



REVIEW ARTICLE

Is gonococcal disease preventable? The importance of understanding immunity and pathogenesis in vaccine development

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Abstract

Gonorrhea is a major, global public health problem for which there is no vaccine. The continuing emergence of antibiotic-resistant strains raises concerns that untreatable *Neisseria gonorrhoeae* may become widespread in the near future. Consequently, there is an urgent need for increased efforts towards the development of new anti-gonococcal therapeutics and vaccines, as well as suitable models for potential pre-clinical vaccine trials. Several current issues regarding gonorrhea are discussed herein, including the global burden of disease, the emergence of antibiotic-resistance, the status of vaccine development and, in particular, a focus on the model systems available to evaluate drug and vaccine candidates. Finally, alternative approaches to evaluate vaccine candidates are presented. Such approaches may provide valuable insights into the protective mechanisms, and correlates of protection, required to prevent gonococcal transmission, local infection and disease sequelae.

Keywords

Candidate vaccine antigens, gonorrhea, infection models, multi-drug resistance, *Neisseria gonorrhoeae*

History

Received 31 July 2015
Revised 1 October 2015
Accepted 6 October 2015
Published online 11 January 2016

Introduction

Infections caused by *Neisseria gonorrhoeae* (the gonococcus) continue to be a global, intractable problem. The absence of a gonococcal vaccine, together with the continuing emergence of antibiotic-resistant and untreatable gonococcal strains, has raised awareness that *N. gonorrhoeae* poses an “urgent” public health threat for which immediate aggressive action is greatly needed (Unemo & Shafer, 2014). Herein, we review the impediments and potential opportunities to gonococcal vaccine development.

The global burden of gonorrhea

The highest burden of *N. gonorrhoeae* disease occurs in men and women between 15 and 29 years of age (CDC, 2013; WHO, 2012). Although infection typically results in urethritis in males and cervicitis in females, mucosal infections of the rectum, pharynx and eye are also commonly reported (Hook & Handsfield, 2008). The World Health Organization (WHO)

reports an estimated global incidence of over 106 million cases per year, with a 21% increase in incidence having occurred between 2005 and 2008 (WHO, 2012). More recent data indicate an 11% increase in the number of reported cases in the United States from 2009 to 2013 (CDC, 2014) and a 90% increase in Australia from 2009 to 2014 (NNDSS, 2015). Incidence is likely underestimated, however, because of inadequate surveillance and diagnostics methods in many regions, as well as the high number of asymptomatic cases. In this regard, if not promptly diagnosed and treated, infection can lead to severe reproductive complications. *Neisseria gonorrhoeae* disease sequelae are predominately observed among women, owing to the higher proportion of asymptomatic infections in females [50–80% (Farley et al., 2003; Hook & Handsfield, 2008; WHO, 2011)] versus males [reported to be from 1% to 40% (Farley et al., 2003; Handsfield et al., 1974; Hook & Handsfield, 2008; Johnson et al., 2010; WHO, 2011)]. Ascending reproductive tract infections lead to pelvic inflammatory disease (PID, i.e. inflammation of the uterus, fallopian tubes, ovaries, etc.) in 10–40% of infected women that, in turn, can cause infertility and ectopic pregnancies. Additional disease sequelae include fetal wastage, neonatal conjunctivitis, adverse pregnancy outcomes and disseminated gonococcal infections (Hook & Handsfield, 2008). Complications of infection are rare in males, but they include urethral stricture and urogenital tract abscesses, as well as inflammation of the prostate gland, epididymis and/or testes (Hook & Handsfield, 2008). In both sexes, infection with *N. gonorrhoeae* imposes an additional increased risk to contract and transmit HIV (Chen et al., 2003; Cohen et al., 1997; Levine et al., 1998).

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In terms of economic burden, gonococcal infections in the United States are estimated to account for annual direct medical costs exceeding \$162.1 million (Owusu-Eduesei et al., 2013) and indirect costs in excess of \$1.1 billion (Aledort et al., 2005).

Current situation for gonococcal control

Presently, the control of *N. gonorrhoeae* largely relies on prompt diagnosis and antibiotic treatment of infected persons and their acknowledged sex partners (CDC, 2013). However, high levels of asymptomatic infections (as described above) and low levels of routine screening (Huppert et al., 2005) hamper prompt diagnosis, even though available diagnostics are highly sensitive (>90%) and specific (>99%) (Papp et al., 2014). Furthermore, there is a heavy reliance upon syndromic disease management (i.e. the treatment of all at risk patients based upon the presence of symptoms rather than a definitive diagnosis) in many of the countries that are most heavily impacted by gonococcal disease. Syndromic disease management is not suitable for the diagnosis and treatment of the high numbers of individuals that present with asymptomatic disease. Moreover, it contributes to antibiotic-resistance by the overuse and misuse of antibiotics. In this regard, the gonococcus has developed resistance to all classes of antibiotics used to treat it over the past seven decades, including the sulphonamides, penicillins, tetracyclines, macrolides and quinolones (reviewed in Unemo & Shafer, 2014). Multi-drug-resistant strains are of the greatest concern and have been identified in Japan (Ohnishi et al., 2011), Europe (Unemo et al., 2011, 2012), South Africa (Lewis et al., 2013), Australia (Lahra et al., 2014) and North America (Barry & Klausner, 2009; Martin et al., 2012). These strains exhibit high-level resistance to the expanded-spectrum cephalosporins, ceftriaxone and cefixime; which are the last remaining options for empirical first-line treatment. Combined antibiotic therapies are currently being evaluated in clinical trials (Kirkcaldy et al., 2014), but there are no new single antibiotic options presently available, or in the pipeline, and it is of concern that even new classes of antibiotics would only offer a short-term therapeutic solution (Tapsall et al., 2009; Unemo & Nicholas, 2012). An example of this rapid change in antimicrobial-resistance is exemplified by the fact that the 2010 Centers for Disease Control and Prevention (CDC) recommendations for gonorrhea treatment had to be revised in 2012 to include the use of double antibiotic therapy (CDC, 2012). Additionally, diminished effectiveness of cefixime, an oral cephalosporin, has prompted the recommended use of ceftriaxone, which is administered by muscular injection (CDC, 2012). Therefore, more aggressive and invasive treatment is now required to treat *N. gonorrhoeae* infections. Recognizing infections with antibiotic-resistant strains and, hence preventing treatment failures, is also becoming increasingly difficult because of the general shift from culture-based diagnostic methods (required for susceptibility testing) to nucleic acid amplification testing. Additionally, most national surveillance bodies only monitor antimicrobial-resistance for a small percentage of reported cases [e.g. fewer than 2% of all reported gonorrhea cases are

sampled by the USA Gonococcal Isolate Surveillance Project (GISP) (Bolan et al., 2012)].

