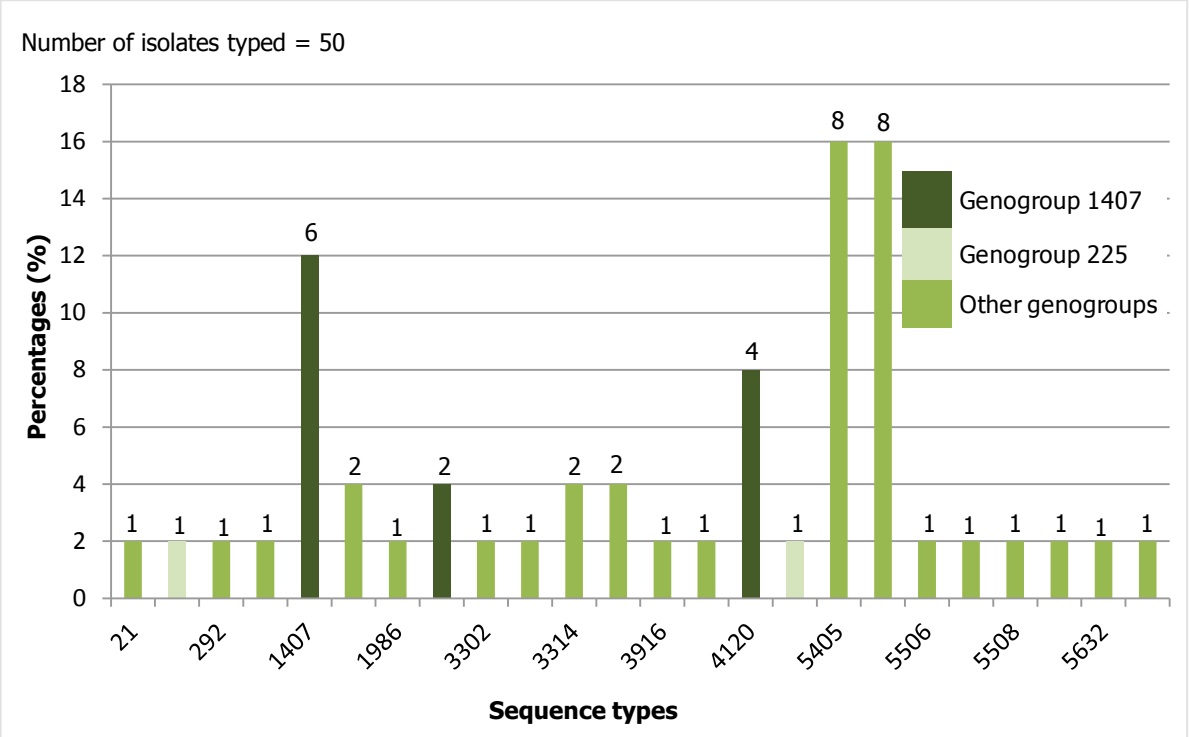

Figure A2.8: Sequence types in Hungary

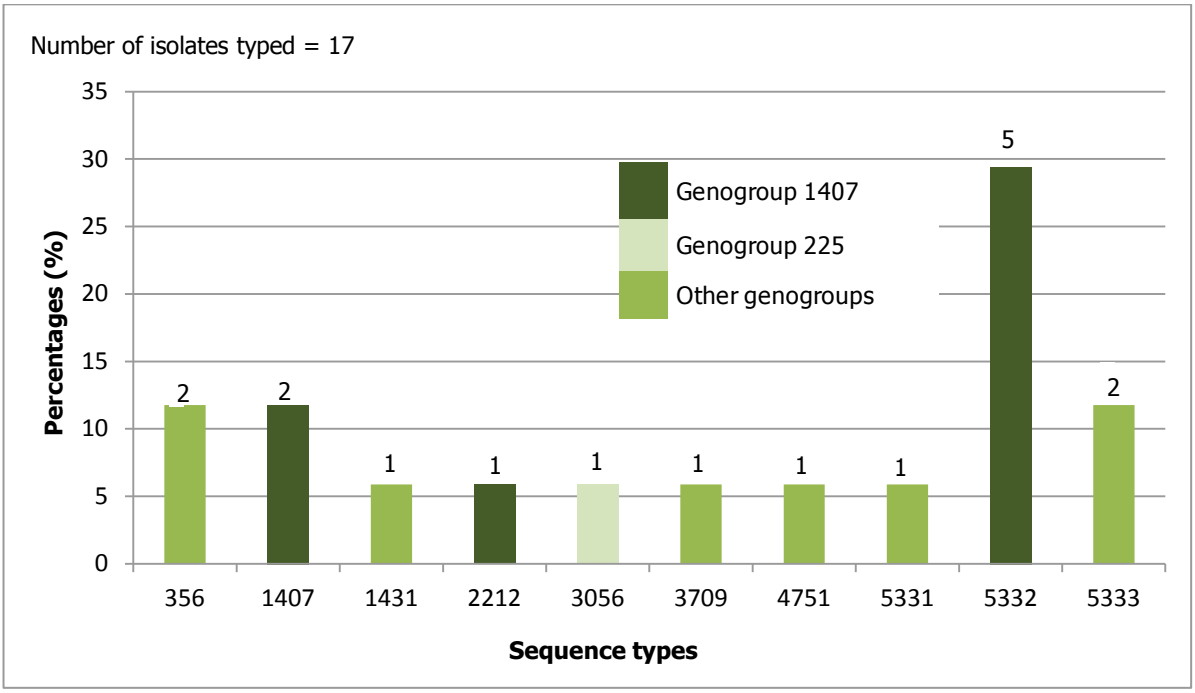

Figure A2.9: Sequence types in Ireland

