Determining the role of inhibitory antibodies on complement-mediated killing of *Neisseria gonorrhoeae* during acute gonorrhoea infection

by

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ABSTRACT

The mechanism of inhibitory antibodies produced by patients during acute gonorrhoea infection is largely unknown, and thus far there has been a lack of large-scale studies of the human immune response to the gonococcus to elucidate this mechanism. During this study, 336 clinical *Neisseria gonorrhoeae* isolates were screened for resistance against their autologous participant serum. Serum bactericidal assays revealed serum samples from nine participants which contained inhibitory antibodies. These isolates were sensitive to healthy serum, but resistant when incubated with the autologous participant serum. Antibody isotypes IgG, IgM and IgA were purified from inhibitory serum. After serum bactericidal assays with the autologous purified antibodies, it was observed that all isolates tested were protected from killing by healthy serum by purified inhibitory IgG and IgM at the highest concentrations trialled. IgA was able to inhibit killing in half of the isolates trialled. Closer analysis of the IgG subclasses involved in inhibition, showed a correlation between increased binding of IgG2 and a decreased binding of IgG3 to isolates demonstrating the inhibitory phenotype. This mechanism is distinct from previous literature on inhibitory antibodies against other Gram-negative organisms. Further analysis of the mechanism would be invaluable for informing gonococcal vaccine development.

DEDICATION

To my wonderful nan,
who sadly passed away during the writing of this thesis.

I know how much you wanted to see me finish my PhD, and it breaks my heart that you can't be here. I know you'll still be here with me in another way, and I hope I have made you proud.

Thank you for all the love and support you have always given me,
All my love.

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LIST OF ABBREVIATIONS

Abbreviation	Definition
AMR	antimicrobial resistance
ANCOVA	analysis of covariance
ATCC	The American Type Culture Collection
BASHH	British Association for Sexual Health and HIV
BHI	brain heart infusion
BRC	baby rabbit complement
BSA	bovine serum albumin
C3c	C3 convertase
C5c	C5 convertase
CDC	Centres for Disease Control and Prevention
CEACAM	carcinoembryonic antigen-related cell adhesion molecule
CFU	colony forming unit
CI	confidence interval
CIS	Clinical Immunology Services
CMP-NANA	cytidine 5'-monophospho-N-acetyl neuraminic acid
CS&T	cytometer setup and tracking
DGI	disseminated gonococcal infection
DNA	deoxyribonucleic acid
ELISA	enzyme-linked immunosorbent assay
ENA	European Nucleotide Archive
EUCAST	European Committee on Antimicrobial Susceptibility Testing
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
FSC	forward scatter
G-ToG	gentamycin for the treatment of gonorrhoea
GalNAc	N-acetylgalactosamine
GAM	IgG, IgA and IgM immunoglobulins
GISP	Gonococcal Isolate Surveillance Project (USA)
GlcNAc	N-acetylglucosamine
gMFI	geometric mean fluorescence intensity
GRASP	Gonococcal Resistance to Antimicrobials Surveillance Programme
GWAS	genome-wide association study
HCS	healthy control serum
Hep	heptose
HI	heat inactivated
HIV	human immunodeficiency virus
HRP	horseradish peroxidase
i-gMFI	integrated geometric mean fluorescence intensity
IFNγ	interferon-γ
Ig	immunoglobulin
IL	interleukin
iTOL	Interactive Tree of Life
KDO	2-keto-3-deoxy-mannooctulosonic acid

LNnT	lacto-N-neotetraose
LOS	lipooligosaccharide
LPS	lipopolysaccharide
MAC	membrane attack complex
maf	minimum allele frequency
MASP	MBL-associated serine proteases
MBL	mannose-binding lectin
MFI	mean fluorescence intensity
MIC	minimum inhibitory concentration
ML	maximum likelihood
MLST	multi-locus sequence type
MSM	men who have sex with men
MSW	men who have sex with women
NAAT	nucleic acid amplification test
NG-MAST	*Neisseria gonorrhoeae* multi-antigen sequence type
NHS	normal human serum
NOD	nucleotide-binding oligomerisation domain-containing protein
OD	optical density
OMV	outer membrane vesicle
o/n	overnight
PAMP	pathogen-associated molecular pattern
p*bla*	β-lactamase plasmid
PBS	phosphate buffered saline
pConj	conjugative plasmid
pCryp	cryptic plasmid
PE	R-phycoerythrin
PEA	phosphoethanolamine
PHE	Public Health England
PID	pelvic inflammatory disease
ROS	reactive oxygen species
SBA	serum bactericidal assay
SNP	single nucleotide polymorphism
SSC	side scatter
ST	sequence type
STD	sexually transmitted disease
TGF-β	transforming growth factor β
TLR	toll-like receptor
UKHSA	UK Health Security Agency
UPEC	uropathogenic *Escherichia coli*
UTI	urinary tract infection
WGS	whole genome sequencing
WHO	World Health Organisation
WSM	women who have sex with men

CHAPTER 1

INTRODUCTION

1.1 The genus *Neisseria*

Species within the *Neisseria* genus are members of the Betaproteobacteria class, that are known to colonise the mucosal surfaces of numerous animals. *Neisseria* comprises of at least 23 species, of which a large number exist as non-pathogenic commensal organisms in humans, including *Neisseria lactamica* and *Neisseria cinerea*, making up a large proportion of the oropharyngeal and nasal flora (Liu *et al.*, 2015). Only two members of the genus are obligate human pathogens, *Neisseria gonorrhoeae*, the cause of the sexually transmitted disease (STD) gonorrhoea and *Neisseria meningitidis*, the etiological agent of meningococcal septicaemia and the main cause of bacterial cerebrospinal meningitis. *N. meningitidis* can also be carried by 10-15% of the general population as a nasopharynx commensal.

1.1.1 Neisserial genetics

Considering the vast differences in the disease pathogenesis of *N. gonorrhoeae* and *N. meningitidis*, both organisms are closely related genomically and share 80-90% deoxyribonucleic acid (DNA) homology (Marjuki *et al.*, 2019) including a highly similar core genome. The major differences between the two organisms are that *N. meningitidis* has loci which encode its surrounding polysaccharide capsule, and it rarely carries plasmids. However, *N. gonorrhoeae* can harbour three different plasmids (Cehovin *et al.*, 2020), the cryptic plasmid (pCryp), a small 4.2 kb multi-copy plasmid with no defined function but highly prevalent in ~95% of gonococcal isolates, the conjugative plasmid (pConj) which can carry *tetM* conferring resistance to tetracycline, and the ß-lactamase plasmid (p*bla*), of which there are several varieties which can confer resistance to ß-lactams.

Both pathogenic *N. gonorrhoeae* and *N. meningitidis* are polyploid, with an least two chromosomal copies per growing monococcal cell (Tobiason and Seifert, 2006). Commensal *N. lactamica* is monoploid (Tobiason and Seifert, 2010), therefore it is thought that polyploidy is a

virulence trait which could facilitate homologous recombination.

Phylogenetic analysis shows that both pathogenic species represent two separate lineages that evolved from a common ancestor. *N. gonorrhoeae* and *N. meningitidis* now occupy diverse separate niches; the genital mucosa and nasopharyngeal mucosa, respectively (Maiden and Harrison, 2016, Bratcher *et al.*, 2014, Maiden, 2008). However, some of these niches are also occupied by commensal *Neisseria* species. Many of the virulence factors associated with pathogenic *Neisseria* are also found in commensal species, such as immunoreactive surface proteins and iron scavenging (Marri *et al.*, 2010, Snyder and Saunders, 2006), presumably because they are essential factors for colonisation, survival and growth within the human host, hence differentiating between colonisation and virulence factors is often challenging. As it has evolved, the gonococcus has retained many features commonly found in commensal species, however it has also acquired specific features that are essential for its pathogenesis (as detailed in a review by Quillin and Seifert (2018)).

N. gonorrhoeae has a small circular chromosome approximately ~2.2 Mbp in size with an average G+C content of 52% (Rotman and Seifert, 2014), equating to 2,200-2,500 protein coding sequences. This small genome reflects the evolutionary changes the bacterium has undergone to adapt to surviving and proliferating in the mucosal niche (Lu *et al.*, 2019).

1.1.2 The bacterium *Neisseria gonorrhoeae*

N. gonorrhoeae are Gram-negative cocci that are approximately 0.6-1 μm in size and typically appear in pairs (diplococci). These bacteria are characteristically non-motile due to the lack of flagella, yet are capable of moving through twitching motility using type IV pili (Craig *et al.*, 2019). These fastidious organisms are identified as capnophiles, as they thrive in the presence of a high concentration of carbon dioxide if there is a small amount of free oxygen present.

1.2 Gonococcal disease

1.2.1 Clinical manifestations

Gonococcal disease is typically an uncomplicated infection of mucosal surfaces. The gonococcus infects the genitourinary tract of both women and men, as well as the ability to penetrate and proliferate other mucosal surfaces, such as the oropharynx and rectum, leading to invasive disease. In infected males, at least 90% of cases are symptomatic (Unemo *et al.*, 2019). There is typically a ~2-8 day incubation period before symptoms begin, taking the form of proctitis, urethritis and/or pharyngitis. However, in women, infections are commonly asymptomatic, with half of women not presenting with symptoms (Martin-Sanchez *et al.*, 2020). If symptoms do appear, they tend to be less severe than in males, with cervicitis being the most common symptom in women. A review by Quillin and Seifert (2018), suggests that the reason infections in women are mostly asymptomatic is largely due to anatomical differences in the urogenital tracts of women and men. The genital tract of men and women are both very distinct microenvironments and the epithelial lining of both are decorated with differing cell surface structures. These act as receptors for the bacterium and result in different mechanisms by which *N. gonorrhoeae* survives in the female and male urogenital tract niches (Edwards and Apicella, 2004). In males, the bacterium infects the urethra and urinary tract, and therefore the symptoms caused by inflammation and immune cell influx are usually more overt and easier to diagnose. In females, the bacterium occupies a niche very different to the urinary tract and is therefore less likely to be painful, resulting in the infection going unnoticed. If symptoms do present, they are more likely to be non-specific (Hook iii, 2012).

On rare occasions, gonococcal infections can lead to one or more complications, usually when left untreated. In males, ascending urethral infection can result in epididymitis (Unemo *et al.*, 2019). In females, 18-20% of untreated cervical infections can transport to the fallopian tubes and endometrium, which might cause pelvic inflammatory disease (PID) leading to fertility

problems (Lenz and Dillard, 2018, Walker and Sweet, 2011). In addition, gonorrhoea is associated with adverse pregnancy outcomes, and babies delivered from infected mothers have 30-45% increased risk of acute conjunctivitis. Rarely, disseminated gonococcal infection (DGI) is another severe complication involving a systemic form of the disease that can occur in 0.5-3% of individuals of either gender (Beatrous *et al.*, 2017). This can involve infection of the skin, joints and synovium resulting in gonococcal arthritis, and the heart resulting in gonococcal endocarditis.

1.2.2 Epidemiology

Gonorrhoea is the second most common bacterial STD in the United Kingdom, with 70,922 diagnosed infections reported in England in 2019 (UKHSA, 2020) representing a 25.6% increase in infections from the previous year. Diagnoses are concentrated among specific population groups. Males experience the highest rates of gonorrhoea diagnoses (Figure 1.1), specifically within the men who have sex with men (MSM) community. In addition, the report also found incidence was largely concentrated among individuals of black Caribbean ethnicity.

1.2.3 Antibiotic resistance

In the absence of a gonococcal vaccine, the control and management of the disease relies heavily on effective, accessible and affordable antimicrobial treatment, supported by adequate diagnostic testing, transmission prevention and epidemiological surveillance. *N. gonorrhoeae* infections have been successfully treated with antibiotics since their introduction. Despite this, there has been a concerning increase in antimicrobial resistance (AMR) reported in gonococcal populations globally (Wi *et al.*, 2017), emphasising the imminent probability of widespread untreatable gonorrhoea. AMR *N. gonorrhoeae* was reported by the Centres for Disease Control and Prevention (CDC) in 2013 and 2019 as one of the five “urgent threats” of concern to human

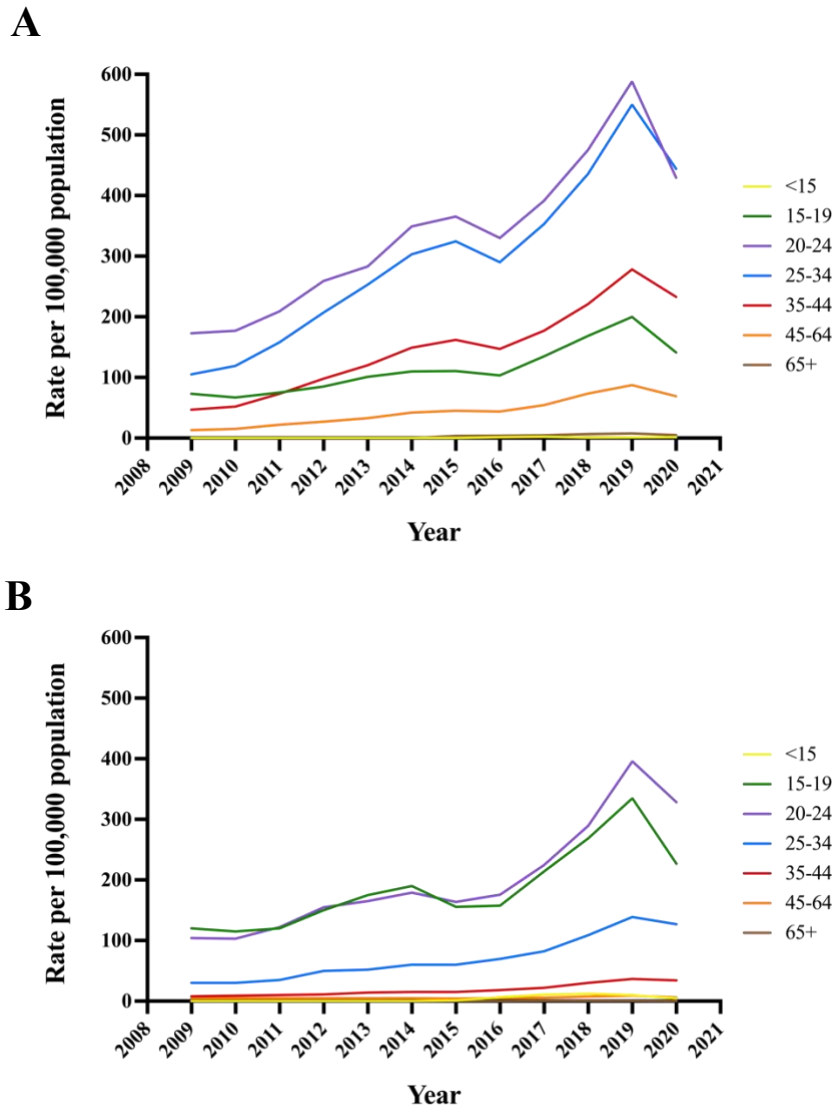


Figure 1.1. Rates of gonorrhoea diagnoses in England. Rates of newly diagnosed gonorrhoea infections among individuals attending sexual health clinics between 2009 and 2020 (rates per 100,000 population). Data taken from PHE (2020) and organised by age-group and gender; **A)** males and **B)** females.

health (CDC, 2019). The World Health Organisation (WHO) have also declared AMR *N. gonorrhoeae* as a high priority pathogen (Tacconelli *et al.*, 2018). There are currently elevated levels of resistance against multiple classes of antibiotics, including fluoroquinolones, tetracyclines and some β-lactams (Lewis, 2010, Workowski *et al.*, 2008), rendering most of these treatment options ineffective for clinical use. Much of this resistance of *N. gonorrhoeae* to cephalosporins and fluoroquinolones are carried on the chromosome. However, plasmids have contributed to tetracycline (Morse *et al.*, 1986) and β-lactam (Eisenstein *et al.*, 1977) resistance. Tetracycline resistance mediated by the *tetM* gene is sometimes carried on pConj (Pachulec and van der Does, 2010) with this plasmid also aiding in the transfer of other plasmids carrying other AMR genes (Pachulec and van der Does, 2010, Roberts, 1989), such as p*bla*, known for carrying β-lactamases. *N. gonorrhoeae* is also able to express up to five efflux pump systems (MtrC-MtrD-MtrE, MacA-MacB-MtrE, NorM, FarA-ForB-MtrE and MtrF) which help the bacterium remove toxic molecules and antimicrobials (Hagman *et al.*, 1995).

From 2018, the British Association for Sexual Health and HIV (BASHH) (Fifer *et al.*, 2020) recommended a regiment of 1 g ceftriaxone delivered intramuscularly as a single dose for the treatment of uncomplicated ano-genital and pharyngeal infection. This is a change from the 2011 guidelines that recommended a dual treatment of 500 mg ceftriaxone with 1 g azithromycin (Bignell *et al.*, 2011). This increase in ceftriaxone was anticipated to be more effective against isolates with increased minimum inhibitory concentrations (MICs). In addition, removal of azithromycin from the therapy was due to the increase transmission of azithromycin-resistant strains since 2011.

1.2.4 Gonococcal vaccine development

The emergence of AMR *N. gonorrhoeae* raises the concern that untreatable gonorrhoea might be inevitable (Unemo and Nicholas, 2012). Therefore, there is an urgent requirement to develop

an effective vaccine. Despite many efforts spanning a century, it has proven very difficult to develop an effective vaccine against *N. gonorrhoeae* (Russell *et al.*, 2019). To date, no FDA-approved vaccine against gonorrhoea is available.

1.2.4.1 Previous vaccine attempts

Only a few gonococcal vaccine candidates have reached human trials to date. There was a whole-cell vaccine, which provoked production of bactericidal antibodies, however it did not induce long term protection (Greenberg *et al.*, 1974, Greenberg, 1975). Subsequently, there were efforts to trial a type IV pilus individual component vaccine (Boslego *et al.*, 1991). However, this was also found to be ineffective, as there were no differences in the protection between the vaccinated and control groups in those that aquired gonorrhoea at least 15 days after vaccination. This and many other antigens considered as vaccine targets, such as lipooligosaccharide (LOS), are highly antigenically variable, rendering them highly infective as vaccine candidates. There is an exhaustive list of other previous single antigen vaccine candidates detailed in a review by Edwards *et al.* (2016). These failed attempts suggest that using a single antigen induces a unidimensional immune response that has proven not to result in protection against *N. gonorrhoeae*. The lack of progress on a gonorrhoea vaccine has also been due to the correlates of protection in humans being unknown and a lack of animal models to identify these protective responses. These hindrances discouraged many further efforts and the potential for a vaccine against gonorrhoea to be biologically feasible were doubted.

1.2.4.2 Outer membrane vesicle vaccines

It has become apparent that for an effective neutralising vaccine, it is essential that it contains multiple pathogen outer membrane structures that the immune system naturally comes into contact with. This ensures the induction of a broad immune response and, in turn, protective

immunity (van der Pol *et al.*, 2015). Therefore, outer membrane vesicles (OMVs) have been described as a potential antigen complex candidate for a vaccine for *N. gonorrhoeae* and other bacterial pathogens due to the molecular diversity of their composition. OMVs are single lipid bilayer spheres produced from the outer lipid bilayer of Gram-negative bacteria (Schwechheimer and Kuehn, 2015), and therefore they typically carry a broad array of outer membrane structures, including phospholipids, lipopolysaccharide (LPS) or LOS and various outer-membrane proteins all existing in their native form (Kulp and Kuehn, 2010).

Surveillance data on an OMV vaccine against meningococcal B (MeNZB) was associated with reduced rates of gonorrhoea in young adults following a mass vaccination campaign in New Zealand (Petousis-Harris *et al.*, 2017). It was estimated that the vaccine provided 31% effectiveness for cross protection against gonococcal infections. It has since been demonstrated that antibodies induced by these OMVs are able to recognise gonococcal outer membrane proteins, such as *Neisseria* heparin binding antigen (NhbA) (Semchenko *et al.*, 2019), and immunisation with the vaccine accelerated the clearance of *N. gonorrhoeae* in a mouse model (Leduc *et al.*, 2020). Numerous proteins integrated in the OMVs share similarities between both *N. gonorrhoeae* and *N. meningitidis* (Marjuki *et al.*, 2019), giving this vaccine a lot of potential as a universal vaccine against pathogenic *Neisseria* species. These were the first observations of a vaccine that induced protection against gonorrhoea. This evidence combined with research into gonococcal molecular pathogenesis, immunoproteomics and whole-genome sequencing, utilising databases such as PubMLST (Jolley *et al.*, 2018), has fuelled recent progress and provided optimism about the feasibility of a gonococcal vaccine (Gottlieb *et al.*, 2020).

1.3 *Neisseria gonorrhoeae* pathogenesis

1.3.1 Virulence factors

The cell envelope of any bacterium is involved in many interactions with the host. Therefore,

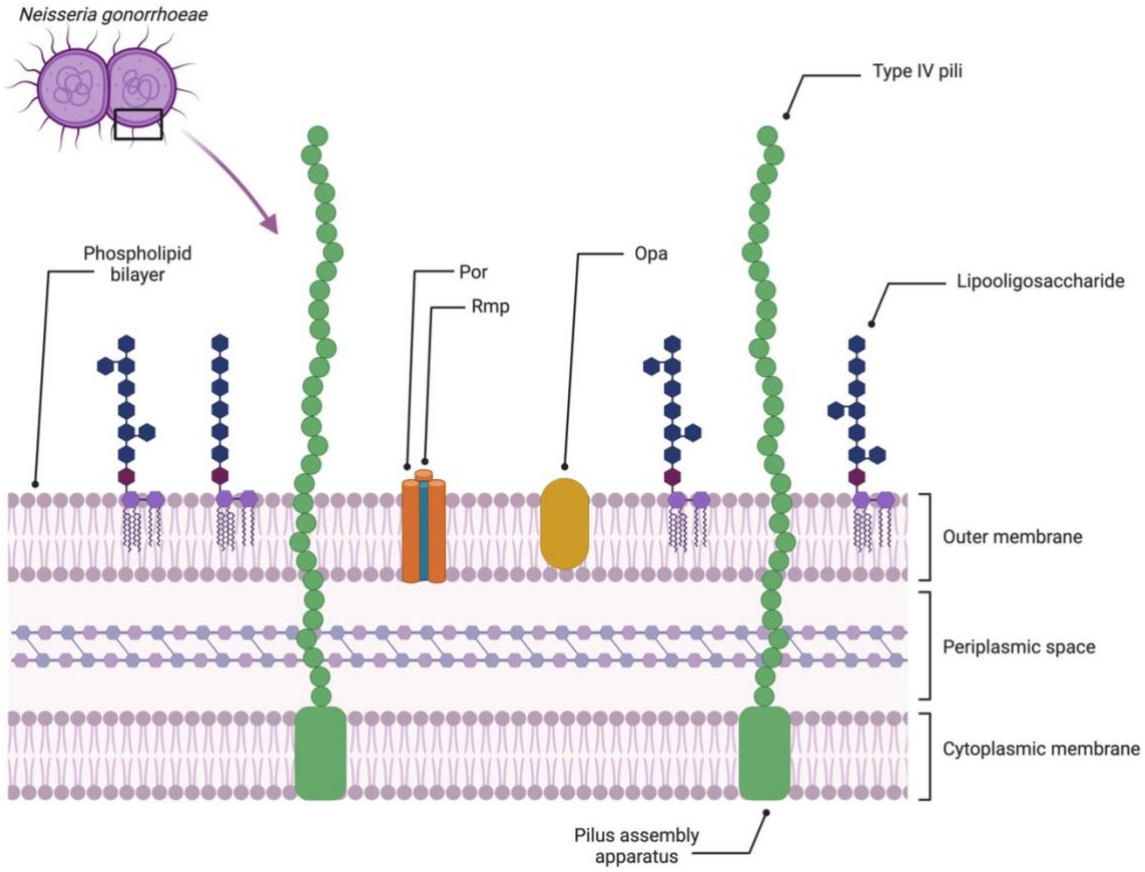


Figure 1.2. Outer membrane surface structures of *Neisseria gonorrhoeae*. *N. gonorrhoeae* uses an array of outer membrane surface structures to adhere to and invade host cells and evade the immune system, including type IV pili, porin proteins (Por), reduction modifiable protein (Rmp), lipooligosaccharide (LOS) and opacity protein (Opa). Image created using BioRender.com.

the gonococcal outer membrane is comprised of a complex mixture of structures that promote survival in the host and facilitates its evasion of the immune system. These include pili, porin proteins (Por), reduction modifiable protein (Rmp), opacity-associated proteins (Opa) and LOS, as detailed in Figure 1.2.

1.3.1.1 Pili

Type IV pili are filamentous appendages on the surface of the gonococcus anchored within the cell membrane. They are composed of several components, a major subunit (PilE) (Meyer *et al.*, 1984) and an adherence-associated subunit (PilC) located at the tip (Rudel *et al.*, 1995, Jonsson *et al.*, 1992). Type IV pili have many functions, including microcolony formation, twitching motility and facilitating in the exchange of DNA between bacteria (Craig *et al.*, 2019). They are also essential for establishing infection (Kellogg *et al.*, 1963, Seifert *et al.*, 1994) by mediating attachment to the epithelial (Swanson, 1972) and endothelial cells, which is supported by the observation that piliated gonococci adhere to and internalise into endothelial cells in higher numbers compare to non-piliated bacteria (Virji *et al.*, 1991). Pili largely bind to the eukaryotic transmembrane glycoprotein, CD46 (Källström *et al.*, 1997) that is expressed on almost all human cells (Liszewski *et al.*, 1991, Blom *et al.*, 2001, Edwards *et al.*, 2002). Clinical isolates are always piliated, however this piliation tends to be lost during lab culture, suggesting pilus expression is under strong selective pressure during infection.

Pili structures undergo antigenic variation allowing the bacterium to modify its antigenic structure, which is crucial for immune evasion (Craig *et al.*, 2004, Obergfell and Seifert, 2016). This is caused by homologous recombination between expressed pilin structural genes (*pilE*) and silent pilin gene homologues (*pilS*) (Vink *et al.*, 2012). The gonococcal genome has up to 19 *pilS* genes scattered around the chromosome in four or five different loci (Hamrick *et al.*, 2001), indicating that a single strain has the potential to construct a vast number of different

immunologically distinct pilin monomers. This results in the ability of bacteria to evade the immune response mounted against a previous pilin variant (Sparling *et al.*, 1986). In addition to this, pilin variation can be produced when piliated gonococci recombine with exogenous DNA taken up from the environment (Seifert *et al.*, 1988).

1.3.1.2 Opacity-associated proteins

The Opa proteins (or 'protein II') are abundant outer membrane structures which function as adhesions that bind to a variety of host cell receptors (Dehio *et al.*, 1998), and facilitate the tight attachment and invasion of host cells (Lambden *et al.*, 1979, James and Swanson, 1978). The majority of Opa proteins bind one or more human carcinoembryonic antigen-related cell adhesion molecules (CEACAMs) or heparan sulfate proteoglycans on epithelial cells (Sadarangani *et al.*, 2011).

Opa^+ gonococci (opaque phenotype) are associated with localised urogenital, rectal and/or cervical infections (Zak *et al.*, 1984). One study by Bos *et al.* (1997), showed that strains with the Opa^+ phenotype have a survival advantage whilst within the host. This occurrence might account for why there is a dominating presence of Opa^+ in fluids excreted by male volunteers during an intraurethral challenge model (Bos *et al.*, 1997, Schmidt *et al.*, 2000). The Opa^+ phenotype also confers a greater resistance to serum upon the bacterium, resulting in a selective survival of Opa^+ strains in a mixed population.

A single strain of *N. gonorrhoeae* can encode ~11-13 *opa* genes (Anderson and Seifert, 2013), and at any one time, strains can express none, one or multiple Opa proteins (Swanson, 1978). Each *opa* gene contains multiple pentameric coding repeats (5′-CTCTT-3′), which each gene can independently switch on and off using slipped-strand mispairing. This results in frameshifting of translated Opa proteins generating proteins that are antigenically distinct (Anderson and Seifert, 2013). This phase variation allows for a low-level continuous cycling of

the whole Opa repertoire during infection. The specific Opa proteins that a strain expresses are dependent on selective pressures that occur once the strain has interacted with the host, and strains that express Opa proteins with greater affinity for certain host receptors become dominant and proliferate (Robertson and Meyer, 1992). For example, OpaK is the most commonly detected Opa protein from clinical isolates and it aids adherence to epithelial cells and mediates invasion. OpaI/H is dominant in isolates obtained from patients with blood type O+, and OpaF from patients with B+ and AB+.

1.3.1.3 Porin protein

Gonococcal Por protein (or 'protein I') is a highly abundant outer membrane pore made from trimer of porin monomers (Weiss *et al.*, 1991, Weiss and Schulz, 1992), and makes up 60% of the gonococcal outer membrane proteins (Johnston *et al.*, 1976). Porins function as hydrophilic channels that facilitate the passage of small molecules across the outer membrane (Nikaido, 1994) allowing access to the periplasm. PorB also increases bacterial attachment and invasion of host cells, resists the action of complement factors, impairs the ability of phagocytes and modulates apoptosis (Massari *et al.*, 2002, Olesky *et al.*, 2006). Unlike the other aforementioned outer membrane structures, PorB is antigenically stable and does not undergo the same high-frequency variation.

There are two major variants of PorB, PorB.IA and PorB.IB with around 80% homology, and each gonococcal strain expresses a single PorB allele (Sun *et al.*, 2010). Each allele is associated with distinctive biological phenotypes. PorB.IA is present in 10-30% of *N. gonorrhoeae* isolates and is typically associated with DGI (Cooke *et al.*, 1998). Strains expressing PorB.IB occur in 70-90% of isolates and tend to cause uncomplicated infections (Brunham *et al.*, 1985, Cannon *et al.*, 1983).

1.3.1.4 Reduction modifiable protein

RmpM (or 'protein III') is a surface-exposed outer membrane protein that occurs in both species of pathogenic *Neisseria* (Judd, 1982), and noncovalently associates with PorB in the outer membrane (Newhall *et al.*, 1980). It is highly conserved and does not exhibit antigenic or phase variation. Rmp is known to elicit the production of antibodies that inhibit the binding of bactericidal antibodies (Rice *et al.*, 1986), which is detailed further in Section 1.5.4.1.

1.3.1.5 Lipooligosaccharide

LPS is a major outer surface membrane component present on almost all Gram-negative bacteria. It is comprised of three genetically, chemically and biologically unique domains. Firstly, the phosphorylated and acylated lipid A is anchored in the outer membrane of the bacterium, that is then linked to the core oligosaccharide followed by the long O-antigen. In contrast, the gonococcus has a low molecular weight version of LPS on its surface, LOS. LOS lack the repeating saccharide units present in LPS (Song *et al.*, 2000), resulting in just the lipid A and a highly branched basal core oligosaccharide structure. The gonococcus might contain these shorter branching oligosaccharides rather than long O-antigen oligosaccharide repeats due to *N. gonorrhoeae* only having to colonise the non-hostile environment of the mucosal lining. In contrast, *Salmonella enterica*, for example, usually inhabit much harsher environments containing bile salts, and therefore the long hydrophobic oligosaccharide repeats protect the bacterial lipid membrane from dispersion. In addition, gonococcal LOS genes are scattered around the genome, suggesting regulation of LOS expression is fundamentally distinct to the regulation of LPS in enteric bacteria, where the genes are in clusters.

Gonococcal LOS is the most abundant molecule in the outer membrane, and accounts for around 50% of the outer membrane mass. It plays an essential role during pathogenesis of the organism, including host cell adherence, invasion and immune cell recognition (Song *et al.*,

2000, Ram *et al.*, 2018). LOS also triggers an intense inflammatory response and is therefore thought to be responsible for the majority of the symptoms, with particular structures associated with severity of symptoms. A general structure of gonococcal LOS is depicted in Figure 1.3. The core portion of the LOS is directly linked to one lipid A molecule *via* a 2-keto-3-deoxy-mannooctulosonic acid (KDO) residue. From here, α-, β- and γ-oligosaccharide branches can be extended by the sequential addition of monosaccharide units of either glucose, galactose, N-acetylgalactosamine (GalNAc) and N-acetylglucosamine (GluNAc). The heptose^{I} (Hep) lactose structure (Hep^{I} -glucose-galactose) of the α-chain is observed in all *Neisseria* species and have been found to mimic human outer membrane lactosylceramide glycosphingolipids, present on most host cells (Harvey *et al.*, 2001b). This allows the gonococcus to evade detection by the host immune system. In addition, the same lactose structure from Hep^{II} (Hep^{II}-glucose-galactose) of the β-chain, is expressed exclusively by *N. gonorrhoeae*, and has been found to be expressed by ~95% of clinical isolates (Gulati *et al.*, 1996a), suggesting a key role in virulence. This has been supported by Gulati *et al.* (2013), as they found that strains with no extension from Hep^{II} (*lgtG* mutants) were less virulent in mice. The addition of a galactose creates a globotriose (Hep^{I}-glucose-galactose-galactose), which is identical to human P^{K} antigen (Mandrell, 1992). A 'Hep^{I}-glucose-galactose-GlcNAc-galactose' α-chain extension generates a tetraose named lacto-N-neotetraose (LNnT). This tetraose structure is considered a mature LOS structure, and very highly conserved among *Neisseria* species (Griffiss *et al.*, 1988). This structure is identical to human paragloboside glycosphingolipids, that again allows the gonococcus to evade the immune system (Mandrell *et al.*, 1988). Strains expressing LNnT LOS are typically associated with symptomatic gonococcal infection. In contrast, strains expressing lactose LOS are usually associated with asymptomatic gonococcal infection.

In addition to the previously discussed pili and Opa proteins, neisserial LOS also exhibits structural and antigenic diversity. The lipid A component is highly conserved, however the

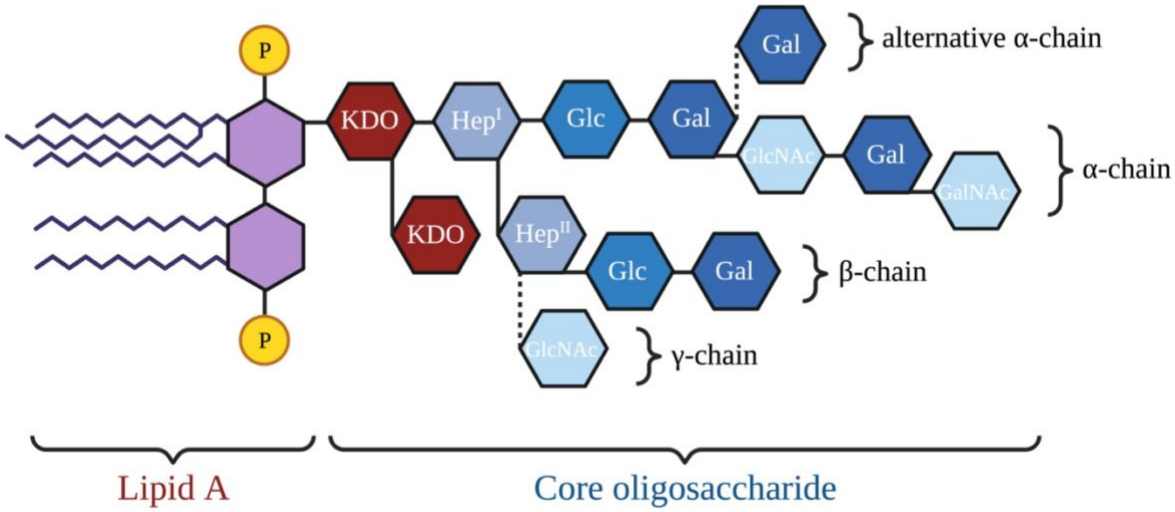


Figure 1.3 General structure of *Neisseria gonorrhoeae* lipooligosaccharide molecules. Lipooligosaccharide **(**LOS) molecules are structurally diverse, and the α-, β- and γ-oligosaccharide branches might be extended by the sequential addition of monosaccharide units of either glucose (glu), galactose (gal), N-acetylgalactosamine (GalNAc) and N-acetylglucosamine (GluNAc). The end of the mature lacto-N-neotetraose (LNnT) structure (Hep^{I}-glu-gal-GlcNAc-gal) is the usual site of sialylation where host sialic acid can bind the bacteria once it establishes infection in the host. Image created using BioRender.com.

composition of the adjacent basal region varies. It can differ in the branches (α-, β- and/or γ-chains) that are present, in oligosaccharide length and in the monosaccharides added to each chain and their concentrations (Apicella *et al.*, 1987, Yamasaki *et al.*, 1991). It is common for one strain of *N. gonorrhoeae* to express numerous distinct LOS species simultaneously, with each having a different antigenic structure (Apicella *et al.*, 1987).

1.3.2 Transmission

N. gonorrhoeae is unable to survive outside the host, therefore transmission between people requires close intimate contact. The infection is easily spread from men to their partner through their ejaculate, as bacteria are excreted in large quantities (Cohen *et al.*, 1994), where ~$7x10^6$ total colony forming units (cfu) can be recovered from semen (Isbey *et al.*, 1997). A study by Harvey *et al.* (2000) showed that the gonococcus attaches to sperm *via* their LOS, binding to asialoglycoprotein receptor 1 on the sperm. However, the mechanism of transmission of *N. gonorrhoeae* from women to men is less apparent. For the bacterium to effectively attach and enter the male urethral epithelial cells, the bacterial surface must not be sialylated. It is therefore thought that *N. gonorrhoeae* is desialylated by secreted cervicovaginal microbiota bacterial sialidases, to facilitate successful transmission from women to men (Ketterer *et al.*, 2016).

1.3.3 Adherence, colonisation and invasion

After transmission, adherence of the bacterium to the microvilli of non-ciliated epithelial cells on host mucosal surfaces is critical for establishing infection, and this initial attachment is mediated by type IV pili. Anderson *et al.* (2014) proposed that during transmission, the exposure of the gonococcus to seminal plasma alters bacterial motility characteristics, with an increase in pili filaments on the bacterial surface. Protein PilC, located at the tip of the mature pili structure, is involved in this adherence (Jonsson *et al.*, 1991, Rudel *et al.*, 1992). After contact, pilus

retraction brings the bacterium closer to the surface of the cell. Once the bacterium reaches the surface of the epithelial cell, PilC is then downregulated in the microaerobic environment, allowing for a tighter attachment mediated by Opa proteins. Opa proteins typically interact with CEACAM receptors on mucosal epithelium cell surfaces (Swanson *et al.*, 1988, Jerse *et al.*, 1994).

After this initial adherence, *N. gonorrhoeae* replicates and can form microcolonies and possibly biofilms (Anderson *et al.*, 2016) on the mucosal epithelium. It is thought that during this colonisation, *N. gonorrhoeae* has to compete with the resident microbiota, where some studies have shown that organisms within the vaginal microbiota, for example lactobacilli producing hydrogen peroxide inhibit the growth of *N. gonorrhoeae* (van der Veer *et al.*, 2019, Wiesenfeld *et al.*, 2003). During this colonisation, bacteria have been found to release fragments of peptidoglycan, OMVs or LOS, collectively described as pathogen-associated molecular patterns (PAMPs), that can trigger cognate innate immune sensors. These include nucleotide-binding oligomerisation domain-containing protein 1 (NOD1) and 2 (NOD2), which detect peptidoglycan, and Toll-like receptor 2 (TLR2) and 4 (TLR4), which detect LOS and lipoproteins, all of which induce host innate immune system signalling (Mavrogiorgos *et al.*, 2014, Fisette *et al.*, 2003, Massari *et al.*, 2002). Detection of colonising bacteria and PAMPs by immune sentinel cells, such as dendritic cells and macrophages (van Vliet *et al.*, 2009, Chateau and Seifert, 2016) stimulates the release of pro-inflammatory cytokines and chemokines, including interleukin-6 (IL-6), IL-8, IL-1β, IL-17 and interferon-γ (IFNγ). This results in a symptomatic gonococcal infection, which has potential to result in inflammatory damage within the epithelial mucosa (Edwards and Butler, 2011).

Also during colonisation of the epithelial surface, the bacterium has the ability to invade non-ciliated cervical or urethral epithelial cells, when LOS is desialylated (Edwards and Apicella, 2004). The interaction with desialylated LOS and a variety of epithelial cell receptors

promotes invasion, including asialoglycoprotein receptors in the urethra of men (Harvey *et al.*, 2001a), complement receptor 3 in the lower cervical genital tract (Edwards *et al.*, 2001) and lutropin-choriogonadotropin hormone receptor in the fallopian and endometrium environments. Actin polymerisation is induced along with extension of microvilli, and the bacteria are internalised into vacuoles at the apical surface of the epithelial cell. Gonococci are capable of replicating in vacuoles and can transcytose across to the basolateral epithelial cell surface. The bacteria are then able to erupt through the basolateral surface and are released into the subepithelial space where infection is established. Here they can then infect other epithelial cells. Infected cervical and vaginal epithelial cells showed increases in IL-1, IL-6 and IL-8 levels *in vitro* (Fichorova *et al.*, 2001), which suggests an inflammatory cytokine response occurs during infection. Gonococci are able to survive in macrophages by regulating apoptosis and production of cytokines (Chateau and Seifert, 2016) and they can polarise macrophages leading to T cell proliferation suppression (Ortiz *et al.*, 2015).

Release of proinflammatory cytokines cause a large influx of neutrophils at the infection site (Criss and Seifert, 2012). Gonococci are able to internalise into neutrophils by interacting with CEACAM1, CEACAM3 and/or CEACAM6 receptors on the surface of neutrophils (Sarantis and Gray-Owen, 2012), with most isolated clinical bacteria predominantly expressing Opa for CEACAM1 (Sintsova *et al.*, 2015). The gonococcus is then able to survive inside by producing enzymes, such as catalase and peptide methionine sulfoxide reductase, which protect the bacterium by detoxifying reactive oxygen species (ROS) that are generated by neutrophils (Stohl *et al.*, 2005). The purulent exudate that symptomatic patients report (Nassif *et al.*, 1999) contains significant amounts of neutrophils, many of which are associated with bound and intracellular gonococci, which facilitates further transmission. In contrast, very little is known about the immune response during asymptomatic infection and whether neutrophils are recruited to the infection site, or just in insufficient amounts. Interestingly, it has been noted that some

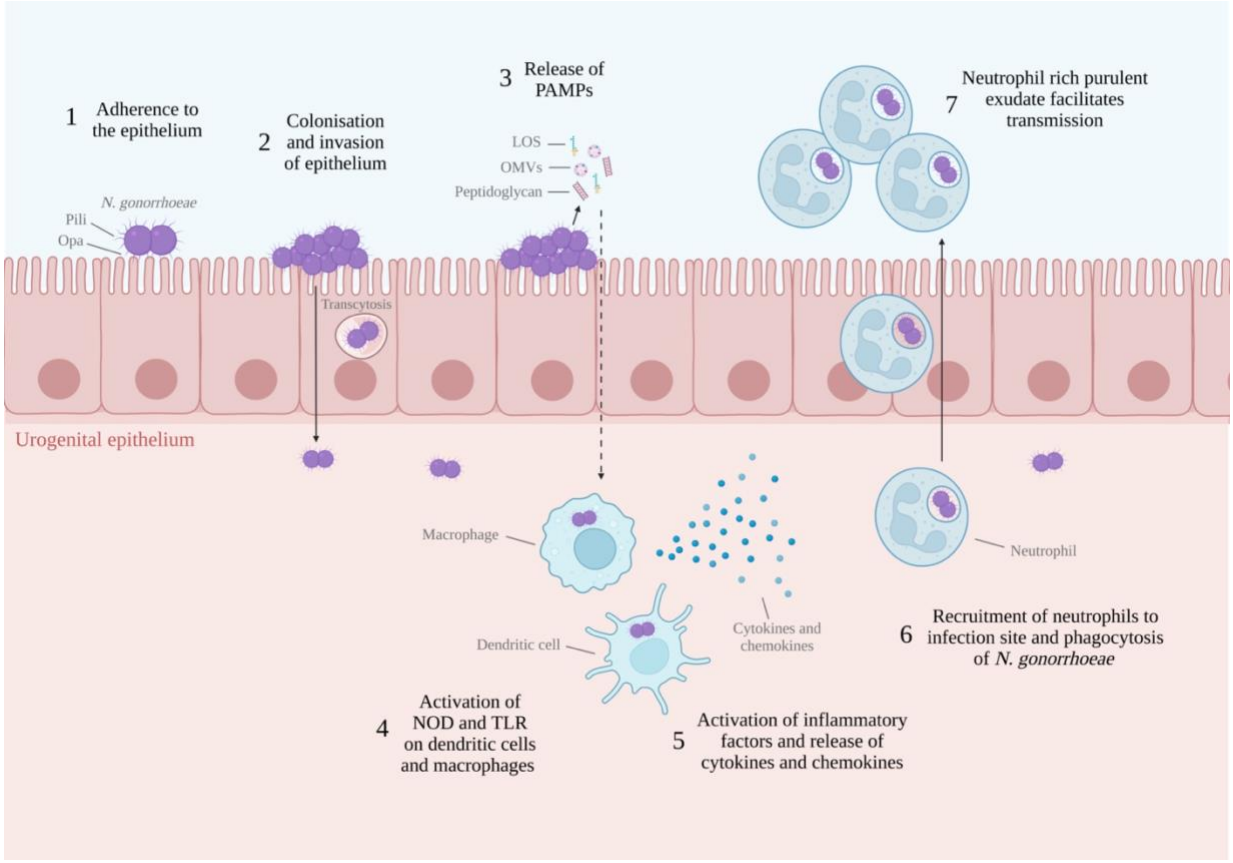


Figure 1.4 Overview of *Neisseria gonorrhoeae* pathogenesis. During infection, *N. gonorrhoeae* adheres to epithelial cells through type IV pili and Opa proteins. The bacteria colonise the epithelium and are able to invade cells and transcytose to the basolateral epithelial cell surface. During colonisation, they are capable of releasing pathogen-associated molecular patterns (PAMPs) that can cause toll-like receptor (TLR) and nucleotide oligomerisation domain-containing protein (NOD) activation on epithelial, dendritic cells and macrophages. This leads to activation of inflammatory factors and the release of chemokines and cytokines, that recruit a large number of neutrophils to the infection site. *N. gonorrhoeae* is able to survive in these cells which facilitates transmission. Image created using BioRender.com and adapted from Quillin and Seifert (2018).

bacteria express Opa that bind CEACAM3 which are in turn killed inside neutrophils (Palmer and Criss, 2018). This is thought to be orchestrated by a subset of bacteria during infection as a decoy to prevent neutrophil influx, and therefore avoiding the development of symptoms, but still colonising other host cells, leading to an asymptomatic infection. Gonococcal pathogenesis is further detailed in a review by Quillin and Seifert (2018). A schematic of gonococcal pathogenesis is shown in Figure 1.4.

1.3.4 Adaptive immune response to *Neisseria gonorrhoeae*

Induction of IL-17 is important for recruiting neutrophils to the site of infection to induce a localised immune response. Studies in mice have demonstrated that during infection with *N. gonorrhoeae,* an enhanced Th17 response is provoked through the induction of transforming growth factor β (TGF-β), and suggested that *N. gonorrhoeae* selectively inhibited the Th1 and Th2 adaptive responses (Liu and Russell, 2011, Liu *et al.*, 2012). This was also demonstrated in a study in humans, where an elevated production of IL-17 was observed, which inversely correlated with a suppression of IFNγ production (Gagliardi *et al.*, 2011). This Th17 weighted immune response provoked by *N. gonorrhoeae* is not effective at clearing the infection as a strong Th1 and Th2 might be (Criss *et al.,* 2009). In support of this, it was demonstrated in mice that blocking the induction of Treg related activities and driving a more robust Th1 and Th2 response enhanced clearance (Liu *et al.*, 2013). This inhibition of Th proliferation in turn results in difficulties for B cells to produce high affinity specific antibodies (Li and Su, 2018). Therefore it could be proposed that a successful vaccine against *N. gonorrhoeae* might need to stimulate a Th1 response in order to confer protection.

1.4 Humoral immune response to *Neisseria gonorrhoeae*

1.4.1 Antibody response

The bloodstream, mucosal surfaces and tissues of the host are all protected from invading pathogens by antibodies, that aim to neutralise and eliminate the pathogen before infection can be established. Different antibody isotypes, immunoglobulin (Ig) G, IgM and IgA, are adapted to implement different functions, and are summarised in Table 1.1.

Naturally occurring IgM antibodies are the most abundant natural antibody in the body and account for 80% of all IgM circulating in the bloodstream (Savage and Baumgarth, 2015). They are non-specific polyvalent molecules with ten antigen binding sites that recognise many bacterial structures, including carbohydrates, proteins, phospholipids, and nucleic acids. Upon antigen binding IgM is able to initiate complement, directly neutralise invading organisms and opsonise for engulfment by phagocytes (Chen *et al.*, 2009). After antigen stimulation, antibody class switching occurs where activated B cells switch from producing IgM to IgG and IgA, which increases antigen-specificity compared to IgM alone (Panda and Ding, 2015). IgG is a strong activator of the complement system and also opsonises invading pathogens for phagocytosis. It is small in size and therefore can easily diffuse out of the blood and into the extracellular fluid. Here, in bodily tissues is where the antibody mainly operates, as accessory cells and molecules are readily available. Elevated levels of adaptive IgG are indicative of an established infection. IgG consists of four subtypes, predominantly IgG1, followed by IgG2, IgG4 and IgG3, where each vary in their antigen binding target, ability to activate the complement system and opsonisation ability. IgA antibodies reside on the mucosal epithelium surface. It is a weak opsonin and activator of complement, as complement and phagocytes are not typically present in the mucosa. Therefore, IgA functions principally as a neutralising antibody, and aims to prevent pathogen attachment to epithelial cells.

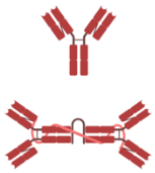
Characteristic	IgA	IgM	IgG1	IgG2	IgG3	IgG4
Size (kDa)	160	970	146	146	170	146
Structure						
Response to polysaccharide antigens	++	++	+	+++	+/-	+/-
Response to protein antigens	+	+	++	+/-	++	++
Activation of complement	+	+++	++	+	+++	-
Neutralisation	++	+	++	++	++	++
Opsonisation	+	-	+++	+/-	++	+
Diffusion into extravascular sites	++	+/-	+++	+++	+++	+++
Average serum concentration (mg/ml)	2.1	1.5	6.98	3.8	0.51	0.56

Table 1.1 Characteristics of the predominant human immunoglobulin isotypes. The three main human antibody isotypes are IgA, IgM and total IgG, which encompasses the four subclasses IgG1-4. All antibodies are adapted to implement different functions to protect the host from invading pathogens before infection can be established, with each differing in major effector functions (+++), lesser functions (++) and minor functions (+). Data taken from Vidarsson *et al.* (2014). Antibody structure images created using BioRender.com.

1.4.1.1 Antibody response to *Neisseria gonorrhoeae*

Infected individuals have been shown to produce antibodies against the gonococcus in sera, cervical secretions and seminal plasma, as detailed in a review by Lovett and Duncan (2018). During natural gonococcal infection, the antibody response is primarily targeted against the outer membrane structures LOS, pili, Rmp, Opa and in reduced amounts, Por (Hook iii *et al.*, 1984, Brooks and Lammel, 1989). Lammel *et al.* (1985) found IgG and IgA against pili, Por and Opa in both vaginal fluids and serum of female patients. However, *N. gonorrhoeae* only elicits low levels of specific antibody that decrease rapidly once the infection is cleared (So *et al.*, 2012).

Recent studies on human infection are limited, however many previous experimental gonococcal human challenge models have enabled more of an understanding of the humoral immune response. Natural killing of sensitive gonococcal strains have been shown to be mediated by IgM (Schoolnik *et al.*, 1979), that recognise epitopes on the gonococcal LOS. The terminal GalNAc expressed in the mature LNnT LOS has been reported to be a target for natural bactericidal IgM antibodies (Griffiss *et al.*, 1991). Antibody against LOS has been shown to facilitate complement activation and bactericidal activity (Apicella *et al.*, 1986) as well as opsonophagocytic activity (Ross and Densen, 1985, Densen *et al.*, 1987), and it has been shown that numerous human IgG antibodies and mouse monoclonal antibodies that recognise LOS epitopes are bactericidal (Yamasaki *et al.*, 2005, Apicella *et al.*, 1986, Gulati *et al.*, 1996b). In a study by Hicks *et al.* (1987) the majority of the participants, who had no previous history of gonorrhoea, developed LOS antibodies after infection with gonorrhoea. In a different study by Hook iii *et al.* (1984), it had been found that the majority of participants with complicated gonococcal infection displayed an antibody response against LOS. Eventually, most patients do produce antibodies that can opsonise and kill the infecting isolate *in vitro* (Brooks *et al.*, 1976), which are usually anti-LOS IgG.

Anti-pilus IgG and IgA antibodies in both serum (Tramont *et al.*, 1981) and vaginal

secretions (Tramont *et al.*, 1980) were found to inhibit the attachment of gonococci to epithelial cells, and therefore prevent infection. Miettinen *et al.* (1989) showed that in participants with uncomplicated gonococcal infections, the mean IgG, IgM and IgA antibody levels against *N. gonorrhoeae* pilus were approximately 2-fold higher in men with urethritis and around 4-fold higher in women with cervicitis, in comparison with sera from uninfected participants. The same study showed antibodies against the pilus were also significantly higher in sera from women with PID when compared to sera from participants with non-gonococcal PID.

Antibodies against Por protein are capable of complement dependent bactericidal activity (Hook iii *et al.*, 1984), although during natural uncomplicated human infection this antibody response is minimal. However, patients with complicated gonococcal infections were found to develop antibodies against Por (Hook iii *et al.*, 1984). Studies by Plummer *et al.* (1993) and Plummer *et al.* (1994) found antibodies against Por in the sera of nearly all commercial sex workers enrolled in a study in Nairobi, Kenya. Antibodies against Opa proteins were also detected in all sex workers. Studies by Hicks *et al.* (1987) and Schmidt *et al.* (2001), both found that after infection with *N. gonorrhoeae*, the majority of participants developed IgG antibodies in the serum against one or more of the previously mentioned outer membrane antigens. However, the antibodies the participants elicited during infection varied between each individual.

There are many studies that show healthy individuals with no previous history of gonorrhoea infection can carry antibodies against *N. gonorrhoeae*. Schmidt *et al.* (2001) observed that in serum of uninfected males with no previous history of *N. gonorrhoeae*, there were IgG antibodies that recognised at least one of the main outer membrane proteins. In separate studies, Hicks *et al.* (1987) and Yamasaki *et al.* (2005) both described subjects with no history of gonococcal infection who carried LOS-directed antibodies. It is presumed that these antibodies have arisen from cross reactivity to commensal *Neisseria* species. It is well known that commensal *N. lactamica* and *N. meningitidis* have many antigenic determinants in common

with pathogenic *N. gonorrhoeae* (Hicks *et al.*, 1987).

1.4.2 The complement system

The complement system comprises of around 60 soluble and membrane bound proteins, that work concurrently to eradicate invading pathogens. This is through direct lysis by membrane attack complex (MAC) formation and/or by increasing opsonophagocytosis (Lewis *et al.*, 2013) by complement components opsonising the pathogen surface and promoting uptake and removal by phagocytes. These complement proteins are synthesised by the liver, and they circulate in the bloodstream as inactive precursors. They only become activated when they are cleaved after interaction with a pathogen. In addition to the bloodstream, there is evidence that hemolytically active complement is present in human female cervical secretions, and 11.5% of that typically found in an equal volume of undiluted serum was found in mid cycle cervical mucus (Price and Boettcher, 1979). In another study, gonococci recovered directly from mouse and human vaginal secretions were coated with antibody and complement C3 and C4 (Gulati *et al.*, 2015b, McQuillen *et al.*, 1999), suggesting that bactericidal antibodies and complement may be protective against female genital tract gonorrhoea infection. There are three main pathways through which complement is initiated; the classical, alternative and lectin pathways (Figure 1.5). The activation for each pathway is different but they all converge to produce the same effector proteins, and ultimately lysis of the bacterium. Deficiencies in complement have previously been shown to correlate with neisserial disease, specifically an increased risk of invasive infection such as DGI, compared to the rest of the population, suggesting that the complement system helps limit survival of the gonococcus in the bloodstream (Densen, 1989).

1.4.2.1 The classical pathway

The classical pathway of complement is instigated when complement-fixing immunoglobulins

bind to an antigen on the bacterial surface of pathogens. The ability of the antibody to activate the pathway is dependent on many factors; the subclass of the antibody, its binding avidity and the distribution, density and accessibility of the target antigen (as detailed in a review by Lewis and Ram (2020)). It has been found that an intact antibody-dependent classical pathway is essential for direct complement-dependent killing of *Neisseria* species (Ingwer *et al.*, 1978, Joiner *et al.*, 1983, Shafer *et al.*, 1982). This essentiality has been demonstrated *in vitro* by the absence of bactericidal activity when using serum depleted of IgG, IgM (Ray *et al.*, 2011, Gulati *et al.*, 2019) and C1q (Lewis *et al.*, 2009). For *N. gonorrhoeae,* it is usually IgG or IgM that initiates the classical pathway, forming an antigen-antibody complex on the surface of the cell. However, typically pathway activation is principally mediated by one pentameric IgM molecule directed against LOS on the surface (Ward and Glynn, 1972, Griffiss *et al.*, 1991, Rice, 1989). One of the first components in the system, the C1 complex, is made up of a C1q molecule that is a globular head made up of six portions and two associated serine proteases (C1s and C1r). Each C1q globular head can bind one immunoglobulin F_c domain, and the binding of two or more globular heads to multiple F_c domain activates the C1 complex. This causes a conformational change to the C1q, that subsequently converts C1r to an active serine protease. The activated C1r in turn cleaves C1s converting it to an active serine protease also. The active C1s cleaves molecule C4 into C4a and C4b, and molecule C2 into C2a and C2b. C4b fragment then covalently attaches, *via* either an amide or ester linkage, to the microbial surface. Major targets on the gonococcal surface for C4b deposition are LOS (Ram *et al.*, 2003), PorB.IB (Lewis *et al.*, 2008) and Opa, especially those expressing A, B, C, D, E and I variants (Lewis *et al.*, 2008). It had been found, however, that C4b does not bind to conserved membrane proteins such as lipoprotein H.8 or RmpM (Lewis *et al.*, 2008). C2a is able to bind onto C4b, forming a molecule (C4b2a) named the C3 convertase (C3c) of the classical pathway. This C3c molecule cleaves C3 into C3a and C3b, *via* the enzymatic activity of the C2a part of the molecule. C3a is

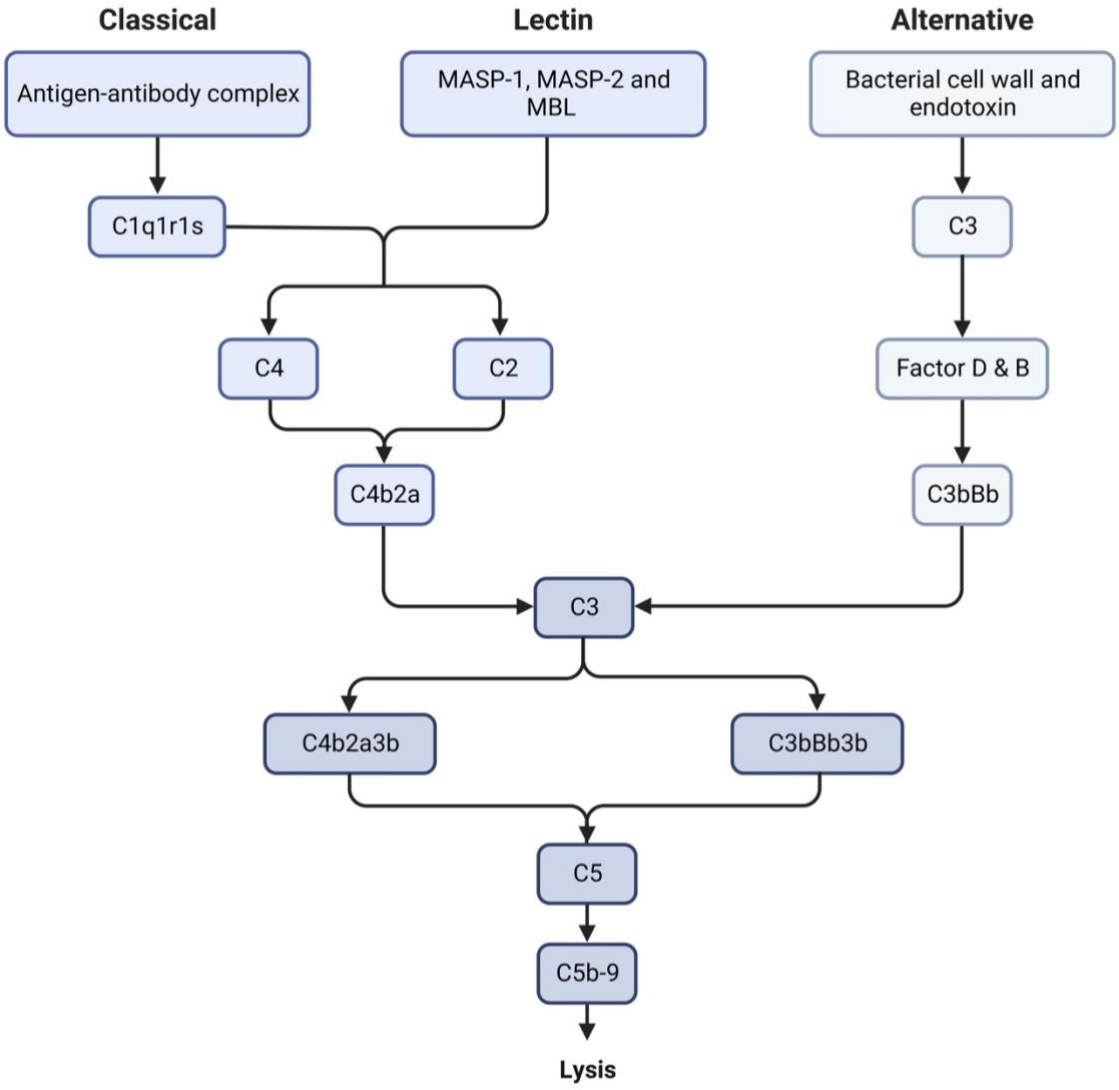


Figure 1.5 Schematic representing activation of the complement cascade. The complement system functions to enhance the ability of antibodies and phagocytic cells to eradicate invading pathogens. Complement is initiated through activation of three pathways; the classical, lectin or alternative pathway. The classical pathway is activated when antibodies bind the pathogen which in turn engages the C1 complex. The lectin pathway is initiated by the binding of mannose binding lectin (MBL) and associated MBL-associated serine proteases (MASPs). The alternative pathway is initiated when factor B binds C3b, produced by spontaneous hydrolysis of C3. All pathways converge at the production of complement C3c, and the enzymatic cascade continues until the formation of the membrane attack complex (MAC), C5b-9. These membrane pores lyse the pathogen and attract immune cells to the site of infection. Image created using BioRender.com.

a potent chemoattractant, that attract mast cells to the complement activation site, and then their binding to these cells and basophils are able to induce the release of histamine and other basoactive amines. The C3b molecule covalently attaches to C3c, creating a trimolecular complex (C4b2a3b) named the C5 convertase (C5c) of the classical pathway. The C5 molecule is cleaved by the C5c molecule into C5a and C5b. C5a is another potent chemoattractant that facilitates opsonophagocytosis by recruiting macrophages and neutrophils. C5b associates with complement molecules C6, C7 and C8 to form a large multimolecular complex that associates with and starts to disturb the cell membrane. Consequently, a molecule of C9 then binds to C8, followed by the binding of multiple C9 molecules resulting in a large pore forming within the bacterial outer membrane. The pore complex involving components C5b-9 is called the MAC and it is large enough to allow water, small molecules and ions into the cell, resulting in bacterial lysis (Müller-Eberhard, 1986).

1.4.2.2 The lectin pathway

The lectin pathway is initiated through serum proteins structurally very similar to C1q, such as mannose-binding lectin (MBL). These complexes have two MBL-associated serine proteases (MASP-1 and MASP-2) attached, similar to C1s and C1r. When these structures bind to mannose residues present on the pathogens surface, MASP-1 and MASP-2 are activated and MASP-2 cleaves complement factors C4 and C2, with fragments C4b and C2a combining to generate C3c (C4b2a) in the same way as the classical pathway. The pathway then continues in the same manner as the classical pathway, ultimately resulting in the formation of the MAC in the bacterial outer membrane. *In vitro*, purified MBL has been shown to bind to *N. gonorrhoeae* (Devyatyarova-Johnson *et al.*, 2000). However, killing of gonococci only occurred when bacteria were pre-incubated with MBL/MASP, and then MBL-deficient serum had been added afterwards, but not when both were added simultaneously (Lewis and Ram, 2020). This suggests

that the lectin pathway alone might not be able to support the direct opsonophagocytosis or complement-dependent killing of *Neisseriae* in serum.

1.4.2.3 The alternative pathway

The alternative pathway can be activated by IgA immune complexes and bacterial PAMPs, including LOS and cell wall components. It is also always active at a very low level. This activation is due to the spontaneous hydrolysis of an internal thioester bond in C3. Hydrolysed C3 ($C3(H_2O)$) has a binding site for complement protein, factor B (Pangburn *et al.*, 1981). The attached factor B is cleaved by factor D that removes cleaved protein factor Ba and leaves factor Bb associated with hydrolysed C3, which is now enzymatically active. This complex is named the alternative pathway C3 initiation convertase ($C3H_2OBb$). The alternative pathway C3 initiation convertase cleaves C3 into C3a and C3b, where the C3b molecule covalently attaches to the microbial surface. In the absence of an appropriate activator for the alternative pathway, complement regulatory proteins inhibit further activation of the pathway. However, if C3b attaches to a microbial surface, factor B will bind to it. The C3b molecule has been found to attach to gonococcal LOS (Edwards and Apicella, 2002), Opa (Lewis *et al.*, 2008) and PorB.IB (Lewis *et al.*, 2008). It had been found, however, that C3b also does not bind to conserved membrane proteins such as RmpM or lipoprotein H.8 (Lewis *et al.*, 2008). Factor B is then cleaved by factor D, leaving cleaved factor Bb attached to C3b. This complex (C3bBb) is named the alternative pathway C3c. This alternative pathway C3c can cleave C3 into C3a and C3b. Some of the C3b produced remains soluble, while some will also covalently bind to the microbial surface, where this again will bind factor B. This is again cleaved by factor D, creating many more alternative pathway C3c on the microbial surface, and therefore rapidly amplifying activation of complement through this pathway. The surface of the cell can rapidly be covered with thousands of C3b molecules as a result of this amplifying effect, which is a unique feature

of the alternative pathway. As with the classical pathway, some of the C3b generated will interact with the C3c, resulting in a trimolecular complex of two C3b molecules and factor Bb (C3bBb3b). This is named the alternative pathway C5c, which can cleave C5. The pathway then continues the in the same manner as the classical pathway, resulting in the formation of the MAC.

