Investigating the Host-Binding Properties of *Neisseria gonorrhoeae* and Newer Therapeutics against this Pathogen

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Supervised by:

Professor Nathlee S. Abbai

PREFACE

The experimental work described in this thesis was conducted at the School of Clinical Medicine Laboratory, Nelson R. Mandela School of Medicine, University of KwaZulu-Natal, Durban, South Africa, by Deshanta Naicker from January 2021 to July 2023, under the supervision of Prof Nathlee S. Abbai.

This work has not been submitted in any form for any degree or diploma to any tertiary institution, where use has been made of the work by others, it is duly acknowledged in the text.

Deshanta Naicker: [redacted] Date: 17 November 2023

Supervisor Prof. N.S. Abbai: Date: 17 November 2023

As the candidate's supervisor, I agree to the submission of this thesis:

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PLAGIARISM DECLARATION

I.....Deshanta Naicker.....declare that

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PERMISSION TO SUBMIT

As the candidate's supervisor, I Professor Nathlee Samantha Abbai have read the thesis and have given our approval for submission for examination.

Supervisor: Professor Nathlee S. Abbai:

Date:

Discipline: Clinical Medicine Laboratory

School of Clinical Medicine

College of Health Sciences

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Durban, South Africa

PRIMARY RESEARCH OUTPUT

Submitted Manuscripts

The following publications have emanated from this study:

1. **Deshanta Naicker**, Ntombifikile Nkwanyana, and Nathlee S Abbai. (2023). Design and Performance of an *Opa* Gene-Based Real-Time PCR Assay as a Potential Diagnostic Test for *Neisseria gonorrhoeae*. Submitted to the African Journal of Laboratory Medicine. Reference number: 2348 (Undergoing Peer review).
2. **Deshanta Naicker**, Rowen Govender, and Nathlee S Abbai. (2023). Busting the Resistance: Antimicrobial Activity of Plant-Infused Nanoemulsions against *Neisseria gonorrhoeae*. Submitted to the International Journal of Microbiology. Article ID: 7084347 (Undergoing Peer review).
3. **Deshanta Naicker** and Nathlee S Abbai. (2023). Combating Multidrug-Resistant *Neisseria Gonorrhoeae*: The Potential of Nanoemulsions as Alternative Therapeutics. Submitted to the International Journal of Microbiology. Article ID: 1383296 (Undergoing Peer review).

OTHER RESEARCH OUTPUT

International Conferences

1. **Deshanta Naicker** and Nathlee S Abbai. Investigating the Host-Binding Properties of *Neisseria gonorrhoeae* in South African Population. Poster presentation at the SciTech Immuno-Microbiology, Women & Nursing 2023 (34th World Summit on Immuno-Microbiology, Women, Health & Nursing) Mauritius- Won Best Poster Presentation (May 2023).

National Conferences

1. **Deshanta Naicker** and Nathlee S Abbai. Investigating the Host-Binding Properties of *Neisseria gonorrhoeae* in South African Population. Poster presentation at the College of Health Science Annual Research Symposium, University of KwaZulu Natal, Durban, South Africa (August 2023).
2. **Deshanta Naicker** and Nathlee S Abbai. Evaluation of an Opa Gene-Based Real-Time PCR Assay for Detection of *Neisseria gonorrhoeae* in South African Populations. Virtual poster presentation at the 45th Global Congress on Infectious Diseases: Research on Diagnosis and Therapeutics- Was to be held in South Africa, now a virtual conference (November 2023).

Co-authored manuscripts emanating from this study

1. Rowen Govender, **Deshanta Naicker**, Swenkile Ndlovu and Nathlee S Abbai. (2023). Synergistic Strategies: Harnessing Plant Nanoemulsions to Combat Metronidazole-Resistant *Trichomonas vaginalis* in South Africa.

DEDICATION

I dedicate this thesis to:

To the one who sculpted my existence, your unwavering guidance and unfaltering support have been the pillars upon which my journey thrives. Countless educational opportunities, generously provided, have enriched my path, for which I am profoundly grateful. Jai Shree Ram.

"Jai Hanuman gyan gun sagar Jai Kapis tihun lok ujaga"

My loving parents Mala and Selvan Naicker for your endless love and support throughout my career, for recognizing the potential in me and encouraging me to strive for excellence.

My beautiful sister Kim Naicker for always encouraging and motivating me throughout my academic career and life.

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ACRONYMS

-	Minus
%	Percentage
>	Greater than
±	plus/minus
µ	Micro
AMR	Antimicrobial Resistance
BHI	Brain Heart Infusion
bp	Base pairs
BREC	Biomedical Research Ethic Committee
BSA	Bovine Serum Albumin
℃	Degree Celsius
CDC	Centre’s of Disease Control and Prevention
CEACAM	Carcinoembryonic Antigen-Related Cell Adhesion Molecule
CO_2	Carbon Dioxide
DGI	Disseminated Gonococcal Infection
DMEM	Dulbecco's modified Eagle's medium
DNA	Deoxyribonucleic Acid
FDA	Food and Drug Administration
g	gram

HIV	Human Immunodeficiency Virus
HSPGs	heparan sulphate proteoglycans
IM	Intramuscular
IPTG	Isopropyl-β-D-thiogalactopyranoside
l	Liters
LB	Luria-Bertani
LOS	Lipooligosaccharide
m	Milli
M	Molar
MDR	Multidrug-Resistant
n	nano
NAATs	Nucleic Acid Amplification test
NaCl	Sodium Chloride
NYC	New York City plates
Opa	Opacity
PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction
PID	Pelvic Inflammatory Disease
PVDF	Polyvinylidene difluoride
ROS	Reactive Oxygen Species

SDS	Sodium dodecyl-sulfate
SDS-PAGE	Sodium dodecyl-sulfate polyacrylamide gel electrophoresis
STI	Sexually Transmitted Infection
TBS-T	Tris-Buffered Saline and Tween
UV	Ultraviolet
WGS	Whole Genome Sequencing
WHO	World Health Organization
x *g*	Times gravitational force
x	times
α	Alpha
β	Beta

ABSTRACT

Background

With 80 million annual cases, gonorrhoea is a common sexually transmitted disease caused by *Neisseria gonorrhoeae* (*N. gonorrhoeae*). The current study investigated a reverse vaccinology approach (determining the CEACAM binding patterns of *N. gonorrhoeae*) and alternative therapeutics (nanoemulsion and plant extracts) to combat this disease as well as evaluated cost-effective assays that target virulence proteins such as the *opa* gene (in-house *opa*-based real-time PCR assay).

Methodology

This was a retrospective laboratory-based study using stored bacterial isolates and primary genital swabs collected from larger studies. For the binding assays, we investigated the host-pathogen associations by determining the CEACAM binding patterns of *N. gonorrhoeae* isolated from symptomatic (G180) versus asymptomatic (G136) pregnant women. To identify the complete repertoire of G136 and G180 Opa proteins, DNA was isolated and a PCR with primers targeting the conserved regions of *opa* genes was performed. Thereafter, *opa* amplicons were ligated into pCR Blunt II TOPO, and single clones from this *opa* amplicon library were sequenced to identify the respective unique *opa* genes. The Opa proteins identified in this study were subjected to test expression and Western blotting was performed to verify these Opa proteins with a monoclonal antibody against *neisserial* Opa proteins. Thereafter, binding assays were performed to analyse the host-binding properties of *N. gonorrhoeae*.

For the objective which involved the development and evaluation of the performance of an in-house *opa*-based real-time PCR assay, three primer sets targeting the *opa* gene of *N. gonorrhoeae* were designed and evaluated against published *opa* gene primers (comparator

assay). The in-house and published primers were tested against laboratory and clinical isolates of *N. gonorrhoeae* as well as non-gonococcal *Neisseria* control isolates.

The antimicrobial properties of *Ocimum tenuiflorum* ("holy basil"), *Moringa oleifera,* and *Azadirachta indica* plants against *N. gonorrhoeae* were then explored. The plants were collected from the Botanical Gardens, Durban, South Africa. Upon collection and transport to the laboratory, the plants were left to dry naturally from sunlight for about 4 to 5 days and then used in the preparation of the aqueous extracts. The nanoemulsions were produced according to published methods. Different concentrations of the extracts (1000 µM, 100 µM, 10 µM, and 1 µM) were tested against *N. gonorrhoeae* isolates using the disk diffusion method. The extracts were also tested for their toxicity against human erythrocytes.

Results

In this study, were able to identify nine distinct Opa proteins from G136 and ten distinct Opa proteins from G180. For isolate G136 (asymptomatic patient), 66.7% of the Opa proteins bound to CEACAM3, 55.6% bound to CEACAM1, and 88.9% bound to CEACAM5. For isolate G180 (symptomatic patient), 30% of the Opa proteins bound to CEACAM3, 80% bound to CEACAM1, and 70% bound to CEACAM5. In this study, it was shown that the *N. gonorrhoeae* Opa proteins from a symptomatic patient bound at a higher frequency to CEACAM1 and 5 which causes the pathogen to invade the host cell and cause infections. However, *N. gonorrhoeae* Opa proteins from the asymptomatic patient bound at a higher frequency to CEACAM3.

According to the real-time PCR evaluation assays, the *opa* 1 primer performed better than *opa* 2, *opa* 3, and the comparator *opa* primer sets. The *opa* 1 assay produced positive amplification for the five WHO and the six *N. gonorrhoeae* clinical isolates, whereas the comparator assay amplified 90.9% of the samples. For the endocervical DNA samples, 82.8% were amplified

with the *opa* 1 assay, while the comparator assay had only amplified 27.6% of the samples. For the vaginal DNA samples, the *opa* 1 assay amplified 95.0% of the samples, whereas the comparator assay amplified 25.0% of the samples. All eleven (100%) urine DNA samples were amplified with the *opa* 1 assay, in contrast to 36.4% with the comparator assay. The *opa* 1 assay showed no cross-reactivity with non-gonococcal isolates, whilst cross-reactivity was observed with the comparator assay. The *opa* 1 assay also had a higher limit of detection when compared to the other assays.

In this study, all six gonococcal isolates exhibited clear zones of inhibition when exposed to the 1000µM concentration of all three of the nanoemulsion-based plant extracts investigated. Conversely, no zones of inhibition were detected at extract concentrations of 100µM, 10µM, and 1µM for five of the isolates. Isolate G176, however, displayed zones of inhibition at both 1000µM and 100µM concentrations when exposed to the nanoemulsion plant-based extracts derived from *Ocimum tenuiflorum*. Additionally, the WHO control strains included in the assay exhibited zones of inhibition at the 1000µM concentration. Notably, the WHO Y strain displayed zones of inhibition at both 1000µM and 100µM concentrations when exposed to nanoemulsion-based extracts from *Ocimum tenuiflorum* and *Azadirachta indica*. Importantly, the analysis revealed 0% haemolytic activity against human erythrocytes, indicating the non-toxic nature of the extracts.

Conclusion

In this study, it was demonstrated that *N. gonorrhoeae* Opa proteins obtained from symptomatic patients exhibited a higher affinity for CEACAM1 and CEACAM5, facilitating the pathogen's invasion of host cells and subsequent infection. Conversely, Opa proteins from asymptomatic patients displayed a higher binding frequency to CEACAM3. These Opa-CEACAM interactions are believed to potentially restrict the dissemination of gonococci by promoting

granulocyte-mediated opsonin-independent phagocytosis. Investigating interactions represents a crucial step forward in the quest for vaccine development against this pathogen. This study contributes valuable insights to the expanding body of knowledge concerning the host-receptor binding profiles of this pathogen. This study demonstrated that the *opa* 1 primer was the superior primer when compared to *opa* 2*, opa* 3, and the comparator *opa* primers from Verma *et al.,* (2012) study. Therefore, this in-house *opa* 1 assay can be potentially used and further evaluated for its use as a diagnostic assay. This study also demonstrated that the combination of nanoemulsions with plant extracts (specifically, *Ocimum tenuiflorum, Moringa oleifera*, and *Azadirachta indica*) presents a promising alternative to traditional antibiotics for combatting *N. gonorrhoeae* infections. This strategy capitalizes on the antimicrobial attributes of natural compounds and utilizes nanoemulsion technology to optimize their delivery and efficacy. Nevertheless, continuous research and development efforts must be undertaken to validate and enhance the viability of this approach for clinical application

Iqoqa

Ugcunsula yisifo esijwayelekile sokuthelelana ngokocansi esibangwa yi- Neisseria gonorrhoeae (N. gonorrhoeae). Abachaphazelwa yilesi sifo bayizigidi ezingama-80 minyaka yonke. Lolu cwaningo lwahlola ngokusetshenziswa kwendlela eyaziwa nge-reverse vaccinology kanye nezinye izindlela ezihlukile zokwelapha ukuze kunqandwe lesi sifo kanye nezindlela zokuhlola ezingabizi kakhulu ezigasela amaphrotheni anegciwane afana nofuzolibo i-opa. Lolu cwaningo lwasebenzisa ama-isolate amagciwane agciniwe e- N. gonorrhoeae kanye namasampuli atholakale kulabo abaqalayo ukutheleleka. Ama-asayi anamathiselayo sahlola ukuhambisana kwegciwane elisemzimbeni ngokuthola amaphethini okunamathelisa e-CEACAM kwi- N. gonorrhoeae ehlukaniswe/esuswe kubantu besifazane abakhombisa ukuba nalo (G180) kunalabo abangatshengisi ukuba nalo (G136).

Kule-asayi eyi-PCR yangaphakathi ocwaningweni kwenziwa lokhu okwaziwa ngama-primer set agasela/ahlasela ufuzolibo i-opa lwe- N. gonorrhoeae, kwase kuhlolwa kuqhathaniswa nama-primer ofuzolibo lwe-opa osekubhaliwe ngawo. Kwahlolwa okukhishwe ezitshalweni okuyi-(Ocimum tenuiflorum, Moringa oleifera, kanye ne Azadirachta indica)kwahlanganiswa nama-emulsion ayi-nano. Lokhu kuhlola kwakuqhathaniswa nama-isolate e-N. gonorrhoeae. Lokhu okukhishwe ezitshalweni kwahlolwa ukuthi akunabo ubuthi kusetshenziswa amaseli egazi lomuntu aziwa ngama-erythrocyte. Sakwazi ukuthola izinhlobo ezihlukile eziyisishiyagalolunye zamaphrotheyni i-Opa ku-G136 kanye namaphrotheyni ahlukile ayishumi e-Opa ku-G180. Kwi-isolate G136 awu-66.7% amaphrotheyni e-Opa anamathela ku-CEACAM3, ama-55.6% anamathela ku-CEACAM1, kanti ama-88.9% anamathela ku-CEACAM5. Kwi-isolate G180 kwanamathela ama-30% amaphrotheyni e-Opa kwi-CEACAM3, ama-80% anamathela ku-CEACAM1, kwathi ama-70% anamathela ku-CEACAM5 Amaphrotheyni e-Opa ye- N. gonorrhoeae eziguli ezingakhombisi ukuphathwa yilesi sifo anamathela ku-CEACAM3 ngokwezinga eliphezulu. Lokhu kuhlangana kwe-Opa-CEACAM kunganciphisa ukwanda kwegciwane logcunsula ngenxa yokwakhelka kohlobo lwe-phygocytosis emahhadla enganamatheleki. Kuyigxathu elincomekayo ukuhlola ukuhlangana kwe-Opa ne-CEACAM emizamweni yokugomela leli gciwane. Kuma-primer ayesetshenziswa ocwaningweni i-opa 1 yasebenza kangcono kune-opa 2, i-opa 3, kanye ne-opa okusetshenzelwa kuyo. I-asayi eyi-opa iyakhombisa ukungasebenzisani nama-isolate angenalo igciwane logcunsula.

Onke ama-isolate ayisithupha ogcunsula akhombisa izindawo ezisobala zokuthiba uma ebhekene nobungakokujiya obuyi-1000μM bako kokuthathu okukhishwe ezitshalweni okunama-emulsion e-nano asetshenziswe ekuhloleni. Ekuhlaziyweni kobuthi, lokho okukhishwe ezitshalweni kwakhombisa u-0% wokufa kwamaseli abomvu egazi uma kuhlolwa kuqhathaniswa namaseli abomvu egazi lomuntu ophile kahle. Lokhu kukhombisa ngokusobala ukuthi kuphephile ekusetshenzisweni emzimbeni kanti futhi kungasebenziseka njengesithako esiphephile ekwelapheni. Luyadingeka ucwaningo oluqhubekayo ukuze kuqiniseke futhi kuthuthukiswe ukusebenza kahle kwalezi zindlela ezintsha kwezokwelapha.

CHAPTER ONE

1. INTRODUCTION

1.1. Background

Globally, gonorrhoea has been reported as the second most prevalent sexually transmitted infection (STI), impacting approximately 80 million individuals each year (1, 2). *Neisseria gonorrhoeae* (gonococcus) is a Gram-negative, diplococcal bacterium, known to cause gonorrhoea. In a large population of infected people, especially women, the infection is asymptomatic which allows for silent transmission (3, 4). Women infected with gonorrhoea can incur more severe complications such as pelvic inflammatory disease (PID) and disseminated gonococcal infection (DGI), resulting in infertility or predisposing women to ectopic pregnancy (3, 5). Untreated *Neisseria gonorrhoeae* (*N. gonorrhoeae*) infections are associated with a range of adverse pregnancy outcomes such as conjunctivitis, foetal growth retardation, spontaneous abortion, stillbirth, prematurity, low birth weight, postpartum endometritis, and increased risk of Human immunodeficiency virus (HIV) transmission from mother to child during birth (6-8). According to the World Health Organization (WHO), approximately 82.4 million individuals contracted gonorrhoea as new cases in 2020 and approximately 11.4 million in the African Region (9). Studies conducted in South Africa have reported prevalence rates for *N. gonorrhoeae* ranging from 3%-11% in women (6, 10, 11).

The “gold standard” for the diagnosis of *N. gonorrhoeae* is culture (12-14). Culture includes microscopy (Gram staining and methylene blue staining) and antimicrobial susceptibility testing. These methods are inexpensive, highly sensitive and specific in well-developed laboratories (7, 14). The disadvantage associated with this technique is the low successful growth of *N. gonorrhoeae* due to the fastidious nature of the microorganism (7, 14). Due to the

limitations associated with culture, some nucleic acid amplification tests (NAATs) for *N. gonorrhoeae* have been Food and Drug Administration (FDA) approved (7). NAATs are the most sensitive techniques to detect *N. gonorrhoeae*. The sensitivity and specificity of *N. gonorrhoeae* NAATs are generally >95% and >99% in swabs respectively. Moreover, NAATs are easier to perform and faster than culture with less hands-on time and the capability of automation allowing high throughput testing (7).

Initial colonization of the mucosa by *N. gonorrhoeae* depends on several virulence factors, which have a high selectivity for human proteins, and which do not recognize determinants in standard model animals, such as rodents or non-human primates. *N. gonorrhoeae* mainly colonizes the genital mucosa, but it can also colonize the ocular, nasopharyngeal and anal mucosa (15-18). Accordingly, progress in developing preventive and/or therapeutic measures against gonococci and other human-restricted bacterial pathogens such as *Haemophilus ducreyi* has been conducted in a limited number of studies using appropriate animal models. For the efficient colonization of human mucosal epithelial cells, *N. gonorrhoeae* has evolved dedicated surface components, including, pili, colony opacity (Opa) proteins, and lipooligosaccharide (LOS). All three components are well-studied virulence factors of these bacteria and undergo phase and/or antigenic variation, allowing gonococci to evade the human immune response.

N. gonorrhoeae is primarily a mucosal colonizer, attaching to various epithelial surfaces. The first step in pathogenesis is bacterial adherence to the epithelium of the mucosa, which is mediated through distinct bacterial surface structures that include type IV pili, Opa proteins, LOS, and the major outer membrane protein porin (also known as PorB) (10). During initial infection, following initial host-cell interaction, *N. gonorrhoeae* attachment and subsequent colonization depend largely on type IV pili forming microcolonies on the epithelial cell surface (19). Type IV pili are outer membrane structures that are crucial for mediating initial cellular adherence, natural transformation competence, twitching motility, and immune evasion

through antigenic variation and phase variation. Type IV pili and Opa proteins are expressed during infection in both women and men and are considered essential for the colonization of the mucosal epithelium of the genital tract and other sites of infection (18, 20). Adherence to the epithelial surface and subsequent pilus retraction brings the gonococci close to the cell surface.

Opa proteins are located on the outer membrane of *N. gonorrhoeae* and facilitate the interaction of the bacteria with the several host cell types (including epithelial cells on mucosal surfaces and various immune cells) (21). Opa proteins form a barrel shape on the outer membrane of the gonococcus cells with approximately eight antiparallel β strands which are linked to the membrane using four extracellular loops (3, 21, 22). *N. gonorrhoeae* chromosomes encode for eleven unique Opa proteins which are distributed throughout the genome (4, 21). Opa proteins can switch on and off their sequence arrangements at any of the eleven *opa* alleles in the genome (4, 21, 22). The expression of these proteins is rapidly changing due to phase variation which is triggered by a pentameric coding repeat sequence which is caused by slipped-strand mispairing during DNA replication (21).

A total of 22 different genes have now been identified in the human Carcinoembryonic Antigen-Related (CEA) family, divided into the Carcinoembryonic Antigen-Related Cell Adhesion Molecules (CEACAM) and the pregnancy-specific glycoprotein subgroups. The CEACAM subgroup, which contains the Opa protein-binding receptors, contains one secreted and eleven cell surface glycoproteins, which have undergone several changes in nomenclature. Only CEACAM1, CEACAM3, CEA (or CEACAM5) and CEACAM6 have been shown to bind to *neisserial* Opa proteins (4, 21, 23). Gonococcal Opa proteins bind specifically to human CEACAM family members and not the CEACAM orthologue of other species (24). The binding of CEACAM1, CEA or CEACAM5 to the epithelial cell, benefits gonococci during mucosal colonization. It also triggers enhanced integrin-mediated host cell adhesion to the

extracellular matrix and counteracts the shedding of infected epithelial cells from tissue. A key host factor mediating rapid phagocytosis and destruction of gonococci is the carcinoembryonic antigen-CEACAM3, a granulocyte membrane glycoprotein (4, 25-27). Recognition by CEACAM3 depends on the presence of Opa protein variants (28). Recognition of Opa protein by CEACAM3 could limit the spread of gonococci because of granulocyte-mediated opsonin-independent phagocytosis (28). The lack of CEACAM3-binding Opa proteins might contribute to the ability of certain gonococcal strains to evade opsonin-independent recognition by granulocytes and to cause disseminated disease. Due to the frequent phase variation of Opa protein expression and the redundancy of *opa* genes in the gonococcal genome, there is a lack of detailed analysis of the CEACAM-binding Opa protein patterns.

Nanoemulsions, which are submicron-sized emulsions, are currently undergoing extensive research as potential drug carriers aimed at enhancing the delivery of therapeutic agents. Regarded as an advanced nanoparticle technique, nanoemulsions facilitate improved systematic delivery of biologically active agents, thereby enhancing and controlling drug delivery more effectively (29). Nanoemulsion refers to a stable, clear dispersion of two liquids that are typically immiscible, like oil and water, maintained by a surfactant film at the interface between them (29-31). Usage of plant extracts and the benefits of their properties to treat pathogens have been used lately to combat the side effects of chemical drugs and the ability of the pathogens to become multi-drug resistant (30). *Azadirachta indica* plants are suggested to have anti-bacterial and anti-inflammatory properties, therefore, seemingly promising against *N. gonorrhoeae* (32-35). Therefore, synergy between plant extracts and nanoemulsions may enhance the effectiveness of the treatment against *N. gonorrhoeae*, potentially reducing the risk of resistance development. Another advantage to this approach is, using nanoemulsions with plant extracts as an alternative treatment can help reduce the selective pressure for antibiotic resistance.

1.2. Literature Review

1.2.1. *Neisseria gonorrhoeae*

N. gonorrhoeae is a Gram-negative, diplococci bacteria, isolated first by Albert Neisser in 1879 (4, 36). *N. gonorrhoeae* is known to cause gonorrhoea. Gonorrhoea is the second most reported STI globally and remains a public health concern (37-40). *N. gonorrhoeae* is transmitted via direct contact, through sexual intercourse, kissing, touching and oral sex. Following transmission, *N. gonorrhoeae* establishes contact with the mucosal epithelium to replicate and ultimately be transmitted to new hosts. Local genital infections in both men and women can result in pain during urination and urethral discharge. In a large population of infected people, especially women, the infection is asymptomatic which allows for silent transmission (3, 4). Women infected with gonorrhoea can incur more severe complications such as pelvic inflammatory disease (PID) and disseminated gonococcal infection (DGI). Thus, resulting in infertility or predisposing women to ectopic pregnancy (3, 5). Untreated *N. gonorrhoeae* infections are associated with a range of adverse pregnancy outcomes such as conjunctivitis, foetal growth retardation, spontaneous abortion, stillbirth, prematurity and more (6-8).

Of global concern is the current appearance of multidrug-resistant gonococcal strains, which render the disease untreatable. Observing the alarming antimicrobial resistance and failure of treatment as well as the severe impact this STI has, more research must be done on vaccine development and alternative treatment for gonorrhoea. The WHO has recently issued a global health alert and urged the international scientific and drug-development community to increase efforts to combat *N. gonorrhoeae* (41-43).

1.2.2. Epidemiology

The WHO estimates that 82.4 million people were newly infected with gonorrhoea in 2020, including 35.2 million in the WHO Western Pacific Region, 11.4 million in the Southeast Asian Region, 11.4 million in the African Region, 11 million in the Region of America, 4.7 million in the European Region, and 4.5 million in the Eastern Mediterranean Region (Figure 1.1) (44). Studies conducted in South Africa have reported prevalence rates for *N. gonorrhoeae* ranging from 3%-11% in women (6, 10, 11, 45). The worldwide clinical management of *N. gonorrhoeae* infections is becoming increasingly challenging due to antimicrobial resistance (AMR) to various classes of available antibiotic therapy (46).

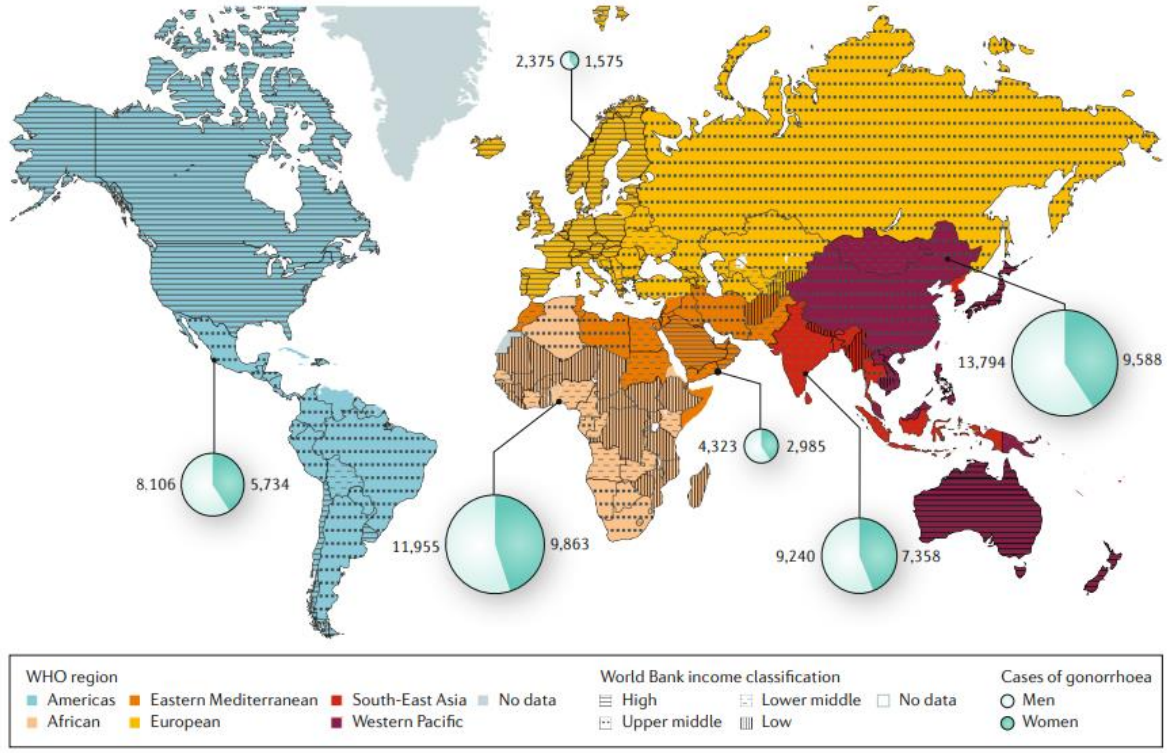

Figure 1.1: Estimated global cases of gonorrhoea in 2016. Estimated numbers (in millions) of incident cases of gonorrhoea in adults (15–49 years of age) by WHO region (36, 47).

1.2.3. Antimicrobial resistance of *Neisseria gonorrhoeae*

Antimicrobial resistance for gonorrhoea has increased over the past 50 years, rendering ineffective many classes of antibiotics including sulphonamides, penicillin, earlier cephalosporins, tetracyclines, macrolides and fluoroquinolones resulting in limited treatment options for this infection (41, 44, 48). The Extended-spectrum cephalosporin is the last option for empirical first-line gonorrhoea monotherapy in most countries worldwide (41, 44). However, decreased susceptibility or resistance to ceftriaxone has been reported and continues to emerge globally (49). Sporadic treatment failures with recommended dual therapy (ceftriaxone plus azithromycin) and ceftriaxone monotherapy have also been confirmed internationally (49). Since 2015, the international spread of ceftriaxone-resistant gonococcal strains has been confirmed and, in 2018, the first strain with ceftriaxone resistance plus high-level resistance to azithromycin was isolated in the United Kingdom and Australia (50, 51).