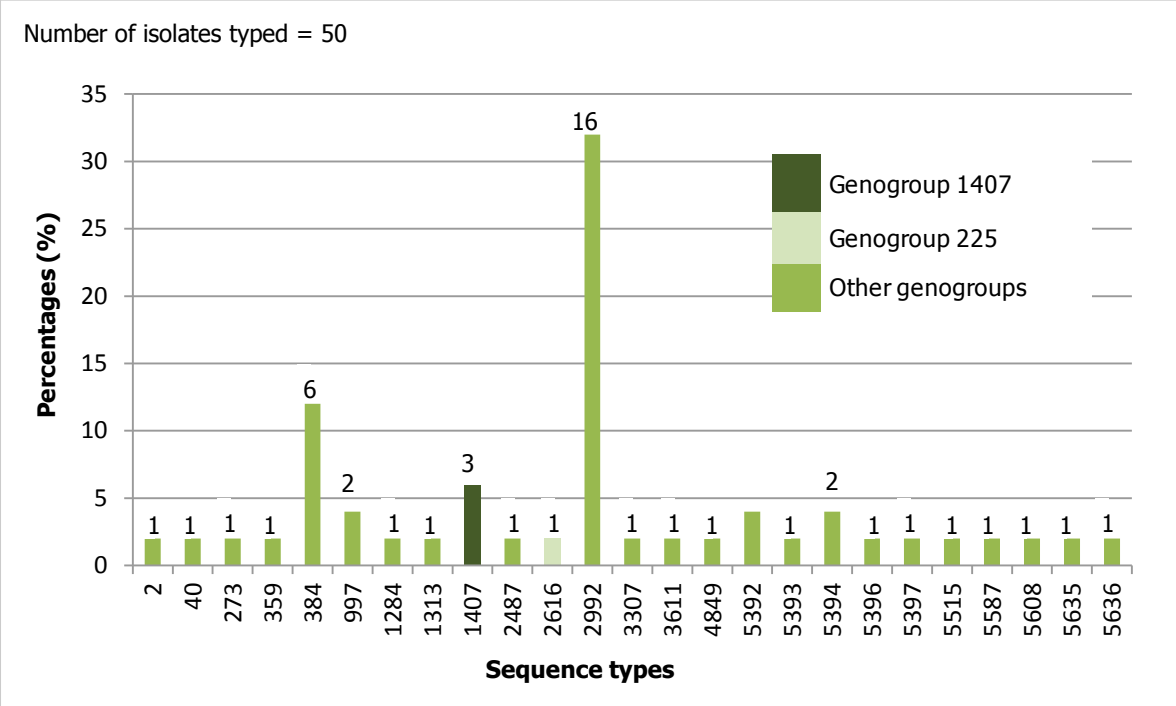

Figure A2.10: Sequence types in Italy

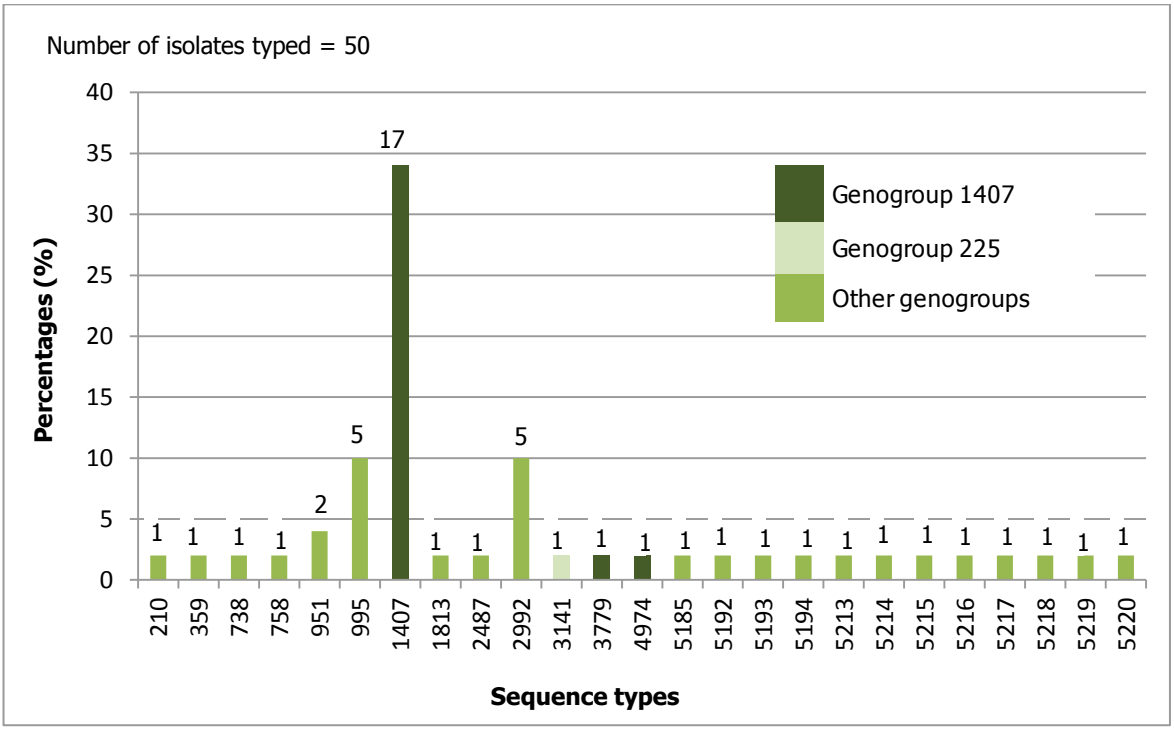

Figure A2.11: Sequence types in Latvia

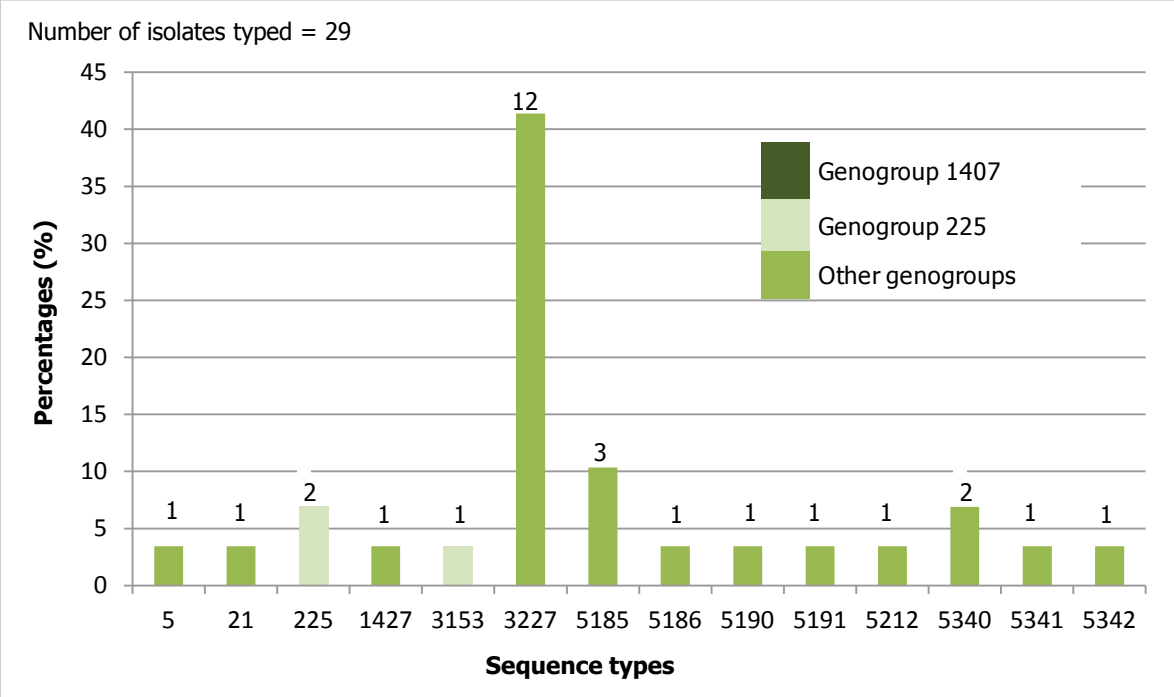