During the absence of antibody, the alternative pathway alone deposits negligible C3 on *Neisseriae* and is therefore unable to sustain complement-dependent killing. This is evident when the alternative pathway is inhibited, as the efficacy of bactericidal antibodies against expressed surface antigens are unaffected (Giuntini *et al.*, 2012). However, if antibody is present, the alternative pathway is important in C3b amplification and deposition through the classical pathway. Antibodies can also directly activate the alternative pathway when C1, C2 or C4 are absent (Ratnoff *et al.*, 1983, Wagner *et al.*, 1999, May and Frank, 1973). All components of the alternative pathway are secreted by primary human cervical and vaginal epithelial cells (Edwards *et al.*, 2002).

1.5 Resistance to serum killing of *Neisseria gonorrhoeae*

The complement system is a significant part of the innate immune response and represents the first host defence mechanism that pathogens will encounter, which causes rapid death of the invading organism. Therefore, unsurprisingly most pathogenic organisms, especially obligate human pathogens such as *Neisseriae*, have evolved a wide range of mechanisms to resist human complement activation on their surfaces (Parsons *et al.*, 1992) and in turn evade the human immune response. The gonococcus evades complement mediated killing through binding host complement regulatory factors (factor H and C4b-binding protein), producing IgA1 proteases, binding and inactivating complement components using factor I, preventing MAC formation and binding inhibitory antibodies. LOS is an important component of neisserial pathogenesis as it also has a significant role in regulating host immune defences, and structural LOS variations

have a large impact on its ability to resist complement. It has been well documented that DGI strains are naturally resistant to complement mediated killing by normal human serum (NHS) (Schoolnik *et al.*, 1976) and are said to exhibit "stable" serum resistance.

1.5.1 Natural lipooligosaccharide resistance

Natural substitutions within the LOS can modulate resistance to complement. Phase variation can result in the final GalNAc not being expressed on the mature LNnT LOS structure, which increases resistance to complement activation (Virji *et al.*, 1996) because the additional GalNAc is a target for bactericidal IgM antibodies (Griffiss *et al.*, 1991). Also, mutants that lack glycan extensions from Hep^{I}, for example *lgtF* and *pgm* mutants, are more susceptible to serum killing (Kahler *et al.*, 1998, Ram *et al.*, 2007). Phosphoethanolamine (PEA) modifications of LOS increase proinflammatory cytokine production, which interestingly does not occur in most commensal *Neisseria*.

1.5.2 Lipooligosaccharide sialylation

Ward *et al.* (1970) demonstrated that all *N. gonorrhoeae* recovered from male uncomplicated urethral infections were completely resistant to complement-mediated killing by NHS. Interestingly, many of these strains became sensitive to NHS after being grown on media *in vitro*, and in turn this had been termed "unstable resistance". The reason for this "unstable resistance" had been discovered almost two decades later, whereby it was observed that the gonococcus scavenges, and binds host derived cytidine monophospho-N-acetylneuraminic acid (CMP-NANA) to its LOS, known as sialylation (Parsons *et al.*, 1988, Nairn *et al.*, 1988). Most host cells are typically sialylated to help the immune system recognise them as "self" cells. Therefore, the gonococcus has evolved to also bind sialic acid when inside the host, to mimic host sialylated glycosphingolipids. It has been reported that sialylated LOS does not stimulate an effective

immune response (Mandrell *et al.*, 1988), therefore helping the gonococcus evade the host immune response. CMP-NANA is available to gonococci *in vivo* within epithelial cells and phagocytes, but at very low concentrations. The inflammation and cell lysis initiated by the gonococcal infection would release larger CMP-NANA quantities, which is beneficial to the organism. Interestingly, adding CMP-NANA to media *in vitro* is able to restore complement resistance in strains that are serum sensitive (unsialylated).

There are many oligosaccharide structures of LOS that can become sialylated. Sialylation occurs most frequently on the mature gonococcal LOS LNnT structure (Hep^{I}-glucose-galactose-GlcNAc-galactose) (Gulati *et al.*, 2005). Another recipient for CMP-NANA is the gonococcal globotriose structure (Hep^{I}-glucose-galactose-galactose) (Rice *et al.*, 2017), which is identical to the human P^{K}-like blood group antigen (Mandrell, 1992). Lactose extensions (Hep^{I}-glucose-galactose and Hep^{II}-glucose-galactose) are also acceptors of sialic acid. This sialylation is an important factor in virulence as it also inhibited C3 complement deposition on gonococci (Ram *et al.*, 2018). There have also been many other reports of LOS sialylation preventing complement components from binding to the bacterium (Jonsson *et al.*, 1992, Blom *et al.*, 2001), particularly C3 binding and inactivation. In addition, the binding of IgG present in healthy serum (Edwards *et al.*, 2002) and the binding of IgM to serum-sensitive gonococci (Källström *et al.*, 1997), were both drastically decreased by LOS sialylation, which reduces C4b deposition and enhances serum resistance (Gulati *et al.*, 2015b). Sialylation of LOS LNnT also inhibited purified MBL from binding *in vitro*, therefore preventing lectin pathway activation (Devyatyarova-Johnson *et al.*, 2000).

1.5.3 Regulation of complement

C3b binds non-specifically to both host and pathogen molecules, therefore complement activation must be tightly regulated to prevent inadvertent host tissue damage (Jozsi and Zipfel,

2008). Factor H is a human complement regulatory protein, that binds sialylated human cells and aids the human immune system identify their cells as "self" cells, to keep complement activation under control (Swanson, 1978). It has been shown that factor H also binds sialylated gonococcal LOS (Lambden *et al.*, 1979) and PorB, therefore allowing bacterial cells to evade the immune system further. Their abundance in the outer membrane results in the gonococcus becoming completely coated in factor H. Factor H binds the bacterium faster than C3b, which prevents alternative pathway C3c formation. This lowers the activation of the alternative pathway, and protects the bacterium against complement mediated killing (James and Swanson, 1978) and enhances serum resistance. Factor H is also a co-factor for Factor I, that cleaves C3b into iC3b, that in turn inactivates the C3b molecule irreversibly (Bhat *et al.*, 1991, Swanson and Barrera, 1983).

C4b-binding protein is another human complement regulatory factor, that is also used by the host to downregulate complement activation to avoid host tissue damage. The gonococcus also binds C4b-binding protein *via* PorB, with about 90% of strains encoding PorB.1A (Bettoni *et al.*, 2019) and only 20% encoding PorB.1B. This allows the gonococcus to lower the activation of the classical pathway and therefore protects the bacterium against complement mediated killing and enhancing serum resistance. It does this by cleaving C4b into inactive C4d and C4c (Swanson, 1978) and therefore prevents classical pathway C3c formation (Swanson, 1982, Stern *et al.*, 1986). Loss of PEA from the lipid A portion of the LOS molecule has been found to diminish binding of both factor H and C4b-binding protein (Lewis *et al.*, 2013), and therefore increases C4b deposition and sensitivity to serum killing.

1.5.4 Inhibitory antibodies

Antibodies are typically associated with protection against pathogens, however there have been some instances reported where antibodies have been directly linked with the exacerbation of viral

and bacterial disease, identified as antibody-dependent enhancement. In Gram-negative bacteria, there have been reports of antibodies that have been produced through natural infection, colonisation, and sometimes immunisation, that appear to inhibit bactericidal antibodies from causing complement-dependent killing. This phenomenon, termed 'inhibitory antibodies', had been first described by Pfeiffer and Issaeff (1894) and has subsequently been described for several pathogenic bacteria.

Inhibitory antibodies against *S. enterica* serovar Typhimurium were originally reported when the occurrence of excess antibodies to *S.* Typhimurium LPS had been found to inhibit the bactericidal activity of serum (Adler, 1953). Waisbren and Brown (1966) found a patient suffering with septicaemia and a splenic abscess had inhibitory antibodies to their infecting *S.* Typhimurium strain. In a larger study, inhibitory antibodies have been observed in a population of human immunodeficiency virus (HIV)-infected adults in Africa, who were incapable of clearing a non-typhoidal *Salmonella* bacteremia infection, specifically *S.* Typhimurium (MacLennan *et al.*, 2010). It had been reported that these particular patients had a high titer of antibody that positively correlated with an impaired killing of *S.* Typhimurium. Further examination found that LPS O-antigen specific IgG were able to inhibit serum-mediated killing of *S.* Typhimurium. Another study by Trebicka *et al.* (2013), found that after analysis of 49 samples of healthy serum, one of the samples contained anti-LPS IgM inhibitory antibodies against a strain of *S.* Typhimurium. In contrast, the remaining 48 samples exhibited strong killing of the same strain of *S.* Typhimurium, with anti-LPS antibodies identified as being significant in bactericidal activity. Therefore, it had been determined that anti-LPS antibodies were able to both inhibit and promote complement-mediated killing, in a concentration dependent manner. Further work by Goh *et al.* (2016) found that LPS-specific IgG2, IgA and IgM were all able to inhibit serum-mediated killing of *S.* Typhimurium.

Pseudomonas aeruginosa commonly affects patients with cystic fibrosis and bronchiectasis. Waisbren and Brown (1966) also reported the first incident of a inhibitory factor in the serum against *P. aeruginosa*, confirmed later to be IgG (Guttman and Waisbren, 1975). Further work showed that the inhibition of serum killing of *P. aeruginosa* had been mediated by high titres of LPS O-antigen-specific IgG2 (Wells *et al.*, 2014) and O-antigen-specific IgA (Pham *et al.*, 2021) antibodies in bronchiectasis patients. From this work, there was great success using plasmapheresis to treat chronic *P. aeruginosa* infections in bronchiectasis patients who had inhibitory antibodies. This treatment involved the removal of all antibodies from the serum, and replacing them with donor immunoglobulins. This resulted in a cease of culturable *P. aeruginosa* within the patient sputum and a significant improvement in patient health (Wells *et al.*, 2017).

Inhibitory antibodies against *Escherichia coli* had previously been described, where a proportion of female patients with urinary tract infections (UTIs) were found to have a factor in the serum that inhibited normal healthy serum killing of the autologous strain (Gower *et al.*, 1972). More recently, in patients with uropathogenic *E. coli* (UPEC) infection (Coggon *et al.*, 2018) 24% of urosepsis patients tested were found to have inhibitory IgG2 antibodies against LPS. As resistance to serum is essential for bacteria to infect the bloodstream, this work suggests that the incidence of inhibitory antibodies has potentially allowed these otherwise serum-sensitive UPEC strains to survive in the bloodstream. Therefore, using plasmapheresis, or another method of removing inhibitory antibodies from patients with urosepsis, has potential in combatting multidrug-resistant pathogens that do not respond to antibiotics.

Lastly, Griffiss (1975) found that IgA purified from sera from different patients each infected with *N. meningitidis* serogroups B, Y or C, inhibited bactericidal IgG and IgM from complement-mediated killing. This inhibition had been found to be greater for IgG than IgM, strain specific and dependent on ratio of inhibitory to bactericidal antibodies. In another study,

it had been found that depleting inhibitory IgA from the sera of 28 patients with meningococcal disease had been found to restore bactericidal action of the infecting strain in 24 of these sera (Griffiss and Bertram, 1977). Jarvis and Griffiss (1991) identified that inhibitory IgA1 antibodies were binding to capsular polysaccharides of meningococcal group C bacteria. Since this work, inhibitory IgA antibodies specific for various capsule serotypes, such as group A, have been reported (Amir *et al.*, 2005). In contrast, it had been reported that serogroup B meningococci have inhibitory antibodies targeting lipoproteins. Sera from three out of 17 healthy individuals contained IgG3 specific for two lipoproteins (Lip/H.8 and Laz) that had the ability to inhibit bactericidal killing (Ray *et al.*, 2011). Expression of both were necessary for maximum inhibition. However, these inhibitory antibodies were not able to inhibit the killing by all bactericidal sera.

1.5.4.1 Inhibitory antibodies to *Neisseria gonorrhoeae*

Inhibitory antibodies against *N. gonorrhoeae* were first described by McCutchan *et al.* (1978). In this study, they observed that gonococci isolated from patients with DGI resisted killing by NHS, but were killed by normal rabbit serum. When both sera were mixed in a ratio of 1:1, killing was reduced. Due to the heat lability of the inhibitory factor above 65°C, it had been suggested it was an antibody in the NHS that had been inhibiting killing by rabbit serum. Further investigation found that when IgG fractions were added to rabbit serum, it reduced killing of DGI strains. The same effect had not been seen for IgM or IgA. They concluded that NHS contains natural IgG inhibitory antibodies that compete with the natural bactericidal antibody, and this protection enables them to disseminate in the blood. Rice and Kasper (1982) found similar findings where a DGI strain that had been sensitive to its matching patient serum, was resistant to complement-mediated killing by seven out of ten different NHS. Continuing from this, Joiner *et al.* (1985) pre-sensitised the DGI strain to inhibitory IgG antibody purified from

NHS, and then after incubation added purified bactericidal antibody from the DGI patient serum along with complement. They found that killing by the bactericidal patient IgG antibody had been partially inhibited by the IgG, and $F(ab')_2$ fragments of IgG in the NHS. Continuing this, the same DGI strain had been found to be resistant to serum from a different DGI patient. When this DGI serum had been depleted of inhibitory antibodies, it was then able to kill the original strain (Rice *et al.*, 1986), indicating inhibitory antibodies in healthy and patient serum have a role in serum resistance. Upon further analysis, they found that most of the inhibitory activity was directed against gonococcal outer membrane 'protein III' (Rice *et al.*, 1986), now known as RmpM. Anti-Rmp antibodies have been found to inhibit bactericidal anti-LOS and anti-Por antibodies in immune serum (Rice *et al.*, 1986). Work by Virji *et al.* (1987) found that reactivity of antibodies against Rmp vary dependent on the epitope recognised. The binding specificities of six different anti-Rmp antibodies against three Rmp epitopes were analysed. They found that only one of the six was able to inhibit when challenged, whereas the other four were bactericidal and one ineffective.

Interestingly, Joiner *et al.* (1985) also showed enhanced complement C3 and C5b-9 deposition on a DGI strain in the presence of inhibitory IgG. One-quarter of C3 that had been deposited in the presence of inhibitory IgG covalently associated with the antibody, resulting in complement deposition at new sites that did not result in serum killing. This indicates that the site of complement deposition has been altered by inhibitory antibody, onto a non-bactericidal target. Further details regarding inhibitory antibodies can be found in a review by Lewis and Ram (2020).

1.5.4.2 Implications of inhibitory antibodies in vaccine design

Inhibitory antibodies are an important consideration when developing a vaccine for several reasons. It is important that any vaccine produced does not elicit the production of inhibitory

antibodies, that might in turn protect the bacteria from complement-mediated killing or might diminish the efficacy of bactericidal antibodies elicited by the vaccine. This has been shown recently in a mouse colonisation model where anti-Rmp antibodies were found to prevent anti-LOS from mediating infection clearance, and in turn increased bacterial burden (Gulati *et al.*, 2015a). In addition to this, there have been a few reports of antibodies to Rmp increasing the risk of acquiring gonorrhoea. In a larger longitudinal study among 243 female commercial sex workers in Nairobi, Kenya, women who possessed antibodies against Rmp in their sera were found to be 3.4-fold more likely to become infected with gonorrhoea from male partners, than women who did not possess anti-Rmp antibodies (Plummer *et al.*, 1993). A greater understanding of bacterial inhibitory antibodies during infection might lead to improvements in the management of the disease and help to inform vaccine development efforts.

1.6 Aims of the project

1.6.1 The G-ToG clinical trial

This PhD project stemmed from a randomised non-inferiority clinical trial (registered under ISRCTN51783227) by Ross *et al.* (2019), which assessed the effectiveness of gentamicin as an alternative treatment for gonorrhoea to ceftriaxone (G-ToG). To be eligible for trial participation, participants must have received a diagnosis of uncomplicated genital, rectal or pharyngeal gonorrhoea, based on a positive nucleic acid amplification test (NAAT). During the initial baseline trial visit, participants had swabs taken from different infection sites for culture and NAAT diagnosis. A blood sample had also been taken for the pre-treatment immune response measurements. Overall, between Oct 2014 and Nov 2016, 1762 patients were assessed and 720 participants were enrolled onto the G-ToG trial. Of these, 362 were assigned ceftriaxone treatment and 358 were assigned gentamicin in a randomised way, both in combination with azithromycin. Participants returned to the clinic two weeks after treatment for assessment of

clearance of *N. gonorrhoeae* from all initially infected sites, defined by a negative NAAT. Another blood sample was taken for post-treatment immune response measurements. At two weeks after treatment, infection had been found to have cleared for 98% (299/306) of participants in the ceftriaxone group, compared with 91% (267/292) of participants in the gentamicin group. It was found that 98% of participants treated with ceftriaxone were able to clear genital site infections, compared to 94% of participants treated with gentamicin. It had also been found that 96% of participants treated with ceftriaxone were able to clear pharyngeal site infections, compared to only 80% of participants treated with gentamicin. Lastly, it had been found that 98% of participants treated with ceftriaxone were able to clear rectal site infections, compared to 90% of participants treated with gentamicin. Therefore, it was not found that gentamicin was non-inferior to ceftriaxone for the treatment of gonorrhoea.

This PhD project was able to take advantage of the large collection of clinical *N. gonorrhoeae* isolates from trial participants, along with matching serum from each participant, allowing for a large study of the immune responses to acute gonococcal infection. We hypothesise that inhibitory antibodies are produced during acute infection with gonorrhoea, which would prevent complement-mediated killing.

1.6.2 Project aims

Initial research into inhibitory antibodies mentioned previously focused on the isolation of inhibitory antibodies from healthy serum. These were observed to be effective at inhibiting the killing of serum-sensitive *N. gonorrhoeae* strains that cause DGI. The majority of gonococcal infections found in clinic are acute uncomplicated mucosal infections, however there is limited research to understand whether patients with acute gonorrhoea produce inhibitory antibodies during infection and the prevalence of this in the population. Therefore, the main aim of this study is to investigate whether inhibitory antibodies occur in patients during infection with

uncomplicated gonorrhoea. The mechanism of inhibitory antibodies is also not fully understood, therefore the second aim is to characterise these antibodies and determine the mechanism underpinning the action of inhibitory antibodies against *N. gonorrhoeae*.

To accomplish the aims, the main project objectives were to:

- Use whole genome sequencing (WGS) to correlate the genomic data with clinical metadata acquired through the trial to gain a greater understanding of the G-ToG *N. gonorrhoeae* isolate collection
- Determine whether any of the participants have inhibitory antibodies in their serum against their infecting isolate, using serum bactericidal assays (SBAs).
- Investigate whether candidates for inhibitory antibodies have increased antibody binding titers as seen in previous studies (Wells *et al.*, 2014, MacLennan *et al.*, 2010) by analysing antibody and complement deposition on the bacterial cell surface, using a flow cytometric assay.
- Use WGS to identify genomic distinctions underpinning differences in serum susceptibility between *N. gonorrhoeae* clinical isolates.

CHAPTER 2

MATERIALS AND METHODS

2.1 *Neisseria gonorrhoeae* isolates and serum

2.1.1 *Neisseria gonorrhoeae* clinical isolates

From the 720 participants recruited to the G-ToG clinical trial (Ross *et al.*, 2019), *N. gonorrhoeae* isolates were successfully collected from 307 individuals, equating to 367 isolates which were the focus of this study. For this study, all isolates were renamed NG001 to NG367 for complete participant anonymity. Upon trial entry, an abundance of clinical and demographic data were collected from participants and recorded in a database developed by The Nottingham Clinical Trial Unit on the G-ToG team, with all data detailed in Appendix I.

2.1.2 *Neisseria gonorrhoeae* MS11

N. gonorrhoeae MS11 had been used as a control strain for some initial optimisation experiments and as a reference strain during genomic analysis. The isolate was obtained from The American Type Culture Collection (ATCC) under ATCC BAA-1833. MS11 had been originally isolated in 1960 from an uncomplicated infection in an outpatient clinic by J Swanson and deposited to ATCC by HS Seifert. The strain was propagated using the manufacturer's instructions for reviving a freeze-dried microorganism.

2.1.3 Human serum

Blood samples from participants were collected during the initial baseline visit and then two weeks later after antibiotic treatment and stored as both serum and plasma by The Clinical Immunology Services (CIS) (The University of Birmingham, UK). Serum isolates from the participant with the indicated isolate numbers were named "NGxxx-serum". Baseline sera selected for experiments were stored at -80°C and were defrosted on ice on the day of the experiment, and never freeze-thawed more than three times to preserve complement function (O'Shaughnessy *et al.*, 2012).

Blood had also been collected from eight healthy human volunteers with no previous history of gonococcal infection. The blood was allowed to clot at room temperature and then centrifuged for 10 minutes at 1000 x g at 4°C, and the serum layer was removed. Equal amounts of each serum were pooled, aliquoted and stored at -80°C. Aliquots of the pooled healthy control serum (HCS) were also defrosted on ice on the day of the experiment, and never freeze-thawed more than three times.

2.2 Growth conditions

2.2.1 Media and media supplements

Glassware used for media preparation and the growth of *N. gonorrhoeae* was thoroughly rinsed out with deionised water to remove any trace detergents before use and then kept separate from general lab glassware. *N. gonorrhoeae* isolate cultures were grown on GC agar. Each litre of agar contained; 36 g of GC agar base (Oxoid), 6 g of bacteriological agar (BD) and 10 ml Vitox supplement (Oxoid). Both agar powders were added to a Duran bottle and made up to 1 L with deionised water and autoclaved at 121°C for 15 minutes. Once the agar had cooled to 50°C, the Vitox supplement was added aseptically, mixed well, and poured into petri dishes. GC agar plates were stored at 4°C for no longer than one week. VCAT selective supplement (Oxoid), containing antibiotics including colistin (kills most Gram-negative organisms except *Neisseria*), vancomycin (targets most Gram-positive organisms), trimethoprim (inhibits some Gram-negative organisms) and amphotericin B (prevents fungal growth), were sometimes used for the isolation of *N. gonorrhoeae* from samples that were heavily contaminated. As per manufacturer's instructions, 20 ml VCAT supplement were added per 1 L of GC agar, at the same time as the Vitox supplement.

For liquid cultures, *N. gonorrhoeae* were grown in brain heart infusion (BHI) broth (Oxoid). The BHI broth was made up according to manufacturer's instructions where each litre

of broth contained 37 g BHI powder. Broth powder was added to a Duran bottle and made up to 1 L with water and autoclaved at 121°C for 15 minutes

2.2.2 Growth of *Neisseria gonorrhoeae*

Gonococcal stock suspensions were kept at -80°C. Every time a specific strain was required, approximately 5-10 µl of the suspension was streaked for single colonies across a pre-warmed GC agar plate using a disposable plastic loop. Care was taken to ensure -80°C stock suspensions did not start to defrost during plate inoculation. Plates were then incubated for 20 hours at 37°C in an anaerobic jar with a moistened paper towel to provide a humid environment as the bacterium is sensitive to dehydration so needs at least 70% humidity whilst growing. The bacterium is a capnophile, and therefore requires an increased concentration of carbon dioxide to 5-10% up from normal atmospheric 0.04% concentration, and a reduced oxygen level from ~20.95% atmospheric down to about 15%. Therefore, a lighted candle was also added to the anaerobic jar, as once the candle had burned out, it provided an atmosphere containing an increased carbon dioxide concentration and a reduced oxygen concentration.

After overnight growth, a few colonies from the streak plate were swabbed onto multiple fresh pre-warmed GC agar plates using disposable sterile swabs to create a lawn of bacteria. The number of lawn plates varied according to the quantity of cultured plates needed for experiments. These lawn plates were incubated for a further 16-18 hours at 37°C in a candle extinction jar as previously described.

For long-term storage of strains, cultures from lawn plates were scraped up using 800 µl BHI and an L-shaped disposable spreader and resuspended into bead tubes from Microbank (Pro-Lab Diagnostics) following manufacturer's instructions for long term microorganism storage. Cultured bead tubes were then snap frozen in liquid nitrogen and stored at -80°C.

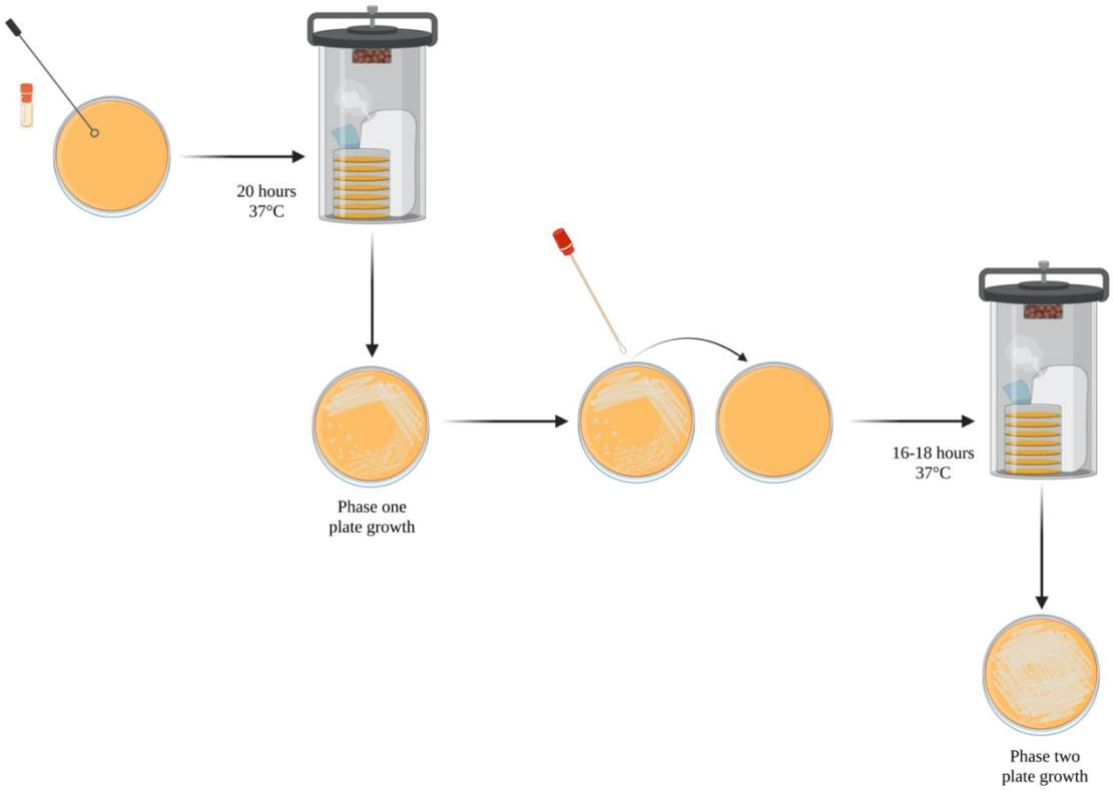


Figure 2.1 Protocol for growing *Neisseria gonorrhoeae*. Schematic of plate growth stages for *Neisseria gonorrhoeae*. On day one, isolates are streaked onto GC agar plates and incubated for 20 hours in a candle extinction jar. The next day, single colonies are swabbed onto fresh GC agar plates to create a lawn and incubated for 16-18 hours in a candle extinction jar. Figure created using BioRender.com.

2.3 Genomic analysis

2.3.1 Whole genome sequencing and genome assembly

Genome sequencing of all clinical isolates was provided by MicrobesNG (http://www.microbesng.com) which is supported by the BBSRC (grant number BB/L024209/1). Each isolate was prepared for sequencing as per MicrobesNG strain submission procedures. Briefly, a single colony from a streak plate was swabbed out on a fresh GC agar plate to form a lawn along with some single colonies to determine purity of the culture. Plates were grown overnight at 37°C in a candle extinction jar as usual. The next day, all growth from the plate was harvested and resuspended in a bead tube containing cryopreservative (Microbank™, Pro-Lab Diagnostics UK) by mixing by inverting the tube ten times. Each tube was barcoded to correspond to the isolate sample number and entered into the MicrobesNG online database. MicrobesNG extracted DNA from isolates, prepared libraries and sequenced using HiSeq next generation sequencing (Illumina, USA) using a 250 bp paired end protocol with a minimum coverage of 30x. They also ran basic bioinformatics using their standard pipeline, including adapter trimming and removal of low quality reads using Trimmomatic (version 0.30) (Bolger *et al.*, 2014) (with a sliding window quality cut off of Q15), and *de novo* assembly of trimmed reads using SPAdes (version 3.7) (Bankevich *et al.*, 2012). Overall, this resulted in the attainment of R1/R2 trimmed raw read files (FASTQ) and assembled FASTA files for each isolate. A schematic of the overall bioinformatics workflow is depicted in Figure 2.2. Of the total 367 *N. gonorrhoeae* isolates, sequencing determined that ten of the cultured samples were different organisms. In addition, two samples were not viable from the original bead tubes and one isolate was never received, further detailed in Table 2.1, therefore WGS data could be produced and analysed for 354 of the clinical isolates. The data for this study have been deposited in the European Nucleotide Archive (ENA) under accession number PRJEB63206.

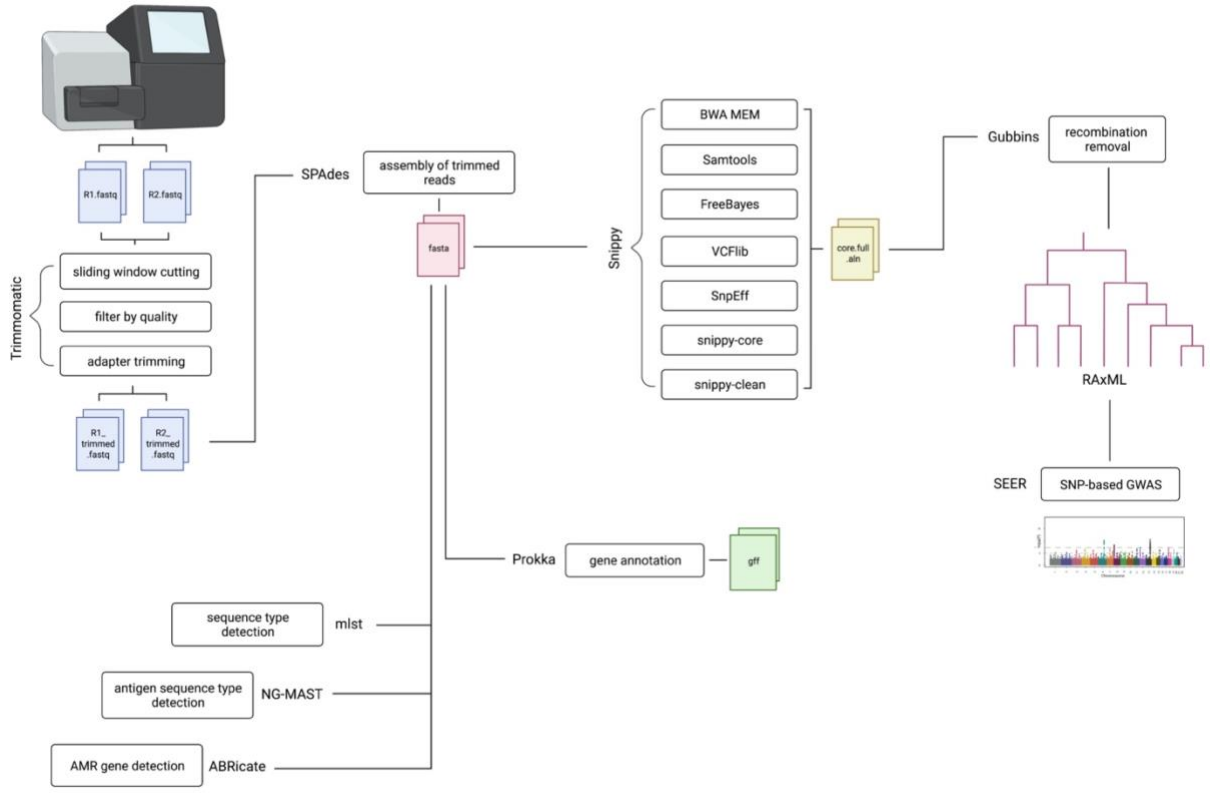


Figure 2.2 Schematic of the bioinformatic workflow. Trimmomatic is used for quality control of pair-end FASTQ reads. SPAdes was used to assemble trimmed reads and resultant FASTA files were used for genotyping. Single nucleotide polymorphism (SNP) variants were called using Snippy, and the multiple sequence alignment file produced were used to construct a recombination removed maximum likelihood phylogenomic tree, using RAxML. Schematic was created using BioRender.com.

2.3.2 Mapping and variant calling

FASTQ trimmed raw read files from the 354 *N. gonorrhoeae* clinical isolates were mapped to reference strain *N. gonorrhoeae* MS11 (Genbank accession: CP003909.1) using Snippy (version 4.4.5) (Seemann, 2015), highlighting variants in the form of substitutions and insertions/deletions. All individual results were combined, using snippy-core, to align loci common to all isolates to generate a core single nucleotide polymorphism (SNP) alignment. The multiple sequence alignment file was then "cleaned" before recombination-removal and tree-building, for phylogenomic analysis.

For SNP comparisons between two similar clinical isolates, one isolate was chosen for input as the 'reference genome' and sequence reads from the other isolate was aligned to that 'reference' isolate.

2.3.3 Recombination removal and phylogenomic construction

The clean core SNP alignment file was used to create a recombination-corrected phylogeny of all isolates through running Gubbins (version 2.3.5) (Croucher *et al.*, 2015), where a maximum likelihood (ML) phylogenomic tree was constructed using RAxML (version 8.2.12) (Stamatakis, 2014) using the GTRGAMMA model of nucleotide substitution, where the branch lengths are in point mutations.

2.3.4 Multi-locus and multi-antigen sequence type assignment

Contigs were annotation using Prokka (version 1.11) (Seemann, 2014). Multi-locus sequence typing (MLST) analysis was completed using MLST (version 2.16.4) (Jolley and Maiden, 2010) on *de novo* assemblies using the PubMLST database (Jolley *et al.*, 2018), scanning for neisserial housekeeping genes; *abcZ, adk, aroE, fumC, gdh, pdhC* and *pgm,* to determine each gene allele and the resultant sequence type of each isolate.

Neisseria gonorrhoeae multi-antigen sequence types (NG-MAST) (Martin *et al.*, 2004) were assigned using NGmaster (version 0.5.8) (Kwong *et al.*, 2016) on *de novo* assemblies by analysing internal fragments of two highly variable and polymorphic outer membrane protein genes, *porB* and *tbpB* and comparing their sequences to an open-access database (http://www.ng-mast.net).

2.3.5 Clinical metadata analysis

Participant clinical metadata was collected upon entry to the G-ToG trial. Phenotypic susceptibility metadata for all isolates against the three G-ToG trial drugs; azithromycin, ceftriaxone and gentamicin, were also obtained. Here MICs were determined by Public Health England (PHE) (Cole, 2019) using an E-test on GC agar base with 1% Vitox. It was found that that as the MIC of azithromycin increased, it resulted in the combined gentamicin and azithromycin treatment becoming less effective than the combined ceftriaxone and azithromycin treatment. It was also found that of the participants who received the combined gentamicin and azithromycin treatment, their isolates had a higher geometric mean azithromycin MIC and did not clear infection *in vivo*, compared with those who did. It was also found that the geometric mean azithromycin MIC was higher in isolates from the pharynx compared with genital sites (Cole, 2019).

All metadata annotations were visualised on the phylogenomic tree using Interactive Tree of Life (iTOL) software (Letunic and Bork, 2021). Associated clinical data for each isolate is listed in Appendix I.

2.3.6 Genome-wide association study

Genome-wide association study (GWAS) was used to identify the association of genomic variants with phenotypes of interest. A pairwise distance matrix of population structure was

created using mash (version 2.3) (Ondov *et al.*, 2016). A scree plot (Appendix II) was produced using multidimensional scaling and the distance matrix, showing the variation for the first 30 dimensions, which allowed for the number of dimensions to retain during the GWAS to be chosen. As there was a drop at around 10 dimensions, these were used during analysis. K-mers present in assembled FASTA files for all samples were counted using fsm-lite (version 1.0) (Välimäki and Lees, 2016). These output files generated combined with binary phenotypic data were used in a fixed effect SNP-based GWAS using SEER (version 1.1.4) (Lees *et al.*, 2016). K-mers found to be significantly associated with the phenotype were filtered using filter-SEER with a minimum allele frequency (maf) of 0.05.

2.4 Serum bactericidal assay

N. gonorrhoeae isolates selected for experiments were grown on GC agar plates from -80°C stocks, as previously described. Bacteria from lawn plates were scraped up using 1 ml of phosphate buffered saline (PBS) into a 1.5 ml microcentrifuge tube and adjusted to an optical density (OD) 600 nm of 3.0 using PBS which contained 7.5 x 10^8 cfu/ml bacteria. Adjusted cultures were added to serum (concentration specified for each experiment) pre-warmed to 37°C in 1.5 ml microcentrifuge tubes, to a final concentration of 1.5 x 10^7 cfu/ml bacteria in a total reaction volume of 50 µl. Samples were then incubated without aeration at 37°C for 3 hours. As a control, the same cultures were added to PBS and incubated under the same conditions. Before incubation and at hourly intervals (0, 60, 120 and 180 minutes), the number of cfu/ml were determined using a modified version of the Miles and Misra method (Miles *et al.*, 1938) for serial dilution and plating of a bacterial suspension. Here, 10 µl from each sample were serially diluted 1:10 into PBS in a 96 well plate. Then 10 µl of each dilution, at 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4} were all plated out in triplicate on GC agar. Once dry, all plates were incubated at 37°C in candle extinction jars for ~18 hours and average cfu/ml were recorded the following day. Survival after

N. gonorrhoeae isolate number	Reason for serum bactericidal assay incompletion	
	Baseline serum	Post-treatment serum
NG003		No post-treatment serum for SBA
NG005	Sequencing determined isolate as *Enterococcus* only	
NG009		No post-treatment serum for SBA
NG010		No post-treatment serum for SBA
NG011		No post-treatment serum for SBA
NG012		No post-treatment serum for SBA
NG013		No post-treatment serum for SBA
NG014		No post-treatment serum for SBA
NG016		No post-treatment serum for SBA
NG022		No post-treatment serum for SBA
NG024		No post-treatment serum for SBA
NG029		No post-treatment serum for SBA
NG031	No baseline serum for SBA	
NG033		No post-treatment serum for SBA
NG034		No post-treatment serum for SBA
NG037		No post-treatment serum for SBA
NG038		No post-treatment serum for SBA
NG046		No post-treatment serum for SBA
NG047		No post-treatment serum for SBA
NG049		No post-treatment serum for SBA
NG050		No post-treatment serum for SBA
NG055		No post-treatment serum for SBA
NG062		No post-treatment serum for SBA
NG063		No post-treatment serum for SBA
NG066		No post-treatment serum for SBA
NG067		No post-treatment serum for SBA
NG074	Sequencing determined isolate as *Escherichia* only	
NG077		No post-treatment serum for SBA
NG089	No growth on VCAT supplemented GC agar	
NG092		No post-treatment serum for SBA
NG104	Sequencing determined isolate as *Capnocytophaga* only	
NG107	No baseline serum for SBA	
NG108		No post-treatment serum for SBA
NG126	No baseline serum for SBA	No post-treatment serum for SBA
NG127		No post-treatment serum for SBA
NG128		No post-treatment serum for SBA
NG131	No baseline serum for SBA	
NG137		No post-treatment serum for SBA
NG141	Sequencing determined isolate as *Moraxella* only	
NG146		No post-treatment serum for SBA
NG147	No baseline serum for SBA	
NG148	No baseline serum for SBA	
NG149	No baseline serum for SBA	

N. gonorrhoeae isolate number	Reason for serum bactericidal assay incompletion	
	Baseline serum	Post-treatment serum
NG150	No baseline serum for SBA	
NG151	No baseline serum for SBA	
NG154		No post-treatment serum for SBA
NG157		No post-treatment serum for SBA
NG162	No growth on VCAT supplemented GC agar	
NG163	Sequencing determined isolate as *N. meningitidis* only	
NG171	Sequencing determined isolate as *Kingella* only	
NG177	No baseline serum for SBA	No post-treatment serum for SBA
NG178	No baseline serum for SBA	No post-treatment serum for SBA
NG181		No post-treatment serum for SBA
NG190		No post-treatment serum for SBA
NG201		No post-treatment serum for SBA
NG217		No post-treatment serum for SBA
NG218		No post-treatment serum for SBA
NG221		No post-treatment serum for SBA
NG222		No post-treatment serum for SBA
NG224		No post-treatment serum for SBA
NG225		No post-treatment serum for SBA
NG226		No post-treatment serum for SBA
NG235	Sequencing determined isolate as *Escherichia* only	
NG241		No post-treatment serum for SBA
NG243	Sequencing determined isolate as *Enhydrobacter* and *Micrococcus* only	
NG256		No post-treatment serum for SBA
NG257		No post-treatment serum for SBA
NG259	No baseline serum for SBA	No post-treatment serum for SBA
NG266	Sequencing determined isolate as *Sphingomonas* only	
NG269	Sequencing determined isolate as *Corynebacterium* only	
NG277	No baseline serum for SBA	
NG278	No baseline serum for SBA	
NG279	No baseline serum for SBA	
NG282		No post-treatment serum for SBA
NG288	Isolate never received	
NG303		No post-treatment serum for SBA
NG308		No post-treatment serum for SBA
NG309		No post-treatment serum for SBA
NG321		No post-treatment serum for SBA
NG325	No baseline serum for SBA	No post-treatment serum for SBA
NG327		No post-treatment serum for SBA
NG328		No post-treatment serum for SBA
NG348		No post-treatment serum for SBA
NG350	No baseline serum for SBA	
NG360	No baseline serum for SBA	No post-treatment serum for SBA

Table 2.1 *Neisseria gonorrhoeae* isolates with incomplete immune analysis. List of *N. gonorrhoeae* clinical isolates where an analysis of immune response could not be completed and the reasons for the incompletion. These included issues with culture species and lack of baseline or post-treatment autologous serum.

incubation with serum was calculated through the number of viable colonies at each time point (60, 120, 180 minutes) relative to the initial colony count at time point 0 minutes, with a decrease in average bacterial counts over time indicating serum killing of bacteria.

When an exogenous complement source was required, baby rabbit complement (BRC) (AbD Serotec) was added at a final concentration of 5% to participant serum, which had been heat inactivated by incubation at 56ºC for 30 minutes to destroy the complement. BRC was reconstituted following manufacturer's instructions by the addition of 2 ml ice cold water to the lyophilised powder, and then aliquoted and stored at -20ºC.

Several isolate-serum pairs from the collection could not be assessed in this project due to various reasons, detailed in Table 2.1. In summary, autologous baseline serum was not available for 18 of the isolates, sequencing determined that ten of the cultured samples were different organisms, two samples were not viable from the original bead tubes and one isolate was never received. Therefore, there were 336 isolates remaining that could be used during this project for baseline serum assay analysis.

2.5 Flow Cytometry

2.5.1 Multi-colour fluorochrome panel design

Flow cytometric assays were developed to determine gonococcal-specific IgG, IgA and IgM immunoglobulin (GAM) binding titers and complement C3c and MAC deposition from autologous serum to resistant and sensitive clinical *N. gonorrhoeae* isolates.

Secondary antibodies for these five serum proteins to be measured were obtained; murine monoclonal anti-human IgG (clone #IgG-R10), IgM (clone #IgM-AF6) and IgA (clone #IgA-2D7) antibodies were kindly provided by Dr. Margaret Goodall (CIS, University of Birmingham, UK). The anti-IgG comprises all four IgG isotypes and therefore binds total IgG. Rabbit polyclonal anti-human C3c antibodies and murine monoclonal anti-human C5b-9 antibodies

were both obtained from Dako (Agilent, California, USA). According to the manufacturer, anti-C3c reacts with both C3c and the C3c parts of the native C3 and C3b, but does not bind to C3d and C3a. The anti-C5b-9 recognises a neo-epitope on the MAC that only forms upon MAC assembly. Murine monoclonal anti-human C5 antibodies were obtained from Hycult Biotech (The Netherlands). The anti-C5/C5b antibody reacts with an epitope on C5 and C5b.

For flow cytometry experiments, five fluorochromes; DyLight 405 (Pacific blue), fluorescein isothiocyanate (FITC), R-phycoerythrin (PE), PerCP-Cy5.5 and APC-Cy7, were chosen as fluorescent conjugates for each secondary antibody based on both the separation distances between their emission spectra (Figure 2.3.A) and on the compatibility with the lasers on the FACSCanto II (BD Biosciences, Oxford, UK) flow cytometer to be used.

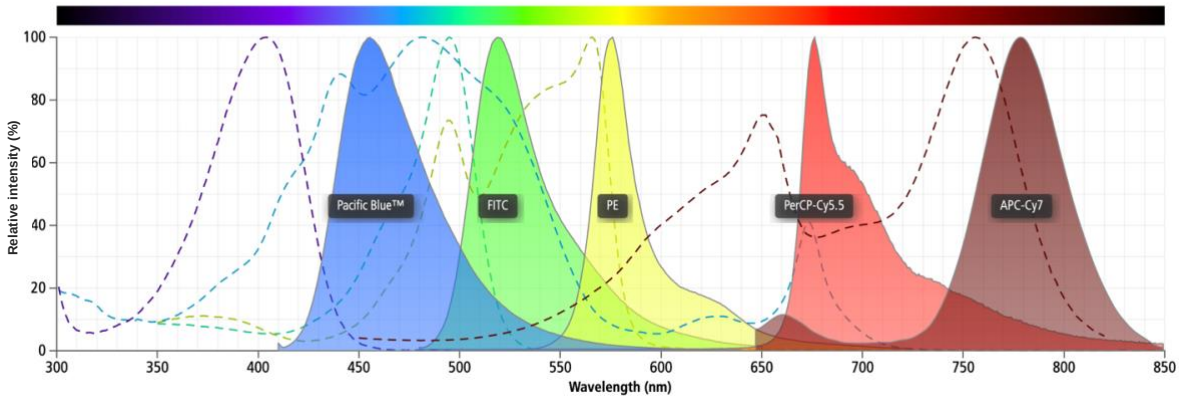
2.5.2 Secondary antibody conjugation

Secondary antibodies for IgG, IgM, IgA, C3c, C5 and C5b-9 were each conjugated to a different fluorochrome, as described in Figure 2.3.B, using Lightning-Link® Rapid Conjugation kits (Innova Biosciences, Cambridge, UK) following manufacturer's instructions. The antibody conjugate constructs were all made up to a final stock concentration of 1 mg/ml. These constructs were stored at 4°C in the dark and are stable for 18 months. Conjugates were never frozen to prevent the donor proteins on tandem antibody conjugates (for example, APC-Cy7) from potentially denaturing.

2.5.3 Preparation of *Neisseria gonorrhoeae* for flow cytometry

Selected *N. gonorrhoeae* isolates were grown on GC agar plates from -80°C stocks as previously described. Bacteria from lawn plates were scraped up using 1 ml of PBS in a 1.5 ml microcentrifuge tube and adjusted to an $OD_{600\ nm}$ of 8.0 using PBS which contained 2 x 10^9 cfu/ml bacteria. Adjusted cultures were added to serum (concentration specified for each

A



B

Fluorochrome	Maximal excitation (nm)	Maximal Emission (nm)	Secondary antibody
DyLight 405 (Pacific blue)	400	420	Anti-IgG
Fluorescein (FITC)	494	520	Anti-C3c / Anti-C5
R-Phycoerythrin (PE)	488/535	575	Anti-C5b-9
PerCP-Cy5.5	482	700	Anti-IgA
APC-Cy7	633	776	Anti-IgM

Figure 2.3 Spectrum range of five fluorochromes used during flow cytometry experiments. A) Spectral view for all five fluorochromes, Pacific Blue, FITC, PE, PerCP-Cy5.5 and APC-Cy7, when using FACSCanto II (BD Biosciences) flow cytometer. The excitation spectra for each fluorochrome is shown through the dotted curves, and the emission spectra for each fluorochrome is shown through the filled curves. This image was created using BD Biosciences Spectrum Viewer. **B)** Maximal absorbance and maximal emission of chosen fluorochromes.

experiment) pre-warmed to 37°C in 1.5 ml microcentrifuge tubes, to a final concentration of 2 x 10^8 cfu/ml bacteria in a total reaction volume of 50 µl. Samples were then incubated without aeration at 37°C for 20 minutes. As a control, the same cultures were added to PBS and incubated under the same conditions. Bacteria were then washed by the addition of 1 ml of PBS, mixed by inverting and spun for 5 minutes at 3,300 x g at room temperature. The supernatant was removed, and the tubes were racked to dislodge the pellet. The cells were then washed a second time in the same way, removing the supernatant to leave approximately 50 µl. The pellet was dislodged again and 2 µl of each fluorochrome conjugated secondary antibody (IgG, IgM, IgA, C3c and C5b-9) at a concentration of 0.5 mg/ml were added to the sample and mixed well by pipetting. Samples were then incubated for 20 minutes at room temperature in the dark, as the fluorochromes are light sensitive and extended exposure can cause photo-bleaching. Therefore, from this point, the samples were always protected from light, by adding them to amber-coloured 1.5 ml microcentrifuge tubes.

The bacteria were then washed twice again in PBS as previously described, leaving 50 µl again after the supernatant had been removed. Bacteria were then fixed by adding 200 µl 4% formaldehyde in PBS (Sigma-Aldrich) to each tube and incubated at room temperature in the dark. Cells were fixed for only 10 minutes to prevent tandem antibody conjugates from breaking in prolonged exposure to paraformaldehyde. Sample volumes were made up to 1 ml with PBS, vortexed and the cells were washed twice again to remove any residual paraformaldehyde. After the final wash and removal of the supernatant, 300 µl cellWASH (BD Biosciences) were added and the bacteria were transferred to cytometer tubes in preparation for flow cytometry analysis. Samples were kept at 4°C and analysed within four hours of paraformaldehyde fixation.

2.5.4 Flow cytometer

Samples were acquired on the FACSCanto II (BD Biosciences, Oxford, UK) flow cytometer

using FACSDiva (BD Biosciences, Oxford, UK) software. Cytometer setup and tracking (CS&T) beads (BD Biosciences, Oxford, UK) were used for daily calibration and instrument monitoring, in order to limit variation from baseline performance. Bacteria were gated based on forward scatter (FSC) and side scatter (SSC). FSC is proportional to the cell-surface area or size, whereas SSC is proportional to cell granularity/internal complexity of the cell. The bacteria were very small therefore a log scale was used upon event collection and laser settings were adjusted so the events were moved away from the axis and could easily be differentiated from the debris. The laser parameters used are detailed in Table 2.2.A.

Fluorochromes have a broad emission spectrum, therefore spectral overlap can occur when two or more fluorochromes are used. Five fluorochromes were required in this experiment, therefore compensation was applied to reduce this spectral overlap between fluorochromes. Initially, compensation was calculated using single colour controls of each fluorochrome secondary antibody attached to the bacterium. However, binding of some serum proteins were generally quite low, therefore it became problematic when creating compensation controls as there were not high enough positive sample sizes to create good compensation controls. Therefore, the compensation was calculated using UltraComp eBeads (Invitrogen). These compensation beads were set up using single colour controls following manufacturer's instructions, with 1 µl neat secondary antibody were added to one drop of beads. Each bead drop contained positive beads which bound the fluorochrome attached secondary antibody and negative beads which did not bind anything, making both populations easily distinguishable on the flow cytometer. The resultant compensation matrix applied to all samples is shown in Table 2.2.B. After compensation and other controls had been applied, prepared clinical isolate samples were then acquired, until 50,000 events were recorded.

A

	Voltage	Log	Area (A)	Height (H)	Width (W)
FSC	445	X	X	X	X
SSC	400	X	X	X	X
FITC	440	X	X		
PE	440	X	X		
PerCP-Cy5	429	X	X		
APC-Cy7	555	X	X		
Pacific Blue	401	X	X		

B

Fluorochrome	% Fluorochrome	Spectral Overlap
PE	FITC	21
PerCP-Cy5	FITC	1
APC-Cy7	FITC	0
Pacific Blue	FITC	0
FITC	PE	1.1
PerCP-Cy5	PE	5.5
APC-Cy7	PE	0
Pacific Blue	PE	1.4
FITC	PerCP-Cy5	0
PE	PerCP-Cy5	0
APC-Cy7	PerCP-Cy5	0.9
Pacific Blue	PerCP-Cy5	0.5
FITC	APC-Cy7	0.2
PE	APC-Cy7	0.2
PerCP-Cy5	APC-Cy7	1.1
Pacific Blue	APC-Cy7	0.8
FITC	Pacific Blue	0
PE	Pacific Blue	0
PerCP-Cy5	Pacific Blue	0
APC-Cy7	Pacific Blue	0

Table 2.2 Laser and compensation metrics used during flow cytometric experiments. A) Adjusted laser parameters on the FACSCanto II (BD Biosciences) flow cytometer used during multi-fluorescence experiments. **B)** Compensation values applied during flow cytometric experiments involving five different fluorochromes, which were calculated using UltraComp eBeads (Invitrogen).

2.5.5 Flow cytometry data analysis

Raw Flow Cytometry Standard data files were analysed using FlowJo software (version 10.8.1) (BD Biosciences, Oxford, UK). A representative gating strategy for analysis of clinical isolates is shown in Figure 2.4. Initially only bacterial cells were gated on FSC and SSC, away from the whole population to remove any cell debris (Figure 2.4.A). For each isolate, the PBS control sample that was ran alongside the serum incubated sample, were used to gate the negative population under each fluorescence (Figure 2.4.B), from which the positive populations in the serum binding samples could be gated (Figure 2.4.C), enabling the negative and positive fluorescent populations to be distinguished.

Histograms were viewed and the percentage of the population of cells that were positively labelled for a specific fluorochrome could be calculated. Here, the geometric mean fluorescence intensity (gMFI) of the positive population could also be calculated, which indicated the level of binding of each serum protein to the population of cells, as the emitted fluorescence intensity is proportional to binding of fluorescent maker. gMFI was used over the median fluorescence intensity (MFI) due to the distribution of the data. Results are determined as either percent positive cells or as gMFI, therefore the integrated gMFI (i-gMFI) (Shooshtari *et al.*, 2010, Darrah *et al.*, 2007) was sometimes used to increase quantitative informational content of data obtained from flow cytometry, by multiplying the relative amount of serum component bound (the gMFI of the positive population) with the relative number of cells stained (the percent of positively labelled cells). Bacteria only populations were also gated with a fluorochrome against another fluorochrome, to compare the binding of both components simultaneously on the population of cells (Figure 2.4.D). The percentage of the population that are binding both serum proteins, or just one and not the other, or neither were calculated, which was made possible through a multi-colour assay.

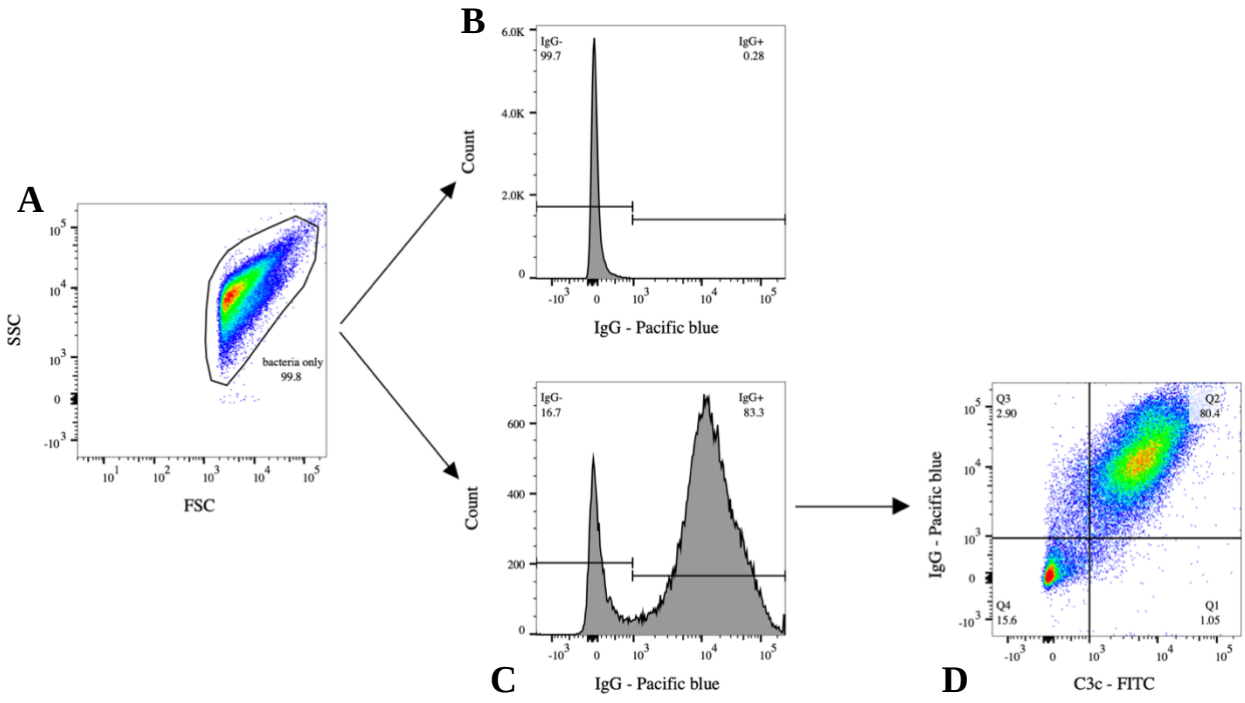


Figure 2.4 Gating strategy for *Neisseria gonorrhoeae* clinical isolates. The gating strategy applied during flow cytometry analysis of isolate NG316, after it was incubated with either autologous serum and PBS as a control. **A)** FSC and SSC plot with gate selecting the bacterial population (bacteria only). **B)** Control sample incubated with PBS, and gating presented as a histogram of the bacteria only population showing IgG binding. **C)** Same gating of the bacteria only population of the sample incubated with serum, showing fluorescence detected in the IgG channel from the Pacific Blue emission spectrum. Indicating a population positively binding IgG. The percentage positively labelled from the whole population can be calculated (83.3%) and the geometric mean fluorescence intensity (gMFI) can also be calculated from this positive population. **D)** Dot plot from the bacteria only population shown as the IgG channel against C3c gating for all four quadrants (Q1 IgG-C3c+ / Q2 IgG+C3c+ / Q3 IgG+C3c- / Q4 IgG-C3c-) and percentage positive from each quadrant shown can be calculated. Image created in layout editor using FlowJo (BD Biosciences) software.

2.6 Antibody depletion

2.6.1 Antibody purification from serum

Participant serum samples were depleted of GAM antibodies by The Binding Site Ltd (Birmingham, UK) from 500 µl samples. First individual serum samples were run through a Protein G column at 1 ml/min and sample injected *via* a 500 µl sample loop. Purified IgG was then eluted at 2 ml/min in a fraction size of 5 ml. The unbound serum (depleted of IgG) was then used as the start material for the next depletion. This serum was then run in the same way through a Capture Select IgA column, with purified IgA eluted in the same way. The unbound (depleted of IgG and IgA) was then used as the start material for the next depletion. Serum was then run in the same way through a Capture Select IgM column, with purified IgM eluted in the same way. Each of the three individual GAM elutions were concentrated back down to 500 µl using Vivaspin 6s. The chromograph (Figure 2.5) from The Binding Site show the results from antibody depletion of NG298-serum.

All purified antibodies were quantified using the Qubit Protein Assay Kit (Thermo Fisher Scientific) following manufacturer's instructions. Each sample was measured in duplicate using the Qubit 2.0 Fluorometer system (Thermo Fisher Scientific), and the average stock concentration was calculated in mg/ml. New standards (1, 2 and 3) were prepared every time using the same master mix (buffer and dye) as used for the samples.

Purified antibodies received from The Binding Site were stored in sodium azide to limit contamination and therefore were buffer exchanged into PBS prior to experiments. Vivaspin 500 µl ultrafiltration spin columns (Sartorius) with a small molecular weight cut-off of 30 kDa was chosen for buffer exchange due to the larger molecular weights of the antibodies (IgG: 150 kDa, IgM: 900 kDa, IgA: 160 kDa) and the small molecular weight of sodium azide (65 Da). The columns were sterilised using ultraviolet light and then PBS was passed through the column prior to use to pre-rinse the membrane, spinning at 12,000 x g. The purified antibody was then added

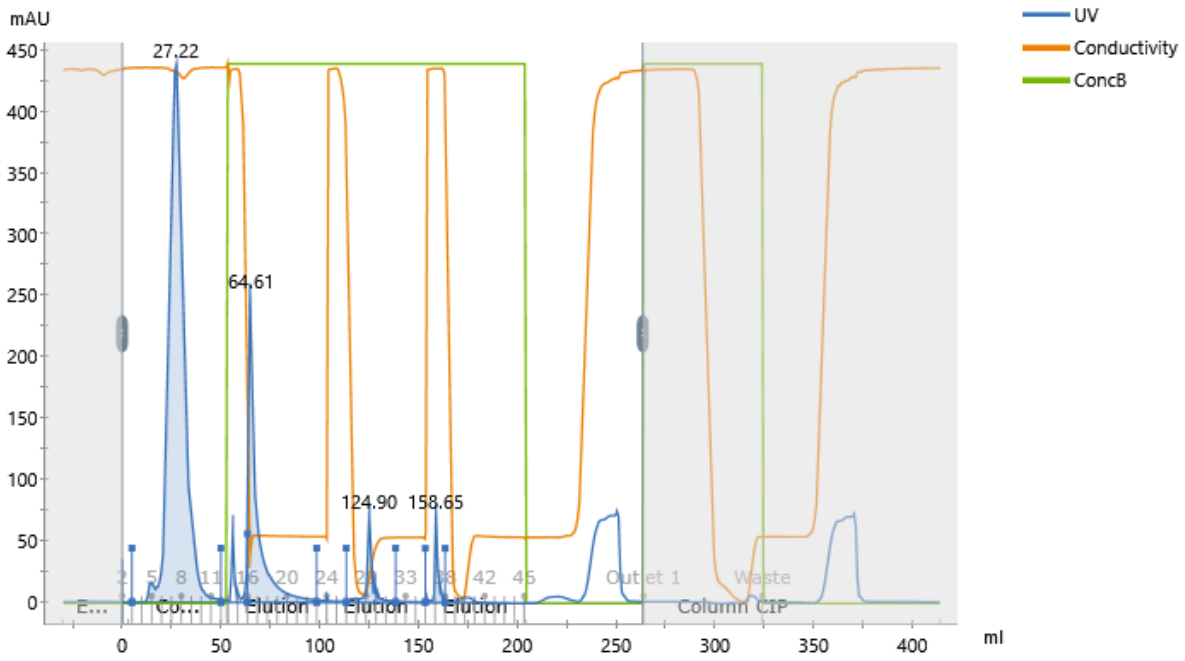


Figure 2.5 Antibody depletion from participant serum. A chromatogram for antibody depletion from NG298- serum produced by The Binding Site (Birmingham, UK). The blue peak at 27.22 ml is the unbound peak, at 64.61 ml is the IgG peak, at 124.9 ml is the IgA peak and at 158.65 ml is the IgM peak. The columns were run in series in buffer A and then eluted separately.

to the column and spun at 4ºC, to remove most of the sodium azide. They were then washed three times with cold PBS and during the final wash, were concentrated down to between 50-100 µl. The purified antibodies were then re-quantified after buffer exchange.

2.6.2 Serum bactericidal assay using purified antibodies

For SBAs involving purified antibody, bacteria were prepared in the same way as previously described and added to a mixture of 5% BRC and each individual purified antibody at varying concentrations in a total volume of 50 µl in PBS. Final concentrations of purified antibody added were 10-fold dilutions ranging from 1 mg/ml to 100 pg/ml for IgG, and 0.1 mg/ml to 100 pg/ml for IgM and IgA. Using BRC as an exogenous complement source allowed for functionality testing of the antibodies after purification.

Other SBA experiments were set up to mirror the 1:1 participant serum/HCS experiments previously completed. Bacteria were prepared for SBA in the same way as previously described and added to a mixture of 50% HCS and purified antibody at the same varying concentrations, in a total volume of 50 µl in PBS.

2.7 Enzyme-linked immunosorbent assay

Bacteria were grown on plates as previously described. Bacteria from lawn plates were scraped up using 2 ml PBS and adjusted to $OD_{600\ nm}$ 0.5. High binding enzyme-linked immunosorbent assay (ELISA) 96 well plates (Nunc-Immuno™ 96-MicroWell™, ThermoScientific) were coated with 100 µl of adjusted culture suspension and incubated overnight at 4ºC in a humid environment. Plates were washed three times with PBS and then blocked with 100 µl of blocking buffer (1% bovine serum albumin (BSA) in PBS) for 1 hour at room temperature. Plates were then emptied and participant serum pre-diluted to 1 in 20 were added to the plate and serially diluted 3-fold in dilution buffer (0.05% Tween20 and 1% BSA in PBS) all to a total volume of

Secondary antibody target	Product details	Dilution in ELISA
Total IgG	SouthernBiotech: goat anti-human IgG-HRP	1:4000
IgG1	SouthernBiotech: mouse anti-human IgG1 Fc-HRP	1:4000
IgG2	SouthernBiotech: mouse anti-human IgG2 Fc-HRP	1:2000
IgG3	SouthernBiotech: mouse anti-human IgG3 hinge-HRP	1:4000
IgG4	SouthernBiotech: mouse anti-human IgG4 Fc-HRP	1:4000
IgM	Merck: goat anti-human IgM Fc5μ-HRP	1:5000
IgA	Merck: goat anti-human IgA α-chain-HRP	1:50,000

Table 2.3 Secondary antibodies used during enzyme-linked immunosorbent assay experiments. Details of the horseradish peroxide (HRP) conjugated secondary antibodies used during enzyme-linked immunosorbent assay (ELISA) experiments, including manufacturer information and dilution of antibody.

100 µl. After incubation for 1 hour at 37ºC in humid conditions, plates were washed with wash buffer (0.05% Tween 20 in PBS) three times and then once with PBS. Next, 100 µl of each secondary antibodies were then added to each well, at varying final concentrations stated in Table 2.3, and incubated at room temperature for 1 hour. Plates were subsequently washed again as above and 100 µl of horseradish peroxidase (HRP) substrate TMB core (AbD Serotec) were added and incubated for 10 minutes at room temperature in the dark. To stop the reaction, 50 µl 0.2 M sulphuric acid were added to each well and the $OD_{450\ nm}$ of each well was read using a CLARIOstar 96-well plate reader (BMG Labtech). Average blank recording at $OD_{450\ nm}$ of 0.05 was subtracted from every resultant well reading.

2.8 Statistical analysis

For flow cytometry experiments, for comparisons of i-gMFI between sensitive and resistant isolates, a Mann-Whitney U statistical test was used to check for statistical significance between the two groups using Prism version 9.2.0 (GraphPad software, San Diego, CA). For the IgG/C3c phenotype comparison between the sensitive and resistant groups, an analysis of covariance (ANCOVA) was used to check for statistical significance. SPSS Statistics version 27 (IMB Corp, Armonk, NY) was used for this statistical analysis.