South Africa has followed the recommendations made by the WHO in 2014 which advocated for the replacement of the first-line treatment with oral cefixime to a single injectable dose (250 mg) of ceftriaxone (37, 44). Treatment failures of ceftriaxone monotherapy led to the WHO recommendation of administering dual antimicrobial therapy with the combination of ceftriaxone (250 mg) and azithromycin (1 g stat) (37). However, decreasing susceptibility of *N. gonorrhoeae* to ceftriaxone has been reported with the proportion of resistance to ceftriaxone varying extensively, from 1.3% to 55.8% (52). In addition, resistance to azithromycin is already prevalent in many settings (53). Therefore, dual antimicrobial therapy cannot ensure long-term effectiveness. This highlights the critical need to develop a vaccine to stop the spread of *N. gonorrhoeae.* To develop such a vaccine, a better understanding of the pathogenesis of *N. gonorrhoeae* is required.

1.2.4. Major colonization factors of *N. gonorrhoeae*

Initial colonization of the mucosa by *N. gonorrhoeae* depends on several virulence factors, which have a high selectivity for human proteins, and which do not recognize determinants in standard model animals, such as rodents or non-human primates. *N. gonorrhoeae* mainly colonizes the genital mucosa, but it can also colonize the ocular, nasopharyngeal and anal mucosa (15-18). Accordingly, progress in developing preventive and/or therapeutic measures against gonococci and other human-restricted bacterial pathogens such as *Haemophilus ducreyi* has been hampered by the lack of appropriate animal models. For the efficient colonization of human mucosal epithelial cells, *N. gonorrhoeae* has evolved dedicated surface components, including, pili, colony Opa proteins, and lipo-oligosaccharides (LOS) (15-18).

N. gonorrhoeae is primarily a mucosal colonizer, attaching to various epithelial surfaces. The first step in pathogenesis is the bacterial adherence to the epithelium of the mucosa, which is mediated through distinct bacterial surface structures that include type IV pili, Opa proteins, LOS and PorB (Figure 1.2) (18). During initial infection, following initial host-cell interaction, *N. gonorrhoeae* attachment and subsequent colonization depend largely on type IV pili forming microcolonies on the epithelial cell surface (19). Type IV pili are outer membrane structures that are crucial for mediating initial cellular adherence, natural transformation competence, twitching motility and immune evasion through antigenic variation and phase variation. Type IV pili and Opa proteins are expressed during infection in both women and men and are considered essential for the colonization of the mucosal epithelium of the genital tract and other sites of infection (Figure 2) (18, 20). Adherence to the epithelial surface and subsequent pilus retraction brings the gonococci close to the cell surface.

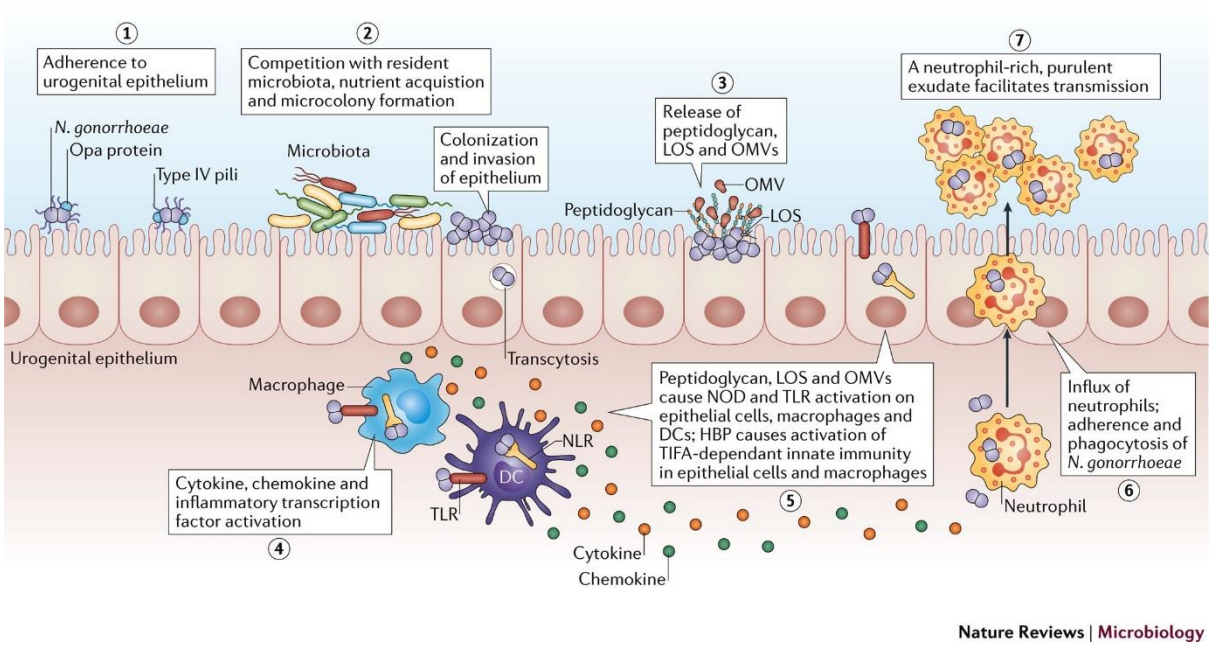

Figure 1.2: diagram describes the pathogenesis of *N. gonorrhoeae* (18).

1.2.5. Opa proteins

Bacterial pathogens have evolved intricate mechanisms to interact with host cells and establish infections. Among these mechanisms, Opa proteins, found primarily in *Neisseria* species, play a pivotal role in bacterial adhesion, immune evasion, and modulation of host cell response (18, 21). Gonococcal Opa proteins play, besides pili, a central role in the establishment and maintenance of gonococcal infection. After the initial pili-mediated attachment, Opa proteins are responsible for a more intimate interaction of gonococci with epithelial cells (54).

1.2.5.1. Structural Diversity of Opa Proteins

Opa proteins are located on the outer membrane of *N. gonorrhoeae* and facilitate the interaction of the bacteria and the several host cell types (including epithelial cells on mucosal surfaces and various immune cells) (21, 27).

Opa proteins form a barrel shape on the outer membrane of the gonococcus cells with approximately eight antiparallel β strands which are linked to the membrane using four

extracellular loops creating a pore-like structure (Figure 1.3) (3, 21, 22). The β-barrel domain is a conserved feature among Opa proteins. This domain not only anchors the Opa protein to the bacterial membrane but also provides stability to the overall structure. The β-barrel forms a channel that allows Opa proteins to traverse the bacterial cell envelope, facilitating their exposure to the extracellular environment (55, 56). The extracellular loops protruding from the β-barrel domain are responsible for the Opa protein's interaction with host cell receptors. These loops exhibit substantial variation in sequence and length, enabling Opa proteins to interact with a diverse range of host cell receptors, particularly CEACAMs (56). The dynamic nature of these loops, influenced by phase variation, results in a "switching" mechanism that alters the Opa protein's specificity for different receptors, contributing to immune evasion and host tropism. Every genetic site seems to undergo continuous transcription, yet the expression of separate Opa proteins is subject to autonomous regulation at the translational stage. This variability in expression arises from distinct pentameric repeat sequences within the 5' coding region (5’-CTCTT-3’) of each *opa* gene, governing the reading frame (57). The process of adding or removing pentameric repeats during bacterial chromosome replication, possibly due to slipped-strand mispairing, rectifies or disrupts the reading frame, leading to the on/off phase of individual Opa proteins (4, 57, 58). *N. gonorrhoeae* chromosomes encode for eleven unique Opa proteins which are distributed throughout the genome (4, 21, 59-61). Opa proteins can switch on and off their sequence arrangements at any of the eleven *opa* alleles in the genome (4, 21, 22). One of the remarkable features of Opa proteins is their high degree of antigenic variation. This allows bacteria to continually alter the appearance of their surface, evading host immune responses (57). Opa proteins mediate bacterial attachment to host cells, triggering a variety of downstream effects. These effects can include alterations in cellular signalling, cytoskeletal rearrangements, and immune evasion strategies, all of which enhance bacterial survival and colonization within the host (11).

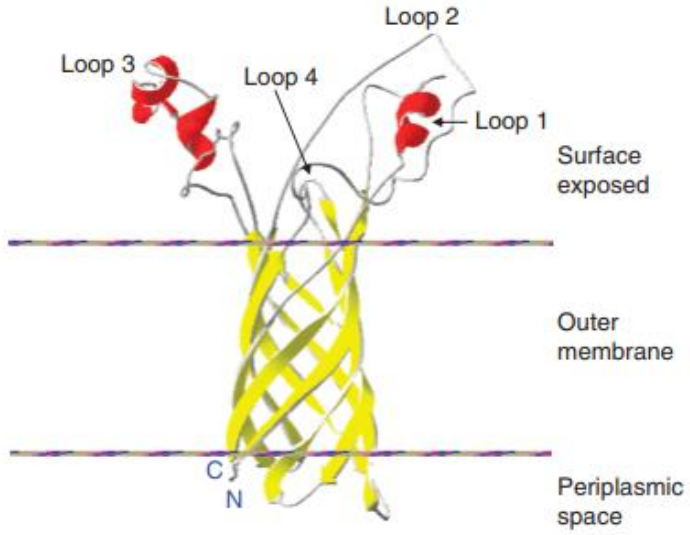

Figure 1.3: Predicted three-dimensional structure of Opa proteins (21).

1.2.5.2. Opa- CEACAM interactions

A total of 22 different genes have now been identified in the human CEA family, divided into the CEACAM and the pregnancy-specific glycoprotein subgroups. The CEACAM subgroup, which contains the Opa protein-binding receptors, contains one secreted and eleven cell surface glycoproteins, which have undergone several changes in nomenclature. Only CEACAM1, CEACAM3, CEA (or CEACAM5) and CEACAM6 have been shown to bind to *neisserial* Opa proteins (4, 21, 23).

Opa proteins play a crucial role as adhesion molecules that facilitate the close attachment of *N. gonorrhoeae* to host epithelial cells (54). Gonococcal Opa proteins bind specifically to human CEACAM family members and not the CEACAM orthologue of other species (24). Through functional investigation of the full range of Opa proteins in the gonococcal strain MS11, it has been demonstrated that one out of the eleven Opa proteins can bind to heparan sulfate proteoglycans (HSPGs) by recruiting vitronectin and fibronectin and can interact with host cell integrins (62-64). Opa proteins binding to HSPGs are alternatively referred to as OpaHSPG, emphasizing their specific binding affinity (54). The remaining ten Opa proteins in the MS11

strain bind to various members of the CEACAM family, notably CEACAM1, CEACAM3, CEA, and CEACAM6 (65, 66), and are accordingly designated as OpaCEA (54). Importantly, the OpaCEA proteins of *N. gonorrhoeae* exclusively bind to human CEACAM family members, distinguishing them from CEACAM orthologs in other species (24).

During mucosal colonization, OpaCEA proteins engage with epithelial CEACAMs, specifically CEACAM1, CEA, or CEACAM6, on the surface of epithelial cells. This interaction leads to advantageous outcomes for gonococci, including enhanced integrin-mediated adhesion of host cells to the extracellular matrix and prevention of the detachment of infected epithelial cells from the tissue (26, 28, 67, 68). Conversely, the recognition of CEACAM binding Opa proteins by CEACAM3 (present in human granulocytes) might curtail the dissemination of gonococci due to granulocyte-mediated opsonin-independent phagocytosis. This is exemplified by the presence of multiple CEACAM3-binding OpaCEA proteins in the genome of the MS11 strain, associated with localized infections (65, 66, 69). A key host factor mediating rapid phagocytosis and destruction of gonococci is carcinoembryonic antigen-CEACAM3, a granulocyte membrane glycoprotein (4, 27). Recognition by CEACAM3 depends on the presence of Opa protein variants (28).

The lack of CEACAM3-binding Opa proteins might contribute to the ability of certain gonococcal strains to evade opsonin-independent recognition by granulocytes and to cause disseminated disease. Due to the frequent phase variation of Opa protein expression and the redundancy of *opa* genes in the gonococcal genome, there is a lack of detailed analysis of the CEACAM-binding Opa protein patterns. Therefore, the present study was initiated to determine the CEACAM-binding profile of the Opa protein repertoire of South African clinical isolates of *N. gonorrhoeae* cultured from pregnant women.

1.2.5.3. Clinical Implications and Research Avenues

Understanding the roles of Opa proteins in bacterial infections has significant implications for public health. The continued rise of antibiotic-resistant *Neisseria* strains underscores the need for alternative therapeutic strategies. Opa proteins, given their direct involvement in virulence, present attractive targets for vaccine development and therapeutic interventions. Researchers are actively exploring the possibility of developing vaccines that target Opa proteins, aiming to elicit immune responses that prevent bacterial attachment and subsequent colonization.

1.2.6. Newer therapeutics against *Neisseria gonorrhoeae*

1.2.6.1 Nanoemulsions

Nanoemulsions which are emulsions with submicron-sized droplets, are currently undergoing extensive research as potential drug carriers to enhance the delivery of therapeutic agents. Nanoemulsions are considered an advanced nanoparticle method that aids in better systematic delivery of biologically active agents for enhanced and controlled drug delivery (29). A nanoemulsion is a thermodynamically stable isotropic clear dispersion of two immiscible liquids, such as oil and water, stabilized by an interfacial film of surfactant molecules (29, 31, 70). The dispersion phase generally consists of diminutive particles or droplets, falling within a size range of 5nm to 200nm, and exhibits exceptionally low interfacial tension between oil and water. Due to the droplet size being less than 25% of the wavelength, the nanoemulsion is formed easily (29, 70). In many cases, a co-surfactant or co-solvent is used in addition to the surfactant, the oil phase, and the water phase. There are three components of a nanoemulsion: oil, surfactant/ co-surfactant, and aqueous phase (29, 31, 70, 71). Nanotechnology has enhanced drug targeting capabilities and minimized the required dosage size and quantity for effective treatment (72).

1.2.6.1.1. Exploring the benefits of nanoemulsion formulations for drug delivery

Nanoemulsion formulations for drug delivery are a highly promising advancement in pharmaceuticals and healthcare. The advantages of using nanoemulsion formulations for drug delivery are manifold. Nanoemulsion include the ability to enhance the solubility of poorly water-soluble drugs, a critical factor for optimal drug absorption and bioavailability (73, 74). Moreover, nanoemulsions improve the bioavailability of drugs by enhancing their absorption and distribution within the body, ensuring a more significant proportion of the administered drug reaches its target site (73). These formulations also enable precise drug targeting, allowing engineered delivery to specific areas, like tumours or infected tissues, thus minimizing off-target effects (73). Furthermore, nanoemulsion can provide controlled and sustained drug release, ensuring a consistent therapeutic effect and potentially reducing dosing frequency (73). Nanoemulsions protect drugs from degradation, enhancing stability and shelf life and minimizing toxicity by delivering drugs directly to the site of action. Additionally, nanoemulsion facilitates combination therapy, intracellular drug delivery, and personalized medicine, making them versatile across various drug types and therapeutic applications (73). By integrating diagnostic and therapeutic functions, nanoemulsions enable real-time disease monitoring alongside drug delivery and can aid in overcoming drug resistance in certain diseases by ensuring a higher concentration of the drug reaches the site.

1.2.6.1.2. Nanoemulsions as antimicrobial agents

Nanoemulsions employ several means to disrupt bacterial membranes. The surfactants within these nanoemulsions can interact with the lipid bilayer of bacterial cell membranes, leading to destabilization and increased permeability. This interaction results in the leakage of intracellular contents, including ions and proteins, ultimately culminating in bacterial cell death. Another significant mechanism involves the solvent action of nanoemulsions. Many nanoemulsions contain lipophilic compounds that can solubilize and extract lipids from

bacterial membranes. This process disrupts the structural integrity and fluidity of the membrane, rendering it more susceptible to damage (73).

Certain nanoemulsions can generate reactive oxygen species (ROS), such as singlet oxygen, upon exposure to light. These highly reactive molecules can damage bacterial membranes and cellular components, eventually leading to cell death. A study conducted by Ahmad *et al.,* 2017 showed that rutin nanoemulsions provoked cell death by inducing ROS production (75). Keeping this in mind, we can assume that if ROS is induced, the damage to bacterial membranes and cellular components will lead to cell death in *N. gonorrhoeae.*

Nanoemulsions have been explored for their potential to disrupt biofilm formation, which is a protective matrix of extracellular polymeric substances that bacteria, including *N. gonorrhoeae*, often form. By disrupting biofilms, nanoemulsions weaken the bacterial community, rendering individual bacteria more susceptible to antimicrobial actions. A study conducted by Shehabeldine *et al.,* 2023 demonstrated that nanoemulsions displayed remarkable antibacterial activity, and nanoemulsions exhibited the lowest minimum inhibitory concentration (MIC) values ranging from 0.31 to 5 mg/mL against pathogenic Gram-negative and Gram-positive bacteria. Furthermore, when assessing antibiofilm efficacy against *S. aureus*, nanoemulsions were notably effective in reducing biofilm development. Since nanoemulsion has the capability of disrupting biofilm formation, it could have the exact mechanism of action for *N. gonorrhoeae (76)*. Furthermore, nanoemulsions offer the advantage of being utilized in combination therapy alongside other antimicrobial agents, such as antibiotics or antimicrobial peptides. Their ability to facilitate efficient delivery of these agents to bacterial cells enhances antimicrobial activity (73).

It is essential to acknowledge that the effectiveness of nanoemulsions as antimicrobial agents, as well as their mechanisms of action, can vary significantly based on factors such as the composition of the nanoemulsion, the specific bacterial strain being targeted, and

environmental conditions. Ongoing research is essential for a comprehensive understanding of the potential of nanoemulsions in combatting *N. gonorrhoeae* and other pathogenic bacteria. Nevertheless, nanoemulsions hold promise as a novel approach in the battle against antimicrobial resistance and infectious diseases, with their membrane-disrupting capabilities serving as a pivotal aspect of their antimicrobial activity.

1.2.6.2. Plant extracts

Plant extracts have properties that can be beneficial against pathogens and have recently been used to combat the side effects of chemical drugs as well as reduce the emergence of multidrug-resistant pathogens (70). *Ocimum tenuiflorum* ("holy basil"), *Moringa oleifera,* and *Azadirachta indica* plants are suggested to have antibacterial and anti-inflammatory properties and may show promising effects against *N. gonorrhoeae* (32-35, 77). There is a lack of data available on the effectiveness of these plants against this STI. Therefore, future research directions should explore this option. While these plants, *Ocimum tenuiflorum*, *Moringa oleifera*, and *Azadirachta indica,* have been traditionally used for their potential medicinal properties, their antimicrobial properties against STIs still require more comprehensive scientific research.

1.2.6.2.1. *Ocimum tenuiflorum* ("Holy Basil")

Holy basil possesses antimicrobial properties that are attributed to its essential oils, including eugenol. These compounds have demonstrated antibacterial, antiviral, and antifungal activities that include activity against many pathogens responsible for human infections (78, 79). While no human trials have been published, there is experimental evidence that holy basil may help treat various human bacterial infections, including urinary tract infections, skin and wound infections, Typhoid fever, gonorrhoea and herpes (80-83).

1.2.6.2.2. *Moringa oleifera*

Moringa extracts have exhibited antibacterial and antiviral activity against various pathogens (84). These properties could contribute to its potential to support the body's defence against STIs. *Moringa's* rich nutrient profile, including vitamins, minerals, and antioxidants, promotes overall health and immune function (85).

1.2.6.2.3. *Azadirachta indica* ("Neem")

Neem's active compounds, such as azadirachtin, have demonstrated antimicrobial properties against bacteria, viruses, and fungi (86). Neem can modulate immune responses, potentially aiding the body in resisting infections (86, 87). Neem's anti-inflammatory and antioxidant properties may help reduce inflammation and oxidative stress associated with infections (88).

Fusing nanoemulsion technology with medicinal plant extracts presents a promising and innovative approach for tackling *N. gonorrhoeae* infections. This amalgamation offers many advantages, starting with an amplification of antimicrobial activity achieved by enhancing the solubility and stability of the bioactive compounds inherent in medicinal plants. Nanoemulsions excel at delivering these compounds precisely to the infection site, ensuring maximal impact on the pathogen while sparing healthy tissues (74, 89-91). Furthermore, medicinal plants typically house a diverse array of bioactive compounds. When these are co-encapsulated within nanoemulsions, synergistic effects can be harnessed to combat *N. gonorrhoeae*, effectively reducing the risk of resistance development. Improved bioavailability, minimal side effects, and the potential for combination therapies further augment the appeal of this approach. Utilizing sustainable and natural sources, aligns seamlessly with the current trend toward eco-friendly alternatives to antibiotics, thereby minimizing environmental impact and the increase in antibiotic resistance of this pathogen. The rich diversity of medicinal plants from various geographical regions opens a vast landscape of possibilities in the battle against drug-resistant strains. However, it is crucial to underscore the necessity for rigorous research

and comprehensive clinical studies to substantiate the safety and efficacy of this cutting-edge treatment strategy for gonorrhoea.

1.2.7. Methods of detection for *Neisseria gonorrhoeae*

1.2.7.1. Culture-based tests

The "gold standard" for the detection of *N. gonorrhoeae* is culture (7, 92, 93). Culture includes microscopy (Gram staining and methylene blue staining) and antimicrobial susceptibility testing (2). These methods are cheap and highly sensitive and specific in well-developed laboratories (93, 94). The disadvantage associated with this technique is the low successful growth of *N. gonorrhoeae* due to the fastidious nature of the microorganism (7, 93).

1.2.7.2. Nucleic acid amplification test

Due to the limitations associated with culture, some NAATs for *N. gonorrhoeae* have been approved by the FDA (Table 1.1) (7). NAATs are the most sensitive techniques to detect *N. gonorrhoeae*. The sensitivity and specificity of *N. gonorrhoeae* NAATs are generally >95% and >99% in swabs respectively. Moreover, NAATs are easier to perform and faster than culture with less hands-on time and the capability of automation allowing high throughput testing (7). One limitation of NAATs is their inability to detect AMR, and certain PCR systems can be expensive leading to issues of accessibility in resource-limited settings. Nevertheless, NAATs have been previously recognized as a remarkable platform for detecting *N. gonorrhoeae* in clinical specimens. These types of tests include different types of PCRs such as *16S rRNA* PCR, and PCR for functional genes such as the *opa* gene (14, 92, 95). Other accurate methods include; *N. gonorrhoeae* multi-antigen sequence typing which is employed for the identification of different strains associated with specific populations and Whole Genome Sequencing (WGS) which provides information on the local and global epidemiology of gonorrhoea infections as well as the ligase chain reaction which was previously used (95, 96).

Table 1.1: Current FDA-approved NAATs for the detection of *N. gonorrhoeae* (7).

Assay (Company)	***Ng* Targets**	**Cleared Specimen Types**
Abbott Real-time CT/NG (Abbott)	*Opa* gene	Women: urine, swabs (vaginal, endocervical) Men: urine, urethral swab
cobas CT/NG (Roche)	Two different targets in the DR 9 region	Women: urine, swabs (vaginal, endocervical) Men: urine
APTIMA Combo 2 Assay (Hologic)	*16S-rRNA*	urine swabs (vaginal, endocervical, urethral, rectal, pharyngeal)
BD MAX GC BD MAX CT/GC BD MAX CT/GC/TV	*OpcA* gene	urine (20-60mL of first morning urine recommended), swabs (vaginal endocervical)
BD ProbeTec *Neisseria gonorrhoeae* (GC) Qx Amplified DNA Assay	*Pilin*-gene inverting protein homologue	Women: urine, swabs (vaginal, endocervical) Men: urine, urethral swab
BDProbeTec ET *Chlamydia trachomatis* and *Neisseria gonorrhoeae* Amplified DNA Assays	*Pilin*-gene inverting protein homologue	Women: urine, endocervical swab Men: urine, urethral swab
Xpert CT/NG (Cepheid)	Two distinct chromosomal targets	urine swabs (vaginal, endocervical, rectal, pharyngeal)
Binx io CT/NG Assay (binx health)	Not specified	vaginal swabs

1.3. Rationale for this study

The emergence of multidrug-resistant gonococcal strains is a grave public health concern. Due to the increasing rise in antimicrobial resistance and clinical consequences of infection, it is of paramount importance to facilitate research involved in the development of vaccines for gonorrhoea. Opa proteins, found primarily in *Neisseria* species, play a central role in bacterial adhesion, immune evasion, and modulation of host cell response (18, 21). The Opa-CEACAM interaction has been an interaction of interest in *N. gonorrhoeae.* Gonococcal Opa proteins bind specifically to human CEACAM family members and not the CEACAM orthologue of other species (24). An understanding of the host-binding properties of *N. gonorrhoeae* will facilitate efforts toward vaccine designs for this pathogen. Therefore, the present study was initiated to determine the CEACAM-binding profile of the Opa protein repertoire of South African clinical isolates of *N. gonorrhoeae* cultured from pregnant women. The study aimed to analyse the

host-pathogen associations by determining the CEACAM binding patterns of *N. gonorrhoeae* in symptomatic versus asymptomatic patients.

The "gold standard" for the detection of *N. gonorrhoeae* is culture. This method has disadvantages such as low successful growth of *N. gonorrhoeae* due to the fastidious nature of the microorganism and its long detection period (3-5 days). Based on these disadvantages, NAATs for *N. gonorrhoeae* which target different genes, such as Opa proteins, *16S rRNA*, porin A (*porA*) pseudogene, complete protein B (*cppB*) gene, Charcot-marie -tooth (*CMT)* gene, and pili (*pil*) gene, are being designed and assessed by laboratories throughout the world. Opa proteins are located on the outer membrane of *N. gonorrhoeae* and facilitate with the interaction of the bacterium and the several host cell types (including epithelial cells on mucosal surfaces and various immune cells) (3, 21, 22). *N. gonorrhoeae* chromosomes encode for eleven unique Opa proteins which are distributed throughout the genome (3, 21, 22). Since *opa* genes are multicopy genes that contain conserved areas and encode proteins with physiological function, *opa* genes are therefore considered suitable target sequences for a real-time PCR amplification assay (93). The objective of the present study was to develop and evaluate the performance of an in-house *opa*-based real-time PCR assay. Three primer sets targeting the *opa* gene of *N. gonorrhoeae* were designed and evaluated against published *opa* gene primers (Verma *et al.*, 2012) (97).

Nanoemulsions serve as submicron-sized emulsions utilized for the improved delivery of therapeutic agents. Nanoemulsion are seen as an advanced nanoparticle technique facilitating more efficient systemic administration of biologically active substances, thereby enhancing and regulating drug delivery (29). In addition, plant extracts have been recently exploited to counteract the adverse effects of pharmaceutical drugs and circumvent the rise of pathogens that are resistant to multiple drugs (70). According to the WHO, traditional medicines derived from medicinal plants continue to provide benefits to 80% of the developing world's population

(98-100). Therefore, combining medicinal plant extracts with nanoemulsions can lead to synergistic effects. This study investigated the antimicrobial properties of nanoemulsions of *Ocimum tenuiflorum* ("holy basil"), *Moringa oleifera* and *Azadirachta indica* plants. While these plants, *Ocimum tenuiflorum*, *Moringa oleifera*, and *Azadirachta indica*, have been traditionally used for their potential medicinal properties, their antimicrobial properties against STIs require more comprehensive scientific research. Therefore, this will be the first study to explore the activity of these nanoemulsion-based plant extracts against *N. gonorrhoeae* in the South African context.

1.4. Aims and Objectives

Aim one

To investigate the host receptor binding capabilities of *N. gonorrhoeae.*

Objectives

- To identify unique *opa* alleles in isolates of *N. gonorrhoeae*.
- To analyse the host-pathogen associations by determining the CEACAM binding patterns of *N. gonorrhoeae*.

Aim two

To design newer nucleic acid amplification detection methods for *N. gonorrhoeae*.

Objectives

- To evaluate the performance of in-house *opa* amplification assays against a published assay for the detection of *N. gonorrhoeae*.
- To determine the cross-reactivity of the in-house assays against non-gonococcal *Neisseria* species.

Aim three

To investigate the antimicrobial properties of plant extracts against *N. gonorrhoeae* using a nanoemulsion method.

Objectives

- To determine the antimicrobial activities of plant nanoemulsions from *Ocimum tenuiflorum*, *Moringa oleifera* and *Azadirachta indica* plants against *N. gonorrhoeae.*
- To determine the toxicity of the plant nanoemulsions using human erythrocytes.

CHAPTER TWO

AIM ONE

Investigating the Host-Binding Properties of *Neisseria gonorrhoeae* Isolated from South African Women

1. INTRODUCTION

Gonorrhoea is the second most reported STI globally and remains a public health concern (37-40). *Neisseria gonorrhoeae* (*N. gonorrhoeae*) is known to cause gonorrhoea and is a Gram-negative, diplococci bacteria, isolated by Albert Neisser in 1879 (4, 36). *Neisseria gonorrhoeae* is spread through direct contact like sexual intercourse, kissing, and touching. After transmission, it attaches to mucosal epithelium to reproduce and spread to others. Symptoms include pain during urination and discharge from the urethra, but many infected individuals, especially women, do not show symptoms, facilitating silent transmission (3, 4). Gonorrhoea can lead to serious complications in women, such as PID and DGI, which can increase the risk of infertility and ectopic pregnancy (3, 5). Untreated infections of *N. gonorrhoeae* can lead to various negative outcomes during pregnancy, including conjunctivitis, impaired foetal growth, spontaneous abortion, stillbirth, premature birth, and other complications (6-8).

The current emergence of multidrug-resistant gonococcal strains, which make the disease impossible to treat, is a matter of global concern. Given the alarming rise in antimicrobial resistance, treatment failures, and the severe impact of this STI, it is imperative to prioritize further research into developing vaccines for gonorrhoea. The Opa- CEACAM interaction has been an interaction of interest in *N. gonorrhoeae.* An understanding of the host-binding properties of *N. gonorrhoeae* will facilitate efforts toward vaccine designs for this STI.

