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Neisseria gonorrhoeae

Methods and Protocols

Edited by

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Cover Caption: *Neisseria gonorrhoeae* infection remodels microvilli in human 3-dimensional (3-D) endometrial cell model. Scanning electron microscopy image of 3-D human cells infected with *N. gonorrhoeae* MS11 at a multiplicity of infection of 10 for 4h. Gonococci were pseudo-colored red using Photoshop C5.1 software (Adobe). Image courtesy of Paweł Łaniewski and Melissa M. Herbst-Kralovetz.

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Preface

Introduction

"Gonorrhoea is as old as the world" (George Luys) [1]. *Neisseria gonorrhoeae* (gonococcus) is an obligate human pathogen that has coexisted with humanity and causes the sexually transmitted disease gonorrhea. Gonococci infect principally the mucosal epithelium of the genitourinary tract and can also infect anorectal and pharyngeal mucosal surfaces [2]. In men, infection of the urethra causes urethritis and painful discharge, and untreated infection may cause epididymitis leading to infertility. In women, localized infection of the ectocervix and endocervix leads to a mucopurulent cervicitis: however, infection is frequently asymptomatic, and in ~10–25% of untreated women, gonococci can ascend into the upper reproductive tract (URT). The host response to ascending gonococcal infection is pelvic inflammatory disease syndrome, which is an umbrella term for severe inflammatory conditions affecting all parts of the URT, e.g., endometritis, pelvic peritonitis (tubal, ovarian), and fallopian tube salpingitis [3]. Disseminated infection is rare but can present as arthritis, perihepatitis, meningitis, or endocarditis. Long-term and permanent sequelae resulting from untreated infection include chronic pelvic pain, tubal damage, ectopic pregnancy, and infertility. Chorioamnionitis, septic abortion, preterm delivery, and premature rupture of membranes can occur in infected pregnant women. Gonococci can also infect neonates by vertical transmission during birth and cause *ophthalmia neonatorum* (neonatal conjunctivitis) that leads to irreparable corneal tissue destruction and blindness [2]. In addition, there is a strong association between maternal gonorrhea with premature delivery and low neonatal birth weight [4]. More rarely observed in neonates are abscesses, meningitis, and sepsis.

The WHO estimates that ~78 million people are infected with gonorrhea worldwide, which is probably an underestimate (http://www.who.int/en/news-room/fact-sheets/detail/sexually-transmitted-infections-(stis)) [5]. Antibiotics have been tremendously successful for treating gonorrhea, but treatment is now severely compromised by the emergence of gonococci resistant to "last-resort" antibiotics [6]. Gonococci are now colloquially referred to as a "superbug" and listed by the WHO as a high-priority pathogen for research and development of new antimicrobials and vaccines. Despite the good intentions of global initiatives and funding for developing antimicrobials, the current pipeline for new anti-gonococcal treatments is dismal. Potentially untreatable gonorrhea has arrived, and prevention through vaccination is now a priority [7], as highlighted by the summary and recommendations of a NIAID workshop on gonorrhea vaccines held in 2015 [8].

A Brief History of Gonorrhea

Sexually transmitted diseases (STDs), known also as venereal diseases (VD), as derived from Venus, the Roman goddess of love, are known to have existed from antiquity and are recorded in many of humanity's earliest surviving texts. The compelling descriptions offered by these texts suggest that sexually transmitted infections such as syphilis, gonorrhea/chlamydia, herpes, and genital warts have been a constant scourge to human health.

However, in the absence of microbiological proof, the provenance of many of these diseases could be attributable to fungal, protozoan, and ectoparasitic infection.

