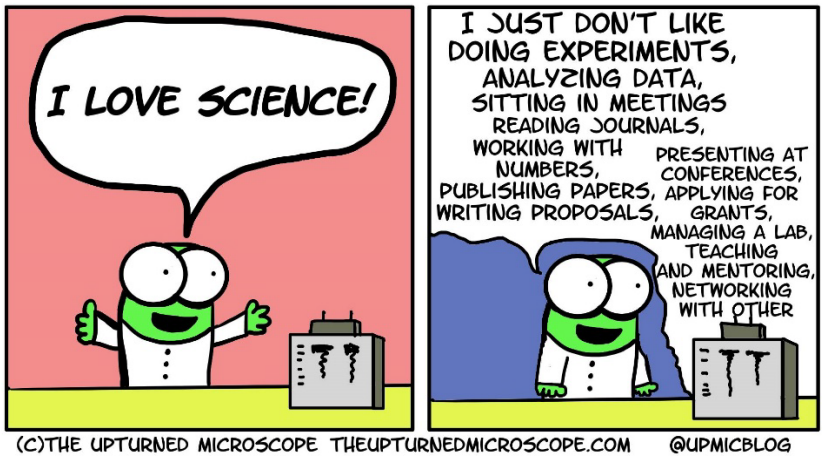
Implementation of strategies for management and prevention of sexually transmitted infections with focus on *Neisseria gonorrhoeae* and *Chlamydia trachomatis*



Örebro Studies in Medicine 267

RONZA HADAD

Implementation of strategies for management and prevention of sexually transmitted infections with focus on *Neisseria gonorrhoeae* and *Chlamydia trachomatis*

Title: Implementation of strategies for management and prevention of sexually transmitted infections with focus on *Neisseria gonorrhoeae* and *Chlamydia trachomatis*
Publisher: Örebro University 2022
www.oru.se/publikationer

Print: Örebro University, Repro 04/2022

ISSN 1652-4063
ISBN 978-91-7529-440-7

Abstract

Ronza Hadad (2022): Implementation of strategies for management and prevention of sexually transmitted infections with focus on *Neisseria gonorrhoeae* and *Chlamydia trachomatis*. Örebro Studies in Medicine 267.

Sexually transmitted infections (STIs) are a public health issue of great importance worldwide, with effects on fertility and reproduction. *Chlamydia trachomatis* and *Neisseria gonorrhoeae*, causative agents of chlamydia and gonorrhoea, respectively, are the most common bacterial STIs with an estimated 127 million new global cases of chlamydia and 87 million new gonorrhoea cases. The continued emergence of antimicrobial resistance (AMR) in *N. gonorrhoeae* may in the future lead to an untreatable infection. Prevention of these infections and controlling the development of AMR rely on several strategies developed by the World Health Organization (WHO). This thesis aimed to implement several of these strategies, including supporting vaccine development for *C. trachomatis* and *N. gonorrhoeae*, evaluating molecular methods for detecting *N. gonorrhoeae*, predicting AMR and supporting surveillance of the spread and prevalence of AMR in *N. gonorrhoeae*. The present studies on a *C. trachomatis* recombinant vaccine antigen and the investigation of similarities of *N. gonorrhoeae* antigen amino acid sequences to the antigens included in the meningococcal vaccine 4CMenB contributed to the field of vaccine development for STIs. The assay SpeeDx ResistancePlus® GC performed well in detecting *N. gonorrhoeae* and predicting ciprofloxacin resistance and could be used in AMR surveillance and individualised treatment. In 2016, the first national genomic surveillance of all *N. gonorrhoeae* isolates in Sweden was performed. This national surveillance study included whole-genome sequencing combined with phenotypic AMR and epidemiological data, which provides valuable information on circulating strains, epidemiology and phylogeny. Greater knowledge of gonorrhoea and gonococcal AMR epidemiology could inform decisions on guidelines and prevention. It is essential to continue to implement WHO strategies at the national and global levels to prevent and control chlamydia and gonorrhoea infections.

Keywords: *Neisseria gonorrhoeae*, epidemiology, whole-genome sequencing, antimicrobial resistance (AMR), *Chlamydia trachomatis*, vaccine, strategies, management and prevention.

Ronza Hadad, School of Medical Sciences, Örebro University, SE-701 82 Örebro, Sweden, ronza.hadad@regionorebrolan.se

Sammanfattning

Sexuellt överförbara infektioner är ett stort folkhälsoproblem världen över som påverkar reproduktiv hälsa. *Chlamydia trachomatis* och *Neisseria gonorrhoeae*, bakterierna som orsakar klamydia och gonorré, är de vanligaste bakteriella sexuellt överförbara infektionerna med 127 miljoner respektive 87 miljoner estimerade nya globala fall bland vuxna per år. Förutom den höga infektionsbördan är den fortlöpande utvecklingen av antibiotikaresistens hos *N. gonorrhoeae* ett stort bekymmer då det kan leda till att gonorré blir obehandlingsbar. Prevention av de här infektionerna, samt att kontrollera utvecklingen av antibiotikaresistens, bygger på flera strategier som tagits fram av Världshälsoorganisationen (WHO). Syftet med denna avhandling var att implementera ett flertal av dessa strategier, specifikt att stödja arbetet med att utveckla vaccin mot *N. gonorrhoeae* och *C. trachomatis*, utvärdera molekylära tester för detektion av *N. gonorrhoeae* och markörer för att förutspå antibiotikaresistens samt att stödja övervakningen av spridningen och förekomsten av antibiotikaresistens hos *N. gonorrhoeae*. Utvärderingen av ett syntetiskt *C. trachomatis* vaccinantigen och undersökningen kring likheterna mellan *N. gonorrhoeae* proteinsekvenser som motsvarar de antigen som är inkluderade i meningokockvaccinet 4CMenB (och ev. korsreaktion), har bidragit till forskningsområdet inom vaccinutveckling för sexuellt överförbara infektioner. Metoden SpeeDx ResistancePlus® GC visade på goda resultat för att kunna detektera *N. gonorrhoeae* och att förutsäga resistens mot antibiotikan ciprofloxacin och kan därför användas för både övervakning av resistens samt för individanpassad behandling. Slutligen, den första nationella genomiska övervakningen av alla *N. gonorrhoeae* isolat i Sverige under ett år genomfördes, inklusive sekvensering av arvsmassan i kombination med antibiotikaresistens och epidemiologiska data. Detta gav värdefull information kring cirkulerande stammar i samhället, epidemiologi och det genomiska släktskapet. Det förbättrade kunskapsläget kring spridningen och förekomsten av antibiotikaresistens hos *N. gonorrhoeae* kan påverka beslut kring rekommendationer och prevention. Det är nödvändigt att kontinuerligt implementera WHO strategier, både nationellt och globalt, för hantering och kontroll av klamydia och gonorré.

Table of Contents

ABBREVIATIONS 12

ORIGINAL PAPERS 14

INTRODUCTION 15
Sexually transmitted infections in a historical perspective 15
Prevalence of sexually transmitted infections 15
Clinical management of gonorrhoea and chlamydia 17
Prevention of sexually transmitted infections 18
Neisseria gonorrhoeae 19
Taxonomy 19
Biology of *N. gonorrhoeae* 19
Emergence of *N. gonorrhoeae* antimicrobial resistance 21
Genome and genetic mechanisms of *N. gonorrhoeae* 24
Antimicrobial resistance determinants in *N. gonorrhoeae* 25
Gonorrhoea treatment 28
Chlamydia trachomatis 29
Taxonomy 29
Biology of *C. trachomatis* 29
Vaccine candidates 31
N. gonorrhoeae vaccine candidates 32
C. trachomatis vaccine candidates 33
Animal models 33
Adjuvants 34
Clinical laboratory diagnostics 34
Molecular methods for detection and characterisation 35
Nucleic acid amplification tests 35
Sanger sequencing 39
Whole-genome sequencing 40
Molecular typing schemes for *N. gonorrhoeae* 42
Strategies for management and prevention 43

AIMS 45

MATERIALS AND METHODS 46
Material and methods used in paper I 46
Culture and purification of *C. trachomatis* elementary bodies 46
Protein production 46

Immunisation and challenge in a mouse model 47
Analysis of immune response, challenge and fertility 49
Material and methods used in papers II-IV 49
Isolates 49
Antimicrobial susceptibility testing 50
DNA extraction 52
PCR 52
Sequencing 52
Bioinformatic analysis 53
Typing methods 53
Statistics 53
Ethical considerations 54

RESULTS AND DISCUSSIONS 55
Implementation of WHO strategies (papers I-IV) 55
Vaccine research (papers I-II) 55
Vaccine candidate for the prevention of *C. trachomatis* (paper I) 55
Vaccine research for the prevention of *N. gonorrhoeae* (paper II) 58
Surveillance of AMR and AMR determinants (papers III-IV) 64
ResistancePlus® GC assay for ciprofloxacin resistance prediction (paper III) 64
C2611 assay for azithromycin resistance prediction (paper III) 66
AMR in *N. gonorrhoeae* isolates (paper IV) 67
AMR determinants in *N. gonorrhoeae* isolates (paper IV) 69
***N. gonorrhoeae* epidemiology (paper IV)** 69
Population characteristics (paper IV) 69
Molecular epidemiology and phylogeny (paper IV) 71
Conclusions and future perspectives 74
Acknowledgements 76

REFERENCES 78

Abbreviations

4CMenB	Four-component meningococcal group B vaccine
AC2	Aptima Combo 2 (NAAT assay)
AMR	Antimicrobial resistance
bla_{TEM}	TEM β-lactamase gene
bp	Base pairs
CEACAM	Carcinoembryonic antigen-related cell adhesion molecule
CI	Confidence interval
CT	Cholera toxin
CTA1	CT subunit A1
CTA1-DD	CTA1 and dimer of the immunoglobulin-binding D region
DNA	Deoxyribonucleic acid
EB	Elementary body
ESC	Extended-spectrum cephalosporin
EUCAST	European Committee on Antimicrobial Susceptibility Testing
EU/EEA	European Union/European Economic Area
fHbp	Factor H binding protein
GNA	Genome-derived neisserial antigens
IFNγ	Interferon γ
IFU	Inclusion forming units
IL	Interleukin
i.n.	Intranasal
i.p.	Intraperitoneal
i.vag.	Intravaginal
kDa	Kilodalton
LGV	Lymphogranuloma venereum
LOS	Lipooligosaccharides
MeNZB	Meningococcal New Zealand group B OMV vaccine
MetQ	Methionine binding component
MIC	Minimum inhibitory concentration
MLST	Multilocus sequence typing
MOMP	Major outer membrane protein
MSM	Men who have sex with men
NAAT	Nucleic acid amplification test
NadA	*Neisseria* adhesin A
NG-MAST	*N. gonorrhoeae* multi-antigen sequence typing

NG-STAR	*N. gonorrhoeae* sequence typing for antimicrobial Resistance
NG-STAR CC	NG-STAR clonal complex
NHBA	Neisserial heparin-binding antigen
OMV	Outer membrane vesicle
Opa	Opacity
PBP	Penicillin-binding protein
PCR	Polymerase chain reaction
RB	Reticulate body
Rmp	Reduction-modifiable protein
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
SNP	Single nucleotide polymorphism
ST	Sequence type
STI	Sexually transmitted infection
Tbp	Transferrin-binding protein
Th	T helper
TMA	Transcription-mediated amplification
UK	United Kingdom
USA	United States of America
VS	Variable segment
WGS	Whole-genome sequencing
WHO	World Health Organization
WT	Wild type

Original papers

This thesis is based on the following four original papers, referred to by their roman numerals I-IV.

I. **Hadad R**, Marks E, Kalbina I, Schön K, Unemo M, Lycke N, Strid Å, Andersson S. Protection against genital tract *Chlamydia trachomatis* infection following intranasal immunization with a novel recombinant MOMP VS2/4 antigen. APMIS 2016;124:1078-86

II. **Hadad R**, Jacobsson S, Pizza M, Rappuoli R, Fredlund H, Olcén P, Unemo M. Novel meningococcal 4CMenB vaccine antigens – prevalence and polymorphism of the encoding genes in *Neisseria gonorrhoeae*. APMIS 2012;120:750-60

III. **Hadad R**, Ebeyan S, Yeen Tan L, Cole MJ, Jacobsson S, Golparian D, Day M, Whiley D, Unemo M, Euro-GASP study group. Evaluation of SpeeDx *ResistancePlus*® GC and the β-version of the SpeeDx 23S rRNA gene molecular assay for prediction of antimicrobial resistance to ciprofloxacin and azithromycin in *Neisseria gonorrhoeae*. J Antimicrob Chemother 2020;76:84-90

IV. **Hadad R**, Golparian D, Velicko I, Ohlsson A-K, Lindroth Y, Ericson E-L, Engstrand L, Fredlund H, Unemo M. First national study of genomic epidemiology of *Neisseria gonorrhoeae* strains spreading across Sweden, 2016. Front Microbiol 2022;12:820998

Introduction

Sexually transmitted infections in a historical perspective

Indications of sexually transmitted infections (STIs) date back to the earliest written narratives. Symptoms have been described in several old writings from ancient Egypt, Greece, and the Old Testament, which mention symptoms of acute urethritis and unclean bodily discharge that may indicate bacterial infection. The origin of the word gonorrhoea, derived from the Greek language, means "flow of seed," referring to the involuntary discharge [1]. The causative agent of gonorrhoea, *Neisseria gonorrhoeae*, was first described in 1879 by Albert Neisser [2]. Similarly, genital chlamydia and trachoma, causative agent *Chlamydia trachomatis*, was indicated in 2700 years BC in China and by ancient Egyptian physicians [3] and was first scientifically described by Halberstaedter and von Prowazek in 1907 [4]. The word chlamydia (meaning cloak) was derived from the original misclassification as a protozoan, which was later changed to a virus. It was not until the beginning of the 1960s that the organism proved to be a bacterium [5]. Data on gonorrhoea cases in Sweden have been reported since 1912 and chlamydia since 1982. However, mandatory partner notification and contact tracing in Sweden, regulated by the Communicable Diseases Act, became effective in 1988 [6, 7].

The World Health Organization (WHO) was officially established in 1948 [8]. The objective of WHO is to promote public health, which includes prevention and reduction of risk factors associated with health risks. Among the priorities was to work towards improved sexual and reproductive health, including collecting accurate epidemiological data to control the spread of STIs. The massive task of reducing the global spread of STIs is managed through defined WHO strategies. Implementing these strategies aims to control the uncontrolled spread of STIs and improve global reproductive health in developing and industrialised countries [9].

Prevalence of sexually transmitted infections

WHO periodically publishes global estimates of four major curable STIs: gonorrhoea, chlamydia, syphilis (*Treponema pallidum* subspecies *pallidum*) and trichomoniasis (*Trichomonas vaginalis*) [10-13]. In the most recent published report from 2016, WHO estimated the incidence of these four STIs to be 376 million new global cases among adults each year (Figure 1),

with gonorrhoea and chlamydia accounting for 87 million and 127 million new cases, respectively, among 15-49-year olds [14]. The true prevalence is difficult to estimate as evidence-based studies in general populations are limited in most areas, and screening, surveillance and reporting methods vary considerably. The estimated incidence of gonorrhoea is the highest in the WHO African Region and generally in low-income countries as opposed to chlamydia, which was the highest in the WHO Region of the Americas and mostly in upper-middle-income countries [14]. Generally, the prevalence of STIs tends to be higher in urban areas, younger age groups [10] and in sub-populations such as men who have sex with men (MSM) and sex workers [15]. Specifically for gonorrhoea, it is most prevalent in the age groups 25-44 years and MSM accounts for a high proportion of reported gonorrhoea cases in most developed settings [16, 17]. Chlamydia is consistently prevalent in young adolescents and adults <25 years, in addition to the association with behavioural risk factors (e.g., inconsistent condom uses and multiple new partners per year) [15, 18].

In Sweden, the gonorrhoea incidence in 2017-2019 increased from 25 to 31 cases per 100 000 inhabitants. The incidence was highest in the age groups 20-34 years in men and 20-24 years in women, with the majority (78% in 2017 to 73% in 2019) diagnosed in men and most frequently in MSM. Reported domestic cases in 2018-2019 were 70-75% and foreign cases about 25% [19]. In 2020 the incidence of reported cases decreased for the first time in a decade (to 25 cases per 100 000 inhabitants). The decrease was significant compared to the average incidence in 2017-2019, which is most likely linked to the implementation of social and physical restrictions, decreased STI testing and travel restraints due to the COVID-19 pandemic [19, 20].

The chlamydia incidence in Sweden in 2017-2019 increased from 333 to 336 cases per 100 000 inhabitants. However, the average chlamydia incidence has decreased by 2.5% per year from 2007 to 2018. The majority of infections (50-60%) were diagnosed in women and the incidence was highest in age groups 20-24 years in men and 15-24 years in women [21]. Similarly to gonorrhoea, reported chlamydia cases decreased in 2020, but this decrease was not significant compared to the average incidence in 2017-2019 [20].

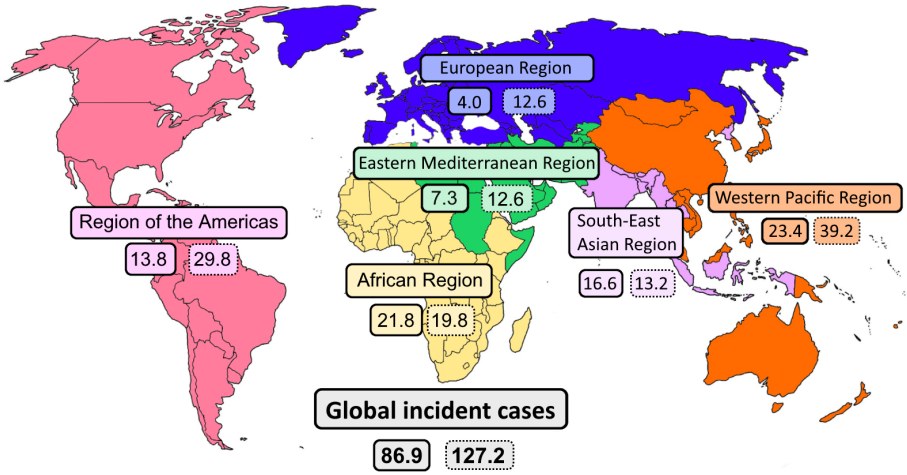


Figure 1. Estimates of the 2016 incidence of gonorrhoea and chlamydia globally and divided into WHO regions [14]. Numbers with solid and dashed lines depict estimates of the number of million gonorrhoea and chlamydia cases, respectively.

Clinical management of gonorrhoea and chlamydia

N. gonorrhoeae causes inflammation of the infected anatomical site, resulting in, for example, urethritis, cervicitis, conjunctivitis, pharyngitis and proctitis. If symptoms are present, they are usually nonspecific. Urogenital infections in men are symptomatic in >90% of cases and the predominant symptoms are urethral discharge (frequently mucopurulent) and dysuria. Endocervical and urethral infections in women may cause symptoms such as mucopurulent and altered vaginal discharge, lower abdominal pain, dysuria or intra-menstrual bleeding. However, most infections (>50%) in women are asymptomatic. Pharyngeal and rectal infections are usually asymptomatic in both sexes [16, 22]. The symptoms of *C. trachomatis* infections in men and women are similar to *N. gonorrhoeae* and asymptomatic infections are usually present in >50% of men and 70-95% of women [18]. If the *N. gonorrhoeae* and *C. trachomatis* urogenital infections remain untreated, they may ascend and cause more severe complications, including

pelvic inflammatory disease, chronic pelvic pain, ectopic pregnancy and infertility in women and epididymitis and epididymo-orchitis in men [16, 18, 22]. Pregnant women with urogenital *N. gonorrhoeae* or *C. trachomatis* infection may transmit the infection to their child during vaginal delivery, causing conjunctivitis or, in case of *C. trachomatis* infection, pneumonia [16, 18]. If untreated, *N. gonorrhoeae* may cause ophthalmia neonatorum, leading to a high risk of blindness [23]. In rare cases, *N. gonorrhoeae* infection may lead to disseminated gonococcal infection with such symptoms as cutaneous lesions, arthritis and arthralgia [16, 24]. Long-term eye infection of *C. trachomatis* serovars/genovars A-C, transmitted by direct or indirect contact, may lead to trachoma that causes scarring and eventual blindness [25]. An additional consequence of urogenital gonorrhoea and chlamydia is the increased risk of HIV transmission [26].

According to European guidelines, the current treatment for gonorrhoea in Sweden is a single dose of ceftriaxone 1 g for uncomplicated urogenital infection [27]. Swedish and international recommendations for treating chlamydia with uncomplicated infection is doxycycline 100 mg twice daily for a week. Swedish guidelines discourage using azithromycin for chlamydia treatment because of the risk of antimicrobial resistance (AMR) selection in *Mycoplasma genitalium*, *N. gonorrhoeae* and *T. pallidum*. There is no evidence of stable genetic or phenotypic AMR in clinical *C. trachomatis* leading to treatment failures [18, 27, 28].

Prevention of sexually transmitted infections

Prevention of transmissions of STIs includes community-based methods achieved through interventions focused on five areas [29]: (i) epidemiologic targeting of populations at high risk, (ii) primary prevention such as education and condom use, (iii) availability of healthcare services, including management of patients, screening programmes and counselling, (iv) building enabling environments, i.e. addressing social, cultural, political and economic factors influencing sexual and reproductive health [29, 30] and (v) reliable surveillance data to guide decisions and management. STI surveillance should be based on routine STI case reporting and monitoring of AMR, risk behaviour and STI services [29].

In Sweden, opportunistic testing of STIs is implemented where testing for gonorrhoea and chlamydia (as well as syphilis and HIV) is offered by healthcare services without cost to the patient. *N. gonorrhoeae* and *C. tra-*

chomatis are mandatory notifiable infections and notified by both laboratories and physicians [7]. In case of a positive test result, sexual contact tracing is initiated to control further transmission.

Neisseria gonorrhoeae

Taxonomy

The taxonomy of *N. gonorrhoeae* is determined by the phylogeny of conserved signature sequences, and *N. gonorrhoeae* belongs to the order of *Neisseriales* and the family *Neisseriaceae*. This family comprises 10 genera; *Alysiella, Bergeriella, Conchiformibius, Eikenella, Kingella, Neisseria, Simonsiella, Stenoxybacter, Uruburuella,* and *Vitreoscilla* [31]. The genus *Neisseria* includes two strictly human pathogens, *N. gonorrhoeae* and *N. meningitidis*. Both pathogens share 80-90% genomic identity [32, 33], however, with distinct disease outcomes and only *N. gonorrhoeae* is an obligate pathogen. Additionally, the genus comprises several commensal species, including *N. sicca, N. lactamica, N. subflava, N. perflava, N. cinerea, N. elongata* and *N. flavescens* [31]. The species mainly consists of Gram-negative aerobic diplococcus with joining sides characteristically flattened. They lack flagella and are non-sporulating bacterium with optimal growth in a humid atmosphere enriched with 5% carbon dioxide at 35-37°C. Owing to the wide range of oxygen levels in the male and female urogenital tract, *N. gonorrhoeae* can grow in aerobic to anaerobic conditions in a host [34].

Biology of *N. gonorrhoeae*

The bacterial cell wall comprises the outer and inner membrane separated by a thin peptidoglycan layer. Various proteins, intra- and extracellular, are present for one or several purposes, such as adherence, colonisation, virulence, immunogenic or immune evasive capacity (Figure 2). The pili and the opacity (Opa) proteins are essential for attachment and subsequent colonisation of the host [35, 36]. *N. gonorrhoeae* carries the type IV pilus involved in initial adherence to the mucosa, cells and tissues, as well as microcolony formation, twitching motility, resistance to neutrophil-mediated killing and transformation [36, 37]. *N. gonorrhoeae* is naturally competent for transformation, meaning they can during all phases of growth acquire extracellular deoxyribonucleic acid (DNA) by horizontal gene transfer, which occurs through type IV pilus [37, 38]. Noteworthy, clinical isolates cultured in laboratories lose the expression of pilus, suggesting selective pressure in

a natural environment [22]. The Opa proteins are involved in enhanced adhesion by binding to various receptors on host cells and tissues, such as the carcinoembryonic antigen-related cell adhesion molecule (CEACAM) receptor family [39]. The most abundant protein on the cell surface is the PorB protein, a porin with multiple functions. This transmembrane porin, encoded by the *porB* gene, regulates the ion exchange and transports small molecules. It also increases attachment, impairs phagocytosis, resists complement factors (through binding of C4 binding protein and factor H) and is involved in AMR [40-44]. Furthermore, lipooligosaccharides (LOS), released together with peptidoglycan, may act as a toxin and facilitate transmission and sialylated LOS inhibits complement activation by binding of factor H and decreased binding of iC3b [45, 46]. Iron acquisition is an essential part of survival for *N. gonorrhoeae* and several iron transport systems exist: for example, the transferrin-binding protein (Tbp) A and B, located in the membrane. TbpA acts as a transporter protein by binding transferrin and transporting iron across the membrane while TbpB substantially increases iron uptake efficiency [47]. Lastly, an important presence in the outer membrane is the main and most characterised efflux pump system, the MtrCDE complex, one of five defined efflux pump systems in *N. gonorrhoeae*. The main function is to export hydrophobic and toxic molecules such as fatty acids, cationic peptides and antimicrobial agents [22, 48]. Although not surface exposed, other important proteins are the penicillin-binding proteins (PBPs). These enzymes are involved in the peptidoglycan assembly by transglycosylase (PBP1) and transpeptidase (PBP1 and PBP2) activity, constituting the cell wall. With the high structural similarity between the substrate of transpeptidase and β-lactam ring present in certain antimicrobials, these proteins have been a long-standing target for several antimicrobials [49].

