Neisseria gonorrhoeae: testing, typing and treatment in an era of increased antimicrobial resistance
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CHAPTER 9

General discussion
Steadily, antimicrobial resistance (AMR) in Neisseria gonorrhoeae has emerged for all types of drugs that have been used as first-line treatment. As mentioned in the introduction of this thesis, AMR to either component of the internationally recommended dual therapy, consisting of azithromycin and ceftriaxone, has been reported since 2011. An outbreak of high-level azithromycin resistance has been observed in the United Kingdom since 2015, which was followed in 2016 by the first report of dual therapy treatment failure. Because no new antimicrobials are expected shortly, these trends threaten the future treatment of patients with gonorrhoea.

The first reports of ceftriaxone resistance prompted the World Health Organization (WHO) to publish an action plan with recommendations to stop resistance in gonorrhoea. The main suggested strategies included: effective diagnosis and systematic monitoring of treatment failures, strengthened AMR surveillance, improved networks of laboratories to perform culture, molecular methods to detect AMR, and new treatment strategies. With the studies in this thesis we aimed to provide knowledge on three main topics recommended by the WHO: 1) the improvement of diagnostics for N. gonorrhoeae, 2) surveillance of AMR, and 3) the improvement of treatment strategies. In this last chapter, the main conclusions of the studies in this thesis are compared to existing literature. The implications are discussed, and recommendations for further steps in research are provided.

Improvement of diagnostics for N. gonorrhoeae
In most settings gonorrhoea is diagnosed using a syndromic approach based on symptoms, culture of N. gonorrhoeae, or a nucleic acid amplification test (NAAT). Currently, the only way to determine resistance in N. gonorrhoeae is to culture the bacteria and expose them to drugs in vitro. Until molecular methods to determine AMR are widely available, and reliable, successful culture techniques are essential to monitor resistance and individualize treatment. The use of culture is complicated by the required logistics and technical expertise. Many low-income countries and remote settings do not have either, and therefore culture results, and the subsequent antimicrobial susceptibility data are very limited. Yet, even in high-income countries the logistic distance between collection site (an STI or general practitioners clinic) and laboratory creates difficulties for successful cultures. To improve success rates of culture and allow time for transport of samples, a medium is needed that keeps the sample viable during storage. Several previous studies have shown that the ESwab

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medium could be used for this purpose, but none used clinical samples to evaluate this.\textsuperscript{24-26}

In Chapter 2 we demonstrated a method of targeted deferred culture from the Eswab medium, which was stored for up to 3 days. After selection of samples positive for \textit{N. gonorrhoeae} in NAAT, culture success rates were 69\% and 56\%, after storage for 1 and 2 days, respectively. After storage for 3 days urogenital samples (cervical and urine) still resulted in 44\% successful cultures, compared to 22\% for rectal samples. These results not only show that \textit{N. gonorrhoeae} samples can be stored and refrigerated prior to culturing, but also that culture from urine samples is successful. All three procedures were previously thought to be detrimental for \textit{N. gonorrhoeae} cultures.\textsuperscript{20,27,28} The method we presented will enable care providers to transport samples from remote locations to laboratory facilities. By obtaining culture and resistance data, these settings could improve their diagnostic methods, individualize treatment if resistance increases, and contribute to AMR surveillance in their population. Our method of targeted deferred culture could also improve diagnostic procedures in settings with fully equipped laboratories on site. Selection of stored samples for culture, based on NAAT results (potentially available within 24 hours), can avoid many unsuccessful cultures, and improve cost-effectiveness of gonorrhoea diagnostics.

To implement this method in routine diagnostics and AMR surveillance, further studies should be performed to confirm our results in routine practice. These studies should include analyses of the transport times, and success rates of deferred culture from samples collected by general practitioners, in remote settings, or in low-income countries. Another aim should be to perform NAAT and culture from the same medium, allowing care providers to obtain only one sample from patients, and limiting discomfort to patients.