For ELISA experiments, for comparisons of binding between sensitive and resistant isolates, an unpaired t-test was used to check for statistical significance between the two groups using GraphPad Prism software.

For genomic analysis, it was determined whether particular datasets were over- or under-represented within any of the MLSTs. An example of this would be to investigate whether the female subcategory within the gender dataset were overrepresented in any MLSTs, and a representation of this is shown in Appendix III. To do this, a comparison was made between the proportion of a subcategory (i.e. females) within each of the 55 MLSTs, and the overall

proportion of this subcategory within the entire population of 354 isolates. This gave a ratio for each data subcategory of the positive proportion within an MLST and the whole population. This would therefore indicate that a particular MLST is enriched for a certain subcategory compared to what would be expected from the whole population. This allowed us to identify collections of isolates that had a genetic link to certain data. The comparison between the two proportions were analysed statistically with a two proportion z-test with a 95% confidence level. A z-score of between -1.96 and 1.96 of a normal distribution shows that the null hypothesis is correct (the proportions are the same). Anything outside this distribution, suggests the alternative hypothesis is correct (the proportions are not the same). Statistical significance was then calculated from the z-score based on a normal distribution using a 2-tailed z-test, with $p<0.05$ indicating statistical significance. This same statistical analysis was applied when comparisons of the proportions the clinical data between the eleven inhibitory isolates and the whole population were made.

All statistical analysis throughout was represented as * ($p\leq0.05$), ** ($p\leq0.01$), *** ($p\leq 0.001$) and **** ($p\leq0.0001$).

CHAPTER 3

WHOLE GENOME SEQUENCING ANALYSIS OF THE *NEISSERIA GONORRHOEAE* G-TOG ISOLATE COLLECTION

3.1 Introduction

This PhD project was able to utilise the large collection of clinical *N. gonorrhoeae* isolates from the G-ToG clinical trial. The availability of matching serum from each participant provided a unique opportunity to study the immune response to acute gonococcal infection. WGS of all the isolates in the collection allowed for association of the genomics to the clinical, epidemiological and AMR data from each isolate collected during the trial. In addition, comparisons can be made to the immune response data produced during experimental work, presented in Chapter 4. WGS data is well known as being useful in investigating bacterial evolution, infection outbreaks, transmission routes and the identification and control of the spread of clones of concern. There have previously been many large scale WGS studies of *N. gonorrhoeae*, however the majority of these typically focused on AMR within *N. gonorrhoeae* isolates all over the world. Past studies have successfully identified resistance determinants in multi-resistant isolates from the USA (Grad *et al.*, 2016) and Europe (Harris *et al.*, 2018) and predicted resistance to various antimicrobials (Eyre *et al.*, 2017, Golparian *et al.*, 2018). However, there are a lack of large scale WGS studies with *N. gonorrhoeae* that associate genomics with human immune response data, such as presentation of symptoms and serum susceptibility assays. Therefore, the aim of this study was to utilise the isolate collection, WGS data and clinical data gathered from participants in the trial to try and understand the collection further and make associations with the human immune response to infection.

3.2 Results

3.2.1 Analysis of clinical data gathered during the G-ToG trial

From the 720 participants recruited to the G-ToG clinical trial (Ross *et al.*, 2019), *N. gonorrhoeae* isolates were successfully collected from 307 individuals from a variety of anatomical sites, equating to 367 isolates that were the focus of this study. They were isolated in

	Men who have sex with men (MSM)	Heterosexual male	Female	Not reported	Total
Individuals (N)	183	69	54	1	307
Age groups (years)					
15-19	3 (1.6%)	10 (14.5%)	18 (33.3%)	0 (0.0%)	31 (10.1%)
20-24	39 (21.3%)	19 (27.5%)	23 (42.6%)	0 (0.0%)	81 (26.4%)
25-34	82 (44.8%)	27 (39.1%)	11 (20.4%)	0 (0.0%)	120 (39.1%)
35-44	39 (21.3%)	7 (10.1%)	2 (3.7%)	0 (0.0%)	48 (15.6%)
≥ 45	20 (10.9%)	6 (8.7%)	0 (0.0%)	1 (100.0%)	27 (8.8%)
Ethnicity					
White	140 (76.5%)	31 (44.9%)	36 (66.6%)	0 (0.0%)	207 (67.4%)
Black	17 (9.3%)	21 (30.4%)	3 (5.5%)	0 (0.0%)	41 (13.4%)
Asian	9 (4.9%)	8 (11.6%)	5 (9.3%)	0 (0.0%)	22 (7.2%)
Other ethnic group	9 (4.9%)	3 (4.3%)	2 (3.7%)	0 (0.0%)	14 (4.6%)
Mixed ethinic group	8 (4.4%)	6 (8.7%)	8 (14.8%)	1 (100.0%)	23 (7.5%)
Patient residence					
Outside London	97 (53.0%)	61 (88.4%)	50 (92.6%)	0 (0.0%)	208 (67.8%)
London	85 (46.4%)	8 (11.6%)	1 (1.9%)	1 (100.0%)	95 (30.9%)
Not reported	1 (0.5%)	0 (0.0%)	3 (5.5%)	0 (0.0%)	4 (1.3%)
HIV status					
Positive	42 (23.0%)	1 (1.4%)	0 (0.0%)	1 (100.0%)	44 (14.3%)
Negative	136 (74.3%)	68 (98.5%)	54 (100.0%)	0 (0.0%)	258 (84.0%)
Not reported	5 (2.7%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	5 (1.6%)
Site of infection					
Urethral	105 (57.4%)	67 (97.1%)	0 (0.0%)	0 (0.0%)	172 (56.0%)
Cervical	0 (0.0%)	0 (0.0%)	44 (81.5%)	0 (0.0%)	44 (14.3%)
Rectal	66 (36.1%)	0 (0.0%)	19 (35.2%)	1 (100.0%)	86 (28.0%)
Pharyngeal	41 (22.4%)	1 (1.4%)	12 (22.2%)	0 (0.0%)	54 (17.6%)
Other site	5 (2.7%)	5 (7.2%)	1 (1.9%)	0 (0.0%)	11 (3.6%)
Multiple sites	30 (16.4%)	3 (4.3%)	18 (33.3%)	0 (0.0%)	51 (16.6%)
Symptoms					
Discharge and/or dysuria	104 (56.8%)	61 (88.4%)	28 (51.9%)	0 (0.0%)	193 (62.9%)
No discharge and/or dysuria	79 (43.2%)	8 (11.6%)	26 (48.1%)	1 (100.0%)	114 (37.1%)
Co-current STIs					
Chlamydia	39 (21.3%)	22 (31.8%)	20 (37.0%)	0 (0.0%)	81 (26.4%)
Syphilis	9 (4.9%)	2 (2.9%)	0 (0.0%)	0 (0.0%)	11 (3.6%)
Herpes	1 (0.5%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	1 (0.3%)
Trichomonas	0 (0.0%)	0 (0.0%)	2 (3.7%)	0 (0.0%)	2 (0.7%)
New HIV diagnosis	3 (1.6%)	1 (1.4%)	0 (0.0%)	0 (0.0%)	4 (1.3%)
Previoulsy diagnosed with gonorrhoea					
Yes	104 (56.8%)	14 (20.3%)	9 (16.6%)	0 (0.0%)	127 (41.4%)
No	77 (42.1%)	55 (79.7%)	45 (83.3%)	1 (100.0%)	178 (58.0%)
Not reported	2 (1.1%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	2 (0.7%)

Table 3.1 Characteristics of individuals in the G-ToG clinical trial by gender and sexual orientation. Demographic, clinical and behavioural characteristics of 307 participants from the G-ToG trial from which *N. gonorrhoeae* isolates had been cultured. Data shown as a percentage of individuals in each category (n (% of N)).

2014 (n=20), 2015 (n=166) and 2016 (n=175). An abundance of epidemiological and clinical data were collected from participants upon trial entry and all data are detailed in Appendix I. The trial included a wide range of ages, variety of ethnic groups, both women and men (including MSM and heterosexual men), asymptomatic and syptomatic individuals, HIV-positive and negative individuals and a variety of anatomical sites of infection, all of which are summarised in Table 3.1. The distribution of these epidemiological and clinical characteristics in the G-ToG trial were highly comparable with larger studies, including the UK Health Security Agency (UKHSA) data in the 2020 Gonococcal Resistance to Antimicrobials Surveillance Programme (GRASP) sentinel surveillance collection (UKHSA, 2020).

Of the 307 participants, 82.3% (252/307) were male and the remaining 17.7% (55/307) were female, which agrees with the UK epidemiology data (Figure 1.1). Of the 252 males, 72.6% (183/252) were MSM. Most individuals were white (67.4%), and the modal age group was 25-34 (39.1%), ranging from 16 to 69 years old across all those in the study. Amongst all individuals, 14.3% were living with HIV, and 95.5% (42/44) of these were MSM. Previously 41.4% of individuals had already been infected with gonorrhoea at least once before and 26.4% of individuals were concurrently diagnosed with chlamydia during the trial. From the 367 isolates collected from the 307 individuals, the anatomical site of specimen collection had been mainly urethral (46.9%), followed by rectal (23.4%), pharyngeal (14.7%) and cervical (12.0%). Among heterosexual males, in 97.1% (67/69) gonorrhoea was located in the urethra, whereas the sites of infection for MSM and females were more varied, ranging between genital, rectal and pharyngeal.

3.2.2 Sequence type assignment

WGS of all isolates were determined using HiSeq next generation sequencing (Illumina, USA). Of the total 367 isolates in the collection, sequences of 354 isolates were analysed as part of this

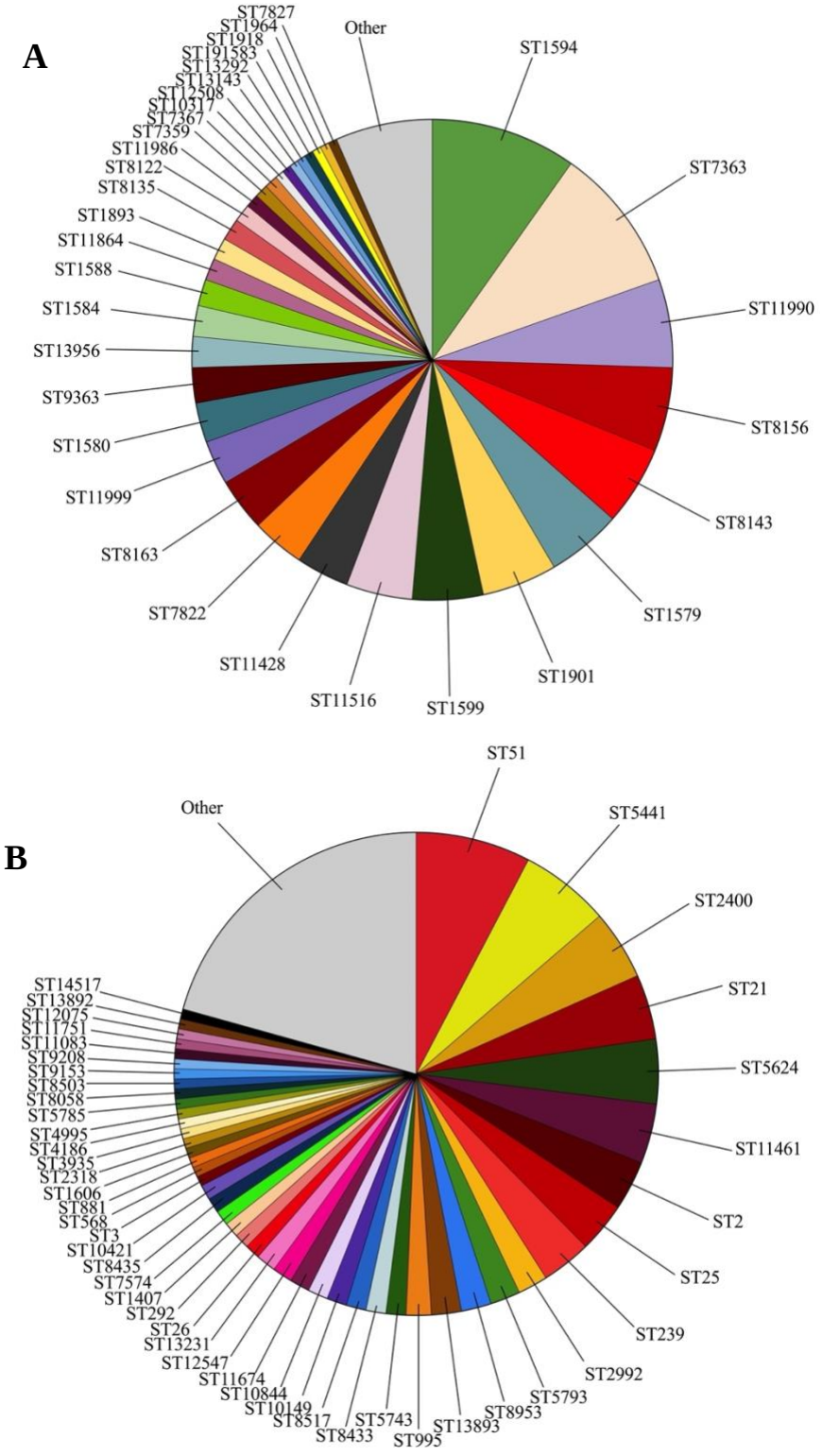


Figure 3.1 Genotype proportions among isolates in the G-ToG trial. A) Multi-locus sequence types (MLSTs) were assigned using MLST (version 2.16.4) (Jolley and Maiden, 2010) on *de novo* assemblies. The 354 isolates that were sequenced represented 55 different MLSTs. Proportion of each MLST are depicted, with 'other' encompassing 22 isolates that each had a single ST. **B)** Multi-antigen sequence types (MASTs) were assigned using NGmaster (version 0.5.8) (Kwong *et al.*, 2016) on *de novo* assemblies. The 354 isolates that were sequenced represented 108 different MASTs. Proportion of each MAST are depicted, with 'other' encompassing 62 isolates that each had a single ST.

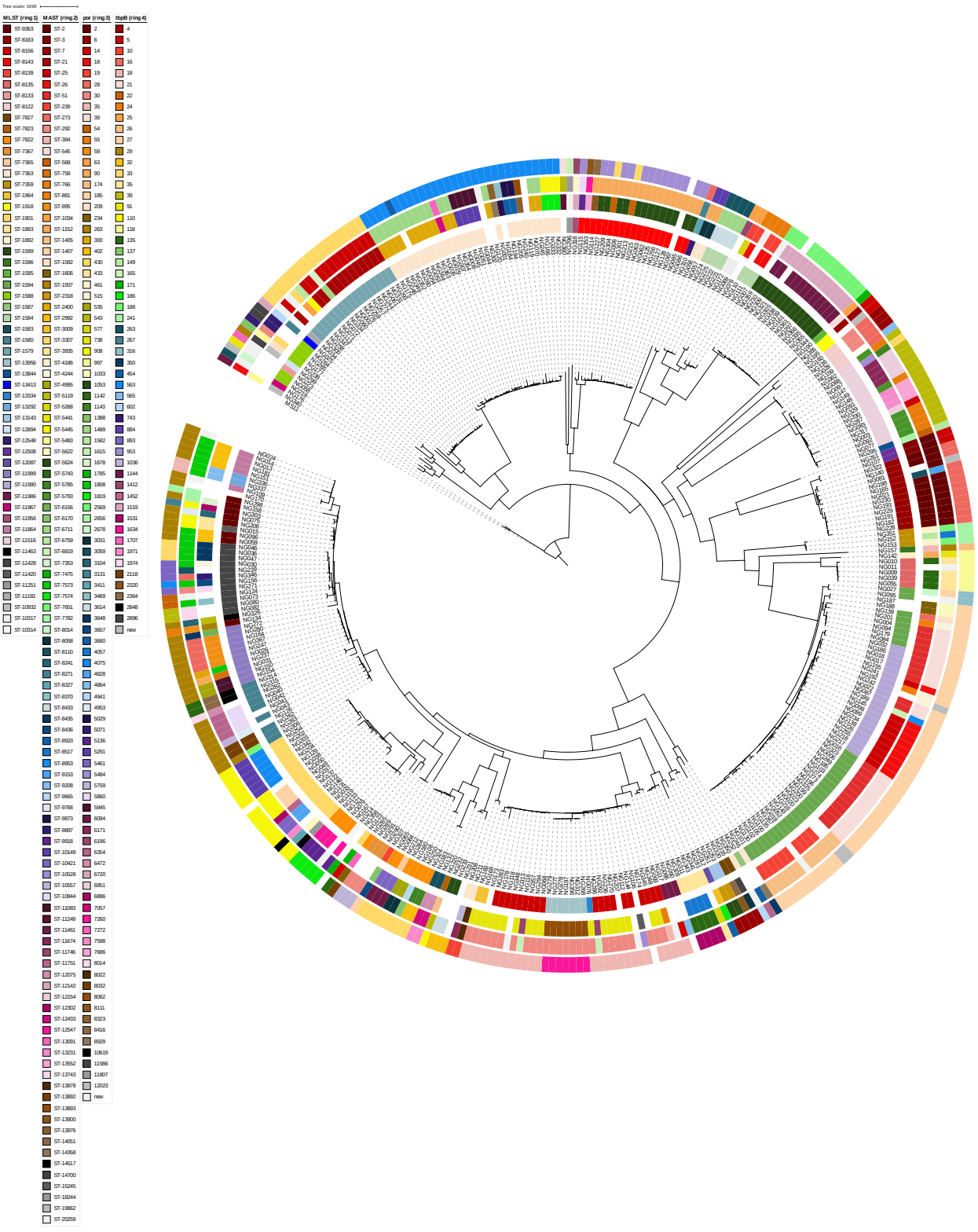


Figure 3.2 Phylogenomic distribution of genotypes among isolates in the G-ToG collection. Recombination corrected maximum likelihood tree of all 354 sequenced *N. gonorrhoeae* isolates was created using RAxML (Stamatakis, 2014). Multi-locus sequence type (MLST) and multi-antigen sequence type (MAST) (along with *por* and *tbpB* allele variants also identified alongside MA typing) were annotated using iTOL software (Letunic and Bork, 2021).

project, as 13 samples were required to be removed due to reasons described in Table 2.1. This therefore resulted in 296 participants in this collection with sequenced isolates, down from the overall 307. From these, a maximum likelihood phylogenomic tree could be constructed that allowed for associations to be made with genotypes, clinical data and other phenotypes.

MLST is a standard tool for characterising bacterial isolates based on their distinct alleles for a set of seven housekeeping genes belonging to that particular species, resulting in a sequence type (ST) for each isolate. The neisserial PubMLST database (Jolley *et al.*, 2018) was used to assign 55 different sequence types (Figure 3.1.A). There were 33 clusters (two or more isolates with the same MLST) identified, and 22 single STs. The most frequently observed STs represented by ≥ 13 isolates were ST7363 (n=33), ST1594 (n=33), ST11990 (n=20), ST8156 (n=19), ST8143 (n=18), ST1901 (n=17), ST1579 (n=17), ST1599 (n=16) and ST11516 (n=15).

Similarly, multi-antigen sequence typing for *Neisseria gonorrhoeae* (NG-MAST) (Martin *et al.*, 2004) is another typing scheme for characterising gonococcal isolates based on distinct alleles for genes *porB* and *tbpB*, again resulting in STs for each isolate. NG-MASTs were assigned, and 108 different STs were identified, representing 96 different *porB* alleles and 54 different *tbpB* alleles (Figure 3.1.B). There were 46 clusters identified, and 62 single STs. The most frequently observed STs represented by ≥ 13 isolates were ST51 (n=23), ST5441 (n=18), ST2400 (n=14), ST21 (n=13) and ST5624 (n=13). Both MLST and NG-MAST identifications for each isolate are also displayed phylogenomically (Figure 3.2).

3.2.3 Phylogenomic analysis of isolates derived from multiple anatomical site infections

Of the sequenced isolates within the collection, 16.6% (49/296) of participants had multiple gonococcal isolates at different anatomical sites; 40 participants had two isolates at different sites and nine participants had three isolates at different sites. The phylogenomic tree has been annotated with this dataset, to investigate any relationships between isolate pairs derived from

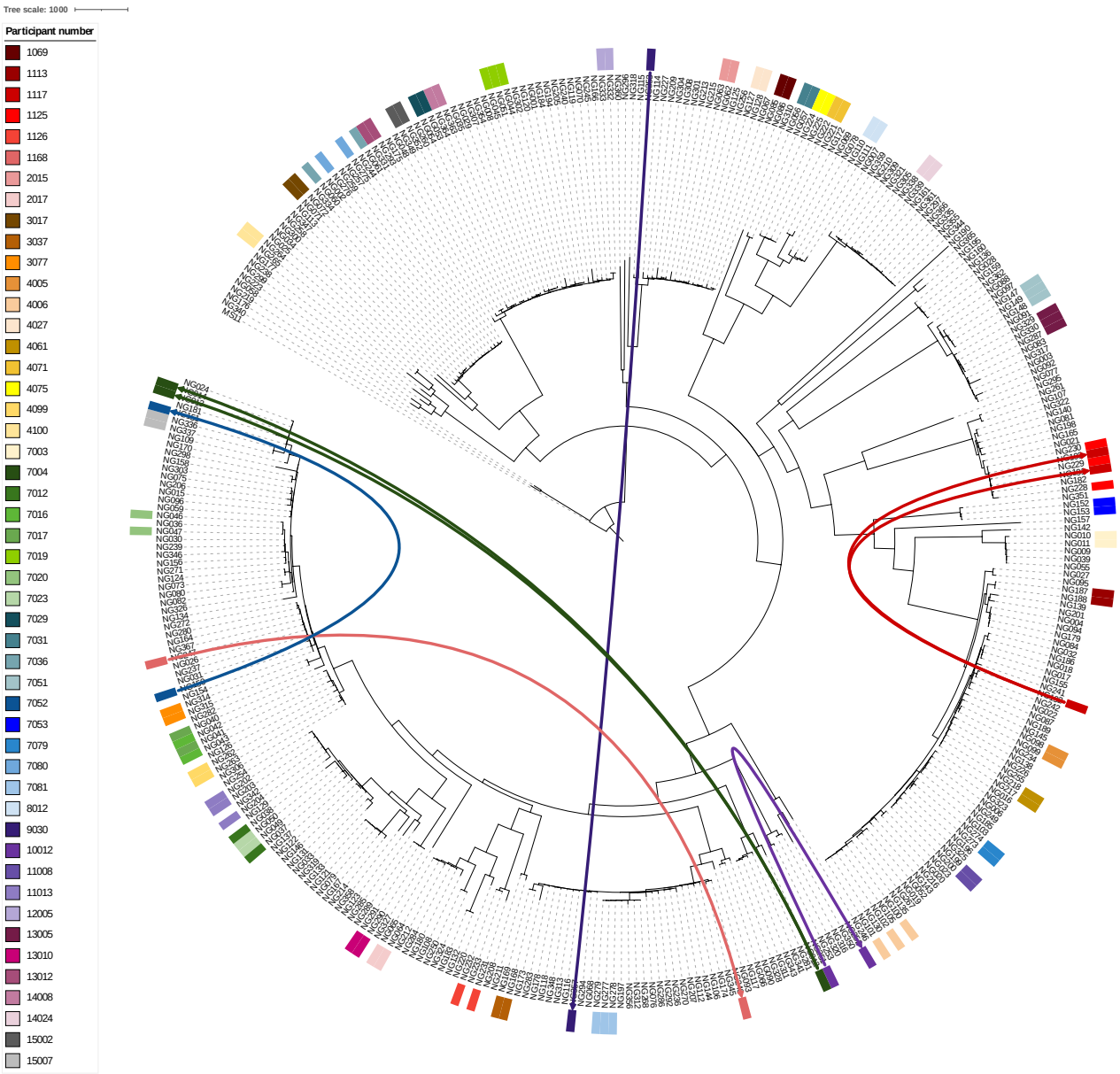


Figure 3.3 Phylogenomic distribution of multiple anatomical sites among isolates derived from the same participant. Recombination corrected maximum likelihood tree of all 354 sequenced *N. gonorrhoeae* isolates was created using RAxML (Stamatakis, 2014). Isolates from participants with multiple anatomical site infections were annotated using iTOL software (Letunic and Bork, 2021), with the addition of arrows indicating isolates from the same participant located in different regions of the tree.

the same host (Figure 3.3). The majority of pairs were highly similar at the genetic level, with the same MLST and NG-MAST profiles. However, a small number of the isolate pairs from the same participant (6/49) were found in different regions of the phylogenomic tree, highlighted by the connecting arrows in Figure 3.3. This suggests that these isolates are genetically very different, and these participants were infected with different isolates at different anatomical sites.

3.2.4 Phylogenomic analysis of clinical metadata derived from participants

To understand whether there were any correlations between the phylogenomics and the different sets of clinical data, analysis was undertaken to determine whether any datasets were enriched within different genetically similar groups of isolates. MLSTs were used to separate all isolates into their 55 ST groups. Next, for each dataset, comparisons were made between proportions within each ST groups and the expected proportions within the overall population of 354 isolates. This determined whether particular sets of data were over- or under-represented in any of the MLSTs, allowing for identification of ST groups that have a genetic link to certain datasets. An example of this is shown in Appendix III, depicting part of an investigation into the gender dataset, to determine whether females were over- or under-represented in any MLSTs, when compared to the average result expected in the overall population.

3.2.4.1 Effect of genetics on epidemiology data from participants

Initially epidemiology data from all participants, such as geographical location, date of isolate collection, participant age and ethnicity, were correlated with the phylogenomic tree of all isolates (Figure 3.4). The geographical location of each participant was analysed to investigate whether there had been an outbreak of a single strain type in a specific region of the country that might assist in tracking the spread of strains (Figure 3.4, ring 2). Initial observations showed that

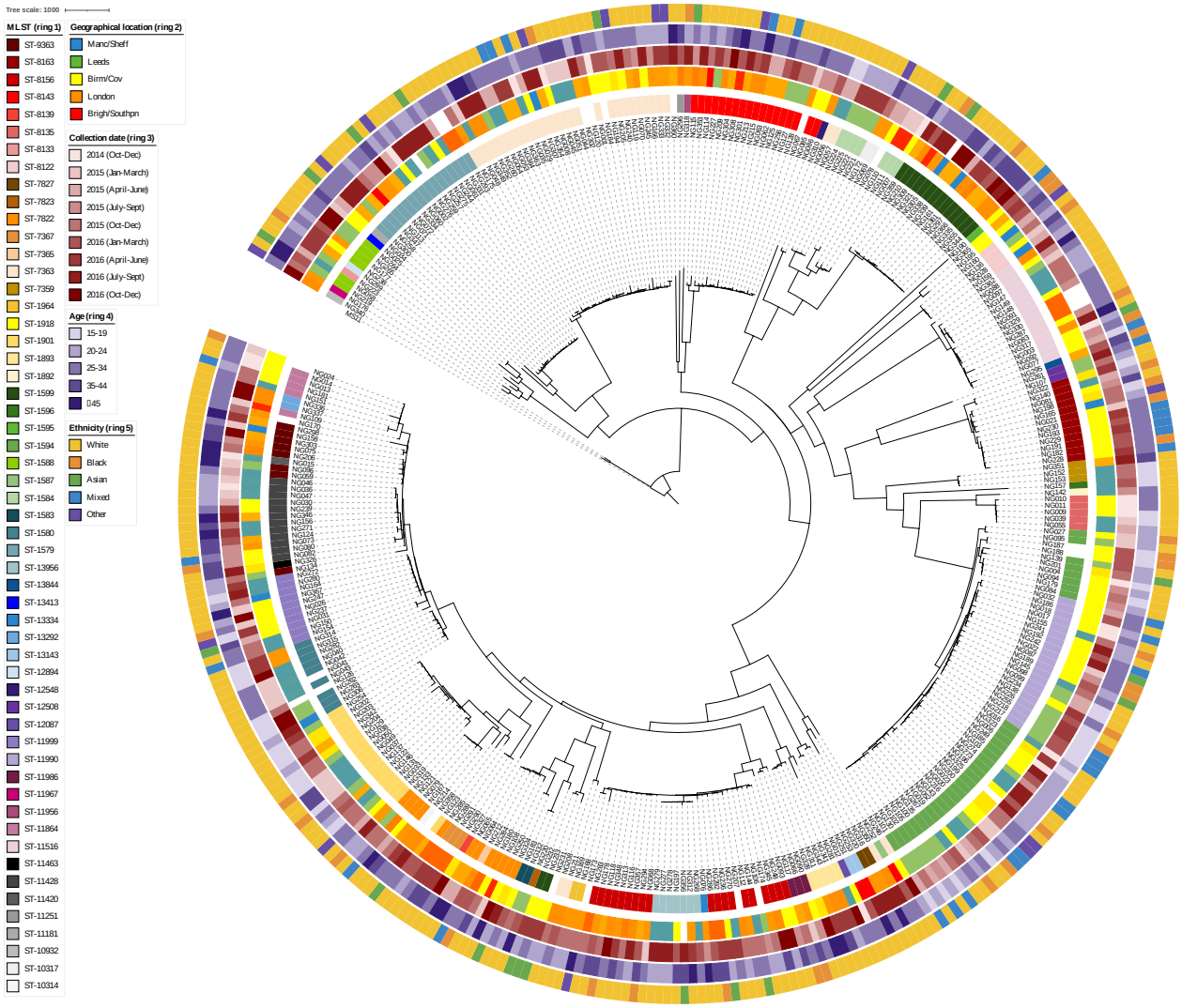


Figure 3.4 Phylogenomic distribution of participant epidemiological characteristics. Recombination corrected maximum likelihood tree of all 354 sequenced *N. gonorrhoeae* isolates was created using RAxML (Stamatakis, 2014). Participant geographical location, date of isolate collection, participant age and ethnicity were annotated using iTOL software (Letunic and Bork, 2021) and compared to MLST data.

the geographical data was spread evenly around the tree, suggesting that there were no outbreaks of a particular clade within any particular regions in the country. This suggests that the isolates in this study are an overview of isolates naturally circulating in the country during 2014-2016. Closer inspection found that within this dataset, MLST groups ST11990, ST1594 and ST8163 were over-represented with isolates derived in Birmingham and Coventry, and under-represented in isolates derived from London, compared to the proportions expected to be found in the overall population of 354 isolates. In contrast, ST1599 and ST8143 were over-represented amongst isolates derived from London.

No significant associations were found with the second data set, the date that each isolate had been collected (Figure 3.4, ring 3). The final data sets analysed were the age of each participant (Figure 3.4, ring 4) and ethnicity (Figure 3.4, ring 5). Both ST1594 and ST1901 were over-represented among isolates derived from the youngest participants in the trial ($\leq$ 24 years). Within the ethnicity dataset, ST11990 and ST8163 were under-represented amongst isolates derived from white participants but over-represented amongst isolates from black and mixed race backgrounds.

3.2.4.2 Effect of genetics on the response of the host immune system

Clinical datasets associated with the host immune response, such as presentation of symptoms, previous gonorrhoea infections, other co-current STDs and HIV status, were next analysed against the phylogenomic tree of all isolates (Figure 3.5). Over half of the participants were presenting symptoms during their infection with gonorrhoea, so possible links between the genetics of the infecting isolate and the causation of symptoms in the participant were investigated. During recruitment to the G-ToG clinical trial, details of the presentation of a wide variety of different symptoms were reported and underwent statistical analysis (Figure 3.5, ring 2). Genital discharge was one of the most reported symptoms by trial participants during *N.*

gonorrhoeae infection. MLST groups ST11864, ST1584 and ST8156 were all over-represented with isolates from participants who reported profuse genital discharge. In addition, there were other MLSTs that were over-represented amongst isolates from participants who reported genital discharge, however these depended on the duration of symptoms. Isolates which caused under one week of genital discharge were over-represented in ST7822, ST13956 and ST1580, and over one week were over-represented in ST8143 and ST1588. In contrast MLST groups ST11428, ST1579 and ST1594 were over-represented amongst isolates that did not cause genital discharge. Dysuria was another symptom reported by participants with *N. gonorrhoeae* infection. Isolates which caused under one week of dysuria symptoms were over-represented in ST1599, ST7822, ST11864 and ST1901, and over one week were over-represented in ST8156, ST1588 and ST1594. In contrast, MLSTs ST11428, ST11990, ST7363 and ST8135 were over-represented amongst isolates that did not cause dysuria.

Prior gonococcal infections were also compared to the phylogenomics (Figure 3.5, ring 3) to understand whether there was a correlation with isolates that were able to re-infect participants who had already been infected before, potentially giving them an advantage when evading the immune system. ST8156 and ST1893 were over-represented in participants with one previous gonorrhoea infection, ST11428 and ST11864 between 2-7 previous infections and ST1599 and ST7822 > 8 previous infections. In contrast, MLST groups ST1580, ST1594 and ST1901 were over-represented amongst isolates infecting a participant with no prior history of gonorrhoea infections.

Infection with another STD, such as chlamydia and syphilis, alongside gonorrhoea is not uncommon. Therefore, the next dataset analysed was co-current STDs to identify whether certain gonococcal isolates were better adapted to co-inhabit with another pathogenic organisms. MLSTs ST1594 and ST8163 were both over-represented amongst isolates co-inhabiting with chlamydia. Whereas ST11999 and ST1579 were both over-represented amongst isolates

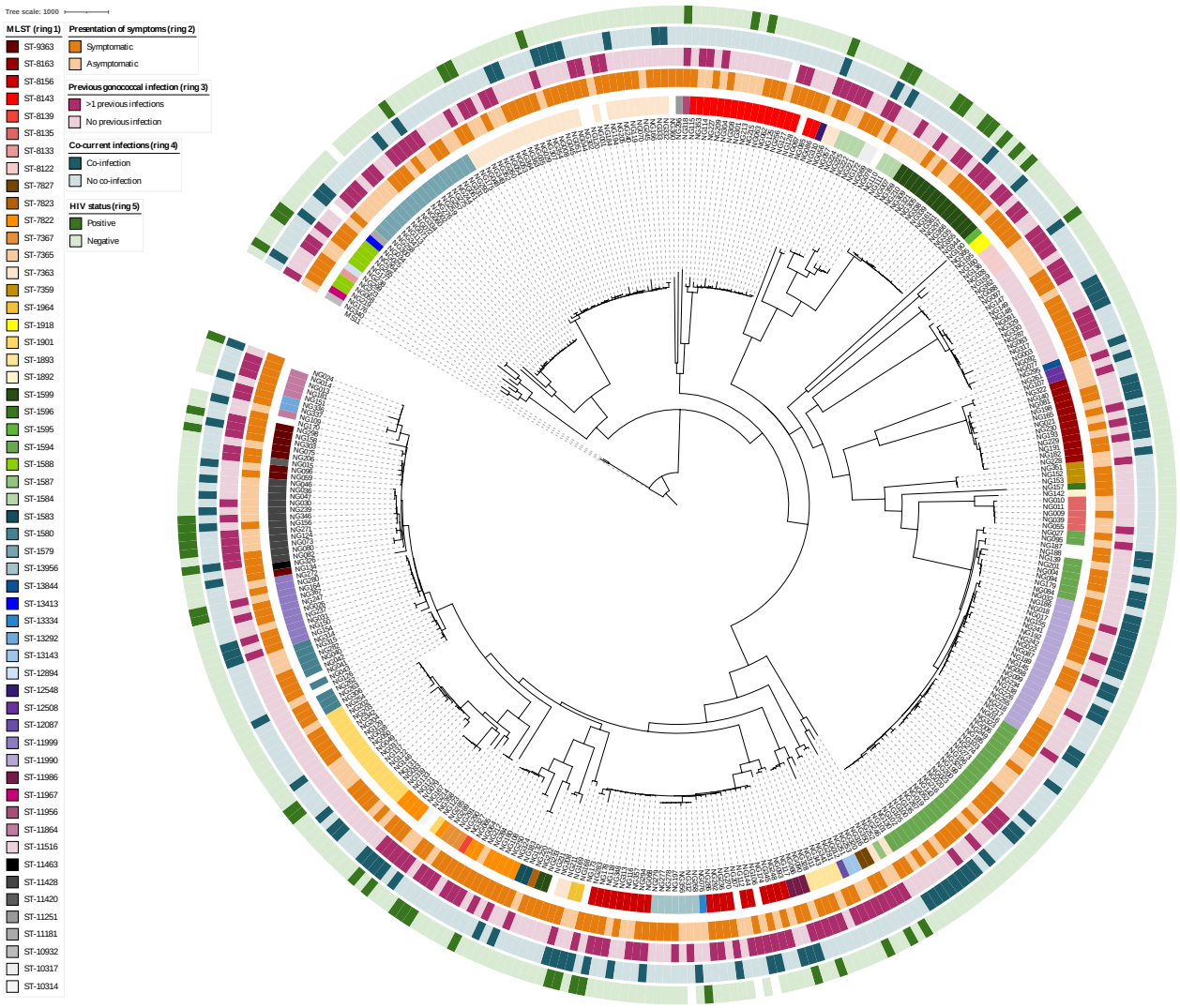


Figure 3.5 Phylogenomic distribution of participant immune response characteristics. Recombination corrected maximum likelihood tree of all 354 sequenced *N. gonorrhoeae* isolates was created using RAxML (Stamatakis, 2014). Participant's presentation of symptoms, prior infection gonorrhoea, other co-current STI infections (including chlamydia and syphilis) and HIV status were all annotated using iTOL software (Letunic and Bork, 2021) and compared to MLST data.

co-inhabiting with syphilis. In contrast, ST1580 and ST8143 were over-represented in participants with no other associated STDs during the time they were infected with gonorrhoea.

3.2.4.3 Effect of genetics on antimicrobial resistance

Isolates underwent antimicrobial susceptibility testing against ceftriaxone and azithromycin by PHE, UK (Cole, 2019). The current MIC clinical breakpoints recommended by The European Committee on Antimicrobial Susceptibility Testing (EUCAST) are 'sensitive ≤ 0.125 mg/L > resistant' for ceftriaxone (EUCAST, 2022). For azithromycin, EUCAST excluded clinical breakpoints in their 2019 report. However previous reported breakpoints were 'sensitive ≤ 0.25 mg/L and 0.5 mg/L > resistant'. For the isolates in this collection, it had been calculated that the average MIC for ceftriaxone was 0.004 µg/ml (41.8%, 148/354) within a range of ≤ 0.002 to 0.125 µg/ml, and the average MIC for azithromycin was 0.25 µg/ml (28.5%, 101/354), within a range of ≤ 0.016 to 4 µg/ml. MIC datasets for each isolate against the two antibiotics were annotated onto the phylogenomic tree (Figure 3.6).

Statistical analysis of MIC differences identified five MLSTs (ST11990, ST8135, ST8163, ST1594, ST1599 and ST8156) all concentrated towards the right hand side of the tree, that were all over-represented amongst isolates with an average MIC for both antibiotics below the expected MIC of the total population. Overall, this suggests these MLSTs are more susceptible to the antibiotics. Further analysis identified that eight MLSTs (ST11428, ST1901, ST1579, ST11999, ST1580, ST7363, ST11864 and ST9363) all concentrated towards the left hand side of the tree, that were all over-represented among isolates with a higher MIC for both or one of the antibiotics. Overall, this suggests these MLSTs are less susceptible to the antibiotics. It has previously been reported that MLSTs ST1901, ST1579 and ST7363 all have higher ceftriaxone resistance (Shimuta *et al.*, 2013). In addition, ST1901 has previously been described as the most common cause of gonococcal infections that exhibit reduced extended spectrum cephalosporin susceptibility (Harris *et al.*, 2018, Shimuta *et al.*, 2015).

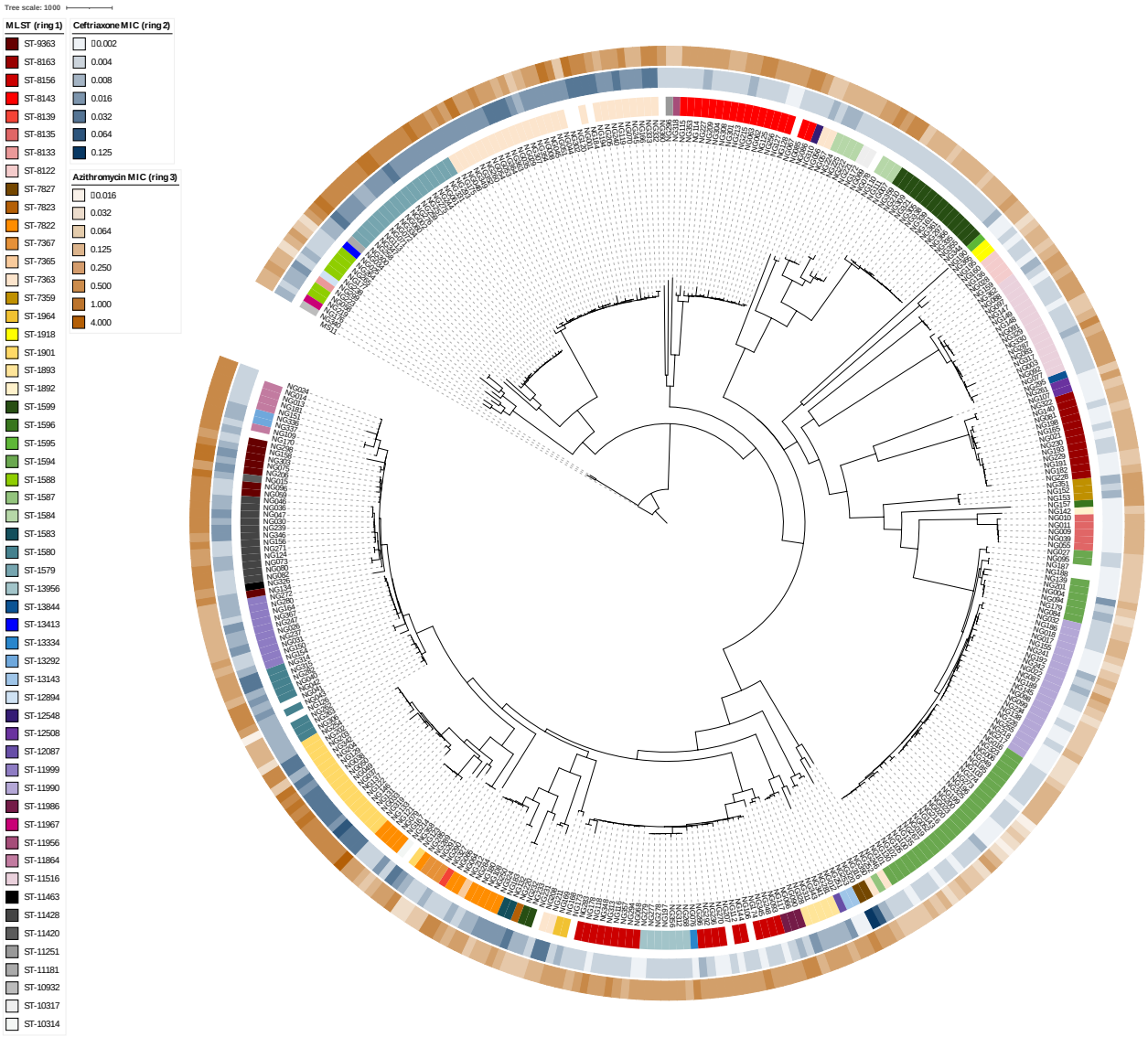


Figure 3.6 Phylogenomic distribution of minimum inhibitory concentrations amongst isolates. Recombination corrected maximum likelihood tree of all 354 sequenced *N. gonorrhoeae* isolates was created using RAxML (Stamatakis, 2014). MICs of ceftriaxone and azithromycin were annotated using iTOL software (Letunic and Bork, 2021) and compared to MLST data.

3.2.4.4 Effect of the behaviour of participants of different genders on genetics

The next analyses focused on gender characteristics and behaviours, such as sexual network and anatomical site of infection, which were correlated to the phylogenomic tree of all isolates (Figure 3.7). Gender was the first dataset analysed to identify whether isolates preferred infecting a particular gender. Initial observation from the phylogenomic tree (Figure 3.7, ring 2) showed the majority of isolates that were derived from female participants were clustering in the bottom right of the tree (47/75, 62.7%). Statistical analysis of gender differences identified five MLSTs (ST11990, ST1594, ST8135, ST8163 and ST1580) that were over-represented amongst isolates derived from females and under-represented amongst isolates derived from males. Four of these five MLSTs were in that bottom right portion of the tree.

Focusing more closely on gender differences, sexual networks within gender categories were able to separate the males into two groups, MSM and men who have sex with women (MSW). Statistical analysis of sexual networks showed the same five MLSTs were also over-represented amongst isolates derived from women who have sex with men (WSM) network. Of the few males found interspersed within each of these five MLSTs, isolates were predominantly derived from the MSW network, and not MSM communities.

The third dataset annotated on the tree was the anatomical site of infection (Figure 3.7, ring 4). This analysis revealed an over-representation of isolates derived from the cervix within four out of the five MLSTs identified earlier as over-represented in females. No similar correlation had been found with any other MLST. Three of these four MLSTs (ST11990, ST1594 and ST8135) were along one lineage on the phylogenomic tree in the bottom right portion of the tree, with 76.2% (32/42) of all cervical isolates collected during the trial clustering together in this portion of the tree. In contrast, urethral, rectal and pharyngeal isolates were spread relatively evenly around the tree.

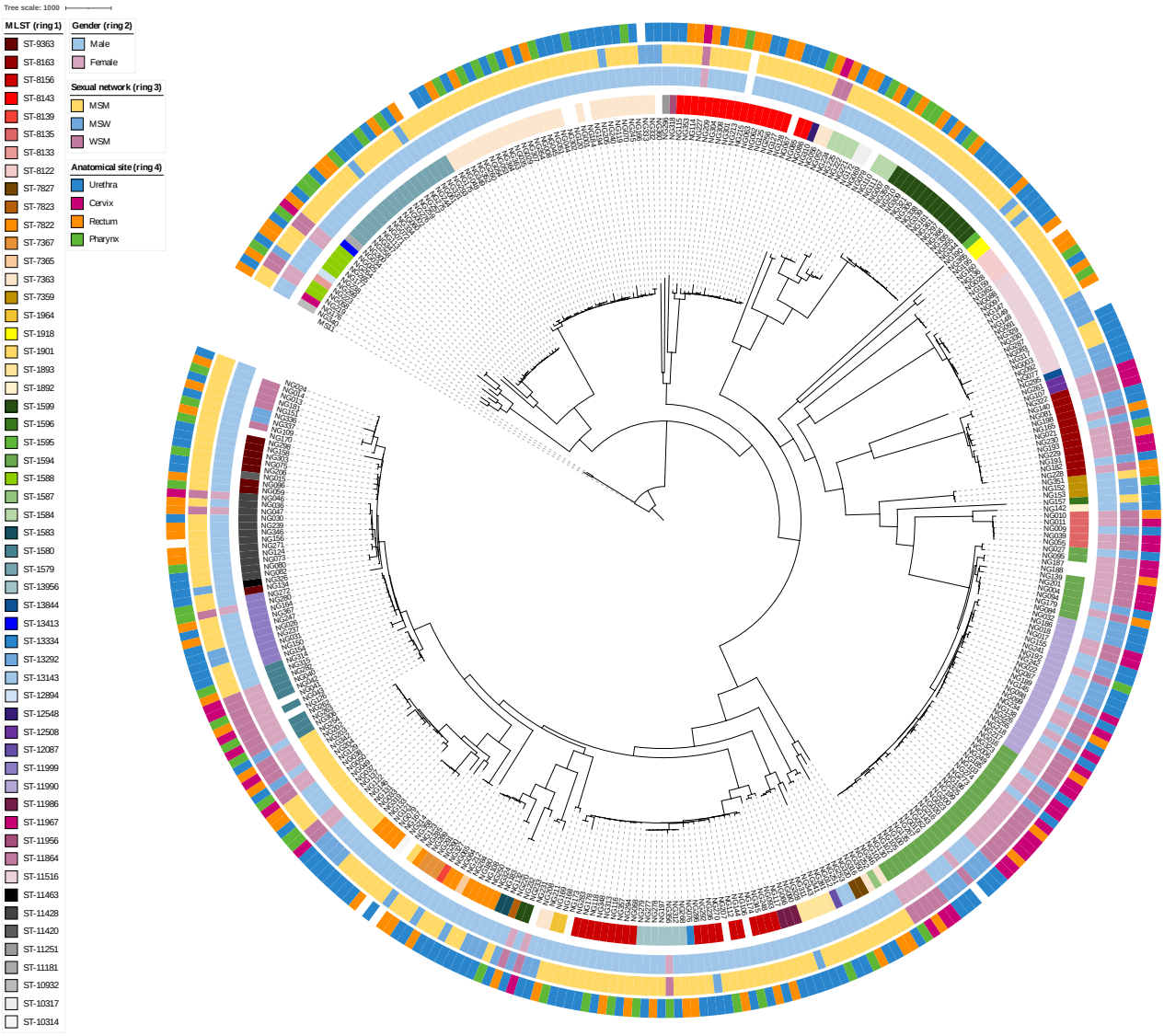


Figure 3.7 Phylogenomic distribution of participant gender characteristics. Recombination corrected maximum likelihood tree of all 354 sequenced *N. gonorrhoeae* isolates was created using RAxML (Stamatakis, 2014). Participant gender, sexual network and isolate anatomical site were annotated using iTOL software (Letunic and Bork, 2021) and compared to MLST data.

3.3 Discussion

Associating the clinical data gathered during the G-ToG clinical trial with the WGS data helped to characterise the isolates within the collection. The analysis of participants with multiple anatomical site infections (Figure 3.3) revealed six participants had been infected with different strains at different sites. The remaining 43 participants had multiple infecting isolates that were highly similar at the genetic level. However, at each of these individual infection sites, it is difficult to confirm the diversity, as alike most clinical laboratories, only a single colony was saved when culturing these isolates. Therefore this has probably led to an underestimation of the incidence of mixed infections at each infection site, as the literature suggests mixed-strain gonorrhoea infections are common (Goire *et al.*, 2017, Martin and Ison, 2003, Lynn *et al.*, 2005). It is suggested that they are common due to genomic signatures found within the genome stemming from the horizontal gene transfer and recombination that occurs within *N. gonorrhoeae* very frequently, which results in a marked inability to establish stable clonal linages.

Further overall observations (summarised in Table 3.2) indicated isolates on the left hand side of the phylogenomic tree have a significant increase in MIC of both or one of the treatment antibiotics. Closer inspection of these isolates with higher antibiotic MICs showed a correlation with the male gender category. There was a significant increase in ceftriaxone MIC relating to the male sexual networks, MSM and MSW, compared to WSM (Figure 3.8.A). This included MLSTs previously mentioned, such as ST7363. This ST has previously been documented to have likely originated in Japan (Ohnishi *et al.*, 2010) and has spread globally to many countries since. A substantial proportion of global isolates have shown decreased susceptibility and resistance to extended-spectrum cephalosporins resulting in treatment failures. In 2009, a subclone of the cefixime-resistant ST7363, strain H041, was the first gonorrhoea “superbug” (Ohnishi *et al.*, 2011). The strain had the capacity to develop high-level ceftriaxone resistance, at 4- to 8-fold

MLST	Gender		Sexual network			Infection site	Ceftriaxone MIC		Azithromycin MIC	
	Male	Female	MSM	MSW	WSM	Cervix	≤ 0.004	≥ 0.008	≤ 0.25	≥ 0.5
ST11990	Decrease **	Increase **	Decrease ****	Decrease ***	Increase **	Increase **	Increase ***	ns	Increase ***	ns
ST1594	Decrease ****	Increase ****	Decrease ****	Decrease *	Increase ****	Increase ****	Increase *	ns	Increase ***	Decrease **
ST8135	Decrease **	Increase **	Decrease **	ns	Increase **	Increase **	Increase ****	ns	ns	ns
ST8163	Decrease ***	Increase ***	Decrease ****	ns	Increase ***	ns	Increase ****	ns	Increase **	ns
ST11428	ns	ns	Increase **	ns	ns	ns	ns	Increase *	ns	Increase ****
ST11864	ns	ns	Increase *	ns	ns	ns	ns	ns	ns	Increase ****
ST1579	Increase *	Decrease *	Increase *	ns	Decrease *	ns	ns	Increase ****	ns	Increase ****
ST7363	Increase *	Decrease *	Increase *	ns	Decrease *	ns	ns	Increase ****	ns	ns
ST9363	ns	ns	Increase *	ns	ns	ns	ns	ns	ns	Increase **

Table 3.2 Associations between MLSTs and clinical data relating to *Neisseria gonorrhoeae* isolates. Multi-locus sequence types (MLSTs) were assigned to *N. gonorrhoeae* isolates using MLST (Jolley and Maiden, 2010). A comparison was made between the proportion of isolates within MLSTs to the overall proportion of expected isolates within the total population, based on different datasets. A two proportion z-test with a 95% confidence level was used to determine over- and under-representations of particular datasets compared to the overall population. Statistical significance was then calculated based on a normal distribution using a 2-tailed z-test, where $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***) and $p \leq 0.0001$ (****).

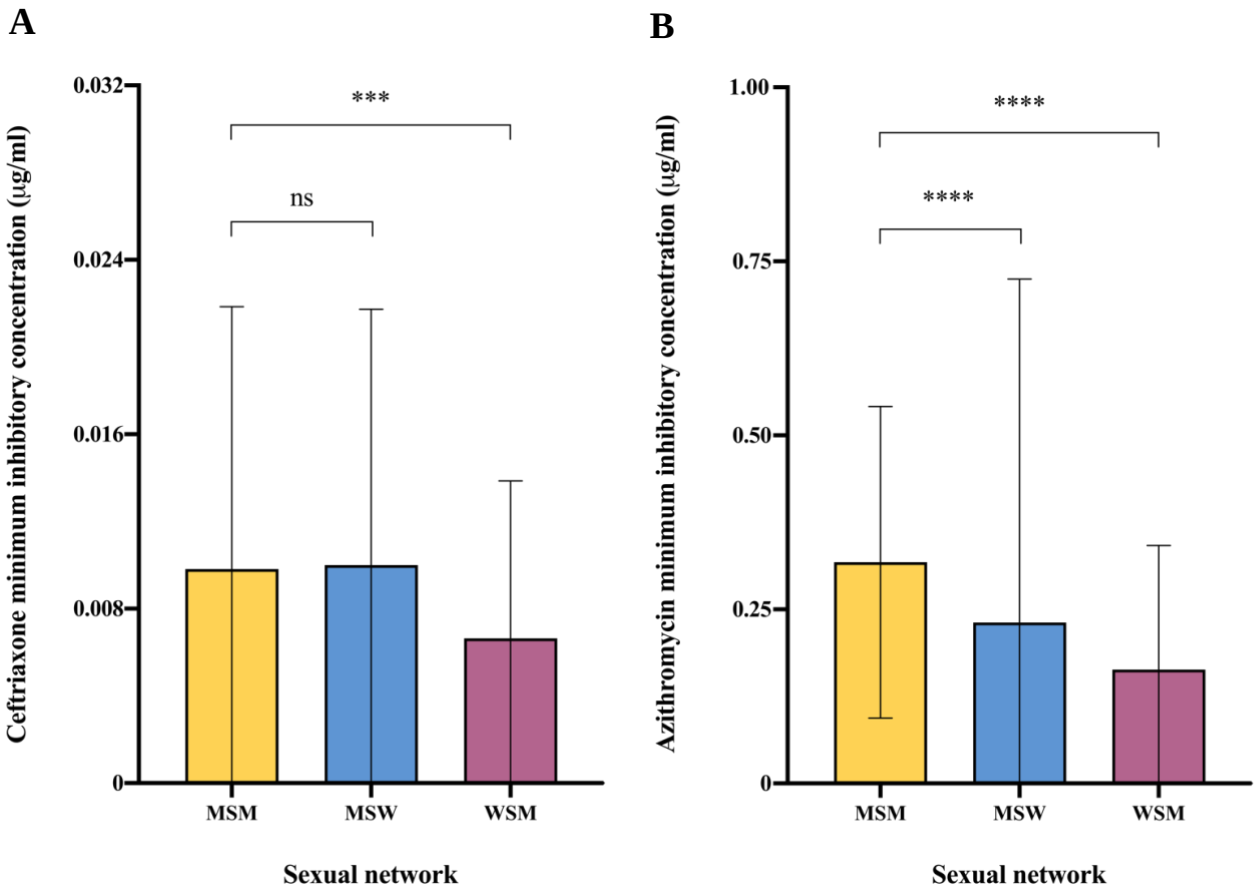


Figure 3.8 Minimum inhibitory concentration of antibiotics against isolates within different sexual networks. Comparison of the minimum inhibitory concentrations (MICs) of **A)** ceftriaxone and **B)** azithromycin between different sexual networks; men who have sex with men (MSM), men who have sex with women (MSW) and women who have sex with men (WSM). Statistical significance was measured using a Mann-Whitney test, where $p \leq 0.001$ (***) and $p \leq 0.0001$ (****).

more resistant than any previously described isolates. It had been thought to be caused by a new *penA* mosaic allele. This MLST was over-represented amongst isolates with high ceftriaxone MIC and over-represented within isolates from the MSM community when compared to the whole population of 354 isolates. This was similar to results from the US Gonococcal Isolate Surveillance Project (GISP) and the UK GRASP that both found that ceftriaxone resistance is strongly associated with MSM communities (Kirkcaldy *et al.*, 2015, Town *et al.*, 2017, Ison *et al.*, 2013). In addition, there was a significant increase in azithromycin MIC relating to MSM sexual network compared to both the MSW and WSM sexual networks (Figure 3.8.B). This included MLSTs previously mentioned, such as ST11864 and ST9363, that were both over-represented within isolates from the MSM community, and they had increased azithromycin MICs. Similarly, the US GISP found significantly higher geometric mean MICs of azithromycin in MSM than heterosexual males (Kirkcaldy *et al.*, 2015). Overall, this correlation suggests that the higher risk sexual behaviour that is generally observed in MSM communities, facilitates the persistence of STDs within the population and assists the spread of isolates with higher resistance to antibiotics.

In contrast, isolates on the right of the tree have a lower MIC to both or one of the antibiotics. Closer inspection of these isolates showed a correlation with the female gender category. There was a decrease in antibiotic MIC relating to the WSM compared to the MSM (Figure 3.7.A and B). This included MLSTs previously mentioned, such as ST1594, ST11990, ST8163 and ST8135, that were over-represented amongst isolates with low MICs to both or one of the antibiotics, and to the WSM sexual network, when compared to the whole population. MLST ST1594 has previously been documented as typically being gonococci susceptible to antibiotics (Ilina *et al.*, 2010). These MLSTs also demonstrated an over-representation within isolates derived from the cervix, suggesting that these isolates are better adapted to infecting and residing in the female genital tract compared to other female or male body sites. Of the few males

found interspersed within these MLSTs, there was an over-representation of isolates derived from the MSW network suggesting that these isolates are mainly circulating within heterosexual individuals, rather than the MSM community. Transmission from females to males is more complicated when compared to the transmission from males to their partners. This is due to numerous factors, including desialylation of the gonococcus required to facilitate successful transmission (Ketterer *et al.*, 2016). Therefore these observations could suggest that this cluster of isolates might have an adaptation advantage for transmitting from females to males. The same link between cervical isolates and increase antibiotic susceptibility had also been found by Ma *et al.* (2020). After an in depth analysis of this correlation, they identified an over-representation of loss of function mutations in the efflux pump mtrCDE operon in the cervical isolates, which increased their susceptibility to antibiotics. This suggests the bacteria experience environmental pressures whilst inhabiting the female genital tract which result in the expression of these pumps incurring a fitness cost on the bacterium.

Of these four MLSTs (ST1594, ST11990, ST8163 and ST8135), an over-representation of asymptomatic infection were found in three; one MLST over-represented for no genital discharge and two over-represented for no dysuria. The remaining MLST, ST1594, in contrast was over-represented for presenting with dysuria. However, this MLST was also over-represented amongst isolates co-inhabiting with chlamydia. Eight of the 13 participants reporting dysuria also had chlamydia, of which dysuria is also a symptom. Therefore, it cannot be concluded that the symptoms were directly caused by the gonococcal infection. Removing this isolate as a potential anomaly would indicate that there would actually be an over-representation of no symptoms amongst this MLST, more comparable to the rest of the MLSTs in this lineage. This is confirmed in the literature that reports that on average 50% of women do not present with any symptoms during gonococcal infection (Martin-Sanchez *et al.*, 2020).

In addition to these observations, it might be of interest to note that of these heterosexual

isolates in the bottom right portion of the tree, 63% (50/80) all have *tbpB* allele 27 annotated from NG-MAST analysis (Figure 3.2). TbpB encodes a transferrin binding protein that enables efficient iron transport from human transferrin, therefore this might suggest that this allele is an advantage for the bacterium when acquiring iron in a female specific environment.

To further expand the analysis of correlating each different unit of metadata with the WGS data undertaken in this Chapter, in future it might be more useful to reanalysis the data in the form of a multivariant analysis. This might reveal more in depth correlations between many different variables that would not have been picked up when doing the univariate analysis.

The main reason for sequencing the isolates had been to determine whether there was an association with the genomics and the susceptibility to isolates to serum. In order to investigate whether there is a specific genetically related group that stimulate the production of inhibitory antibodies. Therefore, the genomics will be further analysed in Chapter 4, after serum susceptibility analyses are complete.

CHAPTER 4

SERUM RESISTANCE OF *NEISSERIA GONORRHOEAE* INCITED BY INHIBITORY ANTIBODIES

4.1 Introduction

Antibodies are an essential part of the human immune system that facilitate the elimination of pathogens. Despite this, it has been well documented that some antibodies have instead appeared to prevent these bactericidal antibodies from causing complement-mediated killing of pathogens, termed 'inhibitory antibodies'. This has been described for many different Gram-negative bacteria, including *Brucella* species. (Glenchur *et al.*, 1959, Hall *et al.*, 1971), non-typhoidal *Salmonella* (MacLennan *et al.*, 2010, Adler, 1953, Waisbren and Brown, 1966), *P. aeruginosa* (Guttman and Waisbren, 1975, Wells *et al.*, 2014), *E. coli* (Taylor, 1972, Coggon *et al.*, 2018), *N. meningitidis* (Griffiss and Bertram, 1977, Griffiss, 1975) and many others.

It has previously been reported that *N. gonorrhoeae* strains acquired from DGI patients are sensitive to the autologous serum, but are resistant to some healthy serum. It was found that these healthy sera contained IgG antibodies against outer membrane protein Rmp (Rice *et al.*, 1986) that blocked bactericidal antibodies against LOS and Por from causing complement-mediated killing. This same inhibitory effect had not been seen for IgM or IgA (McCutchan *et al.*, 1978). Joiner *et al.* (1985) also found enhanced C3 complement deposition on these DGI strains where it covalently associated with the inhibitory IgG, resulting in deposition at non-bactericidal sites.

These previous studies describe inhibitory antibodies in healthy serum against DGI *N. gonorrhoeae* strains. The primary aim of this thesis was to determine whether gonococci can induce the formation of inhibitory antibodies by their host, during acute gonococcal infection. If so, such clinical isolates should be resistant to killing by the sera of the hosts from which they were isolated. Therefore, a serum bactericidal assay (SBA) was used as a screen to detect potential candidates for possessing inhibitory antibodies. The large clinical isolate collection from the G-ToG clinical trial (Ross *et al.*, 2019) will be used during investigations, along with autologous participant serum collected during active infection.

4.2 Results

4.2.1 Development of an assay to screen clinical gonococci for serum resistance

Wells *et al.* (2014) described an SBA for screening clinical isolates of *P. aeruginosa* for their ability to survive during incubation with the serum of the host from which they had been isolated. Using this protocol, a time course of killing of the serum sensitive laboratory strain *N. gonorrhoeae* MS11 had been attempted, whilst incubating with healthy control serum (HCS) made from a pool of eight healthy human volunteers with no previous history of gonococcal infection. This initial attempt was unsuccessful as no colonies were enumerated the next day, even from samples that had been incubated with the positive non-serum control (Figure 4.1.A). Therefore, several parameters were varied to modify the assay for clinical gonococci.

First the density of bacteria added to the serum was varied in the range of a final concentration of 1.5 x 10^7 cfu/ml to 7.5 x 10^7 cfu/ml (Figure 4.1.B-D). Suspensions of strain MS11 were added either to HCS or PBS as a positive non-serum control, to give a final volume of 50 µl. During incubation at 37°C, samples were serially diluted and plated onto GC agar before incubation (0 hours), and every hour for 3 hours. In contrast to the PBS control culture, which survived the 3 hour incubation, an inoculum of 1.5 x 10^7 cfu/ml was progressively killed by the serum (Figure 4.1.B), where no colonies were enumerated after the 3 hour incubation. Whereas higher final concentrations of bacteria oversaturated the HCS (Figure 4.1.C-D), and lower inocula were unable to survive in either HCS or the non-serum control samples (Figure 4.1.A). Therefore, after varying this parameter to modify the assay, it was concluded that a final inoculum concentration of 1.5 x 10^7 cfu/ml will be used in future experiments.

