

Fatty acids and monoglycerides as novel prophylaxis against gonococcal ophthalmia neonatorum

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Abstract

Neonates born to mothers with an active gonorrhoea infection can develop serious sight threatening eye infections. The causative agent, *Neisseria gonorrhoeae*, is passed to the neonate during birth, and infects the eyes of the neonate. The condition, ophthalmia neonatorum, develops 0-14 days after birth and initially presents as a painful inflammation of the eye with yellowish purulent discharge from one or both eyes. One preventative action used by some countries is the use of an ophthalmic prophylaxis which is usually an ophthalmic ointment which contains an antibiotic. This its self can cause chemical conjunctivitis.

This study evaluates the potential to use a fatty acid or fatty acid derivative as the active antimicrobial agent in an ophthalmic prophylaxis. A panel of thirty-seven initial candidates were screened for anti-gonococcal properties. Seven of this panel were selected and tested against for ocular irritation potential using *in vitro* models and anti-gonococcal properties tested further in simulated tear fluid. Finally a single candidate, monocaprin, was selected as the main drug candidate. Ophthalmic formulations of liquid and semi-solid dosage forms were made and evaluated. Liquid dosage forms performed the best in *in vitro* tested and were further evaluated in cell culture and explanted models. The cell culture model suggested that monocaprin could be used to prevent infection 90 minutes after the cell were inoculated with the bacteria. An explanted corneal infection model was used to assess the potential formulations. It was shown that the anti-gonococcal properties of the drug candidate were inhibited on the ocular surface but this this could be countered by increasing the amount of monocaprin in the formulation. The formulations containing 0.188 % and 0.25 % (w/w) monocaprin were in some cases able to totally clear inoculations of higher cell numbers on the surface of the eye.

Passage on agar plates containing monocaprin showed that increasing resistance due to genomic mutation is not likely and that existing mechanisms of fatty acid resistance did not give cross-resistance to monocaprin. However, duplicate samples passaged on monocaprin both acquired identical mutations in the *dksA* gene which may confer a small decrease in susceptibility. Also, work done on the processing of natural sources of fatty acids showed that treatment of coconut oil by use of a purified lipase or a lipase secreting yeast produced powerful anti-gonococcal substances. This could has the potential to be used in developing nations treat gonococcal and other bacterial infection.

Overall, the work in thesis demonstrates that there is potential in the use a fatty acid or fatty acid derivative, most likely monocaprin, to be used as the active antimicrobial agent in an ophthalmic

prophylaxis but more evaluation in terms of in vivo testing is required to demonstrate that the higher levels of monocaprin do not cause irritation to the eye.

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List of Abbreviations

°C	Degree Celsius
.gb	Genbank files
BCOP test	Bovine corneal opacity and permeability test
bp	Base pairs
BSA	Bovine serum albumin
CaCl ₂	Calcium chloride
CAM	Chorioallantoic Membrane
CDC	Centers for Disease Control and Prevention (CDC)
CEACAM	Carcinoembryonic antigen-related cell adhesion molecule
CFUs	Colon forming units
CHV-1	Canine herpes virus type-1
CMC	Carboxymethylcellulose
CO ₂	Carbon dioxide
DAPI	4',6-diamidino-2-phenylindole
DMAc	dimethylacetamide
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPX	Distyrene, plasticiser, dissolved in toluene-xylene mountant
E&R	Evolve and re-sequence
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene-glycol tetraacetic acid
ESC	expanded-spectrum cephalosporins
EURL-ECVAM	European Union Reference Laboratory for Alternatives to Animal Testing
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
g	Gram(s)
GC	Gonococcal
GRAS	Generally Regarded As Safe
GUM	Genitourinary medicine
h	Hour(s)
H&E	Haematoxylin and eosin stain
H ₅₀	Concentration at which lysis of 50 % of the RBCs occurs
HET-CAM	Hen's egg test chorioallanoic membrane
HPLC	High performance chromatography
HPMC	(Hydroxypropyl)methyl cellulose
HSV-1	Herpes simplex virus type-1
ICH	International Conference on Harmonisation
Ig	Immunoglobulin
IMS	Industrial methylated spirit
indels	Insertion or deletion
iNTP	Initial ribonucleotides (iNTP)
KSFM	Keratinocyte serum-free medium
LoD	Limit of detection

log D	Distribution coefficient
log P	Partition coefficient
LoQ	Limit of quantitation
LOS	Lipo-oligosaccharide
LPS	Lipopolysaccharide
LR	Log reduction
MBC	Minimum bactericidal concentration
MF	Major facilitator
MIC	Minimum inhibitory concentration
min	Minute(s)
mm	Millimetre(s)
mM	Millimolar
Moi	Multiplicity of infection
MSM	Men who have sex with men
NaCl	Sodium chloride
NaCMC	Sodium carboxymethylcellulose
NaOH	NaOH
NGS	Next generation sequencing
OD	Optical density
OECD	Organisation for Economic Cooperation and Development
Opa	Opacity-associated
ORFs	Open reading frames
PBS	Phosphate buffer saline
PBSG	PBS with 10 mM glucose
pHCEC	Primary human corneal epithelial cells
PHE	Public Health England
PID	Pelvic inflammatory disease
pKa	Dissociation constant
ppGpp	Guanosine-3',5'-(bis)pyrophosphate (ppGpp) and
PPNG	Penicillin producing gonococci
PVC	Polyvinyl chloride
PVP	Polyvinylpyrrolidone
QC	Quality control
RBC	Red blood cell
rpm	Rotation(s) per minute
RND	resistance-nodulation-cell division
RSD	Relative standard deviations
sec	Second(s)
snp files	snp files
SNPs	Single nucleotide polymorphism
STI	Sexually transmitted infection
T0	Time of infection
T25	25 cm ² flask
T75	75 cm ² flask
T90	90 Minutes post infection
Tfp	Type IV pili

TRITC	Tetramethylrhodamine
UK	United Kingdom
US	United states
USPSTF	The United States Preventive Services Task Force
UV	Ultra violet
v/v	Volume per volume
vcf files	vcf files
VIS	Visible
w/v	Weight per volume
w/w	Weight per weight
WHO	World Health Organisation
x G	Times gravity
μg	Microgram(s)
μl	Microliter(s)
μm	Micrometre(s)
μM	Micromolar
μmol	Micromole(s)

Chapter 1 - Introduction

1.1 *Neisseria gonorrhoeae* and its diseases

1.1.1 History of gonorrhoea

In 1879, a young German physician by the name of Albert Ludwig Sigismund Neisser reported that he had successfully stained a bacteria that was present in 46 smear samples [1]. Thirty-five of the smears were from men and nine from women with purulent urethritis with two of the smears coming from patients with acute ophthalmia. He described a bacterium shaped as a “figures of eight” in methyl violet stained samples. He was also successful in culturing the organism in a meat-extract gelatine medium. The genus of this bacteria would be named after him and the bacteria he isolated became *Neisseria gonorrhoeae*. The discovery was a landmark in the area of sexually transmitted infections (STIs) as so little was known at that time.

One hundred years previous to Albert Neisser, it was thought that gonorrhoea and syphilis were the same disease. Both diseases were sexually transmitted, presenting within days of sexual intercourse. It was also common for the same individual to have both diseases which could be successfully treated at the same time. However, Benjamin Bell, the first Scottish scientific surgeon, published in 1793 a detailed work of clinical presentations of the two diseases, how they differed and how to best treat them [2]. He argued that they were separate diseases and pointed particularly to the fact that syphilis could be treated with mercury while gonorrhoea could not. He also argued that gonorrhoea remained a localised infection while syphilis spread to other parts of the body:

“At present, it is only necessary to observe, that gonorrhoea consists of a discharge of puriform matter from the urethra; which, even by those who support the contrary opinion, is now admitted to be, in almost every instance, a local affection, and that it very rarely contaminates the general habit of the body.”

After Neisser was successful in culturing what he thought was the causative agent, he could not reproduce the disease by infecting healthy individuals. It now seems likely that either what he initially cultured was not *Neisseria gonorrhoeae* but a commensal species of *Neisseria* or that isolate lost virulence upon culture [3]. The first person to successfully infect a patient and replicate clinical presentation was Max Bockhart [4]. He inoculated the urethra of a male patient with creeping paralysis with a pure culture of the bacteria [5]. After three days a small amount of discharge was observed. The patient was then given half a litre of beer and the discharge increased. The patient died ten days post inoculation due to the creeping paralysis. Upon autopsy, the patient had acute

urethral inflammation. Due to the crudeness of this experiment a more refined experiment was needed to fully prove Koch's postulate.

Particular note must go to Ernst Von Bumm who also made a significant contribution. He noted that there were many bacteria that were morphologically similar to the presumed causative agent that were isolated from healthy individuals [3]. He carefully isolated the organism and infected two female patients using a platinum wire [6]. However, later attempts failed. Koch's postulate was finally clearly proven in 1891 by Ernst Wertheim; he noted the problem of the fastidious nature of the gonococcus and used serum-peptone agar to successfully culture the organism [7]. He infected five men with the bacteria and all five developed gonorrhoea.

A few years prior to Neisser's discovery, another major development was made in the area of gonorrhoea knowledge. A German gynaecologist, Emil Noeggerath proposed in 1876 that a "latent gonorrhoea" state existed in women [8]. He initially presented his findings to the American Gynecological Society in New York City and was met with sharp criticism from attendees [9]. His theory was heavily debated for a few years. However, his latent gonorrhoea theory was later more readily accepted, especially in Europe. Bell, in his earlier account of gonorrhoea had commented that he didn't know why gonorrhoea commonly affected men more than women. It is now known that asymptomatic infections are common, especially in women.

Ocular inflammation in gonorrhoea patients has been common in the past. This gonorrhoeal ophthalmia was thought by some to be due to dissemination of the infection from the genitalia to the eyes [10]. Others, such as Jean Astruc, a physician to King Louis XV, believed it was spread from direct contact [11]. He noted that a young man who had acute symptoms of gonorrhoea, had washed his eyes daily in his own fresh urine as he perceived that this would improve his sight [12]. Needless to say he developed gonorrhoeal ophthalmia and had a purulent discharge from his eyes.

1.1.2 Ophthalmia neonatorum and its history

Ophthalmia neonatorum is used to describe conjunctivitis during the first 28 days of life, also referred to as neonatal conjunctivitis [13]. More commonly it is used to describe conjunctivitis that is caused by transfer of infectious agents from the mother to the eyes of the neonate during passage through the birth canal. It causes a painful inflammation of the eye with yellowish purulent discharge (See Figure 1.1). If left untreated or if standard treatment fails, it can cause corneal scarring and corneal perforation which can lead to permanent blindness. Before the 20th century, ophthalmia neonatorum was common and severe infections would lead to blindness [14]. In fact, it is thought that this was the cause of about a quarter of all blindness. However, this trend changed in the latter two decades of the 19th century [14].

A German doctor named Carl Credé, who was chair of obstetrics in Leipzig, came up with a method that would radically reduce the number of cases of ophthalmia neonatorum. In 1874 the incidence rate in his maternity wards was 13.6%. Many of these had to be treated with silver nitrate which had been commonly used since the 1830's. However, this treatment did not always prevent blindness in cases of severe infections. What Credé proposed in his hospital was to wash and thoroughly clean the closed eyes of the new born and then treat with a single dose of 2 % silver nitrate shortly after birth before the onset of ophthalmia neonatorum. After enforcement of this post-natal practice, the incidence rate dropped to 0.15 %.

Today, the two most common aetiological agents of ophthalmia neonatorum are *N. gonorrhoeae* and *Chlamydia trachomatis*. However, it is more likely that *N. gonorrhoeae* was the causative agent in Credé's hospital due its sensitivity to silver nitrate. Gonococcal ophthalmia neonatorum usually has a faster onset than *C. trachomatis* ophthalmia neonatorum and is usually more aggressive [15].



Figure 1.1 Picture of a typical ophthalmia neonatorum case. The discharge from the eye dries to form a yellow crust around the eye. This example was taken from the digital medicine website: <http://digitalmedicine.com.np/>.

1.1.3 Ophthalmia neonatorum prophylaxis in the antibiotic age

Although Credé prophylaxis dramatically reduced the incidence rates of ophthalmia neonatorum for many years, it was later criticised by some for causing chemical conjunctivitis [16]. It caused chemical conjunctivitis in 90 % of infants treated, 3-6 hours after administration [17], although the majority of these cases would clear up after 24 hours. In addition, there were questions about its effectiveness against *C. trachomatis* [18, 19]. Figure 1.2.A shows historical records of the number of cases of ophthalmia neonatorum in England and Wales from 1914 to 1981. These are all cases of ophthalmia neonatorum and not limited to gonococcal ophthalmia neonatorum. The number of cases of ophthalmia neonatorum showed a steady decrease from 1920 onwards. While the use of silver nitrate was justifiable in the times of a high prevalence of gonorrhoea and high incidence rate of ophthalmia neonatorum, the severity of the treatment was less justifiable in times of low prevalence. The use of silver nitrate for prophylactic treatment of ophthalmia neonatorum was discontinued in UK hospitals in the mid-1950s [20, 21]. Despite this, the incident rate of ophthalmia neonatorum continued to fall.

Silver nitrite, despite being a relatively good prophylactic drug, had limited success as a treatment once ophthalmia neonatorum had developed. The potential for antibiotics for use in prevention and treatment of ophthalmia neonatorum was realised early. In August 1930, a doctor working at Sheffield Royal Infirmary, Cecil Paine, used a crude extract from the fungus *Penicillium notatum* to treat a case of ophthalmia neonatorum in a three-week-old male baby [22]. Although certain details are missing, the eyes were described to be clean seven days after initial treatment was started. After the first large-scale release of antibiotics in the mid-1940s, a solution of benzylpenicillin was trialled in 1947 as a possible replacement to silver nitrate [23]. Although the trial was originally successful, it required three daily dosages, was not effective against *C. trachomatis* and could possibly cause complications in penicillin sensitive individuals. Sulphonamides were later trialled at the beginning of the 1950's but this was no more effective than silver nitrate. At about the same time, tetracycline's were also used in trials [24].

After some time in development, it was found that a single dose of 1 % tetracycline ointment was effective to prevent development of ophthalmia neonatorum. The ointment performed better than silver nitrate with an estimated risk of 0.012 % of treatment failure compared with 0.063 % for silver nitrate [25]. Although tetracycline is effective against *C. trachomatis in vitro*, there were concerns that the ointment was less effective at preventing chlamydial ophthalmia neonatorum [18]. However, use of the 1 % tetracycline ointment was included in the Centers for Disease Control and Prevention (CDC) sexually transmitted diseases treatment guidelines of 1982 and last appeared in 2006; recommendation of the treatment was absent from the 2010 guidelines [26-28].

In the 1960s, use of 0.5 % erythromycin appeared. This was identified as having a lower rate of treatment failure than that of silver nitrate [29]. It was also shown to be effective against chlamydial ophthalmia neonatorum [30]. Like the tetracycline ointment, it was included in the 1982 CDCs sexually transmitted diseases treatment guidelines and now is the only recommended treatment in the 2015 treatment guidelines [26, 31].

1.1.4 *Neisseria gonorrhoeae*

N. gonorrhoeae is a Gram negative cocci bacteria which are kidney bean shaped and are usually arranged as diplococci (see Figure 1.3.A). They are catalase and oxidase positive [32]. They are classified as being non-motile as they do not have flagella but possess type IV pili which can cause a twitching motility. The pili are also important for initial attachment to host cells (see section 1.1.10). *N. gonorrhoeae* is an obligate human pathogen as they are not found in any other animal or found surviving in the environment. They are relatively fastidious, requiring excess sugars and iron to maintain growth. One of the most commonly used media for isolation is Thayer-Martin agar [33]. On this medium they form smooth, round, moist, uniform grey/brown colonies (see Figure 1.3.B) [32]. This medium is made from pancreatic digest of casein, selected meat peptone and corn starch. It also contains heat-treated haemoglobin which gives the medium a brown appearance. Haemoglobin-free media are also available. Thayer-Martin agar is supplemented with vancomycin, colistin and nystatin (VPN); with the optional addition of trimethoprim, for primary isolation [34, 35]. Unlike other bacteria, gonococci are often more difficult to culture in liquid medium than with solid agar medium. However, liquid media developed more recently has shown to support growth [36, 37]. *N. gonorrhoeae* is capnophilic requiring an increased amount of CO₂ to grow [38]. Optimal conditions for growth are is 36 °C ±1 °C with 3% to 7% CO₂ [39]. The addition of 0.1 % sodium bicarbonate to solid medium has been shown to be able replace the need for additional CO₂ gas with some isolates [40, 41]. The cells contain autolysins which shorten the cells viability when stationary phase is reached [42, 43].

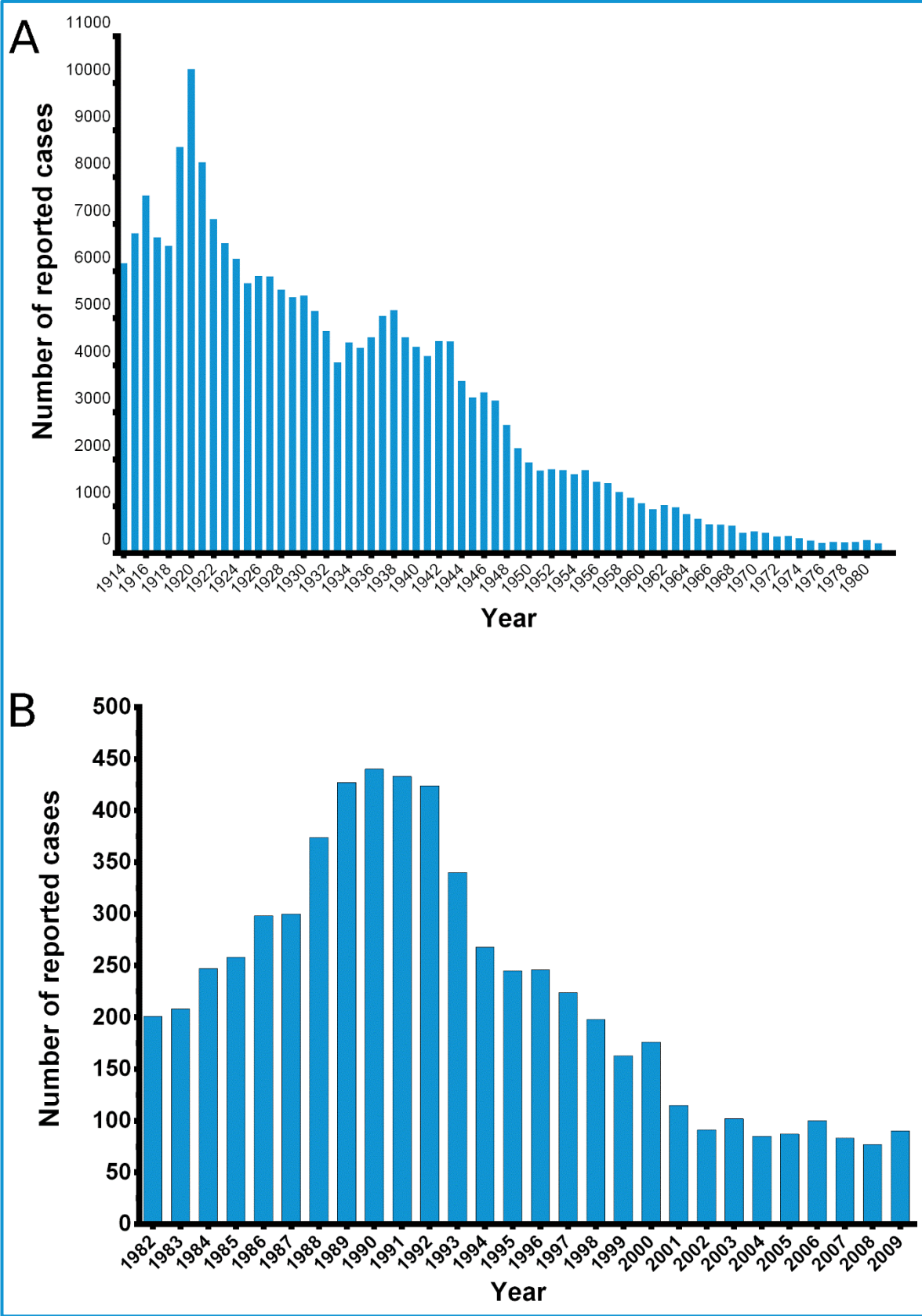


Figure 1.2 Reported cases of ophthalmia neonatorum in England and Wales. (A) Number of reported between 1914 and 1981. (B) Number of cases between 1982 and 2009. Ophthalmia neonatorum ceased to be a notifiable disease after 6th April 2010 [44, 45].

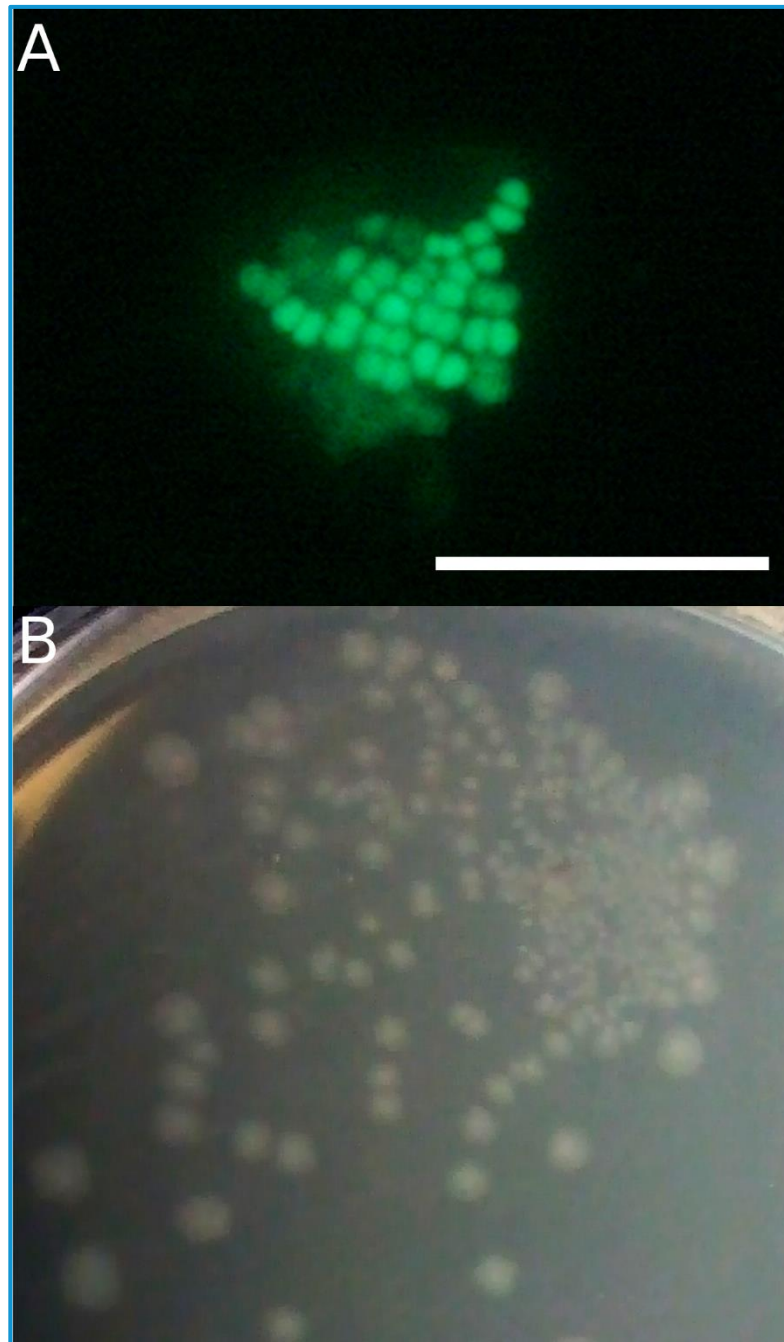


Figure 1.3 Images of *Neisseria gonorrhoeae*. (A) SYTO 9 stained gonococcal cells attached to a human corneal epithelial cell. Notice the kidney bean shape of the cells which prefer to cluster into pairs. The white bar represents 10 μm . (B) Colonies of *N. gonorrhoeae* growing on a GC agar plate after 48 hours incubation. The colonies appear slightly opaque and can be difficult to spot under certain conditions. The larger colonies in this picture are up to 3 mm in diameter. Both of these pictures were produced from work in Chapter 4.

1.1.5 Gonorrhoea and other *N. gonorrhoeae* infections

Although *N. gonorrhoeae* can infect any human mucosal layer, it most commonly infects the adult urogenital tract [46]. In men, it can cause urethritis with symptoms which may include a white, yellow or green discharge from the penis, pain or a burning sensation when urinating (dysuria) and inflammation of the foreskin [47]. In rare cases it can also cause epididymitis, a pain or tenderness of the epididymis, a curved structure at the back of the testicles. If left untreated it can lead to a possible reduction in fertility. Symptoms usually develop 2-5 days post infection but can develop as long as 30 day after infection [48]. Asymptomatic infections occurs in about 10 % of men [48].

In women, symptoms can include inflammation of the cervix (cervicitis) with a thin or watery, green or yellow vaginal discharge, intermenstrual bleeding, itchiness (pruritus), and dysuria [47]. If untreated, the infection can spread to the upper genital tract and cause pelvic inflammatory disease (PID). Symptoms of PID may include: abdominal pain; abnormal discharge; intermenstrual bleeding; postcoital bleeding; fever; lower back pain; and in some cases nausea [49]. PID is estimated to occur in 10-20 % cases of untreated gonorrhoea [50]. PID can lead to long-term pelvic pain, ectopic pregnancies and infertility. The infertility is a result of scarred and narrowed fallopian tubes. Asymptomatic infections are far more common in females than males. The exact percentage of infections which are asymptomatic vary depending upon the population which is sampled. Between 2001 and 2006 in England and Wales more than 50% of women that attended selected genitourinary medicine (GUM) clinics had no symptoms of *N. gonorrhoeae* infection. This is likely to be underestimate as the populations would be biased by people visiting the clinics due to showing symptoms. In another study, 66.7 % to 100 % women from China, India, Peru, Russia, and Zimbabwe reported no symptoms when screened for *N. gonorrhoeae* infection [51]. However, as this study used certain social groups within the general population, it would be biased toward asymptomatic infections as those with symptom would likely seek medical attention. Therefore the actually rate of asymptomatic infections is probably somewhere between 50-100 %.

Anorectal infection can occur in both males and females but are far more prevalent in males. Symptoms include anal pruritus, rectal pain, mucopurulent discharge, and feeling of constantly needing to pass stools (tenesmus). Infection are asymptomatic in 50 % of individuals [48]. Pharyngeal infections are asymptomatic in more than 90 % cases [52]. They can act as an important route of transmission [53].

Adult ocular gonococcal infections are rare but in recent years are presenting more commonly in young adults, especially in males [54, 55]. Clinical signs can include hyperaemia, eye lid swelling and purulent discharge. Infection of the cornea can lead to keratitis, ulceration, descemetocoele

formation and ultimately perforation. Corneal perforation risks blindness but sight may be saved with corneal grafting (keratoplasty) [56, 57]. As these infections are relatively rare compared to other causes of eye infections they are often incorrectly treated [57].

Disseminated gonococcal infection can include acute septic arthritis. This condition is rare in Europe but more common in the US [58]. Ankles, knees, wrists, and hands are the common sites which are affected [55].

1.1.6 Prevalence and incident rates of gonorrhoea

Gonorrhoea is a global problem with 106.1 million cases reported to the World Health Organisation (WHO) in 2008 (see Figure 1.4) [59]. This makes it the most common bacterial sexually transmitted infection (STI) globally, just ahead of *C. trachomatis*. Approximately 40 % of all reported cases were from the Western Pacific WHO reporting region. This region comprises of 37 countries from developed countries such as Australia, Japan, New Zealand, the Republic of Korea, China and Singapore to developing nations such as Viet Nam, Malaysia, and the Philippines [60]. Despite the high number of cases of this reporting region, it doesn't have the highest incidence rate. The African WHO reporting region has the highest incident rate with over 100 cases per 1,000 population. In simple terms, this means that one in ten people will have a gonococcal infection in a year. The African WHO reporting region is made up of 47 countries. Roughly, it consists of all countries on the African continent excluding Morocco, Tunisia, Libya, Egypt, Sudan, and Somalia [61]. In all reporting regions apart from the European region, there are more male cases than female. The reason why this trend is not seen in the European region is unknown but may be due to some of these countries more actively testing female populations.

In England, gonorrhoea is the second most common bacterial STI, with the most common being *C. trachomatis* infections [62]. In 2014, there were 34,958 reported cases to Public Health England. That is an increase of 98 % from 2005 and is the sixth year in a row that there has been an increase in the number cases (see Figure 1.5). The most common age groups for reported gonorrhoea cases is 25-34 year old for males and 20-24 year old females (see Figure 1.6.A).

In England in 2014, 53.23 % of all cases were found in males who are described as "men who have sex with men" (MSM), see Figure 1.6.B. This group contains males who have sex with other males, regardless of whether or not they have sex with women or do not identify with being homosexual or bisexual [63]. Unfortunately, there are no estimates for the size of this population so the incidence rate can't be calculated. However, as MSM are classed as a social minority [63], the prevalence is therefore going to be greater in this population than in either of the heterosexual populations. The level of prevalence in this population highlights the problem if antimicrobial

resistance appears in this group. Interestingly the number of cases in heterosexual males and females are about equal in the England and Wales.

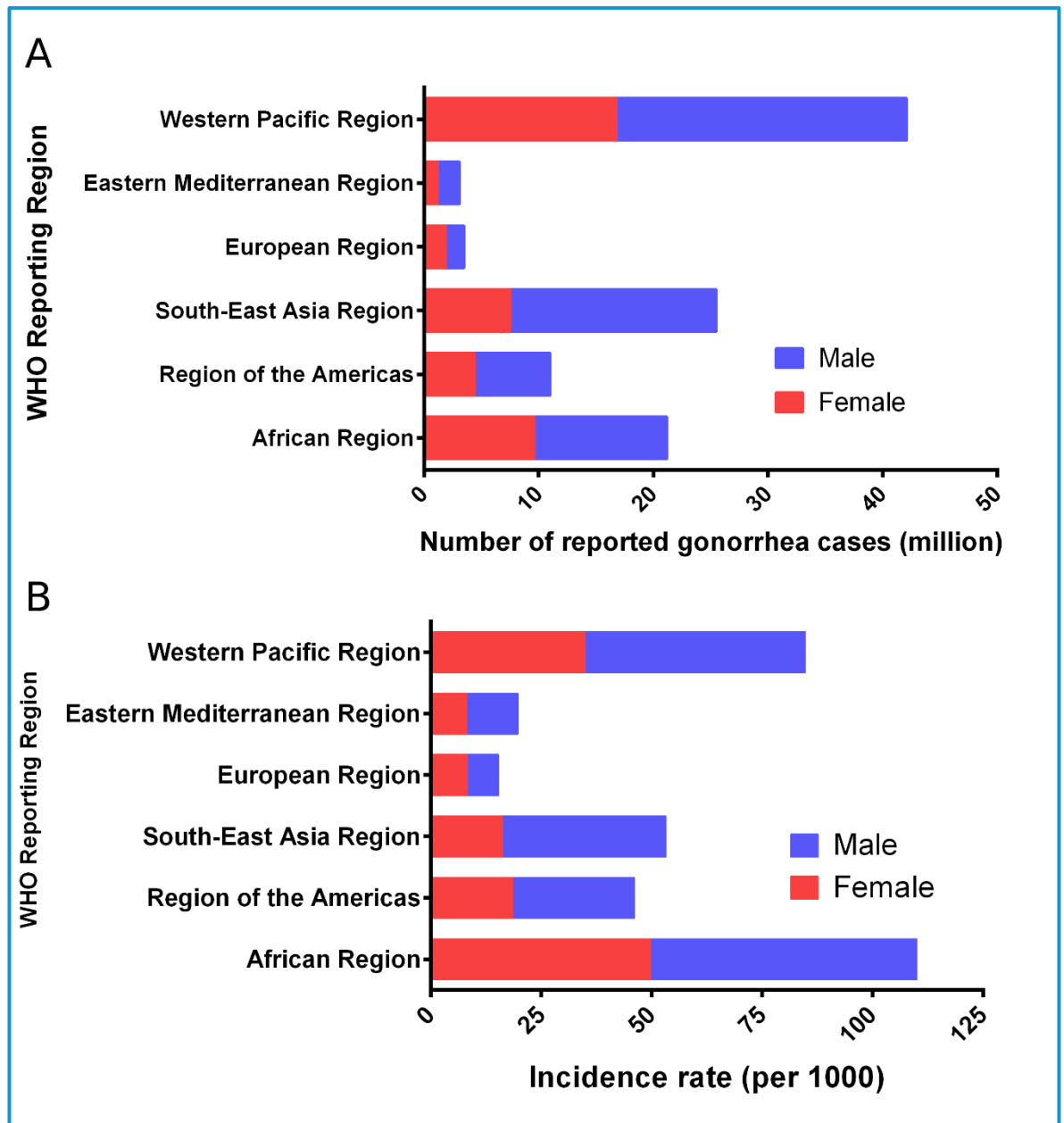


Figure 1.4 Global gonorrhoea cases reported to the World Health Organisation in 2008 categorised into reporting regions [59]. (A) Number of cases reported for each region. (B) Incidence rate, number of cases per 1000 people in the population. The Western Pacific region reported the highest number of cases with over 40,000,000 cases in 2008. However, the African Region has the greatest number of cases for its population size. Unusually, the European region has a greater number of female cases than male cases

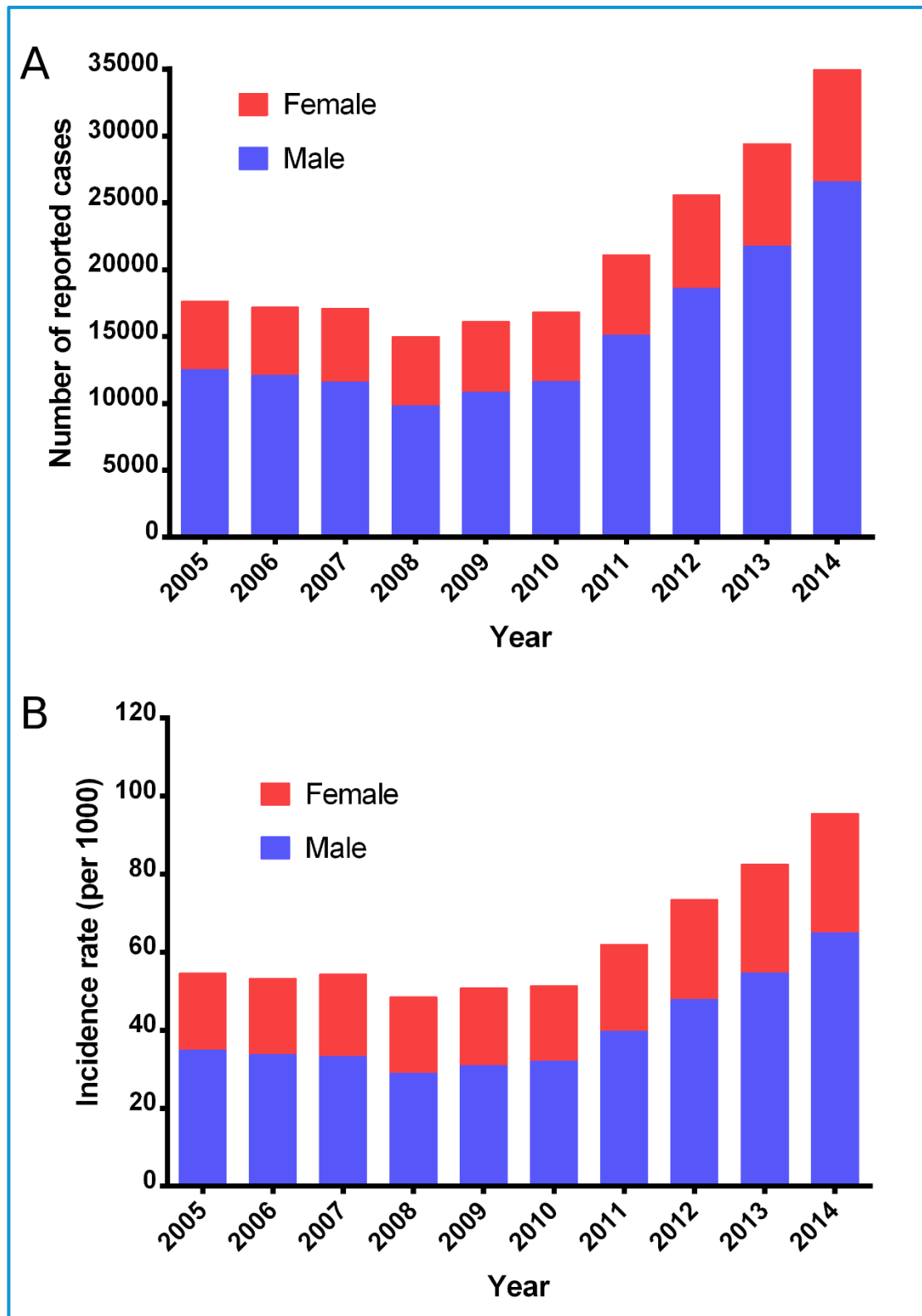


Figure 1.5 Reported cases of gonorrhoea in England between 2005 and 2014 [64]. (A) Number of cases reported. (B) Incidence rate, number of cases per 1000 people in the population.

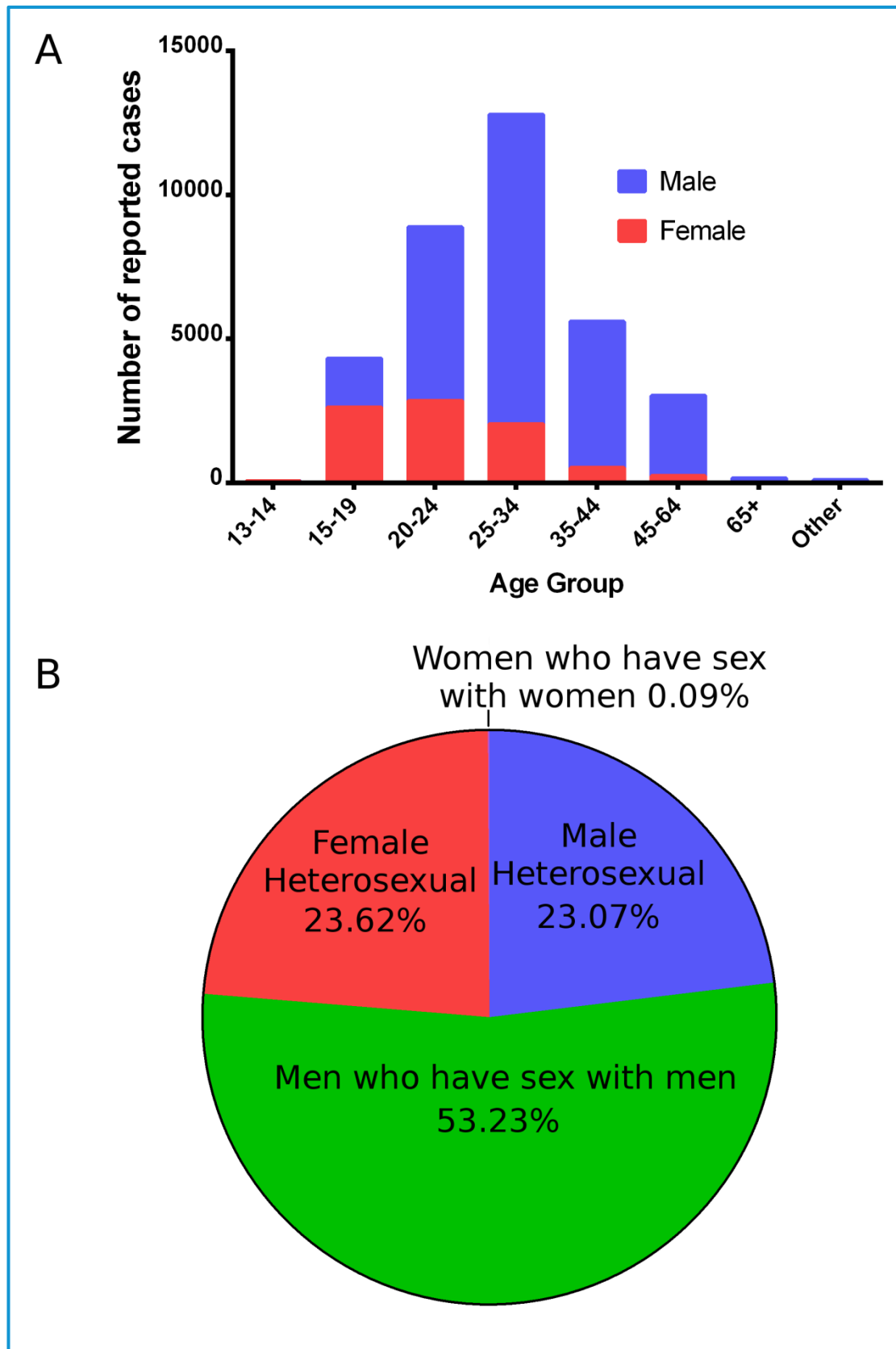


Figure 1.6 Breakdown of reported cases in England in 2014 [65]. (A) Breakdown of number of cases for different age groups for males and females. Note that the greatest number of cases for males is 25-34 and 20-24 for females. (B) Breakdown of cases into sexual orientation. By far, the most cases occurred in men who have sex with men

1.1.7 Antimicrobial resistance

N. gonorrhoeae infections are at a great risk of becoming untreatable [66]. Figure 1.7 shows a timeline of the recommended antimicrobials used for treatment and the development of resistance mechanisms. Resistance to antimicrobials started with sulphonamides which were one of the first antimicrobial used for treatment of gonorrhoea [67]. Sulfanilamide and sulfapyridine were commonly used from the early 1940s. By the end of the decade, over 90 % of isolates were resistant [68]. Penicillin took over from sulphonamides as the best treatment for gonococcal infections. However, low-level resistance due to chromosomal mutations was soon seen leading to a continuous increase in dose. In hindsight, the initial low dosage and incremental increases helped the bacteria develop resistance. Outbreaks would occur in the 1960s of increased resistant isolates and in 1976 plasmid mediated resistance by production a β -lactamase was seen [69]. The main plasmid, *bla*_{TEM-1} encoded a TEM-1-type β -lactamase that would hydrolyse the β -lactam ring of penicillins and cephalosporins. These isolates would be termed penicillin producing gonococci (PPNG). Use of penicillin continued and in 1980 highly resistant non-PPNG were reported [70].

However good β -lactam are at any period in time to treat *N. gonorrhoeae* infections, an alternative is always required for patients with a sensitivity to them. For a long time, since the late 1940s, tetracyclines provided this alternative. However, chromosomal resistance started to develop and by the mid-1980s *tetM* determinants which resulted in high-level resistance were reported [71]. After this, their use was not recommended.

Spectinomycin was another alternative to penicillin which was initially sold as a specific treatment for gonorrhoea in the 1960s. Chromosomal mutation in the 16S rRNA gene resulted in decreased susceptibility [72]. Also, mutations in *rpsE* gene encoding the ribosomal protein S5 also confers a decreased susceptibility [73]. Spectinomycin also has a low efficacy in the treatment of pharyngeal gonorrhoea [74]. Use of spectinomycin as a first-line treatment was abandoned in the 1980s. With the failure of tetracycline and spectinomycin, the new generation of broad spectrum fluoroquinolones quickly filled a gap in the mid to late 80s. Initially they were very effective when they were first introduced. However, increasing resistance in *N. gonorrhoeae* to ciprofloxacin was first reported in 1990 in North America [75, 76]. The decreased susceptibility was due to mutations in the *gyrA* and *parC* genes [77, 78]. Ten years later the CDC no longer recommended fluoroquinolones for treatment in persons who acquired their infections in Asia or the Pacific Islands due to high prevalence of resistance in these regions [79-81]. In 2002, the CDC no longer recommended fluoroquinolones for MSM and by 2006, they no longer recommended fluoroquinolones for treatment of gonococcal infections [79]. The peak prevalence of fluoroquinolones resistant gonococci in 2002 was 52.2 %, by 2006 it had decreased 9.1 % [82].

Unfortunately, this prevalence is still too high to reintroduce its use in first-line treatments, especially considering the ease at which resistance is acquired.

Macrolides have had a little more success than some of the other antimicrobials talked about so far. The macrolides erythromycin and azithromycin are still being used today for the treatment of *N. gonorrhoeae* infections. Erythromycin, as stated previously is now the only recommended prophylactic treatment for ophthalmia neonatorum. It has been reported that azithromycin is a better antimicrobial agent than erythromycin for gonorrhoeae as it is able to treat pharyngeal gonorrhoea, which are normally asymptomatic [83]. Hence, azithromycin is the preferred macrolide for treatment. Resistance to azithromycin has been reported in North America and Europe [84]. However, the use of azithromycin is still used in combination therapy [85]. Its continued use has resulted in high prevalence of resistant isolates in areas of China [86] and there have been recent outbreaks such those in Leeds, England of high-level resistant isolates [87, 88]. This outbreak in Leeds that occurred in September 2015 consisted of 18 heterosexual cases. However, the high-level azithromycin resistance has since been seen in the Midlands and in London. It is now thought that it is in MSM population [89].

Today, the class of antimicrobial most widely used to treat gonococcal infections are cephalosporins. The third generation cephalosporins are effective against PPNG as they are not prone to hydrolysis from the β -lactamase. This class of cephalosporins are often referred to as expanded-spectrum cephalosporins (ESC). This series of ESC carries a negligible risk of cross allergy in patients with penicillin allergies [90]. The most commonly ESCs used to treat gonococcal infections are ceftriaxone which is taken by injection and cefixime which is taken orally. Cefuroxime, which is an oral (or injectable) second generation cephalosporins, is also sometimes used in Europe but is a poor substitute [91]. Ceftriaxone is now the preferred ESC as lower rates of resistance have been seen. Since 2012, the European guidelines have recommended dual treatment of ceftriaxone 500 mg (intramuscular injection) and azithromycin 2 g (oral) for treatment of uncomplicated *Neisseria gonorrhoeae* infections of the urethra, cervix, rectum and pharynx, when the antimicrobial sensitivity is unknown [92].

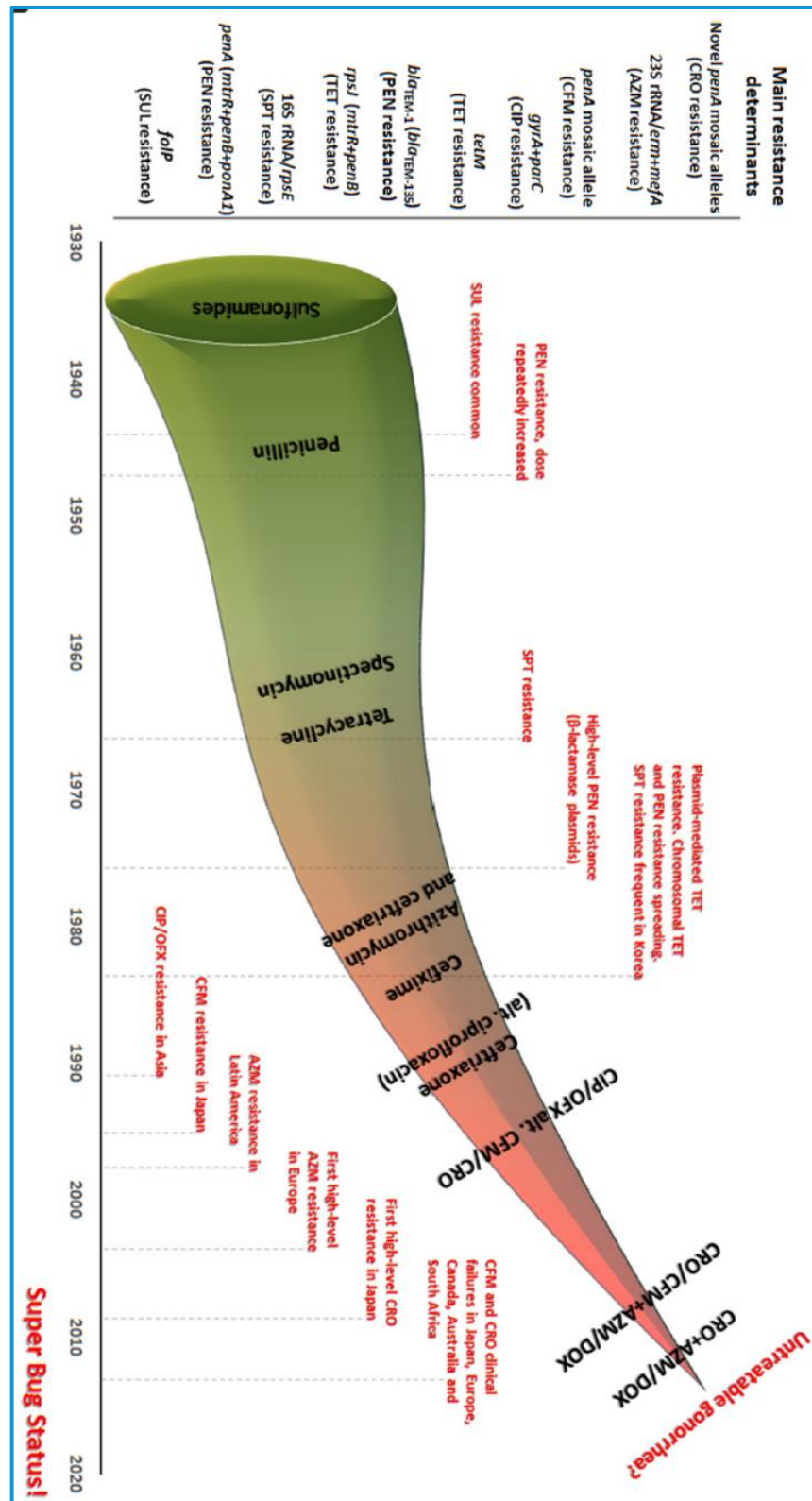


Figure 1.7 History of discovered and recommended antimicrobials and evolution of resistance in *Neisseria gonorrhoeae* [69]. Abbreviations used in figure SUL, sulfonamides; PEN, penicillin; SPT, spectinomycin; TET, tetracycline; CIP, ciprofloxacin; OFX, ofloxacin; CFM, cefixime; CRO, ceftriaxone; AZM, azithromycin; DOX, doxycycline.

1.1.8 Control measures of ophthalmia neonatorum

Prevention of ophthalmia neonatorum can be controlled in three ways: selective treatment of expectant mothers via prenatal screening; blanket prenatal antimicrobial treatment of all expectant mothers; or postnatal application of prophylaxis to all neonates within the first few hours after birth.

Postnatal prophylaxis can be in the form of an eye drop or eye ointment. Current recommended prophylaxis is 0.5 % erythromycin ophthalmic ointment. This is usually given in the first hour after birth. In the United Kingdom there has been no prophylaxis since the 1950s and currently prenatal screening via assessment of risk factors is used [20]. Risk factors include having had known contact with someone with gonorrhoea, having unprotected sex with a new partner(s), recent multiple sexual partners, living in an urban area, or having had a sexual partner from an urban area with a known high prevalence [93]. If the mother is deemed as being at risk then testing for STIs is recommended. However, the expectant mother still has the risk of becoming reinfected after treatment. It is therefore recommended that the mother be re-screened after 28 weeks or at the time of delivery [94].

The use of prophylactic treatment divides medical opinion. The United States Preventive Services Task Force (USPSTF) has graded the use of an ocular prophylaxis for prevention of ophthalmia neonatorum as “Grade A” which means that there is high certainty that the net benefit is substantial [95, 96]. They recommend that all newborns be given a prophylaxis within 24 hour after birth. However, meta-analysis of published data finds that the failure rate of prophylaxis treatment was too great to use in developed countries as the prevalence of disease was too low [15]. Prophylactic treatment is given in Canada where it is mandatory in some provinces and optional in others. The fact that it is mandatory in some provinces, such as Ontario, has been criticised by some health workers as parents that refuse prophylactic treatment are reported to the local children’s aid society [97]. Other concerns for the prophylactic treatment is that it could interrupt mother-infant bonding by blurring the vision of neonate [94]. During a shortage of erythromycin ointment in the United States in 2009, gentamicin ointment was used as prophylactic treatment but resulted in reports of severe ocular reactions [98, 99]. All these factors helps to undermine the use of prophylactic treatment in developed nations.

Table 1.1 Main virulence factors of *Neisseria gonorrhoeae* [55].

Virulence Factor	Host Cell Interactions	Interaction Results	
Pili	Pilus PilC protein subunit binds to CD46* receptors that are present on most human cells [100]	Calcium [101] release and cytoskeletal rearrangements that lead to elongation of microvilli which embrace the microbe, facilitating cell entry.	Provides twitching motility function. Involved in DNA transformation. Undergoes phase variation. May play a role in resistance to granulocyte phagocytosis [102]
Opacity-associated proteins (Opa; each strain can make at least 11 different types, but express no more than five at a time)	Most interact with the CD66 (CEACAM) family of receptors on epithelial cells and neutrophils.	Mediates non-opsonic phagocytosis by neutrophils that is distinct from antibody- and complement-mediated phagocytosis [102, 103]	Undergo antigenic and phase variations. Sequesters pyruvate kinase while within host cells to acquire pyruvate for bacterial metabolic needs [103]
Porin protein (most common outer membrane protein; exists as a trimer in the membrane)	Translocates into the host cell membrane. Able to bind with C4b-binding protein. [104]	Translocation permits a rapid Ca ²⁺ influx into the host cell from the external environment [105]. Disrupts neutrophil degranulation, oxidative burst, and phagosome maturation [106, 107]. Down-regulation of complement activation.	Can induce apoptosis in epithelial cells and neutrophils <i>in vitro</i> . [105] Located in the cell membrane in close proximity to lipopolysaccharides and reduction modifiable proteins. Allows small molecules to pass through the bacterial outer cell membrane.
Iron-scavenging system of proteins [108, 109]	Scavenges iron from transferrin, lactoferrin, and hemoglobin	Allows microbe to maintain metabolic processes in the face of the low free iron environment created by early innate immune responses.	Gonococci do not produce any siderophores. Transferrin- and lactoferrin-binding proteins are required in order for gonococci to cause experimental urethritis [110].
IgA protease	Cleaves IgA, at the hinge region. Cleaves host cell lysosome-associated membrane protein, which is involved in phagosome compartmentalization [111]	May help to evade host IgA at the mucosal surface. Most important function may be to enhance intracellular survival within host cell phagosomes.	
Lipo-oligosaccharide (LOS; up to six variants expressed by a single strain) [112]	LOS moieties mimic human glycosphingolipids; can bind to asialoglycoprotein receptor (ASGP-R). Induces cytokine production (TNF- α , IL-1 β , IL-6, IL-8) from urethral epithelial cells [113].	Can activate the classic complement pathway. Can mediate host defensin-enhanced adherence to epithelial cells [114] ASGP-R interaction mediates endocytosis (internalization of the microbe into epithelial cells).	Consists of a lipid A moiety and a core polysaccharide; lacks the long polymeric sugars lipopolysaccharides. Predominately short LOS appear more sensitive to killing by human serum but also more able to invade eukaryotic cells.

Cell wall peptidoglycan (shed as membrane fragments during exponential growth)		Activation of complement occurs. Modulation of mononuclear cell proliferation	Cause damage to fallopian tube mucosal cells in organ culture [115].
Reduction modifiable protein (Rmp)	Generates host antibodies against itself.	Antibodies that bind to Rmp epitopes block the bactericidal effect of complement-fixing IgM antibodies that recognize LOS.	Women with preexisting anti-Rmp antibodies appear more susceptible to infection than those without such antibodies [116].
Ribosomal protein L12	Binds to lutropin receptors in the upper female genital tract.	Facilitates ascending infection in females.	Ribosomal protein L12 mimics the structure of human chorionic gonadotropin, which is the natural ligand for lutropin receptor [117, 118].

1.1.9 Virulence factors

N. gonorrhoea has been studied for many years and some of its virulence factors have been identified and are listed in Table 1.1. As it is an obligate human pathogen it has developed complex systems for survival inside the human body. These range from complex avoidance of the immune system via antigen variation [103] to acquiring iron [108, 109].

1.1.10 Attachment and internalisation

N. gonorrhoeae is an intracellular pathogen. It possess a Type IV pili (Tfp) which is a retractable appendage known to mediate surface attachment [119]. The retractive force of the *N. gonorrhoeae* Tfp is estimated to be 50–100 piconewtons [120]. However, the pili usually form bundles of 8-10 pili which are capable of retracting up to 1000 piconewtons sustainably [121]. The force generated by Tfp is one of the strongest microscale elements known to date, and is high enough to induce cytoskeletal rearrangements in the host cell [120, 121]. Tfp are made from filamentous polymers of the PilE protein with a diameter of 60-80 Å and several micrometers in length. The PilE expression locus (*pilE*), undergoes phase and antigenic variation by a range of different methods [122]. One of the most productive methods of antigenic variation of PilE is RecA-dependant recombination that replaces the *pilE* sequence with the sequence other silent *pil* loci (*pilS*) [123]. The amount of antigenic variation, which occurs at a rate of 4×10^{-3} events per cell per generation, can also result in a *pilE* variant defective in fibre formation leading to a non-piliated phenotype [124]. The Tfp also helps with microcolony formation [119]. This can occur on the surface of the host cell and the Tfp triggers what's termed a cortical plaque. This is defined as rearrangements in the cortical cytoskeleton and the plasma membrane and helps cluster transmembrane signalling proteins, actin microfilaments and ezrin, which act as receptors for other attachment proteins on the gonococcus [125].

Once *N. gonorrhoeae* has attached to the host cells it can then initiate more intimate binding with surface attachment proteins, as seen in Figure 1.8.A. One such class of proteins well described in the literature is the opacity-associated (Opa) proteins. *E. coli* expressing an Opa protein has been shown to attach and invade cultured human epithelial cells while the non-Opa expressing control was not [126]. *N. gonorrhoeae* possess approximately 11 Opa genes [127]. A bacterial population can contain cells expressing none, one or multiple Opa proteins, with varying levels of expression [128]. This series of proteins have different attachment targets on the host cell and the Opa can be classified by their targets such as Opa_{HS} which bind to heparansulphate proteoglycan (HSPG), and Opa_{CEA}, the most abundant group, which binds to the carcinoembryonic antigen-related cell adhesion molecule (CEACAM) protein family [129]. From this family, only CEACAM1, CEACAM3, CEA (or CEACAM5) and CEACAM6 have been shown to bind to neisserial Opa proteins [130]. Opa_{CEA}-CEACAM interactions can also have an added benefit of bypassing the exfoliation response of

epithelial cells which can be induced by infection [131]. Different host cells types express different CEACAM proteins, for example, CEACAM1, CEACAM5, CEACAM6 can be expressed by epithelial cells but CEACAM3 is normally found expressed in neutrophils [128]. The Opa proteins all have a similar structure, which contains eight antiparallel β strands which form a barrel, this structure sits in the outer membrane, and four extracellular loops extend out from the cell surface (see Figure 1.8.B) [132]. The loops contain variable regions which have specificities for certain host receptors [128]. These variable regions are created by several mechanisms. The main mechanism is recombination between the different *opa* loci. As well as the recombination occurring within the bacteria, it can also occur between organisms [133].

It is clear that both the Tfp and Opa proteins are virulence factors as they are required for infection. Both are highly variable and that is one of the reasons why immunity is not possible with gonococcal infections [134]. Persons infected with gonorrhoea are not protected from reinfection, even when the reinfection introduces the same strain of bacteria back into the host.

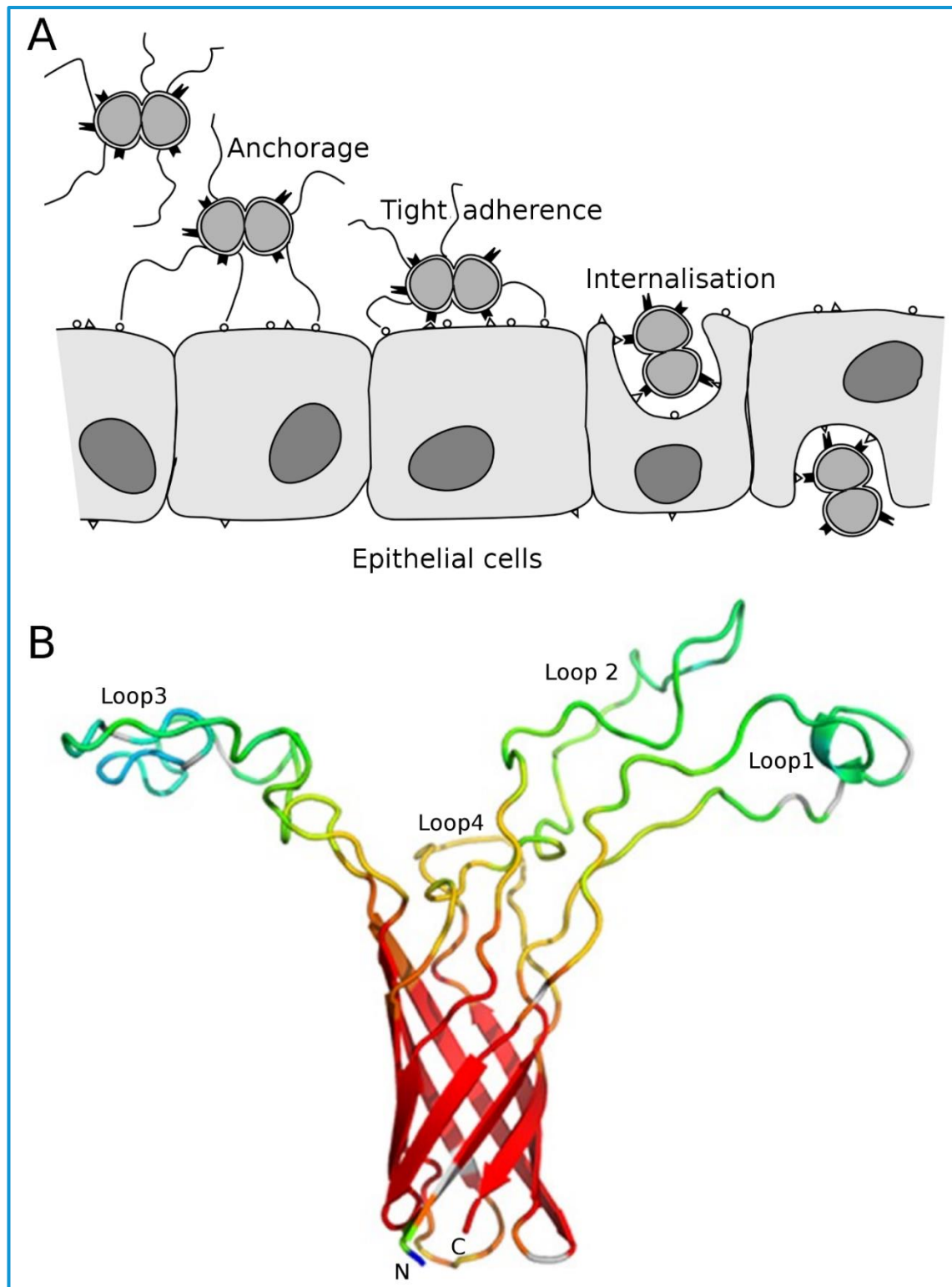


Figure 1.8 How gonococci enter cells. (A) Simplistic image showing the stages used by *N. gonorrhoeae* to enter cells [103]. Type IV pili extending out from the surface of the bacteria allow them to anchor onto the host cells. Retraction of pili allows close interaction between the bacterial and host cell to occur with tight adherence being mediated by Opa proteins. These interactions cause a cytoskeletal change that internalises the gonococcus into the host cell. (B) Graphical representation of the typical Opa 3D structure [132]. The beta-barrel, in red, sits within the outer membrane of the bacteria, whilst the loops extend from the surface and are able to interact with host cells.

1.1.11 Efflux pumps

N. gonorrhoeae processes four drug efflux pumps MtrC-MtrD-MtrE, FarA-Far-B-MtrE, MacA-MacB, and NorM [135]. Efflux systems are an important defence against antimicrobials as well as other toxins. The MtrC-MtrD-MtrE forms a tripartite multidrug efflux pump system that has been well studied. This efflux pump mediates the export of various structurally diverse hydrophobic antimicrobial agents and confers decreased susceptibility to penicillin, macrolides and tetracycline [136, 137]. It is similar to other resistance-nodulation-cell division (RND) -type pumps of Gram-negative bacteria such as the MexA-MexB-OprM efflux pump of *Pseudomonas aeruginosa* [138]. Its name is derived from “multiple transferable resistance”. The pump is made from an inner membrane transporter (MtrD) [139], a membrane fusion protein (MtrC), and an outer membrane channel protein (MtrE) [140]. Transcription of *mtrCDE* is positively regulated by MtrA and negatively regulated by MtrR [141, 142]. Mutants deficient in MtrA have a decreased fitness and mutants deficient in MtrR have an increased fitness in mouse infection models [143]. However, the transcription regulator MtrR has many other roles in gene regulation within the gonococcus. MtrR also regulates other efflux systems and resistance mechanisms, see Figure 1.9. As well as this, it also regulates other “off-target” genes [144-147].

The fatty acid resistance (*far*) efflux pump (FarA-Far-B-MtrE) is a member of the major facilitator (MF) family of efflux pumps and recognises long-chain fatty acids [148]. It confers a 32-fold decreased susceptibility to linoleic acid and oleic acid and an eight-fold decrease in palmitic acid susceptibility [148]. The efflux system shares the outer membrane channel protein, MtrE, with the Mtr efflux system. The FarR protein negatively regulates *farAB* transcription and is member of the MarR transcription regulator family [149, 150]. However, MtrR negatively regulates *farR* transcription, so MtrR therefore can indirectly activate *farAB* transcription [148, 151]. This association is shown in Figure 1.9.

The MacA-MacB efflux system can confer decreased susceptibility to macrolides [152]. Substrates for the other efflux pump system, NorM, are various toxic compounds including several antimicrobial agents, (such as the fluoroquinolones, norfloxacin and ciprofloxacin), ethidium bromide, rhodamine 6G, acriflavine, crystal violet, berberine, doxorubicin, novobiocin, enoxacin, and tetraphenylphosphonium chloride [153].

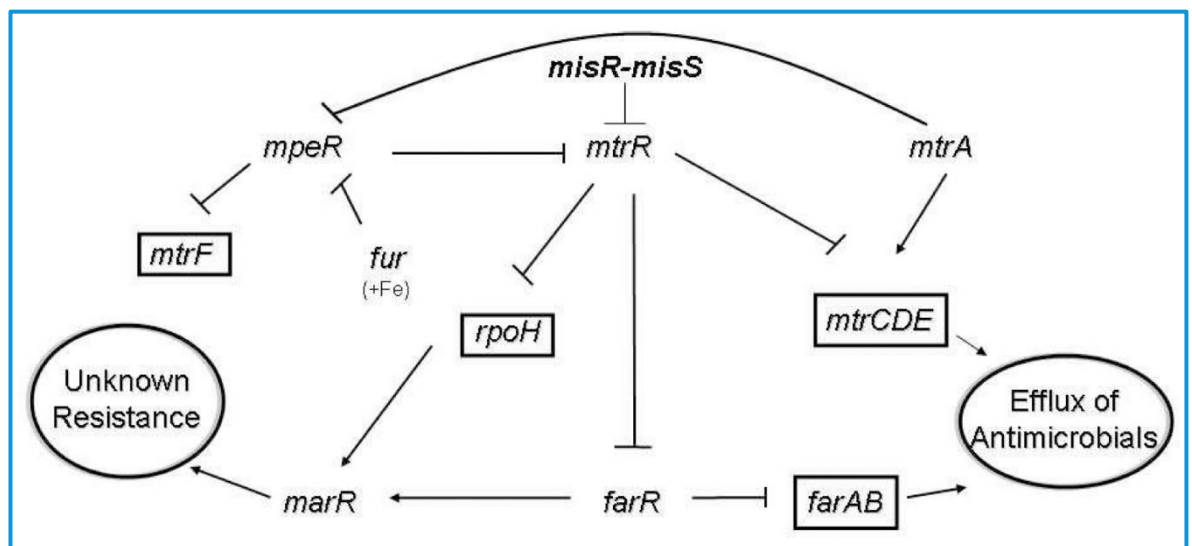


Figure 1.9 Proposed model of the regulatory system in *N. gonorrhoeae* [135]. The lines with bars indicate transcriptional repression and the lines with arrows indicate transcriptional activation. Factors that control *mtrCDE* expression directly or indirectly in gonococci are MtrR, MpeR, MisR-MisS and MtrA. MpeR being a transcriptional repressor *mtrF* in the absence of iron and MisR-MisS being a two-component regulatory system.

1.2 Fatty acids and other novel solutions to antimicrobial resistance

1.2.1 Antimicrobial agents discovery

Antimicrobials are in no doubt lifesaving drugs and have revolutionised our healthcare system. They have not only made a massive difference to the way we treat infections but have made invasive operating procedures possible. They are commonly used to prevent infections as well as to treat current infection. When introduced in the 1940s, antibiotics made a massive change to public health. Infections, including *N. gonorrhoeae* infections, were thought to be diseases that would be condemned to history. Unfortunately, the bacteria fought back and developed methods of evading the action of certain antimicrobials. Starting in the 1940s many different classes of antimicrobial have been discovered with the so called “golden age” of antimicrobial discovery which went on to the late 1960s [154]. However, no new antimicrobial classes were discovered or have been developed in the following three decades [155]. Drugs currently in development are mostly derivatives of older classes’ already in use and therefore are prone to existing resistance mechanisms.

The incentives for pharmaceutical companies in the present day to try to get new antimicrobial agents to market are too small. From a business perspective, it does not make financial sense to develop and market a drug that could potentially be obsolete in a matter of years due to resistance. In 2010, it was estimated that only 1.6 % of drugs in development by major pharmaceutical companies are antimicrobials [156]. The risk of resistance emerging and the cost to develop new antibiotics means that antimicrobial development is not appealing to pharmaceutical companies. Worse still, if they do develop an effective antimicrobial they could risk forced restrictions on its use in front-line medicine to prevent resistance emerging. The initial cost to the company for basic drug development (drug discovery and preclinical development) typically costs \$350m and takes a minimum of 3.5 years [157].

Many antimicrobial agents are discovered by screening large libraries for growth-inhibitory effects [158]. It was hoped that the impact of the genomics era would aid antimicrobial discovery. Researchers believed that next generation sequencing (NGS) technologies combined with comparative genomics would reveal novel drug targets. However, despite large investments by pharmaceutical companies such as GlaxoSmithKline who undertook a 7 year programme from 1995 to 2001, no candidates have successfully progressed to clinical trials [159].

Targets for antimicrobials would ideally not be present in the host and, like most of the successful antimicrobial classes to date, wouldn’t rely on a single target or a target that is encoded by only a single gene. It is imperative that single nucleotide polymorphisms do not lead to decreased susceptibility to the antimicrobial as this form of resistance is more easily acquired. If conventional

scientific development strategies are failing to produce new antimicrobial agents then maybe a more novel approach is required or perhaps revisiting some previously discovered agents or looking back at traditional remedies is required.

1.2.2 Fatty acids

Fatty acids are amphiphilic molecules. They are made up of a saturated or unsaturated carbon chain which is attached to a carboxylic acid. The carboxylic acid is hydrophilic while the carbon chain is hydrophobic. This gives the molecule its amphiphilic nature and means that it is a surfactant, able to act at the interface between polar and non-polar liquids and lower surface tension. The aqueous solubility of the molecule is determined by the length of the non-polar hydrophobic carbon chain. The longer the chain the less water soluble the fatty acid. The number of carbons in the chain are usually in multiples of two for naturally occurring fatty acids. However, fatty acids with an odd number of carbons in their fatty acids can be manufactured artificially. There are some contrasting opinions on the classification of the sizes of the chain. Generally, carbon chain length of ten or less are referred to as short-chain fatty acids, 12-16 are medium chain fatty acids and 18 or greater are known as long-chain fatty acids [160]. However, these length classifications may vary depending on the author's preference.

The carbon chain can also vary in complexity. Here and throughout this thesis we will only discuss unbranched fatty acids. Despite removing branched fatty acids, the level of complexity is still quite large. After carbon chain length, the next distinction of fatty acids is the number of saturated carbon bonds in the carbon chain. Fatty acids with no double bonded carbons in their carbon chains are known as saturated fatty acids. These are the simplest fatty acids. Fatty acids that do have double carbons in their carbon chain are known as unsaturated fatty acids. If only one double bond is present, then the term monounsaturated is sometimes used and fatty acids with more than one double bond can be termed polyunsaturated. The double bond(s) on the carbon chain can occur on any of the carbons but are commonly at certain positions. Chemists usually number the carbons starting from the main functional group of a molecule, see Figure 1.10.A. In the case of fatty acids this would be the carbon in the carboxyl group and then moving along the carbon chain until the final methyl end. However, another nomenclature system is favoured by some scientists where the carboxylic carbon end of the molecule is known as the alpha end and the methyl group end known as the omega end. This system is often favoured by some because the position of double bonds in relation to the omega end is more biologically relevant. For example, fatty acids with a double bond between the third and fourth carbons from omega end are known as omega three fatty acids.

The double bond also creates another level of complexity as the bond could be in either *cis* or *trans* form, see Figure 1.10.B. The difference in physical properties between the two isomers is large. The *trans* form would make the molecule flatter, more like the straight carbon chain of an unsaturated

carbon chain. The *cis* form on the other hand would make a more “bent” molecule, see Figure 1.10.B for an example. Polyunsaturated molecules with *cis* form double bonds therefore do not have straight carbon chains. This hinders the packing of molecules and can affect the melting temperature. An example of this is shown in Table 1.2. The *cis* form of double bond is more common in nature. Although the *trans* form can occur in nature, it is more commonly associated with man-made processing for creating unsaturated fatty acid via hydrogenation.

1.2.3 Lipid nomenclature systems

Lipids normally have common names as well as systematic names (IUPAC standard). For example, the saturated 12 carbon chain fatty acid has the systematic name of dodecanoic acid but is commonly referred to as lauric acid. Man-made fatty acids with an odd number of carbons in their carbon chain do not have common names so are always referred to by their systematic name. The common naming system however, becomes more complex as the number, position, and types of double bonds change. For this reason, lipid numbers are often employed to simplify the naming process. This system consists of one number identifying the number of carbons in its chain followed a second number showing number of double bonds (separated by a colon). Therefore, lauric acid would have the lipid number, 12:0. Further to this, the position and type of double bond can also written after these numbers. Looking back at Table 1.2, oleic acid had the lipid number 18:1 *c*Δ9 as it has a *cis* form double bond on the ninth carbon and elaidic acid has the lipid number 18:1 *t*Δ9 as it had a *trans* form bond at the same position. This system is the most common so will be used from this point forward.

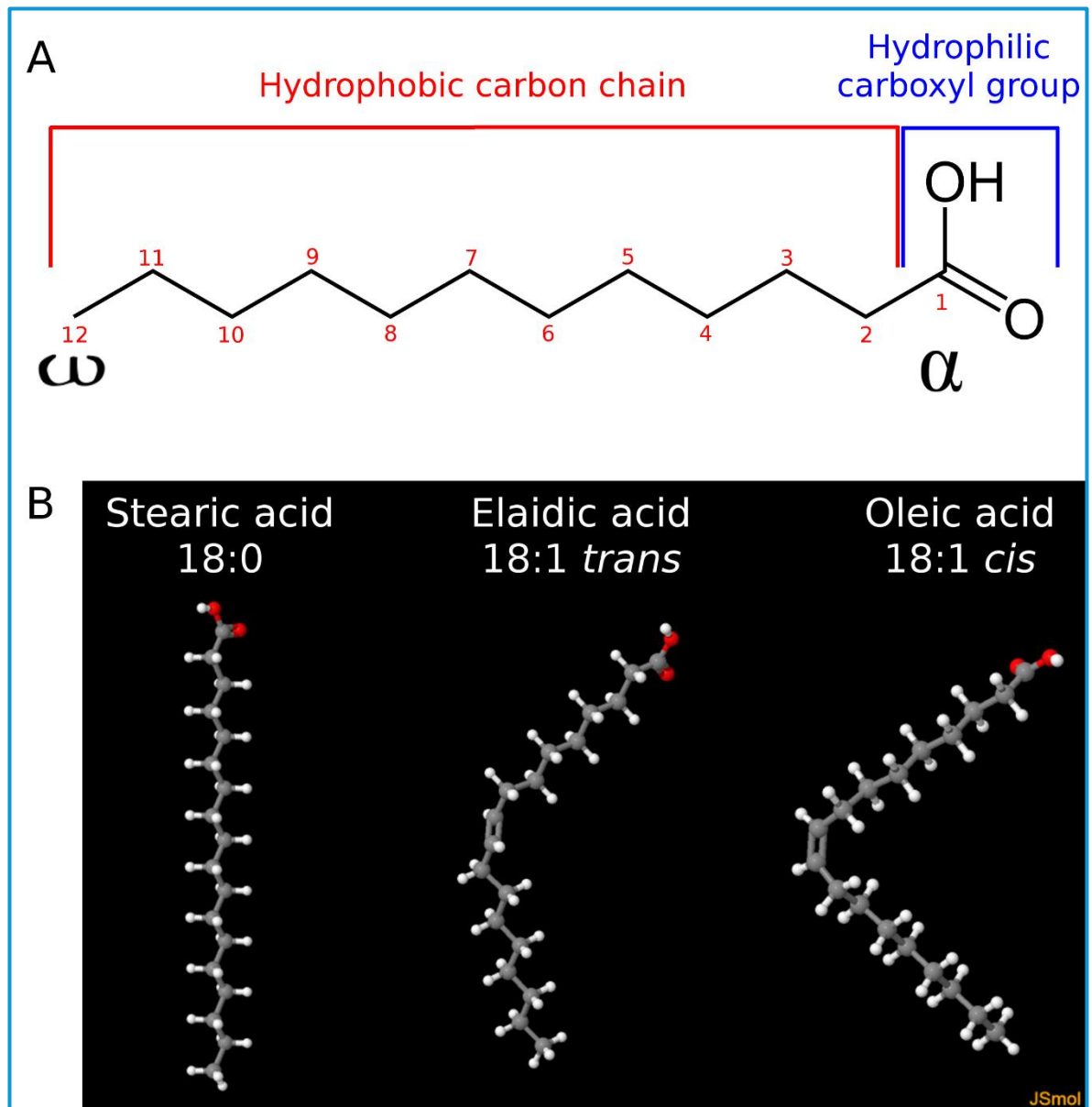


Figure 1.10 Structure of fatty acids. (A) Chemical structure of typical fatty acids, in this case lauric acid, a saturated medium chain fatty acid. The carbons are labelled with their carbon numbers and omega (ω) and alpha (α) ends are also labelled. (B) The differing structures of three 18 carbon chain length fatty acids. The first is stearic acid which has a saturated carbon chain. The other two are *cis/trans* isomers with a double bond on the ninth carbon. Elaidic acid is a *trans* isomer with the carbon chains pointing out from opposite sides of the double bond creating a small kink in the molecule. Oleic acid is the *cis* isomer and has the carbon chains extending out on the same side of the molecule creating a large bend on the molecule. Images in panel B were created using JSmol: an open-source Java viewer for chemical structures in 3D <http://www.jmol.org/>.