Opa proteins, found primarily in *Neisseria* species, play a pivotal role in bacterial adhesion, immune evasion, and modulation of host cell response (18, 21). Gonococcal Opa proteins in addition to pili, play a central role in the establishment and maintenance of gonococcal infection. After the initial pili-mediated attachment, Opa proteins are responsible for a more intimate interaction of gonococci with epithelial cells (54). The Opa proteins adopt a barrel-shaped configuration along the outer membrane of gonococcal cells. This configuration consists of around eight antiparallel β strands, connected to the membrane via four extracellular loops, thereby forming a pore-like structure (3, 21, 22). The β-barrel domain remains a consistent characteristic across Opa proteins. It serves the dual function of securing the Opa protein to the bacterial membrane and enhancing the structural integrity. This domain creates a passage enabling Opa proteins to navigate through the bacterial cell envelope, thereby aiding their interaction with the external surroundings (55, 56). The extracellular loops protruding from the β-barrel domain are responsible for the Opa protein's interaction with the host cell receptors. These loops exhibit substantial variation in sequence and length, enabling Opa proteins to interact with a diverse range of host cell receptors, particularly CEACAMs (56). The ever-changing character of these loops, affected by phase variation, leads to a "switching" mechanism that modifies the Opa protein's affinity for various receptors. This process contributes to immune evasion and the adaptation to different hosts. Although every genetic locus undergoes ongoing transcription, the expression of individual Opa proteins is autonomously controlled during translation. This variability in expression arises from distinct pentameric repeat sequences within the 5' coding region (5’-CTCTT-3’) of each *opa* gene, governing the reading frame (57). The addition or deletion of pentameric repeats during bacterial chromosome replication, potentially caused by slipped-strand mispairing, corrects, or interrupts the reading frame. This alteration results in the activation or deactivation of individual Opa proteins (4, 57, 58). *N. gonorrhoeae* chromosomes encode for eleven unique

Opa proteins which are distributed throughout the genome (4, 21, 59-61). Opa proteins can switch on and off their sequence arrangements at any of the eleven Opa alleles in the genome (4, 21, 22).

The Opa proteins of gonococci exhibit a specific affinity for human CEACAM family members, rather than the CEACAM counterparts found in other species (24). Through functional investigation of the full range of Opa proteins in the gonococcal strain MS11, it has been demonstrated that one out of the eleven Opa proteins can bind to HSPGs or, by recruiting vitronectin and fibronectin, can interact with host cell integrins (62-64). The remaining ten Opa proteins in the MS11 strain bind to various members of the CEACAM family, notably CEACAM1, CEACAM3, CEA (CEACAM5), and CEACAM6 (54, 65, 66).

Apart from humoral elements such as the complement system and antibodies against gonococci, it is believed that innate immune mechanisms involving cells, play a role in constraining gonococcal infections. Granulocytes are extensively mobilized to infection sites and possess the capability to identify and eradicate gonococci without relying on opsonins (101). An essential host factor facilitating the quick phagocytosis and elimination of gonococci is CEACAM3. Recognition by CEACAM3 depends on the presence of Opa protein variants (28). Recognition of Opa proteins by CEACAM3 could limit the spread of gonococci because of granulocyte-mediated opsonin-independent phagocytosis (28).

Understanding the roles of Opa proteins in bacterial infections has significant implications for public health. The continued rise of antibiotic-resistant *Neisseria* strains underscores the need for alternative therapeutic strategies. Opa proteins, given their direct involvement in virulence, present attractive targets for vaccine development and therapeutic interventions. Researchers are actively exploring the possibility of developing vaccines that target Opa proteins, aiming

to elicit immune responses that prevent bacterial attachment and subsequent colonization (102, 103).

Therefore, the present study was initiated to determine the CEACAM-binding profile of the Opa protein repertoire of South African clinical isolates of *N. gonorrhoeae* cultured from pregnant women. The study aimed to analyse the host-pathogen associations by determining the CEACAM binding patterns of *N. gonorrhoeae* in symptomatic versus asymptomatic patients.

2. MATERIALS AND METHODS

2.1. Ethical approval

Ethical approval for this study was obtained from the Biomedical Research Ethics Committee (BREC) of the University of KwaZulu-Natal, (BREC/00005104/2022).

2.2. Study design

This was a retrospective laboratory-based study using stored bacterial isolates collected from a larger study. The larger study was conducted at the School of Clinical Medicine Laboratory, University of Kwa-Zulu Natal. That study included pregnant women attending the antenatal clinic at the King Edward Hospital in Durban, South Africa. Pregnant women, 18 years and older from any gestational age were eligible for participation. At enrolment, women underwent a pelvic examination by a specialist gynaecologist to collect two endocervical swabs for testing. Women who presented with clinical signs of infection were treated using syndromic management. Endocervical swabs were placed immediately in Amies Media with charcoal after collection. The swabs were processed within 6 hours of collection. At the laboratory, the swabs were used to inoculate New York City Agar plates. The plates were incubated for 48 hours at 37℃ in the presence of 5% CO_2. After incubation, *N. gonorrhoeae* appeared as small greyish white to colourless mucoid colonies. The pure isolates were stored at -80℃. For this analysis, two clinical isolates (one from a symptomatic woman [G180] and one from an asymptomatic woman [G136]) were cultured. The women were confirmed to be symptomatic and asymptomatic for signs of STIs (abnormal discharge, vaginal itchiness, burning during urination) by the clinician who had performed the pelvic examinations on these women.

2.3. Culture confirmation of stored *N. gonorrhoeae* isolates

The isolates were retrieved from storage at -80 ℃ and cultured onto chocolate agar plates and incubated for 24 to 48 hours at 37℃ in the presence of 5% CO_2. Following incubation,

suspected colonies were sub-cultured onto chocolate agar plates using a four-way streak technique. The plates were incubated for another 24 to 48 hours in the presence of 5% CO_2. Presumptive tests were conducted which included the oxidase test, Gram-staining, and the Analytical Profile Index (API) test kit (BioMerieux, United States of America) which acted as a means of confirmation.

For the oxidase test, suspected colonies were spotted on filter paper and a dropper was used to add a drop of oxidase reagent thereafter a colour change was observed and noted. The Gram-stain was done by smearing the bacteria on a microscope slide near a flame, thereafter a dropper was used to add crystal violet onto the slide for 1 minute, followed by iodine for 1 minute, alcohol for 10 seconds and carbol fuschin for 1 minute. A microscope (Olympus CX21, New York Microscope Company, United States) was used at 1000x under oil immersion to determine the Gram-stain results.

The API test was done according to the manufacturer's instructions (BioMerieux, 2016). Briefly, a sterile loop was used to pick up isolated colonies from the chocolate agar plates and the colonies were re-suspended into 2 ml of NaCl 0.85% medium, and the turbidity was equivalent to a 4 McFarland standard. Thereafter, the strip was inoculated by distributing the prepared bacterial suspension into each cupule on the strip using a pipette. A total of 50µl of the suspension was pipetted into the first seven microtubes going from PEN to URE and 150µl of the suspension was added to the last three cupules. The first seven tests were covered with mineral oil, the test was closed and put to incubate for 2 to 2 ¼ hours at 36 ℃ under aerobic conditions. After the incubation period, the reactions were read according to the reading table provided by the manufacturer. The results of reactions LIP, PAL and β-GAL were recorded before the addition of the reagent. Thereafter, 1 drop of ZYM B reagent was added to the cupules labelled LIP/ProA and PAL/GGT and 1 drop of JAMES reagent was added to the

microtube labelled β-GAL/IND. Results were read after 3 minutes according to the interpretation guide shown in Figure 2.1. A four-digit profile was generated and compared to the numerical list of profiles provided by the manufacturer (Figure 2.2).

2.4. DNA extraction from cultured isolates

Fresh cultures were removed from the overnight chocolate agar plates, resuspended into 1ml Brain Heart Infusion (BHI) broth, and thereafter incubated for 30 minutes at 37 ℃ aerobically. The DNA extraction procedure was performed on the pure cultures using the Wizard® Genomic DNA Purification Kit (Promega) according to the manufacturer's instructions (Promega Corp., Madison, United States). Very briefly, 1ml of an overnight culture was added to a 1.5ml microcentrifuge tube and centrifuged at 16000 x *g* for 2 minutes to pellet the cells. The supernatant was removed and to the tube with the pellet, 600µl of Nuclei Lysis Solution was added and gently pipetted until the cells were resuspended. The tube was incubated at 80 ℃ for 5 minutes to lyse the cells; then cooled to room temperature. Further 3µl of RNase solution was added to the cell lysate and the tube was then inverted 2–5 times to mix the solution. This was incubated at 37 ℃ for 60 minutes. The sample was cooled at room temperature. To the sample, 200µl of Protein Precipitation Solution was added to the RNase-treated cell lysate and vortexed vigorously at high speed for 20 seconds to mix the Protein Precipitation Solution with the cell lysate. The sample was incubated on ice for 5 minutes and thereafter centrifuged at 16000 x *g* for 3 minutes. The supernatant containing the DNA was transferred to a clean 1.5ml microcentrifuge tube containing 600µl of room-temperature isopropanol. The samples were then gently mixed by inversion until the thread-like strands of DNA formed a visible mass, thereafter, centrifuged at 16000 x *g* for 2 minutes. The supernatant was removed gently, and the tube was drained on clean absorbent paper. Thereafter, 600µl of 70% ethanol (room temperature) was added to the tube and gently inverted several times to wash the DNA pellet. The sample was centrifuged at 16000 x *g* for 2 minutes. The ethanol was

carefully aspirated, the tube was drained on a clean absorbent paper and the pellet was air-dried for 15 minutes. Thereafter, 100µl of DNA Rehydration Solution was added to the tube which rehydrated the DNA by incubating at 65°C for 1 hour. The solution was mixed periodically by gently tapping the tube. A Nanodrop spectrophotometer (ThermoFisher Scientific, South Africa) was used to assess the concentration and purity of the extracted DNA. The extracted DNA was stored at -20℃ until further use.

2.5. Cloning and expression of Opa proteins in *Escherichia coli*

Opa genes were initially amplified through PCR using chromosomal DNA from the two clinical *N. gonorrhoeae* isolates. The specific primers used were Opa-*Xho*I-sense (5'-CCTCTCGAGTCTCTTCTCTTCTCTTCC-3') and Opa1/2-MC58-*Hind*III-anti (5'-GGTCAA AGCTTTCAGAAGCGGTAGCG-3'). Each reaction comprised 12.5 µl DreamTaq master mix (ThermoFisher Scientific, United States), 0.25 µl forward primer (10uM), 0.25 µl reverse primer (10uM) and 0.5 µl of template DNA to a final volume of 25 µl. Amplification included an initial denaturation cycle at 94 ℃ for 2 minutes followed by 30 cycles of denaturation (94 ℃ for 20 seconds), annealing (60 ℃ for 20 seconds), extension (72 ℃ for 1 minute), and final extension (72 ℃ for 5 minutes). Subsequently, the amplified products were cloned into the pCR Blunt II-TOPO vector (Invitrogen) and used to transform *E. coli* JM109 cells (Promega). Single clones from the *opa* amplicon library were sequenced via Sanger sequencing to identify the respective unique *opa* genes. We used the sequence generated in the Roth *et al.,* (2013) study, as a guide when editing the sequences on Clone Manager (4). Thereafter, we performed multiple sequence alignments to select the unique Opa proteins amongst the clones generated. Once we selected the unique Opa proteins, we performed a second PCR on the selected clones. To prevent phase variation, we followed the cloning strategy outlined by Kuespert *et al.,* 2011 (104). Accordingly, the selected Opa proteins were further amplified using the primers Opa-MC58-mitte-sense

(5’ATCGCTTCTATTTAGCTCTTTATTGTTCAGTTCCCTACTCTTCAGCTCCGCAGCGC AGGCGGCAACTGA-3’) and Opa1/2-MC58-*Hind*III-anti. Each reaction mix was the same as the first round of PCR. Amplification included an initial denaturation cycle at 94℃ for 2 minutes followed by 30 cycles of denaturation (94 ℃ for 20 seconds), annealing (57℃ for 20 seconds), extension (72℃ for 1 minute), and final extension (72 ℃ for 5 minutes). The resulting PCR product was used as a template for a third PCR, employing the primers OpaMC58 (pET28)-*Nco*I-sense (5’GGCGCCCATGGAACCAGCCCCCAAAAAACCTTCTCTCCTGT TCTCATCGCTTCTATTTAGCTCTTTA-3’) and Opa1/2-MC58-HindIII-anti. Each reaction mix was the same as the first round of PCR. Amplification included an initial denaturation cycle at 94℃ for 2 minutes followed by 30 cycles of denaturation (94℃ for 20 seconds), annealing (53℃ for 20 seconds), extension (72℃ for 1 minute), and final extension (72℃ for 5 minutes). The products from this third PCR were digested using *Nco*I and *Hind*III enzymes and then inserted into the pET-28a vector (Novagen). Colony PCR was performed to verify and select the appropriate clones. The primers used were the forward (5’-ATAGCGGCCGCGGCCCGTATGTACAGGC-3’) and the reverse (5’-ATAGCGGCCGCGAATTCTCAGAAGCGGTAGCGCACG-3’). Each reaction mix was the same as the first round of PCR. Amplification included an initial denaturation cycle at 94 ℃ for 2 minutes followed by 30 cycles of denaturation (94℃ for 20 seconds), annealing (70℃ for 20 seconds), extension (72℃ for 1 minute), and final extension (72℃ for 5 minutes). The recombinant plasmids were extracted using the Zippy Plasmid Miniprep kit (Inqaba Biotech, South Africa) as per the manufacturer’s instruction. The recombinant plasmids encoding Opa proteins was verified by sequencing and transformed into *E. coli* Single Step (KRX) competent cells (Promega), which was induced for protein expression by IPTG (isopropyl-β-D-thiogalactopyranoside) and incubated at 37°C in Luria-Bertani (LB) medium supplemented

with appropriate antibiotics. All primers for section 2.5 were generously provided by the collaborators from Konstanz University, Germany.

2.6. Cell culture and transfection

Human embryonic kidney cells, specifically the 293T cell line (referred to as 293 cells), were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% calf serum. The cell culture was maintained at 37 ℃ in an environment containing 5% CO_2, and sub-culturing was performed every 2 to 3 days. For transfection of the 293 cells, a calcium phosphate co-precipitation method was employed, using 5µg of plasmid DNA for each 10-centimeter culture dish. The CEACAM plasmids were obtained from the University of Konstanz, Germany. After 6 hours of transfection, the culture medium was replaced with OptimMem (Gibco BRL). Culture supernatants was collected 3 days after transfection and purified from cell debris by centrifugation (2500 x *g*, 4 ℃, 10 minutes). Supernatants were adjusted for equal levels of soluble CEACAMs and used for pull-down assays. Control supernatants for this experiment was CEACAM 8 (Negative control).

2.7. Recombinant plasmid constructs

Mammalian expression plasmids that carry the soluble amino-terminal domain of CEACAM1, CEACAM3, CEACAM5, and CEACAM8 with a GFP tag were detailed in a previous publication and were generously supplied by the University of Konstanz, Germany (105).

2.8. Cell lysis and Western blotting

Overnight colonies were selected and added to 5 ml of LB medium supplemented with appropriate antibiotics and incubated at 37 ℃ on a shaker for ± 4 hours. The samples were then induced with IPTG and incubated on the shaker at 37 ℃ for one hour. The samples were loaded on a 12% Sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel which ran for ±1 hour 30 minutes at 120 volts. Thereafter the western blot was performed for viewing

purposes using the iBlot™ 2 Dry Blotting system (ThermoFisher Scientific, United States). The iBlot™ 2 Gel Transfer Device is pre-programmed with six voltage methods that allow blotting using different combinations of volts and time. For this study, we used the preset programme of P0, 20-25 volts and ran the transfer for around 10 minutes using Polyvinylidene difluoride (PVDF) membrane. Cell lysis and Western blotting was performed using a monoclonal antibody against GFP (clone JL-8; Clontech) or a monoclonal antibody against Opa proteins (clone 4B12/C11), provided by the University of Konstanz, Germany. Secondary antibodies (goat α-mouse IgG HRP-conjugate) were provided by the University of Konstanz, Germany. Blocking was performed on the membrane for one hour using blotto solution (mix 20g Bovine Serum Albumin (BSA), 0.5g sodium azide with 1L TBS-T solution). TBS-T was preprepared as follows: 10X Tris-Buffered Saline (mix 121g Trizma base, 175g sodium Chloride with 2L distilled water to pH 7.5) and 10% Tween (50ml tween-20 and 500ml distilled water) was first prepared. Subsequently, TBS-T solution is prepared by mixing 200ml 10X TBS, 10ml 10% tween with 2L distilled water. After one hour of blocking the membrane, the solution was discarded and fresh blotto with the primary antibody was left shaking overnight at 4℃. The next day, the solution with the primary antibody was removed, and the membrane was washed with TBS-T solution three times for 10 minutes each time. Thereafter, the membrane was left shaking with the secondary antibody for a further one hour at room temperature. After the one hour, the membrane was washed with TBS-T three times for 10 minutes each time. The membrane was viewed using 1-Step™ Ultra TMB-Blotting Solution (ThermoFisher Scientific, United States).

2.9. Pull-down Assay

Expression of the soluble N-terminal domains of human CEACAMs in 293 cells and binding studies with different pathogens was performed as described previously by Kuespert *et al.,*2007 (15). Briefly, 2 x 10^7 bacteria were added to the CEACAM-N domain containing cell culture

supernatant in a total volume of 1 ml and incubated for 1 hour. After incubation, bacteria were washed twice with PBS (Phosphate Buffered Saline) and boiled in SDS (sodium dodecyl sulphate) sample buffer prior to SDS-PAGE and Western blotting. Western blot was performed using a monoclonal antibody against GFP (clone JL-8; Clontech) provided by the University of Konstanz, Germany. Secondary antibody (goat α-mouse IgG HRP-conjugate) were also provided by the University Konstanz, Germany. Similar method was performed for the SDS-PAGE and Western blot as described for the cell lysis.

3. RESULTS

3.1. Culture detection of *N. gonorrhoeae*

To detect and confirm that the isolates were *N. gonorrhoeae,* several tests and procedures were performed such as sub-culturing onto chocolate agar plates, the oxidase test, Gram-staining, and the API test. The isolates were oxidase-positive (purple in colour) and Gram-negative diplococci. Figure 2.3 displays the API test strips which were used to confirm if the isolates were *N. gonorrhoeae*, each isolate had its own test strip, and it was labelled accordingly. Table 2.1 describes the results obtained from this test, overall, all six isolates produced the same results with only a few exceptions which had been noted. Once the results were captured and compared to the numerical profiles provided by the manufacturer, a four-digit profile of 1001 was generated, which matched the numerical profile for *N. gonorrhoeae* according to the manufacturer's insert (Figure 2.2). For this study aim (Chapter Two) the focus was on the results for G136 and G180 only, since the study was to investigate the binding profiles across isolates from a symptomatic versus an asymptomatic patient.

Table 2.1: API test results according to the colour changes that occurred from Figure 1. All six isolates produced the same results with only a few exceptions which have been noted.

Tests:	PEN	GLU	FRU	MAL	SAC	ODC	URE	LIP	PAL	βGAL	ProA	GGT	IND
Results:	+	+	-	-	-	-	-	-	-	-	+	-	-
Exceptions:	G176 G247	G247									G176 G247		

READING TABLE

TESTS	ACTIVE INGREDIENTS	QTY (mg/cup.)	REACTIONS/ENZYMES	RESULTS	
				NEGATIVE	POSITIVE
1) PEN	potassium benzylpenicillin	1.36	PENicillinase	blue (penicillinase absent)	yellow yellow-green yellow-blue (penicillinase present)
2) GLU	D-glucose	0.5	acidification (GLUcose)	red red-orange	yellow orange
3) FRU	D-fructose	0.1	acidification (FRUctose)		
4) MAL	D-maltose	0.1	acidification (MALtose)		
5) SAC	D-saccharose (sucrose)	0.5	acidification (SACcharose)		
6) ODC	L-ornithine	0.552	Ornithine DeCarboxylase	yellow-green grey-green	blue
7) URE	urea	0.41	UREase	yellow	pink-violet
8a) LIP	5-bromo-3-indoxyl-caprate	0.033	LIPase	colorless pale grey	blue (+ precipitate)
9a) PAL	4-nitrophenyl-phosphate 2CHA	0.038	ALkaline Phosphatase	colorless pale yellow	yellow
10a) ßGAL	4-nitrophenyl-ßD-galactopyranoside	0.04	ß GALactosidase	colorless	yellow
8b) ProA	proline-4-methoxy-ß-naphthylamide	0.056	Proline Arylamidase if LIP is +, ProA is always –	ZYM B / 3 min yellow pale orange (brown if LIP +)	orange
9b) GGT	γ-glutamyl-4-methoxy-ß-naphthylamide	0.049	Gamma Glutamyl Transferase	ZYM B / 3 min yellow pale orange (yellow-orange if PAL +)	orange
10b) IND	L-tryptophane	0.036	INDole	JAMES / 3 min colorless	pink

Figure 2.1: Interpretation table for API results.

LISTE DES PROFILS NUMÉRIQUES / LIST OF NUMERICAL PROFILES / LISTE DER NUMERISCHEN PROFILE / LISTA DE PERFILES NUMÉRICOS / LISTA DEI PROFILI NUMERICI / LISTA DE PERFIS NUMÉRICOS / ΚΑΤΑΛΟΓΟΣ ΑΡΙΘΜΗΤΙΚΩΝ ΠΡΟΦΙΛ / LISTA ÖVER NUMERISKA PROFILER / LISTE OVER NUMERISKE PROFILER / LISTA PROFILI NUMERYCZNYCH

0 001	*Neisseria cinerea/Neisseria gonorrhoeae*	(2)	5 162	*Haemophilus aphrophilus/paraphrophilus***	(1)
0 002	*Neisseria meningitidis**		5 320	*Haemophilus parainfluenzae*	(1)
0 010	*Moraxella (Branhamella) catarrhalis***		5 324	*Haemophilus parainfluenzae*	(1)
1 000	*Neisseria gonorrhoeae*		5 360	*Haemophilus parainfluenzae*	(1)
1 001	*Neisseria gonorrhoeae*		5 420	*Haemophilus influenzae/parainfluenzae*	
1 002	*Neisseria meningitidis**		5 424	*Haemophilus influenzae*	(1)
1 003	*Neisseria meningitidis**		5 520	*Haemophilus parainfluenzae*	(1)
1 010	*Moraxella (Branhamella) catarrhalis***		5 620	*Haemophilus influenzae/parainfluenzae*	
1 020	*Haemophilus influenzae*	(1)	5 624	*Haemophilus influenzae*	(1)
1 024	*Haemophilus influenzae*	(1)	5 720	*Haemophilus parainfluenzae*	(1)
1 103	*Neisseria spp*	(2)	5 724	*Haemophilus parainfluenzae*	(1)
1 224	*Haemophilus influenzae*	(1)	5 760	*Haemophilus parainfluenzae*	(1)
1 420	*Haemophilus influenzae*	(1)	7 000	*Neisseria spp*	(2)
1 424	*Haemophilus influenzae*	(1)	7 001	*Neisseria spp*	(2)
1 426	*Haemophilus influenzae*	(1)	7 003	*Neisseria spp*	(2)
1 620	*Haemophilus influenzae*	(1)	7 020	*Haemophilus aphrophilus/paraphrophilus/ parainfluenzae/influenzae***	
1 624	*Haemophilus influenzae*	(1)			
1 626	*Haemophilus influenzae*	(1)	7 022	*Haemophilus aphrophilus/paraphrophilus/ parainfluenzae/influenzae***	
1 720	*Haemophilus parainfluenzae/influenzae*				
3 001	*Neisseria spp*	(2)	7 024	*Haemophilus influenzae/parainfluenzae*	
3 003	*Neisseria spp*	(2)	7 060	*Haemophilus aphrophilus/paraphrophilus***	(1)
3 024	*Haemophilus influenzae*	(1)	7 062	*Haemophilus aphrophilus/paraphrophilus***	(1)
3 026	*Haemophilus influenzae*	(1)	7 100	*Neisseria spp/Haemophilus parainfluenzae*	(2)
3 101	*Neisseria spp*	(2)	7 101	*Neisseria spp*	(2)
3 103	*Neisseria spp*	(2)	7 103	*Neisseria spp*	(2)
3 120	*Haemophilus paragallinarum*		7 120	*Haemophilus aphrophilus/paraphrophilus/parainfluenzae***	(1)
3 122	*Haemophilus paragallinarum/aphrophilus/ paraphrophilus/ parainfluenzae***		7 122	*Haemophilus aphrophilus/paraphrophilus/parainfluenzae***	(1)
			7 124	*Haemophilus parainfluenzae*	(1)
3 160	*Haemophilus aphrophilus/paraphrophilus/parainfluenzae***	(1)	7 160	*Haemophilus aphrophilus/paraphrophilus***	(1)
3 162	*Haemophilus aphrophilus/paraphrophilus***	(1)	7 162	*Haemophilus aphrophilus/paraphrophilus***	(1)
3 200	*Histophilus somni*		7 164	*Haemophilus parainfluenzae*	(1)
3 204	*Histophilus somni*		7 220	*Haemophilus parainfluenzae/influenzae*	
3 220	*Haemophilus influenzae*	(1)	7 224	*Haemophilus influenzae/parainfluenzae*	
3 224	*Haemophilus influenzae*	(1)	7 260	*Haemophilus parainfluenzae*	(1)
3 320	*Haemophilus parainfluenzae*	(1)	7 300	*Haemophilus parainfluenzae*	(1)
3 324	*Haemophilus parainfluenzae/influenzae*		7 320	*Haemophilus parainfluenzae*	(1)
3 360	*Haemophilus parainfluenzae*	(1)	7 322	*Haemophilus parainfluenzae*	(1)
3 420	*Haemophilus influenzae*	(1)	7 324	*Haemophilus parainfluenzae*	(1)
3 422	*Haemophilus influenzae*	(1)	7 326	*Haemophilus parainfluenzae*	(1)
3 424	*Haemophilus influenzae*	(1)	7 340	*Haemophilus parainfluenzae*	(1)
3 426	*Haemophilus influenzae*	(1)	7 360	*Haemophilus parainfluenzae*	(1)

Figure 2.2: Numerical profiles used to interpret API test results.

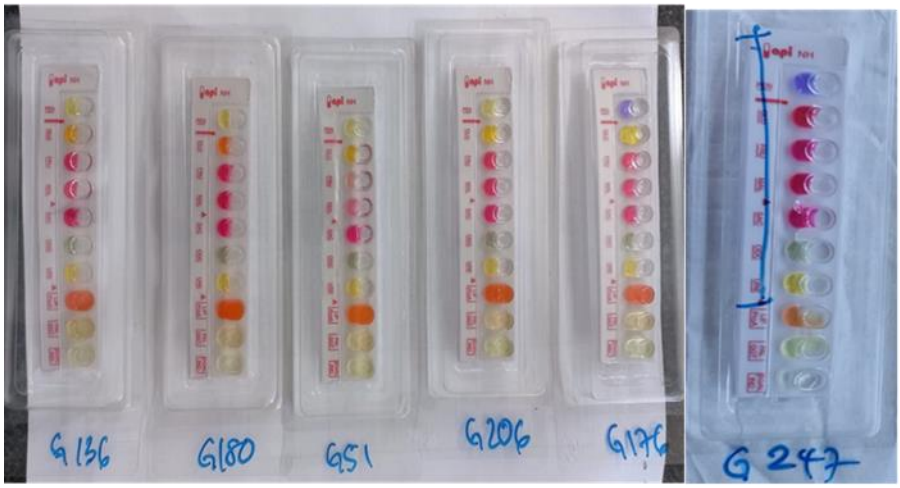

Figure 2.3: API test strip used to detect *N. gonorrhoeae* cultures. This study aim was interested in the readings of G136 and G180.

3.2. Genomic DNA

Figure 2.4 displays an E-gel image of the *N. gonorrhoeae* genomic DNA which was isolated from the six isolates. In this study aim (Chapter two) we were interested in two isolates, G180 and G136. From the figure, it was evident that genomic DNA is a relatively large fragment as each of the bands present were mainly seen at the top of the gel. Each of the six lanes displayed clear, bright single bands which indicated that good quality DNA was extracted.

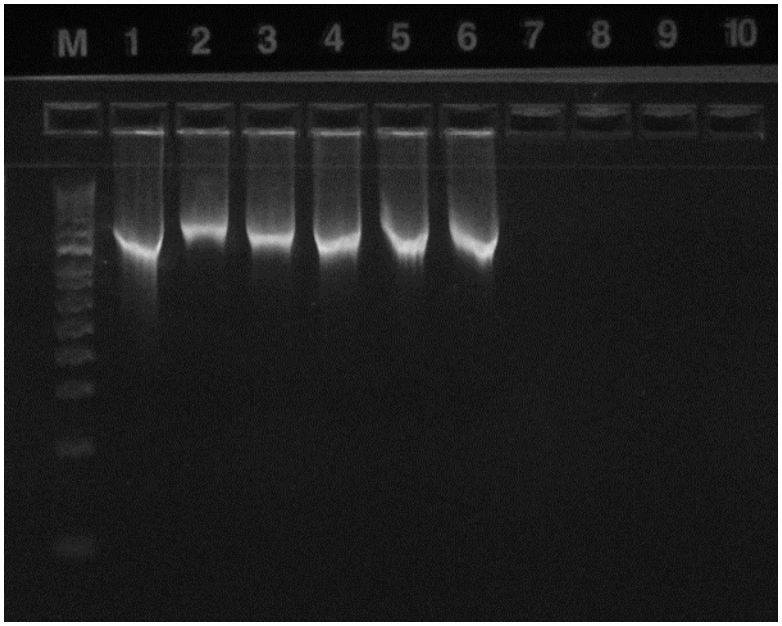

Figure 2.4: E-gel image of *N. gonorrhoeae* genomic DNA. Lanes 1 to 6 were loaded with the genomic DNA from the isolates and lane 7 was the negative control which did not contain any genomic DNA. Lanes 8 to 10 were left empty. Lane M: E-gel 1kb plus express DNA ladder, lane 1: G51, lane 2: G136, lane 3: G176, lane 4: G180, lane 5: G206 and lane 6: G247.