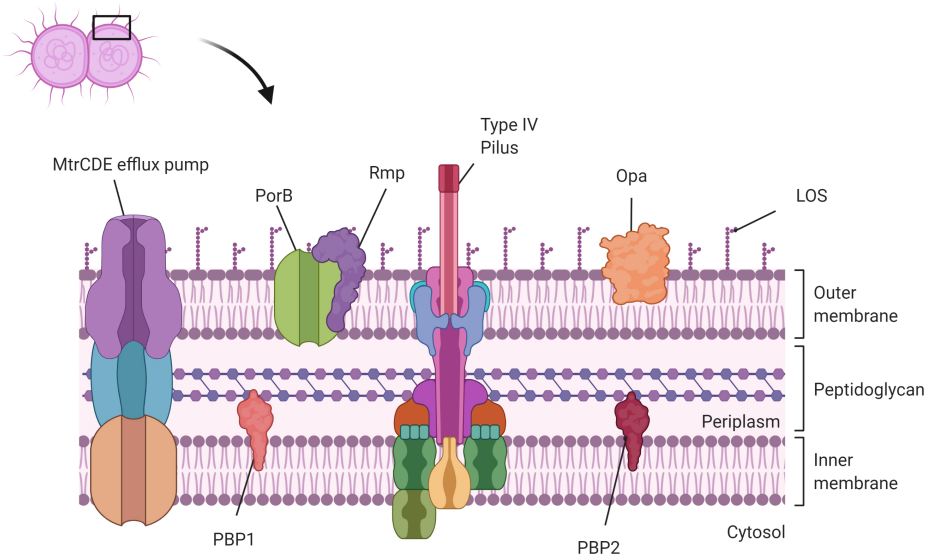


Figure 2. Illustration of structures and proteins present in Neisseria gonorrhoeae membrane. Type IV pilus and opacity (Opa) proteins are involved in adhesion to host cells and lipooligosaccharide (LOS) acts as a toxin. The type IV pilus also facilitates DNA transportation across the membrane. The porin PorB is involved in the influx of molecules and MtrCDE efflux pump in the efflux of molecules. Reduction-modifiable protein (Rmp) is associated with immune evasion by blocking the binding of host antibodies to PorB. The penicillin-binding proteins (PBPs) are involved in peptidoglycan synthesis. Image created in BioRender.com.

Emergence of *N. gonorrhoeae* antimicrobial resistance

In the pre-antimicrobial era, treatment of gonorrhoea embodied lifestyle changes (e.g., fresh air and rest, abstinence from alcohol and sexual activity) and systemic treatment with various balsams, metallic compounds and hyperthermia [23]. In the mid-1930s, antimicrobial treatment of gonorrhoea was introduced using sulfonamides [50]. Different agents of sulfonamides were used during the following decade; however, by the late 1940s, most isolates were resistant [51]. Simultaneously, the development of penicillin was ongoing after its accidental discovery in 1928. Still, it was not until 1943 that penicillin was properly evaluated to treat *N. gonorrhoeae* and

replaced sulfonamides. Low dosage was used due to limited supply and cost, and its use was restricted to severe infections [52, 53]. Treatment of *N. gonorrhoeae* with penicillin continued for many decades after its introduction, although with increasing concentrations due to the rise of resistance [54, 55]. The occurrence of β-lactamase plasmids in 1976 [56], resulting in degradation of the β-lactam structure of penicillin and high-level resistance, caused major concern when it spread internationally. The progression of penicillin resistance continued and chromosomally mediated resistance was described a decade later [57] and is still widespread throughout the globe. Patients who could not receive penicillin treatment were treated with a different compound, tetracycline. The agent inhibits protein synthesis specifically by targeting 30S ribosomal ribonucleic acid (rRNA) [58]. This antimicrobial compound was discovered in 1945 from a fungus found in soil, which was effective in treating both Gram-positive and Gram-negative bacteria. It was later modified and named tetracycline [59, 60]. However, global tetracycline resistance was observed in the 1960s [58, 61], and the high prevalence resulted in its abandonment as an option for treatment by the end of 1980 [1].

The discovery of antimicrobial agents continued when spectinomycin was isolated from *Streptomyces spectabilis* in 1960 [62] and subsequently used for gonorrhoea treatment as an alternative antimicrobial for penicillin-resistant isolates [63]. However, quickly after its introduction, spectinomycin resistance was found in a penicillin-susceptible isolate [64] and over a decade later also in penicillin-resistant isolates [65]. In some local settings where spectinomycin was used as first-line treatment, resistance emerged rapidly [66] and was later reported internationally [67]. Spectinomycin is not frequently used due to limited availability in many countries and fear of emerging resistance [23].

The fluoroquinolone ciprofloxacin, administered as a single oral dose, has been used as a first-line treatment for gonorrhoea since the mid-1980s [23, 68]. Fluoroquinolones inhibit the targets DNA gyrase and topoisomerase IV resulting in bactericidal activity [69]. Quinolone was first discovered in the 1960s as a by-product of chloroquine, which is used against malaria and is a predecessor to the broad-spectrum antimicrobial fluoroquinolone [23]. Clinical failure was reported in 1989 [70], and fluoroquinolone resistance spread quickly. By the end of the 1990s, the Western Pacific region abandoned fluoroquinolones as a first-line empirical treatment [23, 71]. Europe and the USA soon followed [72, 73], and the prevalence of ciprofloxacin-resistant isolates remains high [74-76].

Azithromycin, belonging to the macrolides, inhibits the protein synthesis by binding and disrupting the interaction between 50S ribosomal subunit and 23S rRNA close to the exit tunnel [77]. Azithromycin was developed in 1980 as a synthetic derivative of erythromycin, which proved effective against *N. gonorrhoeae*. Decreased susceptibility to azithromycin first emerged in Latin America [78] and then spread globally [75, 76, 78-81].

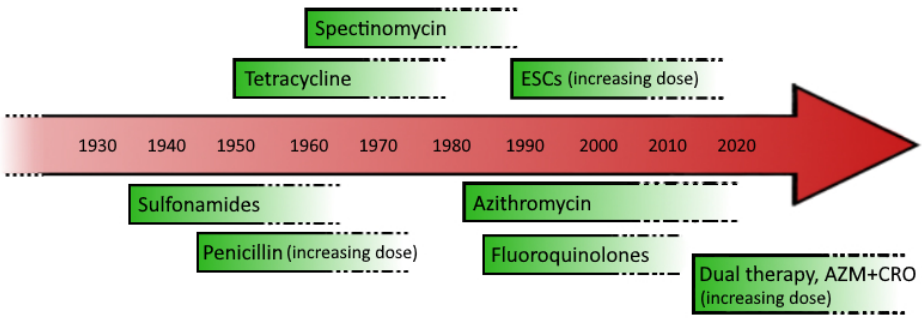


Figure 3. Antimicrobial agents used in treating gonorrhoea since the start of the antimicrobial era. Abbreviations: ESCs, extended-spectrum cephalosporins; AZM, azithromycin; CRO, ceftriaxone.

Extended-spectrum cephalosporins (ESCs) are β-lactam antibiotics that target peptidoglycan synthesis; however, unlike penicillin, they are stable from degradation by β-lactamase. Cephalosporin was first discovered in the fungus *Acremonium chrysogenum* in 1948 but was not commercialised until 1964 [82]. There are five generations of cephalosporins because of different structural alterations to the molecule. The third-generation, i.e. ceftriaxone and cefixime, is effective against *N. gonorrhoeae* and other Gram-negative bacteria [83]. In many countries, ESCs are the last option for empirical first-line monotherapy of gonorrhoea [23, 84], but this may not last. The emergence of decreased susceptibility to ESCs in *N. gonorrhoeae* began in Japan in the late 1990s when subinhibitory concentrations of several different oral cephalosporins were administered [85]. At the beginning of the millennium, reported resistance included treatment failures with cefixime

[86]. Resistance to ESCs has spread internationally with reports of additional treatment failures with ceftriaxone in Japan, Australia and Europe [87-91]. International transmission of ceftriaxone resistance has been associated with the dissemination of strain FC428 and its sublineages, including the mosaic *penA* 60.001 allele, since 2015 [92-95]. Strains with ceftriaxone resistance and high-level azithromycin resistance have been described in the United Kingdom and Australia [96, 97]. However, the first described high-level ceftriaxone resistant *N. gonorrhoeae* strains appeared to have had a fitness cost, making the strains unlikely to spread [91, 98-100].

A summary of antimicrobial agents used for *N. gonorrhoeae* treatment over time is shown in Figure 3. History has shown the remarkable ability and consistency of *N. gonorrhoeae* to survive and adapt to selective pressure for the past century. The emergence of resistance to antimicrobials used for the treatment of gonorrhoea mostly spread internationally within one to two decades of their introduction as recommended first-line therapies. Effective evasion is possible through various genetic mechanisms, such as the inherent ability for extracellular DNA uptake, genetic exchange and recombination.

Genome and genetic mechanisms of *N. gonorrhoeae*

The genome of *N. gonorrhoeae* is approximately 2.2 million base pairs (bp) [101], and most isolates contain several plasmids (e.g., the conjugative and the cryptic plasmid). The conjugative plasmid is the largest with 42 kbp and encodes genes necessary for conjugation [102]. In contrast, the cryptic plasmid lacks known function and virulence traits. The cryptic plasmid comprises 4.2 kbp present in >90% of *N. gonorrhoeae* isolates [102, 103]. Additionally, a β-lactamase plasmid (carrying the β-lactamase TEM (bla_{TEM}) gene) may be present (size ranging from 3-9 kbp), conveying high-level penicillin resistance. Different β-lactamase plasmids with divergent size and DNA sequences have been identified and named after their geographic origin, such as the African, Asian and Rio/Toronto plasmids [22, 102].

Plasmids are easily transferred between isolates by conjugation. However, *N. gonorrhoeae* is also naturally competent for transformation and can acquire extracellular DNA from the environment through the type IV pili or recognize the DNA uptake sequences and then incorporate that DNA in the genome [104]. Similarly, *N. gonorrhoeae* can secrete chromosomal DNA made available for transformation by another cell [105], i.e. exchange genetic material by horizontal gene transfer. Transposons (or "jumping

genes") are elements with inverted repeats that can move within the genome and may incorporate extra genetic material [102]. Additionally, nearly 80% of *N. gonorrhoeae* isolates comprise a 57 kbp region called the gonococcal genetic island. This region contains genes similar to the encoding type IV secretion system and enables DNA exchange between *N. gonorrhoeae* isolates. The gonococcal genetic island is rarely found in commensal *Neisseria* species [22, 102].

Comparison of *N. gonorrhoeae* with other *Neisseria* species has shown high homology between genomes that facilitate recombination and exchange of genetic material [32, 33, 106, 107]. The ability for homologous recombination is a significant attribute for the gain and spread of genetic material, specifically AMR determinants [108]. The co-occurrence of *N. gonorrhoeae* with naturally occurring commensal *Neisseria* species, together with exposure to antimicrobials, drives the development of AMR [22, 108]. Pharyngeal infections may be accountable for the emergence of many AMR mechanisms, where commensal *Neisseria* species act as reservoirs, and exchange of genetic material, including AMR determinants, occurs between commensals and *N. gonorrhoeae* [23, 107-109]. The close homology with commensal species and co-occurrence at infection sites, including the high transformation and recombination rates, make *N. gonorrhoeae* inclined to AMR development and exceptionally adaptable in environments with selective pressure [108, 109].

Antimicrobial resistance determinants in *N. gonorrhoeae*

Phenotypic AMR is mainly achieved through enzymatic destruction or alteration of the antimicrobial, reducing affinity to the target or altering the concentration of antimicrobials in the cell by decreasing the influx or increasing the efflux of molecules. AMR in *N. gonorrhoeae* has emerged through chromosomal and plasmid-mediated changes. Examples of plasmid-mediated resistance are the bla_{TEM} and *tetM* genes [102]. The bla_{TEM} gene results in high-level resistance to penicillin. It is a cause for concern as the $bla_{\text{TEM-135}}$, currently mainly found in the Rio/Toronto plasmid, is only one single nucleotide polymorphism (SNP) from becoming an extended-spectrum β-lactamase and capable of degrading cephalosporins [110]. Another penicillin AMR determinant is the *ponA* gene, coding for PBP1, where the resistance mutation results in an L421P alteration and a two-fold increase in minimum inhibitory concentration (MIC) for penicillin [111]. Similar to penicillin, tetracycline resistance may be mediated through plasmid

and chromosomal changes. High-level resistance to tetracycline is mediated through the *tetM* gene, carried by some conjugative plasmids [112]. Additionally, the SNP in the *rpsJ* gene encoding S10 ribosomal protein confers tetracycline resistance [113].

SNPs have been found to mediate phenotypic AMR to several drugs in *N. gonorrhoeae*. The C2611T and A2059G (*Escherichia coli* numbering) mutations in the 23S rRNA gene, present in four copies in the genome, are associated with moderate and high-level azithromycin resistance, respectively. The level of resistance is dependent on the number of mutated alleles [114]. Spectinomycin activity occurs through interactions with 16S rRNA inhibiting the protein translation [115]. The SNP C1192T in the 16S rRNA gene results in high-level spectinomycin resistance [116, 117]. Additional rare mutations in the ribosomal protein S5, encoded by the *rpsE* gene, result in different amino acid deletions (V25) or alterations (T24 and K26), causing low- to high-level resistance [117]. However, these mutations are rare globally [23, 117]. SNPs in the *gyrA* gene, causing GyrA alternations at positions S91 and D95, result in ciprofloxacin resistance. Additional mutations in the *parC* gene, encoding the topoisomerase IV, result in a high-level ciprofloxacin resistance [118].

The primary resistance determinant for the emerging resistance to ESCs is mosaic *penA* alleles, which code for mosaic PBP2, containing up to 60-70 amino acid substitutions compared to the wild type (WT) allele. The emergence of these mosaic alleles has been due to DNA transformation and recombination with partial *penA* genes from commensal *Neisseria* species, most likely in the pharynx [119, 120]. Certain positions with amino acid substitutions in the PBP2 have been linked to decreasing susceptibility to ESCs, to date at positions 311, 312, 316, 483, 501, 512, 542, 545 and 551 [121-125]. Recently, alterations in RNA polymerase sigma factor (RpoD) and RNA polymerase beta subunit, specifically P157L, G158V and R201H, have been associated with decreased susceptibility or resistance to ceftriaxone [126].

Proteins in the outer membrane that control the import and export of molecules (such as the MtrCDE efflux pump and PorB1b porin) also contribute to the increased AMR in N. gonorrhoeae. MtrR is a key regulatory protein that directly or indirectly affects at least 69 genes. The MtrCDE complex, regulated by the MtrR repressor protein, is coded by the *mtrCDE* operon. Mutations in the repressor gene *mtrR*, i.e. single A-deletion in the promoter or MtrR G45D substitution, result in reduced inhibition of the transcription of the *mtrCDE* operon [127, 128]. Overexpression of the MtrCDE efflux

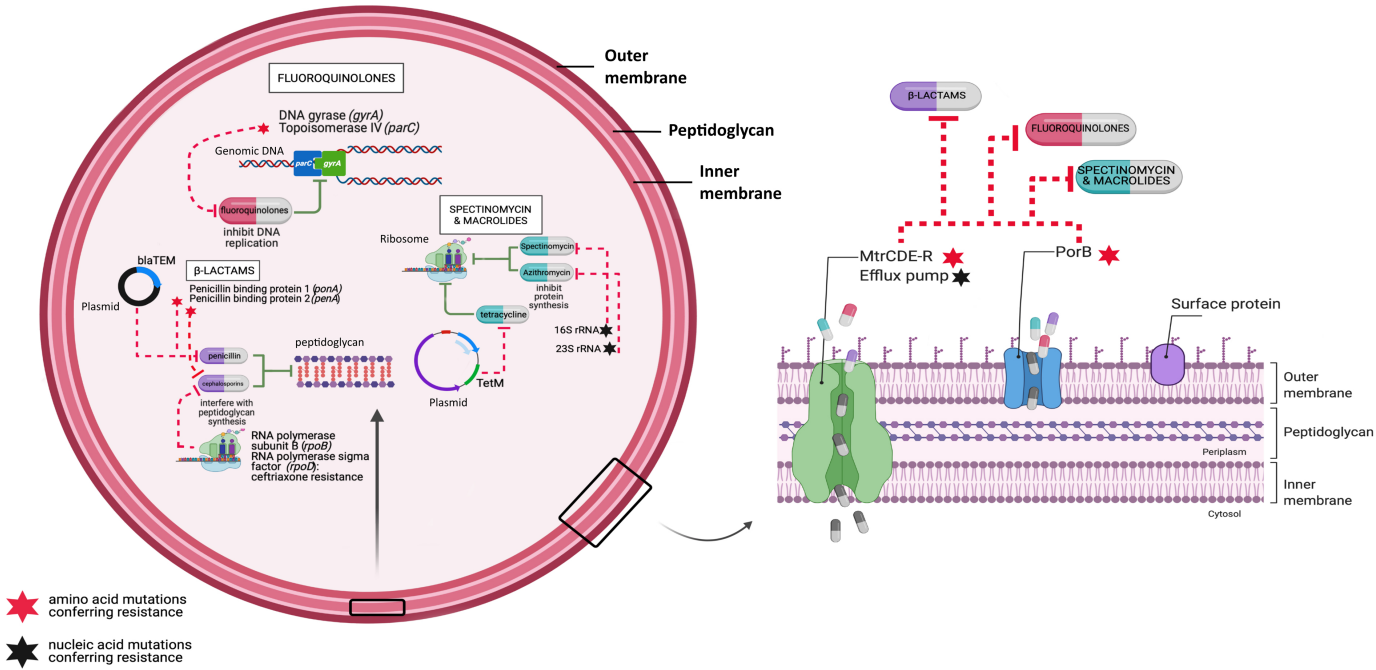


Figure 4. An overview of antimicrobial resistance determinants for different antimicrobials previously and currently used for gonorrhoea treatment [133]. Modified image reprinted with permission according to the Creative Commons Attribution License terms.

pump causes an increased efflux of substrates, including antimicrobials (e.g., penicillins, ESCs, macrolides and tetracyclines) [23, 113, 120, 127]. Mutations in the *porB1b* gene, resulting in *penB* AMR determinant and PorB1b alterations (G120K and G121A/D), decrease the influx of antimicrobials [129]. The *mtrR* and *penB* AMR determinants act synergistically to increase AMR in *N. gonorrhoeae* [42, 120, 130]. The ceftriaxone resistance is more dependent on the combination of *mtrR* and *penB* AMR determinants than cefixime [120]. An additional mechanism, acquired through recombination with other *Neisseria* species, is the mosaic alleles of *mtr* locus. These mosaic alleles are associated with low-level azithromycin resistance [131, 132].

A summary of AMR determinants and the associated antimicrobial is shown in Figure 4.

Gonorrhoea treatment

Traditionally, the criterion for recommended treatment of gonorrhoea has been a single dose with a cure rate of >95% [134]. However, given the lack of antimicrobial alternatives and to minimise the emergence of resistance to ESC, dual antimicrobial therapy for gonococcal infections has been used in the past decade. Currently, European guidelines recommend a dual therapy (ceftriaxone 1 g and azithromycin 2 g), in addition to a test of cure after treatment [16]. Ceftriaxone 1 g monotherapy can be used in well-controlled settings, i.e. where AMR is continuously monitored, ceftriaxone resistance is lacking, and a test of cure is performed. However, recommended treatment of gonorrhoea has not reached a global consensus. WHO [135], Australia [136] and Canada [137] also recommend dual therapy while the USA and the UK recently have returned to monotherapy, specifically recommend ceftriaxone (500 mg) single dose [138] and ceftriaxone (1 g) [139], respectively. Unfortunately, isolates with both ceftriaxone resistance and intermediate to high-level resistance to azithromycin have already been described [97, 140-142].

Chlamydia trachomatis

Taxonomy

Describing the genus belonging and nomenclature of *Chlamydia* species has been an ongoing process. The pathogen was previously considered a virus as it depended on host cells for survival but was later determined to be an obligate intracellular bacteria [5]. Similarly, the taxonomy of the family *Chlamydiaceae* and genera within the family have been subjected to change several times. Before 1980, there were only two *Chlamydia* species, *C. trachomatis* and *C. psittaci* [143]. However, newly developed DNA technologies enabled further classifications and inclusion of species [144, 145]. In 1999, a new classification system was proposed based on ribosomal genes. Accordingly, the species *C. psittaci, C. pneumoniae* and *C. abortus* were reclassified as *Chlamydophila* [144]. A decade later, most of the scientific community had rejected this nomenclature. With the addition of the genome- and protein-based comparison among different species, it was proposed to return to the previous classification [146, 147]. Full consensus on nomenclature has still not been reached [148]. As recognised by the International Committee on Systematics of Prokaryotes, the current taxonomy asserts that the order is *Chlamydiales* and family *Chlamydiaceae*. The family comprises the genera *Chlamydia*, *Candidatus Rubidus* and *Candidatus Amphibiichlamydia,* of which *Chlamydia* includes 15 species, including *C. trachomatis, C. muridarum, C. pneumoniae* and *C. psittaci* [148, 149]. Different species of *Chlamydia* are associated with specific hosts and *C. trachomatis* is a strictly human pathogen; *C. muridarum* and *C. psittaci* are mainly pathogens for mice and birds, respectively [150].

Biology of *C. trachomatis*

C. trachomatis depends on the host cell for energy and necessary nutrients. *C. trachomatis* has a unique replication cycle (Figure 5), enabling alternation between two forms: elementary bodies (EBs) and reticulate bodies (RBs). The EB is the infectious but metabolically inactive form of *Chlamydia* with a size of approximately 0.3 μm. The EB appears dense in an electron microscope. Inside the host, EBs start to transform to the larger (~1 μm) non-infectious but metabolically active RBs and the host cell will contain a mixture of the two forms [151].

Chlamydia is similar to other Gram-negative bacteria with outer and inner cell membranes. A large part of the outer membrane, approximately

60%, consists of the major outer membrane protein (MOMP), a ~40 kilodalton (kDa) transmembrane protein [152] rich in cysteine residues. The protein structure is composed of five conserved regions and four variable segments (VS) [153]. MOMP is expressed in both the EB and RB, and the protein structure is supported by cross-linking of disulphide bonds [154]. The MOMP protein is oxidised and extensively cross-linked in the EB, providing a very stable outer membrane. In contrast, the RBs have a reduced and monomeric form of MOMP, enhancing pore formation and creating an active osmotic membrane [154, 155]. MOMP VS are highly immunogenic and contain T-cell and B-cell epitopes [156, 157]. The MOMP protein forms the basis for traditional typing of *C. trachomatis* by using antibodies for serovar determination. This method has characterised *C. trachomatis* into 15 serovars: A to K and L1 to L3. Serovars A-C are associated with trachoma, D-K with urogenital infections and L1-L3 with the more invasive infection lymphogranuloma venereum (LGV) [158]. Although the MOMP protein is serovar-specific, similarities between several serovars exist, and immunogenic cross-reaction between multiple serovars is possible [159]. The development of molecular methods has allowed the characterisation of *C. trachomatis* by sequencing the *ompA* (*ompI*) gene, encoding the MOMP protein, and enabling discrimination into different genovars [160].

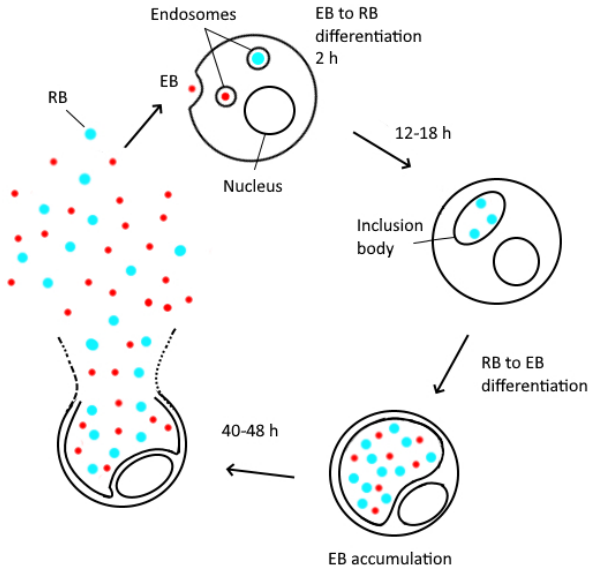


Figure 5. The development cycle of Chlamydia species depends on the host cell for survival and replication. The cell cycle starts with the initial infection by an elementary body (EB), transforming into a larger and metabolically active reticulate body (RB) inside an inclusion body. RBs are converted back to EBs and released within 40-48 hours and can infect new cells [161].

Vaccine candidates

The primary actions for prevention (sex education, condom promotion and sexual contact tracing) have been implemented for decades but have had limited success in combatting STIs [10-12, 14, 162-164]. Low and middle-income countries suffer from the highest prevalence of gonorrhoea. However, despite all available interventions and full access to health services in high and upper-middle-income countries, a substantial increase of gonorrhoea and very high rates of chlamydia infection remain [14, 162, 164]. Vaccines may be the only viable alternative for the long-term effects on the global control of STIs, adverse outcomes and reduction of *N. gonorrhoeae* AMR.

N. gonorrhoeae vaccine candidates

The development of a gonorrhoea vaccine is still in early preclinical phases with unresolved challenges, such as understanding and generating a protective immune response and defining ideal animal models [162, 165]. In the past, only four *N. gonorrhoeae* vaccine candidates have reached clinical trials: specifically whole-cell vaccine [166], partially autolysed *N. gonorrhoeae* strains [167], a pilus-based candidate [168] and a PorB-based candidate [169]. However, none of these vaccine candidates successfully generated protection against *N. gonorrhoeae* infection. Contributing factors to the challenges of vaccine development is the immune evasive characteristics of *N. gonorrhoeae*, including such factors as the inter- and intrastrain variability of antigens (Opa proteins, LOS, pili) [170-172], the presence of antibodies to conserved reduction-modifiable protein (Rmp) that block antiporB bactericidal activity [173] and the inhibition of host complement system [165, 174, 175].

Significant progress has been made in identifying gonococcal candidate antigens in recent decades. Vaccine candidates with immunogenic properties involve the antigens 2C7 epitope of LOS [176], methionine sulfoxide reductase [177], methionine binding component (MetQ) [178, 179], neisserial heparin-binding antigen (NHBA) [180] and outer membrane vesicles (OMVs) that include antigens present in the outer membrane. The prospect of the development of a vaccine for gonorrhoea has been pushed forward based on recent observations using the meningococcal OMV vaccines. OMVs are naturally produced by *Neisseria* species and released to the extracellular compartment [181]. Vaccinations against meningococcal group B epidemics have previously used strains of *N. meningitidis* group B OMVs for mass vaccinations of populations [182-184]. In New Zealand, mass vaccination of a meningococcal New Zealand group B OMV (MeNZB) was administered in response to a meningococcal outbreak. A retrospective study showed significantly decreased rates of gonorrhoea for the first time compared to the unvaccinated. The estimated effect was 31%, and the antibody concentrations were short-lived but provided proof of principle that a large-scale reduction of gonorrhoea incidence is possible [185]. Smaller retrospective studies have also been performed in Canada [186] and Cuba [187], supporting the observations in New Zealand. MeNZB vaccine is no longer available, but the same meningococcal OMV is included in the commercially available Bexsero meningococcal B vaccine. Bexsero also includes the recombinant antigens in the previously named four-component meningococcal group B (4CMenB) vaccine, including genome-derived neisserial

antigens (GNAs) [188]. GNA antigens in the Bexsero vaccine have been selected through reverse vaccinology [189]. They consist of the main immunogenic antigens factor H binding protein (fHbp) and NHBA coupled with accessory proteins GNA1030 and GNA2091, respectively, and *Neisseria* adhesin A (NadA) protein [188].