Before the use of extended-spectrum cephalosporins (ESC), treatment failures caused by antimicrobial resistance could be diagnosed by performing a test of cure (TOC). A TOC consisted usually of a culture obtained around 1–2 weeks after treatment, and a negative result was interpreted as a cured infection.\textsuperscript{29,30} This method was complicated by imperfect sensitivity (around 80\%), and false-negative results of cultures.\textsuperscript{31} When treatment with ceftriaxone was commenced, and based on its high efficacy, TOC were no longer deemed useful.\textsuperscript{32,33} In addition, diagnostics for gonorrhoea had improved and were now performed using high-sensitivity NAATs.\textsuperscript{29,30} With the looming AMR to ceftriaxone, a TOC could again prove helpful. Ideally a TOC would be performed using NAAT.
In Chapter 3 we analysed the appropriate timing of a TOC for *N. gonorrhoeae*, using modern ribonucleic acid (RNA)- and deoxyribonucleic acid (DNA)-based NAATs and daily sampling up to 28 days following routine treatment. The median time to clearance was 2 days, and all patients had cleared RNA within 7 days, and DNA within 15 days. The few previous studies, that used either different molecular tests or single time point testing, showed a median time to clearance of 2 days, or complete clearance between 14 and 21 days.\textsuperscript{34-37} Despite the use of different tests or frequency of testing in these studies, their results are in line with those of Chapter 3. Our results also confirm previous reports of intermittent NAAT-positive results in 18% of gonorrhoea patients after treatment.\textsuperscript{34} Although we used a different molecular test method, we noted these ‘blips’ in 0.8% and 1.5% (RNA and DNA, respectively) of post-clearance samples. This corresponded to 10% and 16% of patients in our study, respectively. Because NAAT only determines the presence of genetic material, positive results could represent viable infections, shedding of dead bacteria, or the deposition of genetic material. We did not consider blips as reinfections, because positive results were often solitary, not persistent, and not positive for both RNA and DNA. We also noted that test levels (relative light units or cycle threshold) of blips were significantly lower compared to pretreatment samples, suggesting lower genetic load in blips. Some blips were preceded by sexual contact, which suggests that these blips could represent depositions by a partner, although power was too small for statistical analysis.

Currently, a TOC is not recommended for anogenital gonorrhoea in routine practice, but this could change if resistance to azithromycin and ceftriaxone continues to increase.\textsuperscript{20,21,29,30} If a TOC is indicated, we recommend performing it at least 1 or 2 weeks after treatment, when using RNA- or DNA-based NAAT respectively. Our results suggested faster clearance in rectal samples, or in those treated with ceftriaxone plus doxycycline, but these differences were not statistically significant, possibly due to insufficient sample size.

Because this study is the first using modern NAATs and daily testing, our results should be confirmed by other studies, preferably including pharyngeal infections, and different commercial types of NAAT. Future studies should have a larger sample size, thereby providing more power to analyse associations of clearance with anatomical site or treatment regime, and of blips and sexual contact. A main focus of future studies should be to determine the origin and meaning of blips, confirming if blips occur, how often, and for how long after treatment. Preferably, a test should be
designed to establish if test results represent viable or dead bacteria, for instance by testing messenger RNA (mRNA). Until further research sheds more light on the origin of blips, we recommend that a positive TOC should not immediately be interpreted as a treatment failure, but is confirmed with a second sample. Especially if no explanation for reinfection exists, will this help to differentiate between a blip and a treatment failure. When a TOC result suggests a treatment failure, it is important to obtain a culture and determine AMR for the infecting strain.

Coinfections of both *N. gonorrhoeae* and *Chlamydia trachomatis* occur in 2–5% of patients at high-risk of having an STI. Among patients with gonorrhoea, the percentage of coinfections with chlamydia is much higher at 10–40%. Therefore, most clinics test for both infections simultaneously, and many NAATs give results for both *N. gonorrhoeae* and *C. trachomatis* from a single test. While AMR has been a longstanding problem for *N. gonorrhoeae*, this has not been reported for *C. trachomatis*. However, there is increasing concern about the effectiveness of azithromycin as first-line treatment in urogenital (and often rectal) chlamydia. Treatment failures of azithromycin for urogenital and rectal chlamydia are occasionally seen, and treatment efficacy is reported to be between 83–97%. A TOC is currently not routinely recommended for *C. trachomatis*, but suggested to be performed 3–4 weeks after treatment, for specific indications. However, if efficacy of azithromycin decreases further in the future, a TOC could be helpful to identify patients with treatment failures.