There is sufficient circumstantial evidence that gonorrhea existed as an STD in early human cultures. As a starting reference point, the George Ebers papyrus, which was found in a temple at the city of Abydos in upper Egypt, is believed to date from 1555 BC but most certainly reflects circumstances dating back to 3000 BC (http://digi.ub.uniheidelberg.de/diglit/ebers1875bd1, http://digi.ub.uni-heidelberg.de/diglit/ebers1875bd2) [9]. Gynecology is a major theme of this Egyptian text, and mention is made of pelvic (inflammatory) disease (e.g., salpingitis, pelvic adhesions), leukorrhea (mucus discharge from the vagina), and "burning micturition," all clinical signs suggestive of gonorrhea infection [10]. Unfortunately, there are no Egyptian records of painful urination accompanying urethral discharge in men, although orchitis, a reference to epididymitis and suggestive of gonorrhea, is mentioned [10]. However, urethral gonorrhea is a recognized disease in the Bible, wherein it is commonly described as the "issue" and explicit measures are provided for disease control. In Leviticus 15:2–12, there is a description of male discharge that can render him "unclean," and in Leviticus 22:4, "any descendent. . .who has a male discharge may not eat any sacred offerings." Recommendations for disease control included social exclusion (quarantine) for 7 days after cessation of the discharge and disinfection of the patient and belongings [11]. In the Book of Numbers (fourth Book of the Law of Moses/Torah or Pentateuch), which chronicles the exodus of Moses and the Israelites from Egypt and their 40-year wandering, mention is made in Chapter 5:2 of the command to "cast (the unclean) out of the camp. . .whosoever hath an issue of seed." There is also the record in Chapter 31: 35 of the army returning from war with the Midianites (who had drawn the Israelites "into sin"), with "32,000 of the female sex, that had not known men," and explicit instructions (31:19–20) to "stay without the camp seven days." Moses' troops were considered to have returned with the "issue," and the chapter recites how Moses dealt with infection by quarantine and disinfection of individuals and their garments and possessions [10].

By the fourth century BC, Hippocrates (460–375 BC) had noted that the introduction of sexual mores and practices from Asia and Egypt into Greece was accompanied by STDs [12]. He described a disease suggestive of a chronic suppurative renal infection or a sexually transmitted urethritis, complicated by renal involvement. Hippocrates termed this "strangury" ("Their urine was copious, thick, varied, mixed with pus, and passed with pain"). Interestingly, gonorrhea in women was not recognized easily. Weatherhead presents a summary of the history of venereal disease, including gonorrhea [13], and cites passages by Herodotus (484–425 BC) that report a "female disease" that is surmised to be gonorrhea. As the Roman Empire superseded Greek dominion, many Greek physicians immigrated to Rome to practice medicine. The Roman Gaius Plinius Secundus, (23–79 AD, Pliny the Elder) in his *Naturalis Historia* describes a *profluvia geniturae viris*, which is generally thought to describe a male urethral discharge. The Greco-Roman gynecologist Soranus of Ephesus (practicing between 98 and 138 AD) in his remarkable book titled *Gynecology* provides instructions for "treating the eyes (of newborns) by an injection of olive oil; for it is good thus to wash off the thickest moisture in them; if it is not done, in most cases the nurslings become dim-sighted" (Book 2; viii)—a possible treatment for gonococcal conjunctivitis (http://hdl.handle.net/2027/fulcrum.n870zr06z). Weatherhead also cites Actuarius' description of a flux and a "profluvium seminis sine voluptate" (i.e., seminal discharge without pleasure) [13]. But it is to Aelius Galen (Claudius Galen, Galen of Pergamon, 129–216 AD), arguably the most important Greek physician in Rome, to whom we owe the first mention of the word "gonorrhea." However, Galen mistook the discharge of urethral pus from a male as semen, hence from the Greek words *gonos* (semen) and *rhoia* (flow).