Figure A2.12: Sequence types in Malta

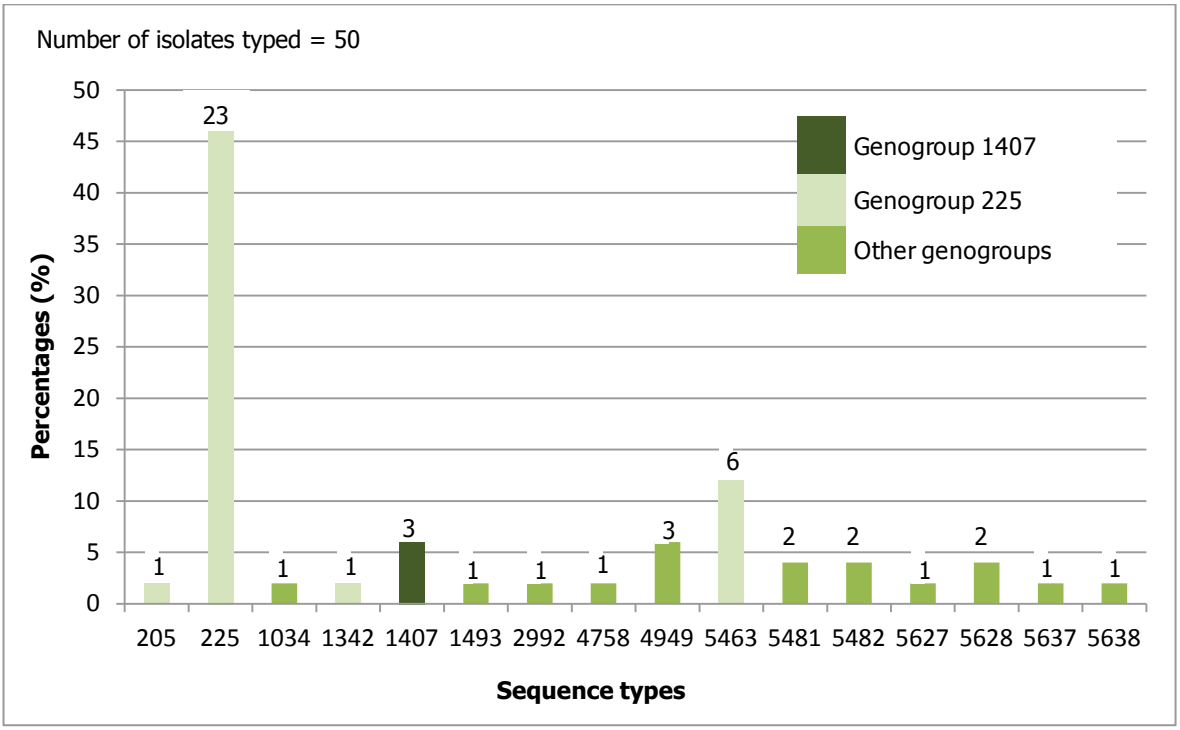

Figure A2.13a: Sequence types in the Netherlands

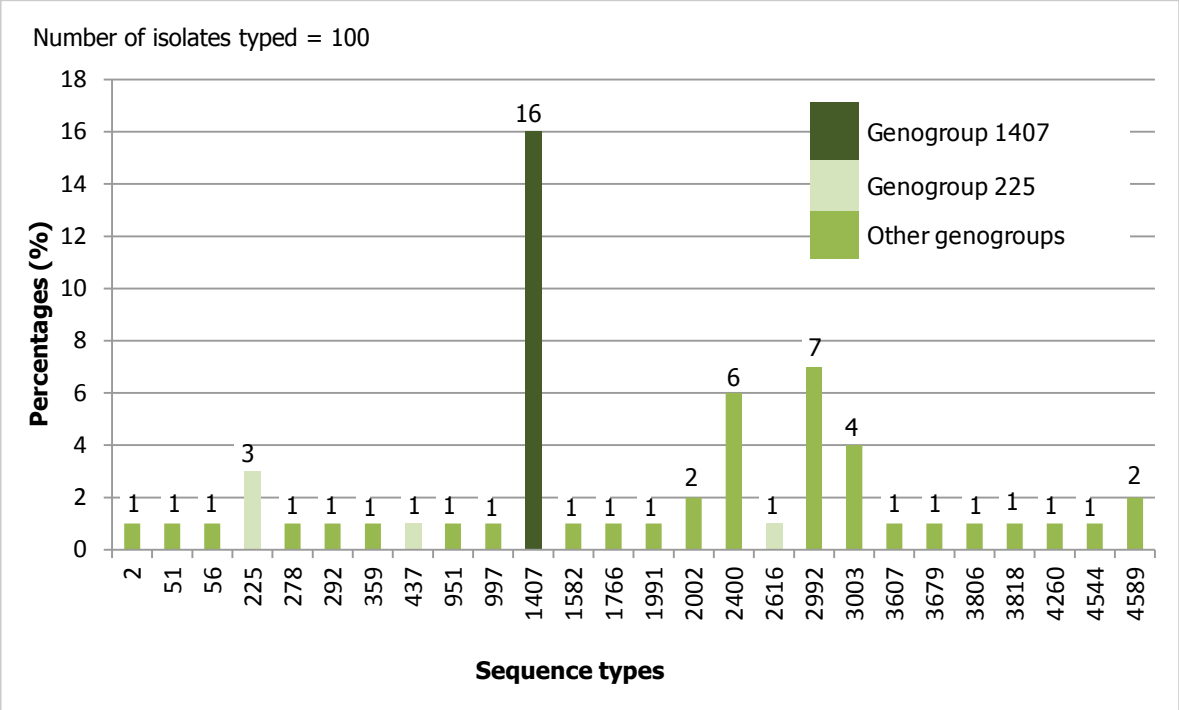