The second parameter to be optimised was the concentration of serum added to the assay. Pooled HCS was diluted with PBS to final concentrations of 10%, 20% or 50% (v/v), and suspensions of MS11 were added. Serial dilutions of samples were plated as previously described

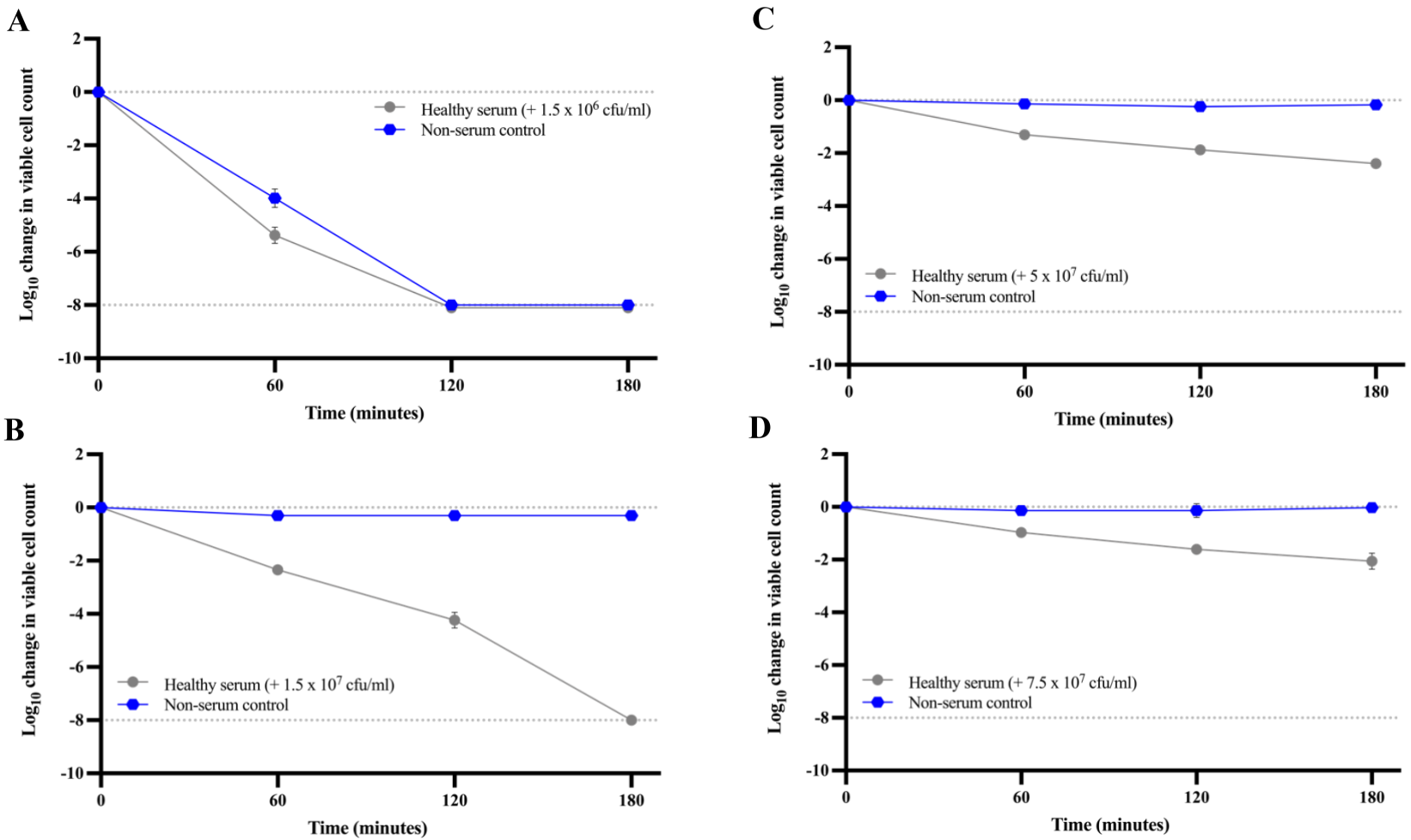


Figure 4.1 Serum bactericidal assay to trial different inoculum concentrations. A serum bactericidal assay (SBA) was used to investigate the effects of different inoculum concentrations on the survival of *N. gonorrhoeae* strain MS11 in HCS and a non-serum control. Final gonococcal inoculum concentrations were **A)** 1.5 x 10^6 cfu/ml, **B)** 1.5 x10^7 cfu/ml, **C)** 5 x 10^7 cfu/ml and **D)** 7.5 x 10^7 cfu/ml. The change in viable colony forming units after 60, 120 and 180 minutes in the HCS was measured, with negative values corresponding to a decrease in viable bacteria when compared to the initial inoculum. Values at -8 indicate the limit of detection for samples from which no colony formed even from the least diluted sample plated. Data shown are the mean and standard deviation from three independent experiments.

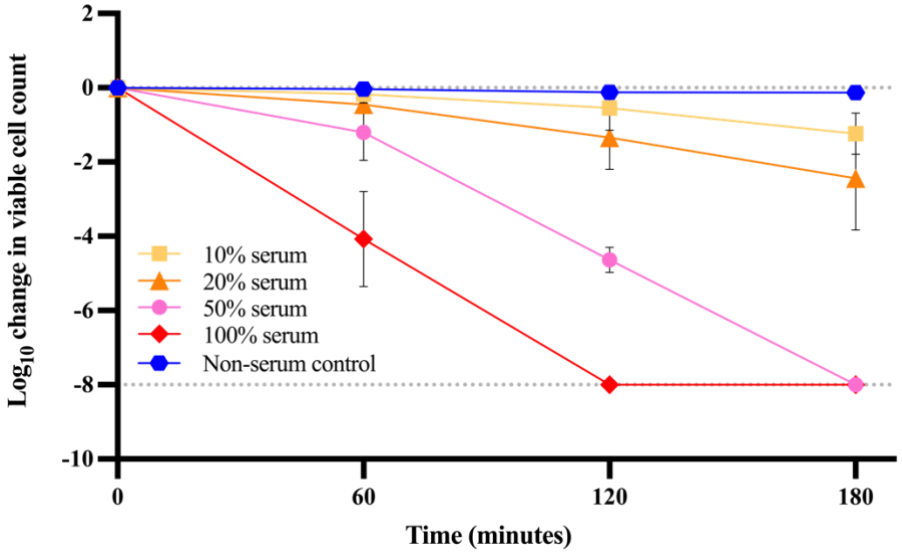


Figure 4.2 Serum bactericidal assay of serum titration. A serum bactericidal assay (SBA) was used to investigate the effects of different HCS concentrations; 100%, 50%, 20% and 10%, on the survival of *N. gonorrhoeae* strain MS11. The change in viable colony forming units after 60, 120 and 180 minutes in the HCS was measured, with negative values corresponding to a decrease in viable bacteria when compared to the initial inoculum. Values at -8 indicate the limit of detection for samples from which no colony formed even from the least diluted sample plated. Data shown are the mean and standard deviation from three independent experiments.

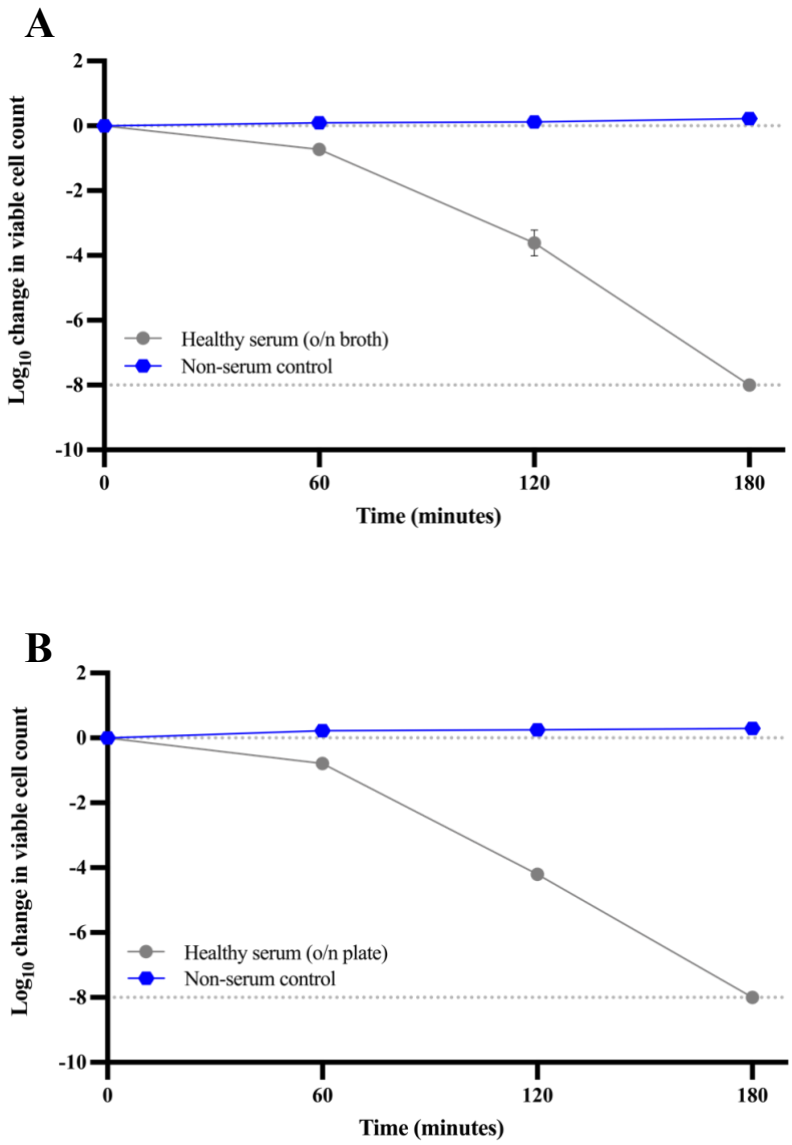


Figure 4.3 Serum bactericidal assay using different growth cultures. A serum bactericidal assay (SBA) was used to trial *N. gonorrhoeae* strain MS11 in HCS and a non-serum control using cultures grown **A)** overnight (o/n) in BHI broth and **B)** overnight on GC agar plates. The change in viable colony forming units for MS11 after 60, 120 and 180 minutes in the HCS after serum bactericidal assay completion was measured with negative values corresponding to a decrease in viable bacteria when compared to the initial inoculum. Values at -8 indicate the limit of detection for samples from which no colony formed even from the least diluted sample plated. Data shown are the mean and standard deviation from three independent experiments.

to determine the viable count before incubation (0 hours) and every hour for 3 hours of incubation at 37°C. Results found that bacterial survival increased with decreasing concentration of HCS in the assay (Figure 4.2). Here, a serum concentration of 50% was the lowest concentration required for killing of sensitive strain MS11, therefore this concentration will be used in future assays.

The third parameter to be optimised was the source of inoculum. Cultures of MS11 were grown overnight on GC agar plates or overnight in BHI broth, and suspensions were added either to HCS or PBS as a non-serum control. Serial dilutions of samples were plated as previously described to determine the viable count before incubation and every hour for 3 hours of incubation at 37°C. Bacterial survival had been the same when using inoculum cultures from either source (Figure 4.3). Therefore, due to the difficulties of growing clinical gonococci in broth overnight, it was decided to use plate grown bacteria in future experiments.

4.2.2 Screening the gonococcal library for isolates resistant to host serum

Gonococcal isolates that had induced the production of inhibitory antibodies in a host should be resistant to killing by its host sera. As a first step to identify sera that might contain inhibitory antibodies, all the available gonococcal isolates were screened for resistance to the serum of the host from which it had been isolated. From the total 367 *N. gonorrhoeae* isolates in the G-ToG collection, only 336 were able to be assayed for various reasons (detailed in Table 2.1). Baseline serum was the focus of these assays, which was the serum taken from the participant's during active infection. After overnight growth on GC agar, cultures were incubated at 37ºC for 3 hours with the autologous host serum at a final concentration of 50% (v/v). As a positive non-serum control, PBS was added to additional cultures that were incubated under the same conditions. Serial dilutions of samples were plated to determine the viable count before incubation (0 hours) and every hour for 3 hours.

As expected, the majority of isolates were sensitive, with 84% (282/336) of the

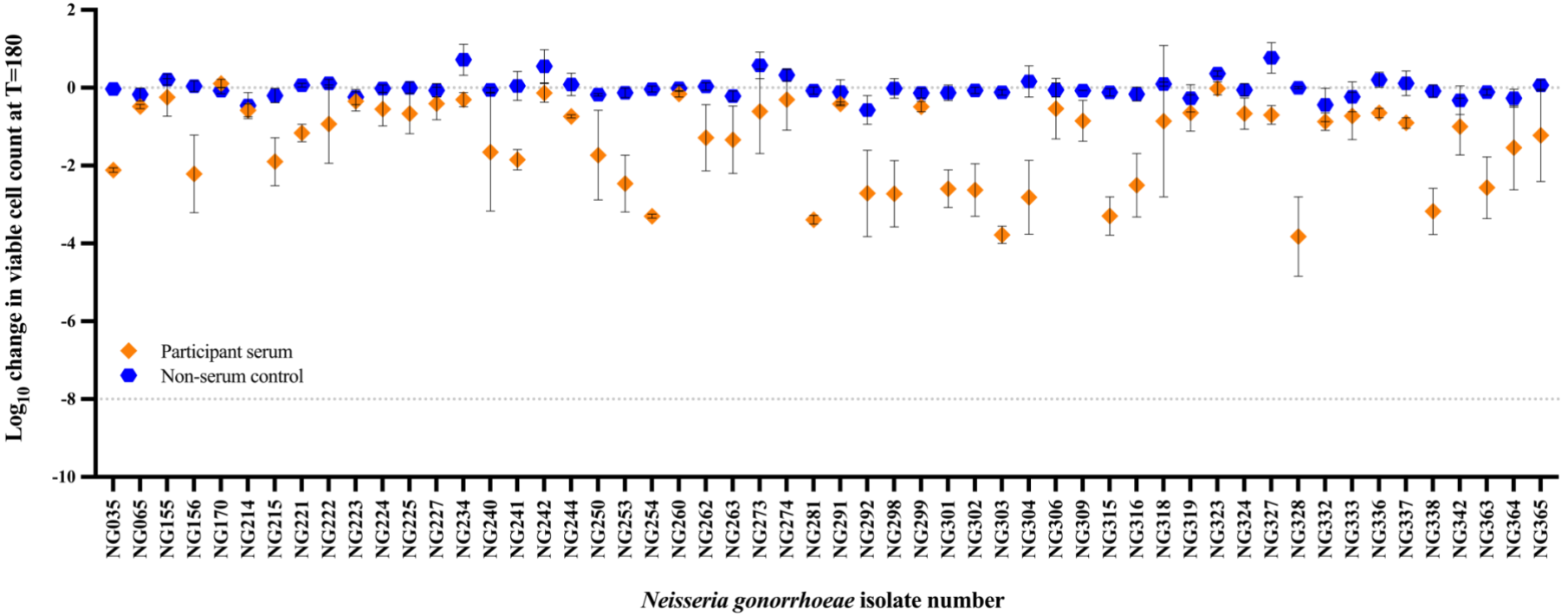


Figure 4.4 *Neisseria gonorrhoeae* clinical isolates that resist killing by autologous host serum. A serum bactericidal assay (SBA) was used to screen the 336 *N. gonorrhoeae* clinical isolates to determine the sensitivity in autologous host serum. Cultures of each isolate were incubated with either the autologous participant serum or a non-serum control. The change in viable colony forming units for 54 *N. gonorrhoeae* clinical isolates after 180 minutes in the autologous serum was measured, with negative values corresponding to a decrease in viable bacteria when compared to the initial inoculum. Values at -8 indicate the limit of detection for samples from which no colony formed even from the least diluted sample plated. Data shown are the mean and standard deviation from three independent experiments.

gonococcal isolates killed rapidly by the autologous host serum, as shown by a $\log_{10}$ kill of greater than 8 after 3 hours. In contrast, 16% (54/336) of the isolates were either completely resistant or killed less than a $\log_{10}$ of 8 after the 3 hour incubation period. The viable counts after incubation for 3 hours for all 54 surviving isolates are shown in Figure 4.4. Bacteria incubated in the non-serum control remained viable throughout the 3 hour incubation period.

4.2.3 Identification of isolates resistant to host serum in the presence of baby rabbit complement.

It was possible that degraded host complement enabled some of the 54 isolates to survive in the matching participant serum. To eliminate such isolates, all 54 resistant isolates were reassayed, but the participant serum was heat inactivated for 30 minutes at 56ºC and an exogenous complement source, baby rabbit complement (BRC) (AbD Serotec), was added. First it was necessary to determine the maximum concentration of BRC that enabled bacteria to survive incubation for 3 hours. Suspensions of serum sensitive isolate NG115 were incubated at 37ºC for 3 hours with various concentrations of BRC diluted with PBS. Bacteria were killed by BRC concentrations of 10% and above (Figure 4.5). In subsequent experiments, 5% (v/v) BRC was added. This is consistent with the concentration of complement used in other studies, for example, Christodoulides *et al.* (1998) used 5% guinea pig complement and Hung *et al.* (2011) also used 5% BRC.

Next, the 54 isolates were assayed for resistance in the presence of 5% BRC and 50% heat inactivated autologous participant serum. Eight isolates were killed by the participant serum, once an exogenous complement source had been added (Figure 4.6), suggesting the human complement in these participant sera could have been degraded during the serum collection process.

Overall, these preliminary SBAs resulted in 14% (46/336) of all isolates that were

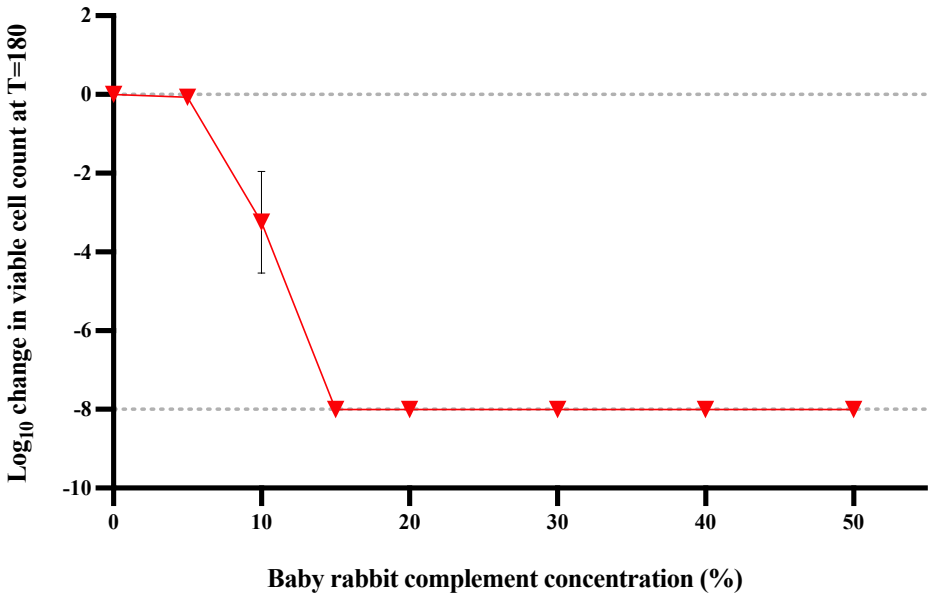


Figure 4.5 Serum bactericidal assay of a sensitive isolate in the presence of baby rabbit complement. A serum bactericidal assay (SBA) was used to investigate the effects of different baby rabbit complement (BRC) concentrations; 0% - 50% in PBS, on the survival of sensitive *N. gonorrhoeae* isolate NG115. The change in viable colony forming units after 180 minutes in the BRC was measured, with negative values corresponding to a decrease in viable bacteria when compared to the initial inoculum. Values at -8 indicate the limit of detection for samples from which no colony formed even from the least diluted sample plated. Data shown are the mean and standard deviation from three independent experiments.

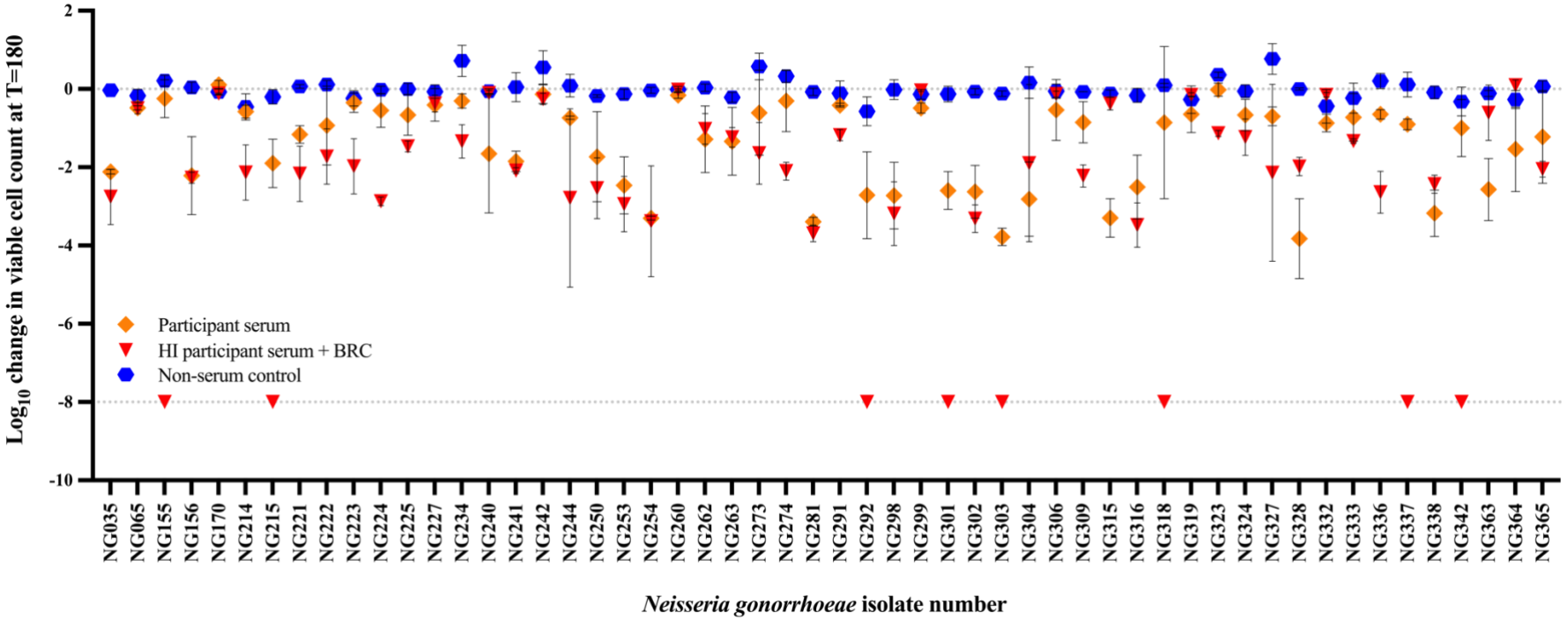


Figure 4.6 Effects of adding an exogenous complement source on isolates that resisted killing by autologous host serum. A serum bactericidal assay (SBA) was used to screen the 54 *N. gonorrhoeae* clinical isolates resistant to autologous participant serum to determine the effect of an addition of an exogenous complement source on survival. Cultures of each isolate were incubated with heat inactivated (HI) autologous participant serum and 5% BRC, autologous participant serum or a non-serum control. The change in viable colony forming units for 54 *N. gonorrhoeae* clinical isolates after 180 minutes in the different sera was measured, with negative values corresponding to a decrease in viable bacteria when compared to the initial inoculum. Values at -8 indicate the limit of detection for samples from which no colony formed even from the least diluted sample plated. Data shown are the mean and standard deviation from three independent experiments.

Neisseria gonorrhoeae isolate number	G-ToG clinical trial participant number	Participant location	Participant age	Participant gender	Participant ethnicity	Participant HIV status	Participant co-infections	Participant previous gonococcal infections	Isolate anatomical site of infection
NG035	7009	Sheffield (Royal Hallamshire)	34.8	male	White	negative	0	0	urethra
NG065	2017	London (St Bartholomew)	53.2	male	White	negative	0	12	rectum
NG156	7056	Sheffield (Royal Hallamshire)	37.3	male	White	positive	syphillis	0	unknown
NG170	3038	London (Guy's Hospital)	25.7	male	White	negative	0	0	pharynx
NG214	9018	London (Chelsea & Westminst)	25.1	male	White	positive	chlamydia	4	rectum
NG221	4071	Leeds (General Infirmary)	23.5	male	White	negative	0	0	urethra
NG222									rectum
NG223	4072	Leeds (General Infirmary)	30.2	male	White	negative	0	1	urethra
NG224	4075	Leeds (General Infirmary)	23.9	male	White	negative	0	1	urethra
NG225									rectum
NG227	4079	Leeds (General Infirmary)	23.2	female	White	negative	0	0	cervix
NG234	1129	Birmingham (Whittall St. Clinic)	34.4	male	Black	negative	0	2	urethra
NG240	1142	Birmingham (Whittall St. Clinic)	25.9	male	White	negative	0	0	urethra
NG241	1147	Birmingham (Whittall St. Clinic)	24.2	male	White	negative	chlamydia	0	urethra
NG242	1152	Birmingham (Whittall St. Clinic)	26.1	male	Asian	negative	chlamydia	0	urethra
NG244	1156	Birmingham (Whittall St. Clinic)	27.1	male	Asian	negative	0	0	urethra
NG250	1170	Birmingham (Whittall St. Clinic)	66.4	male	White	negative	chlamydia	1	urethra
NG253	10014	Brighton (Royal Sussex County)	49.7	male	White	negative	0	1	rectum
NG254	4084	Leeds (General Infirmary)	19.0	female	White	negative	0	0	rectum
NG260	4093	Leeds (General Infirmary)	44.0	male	White	negative	0	0	urethra
NG262	4099	Leeds (General Infirmary)	18.8	female	White	negative	0	0	cervix
NG263									pharynx
NG273	7079	Sheffield (Royal Hallamshire)	20.6	female	White	negative	0	0	cervix
NG274									rectum
NG281	7089	Sheffield (Royal Hallamshire)	33.0	male	White	negative	0	1	urethra
NG291	13010	Reading (Royal Berkshire)	40.3	male	White	positive	0	6	rectum
NG298	2057	London (St Bartholomew)	45.9	male	White	positive	0	1	urethra
NG299	2058	London (St Bartholomew)	23.2	male	Black	positive	0	1	rectum
NG302	2062	London (St Bartholomew)	22.5	male	White	negative	0	1	urethra
NG304	2070	London (St Bartholomew)	38.8	male	White	negative	0	0	urethra
NG306	5026	Manchester (Royal Infirmary)	24.2	male	White	negative	0	0	urethra
NG309	8027	Southampton (University Hosp)	28.3	male	Black	positive	0	1	urethra
NG315	3077	London (Guy's Hospital)	17.8	male	White	negative	0	0	rectum
NG316	3082	London (Guy's Hospital)	49.0	male	White	positive	0	3	urethra
NG319	3090	London (Guy's Hospital)	21.3	male	Black	negative	0	0	urethra
NG323	11021	Coventry (Univ Hosp Coventry & Warw)	18.5	male	White	negative	0	0	urethra
NG324	11026	Coventry (Univ Hosp Coventry & Warw)	24.6	male	Asian	negative	0	0	urethra
NG327	11033	Coventry (Univ Hosp Coventry & Warw)	28.3	male	White	negative	0	0	urethra
NG328	11035	Coventry (Univ Hosp Coventry & Warw)	57.4	male	White	positive	0	1	urethra
NG332	12005	London (Royal Free Trust)	20.0	male	Other	negative	chlamydia	0	urethra
NG333									unknown
NG336	15007	London (Chelsea & Westminst)	29.2	male	White	unknown	0	4	urethra
NG338	14024	London (St Mary's)	35.6	male	White	positive	0	1	pharynx
NG363	14008	London (St Mary's)	31.9	male	Mixed	negative	chlamydia	0	pharynx
NG364									urethra
NG365	14010	London (St Mary's)	26.0	male	Black	negative	0	1	urethra

Table 4.1 Participant demographics of isolates resistant to autologous host serum. Participant information, including gender, HIV status and previous gonococcal infection, for the 40 participants from which the 46 resistant *N. gonorrhoeae* isolates were derived from.

genuinely resistant in the autologous participant serum and therefore these will be of interest going forward in subsequent experiments to identify sera which might containing inhibitory antibodies. Clinical participant information regarding the 46 resistant isolates, including gender, HIV status and previous gonococcal infections, are shown in Table 4.1. Initial observations of this clinical data showed that there were no obvious correlations between the epidemiological data between the participants from which these resistant isolates were isolated.

4.2.4 Development of an assay to measure antibody and complement binding

The preliminary SBA screen of all isolates in the G-ToG collection resulted in 46 isolates that were resistant in the autologous participant serum. These sera were therefore of interest going forward for potentially containing inhibitory antibodies. Many inhibitory antibody studies so far differentiated sera that contained inhibitory antibodies by identifying those containing extremely high antibody titers in the serum (Wells *et al.*, 2014, MacLennan *et al.*, 2010) binding to the bacteria. Wells *et al.* (2014) described a flow cytometric assay for measuring the binding of antibodies IgG, IgM and IgA and complement components C1, C3 and C5b-9 from host serum to clinical isolates of *P. aeruginosa*. In their assay, binding of six different serum components were measured in separate experiments using a FITC conjugated secondary antibody for each component.

To determine whether the 46 candidate sera in this study had the same phenotype, a similar flow cytometry assay was set up measure five different serum components, including the main antibodies found in serum, IgG, IgM and IgA, and complement C3c to show complement had been initiated, and complement C5b-9 to confirm MAC complex formation. Initial experiments were designed to determine whether it was possible to measure the binding of these five serum components to *N. gonorrhoeae* simultaneously in a multi-colour flow cytometric assay. This would facilitate a detailed overview of multiple serum protein interactions with each

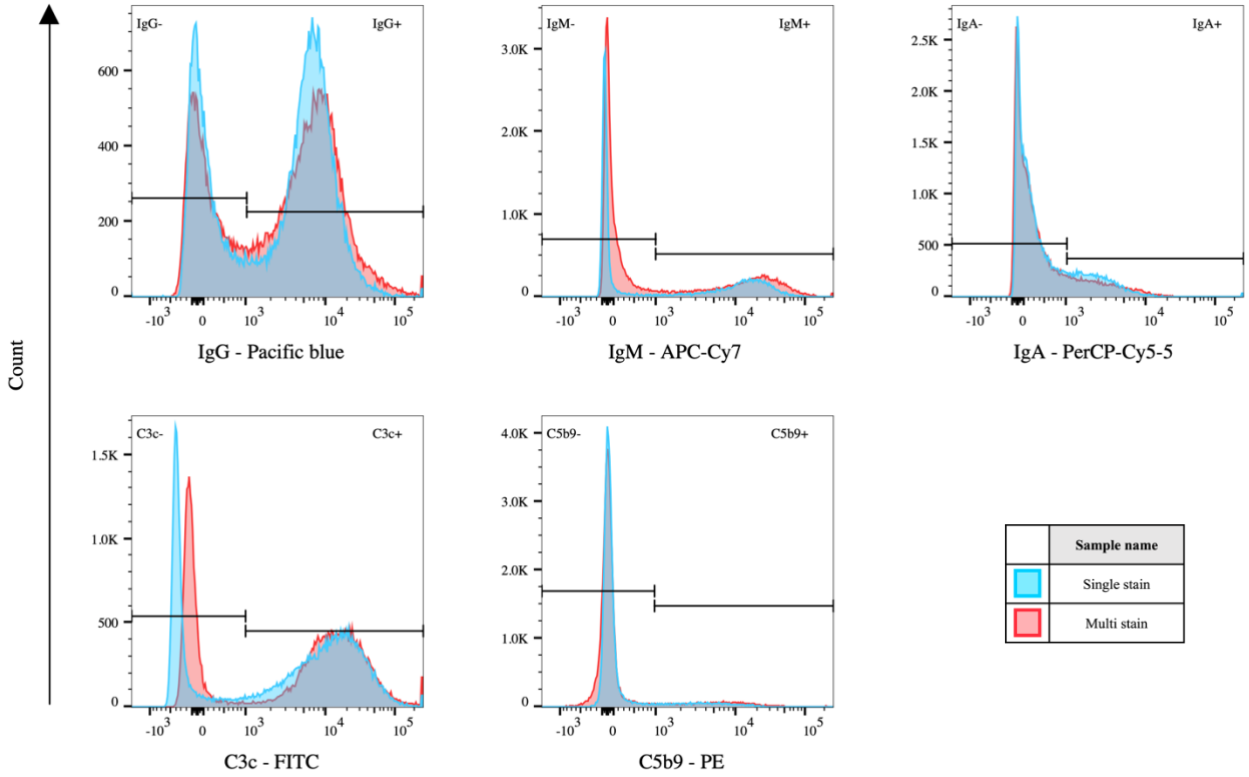


Figure 4.7 Flow cytometry assay for comparing single and multi-component stains. A flow cytometric assay was used to investigate the effects of a single and multi-component secondary antibody stain on the binding of antibody and complement to *N. gonorrhoeae* MS11. Raw flow cytometric histogram plots for each of the five fluorescent channels represent binding of each individual serum proteins, IgG, IgM, IgA, C3c and C5b-9. For each individual fluorochrome, the different stain conditions are overlaid. Each channel was gated based in control samples (block horizontal lines) showing positive and negative signals.

bacterium, which would be a more representative demonstration of *in vivo* interactions. Once the multi-colour fluorochrome panel had been designed (detailed in Section 2.4.1), it was necessary to optimise several parameters before the assay could be applied to the clinical isolates.

To confirm the secondary antibodies were all anti-human, a flow cytometric assay was ran using fetal bovine serum (FBS) in the place of participant serum. This allowed for measurement of binding of secondary antibodies to any of the five serum components that bound that bacterium from FBS. No positive signal was detected from these samples (Appendix IV). Therefore, this confirms that the secondary antibodies do not interact with non-human serum components, and also shows that these secondary antibodies are not binding the bacterium.

To ensure that it was possible to develop a multi-colour assay, data from the five-stain multi-colour assay were compared with results from five individually stained samples. After overnight growth on GC agar, cultures of *N. gonorrhoeae* MS11 were incubated with HCS for 20 minutes at 37°C. As a negative control, PBS was added to additional cultures that were incubated under the same conditions. After the bacteria had been washed, five secondary antibodies for each serum component to be measured were added to one sample and each secondary antibody was added individually to five separate samples. After incubation for 20 minutes at room temperature, cells were washed again and fixed ready for the cytometer. The overall profiles of the raw flow plots for each fluorochrome from the individually stained samples compared to the multi-colour assay were highly similar (Figure 4.7), therefore they will be combined for future assays.

To develop the multi-colour assay further, the first parameter to be optimised was the concentration of fluorochrome conjugated secondary antibody added to the assay. Neat antibody (1 mg/ml) was diluted with PBS to final concentrations of 0.5 mg/ml, 0.25 mg/ml or 0.125 mg/ml. Samples were set up as previously described and all five secondary antibodies at each concentration were added to separate samples. Raw flow plots from the cytometer showed that

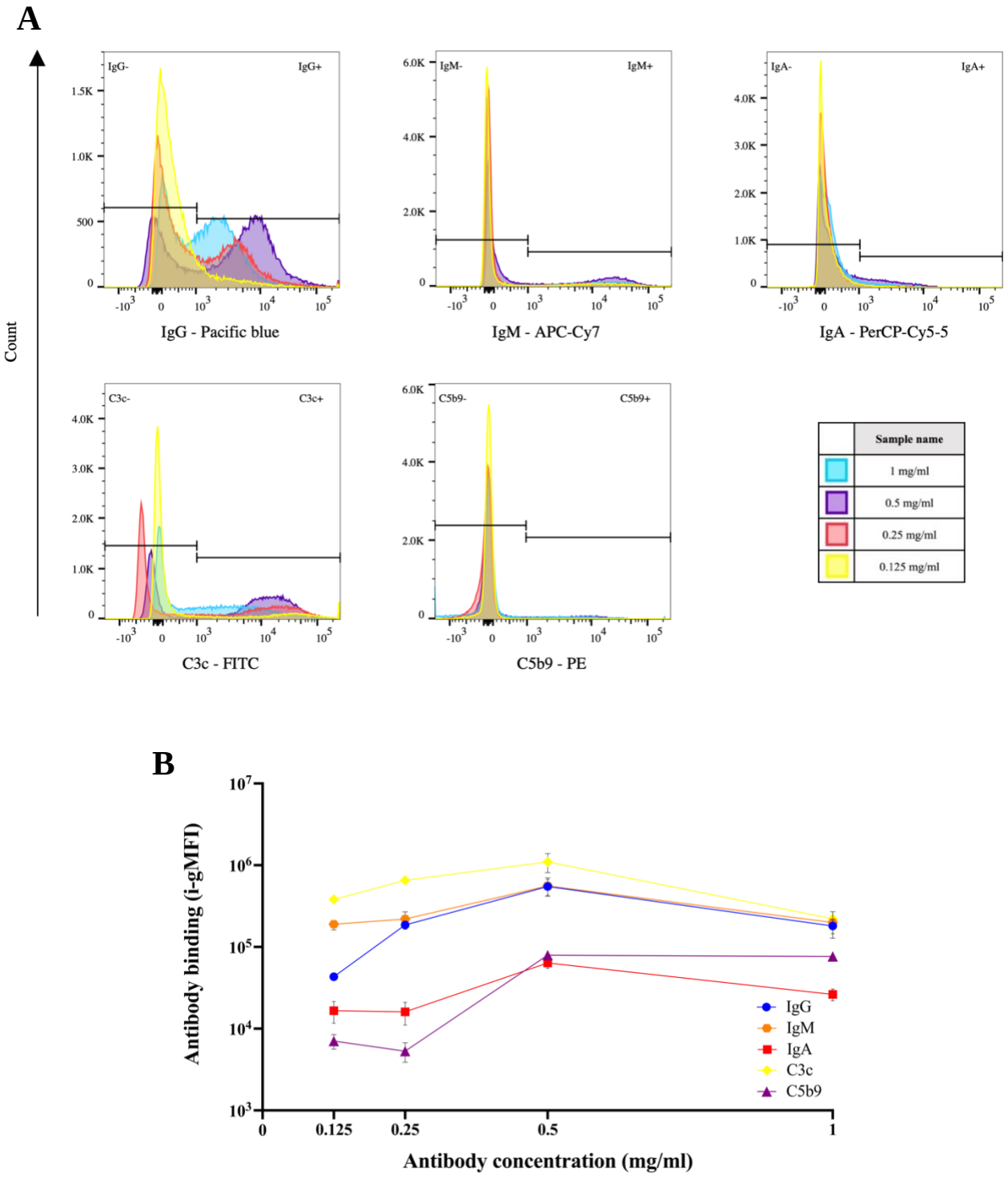


Figure 4.8 Effect of different concentrations of secondary antibody on fluorescence intensity. A flow cytometric assay was used to investigate the effects of different concentrations of secondary antibody on the binding of antibody and complement to *N. gonorrhoeae* MS11. **A)** Raw flow cytometric histogram plots for each of the five fluorescent channels represent binding of each individual serum proteins. For each individual fluorochrome, the different secondary antibody concentrations are overlaid. Each channel was gated (block horizontal lines) showing positive and negative signal based on negative control samples. **B)** The change in integrated geometric mean fluorescence intensity (i-gMFI) for each serum protein was measured at increasing concentrations of secondary antibody. Data shown are the mean and standard deviation from three independent experiments.

as the concentration of the secondary antibodies increased, the overall amount of signal increased (Figure 4.8.A). However, at the highest concentration of 1 mg/ml, the signal decreased in all samples and the fluorescence peak shifted to the left. After calculating the overall effect of increasing the secondary antibody concentration on the integrated geometric mean fluorescence intensity (i-gMFI), the linear response had been lost at a concentration higher than 0.5 mg/ml (Figure 4.8.B), suggesting that the secondary antibodies were at an excessively high and over saturating concentration. This is referred to as a prozone effect. Therefore, a secondary antibody concentration at 0.5 mg/ml was used in subsequent experiments.

The second parameter to be optimised was the concentration of serum added to the assay. Pooled HCS was diluted with PBS to final concentrations of 10%, 20% or 50% (v/v). Prepared bacteria were assayed as described previously, with each sample incubated it a different serum concentration. Raw flow plots from the cytometer showed that as the concentration of serum increased, the overall amount of signal increased (Figure 4.9.A). However, at the highest concentration of 100%, the signal decreased in all samples with peak shifts down and some to the left. After calculating the overall effect on increasing serum concentration on the i-gMFI, the linear response had been lost at a concentration higher than 50% (Figure 4.9.B), suggesting another prozone effect. Therefore, a serum concentration of 50% was used in subsequent experiments.

In all of the assays shown above, a proportion of the bacteria failed to bind the secondary antibody. The temperatures at which the cultures were incubated with the serum and secondary antibodies were therefore varied to see whether it had an effect on the large negative population in the samples. After overnight growth on GC agar, cultures of *N. gonorrhoeae* clinical isolate NG227 were incubated with HCS for either 20 minutes at 37°C (condition 1), 10 minutes at 37°C and then 10 minutes at 4°C (condition 2) or 20 minutes at 4°C (condition 3). After the bacteria had been washed, all five secondary antibodies were added to each sample and incubated for 20

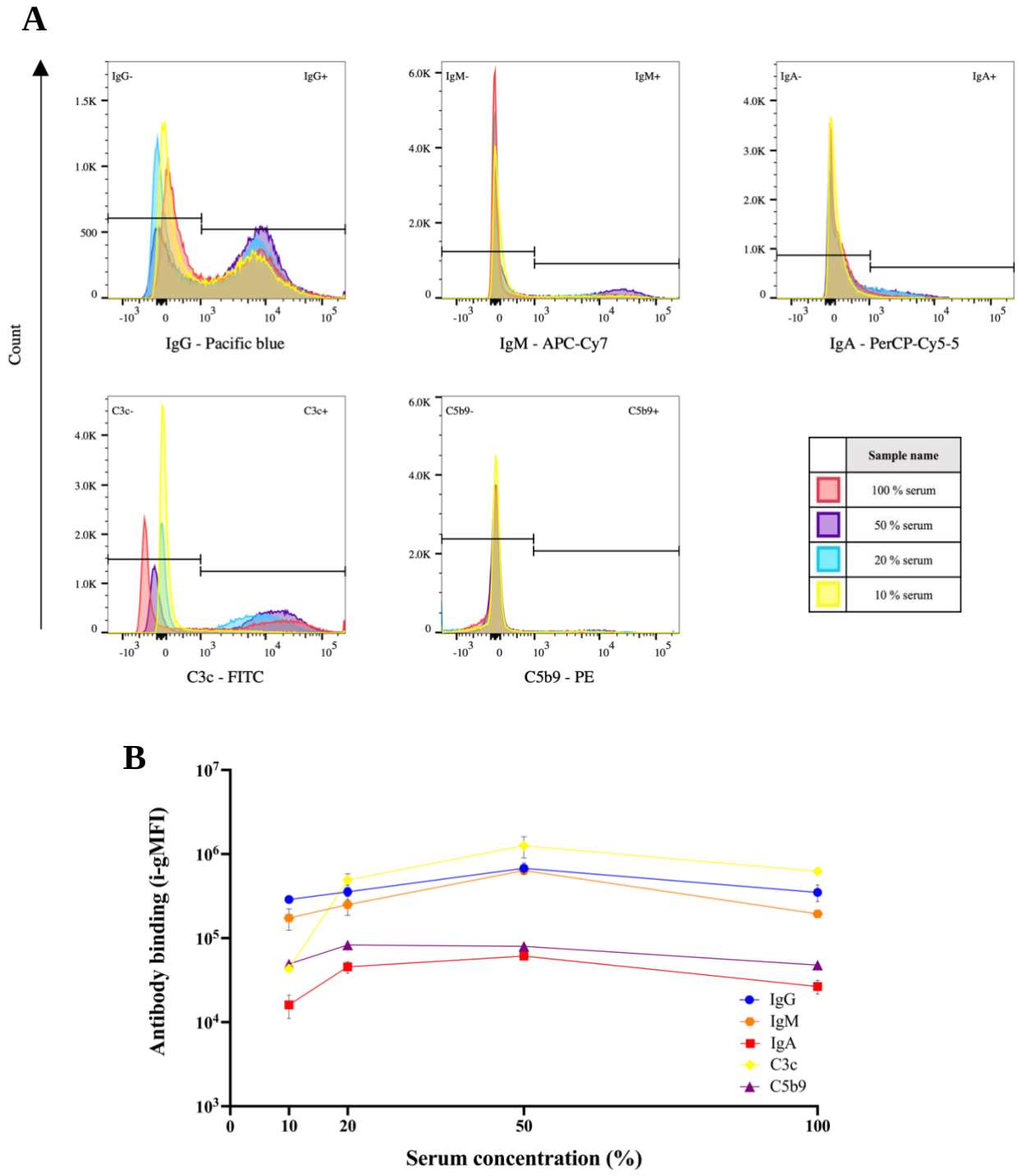


Figure 4.9 Effect of different concentrations of serum on fluorescence intensity. A flow cytometric assay was used to investigate the effects of different concentrations of serum on the binding of antibody and complement to *N. gonorrhoeae* MS11. **A)** Raw flow cytometric histogram plots for each of the five fluorescent channels represent binding of each individual serum proteins. For each individual fluorochrome, the different serum concentrations are overlaid. Each channel was gated showing positive and negative signal based on negative control samples. **B)** The change in integrated geometric mean fluorescence intensity (i-gMFI) for each serum protein was measured at increasing concentrations of serum. Data shown are the mean and standard deviation from three independent experiments.

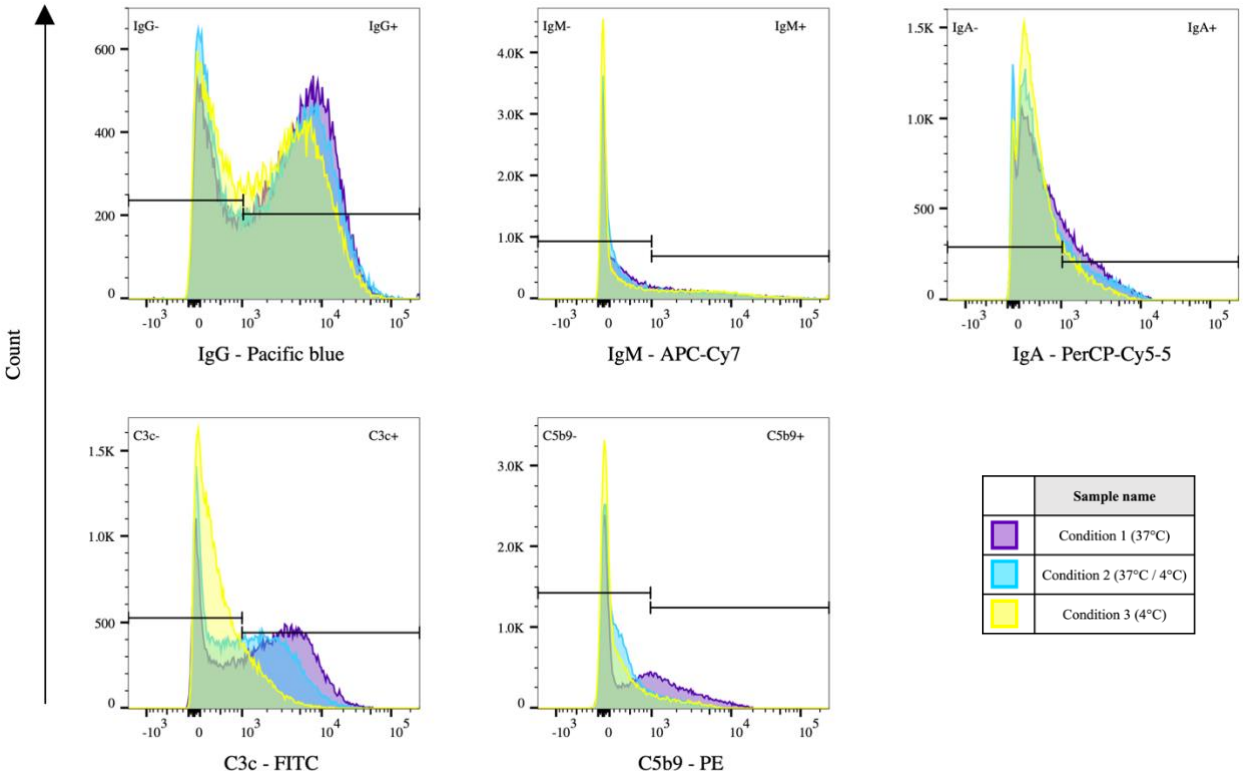


Figure 4.10 Effect of different incubation temperatures on secondary antibody binding. A flow cytometric assay was used to investigate the effects of different incubation temperatures on the binding of antibody and complement to *N. gonorrhoeae* clinical isolate NG227. Raw flow cytometric histogram plots for each of the five fluorescent channels represent binding of each individual serum proteins. For each individual fluorochrome, the different stain conditions are overlaid. Each channel was gated showing positive and negative signal based on negative control samples.

minutes at room temperature (condition 1) or at 4°C (condition 2 and 3). Cells were washed again and fixed ready for the cytometer. Raw flow plots from the cytometer showed that at lower incubation temperatures, the signal intensity decreased (Figure 4.10). The amount of antibody binding was similar at all temperatures, however a large reduction in signal had been seen for complement binding as the temperatures decreased. This was not an unexpected observation, as complement is only active at 37ºC. Therefore, temperature condition 1 was used in subsequent experiments.

In conclusion, based upon the optimisation of these parameters, a serum concentration of 50% and a secondary antibody concentration at 0.5 mg/ml was used in subsequent experiments. Lower concentrations resulted in lower positive signals, suggesting that saturation of protein binding had not been achieved. Higher concentrations oversaturated the assay and therefore a decrease in signal had been observed due to a prozone effect. As the sample incubation temperature had little effect on the proportion of bacteria that failed to bind the secondary antibodies, the original incubation protocol (condition 1) was used for all subsequent experiments.

4.2.5 Binding of individual serum proteins to resistant and sensitive isolates

To understand more about the ability of the 46 isolates to survive in the autologous host serum, experiments were designed to determine the binding titers of different proteins from the matching serum, with particular interest in antibodies IgG, IgM and IgA and complement components C3c and C5b-9. Another set of 46 isolates that were sensitive to the autologous host serum were also included to allow resistant and sensitive isolates to be compared. To decrease any risk of bias in the selection of this control group, sensitive isolates were chosen to mirror the participant demographic of the resistant isolates. After overnight growth on GC agar, cultures were incubated with the autologous host serum, at a final concentration of 50%, for 20 minutes at

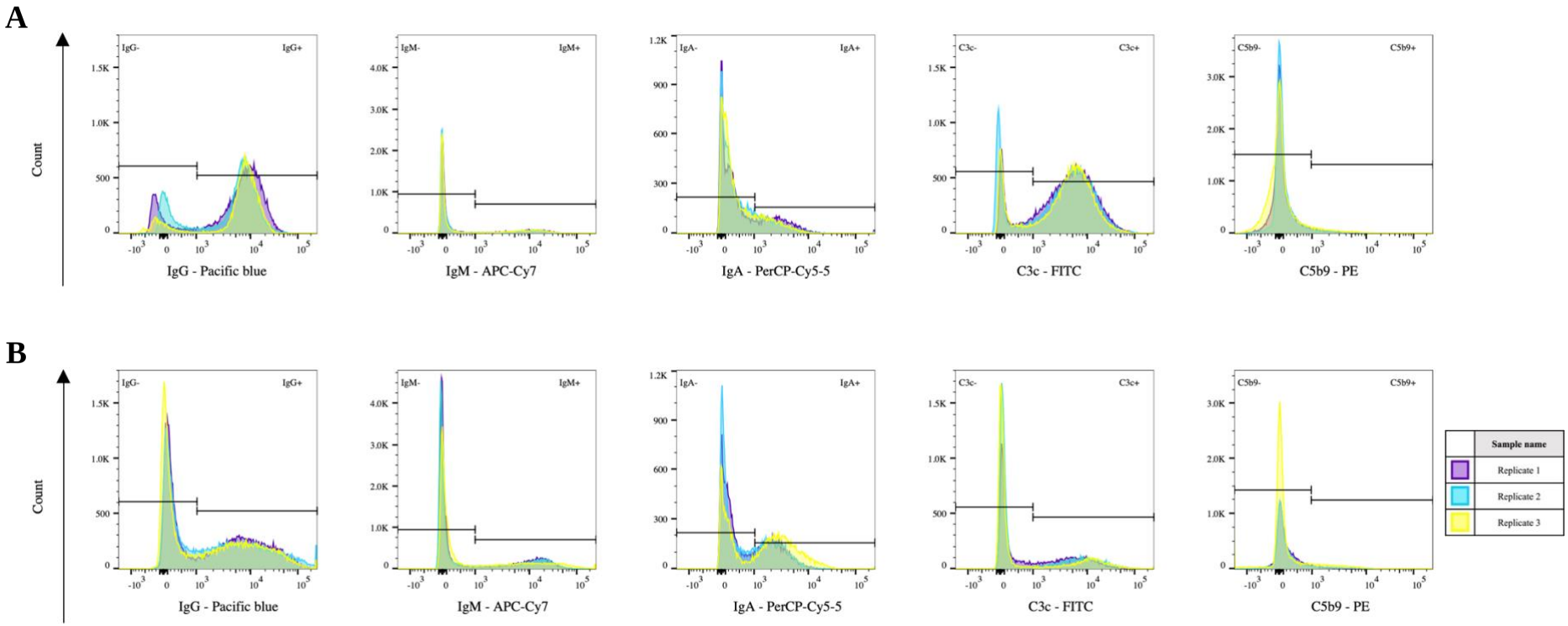


Figure 4.11 Replicates of serum components individually binding to a resistant and sensitive isolate. A flow cytometric assay was used to investigate the binding of antibodies IgG, IgM and IgA and complement C3c and C5b-9 to *N. gonorrhoeae*, **A)** serum resistant isolate NG263 and **B)** serum sensitive isolate NG069. Raw flow cytometric histogram plots for each of the five fluorescence channels represent binding of each individual serum protein. Data shown are from three independent experiments, with each overlaid for each individual fluorochrome.

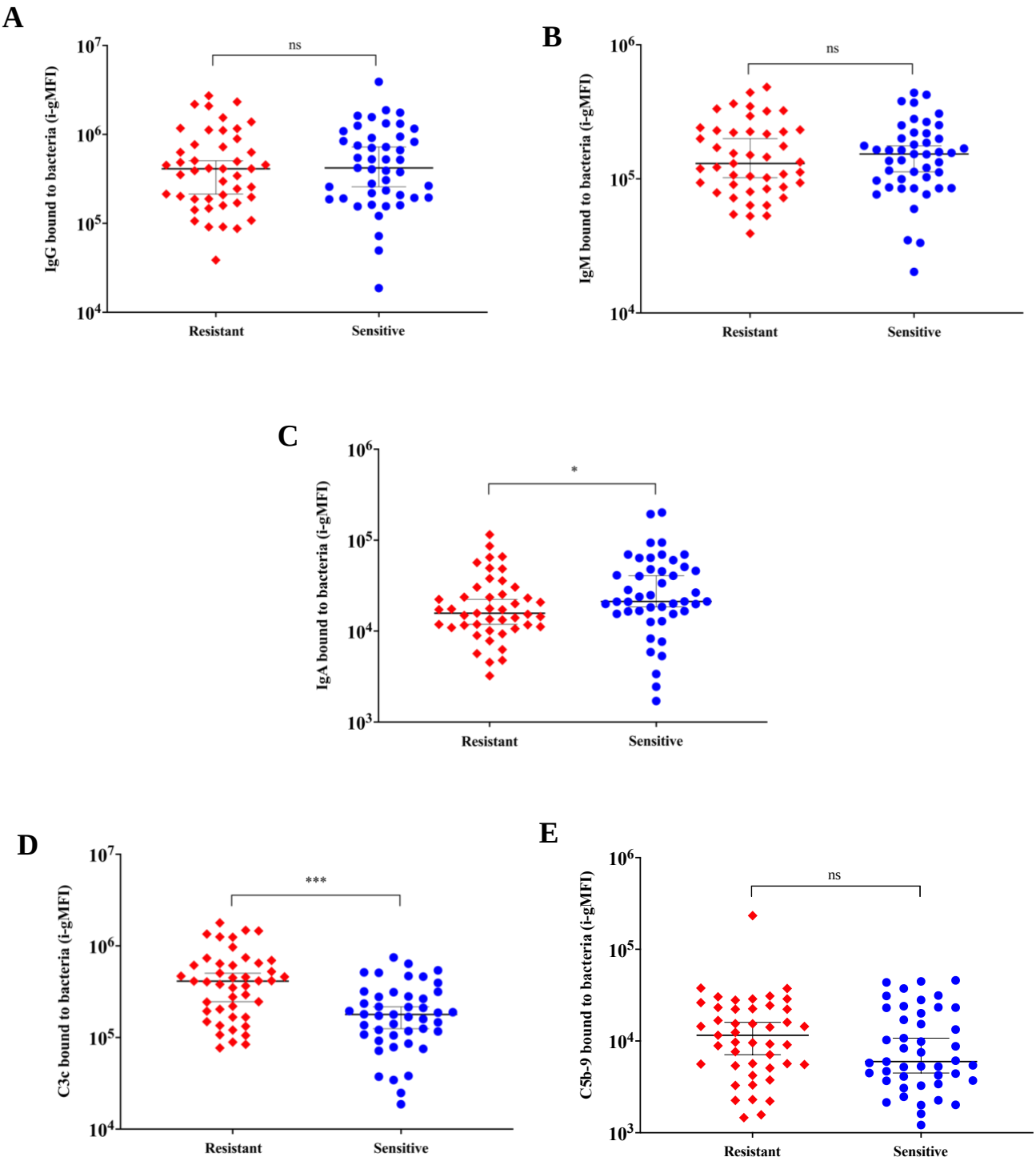


Figure 4.12 Binding of serum components individually to all resistant and sensitive isolates. A flow cytometric assay was used to analyse antibody and complement binding to all 46 isolates resistant to autologous serum, and 46 isolates sensitive to autologous serum. Cultures of each isolate were incubated with autologous serum and binding of **A)** IgG, **B)** IgM, **C)** IgA, **D)** C3c and **E)** C5b-9 were measured by flow cytometry using fluorescently labelled secondary antibodies. Shown is the integrated geometric mean fluorescence intensity (i-gMFI) for each serum component. The median for each group was calculated and a 95% confidence interval (CI) was applied. Statistical significance was measured using a Mann-Whitney test, where * ($p \leq 0.05$) and *** ($p \leq 0.001$).

37°C. As a positive control, PBS was added to additional cultures that were incubated under the same conditions. Fluorochrome conjugated antibodies reactive to the five selected serum proteins were added. Binding of these different proteins to the bacterium during initial serum incubation was assayed by flow cytometry. Biological replicates (n=3) for an example resistant isolate and a sensitive isolate are shown in Figure 4.11. Raw flow cytometry plots show highly similar signals for each fluorochrome from each replicate. Although there are very slight differences between the data on each plot, the overall profiles from all five are highly similar between replicate assays. Therefore, as the replicates gave similar data, it is confirmed this was a reproducible assay.

For all 92 isolates, 46 resistant and 46 sensitive to autologous serum, bacteria positively labelled for each fluorochrome were gated and the percentage of positive cells (Appendix V) and gMFI (Appendix VI) for each serum component, IgG, IgM, IgA, C3c and C5b-9, were calculated. From these the i-gMFI was calculated, allowing the binding of each serum component between the two groups to be compared (Figure 4.12). The binding of antibodies IgG, IgM and IgA were similar for both the sensitive and resistant isolates. Similar results were also found for complement C5b-9 between the two groups. Surprisingly, the main difference between the two groups had been a statistically significant increase in the binding of complement component C3c to resistant isolates compared to sensitive isolates.

4.2.6 Comparison of resistant and sensitive isolates simultaneously binding IgG and C3c

Focussing on this increased binding of complement C3c to the resistant isolates, it had been observed that this increase correlated directly with the binding of antibody IgG to the same bacteria. In contrast, far less C3c bound to the sensitive isolates with the same amount of IgG binding as the resistant isolates, and therefore only a weak correlation had been observed between C3c and IgG binding to these sensitive isolates (Figure 4.13.A).

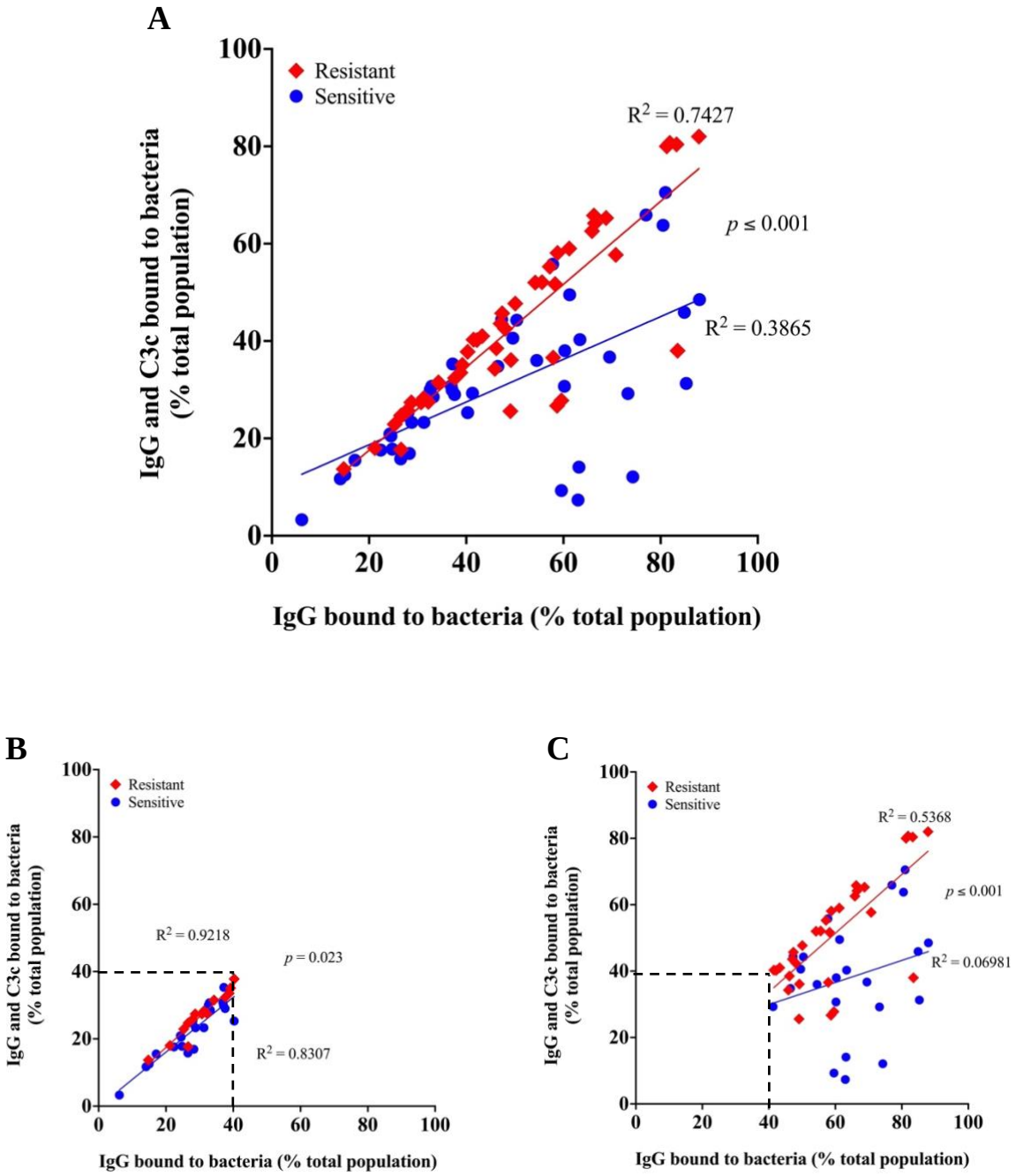


Figure 4.13 Comparison of all resistant and sensitive isolates simultaneously binding IgG and C3c. A flow cytometric assay was used to compare antibody and complement binding to 46 isolates resistant and 46 isolates sensitive to autologous serum. Cultures of each isolate were incubated with autologous serum and binding of IgG and C3c were measured by flow cytometry using fluorescently labelled secondary antibodies. Shown is **A)** the positive population of cells binding antibody IgG and C3c simultaneously compared to the positive population of cells binding antibody IgG. This has then been split to emphasise **B)** below 40% IgG positive populations, and **C)** above 40% IgG positive populations. Statistical significance was measured using an analysis of covariance (ANCOVA) test.

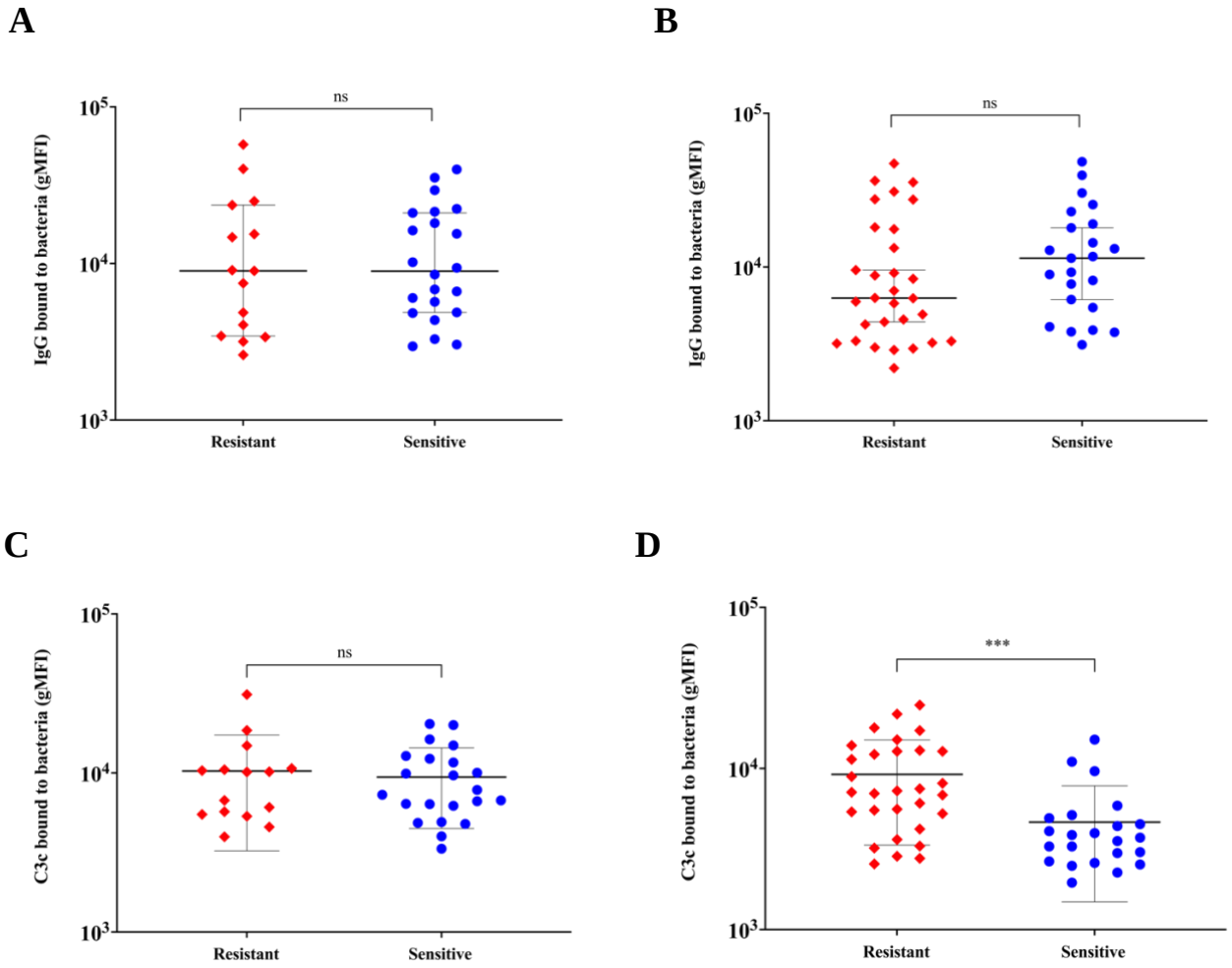


Figure 4.14 Comparison of gMFI between resistant and sensitive isolates simultaneously binding IgG and C3c, split into the above and below 40% IgG populations. A flow cytometric assay was used to compare antibody and complement binding to 46 isolates resistant to autologous serum, and 46 isolates sensitive to autologous serum. Cultures of each isolate were incubated with autologous serum and binding of IgG and C3c were measured by flow cytometry using fluorescently labelled secondary antibodies. Shown is the geometric mean fluorescence intensity (gMFI) comparing all resistant isolates and sensitive isolates for bound IgG; **A)** below 40% IgG positive populations and **B)** above 40% IgG positive populations, and bound C3c; **C)** below 40% IgG positive populations and **D)** above 40% IgG positive populations. The median for each group was calculated and a 95% confidence interval (CI) was applied, and statistical significance was measured using a Mann-Whitney test, where *** ($p \leq 0.001$).