C. trachomatis vaccine candidates

There has been ongoing vaccine development for *C. trachomatis* during the past 70 years, starting with live or attenuated EBs [190], including some recent studies using plasmid-deficient or UV-treated *C. trachomatis* as vaccine candidates [191, 192]. Most vaccine research has moved on to recombinant outer membrane proteins and synthetic peptides, such as the polymorphic membrane proteins [193, 194] and chlamydia protease-like activity factor [195]. However, the antigen that has been most extensively studied is the MOMP [190]. The MOMP has been and remains a promising antigen in vaccine research for *C. trachomatis* due to the immunogenic properties of its B- and T-cell epitopes and its abundance in the outer membrane [156, 196]. A MOMP-based recombinant protein, CTH522, has recently passed the clinical phase 1 trial. The recombinant protein comprises segments of VS4 of the MOMP protein from serovars D, E, F and G. This clinical trial is the first to be performed with genital chlamydia vaccine [197, 198].

Animal models

A significant challenge to properly evaluate vaccine candidates is the lack of animal models that adequately mimic human disease infection as both *N. gonorrhoeae* and *C. trachomatis* are strictly human pathogens. A few experiments have been performed on human volunteers for studies on the natural immunity of *N. gonorrhoeae*. However, these infection models are limited as group sizes are restricted and infection must be terminated at the first sign of disease [199-202]. Non-human primates, such as chimpanzees and orangutans, have been used for gonorrhoea and chlamydial infections but are restricted due to ethical considerations, limited availability and high costs [203, 204]. Murine models, in particular mice, are extensively used in research for *in vivo* experiments and, for genital infections, rely on hormone treatment to enable colonisation of the genital tract [205, 206]. There are many advantages to using mice, including their small size and the ability to

be genetically engineered. In chlamydia research, different species of the genus *Chlamydia* have been used in their natural host to model *C. trachomatis* infections in humans. The most common is *C. muridarum* in mice, which has been used to elucidate disease pathology, protective immunity and infectious challenges after immunisations with vaccine candidates [190]. Transgenic mouse models, expressing human transferrin [207] and CEACAM [208], have been developed to mimic human *N. gonorrhoeae* infection. Recently, *N. gonorrhoeae* infection was able to be sustained in the upper reproductive tract in a mouse model by the addition of human transferrin [209]. These mouse models may be useful for *in vivo* testing of therapeutic products.

Adjuvants

An important aspect to consider in generating immunity is choosing an adjuvant that is safe, non-toxic and can support the induction of a strong immune response. Some currently used adjuvants are formulas based on aluminium salts, detoxified lipopolysaccharides and oil-in-water emulsions [210]. Cholera toxin is a potent adjuvant but toxic if administrated nasally as it may accumulate in the nervous system and cause damage. A new promising adjuvant, cholera toxin A1 subunit (CTA1), has been developed for mucosal administration. It retains the adjuvant properties of the cholera toxin but without the toxic side effect [211].

Clinical laboratory diagnostics

Many developing countries lack resources and laboratory infrastructure and therefore only use syndromic management or possibly microscopy. *N. gonorrhoeae* could be diagnosed based on the microscopical detection of diplococci in urogenital smears from symptomatic males with urethritis. The method, however, is substantially less reliable, particularly for cervical, rectal and pharyngeal samples [212]. Diagnosis by culture provides viable bacteria that can be diagnosed and distinguished from other *Neisseria* species by, for example, a rapid positive oxidase test, carbohydrate/sugar utilisation test (positive for glucose and negative for maltose and fructose) and by using matrix-assisted laser desorption time-of-flight (Maldi-TOF) [213], an easy and fast method for species identification of *N. gonorrhoeae*.

Culturing *C. trachomatis* requires using cell culture, which is both time-consuming and laborious. Enzyme immunoassays were developed in the

1980s, which provided a faster and easier diagnostic method [214]. However, large-scale testing was not performed until the development of automated molecular techniques, i.e. nucleic acid amplification tests (NAATs). NAATs that simultaneously detect *N. gonorrhoeae* and *C. trachomatis* are most frequently used, particularly in developed countries. The transition from culture to using NAATs for diagnostics of *N. gonorrhoeae* and *C. trachomatis* provides many advantages: higher sensitivity, the use of non-invasive samples for screening, easier sample handling (including transportation and storage where viable bacteria is not a requirement) and the ability to perform high-throughput screening [22, 215]. This transition has resulted in increased testing and treatment. It has also led to a transition of diagnostic methods from culture to molecular tests, leading to less availability of viable gonococcal isolates. Another consequence of the transition is the loss of skills to perform culture, reliable phenotypic AMR detection and surveillance in routine clinical settings.

Molecular methods for detection and characterisation

Nucleic acid amplification tests

Several NAATs have been developed for the detection of STIs. Two common techniques are polymerase chain reaction (PCR) and transcription-mediated amplification (TMA) [216, 217]. The first NAAT used was based on PCR, first published in 1985 [218]. PCR is performed by using specific primers to target genes in the genome. Applying different temperatures for denaturation, annealing of primers and DNA extension results in an exponential increase of the target gene and allows for detection by labelled probes, fluorescent dyes or other post-PCR applications. The PCR method is illustrated in Figure 6.

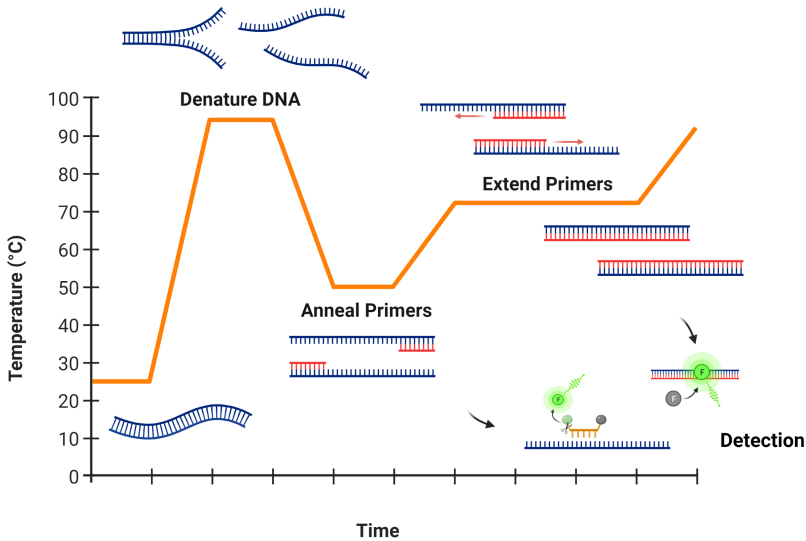


Figure 6. The polymerase chain reaction (PCR) method amplifies target DNA and includes repetition of thermal steps: denaturation, annealing and extension. These steps are run in cycles until the number of synthesised DNA targets reaches detectable levels. Image created in BioRender.com.

TMA (Figure 7) use species-specific rRNA (e.g., 16S and 23S rRNA) as a target for amplification [216, 217]. Briefly, the target rRNA is specifically captured on magnetic beads where reverse transcriptase generates complementary DNA and double-stranded DNA, allowing RNA polymerase to transcript the target back to RNA. The transcripts are detected using labelled probes and re-enter the cycle where reverse transcriptase generates additional complementary DNA. This cycle of regeneration of the target provides very high sensitivity.

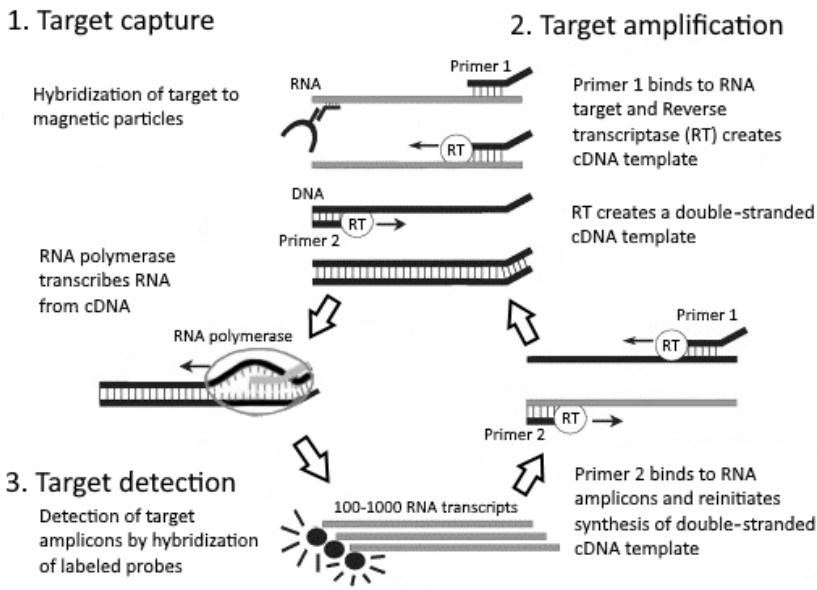


***Figure** 7. Schematic illustration of transcription-mediated amplification (TMA) for detecting specific nucleic acid targets. The process involves the enzymes reverse transcriptase (RT) and RNA polymerase and complementary DNA (cDNA) generation. Modified image reprinted with permission from Elsevier® [219].*

Multiplex assays allow for amplification of multiple targets simultaneously, such as for specific pathogen detection and AMR prediction, as used in PlexPrime™ and PlexZyme™ technology [220]. PlexPrime™ primers are designed with specificity for the pathogen target and AMR mutations but include a mismatch sequence. The synthesised amplicon will be different from the original sequence. Additional sequences (partzymes), specific to the amplicon, form a PlexZyme structure allowing for a universal probe to bind which is cleaved and subsequently detected. An overview of the technology is shown in Figure 8.

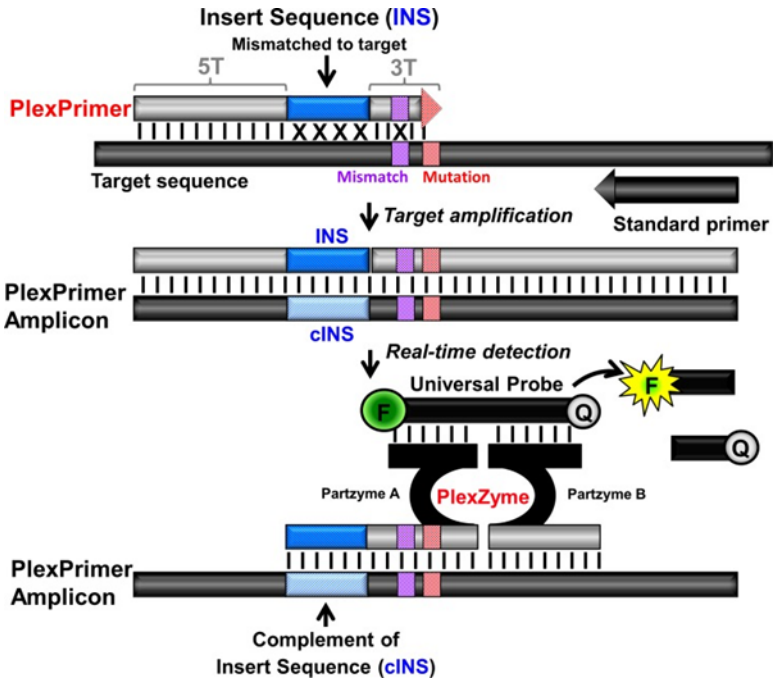


Figure 8. Amplification of a target sequence by PlexPrimeTM and PlexZymeTM technology. The PlexPrimer contains a 5' specific sequence (5T) and a 3' mutation-specific sequence (3T), including an additional mismatch insert sequence (INS) in the middle. The generated amplicons will differ from the original sequence, including a complementary INS (cINS). The amplicon is detected by a specific partzyme sequence, forming PlexZyme, which allows for cleavage of the universal probe. The separation of the fluorophore (F) and quencher (Q) results in a detectable signal [220]. Image reprinted under the terms of Creative Commons Attribution License.

The development of NAATs for diagnosing STIs has revolutionised laboratory testing, providing sensitive and specific automated high-throughput methods [221-223] for diagnosing symptomatic patients and screening asymptomatic populations. Dual detection of *N. gonorrhoeae* and *C. trachomatis* in a single test [221-225] has implications for treatment choice. However, NAATs are more expensive, resource-demanding and mostly used in more developed countries [22, 215]. Furthermore, false-positive results can be obtained due to cross-reaction with similar DNA sequences in other bacteria and because bacterial DNA can be detected in samples from patients up to at least 2-3 weeks after successful treatment. It can also be due to contamination during laboratory processing [226-228]. Special care must be taken to avoid false-positive results. A positive NAAT result for *N. gonorrhoeae* must be confirmed by repeat testing using another target, particularly in pharyngeal specimens [16]. Any use of NAAT for *N. gonorrhoeae* diagnosis must be appropriately validated and quality assured (particularly for specificity in pharyngeal swabs) to verify there is no cross-reaction to non-gonococcal species [215]. A major drawback of NAAT is the loss of phenotypic AMR testing for gaining information on treatment possibilities and for surveillance of AMR, as well as molecular epidemiology and evolution of *N. gonorrhoeae*, which remain challenging to examine using NAAT samples.

Sanger sequencing

The Sanger sequencing method was developed in 1977 [229], and optimisation of the technique allowed for determining a nucleotide sequence of approximately 1000 bp with very high accuracy. The process, illustrated in Figure 9, amplifies a gene sequence by using normal nucleotides and terminating nucleotides, the latter labelled with different fluorescent dyes. Random incorporation of terminating nucleotides generates different fragment lengths, separated by electrophoresis and the order of nucleotides can be detected. This method is suitable for sequencing specific genes but laborious and expensive if the aim is to collect large amounts of sequence data. The first complete bacterial genome to be published was *Haemophilus influenzae* in 1995 [230], using Sanger sequencing methods at great effort and cost.

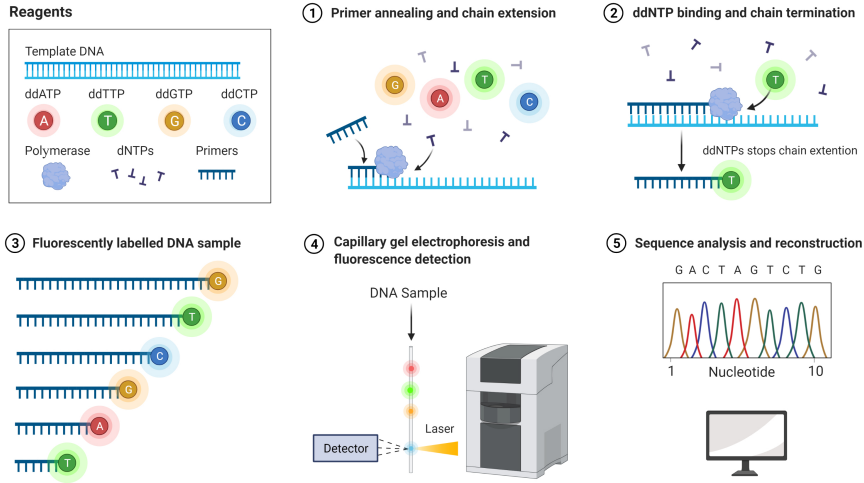


Figure 9. The Sanger sequencing method can determine the DNA sequence of a specific gene or target. The DNA target is amplified using labelled terminating nucleotides (ddNTPs) mixed with standard nucleotides (dNTPs) that generate fragments of different lengths. These fragments are separated by electrophoresis, allowing determination of the sequence. Image created in BioRender.com

Whole-genome sequencing

Sequencing technologies and platforms have undergone dramatic development in the past decades. The introduction of next-generation sequencing transformed the field of molecular biology by offering high-throughput sequencing and with time-efficient and cost-effective methods for whole-genome sequencing (WGS) [231, 232]. Commonly used methods for WGS are the Illumina platforms (Figure 10) which use sequencing of small fragments. Briefly, the genome is analysed through initial genome fragmentation and ligation of specific barcode sequences and adapters to form a sequence library. Sequencing of these fragments is performed using solid-phase bridge amplification with reversible terminating nucleotides labelled with fluorescent dyes. When the correct nucleotide is incorporated, a signal is emitted, detected, and subsequently washed away to incorporate the next nucleotide.

The process is visualised through imaging and interpreted to nucleotide sequences [231]. The generated data can be assembled through various software tools to whole genomes and used in different genomic analyses.

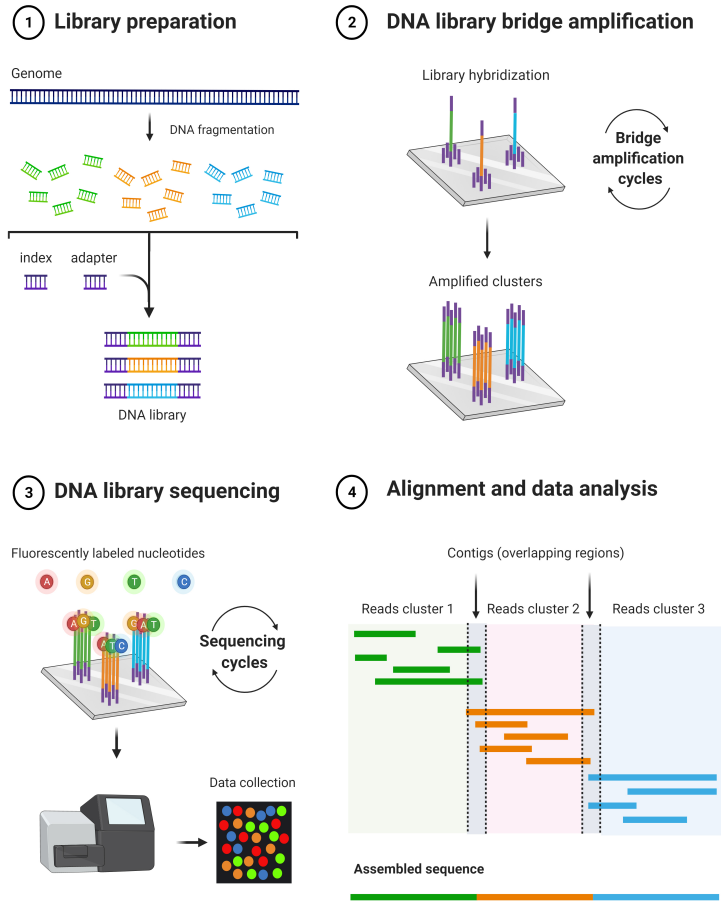


Figure 10. Schematic view of whole-genome sequencing on an Illumina platform. The genome is fragmented and ligated with small barcode sequences (indexes and adapters) in a process called library preparation. These sequences bind to a solid surface and form clusters by bridge amplification. The sequences in every cluster are detected by photography and subsequently assembled into whole genomes in various software tools. Image created in BioRender.com.

Molecular typing schemes for *N. gonorrhoeae*

Several molecular methods have been developed for characterising *N. gonorrhoeae* for epidemiological purposes. Characterisation of isolates increases knowledge, understanding and identification of emerging and circulating strains, enables surveillance of strains with AMR and AMR determinants and informs contact tracing and transmissions of strains in local, national and international settings.

One of the first molecular typing methods for bacterial species was multilocus sequence typing (MLST), initially developed for the molecular typing of *N. meningitidis* [233]. The method consists of the partial sequencing of seven housekeeping genes: *abcZ, adk, aroE, fumC, gdh, pdhC* and *pgm*. The genes are assigned allele types and together generate a sequence type (ST).

Due to frequent horizontal gene transfer and especially suboptimal discriminatory ability in *N. gonorrhoeae*, MLST does not reliably elucidate ancestry between strains collected within a short time [133]. Therefore, it is unsuitable for typing strains that require high resolution, such as in partner notification or outbreaks. For this purpose, another molecular typing method was developed, the *N. gonorrhoeae* multi-antigen sequence typing (NG-MAST) [234]. NG-MAST is based on the partial sequencing of the more variable genes *porB* and *tbpB*, and similar to MLST, the combined sequences (allele types) generate an ST.

To facilitate surveillance of AMR in *N. gonorrhoeae* strains and in response to the need for a uniform molecular nomenclature addressing AMR and AMR determinants, a new typing method was implemented: *N. gonorrhoeae* sequence typing for antimicrobial resistance (NG-STAR) [235]. This typing scheme focuses on AMR determinants related to β-lactam antimicrobial, fluoroquinolone and macrolide resistance through typing of *penA, mtrR, porB, ponA, gyrA, parC* and 23S rRNA. A novel typing method has been developed using the NG-STAR ST and applying it to the eBURST algorithm, used to create MLST clonal complexes (CCs) [236], to create NG-STAR CCs. Briefly, NG-STAR STs are analysed in a minimum spanning tree. The closest founder ST with ≥5 identical alleles and the highest number of links is assigned the CC type. STs with >2 alleles difference are not grouped. The NG-STAR CC has been shown to correspond with genome phylogeny, predict AMR lineages and support gonococcal epidemiological and AMR surveillance [237].

Traditional molecular typing methods use the sequencing of specific genes to describe a strain; however, the shared alleles among strains in the

typing schemes do not always correspond to shared ancestry due to the high genetic exchange in gonococci [108, 238]. On the other hand, WGS provides superior resolution and assessment of emergence, spread and circulating clones as well as relatedness and evolution compared to other strains in close proximity and over time [238]. Surveillance is essential for managing infection and emerging AMR, ideally with clinical, epidemiological and phenotypical data. WGS is therefore an ideal method to support infection and AMR surveillance, as well as short- and long-term molecular epidemiology of *N. gonorrhoeae* AMR [239].

Strategies for management and prevention

Goals leading to improved sexual and reproductive health became a significant WHO priority in 2002 [240]. The exceptionally high prevalence of STIs, combined with AMR development for *N. gonorrhoeae*, illustrates the global problems in sexual and reproductive health, i.e. reducing the global burden of STIs and improving the quality and availability of health services. The systematic development of AMR in *N. gonorrhoeae* to all previously recommended treatments and the limited options for empirical monotherapy heighten the challenge of reducing the high prevalence of infections [9, 23]. The high prevalence of *C. trachomatis* infections, particularly in adolescents, is recognised by WHO. Further research to control the infection is encouraged, particularly as there are similarities in clinical manifestations and coinfection with *N. gonorrhoeae* [9]. WHO has developed and published a global strategy and action plan to control the spread of *N. gonorrhoeae* and mitigate the health impact of AMR through sustainable, evidence-based and collaborative actions. The strategies include the following [9, 241]:

- support healthcare services
- support evidence-based preventive interventions
- increase awareness on antibiotic usage
- recommend adequate diagnosis and treatment
- strengthen AMR surveillance and monitor treatment failure
- support laboratories to perform good quality culture of *N. gonorrhoeae*
- support development of new molecular methods for surveillance and detection of AMR
- support development of alternative treatment options
- support vaccine research

Regular and systematic surveillance of the spread of *N. gonorrhoeae* and AMR is essential for early detection of emerging AMR and crucial for decision making by providing a knowledge base and informing national and international guidelines. Sensitive and specific molecular AMR assays could be performed routinely and may significantly improve detection and surveillance of AMR [241], ultimately enabling individualised treatment. These strategies by WHO aim to help international, national and local healthcare to prevent infection, disease and promote public health. The Swedish strategy to prevent STIs includes sex education, dissemination of knowledge, access to STI clinics, testing and treatment, sexual contact tracing, and counselling without any cost for the patient. Identification of groups at higher risk of contracting STIs, such as adolescents, MSM, commercial sex workers and, in some settings, people with foreign origins, is crucial for directed preventive actions [242].

Aims

The general aim was to implement WHO strategies for managing and preventing *C. trachomatis* and *N. gonorrhoeae* infections by vaccine development, supporting and contributing to surveillance of *N. gonorrhoeae* spread and AMR. Specific aims for the individual studies were as follows:

I. Evaluate the protective immunity of a recombinant MOMP VS2/4 vaccine candidate through intranasal (i.n.) administration against genital *C. trachomatis* infection in mice.

II. Investigate the prevalence and polymorphisms of nucleotide and amino acid sequences of antigens included in the 4CmenB meningococcal group B vaccine, i.e. *fHbp, nhba, gna1020, gna2091* and *nadA*, in *N. gonorrhoeae*.

III. Evaluate molecular assays for prediction of *N. gonorrhoeae* phenotypic resistance/susceptibility to ciprofloxacin using the SpeeDx *ResistancePlus*® GC assay and azithromycin using the SpeeDx GC 23S 2611 (beta) assay.

IV. Perform a national surveillance study by examining all cultured *N. gonorrhoeae* isolates in Sweden during 2016 by WGS in conjunction with phenotypic AMR and epidemiological data of patients.

Materials and methods

Material and methods used in paper I

Culture and purification of *C. trachomatis* elementary bodies

A human genital tract clinical isolate of *C. trachomatis* serovar D was cultured on a confluent cell layer of McCoy cells for 2-3 days. Harvested cells were lysed and Ebs were purified in a density gradient solution and recultured to determine the concentration of inclusion forming units (IFUs).

Protein production

The recombinant vaccine candidate MOMP VS2/4, originating from *C. trachomatis* serovar E, was cloned and expressed in *E. coli* and subsequently used for immunisations, detection of antibodies and restimulations of T cells. The amino acid sequence of the antigen is marked in Figure 11. *E. coli* carrying the MOMP VS2/4 nucleotide sequence were cultured and induced to produce the recombinant protein during the exponential phase. The *E. coli* cells were frozen in liquid nitrogen and the protein was extracted by hydraulic pressure and sonication that disrupted the cells. The protein construct was subsequently purified using histidine residues (later removed) and size exclusion chromatography.

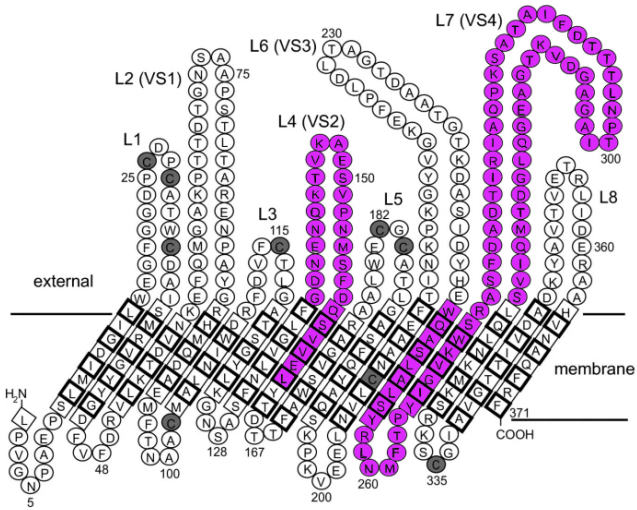


Figure 11. Model of the Chlamydia trachomatis major outer membrane protein (MOMP) where the coloured amino acids depict the sequence of the recombinant vaccine construct MOMP VS2/4. Residues in the transmembrane strands are boxed; bold borders indicate side chains facing the bilayer; and cysteine residues are shaded [153]. Modified image reprinted with permission of Springer Nature.

