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University of Southampton

Faculty of Medicine

Clinical and Experimental Sciences

Controlled Human Infection with *Neisseria lactamica*

by

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**Thesis for the degree of Doctor of Philosophy in
Infection, Inflammation and Immunity**

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University of Southampton

Abstract

Faculty of Medicine
Clinical and Experimental Sciences

For the degree of Doctor of Philosophy

Controlled Human Infection with *Neisseria lactamica*

by

Dr Diane Frances Gbesemete

Meningococcal disease is a globally important cause of mortality and morbidity, particularly in the meningitis belt of Sub-Saharan Africa. The causative agent, *Neisseria meningitidis*, commonly colonises the human nasopharynx asymptotically, but occasionally becomes invasive, resulting in fulminant disease.

Neisseria lactamica is a closely related bacterium which colonises the same niche, but is non-pathogenic. Colonisation with *N. lactamica* may provide protection from colonisation with *N. meningitidis*, and from meningococcal disease. A controlled human infection model has previously been developed, using nasal inoculation to induce nasopharyngeal colonisation with *N. lactamica*, with a subsequent reduction in *N. meningitidis* carriage demonstrated.

This thesis describes four controlled human infection studies designed to optimise and further develop this model. In the first study, the impact of duration of colonisation on immunogenicity was assessed by allowing participants to remain colonised for a short, or longer period after inoculation. The use of an increased inoculum dose, antibiotic clearance of colonisation, and the shedding of bacteria were also investigated.

The second study involved challenge with a genetically modified strain of *N. lactamica* expressing the meningococcal antigen NadA. This study aimed to investigate the immunological impact of the expression of a bespoke, heterologous antigen during asymptomatic colonisation.

Abstract

The final two studies investigated the use of a lyophilised preparation of *N. lactamica*, aiming to make the model feasible for use in a lower resourced setting, beyond a reliable cold-chain. Dose-ranging studies were conducted in the UK, and subsequently in Mali, within the meningitis belt. The impact of lyophilisation on the ability of *N. lactamica* to colonise the nasopharynx, and to induce an immune response, was investigated.

These studies have confirmed that intranasal inoculation with both wild-type and genetically modified strains of *N. lactamica* safely induces long-standing nasopharyngeal colonisation in a high proportion of individuals. This colonisation induces *N. lactamica* specific and cross-reactive *N. meningitidis* specific immune responses. An immune response specific to a heterologous antigen can be induced by expression of that antigen by recombinant *N. lactamica*. Lyophilisation of *N. lactamica* is safe, feasible, and does not reduce colonisation potential or immunogenicity. Use of the model within the meningitis belt is feasible, safe and induces a similar pattern of colonisation and immunogenicity.

Future studies will investigate the impact of *N. lactamica* colonisation on meningococcal carriage and disease in the meningitis belt, and the use of further genetic manipulation to improve cross-reactive immunity to *N. meningitidis*, with the overall aim of developing improved strategies for protection from meningococcal disease.

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- G3 – Effect of colonisation with *Neisseria lactamica* on cross-reactive anti-meningococcal B cell responses: A randomised, controlled, human infection trial

Research Thesis: Declaration of Authorship

Print name: Dr Diane Gbesemete

Title of thesis: Controlled Human Infection with *Neisseria lactamica*

I declare that this thesis and the work presented in it is my own and has been generated by me as the result of my own original research.

I confirm that:

1. This work was done wholly or mainly while in candidature for a research degree at this University;
2. Where any part of this thesis has previously been submitted for a degree or any other qualification at this University or any other institution, this has been clearly stated;
3. Where I have consulted the published work of others, this is always clearly attributed;
4. Where I have quoted from the work of others, the source is always given. With the exception of such quotations, this thesis is entirely my own work;
5. I have acknowledged all main sources of help;
6. Where the thesis is based on work done by myself jointly with others, I have made clear exactly what was done by others and what I have contributed myself;
7. Parts of this work have been published as:

Diane Gbesemete, Jay Robert Laver, Hans de Graaf, Muktar Ibrahim, Andrew Vaughan, Saul Faust, Andrew Gorringer, Robert Charles Read

Protocol for a controlled human infection with genetically modified *Neisseria lactamica* expressing the meningococcal vaccine antigen NadA: a potent new technique for experimental medicine

BMJ Open. 2019; 9(4)

Declaration of Authorship

A recombinant commensal bacteria elicits heterologous antigen-specific immune responses during pharyngeal carriage

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Sci Transl Med. 2021 Jul 7; 13(601)

Effect of colonisation with *Neisseria lactamica* on cross-reactive anti-meningococcal B cell responses: A randomised, controlled, human infection trial

Adam P. Dale, Anastasia A. Theodosiou, **Diane F. Gbesemete**, Jonathan M. Guy, Eleanor F. Jones, Alison R. Hill, Muktar M. Ibrahim, Hans de Graaf, Muhammad Ahmed, Saul N. Faust, Andrew R. Gorringe, Marta E. Polak, Jay R. Laver, Robert C. Read

Lancet Microbe. 2022 Dec 1; 3(12)

Signature:

Date: 19th July 2024

Contributions

Development of the *N. lactamica* (Nlac) controlled human infection model (CHIM), and the potential for protection from meningococcal disease, originated with Professor R. Read and commenced with two previous studies – LACTAMICA 1 and 2.

The concept, research questions and study design for of each of the studies described in this thesis originated with Professor Read and the University of Southampton Controlled Human Infection group (CHIG), of which I am part, with Professor Gorrington (UKHSA) for LACTAMICA 3 (L3) and LACTAMICA 4 (L4), and Professor Heyderman (University College London), Professor Tapia (University of Maryland) and Dr MacLennan (University of Oxford) for LACTAMICA 5 (L5) and LACTAMICA Étape 1 (LE1).

I was responsible for the production of all study documents with input from other members of the CHIG and LE1 study teams as above. I led the ethics approvals application for L3, L4 and L5. Ethics application for LE1 (US and Mali) was led by Prof. Tapia and the CVD Mali team.

The genetically modified (GM) Nlac strains, and lyophilised Nlac, were produced in the University of Southampton laboratories by Dr. Laver. Application for DEFRA approval for use for the GM strains, and QC of lyophilised Nlac (LyoNlac) were led by Dr. Laver.

Public engagement activities around the use of a GM inoculum for L4 were carried out by the BRC public engagement team with Dr. Laver, and for LE1 were carried out by the social and behavioural science team at CVD Mali.

I wrote or co-wrote all clinical standard operating procedures and source documents, and the laboratory standard operating procedures and source documents included in the appendices, for all studies. I developed and optimised the Nlac IgG ELISA as detailed in Chapter 3 of this thesis, with supervision from Dr. Laver. I conducted training sessions for the clinical study staff for each study and was responsible for study set-up both in Southampton and Mali, with the help of interpreters and the CVD Mali team in Mali.

I conducted the majority of screening visits and inoculations in the UK, along with members of the CRF nursing team. I had oversight of all participant admission periods and follow up visits. Clinical samples were collected by members of the Clinical Research Facility (CRF) nursing team. Due to the COVID-19 pandemic, I was unable to travel back

Contributions

to Mali to have on-site input into the conduct of the study. However, I was in almost daily contact with members of the local team during the study, supervising both clinical and laboratory decisions, and led weekly meetings with the study safety committee.

I worked closely with the laboratory team and carried out some of the microbiological processing for L3. I initially processed all serum samples for L5 but these were subsequently repeated by another study team member with samples from LE1 using a single reference serum to allow comparison between the two studies.

I collated and analysed all safety and colonisation data and communicated with the external safety committees. I also analysed all immunological data for L3, L5 and LE1. Immunological data for L4 were initially analysed by Drs Laver and Dale for publication, but subsequently reviewed and re-analysed by me for this thesis. All figures included in this thesis were prepared by me.

I wrote the manuscript for publication of the L4 protocol (Appendix G1), and had input into the publication of L4 results (Appendix G2) and L3 results (Appendix G3). I have drafted a manuscript for the results of L5 and LE1 which is awaiting submission.

Acknowledgements

The set up and delivery of these studies has involved the co-ordinated efforts of a large number of individuals. While it is not possible to name each person, I am very grateful to each one for their hard work and dedication which have made this research programme such a success.

Firstly, to my supervisory team of Professor Robert Read and Professor Saul Faust who have led and supported me throughout this PhD. I am very grateful to Professor Faust for introducing me to research and giving me the opportunity to start my academic career. I have been a part of Professor Read's research group for many years and I am incredibly grateful for the opportunities and support he has given me, and the trust he has put in me to conduct these studies which came out of his many years of experience in the field.

The past and present members of the University of Southampton Controlled Human Infection Group, in particular Jay Laver who has had a pivotal role in each study, particularly in the development of the GM Nlac strains and LyoNlac. Muktar Ibrahim who developed and conducted the microbiological techniques such as the assessment of shedding, some of which were included in his PhD thesis, Adam Dale who led some of the immunological assay development and analysis, Hans de Graaf who co-wrote many of our shared SOPs and study processes, Jon Guy who processed all of the serological samples, Anastasia Theodousiou who developed and conducted the PCR analysis, Rosalind Haig, Eleanor Roche and Carl Webb who processed many of the samples.

Members of UKHSA, particularly Professor Gorringe, for their input into the study design, the pre-clinical testing of the GM Nlac strains, and further immunological sample processing.

The NIHR CRF team members including project managers Filipa Martins, Sara Hughes, lead nurses Tanya Ogrina, Alice Baker, Victoria Buxton, Rachel Miller-Price, Kate Battley, Sarah Horswill and Caroline Grabau. The clinical fellows who supported me including Chris Wilcox, Ben Welham, Alasdair Munro and Dan Owens.

LE1 was conducted by a large team from CVD Mali led by Professor Samba Sow including the study doctors, led by Dr. Fadima Haidara and the laboratory team led by Henry Badji. Other key individuals include Professor Milagritos Tapia and Fleesie Hubbard from the University of Baltimore, Dr. MacLennan from the University of Oxford and

Acknowledgements

Professor Heyderman and the Mucosal Pathogens Research Unit, based at University College London.

The database managers Colin Newell (Southampton) and Uma Onwuchekwa (Mali), The external safety committee for each study, the study sponsors (University Hospital Southampton, University of Southampton and CVD Mali), and the funders – Southampton Biomedical Research Centre, Medical Research Council, Mucosal Pathogens research unit, and support from HIC-Vac and the Meningitis Research Foundation.

To the participants who volunteered their time, and their noses, to each study. Without them all we can do is hypothesise.

And lastly to my family, my husband Dela and my children Maria, Samuel and Anna, who have tolerated me working far too many hours and being away far too much. I am looking forward to spending more time with you now.

Abbreviations

ACRE	Advisory Committee for Release to the Environment
AE	Adverse Event
AESI	Adverse Event of Special Interest
ALP	Alkaline Phosphatase
ALS	Advanced Life Support
ALT	Alanine Aminotransferase
API	Analytic Profile Index
B _{MEM}	Memory B cells
B _{PLAS}	Antigen secreting plasma cells
Bp	<i>Bordetella pertussis</i>
BSA	Bovine serum albumin
CD	Clusters of differentiation
CFU	Colony forming units
CHI	Controlled Human Infection
CHIM	Controlled Human Infection Model
ChV	Challenge volunteer
CI	Confidence Interval
CoV	Contact volunteer
COVID	Coronavirus Disease
CPS	Capsular polysaccharide
CRF	Clinical Research Facility
%CV	Coefficient of variance
CVD-Mali	Centre pour le Développement des Vaccins du Mali
DEFRA	Department for Environment, Food and Rural Affairs
dOMV	Deoxycholate-extracted outer membrane vesicles
ECG	Electrocardiogram
EHPC	Experimental Human Pneumococcal Challenge
ELISA	Enzyme linked immunosorbent Assay
ELISpot	Enzyme linked immunospot
EMA	European Medicines Agency
FCS	Fetal calf serum
fHbp	Factor H binding protein

Abbreviations

FMOS/FAPH	Comite d’Ethique de la Faculte de Medicine et d’odontostomatologie Faculte de Pharmacie, Mali
Fur	Ferric uptake regulator
GCP	Good clinical practice
GM	Genetically modified
GMO	Genetically modified organism
GMP	Good manufacturing practice
HPA	Health Protection Agency
HRA	Health Research Authority
HRP	Horseradish peroxidase
HSE	Health and Safety Executive
Ig	Immunoglobulin
IMP	Investigational medicinal product
Inf HA	Influenza Haemagglutinin
IQR	Interquartile range
IV	Intravenous
KLH	Keyhole limpet haemocyanin
L1	LACTAMICA 1
L2	LACTAMICA 2
L3	LACTAMICA 3
L4	LACTAMICA 4
L5	LACTAMICA 5
LE1	LACTAMICA Etape 1
LMIC	Lower and middle income country
LOS	Lipo-oligosaccharide
LyoNlac	Lyophilised <i>N. lactamica</i>
MALDI-ToF	Matrix-assisted laser desorption/ionisation time of flight
MHRA	Medicines and Healthcare products Regulatory Authority
MLST	Multi-locus sequence typing
MRC	Medical Research Council
NAD	No abnormalities detected
NadA	Neisserial adhesin A
NHBA	Neisseria heparin binding antigen
NHS	National Health Service
NIHR	National Institute for Health and care Research
Nlac	<i>Neisseria lactamica</i>

Nmen	<i>Neisseria meningitidis</i>
NRES	National Research Ethics Service
OD	Optical density
OMV	Outer membrane vesicle
OPD	O-phenylenediamine dihydrochloride
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline + Tween
PCR	Polymerase chain reaction
PHE	Public Health England
PPE	Personal protective equipment
QTc	Corrected QT interval
SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2
SAE	Serious adverse event
SBA	Serum bactericidal activity
SD	Standard deviation
SFU	Spot forming unit
sNadA	Soluble Neisseria adhesin A
SOP	Standard operating procedure
Spn	<i>Streptococcus pneumoniae</i>
SPSS	Stable peroxide substrate solution
ST	Sequence type
TOPS	The over-volunteering prevention system
TT	Tetanus toxoid
UHS	University Hospital Southampton
UK	United Kingdom
UKHSA	United Kingdom Health Security Agency
UMB IRB	University of Maryland, Baltimore Institutional Review Board
UoS	University of Southampton
USA	United States of America
WBC	White blood cell count
WHO	World Health Organisation
X-gal	5-bromo-4-chloro-3-indolyl β -D-galactopyranoside
4PL	4-Parameter logistic

Chapter 1 Introduction

1.1 *Neisseria meningitidis* and meningococcal disease

1.1.1 Meningococcal disease

Meningococcal disease is an important cause of mortality and morbidity globally. It is characterised by an acute onset and rapid progression, typically in previously healthy children and young people, frequently resulting in death or devastating sequelae (1). Cases occur sporadically, in outbreaks and in larger epidemics with the potential to overwhelm health systems. While effective vaccines have substantially reduced the burden of disease in many populations, meningococcal disease remains a globally important health concern (2). In 2021 the World Health Organisation (WHO) published a roadmap aiming to defeat meningococcal disease by 2030, recognising the ongoing need for effective strategies for prevention and control of disease (3).

1.1.2 Clinical features

The initial symptoms of meningococcal disease are often non-specific and difficult to distinguish from of a viral upper respiratory tract infection but can progress to invasive disease with multi-organ failure and death over a matter of hours. Invasive meningococcal disease usually presents predominantly as either meningitis or septicaemia but features of both may be present (1).

Meningitis is a common clinical presentation, seen in approximately 60% of cases in industrialised countries and a higher proportion of cases in low resource settings (4). The classical symptoms of headache, photophobia, neck stiffness, vomiting, altered conscious level and seizures occur as a result of meningeal irritation and raised intracranial pressure. These features may be less apparent in young children who instead present with poor feeding, a bulging fontanelle, and a characteristic change in cry. More generalised features of infection such as a fever are usually present. Long term sequelae of meningitis include hearing and visual impairment, seizures, motor impairment and behavioural difficulties (1).

The most dangerous consequence of meningococcal bacteraemia is severe sepsis, which presents as a febrile illness, classically with a rash. At initial presentation there may be no rash present, or a non-specific blanching rash easily confused with a viral exanthem. The characteristic petechial and then purpuric rash usually becomes apparent as the disease progresses. Shock may develop with signs of peripheral hypoperfusion (cold peripheries, pallor, prolonged capillary refill, and metabolic acidosis) and initial compensation

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(tachycardia, tachypnoea, reduced urine output) progressing to decompensated shock with hypotension and end organ failure (decreased myocardial function, altered conscious level) and a severe coagulopathy (disseminated intravascular coagulation) (1, 4).

Progressive coagulopathy, intravascular thrombosis and hypoperfusion lead to necrotic damage to the skin and underlying structures resulting in purpura fulminans and tissue destruction which may necessitate amputation of affected extremities (1, 5). Long term sequelae of meningococcal sepsis include the effects of tissue destruction such as amputation, scarring, growth plate damage and chronic pain (1).

Occasionally, meningococcal disease follows a less fulminant course as either a more slowly progressive febrile illness, a transient self-resolving bacteraemia or a chronic intermittent febrile illness. Rarely it can cause an isolated localised infection such as a pneumonia, arthritis, pericarditis or myocarditis (1, 4).

The clinical management of invasive meningococcal disease has been well documented in established guidelines (6) and centres around early recognition, early and effective antibiotic treatment to halt the rapid proliferation of meningococci, and good supportive care including fluid resuscitation to limit the pathophysiological consequences of infection such as cerebral oedema, raised intracranial pressure, hypoperfusion and end organ damage.

The case fatality rate is approximately 8-14% worldwide with significant long term sequelae in 11-19% of survivors (1, 7).

1.1.3 Microbiological features

The causative agent of meningococcal disease is *Neisseria meningitidis* (Nmen) or meningococcus. This Gram negative β -proteobacterium is part of the bacterial family *Neisseriaceae* (8, 9). It is an aerobic diplococcus with fastidious growth requirements, growing best on supplemented media (e.g. blood agar) at 35-37°C in 5-10% CO₂. Colonies appear smooth, round and grey-white on blood agar, are oxidase positive and metabolise glucose and often maltose (9). Nmen is adapted to colonise the human oropharynx. This carrier state is its only environmental habitat, with no known animal reservoir. Within the human host Nmen can adhere to host cell surfaces or survive within the host cells (10, 11). Transmission between human hosts sustains the reservoir of carriage which underlies both endemic and epidemic meningococcal disease.

The outer membrane of the Nmen cell envelope is associated with a layer of lipooligosaccharide and is often surrounded by a polysaccharide capsule. Pili extend from the

outer cell membrane to well beyond the capsule. Outer membrane proteins involved in adherence and metabolic functions traverse, or are embedded in the outer membrane (4, 9). Many of these structural features contribute to meningococcal virulence.

1.1.4 Virulence factors

A variety of virulence factors allow Nmen to evade host defences and cause invasive disease. The relative virulence of different strains is a consequence of the variable expression of those factors.

1.1.4.1 Polysaccharide capsule

Nmen is often, although not always, an encapsulated bacterium. There are multiple structural types, which are the basis for serogrouping and traditional vaccines (4, 8, 9). The expression of a capsule is a key determinant of invasion as it confers resistance to host defences, allowing bacterial cells to survive in the hostile environment within tissues and blood. Invasive disease is almost exclusively caused by encapsulated meningococci expressing one of six capsular types – A, B, C, W-135, X and Y (4).

Capsules protect the bacterium from host defences such as opsonisation, phagocytosis and complement-mediated killing (4, 8). In the past they were considered to protect the bacteria from desiccation when outside of the human host and therefore have a role in transmission, although more recent studies have shown that non-capsulate meningococci are no more liable to desiccation than encapsulated meningococci (12, 13). Acapsulate strains are often identified in carriage studies, therefore either they are not necessary for transmission and colonisation, or expression may be switched off after successful colonisation. It has been suggested that the presence of a capsule may hinder prolonged colonisation, as it blocks the outer membrane proteins involved in adhesion, so down regulation of the expression of capsule may then confer an advantage (14).

Nmen is thought to have evolved from a single common non-capsulate ancestor with other *Neisseria* species and acquired the ability to express a capsule, and thus become virulent, as a result of horizontal transfer from another unrelated species (15). Molecular typing of Nmen has shown evidence of capsule switching, where strains with the same sequence type express different capsular groups as a result of horizontal gene transfer between strains (16). As the capsule is highly immunogenic and targeted by traditional vaccines, capsule switching is a mechanism of escape from naturally acquired or vaccine induced immunity. The acquisition and expression of a new capsular polysaccharide by a virulent strain has been identified as the cause of outbreaks (17-19). The use of mass

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vaccination with capsule based vaccines has raised the concern of immunological pressure resulting in a vaccine escape strain with the potential for epidemic spread (9).

1.1.4.2 Bacterial endotoxin

The lipo-oligosaccharide (LOS) surrounding the bacterial cell membrane is a potent endotoxin with an important role in adherence, colonisation, and the induction of an inflammatory response. It is composed of biologically active lipid A and a core oligosaccharide. It has structural similarity to human antigens, using molecular mimicry to evade innate immunity (8).

1.1.4.3 Outer membrane proteins

Pili are complex proteins which are anchored into the outer membrane and extend out beyond the capsule (4, 20). They allow bacterial movement by means of a twitching motility (8, 21). There are two major classes of meningococcal pili but the more important is class 1, also known as Type IV pili. These pili bind to CD46 – a complement regulatory glycoprotein expressed on the outer surface of host cells (8, 21).

Another important class of outer membrane proteins are the Porins; PorA and PorB. These are involved in bacterial interaction with, and adhesion to, host cells. PorB inserts into the host cell membrane, causing an influx of calcium which in turn results in host cell apoptosis via Toll-like receptor 2 (8).

A range of other outer membrane proteins act as adhesins. Important examples include the opacity associated proteins (Opa, Opc), factor H binding protein (fHbp) and Neisserial adhesin A (NadA) (8).

Outer membrane proteins and LOS are released from the cell surface in blebs known as outer membrane vesicles (OMVs) (22). These OMVs are involved in cell-cell interaction and communication. Outer membrane proteins and surface antigens are highly immunogenic and some have been used as subcapsular targets in vaccine development (23).

Meningococci exhibit phase variation of some outer membrane proteins where expression of the surface antigen is up or down-regulated in response to the environment or cell-cell interactions (24). This is an important mechanism of host immune evasion (8, 24).

1.1.4.4 Iron acquisition systems

Iron is essential for the survival and replication of Nmen. Meningococci express lactoferrin and transferrin binding proteins to scavenge iron from host stores and internalise it. The expression of these bacterial surface proteins is under the control of the ferric uptake regulator (Fur) protein – an iron responsive repressor gene (8, 24, 25).

1.1.5 Genetic features

The Nmen genome is approximately 2.0-2.1 megabases and contains approximately 2000 genes. The entire genome has been sequenced for several specific strains and loci encoding the capsule and other virulence factors have been identified (15, 26-28).

The genome is subject to frequent spontaneous chromosomal mutations as well as recombination and transformation from other commensal and pathogenic species. This genomic plasticity results in significant phenotypic and antigenic diversity and the potential for antigenic shift in response to selective immunological pressure (29, 30).

1.1.6 Classification

1.1.6.1 Phenotypical typing

Classification of Nmen strains has traditionally been made phenotypically with the major division being into serogroups and further subdivision into serotype and immunotype. The classification of serogroup is made on the basis of capsular polysaccharide structure with 13 serogroups described (4, 8, 20).

Serotyping is defined by the type of major (class 1) outer membrane protein and sero-subtyping by the type of class 2 and 3 outer membrane protein. The type of LOS defines the immunotype (8, 21, 31).

The use of such phenotyping, particularly at a serogroup level, has provided information about the global epidemiology of different strains and therefore informed the use of vaccines in different epidemiological settings. However, the propensity of Nmen for genetic recombination, particularly of antigenic determinants including capsule type, means that this classification technique is potentially inadequate for tracking transmission during an outbreak (21).

1.1.6.2 Molecular typing

Molecular techniques have been developed to more accurately classify isolates of Nmen including multi-locus sequence typing (MLST) and whole genome sequencing.

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Multi-locus sequence typing is the most commonly used molecular technique in which fragments of 7 “house-keeping” genes are compared. The specific allele at each locus is identified and the combination of all seven loci is summarised as a sequence type (ST). Closely related sequence types are then grouped into clonal complexes (31, 32). Multiple sequence types have been identified and specific clonal complexes are characteristically associated with invasive disease (8, 31). MLST has been used to demonstrate significant antigenic variation including capsule type within an epidemic strain. It allows the tracking of an epidemic strain even when capsular switching has occurred, unlike classification by serogroup (31).

1.1.7 Transmission and acquisition

Humans are the only natural host of Nmen, which is almost exclusively found in the nasopharynx and is widely regarded as a commensal organism. Acquisition of meningococcus is via airborne or directly transferred droplets of saliva or respiratory secretions (4, 24, 33) and the main reservoir of onward transmission is the asymptomatic carrier. The highest risk of transmission is between household contacts and kissing contacts (34, 35) and usually occurs within the first few weeks after acquisition (36).

Studies have shown some environmental survival of Nmen for up to 8 days, on glass and metal surfaces kept at optimal temperature and humidity conditions. This suggests that fomite transmission e.g. via unwashed drinking vessels may be another possible source of transmission (12, 13). Meningococci may rarely be transmitted sexually (4).

When Nmen enters the upper respiratory tract of a new human host, it can either be present transiently and rapidly cleared, it can adhere and result in a more prolonged period of colonisation or it can invade and result in invasive disease (4).

1.1.8 Colonisation

In order to colonise the human nasopharynx, meningococci must penetrate the host mucus barrier and adhere to the host nasopharynx (4). Traversing the mucus barrier is facilitated by the twitching motility action of pili. Pili are also essential for interaction with, and adhesion to cells of the host nasopharynx. Nmen preferentially adheres to non-ciliated columnar epithelial cells or damaged epithelium (10, 24). Bacterial outer membrane proteins such as the porins and adhesins are also involved in the process of adhesion (8).

Once adhered, meningococci multiply to form micro-colonies on the host cell surface and can also invade and survive within host cells. Bacterial-host cell interaction triggers the

production of pseudopodia by the host cell, resulting in internalisation by endocytosis. Intracellular meningococci survive by resisting host defences such as phagosome maturation and lysosomal degradation and are capable of intracellular replication (4, 8, 21, 37).

Colonising meningococci almost always remain as commensals in the nasopharynx, with episodes of asymptomatic carriage lasting for several months prior to spontaneous clearance (38). Individuals will have multiple episodes of Nmen carriage over their lifetime (33).

Asymptomatic meningococcal colonisation is an immunising event, inducing both systemic and mucosal specific humoral responses (39, 40). These responses are discussed further in section 1.1.12.

Oral Ciprofloxacin has been shown to be effective in eradicating carriage of Nmen within 24 hours (41) and is used as prophylaxis in close contacts of meningococcal disease cases (42).

1.1.9 Invasion

Occasionally, acquisition of Nmen is followed by rapid invasion and proliferation resulting in invasive meningococcal disease. The development of disease in this minority of cases is not fully understood but is influenced by pathogen, host and environmental factors. It can be considered an `accidental` consequence of the carrier state, as no biological advantage is conferred to the organism that invades. Specific hypervirulent strains of Nmen are known to be associated with most cases of invasive disease (31, 38). Host and environmental factors are discussed further in sections 1.1.11 and 1.1.12 below.

Invasion occurs when intracellular meningococci traverse the host cell cytoplasm (transcytosis), or adhered meningococci directly invade through epithelial layers that have been damaged by environmental factors e.g. dust, smoke, or low humidity (4, 37). From the submucosa, meningococci can enter the bloodstream where they will usually be cleared by host humoral and phagocytic defences. Occasionally they can evade these defences and multiply rapidly leading to invasive disease. Invasive disease usually occurs within 1-14 days following the acquisition of carriage (21).

1.1.10 Pathophysiology

In meningococcal sepsis, the rapid multiplication of meningococci in the bloodstream results in the release of large amounts of LOS (endotoxin). This is present on the surface

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of bacteria and in their associated outer membrane vesicles and can reach high concentrations in the blood. A high bacterial genomic load in blood correlates with severity of disease (43). The lipid A portion of LOS forms complexes with lipopolysaccharide binding protein, which in turn binds to a receptor complex on the surface of host immune cells. This interaction results in a transmembrane signal via TLR-4, which initiates a pathway of intracellular signals leading to the release of inflammatory mediators such as IL-1, IL-5, IFN γ and TNF α by the host cell (8, 25). These cytokines activate multiple pathways including the coagulation cascade and the complement cascade, leading to an increase in capillary permeability and disordered coagulation. Excessive activation of the coagulation system and reduced fibrinolysis lead to intravascular thrombosis in the skin and organs, and fluid shifts due to capillary leak lead to shock and tissue hypoperfusion. These changes appear clinically as the classical cutaneous lesions progressing to purpura fulminans, and multi-organ dysfunction and ultimately death (4, 8, 25).

When meningococci cross the blood brain barrier, by a mechanism that is not fully understood, they bind to endothelial cells of the cerebral microvasculature and replicate. High levels of endotoxin are found in the CSF, which trigger an inflammatory process in the meninges and subarachnoid space. This inflammation increases the permeability of the blood brain barrier resulting in cerebral oedema and raised intracranial pressure. The resulting reduction in cerebral blood flow results in cortical hypoxia.

Meningeal inflammation, cortical hypoxia and raised intracranial pressure are evident clinically with increasing levels of neurological abnormality. Headaches, photophobia and neck stiffness progress to reduced conscious level, focal neurological deficits and seizures and ultimately cerebral herniation and death (8, 25).

1.1.11 Host factors in disease

Non-bacterial factors can have an impact on the risk of disease, either by increased opportunity for transmission, increased rate of colonisation from a given exposure or by increasing the risk of colonisation leading to invasive disease.

Social and behavioural factors can increase the opportunity for onward transmission from an asymptomatic host. Close social interaction such as attending crowded social environments, dormitory sharing or intimate kissing can increase transmission (44). Increased rates of carriage in students (45, 46), military camps (38, 47) and mass gatherings such as the Hajj pilgrimage (19, 48) are well documented.

Any damage to the integrity of the nasopharyngeal host defences such as the mucus layer and epithelium, can increase the chance of carriage acquisition and of invasion through the epithelial layer. Environmental factors such as decreased humidity, dust and smoke inhalation, as well as concurrent viral infections, can cause such damage (49). In addition, an increase in the temperature of the air within the nasopharynx can alter the expression of genes, resulting in a more virulent phenotype (24).

Specific deficits in host immunity can increase the risk of invasive disease, such as deficiencies in the terminal complement pathway (50), properdin deficiency and asplenia (20).

1.1.12 Immunity

The presence of specific serum bactericidal antibodies (SBA) has been shown to correlate with the risk of invasive disease and the induction of SBA is widely accepted as a correlate of protection in vaccine efficacy studies (23, 51-53).

Studies carried out in the 1960s by Goldschneider et al. showed an age-related pattern in both the incidence of disease and the presence of SBA with an inverse correlation between the two. Antenatally acquired SBAs are present during the first month of life when invasive meningococcal disease is rare. These maternal antibodies are then lost in a physiological period of hypogammaglobulinaemia prior to the development of the infant's own immunity. The trough in SBA occurs between 6 months and 2 years of age, coinciding with a peak in invasive meningococcal disease. There is then a gradual increase in SBA inversely correlating with a gradual decrease in disease incidence (51). A study of military personnel during an outbreak of meningococcal meningitis showed that individuals who went on to have invasive meningococcal disease were significantly less likely to have had Nmen specific SBA at baseline (5.6%) in comparison to matched controls (82.2%) (51).

The majority of the population do not develop invasive disease and yet do develop protective immunity in the form of humoral immunity (SBA) and mucosal immunity, which must therefore be a result of asymptomatic carriage (52). This has been demonstrated by observing a rise in SBA in individuals in response to episodes of asymptomatic carriage (39). This humoral response is mainly strain specific but some cross reactivity has been seen between strains (38, 54). Mucosal immunogenicity has also been demonstrated with a rise in salivary specific IgA in response to colonisation (38, 40). Such local mucosal responses are thought to prevent adhesion to the epithelial surface while allowing the organism to remain in the mucus layer (24).

Individuals will have multiple discrete episodes of meningococcal carriage over their lifetime, including repeated carriage of the same serogroup, each lasting up to several months before spontaneous clearance. This suggests that the development of protective immunity from an episode of carriage does not necessarily terminate carriage (24) and may be short lived or only be protective against invasive disease and not colonisation (33). These repeated periods of carriage appear to induce, develop and maintain natural immunity to invasive meningococcal disease over the course of a lifetime (39, 55).

1.1.13 Vaccines

Attempts at developing meningococcal vaccines began in the first half of the 20th century with the use of killed whole bacteria. These early vaccines were found to be highly reactogenic and due to the availability of antibiotic treatment, further vaccine development was abandoned for some time (55). With the advent of antibiotic resistance and following the increased understanding of meningococcal immunology in the 1960s, the first serogroup specific polysaccharide vaccine directed at the serogroup A capsule was developed in 1970 and was shown to be effective in field trials (55, 56). Further plain capsular polysaccharide vaccines were successfully developed for serogroups A, W, and Y (55). Plain polysaccharide vaccines were found to be safe and immunogenic in adults and children over 2 years, but not effectively immunogenic in infants and children less than two years. There was a reduction in the induced SBA over time and poor induction of immunological memory (55). Polysaccharide vaccines have not been shown to be effective at reducing meningococcal carriage which is required for herd immunity (57).

The use of a highly immunogenic protein conjugated to the polysaccharide capsule has been found to induce a stronger antibody response in all age groups, effective in protection against both disease and colonisation (58, 59). There is some waning of immunity following vaccination in early childhood, with a reduction in SBA after one year. Vaccination in later childhood or adolescence results in a longer duration of protection (60, 61). Such glycoconjugate vaccines were developed in the 1990s and have become a highly effective part of routine immunisation schedules (55). These have had dramatic effects on disease incidence, probably mostly due to herd protection conferred by vaccine-induced modification of colonisation reducing inter-host transmission (62, 63).

Glycoconjugate vaccines are currently available for serogroups A, C, W, and Y. A pentavalent glycoconjugate ACWYX vaccine has recently been developed (64).

A concern regarding serogroup specific vaccines is the potential for immune pressure to induce capsular switching resulting in a virulent vaccine escape strain with epidemic potential (9, 16).

The development of a vaccine directed against Nmen serogroup B has been more complicated. The serogroup B capsule is poorly immunogenic (55, 65) and exhibits molecular mimicry. The Nmen B capsule contains an α 1,8-linked polysaccharide acid which is structurally identical to a neural cell adhesion molecule (55, 66). This has therefore led to concern that a capsular vaccine may induce autoimmune antibodies with the potential to cross react with foetal brain tissue (55). The development of a meningitis B vaccine has therefore focussed on the antigenicity of subcapsular structures such as outer membrane proteins and lipo-oligosaccharide.

Outer membrane vesicles, shed from the surface of meningococci, contain surface proteins and LOS. Vaccines have been developed based on these OMVs, which have been highly immunogenic, but also very strain specific. The outer membrane proteins are very variable between strains and therefore many different subserotypes would need to be included in a vaccine to provide sufficiently broad protection (23, 55).

More recently outer membrane protein-based vaccines have been developed using reverse vaccinology – bioinformatic analysis of the meningococcal genome to identify sufficiently immunogenic proteins with limited variability across strains (55). Two such vaccines have recently been developed.

4CMenB (Bexsero) is licensed for use in several countries and is part of the routine immunisation schedule in the UK at 2, 4 and 12 months (23, 67). This vaccine targets 3 immunogenic Nmen serogroup B surface proteins – Neisseria heparin binding antigen (NHBA), Neisserial adhesin A (NadA) and Factor H binding protein (fHbp) as well as an OMV based vaccine developed for use in an outbreak in New Zealand (23). It induces bactericidal antibodies against a range of strains including serogroup B, and protects vaccinated infants against disease (68). However, unlike the glycoconjugate vaccines, 4CMenB does not have a significant impact on carriage and so will not induce herd immunity (58, 69, 70).

MenB-fHbp (Trumenba) is a bivalent factor H binding protein (fHbp) vaccine containing an antigen from each of the two fHbp subfamilies. fHbp is expressed by virtually all pathogenic strains of serogroup B. It is licensed in the United States for use in 10 to 25 year olds but is not available for use in younger children who are at the highest risk of

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invasive disease, due to a high incidence of vaccine related fever (23). Assessment of the impact of MenB-fHbp on carriage is ongoing (71).

1.1.14 Epidemiology

Meningococcal carriage and disease occur throughout the world but the epidemiology varies dramatically across different geographical locations and over time, with different serogroups and sequence types predominating in different populations (72).

1.1.14.1 Carriage

In Europe, approximately 10% of the overall population are asymptomatic carriers of Nmen but carriage rates vary significantly with age. The lowest rate is seen in infants (4.5%) increasing to a peak in adolescents/young adults (24%) and then dropping throughout adulthood to less than 10% (73, 74). Certain sub-populations can have much higher rates of carriage, such as students (45, 46) and military recruits (38, 47) due to the increased transmission risk in close communities, and with close social behaviour.

1.1.14.2 Disease

The incidence of disease also varies with age although the pattern of this association is quite different to that of colonisation, with the highest risk of disease seen in infants and young children. Invasive disease is uncommon in the first month of life then rises to a peak between 6 months and 2 years of age. Following this there is a progressive decline in the incidence of disease throughout childhood with a second smaller peak in disease in late adolescence coinciding with the peak in carriage. The incidence of disease then steadily declines through adulthood (33, 51). The risk of disease developing in a colonised individual is therefore substantially higher in younger children and decreases with age, presumably with the development of immunity (33).

Meningococcal disease can occur as sporadic cases, small outbreaks or larger epidemics. Cyclical fluctuations of disease are seen in some regions (72). The meningitis belt of sub-Saharan Africa has a particularly high incidence and unique epidemiology and is discussed in the next section.

Different serogroups are seen to cause disease in different regions of the world.

Serogroups B and C have classically caused the majority of disease in Europe, Australasia and the Americas and serogroup A and C in Asia and Sub-saharan Africa. The serogroup distribution is changing in response to vaccination with a decrease in the incidence of serogroups A, B and C and the emergence of serogroups Y and W-135 and X (72, 75).

1.1.15 The meningitis belt

1.1.15.1 Definition and geography

The meningitis belt is a region of Sub-saharan Africa with a high incidence of meningococcal disease. It has an estimated population of 500 million people and stretches from Senegal in the west to Ethiopia in the east (76). It was originally described by Lapeyssonie in 1963 with boundaries defined on the basis of annual rainfall between 300 and 1100 mm. Rainfall within these limits was deemed high enough to allow sufficient population density for transmission and low enough to be below a critical level of humidity (77, 78). The defined area has been extended in 1971, 1992 and 1996 to cover areas observed to have regular epidemics, and on the basis of improved diagnostics and changing climate (49, 78-80). Ongoing climate change has the potential to further alter the geographical definition of the region (78).

1.1.15.2 Epidemiology

The increase in meningococcal meningitis incidence occurs annually with peaks coinciding with the middle of the dry season and abating with the onset of the rainy season (78, 80). This seasonal hyperendemicity is thought to result from dust or low humidity causing damage to the nasopharyngeal mucosa or inhibiting the host mucosal immune responses leading to an increased rate of bloodstream or CNS invasion. Weekly incidence rates increase by a factor of 10-100 in comparison to the baseline rate outside of this season (78, 80).

Sporadic localised epidemics or outbreaks also occur intermittently and more widespread epidemic waves, affecting larger regions over more than one season, occur at irregular periods of approximately every 5-12 years (78, 81). These epidemics are thought to be a result of increased transmission and colonisation at a population level due to changing population immunity as a result of genomic change of the pathogen (78, 81).

1.1.15.3 Typing

The main pathogen responsible for outbreaks and epidemics in the meningitis belt has historically been Nmen serogroup A. With the introduction of a serogroup A vaccine, the disease incidence due to serogroup A has declined but other serogroups of Nmen such as C and X, and other pathogens such as *Streptococcus pneumoniae* have also been identified in localised outbreaks and occasional larger epidemics (75, 78).

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1.1.15.4 Risk factors

The main risk factors for meningococcal disease in the meningitis belt are climate related with an increased incidence at times of lower humidity and rainfall. Dust and carbon monoxide emissions have also been shown to have an association with increased epidemic potential (78, 82). In addition increased population density, viral infections and smoking have been suggested as potential factors (78).

1.1.15.5 Control strategies

Strategies for the control of meningococcal disease include preventative interventions such as mass and routine vaccination, rigorous and co-ordinated disease surveillance, and reactive vaccination in response to outbreaks or epidemics.

An affordable serogroup A glycoconjugate vaccine, MenAfriVac, was developed specifically for the meningitis belt and approved for use in 2010 (83). The strategy for rollout was mass vaccination of 1 to 29 year-olds, followed by introduction of the vaccine into routine infant immunisation schedules, with catch up campaigns where necessary. Between December 2010 and December 2019, mass vaccination campaigns had taken place in 24 of the 26 meningitis belt countries, with coverage of the entire country in approximately half, and targeted high-risk areas for the other half. It has since been introduced into routine immunisation schedules in 14 meningitis belt countries (84). This has had a profound impact on the incidence of serogroup A disease with near elimination of disease and carriage among vaccinated populations (85). Limitations to this strategy are the need for ongoing high vaccine coverage to avoid the resurgence of disease in unvaccinated populations or age groups, and the potential for serogroup replacement, with outbreaks of non-A serogroup disease occurring (75, 86).

Two different models of surveillance are used in the meningitis belt. Enhanced surveillance involves the weekly collection of case and laboratory data at a district population level. This allows for the calculation of weekly incidence rates and the early detection of outbreaks, with pre-defined alert and epidemic thresholds. If the incidence crosses the alert threshold, then surveillance is increased with investigation of the causative organism and serogroup, and preparation is commenced for a potential vaccination campaign. If the epidemic threshold is crossed, reactive vaccination of the population is triggered. All countries within the meningitis belt participate in enhanced surveillance (76).

Case based surveillance uses individual case level data to monitor the impact of vaccination on disease incidence and serogroup epidemiology. Case based surveillance

is used in addition to enhanced surveillance in districts or regions with sufficient resources and vaccine uptake (76).

These surveillance strategies are limited by the ability and choice of individuals to access health care providers when unwell, and the resources available to accurately diagnose meningococcal disease with identification the causative organism and serogroup.

Serogroup specific mass vaccination campaigns should occur within four weeks of a district crossing the epidemic threshold (76). However, there are significant challenges to achieving this aim. During a recent epidemic with serogroup C in Nigeria there were over 12,000 cases and 887 recorded deaths. A review of the control strategies highlighted delays in detection, serogroup identification and notification, the co-ordination of the response and the supply of vaccines, with approximately nine weeks from crossing the epidemic threshold to the commencement of reactive vaccination (87). It has been estimated that each week of delay in vaccination reduces the number of prevented cases of disease by 3-8% (88). Specific limitations to the timely accessibility of vaccines are the requirement for a cold chain, which is logistically difficult in a resource poor setting, and the relatively high cost of effective vaccines for non-A serogroups (89). There is also likely to be a delay between vaccination and the development of a protective adaptive immune response (90).

While antibiotic prophylaxis is currently recommended for household contacts of laboratory confirmed cases in non-epidemic settings, it is not currently recommended in epidemics because although it would effectively reduce carriage within the household, it would not have a significant impact on overall carriage within the population (91). However, a recent trial of the village wide distribution of antibiotic prophylaxis within 72 hours of a case presenting in that village, was effective in reducing the overall attack rate (92).

The use of antibiotics for reactive prophylaxis on a wider scale, such as in whole villages or communities has been suggested, as it has the advantage of requiring no cold chain, and therefore being potentially quicker and cheaper to distribute (92). The effect of antibiotics on carriage is also extremely fast so may halt transmission more quickly than reactive vaccination (41). However, the widespread use of antibiotics has important implications for antibiotic resistance. No change in resistance patterns of enteric organisms was seen by Coldiron et al (92) but this is an important potential hazard given the significant global threat of anti-microbial resistance.

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1.1.15.6 Future developments

The WHO Roadmap “Defeating meningitis by 2030” has a number of strategic goals within the the pillar of “Prevention and epidemic control”. One of these is to “Develop and improve strategies for epidemic prevention and response” (3).

The recently developed pentavalent ACYWXX vaccine is planned for use in mass vaccination campaigns and subsequently to be included in routine immunisation schedules in the meningitis belt (93). This has the potential to have a dramatic impact on the incidence of disease with these serogroups. However, consideration should still be given to the potential for serogroup replacement and the challenges posed by the need for a cold chain and training for administration.

Alternative epidemic control strategies must focus on the speed of implementation and carriage reduction, as well as considering the effects on anti-microbial resistance, the required infrastructure and cost. An ideal form of reactive prophylaxis would be non-serogroup specific, cheap, readily available with no requirement for a cold chain, easy to administer and with no impact on anti-microbial resistance.

1.2 *Neisseria lactamica*

1.2.1 Non-pathogenic *Neisseria*

The genus *Neisseria* contains two important pathogens - *N. meningitidis* and *N. gonorrhoea*. There are multiple other species of *Neisseria* which are not typically associated with disease. These non-meningococcal, non-gonococcal *Neisseria* species are found in abundance in the human upper respiratory tract. Carriage is seen from the neonatal period and is seen in across many geographical regions (94, 95) including the meningitis belt (96). Unlike the two pathogens, many commensal *Neisseria* are not exclusive to humans but also colonise a variety of animal hosts (94, 95).

These typically non-pathogenic *Neisseria* do occasionally cause clinically important infections, with a wide range of clinical presentations described. The majority are opportunistic with underlying factors such as immunocompromise, recent surgery or intravenous drug use (94, 97). A recent report highlighted an association with the use of eculizumab, a terminal complement inhibitor, with seven cases reported (98).

Examples of commensal *Neisseria* include *N. cinerea*, *N. sicca*, *N. flavescens*, *N. polysacchareae*, *N. mucosa* and the species of particular interest in this thesis, *N. lactamica* (Nlac) (94).

1.2.2 Microbiological and genomic features

Nlac is a Gram negative, acapsulate, aerobic diplococcus which is both oxidase and catalase positive. It grows best at 35-37°C in 5% CO₂. Like other *Neisseria* it can metabolise glucose and maltose, but it is the only member of the genus to ferment lactose due to the action of β -D-galactosidase, which is encoded by the gene *lacZ*. This allows Nlac to be differentiated from other *Neisseria* by the appearance of colonies on media containing the chromogenic substrate 5-bromo-4-chloro-3-indolyl β -D-galactopyranoside (X-gal). Colonies of Nlac appear blue whereas colonies of other *Neisseria* species will grow white (99).

The lack of a polysaccharide capsule means that Nlac does not have the ability to withstand human host defences, and that it cannot be classified by serogroup. It can be classified by MLST in the same way as Nmen (99, 100).

The Nlac genome is similar in size to that of Nmen, at approximately 2.2Mb. A high percentage of this is coding content (85.3%), containing genes for almost 2000 proteins (99).

1.2.3 Carriage of *N. lactamica*

Nlac is a common commensal of the human nasopharynx, particularly in young children. The rate of natural carriage of Nlac rises over the first year of life then wanes in toddlers and older children and by adolescence carriage is approximately 1% (100, 101).

Genetic analysis of isolates of Nlac obtained in carriage studies suggests that transmission occurs between household contacts (102) and is more likely to occur between siblings, than between adults and children, within the same household (100).

The efficacy of ciprofloxacin to clear Nlac carriage is likely to be similar as for Nmen, as the organism is acutely sensitive to this antibiotic (103).

1.2.4 *N. lactamica* disease

Disease caused by Nlac is extremely rare, with only a few cases reported, almost always in the context of significant immunocompromise or co-morbidity (97). Table 1.1 details the few reported cases of Nlac disease (104-114). Nlac was not one of the *Neisseria* species identified in the series of infections in patients receiving eculizumab (98).

Disease	Predisposing conditions	Patient	Reference
Septic arthritis and septicaemia	Myeloma Corticosteroids	60 year old man	Everts 2010 (104)
Pneumonia	Hepatitis B Liver cirrhosis	42 year old man	Wang 2006 (105)
Cavitatory lung disease	Organ transplant recipient	64 year old man	Zavascki 2006 (106)
Otitis media	Not reported	Not reported	Orden 1991 (107)
Septicaemia	ALL on chemotherapy	7 year old girl	Schifman 1983 (108)
Septicaemia and otitis media Nlac and Gram positive cocci cultured from ear, Nlac in blood culture	Chromosomal abnormality	23 month old girl	Wilson 1976 (109)
Septicaemia and meningitis	None	7 month old girl	Lauer 1976 (110)
Septicaemia	Cerebral infarcts	15 week old boy	Brown 1987 (111)
Meningitis	Cribiform plate fracture	Adult female	Denning 1991 (112)
Meningitis	Unknown	Male child	Hansman 1978 (113)
Lung cavity	Organ transplant recipient	55 year old man	Changal 2016 (114)

Table 1.1 Nlac disease
Summary of Nlac disease case reports

1.2.5 Epidemiological relationship between *N. lactamica* and *N. meningitidis*

Nlac and Nmen are closely related species and occupy the same niche – the human nasopharynx. They have a high level of homology at a nucleotide level (8) and the majority of genes present in Nmen are also present in Nlac, including many associated with meningococcal virulence (115). Age-related patterns of carriage have been described in sections 1.1.14.1 (Nmen) and 1.2.3 (Nlac). These patterns have been consistently observed, and are inversely proportional, with Nmen carriage increasing over childhood to peak in adolescence and Nlac carriage falling over childhood from a peak in pre-school children (74, 100, 101). Co-colonisation with both species is rare (102, 116).

A negative association between Nlac carriage and both meningococcal carriage and disease was demonstrated during an outbreak in the Faroe Islands. Overall, Nmen colonisation rates were 4.5% in Nlac colonised individuals and 17.3% in those not colonised with Nlac. Comparison was made of Nlac and Nmen carriage in distinct areas of high and low incidence of meningococcal disease. Areas of high disease incidence had higher Nmen and lower Nlac colonisation rates than areas of low disease incidence. The age adjusted odds ratio of Nlac carriage for those living in areas of high incidence was 0.41 (95% CI 0.31-0.53) (117).

These observations have led to the hypothesis that Nlac carriage may provide protection from both Nmen carriage and meningococcal disease. Mathematical modelling has estimated a potential duration of protection of 4-5 years (118).

Gold et al demonstrated the natural acquisition of Nlac carriage over early childhood in the 1970s. A rise in cross-reactive Nmen IgG and bactericidal activity was seen among those who were Nlac colonised in comparison to non-colonised children (101). However, more recent studies have shown that the development of protective levels of SBA does not occur until later in childhood, after Nlac carriage rates have decreased (119). In a murine model of invasive meningococcal disease, mice immunised with either killed whole cell Nlac or Nlac OMVs were protected against challenge with a variety of Nmen strains in comparison to controls. However, bactericidal activity was not demonstrated using sera from the immunised mice (120). Furthermore, a phase 1 human clinical trial of Nlac OMV demonstrated generation of immune responses to Nlac but there was little evidence of cross protective SBA against Nmen (121).

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Other postulated immunological mechanisms include cross-reactive but non-bactericidal antibodies, non-humoral acquired immune responses, or innate immune responses triggered by Nlac colonisation.

Potential non-immunological interactions include competition for resources and direct antagonism by the release of specific toxins, antimicrobial peptides or extracellular DNA fragments (122).

The relationship between Nlac and Nmen has been further investigated in previous controlled human studies (123, 124). These studies and the insights they have provided are discussed in section 1.4.1.

1.3 Controlled Human Infection

1.3.1 Introduction

Controlled human infection, or human challenge, involves the deliberate exposure of volunteers to a micro-organism of interest, with the aim of inducing infection or colonisation. A controlled human infection model (CHIM) is a standard process of exposure developed for a specific organism which has been shown to be safe, effective and reproducible. Such a CHIM can then be used in clinical studies to investigate the bacterial-host interaction, immunological parameters and the efficacy of preventative or therapeutic interventions (125, 126).

1.3.2 History

The practice of deliberate infection of human subjects has a long, and at times unpalatable, history. An early example is the use of cowpox inoculation to prevent smallpox disease, made famous by Jenner in the 1700s. In the early 20th century, thousands of individuals were experimentally infected with a wide range of micro-organisms via respiratory exposure, ingestion and injection. Some of these studies yielded valuable insights into the aetiology, transmission and clinical features of infections, others allowed assessment of vaccines and therapeutic interventions (126, 127). However, many were highly unethical, involving experimentation on non-consenting vulnerable groups such as prisoners and children. Most lacked scientific rigor and appropriate consideration of risk (128).

Over recent decades, controlled human infection has become a valuable, scientifically robust and ethically sound field of clinical research. Great consideration has been given to the justification, design and ethical conduct of CHIM studies (125, 128-130). Current examples include influenza and other respiratory viruses (131), gastrointestinal pathogens

such as *Vibrio cholerae* (132) and *Salmonella typhi* (133), and parasitic infections such as malaria (134) and leishmaniasis (135).

1.3.3 Uses and limitations

The crucial strength of controlled human infection is prospective knowledge of the timing, site, and level of exposure of specific subjects, to the organism of interest. This allows a unique insight into the host-pathogen interaction before, during and after exposure and infection. The dynamics of the host immune response can be mapped in detail and immunological correlates of protection from infection, or disease, can be elucidated. Other factors influencing the response to infection can also be considered, e.g. co-infections, microbiome, host genetics and diet (126). The impact of different challenge doses, the incubation period, microbial dynamics, shedding and transmission to other subjects can be monitored (125). The response to re-challenge can give clues to the potential protection afforded by natural infection (136).

CHIMs can be used to evaluate and compare the efficacy of vaccines and other preventative interventions, as well as therapeutics. Given that many infections occur sporadically and may have very low incidence in the research population, the efficacy of such interventions can be assessed much more quickly, and with a far smaller sample size, than would be required in a conventional field study. During the development of new vaccines, potential candidates can be quickly assessed and either discarded or progressed to further stages of development (125, 126).

A limitation of CHIM studies is that because of the level of control required, it may not accurately represent real life infection. Volunteers are by necessity young healthy adults from the population local to the study site, the process of exposure may be different to natural infection and the fixed dose will not be representative of the wide range of exposure levels experienced in natural infection. These factors limit the generalisability of the results (126).

Safety is of paramount importance and so CHIMs are designed to avoid causing harm to the participants. This may mean limiting the endpoints of the study to microbiological confirmation of infection with no disease, or to clinical endpoints of mild disease which can be quickly terminated with no long-term effects. These limitations are clearly necessary in order for a study to be ethically acceptable, but it does limit the clinical information that can be gained (125, 126, 128).