Table 1.2 Example how the number and form of a double bond in the carbon chain of a fatty acids affects the melting temperature [161].

Name	Carbon chain	Lipid number	Melting temperature
Stearic acid	Unsaturated fatty acid	18:0	67-72 °C
Oleic acid	Monounsaturated, <i>trans</i> form double bond on 9 th carbon	18:1 <i>t</i> Δ9	42-44 °C
Elaidic acid	Monounsaturated, <i>cis</i> form double bond on 9 th carbon	18:1 <i>c</i> Δ9	13-14 °C

1.2.4 Antimicrobial effects of fatty acids

The antimicrobial properties of fatty acids have been demonstrated scientifically for over 100 years [162]. Before this time, metal salts of fatty acids (soaps), had been known to have antibacterial properties. While scientists investigated the action of these soaps, the study of the antimicrobial properties of the fatty acids themselves was somewhat neglected in comparison. This continued with the discovery of antibiotics and then the “golden age” of antimicrobial discovery which went onto the late 1960s. Then, in 1972, Kabara and co-workers published the most comprehensive study of its time on the action of fatty acids on bacteria [163]. This study tested a range of Gram positive and Gram negative bacteria against saturated and unsaturated fatty acids as well as monoglycerides, other esters and amines. The trend of the antimicrobial effects of the different types of fatty acids mirrored that discovered for the soaps. The trend discovered was that lauric acid had the most potent antimicrobial properties of the saturated fatty acids and that linoleic (18:2 c Δ 9,12) and linolenic (18:3 c Δ 9,12,15) were the most potent of the unsaturated fatty acids. However, the exact trend varied between studies and the action of the fatty acids could be different between bacterial species. The monoglycerides were also highlighted as enhancing antimicrobial properties. After this, publications of more defined studies were conducted focusing on a particular species or groups of organisms such as *Mycobacterium* [164], enveloped viruses [165], *C. trachomatis* [166], *N. gonorrhoeae* [167, 168], *Candida albicans* [169], *Helicobacter pylori* [170-172], and *Listeria monocytogenes* [173] to name some examples.

1.2.5 Mechanism of action of fatty acids and monoglycerides

The mechanism of action of the antimicrobial properties of fatty acids and similar molecules is still unclear. However, a number possible targets have been identified [174], see Figure 1.11. Work done by Knapp and co-worker demonstrated that many morphological changes occurred upon the exposure of *N. gonorrhoeae* and *Haemophilus influenzae* to 10 mM arachidonic acid (20:4 c Δ 5,8,11,14) for one hour [175]. It was noted that there was separation of inner and outer membranes, loss of cell shape, and complete cell disorganisation and disruption. It is thought that this was due to the amphiphilic nature of fatty acids, that at high concentrations they act as a detergent and aid the solubilisation of the lipid in the membranes causing cell lysis. Work by Yoon and co-workers used quartz crystal microbalance-dissipation and fluorescence microscopy to examine the action of lauric acid on the destabilisation of the bacterial bilayers [176]. Their action on the outer cell membrane is thought to be the main target of the action of fatty acids. This would easily explain why fatty acids only inactivate enveloped viruses and not non-enveloped viruses [165].

The antibacterial action of fatty acids is seen to be bacteriostatic at lower concentrations and bactericidal at higher concentrations. Therefore, either the fatty acids have to interact with the cell

membrane or another intracellular targets to prevent cell division by interfering with essential cell processes. There are a number of theories to what these processes are that prevents cell division. Inhibition could potentially be due to fatty acids interacting with the cell membrane making it become more permeable and allow the escape of some of the cells' contents without causing cell death. Miller and co-workers predicted that inhibition of the electron transport system was partly responsible for the growth inhibition observed by *N. gonorrhoeae* when exposed to various fatty acids [168]. Membrane-bound protein complexes involved in the electron transport chain could be inhibited by the presence of the fatty acids outside of the cell so cell entry may not be required. Likewise, fatty acids could interfere with other membrane-bound cellular processes such as nutrient uptake or enzyme inhibition [174]. Another possible mechanism of action, that would require entry to the cell, is the inhibition of fatty acid biosynthesis in the bacteria [177, 178]. Inhibition of fatty acid biosynthesis has long been a desired target of drug makers as it offers a target not used by existing antimicrobials so there is little chance of cross resistance [179].

1.2.6 Inhibition of antimicrobial properties

Fatty acids and monoglycerides do not have bactericidal action against all bacterial species [180, 181]. Generally speaking, more Gram positive species are prone to their action than are Gram negatives. As described above, the main target of the fatty acids is the cell membrane. Therefore, anything that prevents access to this target will inhibit their action. For Gram positives, it has been demonstrated that *Streptococcus pneumoniae* is more susceptible than *Staphylococcus aureus*, which is more susceptible than *Bacillus subtilis* [163, 182]. This could be due to the differences within the peptidoglycan layer between these three bacterial species which may inhibit the action of the fatty acids. For example, the amount of teichoic acid present in this layer differs between these species [183].

Gram negatives are more likely to be resistant to effects of fatty acids with only species of *Neisseria*, *Haemophilus*, *Moraxella*, *Helicobacter*, and *Campylobacter* being shown to be susceptible [175, 184]. This may be due to differences in the outer membrane of these bacteria. The protein content and lipopolysaccharide (LPS) structure can vary greatly between Gram negative species as well as the possible production of a capsule in some species. These may interact with the fatty acids preventing them from reaching their targets.

However, some Gram negative bacterial species are susceptible. For example, we know that *Neisseria* and *Haemophilus* species are susceptible to a range of fatty acids and monoglycerides. These bacteria have notable differences to other Gram negative bacteria, for example members of the Enterobacteriaceae, which are intrinsically resistant to fatty acids. One difference in these two genera is that their LPS structures are different. They lack the repeating polysaccharide O-antigen found in other Gram negatives and instead have a highly variable and branched core

oligosaccharide region [185, 186]. They are so different that some researchers differentiate them by referring to them as lipo-oligosaccharides (LOS). As the O-antigen of other Gram negatives are made of a chain of hydrophilic sugars, the longer the chain the less lipophilic the outer membrane. In fact, bacteria expressing a shorter form of O-antigen are more permeable to hydrophobic antimicrobials [187].

Within the genus *Neisseria*, there are two pathogenic species: *N. gonorrhoeae* and *N. meningitidis*. These bacteria are very similar genetically but have different strategies for fatty acid resistance. Both have the FarA-Far-B-MtrE efflux system [188]. This is made up of the inner membrane bound FarA protein (Far being short for fatty acid resistance), the FarB protein which attaches to the FarA protein in the periplasmic space and the MtrE outer membrane protein [148, 151]. However, despite *N. meningitidis* having this efflux system, it also has additional resistance mechanisms due to its core oligosaccharide structure and hexa-acylation of its membrane anchor lipid A [189]. This highlights the importance of the role of the LPS in fatty acid resistance in Gram negative bacteria.

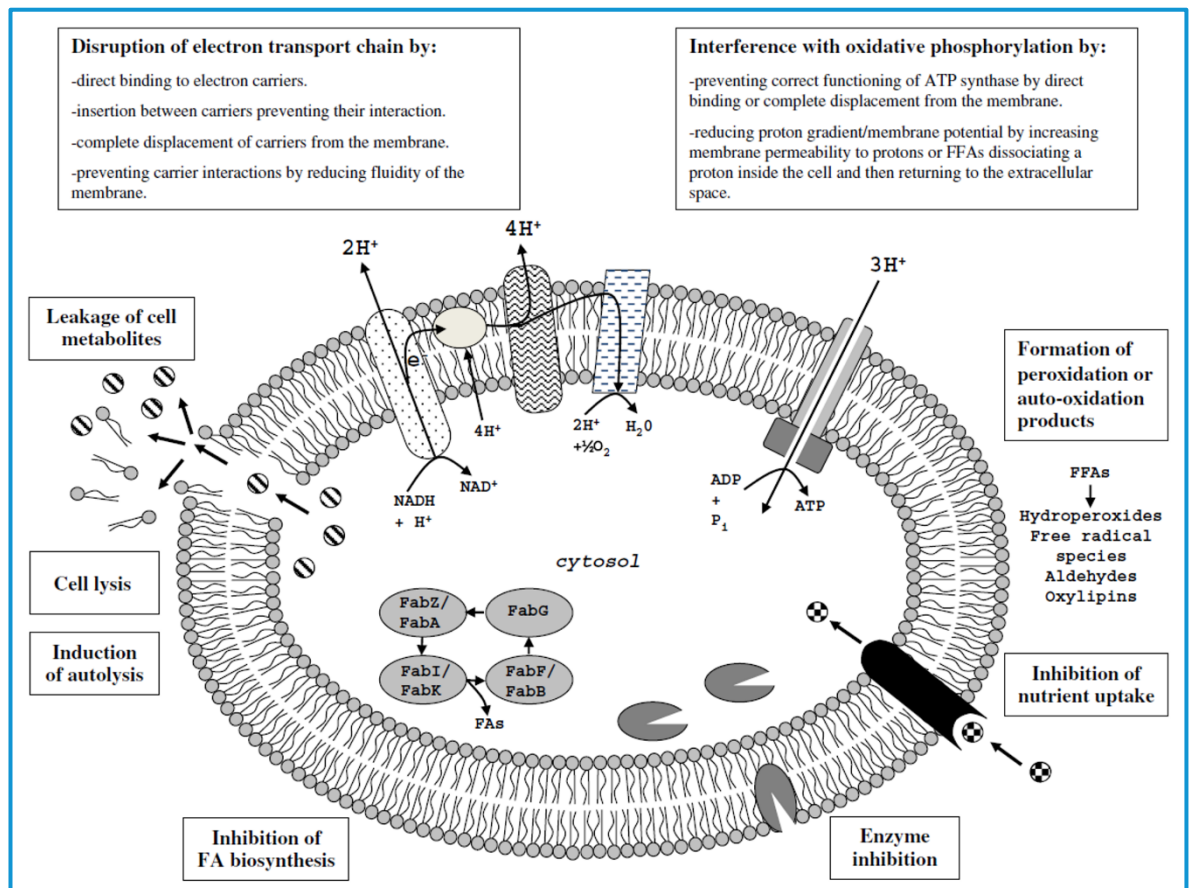


Figure 1.11. Mechanisms of bacterial inactivation by fatty acids [174]. The fatty acids could interact with the cell membrane causing cell lysis, affect bacterial energy production by disrupting the electron transport chain and/or interfering with oxidative phosphorylation, or they may hinder nutrient transport across the cell membrane. FFAs abbreviation in the figure stands for free fatty acids.

1.3 Anatomy of the eye and ophthalmic formulations

1.3.1 Structure and function

The eye is a complex organ. It converts light into electro-chemical signals that the brain can understand. The eye typically uses light wavelengths in the 400 to 700 nm range [190]. This light can be differentiated into around 2.3 million colours [191]. Human eyes are both forward facing giving us a good range of binocular vision. This gives us a good level of depth perception in front of us but restricts the overall visual range. For humans, sight is probably the most important of the five senses as we rely on it so heavily. The complexity of the eye has been driven through sight being a key advantage throughout evolution [192]. The structure of the eye is shown in Figure 1.12.A. As sight is such a driver for evolution it has an array of defensive mechanisms to help protect it from physical injury or infectious diseases. In particular, the exposed parts of the eye (Figure 1.12.B) are very important protective structures.

The outer layers of the eye are the sclera and cornea. The sclera, commonly referred to as “the white of the eye” protects the inner workings of the eye and is mainly made up of a complex structure of collagen fibres [193]. The sclera covers the majority of the eyeball and unlike other mammals (including non-human primates) is not pigmented the same colour as the iris. The other outer part of the eye is the cornea. This is the highly specialised outer structure that is completely transparent. It contains no blood vessels but does contain many nerve endings. The cornea is actually responsible for two thirds of the refraction of the light. The rest is done by the lens. The other function of the cornea is to protect the eye.

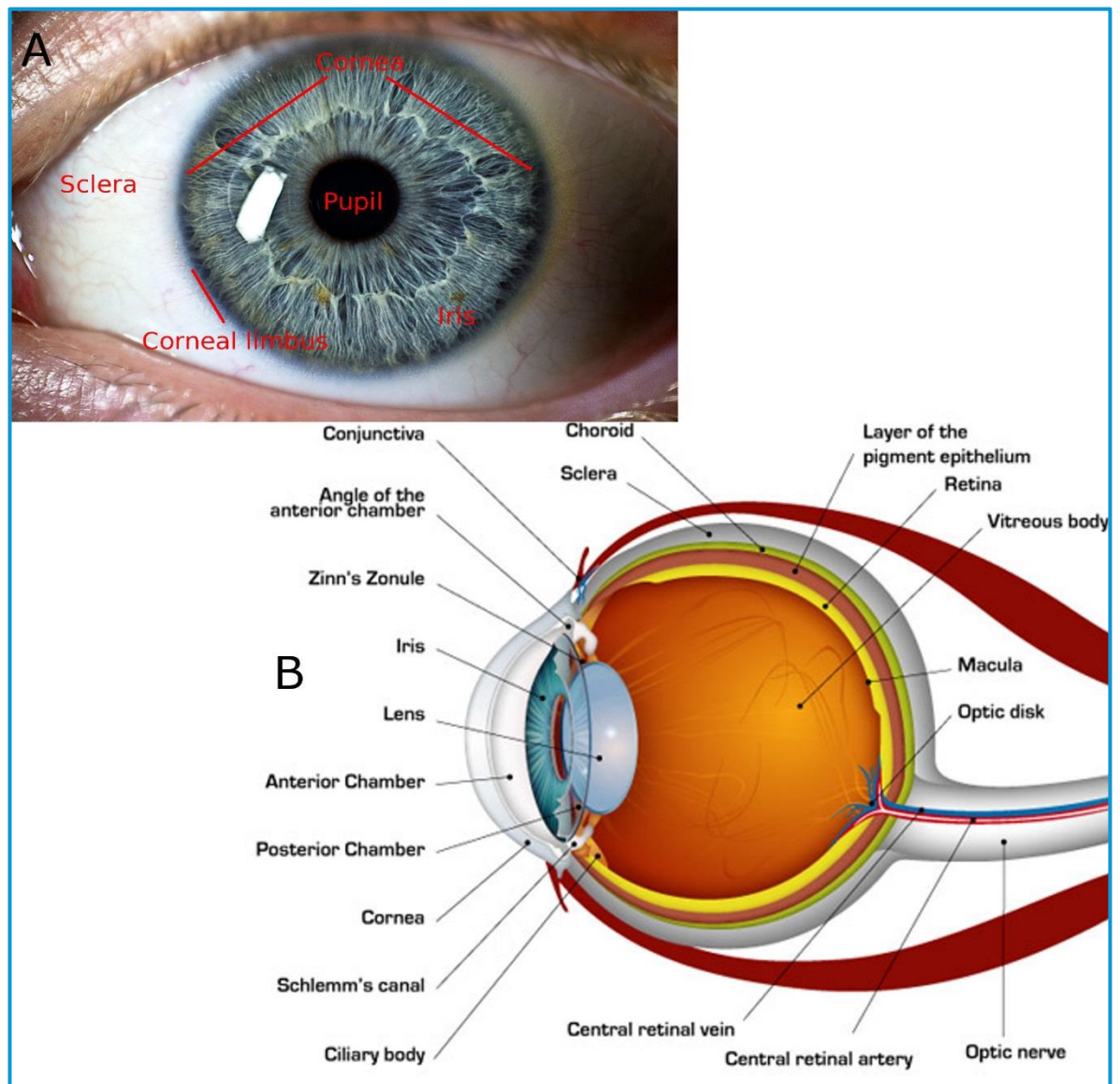


Figure 1.12 Anatomy of the eye. (A) Photograph of an eyeball with visible features labelled. The cornea is completely transparent and therefore the iris and pupil below are visible. (B) Graphical representation of a cross section of an eyeball showing all the main structures. Image for panel A was downloaded from freely from Wikimedia Commons and panel B was freely downloaded from Clipartbest.com.

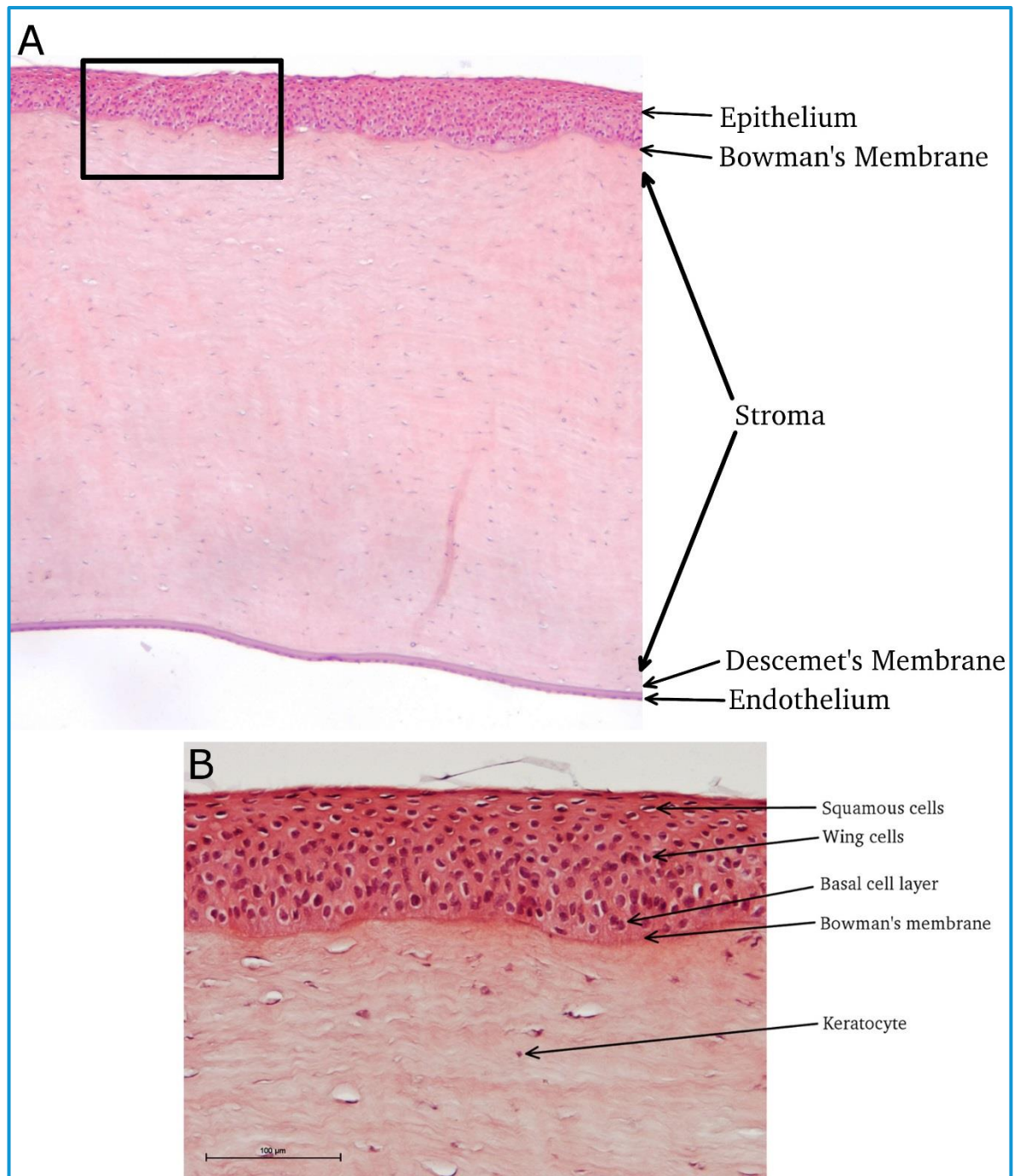


Figure 1.13 Histology of the cornea. (A) Haematoxylin and eosin stained section of a cornea with five distinct layers all labelled. (B) Haematoxylin and eosin stained epithelial layer, Bowman's membrane and some of the stroma. The different morphology of the cells in the epithelial layer is clearly visible in this picture. These pictures were produced in this study, see chapter 2 for details.

1.3.2 The sclera and cornea

The cornea is made of five layers (Figure 1.13.A); the epithelium, Bowman's membrane, stroma, Descemet's membrane and endothelium. The corneal epithelium is made of layers of cells about six cells deep. Corneal epithelial cells are made in the corneal limbus; the region between the cornea and sclera (Figure 1.13.B). The cells continue to divide and move inward toward the centre of the cornea. The cells at the bottom of the epithelium are known as basal cells, cells in the middle are known as wing cells due to their flattened shape (relative to the basal cells) and cells at the top are known as squamous cells. The squamous cells appear almost completely flat with barely visible nuclei. On top of the squamous cells sits the tear film, which is an aqueous layer with a lipid coating to reduce evaporation of the tear film (Figure 1.14.B). Squamous cells slough off into the tear film but are quickly replaced. The epithelial layer is lipid rich and therefore hydrophobic.

The Bowman's membrane is a tough layer of interwoven Type I collagen fibres. This layer is more developed in higher mammals [195]. The stroma is the thickest part of the cornea at about 1 mm thick. It is made up of Type I collagen arranged in complex fibres. The fibres are made by corneal keratocyte cells that are sparsely spaced but interconnected. In contrast to the epithelial layer, the stroma is hydrophilic. The Descemet's membrane is a thin layer composed mainly of type IV collagen fibres. The endothelium layer is made from a thin monolayer of corneal endothelium cells that transport fluid and solutes between the aqueous humor and stroma.

1.3.3 Lacrimation and other defence mechanisms

The eye is an immunologically privileged site which means the presence of leucocytes there is limited. This has the consequence that the eye is particularly vulnerable to infection, therefore relies on other powerful methods to prevent infection. The main defence against infection is lacrimation, commonly referred to as the production of tears. Figure 1.14.A shows the main parts of the eye that are responsible for producing tears and for the drainage of tears. The tear fluid itself is produced in the lacrimal gland. The composition of the tears can vary and tears can be generally being split into three categories: basal tears; reflex tears; and emotional tears.

Basal tears are continually being produced and stop the eyes drying out. These tears remove potentially irritating particles, such as dust, and help to kill infectious agents. Reflex tears are produced when triggered by irritation to the eye. These are generally similar to basal tears but are released at a much greater flow rate to wash out any irritating particle or chemical. Emotional tears are triggered by a strong emotional response such as sadness, stress, happiness or physical pain. This tear type contains much higher levels of hormones. Infants cannot usually produce emotional tears until they are several months old [196].

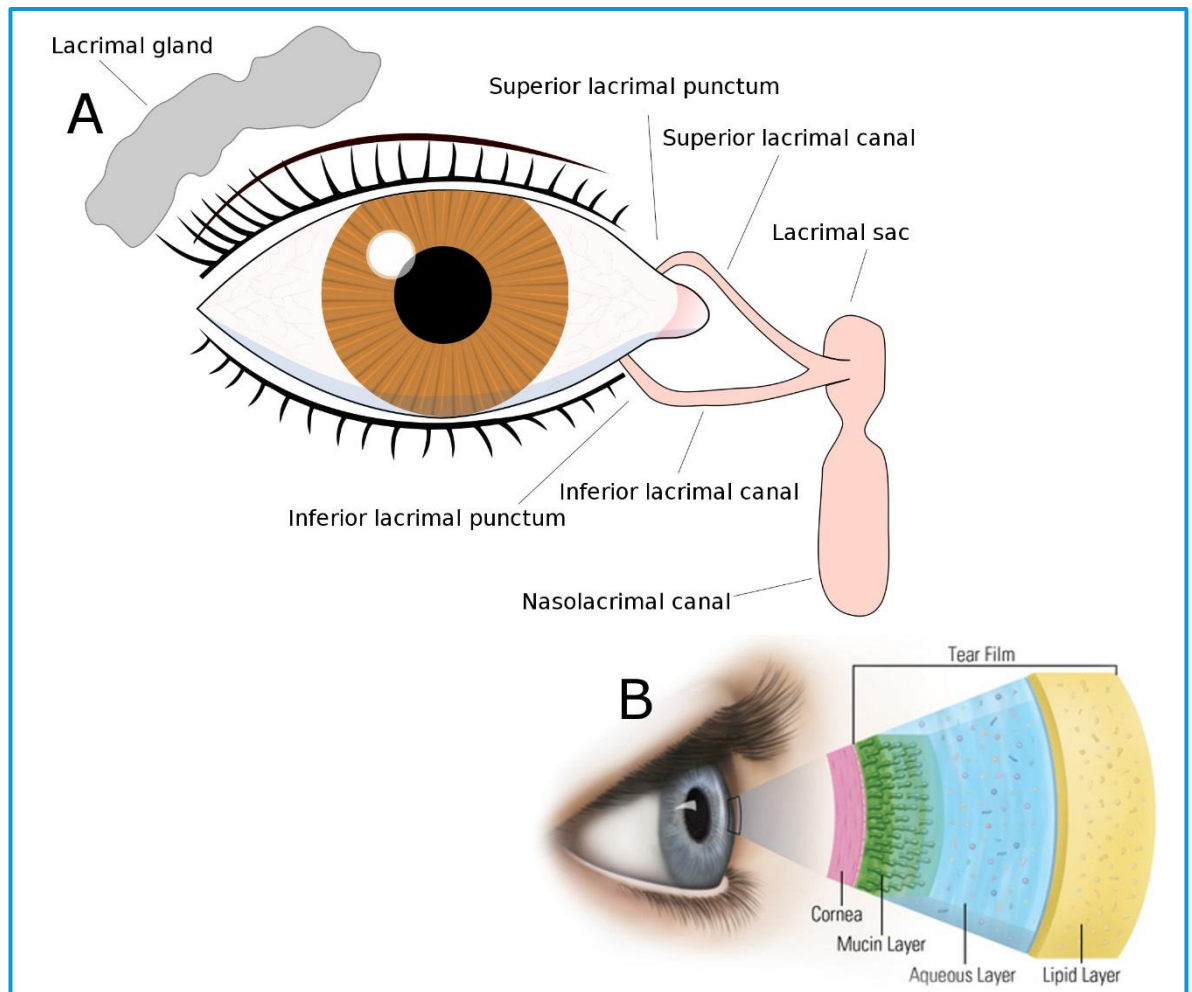


Figure 1.14 Lacrimal system of the eye. (A) Diagrammatic representation of the eye with tissues related to the lacrimal system taken from Lamellar Biomedical website <http://lamellar.dnsblock4.com/wp-content/uploads/2015/01/Tear-film-sharp.jpg>.

(B) Diagrammatic representation of the different layers that make up tear film. This image is based on an image created by User: FML and uploaded to WikiMedia Commons as Image:Sistema lacrimal.svg

The antimicrobial properties of tears have been well studied, see Figure 1.15 [197]. About 20-30 % of the protein content of basal tears is from of lysozyme [198]. Lysozyme attacks bacterial cell walls and is particularly effective at killing Gram positive bacteria [199]. Another 20-30 % of the protein content is made up from lactoferrin [198]. This sequesters iron from the tear fluid which would be essential to bacterial growth. Although this doesn't kill bacteria, iron limitation is an effective part of the innate immune system, as it gives other antimicrobial systems a chance to act on the bacteria before they become overwhelmed. Secretory immunoglobulin A (sIgA) is the main immunoglobulin present in the tear fluid. It is secreted by plasma cells resident in the lacrimal gland and conjunctival associated lymphoid tissue [200]. sIgA is a poor activator of complement but acts as a neutralising antibody, blocking recognised bacterial, viral or fungal binding proteins so they can be flushed away from the eye by the lacrimation system. However, some pathogens such as *N. gonorrhoeae* can produce a IgA protease which cleave the secreted IgA present on body surfaces, such as the eye [201].

Tears are produced in the lachrymal glands. The basal rate of flow of the tears is 1 $\mu\text{l}/\text{min}$ [202]. This liquid is then spread over the surface of the eye by blinking. Lipids are secreted by sebaceous glands and form an oily layer on top of the aqueous layer that helps to prevent evaporation of the tear fluid (see figure 1.14.B). The entire tear fluid volume (sometimes referred to as the precorneal fluid) is 7 μl but the ocular surface is able to hold 20-30 μl before tearing [203]. This volume is likely to be smaller in children, infants and neonates. Most liquid dose formulations (commonly referred to as eye drops) have a droplet size of 25-70 μl [204]. This means that tearing is likely and loss of active substances in ocular formulations is common. Tear fluid is drained from the eye via the superior and inferior lacrimal canals that lead to the nasolacrimal canal. This process is sped up by blinking and by production of either reflex or emotional tears. Application of a liquid dose form would also increase the drainage rate with active compounds drained from the eye rapidly.

A		Mechanism of action
Lysozyme		Attacks cell wall of Gram positive bacteria. In most cases this causes cell lysis
Lactoferrin		Deprives bacteria of iron and therefore creates a limiting factor to bacterial/fungal growth
Lipocalin		Bind to <u>siderophores</u> used by bacteria and fungi to recruit iron. Like <u>lactoferrin</u> it limits the iron source of the pathogen.
Secretory immunoglobulin A (sIgA)		Binds to binding receptors on the pathogen preventing them from attaching.
Secretory phospholipase A ₂ (sPLA ₂)		Binds to bacterial cell wall of Gram positive bacteria and breaks down lipids in cell membrane.
Secretory leukocyte protease inhibitor (SLPI)		Is anti-inflammatory and antimicrobial protein which is effective against both Gram positive and negative bacteria as well as <u>fungi</u> .
Surfactant protein D (SP-D)		Binds to bacterial carbohydrates making them for opsonisation
Antimicrobial peptides (AMP)		Disruption of bacterial cell membranes
Mucin		Helps to trap microbes aiding faster clearance from the ocular surface
Complement		Is in fairly low levels in tear fluid. Can kill bacteria via membrane attack complex which causes membrane lysis.

B

The diagram shows a central tear drop containing various antimicrobial components. These components are produced by different sources:

- Lacrimal Gland:** Lysozyme, Lactoferrin, Lipocalin, sIgA, sPLA₂, SLPI, SP-D
- plasma cell:** IgA
- Neutrophil:** AMPs
- Conjunctival Epithelium:** sPLA₂, SLPI, Mucins, AMPs, SP-D
- Corneal Epithelium:** Mucins, AMPs, SP-D
- goblet cell:** Mucins

The tear drop contains: Lysozyme, Lactoferrin, Lipocalin, Complement, sIgA, sPLA₂, SLPI, Mucins, AMPs, SP-D. The diagram shows these components acting on dead/inhibited pathogens.

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Figure 1.15 Known tear components that have antimicrobial properties and where they are produced [197]. (A) List of tear components and their mechanism of action. (B) Graphical representation of the antimicrobial components and where they are produced.

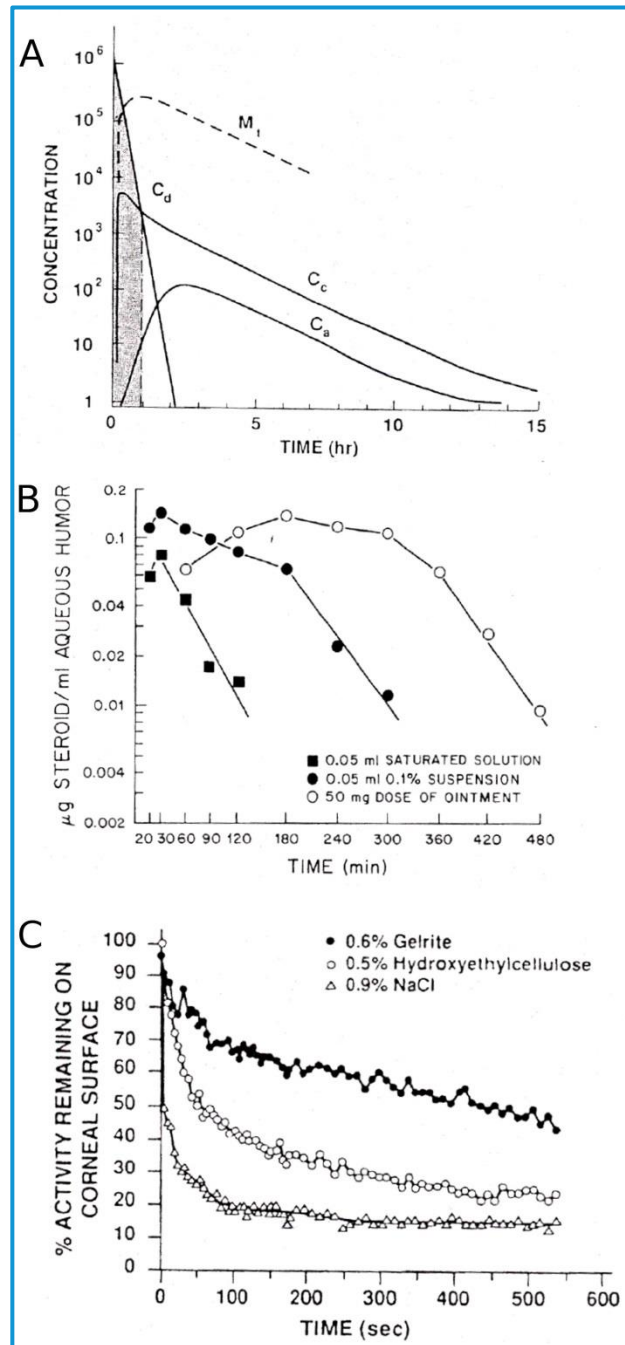


Figure 1.16 Ocular drug release. (A) Example of drug delivery levels to tear fluid (C_d), cornea (C_c) and aqueous humor (C_a) from a topical liquid dosage form [205]. (B) The difference between a solution, suspension and ointment for drug delivery to the aqueous humor [206]. (C) Difference in drug retention on the ocular surface of a liquid dosage form with two bioadhesives compared to saline [207].

1.3.4 Types of ophthalmic dosage forms

There are many types of delivery system options for ophthalmic formulations. The different delivery systems are usually chosen based on what will give the optimal drug delivery to the target site. However, other considerations are also taken into account such as ease of administration and whether the treatment results in blurred vision, as is the case with ointments applied to the eye. Figure 1.16.A. is a graph showing the time and concentration at which a drug reaches different parts of the eye. A pure liquid dosage form such as an eye drop will mix with the tear film instantly on contact but may be washed away from the eye before it has enough time to start to migrate into the cornea in significant quantities to be effective. Therefore, a delivery system that is retained in the eye for an extended time may be better suited.

1.3.5 Traditional liquid dosage forms

Due to factors mentioned above, liquid dosage forms don't have a very long retention time on the ocular surface, see Figure 1.16.B. For drugs which need to penetrate into the eye this is a problem. However, if the purpose of the therapeutic agent is to kill bacteria on the ocular surface then this delivery system may be more suitable as long as the drug is fast acting. The drug candidate would need to have a good level of solubility in an aqueous solution as high levels of other solvents may not be tolerated. Suspensions could be used for poorly soluble drugs but this could potentially lead to dose uniformity problems in some cases. Thickening agents or viscosity enhancers may be added to decrease drainage rates and therefore potentially increasing the retention time of the active drug. Agents such as hydroxypropyl methylcellulose (HPMC), polyvinyl alcohol, povidone or dextran can be used for this purpose. Some viscosity enhancers double as bioadhesive agents which interact with the ocular surface (such as carboxymethylcellulose (CMC) and carbopol). This can increase retention time, see Figure 1.16.C. Ophthalmic liquid dosage forms always require a tonicity agent, antimicrobial preservative and preferably a buffering agent [208].

1.3.6 Phase-change liquid dosage forms

These are dosage forms that are liquid upon administration but change to a semi-solid when exposed to the ocular surface; these can also be referred to as *in situ* hydrogels. This phase-shift can be triggered by temperature, pH or ionic strength. This is caused by crosslinking of polymers in the formulation and results in increased retention of the active drug.

1.3.7 Semi-solid dosage forms

The semi-solid dosage forms usually have the best retention times of any delivery system. The majority are based on blends of white/yellow petrolatum and liquid petrolatum (mineral oil). The addition of the liquid petrolatum would lower the softening point of the white/yellow petrolatum closer to the temperature of the ocular surface. Common ophthalmic ointments, such as chloramphenicol (1 % (w/w)), are produced in this way and are available over-the-counter in

pharmacies in the UK. When treatment with an ointment is advised, it is often administered in adults just before sleep due to the side effect of blurred vision caused by the semi-solid formulation. As blurred vision is not of concern with neonates, where vision is not fully developed and where the eyes are closed for long periods. Therefore, ointments are often preferred in neonatal ophthalmic delivery systems.

1.3.8 Ocular inserts

A newer method of drug delivery is the application of ocular inserts. These can either be in soluble form where the insert slowly dissolves into the tear film over the period of up to 24 hours or an insoluble form where the inserts can release the active drug over a longer period. These delivery systems are not commonly used for antimicrobial drug delivery.

1.4 Aims of this thesis and structure

The aim of this thesis is to assess the suitability of using a fatty acid or fatty acid derivative as the active component in the development of an alternative prophylaxis and/or treatment for ophthalmia neonatorum. The rise in antimicrobial resistance may make current antibiotic based prophylaxis and treatments ineffective, while the use of other non-antibiotic drugs may cause an increase in the rate of chemical conjunctivitis.

The main characteristics desired of such a formulation are that it is effective, safe, stable, easy to use, and cost effective. To address effectiveness, the active fatty acid must be able to kill *N. gonorrhoeae* cells quickly on the ocular surface. Also, the formulation must remain effective in the future so mechanisms of resistance should not already exist nor should they be easily acquired. The safety of any medicine used on neonates must be demonstrated. The drug and its formulation must not cause harm. Any damage to the cornea in neonates could have lasting effects. Furthermore, any adverse reaction would undermine use of the prophylaxis, as has been experienced with silver nitrate use. Also, the rate of adverse side effects of the treatment, such as chemical conjunctivitis, must be lower than the incidence rate of ophthalmia neonatorum. Formulations must be stable for a long shelf life. This ensures that the formulation can be stockpiled before use and will still retain its effectiveness. Consideration must also be given to how the formulation will be stored and used. The treatment should be formulated with the end user in mind, as well as where it will be used. Any mandatory medicine has to be cost effective. There were 695,233 live births in England and Wales in 2014 [209]. The lower the cost of the treatment, the greater the chance of uptake.

This thesis will try to address all of these characteristics. First a suitable candidate must be identified. This suitability will be deemed as being able to kill gonococcal cells quickly but to also not be damaging to the ocular surface. This is addressed in Chapter 2. Pre-formulation testing and formulation is presented in Chapter 3. In this chapter an analytical technique that was developed

will be described. The physical and chemical properties of the candidates for drug formulation are reviewed. The formulation is described in this chapter to look at ease of use and cost effectiveness. Chapter 4 explores *in vitro* and *ex vivo* models to determine the effectiveness of the active candidate and possible formulations. Chapter 5 investigates whether any resistance to the candidate drugs exist and whether genetic mutations can lead to decreased susceptibility to the drug candidate. Chapter 6 looks at natural sources of fatty acids that have been shown to have good anti-gonococcal activity. These could offer a cheap alternative source of antimicrobial agents for treatment of ophthalmia neonatorum in under-developed areas of the world.

Chapter 2 - Identification of possible drug candidates

2.1 Introduction

2.1.1 Drug candidate discovery

As mentioned in Chapter 1, certain fatty acids and fatty acid derivatives have been identified by previous studies to be bactericidal to *N. gonorrhoeae*. Those studies although comprehensive, may be out of date as they don't use recent isolates. Bacteria are continuously changing with their environment and antimicrobial resistance profiles of *N. gonorrhoeae* change within the space of a few years, especially if mechanisms of resistance easily acquired. Furthermore, it is important to repeat work by other researchers to ensure reproducibility and also to compare newly found antimicrobials against existing ones. For these reasons examination of previously tested fatty acids and fatty acid derivatives using recent gonococcal isolates as well as testing of more novel fatty acid derivative groups is important. Many antimicrobial agents are discovered by screening libraries for growth-inhibitory effects [158]. This is the starting approach that this study has also taken. However, the action of any drug in the ophthalmic formulation should be to kill gonococcal cells not just to inhibit growth. For this reason the bactericidal properties will also be needed to be measured. This will identify drug candidates that will go on to be further evaluated.

2.1.2 Irritation assays

Irritation potential of drug candidates or ophthalmic formulation can be assessed in a number of different ways. Currently, the gold standard test is the Draize test which involves the use of live New Zealand white rabbits [210]. However, *in vitro* testing is commonly used by pharmaceutical companies and mandatory use of the Draize test is no longer required by the European Union [211]. There are many available and are especially useful to initially screen chemicals and formulations to remove moderate to severe ocular irritants [212]. However, they can lack the sensitivity to identify mild to moderate irritants [213, 214]. It is therefore recommended that a battery of *in vitro* tests be used and not one single test alone [213, 215, 216].

This study uses three irritation assays to establish irritation potential. The first test is the bovine corneal opacity and permeability (BCOP) test. This is a model for damage to the cornea and involves the testing of substances on excised bovine eyes [217]. The test evaluates corneal swelling, wrinkling, opacity and fluorescein retention. Histopathological damage can also be assessed to grade damage to the corneal epithelia for enhanced discrimination of mild to moderate irritants [218]. The Hen's Egg Test – Chorioallantoic Membrane (HET-CAM) is a model of conjunctival irritation which has been used in this study [217, 219, 220]. Conjunctival hyperaemia is a common sign of ocular irritation and the vascular CAM of a hen's egg is therefore an ideal model. The capillaries located just under the epithelium of the CAM will have a rapid response to any substance that causes damage to the vascular system. The third test is the red blood cell (RBC) lysis assay

which is a more general test that determines whether the substances is capable of lysing cells membranes [221]. This test has been reported to offer a higher level of sensitivity [222]. These tests combined will give a good level of certainty of the irritation potential of any drug candidate.

2.1.3 Aims of this chapter

The main aim of this chapter is to identify fatty acids and/or fatty acid derivatives which are suitable for further development into ophthalmic formulation in terms of anti-gonococcal properties and also ocular safety. The anti-gonococcal properties will be tested in terms growth inhibition first and then killing abilities. Candidates with the fast killing properties will be selected further. The ocular safety of the candidates will be evaluated via the use of three *in vitro* models. Finally, the killing action of the candidates will also be measured in simulated tear fluid to assess their ability to act in the eye.

2.2 Materials and methods

2.2.1 Bacterial strains and culture conditions

GC agar was used to grow all *Neisseria* isolates. This was produced by adding 18 g/L GC base (15 g/L Special peptone, 1 g/L corn starch, 5 g/L sodium chloride, 4 g/L dipotassium hydrogen phosphate, 1 g/L, potassium dihydrogen phosphate, and 10 g/L agar) (Oxoid Limited, Hampshire, UK) to deionised water and sterilising by autoclaving at 121 °C for 15 minutes. Once cooled to approximately 50-60 °C, Kellogg supplements (final concentrations: 12.3 µM Fe(NO₃)₃, 22.2 mM D-glucose, 0.68 mM L-glutamine and 0.43 µM thiamine pyrophosphate) were added and poured into either 55 or 90 mm sterile petri-dishes [101]. Isolates were often maintained in GC broth (15 g/L protease peptone, 4 g/L potassium phosphate dibasic (K₂HPO₄), 1 g potassium phosphate monobasic (KH₂PO₄), and 5 g/L NaCl) which had been sterilised by autoclaving as above [223]. However, gonococcal isolates were always grown on solid agar medium. Growth conditions were 37 °C, 5% CO₂ which was maintained by a laboratory CO₂ laboratory incubator. Trays of water containing copper sulphate were placed in the incubator to increase humidity to prevent agar plates drying out.

After receiving isolates for the first time, a swab was used to inoculate GC agar plates. A continuous streak was done over an entire 90 mm agar plate and incubated for 24 hours at 37 °C, 5% CO₂. Cells were then scraped off the surface of the plate and placed into GC broth containing 15 % (v/v) glycerol. Aliquots were then stored at -80 °C freezer for long term storage. To revive a culture, stocks were briefly taken out the -80 °C freezer, a sterile cotton wool swab used to take cells off the surface of stock and then returned to the freezer before the stock had defrosted. The swabs were used to inoculate GC plates and these were incubated at 37 °C, 5% CO₂. Growth was checked to ensure no contamination present. Plates containing bacterial growth that differed from the

characteristic gonococcal growth (see Figure 1.3.B) where discarded. Cultures could be maintained by passage every 24 hours on GC agar but no cultures were maintained any further than four passages from the glycerol stock. This was done to maintain genotype and phenotypic characteristics of the isolate.

The well characterised *N. gonorrhoeae* strain NCCP11945 was used in the screening stage of this experiment [224]. Eight isolates that were obtained from PHE from various European locations were used in the log reduction assays (designated HPA 1-8). All isolates were first cultured for 24 hours prior to any testing by plating on GC agar and incubating at 37 °C, 5% CO₂.

2.2.2 Fatty acid and fatty acid derivatives

A full list of all the fatty acid and fatty acid derivatives used in this study is in Table 2.1. The tested compounds include saturated and unsaturated fatty acids, monoglycerides, dicarboxylic acids, and fatty acid sodium salts. All compounds investigated were resuspended at a concentration of 100 mM in ethanol unless stated, these were stored at -20 °C. All chemicals were obtained from Sigma-Aldrich (Poole, Dorset, UK).

2.2.3 Screening of the fatty acids/ fatty acid derivatives for bactericidal action

A diagrammatical representation of the screening stage can be found in Figure 2.1. GC plates were prepared with a concentration 1 mM of the test compounds. This was done by adding a 1/100th volume of the 100 mM stock to the molten agar with Kellogg supplements (see 2.2.1) before pouring. For this stage of screening, 55 mm petri dishes were used. *N. gonorrhoeae* isolate NCCP11945 was grown overnight on GC agar at 37 °C with 5 % CO₂ and cells scrapped into GC broth medium. It was previously determined that an optical density (OD) at 520 nm of between 0.25 and 0.3 was equivalent to approximately 10⁷ gonococcal cells. Therefore, suspensions were made to that value and a sample was also diluted 1/1,000, therefore producing a 10⁴ CFU/ml suspension. An aliquot of 100 µl of the undiluted cell suspension and the dilution were then plated on separate plates containing the different test candidates and spread using an L shaped spreader. This would challenge the candidates with approximately 10⁶ and 10³ cells per plate. The plates were incubated for 48 hours at 37 °C with 5 % CO₂. Any test candidates that had inhibited growth of the bacteria were selected for the next stage of selection.

Table 2.1 A list of fatty acids and fatty acid derivatives with their chemical formulae used in this study.

Chemical Class	Candidate	Formula
Saturated Fatty Acids	Caprylic (8:0)	$\text{CH}_3(\text{CH}_2)_5\text{COOH}$
	Capric acid (10:0)	$\text{CH}_3(\text{CH}_2)_8\text{COOH}$
	Undecanoic acid (11:0)	$\text{CH}_3(\text{CH}_2)_9\text{COOH}$
	Lauric acid (12:0)	$\text{CH}_3(\text{CH}_2)_{10}\text{COOH}$
	Tridecanoic acid (13:0)	$\text{CH}_3(\text{CH}_2)_{11}\text{COOH}$
	Myristic acid (14:0)	$\text{CH}_3(\text{CH}_2)_{12}\text{COOH}$
	Pentadecylic acid (15:0)	$\text{CH}_3(\text{CH}_2)_{13}\text{COOH}$
	Palmitic acid (16:0)	$\text{CH}_3(\text{CH}_2)_{14}\text{COOH}$
	Heptadecylic acid (17:0)	$\text{CH}_3(\text{CH}_2)_{15}\text{COOH}$
	Stearic acid (18:0)	$\text{CH}_3(\text{CH}_2)_{16}\text{COOH}$
Mono-Unsaturated Fatty Acids	Myristoleic acid (14:1 $c\Delta 9$)	$\text{CH}_3(\text{CH}_2)_3\text{CH}=\text{CH}(\text{CH}_2)_7\text{COOH}$
	Palmitoleic acid (16:1 $c\Delta 9$)	$\text{CH}_3(\text{CH}_2)_5\text{CH}=\text{CH}(\text{CH}_2)_7\text{COOH}$
	Oleic acid (18:1 $c\Delta 9$)	$\text{CH}_3(\text{CH}_2)_7\text{CH}=\text{CH}(\text{CH}_2)_7\text{COOH}$
	Elaidic acid (18:1 $t\Delta 9$)	$\text{CH}_3(\text{CH}_2)_7\text{CH}=\text{CH}(\text{CH}_2)_7\text{COOH}$
	Petroselinic acid (18:1 $c\Delta 6$)	$\text{CH}_3(\text{CH}_2)_{10}\text{CH}=\text{CH}(\text{CH}_2)_3\text{COOH}$
	trans-Vaccenic acid (18:1 $t\Delta 11$)	$\text{CH}_3(\text{CH}_2)_5\text{CH}=\text{CH}(\text{CH}_2)_9\text{COOH}$
	cis-Vaccenic acid (18:1 $c\Delta 11$)	$\text{CH}_3(\text{CH}_2)_5\text{CH}=\text{CH}(\text{CH}_2)_9\text{COOH}$
Poly-Unsaturated Fatty Acids	Erucic acid (22:1 $c\Delta 13$)	$\text{CH}_3(\text{CH}_2)_7\text{CH}=\text{CH}(\text{CH}_2)_{11}\text{COOH}$
	Sorbic acid (6:2 $t\Delta 2,4$)	$\text{CH}_3\text{CH}=\text{CHCH}=\text{CHCOOH}$
	Linoleic acid (8:2 $c\Delta 9,12$)	$\text{CH}_3(\text{CH}_2)_4\text{CH}=\text{CHCH}_2\text{CH}=\text{CH}(\text{CH}_2)_7\text{COOH}$
	Linolenic acid (18:3 $c\Delta 9,12,15$)	$\text{CH}_3(\text{CH}_2\text{CH}=\text{CH})_3(\text{CH}_2)_7\text{COOH}$
Monoglycerides	Arachidonic Acid (20:4 $c\Delta 5,8,11,14$)	$\text{CH}_3(\text{CH}_2)_4(\text{CH}=\text{CHCH}_2)_4\text{CH}_2\text{CH}_2\text{COOH}$
	Monocaprylin (8:0MG)	$\text{H}_3\text{C}(\text{CH}_2)_6\text{COOCH}_2\text{CH}(\text{OH})\text{CH}_2\text{OH}$
	Monocaprin (10:0MG)	$\text{H}_3\text{C}(\text{CH}_2)_8\text{COOCH}_2\text{CH}(\text{OH})\text{CH}_2\text{OH}$
	Monolaurin (12:0MG)	$\text{H}_3\text{C}(\text{CH}_2)_{10}\text{COOCH}_2\text{CH}(\text{OH})\text{CH}_2\text{OH}$
Dicarboxylic Acids	Monomyristin (14:0MG)	$\text{H}_3\text{C}(\text{CH}_2)_{12}\text{COOCH}_2\text{CH}(\text{OH})\text{CH}_2\text{OH}$
	Succinic acid	$\text{HOOCCH}_2\text{CH}_2\text{COOH}$
	Adipic acid	$\text{HOOC}-(\text{CH}_2)_4-\text{COOH}$
	Pimelic acid	$\text{HOOC}-(\text{CH}_2)_5-\text{COOH}$
	Suberic acid	$\text{HOOC}-(\text{CH}_2)_6-\text{COOH}$
	Azelaic acid	$\text{HOOC}-(\text{CH}_2)_7-\text{COOH}$
Ricinoleic acid	Sebacic acid	$\text{HOOC}-(\text{CH}_2)_8-\text{COOH}$
	Ricinoleic acid	$\text{CH}_3(\text{CH}_2)_5\text{CH}(\text{OH})\text{CH}_2\text{CH}=\text{CH}(\text{CH}_2)_7\text{COOH}$
	Isopropyl myristate	$\text{CH}_3(\text{CH}_2)_{12}\text{COOCH}(\text{CH}_3)_2$

Esters	Isopropyl palmitate	$\text{CH}_3(\text{CH}_2)_{14}\text{COOCH}(\text{CH}_3)_2$
Fatty Acid Sodium Salt	Sodium dodecanoate	$\text{CH}_3(\text{CH}_2)_{10}\text{COONa}$
	Sodium myristate	$\text{CH}_3(\text{CH}_2)_{12}\text{COONa}$

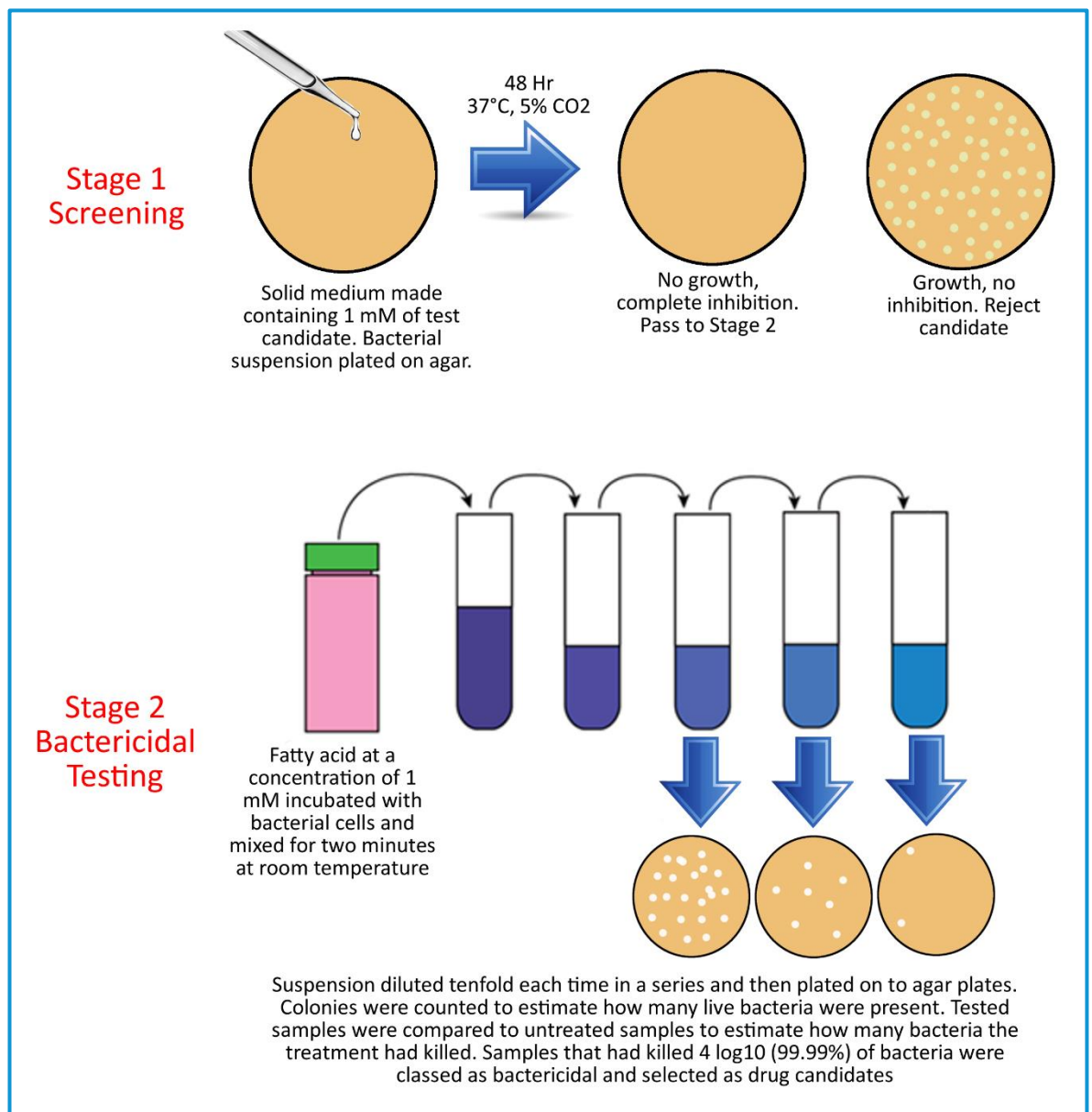


Figure 2.1 Diagrammatical representation of fatty acid/ fatty acid derivative drug candidate selection process. This phase of the selection process focused on selection based on anti-gonococcal properties of the tested substances. The first stage (screening) identified whether the candidates inhibited growth. Ones that did inhibit growth were then tested for fast killing (bactericidal) properties by log reduction experiments.

2.2.4 Measurement of bactericidal action of the fatty acids

Log reductions were done using a modified method of Bergsson and co-workers [167]. A diagrammatical representation of this process can be found in Figure 2.1. Briefly, cells were suspended in GC broth to an optical density of between 0.25-0.3 at 520 nm which is approximately equivalent to 10^7 cells per millilitre. Five hundred microliters of the suspension was then added to 5 μ l of the 100 mM stocks to give a final concentration of 1 mM. A negative control was also done to calculate the reduction in bacterial titre by adding 500 μ l to 5 μ l ethanol. The tubes were then mixed at room temperature for two minutes and immediately diluted in a 10 fold dilution series down to 10^{-4} . The dilutions were plated in duplicate by spreading 10 μ l on a quarter of a GC agar plate and 100 μ l of the neat solution was also plated over a whole plate, again in duplicate. Plates were incubated at 37 °C, 5% CO₂ for 48 hours. All experiments were repeated on separate days to ensure that results were reproducible. A reduction in viable cell count by more than 4 log₁₀ was deemed to be bactericidal and was selected for further investigation. Bactericidal compounds were then tested at concentrations of 0.75, 0.5 and 0.25 mM (0.125 mM was also used in some potent compounds) to find the minimum bactericidal concentration (MBC). These MBC calculation experiments were done using eight clinical gonococcal strains as well as the NCCP 11945 isolate that was used for the screening stage.

2.2.5 Bovine corneal opacity and permeability test

Bovine eyes were collected from a slaughter house (ABP, Guildford, Surrey, UK) and were transported in cold saline for testing at the laboratory. Each eye was first examined for signs of damage before use. Plastic weighing boats were used to hold individual eyes in a closed shaking water bath at 37°C, 90 rpm. The eyes were incubated in the water bath for 10 minutes and then a rubber 8 mm O-ring was carefully placed on the centre of the cornea. One drop of saline was added to the test area inside the O-ring and incubated for 5 minutes. The saline was then removed and the test substance added for 30 seconds. Bactericidal compounds were tested at a concentration of 1 mM in saline. Overall concentration of ethanol from carry-over from the stock was therefore 1 %. A negative control of saline with 1 % ethanol, an irritant control of 100% acetone and a strong irritant control of 0.5 M sodium hydroxide were all tested in each experiment. The test area of the cornea was then washed with 10 ml saline and the eyes were incubated for 10 minutes in the water bath.

Corneal damage to the test area was assessed by fluorescein examination by adding a few drops of 2 % sodium fluorescein and washing with 10 ml saline. The eyes were examined under a cobalt blue filter and pathological scoring based on opacity, epithelial integrity and epithelial detachment was done in accordance with previous publications (see Table 2.2) [217]. Each of the seven

candidates were tested in triplicate, each test was performed on a separate day from a different batch of eyeballs.

2.2.6 BCOP - Cornea Histology

After BCOP experiments, the corneas were excised from the eyeballs. These were trimmed, put into histological cassettes and placed in neutral buffered formalin (Sigma-Aldrich) for 24 hours. The formalin was removed and replaced with 70% industrial methylated spirit (IMS) and stored for a minimum of 48 hours until the samples were ready to test. The samples were put into a Thermo-Fisher Scientific (Loughborough, Leicestershire, UK) Shandon-Citadel 1000 automatic tissue processor where they were processed as stated in Figure 2.2.A The samples were embedded in paraffin in 37 X 24 X 9 mm stainless steel moulds (Thermo-Fisher Scientific) using a Histostar embedding centre (Thermo-Fisher Scientific) and stored at 4 °C overnight. Excess wax was removed using a scalpel and the block placed on a Leica Biosystems (Milton Keynes, Buckinghamshire, UK) rotary microtome (model RM2255) and sections taken at a thickness of 5-7 µm. The sections were mounted on polysine coated microscope slides (Thermo-Fisher Scientific) and incubated at 40 °C overnight. After this, the slides were stained with Haematoxylin and Eosin (H&E) using standard protocols outlined in Figure 2.2.B. After soaking in HistoClear II (National Diagnostics, Atlanta, Georgia, USA) for 15 minutes after staining, a coverslip was mounted on the slides using DPX mountant (BDH Chemicals, VWR International, Lutterworth, Leicestershire, UK) and the slides dried at 40 °C for 24 hours. The slides were examined using a Nikon Eclipse i80 (Nikon UK Limited, Kingston-Upon-Thames, Surrey, UK) light microscope and images captured using NIS-Elements BR 3.0 SP7 (Nikon UK Limited) software.

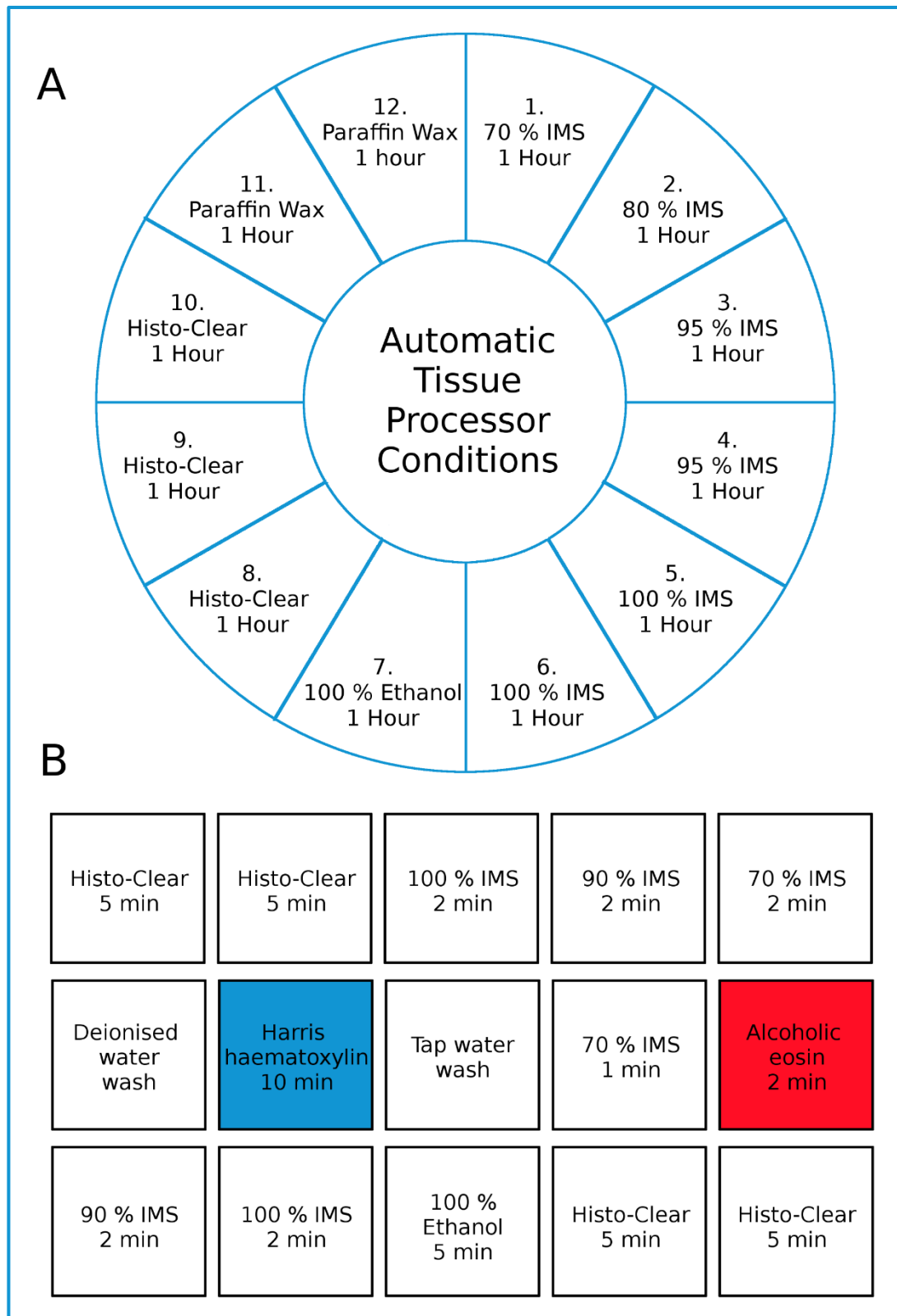


Figure 2.2 Conditions for automated tissue processing and H&E staining. (A) Conditions for the automated tissue processor which was run overnight. The paraffin baths (stages 11 and 12) were done at 65 °C. (B) Order and length of incubation of slides for H&E staining. Staining was done glass staining dishes in batches of up to twelve. Abbreviations used: IMS, Industrial Methylated Spirit.

Table 2.2 Scoring table of bovine cornea opacity permeability test.

Opacity	Score	Epithelial integrity	Score	Epithelial detachment	Score	Cumulative score	Score
None	0	None	0	No gross abnormalities	0	≤0.5	None
Slight	1	Diffuse and weak	0.5	Wrinkling of corneal surface	2	0.6-1.9	Slight
Marked	2	Confluent and weak	1	Loosening of epithelium	3	2.0-4.0	Moderate
Severe	3	Confluent and intense	1.5	Epithelium absent	4	>4	Severe
Opaque	4						

2.2.7 Hen's Egg Test – Chorioallantoic Membrane (HET-CAM)

The HET-CAM was performed as Alany and co-workers [219]. Fertilised White Leghorn eggs were purchased from Henry Stewart & Co. Ltd (Fakenham, Norfolk, UK) and delivered to Kingston University. If the eggs were not being used immediately then they were stored at 10-15 °C but for no longer than one week. All eggs were checked for damage and any which were overly misshaped were discarded. Eggs were cleaned with 70 % IMS and incubated at 37.5 °C ± 1°C with 60-70 % relative humidity. Conditions were monitored by an Ebro (Ingolstadt, Germany) EBI20-TH1-se temperature and humidity data-logger. The eggs were turned three times a day to prevent shell membranes adhering to the shell.

After four days of incubation the eggs were again cleaned. A growth chamber was made by placing cling film over a white PVC tube with a 10 cm diameter and a depth of 10 cm. The cling film was pushed half way into the tube so it would be able to hold the contents of the egg. The cling film was secured to the holder with a circular PVC clip which had the same diameter as the holder but only had a depth of 1 cm. The eggs were broken open carefully and poured into the growth chambers (see Figure 2.3). A sterile glass 120 mm petri-dish half was then placed over the chamber. Any eggs that didn't have a viable chorioallantoic membrane or that had a broken yolk sac were discarded. All viable eggs in chambers were returned to the incubator.

At day nine, the CAMs were tested. This was done by applying 200 µl of the testing material and recording results at 30, 120, and 300 seconds. At each of these time-points the effect of the substance on the CAM was evaluated for signs of hyperaemia, haemorrhaging and coagulation (examples are shown in Figure 2.4) and assessed using a scoring system as shown in Table 2.3 [217]. Photographs were also taken before exposure and at each time-point. The negative control used in this experiment was saline solution. Positive controls were 0.1 M NaOH, acetone, and propylene glycol.

2.2.8 Red Blood Cell (RBC) lysis assay

RBC lysis assay testing was done following European Union Reference Laboratory for Alternatives to Animal Testing (EURL-ECVAM) guidelines [225]. A sterile 60 ml pot (Sterilin Ltd, Newport, Gwent, UK) containing 10 ml citrate anticoagulation buffer (110 mmol/L citrate (66 mmol/L sodium citrate, 44 mmol/L citric acid)) was couriered to a slaughter house (ABP, Guildford). The pot was filled up to 100 ml with fresh bovine blood and mixed well. The pot was transported back to Kingston University and RBCs were isolated the same day. The blood was diluted in four volumes to ten in PBS with 10 mM glucose (PBSG). This was centrifuged at 1,500 x g for 10 minutes. The supernatant (serum and white "buffy coat") were discarded and RBCs resuspended by adding PBSG. A total of five washes were completed. Finally, the pellet was resuspended in PBSG. A sample of 500 µl was diluted in 5 ml distilled water and the OD measured at 541 nm. Extra PBSG was added to the RBCs

until a sample gave an OD value of 0.5 using the Equation 2.1. This would give a 2 % suspension of RBCs which could then be stored at 4 °C and used within two weeks.

Equation 2.1

$$\frac{\text{Observed OD} \times \text{original volume of buffer added}}{\text{Desired OD}} = \text{Desired final volume of buffer}$$

The fatty acids stocks were made up in dimethyl sulfoxide (DMSO) at a concentration of 13,333 mg/L. Before accurate testing, a range-finding experiment was conducted to know the approximate testing concentration range. The stocks were diluted one in ten with PBS and then diluted in a ten-fold dilution series to 0.01 mg/L with PBSG with 10 % DMSO. Tests were set up by incubating 750 µl of the test concentration with 250 µl of the 2 % RBC suspension at room temperature for one hour with shaking at 125 rpm. Controls used were a complete lysis control (deionised water) and a fragility control (PBSG + 10 % DMSO). Samples were centrifuged at 10,000 x g for 2 minutes. An aliquot of 750 µl of the supernatant was taken, placed in a plastic cuvette and absorbance at 451 nm measured. The testing range lay between the lowest dilution to cause haemolysis and the dilution below.

Once the testing range had been identified, at least five concentrations within the range were tested as above but this time testing was done in triplicate. The OD_{451nm} of the mean fragility control was subtracted from the means of all samples and percentage haemolysis estimated for each sample by comparison of the complete lysis control. By plotting these values the concentration at which lysis of 50 % of the RBC occurs (H₅₀) could be estimated.

2.2.9 Testing in simulated tear fluid

A simulated tear fluid based on published work was made up as Table 2.4 [226]. The stock solution were combined before use and diluted to working concentration by the addition of deionised water. The simulated tear fluid was filter sterilised through a 0.22 µm filter (Nalgene, Rochester, New York, USA) before use. The fatty acid candidates were tested in the simulated tear fluid to demonstrate that they were still able to kill the bacteria in this environment. Log reductions were tested as 2.2.4 with one exception; after adjusting the bacterial suspension to OD_{520nm} of 0.25-0.3, the cell suspension was centrifuged at 14,000 x G for 3 minutes, culture medium removed and bacteria resuspended in the simulated tear fluid. This was then added to the concentrated fatty acid candidates as 2.2.4

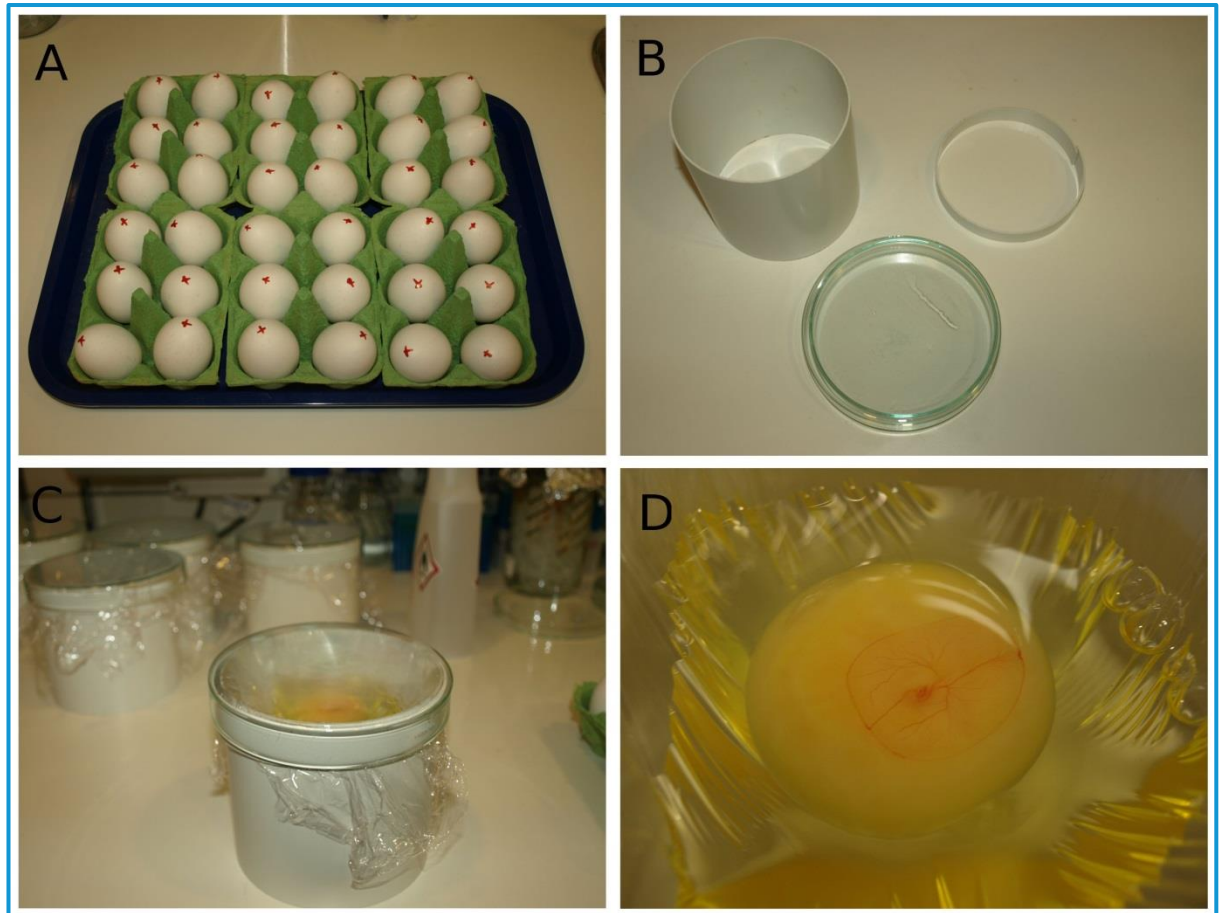


Figure 2.3 HET-CAM method. (A) The cleaned eggs in the first few days of the experiment. The crosses on the eggs make it easier to tell if the eggs have been turned. (B) Example of a HET-CAM holder, clip and top (glass petri-dish lid). (C) The holder after the egg has been added with the cling film holding the egg. (D) The egg inside the holder at either day 3 or 4. At this stage of the experiment the heart can be seen beating

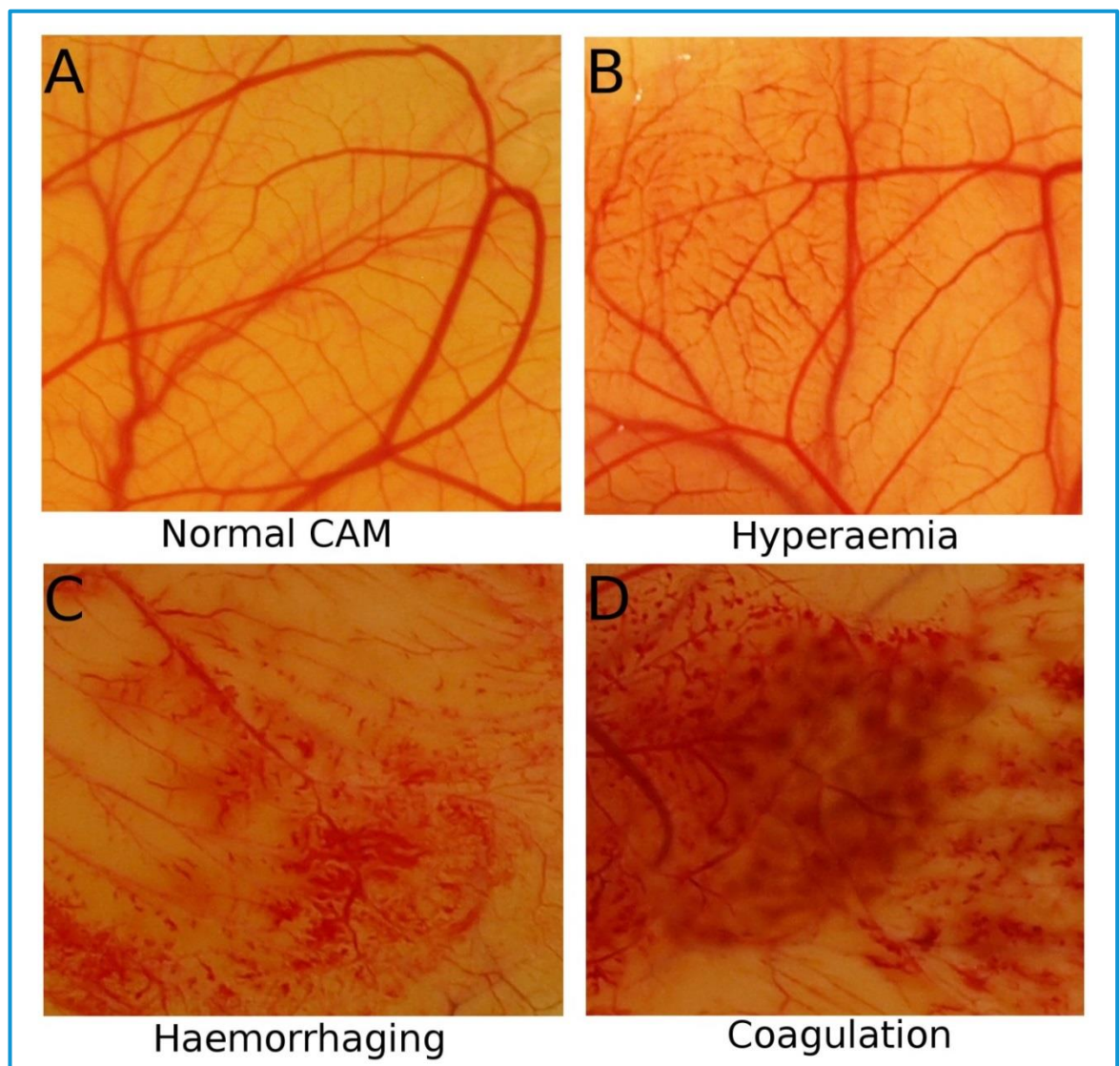


Figure 2.4 Examples of different reactions of the HET-CAM test. (A) A normal CAM before exposure. (B) Typical hyperaemia reaction. This is the increase of blood flow to different areas caused by vasodilation. The blood vessels are clearly being affected by the presence of the testing substance. (C) Haemorrhaging is where the blood vessels fail to contain the blood and it leaks into the tissue. (D) CAM exhibits haemorrhaging but here the blood starts to coagulate (clot) forming darker spots. Images are from this study.

Table 2.3 Scoring scheme for the HET-CAM with the classification of the cumulative score.

Effect	Score		
	0.5 min	2 min	5 min
Hyperaemia	5	3	1
Haemorrhage	7	5	3
Clotting/coagulation	9	7	5

Cumulative Score	Interpretation
0-0.9	None
1.0-4.9	Slight
5.0-8.9	Moderate
9.0-21.0	Severe

Table 2.4. Components and concentrations of the simulated tear fluid.

