

***Neisseria gonorrhoeae* sequence types and antimicrobial resistance in non-cultured clinical samples from females in British Columbia: a pilot study**

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September 2019

ABSTRACT

Background: In British Columbia (BC), enhanced gonorrhoea surveillance to monitor sequence types and antimicrobial resistance (AMR) is based on cultures which are typically performed on samples from symptomatic individuals and extra-genital sites, leaving certain subpopulations that are usually screened using nucleic acid amplification tests (NAAT) only under-represented, such as females.

Objective: Determine *Neisseria gonorrhoeae* multi-antigen sequence types (NG-MASTs) and AMR in NAAT samples from females in BC.

Methods: The first 30-40 NAAT positive female gonorrhoea samples received at the BC Centre for Disease Control Public Health Laboratory from each month of October, November, and December 2015 were sent to the National Microbiology Laboratory for further characterization. Sequence types were classified by NG-MAST and AMR associated genetic markers were determined using real-time PCR.

Results: Of the 112 collected samples, 77 (68.8%) had viable typing. The most common NG-MAST were ST-5985 (25/77, 32.5%), ST-7638 (26/77, 20.7%) and ST-4637 (8/77, 10.3%). Genomic-based AMR results were available for 52 (67.8%) samples. Nine (17.3%) predicted ciprofloxacin resistance and 3 (5.8%) predicted intermediate susceptibility to cephalosporins.

Conclusion: A large proportion of samples had NG-MASTs rarely identified in BC (ST-7638 and ST-4637). Whether this is due to sequence replacement or under-sampling of females is not clear. These results highlight the need to improve representativeness of gonorrhoea surveillance, and the potential of molecular methods to address this.

INTRODUCTION

Gonorrhoea, caused by the bacterium *Neisseria gonorrhoeae*, is the second most common bacterial sexually transmitted infection (STI) in Canada (1). If left untreated, gonorrhoea can lead to serious complications, including pelvic inflammatory disease and ectopic pregnancy in females, and epididymitis in males (2-5). In British Columbia (BC), the rate of gonorrhoea increased by 72% from 43.9 per 100,000 population in 2014, to 74.6 per 100,000 population (6) in 2015, surpassing the national average. The increase was greatest among females, increasing by 114% from 22.3 in 2014 to 47.7 per 100,000 in 2015 (Appendix Figure A1, A2). Substantial increases of gonorrhoea have also been observed in other jurisdictions across Canada, the USA and Europe (7-10).

These increases are particularly concerning because of the organisms' ability to easily develop antimicrobial resistance (AMR), which presents challenges for treatment and control. Currently national and provincial STI guidelines recommend cefixime or ceftriaxone, plus azithromycin as first-line treatment for gonorrhoea (11, 12). In BC, reduced susceptibility to cefixime, ceftriaxone and resistance to azithromycin increased from 2007-2010. The trend reversed from 2011-2015, possibly due to more effective regimens including increased cefixime dosage and improved medication adherence due to single dosage (13). However, the potential for untreatable gonorrhoea remains a concern. Nationally, the proportion of isolates resistant to azithromycin also increased between 2012 – 2016, as did the rate of multi-drug resistance (MDR), defined as decreased susceptibility/resistance to one currently recommended therapy and resistance to at least two other antimicrobials (14).

As in other jurisdictions across Canada, over 70% of gonorrhoea diagnoses in BC are based on commercial nucleic acid amplification test (NAAT) systems (15). While NAAT-based systems provide greater ease of sample collection, transport, storage and detection (16, 17), it is unable to provide subtypes (i.e. *Neisseria gonorrhoeae* multi-antigen sequence types [NG-MAST]) or antimicrobial resistance profiles which are useful in outbreak investigations (18) and for informing empirical treatment guidelines (19), respectively. In order to monitor for these characteristics, the STI clinics operated by BC Centre for Disease Control (BCCDC) are sentinel sites for enhanced surveillance of gonorrhoea where culture is performed in parallel with NAAT testing for samples with a high pre-test probability for being positive (e.g. client is symptomatic), for samples where NAAT methods are potentially less specific (e.g. extra-genital samples), or if there is concern for treatment failure following an initial NAAT positive result. Samples are sent to the BCCDC Public Health Laboratory (PHL) who performs susceptibility testing on the cultured isolates. Isolates found to be resistant to at least one antibiotic are further sent to the National Microbiology Laboratory (NML) for sequence typing using the NG-MAST method. As a result of these routine practices, surveillance of antimicrobial susceptibilities and NG-MAST in BC is likely over-represented by gonorrhoea cultures from symptomatic individuals, and from non-genital sites (which are most commonly from gay, bisexual, and other men who have sex with men [gbMSM]), and may not reflect of the circulating strains in the general population. For example, in 2015, female case-isolates made

up 17% of all case-isolates sent to NML for resistance testing and sequence typing but represented 32% of all cases of gonorrhoea diagnosed that year.