1.3.4 Regulations and standards

In the United Kingdom, CHIM studies are subject to the same regulatory requirements as other clinical research studies. They therefore require approval from the National Research Ethics Service (NRES) and Health Research Authority (HRA) and must adhere to the principles of Good Clinical Practice (GCP). In contrast to the United States and the European Union, challenge agents are not considered to be Investigational Medicinal Products (IMPs), and so Medicine and Healthcare Regulatory Agency (MHRA) approval is not required, unless an IMP is included elsewhere in the protocol, for example as a candidate vaccine. Although Good Manufacturing Practice (GMP) production of challenge agents is desirable, it is not a requirement, and often not logistically practical, so a GMP-like approach to production is often used. Any challenge agent which is genetically modified will require approval from the Department of Food and Rural Affairs (DEFRA) prior to release (130).

However, standards have been published regarding the design and conduct of CHIM studies (128-130, 137). Robustly designed studies will minimise the risks and burdens to the participants and local population, while optimising social and scientific value (128).

1.3.5 Public Engagement

Public and participant involvement and engagement activities are an important part of the ethical design and conduct of clinical research and particularly so in CHIMs due to the potentially higher level of risk and burden to participants and third parties, and the potential for public scrutiny and misinformation. Consultation with potential, current and past participants and representatives of the local population as well as other key stakeholders such as community leaders and expert groups can help to inform study design and to optimise communication with the public, ensuring accountability and transparency (128-130). A variety of different activities can be employed e.g. focus groups, surveys, and consultation with specific individuals and groups (138).

The level of public engagement required will vary between different studies and situations. This may be relatively minimal in a well characterised CHIM with a low risk or burden, but will be more comprehensive in a novel CHIM with a higher level of risk or public interest. (128).

1.3.6 Controlled human infection in low and middle income countries

In order to address ethical requirements and safety concerns, CHIM studies are designed to avoid higher risk or more vulnerable populations. In addition, the infrastructure and resources required to maintain scientific robustness have generally limited their use to well-resourced research centres. As a result, the vast majority have been conducted in high income countries. However, many infections have the highest, or only, burden of disease in lower income countries. Differences between populations, such as previous exposure levels, baseline immunity, co-infections, microbiome, environment and social behaviour mean that the study findings may not be generalisable to the populations with the highest burden of disease. In fact, naïve individuals within a research population may be at higher risk of disease than those within an endemic setting.

There has therefore been a recent move towards translating established CHIMs to low- and middle-income countries (LMICs) where the infection of interest is endemic. When designing such a study, public engagement activities are vital to establish trust and ensure acceptability to the local population and community leaders. Care must be taken not to exploit vulnerable individuals, and a careful balance needs to be struck between fair compensation and undue inducement. The resources, processes and infrastructure must be put in place to ensure safety and appropriate access to health care for participants, and to maintain scientific integrity. With such safeguards in place, CHIM studies within endemic settings can be conducted safely and ethically and can yield results and benefits relevant to that population (128, 139).

1.4 The *Neisseria lactamica* controlled human infection model

1.4.1 Previous studies

Controlled human infection with Nlac can be used to investigate and potentially exploit the relationship between Nlac and Nmen. Two studies were conducted during the initial development of the model – LACTAMICA 1 (L1) (123) and LACTAMICA 2 (L2) (124). Healthy adult volunteers were challenged intranasally with Nlac using an automated pipette, with the aim of deliberately inducing nasopharyngeal colonisation. The inoculum used was a suspension of wild type Nlac strain Y92-1009 in phosphate buffered saline (PBS), prepared from frozen bacterial stocks. A dose of 10^4 colony forming units (CFU) was used for the majority of challenges.

The colonisation fraction (the proportion of challenged volunteers who were successfully colonised) was 58.5% by two weeks and 63.4% over the whole study period of 24 weeks in L1, and 33.6% by two weeks, 41% over the whole study period of 26 weeks in L2. This

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difference in colonisation fraction was partly due to an inhibitory effect of Nmen carriage on Nlac acquisition. Carriage of Nmen at baseline was an exclusion criterion in L1 but not in L2. In L2, 24% of challenged volunteers were colonised with Nmen at baseline and the Nlac colonisation fraction by two weeks was 21% in those with, and 37% in those without baseline Nmen colonisation. In addition, the exclusion of smokers from L1 but not L2 may also have had an impact on colonisation fraction. Acquisition of colonisation with Nlac predominantly occurred within the first two weeks following inoculation, but some new acquisition continued to occur up to 4 weeks (L1) and 26 weeks (L2) (123, 124).

There was some spontaneous loss of carriage of Nlac throughout the follow up periods but long-standing colonisation was achieved in the majority of those successfully colonised with a colonisation fraction of 41% after 24 weeks (L1) and 24% after 26 weeks (L2) (123, 124).

Successful colonisation with Nlac was seen to have an inhibitory effect on the acquisition of Nmen carriage in both studies, and to displace pre-existing Nmen carriage in L2, supporting the role of Nlac carriage in protection from meningococcal carriage and therefore disease (123, 124).

Successful colonisation with Nlac was also shown to be immunogenic with the production of Nlac specific systemic and mucosal antibody responses by 4 weeks. Some cross-reactive Nmen specific opsonophagocytic antibody production occurred but there was not a significant production of cross-reactive bactericidal antibodies to Nmen (123).

Colonisation following rechallenge with Nlac at 26 weeks differed between the two studies. In L1 there was evidence of resistance to rechallenge following spontaneous loss of carriage or failure to colonise. This resistance to rechallenge was not seen in L2. In L1 six volunteers underwent a third challenge at a higher dose of 10^5 CFU. These volunteers had not been successfully colonised at either of the first two challenges at 10^4 CFU. The colonisation fraction at this higher dose was 50%. There were no significant safety concerns in either study, at a dose of 10^4 in over 340 volunteers, or at a dose of 10^5 CFU in six volunteers (123, 124).

1.4.2 Further development of the model

The studies described above demonstrated that challenge with wild type Nlac is safe and induces long standing immunogenic colonisation in a reasonable proportion of healthy adults. However, some aspects of the model could be further optimised to improve the

colonisation fraction, the logistics and transferability of the model and the cross-reactive Nmen specific immunogenicity.

Six participants were safely challenged with a dose of 10^5 CFU in L1. The colonisation fraction in this subgroup, apparently resistant to challenge at the lower dose, was 50% (123). This suggests that this higher dose could be used safely in a less selected population, and is likely to substantially improve the colonisation fraction.

The preparation of the inoculum from frozen bacterial stocks is effective, but requires a temperature monitored -80°C freezer. The resulting inoculum dose may be inaccurate due to variability and gradual decline in the batch viability over time. The need for precise and complex dilutions adds further logistical difficulty and potential inaccuracy. Modification of these storage and preparation processes using lyophilised stocks of Nlac could improve the reliability and accuracy of the model and make it a feasible model to use outside of the highly resourced research environment. This is discussed further in section 1.4.3.

Experimental colonisation with Nlac induces some cross-reactive Nmen specific antibodies, but does not result in a significant rise in serum bactericidal activity (123), which is the accepted correlate of protection against meningococcal disease (23, 51-53). Transformation of Nlac to express a meningococcal-specific antigen could potentially improve the Nmen specific immunogenicity. This is discussed further in section 1.4.4 and 1.4.5.

1.4.3 Lyophilisation

Lyophilisation, or freeze-drying is a well-established method used to store specific strains of micro-organisms. Large counts of bacteria can be produced at high concentrations in small volumes and then stored or transported at ambient temperatures. These can then be reconstituted when required to yield viable cells.

The process of lyophilisation is variable but involves initial culture of the bacteria followed by the addition of protective agents, freezing, dessication and storage (140).

A disadvantage of lyophilisation has been the occurrence of cell damage during the freezing process leading to the loss of a proportion of the original number of bacterial cells. Many variables have an impact on this cell loss and the final viable cell count. Such variables include the species and strain of bacteria, the original cell count, the growth media and protective agents used, the speed of freezing and drying, the method of storage and the fluid and temperature of reconstitution (140). With optimisation of these variables, the recovery of a high and reproducible proportion of the original cell count is

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possible, although the effect of lyophilisation on the ability of micro-organisms to interact with host cells is unknown (141).

The optimal cell count to lyophilise has traditionally been taken as greater than 10^8 CFU. Protective agents include oligosaccharides and proteins such as skimmed milk. The speed of freezing influences the size and shape of the ice crystals formed, which then influences the ability to remove water vapour during the drying phase, and the direct cell damage. The shelf life and recoverable cell count is improved with packaging that does not allow interaction with moisture or oxygen. Vacuum sealed glass vials or ampoules are commonly used (140).

Two controlled human infection studies using a lyophilised Nlac inoculum are detailed in Chapter 6 of this thesis.

1.4.4 Genetically modified Nlac

Colonisation with a strain of Nlac expressing a meningococcal antigen could potentially impact both the colonisation fraction and duration, and the Nmen specific immunogenicity, in comparison to wild type Nlac. A CHIM incorporating such a genetically modified organism (GMO) would allow evaluation of the impact and host response to that specific antigen. A small number of CHIMs with “knock out” GMOs are in progress or have been published (133, 142, 143). To our knowledge, no previous controlled human infection studies involving a “knock-in” GMO have been undertaken.

1.4.5 The use of NadA as the antigen of interest

NadA is a surface-expressed meningococcal antigen with a role in adhesion and invasion into human epithelial cells (144). It is variably expressed by strains possessing the *nadA* gene, found in 30-50% of disease isolates. It is associated with hypervirulent clonal complexes and is present in only a small proportion of carriage strains (145, 146). NadA is potentially immunogenic and is one component of the 4CMenB vaccine (146). NadA has been shown to induce bactericidal antibodies in a murine model with mice inoculated with *Streptococcus gordonii* expressing NadA (147) or purified NadA protein with adjuvant (148).

NadA expression by a genetically modified strain of Nlac may therefore impact the colonisation fraction following challenge, and may improve the induction of cross-reactive Nmen immune responses. However, due to its potent immunogenicity, it may in fact reduce the duration of colonisation due to enhanced clearance.

A controlled human infection study with GM Nlac expressing NadA is detailed in Chapter 5 of this thesis.

1.5 Aims of this thesis

This thesis describes the design, conduct and results of four Nlac CHI studies, each addressing different research questions, and each progressing the Nlac CHI research programme towards the overall aim of improving protection from meningococcal disease.

Chapter 2 is an overview of Nlac CHIM methods. The common processes and procedures used across all four studies are described, and differences between the studies noted.

For each study, a key endpoint was Nlac specific IgG, with comparison made from baseline to post challenge timepoints. Chapter 3 describes the development, optimisation and validation of an in-house ELISA used to determine titres of serum IgG specific to outer membrane vesicles derived from the inoculum strain of Nlac – Y92-1009.

Chapter 4 describes LACTAMICA 3 (L3), a study designed to assess the impact of duration of colonisation on immunogenicity. Volunteers were inoculated with wild type Nlac and received antibiotic eradication therapy at day 4 (Group 1) or day 14 (Group 2). In order to optimise the colonisation fraction, the dose of inoculum was increased from that used in previous studies, and methods for assessing shedding were developed. The information gathered during this study helped to inform the design of the subsequent studies.

Chapter 5 describes LACTAMICA 4 (L4), in which volunteers received one of two strains (intervention or control) of genetically modified (GM) Nlac. As a world first controlled human infection study using a “knock in” genetically modified organism (GMO), safety and infection control were of paramount importance in the design of this study. Volunteers were therefore observed as inpatients for 4.5 days and then discharged and followed up until carriage was cleared after approximately 3 months. Safety, colonisation and shedding were closely monitored. A further group of ‘contact volunteers’ were also enrolled to assess potential onward transmission to bedroom sharers of the inoculated challenge volunteers following discharge. The immunological responses among those colonised with the different strains were compared.

All studies to this point used inocula prepared from frozen stocks of Nlac. This was effective, but limited the use of the model to centres equipped with the resources to reliably store and prepare the inoculum immediately prior to administration. In order to

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develop the model for potential use in a resource-limited setting, such as in the meningitis belt of Sub-Saharan Africa, the method of inoculum storage and preparation was modified. Stocks of Nlac were freeze-dried, or lyophilised, and then reconstituted shortly prior to administration. To demonstrate that this method was equivalent to using frozen stocks, two further studies were carried out. The first, LACTAMICA 5 (L5), recruited healthy adult volunteers in the UK, and following this, the second, similar study was carried out in a setting within the meningitis belt, in Bamako, Mali, LACTAMICA Étape 1 (LE1). Both studies used a dose-ranging strategy to identify the optimal dose of inoculum derived from lyophilised bacterial stocks, and then assessed safety, colonisation duration and immunogenicity of inoculated volunteers. These two studies are described in Chapter 6.

Chapter 2 The *Neisseria lactamica* controlled human infection model – an overview of methods

2.1 Overview

The *Neisseria lactamica* controlled human infection model (Nlac CHIM) has been established to safely and consistently induce nasopharyngeal colonisation with Nlac. Eligible volunteers are inoculated nasally with a suspension containing live Nlac, following which they can be monitored for endpoints such as colonisation dynamics and specific immunogenicity. Colonisation can be cleared with antibiotic eradication therapy if required by protocol. The four clinical studies presented in this thesis build on the previous work described in the introduction (123, 124), to further develop and optimise this model.

This chapter describes the common methods used across all four studies. An example study timeline is shown in Figure 2.1. Study specific methods including the study schedule, objectives and endpoints for each study are detailed in study specific chapters 4, 5 and 6. The study protocols are included as Appendices A1 to A4.

2.2 Regulatory requirements and approvals

All four studies were conducted in accordance with the Declaration of Helsinki (1996) and the International Conference on Harmonisation Guidelines for Good Clinical Practice and were registered with ClinicalTrials.gov.

The sponsor for L3 was University Hospital Southampton Trust and for L4 and L5 was the University of Southampton. These three studies were considered and approved by the National Research Ethics Service and the Health Research Authority. The two genetically modified strains of Nlac used in L4 were approved by the responsible government agency – the Department for Environment, Food and Rural Affairs (DEFRA) for use in this study (149).

LE1 was sponsored by the Centre pour le Développement des Vaccins du Mali (CVD Mali) and received ethical approval from the University of Maryland, Baltimore Institutional Review Board (UMB IRB) and the Comité d’Ethique de la Faculté de Médecine et d’odontostomatologie Faculté de Pharmacie, Mali. (FMOS/FAPH). Approval was also given by the University of Southampton Research Governance Organisation.

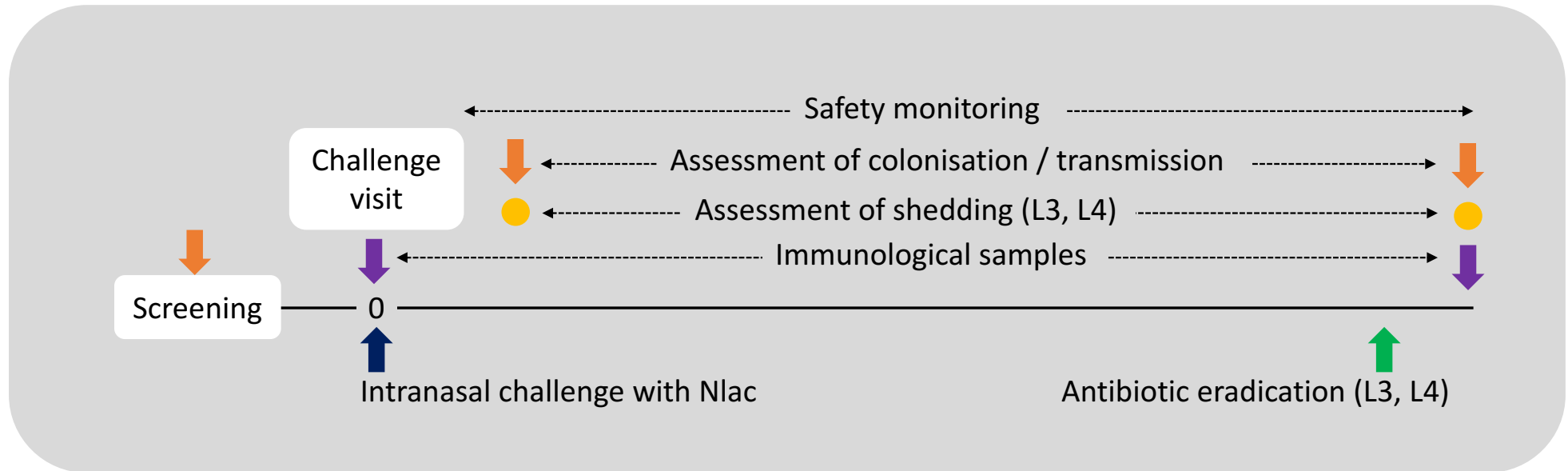


Figure 2.1 Nlac CHI study schedule

Overview of a generic Nlac CHI study schedule with key interventions and monitoring processes. Specific timepoints are variable for each study – see study specific chapters 4-6.

2.3 Study setting and study team

L3, L4 and L5 were carried out in Southampton, UK. All study visits and procedures, including admission for L4 challenge volunteers, took place at the Southampton NIHR Clinical Research Facility (CRF) located within University Hospital Southampton NHS Foundation Trust.

The NIHR CRF is a world class research facility with experience of vaccine, early phase and human challenge studies. It is staffed with a team of GCP trained research personnel including dedicated nurses, doctors and laboratory technicians. Management systems are in place for training, standard operating procedures, and sample tracking. The facility has multiple individual rooms suitable for inpatient or outpatient use, with additional facilities for inpatients such as a recreation room and designated toilets and showers. Laboratory facilities include an environmental chamber with category 2 environmental hood, level 2 microbiological safety cabinets, temperature monitored freezers and incubators. The facility is within the main hospital building with access to a resuscitation team and intensive care facilities if required. Standard infection control precaution policy is followed as per NHS and PHE policy.

For these studies, study team members received study specific training and were delegated to appropriate tasks. Informed consent, screening and inoculation procedures were all carried out by ALS trained study doctors. Other study visits and procedures were carried out by research nurses with ALS trained doctors on site.

LE1 was designed to translate the model to a setting within the meningitis belt. For this purpose, we collaborated with investigators from several different institutions, as detailed in Chapter 6, and the study was carried out at CVD Mali which is a research facility in Bamako, Mali, West Africa. CVD Mali has extensive experience in conducting clinical trials and is equipped with clinical and laboratory facilities including microbiological safety cabinets and temperature monitored freezers and incubators. Dedicated research staff were trained in the study specific clinical and laboratory procedures. CVD Mali is partnered with the University of Maryland School of Medicine Center for Vaccine Development in Baltimore, USA so collaborators from this institute were also involved in the design and conduct of LE1.

2.4 Data collection and storage

For the Southampton studies, all study specific data were collected onto study specific source documents including case report forms and laboratory source documents which can be found in Appendices C1-7 and D1-3. These were link anonymised and stored securely within the CRF or university premises with restricted access. Link anonymised data were transcribed into a study specific database stored on the UHS trust (L3 and L5) or University of Southampton (L4) systems. In CVD Mali, LE1 link-anonymised clinical data were entered directly into a password protected study database stored on a server in CVD Mali. Laboratory data were collected onto study specific source documents and transcribed into the database. Data were anonymised prior to electronic transfer to the University of Southampton for analysis.

2.5 Sample size

For all four studies, which all included serological response to Nlac as a primary or co-primary outcome measure, the sample size was calculated from the results of a previous Nlac CHIM study, in which a significant rise in Nlac specific IgG was seen 2 weeks following inoculation (123). Using the SD of 0.26 seen in that study, it was estimated that a sample size of 10 colonised individuals should be sufficient to confirm a 4-fold rise of specific IgG with 90% power using analysis of variance.

We therefore aimed to achieve colonisation in 10 participants for each group in L3 and L4, and at a final “standard inoculum dose” for L5 and LE1, with recruitment planned to continue until that had been achieved.

For L3 and L4, we estimated a colonisation fraction of approximately 50% at the planned inoculation dose of 10^5 CFU, based on the colonisation fraction seen previously at this dose (123), and allowed for a drop-out rate of 10%. We therefore anticipated a total sample size of up to 22 participants per group, so 44 participants challenged in total. In L4 we also planned to recruit a maximum of 1 contact volunteer per challenge volunteer, so a maximum of 44 contact volunteers.

In L5 and LE1, the study design included a dose-ranging process with 5 participants per dose cohort. The estimated total sample size was therefore dependant on the number of cohorts required to find the standard inoculum dose, but we estimated it to be 15-35 participants in total for L5. For LE1 the initial sample size was expected to be up to 25 participants, but this was increased to up to 100 participants following the early part of the study in which some participants needed to be replaced. This is detailed further in Chapter 6.

2.6 Safety considerations

The priority in conducting these studies was to avoid causing harm. As in all controlled human infection studies, it was important to consider the potential for the inoculum strain to cause disease to the challenged participants and the potential for onward transmission to their contacts or to the wider population.

Wild type Nlac is a non-virulent commensal organism and has been shown to be safe in previous controlled human infection studies. The likelihood of causing disease in L3, L5 or LE1 was therefore considered to be negligible. However, the eligibility criteria were carefully considered to minimise any risk to immunosuppressed individuals. Volunteers with a personal history, or close contacts with known or suspected immunocompromise were excluded from participation. HIV testing was not routinely conducted at screening after consideration of the local incidence and potential risk to asymptomatic individuals.

Staggered inoculations and maximum cohort sizes were built into the protocols for L5 and LE1 for the first individuals challenged at each escalated dose of lyophilised Nlac. These are detailed in Chapter 6.

The genetically modified strains used in L4 were designed to be non-virulent, but as novel GM strains, with one strain expressing a protein normally expressed by a pathogen, it was vital to fully assess their pathogenic potential and to minimise any onward transmission.

Following the production of the two strains, extensive pre-clinical testing was carried out, and the resulting data were submitted to DEFRA (150). Approval was given for their deliberate release in a controlled human infection study with the acknowledgement that onward transmission was possible, but that the risk of this would be minimised. This required strict eligibility criteria and infection control procedures, monitoring of shedding and transmission to bedroom contacts, and antibiotic clearance of colonisation at the end of the study (149). Safety considerations for minimising the risk of disease in challenge volunteers included a 4.5-day period of inpatient monitoring following challenge and staggering of the first challenges with regular safety reviews and strict stopping / holding rules. These are discussed in detail in Chapter 5. Participants were able to contact the study team via a 24-hour telephone number throughout their involvement in the study.

L3, 4 and 5 each had an independent external safety committee who reviewed the study protocol prior to study commencement, and reviewed safety data at pre-agreed intervals and in the event of any safety concerns. For LE1 the safety committee consisted of the

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investigators from each collaborating institution who met online regularly during the study to review all safety, eligibility and colonisation data.

Clearance of Nlac colonisation requires a single dose of Ciprofloxacin, either routinely as per protocol (L3 and L4) or in the event of any safety concerns. Eligibility and screening for all studies therefore included consideration of the potential risk of Ciprofloxacin to participants.

Additional safety measures were implemented to mitigate the risk of SARS-CoV-2 infection, which emerged during this research programme. These are detailed in Appendices E18-19.

2.7 Recruitment and consent

The recruitment strategy, all advertising material and information sheets were approved by the relevant ethics committee for each study.

In Southampton, the studies were advertised using a variety of media including social media, posters and articles within literature for circulation. Individuals registered with the NIHR CRF Healthy Volunteers Database were contacted directly by email. Individuals who responded to these advertisements were provided with a volunteer information sheet containing full information about the requirements, potential risks and benefits of the study. For L4, potential challenge volunteers were asked to inform their bedroom sharer (if applicable) about the study and if willing, they were provided with a contact volunteer information sheet.

All potential volunteers, including contact volunteers for L4, had a short pre-screening telephone call and if willing and potentially eligible, they were invited to attend a screening visit at the NIHR CRF. Fully informed written consent was taken by a study doctor at the screening visit, prior to any study procedures taking place. For volunteers in L4, a pre-consent questionnaire, infection control questionnaire and infection control agreement were completed to ensure the volunteers understood and agreed to the requirements, risks and benefits of the study. This is detailed further in Chapter 5.

The recruitment and consent strategy in Bamako differed from that in Southampton as the population and setting required a different approach including seeking community permission and consideration of lower literacy rates. This is discussed further in Chapter 5. Volunteer information sheets, informed consent forms and questionnaires (where applicable) for each study are included in Appendix B1-11.

2.8 Eligibility

Following informed consent, a detailed medical history and physical examination were conducted by a study doctor, ensuring that all inclusion criteria, and no exclusion criteria were met. Screening investigations included baseline safety bloods, urinalysis, urine drug screen and pregnancy test with details for each study in the specific study chapters. An ECG was performed (L3, L4 and L5 only) as a prolonged QT interval was considered a contra-indication for the use of ciprofloxacin. Results were considered on an individual basis, using the reference ranges in Table 2.1 as a guideline for L3, L4 and L5 and local normal ranges for LE1. Investigations could be repeated at the discretion of the study investigators.

All four studies required challenge volunteers to be healthy adults, aged 18-45 years. Care was taken to avoid recruiting vulnerable or immunosuppressed individuals or those with close contacts who were immunosuppressed. Smoking and pre-existing *Neisseria* carriage are known to influence the acquisition of Nlac. Current smokers were therefore excluded from all three studies in the UK, whereas in Mali it was felt to be culturally unacceptable to exclude smoking. Nasal wash and/or throat swab samples were taken for assessment of pre-existing Nlac and Nmen carriage at the screening visit, and at a pre-challenge visit 5-7 days prior to inoculation for challenge volunteers in L4. Nlac carriers were excluded from all four studies, and Nmen carriers were excluded from L3 and L4. Details of the screening investigations for each study are in the relevant chapter, and the eligibility criteria are summarised in Table 2.2 and 2.3.

	Lower limit	Upper limit
Haematology		
Haemoglobin [g/L]	Male: 130, Female: 120	Male: 170, Female: 150
White Cell Count [$\times 10^9/L$]	4	11
Platelet Count [$\times 10^9/L$]	150	450
Neutrophil count [$\times 10^9/L$]	2	7.5
Lymphocyte count [$\times 10^9/L$]	1.5	4.0
Biochemistry		
Sodium [mmol/L]	133	146
Potassium [mmol/L]	3.5	5.3
Urea [mmol/L]		7.8
Creatinine [$\mu\text{mol/L}$]		97
Albumin [g/L]	35	
Total bilirubin [$\mu\text{mol/L}$]		20
ALT [IU/L]		Male: 50, Female: 43
ALP [IU/L]		130
C reactive protein [mg/l]		10
Urine pregnancy test	Excluded if positive	
QTc	Excluded if ≥ 440 ms (Male) or ≥ 460 ms (Female)	

Table 2.1 Screening investigation reference ranges

Reference ranges for screening investigations for Nlac CHI study inclusion – to be used for guidance but investigator discretion is allowed. Based on University Hospital Southampton laboratory reference ranges.

Adapted from L3, L4 and L5 study protocols (Appendix A1, A2, A3).

Inclusion criteria	L3	L4 ChV	L4 CoV	L5	LE1
Healthy adults aged 18 to 45 years inclusive on the day of enrolment	✓	✓		✓	✓
Healthy adults aged 18 years or over on the day of enrolment			✓		
Residing outside the demographic surveillance area					✓
Fully conversant in the English language	✓	✓	✓	✓	
Able and willing (in the investigator's opinion) to comply with all study requirements	✓	✓	✓	✓	✓
Provide written informed consent to participate in the trial	✓	✓	✓	✓	✓
Provide written agreement to abide by infection control guidelines during the study		✓	✓		
Provide written consent to allow the study team to discuss the volunteer's medical history with the General Practitioner		✓			
Written informed contact volunteer consent provided by any bedroom contact		✓			
Agreement to be admitted to Southampton NIHR-CRF for 4.5 days following inoculation		✓			
For females only, willingness to practice continuous effective contraception during the study and a negative pregnancy test on the day(s) of screening and inoculation	✓	✓	✓	✓	✓ (to D28)
Able to correctly answer all questions in the pre-consent questionnaire		✓			
Able to correctly answer all questions in the infection control questionnaire		✓	✓		
Agreement to take antibiotic eradication therapy according to the study protocol	✓	✓	✓		
TOPS registration completed no conflict found	✓	✓	✓	✓	

Table 2.2 Inclusion criteria for NIAC CHI studies

ChV = Challenge volunteer, CoV = Contact volunteer, Adapted from study protocols (Appendix A).

Exclusion criteria	L3	L4 ChV	L4 CoV	L5	LE1
Current active smokers defined as having smoked a cigarette or cigar in the last four weeks	✓	✓		✓	
<i>N. lactamica</i> or <i>N. meningitidis</i> detected on throat swab or nasal wash taken at screening or at the pre-challenge visit	✓	✓			
<i>N. lactamica</i> detected on throat swab taken at the screening visit					✓
Individuals who have a current infection at the time of inoculation	✓	✓			✓
Individuals who have been involved in other clinical trials involving receipt of an investigational product over the last 12 weeks or if there is planned use of an investigational product during the study period	✓	✓	✓	✓	✓
Individuals who have previously been involved in clinical trials investigating meningococcal vaccines or experimental challenge with <i>N. lactamica</i>	✓	✓		✓	
Individuals who have received one or more doses of the meningococcus B vaccine Bexsero		✓			
Use of systemic antibiotics within the period 30 days prior to the challenge	✓	✓		✓	✓
Any confirmed or suspected immunosuppressive or immune-deficient state, including HIV infection; malignancy, asplenia; recurrent, severe infections and chronic (more than 14 days) immunosuppressant medication within the past 6 months (topical steroids are allowed)	✓	✓	✓	✓	✓
Use of immunoglobulins or blood products within 3 months prior to enrolment.	✓	✓		✓	✓
History of allergic disease or reactions likely to be exacerbated by any component of the inoculum	✓	✓		✓	✓

Exclusion criteria	L3	L4 ChV	L4 CoV	L5	LE1
Contraindications to the use of ciprofloxacin, specifically a history of epilepsy, prolonged QT interval, hypersensitivity to quinolones or a history of tendon disorders related to quinolone use	✓	✓	✓	✓	
Contraindications to the use of ceftriaxone, specifically hypersensitivity to any cephalosporins		✓	✓		
Any clinically significant abnormal finding on clinical examination or screening investigations	✓	✓		✓	✓
Any other significant disease, disorder, or finding which may significantly increase the risk to the volunteer because of participation in the study, affect the ability of the volunteer to participate in the study or impair interpretation of the study data, for example recent surgery to the nasopharynx	✓	✓	✓	✓	✓
Occupational, household or intimate contact with immunosuppressed persons, specifically HIV infection with a CD4 count <200 cells/mm³; asplenia; any malignancy, recurrent, severe infections and chronic (more than 14 days) immunosuppressant medication within the past 6 months (topical steroids are allowed)	✓	✓	✓	✓	✓
Occupational or household contact with children under 5 years or an older child with a tendency to co-sleep with the volunteer		✓	✓		
Pregnancy, lactation or intention to become pregnant during the study	✓	✓	✓	✓	✓
Inability of the study team to contact the volunteer's GP to confirm medical history and safety to participate		✓			

Table 2.3 Exclusion criteria for Nlac CHI studies

ChV = Challenge volunteer, CoV = Contact volunteer

Adapted from study protocols (Appendix A).

2.9 Inoculum

2.9.1 Inoculum strain

Nlac Y92-1009 was originally isolated in 1992 during a school-aged carriage study in Londonderry, Northern Ireland. It has been characterised using MLST analysis (ST-3493, CC 613) and whole genome sequencing (99). It has been used in previous Nlac CHIM studies (123, 124), and to develop an OMV based meningococcal vaccine (121).

2.9.2 Inoculum production, storage, and quality control

Bacterial stock vials were produced under GMP conditions at Public Health England (Porton Down, UK) and securely transferred under temperature monitored conditions to the University of Southampton. These stock vials were then diluted in PBS, glycerol and Franz medium and stored in aliquots.

Two strains of genetically modified Nlac were produced for use in L4, using Y92-1009 as a parent strain. This strain was also used to produce vials of lyophilised Nlac (LyoNlac) for use in L5 and LE1. These processes were designed and undertaken by Dr Jay Laver in the University of Southampton laboratories, using GMP-like techniques and specific standard operating procedures.

Frozen bacterial aliquots were stored in a -80°C freezer and lyophilised vials were stored in a refrigerator. Each was temperature monitored and located in secured facilities with restricted access either within the University of Southampton or NIHR Clinical Research Facility. Aliquots were assessed regularly for viability and contamination using standard microbiological procedures.

2.9.3 Inoculum preparation

Each dose of inoculum was prepared immediately prior to inoculation, once all pre-inoculation checks and investigations had been completed for that participant. The study team and laboratory team communicated directly to ensure the inoculum was prepared at the correct time.

A suitable aliquot or vial was identified from the refrigerator or freezer and transferred to the laboratory where it was prepared in a category 2 microbiological safety cabinet. Frozen aliquots of inoculum were thawed and then diluted in phosphate-buffered saline (PBS) (L3 and L4), and lyophilised vials were reconstituted and diluted in sterile water (L5) or PBS (LE1) according to a pre-specified dilution calculation. This calculation was based on the mean viability of the batch of inoculum, aiming for the viability that would achieve

the intended dose, expressed in colony forming units (CFU) in 1 millilitre (ml) of inoculum. The prepared inoculum dose was handed directly to the study doctor and 1 ml of inoculum administered to the participant (see section 2.11). Following inoculation, the residual inoculum was immediately returned to the laboratory for assessment of the residual inoculum viability using a Miles and Misra technique (CFU/ml). This CFU count was then recorded as the actual inoculum dose administered for that subject. While some variability in viability was expected, if administered dose was substantially or repeatedly different to the intended dose then further batch viable counts were performed and the dilution calculation adjusted appropriately.

For L3, L4 and L5 the laboratory was located within the NIHR CRF, within a few metres of the clinical room used for inoculation. For LE1 the laboratory was located on the same site as the clinical area, but required a 2-3 minute walk in notably higher ambient temperatures than the UK. After some initial unexpected variability in the inoculum count, a check of inoculum viability was added in immediately after dilution (i.e. pre-inoculation) as well as post-inoculation.

The residual inoculum was also assessed for purity following each inoculation, in each study. The study specific standard operating procedures (SOPs) for preparation and monitoring of the inoculum are detailed in Appendices E1-5.

2.10 Inoculum dose

The previous Nlac CHIM studies routinely used a dose of 10^4 CFU for challenge, all prepared from frozen stocks. This achieved a colonisation fraction of 34-63% by two weeks (123, 124). In a small subgroup of volunteers who had already been challenged twice, but not colonised, with a dose of 10^4 CFU, the dose was increased to 10^5 CFU. The resulting colonisation fraction in this very select subgroup was 50%, with no safety concerns (123). A dose of 10^5 CFU was therefore felt to be safe and likely to increase the colonisation fraction, and so was chosen as the appropriate dose for L3 and L4, anticipating a colonisation fraction of at least 50%.

The method of inoculum storage and preparation was then altered for L5, with the use of lyophilisation. It was therefore felt that the dose of inoculum may not be comparable and so a dose-ranging strategy was designed, starting with the same dose of 10^5 CFU and escalating or de-escalating the dose to find the optimal “standard inoculum dose” which resulted in a colonisation fraction of approximately 80%. For LE1, a similar dose-escalating strategy was used to identify the standard inoculum dose for Mali. Given the different population, with differing social behaviours, environment, diet and

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nasopharyngeal flora, the dose required to achieve colonisation was considered not to be generalisable between the two settings. The standard inoculum dose identified in L5 was used as a starting dose in LE1. The dose-ranging strategies are detailed in Chapter 6.

A colonisation fraction of 50-80% was chosen as the aim, in order to limit the overall sample size required, but to avoid using a higher saturating and potentially physiologically inappropriate dose.

2.11 Inoculation

On the day of inoculation, the identity, ongoing eligibility and informed consent of participants were confirmed. Baseline microbiological, immunological and safety investigations were performed including a pregnancy test for females. These are detailed in the schedule of procedures in each study specific chapter.

The inoculation procedure was performed by a study doctor and observed by a study nurse. Participants were positioned supine with a pillow under their shoulders and neck extended. The inoculum was administered slowly into their nose via a pipette, 0.5ml per nostril. During administration the participant was asked to breathe slowly through their mouth and avoid swallowing. In L3 and L4, an automatic pipette with sterile tip was used, and the participant was asked to remain supine for 15 minutes after inoculation. For L5 and LE1, a single use Pasteur pipette was used, and the volunteer was asked to remain supine for 5 minutes following inoculation. This change in inoculation procedure was made to improve the feasibility of the model for future field studies, while considering any possible impact on colonisation fraction.

SOPs for the inoculation procedure are detailed in Appendices E6-E8.

2.12 Follow up period

Following inoculation, volunteers were monitored for safety, colonisation and immunogenicity for the remainder of their enrolment in the study. For L3, L5 and LE1, volunteers attended outpatient follow up visits according to the study schedule. For L4, volunteers remained in the NIHR CRF as inpatients for the first 4.5 days following inoculation, following which they were discharged and completed their follow up period on an outpatient basis with specific infection control procedures in place. This period of admission and the infection control procedures were a requirement of the DEFRA approval for deliberate release of the GM Nlac strains, and are discussed in detail in Chapter 5.

Antibiotic eradication therapy was administered to all volunteers in L3 and L4 prior to completing or withdrawing from the study as per protocol, and was available for use in the event of significant safety concerns in all of the studies. A single dose of 500mg Ciprofloxacin was used for this purpose. Potential volunteers with known contra-indications for Ciprofloxacin use were excluded at screening and all female volunteers took a pregnancy test prior to administration. In the event of a positive pregnancy test, Rifampicin 600mg bd for 48 hours was planned as an alternative eradication therapy. The dose was administered and directly observed by a member of the study team.

The common methods for monitoring safety, microbiological and immunological endpoints are discussed in the following sections and the schedule of procedures and specific endpoints for each study are detailed in the study specific chapters. All clinical samples were collected and processed according to standard operating procedures, which are either study specific, used across the human challenge programme or generic Southampton NIHR CRF SOPs. Relevant study or programme specific SOPs are included in appendices E9-E15.

2.13 Safety monitoring

Volunteers were directly observed for a period of approximately 30 minutes following inoculation. Any symptoms reported were recorded as unsolicited adverse events for L3, L4 and L5. For LE1, volunteers were asked about specific symptoms during this period of observation (nasal irritation/stinging, sneezing, coughing, headache) and these were recorded as reactogenicity.

If an administered dose was found to be greater than 5 times the intended dose then it was recorded as an adverse event (AE), and if greater than 10 times the intended dose, it was recorded as an adverse event of special interest (AESI) and reported as a serious adverse event (SAE).

Volunteers were monitored for safety parameters at each scheduled follow up visit, and at four-hourly intervals throughout the period of admission for L4. Safety parameters included solicited symptoms for L4 and L5 (see Table 2.4), unsolicited symptoms or adverse events, safety blood tests taken at the time points specified in the study schedules, and clinical observations. Clinical observations and laboratory results were graded according to Tables 2.5 and 2.6 for L3, 4 and 5, and according to locally agreed parameters for LE1, shown in Table 2.7. Grading of unsolicited AEs and causality assessments were undertaken by a study doctor according to criteria in the study protocols.

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In the event of any safety concerns, volunteers were assessed by a study doctor. In between scheduled study visits, volunteers were able to contact a study doctor via a 24-hour telephone number, and additional visits were arranged if required. Early antibiotic eradication therapy was available if required, and a plan for further investigation and escalation to admission and IV antibiotics was included in the protocol for each study.

AE data was collected throughout the study period for L3, L4 and L5, and until Day 28 post challenge for LE1. SAE data was recorded throughout the study period for all studies and reported according to the relevant regulations. Safety data was reported to the external safety committee for L3, L4 and L5, and discussed at regular study safety committee meetings for LE1. The safety committee gave approval for ongoing recruitment and / or dose escalation where relevant, or advised in the case of any safety concerns. In L4, safety stopping and holding rules were specified in the protocol – these are discussed in Chapter 5.

Solicited symptoms Graded as No / Mild / Moderate / Severe	L4	L5
Rhinorrhoea	✓	✓
Nasal congestion	✓	✓
Epistaxis		✓
Sneezing	✓	✓
Ear pain		✓
Eye pain		✓
Sore throat	✓	✓
Cough	✓	✓
Dyspnoea	✓	✓
Feeling generally unwell	✓	✓
Tiredness	✓	✓
Headache	✓	✓

Table 2.4 Solicited symptom checklist – L4 and L5

Symptom data collected from participants at each follow up visit post challenge for L4 and L5, and four-hourly during admission for L4. Adapted from L4 and L5 protocols (Appendix A2 and A3) and case report forms (Appendices C3-5).

Clinical Observations	Normal values	Not an AE	Grade 1	Grade 2	Grade 3
Tachycardia – beats per min	40-100	NA	*101-115	116-130	>130
Hypotension (systolic) mm Hg	<100	>90	*85-89	80-84	<80
Hypertension (systolic) mm Hg	90-140	NA	*141-159	160-179	>180
Hypertension (diastolic) mm Hg	40-80	81-90	*91-99	100-109	>110
Fever °C	36.0-37.5	NA	37.6 – 38.0	>38.0	>39.0
Respiratory rate /min	10-18	19-22	*23-25	26-30	>30
O ₂ saturation %	95-100%	NA	94-92%	91-88%	<88%

Table 2.5 Severity grading for clinical adverse events - L3, L4 and L5

Observations recorded from participants at each follow up visit post challenge for L3, L4 and L5, and four-hourly during admission for L4. Adapted from study protocols (Appendices A1-A3) and case report forms (Appendices C3-5).

*During the inpatient period for L4, measurements of heart rate, respiratory rate and blood pressure, which fall into the Grade 1 AE range, are only graded as AEs if they remain in that range for 2 consecutive sets of observations

		Lab range	Not an AE	Grade 1	Grade 2	Grade 3
Haematology						
Haemoglobin [g/L]	Male	130-170	126-129	115-125	100-114	<100
	Female	120-150	114-119	105-113	90-104	<90
White Cell Count [x 10⁹/L]		4.0-11.0	11.0-11.49	11.50-15.00	15.01-20.00	>20
			3.51-3.99	2.50-3.50	1.50-2.49	<1.50
Platelet Count [x 10⁹/L]		150-450	136-149	125-135	100-124	<100
Neutrophil Count [x 10⁹/L]		2-7.5	1.5-1.9	1.0-1.49	0.50-0.99	<0.5
Lymphocyte Count [x 10⁹/L]		1.5-4.0	1.0-1.49	0.75-0.99	0.50-0.74	<0.5
Biochemistry						
C reactive protein [mg/l]		0-5	5-9	10-19	20-50	>50

Table 2.6 Severity grading for laboratory adverse events – L3, L4, L5
Safety bloods taken at scheduled timepoints for L3, L4 and L5. Adapted from study protocols (Appendices A1-A3).

		Mild (Grade 1)	Moderate (Grade 2)	Severe (Grade 3)	Potentially Life Threatening
Haematology					
Haemoglobin (gm/dL)	Female (absolute)	10.0 – 10.9	9.0 – 9.9	8.0 – 8.9	< 8.0
	Female decrease		1.6 – 2.0	2.1 – 5.0	> 5.0
	Male – absolute	11.5 – 12.4	10.5 – 11.4	9.5 – 10.4	< 9.5
	Male – decrease		1.6 – 2.0	2.1 – 5.0	> 5.0
WBC (10³ / μL)	Increase	11.6 – 15.0	15.1 – 20.0	20.1 – 25.0	> 25.0
	Decrease	2.35 – 3.20	1.50-2.34	1.00 – 1.49	< 1.0
Lymphocytes (10³ / μL)	Decrease	0.75 – 0.99	0.50 – 0.74	0.49 – 0.25	< 0.25
Neutrophils (10³ / μL)	Decrease	1.19 – 0.85	0.84 – 0.50	0.49 – 0.40	< 40
Eosinophils (10³ / μL)	Increase	0.50 – 1.50	1.51 – 5.00	> 5.00	Hypereosinophilic
Platelets (10³ / μL)	Decrease	125 – 135	124 – 100	99 – 25	< 25
Biochemistry					
Creatinine (μmol/l)		132-150	151-176	177-221	> 221 or requires dialysis
ALT (IU/L)		50-112	113-225	226-450	> 450

Table 2.7 Severity grading for laboratory adverse events – LE1

Safety bloods taken at scheduled timepoints for LE1. Adapted from study protocol (Appendix A4).

2.14 Microbiological endpoints

2.14.1 Colonisation

Nasopharyngeal colonisation with Nlac and Nmen was assessed by culture of throat swabs (all volunteers) and nasal wash samples (L3, L4 Challenge volunteers and L5) at specified time-points. Selective GC agar plates supplemented with 5-bromo-4-chloro-3-indolyl-galactopyranoside (X-gal) were used and colonies which were morphologically in-keeping with *Neisseria* species were identified as putative Nlac colonies if they were blue, and Nmen colonies if they were white. Colony counts were recorded and putative colonies were sub-cultured. Species identification was confirmed by analytical profile index (API) or Matrix-assisted laser desorption/ionisation time of flight (MALDI-ToF) for Nmen and Nlac at pre-challenge timepoints. The SOPs for microbiological sample collection and processing are detailed in appendices E9-14.

Putative Nlac colonies at post-challenge time points were confirmed to be the inoculum strain by strain specific PCR (L4, L5, LE1).

Successful colonisation with Nlac was defined as the culture of at least one colony of Nlac from a throat swab or nasal wash sample (where appropriate) taken between challenge and the Day 14 post challenge visit. In LE1, samples up to the Day 7 visit were used to define colonisation fraction for the purpose of dose-ranging decisions, but samples up to Day 14 were used for further analysis.

Efficacy of antibiotic eradication therapy was assessed by culture of throat swabs taken 1-2 days post eradication for all volunteers in L3 and L4.

2.14.2 Shedding and transmission

A requirement of DEFRA approval for the deliberate release of the GM Nlac strains in L4, was that challenged volunteers were monitored for environmental shedding of the strains. Methods for monitoring shedding were therefore developed and optimised for L3, and subsequently used for L4 (Appendix E9).

Two different “shedding check” processes were developed, a basic shedding check and an extended shedding check. The basic shedding check was used for all volunteers in L3 at each visit following inoculation, until after they had received antibiotic eradication therapy and been shown to be non-colonised. This was also used in L4 for all participants each day of the admission period and for at each outpatient visit until antibiotic eradication therapy had been given. The extended shedding check was used for volunteers in L4 who

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had evidence of shedding at the basic shedding check to assess any substantial increase in shedding.

The basic shedding check included a mask and air sample. The mask sample was collected by asking the volunteer to wear a simple surgical mask over their mouth and nose for 30 minutes. The air sample was collected by sitting the volunteer within an environmental chamber, asking them to cough intermittently for two minutes and collecting exhaled air into a Coriolis μ air sampler.

The extending shedding check included the mask and air samples as above, plus additional air samples collected with further aerosol-producing activities (speaking/singing/shouting). During the admission period this also included a sample of the volunteer's personal room air and contact samples from specific surfaces within their personal room.

These samples were then cultured on GC-Xgal plates, with identification of any resulting colonies as described in section 2.14.1.

A further DEFRA requirement for L4 was the assessment of onward transmission to close contacts. This was achieved by monitoring contact volunteers for nasopharyngeal colonisation using throat swab samples taken at specified time points. This is discussed further in Chapter 5.

2.15 Immunological endpoints

The immunological response to Nlac colonisation was assessed by analysis of blood samples taken at baseline and at post-challenge timepoints as specified in each study specific chapter. Comparison was made between participants colonised for a longer versus shorter duration (L3), participants colonised with NadA-expressing GM Nlac versus control GM Nlac (L4), and colonised versus non-colonised participants (L5 and LE1).

Nlac specific IgG titres were assessed for all studies. In addition, titres of Nmen specific IgG were measured in L3, L5 and LE1.

In L4, titres of IgG with specificity for the meningococcal antigen NadA, B cell responses with specificity to Nlac, Nmen and NadA, and meningococcal serum bactericidal activity (SBA) were also assessed. Methods for assays used for L4 are described briefly below and are detailed further in Laver et al 2021 (151), included as Appendix G2.

2.15.1 Specific IgG titres

Specific IgG titres were measured by enzyme-linked immunosorbent assay (ELISA) performed on serum samples. General ELISA methods are outlined in Chapter 3, with additional details for individual assays included below.

For L4, an Nlac-specific IgG ELISA was performed at an external laboratory. 96-well plates were coated with deoxycholate-extracted outer membrane vesicles (dOMV) derived from Nlac strain Y92-1009 (the inoculum strain). Participant and reference serum samples were serially diluted and added to the plate in duplicate. Secondary antibody (goat anti-human IgG Fc γ -fragment-specific alkaline phosphatase conjugate) and substrate (p-nitrophenyl phosphate) were added with incubation and wash steps between each addition. The reaction was stopped with 3M sodium hydroxide and optical density (OD) at 405nm determined. Test sample titres were then calculated by interpolating from the reference serum. Serial three-fold dilutions of each serum sample were analysed with the mean of all acceptable values from all dilutions used as the final titre for each serum sample.

Due to the COVID pandemic, this assay was not available for analysis of samples obtained from L3, L5 or LE1. Therefore an Nlac-specific IgG ELISA was developed for use within the University of Southampton laboratories and optimised for use in these studies. This development and optimisation process is detailed in Chapter 3 of this thesis and the resulting standard operating procedure is included as Appendix E15. This ELISA was also modified to measure Nmen specific IgG by coating wells with dOMV derived from the meningococcal strain H44/76.

In L4, sNadA specific IgG titres were determined by endpoint ELISA. 96-well plates were coated with sNadA in calcium carbonate buffer, with negative control wells coated with bovine serum albumin (BSA) or calcium carbonate buffer alone. Serum samples were serially diluted and added in duplicate with a positive control serum. Primary (biotinylated, rat-derived anti-human IgG mAb) and secondary (streptavidin-horseradish peroxidase) antibodies and substrate (o-phenylenediamine dihydrochloride (OPD)) were added with incubation and wash steps between each addition. The reaction was stopped with sulphuric acid, and OD at 490nm determined. Titres were expressed as reciprocal endpoint titres, with the endpoint threshold set at an optical density at 490nm (OD_{490nm}) of ≥ 1.4 , a threshold derived from reference curves generated by repeated dilutions of a reference serum. Serial two-fold dilutions of each serum sample were analysed and the

Chapter 2

result for each sample was the reciprocal of the last dilution of that sample to generate a positive response (OD_{490nm} of ≥ 1.4).

2.15.2 B cell responses

Enzyme-linked immunospot (ELISpot) assays were performed on peripheral blood mononuclear cells (PBMCs) to assess antibody (IgG, IgA and IgM) secreting plasma cells (B_{PLAS}) with specificity to Nlac and Nmen strains and to NadA, and IgG memory B cells (B_{MEM}) with specificity to Nlac and NadA. PBMCs were isolated from participant whole blood samples by density gradient centrifugation. B_{PLAS} assays were conducted on freshly isolated PBMCs and B_{MEM} assays on PBMCs which had been stored in liquid nitrogen and polyclonally stimulated for 5 days.

96-well ELISpot plates were activated, washed and coated with antigens as detailed below:

B_{PLAS} ELISpot:

- Keyhole limpet haemocyanin (KLH) – negative control to determine background signal
- Tetanus toxoid (TT) – negative control to determine any non-specific response to colonisation
- Test antigens:
 - 4NB1 dOMV (NadA expressing GM Nlac strain)
 - 4YB2 dOMV (control GM Nlac strain)
 - sNadA
 - N54.1 dOMV (Nmen serogroup Y, high NadA expressing)
 - N54.1 Δ nadA (N54.1 with coding sequence for NadA removed)

B_{MEM} ELISpot:

- Keyhole limpet haemocyanin (KLH) – negative control to determine background signal
- Influenza haemagglutinin (Infl HA) – negative control to determine any non-specific response to colonisation
- Test antigens:
 - 4NB1 dOMV (NadA expressing GM Nlac strain)
 - 4YB2 dOMV (control GM Nlac strain)
 - sNadA
- Rat anti-human IgG monoclonal antibody – to determine total circulating IgG B_{MEM}

Plates were incubated overnight, washed and blocked prior to the addition of PBMCs in duplicate. For the B_{PLAS} ELISpot, 2×10^5 PBMCs were added to each well. For B_{MEM}, 2, 4 and 10×10^5 cells were added to each 4NB1, 4YB2 and sNadA well, with 5×10^4 for Infl HA and 5×10^2 for total IgG. For each, an equivalent number were added to KLH coated wells. After incubation and washing steps, cells were lysed and plates were incubated with secondary antibody (alkaline phosphatase-conjugated anti-human IgG (B_{PLAS} and B_{MEM}) or IgA / IgM (B_{PLAS}). After further wash and incubation steps, substrate (5-bromo-4-chloro-3-indolyl phosphate) was added. Wells were then washed and dried and the number of spots per well was determined using an AID ELISpot reader, expressed as spot-forming units (SFU) with the assumption that one SFU indicated the presence of one B_{PLAS} or B_{MEM} cell. For each antigen, the mean of duplicates was determined and the background signal from equivalent KLH coated wells was subtracted. Antigen-specific IgG, IgA and IgM B_{PLAS} were expressed as total SFU after subtraction of KLH background. For B_{MEM}, antigen-specific IgG SFU were determined from the lowest input well with a mean of one to 20 SFU after KLH subtraction, and expressed as a percentage of the total IgG SFU.

2.15.3 Serum bactericidal activity

Serum bactericidal assays were performed at an external laboratory, against Nmen strain 5/99. Serum samples were serially diluted, heat inactivated and added to micro-titre plates. Nmen strain 5/99 in bactericidal buffer was added to each well. Human complement (IgG depleted pooled human plasma) was added, and plates incubated for 1 hour after which each well was plated and incubated overnight. Colonies were then counted for each dilution of each serum sample and compared to a t = zero control plate. Plates with colony counts $\leq 50\%$ of the control plate were considered to demonstrate bactericidal activity. SBA titres for test samples were expressed as the reciprocal of the highest dilution factor to demonstrate bactericidal activity.

2.16 Data analysis

Statistical analysis was carried out using GraphPad Prism version 9 for Mac. Appropriate parametric and non-parametric tests were used as detailed in the results sections. *P*-values were two-tailed and values of ≤ 0.05 were considered to be statistically significant.

Chapter 3 Nlac IgG ELISA development

3.1 Introduction

3.1.1 Nlac IgG ELISA background

A specific humoral immune response to Nlac has previously been demonstrated following controlled human infection with Nlac Y92-1009 (123), and following vaccination with outer membrane vesicles (OMV) derived from the same strain (121). For both studies, titres of IgG with specificity to OMV derived from Nlac strain Y92-1009 (hereafter Nlac IgG) were determined by analysis of serum using an enzyme linked immunosorbent assay (ELISA). This assay was developed and conducted at the Centre for Emergency Preparedness and Response, Porton Down, Salisbury by the Health Protection Agency (HPA), now known as the United Kingdom Health Security Agency (UKHSA), hereafter termed the Porton Down ELISA.