Stock Name	Tear Component	Sigma code	Final Concentration (g/L)	Stock concentration
A - Proteins 1	Lysozyme, human, rice recombinant	L1667	1.9	10 X
	Lactoferrin, human, rice recombinant	L1294	1.8	
	α 1-Acid Glycoprotein from bovine plasma	G3643	0.5	
B - Proteins 2	Albumin from human serum	A9511	0.2	10 X
	Mucin from bovine submaxillary glands	M3895	0.15	
	γ -Globulins from human blood	G4386	0.1	
C - Lipids	Cholesteryl linoleate	C0289	0.024	10 X
	Linalyl acetate	W263605	0.02	
	Glyceryl trioleate	T7140	0.016	
	Cholesterol	C8667	0.0016	
D - Salts and buffer agents	Sodium chloride	S5886	6.626	2 X
	Potassium chloride	P9541	1.716	
	Sodium bicarbonate	S6014	1.376	
	Lactic acid	252476	0.27	
	Calcium chloride, dihydrate	C3306	0.147	
	Sodium dihydrogen phosphate, monohydrate	S9638	0.1	
	MOPS	M3183	4.18	

2.3 Results

2.3.1 Screening of the fatty acids/ fatty acid derivatives

In total thirty-seven fatty acids/ fatty acid derivatives were tested in the screening process. With the compounds added directly to the solid agar media to a concentration of 1 mM, 20 (54 %) of the compounds completely inhibited growth of the test gonococcal strain (NCCP11945). The full list of results are listed in Table 2.5. For the saturated fatty acids, only medium chain (11-15) fatty acids were inhibitory. For monounsaturated fatty acids medium to long (14-18) were inhibitory. However, only oleic acid and elaidic acid were inhibitory from the 18 carbon chain length monounsaturated fatty acids which may suggest that the position and/or type of double bond are important. Poly-unsaturated fatty acids with a chain length of 18 and 20 were inhibitory. All monoglycerides were inhibitory. None of the dicarboxylic acids were inhibitory and in fact the bacteria grew very well in their presence. This indicates that the hydrophobic carbon chain plays a vital role in the inhibitory properties of the fatty acids. None of the propyl esters were inhibitory but the tested saturated fatty acid sodium salts were.

2.3.2 Measurement of bactericidal action of the fatty acids

Log reductions were completed using a concentration of 1 mM on the twenty compounds that had passed the screening. Only seven of these compounds were classed as being bactericidal at this concentration. The seven compounds were lauric acid, tridecanoic acid, myristoleic acid, palmitoleic acid, linolenic acid, monocaprin and sodium dodecanoate. Results of the log reductions can be found in Table 2.5. The candidates were further tested at lower concentrations against a total of nine gonococcal isolates, these results can be found in Figure 2.5 and Figure 2.6. The mean MBCs were estimated using the log reductions averaged over all nine isolates tested and are given in Table 2.5. The results indicate that myristoleic acid, palmitoleic acid, linolenic acid, monocaprin (all had MBC of 0.5) performed slightly better than lauric acid, tridecanoic acid, and sodium dodecanoate (MBC of 0.75). However, this may not be the case. Figure 2.7 displays the log reduction graphs for the seven candidates, with the bars representing the mean log reduction and the error bars the standard deviation over the nine isolates investigated. Any candidate that caused complete killing of all bacterial cells in the cell suspension would be given a log reduction score of six (scores of seven could not be given due to measurement limitation of the experiment) so therefore if one concentration caused total killing of all isolates testing no error bars would be present (as all log reduction values would be 6.0). Lauric acid, myristoleic acid, monocaprin, and sodium dodecanoate when tested at a concentration of 1 mM killed all gonococcal cells for all isolates. However, palmitoleic acid, linolenic acid, and tridecanoic acid did not. Two isolates (namely HPA 3 and HPA 4) in particular had some level of tolerance to palmitoleic acid and linolenic acid and were not completely inhibited by a 1 mM concentration of these compounds. Log reductions were

completed in triplicate with new batches of stock fatty acid of these fatty acids on these isolates to confirm this result. The results are shown in Figure 2.8.

Table 2.5 Results of all stages of the investigation of anti-gonococcal properties of fatty acids and derivatives tested.

Chemical Class	Organic Acid	Growth inhibition	Log reduction at 1 mM	MBC
Saturated Fatty Acids	Caprylic/Octanoic acid 8:0	No		
	Capric Acid/ Decanoic acid 10:0	No		
	Undecanoic acid 11:0	Yes	No - 0.2	
	Lauric acid 12:0	Yes	Yes >6	0.75 mM
	Tridecanoic acid 13:0	Yes	Yes >6	0.75 mM
	Myristic acid 14:0	Yes	No - 1.5	
	Pentadecylic acid 15:0	Yes	No - 0.5	
	Palmitic acid 16:0	No		
	Heptadecylic acid 17:0	No		
	Stearic acid 18:0	No		
Mono-unsaturated Fatty Acids	Myristoleic acid 14:1 c Δ 9	Yes	Yes - >6	0.5 mM
	Palmitoleic acid 16:1 c Δ 9	Yes	Yes - >6	0.5 mM
	Oleic acid 18:1 c Δ 9	Yes	No - 1.0	
	Elaidic acid 18:1 t Δ 9	Yes	No - 0.5	
	Petroselinic acid 18:1 c Δ 6	No		
	trans-Vaccenic acid 18:1 t Δ 11	No		
	cis-Vaccenic acid 18:1 c Δ 11	No		
	Erucic acid 22:1 c Δ 13	Yes	No - 0.0	
Poly-Unsaturated Fatty Acids	Sorbic acid 6:2 t Δ 2,4	No		
	Linoleic acid 18:2 c Δ 9,12	Yes	No - 1.6	
	Linolenic acid 18:3 c Δ 9,12,15	Yes	Yes - >6	0.5 mM
	Arachidonic Acid 20:4 c Δ 5,8,11,14	Yes	NT	
Monoglycerides	1-Octanoyl-rac-Glycerol (C8MG)	Yes	No - 0.0	
	Monocaprin (C10MG)	Yes	Yes - >6	0.5 mM
	Monolaurin (C12MG)	Yes	No - 3.4	
	Monomyristin (C14MG)	Yes	No - 0.6	
Dicarboxylic Acids	Succinic acid	No		
	Adipic acid	No		
	Pimelic acid	No		
	Suberic acid	No		
	Azelaic acid	No		
	Sebacic acid	No		
Ricinoleic acid	Ricinoleic acid	Yes	No - 3.5	
Esters	Isopropyl myristate	No		
	Isopropyl palmitate	No		
Fatty Acid Sodium Salt	Sodium dodecanoate	Yes	Yes - >6	0.75 mM
	Sodium myristate	Yes	No - 0.5	

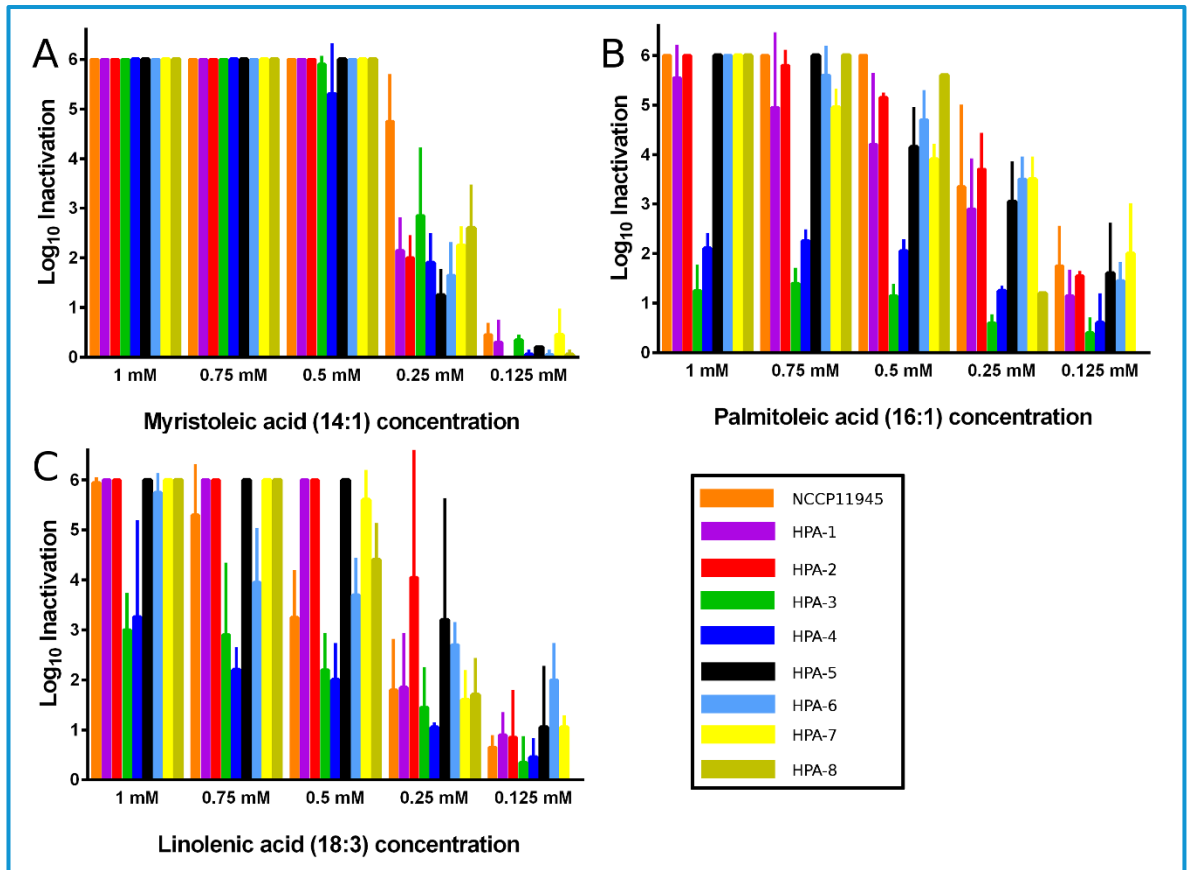


Figure 2.5 Individual log reduction results of the nine *N. gonorrhoeae* isolates when tested against the unsaturated carbon-chain drug candidates. (A) Myristoleic acid log reductions. (B) Palmitoleic acid log reductions. (C) Linolenic acid log reductions. The error bars represent the standard deviation over the duplicate experiments. Note that HPA-2 and HPA-3 had decreased susceptibility to palmitoleic acid and linolenic acid.

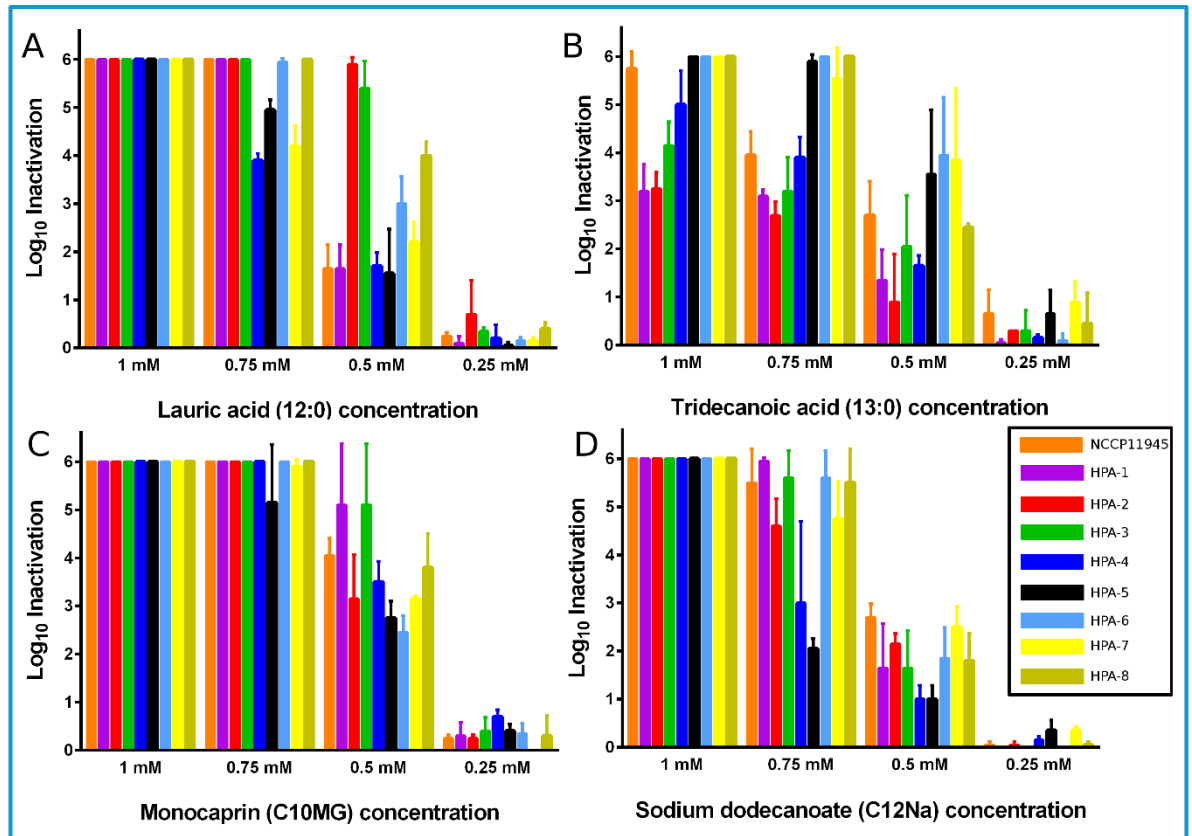


Figure 2.6 Individual log reduction results of the nine *N. gonorrhoeae* isolates when tested against the saturated carbon-chain drug candidates. (A) Lauric acid log reductions. (B) Tridecanoic acid log reductions. (C) Monocaprin log reductions. (D) Sodium dodecanoate log reductions. The error bars represent the standard deviation over the duplicate experiments.

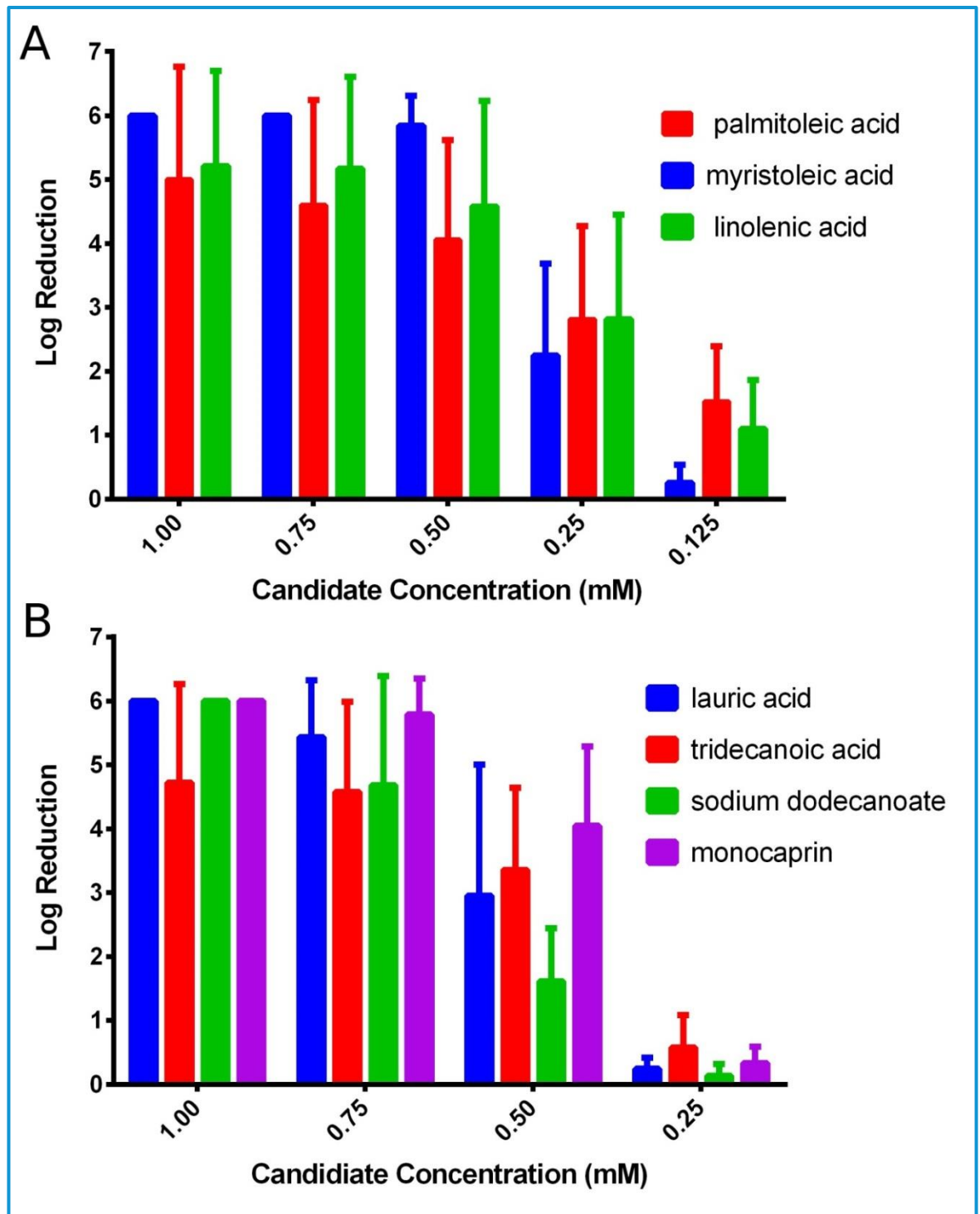


Figure 2.7 Summary of log reduction assays conducted in culture medium. The results shown are mean log reductions with standard deviations for each of the seven candidates assayed against nine different gonococcal isolates. (A) Results for the unsaturated carbon chain fatty acids. (B) Results for the saturated-carbon chain fatty acids and fatty acid derivatives. The maximum value for the log reduction was six, so samples at this value effectively killed all bacteria in the sample. The error bars for some samples cover over 2 log₁₀, this demonstrates the variation of susceptibility between isolates.

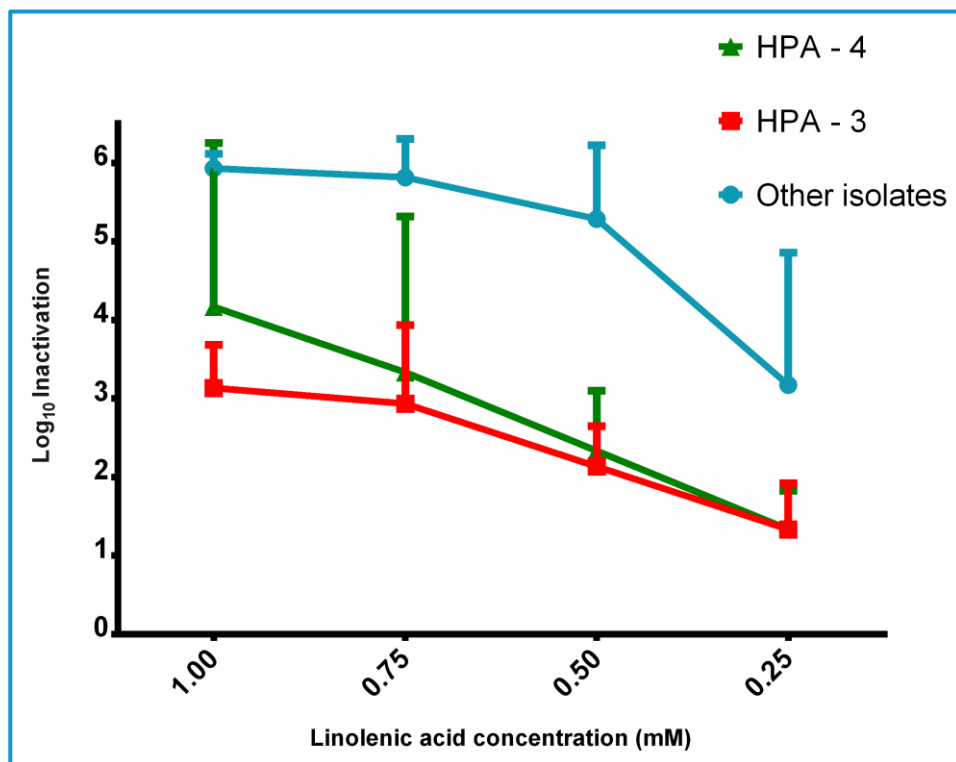


Figure 2.8 Summary of repeated log reduction assays of linolenic acid. Comparison of linolenic acid log reductions of two clinical isolates tested in triplicate against mean of all other gonococcal isolates ($n=7$). Error bars represent positive Standard Deviation. The susceptibility to linolenic acid appeared to be highly variable as demonstrated by size of the error bars of isolate HPA-4. The reason for this variation is unknown but may be due to the state of the bacterial suspension before testing.

2.3.3 BCOP test

The cornea's primary function is to protect the eye. This means that it is in fact impermeable to many substances. The stroma in particular will not allow lipids to permeate through the cornea. The fluorescein dye as well does not permeate the cornea making it ideal to assess corneal damage as any increase in permeability is due to tissue damage of the outer layers of the cornea.

Each of the seven drug candidates were tested on three separate occasions. Photographs of the post-exposure eye balls, fluorescein staining and histology of the cornea are shown in Figure 2.9 to Figure 2.18. The mean cumulative score of each of the compounds and the controls are shown in Figure 2.19. As expected, the sodium hydroxide and acetone controls caused significant tissue damage to the cornea. The sodium hydroxide control caused visible opacity of the cornea that could be viewed even before fluorescein staining. Post-staining showed that the fluorescein was able to penetrate the cornea indicating extensive corneal tissue damage. The acetone control caused less marked opacity which was only visible pre-staining via close examination.

The eyeballs which were exposed to the seven candidates on the other hand were similar to the negative control. These had no visible opacity and had little fluorescein permeability. However, tridecanoic acid, linolenic acid, monocaprin and sodium dodecanoate did give a small amount of fluorescein staining around where the rubber application ring was placed. With tridecanoic acid, and sodium dodecanoate this only occurred in one of the three experiments but twice for linolenic acid and monocaprin. This damage could have been caused when removing the application ring and the mean BCOP cumulative score of these four candidates was still lower than 0.5 which means they are classed as non-irritant.

2.3.4 Histological examination of bovine corneas

The bovine corneas from all tested eyeballs were removed and prepared for histological examination by H&E staining. Histological examination of the sodium hydroxide and acetone samples were also completed to show tissue damage caused by strong and moderate irritant. These two controls had different cytopathic effects (see Figure 2.9 to Figure 2.18). The sodium hydroxide caused a breakdown in the epithelial layer integrity with the layer become thinner, more condensed with more intensive haematoxylin staining. Cellular structures were also hard to make out in comparison to the negative control. The epithelial layer had also detached from the stroma in places and was completely absent in other places.

The sodium hydroxide control also caused stromal swelling with an observed increased thickness in comparison with non-treated sections of the cornea and in relation to negative controls. However, this was only an observation and not proven statistically as stroma thickness varies greatly between animals. In contrast the acetone control did not cause epithelial layer detachment.

The main cytopathic effect of exposure to the acetone to the epithelial cell types was cytoplasmic vasuolation predominantly in basal and wing cells. There was none of these cytopathic effects seen in any of the slides of the seven tested candidates. The test samples appear identical to the negative controls.

2.3.5 HET-CAM

The candidates were applied to CAMs and left for 5 minutes. The samples were all tested at a concentration of 1 mg/L. This would give a concentration range from 3.6 mM for linolenic acid to 5 mM for lauric acid. As all candidates were totally bactericidal at a concentration of 1 mM, the testing concentration would demonstrate the safety of the candidates. Concentrations higher than this could not be used as the candidates would not be soluble at higher concentrations and even dispersal of the candidate would not be achieved.

Photographs of the CAM before exposure and at each time point for all candidates are given Appendix 1.1-1.11 but a summary of pictures are given in Figure 2.20. The cumulative scores of all the controls and test candidates are given in Figure 2.21. Sodium hydroxide produced the most notable effect upon the CAM with hyperaemia and haemorrhaging seen within the first 30 seconds. The acetone also caused hyperaemia and haemorrhaging in the first 30 seconds but not to the same extent as the sodium hydroxide and did not cause any coagulation. Sodium hydroxide caused coagulation but only after the two minute time-point. Propylene glycol was a good control as it only caused tissue damage at the 2 minute time-point onward. None of the candidates caused any visible tissue damage to the CAM over the five minute exposure time.

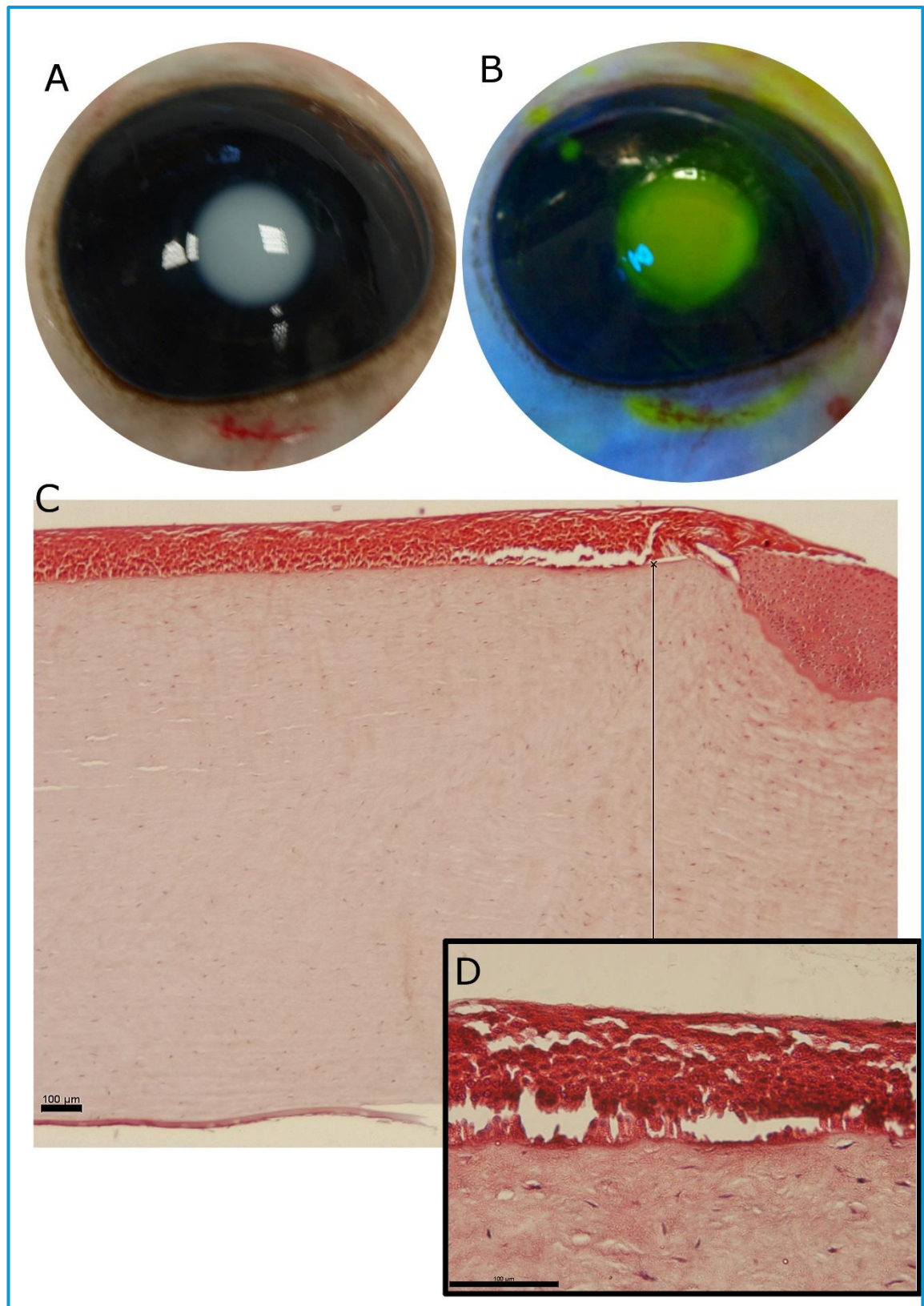


Figure 2.9 Bovine corneal opacity and permeability test results for 0.5 M sodium hydroxide. (A) Photograph of the eyeball after the exposure. The cornea appears completely opaque. (B) Photograph of the eye after fluorescein staining under a cobalt blue filter light. The fluorescein has completely penetrated into the cornea indicating extensive damage. (C and D) Photomicrographs of H&E stained section of exposure site. The bars in the bottom left hand corner of each panel are a 100 µm bar. The corneal epithelial layer has become thinner and has detached from the stroma in places.

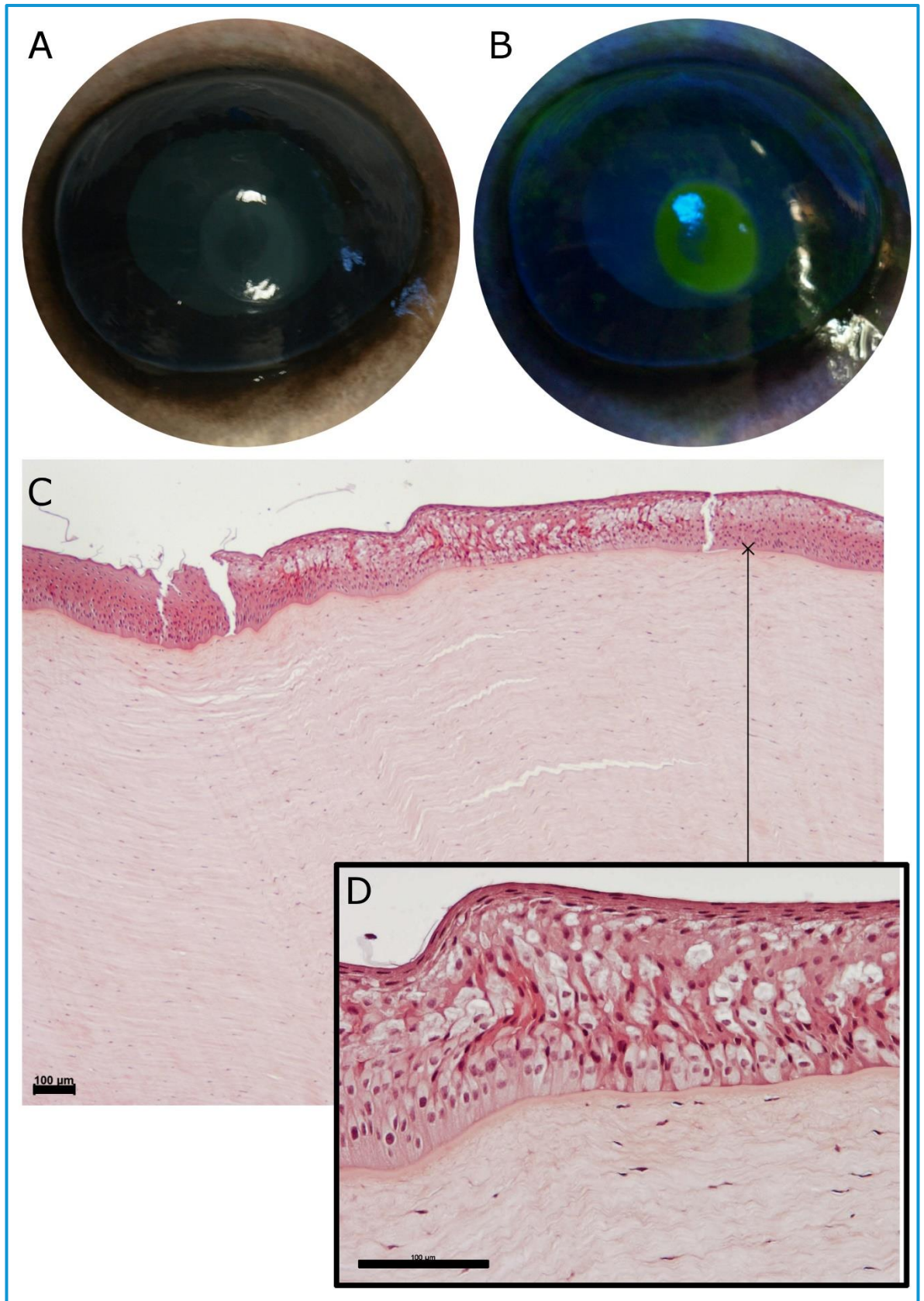


Figure 2.10 Bovine corneal opacity and permeability test results for acetone. (A) Photograph of the eye ball after the exposure. The cornea appears slightly opaque. (B) Photograph of the eye after fluorescein staining under a cobalt blue filter light. The fluorescein has penetrated into the cornea indicating loss of integrity. (C and D) Photomicrographs of H&E stained section of exposure site. The bars in the bottom left hand corner of each panel are a 100 μm bar. Cell of corneal epithelial layer now have vacuoles present.

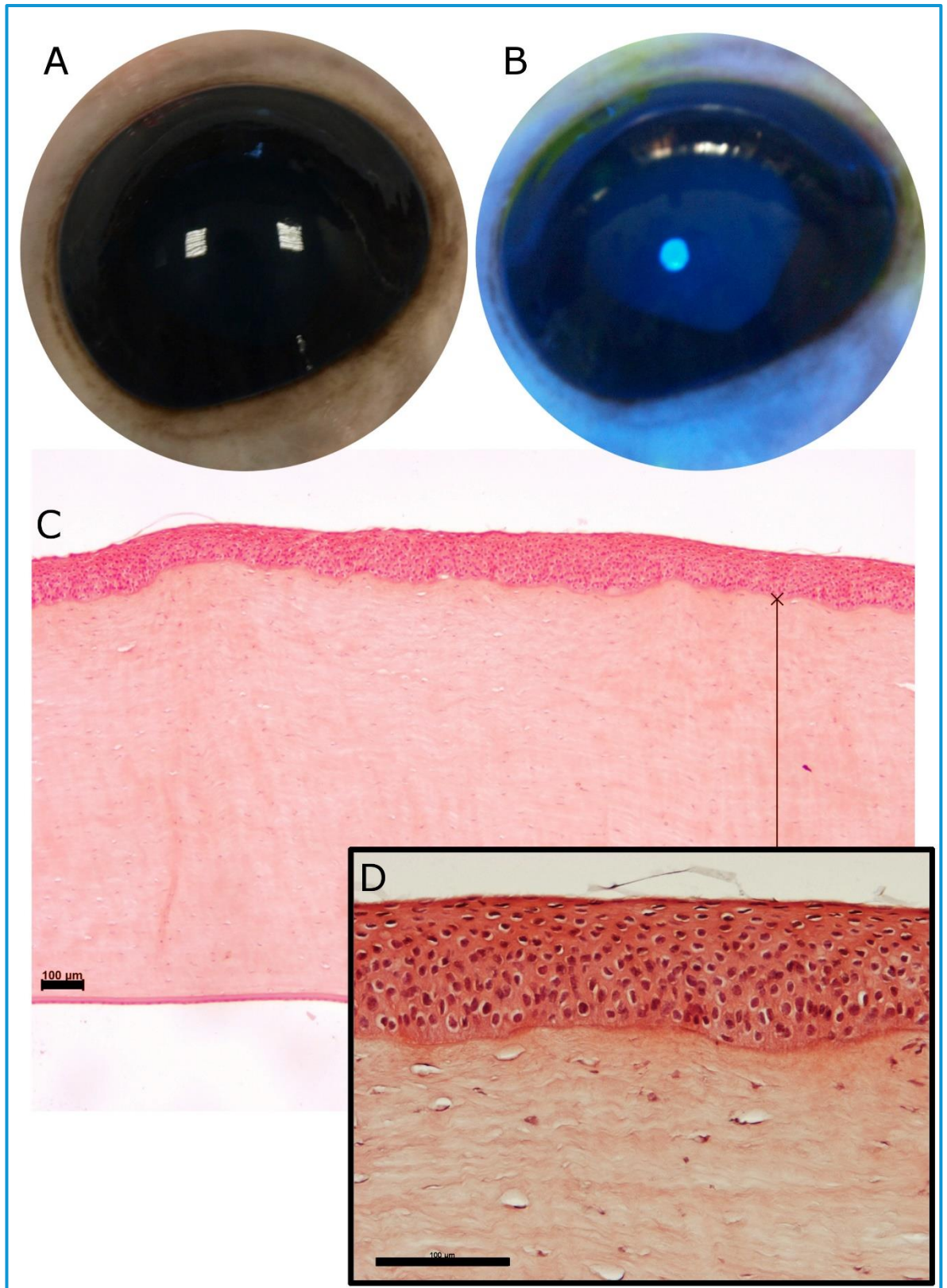


Figure 2.11 . Bovine corneal opacity and permeability test results for saline. (A) Photograph of the eye ball after the exposure. The cornea appears normal. (B) Photograph of the eye after fluorescein staining under a cobalt blue filter light. The fluorescein has been washed off and couldn't penetrate the cornea. (C and D) Photomicrographs of H&E stained section of exposure site. The bars in the bottom left hand corner of each panel are a 100 μm bar. Cell of corneal epithelial layer look normal.

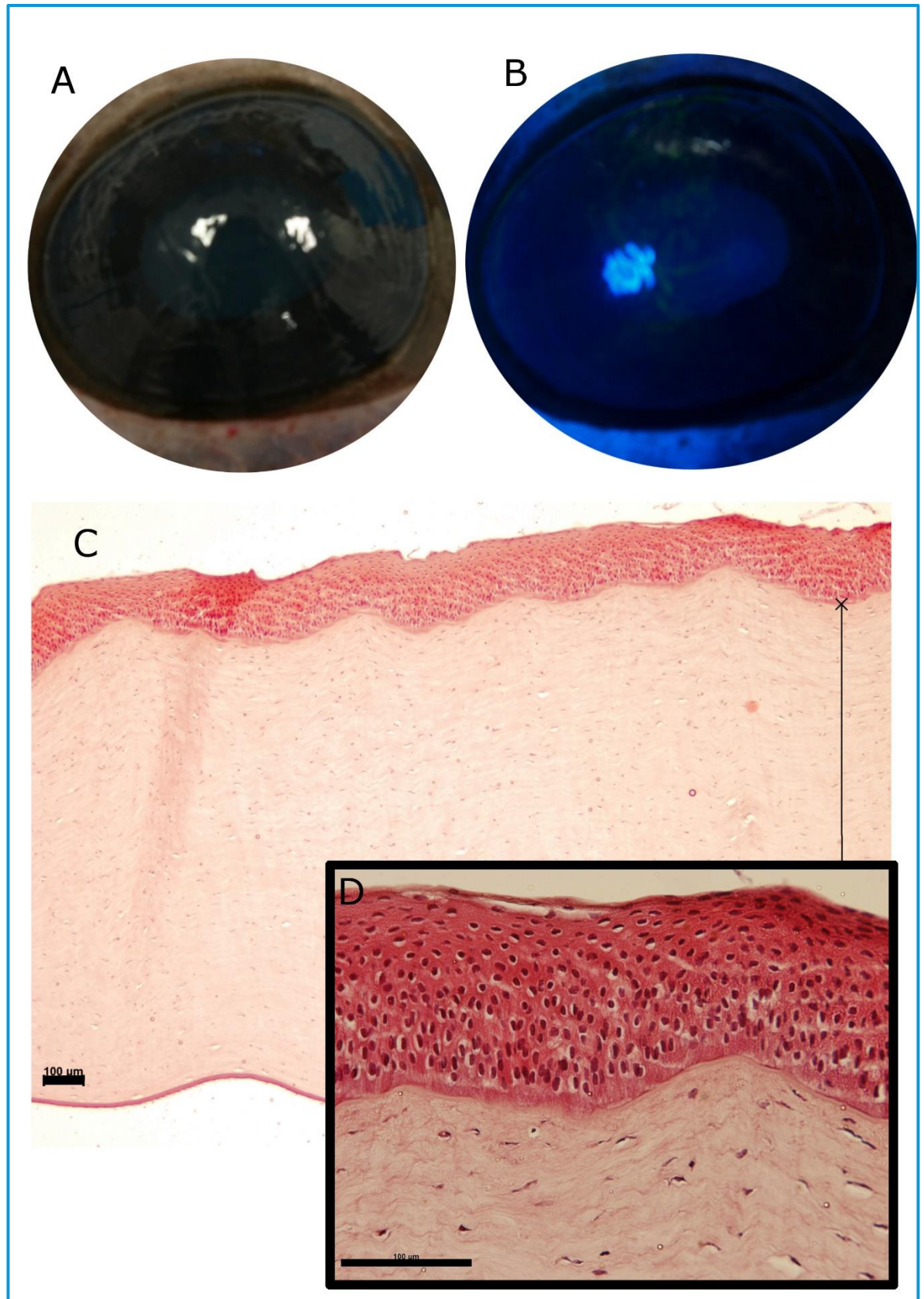


Figure 2.12 Bovine corneal opacity and permeability test results for lauric acid. (A) Photograph of the eye ball after the exposure. The cornea appears normal. (B) Photograph of the eye after fluorescein staining under a cobalt blue filter light. The fluorescein has been washed off and couldn't penetrate the cornea. (C and D) Photomicrographs of H&E stained section of exposure site. The bars in the bottom left hand corner of each panel are a 100 μm bar. Cell of corneal epithelial layer look normal.

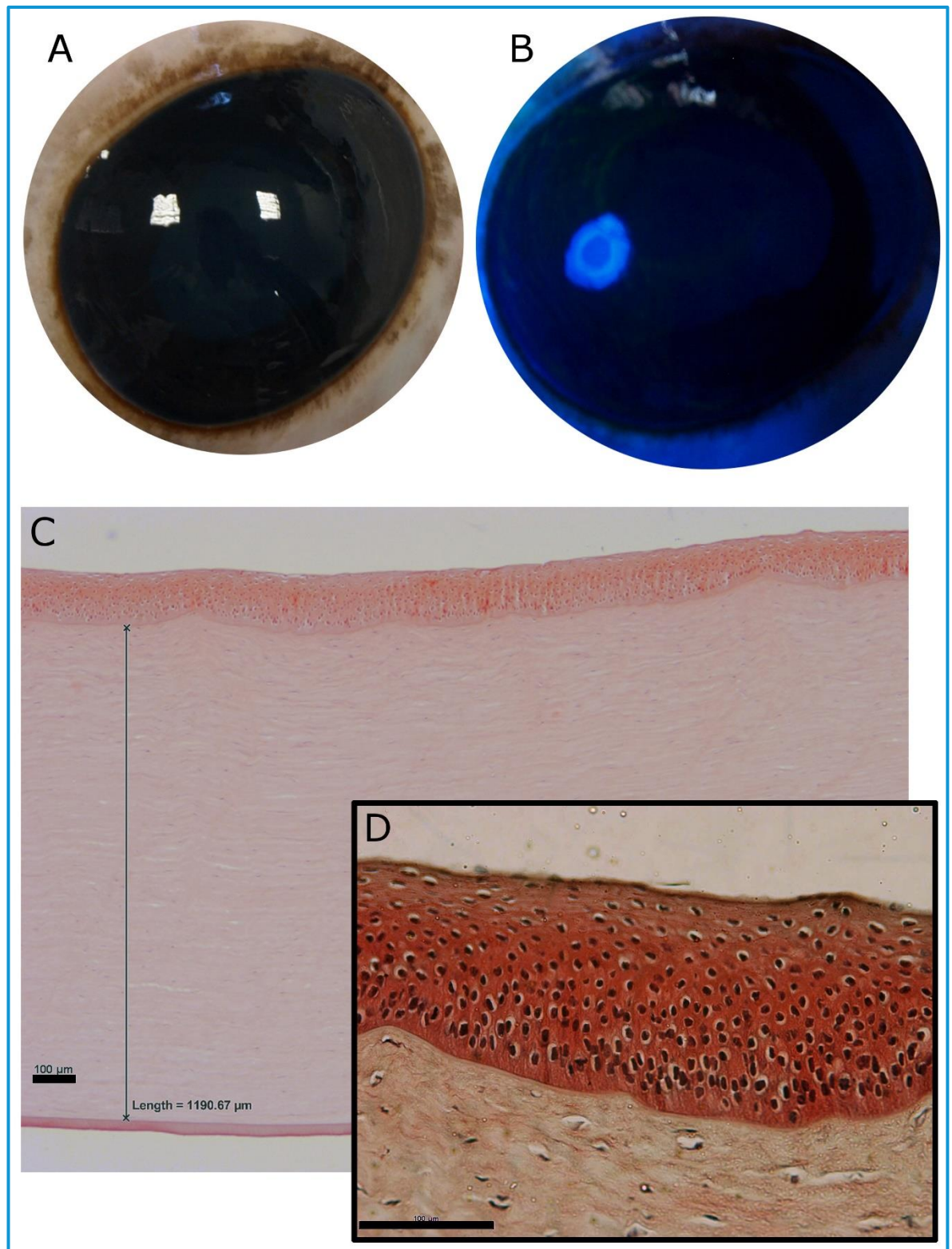


Figure 2.13 Bovine corneal opacity and permeability test results for tridecanoic acid. (A) Photograph of the eye ball after the exposure. The cornea appears normal. (B) Photograph of the eye after fluorescein staining under a cobalt blue filter light. The fluorescein has been washed off and couldn't penetrate the cornea. (C and D) Photomicrographs of H&E stained section of exposure site. The bars in the bottom left hand corner of each panel are a 100 μm bar. Cell of corneal epithelial layer look normal.

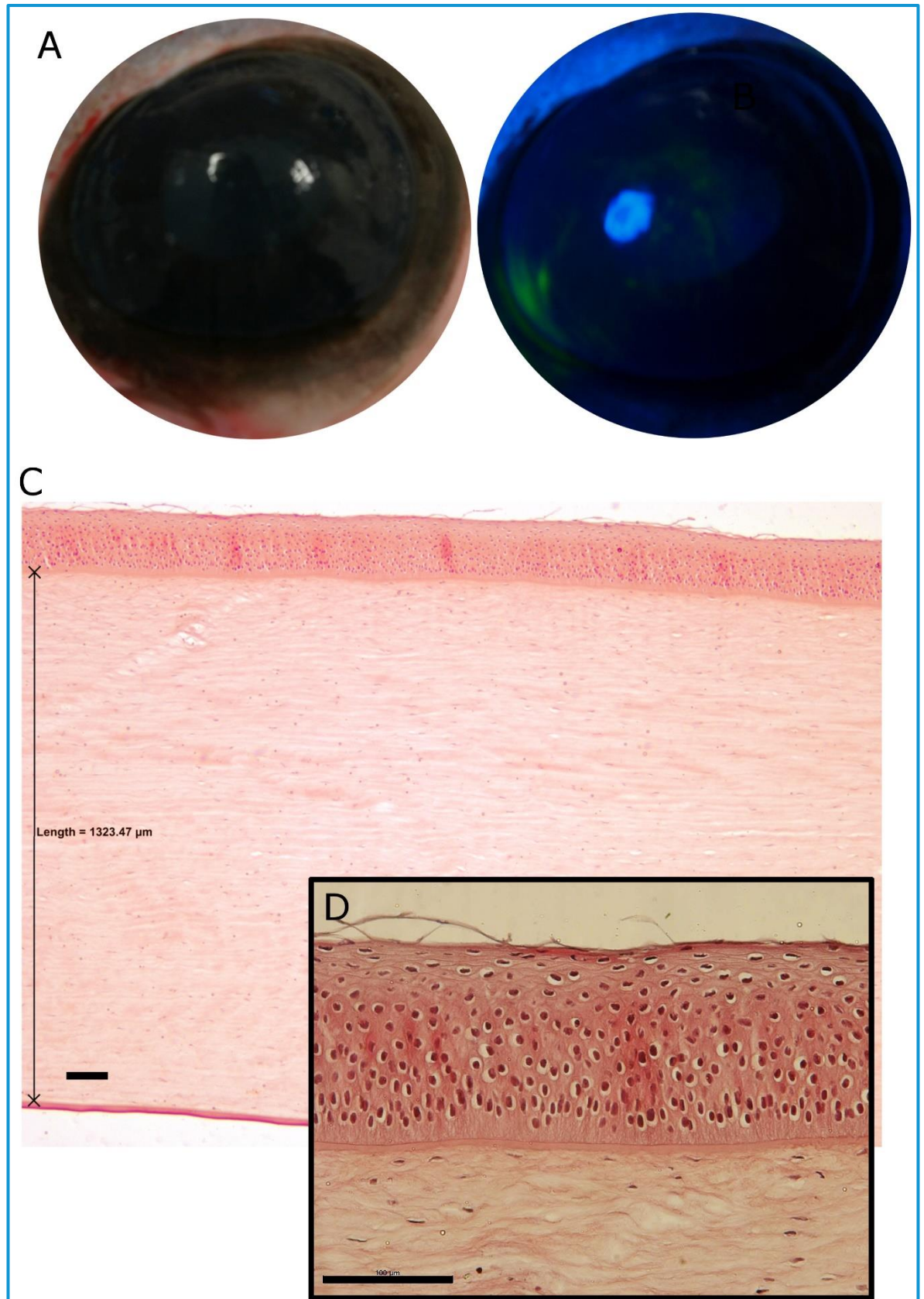


Figure 2.14 Bovine corneal opacity and permeability test results for myristoleic acid (14:1). (A) Photograph of the eye ball after the exposure. The cornea appears normal. (B) Photograph of the eye after fluorescein staining under a cobalt blue filter light. The fluorescein has been washed off and couldn't penetrate the cornea. (C and D) Photomicrographs of H&E stained section of exposure site. The bars in the bottom left hand corner of each panel are a 100 μm bar. Cell of corneal epithelial layer look normal.

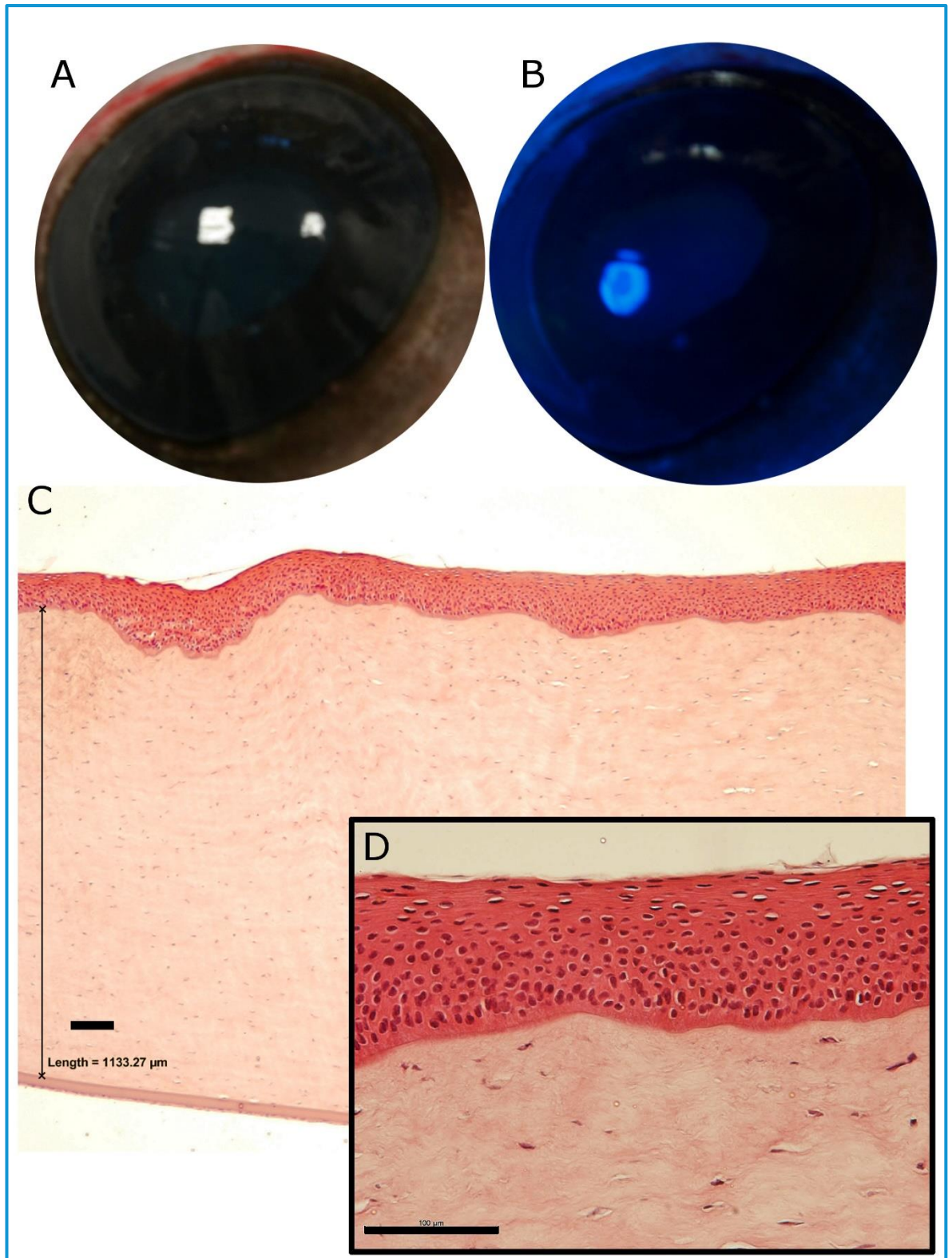


Figure 2.15 Bovine corneal opacity and permeability test results for palmitoleic acid (16:1). (A) Photograph of the eye ball after the exposure. The cornea appears normal. (B) Photograph of the eye after fluorescein staining under a cobalt blue filter light. The fluorescein has been washed off and couldn't penetrate the cornea. (C and D) Photomicrographs of H&E stained section of exposure site. The bars in the bottom left hand corner of each panel are a 100 μm bar. Cell of corneal epithelial layer look normal.

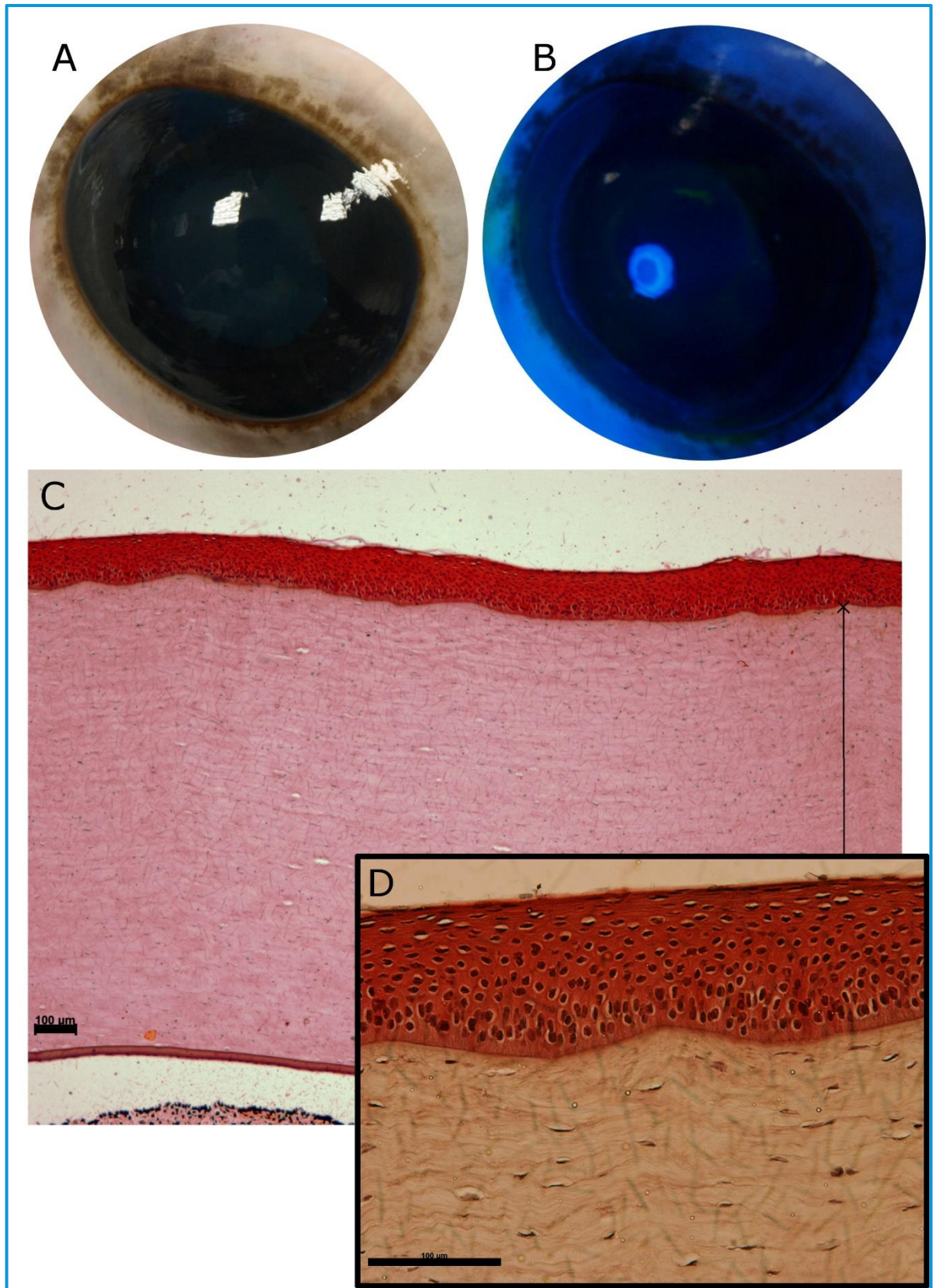


Figure 2.16 Bovine corneal opacity and permeability test results for linolenic acid (18:3). (A) Photograph of the eye ball after the exposure. The cornea appears normal. (B) Photograph of the eye after fluorescein staining under a cobalt blue filter light. The fluorescein has been washed off and couldn't penetrate the cornea. (C and D) Photomicrographs of H&E stained section of exposure site. The bars in the bottom left hand corner of each panel are a 100 µm bar. Cell of corneal epithelial layer look normal.

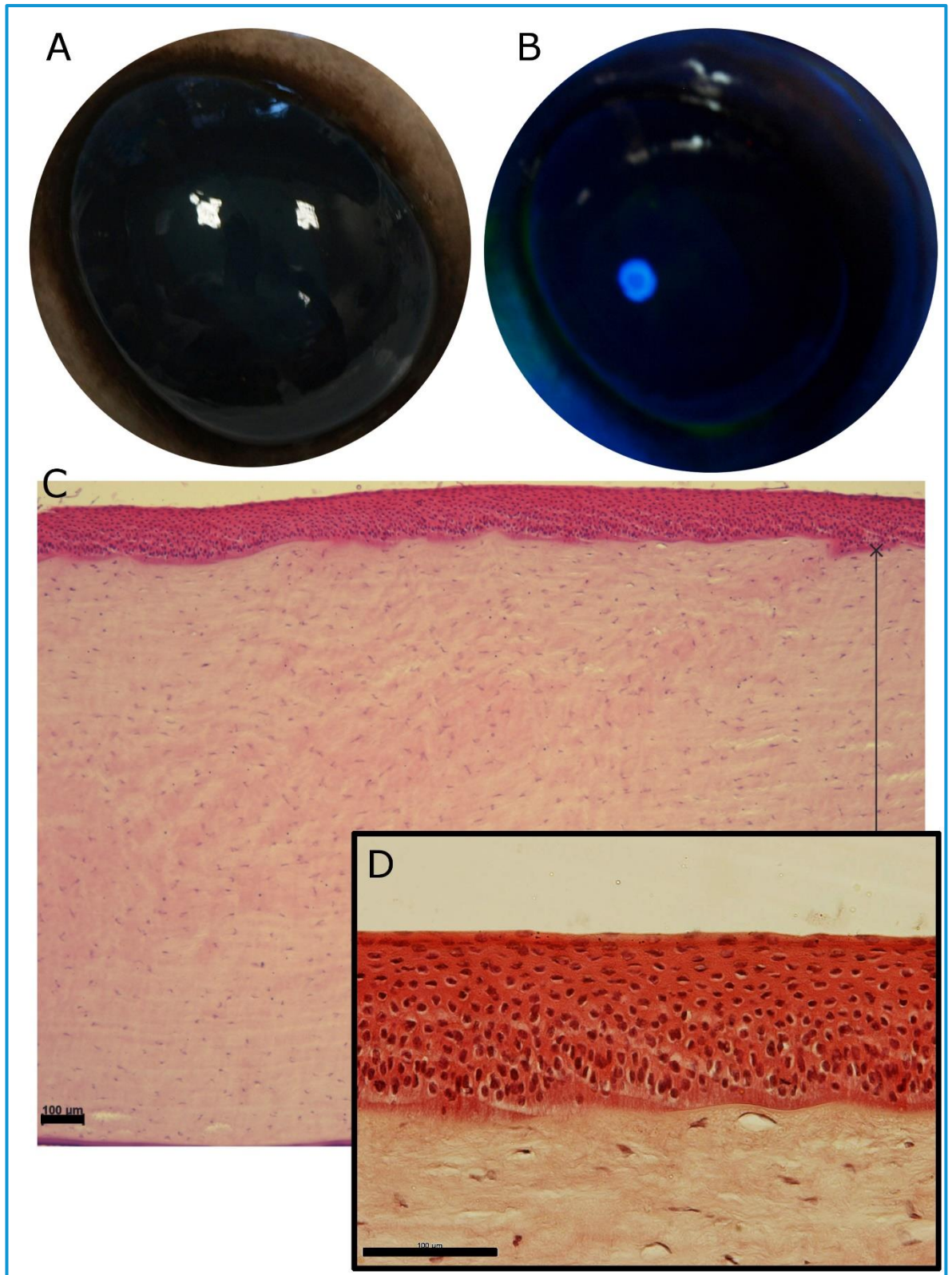


Figure 2.17 Bovine corneal opacity and permeability test results for monocaprin (10:0MG). (A) Photograph of the eye ball after the exposure. The cornea appears normal. (B) Photograph of the eye after fluorescein staining under a cobalt blue filter light. The fluorescein has been washed off and couldn't penetrate the cornea. (C and D) Photomicrographs of H&E stained section of exposure site. The bars in the bottom left hand corner of each panel are a 100 μm bar. Cell of corneal epithelial layer look normal.

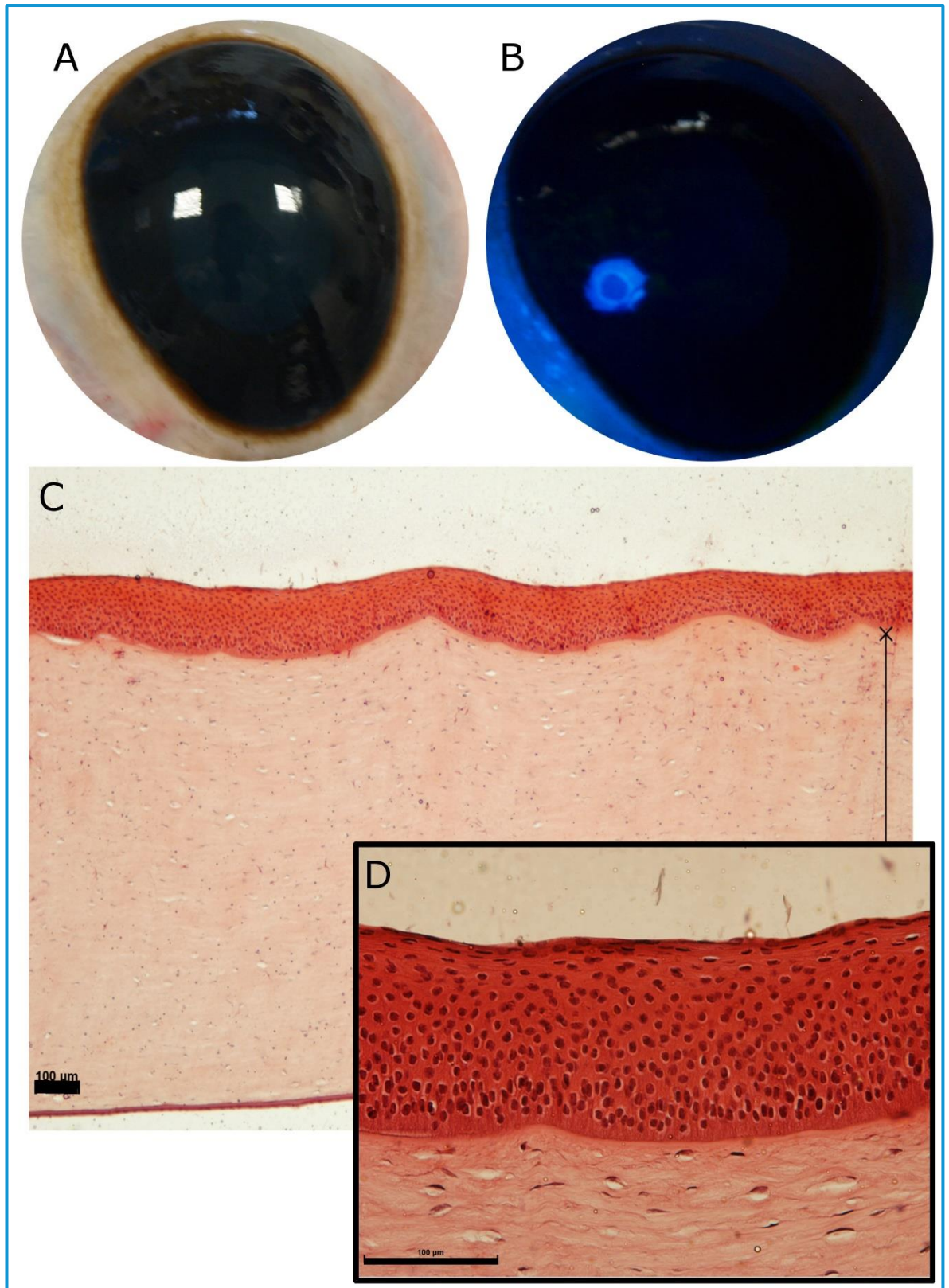


Figure 2.18 Bovine corneal opacity and permeability test results for sodium dodecanoate (12:0Na). (A) Photograph of the eye ball after the exposure. The cornea appears normal. (B) Photograph of the eye after fluorescein staining under a cobalt blue filter light. The fluorescein has been washed off and couldn't penetrate the cornea. (C and D) Photomicrographs of H&E stained section of exposure site. The bars in the bottom left hand corner of each panel are a 100 μm bar. Cell of corneal epithelial layer look normal.

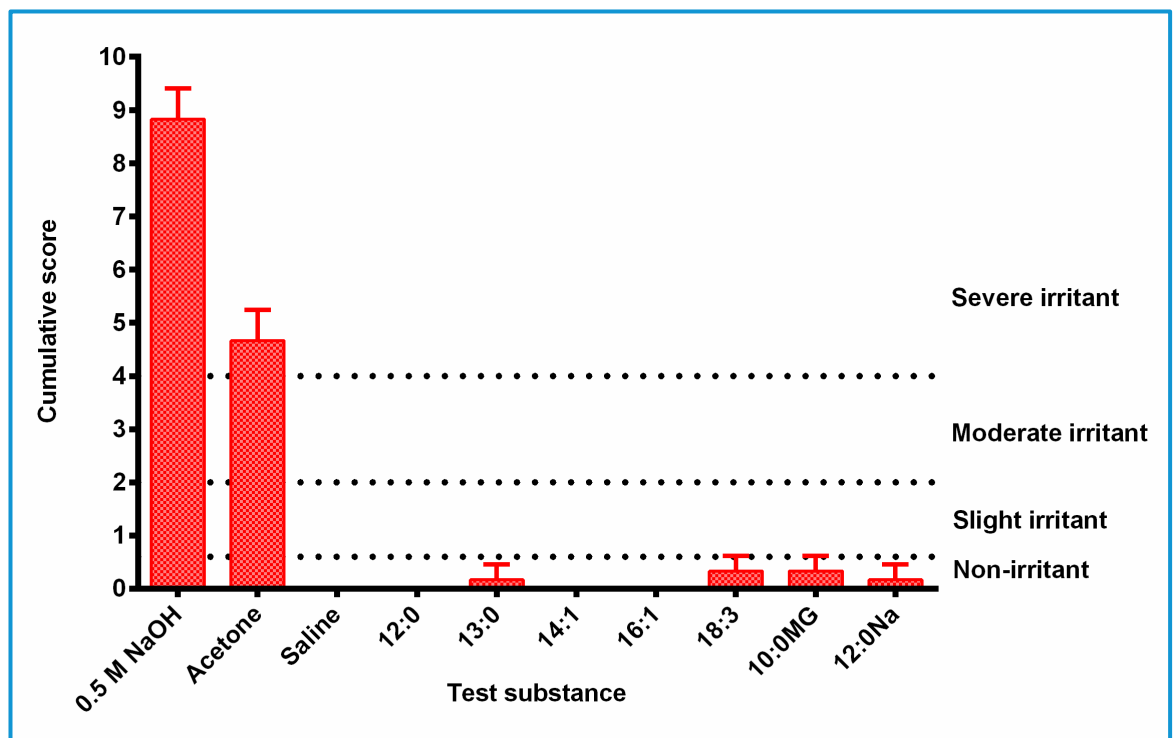


Figure 2.19 Summary of the cumulative BCOP scores of the seven selected candidates and the controls. Error bars represent standard deviation (n=3). Abbreviations are; lauric acid (12:0), tridecanoic acid (13:0), myristoleic acid (14:1), palmitoleic acid (16:1), linolenic acid (18:3), monacprin (12:0MG), and sodium dodecanoate (12:0Na).

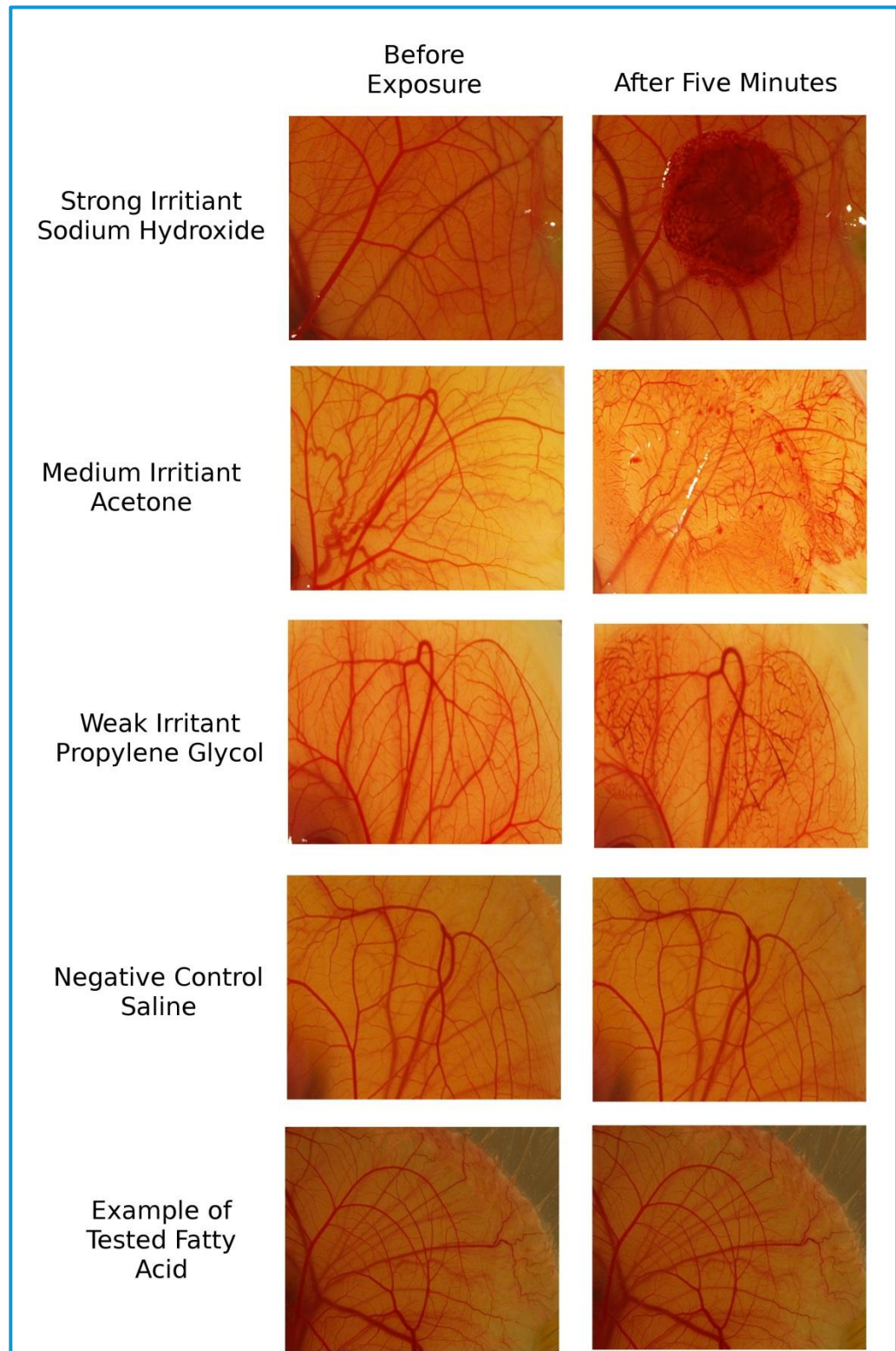


Figure 2.20 Example photographs from HET-CAM. Photographs on the left were taken before exposure and the ones on the right were taken of the same spot five minutes after exposure. The top three pictures are positive controls as they cause hyperaemia and haemorrhaging at the site of application. The tested candidates (bottom picture is an example) did not cause a reaction.

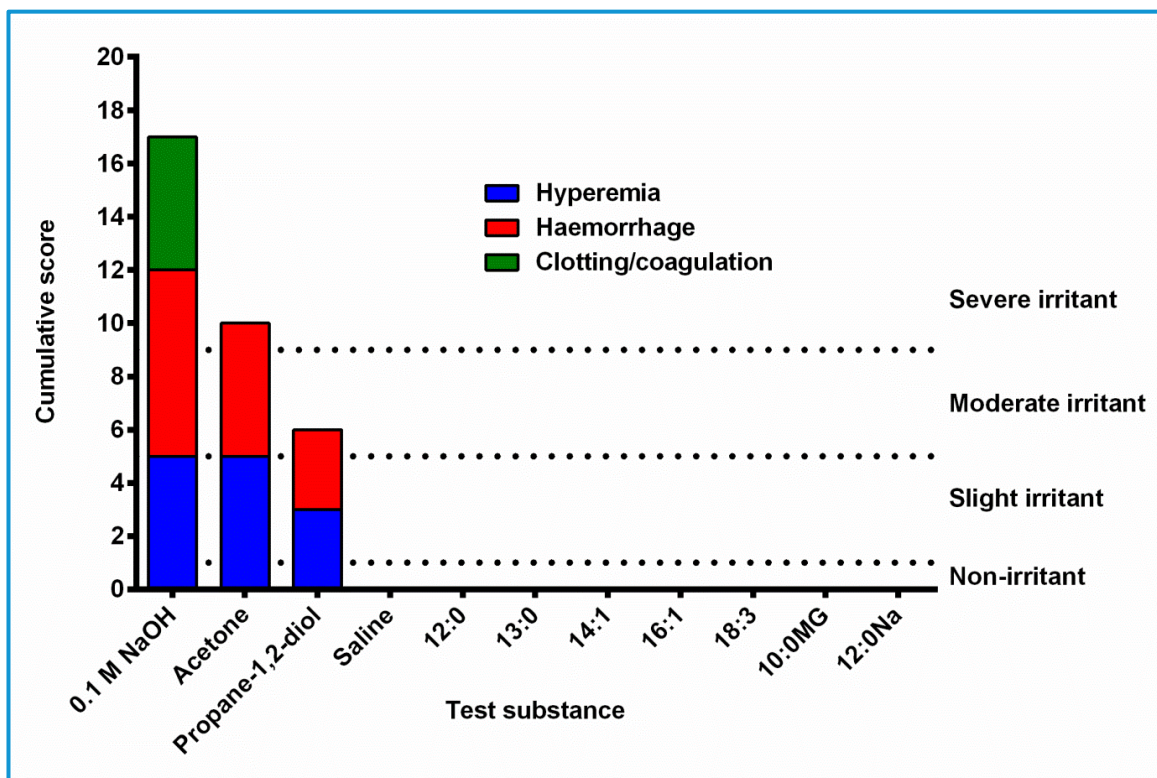


Figure 2.21 HET-CAM results. A graph of the cumulative scores of controls and test substances from the HET-CAM. The positive controls caused visible damage to the CAM but the negative control and samples did not. All samples were tested in triplicate. Abbreviations are; lauric acid (12:0), tridecanoic acid (13:0), myristoleic acid (14:1), palmitoleic acid (16:1), linolenic acid (18:3), monacaprln (10:0MG), and sodium dodecanoate (12:0Na).

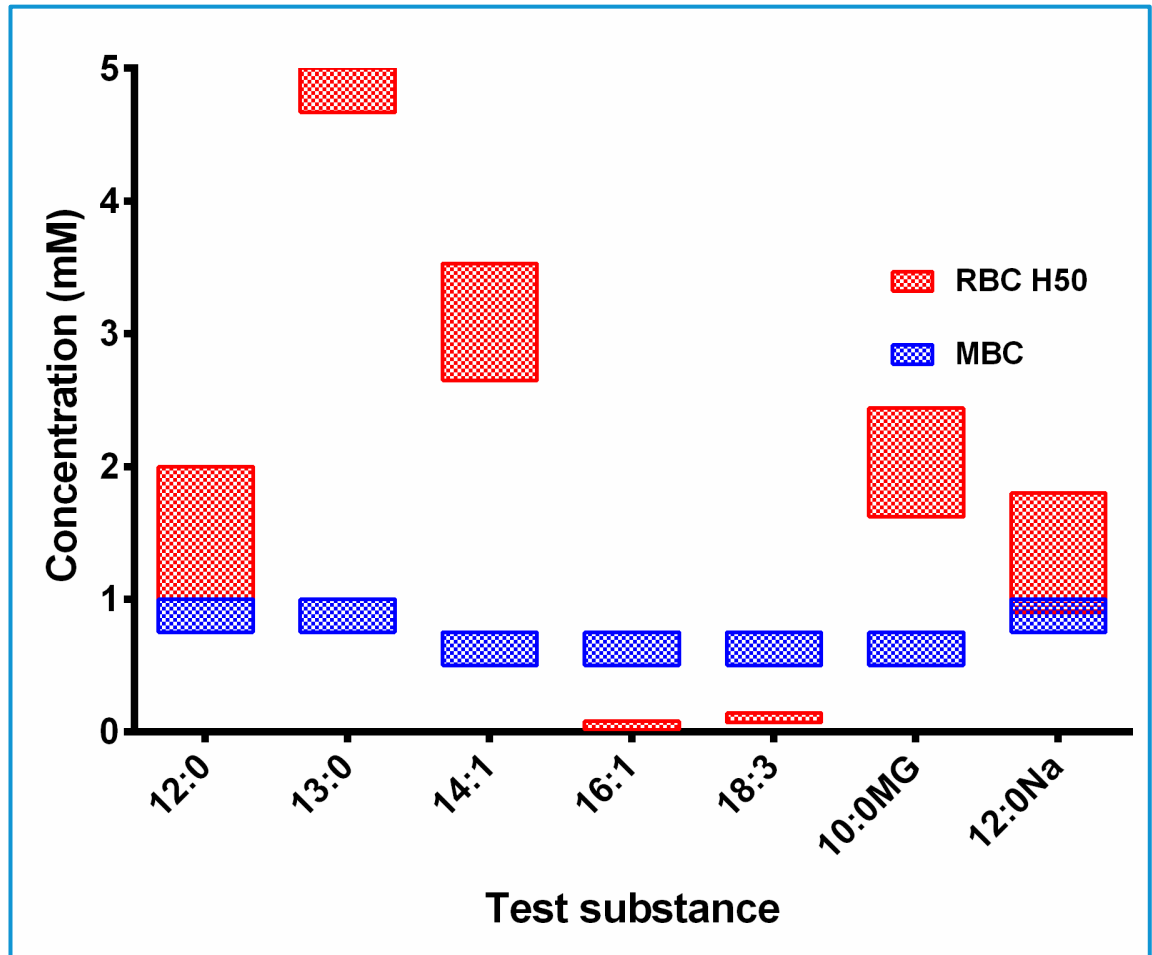


Figure 2.22 Comparison of red blood cell lysis assay results against minimum bactericidal concentration of the drug candidates. H₅₀ values are defined by the concentration range to lyse 50 % of the red blood cells. Two candidates had MBC greater than their H₅₀ results (16:1 and 18:3), all others had H₅₀ greater than their MBCs. Abbreviations are; lauric acid (12:0), tridecanoic acid (13:0), myristoleic acid (14:1), palmitoleic acid (16:1), linolenic acid (18:3), monolaurin (12:0MG), and sodium dodecanoate (12:0Na).