3.3. Identification of the distinct unique Opa proteins from the *N. gonorrhoeae* isolates

There are eleven distinct Opa proteins encoded by the genome of *N. gonorrhoeae* (4, 21, 59-61). To identify the complete repertoire of G136 and G180 Opa proteins, DNA was isolated and a PCR with primers targeting the conserved regions of *opa* genes was performed. Thereafter, *opa* amplicons were ligated into pCR Blunt II TOPO cloning vector, and single clones from this *opa* amplicon library were sequenced via Sanger sequencing to identify the respective *opa* genes. We were able to identify nine distinct Opa proteins from G136 and ten distinct Opa proteins from G180, despite sequencing over 80 clones from each of these isolates (Figures 2.5 and 2.6).

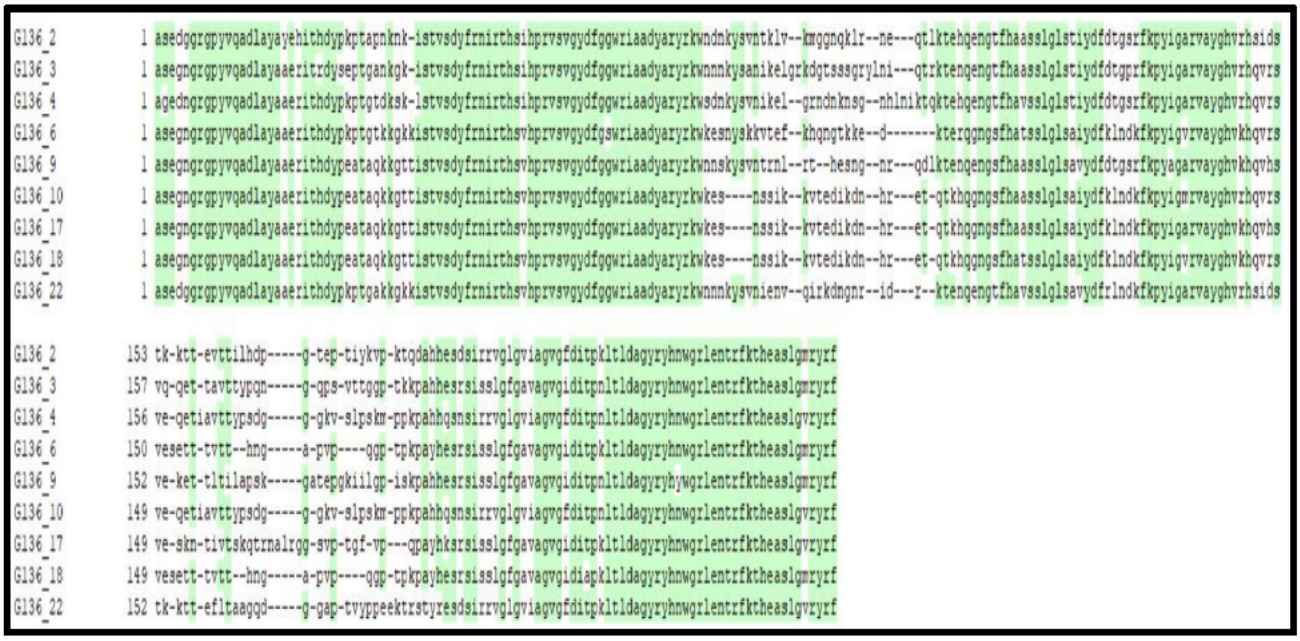

Figure 2.5: Amino acid sequence alignment of *N. gonorrhoeae* G136 Opa proteins expressed in *E. coli*. The isolates Opa protein sequences were aligned by multiple sequence alignment using Clone Manager software. Identical amino acids are shaded in green.

Figure 2.6: Amino acid sequence alignment of *N. gonorrhoeae* G180 Opa proteins expressed in *E. coli*. The isolates Opa protein sequences were aligned by multiple sequence alignment using Clone Manager software. Identical amino acids are shaded in green.

3.4. Colony PCR

Colony PCR was performed to verify that the *opa* genes were inserted into the pET-28a vector and that the cloning was successful by choosing the appropriate clones. The base size of the inserted amplicon was around ~750bp. Figure 2.7-2.8 illustrates the results from the colony PCR. A positive band around 750bp indicated a positive and successful clone. Figure 2.8 only represented a select few of the clones for viewing purposes for G180.

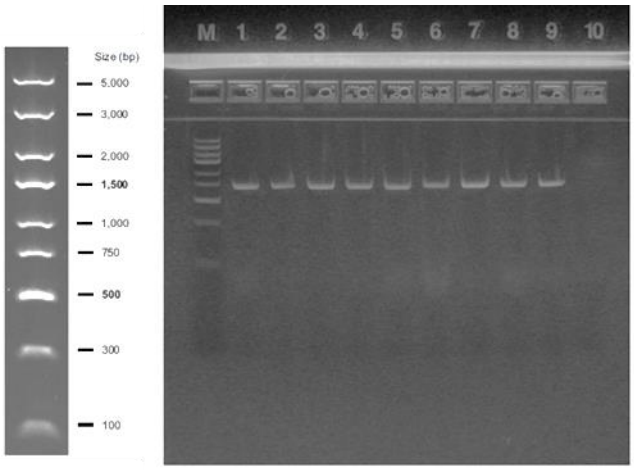

Figure 2.7: Results of the Colony PCR. M was the E-gel 1kb plus express DNA ladder. Lane 1: G136-2, lane 2: G136-3, lane 3: G136-4, lane 4: G136-6, lane 5: G136-9, lane 6: G136-10, lane 7: G136-17, lane 8: G136-18, lane 9: G136-22 and lane 10: negative control.

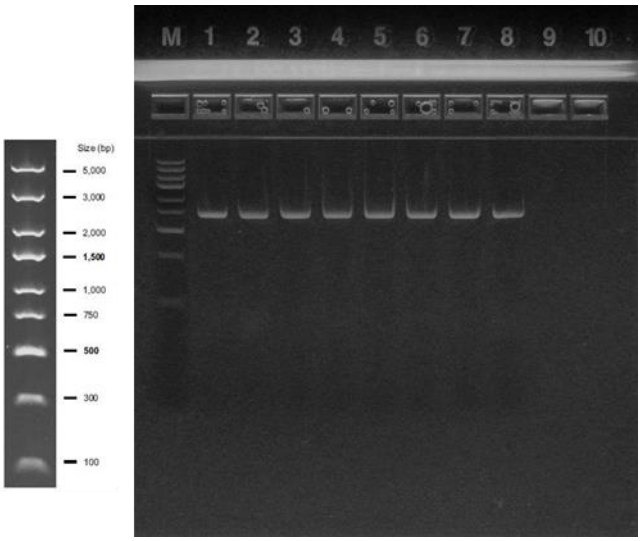

Figure 2.8: Results of the Colony PCR. M was the E-gel 1kb plus express DNA ladder. Lane 1: G180-1, lane 2: G180-15, lane 3: G180-18, lane 4: G180-33, lane 5: G180-35, lane 6: G180-37, lane 7: G180-38, lane 8: G180-43 and lane 9: negative control.

3.5. Opa proteins of clinical isolates bind to soluble domains of human CEACAMs

This study aimed to compare the CEACAM-binding properties of *N. gonorrhoeae* across symptomatic and asymptomatic patients. The soluble domains of CEACAMs that were expressed in 293 cells were adjusted to the same concentration (Figure 2.9). The Opa proteins identified in this study were subjected to test expression and Western blot was performed to verify these Opa proteins with a monoclonal antibody against neisserial Opa proteins (Figure 2.10 and 2.11). As expected, the uninduced negative control was not expressed (Figures 2.10 and 2.11).

Thereafter, binding assays were performed to analyse the host-binding properties of *N. gonorrhoeae* (Figure 2.12-2.19). There was no distinct Opa protein band observed for the Opa proteins found in G136 and G180 for CEACAM8 which was expected. However, bands were observed for CEACAM1, CEACAM5 and CEACAM3. Table 2.2 summarizes the results of the CEACAM-Opa protein interaction. Where the "+" indicates a positive binding and "-" indicates no binding was identified.

For isolate G136 (asymptomatic patient), 6/9 (66.7%) of the Opa proteins bound to CEACAM3, 5/9 (55.6%) bound to CEACAM1, and 8/9 (88.9%) bound to CEACAM5.

For isolate G180 (symptomatic patient), 3/10 (30%) of the Opa proteins bound to CEACAM3, 8/10 (80%) bound to CEACAM1, and 7/10 (70%) bound to CEACAM5.

It can be suggested that *N. gonorrhoeae* isolated from an asymptomatic patient is more likely to bind to CEACAM3 when compared to *N. gonorrhoeae* isolated from a symptomatic patient. However, this still requires further investigation.

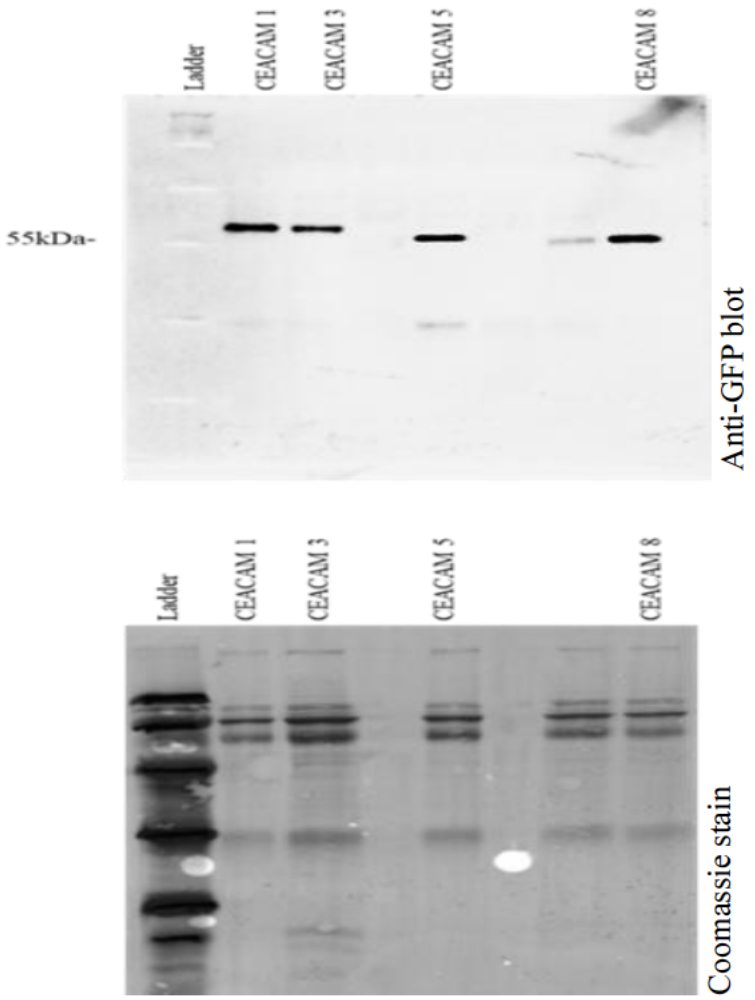

Figure 2.9: Supernatants from cell cultures containing the amino-terminal domains of specific human CEACAMs fused with GFP were gathered. The quantity of GFP fusion proteins was assessed through Western blot analysis using a monoclonal anti-GFP antibody.

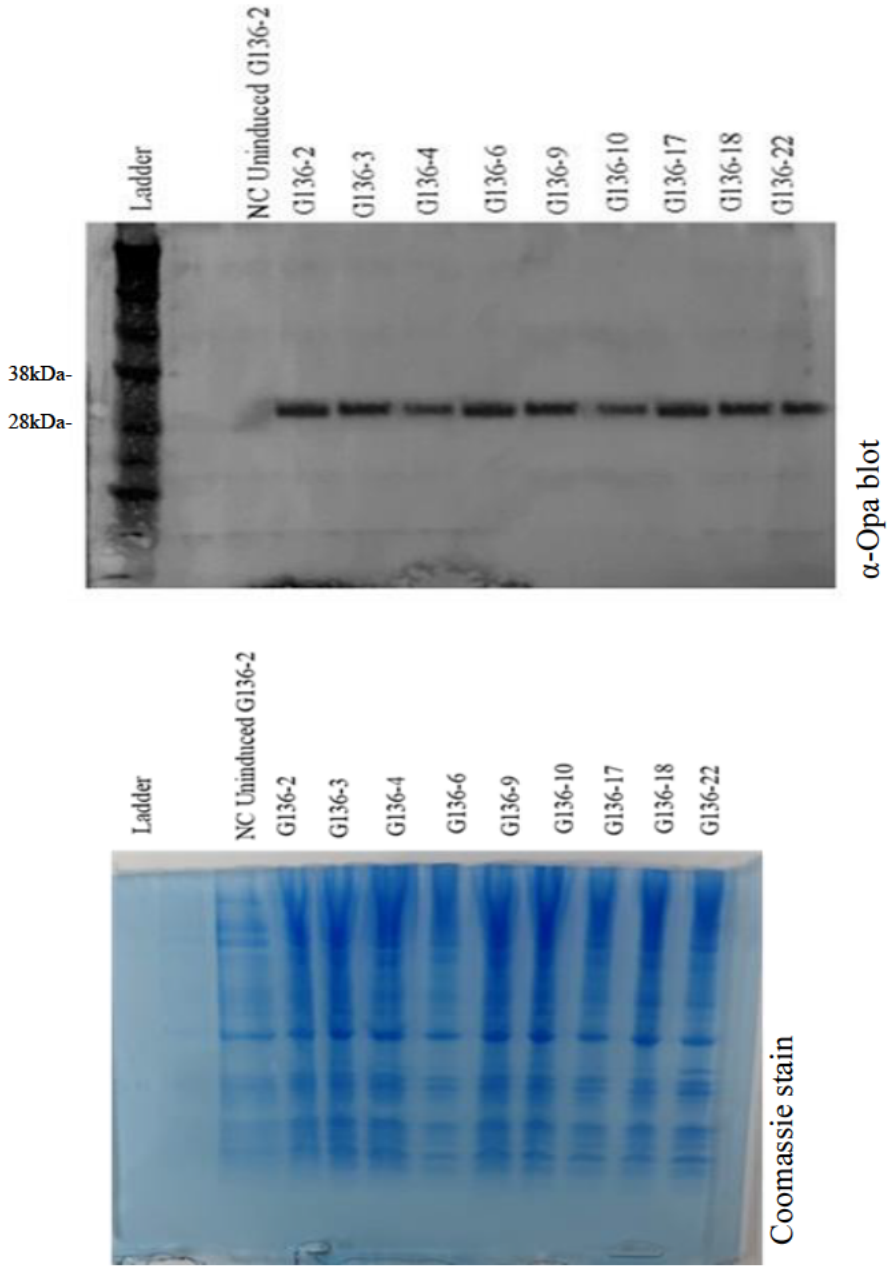

Figure 2.10: *N. gonorrhoeae* isolates G136 lacking Opa protein expression or expressing a single defined Opa protein. The confirmation of Opa protein expression was carried out through Western blot analysis, utilizing a monoclonal antibody against Opa proteins.

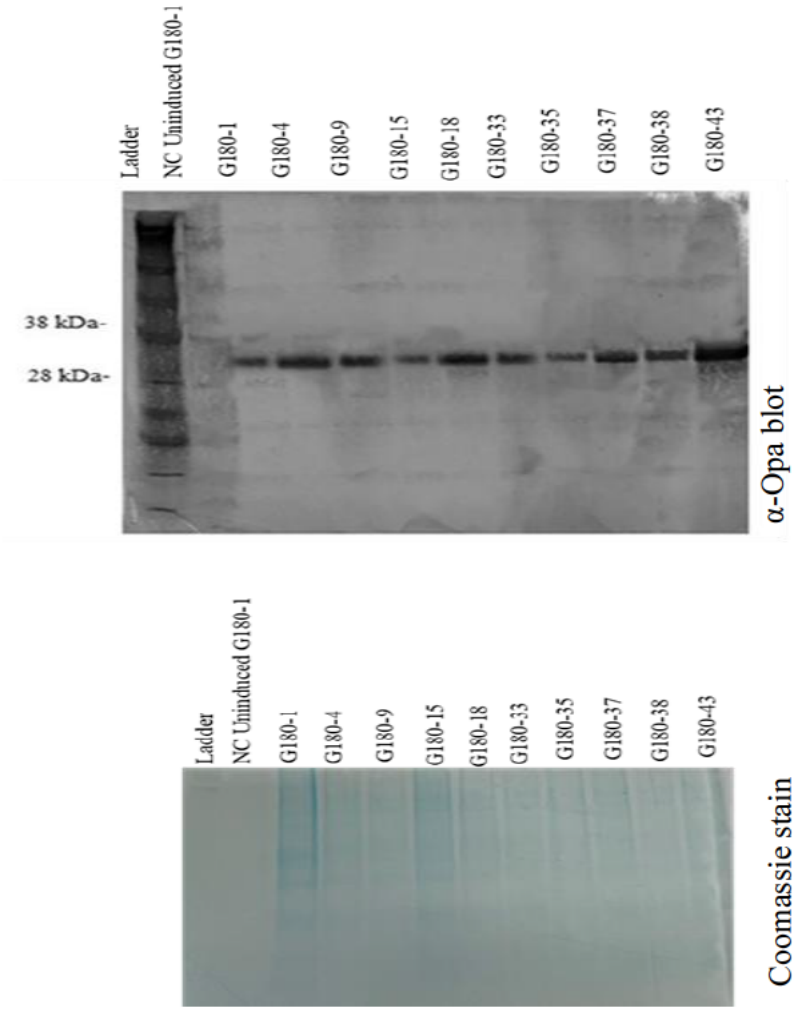

Figure 2.11: *N. gonorrhoeae* isolates G180 lacking Opa protein expression or expressing a single defined Opa protein. The confirmation of Opa protein expression was carried out through Western blot analysis, utilizing a monoclonal antibody against Opa proteins.

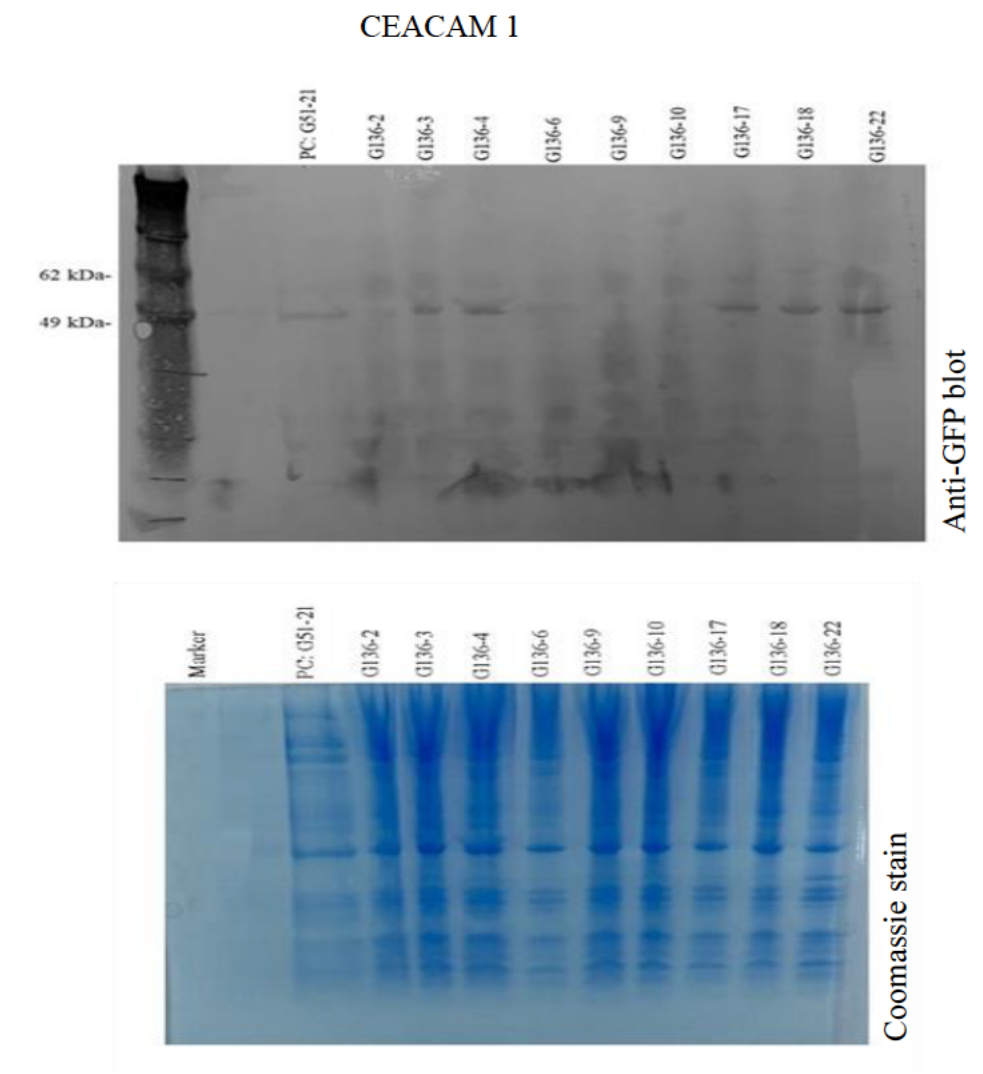

Figure 2.12: The pulldown experiments involved utilizing soluble CEACAM1-GFP fusion proteins along with *N. gonorrhoeae* G136 isolate that expressed the Opa proteins. Precipitates were probed with a monoclonal anti-GFP antibody to detect CEACAMs co-precipitating with *E. coli*.

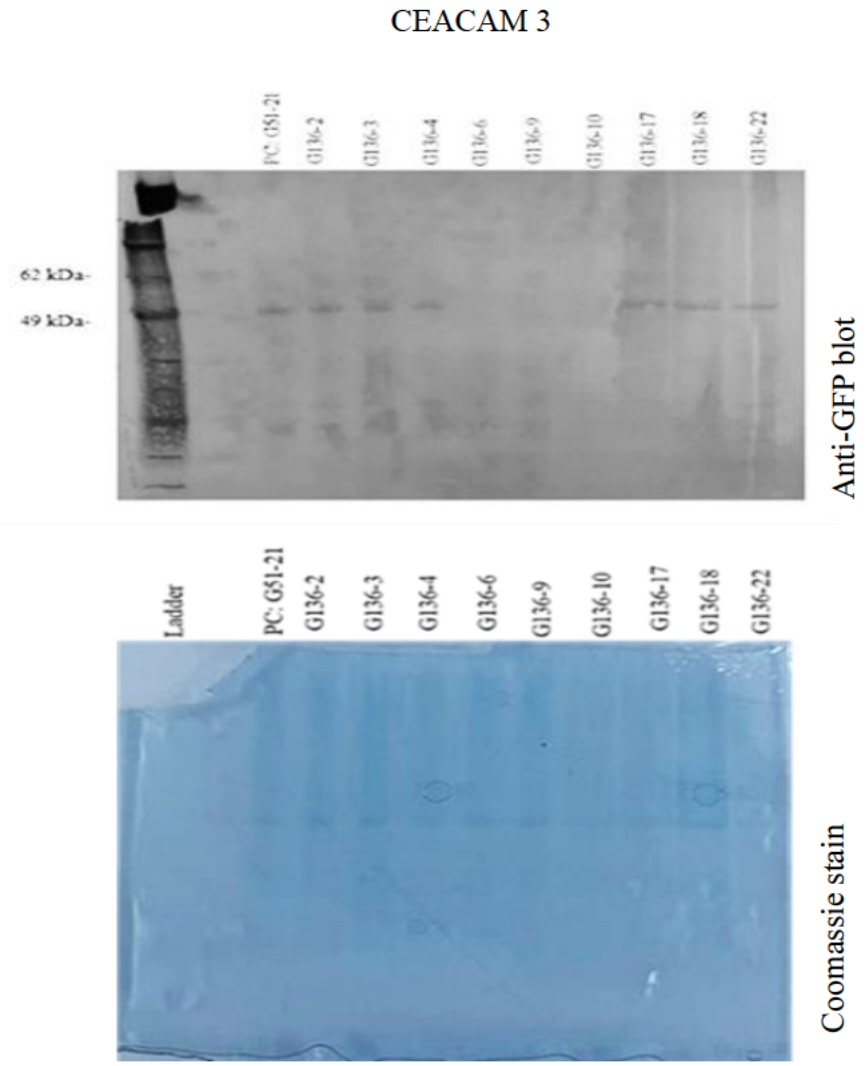

Figure 2.13: The pulldown experiments involved utilizing soluble CEACAM3-GFP fusion proteins along with *N. gonorrhoeae* G136 isolate that expressed the Opa proteins. Precipitates were probed with a monoclonal anti-GFP antibody to detect CEACAMs co-precipitating with *E. coli*.

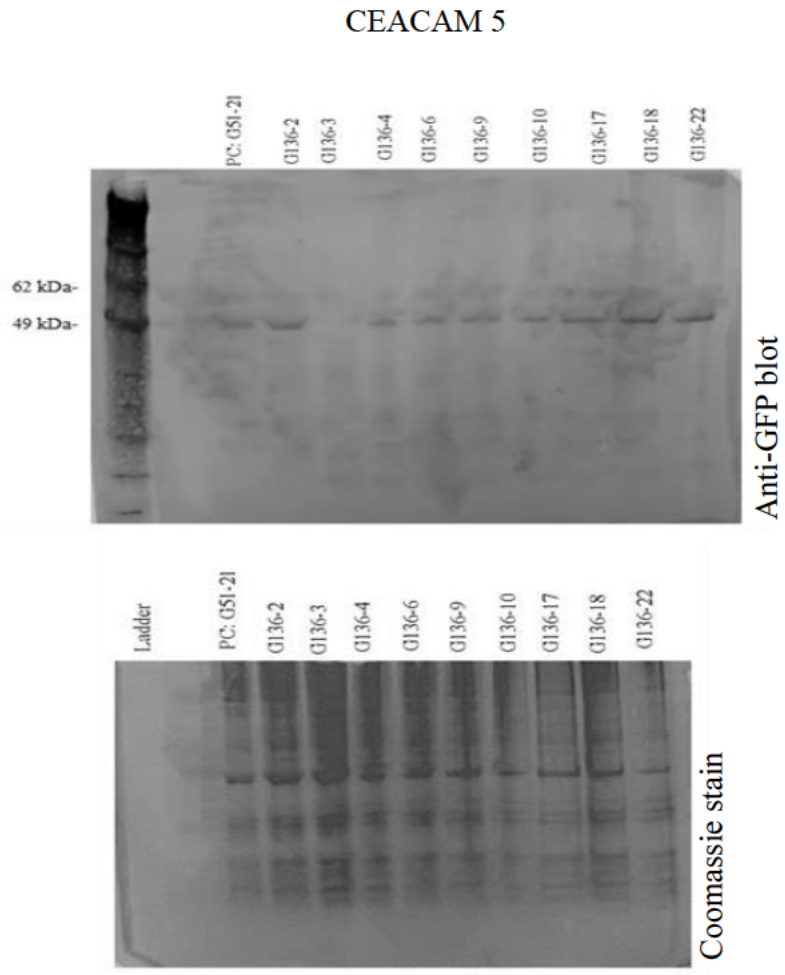

Figure 2.14: The pulldown experiments involved utilizing soluble CEACAM5-GFP fusion proteins along with *N. gonorrhoeae* G136 isolate that expressed the Opa proteins. Precipitates were probed with a monoclonal anti-GFP antibody to detect CEACAMs co-precipitating with *E. coli*.

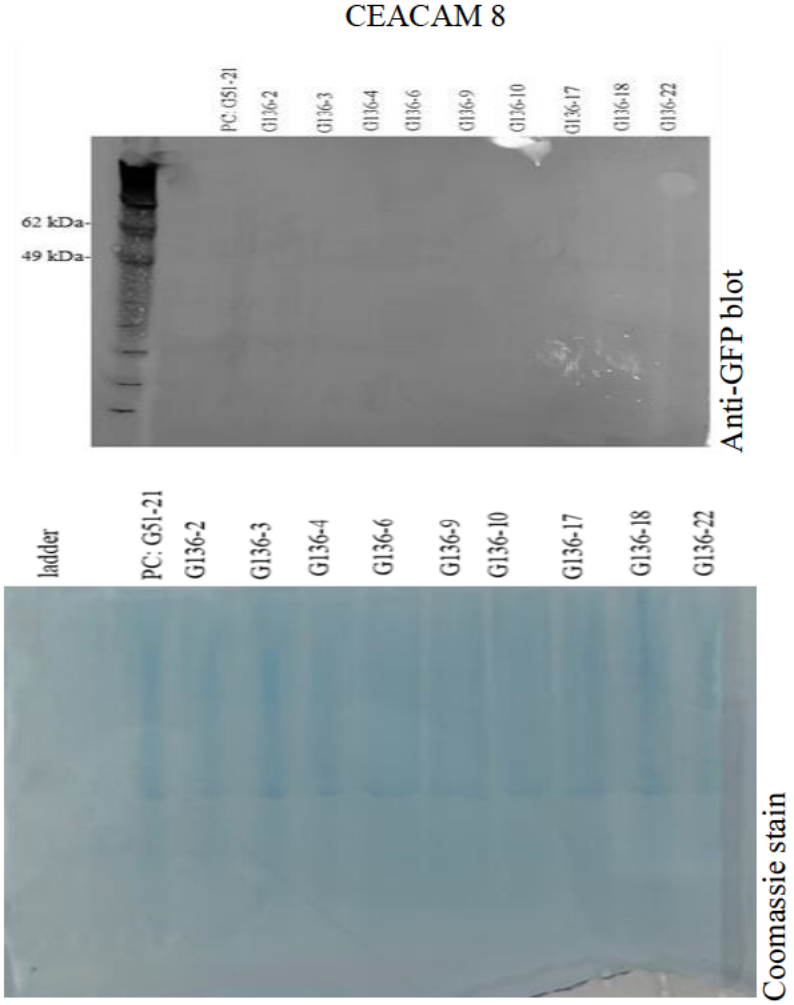

Figure 2.15: The pulldown experiments involved utilizing soluble CEACAM8-GFP fusion proteins along with *N. gonorrhoeae* G136 isolate that expressed the Opa proteins. Precipitates were probed with a monoclonal anti-GFP antibody to detect CEACAMs co-precipitating with *E. coli*.