In Chapter 4 we analysed the NAAT results of a subpopulation from Chapter 3, coinfectected with *C. trachomatis* and *N. gonorrhoeae*. The median time to clearance of *C. trachomatis* was 7 days (RNA) or 6 days (DNA), and all patients cleared within 13 days (RNA) or 15 days (DNA). These results are in line with previous studies showing clearance within 3–4 weeks, although these studies used different molecular tests or frequency of sampling. However, some reports describe persistence or intermittent positivity of *C. trachomatis* up to 51 days. Because in those studies testing was not daily, it is unclear whether positive test results represent persistent infection, reinfection, or intermittent positive results (blips) without clinical significance. In some studies 5–18% of patients had positive results preceded by negative results, suggesting that these represent blips. This is similar to our results of 2% and 4% blips in post-clearance samples, representing 22% and 35% of patients (DNA and RNA, respectively). However, because we performed daily sampling, we could exclude that chlamydia reinfections occurred. Since blips in our study were preceded and followed by a large number of negative test result, persistent infections were also less likely.
Even though our results are similar to previous studies, and add new knowledge by using modern RNA- and DNA-based NAATs and daily sampling, the sample size of our study was small. Future studies aimed at confirming our results need to have a larger sample size, and include monoinfections with *C. trachomatis*. It is also important for future studies to assess possible associations between clearance and anatomical site, human immunodeficiency virus (HIV) status, or treatment regime (azithromycin or doxycycline), and between blips and sexual contact, coinfections or immune status.

Because *C. trachomatis* is an intracellular growing organism, it is important that the origin of blips is studied in more detail. Especially whether blips represent viable or dead bacteria, and if they are caused by genetic, but non-viable, deposits from a sex partner, or are the result of shedding in the patient. The viability of bacteria could be determined by testing for mRNA. Currently such tests are not yet available, but studies are being performed in this field. Furthermore, it is important to affirm the effectiveness of azithromycin and doxycycline in anogenital chlamydia, by double blind randomized controlled trials. If indicated, we recommend performing TOC for chlamydia at least 2 weeks after treatment. If a TOC is positive, and a reinfection is unlikely, it needs to be confirmed with a second sample.

**Surveillance of antimicrobial resistance in *N. gonorrhoeae***

As mentioned before, in recent years AMR to ceftriaxone and/or azithromycin has increased. Most worrying is the first reported treatment failure to dual therapy of azithromycin and ceftriaxone. In *Chapter 5* we determined susceptibility to azithromycin and ceftriaxone in *N. gonorrhoeae* isolates from patients of the STI Clinic Amsterdam, between 2012 and 2015. We found no resistance to ceftriaxone in Amsterdam yet. Resistance to azithromycin was stable at around 1.2%, while this was 5% across Europe in 2013. Although the lower resistance in Amsterdam is a fortunate situation, we did see a rise in isolates with decreased susceptibility to azithromycin (from 4% in 2012, to 9% in 2015), or to ceftriaxone (from 4% in 2012, to 8% in 2015). This rise of decreased susceptibility was significantly associated with more recent year of infection for both azithromycin and ceftriaxone in MSM, but only for ceftriaxone in heterosexuals. Among heterosexuals, decreased susceptibility to either drug was associated with high-risk behaviour, i.e. by more sex partners in the previous 6 months.

Our results confirm the need for continued monitoring of AMR. Many countries have already established national gonococcal AMR surveillance programs, but in others this needs to be set up as well. It is important that methods and results of surveillance programs are comparable. Currently, the isolates that are selected for surveillance, and
the testing methods can differ per county, and sometimes per laboratory. For instance, two
different growth media are used (Mueller-Hinton and GC chocolate agar),
antimicrobial susceptibility can be determined with agar dilution or Etest methods,
and resistance breakpoints used in Europe are not identical to those used in the United
States. For the future it is very important that surveillance is improved, increased
and standardised. The effort to establish national surveillance programs in developing
countries needs to be continued and expanded. In countries with strong first-line care
such as the Netherlands, AMR surveillance should not be limited to STI clinics, but
also include data from general practitioners and outreach programs. Future research
should aim at continued reporting of surveillance data, and include essential patient
characteristics such as age, gender, sexual orientation, HIV status, and data on sexual
risk behaviour. These data are necessary to assess which patients are at highest risk
for resistance. Future studies could also help to determine which methods to collect,
culture, and determine resistance are most suitable to use for surveillance.