Galen's ideas on medicine prevailed into the Middle Ages and were preserved along with the major works of the Greco-Roman period in Byzantine medicine. Gonorrhea and other venereal diseases were rife in the Middle Ages and are mentioned in both Christian and Arabic texts [14]. The surgeon John Arderne (English, 1307–1392), in 1376, described a condition called *la chaude-pisse* (literally "hot piss"), i.e., a burning sensation during micturition in both men and women, and the term has been colloquially adopted to describe gonorrhea. Other colloquialisms include "the clap," which may originate from "le clapier," French for "house of prostitutes." It is interesting to note that until the sixteenth century, syphilis and gonorrhea were considered different diseases. In 1496, the humanist and physician Joseph Grünpeck (German, 1473–1532) gave clear descriptions of mixed infections of syphilis and gonorrhea, or colloquially known as the "French disease" [15]. VD has been a common subject in (nonmedical) literature from Greco-Roman times to the modern day [16, 17]. Gonorrhea does not escape even the attentions of Shakespeare (1564–1616); it is probably no accident that in *King Lear* (written in 1603–1606) the evil daughter is named Goneril (the name itself a reference back to Geoffrey of Monmouth's character name Gonorilla in *The History of the Kings of Britain*, written in 1136). Shakespeare intended Goneril to be like a "disease full of venom," and the imagery of STDs, putrescence, and pathogenic bodies is very intense in this particular play.

Later, distinguished scientists/clinicians, such as Ambroise Pare (French, 1510–1590), Thomas Sydenham (English, 1624–1689), and John Hunter (Scottish, 1728–1793), all thought that syphilis and gonorrhea were one and the same [18–20]. However, the surgeon Benjamin Bell (Scottish, 1749–1806) published in 1793 *A Treatise on Gonorrhoea Virulenta and Lues Venerea* in which he contended that the signs and symptoms of syphilis and gonorrhea were indeed distinct and the diseases separate [21], a conclusion supported by the physician Philippe Ricord (French, 1800–1889) in 1838. In 1815, the venereologist Francois Swediaur (Austrian, 1748–1824) coined the terms blennorrhagia and blennorrhea to describe acute and chronic gonorrhea [22]. Interestingly, Ricord recommended the rapid application of silver nitrate to preserve sight when "gonorrheal matter" was found in the conjunctiva of the eyes, thereby laying down the first recorded use of this compound for the treatment of gonococcal ocular infection [23]. Both Bell and Ricord also argued that mercury was effective for treating syphilis, but not gonorrhea. For the interested reader, Benedek provides an excellent review of research on ocular inflammation associated with gonorrhea and the studies in the eighteenth to early twentieth century on the experimental induction of gonorrheal ophthalmia in humans (including the ethically unacceptable practice of application of gonorrheal cultures to the eyes of sick children), following the observations that animals were refractory to gonococcal infection [24]. It is noteworthy that the ophthalmologist Joseph F. Piringer (Austrian, 1800–1879) inoculated the eyes of blind people with blennorrheal (gonorrheal) pus, some 38 years before the discovery of the gonococcus, as an attempt to cure those patients with eyes damaged by trachoma [24, 25]. In 1872, the gynecologist Emil Jacob Noeggerath (American, 1827–1895) described latent gonorrhea in women and subsequently on its influence especially on female fertility [26, 27].

Albert Ludwig Siegmund Neisser (German, 1855–1916) is credited with the discovery of the gonococcus and is arguably the first microbiologist to attribute a chronic human disease to a microorganism. In his seminal paper of 1879, Neisser used Robert Koch's (German, 1843–1910) methyl violet staining technique to study smears from 35 men and

9 women with purulent urethritis and 2 patients with acute ophthalmia. Neisser noted that "If gonorrheal pus is spread out in a layer, allowed to dry, stained by methyl violet, a number of masses of micrococci are seen. They have a characteristic, typical form. These characteristic micrococci appear to be a constant mark of all gonorrheal affections" [28]. The organism was named *Neisseria gonorrhoeae* to respect Neisser [19, 29].

Progress in gonococcal biology was rapid over the following decade. Following Neisser's first identification and isolation, Frédéric Weiss (French) isolated *Neisseria gonorrhoeae* in 1880 [30], and Leo Leistikow (German, 1847–1917) followed suit in 1882 [31, 32]. In 1880–1881, the surgeon Alexander Ogston (Scottish, 1844–1929) examined a variety of lesions associated with gonorrhea, soft chancre, sycosis (hair follicle inflammation), sputa from pulmonary tuberculosis, and discharges from wounds and ulcers [33, 34]. He reported the presence of micrococci within gonorrhea lesions, which, reportedly, he was able to culture in fresh eggs. The surgeon and pioneering microbiologist George Sternberg (American, 1838–1915) also confirmed that no microorganism other than the gonococcus (micrococcus) was detectable in gonorrhea secretions [35].