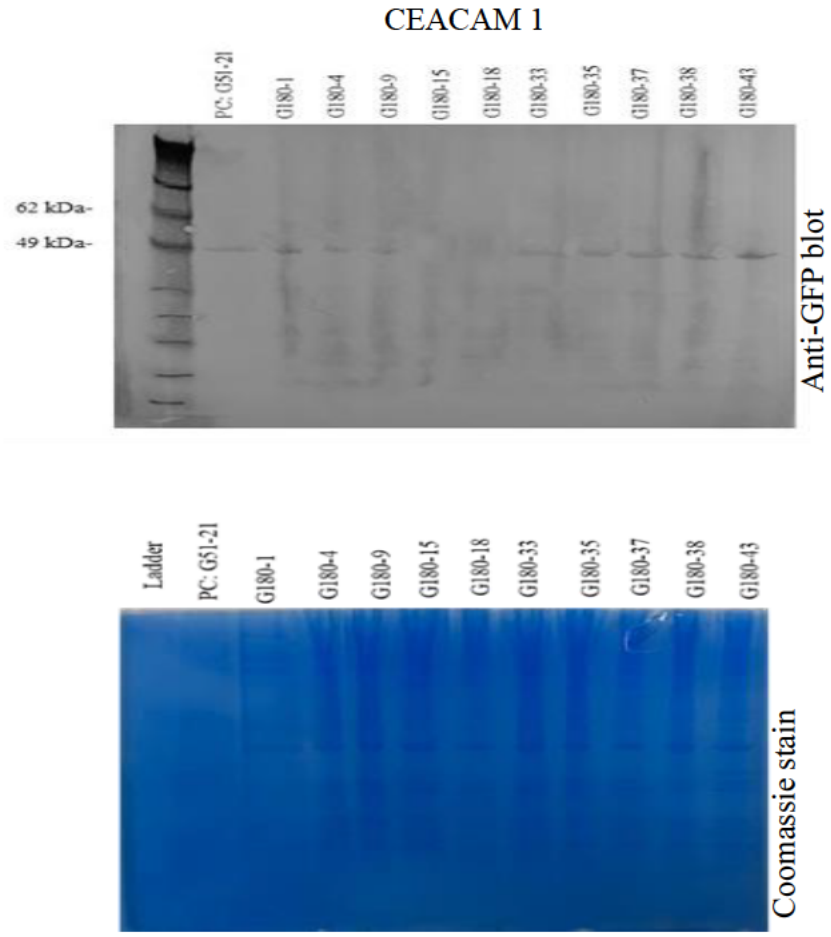

Figure 2.16: The pulldown experiments involved utilizing soluble CEACAM1-GFP fusion proteins along with *N. gonorrhoeae* G180 isolate that expressed the Opa proteins. Precipitates were probed with a monoclonal anti-GFP antibody to detect CEACAMs co-precipitating with *E. coli*.

CEACAM 3

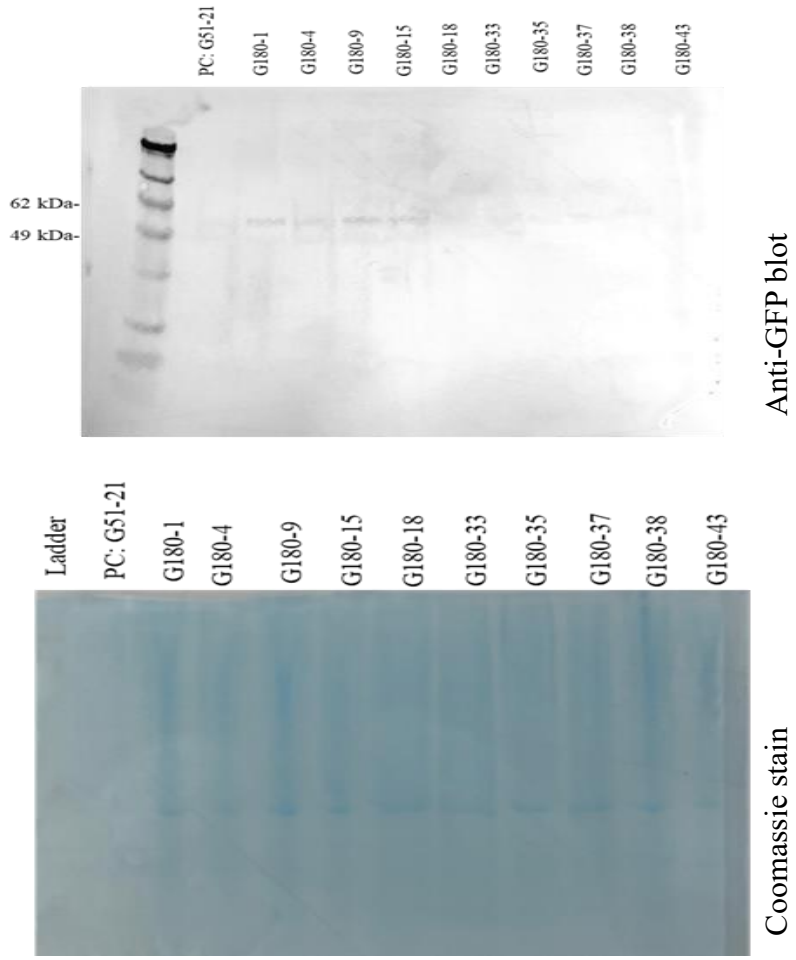

Figure 2.17: The pulldown experiments involved utilizing soluble CEACAM3-GFP fusion proteins along with *N. gonorrhoeae* G180 isolate that expressed the Opa proteins. Precipitates were probed with a monoclonal anti-GFP antibody to detect CEACAMs co-precipitating with *E. coli.*

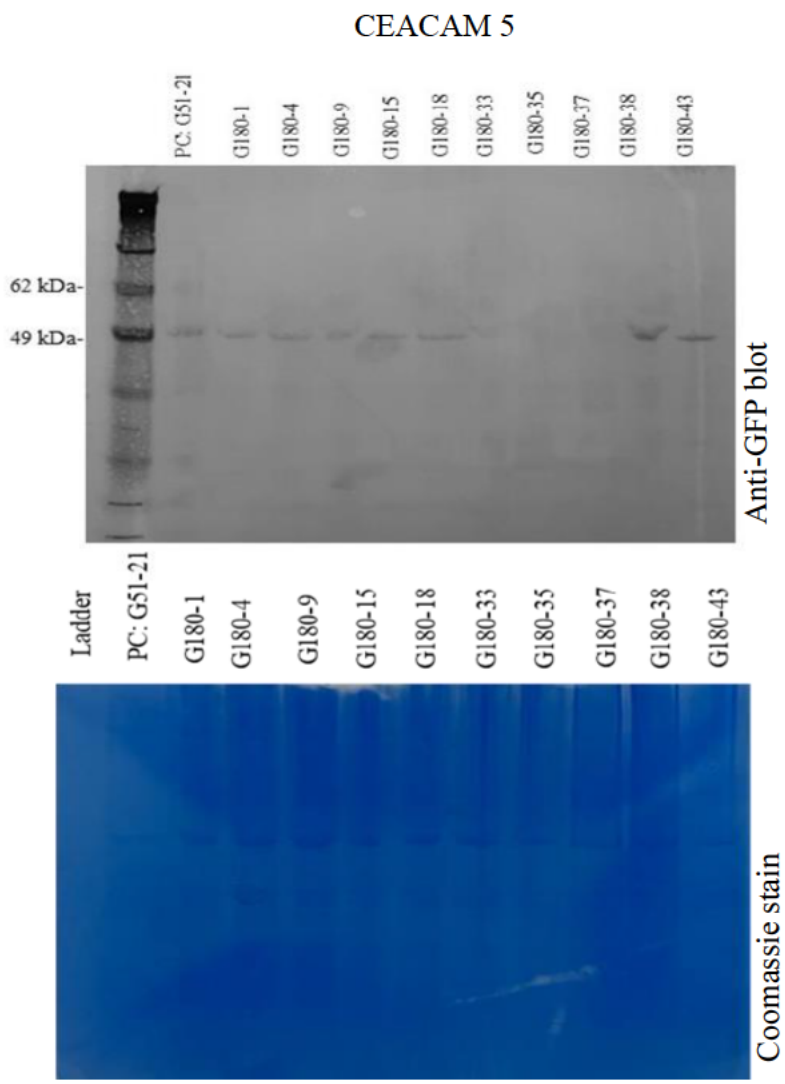

Figure 2.18: The pulldown experiments involved utilizing soluble CEACAM5-GFP fusion proteins along with *N. gonorrhoeae* G180 isolate that expressed the Opa proteins. Precipitates were probed with a monoclonal anti-GFP antibody to detect CEACAMs co-precipitating with *E. coli.*

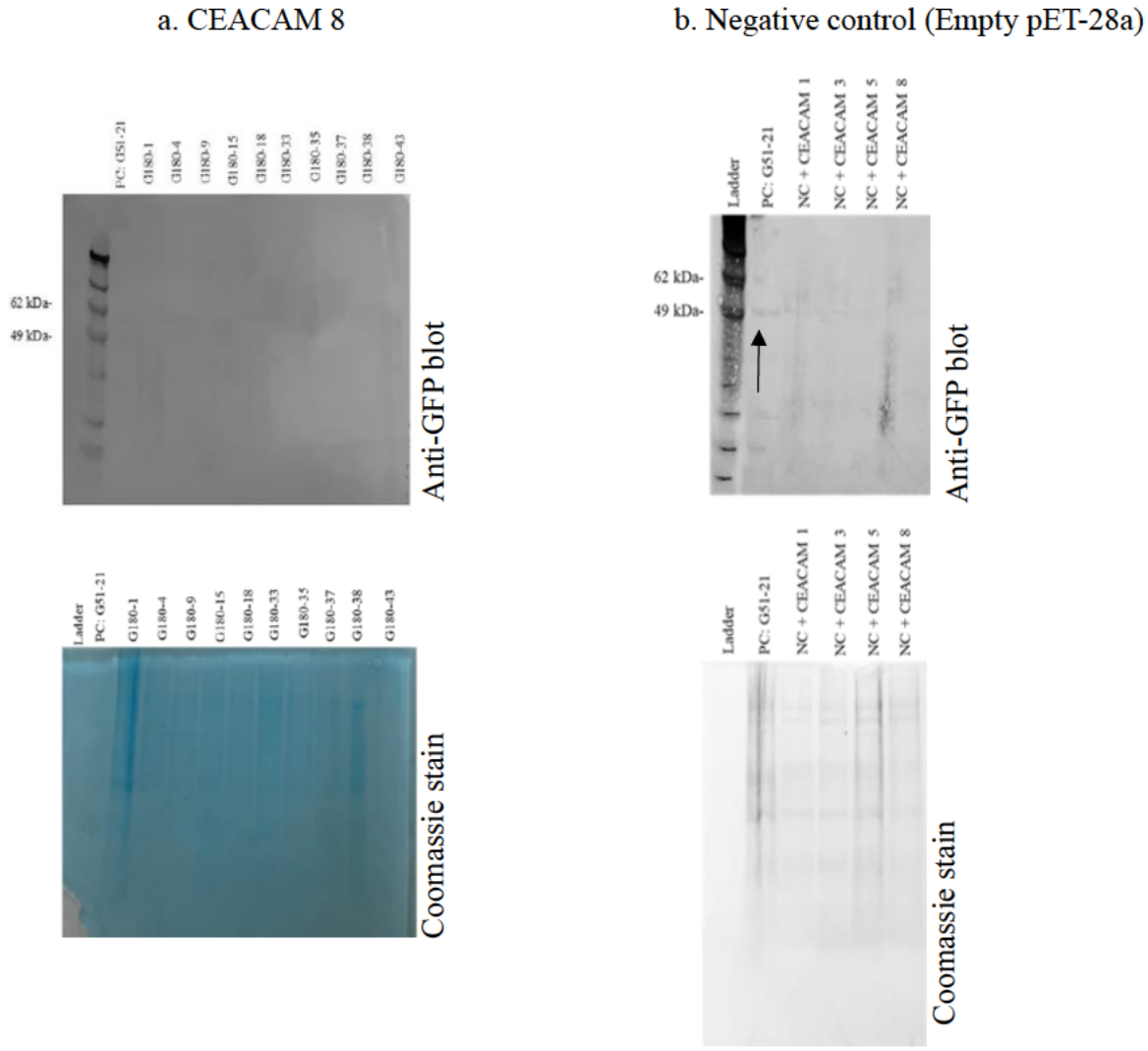

Figure 2.19: (a) The pulldown experiments involved utilizing soluble CEACAM8-GFP fusion proteins along with *N. gonorrhoeae* G180 isolate that expressed the Opa proteins. Precipitates were probed with a monoclonal anti-GFP antibody to detect CEACAMs co-precipitating with *E. coli.* (b) The pulldown experiments involved utilizing soluble CEACAM-GFP fusion proteins along with *N. gonorrhoeae* isolate that expressed the Opa proteins and the controls. Precipitates were probed with a monoclonal anti-GFP antibody to detect CEACAMs co-precipitating with *E. coli.*

Table 2.2: CEACAM and Opa binding profiles for *N. gonorrhoeae* for isolates G136 and G180 expressed in *E. coli*.

	G136									
Receptor	G136-2	G136-3	G136-4	G136-6	G136-9	G136-10	G136-17	G136-18	G136-22	
CEACAM 1	-	+	+	-	-	-	+	+	+	
CEACAM 3	+	+	+	-	-	-	+	+	+	
CEACAM 5	+	-	+	+	+	+	+	+	+	
CEACAM 8	-	-	-	-	-	-	-	-	-	
	G180									
Receptor	G180-1	G180-4	G180-9	G180-15	G180-18	G180-33	G180-35	G180-37	G180-38	G180-43
CEACAM 1	+	+	+	-	-	+	+	+	+	+
CEACAM 3	+	-	+	+	-	-	-	-	-	-
CEACAM 5	+	+	+	+	+	-	-	-	+	+
CEACAM 8	-	-	-	-	-	-	-	-	-	-

4. DISCUSSION

Neisseria gonorrhoeae is responsible for the second most common STI known as gonorrhoea (1, 2). The pathogenesis of *N. gonorrhoeae* involves several factors, including the role of Opa proteins (15, 16, 18, 20, 106). These proteins are found on the outer membrane of the bacterium and play a crucial role in its interactions with host cells (18, 21, 27). The first step of the pathogenesis involves attachment and adhesion. Opa proteins are involved in the initial attachment and adhesion of *N. gonorrhoeae* to host epithelial cells and interact with specific receptors (CEACAMs) on the surface of host cells, promoting the binding of the bacterium to the mucosal surfaces of the genital tract, rectum, and oropharynx (56). Thereafter, *N. gonorrhoeae* has a mechanism of phase variation for Opa proteins. This means that different strains of the bacterium can express different Opa proteins, and the expression can switch on or off over time. This variation allows the bacterium to adapt to changing host environments and immune responses (57). Following this is immune evasion. The Opa proteins are highly variable and can undergo antigenic variation, which helps *N. gonorrhoeae* evade the host immune system. The bacterium can switch its Opa protein expression, making it difficult for the immune system to develop long-lasting immunity against it (57). The current study aimed to investigate the host-pathogen associations by determining the Opa-CEACAM binding patterns of *N. gonorrhoeae* in symptomatic versus asymptomatic pregnant women in a South African setting.

N. gonorrhoeae chromosomes encode for eleven unique *Opa* proteins which are distributed throughout the genome (4, 21, 59-61). In the Roth *et al.,* (2013) study, *N. gonorrhoeae* strain MS11-B2.1 was used to investigate CEACAM-Opa binding properties (4). MS11 (id:27228), is suggested to date back to 1970 and was isolated from a patient with uncomplicated anterior urethritis from the North American region (107, 108). MS11 strain is now considered to be a

laboratory-based strain (4, 109). Using MS11, all eleven distinct Opa proteins were identified and used to investigate host-pathogen binding properties (4). In the present study, we used pure clinical isolates cultured from pregnant women. These isolates had not undergone any genetic manipulations. Despite having obtained over 80 clones for the isolates, we were not able to retrieve the full 11 distinct Opa proteins for any of our isolates. For G136 (isolated from an asymptomatic woman), we were only able to express nine distinct Opa proteins. For G180 (isolate from symptomatic woman), we identified ten distinct Opa proteins. Since this was the first study to express Opa proteins for these isolates, we were unable to know for certain if the eleven complete distinct Opa proteins could be expressed. We suggest that the differences in the number of unique Opa proteins identified for MS11 versus the isolates in this study could be due to the following; firstly, the MS11 strain was a laboratory strain from North America and the current study had pure clinical isolates from South African pregnant women (no prior manipulations performed), secondly, geographical location could contribute to the genetic differences observed between the isolates.

The interactions between CEACAM and the pathogenic *N. gonorrhoeae* serve multiple functions through the binding of the Opa proteins found in *N. gonorrhoeae*. They not only enable the bacteria to attach to and inhabit human cells but can also trigger the engulfment of the bacteria. These interactions have been extensively examined in both epithelial cells and neutrophils (polymorphonuclear leukocytes). Epithelial cells express certain CEACAMs, namely CEACAMs 1, 5, and 6, which bind to the pathogen, as well as CEACAM7, which does not interact with pathogens (110, 111). In contrast, primary human neutrophils express CEACAMs 1, 3, and 6, alongside non-pathogen binding CEACAMs 4 and 8 (112, 113). Of particular significance are CEACAMs 1 and 3, as both contain cytoplasmic domains that play a role in signalling and can lead to the internalization of the bacterium. However, they often trigger opposing responses within the cells. CEACAM1 contains two immunoreceptor

tyrosine-based inhibition motifs (ITIMs) that, when activated, recruit the phosphatase SHP-1 (106, 114). This recruitment suppresses phosphotyrosine-based signalling cascades. On the other hand, CEACAM3 contains an immunoreceptor tyrosine-based activation motif (ITAM), which, upon activation, recruits kinases such as Src family kinases (e.g., Syk) to propagate pro-inflammatory signalling cascades (115, 116). Interestingly, when neutrophils express CEACAM1 and CEACAM6, they bind more bacteria, but the process of *Neisseria* uptake through these two receptors surprisingly occurs without causing significant activation of the neutrophils (117). CEACAM3, found exclusively on human neutrophils and other granulocytes, is believed to have evolved as an innate immune defence mechanism. It has no known endogenous ligand but interacts specifically with proteins on the surface of human-specific bacterial pathogens such as *Neisseria* (117). These CEACAM3 interactions facilitate the uptake of the pathogen, resulting in an oxidative burst and the release of toxic granules to effectively eliminate the pathogen (111, 117, 118).

In the Roth *et al.,* (2013) study, each Opa protein expressed by the MS11 strain demonstrated the capability to bind at least one of the recombinant CEACAM domains (4). Based on the binding patterns identified, it became evident that Opa proteins in strain MS11, which recognize CEACAMs, could be categorized into two groups. The first group consists of a substantial number of Opa proteins that exclusively bind to CEACAMs present on epithelial cells. In contrast, a smaller group of Opa proteins in MS11, binds to epithelial CEACAMs as well as to the CEACAM3 found exclusively on granulocytes. There was a higher frequency of the MS11 Opa proteins binding to CEACAM5 and CEACAM1 as opposed to CEACAM3 (4). In this study we investigated the interactions of *neisserial* Opa proteins with CEACAMs 1, 3, 5, and 8. As expected, there was no interaction between the Opa proteins and CEACAM8 as CEACAM8 has been reported not to bind with any Opa proteins (112). G180 had a higher frequency of its unique Opa proteins binding to CEACAM1 and CEACAM5 and the least

binding to CEACAM3. Whilst the unique Opa proteins from G136 bound more with CEACAM3. During symptomatic disease/inflammation, the presence of neutrophils and CEACAM3-mediated phagocytosis might select against Opa-proteins binding to CEACAM3. This might be the reason, why the isolate selected from the symptomatic patient has a lower percentage of CEACAM3-binding opa proteins (in line with the previously characterized strain MS11), while the one from the asymptomatic patient (where presumably the bacteria are not in contact with neutrophils/no inflammation) are not negatively selected for the absence of CEACAM3-binding Opa. Currently, there are no studies conducted in Africa investigating the binding profiles of clinical isolates of *N. gonorrhoeae*, thereby limiting our study comparisons.

The limitation of this study is that the isolates investigated were obtained from patients with most probably a localized infection. However, we can only assume this, since samples were not collected from other sites such as the rectum or pharynx. Future studies conducted in our setting should include localized and disseminated infections. Another limitation is the population sampled, the only isolates available for the analysis were of pregnant women, a much more diverse population can be used for future studies. The last limitation of the study was the insufficient statistical power to draw sound conclusions due to the number of strains. However, this was a novel study and future research direction can be on a larger scale. The strength of the study is that this is the first South African study to report on the host-receptor binding profiles of *N. gonorrhoeae.*

Conclusion

In this study, it was shown that the *N. gonorrhoeae* Opa proteins from a symptomatic patient bound at a higher frequency to CEACAM1 and 5 which causes the pathogen to invade the host cell and cause infections. However, *N. gonorrhoeae* Opa proteins from the asymptomatic patient bound at a higher frequency to CEACAM3. This Opa-CEACAM interactions are said

to possibly limit the spread of gonococci due to granulocyte-mediated opsonin-independent phagocytosis (4). Investigating Opa-CEACAM interactions is a step in the right direction towards reverse vaccinology for this pathogen. This study now adds to the growing body of knowledge on the host-receptor binding profiles of this pathogen.

In Chapter Two, the Opa-CEACAM binding profiles of *N. gonorrhoeae* were described as a potential to be used for reverse vaccinology. The *opa* gene is described as a multicopy gene that contains conserved areas and encodes proteins with physiological function to *N. gonorrhoeae*. *Opa* genes are therefore considered as suitable target sequences for a real-time PCR amplification assay to detect *N. gonorrhoeae.* Thus, in Chapter Three, the objective was to develop and evaluate the performance of an in-house *opa*-based real-time PCR assay. Three primer sets targeting the *opa* gene of *N. gonorrhoeae* were designed and evaluated against published *opa* gene primers. This could potentially be utilized to develop better diagnostic methods for *N. gonorrhoeae.*

CHAPTER THREE

AIM TWO

Design and Performance of an *Opa* Gene-Based Real-Time PCR Assay as a Potential Diagnostic Test for *Neisseria gonorrhoeae*

1. INTRODUCTION

With an estimated 80 million people affected annually, gonorrhoea is the second most prevalent STI globally (3, 4). In the male population, the infection is usually symptomatic. However, in the female population, gonorrhoea is mostly asymptomatic which allows for silent transmission (3, 4). Women infected with gonorrhoea can incur more severe complications, if left untreated, such as PID and DGI, resulting in infertility or predisposing women to ectopic pregnancy (3-5). The prevalence of *N. gonorrhoeae* infections going undiagnosed and/or untreated, combined with the high incidence rates of these infections, emphasizes the necessity for effective identification of both symptomatic and asymptomatic infections (94).

The "gold" standard method for detecting *N. gonorrhoeae* involves culture, which encompasses techniques such as microscopy (utilizing Gram staining and methylene blue staining) and antimicrobial susceptibility testing (5, 8). The drawbacks related to this method include the limited success in cultivating *N. gonorrhoeae* owing to the demanding characteristics of the microorganism and its extended period of detection. Due to the limitations associated with culture, some NAATs for *N. gonorrhoeae* have been FDA-approved (5). The use of NAATs has been shown to provide an enhanced diagnosis of gonorrhoea. However, it is recommended that an ongoing assessment of the test assays should be performed to check for any probable sequence variation occurring in the targeted region (94).

Opa proteins are located on the outer membrane of *N. gonorrhoeae* and facilitate with the interaction of the bacterium and the several host cell types (including epithelial cells on mucosal surfaces and various immune cells) (3, 21, 22). Opa proteins adopt a barrel-shaped configuration on the outer membrane of gonococcal cells, featuring roughly eight antiparallel β strands. These strands are anchored to the membrane through four extracellular loops (3, 21, 22). *N. gonorrhoeae* chromosomes encode for eleven unique Opa proteins which are distributed throughout the genome (3, 21, 22). Opa proteins can switch on and off their sequence arrangements at any of the eleven *opa* alleles in the genome. The expression of these proteins is rapidly changing due to phase variation which is triggered by a pentameric coding repeat sequence which is caused by slipped-strand mispairing during DNA replication (4). Additionally, Opa proteins enhance resistance to complement-mediated killing (119). Since, *opa* genes are multicopy genes that contain conserved areas and encode proteins with physiological function, *opa* genes are therefore considered as suitable target sequences for a real-time PCR amplification assay (93).

The objective of the present study was to develop and evaluate the performance of an in-house *opa*-based real-time PCR assay. Three primer sets targeting the *opa* gene of *N. gonorrhoeae* were designed and evaluated against published *opa* gene primers (Verma *et al.*, 2012) (97).

2. MATERIALS AND METHODS

2.1. Ethical approval

Full ethics approval for this study was granted by the Biomedical Research Committee (BREC) of the University of Kwa-Zulu Natal (UKZN), (BREC/00005104/2022).

2.2. Study population and samples

This study was a retrospective laboratory-based study using stored DNA from bacterial isolates and clinical samples. The *N. gonorrhoeae* WHO references strains (O, N, X, Y, and Z) used in this study were generously provided by the National Institute of Communicable Diseases (NICD), South Africa. Clinical isolates cultured from pregnant women (n=6) and confirmed to be *N. gonorrhoeae* were used. The non-gonococcal *Neisseria* control isolates (*N. cinerea*, *N. weaveri* and *N. sicca*) were donated by the Department of Medical Microbiology from National Health Laboratory Service South Africa. These samples were randomly selected as they were readily available in the Clinical Medicine repository.

The clinical DNA samples were from endocervical swabs (n= 307), self-collected vaginal swabs (n=385), and urine samples (n=200). The swabs were collected from pregnant women and the urine was obtained from males. The pregnant women were recruited from King Edward VIII Hospital, Durban, South Africa between October 2018, and April 2021. These women provided self-collected vaginal swabs, however, the endocervical swabs were collected by a clinician. The male urine samples were collected from men enrolled at the Aurum Institute in Durban, South Africa between October 2021 to July 2022. The extracted DNA from these samples were subjected to the TaqMan Assay which was used to detect *N. gonorrhoeae* using primers and probes specific for *N. gonorrhoeae* (Assay ID: Ba04646252_s; ThermoFisher Scientific, United States of America). The Department of Medical Microbiology at the University of KwaZulu-Natal utilized the TaqMan assay for detection purposes. This assay was

compared with the AnyPlex assay (SeeGene), an approved diagnostic test for *N. gonorrhoeae* (120). The evaluation study revealed a strong correlation between these two assays regarding sensitivity, specificity, as well as positive and negative predictive values (120). The TaqMan qPCR exhibited a sensitivity of 91.6%, specificity of 100%, positive and negative predictive values of 100% and 99.58%, respectively (120), and was therefore used for the detection of *N. gonorrhoeae* in the clinical samples.

2.3. DNA isolation

DNA was extracted from the urine, self-collected vaginal swabs, and endocervical swabs using the PureLink Microbiome Kit (ThermoFisher Scientific, United States) according to the manufacturer's instructions. Briefly, 10ml of urine and 2ml of the swabs resuspended in phosphate-buffered saline (PBS) were centrifuged for 30 minutes at 14 000 x *g* to pellet the cells. The supernatant was discarded and 800µl of S1 lysis buffer was added to the pellet and pipetted up and down to mix the sample. The sample was then transferred to the bead tube and 100µl of S2 lysis enhancer was added to the bead tube, capped, and vortexed briefly. This was incubated at 95℃ for 10 minutes, followed by vortexing at a maximum speed for 7 minutes and further centrifuged at 14 000 x *g* for 1 minute. Thereafter, 500µl of the supernatant was transferred to a clean microcentrifuge tube, avoiding the bead pellet and any cell debris. To bind DNA to the column, 900µl of binding buffer was added and vortexed briefly. Following this, 700µl of the sample mixture was loaded onto a spin column-tube and centrifuged at 14 000 x *g* for 1 minute. The flow-through was discarded and the spin column was centrifuged at 14 000 x *g* for 30 seconds. The spin column was placed in a clean tube and 50µl of S6 elution buffer was added. The tube was incubated at room temperature for 1 minute. After 1 minute, the spin column was centrifuged at 14 000 x *g* for 1 minute and the column was discarded. The purified DNA was stored at -20℃. The concentration and purity of the DNA were assessed using a Nanodrop Spectrophotometer (ThermoFisher Scientific, United States).

2.4. Culture detection of *N. gonorrhoeae*

Methods were performed as described in chapter two, section 2.3, pages 27-29 for all 6 isolates (G51, G136, G176, G180, G206, and G247) and WHO control strains (WHO O, N, X, Y, and Z) of this thesis. The results are shown in Chapter Two, section 3.1, pages 35-38 of this thesis.