During the design and set up of the controlled human infection (CHI) studies detailed in this thesis, the intention was to transport participant serum samples for analysis using the Porton Down ELISA. Unfortunately, due to the COVID-19 pandemic, laboratory time and resources were necessarily diverted to the pandemic response, limiting their ability to conduct other work. Serum samples for L4 (Chapter 5) were analysed using the Porton Down ELISA as planned, but it was not possible to analyse serum samples for L3 (Chapter 4), L5 and LE1 (Chapter 6) within a reasonable time frame. Therefore, an Nlac IgG ELISA was developed, optimised and conducted at the University of Southampton Medical School Laboratory LC67 / LC70 in order to analyse these samples (hereafter UoS ELISA). This chapter details the development, optimisation and validation of the UoS ELISA.

3.1.2 Principles of ELISA design and analysis

3.1.2.1 Generalised ELISA reagents and ELISA protocol

ELISA capitalises on the specific bond between antigen and antibody, and the catalytic action of an enzyme upon a substrate, to detect and quantify the presence of specific antigens or antibodies in clinical or test samples (152).

The steps involved in an ELISA designed to detect a specific antibody/combination of antibodies (hereafter, target antibody), such as Nlac IgG, are outlined below. A wash step between the addition of each reagent ensures that any unbound molecules are removed from the reaction.

Chapter 3

- Coating – a capture antigen that will bind the target antibody is bound to an absorbent surface.
- Blocking – a blocking buffer, usually containing animal proteins, is added to the surface to block any unbound sites. This minimises non-specific binding of further reagents to the capture surface.
- Addition of the test sample – target antibody will bind to the capture antigen. Serial dilutions of the test sample are added to separate wells.
- Addition of a secondary antibody – selected to bind to target antibody (typically via the Fc domain) bound to the capture antigen. The secondary antibody may or may not be conjugated to an enzyme capable of catalysing a reaction with a quantifiable product, typically via a colour change. If the secondary antibody is not conjugated to such an enzyme, a third component, conjugated to the appropriate enzyme can be added in a separate step.
- Addition of substrate – this will be catalysed by the aforementioned enzyme, resulting in a signal, usually a colour change, which develops over time. An additional reagent is usually added to stop the reaction after a specified time.
- Quantification of the signal produced by the substrate reaction by measuring the optical density (OD) (152-154).

The readout of the ELISA is the absorbance of light by the enzymatically-derived reaction product at a specific wavelength, expressed as an OD. The OD is reported for each reciprocal dilution of a test sample and is assumed to be related to the number of target antibody molecules bound in the reaction at that specified dilution (152).

3.1.2.2 Antibody titre and reference curve

The antibody titre, or relative concentration of antibodies in a test sample is determined by comparison to a standard, or reference sample. Comparison of different test samples to the same reference sample allows comparison of the antibody titre, as they are relative to the same absolute concentration.

ELISA of the reference sample generates an OD for each dilution. These reported ODs are used to generate a four-parameter logistic (4PL) curve for the reference serum, termed the reference curve. The reference curve should be sigmoidal, with a reduction in OD seen at each increased reciprocal dilution, and with an $R^2 > 0.98$ to demonstrate a good fit (153). 4PL curves can also be generated for test samples.

3.1.2.3 Interpolation from the reference curve

The OD measured at a given dilution of the test sample can be interpolated from the reference curve (Figure 3.1). This determines the reciprocal dilution of the reference serum which has the same OD, and therefore number of bound target antibody molecules, as that dilution of the test sample. Correction for the two dilution factors allows the concentration of antibody, relative to the reference curve, to be generated for that test sample dilution, using the calculation:

$$\frac{\text{Test sample reciprocal dilution}}{\text{Reciprocal dilution interpolated from reference curve}} \times 100$$

In the example shown in Figure 3.1, the relative antibody concentration is:

$$\frac{\text{Test sample reciprocal dilution} = 8}{\text{Reciprocal dilution interpolated from reference curve} = 23.01}$$

$$(8 / 23.01) \times 100 = 34.77$$

ODs are generated for all reciprocal dilutions of the test sample and the final antibody titre is determined by calculating the mean result for all dilutions for which there is an acceptable result (i.e. within the range of detection) (153, 155). Alternatively in a standardised ELISA in which the reference and test curves are known to be parallel, a single pre-specified OD may be used, with interpolation on the test sample 4PL curve to identify the dilution factor which results in that OD (155).

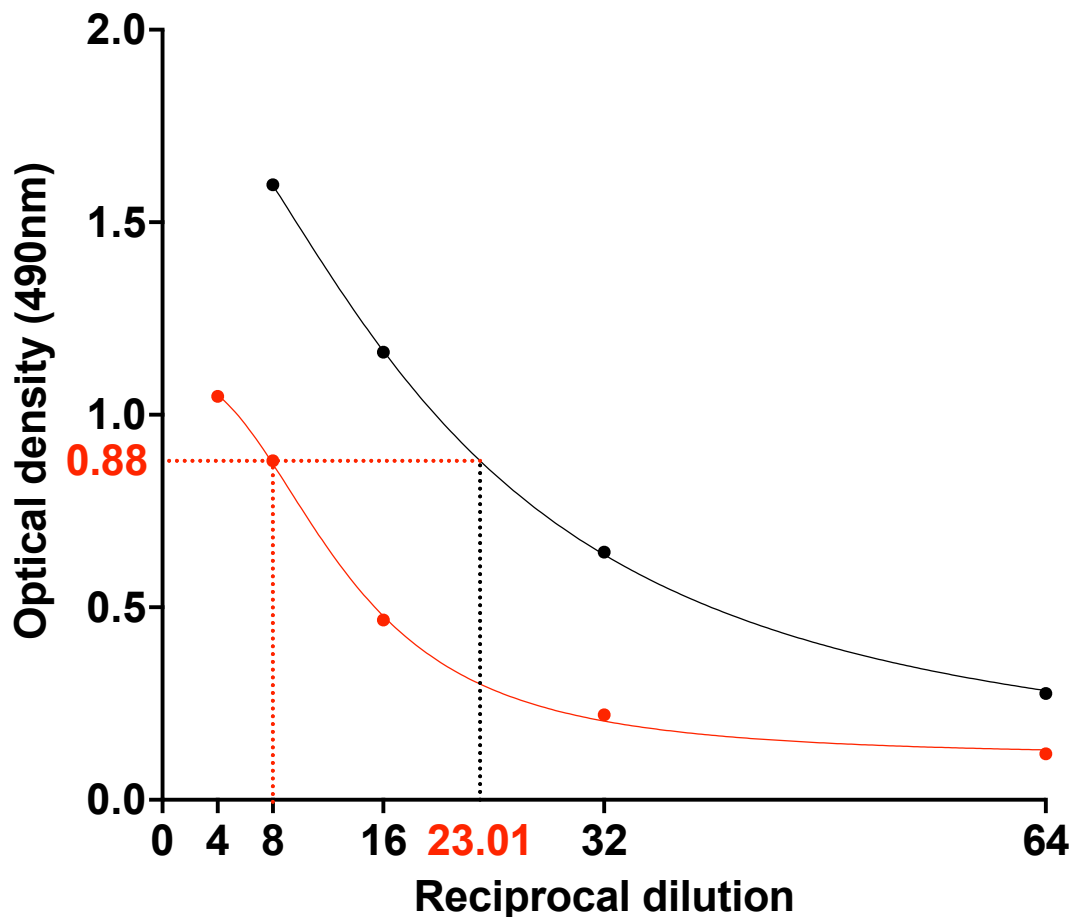


Figure 3.1 Interpolation from a reference curve

Stylised example of a test sample curve (red curved line) and reference curve (black curved line) derived from ELISA of test and reference serum samples. The test sample dilution x8 (red vertical dotted line) meets the test curve at an OD of 0.88 (red horizontal dotted line) which is interpolated on the reference curve to give an equivalent dilution factor of 23.01 (black vertical dotted line). In this example, measuring the x8 dilution of the test serum, this material has an interpolated, antigen-specific, reciprocal immunoglobulin titre of 34.77.

3.1.2.1 Endpoint ELISA

An alternative method of estimating test sample concentration is to determine the endpoint titre. Serial dilutions of the test sample are assayed, and the reciprocal of the last dilution resulting in a detectable level of target antigen, i.e. resulting in an OD above a pre-defined threshold, is reported as the endpoint titre (156).

3.1.2.2 Potential causes of inaccuracy

Occasionally an artefactually low OD is seen when very high levels of antibody are present in a sample. Binding of antibody and antigen is inhibited due to a phenomenon known as steric hindrance, or the hook effect, in which low affinity non-specific binding occurs and prevents correct binding in the correct orientation and thus completion of the ELISA reaction (157). This may result in a non-sigmoidal curve with a “hook” seen at the lowest reciprocal dilution and a poor fit (Figure 3.2). Therefore, if an OD result at a given dilution factor is lower than that at the next dilution, this is likely to be falsely low and should therefore be excluded from the analysis.

“Edge effect” is a previously demonstrated cause of ELISA error and inaccuracy, where a higher OD is seen in the outermost wells of a microtitre plate, thought to be due to thermal differences (158).

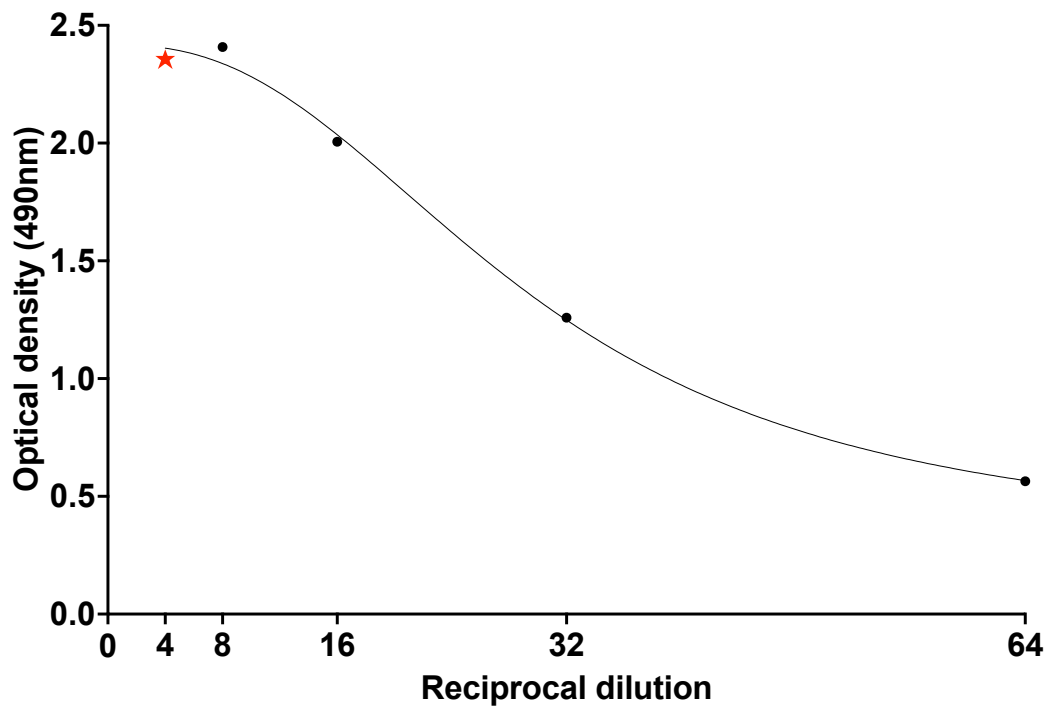


Figure 3.2 The hook effect

Stylised example of the "hook effect" in a 4PL curve in which the OD measured by ELISA of the x4 dilution (indicated by the red star), is lower than the OD measured for the x8 dilution of the same material due to steric hindrance. This single OD result should be excluded from further analysis.

3.1.2.3 ELISA validation

ELISA protocols must be validated for use, including assessment of the range and limits of detection, and assay precision.

The lower limit of detection is the point at which a signal is detected that is robustly distinguishable from any background signal. This background signal is determined by the ODs of negative control wells which should be consistent and low. The threshold for differentiating between a negative and a detectable positive signal is commonly set at the mean plus three standard deviations (SD) of repeated negative control results (153).

The precision of the ELISA is defined as the closeness of results from repeated measurements of the same sample. An appropriate measurement is the co-efficient of variance (%CV) which is the standard deviation (SD) expressed as a proportion of the mean (153). This can be split into agreement between duplicate measurements (measurements of a single dilution series from one test sample on a single plate), intra-assay variance (repeated dilution series of the same sample on a single plate), inter-assay variance (assay of the same sample on different plates and/or different days), and inter-operator variance (assay of the same sample by different operators). The threshold for an acceptable %CV is arbitrary, with different authors suggesting thresholds between 10 and 20%. A median %CV of $\leq 10\%$ has been suggested for duplicate ELISA ODs (154). The European Medicines Agency (EMA) suggests a %CV of $\leq 15\%$ for both intra- and inter-assay variance for the validation of bioanalytic methods, with up to 20% at the lower limit of quantification (159).

3.2 Methods

3.2.1 Nlac IgG ELISA protocol development

A draft standard operating procedure (SOP) for the UoS ELISA was developed from the Porton Down ELISA protocol described by Gorringer et al (121) with additional details and modifications following personal communication and considering the resources and expertise available.

In summary:

96-well microtitre plate layout:

- Exclude outermost rows and columns to avoid “edge effect”
- Paired individual test samples requiring direct comparison (e.g. samples from a single participant at two timepoints) should be run on a single 96-well plate with a single reference serum dilution series.
- Test samples requiring comparison of summary data (e.g. all participants from a single study) may be run on separate plates but must all be compared to the same reference serum.
- A negative control well (no secondary antibody) should be included for each sample dilution.

Coating:

- Capture antigen: Nlac Y92-1009-derived OMV
- Dilute to 20 mcg/ml in coating buffer:
 - a. 0.32g Na_2CO_3 + 0.59g NaHCO_3
 - b. Dissolve in water to 200ml
 - c. pH adjusted to 9.6
- 50 μl per well
- Incubate overnight at 2-8 °C
- Wash x 5 with Phosphate buffered saline (PBS)

Blocking:

- PBS + 5% (v/v) fetal calf serum (PBS-FCS)
- 200 μl per well
- Incubate for 2 hours at 37 °C
- Wash x 5 with PBS + 0.1% (v/v) Tween 20 (PBST)

Test sample preparation and addition:

- Serial dilution of test and reference serum samples in PBS-FCS
- 50 µl of each dilution added to coated 96 well plate in duplicate
- Incubate for 1 hour at 37 °C
- Wash x 5 with PBST

Addition of secondary antibody:

- Biotinylated, rat derived anti-human IgG (M1310G05) in PBS-FCS (1:1000)
- 50 µl per well
- Incubate for 1 hour at 37 °C
- Wash x 5 with PBST

Addition of detection reagent:

- Streptavidin horseradish peroxidase (HRP) in PBS-FCS (1:1000)
- 50 µl per well
- Incubate for 1 hour at 37 °C
- Wash x 5 with PBST, x1 with PBS

Addition of substrate:

- O-phenylenediamine dihydrochloride (OPD) – 1 tablet dissolved in 9ml sterile water / 1ml stable peroxide substrate solution (SPSS), protected from light
- 100 µl per well
- Incubate for 20 mins at 32 °C

Stop reaction:

- 1 M sulphuric acid
- 50 µl per well

Signal measurement:

- Determine OD at 490nm using Biorad iMark microtitre plate reader

3.2.2 Calculation of the Nlac IgG titre

A standardised method for calculating the relative value for Nlac IgG titre present in a given test sample was established following communication about the methods used in the Porton Down ELISA (A.Gorringe, J. Laver, Personal communication).

- Determine the mean and CV% of duplicate ODs
- Exclude non-valid results (see section 3.2.3)
- Generate a reference curve using the mean ODs for each dilution of the reference sample
- Interpolate the mean OD for each test sample dilution on the reference curve
- Derive a value for relative test sample Nlac IgG concentration for that test sample dilution factor by correcting for the test and reference reciprocal dilutions (see section 3.1.2.3)
- For each test sample, calculate the Nlac IgG titre as the mean of all dilution factors for which there is a valid result
- This will determine the relative test sample Nlac IgG concentration as a proportion of the reference sample concentration

3.2.3 Non-valid results

To ensure validity of the ELISA and final Nlac IgG titre results, limits were set for valid and non-valid individual results which would need to be excluded from the analysis or repeated.

Firstly, duplicate OD values should be in close agreement with each other. Following personal communication regarding the Porton Down ELISA, the threshold for acceptable variance between individual duplicate pairs was set at a %CV of 35%. (A.Gorringe, J. Laver, Personal communication). Any individual OD result with %CV between duplicates > 35% should be excluded from the analysis.

Secondly, considering the possibility of a falsely low OD due to the “hook” effect, an OD at one reciprocal dilution which is lower than the OD of the next reciprocal dilution should be excluded from the analysis.

Lastly, the negative control wells should have a low OD value. Higher than expected OD in the negative control wells suggests a high background signal for the whole plate, which should therefore be excluded and repeated. The specific threshold was set following repeated runs of the ELISA and assessment of the resulting negative control values.

In addition to these rules, some test sample ODs may not provide a result when interpolated on the reference curve, if the OD value falls outside of the range of the reference curve. These results would therefore not be able to be included in the final calculation. If the test sample Nlac IgG concentration is much higher or lower than that of the reference serum, this may occur for some or all dilution factors of the test sample. To determine a result for such a test sample, an extended dilution series of either the reference sample (if test sample Nlac IgG concentration is too low), or of the test sample (if test sample Nlac IgG concentration is too high), may be conducted.

3.2.4 Identification of an appropriate reference serum

An appropriate reference sample must have sufficient concentration of Nlac IgG to be detectable above the background signal at a high dilution factor, and to generate a reference curve against which a wide range of test sample OD values can be interpolated.

Four potential reference sera were identified, denoted Serum A to D. These were analysed and compared using the draft UoS ELISA protocol.

For each serum sample, the OD at each dilution factor (as a mean of duplicates) was compared to negative control wells on the same plate, looking for an Nlac IgG signal detectable above background, nominally set at the mean + 3 standard deviations (SD) of the negative control OD values.

The relative Nlac IgG concentrations of each serum sample were compared by generating a 4PL curve from the mean OD for each dilution. The interpolated reciprocal dilution of each sample was compared at a nominal OD of 1.0.

3.2.5 Analysis of variance in the draft UoS ELISA

Repeat analyses of two serum samples (serum A and serum B) were conducted to assess the precision of the UoS ELISA.

Twenty dilution series of serum A, ranging from x8 dilution to x128 dilution were made at the same time and run in duplicate on a single day on a total of 4 x 96-well plates.

Subsequently, six dilution series were made of serum B ranging from x8 dilution to x128 dilution, two per day over three days. Each dilution series was run in triplicate on two separate plates, using a total of 6 x 96-well plates.

Each plate included a negative control for each serum dilution, in which no secondary antibody was added. Potential edge effects were eliminated by excluding use of the outermost rows and columns of each plate.

The OD resulting from each dilution of each series was measured, in duplicate or triplicate and the variance between duplicates or triplicates was assessed, aiming for a median %CV of $\leq 10\%$ (154).

Negative control ODs were assessed for agreement between duplicates/triplicates and variance within and between plates. Comparison was also made between the upper limit of background signal (mean + 3SD of negative control wells) for each serum sample.

The mean OD for each dilution of each dilution series on an individual plate was used to construct a 4PL curve, which was assessed for goodness of fit, aiming for an $R_2 > 0.98$.

Intra and interassay variance were assessed by calculating the %CV between reported OD values (as a mean of duplicates / triplicates) between different dilutions, plates and days, aiming for a median %CV of $\leq 15\%$ (159).

3.2.6 Finalised standard operating procedure

Following this analysis, the standard operating procedure for the Nlac IgG ELISA was finalised, with minimal amendments to optimise the precision of the assay:

- Pre-warming of all reagents and washes to the temperature used for the next incubation step
- PBS-FCS added into negative control wells when secondary antibody added to the other wells
- Clarification of each incubation step timing as intended time \pm 15 minutes
- Upper limit of valid negative control set at OD of 0.21

The final SOP is included as Appendix E15. This includes the ELISA protocol, data interpretation including GraphPad Prism analysis instructions, and appendices with volumes required for the dilution of reagents and clinical samples.

3.2.7 Analysis of paired samples and intra-operator variability

The finalised SOP was used to analyse 58 samples, which were paired samples from 29 individuals at two timepoints. This analysis was conducted by a single operator with comparison to a single reference serum (serum A), and subsequently by a second operator with comparison to a second reference serum (serum X). Paired samples were always analysed on a single plate with the reference serum.

The usability and precision of the ELISA was assessed for the first set of OD results with consideration of the number of invalid or repeated samples and the variance between duplicates, aiming for a median %CV between duplicate ODs of \leq 10%. The range of Nlac IgG titres detected and any change in titre demonstrated between timepoints was considered.

Intra-operatory variability was assessed by comparison of the final Nlac IgG titre reported for each sample following ELISA conducted by the two operators.

3.3 Results

3.3.1 Initial analysis of potential reference sera

The reported ODs for each duplicate measurement of each dilution of sera A to D are shown in Figure 3.3 demonstrating that a signal was detected for each serum sample, with decreasing OD seen as the dilution factor increased, as would be expected. One exception to this was the x4 dilution of serum A which had a lower OD than the x8 dilution, suggesting that steric hindrance had occurred at x4 dilution. The background level of signal was considered to be the mean + 3 SD of the negative control ODs = 0.057, shown as a black dotted line. An OD in excess of this background level was seen for each of the four serum samples even at the highest dilution factor of x128.

4PL curves for each sample using the mean value for each duplicate are shown Figure 3.4. The x4 value for serum A has been excluded. Each 4PL curve had a good fit, with $R_2 > 0.99$.

The relative concentrations of Nlac IgG in each serum sample were estimated and compared by comparing the interpolated reciprocal dilution of each sample at a nominal OD of 1.0. This arbitrary OD was chosen as a point which lay on the steep part of the 4PL curve for each sample:

Serum A: 27.0

Serum B: 11.1

Serum C: 17.3

Serum D: 31.9

This demonstrated that each of the four serum samples had sufficient Nlac IgG concentration to be appropriate for use as a reference serum, but with serum D having the highest, and serum B the lowest concentration of Nlac IgG.

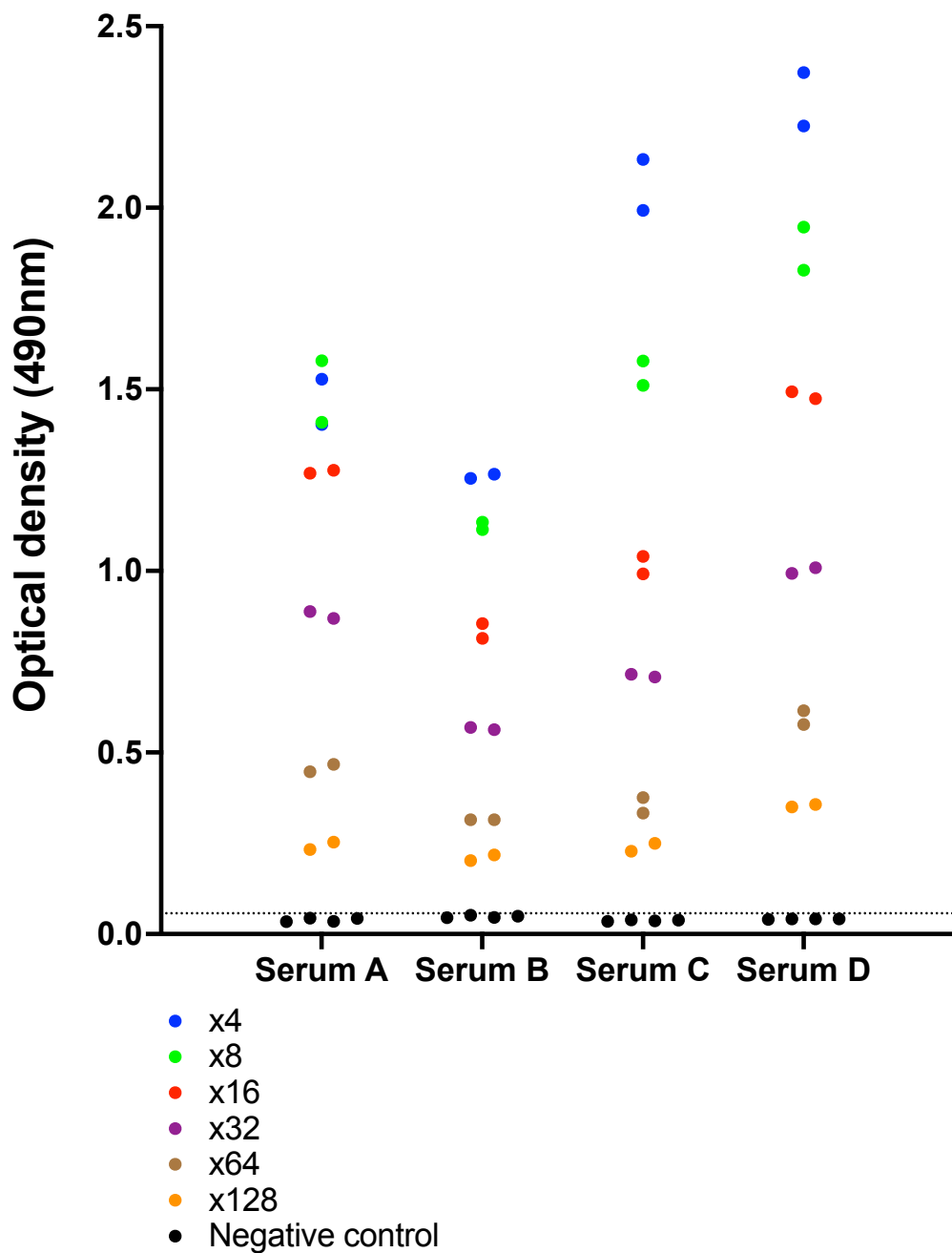


Figure 3.3 OD results for serum samples A-D

Two duplicate OD results shown for each dilution of sera A-D (coloured circles – see key) with negative control OD results in black. Black dotted line indicates the background signal estimated as the mean+3SD of negative control well OD.

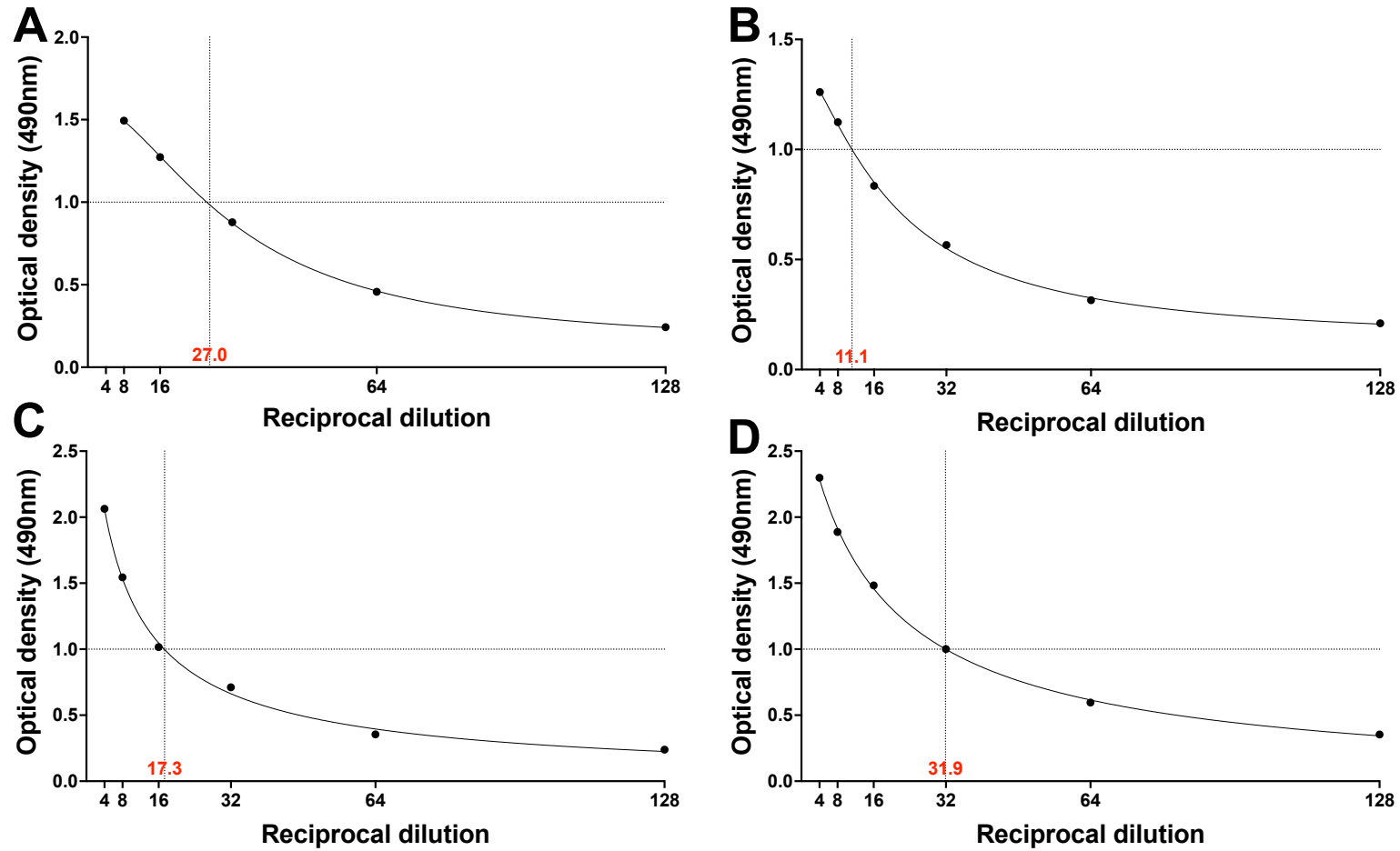


Figure 3.4 Potential reference sera 4PL curves

4PL curves generated for serum samples A-D with interpolated reciprocal dilution (red) shown for each sample at an OD of 1.0 (black dotted lines).

3.3.2 Agreement between duplicates / triplicates

Repeated ELISA of serum A and B was conducted as described in section 3.2.5. The agreement between duplicate/triplicate results was assessed by calculating the coefficient of variance (%CV) between each two duplicate (serum A) or three triplicate (serum B) OD values resulting from ELISA of a single dilution series assayed in separate wells of a single plate.

Figure 3.5A shows individual %CV for duplicate ODs at each reciprocal dilution of serum A. The median %CV and 95% confidence intervals were well below the stated aim of 10% for each reciprocal dilution, but there were a few outlying data points above 10%. The median %CV across all reciprocal dilutions for serum A was 3.97% (95% CI 3.14-5.05%).

For serum B, the median %CV of triplicates ODs across all reciprocal dilutions for serum B was 5.57% (95% CI 4.21-7.42%) and for each reciprocal dilution the median %CV was below 10% as shown in Figure 3.5B. However, there were several individual outlying data points with notably higher %CV, up to a maximum of 28.5%. Upon visual assessment of the raw data there was a notable difference between the OD values in column 2 in comparison to the corresponding triplicates in columns 3 and 4, and values in column 7 in comparison the corresponding triplicates in 5 and 6. While columns 2 and 7 were not “edge” columns, they were the outermost wells in use, with the surrounding wells being empty. Exclusion of these outermost triplicates, with analysis of the remaining duplicate results improved the overall variance with the median %CV and 95% CI below 10% for all reciprocal dilutions, and an overall %CV of 2.98% (95% CI 1.80-4.06%). This is shown in Figure 3.5C.

This highlighted the potential impact of the “edge effect” as discussed in section 3.1.2.2. To minimise this impact, the SOP was amended to include the addition of warmed PBS into the empty external wells at the same time as the addition of substrate and for the final incubation period.

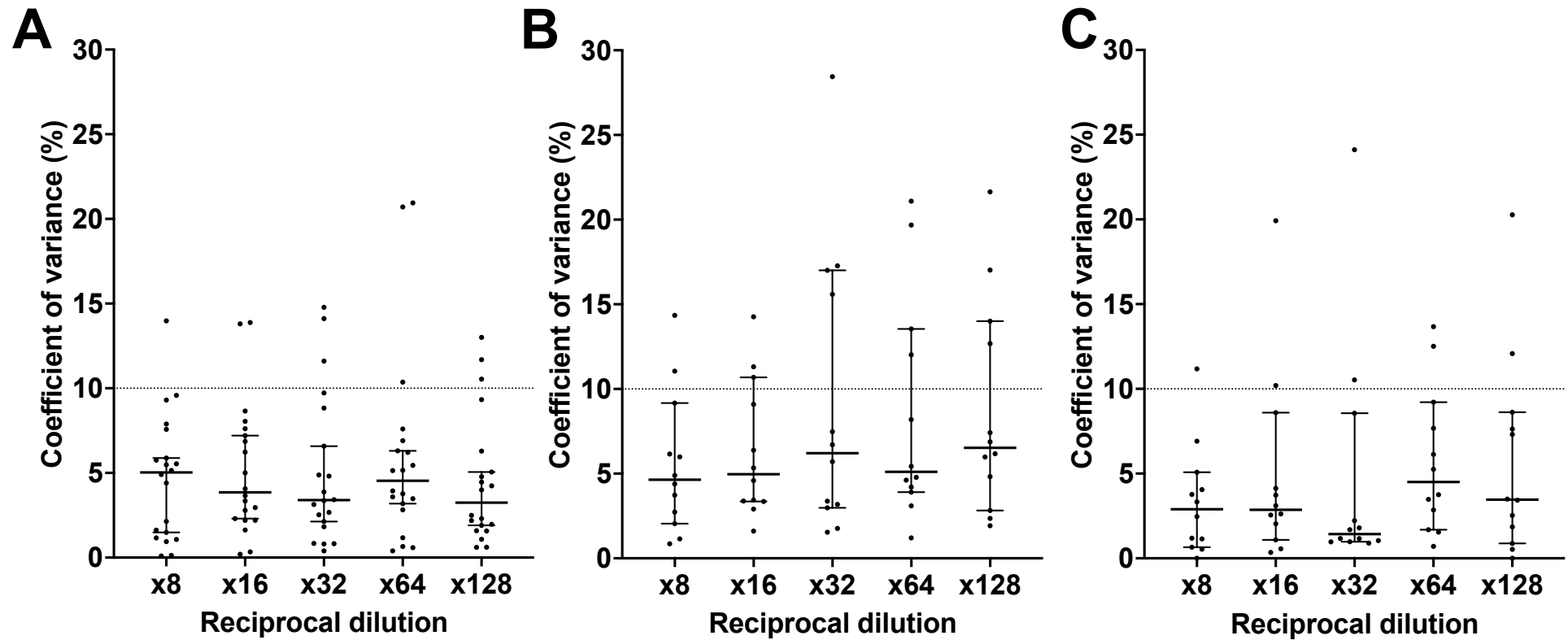


Figure 3.5 Variance between duplicate and triplicate OD values

%CV of duplicate or triplicate OD values derived from UoS Nlac IgG ELISA at each reciprocal dilution. of repeat dilutions of serum A and B. Black circles indicate the %CV of two or three duplicate/triplicate ODs (same dilution series, dilution factor and plate), median and 95% confidence interval shown at each reciprocal dilution.

A: Serum A duplicates, B: Serum B triplicates, C: Serum B duplicates when outermost wells excluded.

3.3.3 Negative control background signal

One negative control well per duplicate or triplicate dilution series was included on each plate, identical to the x8 well except for the exclusion of secondary antibody. The negative control OD values are shown in Figure 3.6 separated into plates for serum A (Figure 3.6A) and plates / days for serum B (Figure 3.6B).

The nominal “background signal” of mean + 3 SD of negative control ODs is shown as a dotted line for each serum sample. This is notably higher for serum A (0.59) than for serum B (0.21). The serum B value of 0.21 was used as a limit for an acceptable background signal for the finalised SOP, meaning that any plate with negative controls above that limit would be excluded and repeated.

Within each plate, for both serum A and B, the negative control ODs were consistent, with a median %CV per plate of 6.1%.

For serum A, there was a high level of variance between the four plates, with an overall %CV of 48.1%. These four plates were all assayed on a single day by a single operator using the same reagents and equipment. However, the plates were processed in parallel, with the incubation times taken as a minimum requirement. There may therefore have significant variation in the time between each step, or variation in the time taken to complete each step and thus in incubation time, and / or time out of the incubator. There may also have been variation in the temperature of reagents used over the four plates. Some of these potential inaccuracies were mitigated for serum B with more precise timing and warming of reagents prior to use, to try to reduce the variation in background signal.

For serum B, there was increased consistency between the background signal seen on separate plates run on one day, with a median %CV of 16.3%. However, there was a high level of variance between the three days, with higher ODs seen on Day 2 and an overall %CV of 53.3%. Some of this variation may have been due to ambient temperature differences or to the use of a different hood and/or pipette set on different days, as well as the “edge effect” in the outermost wells already discussed in section 3.1.2.2.

This variance in background signal between plates and days is higher than would be optimal. However, the potential impact of this variance on test sample ODs may be small as the absolute background signal is very small, and plates with a particularly high background signal would be excluded as per the SOP. The variance between test samples ODs on different plates and days is considered in section 3.3.4.

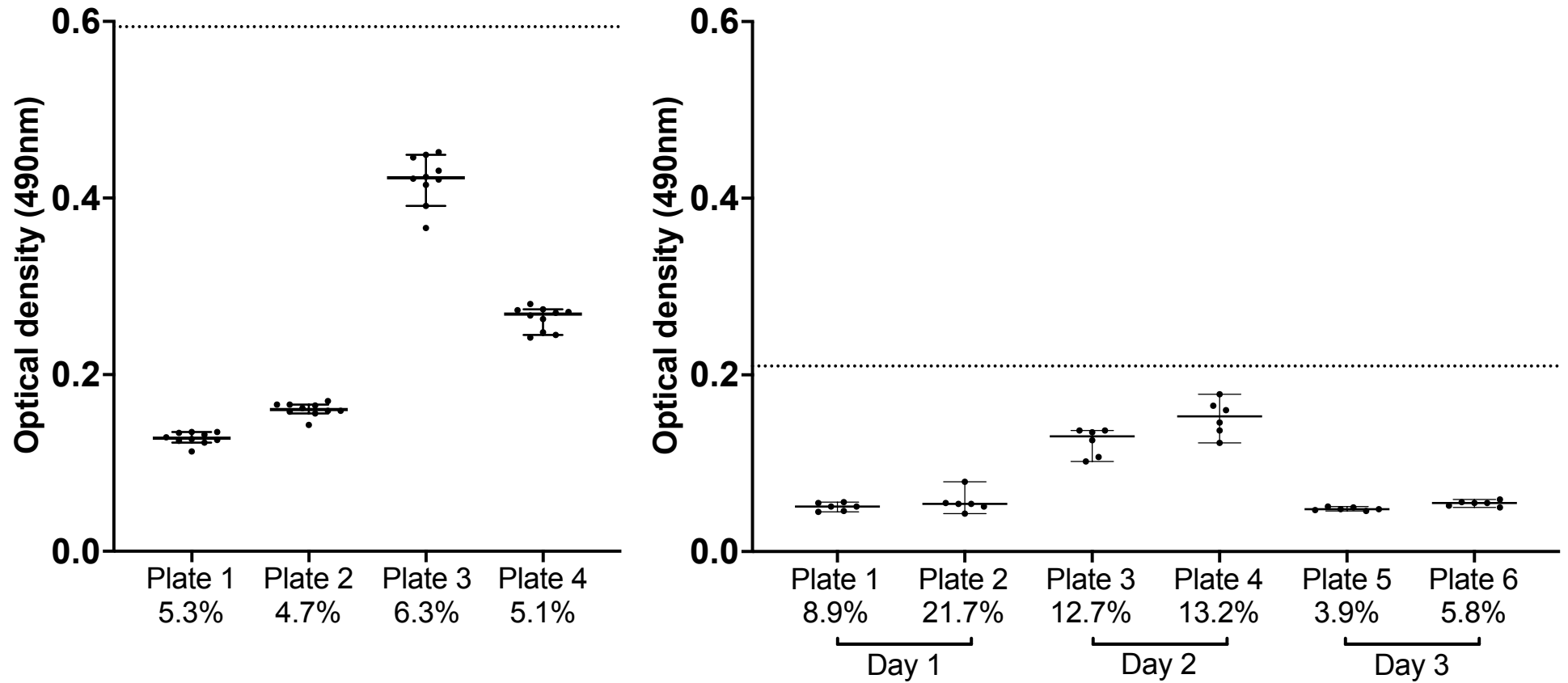


Figure 3.6 Negative control ODs for serum A and B

OD values from negative control wells. Individual ODs shown with median and 95% CI per plate. %CV of values on each plate indicated.

Black dotted line indicates mean + 3 SD of all negative control OD values. A: Serum A, B: Serum B.

3.3.4 Analysis of assay variance

The precision of the ELISA was analysed by considering the variance in OD values reported for repeated analysis of serum A and B. The mean value for each pair of duplicates (serum A) or triplicates (serum B) was used to generate a 4PL curve for each dilution series assayed on an individual plate. Figure 3.7 shows the 4PL curves for twenty dilutions of serum A, run across four plates. Figure 3.8 shows six dilutions of serum B, each run on two different plates. All 4PL curves for serum A, and all except one for serum B had a good fit with $R^2 > 0.98$. Visually, good agreement is seen for different dilutions on one plate (Figure 3.7) and each single dilution run on two plates (Figure 3.8).

The intra and inter-assay variance was analysed by calculating the %CV between dilutions, plates and on different days, aiming for a median %CV $\leq 15\%$ (159).

Figure 3.9 shows the %CV at each reciprocal dilution of serum A. Coloured circles indicate the %CV of different dilutions assayed on a single plate, which were $<15\%$ for all except two plates at x32 dilution. The median %CV across all reciprocal dilutions was 10.2%. The %CV across all four plates (twenty dilution series) are shown as black stars. This increased with increasing reciprocal dilution from $<15\%$ at lower dilution factors up to almost 35% at a x128 dilution with a median of 18.6%.

Figure 3.10 shows the variance between dilutions, between plates and between days for serum B. For individual dilution series assayed on two separate plates, each %CV was below 15% (for all dilution series at all reciprocal dilutions), with a median %CV of 3.7% overall (Figure 3.10A).

For different dilution series assayed on a single plate, the median %CV at each reciprocal dilution was $<15\%$ with almost all individual values $<15\%$. Individual values with %CV $>15\%$ were from the highest reciprocal dilutions, and therefore the lowest concentrations of Nlac IgG. The median %CV was 5.5% overall (Figure 3.10B).

Figure 3.10C shows the %CV for all four results on a single day (two dilutions, each on two plates). All individual CV% values were $<15\%$ with a median %CV of 6.4% overall.

Figure 3.10D shows the %CV for all OD values derived from all six dilutions on all plates over all three days. The %CV was approximately 15% for all reciprocal dilutions (range 13.5–15.4%) with a median of 15.2%

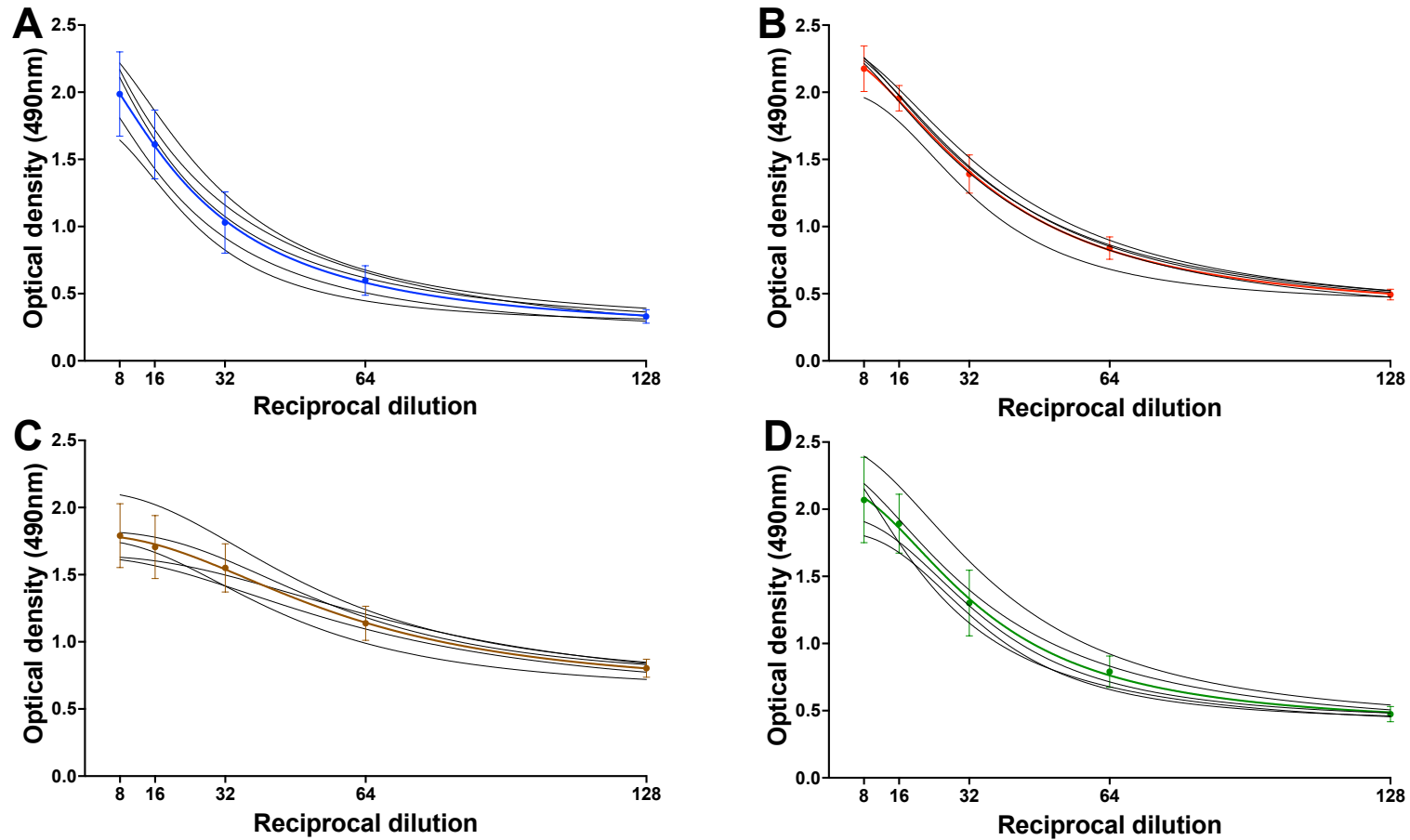


Figure 3.7 Serum A 4PL curves
4PL curves generated by ELISA of twenty dilution series of serum A.

A-D = Plate 1-4, five dilution series run on each plate (black lines) with combined curve per plate with 95% confidence interval (coloured lines).

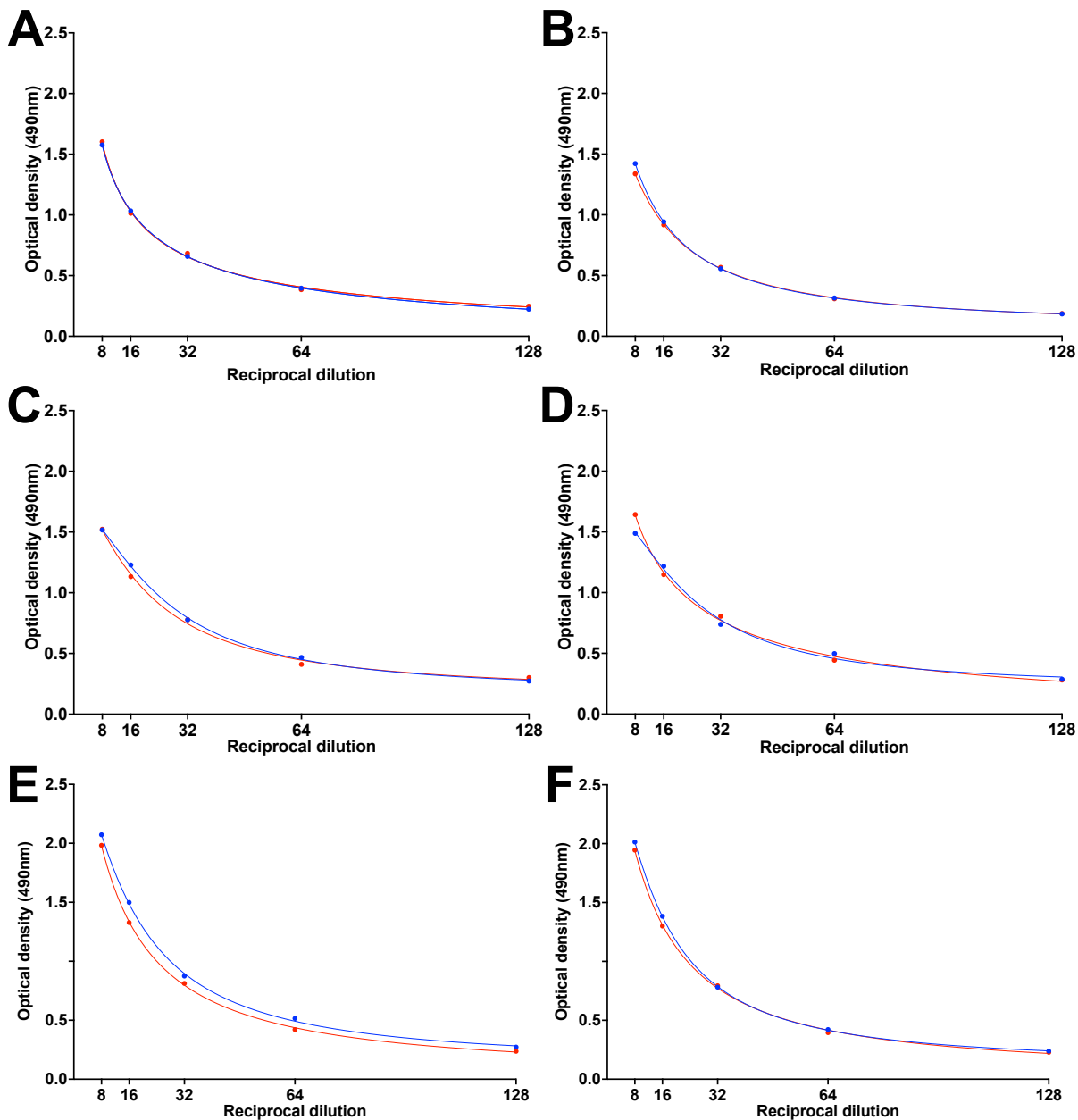


Figure 3.8 Serum B 4PL curves

4PL curves generated by ELISA of six dilutions of serum B. Each graph (A-F) shows a single dilution series of serum D assayed on two different plates (indicated in red and blue).

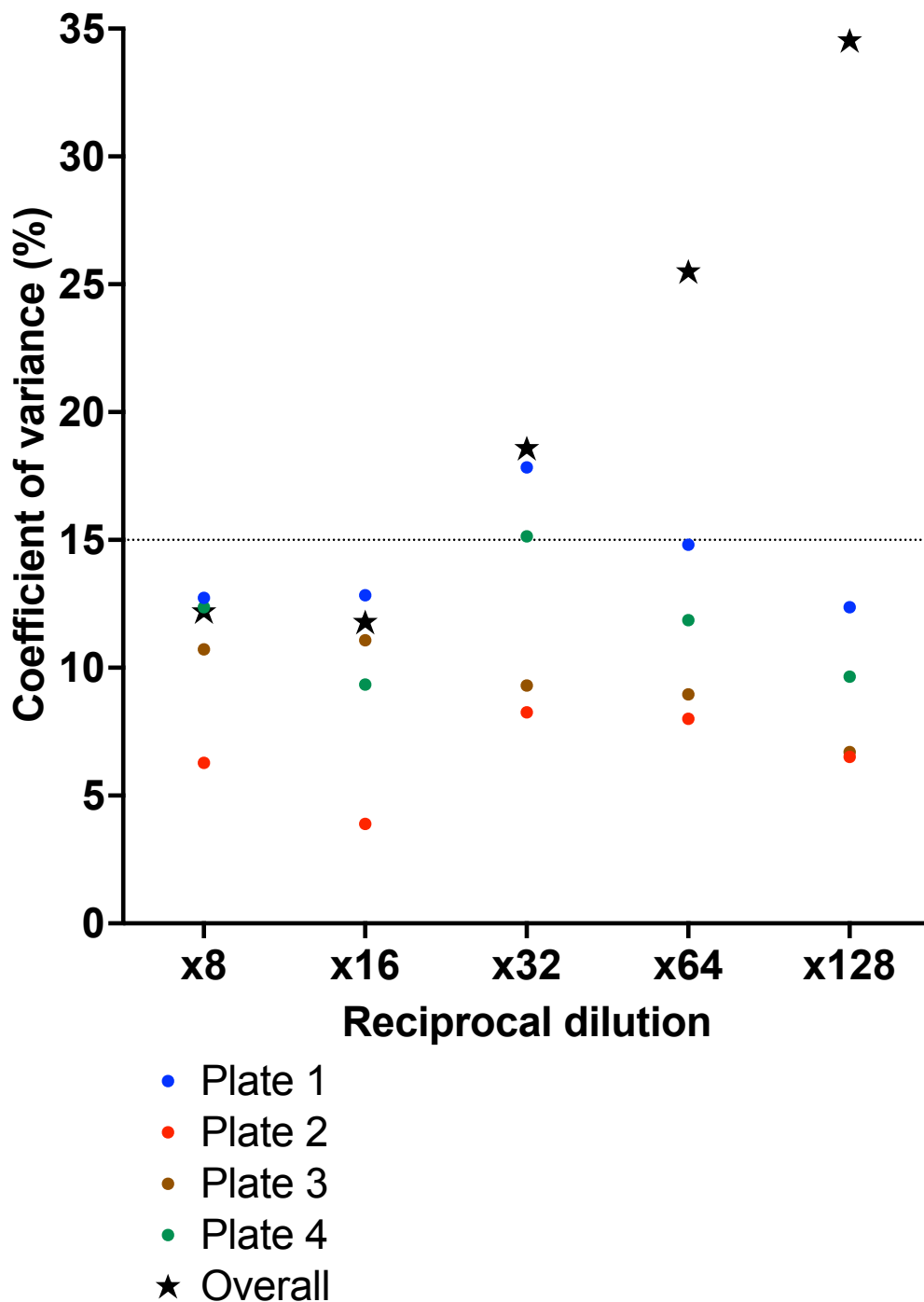


Figure 3.9 Intra and inter-assay variance – Serum A

%CV of OD values generated by repeat ELISA of Serum A. Coloured circles denote %CV of five OD values from five dilution series on a single plate, shown for each different reciprocal dilution (Plates 1-4, see colour key). Black stars indicate the %CV of 20 OD values from 20 dilution series on all four plates, shown for each reciprocal dilution.