The CDC has listed *N. gonorrhoeae* as one of the three microorganisms that pose an urgent threat with regard to antibiotic-resistance – highlighting it as an “immediate public-health threat that requires urgent and aggressive action” (CDC, 2013). This threat assessment also included the evaluation of the clinical and economic impact, incidence, 10-year projection of incidence, transmissibility, availability of effective antibiotics and barriers to prevention for gonorrhea. Projection of these data indicates there will be an additional 75 000 cases of PID, 15 000 cases of epididymitis and 222 HIV infections during a 10-year period in the United States alone, if cephalosporin-resistant *N. gonorrhoeae* strains become widespread. Furthermore, direct medical costs will more than double, with greater than \$200 million in additional budget being required for increased monitoring and case management, as well as for additional courses of antibiotics (CDC, 2013).

Current status of vaccine development

Vaccination is generally considered the best long-term approach to control infectious diseases. However, efforts over the past century to develop a gonococcal vaccine have proven particularly arduous. Only four candidate vaccines have progressed to clinical trials, (1) a whole cell (therapeutic) vaccine tested during the early 1900s (Eyre & Stewart, 1909), (2) a partially autolyzed vaccine tested in 1974 (Greenberg et al., 1974), (3) pilus-based vaccines tested in the 1990s (Boslego et al., 1991) and (4) a protein I-based vaccine derived by differential centrifugation of disrupted gonococci (reviewed in Tramont, 1989). None of these vaccines were successful, despite preliminary evidence to suggest that (1) whole cell (mixed strain) vaccination therapy could improve infection outcomes in patients (Eyre & Stewart, 1909) and (2) human anti-pilus antibodies, and (antibody-containing) genital secretions, could block subsequent *in vitro* adherence to human buccal epithelial cells (Tramont et al., 1980, 1981). These latter data potentially highlight the importance of the use of appropriate infection models for pre-clinical *N. gonorrhoeae* vaccine evaluation, in that gonococci do not naturally colonize buccal cells. Moreover, these studies provided the first insight into the impending numerous difficulties in *N. gonorrhoeae* vaccine development, which are primarily the result of the variability of gonococcal antigens, production of “blocking” antibodies to conserved gonococcal antigens (Rice et al., 1986), a lack of knowledge of what immune response might confer protective immunity and the absence of robust animal models in which to study this obligate human pathogen, as is discussed further below (reviewed in Jerse et al., 2014; Mietzner et al., 2004; Seib & Rappuoli, 2010).

Candidate gonococcal vaccine antigens

The majority of gonococcal antigens studied to date are extremely variable in the amino acid sequence of protein antigens and in the structure of oligosaccharide antigens. This is true both among strains and within a given strain. High levels of natural transformation, antigenic variation and phase

variation drive this variation, which is characteristic of the pathogenic *Neisseria* (Rotman & Seifert, 2014). The impact of these factors on vaccine development is clear from clinical trials in which gonococci expressing multiple variants of the major surface appendages, pilin (Seifert et al., 1994; Swanson et al., 1987), lipooligosaccharide (LOS) (Schneider et al., 1991) and the opacity-associated outer membrane proteins (Opa) (Jerse et al., 1994; Swanson et al., 1988), were recovered from human male volunteers shortly after infection with a predominantly antigenically-homogenous inoculum. However, several other immunogenic proteins are also characterized that could serve as potential vaccine targets resulting from their high level of antigenic conservation and stable expression within and between strains. These targets include: the nitrite reductase, AniA (Shewell et al., 2013); phospholipase D (PLD) (Apicella & Edwards, 2007; Edwards & Apicella, 2006); transferrin-binding proteins, TbpAB (Price et al., 2007); MtrE of the Mtr efflux pump complex (DeRocco and Jerse, unpublished data reported in Jerse & Deal, 2013), the outer membrane porin, PorB (Garvin et al., 2010; Massari et al., 2003), as well as the conserved 2C7 epitope of LOS (Gulati et al., 2013; Ngampasutadol et al., 2006) [Table 1; (reviewed in Jerse et al., 2014; Jerse & Deal, 2013)]. New gonococcal candidate antigens could also be realized through improved vaccine technologies that support genome wide analyses as well as the rational engineering and redesigning of promising targets (as outlined in Rinaudo et al., 2009). Additionally, novel antigen presentation strategies [e.g. outer membrane vesicles (OMVs) that present several target antigens, as used for serogroup B meningococcal vaccines (Holst et al., 2013)] may facilitate gonococcal vaccine development.