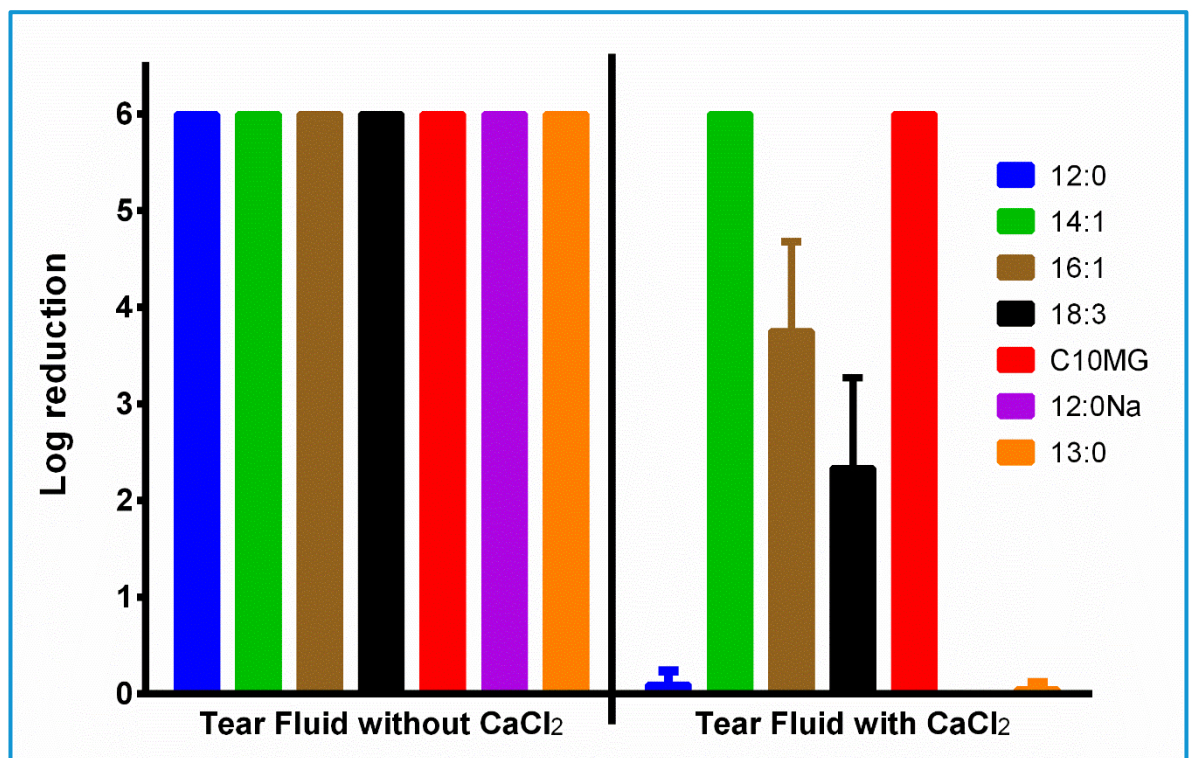


Figure 2.23 Log reductions in simulated tear fluid with or without calcium. Calcium ions (CaCl_2 , 1 mM) present in the simulated tear fluid inactivated the antimicrobial effects of lauric acid, tridecanoic acid, and sodium dodecanoate. The calcium ions also effected palmitoleic acid and linolenic acid but to a lesser extent. Myristoleic acid and moncaprin were unaffected. Abbreviations are; lauric acid (12:0), tridecanoic acid (13:0), myristoleic acid (14:1), palmitoleic acid (16:1), linolenic acid (18:3), moncaprin (10:0MG), and sodium dodecanoate (12:0Na). Error bars represent one standard deviation based on two separate experiments.

2.3.6 RBC lysis assay

The RBC lysis assay gave considerably different results to the HET-CAM and BCOP. The RBC lysis assay results are shown in Figure 2.22. The larger unsaturated fatty acids, palmitoleic acid and linolenic acid, required the lowest concentrations to cause haemolysis in 50 % of the RBCs. The best performer was tridecanoic acid which didn't cause any haemolysis even at the highest concentration tested. Surprisingly, the twelve carbon-chain unsaturated sodium salt gave very similar results to that of lauric acid. Also presented in Figure 2.22 are the MBC values of each of the candidates. This allows the comparison of the anti-bacterial properties of the candidate to possible ocular irritation effects. It is clear that tridecanoic acid, myristoleic acid and monocaprin performed the best with H_{50} values over their MBC values.

2.3.7 Log reductions of selected fatty acids in simulated tear fluid

The anti-gonococcal properties of the seven selected fatty acids were tested in the simulated tear fluid. The addition of 1 mM of lauric acid, tridecanoic acid, or sodium dodecanoate gave no reduction in cell number after exposure, a complete reversal from when they were tested in culture medium (see Figure 2.23). Palmitoleic acid and linolenic acid were also affected but to a lesser extent. Upon addition of some the candidates, lauric acid, tridecanoic acid, or sodium dodecanoate in particular, it was observed that the clear simulated tear fluid would turn cloudy. This cloudiness was obviously the fatty acids precipitating out of solution when added to the simulated tear fluid. After some research calcium ions were identified as the possible cause. The simulated tear fluid contains a concentration of 1 mM $CaCl_2$. A batch of simulated tear fluid was made omitting calcium chloride and the anti-gonococcal properties were completely restored (Figure 2.23). All candidates caused complete killing in the simulated tear fluid without calcium chloride but only myristoleic acid and monocaprin caused complete killing in the full simulated tear fluid.

2.4 Discussion

2.4.1 Selection of fatty acid candidates

During the course of this study, thirty-seven fatty acid or fatty acid derivatives have been screened for anti-gonococcal properties. From this, seven were selected for further analysis. These candidates were lauric acid, tridecanoic acid, myristoleic acid, palmitoleic acid, linolenic acid, moncaprin and sodium dodecanoate. All these candidates demonstrated they possess powerful, fast-acting bactericidal activity against *N. gonorrhoeae*. However, further log reductions on European clinical isolates showed that two of these isolates were not completely inactivated by 1 mM palmitoleic acid (16:1) or linolenic acid (18:3). This may suggest that these two isolates (HPA 3 and HPA 4) have protection mechanisms against these two fatty acids. The exact mechanism for this increased tolerance to these fatty acids is not known and was not investigated further. However, as the *farAB*-encoded efflux pump of *N. gonorrhoeae* mediates resistance certain long chain fatty acids such as linoleic acid (18:2) and palmitic acid (16:0), the increased tolerance maybe linked to this efflux system [148]. The log reduction for these isolates against linolenic acid was still between 3 and 4 log₁₀ so they still have good activity against *N. gonorrhoeae*. Also, the two isolates were still susceptible to other fatty acids.

2.4.2 Irritation assays

The seven candidates were tested in three irritation assays, all candidates were classed as non-irritants by HET-CAM and BCOP. However, for the RBC lysis assay, the H₅₀ value of palmitoleic acid and linolenic acid was lower than their MBC. These two values are not directly comparable as the methods they use are very different. The log reductions (used to measure the MBC) uses an incubation time of two minutes while the RBC lysis assays H₅₀ value incubates the drug candidate with the RBCs for one hour. If a liquid dosage form is used in formulation then the drug may only be in contact with the ocular surface for a few minutes so the one hour incubation time of the RBC lysis assay is not representative. The RBC lysis assay was performed in accordance to EURL ECVAM guidelines. However, EURL ECVAM recommends two different protocols for performing the assay [225, 227]. The protocol used in this study uses an incubation time of 1 hour at room temperature with shaking while another protocol uses an incubation time of 10 minutes at room temperature with shaking. The protocol selected for this study was preferred as it gave more experiment detail on use of a vehicle (DMSO was used in these experiments) to aid the solubility of candidates. Some researchers have chosen to use this protocol but reduce the incubation time [214]. However, as these two fatty acids that performed poorly in the RBC lysis assay also had some form of tolerance in some gonococcal isolates, they were excluded from further consideration.

2.4.3 Testing in simulated tear fluid

Testing in simulated tear fluid revealed a problem with the use of the saturated fatty acids candidates (lauric acid, tridecanoic acid, and sodium dodecanoate). These fatty acids were completely ineffective in the presence of calcium ions. On further research, these metal ions would cause dimerization of fatty acid molecules forming a highly hydrophobic molecule that would precipitate out of solution. This would appear to make them completely ineffective against gonococcal cells. The inhibition of the antimicrobial effects of fatty acids by calcium ions has also been reported previously but could be limited by lowering the pH [164, 181]. Lowering the pH could be used in a liquid dosage form but would not be suitable for any formulation that would have extended contact time with the ocular surface. Why mainly saturated carbon-chain fatty acids were affected is unknown. The effect on unsaturated carbon chain fatty acids is curious, the worst affected was linolenic acid while myristoleic acid was not affected at all. At first glance there appears to be some correlation between the carbon chain length of the unsaturated fatty acids and its effectiveness in the presence of calcium ions with longer chains worse affected than short chains. As monocaprin has no carboxylic acid, this candidate was not affected by the presence of the calcium.

It is possible that a chelating agent such as ethylenediaminetetraacetic acid (EDTA) or the more calcium specific ethylene-glycol tetraacetic acid (EGTA) could reverse the effect of the calcium. EDTA is not an uncommon excipient in ophthalmic formulations but its concentration is limited to 0.1 % (equivalent to 3.4 mM). Further testing would be required to determine whether this concentration is sufficient to reverse the effects of the calcium present in tear fluid. Also, consideration must be given to what other effects the chelating agent would have; would it increase irritation or affect the eye's natural defences against bacterial infection? These points would have to be addressed if they were to be included in any formulation.

2.4.4 Selection of drug candidates for further analysis

Initially seven candidates that were selected based on antibacterial properties. However, it soon became clear that palmitoleic acid and linolenic acid should be withdrawn from further selection. These both performed poorly in the RBC lysis assay and did not cause total killing in some isolates. Also linolenic acid, having three unsaturated bonds in its carbon chain is more prone to oxidation reactions than other candidates and is therefore less stable. During testing the stock for this fatty acid was stored at -20 °C and was regularly replaced. The activity in simulated tear fluid has also raised problems with some candidates. All candidates apart from monocaprin and myristoleic acid were affected by the presence of calcium. As there are candidates that are unaffected by the calcium it would be easier to examine these candidates rather than trying to remove the free calcium ions from the tear fluid.

This means that only two candidates remain: myristoleic acid and monacaprins. Out of these two remaining candidates, two main factors were considered for their inclusion into a formulation. These were: cost and amount of literature on bactericidal activity. The cost of myristoleic acid is £511 per gram [228]. The cost of monacaprins is £57.70 per gram [229]. Monacaprins have already been reported to have strong anti-gonococcal properties but myristoleic acid has never been tested [167]. However, myristoleic acid was found to be ineffective against *C. trachomatis*, while monacaprins have again been shown to be bactericidal [166]. This means that monacaprins would have the advantage that they are active against other causes of ophthalmia neonatorum. Monacaprins have also reportedly been used to make hydrogel based formulations and have been proposed for the treatment of cold sores and as protection against sexually transmitted infections [230-232]. Monacaprins based hydrogels have been used in a mouse model for protection against herpes simplex-2 virus infection [231]. In that study no irritation was observed of the vaginal mucosa on application of the hydrogel.

The mode of action of the monacaprins and myristoleic acid is unknown but it has been suggested to be cell membrane disruption (see 1.2.5) [165, 167, 174]. This may help to explain why the candidates caused lysis of RBCs. However, some candidates had MBCs far below their H₅₀ result. This demonstrates that there exists some level of selective toxicity in these candidates. This is a valid target for inhibition or cidal activity against bacteria but is not the best [233]. For example, polymyxin B is an antimicrobial that targets the cell membrane of bacteria that is very effective against Gram negative bacteria (in particular *Pseudomonas aeruginosa*) [234, 235]. It is usually used as a drug of last resort; this is because of the similarities between the bacterial and eukaryotic cell membranes. However, this mode of action would theoretically also have the added advantage that it is less likely that the bacteria could develop resistance in comparison to antimicrobials that act on single targets inside the cell. One possible way it could develop resistance is if modification to existing efflux systems (e.g. *mtrCDE* or *farAB* encoded systems) enabled the transport out of the cell [148, 236]. Despite *N. gonorrhoeae* strain NCCP11945 possessing these efflux systems, in this case it was unable to transport the fatty acid outside the cell quick enough to prevent membrane lysis. However, use of these efflux pumps will be investigated further in Chapter 5.

2.4.5 Further work

To fully establish whether either monacaprins and/or myristoleic acid can be used in a formulation of an ophthalmic formulation to prevent ophthalmia neonatorum, the safety has to be evaluated. Three irritation assays have been completed in this study but an *in vivo* test will have to be conducted to be sure that no irritation occurs. There are no doubts that these two candidates have powerful antimicrobial properties that will not be inactivated by tear fluid but their effectiveness on the surface of the eye or in an ointment needs to be fully evaluated. However, this study has

demonstrated that there are fatty acid candidates with the potential to be developed into alternative prophylaxis options to help prevent ophthalmia neonatorum.

Chapter 3 - Preformulation and formulation studies

Outputs:

Patent application: Ophthalmia neoatorum prophylaxis. 2016. Status Pending

3.1 Introduction

3.1.1 Preformulation studies

Preformulation studies are fundamental to drug development [237]. Once a drug has been discovered then preformulation is the next step in development. This can comprise of literature searches on use of that particular drug candidate or similar compounds in relation to: degradation processes, any published cases of adverse effects relevant to the drug, bioavailability, pharmacokinetics and formulation of similar compound [238]. As well as these literature searches, experiments can also be done to initially assess the physical and chemical properties of the drug candidate such as:

- I. Solubility – solubility in different solvents, dissociation constant (pKa), salt formation, partition or distribution coefficient (log P or log D) and pH solubility profile.
- II. Permeability – permeability of the drug candidate to ensure that the drug can be delivered to the target site.
- III. Physical properties – Solid state properties like solid form and melting temperature.
- IV. Stability – Stability of drug candidate in pure form or with other excipients, pH stability profile and photo-stability are studied.

These properties will aid formulation of the drug into a form which ensures it can be delivered to the point of action in an active form with no toxic effects or that harmful products have not been built up over the storage life of the drug-form.

First consideration must be given to site of action. Our aim is to kill the bacteria on the ocular surface before they can infect the eye. The ophthalmic formulation would be given in the first hour(s) of life. It therefore must reach all the areas of the eye where the bacteria are present at that time. If the bacteria mix with the tear fluid this would mean that they are present everywhere where the tear fluid can reach. That would mainly be the corneal surface, conjunctiva and would also include tear drainage ducts such as the inferior lacrimal punctum. Any formulation must cover these areas for long enough for the active drug to kill any of the gonococcal cells present. During the time of birth to the time of applying the prophylaxis the gonococcal cells may have invaded the first layer of epithelial cells of the cornea. The ability of the active drug to treat these cells will be fully addressed by *in vitro* models in Chapter 4. So, in the case of our study, the drug is applied directly to the ocular surface and has to kill bacteria on this surface, it has to mix with the tear fluid to kill all the bacteria in the tear fluid and be able to reach all the areas in contact with the tear fluid. For this reason, delivery of sufficient active drug to the target site is not so much of a problem as to say a drug that has to reach the vitreous humour as the drug doesn't have to permeate through complex tissue structures such as the stroma.

3.1.2 Physical and chemical properties of monocaprin

Monocaprin is a monoglyceride (sometimes referred to as monoacylglycerols) which are made up of a single fatty acid attached to a glycerol molecule by an ester bond (see Figure 3.1.A). In the case of monocaprin, this fatty acid has a saturated carbon chain with a length of ten carbons. The empirical formula of monocaprin is $C_{13}H_{26}O_4$ and it has a molecular weight of 246.34 g/mol. Other synonyms for monocaprin include 1-decanoyl-rac-glycerol and glycerol monocaprate. The synonym 1-decanoyl-rac-glycerol describes more accurately that the monocaprin used in this study has the fatty acid chain on the first carbon of the glycerol molecule as opposed to the second. 2-decanoyl-rac-glycerol was not tested in this study as it was not readily available from a supplier. Studies of monolaurin have demonstrated that there was little observed difference in bactericidal activity against *Streptococcus pyogenes* between the two isomers [239].

Just like free fatty acids, monoglycerides have both hydrophobic and hydrophilic regions with the fatty acid tail being highly hydrophobic and the ester bond and two hydroxyl groups on the glycerol molecule being hydrophilic (see Figure 3.1.A). Monoglycerides are more water soluble than their constituent free fatty acids alone. This also means that monoglycerides have a higher hydrophilic-lipophilic balance (HLB). Figure 3.1.B shows a table which contains the values (calculated via Davies' method [240]) of each of the original seven drug candidates from Chapter 2. With the exception of sodium dodecanoate, monocaprin has the highest HLB value. This suggests that it is more water soluble than most of the other original candidates. This could potentially be an advantage when formulating an eye drop or other aqueous based ophthalmic formulation.

Monocaprin is a waxy solid powder at room temperature. It has a melting temperature of around 51-55 °C [241]. It is poorly water soluble but highly soluble in ethanol [241]. As seen in Chapter 2, monoglycerides are not prone to inactivation by calcium or magnesium ions like fatty acids. However, the ester bond could be attacked by a strong base (such as sodium hydroxide) with the hydroxide anion cleaving the ester bond forming glycerol and a fatty acid metal salt as products. This process is called saponification and is used in industry to make soap [242]. As well as bases, esters bonds are also prone to hydrolysis. The rate that this occurs in water is very slow (via non-enzymatic hydrolysis). However, the process can be sped up with either the use of lipase enzymes or even a weak acid. This process would split the monoglyceride back into its fatty acid and glycerol constituents.

3.1.3 Analytical methods for detection and quantification of monocaprin

An analytical method for detection and quantification of a drug candidate is an important part preformulation. It can be used to obtain information of the chemical properties of the candidate such as saturation solubility, Log P and Log D. It can also aid in estimating drug permeability through biological barriers, measure chemical breakdown in stability trials [243] and ensure drug uniformity

of dosage units [244]. As well as the active drug, methods of quantification of the drugs possible breakdown products for possible drug impurities are required [245].

There are three published analytical methods for the direct detection of monocaprin. All of these methods have the advantage over other indirect methods that they don't require prior derivatisation before analysis. These methods have been summarised in **Error! Reference source not found.** Two of these methods [230, 246] have already been used for development of pharmaceutical products. The saturated fatty acid carbon chain means that the molecule would have poor UV absorbance at anything but the lowest UV wavelengths. For this reason Chu and co-workers [247] developed a high performance liquid chromatography (HPLC) method based on mass spectrometry detection. Their method is claimed to be able to detect monoglycerides down to 1–30 ppm.

3.1.4 Monocaprin in pharmaceuticals

Pharmaceutical products containing monocaprin have been proposed previously. The first proposed product developed was a hydrogel to protect against sexually transmitted infections [230-232, 248]. This formulation used monocaprin at a concentration of 20 mM (approximately equivalent to 0.5 % w/w). The authors originally tested various co-solvents and polymer mixes. From the solvents, Glycofurol 75 (tetraglycol) produced the best anti-microbial effects. The two blends of polymers used were either NaCMC and PVP (2 % (w/w) and 1 % (w/w)) or carbomer and HPMC (0.5 % (w/w) and 1 % (w/w)). From these polymers, the carbomer and HPMC mix gave the fastest release profile [230]. The fee status of the patent for this formulation is classified as lapsed which suggests that the patent is no longer valid [248].

The second pharmaceutical product is a virucidal cream which has been proposed to be a treatment for cold sores [246, 249]. The oil in water emulsion also uses monocaprin at a final concentration 20 mM. The aqueous phase contains 5 % propane-1,2-diol and 1 % Polysorbate 20 with the monocaprin and this is mixed with the oil phase to create an oil in water emulsion. Formulations with oil phases comprising 2.5, 5, 7.5 or 10 % (v/v) were tested in conjunction with varying amount the polymer carbomer. The smaller volumes of oil phases gave better release of the monocaprin with 2.5 % carbomer performing the best with greater than 40 % release within 50 minutes. Also, 0.33 % carbomer gave a more rapid drug release profile than 0.5 %.

Monocaprin, as well as other fatty acids and monoglycerides, has also been suggested for the use of disinfecting contact lenses [250]. Details of concentrations and other excipients are not described but the patent does suggest that Tween[®] 80 could be used in the finalised formulation. No contact lens solution products containing monocaprin have ever come to market and the patent status is lapsed.

Use of monocaprin to kill *Helicobacter* have been described in the literature [170-172, 251]. A couple of patents also covers their use in pharmaceutical products. The earliest of the patents is very non-descript and gives very little detail [252]. This patent lapsed in 2011. The second patent gives more detail and combines the active fatty acid or monoglyceride with a proton pump inhibitor, solubilising agent, density modifier and a viscosity enhancer [253]. The preferred solubilising agent is a non-ionic surfactant (Tween® 20 or 80 suggested), named possible viscosity enhancers include CMC and HPMC and peppermint oil is the preferred density modifier.

3.1.5 Potential for ophthalmic formulations

There are two simple formulation options that could be proposed first. A liquid dosage form and a semi-solid dosage form. Currently, the recommended prophylaxis is in the form of a semi-solid ointment [31]. Therefore this dosage form maybe preferred by hospitals using the current prophylaxis. The application process between the use of eye drops and ointment on newborns is similar as both are applied under the lower eyelid [254, 255]. Therefore, a change in the dosage form shouldn't cause too much disruption. In terms infection control, single-use applicators maybe preferred by healthcare workers. Both dosage forms would be compatible with this form of packaging. Single-use containers are not required to contain extra antimicrobials but still must meet sterility requirements and pass testing for leakages and presence of metal particles [256]. This means that both dosage forms would be suitable to use as an ophthalmia neonatal prophylaxis and both should be investigated.

3.1.6 Ophthalmic semi-solid dosage form

The use of semi-solid dosage forms is common in anti-inflammatory and antimicrobial ophthalmic products [257]. The base of the semi-solid ointment is primarily petrolatum [258]. As stated in Chapter 1, it has many advantages over liquid dosage forms, mainly that it is retained in the eye for a much longer period improving ocular drug levels [259]. White Petrolatum USP is usually preferred for ophthalmic formulation but even within this type the grade of Petrolatum can vary significantly in terms of colour, clarity and hardness [258]. The addition of mineral oil, surfactants and/or preservatives can often lower the viscosity and change other physical properties. Due to the lack of water, microbial contamination would be less likely but still possible [260]. The ointment base is a non-aqueous environment, so lack of aqueous solubility would not be a problem. Furthermore, drug breakdown by non-enzymatic hydrolysis would not be an issue. This dosage form needs to be explored further as a possible formation option.

3.1.7 Use texture profile analysis in semi-solid dosage form development

Texture profile analysis is a test of the physical properties of a product. The product could be a food substances, such as, for example, ice creams [261], cheeses [262] or bread product [263, 264]. Although texture profile analysis is mainly done in the food industry, it has also been used in the

development of pharmaceutical products. For example, testing varying concentration of polymers in polymeric semi-solid dosage forms [265, 266]. In the testing of pharmaceutical semi-solid dosage forms, the main texture properties examined are cohesiveness, adhesiveness, and hardness [267]. These can be estimated by texture profile analysis where the force taken to insert and withdraw a probe a set distance into the semi-solid is measured. An example of typical output data produced in such an experiment is give in Figure 3.2. The maximum force to enter the semi-solid (positive peak 1 height), is used to measure the hardness. The total force used to enter the semi-solid (positive peak 1 area), is a measure of the cohesiveness and the total force used to withdraw from the semi-solid (negative peak 1 area) used to measure adhesiveness. The testing procedure is usually repeated after a rest of a few seconds and a second positive and negative peak produced as further properties can be measured by the comparing the differences between the initial and repeated test cycles.

As mentioned above, the physical properties of ointments varies depend upon the type of White Petrolatum used and what other excipients are used in the ointment base. It should be possible to imitate the physical properties of an unknown ointment by comparing its texture profile analysis against a panel of known blends of excipients. These blends could be fine-tuned to replicate the cohesiveness, adhesiveness, and hardness of the unknown ointment.

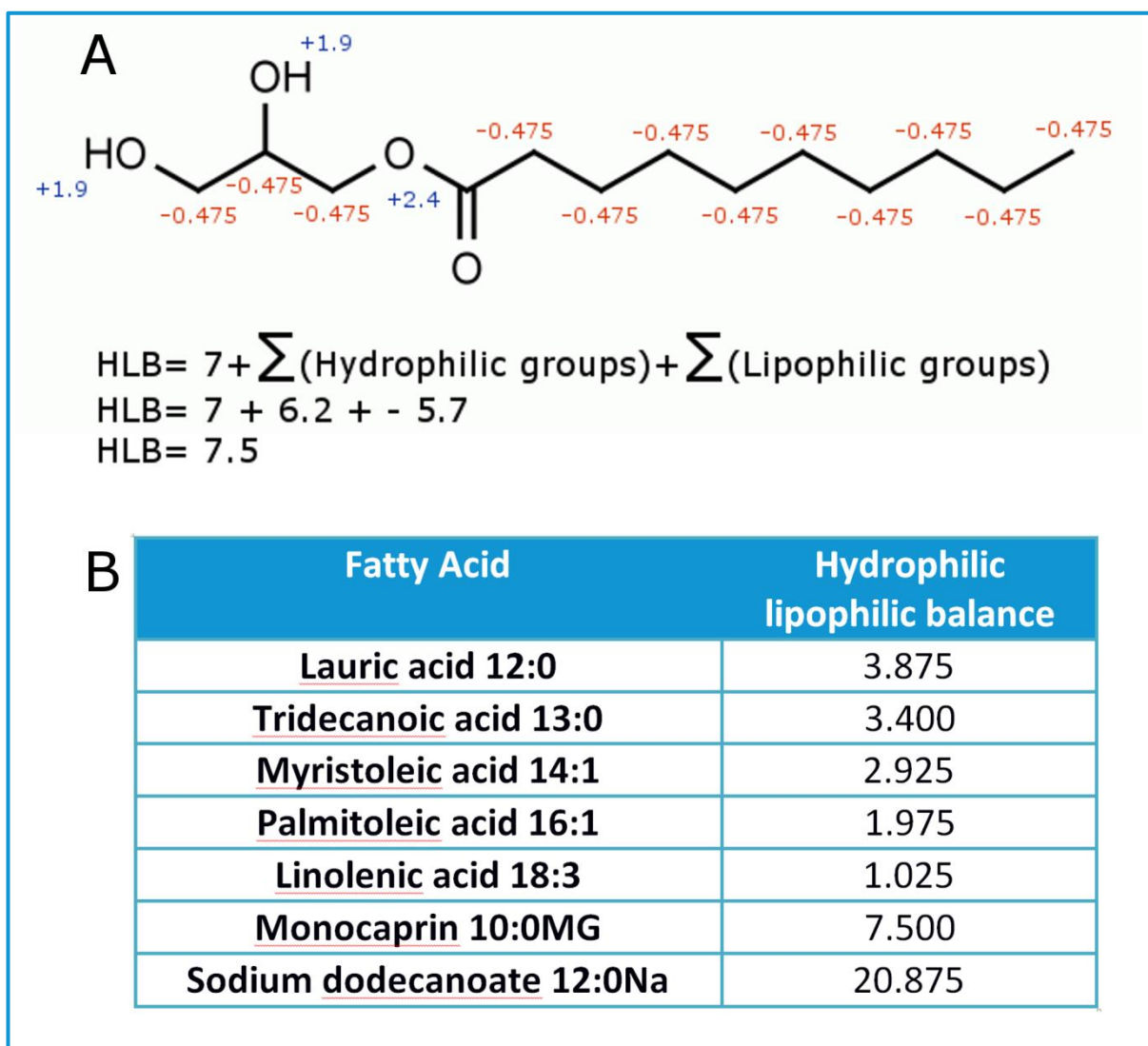


Figure 3.1 Hydrophilic lipophilic balance of monocaprin. (A) Breakdown of Davies' hydrophilic lipophilic balance values for monocaprin with calculations of overall value. (B) Table of Davies' hydrophilic lipophilic values of monocaprin and all of the other first seven drug candidates.

Table 3.1. High performance liquid chromatography methods for detection and quantification of monocaprin

	Chu <i>et al.</i> [247]	Kristmundsdoöttir <i>et al.</i> [230]	Thorgeirsdóttir <i>et al.</i> [246]
Equipment	Thermo Accela 1250 system	Thermo Separation Products Spectra Series P200 with SP4400 autosampler	Thermo Separation Products Spectra Series P200 with SP4400 autosampler
Column	Two Waters Atlantis C18 columns, 3 µm particle size, 2.1 mm × 150 mm (run in series)	Waters µBondapak C18, 10 µm particle size, 3.9 × 300 mm column	Cosmosil C18, 5 µm particle size, 4.6 mm, 150 mm column
Detection	TSQ Quantum Access MAX mass spectrometer	Spectra Series UV150 detector, 218 nm	Spectra Series UV150 detector, 208 nm
Injection volume	25 µl	Not stated	Not stated
Mobile phase	Gradient consisting of: (A) water, (B) 1 mM ammonium-formate in methanol, and (C) isopropanol:n-hexane 1:1.	Isocratic: (A) acetonitrile, (B) water, and (C) tetrahydrofuran (57:42:1)	Isocratic: (A) acetonitrile and (B) water (58 : 42)
Flow rate	Variable, 0.25-0.4 ml/min	1.25 ml/min	1.5 ml/min
Retention time	N/A	2.1 min	4.00 min
Notes	Lower limit of quantification 1–30 ppm for monoglycerides	Used in development and evaluation of monocaprin based hydrogel	Used in development and evaluation of virucidal cream for treatment of cold sores
Pros and cons	Pros: highly sensitive Cons: requires mass spectrometry which can be expensive and more difficult to optimise	Pros: rapid method Cons: low sensitive	Pros: rapid and simple method Cons: low sensitive

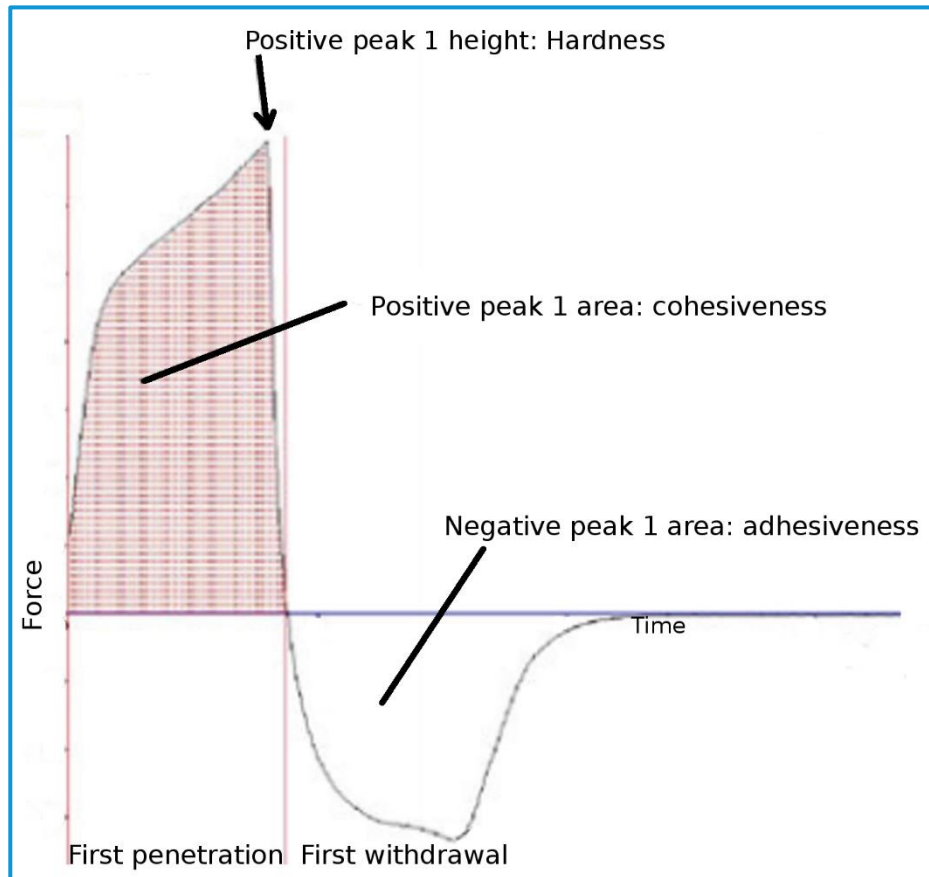


Figure 3.2 Typical texture analysis profile of a single testing cycle of an ophthalmic semi-solid dosage form.

3.1.8 Ophthalmic liquid dosage forms

Liquid dosage forms are normally aqueous based but novel oil based liquid dosage forms have been described [268, 269]. It is likely that monocaprin will have poor aqueous solubility. More than 40 % of new chemical entities developed by the pharmaceutical industry are classed as practically insoluble [270]. This can pose a major challenge when formulating products such as ophthalmic liquid dosage forms. However, solubility can be improved with the addition certain excipients [271]. One solution is to add a co-solvent that is miscible with water which can increase solubility of the drug [272]. The drug will have to have a high solubility in the co-solvent for it to significantly improve solubility in the overall mixture. Use of co-solvents can increase the solubility by several thousand times compared to the aqueous solubility of the drug in water alone [273]. The amount of co-solvent is important, the higher the concentration of co-solvent then the higher the solubility of the drug. However, the co-solvent could be an irritant at higher concentrations so guidelines should be checked regarding acceptable levels and irritation assays (such as in Chapter 2) should be conducted on the finalised formulation.

3.1.9 Aiding drug solubility in liquid dosage forms

It is likely that monocaprin will have poor aqueous solubility. More than 40 % of new chemical entities developed by the pharmaceutical industry are classed as practically insoluble [270]. This can pose a major challenge when formulating products such as ophthalmic liquid dosage forms. However, solubility can be improved with the addition of certain excipients [271]. One solution is to add a co-solvent that is miscible with water which can increase solubility of the drug [272]. The drug will have to have a high solubility in the co-solvent for it significantly improve solubility in the overall mixture. Use of co-solvents can increase the solubility by several thousand times compared to the aqueous solubility of the drug in water alone [273]. The amount of co-solvent is important, the higher the concentration of co-solvent then the higher the solubility of the drug. However, the co-solvent could be an irritant at higher concentrations so guidelines should be checked regarding acceptable levels and irritation assay (such as in Chapter 2) should be conducted on the finalised formulation.

Micellar solubilisation is term used when a surfactants is used aid drug solubility by forming micelles. At low concentrations in an aqueous solution, a surfactant will accumulate at the air-liquid interface and lower the surface tension [274]. Once the interface is saturated, any extra surfactant will be forced into the bulk of the aqueous phase. This continues until a certain concentration is reached where the surfactant molecules form a single layered sphere with hydrophilic regions on the outside and the hydrophobic regions pointing inward. This concentration is known as the critical micelle concentration. The micelle can then entrap the insoluble drug either inside the micelle or if the drug also has amphiphilic properties it can be contained in the surfactant monolayer.

Conventional surfactants which are commonly used include polysorbates (Tween® 20 and Tween® 80), polyoxyethylated castor oil (Kolliphor® EL), lauroyl macroglycerides and mono and di-fatty acid ester of low molecular weight [270]. More recently, higher molecular weight surfactants made from polymers such as polyethylene glycol (PEG), polyvinylpyrrolidone (PVP), or Poloxamers (such as Pluronic® F127 and Pluronic® P85) have been used [275, 276]. These have lower critical micelle concentration values and are more thermally and kinetically stable [275].

3.1.10 Viscosity enhancing agents in liquid dosage forms

In Chapter 1, we discussed briefly about viscosity enhancers used in ophthalmic formulations. In this chapter, we will be using two particular types of viscosity enhancers; CMC and HPMC. The role of the viscosity enhancer is to increase the viscosity of the formulation to prolong the residence time on the ocular surface. However, although highly viscous formulations have better residence times, they may increase blinking, lacrimation, hinder drug release and generally be less tolerated [277]. This is why it is important to get the viscosity of the formulation right so the drug is released at a therapeutic level and maintain this level for long enough to be completely effective.

Both CMC and HPMC are long chain polymers derived from cellulose. With CMC, carboxymethyl groups replace the hydroxyl groups on carbons 2, 3 and 6 of the glucopyranose sugars. In the case of HPMC, 2-hydroxypropyl (isopropanol) groups replace these same hydroxyl groups. This is shown in Figure 3.3. With CMC, the carboxyl group is often converted to a sodium salt to further increase aqueous solubility (NaCMC). The polymer chain length of both polymers can be varied, with longer chains used to create stiffer gels. As the polymers are cellulose based they are prone to hydrolysis by cellulases that could be produced from a number of sources including bacteria [278]. Both polymers have excellent safety records. CMC (E number E466) is commonly used in foods and pharmaceutical products such as toothpastes and ophthalmic preparations [279]. Similarly, HPMC (E number E464) is also found in foods and pharmaceutical products such as cosmetics as well as in paints and adhesives [279].

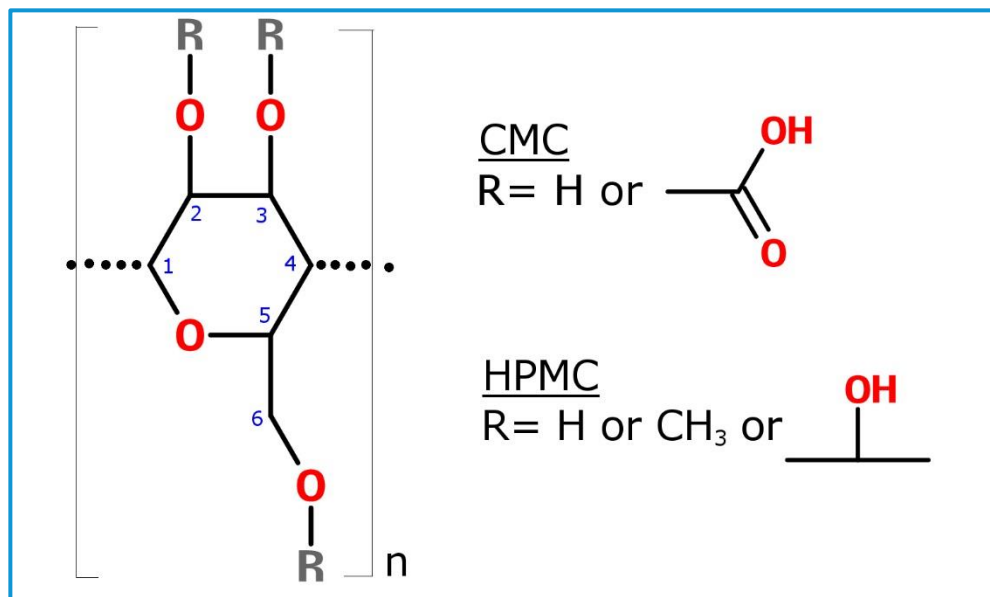


Figure 3.3 Graphical representation of chemical structure of cellulose based polymers CMC and HPMC.

3.1.11 Aim of this chapter

In the last chapter, we discovered one drug candidate, which was the monoglyceride monocaprin, had fast acting antigonococcal properties and was deemed by *in vitro* testing methods, to have low ocular irritation potential. There are two main aims of this chapter: the first is further testing of the physical and chemical properties of monocaprin to determine its suitability in an ocular formulation (preformulation studies) and the second is to develop potential ophthalmic formulations.

3.2 Materials and methods

3.2.1 Chemical and reagents

Monoglycerides: monocaprin, monolaurin and monomyristin were obtained from Tokyo Chemicals Industry UK Ltd (Oxford, UK). All solvents used in HPLC and solubility testes such as acetonitrile, dimethylacetamide (DMAc), ethanol, methanol, propane-1,2-diol and butane-1,3-diol were HPLC grade reagents (BDH Chemicals, VWR International).

For the formulation work, Vaseline[®] (Sigma-Aldrich) was used as petroleum jelly and light (0.84 g/mL at 25 °C) mineral oil (Sigma-Aldrich) used for to make the ointment base. The polymers for the thickened eye drops used carboxymethylcellulose sodium salt (Sigma-Aldrich), medium viscosity (400-800 cP, 2 % in H₂O at 25 °C) (Sigma-Aldrich) and (Hydroxypropyl)methyl cellulose with an average polymer molecular weight of 90,000 (15,000 cP, 2 wt. % in H₂O at 20 °C) (Sigma-Aldrich). The Polysorbate 20 (BDH Chemicals, VWR International) was used as the surfactant.

3.2.2 Ultra violet spectrophotometry

A solution of 1 mg/mg monocaprin was made up in acetonitrile. An aliquot of 2 ml was transferred into a quartz UV cuvette and placed in a Varian Cary 100 Scan (Agilent Technologies LDA UK Limited, Stockport, Cheshire, UK) UV spectrophotometer. A scan of absorbance in the range of 200-400 nm was completed for the sample using acetonitrile as a blank. Data was acquired using Varian Cary WinUV Version 3.00 software (Agilent Technologies LDA UK Limited).

After the initial scan, individual readings at a set absorbance were done on a concentration range of monocaprin at 1000, 750, 500, 250, 125 and 62.5 µg/ml. All dilutions were done in acetonitrile. The data was inputted into Graphpad 6 software and a regression line produced.

3.2.3 High performance liquid chromatography

The HPLC method of Thorgeirsdottir *et al.* was used as the basis for method development [246]. A Perkin Elmer 200 series HPLC system with a series 225 auto-sampler and series 200EP diode array detector (UV/VIS) was used in all experiments. Data was acquired using Perkin Elmer TotalChrom Workstation (version 6.3.1). A 4.6 X 250 mm C18 column packed with 5 µm Kinetex solid core particles (Phenomenex, Macclesfield, Cheshire, UK) was used to analyse samples. An isocratic

mobile phase of 80:20 (v/v) of acetonitrile/water was used at a flow rate of 1 ml/min. Samples were injected at a volume of 50 µl. Monocaprin was detected by UV absorption at 208 nm.

3.2.4 Validation

A stock of 2 mg/ml monocaprin was made in acetonitrile. This was diluted in acetonitrile to monocaprin concentrations of 1000, 750, 500, 250, 125 and 62.5 µg/ml. Five injection of each of these standards was made on the optimised method followed by five injection of acetonitrile (blank).

For the intra-day and inter-day testing, monocaprin concentrations of 750, 250, 125 and 62.5 µg/ml were tested. Three injections of a 1000 µg/ml monocaprin (standard) followed by a blank and then a sequence of the test samples with each being tested in triplicate and a standard and blank being run after every six injections.

3.2.5 Limits of detections and quantification

The limits of detection (LoD) and quantification (LoQ) were determined by a signal-to-noise approach. A signal-to-noise ratio of 3:1 was used to determine the LoD and 10:1 used for LoQ [280].

3.2.6 Saturation solubility

Solubility was determined by shaking flask method [281]. Common solvents tested were: acetonitrile, ethanol, methanol and water. Solvents that are commonly used in pharmaceuticals products and appearing British Pharmacopoeia were also selected: butane-1,3-diol, DMAc, propane-1,2-diol and tetraglycol. A given amount of monocaprin known to be above the saturation point of the solvent was added to a 100 ml conical flask containing either 5 or 10 ml of the specified solvent (5 ml was used for solvents capable to dissolving large amounts of monocaprin). Flasks were placed on a shaker at room temperature and shaken for 16-18 hours at 100 rpm. Flasks were left at room temperature without shaking for a further five hours. The solutions were passed through a 0.22 µm disposable syringe filter (Nalgene) to remove any undissolved monocaprin. Samples that required dilution were done so in acetonitrile so that a concentration was within the HPLC validation zone was obtained. Monocaprin was quantified using HPLC. Experiments were done in duplicate.

3.2.7 Texture analysis

Blends of light mineral oil and white petroleum were made by adding 1, 2, 3, 4 or 5 parts light mineral oil to 10 parts white petroleum blends. The light mineral oil was mixed into the white petroleum on a black pill tile and sheered together using two 30 cm spatulas until an even consistency was obtained. To compare these to commercially available ophthalmic ointments, 1 % (w/w) chloramphenicol ophthalmic ointment (GoldenEye®, Typharm Limited, Norwich, Norfolk, UK) was used.

Semi-solid samples for texture analysis were first put into 10 ml wide-necked glass vials and packed into the bottom of the vial ensuring no air bubbles were present. The vials were placed on a Stable Microsystems TA.XTplus Texture Analyser testing stage and clamped into position. The testing probe was positioned just above the surface of the sample and two testing cycles automatically run one after the other. Each sample was tested five times, after each reading the sample was removed and the sample surface flattened before testing again. Stable Microsystems Exponent Lite (version 6.1.4.0) software was used to obtain data and to combine each of the five replicate into representative plots.

3.2.8 Production of ophthalmic ointments

Monocaprin was warmed on a heat-block in a centrifuge tube to 55 °C until melted. This was mixed with the light mineral oil (warmed to 37 °C to prevent precipitation of monocaprin) and added to the required amount of white petroleum on a black pill tile. The mineral oil was mixed and then sheered into the white petroleum using two 30 cm spatulas until an even consistency was obtained and the drug was evenly distributed throughout the ointment. Ointments were stored in 60 ml plastic pots (Sterilin Ltd) at room temperature until required.

3.2.9 Production of CMC polymer based thickened eye drops

A monocaprin stock of 100 g/L in propan-1,2-diol was made. The required amount of CMC powder was added to half the required final volume of sterile deionised water (for example, if 100 g formulation wanted, then 50 ml of water added) in a sterile conical flask and placed on an orbital shaker at 200 rpm overnight at room temperature. A double concentrate of Polysorbate 20 was made up in sterile deionised water. The monocaprin stock was then added at double the required overall concentration and extra propan-1,2-diol was added (if required) to bring the concentration up to 10 %. After briefly mixing this solution, the double concentrate of the polymer was added to the double concentrate of Polysorbate 20, monocaprin and propan-1,2-diol to the required weight. This thickened solution was mixed at room temperature on a Labnet Mini LabRoller (Edison, NJ, United States) with a 5 X 50 ml tube rotisserie.

3.2.10 Production of HPMC polymer based thickened eye drops

HPMC based formulations were produced using the same method as CMC based formulation apart from the preparation of the polymer. To produce the polymer gel, the required amount of HPMC powder was added to the required amount of sterile deionised water heated to 90 °C. The double concentrate polymer was shaken on an orbital shaker at room temperature at 200 rpm until cooled. The polymer was then placed at 4 °C for an hour and then added to the double concentrate of Polysorbate 20, monocaprin and propan-1,2-diol as above.

3.2.11 Assessing growth inhibition properties of formulations via growth inhibition tests

N. gonorrhoeae strain NCCP11945 was cultured as in Chapter 2 overnight on a GC plate at 37 °C, 5 % CO₂. A cell suspension in PBS equal in the opacity to a 0.5 McFarland standard was made. A sterile cotton-wool swab was dipped into the bacterial suspension and excess liquid removed by pressing it against the side of the glass universal tube. Three continuous streaks were made on a GC plate, each one starting at the top of the plate continuing down the plate to the bottom and then plates were turned plate 90 ° before starting the next streak. After allowing a few minutes for the plates to dry, a sterilised number 5 cork borer was used to make a well in the agar with a diameter of 8 mm. Approximately 150 µl of the testing substance was placed in the well. For testing of ointments, the semi-solid material was used to fill the well until full. Plates were incubated at 37 °C, 5 % CO₂ for 16-18 hours. The diameter of the inhibition zones were measured if present.

3.2.12 Assessing bactericidal properties of formulations via log reductions

Log reductions as in Chapter 2 were used with slight modification. A double concentration of bacteria suspension (2×10^7) was used as compared to in Chapter 2, and only 250 µl was added (instead of 495 µl) to 0.25 g of the testing formulation. All formulations were tested for the standard 2 minutes and ointments were additionally tested at 1 hour exposure as well. For the negative control, formulations minus the monacaprins were used. All plate counts were and calculations were done as in Chapter 2.

3.3 Results

3.3.1 Ultra violet - Spectrophotometric scan and calibration curve

As expected, monacaprins poorly absorbed UV light at anything but the lowest wavelengths (See Figure 3.4.A). Due to this, the wavelength 208 nm was chosen for further testing as it has previously been used to detect monacaprins in a HPLC method. Regression analysis of the calibration curve of the standard concentrations tested at 208 nm revealed a straight line relationship ($R^2 = 0.9992$) (Figure 3.4.B).

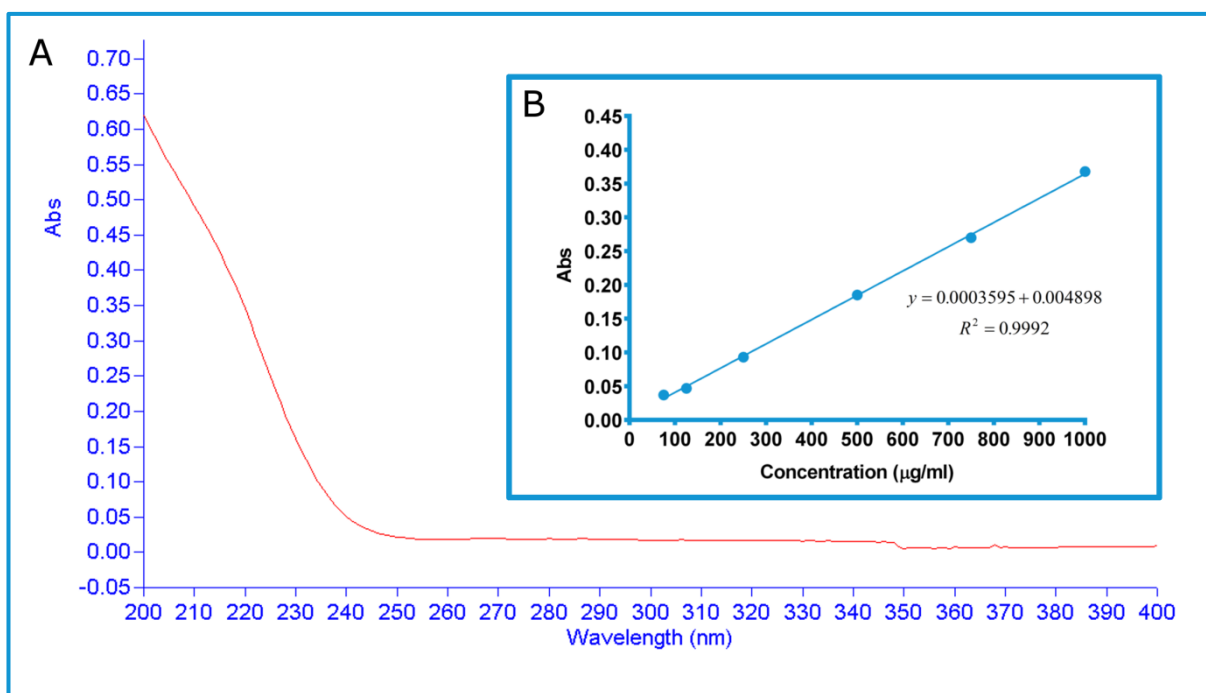


Figure 3.4 Ultra violet absorbance properties of monocaprin. (A) Ultra violet spectrophotometric scan of 1 mg/ml monocaprin in acetonitrile from 200-400 nm. (B) Ultra violet calibration curve of monocaprin in acetonitrile at 208 nm.

3.3.2 High performance liquid chromatography - method development

Some modifications had to be made from the published method of Thorgeirsdottir and co-workers [246]. The peaks produced with the published method were wide and would give poor resolution at lower concentrations. It was decided to switch to a column based on solid core particles (still 5 μm in diameter) instead of fully porous. This increased the resolution of technique (see Figure 3.5.A for an example of chromatogram). The column length was also extended from 150 mm to 250 mm to further increase the resolution over the original method. These changes increased the retention of the monocaprin with the stationary phase. Therefore, the concentration of acetonitrile in the mobile phase was increased to 80 % to reduce the retention time. The final retention time of monocaprin using this method was approximately 3.4 minutes.

3.3.3 High performance liquid chromatography - calibration curve

The estimated concentrations of the samples run in the HPLC calibration curve are shown in Table 3.2. Example chromatograms and the regression analysis are shown in Figure 3.5.A and 3.5.B respectively. The test performed well in terms regression analysis with a R^2 value of 0.9997. However, the relative standard deviations for lower concentrations were slightly high (Table 3.2). The relative standard deviations (RSD) for the 62.5 and 125 $\mu\text{g}/\text{ml}$ monocaprin solutions were 5.9 % and 2.9 % respectively. However, the mean estimated concentrations are 62.6 $\mu\text{g}/\text{ml}$ (100.2 %) and 124.5 $\mu\text{g}/\text{ml}$ (99.6 %) respectively. This would indicate the method is accurate but not precise at these lower concentrations. At concentrations of 500 $\mu\text{g}/\text{ml}$ and above, the RSD was 1 or less demonstrating that the test had a good level of repeatability at higher concentrations. Figure 3.5.A shows an overlay of the chromatograms of the concentrations of the tested concentrations. The baseline readings are not completely flat which could be due a consequence of the low wavelength used for detection.

3.3.4 Precision and accuracy

To test the precision and accuracy of the HPLC method, test samples were tested in three different run in the same day (intra-day) and in three runs on separate days (inter-day). The results of the intra-day and inter-day testing are given in

Table 3.3. One immediate observation of the data was that the majority of the readings gave a small overestimate of the concentration. This could be due to a small inaccuracy with the validation. Despite this, the method still gave accurate results. Overall, for the tested samples in the intra-day testing, the mean recovery was 102.4 % with a RSD of 2.7 %, and for the inter-day testing the mean recovery was 102.5 % with a RSD of 3.0 %.

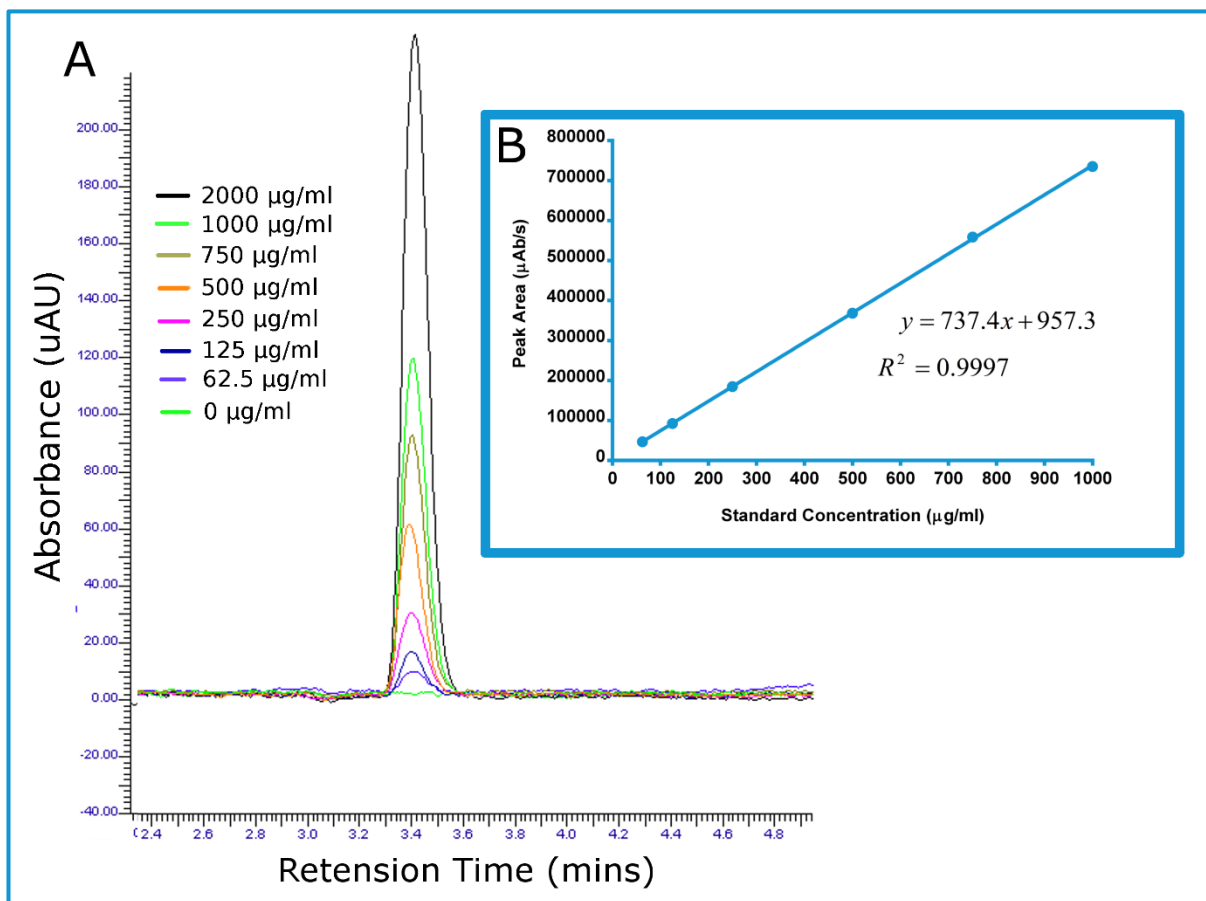


Figure 3.5 Results of high performance liquid chromatography validation of monocaprin. (A) Overlaid chromatograms from HPLC validation of monocaprin tested at different concentrations. (B) Calibration curve of the different concentration of monocaprin and area under the curve of their resulting chromatogram peak.

Table 3.2 Estimated concentrations of HPLC validation samples.

Sample	Estimated Concentration ($\mu\text{g/ml}$)						Regression Coefficient	Slope	Intercept
	62.5	125	250	500	750	1000			
Replicate 1	61.11	123.08	248.60	507.25	756.44	1001.38	0.9999	1.0065	-1.2028
Replicate 2	65.36	129.14	255.84	494.25	763.11	985.91	0.9995	0.9888	6.0198
Replicate 3	70.10	123.31	255.97	500.97	753.38	996.64	0.9999	0.9937	4.9647
Replicate 4	67.42	131.28	247.67	504.72	765.63	1007.79	0.9998	1.0084	2.404
Replicate 5	62.21	128.47	252.96	498.44	760.21	1003.83	0.9999	1.0051	0.8189
Mean	62.6	124.5	249.6	498.5	757.2	996.5	0.9999	1.0005	-0.0044
S.D.	3.7	3.6	3.9	5.1	4.9	8.4			
RSD (%)	5.9	2.9	1.6	1.0	0.7	0.8			
Accuracy (%)	100.2	99.6	99.8	99.7	101.0	99.7			

*

Table 3.3 Summary of inter and intra day testing results of a selection of standards of monocaprin.

Standards	Intra-day testing (n=3)				Inter-day testing (n=3+3+3)			
	Mean estimate (µg/ml)	S.D. (µg/ml)	RSD (%)	Accuracy (%)	Mean estimate (µg/ml)	S.D. (µg/ml)	RSD (%)	Accuracy (%)
750	761.2	18.5	2.5	101.5	751.0	8.5	1.1	100.1
250	255.5	5.6	2.2	102.2	257.3	6.4	2.6	102.9
125	127.7	1.0	0.8	102.1	130.3	3.0	2.4	104.2
62.5	65.0	3.3	5.2	103.9	64.2	3.8	6.0	102.7

3.3.5 Limits of detection and quantification

The International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) guidelines only give a brief outline of how to set the LoD and LoQ as there are a few methods that can be used [280]. The optimised method has already demonstrated that it has a good level of repeatability, precision, and accuracy. However, the baseline of chromatograms is slightly noisy. Due to this, it was decided that the LoD and LoQ should be estimated taking this into account and a signal-to-noise ratio based method should be used.

The LoD and LoQ of the optimised method was determined to be 13.4 and 40.6 µg/ml respectively from analysis of the calibration curve. These limits have values outside our calibration range which is encouraging.

3.3.6 Testing of other monoglycerides and possible breakdown products

Other monoglycerides as well as monocaprin were tested using the optimised HPLC method. This was done to make sure that the test could detect any possible impurities, contamination or incorrect labelling of samples. Examples of chromatograms are shown in Figure 3.6.A. Monolaurin (12:0MG), a saturated twelve carbon chain length monoglyceride and monomyristin a saturated fourteen carbon chain length monoglyceride were tested. The retention times of the monoglycerides increased with carbon chain length as would be expected. The retention of monocaprin was approximately 3.38 min with monolaurin having a 1.27 min longer retention time at approximately 4.65 min. The difference in retention time between monolaurin and monomyristin was 2.66 min with monomyristin having a retention time of 7.31 min. Therefore, the difference between the 12 and 14 carbon length is double that of the difference between the 10 and 12 carbon length monoglycerides. More testing would be required to confirm if this trend of double retention time differences continues.

One of the possible breakdown pathways is hydrolysis. Breakdown of monocaprin by hydrolysis would lead to the formation of capric acid and glycerol. Therefore, these were run on the HPLC method to be sure that the method could detect this possible breakdown route. The resulting chromatograms from the testing of these products are shown in Figure 3.6.B. Testing of capric acid resulted in a peak at 4.28 mins. The increase in retention time over monocaprin would be expected due to the loss of the ester bond and two hydroxyl groups resulting in greater net hydrophobicity of the molecule despite the gain of the hydrophilic carboxylic acid group.

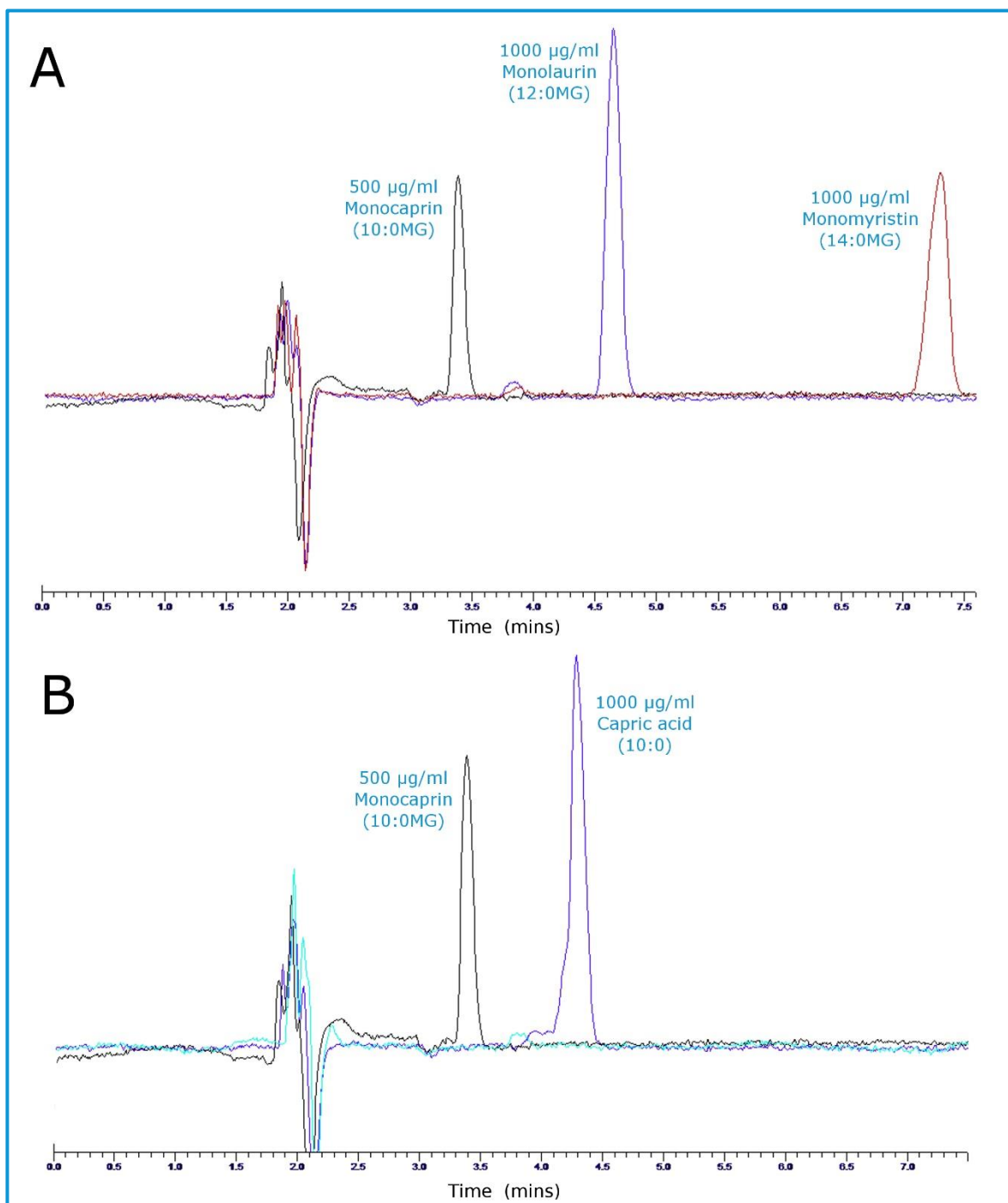


Figure 3.6 Overlaid single representative HPLC chromatograms (UV detection at 208 nm) of monocaprin and closely related chemicals. (A) Comparison of monocaprin (3.38 min) to other monoglycerides; monolaurin (4.65 min) and monomyristin (7.31 min). (B) Comparison of monocaprin (3.38 min) to capric acid (4.28 min). Chromatogram for glycerol also displayed (light blue line) but didn't result in any peaks. Capric acid and glycerol would be products from the potential breakdown of monocaprin by hydrolysis.

Table 3.4 Experiment solubility of monocaprin in selected solvents at 20 °C.

Solvent	Experimental saturation solubility (g/L)	SD (g/L)
Acetonitrile	99.6	2.3
Butane-1,3-diol	184.71	0.6
DMAc	693.53	22.0
Ethanol	331.4	9.3
Methanol	432.4	5.2
Propane-1,2-diol	303.38	1.7
Tetraglycol	439.00	19.1
Water	0.26	0.008

3.3.7 Monocaprin solvent solubility

Results of the solubility of monocaprin in the tested solvents are displayed in Table 3.4. Monocaprin was highly soluble in the two alcohol based solvents tested (ethanol and methanol). Experiment results confirm that monocaprin is insoluble in water with an estimate of solubility of 0.26 g/L.

3.3.8 Texture analysis of commercially available ointments

The commercially available 1 % (w/w) chloramphenicol ophthalmic ointment was tested against different blends of light mineral oil and white petroleum. This was done to reverse-engineer an ointment based from an existing ophthalmic ointment. The results of the texture analysis are displayed in Table 3.5. Also, the data is graphically represented in Figure 3.7. From this data, it estimates that it should be possible to replicate the texture properties of the ophthalmic ointment with a blend of two parts light mineral oil to ten parts white petroleum. The initial measure of parts for light mineral oil was by volume and the measure of parts for white petroleum was by weight. As light mineral oil has a density of 0.84 g/cm³, this means that this mixture is equivalent to approximately 15 % by weight light mineral oil and 85 % by weight white petroleum.

Unfortunately, the texture analysis graph for chloramphenicol in Figure 3.7.B doesn't completely match that to the blends of the light mineral oil and white petroleum. The negative peak height doesn't match the values given by the known standard. However, this blend of excipients does match the chloramphenicol ophthalmic ointment in terms the physical properties of hardness (positive peak 1 height), cohesiveness (positive peak 1 area) and adhesiveness (negative peak 1 area). The reason for this is unknown but may be because of the contribution of the chloramphenicol on the texture properties of the ointment. Despite this, 15 % by weight light mineral oil and 85 % by weight white petroleum was clearly the closest match to the ointment especially in terms of hardness.

3.3.9 Development of ointment based ophthalmic ointments

The 15 % by weight light mineral oil and 85 % by weight white petroleum was used as the ointment base. The monocaprin was incorporated into the base using standard ointment manufacturing procedures (also outlined in the methods section). The monocaprin concentrations chosen to be used in the formulations were 0.068 %, 0.125 %, 0.188 %, and 0.25 % by weight. The monocaprin was evenly distributed within the ointment with no sign of clumping upon storage.

3.3.10 Initial development of CMC and HPMC polymer based liquid dosage forms

The development of the liquid dosage forms was much more complex than with the ointment. As seen in previous results in this chapter, monocaprin has poor aqueous solubility. Therefore, co-solvents had to be used to aid solubility. The concentrations of monocaprin chosen for the

formulations were 0.068 %, 0.125 %, 0.188 %, and 0.25 % by weight to be comparable to the ointment. However, to aid solubility of the drug, monocaprin was first dissolved in propane-1,2-diol and Polysorbate 20 was also added to the formulation. Initially 0.1 % (v/v) Polysorbate 20 was added to these monocaprin concentrations. Figure 3.8 shows photographs of these formulations and demonstrates the action of the Polysorbate 20 on the solubility of the monocaprin. At a concentration of 0.1 % Polysorbate 20, the monocaprin was completely in solution in the HPMC polymer based formulation. This however, was not the case in the CMC based formulation at the same concentration of surfactant and drug. The reason for the decreased solubility of monocaprin in CMC based formulations is unknown. Without the Polysorbate 20, the monocaprin in both the CMC and HPMC polymer based formulations was not solubilised. Interestingly, the visual form of the precipitate in the two polymer type based formulations were different with the CMC based formulations having clumps of monocaprin and the HPMC precipitate forming a cloudy precipitate at the top of the vessel just below the meniscus (as shown in Figure 3.8). However, with the Polysorbate 20, all the tested concentrations of monocaprin in both polymer types formed a stable cloudy suspension.

3.3.11 Antimicrobial testing of ointments and CMC and HPMC polymer based liquid dosage forms

The antimicrobial testing was done by growth inhibition testing (bacteriostatic) and also by performing log reductions (bactericidal). The growth inhibitions for liquid dosage forms based formulations was slightly more complex than the ointments. This was because the excipients alone (formulations with no drug added) caused some inhibition of growth. It was possible that Polysorbate 20 was inhibiting the GC growth. Therefore, all the liquid dosage forms; no drug, 0.068 %, 0.125 %, 0.188 %, and 0.25 % monocaprin for both polymer types were tested with and without the added 0.1 % Polysorbate 20. The only difference in the results between the formulations with or without Polysorbate 20 was for the negative control where the no-drug control would cause a mean 13 mm zone inhibition compared to no zone for formulations without Polysorbate 20. This indicates that the Polysorbate 20 has some bacteriostatic properties but these are hidden when the more powerful bacteriostatic agent monocaprin has been included in the formulation.

Graphical representations of the growth inhibition testing are displayed in Figure 3.9. It is clear that the ointment gave the smallest zones of inhibition. This is not surprising as the diffusion rate of the monocaprin from the well would be much slower than that from the liquid dosage forms due to the difference in viscosity. Furthermore, the base of the ointment is very hydrophobic so it would be more difficult for the monocaprin to travel from this matrix into a more aqueous based matrix. For the liquid dosage forms, the HPMC based formulations performed slightly better than CMC based

formulations. However, the viscosity of the CMC based formulations was slightly greater than that of the HPMC formulations.

The log reductions were done for the ointments at 0 %, 0.068 %, 0.125 %, 0.188 %, and 0.25 % monocaprin concentrations. There was no reduction in bacterial count after the standard two minute exposure time despite rigorous mixing. However, this would not be a fair test for the ointment as it intended to release the drug over an extended period. Therefore, the bacterial counts were also done on the ointments after an hour of exposure. There was again no reduction in bacterial counts. Any extension of this exposure time would have an intrinsic effect on the bacteria as they would have been starved of nutrients and optimal temperature conditions for a prolonged period.

The log reduction results of the liquid dosage forms was interesting. Initial testing demonstrated that there was no difference between the log reductions of the two polymer types. However, it was clear that there was difference in Polysorbate 20 concentration. Polysorbate 20 concentrations of 0 %, 0.1 %, 0.2 %, 0.5 %, 1 %, and 2 % were tested with or without drug. The results of these for the HPMC based formulations are given in the graph in Figure 3.10. All tested formulations without monocaprin didn't cause any killing of GC cell even at a concentration of 3 % Polysorbate 20 indicating that the action of the Polysorbate 20 is bacteriostatic only not bactericidal. Surprisingly, the higher the concentration of Polysorbate 20 the lower the anti-gonococcal properties of the monocaprin. This inhibition of the monocaprin by the surfactant appears to be concentration dependant – the greater the monocaprin concentration in the formulations the more Polysorbate 20 required to inhibit its action. If a log reduction value of four or great was deemed bactericidal, then the maximum concentration of Polysorbate 20 of 0.2 % should be used for 0.062 and 0.125 % monocaprin formulations; 0.5 % for 0.188 % monocaprin formulations; and 2 % for 0.25 % monocaprin formulations.

Table 3.5 Amounts of force (mN) required for TA.XTplus Texture Analyser to complete testing cycle of ophthalmic chloramphenicol ointment in comparison to blends of light mineral oil and white petroleum.

Parts Mineral Oil	Parts Petroleum Jelly	Positive Peak Height 1		Positive Peak Height 2		Negative Peak Height 1		Negative Peak Height 2	
		Mean*	S.D.*	Mean	S.D.	Mean	S.D.	Mean	S.D.
0	10	329.82	25.72	307.06	20.08	-217.79	21.1	-200.81	21.1
1	10	259.62	30.28	246.33	23.47	-189.54	24.32	-174.62	20.53
2	10	184.14	16.59	171.65	15.64	-128.21	13.41	-120.78	10.41
3	10	151.62	4.83	146.24	4.56	-111.30	4.41	-107.18	4.43
4	10	137.93	9.5	133.13	9.32	-97.24	11.91	-94.72	9.96
5	10	123.68	9.39	120.20	7.3	-87.68	8.30	-85.02	7.06
Chloramphenicol ointment		184.89	5.69	170.55	5.22	-112.98	3.10	-110.15	5.22
Parts Mineral Oil	Parts Petroleum Jelly	Positive Peak Area 1		Positive Peak Area 2		Negative Peak Area 1		Negative Peak Area 2	
		Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.
0	10	537.52	80.05	433.05	75.11	-425.79	73.81	-486.84	104.87
1	10	495.76	88.31	388.08	63.78	-397.03	60.64	-463.41	85.60
2	10	331.86	21.62	256.85	23.76	-265.12	23.35	-311.37	14.30
3	10	293.10	9.92	241.85	13.99	-236.21	12.50	-304.14	34.28
4	10	273.70	22.83	227.53	19.84	-209.17	22.35	-305.78	27.88
5	10	247.46	25.09	200.17	15.89	-190.54	17.25	-264.90	23.78
Chloramphenicol ointment		321.23	11.93	257.59	10.02	-259.66	8.78	-369.56	19.63

* Means and standard deviations are based on five separate readings.

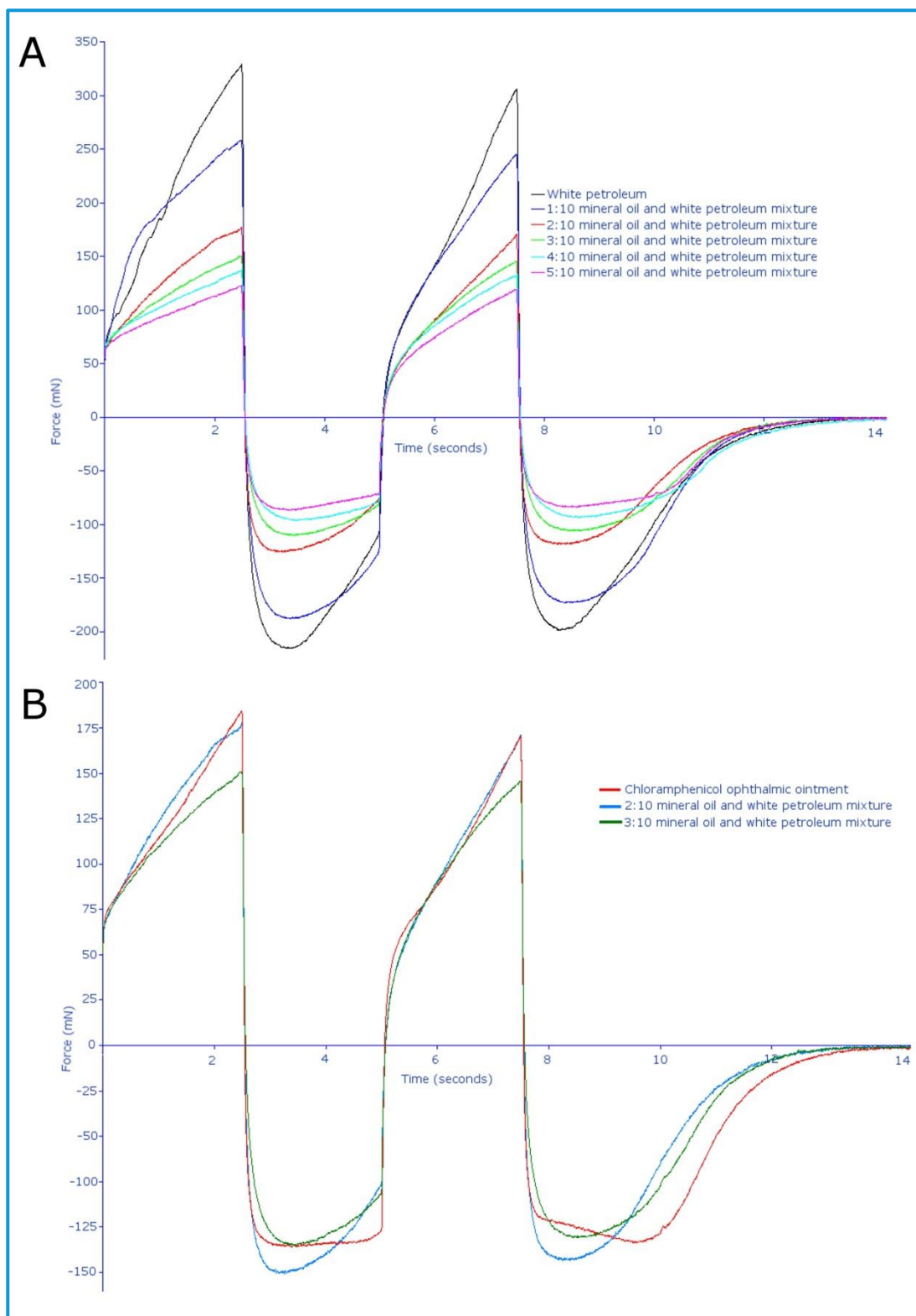


Figure 3.7 Texture analysis of ointments. (A) Comparison of texture of different light mineral oil and white petroleum blends (1-5:10). (B) Comparison of chloramphenicol ophthalmic ointment to the blends of different light mineral oil and white petroleum. The chloramphenicol ophthalmic ointment closely resembles the 2:10 light mineral oil and white petroleum blend profile. Graphs represent a mean of five tests on the each sample.