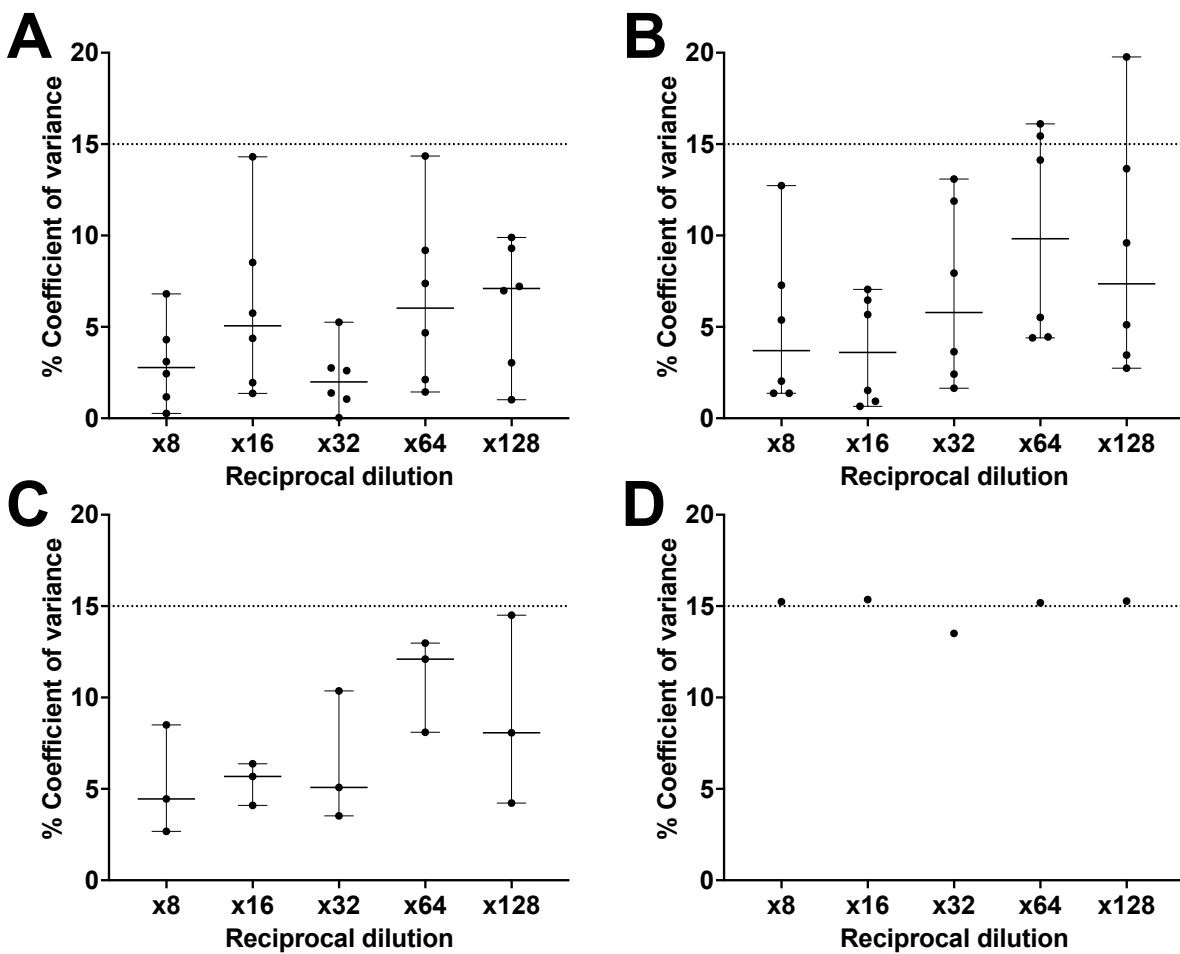


Figure 3.10 Intra and inter-assay variance - Serum B

%CV of OD values derived from ELISA of repeat dilutions of serum B.

Individual %CV values with median and 95% confidence interval shown for each reciprocal dilution.

A: Variance of a single dilution series assayed on two different plates

B: Variance of two dilution series assayed on a single plate

C: Variance of two dilution series each assayed on two plates in a single day

D: Variance of all dilution series on all plates over three days - single value shown

3.3.5 Paired sample analysis

58 test samples, from 29 individuals at two timepoints, were analysed to determine Nlac IgG titres and detect any change in titre between the two timepoints, using serum A as a reference serum on each plate.

Of these, two plates (four test sample pairs) were repeated due to a high background signal and/or inadequate 4PL curves. Seven test sample pairs were repeated to alter the dilution series due to low levels of Nlac IgG in the test sample. 13 individual OD values from 11 test samples were excluded for apparent hook effect and one individual OD value was excluded for %CV >35% between duplicates.

Each test pair therefore had one set of OD values which met the validity criteria. Of these, the median negative control OD was 0.047 (Range 0.034-0.068, overall %CV 10.2%). The agreement between duplicates at each reciprocal dilution is shown in Figure 3.11 with a median %CV <10% for each reciprocal dilution and 3.9% overall (95% CI 3.2-4.3%).

The range of determined Nlac IgG titres relative to serum A was 4.92 – 670.0 with a median of 36.32. Example 4PL curves for individuals who demonstrated an increase in Nlac IgG titre from the first to the second timepoint are shown in Figure 3.12, and for individuals who did not are shown in Figure 3.13. In both figures an example of extended dilution of the reference sample is included as the Nlac IgG titres in the test samples were very low (Figure 3.12D and 3.13D). For one (Figure 3.13D) it was not possible to generate 4PL curves for the test samples as only 3 dilutions yielded valid ODs, but interpolation of each of these ODs on the reference curve still allowed the Nlac IgG titre to be determined.

3.3.6 Comparison with a second operator and reference serum

The same 58 test samples were analysed by a second operator, with comparison to a second reference serum X.

Due to the different absolute concentration of Nlac IgG in the two reference sera, the values obtained for each test sample would inevitably be different, but should be directly proportional. There was a significant correlation between the two sets of results with $R_S = 0.917$ (95% CI 0.86-0.95) and $p < 0.0001$ (Spearman correlation). Simple linear regression of this relationship is shown in Figure 3.14.

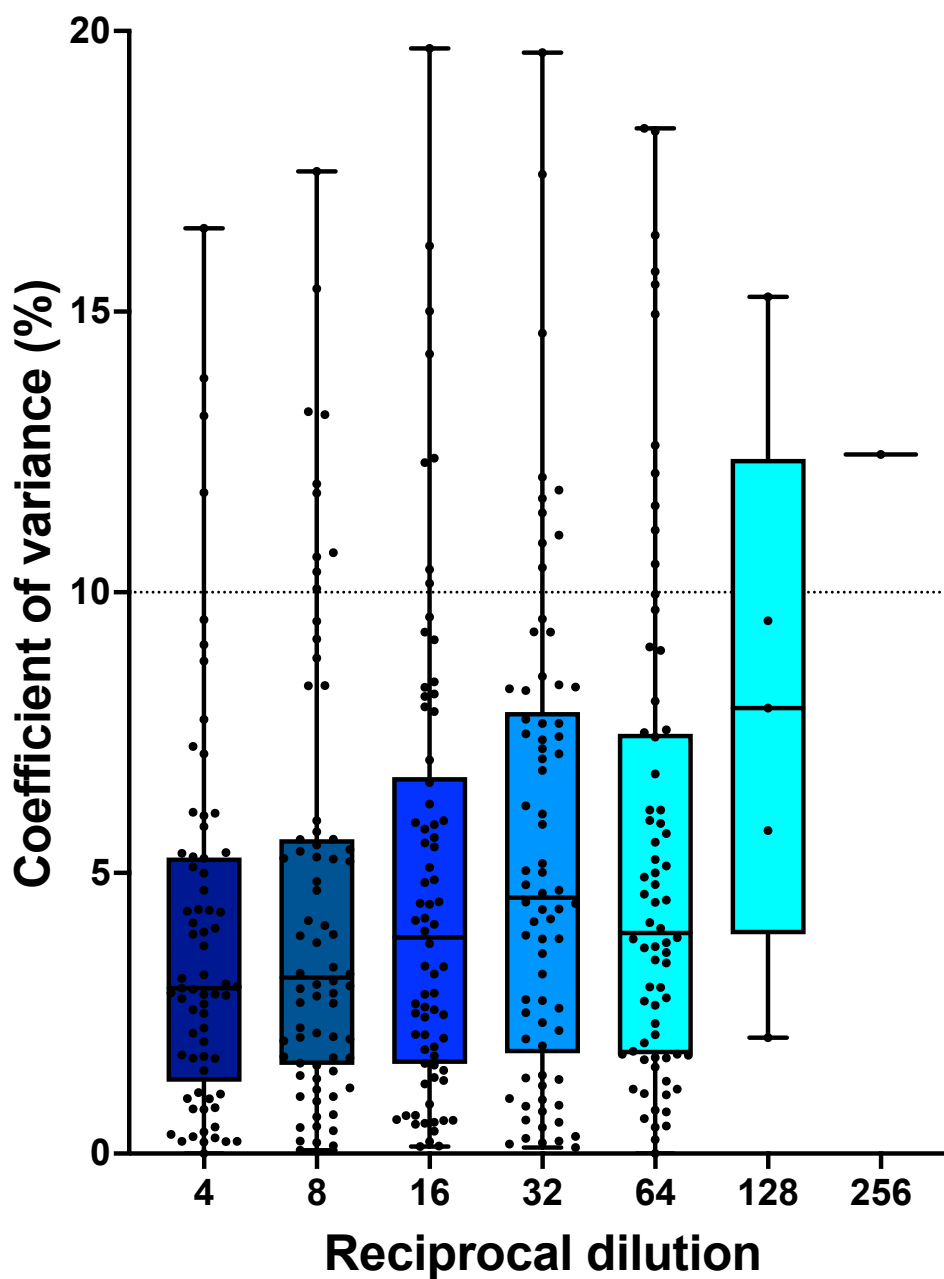


Figure 3.11 Variance between duplicates - test samples

%CV of duplicate OD values derived from ELISA of test samples. Individual %CV values, median, IQR and range shown for each reciprocal dilution.

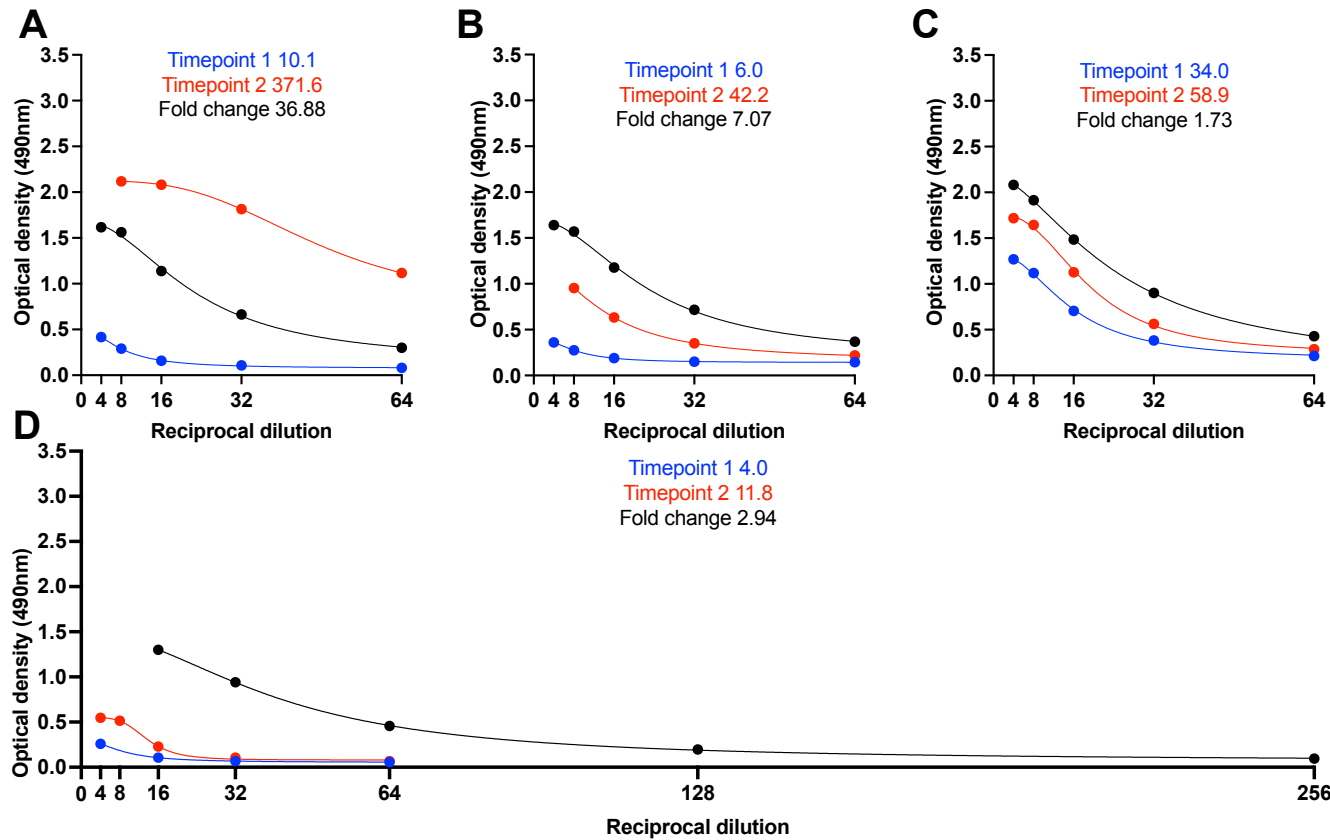


Figure 3.12 ELISA results for paired test samples demonstrating a rise in Nlac IgG

OD at each reciprocal dilution (circles) and 4PL curves shown for timepoint 1 (blue) and timepoint 2 (red) test samples in comparison to reference serum C (black). Relative Nlac IgG titres and fold change shown.

A-C: 4x – 64x dilution of test and reference samples, D: 4x – 64x dilution of test samples, 16x – 256x dilution of reference sample (extended dilution)

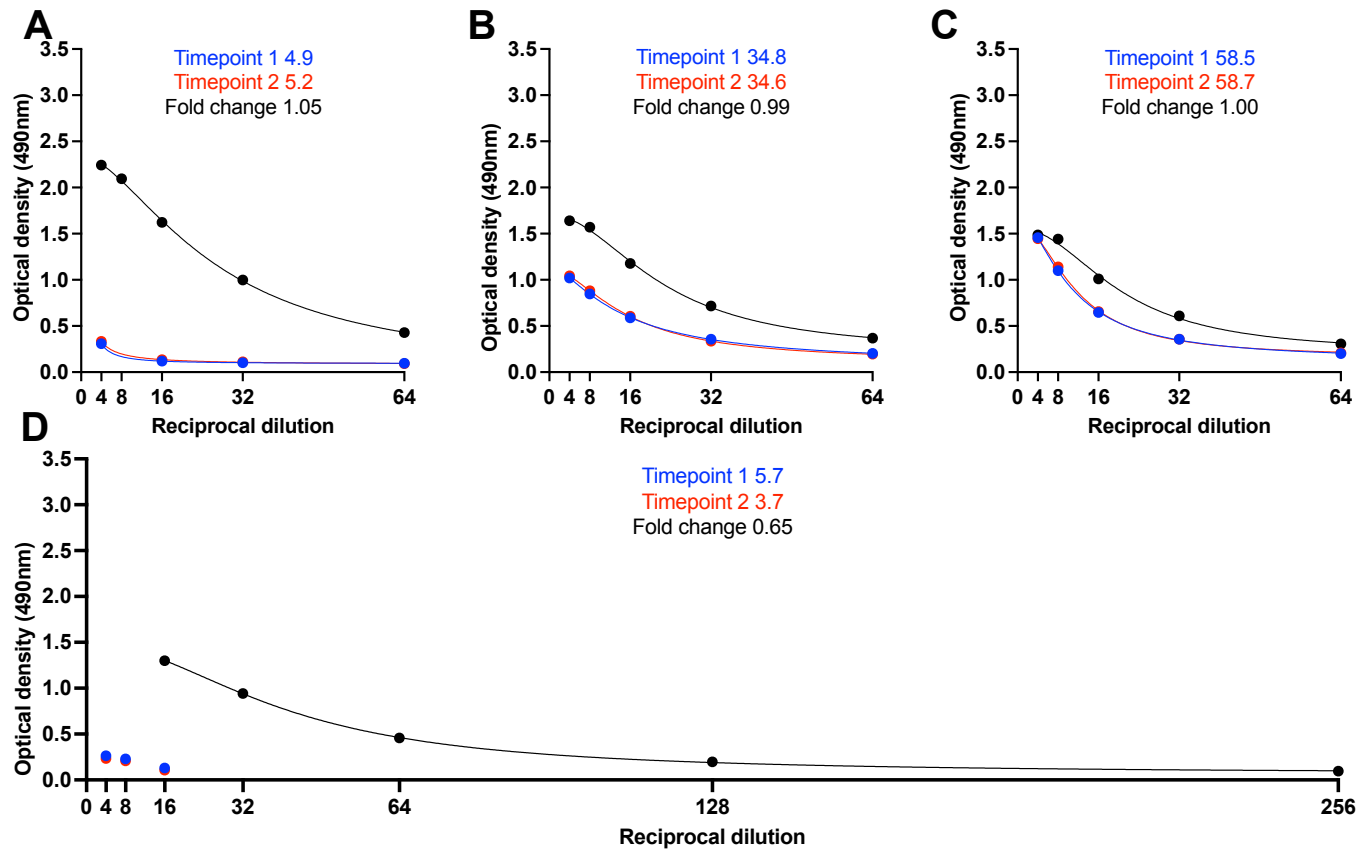


Figure 3.13 ELISA results for test samples demonstrating no change in Nlac IgG

OD at each reciprocal dilution (circles) and 4PL curves shown for timepoint 1 (blue) and timepoint 2 post challenge (red) test samples in comparison to reference serum C (black). Relative Nlac IgG titres and fold change shown.

A-C: 4x – 64x dilution of test and reference samples, D: 4x – 64x dilution of test samples, 16x – 256x dilution of reference sample (extended dilution). No 4PL curve generated from ELISA of test samples as only 3 valid OD results available.

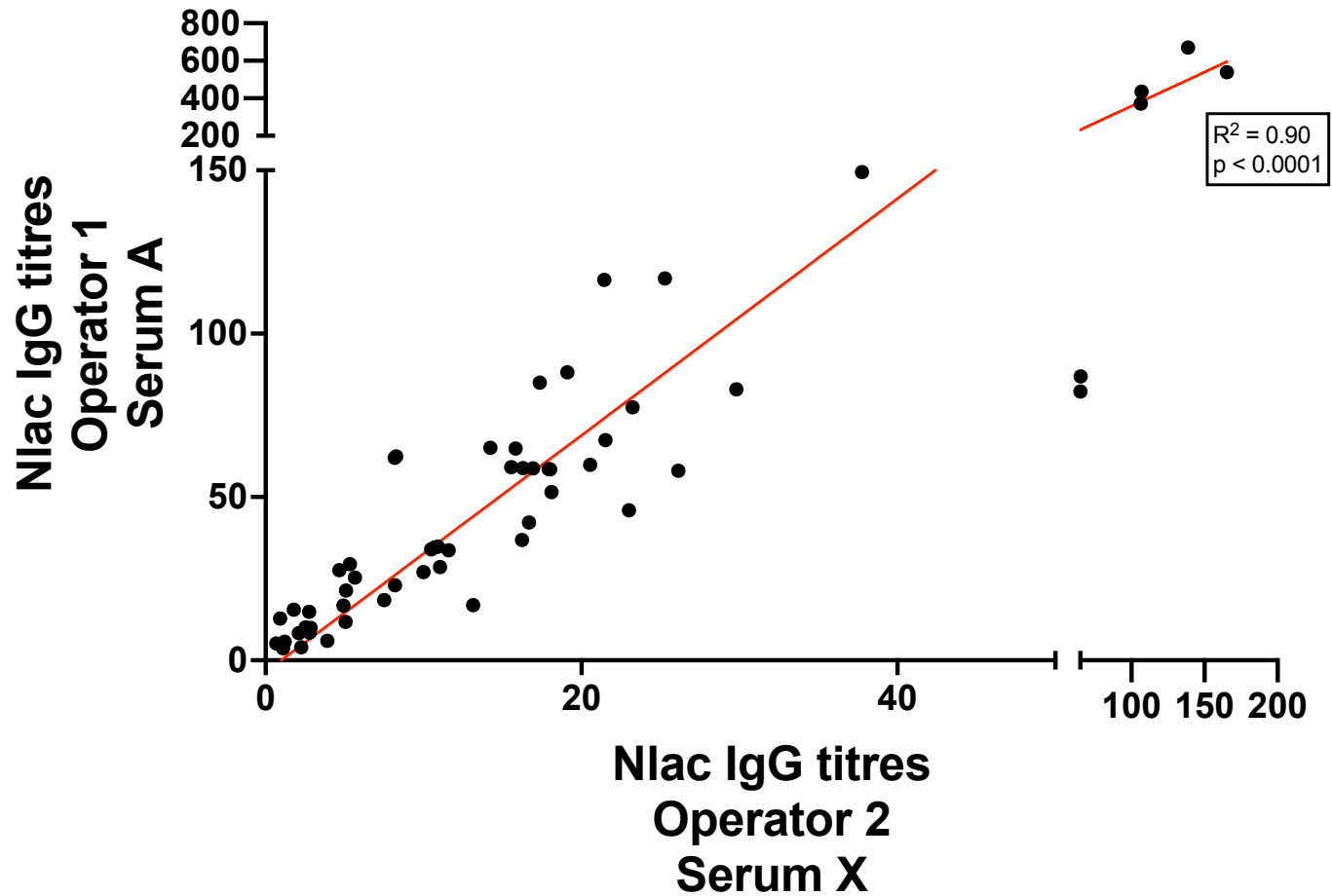


Figure 3.14 Comparison of Nlac IgG titres generated by two operators

Simple linear regression analysis of Nlac IgG titres derived from UoS Nlac IgG ELISA performed by two different operators, with two different reference samples. Black circles indicate individual test samples, p and R_s derived from Spearman correlation.

3.4 Discussion

This chapter describes the development, optimisation and validation of an in-house ELISA to determine the relative concentration of Nlac Y92-1009 dOMV specific IgG in serum samples. A draft UoS ELISA protocol (Section 3.2.1) was based on the Porton Down ELISA, used for previous Nlac CHI studies, but modified for use within the University of Southampton laboratories. It was optimised in a step-wise fashion with repeated comparison of the variance between duplicates, between different dilutions, between plates, and between days.

Good agreement was seen between duplicate and triplicate values, particularly once the impact of “edge effect” was considered and affected values excluded. There was notable variation in the background signal detected on different plates and different days, potentially due to differences in ambient temperature, temperature of reagents or timing of incubation steps. Despite this, the precision of the assay was within acceptable limits with a median %CV < 15% when comparing the same serum sample run both on the same plate and on different plates. The variance over different days was marginally over the nominal 15% threshold at 15.2%.

The draft protocol was amended to mitigate potential causes of variation with the addition of warmed PBS in the outermost, empty wells of each assay plate and increased control reagent temperature and incubation step timings. The SOP was worded to ensure that all samples from a single participant would be run on a single plate, and an upper acceptable limit of negative control OD was set so that plates with a high background reading would be excluded and the ELISA repeated for those samples. Individual OD values with a high %CV between duplicates, or with evidence of steric hindrance, would be excluded from analysis.

This finalised ELISA protocol was then assessed across multiple serum samples and shown to be usable, with a minority of samples requiring repeat analysis, and good agreement between duplicate results. A wide range of Nlac IgG titres were determined, with a change in titre across time-points demonstrated. Repeat analysis of the same samples by a second operator with a second reference serum correlated significantly.

The UoS Nlac IgG ELISA was therefore determined as fit-for-purpose for use in Nlac CHI studies, to measure antibody titres in sera sampled longitudinally from baseline to post-challenge.

Chapter 4 Optimising the *N. lactamica* controlled human infection model: Dose and duration of colonisation

4.1 Introduction

The aims of LACTAMICA 3 (L3) were to optimise the previously developed Nlac CHIM and to answer specific questions to inform the design of a planned study with a genetically modified strain of Nlac (GM Nlac). This MRC funded study – LACTAMICA 4 (L4) was expected to have stringent control requirements such as the complete containment of participants for a specified period of colonisation, the reliable clearance of colonisation at the end of that period, and careful monitoring of bacterial shedding throughout colonisation to identify and clear “supershedders”. The design, conduct and results of L4 are detailed in Chapter 5.

L3 was primarily designed to evaluate the immunogenicity of two different durations of Nlac colonisation, comparing a short duration of 4 days with a longer duration of 14 days. The effect of duration of nasopharyngeal bacterial colonisation on immunogenicity is unknown, and this applies to all bacterial colonisers of the upper respiratory tract, including *Haemophilus spp.*, and the pneumococcus as well as *Neisseria spp.* The practical value of L3 was to allow the period of colonisation in L4, and therefore the period of containment and follow-up of participants, to be minimised with considerable advantages to the cost, logistics and participant experience.

Pre-clinical testing of the GM Nlac strain was sufficiently reassuring that strict containment of participants in L4 was not anticipated to be required by the regulatory authorities (The Department of Environment, Food and Rural Affairs, responsible for approval of all deliberate release of genetically modified organisms) (150). L4 could therefore be designed to allow the optimal reasonable period of potential colonisation to generate a measurable immune response and was therefore able to commence prior to the completion of L3.

In the original Nlac CHIM studies, the standard inoculum dose was 10^4 CFU (123, 124). A small group of subjects who had not been colonised by challenge with this standard dose, had received a higher dose of 10^5 CFU with no safety concerns, and a colonisation fraction of 50% (123). Another aim of L3 was to further assess the safety and colonisation fraction of this higher inoculum dose in a less selected group of healthy volunteers.

A reliable and rapid method of clearing Nlac carriage was required for the safe design of L4. Ciprofloxacin clears Nmen carriage within 24 hours (41) and as Nlac is highly sensitive to ciprofloxacin (103) it was also expected to rapidly clear Nlac carriage. A further aim of L3 was to assess the efficacy and speed of a single dose of oral ciprofloxacin in clearing Nlac colonisation. The assessment of environmental shedding from colonised subjects would also help inform the design and safe conduct of L4.

4.2 Study specific methods

4.2.1 Study design

In this prospective controlled human infection study, participants were inoculated intranasally with wild type Nlac at an intended dose of 10^5 CFU. Eradication (ciprofloxacin) therapy was given at Day 4 (Group 1) or Day 14 (Group 2) post challenge to terminate colonisation with confirmation of clearance after 24 hours. Colonisation, shedding, immunogenicity and efficacy of eradication were assessed, and participants were followed up until Day 28 post eradication. The intended sample size was 10 colonised participants per group. Participants and investigators were not blinded to group allocation which was on a convenience basis. Laboratory staff processing serum samples were blinded to group allocation.

An overview of the study timelines for Groups 1 and 2 are shown in Figure 4.1. The objectives and endpoints of this study are summarised in Table 4.1 and the schedule of procedures for Groups 1 and 2 are shown in Tables 4.2 and 4.3. The eligibility criteria are detailed in Chapter 2.

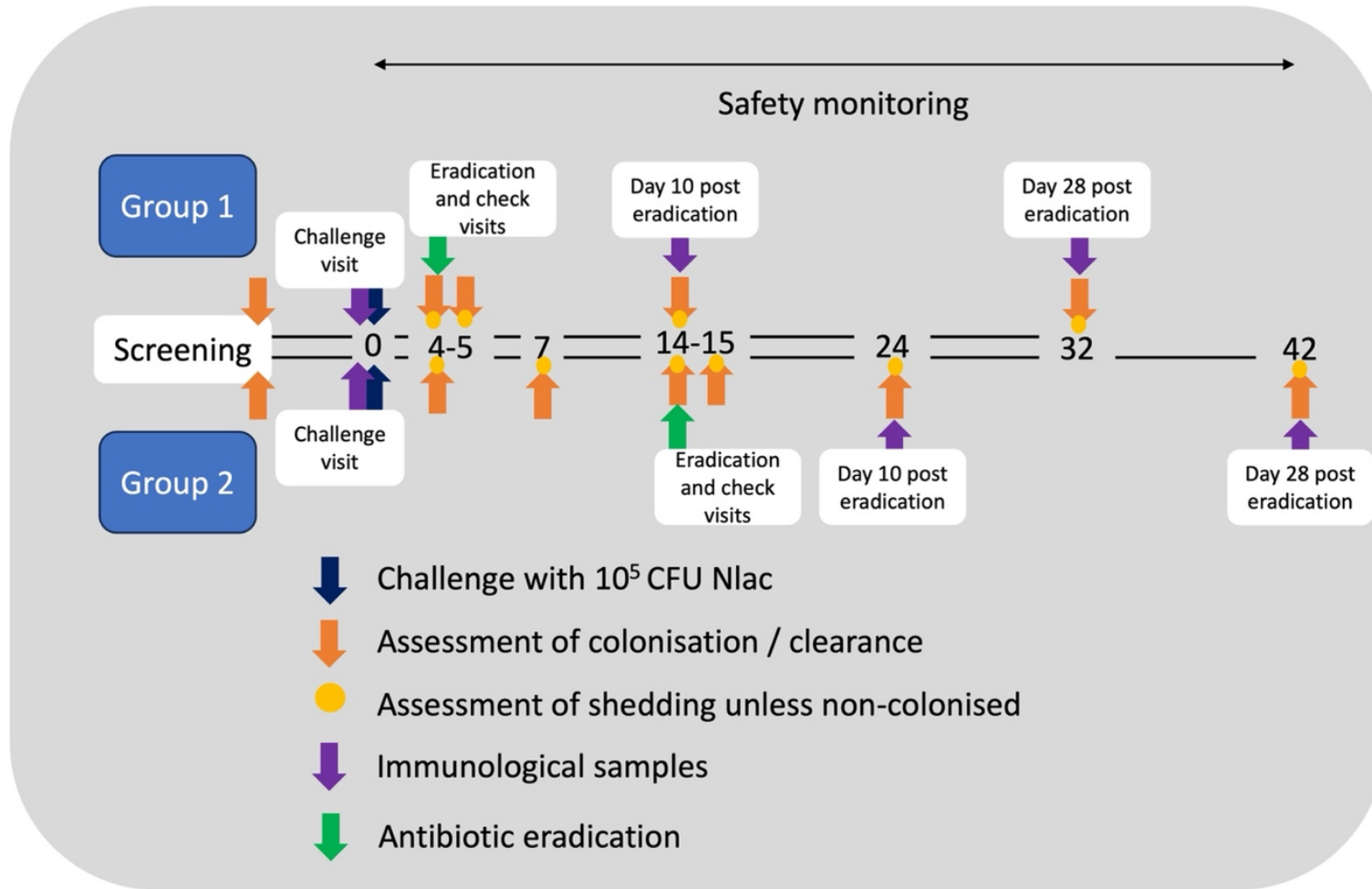


Figure 4.1 L3 Study timeline
 Schematic presentation of study timeline for L3 participant visits, interventions and monitoring with comparison between Group 1 and 2.

Objectives		Endpoints
Primary objective	To compare the effect of short (4 days) versus longer (14 days) oropharyngeal carriage on systemic humoral immunogenicity following nasal inoculation with Nlac	Rise in serological specific antibody titre comparing Day 0 versus Day 14 and 28 days post eradication and comparing early (Day 4) versus late (Day 14) eradication
Secondary objective	To assess the efficacy of oral ciprofloxacin in eradicating nasal Nlac colonisation by 24 hours after treatment	Culture of Nlac from throat swabs on the day of eradication, one day, 10 days and 28 days after eradication
Additional microbiological endpoints		Assessment of colonisation – culture of Nlac from throat swabs at Day 4 for Group 1 and Days 4, 7 and 14 for Group 2
		Difference in environmental shedding between short (Group 1) and longer (Group 2) periods of colonisation.
Safety endpoints		Occurrence of unsolicited adverse events within the study period
		Occurrence of serious adverse events within the study period

Table 4.1 L3 Objectives and endpoints

Adapted from L3 study protocol (Appendix A1)

Timeline (days)	Screening	Inoculation and follow up				
	≤ 90	0	4	5	14	32
TOPS confirmation	+					
Volunteer Information Sheet	+					
Informed consent	+					
Vital signs	+	+	(+)	(+)	(+)	(+)
Medical history	+					
Physical examination	+	(+)	(+)	(+)	(+)	(+)
Pregnancy test (females only)	+	+	+			
Review eligibility		+				
Inoculation		+				
Eradication			+			
Review of AEs / concomitant medications			+	+	+	+
Nasal wash	+				+	+
Throat swab	+	+	+	+	+	+
Environmental samples			+	+	+	+
Immunological blood tests (ml)		20			20	20
Cumulative blood volume (ml)		20			40	60

Table 4.2 L3 Schedule of procedures - Group 1

+ Procedure at this timepoint, (+) if clinically indicated

Adapted from L3 study protocol (Appendix A1)

Timeline (days)	Screening	Inoculation and follow up						
	≤ 90	0	4	7	14	15	24	42
TOPS confirmation	+							
Volunteer Information Sheet	+							
Informed consent	+							
Vital signs	+	+	(+)	(+)	(+)	(+)	(+)	(+)
Medical history	+							
Physical examination	+	(+)	(+)	(+)	(+)	(+)	(+)	(+)
Pregnancy test (females only)	+	+			+			
Review eligibility		+						
Inoculation		+						
Eradication					+			
Review of adverse events and concomitant medications			+	+	+	+	+	+
Nasal wash	+				+			+
Throat swab	+	+	+	+	+	+	+	+
Environmental samples			+	+	+	+	+	+
Immunological blood tests (ml)		20			20			20
Cumulative blood volume (ml)		20			40			60

Table 4.3 L3 Schedule of procedures - Group 2

+ Procedure at this timepoint, (+) if clinically indicated, adapted from L3 study protocol (Appendix A1)

4.3 Results

4.3.1 Recruitment

All required approvals were gained in early 2017 and recruitment was commenced in April 2017. Due to competition with other studies, recruitment was paused from October 2017 to May 2018. With the approval and commencement of L4 in October 2018, recruitment was again paused between August 2018 and August 2019 to prioritise recruitment to L4. Further recruitment then continued until early 2020 when the study was paused due to the emerging SARS-CoV-2 pandemic. The planned sample size of 11 colonised participants per group had not been met, but due to ongoing difficulties with recruitment, an interim data analysis was carried out late 2021. The immunological data available at this point were sufficient to meet the primary objective and so the recruitment was closed, following consultation with the external safety committee.

Details of participant screening, enrolment, group allocation and inclusion in analyses are shown in Figure 4.2.

Demographic details for challenged participants with comparison between Group 1 and Group 2 are shown in Table 4.4. There was a significant difference in the proportions of males and females between the two groups, with all participants in Group 2 being female.

4.3.1 Inoculum dose and purity

The confirmed doses of inoculum administered to each participant are shown in Figure 4.3, comparing Group 1 and Group 2. Most participants received a dose higher than the intended dose of 1×10^5 CFU. The median dose for Group 1 was 1.15×10^5 CFU (IQR $9.1 \times 10^4 - 1.8 \times 10^5$ CFU) and for Group 2 was 1.53×10^5 CFU (IQR $1.2-4.7 \times 10^5$ CFU), however there was not a statistically significant difference between the two groups. There were some notable outliers who received a much higher than intended dose (maximum 5.9×10^5 CFU in Group 2). Culture of all residual inocula resulted in a pure growth of Nlac.

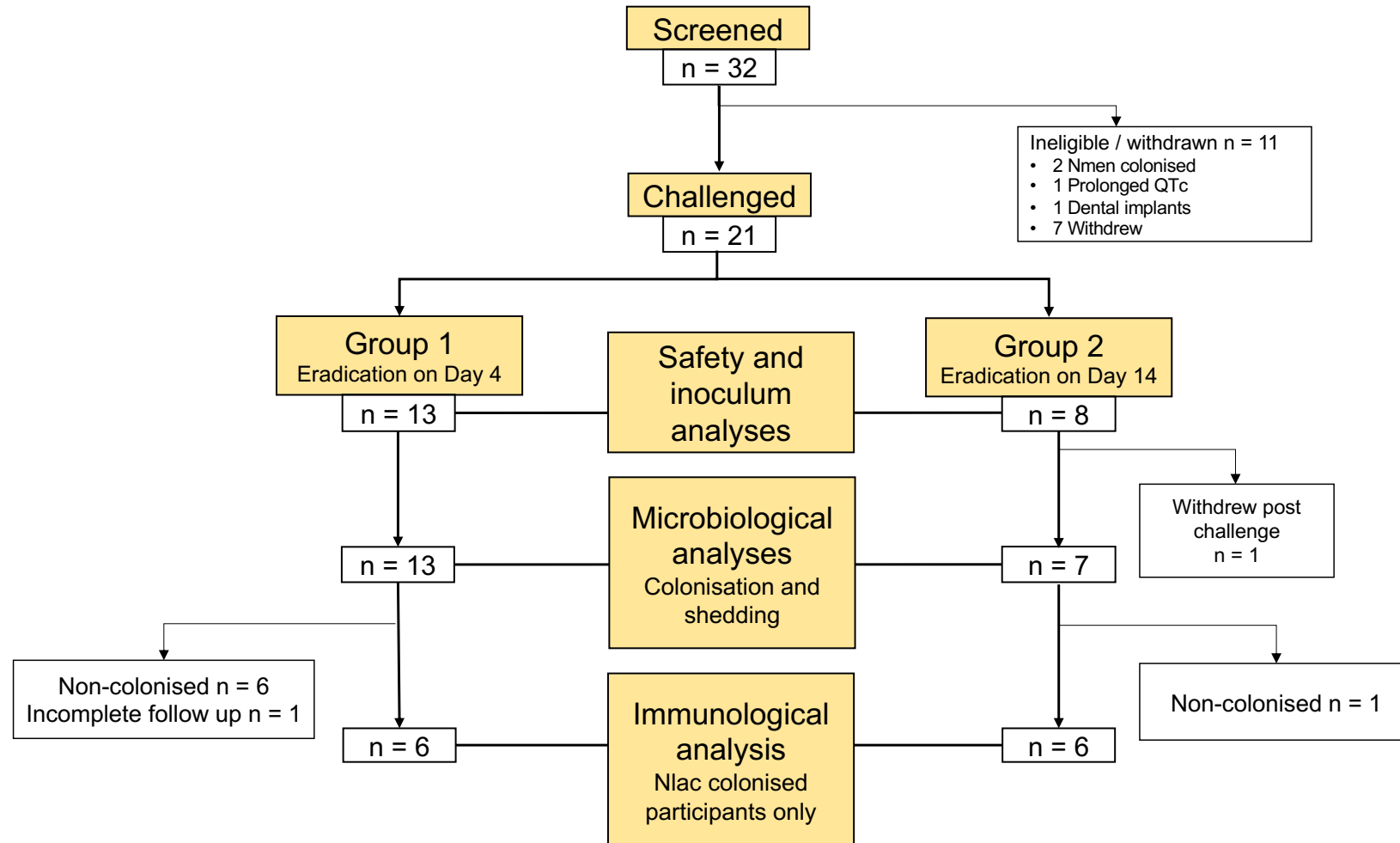


Figure 4.2 L3 Participant recruitment

Overview of participant recruitment for L3 with number of participants (n) screened, enrolled and included in each analysis set.

	Number of participants n	Sex Male n (%) Female n (%)	Age Median (IQR)	BMI Median (IQR)
Group 1	13	8 (38.5) 5 (61.5)	33.0 (27.5 – 42.5)	26.5 (22.3 – 30.2)
Group 2	8	0 (0) 8 (100)	29.0 (28.3 – 33.0)	26.6 (22.1 – 31.9)
<i>p</i> value		0.007 **	0.24 (NS)	0.97 (NS)

Table 4.4 L3 Participant demographics

Comparison of demographics for participants challenged in Group 1 (Eradication on Day 4) and Group 2 (Eradication on Day 14)

Sex – *p* value calculated using Fisher’s exact test

Age and BMI – *p* values calculated using Mann Whitney test.

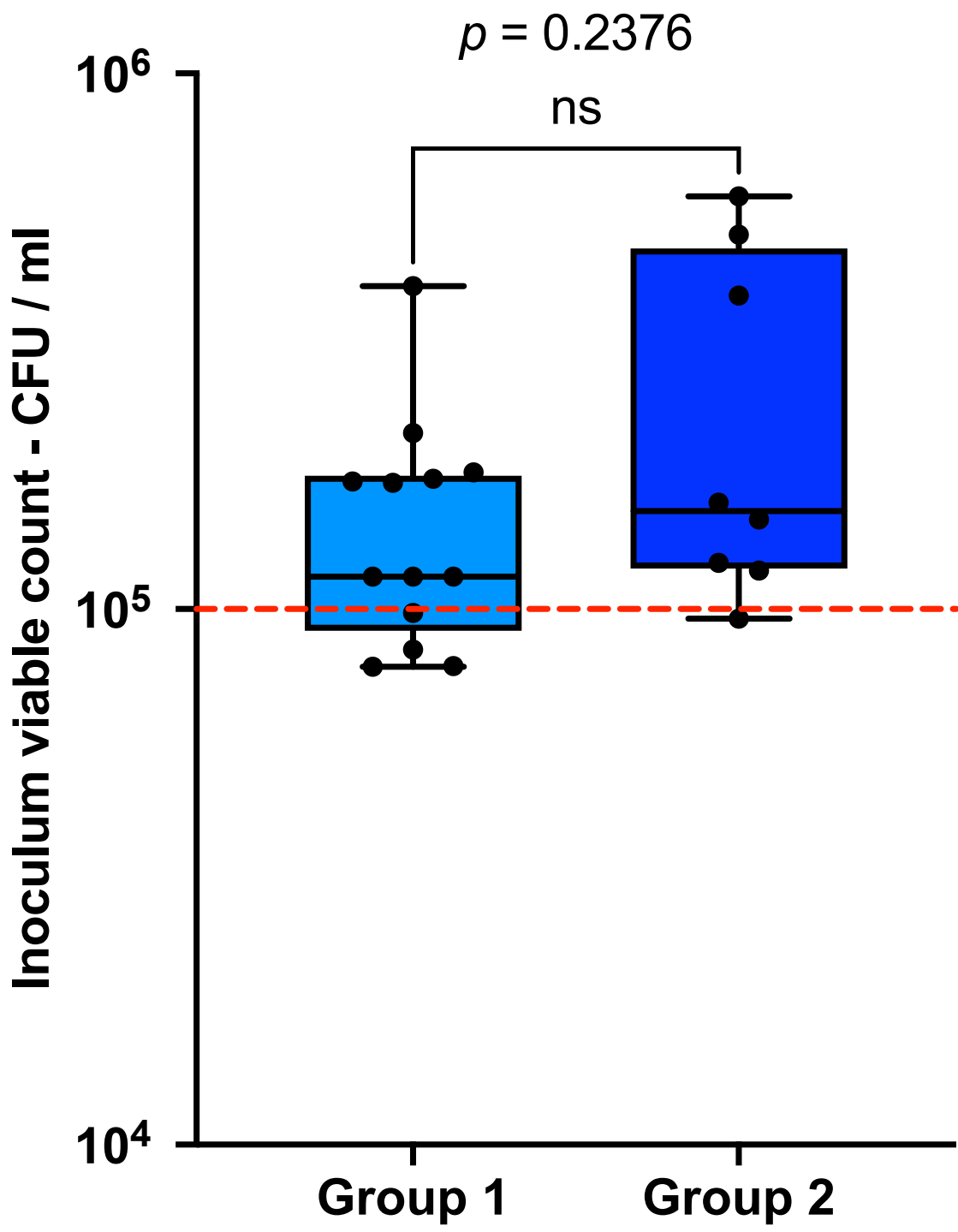


Figure 4.3 L3 Inoculum doses

Confirmed doses calculated by viable count of the residual inoculum following inoculation. Individual values, median and range shown, red dotted line indicates the intended dose of 1×10^5 CFU. p value derived from Mann Whitney test.

4.3.2 Colonisation

The colonisation fraction (proportion of participants colonised with Nlac) at Day 4 was 0.54 (7 of 13, 95% CI 0.29-0.77) in Group 1 and 0.43 (3 of 7, 95% CI 0.16-0.75) in Group 2.

Those in Group 1 then received eradication therapy as per protocol and no further acquisition of colonisation was seen. In Group 2 the colonisation fraction rose to 0.86 (6 of 7, 95% CI 0.49-0.99) by Day 7. One participant showed spontaneous loss of carriage prior to eradication on Day 14. Eradication therapy was seen to be effective with no carriage detected from one day post eradication therapy onwards in either group. The colonisation status over time for individual participants is shown for Group 1 and Group 2 in Figure 4.4.

4.3.3 Shedding

Environmental samples were obtained at each study visit after challenge until participants had already been shown to be non-colonised following eradication. No Nlac was detected in any environmental samples from any participants, whether colonised or not. This may suggest that shedding does not occur, or that it is outside the limit of detection with the sampling techniques used. Of note, other respiratory commensal organisms such as viridans streptococci and commensal *Neisseria* such as *N. flavescens* and *N. subflava* were detected from these samples.

4.3.4 Safety

There were no significant safety concerns or serious adverse events. No events were assessed as likely to be related to challenge or colonisation with Nlac.

Two participants reported symptoms which were assessed as possibly related to challenge due to the temporal relationship with challenge. The first of these was mild nasal congestion from Day 1 post challenge, which resolved following the use of over-the-counter medication. The other was a participant who reported a sore throat for seven days from the day of challenge, and lethargy which continued until Day 14 post challenge, although of note this was during a period of unusually hot weather. Both of these symptoms were mild and self-resolved. One AE was assessed as likely related to a study procedure (onset of sore throat immediately following a throat swab). This self-resolved within 24 hours.

All adverse events are summarised in Tables 4.5 and 4.6.

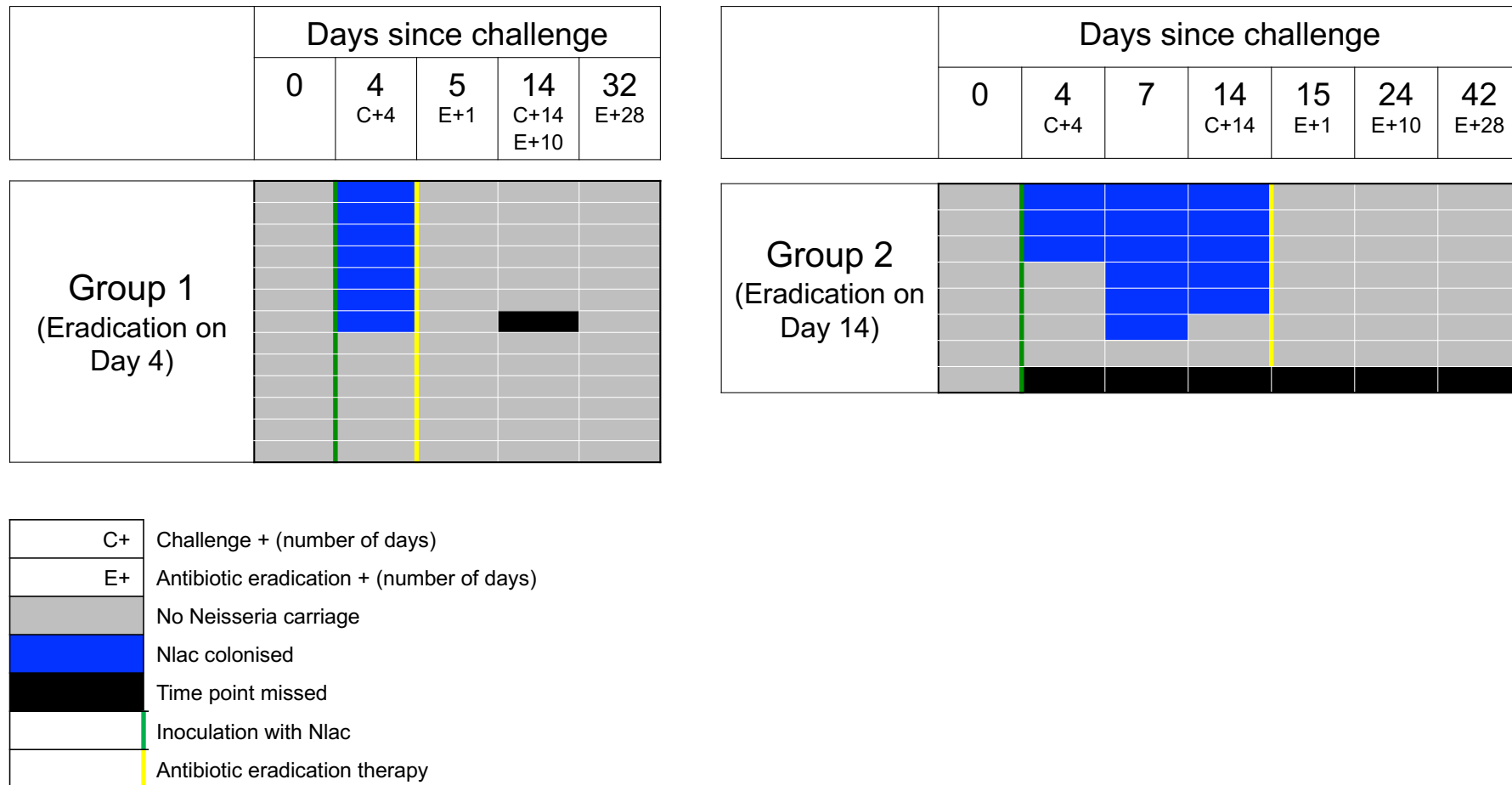


Figure 4.4 Colonisation status over time

Colonisation with Nlac at each timepoint for individual participants. Each row represents one participant, colonisation defined as the culture of at least one colony of Nlac from a throat swab or nasal wash taken at the time points shown. No Nmen colonisation detected.

Start day	End day	Symptoms	Investigations	Treatment	Comments	Severity	Relatedness	Serious
Pre challenge	14	Foot pain		Ibuprofen	Onset when running	II	No relationship	N
1	2	Nasal congestion		Antihistamine	Contacts with similar symptoms	II	Possibly related	N
4	5	Sore throat			Onset after throat swab	I	Likely related	N
12	14	Coryzal, cough		Paracetamol		II	Unlikely related	N
16	43	Coryzal, cough	Viral PCR NAD			I	Unlikely related	N

Table 4.5 Adverse events – Group 1

Adverse events reported by participants in Group 1 during follow up period.

Start day	End day	Symptoms	Investigations	Treatment	Comments	Severity	Relatedness	Serious
Pre-challenge	14	Cyst behind ear		Drainage		II	No relationship	N
23	23	Malaise		Ibuprofen		II	No relationship	N
0	15	Lethargy	Safety bloods / viral swab NAD		Heatwave	I	Possibly related	N
0	7	Sore throat	Safety bloods / viral swab NAD			I	Possibly related	N
14	15	Headache		Paracetamol	Onset before eradication	II	Unlikely related	N
14	15	Vomiting		Ibuprofen, dioralyte, Antiemetics	Onset before eradication	III	Unlikely related	N
14	15	Diarrhoea			Onset before eradication	III	Unlikely related	N
15	15	Sore throat				I	Unlikely related	N
17	24	Sore throat				II	Unlikely related	N
41	42	Dysuria/cystitis		Paracetamol		II	No relationship	N

Table 4.6 Adverse events - Group 2

Adverse events reported by participants in Group 2 during follow up period.

4.3.5 Serological responses

Nlac and Nmen dOMV specific IgG titres were compared at baseline (D0), Day 14 post challenge (C14) and Day 28 post administration of antibiotic eradication therapy (E28) for participants who were challenged, colonised and had serum samples collected at each time point, comparing Group 1 (eradication therapy at Day 4, n=6) and Group 2 (eradication therapy at Day 14, n=6).

No difference was seen between Group 1 and Group 2 in baseline titres of either Nlac or Nmen IgG, as shown in Figure 4.5. The median baseline Nlac IgG titre was 5.3 arbitrary units (AU) for Group 1 and 4.6 AU for Group 2 and the median baseline Nmen IgG titre was 22.6 AU for Group 1 and 10.4 AU for Group 2.

Nlac and Nmen IgG titre trends for each participant are shown in Figure 4.6. A fold change of ≥ 2 , indicated by a star, was seen by C14 in three participants in Group 2 (50%) for Nlac IgG. For each of these the titre reduced by E28 but remained ≥ 2 -fold above baseline. Two participants in Group 2 (33.3%) had a ≥ 2 -fold increase in Nmen IgG from baseline. By E28 these had reduced to 1.52 and 1.63 times baseline. No participants in Group 1 had a ≥ 2 -fold rise in either titre. Median IgG titre fold changes for each group are shown in Table 4.7.

Summary data for each group are shown in Figure 4.7. A significant increase was seen in both Nlac and Nmen specific titres by Day 14 in Group 2 ($p = 0.003$ for Nlac IgG and $p = 0.008$ for Nmen IgG). By Day 28 post eradication, this remained a significant increase above baseline for Nmen specific IgG ($p = 0.04$) but was no longer significant for Nlac IgG ($p = 0.09$). No increase in either Nlac or Nmen specific IgG titres was seen in Group 1. This demonstrates an Nlac specific and cross-reactive Nmen specific humoral immune response in participants colonised with Nlac for 14 days (Group 2), but not in those colonised for 4 days (Group 1).

The relationship between Nlac and Nmen specific humoral immune responses to Nlac colonisation was assessed by comparing fold change in Nlac and Nmen IgG titre from baseline to C14 each individual Nlac colonised participant. A significant correlation was seen between the two (Spearman correlation coefficient = 0.83, $p = 0.0013$) as shown in Figure 4.8.