Immune response to gonococcal infection

Traditional vaccine approaches are based on inactivated or attenuated organisms, or purified antigens, and they aim to replicate natural immunity while simultaneously deterring disease (Rappuoli, 2007). However, natural infection with *N. gonorrhoeae* does not induce a protective immune response (Fox et al., 1999; Hedges et al., 1999; Schmidt et al., 2001; Ross et al., 1995). Indeed, the Scottish essayist and biographer of Samuel Johnson, James Boswell, wrote detailed diary accounts of his 18 episodes of gonococcal urethritis between 1760 and 1790 (Ober, 1970). It is not clear if this suggests natural clearance of the organism may have occurred in the pre-antibiotic era and, thus, would eventually occur today, if treatment were withheld. However, given the high prevalence of asymptomatic and sub-clinical gonococcal disease, it is unknown whether, in the pre-antibiotic era when diagnosis was based on symptomatology, patients did actually clear the infection. Regardless, it is well known among STI clinicians that patients with gonorrhea frequently have recurrent gonococcal infections (Fowler et al., 2010), indicating a lack of natural immunity. Furthermore, experimental gonococcal infections of human volunteers demonstrated that protection was not conferred, even when subjects were challenged six months after the first infection with the identical strain (Schmidt et al., 2001). Such experiences should tell us that, if natural infection does not provide protection against

reinfection, vaccine development will require the generation of a non-native immune response. Therefore, a full understanding of gonococcal pathogenesis is needed to identify possible, and novel, approaches to solve the problem. An increased understanding of the impact of the female genital tract microbiome and co-infection with other STIs on the immune response would also be valuable, as both these factors may alter transmission and disease progression of *N. gonorrhoeae* (Gallo et al., 2012; Saigh et al., 1978).

The observations that gonococcal infections are often persistent and that reinfection is common are consistent with a growing body of literature indicating that *N. gonorrhoeae* is able to avoid (Kilian & Reinholdt, 1987; Lewis et al., 2010; Mandrell & Apicella, 1993; Pettit & Judd, 1992; Rice et al., 1986), and actively suppress (Liu et al., 2014; Liu & Russell, 2011), the immune response. Strain-specific antibodies are detected in serum, urethral exudates (Kearns et al., 1973; Tramont, 1977) and vaginal/cervical secretions (Hedges et al., 1999; O'Reilly et al., 1976; Tramont et al., 1980) of infected males and females. However, antibody production is low, it is not protective, and, with few exceptions, it is not significantly different among infected versus uninfected individuals (Hedges et al., 1999). The finding that antibodies directed against the gonococcal antigen RmpM (reduction modifiable protein M) block the bactericidal activity of other antibodies directed against *N. gonorrhoeae* (Rice et al., 1986), may explain, to a certain extent, the lack of protection seen. The presence and duration of antibody are also highly variable among individuals as well as between males and females (Hedges et al., 1999; Welch & O'Reilly, 1973).

Studies of the innate immune response during gonococcal infection provide additional clues as to how the gonococcus evades host defenses. Elegant studies demonstrate a role for complement inactivation and down regulation (Ngampasutadol et al., 2005, 2008a,b; Ram et al., 2001) in mediating infection (Edwards & Apicella, 2002; Jarvis, 1994; McQuillen et al., 1999). Gonococcal membrane-associated antimicrobial peptide efflux pumps (e.g. the Mtr protein complex) (Warner et al., 2007, 2008) are also shown to promote gonococcal survival during infection. However, the role of human phagocytic cells [neutrophils (PMNs) and macrophages] in gonococcal pathobiology is less well understood. The human neutrophil is clearly ineffective in resolving gonococcal infection, albeit evidence of stimulation of a respiratory burst, as well as a robust and prolonged response during gonococcal urethritis, exists (Apicella et al., 1996; Criss & Seifert, 2012; Seib et al., 2005; Simons et al., 2005). Infection studies performed *in vitro* using human PMNs indicate that there is an initial oxidative burst within the PMN upon infection resulting in death of 30–40% of the population (Simons et al., 2005). This is followed by replication of the resistant gonococci within the PMN and is associated with induction of anti-apoptosis, which prevents PMN death until 21–24 h after infection (Simons et al., 2006). At that time, the infected PMN is little more than a sac containing large numbers of gonococci (Simons et al., 2005). Understanding how the gonococcus evades PMN killing may lead to novel vaccine and therapeutic approaches. For example, data indicate that gonococcal surface proteins; porin (Bjerknes et al., 1995; Dehio et al., 2000; Haines et al., 1988, 1991;

Table 1. Potential gonorrhoea vaccine antigens.

Name	Role	Variability	Antibodies	Effect of candidate-directed antibody	References
Colonization PLD	Phospholipase D, regulator of gonococcal invasion of and survival within cervical epithelia	Highly conserved	FI	Antibodies inhibiting NgPLD decrease adherence to and invasion of primary cervical cells	Apicella & Edwards (2007) and Edwards et al. (2003)
Pilin	Major outer membrane protein, mediates adherence to epithelial cells	Antigenically variable	FI	Antibodies to pili block attachment to human cells, but are directed at variable epitopes	Rothbard et al. (1985), Siegel et al. (1982), Schoolnik et al. (1983), Tramont et al. (1981), and Virji & Heckels (1984)
PilQ	Outer membrane channel through which pili are extruded	Stable expression	B*	N/A	Haghi et al. (2012)
PorB	Major porin, outer membrane pore; involved in gonococcal invasion of cervical cells through CR3, PorB1A mediates epithelial invasion through the SREC-1 receptor	Stable expression, two serogroups (PorB1A and PorB1B)	B (Cyclic loop peptides); B*	N/A	Edwards et al. (2002), Rechner et al. (2007), Garvin et al. (2010), and Massari et al. (2003)
Opa proteins	Mediates adherence to immune cells	Phase variable, several antigenically distinct Opa proteins per strain	B*, M*	N/A	Callaghan et al. (2011), Cole & Jerse (2009), and de Jonge et al. (2004)
OmpA	Mediates invasion of malignant cervical and endometrial cell lines	Stably expressed, highly conserved	B	N/A	Serino et al. (2007)
Nutrient acquisition and metabolism TbpA, TbpB	Transferrin (Tf) receptor	TbpA and TbpB are highly and semiconserved, respectively	B, FI	Tf required for infection of male volunteers by strains lacking LbpA and LbpB Antibodies in mice block growth in the presence of Tf as a sole iron source	Price et al. (2007) and Hobbs et al. (2011)
LbpA, LpbB	Lactoferrin receptor	LbpA and LpbB are semiconserved*, not expressed by all gonococcal strains	B*	N/A	Pettersson et al. (2006), Adamiak et al. (2012), Biswas et al. (1999) and Mickelsen et al. (1982)
Tdfl (ZnuD*)	TonB-dependent zinc transporter	Conserved, iron induced	B*	N/A	Comelissen & Hollander (2011) and Stork et al. (2010)
AniA	Nitrite reductase, required for anaerobic growth and biofilm formation	Conserved, induced by low O ₂ tension and the presence of nitrite	FI	Antiserum to truncated non-glycosylated recombinant protein blocks AniA nitrite reductase activity	Clark et al. (1988), Falsetta et al. (2009) and Shewell et al. (2013)
Evasion of innate defenses MtrE	Surface-exposed channel of the MtrC-MtrD-MtrE and FarA-FarB-MtrE active efflux pumps	Stable expression and highly conserved	B	N/A	Jerse & Deal (2013) (DeRocco and Jerse, unpublished data)
Lst	α 2,3 sialyltransferase; catalyzes the addition of host-derived sialic acid to the lacto-N-neoteosae moiety of LOS; protects gonococci from complement, non-opsonic uptake by neutrophils, and antimicrobial peptides	Conserved, variable levels of activity between strains, repressed by CrgA	FI	Antibodies reduce sialylation	Smith et al. (1995), Shell et al. (2002) and Packiam et al. (2006)