Resistance to azithromycin has been strongly associated with specific genetic
mutations. As a result of mutations in any of the four 23S rRNA genes, azithromycin
can no longer bind effectively to 23S rRNA, and its inhibitory effect on protein
synthesis is limited. Mutations at position 2059 or 2058 are associated with high-
level azithromycin resistance, while mutations at position 2611 are associated with
moderate-level resistance. Furthermore, mutations in one allele are associated
with the rapid introduction of mutations in other alleles, and the cumulative number
of C2611T mutated alleles is associated with higher MICs. Specific strains have also
been associated with resistance, as demonstrated by the occurrence of ESC resistance
in sequence type (ST) ST1407 strains from different geographical regions.

In Chapter 8 we showed that 90% of azithromycin resistant N. gonorrhoeae isolates
had at least one C2611T mutation, while all susceptible isolates had a non-mutated
wild-type 23S rRNA; this difference was highly significant. These results confirm
the previously reported association between azithromycin resistance and 23S
rRNA mutations. Using N. gonorrhoeae multilocus variable-number tandem
repeat analysis (NG-MLVA), we noted that resistant isolates were significantly more
often included in any NG-MLVA hierarchical cluster, and three of the five NG-MLVA
clusters consisted predominantly of resistant isolates. This could indicate that these
clusters represent sexual networks in which resistant isolates are transmitted.
However, four clusters included both resistant and susceptible isolates, suggesting
that resistance occurs independently from NG-MLVA type. NG-MLVA is an in-house developed PCR method that is not widely used, which complicates the comparison of results to those of other studies. A more commonly used typing method is *N. gonorrhoeae* multiantigen sequence typing (NG-MAST). Previous studies have linked azithromycin resistance (among others) to NG-MAST genogroups G2992, G2400 and G1407. No correlation has been reported for G5108 or G359. In *Chapter 8*, azithromycin resistance was significantly associated with genogroups G2992, G5108, and G359, whereas G2400 was significantly associated with susceptibility. We found no association of G1407 with either resistance or susceptibility. Our results, and those from previous studies, suggest that common STs differ by geographical region, and that azithromycin resistance develops independently from the 'background' genetic profile, but that such resistant strains can subsequently spread clonally.

This again stresses the need for continued and improved regional AMR surveillance, which should include epidemiological characteristics to identify risk groups. The association between azithromycin resistance and 23S rRNA mutations allows for fast and targeted analyses of resistance. Other molecular markers are associated with resistance to other antimicrobials. For instance, *PenA* mosaic types are associated with ESC resistance, and *gyrA* mutations with ciprofloxacin resistance. However, these associations are not always very strong, and for many antimicrobials molecular markers associated with resistance have not yet been described. Future studies could help to identify molecular markers, or a combination of markers, in strong association with resistance to different antimicrobial drugs. Combining this knowledge with improving techniques such as whole genome sequencing (WGS) could improve the quality and efficiency of AMR surveillance in gonorrhoea. When high-definition methods like WGS are used for genetic linkage studies the discriminatory power should be taken into consideration. Too much discriminatory power could limit the possibility to identify clusters and determine risk groups. Another important factor is that many current molecular methods require large quantities of DNA, that are only obtainable after culture. It would be a great improvement to be able to perform cluster analysis on samples directly derived from patients, for instance from NAAT samples, without the need for culture. The determination of AMR by molecular markers also still requires cultured strains. Even when molecular AMR markers will be determined and available for routine testing in the future, this will never cover new mutations that occur spontaneously in the population. Genetic markers of resistance to new gonorrhoea treatment strategies will require cultured strains, that are exposed to antimicrobial
drugs, to detect the phenotypic AMR. To allow rapid AMR determination, molecular methods need to be improved and expanded, but culture should not be discarded from AMR surveillance.