Recent advancements in molecular technologies have resulted in assays that can use gonorrhoea-positive NAAT samples to determine NG-MAST and predict AMR. These assays have been previously validated in Canada, and show high levels of sensitivity and specificity compared with cultures (20, 21), and a valuable supplement to culture-based surveillance. However, such NAAT-based assays are not widely available outside of research settings, despite this information being critical to inform empiric treatment recommendations and to better understand gonorrhoea transmission (16, 22). Given the potential gap in the enhanced surveillance of gonorrhoea and the recent rise in rates of gonorrhoea among females, we sought to use these novel methods to describe the NG-MAST and AMR profiles in females, particularly those tested outside BCCDC-operated STI clinics.

METHODOLOGY

Collection of gonococcal samples

Between October to December 2015, The first 30-40 NAAT positive gonorrhoea samples that were received at the BCCDC PHL were forwarded to the NML for sequence typing and AMR prediction. For this project, the BCCDC PHL also requested community laboratories around BC send them leftover samples that were positive for *N. gonorrhoeae* by APTIMA® NAATs that were collected from females (urine, vaginal or cervical).

Sequence Type

DNA was extracted from APTIMA® NAAT sample using the QIAamp® Viral RNA Mini kit as per manufacturer's instructions (Qiagen®, Toronto, Ontario). Patient samples were characterized by NG-MAST based on the sequence of the *porB* and *tbpB* genes. The NG-MAST procedure uses these two variable gonococcal genes to provide a method that is able to discriminate gonococcal strains (23). Sequence type was determined using the NG-MAST website (www.ng-mast.net). Samples which had DNA concentrations that were too low to be detected in these assays were classified as non-typeable.

Antimicrobial Susceptibility Prediction from clinical samples

Antimicrobial susceptibility prediction was conducted on patient samples for which a sequence type was determined. Susceptibility to the currently recommended therapies, cefixime or ceftriaxone (cephalosporins), azithromycin, and ciprofloxacin (a former preferred treatment) were predicted using real-time PCR (RT-PCR) assays that detect single nucleotide polymorphisms (SNPs) validated to be associated with resistance (21, 24-26).

Samples were classified as having decreased susceptibility (DS) to cephalosporin if all of the following four SNPs targets were present: *ponA* L421P, *mtrR* 35delA, *porB* G120/A121, and

penA mosaic allele. Samples with 3/4 SNPs were classified as intermediate susceptibility, while those with 2 or fewer were classified as susceptible (21). Samples were classified as resistant to azithromycin if either 23S rRNA and A2059G, or 23S rRNA and C2611T SNPs were present. Otherwise they were classified as susceptible (24). Samples were classified as resistant to ciprofloxacin if any of the following three SNPs were present (*gyrAS91*, or *parC* D86/S87/S88 and D95). Otherwise they were classified as susceptible (25). If any of the alleles being tested did not produce a result, AMR results were considered inconclusive.

Descriptive analysis of samples by NG-MAST sequence types (number and proportion) was conducted. Antimicrobial susceptibility was examined for each antimicrobial drug overall and by NG-MAST. Analysis was conducted using RStudio, Version 1.0.153 (Boston, USA).