2.5. DNA extraction from cultured isolates

Culture-confirmed clinical isolates were re-plated onto Chocolate Agar plates. A set of WHO control strains (WHO O, N, X, Y, and Z) were also plated out. The culture plates were incubated for 24 hours at 35 ℃ - 37 ℃ in the presence of 5% CO_2. Following growth on the agar plate, an inoculum with 1 ml Brain-Heart Infusion Broth (LASEC, South Africa) was then prepared for the clinical isolates and WHO control strains. The samples were incubated overnight at 35 ℃ - 37 ℃ in 5% CO_2. The inoculum was then used for the isolation of genomic DNA using the Wizard Genomic DNA Purification Kit (Promega Corp., Madison, United States) according to the manufacturer's guide. The detail of this method is described in Chapter Two, section 2.4, pages 29-30 of this thesis. The results are shown in Chapter Two, section 3.2 pages 38-39 for all 6 isolates (G51, G136, G176, G180, G206, and G247) of this thesis.

2.6. Design of the in-house *opa* primers

The template used for the design of the primers was based on a conserved region of the *opa* region which includes all eleven *opa* types as described by Roth *et al.*, (2013) (GenBank accession number: KC503485.1) (4). The real-time PCR primers were designed using the freely available software from GenScript Biotech Corporation (Piscataway, New Jersey, United States). The positions of the primers on the *opa* gene are shown in Figure 3.1. The sequences of the primer sets are shown in Table 3.1.

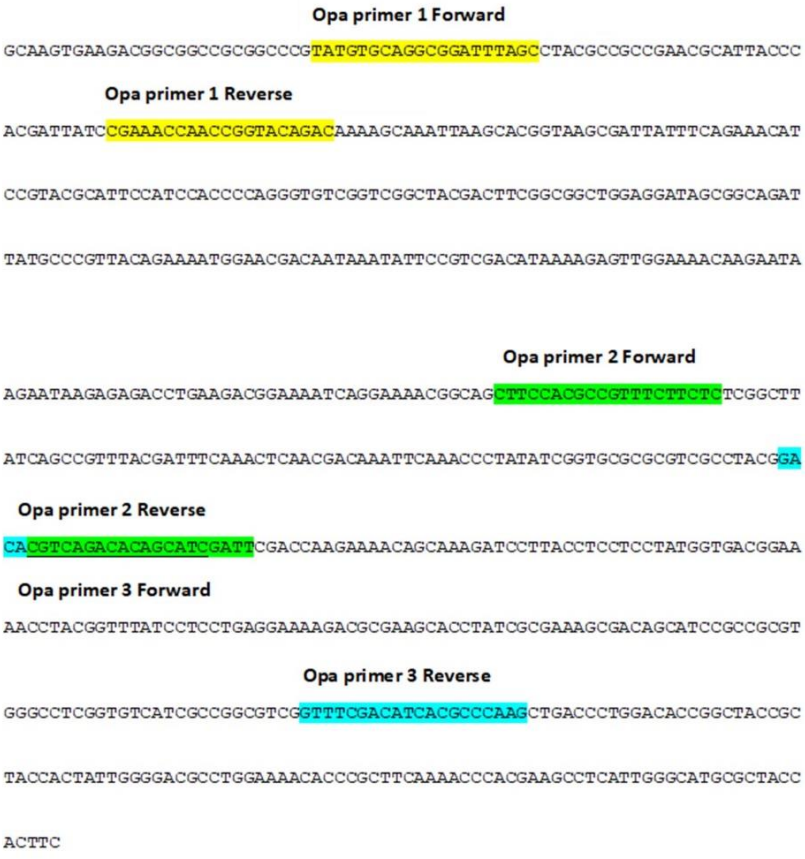

Figure 3.1: The template used for the design of the primers was based on a conserved region of the *opa* region which includes all eleven *opa* types as described by Roth *et al.*, (2013) (GenBank accession number: KC503485.1) (4). The positions of the primer pairs are on the *opa* gene. There is an overlap between the *opa* 2 reverse primer and *opa* 3 forward primer. The region where the overlap (forward and reverse primers share the same DNA sequence) occurs is underlined.

Table 3.1: Showing the *in-house opa* primer designed using the GenScript software.

Primer Name	Sequence	Amplicon size (bp)	Start position strand	Length	Primer Melting temperature (Tm)
***Opa* 1 RT Forward**	5' TAT GTG CAG GCG GAT TTA GC 3'	72bp	28	Forward 20	55.49
***Opa* 1 RT Reverse**	5' GTC TGT ACC GGT TGG TTT CG 3'		80	Reverse 20	56.00
***Opa* 2 RT Forward**	5' CTT CCA CGC CGT TTC TTC TC 3'	119bp	324	Forward 20	56.35
***Opa* 2 RT Reverse**	5' AAT CGA TGC TGT GTC TGA CG 3'		423	Reverse 20	55.50
***Opa* 3 RT Forward**	5' GAC ACG TCA GAC ACA GCA TC 3'	188bp	419	Forward 20	56.09
***Opa* 3 RT Reverse**	5' CTT GGG CGT GAT GTC GAA AC 3'		587	Reverse 20	56.98

2.7. Qualitative PCR of *opa* genes

2.7.1. Comparator *opa* primers

The comparator primer set for this study was the *opa* primers from the Verma *et al.,* (2012) study. The primer sequences were as follows; forward primer 5`-CGG TGC TTC ATCACC TTA G-3` and reverse primer 5`-GGATTC ATT TTC GGC TCC TT-3' (97). The Verma *et al.,* (2012) study used classical PCR for the detection of the *opa* gene (188 bp) from *N. gonorrhoeae* which requires endpoint analysis (agarose gel electrophoresis to visualise results). However, the current study adapted the conditions described by Verma *et al.,* (2012) to perform a real-time SYBR Green assay instead of classical PCR. While classical PCR is commonly utilized, it is constrained by the necessity of endpoint analysis, leading to a lengthier wait for results. In contrast, real-time PCR yields faster results, significantly reducing the turnaround time (121). To standardize the methodology used in the current study, only SYBR Green assays were performed.

The amplification of the *opa* region was carried out as follows; the assay included: 5ul of PowerUp SYBR Green Master Mix (ThermoFisher Scientific, United States), 0.2nm of the

forward and reverse primers, 1.5ul template DNA which was brought up to a final volume of 10ul with nuclease free water. The reactions were run on a Quant Studio 5 PCR instrument (ThermoFisher Scientific, United States).

The cycling conditions comprised of a UDG activation stage for 2 minutes at 50℃, initial denaturation for 2 minutes at 95℃, denaturation for 15 seconds at 95℃, annealing 15 seconds at 52℃ with extension for 1 minute at 72℃ for 40 cycles. To assess any contamination and efficiency of the PCR, a negative control was included in all PCR runs.

2.7.2. In-house *opa* primers

The in-house assay made use of the primer sets described in Table 1. The assay included: 5ul of PowerUp SYBR Green Master Mix (ThermoFisher Scientific, United States), 0.2nm of the forward and reverse primers, 1.5ul template DNA brought up to a final volume of 10ul with nuclease free water. The reactions were run on a Quant Studio 5 PCR instrument (ThermoFisher Scientific, United States).

Since all three primer sets had very similar melting temperatures, an annealing temperature of 56℃ was used. The cycling conditions of the assays were as follows: The cycling conditions comprised of a UDG activation stage for 2 minutes at 50℃, initial denaturation for 2 minutes at 95℃, denaturation for 15 seconds at 95℃, annealing 15 seconds at 56℃ with extension for 1 minute at 72℃ for 40 cycles. To assess any contamination and efficiency of the PCR, a negative control was included in all PCR runs.

2.8. Limit of detection

Ten-fold serial dilutions of the genomic DNA from the bacterial isolates and clinical samples were prepared. The dilution series ranged from a 1:10 - 1:100 000. The comparator *opa* and in-house *opa* primers were performed on each dilution series. The samples were run in replicates

of six. The DNA concentration of each dilution was determined using a Nanodrop spectrophotometer (ThermoFisher Scientific, South Africa).

The limit of detection of the assays was determined by calculating the copy numbers yielding positive amplification for each gene investigated. To calculate the copy number, the following formula was used, where the length of the base was obtained from the GenScript software for in-house *opa* primers 1-3 and comparator *opa* from Verma *et al.*, (2012) (97). The limit of detection was only determined for the endocervical swab, vaginal swab, and urine sample. The rationale for this, was selecting one sample for each sample type.

$$\text{Number of copies} = \frac{Amount\ of\ amplicons\ X\ 6.0221\ X\ 10^{23}\ molecules/mole}{Lenghth\ of\ ds\ DNA\ amoplicons\ X\ 1\frac{X1O^{9}NG}{G} X\ 650\ G/mole}$$

2.9. Statistical analysis

The data was analysed using IBM SPSS Statistics version 27. For this study, sensitivity was calculated as the probability that the in-house *opa* primers produced positive amplification for culture isolates, endocervical swabs, vaginal swabs, and urine DNA samples.

3. RESULTS

Summary

Of the 307 endocervical swabs, *N. gonorrhoeae* was detected in 29 samples. For the 385 vaginal swabs, 20 tested positive for *N. gonorrhoeae*. Finally, of the 200 urine samples, only eleven tested positive for *N. gonorrhoeae*. For this analysis, DNA from the 29 endocervical swabs, 20 vaginal swabs, and eleven urine samples were used.

3.1. Amplification of the *opa* genes from study samples

3.1.1. Comparator *opa* primers

For the *N. gonorrhoeae* isolates (five WHO and six clinical isolates), the comparator assay had amplified 10/11 (90.9%) of the samples. One sample was classified as a false negative (9.1%). Of the 29 endocervical DNA samples, eight samples (27.6%) produced positive amplification whilst 72.4% were classified as false negatives. For the vaginal DNA samples, 5/20 (25.0%) samples produced positive amplifications for *N. gonorrhoeae*, however, 15/20 (75%) samples produced false negative results. Lastly, for the urine DNA samples, 4/11 (36.4%) samples produced positive amplifications for *N. gonorrhoeae* and there were 7/11 (63.6%) samples which were false negatives. The comparator assay produced positive amplification for *N. sicca* indicating cross reactivity with this bacterium (Table 3.2, 3.3, 3.4).

3.1.2. In-house *opa* 1 primers

The *opa* 1 primer set produced positive amplification for all (100%) five WHO and the six *N. gonorrhoeae* clinical isolates. For the endocervical DNA samples, 24/29 (82.8%) samples were amplified, and 5 samples were not amplified (17.2%) and classified as false negatives. For the vaginal DNA samples, 19/20 (95.0%) samples were amplified and only one sample was misclassified as a negative (5%). All 11 (100%) of the urine DNA samples produced positive

amplification. There was no cross-reactivity with this primer set and the non-gonococcal isolates (Table 3.2, 3.3, 3.4).

3.1.3. In-house *opa* 2 primers

The *opa* 2 primer set produced positive amplification for all (100%) five WHO and the six *N. gonorrhoeae* clinical isolates. For the endocervical DNA samples, 21/29 (72.4%) samples produced positive amplification and 8/29 (27.6%) were false negatives. For the vaginal DNA samples, 19/ 20 (95%) samples produced positive amplification with one sample classified as a false negative (5%) and for the urine DNA samples, 9/11 samples (81.8%), produced positive amplification with two samples (18.2%) being classified as false negatives. This primer set produced positive amplification for *N. sicca* indicating cross reactivity with this bacterium (Table 3.2, 3.3, 3.4).

3.1.4. In-house *opa* 3 primers

The *opa* 3 primer set produced positive amplification for the five WHO and the six *N. gonorrhoeae* clinical isolates. For the endocervical DNA samples, 17/29 (58.6%) samples produced positive amplification, however, 12 samples (42.4%) were classified as false negatives. For the vaginal DNA samples, 18/20 (90.0%) samples produced positive amplification for *N. gonorrhoeae* with two samples (10%) being classified as false negatives. Lastly, for the urine DNA samples, 8/11 (72.7%) samples produced positive amplification and three (27.3%) samples were false negatives. This primer set showed cross reactivity to both *N. cinerea* and *N. sicca* (Table 3.2, 3.3, 3.4).

Overall, the *opa* 1 primer set, performed the best when compared to the comparator *opa*, *opa* 2 and *opa* 3 primers. For the endocervical DNA samples, *opa* 1 performed better than *opa* 2 and *opa* 3. For the vaginal DNA samples, *opa* 1 and *opa* 2 both performed well and for the urine DNA samples, *opa* 1 performed the best.

Table 3.2: Showing the data for positive and negative amplification for the comparator *opa*, in-house *opa* 1, *opa* 2, and *opa* 3 *primers* across the various sample types.

Opa type	Sample type			Negative	Positive	Total
Comparator *opa*	Culture	Negative	Count	2	1	3
			% within the sample	66.7 %	9.1 %	21.4 %
		Positive	Count	1	10	11
			% within the sample	33.3 %	90.9 %	78.6 %
	Endocervical swabs	Negative	Count	2	21	23
			% within the sample	66.7 %	72.4%	71.9 %
		Positive	Count	1	8	9
			% within the sample	33.3 %	27.6 %	28.1 %
	Vaginal swabs	Negative	Count	2	15	17
			% within the sample	66.7 %	75.0 %	73.9 %
		Positive	Count	1	5	6
			% within the sample	33.3 %	25.0 %	26.1 %
	Urine	Negative	Count	2	7	9
			% within the sample	66.7 %	63.6 %	64.3 %
		Positive	Count	1	4	5
			% within the sample	33.3 %	36.4 %	35.7 %
In-house opa 1	Culture	Negative	Count	3	0	3
			% within the sample	100 %	0.0 %	21.4 %
		Positive	Count	0	11	11
			% within the sample	0.0 %	100 %	78.6 %
	Endocervical swabs	Negative	Count	3	5	8
			% within the sample	100 %	17.2 %	25.0 %
		Positive	Count	0	24	24
			% within the sample	0.0 %	100 %	100 %
	Vaginal swabs	Negative	Count	3	1	4
			% within the sample	100 %	5.0 %	17.4 %
		Positive	Count	0	19	19
			% within the sample	0.0 %	100 %	100 %
	Urine	Negative	Count	3	0	3
			% within the sample	100 %	0.0 %	100 %
		Positive	Count	0	11	11
			% within the sample	0.0 %	100 %	100 %
In-house opa 2	Culture	Negative	Count	2	0	2
			% within the sample	66.7 %	0.0 %	14.3 %
		Positive	Count	1	11	12

			% within the sample	33.3 %	100 %	85.7 %
	Endocervical swabs	**Negative**	**Count**	2	8	10
			% within the sample	66.7 %	27.6 %	31.3 %
		Positive	**Count**	1	21	22
			% within the sample	33.3 %	72.4 %	68.8 %
	Vaginal swabs	**Negative**	**Count**	2	1	3
			% within the sample	66.7 %	5.0 %	13.0 %
		Positive	**Count**	1	19	20
			% within the sample	33.3 %	95.0 %	100 %
	Urine	**Negative**	**Count**	2	2	4
			% within the sample	66.7 %	18.2 %	28.6 %
		Positive	**Count**	1	9	10
			% within the sample	33.3 %	81.8 %	71.4 %
In-house opa 3	**Culture**	**Negative**	**Count**	1	0	1
			% within the sample	33.3 %	0.0 %	7.1 %
		Positive	**Count**	2	11	13
			% within the sample	66.7 %	100 %	92.9 %
	Endocervical swabs	**Negative**	**Count**	1	12	13
			% within the sample	33.3 %	41.4 %	40.6 %
		Positive	**Count**	2	17	19
			% within the sample	66.7 %	58.6 %	59.4 %
	Vaginal swabs	**Negative**	**Count**	1	2	3
			% within the sample	33.3 %	10.0 %	13.0 %
		Positive	**Count**	2	18	20
			% within the sample	66.7 %	90.0 %	87.0 %
	Urine	**Negative**	**Count**	1	3	4
			% within the sample	33.3 %	27.3 %	28.6 %
		Positive	**Count**	2	8	10
			% within the sample	66.7 %	72.7 %	71.4 %

Table 3.3: Showing the CT values for the controls across the different primer sets.

Sample Name	**Target Name**	**CT**
NC	Reference *Opa*	Undetermined
WHO O	Reference *Opa*	Undetermined
WHO N	Reference *Opa*	23,671
WHO X	Reference *Opa*	23,247
WHO Y	Reference *Opa*	12,607
WHO Z	Reference *Opa*	22,333
NC	Reference *Opa*	Undetermined
N. cinera	Reference *Opa*	Undetermined
N. weaveri	Reference *Opa*	Undetermined
N. sica	Reference *Opa*	25,867
NC	*Opa* 1	Undetermined
WHO O	*Opa* 1	9,581
WHO N	*Opa* 1	11,799
WHO X	*Opa* 1	9,841
WHO Y	*Opa* 1	9,453
WHO Z	*Opa* 1	9,466
N. cinerea	*Opa* 1	Undetermined
N. weaveri	*Opa* 1	Undetermined
N. sicca	*Opa* 1	Undetermined
NC	*Opa 2*	Undetermined
WHO O	*Opa 2*	9,393
WHO N	*Opa 2*	10,883
WHO X	*Opa 2*	10,008
WHO Y	*Opa 2*	9,705

Sample Name	**Target Name**	**CT**
WHO Z	*Opa 2*	10,802
N. cinerea	*Opa 2*	31,427
N. weaveri	*Opa 2*	Undetermined
N. sicca	*Opa 2*	Undetermined
NC	*Opa 3*	Undetermined
WHO O	*Opa 3*	9,300
WHO N	*Opa 3*	9,994
WHO X	*Opa 3*	7,988
WHO Y	*Opa 3*	7,890
WHO Z	*Opa 3*	7,257
N. cinerea	*Opa 3*	34,024
N. weaveri	*Opa 3*	Undetermined
N. sicca	*Opa 3*	25,444

Table 3.4: Showing the CT values for the clinical samples and clinical isolates for the different primer sets.

Sample Name	**Sample type**	**CT: Comparator *opa***	**CT: In-house *opa* 1**	**CT: In-house *opa 2***	**CT: In-house *opa 3***
NC		Undetermined	Undetermined	Undetermined	Undetermined
G003	Endocervical swab	Undetermined	30,368	34,799	34,12
G007	Endocervical swab	Undetermined	32,545	37,932	Undetermined
G51	Endocervical swab	Undetermined	25,144	24,92	23,096
G61	Endocervical swab	Undetermined	Undetermined	29,942	28,061
G79	Endocervical swab	37,000	27,908	26,607	26,045
G132	Endocervical swab	Undetermined	Undetermined	Undetermined	Undetermined
G133	Endocervical swab	37,002	25,177	26,836	35,546
G134	Endocervical swab	Undetermined	33,662	Undetermined	Undetermined
G136	Endocervical swab	Undetermined	35,188	28,407	33,543
G138	Endocervical swab	Undetermined	34,970	Undetermined	Undetermined
G141	Endocervical swab	Undetermined	Undetermined	30,743	Undetermined
G142	Endocervical swab	Undetermined	35,366	37,236	39,851
G143	Endocervical swab	Undetermined	34,427	36,119	37,174
G144	Endocervical swab	Undetermined	34,012	35,794	Undetermined
G145	Endocervical swab	Undetermined	33,455	Undetermined	Undetermined
G148	Endocervical swab	Undetermined	Undetermined	38,522	Undetermined

Sample Name	**Sample type**	**CT: Comparator o*pa***	**CT: In-house o*pa* 1**	**CT: In-house o*pa 2***	**CT: In-house o*pa 3***
G151	Endocervical swab	Undetermined	34,783	Undetermined	Undetermined
G153	Endocervical swab	Undetermined	33,398	Undetermined	Undetermined
G154	Endocervical swab	Undetermined	33,986	Undetermined	Undetermined
G160	Endocervical swab	Undetermined	Undetermined	Undetermined	Undetermined
G176	Endocervical swab	Undetermined	36,080	21,4	21,911
G180	Endocervical swab	Undetermined	21,133	21,989	22,969
G206	Endocervical swab	37,377	26,767	27,009	25,633
G247	Endocervical swab	36,642	35,147	27,586	25,127
NC		Undetermined	Undetermined	Undetermined	Undetermined
BN42	Vaginal Swab	Undetermined	Undetermined	Undetermined	Undetermined
BN117	Vaginal Swab	Undetermined	28,990	29,831	29,018
BN193	Vaginal Swab	Undetermined	25,810	27,637	28,329
BN214	Vaginal Swab	Undetermined	32,353	38,721	32,372
BN243	Vaginal Swab	Undetermined	28,466	32,253	31,083
BN251	Vaginal Swab	Undetermined	32,687	33,456	32,557
BN261	Vaginal Swab	Undetermined	34,433	30,844	30,53
BN292	Vaginal Swab	Undetermined	33,384	33,445	32,451
BN309	Vaginal Swab	Undetermined	34,430	31,408	29,739
BN311	Vaginal Swab	Undetermined	33,452	34,023	33,987
BN335	Vaginal Swab	Undetermined	29,509	31,591	31,73
BN362	Vaginal Swab	Undetermined	25,783	32,006	32,016
BN363	Vaginal Swab	34,166	33,202	23,031	22,551
BN375	Vaginal Swab	Undetermined	35,285	32,877	31,794
BN377	Vaginal Swab	Undetermined	27,675	32,247	Undetermined
NC		Undetermined	Undetermined	Undetermined	Undetermined
KM12	Urine	Undetermined	32,428	35,556	Undetermined
KM60	Urine	Undetermined	36,410	Undetermined	36,151
KM69	Urine	Undetermined	33,278	Undetermined	Undetermined

Sample Name	**Sample type**	**CT: Comparator o*pa***	**CT: In-house o*pa* 1**	**CT: In-house o*pa 2***	**CT: In-house o*pa 3***
KM79	Urine	Undetermined	34,071	35,35	Undetermined
KM94	Urine	Undetermined	28,963	29,06	28,658
KM97	Urine	Undetermined	34,891	34,868	35,182
NC		Undetermined	Undetermined	Undetermined	Undetermined
G51	Culture isolates	16,025	10,726	12,498	20,045
G136	Culture isolates	23,469	15,215	10,84	11,593
G176	Culture isolates	28,809	12,657	12,995	10,637
G180	Culture isolates	17,298	11,820	12,11	10,124
G206	Culture isolates	21,457	12,204	13,255	12,764
G247	Culture isolates	21,886	26,102	16,109	13,498

3.2. Limit of detection

The limit of detection was determined for each sample type using the different primer sets. For the endocervical DNA samples, at the highest DNA dilution of 1:100 000, *opa* 1 was able to detect 5.15 x 10^9 copies of the gene, o*pa* 2 was able to detect 3.11 X 10^9 copies of the gene and *opa* 3 was able to detect 1.97 x 10^9 copies of the gene (Table 3.5).

For the vaginal DNA samples, at the highest DNA dilution of 1:100 000, *opa* 1 was able to detect a 5.15 x 10^9 copies of the gene, *opa* 2 was able to detect 3.11 X 10^9 copies and *opa* 3 was able to detect 1.97 x 10^9 copies (Table 3.5).

For the urine DNA samples, at the highest DNA dilution of 1:100 000, *opa* 1 was able to detect 1.29 x 10^{10} copies of the gene, *opa* 2 was able to detect 7.79 X 10^9 copies and *opa* 3 was able to detect 4.93 x 10^9 copies of the gene (Table 3.5).

Overall, *opa* 1 was able to detect the highest number of copies of the target gene at the highest DNA dilution when compared to primer sets, *opa* 2 and *opa* 3.

Table 3.5: Showing the results for the number of copies calculated to determine the limit of detection for the different sample types across the different primer sets.

Sample number	Sample type	DNA concentration (ng/µl)	Dilution	*Opa primer*	No. of copies of target gene (*opa*)
G180	Endocervical swab	5.5	1:10	*Opa* 1	$7.08\ X10^{10}$
G180	Endocervical swab	3.5	1:100	*Opa* 1	$4.50\ X10^{10}$
G180	Endocervical swab	3.2	1:1000	*Opa* 1	$4.12\ X10^{10}$
G180	Endocervical swab	0.4	1:10 000	*Opa* 1	$7.72\ X10^{9}$
G180	Endocervical swab	0.4	1:100 000	*Opa* 1	$5.15\ X10^{9}$
G180	Endocervical swab	5.5	1:10	*Opa* 2	$4.28\ X10^{10}$
G180	Endocervical swab	3.5	1:100	*Opa* 2	$2.72\ X10^{10}$
G180	Endocervical swab	3.2	1:1000	*Opa* 2	$2.49\ X10^{10}$
G180	Endocervical swab	0.4	1:10 000	*Opa* 2	$4.67\ X10^{9}$
G180	Endocervical swab	0.4	1:100 000	*Opa* 2	$3.11\ X10^{9}$
G180	Endocervical swab	5.5	1:10	*Opa* 3	$4.28\ X10^{10}$
G180	Endocervical swab	3.5	1:100	*Opa* 3	$1.72X10^{10}$
G180	Endocervical swab	3.2	1:1000	*Opa* 3	$2.49\ X10^{10}$
G180	Endocervical swab	0.4	1:10 000	*Opa* 3	$2.69\ X10^{9}$
G180	Endocervical swab	0.4	1:100 000	*Opa* 3	$1.97\ X10^{9}$
BN117	Vaginal swab	1.7	1:10	*Opa* 1	$2.19\ X10^{10}$
BN117	Vaginal swab	1.2	1:100	*Opa* 1	$1.54\ X10^{10}$
BN117	Vaginal swab	1.2	1:1000	*Opa* 1	$1.54X10^{10}$
BN117	Vaginal swab	0.5	1:10 000	*Opa* 1	$6.43\ X10^{9}$
BN117	Vaginal swab	0.4	1:100 000	*Opa* 1	$5.15\ X10^{9}$
BN117	Vaginal swab	1.7	1:10	*Opa* 2	$1.32X10^{10}$
BN117	Vaginal swab	1.2	1:100	*Opa* 2	$9.34\ X10^{9}$
BN117	Vaginal swab	1.2	1:1000	*Opa* 2	$9.34\ X10^{9}$
BN117	Vaginal swab	0.5	1:10 000	*Opa* 2	$3.89\ X10^{9}$
BN117	Vaginal swab	0.4	1:100 000	*Opa* 2	$3.11\ X10^{9}$

Sample number	Sample type	DNA concentration (ng/µl)	Dilution	*Opa primer*	No. of copies of target gene (*opa*)
BN117	Vaginal swab	1.7	1:10	*Opa* 3	$8.38\ X10^{9}$
BN117	Vaginal swab	1.2	1:100	*Opa* 3	$5.91X10^{9}$
BN117	Vaginal swab	1.2	1:1000	*Opa* 3	$5.91\ X10^{9}$
BN117	Vaginal swab	0.5	1:10 000	*Opa* 3	$2.46\ X10^{9}$
BN117	Vaginal swab	0.4	1:100 000	*Opa* 3	$1.97\ X10^{9}$
KM 94	Urine	2.3	1:10	*Opa* 1	$2.96\ X10^{10}$
KM 94	Urine	1.6	1:100	*Opa* 1	$2.06\ X10^{10}$
KM 94	Urine	1.2	1:1000	*Opa* 1	$1.54X10^{10}$
KM 94	Urine	1.1	1:10 000	*Opa* 1	$1.42X10^{10}$
KM 94	Urine	1.0	1:100 000	*Opa* 1	$1.29\ X10^{10}$
KM 94	Urine	2.3	1:10	*Opa* 2	$1.79X10^{10}$
KM 94	Urine	1.6	1:100	*Opa* 2	$1.25\ X10^{10}$
KM 94	Urine	1.2	1:1000	*Opa* 2	$9.34\ X10^{9}$
KM 94	Urine	1.1	1:10 000	*Opa* 2	$8.56\ X10^{9}$
KM 94	Urine	1.0	1:100 000	*Opa* 2	$7.79\ X10^{9}$
KM 94	Urine	2.3	1:10	*Opa* 3	$1.13\ X10^{10}$
KM 94	Urine	1.6	1:100	*Opa* 3	$7.88X10^{9}$
KM 94	Urine	1.2	1:1000	*Opa* 3	$5.91\ X10^{9}$
KM 94	Urine	1.1	1:10 000	*Opa* 3	$5.42\ X10^{9}$
KM 94	Urine	1.0	1:100 000	*Opa* 3	$4.93X10^{9}$

4. DISCUSSION

The standard approach for *N. gonorrhoeae* detection involves culturing. Nevertheless, the cultivation of *N. gonorrhoeae* poses difficulties due to the fastidious nature of the microorganism (5). Due to the constraints related to cultivation, certain NAATs for *N. gonorrhoeae* have received approval from the FDA. There are numerous genes which are used for the detection of *N. gonorrhoeae.* Examples of these genes are *opa, 16S rRNA, porA, cppB,* and *CMT* genes (93). The conserved region of the *opa* gene encodes proteins with physiological functions. Additionally, *N. gonorrhoeae* has eleven unique *opa* genes. Geraats-Peters *et al.,* (2005) reported that an *opa* gene-based PCR assay is suggested to be more sensitive than a *16S rRNA* PCR assay (93). Other studies also reported that the *opa* gene assays have a higher sensitivity than *porA* pseudogene assays (97). The *cppB* gene and *CMT* gene exhibit broad sensitivity due to the absence of the genes in specific strains of *N. gonorrhoeae*. The *CMT* gene demonstrates reduced specificity as it can react with commensal *Neisseria* species. Consequently, the *cppB* and *CMT* genes are unsuitable for the detection of *N. gonorrhoeae* (122).

In this study, three in-house real-time PCR assays targeting the *opa* gene were compared against the *opa* primer set published by Verma *et al.,* (2012) (97). Cross-reactivity tests confirmed that the in-house *opa* 1 was specific for *N. gonorrhoeae* only. However, cross-reactivity was observed with primer sets *opa* 2 and *opa* 3 as well as the comparator *opa* primer set, since positive amplification was obtained with the DNA from *N. cinerea* and *N. sicca.* These results were in accordance with the cross-reactivity tests conducted in the Verma *et al.,* (2012) study (97). In that study, the *porA* pseudogene and the *opa*-based PCR assays proved to be highly specific for gonococcal DNA, however, cross-reaction with *N. sicca* was evident with the *16S rRNA* assay (97).