**Improvement of treatment strategies in N. gonorrhoeae**

To overcome AMR and subsequent treatment failures, various strategies have been used in the past. Treatment options proved effective for a while, but eventually AMR against the new regime emerged, leading again to treatment failures. This was followed by steps to increase the antimicrobial dosage, and then to switch from antimicrobial class. Currently, dual therapy consisting of ceftriaxone plus azithromycin is recommended. Several reasons were put forward to motivate dual therapy. First, in chronic infections such as human immunodeficiency virus (HIV), leprosy and tuberculosis, combination therapy is effective against AMR, because it is harder for a pathogen to develop resistance to multiple drugs with different modes of action at once. Second, if the bacterial strain is resistant to only one of the components of dual therapy, it will still be treated effectively by the other component. Third, gonorrhoea often coincides with chlamydia, for which the preferred treatment is azithromycin. Therefore the current dual therapy could also treat a possible chlamydia coinfection. Finally, some drug combinations have shown to act synergistically, each drug enhancing the effect of the other, and the combination is more effective than the mere sum of both effects. This effect had been reported for ceftriaxone and azithromycin in *N. gonorrhoeae* in one study prior to the recommendation of this dual therapy. However, in Chapter 6 we found different results.

Using various strains and two testing methods, we determined that the combined effect of ceftriaxone and azithromycin was indifferent, meaning that there was no synergy. However, there was no antagonism either, indicating that the combined effect was not less than the effects of monotherapy. Several other studies have since confirmed our results, refuting the hypothesis that synergy could be a justification for using ceftriaxone and azithromycin. We also tested 65 other antimicrobial dual combinations, and found no synergy in any of them. For some of the combinations we tested, and several new combinations, the lack of synergy was confirmed by other *in vitro* studies. However, we did identify *in vitro* efficacy for several existing antibiotics, that have not yet been first-line choices for gonorrhoea, such as ertapenem, fosfomycin and gentamicin. These drugs are good candidates for future therapy in gonorrhoea, and need to be evaluated further in clinical trials.
Although the lack of synergy between ceftriaxone and azithromycin has been confirmed by several studies, and resistance to either drug is increasing, the addition of azithromycin is still recommended by international guidelines.29,30 Azithromycin is widely used; not only for chlamydia, but also for the syndromic management of urethritis, and for many respiratory and cutaneous infections.30,93,94 Therefore, the population exposure to azithromycin is high, especially in patients at risk of N. gonorrhoeae infections. Exposure to antimicrobial drugs is the strongest risk factor to develop resistance.95 The correlation between exposure to macrolides and resistance has been demonstrated for azithromycin and erythromycin in different microorganisms.96-98

In Chapter 7 we showed that N. gonorrhoeae isolates from patients who were treated with azithromycin in the 30 days before diagnosis, had minimum inhibitory concentrations (MICs) that were 2.7 times higher than those of strains isolated from patients who had not been treated with azithromycin. This effect was significant, also when adjusted for year of infection, age, ethnic origin, and anatomical site of infection. These results suggest that exposure to azithromycin induces or selects for azithromycin resistance in N. gonorrhoeae. The causative factors for this effect of exposure on MIC remain unresolved. We performed WGS for a subset of samples and noted significantly more mtrR A39T and G45D mutations in those recently exposed. Despite the low number of resistant isolates in our study, these mtrR mutations could possibly explain the increase in MIC. Future studies are needed to determine the effect of exposure to azithromycin on resistance, and the mechanisms by which this effect is achieved. Whether exposure causes selection of resistant isolates that are already present, or if exposure induces resistance in susceptible strains needs to be elucidated. Especially the influence of exposure on 23S rRNA and on mtrR mutations needs to be examined, as well as the effect of mtrR mutations on azithromycin MICs.