Neisser was unable to satisfy Koch's postulates with the organism he had isolated on a meat extract—gelatin medium, but his identification of the pathogen led to decades of experimental gonococcal infection studies in humans that, in retrospect, are central to the development of clinical research ethics [24]. Koch's postulates were subsequently satisfied by the experiments of the physicians Arpad Bokai (Hungarian, 1856–1919) and Max Bockhart (German, 1883–1921) and the gynecologists Ernst von Bumm (German, 1858–1925) and Ernst Wertheim (Austrian, 1864–1920). In 1880, Bokai confirmed Neisser's microscopy observations and also inoculated six students urethrally with gonococcal culture fluid and reported that three of the patients developed acute gonorrhea [36]. In 1883, Bockhart inoculated the urethra of a man and reported the development of classical gonococcal urethritis after 3 days [37]. In 1885, von Bumm grew axenic cultures of Neisser's gonococcus, *Neisseria gonorrhoeae*, and proved by inoculations of humans that it causes gonorrhea (urethritis) [38, 39]. This experimental urethritis was reproduced by Wertheim in 1891 [40]. In 1890, Wertheim had already demonstrated the existence of gonococci in fallopian tube tissue, and in 1892, he established the hypothesis that gonococci could ascend the female reproductive tract [41]. In 1895, he further demonstrated the presence of gonococci in acute cystitis (bladder gonorrhea) [42] and, a year later, the importance of latent uterine gonorrhea [43]. In 1893, Steinschneider, working in Neisser's laboratory, was able to induce gonorrhea in a colleague following urethral instillation of bacteria [44]. In the same year, the dermato-venereologist Ernst Finger (Austrian, 1856–1939) and his colleagues asked the question as to whether previous gonorrhea infection conferred immunity against reinfection. In this pioneering experiment, he instilled gonococci into the urethra of six men who had a history of gonorrhea but were currently "healthy." Each subject subsequently developed gonorrhea, leading him to conclude that "the gonorrheal process is capable of re-infection and super-infection" [45]. Other inoculation experiments by the surgeon Edward Martin (American, 1859–1938) in 1982 [46], the pediatrician Henry Heiman (American) in 1895 [47], and Jundell and Ahman (Swedish) in 1897 [48] served to reinforce the now obvious conclusion that gonococci experimentally instilled into the urethra can induce gonorrhea. By the turn of the twentieth century, nothing further could be learned about gonococcal pathogenesis from these human inoculation experiments, and they were essentially abandoned. Ethical criticism of some of these studies was probably also a major factor in their discontinuation, given that some were particularly unpalatable, e.g., Heiman's instillation of gonococci into the urethra of intellectually disabled children aged 4 and 16.

Gonorrhea was now accepted as an STD caused by a singular pathogen, *N. gonorrhoeae*, and attention turned to the pathogen's involvement in extragenital infections and to pathological studies. Gonococcal conjunctivitis was well known, and there were anecdotal reports of gonococci associated with arthritis, which was eventually proved in 1894 by the clinician Guido Bordoni-Uffreduzzi (Italian, 1859–1943), who was able to culture gram-negative cocci from pus obtained from the ankle of a woman with gonorrhea. Incontrovertible proof that this organism was a gonococcus was provided by his subsequent inoculation of the urethra of a healthy male volunteer, with no history of STDs, with the cultured organism, which resulted in typical gonorrhea [49]. By the end of this decade, the neurosurgeon and pathologist Harvey Williams Cushing (American, 1869–1939) had reported also that a vaginal gonococcus infection could eventually lead to acute diffuse peritonitis, demonstrating further the ability of the gonococcus to disseminate [50]. An extensive historical literature is also available for pathological findings from studying patient cadavers and biopsy materials from diseased organs [25].