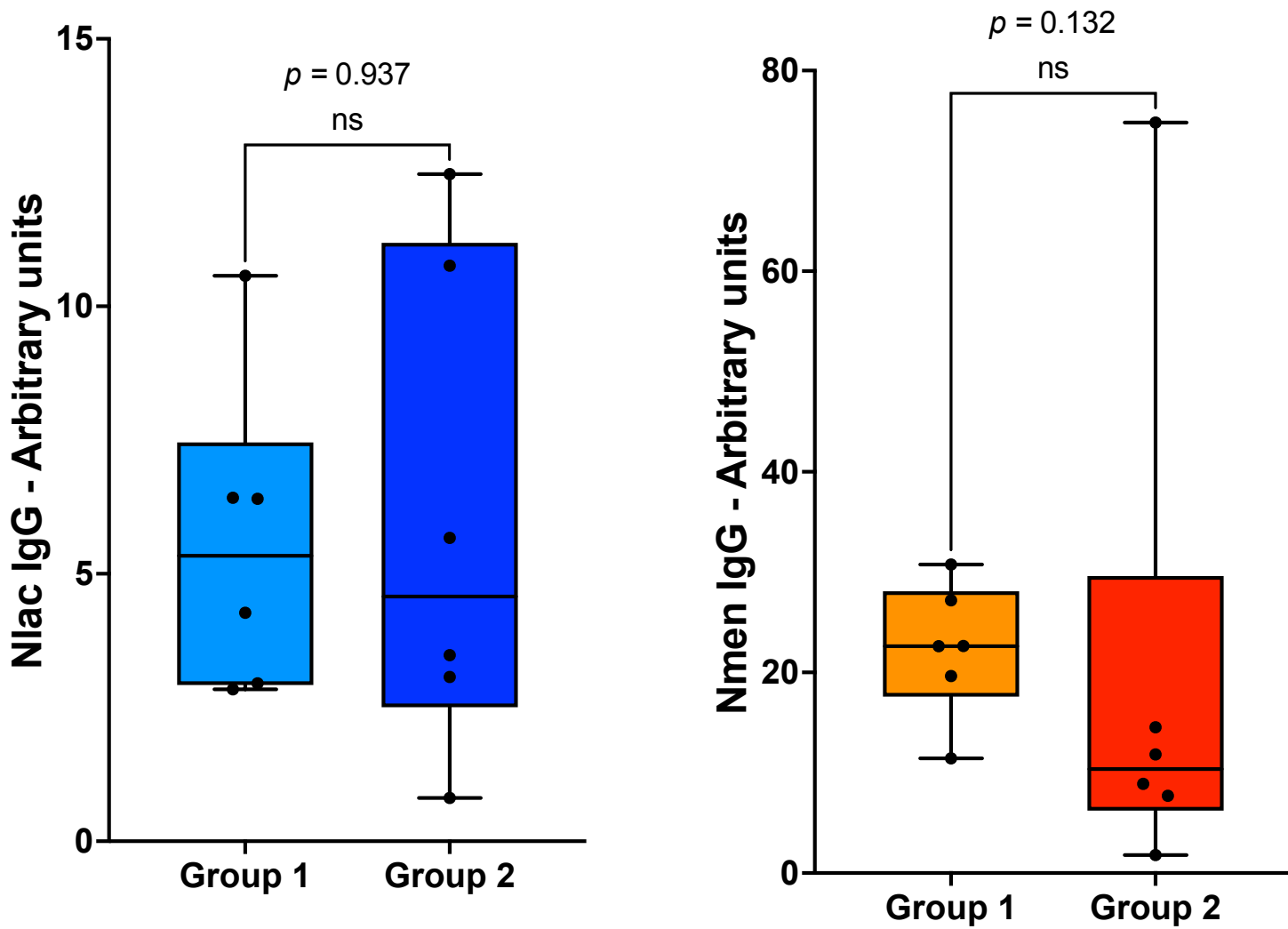


Figure 4.5 Baseline Nlac and Nmen IgG titres

Nlac (blue) and Nmen (red) IgG titres at baseline for Group 1 and Group 2.

Individual values, median, IQR and range shown, *p* values derived from Mann-Whitney test.

Group 1

Group 2

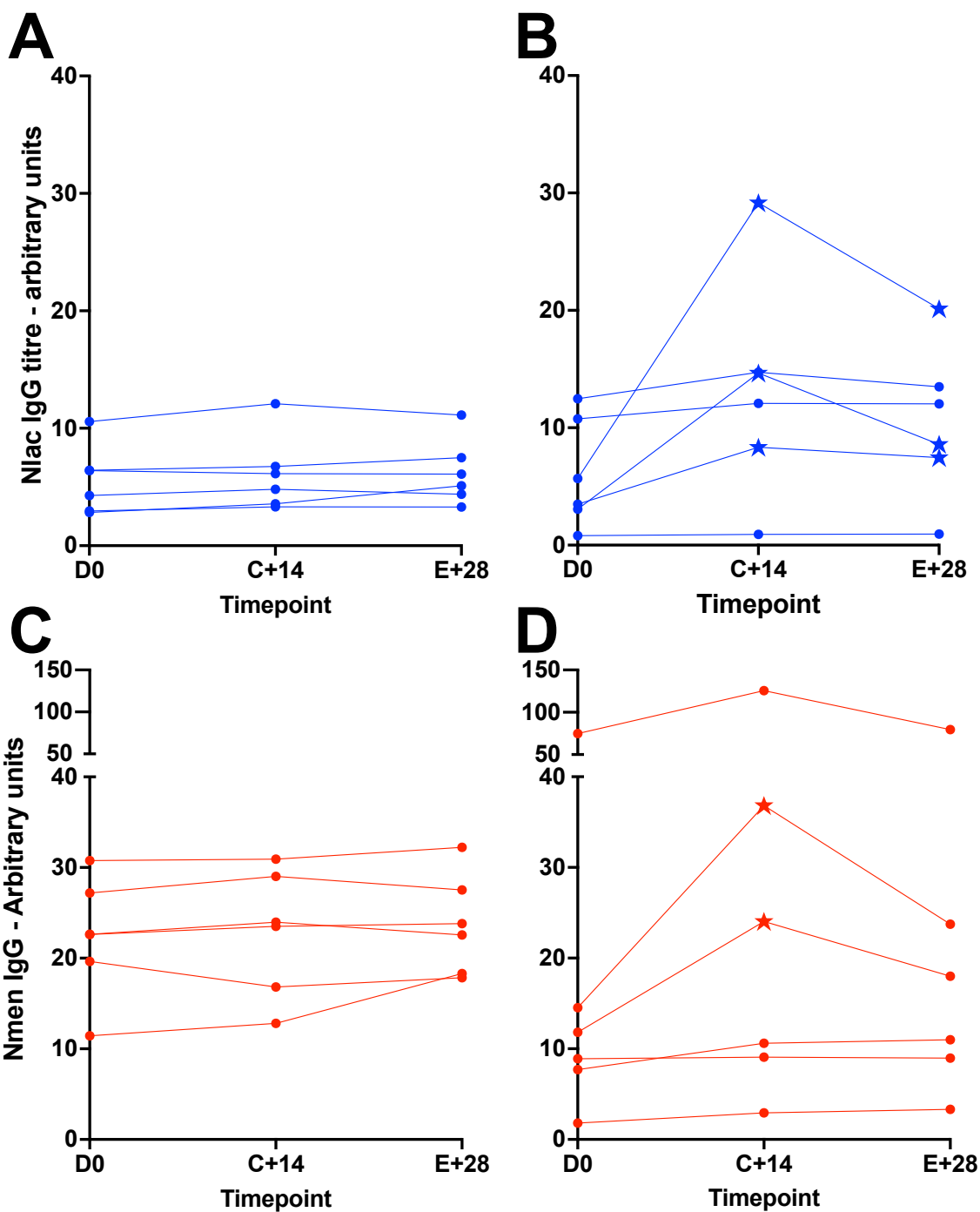


Figure 4.6 Niac and Nmen IgG trends following Niac colonisation

Individual participant titres shown at baseline (D0), 14 days post challenge (C14) and 28 days post antibiotic eradication therapy (E28). Stars indicate ≥ 2 -fold rise from baseline.

A = Niac IgG, Group 1, B = Niac IgG, Group 2, C = Nmen IgG, Group 1, D = Nmen IgG, Group 2.

	Nlac IgG		Nmen IgG	
	Group 1	Group 2	Group 1	Group 2
C+14 Median (IQR)	1.13 (1.03 – 1.17)	1.79 (1.14 – 4.86)	1.05 (0.97 – 1.08)	1.66 (1.28 – 2.16)
E+28 Median (IQR)	1.09 (1.01-1.33)	1.67 (1.11 – 2.98)	1.03 (0.98 – 1.19)	1.48 (1.05 – 1.68)

Table 4.7 Nlac and Nmen IgG fold change following Nlac colonisation

Fold change from baseline to C+14 = 14 days post challenge and E+28 = 28 days post antibiotic eradication therapy, median and IQR shown per group.

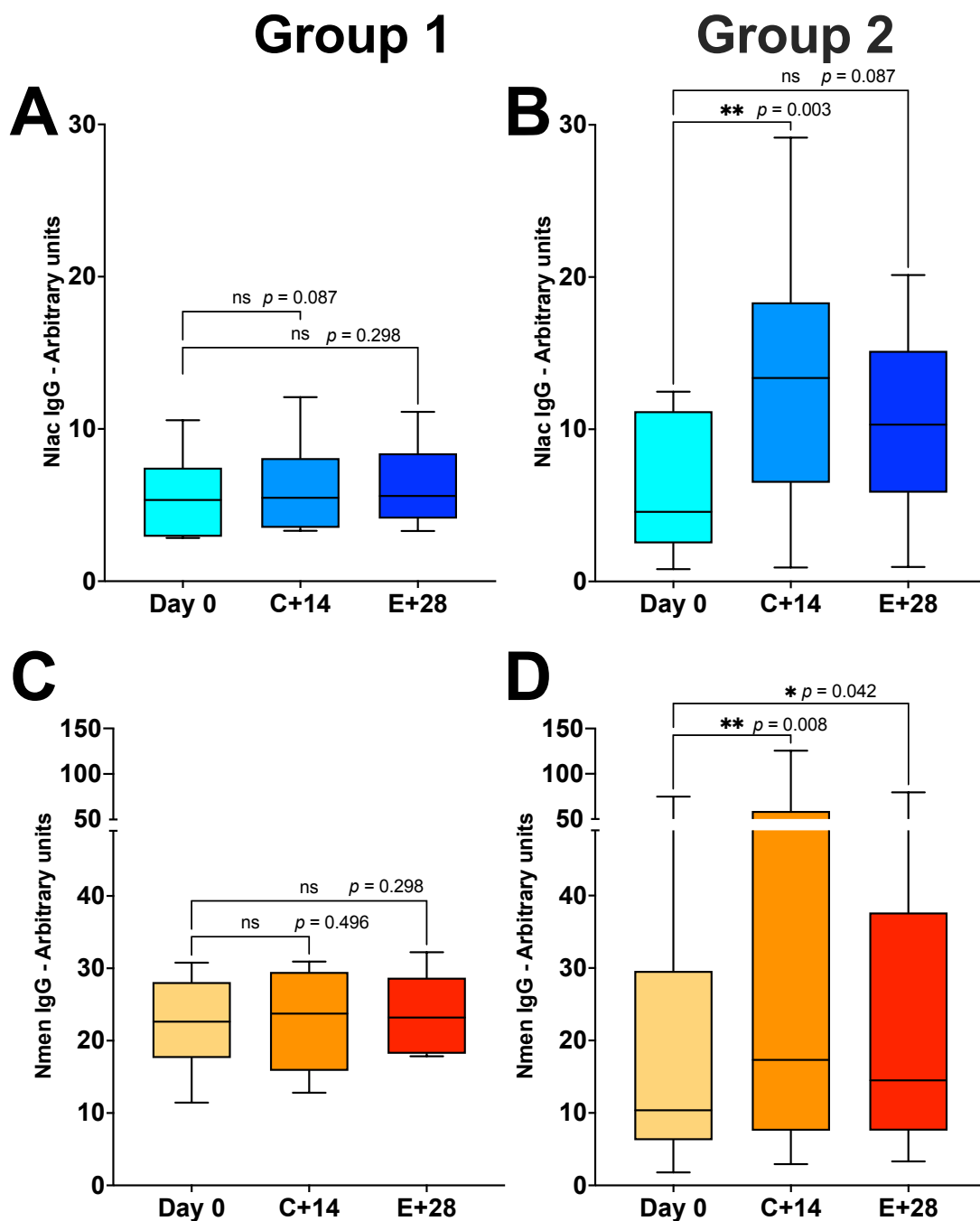


Figure 4.7 Niac and Nmen specific IgG titres following Niac colonisation
 Median, IQR and range shown for each group at each timepoint, Day 0 = Day of Niac challenge, C+14 = 14 days post challenge, E+28 = 28 days post antibiotic eradication therapy.

p values derived from Friedman test with Dunn's multiple comparisons test

A = Niac IgG, Group 1, B = Niac IgG, Group 2, C = Nmen IgG, Group 1,

D = Nmen IgG, Group 2.

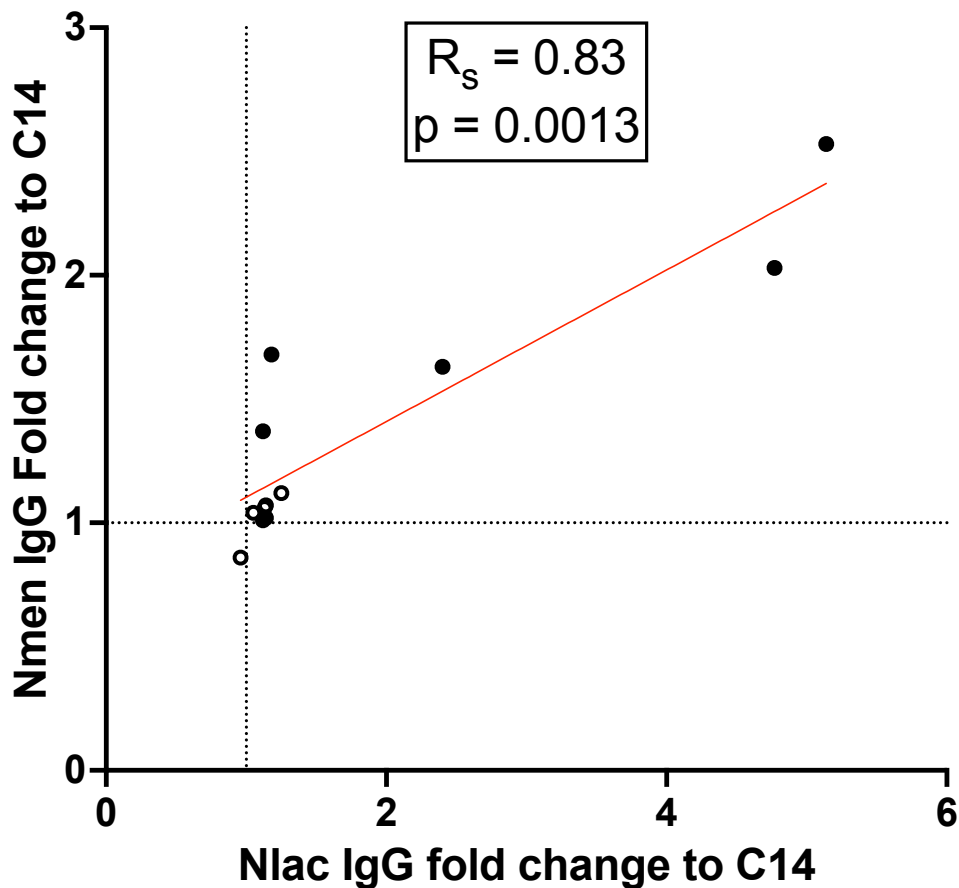


Figure 4.8 Simple linear regression analysis of Nlac IgG vs Nmen IgG fold change IgG fold change from D0 to C14 with Spearman correlation analysis.

Open circles = Group 1, filled circles = Group 2.

Dotted lines indicate no change from baseline.

4.4 Discussion

The aim of this study was to optimise the previously developed Nlac CHIM, specifically to assess the use of an increased inoculum dose, the impact of limiting the duration of colonisation upon immunogenicity, the efficacy of antibiotic eradication therapy, and to assess shedding of Nlac from colonised participants.

The challenge dose in L3 (10^5 CFU) was ten-fold higher than that used for the majority of challenges in previous Nlac CHIM studies (123, 124). This higher dose had previously been used to rechallenge a small group of participants ($n=6$) who had not been colonised following two challenges with the lower inoculum dose (123), but not as an initial challenge dose in an unselected group of participants.

In L3, the colonisation fraction of 0.86 by Day 14 among those who had not received antibiotics prior to that point (Group 2). This compares to a colonisation fraction of 0.34-0.59 by Day 14 at a dose of 10^4 CFU in the previous Nlac CHIM studies (123, 124). There were no safety concerns in L3 following the use of this increased dose.

Although the sample size in this study is small, it suggests that the use of this increased dose is safe, well tolerated, and results in an improved colonisation fraction, making it an appropriate dose to use in future Nlac CHIM studies.

This study has confirmed the previous findings that experimental nasopharyngeal colonisation with Nlac is immunogenic (123), with the induction of humoral responses specific to both Nlac and Nmen. Although the serological responses appear modest, it should be noted that this was a bespoke ELISA assay, and the levels of response cannot be reliably compared with those observed in other studies.

We have demonstrated that the duration of colonisation influences immunogenicity. An increase in Nlac and Nmen specific IgG was only seen in participants in whom colonisation was allowed to continue to Day 14 post challenge. The short colonisation duration of four days was chosen for logistical reasons, allowing a potential inpatient period to fit into a Monday-Friday working week. The longer duration of 14 days was chosen as the nominal time taken for the induction of an acquired immune response.

In Group 2, Nlac and Nmen IgG titres peaked at Day 14 post challenge (at which point carriage was cleared) and then reduced by Day 28 post eradication. At this point Nlac IgG titres were no longer significantly above those at baseline. In a previous Nlac CHIM study

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with a prolonged period of colonisation, Nlac IgG rose approximately 2-fold from baseline by Day 14 post challenge and was then relatively stable over twelve weeks following challenge (123). This suggests that 14 days is a sufficient duration of colonisation to be immunogenic, but that prolonged colonisation allows ongoing exposure to antigens, resulting in a sustained humoral response. Further studies could interrogate the impact of colonisation duration further, potentially looking for a threshold duration at which an immune response is induced. The impact of colonisation duration on other immunological endpoints could be investigated, for example using functional assays, and also protection against rechallenge.

This finding is supported by a previous murine study of nasopharyngeal colonisation with a mutant strain of *Streptococcus pneumoniae*. A significantly higher serum IgG was seen at day 14 post challenge in mice colonised for a longer duration (> 5 days) in comparison to a shorter duration (< 48 hours). However, by day 28 this difference was no longer evident (160). In contrast, the live attenuated influenza vaccine (LAIV) is potentially immunogenic despite only a brief period of upper respiratory tract infection with restricted replication ability (161, 162).

In this study, a single dose of Ciprofloxacin was shown to be effective in clearing Nlac carriage within 24 hours in 100% of colonised participants. This is of vital importance in providing evidence for the safe and robust design of future studies where effective Nlac clearance is required, for example for a GM strain of Nlac where clearance of carriage is a pre-requisite for approval, or if clearance of carriage is required prior to rechallenge.

No shedding of Nlac was detected from colonised participants in this study. This may be because nasopharyngeal carriage of Nlac by adults does not result in shedding in respiratory droplets, or that the carriage density was below a threshold required for shedding of respiratory exhaled droplets, or because the techniques used were not sufficiently sensitive to detect shedding. However, the shedding of other respiratory organisms was identified using these methods. In addition, mechanically aerosolised Nlac was successfully detected using the Coriolis air sampler and culture method during the development of this technique, suggesting that negligible Nlac shedding from participants occurred.

The main limitations to this study were the difficulties with recruitment leading to a smaller than planned sample size. Recruitment occurred over three distinct time periods (April-Oct 2017, May-Aug 2018, Jan-March 2020). The main factor limiting recruitment was competition with other similar studies for clinical, nursing and laboratory time, and for

volunteer willingness to be screened and enrolled, as competing studies were offering a higher level of reimbursement. In March 2020 the study was paused due to the COVID-19 pandemic, following which an interim data analysis was performed. As the primary endpoint had been met, the study was formally closed, having completed six colonised participants per group, instead of the planned 10. If a larger sample size had been recruited then the resulting data may have been more robust, for example the Nlac IgG titres Day 28 post eradication may have remained statistically significant. In addition, this would have increased the opportunity to detect shedding from Nlac colonised participants.

In addition to this, the two groups were not ideally matched with a statistically significant differences in male : female proportions between the two groups, and an apparent but not statistically significant difference between the actual dose of inoculum administered. Recruitment of an increased sample size may have resolved these differences.

This study has demonstrated a relationship between duration of colonisation and induction of a specific immunological response. Future studies may build on this initial evidence, both with Nlac and potentially with other nasopharyneal commensals and pathobionts, to better understand the kinetics of the host immunological response to transient versus prolonged interaction with a micro-organism or antigen of interest.

Importantly this study provided evidence for an optimised Nlac CHIM, safely achieving colonisation of a high proportion of participants, with tools to assess for shedding and to quickly and reliably clear carriage. These will be invaluable in progressing the Nlac CHIM research programme.

Chapter 5 Colonisation with recombinant *N. lactamica* expressing the meningococcal adhesin NadA

5.1 Introduction

5.1.1 Study overview

LACTAMICA 4 (L4) was the first ever controlled human infection study using a knock-in genetically modified organism inoculated into the upper respiratory tract. It was designed to investigate the impact of the expression of the meningococcal antigen NadA on colonisation and immunogenicity, using the established Nlac CHIM already described.

Two genetically modified strains of Nlac were used. The intervention strain (designated 4NB1) was transformed to express NadA, while the control strain (designated 4YB2) was an empty vector strain. This control strain was exposed to the same process of genetic manipulation but without the addition of the *nadA* gene. It was therefore a wild-type equivalent strain allowing comparison of NadA expression alone, removing the potential for the transformative process itself to alter the behaviour of the organism.

As this study involved challenge with GMOs, it required UK Government Department of Environment, Food and Rural Affairs (DEFRA) approval and stringent safety and infection control measures, detailed in the sections below. The highest risk of development of invasive meningococcal disease is known to be the first 48 hours following acquisition (163, 164) so in the unlikely event of any participant developing disease it was anticipated that this would be most likely within the first few days following challenge. Participants were therefore admitted to the research facility for a period of 4.5 days following challenge and were closely observed for the development of any signs or symptoms of disease, with a plan for clearance of carriage or treatment of disease if it occurred.

Following the 4.5-day admission period, the remainder of the study was conducted on an outpatient basis. However, we aimed to limit the potential for transmission to close contacts of the volunteers, study team members and the wider population. Participants with potentially vulnerable close contacts were therefore excluded, and all participants were required to abide by infection control rules for the duration of their involvement in the study. Shedding of GM Nlac was monitored using environmental sampling and onward transmission to bedroom sharers was assessed. All participants received eradication

therapy at 90 days post challenge, or upon study withdrawal, regardless of their colonisation status, with confirmation of clearance after 24 – 48 hours.

5.1.2 GM Nlac strains

Wild-type Nlac is able to ferment lactose using β -D-galactosidase, encoded by the *lacZ* gene. Colonies of Nlac therefore appear blue when grown on media containing X-gal, allowing for their easy identification and distinction from other *Neisseria* species which do not possess *lacZ* (99). This characteristic was utilised in the production of the GM Nlac strains used in this study. A *lacZ* deficient strain of Nlac Y92-1009 (Δ *lacZ*) was initially produced, which resulted in white colonies when grown on X-gal-containing medium (150).

The intervention GM strain (4NB1) was derived from Δ *lacZ* by integrating the *Nmen* gene *nadA* linked to the Nlac gene *lacZ*. Successful transformation would result in the expression of the protein NadA, due to the presence of *nadA*, and the production of blue colonies on X-gal containing media due to the re-integration of *lacZ*. Colony colour could therefore be used as a marker of successful transformation. This phenotypic marker allowed the complete avoidance of antimicrobial resistance genes, which might otherwise have been used as a marker of transformation, but would have risked the dissemination of antimicrobial resistance genes into the nasopharyngeal microbiome. The control GM strain (4YB2) was also derived from Δ *lacZ* and transformed in the same way with re-integration of *lacZ*, but without the coding sequence for *nadA*. The gene content and in vitro behaviour of the control strain was very similar to wild type Nlac. However, changes to the genetic architecture and gene regulation as a result of the transformation process would be equivalent to 4NB1, making it a better control strain than wild type Nlac (150).

Both GM Nlac strains are non-capsulate, which is the key virulence factor in *Nmen*, and both are highly susceptible to killing by human serum (150). NadA has a role in adhesion and invasion into epithelial cells, and the presence of the *nadA* gene in the genome is associated with hypervirulent lineages of *Nmen*. However, NadA surface expression has not been shown to be necessary or sufficient for increased virulence (144, 145).

Therefore, neither the intervention nor control strains were considered more likely than wild type Nlac to cause invasive disease. Extensive pre-clinical testing of both strains was undertaken prior to submission to DEFRA. This indicated that the GM strains were stable and did not undergo recombination events at higher frequency than wild type Nlac. They were resistant to the uptake of *Nmen* capsule-associated genes which could confer virulence. Studies in a murine model demonstrated that neither strain was virulent following inoculation (150). All preclinical studies satisfied the Advisory Committee for

Release to the Environment (ACRE) which informs DEFRA on safety of deliberate release of GMOs (149). Vials of both GM Nlac strains were produced to GMP-like standards at the University of Southampton Medical School Laboratory LC67 / LC70 by Dr.Jay Laver according to specific SOPs.

5.1.3 DEFRA approval

DEFRA approval for the deliberate release of the GM strains was gained in October 2017. This approval was given with the conditions that there should be enhanced monitoring of colonisation and shedding, and clear rules about actions to be taken in the event of any disease, enhanced colonisation or shedding. These conditions were written into the protocol and are detailed in sections 5.2.6 and 5.2.8. There was no requirement for complete containment of colonised participants, so outpatient follow up after the initial 4.5-day admission period was permitted (149). A Health and Safety Executive (HSE) inspection of the study processes was undertaken in December 2018 which was found to be satisfactory with no further recommendations or inspections required.

5.1.4 Public engagement

Given the novel nature of this study, and the potential for strong public opinion regarding use of a GMO, public engagement activities were conducted to inform the local community, and to gather opinions, advice and feedback. Information was provided in a press release, and a focus group was conducted to discuss attitudes to genetic modification and its use in medicine and clinical research. Advice and feedback was sought regarding the volunteer information sheets (VIS) (Appendices B3 and B6) and recruitment materials. Feedback from participants involved in previous studies requiring an inpatient stay was used to inform and improve the admission period experience.

5.2 Study specific methods

5.2.1 Study design

In this prospective controlled human infection study, participants were inoculated intranasally with either the intervention or control strain of GM Nlac at an intended dose of 10^5 CFU. Following inoculation, these challenge volunteers (ChV) were admitted to Southampton National Institute for Health Research Clinical Research Facility (NIHR CRF) for 4.5 days. ChV were then discharged to their normal residence and followed up as outpatients for a period of approximately 3 months. Safety parameters, colonisation, shedding and immunogenicity were assessed during the admission period and outpatient follow up period. A separate group of contact volunteers (CoV) who were bedroom sharers of ChV were enrolled to assess for onward transmission and safety from the time of ChV discharge until Day 90 post challenge. Colonisation was terminated with antibiotic eradication therapy on Day 90, for all challenge and contact volunteers, or prior to study withdrawal. Efficacy of eradication was confirmed at 24 – 48 hours post eradication. The intended sample size was 10 colonised ChV per group (intervention or control strain). The study team was not blinded to group allocation during the study. Immunology samples were coded prior to processing and analysis so laboratory staff and investigators were blinded to group allocation during those processes.

Overviews of the study timelines for ChV and CoV are shown in Figure 5.1. The objectives and endpoints of this study are summarised in Table 5.1 and the schedule of procedures for ChV and CoV are shown in Tables 5.2 and 5.3. The eligibility criteria are detailed in Chapter 2.

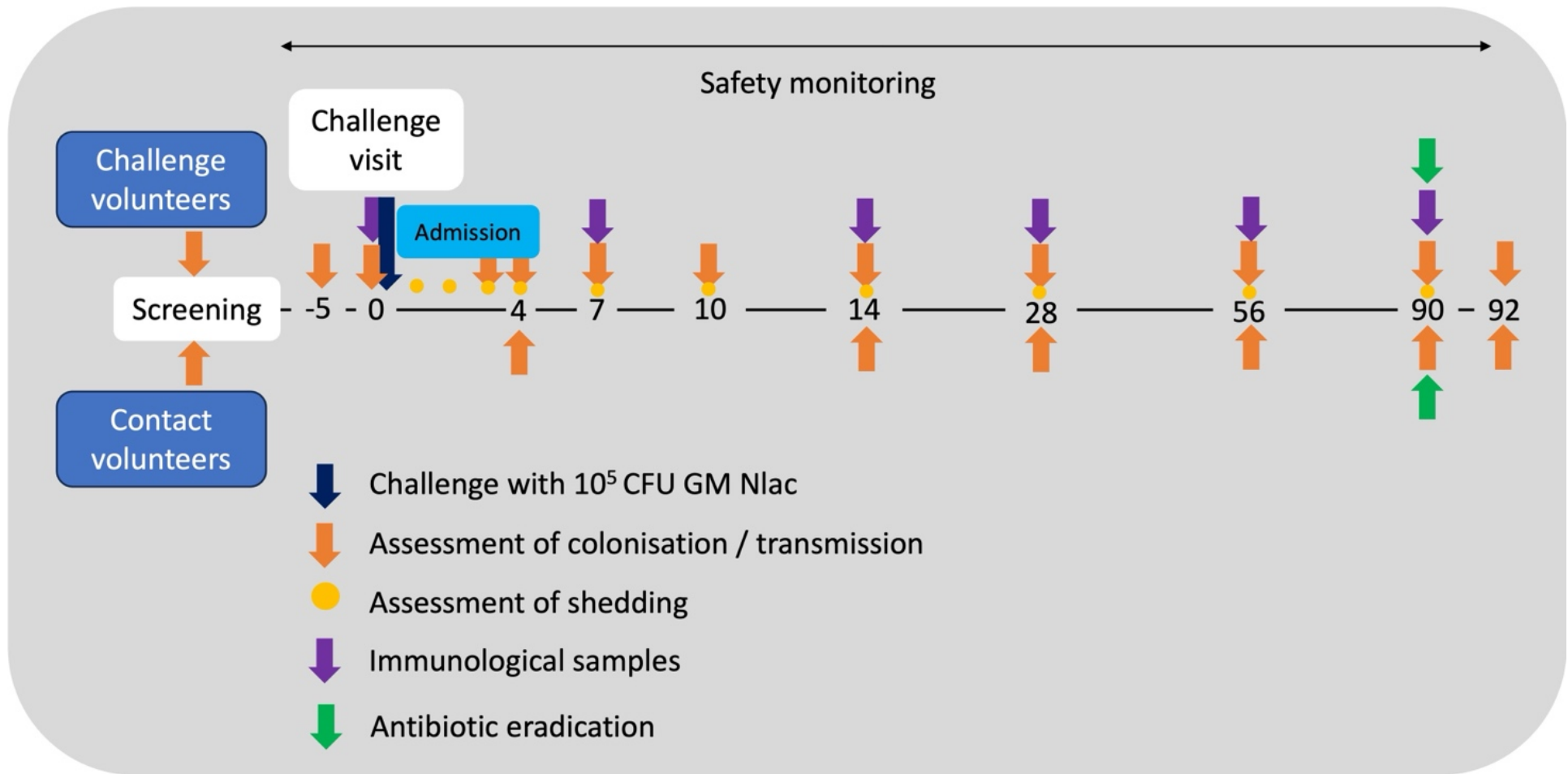


Figure 5.1 L4 study timeline

Schematic presentation of study timeline for L4 participant visits, interventions and monitoring with comparison between challenge and contact volunteers.

Objectives		Endpoints
Co-primary objectives	To establish the safety of nasal inoculation of healthy volunteers with GM Nlac expressing NadA	Occurrence of unsolicited adverse events within the study period
		Occurrence of serious adverse events within the study period
	To assess the NadA specific immunity in healthy volunteers following nasal inoculation with GM Nlac expressing NadA	Rise in serological specific IgG titre (anti-NadA) comparing day 0 versus days 14 to 90 comparing volunteers colonised with each GM Nlac strain
Secondary objectives	To assess the shedding of GM Nlac following nasal inoculation	Culture of GM Nlac from environmental samples – comparing intervention and control groups
	To assess the transmission of GM Nlac to bedroom contacts of inoculated volunteers	Culture of GM Nlac from throat swabs taken from contact volunteers from day 4 until day 90 – comparing intervention and control groups
	To assess the efficacy of a single dose of Ciprofloxacin in eradicating carriage of GM Nlac	Culture of GM Nlac from throat swabs taken at the eradication visit in comparison to post-eradication visit in challenge and contact volunteers

Table 5.1 L4 Objectives and endpoints

Adapted from L4 study protocol (Appendix A2).

Timeline (days)	Screening	Pre challenge	Admission					Follow up							Potential additional visits		
	≤ 90	-5	0	1	2	3	4	7	10	14	28	56	90	92	Additional shedding check ^b	Triggered eradication ^c	Post triggered eradication check
Day		W	M	Tu	W	Th	F	M	Th	M	M	M		-1 to 0 ^a			
Visit window		+/-2	0	0	0	0	0	+/-1	+/-1	+/-2	+/-3	+/-5	+/-7		0 ^d	0 ^d	0 ^a
Informed consent	+																
Infection control training	+						+										
Vital signs	+	(+)	+	+	+	+	+	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)
Medical history	+																
Physical examination	+	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)
Pregnancy test (females only)	+		+										+				
Urinalysis	+																
Electrocardiogram	+																
Review eligibility		+	+														
Inoculation			+														
Eradication													+		+		
Review of adverse events and concomitant medications		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Throat swab	+	+	+		+	+	+	+	+	+	+	+	+	+	+	+	+
Nasal wash		+				+				+	+	+	+		+		
Environmental samples				+	+	+	+	+	+	+	+	+	+		+	+	
Safety bloods	8		8			8		8		8	8	8	8		8		
Immunological blood tests (ml)			70					70		70	70	70	70		70		
Cumulative blood volume (ml)	8		86			94		172		250	328	406	484				

Table 5.2 L4 schedule of procedures - Challenge volunteers

+ Procedure at this timepoint, (+) if clinically indicated, ^a1-2 days after eradication, ^bIf increased shedding seen at one timepoint from Day 14, ^cIf early eradication triggered, ^dAs soon as possible after triggering results are known. Adapted from L4 study protocol (Appendix A2)

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	Screening	Challenge volunteer discharge	Follow up					Potential additional visits	
			14	28	56	90	92	Early eradication ^c	Early eradication check
Timeline (days)	≤ 90	4	14	28	56	90	92	Early eradication ^c	Early eradication check
Day		F	M	M	M	+/-7 ^a	-1 to 0 ^c	0 ^d	-1 to 0 ^b
Visit window		0	+/-2	+/-3	+/-5				
Informed consent	+								
Reconfirm eligibility		+							
Infection control training	+	+							
Vital signs	+		(+)	(+)	(+)	(+)	(+)	(+)	(+)
Medical history	+								
Physical examination	+		(+)	(+)	(+)	(+)	(+)	(+)	(+)
Pregnancy test (females only)	+	+				+		+	
Urinalysis	+								
Electrocardiogram	+								
Eradiation						+		+	
Review of adverse events and concomitant medications			+	+	+	+	+	+	+
Throat swab	+	+	+	+	+	+	+	+	+

Table 5.3 L4 Schedule of procedures - Contact volunteers

+Procedure at this timepoint, (+) if clinically indicated, ^aSame day as corresponding challenge volunteer, ^b1-2 days after eradication, ^cIf early eradication triggered, ^dAs soon as possible after triggering results are known. Adapted from L4 study protocol (Appendix A2).

5.2.2 Safety measures

The first five participants to receive each strain were staggered for safety. The first was challenged individually and the remainder individually or in pairs. Further participants were challenged in groups of up to five.

ChV were closely monitored during admission and safety data was collected at each follow up visit for ChV and CoV. All participants were encouraged to contact the study team via the 24-hour telephone number if any symptoms developed between follow up visits. Any participants who developed solicited or unsolicited symptoms or abnormal vital signs were discussed with a study doctor. Further clinical assessment and investigations were arranged if required.

In the event of symptoms suggestive of possible GM Nlac disease, a clear management plan was in place for early eradication with a single dose of ciprofloxacin, or treatment with a longer course of oral ciprofloxacin or IV ceftriaxone if required.

A safety review was completed between each set of inoculations, including clinical data and safety bloods up to at least day 4. The external safety committee reviewed the safety data after the first five volunteers to be inoculated with each strain and in the event of any significant safety concerns.

5.2.3 Infection control measures

Participants and study team members were provided with appropriate PPE and infection control training. Infection control procedures such as the use of PPE, cleaning of designated areas, fomite control and disposal of GM waste were detailed in standard operating procedures (see Appendices E16 and E17). Any potentially aerosol-producing procedures, such as the inoculation or respiratory sampling, were carried out in the level 2 environmental chamber with trained staff members wearing PPE including FFP3 respirators.

Participants were required to abide by infection control rules during admission (ChV only) and throughout the outpatient follow up period (ChV and CoV), as detailed in Table 5.4. These rules were detailed in the VIS (Appendices B3 and B6) and during consent and all participants completed an infection control questionnaire and signed an infection control agreement at the time of consent (Appendices B8 and B9). The rules were then reiterated prior to inoculation and discharge of the challenge volunteer.

Admission period (ChV only)	Volunteers are not allowed to enter the personal rooms of other volunteers
	The volunteer must wash hands before leaving their personal room
	The volunteer is not allowed to leave the NIHR-CRF without permission of the clinical team
	Volunteers are allowed to leave the NIHR-CRF for a maximum of two hours twice a day, between 08.00-18.00
	The volunteer will be escorted by a member of the study team when walking through non-designated areas of the NIHR-CRF
	The volunteer must not have contact with immunosuppressed individuals
	The volunteer must not have any direct contact that could involve transfer of respiratory secretions to anyone during the admission period
	The volunteer must not use the main entrance of the hospital or shops or cafes within the hospital building
	When outside of the NIHR-CRF the volunteer must be contactable by mobile phone at all times and have study emergency phone number stored on their phone to contact the clinical study team if necessary
	The volunteer must be able to return to the NIHR-CRF within 30 minutes
	The volunteer may receive a maximum of two guests at a time between 8.00 and 22.00, who must wear masks covering nose and mouth while in close proximity to the volunteer and must adhere to strict infection control procedures.
Outpatient follow up (ChV and CoV)	Volunteers must avoid crowded social environments such as pubs and clubs for two weeks after discharge
	Volunteers must not have any contact with high risk of transmission with any individuals other than their declared and consented bedroom contact/corresponding challenge volunteer – such contact includes: <ul style="list-style-type: none"> • Bed sharing • Intimate/sexual contact • Contact that may involve transfer of respiratory secretions e.g. kissing • Sharing cutlery or drinking vessels
	Volunteers must not engage in oral sex
	Volunteers must avoid contact with immunosuppressed individuals

Table 5.4 Infection control rules

Infection control rules for Challenge volunteers (ChV) during admission and follow up and Contact volunteers (CoV) during outpatient follow up. Adapted from L4 study protocol (Appendix A2).

5.2.4 Admission period

ChV were admitted to the NIHR-CRF for approximately 4.5 days following inoculation. A maximum of 5 participants were admitted at any time, with individual bedrooms, shared toilets, washing facilities and a recreational area. Participants were allowed to leave the unit for exercise, and to have visitors as detailed in the infection control rules (Table 5.4). Two study team members were available on-site for the duration of the admission period.

Participants were reviewed by a study nurse six times per day at approximately four hourly intervals. This review included vital signs and recording of any solicited or unsolicited AEs. They were also reviewed by a study doctor twice a day, either face to face or by telephone, and in the event of any safety concerns. Study procedures were conducted according to Table 5.2.

CoVs attended on the day of ChV discharge to reconfirm eligibility. All participants were reminded of the infection control rules for the remainder of the study and were provided with a 24-hour telephone number to contact the study team if required.

5.2.5 Outpatient follow up period

Participants attended for follow up visits as detailed in Tables 5.2 and 5.3 for clinical review and assessment of colonisation, shedding, immunogenicity and transmission. Additional visits were arranged if required.

5.2.6 Shedding assessment

According to the DEFRA conditions for approval of deliberate release, ChV were assessed for shedding of GM Nlac throughout the study. A basic shedding check including air and mask samples was conducted each day during admission and at each follow up visit until antibiotic administration at Day 90 as detailed in Table 5.2. If increased shedding was detected, then an enhanced shedding check including further air and contact samples was conducted according to the SOP (Appendix E9).

Increased shedding was defined by comparing colonised volunteers in Group 1 (NadA expressing strain) with those in Group 2 (Control strain), with a threshold of 10-fold greater shedding in a single ChV in Group 1, in comparison to the average shedding at the same time point in colonised ChV in Group 2. If increased shedding was detected at any point from Day 14, then the ChV would be asked to reattend as soon as possible for an additional shedding check. Enhanced shedding was defined as increased shedding at two consecutive timepoints in a Group 1 ChV. Any ChV with enhanced shedding, and

their corresponding CoV (unless pre-discharge) would be given triggered eradication a maximum of 72 hours after detection.

5.2.7 Eradication

A single dose of oral ciprofloxacin was given to all participants (ChV and CoV) to eradicate carriage of GM Nlac, with a throat swab to confirm successful eradication after a maximum of 48 hours. Standard eradication was given to all volunteers at Day 90, regardless of colonisation status, with a confirmatory throat swab on Day 92.

Triggered eradication was given to ChV and CoV (if applicable) on the same day and both were then withdrawn from the study, in the following circumstances:

- Safety concerns in either ChV or CoV (investigator discretion)
- Enhanced shedding (ChV)
- Study withdrawal of either volunteer for any other reason

Early eradication was given to CoV only if transmission of GM Nlac was detected, as ongoing colonisation of CoV was not required to fulfil the study objectives. Both ChV and CoV would continue to participate in the study.

5.2.8 Safety stopping and holding rules

The following stopping and holding rules were established in response to the DEFRA approval conditions:

- Individual stopping rules:
 - ChV and CoV pairs to be withdrawn from the study if either required triggered or early antibiotic eradication for safety reasons or enhanced shedding
- Study holding rules:
 1. Enhanced shedding:
 - Enhanced shedding triggering eradication in 3 or more of the first 5 ChV or in >50% of ongoing ChV in Group 1.
 2. Enhanced transmission:
 - Transmission of either strain of GM Nlac to 3 of the first 5 or >50% of ongoing CoV.
 3. GM Nlac disease:
 - Antibiotic treatment (IV ceftriaxone or IV chloramphenicol) given to any volunteer due to possible GM Nlac disease.

If any study holding rules were met, the study would be paused for a safety review, with no further challenges until the data had been reviewed by the external safety committee and study continuation approved.

5.3 Results

5.3.1 Recruitment

All required approvals were gained and recruitment was commenced in October 2018. Recruitment was continued until June 2019 by which time 11 challenge volunteers had been successfully colonised with each GM Nlac strain and the study was completed, as per protocol. Details of volunteer screening, participant enrolment, group allocation and inclusion of subjects in different analyses are shown in Figure 5.2.

Demographic details for enrolled Challenge volunteers with comparison between Group 1 and Group 2 are shown in Table 5.5. There were no significant differences, but there was a non-significant trend towards younger age in Group 2.

5.3.2 Inoculum count and purity

The confirmed doses of inoculum administered to each participant are shown in Figure 5.3, comparing Group 1 and Group 2. The median dose for Group 1 was 3.38×10^5 CFU (IQR $2.9\text{-}4.8 \times 10^5$) and for Group 2 was 3.57×10^5 CFU (IQR $2.8\text{-}3.8 \times 10^5$), both higher than the intended dose of 1×10^5 CFU, with no difference between the two groups. Culture of all residual inocula resulted in a pure growth of Nlac.

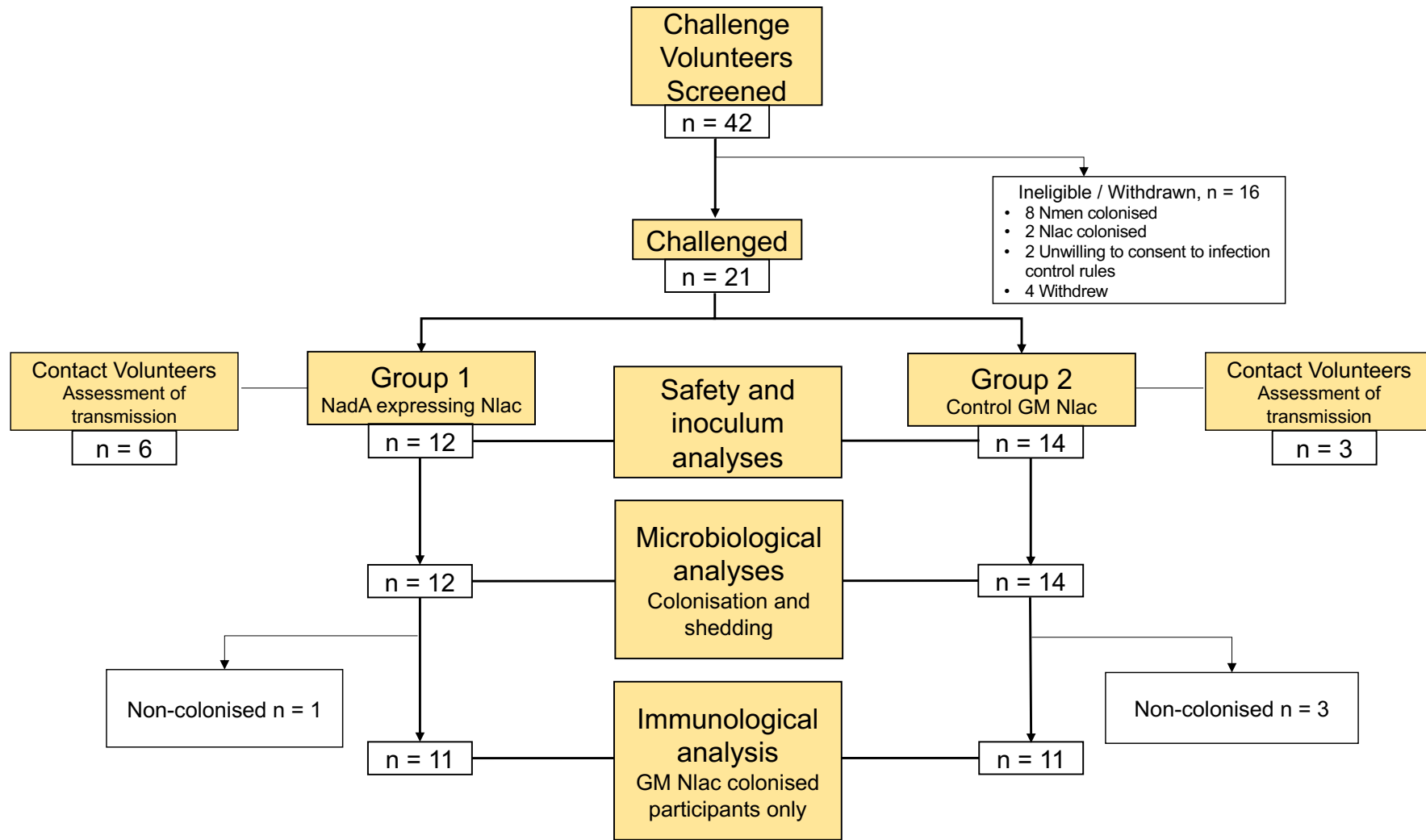


Figure 5.2 L4 Participant recruitment

Overview of participant recruitment for L4 with number of participants (n) screened, enrolled and included in each analysis set.

	Number of participants n	Sex Male n (%) Female n (%)	Age Median (IQR)	BMI Median (IQR)
Group 1 NadA expressing Nlac	12	7 (58.3) 5 (41.7)	30.9 (23.3 – 42.8)	25.7 (22.4 – 34.0)
Group 2 Control GM Nlac	14	7 (50.0) 7 (50.0)	23.3 (20.2 – 29.0)	24.6 (21.8 – 34.0)
p value		0.7 (NS)	0.07 (NS)	0.95 (NS)

Table 5.5 L4 Participant demographics

Comparison of demographics for participants challenged in Group 1 (NadA expressing Nlac) and Group 2 (Control GM Nlac)

Sex – p value derived from Fisher’s exact test, NS = Non-significant

Age and BMI – p values derived from Mann Whitney test.

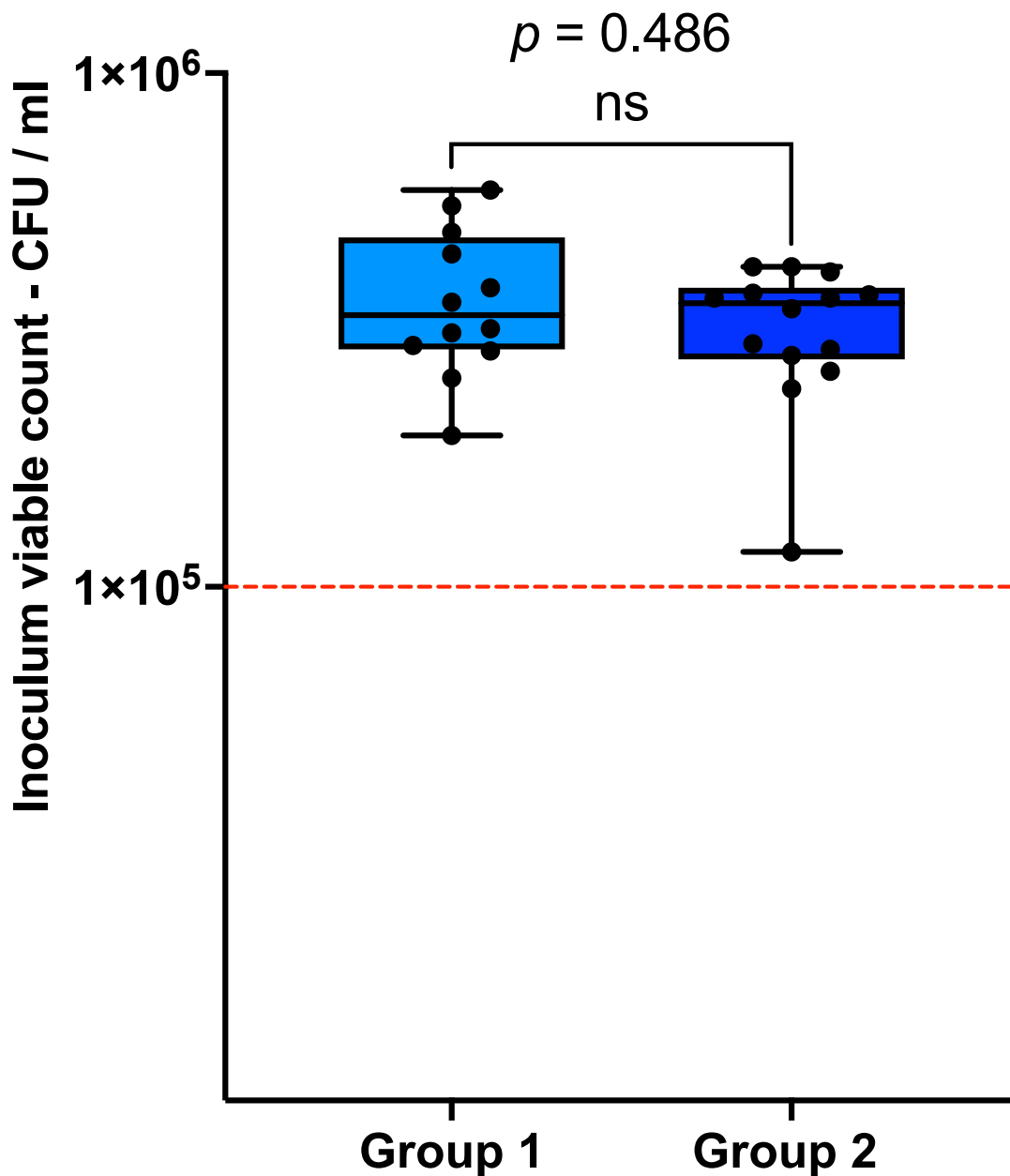


Figure 5.3 L4 inoculum doses

Confirmed doses calculated by viable count of the residual inoculum following inoculation. Individual values, median and range shown, red dotted line indicates the intended dose of 1×10^5 CFU, p value derived from Mann Whitney test.

5.3.3 Colonisation

The overall colonisation fraction was 0.92 (11 of 12, 95% CI 0.65-0.996) in Group 1 (NadA expressing GM Nlac) and 0.79 (11 of 14, 95% CI 0.52-0.92) in Group 2 (Control GM Nlac). Successful colonisation was defined as the culture of at least one colony of Nlac from throat swab or nasal wash samples between challenge and Day 14 post challenge. Isolates were confirmed by PCR to be the inoculum strain of GM Nlac (4NB1 for Group 1 and 4YB2 for Group 2).

Colonisation was detected by Day 2 in 91% (10 of 11) of those colonised overall in Group 1 and 72% (8 of 11) colonised overall in Group 2, in the remainder colonisation was detected on Day 3.

The only participant in Group 1 who was not successfully colonised with the inoculated strain of Nlac was found to be Nmen colonised on Day 0. This participant had negative screening throat swabs and nasal wash but the pre-inoculation throat swab on Day 0 was positive for Nmen. He then remained Nmen colonised until Day 7 when he received triggered eradication having chosen to withdraw from the study for personal reasons. One other participant in Group 1 withdrew for personal reasons on Day 14 post challenge and received triggered eradication. Both of these participants returned for a triggered eradication check after 48 hours and had cleared carriage of GM Nlac / Nmen.

One participant in the Group 2 who was successfully colonised with GM Nlac had that colonisation displaced with Nmen between days 28 and 56 and then remained Nmen colonised until eradication treatment at Day 90. This was shown to be a non NadA-expressing strain of Nmen by western blot of whole bacterial lysate, using an anti-NadA antiserum. One participant in Group 1 spontaneously cleared Nlac carriage after Day 28 post challenge. All other colonised participants remained colonised with Nlac until Day 90. Therefore, of those colonised participants who completed follow up to Day 90, nine of 10 participants (90%) in Group 1 and 10 of 11 (90.9%) continued to be colonised for the duration of the study. Carriage of the GM Nlac strains and Nmen was cleared by 48 hours following eradication in all participants.

The colonisation status over time for individual participants is shown for Group 1 and Group 2 in Figure 5.4.