Immunisation and challenge in a mouse model

Female C57BL/6 mice, 6-8 weeks old, were used and contained under pathogen-free conditions at the Department of Experimental Biomedicine at Gothenburg University, Sweden. The mice (n=10) were immunised by i.n. administration three times with the MOMP VS2/4 antigen construct with 7-10 day intervals and boosted once with the same antigen through intravaginal (i.vag.) administration. Together with the antigen MOMP VS2/4 antigen, the cholera toxin (CT) adjuvant was administered. However, in some experiments, the mice were boosted i.vag. with antigen alone. To test the immunogenic properties of the MOMP VS2/4 construct on a different major histocompatibility complex (MHC), C3H/HeN mice were used with the same immunisation protocol as above. For the analysis of the cytokine

response, mice (n=3) were immunised twice i.n. with CTA1-DD as an adjuvant (cholera toxin subunit A1 coupled with a dimer of the immunoglobulin-binding D region) [211], chosen due to its similarities to CT and to show the effect of a non-toxic adjuvant together with MOMP VS2/4. All mice were treated with medroxyprogesterone subcutaneously 7 days before i.vag. boost or infection. The infectious dose was 3×10^4 IFUs of *C. trachomatis* EBs 2-3 weeks after the last immunisation. The timeline is illustrated in Figure 12.

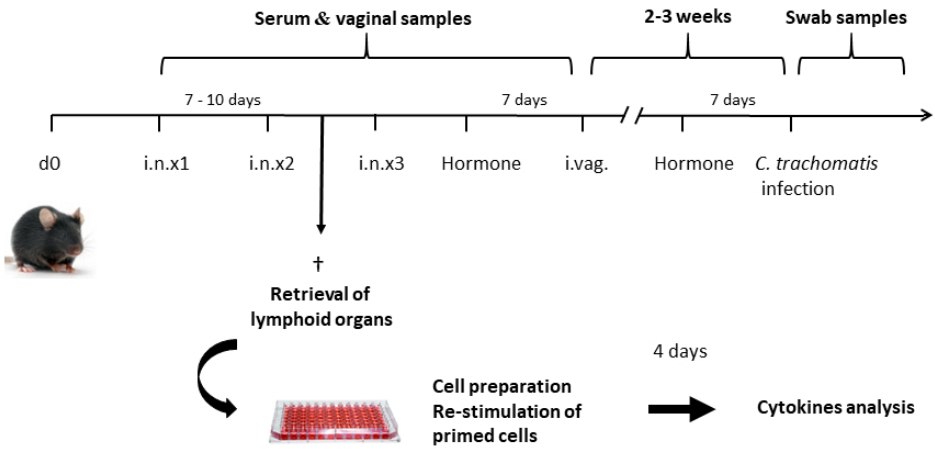


Figure 12. Timeline scheme used in paper I for immunisation, infection challenge of C57BL/6 mice and sampling. Abbreviations: i.n., intranasal; i.vag., intravaginal.

Analysis of immune response, challenge and fertility

The antibody responses (IgG, IgG subclasses, IgA) in serum and vaginal washes were analysed using the MOMP VS2/4 antigen. Briefly, MOMP VS2/4 protein was coated onto microwell plates and unspecific binding was blocked with bovine serum albumin. Serum and vaginal washes were added to allow attachment to the antigen, followed by enzyme-labelled secondary antibodies and a substrate to enable measurement using a spectrophotometer. Bacterial shedding was monitored by a similar method using a commercial kit. Regular swab samples were collected at 3-day intervals starting at day 4 post-infection. *C. trachomatis* was detected using species-specific antibodies to lipopolysaccharides coated on microwell plates. The antibody-antigen complex was detected by enzyme-labelled antibodies and measured using enzymatic degradation of a substrate.

Two weeks after the mice had tested negative for *C. trachomatis* infection twice, the fertility of the mice was evaluated. A mating male was introduced to a group of five females for up to 3 weeks. Three days of consistent weight gain was interpreted as a successful pregnancy and the pregnant mice were removed from the breeding cages. Mice that did not show signs of pregnancy were mated with another male for up to 3 weeks. Pregnant mice were euthanised and the number of implantations in the uterine horns was counted.

For cytokine response to MOMP VS2/4, a separate group of mice (n=3) was used. These mice were euthanised 7 days after the last immunisation and spleen and mediastinal lymph nodes were collected, homogenised and the cells were seeded into culture plates. The MOMP VS2/4 antigen and negative controls were added before incubation. The supernatant was removed and interferon-γ (IFNγ), interleukin (IL) 13, and 17 were analysed.

Material and methods used in papers II-IV

Isolates

For paper II, 103 clinical *N. gonorrhoeae* isolates from 28 countries, eight WHO 2008 *N. gonorrhoeae* reference strains [243] and the meningococcal reference serogroup B strain MC58, were included. The selected *N. gonorrhoeae* isolates represented all world continents and as many phenotypes as possible.

For paper III, previously WGS *N. gonorrhoeae* isolates from 20 European countries [74] and clinical *N. gonorrhoeae* NAAT positive samples (one sample per patient and episode), collected from November 2014 to May 2018, were included. To further evaluate specificity, *N. gonorrhoeae* NAAT negative samples, isolates from eight non-pathogenic commensal *Neisseria* species, closely related *Moraxella* species and *N. meningitidis* reference strains (serogroups A, B, C, W, X and Y) were included. The clinical samples were analysed using the TMA assay Aptima Combo 2 (AC2; Hologic, Inc) and confirmed using culture or another target in the Aptima GC assay (n=106). An overview of all the samples is summarised in Table I.

All cultured clinical *N. gonorrhoeae* isolates (n=1279, one per patient and reported case) in Sweden in 2016 were included for paper IV. Patients with multiple gonorrhoea episodes were treated as separate cases if the infections were >3 weeks apart. When multiple isolates from the same gonorrhoea episode were available, isolates from extragenital sites were prioritised.

All isolates in papers II-III were collected at the Department of Laboratory Medicine, Clinical Microbiology, Örebro University Hospital, and additionally for paper IV, from the Departments of Clinical Microbiology at Karolinska University Hospital and Skåne University Hospital. All isolates were cultured on non-selective gonococcal agar plates overnight at 37°C in a humid 5% CO_2-enriched atmosphere.

Antimicrobial susceptibility testing

Phenotypic AMR testing was performed using Etest for ciprofloxacin and azithromycin (papers III and IV) and additionally ceftriaxone, cefixime and spectinomycin (paper IV). Antimicrobial MIC was interpreted according to the clinical breakpoints recommended for *N. gonorrhoeae* in 2019 by the European Committee on Antimicrobial Susceptibility Testing (EUCAST; www.eucast.org/clinical_breakpoints), where available. Azithromycin lacks clinical breakpoints and therefore the epidemiological cut-off value of MIC >1 mg/mL was used to indicate isolates with azithromycin resistance mechanisms (hereafter referred to as resistant). The same EUCAST 2019 *N. gonorrhoeae* breakpoints were applied to non-pathogenic *Neisseria* species.

Table I. *Overview of the number of isolates and samples included in paper III. The bacterial isolates include previously whole-genome sequenced (WGS) Neisseria gonorrhoeae isolates, commensal isolates, and Neisseria meningitidis reference strains. Additional clinical N. gonorrhoeae positive samples, with and without confirmed culture, and N. gonorrhoeae negative samples diagnosed using the nucleic acid amplification test (NAAT) Aptima Combo 2 (AC2) assay (Hologic, Inc).*

	Samples	Ciprofloxacin			Azithromycin	
		S	I	R	S	R
***N. gonorrhoeae* isolates**						
WGS isolates	967*	446	1	520	951	13
Commensal isolates	137					
N. flavescens	52	21	12	19	1	51
N. perflava	27	12	4	11	1	26
N. macacae	11		2	9		11
N. mucosa	5	2	2	1		5
N. sicca	3		1	2		3
N. cinerea	2			2	1	1
N. animalis	1			1		1
Neisseria species	34	7	16	11	2	32
M. osloensis	1		1		1	
M. catarrhalis	1		1		1	
N. meningitidis	6	6			6	
Clinical NAAT AC2 samples						
Confirmed culture	37	17		20	36	1
No confirmed culture	106					
Negative	167					

Abbreviations: S, susceptibility; I, intermediate; R, resistant; WGS, whole-genome sequenced; NAAT, nucleic acid amplification test; AC2, Aptima Combo 2. * 964 isolates included for azithromycin.

DNA extraction

All isolates in paper III were extracted from single colonies or cryobeads by boiling, followed by centrifugation. DNA isolation was performed on MagNA Pure systems in papers II and III (clinical AC2 samples) and QiaSymphony platform for some indeterminate results in paper III and all isolates in paper IV. Both extraction systems are silica-based purification methods using magnetic beads.

PCR

PCR was performed in papers II and III. The genes included in the 4CMenB vaccine antigen (paper II), i.e. the *fHbp*, *nhba*, *gna1030*, *gna2091* and *nadA* genes, were amplified using real-time or conventional PCR. These genes were then purified using spin column purification before sequencing. *In silico* PCR was performed for different *N. meningitidis nadA* in all available *N. gonorrhoeae* genome sequences. All isolates and clinical AC2 samples (paper III) were analysed in *ResistancePlus*® GC assay and GC 23S 2611 (beta) assay (hereafter called C2611 assay), combining detection of *N. gonorrhoeae* and AMR determinant or WT in one assay. The *ResistancePlus*® GC assay targets the *opa*, *porA* pseudogene for *N. gonorrhoeae* detection, GyrA S91 WT mutation for ciprofloxacin susceptibility, GyrA S91F mutation for resistance and an internal control. The C2611 assay targets the *porA* pseudogene for *N. gonorrhoeae* detection, 23S rRNA C2611 WT and C2611T mutation for azithromycin susceptibility and resistance, respectively. All *N. gonorrhoeae* positive AC2 samples and cross-reactive non-gonococcal *Neisseria* isolates in the ResistancePlus® GC assay were further analysed using an in-house *N. gonorrhoeae* GyrA S91 WT and S91F PCR [244].

Sequencing

In paper II, all sequences were determined using Sanger sequencing. In paper IV, WGS of *N. gonorrhoeae* genomic DNA was performed on an Illumina HiSeq X platform using paired-end sequencing with 50-100X coverage.

Bioinformatic analysis

Paper II analyzed nucleotide sequences and the deduced corresponding amino acid sequences in multiple sequence alignments using BioEdit software (v7.0.9.0) with manual adjustments. The phylogenetic analyses of the nucleotide and amino acid sequences of the mature proteins were performed with TREECON software (v1.3b).

For paper IV, sequencing data were analysed using a customised workflow in CLC Genomic Workbench, which includes quality control, *de novo* assembly and subsequent genetic characterisation of AMR determinants. Reads were also mapped to the reference genome before molecular characterisation. Furthermore, the mapped reads were used to create a multiple sequence alignment for phylogenomic analysis. Recombination events in the alignments were removed before creating the phylogenomic tree. The tree was midpoint rooted and visualised through a web-based application (microreact) [245].

Typing methods

N. gonorrhoeae molecular typing schemes were used to characterise isolates: NG-MAST in paper II and MLST, NG-MAST, NG-STAR and NG-STAR CC in paper IV. Phenotypic characterisation of clinical isolates was performed using antimicrobial susceptibility testing in papers II-IV, including PorB serovar determination in paper II.

Statistics

In paper I, statistical analysis of infectious burden was calculated by logistic regression with 95% confidence interval (CI). The Mann-Whitney test was computed to determine differences in cytokine response and the Kruskal-Wallis test to detect differences in fertility rate (between un-immunised and immunised groups).

For paper IV, the correlation between *N. gonorrhoeae* isolates (NG-STAR CC and phenotypic AMR) and epidemiological data was calculated using logistic regression with a 95% CI and odds ratio. Pearson's $\chi 2$ test was used to calculate significance on the most common STs and epidemiological and phenotypic AMR.

Ethical considerations

Paper I was performed in accordance with the guidelines of the Federation for Laboratory Animal Science Association. The Animal Ethics Committee of Gothenburg granted the laboratory, in which the mice experiments were performed, a collective ethical approval (permit number N146/12) in which paper I was included. Studies II and III were performed on bacterial isolates collected in routine diagnostics (standard care) without patient identification and ethical approval was not required. Ethical approval for paper IV was given by the Swedish Ethical Review Authority in Uppsala (approval number 2020-05008).

Results and discussions

Implementation of WHO strategies (papers I-IV)

The studies in papers I-IV have successfully implemented the WHO strategies of supporting the development of vaccine research (papers I-II), development of new molecular methods for surveillance (paper III), strengthening AMR surveillance (III-IV) and supporting healthcare services and evidence-based preventive interventions (paper IV). The results of these implementations are presented and discussed below.

Vaccine research (papers I-II)

Vaccine candidate for the prevention of *C. trachomatis* (paper I)

The MOMP VS2/4 construct, together with CT adjuvant, effectively generated MOMP-specific antibodies in serum (and vaginal washes, Figures 13A and B) in C57BL/6 mice. Antigen-specific IgA and substantial IgG antibodies were noted in vaginal washes. In addition, IgG subclasses (IgG1 and IgG2c) were analysed in serum and showed a strong IgG2c presence, indicating a more potent T helper (Th) 1 response by the immunisations. Additionally, the MOMP VS2/4 antigen also immunised C3H mice, showing that the antigen was similarly immunogenic when immunising mice expressing a different MHC.

The role of antibodies in *C. trachomatis* protective immunity is still not fully understood. Some studies suggest a lack of protective properties of human antibodies generated by *C. trachomatis* infection to reinfection and endometrial ascension [246-248]. Nevertheless, vaccine candidates have been shown to generate *in vitro* neutralising antibodies [249, 250] and the transfer of neutralising antibodies to knock-out mice lacking B and T cells, induced protection [251].

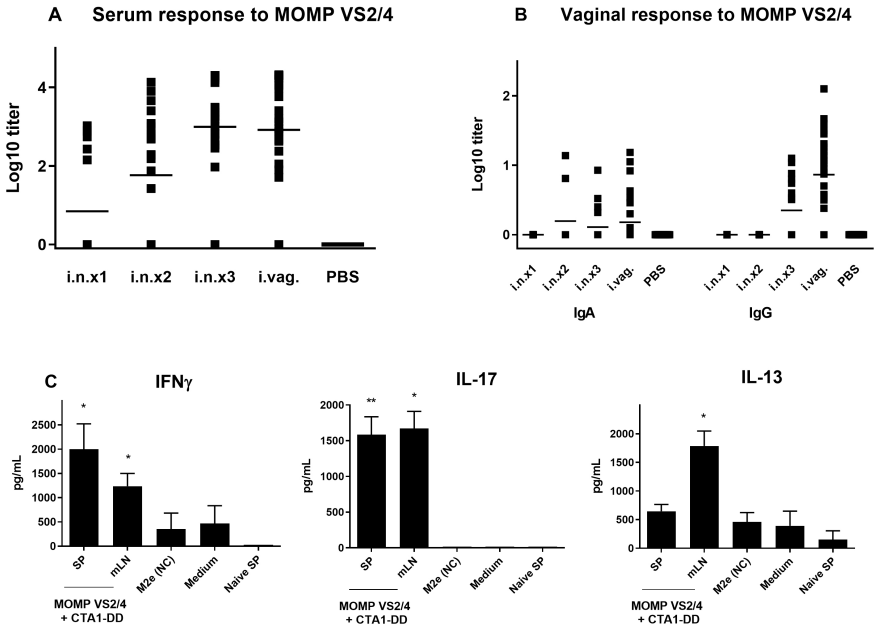


*Figure 13. IgG antibody immune response in serum (A) and vaginal washes (B) against the MOMP VS2/4 vaccine candidate following immunisations in C57BL/6 mice. Mice were immunised intranasally (i.n.) three times and intravaginally (i.vag.) once using cholera toxin as an adjuvant. In the last repeat experiment, the i.vag. boost was administered with antigen only. Analysis of cytokine production (C) performed on mice with two immunisations i.n. using cholera toxin subunit A1 (CTA1-DD) as an adjuvant. Concentrations of interferon-gamma (IFNγ), interleukin (IL)-17 and IL-13 in the spleen (SP) and mediastinal lymph node (mLN) are shown. Influenza antigen M2e and culture medium represent negative controls. Significance was analysed using the Mann-Whitney test, *p<0.05 and **p<0.01 (paper I).*

Immunity was further assessed by analysing the cytokine response. The MOMP VS2/4 antigen-stimulated lymphocytes isolated from the spleen and mediastinal lymph node produced significant levels of IFNγ, IL-13 and IL-17 (Figure 13C), indicative of Th1, Th2 and Th17 immune response, respectively. IFNγ has a central role in clearance and protection from *C. trachomatis*, as shown in both human and murine models [252-255]. Of note, reducing infectious load may not correlate to the prevention of immunopathology. A study performed with *C. muridarum* challenge in the respiratory tract on immunised mice showed that (i) immunopathology was generated independently of bacterial load and (ii) the CTA1-DD adjuvant was associated with reduction of immunopathology. This finding suggests the development may be independent of bacterial load but driven by the immune response [256]. However, more studies on protective immunity are needed to define the underlying mechanisms of *C. trachomatis* pathology, particularly in humans.

The recombinant antigen MOMP VS2/4, designed with a flexible amino acid sequence to facilitate antigen presentation and soluble for production and purification ease [257], clearly stimulates the humoral and adaptive immune responses. The MOMP protein is known for its immunogenicity and has been the subject of many vaccine candidates, mainly subunit proteins [258]. The most successful vaccine candidates to date have been derivatives of the multivalent VS4 domain with similar sequences as the VS4 sequence of MOMP VS2/4, including the non-conformational and conserved epitope LNPITIAG [197, 198]. Although the antibodies generated by MOMP VS2/4 have not been proven to be protective, the findings are comparable with studies on multivalent VS4 vaccine candidates in mouse models showing cross-protection to other serovars, reduced infectivity in early stages of *C. trachomatis* infection and protection against pathology [198].

Significant protection from *C. trachomatis* infection was observed in immunised mice when challenged with *C. trachomatis* serovar D, especially in the early stages of the infection. To assess the protection from pathology, the fertility level was observed by introducing a male mouse following clearance of the infection challenge. All immunised mice achieved pregnancy compared to 60% in the un-immunised group. Moreover, the immunised group showed a significantly higher fertility rate ($p<0.05$), i.e. number of implantations per pregnant mouse, than the un-immunised group after clearance of infection. A vaccine should primarily prevent adverse outcomes caused by *C. trachomatis* infection but ideally with simultaneous reduction

of infectious burden and reproductive tract pathology, ultimately preventing both transmission and adverse outcomes. Results from the MOMP VS2/4 immunised mice showed reductions in bacterial load in early infection and infertility. Encouragingly, other studies using the MOMP subunit antigen have found similar results with strong IFNγ, IL-17, serum IgG and local IgA production in a minipig model [249, 259] using intramuscular, subcutaneous and i.n. administration with significant protection after *C. trachomatis* challenge. The MOMP VS2/4 antigen, based on serovar E MOMP sequence, conferred protection against serovar D, demonstrating cross-protective properties. These cross-protective properties are most likely due to similarities in sequences between the serovars. Cross-protection to other serovars warrants further investigation. The novel MOMP VS2/4 vaccine construct appears to be a promising *C. trachomatis* vaccine candidate.

Vaccine research for the prevention of *N. gonorrhoeae* (paper II)

All genes coding for 4CMenB antigens, except *nadA*, were present in all clinical *N. gonorrhoeae* isolates and the WHO reference strains. The prevalence and polymorphisms of the remaining genes and amino acid sequences within the *N. gonorrhoeae* population and compared to the *N. meningitidis* reference strain MC58 are summarised in Table II. Single nucleotide insertions causing frame shifts in the open reading frame were seen in fHbp (n=9) and NHBA (n=1), in addition to premature stop codons in NHBA (n=17) and GNA1030 (n=4). Both the insertions and premature stop codons caused truncated proteins. When excluding isolates with truncated sequences, the isolates showed a high identity in the nucleotide and amino acid sequences with three fHbp, 18 NHBA, four GNA1030 and two GNA2091 amino acid sequence types (Figures 14 and 15). However, compared to *N. meningitidis*, a considerable decrease in sequence identity to the corresponding meningococcal sequences was observed, particularly for fHbp and NHBA. The high homology in fHbp sequences in *N. gonorrhoeae* may be partly due to the location of fHbp. In *N. meningitidis*, the fHbp is a surface-exposed protein that binds host complement factor H [260]. All the gonococcal fHbp sequences had differences in the N-terminal signalling peptide, both the lipidation signal and partial differences in the hydrophobic region (Figure 14). These alterations indicate that the *N. gonorrhoeae* fHbp may not be surface-exposed, which was later confirmed in addition to lack of binding to human factor H [261]. Even so, several epitopes recognised by monoclonal antibodies against *N. meningitidis* fHbp variant 1 are conserved, specifically

D25-K27 [262], A174 and K180 [263]. However, the epitopes E192, S216 [262, 263], G120, K121, E146-R149 [264], L36-L48, I82-L88 and T107-M123 [265] differed compared to *N. gonorrhoeae* (Figure 14), although conserved within the population.

Table II. *Prevalence and polymorphisms of the nucleic acid and deduced amino acid sequences of the genome-derived antigens (GNAs) of the 4CMenB vaccine, factor H binding protein (fHbp), neisserial heparin-binding protein (nhba), gna1030 and gna2091. The results are compared to other Neisseria gonorrhoeae isolates and Neisseria meningitides (Nm) (paper II).*

Gene	Isolates (%)	Alignment (bp)	Nucleic acid (amino acid) polymorphic sites	Sequence identity		Alleles gene/protein	Mutations causing Truncated proteins (no of isolates)
				Nucleic acid %	Amino acid %		
fHbp	102 (91.9)	837	2 (2)	99.8	99.3	3/3	G insertion (9)
fHbp_Nm		843	278 (110)	67.0	60.9		
nhba	93 (83.8)	1290	91 (42)	92.9	90.2	18/18	T insertion (1)
nhba_Nm		1494	437 (170)	70.7	65.9		Premature stop codons at amino acid positions 200-300 (17)
gna1030	107 (96.4)	558	4 (3)	99.3	98.4	5/4	Premature stop codons at amino acid positions 116 (3) and 50 (1)
gna1030_Nm		561	37 (15)	93.4	92.0		
gna2091	111 (100)	591	3 (1)	99.5	99.5	4/2	
gna2091_Nm		591	27 (10)	99.4	94.9		

Abbreviations: bp, base pairs; fHbp, factor H binding protein; Nm, *Neisseria meningitidis*; nhba, neisserial heparin-binding protein.

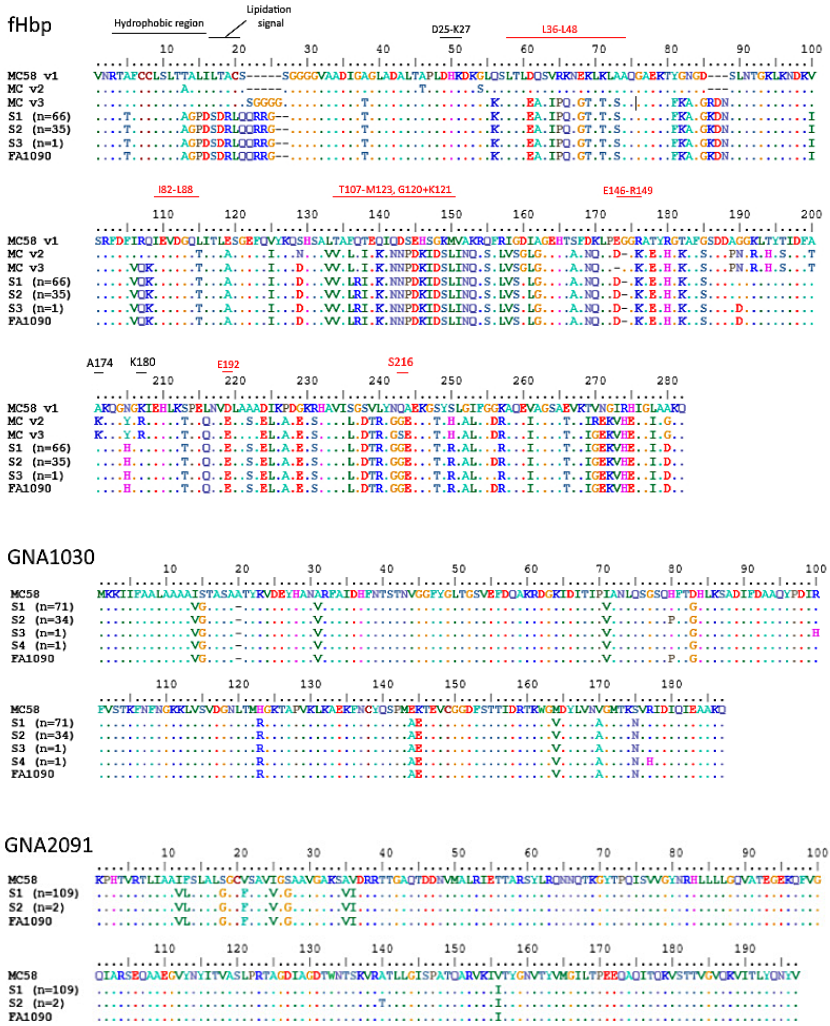


Figure 14. Amino acid sequence types of Neisseria gonorrhoeae factor H binding protein (fHbp), genome-derived neisserial antigen (GNA) 1030 and GNA2091 compared to Neisseria meningitidis reference strain MC58. All alignments include N. gonorrhoeae reference strain FA1090 and, for the fHbp alignment, the meningococcal fHbp variant 2 and 3 sequence types (MC v2 and v3). Known N. meningitidis epitopes compared to the gonococcal sequences are in black (conserved) and red (mismatch) [261-265]. The modified image is reprinted with permission from John Wiley and Sons.