(continued)

Table 1. Continued

Name	Role	Variability	Antibodies	Effect of candidate-directed antibody	References
NspA	Factor H binding and serum resistance	Stably expressed, highly conserved	B, O	N/A	Li et al. (2011) and Martin et al. (1997)
PorB	PorB binds C4b-binding protein and factor H to down-regulate complement activation at the gonococcal surface	Described above			
Other					
2C7 epitope	The 2C7 epitope is a common oligosaccharide structure within gonococcal LOS that is recognized by a murine monoclonal antibody, called 2C7	Phase variable but expressed by >95% of isolates	B, O, M	N/A	Gulati et al. (1996, 2012, 2013) and Ngampasutadol et al. (2006)

B, bactericidal; O, opsonic; M, protective in mouse model of infection; FI, function inhibiting.

*Data for *N. meningitidis* homologue; N/A, not applicable/no data available.

Lorenzen et al., 2000; Mosleh et al., 1998), pili (Stohl et al., 2013) and Opa (Ball & Criss, 2013); may have a substantive impact on the PMN response to infection. There are also data to indicate that a secreted gonococcal nuclease (Nuc) is involved in gonococcal survival by promoting escape from PMN nets (Juneau et al., 2015). Although the utility of porin, pili and Opa proteins as vaccine candidates is problematic (Jerse et al., 1994; Seifert et al., 1994; Swanson et al., 1987, 1988), targeting these (or other surface constituents) as an approach to treat gonococcal disease has not been examined. Similarly, the potential of Nuc as a vaccine antigen or a therapeutic target has not been examined. The ongoing development of multi-organ culture systems (outlined below) may provide novel ways to evaluate natural, and vaccine-induced, immune responses that involve phagocytic cells.

It is not clear if a single vaccine preparation, or elicitation of a single type of immune response, will be effective in both men and women (Edwards & Apicella, 2004). However, it is apparent that novel vaccination approaches that target and optimize specific aspects of the human response(s) to infection, in combination with scrupulous antigen selection, will be needed and may enable vaccine(s) development, provided a physiologically relevant model(s) of human disease and/or correlates of protection are established.

Models of infection and vaccine evaluation

Neisseria gonorrhoeae is an obligate human pathogen; therefore, humans serve as the only known natural reservoir for gonococcal infections. The *in vivo* human environment is difficult, if not impossible, to recapitulate in the laboratory, particularly given our incomplete understanding of the pathobiology of *N. gonorrhoeae* and the human response(s) to infection. Nevertheless, several infection models have been developed to investigate certain aspects of gonococcal infection, colonization and disease pathology (Arko, 1989; Edwards et al., 2000; Harvey et al., 1997; Jerse, 1999; Morales et al., 2006; Simons et al., 2005; Stephens, 1989; Stohl et al., 2005; Timmerman et al., 2005) (Table 2).

The best system in which to study gonococcal pathogenesis is, of course, infected humans. In this regard, a human challenge model exists and involves experimental urethral infection of male volunteers. This model has been used to evaluate the protective role of vaccine candidates, to investigate the immune response(s) to infection and to examine the role of specific gonococcal constituents in promoting infection/disease (Cohen & Cannon, 1999; Cohen et al., 1994; Hobbs et al., 2011, 2013; Ramsey et al., 1994; Schmidt et al., 2001). This is also the optimal (in terms of mimicking natural infection) system available to test vaccine efficacy as well as to evaluate gonococcal antigens that only may be highly expressed (in men) *in vivo*. However, as with any scientific model, there are certain constraints that limit the utility of this infection model, including: (1) high associated costs, (2) a restriction to small group sizes and (3) infection is terminated at the first signs of disease and, thus, can only assess early stages of infection. Also of importance is that data obtained from infection in men likely does not translate to infection of women, as some pathways of infection are sex-specific

Table 2. Model systems for investigation of gonococcal infections and vaccine development.