Ethics Approval

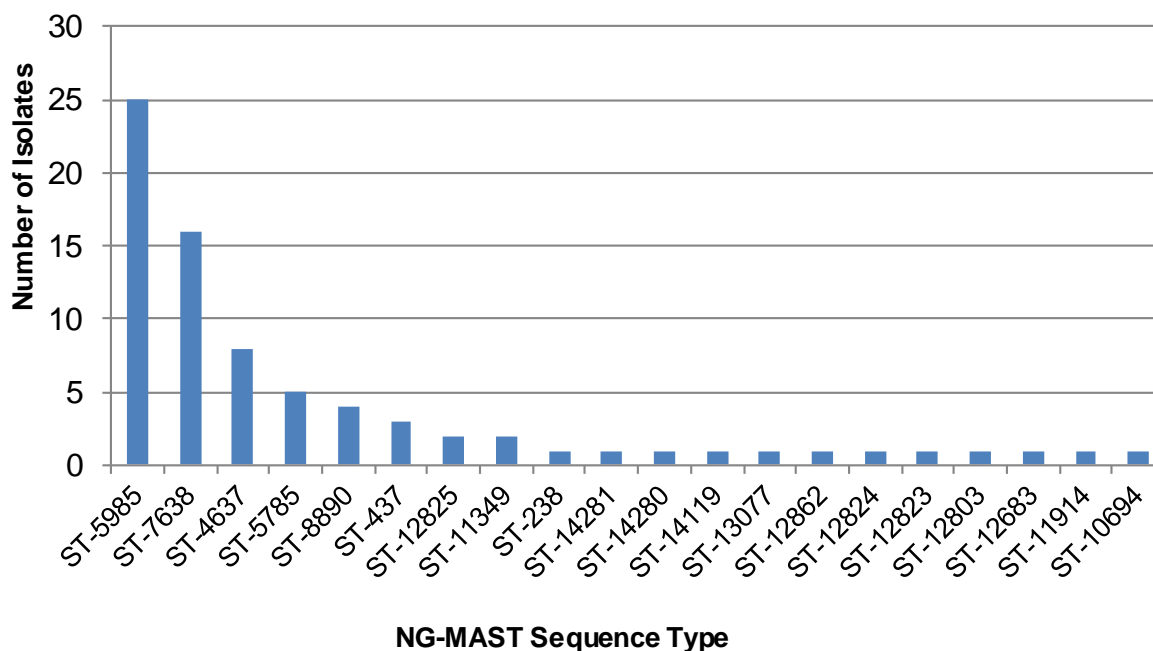
This study falls within BCCDC's public health mandate thus, ethics approval was not needed.

RESULTS

Evaluation of NG-MAST Sequence Typing and Antimicrobial Resistance

A total of 112 NAAT positive gonorrhea samples were forwarded to NML for characterization. Of those, 77 (68.8%) were typeable and are presented in Figure 1. There were twenty different sequence types identified. The most common sequence type identified was ST-5985 (n=25, 32.5%) followed by ST-7638 (n=16, 20.7%) and ST-4637 (n=8, 10.3%).

Figure 1: NG-MAST of the NAAT samples that were typeable, Oct to Dec 2015 (n=77)^a



^aST-5985, ST-14281, and ST-12823 differ by one base pair.

Antimicrobial resistance prediction

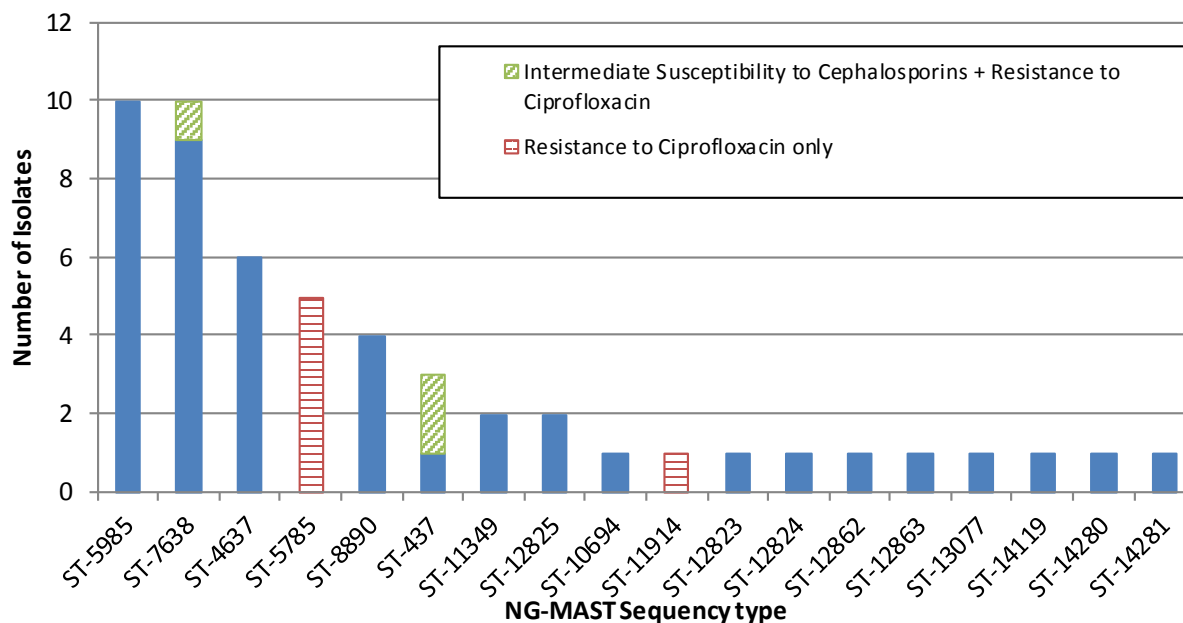
Of the 77 NAAT samples that were typeable, 52 (67.5%) had sufficient DNA for resistance testing. Among the 52 samples, 9 (17.3%) were predicted to be resistant to ciprofloxacin. Three (5.8%) were predicted to have intermediate susceptibility to cephalosporins, no samples exhibited DS to cephalosporins, nor resistance to azithromycin (Table 1).