This highly positive correlation between IgG and C3c bound to resistant isolates compared to sensitive isolates is more apparent when over 40% of the total population is binding IgG. In sensitive isolates when over 40% of the population bound IgG, on average only half of this IgG positive population bound C3c. In contrast, for the resistant isolates, the IgG positive population all also bound C3c. This split is depicted in Figure 4.13.B and C. The gMFI values, which indicated the amount of protein bound to each positive cell, for IgG and C3c for each group were calculated. There was no difference between each group (above and below 40% population binding IgG) between resistant and sensitive isolates when the gMFI for IgG had been compared (Figure 4.14.A and C). However, when the same comparison for C3c were made, an increased gMFI in resistant isolates in the over 40% IgG positive population group had been observed (Figure 4.14.D). Overall, on the IgG positive cells, the resistant isolates have an increased population binding C3c compared to sensitive isolates, and an increased gMFI per cell binding C3c compared to sensitive isolates. This result was unexpected as it would be anticipated that more complement C3c would have bound the sensitive isolates.

4.2.7 Comparison of resistant and sensitive isolates binding C5

The lower binding of complement C3c to the sensitive isolates could potentially have been a result of these isolates being further on in the complement cascade compared to the resistant isolates. To investigate this further, a complement component further in the pathway, C5, was analysed for binding to all isolates in both groups, sensitive and resistant to autologous serum. Flow cytometric assays were set up as previously described, but with a C5 antibody conjugated to FITC replacing the C3c-FITC conjugated antibody. For all isolates, the data were gated in the same way. This allowed the binding of C5 in the two groups to be compared (Figure 4.15.A). There was no statistically significant difference in the binding of C5 between the two groups. This suggests that the hypothesis that the lower C3c population in the sensitive isolates is due to

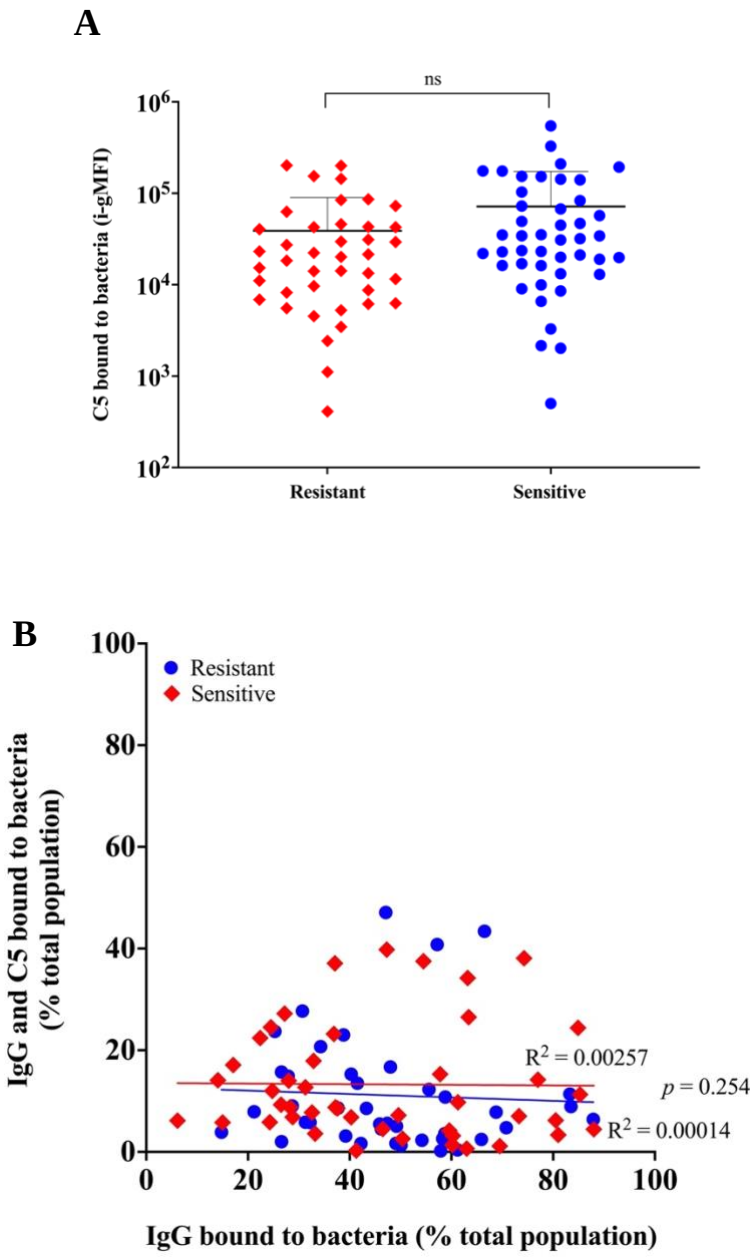


Figure 4.15 Comparison of all resistant and sensitive isolates simultaneously binding IgG and C5. A flow cytometric assay was used to analyse C5 complement binding to 46 isolates resistant and 46 isolates sensitive to autologous serum. Cultures of each isolate were incubated with autologous serum and binding of IgG and C5 was measured by flow cytometry using fluorescently labelled secondary antibodies. Shown is **A)** the integrated mean geometric fluorescence intensity (i-gMFI) comparing all resistant isolates and sensitive isolates. Median was calculated with 95% CI, and statistical significance was measured using a Mann-Whitney test and **B)** the positive population of cells binding antibody IgG and C5 simultaneously compared to the positive population of cells binding antibody IgG. Statistical significance was measured using an analysis of covariance (ANCOVA) test.

them being further along in the complement cascade is unlikely to be correct.

A comparison between sensitive and resistant bacteria simultaneously binding IgG and C5 was made (Figure 4.15.B), in the same way to the previous comparison of IgG and C3c binding. The same strong positive correlation between IgG and C3c in the resistant isolates had not been seen between IgG and C5 in either group. There was no change in either group in complement C5 binding bacteria as the amount of binding of IgG increased. This further confirms that the hypothesis that the lower C3c population in the sensitive isolates is down to them being further along in the complement cascade is unlikely to be correct.

Overall, this increase in C3c binding the resistant isolates was unexpected as it would be anticipated that more complement C3c would have bound the sensitive isolates. This could suggest that this excess binding of complement might be promoting resistance in these isolates, and would be an interesting project to follow up at a later date. However, these results from the flow cytometric antibody and complement deposition experiments did not inform the main aim of the project, which is to determine whether inhibitory antibodies occur during acute infection with gonorrhoea. Thus, using flow cytometric based methods to determine antibody binding titers, based on previous methods (Wells *et al.*, 2014, MacLennan *et al.*, 2010) had not been as informative in this study. These data instead indicated there were no differences in antibody binding between the resistant inhibitory antibody candidates and sensitive isolates, potentially suggesting there could be a different mechanism for inhibitory antibodies against *N. gonorrhoeae*.

4.2.8 Effects of healthy serum on isolates that resisted killing by autologous host serum

To gain a greater understanding of the 46 isolates resistant to participant serum, further investigations into the serum resistance of these isolates were warranted. There are various reasons why bacteria survive incubation with the serum from the autologous host. For example,

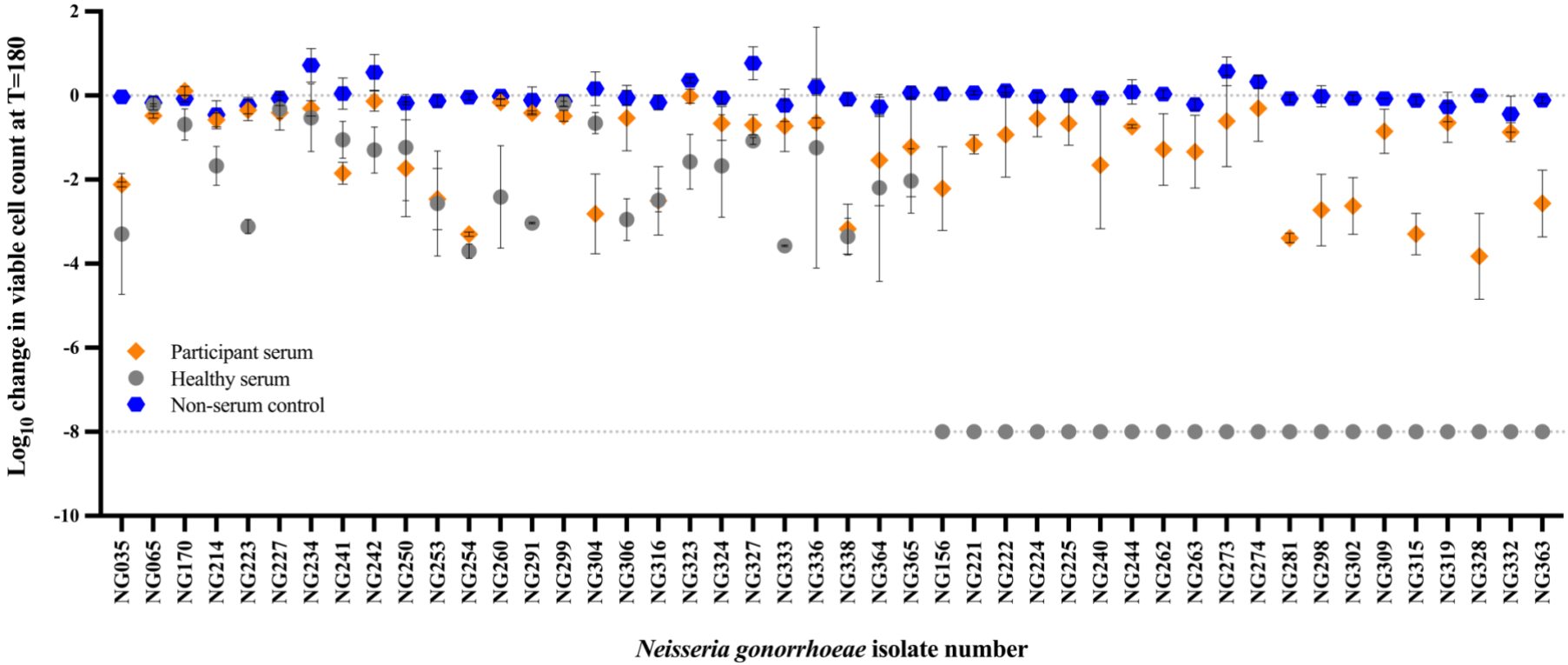


Figure 4.16 Effects of healthy sera on isolates that resisted killing by the autologous host serum. A serum bactericidal assay (SBA) was used to screen the 46 *N. gonorrhoeae* clinical isolates resistant to autologous serum to determine the effect of a pool of human healthy control sera (HCS) on survival. Cultures of each isolate were incubated with the autologous serum, HCS or a non-serum control. The change in viable colony forming units for 46 *N. gonorrhoeae* clinical isolates after 180 minutes in each sera was measured, with negative values corresponding to a decrease in viable bacteria when compared to the initial inoculum. Values at -8 indicate the limit of detection for samples from which no colony formed even from the least diluted sample plated. Data shown are the mean and standard deviation from three independent experiments. The left portion of the graph shows isolates resistant to HCS, and the right side shows isolates sensitive to HCS.

some of the 46 gonococcal isolates might have acquired mutations that made them resistant to complement mediated killing. Such isolates should also be resistant to killing by a pool of HCS from uninfected individuals with no known previous gonococcal infection. Serum bactericidal assays were therefore repeated as above except that the autologous host serum was replaced by pooled HCS. The 46 isolates screened essentially fell into two groups, with 26 of the isolates resisting killing by the pooled healthy serum, which suggests that these isolates were potentially intrinsically resistant to serum killing. The remaining 20 isolates were completely killed by the pooled HCS (Figure 4.16). As these 20 isolates are serum sensitive, the sera from their autologous hosts merited further investigation for the presence of inhibitory antibodies.

4.2.9 Binding of individual serum proteins to isolates resistant and sensitive to healthy serum, that were resistant to autologous host serum

The main aim of this project had been to determine whether inhibitory antibodies were present against resistant clinical isolates of *N. gonorrhoeae* from autologous participant serum. In previous inhibitory antibody studies with other Gram-negative organisms, this had been determined through increased titers of antibody binding to bacteria. No differences were so far observed in the binding of antibody between gonococcal isolates that were resistant or sensitive to autologous serum. Therefore, further investigations were required to determine whether there were differences in binding of antibody and complement to subsets of resistant isolates that were categorised through previous SBA experiments with HCS resulting in two groups; isolates resistant and sensitive to HCS. This analysis would also begin to help understand whether the increased C3c deposition on the resistant isolates was directly related to isolates that were candidates for inhibitory antibodies.

The flow cytometric binding data were split into the two HCS groups, however no differences were observed in the i-gMFI between the binding of antibodies IgM and IgA and

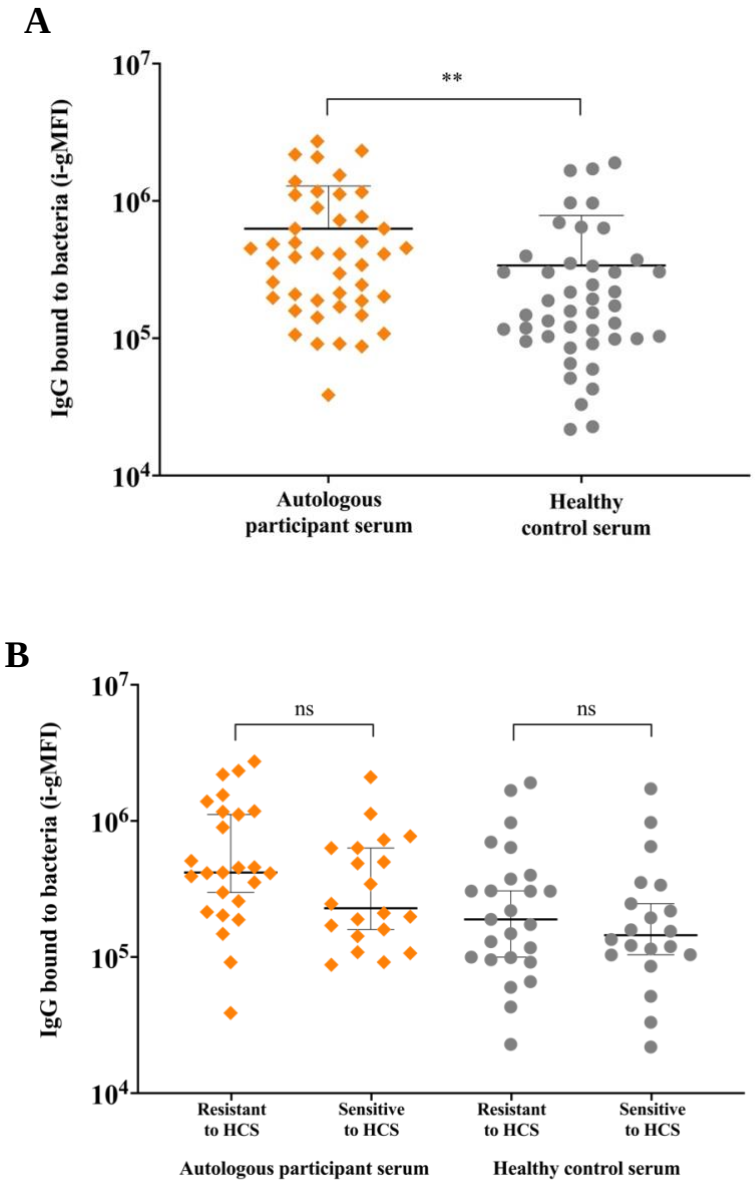


Figure 4.17 Binding of IgG from healthy or autologous host serum to isolates that resisted killing by host serum. A flow cytometric assay was used to analyse IgG binding to 46 isolates resistant to autologous serum, when incubated with autologous participant serum or healthy control serum (HCS). The integrated geometric mean fluorescence intensity (i-gMFI) were measured for **A)** all 46 resistant isolates binding IgG from either serum and **B)** isolates resistant or sensitive to HCS binding IgG from either serum. The median for each group was calculated and a 95% confidence interval (CI) was applied. Statistical significance was measured using a Mann-Whitney test, where ** ($p \leq 0.01$).

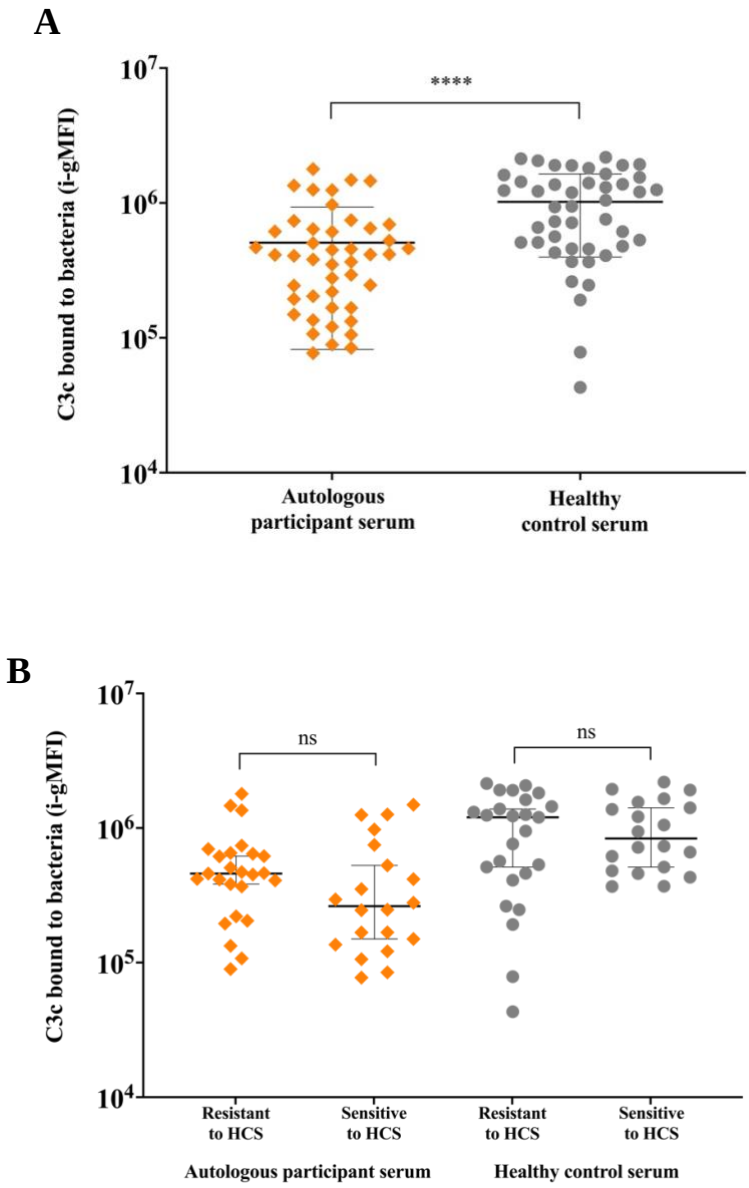


Figure 4.18 Binding of C3c from healthy or autologous host serum to isolates that resisted killing by host serum. A flow cytometric assay was used to analyse C3c binding to 46 isolates resistant to autologous serum, when incubated with autologous participant serum or healthy control serum (HCS). The integrated geometric mean fluorescence intensity (i-gMFI) were measured for **A)** all 46 resistant isolates binding C3c from either serum and **B)** isolates resistant or sensitive to HCS binding C3c from either serum. The median for each group was calculated and a 95% confidence interval (CI) was applied. Statistical significance was measured using a Mann-Whitney test, where **** ($p \leq 0.0001$).

deposition of C5b-9 (data not shown). Earlier analysis showed that the main differences in these isolates resistant to autologous serum were between the binding of antibody IgG and complement C3c, therefore these were the focus to see whether there were differences between the resistant isolates that were also resistant to HCS and those sensitive to HCS. Binding of antibody IgG and complement C3c to all 46 isolates was measured as previously described after incubation with either serum; autologous participant and HCS. There was a statistically significant decrease in the isolates binding of IgG from HCS compared with IgG from autologous participant serum (Figure 4.17.A). However, there was a statistically significant increase in the isolates binding of C3c from HCS compared with C3c from autologous participant serum (Figure 4.18.A).

To understand these results further, a comparison of the two groups; those resistant to HCS and those sensitive to HCS, revealed that there was no significant difference in binding of IgG when isolates were incubated in autologous participant serum, or when incubated with HCS (Figure 4.17.B). This suggests that even though there are differences in serum bactericidal activity, these differences are not due to antibody binding. An additional comparison of the two serum assay groups revealed that there was no significant difference in binding of C3c when isolates were incubated in autologous participant serum, or when incubated with HCS (Figure 4.18.B). This again suggests that even though there are differences in serum bactericidal activity, these differences are not due to complement binding. Individual percentage positive and gMFI data for IgG and C3c are shown in Appendix VII and VIII, respectively.

4.2.10 Effects of a mixture of healthy serum and autologous host serum on isolates that resisted killing by the autologous host serum, but were sensitive to healthy serum

Focussing on the 20 serum sensitive isolates which survived incubation with the autologous host serum, survival in participant serum might have been because the autologous host failed to generate an effective immune response to the infection, or patients had no/low antibody

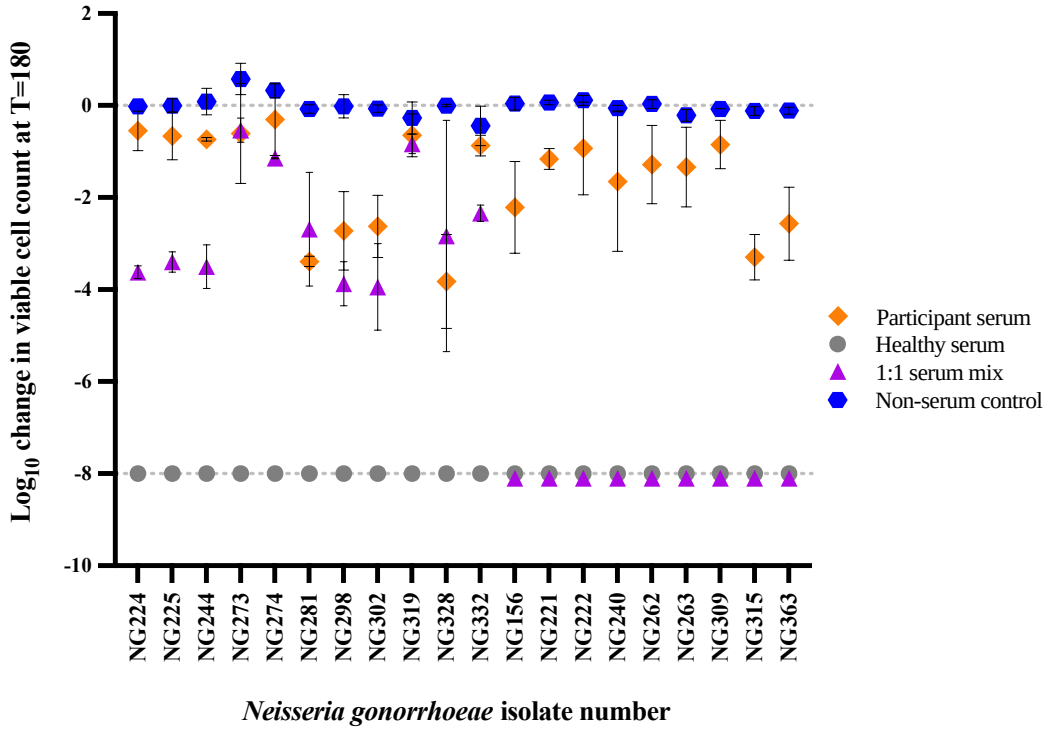


Figure 4.19 Effects of a 1:1 mixture of healthy and autologous host serum on isolates that resisted killing by host serum but were sensitive to healthy serum. A serum bactericidal assay (SBA) was used to screen the 20 *N. gonorrhoeae* clinical isolates resistant to autologous serum and sensitive to HCS, to determine the effect of a 1:1 mix of both sera on survival. Cultures of each isolate were incubated with the autologous serum, HCS, 1:1 sera mix or a non-serum control. The change in viable colony forming units after 180 minutes in each sera were measured, with negative values corresponding to a decrease in viable bacteria when compared to the initial inoculum. Values at -8 indicate the limit of detection for samples from which no colony formed even from the least diluted sample plated. Data shown are the mean and standard deviation from three independent experiments. The left side of the graph shows isolates that were resistant to the 1:1 mix of both sera, and the right side shows isolates sensitive to the 1:1 mix.

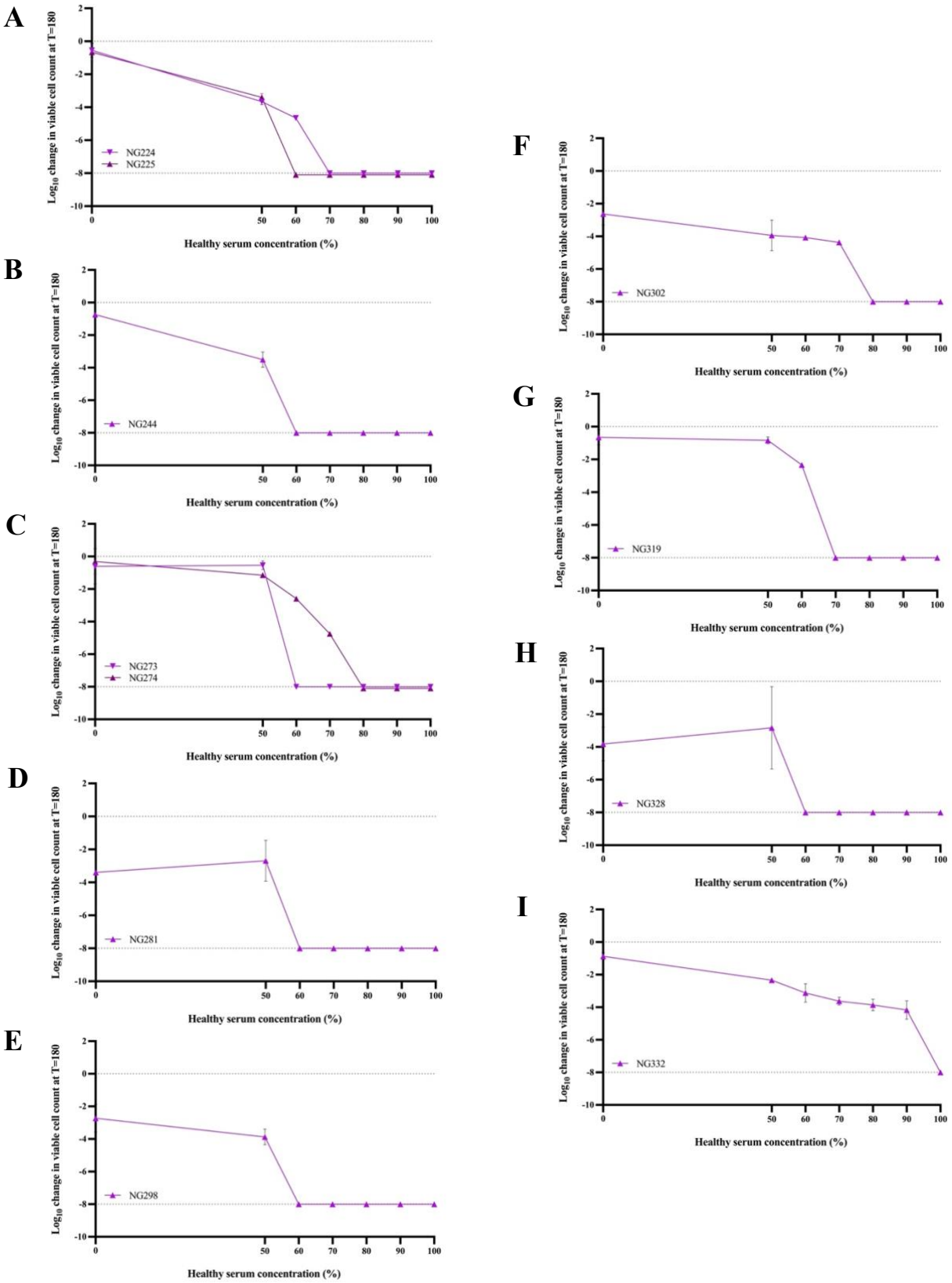


Figure 4.20 Effects of increasing the amount of healthy serum in the mixture of healthy and autologous host serum on isolates that resisted killing by the 1:1 serum mixture. A serum bactericidal assay (SBA) was used to investigate the effects of increasing the concentration of HCS (50-90%) on the survival of *N. gonorrhoeae* clinical isolates resistant to a 1:1 mix of HCS and autologous host serum. Total volume for each sample was made up to 100% with autologous participant serum. The change in viable colony forming units after 180 minutes was measured, with negative values corresponding to a decrease in viable bacteria when compared to the initial inoculum. Values at -8 indicate the limit of detection for samples from which no colony formed even from the least diluted sample plated. Data shown are the mean and standard deviation from two independent experiments.

potentially due to a recent infection, as patients tend to only have a protective and specific antibody level after 3-4 weeks. If so, the host serum would be unable to protect the gonococcal isolate from killing by the pooled HCS. Conversely, any sera that contained inhibitory antibodies would protect the gonococci from killing by the pooled HCS. Each of the 20 isolates were therefore incubated with a 1:1 mixture of the pooled HCS and the autologous participant serum (Figure 2.19). Nine of the 20 isolates were killed by the sera mixture, so the autologous serum had been unable to provide protection. In contrast, eleven isolates were either completely or significantly more resistant to the mixed sera than to the pool of HCS. The autologous sera therefore protected these eleven isolates from complement mediated killing. Therefore, these isolates are the strongest candidates for potentially having the ability to induce the production of inhibitory antibodies and are the focus of further study.

To determine the potency of the inhibitory effects from the participant serum to these eleven isolates, and to identify whether the effects could be diluted out with more HCS, the same 1:1 mix assay was repeated but with increasing concentrations of HCS in the range of 50-90% of the total volume, with the remaining volume being made up to 100% with autologous participant serum. Isolates NG244, NG273, NG281, NG298, and NG328 were all unable to survive as HCS was increased to 60% and higher (Figures 4.20.B, C, D, E and H respectively). However, isolates NG274, NG302, NG319 and NG332 were able to survive in higher concentrations of HCS, showing that the inhibitory effects of these participant serum was more potent (Figures 4.20.C, F, G and I respectively). NG332 showed the strongest potency as it had been able to survive with only 10% participant serum and 90% of the killing HCS.

4.2.11 Clinical implications of *Neisseria gonorrhoeae* inhibitory antibodies

The vast majority of the inhibitory antibody studies described thus far are all very clearly linked to patient outcomes. Inhibitory antibodies in patients with non-typhoidal *Salmonella* bacteremia

infection were found to be positively correlated with infection with HIV (MacLennan *et al.*, 2010). Inhibitory antibodies in patients with persistent *P. aeruginosa* infection were also burdened with a chronic lung condition, such as bronchiectasis (Wells *et al.*, 2014). In patients with *E. coli* urinary tract infections, inhibitory antibodies allowed the bacterium to infect the bloodstream, causing urosepsis (Coggon *et al.*, 2018). To determine whether the inhibitory antibodies observed in this study correlated with patient clinical outcome, an analysis of clinical metadata had been undertaken, to compare the whole population with the population of isolates deemed to elicit inhibitory antibodies. Clinical participant information regarding the eleven 1:1 isolates, including gender, HIV status and previous gonococcal infections, are shown in Table 4.2.

The correlation between HIV and inhibitory antibodies during *S.* Typhimurium infection (MacLennan *et al.*, 2010) had not been observed during *N. gonorrhoeae*, as the proportions of HIV positive participants were similar in both groups (Figure 4.21.D), with 16% HIV positive participants in total population (left graph) compared with 22% HIV positive participants in the inhibitory antibody group (right graph). Participant geographical location, ethnicity and their presentation of symptoms were all also analysed for correlations when comparing the two groups, but no significant findings were reported (Figure 4.21.A, B, and C respectively). There were also no differences seen within the gender category between the two groups also (Figure 4.21.E), however there was a difference in the anatomical site of infection within these groups (Figure 4.21.G). There was a statistically significant larger proportion of urethral isolates in the inhibitory antibody population (right graph) compared to total population (left graph), no isolates from the pharynx and a slight reduction in rectal isolates. These differences could be linked to the results seen within the sexual network dataset, as here the proportion of males are the same, however there was a statistically significant increase of MSW and fewer from the MSM community in the inhibitory antibody population compared to the total population (Figure

Neisseria gonorrhoeae isolate number	G-ToG clinical trial participant number	Participant location	Participant age	Participant gender	Participant ethnicity	Participant HIV status	Participant co-infections	Participant previous gonococcal infections	Isolate anatomical site of infection
NG224	4075	Leeds (General Infirmary)	23.9	male	White	negative	0	1	urethra
NG225									rectum
NG244	1156	Birmingham (Whittall St. Clinic)	27.1	male	Asian	negative	0	0	urethra
NG273	7079	Sheffield (Royal Hallamshire)	20.6	female	White	negative	0	0	cervix
NG274									rectum
NG281	7089	Sheffield (Royal Hallamshire)	33.0	male	White	negative	0	1	urethra
NG298	2057	London (St Bartholomew)	45.9	male	White	positive	0	1	urethra
NG302	2062	London (St Bartholomew)	22.5	male	White	negative	0	1	urethra
NG319	3090	London (Guy's Hospital)	21.3	male	Black	negative	0	0	urethra
NG328	11035	Coventry (Univ Hosp Coventry & Warw)	57.4	male	White	positive	0	1	urethra
NG332	12005	London (Royal Free Trust)	20.0	male	Other	negative	chlamydia	0	urethra

Table 4.2 Participant demographics of isolates resistant to a 1:1 mixture of healthy and autologous host serum. Participant information, including gender, HIV status and previous gonococcal infection, for the nine participants from which the eleven resistant *N. gonorrhoeae* inhibitory isolates were derived from.

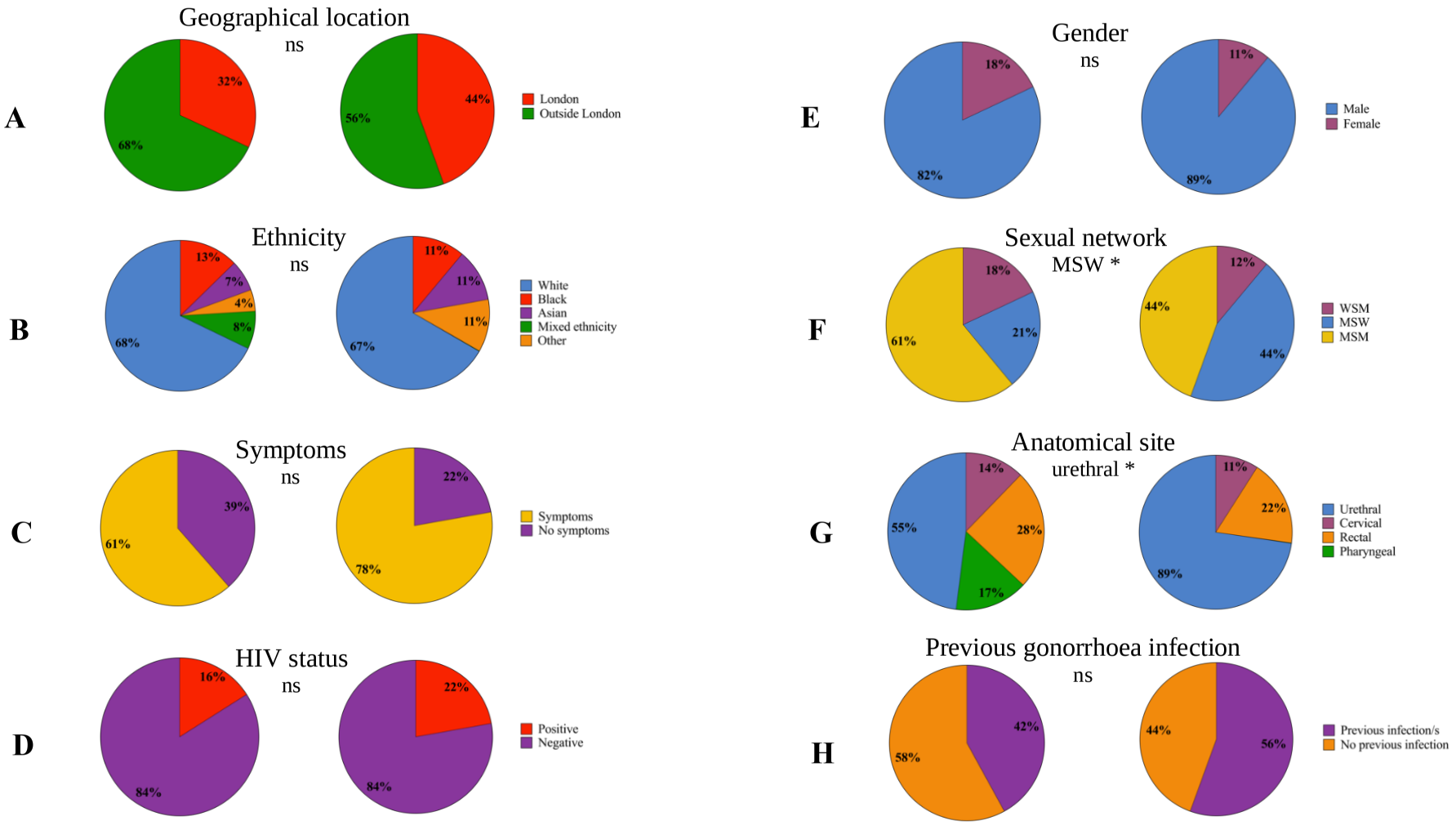


Figure 4.21. Comparison of clinical data between the proportions of participants within the whole population and the inhibitory serum population. Comparison of the proportions within the total population within the clinical trial of 286 participants from which all 336 isolates were derived (left hand side graphs) and the population of nine participants from which the eleven inhibitory isolates were derived (right hand side graphs), for a variety of clinical datasets including **A)** geographical location, **B)** ethnicity, **C)** symptoms, **D)** HIV status **E)** gender, **F)** sexual network, **G)** anatomical site and **H)** previous gonorrhoea infection. A two proportion z-test with a 95% confidence level were used when comparing populations within each dataset. Statistical significance was then calculated using a 2-tailed z-test, where * ($p<0.05$).

4.21.F). This could explain the differences in anatomical site between the two groups, as rectal and pharyngeal male infections are associated with the MSM community, leading to an increased proportion of urethral infections within the MSW network.

It has also been considered that inhibitory antibodies elicited during infection with *N. gonorrhoeae* might be linked to recurrent infections. After initial infection, these antibodies could remain after antibiotic treatment, and therefore protect a future gonococcal isolates upon infection. This would therefore aid in explaining why a large proportion of patients succumb to repeat infections. When comparing the total population and the inhibitory antibody population in this study with regards to previous gonococcal infections, it had been found that there was a slight increase when having previously had gonorrhoea before and eliciting inhibitory antibodies (Figure 4.21.H), however this again was not statistically significant. Interestingly, all participants in the inhibitory antibody group that had previously had gonorrhoea reported only one previous infection. Therefore a larger sample size of participants with inhibitory antibody would be required to suggest that previous infection with gonorrhoea increases chances of eliciting inhibitory antibody and therefore would hinder the patient in naturally clearing a future infection.

4.2.12 Binding of individual serum proteins to isolates resistant and sensitive to a 1:1 of healthy serum and autologous host serum

As detailed in Section 4.2.9, there were no significant differences observed in the binding of antibody and complement between the 46 participant serum resistant gonococcal isolates, that were also either resistant or sensitive to HCS. As mentioned previously, of these two groups, the isolates sensitive to HCS were candidates for instigating the production of inhibitory antibodies. Therefore, further investigations on these isolates were required to determine whether there were differences in binding of antibody and complement to subsets that were categorised through SBA

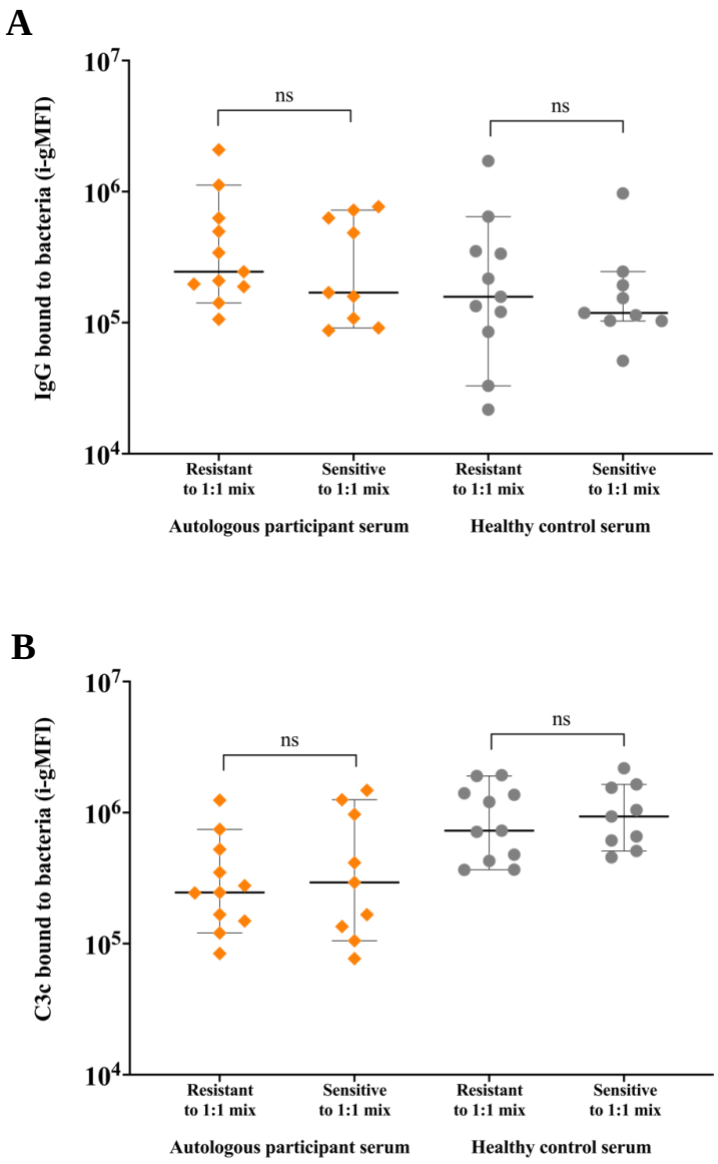


Figure 4.22 Binding of IgG and C3c from healthy or autologous host serum to isolates that were sensitive to killing by healthy serum. A flow cytometric assay was used to analyse **A)** IgG and **B)** C3c binding to 20 isolates that were resistant or sensitive to a 1:1 mixture of healthy control serum (HCS) and autologous host serum, when incubated with either autologous serum or HCS. The integrated geometric mean fluorescence intensity (i-gMFI) were measured. The median for each group was calculated and a 95% confidence interval (CI) was applied. Statistical significance was measured using a Mann-Whitney test.

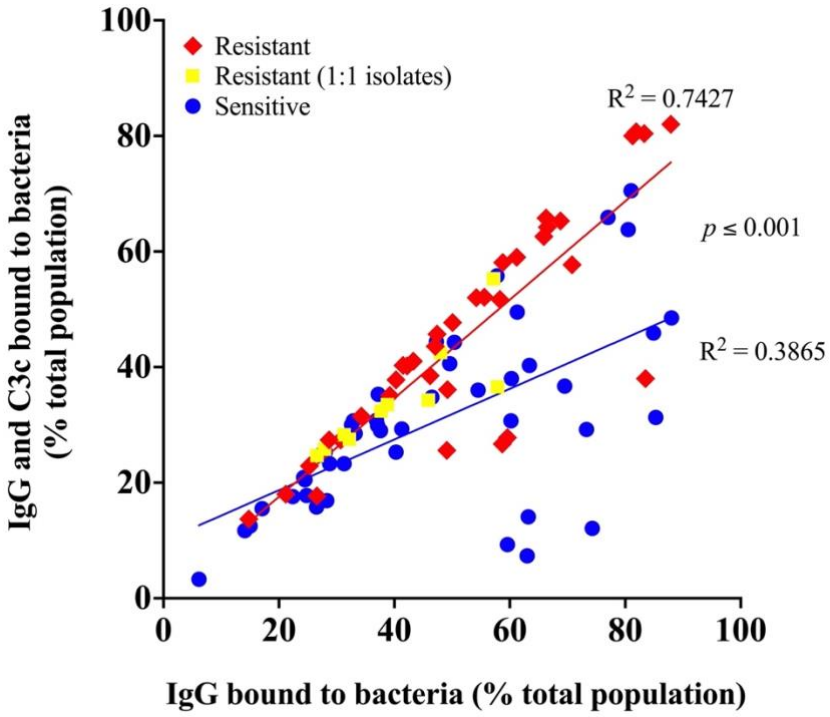


Figure 4.23 Comparison of all resistant and sensitive isolates simultaneously binding IgG and C3c, highlighting the inhibitory antibody candidates. A flow cytometric assay was used to compare antibody and complement binding to 46 isolates resistant and 46 isolates sensitive to autologous serum. Cultures of each isolate were incubated with autologous serum and binding of IgG and C3c were measured by flow cytometry using fluorescently labelled secondary antibodies. The positive population of cells binding antibody IgG and C3c simultaneously compared to the positive population of cells binding antibody IgG, with resistant isolates found to be candidates for inhibitory antibodies shown in yellow. Statistical significance was measured using an analysis of covariance (ANCOVA) test.

experiments with the 1:1 mix of both sera. These experiments resulted in two groups; isolates resistant to 1:1 mix and isolates sensitive to 1:1 mix.

The flow cytometric binding data were split in to the two groups, however, no differences were observed in the i-gMFI between the binding of antibodies IgM and IgA and deposition of C5b-9 (data not shown). The focus was again on the binding of antibody IgG and complement C3c to see whether there were differences between the resistant isolates that were resistant to 1:1 mix and those sensitive to 1:1 mix. Binding of antibody IgG and complement C3c to all 20 isolates was measured as previously described after incubation with either serum, autologous participant and HCS. Individual percentage positive and gMFI data for IgG and C3c are shown in Appendix IX. There were no differences in the isolates binding IgG or C3c from either sera within the two groups (Figure 4.22.A and B respectively). The lack of differences in C3c binding suggests that the increased binding of C3c previously found to all isolates resistant to autologous participant serum compared to sensitive, might not necessarily be linked to the inhibitory antibodies. It could be assumed that this is potentially a separate serum resistance mechanism. This is further depicted in Figure 4.23, where the isolates representing those that were resistant to the 1:1 serum mix are highlighted on the IgG/C3c correlation graph in yellow.

4.2.13 Phylogenomic analysis of the susceptibility of isolates to autologous serum

To further help in understanding the isolates susceptibility to different serum, the relationship between this data and the genetics were analysed, which might inform whether it is the isolate or the serum causing the different resistance phenotypes. Serum susceptibility, including sensitivity of isolates to autologous serum, HCS and a 1:1 mix of both sera, were correlated with the phylogenomic tree of all isolates (Figure 4.24). It is immediately evident that isolates that are resistant to autologous serum are spread evenly around the tree, and there is not a particular lineage causing serum resistance. To further understand whether the genomic data correlated

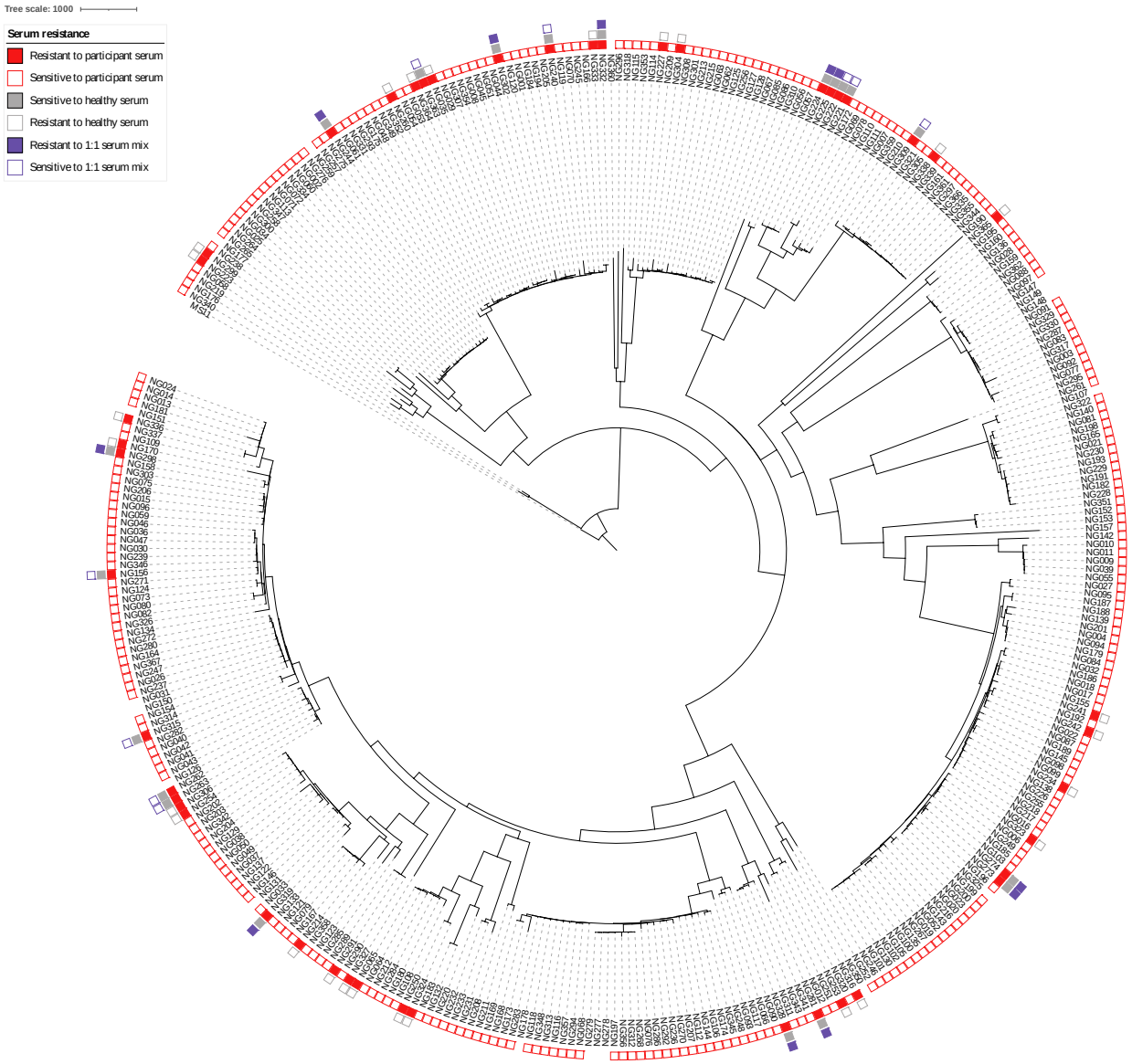


Figure 4.24 Phylogenomic distribution of serum susceptibility among isolates. Recombination corrected maximum likelihood tree of all 354 sequenced *N. gonorrhoeae* isolates was created using RAxML (Stamatakis, 2014). Susceptibility of isolates to autologous participant serum, healthy control serum (HCS) and a 1:1 mixture of both sera were annotated using iTOL software (Letunic and Bork, 2021).

with the phenotypic serum data, a genome-wide association study (GWAS) had been ran using SEER (Lees *et al.*, 2016). Initial results from the SNP-based GWAS found 19842 k-mers associated with sensitivity to serum. However, after vigorous filtering and analysis of hits, there were no k-mers that showed a significant association with susceptibility to serum within these isolates. Overall this indicates that within this dataset, there are no particular genomic links between the serum resistant isolates.

4.2.14 Phylogenomic analysis of the susceptibility to serum of isolates derived from multiple anatomical site infections

Within the sequenced G-ToG collection of isolates, 16.6% (49/296) of participants had multiple gonococcal isolates at different anatomical sites, with the annotated phylogenomic tree shown previously in Figure 3.3. These isolates had been assayed for serum susceptibility in their matching participants sera, in an SBA as previously described, to discover whether there were differences in the immune response of one participant towards their multiple different infecting isolates. Overall, the phylogenomic tree indicated that most isolate pairs were either both sensitive or both resistant to the autologous serum (Figure 4.26.A). This again suggested that each participants isolates were most likely to be either very similar or the same strain, just residing at different anatomical sites within the host. Interestingly, five of the participants, each with two infecting isolates at different anatomical sites, had a different serum response to each of their isolates. The first participant had a sensitive pharyngeal isolate NG314, but a resistant rectal isolate NG315 (Figure 4.25.A), the second participant had a sensitive urethral isolate NG290, but a resistant rectal isolate NG291 (Figure 4.25.B), the third participant had a sensitive pharyngeal isolate NG337, but a resistant urethral isolate NG336 (Figure 4.25.C), the fourth participant had a sensitive urethral isolate NG064, but a resistant rectal isolate NG065 (Figure 4.25.D) and the fifth participant had a sensitive rectal isolate NG339, but a resistant pharyngeal

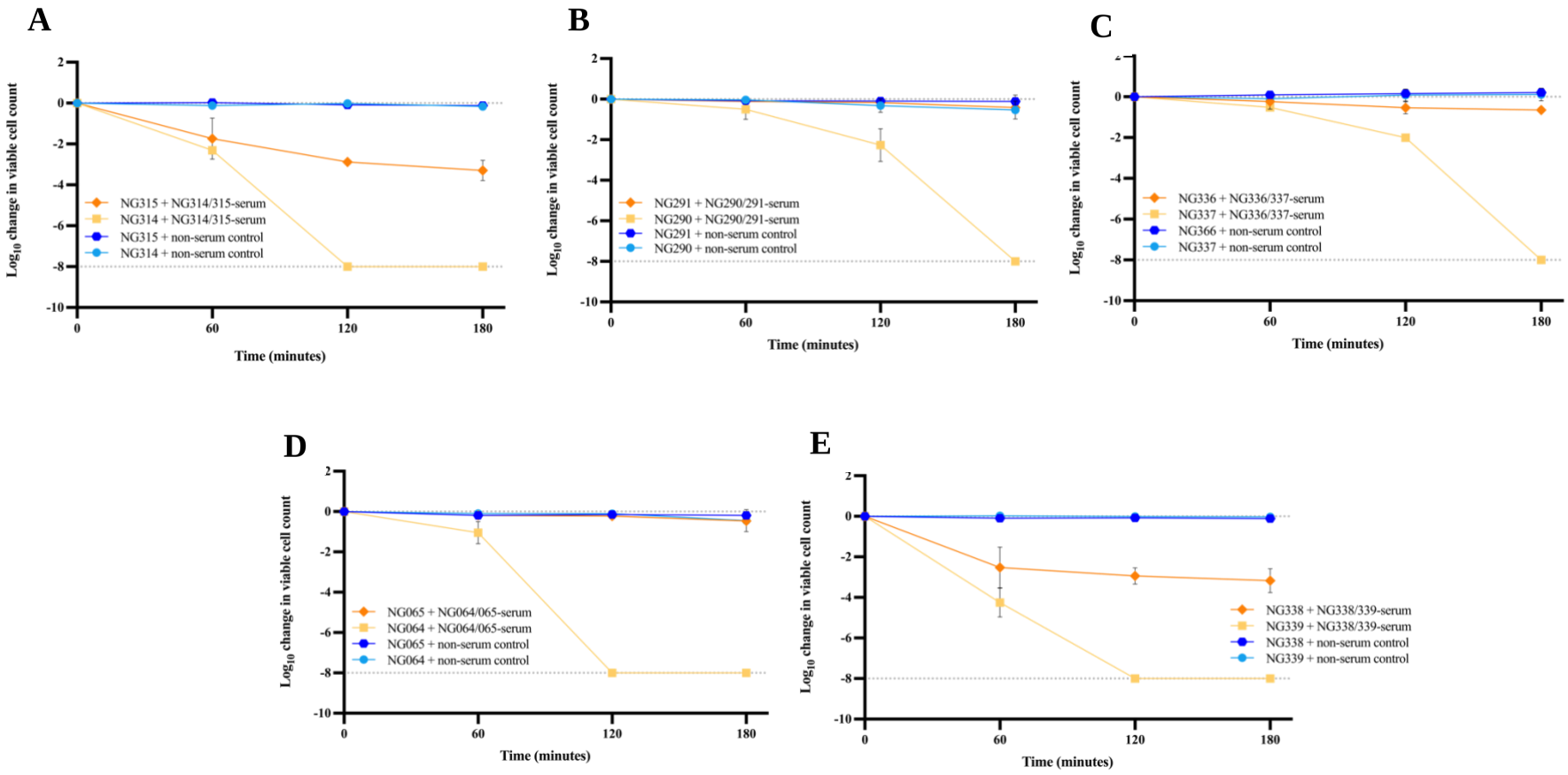
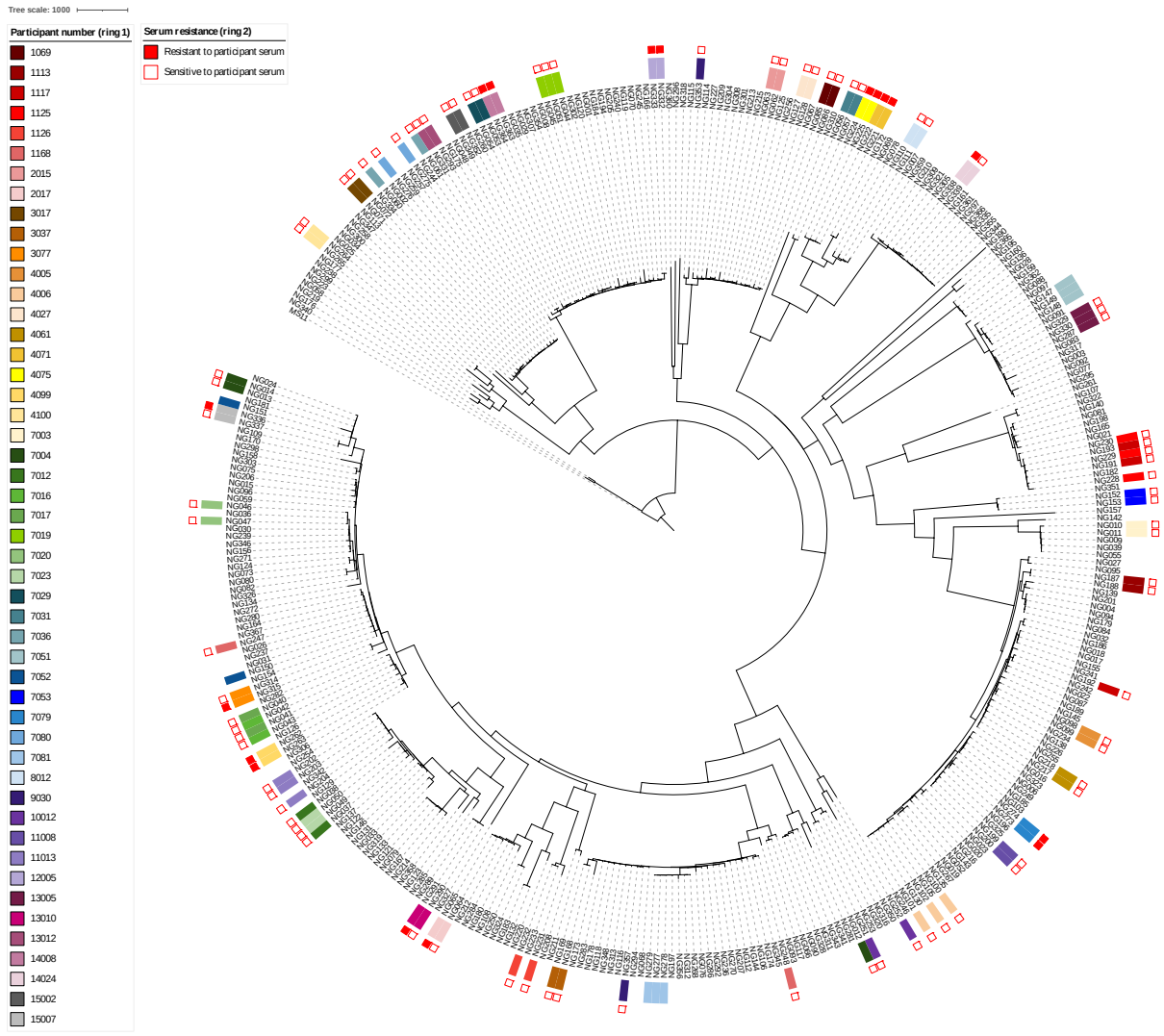


Figure 4.25 Serum bactericidal assay of isolates derived from multiple anatomical sites on the same participant. A serum bactericidal assay (SBA) was used to investigate the serum susceptibility of multiple isolates derived from individual participants, including isolates **A)** NG314 and NG315, **B)** NG290 and NG291, **C)** NG336 and NG337, **D)** NG064 and NG065 and **E)** NG338 and NG339. The change in viable colony forming units each isolate after 60, 120 and 180 minutes in autologous participant was measured with negative values corresponding to a decrease in viable bacteria when compared to the initial inoculum. Values at -8 indicate the limit of detection for samples from which no colony formed even from the least diluted sample plated. Data shown are the mean and standard deviation from three independent experiments

A



B

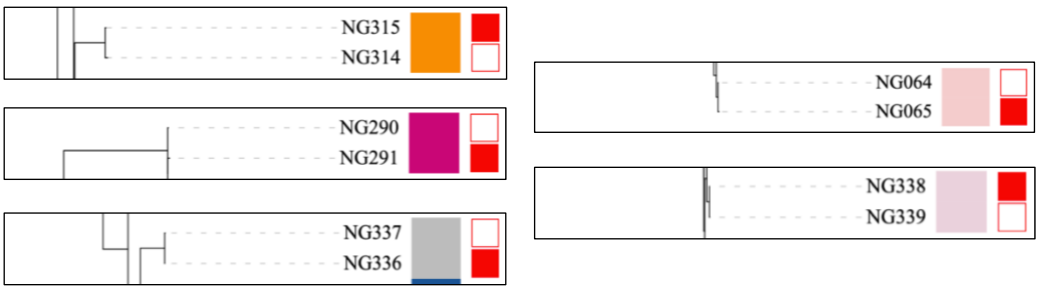


Figure 4.26 Phylogenomic distribution of serum susceptibility among isolates from multiple anatomical sites on the same participant. Recombination corrected maximum likelihood tree of all 354 sequenced *N. gonorrhoeae* isolates was created using RAxML (Stamatakis, 2014). Isolates from participants with multiple anatomical site infections along with their susceptibility to autologous serum were annotated using iTOL software (Letunic and Bork, 2021). **B)** Focus on the isolate pairs with different serum susceptibilities.

Isolates	Type of variant and effect	Gene affected	Gene product
NG314 and NG315	del (frameshift variant)	gspA	general stress protein A
	snp (missense variant)	mfd	transcription-repair-coupling factor
	snp (missense variant)	pilE_1	fimbrial protein
NG290 and NG291	snp (synonymous variant)		hypothetical protein
	snp (missense variant)	adh	alcohol dehydrogenase
	snp (missense variant)		hypothetical protein
	ins (frameshift variant)		hypothetical protein
NG336 and NG337	complex (missense variant)		hypothetical protein
	snp (synonymous variant)		hypothetical protein
	ins (frameshift variant)	gspA	general stress protein A
	complex (missense variant)	piiC_2	outer membrane protein P.IIC
	del (frameshift variant)		hypothetical protein
	snp (synonymous variant)		hypothetical protein
	snp (missense variant)	pilE_4	fimbrial protein
NG064 and NG065	del (frameshift variant)	piiC_3	outer membrane protein P.IIC
	snp (missense variant)		hypothetical protein
NG338 and NG339	snp (intergenic region)		tRNA-Thr
	ins (disruptive inframe insertion)	piiC_6	outer membrane protein P.IIC
	snp (missense variant)		hypothetical protein
	snp (missense variant)		hypothetical protein
	snp (missense variant)	mutS	DNA mismatch repair protein MutS

Table 4.3 Analysis of single nucleotide polymorphism differences between genetically similar isolate pairs derived from the same participant. Snippy (Seemann, 2015) was utilised to generate single nucleotide polymorphism (SNP) analysis of isolate pairs derived from the same participant that were genetically similar on the phylogenomic tree, but had phenotype differences in serum susceptibility. Highlighting variants in the form of substitutions and insertions/deletions and the genes affected.

isolate NG338 (Figure 4.25.E). Due to the differences in serum susceptibility of these isolates derived from the same participant, the phylogenomic tree was annotated with serum susceptibility of isolates in multiple site infections (Figure 4.26.A) to determine whether the differences were due to phylogeny. Surprisingly, the five isolate pairs were each genetically very similar to one another (Figure 4.26.B) and each pair had the same MLST and NG-MAST. To try to understand the reason for the differences in phenotype of these genetically similar isolates, Snippy (Seemann, 2015) was ran on the pairs to identify any SNP differences (Table 4.3). Many of the resultant SNP differences included piiC, an outer membrane protein, and pilE, a fimbrial protein. However, both of these genes are known to be hyper-variable, and are usually excluded from this kind of analysis (Ma *et al.*, 2020, Osnes *et al.*, 2021). The majority of remaining genes with SNP differences between the pairs unfortunately encoded unknown hypothetical proteins, and therefore further investigations would be required to understand what they are and whether they have any involvement in serum susceptibility.

4.3 Discussion

Overall, the investigation of clinical *N. gonorrhoeae* isolates from the G-ToG collection, using an analysis of serum susceptibility, revealed eleven inhibitory antibody candidate isolates, derived from nine different participants. This equates to about 3% of the population that produce inhibitory antibodies during acute infection with gonorrhoea. In contrast, Pham *et al.* (2021) determined that one in three adults with cystic fibrosis and *P. aeruginosa* infection had serum that contain inhibitory antibodies, and Wells *et al.* (2014) reported the prevalence in patients with non-cystic fibrosis bronchiectasis was 20%. In addition, Coggon *et al.* (2018) determined that 24% of patients with *E. coli* urosepsis had inhibitory antibodies. These data suggest that the prevalence of inhibitory antibodies during chronic infection is relatively high. In this study, however, the gonococcal infections are all acute and the incidence of inhibitory antibodies are

relatively low. Previous gonococcal anti-Rmp inhibitory antibody work has mainly focussed on chronic DGI strains with inhibitory healthy serum (Rice and Kasper, 1982). The occurrence of DGI infections within the population is extremely low nowadays, therefore understanding inhibitory antibody elicitation during acute infection is very important. However, interestingly in this study, it was observed that participants with urethral infections and within the MSW sexual network were significantly more likely to have protective sera (Figure 4.21). Thus, it would be of great interest to determine the clinical implications of inhibitory antibodies on recurrent gonorrhoea and patient outcomes in targeted follow up studies.