Chapter 5

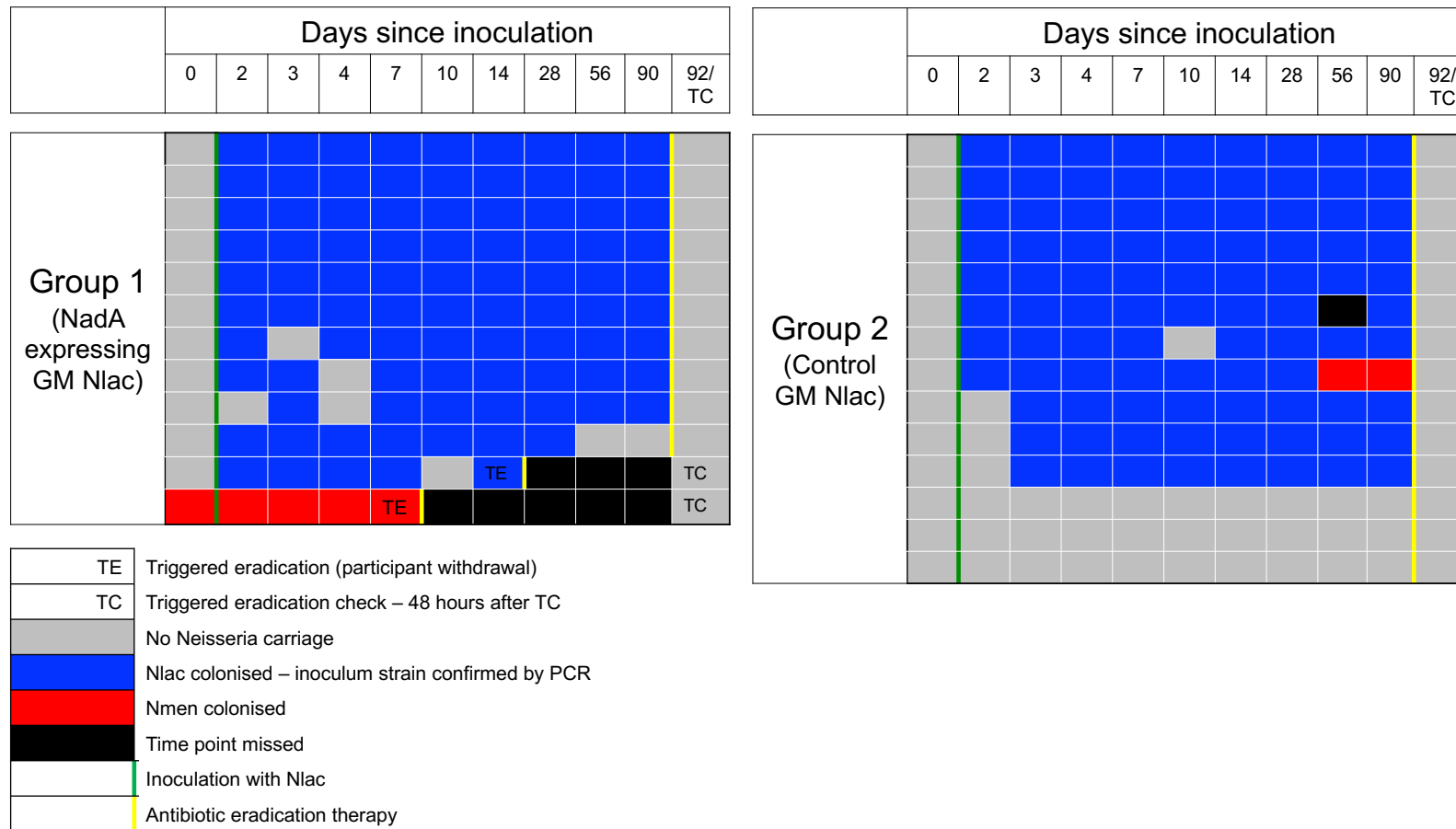


Figure 5.4 Colonisation status over time

Colonisation with Nlac / Nmen at each timepoint for individual participants. Each row represents one participant, colonisation defined as the culture of at least one colony of Nlac / Nmen from a throat swab or nasal wash taken at the time points shown.

5.3.4 Comparison of throat swab and nasal wash samples

Comparison was made between the sensitivity of throat swabs and nasal washes in the detection of GM Nlac carriage. In calculating this, the following assumptions were made:

- All positive cultures were considered to be true positive
- Negative cultures were considered to be false negatives if culture of the other sample type at the same time point was positive
- Negative cultures were considered to be false negative if the participant had a positive culture (of any sample type) at both the preceding and the following time point
- All other negative culture results were considered to be true negative

Using these assumptions, and only looking at time points when both sample type was taken, the estimated sensitivity of throat swabs in detecting GM Nlac carriage was 96.0% whereas that of nasal washes was only 52.4%, as shown in Table 5.6. This difference in sensitivity was statistically significant ($p < 0.0001$, Fisher's exact test).

	True positive	False negative	Sensitivity
Throat swab	97	4	96.0%
Nasal wash	53	48	52.4%

Table 5.6 Comparison of sample type for GM Nlac culture

5.3.5 Shedding

Air and mask samples were obtained at each study visit up to and including Day 90 or triggered eradication. No Nlac was detected in any environmental samples from any participants, whether colonised or not. No participants required an extended shedding check.

5.3.6 Transmission

For contact volunteers, potential exposure to GM Nlac began on Day 4 when the corresponding challenge volunteer was discharged. A throat swab was taken from contact volunteers on Day 4 to look for baseline carriage, and at all visits from Day 14 to 90 to look for transmission. Six contact volunteers were enrolled for Group 1 and three contact volunteers were enrolled for Group 2. For all enrolled contact volunteers, their corresponding challenge volunteer had been successfully colonised with GM Nlac. One contact volunteer in Group 1 received a dose of ciprofloxacin and was withdrawn after their Day 14 visit as their challenge volunteer had chosen to withdraw for personal reasons, so early eradication therapy for the contact volunteer was triggered per-protocol. One contact volunteer in Group 2 acquired Nmen carriage between Day 4 and Day 14 and then spontaneously cleared this carriage by Day 90. Their corresponding challenge volunteer was not colonised with Nmen at any timepoint. Another contact volunteer in Group 2 was found to be colonised with Nlac at Day 4 (prior to exposure to GM Nlac). PCR confirmed that this was wild type Nlac rather than transmission of GM Nlac. This colonisation with wild type Nlac continued until Day 90 and was then cleared following eradication therapy.

No transmission of either GM Nlac strain was detected to any contact volunteer at any time point.

5.3.7 Safety

No serious adverse events occurred during the study. The inoculation procedure was well tolerated with no adverse events or symptoms reported at the time of inoculation for any participant.

Participants were reviewed for the occurrence of solicited symptoms or other adverse events every four hours during admission. The proportion of participants in each group reporting symptoms during admission are shown in Figure 5.5 A and B.

5 of 12 participants in Group 1 and 7 of 14 participants in Group 2 reported mild or moderate solicited symptoms at some point during the admission. Unsolicited symptoms reported during admission are recorded as “other”, none of which were considered to be likely or definitely related to challenge or colonisation with Nlac. All reported symptoms all self-resolved or resolved with simple analgesia.

Symptoms and adverse events reported during the outpatient follow up period are shown in Figure 5.5 C and D for Challenge Volunteers and Figure 5.5 E and F for Contact Volunteers. Symptoms are categorised as upper respiratory tract, lower respiratory tract, generalised (e.g. feeling generally unwell, headache) and other. All upper respiratory tract,

lower respiratory tract and generalised symptoms were mild to moderate, all resolved spontaneously or with non-antibiotic treatment (e.g. analgesia, anti-histamines), and no difference was seen between Group 1 and 2. All “Other” adverse events were unrelated or unlikely to be related to challenge or colonisation with Nlac and all were mild to moderate with the exception of one Challenge Volunteer in Group 2. This participant had a worsening of pre-existing syncopal episodes for which she was referred for cardiac investigations during the study period. This was not felt to be related to the study interventions or procedures and remained under investigation at the end of the study.

Challenge volunteer clinical observations were recorded four-hourly during admission and at each follow up visit and were graded as per protocol. Several participants in each group had raised blood pressure but were either entirely well or reported non study-related anxieties at the time. One participant in each group had low oxygen saturations (minimum 94% - Grade 1) and one had a mildly raised temperature at 37.6 °C. All were systemically well with no other symptoms and all resolved spontaneously. Abnormal clinical observations are summarised in Figure 5.5 G and H.

Challenge volunteers had safety blood samples taken at protocol defined timepoints with results graded as per protocol. Mildly out of range results were seen for participants in both groups but all participants were asymptomatic or had mild upper respiratory tract symptoms only. Abnormal safety blood results are summarised in Figure 5.5 I and J.

5.3.8 Triggered/early eradication

No participants were given triggered or early eradication therapy due to safety reasons, or transmission to the contact volunteer. Two challenge volunteers and one contact volunteer from the intervention group withdrew from the study for personal reasons and therefore received triggered eradication therapy.

Group 1 - NadA expressing strain

Group 2 - Control GM Nlac strain

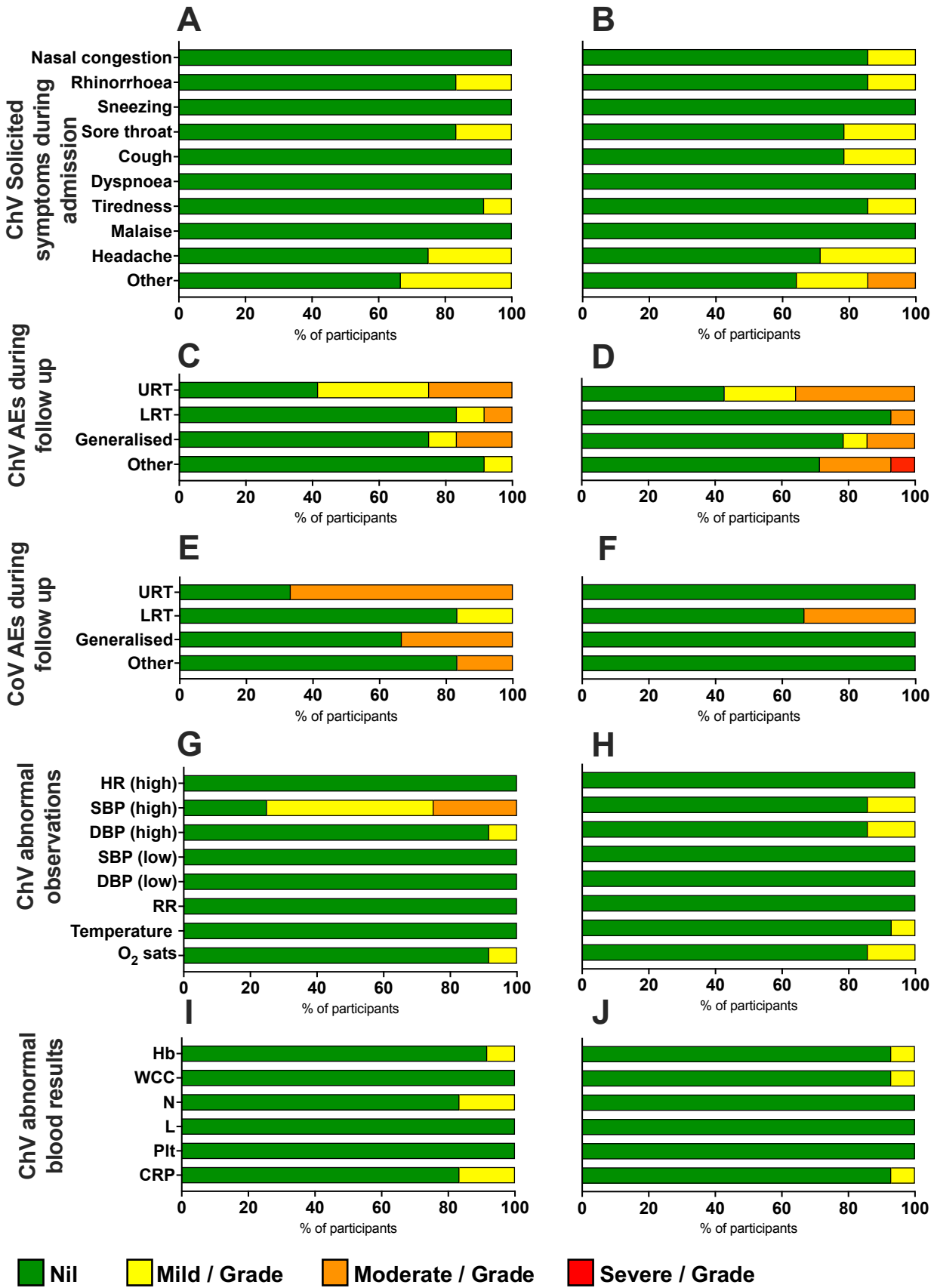


Figure 5.5 L4 Adverse events

Percentage of participants reporting any adverse events in each category with maximum severity with comparison of Group 1 (A, C, E, G, I) and Group 2 (B, D, F, H, J)

A and B: Solicited and other symptoms reported by Challenge Volunteers at four hourly reviews during admission period

C and D: Adverse events reported by Challenge Volunteers during follow up

E and F: Adverse events reported by Contact Volunteers during follow up (C-F categorised as URT = Upper respiratory tract symptoms, LRT = Lower respiratory tract symptoms, Generalised = Systemic illness or Other)

G and H: Challenge Volunteer observations (HR = Heart Rate, SBP = Systolic Blood Pressure, DBP = Diastolic Blood Pressure, RR = Respiratory Rate, O₂ sats = oxygen saturations)

I and J: Challenge Volunteer blood results (Hb = Haemoglobin, WCC = White cell count, N = Neutrophil count, L = Lymphocyte count, CRP = C-reactive protein).

ChV = Challenge Volunteer, CoV = Contact Volunteer, AE = Adverse event.

5.3.9 Immunogenicity

Participants who were colonised with either GM Nlac strain are included in the immunological analysis, a total of 11 participants per group. However, some participants had incomplete immunological sampling due to early withdrawal or missed visits and so are excluded from some analyses as detailed below. One participant in Group 2 lost GM Nlac carriage and acquired non-NadA expressing meningococcal carriage between the Day 28 and Day 56 visit. This participant is included in the analysis as per protocol, but where relevant their results are highlighted, as acquisition of meningococcal carriage is likely to have impacted their subsequent immunological results.

5.3.9.1 Nlac specific IgG

Participant samples were taken at baseline and Day 28, 56 and 90 post challenge and only participants who attended each of these visits are included in the analysis – a total of 10 participants per group. Every colonised participant in both groups had a relative increase in titre from baseline by Day 28 post challenge, which was sustained to Day 90. This increase was statistically significant at each post challenge timepoint for both groups. In Group 1, six participants had a ≥ 2 -fold rise by Day 28 which was sustained to Day 90. In Group 2, nine participants had a ≥ 2 -fold rise by Day 28 or 56, this was sustained to Day 90 for seven participants. Trends in titre for each individual participant with summary data for each group are shown in Figure 5.6.

Comparison of absolute titres at baseline and at each post challenge timepoint showed no difference between the two groups at any timepoint: Day 0 $p=0.9014$, Days 28, 56 and 90 $p > 0.999$ (Kruskal Wallis test with Dunn's multiple comparisons) as shown in Figure 5.7. There was no difference between the maximum fold change from baseline to any post challenge timepoint between the two groups, with a median maximum fold change of 2.82 for Group 1 and 3.04 for Group 2 ($p > 0.999$, Mann Whitney test) as shown in Figure 5.8.

These results are similar to those seen during prolonged colonisation with wild type Nlac (123), confirming that neither the genetic manipulation itself, nor the expression of NadA, impacts the ability of Nlac to induce an Nlac specific humoral immune response.

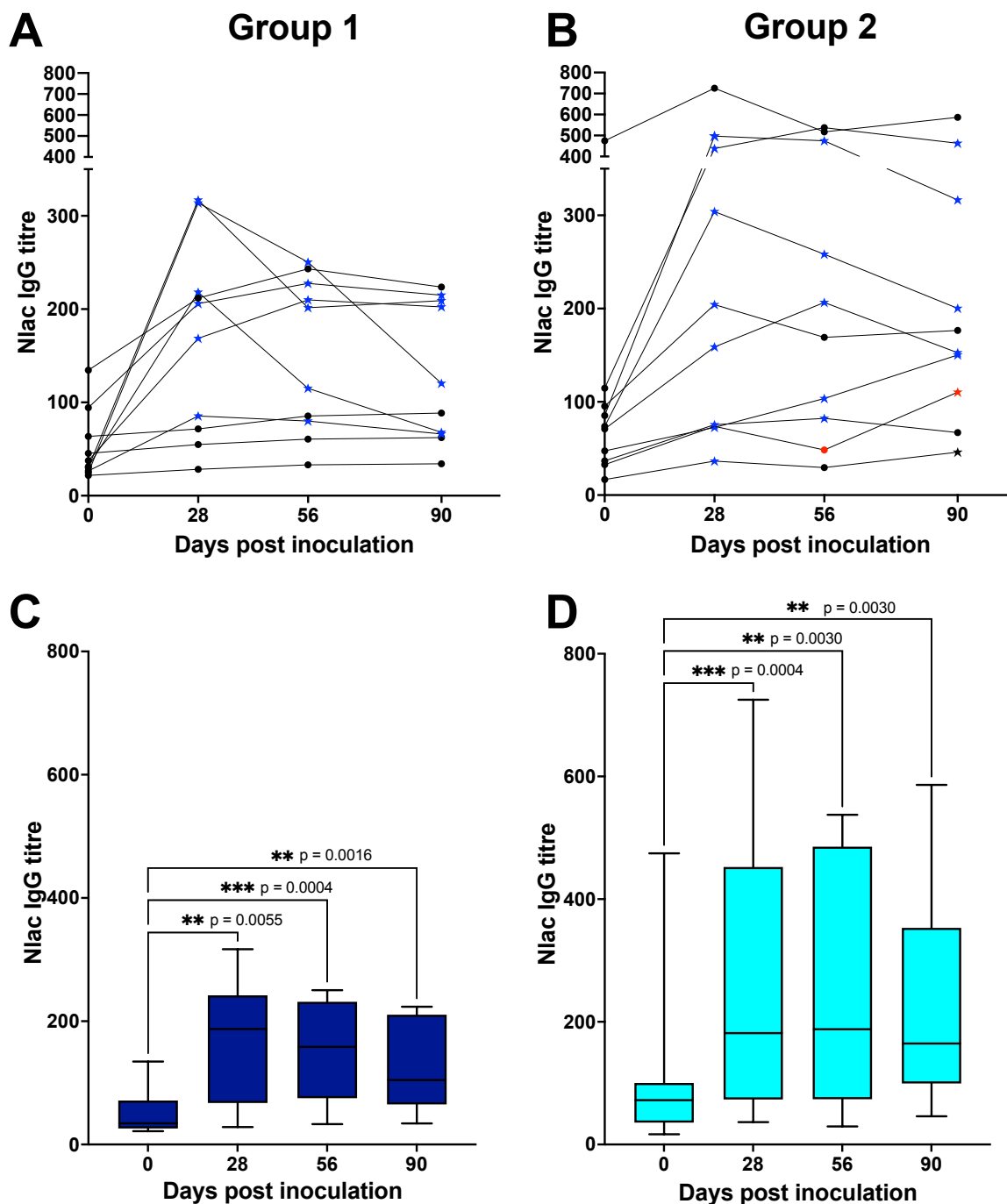


Figure 5.6 Nlac specific IgG trends

Trend for individual participants per in Group 1 (A) and Group 2 (B). Participant timepoints with a ≥ 2 -fold rise from baseline are indicated with blue stars, participant colonised with Nmen indicated in red

Summary data for each timepoint for Group 1 (C) and Group 2 (D) – median, IQR and range shown (C+D). *P* values derived from Friedman test with Dunn's multiple comparisons.

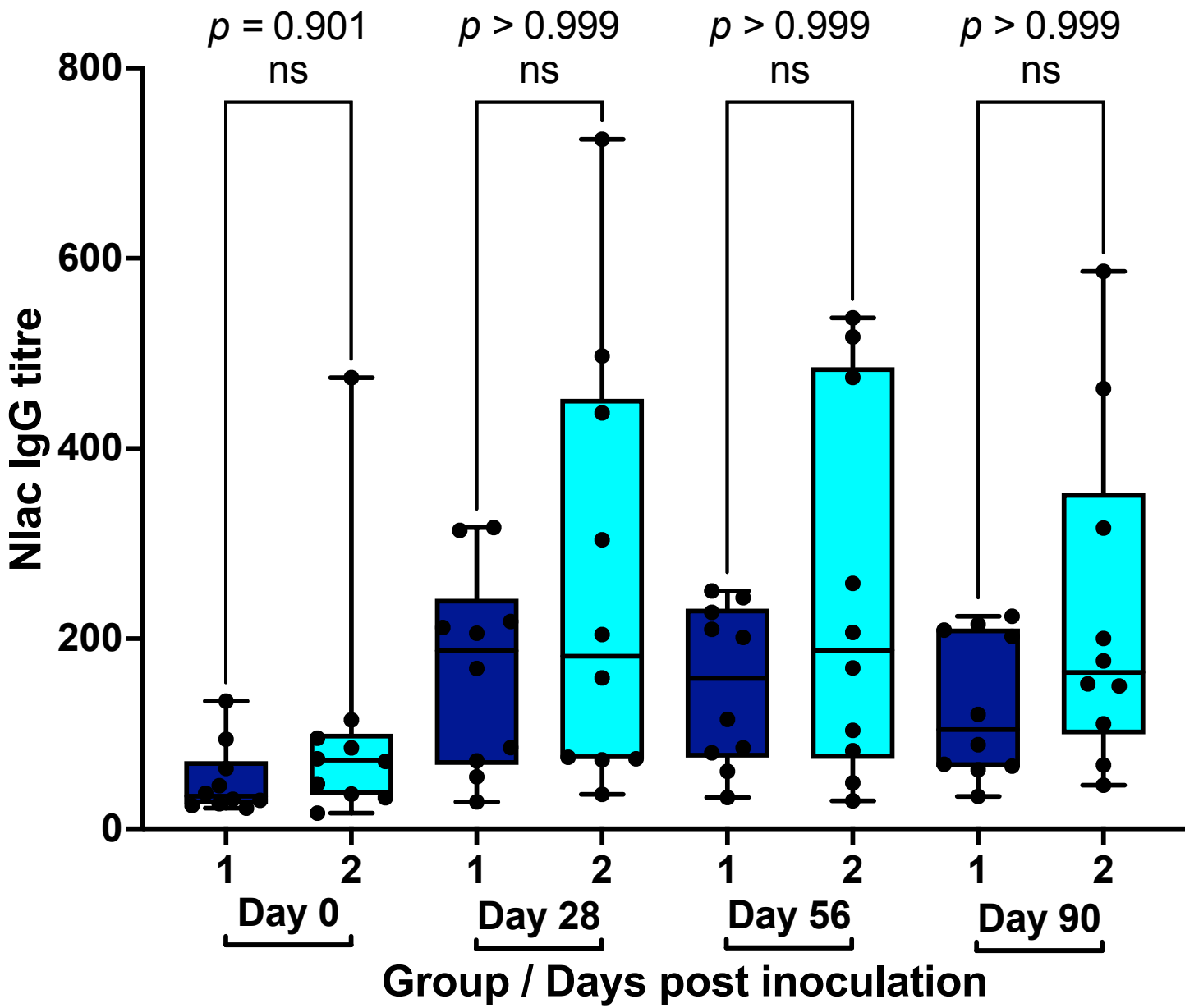


Figure 5.7 Nlac specific IgG titres per timepoint

Nlac specific IgG titres – Comparison of Group 1 vs Group 2 Nlac specific IgG titres at each timepoint. Individual values, median, IQR and range shown, *p* values derived from Kruskal-Wallis test with Dunn’s multiple comparisons.

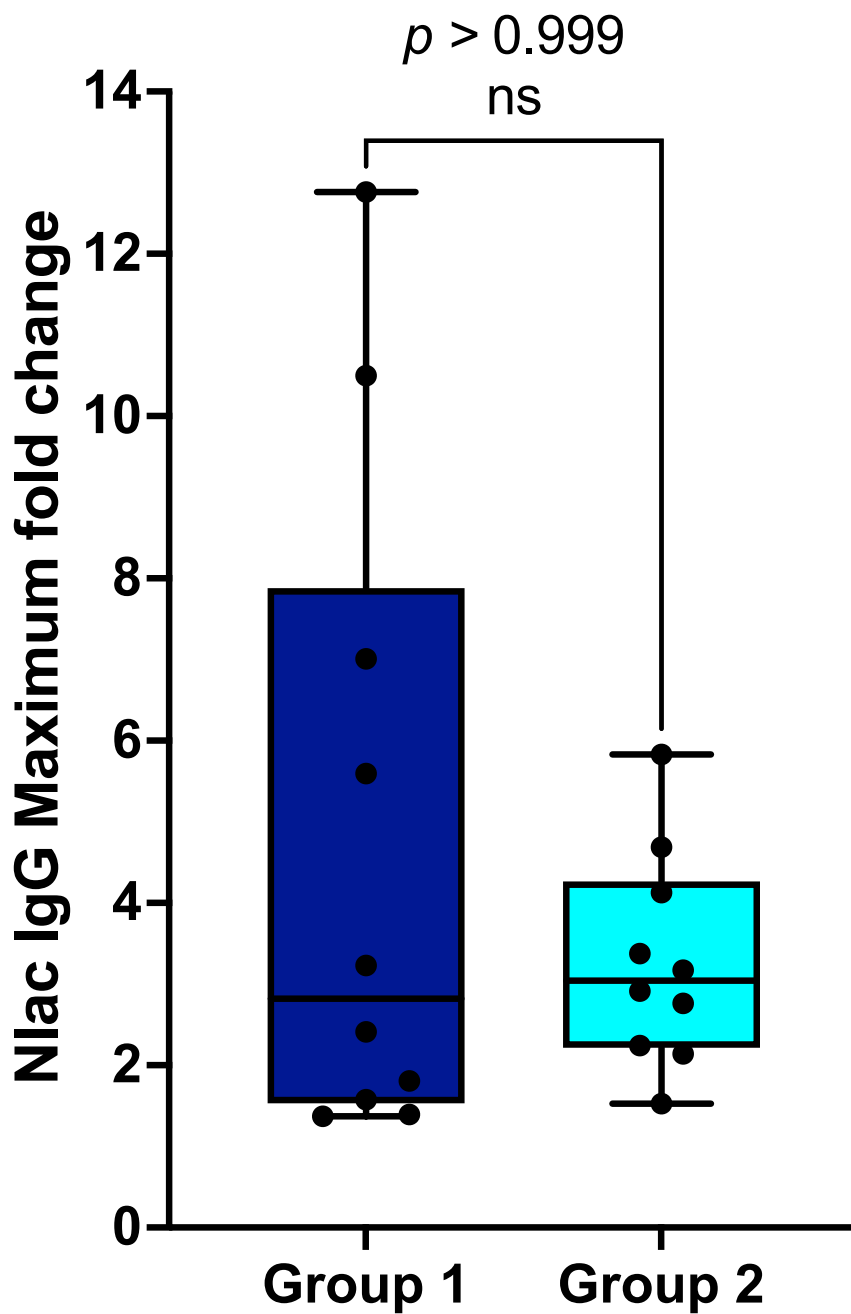


Figure 5.8 Nlac specific IgG fold change

Maximum fold change per participant from baseline to Day 28, 56, or 90. Individual values, median, IQR and range shown, p value derived from Mann Whitney test.

5.3.9.2 sNadA specific IgG

Anti-sNadA specific IgG titres were determined by endpoint ELISA of participant serum samples taken at baseline, Day 28, 56 and 90. One participant who withdrew from the study prior to Day 28 is excluded from this analysis.

Reciprocal endpoint titres for each participant timepoint are shown in Table 5.7.

One of 10 participants in Group 1 and two of 10 participants in Group 2 had a detectable level of sNadA specific IgG at baseline. ≥ 2 -fold increase from baseline in reciprocal endpoint titre at one or more post-challenge timepoints was considered to indicate seroconversion. In Group 1, only one participant had detectable sNadA IgG at baseline. This participant and four others seroconverted (5 of 10, 50%) and for three participants this increase was sustained to Day 90. In Group 2, two participants had detectable sNadA IgG at baseline but had no change in the reciprocal endpoint titre following challenge, and all other participants had no detectable titres at any timepoint, so no participants seroconverted (0 of 11, 0%). This increase in the proportion of participants who seroconverted (≥ 2 -fold change) is statistically significant ($p = 0.0124$, Fisher's exact test), showing that the expression of NadA by colonising Nlac can induce a NadA specific humoral immune response. The participant who acquired Nmen carriage did not have an increase in sNadA IgG as expected, as the Nmen strain was non-NadA expressing.

Participant	Day 0	Day 28	Day 56	Day 90	Max fold change
Group 1 – NadA expressing GM Nlac					
1	<2	8	8	8	8
2	<2	<2	<2	<2	1
3	<2	<2	4	4	4
4	<2	<2	<2	<2	1
5	<2	8	8	8	8
6	<2	<2	<2	<2	1
7	4	8	4	4	2
8	<2	<2	<2	<2	1
9	<2	4	<2	<2	4
10	<2	<2	<2	<2	1
Group 2 – Control GM Nlac					
1	16	16	16	16	1
2	<2	<2	<2	<2	1
3	<2	<2	<2	<2	1
4	8	8	8	8	1
5	<2	<2	<2	<2	1
6	<2	<2	<2	<2	1
7	<2	<2	<2	<2	1
8	<2	<2	-	<2	1
9	<2	<2	<2	<2	1
10	<2	<2	<2	<2	1
11	<2	<2	<2	<2	1

Table 5.7 sNadA specific IgG - Reciprocal endpoint titres

Reciprocal endpoint titre of sNadA IgG per participant in each group. ≥ 2 -fold increase from baseline shaded in blue, participant who became Nmen colonised is shaded in red.

5.3.9.1 Nlac and NadA specific antibody secreting plasma cells

IgG, IgA and IgM secreting antibody secreting plasma cells (B_{PLAS}) with specificity to relevant Nlac and Nmen antigens as detailed below, were determined by ELISpot of PBMCs isolated from whole blood taken from participants at baseline and at Day 7, 14, 28, 56 and 90. All colonised participants are included in this analysis ($n=11$ per group) although some participants have missing values due to missed visits. Results are expressed as the number of spot forming units (SFU) per 2×10^5 PBMCs, with the assumption that one SFU indicates the presence of one B_{PLAS} cell.

B_{PLAS} with specificity to Tetanus toxoid (TT) were also determined at each time point to ensure that any B_{PLAS} increase detected was a response to exposure to specific antigens rather than a non-specific response to colonisation. Figure 5.9 shows the comparison of baseline and peak IgG, IgA or IgM TT specific B_{PLAS} . There is a small but significant rise in IgA B_{PLAS} in Group 1, but this is non-significant when considering the increase from baseline to each timepoint individually for those participants in whom a full data set is available (Friedman test with Dunn's multiple comparisons, $p > 0.99$ for each of Day 7, 14 and 28 in comparison to baseline). At baseline, the majority of participants had no, or very few B_{PLAS} cells detected with specificity for any of the assessed antigens, and there were no significant differences between Group 1 and 2 for any antigen (all baseline comparisons $p > 0.999$, Kruskal-Wallis test with Dunn's multiple comparisons).

For each antigen, individual participant trends over time and comparison of baseline and peak values per group are shown in Figures 5.10-14. The peak value reported per participant is the highest number of B_{PLAS} detected at Day 7, 14 or 28 post challenge, as this has been shown to be the timeframe in which B_{PLAS} cells peak following exposure to an antigen (165). In the majority of cases, this highest value was detected at Day 7 or 14 post challenge. Where higher values were detected at Day 56 or 90 post challenge these are shown in the individual trend lines below but are not reported as a peak value. One participant in Group 2 acquired carriage of a non-NadA expressing strain of Nmen between Day 28 and 56. This may have impacted their Day 56 or Day 90 values which are highlighted in the trend lines, but this will not have affected their peak values as Nmen acquisition was after the Day 28 visit. Comparison is also made between the change from baseline to peak between Group 1 and 2, for each antigen.

Figures 5.10 and 5.11 show results for B_{PLAS} with specificity for OMV derived from the two GM Nlac strains, 4NB1 (Figure 5.10) and 4YB2 (Figure 5.11). Participants in both groups had a significant rise in IgG, IgA and IgM secreting B_{PLAS} to both 4NB1 and 4YB2 dOMV, with the exception of 4YB2 dOMV IgG B_{PLAS} which did not quite reach statistical

significance for Group 1 ($p=0.0625$). As both strains are derived from a single parent strain (Y92-1009), all epitopes apart from the expression of NadA by 4NB1, should be identical. This confirms the ability of each strain to induce a specific humoral immune response. When comparing the absolute change for each from baseline for Group 1 and 2, there was no significant difference between the two groups.

B_{PLAS} specific for sNadA are shown in Figure 5.12. Participants in Group 1 had a significant increase from baseline in sNadA specific IgG and IgA B_{PLAS} , although not for IgM B_{PLAS} . No change was seen from baseline to peak for participants in Group 2. Comparison of the absolute change from baseline to peak for sNadA specific IgA B_{PLAS} was significantly higher for Group 1 than Group 2, although for IgG B_{PLAS} this trend did not reach statistical significance and for IgM there was no difference seen. These data suggest that the expression of NadA during colonisation by 4NB1 induces NadA specific B_{PLAS} , although a larger sample size may have shown this more robustly. Of note, the participant who acquired non-NadA expressing Nmen prior to the Day 56 visit did have a transient increase in sNadA specific IgG, IgA and IgM at Day 56. This was no longer apparent by Day 90.

B_{PLAS} with specificity for Nmen dOMV were also determined. OMV derived from meningococcal strain N54.1 (a high NadA expressing serogroup Y strain) and from the same strain with the coding sequence for NadA removed (N54.1 Δ nadA) were used, as shown in Figures 5.13 and 5.14 respectively. For N54.1, there was a significant increase in IgG, IgA and IgM B_{PLAS} from baseline to peak for both groups, with no difference seen between the two groups. For N54.1 Δ nadA, there was a similar trend but this did not reach statistical significance for IgG (either group) or for IgM in Group 2. Again, there was no difference between the two groups. This suggests that colonisation with Nlac, with or without the expression of NadA, induces anti-meningococcal B_{PLAS} production, in keeping with previous CHIM results where a Nmen specific IgG response has been demonstrated (Evans). However, the inclusion of a non-Nlac colonised control group and a larger sample size could have demonstrated this more conclusively. The expression of NadA did not impede the B_{PLAS} response by being immunologically dominant. A larger sample size would be required to demonstrate any positive impact of NadA expression on the cross-reactive immune response to NadA expressing meningococci, on top of that induced by Nlac colonisation.

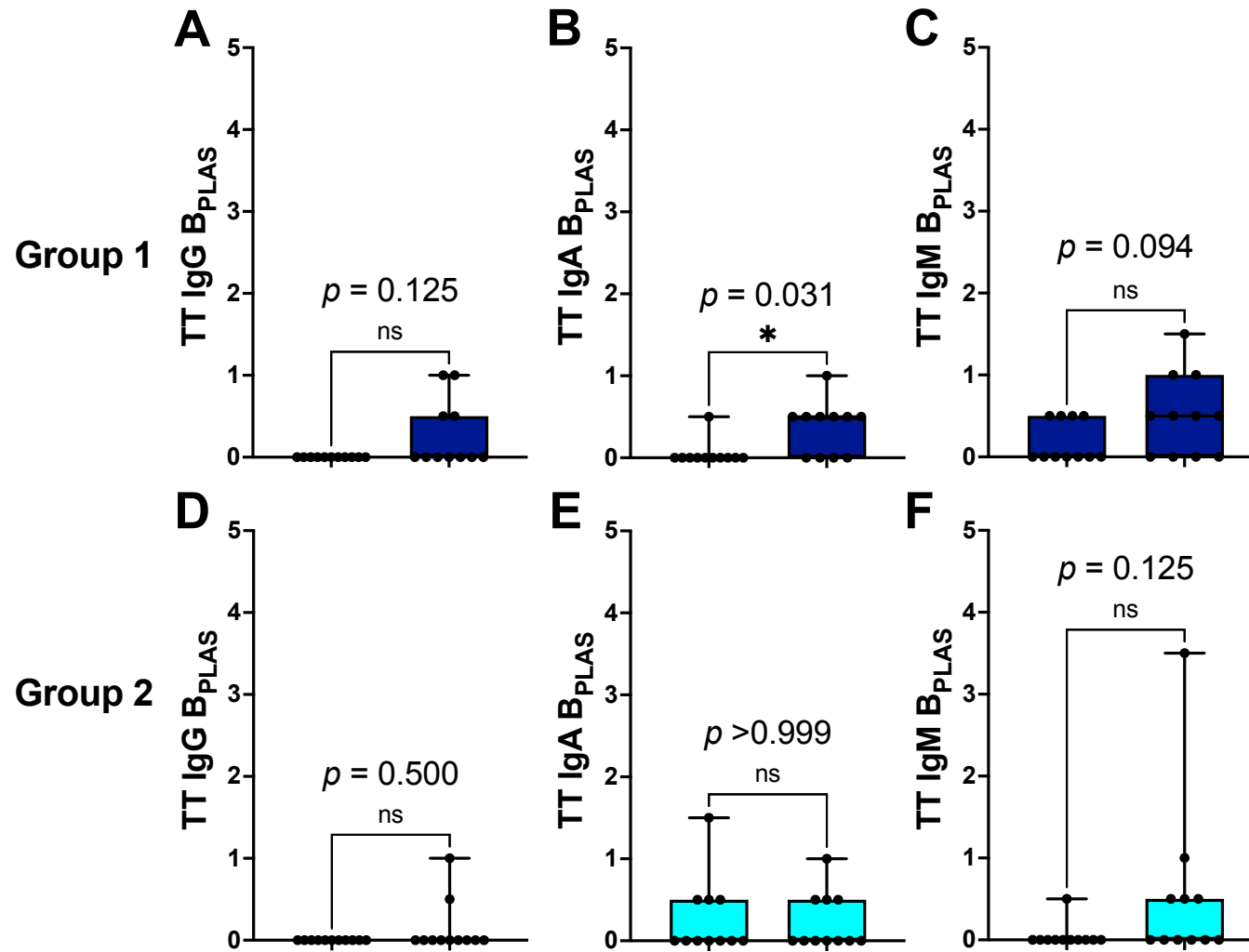


Figure 5.9 B_{PLAS} negative control

Antibody secreting plasma cells with specificity for tetanus toxoid (TT)

Summary data per group comparing baseline and peak (to Day 28) values

Median, IQR and range shown, *p* values derived from Wilcoxon signed ranks test.

A-C: Group 1, D-F: Group 2

A+D: IgG, B+E: IgA, C+F: IgM

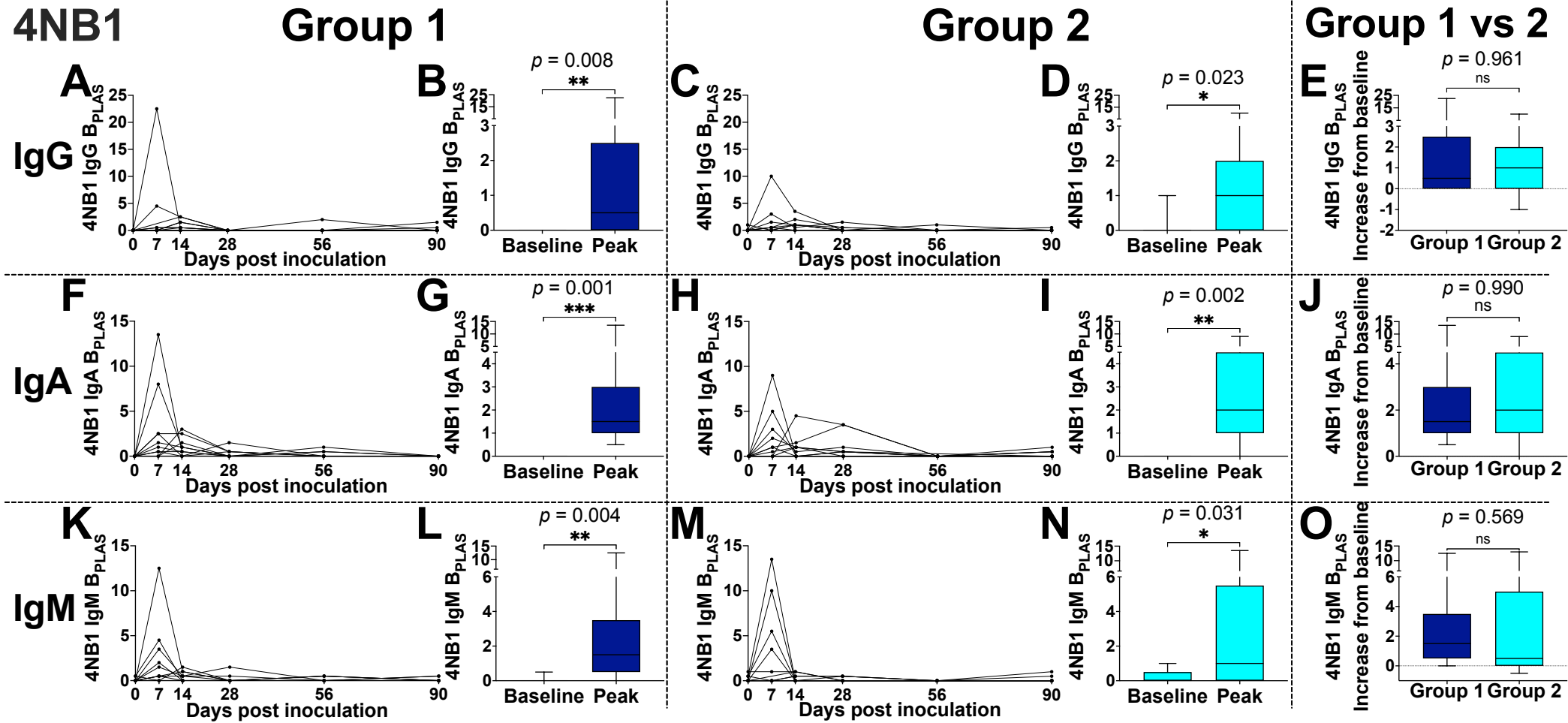


Figure 5.10 B_{PLAS} : 4NB1

Antibody secreting plasma cells with specificity for 4NB1 dOMV (NadA expressing GM Nlac)

A-E: IgG B_{PLAS}, F-J: IgA B_{PLAS}, K-O: IgM B_{PLAS}.

A,B,F,G,K,L: Group 1, C,D,H,I,M,N: Group 2.

A,C,F,H,K,M: Individual participant trends over time per group.

B,G,L,D,I,N: Summary data per group comparing baseline and peak (to Day 28) values, median, IQR and range shown, p values derived from Wilcoxon signed ranks test.

E,J,O: Comparison of Group 1 and 2 increase in B_{PLAS} seen from baseline to peak, median, IQR and range shown, p values derived from Mann Whitney test.

4YB2

Group 1

Group 2

Group 1 vs 2

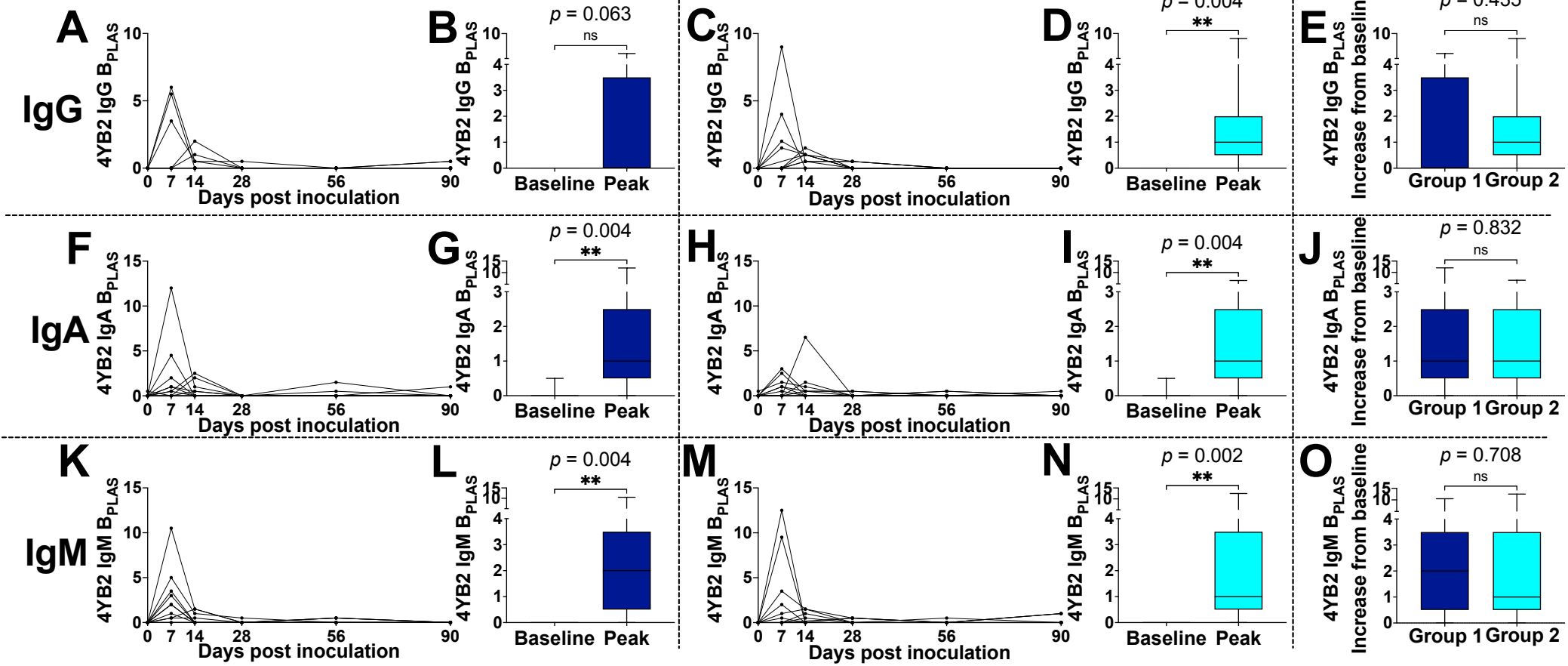


Figure 5.11 B_{PLAS} : 4YB2

Antibody secreting plasma cells with specificity for 4YB2 dOMV (non NadA-expressing GM Nlac)

A-E: IgG B_{PLAS}, F-J: IgA B_{PLAS}, K-O: IgM B_{PLAS}.

A,B,F,G,K,L: Group 1, C,D,H,I,M,N: Group 2.

A,C,F,H,K,M: Individual participant trends over time per group.

B,G,L,D,I,N: Summary data per group comparing baseline and peak (to Day 28) values, median, IQR and range shown, p values derived from Wilcoxon signed ranks test.

E,J,O: Comparison of Group 1 and 2 increase in B_{PLAS} seen from baseline to peak, median, IQR and range shown, p values derived from Mann Whitney test.

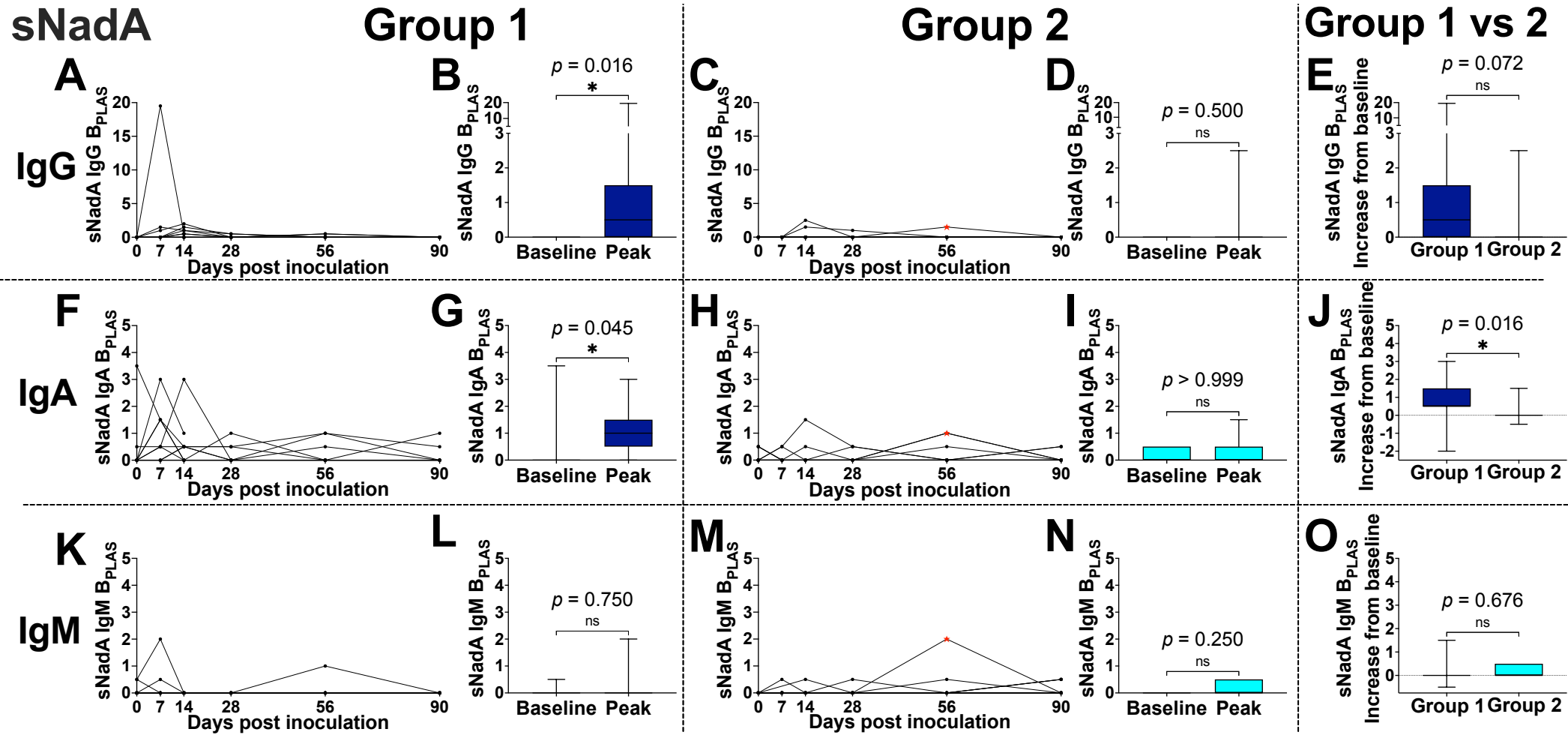


Figure 5.12 B_{PLAS} : sNadA

Antibody secreting plasma cells with specificity for sNadA**A-E: IgG B_{PLAS}, F-J: IgA B_{PLAS}, K-O: IgM B_{PLAS}.****A,B,F,G,K,L: Group 1, C,D,H,I,M,N: Group 2.****A,C,F,H,K,M: Individual participant trends over time per group.****B,G,L,D,I,N: Summary data per group comparing baseline and peak (to Day 28) values, median, IQR and range shown, p values derived from Wilcoxon signed ranks test.****E,J,O: Comparison of Group 1 and 2 increase in B_{PLAS} seen from baseline to peak, median, IQR and range shown, p values derived from Mann Whitney test.****Nmen colonised individual indicated with red star when above zero.**

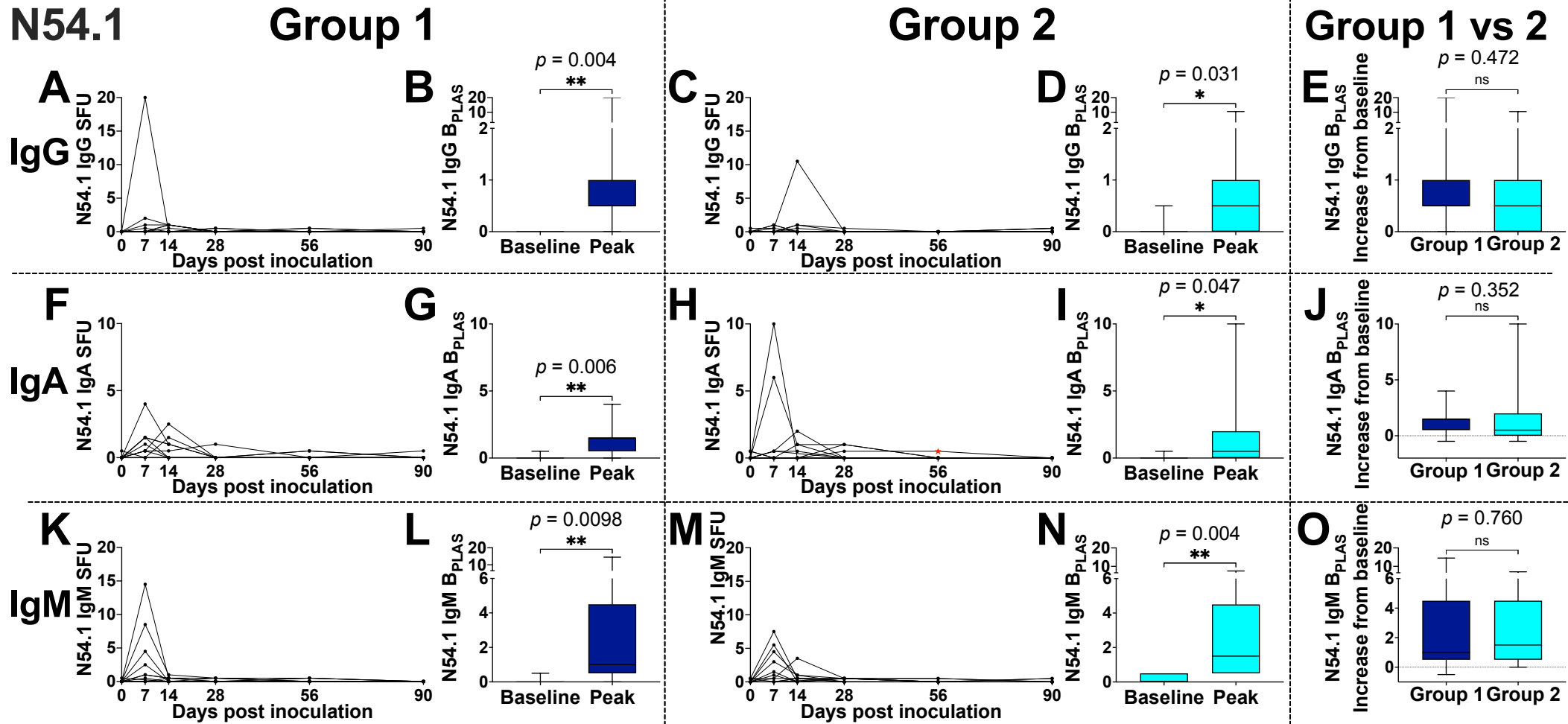


Figure 5.13 B_{PLAS} : N54.1

Antibody secreting plasma cells with specificity for N54.1 (NadA expressing Nmen)

A-E: IgG B_{PLAS}, F-J: IgA B_{PLAS}, K-O: IgM B_{PLAS}.

A,B,F,G,K,L: Group 1, C,D,H,I,M,N: Group 2.

A,C,F,H,K,M: Individual participant trends over time per group.

B,G,L,D,I,N: Summary data per group comparing baseline and peak (to Day 28) values, median, IQR and range shown, *p* derived from Wilcoxon signed ranks test.

E,J,O: Comparison of Group 1 and 2 increase in B_{PLAS} seen from baseline to peak, median, IQR and range shown, *p* values derived from Mann Whitney test.