Table 1: Prediction of antimicrobial resistance for female gonorrhea samples with sufficient DNA, 2015

Interpretation	Antimicrobials		
	Cephalosporins ^b	Ciprofloxacin	Azithromycin
Susceptible	24 (46.1%)	26 (50.0%)	36 (69.2%)
Intermediate	3 (5.8%)	0 (0.0%)	0 (0.0%)
Resistant/Decreased Susceptibility	0 (0.0%)	9 (17.3%)	0 (0.0%)
Inconclusive ^b	25(48.1%)	17(32.7%)	16 (30.8%)
Total	52(100.0%)	52 (100.0%)	52 (100.0%)

^a Cephalosporin (ceftriaxone or cefixime)
^b Inconclusive - SNP assay results were unclear therefore AMR profile could not be determined

Figure 2: Antimicrobial resistance^a profiles by NG-MAST (n=52)^b



^a Cephalosporin (ceftriaxone or cefixime)

^b Denominator includes samples with inconclusive results

Resistance was confined to four sequence types (Figure 2). Resistance to ciprofloxacin only was observed in all ST-5785 (5/5), as well as ST-11914 (1/1). Three samples with the sequence types ST-7638 (1/9) and ST-437(2/3) showed both resistance to ciprofloxacin and intermediate susceptibility to cephalosporin. Resistance results were inconclusive for 30.8- 48.1% of samples tested, depending on the assay used.

DISCUSSION

Our study demonstrated that NAAT samples could be used to characterize sequence types and predict antimicrobial resistance profiles. The majority of samples were typeable and of these, a large proportion was successfully assayed to predict AMR. Together, these results suggest that NAATs could supplement the current culture-based enhanced surveillance for gonorrhea in BC.

We found ST-5985 to be the most prevalent NG-MAST, consistent with enhanced provincial surveillance (15). However, we also found a high prevalence of two sequences, ST-7638 and ST-4637, which have rarely been identified in BC; ST-4637 was identified once in 2014, from a cultured female sample (Appendix Figure A3). These sequences may be novel or may be previously circulating in BC but not detected through routine surveillance.

Both ST-7638 and ST-4637 have been identified in the neighbouring province of Alberta (15, 27, 28). In fact, ST-7638 was the most common sequence type in Alberta from 2012-2016, and was more common among females than other circulating sequence types (28). Travel and sexual mixing between Alberta and BC could have introduced these strains to BC. On the other hand, gonorrhea sequence types are known to be different in different sexual networks (18, 29) and so may have been circulating in BC but not detected through routine culture-based enhanced surveillance. This highlights the need for representativeness to better understand the epidemiology of gonorrhea in BC. Representativeness of enhanced gonorrhea surveillance is a challenge across Canada. The selection criteria for gonorrhea isolates is not always consistent, leading to results, specifically of sequences, which may not be representative of the gonorrhea circulating in Canada (15).