Figure A2.13b: Sequence types in the Netherlands

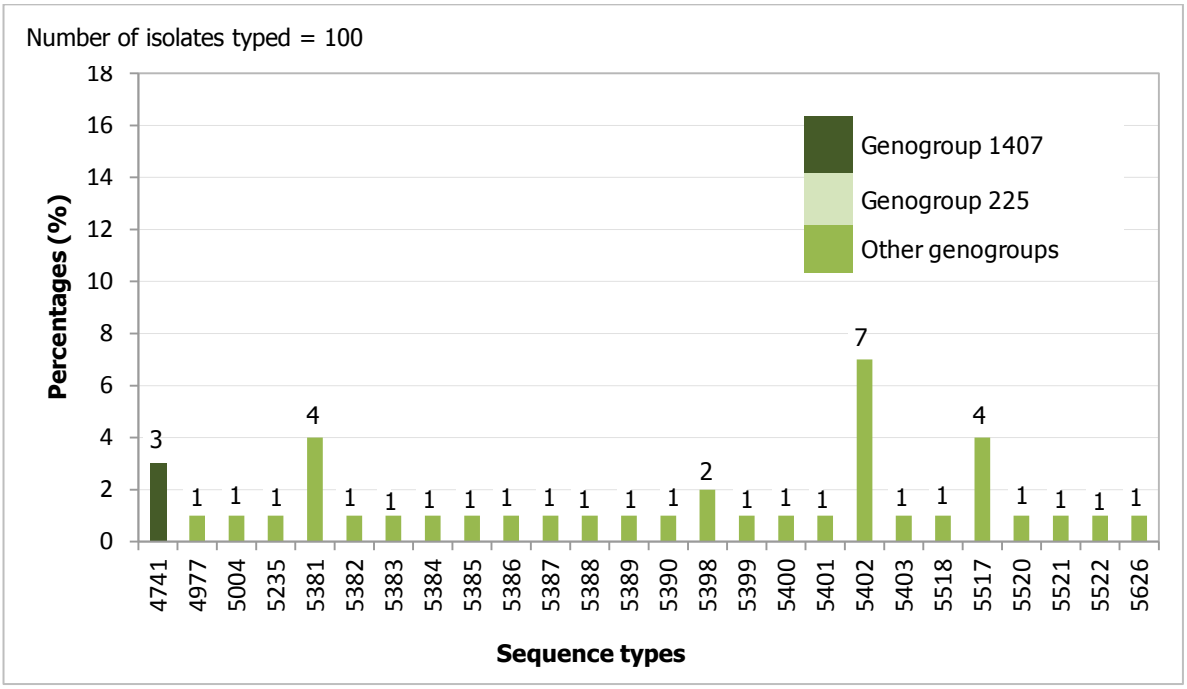

Figure A2.14: Sequence types in Norway

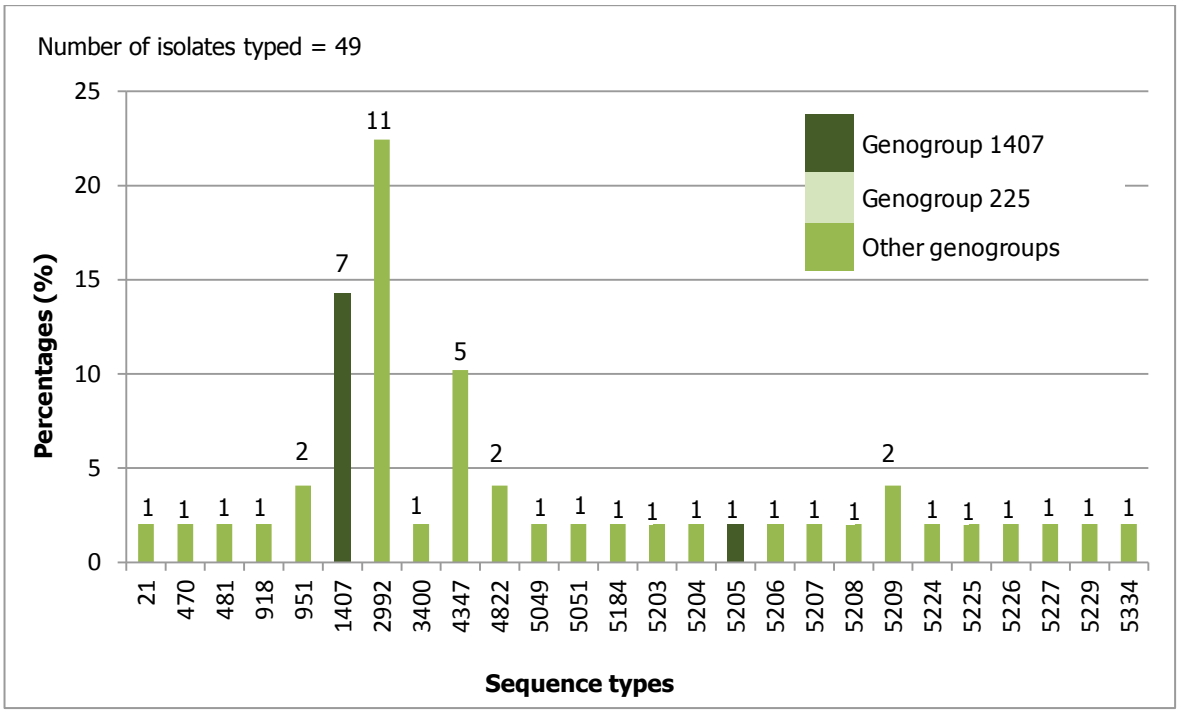