Nmen colonised individual indicated with red star when above zero.

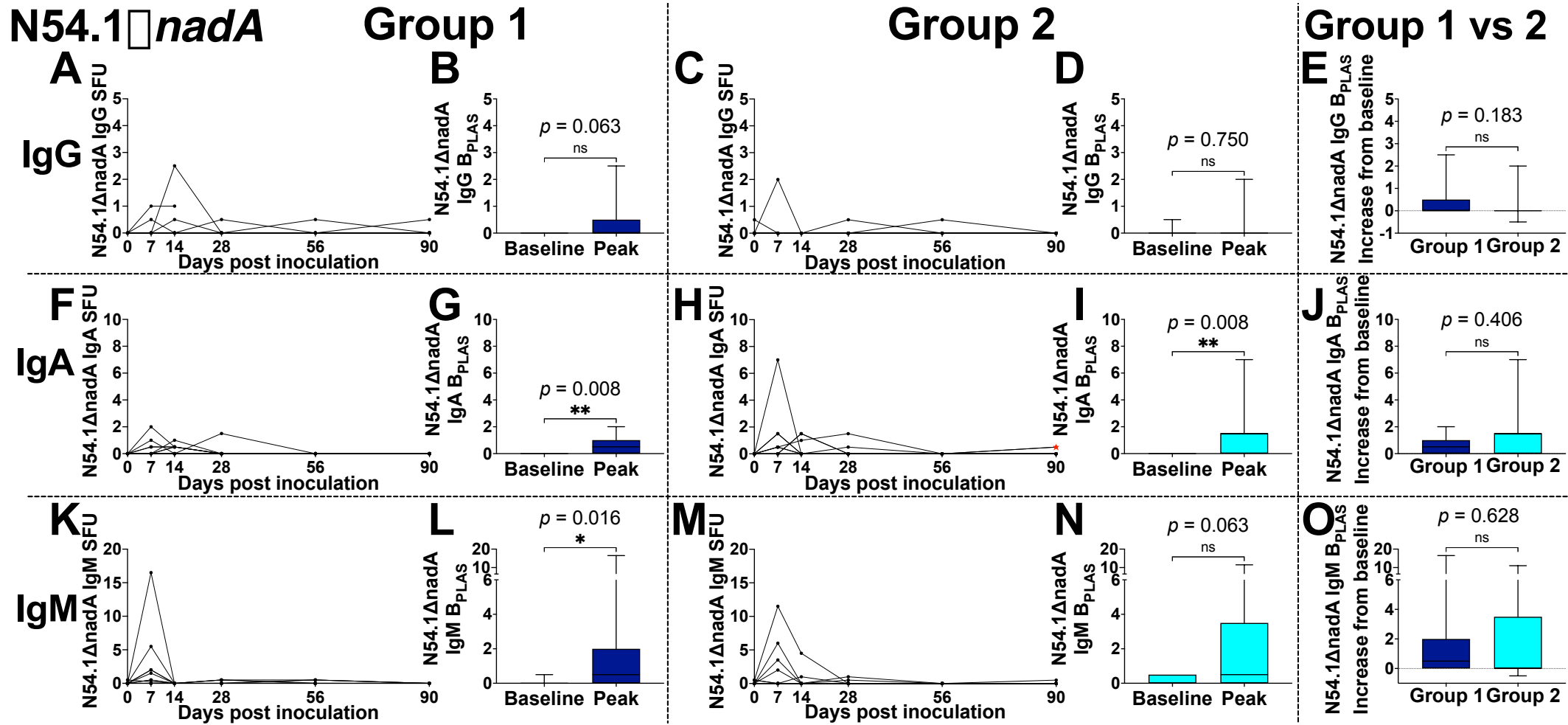


Figure 5.14 B_{PLAS} : N54.1 Δ *nadA*

Antibody secreting plasma cells with specificity for N54.1 Δ *nadA* (Nmen strain with *nadA* gene deleted).

A-E: IgG B_{PLAS}, F-J: IgA B_{PLAS}, K-O: IgM B_{PLAS}.

A,B,F,G,K,L: Group 1, C,D,H,I,M,N: Group 2.

A,C,F,H,K,M: Individual participant trends over time per group.

B,G,L,D,I,N: Summary data per group comparing baseline and peak (to Day 28) values, median, IQR and range shown, *p* values derived from Wilcoxon signed ranks test.

E,J,O: Comparison of Group 1 and 2 increase in B_{PLAS} seen from baseline to peak, median, IQR and range shown, *p* values derived from Mann Whitney test.

Nmen colonised individual indicated with red star when above zero.

Chapter 5

5.3.9.2 Nlac and NadA specific memory B cells

Memory B cells (B_{MEM}) with specificity to OMV derived from both GM strains (4NB1 and 4YB2) and to sNadA were determined by ELISpot of PBMCs isolated from whole blood taken from participants at baseline and at Day 28, 56 and 90 post challenge time points. B_{MEM} were not measured at earlier post challenge time points as B_{MEM} circulation has been shown to occur from 14-28 days following exposure to an antigen (165). Only participants who attended each of these visits are included in the analysis – a total of 10 participants per group. Results are expressed as the percentage of circulating IgG memory B cells with specificity to each antigen, with the assumption that one SFU indicates the presence of one memory B cell.

B_{MEM} with specificity to Human Influenza Haemagglutinin (Inf HA) were also determined at each time point to ensure that any change in the composition of the circulating memory B cell pool was a response to exposure to specific antigens rather than a non-specific response to colonisation. No change in the percentage of circulating memory B cell pool with specificity to Infl HA was seen at any timepoint for either group as shown in Figure 5.15.

At baseline, Group 1 had a higher percentage of 4YB2 B_{MEM} than Group 1 ($p=0.0007$) but there were no differences between the two groups for 4NB1 ($p=0.537$) or sNadA ($p=0.201$) specific B_{MEM} (all Mann Whitney test).

Figure 5.16-5.18 show results for 4NB1, 4YB2 and sNadA specific B_{MEM} with individual trends over time, summary data for each group at each time point, and a comparison of the maximum % B_{MEM} change for each antigen (the maximum percentage change from baseline at any timepoint for each participant, summarised for each group).

In both groups, a significant increase was seen in the percentage of B_{MEM} with specificity to 4YB2 and 4NB1 dOMV by Day 28 in comparison to baseline. For each this is sustained to Day 56, but is no longer significantly increased from baseline by Day 90.

For Group 1, there was a significant increase in the percentage of B_{MEM} with specificity to sNadA, apparent by Day 28 post challenge, and each individual participant had a relative increase in percentage at this timepoint. At Day 56 post challenge, this is not statistically significant, although by Day 90 post challenge, it is again statistically significant. No increase in the percentage of B_{MEM} with specificity to sNadA was seen in Group 2.

There was no difference between the two groups in the maximum titre change at any timepoint for the percentage of B_{MEM} with specificity for either 4NB1 or 4NB1 dOMV but a significantly higher change was seen for those with specificity for sNadA in Group 1.

These results again show that colonisation with either strain induces a specific B_{MEM} response to Nlac, but the induction of a NadA specific B_{MEM} response was only seen in the group colonised with the GM Nlac strain expressing NadA.

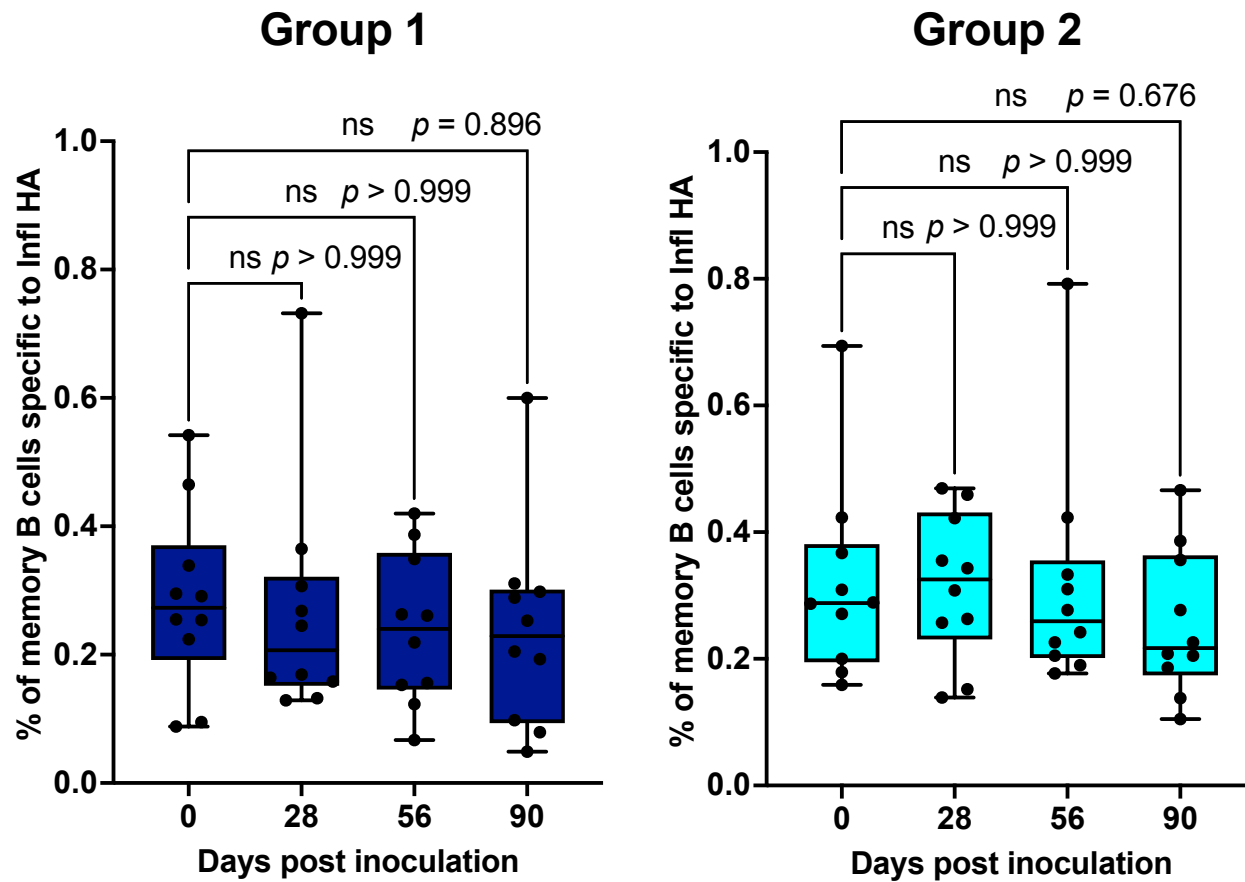


Figure 5.15 B_{MEM} : Influenza Haemagglutinin

Memory B cells with specificity to Influenza Haemagglutinin expressed as a percentage of total circulating IgG memory B cells at each timepoint post challenge from participants in Group 1 and Group 2. Individual values, median, IQR and range shown, p values derived from Friedman test with Dunn's multiple comparisons.

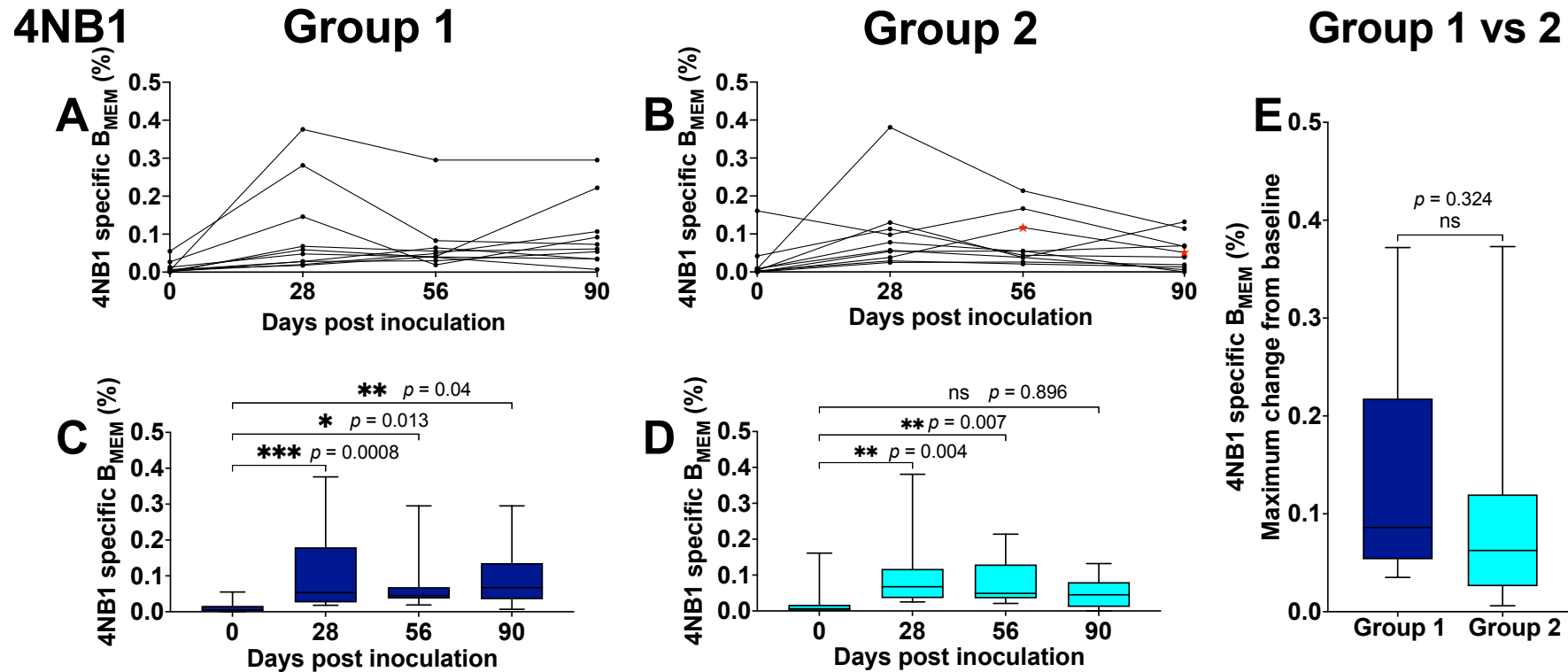


Figure 5.16 B_{MEM} : 4NB1

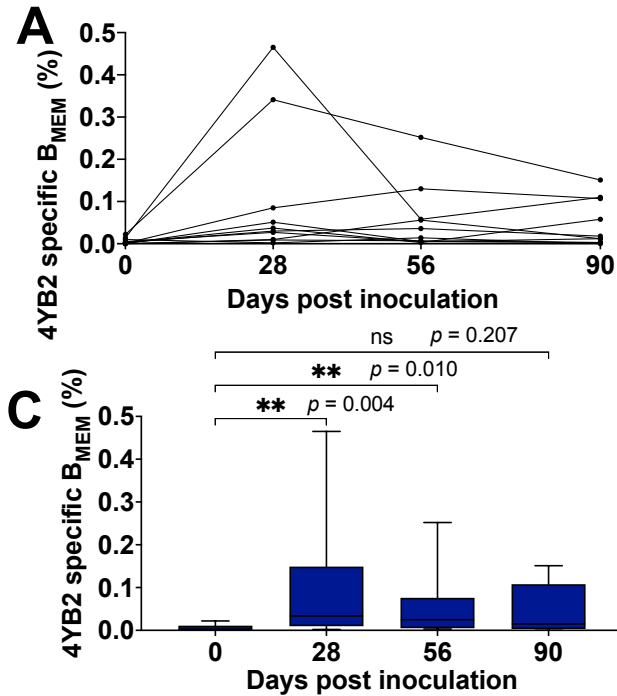
Memory B cells with specificity for 4NB1 dOMV (NadA expressing GM NIac)

B_{MEM} expressed as a percentage of total circulating IgG memory B cells at each timepoint post challenge from participants in Group 1 (A+C) and Group 2 (B+D). A+B: Individual participant trends over time per group, Nmen colonised individual indicated with red star. C+D:

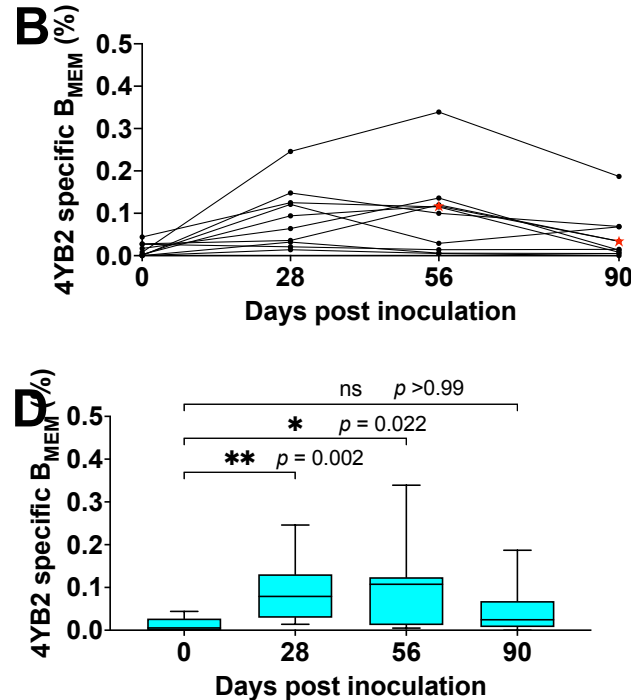
Summary data per group comparing baseline with each timepoint post challenge, median, IQR and range shown, p values derived from Friedman test with Dunn's multiple comparisons, E: Comparison of maximum change from baseline between Group 1 and 2, p value derived from Mann Whitney test.

4YB2

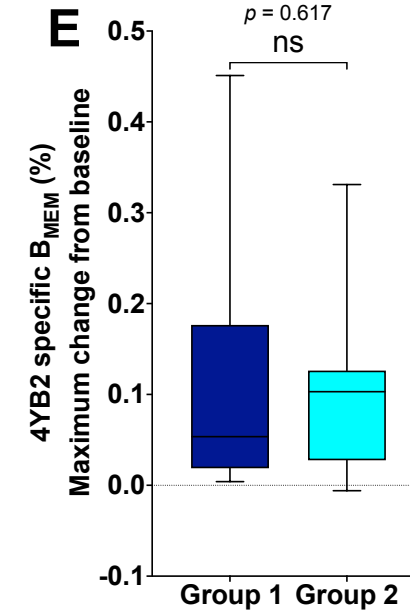
Group 1



Group 2



Group 1 vs 2

Figure 5.17 B_{MEM} : 4YB2

Memory B cells with specificity for 4YB2 dOMV (Non NadA expressing GM Nlac)

B_{MEM} expressed as a percentage of total circulating IgG memory B cells at each timepoint post challenge from participants in Group 1 (A+C)

and Group 2 (B+D). A+B: Individual participant trends over time per group, Nmen colonised individual indicated with red star. C+D:

Summary data per group comparing baseline with each timepoint post challenge, median, IQR and range shown, p values derived from

Friedman test with Dunn's multiple comparisons, E: Comparison of maximum change from baseline between Group 1 and 2, p value derived from Mann Whitney test.

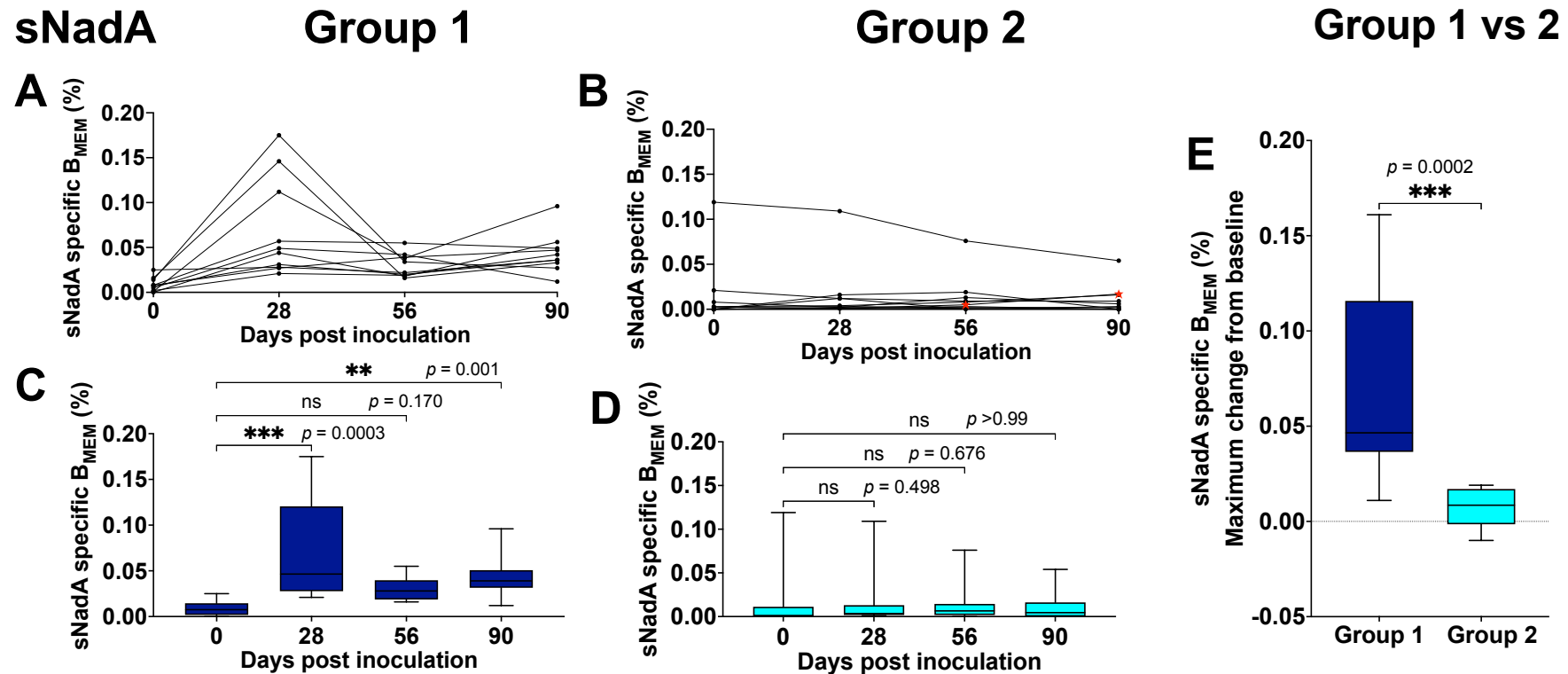


Figure 5.18 B_{MEM} : sNadA

Memory B cells with specificity for sNadA

B_{MEM} expressed as a percentage of total circulating IgG memory B cells at each timepoint post challenge from participants in Group 1 (A+C) and Group 2 (B+D). A+B: Individual participant trends over time per group, Nmen colonised individual indicated with red star. C+D: Summary data per group comparing baseline with each timepoint post challenge, median, IQR and range shown, p values derived from Friedman test with Dunn's multiple comparisons, E: Comparison of maximum change from baseline between Group 1 and 2, p value derived from Mann Whitney test.

Chapter 5

5.3.9.3 Serum bactericidal activity

Serum bactericidal activity to Nmen strain 5/99 was determined at baseline and Day 28, 56 and 90 following challenge. This Nmen strain is known to express NadA at high levels. Serial two-fold dilutions of each serum sample were analysed and the SBA titre reported for each sample is the reciprocal of the last dilution of that sample deemed to be bactericidal ($\leq 50\%$ Nmen colony survival in comparison to control). One colonised participant who withdrew from the study prior to Day 28 is excluded from this analysis.

SBA titres for each participant timepoint are shown in Table 5.8. An SBA titre ≥ 4 is the internationally accepted correlate of protection against meningococcal disease (53). Participants were therefore considered “Unprotected at baseline” (from meningococcal disease) if they had an SBA titre < 4 at baseline, and of those participants, they were considered to be “Newly protected post challenge” if they had an SBA titre ≥ 4 at any post challenge timepoint, and “Newly protected at Day 90” if they had an SBA titre ≥ 4 at Day 90 post challenge.

Of those participants who were unprotected at baseline, 5 of 7 in Group 1 (71.4%) and 4 of 9 in Group 2 (44.4%) became protected (SBA titre ≥ 4) at some point post challenge, and 4 of 7 in Group 1 (57.1%) and 3 of 9 in Group 2 (33.3%) were protected at Day 90 post challenge. This suggests that the expression of NadA may have had some impact on the induction of SBA but does not reach statistical significance ($p=0.358$ for newly protected post challenge, $p=0.615$ for newly protected at Day 90, Fisher’s exact test).

The participant in Group 2 who lost GM Nlac colonisation and acquired Nmen carriage between Day 28 and 56 post challenge is highlighted in red. His SBA titres were < 2 until Day 90 at which point they were 8, i.e. he became newly protected following the acquisition of Nmen. It is likely that this induction of a protective SBA response was due to the acquisition of Nmen rather than carriage of GM Nlac. Exclusion of this participant from the analysis does not alter the statistical significance (newly protected post challenge $p=0.315$, newly protected at Day 90 $p=0.590$, Fisher’s exact test).

Participant	Day 0	Day 28	Day 56	Day 90	Unprotected at baseline	Newly protected post challenge	Newly protected at Day 90
Group 1							
1	8	16	16	16	N		
2	<2	<2	2	<2	Y	N	N
3	8	8	8	8	N		
4	8	8	8	8	N		
5	<2	4	8	8	Y	Y	Y
6	<2	<2	4	<2	Y	Y	N
7	<2	<2	4	4	Y	Y	Y
8	2	4	4	4	Y	Y	Y
9	<2	<2	<2	<2	Y	N	N
10	2	8	32	16	Y	Y	Y
Total per group n (%)					7 (70.0)	5 (71.4)	4 (57.1)
Group 2							
1	8	8	8	16	N		
2	<2	<2	<2	<2	Y	N	N
3	4	4	8	4	N		
4	<2	<2	<2	8	Y	Y	Y
5	<2	2	2	2	Y	N	N
6	2	2	4	2	Y	Y	N
7	2	<2	<2	<2	Y	N	N
8	2	8	-	4	Y	Y	Y
9	<2	<2	2	<2	Y	N	N
10	<2	<2	2	<2	Y	N	N
11	2	4	4	4	Y	Y	Y
Total per group n (%)					9 (81.8)	4 (44.4)	3 (33.3)

Table 5.8 Serum bactericidal activity

Serum bactericidal activity against Nmen strain 5/99

Participants already protected at baseline shaded in red. SBA titre ≥ 4 in a previously unprotected participant shaded in blue, Nmen colonised participant indicated with red font.

5.4 Discussion

The primary research questions addressed by this study were whether nasopharyngeal colonisation could be safely induced with a “knock in” genetically modified strain of Nlac expressing the meningococcal antigen NadA, and whether that colonisation would result in a specific immunological response to NadA, thus potentially strengthening the known protective relationship between Nlac colonisation, and Nmen colonisation and disease.

However, demonstrating that it is possible and safe to induce a specific immune response to a bespoke antigen using a colonising bacterial vector is also of wider relevance. This approach could be translated to other antigens, either from Nmen or from other pathogens, to facilitate the study of specific mucosal immunogenicity, or even to use “bacterial medicine” to provide protection from other infections.

The nasopharynx is known to be a site capable of producing a significant immune response. The live attenuated influenza vaccine is administered via the nasal mucosa. Despite restricted replication in the respiratory tract (161), it is potently immunogenic (162) and provides long standing protection against infection (166). The candidate *Bordetella pertussis* vaccine BPZE1 is a live attenuated strain of *B. pertussis* with four toxins removed or inactivated (142). Nasal administration of the vaccine strain results in transient nasopharyngeal colonisation (167), but long-standing specific immunity and protection from re-colonisation with the same strain (168). Both of these examples are organisms rendered non-virulent by genetic manipulation, whereas the intervention strain in this study has instead been manipulated to express a heterologous antigen associated with virulence. Despite the theoretical and pre-clinical evidence that this would not result in gain of function and pathogenicity, this was to our knowledge the first controlled human infection study to use this approach, and was therefore both a novel and ground-breaking study, and also one requiring very careful consideration and management of safety and infection control measures, including the specific requirements set out by DEFRA (149).

There were no significant safety concerns in this study. Although some adverse events were reported, none were serious, no difference was seen between the two groups, and no adverse events were felt to be related to colonisation with either GM Nlac strain. No participants received early eradication therapy due to symptoms or safety concerns. Although our study had a small sample size, this would suggest that the expression of NadA did not result in increased virulence in these individuals.

The infection control measures were communicated effectively to all participants prior to enrolment, both verbally and in written form, with full understanding being confirmed by completion of the infection control questionnaire. These measures were adhered to throughout the admission period, and to our knowledge also during the outpatient follow up period. No shedding was detected from any participant, and no transmission was detected even to their closest contacts. Eradication with a single dose of ciprofloxacin was effective in 100% of colonised participants within 48 hours.

This study has therefore been very successful in establishing a safe model of controlled human infection with GM Nlac, with robust and effective safety and infection control measures. The design and conduct of this study will set the standard for future similar studies.

The genetic manipulation of both strains, and the expression of NadA by 4NB1, may have influenced their ability to colonise the nasopharynx and to induce an immune response. As NadA functions as an adhesin (144, 145), it was possible that the expression of NadA would result in an increased colonisation fraction or duration. On the other hand, the potent immunogenicity of NadA (146) may have exerted immunological dominance, thus impacting the Nlac specific immunogenicity, or may have resulted in more rapid clearance of this strain.

The colonisation fraction of both strains was consistent with that seen with wild type Nlac in L3 (0.85 when colonisation was allowed to continue to Day 14) vs 0.92 for Group 1 and 0.79 for Group 2. The majority of participants in both groups remained colonised for the 90 day follow up period, with no difference in duration of colonisation seen between the two groups. Nlac specific humoral immunogenicity was seen in participants colonised with both strains, with a sustained increase seen in IgG specific to Nlac Y92-1009 dOMV, similar to that seen following colonisation with wild type Nlac (123). An increase in B_{PLAS} and B_{MEM} with specificity to OMV derived from each of the GM Nlac strains. The induction of B-cell mediated immune responses have also been demonstrated in response to nasopharyngeal challenge with *Streptococcus pneumoniae* (169).

These findings suggest that neither the genetic manipulation itself, nor the expression of NadA significantly altered the ability of the organism to colonise the nasopharynx or to induce an Nlac specific immune response. However, given the high colonisation fraction in both groups, it is possible that a small difference in colonisation may have become apparent with a larger sample size.

NadA specific immunogenicity differed between the two groups, with colonised participants in Group 1 (colonised with the NadA expressing strain 4NB1) showing a NadA specific humoral immune response, in contrast to colonised participants in Group 2 (colonised with the non-NadA expressing strain 4YB2). 50% of colonised participants in Group 1 seroconverted to NadA with a ≥ 2 -fold increase in reciprocal endpoint titre of sNadA specific IgG versus 0% in Group 2. An increase in sNadA specific IgG and IgA B_{PLAS} and B_{MEM} was seen in Group 1 but not Group 2. This demonstrates that the genetically modified strain does indeed express the heterologous antigen *in vivo*, and that this expression results in a specific humoral immune response.

Colonisation with wild type Nlac has previously been shown to induce some cross-reactive immunogenicity to Nmen, but crucially not an SBA response sufficient to meet the international accepted correlate of protection against meningococcal disease, namely a reciprocal SBA titre of ≥ 4 . In this study we have demonstrated a B_{PLAS} response to a representative NadA expressing Nmen strain, seen in both Groups, so likely due to cross-reaction between Nlac and Nmen epitopes, rather than due to the expression of NadA.

Colonisation with either strain of Nlac may have had some impact on the induction of SBA with 71.4% of those colonised and previously unprotected becoming newly protected in Group 1, in comparison to 44.4% in Group 2. This suggests that colonisation with Nlac itself, and expression of NadA, may each have an impact on anti-meningococcal SBA. However, in order to demonstrate this conclusively, a non Nlac colonised control group would be required to prove that Nlac colonisation had an impact, and a much larger sample size would be required to prove that expression of NadA increased that impact.

The small sample size is a notable limitation to this study. A larger sample size could have provided even more reassuring safety data, as although no safety signal was identified in this study, it does not definitively prove that inoculation with these GMOs would be safe in the wider population. We have not detected any shedding or transmission which is reassuring, but again with this small sample size, it is not possible to be definitive that shedding and transmission do not ever occur. A larger sample size could have uncovered an impact of NadA expression on the ability of Nlac to colonise the nasopharynx, or the rate of natural clearance, especially if carriage had been allowed to continue for a longer duration. It may also have increased the significance of some of the immunological comparisons, allowing more robust conclusions to be drawn, for example about the impact of NadA expression on SBA induction.

However, the sample size was deliberately and necessarily limited. As a pathfinding study involving an inpatient stay, the cost and staffing resources required per participant were high. The study was powered to demonstrate that safe colonisation could be achieved, and to detect significant differences in NadA-specific seroconversion, both aims which were achieved.

A further limitation was the inaccuracy and variability of the actual dose of inoculum administered. The intended dose was 10^5 CFU, but in both groups the actual dose administered was notably higher than this. As there was not a significant difference between the two groups, this will not have impacted any conclusions drawn by comparison between the two strains. However, for future studies it may be vital to achieve the intended inoculum dose with more accuracy, especially if comparisons between different doses are required. Further consideration and optimisation of the inoculum preparation process should therefore be undertaken prior to future studies. Any modification to this process during the conduct of this study could have resulted in an inequality between the groups and so was not attempted.

In conclusion, we have designed and conducted a novel, pathfinding controlled human infection study, safely and reproducibly achieving long-standing immunogenic colonisation with two GM Nlac strains. We have demonstrated a specific immunological response to the expression of a heterologous antigen during colonisation. Future use of this model could include a vaccine-challenge study, investigating the impact of vaccination with specific antigens upon the acquisition and carriage of bacteria expressing those antigens, so for example investigating the impact of Bexsero vaccination on the carriage of 4NB1, and by extension NadA expressing Nmen strains. The model could also be further developed to use transformants expressing other meningococcal antigens, and potentially with antigens originating from other pathogens. Such studies will lead to a greater understanding of mucosal immune responses to colonisation, and the impact of duration and density of colonisation on those immune responses, and may lead to the development of novel bacterial medicines.

Chapter 6 Efficacy of lyophilised *N. lactamica* to induce colonisation in UK and African participants

6.1 Introduction

6.1.1 Study overview

To this point, the Nlac CHIM has used inocula prepared from frozen stocks. This has been effective in reliably inducing immunogenic colonisation in healthy participants within a highly resourced research centre. However, there are some practical disadvantages to this method of storage and preparation which limit the utility of the model.

The bacterial stocks need to be stored in a reliable, temperature monitored minus 80 °C freezer. Even under optimal storage conditions there is a gradual decline in the viability of the stocks. Preparation of the inoculum therefore requires knowledge of the current mean batch viability and a potentially complicated dilution calculation and technique to achieve the intended inoculum dose. Variability in the viability of different vials, plus any error in the precise dilution steps required results in a notable variation in the administered dose of inoculum. Once prepared, the viability of the inoculum drops rapidly so inoculation must take place within 30 minutes of inoculum preparation.

This limits the use of the model to settings with sufficient resources and expertise to store, prepare and rapidly administer the inoculum along a cold chain. Even in such a setting, the dose accuracy is sub-optimal. As a potential use for the model is in an outbreak setting within the meningitis belt, it was necessary to overcome some of these limitations.

A potential resolution was to change the method of storage to a lyophilised preparation of the bacterial stocks. Lyophilisation (freeze drying) is a process in which bacteria are desiccated to form a powder with efficient reconstitution to a viable state on re-addition of an appropriate diluent. Lyophilised bacteria can remain stable for long periods of time without the need for strict temperature control (140). Long term storage and transport to a resource-poor setting would therefore be logistically feasible, and the reliability of the batch viability could allow a simplified and more accurate process of inoculum preparation. This would make the use of this model feasible in low resource settings, where the incidence of meningococcal disease is highest.

Other modifications to the model considered likely to improve transferability to a low resource setting include the use of a single use Pasteur pipette rather than an automated

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pipette, change of the diluent from PBS to water, and reducing the time the participant is required to remain supine post inoculation from 15 minutes to five minutes.

Two studies were conducted to assess the use of lyophilised Nlac (LyoNlac) in a CHIM. Both studies were designed as dose-ranging studies as the impact of prior lyophilisation on colonisation potential, and the speed of reanimation of bacterial cells within the nasopharyngeal environment Nlac were unknown.

The first study LACTAMICA 5 (L5) was conducted in a healthy adult population within the UK. Once this had been completed a similar study LACTAMICA Etape 1 (LE1) was conducted in a population within the meningitis belt, in Bamako, Mali. The aim of both studies was to assess the dose of LyoNlac required to reliably induce nasopharyngeal colonisation, as well as the safety, duration and immunogenicity of such colonisation.

6.1.2 LyoNlac

Ampoules of LyoNlac were produced to GMP-like standards at the University of Southampton Medical School Laboratory LC67 / LC70 by Dr. Jay Laver according to specific SOPs. Each ampoule contained a defined amount of reconstitutable bacteria in powder form. These ampoules could be reconstituted in sterile water or PBS to yield cultivable Nlac, phenotypically and genetically identical to the frozen bacterial stocks (JR Laver, Personal Communication).

Pre-clinical work assessed the viability of ampoules over time at a range of cycling temperatures, intended to mimic day/night ambient temperatures or unreliable refrigeration conditions in a tropical climate. Viability above a minimal viable product (10^7 CFU) was maintained for 3 months in the worst performing scenario (cycling 16 hours at 37 °C / 8 hours at 25 °C) and was maintained beyond that for other temperature cycling patterns (JR Laver, unpublished data). Further information about LyoNlac can be found in Appendix F.

6.1.3 COVID-19 pandemic impact

The COVID-19 pandemic began as the final participants were recruited to L5, and during the set-up phase for LE1. For L5, this resulted in some missed visits or telephone visits and therefore missed clinical samples for the final participants.

For LE1, the pandemic had a substantial impact on the way the study was conducted. Having completed an initial site visit and local study team training in February 2020, the intention was for the UK study team to return to Mali in May 2020 to refresh training, commence recruitment and oversee the challenge and follow up periods for the first few cohorts. This was no longer possible, and the study was initially put on hold until travel was permitted, but after much discussion and further training online and via transfer of detailed SOP documents and videos, the decision was taken to commence the study in March 2021 with close virtual supervision from the UK study team, and weekly online meetings with the whole study safety committee.

Recruitment and screening processes were carried out quickly and efficiently by the local study team but there were two notable protocol deviations during challenge of the first cohorts due to administrative and technical errors, likely due to the limitations of virtual supervision. These incidents are discussed in more detail in sections 6.3.1 and 6.3.5 and the COVID risk mitigation SOPs are detailed in Appendices E18-19.

6.1.4 LE1 – Collaborating institutions

Translation of the Nlac CHIM to Mali required additional expertise and resources, therefore several different institutions collaborated in the design, set up, conduct and safety monitoring of this study:

- University of Southampton – LyoNlac production, study design and oversight, study safety committee, immunological sample processing and PCR of Nlac isolates, data analysis
- Centre pour le Développement des Vaccins du Mali (CVD Mali) – Study sponsor, recruitment and all study visits, data collection, inoculum preparation, culture of microbiological samples, serum storage
- University College London and the Mucosal Pathogens Research Unit – funding, input into study design, study safety committee
- University of Maryland – Input into study design, study safety committee
- University of Oxford – Expertise in *Neisseria* epidemiology in meningitis belt, input into study design, study safety committee

6.1.5 LE1 Community engagement and recruitment

Recruitment to LE1 followed the standard process at CVD Mali, with consideration given to the need for community engagement and permission, and the differing needs and expectations of potential volunteers. In order to obtain community permission, meetings were held with community stakeholders such as local religious and cultural leaders. The study purposes, risks and benefits were explained and audio recordings of the information sheet were played, followed by a question and answer session. Community liaisons were appointed at local health centres who were able to identify potential participants and link interested persons with the study team.

Potential volunteers were invited to a screening visit at CVD Mali. Adult literacy is approximately 40% with several different languages spoken, so volunteers were provided with written and/or audio versions of the information sheet and consent form in an appropriate language. All volunteers were asked to sign or mark the consent form but if illiterate, an independent literate witness would also sign and date the form to confirm that informed consent had taken place.

6.2 Study specific methods

6.2.1 Study design

This chapter describes two dose-ranging prospective controlled human infection studies. L5 was conducted in the UK and LE1 in Bamako, Mali. In both studies, participants were inoculated intranasally with LyoNIac. The dose ranging strategy for each study is described in section 6.2.2. Safety and colonisation were monitored until Day 168 following challenge and immunogenicity was assessed at Day 28 post challenge in comparison to baseline. In L5, participants were invited to an optional visit at 18 months to 3 years following inoculation to assess for ongoing colonisation.

Overviews of the study timelines for each study are shown in Figure 6.1. The objectives and endpoints of each study are summarised in Table 6.1 and 6.2, and schedule of procedures in Table 6.3. The eligibility criteria are detailed in Chapter 2.

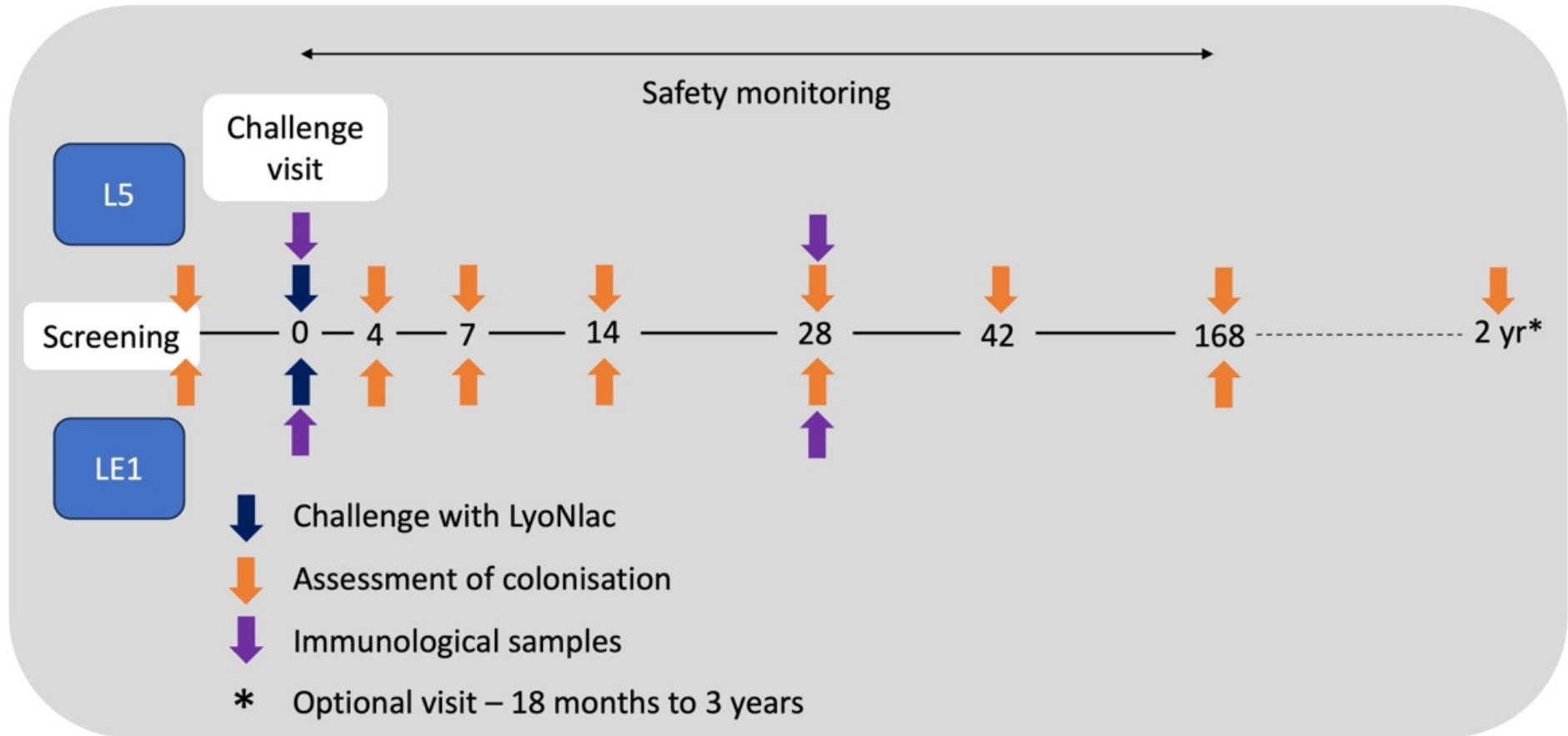


Figure 6.1 L5 and LE1 Study timeline

Schematic presentation of study timeline for L5 and LE1 participant visits, interventions and monitoring.

Objectives		Endpoints
Primary objective	To assess the safety of nasal inoculation of healthy volunteers with LyoNlac	Occurrence of unsolicited adverse events within the study period
		Occurrence of serious adverse events within the study period
Secondary objectives	To establish the dose of nasally administered LyoNlac required to induce nasopharyngeal colonisation in 80% of inoculees	Assessment of colonisation – culture of Nlac from throat swabs taken between day 4 and day 14 post challenge
	To demonstrate the immunogenicity of colonisation induced by LyoNlac	Rise in serological specific antibody titre comparing day 0 versus day 28 post challenge

Table 6.1 L5 Objectives and endpoints

Adapted from L5 study protocol (Appendix A3).

Objectives		Endpoints
Primary objective	To demonstrate successful nasopharyngeal colonisation of healthy Malian adults with nasally administered LyoNlac	Culture and PCR of colonies isolated from throat swabs taken at any study visit
Secondary objectives	To assess the safety of nasal inoculation of healthy Malian adults with LyoNlac	Occurrence of solicited reactogenicity within 25 minutes post-inoculation
		Occurrence of unsolicited adverse events within 28 days post-inoculation
		Occurrence of serious adverse events within the study period
	To establish the dose of LyoNlac required to induce nasopharyngeal colonisation in at least 70% of healthy Malian adults	Culture of Nlac from throat swabs taken between Day 4 and Day 7 following inoculation
To demonstrate the immunogenicity of colonisation induced by nasal inoculation with LyoNlac in healthy Malian adults	Rise in serological specific antibody titre comparing Day 0 versus Day 28 post inoculation	
Exploratory objective	To assess the duration of colonisation induced by nasal inoculation with LyoNlac in healthy Malian adults	Culture and PCR of colonies isolated from throat swabs taken at any study visit

Table 6.2 LE1 Objectives and endpoints

Adapted from LE1 study protocol (Appendix A4).

	Screening	Challenge		Follow up					
Timeline (days)	≤ 30 (L5) -2 to -17 (LE1)	0	4	7	14	28	42*	168	2yr**§
Informed consent	+								
Vital signs	+	+	+	+	+	+	+	+	+
Medical history	+								
Physical examination	+	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)
Pregnancy test (females)	+	+							
Electrocardiogram*	+								
Review eligibility		+							
Challenge		+							
Review of AEs / concomitant medications		+	+	+	+	+	+	+	+
Nasal wash*	+		+	+	+	+	+	+	+
Throat swab	+	+	+	+	+	+	+	+	+
Safety bloods	+		+						
Immunological bloods		+				+			

Table 6.3 L5 and LE1 Schedule of procedures

+ Procedure at this timepoint, (+) if clinically indicated, * and grey shading – L5 only, § 18 months to 3 years post challenge. Adapted from L5 and LE1 study protocols (Appendices A3 and A4).

6.2.2 Dose ranging strategy

Dose ranging strategies were used to establish the optimum dose, or standard inoculum for each population. For both studies, participants were challenged in dose cohorts of 5, with dose adjustments to achieve the desired colonisation fraction and then continued challenge of participants until 10 individuals had been colonised at that dose.

For L5, the starting dose was 10^5 CFU, the dose used with inocula prepared from frozen stocks in L3 and L4. The dose was escalated and de-escalated to achieve a colonisation fraction of approximately 80%, as detailed in Figure 6.2A. For LE1, the starting dose was the standard inoculum dose established in L5. The dose was then escalated only, to achieve a colonisation fraction of at least 70%, as detailed in Figure 6.2B.

6.2.1 Safety considerations

The first participant to be challenged with LyoNlac, in L5, was challenged individually with a safety review following the Day 4 visit. The remainder of the first cohort were challenged in groups of 1-3. For the remainder of L5 and subsequently in LE1 the first participants challenged at each escalated dose were challenged in groups of up to 5. The external safety committee (L5) or study safety committee (LE1) reviewed the safety data to at least Day 4 post challenge and gave permission for dose escalation. In the event of any significant safety concerns, participants could be reviewed with eradication therapy with ciprofloxacin or further investigation and treatment if required.

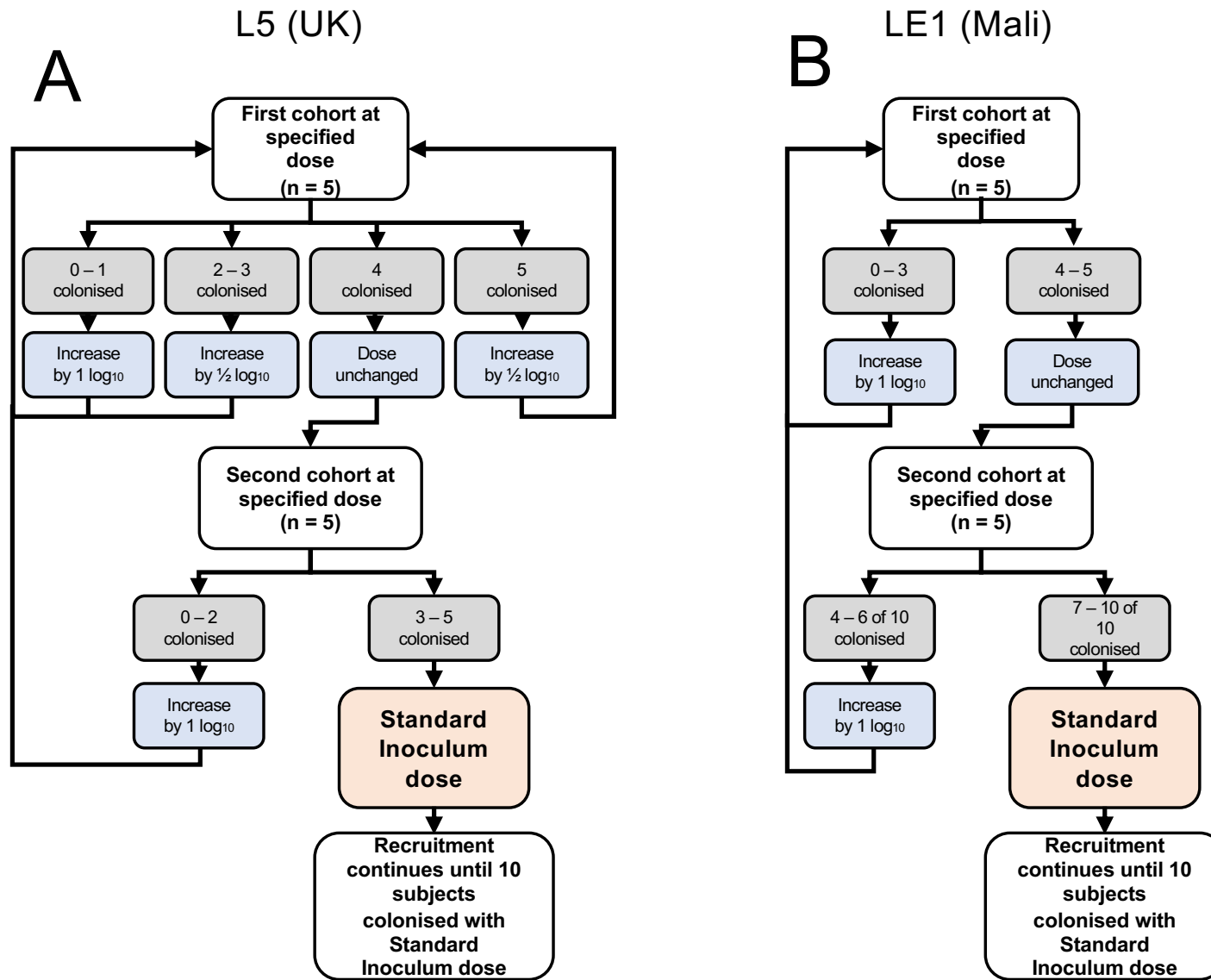


Figure 6.2 Dose ranging processes

6.3 Results

6.3.1 Recruitment and study progress

For L5, 31 healthy adult volunteers were screened in Southampton between May 2019 and March 2020. 30 eligible participants were enrolled and challenged according to the dose ranging process. Five participants withdrew during the study, one prior to the Day 28 immunological assays. All of these were due to non study-related reasons. 20 participants attended the optional two year follow up visit.

LE1 was conducted in Bamako, Mali. 80 volunteers were screened between March and June 2021 in a total of 95 screening visits, as some volunteers were re-screened having gone past the permitted screening window. A total of 55 participants were enrolled and challenged, initially in weekly cohorts of five.

For the first three cohorts of participants ($n = 15$), the viable count of the residual inoculum was substantially lower than expected, resulting in a very low colonisation fraction. This was found to be due to an error in the inoculum preparation resulting in an inadequate inoculum. During this review, it was also noted that one participant had been inadvertently enrolled and challenged, when they were ineligible due to baseline Nlac carriage. The cause of this error was inadequate documentation and no formal eligibility check.

These events were immediately reported to the IRB as protocol violations. The 15 affected participants were followed up for safety only, and are therefore not included in any further analyses.

Re-training was conducted for inoculum preparation and eligibility assessment, and additional safeguards were implemented for robust documentation and communication of screening results. The process of approval for inoculation was changed to include discussion at the study safety committee meeting with approval given for each eligible volunteer. Once permission had been given by the IRB and study safety team to restart, recruitment was continued, starting at the beginning of the dose ranging process. Following this restart, a further 40 subjects were enrolled. One participant was lost to follow up prior to the final visit at Day 168.

Participant recruitment and inclusion in each analysis set is shown in Figure 6.3 for both studies.