Model	Key features	Pros	Cons	Key information to be gained
Human male challenge	<ul style="list-style-type: none"> • Urethral infection of male volunteers 	<ul style="list-style-type: none"> • Mimics natural infection • Reflects disease in males • Evaluate gonococcal antigens that may only be highly expressed (in men) <i>in vivo</i> 	<ul style="list-style-type: none"> • May not reflect disease in females • Cost • Group size • Infection is terminated at the first signs of disease • Inability to investigate long term infections 	<ul style="list-style-type: none"> • Immunogenicity of antigens • Protective efficacy • Immune response(s) to infection • Role of specific gonococcal constituents in promoting infection/disease
Non-human primates	<ul style="list-style-type: none"> • Infection of, and transmission between, males and females 	<ul style="list-style-type: none"> • Less subject to gonococcal host restrictions than are other laboratory animals 	<ul style="list-style-type: none"> • Ethical considerations • Limited availability • High costs 	<ul style="list-style-type: none"> • Immunogenicity of antigens • Protective efficacy
E2-mice	<ul style="list-style-type: none"> • Administration of 17β-estradiol (E2) and antibiotics to female mice • Gonococcal infection of genital tract, and recovery of gonococci from infected animals for approximately 5–12 days 	<ul style="list-style-type: none"> • Abundant reagents available • More closely aligned with infection of males 	<ul style="list-style-type: none"> • Structural differences between mouse and female reproductive tract • Differences in the development, the activation, and the response to challenge between the murine and human immune systems • Less closely aligned with females • Absence of select human constituents as well as human-specific features of select human-murine orthologs 	<ul style="list-style-type: none"> • Immunogenicity of antigens • Compare adjuvants and routes of immunization
Guinea pigs and rabbits	<ul style="list-style-type: none"> • Subcutaneous chambers implanted in animal 	<ul style="list-style-type: none"> • Reported to be better than mice when measuring immunogenicity of gonococcal antigens 	<ul style="list-style-type: none"> • Not been used in recent years 	<ul style="list-style-type: none"> • Immunogenicity of antigens • Compare adjuvants and routes of immunization
Primary Organ culture	<ul style="list-style-type: none"> • E.g. fallopian tube, endometrium 	<ul style="list-style-type: none"> • Closely reflect natural sites of infection • Interactions between several host cell types can be evaluated 	<ul style="list-style-type: none"> • Typically does not allow the simultaneous analysis of innate and adaptive immune responses • Limited availability of human specimens • Technical expertise required to reproducibly procure these models • Cannot fully assess the contiguous variation in environmental factors occurring <i>in vivo</i> throughout the course of infection/disease 	<ul style="list-style-type: none"> • Ability of antibodies to inhibit antigen function (e.g. adherence, an intracellular survival, nutrient uptake)
Primary cell culture	<ul style="list-style-type: none"> • E.g. cervical, endometrial, fallopian tube or male urethral epithelial cells, immune cells 	<ul style="list-style-type: none"> • Closely reflect natural sites of infection • Interactions between several host cell types can be evaluated 	<ul style="list-style-type: none"> • Typically does not allow the simultaneous analysis of innate and adaptive immune responses • Limited availability of human specimens • Technical expertise required to reproducibly procure these models • Cannot fully assess the contiguous variation in environmental factors occurring <i>in vivo</i> throughout the course of infection/disease 	<ul style="list-style-type: none"> • Ability of antibodies to inhibit antigen function • Ability to assess local production of innate immune effectors (cytokine responses, complement, antimicrobial peptides, etc.)
Immortalized/cancer cell lines	<ul style="list-style-type: none"> • E.g. cervical, endometrial or male urethral epithelial cells, immune cells 	<ul style="list-style-type: none"> • Relatively low cost • Readily available • Easily maintained • Interactions between several host cell types 	<ul style="list-style-type: none"> • Typically do not allow the simultaneous analysis of innate and adaptive immune responses • Altered protein 	<ul style="list-style-type: none"> • Ability of antibodies to inhibit antigen function

(continued)

Table 2. Continued

Model	Key features	Pros	Cons	Key information to be gained
		<ul style="list-style-type: none"> can be evaluated • Readily adaptable to high-throughput analyses 	<ul style="list-style-type: none"> expression profiles relative to primary cells • Altered responses generated upon infection relative to the <i>in vivo</i> condition • Cannot fully assess the contiguous variation in environmental factors occurring <i>in vivo</i> throughout the course of infection/disease 	
Human/patient fluids and tissues	<ul style="list-style-type: none"> • Proteomic, genomic, metabolomic analyses of human specimens 	<ul style="list-style-type: none"> • Can be non-invasive, human-specific models 	<ul style="list-style-type: none"> • Limited availability of human specimens • Data obtained are restricted to a specific ‘snap shot’ of infection/disease related to when the specimen is obtained 	<ul style="list-style-type: none"> • Identify human factors generated during infection and disease • Identify critical virulence determinants involved in immune induction or its suppression
‘‘Human-on-a-chip’’	<ul style="list-style-type: none"> • Microfluidic cell culture chips that mimic multiple organs in the body 	<ul style="list-style-type: none"> • Non-invasive, human-specific models 	<ul style="list-style-type: none"> • Cost • Untested for gonorrhea, standardization likely will be required • System complexity • Cost • Untested for gonorrhea 	<ul style="list-style-type: none"> • Human response(s) generated to infecting organisms
Immunotherapy	<ul style="list-style-type: none"> • Treatment of infection using monoclonal antibodies or immune serum 	<ul style="list-style-type: none"> • Feasibility shown for delivery and targeting of antibodies to the genital tract • Targets available that are known to block colonization and essential metabolic activities of the bacteria 		<ul style="list-style-type: none"> • Define the target, type, and level of antibody response needed to protect against, or reduce, mucosal infection or sequelae

(Edwards & Apicella, 2004). A similar model for investigations of gonococcal disease in women is ethically prohibited because of the potential for complications resulting from infection in females.