The current study demonstrated that the in-house *opa* 1 primer set had yielded the highest number of positives for the respective sample types. The in-house *opa* 1 primer set performed better than the *opa* primers published by Verma *et al.,* (2012). None of the non-gonococcal isolates were amplified with the in-house *opa* 1 primer set, indicating that there was no cross-reactivity. The in-house *opa* 1 was a more reliable primer set to use across all sample types. The in-house *opa* 1 primer was also more sensitive, producing a stronger amplification at the highest DNA dilution (1: 100 000) when compared to the in-house *opa* 2 and *opa* 3 primer sets. As this study is novel, there are no other studies to which we can compare our findings to. This adds to the novelty of the study and these results can be added to the growing body of literature.

A cost-effective qPCR (SYBR green) assay was used in this study. Similar findings were reported by Yasmon *et al.,* (2022), who used a SYBR green assay for the detection of *N. gonorrhoeae* from clinical samples. Their SYBR green assay was also highly specific since no cross-reactivity with other bacteria, viruses, fungi, and protozoa which might cause possible false-positive results was noted (122).

Single-tube qPCR assays have been recognized for their precision and sensitivity in detecting *N. gonorrhoeae* from clinical specimens (93). Other diagnostic assays such as the BD MAX (Becton Dickinson), GeneXpert (Cepheid), Allplex (SeeGene), and Anyplex (SeeGene) have been used in our local setting. These multiplex assays, whilst highly sensitive, are expensive and are designed to simultaneously screen for various STIs not just *N. gonorrhoeae*. The commercially available TaqMan assay is an economical option that specifically targets *N. gonorrhoeae* only (123-125). However, SYBR green assays can be as sensitive and specific as the TaqMan assays. The ideal screening assay should be specific, sensitive, and easy to use. Real-time PCR assays allow for rapid, sensitive, and specific diagnosis for *N. gonorrhoeae* (7, 95). The SYBR green assays are cheaper than TaqMan assays and may be suitable for routine testing of STIs (126). Therefore, the in-house *opa* 1 assay described in this study has the

potential to be developed into a screening assay for the detection of *N. gonorrhoeae* from various sample types.

The limitation of the study is the small sample size. Despite this, we tested the *opa-based* real-time PCR across different sample types: endocervical swabs, vaginal swabs, urine samples, and clinical isolates. Other studies such as the Yasmon *et al.,* 2022 study used only cervical samples and the Geraats *et al.,* (2005) study used either urethral, cervical, proctal, or tonsil specimens, thereby limiting their study (93, 127). Our study used self-collected vaginal swab samples as well, and despite the possible limited material on the swab, we were still able to detect a positive amplification with DNA diluted at 1:100 000 for the in-*house opa* 1 primer. This study used a single tube, low cost (cheaper than probe-based assays), and less time-consuming qPCR assay (when compared to classical PCR) to detect *N. gonorrhoeae* which may have future clinical utility.

Conclusion

In conclusion, the study demonstrated that the *opa* 1 primer was the superior primer when compared to *opa* 2, *opa* 3, and the comparator *opa* primers from the Verma *et al.,* (2012) study. Therefore, this in-house *opa* 1 assay can be potentially used and further evaluated for its use as a diagnostic assay for *N. gonorrhoeae*.

In chapter Two, the Opa-CEACAM binding profiles of *N. gonorrhoeae* was described as a potential method for reverse vaccinology development for *N. gonorrhoeae*. Since, *N. gonorrhoeae* has shown resistance to a wide range of antibiotics, in Chapter Four alternative therapeutics for *N. gonorrhoeae* was explored. The study investigated a more advanced alternative therapeutic, such as nanoemulsion of plant extracts as a potential therapeutic option.

CHAPTER FOUR

AIM THREE

Busting the Resistance: Antimicrobial Activity of Plant-Infused Nanoemulsions against *Neisseria gonorrhoeae*

1. INTRODUCTION

Sexually transmitted infections continue to be a global public health concern, with *N. gonorrhoeae)*, the Gram-negative bacterium responsible for gonorrhoea, posing challenges due to its ability to develop antibiotic resistance (4, 37, 39, 40, 49). South Africa has followed the recommendations made by WHO in 2014 which advocated for the replacement of the first-line treatment with oral cefixime to a single injectable dose (250 mg) of ceftriaxone (37, 44). Treatment failures of ceftriaxone monotherapy led to the WHO recommendation of administering dual antimicrobial therapy with the combination of ceftriaxone (250 mg) and azithromycin (1 g stat) (37). Nevertheless, there have been reports of reduced susceptibility of *N. gonorrhoeae* to ceftriaxone, with the level of resistance ranging widely, from 1.3% to 55.8% (52). In addition, resistance to azithromycin is already prevalent in many settings (53). Therefore, dual antimicrobial therapy cannot ensure long-term effectiveness. This highlights the critical need to develop an alternative therapeutic to reduce the spread of *N. gonorrhoeae.* The emergence of multidrug-resistant strains of *N. gonorrhoeae* has heightened the urgency for innovative and effective treatment strategies. In this study, the fusion of two promising fields of research, nanoemulsion technology, and plant-based therapeutics, presents a novel approach to combating *N. gonorrhoeae* infections.

Nanoemulsions, which are currently the subject of extensive investigation, serve as submicron-sized emulsions utilized for the improved delivery of therapeutic agents. They are recognized

as an advanced nanoparticle technique that contributes to the more efficient systemic administration of biologically active substances, ultimately enhancing and regulating drug delivery (29). A nanoemulsion is characterized by its stability and clarity, resulting from the thermodynamically balanced dispersion of two immiscible liquids, commonly oil and water. This stability is ensured by the formation of an interfacial layer composed of surfactant molecules (29, 31, 70). The dispersed phase typically comprises tiny particles or droplets, ranging in size from 5nm to 200nm, and exhibits an exceptionally low interfacial tension between the oil and water phases. This formation is aided by the droplets' size, which is less than 25% of the wavelength (29, 70). Often, a co-surfactant or co-solvent is added alongside the surfactant, oil phase, and water phase, constituting three vital components of a nanoemulsion in many cases (29, 31, 70-72). Nanotechnology has greatly augmented the potential for drug targeting, reducing the necessary dosage size and quantity for effective treatment (72). Consequently, the study's objective is to explore the utilization of nanotechnology in conjunction with plant extracts to identify an alternative therapeutic approach for combating *N. gonorrhoeae* infections. By combining the unique properties of nanoemulsions, such as enhanced drug delivery and stability, with the diverse bioactive compounds found in plant extracts, it is hoped to unlock new solutions (alternative therapeutics to treat *N. gonorrhoeae* as opposed to antibiotics) in the battle against this persistent and adaptable bacterial pathogen (72).

The properties found in plant extracts have been harnessed in recent times to counteract the adverse effects of pharmaceutical drugs and mitigate the rise of pathogens that are resistant to multiple drugs (70). This study will investigate the antimicrobial properties of *Ocimum tenuiflorum* (“holy basil”), *Moringa oleifera* and *Azadirachta indica* plants. These plants are suggested to have anti-bacterial and anti-inflammatory properties and may show promising effects against *N. gonorrhoeae* (32-35, 77). While these plants, *Ocimum tenuiflorum*, *Moringa*

oleifera, and *Azadirachta indica* have been traditionally used for their potential medicinal properties, their antimicrobial properties against STIs requires more comprehensive scientific research. Therefore, this will be the first study to explore the activity of these plants against *N. gonorrhoeae* in South Africa.

This study aimed to determine the antimicrobial activities of plant nanoemulsions from *Ocimum tenuiflorum* ("holy basil"), *Moringa oleifera* and *Azadirachta indica* plants against *N. gonorrhoeae* and to determine the toxicity of the plant nanoemulsions using human erythrocytes.

2. MATERIALS AND METHODS

2.1. Ethical approval

Ethical approval for this study was obtained from the Biomedical Research Ethics Committee (BREC) of the University of KwaZulu-Natal, (BREC/00005104/2022).

2.2. Study design

This study was a retrospective laboratory-based study using stored clinical isolates. For this analysis, six clinical isolates (G51, G136, G176, G180, G206, G247) testing positive for *N. gonorrhoeae* as well as two WHO strains (WHO Y and WHO Z) were used. The WHO Y and WHO Z isolates are both ceftriaxone and azithromycin-resistant (128).

2.3. Culture detection of *N. gonorrhoeae*

The stored isolates were plated out onto chocolate agar plates and incubated for 24 to 48 hours, at 37℃ in the presence of 5% CO_2. After this incubation process, suspected colonies were sub-cultured onto chocolate agar plates using a four-way streak. The plates were incubated for another 24 to 48 hours in the presence of 5% CO_2. Presumptive tests were conducted which included the oxidase test, Gram-staining, and the API test kit (BioMerieux) which acted as a means of confirmation (129). The methods are described in detail in Chapter Two, section 2.3, pages 27-29, and the results can also be found in Chapter Two, section 3.1, pages 35-38 of this thesis.

2.4. Plant collection and preparation of extracts

The plant leaves (*Ocimum tenuiflorum*, *Moringa oleifera* and *Azadirachta indica*) were collected from the Botanical Gardens in Durban, South Africa. After removing any dust and dirt, the leaves underwent an initial cleaning step by immersing the leaves in deionized water for one minute. Subsequently, the leaves were left to naturally air dry for a period of 4-5 days, shielded from direct sunlight for the preparation of aqueous extracts (Figure 4.1).

For the preparation of the plant extracts, the dried leaves were cut into small pieces using sterilized scissors and approximately 100g of each type of leaf material was combined with 500ml of distilled water and boiled for 30 minutes at 100°C. Following boiling, the mixture was allowed to cool and then filtered to obtain the aqueous extract (Figure 4.2a). The extracts were further filtered and subjected to centrifugation to remove any remaining solid particles and were stored at -20°C until ready for use.

Figure 4.1: (a) Images of the cleaned and dried leaves of *Ocimum tenuiflorum*, (b) *Moringa oleifera*, and (c) *Azadirachta indica* plants.

2.5. Nanoemulsion of the plants

Preparation of the oil phase involved creating a homogeneous organic solution (referred to as S1) comprising 400µl of isopropyl myristate (Sigma-Aldrich, Germany) and 86µl of span 80 (Sigma-Aldrich, Germany), which is lipophilic surfactant, dissolved in a water-miscible solvent (Figure 4.2b).

To prepare the aqueous phase, a homogeneous solution (S2) was formed by mixing 80 ml of water with 136µl of Tween 80 (Sigma-Aldrich, Germany), a hydrophilic surfactant. Into this aqueous phase, 30ml of the plant extract was introduced while subjecting the mixture to magnetic stirring. This resulted in the nearly instant formation of an oil-in-water (o/w) emulsion as the organic solvent diffused into the external aqueous phase, creating nano-sized

droplets. Magnetic stirring was continued for 30 minutes to allow the system to reach equilibrium (Figure 4.2c).

To remove all the water-miscible solvent, the emulsion was subjected to evaporation for 45 minutes under reduced pressure while being centrifuged at 1000rpm. Subsequently, the emulsion was cooled by immersing it in an ice bath for 10 minutes. Various nanoemulsion concentrations, namely 1000µM, 100µM, 10µM and 1µM, were prepared as part of the process and stored at 4℃ (Figure 4.2d).

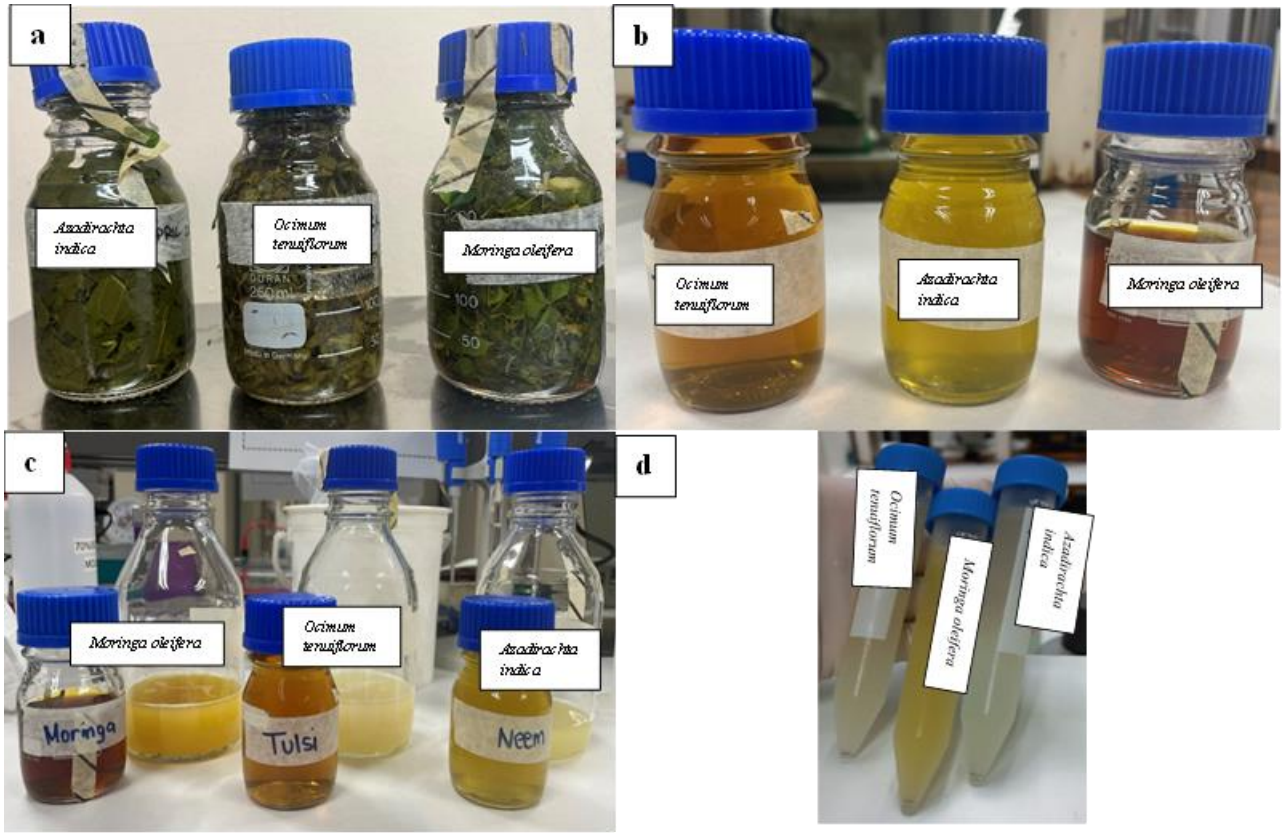

Figure 4.2: Preparation of the nanoemulsions of the three plants: (a) Image showing the leaves after they had been boiled and cooled down during the plant preparation step. (b) Image showing the leaves after the S1 preparation step. (c) Image showing the nanoemulsion after the S2 preparation step. (d) Image showing the final nanoemulsion with the leave extracts that can be stored at 4℃.

2.6. Antimicrobial activity of the nanoemlusions

A disk diffusion method was adapted for this experiment. Punched Whatman filter paper (no. 1) was baked at 160 ℃ for 1 hour to sterilize the paper and thereafter cooled until ready for use at room temperature. Overnight cultures of the isolates were used to make a 0.5 McFarland using deionised water. Thereafter, 100µl of the McFarland solution was plated out on chocolate agar plates. The sterile disks were saturated with the nanoemulsions: 5µl of different concentrations (1000µM, 100µM, 10µM and 1µM) of the nanoemulsions were added to the disks aseptically. The disks were then placed on the chocolate agar plate using aseptic techniques. The agar plates were incubated for 24 hours in a 37℃ CO_2 incubator. Antimicrobial activity was recorded as zones of clearance around the disks. Positive control strains, WHO Y and Z were generously provided by NICD, South Africa. These strains are known to be resistant to the currently used dual therapy for *N. gonorrhoeae* infections (128).

2.7. Haemolytic activity of the nanoemulsions

Preparation of the erythrocyte suspension employed in this study was similar to the methods reported by Kumar *et al.,* (2011) (130). Blood was collected from a healthy individual by venepuncture. The blood was immediately processed by centrifugation at 1500 rpm for 10 minutes. The supernatant (plasma) was discarded, and the pellet which contained the erythrocytes was washed three times with sterile phosphate buffer saline (PBS) solution (pH 7.2±0.2) and centrifuged at 1500rpm for 5 minutes. A 5% cell suspension was then made using normal saline.

In vitro haemolytic activity was measured by spectrophotometry as described by Yang *et al.,* (2005) (131). A volume of 100µl of the cell suspension was mixed with 100 µl of the individual plant extracts. The mixtures were incubated for 30 minutes at 37˚C. Following incubation, the mixture was centrifuged at 1500rpm for 10 minutes. The free haemoglobin in the supernatant was measured in an Ultraviolet (UV) spectrophotometer (Biochrom Libra S12, England) at

540nm. The haemolytic controls for this experiment were a PBS control (100µl of cell suspension and 100µl PBS) and a water control (100µl cell suspension and 100µl sterile water). Each experiment was performed in triplicate at each concentration across the individual plant types.

Percentage haemolysis was calculated according to the following formula:

$$\% \text{ Haemolysis} = \frac{AT - AN}{AC - AN} \text{ X } 100$$

Key:

AT is the absorbance of test sample.

AN is the absorbance of the control (saline control)

AC is the absorbance of the control (water control)

3. RESULTS

3.1. Antimicrobial activity of the nanoemulsions

All six isolates had zones of inhibition for the 1000 µM concentration for all three nanoemulsion based plant extracts. There were no zones of inhibition observed for 100µM, 10 µM and 1 µM (Figure 4.3-4.5) nanoemulsion concentrations for five of the isolates. Isolate G176 had zones of inhibition at both 1000µM and 100µM concentrations for the nanoemulsions of *Ocimum tenuiflorum* (Figure 4.3). WHO Y and WHO Z were used as a positive control (both resistant to azithromycin and ceftriaxone). Both the WHO strains had zones of inhibition appearing at the 1000µM concentration (Figure 4.6). For the WHO Y strain, zones of inhibition for both 1000µM and 100µM concentrations were observed for the nanoemulsions of *Ocimum tenuiflorum* and *Azadirachta indica.* The radius of the zone of inhibition was recorded (Table 4.1), however, since this was a novel study, there was no breakpoint data to compare with. The data described in this study may be used for future studies.

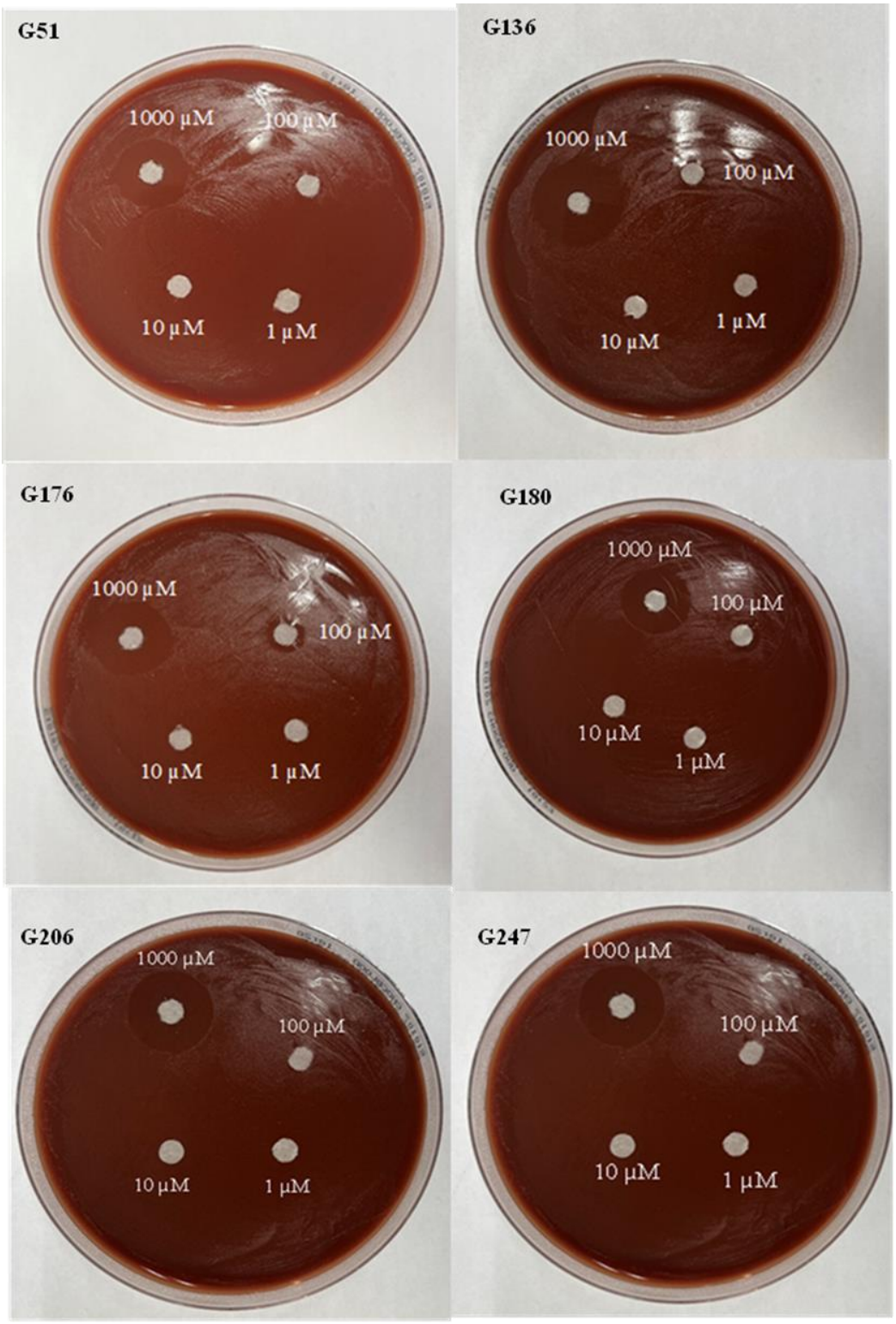

Figure 4.3: Antimicrobial activity of the nanoemulsions (different concentrations) of *Ocimum tenuiflorum*. At a concentration of 1000µM of the nanoemulsion, antimicrobial activity against the isolates of *N. gonorrhoeae* was observed. However, for isolate G176, a small zone of inhibition was observed at 100µM as well.

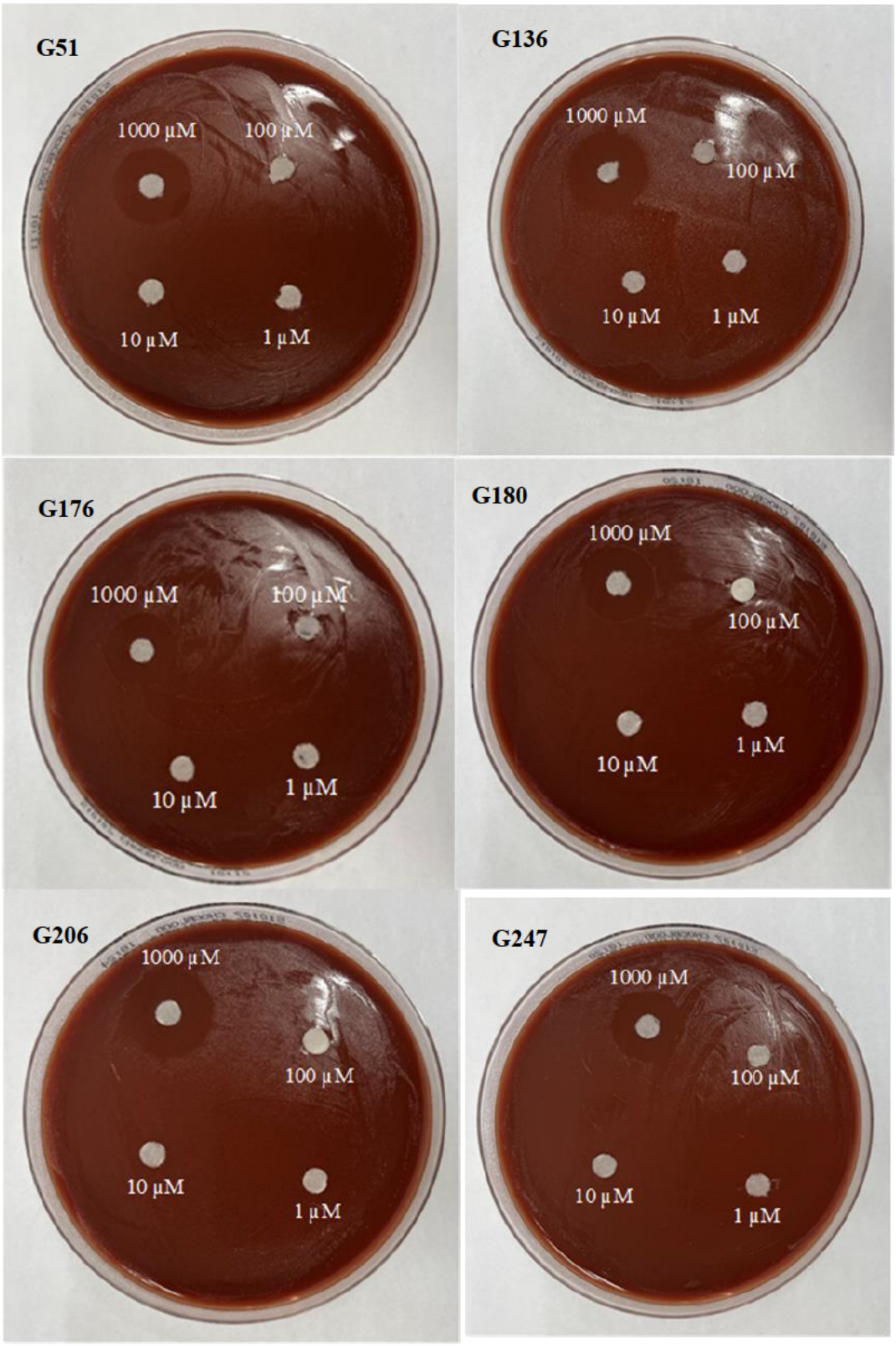

Figure 4.4: Antimicrobial activity of the nanoemulsions (different concentrations) of *Moringa oleifera*. At a concentration of 1000µM of the nanoemulsion, antimicrobial activity against the isolates of *N. gonorrhoeae* was observed.

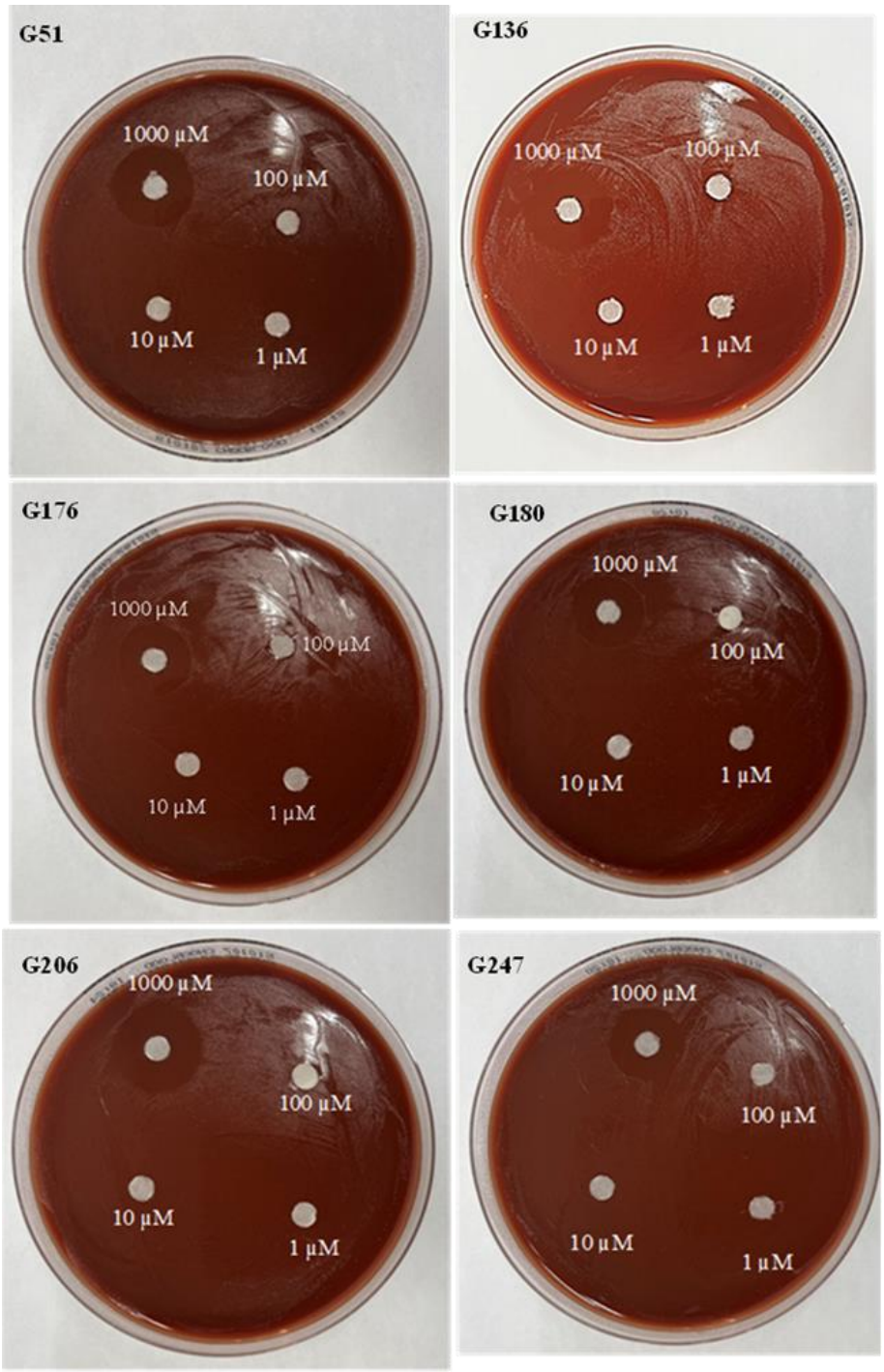

Figure 4.5: Antimicrobial activity of the nanoemulsions (different concentrations) of *Azadirachta indica*. At a concentration of 1000µM of the nanoemulsion, antimicrobial activity against the isolates of *N. gonorrhoeae* were observed.