The lack of synergy between ceftriaxone and azithromycin, the long half-life of azithromycin, and the evidence that exposure to azithromycin could induce resistance, calls for a re-evaluation of the use of azithromycin in dual therapy for gonorrhoea.99 Future studies should focus on in vitro identification of antimicrobial drugs that are effective against N. gonorrhoeae, and on the correlation between in vitro susceptibility and clinical effect. Antimicrobial drugs with promising in vitro results should be clinically evaluated as soon as possible. Several trials of currently existing drugs have recently been completed, showing clinical effect of gentamicin, gemifloxacin
and fosfomycin on *N. gonorrhoeae*.\(^{100,101}\) Randomized controlled trials should first evaluate promising antimicrobials as monotherapy to establish their effectiveness and adverse events, and only subsequently evaluate these in combination with other drugs. In addition to evaluating existing treatments,\(^{102}\) it is very important that new antimicrobial drugs are developed and tested for gonorrhoea.

Several new drugs, such as modithromycin, delafloxacin, and a new fluoroquinolone (WQ3810), are being investigated for *in vitro* efficacy in *N. gonorrhoeae*.\(^{103-105}\) Currently, only three new drugs have progressed from *in vitro* studies to clinical evaluations. The oral ETX0914 (AZD0914) is a new class antimicrobial drug, acting as a DNA gyrase/topoisomerase II inhibitor. Promising *in vitro* results have been reported by several studies,\(^{106-111}\) and a phase 1 clinical study in human volunteers showed favourable pharmacokinetic and -dynamic profiles, whereas adverse events were mild.\(^{112}\) The results of a completed open-label phase 2 study in patients with gonorrhoea have not yet been published (clinicaltrials.gov identifier: NCT02257918). This is similar to the progress of gepotidacin; after promising *in vitro* results,\(^{113}\) an open-label phase 2 study is now completed (NCT02294682). The development of solithromycin, a new fluoroketolide, is most advanced. After showing promising *in vitro* results,\(^{114,115}\) and good clinical effectiveness in a phase 2 study,\(^{116}\) an open-label phase 3 trial is currently ongoing (NCT02210325).

Because *N. gonorrhoeae* is such a fast changing organism, it is to be expected that AMR will occur in the future for new types of drugs. A vaccine reducing or preventing infections in high-risk populations, might prove more effective in stopping the spread of gonorrhoea without causing the induction of AMR. Several bacterial antigens have been identified as possible targets for a vaccine,\(^{117-120}\) enhanced by the use of proteome mining,\(^{121}\) but this is complicated by the variability of gonococcal surface antigens.\(^{119,121-123}\) A transgenic mouse-model is also available to test potential vaccines, but clinical tests in humans have so far been unsuccessful.\(^{119,121,122}\) The lack of an innate immune response after infections with *N. gonorrhoeae*, and insufficient knowledge of protective immune responses further complicate vaccine development.\(^{119,122,123}\)

**Concluding remarks**

Based on previous research and the results of studies in this thesis it is to be expected that antimicrobial resistance in *N. gonorrhoeae* will further increase in the near future. To keep up with, and preferably stay ahead of, this development it is crucial that
AMR surveillance is intensified across the world. Surveillance should focus on the identification of molecular markers for resistance, and rapid, effective test methods to determine their presence in circulating strains. However, while molecular methods are increasingly used, the knowledge and implication of culture should not be abandoned. Research on alternative treatment options should focus on three topics: reappraisal of existing antimicrobial drugs and their effectiveness against *N. gonorrhoeae*, determined both *in vitro* and in clinical trials, the development of new antimicrobial drugs, and the development of a vaccine for high-risk groups. This requires laboratory techniques, time, and effort. Moreover, the required funding for large studies and the development of new techniques and treatments is absolutely indispensable. By improving international collaborations, and gaining the attention of governments, the funding for AMR research in *N. gonorrhoeae* could be improved.

Before novel treatment strategies are available, we could already improve the care of patients with gonorrhoea by focussing on individualized treatment. By using culture or molecular testing, we could identify strains that are still susceptible to previously used antibiotics, such as penicillin, spectinomycin, or ciprofloxacin, and treat patients accordingly. Finally, education of high-risk populations, and continued promotion of condom use could prevent many infections, and thereby limit the spread of antimicrobial resistance.
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