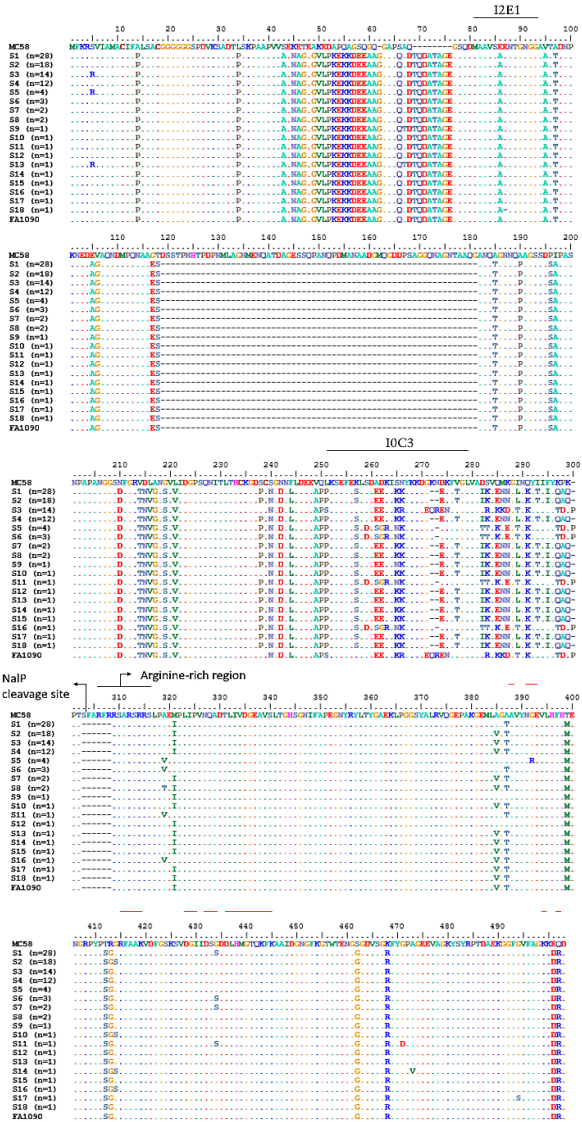


Figure 15. *Amino acid sequence types of neisserial heparin-binding antigen (NHBA) in Neisseria gonorrhoeae isolates compared to Neisseria meningitidis reference strain MC58, including N. gonorrhoeae reference strain FA1090. Known meningococcal epitopes, the NalP cleavage site and the arginine-rich region are marked in black and a conformational epitope is marked by red lines [266-268]. The modified image is reprinted with permission from John Wiley and Sons.*

In recent years, a large number of *N. gonorrhoeae* genomes have been made public and bioinformatic studies show highly conserved 4CMenB GNA genes within *N. gonorrhoeae* isolates and the absence of *nadA* [269, 270]. The *nhba* gene in *N. gonorrhoeae* shows very high conservation and has almost one-tenth of variants compared to *N. meningitidis nhba* [269]. The NHBA protein in *N. meningitidis* has been named for its ability to bind heparin through an arginine-rich domain and increase serum resistance. This process is achieved through proteases, both human lactoferrin and meningococcal NalP, which cleave and release a fragment of NHBA [188]. Although the *nalP* protease gene and the cleavage site in the *N. gonorrhoeae* NHBA sequences are missing, the protein has been shown to bind glycans, including heparin [268]. Recent studies show involvement in adherence to epithelial cells, microcolony formation and serum resistance [268] and also high immunogenic properties by induceing serum bactericidal and opsonophagocytic activity [180]. The generated gonococcal NHBA antibodies reduce the ability to bind heparin by 90% and *in vitro* adherence to cervical and urethral epithelial cells [180]. Monoclonal antibodies from human 4CMenB vaccinations bind mapped epitopes on meningococcal NHBA (I2E1, I0C3 and conformational epitope 5H2, Figure 15) [266, 267]. These epitopes are also present in the *N. gonorrhoeae* isolates, though not fully conserved compared to *N. meningitidis*, and cross-reaction of these antibodies is uncertain. Encouragingly, serum from Bexsero-vaccinated humans show cross-reaction to *N. gonorrhoeae* whole cell lysates, OMVs and gonococcal recombinant NHBA [269]. A similar study, performed in mice, showed that administration of meningococcal 4CMenB vaccine reduced the bacterial burden, accelerated the clearance of *N. gonorrhoeae* infection and that serum IgG and vaginal IgA and IgG recognised several proteins on gonococcal OMVs [271]. Additionally, meningococcal OMVs lacking major outer membrane proteins, such as PorA and PorB, showed enhanced clearance of *N. gonorrhoeae* in mice [272]. These studies show the potential of gonococcal NHBA and OMVs as promising vaccine candidates. Observations from the population-wide vaccination in New Zealand with meningococcal MeNZB, with subsequent reduction in gonorrhoea cases, showed that the effectiveness of MeNZB vaccinations against gonorrhoea was only 31% [185]. However, using modelling simulations, a vaccine with low effectiveness may still substantially impact gonorrhoea prevalence depending on vaccination coverage and duration of protection [273].

Surveillance of AMR and AMR determinants (papers III-IV)

ResistancePlus® GC assay for ciprofloxacin resistance prediction (paper III)

The transition from culture to molecular assays for detecting *N. gonorrhoeae* is becoming more common and with it the loss of phenotypic AMR determination and the collection of viable isolates. Molecular assays for the prediction of phenotypic AMR, based on detection of known AMR determinants, are needed and could support AMR surveillance and individualised treatment. However, with the high similarity between *Neisseria* species [106, 107] and the prevalence of commensal *Neisseria* species at the site of infection, particularly at extragenital sites such as the pharynx, it is essential that these assays are properly evaluated and quality assured.

The ResistancePlus® GC assay, a commercial assay for predicting ciprofloxacin resistance, correctly detected all *N. gonorrhoeae* isolates and *N. gonorrhoeae* positive clinical AC2 NAAT samples except two (one urine sample and one rectal sample missed). Moreover, the *N. gonorrhoeae* negative result was correctly called in all non-gonococcal isolates except two and in all *N. gonorrhoeae* negative clinical AC2 samples (two were invalid and excluded from calculations). The overall sensitivity was 98.6% and specificity 100% (Table III) for detecting *N. gonorrhoeae* by ResistancePlus® GC assay compared to the AC2 assay used in routine diagnostics.

The ResistancePlus® GC assay correctly identified the GyrA S91 WT and GyrA S91F AMR determinant in all previously WGS isolates and 131 (92.9%) of 141 *N. gonorrhoeae*-positive clinical AC2 samples showed concordant results with the in-house *gyrA* PCR [244]. The positive and negative per cent agreement (indeterminate results excluded, n=9) in the detection of GyrA S91F was 98.2% for ResistancePlus® GC assay and 100% for the in-house *gyrA* PCR assay [244].

The GyrA S91 WT signal was visually observed in 4.8% of *N. gonorrhoeae*-negative clinical AC2 samples from the pharynx and the GyrA S91F and GyrA S91 WT signal was observed in several non-gonococcal *Neisseria* species, where two isolates had simultaneous cross-reaction to *N. gonorrhoeae* (*opa* signal) and GyrA S91 WT. Although these samples and most isolates were correctly reported as *N. gonorrhoeae* negative, it displays a concern in the analysis of clinical samples, particularly in pharyngeal samples, as to whether the results reflect the pathogen or commensal bacteria. Pharyngeal sites are particularly challenging in developing molecular assays for *N. gonorrhoeae* due to the presence of non-gonococcal species. The

Neisseria species share high genome similarity [106, 107] and the cross-reaction of non-gonococcal species in the pharyngeal samples underscores the challenges with molecular prediction of AMR in *N. gonorrhoeae*. The high bacterial load of the examined non-gonococcal *Neisseria* isolates does not reflect clinically relevant concentrations, and it may have affected the cross-reaction seen in our evaluation. However, the cross-reactions are comparable to previous studies in non-gonococcal isolates and pharyngeal samples [274] in addition to higher *gyrA* indeterminate results at anorectal and pharyngeal sites [275].

The sensitivity and specificity (Table III) for the ResistancePlus® GC assay to predict phenotypic ciprofloxacin resistance was 99.8%. The high sensitivity and specificity support using ResistancePlus® GC assay for AMR surveillance and individualised treatment, especially in urogenital infections. Depending on the local prevalence of ciprofloxacin resistance, cost of the assay, test frequency and the number of tests that can be evaluated [276], an assay that predicts the success of ciprofloxacin treatment may be a cost-effective alternative to empirical treatment. Ciprofloxacin is easily administrated in a single oral dose. Moreover, alternatives to empirical treatment would reduce the unnecessary use of ceftriaxone and azithromycin and limit the opportunity for further AMR development against these antimicrobials.

Table III. The sensitivity and specificity of two SpeeDx assays for detection of Neisseria gonorrhoeae, prediction of phenotypic resistance and antimicrobial resistance markers. ResistancePlus® GC assay includes the GyrA S91F target for predicting phenotypic ciprofloxacin resistance and the GC 23S 2611 (beta) assay (C2611 assay) consists of 23S rRNA C2611T target for prediction of phenotypic azithromycin resistance.

	Sensitivity (%)	Specificity (%)
ResistancePlus® GC assay		
N. gonorrhoeae detection	98.6	100
Predict ciprofloxacin resistance	99.8	99.8
C2611 assay		
N. gonorrhoeae detection	95.8	100
Predict azithromycin resistance	64.3	99.9
C2611T detection	100	99.9

C2611 assay for azithromycin resistance prediction (paper III)

The C2611 assay correctly detected all but one *N. gonorrhoeae* isolate and 137 of 143 *N. gonorrhoeae*-positive clinical AC2 samples (three rectal, two urine and one pharyngeal samples were missed). The C2611 assay also correctly called all *N. gonorrhoeae*-negative clinical AC2 samples and non-gonococcal *Neisseria* species as *N. gonorrhoeae* negative. The sensitivity for detecting *N. gonorrhoeae* was 95.8% and the specificity was 100% compared to the AC2 assay used in routine diagnostics (Table III). The C2611 assay remains under development and, unlike the ResistancePlus® GC assay, does not include an internal control or the *opa* gene for *N. gonorrhoeae* detection. In previously WGS *N. gonorrhoeae* isolates [74], the C2611 assay showed 100% concordance in detecting the 23S rRNA C2611 WT and 88.9% concordance in detecting the C2611T mutation.

A substantial proportion (47.6%) of non-gonococcal *Neisseria* isolates and most *N. gonorrhoeae*-negative clinical AC2 samples from the pharynx showed a C2611 WT signal. The detection of C2611 targets in non-gonococcal *Neisseria* isolates may reflect a high bacterial load. However, similar findings have been observed in extragenital samples in other assays [277, 278] with cross-reaction to C2611 WT. The C2611 assay also showed dual amplifications of C2611 WT and C2611T targets in *N. gonorrhoeae*-positive clinical AC2 samples and non-gonococcal *Neisseria* species. This dual amplification could be correct as the 23S rRNA gene is present in four copies and only a partial number of these alleles may be mutated [114]. These aspects underline the importance of using appropriate analysis software to interpret the amplification.

Compared to available data on phenotypic AMR testing, the sensitivity of the C2611 assay to predict phenotypic azithromycin resistance by detecting the 23S rRNA C2611T mutation was 64.3% and specificity 99.9% (Table III). However, the sensitivity of the C2611 assay to detect the 23S rRNA mutation C2611T was 100% and specificity was 99.9%.

The main limitation of the C2611 assay for the prediction of azithromycin resistance is that several other AMR determinants may cause resistance to azithromycin, including the 23S rRNA A2059G mutation [114], rRNA methylase (*erm*) genes [114, 279], overexpressed MacAB, and especially, MtrCDE efflux pump (including different *mtrR* mutations and mosaic alleles) [22, 132]. The multiple mechanisms by which *N. gonorrhoeae* acquire antimicrobial resistance represent an inherent limitation for many molecular assays. A multi-target assay may be required to predict azithromycin

resistance accurately. The C2611 assay shows promising potential and encourages further development (e.g., by adding additional AMR targets) of molecular assays to predict antimicrobial susceptibility (or resistance) to aid AMR surveillance and individualised treatment.

AMR in *N. gonorrhoeae* isolates (paper IV)

The first national genomic *N. gonorrhoeae* study, including nearly all viable isolates diagnosed in Sweden in 2016, was performed to fully describe the gonococcal population and prevalence of AMR and AMR determinants in Sweden. All regions in Sweden but one (Västerbotten) were represented. No resistance to ceftriaxone or spectinomycin was found and there was a low prevalence of cefixime (1.7% of isolates) and azithromycin resistance (1.3% of isolates). Ciprofloxacin resistance had a prevalence of 51.1%. A decreased susceptibility to ceftriaxone and cefixime was observed in 9.3% and 10.5% of isolates, respectively. The results of the susceptibility testing and the main molecular AMR determinants are summarised in Figure 16. In 2015-2019, no ceftriaxone resistance was reported in the annual Swedish surveillance of AMR; however, cefixime resistance decreased from 2.0% in 2015 to 1.0% in 2019. In contrast, the prevalence of azithromycin resistance increased from 10.0 in 2015 to 12.0% in 2019 [280]. Similar to Swedish data, the 2019 prevalence in the European Union/European Economic Area (EU/EEA) of ceftriaxone resistance has remained low at 0.1%. The cefixime resistance prevalence has decreased to 0.9% and the azithromycin resistance prevalence has increased to 10.1% of isolates [281]. The decrease of AMR prevalence may reflect the use of recommended antimicrobial treatment. The recommended dual therapy for gonorrhoea treatment is implemented to inhibit the development of AMR primarily to ESCs.

A significant association was found in Sweden between decreased susceptibility to ceftriaxone and the 16-44-year age groups and between resistance and decreased susceptibility to cefixime and the age groups 16-24 and 35-44 years. Ciprofloxacin resistance was significantly associated with heterosexual transmissions and ciprofloxacin susceptibility with the 16-35-year age groups, MSM and domestic infections.

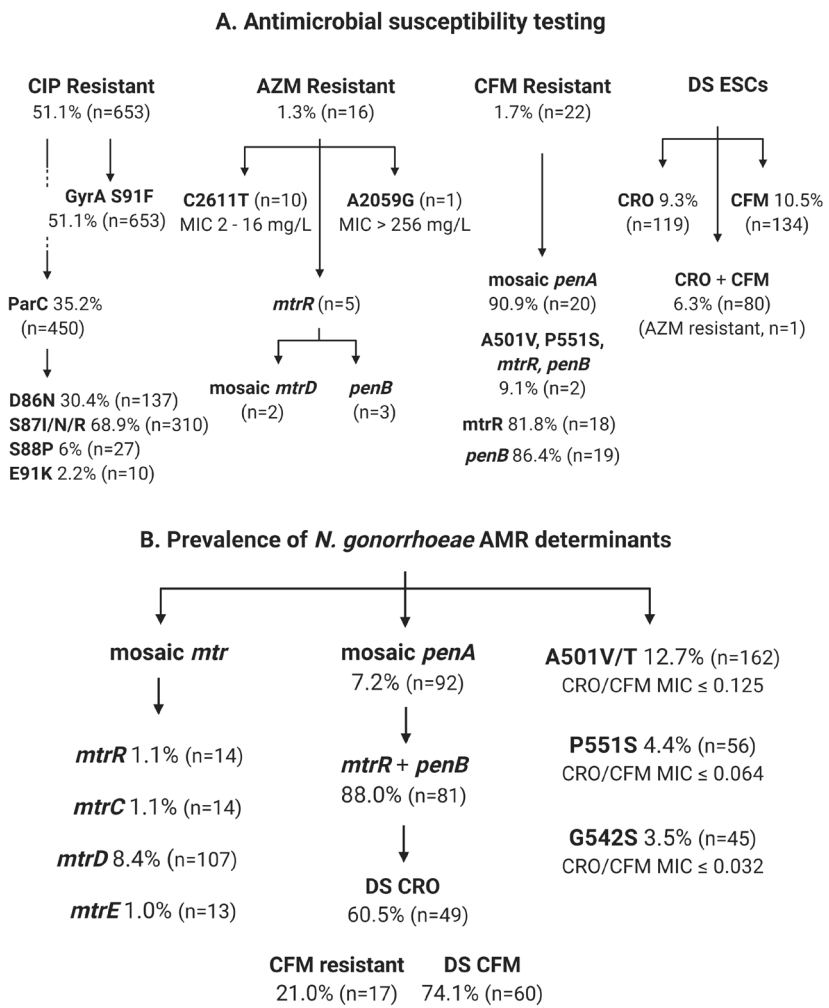


Figure 16. Flowchart of antimicrobial resistance (AMR), decreased susceptibility (DS) and AMR determinants in the Swedish 2016 Neisseria gonorrhoeae isolates. (A) Phenotypic susceptibility testing of ciprofloxacin (CIP), azithromycin (AZM) and extended-spectrum cephalosporins (ESCs), i.e. ceftriaxone (CRO) and cefixime (CFM), and associated AMR determinants. (B) Prevalence of AMR determinants penB, mtrR, mosaic mtr, penA and single amino acid alterations of penA (penicillin-binding protein (PBP) 2 positions A501, P551 and G542) and the susceptibility to CRO and CFM.

AMR determinants in *N. gonorrhoeae* isolates (paper IV)

Known AMR determinants for phenotypic resistance and decreased susceptibility to relevant antimicrobials were extracted from WGS data. Forty-four *penA* alleles were found, of which six were mosaic *penA* (represented by 92 isolates): *penA*-34.001 (n=60), *penA*-10.001 (n=23), *penA*-34.008 (n=6), *penA*-72.001, *penA*-105.001 and *penA*-180.001 (n=1 each). Azithromycin AMR determinants, 23S rRNA C2611T and A2059G mutations, were found in 10 isolates and one isolate, respectively, and *mtrR* and *porB1b* AMR mutations were found in 540 and 356 isolates, respectively. All ciprofloxacin-resistant isolates (n=653) harboured the GyrA S91F alteration. AMR, decreased susceptibility and the associated AMR determinants are summarised in Figure 16. Several AMR determinants (e.g., *mtrR* and *porB1b* mutations together with mosaic *penA* and mosaic *mtr*) can collectively result in phenotypic resistance. However, the absence of a known AMR determinant does not exclude phenotypic AMR. Unknown mechanisms may cause decreased phenotypic susceptibility or AMR. Therefore, WGS together with susceptibility testing are ideal methods to comprehensively evaluate the prevalence and emergence of new AMR determinants, and additionally providing a complete molecular characterisation.

N. gonorrhoeae epidemiology (paper IV)

Population characteristics (paper IV)

Most gonorrhoea cases were diagnosed in regions of the three major metropolitan areas: Stockholm (55.4%), Västra Götaland (15.6%) and Skåne (10.2%). The origin of infection was reported as domestic in 68.4% and foreign in 29.6% of cases, with a higher proportion of domestic infections in women and MSM (Table IV). The data are consistent with previous annual reports from the Public Health Agency of Sweden [19], with a higher proportion of infected men and domestic infections and a high burden among MSM. The high proportion of domestic infections in MSM has been previously reported, with MSM showing higher risk behaviour in local or domestic areas than when traveling abroad [282, 283]. In addition, most cases are observed in patients attending STI clinics, demonstrating the importance of these clinics in healthcare services.

Table IV. *Population characteristics of Swedish gonorrhoea cases in 2016 in men, women and reported sexual orientation in men.*

Population	Total	Age mean (median)	Symptomatic (%)	STI clinic attendance (%)*	Domestic infection (%)
Men	1027	32.1 (30)	655 (63.8)	898 (87.4)	684 (66.6)
Women	252	27.4 (24)	127 (50.4)	182 (72.2)	191 (75.8)
MSM	643	31.6 (30)	321 (49.9)	609 (94.7)	490 (76.2)
Heterosexual men	363	33.2 (30)	323 (89.0)	286 (78.8)	191 (52.6)
Unknown	21	29.5 (25)	12 (50.0)	4 (16.7)	4 (16.7)

Abbreviations: STI clinic, sexually transmitted infection clinic; MSM, men who have sex with men. *1080 patients attending STI clinic in total

Table V. *The most common molecular sequence types (ST) or clonal complexes (CC) found in the Swedish Neisseria gonorrhoeae isolates in 2016 using four typing schemes.*

	Most common STs / CCs				
Typing scheme (no of STs / CCs)	1st	2nd	3rd	4th	5th
MLST (n=133)	ST8156 (n=133)	ST7363 (n=125)	ST1901 (n=93)	ST1588 (n=89)	ST7359 (n=67)
NG-MAST (n=422)	ST5441 (n=89)	ST5793 (n=56)	ST2992 (n=41)	ST1461 (n=39)	ST387 (n=35)
NG-STAR (n=280)	ST442 (n=133)	ST55 (n=63)	ST158 (n=63)	ST231 (n=61)	ST520 (n=52)
NG-STAR CC (n=92)	CC442 (n=134)	CC158 (n=100)	CC63 (n=91)	CC42 (n=70)	CC390 (n=69)

Abbreviations: ST, sequence type; CC, clonal complex; MLST, multi-locus sequence typing; NG-MAST, *Neisseria gonorrhoeae* multi-antigen sequence typing; NG-STAR; *Neisseria gonorrhoeae* sequence typing for antimicrobial resistance.

Molecular epidemiology and phylogeny (paper IV)

Four molecular typing schemes were used to describe the Swedish 2016 *N. gonorrhoeae* population. The five most prevalent STs are presented in Table V. The MLST ST8156 and ST7359, among the most common MLST ST in the Swedish isolates, were also common in a recent Norwegian national study in 2016-2017 [284]. All of the STs described in Norway with ≥10 isolates were also present in the Swedish population. Notably, common MLST STs in Norway and Sweden (i.e. ST8156 and ST7359) are also common STs in Japan (ST7359) [119, 285], Australia and New Zealand (ST8156 and ST7359) [286-288] but appear less prevalent globally with relatively few observations in the EU/EEA and the USA [288, 289]. Three other common Swedish MLST STs (ST7363, ST1901, ST1588) have been shown to also be among the most prevalent MLST STs in the EU/EEA [74]. The emergence of mosaic *penA* in Japan, with decreased susceptibility and resistance to cefixime and other oral ESCs, has been linked to ST7363 and ST1901 [290, 291]. These lineages have since been disseminated globally, and consequently, the resistance and decreased susceptibility to ESCs in Europe and North America [74, 292]. In Sweden, 85% of isolates with mosaic *penA* alleles belonged to MLST ST7363 or ST1901, spread across 13 (61.9%) of 21 Swedish regions. Most cefixime-resistant isolates (81.0%, 17/21) belonged to MLST ST7363. These isolates were cultured in Stockholm (n=15) and spread through domestic transmissions (n=12). The isolates with azithromycin resistance (n=16) were heterogeneous, evenly distributed among eight MLST STs, of which four were also recently described in Norway [284].

Phylogenetic analysis of all *N. gonorrhoeae* isolates revealed two main genomic lineages (A and B), where lineage A was subdivided into two sublineages (A1 and A2), as seen in Figure 17. Most isolates with phenotypic resistance to ESCs and azithromycin and decreased susceptibility to ESCs, including associated AMR determinants, were located in sublineage A2. The Swedish gonococcal phylogeny showed a remarkable resemblance to a global phylogenomic analysis performed on 413 *N. gonorrhoeae* isolates collected from all continents during five decades [108]. This global population showed a similar division of lineages with most AMR and AMR determinants in one lineage, which was further subdivided into two distinct sublineages and a second main lineage with overall antimicrobial susceptible isolates.

Most MSM (60.5%) were associated with sublineage A2; however, cefixime resistance was associated with heterosexual transmissions, which was

also observed in a recent European study [293]. Conversely, decreased susceptibility to ESCs in sublineage A2 was more prevalent among MSM (57.1%) compared to heterosexual patients (38.1%). In sublineage A1, over 90% of isolates were resistant to ciprofloxacin. Notably, lineage B contained most of the antimicrobial-susceptible isolates with very limited AMR and AMR determinants.

Molecular epidemiology (i.e. molecular typing and phylogenomic analysis) provides comprehensive surveillance on the dissemination and relatedness of strains that, together with phenotypic and epidemiological data, offer valuable information on the emergence and transmission of new or previously described gonococcal strains. Continuous national and global genomic surveillance of *N. gonorrhoeae* and its AMR is essential for maintaining knowledge of circulating strains.

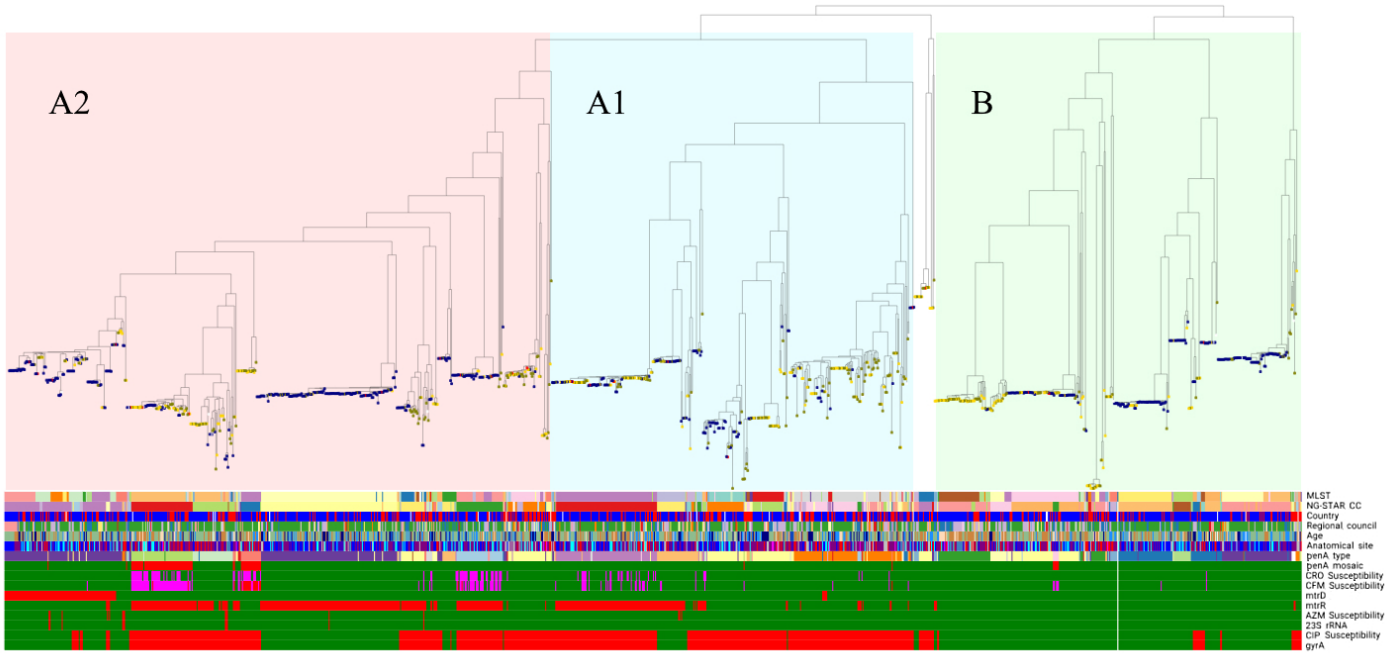


Figure 17. *A phylogenetic tree of Neisseria gonorrhoeae isolates in Sweden in 2016 with data on molecular characterisation, country of infection (domestic/foreign), regional council, age, anatomical site, phenotypic antimicrobial resistance (AMR) and their main determinants. The tree is divided in two lineages (A and B) and sublineages A1 and A2. The nodes represent sexual orientation (blue, homosexual men; yellow, females; green, heterosexual males; red, bisexual males; white, not reported). The colours of the bars for AMR and AMR determinants denotes phenotypic susceptibility and presence or absence, respectively (red, resistance or presence of AMR determinant; green, susceptible or absence of AMR determinant; pink, decreased susceptibility).*

Conclusions and future perspectives

Paper I showed successful priming of Th1, Th2 and Th17 cells in mice using the MOMP VS2/4 antigen with CT adjuvant, with promising protective properties against urogenital *C. trachomatis* infection and infertility. The high estimates of *C. trachomatis* infections globally and the high incidence in Sweden [14, 21] indicate that preventive actions, such as in Sweden with mandatory notification, contact tracing and opportunistic testing, are not sufficiently effective. The MOMP VS2/4 antigen construct showed promise. However, further studies are needed to assess immune responses and neutralising antibodies, cross-protection to other serovars and use of different doses and adjuvants. The promising CTH522 vaccine candidate [197] provides hope for a *C. trachomatis* vaccine. However, more studies to evaluate the efficacy of CTH522 against *C. trachomatis* infection are needed, in addition to vaccine safety concerning the potential impact of a previous *C. trachomatis* infection.