Isolation and culture of the gonococcus now provided an opportunity in the twentieth century for fundamental studies to understand the ultrastructure of the pathogen, its virulence factors, the immunobiology of gonococcal interactions with the host, the nature of the host innate, and adaptive immune responses and to develop effective treatments and potential prophylactics. But it was not until 1938 that the first drug to reliably cure gonorrhea was introduced, sulfanilamide; however, gonococcal resistance rapidly emerged to this drug, a portent of the ensuing and enduring arms race of new antimicrobial chemotherapies and gonococcal adaptability. In addition, vaccines for gonorrhea remain an elusive goal.

Book Synopsis

The first chapter of *Neisseria gonorrhoeae: Methods and Protocols* is a review of the biology of the gonococcus, and this is followed by a brief description of an algorithm for examining infection in women. Antimicrobial resistance (AMR) in gonococci is a major global health problem, and a review examines comprehensively the development of AMR in this pathogen and the strategies for treatment going forward. This links with a chapter that provides a protocol for whole-genome sequencing to predict antimicrobial resistance in gonococci. A seminal chapter follows on colony phenotyping of gonococci, which is an essential method that underpins many laboratory studies. Methods chapters are also provided for producing major gonococcal antigens to high purity, i.e., peptidoglycan, pilus, and lipooligosaccharide (LOS), and outer membranes (OM). High-purity antigens are useful for structural and biochemical studies, for vaccine studies, and for examining their roles in pathogen interactions with the host.

A couple of chapters follow that provide protocols for genetic transformation of gonococci and strategies for global RNA sequencing, in order to study the gonococcal regulatory responses to specific host environments. With no vaccines currently available for *Neisseria gonorrhoeae*, much effort has gone toward identifying potential vaccine candidates. In this book, we have chapters that provide protocols for using high-throughput processes, e.g., bioinformatics workflows for gonococcal proteomics and phenotypic microarray screening of gonococci in chemically defined liquid medium, to evaluate potential vaccine/drug targets and the assessment of hypothetical protein function. There is a companion chapter describing how to grow gonococci in metal-depleted conditions, which leads to the expression of virulence-associated factors that may be important vaccine targets. [Other vaccine strategies, such as immuno-proteomics, antigen identification starting from the genome (reverse vaccinology), and DNA vaccination, can be found in a previous volume on the sister organism *N. meningitidis*, and the interested reader is directed there for methods that are transferable to the gonococcus [51].] In anticipation that new gonococcal vaccines probably will contain recombinant protein antigens, a chapter is provided that comprehensively guides the reader through all the steps required for protein production. One potential measure of vaccine efficacy will be the demonstration of an induced bactericidal response, and a following chapter is provided that explains how to do gonococcal human serum bactericidal assays (hSBA).

Finally, a large proportion of the book is devoted to methods and protocols for studying the biological interactions of the gonococcus and the host. A great deal is still unknown about how gonococci cause disease, and methods and protocols are provided for cell culture models (with primary human macrophages, neutrophils, epithelial cells in three dimensions), ex vivo tissue (human fallopian tubes, bovine eye tissue), in vivo murine models, and finally a guide to the experimental human challenge model.

Acknowledgments

Many of the techniques described herein should have broad appeal not only to the experienced *Neisseria* researcher but also to new researchers seeking to work with this pathogen. Many of the methods may appear daunting and/or require specialist training, but I do hope that they offer an opportunity for inquisitive researchers to engage and collaborate. The book could not have been possible without the contributions of many: principally, I would like to express my gratitude to all authors, all of whom contributed their articles with enthusiasm and showed patience with my editing; to the staff at Humana Press for commissioning this volume; and to the series editor, John Walker, who provided prompt guidance and advice.

The First International Pathogenic Neisseria Conference, held in San Francisco, CA, USA, on 18th–20th January, 1978, was devoted to the immunobiology of *Neisseria gonorrhoeae* [52]. Yet, 40 years on, gonorrhea continues to prove as an intractable disease, compounded by the emergence of antibiotic-resistant strains. However, the continual development and application of new methods and protocols to understand the biology of this pathogen, such as those described in this book, should provide a basis for eventual control of the disease and a reduction in global case numbers.

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