Several cell and organ culture systems have been used to study *N. gonorrhoeae* infection and offer an opportunity to define the potential of vaccine-induced antibodies to inhibit the function of the target protein (i.e. function inhibiting antibodies), such as by inhibiting adherence, invasion, colonization and/or biofilm formation. Primary human organ [e.g. fallopian tube (McGee et al., 1981), endometrium (Timmerman et al., 2005)] and epithelial cell [e.g. cervical (Edwards et al., 2001), endometrial (Timmerman et al., 2005), fallopian tube (Morales et al., 2006) or male urethral (Harvey et al., 1997)] cultures closely reflect natural sites of infection. However, it is impossible to simulate the continuum of numerous environmental changes (e.g. variable oxygen tensions, hormone levels, levels and presence of select proteins and/or cell types) that occur during *in vivo* infection in cell culture models. Moreover, the inability to simultaneously model aspects of the innate and the adaptive immune response in these systems has been a major drawback in vaccine research. These drawbacks can potentially be overcome in 3D cell culture systems which are being developed (Choi et al., 2015). The limited availability of human specimens and the technical expertise required to reproducibly procure these

models has, however, resulted in the predominant use of immortalized or malignant human epithelial cell lines to study gonococcal–host interactions. Although cell lines are readily available and easily maintained, data obtained from the use of such cells may not be reflective of the epithelia encountered by the gonococcus *in vivo*. Continued laboratory maintenance, as well as cellular transformation, can alter the protein expression profiles of these cells as well as the responses generated upon infection [e.g. the absence of CR3 expression by immortal cervical and endometrial cell lines (Edwards et al., 2001)], and the altered production of pro-inflammatory mediators in immortalized human cervical and vaginal epithelial cells in response to gonococcal infection (Fichorova et al., 2001; Hedges et al., 1998).

It is generally believed that the limited host range exhibited by *N. gonorrhoeae* results largely from the specificity of an array of gonococcal proteins for human-specific targets that include: receptors for colonization [e.g. CR3 (Edwards et al., 2002); various CEACAMs (Sadarangani et al., 2011; Virji et al., 1996)]; iron-transport proteins [e.g. transferrin and lactoferrin (Lee & Schryvers, 1988)] and components of the immune system [e.g. C4BP and factor H (fH) (Ngampasutadol et al., 2008a,b)]. In this regard, our closest living relatives are the great apes (chimpanzees, orangutans and gorillas). Although, these non-human primates are less subject to gonococcal–host restrictions than are other

laboratory animals, these models are rarely used because of ethical considerations, limited availability and high costs. Therefore, in the absence of a laboratory animal that is more closely related to humans, and with the abundance of reagents currently available to allow scientific analyses in mice, the use of a murine model to study gonococcal pathogenesis has gained increasing popularity. This model relies on the administration of 17 β -estradiol (E2) and antibiotics to female mice, which enables gonococcal infection of the genital tract and recovery of gonococci from infected animals for approximately 5–12 days (reviewed in Jerse et al., 2011). The development of the estradiol-treated mouse infection model (here after referred to as the E2-mouse model) has provided an opportunity to test the immunogenicity of gonococcal antigens as well as to differentially compare adjuvants and routes of immunization.

The E2-mouse model of *N. gonorrhoeae* infection mirrors some aspects of human disease; however, it does not recapitulate infection of the human female reproductive tract or the immunological responses generated by infected women. Indeed, in many ways, data obtained from the use of this model are more closely aligned with infection of males than they are with females. For example, data obtained from experimental infections indicate that the pro-inflammatory cytokines interleukin (IL)-1 β , IL-6, IL-8 and tumor necrosis factor (TNF)- α are elevated in infected males when compared to uninfected control volunteers (Ramsey et al., 1995). Similar data are obtained upon analyses of urethral exudates from men with culture-documented gonorrhea, from cell culture supernatants collected from gonococci-infected primary (human) male urethral epithelial cells (Harvey et al., 2002), and from genital secretions of E2-treated female mice (Packiam et al., 2010). Conversely, analyses of vaginal and cervical fluids collected from women with culture-documented gonococcal cervicitis reveal that local levels of these inflammatory mediators are not elevated during natural infection (Hedges et al., 1998). *Neisseria gonorrhoeae* infection of primary (Edwards & Apicella, 2004), but not immortalized (Fichorova et al., 2001), human cervical epithelial cells yields results comparable to those observed in naturally-infected females (Hedges et al., 1998). More recently, Russell and co-workers have shown that infection of mice, or mouse vaginal explants, results in increased levels of IL-17A, IL-23, IL-6 and TNF- α , whereas IL-12 (associated with the development of a Th1-mediated response) was not detected (Feinen et al., 2010). Based on these data, this group has proposed that (predominately $\alpha\beta$) T-cells of the Th17 lineage play a central role in the immune response to *N. gonorrhoeae*, resulting in the production of proinflammatory cytokines and chemokines, and the recruitment of neutrophils and other innate defense factors to the site of infection. Consistent with these data, Gagliardi et al. (2011) report increased serum concentrations of IL-17A and IL-23 (as well as IFN- γ) in infected humans; however, it is important to note that of the 27 patients with gonorrhea examined, 26 of these were men, as were all of the control (uninfected) subjects. To our knowledge, the levels of Th17-related cytokines have not been measured in *N. gonorrhoeae*-infected women. However, IL-1 β , IL-6 and TGF- β (associated with Th17 induction) are not elevated in vaginal and cervical fluids of infected women

(Hedges et al., 1998). Thereby, these data suggest that, unlike infection in mice, Th17 cells likely may not play a substantive role in the immune response to gonococcal infection in women, albeit Th17 cell responses do appear to play a role in the immune response to infection in men.