For detection of inhibitory antibodies, it might have been beneficial to do similar analysis using the two week post-treatment serum, as the generation of an antibody response during a new infection usually takes several weeks. Therefore depending on how long they had had the infection before the baseline trial visit, the post treatment sera might be a more accurate representation of the antibody response.

Previous studies have reported that the main way of detecting inhibitory antibodies in serum had been by identifying those containing high antibody binding titers (Wells *et al.*, 2014, MacLennan *et al.*, 2010). In our study, antibody deposition data from flow cytometry experiments suggested there were no differences in the binding of any antibody classes between resistant and sensitive isolates, and therefore this same elevation in antibody binding titer was not observed. This concentration dependence however had been found to be very important in the case of *S.* Typhimurium (Goh *et al.*, 2016). Here in both whole serum and purified antibodies, elevated antibody titers determined inhibition of *S.* Typhimurium. Therefore, it can be assumed that the high antibody titers typically associated with inhibitory antibodies in other Gram-negative organisms is not the same mechanism for these particular *N. gonorrhoeae* isolates. A past study on *P. aeruginosa* had also claimed that titer determined inhibition (Wells *et al.*, 2014). However, in their most recent work they had discovered that this inhibition of killing had not

been solely dependent on titer of antibody, but antibody affinity also played a key role. They found that even low antibody titers were able to maintain inhibition if antibodies were high affinity. While high titers still played a role, if these antibodies were low affinity, there was potential that inhibition might still be ineffective (Pham *et al.*, 2021). This could suggest that low-affinity antibodies might be dislodged by antibodies or complement that are more specific. These differences were not reported in *S.* Typhimurium (Goh *et al.*, 2016), which might suggest that the structure of *S.* Typhimurium LPS naturally induces antibodies with high-affinity, which has previously been reported in chlamydial LPS (Muller-Loennies *et al.*, 2000). Therefore, there is potential that affinity could be an important factor in this study, so a closer look at antibody isotypes and their affinity for their target antigen could inform the mechanism.

Additionally, from our flow cytometry data, it was also concluded that there was an increased binding of complement C3c associated with IgG bound to resistant isolates, compared to sensitive isolates. The C3c phenotype was found to not to be directly linked to the inhibitory antibody candidates. Therefore, the assumption can be made that increased C3c deposition is potentially involved in a mechanism of resistance for the gonococcus, but not involved in the mechanism of inhibitory antibodies for these particular isolates. However further investigation is required, because previous inhibitory antibody studies have reported an association with these antibodies and enhanced complement deposition. Joiner *et al.* (1985) found enhanced complement C3 deposition on a DGI *N. gonorrhoeae* strain in the presence of inhibitory IgG, where it covalently associated with the antibody and also redirected C3 to deposit on different bacterial structures, such as LOS. Both of these suggested that inhibitory antibodies altered the site of complement deposition to new non-bactericidal targets that do not result in serum killing and potentially implicate this as a mechanism for inhibition. However, there is a possibility that inhibitory IgG might be obstructing the usual C3 acceptor sites, which could be dictating the redirection of complement. Joiner *et al.* (1985) also found that $F(ab')_2$ fragments of inhibitory

IgG also activated and deposited C3. Interestingly, these fragments cannot activate the classical pathway of complement, so instead they must be activating the alternative pathway (Ratnoff *et al.*, 1983, Densen *et al.*, 1982). Once activated, the alternative pathway C3c cleaves C3 which initiates a positive feedback loop, that in turn amplifies C3 deposition. A lack of prevention by complement inhibitors could result in excessive C3 deposition. In contrast to the studies on C3 mentioned thus far, recent data by Gulati *et al.* (2015a) found that *N. gonorrhoeae* recovered from infected mice showed that inhibitory Rmp antibodies were associated with a reduction of C3 deposition. However, this contrast in results might be due to the differences in *in vitro* and *in vivo* systems and assays used. *N. gonorrhoeae* is an obligate human pathogen, therefore animal models do not always mimic many of the key aspects of human infection. Therefore, the effects of inhibitory antibodies on complement deposition *in vivo* is yet to be determined.

From all eleven of the inhibitory antibody candidates in this study, three isolates stood out as having the strongest inhibitory phenotype. This is evident in Figure 4.20 where the amount of HCS in the 1:1 serum mixture was increased, with the aim of diluting out the inhibitory effects of the autologous participant serum. Isolates NG302 and NG274 were still protected by participant sera at 30% and NG332 was able to still survive at just 10% participant serum. A similar titration of inhibitory serum and HCS by Wells *et al.* (2014) had been undertaken on three clinical *P. aeruginosa* isolates with autologous inhibitory serum that inhibited the bactericidal activity of HCS. The amount of HCS in the 1:1 serum mixture was increased here also, and they found that the three isolates were killed once patient serum was reduced down to 6, 20 and 30% respectively for each of the three strains. This indicated that the patients serum had a potent capacity to inhibit killing, which showed similarities to the results in this study. To help gain more of an understanding of the inhibitory phenotype identified in these experiments, further investigations into the inhibitory factor in the autologous serum are needed, in order identifying the mechanisms underpinning this phenotype.

CHAPTER 5

ELUCIDATING THE MECHANISM UNDERPINNING THE INHIBITION OF *NEISSERIA GONORRHOEAE* COMPLEMENT-MEDIATED KILLING

5.1 Introduction

The results presented in Chapter 4 showed that there were a proportion of sensitive isolates that were resistant to autologous serum, and therefore their sera were candidates for containing inhibitory antibodies. The next objective was to determine whether it had been an antibody, or a different inhibitory factor, that was driving the protective phenotype. When comparing studies with similar phenotypes to those observed in this project, there was a noticeable association with antibody-dependent inhibition. Previous work had discovered that during some bacterial infections, the inhibitory antibodies elicited were a variety of antibody isotypes with specificities for varied target antigens on the outer membrane. Studies by Griffiss and Bertram (1977), Kayhty (1980) and Amir *et al.* (2005) found that inhibitory antibodies against *N. meningitidis* were isotype IgA directed against the capsular polysaccharide of meningococcal group C bacteria, and isotype IgG3 against lipoproteins in serogroup B meningococci (Ray *et al.*, 2011). Hall *et al.* (1971) and Corbeil *et al.* (1988) reported that IgA was mainly responsible for bactericidal inhibition of *Brucella* species, with IgG2 antibodies against O-antigen LPS also involved. Studies by Wells *et al.* (2014) and Pham *et al.* (2021) showed the inhibition of serum killing of *P. aeruginosa* was mediated by IgG2 and IgA directed against the O-antigen of the LPS. In addition, studies by MacLennan *et al.* (2010) and Goh *et al.* (2016) reported that *S.* Typhimurium induced LPS O-antigen specific IgG2, IgA and IgM antibodies that were all able to inhibit serum killing.

IgG is well known for its ability to activate the complement system, but many of these studies have revealed specific subclasses of IgG that inhibited this pathway. IgG subclasses, IgG1, IgG2, IgG3 and IgG4, share over 90% amino acid sequence homology, but each have a unique profile in respect to hinge region length, Fc effector functions and number of inter-chain disulfide bond, rendering them functionally distinct (Vidarsson *et al.*, 2014). Each subclass is different in regard to immune complex formation, effector cell activation, antigen binding and

complement activation (detailed in Table 1.1).

IgG1 is typically induced in response to proteins, including membrane and soluble antigens. IgG1 is the most abundant of the subclasses (representing 60–70% of the total IgG), followed by IgG2 (20–30%), then IgG3 (4–8%) and least abundant IgG4 (5%) (Schur, 1987).

IgG2 is a weak mediator of complement fixation, due to its C1q-binding sites not being frequently exposed (Bruggemann *et al.*, 1987, Schroeder and Cavacini, 2010). The IgG response to bacterial polysaccharide antigens are almost exclusive to IgG2 (Siber *et al.*, 1980, Schauer *et al.*, 2003). Very little is known about oligosaccharide structures of gonococcal LOS that are recognised by human antibodies, however Yamasaki *et al.* (2010) found that a preparation of purified IgG from healthy serum contained three different IgG2 species that each recognised a different oligosaccharide epitope on core LOS structures. These epitopes resided within branched oligosaccharides, and were not directed towards gonococcal LOS epitopes that mimicked human carbohydrates. They also found that this combined IgG2 preparation had been able to facilitate the killing of a serum-sensitive and resistant strain under certain conditions (Yamasaki *et al.*, 2010). Deficiencies in IgG2 have been found to be associated with an increased susceptibility to pathogens, particularly encapsulated bacteria, suggesting IgG2 has a role in defence against these pathogens (Kuijpers *et al.*, 1992).

IgG3 is the dominant subclasses involved in responses to protein antigens. IgG3 is the strongest mediator of complement fixation, due to binding FcγRIII and C1q with higher affinities, compared to IgG1 which closely follows. It has been suggested that this is due to the long hinge, at 62 amino acids, that makes the C1q binding site more accessible (Tan *et al.*, 1990). IgG3 deficiencies are found in a small percentage of individuals with recurrent gonococcal infections.

Allergens invoke the production of IgG4 and sometimes also IgG1. IgG4 are usually induced following repeated exposure to non-infectious antigens. A switch to producing IgG4 is

suggested to be modulated by IL-10, which is involved with downregulation of immune responses (Aalberse *et al.*, 2009, Jutel and Akdis, 2011). The ability of IgG4 to fix complement is minimal.

The variability in inhibitory antibody and antigen targets suggests it is highly likely that there could be multiple mechanisms generating antibody-mediated inhibition of serum killing. Here, GAM antibody isotypes were purified from candidate participants sera, in order to elucidate the mechanisms driving the inhibition of complement-mediated killing observed in Chapter 4.

5.2 Results

5.2.1 Measuring the ability of purified antibody isotypes to inhibit complement-mediated killing

Eleven participant sera were determined to be most likely to contain inhibitory antibodies, based on the isolates sensitivity to HCS, and their ability to survive in autologous participant serum (detailed in Section 4.2.10). It can be assumed that inhibitory antibodies would still be present within the few weeks after the infection is cleared. To understand whether this occurs against the eleven candidate isolates, each isolate was challenged with post-treatment serum (Figure 5.1), which had been taken two weeks after the start of treatment. Post-treatment serum was unavailable for isolates NG224/225 and NG328. Isolate NG281 was not able to survive in post-treatment serum from the same participant. This indicated that sera from this participant was less likely to contain inhibitory antibodies. All other isolates survived just as well in the post-treatment serum.

To investigate whether antibodies were the inhibitory factor in the participant serum that was preventing killing of the isolates, total IgG, IgM and IgA were purified from these five selected sera, including NG244-serum, NG273/274-serum, NG298-serum, NG302-serum and

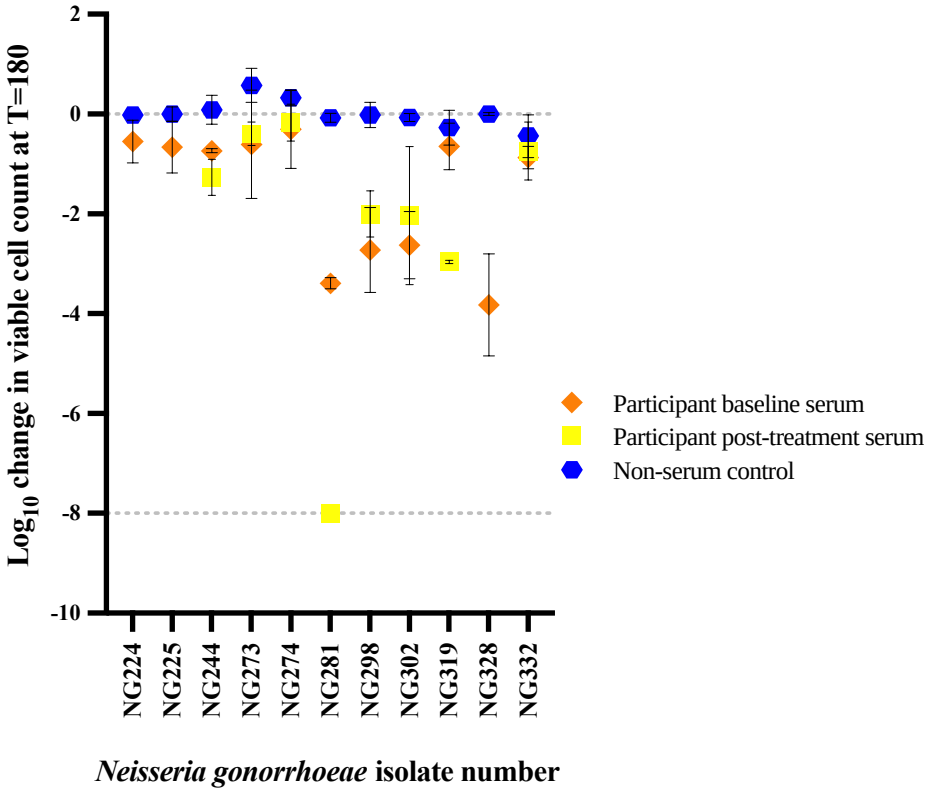


Figure 5.1 Effects of autologous host 'post-treatment' serum on isolates that resisted killing by the 1:1 mixture of healthy serum and autologous host 'baseline' serum. A serum bactericidal assay (SBA) was used to screen the eleven *N. gonorrhoeae* clinical isolates inhibitory antibody candidates to determine the effect of autologous post-treatment serum. Cultures of each isolate were incubated with the autologous baseline serum, post-treatment serum or a non-serum control. The change in viable colony forming units after 180 minutes in each sera was measured, with negative values corresponding to a decrease in viable bacteria when compared to the initial inoculum. Values at -8 indicate the limit of detection for samples from which no colony formed even from the least diluted sample plated. Data shown are the mean and standard deviation from three independent experiments.

NG332-serum. Insufficient serum was available for purification from NG319-serum, so this could not be selected. As a control, GAM antibodies were also purified from a killing serum matching urethral isolate NG115, which was sensitive to killing by the autologous serum and HCS (Figure 5.2.A). NG115-serum had also been able to kill all six candidate isolates selected for inhibitory antibody purification (Figure 5.2.B). These results show that this is a serum that contains bactericidal antibodies and was therefore chosen for antibody purification control experiments. These results also re-confirmed that all the six inhibitory antibody candidate isolates chosen for antibody purification are all sensitive isolates, and it is their autologous participant serum that is promoting their resistance.

Once the total IgG, IgM and IgA antibodies had been purified from bactericidal NG115-serum, their bactericidal properties were tested in a modified SBA with the matching sensitive isolate. Control experiments were set up in which cultures of NG115 were incubated at 37°C for 3 hours with 5% BRC plus each purified antibody individually at different 10-fold concentrations diluted with PBS. Antibodies were quantified before experiments and the average stock concentrations at detailed in Appendix X. The range of IgG concentrations tested were from 100 pg/ml to 1 mg/ml, and the range of IgM and IgA concentrations tested were from 100 pg/ml to 0.1 mg/ml, similar to the concentration range described by Goh *et al.* (2016). Note that the highest antibody concentrations tested were all below normal physiological levels in blood; IgG 6-16 mg/ml, IgM 0.4-2.5 mg/ml and IgA 0.8-3 mg/ml.

Individually added purified total IgG and IgM were bactericidal against isolate NG115 at high concentrations down to 0.1 µg/ml (Figure 5.3). At 1 ng/ml and lower concentrations of both purified antibodies, NG115 was able to survive suggesting this concentration was too low for killing. As a positive non-serum control, PBS was added to additional cultures that were incubated under the same conditions. In contrast, isolate NG115 survived in all concentrations of purified IgA, suggesting that IgA is not bactericidal against NG115. Therefore, this control

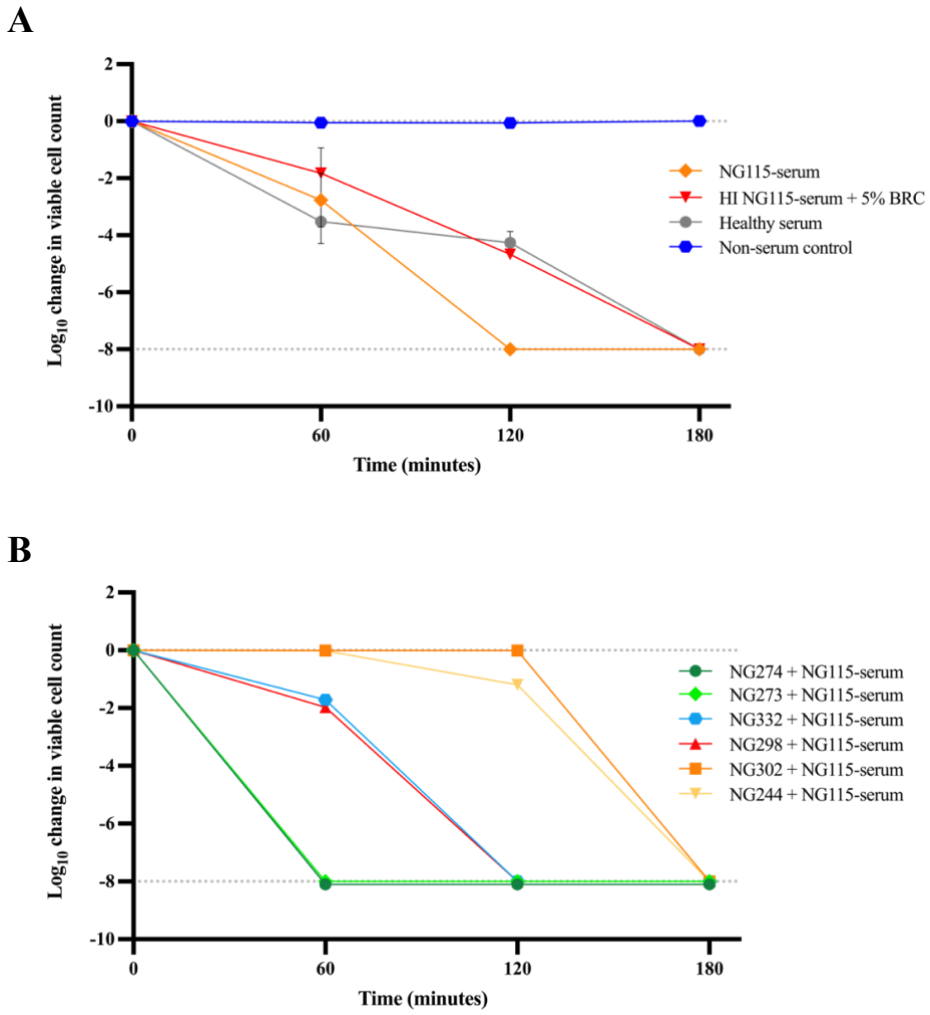


Figure 5.2 Serum bactericidal assay of inhibitory isolates with killing NG115-serum. A serum bactericidal assay (SBA) was used to determine the sensitivity of **A)** isolate NG115 in autologous host serum and healthy serum, and **B)** all isolates selected as inhibitory antibody candidates in NG115-serum. The change in viable colony forming units after 60, 120 and 180 minutes was measured, with negative values corresponding to a decrease in viable bacteria when compared to the initial inoculum. Values at -8 indicate the limit of detection for samples from which no colony formed even from the least diluted sample plated. Data shown are the mean and standard deviation from two independent experiments.

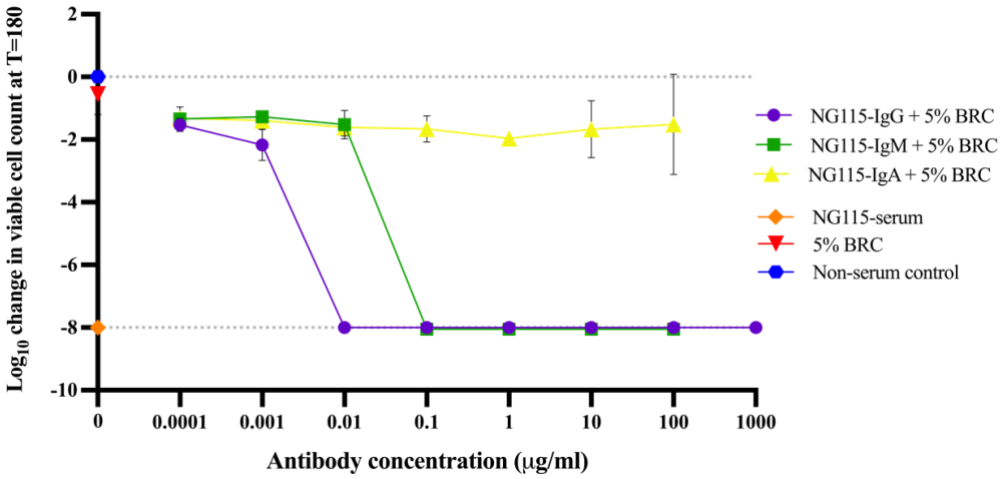


Figure 5.3 Effect of total IgG, IgM and IgA antibodies purified from killing NG115-serum on the autologous isolate. A serum bactericidal assay was used to determine the effect of antibodies IgG, IgM and IgA purified from NG115-serum on the matching *N. gonorrhoeae* sensitive isolate. Cultures of NG115 were incubated with 5% baby rabbit complement (BRC) and each purified antibody individually made up to final concentrations between 1000 µg/ml and 0.0001 µg/ml with PBS. The change in viable colony forming units after 180 minutes was measured, with negative values corresponding to a decrease in viable bacteria when compared to the initial inoculum. Values at -8 indicate the limit of detection for samples from which no colony formed even from the least diluted sample plated. Data shown are the mean and standard deviation from two independent experiments.

experiment with a sensitive isolate showed that the antibodies were still functional after purification and indicated the limit of IgG and IgM killing concentrations.

Purified total IgG, IgM and IgA antibodies from inhibitory NG273/274-serum, NG244-serum, NG298-serum, NG302-serum and NG332-seurm were next incubated in the modified SBA with each autologous isolate. Cultures were incubated at 37°C for 3 hours with 50% HCS plus each purified antibody individually at various concentrations diluted with PBS. These experiments were designed to mimic the 1:1 ratio experiment previously described (Figure 4.19), with individual purified antibody replacing matching participant serum. As HCS alone was able to kill all of these isolates, the addition of purified antibody would therefore measure the ability of different isotypes to inhibit killing by HCS. The range of antibody concentrations tested was from 10 ng/ml to 1 mg/ml for IgG and 10 ng/ml to 0.1 mg/ml for IgM and IgA. Similar separate experiments were also set up in which cultures were incubated at 37°C for 3 hours with 5% BRC plus each purified antibody individually at various concentrations, to remove the complement variable of HCS. The addition of purified antibody in these experiments would measure the ability of different isotypes to initiate complement mediated killing.

Initially, isolates NG244, NG298 and NG302 were assayed with the antibodies purified from their matching sera. In a 1:1 mixture with HCS, only the highest concentrations of IgG (1000 µg/ml) and IgM (100 µg/ml) were able to inhibit killing of all three isolates, NG244, NG298 and NG302 (Figure 5.4.A, C and E respectively), and concentrations of 10 µg/ml and below of either purified antibody were no longer protective against HCS. This suggest that IgG and IgM are able to inhibit killing by HCS, but inhibition is concentration dependent. In contrast, autologous purified IgA did not protect the bacteria from killing, even at the highest concentrations tested (Figure 5.4.A, C and E). It was concluded that IgA had not been able to inhibit killing of HCS for these isolates.

For all three isolates, NG244, NG298 and NG302, low concentrations of autologous

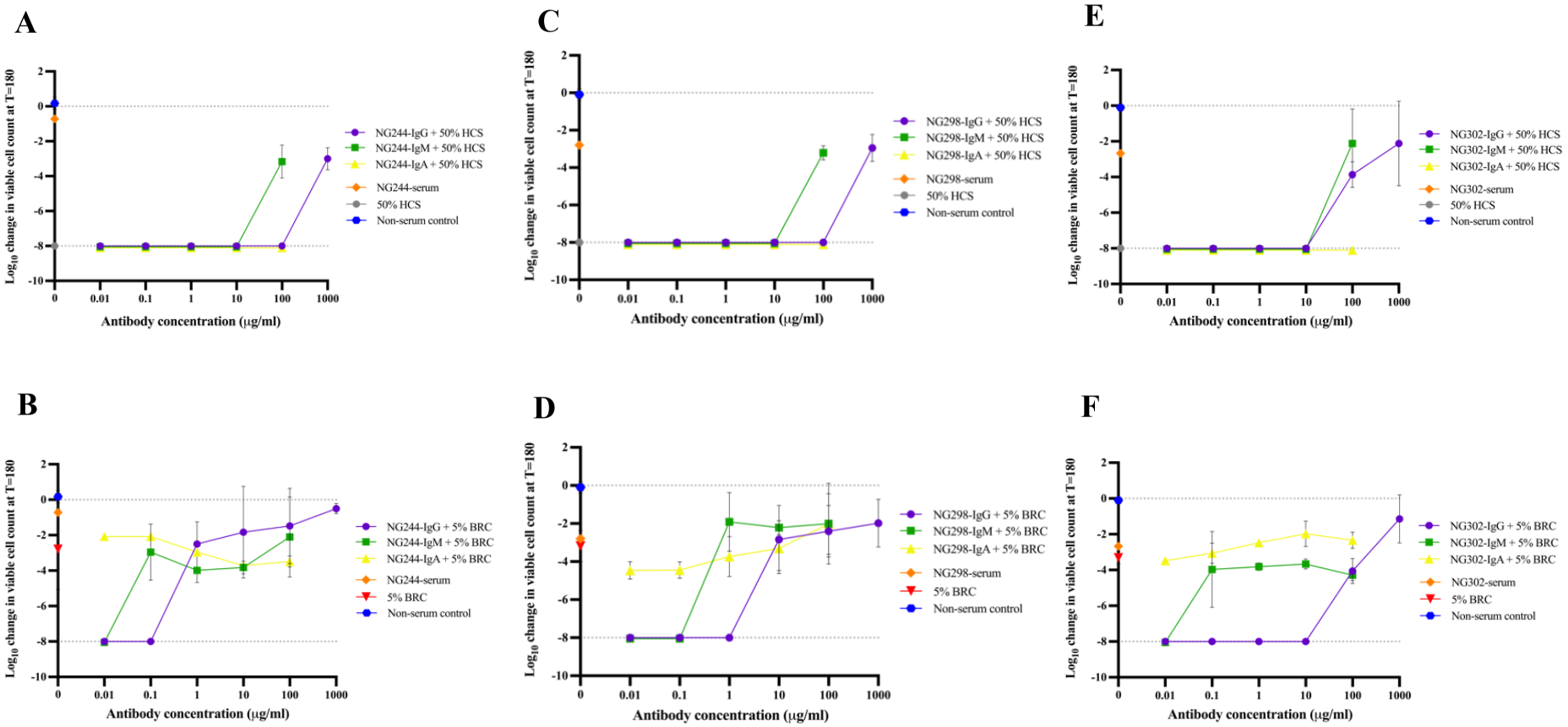


Figure 5.4 Effect of total IgG, IgM and IgA antibodies purified from inhibitory NG244-serum, NG298-serum and NG302-serum on autologous isolates. A serum bactericidal assay (SBA) was used to determine the effect of antibodies IgG, IgM and IgA purified from inhibitory serum on the matching *N. gonorrhoeae* isolates. Cultures of isolates NG244, NG298 and NG302 were incubated with a mixture of 50% HCS and 50% of different concentration of each autologous purified antibody (**A**, **C** and **E**); or with a mixture of 5% baby rabbit complement (BRC) and 95% of each autologous purified antibody at different concentrations (**B**, **D** and **F**). The change in viable colony forming units after 180 minutes was measured, with negative values corresponding to a decrease in viable bacteria when compared to the initial inoculum. Values at -8 indicate the limit of detection for samples from which no colony formed even from the least diluted sample plated. Data shown are the mean and standard deviation from two independent experiments.

purified IgG and IgM were able to kill the bacteria in the presence of 5% BRC (Figure 5.4.B, D and F respectively). In contrast, autologous purified IgA was unable to initiate complement mediated killing of isolates NG244, NG298 and NG302 in the presence of 5% BRC (Figure 5.4.B, D and F respectively). Overall, when combining the data from the inhibitory HCS experiments and the killing BRC experiments, it suggests that inhibition of HCS mediated-killing occurs at high IgG and IgM antibody concentrations, but the same antibodies are able to initiate complement-mediated killing at low concentrations in the presence of BRC. In addition, when combining these data, it also suggests that IgA is unable to inhibit or induce killing these three isolates.

Next, the remaining three isolates from which antibodies were purified from the autologous sera, NG273 and NG274 (from the same participant at different anatomical sites) and NG332, were also assayed in the same way. In a 1:1 mixture with HCS, all purified antibodies isotypes, IgG, IgM and IgA, were able to inhibit killing of HCS in all three autologous isolates (Figure 5.5.A, C and E respectively). However, for two of these isolates, NG274 and NG332, inhibition still occurred with all antibodies at very low concentrations (Figure 5.5.C and E), suggesting a very strong inhibitory phenotype within these sera. This result was consistent with the observations from Figure 4.20, where during a titration of the mix between participant serum and HCS, both of these isolates were still protected by their autologous serum at very low amounts. In the presence of BRC, all three isolates, NG273, NG274 and NG332, were only killed by very low concentrations of purified IgG (Figure 5.5.B, D and F respectively). IgM was found to not be able to kill some of these isolates when mixed with BRC, which could suggest that IgM in these sera are not bactericidal. However, for NG332, killing did occur at the lowest concentration trialled, 0.01 µg/ml, therefore it is highly likely that much lower concentrations are required to be tested for the other two isolates in order to find the concentration required for killing. Autologous purified IgA was unable to initiate complement mediated killing of isolates

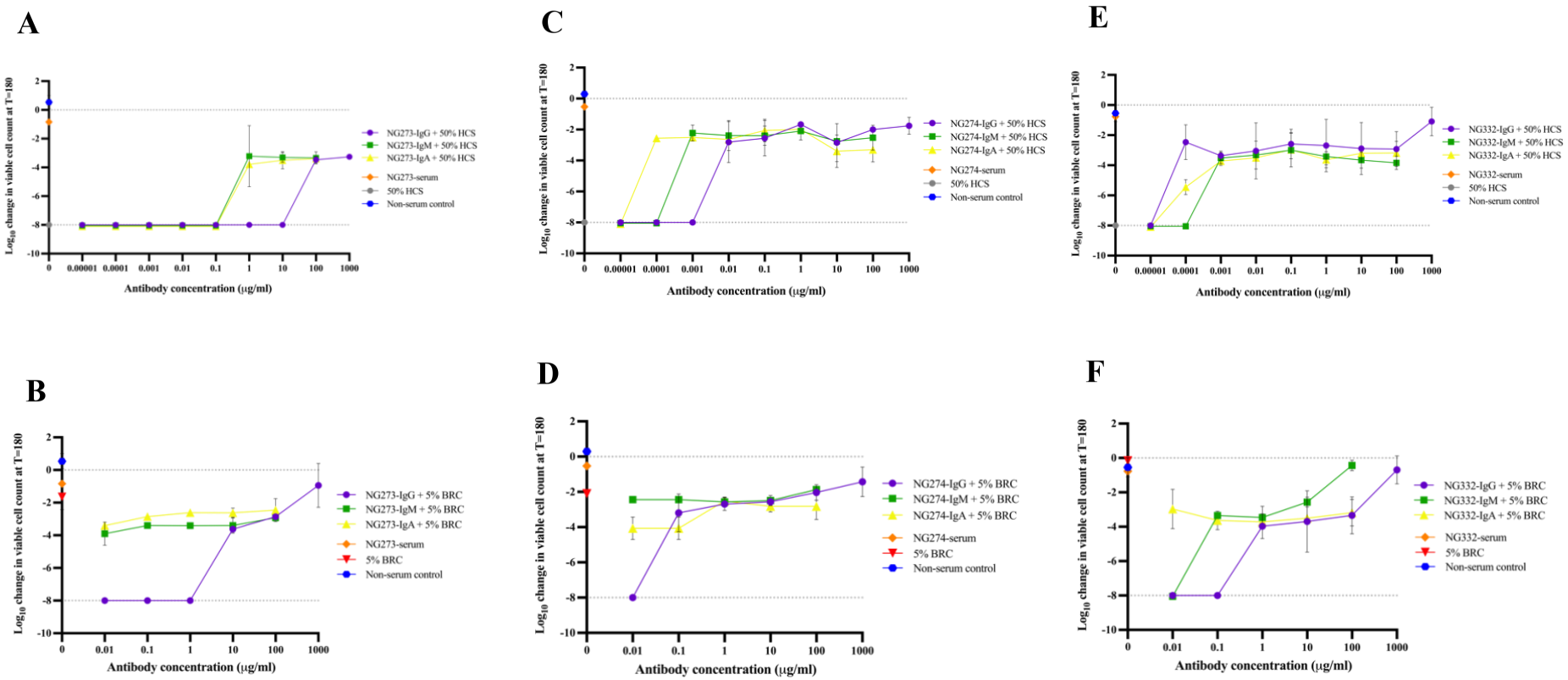


Figure 5.5 Effect of total IgG, IgM and IgA antibodies purified from inhibitory NG273/274-serum and NG332-serum on autologous isolates. A serum bactericidal assay (SBA) was used to determine the effect of antibodies IgG, IgM and IgA purified from inhibitory serum on the matching *N. gonorrhoeae* isolates. Cultures of isolates NG273, NG274 and NG332 were incubated with a mixture of 50% HCS and 50% of different concentration of each autologous purified antibody (**A**, **C** and **E**); or with a mixture of 5% baby rabbit complement (BRC) and 95% of each autologous purified antibody at different concentrations (**B**, **D** and **F**). The change in viable colony forming units after 180 minutes was measured, with negative values corresponding to a decrease in viable bacteria when compared to the initial inoculum. Values at -8 indicate the limit of detection for samples from which no colony formed even from the least diluted sample plated. Data shown are the mean and standard deviation from two independent experiments.

NG273, NG274 and NG332 in the presence of 5% BRC (Figure 5.5.B, D and F respectively).

Overall, when combining the data from the inhibitory HCS experiments and the killing BRC experiments, it again suggests that inhibition of HCS mediated-killing occurs at high antibody concentrations, but the same antibodies are able to initiate complement-mediated killing at low concentrations in the presence of BRC. NG274 and NG332 in particular showed a very strong inhibitory phenotype with inhibition of HCS occurring at very low concentrations with all isotypes of purified antibodies. However, even for these isolates, IgG was still able to kill autologous isolates at very low concentrations. The same was found with IgM for NG332, but lower concentrations of NG273/274-IgM might need to be trialled with BRC to determine the concentrations required for killing. In addition, when combining the data, it also suggests that IgA is able to inhibit killing by HCS but is not able to initiate complement-mediated killing for these three isolates.

5.2.2 The effect of different IgG subclasses on inhibitory activity

In all samples, IgG seemed to be able to inhibit killing of all isolates by HCS, therefore the next step was to determine the subclass of the inhibitory IgG. A whole cell enzyme-linked immunosorbent assay (ELISA) was used to investigate binding titers of inhibitory IgG subclasses. In brief, ELISA plates were coated with whole cells of each *N. gonorrhoeae* isolate to be tested. Diluted serum was added and incubated to enable antibody binding to the bacteria. Secondary antibodies were added for specific antibodies of interest; including total IgG, IgG1, IgG2, IgG3 and IgG4. Finally, the HRP substrate was added, and the $OD_{450\,nm}$ of each well was measured.

The first step to optimise the experiment was to determine the amount of secondary antibody to use. For total IgG, IgG1, IgG2, IgG3 and IgG4 secondary antibodies, the manufacturer recommended using dilutions of 1:4000-1:8000, 1:4000-1:8000, 1:2000-1:8000,

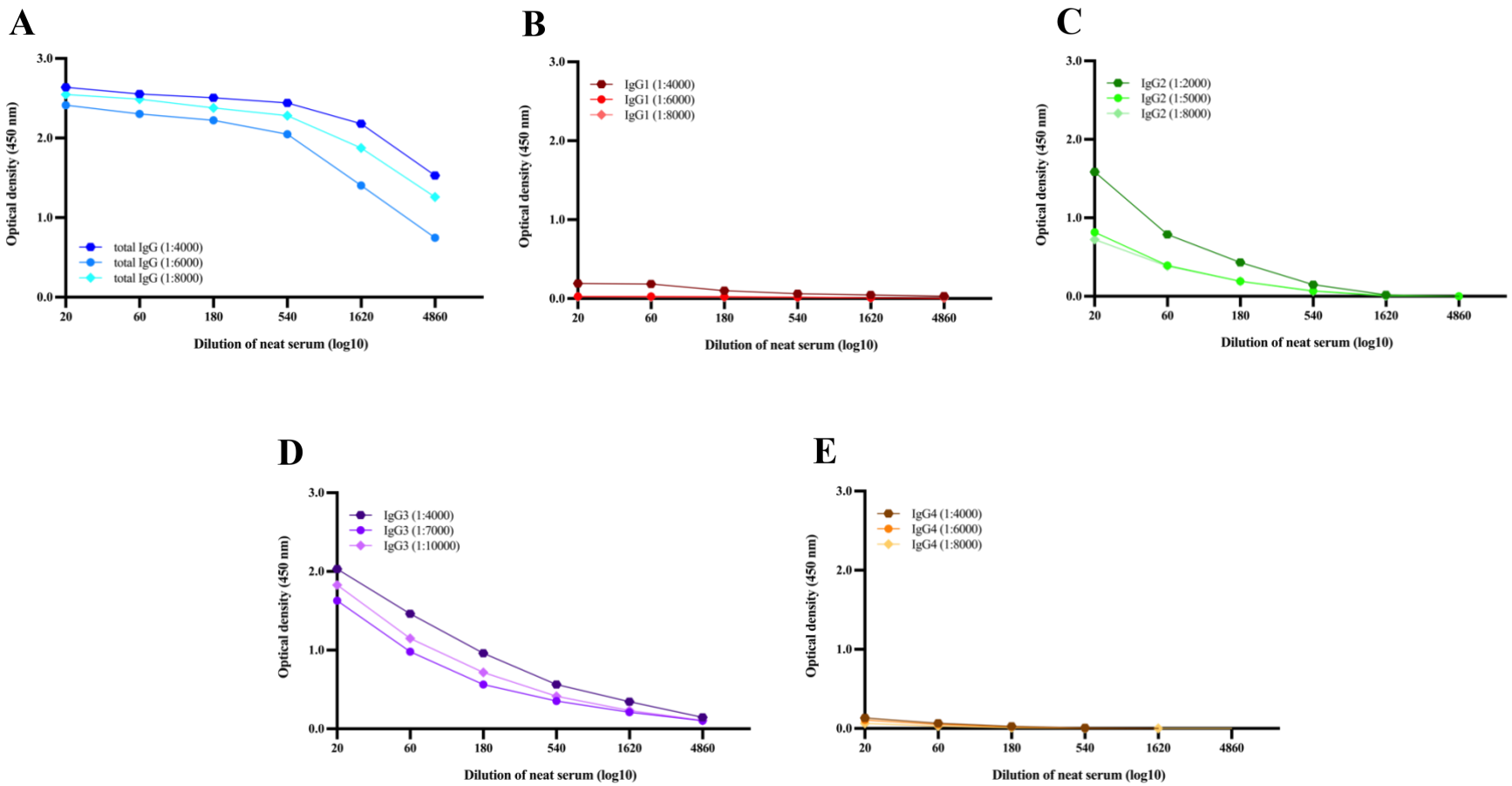


Figure 5.6 Titration of secondary antibody concentrations for measurement of IgG subclass antibodies. An ELISA was used to investigate the effects of different concentrations of secondary antibody on detecting binding of **A)** total IgG, **B)** IgG1, **C)** IgG2, **D)** IgG3 and **E)** IgG4 to whole bacteria of sensitive isolate NG027. The range of concentrations of secondary antibodies were used based on those recommended by the manufacturer. Serum was diluted 1 in 20 initially and 3-fold thereafter, with an $OD_{450\ nm}$ measured upon assay completion. Data shown are from one experiment.

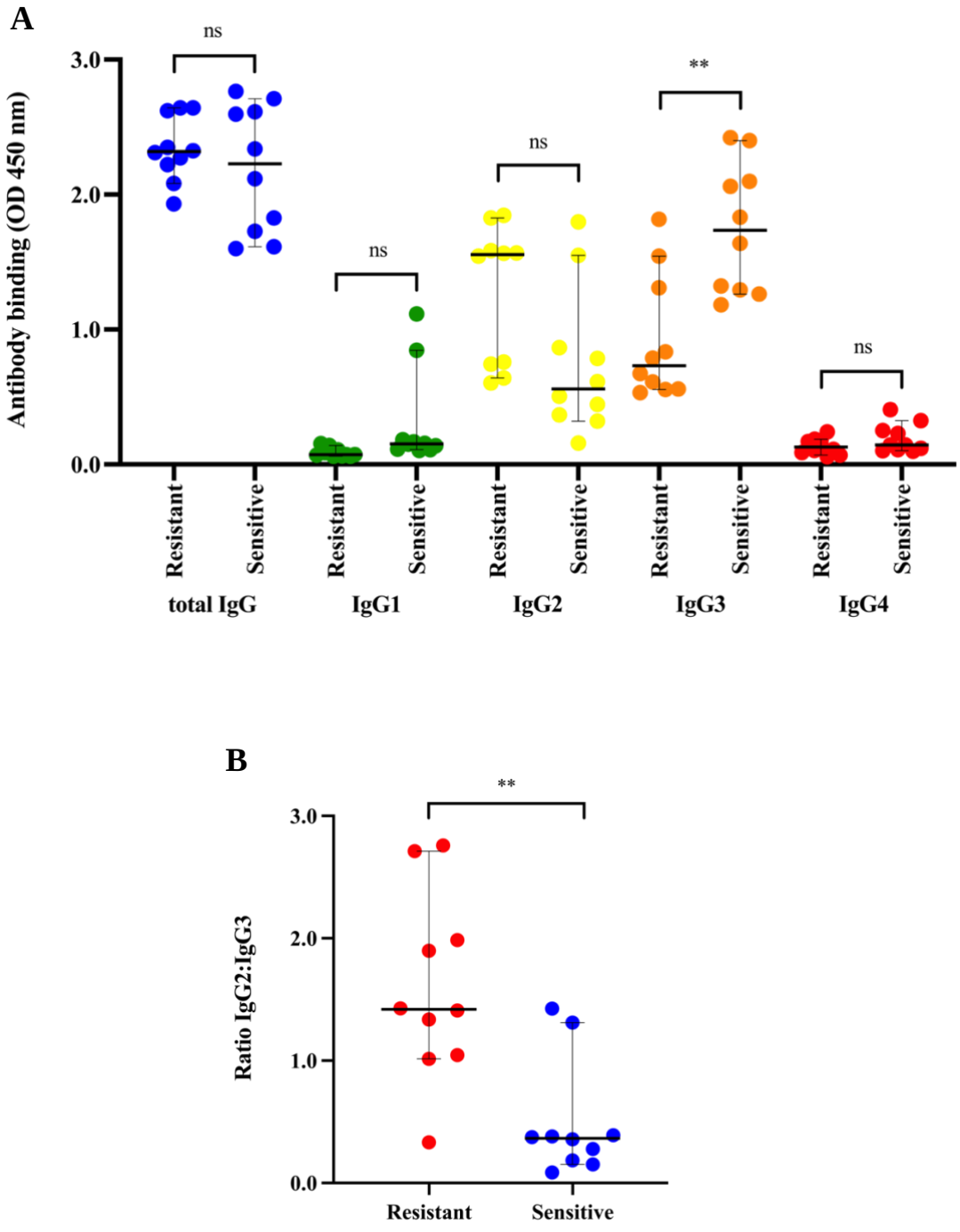


Figure 5.7 Comparison of IgG subclasses binding to serum-resistant and serum-sensitive isolates. An ELISA was used to investigate the amount of different IgG subclasses that bound to whole cell *N. gonorrhoeae*. **A)** Total IgG, IgG1, IgG2, IgG3 and IgG4 that bound to ten resistant isolates from autologous inhibitory serum were measured and compared to the binding of the same subclasses to ten isolates sensitive to autologous serum, at a 1 in 20 serum dilution, with an $OD_{450\ nm}$ measured upon assay completion. **B)** The ratios between IgG2:IgG3 that bound each of the ten resistant and sensitive isolates were calculated and compared. Data shown are the mean and standard deviation from two independent experiments. Statistical significance was measured using an unpaired t-test, where ** ($p \leq 0.01$).

1:4000-1:10,000 and 1:4000-1:8000, respectively. Therefore, ELISAs were set up using sensitive isolate NG027 and autologous serum, using three dilutions of each secondary antibody, including the lowest and highest recommended dilutions and one dilution in between each of these. The lowest dilution of each secondary antibody gave the highest signal and was therefore selected for subsequent use (Figure 5.6).

These same ELISAs were then used to investigate the subclass of the inhibitory IgG binding to all eleven inhibitory candidates found to be resistant to the 1:1 serum mix (see Figure 4.19). Isolate NG319 was not assayed due to the lack of sufficient serum. The IgG subclass binding titers of autologous inhibitory serum to these ten isolates were compared to ten sensitive isolates with their matching sensitive serum, chosen to mirror the participant demographics of the resistant isolates. There were no significant differences of binding of total IgG, IgG1 and IgG4 between the resistant and sensitive isolates (Figure 5.7.A). This similarity between the total IgG binding to resistant and sensitive isolates was no surprise as this was consistent with the flow cytometry data previously described (Figure 4.12.A). However, there had been an increase in binding of IgG2 to resistant inhibitory isolates, but due to the limited data available, this was not statistically significant. There was a statistically significant decrease in binding of IgG3 in the inhibitory serum to resistant isolates compared to sensitive isolates with their killing serum. IgG3 is the main classical complement pathway activator of all the IgG subclasses, so this could explain why there are increased binding of this subclass to sensitive isolates that are being killed by their autologous serum. IgG2 is not a strong complement activator, so this increased binding to the resistant isolates could be linked to the inhibitory phenotype, suggesting that IgG2 might be the inhibitory antibody. There is also less IgG3 on these isolates, which could suggest the IgG2 is binding first and preventing IgG3 from binding or sterically hindering the binding of IgG3, also resulting in this inhibitory phenotype. A closer examination of the ratio between IgG2:IgG3, found a statistically significant increase amongst resistant isolates compared with

sensitive isolates (Figure 5.7.B), suggesting that the proportions of these antibodies binding to the bacterial cell surface may determine serum susceptibility.

5.2.3 The effect of inhibitory serum on isolates that were sensitive to their autologous serum

To understand whether the inhibitory serum is able to protect only the autologous isolates derived from the same host, a 'serum-swap' experiment was set up to assay other isolates against the inhibitory serum. NG332-serum and NG274-serum were used as they had the strongest inhibitory antibody phenotype. Isolates previously found to be sensitive to their matched serum, NG233, NG006 and NG297, were incubated at 37ºC for 3 hours with NG332-serum (Figure 5.8.B) and NG274-serum (Figure 5.9.B). Sensitive isolates NG233 and NG006 survived in both inhibitory sera. This confirmed that it is the sera that is protecting these sensitive isolates from killing and that the inhibitory sera is not specific to the autologous isolate from the participant, but it is also able to inhibit other isolates that have not previously infected the host. However, isolate NG297 was killed by both inhibitory sera. This shows that these inhibitory sera are not able to protect all isolates. It also suggests that there must be bactericidal antibodies within the inhibitory serum as well. Potentially some isolates might bind greater amounts of the inhibitory antibodies, and others might bind more bactericidal antibodies.

Inhibitory isolates NG332 and NG274 were able to be killed by NG233-serum, NG006-serum and NG297-serum (Figure 5.8.A and Figure 5.9.A respectively). This again confirms that these 'resistant' isolates are actually serum sensitive, and it is their autologous serum that is inhibiting killing.

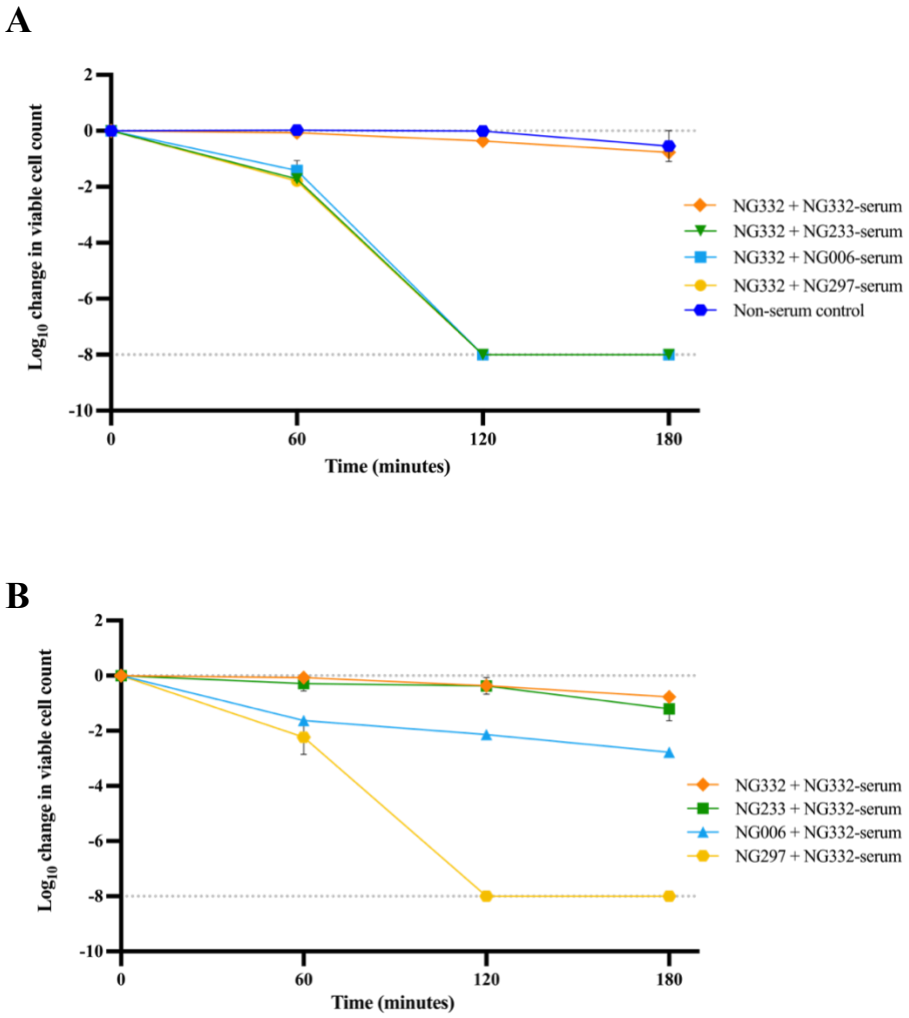


Figure 5.8 Serum bactericidal assay of inhibitory NG332-serum and the autologous isolate. A serum bactericidal assay (SBA) was used to determine **A)** the sensitivity of isolate NG332 in NG233-serum, NG006-serum and NG297-serum, and **B)** inhibitory properties of NG332-serum on sensitive isolates NG233, NG006 and NG297. The change in viable colony forming units after 60, 120 and 180 minutes was measured, with negative values corresponding to a decrease in viable bacteria when compared to the initial inoculum. Values at -8 indicate the limit of detection for samples from which no colony formed even from the least diluted sample plated. Data shown are the mean and standard deviation from two independent experiments.

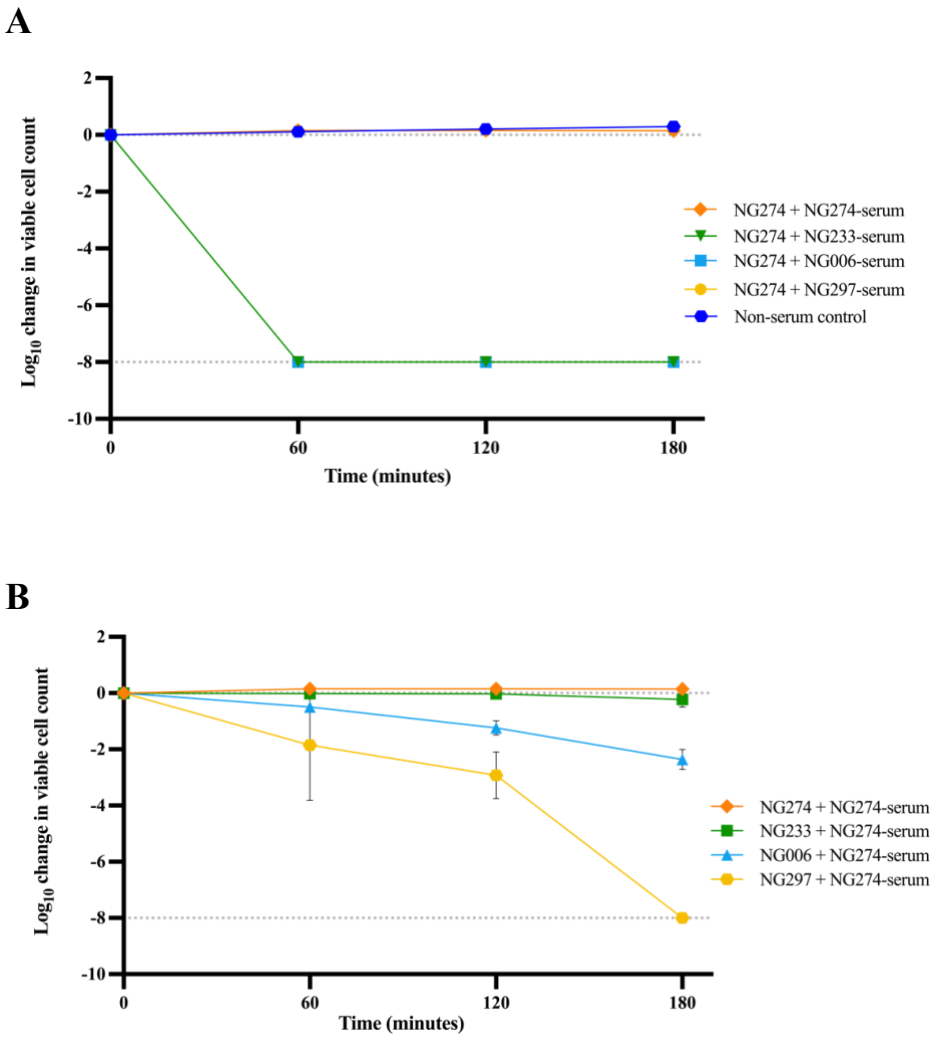


Figure 5.9 Serum bactericidal assay of inhibitory NG274-serum and the autologous isolate. A serum bactericidal assay (SBA) was used to determine **A)** the sensitivity of isolate NG274 in NG233-serum, NG006-serum and NG297-serum, and **B)** inhibitory properties of NG274-serum on sensitive isolates NG233, NG006 and NG297. The change in viable colony forming units after 60, 120 and 180 minutes was measured, with negative values corresponding to a decrease in viable bacteria when compared to the initial inoculum. Values at -8 indicate the limit of detection for samples from which no colony formed even from the least diluted sample plated. Data shown are the mean and standard deviation from two independent experiments.

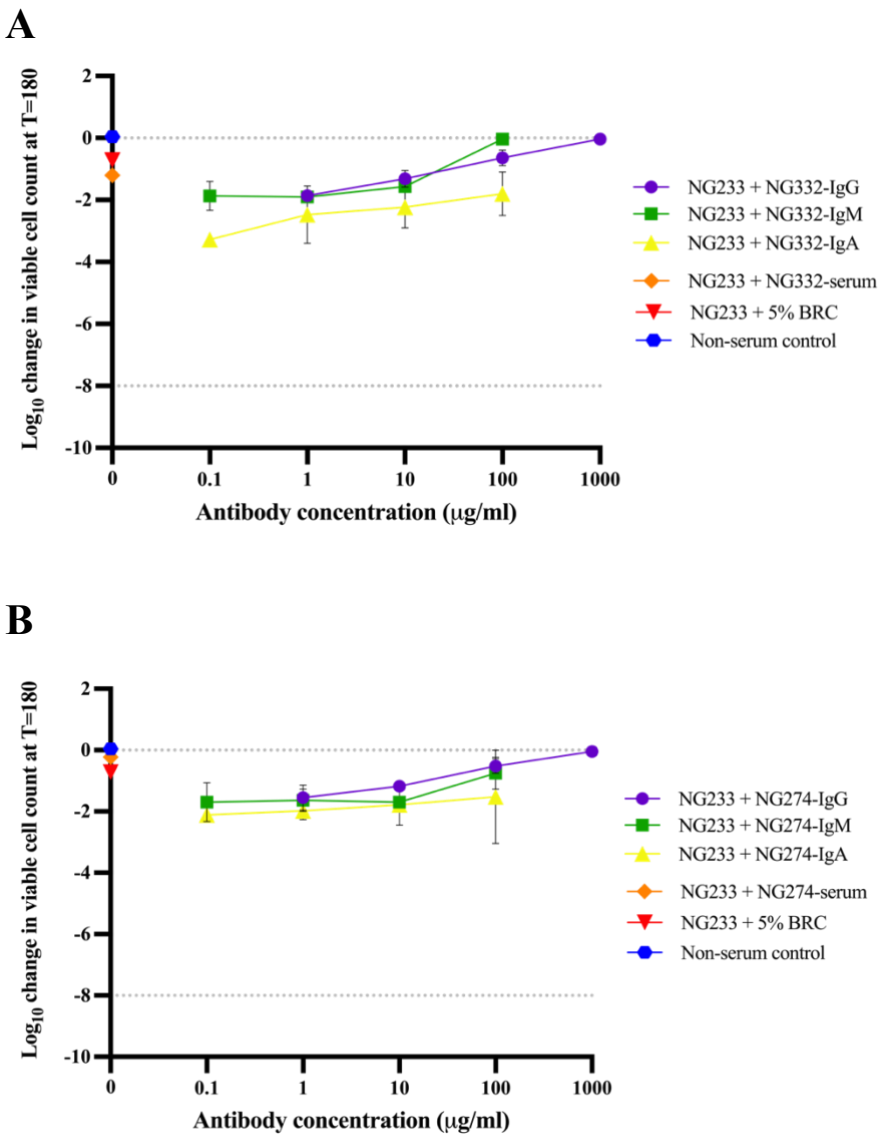


Figure 5.10 Effect of total IgG, IgM and IgA purified from inhibitory NG332-serum and NG274-serum on isolate NG233. A serum bactericidal assay (SBA) was used to determine the effect of antibodies IgG, IgM and IgA purified from inhibitory **A)** NG332-serum and **B)** NG274-serum, on sensitive isolate NG223 which was found to be resistant in both inhibitory sera. Cultures of isolate NG233 were incubated with a mixture of 5% baby rabbit complement (BRC) and 95% of each autologous purified antibody at various concentrations. The change in viable colony forming units after 180 minutes was measured, with negative values corresponding to a decrease in viable bacteria when compared to the initial inoculum. Values at -8 indicate the limit of detection for samples from which no colony formed even from the least diluted sample plated. Data shown are the mean and standard deviation from two independent experiments.

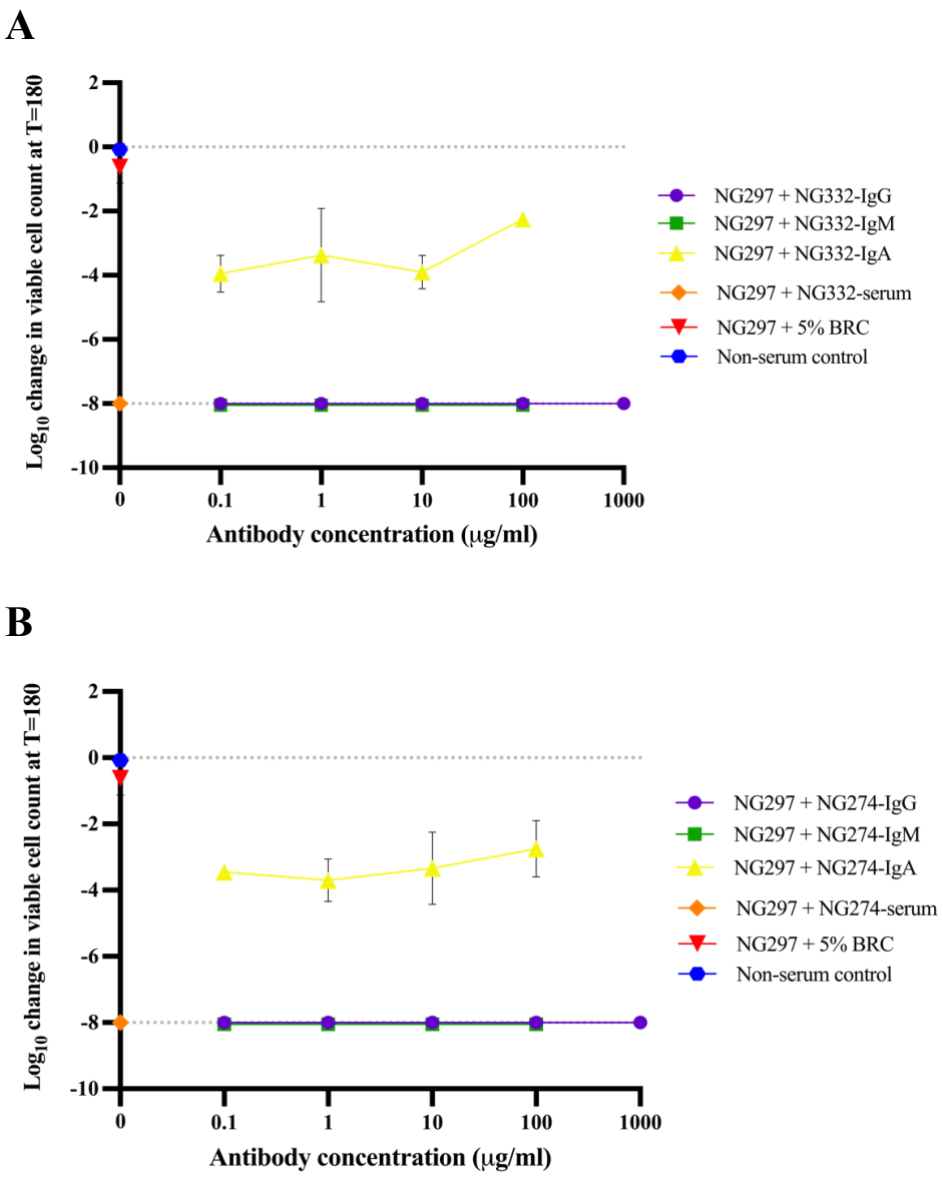


Figure 5.11 Effect of total IgG, IgM and IgA purified from inhibitory NG332-serum and NG274-serum on isolate NG297. A serum bactericidal assay (SBA) was used to determine the effect of antibodies IgG, IgM and IgA purified from inhibitory **A)** NG332-serum and **B)** NG274-serum, on sensitive isolate NG297 which was found to be resistant in both inhibitory sera. Cultures of isolate NG297 were incubated with a mixture of 5% baby rabbit complement (BRC) and 95% of each autologous purified antibody at various concentrations. The change in viable colony forming units after 180 minutes was measured, with negative values corresponding to a decrease in viable bacteria when compared to the initial inoculum. Values at -8 indicate the limit of detection for samples from which no colony formed even from the least diluted sample plated. Data shown are the mean and standard deviation from two independent experiments.

5.2.4 The effect of purified inhibitory antibodies on isolates that were sensitive to their autologous serum

To understand whether antibodies were also the inhibitory factor in this serum that were protecting the sensitive isolates NG233 and NG006 from killing, another modified SBA was set up like those described in Section 5.2.1. Cultures of one of these isolates, NG233, were incubated at 37ºC for 3 hours with 5% BRC and each individual purified antibody from both inhibitory NG332- serum and NG274-serum, at antibody concentrations from 1 ng/ml to 1 mg/ml for IgG and 1 ng/ml to 0.1 mg/ml for IgM and IgA, all diluted in PBS.

Isolate NG223 was able to survive after the addition of either of the three antibody isotypes purified from inhibitory NG332-serum and NG274-serum, at all concentrations trialled (Figure 5.10). This therefore suggests these antibodies are able to protect a different sensitive isolate from killing.

The same experiment was also set up to understand whether it had been the antibodies in the inhibitory NG332-serum and NG274-serum that did not protect the sensitive isolate NG297 from killing. The same experiment was set up with cultures of NG297. Isolate NG297 was not able to survive after the addition of either of the three antibody isotypes purified from inhibitory NG332-serum and NG274-serum, at all concentrations trialled (Figure 5.11). This therefore suggests these antibodies were not able to protect this particular sensitive isolate from killing, and were able to induce killing.