Figure A2.15: Sequence types in Portugal

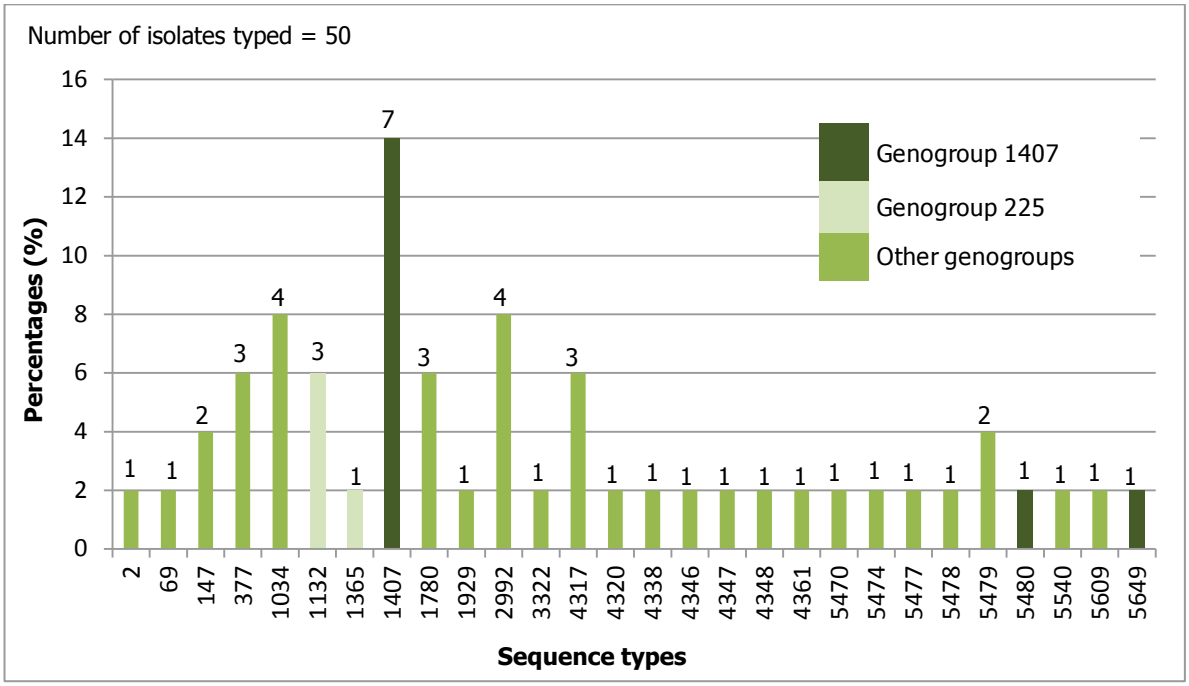

Figure A2.16: Sequence types in Romania

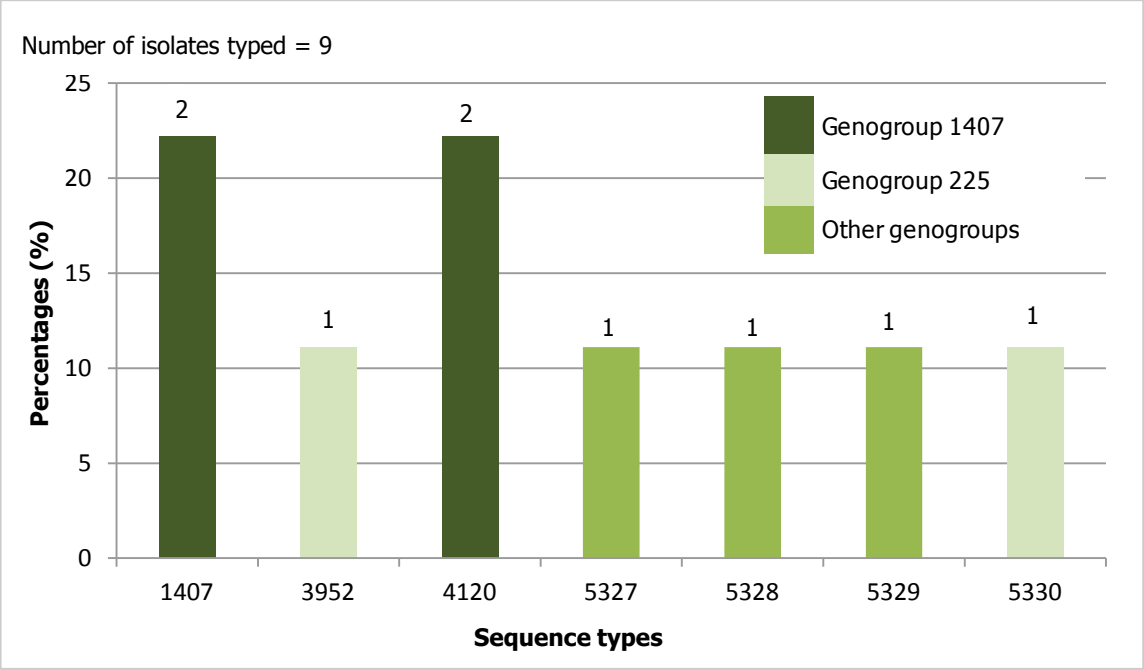