Important structural/functional differences exist between the female reproductive tract of women and that of mice (Rendi et al., 2012). Such differences also may, in part, contribute to difficulties in translating data obtained from the E2-mouse model of infection to females. Of note, is the presence of the squamocolumnar junction/transformation zone (i.e. the distinctly abrupt area of transition from the columnar, to the stratified squamous, epithelia of the endo- and ectocervix, respectively) in humans and its absence in mice. Although the epithelia of the female reproductive tract lack mucosa-associated lymphoreticular tissue (i.e. MALT), organized areas of lymphoid aggregates (comprised of Langerhans, plasma, T- and B-cells) are concentrated within the transformation zone as well as within the cervix and endometrium (Edwards & Morris, 1985; Pudney et al., 2005; Yeaman et al., 2001). Therefore, it is suggested that the transformation zone exists as a unique immunologically active site that likely plays an important role in antigen uptake, lymphocyte recruitment and differentiation, as well as in prohibiting ascension of potential pathogens to the uterine cavity. Similar studies performed in mice indicate that “lymphoid nodules” and B-cells are not present within the epithelia of the murine female reproductive tract (Parr & Parr, 1991). Further investigations should be performed to verify these data, as this may have important implications with regard to the utility of the mouse model for antigen screening and pre-clinical vaccine studies, if women are to be the targeted population. For example, it is suggested that vaccines intended to induce protective immunity within the female reproductive tract should be targeted to the transformation zone/ectocervix for a cytotoxic T-cell-mediated response or to the endocervix for a local antibody-mediated response, neither of which may be possible to evaluate in a mouse model (Pudney et al., 2005). It is also important to note that there are substantive differences in the development, the activation and the response to challenge between the human and murine immune systems (Mestas & Hughes, 2004). Whereas mice have orthologs of many immunologically important human molecules, similarity in function does not always relate to causality. That is, immunological function/outcomes can be achieved in different species by divergent routes, and the potential protective effects of vaccines against targets that interact with host-restricted molecules might be under- or over-estimated in normal mice.

Looking forward

Pre-clinical development and evaluation of vaccines are typically based on protection (e.g. decreases in bacterial load or in illness/symptoms) in animal models, or by using correlates/surrogates of protection (i.e. measureable evidence of protection from infection or disease, e.g. altered mucosal and cellular immune responses). This has led to an over-reliance on the use of animals, in particular mice, by which to study human (-specific) diseases. This is despite the fact that

most trials in animals do not show a similar outcome in human clinical trials (Hackam & Redelmeier, 2006; Knight, 2007; Pound et al., 2004), a problem that is particularly true for those trials examining inflammation (Seok et al., 2013). This can be attributed, at least in part, to the inability of some animal models to accurately replicate the human disease that the animal is intended to model. Given that correlates of protection for *N. gonorrhoeae* are unknown, and there is no animal model that accurately mimics the complexity of human disease in males and females, this begs the question, where do we go from here when there are valid concerns that a time of untreatable gonorrhea may be quickly approaching?

In terms of models systems for vaccine development and pre-clinical trials for exclusive human pathogens, such as the gonococcus, it is important that the model is selected, and data are interpreted, with specific questions and intended outcomes in mind [i.e. relating to immunogenicity, toxicity or protection, and the group (male and/or female) being targeted]. Therefore, data obtained from the use of laboratory models should mimic as closely as possible and, thereby, complement data obtained from human sources. Together, this will ensure that the data obtained from such models are relevant and can be translated to the human male and/or female as effectively as possible. For example, the use of IL-12-encapsulated microspheres has been proposed as a therapeutic option for the treatment of gonorrhea based on data obtained from studies performed in mice. Among the functions ascribed to IL-12 is its ability to antagonize some of the effects of IL-10 and TGF- β (Liu et al., 2013). In that IL-10 and TGF- β are shown to be already low in women with gonococcal disease (Hedges et al., 1999), it seems prudent to question whether such a treatment might exacerbate infection in women, rather than aid in its resolution. Whereas IL-12 therapy may, in theory, provide one solution to treat gonorrhea in males, in practice it may not be well accepted because of patient discomfort and, thereby, patient compliance.

The E2-mouse model permits investigations in a mammalian host and provides a valuable way to test antigen immunogenicity, using different adjuvants and routes of immunization. Subcutaneous chambers implanted in Guinea pigs and rabbits have also been used for such studies and are reported to be comparatively better than mice when measuring immunogenicity of gonococcal antigens (Arko, 1972). Although these models have not been used in recent years, they could be re-evaluated for their utility in determining correlates of protection, the potential immunogenicity of select gonococcal antigens and/or to conduct preclinical toxicity studies for new therapeutic regimens to treat gonococcal disease. The subcutaneous Guinea pig infection model, as described, does not require the administration of E2 and antibiotics to establish infection (Arko, 1972). Therefore, the comparative use of these models could provide insights into any extraneous effects resulting from E2 (or antibiotic) administration to mice (e.g. immune suppression), which have the potential to skew data interpretation and, in turn, the ability to translate such data to *N. gonorrhoeae* vaccine development for use in humans.

Given that currently used mammalian models (the E2-mouse model and male human volunteers) may not be

physiologically relevant models of female infection, it may also be important to take the focus off targeting human females for vaccination. Recently, the potential impact of “hypothetical” vaccines was modeled (Craig et al., 2015). This study indicated that the sex of those vaccinated was unimportant provided the same percentage of the eligible population is vaccinated. That is, a similar reduction in gonorrhea prevalence was predicted for potential vaccines (with 50% or 100% efficacy and 20 years duration of protection) whether 50% of all 13-year-olds, or 100% of male or female 13-year-olds, were vaccinated (Craig et al., 2015).

In light of the anatomical and physiological differences that exist between humans and mice (the only animal model presently used), there is a need for the increased use of humanized models, albeit even humanized animal models of infection may not be sufficient to overcome the numerous obstacles associated with gonococcal vaccine development and will not compensate for anatomical differences among species. Given the propensity of the gonococcus to subvert human-specific molecules to promote infection and disease, data obtained from human sources could, and should, be used to guide the development of such animal models. Total proteomic, genomic, metabolomic analyses of human specimens (e.g. patient fluids and tissues) offer a means of identifying human factors contributing to infection but also the human response(s) generated to infecting organisms. Similar analyses of bacteria isolated from these patients would also identify critical virulence determinants promoting bacterial survival/proliferation and/or that play a role in immune induction or its suppression. The increased speed and lowered costs now associated with these “big data” techniques presently make these approaches more accessible to investigators and, thereby, increase their feasible contribution to vaccine development. Moreover, the relevance of knowledge gained through the *ex vivo* analysis of human subjects cannot be under stated. Focused quantitative analyses to verify/support data obtained by *ex vivo* analyses of human subjects can be achieved by using primary human cell and organ cultures as well as animal models. Moreover, multi-organ cultures (MOCs) could be adapted to incorporate the combined use of primary human epithelial and immune cells (or tissues) into a single experimental model. MOC models are currently being used in pre-clinical drug toxicity studies and offer an excellent opportunity to study the human immune responses generated during infection. The development of MOCs and “organs-/humans-on-chips” also provide alternative non-invasive, human-specific models by which data obtained from patient specimens can be further evaluated (Bhatia & Ingber, 2014).