In paper II, the *N. gonorrhoeae* amino acid sequences of the meningococcal 4CMenB antigens were highly homogenous within the gonococcal population. The gonococcal amino acid sequences of fHbp and NHBA differed substantially from the corresponding meningococcal sequences, which, together with the lack of a universal expression of these antigens and the absence of gonococcal *nadA*, limit the use of the meningococcal GNA antigens in 4CMenB to target *N. gonorrhoeae* appropriately. Since the publication of paper II and the observation of the cross-reaction of meningococcal vaccine on gonorrhoea, several studies have been performed on the cross-reactivity of meningococcal NHBA and OMVs [269, 271], in addition to the immunity and function [180, 268] of gonococcal NHBA. More research is needed on the gonococcal NHBA function and its ability to generate immunity. The surface-exposed antigens (e.g., 2C7 epitope, methionine sulfoxide reductase and MetQ) [176-178] and gonococcal OMVs need further investigation as potential vaccine candidates. Understanding what is required to generate protective immunity against gonorrhoea in humans for future vaccine development is essential.

The evaluation of the molecular diagnostic assays (paper III) showed that the SpeeDx ResistancePlus® GC assay could be effectively used for ciprofloxacin resistance/susceptibility prediction, i.e. in AMR surveillance and ultimately individualised treatments, particularly in testing of urogenital samples. Although the 23S rRNA C2611 assay performed well in detecting C2611T or WT alleles, it failed to predict phenotypic azithromycin re-

sistance accurately. To make an accurate prediction of phenotypic resistance to azithromycin and decreased susceptibility and resistance to ESCs, several AMR determinants need to be simultaneously detected and appropriate algorithms identified. Studies on multiplexed NAATs and algorithms for WGS data to predict decreased susceptibility or resistance to many antimicrobials are underway [294] but further validation and research are warranted. Rapid, accurate and cost-effective molecular tests, i.e. affordable to developing countries and used at the point of care to detect *N. gonorrhoeae* and simultaneously predict AMR, are highly needed. These tests would be most valuable to guide individualised treatment, spare first-line empiric antimicrobial treatment and avoid unnecessary antimicrobial usage.

The Swedish *N. gonorrhoeae* population in 2016 (paper IV) had a low prevalence of resistance to azithromycin and ESCs. In addition, isolates with azithromycin and cefixime AMR were predominantly associated with domestic transmission. The isolates could be divided into two main genomic lineages, A and B, where the phenotypic AMR and AMR determinants were associated with lineage A. Continuous surveillance of phenotypic AMR, molecular typing and genomic phylogeny is necessary for enhanced understanding of the epidemiology of gonorrhoea and evolution and transmission of *N. gonorrhoeae* and its AMR. This knowledge may also support revisions of effective evidence-based guidelines regarding management and control of gonorrhoea in local, national and international settings. It is imperative to maintain and strengthen the phenotypic AMR surveillance to detect new AMR determinants. Importantly, enhanced phenotypic and genomic surveillance is needed in developing countries with a high burden and limited available information on *N. gonorrhoeae* epidemiology and AMR.

Sustained implementation of WHO strategies, as mentioned in this thesis, are needed in conjunction with additional actions, i.e. improving early detection, strengthening preventive measures, advocacy of antimicrobial stewardship and developing new effective antimicrobials for treating gonorrhoea. Collectively, implementations of these actions could effectively halter the increasing burden of STIs and the AMR development in *N. gonorrhoeae*.

Acknowledgements

The journey to this point has been long and the process of learning, including producing this thesis, has only been possible with the help and support of colleagues, friends and family. I would especially like to thank:

Professor Magnus Unemo, my principal supervisor, for allowing me to be a part of this research endeavour and always being supportive, kind and offering the best scientific and ethical guidance by example.

Associate Professor Hans Fredlund, my co-supervisor, for providing an insight into the clinical aspects and patient perspective, teaching me to look at the bigger picture, always supported me and did all this with a smile.

Susanne Jacobsson, my co-supervisor, for supporting me in the laboratory and all the many little things needed.

Professor Åke Strid and Professor Sören Andersson, my supervisors on paper I, for the opportunity to do the research and the learning experiences with cloning and protein production.

Professor Nils Lycke and his group, particularly Karin Schön, for helping me with the animal trials in Gothenburg.

Daniel Golparian, the one and only WHO scientific officer, for helping me with the bioinformatic analysis.

Alvida Qvick, a fellow PhD student and my sister from another mister, for helping and guiding me with statistical analysis and photo editing. I greatly appreciate all your constructive comments on my written texts and always being so positive and enthusiastic.

Sara Thulin-Hedberg, head of the Molecular biology section at the Department of Laboratory Medicine, Clinical Microbiology, for providing me with the time to write this thesis with very few disruptions and many cheers along the way.

Anna Fagerström, my colleague, thank you for writing your thesis first and helping me with mine.

Bengt Hellmark, for always supporting and listening when needed.

Inga Velicko, a researcher at the Public Health Agency, for helping collect patient epidemiological data for paper IV.

Paul Pettersson-Pablo, for all the interesting conversations and help with the references. Always a true Hufflepuff!

My research colleagues at the office, past and present: Malin Kaliff, Kerstin, Malm, Amelia Björnberg, Emma Adolfsson, Lorraine Eriksson, Bianca Stenmark and Amaya C Lagos, for the time spent together, heated salary discussions and the fun times in and outside of work.

My colleagues at the Department of Laboratory Medicine, one cannot ask for better co-workers.

My friends from University, Huthayfa Mujahed and Berolla Sahdo, for support, encouragement and all the coffee breaks during the university years.

To my friends in NATS, the amateur theatre group, for letting me be part of something so far off my comfort zone but also fun!

To Anna-Maria, Katrin and Pär, I love you guys!

My friends outside of academia and work, Alex and Ida: It is always a special treat to spend time with you guys.

To my family, Rimonde, Rawad, Rabie, Sara and my three adorable nephews (Geo, Lucas and Eddie) and my bonus family, Tony, Natalie, Tony Jr and Thomas, for the support. Above all, I express special gratitude to my mother for her unconditional love, always advocating education and giving her complete support no matter what.

الى ست الحبايب
.امي بشكرك على محبتك وتربيتك وتضحيتك الي وعلى وقفتك حدي بكل ظروف حياتي
.واهم شي اكلاتك الطيبين
.من بنتك المحبة

References

1. Unemo M, Shafer WM. Antibiotic resistance in *Neisseria gonorrhoeae*: origin, evolution, and lessons learned for the future. Ann N Y Acad Sci 2011;1230:E19-28.
2. Neisser A. Ueber eine der Gonorrhoe eigentümliche Micrococcusform. Med Wissenschaften 1879;No. 28:497-500.
3. al-Rifai KM. Trachoma through history. Int Ophthalmol 1988; 12(1):9-14.
4. Halberstaedter L, von Prowazek S. Über zelleinschlüsse parasitärer natur beim trachom. Arbeiten aus dem Kaiserlichen Gesundheitsamte 1907;26:44-7.
5. Moulder JW, Novosel DL, Officer JE. Inhibition of the growth of agents of the psittacosis group by D-cycloserine and its specific reversal by D-alanine. J Bacteriol 1963;85(3):707-11.
6. Danielsson D. Gonorrhoea and syphilis in Sweden--past and present. Scand J Infect Dis Suppl 1990;69:69-76.
7. Smittskyddslag (1988:1472), SFS 1988:1472 SmL(1988).
8. Charles J. Origins, history, and achievements of the World Health Organization. Br Med J 1968;2(5600):293-6.
9. World Health Organization (WHO). Global health sector strategy on sexually transmitted infections, 2016-2021. 2016 [Accessed 2021-06-03]. Available from: https://www.who.int/reproductivehealth/publications/rtis/ghss-stis/en/.
10. World Health Organization (WHO). Global prevalence and incidence of selected curable sexually transmitted infections: overview and estimates. 2001 [Accessed 2021-06-03]. Available from: https://www.who.int/reproductivehealth/publications/rtis/HIV_AIDS_2001_2/en/.
11. World Health Organization (WHO). Prevalence and incidence of selected sexually transmitted infections. *Chlamydia trachomatis*, *Neisseria gonorrhoeae*, syphilis and *Trichomonas vaginalis*. Methods and results used by WHO to generate 2005 estimates. 2011 [Accessed 2021-06-03]. Available from: https://www.who.int/reproductivehealth/publications/rtis/9789241502450/en/.
12. World Health Organization (WHO). Global incidence and prevalence of selected curable sexually transmitted infections - 2008. 2012 [Accessed 2021-06-03]. Available from:

https://apps.who.int/iris/bitstream/handle/10665/75181/9789241503839_eng.pdf;jsessionid=F11159911B29B032D1B0E5244F3D3450?sequence=1.
13. World Health Organization (WHO). Report on global sexually transmitted infection surveillance 2018. 2018 [Accessed 2021-03-07]. Available from: https://www.who.int/reproductivehealth/publications/stis-surveillance-2018/en/.
14. Rowley J, Vander Hoorn S, Korenromp E, Low N, Unemo M, Abu-Raddad LJ, et al. Chlamydia, gonorrhoea, trichomoniasis and syphilis: global prevalence and incidence estimates, 2016. Bull World Health Organ 2019;97(8):548-62p.
15. Unemo M, Bradshaw CS, Hocking JS, de Vries HJC, Francis SC, Mabey D, et al. Sexually transmitted infections: challenges ahead. Lancet Infect Dis 2017;17(8):e235-e79.
16. Unemo M, Ross J, Serwin AB, Gomberg M, Cusini M, Jensen JS. 2020 European guideline for the diagnosis and treatment of gonorrhoea in adults. Int J STD AIDS 2020.
17. Hernando RC, Spiteri G, Sabidó M, Montoliu A, Gonzalez V, Casabona J, et al. Antimicrobial resistance in *Neisseria gonorrhoeae* isolates from foreign-born population in the European Gonococcal Antimicrobial Surveillance Programme. Sex Transm Infect 2020;96(3):204-10.
18. Lanjouw E, Ouburg S, de Vries HJ, Stary A, Radcliffe K, Unemo M. 2015 European guideline on the management of *Chlamydia trachomatis* infections. Int J STD AIDS 2016;27(5):333-48.
19. Public Health Agency of Sweden. Gonorrhoea infections. [Accessed 2022-01-21]. Available from: https://www.folkhalsomyndigheten.se/folkhalsorapportering-statistik/statistik-a-o/sjukdomsstatistik/gonorre/?t=county.
20. Saarentausta K, Ivarsson L, Jacobsson S, Herrmann B, Sundqvist M, Unemo M. Potential impact of the COVID-19 pandemic on the national and regional incidence, epidemiology and diagnostic testing of chlamydia and gonorrhoea in Sweden, 2020. APMIS 2022;130(1):34-42.
21. Public Health Agency of Sweden. Chlamydia infections. [Accessed 2022-01-21]. Available from: https://www.folkhalsomyndigheten.se/folkhalsorapportering-statistik/statistik-a-o/sjukdomsstatistik/klamydiainfektion/?p=93949#statistics-nav.
22. Unemo M, Seifert HS, Hook EW 3rd, Hawkes S, Ndowa F, Dillon JR. Gonorrhoea. Nat Rev Dis Primers 2019;5(1):79.

23. Unemo M, Shafer WM. Antimicrobial resistance in *Neisseria gonorrhoeae* in the 21st century: past, evolution, and future. Clin Microbiol Rev 2014;27(3):587-613.
24. Beatrous SV, Grisoli SB, Riahi RR, Matherne RJ, Matherne RJ. Cutaneous manifestations of disseminated gonococcemia. Dermatol Online J 2017;23(1).
25. West SK. Trachoma: new assault on an ancient disease. Prog Retin Eye Res 2004;23(4):381-401.
26. Galvin SR, Cohen MS. The role of sexually transmitted diseases in HIV transmission. Nat Rev Microbiol 2004;2(1):33-42.
27. Swedish Society for Dermatology and Venereology (SSDV). Gonorré. [Accessed 2021-04-21]. Available from: https://ssdv.se/venereologi/riktlinjer.
28. Clarke IN. Evolution of *Chlamydia trachomatis*. Ann N Y Acad Sci 2011;1230:E11-8.
29. Steen R, Wi TE, Kamali A, Ndowa F. Control of sexually transmitted infections and prevention of HIV transmission: mending a fractured paradigm. Bull World Health Organ 2009; 87(11):858-65.
30. Svanemyr J, Amin A, Robles OJ, Greene ME. Creating an enabling environment for adolescent sexual and reproductive health: a framework and promising approaches. J Adolesc Health 2015; 56:S7-14.
31. Adeolu M, Gupta RS. Phylogenomics and molecular signatures for the order *Neisseriales*: proposal for division of the order *Neisseriales* into the emended family *Neisseriaceae* and *Chromobacteriaceae* fam. nov. Antonie Van Leeuwenhoek 2013;104(1):1-24.
32. Perrin A, Bonacorsi S, Carbonnelle E, Talibi D, Dessen P, Nassif X, et al. Comparative genomics identifies the genetic islands that distinguish *Neisseria meningitidis*, the agent of cerebrospinal meningitis, from other *Neisseria* species. Infect Immun 2002;70(12):7063-72.
33. Tinsley CR, Nassif X. Analysis of the genetic differences between *Neisseria meningitidis* and *Neisseria gonorrhoeae*: two closely related bacteria expressing two different pathogenicities. Proc Natl Acad Sci USA 1996;93(20):11109-14.
34. Cole JA. Legless pathogens: how bacterial physiology provides the key to understanding pathogenicity. Microbiology 2012;158:1402-13.
35. Swanson KV, Jarvis GA, Brooks GF, Barham BJ, Cooper MD, Griffiss JM. CEACAM is not necessary for *Neisseria gonorrhoeae*

to adhere to and invade female genital epithelial cells. Cell Microbiol 2001;3(10):681-91.
36. Stohl EA, Dale EM, Criss AK, Seifert HS. *Neisseria gonorrhoeae* metalloprotease NGO1686 is required for full piliation, and piliation is required for resistance to H_2O_2- and neutrophil-mediated killing. mBio 2013;4(4).
37. Obergfell KP, Seifert HS. The pilin N-terminal domain maintains *Neisseria gonorrhoeae* transformation competence during pilus phase variation. PLoS Genet 2016;12(5):e1006069.
38. Biswas GD, Sox T, Blackman E, Sparling PF. Factors affecting genetic transformation of *Neisseria gonorrhoeae*. J Bacteriol 1977;129(2):983-92.
39. Sadarangani M, Pollard AJ, Gray-Owen SD. Opa proteins and CEACAMs: pathways of immune engagement for pathogenic *Neisseria*. FEMS Microbiol Rev 2011;35(3):498-514.
40. Deo P, Chow SH, Hay ID, Kleifeld O, Costin A, Elgass KD, et al. Outer membrane vesicles from *Neisseria gonorrhoeae* target PorB to mitochondria and induce apoptosis. PLoS Pathog 2018;14(3): e1006945.
41. Massari P, Ram S, Macleod H, Wetzler LM. The role of porins in neisserial pathogenesis and immunity. Trends Microbiol 2003;11(2):87-93.
42. Olesky M, Zhao S, Rosenberg RL, Nicholas RA. Porin-mediated antibiotic resistance in *Neisseria gonorrhoeae*: ion, solute, and antibiotic permeation through PIB proteins with *penB* mutations. J Bacteriol 2006;188(7):2300-8.
43. Bauer FJ, Rudel T, Stein M, Meyer TF. Mutagenesis of the *Neisseria gonorrhoeae* porin reduces invasion in epithelial cells and enhances phagocyte responsiveness. Mol Microbiol 1999;31(3): 903-13.
44. Madico G, Ngampasutadol J, Gulati S, Vogel U, Rice PA, Ram S. Factor H binding and function in sialylated pathogenic *Neisseriae* is influenced by gonococcal, but not meningococcal, porin. J Immunol 2007;178(7):4489-97.
45. Ram S, Sharma AK, Simpson SD, Gulati S, McQuillen DP, Pangburn MK, et al. A novel sialic acid binding site on factor H mediates serum resistance of sialylated *Neisseria gonorrhoeae*. J Exp Med 1998;187(5):743-52.
46. Jarvis GA. Analysis of C3 deposition and degradation on *Neisseria meningitidis* and *Neisseria gonorrhoeae*. Infect Immun 1994;62(5):1755-60.

47. Noinaj N, Buchanan SK, Cornelissen CN. The transferrin-iron import system from pathogenic *Neisseria* species. Mol Microbiol 2012;86(2):246-57.
48. Hagman KE, Pan W, Spratt BG, Balthazar JT, Judd RC, Shafer WM. Resistance of *Neisseria gonorrhoeae* to antimicrobial hydrophobic agents is modulated by the *mtrRCDE* efflux system. Microbiology 1995;141:611-22.
49. Sauvage E, Kerff F, Terrak M, Ayala JA, Charlier P. The penicillin-binding proteins: structure and role in peptidoglycan biosynthesis. FEMS Microbiol Rev 2008;32(2):234-58.
50. Cokkinis AJ, McElligott GL. Sulfanilamide in gonorrhoea. An analysis of 633 cases. Lancet 1938;2:355-62.
51. Dunlop EM. Gonorrhoea and the sulphonamides. Br J Vener Dis 1949;25(2):81-3.
52. Wainwright M, Swan HT. C.G. Paine and the earliest surviving clinical records of penicillin therapy. Med Hist 1986;30(1):42-56.
53. Van Slyke CJ, Arnold RC, Buchholtz M. Penicillin therapy in sulfonamide-resistant gonorrhea in men. Am J Public Health Nations Health 1943;33(12):1392-4.
54. Amies CR. Development of resistance of gonococci to penicillin: an eight-year study. Can Med Assoc J 1967;96(1):33-5.
55. Willcox RR. A survey of problems in the antibiotic treatment of gonorrhoea. With special reference to South-East Asia. Br J Vener Dis 1970;46(3):217-42.
56. Percival A, Rowlands J, Corkill JE, Alergant CD, Arya OP, Rees E, et al. Penicillinase-producing gonococci in Liverpool. Lancet 1976;2(8000):1379-82.
57. Faruki H, Sparling PF. Genetics of resistance in a non-beta-lactamase-producing gonococcus with relatively high-level penicillin resistance. Antimicrob Agents Chemother 1986;30(6): 856-60.
58. Nelson ML, Levy SB. The history of the tetracyclines. Ann N Y Acad Sci 2011;1241:17-32.
59. Duggar BM. Aureomycin; a product of the continuing search for new antibiotics. Ann N Y Acad Sci 1948;51(Art. 2):177-81.
60. Boothe JH, Morton J, Petisi JP, Wilkinson RG, Williams JH. Tetracycline. J Am Chem Soc 1953;75(18):4621.
61. Amies CR. Sensitivity of *Neisseria gonorrhoeae* to penicillin and other antibiotics. Studies carried out in Toronto during the period 1961 to 1968. Br J Vener Dis 1969;45(3):216-22.
62. Reyn A, Schmidt H, Trier M, Bentzon MW. Spectinomycin hydrochloride (Trobicin) in the treatment of gonorrhoea.

Observation of resistant strains of *Neisseria gonorrhoeae*. Br J Vener Dis 1973;49(1):54-9.
63. Easmon CS, Forster GE, Walker GD, Ison CA, Harris JR, Munday PE. Spectinomycin as initial treatment for gonorrhoea. Br Med J 1984;289(6451):1032-4.
64. Stolz E, Zwart HG, Michel MF. Activity of eight antimicrobial agents *in vitro* against *N. gonorrhoeae*. Br J Vener Dis 1975;51(4):257-64.
65. Ashford WA, Potts DW, Adams HJ, English JC, Johnson SR, Biddle JW, et al. Spectinomycin-resistant penicillinase-producing *Neisseria gonorrhoeae*. Lancet 1981;2(8254):1035-7.
66. Boslego JW, Tramont EC, Takafuji ET, Diniega BM, Mitchell BS, Small JW, et al. Effect of spectinomycin use on the prevalence of spectinomycin-resistant and of penicillinase-producing *Neisseria gonorrhoeae*. N Engl J Med 1987;317(5):272-8.
67. Ison CA, Littleton K, Shannon KP, Easmon CS, Phillips I. Spectinomycin resistant gonococci. Br Med J 1983;287(6408): 1827-9.
68. Shanmugaratnam K, Sprott MS, Pattman RS, Kearns AM, Watson PG. Single dose ciprofloxacin to treat women with gonorrhoea. Genitourin Med 1989;65(2):129.
69. Chen CR, Malik M, Snyder M, Drlica K. DNA gyrase and topoisomerase IV on the bacterial chromosome: quinolone-induced DNA cleavage. J Mol Biol 1996;258(4):627-37.
70. Gransden WR, Warren CA, Phillips I, Hodges M, Barlow D. Decreased susceptibility of *Neisseria gonorrhoeae* to ciprofloxacin. Lancet 1990;335(8680):51.
71. Tanaka M, Nakayama H, Haraoka M, Saika T. Antimicrobial resistance of *Neisseria gonorrhoeae* and high prevalence of ciprofloxacin-resistant isolates in Japan, 1993 to 1998. J Clin Microbiol 2000;38(2):521-5.
72. Tyson M. Guidance for first-line treatment of anogenital gonorrhoea infection. Br J Nurs 2005;14(12):646-8, 650-2.
73. Centers for Disease Control and Prevention (CDC). Update to CDC's sexually transmitted diseases treatment guidelines, 2006: fluoroquinolones no longer recommended for treatment of gonococcal infections. Morb Mortal Wkly Rep 2007;56(14):332-6.
74. Harris SR, Cole MJ, Spiteri G, Sanchez-Buso L, Golparian D, Jacobsson S, et al. Public health surveillance of multidrug-resistant clones of *Neisseria gonorrhoeae* in Europe: a genomic survey. Lancet Infect Dis 2018;18(7):758-68.

75. Unemo M, Lahra MM, Cole M, Galarza P, Ndowa F, Martin I, et al. World Health Organization Global Gonococcal Antimicrobial Surveillance Program (WHO GASP): review of new data and evidence to inform international collaborative actions and research efforts. Sex Health 2019;16(5):412-25.
76. Unemo M, Lahra MM, Escher M, Eremin S, Cole MJ, Galarza P, et al. WHO global antimicrobial resistance surveillance for *Neisseria gonorrhoeae* 2017–18: a retrospective observational study. The Lancet Microbe 2021;2(11):e627-e36.
77. Jelić D, Antolović R. From erythromycin to azithromycin and new potential ribosome-binding antimicrobials. Antibiotics 2016;5(3).
78. Dillon JA, Ruben M, Li H, Borthagaray G, Márquez C, Fiorito S, et al. Challenges in the control of gonorrhea in South America and the Caribbean: monitoring the development of resistance to antibiotics. Sex Transm Dis 2006;33(2):87-95.
79. McLean CA, Wang SA, Hoff GL, Dennis LY, Trees DL, Knapp JS, et al. The emergence of *Neisseria gonorrhoeae* with decreased susceptibility to azithromycin in Kansas City, Missouri, 1999 to 2000. Sex Transm Dis 2004;31(2):73-8.
80. Palmer HM, Young H, Winter A, Dave J. Emergence and spread of azithromycin-resistant *Neisseria gonorrhoeae* in Scotland. J Antimicrob Chemother 2008;62(3):490-4.
81. Zarantonelli L, Borthagaray G, Lee EH, Shafer WM. Decreased azithromycin susceptibility of *Neisseria gonorrhoeae* due to *mtrR* mutations. Antimicrob Agents Chemother 1999;43(10):2468-72.
82. Abraham EP. Cephalosporins 1945-1986. Drugs 1987;34 Suppl 2:1-14.
83. Chaudhry SB, Veve MP, Wagner JL. Cephalosporins: A focus on side chains and β-lactam cross-reactivity. Pharmacy 2019;7(3).
84. Lewis DA. The Gonococcus fights back: is this time a knock out? Sex Transm Infect 2010;86(6):415-21.
85. Ito M, Yasuda M, Yokoi S, Ito S, Takahashi Y, Ishihara S, et al. Remarkable increase in central Japan in 2001-2002 of *Neisseria gonorrhoeae* isolates with decreased susceptibility to penicillin, tetracycline, oral cephalosporins, and fluoroquinolones. Antimicrob Agents Chemother 2004;48(8):3185-7.
86. Yokoi S, Deguchi T, Ozawa T, Yasuda M, Ito S, Kubota Y, et al. Threat to cefixime treatment for gonorrhea. Emerg Infect Dis 2007;13(8):1275-7.
87. Read PJ, Limnios EA, McNulty A, Whiley D, Lahra MM. One confirmed and one suspected case of pharyngeal gonorrhoea treatment failure following 500mg ceftriaxone in Sydney, Australia. Sex Health 2013;10(5):460-2.