The three isolates NG332 (isolate with matched inhibitory sera), NG233 (isolate protected by inhibitory sera) and NG297 (isolate killed by inhibitory sera) were all assayed with inhibitory NG332-serum to determine binding of total IgG, IgG1, IgG2, IgG3 and IgG4 (Figure 5.12.A). A comparison between the protected isolates (NG332 and NG233) and the killed isolate (NG297) revealed no differences of binding of total IgG, IgG1 and IgG4 between any of the three isolates. However, there was a statistically significant decrease in binding of IgG2 to killed

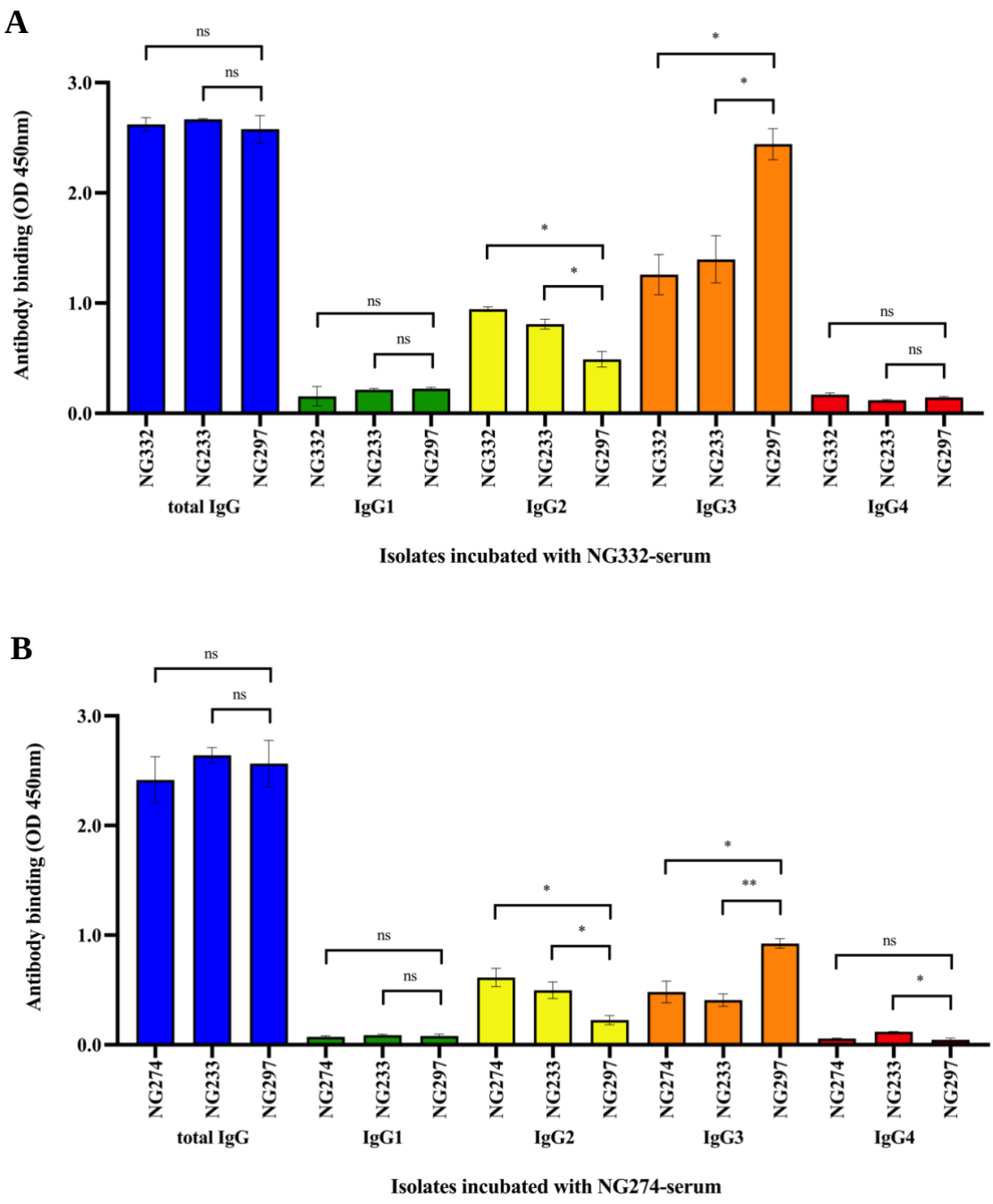


Figure 5.12 Comparison of binding of IgG subclasses from inhibitory sera to isolates NG233 and NG297. An ELISA was used to investigate the amount of different IgG subclasses, including total IgG, IgG1, IgG2, IgG3 and IgG4, binding to whole cell *N. gonorrhoeae* isolates **A)** NG332, NG233, NG297 with inhibitory NG332-serum and **B)** NG274, NG233, NG297 with inhibitory NG274-serum. A 1 in 20 serum dilution, with an $OD_{450\ nm}$ measured upon assay completion. Data shown are the mean and standard deviation from two independent experiments. Statistical significance was measured using an unpaired t-test, where * ($p \leq 0.05$) and ** ($p \leq 0.01$).

isolate NG297 compared to NG332 and NG233 that were protected by the inhibitory sera. There was also a statistically significant increase in binding of IgG3 to killed isolate NG297 compared to the other isolates that were protected by the inhibitory sera. Intriguingly, these results suggest that the inhibitory phenotype might be induced by an increase in subclass IgG2 binding and a decrease in subclass IgG3 binding. Very similar results were obtained with inhibitory NG274-serum (Figure 5.12.B), which adds weight to the hypothesis that the inhibitory phenotype is induced by an increase in subclass IgG2 and a decrease in subclass IgG3.

Previously, during the serum swap experiments, inhibitory isolates NG332 and NG274 were also assayed in the sensitive killing NG233-serum and NG297-serum, and the results showed that both isolates were killed in both of these sera (Figures 5.8.A and Figure 5.9.A respectively). Therefore, a whole cell ELISA was used again to determine binding of total IgG, IgG1, IgG2, IgG3 and IgG4 from the killing sera to inhibitory isolates NG332 and NG274. The binding titers were compared with the binding of these isolates to their autologous inhibitory sera. There was no difference of binding of total IgG, IgG1 and IgG4 from any of the three sera to isolate NG332 (Figure 5.13.A). However, there was a statistically significant decrease in binding of IgG2 to NG332 when incubated with both killing sera compared to its autologous inhibitory sera. There was also a statistically significant increase in binding of IgG3 from killing serum (autologous to NG297) to isolate NG332 when compared to its autologous inhibitory serum. There was also a slight increase in binding of IgG3 from killing serum (autologous to NG233) compared to its autologous inhibitory serum, but this difference was not statistically significant. This suggests that increased binding of IgG3 and decreased binding of IgG2 to the bacterium results in killing of that isolate, which also confirms the principle of the inhibitory phenotype. Similar results were obtained with isolate NG274, which adds weight to the hypothesis that the inhibitory phenotype is induced by an increase in subclass IgG2 and a decrease in subclass IgG3 (Figure 5.13.B). This result shows that these inhibitory isolates

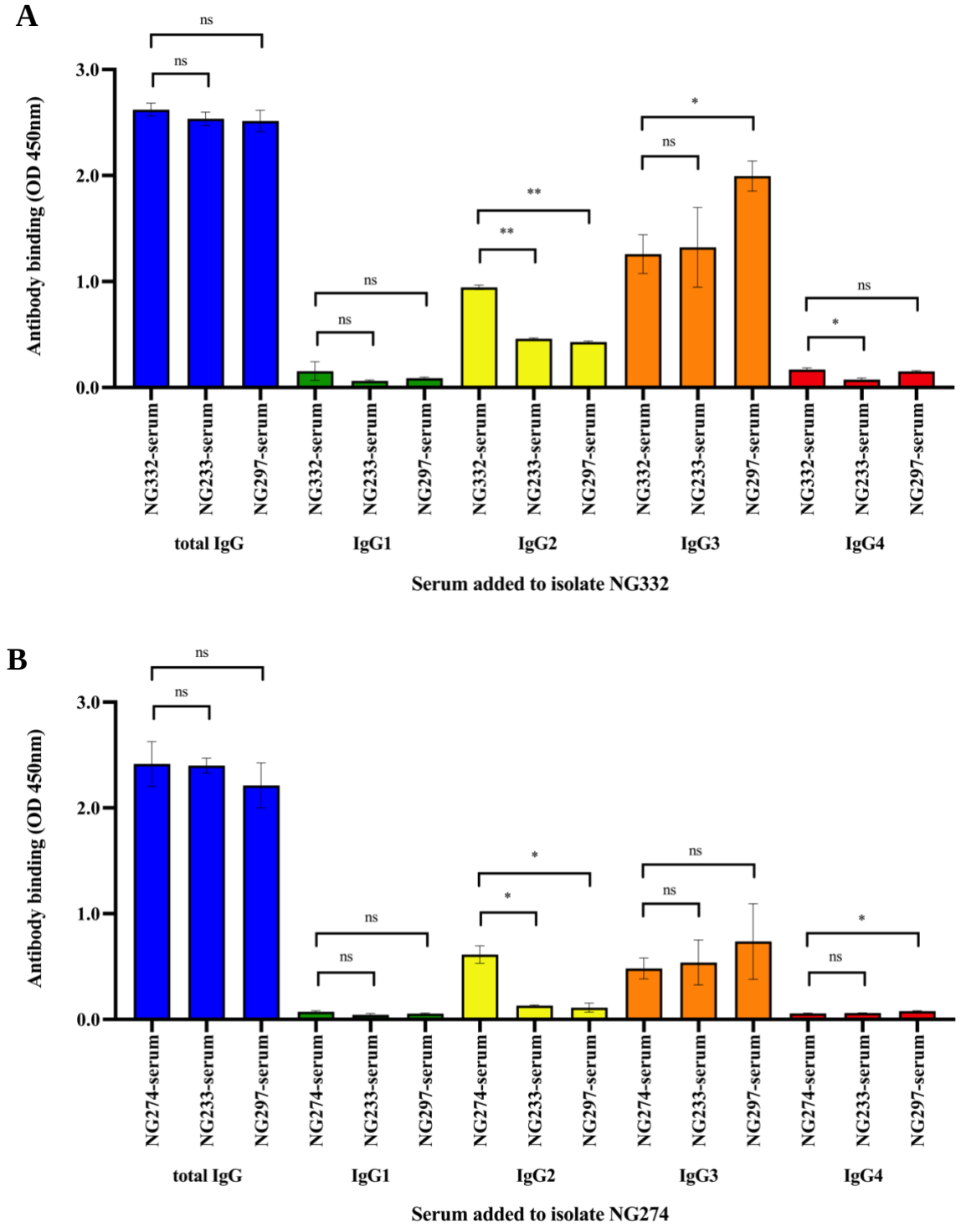


Figure 5.13 Comparison of binding of IgG subclasses from NG233-serum and NG297-serum to inhibitory isolates. An ELISA was used to investigate the amount of different IgG subclasses, including total IgG, IgG1, IgG2, IgG3 and IgG4, binding to whole cell *N. gonorrhoeae* isolates **A)** NG332 incubated with autologous serum, NG233-serum and NG297-serum, and **B)** NG274 incubated with autologous serum, NG233-serum and NG297-serum. A 1 in 20 serum dilution, with an OD $_{450\ nm}$ measured upon assay completion. Data shown are the mean and standard deviation from two independent experiments. Statistical significance was measured using an unpaired t-test, where * ($p \leq 0.05$) and ** ($p \leq 0.01$).

(NG332 and NG274) are both able to be killed by binding IgG3 from both the killing NG223-serum and NG297-serum. The amounts of IgG3 bound to the isolates from the two sensitive sera are not very different to the amount bound from their own inhibitory sera. Therefore, this suggests that the binding of less IgG2 is a major factor in the killing of isolates by both killing sera. This therefore again suggests that high binding of IgG2 is responsible for the inhibitory phenotype, which might prevent IgG3 reaching the surface and initiating complement-mediated killing.

5.3 Discussion

To summarise, experiments with antibodies purified from inhibitory candidate serum confirmed that antibodies were the inhibitory factor in the serum which protected *N. gonorrhoeae* from HCS-mediated killing. This study provided evidence that all three antibody isotypes, IgG, IgM and IgA were able to inhibit killing of *N. gonorrhoeae*. In agreement, past studies have demonstrated that during bacterial infection, some patients can develop inhibitory antibodies comprising of a variety of antibody isotypes, also including IgG, IgM and IgA (Amir *et al.*, 2005, Corbeil *et al.*, 1988, Pham *et al.*, 2021, Goh *et al.*, 2016).

In this study, it was found that inhibition of all isotypes had been concentration dependent. High concentrations of purified IgG and IgM inhibitory antibodies were able to inhibit killing by HCS. However, at low antibody concentrations with BRC, these antibodies were able to kill autologous isolates. Goh *et al.* (2016) also found that antibody titer was critical for inhibition, as lower concentrations of purified anti-LPS IgG and IgM antibodies were able to achieve normal bactericidal activity of *S.* Typhimirium, whereas 10-fold higher concentrations of IgG, IgM and IgA all inhibited HCS-mediated killing. To further this, serum swap experiments in this study demonstrated that inhibitory sera and purified antibodies (from NG332-serum and NG274-serum) were able to protect some different gonococcal isolates (NG233 and NG006),

but not others (NG297). This again suggested that the inhibitory serum contains both inhibitory and bactericidal antibodies that bind differently to varying isolates. Overall, these findings suggest that the ratio of inhibitory to bactericidal antibodies within the serum is an important factor for inhibition.

In Chapter 4, data from flow cytometry experiments initially found no differences in binding titers of any antibody isotypes to resistant isolates, when compared with sensitive isolates. Previous studies found that high binding titers of antibodies were an important factor in determining the occurrence of inhibitory antibodies (Goh *et al.*, 2016, Wells *et al.*, 2014), however thus far similar findings had not been detected in this study. Data from ELISA experiments also showed no differences in binding of total IgG between resistant and sensitive isolates. However, when individual IgG subclasses were analysed, the differences in binding titer between resistant and sensitive isolates could now be observed. It was demonstrated that there had been an overall increase of binding of IgG2 and a decrease in IgG3 binding to resistant inhibitory isolates, and conversely, that sensitive isolates bind decreased levels of IgG2 and increased levels of IgG3. There are many other past studies which attribute the IgG2 subclass to causing the inhibition of complement-mediated killing of other Gram-negative pathogens (Pham *et al.*, 2021, Goh *et al.*, 2016, Coggon *et al.*, 2018), but in these studies they did not observe any differences in binding of IgG3 (Wells *et al.*, 2014, Goh *et al.*, 2016, Coggon *et al.*, 2018). Therefore, this study reveals a novel mechanism relating to the ratios of IgG2 versus IgG3 binding on the gonococci surface. Our whole cell ELISA data using inhibitory isolates (NG332 and NG274) and sensitive isolates (NG223 and NG297) shows that as the concentration of IgG2 binding increases, survival of the bacterium increases**,** however, as the concentration of IgG3 binding increases, survival of the bacterium decreases (summarised in Figure 5.14).

Previous studies have mentioned the varied differences in inhibitory antibody specificities for target antigens on the outer membrane, including LPS and polysaccharide

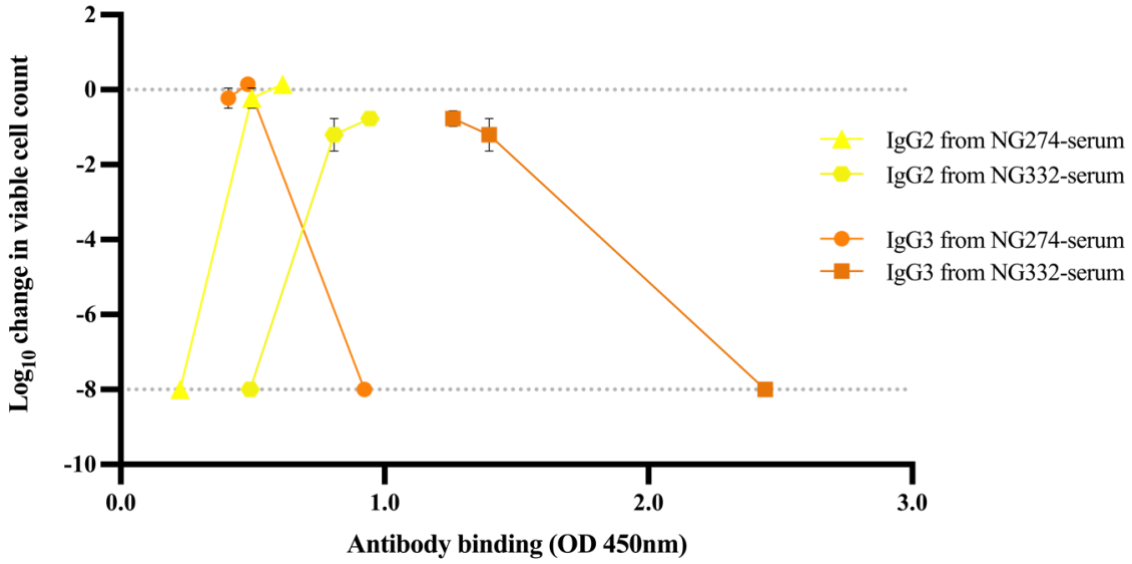


Figure 5.14 Effect of binding IgG2 and IgG3 from inhibitory serum on *Neisseria gonorrhoeae* survival. Data from ELISA and SBA experiments showing the effects on the survival of each autologous inhibitory isolates, NG223 and NG297 after binding IgG2 and IgG3 from inhibitory NG332-seurm and NG274-serum. The change in viable colony forming units after 180 minutes was measured, with negative values corresponding to a decrease in viable bacteria when compared to the initial inoculum. Values at -8 indicate the limit of detection for samples from which no colony formed even from the least diluted sample plated. Data shown are the mean and standard deviation from two independent experiments.

capsule (Wells *et al.*, 2014, MacLennan *et al.*, 2010, Griffiss and Bertram, 1977). In this study the target of the inhibitory antibodies is unknown. Given that there is an increase in binding of IgG2, we hypothesise that this mechanism is distinct to the Rmp inhibitory antibodies previously described (Rice *et al.*, 1986), given that IgG2 largely binds to polysaccharide targets (Siber *et al.*, 1980, Schauer *et al.*, 2003). Therefore, these IgG2 antibodies could potentially be binding LOS, similar to the LPS studies described previously (Goh *et al.*, 2016, Pham *et al.*, 2021). The variability in inhibitory antibody isotypes and potential antigen targets suggests there could be multiple mechanisms inciting antibody mediated inhibition. Further investigations are required to understand the targets for the inhibitory IgA, IgM, IgG2 and bactericidal IgG3 antibodies against *N. gonorrhoeae.*

CHAPTER 6

FINAL DISCUSSION

6.1 General summary

Overall, the main aim of this PhD project was to investigate whether inhibitory antibodies occur in patients during infection with acute gonorrhoea. This had been undertaken utilising the large collection of clinical *N. gonorrhoeae* isolates from participants of the G-ToG clinical trial, along with matching serum from each participant, allowing for a large study of immune responses during gonococcal infection. All 336 *N. gonorrhoeae* isolates were screened for resistance to serum against the autologous participant serum. Initial serum assays determine that 14% (46/336) of all isolates were resistant to serum killing against their matching participant's serum. These 46 resistant isolates were then screened against a pool of HCS from individuals with no previous history of gonococcal infection, where interestingly, 43% (20/46) of these participant resistant isolates were sensitive to HCS. These 20 isolates were challenged with a 1:1 mixture of both sera, where eleven isolates remained resistant. These eleven isolates were candidates for inducing the production of inhibitory antibodies.

Fluorescent flow cytometry was then utilised to measure antibody and complement deposition to resistant isolates, and data surprisingly revealed that higher levels of C3c bound simultaneously with IgG to the majority of resistant isolates when compared to the sensitive isolates. However, this correlation did not directly relate to the inhibitory antibody candidate isolates. Therefore, to investigate the eleven candidates further, GAM antibodies were each individually purified from autologous participant serum. These isolates were assayed in the 1:1 serum mixture assay again, with each purified antibody replacing participant serum. Of the six isolates tested, all were protected by IgG and IgM at the highest concentrations trialled. Two of these isolates (NG332 and NG274) were protected even at low antibody concentrations. IgA was able to protect half of the isolates trialled. This suggests that any antibody isotype can inhibit killing of HCS, but this blocking is concentration dependent. Further investigation of inhibitory IgG antibodies suggested there was increase in binding of IgG2 and decrease in binding of IgG3

to inhibitory isolates. Assaying a different sensitive isolate (NG233) in inhibitory NG332-serum and NG274-serum found that these sera were also able to protect it from killing. This suggests the inhibitory serum is not isolate specific. Purified inhibitory IgG and IgM antibodies from the same sera (NG332-serum and NG274-serum), were also able to protect this isolate. However, when assaying a different sensitive isolate (NG297) in inhibitory NG332-serum and NG274-serum, it was discovered that these sera were not able to protect NG297 from killing. This suggested inhibitory serum is not able to protect all isolates, and bactericidal antibodies must be present in these inhibitory sera. Purified inhibitory IgG and IgM antibodies from the same sera were not able to protect this isolate either.

Further IgG subclass analysis of bound inhibitory NG332-serum to these two different sensitive isolates (NG233 and NG297) revealed there was a decrease in binding of IgG2 to NG297 (killed isolate) compared to NG332 and NG233 (isolates protected by the inhibitory sera). There were also increases in binding of IgG3 to NG297 compared to the other two isolates that were protected by the inhibitory sera. Very similar results were obtained during the same experiment with inhibitory NG274-serum. This suggests that the inhibitory IgG phenotype is a result of an increased binding of IgG2 and a decreased in binding of IgG3 to the bacterial cell surface.

All isolates were also whole genome sequenced, and phylogenomic data was correlated with the immune response data. Initial annotation analysis of isolates resistant to autologous serum on the phylogenomic tree found no obvious genetic link between the isolates. Further GWAS analysis to determine SNP relationships between resistant isolates also gave no significant findings. However, the genomics analysis did yield some interesting results, which were unrelated to the inhibitory antibody phenotype. Several MLSTs that resided adjacently on the phylogenomic tree, such as ST7363, ST11864 and ST9363, were found to have significantly higher MICs to both or one of the treatment antibiotics, ceftriaxone and azithromycin. These

isolates also correlated with the male participants, with a significant increase in isolates with a higher MIC to azithromycin within the MSM network. In contrast, a different set of isolates that clustered near each other on the phylogenomic tree, including MLSTs ST1594, ST11990, ST8135 and ST8163, were found to have significantly lower MICs to one or both of the treatment antibiotics. The isolates within these groups also correlated with a reduction in reported symptoms, which directly related to the observation that these isolates were also associated with female participants, who are known in the literature to be more likely to be asymptomatic compared to males. Upon closer inspection, the majority of these isolates had been derived from the cervical anatomical site, which encompassed the majority of cervical isolates within the whole population that had clustered in one region of the tree. Within this cluster, the few males interspersed between these female isolates were of the MSW sexual network, which suggests that these isolates were predominantly circulating within heterosexual individuals. This could imply that these isolates have an adaptation advantage for infecting and residing within females in the genital tract environment, or that they have evolved to have advantages in transmission from females to males.

6.2 Isotypes of inhibitory antibodies

A recent review by Torres *et al.* (2021) detailed the role of inhibitory antibodies during bacterial disease and described many of the previously mentioned studies, where the production of inhibitory antibodies have been induced by Gram-negative bacteria. These earlier studies have demonstrated that various antibody isotypes are able to inhibit complement-mediated killing in bacteria. Antibodies IgA, IgM and IgG have all been implicated in the inhibition of killing (Wells *et al.*, 2014, MacLennan *et al.*, 2010, Ray *et al.*, 2011, Goh *et al.*, 2016, Pham *et al.*, 2021, Amir *et al.*, 2005, Corbeil *et al.*, 1988). It had also been found that these antibodies have specificities for a variety of target antigens on the bacterial surface, including LPS, capsule, lipoproteins and

other outer membrane proteins (MacLennan *et al.*, 2010, Amir *et al.*, 2005, Ray *et al.*, 2011). Consistent with this previous work, this study has shown that all three antibody isotypes, IgA, IgM and IgG (specifically IgG2) were able to inhibit killing of *N. gonorrhoeae* by HCS-mediated killing.

6.2.1 Inhibitory IgA

IgA antibodies are known to bind both polysaccharide and protein antigens (detailed in Table 1.1). This isotype had been found to be inhibitory in studies involving *N. meningitidis* towards capsular polysaccharides (Jarvis and Griffiss, 1991) and with both *S.* Typhimurium and *P. aeruginosa* towards LPS (Goh *et al.*, 2016, Pham *et al.*, 2021). IgA was able to inhibit killing of HCS against half of the *N. gonorrhoeae* isolates tested in this study. However, in contrast to both previous studies mentioned, *N. gonorrhoeae* does not have a capsule like *N. meningitidis*, nor does it have O-antigen LPS like *S.* Typhimurium and *P. aeruginosa*. Therefore, it can be assumed that inhibitory IgA is binding a different antigen target.

Further, Griffiss (1975) found that IgA purified from *N. meningitidis*-infected patients, inhibited both bactericidal IgG and IgM from complement-mediated killing. This inhibition was dependent on the ratio of inhibitory to bactericidal antibodies, and found to be greater for IgG than IgM. Future experiments to determine the ratios of different antibody isotypes required for inhibition or killing of *N. gonorrhoeae*, utilising the purified antibodies from gonococcal inhibitory participant serum from this study, would aid in the understanding of antibody elicitation required during vaccination for a killing response to be produced.

6.2.2 Inhibitory IgM

Similarly to IgA, IgM antibodies are also known to bind both polysaccharide and protein antigens (detailed in Table 1.1). A study by Trebicka *et al.* (2013) showed that some healthy serum

contained IgM inhibitory antibodies against *S.* Typhimurium. However, they also showed that other anti-LPS antibodies were important in bactericidal activity. This suggested that anti-LPS antibodies both inhibit and promote complement-mediated killing. To further this, Goh *et al.* (2016) also demonstrated that IgM had been able to inhibit killing of *S.* Typhimurium, however the majority of evidence indicated that anti-LPS IgM is bactericidal in serum.

This is very similar to results found in this study. In all *N. gonorrhoeae* isolates trialled, purified IgM was found to inhibit killing of HCS at high concentrations. IgM is a known bactericidal antibody against *N. gonorrhoeae*, and when purified, these antibodies were able to initiate complement-mediated killing at low concentrations in the presence of BRC. Therefore, given their ability to protect the bacterium, it suggests that inhibition of HCS mediated-killing is concentration dependent, and predominantly occurs at high IgM antibody concentrations.

6.2.3 Inhibitory IgG

IgG antibodies are also known to bind both polysaccharide and protein antigens, in a subclass-dependent manner (detailed in Table 1.1). Polysaccharides are the binding preference for IgG2, and given the limited amount of polysaccharide structures on the gonococcal surface, it can be assumed its main targets might be LOS or lipoproteins. In contrast IgG1, IgG3 and IgG4 all prefer binding protein structures, which include Opa, pili, Por and Rmp, as example antigen targets on the gonococcal surface.

Literature concerning *P. aeruginosa* (Wells *et al.*, 2014), *S.* Typhimurium (Goh *et al.*, 2016) and *E. coli* (Coggon *et al.*, 2018), all demonstrated that the inhibitory antibody was identified as IgG2 specifically targeting the O-antigen of LPS. In all three studies, inhibition had been caused by excessively high titers of IgG2 inhibitory antibody, where as little as 6% of inhibitory patient serum added to HCS had the ability to inhibit complement-mediated killing (Wells *et al.*, 2014). In this study, these excessively higher IgG titers binding *N. gonorrhoeae*

were not seen when total IgG binding to resistant isolates were measured using flow cytometry. However, at the IgG subclass level, similar findings could be seen, where elevations in IgG2 antibody binding to gonococcal isolates correlated with inhibition and survival.

Interestingly, Goh *et al.* (2016) further found that concentrated anti-LPS IgG from HCS resulted in the inhibition of complement-mediated killing of *S.* Typhimurium, however, the same anti-LPS IgG could be bactericidal when sufficiently diluted. Goh *et al.* (2016) also found that different epitopes on the O-antigen were potentially targets for bactericidal or inhibitory IgG antibodies, including O:4 and O:5 antigens, respectively. This again is very similar to the results found in this study, where low concentrations of purified IgG with BRC were able to kill autologous gonococcal isolates that were previously inhibited by the same antibodies at higher concentrations. *N. gonorrhoeae* does not have LPS, but instead a truncated version called LOS in which the same principle could be applied, where different LOS epitopes are targeted by bactericidal or inhibitory antibodies. Previous studies have shown that IgG2 recognises at least three distinct epitopes on *N. gonorrhoeae* LOS (Yamasaki *et al.*, 2010). LOS present on the surface of the gonococcus are very structurally diverse, due to antigenic and phase variation. Further investigations are required to determine whether there are specific epitopes of LOS that inhibitory IgG2 targets.

Previous work on serogroup B meningococci have reported sera from three out of 17 healthy individuals contained inhibitory IgG3 antibodies that were specific for two lipoproteins (Ray *et al.*, 2011), which had the ability to inhibit bactericidal killing. This is very contrasting to the results observed in this study, where it had been found that increased binding of IgG3 correlated with killing of gonococcal isolates. Further investigations are required to determine the target antigen of bactericidal IgG3.

6.3 Mechanism of inhibitory antibodies

The antibody subclass is one factor that determines an antibodies ability to activate complement. Other factors include the avidity of antibody binding and the distribution, density and accessibility of the target antigen. Due to the variety of inhibitory antibody isotypes and potential target antigens under consideration, it is highly likely that there are multiple different mechanisms underpinning the antibody-mediated inhibition.

6.3.1 Current mechanisms of inhibition by antibody

Previously, there have been two mechanisms of inhibition by antibody proposed for *P. aeruginosa* and *S.* Typhimurium (Wells *et al.*, 2014, MacLennan *et al.*, 2010). In both, high antibody titers bind to the O-antigen of LPS and then employ their inhibitory effect by either depositing complement away from the bacterial membrane or creating a physical antibody "blockade" so that access for bactericidal antibodies and MAC is obstructed. The exact mechanism is still unclear, but current evidence favours the second explanation, as many studies have found this inhibition to be dependent on high antibody titers. In agreement, during the inhibition of antibody-mediated killing of *N. meningitidis,* it had been found that if enough inhibitory IgA was present to form this "blockade", then no amount of IgM was able to restore bactericidal activity (Griffiss and Goroff, 1983).

Recently Torres *et al.* (2021) termed the proposed mechanism of inhibition of *P. aeruginosa* as "cloaking antibodies", which described the complete coverage of antibody that in turn provided protection. They proposed that the high affinity of antibody is most important for inhibition. These, or high titers of low affinity antibodies, can completely "cloak" the bacteria by binding an abundant antigen that is distal from the cell surface. This in turn created a physical blockade that prevented MAC insertion into the membrane (Figure 6.1.A). Therefore, low titers of antibody are unable to cover the entire bacterial cell surface resulting in bactericidal activity

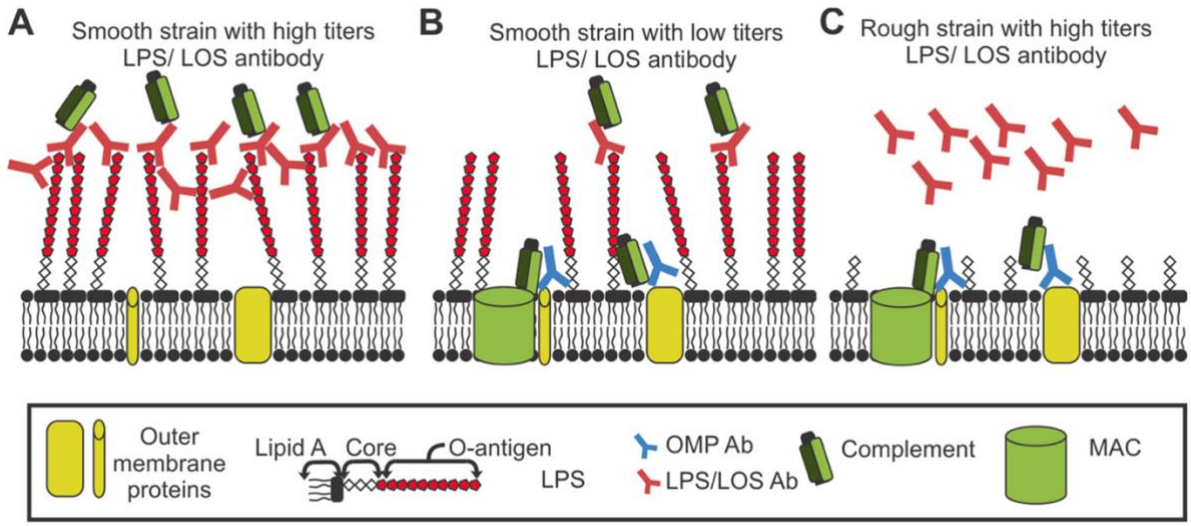


Figure 6.1 Mechanism of "cloaking antibody" as proposed by Torres *et al.* (2021). A) High-titer of O-antigen specific antibodies form a steric barrier, which prevents the membrane attack complex (MAC) from inserting into the bacterial membrane. **B)** Low titer of O-antigen specific antibodies do not prevent both bactericidal antibodies and MAC from accessing the membrane. **C)** Lack of O-antigen allows bactericidal antibodies and complement access to the membrane. Image taken from Torres *et al.* (2021).

(Figure 6.1.B). Additionally, the lack of O-antigen target restored killing by complement (Figure 6.1.C). A similar mechanism had also been assumed for *E. coli* where they suggested that high titers of "cloaking antibodies" were necessary for the bacterium to survive in the bloodstream (Coggon *et al.*, 2018). In addition, the majority of IgM is bactericidal against *S.* Typhimurium, however where IgM was found to inhibit serum killing (Goh *et al.*, 2016), they found that high titers of this low affinity molecule bound the abundant O-antigen which completely "cloaked" the bacteria. Torres *et al.* (2021) has suggested that this mechanism could apply to any antigen that is numerous and distal from the cell surface, including capsule, LOS and LPS.

The exact mechanism of inhibition for *N. gonorrhoeae* by inhibitory antibodies is yet to be determined, however there have been two proposed mechanisms for DGI strains, very similar to those previously suggested for other Gram-negative species. The first proposed mechanism involves RmpM, a conserved gonococcal outer membrane protein that binds antibody and promotes the deposition of complement, however does not induce killing. It is proposed that anti-Rmp competes with bactericidal antibodies, such as anti-LOS and anti-Por antibodies in immune serum (Rice *et al.*, 1994), for binding sites on the gonococcal cell surface (Joiner *et al.*, 1985). As the binding sites for both antibody types are in close proximity, binding of the inhibitory antibody might sterically hinder bactericidal antibodies or potentially displace the bactericidal antibody altogether. The second proposed mechanism involves the diversion of effective complement fixation onto non-bactericidal targets on the surface of the organism in the presence of anti-Rmp, potentially rendering complement function ineffective (Joiner *et al.*, 1985).

6.3.2 Proposed mechanism of inhibition for *Neisseria gonorrhoeae*

Previous studies on *N. gonorrhoeae*, found that total IgG antibody is able to both kill and block killing (Rice and Kasper, 1982). It had been suggested that this was due to either the specificity of the recognised antigen epitope or just the nature of the antibody (Rice *et al.*, 1986).

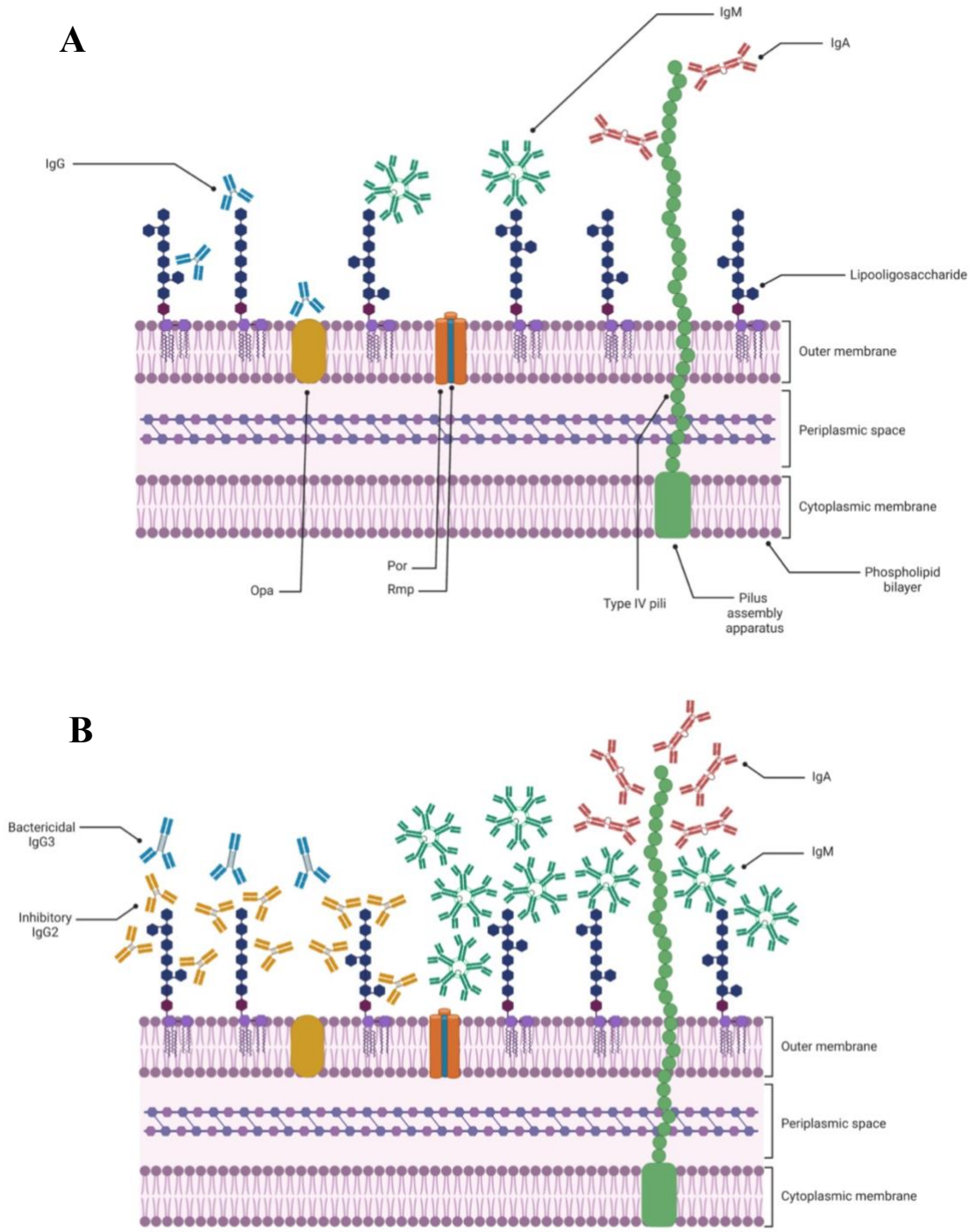


Figure 6.2 Proposed mechanism for inhibition of *Neisseria gonorrhoeae* killing by antibody. A) Typical antibody response during natural infection with *N. gonorrhoeae* targeted against outer membrane structures, where IgG usually targets pili, Por, Opa and/or lipooligosaccharide (LOS) (Lammel *et al.*, 1985), IgA usually targets pili, Por and/or Opa (Lammel *et al.*, 1985), and IgM usually targets LOS (Apicella *et al.*, 1986). **B)** Proposed inhibitory mechanism, where an increased concentration of inhibitory IgG2 binding blocks bactericidal IgG3 from binding, and a high concentration of IgM and IgA antibodies binding result in inhibition of killing.

Rice *et al.* (1986) also suggested that different subclasses of IgG bind different outer membrane antigens that in turn have varying abilities in activating complement. Therefore, they suggested that inhibitory IgG for *N. gonorrhoeae* might be predominantly subclasses that activate complement poorly, in a similar way that inhibitory IgA blocks killing of *N. meningitidis* (Griffiss, 1975).

In agreement with these assumptions, we propose a different mechanism of inhibition (Figure 6.2.B) than those previously described. This study shows that the ratio of inhibitory and bactericidal antibodies is an important factor in isolate survival, and suggests that the high binding of IgG2 antibodies are able to protect isolates from complement-mediated killing by binding lower amounts of IgG3. However, further experiments are required to confirm that IgG2 directly inhibits killing by IgG3 to strengthen the mechanism. Both IgG2 and IgG3 antibodies could be purified from serum using affinity chromatography with specificities for these IgG subclasses. Next experiments could be designed to determine levels of inhibition or killing when the bacteria are incubated with different ratios of IgG2 to IgG3.

The previously proposed mechanism of inhibitory antibodies to *N. gonorrhoeae* involved inhibitory antibodies produced against protein RmpM (Rice *et al.*, 1994, Joiner *et al.*, 1985). It is however unlikely that inhibitory IgG2 identified in this study has the same target, because this IgG subclass preferentially targets polysaccharide antigens, so it might be less likely to bind RmpM, suggesting a distinct mechanism is at play. Therefore, further experiments are required to determine the target antigen of inhibitory IgG2, and also bactericidal IgG3. Target antigens could potentially be determined by designing a pull down experiment, which would detect the physical interaction between the antibodies of interest and their binding targets, after the selected isolates have been incubated with autologous inhibitory sera. Mass spectrometry of these target structures might then provide more clarity on what the target antigens are.

Furthermore, in the same way that has been observed with IgM against *S.* Typhimurium (Goh *et al.*, 2016), concentration is a very important determinant as to whether antibodies will inhibit or kill the gonococcus. Purified IgM was found to inhibit killing of HCS at high concentrations in all isolates trialled. However, it had also been able to initiate complement-mediated killing at low concentrations in the presence of BRC, suggesting that the inhibition mechanism of HCS mediated-killing involving IgM is highly concentration dependent (Figure 6.2.B).

Recent work on *P. aeruginosa* inhibitory antibodies by Pham *et al.* (2021) suggested that antibody affinity played a more important role in inhibition than antibody titer. While titer was still an important factor, if the antibodies were low affinity, inhibition might be rendered ineffective. They also determined that low antibody titers were able to retain inhibition if the antibodies were high affinity. Overall this suggested that mechanistically, low affinity antibodies might be displaced by antibodies or complement that have a higher specificity (Pham *et al.*, 2021). To elucidate whether affinity is a factor in this study, once antigen targets for antibody binding on the gonococcus have been established, it would be interesting to calculate the affinity of inhibitory and bactericidal antibodies to these targets. This could be done in a simple assay involving a modified ELISA coated with the antigen of interest at a variety of different concentrations, and these results would help further inform the mechanism of inhibition.

The final factor with an influence on the mechanism of inhibitory antibodies, is the role of complement deposition. Previously Joiner *et al.* (1985) showed enhanced complement C3 deposition in the presence of inhibitory IgG on a DGI gonococcal strain, at new sites that do not result in serum killing. Similar results were seen in this study, as flow cytometry experiments revealed higher levels of C3c bound simultaneously with IgG to the majority of resistant isolates when compared to the sensitive isolates. Upon further analysis, this correlation however did not directly relate to the eleven inhibitory antibody candidate isolates, therefore this C3c phenotype

might only relate to a general serum resistance mechanism among *N. gonorrhoeae* isolates. However, more recent data in this study has detailed the IgG subclasses potentially involved in inhibition, therefore additional investigations could be done to determine whether these particular subclasses relate more specifically to the C3c phenotype observed.

In summary, in this study, it was established that the concentration of IgG subclasses is an important factor in the protection or killing of *N. gonorrhoeae*, and the outcome depended on the ratio of IgG2 and IgG3 binding the bacteria. If the concentration of IgG2 binding was high, it prevented IgG3 binding and therefore inhibition of killing was observed. However the opposite was witnessed when binding of IgG3 was higher. It is highly likely that IgG2 was found to be inhibitory due to it being a subclass of IgG that is a poor activator of complement. In addition to these findings, it could also be concluded that IgM, which is usually a bactericidal antibody against *N. gonorrhoeae*, could become inhibitory only when bound at high concentrations. The same was seen with IgA which could also be thought to create a "blockade" at high concentrations of binding, resulting in an inhibition of killing. Overall, it still remains unclear whether other factors contribute to this effect, such as an imbalance of antibodies produced by the host during infection, antibody affinity and binding preference or the target antigen distribution, density and accessibility. Therefore further work is required to understand these factors which will be invaluable to understanding the mechanism of inhibition.

6.4 Considerations of inhibitory antibodies in vaccine design

This study suggests that the specificity of antibody isotypes produced by the host following vaccination is an important consideration. Goh *et al.* (2016) also suggest that the protection afforded by different antibody isotypes during vaccination has implications on invasive *Salmonella* disease. A vaccine that induces antibody responses consisting mainly of IgG1 and IgM is more likely to elicit bactericidal antibodies to counter bacteremia, while induction of IgA

is likely to be significant in preventing the initial invasion of *Salmonella* in the gastrointestinal tract.

Goh *et al.* (2016) also mentioned the titer elicited was important. They found that concentrations of over 500 μg/ml and 125 μg/ml of purified IgG and IgM respectively were required to inhibit normal serum killing of *S.* Typhimurium. Overall, all sera that inhibited killing contained over 500 μg/ml of anti-LPS antibody, whereas bactericidal sera contained under 500 μg/ml of anti-LPS antibody. Immunisation has previously been reported to elicit ~15 μg/ml of specific IgG antibody in healthy adults (Naess *et al.*, 1999, Shahid *et al.*, 1995). Therefore, it had been suggested that an O-antigen-based vaccine for *S*. Typhimurium should induce antibodies at low bactericidal concentrations rather than high inhibitory concentrations. This might be an important consideration for most bacterial vaccines, as inducing the production of high antibody amounts could cause inhibition.

6.4.1 Development of an antigen-modified outer membrane vesicle for vaccination against *Neisseria gonorrhoeae*

There is a critical requirement to develop an effective vaccine for gonorrhoea, which induces a greater protective immune response than that observed during natural infection. Past vaccine development attempts have not resulted in protective immunity, where bactericidal antibodies produced during vaccination might have been hindered by the inhibitory action of other antibodies, or vaccination elicited the production of inhibitory antibodies. Therefore, future vaccine development efforts should consider the potential of inhibitory antibodies enhancing infection. It has previously been proposed by Gulati *et al.* (2015a) that the ratio of bactericidal antibodies to inhibitory antibodies defines protective immunity. Therefore, eliciting the correct ratio in the host during vaccination would prevent production of inhibitory antibodies that will protect the bacterium and elicit the production of bactericidal antibodies to a particular antigen targets.

Surveillance data on the MeNZB OMV vaccine was found to provide 31% effectiveness in cross protection against gonococcal infections (Petousis-Harris *et al.*, 2017), which have been the first observations of a vaccine that induced protection against gonorrhoea. With considerations of the results in this study, controlling the ratio of bactericidal antibodies to inhibitory antibodies that are produced by the host after vaccination could potentially be achieved by modifying the OMVs used in the vaccine (Figure 6.3). Once further studies have identified the target antigen for IgG3 and IgG2 binding, the *N. gonorrhoeae* strain utilised in OMV production could be genetically manipulated to increase production of the outer membrane antigen target for bactericidal antibody IgG3 and knock out the gene encoding the antigen target for inhibitory IgG2. The OMVs produced by the genetically modified *N. gonorrhoeae* strain would therefore have an enrichment of the antigen eliciting production of bactericidal IgG3 antibodies and depletion of the antigen eliciting production of inhibitory IgG2 antibodies. This in turn could potentially stimulate the host immune system to only elicit the production of effective bactericidal antibodies. It is also possible that only specific epitopes on these antigen targets that are required for recognition by inhibitory or bactericidal antibodies, therefore a greater understanding of the surface antigens that inhibitory and bactericidal antibodies bind would have significant implications for designing a more effective vaccine. This modified OMV could then be used in a vaccination challenge model to investigate whether immunogenicity is produced and whether the modified OMVs were more protective than the unmodified OMVs.

A recent study by Matthias *et al.* (2022) found that modifying OMVs can improve the outcome of gonococcal infection in mice. Here, OMVs from *N. meningitidis* were produced with outer membrane proteins PorA, PorB and RmpM knocked out. Mice immunised with these modified OMVs resulted in the production of antibodies that recognised a wider range of gonococcal antigens, including GuaB, MtrE, NlpD and PilQ. The modified OMVs were also found to enhance gonococcal clearance compared to wild type OMVs. Therefore, modified OMVs might

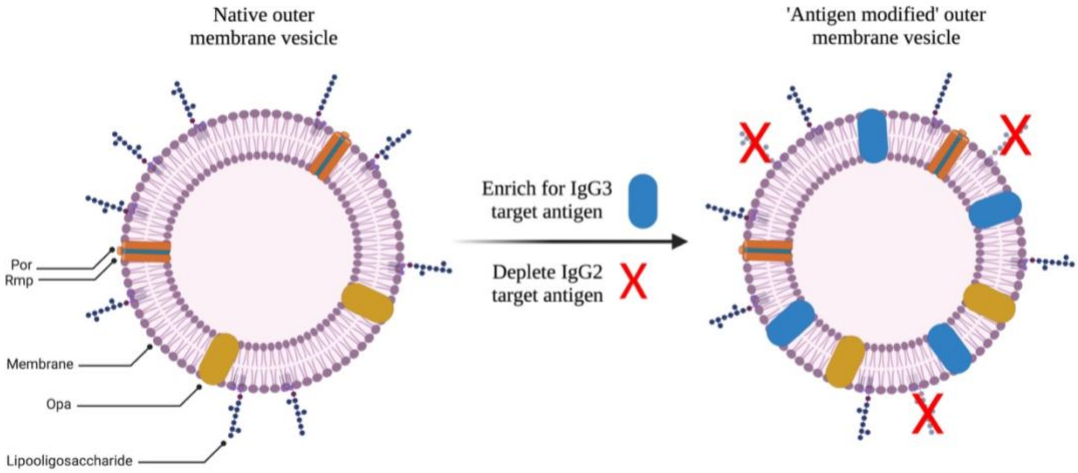


Figure 6.3 Modifications of *Neisseria gonorrhoeae* outer membrane vesicles for vaccination. Antigen modifications to be made to the native gonococcal outer membrane vesicle (OMV) used during vaccination, to control the ratio of bactericidal and inhibitory antibodies elicited by the host immune system during vaccination. This includes an enrichment for the target antigen of IgG3 (depicted by the blue structures) and a depletion of the target antigen for IgG2 (depicted by the red crosses). This would result in the production of an 'antigen modified' OMV, which should stimulate the host immune system to only elicit the production of effective bactericidal antibodies after vaccination.

provide an improved vaccine against gonorrhoea.

Studies have shown that during gonococcal infection, production of IFNγ is low (Gagliardi *et al.*, 2011, Liu and Russell, 2011, Liu *et al.*, 2012), suggesting suppression of the Th1 response. Plasma cells are likely to secrete IgG3 if matured in the presence of IFNγ. This supports the results observed in this study, where a decrease in IgG3 binding correlated with bacterial survival in serum, suggesting IgG3 production and binding is an important correlate of protection against infection, reinforcing the theory that Th1 response might be required to confer protection against *N. gonorrhoeae*. Further exploration by investigating IFNγ cytokine levels in participant serum would help investigate whether the concentrations support this hypothesis. Induction of this Th1 response has been shown to induce immune memory and clear gonococcal infection (Liu *et al.*, 2013, Criss *et al.*, 2009), however this response is not produced during natural infection. Therefore Th1 inducing adjuvants might be crucial for vaccine-induced protection. To assist in this objective, a comprehensive understanding of the correlates of protection is required, which includes further understanding of the humoral immune response. This study suggests the importance of antibody ratio on the outcome of protection, particularly the relative proportion of inhibitory and bactericidal antibodies. Utilising this information, OMV vaccine antigens could be manipulated to provoke production of the desired ratio of antibodies for host protection, and not inhibition, which might be crucial to giving the desired protective effect and therefore a successful gonococcal vaccine.

APPENDICES

G-TOG participant number	Gender	History of same sex partners	Age	Ethnicity	Location	HIV status	Previous gonorrhoea episodes	Co-current STI				Symptoms		Culture information		
								Chlamydia	Genital herpes	Syphillis	Trichomonas	Genital discharge (days)	Dysuria (days)	Lab isolate number	Lab ID	Source
1002	male	yes	29.42	White	Whittall St. Clinic	negative	no	negative		negative		no	no	NG001	RB14002901	pharynx
1004	male	yes	25.43	Other	Whittall St. Clinic	negative	1	negative		positive		7	7	NG002	RB14002903	urethra
1005	male	yes	22.79	Asian	Whittall St. Clinic	negative	1	negative		negative	negative	1	1	NG003	RB14002904	urethra
1006	female	no	16.71	White	Whittall St. Clinic	negative	no	negative		negative	negative	21	no	NG004	RB14002905	cervix
1009	female	no	21.05	White	Whittall St. Clinic	negative	no	negative		negative		no	no	NG006	RB14002907	cervix
1011	male	yes	36.19	Asian	Whittall St. Clinic	negative	1	negative		negative		no	no	NG007	RB14002908	pharynx
1012	male	yes	29.15	Black	Whittall St. Clinic	negative	no	positive		negative		3	3	NG008	RB14002909	urethra
1014	female	no	26.49	White	Whittall St. Clinic	negative	no	negative		negative	positive	16	no	NG017	RB15000534	cervix
1016	female	no	21.78	Mixed	Whittall St. Clinic	negative	2	positive				no	no	NG018	RB15000536	cervix
1018	male	no	23.66	Black	Whittall St. Clinic	negative	no	positive		negative		no	21	NG019	RB15000538	urethra
1019	male	no	25.56	Mixed	Whittall St. Clinic	negative	no	negative		negative		8	no	NG020	RB15000539	urethra
1024	male	no	27.60	Black	Whittall St. Clinic	negative	1	negative		negative	negative	1	1	NG021	RB15000544	urethra
1025	male	no	26.18	Black	Whittall St. Clinic	negative	no	negative				no	no	NG022	RB15000545	urethra
1026	female	no	22.52	Mixed	Whittall St. Clinic	negative	1	negative		negative	negative	no	no	NG023	RB15000568	cervix
1027	male	yes	25.63	Black	Whittall St. Clinic	positive	no	positive		positive		3	3	NG024	RB15000569	urethra
1030	male	yes	44.16	White	Whittall St. Clinic	negative	not known	positive		negative		no	no	NG025	RB15000572	rectum
1032	male	yes	28.61	Black	Whittall St. Clinic	negative	no	negative		negative		1	1	NG026	RB15000573	urethra
1033	male	no	21.55	Black	Whittall St. Clinic	negative	no	negative		negative		no	17	NG027	RB15000574	urethra
1036	male	yes	35.22	Black	Whittall St. Clinic	negative	1	negative		negative		no	no	NG028	RB15000576	rectum
1037	male	yes	20.09	Mixed	Whittall St. Clinic	negative	no	negative		negative		7	7	NG029	RB15000577	pharynx
1042	male	yes	22.17	White	Whittall St. Clinic	negative	3	negative		negative		no	no	NG030	RB15000579	urethra
1046	male	no	35.58	Asian	Whittall St. Clinic	negative	1	negative		positive	negative	2	2	NG031	RB15000582	urethra
1047	male	no	28.28	Mixed	Whittall St. Clinic	negative	no	positive		negative		42	no	NG032	RB15000583	urethra
1049	male	no	29.94	White	Whittall St. Clinic	negative	no	negative		negative		no	no	NG033	RB15000585	urethra
1051	male	yes	32.00	Asian	Whittall St. Clinic	negative	no	positive		negative		4	2	NG034	RB15000587	urethra
1054	male	no	19.08	Mixed	Whittall St. Clinic	negative	no	negative		negative		4	5	NG077	RB15003855	urethra
1056	male	yes	28.05	White	Whittall St. Clinic	negative	no	positive		positive		3	2	NG078	RB15003856	urethra
1057	male	yes	48.59	White	Whittall St. Clinic	positive	3	positive		negative		1	1	NG079	RB15003857	urethra
1061	male	yes	33.60	Mixed	Whittall St. Clinic	negative	1	negative		negative		2	no	NG080	RB15003859	urethra
1062	male	no	44.99	White	Whittall St. Clinic	negative	no	positive				2	no	NG081	RB15003860	urethra
1064	male	yes	35.67	Black	Whittall St. Clinic	positive	3	positive		negative		5	5	NG082	RB15003862	urethra
1065	male	no	28.84	Black	Whittall St. Clinic	negative	1	positive		negative		14	14	NG083	RB15003863	urethra
1066	male	no	23.85	White	Whittall St. Clinic	negative	no	negative		negative		3	3	NG084	RB15003864	urethra
1069	male	yes	26.77	White	Whittall St. Clinic	negative	no	negative		negative		2	2	NG085	RB15003867	urethra
1069	male	yes	26.77	White	Whittall St. Clinic	negative	no	negative		negative		2	2	NG086	RB15003868	pharynx
1072	male	no	23.71	White	Whittall St. Clinic	negative	no	positive		negative		7	no	NG094	RB15003880	urethra
1073	male	no	29.03	Black	Whittall St. Clinic	negative	1	positive		negative		1	no	NG087	RB15003870	urethra
1075	male	yes	48.84	White	Whittall St. Clinic	negative	2	negative		negative		no	no	NG088	RB15003871	rectum
1078	male	yes	32.25	White	Whittall St. Clinic	negative	1	negative		negative		no	no	NG090	RB15003873	urethra
1081	male	yes	27.62	White	Whittall St. Clinic	negative	no	negative		negative		no	no	NG091	RB15003875	rectum
1085	male	yes	27.00	Black	Whittall St. Clinic	negative	1	negative				28	28	NG092	RB15003877	urethra
1086	male	yes	36.75	White	Whittall St. Clinic	negative	no	negative		positive		no	no	NG093	RB15003878	rectum
1089	female	no	18.42	White	Whittall St. Clinic	negative	no	positive		negative	negative	no	no	NG179	RB16000904	rectum
1094	male	yes	37.89	White	Whittall St. Clinic	positive	2	negative		negative		5	no	NG180	RB16000906	urethra
1095	male	yes	23.74	Mixed	Whittall St. Clinic	negative	4	negative		negative		4	4	NG181	RB16000907	urethra
1099	male	no	25.91	Black	Whittall St. Clinic	negative	no	negative		negative		2	2	NG182	RB16000909	urethra
1100	male	no	25.52	White	Whittall St. Clinic	negative	no	positive		negative		5	5	NG183	RB16000910	urethra
1105	male	yes	29.19	White	Whittall St. Clinic	negative	1	negative		negative		6	6	NG184	RB16000911	urethra
1108	male	no	21.09	Mixed	Whittall St. Clinic	negative	no	negative		negative		21	no	NG185	RB16000912	urethra
1112	male	no	27.23	White	Whittall St. Clinic	negative	no	positive		negative		196	196	NG186	RB16000914	urethra
1113	female	no	19.39	Black	Whittall St. Clinic	negative	no	positive		negative		3	no	NG187	RB16000915	cervix
1113	female	no	19.39	Black	Whittall St. Clinic	negative	no	positive		negative		3	no	NG188	RB16000916	rectum
1115	female	no	20.95	Asian	Whittall St. Clinic	negative	no	positive		negative	negative	no	7	NG189	RB16000917	cervix
1116	male	no	24.35	Mixed	Whittall St. Clinic	negative	no	negative		negative		3	3	NG190	RB16000918	urethra
1117	female	no	28.09	Mixed	Whittall St. Clinic	negative	1	positive		negative	negative	7	no	NG191	RB16000919	cervix
1117	female	no	28.09	Mixed	Whittall St. Clinic	negative	1	positive		negative	negative	7	no	NG192	RB16000920	pharynx
1117	female	no	28.09	Mixed	Whittall St. Clinic	negative	1	positive		negative	negative	7	no	NG193	RB16000921	rectum
1118	male	yes	24.59	Asian	Whittall St. Clinic	negative	1	negative		negative		2	2	NG194	RB16000922	urethra
1121	male	no	39.50	Black	Whittall St. Clinic	negative	1	negative		negative		2	7	NG195	RB16000923	urethra
1122	female	no	24.19	Black	Whittall St. Clinic	negative	no	positive		negative	negative	no	3	NG196	RB16000924	cervix
1123	male	yes	43.45	White	Whittall St. Clinic	negative	2	negative		negative		7	4	NG197	RB16000925	urethra
1125	female	no	27.04	Mixed	Whittall St. Clinic	negative	no	positive		negative	negative	no	no	NG228	RB16001813	rectum
1125	female	no	27.04	Mixed	Whittall St. Clinic	negative	no	positive		negative	negative	no	no	NG229	RB16001814	urethra
1125	female	no	27.04	Mixed	Whittall St. Clinic	negative	no	positive		negative	negative	no	no	NG230	RB16001815	pharynx
1126	female	no	21.98	Asian	Whittall St. Clinic	negative	no	negative		negative	negative	175	28	NG231	RB16001816	cervix
1126	female	no	21.98	Asian	Whittall St. Clinic	negative	no	negative		negative	negative	175	28	NG232	RB16001817	rectum
1127	male	no	26.87	Asian	Whittall St. Clinic	negative	no	negative		negative		2	2	NG233	RB16001818	urethra
1129	male	no	34.41	Black	Whittall St. Clinic	negative	2	negative		negative		no	no	NG234	RB16001820	urethra
1134	male	no	20.94	Asian	Whittall St. Clinic	negative	no	negative		negative		14	14	NG236	RB16001824	urethra
1135	male	no	19.77	Other	Whittall St. Clinic	negative	no	negative		negative		no	no	NG237	RB16001825	urethra
1137	male	yes	26.40	White	Whittall St. Clinic	positive	no	negative				no	no	NG238	RB16001826	rectum
1138	male	yes	25.02	White	Whittall St. Clinic	negative	no	positive				no	no	NG239	RB16001827	rectum
1142	male	no	25.89	White	Whittall St. Clinic	negative	no	negative		negative		2	3	NG240	RB16001828	urethra
1147	male	no	24.16	White	Whittall St. Clinic	negative	no	positive		negative		no	7	NG241	RB16003861	urethra
1152	male	no	26.06	Asian	Whittall St. Clinic	negative	no	positive		negative		7	7	NG242	RB16003863	urethra
1156	male	no	27.11	Asian	Whittall St. Clinic	negative	no	negative		negative		3	3	NG244	RB16003866	urethra
1160	female	no	21.08	White	Whittall St. Clinic	negative	no	positive			negative	no	no	NG249	RB16003873	cervix
1162	male	yes	21.76	White	Whittall St. Clinic	negative	no	negative				no	no	NG245	RB16003867	pharynx
1167	male	yes	22.05	White	Whittall St. Clinic	negative	no	negative		negative		2	4	NG246	RB16003870	urethra
1168	male	yes	21.21	White	Whittall St. Clinic	negative	1	negative		negative		8	no	NG247	RB16003871	rectum
1168	male	yes	21.21	White	Whittall St. Clinic	negative	1	negative		negative		8	no	NG248	RB16003872	urethra
1170	male	no	66.39	White	Whittall St. Clinic	negative	1	positive		negative	negative	3	no	NG250	RB16003874	urethra
2015	male	yes	30.06	White	St Bartholomew	negative	no	negative		negative		4	6	NG062	RB15000622	urethra
2015	male	yes	30.06	White	St Bartholomew	negative	no	negative		negative		4	6	NG063	RB15000623	rectum
2017	male	yes	53.20	White	St Bartholomew	negative	12	negative		negative		7	no	NG064	RB15000624	urethra
2017	male	yes	53.20	White	St Bartholomew	negative	12	negative		negative		7	no	NG065	RB15000625	rectum
2019	male	yes	31.41	Asian	Blizard Institute, Barts	negative	no	negative		negative		8	1	NG115	RB16000522	urethra
2030	male	yes	30.83	White	Blizard Institute, Barts	negative	no	negative		negative		no	no	NG116	RB16000573	rectum
2031	male	yes	34.84	Black	St Bartholomew	positive	no	positive		negative		3	1	NG117	RB16000574	urethra
2035	male	yes	41.64	Black	St Bartholomew	negative	1	negative		negative		2	2	NG118	RB16000575	urethra
2036	male	yes	26.12	White	St Bartholomew	negative	no	negative		negative		1	1	NG119	RB16000576	urethra
2039	male	yes	36.60	White	London, Royal London Hosp	negative	2	negative	negative	negative		1	no	NG120	RB16000577	urethra
2040	male	yes	28.15	Mixed	Blizard Institute, Barts	negative	no	positive		negative		1	2	NG121	RB16000578	urethra
2041	female	no	24.75	White	St Bartholomew	negative	no	negative		negative	negative	no	14	NG122	RB16000579	cervix
2044	male	yes	29.66	White	St Bartholomew	negative	2	positive		negative		4	4	NG123	RB16000580	urethra
2045	male	yes	43.91	White	St Bartholomew	positive	1	negative		negative		no	no	NG124	RB16000581	rectum
2046	male	yes	41.83	Mixed	St Bartholomew	negative	no	negative		negative		1	2	NG125	RB16000582	urethra
2050	male	yes	37.48	Black	St Bartholomew	negative	2	negative		negative		7	7	NG294	RB16003974	urethra
2051	male	no	41.84	Black	St Bartholomew	negative	no	positive		negative		1	3	NG295	RB16003975	urethra
2054	male	yes	36.16	White	Blizard Institute, Barts	negative	no	negative		negative		1	4	NG296	RB16003976	urethra

Appendix I. Participant demographics for all *Neisseria gonorrhoeae* isolates collected during the G-ToG clinical trial, that were used in this study. Participant information for the participants from which the 354 *N. gonorrhoeae* isolates were derived from.