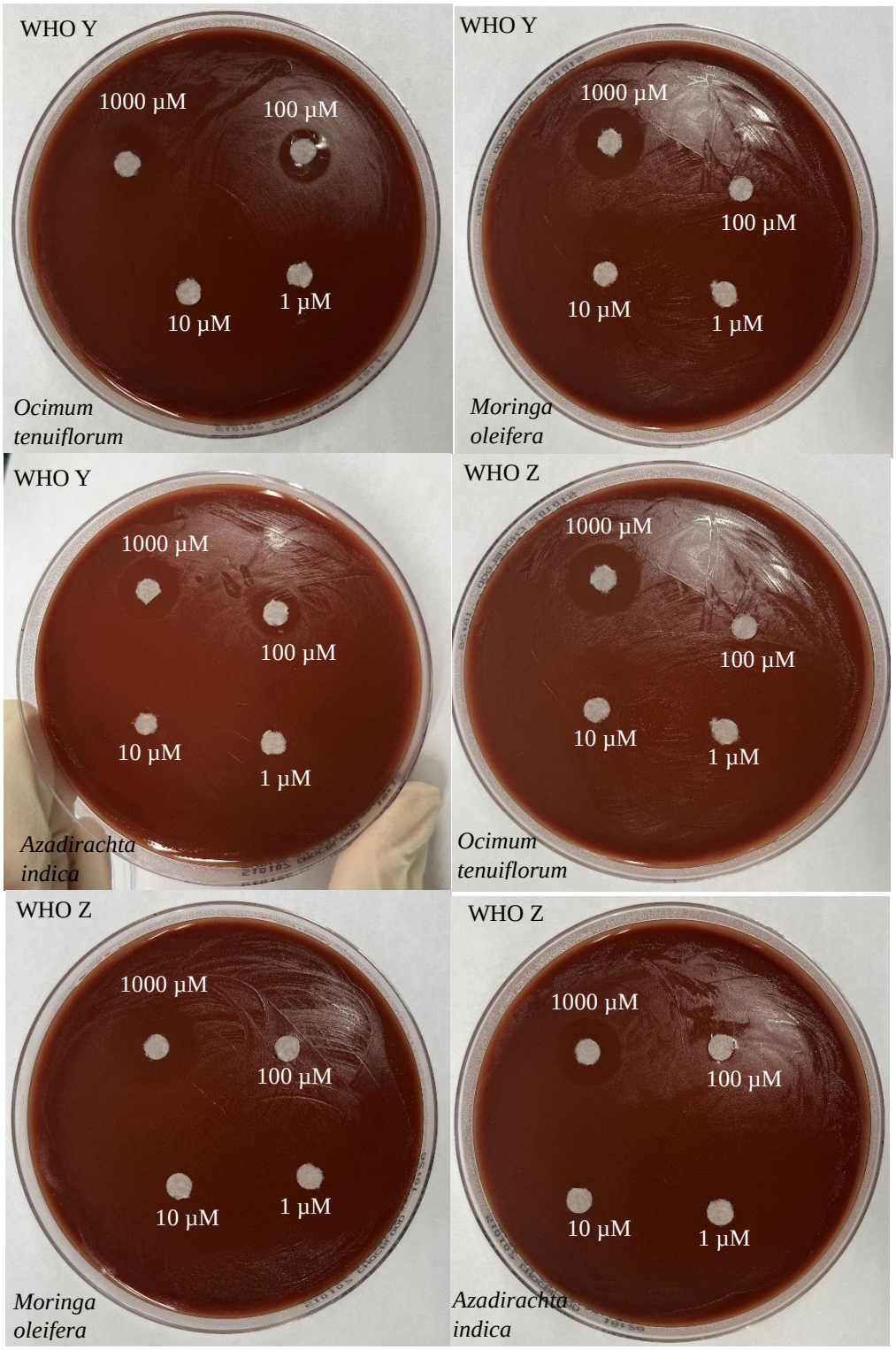

Figure 4.6: Antimicrobial activity of the nanoemulsions against the WHO control strains.

Table 4.1: Primary antimicrobial screening results using disk diffusion method (mm) for the nanoemulsion and plant extracts.

		Zone of inhibition (mm)		
		Plant name		
Isolates	Concentration (µM)	*Ocimum tenuiflorum*	*Moringa oleifera*	*Azadirachta indica*
G51	1000	8	8	10
	100	-	-	-
	10	-	-	-
	1	-	-	-
G136	1000	14	10	15
	100	-	-	-
	10	-	-	-
	1	-	-	-
G176	1000	10	8	10
	100	5	-	-
	10	-	-	-
	1	-	-	-
G180	1000	10	10	10
	100	-	-	-
	10	-	-	-
	1	-	-	-
G206	1000	15	14	10
	100	-	-	-
	10	-	-	-
	1	-	-	-
G247	1000	10	10	10
	100	-	-	-
	10	-	-	-
	1	-	-	-
Control WHO Y	1000	10	10	8
	100	5	-	5
	10	-	-	-
	1	-	-	-
Control WHO Z	1000	8	11	8
	100	-	-	-
	10	-	-	-
	1	-	-	-

3.2. Haemolytic activity of the nanoemulsions

The haemolytic activity of the nanocomposite of the three plant leaf extracts (*Ocimum tenuiflorum*, *Moringa oleifera*, *Azadirachta indica*) were investigated using human erythrocytes at a concentration 1000 µM. This concentration was selected since it produced the best results for inhibiting the growth of *N. gonorrhoeae*. According to the analysis, there was

0% haemolytic activity observed which suggests the non-toxic nature of the extracts (Table 4.2).

Table 4.2: showing the haemolytic activity of plant extracts against human erythrocytes.

OD 540nm of plants	***Ocimum tenuiflorum***	***Moringa oleifera***	***Azadirachta indica***
Concentration (µM)			
1000	0.101	0.100	0.107
	0.100	0.112	0.105
	0.100	0.100	0.100
Average	0.1	0.1	0.1
Haemolysis (%)	0	0	0

The OD_{540} reading for the PBS control was 0.1 and for the water control the reading was 0.291.

4. DISCUSSION

The prevalence of antibiotic-resistant cases of *N. gonorrhoeae* surged by 124% between 2013 and 2019 (132). According to the 2019 antimicrobial resistance report from the Centres for Disease Control and Prevention (CDC), *N. gonorrhoeae* has attained the status of a global urgent threat (133). Furthermore, WHO classified cephalosporin-resistant and fluoroquinolone-resistant *N. gonorrhoeae* strains as high-priority antibiotic-resistant pathogens in 2020 (134). Recent research has shown that the resistance rates for *N. gonorrhoeae* have reached alarming levels, with up to 21% resistance to ceftriaxone, 22% to cefixime, 60% to azithromycin, and a staggering 100% resistance to ciprofloxacin (44, 135). Moreover, various studies, including those by Martin *et al.,* (2019), Kueakulpattana *et al*., (2021), and Pleininger *et al.,* (2022), have documented sporadic instances of multidrug-resistant (MDR) and extensively drug-resistant (XDR) *N. gonorrhoeae* strains (136-138). In response to this escalating global antibiotic resistance crisis, the recommended treatment regimen for non-complicated gonococcal infections was revised in 2020 to include high-dose intramuscular ceftriaxone as the sole option. However, even this last resort is experiencing an increase in resistance rates (139).

Given the upward trajectory of antibiotic resistance observed in clinical *N. gonorrhoeae* strains and the extensive complications associated with asymptomatic gonorrhoea, the creation of a vaccine stands out as the most promising avenue for long-term protection against *N. gonorrhoeae.* Despite concerted efforts, the absence of a reliable vaccine or effective drug against highly drug-resistant *N. gonorrhoeae* remains a glaring reality. Consequently, it is imperative to continue striving for the development of novel and effective strategies to prevent and manage this pathogen. Due to the unsatisfying outcomes of newly developed antibiotics and the diminishing efficacy of repurposed older antibiotics, the pursuit of a new, potent

antibiotic against *N. gonorrhoeae* becomes an unavoidable imperative. In response to increasing antibiotic resistance, researchers and healthcare professionals have been exploring alternative treatment options and strategies to combat gonorrhoea. Therefore, this study aimed to determine the antimicrobial activities of plant nanoemulsions from *Ocimum tenuiflorum* ("holy basil"), *Moringa oleifera,* and *Azadirachta indica* plants against *N. gonorrhoeae* and to determine the toxicity of the plant nanoemulsions using human erythrocytes.

Many medicinal plants contain bioactive compounds with antimicrobial properties. Examples illustrate the diversity of plant-derived antimicrobial peptides and their potential applications in combating microbial infections. Thionins are cysteine-rich antimicrobial peptides found in various plant species which are known for their potent antimicrobial activity against bacteria, fungi, and other microorganisms. Thionins disrupt microbial cell membranes, leading to cell death. One example is α-hordothionin, which is found in barley seeds (140). Defensins are a class of small cationic peptides found in various plant species that possess broad-spectrum antimicrobial activity and contribute to plant defence against pathogens. Defensins exert their antimicrobial effects by disrupting microbial cell membranes or interfering with intracellular processes. Examples include RsAFP2 (Raphanus sativus antifungal protein 2) and NaD1 (Nicotiana alata defensin 1) (140). The bioactive compounds found in medicinal have been used in traditional medicine for centuries and are often considered safer and less likely to lead to antibiotic resistance when compared to synthetic antibiotics. According to the WHO, traditional medicines derived from medicinal plants continue to provide benefits to 80% of the developing world's population (98-100). Therefore, combining medicinal plant extracts with nanoemulsions can lead to synergistic effects. This study showed that at a concentration of 1000 µM, the nanocomposites of *Ocimum tenuiflorum*, *Moringa oleifera,* and *Azadirachta indica* inhibited the growth of *N. gonorrhoeae.* The haemolytic activity indicated that there was 0% haemolysis at 1000 µM across all three plants. This showed that the nanoemulsion and

plant mix, may potentially be used as a safer alternative to treat gonorrhoea. Since, this was the first study to investigate nanoemulsions combined with plant extracts against *N. gonorrhoeae*, our comparison with other studies is limited.

While the concept of using nanoemulsions combined with medicinal plant leaf extracts as an alternative treatment for *N. gonorrhoeae* is promising, it is important to note that further research is needed to determine the most effective plant extracts, the optimal nanoemulsion formulations, and the appropriate dosing regimens. Whilst the plant extracts used in this study (*Ocimum tenuiflorum*, *Moringa oleifera* and *Azadirachta indica*) are suggested to have anti-bacterial and anti-inflammatory properties and may show promising effects against *N. gonorrhoeae*, these plants, still requires more comprehensive scientific research on their impact on *N. gonorrhoeae* and effect on the human body (32-35, 77) . Although there are no human trials that have been published, there is experimental evidence that show that the holy basil may help in the treatment of various human bacterial infections including urinary tract infections, gonorrhoea, and Herpes (80-83). Clinical trials and rigorous testing are necessary to ensure safety and efficacy before these alternative treatments can be widely adopted. However, the advantages of nanoemulsions can improve the solubility and stability of plant-derived compounds, while the plant extracts can provide potent antimicrobial activity. Synergy between plant extracts and nanoemulsions may enhance the effectiveness of the treatment against *N. gonorrhoeae*, potentially reducing the risk of resistance development. Another advantage to this approach is, using nanoemulsions with plant extracts as an alternative treatment can help reduce the selective pressure for antibiotic resistance. By relying less on conventional antibiotics, we can slow down the evolution of antibiotic-resistant strains. Medicinal plant extracts are often considered safer and have fewer side effects when compared to some antibiotics. They may be better tolerated by patients, reducing the risk of adverse reactions. Medicinal plants are often readily available and sustainable, therefore are an

attractive option for global healthcare, especially in regions where access to antibiotics is limited.

The current treatment of gonococcal infection according to the WHO guidelines for genital and anorectal infection is a dual therapy of ceftriaxone 250 mg intramuscular (IM) as a single dose plus azithromycin 1 g orally as a single dose; or cefixime 400 mg orally as a single dose together with azithromycin 1 g orally as a single dose (37). Therefore, keeping in line with the same treatment formulation, this study recommends that this formulation be administered as either an oral pill or intramuscular injection as this STI is known to cause local and systemic infection (141, 142). When the nanoemulsion with the plant extracts were tested for haemolysis, there was 0% haemolytic activity, therefore indicating, this approach as a possible safe alternative for the treatment of gonorrhoea. Therefore, administration of this nanocomposite in the form of an oral pill or intramuscular injection may be safe for human prescription.

One limitation of this study lies in its relatively small number of isolates analysed. To enhance the robustness of future research, it is advisable to focus on a larger number of isolates, including a broader spectrum of gonococcal resistant strains, to facilitate more comprehensive analysis. Additionally, for a more thorough understanding, future investigations should explore a wider range of concentrations to determine the optimal treatment dosage. A notable strength of this study lies in the utilization of the WHO strains resistant to both azithromycin and ceftriaxone. Despite the broad resistance profiles of these strains, the nanoemulsion incorporating plant extracts effectively demonstrated the inhibition of growth of this pathogen in these gonococcal strains. This promising outcome suggests that the nanocomposite holds potential as an alternative treatment option for MDR gonococcal strains.

Conclusion

In conclusion, nanoemulsions paired with plant extracts (*Ocimum tenuiflorum*, *Moringa oleifera,* and *Azadirachta indica*) offer a potential alternative to antibiotics for the treatment of *N. gonorrhoeae*. This approach harnesses the antimicrobial properties of natural compounds while leveraging nanoemulsion technology to enhance their delivery and effectiveness. However, ongoing research and development are essential to validate and refine this approach for clinical use.

CHAPTER FIVE

Synthesis (Discussion, Conclusions, and Recommendations)

1. DISCUSSION

The global burden of *N. gonorrhoeae* cases remains a pressing concern, intensified by the emergence of multidrug resistance (9, 143). This bacterium, a causative agent of gonorrhoea, has exhibited a troubling trend of evading conventional antibiotic treatments, posing a significant challenge to public health systems worldwide (144). Reports indicate a surge in cases, with many regions experiencing a much higher prevalence of infections compared to what official data suggests. Contributing factors include the under-reporting of cases due to limited access to accurate diagnostic tools, as well as a substantial number of asymptomatic cases (particularly in women) that often go undetected (145). The relentless adaptability of *N. gonorrhoeae* has spurred a dire need for more efficient diagnosis, the development of a viable vaccine, and alternative treatment strategies. Given the decreasing efficacy of existing antibiotics, the exploration of novel therapeutic approaches has become imperative. Efforts are underway to explore various avenues, including the development of targeted vaccines to provide long-term protection and innovative treatment methods that can combat the pathogen's resistance mechanisms effectively.

CEACAM3 (expressed by human granulocytes), results in the uptake and destruction of Opa-expressing *N. gonorrhoeae*, hence limiting the spread of gonococci. The current study aimed to investigate the host-pathogen associations by determining the CEACAM binding patterns of *N. gonorrhoeae* isolated from symptomatic (G180) versus asymptomatic (G136) pregnant women in a South African setting. Primers targeting the conserved regions of the *opa* genes were used to identify the complete repertoire of G136, and G180 Opa proteins. The expressed

Opa proteins were visualized by Western blotting using a monoclonal antibody against *neisserial* Opa proteins. Binding assays were performed to analyse the host-binding properties of *N. gonorrhoeae*. We were able to identify, nine distinct Opa proteins from G136, and ten distinct Opa proteins from G180. We were unable to know for certain if the eleven complete distinct Opa proteins could be expressed in our study isolates since this was the first time these isolates were investigated for Opa expression. The study by Roth *et al.,* (2013) reported on the presence of all eleven distinct Opa proteins in a laboratory strain, MS11 (4). We suggest that the differences in the number of unique Opa proteins identified for MS11 when compared to the isolates in this study could be due to the following; firstly, the MS11 strain was a laboratory strain from North America and our study was conducted on clinical isolates (no prior laboratory manipulations performed on these isolates) from South African pregnant women. The binding patterns showed that for isolate G136 (asymptomatic patient), 66.7% of the Opa proteins bound to CEACAM3, 55.6% bound to CEACAM1, and 88.9% bound to CEACAM5. For isolate G180 (symptomatic patient), 30% of the Opa proteins bound to CEACAM3, 80% bound to CEACAM1, and 70% bound to CEACAM5. We found that the *N. gonorrhoeae* Opa proteins from a symptomatic patient bound at a higher frequency to CEACAM1 and 5. These CEACAMs enable the pathogen to invade the host cell and cause infections. During symptomatic disease/inflammation, the presence of neutrophils and CEACAM3-mediated phagocytosis might select against Opa-proteins binding to CEACAM3. This might be the reason, why the bacterium from the symptomatic patient has a lower percentage of CEACAM3-binding opa proteins. The *N. gonorrhoeae* Opa proteins from the asymptomatic patient bound at a higher frequency to CEACAM3. This Opa-CEACAM interaction is said to possibly limit the spread of gonococci due to granulocyte-mediated opsonin-independent phagocytosis. Currently, there are no studies conducted in Africa investigating the binding profiles of clinical isolates of *N. gonorrhoeae*, thereby limiting our study comparisons.

Investigating Opa-CEACAM interactions is a step in the right direction towards vaccinology for this pathogen. By studying the interaction between Opa proteins and CEACAM receptors, researchers can gain insights into how the pathogen adheres to and interacts with host cells, which is crucial for understanding the infection process. Identification of Vaccine Targets: CEACAM receptors are part of the host immune system and play roles in immune cell activation and regulation. By understanding how Opa proteins interact with CEACAM receptors, researchers can identify potential vaccine targets. Targeting these interactions could disrupt the pathogen's ability to adhere to and infect host cells, thus preventing infection or reducing its severity. Enhanced Immunogenicity: By targeting Opa-CEACAM interactions, vaccines may induce immune responses that specifically target these interactions, leading to enhanced immunogenicity and potentially broader protection against diverse strains of the pathogen. Overall, investigating Opa-CEACAM interactions provides valuable insights into the host-pathogen interaction, identifies potential vaccine targets, informs vaccine design, and may lead to the development of new therapeutic strategies, all of which are crucial steps in the field of vaccinology for combating infectious diseases caused by the pathogen.

The use of NAATs has been shown to provide an enhanced diagnosis of gonorrhoea (5, 21, 94). Since *opa* genes are multicopy genes that contain conserved areas and encode proteins with physiological functions, *opa* genes are therefore considered suitable target sequences for a real-time PCR amplification assay (93). In this study, we developed and evaluated the performance of an in-house *opa*-based real-time PCR assay for the detection of *N. gonorrhoeae*. Three primer sets targeting the *opa* gene of *N. gonorrhoeae* were designed and evaluated against published *opa* gene primers (comparator assay) from the Verma *et al.,* (2012) study (97). The in-house and published primers were tested against DNA from endocervical swabs, vaginal swabs, urine samples, clinical and control isolates of *N. gonorrhoeae* as well as non-gonococcal *Neisseria* isolates. Among the in-house *opa* primers, *opa* 1 performed better than *opa* 2, *opa* 3

and the comparator *opa* primer sets. The *opa* 1 assay produced positive amplification for the five WHO control strains and the six *N. gonorrhoeae* clinical isolates, whereas the comparator assay amplified 90.9% of the samples. For the endocervical DNA samples, 82.8% were amplified with the *opa* 1 assay, while the comparator assay had only amplified 27.6% of the samples. For the vaginal DNA samples, the *opa* 1 assay amplified 95.0% of the samples, whereas the comparator assay amplified 25.0% of the samples. All eleven (100%) urine DNA samples were amplified with the *opa* 1 assay, in contrast to 36.4% with the comparator assay. The *opa* 1 assay showed no cross-reactivity with non-gonococcal isolates, while cross-reactivity was observed with the comparator assay. In this study, a budget-friendly qPCR (SYBR green) test was employed. Comparable results were noted in a study by Yasmon *et al.,* (2022), where they similarly utilized a SYBR green test for identifying *N. gonorrhoeae* in clinical samples. Their SYBR green approach demonstrated high specificity, as it did not exhibit any cross-reactivity with other bacteria, viruses, fungi, or protozoa that could potentially lead to false-positive outcomes (127). The *opa* 1 assay, in the current study, also had a higher limit of detection when compared to the other assays in the current study. Acknowledged for their accuracy and sensitivity in detecting *N. gonorrhoeae* from clinical samples, single tube qPCR assays have gained recognition (93). In our local context, other diagnostic methods such as the BD MAX (Becton Dickinson), GeneXpert (Cepheid), Allplex (SeeGene), and Anyplex (SeeGene) have been utilized. While these multiplex assays are highly sensitive, their comprehensive nature makes those assays costly, as they are designed to simultaneously screen for various STIs, not solely *N. gonorrhoeae.* In contrast, the commercially available TaqMan assay, specifically targeting *N. gonorrhoeae*, presents a cost-effective option (123-125). However, SYBR green assays, which demonstrate sensitivity and specificity comparable to TaqMan assays, offer an affordable alternative. An ideal screening assay should possess specificity, sensitivity, and user-friendliness. Real-time PCR assays

enable swift, sensitive, and precise diagnoses for *N. gonorrhoeae* (7, 95). Given their cost-effectiveness, SYBR green assays hold promise for routine STI testing (82).

The escalating antibiotic resistance rates in *N. gonorrhoeae* are now of critical concern (44). There is an urgent need for alternative treatment options for infection since *N. gonorrhoeae* has developed resistance to multiple antibiotics used for treatment. In this study, plant nanoemulsions from *Ocimum tenuiflorum, Moringa oleifera*, and *Azadirachta indica* were tested for their antimicrobial properties against *N. gonorrhoeae.* Various nanoemulsion concentrations, namely 1000 μM, 100 μM, 10 μM and 1 μM, were prepared. This study also assessed the toxicity of these plant nanoemulsions using human erythrocytes. All six isolates had zones of inhibition for the 1000 μM concentration for all three nanoemulsion based plant extracts. There were no zones of inhibition observed for 100 μM, 10 μM and 1 μM nanoemulsion concentrations for five of the isolates. Isolate G176 had zones of inhibition at both 1000 μM and 100 μM concentrations for the nanoemulsions of *Ocimum tenuiflorum*. Both the WHO strains had zones of inhibition appearing at the 1000 μM concentration. For the WHO Y strain, zones of inhibition for both 1000 μM and 100 μM concentrations were observed for the nanoemulsions of *Ocimum tenuiflorum* and *Azadirachta indica.* According to the analysis, there was 0% haemolytic activity observed which suggests the non-toxic nature of the extracts. The combination of plant extracts and nanoemulsions exhibits a synergistic potential that can support the efficacy of *N. gonorrhoeae* treatment, thereby potentially mitigating the emergence of resistance. A notable advantage of this strategy lies in the use of nanoemulsions alongside plant extracts, which serve as an alternative therapeutic option, effectively curbing the selective pressure for antibiotic resistance. By reducing dependence on traditional antibiotics, this approach could decelerate the development of antibiotic-resistant strains. Moreover, medicinal plant extracts are often perceived as a safer alternative with fewer associated side effects compared to certain antibiotics. This characteristic not only enhances patient tolerance but also

minimizes the likelihood of adverse reactions. This study showed that the nanoemulsion and plant mix may potentially be used as a safer alternative to treat gonorrhoea, however, ongoing research needs to still be performed.

2. CONCLUSION

In this study, the pressing global burden of *N. gonorrhoeae*, compounded by multidrug resistance, highlighted the urgent need for more efficient diagnosis, the development of a viable vaccine, and alternative treatment strategies. Investigating Opa-CEACAM interactions is a step in the right direction towards reverse vaccinology for this pathogen. This study now adds to the growing body of knowledge on the host-receptor binding profiles of this pathogen. The study successfully developed and evaluated an in-house *opa*-based real-time PCR assay, demonstrating its superior performance compared to a published comparator assay. The in-house *opa* assay described in this study has the potential to be refined into a screening tool for detecting *N. gonorrhoeae* across various sample types. This is the first study to be performed in South Africa and therefore will add to the growing body of literature. As the global challenge of antibiotic resistance escalates, the combination of plant extracts and nanoemulsions presents a synergistic solution that could slow down the development of antibiotic-resistant strains. This study showed that at a concentration of 1000 µM, the nanocomposites of *Ocimum tenuiflorum*, *Moringa oleifera,* and *Azadirachta indica* inhibited the growth of *N. gonorrhoeae.* Furthermore, the study's findings emphasize the safer nature of medicinal plant extracts, positioning medicinal plants as a well-tolerated and potentially effective alternative in the treatment of *N. gonorrhoeae*. While these initial results suggest promise, continued research is imperative to fully elucidate the scope of application and effectiveness of this novel therapeutic approach.

3. LIMITATION AND FUTURE RECOMMENDATIONS

- Geographic Scope: The research primarily focused on a specific South African setting, which limits the broader applicability of the results to other regions or populations with different genetic backgrounds and environmental factors.
- Sample Size: The study was limited by a small sample size. To enhance the robustness of future research, it is advisable to focus on a larger number of isolates collected from a more diverse population group to draw more sound conclusions.
- The limitation of this study for the host-binding objective was that the isolates investigated were obtained from patients with possibly a localized infection only. However, we can only assume this, since samples were not collected from other sites such as the rectum or pharynx. Future studies conducted in our setting should include localized and disseminated infections.
- The in-house *opa* assay was evaluated on samples collected from our local setting only using targeted populations. There is a need for continued research focusing on the development and application of cost-effective, sensitive, and specific diagnostic tools, such as SYBR green-based assays, for routine screening of *N. gonorrhoeae*, with an emphasis on expanding the assay's applicability to diverse sample types and settings.

Acknowledging the constraints outlined above, forthcoming research endeavours will integrate these limitations into future research. However, despite these challenges, the study demonstrated the following key strengths:

Strengths of this study:

- The strength of the study was that this was the first South African study to report on the host-receptor binding profiles of *N. gonorrhoeae.* The data generated from this study may contribute to efforts towards vaccine design for this pathogen.

- We also reported on the performance an in-house *opa*-based real-time PCR assay for the detection of *N. gonorrhoeae*. The *opa*-based real-time PCR was tested across different sample types: endocervical swabs, vaginal swabs, urine samples, and clinical isolates. The current study used self-collected vaginal swab samples and despite the possible limited material on the swab, we were still able to detect a positive amplification with DNA diluted at 1:100 000 for the in-*house opa* assay.
- This study used a single tube, low-cost (cheaper than probe-based assays), and less time-consuming qPCR assay (compared to classical PCR) to detect *N. gonorrhoeae* which may have future clinical utility in settings where laboratory-based assays are too expensive to conduct.
- This study was the first to use plant nanoemulsions from *Ocimum tenuiflorum, Moringa oleifera*, and *Azadirachta indica* to test for their antimicrobial properties against *N. gonorrhoeae.* The nanoemulsions were tested against WHO strains which were resistant to both azithromycin and ceftriaxone. Despite the broad resistance profiles of these strains, the nanoemulsion incorporating plant extracts effectively demonstrated the inhibition of growth in these gonococcal strains. This promising outcome suggests that the nanocomposite holds potential as an alternative treatment option for multidrug-resistant gonococcal strains. However, future investigations should explore a wider range of concentrations to determine the optimal treatment dosage for the nanoemulsions. In addition, there will be a need for *in vivo* studies and clinical trials to evaluate the therapeutic efficacy of the nanoemulsions.

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APPENDICES

1. University of KwaZulu-Natal Biomedical Research Ethics Committee approval

UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

15 December 2022

Miss Deshanta Naicker (214502981)
School of Clinical Medicine
(College of HS)

Dear Miss Naicker,

Protocol reference number: BREC/00005104/2022
Project title: Investigating the Host-Binding Properties of Neisseria Gonorrhoeae and Newer Therapeutics against this Pathogen
Degree: PhD

EXPEDITED APPLICATION: APPROVAL LETTER

A sub-committee of the Biomedical Research Ethics Committee has considered and noted your application.

The conditions have been met and the study is given full ethics approval and may begin as from 15 December 2022. Please ensure that any outstanding site permissions are obtained and forwarded to BREC for approval before commencing research at a site.

This approval is valid for one year from 15 December 2022. To ensure uninterrupted approval of this study beyond the approval expiry date, an application for recertification must be submitted to BREC on RIG on the appropriate BREC form 2-3 months before the expiry date.

Any amendments to this study, unless urgently required to ensure safety of participants, must be approved by BREC prior to implementation.

Your acceptance of this approval denotes your compliance with South African National Research Ethics Guidelines (2015), South African National Good Clinical Practice Guidelines (2020) (if applicable) and with UKZN BREC ethics requirements as contained in the UKZN BREC Terms of Reference and Standard Operating Procedures, all available at http://research.ukzn.ac.za/Research-Ethics/Biomedical-Research-Ethics.aspx.

BREC is registered with the South African National Health Research Ethics Council (REC-290408-009). BREC has US Office for Human Research Protections (OHRP) Federal-wide Assurance (FWA 678).

The sub-committee's decision will be noted by a full Committee at its next meeting taking place on 14 February 2023.

Yours sincerely,

[redacted]

Prof D Wassenaar
Chair: Biomedical Research Ethics Committee

Biomedical Research Ethics Committee
Chair: Professor D R Wassenaar
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