88. Unemo M, Golparian D, Potočnik M, Jeverica S. Treatment failure of pharyngeal gonorrhoea with internationally recommended first-line ceftriaxone verified in Slovenia, September 2011. Euro Surveill 2012;17(25).
89. Unemo M, Golparian D, Hestner A. Ceftriaxone treatment failure of pharyngeal gonorrhoea verified by international recommendations, Sweden, July 2010. Euro Surveill 2011;16(6).
90. Poncin T, Fouere S, Braille A, Camelena F, Agsous M, Bebear C, et al. Multidrug-resistant *Neisseria gonorrhoeae* failing treatment with ceftriaxone and doxycycline in France, November 2017. Euro Surveill 2018;23(21).
91. Ohnishi M, Golparian D, Shimuta K, Saika T, Hoshina S, Iwasaku K, et al. Is *Neisseria gonorrhoeae* initiating a future era of untreatable gonorrhea?: detailed characterization of the first strain with high-level resistance to ceftriaxone. Antimicrob Agents Chemother 2011;55(7):3538-45.
92. Chen SC, Han Y, Yuan LF, Zhu XY, Yin YP. Identification of internationally disseminated ceftriaxone-resistant *Neisseria gonorrhoeae* strain FC428, China. Emerg Infect Dis 2019;25(7): 1427-9.
93. Golparian D, Rose L, Lynam A, Mohamed A, Bercot B, Ohnishi M, et al. Multidrug-resistant *Neisseria gonorrhoeae* isolate, belonging to the internationally spreading Japanese FC428 clone, with ceftriaxone resistance and intermediate resistance to azithromycin, Ireland, August 2018. Euro Surveill 2018;23(47).
94. Lahra MM, Martin I, Demczuk W, Jennison AV, Lee KI, Nakayama SI, et al. Cooperative recognition of internationally disseminated ceftriaxone-resistant *Neisseria gonorrhoeae* strain. Emerg Infect Dis 2018;24(4):735-40.
95. Lee K, Nakayama SI, Osawa K, Yoshida H, Arakawa S, Furubayashi KI, et al. Clonal expansion and spread of the ceftriaxone-resistant *Neisseria gonorrhoeae* strain FC428, identified in Japan in 2015, and closely related isolates. J Antimicrob Chemother 2019;74(7):1812-9.
96. Jennison AV, Whiley D, Lahra MM, Graham RM, Cole MJ, Hughes G, et al. Genetic relatedness of ceftriaxone-resistant and high-level azithromycin resistant *Neisseria gonorrhoeae* cases, United Kingdom and Australia, February to April 2018. Euro Surveill 2019;24(8).
97. Eyre DW, Sanderson ND, Lord E, Regisford-Reimmer N, Chau K, Barker L, et al. Gonorrhoea treatment failure caused by a *Neisseria gonorrhoeae* strain with combined ceftriaxone and high-level

azithromycin resistance, England, February 2018. Euro Surveill 2018;23(27).
98. Unemo M, Golparian D, Nicholas R, Ohnishi M, Gallay A, Sednaoui P. High-level cefixime- and ceftriaxone-resistant *Neisseria gonorrhoeae* in France: novel *penA* mosaic allele in a successful international clone causes treatment failure. Antimicrob Agents Chemother 2012;56(3):1273-80.
99. Vincent LR, Kerr SR, Tan Y, Tomberg J, Raterman EL, Dunning Hotopp JC, et al. *In vivo*-selected compensatory mutations restore the fitness cost of mosaic *penA a*lleles that confer ceftriaxone resistance in *Neisseria gonorrhoeae*. mBio 2018;9(2).
100. Camara J, Serra J, Ayats J, Bastida T, Carnicer-Pont D, Andreu A, et al. Molecular characterization of two high-level ceftriaxone-resistant *Neisseria gonorrhoeae* isolates detected in Catalonia, Spain. J Antimicrob Chemother 2012;67(8):1858-60.
101. Unemo M, Golparian D, Sanchez-Buso L, Grad Y, Jacobsson S, Ohnishi M, et al. The novel 2016 WHO *Neisseria gonorrhoeae* reference strains for global quality assurance of laboratory investigations: phenotypic, genetic and reference genome characterization. J Antimicrob Chemother 2016;71(11):3096-108.
102. Cehovin A, Lewis SB. Mobile genetic elements in *Neisseria gonorrhoeae*: movement for change. Pathog Dis 2017;75(6).
103. Korch C, Hagblom P, Ohman H, Göransson M, Normark S. Cryptic plasmid of *Neisseria gonorrhoeae*: complete nucleotide sequence and genetic organization. J Bacteriol 1985;163(2):430-8.
104. Elkins C, Thomas CE, Seifert HS, Sparling PF. Species-specific uptake of DNA by gonococci is mediated by a 10-base-pair sequence. J Bacteriol 1991;173(12):3911-3.
105. Ramsey ME, Woodhams KL, Dillard JP. The gonococcal genetic island and type IV secretion in the pathogenic *Neisseria*. Front Microbiol 2011;2:61.
106. Diallo K, MacLennan J, Harrison OB, Msefula C, Sow SO, Daugla DM, et al. Genomic characterization of novel *Neisseria* species. Sci Rep 2019;9(1):13742.
107. Marri PR, Paniscus M, Weyand NJ, Rendón MA, Calton CM, Hernández DR, et al. Genome sequencing reveals widespread virulence gene exchange among human *Neisseria* species. PLoS One 2010;5(7):e11835.
108. Sanchez-Buso L, Golparian D, Corander J, Grad YH, Ohnishi M, Flemming R, et al. The impact of antimicrobials on gonococcal evolution. Nat Microbiol 2019;4(11):1941-50.

109. Barry PM, Klausner JD. The use of cephalosporins for gonorrhea: the impending problem of resistance. Expert Opin Pharmacother 2009;10(4):555-77.
110. Muhammad I, Golparian D, Dillon JA, Johansson A, Ohnishi M, Sethi S, et al. Characterisation of *bla*TEM genes and types of β-lactamase plasmids in *Neisseria gonorrhoeae* - the prevalent and conserved *bla*TEM-135 has not recently evolved and existed in the Toronto plasmid from the origin. BMC Infect Dis 2014;14:454.
111. Ropp PA, Hu M, Olesky M, Nicholas RA. Mutations in *ponA*, the gene encoding penicillin-binding protein 1, and a novel locus, *penC*, are required for high-level chromosomally mediated penicillin resistance in *Neisseria gonorrhoeae*. Antimicrob Agents Chemother 2002;46(3):769-77.
112. Morse SA, Johnson SR, Biddle JW, Roberts MC. High-level tetracycline resistance in *Neisseria gonorrhoeae* is result of acquisition of streptococcal *tetM* determinant. Antimicrob Agents Chemother 1986;30(5):664-70.
113. Hu M, Nandi S, Davies C, Nicholas RA. High-level chromosomally mediated tetracycline resistance in *Neisseria gonorrhoeae* results from a point mutation in the *rpsJ* gene encoding ribosomal protein S10 in combination with the *mtrR* and *penB* resistance determinants. Antimicrob Agents Chemother 2005;49(10):4327-34.
114. Chisholm SA, Dave J, Ison CA. High-level azithromycin resistance occurs in *Neisseria gonorrhoeae* as a result of a single point mutation in the 23S rRNA genes. Antimicrob Agents Chemother 2010;54(9):3812-6.
115. Davies C, Bussiere DE, Golden BL, Porter SJ, Ramakrishnan V, White SW. Ribosomal proteins S5 and L6: high-resolution crystal structures and roles in protein synthesis and antibiotic resistance. J Mol Biol 1998;279(4):873-88.
116. Galimand M, Gerbaud G, Courvalin P. Spectinomycin resistance in *Neisseria* spp. due to mutations in 16S rRNA. Antimicrob Agents Chemother 2000;44(5):1365-6.
117. Unemo M, Golparian D, Skogen V, Olsen AO, Moi H, Syversen G, et al. *Neisseria gonorrhoeae* strain with high-level resistance to spectinomycin due to a novel resistance mechanism (mutated ribosomal protein S5) verified in Norway. Antimicrob Agents Chemother 2013;57(2):1057-61.
118. Belland RJ, Morrison SG, Ison C, Huang WM. *Neisseria gonorrhoeae* acquires mutations in analogous regions of *gyrA* and *parC* in fluoroquinolone-resistant isolates. Mol Microbiol 1994;14(2):371-80.

119. Hanao M, Aoki K, Ishii Y, Shimuta K, Ohnishi M, Tateda K. Molecular characterization of *Neisseria gonorrhoeae* isolates collected through a national surveillance programme in Japan, 2013: evidence of the emergence of a ceftriaxone-resistant strain from a ceftriaxone-susceptible lineage. J Antimicrob Chemother 2021;76(7):1769-75.
120. Zhao S, Duncan M, Tomberg J, Davies C, Unemo M, Nicholas RA. Genetics of chromosomally mediated intermediate resistance to ceftriaxone and cefixime in *Neisseria gonorrhoeae*. Antimicrob Agents Chemother 2009;53(9):3744-51.
121. Tomberg J, Fedarovich A, Vincent LR, Jerse AE, Unemo M, Davies C, et al. Alanine 501 mutations in penicillin-binding protein 2 from *Neisseria gonorrhoeae*: Structure, mechanism, and effects on cephalosporin resistance and biological fitness. Biochemistry 2017;56(8):1140-50.
122. Tomberg J, Unemo M, Davies C, Nicholas RA. Molecular and structural analysis of mosaic variants of penicillin-binding protein 2 conferring decreased susceptibility to expanded-spectrum cephalosporins in *Neisseria gonorrhoeae*: role of epistatic mutations. Biochemistry 2010;49(37):8062-70.
123. de Laat MM, Wind CM, Bruisten SM, Dierdorp M, de Vries HJC, Schim van der Loeff MF, et al. Ceftriaxone reduced susceptible *Neisseria gonorrhoeae* in the Netherlands, 2009 to 2017: From *penA* mosaicism to A501T/V nonmosaicism. Sex Transm Dis 2019;46(9):594-601.
124. Tomberg J, Unemo M, Ohnishi M, Davies C, Nicholas RA. Identification of amino acids conferring high-level resistance to expanded-spectrum cephalosporins in the *penA* gene from *Neisseria gonorrhoeae* strain H041. Antimicrob Agents Chemother 2013;57(7):3029-36.
125. Deng X, Klausner JD. Six *penA* codons accurately and reliably predict cefixime-decreased susceptibility in *Neisseria gonorrhoeae*. J Infect Dis 2020;221(5):851-2.
126. Palace SG, Wang Y, Rubin DH, Welsh MA, Mortimer TD, Cole K, et al. RNA polymerase mutations cause cephalosporin resistance in clinical *Neisseria gonorrhoeae* isolates. eLife 2020;9:e51407.
127. Folster JP, Johnson PJ, Jackson L, Dhulipali V, Dyer DW, Shafer WM. *MtrR* modulates *rpoH* expression and levels of antimicrobial resistance in *Neisseria gonorrhoeae*. J Bacteriol 2009;191(1):287-97.
128. Hagman KE, Shafer WM. Transcriptional control of the *mtr* efflux system of *Neisseria gonorrhoeae*. J Bacteriol 1995;177(14):4162-5.

129. Olesky M, Hobbs M, Nicholas RA. Identification and analysis of amino acid mutations in porin IB that mediate intermediate-level resistance to penicillin and tetracycline in *Neisseria gonorrhoeae*. Antimicrob Agents Chemother 2002;46(9):2811-20.
130. Veal WL, Nicholas RA, Shafer WM. Overexpression of the MtrC-MtrD-MtrE efflux pump due to an *mtrR* mutation is required for chromosomally mediated penicillin resistance in *Neisseria gonorrhoeae*. J Bacteriol 2002;184(20):5619-24.
131. Wadsworth CB, Arnold BJ, Sater MRA, Grad YH. Azithromycin resistance through interspecific acquisition of an epistasis-dependent efflux pump component and transcriptional regulator in *Neisseria gonorrhoeae*. mBio 2018;9(4).
132. Rouquette-Loughlin CE, Reimche JL, Balthazar JT, Dhulipala V, Gernert KM, Kersh EN, et al. Mechanistic basis for decreased antimicrobial susceptibility in a clinical isolate of *Neisseria gonorrhoeae* possessing a mosaic-like *mtr* efflux pump locus. mBio 2018;9(6).
133. Harrison OB, Maiden MC. Recent advances in understanding and combatting *Neisseria gonorrhoeae*: a genomic perspective. Fac Rev 2021;10:65.
134. Tapsall JW, Ndowa F, Lewis DA, Unemo M. Meeting the public health challenge of multidrug- and extensively drug-resistant *Neisseria gonorrhoeae*. Expert Rev Anti Infect Ther 2009;7(7):821-34.
135. World Health Organization (WHO). WHO guidelines for the treatment of *Neisseria gonorrhoeae*. 2016 [Accessed 2021-02-14]. Available from: http://www.who.int/reproductivehealth/publications/rtis/gonorrhoea-treatment-guidelines/en/.
136. Australasian Sexual Health Alliance (ASHA). Gonorrhoea. In: Australian STI management guidelines for use in primary care. 2016 [Accessed 2021-02-14]. Available from: www.sti.guidelines.org.au/sexually-transmissible-infections/gonorrhoea#management.
137. Romanowski B, Robinson J, Wong T. In: Canadian guidelines on sexually transmitted infections. Gonococcal infections chapter. Public Health Agency of Canada Ottawa, ON. 2013 [Accessed 2021-02-14]. Available from: https://www.phac-aspc.gc.ca/std-mts/sti-its/cgsti-ldcits/assets/pdf/section-5-6-eng.pdf.
138. Workowski KA, Bachmann LH, Chan PA, Johnston CM, Muzny CA, Park I, et al. Sexually transmitted infections treatment guidelines, 2021. MMWR Recomm Rep 2021;70(4):1-187.

139. Fifer H, Saunders J, Soni S, Sadiq ST, FitzGerald M. 2018 UK national guideline for the management of infection with *Neisseria gonorrhoeae*. Int J STD AIDS 2020;31(1):4-15.
140. Eyre DW, Town K, Street T, Barker L, Sanderson N, Cole MJ, et al. Detection in the United Kingdom of the *Neisseria gonorrhoeae* FC428 clone, with ceftriaxone resistance and intermediate resistance to azithromycin, October to December 2018. Euro Surveill 2019;24(10).
141. Terkelsen D, Tolstrup J, Johnsen CH, Lund O, Larsen HK, Worning P, et al. Multidrug-resistant *Neisseria gonorrhoeae* infection with ceftriaxone resistance and intermediate resistance to azithromycin, Denmark, 2017. Euro Surveill 2017;22(42).
142. Yuan Q, Li Y, Xiu L, Zhang C, Fu Y, Jiang C, et al. Identification of multidrug-resistant *Neisseria gonorrhoeae* isolates with combined resistance to both ceftriaxone and azithromycin, China, 2017-2018. Emerg Microbes Infect 2019;8(1):1546-9.
143. In: Skerman VBD, McGowan V, Sneath PHA, editors. Approved Lists of Bacterial Names (Amended). Washington (DC): ASM Press; 1989.
144. Everett KD, Bush RM, Andersen AA. Emended description of the order *Chlamydiales*, proposal of *Parachlamydiaceae* fam. nov. and *Simkaniaceae* fam. nov., each containing one monotypic genus, revised taxonomy of the family *Chlamydiaceae*, including a new genus and five new species, and standards for the identification of organisms. Int J Syst Bacteriol 1999;49 Pt 2:415-40.
145. Nicosia R, Pustorino R, Sessa R, Santino I, Simonetti G, Del Piano M. TWAR: a new chlamydial species? Boll Ist Sieroter Milan 1990;69(2):441-5.
146. Stephens RS, Myers G, Eppinger M, Bavoil PM. Divergence without difference: phylogenetics and taxonomy of *Chlamydia* resolved. FEMS Immunol Med Microbiol 2009;55(2):115-9.
147. Sachse K, Bavoil PM, Kaltenboeck B, Stephens RS, Kuo CC, Rosselló-Móra R, et al. Emendation of the family *Chlamydiaceae*: proposal of a single genus, *Chlamydia*, to include all currently recognized species. Syst Appl Microbiol 2015;38(2):99-103.
148. Borel N, Greub G. International Committee on Systematics of Prokaryotes (ICSP) Subcommittee on the taxonomy of *Chlamydiae*, minutes of the closed meeting, 10 September 2020, via Zoom. Int J Syst Evol Microbiol 2021;71(2).
149. Schoch CL, Ciufo S, Domrachev M, Hotton CL, Kannan S, Khovanskaya R, et al. NCBI Taxonomy: a comprehensive update on curation, resources and tools. Database (Oxford) 2020;2020.

150. Cheong HC, Lee CYQ, Cheok YY, Tan GMY, Looi CY, Wong WF. *Chlamydiaceae*: Diseases in Primary Hosts and Zoonosis. Microorganisms 2019;7(5).
151. Abdelrahman YM, Belland RJ. The chlamydial developmental cycle. FEMS Microbiol Rev 2005;29(5):949-59.
152. Caldwell HD, Kromhout J, Schachter J. Purification and partial characterization of the major outer membrane protein of *Chlamydia trachomatis*. Infect Immun 1981;31(3):1161-76.
153. Findlay HE, McClafferty H, Ashley RH. Surface expression, single-channel analysis and membrane topology of recombinant *Chlamydia trachomatis* major outer membrane protein. BMC Microbiol 2005;5:5.
154. Hatch TP. Disulfide cross-linked envelope proteins: the functional equivalent of peptidoglycan in chlamydiae? J Bacteriol 1996;178(1):1-5.
155. Wyllie S, Ashley RH, Longbottom D, Herring AJ. The major outer membrane protein of *Chlamydia psittaci* functions as a porin-like ion channel. Infect Immun 1998;66(11):5202-7.
156. Ortiz L, Angevine M, Kim SK, Watkins D, DeMars R. T-cell epitopes in variable segments of *Chlamydia trachomatis* major outer membrane protein elicit serovar-specific immune responses in infected humans. Infect Immun 2000;68(3):1719-23.
157. Stephens RS, Wagar EA, Schoolnik GK. High-resolution mapping of serovar-specific and common antigenic determinants of the major outer membrane protein of *Chlamydia trachomatis*. J Exp Med 1988;167(3):817-31.
158. Persson K. The role of serology, antibiotic susceptibility testing and serovar determination in genital chlamydial infections. Best Pract Res Clin Obstet Gynaecol 2002;16(6):801-14.
159. Batteiger BE, Lin PM, Jones RB, Van Der Pol BJ. Species-, serogroup-, and serovar-specific epitopes are juxtaposed in variable sequence region 4 of the major outer membrane proteins of some *Chlamydia trachomatis* serovars. Infect Immun 1996;64(7):2839-41.
160. de Vries HJ, Schim van der Loeff MF, Bruisten SM. High-resolution typing of *Chlamydia trachomatis*: epidemiological and clinical uses. Curr Opin Infect Dis 2015;28(1):61-71.
161. Brunham RC, Rey-Ladino J. Immunology of *Chlamydia* infection: implications for a *Chlamydia trachomatis* vaccine. Nat Rev Immunol 2005;5(2):149-61.
162. Gottlieb SL, Ndowa F, Hook EW, 3rd, Deal C, Bachmann L, Abu-Raddad L, et al. Gonococcal vaccines: Public health value and preferred product characteristics; report of a WHO global

stakeholder consultation, January 2019. Vaccine 2020;38(28): 4362-73.
163. Mohammed H, Blomquist P, Ogaz D, Duffell S, Furegato M, Checchi M, et al. 100 years of STIs in the UK: a review of national surveillance data. Sex Transm Infect 2018;94:553-8.
164. Newman L, Rowley J, Vander Hoorn S, Wijesooriya NS, Unemo M, Low N, et al. Global estimates of the prevalence and incidence of four curable sexually transmitted infections in 2012 based on systematic review and global reporting. PLoS One 2015; 10(12):e0143304.
165. Russell MW, Jerse AE, Gray-Owen SD. Progress toward a gonococcal vaccine: The way forward. Front Immunol 2019;10: 2417.
166. Eyre JWH, Stewart BH. The treatment of gonococcus infections by vaccines. Lancet 1909;174:76-81.
167. Greenberg L, Diena BB, Ashton FA, Wallace R, Kenny CP, Znamirowski R, et al. Gonococcal vaccine studies in Inuvik. Can J Public Health 1974;65(1):29-33.
168. Boslego JW, Tramont EC, Chung RC, McChesney DG, Ciak J, Sadoff JC, et al. Efficacy trial of a parenteral gonococcal pilus vaccine in men. Vaccine 1991;9(3):154-62.
169. Tramont EC. Gonococcal vaccines. Clin Microbiol Rev 1989;2 (Suppl):S74-7.
170. Jerse AE, Cohen MS, Drown PM, Whicker LG, Isbey SF, Seifert HS, et al. Multiple gonococcal opacity proteins are expressed during experimental urethral infection in the male. J Exp Med 1994;179(3):911-20.
171. Fussenegger M, Rudel T, Barten R, Ryll R, Meyer TF. Transformation competence and type-4 pilus biogenesis in *Neisseria gonorrhoeae*--a review. Gene;192(1):125-34.
172. Shafer WM, Datta A, Kolli VS, Rahman MM, Balthazar JT, Martin LE, et al. Phase variable changes in genes *lgtA* and *lgtC* within the *lgtABCDE* operon of *Neisseria gonorrhoeae* can modulate gonococcal susceptibility to normal human serum. J Endotoxin Res 2002;8(1):47-58.
173. Rice PA, Vayo HE, Tam MR, Blake MS. Immunoglobulin G antibodies directed against protein III block killing of serum-resistant *Neisseria gonorrhoeae* by immune serum. J Exp Med 1986;164(5):1735-48.
174. Ngampasutadol J, Ram S, Gulati S, Agarwal S, Li C, Visintin A, et al. Human factor H interacts selectively with *Neisseria gonorrhoeae* and results in species-specific complement evasion. J Immunol 2008;180(5):3426-35.

175. Ram S, Cullinane M, Blom AM, Gulati S, McQuillen DP, Monks BG, et al. Binding of C4b-binding protein to porin: a molecular mechanism of serum resistance of *Neisseria gonorrhoeae*. J Exp Med 2001;193(3):281-95.
176. Gulati S, Pennington MW, Czerwinski A, Carter D, Zheng B, Nowak NA, et al. Preclinical efficacy of a lipooligosaccharide peptide mimic candidate gonococcal vaccine. mBio 2019;10(6).
177. Jen FE, Semchenko EA, Day CJ, Seib KL, Jennings MP. The *Neisseria gonorrhoeae* methionine sulfoxide reductase (MsrA/B) is a surface exposed, immunogenic, vaccine candidate. Front Immunol 2019;10:137.
178. Semchenko EA, Day CJ, Seib KL. MetQ of *Neisseria gonorrhoeae* is a surface-expressed antigen that elicits bactericidal and functional blocking antibodies. Infect Immun 2017;85(2).
179. Sikora AE, Gomez C, Le Van A, Baarda BI, Darnell S, Martinez FG, et al. A novel gonorrhea vaccine composed of MetQ lipoprotein formulated with CpG shortens experimental murine infection. Vaccine 2020;38(51):8175-84.
180. Semchenko EA, Day CJ, Seib KL. The *Neisseria gonorrhoeae* vaccine candidate NHBA elicits antibodies that are bactericidal, opsonophagocytic and that reduce gonococcal adherence to epithelial cells. Vaccines 2020;8(2).
181. Balhuizen MD, Veldhuizen EJA, Haagsman HP. Outer membrane vesicle induction and isolation for vaccine development. Front Microbiol 2021;12:629090.
182. Sierra GV, Campa HC, Varcacel NM, Garcia IL, Izquierdo PL, Sotolongo PF, et al. Vaccine against group B *Neisseria meningitidis*: protection trial and mass vaccination results in Cuba. NIPH Ann 1991;14(2):195-210.
183. Thornton V, Lennon D, Rasanathan K, O'Hallahan J, Oster P, Stewart J, et al. Safety and immunogenicity of New Zealand strain meningococcal serogroup B OMV vaccine in healthy adults: beginning of epidemic control. Vaccine 2006;24(9):1395-400.
184. Rosenqvist E, Høiby EA, Wedege E, Bryn K, Kolberg J, Klem A, et al. Human antibody responses to meningococcal outer membrane antigens after three doses of the Norwegian group B meningococcal vaccine. Infect Immun 1995;63(12):4642-52.
185. Petousis-Harris H, Paynter J, Morgan J, Saxton P, McArdle B, Goodyear-Smith F, et al. Effectiveness of a group B outer membrane vesicle meningococcal vaccine against gonorrhoea in New Zealand: a retrospective case-control study. Lancet 2017;390(10102):1603-10.

186. Longtin J, Dion R, Simard M, Betala Belinga JF, Longtin Y, Lefebvre B, et al. Possible impact of wide-scale vaccination against serogroup B *Neisseria meningitidi*s on gonorrhea incidence rates in one region of Quebec, Canada. Open Forum Infect Dis 2017; 4:S734-5.
187. Reyes Díaz LM, Lastre González M, Cuello M, Sierra-González VG, Ramos Pupo R, Lantero MI, et al. VA-MENGOC-BC vaccination induces serum and mucosal anti *Neisseria gonorrhoeae* immune responses and reduces the incidence of gonorrhea. Pediatr Infect Dis J 2021;40(4):375-81.
188. Serruto D, Bottomley MJ, Ram S, Giuliani MM, Rappuoli R. The new multicomponent vaccine against meningococcal serogroup B, 4CMenB: immunological, functional and structural characterization of the antigens. Vaccine 2012;30(0 2):B87-97.
189. Mora M, Veggi D, Santini L, Pizza M, Rappuoli R. Reverse vaccinology. Drug Discov Today 2003;8(10):459-64.
190. Phillips S, Quigley BL, Timms P. Seventy years of *Chlamydia* vaccine research - limitations of the past and directions for the future. Front Microbiol 2019;10:70.
191. Kari L, Whitmire WM, Olivares-Zavaleta N, Goheen MM, Taylor LD, Carlson JH, et al. A live-attenuated chlamydial vaccine protects against trachoma in nonhuman primates. J Exp Med 2011;208(11):2217-23.
192. Stary G, Olive A, Radovic-Moreno AF, Gondek D, Alvarez D, Basto PA, et al. A mucosal vaccine against *Chlamydia trachomatis* generates two waves of protective memory T cells. Science 2015;348(6241):aaa8205.
193. Russi RC, Bourdin E, García MI, Veaute CMI. *In silico* prediction of T- and B-cell epitopes in PmpD: First step towards to the design of a *Chlamydia trachomatis* vaccine. Biomed J 2018;41(2):109-17.
194. Vasilevsky S, Stojanov M, Greub G, Baud D. Chlamydial polymorphic membrane proteins: regulation, function and potential vaccine candidates. Virulence 2016;7(1):11-22.
195. Murthy AK, Li W, Guentzel MN, Zhong G, Arulanandam BP. Vaccination with the defined chlamydial secreted protein CPAF induces robust protection against female infertility following repeated genital chlamydial challenge. Vaccine 2011;29(14):2519-22.
196. Zhu S, Chen J, Zheng M, Gong W, Xue X, Li W, et al. Identification of immunodominant linear B-cell epitopes within the major outer membrane protein of *Chlamydia trachomatis*. Acta biochimica et biophysica Sinica 2010;42(11):771-8.