G-TOG participant number	Gender	History of same sex partners	Age	Ethnicity	Location	HIV status	Previous gonorrhoea episodes	Co-current STI				Symptoms		Culture information		
								Chlamydia	Genital herpes	Syphillis	Trichomonas	Genital discharge (days)	Dysuria (days)	Lab isolate number	Lab ID	Source
2056	male	yes	26.66	White	Blizard Institute, Barts	negative	1	negative				3	3	NG297	RB16003977	urethra
2057	male	yes	45.91	White	St Bartholomew	positive	1	negative		negative		2	1	NG298	RB16003978	urethra
2058	male	yes	23.18	Black	St Bartholomew	positive	1	negative		negative		no	no	NG299	RB16003979	rectum
2059	male	yes	34.56	White	St Bartholomew	negative	1	negative				no	no	NG300	RB16003980	pharynx
2061	male	yes	31.50	White	St Bartholomew	positive	no	negative				no	no	NG301	RB16003981	rectum
2062	male	yes	22.48	White	St Bartholomew	negative	1	negative		negative		5	5	NG302	RB16003982	urethra
2069	male	yes	39.90	White	St Bartholomew	positive	1	negative		negative		6	no	NG303	RB16003983	urethra
2070	male	yes	38.78	White	St Bartholomew	negative	no	negative		negative		49	49	NG304	RB16003984	urethra
3003	male	yes	35.44	Black	London, Guy's Hospital	negative	2	positive		negative		21	21	NG066	RB15003295	urethra
3004	male	yes	43.52	White	London, Guy's Hospital	negative	no	negative		negative		2	3	NG067	RB15003296	urethra
3008	male	yes	34.00	White	London, Guy's Hospital	negative	1	positive		negative		no	1	NG068	RB15003297	rectum
3011	male	yes	27.71	White	London, Guy's Hospital	positive	5	negative		negative		no	no	NG069	RB15003298	rectum
3016	male	yes	28.05	White	London, Guy's Hospital	negative	no	negative		negative		7	7	NG070	RB15003299	urethra
3017	male	yes	34.58	White	London, Guy's Hospital	negative	1	negative				no	no	NG071	RB15003300	rectum
3017	male	yes	34.58	White	London, Guy's Hospital	negative	1	negative				no	no	NG072	RB15003301	pharynx
3018	male	yes	38.13	White	London, Guy's Hospital	positive	7	negative				no	no	NG073	RB15003302	pharynx
3021	male	yes	38.85	White	London, Guy's Hospital	negative	no	negative		negative		no	no	NG075	RB15003304	pharynx
3022	male	yes	48.84	White	London, Guy's Hospital	positive	no	negative				no	no	NG076	RB15003305	rectum
3030	male	yes	22.10	White	London, Guy's Hospital	negative	no	negative		negative		3	4	NG166	RB16000859	urethra
3034	male	yes	41.19	White	London, Guy's Hospital	negative	3	positive		negative		2	2	NG167	RB16000861	urethra
3037	male	yes	23.49	White	London, Guy's Hospital	positive	no	positive		negative		no	no	NG168	RB16000862	pharynx
3037	male	yes	23.49	White	London, Guy's Hospital	positive	no	positive		negative		no	no	NG169	RB16000863	rectum
3038	male	yes	25.72	White	London, Guy's Hospital	negative	no	negative		negative		2	no	NG170	RB16000864	pharynx
3039	male	yes	44.55	Other	London, Guy's Hospital	positive	2	positive		positive		2	2	NG172	RB16000866	pharynx
3042	male	yes	51.94	Other	London, Guy's Hospital	negative	3	positive		negative		3	4	NG173	RB16000867	urethra
3043	male	yes	22.65	White	London, Guy's Hospital	negative	2	positive		negative		3	2	NG174	RB16000868	urethra
3044	male	yes	30.51	White	London, Guy's Hospital	negative	4	negative				2	no	NG175	RB16000869	urethra
3045	male	yes	49.55	White	London, Guy's Hospital	positive	no	positive		negative		3	1	NG176	RB16000871	urethra
3053	male	yes	21.87	White	London, Guy's Hospital	negative	2	positive		positive		6	6	NG177	RB16000872	urethra
3054	male	yes	39.68	White	London, Guy's Hospital	positive	6	positive		negative		6	6	NG178	RB16000873	urethra
3063	male	yes	32.04	White	London, Guy's Hospital	positive	1	negative	positive	negative		no	no	NG310	RB16004000	rectum
3069	male	yes	50.93	White	London, Guy's Hospital	negative	1	negative		negative		3	4	NG311	RB16004001	urethra
3071	male	yes	55.96	White	London, Guy's Hospital		2	negative				no	no	NG312	RB16004002	urethra
3072	male	yes	37.83	White	London, Guy's Hospital	negative	no	positive		negative		5	4	NG313	RB16004003	urethra
3077	male	yes	17.78	White	London, Guy's Hospital	negative	no	negative				no	no	NG314	RB16004006	pharynx
3077	male	yes	17.78	White	London, Guy's Hospital	negative	no	negative				no	no	NG315	RB16004007	rectum
3080	male	yes	25.78	Asian	London, Guy's Hospital	negative	no	negative				no	no	NG321	RB16004015	rectum
3082	male	yes	49.04	White	London, Guy's Hospital	positive	3	negative		negative		2	2	NG316	RB16004008	urethra
3083	male	yes	49.54	White	London, Guy's Hospital	negative	2	negative		negative		2	2	NG317	RB16004009	urethra
3084	male	yes	24.92	Black	London, Guy's Hospital	positive	4	negative		negative		1	4	NG318	RB16004010	urethra
3086	male	yes	32.54	White	London, Guy's Hospital	negative	3	negative		negative		3	2	NG320	RB16004014	urethra
3090	male	no	21.33	Black	London, Guy's Hospital	negative	no	negative		negative		8	8	NG319	RB16004012	urethra
4001	female	no	22.28	White	Leeds General Infirmary	negative	1	negative		negative		no	no	NG095	RB15003881	cervix
4002	male	yes	69.46	White	Leeds General Infirmary	negative	no	positive				no	no	NG096	RB15003882	rectum
4003	male	yes	27.57	White	Leeds General Infirmary	negative	no	negative				no	no	NG097	RB15003883	pharynx
4005	female	no	19.13	White	Leeds General Infirmary	negative	no	positive		negative	negative	no	no	NG098	RB15003884	cervix
4005	female	no	19.13	White	Leeds General Infirmary	negative	no	positive		negative	negative	no	no	NG099	RB15003885	rectum
4006	female	no	17.07	Mixed	Leeds General Infirmary	negative	no	positive		negative	negative	28	28	NG100	RB15003886	cervix
4006	female	no	17.07	Mixed	Leeds General Infirmary	negative	no	positive		negative	negative	28	28	NG101	RB15003887	pharynx
4006	female	no	17.07	Mixed	Leeds General Infirmary	negative	no	positive		negative	negative	28	28	NG102	RB15003888	rectum
4011	female	no	22.56	Mixed	Leeds General Infirmary	negative	no	negative		negative	negative	no	no	NG103	RB15003891	cervix
4014	female	no	21.72	White	Leeds General Infirmary	negative	no	positive		negative	negative	no	no	NG105	RB15003893	cervix
4015	male	yes	21.80	White	Leeds General Infirmary		1	positive				no	no	NG106	RB15003894	pharynx
4022	female	no	21.42	White	Leeds General Infirmary	negative	no	positive		negative	negative	no	no	NG107	RB15003897	cervix
4026	female	no	18.26	White		negative	no	positive		negative	negative	no	no	NG126	RB16000607	pharynx
4027	male	yes	27.97	White	Leeds General Infirmary	negative	1	negative		negative		10	7	NG127	RB16000608	rectum
4027	male	yes	27.97	White	Leeds General Infirmary	negative	1	negative		negative		10	7	NG128	RB16000609	urethra
4034	female	no	19.69	White	Leeds General Infirmary	negative	no	positive		negative	negative	5	7	NG129	RB16000610	cervix
4035	female	no	20.96	White	Leeds General Infirmary	negative	no	positive		negative	negative	no	no	NG130	RB16000611	cervix
4038	male	no	27.64	Other	Leeds General Infirmary	negative	no	positive		negative		6	2	NG131	RB16000612	urethra
4041	male	yes	20.61	White	Leeds General Infirmary	negative	1	negative		negative		no	no	NG132	RB16000613	pharynx
4044	male	yes	20.96	White	Leeds General Infirmary	negative	no	negative		negative		no	no	NG133	RB16000615	urethra
4045	male	yes	24.34	White	Leeds General Infirmary	negative	no	positive		negative		252	no	NG134	RB16000616	urethra
4046	male	no	20.25	White	Leeds General Infirmary	negative	no	positive		negative		no	no	NG135	RB16000617	urethra
4047	male	yes	21.88	White	Leeds General Infirmary	negative	no	negative		negative		3	2	NG136	RB16000618	urethra
4049	female	no	29.20	White	Leeds General Infirmary	negative	no	positive		negative	negative	no	5	NG137	RB16000619	pharynx
4051	female	no	18.57	White	Leeds General Infirmary	negative	no	negative				no	no	NG138	RB16000620	cervix
4053	female	no	24.14	White	Leeds General Infirmary	negative	no	negative		negative	negative	no	no	NG139	RB16000623	cervix
4060	male	no	22.18	Black	Leeds General Infirmary	negative	no	negative				no	no	NG216	RB16001561	urethra
4061	female	no	16.46	Mixed	Leeds General Infirmary	negative	no	positive		negative	negative	7	no	NG217	RB16001562	cervix
4061	female	no	16.46	Mixed	Leeds General Infirmary	negative	no	positive		negative	negative	7	no	NG218	RB16001563	rectum
4062	female	no	49.07	Asian	Leeds General Infirmary	negative	no	negative		negative	negative	28	28	NG219	RB16001564	rectum
4063	male	no	63.07	White	Leeds General Infirmary	negative	no	negative		negative		6	6	NG220	RB16001565	urethra
4071	male	yes	23.49	White	Leeds General Infirmary	negative	no	negative		negative		6	6	NG221	RB16001566	urethra
4071	male	yes	23.49	White	Leeds General Infirmary	negative	no	negative		negative		6	6	NG222	RB16001567	rectum
4072	male	no	30.21	White	Leeds General Infirmary	negative	1	negative		negative		7	4	NG223	RB16001568	urethra
4075	male	yes	23.87	White	Leeds General Infirmary	negative	1	negative		negative		no	no	NG224	RB16001570	urethra
4075	male	yes	23.87	White	Leeds General Infirmary	negative	1	negative		negative		no	no	NG225	RB16001571	rectum
4076	male	no	19.32	Black	Leeds General Infirmary	negative	no	negative		negative		no	no	NG226	RB16001572	urethra
4079	female	no	23.21	White	Leeds General Infirmary	negative	no	negative		negative	negative	7	7	NG227	RB16001573	cervix
4084	female	no	19.03	White	Leeds General Infirmary	negative	no	negative		negative	negative	no	no	NG254	RB16003927	rectum
4087	male	yes	31.74	Other	Leeds General Infirmary	negative	1	negative				no	no	NG258	RB16003931	pharynx
4088	female	no	18.63	Mixed	Leeds General Infirmary	negative	no	negative		negative	negative	14	no	NG255	RB16003928	cervix
4089	male	yes	26.85	White	Leeds General Infirmary	negative	not known	negative		negative		no	no	NG256	RB16003929	rectum
4091	male	yes	23.64	White	Leeds General Infirmary	positive	2	negative		positive		no	no	NG257	RB16003930	rectum
4092	male	yes	26.92	White		negative	1	negative		negative		no	4	NG259	RB16003932	urethra
4093	male	yes	44.01	White	Leeds General Infirmary	negative	no	negative		negative		5	5	NG260	RB16003933	urethra
4094	male	no	28.52	Mixed	Leeds General Infirmary	negative	1	negative		negative		no	no	NG261	RB16003934	urethra
4099	female	no	18.81	White	Leeds General Infirmary	negative	no	negative		negative	negative	7	7	NG262	RB16003935	cervix
4099	female	no	18.81	White	Leeds General Infirmary	negative	no	negative		negative	negative	7	7	NG263	RB16003936	pharynx
4100	female	no	28.56	White	Leeds General Infirmary	negative	no	negative		negative	negative	21	21	NG264	RB16003937	cervix
4100	female	no	28.56	White	Leeds General Infirmary	negative	no	negative		negative	negative	21	21	NG265	RB16003938	pharynx
5002	male	yes	61.65	White	Whittall St. Clinic	positive	no	negative				5	3	NG367	RB17003293	urethra
5007	male	yes	30.41	White	Manchester Royal Inf.	negative	1	positive		negative		2	2	NG158	RB16000849	urethra
5008	male	yes	28.97	White	Manchester Royal Inf.	positive	1	positive				no	no	NG159	RB16000850	unknown
5010	male	yes	33.12	Other	Manchester Royal Inf.	negative	no	negative		negative		2	2	NG160	RB16000851	urethra
5011	male	yes	22.96	White	Manchester Royal Inf.	negative	no	negative		negative		6	6	NG161	RB16000852	urethra
5015	male	yes	20.69	White	Manchester Royal Inf.	positive	1	negative				no	no	NG164	RB16000856	rectum
5020	female	no	20.48	Other	Manchester Royal Inf.	negative	no	positive				no	no	NG165	RB16000858	rectum
5023	male	yes	25.41	White	Manchester Royal Inf.	negative	no	negative		negative		2	2	NG305	RB16003988	urethra

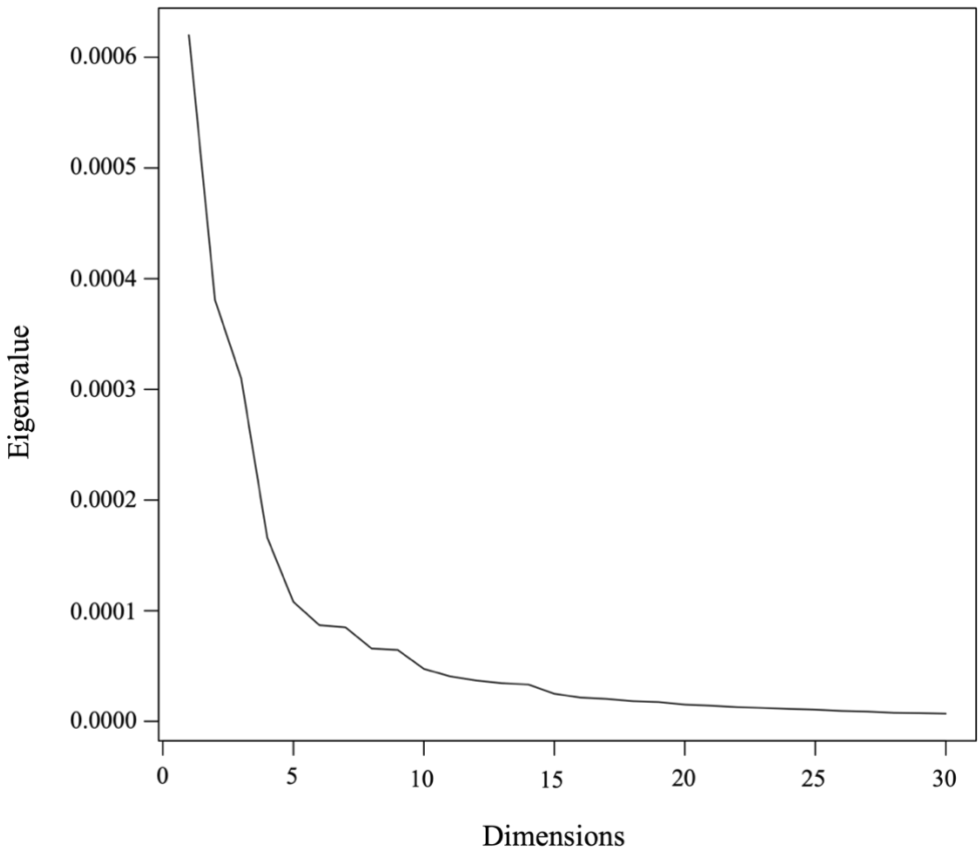
Appendix I. Participant demographics for all *Neisseria gonorrhoeae* isolates collected during the G-ToG clinical trial, that were used in this study. Participant information for the participants from which the 354 *N. gonorrhoeae* isolates were derived from (continued).

G-TOG participant number	Gender	History of same sex partners	Age	Ethnicity	Location	HIV status	Previous gonorrhoea episodes	Co-current STI				Symptoms		Culture information		
								Chlamydia	Genital herpes	Syphillis	Trichomonas	Genital discharge (days)	Dysuria (days)	Lab isolate number	Lab ID	Source
5026	male	no	24.17	White	Manchester Royal Inf.	negative	no	negative		negative	negative	3	2	NG306	RB16003990	urethra
5030	male	yes	25.54	White	Manchester Royal Inf.	negative	no	negative		negative		14	14	NG307	RB16003993	urethra
7002	male	no	19.96	White	Whittall St. Clinic	negative	no	negative		negative		7	7	NG009	RB14002910	urethra
7003	female	no	32.32	White	Whittall St. Clinic	negative	no	negative		negative	negative	no	no	NG010	RB14002911	rectum
7003	female	no	32.32	White	Whittall St. Clinic	negative	no	negative		negative	negative	no	no	NG011	RB14002912	cervix
7004	male	yes	32.52	White	Whittall St. Clinic	negative	3	negative		negative		4	4	NG012	RB14002913	urethra
7004	male	yes	32.52	White	Whittall St. Clinic	negative	3	negative		negative		4	4	NG013	RB14002914	pharynx
7004	male	yes	32.52	White	Whittall St. Clinic	negative	3	negative		negative		4	4	NG014	RB14002915	rectum
7005	male	yes	57.94	White	Sheffield Northern Gen. H	negative	2	negative		negative		1	1	NG015	RB14002916	urethra
7006	female	no	24.71	White	Sheffield Northern Gen. H	negative	1	negative		negative	negative	7	7	NG016	RB14002917	cervix
7009	male	yes	34.77	White	Sheffield, Royal Hallam'	negative	no	negative		negative		7	7	NG035	RB15000588	urethra
7010	male	yes	21.19	White	Sheffield, Royal Hallam'	negative	no	negative		negative		no	no	NG036	RB15000589	rectum
7012	male	yes	18.75	Black	Sheffield, Royal Hallam'	negative	no	negative	negative	negative		no	no	NG037	RB15000590	pharynx
7012	male	yes	18.75	Black	Sheffield, Royal Hallam'	negative	no	negative	negative	negative		no	no	NG038	RB15000591	rectum
7013	female	no	17.18	White	Sheffield, Royal Hallam'	negative	no	negative		negative	negative	no	no	NG039	RB15000592	cervix
7016	female	no	28.33	White	Sheffield Northern Gen. H	negative	no	negative		negative	negative	14	no	NG042	RB15000597	pharynx
7016	female	no	28.33	White	Sheffield Northern Gen. H	negative	no	negative		negative	negative	14	no	NG043	RB15000598	cervix
7017	female	no	29.13	White	Sheffield, Royal Hallam'	negative	no	negative		negative	negative	7	7	NG040	RB15000594	cervix
7017	female	no	29.13	White	Sheffield, Royal Hallam'	negative	no	negative		negative	negative	7	7	NG041	RB15000595	rectum
7019	male	yes	36.94	White	Sheffield, Royal Hallam'	negative	no	positive		negative		7	7	NG044	RB15000599	urethra
7019	male	yes	36.94	White	Sheffield, Royal Hallam'	negative	no	positive		negative		7	7	NG045	RB15000600	rectum
7019	male	yes	36.94	White	Sheffield, Royal Hallam'	negative	no	positive		negative		7	7	NG051	RB15000607	pharynx
7020	female	no	21.26	White	Sheffield Northern Gen. H	negative	no	negative		negative	positive	no	no	NG046	RB15000602	cervix
7020	female	no	21.26	White	Sheffield Northern Gen. H	negative	no	negative		negative	positive	no	no	NG047	RB15000603	rectum
7022	male	yes	20.43	White	Sheffield, Royal Hallam'	negative	no	negative		negative		5	5	NG048	RB15000604	urethra
7023	male	yes	41.93	White	Sheffield, Royal Hallam'	positive	no	negative				no	no	NG049	RB15000605	urethra
7023	male	yes	41.93	White	Sheffield, Royal Hallam'	positive	no	negative				no	no	NG050	RB15000606	rectum
7026	female	no	33.29	White	Sheffield, Royal Hallam'	negative	no	negative		negative	negative	no	42	NG052	RB15000608	unknown
7029	male	yes	34.89	White	Sheffield, Royal Hallam'	negative	1	negative		negative		no	no	NG053	RB15000609	pharynx
7029	male	yes	34.89	White	Sheffield, Royal Hallam'	negative	1	negative		negative		no	no	NG054	RB15000610	rectum
7030	female	no	21.60	White	Sheffield, Royal Hallam'	negative	1	negative		negative	negative	no	no	NG055	RB15000612	cervix
7031	female	no	19.06	White	Sheffield, Royal Hallam'	negative	1	negative		negative	negative	70	70	NG056	RB15000614	cervix
7031	female	no	19.06	White	Sheffield, Royal Hallam'	negative	1	negative		negative	negative	70	70	NG057	RB15000615	rectum
7033	female	no	27.67	White	Sheffield, Royal Hallam'	negative	no	negative		negative		15	15	NG058	RB15000616	pharynx
7034	male	yes	25.54	White	Sheffield, Royal Hallam'	negative	no	negative		negative		2	2	NG059	RB15000618	pharynx
7036	male	yes	24.77	White	Sheffield, Royal Hallam'	negative	no	negative		negative		3	3	NG060	RB15000619	urethra
7036	male	yes	24.77	White	Sheffield, Royal Hallam'	negative	no	negative		negative		3	3	NG061	RB15000620	rectum
7037	female	no	23.41	Black	Sheffield, Royal Hallam'	negative	no	negative		negative		no	no	NG140	RB16000624	urethra
7038	male	no	30.08	White	Sheffield, Royal Hallam'	negative	no	negative		negative		8	8	NG142	RB16000627	urethra
7043	male	no	19.63	White	Sheffield, Royal Hallam'	negative	1	positive		negative		no	28	NG143	RB16000629	urethra
7044	male	yes	34.46	Asian	Sheffield, Royal Hallam'	negative	1	negative		negative		1	2	NG144	RB16000630	urethra
7046	male	no	28.83	White	Sheffield, Royal Hallam'	negative	no	negative		negative		no	14	NG145	RB16000632	urethra
7050	male	no	19.05	White	Sheffield, Royal Hallam'	negative	no	positive		negative		1	no	NG146	RB16000635	urethra
7051	male	yes	23.43	White	Sheffield, Royal Hallam'	negative	1	negative		negative		no	3	NG147	RB16000636	urethra
7051	male	yes	23.43	White	Sheffield, Royal Hallam'	negative	1	negative		negative		no	3	NG148	RB16000637	pharynx
7051	male	yes	23.43	White	Sheffield, Royal Hallam'	negative	1	negative		negative		no	3	NG149	RB16000638	rectum
7052	male	yes	29.39	White	Sheffield, Royal Hallam'	negative	no	positive		negative		2	2	NG150	RB16000639	urethra
7052	male	yes	29.39	White	Sheffield, Royal Hallam'	negative	no	positive		negative		2	2	NG151	RB16000640	rectum
7053	male	no	19.16	White	Sheffield, Royal Hallam'	negative	no	negative		negative		21	no	NG152	RB16000641	pharynx
7053	male	no	19.16	White	Sheffield, Royal Hallam'	negative	no	negative		negative		21	no	NG153	RB16000642	urethra
7055	male	no	23.47	Black	Sheffield, Royal Hallam'	negative	no	positive		negative		7	7	NG155	RB16000644	urethra
7056	male	yes	37.35	White	Sheffield, Royal Hallam'	positive	no	negative		positive		7	7	NG156	RB16000646	unknown
7058	male	yes	32.54	Mixed	Sheffield, Royal Hallam'	negative	3	negative		positive		7	7	NG154	RB16000643	urethra
7061	male	yes	17.65	White	Sheffield, Royal Hallam'	negative	no	negative		negative		yes	yes	NG157	RB16000647	urethra
7064	male	no	26.59	White	Sheffield, Royal Hallam'	negative	1	positive		negative		4	14	NG267	RB16003942	urethra
7065	male	yes	25.39	White	Sheffield, Royal Hallam'	negative	no	positive				no	no	NG268	RB16003943	rectum
7073	male	yes	28.70	White	Sheffield, Royal Hallam'	negative	1	positive		negative		8	8	NG270	RB16003945	urethra
7075	male	yes	50.60	White	Sheffield, Royal Hallam'	positive	5	negative				no	no	NG271	RB16003946	rectum
7076	male	yes	21.11	White	Sheffield, Royal Hallam'	negative	no	negative		negative		no	no	NG272	RB16003947	pharynx
7079	female	no	20.55	White	Sheffield, Royal Hallam'	negative	no	negative		negative		no	no	NG273	RB16003949	cervix
7079	female	no	20.55	White	Sheffield, Royal Hallam'	negative	no	negative		negative		no	no	NG274	RB16003950	rectum
7080	male	yes	23.25	White	Sheffield Northern Gen. H	negative	1	negative		negative		no	no	NG275	RB16003952	pharynx
7080	male	yes	23.25	White	Sheffield Northern Gen. H	negative	1	negative		negative		no	no	NG276	RB16003953	pharynx
7081	male	yes	20.31	White	Sheffield, Royal Hallam'	negative	no	negative		negative		4	1	NG277	RB16003954	urethra
7081	male	yes	20.31	White	Sheffield, Royal Hallam'	negative	no	negative		negative		4	1	NG278	RB16003955	rectum
7081	male	yes	20.31	White	Sheffield, Royal Hallam'	negative	no	negative		negative		4	1	NG279	RB16003956	pharynx
7083	female	no	19.59	White	Sheffield, Royal Hallam'	negative	no	negative		negative		no	7	NG280	RB16003957	pharynx
7089	male	yes	32.95	White	Sheffield, Royal Hallam'	negative	1	negative		negative		1	1	NG281	RB16003961	urethra
7090	female	no	18.78	White	Sheffield, Royal Hallam'	negative	no	negative		negative		no	no	NG282	RB16003962	cervix
8005	male	yes	27.06	White	Southampton, University H	negative	no	positive	negative	negative		no	no	NG109	RB15005416	rectum
8006	male	yes	22.23	White	Southampton, University H	negative	no	negative		negative		5	5	NG108	RB15005415	urethra
8012	male	yes	31.17	White	Southampton, University H	negative	no	negative				no	no	NG110	RB15005420	pharynx
8012	male	yes	31.17	White	Southampton, University H	negative	no	negative				no	no	NG111	RB15005421	rectum
8020	male	yes	55.20	White	Southampton, University H	negative	no					no	no	NG308	RB16003994	rectum
8027	male	yes	28.35	Black	Southampton, University H	positive	1	negative		negative		5	5	NG309	RB16003995	urethra
9001	male	yes	45.24	White	London, Charing Cross	negative	2	negative		negative		4	4	NG206	RB16001405	urethra
9003	male	yes	22.76	White	London, Chelsea & W'minst	negative	no	negative		negative		no	no	NG207	RB16001406	pharynx
9004	male	no	24.04	White	London, Chelsea & W'minst	negative	no	negative		negative		14	no	NG208	RB16001407	urethra
9005	male	yes	32.97	White	London, Chelsea & W'minst	negative	1	negative		negative		no	no	NG209	RB16001408	rectum
9007	male	yes	26.25	White	London, Chelsea & W'minst	positive	no	negative		negative		no	no	NG210	RB16001410	rectum
9008	male	no	33.42	White	London, Chelsea & W'minst	negative	1	positive		negative		3	3	NG211	RB16001411	urethra
9011	male	yes	30.30	Mixed	London, Chelsea & W'minst	negative	1	negative		negative		4	4	NG212	RB16001415	urethra
9015	male	yes	32.89	Other	London, Chelsea & W'minst	negative	no	negative		negative		no	no	NG213	RB16001416	pharynx
9018	male	yes	25.10	White	London, Chelsea & W'minst	positive	4	positive				no	no	NG214	RB16001418	rectum
9019	other	yes	45.50	Mixed	London, Chelsea & W'minst	positive	no	negative				no	no	NG215	RB16001420	rectum
9024	male	yes	24.76	White	London, Chelsea & W'minst	negative	no	negative		negative		no	no	NG341	RB16004155	pharynx
9025	male	yes	38.93	White	London, Chelsea & W'minst	negative	1	negative		negative		1	no	NG343	RB16004157	urethra
9026	male	yes	44.21	White	London, Chelsea & W'minst	positive	3	positive		negative		3	3	NG361	RB16004181	urethra
9027	male	yes	21.94	White	London, Chelsea & W'minst	negative	1	negative		negative		no	no	NG362	RB16004182	rectum
9028	male	yes	25.72	Asian	London, Chelsea & W'minst	negative	1	negative		negative		no	no	NG358	RB16004177	Unknown
9030	male	yes	23.90	White	London, Chelsea & W'minst	negative	1	negative		negative		no	no	NG353	RB16004171	rectum
9030	male	yes	23.90	White	London, Chelsea & W'minst	negative	1	negative		negative		no	no	NG357	RB16004176	pharynx
9032	male	yes	32.43	White	London, Chelsea & W'minst	negative	1	negative		negative		no	no	NG350	RB16004168	rectum
9037	male	yes	39.71	White	London, Chelsea & W'minst	negative	1	positive		negative		no	no	NG344	RB16004158	rectum
10003	male	yes	33.08	White	London, Guy's Hospital		1	negative				no	no	NG112	RB16000477	rectum
10005	male	yes	22.73	White	Brighton, Royal Sussex C.		no	negative				no	no	NG113	RB16000478	rectum
10006	male	yes	29.07	White	Brighton, Royal Sussex C.	negative	3	negative		negative		no	no	NG114	RB16000479	rectum
10012	male	yes	29.45	White	Brighton, Royal Sussex C.	positive	1	negative		negative		no	no	NG251	RB16003897	pharynx
10012	male	yes	29.45	White	Brighton, Royal Sussex C.	positive	1	negative		negative		no	no	NG252	RB16003898	rectum
10014	male	yes	49.68	White	Brighton, Royal Sussex C.	negative	1	negative		negative		no	no	NG253	RB16003899	rectum

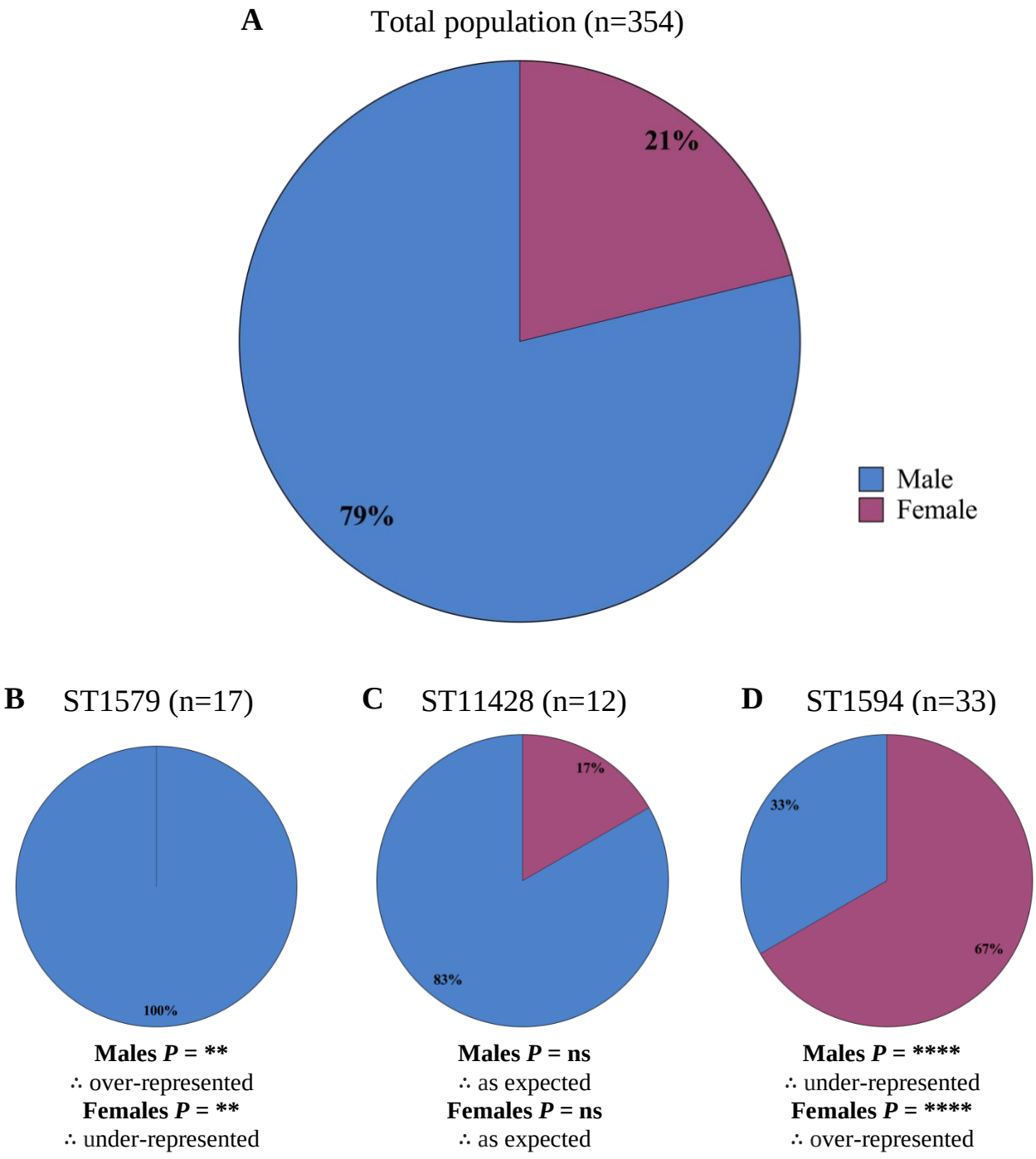
Appendix I. Participant demographics for all *Neisseria gonorrhoeae* isolates collected during the G-ToG clinical trial, that were used in this study. Participant information for the participants from which the 354 *N. gonorrhoeae* isolates were derived from (continued).

G-TOG participant number	Gender	History of same sex partners	Age	Ethnicity	Location	HIV status	Previous gonorrhoea episodes	Co-current STI				Symptoms		Culture information		
								Chlamydia	Genital herpes	Syphillis	Trichomonas	Genital discharge (days)	Dysuria (days)	Lab isolate number	Lab ID	Source
11002	male	no	19.28	Black	Univ Hosp Coventry & Warw	negative	no	positive		negative		no	28	NG198	RB16000927	urethra
11008	female	no	25.54	Other	Univ Hosp Coventry & Warw	negative	1	positive		negative	negative	yes	no	NG199	RB16000930	rectum
11008	female	no	25.54	Other	Univ Hosp Coventry & Warw	negative	1	positive		negative	negative	yes	no	NG200	RB16000931	cervix
11011	female	no	20.61	Asian	Univ Hosp Coventry & Warw	negative	no	positive		negative	negative	no	7	NG201	RB16000933	cervix
11013	female	no	19.32	White	Univ Hosp Coventry & Warw	negative	no	negative		negative		no	5	NG202	RB16000935	cervix
11013	female	no	19.32	White	Univ Hosp Coventry & Warw	negative	no	negative		negative	result not availa	no	5	NG203	RB16000936	rectum
11013	female	no	19.32	White	Univ Hosp Coventry & Warw	negative	no	negative		negative	result not availa	no	5	NG204	RB16000937	pharynx
11016	male	yes	31.72	Other	Univ Hosp Coventry & Warw	negative	no	positive		negative		21	21	NG205	RB16000938	urethra
11020	female	no	17.02	White	Univ Hosp Coventry & Warw	negative	1	negative				no	no	NG322	RB16004016	cervix
11021	male	no	18.47	White	Univ Hosp Coventry & Warw	negative	no	negative		negative		3	3	NG323	RB16004017	urethra
11026	male	no	24.58	Asian	Univ Hosp Coventry & Warw	negative	no	negative		negative		1	1	NG324	RB16004018	urethra
11031	female	no	21.81	White		negative	no	negative			negative	7	no	NG325	RB16004019	cervix
11032	male	no	35.17	White	Univ Hosp Coventry & Warw	negative	no	negative		negative		yes	no	NG326	RB16004020	urethra
11033	male	no	28.27	White	Univ Hosp Coventry & Warw	negative	no	negative		negative		7	no	NG327	RB16004021	urethra
11035	male	no	57.42	White	Univ Hosp Coventry & Warw	positive	1	negative		negative		4	no	NG328	RB16004022	urethra
12005	male	no	20.04	Other	London, Royal Free Trust	negative	no	positive		negative		3	3	NG332	RB16004122	urethra
12005	male	no	20.04	Other	London, Royal Free Trust	negative	no	positive		negative		3	3	NG333	RB16004123	unknown
13001	male	yes	31.03	White	Reading, Royal Berkshire	positive	no	negative				3	5	NG283	RB16003963	urethra
13002	male	no	25.50	Black	Reading, Royal Berkshire	negative	no	negative		negative		8	3	NG284	RB16003964	urethra
13003	male	no	20.12	White	Reading, Royal Berkshire	negative	no	negative		negative		2	3	NG285	RB16003965	Unknown
13004	male	yes	41.22	White	Reading, Royal Berkshire	negative	3	negative		negative		3	1	NG286	RB16003966	urethra
13005	male	no	25.94	White	Reading, Royal Berkshire	negative	no	positive		negative		7	6	NG287	RB16003967	Unknown
13005	male	no	25.94	White	Reading, Royal Berkshire	negative	no	positive		negative		7	6	NG329	RB16004023	Unknown
13005	male	no	25.94	White	Reading, Royal Berkshire	negative	no	positive		negative		7	6	NG330	RB16004024	Unknown
13009	male	yes	26.10	White	Reading, Royal Berkshire	positive	1					no	no	NG289	RB16003969	rectum
13010	male	yes	40.32	White	Reading, Royal Berkshire	positive	6	negative		negative		1	1	NG290	RB16003970	urethra
13010	male	yes	40.32	White	Reading, Royal Berkshire	positive	6	negative		negative		1	1	NG291	RB16003971	rectum
13011	male	yes	42.04	Black	Reading, Royal Berkshire	negative	1	negative		negative		2	2	NG292	RB16003972	urethra
13012	male	yes	27.11	White	Reading, Royal Berkshire	negative	no	negative				no	no	NG293	RB16003973	Unknown
13012	male	yes	27.11	White	Reading, Royal Berkshire	negative	no	negative				no	no	NG331	RB16004025	Unknown
14008	male	yes	31.91	Mixed	London, St Mary's	negative	no	positive		negative		no	2	NG363	RB16004185	pharynx
14008	male	yes	31.91	Mixed	London, St Mary's	negative	no	positive		negative		no	2	NG364	RB16004186	urethra
14010	male	yes	25.98	Black	London, St Mary's	negative	1	negative		negative		2	no	NG365	RB16004189	urethra
14011	male	yes	38.44	Black	London, St Mary's	positive	25	result not available			negative	1	1	NG366	RB16004190	urethra
14013	male	no	20.31	White	London, St Mary's	negative	no	negative		negative		3	4	NG342	RB16004156	urethra
14015	male	yes	22.65	White	London, St Mary's	negative	no	negative		negative		no	no	NG359	RB16004178	pharynx
14016	male	no	68.40	White	London, St Mary's	negative	no	negative		negative		7	7	NG360	RB16004179	urethra
14017	female	yes	38.12	Asian		negative	no	negative		negative		no	no	NG356	RB16004175	pharynx
14019	male	yes	31.87	Other	London, St Mary's	positive	3	negative				no	no	NG354	RB16004172	rectum
14020	male	yes	44.11	White	London, St Mary's	negative	4	negative		negative		no	no	NG351	RB16004169	rectum
14021	male	yes	46.97	White	London, St Mary's	positive	1	negative		negative		no	no	NG346	RB16004162	rectum
14022	male	yes	33.97	White	London, St Mary's	negative	5	negative				no	no	NG347	RB16004163	urethra
14024	male	yes	35.63	White	London, St Mary's	positive	1	negative		negative		no	no	NG338	RB16004152	pharynx
14024	male	yes	35.63	White	London, St Mary's	positive	1	negative		negative		no	no	NG339	RB16004153	rectum
14025	male	yes	35.66	White	London, St Mary's	negative	no	negative		negative		3	2	NG335	RB16004149	urethra
14026	male	no	36.06	Asian	London, St Mary's	negative	no	negative		negative		7	7	NG334	RB16004148	urethra
15001	male	yes	38.03	White	London, Chelsea & W'minst	positive	1	negative		negative		1	3	NG355	RB16004173	urethra
15002	male	yes	45.82	White	London, Chelsea & W'minst	positive	4					no	no	NG349	RB16004165	rectum
15002	male	yes	45.82	White	London, Chelsea & W'minst	positive	4					no	no	NG352	RB16004170	pharynx
15004	male	yes	44.56	White	London, Chelsea & W'minst	negative	2	negative				no	no	NG345	RB16004159	rectum
15005	male	yes	26.40	White	London, Chelsea & W'minst	negative	1	negative				no	no	NG348	RB16004164	pharynx
15006	male	yes	33.43	Other	London, Chelsea & W'minst	negative	2	negative				no	no	NG340	RB16004154	rectum
15007	male	yes	29.22	White	London, Chelsea & W'minst		4	negative				3	4	NG336	RB16004150	urethra
15007	male	yes	29.22	White	London, Chelsea & W'minst		4	negative				3	4	NG337	RB16004151	pharynx

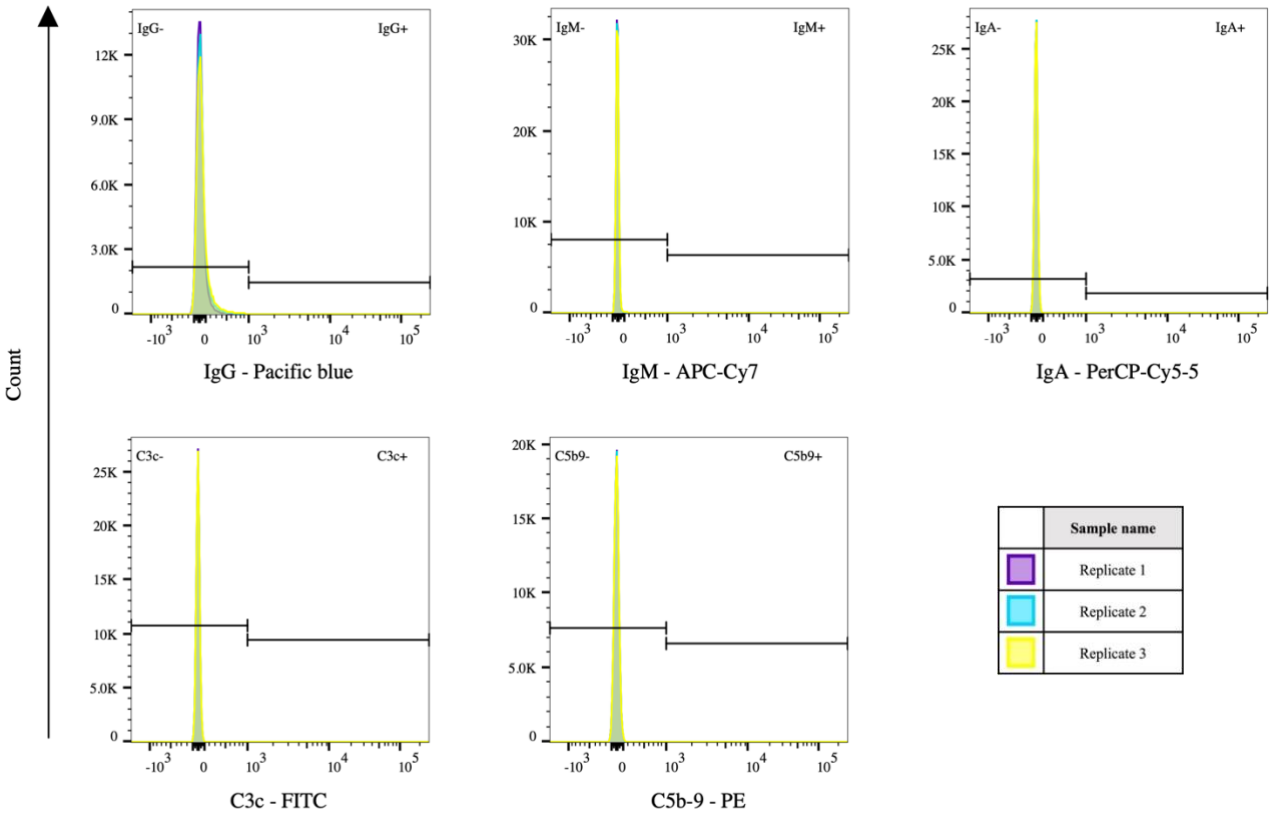
Appendix I. Participant demographics for all *Neisseria gonorrhoeae* isolates collected during the G-ToG clinical trial, that were used in this study. Participant information for the participants from which the 354 *N. gonorrhoeae* isolates were derived from (continued).



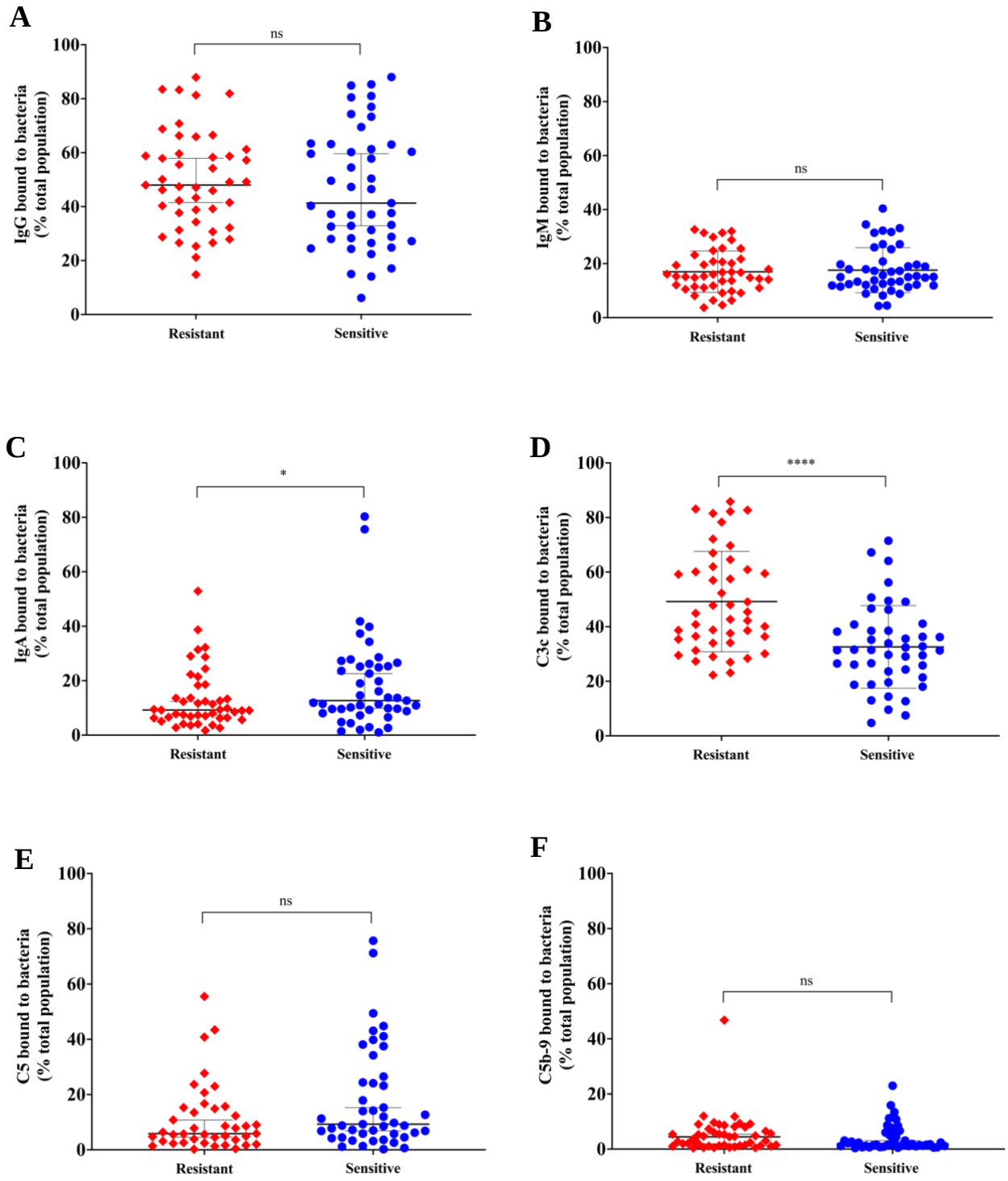
Appendix II. Multidimensional scaling from pairwise population distances of all sequenced G-ToG isolates, depicted as a scree plot. A pairwise distance matrix was generated from population structure of all 354 *N. gonorrhoeae* samples using FASTA files. From this distance matrix and multidimensional scaling, a scree plot was produced, allowing for the number of dimensions to retain during genome wide association study (GWAS) to be chosen.



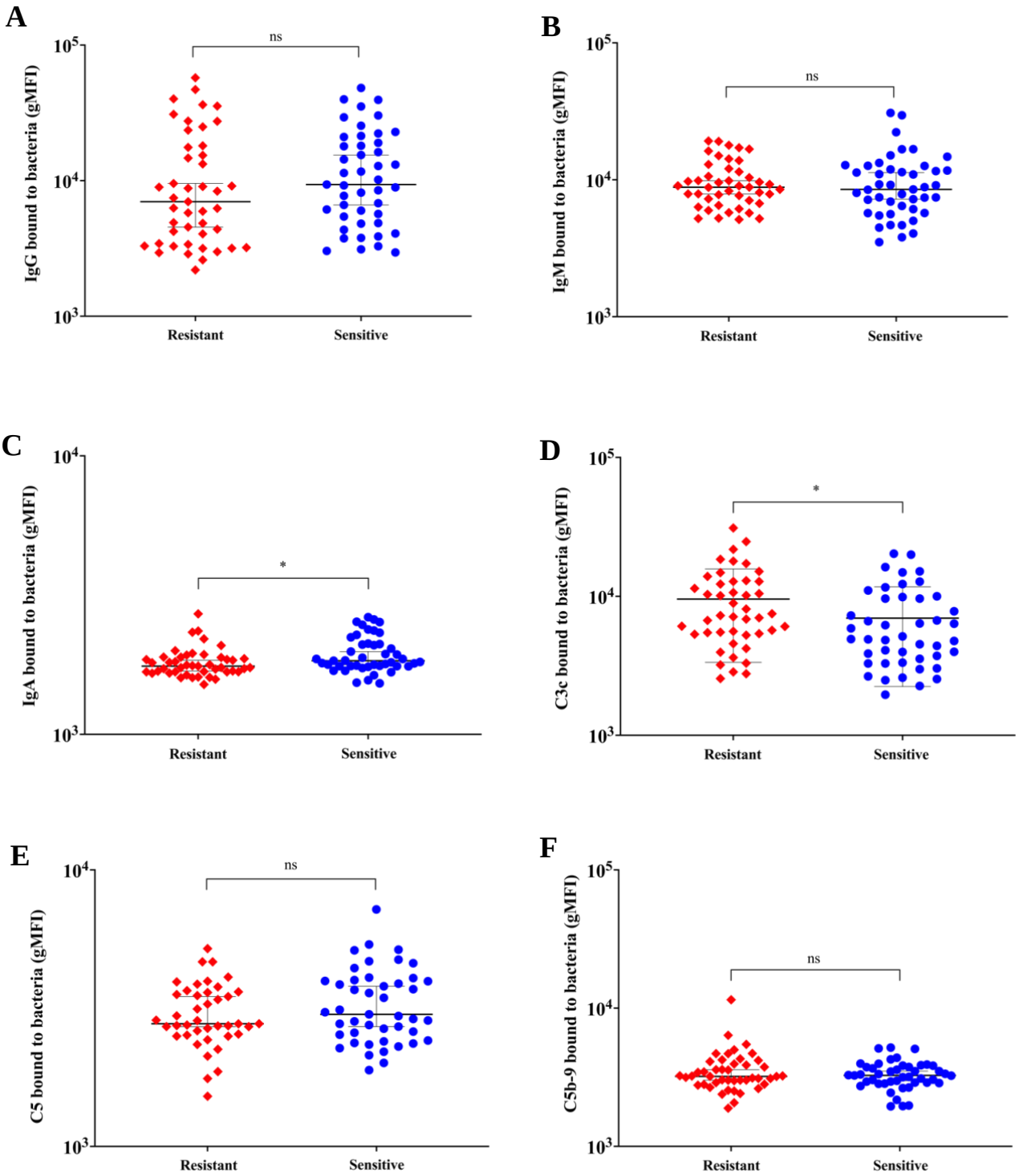
Appendix III. Representation of statistical analysis for genomics in relation to clinical datasets. Statistical analysis for gender dataset comparing two subcategories, male and female. **A)** Proportions of males and females in the total population of 354 isolates. **B)** A statistically significant over-representation of males and under-representation of females in MLST ST1579 compared to the overall population. **C)** No significant difference in males or females in MLST ST11428 compared to the overall population. **D)** A statistically significant under-representation of males and over-representation of females in MLST ST1594 compared to the overall population. A two proportion z-test with a 95% confidence level were used when comparing proportions within each MLST and whole population. Statistical significance was then calculated using a 2-tailed z-test, where ** ($p \leq 0.01$) and **** ($p \leq 0.0001$).



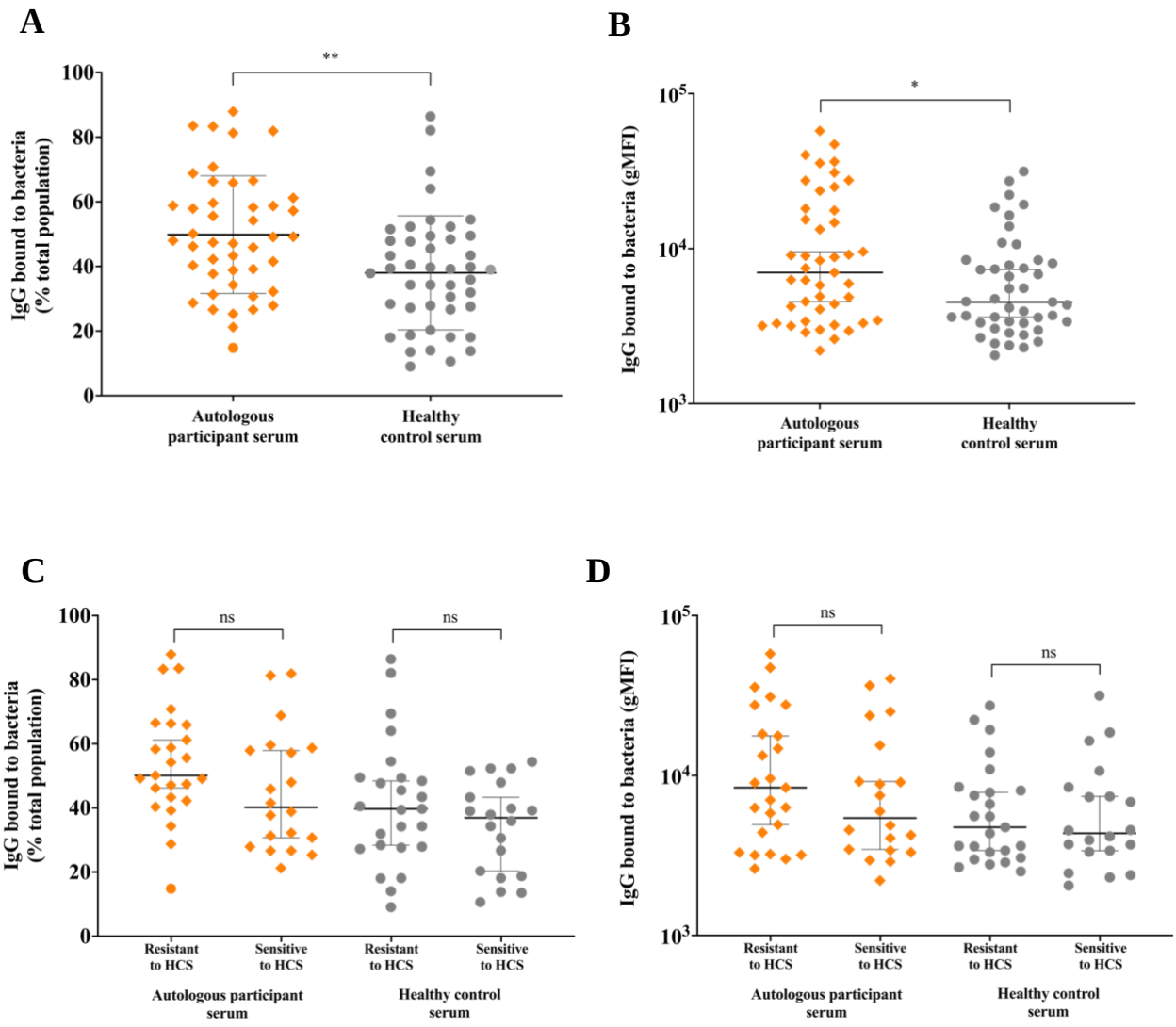
Appendix IV. Effect of fetal bovine serum on secondary antibody binding. A flow cytometric assay was used to investigate the effects of incubation of *N. gonorrhoeae* MS11with fetal bovine serum (FBS) on the binding of secondary antibodies targeting IgG, IgM and IgA and complement C3c and C5b-9. Raw flow cytometric histogram plots for each of the five fluorescent channels represent binding of each individual serum protein. Experiments were repeated three times independently, with each replicate overlaid on the plots. Each channel was gated showing positive and negative signal based on negative control samples.



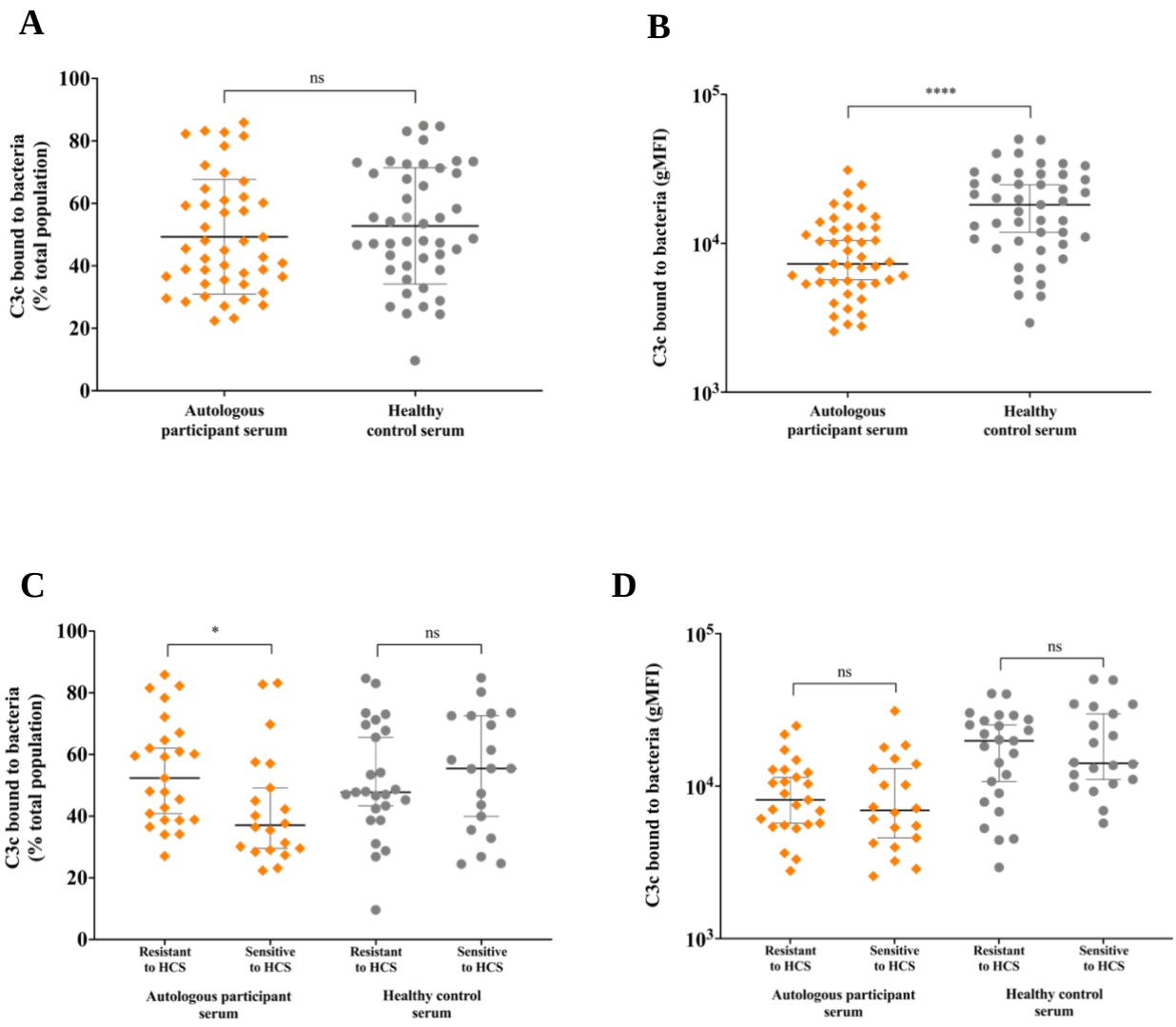
Appendix V. Binding of serum components individually to all resistant and sensitive isolates, presented as percent positive cells. A flow cytometric assay was used to analyse antibody and complement binding to all 46 isolates resistant to autologous serum, and 46 isolates sensitive to autologous serum. Cultures of each isolate were incubated with autologous serum and binding of **A)** IgG, **B)** IgM, **C)** IgA, **D)** C3c, **E)** C5 and **F)** C5b-9 were measured by flow cytometry using fluorescently labelled secondary antibodies. Shown is the percentage of positively labelled bacteria for each serum component. The median for each group was calculated and a 95% confidence interval (CI) was applied. Statistical significance was measured using a Mann-Whitney test, where * ($p \leq 0.05$) and **** ($p \leq 0.0001$).



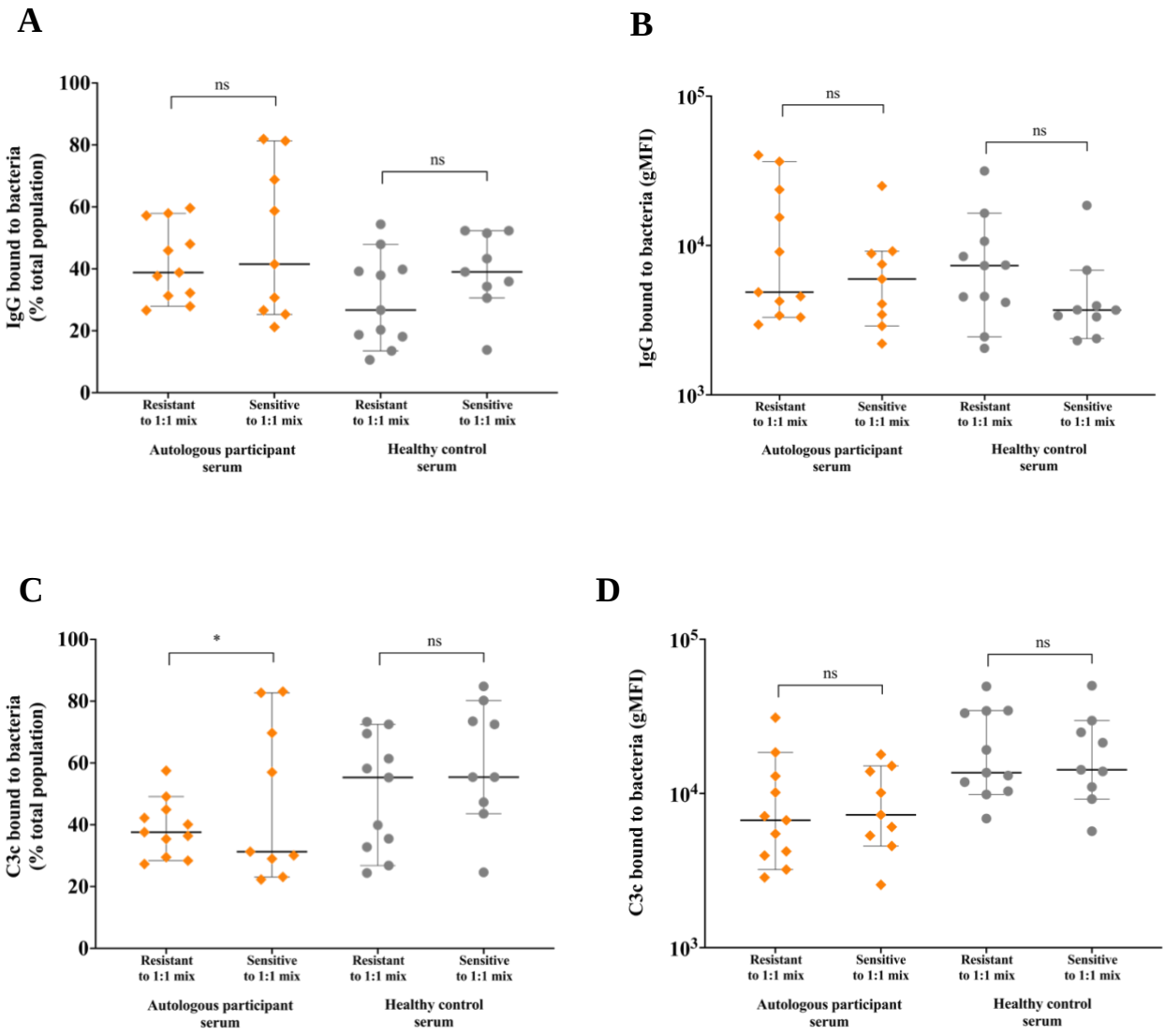
Appendix VI. Binding of serum components individually to all resistant and sensitive isolates, presented as gMFI. A flow cytometric assay was used to analyse antibody and complement binding to all 46 isolates resistant to autologous serum, and 46 isolates sensitive to autologous serum. Cultures of each isolate were incubated with autologous serum and binding of **A)** IgG, **B)** IgM, **C)** IgA, **D)** C3c, **E)** C5 and **F)** C5b-9 were measured by flow cytometry using fluorescently labelled secondary antibodies. Shown is the geometric mean fluorescence intensity (gMFI) for each serum component. The median for each group was calculated and a 95% confidence interval (CI) was applied. Statistical significance was measured using a Mann-Whitney test, where * ($p \leq 0.05$).



Appendix VII. Binding of IgG from healthy or autologous participant serum to isolates that resisted killing from participant serum, presented as percent positive cells and gMFI. A flow cytometric assay was used to analyse IgG binding to 46 isolates resistant to autologous serum, when incubated with autologous participant serum or healthy control serum (HCS). For each sera, the **A)** overall percentage positive population binding IgG and **B)** overall gMFI of the positive cells, were measured for all 46 isolates. These 46 participant resistant isolates were then split into isolates that were resistant to healthy serum, and those that were sensitive and the **C)** overall percentage positive population binding IgG and **D)** overall gMFI of the positive cells were measured when incubated in each sera. The median for each group was calculated and a 95% confidence interval (CI) was applied. Statistical significance was measured using a Mann-Whitney test, where * ($p \leq 0.05$) and where ** ($p \leq 0.01$).



Appendix VIII. Binding of C3c from healthy or autologous participant serum to isolates that resisted killing from participant serum, presented as percent positive cells and gMFI. A flow cytometric assay was used to analyse C3c binding to 46 isolates resistant to autologous serum, when incubated with autologous participant serum or healthy control serum (HCS). For each sera, the **A)** overall percentage positive population binding C3c and **B)** overall gMFI of the positive cells, were measured for all 46 isolates. These 46 participant resistant isolates were then split into isolates that were resistant to healthy serum, and those that were sensitive and the **C)** overall percentage positive population binding C3c and **D)** overall gMFI of the positive cells were measured when incubated in each sera. The median for each group was calculated and a 95% confidence interval (CI) was applied. Statistical significance was measured using a Mann-Whitney test, where * ($p \leq 0.05$) and where **** ($p \leq 0.0001$).



Appendix IX. Binding of IgG and C3c from healthy or autologous participant serum to isolates that succumb to killing by healthy serum, presented as percent positive cells and gMFI. A flow cytometric assay was used to analyse IgG and C3c binding to 20 participant resistant isolates found to be sensitive to healthy control serum (HCS). Isolates were split into two groups, those resistant and sensitive to the 1:1 mixture of both sera and binding of IgG was measured as **A)** percent positively labelled bacteria and **B)** gMFI, and C3c was measured as **C)** percent positively labelled bacteria and **D)** gMFI, when incubated with autologous participant serum or HCS. The median for each group was calculated and a 95% confidence interval (CI) was applied. Statistical significance was measured using a Mann-Whitney test, where * ($p \leq 0.05$) and where **** ($p \leq 0.0001$).

Participant serum	Serum component	Average stock concentration (mg/ml)
NG115-serum	Purified IgG	4.22
	Purified IgM	0.287
	Purified IgA	1.185
NG273/274-serum	Purified IgG	1.725
	Purified IgM	0.129
	Purified IgA	0.384
NG332-serum	Purified IgG	6.58
	Purified IgM	0.169
	Purified IgA	0.718
NG302-serum	Purified IgG	1.71
	Purified IgM	0.08
	Purified IgA	0.51
NG298-serum	Purified IgG	2.3
	Purified IgM	0.176
	Purified IgA	0.376
N244-serum	Purified IgG	3.14
	Purified IgM	0.07
	Purified IgA	0.948

Appendix X. Concentration of IgG, IgM and IgA antibodies purified from participant serum. Participant serum samples most likely to contain inhibitory antibodies were depleted of IgG, IgM and IgA antibodies by The Binding Site Ltd (Birmingham, UK)**.** Protein concentrations of each purified antibody isotypes were quantified using the Qubit Protein Assay Kit (Thermo Fisher Scientific).

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