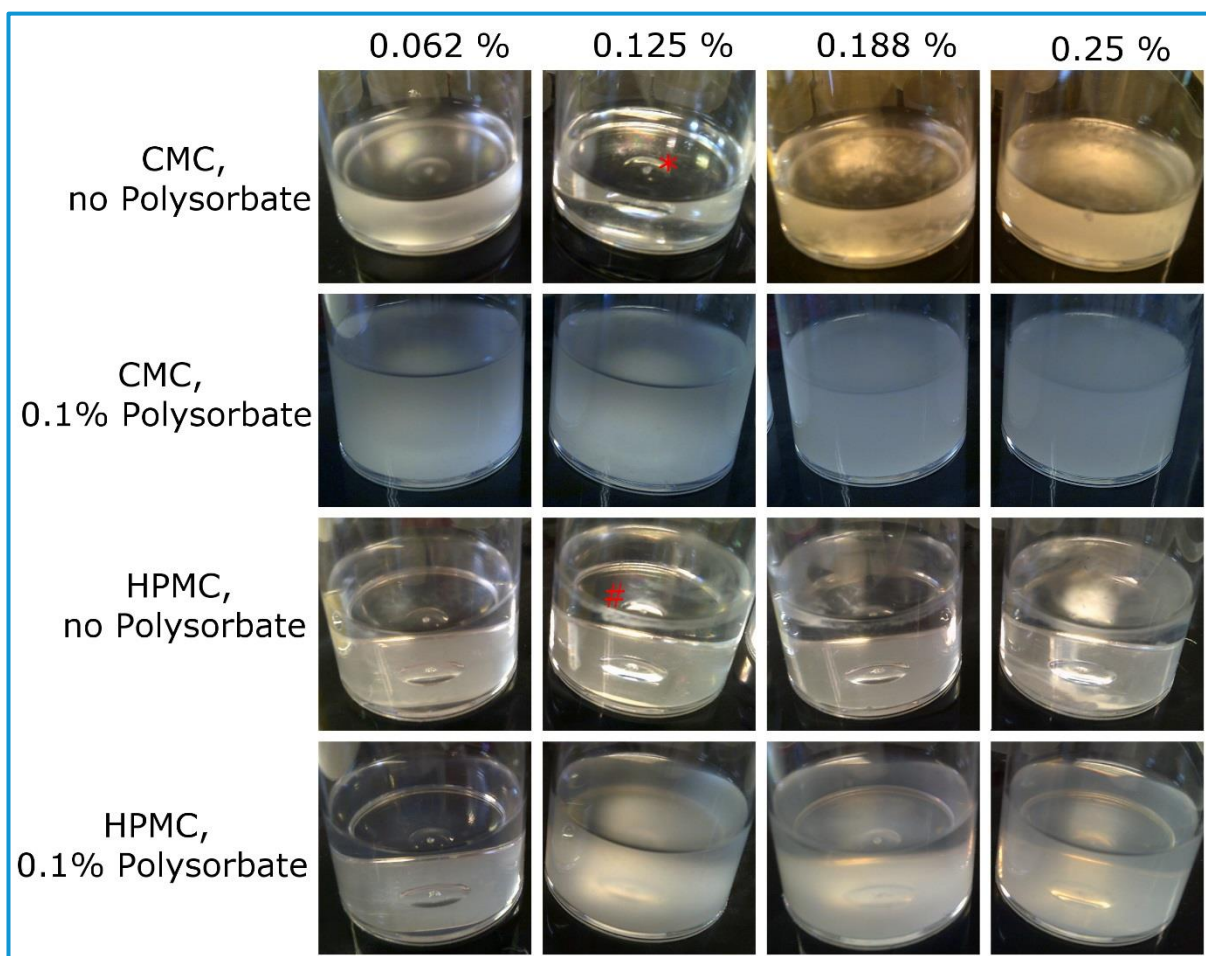


Figure 3.8 Comparison of the effects of polymer and addition of Polysorbate 20 on the solubility of monocaprin. For the CMC polymer based formulations, if the monocaprin at concentrations were above 0.125 % without Polysorbate 20 would form clumps (marked by red asterisk). With the addition of 0.1 % Polysorbate 20, the monocaprin would not be fully soluble but would at least evenly distributed. For HPMC based formulations, without the addition of Polysorbate 20, the monocaprin would form a fine precipitate just under the meniscus of the formulation at concentrations of 0.125 % and above (red hash).

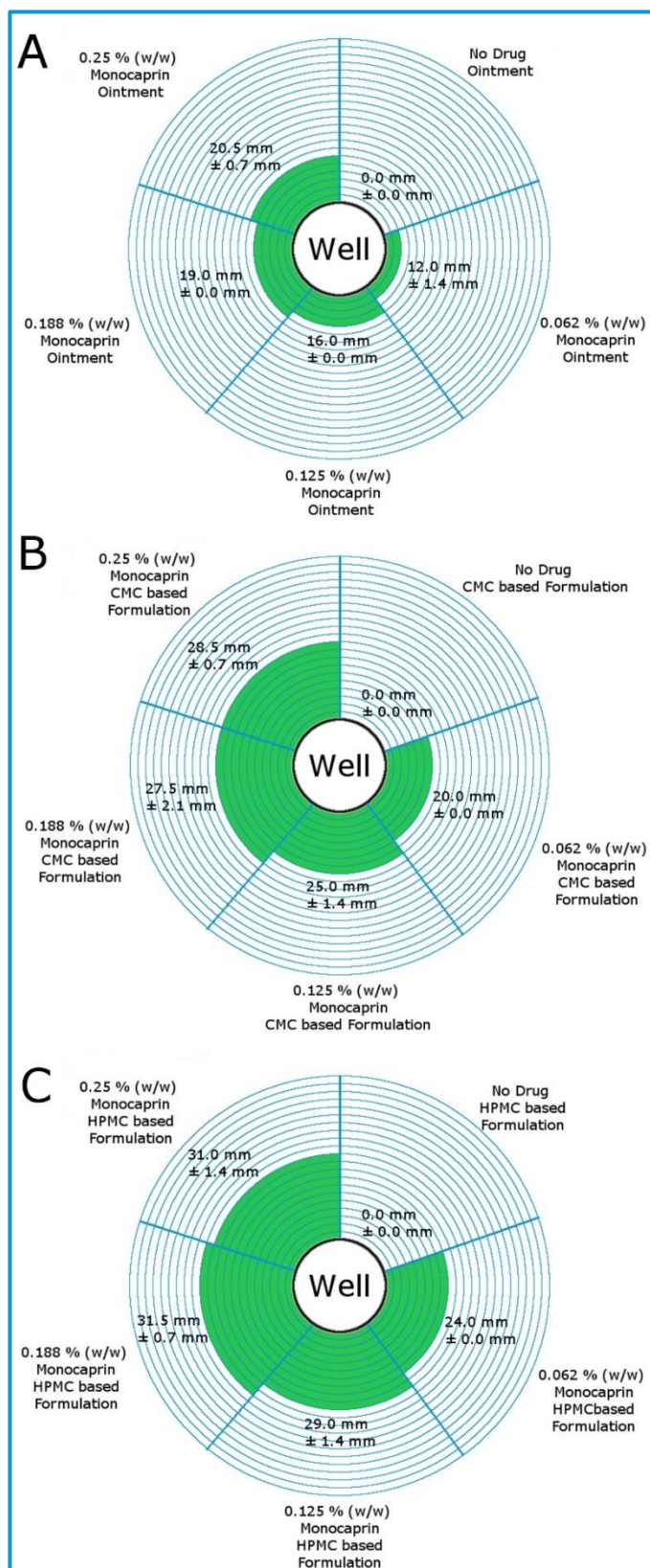


Figure 3.9 Growth inhibition zones of all formulations. (A) Growth inhibition zones of the ointments, (B) Growth inhibition zones of the CMC based formulations. (C) Growth inhibition zones of the HPMC based formulations. Each ring represents a 2 mm zone of inhibition; the diameter of the well is 8 mm. Numerical zone sizes are displayed as mean results with standard deviation over two repeated experiments.

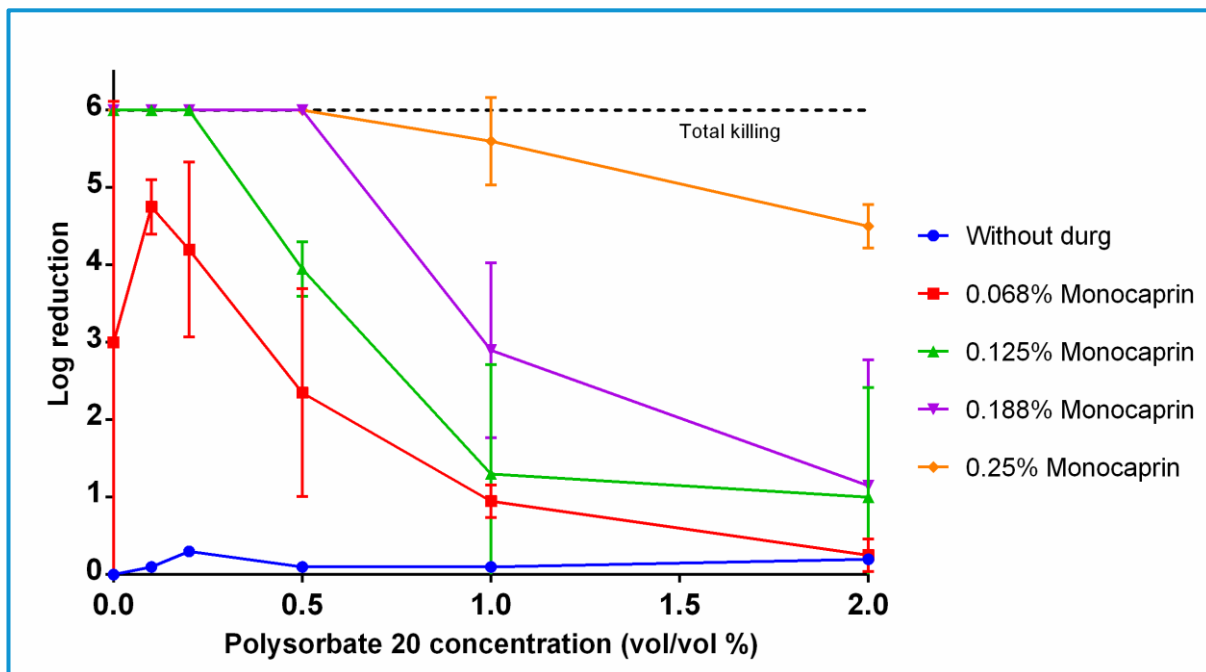


Figure 3.10 Effect of Polysorbate 20 concentration on the anti-gonococcal properties of monocaprin. Formulations without monocaprin were not bactericidal even at a concentration of 2 % Polysorbate. All formulations were strongly bactericidal at low concentrations (> 0.2) or without Polysorbate 20. At higher concentrations of Polysorbate 20, antibacterial properties were diminished and at 2% only 0.25 % monocaprin was effective. Results are displayed means of two separate experiments, error bars represent standard deviation. The large standard deviation for 0.068 % monocaprin without Polysorbate 20 is a result of in one experiment causing complete killing (LR = 6) and in the other causing no killing (LR = 0). The reason why it may not have been bactericidal could be due to the monocaprin precipitating out of the formulation.

3.4 Discussion

The chemical properties of monacaprins have been explored both via literature and by experimental research. As expected, monacaprins have poor aqueous solubility which did hinder formulation. However, monacaprins solubility in other solvents highlight that a co-solvent based formulation could work. Propane-1,2-diol was in the end selected for later development of the liquid dosage form due to common use in ophthalmic formulations over some of the other solvents. The development of an analytical method aided formulation and could be used in future. The lower detection limit of the method was relatively high for a HPLC method which was due to the low UV absorbance of the monacaprins. Mass spectrometry could be used as Chu and co-workers [247] and this would vastly decrease the lower detection limit but extensive validation of the method would be required. The method validated here was sufficiently sensitive for the purposes of this study.

The semi-solid dosage form developed in this study was based on an ointment. The ointment base of the formulation used a blend of petrolatum and mineral oil to replicate the physical properties of an existing ophthalmic ointment already commercially available. The formulation of the ointment after this was straightforward with incorporation of the monacaprins into the ointment base.

The formulation of the liquid dosage form was based on use of a co-solvent and a surfactant to aid aqueous solubility. The power of the surfactant to aid solubility was much greater than that of the solvents with very small amounts drastically aiding the aqueous solubility of the monacaprins. Like the ointment four concentrations (0.068 %, 0.125 %, 0.188 %, and 0.25 %) were selected going forward as potential formulation.

The performance of the two dosage forms were compared in terms of their action against gonococcal cells. The growth inhibition test which measures bacteriostatic activity demonstrated that both formulations had strong bacteriostatic properties. However, when the bactericidal activity was tested via log reductions only the liquid dosage forms were bactericidal. This could mean that the ointment, when applied to the eye may not be able to kill gonococcal cells in the tear fluid. It appears that release of the monacaprins from the ointment base is insufficient to reach a level where it would actively kill the bacterial cells. In comparison, the thickened eye drop formulation which blended with the simulated tear fluid during log reduction assay, killed all the gonococcal cells present. At this point it would have been beneficial to compare the action of the monacaprins ointments to that of other antimicrobial ointments. The action of erythromycin is mostly bacteriostatic and this is already used to successfully prevent development of ophthalmia neonatorum [282]. Therefore, the use of the monacaprins ophthalmic ointment cannot be discounted completely.

The surfactant used in the formulation of the thicken eye drop was Polysorbate 20. This surfactant is highly soluble in water and is a strong solubilising agent. The results from this study show that the Polysorbate 20 had a strong effect on the level of solubility of the monocaprin. However, the Polysorbate 20 also had a negative on the bactericidal properties of the monocaprin. This has also been reported in another study [283]. Without any surfactant present, the monocaprin would precipitate into clumps at concentrations past the maximum solubilising capability of the matrix. This would render the formulation ineffective as the drug is not evenly distributed throughout the formulation. Fortunately, it has been discovered that if only a small amount of surfactant were added, even if the maximum solubilising capability of matrix was still not enough to completely solubilise all of the monocaprin, a stable suspension would be formed. Therefore, the drug would be evenly distributed throughout the formulation without being completely solubilised. The stability of this suspension can also be enhanced with the use of the viscosity enhancers such as that we have used in this study (CMC and HPMC). As long as the surfactant is kept at a high enough level to completely solubilise the monocaprin or to create a stable suspension but is not high enough to inhibit the action of the surfactant then it can be included in the formulation. The only alternative to the use of co-solvents and surfactants which has not been tried is the use of a complexing agent [270]. A complexing agent such as cyclodextrin can aid the solubility of hydrophobic drugs by complexing with them [284]. The drug would interact with hydrophobic regions on the inside of the ring structure of the cyclodextrin while the hydrophilic groups on the outside of the ring structure would aid the solubility of complex. The reason why this option was not explored was due to cost of the complexing agent. Cost of the treatment has to be as low as possible due to the number treatments that would be required. Also, complexing in this manner may also have an effect on the antimicrobial properties of the monocaprin in a similar way to the Polysorbate 20.

This study focused on the use of two commonly used ophthalmic viscosity enhancing polymers; CMC and HPMC. Both are widely used in ophthalmic preparations and have good safety records. HPMC is thickening agent which would also act as ophthalmic lubricant [285, 286]. The benefit of having a thickening agent in the formulation is to control the release of the drug. The thicker the polymer matrix the greater the ocular residence time of the drug. This would stop the natural removal of the drug before it has had a chance to take effect. However, as monocaprin is a more concentration dependant drug rather than a time-dependant drug, if the release of the drug is to slow then there will a minimal effect from the drug. Data from the first chapter demonstrates that the drug must be delivered in an active form at a concentration of 0.5 mM or above to be bactericidal. Anywhere below this level and the drug would not be bactericidal and would be

removed too rapidly via the natural draining mechanisms of the eye to remain at MIC level or above for any length of time.

What could be tested is a measure of drug release? Franz diffusion cell experiments can be used to estimate drug release rates by measuring the diffusion rates from the semisolid to a receptor compartment through a synthetic membrane [287]. Franz diffusion cell experiments were attempted in this study but no meaningful drug release was detected probably due to the monocaprins forming micelles with the Polysorbate 20 and thus not being able to pass through dialysis pores used in the experiment (data not shown). Franz diffusion cells are popular to study diffusion of transdermal drug delivery systems through membranes [288, 289]. So their relevance to estimate rate of release from a formulation to tear fluid and ocular surface is questionable. During the development of the monocaprins based cream for treatment of cold sores referenced in the introduction of this chapter, a membrane-less diffusion cell was used [246]. The exact method used in that study is not given but other studies do give details for the use of membrane-less in drug release studies [290-292].

There were two observed benefits of the use of HPMC over the CMC. The first was the slight increase in the solubility of the monocaprins in HPLMC formulations in comparison to the CMC based formulations. The second is the increased stability that was observed in the formulation over long-term storage (data not shown). Upon storage the thickened eye drop solution would decrease in viscosity after a few months. This was presumably due to breakdown of the polymers over time. The reason why this affected the CMC based formulations only is unknown but must be due to enzymatic breakdown. The rate of enzymatic breakdown would likely be increased by the presence of the Polysorbate as non-ionic surfactants have been well documented to increase the rate of reactions of cellulases [293, 294].

At the beginning of the chapter the aims were stated and this work has gone some way to achieve those aims. Working formulations have been developed but questions still remain unanswered such as which concentration of monocaprins should the final formulation contain? Working in the laboratory killing purified gonococcal cells in simulated tear fluid has been demonstrated that a monocaprins concentration greater than 0.5 mM will be sufficient to kill all gonococcal cells present. Whether this concentration is sufficient or not will be addressed in the next chapter. For now the thickened eye drop formulations will be used going further as many questions remain about how effective the ointment based formulation will be. *In vitro* testing suggests that the monocaprins are not released quick enough to be effective. Whether this testing reflects how effective it would be *in vivo* remains to be seen and no ending of *in vitro* testing could possibly completely answer this

question. It would therefore need some sort of animal testing or clinical trial to fully answer that question.

More work could also be done to further develop the thickened eye-drop formulation. The comparisons of the polymers was arbitrary as no standardisation between the viscosities of the two polymer types was done. This would better compare the two polymers in terms of drug release but at this point use of HPMC is preferable to CMC for the reasons already mentioned above. Study of the drug release should also be conducted using a membrane-less diffusion cell method. This would give more confidence that the drug candidate is being released at the correct levels before the formulation has been removed from the eye by natural processes.

Questions still remain about stability. Testing of 9 month old formulations after storage at room temperature in the dark show that thicken eye drop formulations are still as effective as newly made formulations and HPLC testing cannot detect any decrease in monacaprין or presence of any breakdown products (data not shown). However, full analysis in accordance with ICH guidelines should be done to fully assess the stability of the formulations. These tests should be done on the finished formulation as well as a repeat of the irritation testing to demonstrate that the formulation as a whole does not cause irritation not just the active components.

Overall we have potential formulations to test in other models to prove their effectiveness. A thicken eye drop based formulation has been selected over the ointment based formulation. In the development of the thicken eye drop formulation the use HPMC as a thickening agent is preferred. The use of Polysorbate 20 must be balanced with the amount of monacaprין used in the formulation. In the formulation containing 0.068 and 0.125 % monacaprין, 0.2 % Polysorbate 20 must be used and in 0.188 and 0.25 % monacaprין formulations, 0.5 % Polysorbate 20 must be used. Despite the lack aqueous solubility of the monacaprין, the application of the thickened eye drop formulation is at this point the most suited formulation option given the properties of the killing action of the monacaprין.

Chapter 4 - Models of ocular gonococcal infections

Outputs:

Poster: **Colin P. Churchward**, Anna Tran, Raid G. Alany, Ruth S. Kirk and Lori A. S. Snyder. 2015. Comparison of Cell Culture Methods for Evaluating the Efficacy of the Bactericidal Activities of Drugs. IhSHA. Kingston University June 2015. **Third prize awarded**

Talk: RAG da Silva, CP Churchward, AV Karlyshev, O Eleftheriadou, AK Snabaitis, MR Longman, A Ryan, R Griffin. 2016. The role of apolipoprotein N-acyl transferase, Lnt, in the lipidation of factor H binding protein of *Neisseria meningitidis* strain MC58 and its potential as a drug target. 20th International Pathogenic Neisseria Conference. Manchester, UK. September 2016.

Paper: da Silva RAG, **Churchward CP**, Karlyshev AV, Eleftheriadou O, Snabaitis AK, Longman MR, Ryan A, Griffin R. 2016. The role of apolipoprotein N-acyl transferase, Lnt, in the lipidation of Factor H binding protein of *Neisseria meningitidis* strain MC58 and its potential as a drug target. British Pharmacological Society. doi: 10.1111/bph.13660.

4.1 Introduction

At this point we have demonstrated that we have identified a highly effective drug candidate for the prevention of ophthalmia neonatorum. We have developed potential formulations which prevent growth and kill gonococcal cells. These have to be further tested to establish whether they are suitable for their intended purpose. The treatment being developed in this thesis is a post-exposure prophylaxis. It is deemed as post-exposure as passage through the birth canal can be classed as an event which significantly increases the risk of developing a particular disease, in this case ophthalmia neonatorum. This prophylaxis would normally be given within the first one hour of life. For this reason it would be logical to test the formulations ability to prevent the progression of infection after the bacteria have had a chance to initially infect tissues. The performance of any formulation can be tested with clinical trials but there are experiments involving the use of *in vitro* models which can try to replicate what is otherwise achieved *in vivo*.

4.1.1 Cell culture based models of gonococcal infection

One way to test the effectiveness of the prophylaxis would be in a cell culture based model. The host cells would be exposed to the bacteria, then after a defined time, the drug could be added to test its ability to prevent progression of the infection. This is particularly important in the case of gonococcal infection as *N. gonorrhoeae* is an intracellular pathogen. Once the bacteria has been internalised, it may be protected from the action of the drug. In that case, use of this drug in the formulation would not prevent the development of ophthalmia neonatorum. This is why this type of model can help answer such questions which the antimicrobial testing done so far has not.

There are many studies that have used cell culture infection models. Most of the studies focus on gonococcal attachment and internalisation to host cells. Use of cell culture based methods to study gonococcal infection became popular in the 1990's. Some of the pioneering work at that time was conducted at the University of Amsterdam. Researches originally used Chang "conjunctiva epithelial" cells (a known HeLa contaminated cell line [295]), HeLa cells (cervical cancer cell line) and HEC-1B cells (human uterus epithelial cell line) as host cells [296, 297]. Following work by this research group also used primary human cornea epithelial cells [298, 299]. The work conducted in these studies focused on cell entry, particularly the use of the gonococcal type IV pilus and Opa protein family [296]. Staining of the host cells and the gonococci at this time was done by using crystal violet and then immunogold-silver tag monoclonal antibodies used to help differentiate between bacteria and host [300].

One area of research conducted at the University of Virginia has focused more on gonococcal invasion of polymorphonuclear leukocytes [301-305]. Studies there have developed methods and techniques to aid infection such as selection of pili expressing variants and specialised fluorescence

microscopy methods [306]. Fluorescent labelling of gonococcal cells and host cells have made major advancements in the study of cell culture based infections and the study of host-pathogen interactions.

A number of studies have also been conducted at the Oregon Health and Science University which has published articles on gonococcal infection of epithelial cells (Chang and A431 (epidermal) cell lines) [102, 307-311]. Their work focuses on the role of the gonococcal pili and Opa protein family to aid attachment of the bacterial cell to the cell culture. All of these publications, plus the work of others, have helped to almost standardise infection procedures. However, certain variables still differ due to the preferences of the researcher and experimental aim. For example, the multiplicity of infection (Moi), which is the bacteria to host cell ratio differs between studies. The value is usually between 10 and 100. An infection time of 3 hours is commonly used but this does depend on the aim of the study [297, 301].

4.1.2 *Ex vivo* tissue based models of infection

There is only a single scientific article describing the infection of any excised or explanted ocular tissue with *N. gonorrhoeae* [312]. This publication describes the infection of human corneas with *N. gonorrhoeae*. The experiment used electron microscopy to describe the stages of infection and pathological effect on the tissue during the infection. It notes that attachment to the cornea surface was only achieved by piliated cells and that internalised gonococci cells were observed after just one hour. The article was published in 1988 and a lot of technologies and techniques with explanted tissue have since moved on but it does demonstrate the viability of the experiment. Other, more recent, scientific articles have been published on ocular tissue models involving different species of bacteria. One such publication on the subject describes the infection of explanted bovine conjunctiva samples with *L. monocytogenes* [313]. After the explantation of the bovine conjunctiva samples, they were infected with 10^7 *L. monocytogenes* cells, a Moi of approximately 100. The infection time used in the experiments was 20 hours after which any non-internalised cells were killed by gentamycin treatment. Internalised cells were recovered for CFU counts by homogenising the tissue in PBS with stainless steel beads.

Moving away from bacteria models, a review of the literature does show that excised corneas have been used as models of infection with herpes viruses, firstly using herpes simplex virus type 1 (HSV-1) on rabbit and human excised corneas [314]. The method describing the infection process was published in great detail by the authors and a video demonstrating the technique is available along with written instruction. The authors have since published three scientific articles using the technique [315-317] as well as it being used by other researchers [318]. The application of the technique to has also been used successfully with canine herpes virus type 1 (CHV-1) [319]. The

corneas were removed from the eyeball, explanted in cell culture medium and could be maintained for over one week. The corneas were infected with HSV-1 or CHV-1, incubated for an hour and virus removed. The tissue could then be tested directly or further incubated depending on the aim of the experiment.

4.2 Materials and methods

4.2.1 Host cells and cultivation

Primary human corneal epithelial cells (pHCEC) (Gibco, Thermo Fisher Scientific Inc) were selected to be host cells for infection. Tissue culture flasks or plates used to grow these cells were first coated with recombinant human type-1 collagen. Coating was done using a Coating Matrix Kit (Gibco). Between 60-70 μ l of Dilution Medium from this kit was used per cm^2 of vessel surface, this was added directly to the flask/plate, for example: 5 ml for 75 cm^2 flask (T75) and 1.7 ml for 25 cm^2 flask (T25). The Coating Matrix was added at a volume $1/100^{\text{th}}$ of the volume of the Dilution Medium. This was then mixed briefly and then incubated at room temperature for at least 30 minutes. After this time, the coating solution was removed and plates or flasks used immediately or stored at 4 °C for a maximum of two weeks.

When initially reviving cells from the supplier or using in-house liquid-nitrogen stocks, cryovials were first dipped into a waterbath at 37 °C for two minutes. The contents were removed using a sterile transfer pipette and put into 20 ml pre-warmed keratinocyte serum free medium (KSFM) (Gibco) in a 50 ml centrifuge tube. Antimicrobial treatment was only used on the first passage when Antibiotic-Antimycotic (Gibco) (100 units/mL of penicillin, 100 μ g/mL of streptomycin and 0.25 μ g/mL of Fungizone Antimycotic) was used. The cells were moved into a pre-coated T75 flask and incubated at 37 °C, 5 % CO_2 . The medium was changed every 2-3 days and cells checked under an inverted microscope (model AE31, Motic, Wetzlar, Germany).

When cells had become 80 % confluent, the medium was removed and cells washed with 7 ml trypsin solution (Gibco, 5.3 mM KCl, 0.44 mM KH_2PO_4 , 4.2 mM NaHCO_3 , 137.9 mM NaCl, 0.34 mM Na_2HPO_4 , 5.6 mM D-glucose, 0.48 mM EDTA, phenol red 25 μ M and 21 μ M (0.5 g/L) trypsin). After washing, a further 2 ml of the pre-warmed trypsin solution was added and incubated at room temperature for up to 15 minutes until all cells had un-adhered from the flask. Progress was monitored using an inverted microscope. After this, 8 ml trypsin neutraliser (KSFM plus 10 % fetal bovine serum (FBS) (Gibco)) was added and the flask was rocked for a few seconds. The cells were transferred to 15 ml centrifuge tubes and a 20 μ l sample taken for a cell count (see 4.2.3) before centrifuging at 125 x G for 10 minutes. The supernatant was discarded and cells resuspended in pre-warmed KSFM. A subcultivation ratio of 1:3 was recommended for this cell type but may have

been altered due to the results of the cell count, a value close to 5000 cells/cm² was required to seed a new vessel. Precoated T75 flasks were seeded with 20 ml of cells.

4.2.2 Preparing liquid nitrogen frozen stocks

As primary cells only have a limited life expectancy, freezer stocks had to be made. This was only done after the original first passage after receiving the cells from the suppliers. The cells were grown in a T75 and trypsinised as above. After centrifuging and removing the supernatant, the cells were resuspended in 8 ml freezing medium (85% KSFM, 10% FBS and 5% DMSO). This was then aliquotted into 1 ml volumes in 1.2 ml cryovials, put in a Mr Frosty freezing container (Nalgene) containing 2-propanol and moved carefully to a -80 °C freezer and frozen overnight. The samples were placed in liquid nitrogen vapour phase until required.

4.2.3 Cell counts

Cell counts were performed after trypsinising cells to ensure that flasks/plates were seeded with sufficient cells to ensure growth. For this, a 20 µl aliquot of cells was added to 20 µl 0.4 % trypan blue solution (Sigma-Aldrich) and briefly mixed. A cleaned Bright-Line™ haemocytometer slide (Hausser Scientific, Horsham, PA, USA) was prepared with a coverslip over the counting surface and 10 µl of the sample put into each sampling port of the slide. After a minute to allow for the sample to completely cover the counting surface, the haemocytometer was examined under an inverted microscope. Four four-by-four squares, each in the corners of the counting surface were counted. Cells on the top and left side lines were counted and cells on the bottom and right side lines were not counted, see Figure 4.1. Both live (clear) and dead (blue) cells were counted. Viable cell density and health estimates were estimated as equation:

Equation 4.1

$$\begin{aligned} \text{Cell density (cells.ml}^{-1}\text{)} \\ = \text{Dilution factor} \times \text{mean number of cells in one square} \times 10,000 \end{aligned}$$

4.2.4 Preparing 24-well plates for infection

Twenty-four well plates were used for the infection experiments. For each infection experiment, four wells would be set up for each sample. Two of these would be utilised for microscopy and two for bacterial cell counts. Wells for microscopy had to contain 13 mm round glass coverslips (Thermo-Fisher Scientific) so they could be easily mounted on glass slides. These were omitted from the cell count plates. The plates were collagen coated as above. T75 flasks were prepared as above to 80 % confluency and then trypsinised. However, after centrifugation the cells were resuspended

in 24 ml of medium per T75 flask of cells. The resuspended cells were then aliquoted 500 µl into each well on the plate. This would mean that one T75 flask would provide enough cells for two 24-well plates. The plates were then incubated at 37 °C, 5 % CO₂ and inspected the next day. Cells were either infected 24 or 48 hrs after trypsinising once the cells reached at least 80 % confluence.

4.2.5 Infecting pHCEC

Gonococcal strain NCCP11945 was selected for the infection. The bacteria were plated on to a GC agar plate at an approximate concentration of 10³ CFU/ml by using methods described in Chapter 2. The plates were incubated at 37 °C, 5 % CO₂ for 24 hours. This produced a plate of individual gonococcal colonies which were observed using a dissecting microscope. Piliated (P+) and Opa expressing colonies (Opa+) were then selected by colony appearance [320]. These colonies were placed in GC liquid medium with 15 % glycerol and frozen at -80 °C until required. To produce the inoculum for infecting the pHCEC, GC plates were inoculated with the P+, Opa+ freezer stocker the day before infection and incubated at 37 °C, 5 % CO₂ overnight. P+, Opa+ cells were again selected and washing into GC broth and OD_{520nm} adjusted to between 0.25 and 0.50 (equivalent to approximately 10⁷ CFU/ml). A MoI of 10 was used for the infection meaning that 50 µl of the bacterial suspension was added to positive control and sample wells. The course of the infection was run for three hours after which all wells were washed five times in sterile 0.1 M PBS and processed as below.

4.2.6 Controls and samples tested

For each experiment a range of controls were used, these are displayed in Table 4.1. A Non-Infection Control was used to show the morphology of healthy cells, Non-Infected Treated Control was used to observe the effect of the drug on the HCECs. The Infection Control samples were used as positive control; this would be used in log reductions to measure the effect of the drug and also to be sure that the fluorescence microscopy had worked. The Gentamycin Control was used for the microscopy so a comparison between the action of gentamycin and monacaprins could be made.

Monocaprins were tested at various concentrations: 1; 0.5; 0.25; and 0.125 mM. These concentrations were added to the wells just before the addition of the bacteria at time zero (T₀) but also at 90 minutes post infection (T₉₀). This tested whether the monocaprins would be able to clear the infection even if it was administered an hour or so after birth.

Table 4.1 Controls used in gonococcal infection of human cornea epithelial cells experiments.

Control	Contents
Non-Infected Control	HCEC only
Non-infected Treated Control	HCEC plus 1 mM monocaprin
Infection Control	HCEC infected with GC (Moi = 100), no 10:0MG
Gentamycin Control	HCEC infected with GC (Moi = 100), treated with 50 µg/ml gentamycin

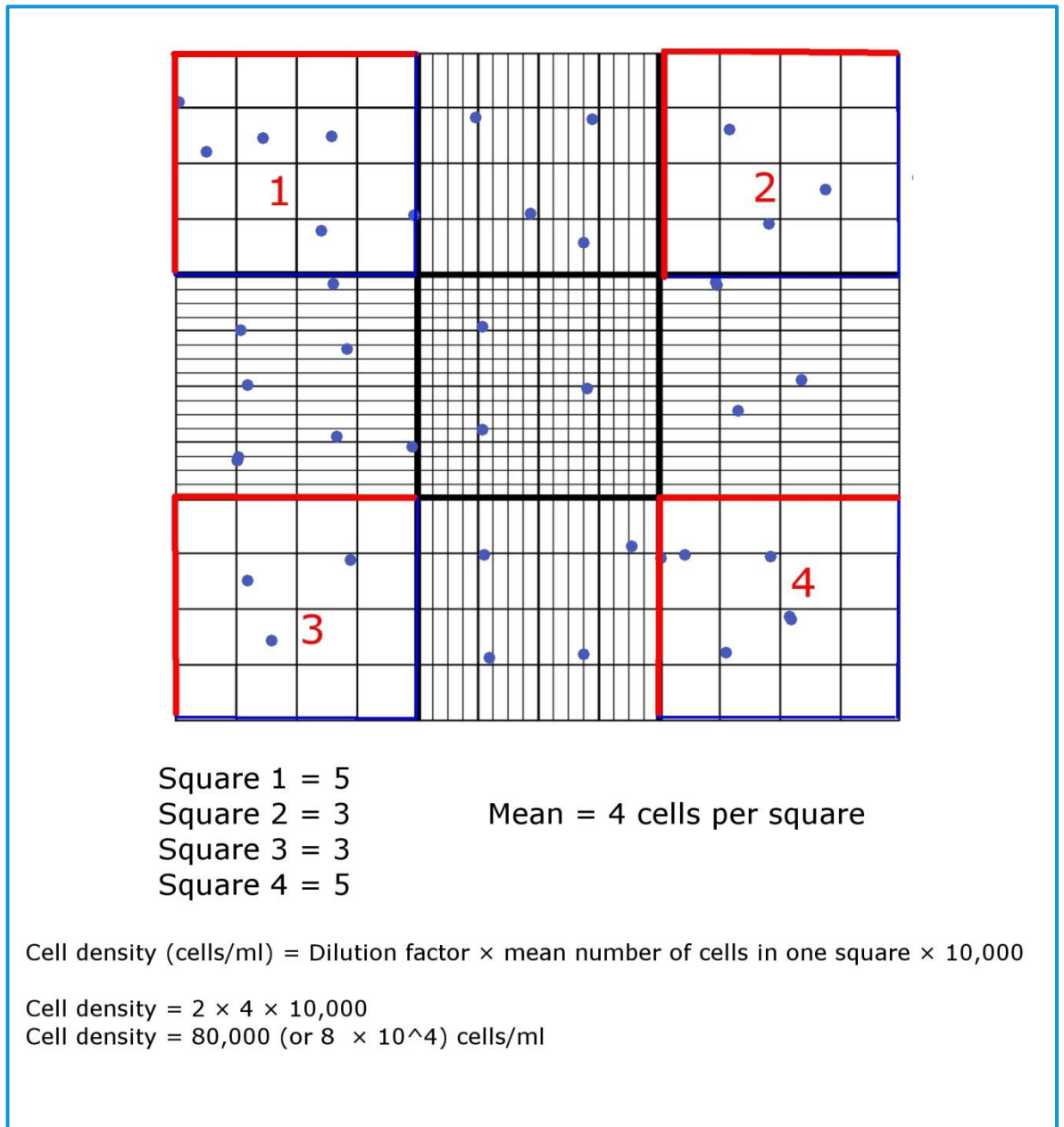


Figure 4.1 Graphical representation of cell counting using a haemocytometer slide. The cells within the four marked squares were counted. Cells touching the top or left lines (marked in red) were counted while cells touching the lower or right lines (marked in blue) were not counted.

4.2.7 Processing of fluorescence microscopy samples post-infection

The wells used for microscopy all contained 13 mm round glass coverslips. After the washing in 0.1 M PBS, the cells were fixed in 4 % paraformaldehyde for 20 minutes at room temperature. Paraformaldehyde was removed and wells washed once in PBS. The samples were blocked in 300 μ l 5 % bovine serum albumin (BSA) in PBS and incubated for 30 minutes at room temperature. The wells were washed, each with 1 ml PBS four times. To each well, 200 μ l rabbit polyclonal IgG anti-gonococcal antibody (Abcam plc., Cambridge, UK) at a dilution of 1/400 in 0.2% BSA in PBS was added and plates incubated at 4 °C overnight. The wells were washed again and 200 μ l of goat polyclonal IgG anti-Rabbit-IgG conjugated to Alexa Fluor 555 (Abcam plc.) at a dilution of 1/500 and phalloidin-Fluor 488 at a dilution of 1/1000 in 0.2 % BSA in PBS was added. The plates were incubated at room temperature for 1 hour in the dark and then wells washed again. The glass coverslips were carefully removed, dipped in deionised water and mounted cells facing down on a spot of Fluoroshield Mounting Medium with 4',6-diamidino-2-phenylindole (DAPI) (Abcam plc.) on a glass slide and allowed to set at room temperature for five minutes. The edges were then sealed using nail varnish and, once dried, viewed under a Nikon Eclipse i80 (Nikon UK Limited) fluorescence microscope and images captured under standard DAPI, FITC and TRITC filters using NIS-Elements BR 3.0 SP7 (Nikon UK Limited) software. The images were later merged using ImageJ [321].

4.2.8 Log reduction experiments of attached and internalised gonococci

After the infection and washing in PBS to remove non-attached cells, the HCEC were lysed by adding 100 μ l pre-warmed 1 % (w/v) saponin (Sigma-Aldrich) and incubating at 37 °C for ten minutes. After lysis, 900 μ l of GC broth was added and samples diluted ten-fold another three times. Each dilution was plated in duplicate, dropping 10 μ l of the dilution on a GC plate and spreading over a quarter of the plate using a loop. Plates were then incubated at 37 °C, 5 % CO₂ for 48 hours. CFU counts were estimated by averaging all four replicates (duplicate plating for duplicate wells for each sample). The CFUs of the drug exposed wells were compared to the CFU counts of the Infection Controls to estimate the log reduction caused by the action of the monacaprin. Comparisons of the log reductions at different drug administration times at given concentrations was analysed in an unpaired t test using GraphPad Prism (version 6.01).

4.2.9 Gentamycin protection assays

After the infection and washing in PBS to remove non-attached cells, 100 μ l 10 mg/ml gentamycin (Gibco) in KSFM was added to all wells and incubated at 37 °C for two hours. After the removal of the gentamycin and washing twice in 1 ml 0.1 M sterile PBS, cells were lysed and treated as the above (method for the attached and internalised cells), apart from 100 μ l of the dilutions were

plated over a whole GC plate instead of only 10 µl over a quarter of a plate. This was done as cell numbers of the “internalised only” were much lower than those of the “attached and internalised”.

4.2.10 Live dead staining

Live/dead staining was done using LIVE/DEAD® BacLight™ Bacterial Viability Kit for microscopy (Life Technologies) according to manufacturer’s instructions. After the course of infection the wells were washed five times, as before, but washes were done with sterile 0.85 % NaCl (approx. 1 ml per wash) to remove non-adhered bacterial cells.

Staining solution was made by adding an equal amount of Component A (1.67 mM SYTO 9 + 1.67 mM propidium iodide) to Component B (1.67 mM SYTO 9 + 18.3mM Propidium iodide); this was then diluted 1/333 in saline solution and 0.5 ml added to each well. The plate was incubated at room temperature in the dark for 15 minutes and then washed once in 0.85 % saline solution. Glass cover slips were carefully removed and mounted cell side down on a drop of BacLight mounting oil (provided with kit). The slides were then examined in the following 30 minutes. Due to the limited time frame, only the infection control was compared to the 1 mM treated sample. Slides were visualised on a Nikon Eclipse i80 (Nikon UK Limited). Due to the narrow band emission filter on this microscope, images were taken of samples on standard FITC and TRITC filters using NIS-Elements BR 3.0 SP7 (Nikon UK Limited) software and then merged later using ImageJ.

4.2.11 Preparation of corneas for *ex vivo* infection

The method used in this study for the preparation of the corneas and the infection was based on a method used of infecting corneas with Herpes Simplex virus [314, 316, 319]. Bovine eyes were collected from a local slaughter house (ABP, Guildford, Surrey, UK) and were transported in cold PBS plus Antibiotic-Antimycotic solution (Gibco). Once back in the laboratory, each eye was first examined for signs of damage before use. The eyeballs were then placed in a sterile 90 mm petri-dish and a sharp scalpel used to make an incision just outside the edge of the cornea. A pair of scissors inserted into the incision and the cornea removed with a small amount of excess sclera with it. The sclera was trimmed back to 3-4 mm from the edge of the cornea if required. The lens was carefully peeled away from the back of the cornea using two forceps. The corneas were rinsed in PBS plus Antibiotic-Antimycotic solution, again in PBS without antibiotics and placed on a sterile spot-plate epithelial layer facing down.

A scaffold medium was made that would maintain the shape of the cornea while also providing sufficient nutrients to maintain the cornea. To produce this medium, 0.2 g agarose was added to 20 ml Minimum Essential Media (MEM) (Gibco) and heated in a microwave just until it started to boil. This was mixed well and left to cool to approximately 55 °C before use. The medium was added to the corneas until they were filled (between 1.5 and 2 ml) and allowed set at room temperature

for a couple of minutes. The corneas were moved to sterile 6-well cell culture plates, this time with the epithelial side up. MEM plus non-essential amino acids (Gibco) medium with 50 µg/ml gentamycin was added to the wells containing cornea, enough to sufficiently to cover the whole of the cornea (approximately 5 ml). The plates were incubated at 37 °C, 5 % CO₂. The corneas would be stable for up to one week but infections were all done within 24 hours.

4.2.12 Infecting the corneas

N. gonorrhoeae strain NCCP11945 was cultured as in Chapter 2 overnight on a GC plate at 37 °C, 5 % CO₂. Cells were then scraped off the plate into GC broth and OD_{520nm} adjusted to between 0.25 and 0.50 (equivalent to approximately 10⁷ CFU/ml). To the centre of a sterile 55 mm petri-dish, a 100 µl spot of the bacterial suspension was added. The corneas were place epithelial side down on to the spot. Care was taken so that the bacteria suspension did not come into contact with any other part of the corneal button. A negative control made with a cornea incubated with 100 µl uninoculated GC medium was also done. The samples were then incubated for 1 hour at 37 °C, 5 % CO₂.

After the incubation the corneas were removed from the 6 well plates, washed five times by pipetting 1 ml of sterile PBS on to the contact points of the cornea and then the whole corneal button was dipped into sterile PBS five times taking care so that the scaffold medium did not become dislodged from the back of the cornea. The corneas were placed scaffold medium down on a new sterile 55 mm petri-dish and 500 µl of the thicken eye-drop formulations added. A summary of the formulations tested is given in Table 4.2. The formulations were left on the cornea for two minutes after which they were washed by dipping them in sterile PBS ten times.

Two methods were used to evaluate the effects of the formulations on infected corneas. Both of these methods are summarised in Figure 4.3. The first method was based on CFU counts on surviving gonococcal cells. To help recover the gonococcal cells, the corneal buttons were incubated epithelial layer down on a 100 µl spot of pre-warmed 1 % saponin in PBS in 6-well plates at 37 °C for 10 minutes. A clean scalpel blade was passed over the epithelial layer and then the saponin was used to wash the epithelial layer of any loose cells. The 100 µl of saponin was recovered and diluted in GC broth and then diluted one in ten again in GC broth. CFU counts were done by plating duplicate samples of 100 µl over a whole GC plate for both dilutions. The plates were incubated at 37 °C, 5 % CO₂ for 48 hours.

The second testing method was a qualitative method. After the corneal button had been washed after formulation treatment, they were then pressed against a GC plate and then left for 30 seconds. The samples were then discarded and the plates were incubated for 48 hours.

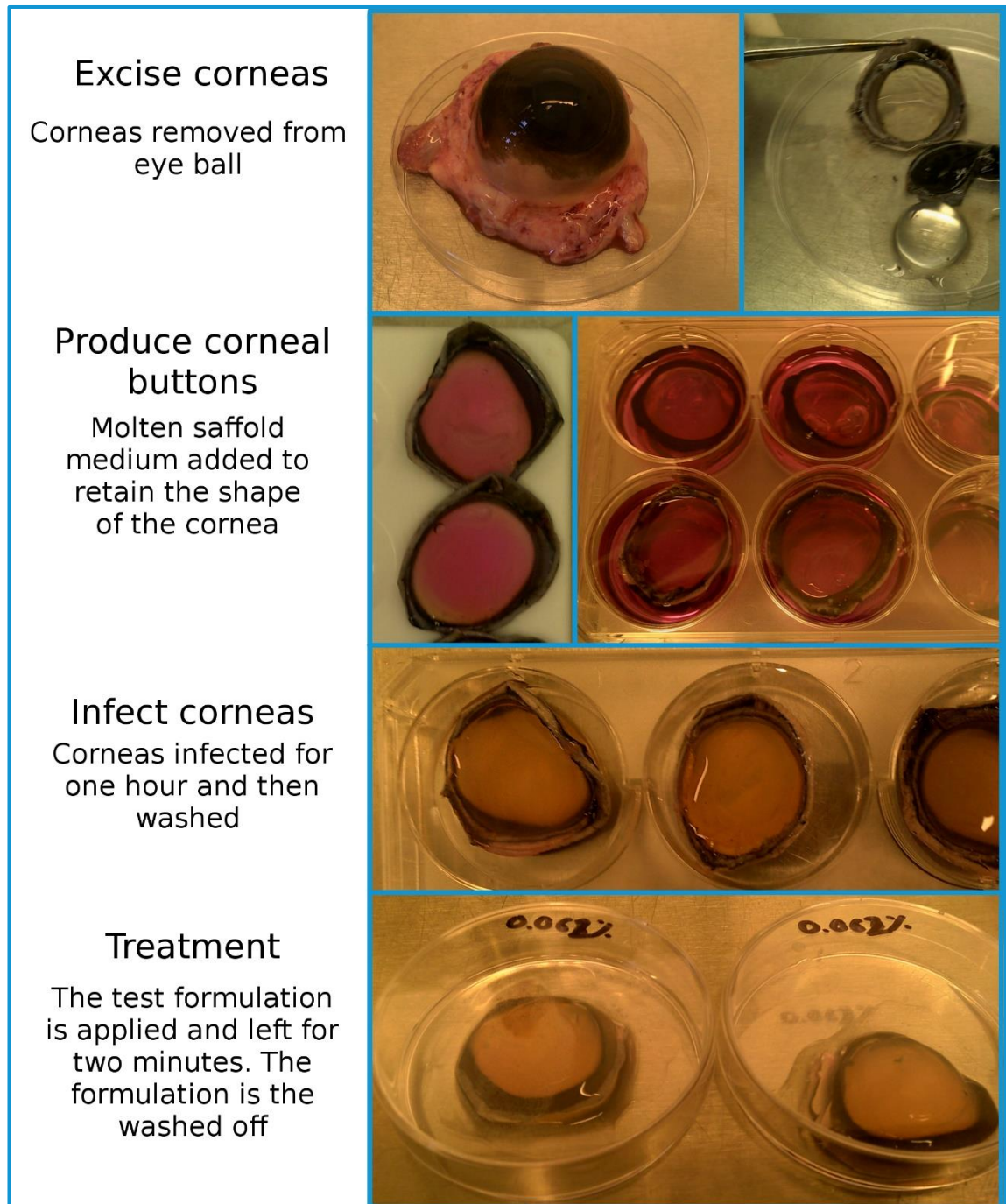
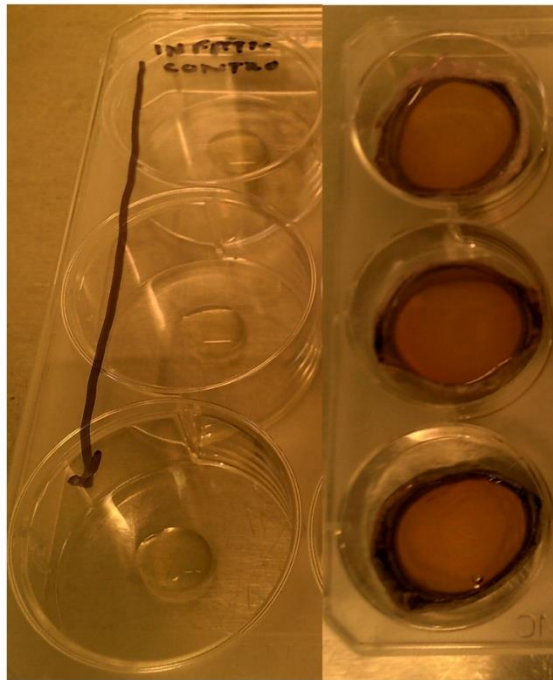


Figure 4.2 Explanted bovine cornea infection model. Photographs of the different stages of infection experiment starting from the excision of the bovine corneas. Once the corneas were removed they were infected and then treated with the test formulation.

Method 1: CFU count

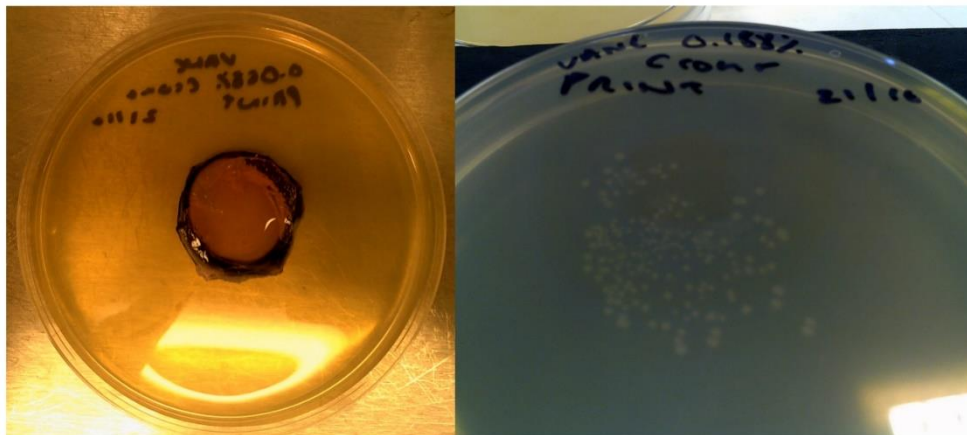


Corneal buttons removed and scalpel used to scrape the epithelial surface of the cornea. GC medium used to dilute covered cells and CFU done

Spot 100 μ l saponin in centre of wells

Incubate the corneal buttons on top of the saponin for 10 minutes

Method 2: Corneal print



Washed corneas were placed on a GC plate for 30 seconds

GC plates were incubated for 48 hours to allow the growth of recovered GC cells

Figure 4.3 Methods of assessing the effectiveness of the treatment. The first method uses a saponin treatment to release attached and internalised cells which were then counted by CFU. The second method was more qualitative. A “print” of the cornea was made on a GC which was then incubated for 48 hours. The amount of growth on the plate represents the level of gonococcal contamination on the cornea.

Table 4.2 Summary of formulations tested in the *ex vivo* cornea infection study

Formulation Name	Amount of monacaprins	Polymer used	Polysorbate 20 amount
No drug control	0 %	HPMC , 1.25 %	0.5 %
0.068 % C10MG	0.068 %	HPMC , 1.25 %	0.2 %
0.125 % C10MG	0.125 %	HPMC , 1.25 %	0.2 %
0.188 % C10MG	0.188 %	HPMC , 1.25 %	0.5 %
0.250 % C10MG	0.250 %	HPMC , 1.25 %	0.5 %

4.3 Results and discussion

4.3.1 Development of cell culture based model

Primary HCEC were used as host cells in the infection model. A continuous cell line (ATCC HCE-2 [50.B1] (ATCC® CRL-11135™)) was also successfully infected but its growth rate was much slower than that of the primary cells (data not shown). Also, these cells required pre-coating with fibronectin which is known to have an affinity with gonococcal cells [296]. The pHCECs were infected in three separate experiments and analysis by log reduction and fluorescence microscopy was done after each one.

4.3.2 CFU counts of attached and internalised gonococci

The drug candidate was tested in two fold dilution series from 1 mM to 0.125 mM. The drug was also challenged at two different time-points, at the time of the inoculation and 90 minutes after. After the course of the infection experiment, CFU counts of the drug candidate treated wells were compared to that of the Infection Control to estimate the killing effect of the drug by calculating the log reductions. A summary of the results are displayed in Figure 4.4. and the raw data is given in Tables 4.3 to 4.9. The log reduction results obtained are similar to the log reduction results obtained in Chapter 2 (Figure 2.7) when the monocaprin was tested on bacterial cell suspensions. This suggests that action of the drug candidate is concentration dependant rather than time dependant as the action of the drug when exposed for 3 hours appears to have no greater effect than the original 2 minutes log reduction experiments that were conducted in Chapter 2. However, as the experiments in this chapter and Chapter 2 are fundamentally different, a direct comparison should be conducted to prove this trend.

There is a clear difference between the log reduction results of the drug when added at the same time as the bacteria or 90 minutes after. When the drug is added at a concentration of 1 mM there is no difference in log reduction between the two different times at which the drug candidate was added. At that concentration, the drug candidate caused complete killing in both insitences. Conversely, at a concentration 0.125 mM the drug was completely ineffective, so again there was no difference between the administration time-points T_0 and T_{90} . However, the log reductions at 0.25 and 0.5 mM between the two drug administration times do appear to be different with the administration of the drug at T_0 being more effective. In the case the drug concentration at 0.25 mM, this difference was found to be statistically significant ($p = 0.0422$), while the results from the 0.5 mM concentration were not statistically significant ($p = 0.0545$). However, the latter's P value is indeed close to the 95 % confidence cut-off of 0.05 and only appears to be outside this value due to its larger standard deviation. When the drug candidate was added at a concentration of 0.5 mM,

the mean log reduction values were 2.9 and 4.3 for the administration of the drug after 90 minutes and administration at the same time as the bacteria, respectively.

The number of bacterial cells recovered after performing the gentamycin sensitivity assay (to determine internalised cell numbers) was very low. In fact, one of the experiments failed to recover any bacteria at all from any sample (Experiment 2, Tables 4.5 and 4.6), while the other experiments resulted in a number of colonies so low that they weren't statistically significant to count. Only the No Drug Control of the experiment 3 gave a statistically significant CFU count. The estimated number of internalised cells from this sample was 2.4×10^2 . This would represent 0.024 % of the original 10^6 cells that were added to the wells. The number of cells recovered from the saponin treated wells which were not subjected to the gentamycin sensitivity assay (to determine attached plus internalised cell numbers), was estimated to be 1.2×10^6 in the case of experiment 3. This would mean that close to all of the cells that were added to the wells were either attached to the host cells, attached elsewhere on the well (either directly to the well or the coating matrix) or internalised. Furthermore, all the bacteria were recovered by the saponin treatment and the succeeding recovery processes. The number of bacteria recovered from experiments 1 and 2 was 2×10^6 for both experiments. This estimate is double the estimate that was originally added to the wells in which OD_{520} was used to standardise the inoculum. This standardisation method is crude but little alternative is available as CFU counts are retrospective. A CFU count of the inoculum was not done as the number of cells that are competent to attach or internalise (numbers recovered from Infection Control) is more important than the total number of cells added.

4.3.3 Fluorescence microscopy

Fluorescence microscopy was done to visualise the gonococcal cells attached to the HCECs. This is a common technique and has been used in many studies (as previously mentioned). It was hoped that this would show some differences between untreated (Infection Control samples) and the treated samples. The gonococci cells were fluorescently labelled indirectly by using a rabbit polyclonal antibody raised against whole cell *N. gonorrhoeae* as primary antibody. This reagent had not been tested by the company for its intended use in this study so first the concentration had to be optimised. After an acceptable concentration was found by testing various concentrations (data not shown), it was used on further Infection Control and Non-Infection Control samples. The fluorescence microscopy images obtained were compared to phase contrast images, these images are displayed in Figure 4.5. As expected, it was clear that the fluorescence microscopy was a much more powerful tool in detecting the gonococcal cells. The gonococcal cells would be shown as black spots on the phase contrast images but many more cells would be shown on the fluorescence microscopy images.

Fluorescence microscopy images of all the control types and drug candidate samples were taken. Some of these images are shown in Figure 4.6. Comparing the images of the Non-Infected Control (Figure 4.6.A.) and Non-Infected Treated Control, we can see that the drug has had an effect on the morphology of the cells. The treated cells appear to be slightly smaller and with less spread (more spherical). This phenomenon was also shown in test wells infected with bacteria and treated with the higher drug concentrations, 1 mM (Figure 4.6.E.) and 0.5 mM (Figure 4.6.F.). This morphology change was not seen at lower drug concentrations in the test wells. Morphology changes due to exposure to high levels of monocaprin have previously been reported [165, 322]. However, one study that looked at the effect of 1.3 mM monocaprin exposure on the permeability of a Caco-2 monolayer demonstrated that the cells later returned to normal after 30 hours [322].

Comparing the images of the controls containing bacteria, Infection Control and Gentamycin Control (4.6.C and 4.6.D respectively), there doesn't appear to be much of a difference between them. The bacteria seemed to attach to the pHCECs in clumps. However, some bacteria were also attached to the surface of the plate, between cells. In an attempt to avoid the latter, plates were coated with BSA or serum before the infection, however there was no significant improvement (data not shown). The difference between the Gentamycin Control and the Infection Control is small, with gonococcal cells being attached to the HCECs in both cases. This may mean that the binding of the bacterial cells to the pHCECs is a passive event and does not require the cells to be alive to do so. However, the images of the test wells were slightly different to those of the Gentamycin Treated Control. The bacterial cells appeared to be in larger clumps and a greater number appear to be attached to the surface of the glass coverslip rather than the pHCEC (see Figure 4.6.E and 4.6.F for examples of this phenomenon).

4.3.4 Live/Dead staining of treated and untreated samples

The drawback to the fluorescence microscopy was that treated samples appeared too similar to untreated samples. This was despite the difference in the results from the log reduction. It was decided that Live/Dead staining should be tried. Therefore, the Live/Dead staining was done as an additional experiment, not on one of the triplicate experiments. The Live/Dead staining technique should be able to differentiate between bacterial cells which have been killed by the action of the drug and those which remained alive. Images taken of the Live/Dead staining are shown in Figure 4.7. The Infection Control samples contained a mixture of live and dead. This is not surprising, as the original inoculum used to infect the HCECs was from an overnight culture. This culture, due to its age, would probably have contained many dead cells. This was found to be the case when the kit was first trialled before these experiments on 24 hr incubated cultures of *Escherichia coli* and *N.*

gonorrhoeae (data not shown). These cultures contained a mixture of live and dead cells even through the culture would be viewed as highly viable.

In contrast to the Infection Control, the treated test samples only contained red (dead) bacterial cells. The contrast between the treated and untreated samples is striking and on the results of this study, is a better tool to use rather than the just fluorescently labelling the bacteria. This also explains why the fluorescence microscopy images mainly contain host cells which have more than ten cells attached, despite the Mol being ten. As the OD₅₂₀ is used as an estimate of how many viable cells are present, the number of dead gonococcal cells, which are now known to be capable of binding to their host, is unknown. As stated in the materials and methods, the health estimates of the host cells is checked but the equivalent value for the inoculum is not. Although the Live/Dead staining technique gave far more useful information, it is still of limited use. It would be difficult to process many samples in one experiment as the dyes tend to degrade rapidly. Likewise, the viability of the gonococcal cells would decrease over time after the samples have been prepared for microscopy.

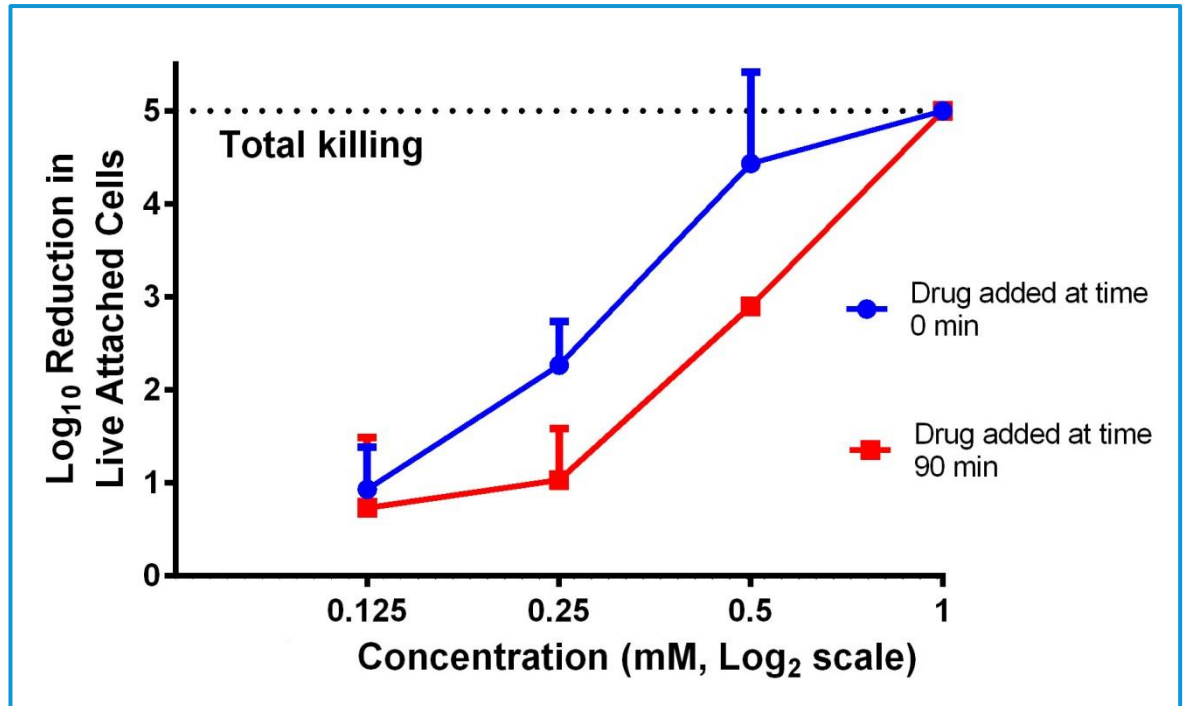


Figure 4.4 Log reduction results of attached gonococci cells at different drug candidate concentrations. The drug was either added at the same time as the bacteria (blue line) or 90 minutes after inoculation (red line). At a drug concentration of 1 mM the drug was completely inhibitory, killing all bacteria regardless of when the drug was added.

Table 4.3 Table of raw data from experiment 1 of CFU counts of attached and internalised gonococcal cells after three infections of HCECs.

Sample	T0							T90						
	R1*	R2	R3	R4	D [#]	CFU	Log Reduction	R1	R2	R3	R4	D	CFU	Log Reduction
50 µg/ml Gentamicin	0	1	0	1	0	0	>5	0	3	0	2	0	0	>5
1mM C10MG	0	0	1	0	0	0	>5	0	0	0	0	0	0	>5
0.5mM C10MG	0	0	0	0	0	0	>5	4	11	33	25	0	1.8 X 10 ³	3.0
0.25mM C10MG	4	37	6	52	-1	2.5 X 10 ⁴	1.9	26	73	130	90	-1	8.0 X 10 ⁴	1.4
0.125mM C10MG	90	44	43	153	-1	8.3 X 10 ⁴	1.4	15	71	36	68	-1	4.6 X 10 ⁴	1.6
No Drug	10	28	25	18	-3	2.0 X 10 ⁶	N/A	17	24	23	38	-3	2.6 X 10 ⁶	N/A

Key: * denotes replicate number. # denotes dilution factor in terms of 10^D.

Table 4.4 Table of raw data from experiment 1 of CFU counts from gentamycin protection assay for internalised gonococcal cells after three infections of HCECs.

Sample	T0							T90						
	R1*	R2	R3	R4	D [#]	CFU	Log Reduction	R2	R3	R4	D [#]	CFU	CFU	Log Reduction
50 µg/ml Gentamicin	0	0	0	0	0	0	0	0	0	0	0	0	0	0
1mM C10MG	0	0	0	0	0	0	0	0	0	0	0	0	0	0
0.5mM C10MG	0	0	0	0	0	0	0	0	0	0	0	0	0	0
0.25mM C10MG	0	0	0	0	0	0	0	0	0	0	0	0	0	0
0.125mM C10MG	1	2	0	0	0	0	0	1	0	0	0	0	0	0
No Drug	1	2	0	0	0	0	N/A	2	4	7	8	0	0	N/A

Key: * denotes replicate number. # denotes dilution factor in terms of 10^D.

Table 4.5 Table of raw data from experiment 2 of CFU counts of attached and internalised gonococcal cells after three infections of HCECs.

Sample	T0							T90						
	R1*	R2	R3	R4	D [#]	CFU	Log Reduction	R2	R3	R4	D [#]	CFU	CFU	Log Reduction
50 µg/ml Gentamicin	0	0	0	0	0	0	>5	0	0	0	0	0	0	>5
1mM C10MG	0	0	1	0	0	0	>5	0	0	0	0	0	0	>5
0.5mM C10MG	5	4	18	14	0	1.0 X 10 ³	3.3	37	40	11	18	0	2.7 X 10 ³	2.8
0.25mM C10MG	8	9	50	51	0	3.0 X 10 ³	2.8	3	8	13	12	-2	9.0 X 10 ⁴	1.3
0.125mM C10MG	29	24	35	24	-2	2.8 X 10 ⁵	0.9	73	76	68	64	-2	7.0 X 10 ⁵	0.4
No Drug	20	19	18	21	-3	2.0 X 10 ⁶	N/A							

Key: * denotes replicate number. # denotes dilution factor in terms of 10^D.

Table 4.6 Table of raw data from experiment 2 of CFU counts from gentamycin protection assay for internalised gonococcal cells after three infections of HCECs.

Sample	T0							T90						
	R1*	R2	R3	R4	D [#]	CFU	Log Reduction	R2	R3	R4	D [#]	CFU	CFU	Log Reduction
50 µg/ml Gentamicin	0	0	0	0	0	0	0	0	0	0	0	0	0	0
1mM C10MG	0	0	0	0	0	0	0	0	0	0	0	0	0	0
0.5mM C10MG	0	0	0	0	0	0	0	0	0	0	0	0	0	0
0.25mM C10MG	0	0	0	0	0	0	0	0	0	0	0	0	0	0
0.125mM C10MG	0	0	0	0	0	0	0	0	0	0	0	0	0	0
No Drug	0	0	0	0	0	0	N/A	0	0	0	0	0	0	N/A

Key: * denotes replicate number. # denotes dilution factor in terms of 10^D.

Table 4.7 Table of raw data from experiment 3 of CFU counts of attached and internalised gonococcal cells after three infections of HCECs.

Sample	T0							T90						
	R1*	R2	R3	R4	D [#]	CFU	Log Reduction	R2	R3	R4	D [#]	CFU	CFU	Log Reduction
50 µg/ml Gentamicin	0	0	0	0	0	0	>5	1	1	0	0	0	0	>5
1mM C10MG	0	0	0	0	0	0	>5	6	9	0	4	0	0	>5
0.5mM C10MG	0	0	0	0	0	0	>5	14	25	16	9	0	1.6 X 10 ³	2.9
0.25mM C10MG	8	6	11	10	-1	8.8 X 10 ³	2.1	34	42	51	76	-2	5.1 X 10 ⁵	0.4
0.125mM C10MG	55	56	18	26	-2	3.9 X 10 ⁵	0.5	68	93	65	84	-2	7.8 X 10 ⁵	0.2
No Drug	12	15	10	11	-3	1.2 X 10 ⁶	N/A							

Key: * denotes replicate number. # denotes dilution factor in terms of 10^D.

Table 4.8 Table of raw data from experiment 3 of CFU counts from gentamycin protection assay for internalised gonococcal cells after three infections of HCECs.

Sample	T0							T90						
	R1*	R2	R3	R4	D [#]	CFU	Log Reduction	R2	R3	R4	D [#]	CFU	CFU	Log Reduction
50 µg/ml Gentamicin	0	0	0	0	0	0	1	0	1	0	0	0	0	1
1mM C10MG	0	0	0	0	0	0	1	0	0	0	0	0	0	1
0.5mM C10MG	0	0	0	0	0	0	1	0	0	0	0	0	0	1
0.25mM C10MG	0	0	0	0	0	0	1	2	4	1	0	0	0	1
0.125mM C10MG	3	1	1	0	0	0	1	1	2	1	8	0	0	1
No Drug	22	39	11	23	0	2.4 X 10 ²	N/A							N/A

Key: * denotes replicate number. # denotes dilution factor in terms of 10^D.

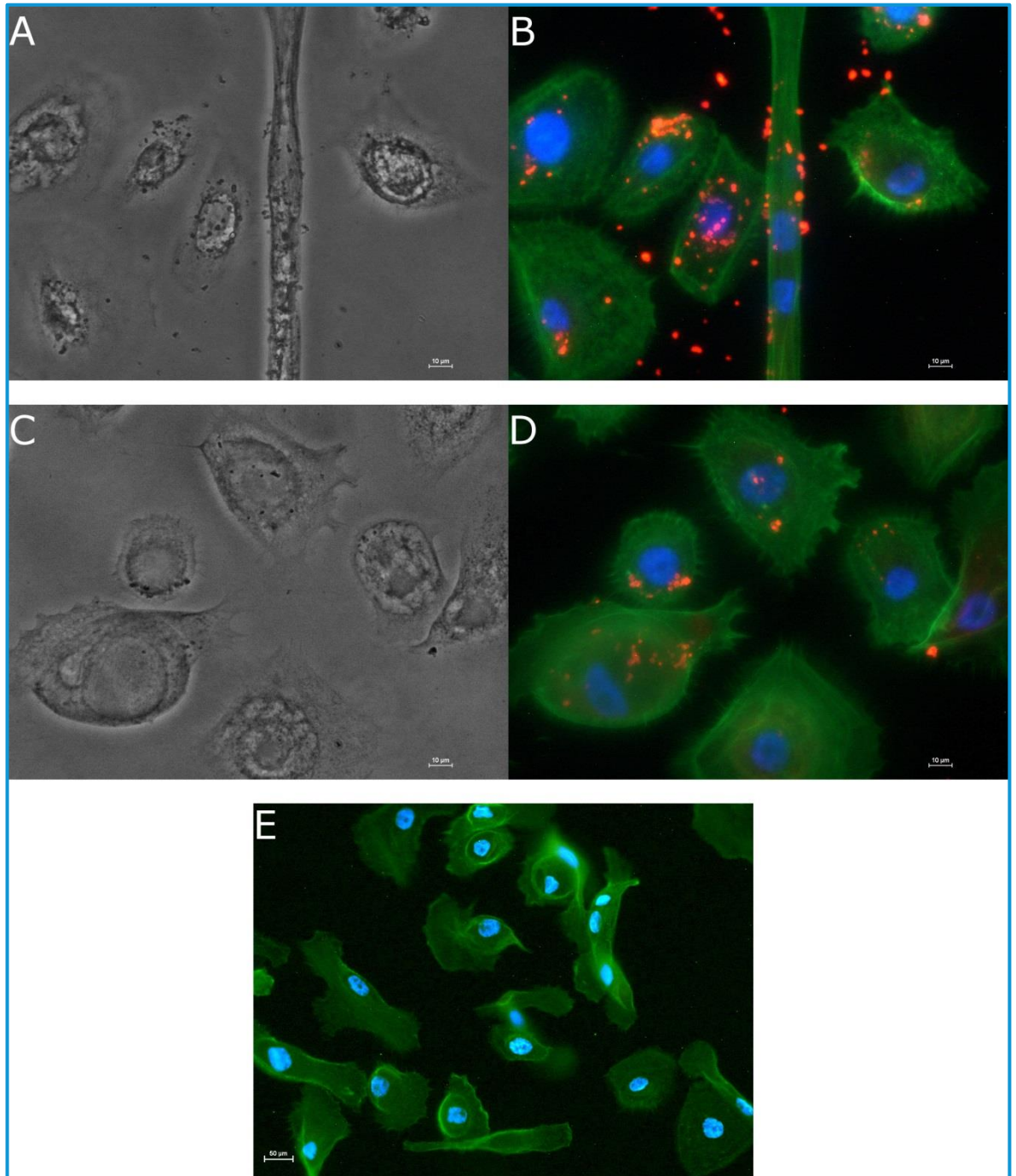


Figure 4.5 Optimisation of fluorescent labelling to detect gonococci. Infection Controls (A-D) were used to optimise the fluorescent labelling. Merged images of the fluorescence labelled samples (B and D) were compared to images taken using phase contrast (A and C). Gonococci are shown as small black dots on the phase contrast images but are clearly shown as red spots in the fluorescence labelled images. Fluorescence labelled Non-Infected Controls (E), show no signs of background staining. Scale bars for panels A-D represent 10 μm and the scale bar in panel E represents 50 μm.

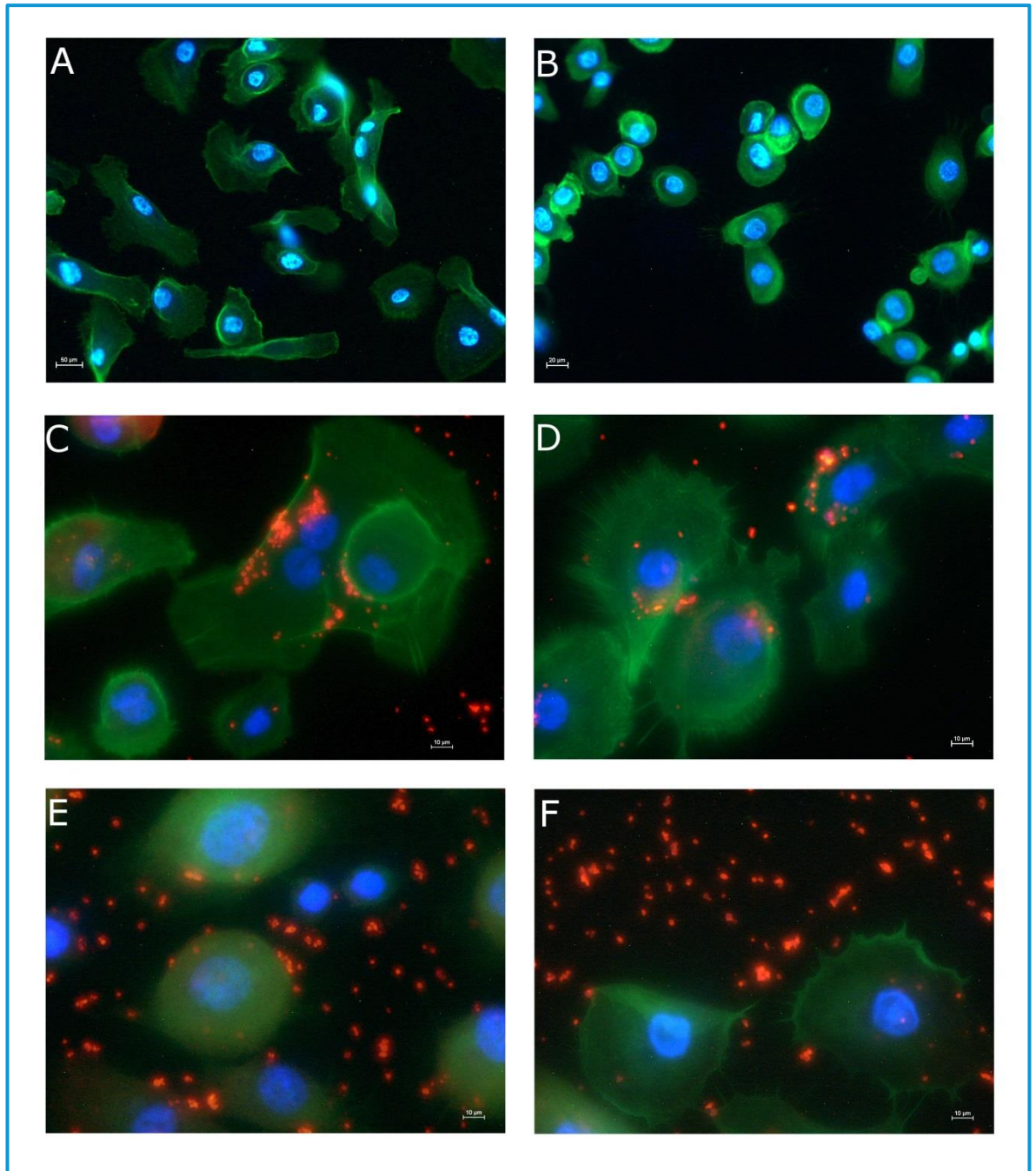


Figure 4.6 Immunofluorescent staining of HCEC infections. (A) Controls used were Non-Infected, (B) Non-Infected Treated, (C) Infection Control and (D) Gentamycin Treated. These were compared to wells treated with the drug candidate (E, 1 mM monocaprin and F, 0.5 mM monocaprin). Scale bars in panel A, B and C-F represent distances of 50, 20 and 10 μm respectively.