Decreased susceptibility to first-line drugs, cefixime or ceftriaxone (i.e. presence of all four mosaic alleles: *ponA* L421P, *mtrR* 35delA, *porB* G120/A121, and *penA*), was not observed among any of the samples, although we did find intermediate susceptibility (i.e. presence of three of the four aforementioned mosaic alleles) to ceftriaxone or cefixime in 6% of tested samples. This compares with 1.7% and 1.9% of samples in BC with a minimum inhibitory concentration [MIC] greater than or equal to 0.064 mcg/mL to ceftriaxone and cefixime, respectively, in 2015 (13). One quarter of the female samples exhibited resistance to ciprofloxacin, a previous first-line drug, compared with 38.9% of isolates in Canada in 2015 (15). Unlike routine surveillance in which about 10% of isolates had a MIC greater than or equal to 1.0 mcg/mL to azithromycin in BC (13), our study found no instances of azithromycin resistance, which may be due to the small sample of female samples investigated or differences in sequence type.

Resistance was uncommon in ST-7638 and ST-4637; only one sample (ST-7638) was predicted to have intermediate susceptibility to cephalosporins and resistance to ciprofloxacin. Similarly, a recent study from Alberta found a low prevalence of resistance in ST-7638 and other closely related sequence types (28). Subtypes which are fully susceptible to antimicrobial drugs (i.e. no resistance) have been shown to have improved fitness, and may be better adapted to cause

infection (30). Whether this may have led to the increased incidence seen in BC, especially among females, is a hypothesis that requires more exploration.

Antimicrobial resistance of gonorrhoea continues to be a public health priority both globally and in Canada (15, 27, 31, 32), and monitoring is key in developing treating guidelines and informing empiric treatment choices. In BC, routine monitoring of resistance to azithromycin and decreased susceptibility to ceftriaxone and cefixime have supported the continued use of these agents as recommended for first-line treatment (6, 11). However, resistance to azithromycin

increased in BC and nationally from under 1% in 2012 to between 3-5% in recent years (2015-2017). Driven by that, the national rate of MDR has also increased between 2012 and 2016 (14). Moreover, the recent detection of a ceftriaxone-resistant strain in an asymptomatic Quebec woman indicates that there is ongoing concern that mixing across international boundaries may introduce resistant strains locally (32). This case highlights the need for strong surveillance systems to support treatment guidelines, and to guide public health action (10, 31, 32). The present study is not without limitations. Firstly, while over two-thirds of samples could be sequenced using residual NAAT DNA, half of *N. gonorrhoeae* NAAT-positive clinical samples had inconclusive AMR results. This is likely due to insufficient DNA in the collected samples. The high proportion of inconclusive AMR results should be noted as a potential limitation if we move towards AMR surveillance based on molecular methods instead of culture. Similar studies from Australia and New Zealand which used molecular methods to characterize AMR found up to one third of samples could not be characterized with the AMR assays (33, 34). This was more likely for pharyngeal samples since *N. gonorrhoeae* DNA loads tend to be lower in the pharynx compared with other sites (33). Understanding differences in the performance of the AMR assays by different epidemiological factors, such as age group or site of collection, would be useful for future study. As this project was a test of principle to determine the value of direct NG-MAST and AMR prediction from gonorrhoea-positive NAAT samples, the sample size was relatively small. A larger investigation using these novel methods to examine ST and AMR across a provincially representative sample of gonorrhoea cases is currently underway. This study will include linkage to epidemiological information, and will allow us to characterize AMR across body site.

The increasing incidence of gonorrhoea combined with the continued risk of antimicrobial resistance in Canada underscores the importance of enhanced monitoring of the disease. Our findings support the feasibility of using NAAT samples to supplement the existing culture-based provincial surveillance of AMR and NG-MAST. We recommend that a NAAT based system is considered as complement to the current enhanced surveillance of gonorrhoea in BC.

ACKNOWLEDGEMENTS:

We'd like to thank Elsie Wong for her role in coordinating this project. We would also like to acknowledge Rob Azana and Ana Paccagnella for coordinating the samples at the BCCDC PHL.