Figure A2.17: Sequence types in Slovakia

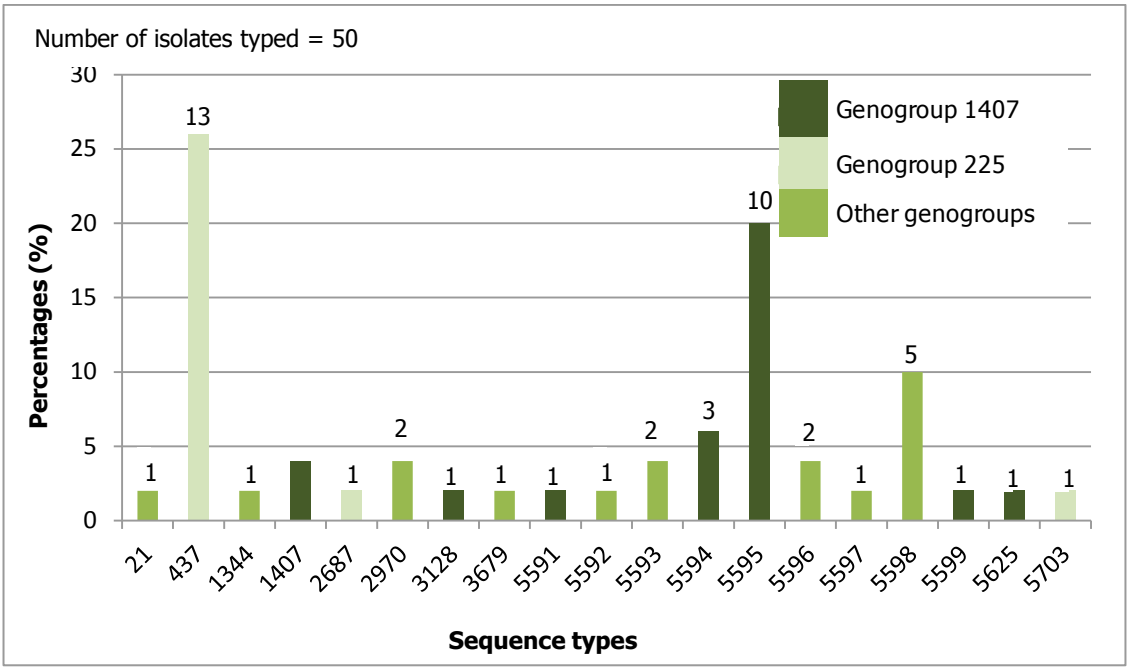

Figure A2.18: Sequence types in Slovenia

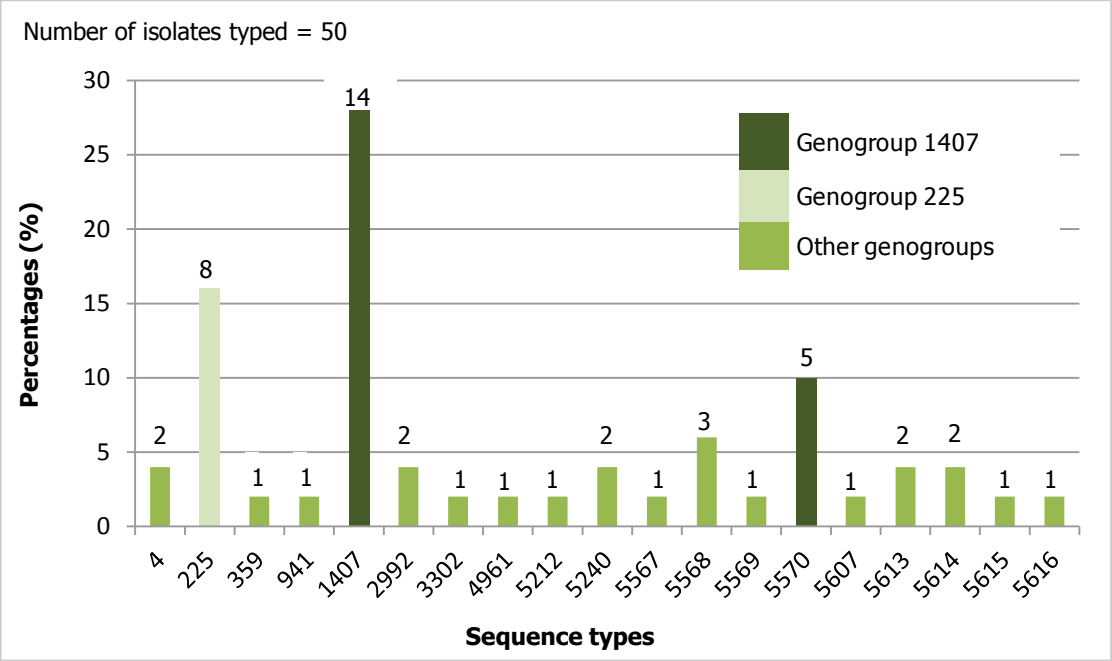