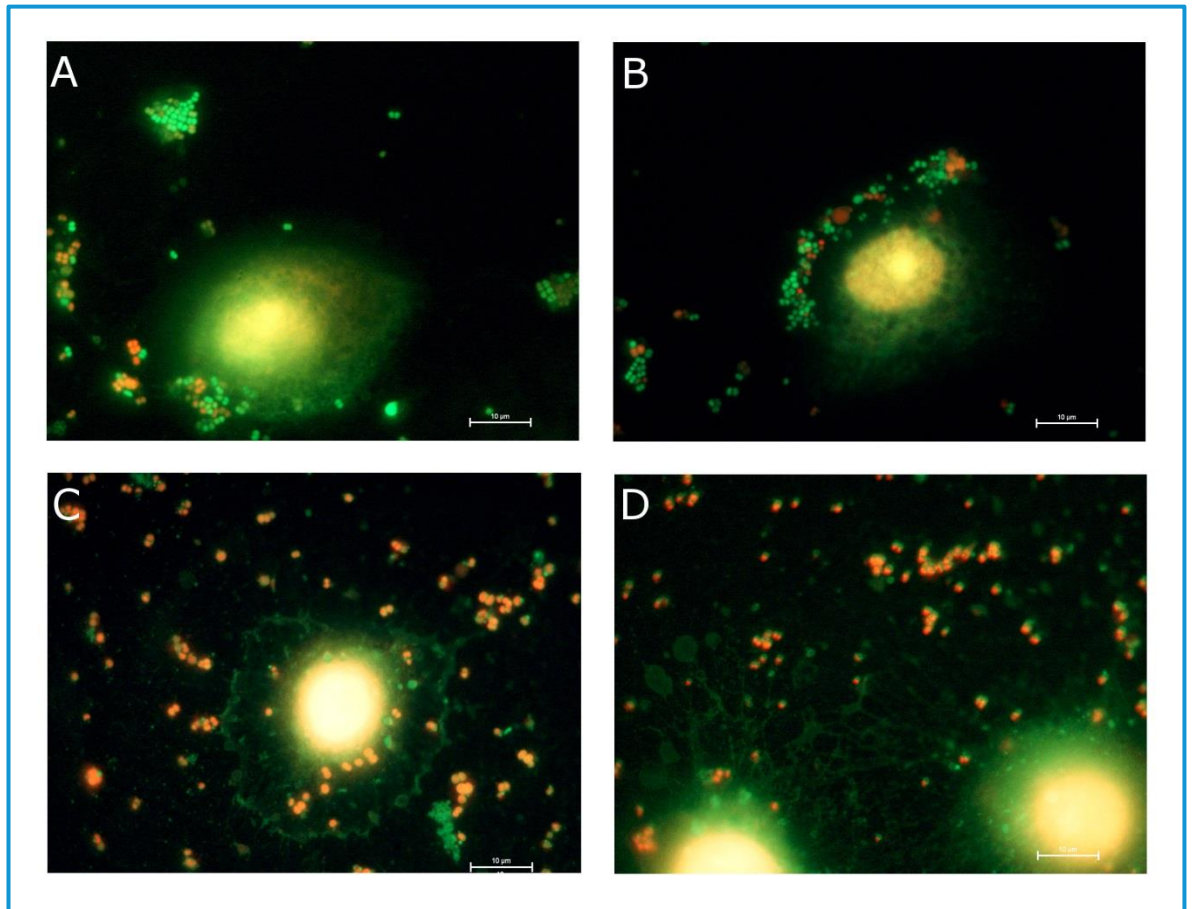


Figure 4.7 Live/Dead staining of gonococcal infected pHCECs. Live cells appear green while dead cells appear red. (A and B) Untreated Infection Control samples. (C and D) HCECs treated with 1 mM monacaprin. The Infection Control sample has many live bacteria and a few dead cells as well, while the monacaprin treated samples contain mostly dead bacterial cells, some of which are still attached to the pHCECs. Some green structures also appear in the treated samples, they appear darker and slightly less defined than the live cells in infection controls. No viable bacteria were recovered from these wells. The scale bar represents a distance of 10 µm.

4.3.5 Explanted cornea infection model

The explanted infection model was used to test the proposed formulations as opposed to the active drug candidate which was tested in the cell culture method. The experimental procedures of the explanted method were far more complex than the cell culture based model. Firstly, the samples were received from the slaughter house where the eyeballs were not aseptically handled. Use of a combination of antibiotics helped to reduce contamination, but contamination was encountered. Ideally, a model based on infecting human eyes would have been preferable. However, human eyes are difficult to obtain. Porcine eyes were sourced but these did not come directly from a slaughter house so problems were encountered with contamination and the integrity of the epithelial layer of the cornea. Due to this, bovine eyes were chosen for use in the model. The bovine corneas were indeed larger in size than either human or porcine corneas so were therefore easier to remove. However, the drawback of this was that the corneas had less curvature to them and more scaffold medium was required to fill them. Due to this, it was sometimes difficult to retain the scaffold medium within the cornea during the experiment, particularly during the extensive washing that was required post-infection and post-treatment.

The full results of the explanted cornea infection model are given in Figure 4.9 and **Error! Reference source not found.** The effect that the formulation had on the treated corneas was tested in two ways. The first was the standard CFU count method. This would estimate the number of gonococcal cells that could be recovered from the cornea after saponin treatment. The saponin was used to lyse the cells of the epithelial layer to release as many of the attached and internalised gonococci as possible. The corneas were infected with approximately 1×10^6 gonococcal cells. From the positive infection control, approximately 5.7×10^4 gonococcal cells were recovered; this represents around 6 % of the original bacterial inoculum. The remaining cells either did not attach, were washed off in the washing procedures or did not survive the recovery process. A number of trial runs of the experiment were conducted. The problems that were originally encountered were that this rate of infection was originally even lower. This would mean that all of the formulations would be effective, even the formulation without the drug candidate would lower the cell numbers to a level close to the lowest level of statistical significance. Lower cell numbers means that the true effectiveness of each of the formulations could not be statistically tested.

The CFU experiment does demonstrate that even treatment without the active drug candidate does yield a drop in the number of bacteria recovered post-treatment. This may be due to the amount of Polysorbate 20 present in the no monocaprin control. The surfactant may interfere with the attachment of the gonococcal to the epithelial layer. The log reduction results between the no monocaprin control and 0.068 % monocaprin formulation are very close and the images from the

corneal prints are also similar in the number of bacteria recovered. This is surprising because in Chapter 3 it was shown that the action of Polysorbate 20 was bacteriostatic not bactericidal. However, this model suggests that the inclusion of the surfactant in the formulation would be beneficial not just in terms of improving the solubility of the monocaprin but in the prevention of gonococcal infection by interfering with attachment and internalisation.

The results in Figure 4.9 and Figure 4.9 show that the action of the monocaprin was diminished somewhat in the explanted corneal model in comparison to previous testing done so far. This model represents a closer environment to the formulations intended end-use than previous experiments. It could be that the presence of the complex organic tissue somewhat hinder the action the monocaprin. However, despite this inactivation, activity of the monocaprin was restored when used at the higher concentrations and it was still able to clear the infection. When the monocaprin was used at a concentration of 0.188 % or 0.25 % it was able to completely clear the infection in some cases. The model is laborious but at present has given a closer representation of how the formulation would function in the eye.

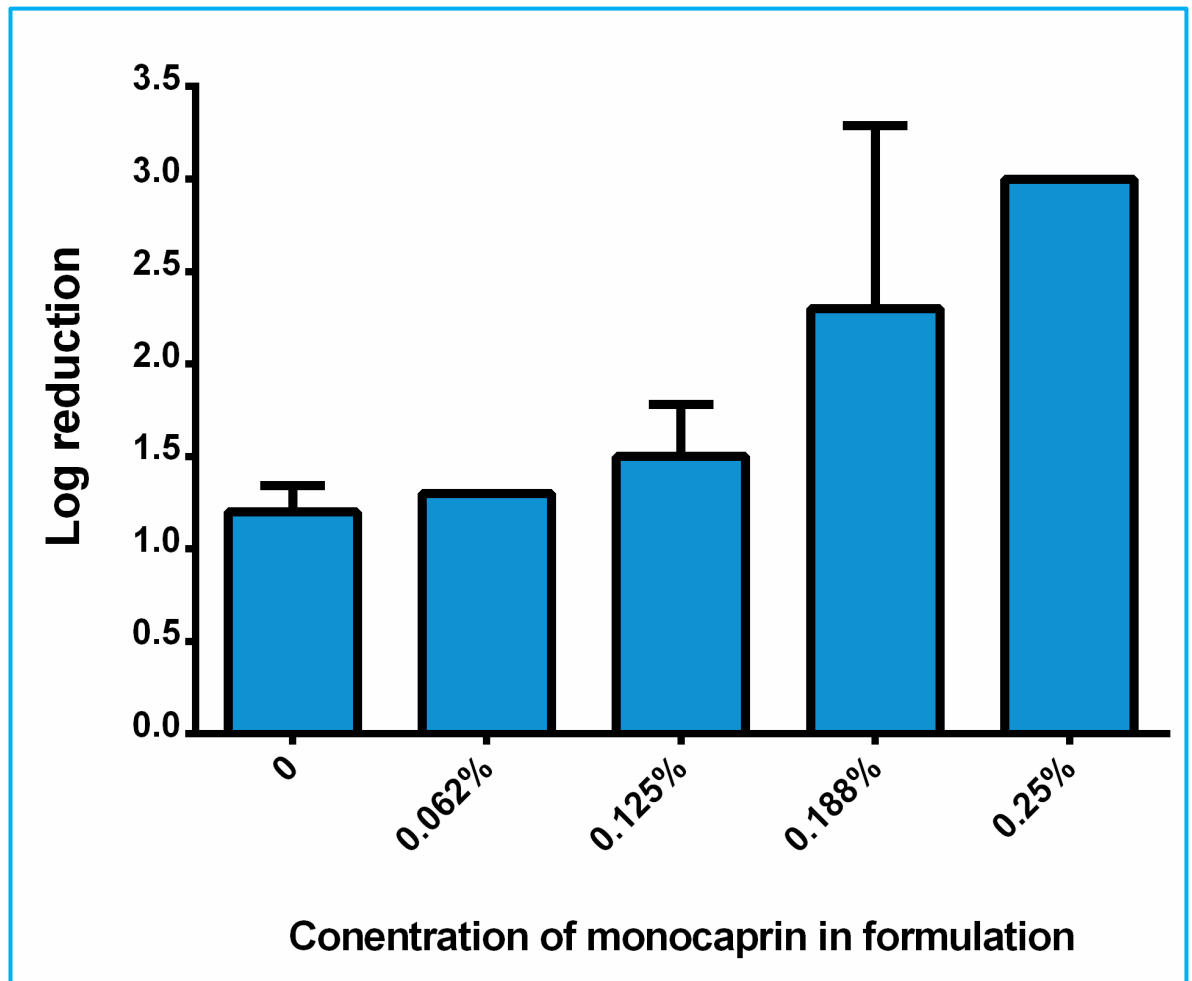


Figure 4.8 The effect of the concentration of monocaprin present in the formulations to reduce the number of live *N. gonorrhoeae* cells attached to a cornea. Different concentrations of monocaprin were used to treat infected corneas in an explanted corneal infection model. The formulation without any monocaprin added (0%), reduced the bacterial load by 1 log₁₀. Formulations with 0.062% and 0.125% monocaprin only made marginal improvements over the formulation without any monocaprin. However, the formulations containing 0.188% and 0.25% monocaprin reduced bacteria load by over 2 log₁₀. Due to the statistical limits of this test the maximum reduction possible was 3 log₁₀. Error bars represent the standard deviation over two experiments (0.25% was no error bars as it caused complete killing in both experiments).

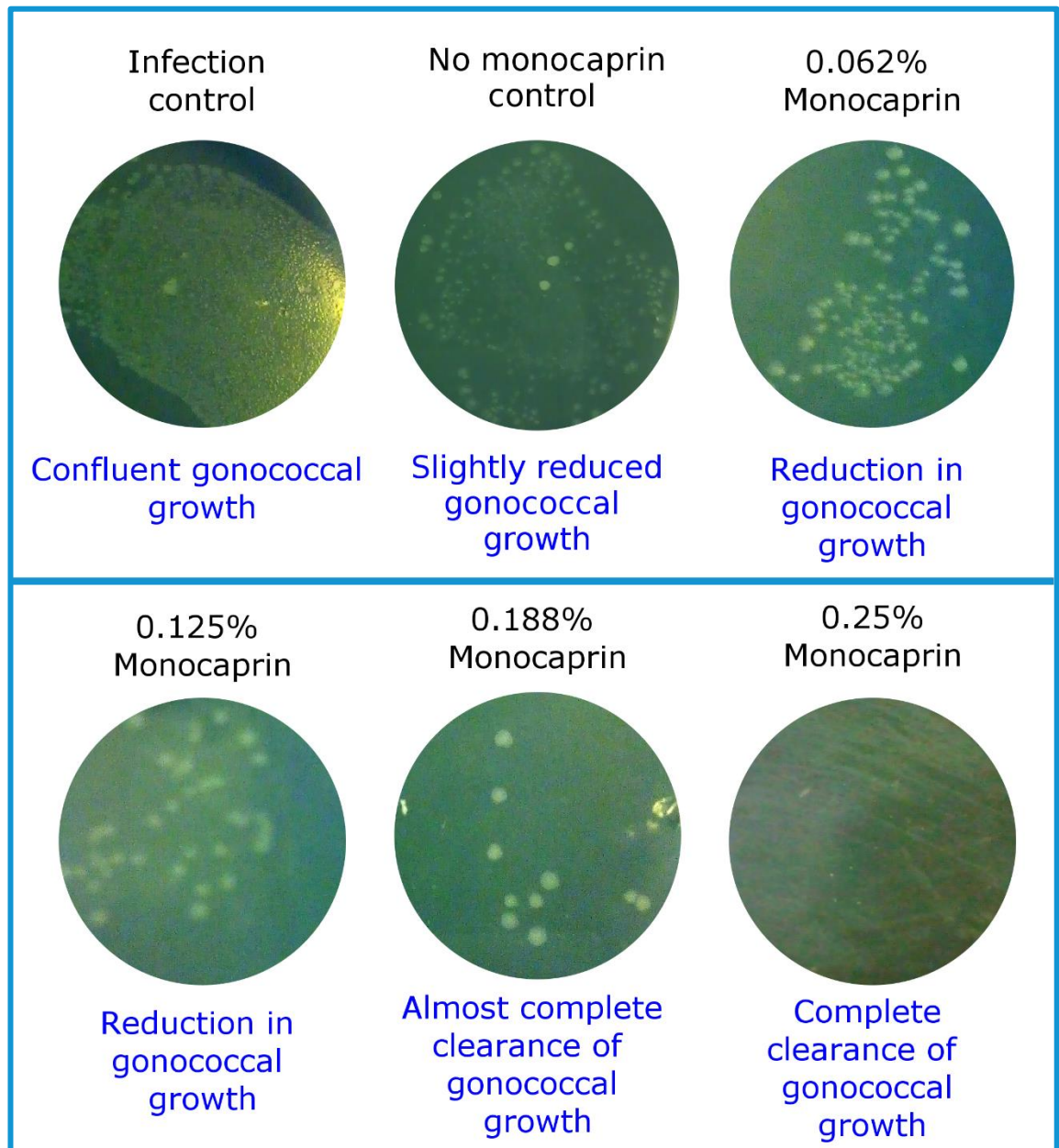


Figure 4.9 Results of the explanted corneal infection model using the corneal print method. The amount of growth present in the Infection control is the greatest. Surprisingly, the No Monocaprin Control had less gonococcal growth. The amount of growth of the test formulations decreased with monocaprin concentration

4.4 Conclusion

The cell culture infection model presented in this chapter used three methods to analyse the effectiveness of the drug candidate. The first method was the CFU count method, which proved laborious but very effective. The second method used fluorescently tagged antibodies to visualise the infecting bacteria on the host cell surface. This method has found to be unsuitable for visualising the effect of the drug candidate because infected untreated wells appeared similar to those that had been treated. Therefore, this method was only able to demonstrate that killed gonococci were still capable of binding to host cells. The third method, which used a live-dead differentiating fluorescent dye kit, proved to be a better tool to visualise the killed bacteria. However, like many qualitative tests, it could suffer from bias as results are open to the interpretation of the researcher. Overall, the CFU count method proved to be best as it produced reliable quantitative data.

Two models of infection have been trialled in this chapter. One was based on cell culture of corneal epithelial cells, the other used explanted corneal tissue. There are positive and negative attributes to each of the models. For a start, the cell culture model doesn't require the use of fresh excised animal material. As such, the experiment is more flexible, and can be conducted whenever the cells are ready. The explanted infection model is also far more laborious and has a greater risk of failing due to contamination. Furthermore, cell culture based models of infections are far more established than infection models based on explanted material. It could be argued that the explanted infection model offers a far closer representation to the conditions leading to ophthalmia neonatorum than the cell culture based model. Due to this, this model has given us important information about effectiveness of the drug candidate and the formulations.

The cell culture returned results similar to the "bacteria suspended in medium" method in Chapter 2; that a concentration of 0.5 mM monacaprins would be sufficient to reduce the bacterial load by at least 10^3 . It also demonstrated that the drug would be effective if applied 90 minutes after infection. However, in the explanted model, the formulations underperformed to what was expected. The formulations tested in the explanted model (which contained active drug candidate) were 0.062 %, 0.125 %, 0.188 %, and 0.25 %, these are approximately equivalent to 2.5 mM, 5 mM, 7.5 mM, and 10 mM respectively. The lowest of these concentrations, based on data of this thesis thus far, should be more than adequate to kill all of the bacteria on the surface of the cornea. However, the 0.062 % monacaprins formulation only performed marginally better than the No Monacaprins Control. This indicates that although monacaprins has powerful anti-gonococcal properties when mixed in a solution directly with the bacteria, when used in an environment replicating the condition of the eye, those anti-gonococcal properties are diminished. As stated in Chapter 1, there are substances that can inhibit the antimicrobial action of fatty acids and

monoglycerides. It could be possible that such inhibitors are present in the tissue of the cornea. For this reason, formulations with higher concentrations of monolaurin were used in the explanted corneal infection model than had been tested before. Despite the diminished activity of the monolaurin, when used at the higher concentrations it was still able to clear the infection. Therefore, 0.188 % or 0.25 % should provide better protection against the development of ophthalmia neonatorum.

Chapter 5 - Molecular evolution due to experimental exposure to fatty acids

Outputs

Paper: Marta A Zelewska, Madhuri Pulijala, Russell Spencer-Smith, A Mahmood Hiba-Tun-Noor, Billie Norman, Colin P Churchward, Alan Calder, Lori AS Snyder. 2016. Phase variable DNA repeats in *Neisseria gonorrhoeae* influence transcription, translation, and protein sequence variation. *Microbial Genomics*. 2016. 2 (8)

Poster: Colin P. Churchward, Alan Calder, and Lori A. S. Snyder. Induced mutations in *Neisseria gonorrhoeae* upon repeated passage on media containing a fatty acid and a monoglyceride. Poster Presentation. 20th International Pathogenic *Neisseria* Conference. Manchester, UK. September 2016.

Poster: Marta A. Zelewska, Madhuri Pulijala, Colin P. Churchward, Hiba-Tun-Noor-Afshan-Mahmood, and Lori A. S. Snyder. 2015. Identification of phase variable repeat changes in *Neisseria gonorrhoeae* using sequencing technologies 20th International Pathogenic *Neisseria* Conference. Manchester, UK. September 2016.

Acknowledgements of practical assistance

All of the isolates in this chapter were passaged by Alan Calder under supervision.

5.1 Introduction

5.1.1 Antimicrobial resistance

An overview of antimicrobial resistance in *N. gonorrhoeae* has already been given in Chapter 1. However, it is necessary to revisit this subject in context of this chapter. So far in this thesis we have established that monocraprin is a powerful bactericidal agent against *N. gonorrhoeae*, which has been demonstrated on a selection of strains that has been tested so far. However, with the development of any antimicrobial containing formulation, attention must be given to possible resistance mechanisms. There are many ways that bacteria can acquire resistance mechanisms and some of these mechanisms which *N. gonorrhoeae* uses has been discussed. Some resistance mechanisms can be induced in the laboratory experimentally. For example, growing the bacteria on media containing a sub-lethal concentration of the antimicrobial will select genomic mutations that confer an additional level of fitness in this environment to out-compete non-mutated cells. Experimentally inducing resistance to antimicrobials has been done in *N. gonorrhoeae* previously. In 1994, Belland and co-workers passaged *N. gonorrhoeae* on media containing increasing amounts of ciprofloxacin, a fluoroquinolone. The mutations from these passages resulted in an isolate that had a MIC value that was 10,000 times greater than of the parental isolate [323]. In this case, mutation in the *gyrA* and *parC* genes were identified to confer this decreased susceptibility. Mutations in the same genes were also seen in naturally occurring clinical isolates [77, 78]. Increasing resistance in *N. gonorrhoeae* to some of the newer fluoroquinolones such as ciprofloxacin was first reported in 1990 in North America [75, 76]. Ten years later the CDC no longer recommended fluoroquinolones for treatment in persons who acquired their infections in Asia or the Pacific Islands due to high prevalence of resistance in these regions [79-81]. In 2002, the CDC no longer recommended fluoroquinolones for MSM and by 2006, they no longer recommended fluoroquinolones for treatment of gonococcal infections [79].

One way to help prevent decreased susceptibility by this mechanism would be to increase the concentration of the antibiotic to a level (as long as this level can be tolerated by the human body) where the microbe is unable to escape the action of the antimicrobial, this is known as the mutant prevention concentration (MPC). This can be determined in the laboratory and can be taken into account when setting treatment guidelines. However, the difference in MIC and MPC can be fairly large in some cases. With the example of ciprofloxacin, the MPC for a non-resistant strain is about 4-fold the MIC and 16-fold the MIC for a strain with an existing *gyrA* mutation [324].

5.1.2 Experimental evolution

A field of research termed experimental evolution has been developed for the monitoring of evolution in the laboratory [325]. How the genotypic and phenotypic properties evolve over

hundreds and even thousands of generations due to a change in environment can be evaluated in detail. The most well-known experimental evolution conducted on a microorganism is the long-term evolution experiment being conducted by Richard Lenski which started in 1988 is still continuing today [326, 327]. The experiment involves the passage of twelve initially identical populations of *E. coli* in standard growth medium. The cultures are passaged every 22-26 hours by sub-culture of 1 % of the bacteria suspension [328]. The initially identical populations have diverged from each other considerably since the experiment started [329]. One of the most notable phenotypic changes is that one of the replicates is able to aerobically utilise citrate [330, 331]. *E. coli* is not normally able to do this and indeed this is a phenotype that is used to distinguishing *E. coli* from pathogenic *Salmonella*. This phenotype was not the result of one mutation but has been achieved by an accumulation of mutations [330]. These types of experiments are useful as they can tell us how a microbe is likely to change in response to a change in their environment.

5.1.3 Evolve and re-sequence

Next generation sequencing (NGS) has revolutionised DNA sequencing [332]. NGS is the term used to describe multiple technologies (produced by competing manufacturers) for the high-throughput, low-cost generation of DNA sequencing [333, 334]. The cost of sequencing is now so low that it is being considered as a diagnostic tool for the identification of human [335-339] and veterinary [340, 341] pathogens. Identification in this way would have other benefits such as predicting the presence of antimicrobial resistance mechanisms, toxin production, virulence factors and genotyping.

The low cost means that it can be considered for analysis of experimental evolution studies. The thought of sequencing the whole genome of a bacteria that had previous been sequenced to map mutations would have been unthinkable a few years ago. Today, masses of genomic data can be produced from experiments and a non-targeted, non-biased approach can be taken. So popular has this approach become that the term “evolve and re-sequence” (E&R) has been coined [342, 343]. E&R has been used not just on bacteria but also on higher organisms such as *Saccharomyces cerevisiae* [344] and *Drosophila melanogaster* [345, 346]. The E&R approach is a powerful one and could identify mutations in unexpected regions of the genome.

5.1.4 NGS – Illuminia

The main NGS technology used in this study is Illuminia so therefore the technology behind this method will be briefly explained here. The technology was originally developed by the company Solexa which was formed by Cambridge scientists Shankar Balasubramanian and David Klenerman in 1998 [347]. By 2005, they had produced a prototype DNA sequencer that was based on sequencing by synthesis technology which showed great promise [348]. In 2006 the Genome

Analyzer was launched. In 2007 Solexa was acquired by Illumina and continued to produce DNA sequencers based on sequencing by synthesis technology.

An overview of the sequencing by synthesis used by Illumina is given in Figure 5.1. More detailed explanations of how the sequencing technology works can be found on Illumina's website [349, 350]. Illumina is one of the best NGS technologies for bacterial genome re-sequencing as it has one of the highest throughputs per run, lowest error rates and does not create homopolymer-associated errors in comparison to some other high-throughput sequencing platforms [351, 352].

5.1.5 Variant calling

NGS typically produces a higher error rate than conventional Sanger sequencing. However, each base in a genome is typically sequenced many times in different reads. The number of times a single base has been sequenced in different reads is termed the coverage. The higher the coverage, the higher the confidence that can be given in the generated data. However, the sequences generated from the different reads have not come from one single DNA molecule. The starting material used in the library preparation is from a population of bacteria which may have differences between each cell. This is typical of viral and bacterial population which have a relatively high mutation rate and is referred to as a quasispecies model. Therefore, at a particular nucleotide in the genome, some of the population may have a different base at this position and therefore be a different genotype. This could be represented in the read data as long as the genotype is not a very small minority and that there is enough coverage to identify it. These polymorphic sites can be identified by using software. Due to the error rate of NGS, all potential polymorphic sites have to go through a strict filter process [353-356]. An example of a work-flow showing the steps from NGS to variant calling and the QC processes is given in Figure 5.2 [356]. There is a dedicated variant calling file format which was originally created by the 1000 Genomes Project [357]. This standard is now maintained by the Global Alliance Data Working Group File Formats Task Team [358].

Although some of mutations may occur randomly, or be in more variable regions, some may arise as they offer a survival benefit in the changed environment. Cells with this mutation could out-compete other non-mutated cells to become the dominant genotype. As with mutations in *N. gonorrhoeae* on exposure to fluoroquinolones, a number of mutations, some conferring greater resistance could occur in specific regions or genes.

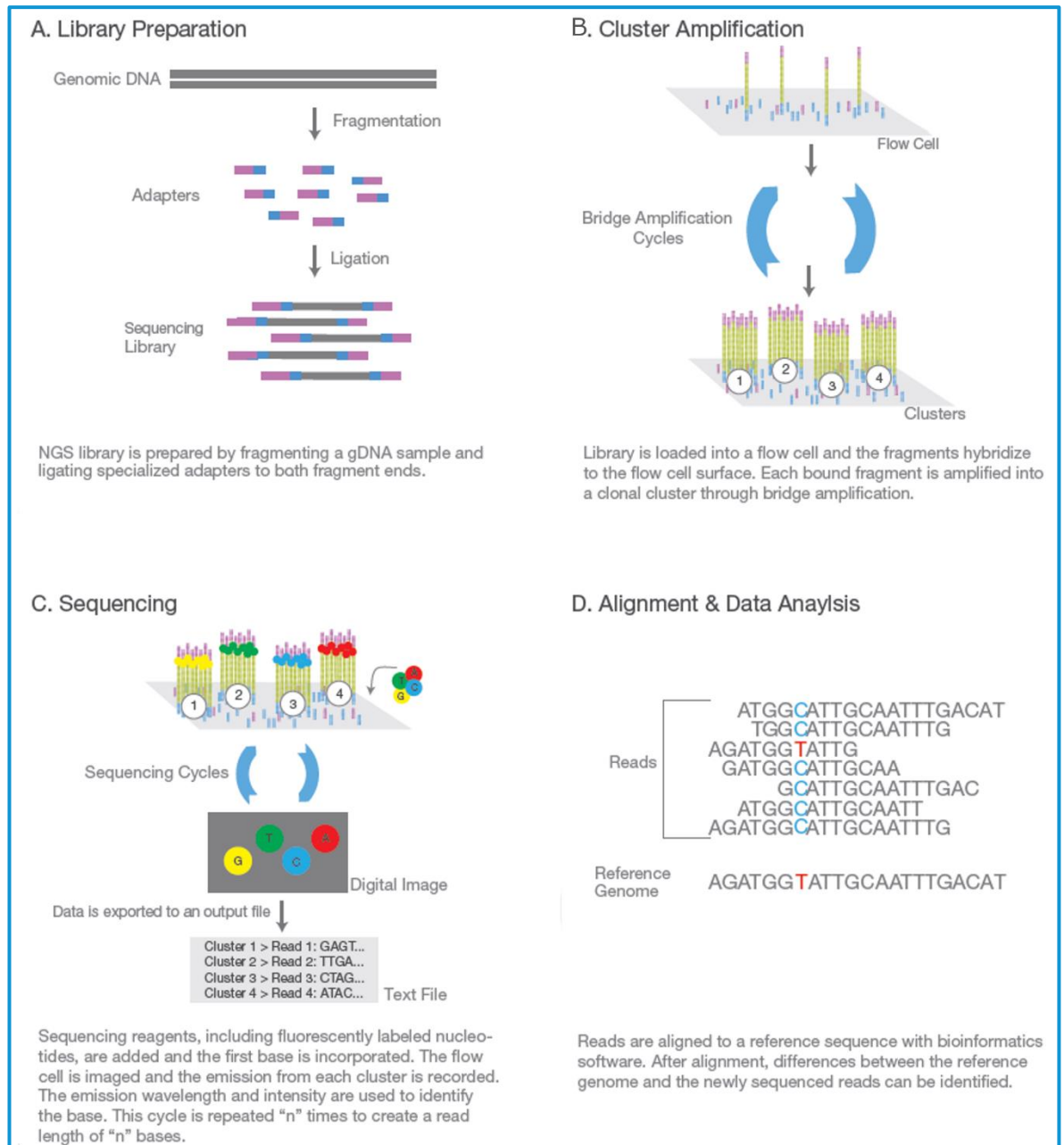


Figure 5.1 An overview of the sequencing chemistry used by Illumina sequencing by synthesis technologies [350]. (A) Library preparation, shows how the template DNA is prepared with the addition of adaptors to bind the DNA to the flow cell. (B) Cluster amplification, once a DNA strand has bonded to the flow cell, that strand is amplified by bridge amplification to form a clonal cluster. (C) Sequencing, labelled nucleotides are added and only a single nucleotide complementary to the template is added. A light source excites the fluorophore on the newly added nucleotide and light is emitted. A sensor detects the light and after, the fluorophore is removed and the cycle starts again. After the read is complete the strand is amplified again by bridge amplification and the strand just sequenced is removed leaving the reverse strand. This is then sequenced as the previous strand. This method is called paired end sequencing and enables more accurate read alignment and helps to cover repetitive regions. (D) Alignment and data analysis, the reads from the different clonal clusters are aligned using software to form an assembly and a consensus sequence is built from this.

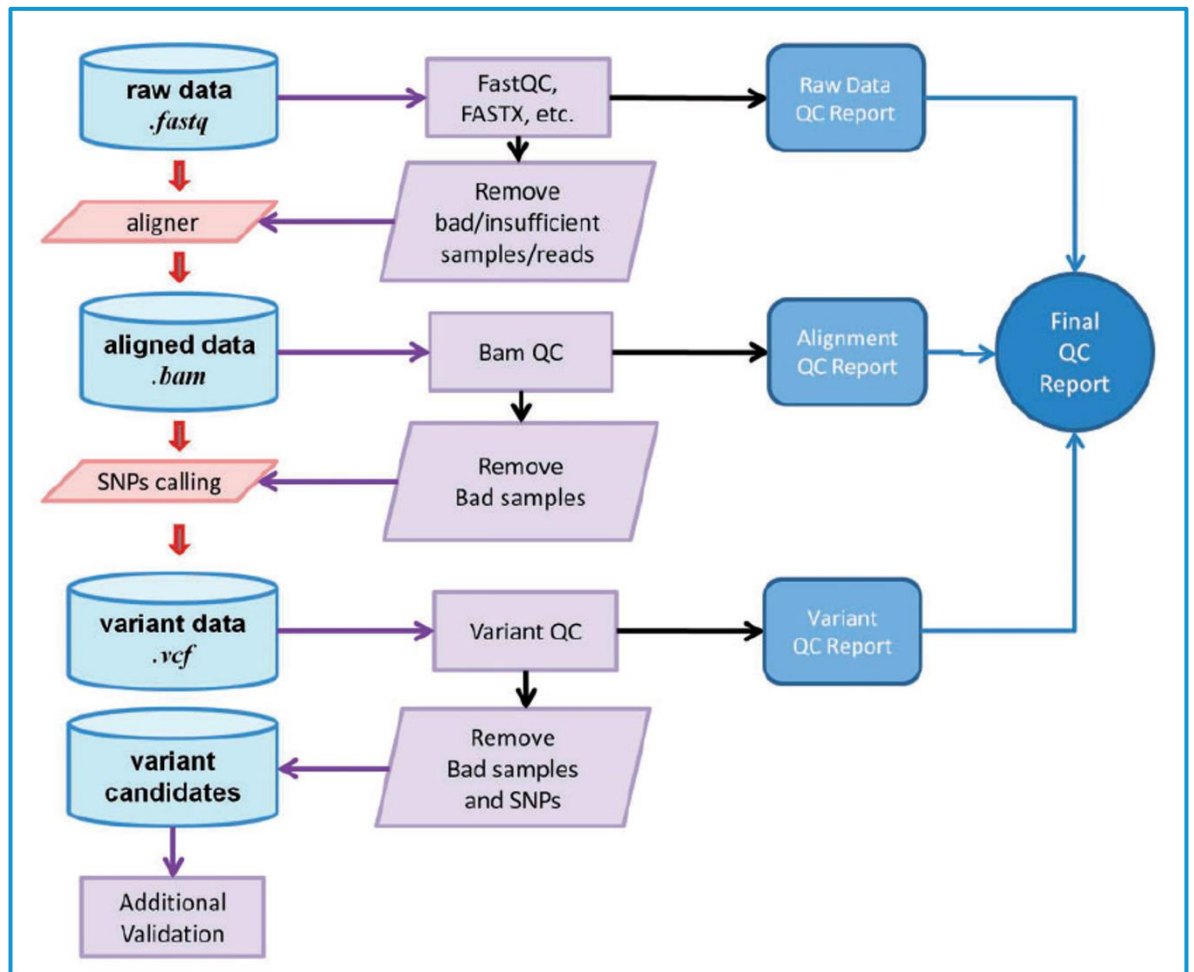


Figure 5.2 Typical work-flow of DNA sequencing experiment with QC reports [356].

5.1.6 Fatty acid resistance in *N. gonorrhoeae*

Efflux pumps and fatty acid resistance in *N. gonorrhoeae* have been outlined in Chapter 1. However, here it is necessary to describe in more detail these systems and how changes in these systems can result in a change in resistance. The FarA-FarB-MtrE efflux pump has been demonstrated to confer decreased sensitivity to certain fatty acids [148]. Transcription of *farAB* is controlled by the FarR protein and integration host factor [150]. Therefore, mutations in *farAB* or *mtrE* open reading frames (ORFs) or promoters; or mutations in any of their regulators could have an effect on the resistance profile of the bacteria. For example, mutation in the promoter of *mtrR* have been shown to exhibit higher levels of resistance to MtrC-MtrD-MtrE efflux pump substrates [359, 360].

5.1.7 Aim of this chapter

The aim of this chapter is to try to experimentally induce resistance to our main drug candidate and thus to identify possible mechanisms of resistance. This will be done by using an experimental evolution study using an E&R strategy to map any resulting mutations that could cause an increase in resistance to our drug candidate and other fatty acids.

5.2 Materials and methods

5.2.1 Bacterial growth and molecular evolution

For the experimental evolution, three conditions were used: two with inhibitors monocaprin (10:0MG) and myristic acid (14:0) and the other without any inhibitor (non-selective). Monocaprin was chosen as it is the main drug candidate in this study and myristic acid was chosen as it has very good bacteriostatic properties against *N. gonorrhoeae* but also has a known mechanism of resistance in the *farAB-mtrE* encoded efflux pump system [148].

A diagram representing the outline of the experimental evolution experiment is shown in Figure 5.3. Duplicate samples were done for each sample condition. The inhibitors were present in GC solid media at sub-MIC levels as determined below. The GC plates were prepared as in methods section 2.2.1 with slight modification: 90 mm petri-dishes were used instead of 55 mm. To start the experiment, a stock of *N. gonorrhoeae* NCCP11945 which was made from the original NCCP11945 received by Kingston University, was removed from -80 °C storage and defrosted at room temperature. From this, 100 µl was plated over a whole agar plate using an 'L' shaped spreader. The plate was incubated at 37 °C, 5 % CO₂ for 48 hours. To passage the cells, a sterile plastic inoculation loop was used to scrape up cells off the plate and put into 1 ml GC broth medium in a 1.5 ml centrifuge tube. Cells were added until a turbidity equivalent to a 0.5 McFarland standard was produced. The tube was then shaken to produce a homogeneous mixture. A sterile cotton wool swab was dipped into the bacterial suspension and a continuous streak done over an entire GC agar

plate. For the first passage, this process was done on six GC plates, two ordinary GC plates (non-selective), two GC plates containing monocaprin and two containing myristic acid.

Passages were done every 48-72 hours until a total of twenty passages were complete. Duplicate samples for each passage condition were labelled 1 and 2 and kept separate from each other. At every third passage a Gram stain was done, to check purity, DNA was extracted, and three glycerol stocks were made (as 2.2.1) for storage at -80 °C. At the final twentieth passage all these processes were done to check the final passages.

5.2.2 Minimum inhibitory concentration estimation

Stocks of 0.1 M monocaprin and myristic acid were made up in ethanol as described in Chapter 2. GC agar was made and sterilised as 2.2.1 in 500ml volumes. The iron and glucose supplements were added and then 40 ml of the molten agar added to a 50 ml centrifuge tube containing stock monocaprin or myristic acid to give the desired overall concentration. The plates were then poured into 90 mm petri-dishes and cooled to room temperature before storage at 4 °C. The tested concentrations of the inhibitors were 500, 250, 125, 62.5, 32.25 and 16.125 mM.

A cell suspension in GC broth equal in opacity to a 0.5 McFarland standard was made. A sterile cotton-wool swab was dipped into the bacterial suspension and used to make a single continuous streak on the agar plate. Plates were incubated at 37 °C, 5 % CO₂ for 48 hours. The lowest concentration of inhibitor which prevented growth was deemed the MIC. The highest concentration of inhibitor which did not prevent growth (sub-MIC concentration), was used in experimental evolution experiment to try to induce resistance. This whole MIC process was then repeated after the experimental evolution process was completed to assess their phenotypic changes.

5.2.3 DNA extraction

DNA was extracted from 500 µl of a bacterial suspension in GC broth equivalent to a 0.5 McFarland standard. A Qiagen (Manchester, UK) Genra Puregene Yeast/Bac kit was used to extract DNA using an in-house method which had been developed from the original manufacturer's instructions with slight modification to increase the yield of DNA from *Neisseria* species. The bacterial suspension was centrifuged at 13,000 x G for 30 seconds, pellet resuspended in 500 µl cell lysis solution and incubated at 80 °C for 10 minutes. The tubes were removed and allowed to cool briefly at room temperature. Then, 1.5 µl RNase A solution was added and mixed thoroughly by inverting the tubes 25 times. The tubes were incubated at 37 °C for one hour.

Once the tubes were removed from the incubator, they were cooled on ice for one minute. After this, 100 µL protein precipitation solution was added and the tubes were shaken on vortex mix for

20 seconds. The samples were centrifuged at 14,500 x G for three minutes. The supernatant was removed and added to a new 1.5 ml centrifuge tube containing 300 µl isopropanol. The tubes were vigorously mixed by inverting 50 times and then centrifuged at 14,500 x G for one minute. The supernatant was discarded and 300 µl 70 % isopropanol added. The tubes were inverted five times and centrifuged again at 14,500 x G for one minute. The supernatant was discarded and the remaining isopropanol was removed by inverting the open tubes on absorbent paper and leaving for five minutes at room temperature. The pellets were resuspended in nuclease-free water (Thermo-Fisher Scientific) and were mixed by using a vortex mixer for 10 seconds. The samples were then incubated at 65 °C for one hour and vortexed again. Samples were stored at -20 °C until required.

5.2.4 Gram staining

A plastic 10 µl loop was dipped into the bacterial suspension used at each third passage and pressed against a glass microscope slide (Thermo-Fisher Scientific). The resulting droplet was spread over a 1 cm² area of the slide and allowed to dry for five minutes at room temperature. Once dry, the cells were then fixed to the slide by passing over a 'roaring' Bunsen burner flame three times and allowed to cool. On a flat surface over a bowl, 0.1 % crystal violet was added, enough to cover the 1 cm² where the suspension was applied. The slides were left for one minute and then washed with deionised water. Gram's iodine was then added, left for one minute, and again washed off with deionised water. Ethanol was added using a glass Pasteur pipette in a continuous stream over the slide for 5-10 seconds. The slide was washed briefly with deionised water, carbol fuchsin added and left for two minutes. The counter-stain was removed, the slide finally washed with deionised water and blotted dry on absorbent paper. After this, the samples were checked under a light microscope at up to 1,000 times magnification. Any sample that had bacteria present that weren't small Gram negative (pink) cocci-diplococci were discarded.

5.2.5 Illumina DNA sequencing

DNA extractions from the six experimental evolution samples 20th passage, as well as the DNA extraction from the original low passage *N. gonorrhoeae* strain NCCP11945 were chosen for DNA sequencing. The DNA concentrations of the samples were determined using a NanoVue Spectrophotometer (GE Healthcare Life Sciences, Little Chalfont, Buckinghamshire, UK). The spectrophotometer was first calibrated against 1.5 µl nuclease-free water. For testing, 1.5 µl of sample added. The DNA concentrations were recorded, and the absorbance ratios at 260/280 nm and 260/230 nm were also recorded to give an indication of purity. After removal of the sample, the target plate was cleaned using a lens tissue paper before the next sample was loaded.

Sample details were sent to MicrobesNG (University of Birmingham, Birmingham, UK, supported by the BBSRC, grant number BB/L024209/1) and barcoded sample stickers were received through the post. The DNA samples were transferred to 1.2 ml screw-capped vials (Nalgene) and the appropriate barcode added. The samples were sent next-day, recorded delivery to MicrobesNG. The samples were DNA sequenced using 2x250 bp paired-end reads on an Illumina HiSeq 2500.

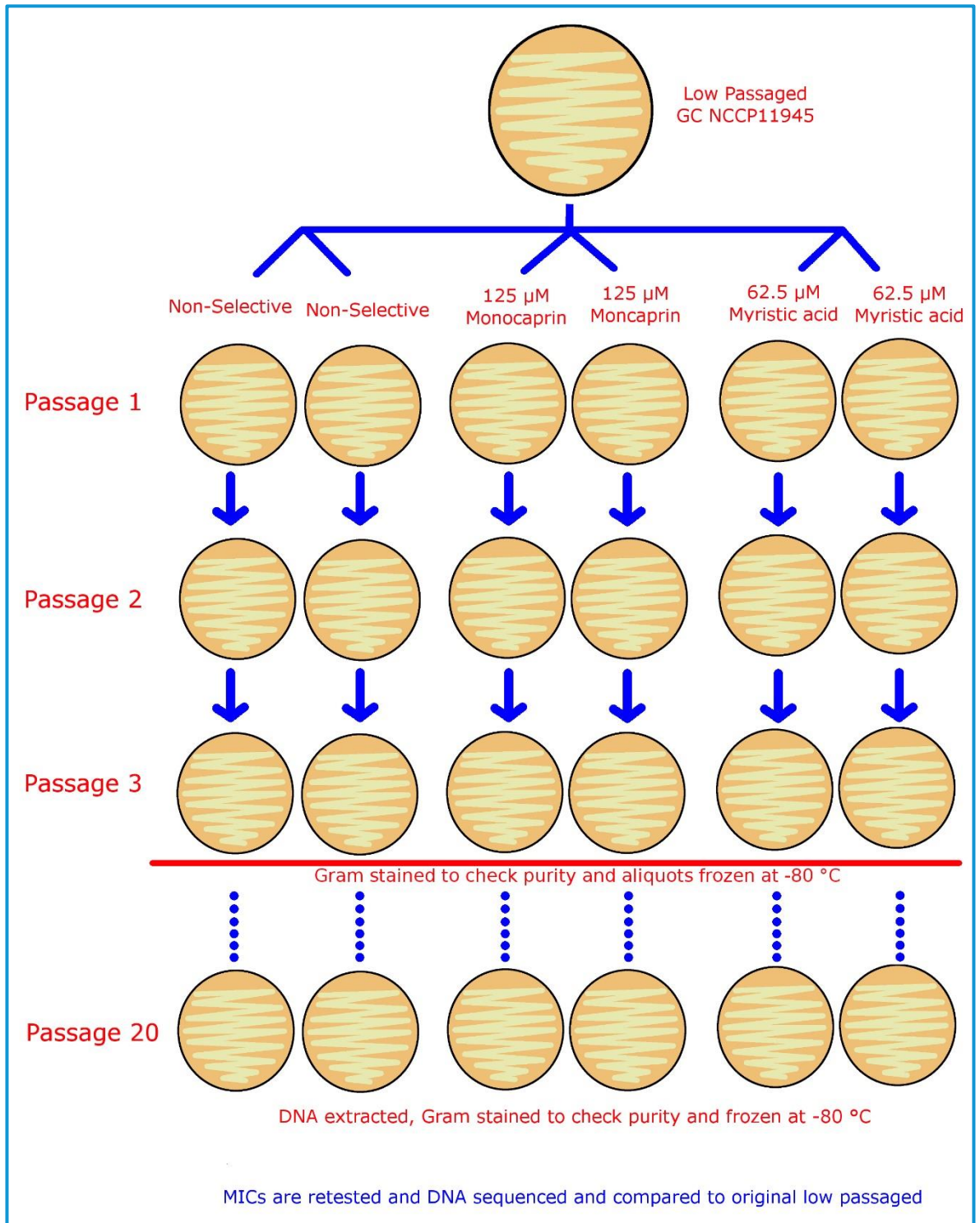


Figure 5.3 Diagram representing an outline of the experimental evolution study.

5.2.6 Bioinformatics – quality control and genome assembly

Trimmed read data in the form of FastQ files were downloaded from the MicobesNG server (<http://microbesng.climb-radosgw01.bham.ac.uk>). The reads had already been trimmed to remove adapters and index sequences. Also, in the automated post-run processing, reads of less than 36 bases were discarded and very long reads trimmed to 251 bp. Only reads that had a matching pair were used in the analysis. The unpaired reads were also provided but were not used in the analysis.

All bioinformatic analysis was done on a Hewitt Packard Pavilion series 7 laptop, with a Pentium dual-core T4300 @2.10 GHz CPU, 4 GB of RAM running Linux 64 Bit Ubuntu 14.04.

All the read files were checked using FastQC version 0.11.2 [361] and output files stored as html files. The software package UGENE version 1.20.0 [362, 363] was used to align the read files. UGENE used a Bowtie2 plug-in [364] to assemble the upstream and downstream paired read files against the published sequence of NCCP11945 which was downloaded from Genbank as a fasta file (accession number NC_011035.1) [224]. The alignments were saved as BAM files. Coverage data was exported from UGENE and converted into histograms using LibreOffice Calc 5.0.

5.2.7 Consensus SNP and variant calling

Each mapped BAM files was aligned against the NCCP11945 Genbank fasta sequence as reference. Consensus SNPs were exported from UGENE as snp files. Variant calling was performed in UGENE using SAMtools plug-ins mpileup and bcftools [365]. Reads that had a base quality lower than 13 were discounted and a minimum coverage of two at the site of interested was used to filter results. Results were exported as vcf files.

Assemblies in UGENE were checked manually for the positions in the snp file. Any positions that had a coverage less than two reads were noted and processed further but were excluded from the final analysis. Genbank files (.gb) for NCCP11945 (NC_011035.1) were downloaded. Artemis version 16.0.0 [365, 366] was used to load the genbank files which displays the genome annotation. The SNP locations were located on the annotated genome and genomic feature at that position was recorded such as if present in an ORF, repetitive region or non-coding region. If located in an ORF, then the gene was identified and any predict consequence from that mutation (such as an amino acid substitution) was also recorded. All SNPs and indels were tabulated and then separated into coding and non-coding regions. They were further separated into mutations that had occurred in all samples (compared to Genbank reference), present in only non-selectively passaged cells in comparison to unpassaged cells (defined as low passage sample), mutations occurring in myristic acid passaged samples, mutations occurring in monocaprin passaged samples and mutations occurring in multiple samples but across sample groups. Also, particular attention was paid to the

promoter regions of *farR*, *farAB*, *mtrR* and *mtrCDE*. For this, the vcf files were checked and loaded against the assemblies in UGENE.

5.2.8 Testing of SNP effects

The position of any mutation was checked in Artemis with the loaded NCCP11945 Genbank annotation. The position of the mutation in terms of the change in codon was also record and any nonsynonymous mutations checked further. The online bioinformatic tool SNAP2 [367] was used to predict the impact of a single amino acid substitution on protein function. The program would return a result of with either “effect” (score >0) if it predicted that the substitution may have a functional effect on the protein or “neutral” (score <0) if it predicted that the substation wouldn't have an effect. For each of these predictions it would score the substation and give and an estimations of accuracy of the prediction.

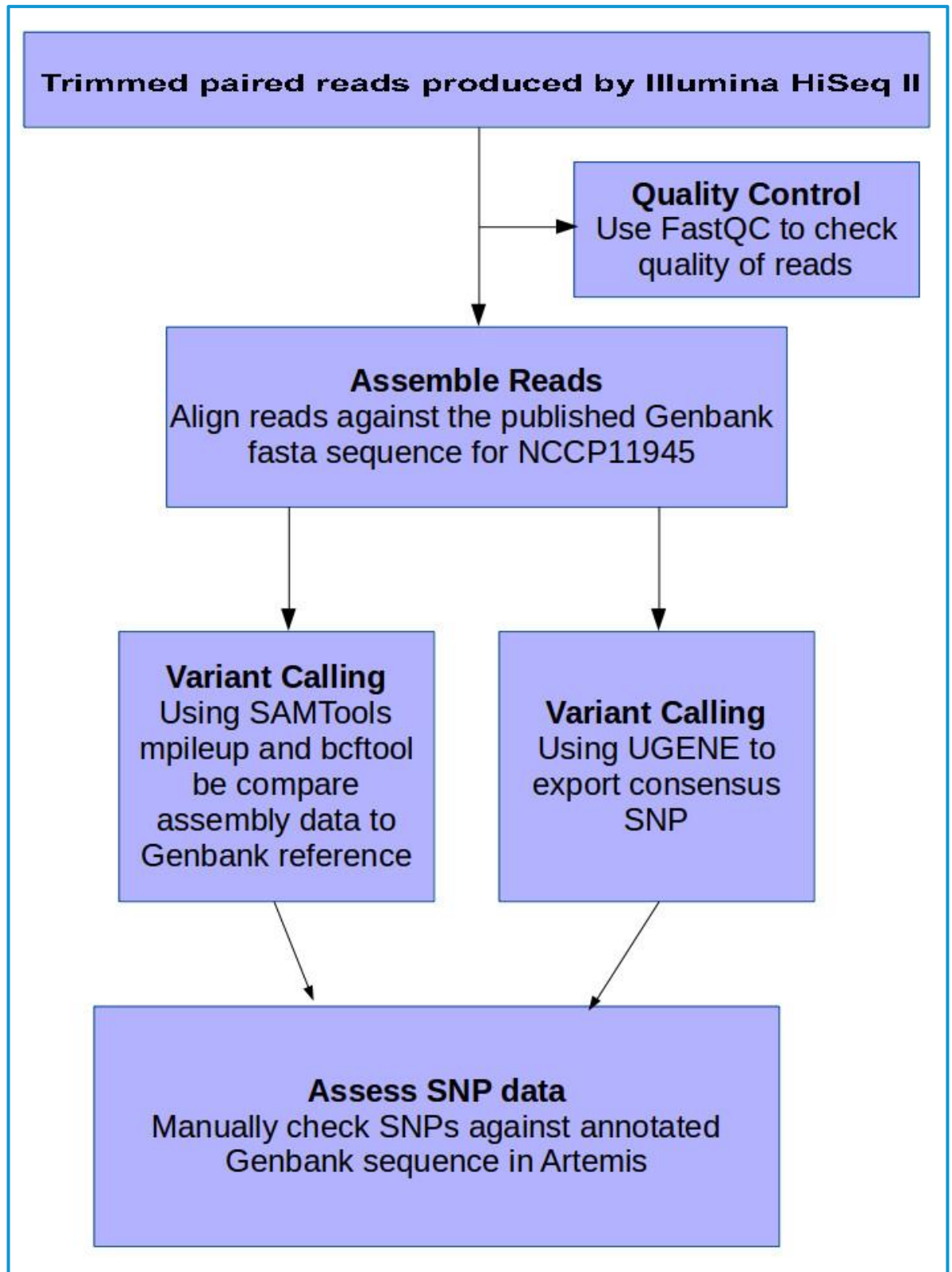


Figure 5.4 Work-flow diagram of bioinformatics approach to identify mutations occurring in passaged samples.

5.3 Results and discussion

5.3.1 Initial MIC testing

The results of initial MIC testing are shown in Table 5.1. The MIC values were 125 and 250 μM for myristic acid and monocaprin, respectively. Therefore, concentrations of 62.5 and 125 μM were used in the experimental evolution for myristic acid and monocaprin, respectively.

5.3.2 Experimental evolution

The non-selective, monocaprin and myristic acid passage samples were successfully passaged for a total of twenty passages each. Growth on the plates containing inhibitors were observed to be slower than those on non-selective plates. This was especially true for the first few passages on the myristic acid plates. This could be due to the concentration of myristic acid being more of a challenge than that of the monocaprin as the experimentally determined MIC for myristic acid may be closer to its true MIC than the monocaprin samples. The samples were passaged every 48-72 hours. The cells had to be passaged for up to 72 hours due to practical limitation, i.e. they couldn't be passaged on weekends. It was noted that on occasion the monocaprin or myristic acid passaged samples were deemed non-viable due to this increased incubation time on the plates containing the inhibitors. This was never the case with the non-selective sample despite their presumed higher growth rate. However, during the experiment, the growth rates of the selective plates were observed to increase, particularly for the initially slow myristic acid samples.

5.3.3 Gram staining

The Gram stains during and after the experiment all showed small Gram negative cocci or diplococci. No other bacteria were observed at any point.

5.3.4 Post-experiment MIC testing

The results of the MIC testing on post-experimental evolution are displayed in Table 5.1. The results of monocaprin MIC for the samples passaged on plates containing inhibitors were all the same. The values were double (250 μM) that of the non-selectively passaged cells and the low passage cells. This indicates that passage on myristic acid prepares the bacterial cells well for growth on monocaprin. This could be due that their resistance mechanisms being the same or similar. The results of the myristic acid MIC testing were more surprising as the increase in MICs were far greater than those seen with monocaprin. The monocaprin passaged cells had an eight-fold increase in MIC value for both duplicate samples. The myristic acid passaged samples had a sixteen-fold increase in MIC value. The sample replicate designated "14:0-1" appeared to have a distinct growth rate advantage over the second replicate but despite this failed to grow on a higher concentration plate (2 mM). They were therefore given the same MIC value. It seems that the growth on monocaprin

has conditioned the cells well for growth on myristic acid with a large increase in MIC. However, unlike the testing of monocaprin, the cells grown on that particular inhibitor do appear to have a slight advantage over the cells passaged on the other inhibitor.

Table 5.1 Minimum inhibitory concentrations for monocaprin and myristic acid of the original isolate (Low passage) and of the six isolates after the twenty passages on non-selective and media containing inhibitors.

Isolate	Monocaprin MIC (μM)	Myristic acid MIC (μM)
Low Passage	125	62.5*
Non-selective 1	125	62.5
Non-selective 2	125	62.5
Monocaprin 1	250	500
Monocaprin 2	250	500
Myristic acid 1	250	1000
Myristic acid 2	250	1000*

* denotes weak growth plate with highest antimicrobial plate allowing growth

5.3.5 DNA extractions.

DNA was extracted and DNA concentrations estimated. The DNA concentration estimates and purity ratios are given in Table 5.2.

5.3.6 NGS sequencing and assembly

Links to data files from the sequencing were emailed 107 days after submission of the samples. The data was downloaded and analysed. The quality of the paired read files was performed with FastQC and a summary is given in Appendix 5.1. The coverage of the samples varied. The sequencing service aimed to obtain a mean coverage of 30. Indeed, an average coverage of 30 is required to gain a high level confidence in a completed genome for publication [368]. Some of the samples were a little below this value but was still of a good enough quality to conduct this study. The coverage summaries of the sequence data are given in Table 5.3. The full coverage histograms are given in Appendix 5.8-5.14. Non-selective 1 had the lowest average coverage at 16.22. The quality of the DNA judged by the 260/280 and 260/230 (Table 5.1) appear to be good so the reason for the lower coverage may not be due to the DNA quality.

The paired sequencing reads were successfully mapped to the Genbank NCCP11945 reference genome. There were however, some gaps in the assemblies. Table 5.4 lists the positions and features of these gaps for each of the samples. There were a few reoccurring regions of the genome where these gaps were present such as: NGK_RS03237 – Peptidase, NGK_RS03380 – hypothetical protein, NGK_RS05170 – integrase, and Non-coding repeat region containing 1154312. Low coverage in some areas of the genome was found in the original sequencing that required primer walking to fully complete [224]. These areas could be due to repetitive regions as the *N. gonorrhoeae* genome is known to have an abundance of repetitive DNA uptake sequences, neisseria intergenic mosaic elements and Correia elements which could hinder the genome assembly process [224]. Repetitive regions cause ambiguities in alignment and assembly which cause produce biases and errors when interpreting results [369].

Table 5.2 Estimated DNA concentrations of passaged samples before sending for DNA sequencing

Sample	Estimated Concentration (ng/ μ L)	A260/280 ratios	A260/230 ratios
Low Passage	634.5	1.970	1.923
Non-selective 1	643.0	1.991	1.877
Non-selective 2	498.0	1.972	1.883
Monocaprin 1	456.0	1.961	2.022
Monocaprin 2	1815	2.023	1.881
Myristic acid 1	141.5	1.814	1.497
Myristic acid 2	146.0	1.814	1.669

Table 5.3 Number of reads and coverage statistics of the sequence assemblies of the Illumina data

Sample	Number of reads	Mean coverage	Mode coverage	Median coverage
Low Passage	429,999	34.46	34	34
Non-selective 1	343,345	26.78	25	26
Non-selective 2	343,345	16.22	15	15
Monocaprin 1	431,099	34.49	34	33
Monocaprin 2	432,281	34.89	35	35
Myristic acid 1	287,133	21.77	20	22
Myristic acid 2	486,881	38.96	27	35

Table 5.4 Regions of NCCP 11945 genome not represented in the NGS data for each sample

Sample	Region of no coverage	Gene locus	Gene product	
Low passage	595990-595995	NGK_RS03237	Peptidase	*
	82331-823364	NGK_RS04550	hypothetical protein	
	940920-941056	NGK_RS05170	integrase	#
	1154310-1154331	Non-coding repeat region	N/A	\$
Non-selective 1	595974-596000	NGK_RS03237	peptidase	*
	621405-621417	NGK_RS03380	hypothetical protein	&
	940932-941039	NGK_RS05170	integrase	#
	1154296-1154332	Non-coding repeat region	N/A	\$
	1724334-1794913	Non-coding repeat region	N/A	
	2128304-2128320	NGK_RS11605	pseudogene, glycosyl transferase family 1	
	2212388-2212419	Non-coding repeat region	N/A	
	2230855-2230877	NGK_RS12140	protein translocase component YidC	
Non-selective 2	549722-549743	NGK_RS03010	hypothetical protein	
	595974-596071	NGK_RS03237	peptidase	*
	621391-621485	NGK_RS03380	hypothetical protein	&
	709564-709568	Non-coding	N/A	
	745877-745884	NGK_RS04105	hypothetical protein	
	940960-941029	NGK_RS05170	integrase	#
	941042-941051	NGK_RS05170	integrase	#
	960561-960609	Intergenic spacer and into NGK_RS05310	ketohydroxyglutarate aldolase	
	977794-977832	NGK_RS05385	hypothetical protein	
	1041361-1041386	NGK_RS05705	type III restriction endonuclease subunit M	
	1102548-1102556	NGK_RS06005	primosomal replication protein N	
	1154306-1154371	Non-coding repeat region	N/A	\$
	1272349-1272363	NGK_RS06920	guanosine-3',5'-bis(diphosphate) 3'-pyrophosphohydrolase	
	1404473-1404578	NGK_RS07615	hypothetical protein	
	2128340-2128351	NGK_RS11605	pseudo gene, glycosyl transferase family 1	
	2187846-2187869	NGK_RS11895	membrane protein	

Monocaprin 1	621416-621435	NGK_RS03380	hypothetical protein	&
	1154312-1154331	Non-coding repeat region	N/A	\$
	2187836-2187854	NGK_RS11895	membrane protein	
	2212393-2212412	Non-coding repeat region	N/A	
Monocaprin 2	621405-621449	NGK_RS03380	hypothetical protein	&
	940948-941027	NGK_RS05170	integrase	#
	1154301-1154381	Non-coding repeat region	N/A	\$
	1794878-1794909	NGK_RS09750	pilS cassette	
	2212403-2212410	Non-coding repeat region	N/A	
Myristic acid 1	595981-596000	NGK_RS03238	peptidase	*
	621387-621482	NGK_RS03380	hypothetical protein	&
	940930-941033	NGK_RS05170	integrase	#
	960499-960599	Intergenic spacer and into NGK_RS05310	ketohydroxyglutarate aldolase	
	1105201-1105208	NGK_RS06020	copper-transporting ATPase	
	1105235	NGK_RS06020	copper-transporting ATPase	
	1105250	NGK_RS06020	copper-transporting ATPase	
	1136543-1136545	Non-coding	N/A	
	1154311-1154377	Non-coding repeat region	N/A	\$
	1253885-1254020	Non-coding	N/A	
	1794878-179491	NGK_RS09750	pilS cassette	
	2073533-2073540	NGK_RS11340	membrane protein	
	2083280-2083281	NGK_RS11385	ribonuclease E	
	2109802-2109836	Non-coding	N/A	
Myristic acid 2	122444	NGK_RS00595	citrate transporter	
	621405-621435	NGK_RS03380	hypothetical protein	&
	848553-848641	Non-coding	N/A	
	855390-855406	Non-coding	N/A	
	858540-858656	NGK_RS04750	hypothetical protein	
	859260	NGK_RS04755	hypothetical protein	
	859548-859586	Non-coding	N/A	
	860342-860354	NGK_RS04760	hypothetical protein	
	864143-864154	NGK_RS04790	hypothetical protein	
864210	NGK_RS04790	hypothetical protein		

	864291-864307	NGK_RS04790	hypothetical protein	
	866518	NGK_RS04805	conjugal transfer protein TraG	
	866546-866628	NGK_RS04805	conjugal transfer protein TraG	
	867196-867201	NGK_RS04805	conjugal transfer protein TraG	
	941031-941036	NGK_RS05170	integrase	#
	972861-972866	NGK_RS1223	hypothetical protein	
	1154296-1154321	Non-coding repeat region	N/A	\$
	1272331-1272347	NGK_RS06920	guanosine-3',5'-bis(diphosphate) 3'-pyrophosphohydrolase	
	2039081-2039198	NGK_RS11155	30S ribosomal protein S8	
	2212383-2212425	Non-coding repeat region	N/A	

* NGK_RS03237, Peptidase (4/7)

& NGK_RS03380, hypothetical protein (6/7)

NGK_RS05170, integrase (6/7)

\$ Non-coding repeat region containing 1154312 (7/7)

5.3.7 Consensus mutations and variant calling

The total number of consensus mutations and variant callings are given in Table 5.5. The non-selective 2 sample had the greatest number of both consensus mutations and variant callings. This sample also had the lowest coverage values. It could be that these facts are linked. However, the two schemes work in different ways. Assigning the consensus sequence complex but is usually assigned to the majority nucleotide present at a given location [368]. Consensus sequences can be based on a single read. This would therefore be far more prone to contain errors than other regions with better coverage. Therefore, consensus mutations based on two or fewer reads were manually discarded from the analysis. However, this system would still be prone to errors in regions of poor coverage. Variant calling on the other hand, reports a variant population if two or more reads are present that are different from the reference. Therefore, it can report on a mixed genotype in the sample even if the mutation is in a minority. All reads have to pass an additional quality filter so the stringency is increased. However, areas of greater coverage have more chance of containing two reads containing the same mutation. Therefore, consensus mutations have a bias toward regions of low coverage while variant calling is more biased to areas of higher coverage.

All consensus mutations of the seven samples in relation to the published Genbank DNA reference sequence were identified. A total of fifteen mutations were found to be present in open reading frames of all the isolates sequenced in this study in comparison to Genbank sequence. These mutations are displayed in Table 5.6. These differences are either the result of sequence errors from the original sequencing, sequencing errors in our data or mutations that have occurred in the NCCP11945 isolate between the time it was sequenced and reaching our laboratory. The original sequencing was done by Sanger sequencing with an eight-fold coverage and predicted error rate of 0.15 per 10,000 bases [224]. If the error rate is correct then approximately 33 errors are present in the 2,232,025 bp genome. All the mutations that occurred in ORFs were either in pseudogenes or were not judged to have a functional change by the Jpred 4 protein secondary structure prediction server (see Table 5.6).

Eleven unique mutations were found in ORFs of the non-selectively passage cells and are displayed in Table 5.7. No one mutation occurred in both replicates. Five mutations occurred in the ORFs of functional genes that were judged to cause a functional effect by Jpred 4. These mutations occurred in: NGK_RS00080 - preprotein translocase subunit SecG, NGK_RS00215 – ribosomal protein L11 methyltransferase, NGK_RS06935 – adenine phosphoribosyltransferase, as well as in NGK_RS03680 a membrane protein and NGK_RS00290 – a hypothetical protein.

Differences that were found in the open reading frames of the mystric acid passaged samples and monocaprin passaged samples were also located and are displayed in Tables 5.7 and 5.8,

respectively. The most striking of mutation that occurred in a myristic acid passaged isolate was a SNP mutation that would result in the 24th codon in the *farR* (annotated as MarR family transcriptional regulator) ORF being changed from a glutamine (Q) codon to an *Ochre* stop codon. The sequence assemblies and translation of the ORF of *farR* for the mutant (myristic acid 1) and the non-mutated replicate and shown in Figure 5.6. The mutation which occurs at position 61,543, is present in all sequence reads. In contrast, the mutation is not present in any of the sequence reads of the duplicate sample. A mutation resulting in the absence of FarR would increase *farAB* transcription [150]. This may suggest that the mutation is rare but gives a significant advantage when it does occur. So, cells that would have this mutation could have an advantage over the bacterial population that has the wild type form of the gene. This sample was observed to have a growth advantage over its other replicate but didn't actually increase its MIC value. It would be interesting to go back and re-sequence some of the earlier passages to find out when this mutation occurred and compare the growth rates of the pre-mutation and post-mutation isolates. It is clear that this is a non-fatal mutation but it is unclear how this mutation would affect the cell in normal growth condition. The FarR transcriptional regulator does perform some off-target gene regulation. It been demonstrated that it can activate *glnA* transcription in *N. gonorrhoeae*, responsible for glutamine biosynthesis [145], and *nadA* in *N. meningitidis* [370].

The monocaprin passaged isolates had two non-synonymous SNP mutations, both of which were predicted to have a functional effect. The first only occurred in replicate 1 and was in the phosphate permease protein. This is a membrane protein which is responsible for passively allowing entry of phosphate ions into the cell. It is not clear what effect this mutation would have on the cells ability to protect it from monocaprin.

The second SNP mutation is in the ORF of the *dnaK* which encodes a suppressor protein (DksA) (NGK_RS106025) and is present in both replicates. DksA is a zinc-containing multi-functional protein, its main role is as a transcription factor. Instead of binding to DNA to help recruit RNA polymerase, DksA binds directly to RNA polymerase. By doing so it; negatively regulates rRNA expression by destabilizes rRNA promoter complexes [371], positively regulates several amino acid biosynthesis genes [372] and regulates *fis* expression (an important gene regulator) [373]. It also inhibits transcript elongation, exonucleolytic RNA cleavage and pyrophosphorolysis; and increases intrinsic termination [374] as well as being involved with RecN in repair of DNA double-strand breaks [375] and suppression of *dnaK* [376].

As with the *farR* mutation, the *dksA* mutation is present in all assembled reads at that position in both replicates. Figure 5.7 gives the details of the mutation. The mutation causes a substitution of the 75th amino acid from a threonine to an isoleucine. This was predicted to have a functional effect

on the protein with a change in the predicted secondary structure (see Figure 5.7.B). The role of D71, D74 or A76 residues of the *E. coli* homolog forms part of the coiled-coil tip that is responsible for the DksA-specific effects on open complex formation [377, 378]. Part of the *N. gonorrhoeae* homolog protein can be seen aligned against the *E. coli* version in Figure 5.7.C. The mutation T75I is 11 amino acids away from these important residues. Although this mutation appears to be in a region that is highly conserved, that particular base is not as conserved as D71, D74, A76 and some other surrounding residues [379]. The protein appears to be highly conserved within *N. gonorrhoeae*; at the time of writing, 322 DksA protein sequences are present on the GenPept database. From these, 319 (99%) have an amino acid sequence identical to NCCP11945, while the other three have separate single amino acid substitutions. None of these substitutions were of the 75th residue.

Cellular levels of DksA in *E. coli* are relatively constant irrespective of changes in growth rate or growth phase as transcriptional control is varied by its cofactors, guanosine-3',5'-(bis)pyrophosphate (ppGpp) and initial ribonucleotides (iNTP) [380, 381]. However, research in other bacteria such as *Chlamydia pneumoniae* [382] and *Pseudomonas aeruginosa* [383] have found that the levels do increase upon certain cellular events.

Transcriptional control of *dksA* in *E. coli* is controlled by DksA (and ppGpp), negatively regulating its own transcription causing a negative feedback [381]. DksA is involved in the 'stringent response' which is the stress response that occurs when bacteria are starved of essential cellular components such as amino acids, fatty acids or iron; or exposed to heat shock, or other stress conditions [384]. It is possible this is the reason why the mutation is favourable but what effect the mutation has in enhancing or suppressing the stringent response is unknown.

Table 5.9 shows mutations that occurred within ORFs in multiple but unrelated samples. Therefore, any mutations shared between the groups of samples that were passaged on plates containing inhibitors would be present in this table. The only mutation that occurred in both of the inhibitor groups but not any of other samples was in a membrane protein (NGK_RS11245). However, all other samples apart from the low passage strain had another mutation present in the same codon. It is likely that this mutation is unrelated to the selective pressure of the inhibitor. All four samples passaged on inhibitors gained increases in MIC values against both inhibitors, this does suggest that mechanisms of survival from one inhibitor gave cross-resistance to the other. However, no common mutations in ORFs were present that could have caused this difference in the MICs.

Table 5.5 Number of variant callings and consensus mutations calculated for each sample

Sample	Variant Calling	Consensus Mutations
Low Passage	452	22
Non-selective 1	475	42
Non-selective 2	550	47
Monocaprin 1	309	31
Monocaprin 2	314	35
Myristic acid 1	530	46
Myristic acid 2	314	43

Table 5.6 Mutation present in ORFs of all sequenced isolates but not in published genome.

Coordinates	Codons	Gene locus ID	Protein ID	Gene product	Substitution	SNP Type	Prediction
2329, A/C	GAC – GcC	NGK_RS00010	WP_009174415.1	Pseudogene, DNA polymerase III subunit beta	D179L	Nonsynonymous	Effect, 46, 71%
37377, C/G	GCC – GgC	NGK_RS00195	WP_003692485.1	glutamate-1-semialdehyde 2,1-aminomutase	A290G	Nonsynonymous	Neutral, -92, 97%
1287872, A/G	ACG – gCG	NGK_RS07005	WP_012503780.1	glycine dehydrogenase (decarboxylating)	T336A	Nonsynonymous	Neutral, -45, 72%
1395910, T/C	AAC – AgC	NGK_RS07575	WP_012503823.1	ubiquinone biosynthesis regulatory protein kinase UbiB	N382S	Nonsynonymous	Neutral, -74, 87%
1670097, C/-	GGT – G-T	NGK_RS09065	N/A	pseudogene, hypothetical protein	Frameshift	N/A	N/A
1718262, C/-	GGT – G-T	NGK_RS09355	N/A	pseudo gene, autotransporter	Frameshift	N/A	N/A
1926831, T/G	ATC – AgC	NGK_RS10485	WP_012504019.1	DNA mismatch repair protein MutS	I681S	Nonsynonymous	Neutral, -63, 82%
2031372, T/C	TTT – TcT	NGK_RS11075	WP_012504051.1	30S ribosomal protein S10	F21S	Nonsynonymous	Neutral, -26, 61%
2086880, T/A	GAT – GAa	NGK_RS11400	WP_012504070.1	lipid-A-disaccharide synthase	D114E	Nonsynonymous	Neutral, -96, 97%
2086888, T/A	ATA – AaA	NGK_RS11400	WP_012504070.1	lipid-A-disaccharide synthase	I117K	Nonsynonymous	Neutral, -93, 97%
2155020, CAGGTC/GATAAG	GAC – ctt, CTG – aTc	NGK_RS11740	WP_010356219.1	diaminopimelate decarboxylase	D355L, L356I	Nonsynonymous	Neutral, -1, 53%, Neutral, -93, 97%

Table 5.7 Mutations in ORFs unique to non-selectively passaged cells.

Coordinates	Codons	Gene locus ID	Protein ID	Gene product	Substitution	SNP Type	Prediction	NS1	NS2	Reads
14279, G/A	CCG – CtG	NGK_RS00080	WP_003687220.1	preprotein translocase subunit SecG	A51V	Nonsynonymous	Effect, 63, 80%		X	15/18
39992, C/A	CGG – CtG	NGK_RS00215	WP_003696642.1	ribosomal protein L11 methyltransferase	G191V	Nonsynonymous	Effect, 69, 80%		X	13/13
57720, A/G	AAG – gAG	NGK_RS00290	WP_041421210.1	hypothetical protein	K86G	Nonsynonymous	Effect, 64, 80%	X		15/29
124256, A/T	ATG – tTG	NGK_RS00605	WP_025456235.1	two-component sensor histidine kinase	M1STOP	Nonsynonymous	NT		X	2/3
668244, G/T	GAC – tAC	NGK_RS03680	WP_003691051.1	membrane protein	D84Y	Nonsynonymous	Effect, 50, 75%	X		18/18
978753, G/A	CAA – tAA	NGK_RS05395	WP_003691244.1	type I restriction endonuclease subunit S"	Q351STOP	Nonsynonymous	NT		X	2/2
1273919, G/A	GGC – GaC	NGK_RS06935	WP_002232954.1	adenine phosphoribosyltransferase	G135D	Nonsynonymous	Effect, 79, 85%		X	15/16
1795528, C/A	CAA – aAA	NGK_RS09755	N/A	fimbrial protein	Q90T	Nonsynonymous	Neutral, -33, 66%		X	4/7
1795532, A/C	AAG – AcG	NGK_RS09755	N/A	fimbrial protein	K91T	Nonsynonymous	Effect, 37,66%		X	4/7
2118245, C/T	CAA – tAA	NGK_RS11565	WP_003697648.1	bifunctional glutamine synthetase adenyltransferase/deadenyltransferase	Q364STOP	Nonsynonymous	NT	X		34/34

Table 5.8 Mutations in ORFs unique to myristic acid passaged cells.

Coordinates	Codons	Gene locus ID	Protein ID	Gene product	Substitution	SNP Type	Prediction	14:0 -1	14:0 -2	Reads
61543, C/T	CAA – tAA	NGK_RS00315	WP_003687296.1	MarR family transcriptional regulator	Q24STOP	Nonsynonymous	Truncation	X		40/40
669035, TTTTTCGGCGGA / -----	TTT ----, TTC ----, GGC ----, GGA ----	NGK_RS03680	WP_003691051.1	membrane protein	Deletion	Deletion	NT	X		13/15
1428182, G/A	CCG – tCG	NGK_RS07715	WP_012503835.1	lysine--tRNA ligase	P151S	Nonsynonymous	Effect, 8, 53%	X		24/24
1795512, C/A	TTC – TTA	NGK_RS09755	N/A	fimbrial protein	F84L	Nonsynonymous	Effect, 62, 80%	X	*	9/18
1795541, T/C	TTA – TcA	NGK_RS09755	N/A	fimbrial protein	L94S	Nonsynonymous	Effect, 49, 71%	X	*	9/18
2055439, C/T	GTG – aTG	NGK_RS11260	WP_003690041.1	valine--tRNA ligase	V187M	Nonsynonymous	Neutral, 0, 53%		X	26/26

* - mutation present in 6/38 reads (therefore not classified as consensus).

Table 5.9 Mutations in ORFs unique to monocaprin passaged cells.

Coordinates	Codons	Gene locus ID	Protein ID	Gene product	Substitution	SNP Type	Prediction	C10MG-1	C10MG-2	Reads
668977, GGTTTTATCG/-----	TGG – T--, TTT – ---, TTA – ---, TCG – --G	NGK_RS03680	WP_003691051.1	membrane protein	Deletion	Deletion	NT		X	26/31
1402968, C/T	AGC – AGt	NGK_RS07610	WP_003705779.1	iron complex outer membrane receptor protein	S21S	Synonymous	Neutral, -99, 97%		X	40/40
1565103, G/A	GCC – GtC	NGK_RS08405	WP_003693510.1	phosphate permease	A509V	Nonsynonymous	Effect, 71, 85%	X		16/31
1635913, G/A	CCG – CCa	NGK_RS08890	WP_003689618.1	magnesium transporter	P48P	Synonymous	Neutral, -99, 97%	X		31/59
1953691, C/T	ACC – AtC	NGK_RS10625	WP_003692108.1	molecular chaperone DnaK [#]	T75I	Nonsynonymous	Effect, 18, 59%	X	X	53/53, 62/62

- pBLAST analysis reveals that protein is actually the RNA polymerase-binding protein DksA.

Table 5.10 Mutation in ORFs found in multiple but unrelated sample types.

Coordinates	Codons	Gene locus ID	Protein ID	Gene product	Substitution	SNP Type	Prediction	lp	NS 1	NS 2	MG 1	MG 2	14:0- 1	14:0- -2
57656, T/G	TTT – TTg	NGK_RS00290	WP_041421210.1	hypothetical protein	F64L	Nonsynonymous	Neutral, -10, 53%		X		X			
958893, G/A	GGC – AGC	NGK_RS05305	WP_003691226.1	phosphogluconate dehydratase	G124S	Nonsynonymous	Effect, 66, 80%	X			X		X	
1003614, C/-	CGC – C-C	NGK_RS05495	WP_003691051.1	glutamate--cysteine ligase	Frameshift	Frameshift	NT	*	X					X
1849149, G/A	CCC – CtC	NGK_RS10035	WP_003692001.1	amino acid ABC transporter permease	P126L	Nonsynonymous	Neutral, -19, 57%			X	X			
2050038, C/A	AAG – AAt	NGK_RS11245	WP_012504064.1	membrane protein	K120N	Nonsynonymous	Neutral, -41, 72%		X	X	X		X	X
2050040, T/C	AAG – gAG	NGK_RS11245	WP_012504064.1	membrane protein	K120E	Nonsynonymous	Neutral, -47, 72%					X		X

*present in 14/35 reads (therefore not classified as consensus).