197. Abraham S, Juel HB, Bang P, Cheeseman HM, Dohn RB, Cole T, et al. Safety and immunogenicity of the chlamydia vaccine candidate CTH522 adjuvanted with CAF01 liposomes or aluminium hydroxide: a first-in-human, randomised, double-blind, placebo-controlled, phase 1 trial. Lancet Infect Dis 2019;19(10):1091-100.
198. Olsen AW, Follmann F, Erneholm K, Rosenkrands I, Andersen P. Protection against *Chlamydia trachomatis* infection and upper genital tract pathological changes by vaccine-promoted neutralizing antibodies directed to the VD4 of the major outer membrane protein. J Infect Dis 2015;212(6):978-89.
199. Hobbs MM, Sparling PF, Cohen MS, Shafer WM, Deal CD, Jerse AE. Experimental gonococcal infection in male volunteers: cumulative experience with *Neisseria gonorrhoeae* strains FA1090 and MS11mkC. Front Microbiol 2011;2:123.
200. Hobbs MM, Anderson JE, Balthazar JT, Kandler JL, Carlson RW, Ganguly J, et al. Lipid A's structure mediates *Neisseria gonorrhoeae* fitness during experimental infection of mice and men. mBio 2013;4(6):e00892-13.
201. Cohen MS, Cannon JG, Jerse AE, Charniga LM, Isbey SF, Whicker LG. Human experimentation with *Neisseria gonorrhoeae*: rationale, methods, and implications for the biology of infection and vaccine development. J Infect Dis 1994;169(3):532-7.
202. Cohen MS, Cannon JG. Human experimentation with *Neisseria gonorrhoeae*: progress and goals. J Infect Dis 1999;179:S375-9.
203. Edwards JL, Jennings MP, Apicella MA, Seib KL. Is gonococcal disease preventable? The importance of understanding immunity and pathogenesis in vaccine development. Crit Rev Microbiol 2016;42(6):928-41.
204. Mabey DC, Hu V, Bailey RL, Burton MJ, Holland MJ. Towards a safe and effective chlamydial vaccine: lessons from the eye. Vaccine 2014;32(14):1572-8.
205. Jerse AE, Wu H, Packiam M, Vonck RA, Begum AA, Garvin LE. Estradiol-treated female mice as surrogate hosts for *Neisseria gonorrhoeae* genital tract infections. Front Microbiol 2011;2:107.
206. Tuffrey MA, Taylor-Robinson D. Progesterone as a key factor in the development of a mouse model for genital-tract infection with *Chlamydia trachomatis*. FEMS Microbiol Lett 1981;12:111-5.
207. Zarantonelli ML, Szatanik M, Giorgini D, Hong E, Huerre M, Guillou F, et al. Transgenic mice expressing human transferrin as a model for meningococcal infection. Infect Immun 2007;75(12): 5609-14.

208. Gu A, Zhang Z, Zhang N, Tsark W, Shively JE. Generation of human CEACAM1 transgenic mice and binding of *Neisseria* Opa protein to their neutrophils. PLoS One 2010;5(4):e10067.
209. Connolly KL, Pilligua-Lucas M, Gomez C, Costenoble-Caherty AC, Soc A, Underwood K, et al. Preclinical testing of vaccines and therapeutics for gonorrhea in female mouse models of lower and upper reproductive tract infection. J Infect Dis 2021;224(S2):S152-60.
210. Del Giudice G, Rappuoli R, Didierlaurent AM. Correlates of adjuvanticity: A review on adjuvants in licensed vaccines. Semin Immunol 2018;39:14-21.
211. Agren LC, Ekman L, Lowenadler B, Lycke NY. Genetically engineered nontoxic vaccine adjuvant that combines B cell targeting with immunomodulation by cholera toxin A1 subunit. J Immunol 1997;158(8):3936-46.
212. Vallely LM, Toliman P, Ryan C, Rai G, Wapling J, Gabuzzi J, et al. Performance of syndromic management for the detection and treatment of genital *Chlamydia trachomatis*, *Neisseria gonorrhoeae* and *Trichomonas vaginalis* among women attending antenatal, well woman and sexual health clinics in Papua New Guinea: a cross-sectional study. BMJ Open 2017;7(12):e018630.
213. Ison CA, Golparian D, Saunders P, Chisholm S, Unemo M. Evolution of *Neisseria gonorrhoeae* is a continuing challenge for molecular detection of gonorrhoea: false negative gonococcal *porA* mutants are spreading internationally. Sex Transm Infect 2013;89(3):197-201.
214. Schachter J. DFA, EIA, PCR, LCR and other technologies: what tests should be used for diagnosis of chlamydia infections? Immunol Invest 1997;26(1-2):157-61.
215. Unemo M, Ross J, Serwin AB, Gomberg M, Cusini M, Jensen JS. Background review for the '2020 European guideline for the diagnosis and treatment of gonorrhoea in adults'. Int J STD AIDS 2021;32(2):108-26.
216. Moncada J, Schachter J, Hook EW 3rd, Ferrero D, Gaydos C, Quinn TC, et al. The effect of urine testing in evaluations of the sensitivity of the Gen-Probe Aptima Combo 2 assay on endocervical swabs for *Chlamydia trachomatis* and *Neisseria gonorrhoeae*: the infected patient standard reduces sensitivity of single site evaluation. Sex Transm Dis 2004;31(5):273-7.
217. Masek BJ, Arora N, Quinn N, Aumakhan B, Holden J, Hardick A, et al. Performance of three nucleic acid amplification tests for detection of *Chlamydia trachomatis* and *Neisseria gonorrhoeae* by

use of self-collected vaginal swabs obtained via an Internet-based screening program. J Clin Microbiol 2009;47(6):1663-7.
218. Saiki RK, Scharf S, Faloona F, Mullis KB, Horn GT, Erlich HA, et al. Enzymatic amplification of beta-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. Science 1985;230(4732):1350-4.
219. Hofmann WP, Dries V, Herrmann E, Gärtner B, Zeuzem S, Sarrazin C. Comparison of transcription mediated amplification (TMA) and reverse transcription polymerase chain reaction (RT-PCR) for detection of hepatitis C virus RNA in liver tissue. J Clin Virol 2005;32(4):289-93.
220. Tabrizi SN, Tan LY, Walker S, Twin J, Poljak M, Bradshaw CS, et al. Multiplex assay for simultaneous detection of *Mycoplasma genitalium* and macrolide resistance using PlexZyme and PlexPrime technology. PLoS One 2016;11(6):e0156740.
221. Unemo M, Hansen M, Hadad R, Puolakkainen M, Westh H, Rantakokko-Jalava K, et al. Sensitivity, specificity, inclusivity and exclusivity of the updated Aptima Combo 2 assay, which provides detection coverage of the new diagnostic-escape *Chlamydia trachomatis* variants. BMC Infect Dis 2020;20(1):419.
222. Van Der Pol B, Fife K, Taylor SN, Nye MB, Chavoustie SE, Eisenberg DL, et al. Evaluation of the performance of the Cobas CT/NG Test for use on the Cobas 6800/8800 systems for detection of *Chlamydia trachomatis* and *Neisseria gonorrhoeae* in male and female urogenital samples. J Clin Microbiol 2019;57(4).
223. Van Der Pol B, Hook EW 3rd, Williams JA, Smith B, Taylor SN. Performance of the BD CTQx and GCQx amplified assays on the BD Viper LT compared with the BD Viper XTR system. Sex Transm Dis 2015;42(9):521-3.
224. Creighton S, Tenant-Flowers M, Taylor CB, Miller R, Low N. Co-infection with gonorrhoea and chlamydia: how much is there and what does it mean? Int J STD AIDS 2003;14(2):109-13.
225. Dicker LW, Mosure DJ, Berman SM, Levine WC. Gonorrhea prevalence and coinfection with chlamydia in women in the United States, 2000. Sex Transm Dis 2003;30(5):472-6.
226. Lewis N, Dube G, Carter C, Pitt R, Alexander S, Ison CA, et al. Chlamydia and gonorrhoea contamination of clinic surfaces. Sex Transm Infect 2012;88(6):418-21.
227. Sultan B, Benn P, Schembri G, Patel H, Brima N, Alexander S, et al. Test of cure study: a feasibility study to estimate the time to test of cure (TOC) for *Neisseria gonorrhoeae* and *Chlamydia trachomatis* infections. Sex Transm Infect 2020;96(6):402-7.

228. Wind CM, Schim van der Loeff MF, Unemo M, Schuurman R, van Dam AP, de Vries HJ. Time to clearance of *Chlamydia trachomatis* RNA and DNA after treatment in patients coinfected with *Neisseria gonorrhoeae* - a prospective cohort study. BMC Infect Dis 2016;16(1):554.
229. Sanger F, Nicklen S, Coulson AR. DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci USA 1977;74(12):5463-7.
230. Fleischmann RD, Adams MD, White O, Clayton RA, Kirkness EF, Kerlavage AR, et al. Whole-genome random sequencing and assembly of *Haemophilus influenzae* Rd. Science 1995;269(5223): 496-512.
231. Loman NJ, Constantinidou C, Chan JZ, Halachev M, Sergeant M, Penn CW, et al. High-throughput bacterial genome sequencing: an embarrassment of choice, a world of opportunity. Nat Rev Microbiol 2012;10(9):599-606.
232. Metzker ML. Emerging technologies in DNA sequencing. Genome Res 2005;15(12):1767-76.
233. Maiden MC, Bygraves JA, Feil E, Morelli G, Russell JE, Urwin R, et al. Multilocus sequence typing: a portable approach to the identification of clones within populations of pathogenic microorganisms. Proc Natl Acad Sci USA 1998;95(6):3140-5.
234. Martin IM, Ison CA, Aanensen DM, Fenton KA, Spratt BG. Rapid sequence-based identification of gonococcal transmission clusters in a large metropolitan area. J Infect Dis 2004;189(8):1497-505.
235. Demczuk W, Sidhu S, Unemo M, Whiley DM, Allen VG, Dillon JR, et al. *Neisseria gonorrhoeae* sequence typing for antimicrobial resistance, a novel antimicrobial resistance multilocus typing scheme for tracking global dissemination of *N. gonorrhoeae* strains. J Clin Microbiol 2017;55(5):1454-68.
236. Feil EJ, Li BC, Aanensen DM, Hanage WP, Spratt BG. eBURST: inferring patterns of evolutionary descent among clusters of related bacterial genotypes from multilocus sequence typing data. J Bacteriol 2004;186(5):1518-30.
237. Golparian D, Sánchez-Busó L, Cole M, Unemo M. *Neisseria gonorrhoeae* Sequence Typing for Antimicrobial Resistance (NG-STAR) clonal complexes are consistent with genomic phylogeny and provide simple nomenclature, rapid visualization and antimicrobial resistance (AMR) lineage predictions. J Antimicrob Chemother 2021;76(4):940-4.
238. Harrison OB, Cehovin A, Skett J, Jolley KA, Massari P, Genco CA, et al. *Neisseria gonorrhoeae* population genomics: use of the

gonococcal core genome to improve surveillance of antimicrobial resistance. J Infect Dis 2020;222(11):1816-25.
239. Golparian D, Unemo M. Now is the time to implement whole genome sequencing in the global antimicrobial resistance surveillance for *Neisseria gonorrhoeae*? EClinicalMedicine 2019; 7:11-2.
240. World Health Organization (WHO). Fifty-fifth World Health Assembly, resolution on prioritizing goals leading to im-proved reproductive health. 2002 [Accessed 2021-11-20]. Available from: https://apps.who.int/iris/bitstream/handle/10665/259364/WHA55-2002-REC1-eng.pdf.
241. World Health Organization (WHO). Global action plan to control the spread and impact of antimicrobial resistance in *Neisseria gonorrhoeae*. 2012 [Accessed 2020-01-22]. Available from: https://apps.who.int/iris/bitstream/handle/10665/44863/9789241503501_eng.pdf;jsessionid=19A2B806B71525B9B8C97924761AAD75?sequence=1.
242. Regeringskansliet. Nationell strategi mot hiv/aids och andra smittsamma sjukdomar. 2017 [Accessed 2020-05-13]. Available from: https://www.regeringen.se/4ad776/globalassets/regeringen/dokument/socialdepartementet/fokhalsa-och-sjukvard/nationell-strategi-mot-hiv-aids-och-vissa-andra-smittsamma-sjukdomar.pdf.
243. Unemo M, Fasth O, Fredlund H, Limnios A, Tapsall J. Phenotypic and genetic characterization of the 2008 WHO *Neisseria gonorrhoeae* reference strain panel intended for global quality assurance and quality control of gonococcal antimicrobial resistance surveillance for public health purposes. J Antimicrob Chemother 2009;63(6):1142-51.
244. Buckley C, Trembizki E, Donovan B, Chen M, Freeman K, Guy R, et al. A real-time PCR assay for direct characterization of the *Neisseria gonorrhoeae* GyrA 91 locus associated with ciprofloxacin susceptibility. J Antimicrob Chemother 2016;71(2):353-6.
245. Argimón S, Abudahab K, Goater RJE, Fedosejev A, Bhai J, Glasner C, et al. Microreact: visualizing and sharing data for genomic epidemiology and phylogeography. Microb Genom 2016;2(11): e000093.
246. Collar AL, Linville AC, Core SB, Wheeler CM, Geisler WM, Peabody DS, et al. Antibodies to variable domain 4 linear epitopes of the *Chlamydia trachomatis* major outer membrane protein are not associated with chlamydia resolution or reinfection in women. mSphere 2020;5(5).

247. Gupta VK, Waugh CA, Ziklo N, Huston WM, Hocking JS, Timms P. Systemic antibody response to *Chlamydia trachomatis* infection in patients either infected or reinfected with different *Chlamydia* serovars. Indian J Med Microbiol 2017;35(3):394-401.
248. Liu C, Hufnagel K, O'Connell CM, Goonetilleke N, Mokashi N, Waterboer T, et al. Reduced endometrial ascension and enhanced reinfection associated with IgG antibodies to specific *Chlamydia trachomatis* proteins in women at risk for chlamydia. J Infect Dis 2022;225(5):846-55.
249. Bøje S, Olsen AW, Erneholm K, Agerholm JS, Jungersen G, Andersen P, et al. A multi-subunit *Chlamydia* vaccine inducing neutralizing antibodies and strong IFN-γ^+ CMI responses protects against a genital infection in minipigs. Immunol Cell Biol 2016;94(2):185-95.
250. Yu H, Karunakaran KP, Jiang X, Chan Q, Rose C, Foster LJ, et al. Comparison of *Chlamydia* outer membrane complex to recombinant outer membrane proteins as vaccine. Vaccine 2020;38(16):3280-91.
251. Olsen AW, Lorenzen EK, Rosenkrands I, Follmann F, Andersen P. Protective effect of vaccine promoted neutralizing antibodies against the intracellular pathogen *Chlamydia trachomatis*. Front Immunol 2017;8:1652.
252. Eleutério J, Jr., Teles RA, Linhares IM, Normand N, Witkin SS. Interferon-gamma gene polymorphism influences the frequency of a *Chlamydia trachomatis* cervical infection in young women. Int J STD AIDS 2015;26(13):960-4.
253. Labuda JC, McSorley SJ. Diversity in the T cell response to *Chlamydia*-sum are better than one. Immunol Lett 2018;202:59-64.
254. Redgrove KA, McLaughlin EA. The role of the immune response in *Chlamydia trachomatis* infection of the male genital tract: A double-edged sword. Front Immunol 2014;5:534.
255. Gondek DC, Roan NR, Starnbach MN. T cell responses in the absence of IFN-gamma exacerbate uterine infection with *Chlamydia trachomatis*. J Immunol 2009;183(2):1313-9.
256. O'Meara CP, Armitage CW, Harvie MC, Timms P, Lycke NY, Beagley KW. Immunization with a MOMP-based vaccine protects mice against a pulmonary *Chlamydia* challenge and identifies a disconnection between infection and pathology. PLoS One 2013;8(4):e61962.
257. Kalbina I, Wallin A, Lindh I, Engstrom P, Andersson S, Strid K. A novel chimeric MOMP antigen expressed in *Escherichia coli*, *Arabidopsis thaliana*, and *Daucus carota* as a potential *Chlamydia*

trachomatis vaccine candidate. Protein Expr Purif 2011;80(2):194-202.
258. Murray SM, McKay PF. *Chlamydia trachomatis*: Cell biology, immunology and vaccination. Vaccine 2021;39(22):2965-75.
259. Lorenzen E, Follmann F, Bøje S, Erneholm K, Olsen AW, Agerholm JS, et al. Intramuscular priming and intranasal boosting induce strong genital immunity through secretory IgA in minipigs infected with *Chlamydia trachomatis*. Front Immunol 2015;6:628.
260. Schneider MC, Exley RM, Chan H, Feavers I, Kang YH, Sim RB, et al. Functional significance of factor H binding to *Neisseria meningitidis*. J Immunol 2006;176(12):7566-75.
261. Jongerius I, Lavender H, Tan L, Ruivo N, Exley RM, Caesar JJ, et al. Distinct binding and immunogenic properties of the gonococcal homologue of meningococcal factor h binding protein. PLoS Pathog 2013;9(8):e1003528.
262. Beernink PT, LoPasso C, Angiolillo A, Felici F, Granoff D. A region of the N-terminal domain of meningococcal factor H-binding protein that elicits bactericidal antibody across antigenic variant groups. Mol Immunol 2009;46(8-9):1647-53.
263. Beernink PT, Welsch JA, Bar-Lev M, Koeberling O, Comanducci M, Granoff DM. Fine antigenic specificity and cooperative bactericidal activity of monoclonal antibodies directed at the meningococcal vaccine candidate factor H-binding protein. Infect Immun 2008;76(9):4232-40.
264. Scarselli M, Cantini F, Santini L, Veggi D, Dragonetti S, Donati C, et al. Epitope mapping of a bactericidal monoclonal antibody against the factor H binding protein of *Neisseria meningitidis*. J Mol Biol 2009;386(1):97-108.
265. Faleri A, Santini L, Brier S, Pansegrau W, Lo Surdo P, Scarselli M, et al. Two cross-reactive monoclonal antibodies recognize overlapping epitopes on *Neisseria meningitidis* factor H binding protein but have different functional properties. FASEB J 2014;28(4):1644-53.
266. Maritan M, Cozzi R, Lo Surdo P, Veggi D, Bottomley MJ, Malito E. Crystal structures of human Fabs targeting the Bexsero meningococcal vaccine antigen NHBA. Acta Crystallogr F Struct Biol Commun 2017;73(Pt 6):305-14.
267. Maritan M, Veggi D, Cozzi R, Dello Iacono L, Bartolini E, Lo Surdo P, et al. Structures of NHBA elucidate a broadly conserved epitope identified by a vaccine induced antibody. PLoS One 2018;13(8):e0201922.
268. Semchenko EA, Mubaiwa TD, Day CJ, Seib KL. Role of the gonococcal neisserial heparin binding antigen in microcolony

formation, and serum resistance and adherence to epithelial cells. J Infect Dis 2020;221(10):1612-22.
269. Semchenko EA, Tan A, Borrow R, Seib KL. The serogroup B meningococcal vaccine Bexsero elicits antibodies to *Neisseria gonorrhoeae*. Clin Infect Dis 2019;69(7):1101-11.
270. Muzzi A, Mora M, Pizza M, Rappuoli R, Donati C. Conservation of meningococcal antigens in the genus *Neisseria*. mBio 2013;4(3):e00163-13.
271. Leduc I, Connolly KL, Begum A, Underwood K, Darnell S, Shafer WM, et al. The serogroup B meningococcal outer membrane vesicle-based vaccine 4CMenB induces cross-species protection against *Neisseria gonorrhoeae*. PLoS Pathog 2020;16(12): e1008602.
272. Matthias KA, Connolly KL, Begum AA, Jerse AE, Macintyre AN, Sempowski GD, et al. Meningococcal detoxified outer membrane vesicle vaccines enhance gonococcal clearance in a murine infection model. J Infect Dis 2022;225(4):650-60.
273. Craig AP, Gray RT, Edwards JL, Apicella MA, Jennings MP, Wilson DP, et al. The potential impact of vaccination on the prevalence of gonorrhea. Vaccine 2015;33(36):4520-5.
274. Ebeyan S, Windsor M, Bordin A, Mhango L, Erskine S, Trembizki E, et al. Evaluation of the ResistancePlus GC (beta) assay: a commercial diagnostic test for the direct detection of ciprofloxacin susceptibility or resistance in *Neisseria gonorrhoeae*. J Antimicrob Chemother 2019;74(7):1820-4.
275. Lee DYJ, Ashcroft MM, Chow EPF, Sait M, De Petra V, Tschaepe M, et al. Reflex detection of ciprofloxacin resistance in *Neisseria gonorrhoeae* by use of the SpeeDx ResistancePlus GC assay. J Clin Microbiol 2021;59(5).
276. Allan-Blitz LT, Hemarajata P, Humphries RM, Wynn A, Segura ER, Klausner JD. A cost analysis of *gyrase A* testing and targeted ciprofloxacin therapy versus recommended 2-drug therapy for *Neisseria gonorrhoeae* infection. Sex Transm Dis 2018;45(2):87-91.
277. Dona V, Smid JH, Kasraian S, Egli-Gany D, Dost F, Imeri F, et al. Mismatch amplification mutation assay-based real-time PCR for rapid detection of *Neisseria gonorrhoeae* and antimicrobial resistance determinants in clinical specimens. J Clin Microbiol 2018;56(9).
278. Trembizki E, Buckley C, Donovan B, Chen M, Guy R, Kaldor J, et al. Direct real-time PCR-based detection of *Neisseria gonorrhoeae* 23S rRNA mutations associated with azithromycin resistance. J Antimicrob Chemother 2015;70(12):3244-9.

279. Roberts MC, Chung WO, Roe D, Xia M, Marquez C, Borthagaray G, et al. Erythromycin-resistant *Neisseria gonorrhoeae* and oral commensal *Neisseria* spp. carry known rRNA methylase genes. Antimicrob Agents Chemother 1999;43(6):1367-72.
280. Swedres-Svarm. Sales of antibiotics and occurrence of resistance in Sweden. 2019. Public Health Agency of Sweden and National Veterinary Institute. Solna/Uppsala ISSN1650-6332.
281. European Centre for Disease Prevention and Control. Data from the ECDC Surveillance Atlas of Infectious Diseases. [Accessed 2022-01-31]. Available from: https://atlas.ecdc.europa.eu/public/index.aspx?Dataset=27&HealthTopic=4.
282. Truong HM, Fatch R, Grasso M, Robertson T, Tao L, Chen YH, et al. Gay and bisexual men engage in fewer risky sexual behaviors while traveling internationally: a cross-sectional study in San Francisco. Sex Transm Infect 2015;91(3):220-5.
283. Zablotska IB, Holt M, de Wit J, Mao L, Down I, Prestage G. At home and away: gay men and high risk sexual practices. AIDS Behav 2014;18(8):1436-42.
284. Alfsnes K, Eldholm V, Olsen AO, Brynildsrud OB, Bohlin J, Steinbakk M, et al. Genomic epidemiology and population structure of *Neisseria gonorrhoeae* in Norway, 2016-2017. Microb Genom 2020;6(4).
285. Shimuta K, Unemo M, Nakayama S, Morita-Ishihara T, Dorin M, Kawahata T, et al. Antimicrobial resistance and molecular typing of *Neisseria gonorrhoeae* isolates in Kyoto and Osaka, Japan, 2010 to 2012: intensified surveillance after identification of the first strain (H041) with high-level ceftriaxone resistance. Antimicrob Agents Chemother 2013;57(11):5225-32.
286. Buckley C, Forde BM, Trembizki E, Lahra MM, Beatson SA, Whiley DM. Use of whole genome sequencing to investigate an increase in *Neisseria gonorrhoeae* infection among women in urban areas of Australia. Sci Rep 2018;8(1):1503.
287. Straub C, Thirkell C, Tiong A, Woodhouse R, Szeto J, Dyet KH. The antimicrobial resistance landscape of *Neisseria gonorrhoeae* in New Zealand from November 2018 to March 2019 and the role of sexual orientation in transmission. Microb Genom 2021;7(11).
288. Pathogenwatch. Global platform for genomic surveillance. [Accessed 2022-02-08]. Available from: https://pathogen.watch/.
289. Jolley KA, Bray JE, Maiden MCJ. Open-access bacterial population genomics: BIGSdb software, the PubMLST.org website and their applications. Wellcome Open Res 2018;3:124.

290. Ohnishi M, Watanabe Y, Ono E, Takahashi C, Oya H, Kuroki T, et al. Spread of a chromosomal cefixime-resistant *penA* gene among different *Neisseria gonorrhoeae* lineages. Antimicrob Agents Chemother 2010;54(3):1060-7.
291. Shimuta K, Watanabe Y, Nakayama S, Morita-Ishihara T, Kuroki T, Unemo M, et al. Emergence and evolution of internationally disseminated cephalosporin-resistant *Neisseria gonorrhoeae* clones from 1995 to 2005 in Japan. BMC Infect Dis 2015;15:378.
292. Thomas JC, Joseph SJ, Cartee JC, Pham CD, Schmerer MW, Schlanger K, et al. Phylogenomic analysis reveals persistence of gonococcal strains with reduced-susceptibility to extended-spectrum cephalosporins and mosaic *penA*-34. Nat Commun 2021;12(1):3801.
293. Jacobsson S, Cole MJ, Spiteri G, Day M, Unemo M. Associations between antimicrobial susceptibility/resistance of *Neisseria gonorrhoeae* isolates in European Union/European Economic Area and patients' gender, sexual orientation and anatomical site of infection, 2009-2016. BMC Infect Dis 2021;21(1):273.
294. Golparian D, Unemo M. Antimicrobial resistance prediction in *Neisseria gonorrhoeae*: Current status and future prospects. Expert Rev Mol Diagn 2022;22(1):29-48.