Figure A2.19a: Sequence types in Spain

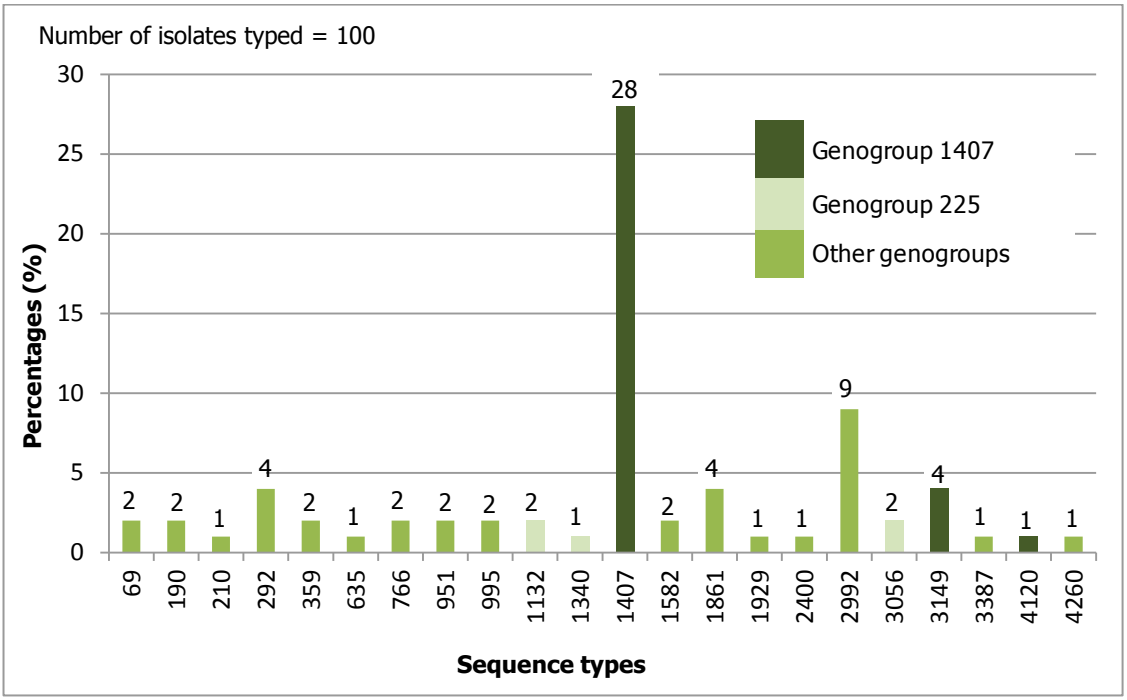

Figure A2.19b: Sequence types in Spain

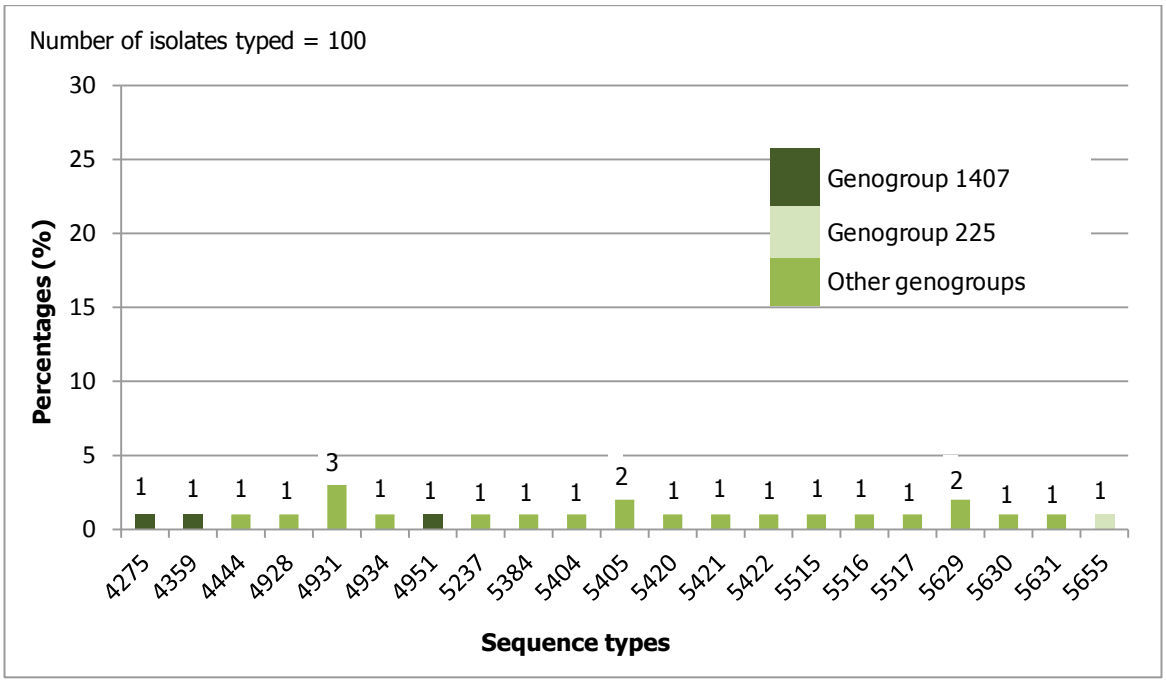

Figure A2.20: Sequence types in Sweden