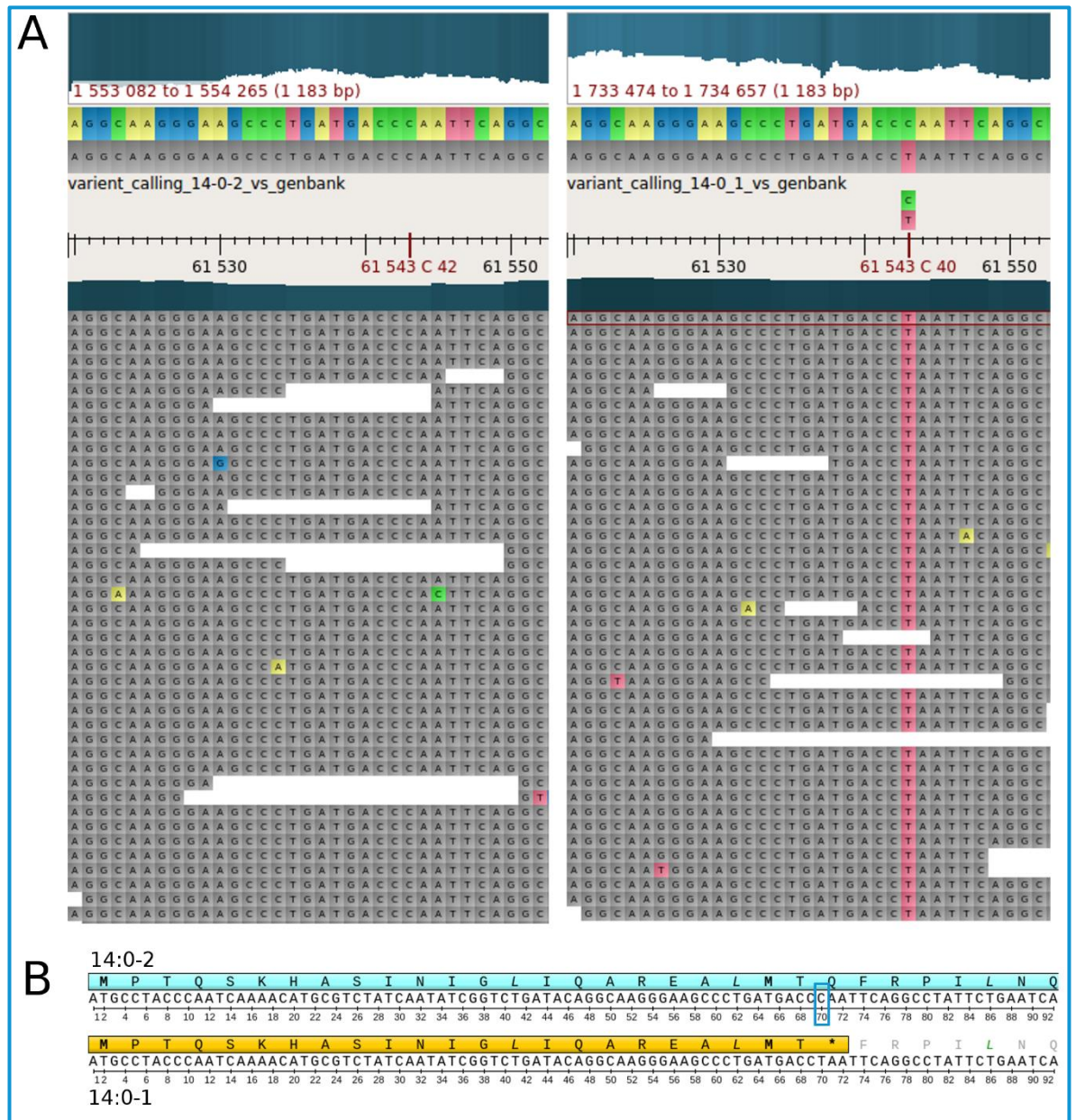


Figure 5.5 Mutation in 14:0-1 result in truncation of FarR protein. (A) Alignment of sequence reads from 14:0-2 (left) and 14:0-1 (right) samples aligned against genbank reference. The SNP mutation at position 61,543 in 14:0-1 is present in all sequence reads in this sample. (B) The SNP is present at the 70th nucleotide into the ORF and results in the formation of a stop codon at the 24th codon. The full protein length in the wild type is 146 amino acids.

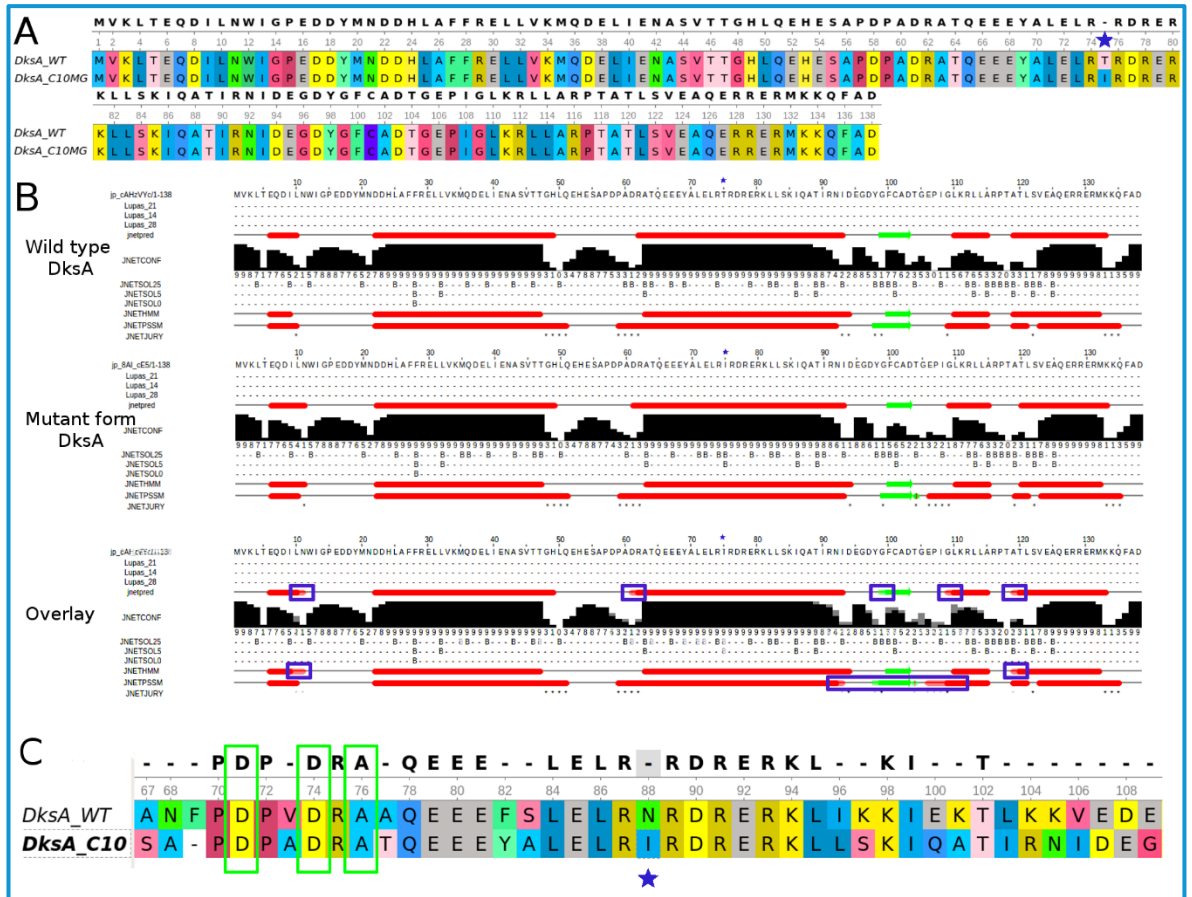


Figure 5.6 Nonsynonymous SNP occurring in *dksA* in both monocaprin passaged samples. (A) Alignment of the original NCCP11945 *DksA* amino acid sequence against the resultant mutation (T75I) in both monocaprin passaged samples. (B) JPred 4 output of predicted secondary protein structure of the wild type and the mutant forms of *DksA*. The top two graphics are the outputs for the wild type (top) and mutant (bottom). As these graphics appear so similar, they were overlaid (third graphic) to show the differences (highlighted with purple boxes). (C) An alignment of a portion of *DksA* from *E. coli* (*DksA_WT*) and monocaprin induced mutation (*DksA_C10*). The green boxes indicate residues important in RNA polymerase binding and mutation labelled with purple star.

5.3.8 Analysis of promoters

The promoters of *farR*, *farAB*, *mtrR* and *mtrCDE* were examined for any signs of mutation by analysing variant calling data. As stated previously, there are mechanisms by which *N. gonorrhoeae* has modified promoter regions to enhance the expression of efflux pumps. For example, a mutation in the sequence upstream of the *mtrC* ORF acts as an alternative promoter region which can enable free transcription of *mtrCDE* without MtrR control [359]. Figure 5.8 shows the sequence of these promoters and binding sites of repressor sites by which the cell could possibly gain an advantage if a mutation was to occur there. In this study, no mutations in these regions were observed.

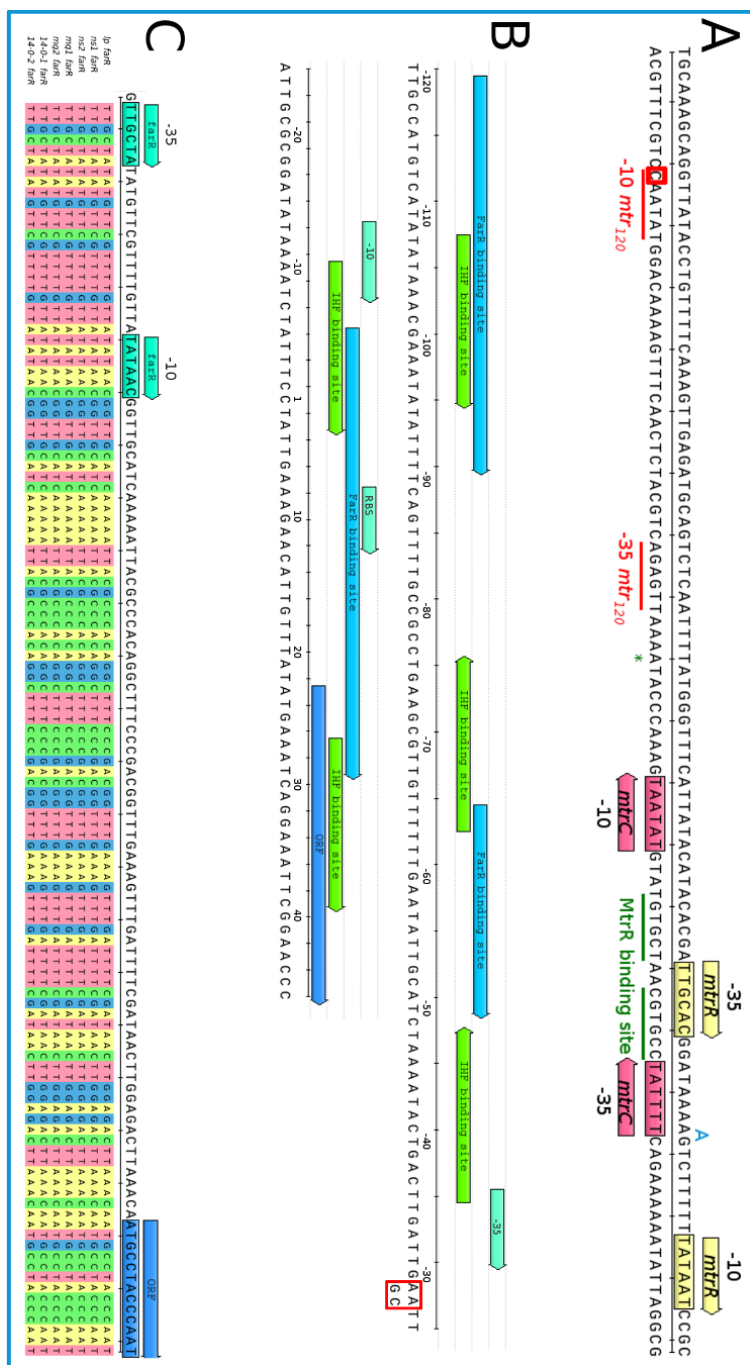


Figure 5.7 . Analysis of some promoter regions of efflux pump proteins and regulators. (A) Analysis of *mtrR* and *mtrC* intergenic spacer based on promoter characterisations done by Ohneck and co-workers [359]. The standard -10 and -35 promoter regions labelled as well as the alternative *mtr*₁₂₀ promoters (in red). This promoter is used to transcribe *mtrCDE* if a SNP mutation occurs upstream of the standard *mtrC* promoter. This new promoter is not repressed by MtrR binding. The position of the required mutation for this to happen is labelled by the red box and requires a C→T mutation. The blue A in this panel marks the additional adenine which strains such as MS11 have but NCCP11945 doesn't have. (B) Analysis of *farAB* promoter region based on characterisation by Lee and co-workers [150]. This panel describes all of the FarR and IHF binding sites. All samples had identical sequences for this region. NCCP11945 does have two SNP mutations as opposed to the characterised strain which is labelled with the red box. The two nucleotides at this positions are adenines in NCCP11945 (top sequence). (C) Analysis of *farR* promoter region. This is the least characterised promoter. The aligned of all the sequences is shown here and shows no differences to reference or other strains.

5.4 Conclusions

N. gonorrhoeae NCCP11945 did adapt to growth in medium containing sub-lethal concentrations of fatty acids. Passage on any of the two inhibitors resulted in a doubling of the MIC value of monocaprin. However, this pales into incognisance when compared to the eight and sixteen fold increase in myristic acid MIC value for the monocaprin and myristic acid passaged cells respectively. It appears that the bacteria were able to increase their ability to withstand the presence of myristic acid just by being in an environment surrounded by a similar hydrophobic agent. The bacteria's resistance to monocaprin did not increase in the same way it did for myristic acid. Even the *farR* mutant had the same MIC level for monocaprin as all the other samples. This does suggest that the *farAB-mtrE* encoded efflux pump does not confer a major increase in resistance to monocaprin.

These results are encouraging in terms of our formulation. No mutations were found that conferred a significant increased resistance to monocaprin, the main drug candidate in our formulation. Also, as ophthalmia neonatorum is a spill-over infection, development of resistance would be difficult. The bacteria causing the infection would have little chance of infecting another host as its usual mode of transmission will not be available. *N. gonorrhoeae* has a limited survival ability outside a host, so it is unlikely to be able to persist in the environment until it reaches a new host. This is a major benefit in the fight against the development of resistance mechanisms. The only downside would be that monocaprin could not be used in any other types of formulations and in particular in the treatment or prevention of sexually transmitted infections.

There is possibly further work that could be done based on the results of this study. The development of *farR* mutant is interesting. What effect would this mutation have on the bacteria's ability to function without the presence of the inhibitor? Is the mutation reversible? Also, what effect does this have on the MIC of other fatty acids? The *dksA* mutation is also of interest. How does this mutation confer an increased rate of survival in the monocaprin passaged cells? These are complex questions and may be difficult to answer. As stated previously, it would be interesting to see when in the experimental evolution study this mutation occurred and whether it is reproducible if the experiment was repeated with more replicates. In this study, changes in MIC values were used to measure the increase in resistance. However, has the passaging effected the MBC? Log reductions could be done to answer this question. We already have a lot of MBC data from Chapter 2 so the new data could be compared directly to this.

The mechanism for the decreased susceptibility of monocaprin (two-fold decrease) in this study is unknown. It appears that passage on a similar fatty acid type inhibitor promotes this decreased susceptibility. No mutation was found in this study to cause this so the mechanism could be a result

of phenotypic changes and not genotypic. If the isolates at the end of the experiment were later given a passage on non-selective medium, would they retain this decreased susceptibility?

Chapter 6 - Sources of hydrophobic antimicrobials from the processing of natural products

Outputs

Poster: Colin P. Churchward, Pollianne Arruda, Andrew K. Sears, and Lori A. S. Snyder. 2015. Treatments of natural oils to produce bactericidal products against *Neisseria gonorrhoeae*. Society of General Microbiology annual conference. Birmingham ICC. April 2015.

Poster: Colin P. Churchward, Andrew K. Sears, Polliane Arruda, and Lori A. S. Snyder. 2014. Novel antimicrobial agents against *Neisseria gonorrhoeae* from extracts of natural products. 19th International Pathogenic *Neisseria* Conference, Asheville, North Carolina, USA. Oct 2014

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The lipase treatment and disc diffusion assays of the natural oils were conducted Andrew Sears. The disc diffusion assays of the essential oils were done by Polliane Arruda. Polliane also assisted with preliminary optimisation of the *Yarrow lipolytica* treatment.

6.1 Introduction

6.1.1 Background

So far in this thesis, we have concentrated on developing and evaluating formulations for the prophylactic treatment of ophthalmia neonatorum based on a purified fatty acid based active ingredient. There are natural sources of fatty acids which could be used to produce some of the antimicrobial fatty acids discovered in Chapter 2 and could also be used as antimicrobial agents. Certain natural products may require a processing step to release the fatty acids but it may offer a cheap and relatively simple way to produce antimicrobial fatty acids.

6.1.2 Lipases

Triacylglycerol acylhydrolases (EC 3.1.1.3) are commonly referred to as lipases. They are a protein family which act on carboxylic ester bonds. Normally these proteins catalyse the hydrolysis of triglycerides into diglycerides, monoglycerides, free fatty acids, and glycerol. However, they are actually more diverse and can act on carboxylic ester bonds by esterification, interesterification, acidolysis, alcoholysis or aminolysis [385, 386]. Lipases are common in microbial species and many have been isolated and characterised [387]. The lipases from these species may have different origins and may differ considerably in terms of activity, positional specificity, fatty acid specificity, thermostability, and optimum pH and temperature [388]. Microbial lipases are most commonly added to household detergents for use in washing machines and dishwashers but are also used in the food industry to produce specific triglycerides and in the paper industry to remove hydrophobic components from wood before pulping [389]. Lipases are distinct from esterases as they are only active when adsorbed to an oil–water interface [390]. Therefore, the kinetics of lipase reactions don't conform exactly to the standard Michaelis-Menten model [391]. For many applications, the lipases have been immobilised to enhance enzyme stability and activity [392]. This also has the added benefit that they are reusable.

It is clear to see that lipases could be used to create antimicrobial fatty acids from natural sources of fats and oils. Certain sources of fats and oils would release different types of fatty acids. Therefore, the fatty acid content of the natural source must be examined to be sure that lipase treatment would release a mixture of free fatty acids which would be antimicrobial. The treatment could consist of incubation with a purified lipase, by a purified lipase which has been immobilised or by incubation in a medium containing a microorganism which secretes a lipase [393, 394].

6.1.3 *Yarrowia lipolytica*

Yarrowia lipolytica is a yeast species which as its name suggests, produces a lipase. It has previously been classed in other genera such as *Candida* but now has its own genus, of which four other

species have recently been added [395]. *Y. lipolytica* can be isolated from a variety of sources and can be found in dairy products such as soft cheeses, yoghurts and sausages. *Y. lipolytica* strains are unable to grow above 32 °C and are strictly aerobic [396]. When exposed to lipids, *Y. lipolytica* secretes extracellular lipases which are encoded by *LIP* genes [397, 398]. *Y. lipolytica* also possess membrane-bound and intracellular lipases [399]. *Y. lipolytica* is considered to be non-toxicogenic, non-pathogenic and has been classified as “Generally Regarded As Safe” (GRAS) by the American Food and Drug Administration (FDA) for citric acid production [400]. This yeast species has been used previously to breakdown coconut oil into free fatty acids by growth on solid medium containing the oil to produce antimicrobial products [401].

6.1.4 Essential oils

Essential oils are hydrophobic volatile extracts from natural plant sources. They are usually extracted by distillation as the volatile compounds are released from its source material with steam but then separates from water when the distillate is collected. They can also be extracted via cold press or solvent extraction as well as other less common methods. The plant sources which can be used include leaves, petals, fruits, seeds, root and bark. About 3000 types of essential oils are known and from these 300 are regarded as commercially important to the pharmaceutical, agronomic, food, sanitary, cosmetic and perfume industries [402]. They are called essential oils as they are said to take the essence of the plants fragrance, for this reason they are commonly used to enhance the aroma of a certain product. The most popular essential oils include: lavender, peppermint, spearmint, black pepper, rosemary, tea tree, eucalyptus, rosewood, orange, lemon, lime, pine, ginger, and oregano.

The essential oils are made up of a complex mixture of low molecular weight hydrocarbons and their oxygenated derivatives [403]. Examples of the components of essential oils are given in Figure 6.1. [404]. The mixture is usually comprised of anything from 20 to 100 compounds [405]. The oils are poorly water soluble but may be soluble in fats, alcohol, and other non-polar solvents.

The antimicrobial properties of essential oils have long been known [402]. A range of Gram positive and negative bacteria have been tested with a range of different essential oils. In one such study, 52 different oils and extracts were tested on nine bacterial and one fungal species [406]. In this study, lemongrass, oregano, and bay extracts were found to inhibit all organisms at a concentration of 2 % (v/v) or below. Although many of the other essential oils had antimicrobial properties, only these three extracts had any activity against *P. aeruginosa*. Some of the extracts were particularly effective at inhibiting certain groups of organisms. For example, vetiver extract had the lowest MIC value against *S. aureus* and was very effective against other Gram positive bacteria and *Candida* but

had no inhibitory effects against Gram negative bacteria. However, vetiver extract potency against *S. aureus* has been questioned by other studies [407-409].

6.1.5 Essential oils mechanisms of action

The proposed mechanisms of action of essential oils are similar to the mechanism of action already given in Chapter 1 for fatty acids and monoglycerides. Membrane disruption or membrane interaction is the main accepted mechanism of action. This mechanism of action has been demonstrated for a few of the essential oils on various bacteria [410-412]. However, as the variety of the components of the essential oils differ between each plant extract, and the fact that there is variation in potency and susceptibility, it is possible there could also be additional mechanisms of action [404]. For example, cinnamaldehyde (seen in Figure 6.1) which makes up 98 % of the essential oils extracted from the bark of cinnamon trees [413], has been shown to interfere with the action of the cell division protein FtsZ in *Bacillus cereus* [414].

6.1.6 Aim of this chapter

The aim of this chapter is to identify natural sources of anti-gonococcal agents. Two approaches will be used to find these. The first will look at the treatment of natural oils to produce antimicrobial free fatty acids. The method of releasing the fatty acids will also be investigated by comparing treatment with a purified lipase with the incubation of the natural product in culture containing the lipase secreting yeast *Y. lipolytica*. The natural sources of the fatty acids will be hemp oil and flaxseed oil as both contain high levels of linolenic acid (18:3) [415, 416] and coconut oil for which lauric acid makes up about 50% of the total fatty acid content as well as containing caprylic (C8:0) and capric (C10:0) acids [417]. The second approach is to use manufactured essential oils. Black pepper, citronella, lemongrass, marjoram and spearmint essential oils will be tested for anti-gonococcal properties.

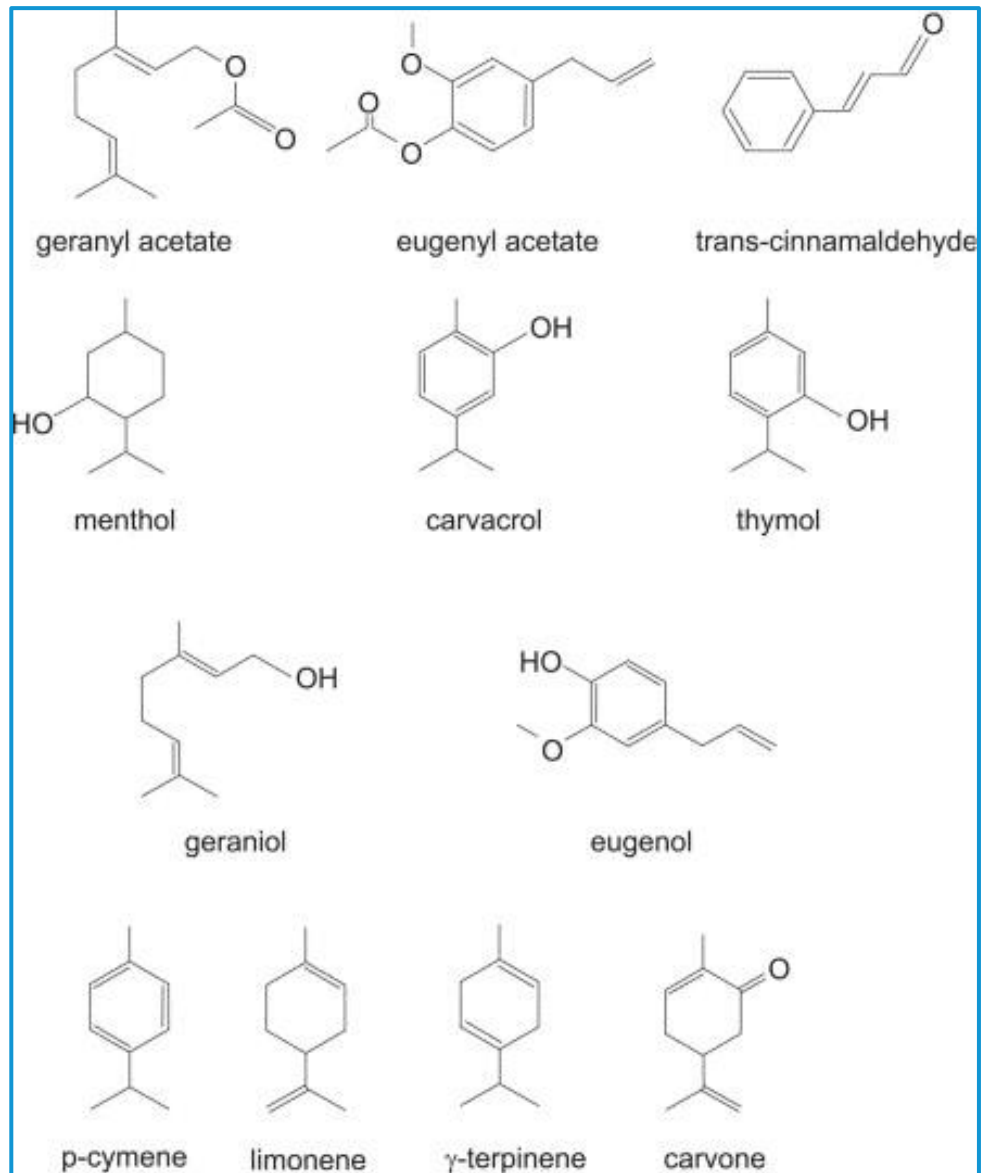


Figure 6.1 Figure 6.1. Structural formulae of selected components of some essential oils taken from Burt (2004) [404].

6.2 Materials and methods

6.2.1 Yeast culture and maintenance

A freeze-dried culture of the yeast *Yarrowia lipolytica* type strain CBS 6124 (deposited as NCYC2904) was obtained from the National Collection of Yeast Cultures (NCYC) (Institute of Food Research, Norwich, UK). Growth medium used for the yeast was either Sabouraud dextrose agar (SDA) or Sabouraud dextrose broth (SDB). SDA was prepared by dissolving 65 g of Oxoid's Sabouraud dextrose agar dehydrated culture medium (10.0 g/L mycological peptone, 40.0 g/L glucose, 15.0 g/L agar pH 5.6 ± 0.2 at 25°C) in 1 L deionised water and autoclaving at 121 °C for 15 minutes. SDB was prepared by adding 30 g Oxoid's Sabouraud dextrose liquid dehydrated culture medium (20.0 g/L glucose, 5 g/L pancreatic digest of casein and 5.0 g/L peptic digest of animal tissue, pH 5.6 ± 0.2 at 25°C) in 1 L deionised water and autoclaving at 121 °C for 15 minutes. No extra antibacterial or antifungal supplements were added as these might have affected later results.

To revive the culture, 300 µl of sterile deionised water was added to the freeze-dried ampoule and left for 10 minutes at room temperature. This was added to 700 µl SDB and split between four SDA plates, spread using an L-shaped spreader and incubated at 28 °C for 72 hours. Stock cultures for long term storage were made by scraping colonies with a 10 µl loop and putting the released cells into SDB plus 15 % glycerol, these were stored at -80 °C. For shorter term storage (< 3 months), slopes were prepared by inoculating a 7 ml SDA slope in a plastic universal tube with a loop of yeast, incubating at 28 °C for 48 hours and then storing at 4 °C.

6.2.2 Sources of natural products

Volatile oils of black pepper, citronella, lemongrass, marjoram and spearmint were all manufactured by Vitamin World Inc. (Ronkonkoma, NY, USA). Non-volatile oils of flax seed, hemp and coconut oil were all produced by Biona (Kingston-upon-Thames, Surrey, UK). Flax seed and hemp oil had been stored at 4 °C prior to purchase to help prevent the breakdown linolenic acid.

6.2.3 Purified lipase reaction

Natural oils (flax seed, hemp and coconut oil) were all used in a lipase reaction to break fats down into free fatty acids. Reactions were set up in 20 ml volumes containing 0.2 g purified *Candida rugosa* lipase (catalogue number L1754, Sigma-Aldrich), 1.4 ml sterile deionised water, 8 ml of oil (flax seed, hemp or coconut oil) and 10.4 ml n-hexane. Reactions were shaken at 120 rpm at 40 °C for 24 hours. These conditions had previously been shown to be optimum for this reaction [418]. The samples were moved to 50 ml centrifuge tubes and centrifuged at 1,500 x G for 5 minutes. The organic (oil) and aqueous phase were then removed separately and the organic phase used for antimicrobial testing.

6.2.4 *Yarrow lipolytica* hydrolysis of coconut oil

A set of ten sterile 150 ml Erlenmeyer flasks were set up containing 50 ml SDB. To five of these flasks, 1.5 g coconut oil was added, making an overall concentration of 30 g/L. Approximately 10^4 *Y. lipolytica* was added to all flasks and shaken at 125 rpm at 28 °C. Two flasks, one containing coconut oil and the other without, were immediately taken and stored at 4 °C for 1 hour to solidify low melting point oils present. The solid oils were collected by filtration first through a Büchner funnel and then through a 3M no. 1 filter (3M United Kingdom PLC, Bracknell, Berkshire, UK). The filtrate and pooled solid fractions were retained in 50 ml centrifuge tubes and stored at -80 °C until testing. Pairs of flasks were removed at day 2, 4 and 7 and treated as above. The two spare flasks were prepared as backups if any other flasks were to show signs of contamination. CFU counts were done daily from day 0 to day 4 and then at day 7. Counts were done by diluting the broths in a ten-fold dilution series in SDB and then plating duplicate 100 µl samples over a whole SDA plate for dilution 10^{-3} to 10^{-6} . Plates were incubated for 72 hours at 28 °C before doing plate counts.

6.2.5 Disc diffusion test

The disc diffusion test was done according to the British Society for Antimicrobial Chemotherapy (BSAC) 2014 guidelines for antimicrobial susceptibility testing [419]. Briefly, 10 µl of the products were spotted onto 6 mm antimicrobial testing discs (Oxoid) and dried overnight in sterile glass petri-dishes. For samples that had solidified, these were gently warmed in a waterbath at 45 °C until melted. Any samples that need diluting (especially the volatile oils), were done so in ethanol. A further negative control was done in experiments involving diluted samples which consisted of using a disc which contained 10 µl ethanol (after drying as samples described above).

N. gonorrhoeae strain NCCP11945 was cultured as in Chapter 2 overnight on a GC plate at 37 °C, 5 % CO₂. A cell suspension in PBS equal in the opacity to a 0.5 McFarland standard was made. A sterile cotton-wool swab was dipped into the bacterial suspension and excess liquid removed by pressing it against the side of the glass universal tube. Three continuous streaks were made on a GC plate, each one starting at the top of the plate continuing down the plate to the bottom and then plates were turned plate 90 ° before starting the next streak. After allowing a few minutes for the plates to dry, the discs were aseptically applied using forceps. A maximum of four samples were tested per plate to ensure that zones did not overlap. All samples were tested in duplicate on three different days. Plates were incubated at 37 °C, 5 % CO₂ for 16-18 hours. The diameter of the inhibition zones were measured if present.

6.2.6 Log reduction assay

The log reduction method was the same as used in Chapter 2. Any sample that was diluted before testing was done so in ethanol. As with previous work, the volume of sample used was 5 µl of

sample to 495 μ l bacterial suspension. Negative controls were tested by adding the 495 μ l bacterial suspension to 5 μ l ethanol.

6.3 Results

6.3.1 Disc diffusion test results of volatile oils

Volatile oils by their nature of their manufacture are highly concentrated solutions of hydrophobic chemicals. It therefore comes as little surprise that these required dilution for testing. The compounds were so potent in undiluted form that some completely inhibited bacterial growth on the whole of the agar plate (data not shown). The samples were therefore diluted 1/10 in ethanol for testing purposes. At this dilution all of the volatile oils still inhibited gonococcal growth to some extent. Figure 6.2 shows a graphical representation of the sizes of the inhibition zones produced. Lemongrass produced the largest zones of inhibition at 39.7 mm which were much larger than any of the other oils. Citronella also produced a large zone (24.8 mm), while spearmint, marjoram, and black pepper all had smaller zones.

6.3.2 Disc diffusion test results of undiluted non-volatile oils and lipase treated non-volatile oils

None of the untreated natural oils inhibited growth. These products only become bacteriostatic when treated with the lipase. After treatment, the zones of inhibition ranged from approximately 23.0 to 35.7 mm. Figure 6.2 shows a graphical representation of the sizes of the inhibition zones produced.

6.3.3 Bactericidal activity of volatile oils

The bactericidal activity of the volatile oils was measured by log reduction. Figure 6.3 shows the highest dilution factor which caused a greater than 4 \log_{10} reduction. It was found that the black pepper wasn't bactericidal even when used at the lowest dilution factor tested which was at 1/100. In contrast, lemongrass and citronella (both extracts from the plant genus *Cymbogon*) were effective down to a dilution factor of 1/2000.

6.3.4 Bactericidal activity of lipase treated natural oils

As the untreated natural oils were not bacteriostatic, they were not tested for bactericidal activity. Only the purified lipase treated oils were tested in the log reduction experiments. The flaxseed oil and hemp oil were not bactericidal as they didn't cause a 4 \log_{10} reduction in viable bacteria. However, coconut oil had strong bactericidal activity. Figure 6.4 shows the results for these tests.

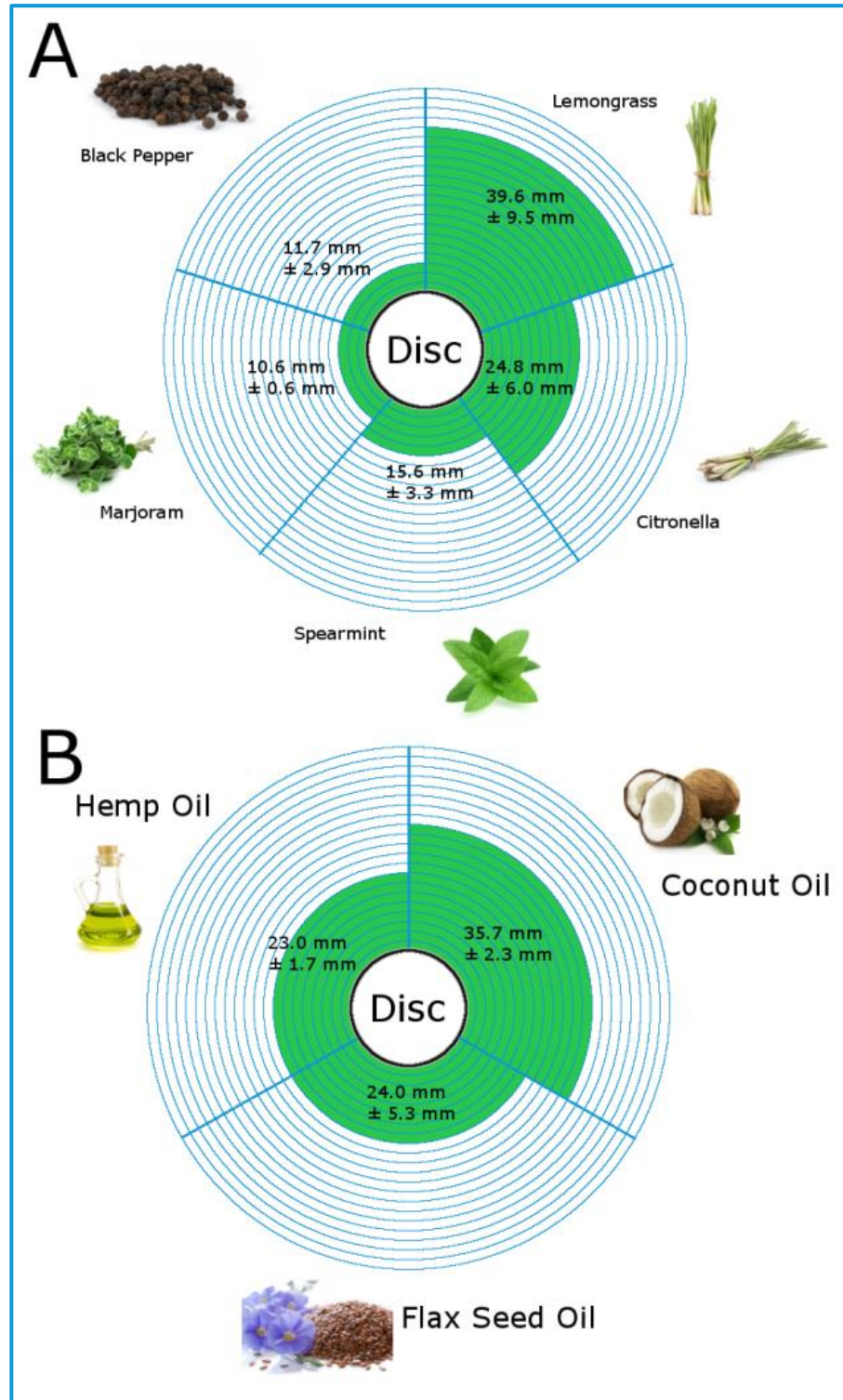


Figure 6.2 Results of disc diffusion tests. (A) Sizes of zones of volatile oils diluted 1/10. (B) Sizes of inhibition zones of undiluted lipase treated natural oils. Each ring represents a 2 mm zone of inhibition; the diameter of the disc is 6 mm. Numerical zone sizes are displayed as mean results with standard deviation over three repeated experiments. Negative control experiments produce no zones of inhibition.

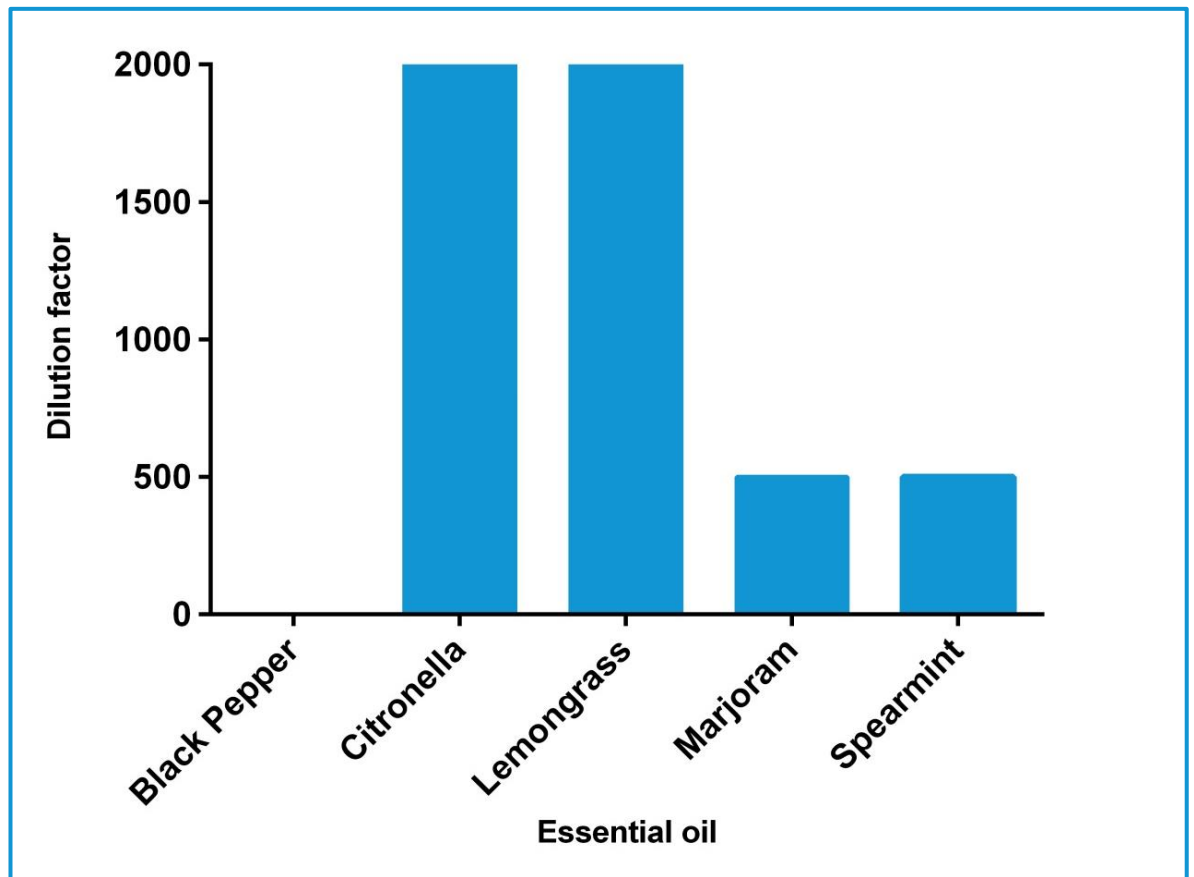


Figure 6.3 Overall maximum dilution factor of volatile oils that resulted in bactericidal activity. Minimum dilution tested was 1/100. Bactericidal activity was defined as causing a greater than 4 \log_{10} reduction in the number bacterial cells. Log reductions of dilutions were tested twice on separate days and gave identical results.

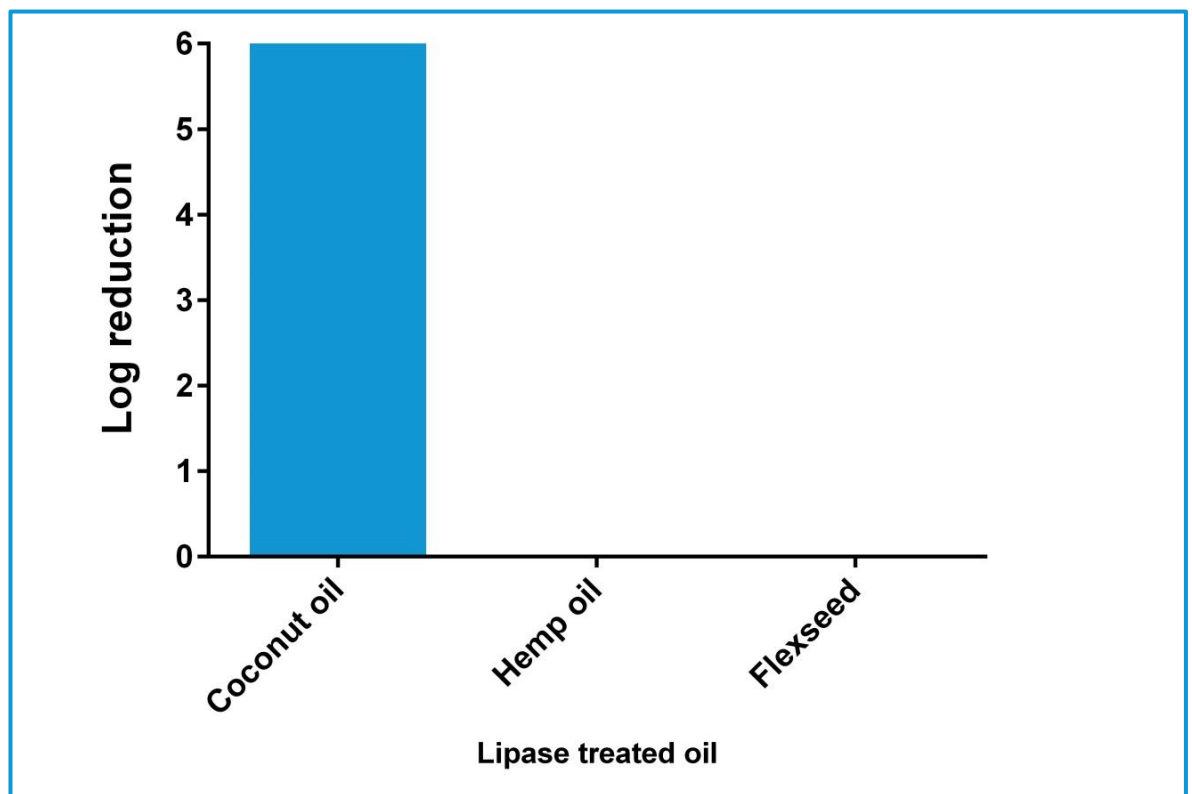


Figure 6.4 Log reduction results of the lipase treated non-volatile oils. Only coconut oil was deemed to be bactericidal, although all oils were bacteriostatic.

6.3.5 *Yarrow lipolytica* hydrolysis reactions of coconut oil

As only coconut oil from the natural oils was bactericidal, this was the only oil to be used in the *Y. lipolytica* hydrolysis experiments. The melting temperature of the coconut oil was close to the optimum temperature for yeast growth and also provided a good temperature for the activity of lipase. At the reaction temperature of 28 °C without shaking the coconut oil would solidify. However, with the addition of shaking, not only would yeast growth rate be improved but the coconut oil would also be in a liquid state and thus improving the mixing of the extracellular lipase enzyme and the coconut oil. This also had the added bonus of ease of extraction as the coconut oil could be solidified after the lipase treatment by a short incubation at 4 °C and solid oil removed by simple filtration. Viable cell densities of the flasks containing yeast with the coconut oil was compared to flasks without the added coconut. These results are displayed in Figure 6.5. It is clear that the yeast's growth rate was negatively affected by the presence of the coconut oil.

6.3.6 Anti-gonococcal properties of the yeast treated coconut oil

The anti-gonococcal properties of the *Y. lipolytica* hydrolysis experiment flasks were tested at 0, 2, 4 and 7 days post inoculation. Again, disk diffusion tests and log reduction experiments were conducted. A summary of the results of these experiments are presented in Figure 6.6. None of the flasks without the coconut oil (SDB medium and yeast only) resulted in inhibition zones on the disc diffusion test. Incubated cultures containing the coconut oil which had been incubated for two days or more were bacteriostatic. However, the zones of inhibition for the days 2, 4 and 7 did not increase for the longer incubated flasks as might be expected. All these flasks had approximately the same sized zones of inhibition (19.7-20.0 mm). For the bactericidal testing, only day 7 was bactericidal. Day 4 had some killing effect with a log reduction value of 1.4 but only day 7 had a greater than 4 log₁₀ reduction in viable cell count.

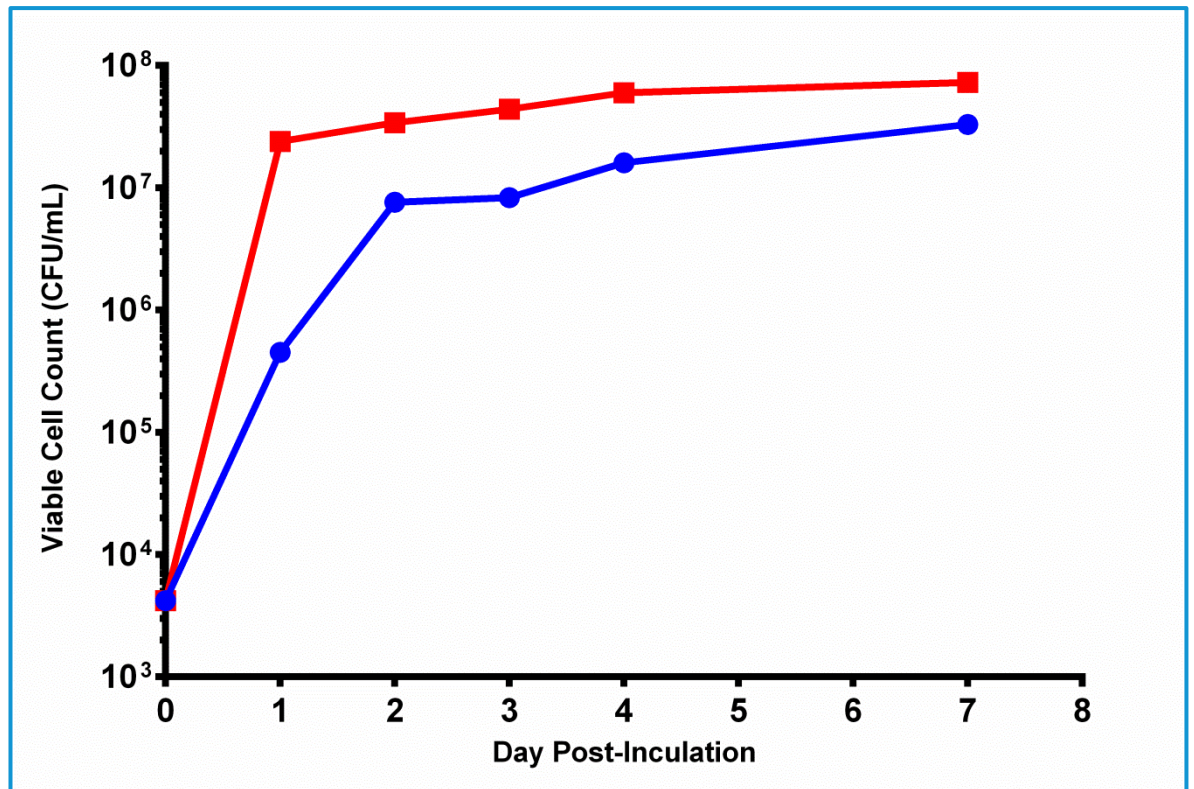


Figure 6.5 *Yarrowia lipolytica* CFU counts. The red line represents the growth of *Y. lipolytica* in medium flasks without coconut oil and the blue line growth in flasks containing coconut oil.

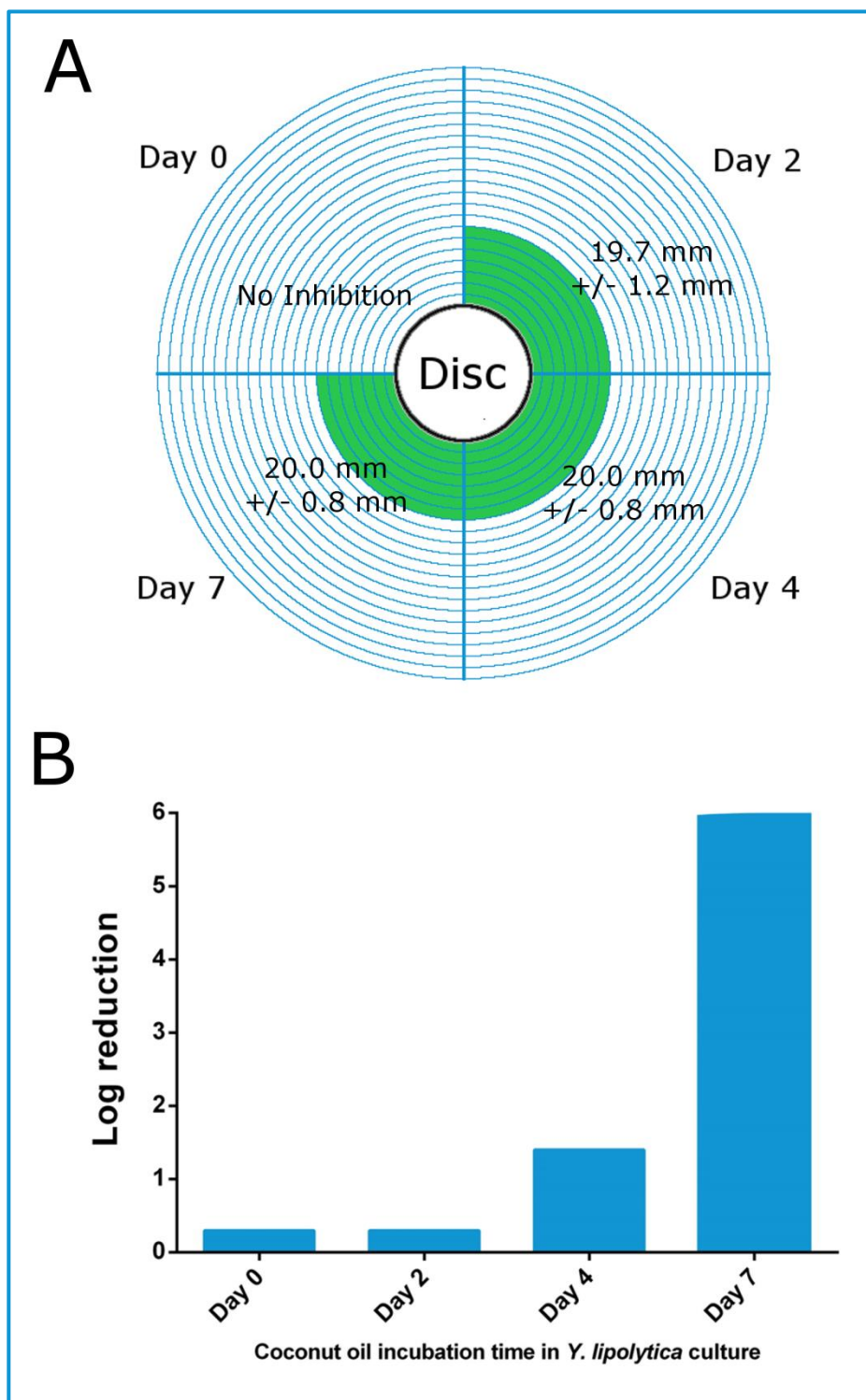


Figure 6.6 Antimicrobial properties of oils incubated with *Yarrowia lipolytica* cultures. (A) Sizes of inhibition zones from oils extracted from broth cultures at different timepoints. Each ring represents a 2 mm zone of inhibition; the diameter of the disc is 6 mm. Numerical zone sizes are displayed as mean results with standard deviation over two repeated experiments. (B) Results of log reduction experiments from oils extracted at different timepoints. Negative control experiments with yeast grown with no coconut oil produced no inhibition zones.

6.4 Discussion

6.4.1 Antimicrobial action of essential oils

All of the essential oils tested in this study had some anti-gonococcal properties. All were growth inhibiting to some extent causing zones of inhibition. Lemongrass and citronella had the greatest anti-gonococcal properties in terms of both bactericidal and bacteriostatic activities. These results are not surprising as lemongrass [402, 404, 406, 408, 420] and citronella [406, 421] essential oils have previously been proven to have antimicrobial properties. The exact antimicrobial agent in these two essential oils is unknown but a list of their main constituents is given in Table 6.1. The chemical components of the essential oils of lemongrass and citronella are similar. Both contain geranial (citral E-isomer), neral (citral Z-isomer), geranyl acetate, and geraniol. This is unsurprising as both plants are of the *Cymbopogon* genus. Lemongrass essential oils are made from the leaves of the plant *Cymbopogon citratus*. The source of citronella essential oils is a bit more complex but have two main types: Ceylon type which is made from extracts of the leaves of the species *Cymbopogon nardus* and Java type which is extracts of the leaves of the species *Cymbopogon winterianus* [422]. The type used in this study was Ceylon type. It is likely that one or more of the components present in both of the essential oils is responsible for their antimicrobial properties. However, this would require fractionation of the essential oils to prove. It could be possible that a minor component that is unique to the individual oils is responsible for their antimicrobial properties. For example, in the case of the citronella essential oil, citronellal (which is not present in the lemongrass essential oil), could be responsible for the antimicrobial activity. This compound has previously been shown to have antifungal activity [423].

There is however, an issue with the use of essential oils for therapeutic uses. They are highly hydrophobic so are therefore difficult to formulate. They also have some cytotoxic effects. This cytotoxic effect is thought to be due to membrane damage as seen in bacteria and yeasts. A modified HET-CAM analysis that measured the irritation threshold (as a percentage) at which the essential oil caused haemorrhaging of the CAM found that certain essential oils were greater mucosal irritants than others [424]. That study demonstrated that camomile, lemon and oranges carp oil were the least irritating (no haemorrhaging at 100 % (v/v)) and that rose, clove and cinnamon oil were the greatest irritants (haemorrhaging at less than 30 % (v/v)). A full analysis of the cytotoxicity, as that conducted in Chapter 2, of lemongrass and citronella should be conducted to assess their suitability in any form of therapeutic formulation.

6.4.2 Antimicrobial properties of natural oils

As stated previously, the natural oils by themselves have no antimicrobial properties. They require a lipase treatment to release free fatty acids for them to become antimicrobial. The fatty acids that

were released by hemp and flaxseed oil were inhibitory to the growth of *N. gonorrhoeae* but did not have any killing action. However, treated coconut oil did have cidal activity. According to one study, coconut oil contains 51.0 % lauric acid (12:0), 18.9 % myristic acid (14:0), 8.6 % palmitic acid (16:0), 6.2 % caprylic acid, 6.1 % capric acid (10:0), and 5.8 % oleic acid (18:1) [425]. As shown in Chapter 2, lauric acid has powerful anti-gonococcal properties, this is most likely the cause of the activity of the lipase treated oil. The hemp and flaxseed oil were originally chosen for this study as they contain linolenic acid. However, their lack of activity after lipase treatment may be because any linolenic acid in these oils is not present in significant levels or that this fatty acid has degraded within the oil. Linolenic acid is more prone to oxidation and thus breaks down quicker than other fatty acids, so this could be the reason why these oils were ineffective despite supposedly containing a powerfully anti-gonococcal agent in linolenic acid.

The purified lipase reactions worked well considering the treatment had not significantly been optimised. The lipase reaction was done easily overnight and produced a mixture which had powerful anti-gonococcal properties. If the lipase had been immobilised, the enzyme could have been recovered and reused to save on costs making this method of treatment both quick and inexpensive.

As coconut oil was the only natural oil which was found to have a killing effect on gonococcal cells, this was the only oil to be tested via *Y. lipolytica* treatment. The method used in this study used liquid medium as opposed to solid medium which had been used previously in another study [401]. This had a few advantages, firstly cost, the broth medium is cheaper than that of the solid medium. The second advantage is that in theory it should be more efficient as lipase reaction happens at the interface between aqueous and organic phases. The third advantage is that the oil can be easily extracted by incubation at 4 °C for one hour. This causes the molten oil which has been incubated with the yeast at 28 °C to solidify so it can easily be removed by filtration.

The growth of the yeast was inhibited by the presence of coconut oil. This may be due to the yeast putting its energy resources into producing the lipases instead of cell division. It could be possible that released free fatty acids had some growth inhibiting properties and therefore slowed the growth rate as well. The only surprising result from the yeast treatment experiment was that despite good yeast growth, it took seven days to produce a bactericidal mixture. The results show that there is very little difference on the bacteriostatic properties of the reactions at days 2, 4 and 7 with comparable inhibition zones. However, only day 7 contains a bactericidal mixture. The reason for this is unknown and analytical analysis of the fatty acid content of the reaction at the different time-points is required to find out what fatty acids are being released. It is possible that another metabolite other than a fatty acid is responsible for the inhibition of growth. As the negative control

showed no inhibition it is assumed that the inhibition must be as a result of the breakdown of the coconut oil. Full analytical analysis is required to define the chemical composition of the crudely extracted filtrate.

6.4.3 Best lipase treatment method

The yeast treatment was done as it was thought that it could be more cost effective alternative to the purified lipase reaction. However, use of an immobilised enzyme reaction could offer a better solution once the initial layout for the enzyme has been paid for. The incubation time is much shorter and the reactions don't require a complex microbiological medium which could be expensive in the long term. Also, there would be fewer concerns about the presence of live microorganism in the antimicrobial product. Both methods would have to be fully optimised and costed in terms of yield produced to fully evaluate the best method.

6.4.4 Final comments

This chapter has shown that there are potential natural sources of anti-gonococcal agents. More work needs to be done to determine whether components of lemongrass and citronella essential oil can be used for any therapeutic application. The use of coconut oil to treat gonococcal or other infections is a novel idea. As stated in Chapter 1, the WHO West Pacific reporting region has the greatest number of gonorrhoea cases of any reporting region. This region also contains some of the world's biggest coconut producers (see Figure 6.7) [426]. They would therefore have relatively easy access to the raw material for processing. However, the practicality of processing it, cost, and effectiveness to treat infections need to be examined.

Table 6.1. The main chemical components of lemongrass [427] and citronella [428]

Compounds	Relative contents*	Notes
Lemongrass		
Geranial	52.0 %	Citral E-isomer. Geranial has a strong lemon odour. Common in oils of citrus fruits and some citrus smelling herbs.
Neral	28.0 %	Citral Z-isomer. Geranial has a lemon odour, slightly sweeter than geranial. Common in oils of citrus fruits and some citrus smelling herbs.
Geranyl acetate	3.6 %	Classified as a monoterpene. Has a pleasant floral or fruity rose aroma. Structure shown in Figure 6.1.
Geraniol	2.0 %	Effective plant-based mosquito repellent and also commonly used in perfumes. Structure shown in Figure 6.1.
Limonene	5.4 %	Classified as a cyclic terpene. Commonly found in the rinds of citrus fruits. Used in cleaning products such as hand cleansers and also mechanical degreasers. Used in histology to remove wax.
Methyl heptenone	1.4 %	Classed as a ketone.
Identified compounds	92.4 %	
Citronella		
Geraniol	35.7 %	Effective plant-based mosquito repellent and also commonly used in perfumes.
Neral	22.7 %	Citral Z-isomer. Geranial has a lemon odour, slightly sweeter than geranial. Common in oils of citrus fruits and some citrus smelling herbs.
Geranial	14.2 %	Citral E-isomer. Geranial has a strong lemon odour. Common in oils of citrus fruits and some citrus smelling herbs.
Geranyl acetate	9.7 %	Classified as a monoterpene. Has a pleasant floral or fruity rose aroma. Structure shown in Figure 6.1.
Citronellal	5.8 %	Gives citronellal its distinctive lemon scent. Has insect repellent properties and fungal growth inhibition properties.
Citronellol	4.6 %	Citronellol is a good mosquito repellent and is also found in perfumes. Structure shown in Figure 6.1.
Linalool	1.3 %	Terpene alcohol. Commonly used in perfumed hygiene products
β -Caryophyllene	0.8 %	Common constitute of essential oils such as black pepper, cloves and hops.
Identified compounds	94.8 %	

* Relevant contents is estimated from publications determining the content from similar products. These amounts could vary between particular cultivar used, extraction method, batch-to-batch variation and storage conditions.

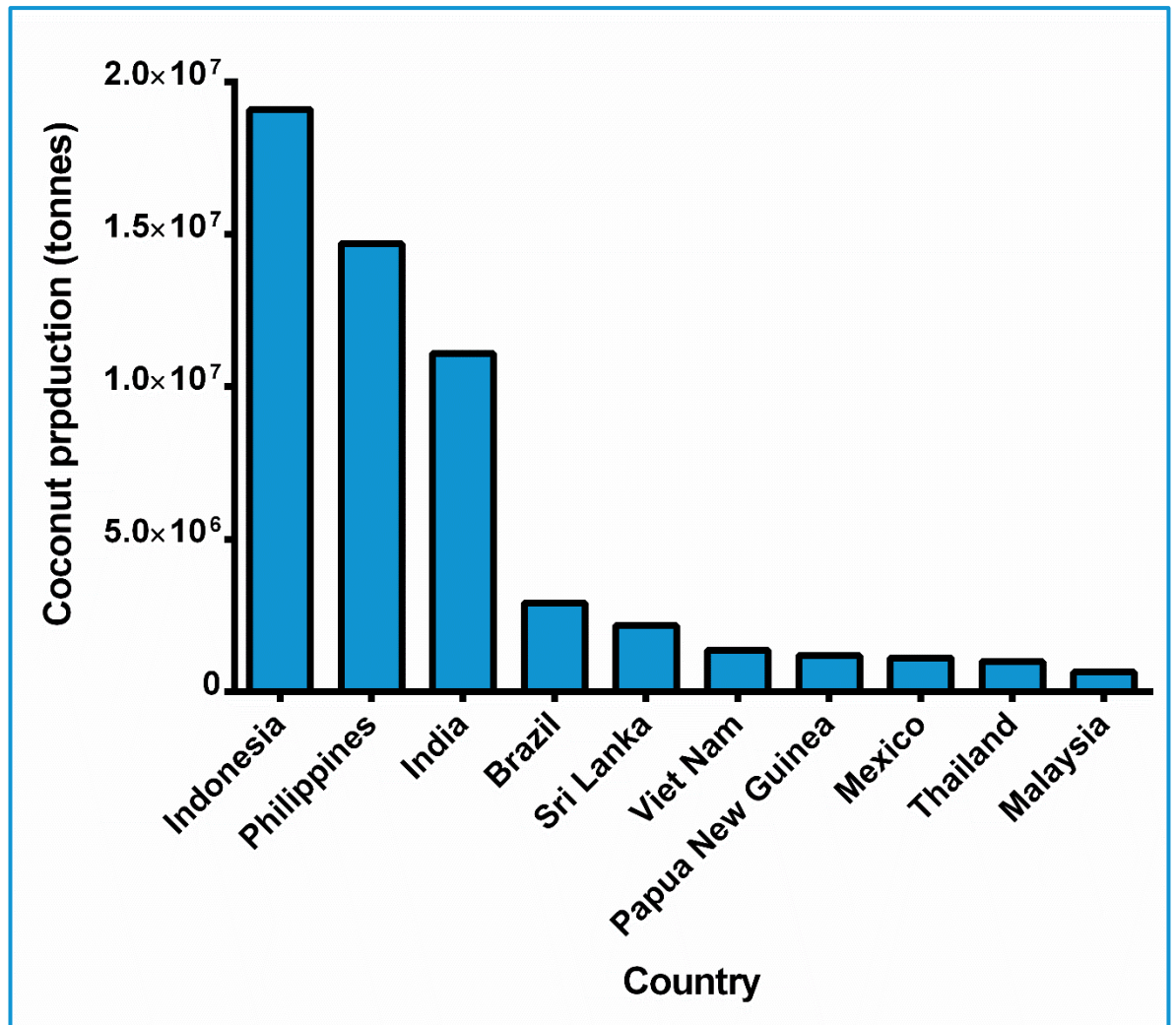


Figure 6.7. Top ten coconut producers in 2014 ranks by weight produced in tonnes.

Chapter 7 - Discussion

The aim of this thesis was to assess the suitability of a single or combination of fatty acids and/or fatty acid derivative to be the active component in a prophylaxis for the prevention of ophthalmia neonatorum. Early work in this study identified seven candidates that had fast acting anti-gonococcal properties. However, two clinical gonococcal isolates had reduced susceptibility to two of these fatty acid candidates. The mechanism for this tolerance is unknown and was not investigated further. The activity of the drug candidates were tested in simulated tear fluid. The results from this work produced results that at the time were unexpected. It was found that simulated tear fluid inhibited the bactericidal action of some of the candidates. This inhibition effect was more pronounced for the saturated fatty acids. However, the monoglyceride monocaprin and the mono-unsaturated fatty acid myristoleic acid were unaffected by the presence of the simulated tear fluid. It was predicted that it was the calcium chloride in the simulated tear fluid that was responsible for this inhibition of the antimicrobial properties. This was confirmed by repeating the log reduction assays in simulated tear fluid made without the calcium chloride. This left a decision to be made to either continue development of a formulation that was based on monocaprin and/or myristoleic acid or try to either compensate or neutralise the effects of the calcium ions that was inhibiting the other drug candidates.

The decision was taken that formulation should begin with monocaprin. The preference of monocaprin over myristoleic acid was mainly due to cost but was also due to the fact that monocaprin was known to work on another causative agent of ophthalmia neonatorum, namely *C. trachomatis*. Cost was a major consideration as the ultimate aim of this prophylaxis would be to be used on a national scale. However, if the cost of myristoleic acid were to fall then it may be included in the formulation. The other candidates, namely; lauric acid, tridecanoic acid, and sodium dodecanoate would remain as possible candidates if an effective formulation was not successfully achieved with either monocaprin or myristoleic acid.

Monocaprin had been proven to have fast acting anti-gonococcal properties when tested in culture medium as well as in simulated tear fluid and also has low irritation potential. It was therefore taken forward for formulation. Two different dosage forms were chosen for the formulation. The first was a semi-solid dosage form. The monocaprin was melted, added to mineral oil and mixed with white petroleum. Semi-solid dosage forms have an advantage over liquid dosage forms in that they have greater contact time on the ocular surface. However, the formulations did not perform well in growth inhibition tests and had no bactericidal activity (as tested in the log reductions) in this form. A liquid dosage form was therefore developed as an alternative formulation to the semi-solid dosage form. A viscosity enhancer was used in this formulation to increase the retention time on the ocular surface. A co-solvent (propane-1,2-diol) and surfactant (Polysorbate 20) was included in the formulation to increase the solubility of the monocaprin. The presence of the surfactant

significantly increased the solubility of the monocaprin but excess surfactant decreased the anti-gonococcal action of the monocaprin. Therefore, the amount of surfactant had to be optimised for the amount of monocaprin in the formulation.

The liquid dosages performed much better than the semi-solid dosage forms in the growth inhibition tests and the log reduction assays. However, how significant is this? Do these *in vitro* experiments really replicate the formulations end use? The reason for the use of the formulation is to prevent the onset of ophthalmia neonatorum and to prevent damage of the cornea that an ocular gonococcal infection would cause. Would the presence of the antimicrobial semi-solid prevent infection? This is a difficult question to answer without *in vivo* experiments. The semi-solid formulation did not have a killing action in log reduction experiments but would it be able to kill cells on the occur surface when in direct contact with them. The liquid dosage was later tested for this in Chapter 4 where the bacteria were allowed to infect excised corneas and then the formulation applied. However, it would be difficult to do this with a semi-solid dosage form as the ointment would be difficult to remove without physically scrapping the ointment off the eye which may in turn remove the bacteria. This does then present experimental challenges. If the growth inhibition tests were done in parallel with antibiotic ointments already on the market then a comparison of its performance could be made. This could be used to either say that the proposed formulation was not as effective or that it was comparable to the antibiotic ointment and the use of this method in this way is a poor indicator of *in vivo* effectiveness.

The reason why the semi-solid dosage form did not perform well is probably due to the fact that drug release from this matrix was too slow. As monocaprin is poorly water soluble, the drug would more than likely remain contained in the ointment rather than being released into an aqueous environment. Work in Chapter 2 showed that monocaprin has fast acting gonococcal killing activity with a MBC of 0.5 mM. However, its killing ability rapidly diminishes below this concentration. In the cell culture infection work in Chapter 4, the bacteria were exposed to monocaprin for up to 3 hours. In these samples, the MBC was also 0.5 mM. Although these experiments were fundamentally different, they do suggest that the action of the drug is concentration dependant and not time dependant. A full study should be done to investigate the pharmacodynamic properties of monocaprin. Such studies have already been conducted on *N. gonorrhoeae* with other possible antimicrobial agents [429]. The pharmacodynamics properties of monocaprin would aid in the formulation of an appropriate treatment form as an ointment base may not be suitable in terms of drug delivery.

Chapter 4 investigated the use of models of gonococcal eye infections to assess the suitability of the drug or formulation. The first experiment used a cell culture infection model was primarily to

be used to check if the monacaprins could clear the infection if the bacteria were introduced to host cells previous to application of the drug. As *N. gonorrhoeae* is an intracellular pathogen this was a valid question. In a hospital setting, the prophylaxis would be used within the first hour of life. It would need to be able to kill bacteria that may have already entered cells. Although the monacaprins applied 1.5 hours after inoculation did not perform as well as when added immediately after inoculation, it still worked effectively and at a concentration of 1 mM completely cleared the infection.

The results of the explanted bovine corneas did raise a lot of questions as well as answer a few. Preliminary data on developing this technique (not shown in this thesis) demonstrated that a formulation made with a monacaprins concentration of 1 mM (2 x MBC) was not sufficient to clear the infection. In fact, this concentration had little effect on reducing the bacterial load. Therefore, formulation work had to be repeated with increased concentrations of monacaprins of 0.068 % (w/w), 0.125 % (w/w), 0.188 % (w/w), and 0.25 % (w/w), these concentrations were approximately equivalent to 2.5 mM, 5 mM, 7.5 mM and 10 mM respectively. From these tested concentrations, 2.5 mM had little or no improvement over the no monacaprins control formulation, 5 mM reduced bacterial load slightly while 7.5 mM and 10 mM showed a very good reduction in bacterial load. This indicates that the ability of monacaprins to function as an antimicrobial on the ocular surface is diminished. This can be countered by increasing the concentration of the monacaprins. However, is the irritation potential of monacaprins also reduced in this environment? If so, then the concentration can be increased. The explanted cornea model has shown that monacaprins can be used but at a higher concentration. However, what is our target that we should be aiming to achieve? The inoculum used in that experiment was a high bacterial load. Would a simple reduction in bacterial load prevent onset of ophthalmia neonatorum, or would only total bacterial clearance prevent onset?

The initial irritation assays done in Chapter 2 used three methods. The first was the BCOP which was only tested the drug candidates at a concentration of 1 mM. The HET-CAM tested all of the candidates at a concentration of 1 mg/ml which would be approximately equivalent to a concentration of 4 mM in the case of monacaprins, this was the maximum concentration that could be tested without the use of a solubility enhancer. These tests showed no irritation at these concentrations. However, the RBC lysis assay predicted that monacaprins would cause cell lysis at a concentration of around 2 mM (4 x MBC). The estimation of the RBC lysis assay does therefore indicate that the minimum toxicity level is close to the effective concentration of the drug. The therapeutic window, is the concentration between the minimum effective concentration and the minimum toxic concentration (see Figure 7.1) [430]. If the RBC lysis assay is an accurate measure of

ocular irritation then the therapeutic window in this case is small. There are antimicrobial drugs, such as gentamicin and amphotericin B which have narrow therapeutic windows [431, 432].

If the minimum effective and minimum toxicity concentrations are affected by their environment, then this therapeutic window is constantly shifting according to the environment in which it is being used. Other drugs with narrow therapeutic window/index can be carefully controlled. In the case of the monocaprin liquid-dosage forms, variables such as uneven drug dispersal within the packaging which could cause application of excess or insufficient drug to the eye. Also, the volume of the application would be difficult to control, how many drops would be applied in one application. It is unclear whether at this stage if this over-application of the ocular treatment would cause an increased risk of irritation as only the drug concentration has been considered so far. Moreover, the eye has a maximum capacity of how much fluid it can hold with any excess applied presumably tearing away from the eye. The cause of the inhibition of the anti-gonococcal properties of monocaprin should be investigated as it may fluctuate between individuals. If this does occur then this makes the choice of which concentration of monocaprin to use in the formulation all the more difficult.

The number of births in England and Wales in 2014 was 695,233 [209]. The incidence rates of ophthalmia neonatorum in England and Wales has not been recorded since 2009. However, if we assume that the incident rate has not changed significantly since then, then the number of reported cases is approximately 100 cases a year. This is putting aside that all cases may not have been reported as suggested in Chapter 1. This would mean that the rate of ophthalmia neonatorum would be 0.014 % or 0.14 per 1,000 births. If the decision to use this prophylaxis was solely based on cost, then the use of the prophylaxis would have to be 6,952 times cheaper than the cost of treatment for ophthalmia neonatorum. The actual cost of the prophylaxis in this study has not been calculated as the formulation has not been finalised. The cheapest proposed currently is thought to be povidone-iodine which is significantly cheaper than erythromycin [433]. However, there are more factors to consider than simply cost, there is a risk of visual impairment or even blindness to consider. Use of the prophylaxis could be restricted to use in high risk areas. In England, 50.1 % of reported gonorrhoea came from PHE Region London [64]. In these areas, the use of the prophylaxis becomes more justifiable. Also, if the decreased susceptibility to the current firstline antimicrobials used to treat ophthalmia neonatorum occurs, then post exposure prophylaxis becomes a more appropriate action rather than treatment of the disease. As discussed previously, this could well be the case in the post-antibiotic area. For this prophylaxis to be successfully used, the rate that it causes adverse side effects such as chemical conjunctivitis must be low.

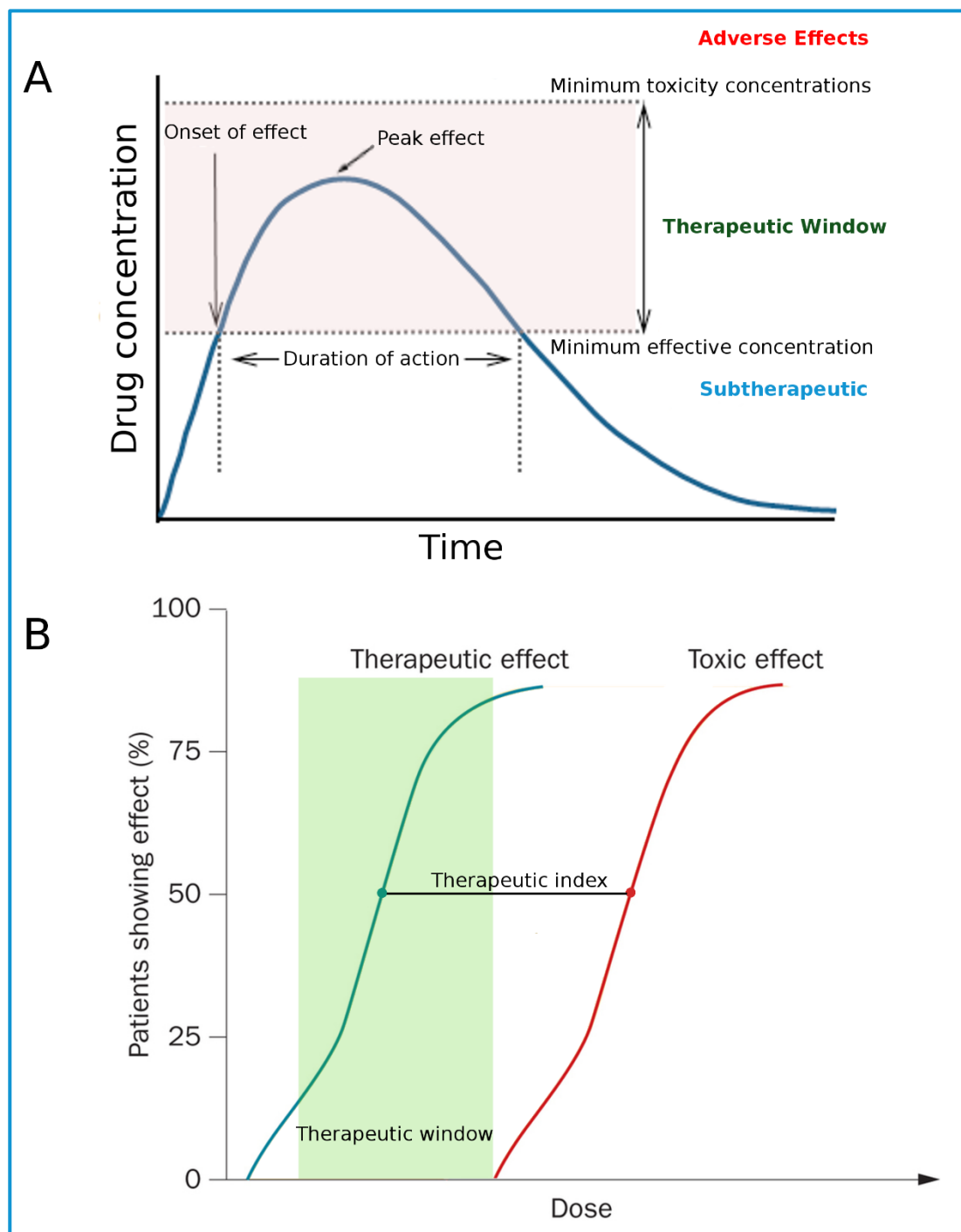


Figure 7.1 Therapeutic windows in an individual and of populations. (A) Active drug level at target site against time. In this case the drug exceeds the minimum effective concentration without reaching the minimum toxicity concentration. (B) Therapeutic windows within populations. The minimum effective and toxicity concentrations may be different within a population. In this example, the minimum therapeutic effect is not reached in 100 % of the population before a small fraction of population will also exhibit some signs of toxicity. The therapeutic window in this case has been placed where the maximum concentration would mean that few or no patients would show signs of toxic effects but there is a risk of treatment failures. The further the two plots of therapeutic effect and toxic effects are, the better the efficacy. The therapeutic index is used to measure this, it is the difference between the concentrations of the therapeutic and toxic effects at 50 % of the population.