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APPENDIX
Figures A1-A3

Figure A1: Rate of gonorrhoea in BC from 2006-2015 in females, by age group

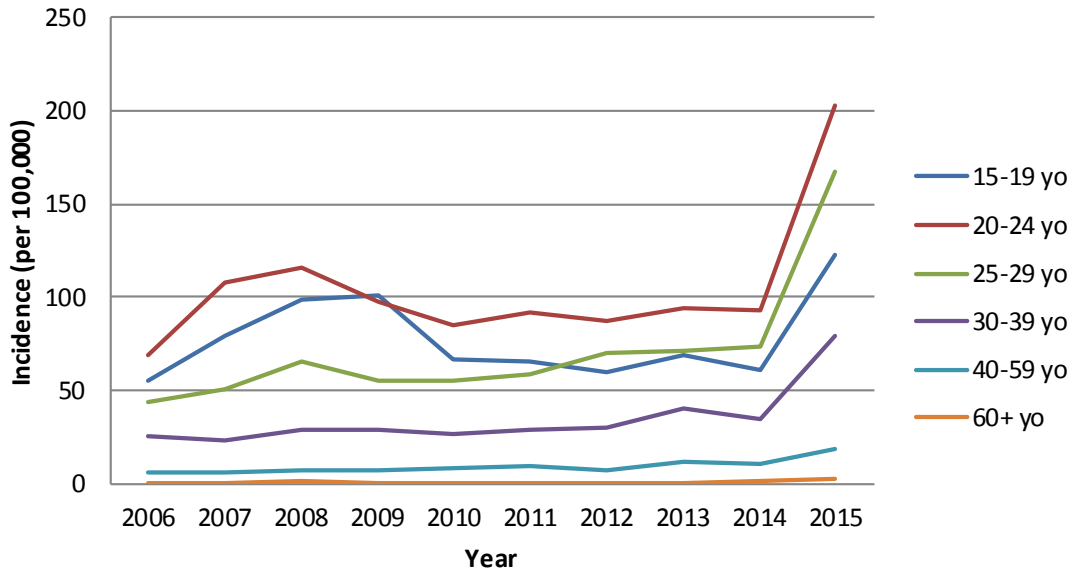


Figure A2: Rate of gonorrhoea in BC from 2006-2015 in males, by age group

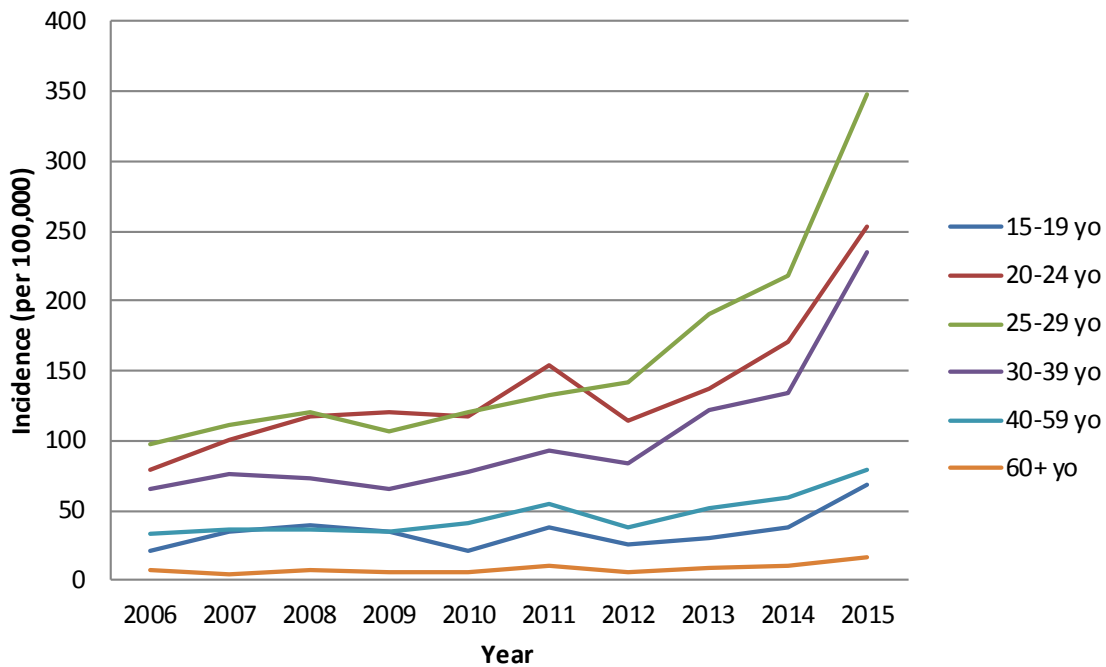
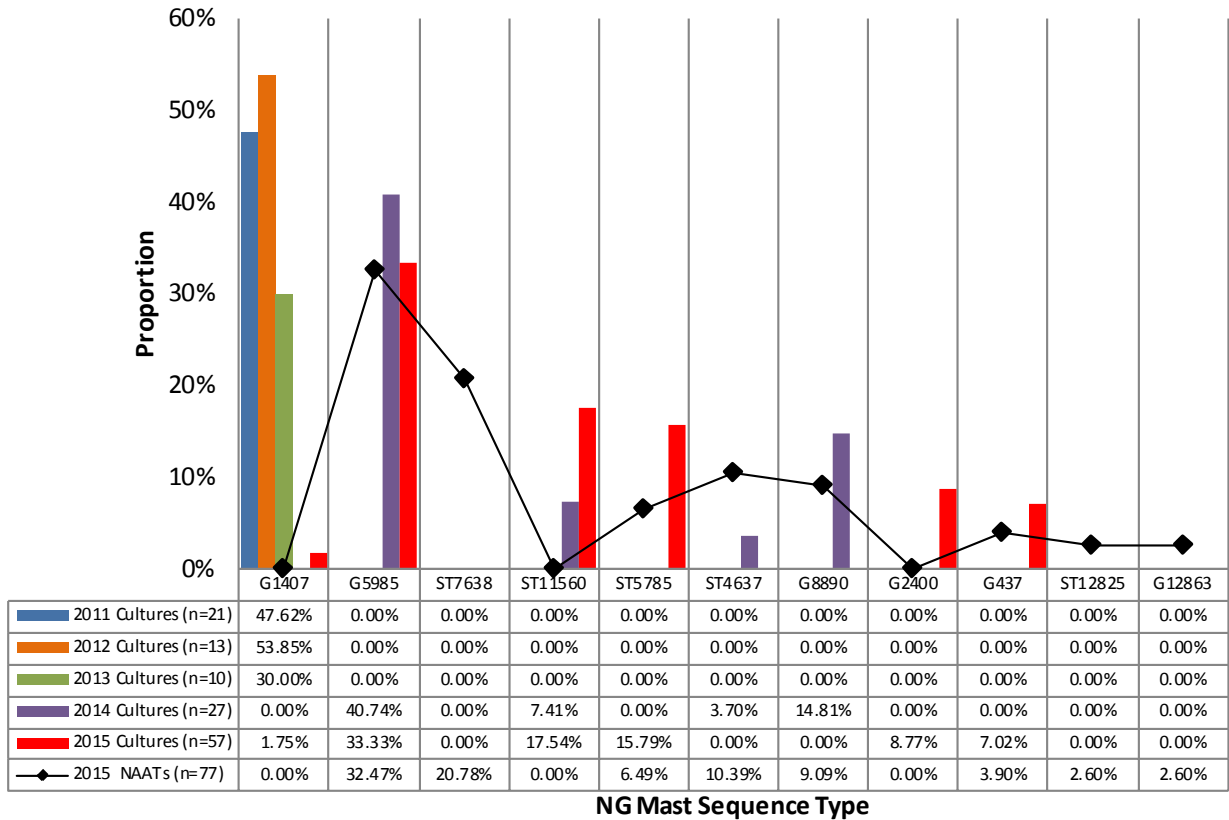


Figure A3: NG-MAST sequence types of *N. gonorrhoeae* from females cultures (2011–2015)^{ab} and NAAT samples (2015)^c, BC.



^aG1407 includes ST1407, ST3149, ST3158, ST4461, and ST10451; G5985 includes ST5985, ST12124, ST12823 and ST14281; G8890 includes ST8890, ST12862, and ST11349; G2400 includes ST2400 and ST6360; G437 includes ST437, ST225, and ST11881.
^bCultures not represented in this graph: 11 isolates from 2011 dispersed among 8 STs; 6 isolates from 2012 in 6 STs; 7 isolates from 2013 in 7 STs; 10 isolates from 2014 in 10 ST; and 8 isolates from 2015 in 8 STs
^cNAATs not represented in this graph: 7 samples dispersed among 7 different STs