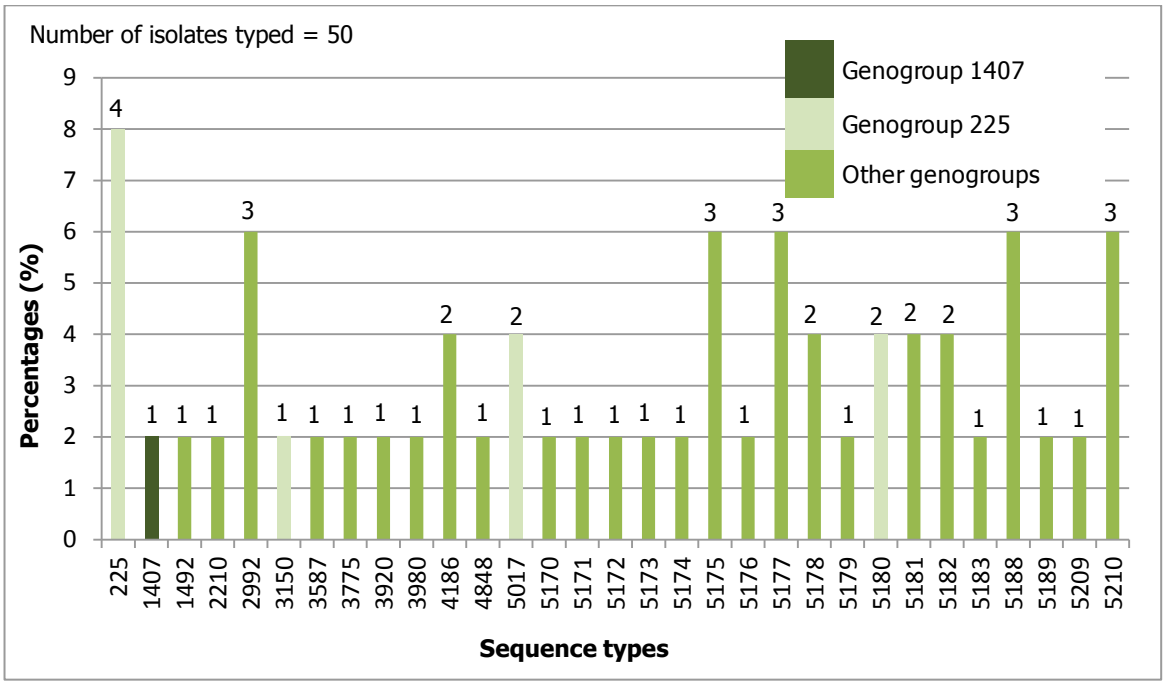

Figure A2.21a: Sequence types in the UK

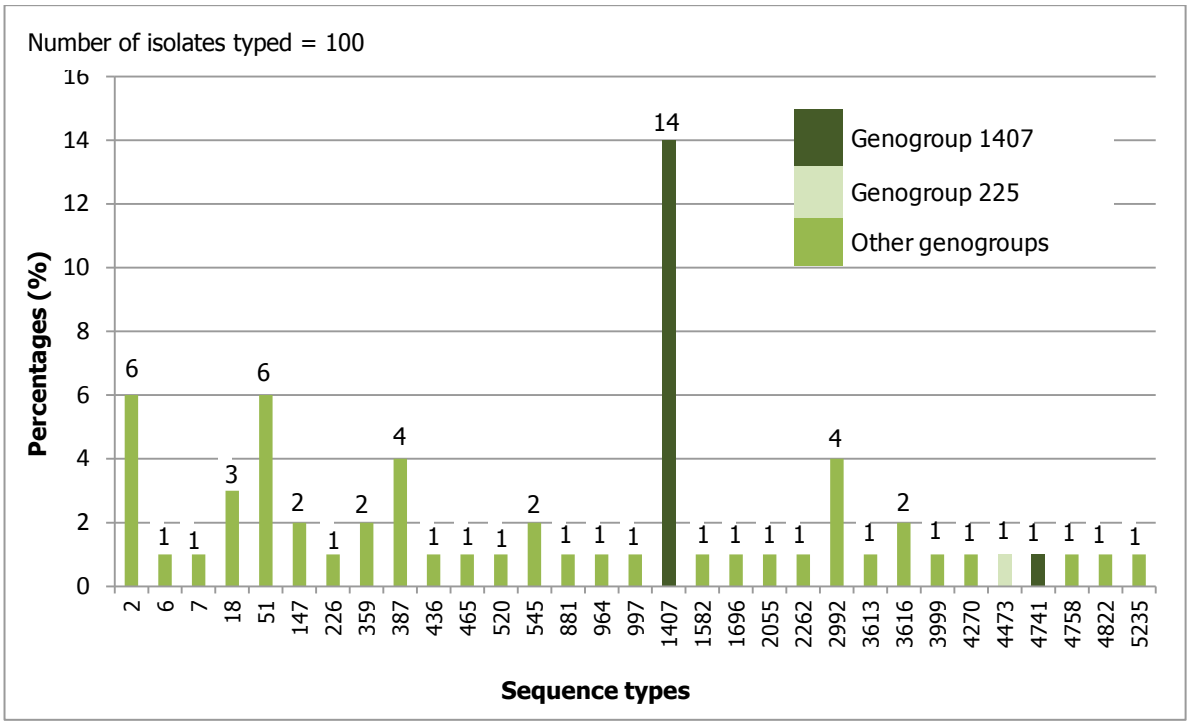

Figure A2.21b: Sequence types in UK