Work done in Chapter 5 has shown that *N. gonorrhoeae* cannot easily develop resistance to monacaprins. Passage on media containing another fatty acid demonstrated that decreased susceptibility to certain fatty acids can develop. However, this was not seen in the case of monacaprins. Furthermore, this decreased susceptibility was achieved by constant exposure to the fatty acid. The one added advantage for the use of a novel antimicrobial agent in the way that we are proposing is that there is little chance of survival of any bacteria that do develop decreased susceptibility mechanisms. The fate of any *N. gonorrhoeae* cell that is passed to the neonate is that its chances of survival are extremely slim in the long-term. *N. gonorrhoeae* does not persist in the environment for any great length of time and its chance of infecting another host apart from the neonate are unlikely. Even if the bacteria were able to infect the neonate its chances of moving to another host are slim as transmission would be highly unlikely and the infection would be quickly treated. *N. gonorrhoeae* has successfully developed resistance to many antimicrobial agents in the past as it acquires or develops decreased susceptibility while in its main sites of transmission. The only way that it would likely gain decreased susceptibility would be to acquire it from another bacteria and that it would gain some advantage by it. Common use of monacaprins is not found at present. However, if it were used, as suggested by one study mentioned in Chapter 1, to prevent STIs then *N. gonorrhoeae* would gain an advantage by acquiring or developing resistance mechanisms. How it would gain resistance mechanisms is yet unknown but *N. gonorrhoeae* has already gained mechanisms of resistance to an array of previously effective antimicrobials. However, as stated before, as long as monacaprins use can be restricted to for prophylaxis then decreased susceptibility is unlikely.

Throughout this thesis, monacaprins will be defined by the broad term; antimicrobial agent. However, which type of antimicrobial agent is it? In the Introduction chapter examples of antibiotic resistance were given as examples of antimicrobial resistance. Antibiotic classically refers to a chemical substance produced by a microorganism that has the capacity, in dilute solutions, to kill other microorganisms or inhibit their growth [434]. However, this term has now been extended to include synthetic chemicals which are based on existing antibiotics. It is also more commonly used to describe a drug which has a high level of selective toxicity so that it can be taken internally. An antiseptic, would therefore be a more fitting term to describe monacaprins. This is a broad term that includes both disinfectants and antibiotics [435]. One form of prophylaxis which has not been described in detail so far is the use of povidone-iodine ophthalmic solution which is commonly used at a concentration of either 1.25, 2.5 or 5 % [436]. Povidone-iodine is an antiseptic which is commonly used to dress wounds and to help prevent contamination of wounds during surgery. This method was thought to be a good alternative to silver nitrate and it has been proven not to affect thyroid hormone levels [437]. Studies on the performance of the povidone-iodine compared to

antimicrobial ointments have given mixed results [433, 438, 439]. The cost of treatment has also been questioned as treatment with erythromycin ointment is more cost effective [440]. As with other prophylaxis, povidone-iodine has been associated with cases of chemical conjunctivitis with one study recording a level of 5 % of applications (n = 201) resulting with some form of chemical conjunctivitis [439]. Whether or not a monacaprins based ophthalmic treatment would be more effective or would cause a lower level of chemical conjunctivitis is unknown until a direct comparison is made. However, in the face of increasing antimicrobial resistance it is imperative that alternative treatments are available and that they have been assessed for efficacy and safety.

Overall, monacaprins has shown that it can be used as the active component in an ophthalmic treatment. More work is required to fully characterise a formulation. However, work here indicates that a liquid dosage form maybe more effective than semi-solid formulation. The use of monacaprins is novel and it is unlikely that *N. gonorrhoeae* would easily develop resistance mechanisms. Before human trials can be conducted some level of animal testing should be done to fully confirm ocular safety. Only then can its full effectiveness be compared to existing treatments. However, this study suggests that it can be used as a prophylaxis in the prevention of ophthalmia neonatorum.

Chapter 8 - Bibliography

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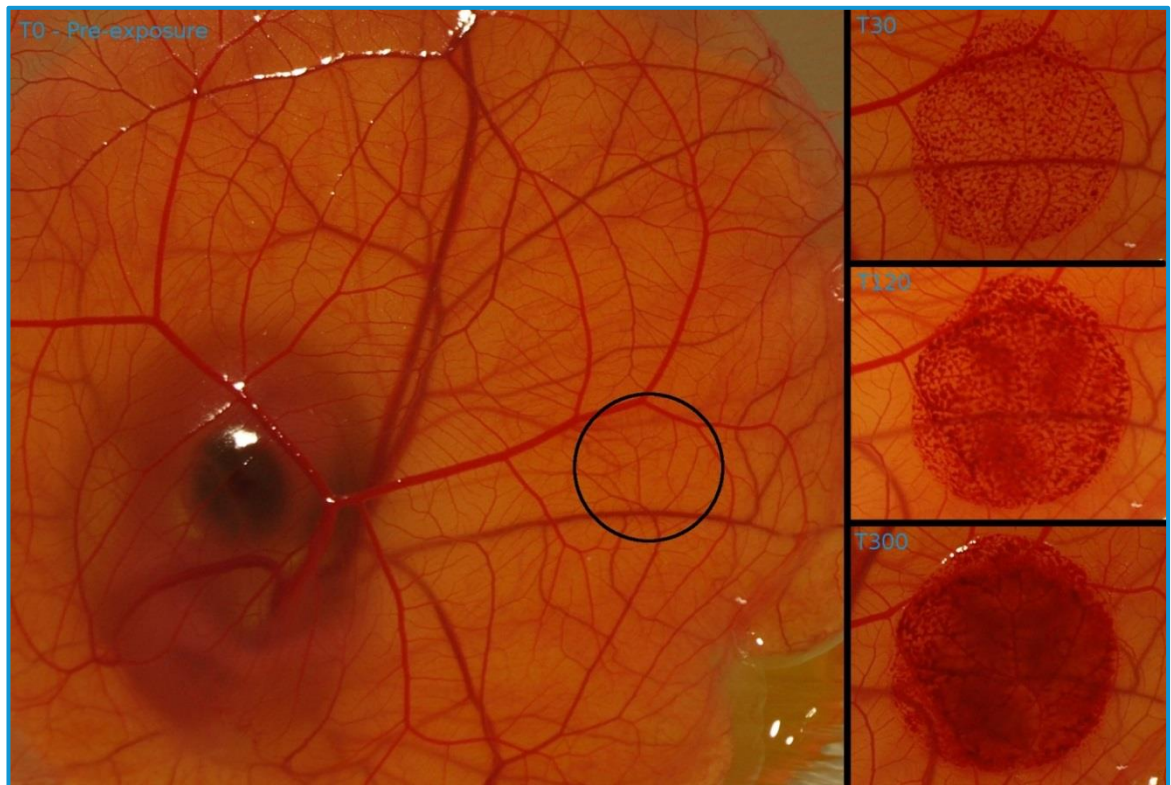
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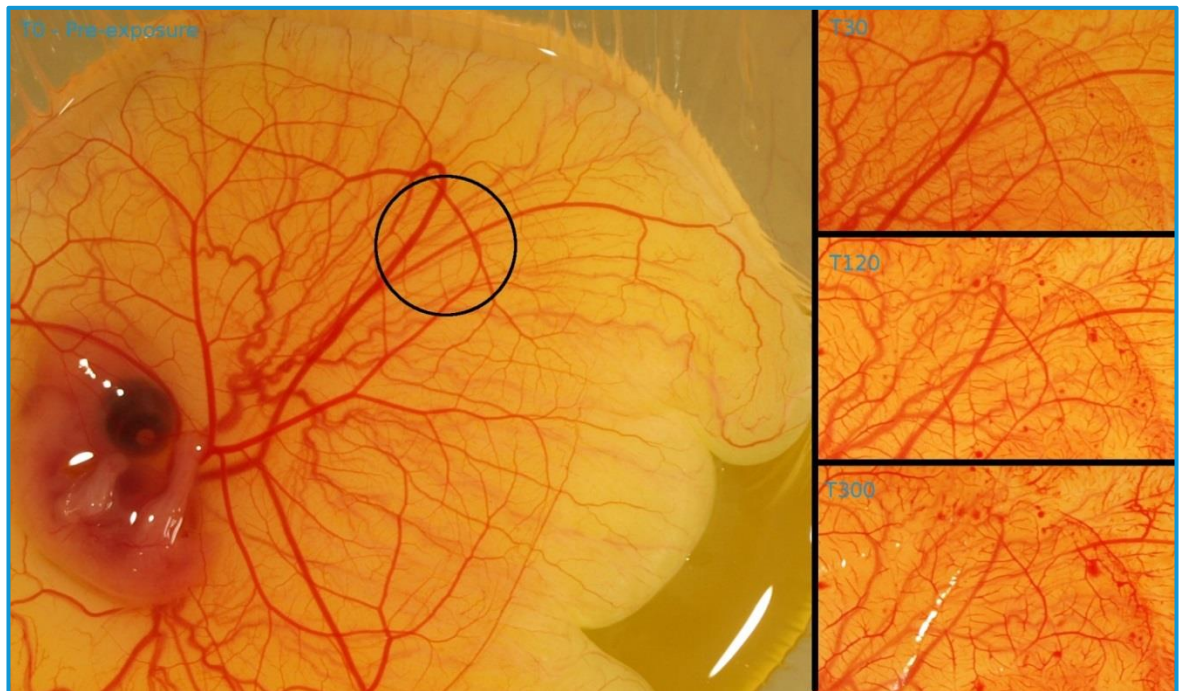
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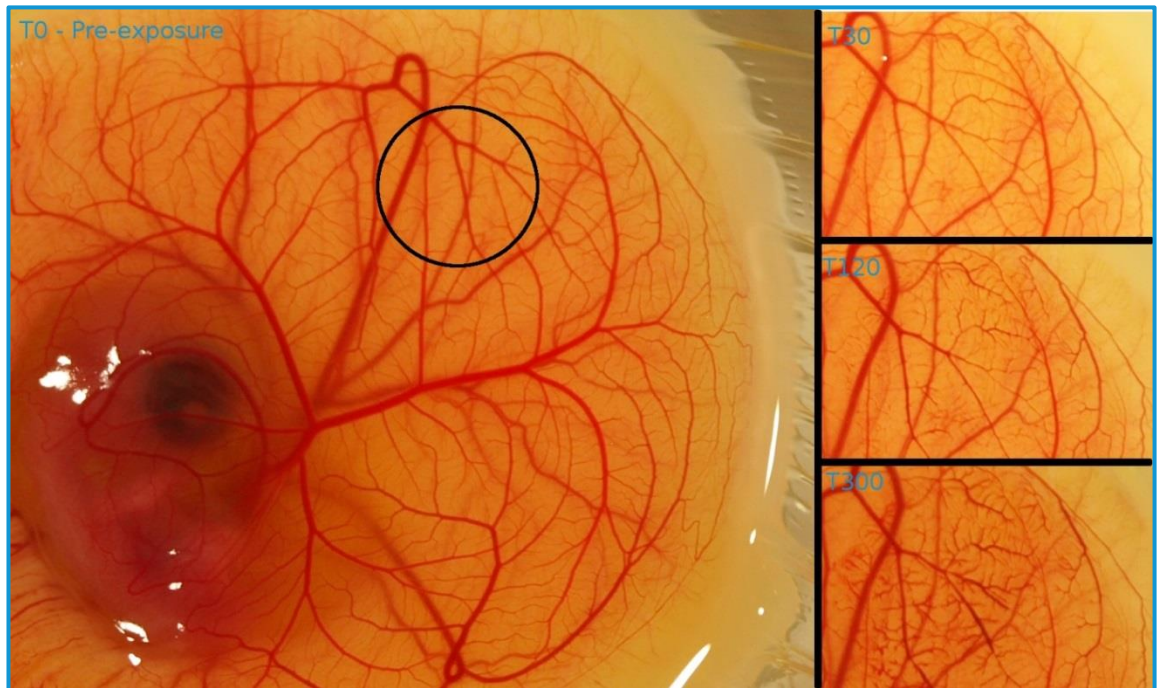
Chapter 9 - Appendix



Appendix 1.1. Photographs of time-points from HET-CAM experiment for 0.1 M sodium hydroxide. The black ring in the main pre-exposure panel marks where the test substance was applied. In this sample, hyperaemia and haemorrhaging started within the first 30 seconds. Coagulation was only clearly observed in the five minute (300 sec) time-point.



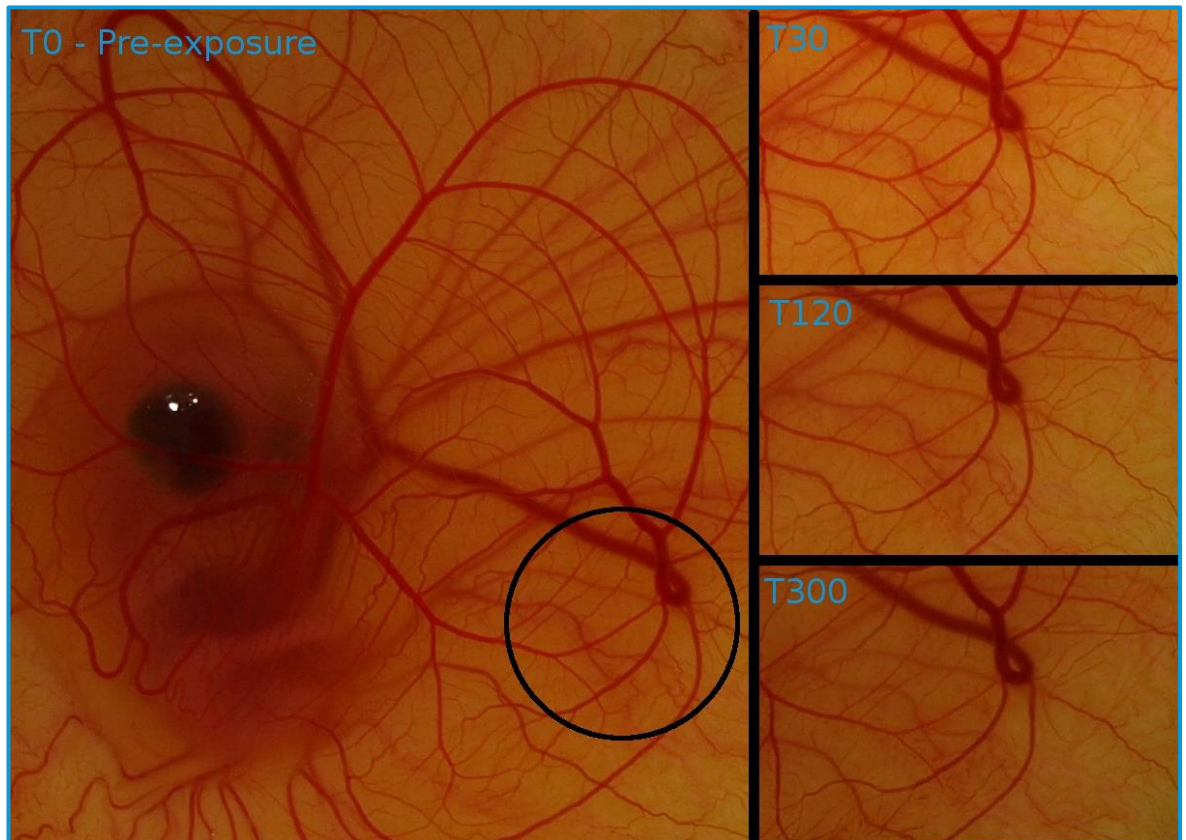
Appendix 1.2 Photographs of time-points from HET-CAM experiment for acetone. The black ring in the main pre-exposure panel marks where the test substance was applied. In this sample, hyperaemia started within the first 30 seconds followed by haemorrhaging at 120 seconds. Coagulation was not observed in this control.



Appendix 1.3. Photographs of time-points from HET-CAM experiment for proylene glycol. The black ring in the main pre-exposure panel marks where the test substance was applied. In this sample, hyperaemia was observed at 120 second and haemorrhaging at 300 seconds. Coagulation was not observed in this control.



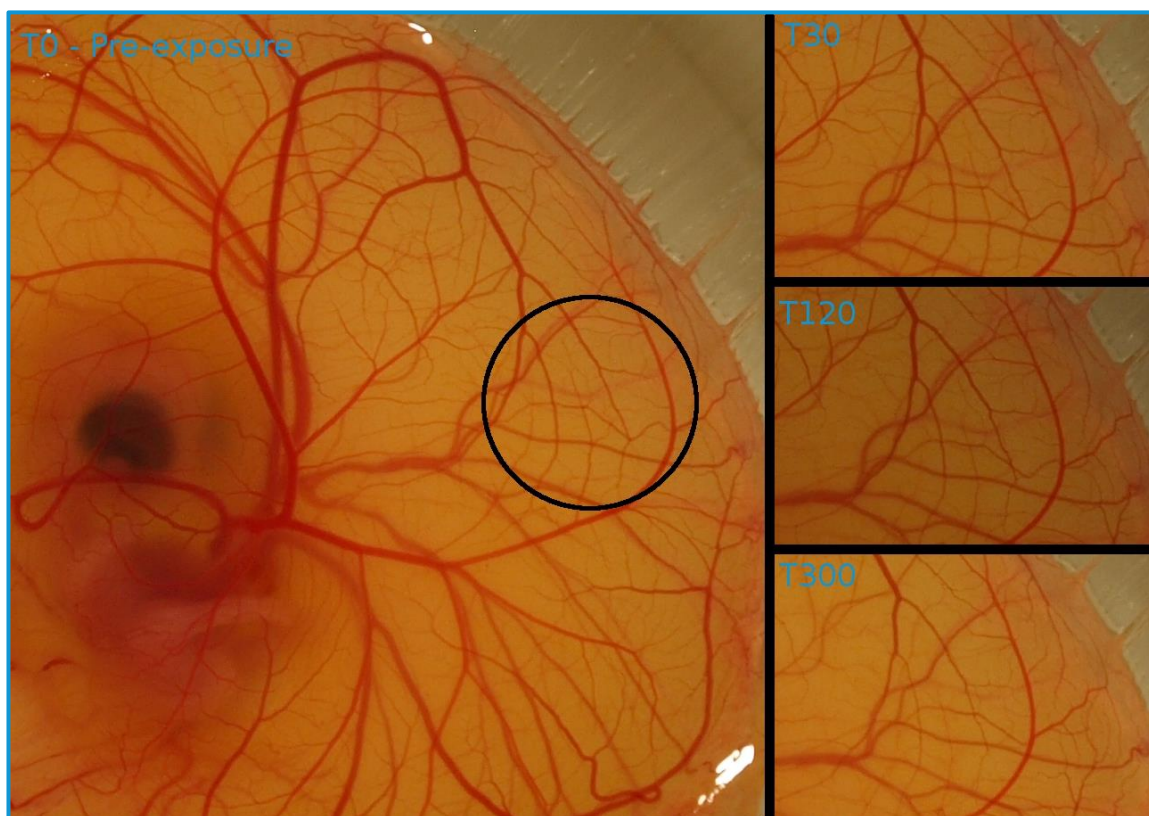
Appendix 1.4. Photographs of time-points from HET-CAM experiment for saline. The black ring in the main pre-exposure panel marks where the test substance was applied. No reaction of the testing substance was seen with this sample.



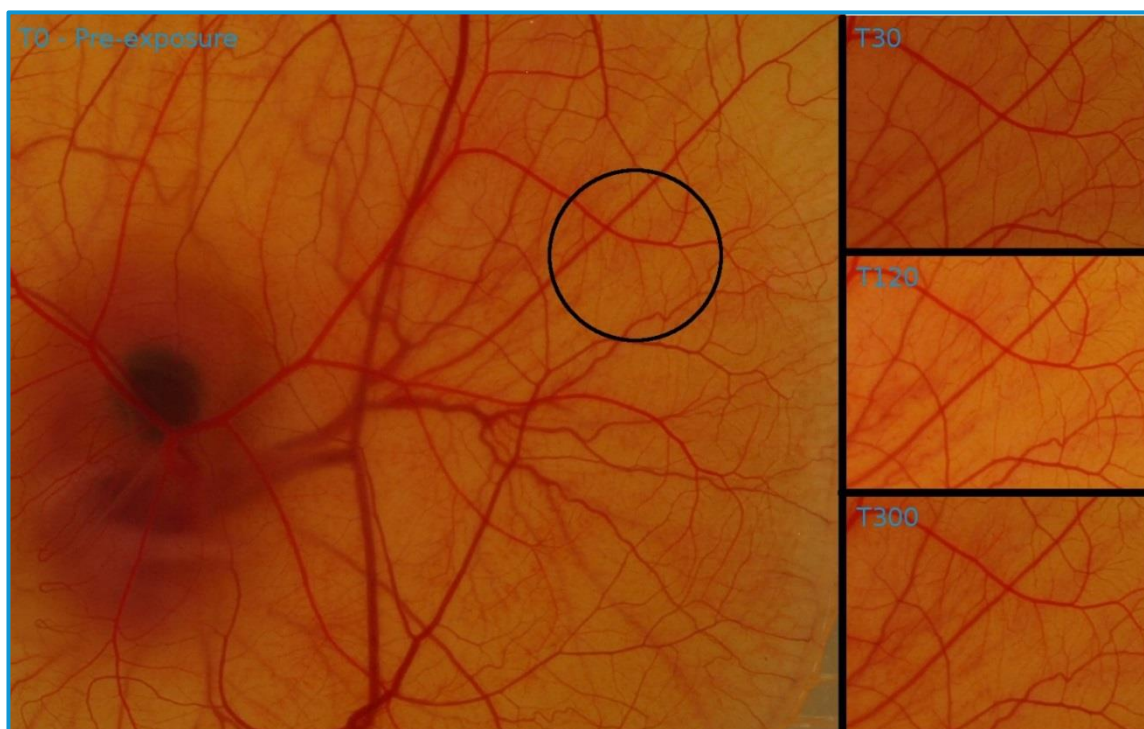
Appendix 1.5. Photographs of time-points from HET-CAM experiment for lauric acid (12:0). The black ring in the main pre-exposure panel marks where the test substance was applied. No reaction of the testing substance was seen with this sample.



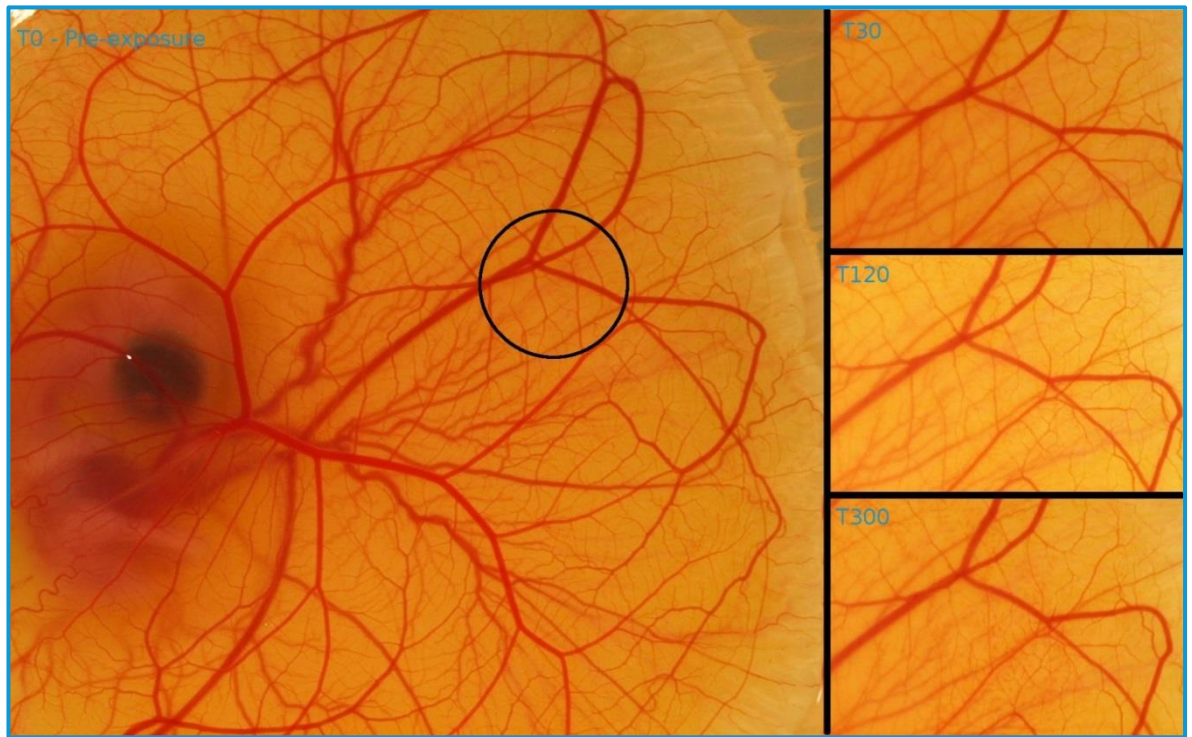
Appendix 1.6. Photographs of time-points from HET-CAM experiment for tridecanoic acid (13:0). The black ring in the main pre-exposure panel marks where the test substance was applied. No reaction of the testing substance was seen with this sample.



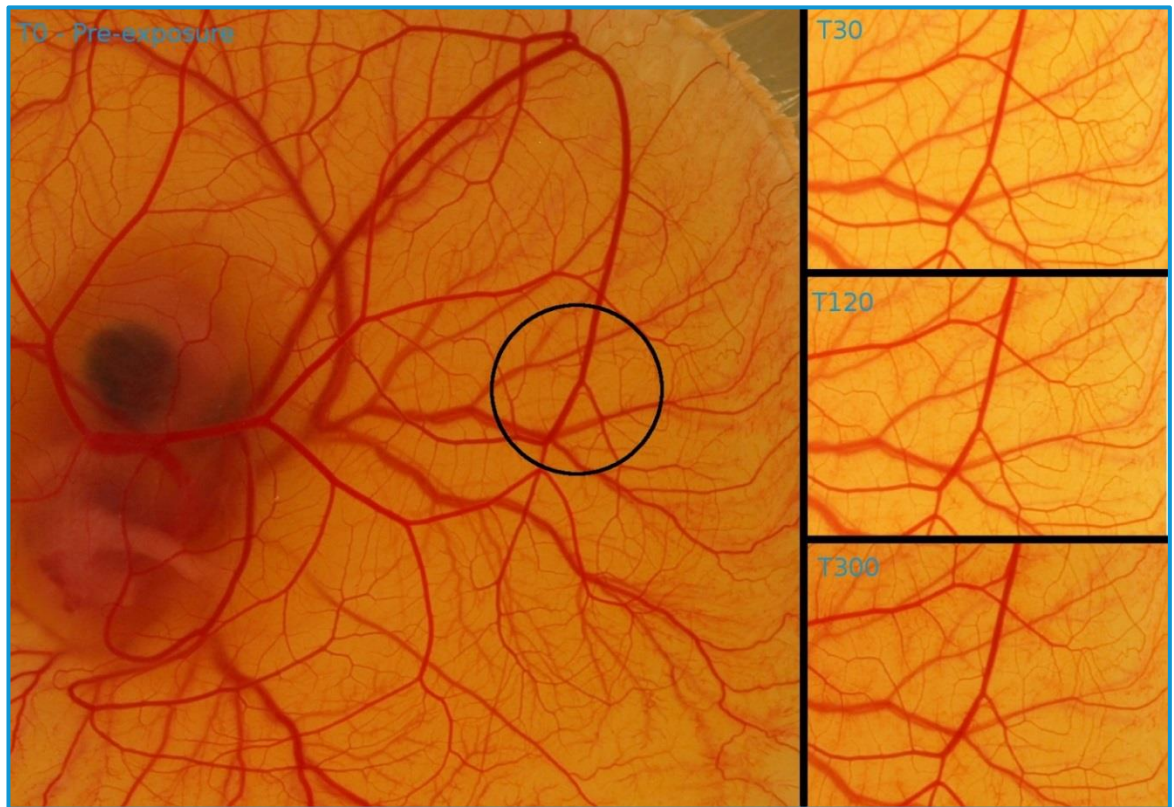
Appendix 1.7. Photographs of time-points from HET-CAM experiment for myristoleic acid (14:1). The black ring in the main pre-exposure panel marks where the test substance was applied. No reaction of the testing substance was seen with this sample.



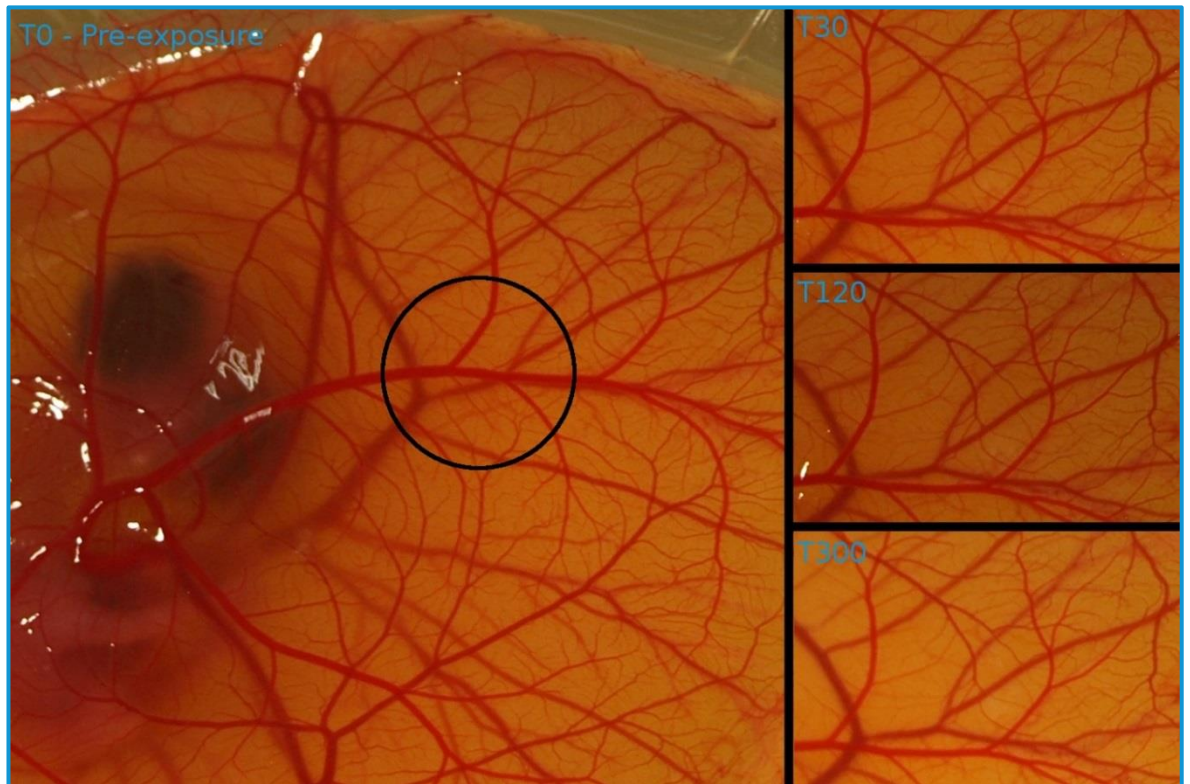
Appendix 1.8. Photographs of time-points from HET-CAM experiment for palmitoleic acid (16:1). The black ring in the main pre-exposure panel marks where the test substance was applied. No reaction of the testing substance was seen with this sample.



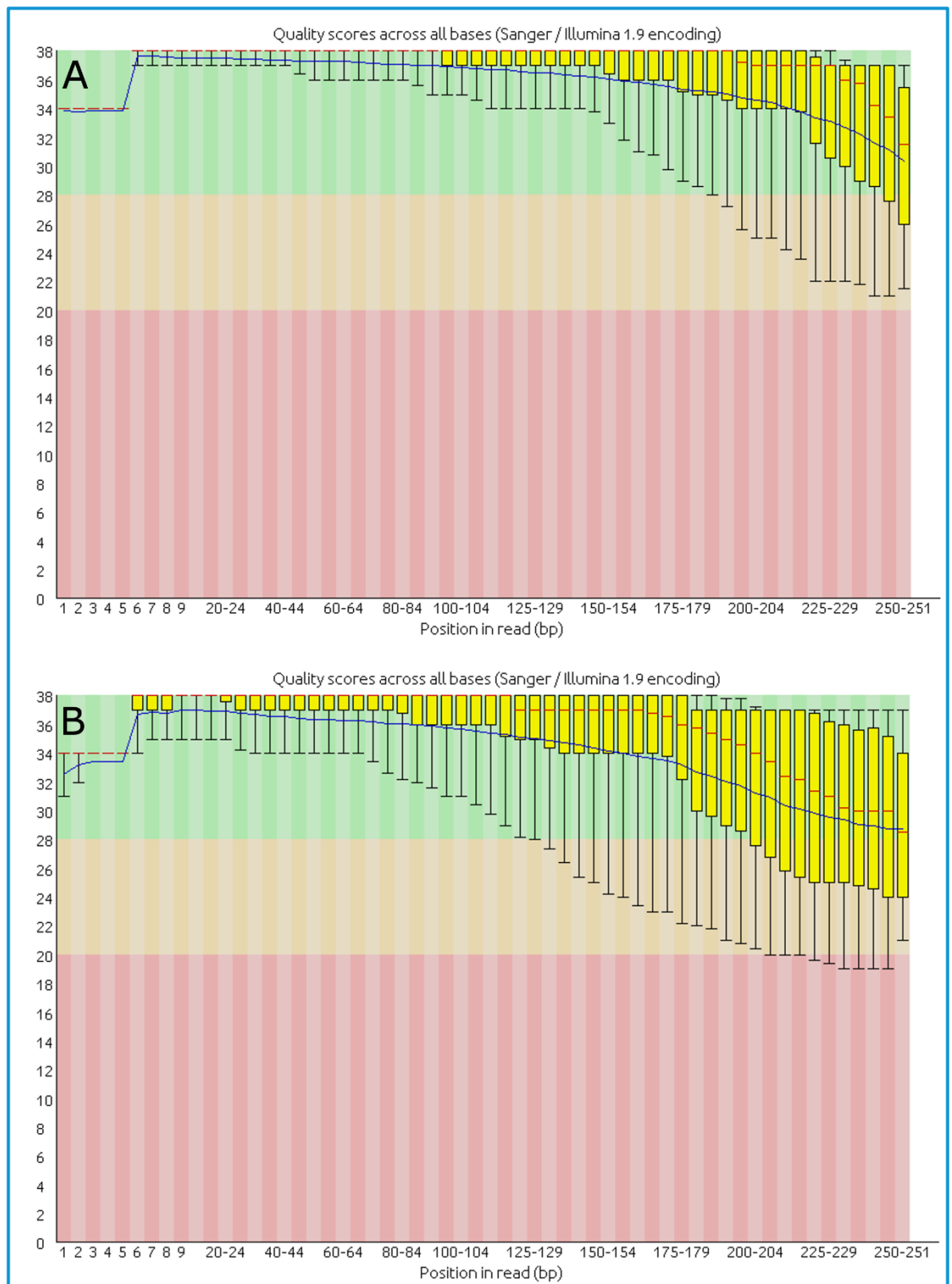
Appendix 1.9. Photographs of time-points from HET-CAM experiment for linolenic acid (18:3). The black ring in the main pre-exposure panel marks where the test substance was applied. No reaction of the testing substance was seen with this sample.



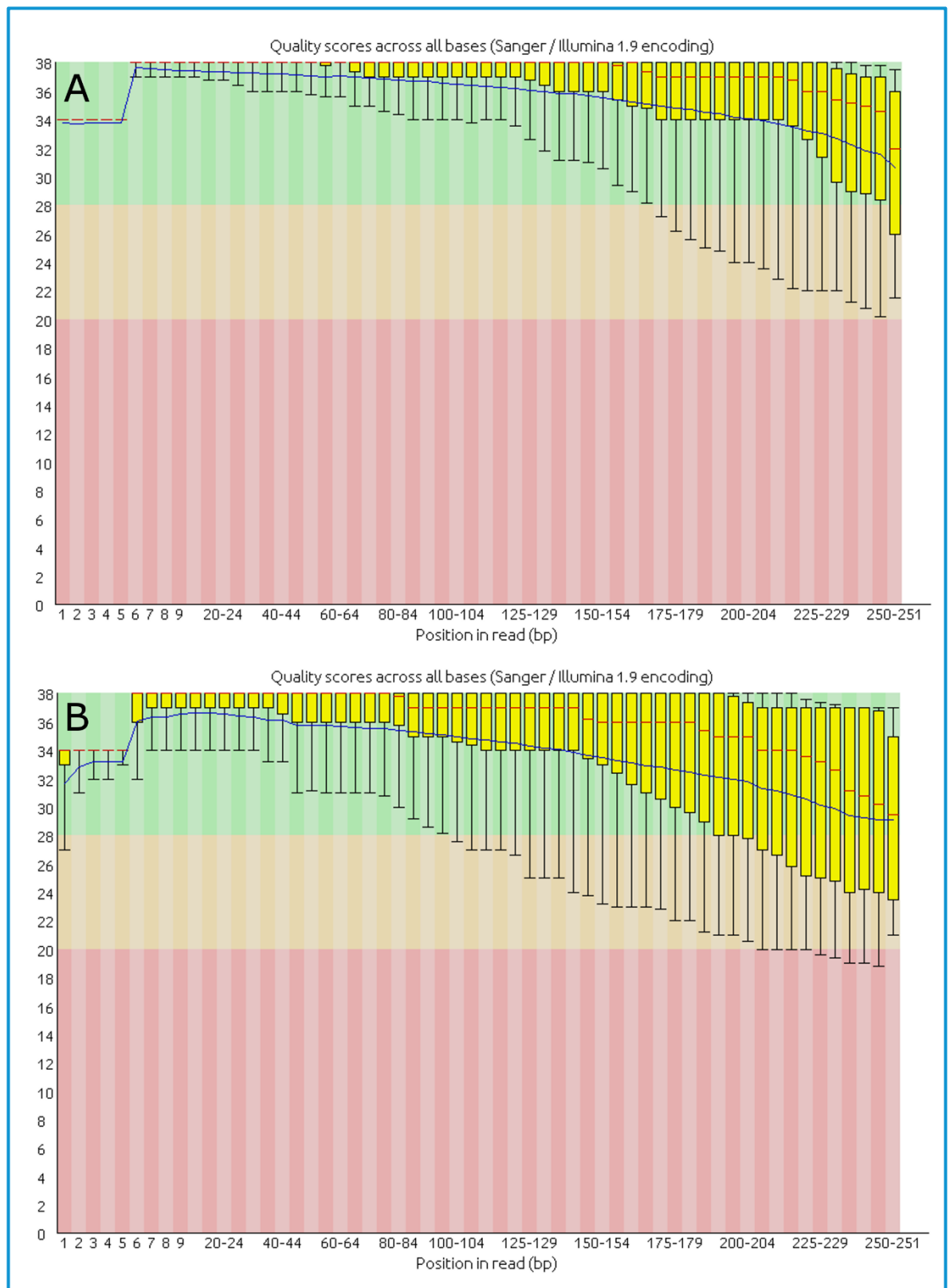
Appendix 1.10. Photographs of time-points from HET-CAM experiment for monocaprin (10:0MG). The black ring in the main pre-exposure panel marks where the test substance was applied. No reaction of the testing substance was seen with this sample.



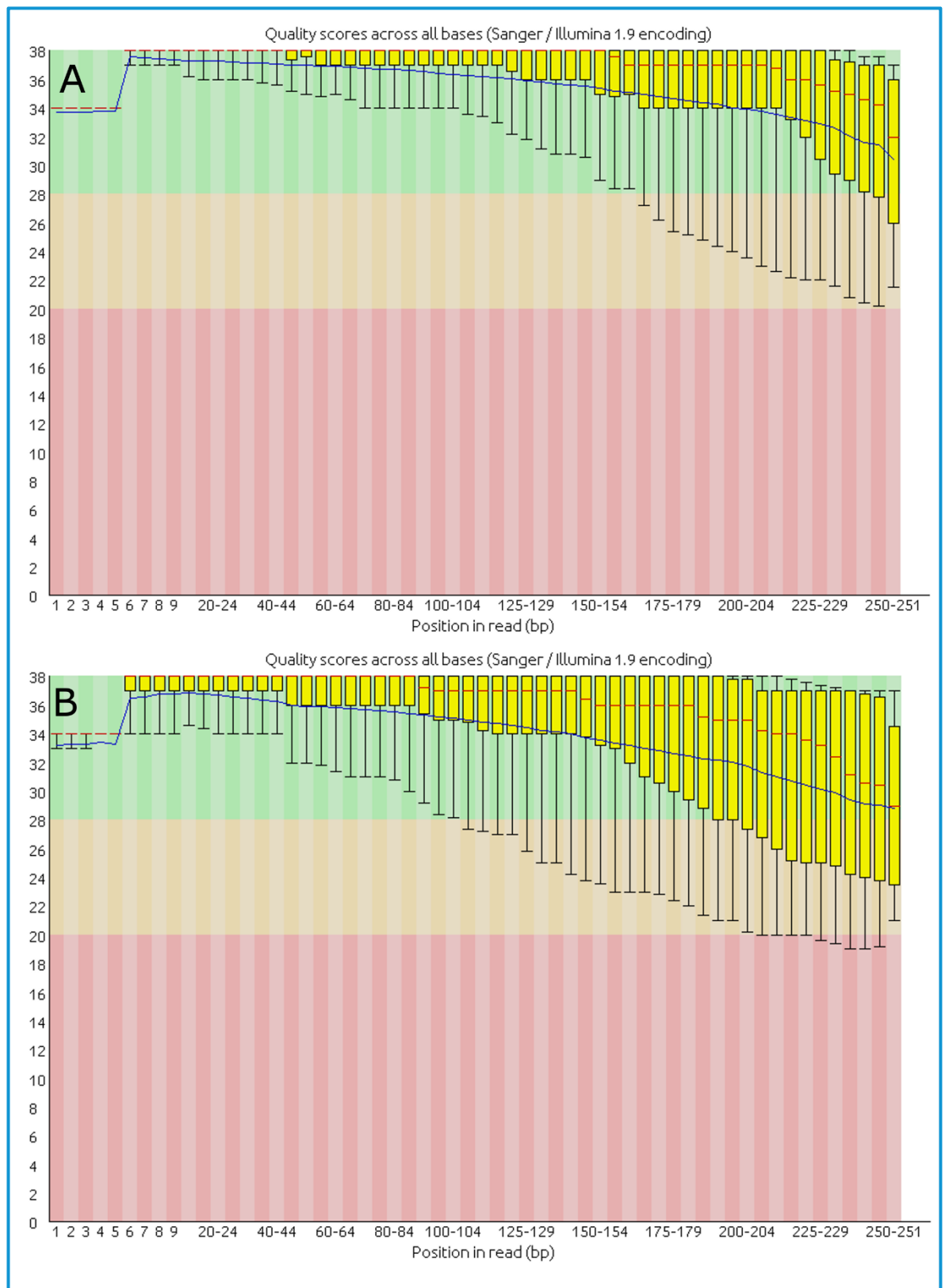
Appendix 1.11. Photographs of time-points from HET-CAM experiment for sodium dodecanoate (12:0Na). The black ring in the main pre-exposure panel marks where the test substance was applied. No reaction of the testing substance was seen with this sample.



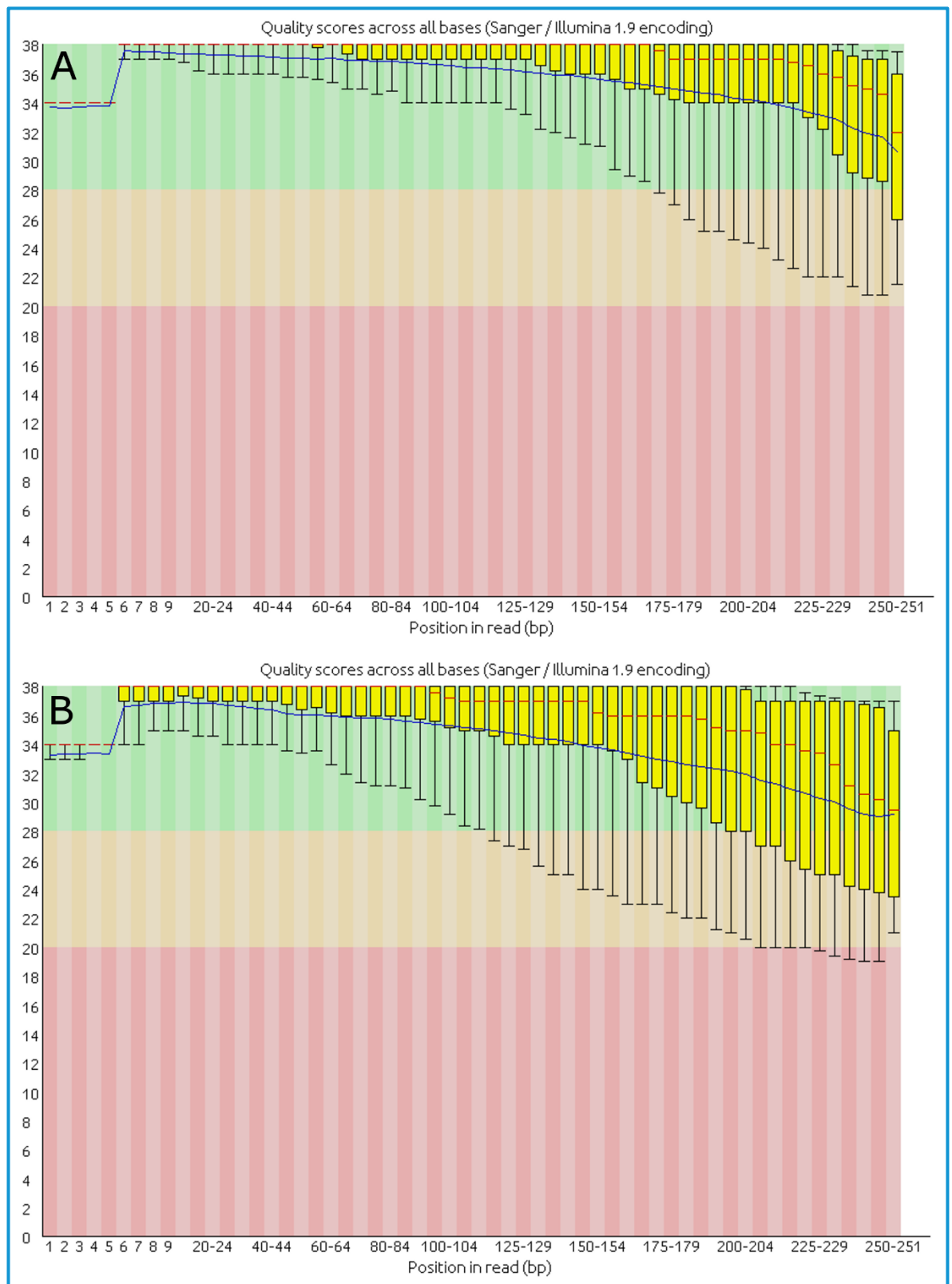
Appendix 5.1. FastQC report of GC NCCP11945 Low Passage samples from Illumina NGS sequencing. Quality scores on y axis are plotted against base position (x axis). The two panels represent the forward and reverse paired reads.



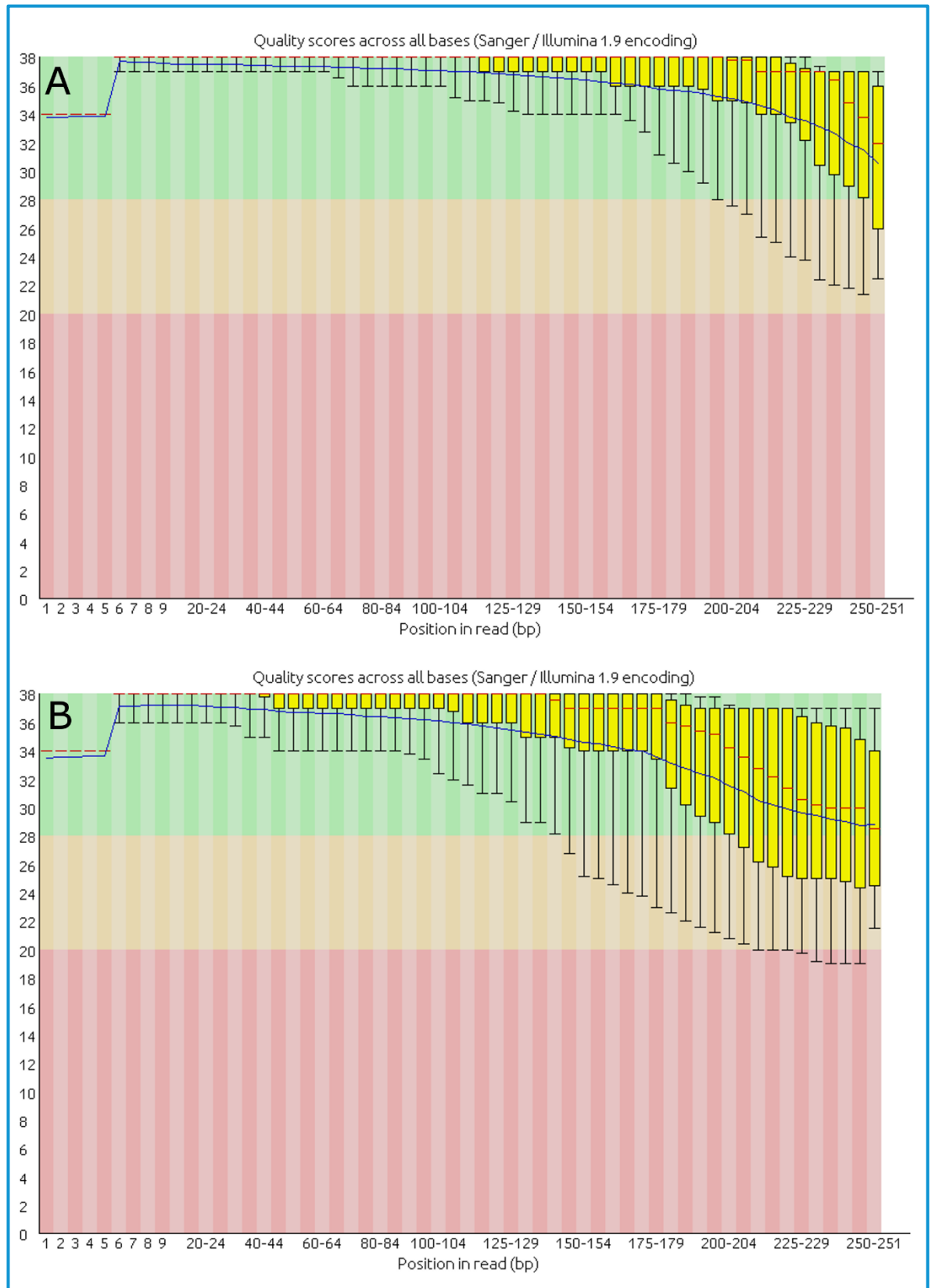
Appendix 5.2. FastQC report of GC NCCP11945 Non-selective 1 sample from Illumina NGS sequencing. Quality scores on y axis are plotted against base position (x axis). The two panels represent the forward and reverse paired reads.



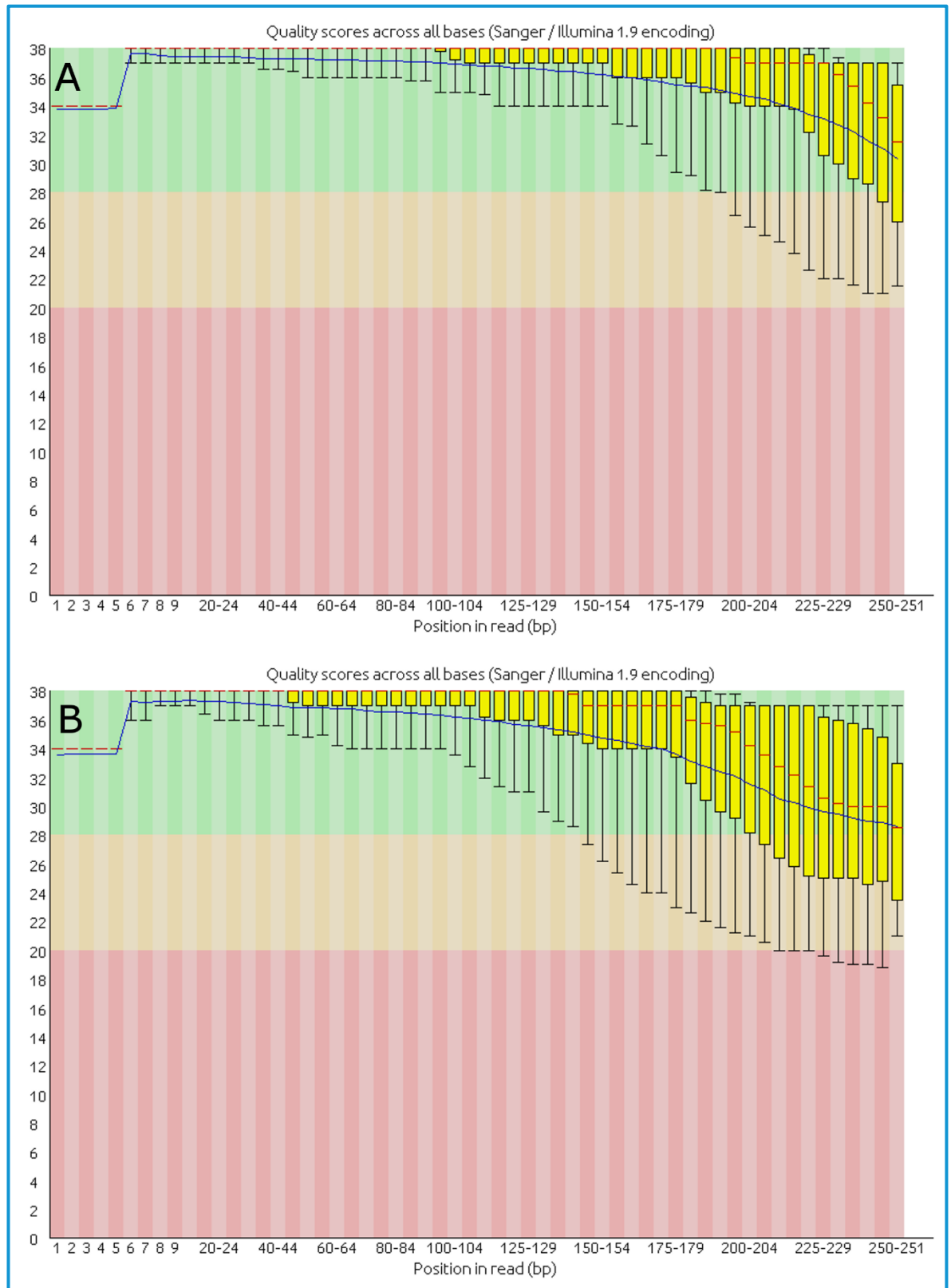
Appendix 5.3. FastQC report of GC NCCP11945 Non-Selective 2 sample from Illumina NGS sequencing. Quality scores on y axis are plotted against base position (x axis). The two panels represent the forward and reverse paired reads.



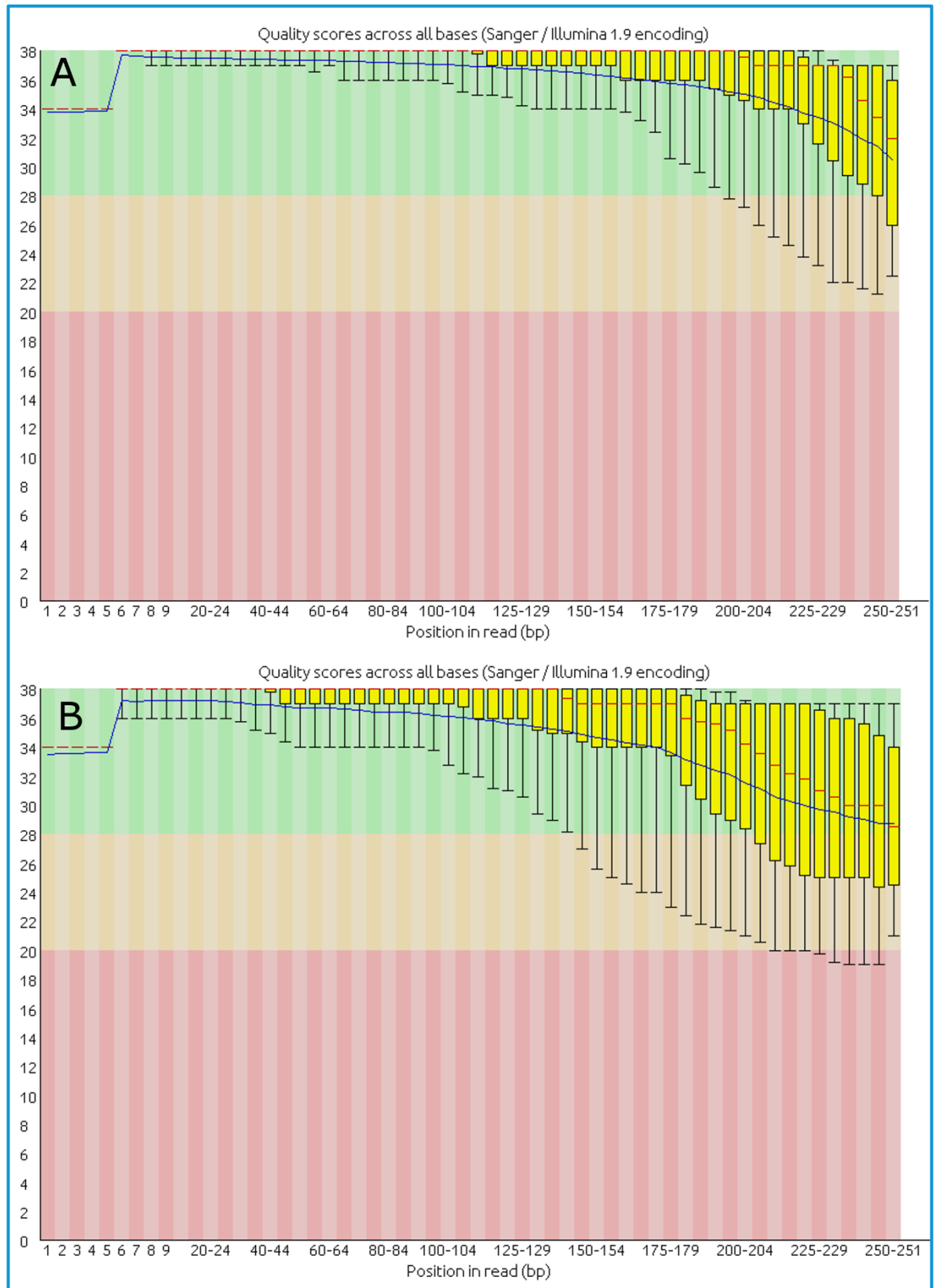
Appendix 5.4. FastQC report of GC NCCP11945 Myristic acid 1 sample from Illumina NGS sequencing. Quality scores on y axis are plotted against base position (x axis). The two panels represent the forward and reverse paired reads.



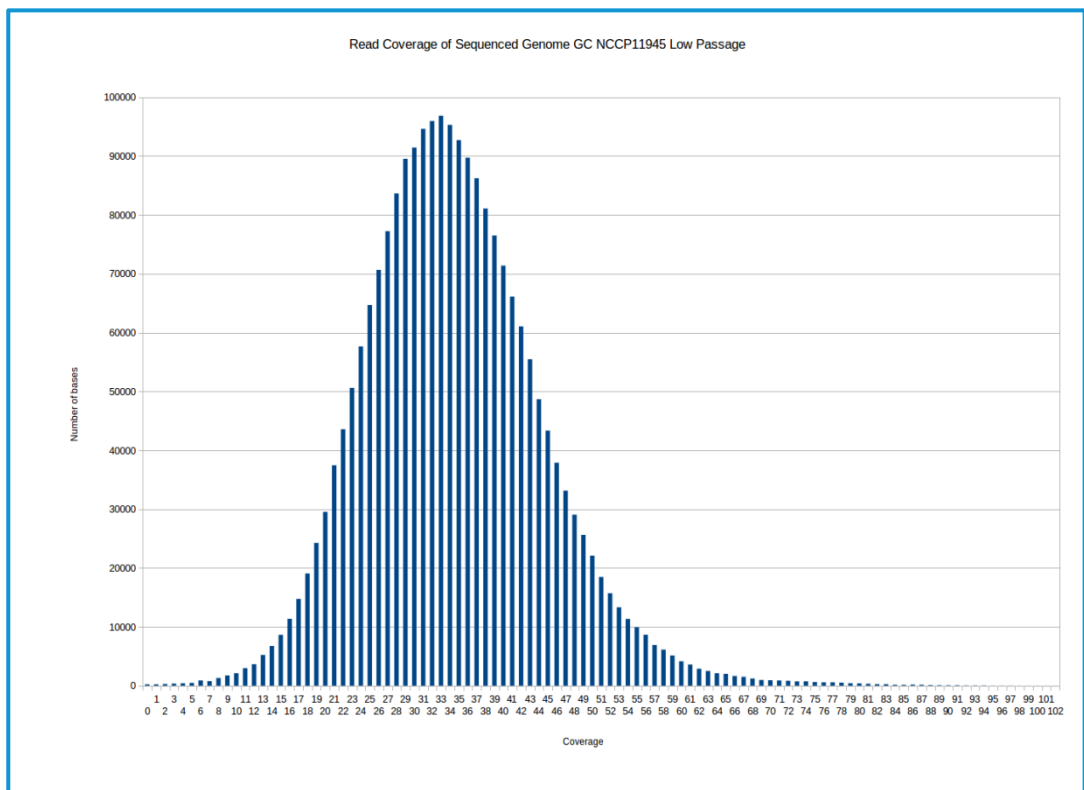
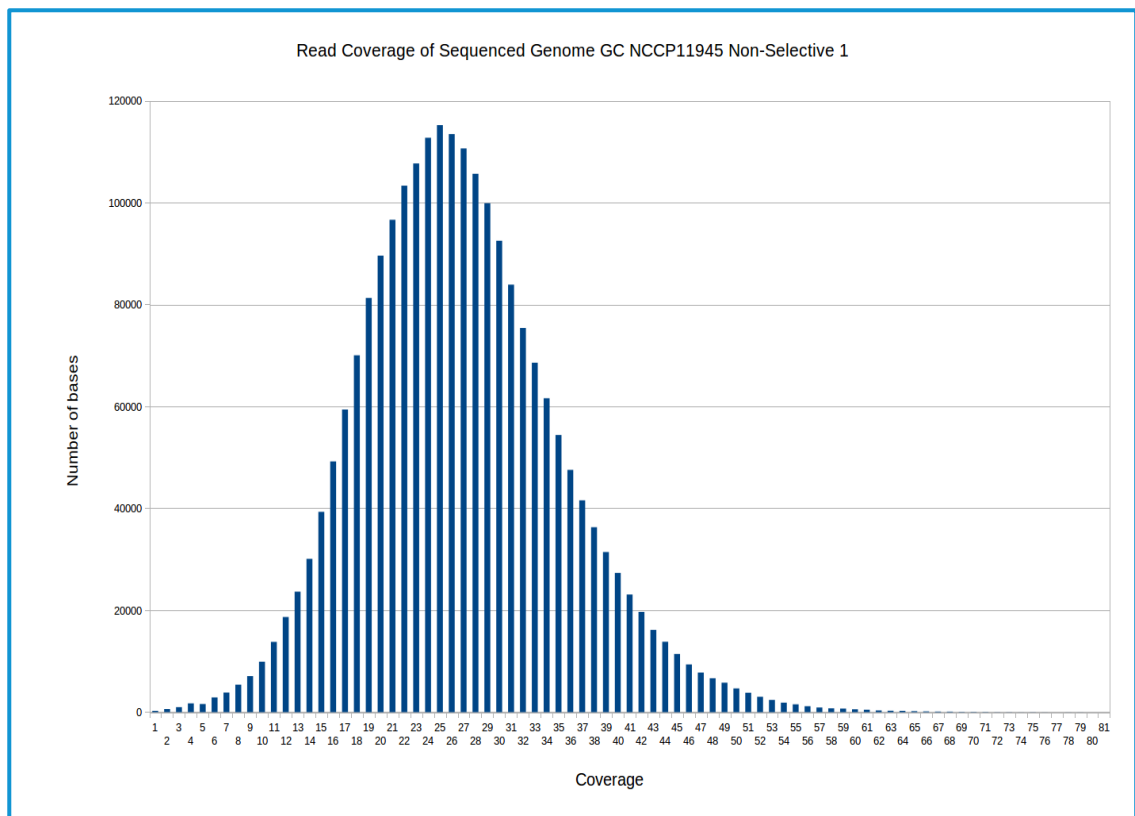
Appendix 5.5. FastQC report of GC NCCP11945 Myristic acid 2 sample from Illumina NGS sequencing. Quality scores on y axis are plotted against base position (x axis). The two panels represent the forward and reverse paired reads.

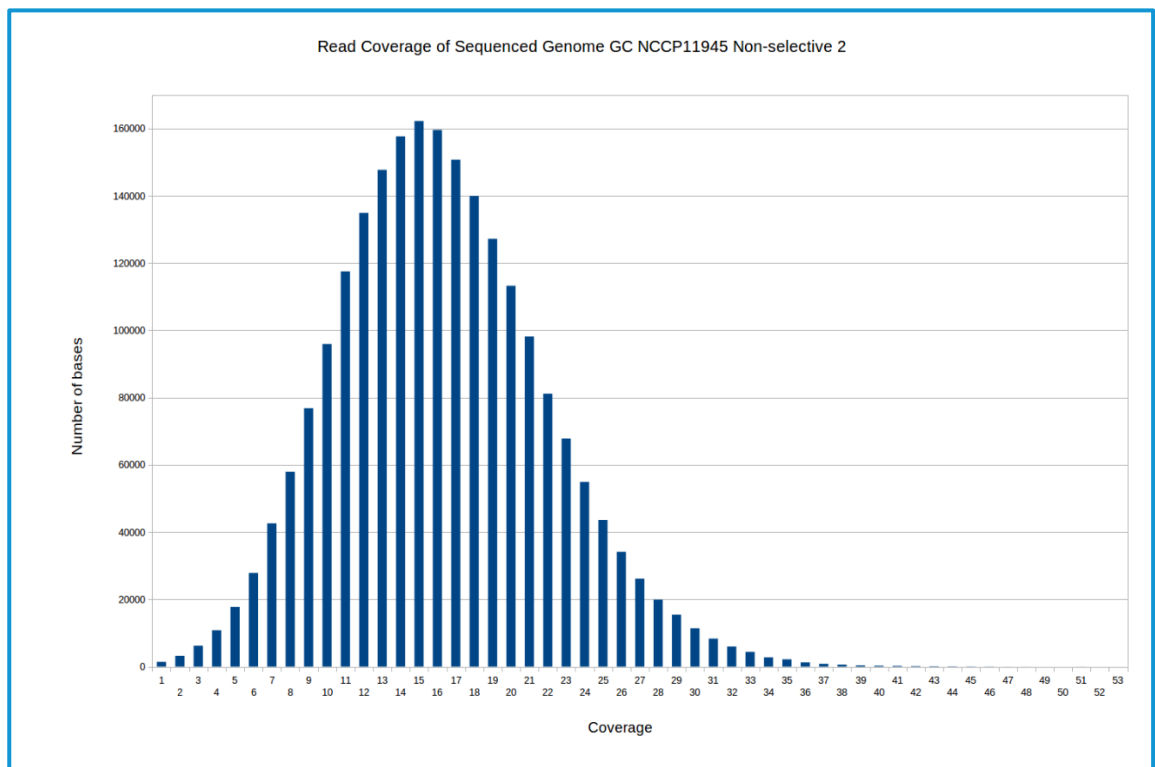


Appendix 5.6. FastQC report of GC NCCP11945 Monocaprin acid 1 sample from Illumina NGS sequencing. Quality scores on y axis are plotted against base position (x axis). The two panels represent the forward and reverse paired reads.

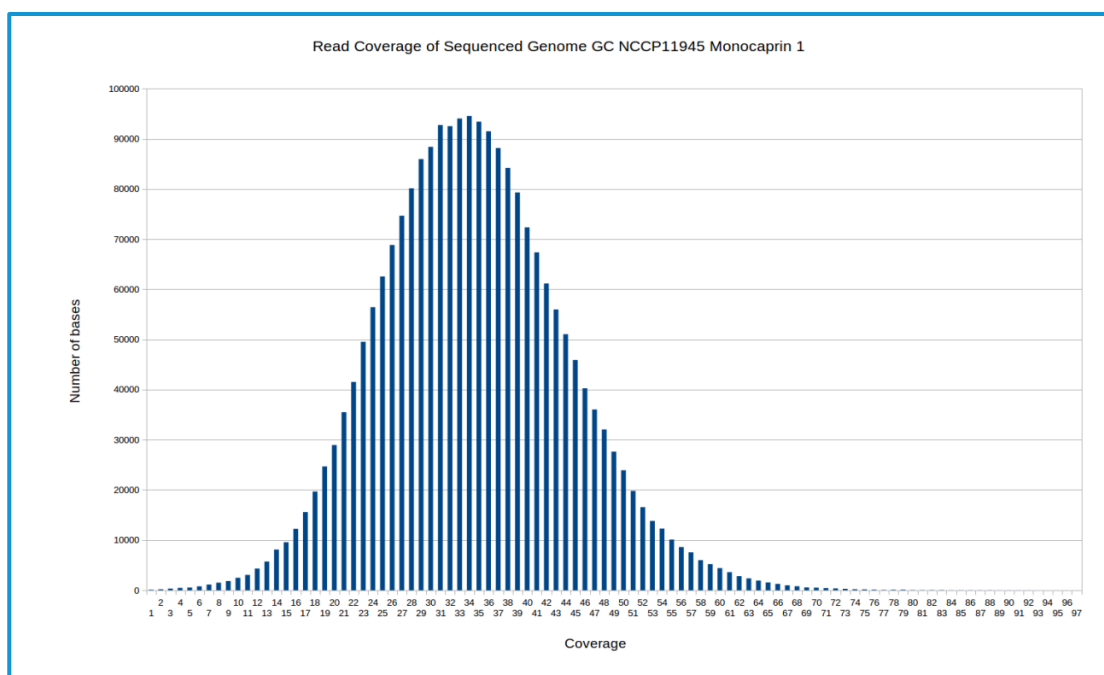


Appendix 5.7. FastQC report of GC NCCP11945 Monocaprin acid 2 sample from Illumina NGS sequencing. Quality scores on y axis are plotted against base position (x axis). The two panels represent the forward and reverse paired reads.

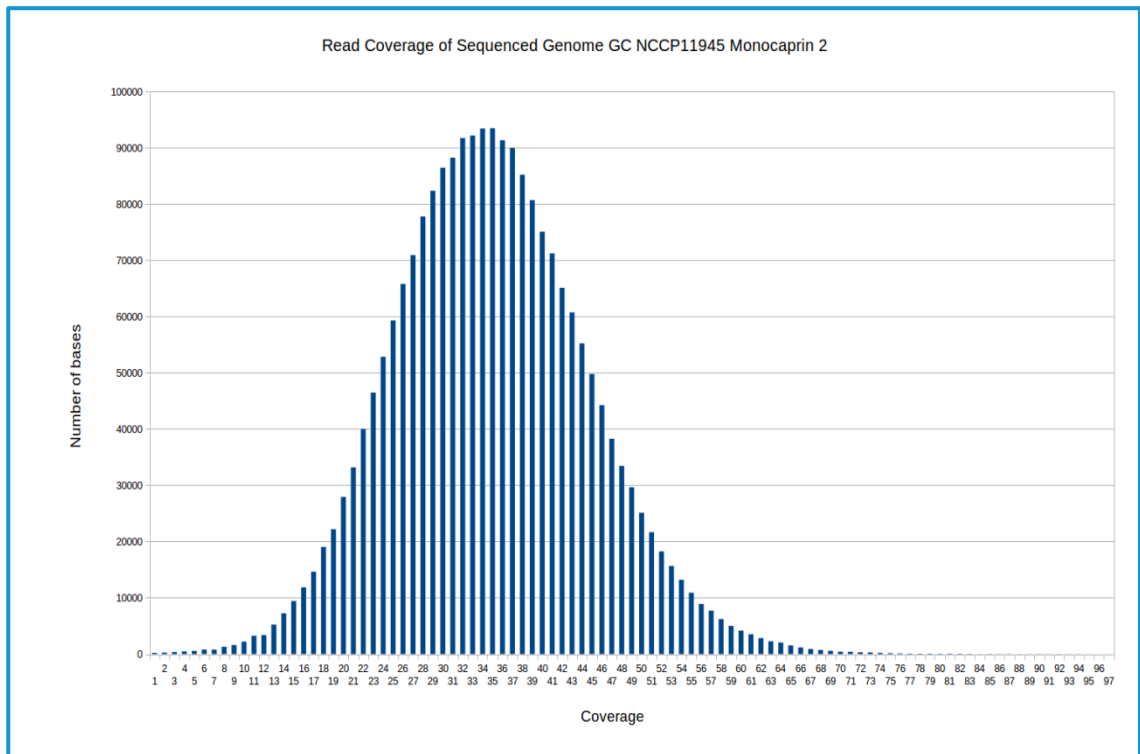
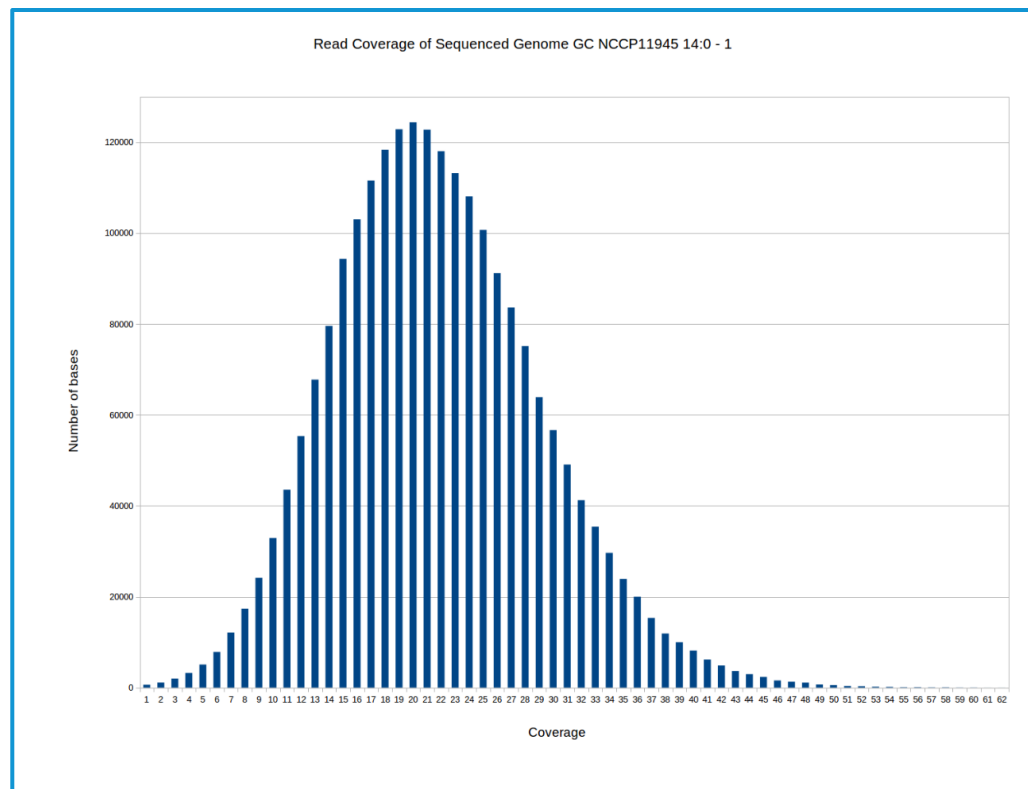
Appendix 5.8. Read coverage of Illumina run on *N. gonorrhoeae* NCCP11945 Low Passage isolateAppendix 5.9. Read coverage of Illumina run on *N. gonorrhoeae* NCCP11945 Non-selective 1 isolate

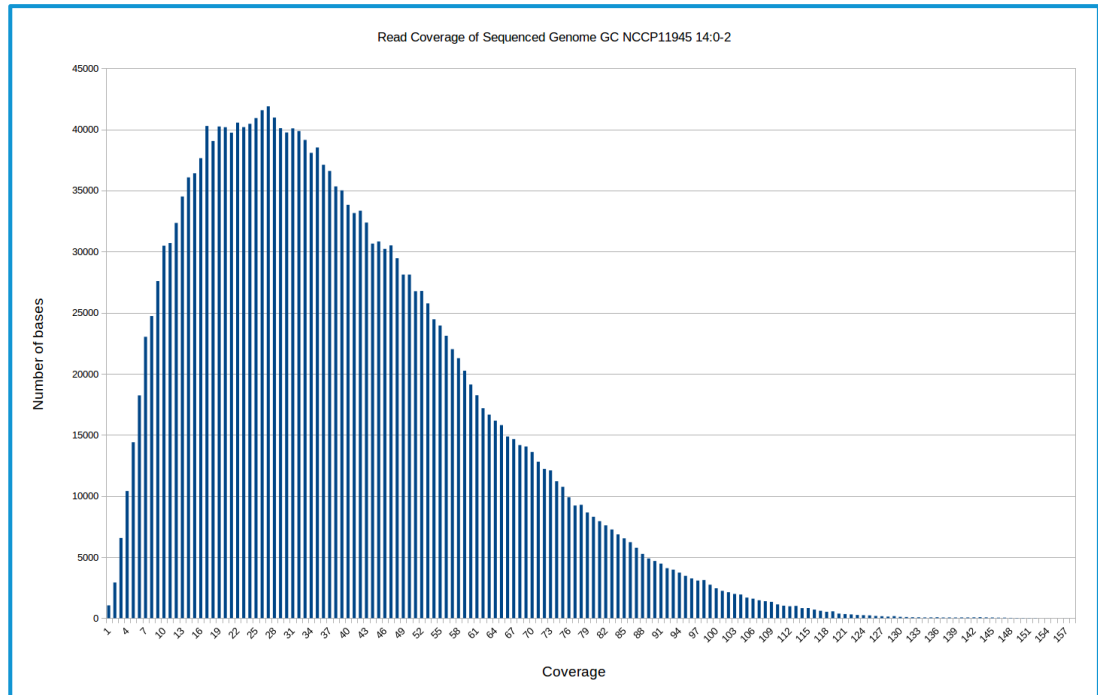


Appendix 5.10. Read coverage of Illumina run on *N. gonorrhoeae* NCCP11945 Non-Selective 2 isolate



Appendix 5.11. Read coverage of Illumina run on *N. gonorrhoeae* NCCP11945 Monocaprin 1 isolate

Appendix 5.12. Read coverage of Illumina run on *N. gonorrhoeae* NCCP11945 Monocaprin 2 isolateAppendix 5.13. Read coverage of Illumina run on *N. gonorrhoeae* NCCP11945 Myristic 1 isolate



Appendix 5.14. Read coverage of Illumina run on *N. gonorrhoeae* NCCP11945 Myristic 2 isolate