An increased focus on human samples and humanized animal models may provide insights into what is required for protective immunity against gonorrhea and help define a correlate of protection. Evaluating immunotherapy may enable us to begin to define the target, type and level of antibody response needed to protect against, or reduce, mucosal infection or sequelae. Several studies support the feasibility of immunotherapy to treat sexually transmitted infections, in terms of effective delivery and targeting of antibodies to the genital tract, leading to clearance of

infection and/or prevention of disease (reviewed in Naz, 2012). For example, mice given prophylactic or therapeutic intravenous hu2c monoclonal antibodies (mAbs) were completely protected from infection-related mortality after vaginal challenge with a lethal dose of HSV-1. Almost complete protection was also seen when hu2c was given intraperitoneally after infection with a multidrug-resistant HSV-1 patient isolate (Krawczyk et al., 2013). Rhesus macaques given neutralizing mAb intravenously were protected against infection or disease manifestations after vaginal challenge with a pathogenic HIV-1/SIV chimeric virus (Mascola et al., 2000). In humans, combined immunochemotherapy with the chimeric human/mouse mAb rituximab has been used to successfully treat primary non-Hodgkin's lymphoma of the uterus, cervix and parametrium (Su et al., 2008), and the humanized mAb Trastuzumab provided relief of symptoms, and prolonged survival, in patients with endometrial carcinoma (Santin et al., 2008). Various delivery systems (e.g. liposomes, microspheres, nanoparticles, nanogels, bionanocapsules) are under investigation (Buss et al., 2012) that may enable monoclonal antibodies to be directly delivered to the genital tract in the future. Potential gonococcal antigen targets that can be exploited for antibody therapy also exist. For example, antibodies to gonococcal phospholipase D (PLD), a key regulator of infection of human cervical epithelia (Apicella & Edwards, 2007), are able to decrease adherence to and invasion of primary cervical cells *in vitro* (Edwards et al., 2003). Antibodies against a truncated, non-glycosylated, recombinant form of the AniA protein are able to block its nitrite reductase function in a whole cell assay (Shewell et al., 2013). Several other examples are listed in Table 1, and a combination of antibodies that are able to block colonization and essential metabolic activities of the bacteria may have a significant impact in preventing infection, transmission and/or disease. Even a modest reduction in infection or sequelae by immunotherapy may provide a significant impact on prevalence, similar to what has been reported for "hypothetical" vaccines, in which even a vaccine with modest efficacy could have a substantial impact on gonorrhea prevalence (Craig et al., 2015). Although monoclonal antibody therapy is expensive and likely to be of limited utility for the population that needs it most, it may aid in understanding required correlates of protection and may serve as a last line of therapy for untreatable *N. gonorrhoeae* strains.

Conclusions

To prevent the increased prevalence of gonorrhea, and its associated disease sequelae, there is an urgent need for increased investment in antibiotic and vaccine development. Future research should also focus on the development of new models of infection and the use of all available tools without an over-reliance on one particular model or method. No infection model is without its limitations, but it may be possible to overcome many of the current limitations by using a combined approach and by placing an emphasis on collecting and incorporating human (or human source)-derived data. Novel approaches may also be required, such as the use of "organs/humans-on-chips" or the evaluation of immunotherapy, to help address the many unanswered

questions regarding the mechanisms of protective immunity and what is required to combat the human specific pathogen *N. gonorrhoeae*. In the absence of a correlate of protection, it is difficult to predict the success of candidate gonococcal antigens based on pre-clinical data. Therefore, it may be that human clinical trials are needed, using the best available antigens (based on immunogenicity and antibody function), to provide evidence of effectiveness and provide a pathway to vaccine licensure. A similar approach was used during the development of the acellular pertussis vaccine, for which the protective mechanism has not been fully elucidated (Farizo et al., 2014). Given the high cost of this approach, it is likely that public-private partnerships will be required to drive development of a gonococcal vaccine. In this regard, the recent emergence of untreatable *N. gonorrhoeae* strains may fuel collaborative efforts towards vaccine development. An increase in the number of untreatable gonorrhea cases may, in the future, also force changes in the way gonococcal vaccines are brought to the public; similar to the way the outbreaks of *N. meningitidis* infections at Princeton University and the University of California, Santa Barbara resulted in FDA approval of the meningococcal vaccine Bexsero to at-risk populations at these universities (only) (McNamara et al., 2015; Seib et al., 2015).

Acknowledgements

JLE and KLS contributed equally to this paper and are co-senior authors.

Declaration of interest

This work was supported by the Australian National Health and Medical Research Council (NHMRC) [Project Grant 1028326 and Career Development Fellowship to KLS; Program Grant 565526 to MPJ]; and the National Institutes of Health (USA) [NIAID Grants 1R01AI076398 to JLE; and R01AI108255 and R01AI09320 to MAA]. The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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Is gonococcal disease preventable? The importance of understanding immunity and pathogenesis in vaccine development

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To cite this article: Jennifer L. Edwards, Michael P. Jennings, Michael A. Apicella & Kate L. Seib (2016) Is gonococcal disease preventable? The importance of understanding immunity and pathogenesis in vaccine development, *Critical Reviews in Microbiology*, 42:6, 928-941, DOI: [10.3109/1040841X.2015.1105782](https://doi.org/10.3109/1040841X.2015.1105782)

To link to this article: <https://doi.org/10.3109/1040841X.2015.1105782>



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