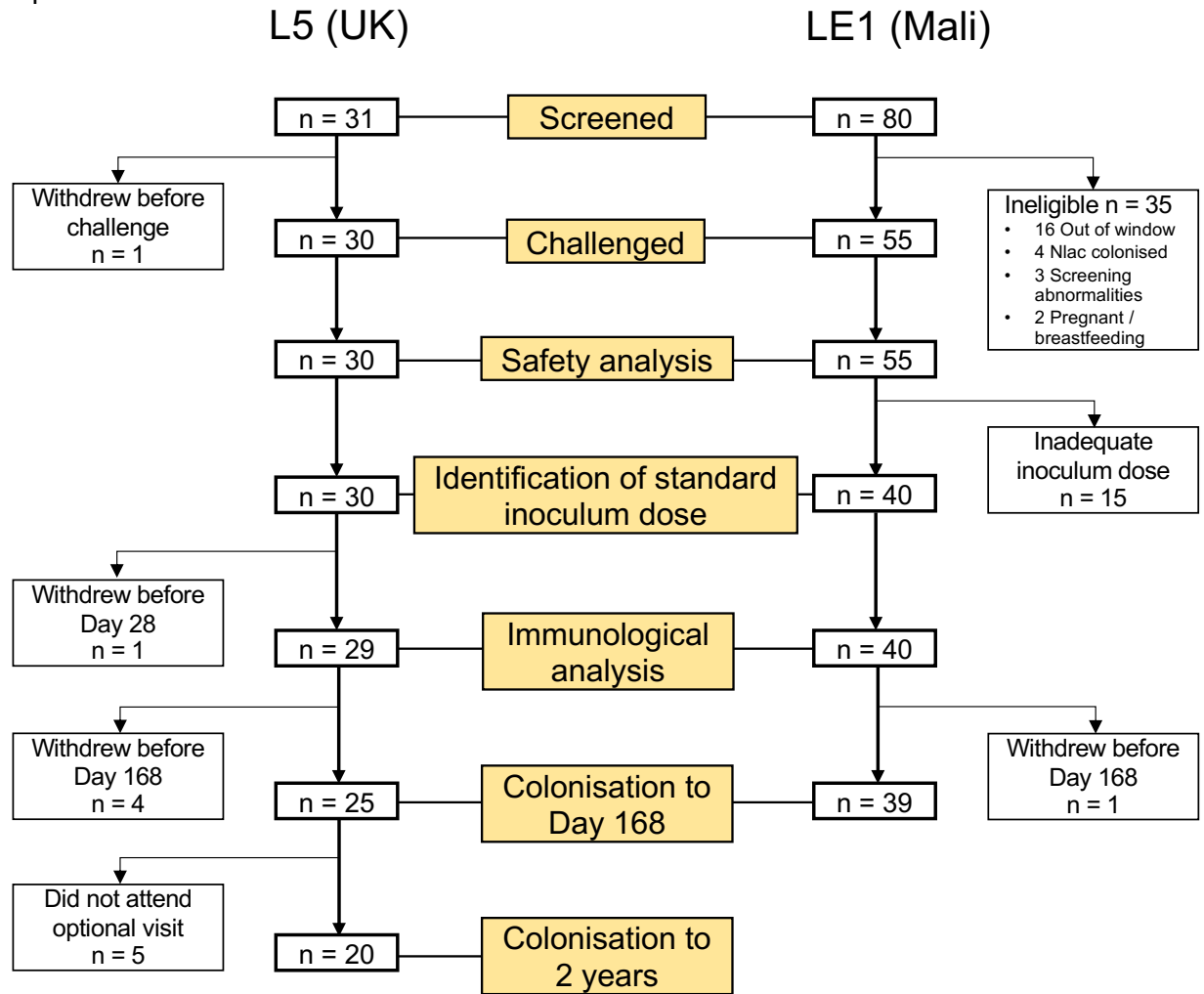


Figure 6.3 Participant recruitment

Overview of participant recruitment for L5 and LE1 with number of participants (n) screened, enrolled and included in each analysis set.

6.3.2 Assessment of colonisation status

For L5, participants were assessed for colonisation by culture of throat swabs and nasal wash samples, with successful colonisation being defined as the culture of at least one colony of Nlac after challenge and up to Day 14 post challenge.

Comparison was made between the sensitivity of throat swabs and nasal washes in the detection of Nlac carriage. In calculating this, the following assumptions were made:

- All positive cultures were considered to be true positive
- Negative cultures were considered to be false negatives if culture of the other sample type at the same time point was positive
- Negative cultures were considered to be false negative if the participant had a positive culture (of any sample type) at both the preceding and the following time point
- All other negative culture results were considered to be true negative

Using these assumptions, and only looking at time points when both sample types were taken, the estimated sensitivity of throat swabs in detecting wild type Nlac carriage was 99.2% whereas that of nasal washes was only 70.8%, as shown in Table 6.4. This difference in sensitivity was statistically significant ($p < 0.0001$, Fisher's exact test).

For LE1, nasal wash samples were not collected, and successful colonisation was defined as the culture of at least one colony of Nlac after challenge and up to Day 14 post challenge. However, for the purpose of dose ranging decisions, the colonisation fraction of each cohort was assessed following the Day 7 visit. Three participants in the final cohort of LE1 had colonisation detected on Day 14 and are included as colonised participants for all further analyses. As the dose-ranging process had already been completed at this point, the exclusion of Day 14 results from dose-ranging decisions did not have any impact on those decisions or the identification of the standard inoculum dose.

For both studies, successful colonisation with the inoculum strain was confirmed by Y92-1009 specific PCR of at least one isolate recovered from each participant following challenge. Isolates from all participants found to be Nlac colonised at screening and one previously non-colonised participant in LE1 who became Nlac colonised at Day 168, were confirmed by PCR to be Nlac, but not Y92-1009.

	True positive	False negative	Sensitivity
Throat swab	119	1	99.2%
Nasal wash	85	35	70.8%

Table 6.4 Comparison of sample type for Nlac culture

Sensitivity of throat swab vs nasal wash culture in the detection of Nlac colonisation in L5

6.3.3 Identification of the standard inoculum dose : L5

The initial dose for L5 was 1×10^5 CFU which achieved a colonisation fraction of 1.0 in the first cohort. Following the pre-defined dose ranging plan (Figure 6.2A), the dose was de-escalated to 1×10^4 CFU and then re-escalated to 1×10^5 CFU, again with a colonisation fraction of 1.0. Although this was higher than the intended colonisation fraction of 0.8, the decision was made to use this dose as the standard inoculum dose, as the colonisation fraction at the lower doses was insufficient. Details of this process are shown in Figure 6.4 and the overall colonisation fractions for each intended dose are shown in Table 6.5.

6.3.4 Inoculum dose and purity : L5

The actual dose of inoculum administered to each participant was assessed by viable count of the residual inoculum following each inoculation. Viable counts for the first two participants were much higher than anticipated at 5.3×10^5 and 1.3×10^6 CFU/ml i.e. 5 and 13 times higher, respectively, than the intended dose. As per protocol, these were defined and reported as an AE and an AESI respectively. Both participants remained entirely asymptomatic. Potential reasons for this apparent inaccuracy in dosing were explored and further checks were made of the batch viability. All calculations were rechecked and a further 4 vials from the batch were diluted and counted by two individuals and found to be exactly as expected, with the intended inoculum viability being achieved with the planned dilution. Following this, the next three participants were inoculated with their inoculum being prepared in exactly the same way as the first two i.e. with the same dilution. The resulting residual inoculum counts were much closer to the intended dose (1.15 , 1.05 and 1.11×10^5 CFU/ml). It is possible that an error occurred during the viable count process for the first two participants, but it was not possible to confirm this. Further inoculum doses were found to be more accurate and reproducible with median doses of 9.7×10^3 for the 1×10^4 participants, 4.4×10^4 for the 5×10^5 participants, and 8×10^4 for the 1×10^5 participants. This is shown in Figure 6.5.

The residual inocula were also assessed for purity by culture on non-selective media. In the first two cohorts, the residual inocula for four participants had a scanty growth of non-*Neisseria* species, consistent with contamination with upper respiratory tract flora. For these participants, one single use Pasteur pipette had been used to inoculate both nostrils, potentially transferring upper respiratory tract flora from the participant's first nostril into the inoculum when withdrawing the second half of the inoculum. Of note each inoculum dose was used for a single participant. Following this, a change to the SOP was made, to use a separate Pasteur pipette for each nostril. Following this, all residual inoculum cultures yielded a pure growth of Nlac.

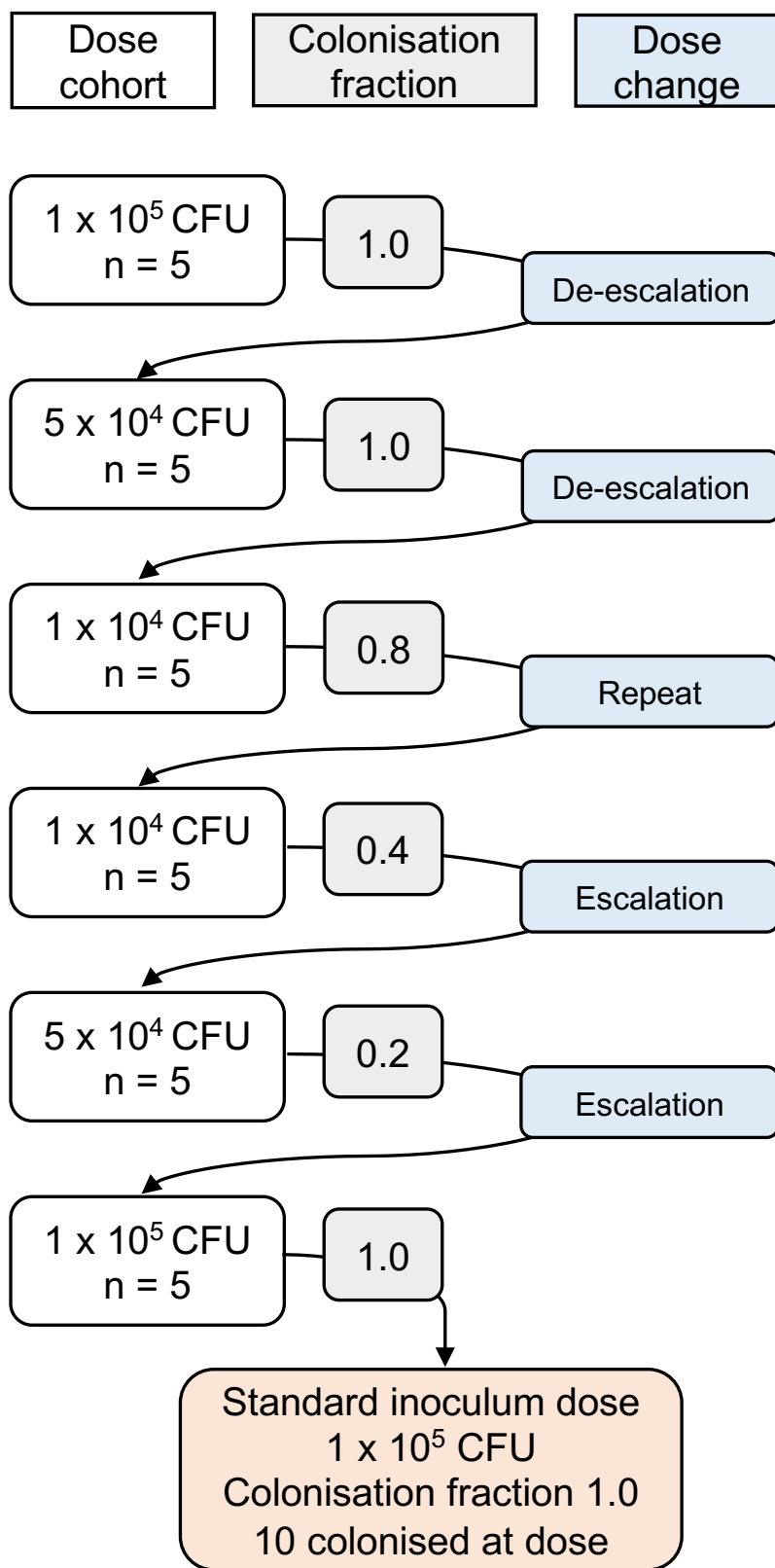


Figure 6.4 Identification of the standard inoculum dose for L5

Process of identifying the standard inoculum as per the dose ranging plan, with number of participants, colonisation fraction and subsequent dose change per cohort.

Intended dose (CFU)	1×10^4	5×10^4	1×10^5
Number of subjects	10	10	10
Colonisation fraction (95% CI)	0.60 (0.31-0.83)	0.60 (0.31-0.83)	1.00 (0.72-1.00)

Table 6.5 Overall colonisation fraction per intended dose for L5

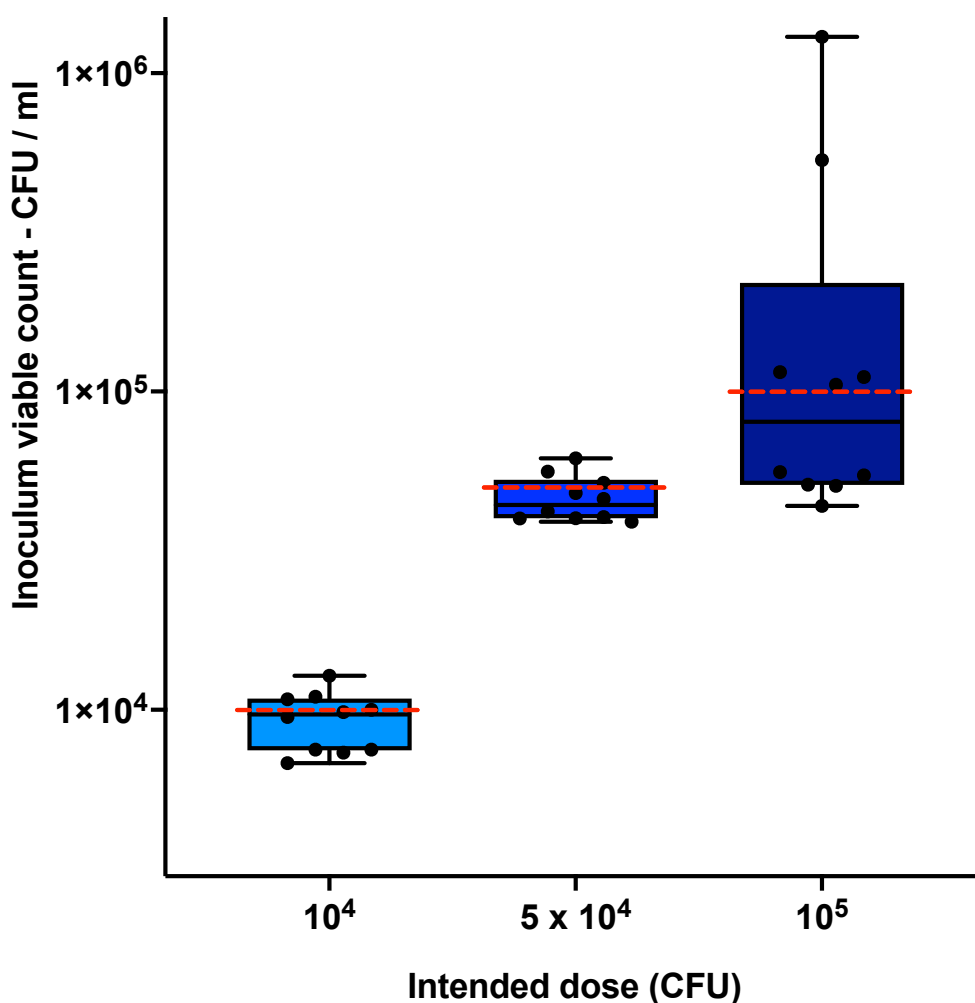


Figure 6.5 Inoculum dose for each intended dose cohort - L5

Confirmed doses calculated by viable count of the residual inoculum following inoculation. Individual values, median and range shown, red dotted line indicates the intended dose.

6.3.5 Identification of the standard inoculum dose : LE1

The initial intended inoculum dose for LE1 was 1×10^5 CFU, as this had been identified as the standard inoculum dose for L5. As discussed in section 6.3.1, viable counts of the residual inocula for the first cohort of participants was much lower than anticipated, with a median of 1×10^4 CFU, and only one participant was colonised.

Potential causes of this were considered. Firstly, the dilution calculation was checked and confirmed by several members of the study team and the inoculum preparation process was reviewed with the laboratory team.

Secondly, the process of transfer from the laboratory to the participant was considered. The distance between the laboratory and clinical areas was significantly further than in Southampton, taking over ten minutes to transfer the inoculum to the participant, in comparison to approximately two minutes in Southampton. The transfer process also involved a brief walk outside where the ambient temperature was close to 40 °C. It was felt that this additional delay, and the exposure to a high ambient temperature, may have resulted in a significant drop in the residual count. For the second cohort, the inoculum viable count was therefore checked immediately after preparation to assess for an impact of transfer time on the viability. Cool transport boxes were used to ensure that the inoculum did not rise above “air-conditioned” room temperature of the laboratory. For the second cohort this pre-inoculation count was improved, but still remained lower than expected, with a median of 5×10^4 CFU. No participants were colonised in that cohort.

After discussion with the study safety committee, the intended dose was escalated as per protocol, to 1×10^6 CFU. The median dose for the third cohort was 1.4×10^5 CFU (post inoculation). Two participants were Nlac colonised following inoculation, but on review, one of these had been incorrectly enrolled having been Nlac colonised at screening. Further assessment with PCR concluded that the strain of Nlac was not Y92 so this participant had not been successfully colonised.

After inoculation of these three cohorts (15 participants), the study was paused for further review. The inoculum viable counts for these three cohorts are shown in Figure 6.6.

During the review process, it was noted that a concentrated solution of PBS at 10 x normal concentration had been used for inoculum preparation. This hypertonic solution had caused a sudden drop in the inoculum viability, a subsequent very reduced colonisation fraction. Additionally, higher levels of reactogenicity had been reported in Mali

than in Southampton, again this was likely to be due to the hypertonic inoculum. Mock inoculum preparation using standard concentration PBS achieved an inoculum of the correct viability.

Following appropriate reporting, retraining, the addition of safeguards, and permission from the IRB and study safety committee, the study was re-opened. The dose-ranging process was restarted at 1×10^5 CFU with the dose escalated to 1×10^6 and then 1×10^7 CFU. This process is shown in Figure 6.7, with the overall colonisation fraction for each intended dose shown in Table 6.6. The colonisation fraction at the final dose of 1×10^7 CFU only reached 0.65 overall, but this was identified as the standard inoculum dose as per the pre-defined dose-ranging process (Figure 6.2B).

Each inoculum was assessed for viable count both immediately after preparation (pre) and on return to the laboratory after inoculation (post), as shown in Figure 6.8. A drop in viable count was seen between these two measurements. This drop did not quite reach significance for the 1×10^5 cohort due to the small cohort size, but was significant for the higher intended doses. The actual dose administered to the participant is likely to have been somewhere between these two measurements.

Culture of all residual inocula resulted in a pure growth of Nlac.

6.3.6 Comparison of colonisation fraction in LE1 vs L5

The standard inoculum dose identified for LE1 was higher (10^7 CFU) than that identified in L5 (10^5 CFU), with a lower colonisation fraction seen at each dose in LE1 in comparison to that seen using the standard inoculum dose in L5. This apparent difference in colonisation fraction between the two settings did not reach statistical significance, both when comparing the colonisation fraction at 10^5 CFU ($p = 0.095$) or comparing the colonisation fraction at the study-specific standard inoculum dose ($p = 0.064$, both Fisher's exact test). A larger sample size may confirm a difference in colonisation fraction between the two settings.

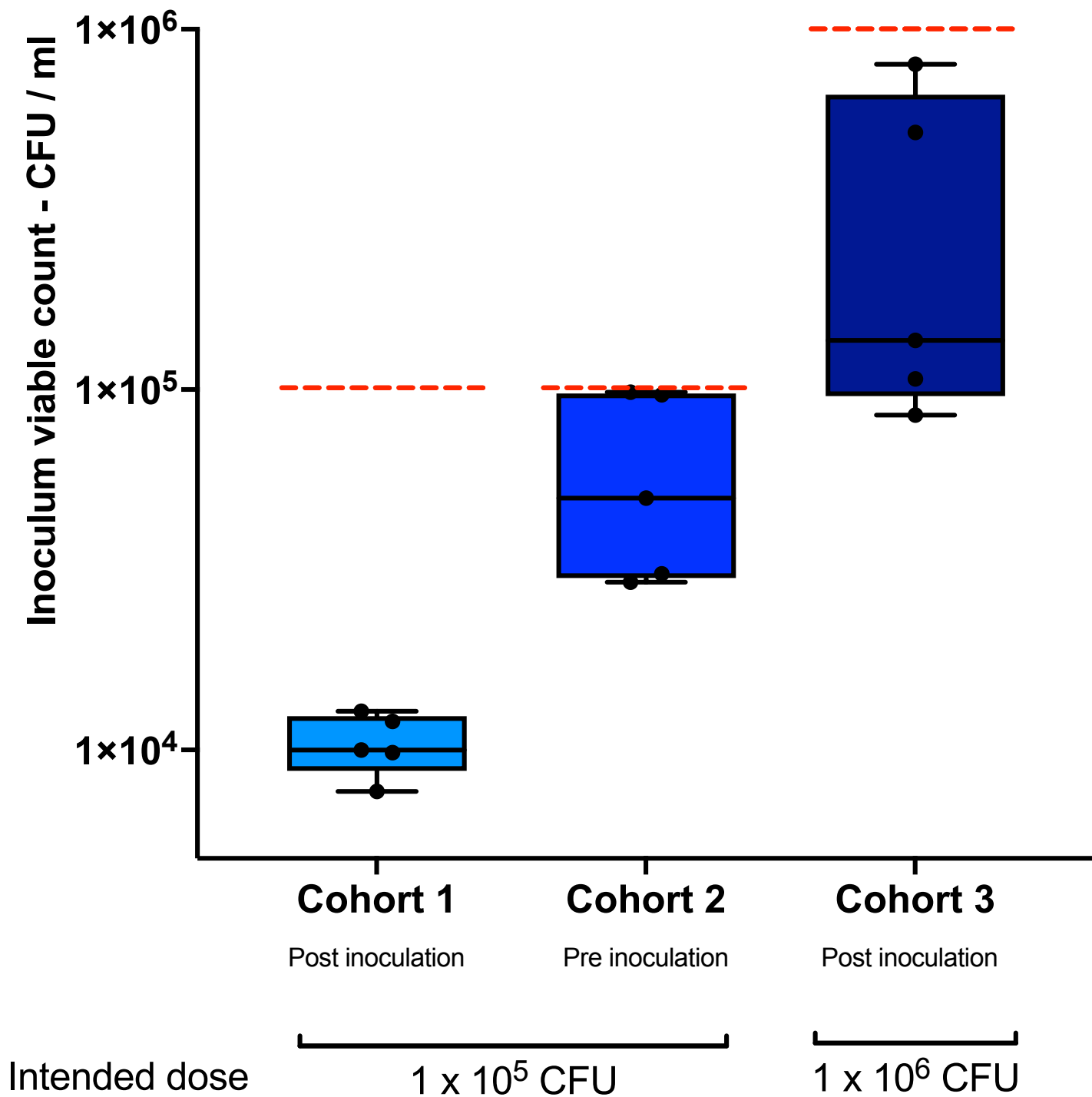


Figure 6.6 LE1 inoculum doses - Cohorts 1-3

Confirmed doses calculated by viable count of the residual inoculum following inoculation (Cohort 1 and 3), or prior to inoculation (Cohort 2). Individual values, median and range shown, red dotted line indicates the intended dose.

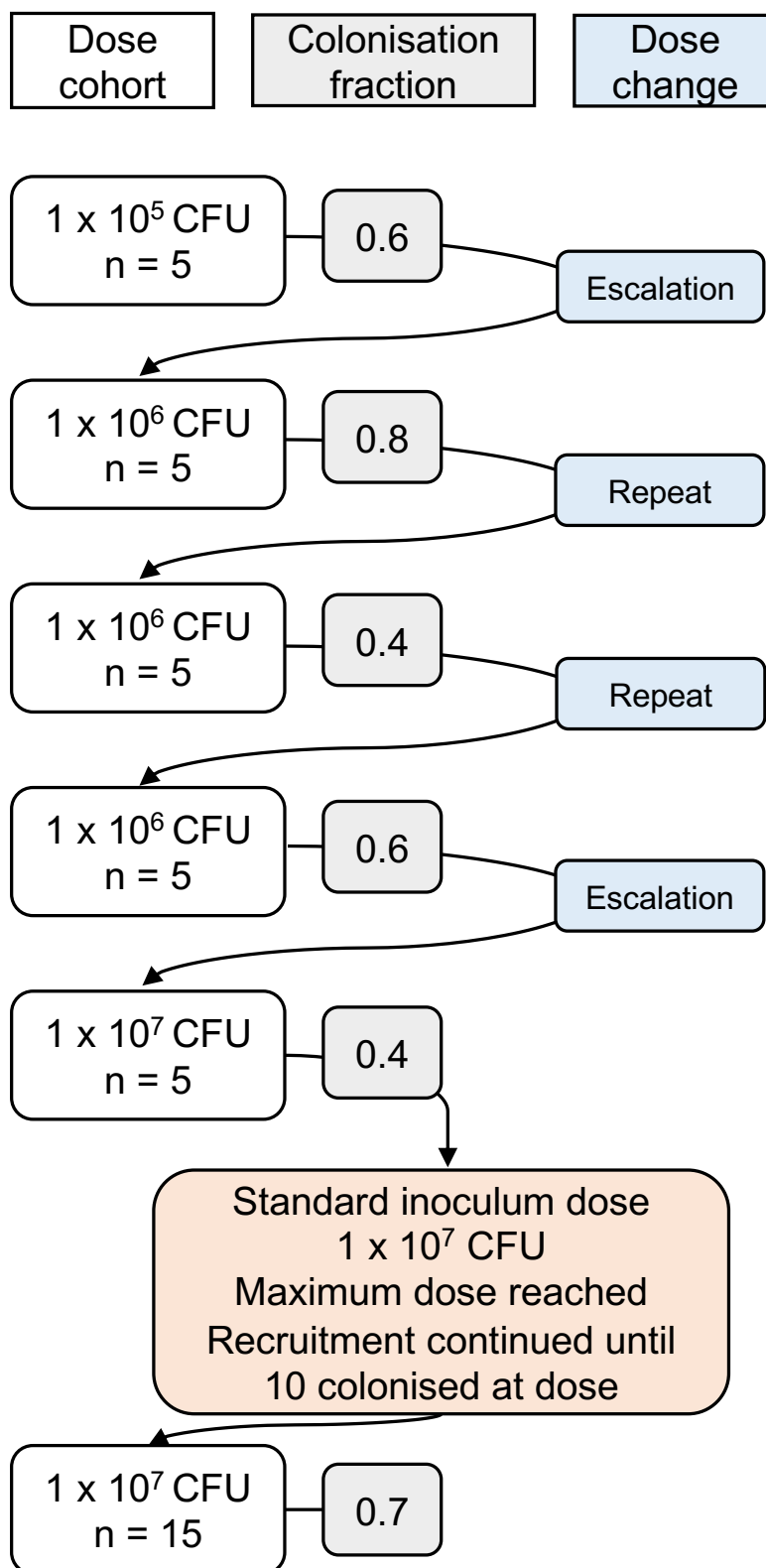


Figure 6.7 Identification of the standard inoculum for LE1

Process of identifying the standard inoculum as per the dose ranging plan, with number of participants, colonisation fraction and subsequent dose change per cohort.

Intended dose (CFU)	1×10^5	1×10^6	1×10^7
Number of subjects	5	15	20
Colonisation fraction (95% CI)	0.60 (0.23-0.93)	0.60 (0.36-0.80)	0.65 (0.43-0.82)

Table 6.6 Overall colonisation fraction per intended dose - LE1

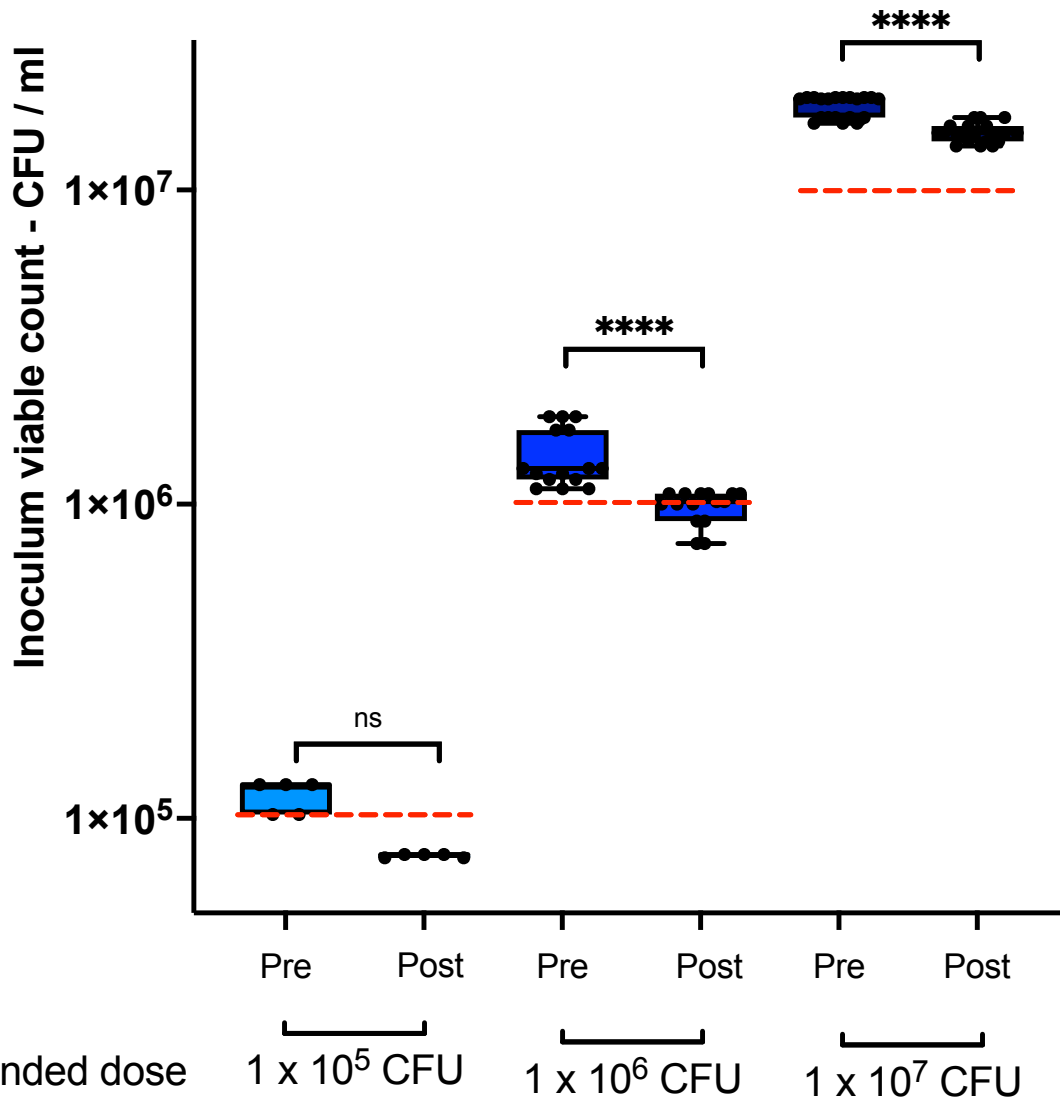


Figure 6.8 LE1 inoculum doses

Confirmed doses calculated by viable count of the residual inoculum immediately post preparation (Pre) and following inoculation (Post), or prior to inoculation (Cohort 2). Individual values, median, IQR and range shown, red dotted line indicates the intended dose. Comparison between pre and post, *p* value derived from Mann Whitney test.

6.3.7 LyoNlac dose accuracy in comparison to frozen stocks

The use of lyophilisation was expected to increase the accuracy of dose preparation. Table 6.7 summarises the dose accuracy achieved in L5 and LE1 (excluding cohorts 1-3) in comparison to L3 and L4 in which frozen stocks of Nlac were used for inoculum preparation. This is expressed as the median value for actual dose / intended dose and the variance (%CV) of residual inoculum viable count for each intended dose cohort.

The median dose / intended dose factor ranged from 0.80 – 1.95 for LyoNlac in comparison to 1.47 and 3.53 for L3 and L4. High variance was seen in one cohort in L5 due to two outlying participants who received apparent overdoses of LyoNlac as discussed in section 6.3.4. In the other LyoNlac dose cohorts the %CV ranged from 7.94 to 21.78% in comparison to 74.2% for L3 and 29.9% for L4. This suggests that LyoNlac may provide more accurate dosing than frozen stocks of LyoNlac, as long as the dose preparation and counting processes are completed correctly.

Study	Inoculum type	N	Intended dose	Median actual dose	Median actual dose / intended dose	CV% of actual dose
L3	Frozen	21	100000	147000	1.47	74.2%
L4	Frozen	26	100000	353000	3.53	29.9%
L5	LyoNlac	10	10000	9665	0.97	21.1%
L5	LyoNlac	10	50000	44000	0.88	16.85%
L5	LyoNlac	8	100000	80400	0.80	165.2%
LE1	LyoNlac	5	100000	128000	1.28	11.6%
LE1	LyoNlac	15	1000000	1300000	1.30	21.78%
LE1	LyoNlac	20	10000000	19500000	1.95	7.94%

Table 6.7 Accuracy of dose preparation per study

6.3.8 Comparison of *Neisseria* carriage at screening – UK vs Mali populations

Carriage of *Neisseria* species was assessed by culture of throat swab specimens taken at screening of all volunteers in both studies. Nlac carriage rates at screening were 0% in the UK (0 of 31) and 5% in Mali (4 of 80). All four Nlac carriers in Mali were female. Nmen carriage rates were 6.4% in the UK (3 of 31) and 8.8% in Mali (7 of 80). Comparison of carriage rates between the two populations showed no significant difference (Nlac $p = 0.57$, Nmen $p > 0.99$, Fisher's exact test).

6.3.9 Comparison of baseline characteristics of challenged participants

Baseline demographic details, Nmen carriage, Nlac and Nmen specific IgG titres for all challenged participants with comparison between between different intended dose groups for each study, and between the two studies, are shown in Table 6.8. One participant in LE1 (1×10^7 cohort) did not have a recorded age and so is not included in the age data. Demographic details and baseline Nmen carriage are included for the initial 15 participants in LE1 who received an inadequate inoculum, to allow comparison with the remainder of the participants enrolled in LE1. However, they are not included in the LE1 total, as they were not challenged as per protocol, and are not included in any analyses apart from safety.

Nlac and Nmen specific IgG titres are not available for these participants, nor for one participant in the 1×10^5 cohort for L5, who withdrew prior to the Day 28 visit.

There was a non-significant trend towards younger age in LE1. In both studies the majority of participants were female, with no difference between the proportions in different cohorts, nor between the two studies. A higher proportion of baseline Nmen carriage was seen in the LE1 1×10^5 cohort than the other cohorts in both studies. Of the two Nmen carriers in this cohort, one became colonised with Nlac and one did not.

There were no significant differences in baseline Nlac and Nmen specific IgG titres between the different dose cohorts in each study. However, baseline titres were higher overall in LE1. This difference reached statistical significance for Nmen specific IgG, but not for Nlac specific IgG. This comparison is also shown in Figure 6.9.

	L5 (UK)					LE1 (Mali)					Mali Total (per protocol)	<i>p</i> (UK vs Mali)
	Dose cohorts				UK Total	Dose cohorts						
	1 x 10 ⁴ CFU	5 x 10 ⁴ CFU	1 x 10 ⁵ CFU	<i>p</i> (comparing dose cohorts)		Inadequate inoculum	1 x 10 ⁵ CFU	1 x 10 ⁶ CFU	1 x 10 ⁷ CFU	<i>p</i> (comparing dose cohorts)		
n	10	10	10		30	15	5	15	20		40	
Age: Median (IQR)	30.6 (26.7-32.8)	27.4 (22.0-30.8)	31.0 (20.6-38.8)	0.52	29.0 (22.8-33.5)	27.8 (20.8-38.3)	21.3 (18.9-22.5)	29.3 (22.6-38.3)	24.4 (21.3-32.0)	0.13	24.3 (21.3-33.9)	0.13
Sex: Male n (%) Female n (%)	3 (30.0) 7 (70.0)	1 (10.0) 9 (90.0)	4 (40.0) 6 (60.0)	0.45	8 (26.7) 22 (77.3)	1 (6.7) 14 (93.3)	2 (40.0) 3 (60.0)	3 (20.0) 12 (80.0)	8 (40.0) 12 (60.0)	0.5	13 (32.5) 27 (62.5)	0.79
Nmen carriage at baseline: n (%)	0 (0.0)	1 (10.0)	1 (10.0)	>0.99	2 (6.7)	0 (0.0)	2 (40.0)	0 (0.0)	2 (10.0)	0.02	4 (10.0)	0.69
Nlac IgG titre baseline: Median (IQR)	5.0 (2.5-16.7)	5.7 (2.5-15.2)	10.5 (4.2-16.5)	0.67	7.5 (2.7-15.9)		10.1 (6.1-16.1)	7.9 (5.4-20.4)	11.4 (6.3-24.3)	0.63	11.0 (5.9-20.9)	0.07
Nmen IgG baseline: Median (IQR)	15.4 (7.3-23.8)	24.6 (5.3-41.1)	18.4 (14.4-23.4)	0.71	18.7 (10.5-28.0)		31.5 (15.0-103.0)	29.6 (12.8-38.1)	24.5 (12.8-38.1)	0.81	25.0 (12.9-44.0)	0.04

Table 6.8 L5 and LE1 participant demographics

Comparison of demographics for participants challenged in L5 (UK) and LE1 (Mali). Sex and Nmen carriage – *p* values derived from Fisher’s exact test, age and IgG titres – *p* values derived from Kruskal-Wallis test when comparing dose groups and Mann Whitney test when comparing UK and Mali. Mali Total excludes those participants who received an inadequate inoculum .

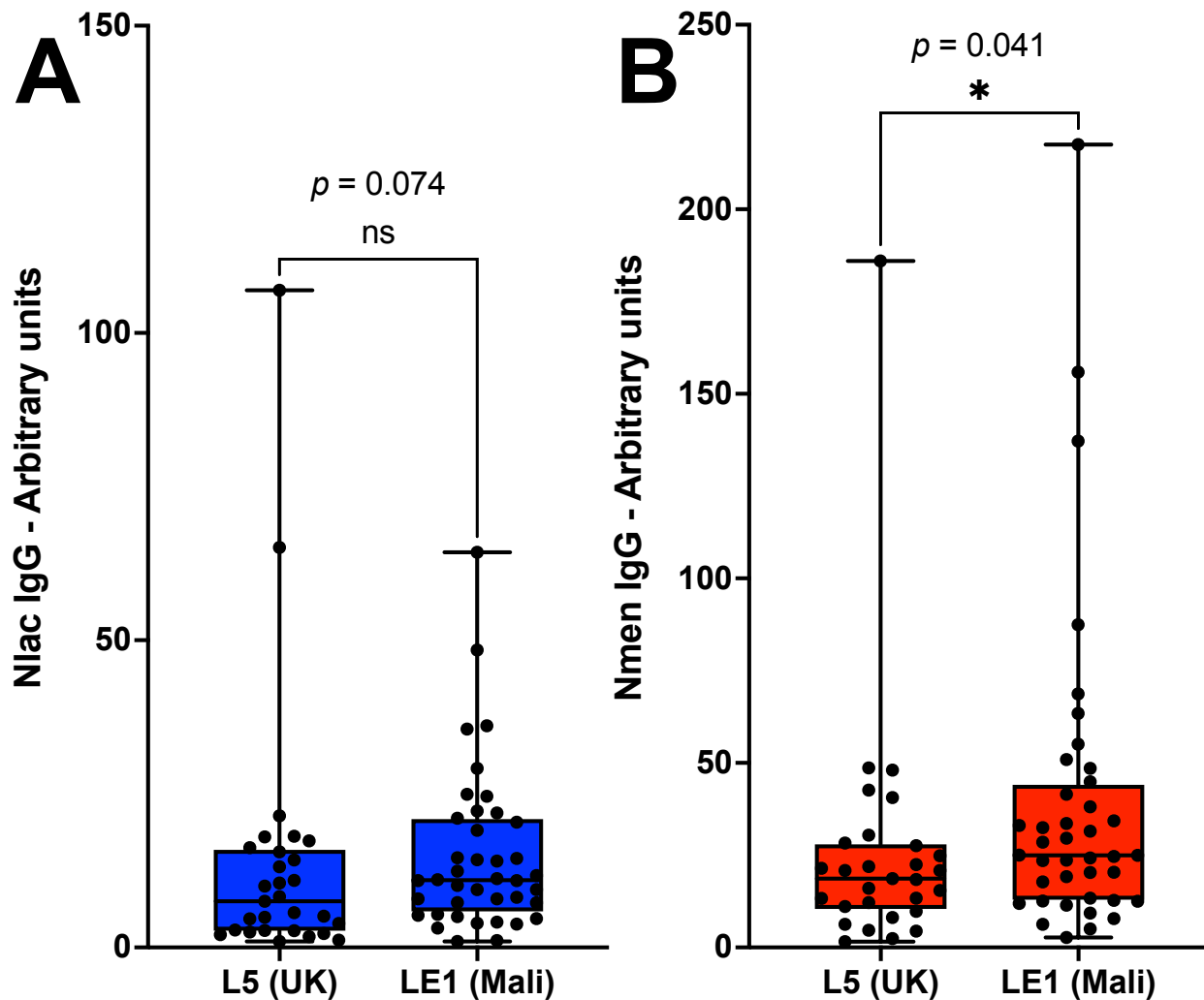


Figure 6.9 Baseline Nlac and Nmen IgG titres: L5 and LE1

Comparison of baseline Nlac and Nmen specific IgG titres between UK (n=29) and Mali (n=40) populations.

Individual values, median, IQR and range shown, *p* values derived from Mann Whitney test.

A = Nlac IgG, B = Nmen IgG.

6.3.10 Comparison of baseline characteristics – colonised vs non-colonised

Baseline demographics, Nmen carriage, Nmen and Nlac IgG titres were compared for colonised and non-colonised participants in each study as shown in Table 6.9. There was a non-significant trend towards an association between age and Nlac colonisation status in both studies. However, in L5, successfully colonised participants tended to be older, whereas in LE1 they tended to be younger. There was a marginal trend towards reduced colonisation in those with pre-existing Nmen carriage in LE1 but the number of baseline Nmen carriers was too small to be conclusive (n=4).

Nmen IgG titres were significantly lower among colonised participants than non-colonised participants in LE1. This was not seen in L5. Nlac IgG titres were not significantly different between colonised and non-colonised participants in either study, and the non-significant trend was in different directions in the two studies, as shown in Figure 6.10.

A further factor which was considered was the potential impact of season upon colonisation, as there is a known association between season and Nmen carriage and disease within the meningitis belt (170). However, all challenges in Mali took place during the dry season (March-June) and so no comparison was possible for this study.

	L5 (UK)			LE1 (Mali)		
	Colonised	Non-colonised	<i>p</i>	Colonised	Non-colonised	<i>p</i>
n	22	8		25	15	
Age:						
Median (IQR)	30.2 (25.5-37.5)	26.0 (21.7-30.0)	0.09	22.5 (19.5-32.3)	28.6 (23.1-36.3)	0.09
Sex:						
Male n (%)	5 (22.7)	3 (37.5)	0.47	9 (36.0)	4 (26.7)	0.73
Female n (%)	17 (77.3)	5 (62.5)		16 (64.0)	11 (73.3)	
Nmen carriage:						
n (%)	1 (4.5)	1 (12.5)	0.47	1 (4.0)	3 (20.0)	0.14
Nlac IgG:						
Median (IQR)	10 (3.3-16.8)	3.8 (1.6-10.3)	0.15	11.0 (5.1-16.8)	11.2 (7.3-35.6)	0.2
Nmen IgG:						
Median (IQR)	18.7 (12.2-26.6)	16.5 (4.2-29.7)	0.65	23.6 (12.2-30.6)	38.1 (24.7-68.7)	0.004

Table 6.9 Baseline characteristics of colonised vs non-colonised participants

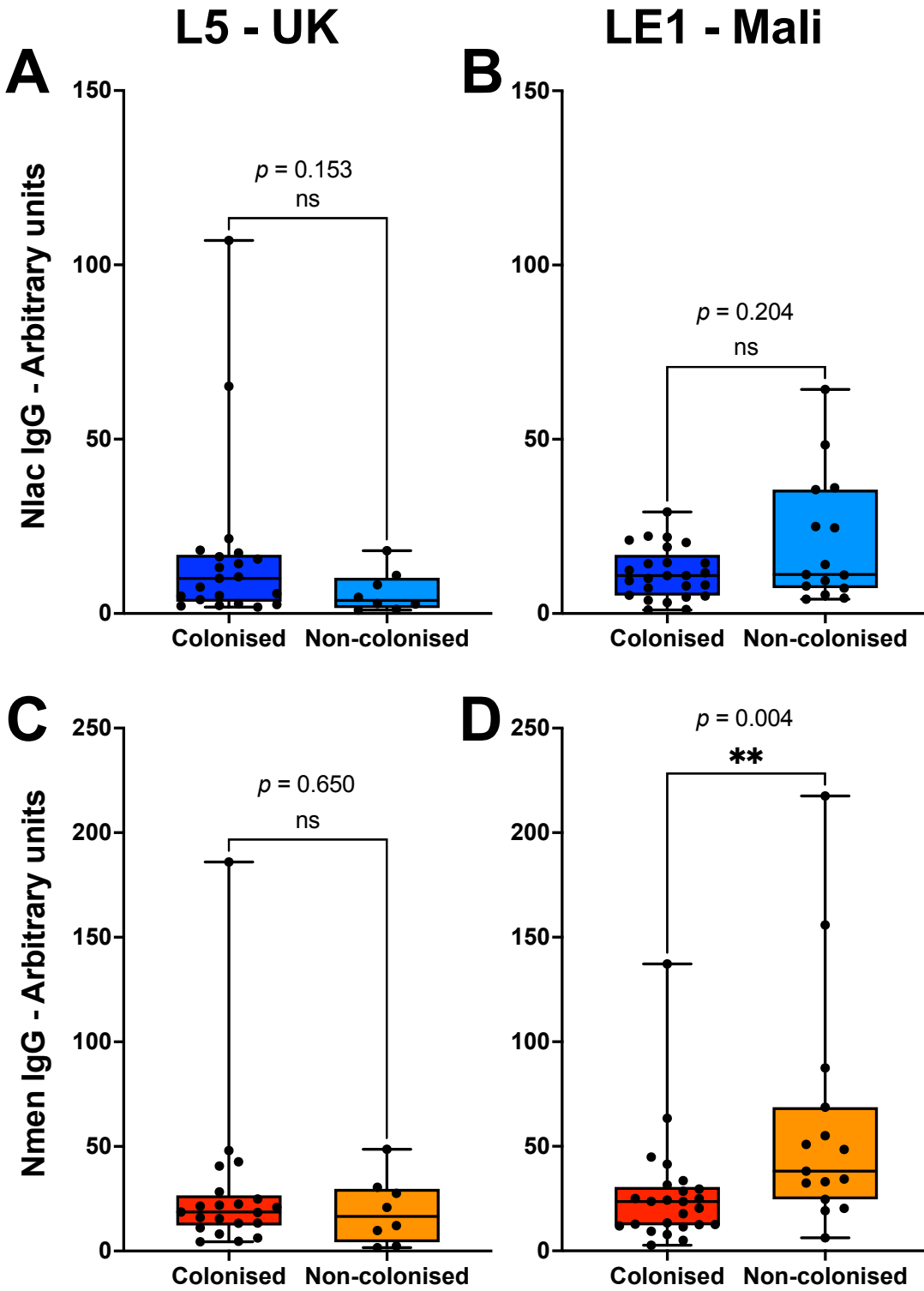


Figure 6.10 Baseline Nlac and Nmen IgG titres: colonised vs non-colonised participants

Comparison of baseline Nlac and Nmen specific IgG titres between subsequently colonised and non-colonised participants, individual values, median, IQR and range shown, p values derived from Mann Whitney test. A = Nlac IgG – L5, B = Nlac IgG – LE1, C = Nmen IgG – L5, D = Nmen IgG – LE1.

6.3.11 Duration of colonisation

Colonisation with Nlac and Nmen over the duration of the study is shown for each participant for L5 (Figure 6.11A) and LE1 (Figure 6.11B).

In L5, Nlac colonisation was detected by Day 7 in all participants who became colonised. Colonisation was sustained to Day 28 in 90% (20 of 21) of colonised participants who attended that visit and to Day 168 in 79% (15 of 19) who attended that visit. Participants were invited to an optional extra visit at 18 months to 3 years post inoculation to assess longer term carriage. 15% (2 of 13) of previously colonised participants who attended that visit remained colonised, both confirmed by PCR to still be the inoculum strain.

In LE1, Nlac colonisation was detected by Day 7 in 88% (22 of 25) of participants who became colonised, with colonisation first detected at Day 14 in three participants. Colonisation was sustained to Day 28 in 76% (19 of 25) of colonised participants and to Day 168 in 33% (8 of 24) colonised participants who attended that visit. One participant had Nlac carriage detected for the first time at Day 168. This was confirmed by PCR to be a wild type Nlac and not the inoculum strain. Several participants received antibiotics during the study for non-study related reasons which in one case appeared to be associated with clearance of Nlac colonisation.

Comparing the two studies, the proportion of colonised participants with colonisation sustained to Day 168 was significantly smaller in LE1 than L5 ($p = 0.005$, Fisher's exact test). Sustained colonisation to Day 28 was not significantly different ($p = 0.106$, Fisher's exact test).

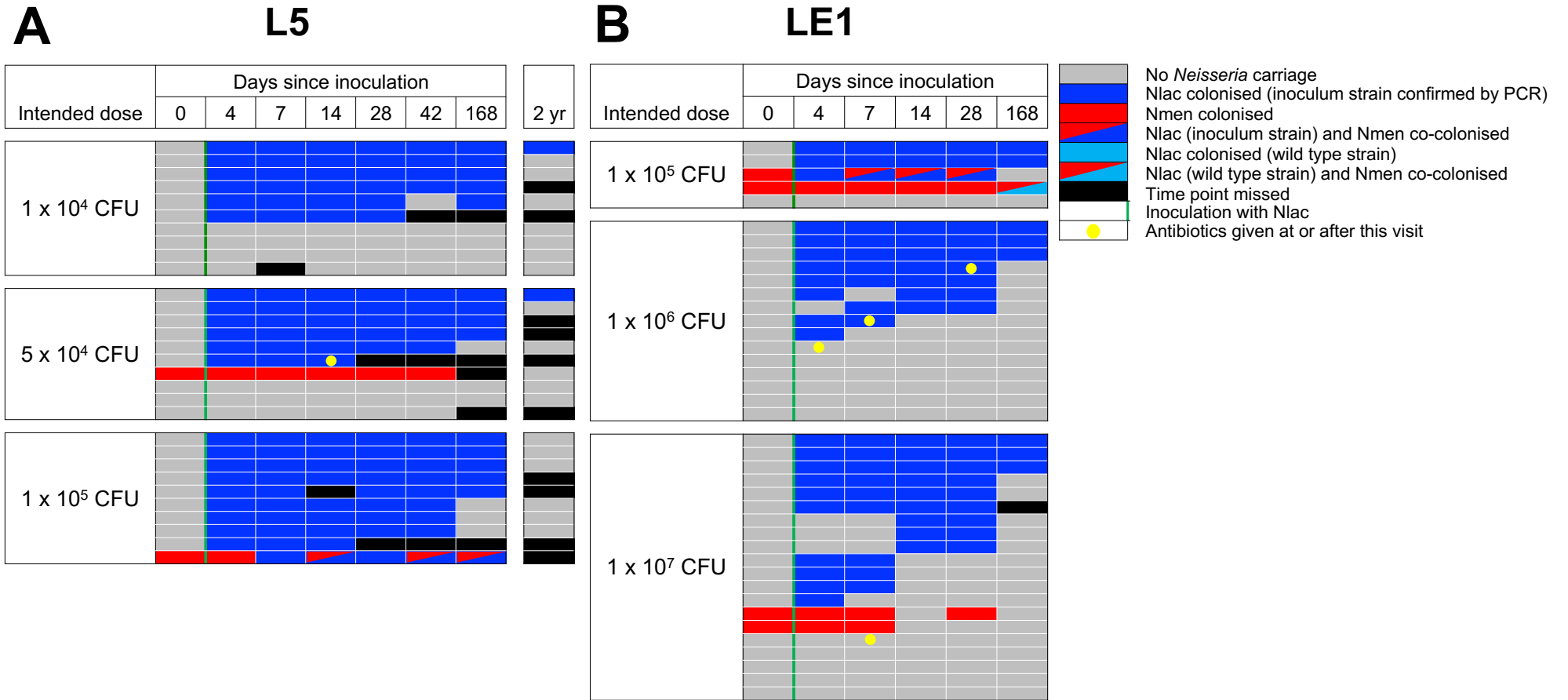


Figure 6.11 Colonisation status over time

Colonisation with Nlac / Nmen at each timepoint for individual participants. Each row represents one participant, colonisation defined as the culture of at least one colony of Nlac / Nmen from a throat swab or nasal wash taken at the time points shown.

6.3.12 Safety

As discussed in section 6.3.4, for two participants in L5 the apparent inoculum dose was found to be notably higher than intended, and so as per protocol they were reported as an AE (greater than five times) and an AESI / SAE (greater than ten times the intended dose). Both participants were asymptomatic. No other SAEs occurred during either study.

In L5, eight of 30 participants (27%) spontaneously reported nasal irritation or stinging immediately following inoculation, with spontaneous resolution within five to ten minutes, as shown in Figure 6.12A. There was no association with inoculum dose, and this was assumed to be due to the use of sterile water as an inoculum diluent. Such reactogenicity had not been reported in previous Nlac CHIMs where PBS was used as the diluent, so for LE1 the diluent was changed back to PBS.

Participants in LE1 were also asked about potential symptoms of reactogenicity in the post inoculation period of observation. Unfortunately, as discussed in section 6.3.5, an error in the concentration of PBS used during preparation of the inocula led to both an inadequate dose of Nlac, and a hypertonic solution being administered to the first 15 participants. 12 of these participants (80%) reported mild to moderate symptoms. In most participants these spontaneously resolved within 15 minutes, but two participants reported a headache lasting 2-3 hours. Following the correction of the inoculum diluent concentration, only two of the remaining 40 participants reported any reactogenicity, both headaches, lasting 15 minutes and 3 hours respectively. Reported reactogenicity for LE1 is shown in Figure 6.12B.

Adverse events reported by participants from inoculation to Day 28 post inoculation are summarised in Figure 6.13 A-D, with comparison between colonised and non-colonised participants for each study. Participants in both studies reported mild to moderate upper and lower respiratory tract symptoms, with no difference seen between colonised and non-colonised participants. Other mild to moderate adverse events occurred in a small number of participants in each study, but none were assessed as being likely to be related to challenge or colonisation with Nlac, and all resolved. One participant in L5 and seven in LE1 received antibiotics for non-study related reasons, prescribed by the study team, which may have been associated with loss of Nlac carriage in the one participant in L5 and two in LE1.

Chapter 6

Safety bloods taken on Day 4 post challenge were graded according to pre-agreed reference ranges. These ranges differed for the two studies as the local normal ranges were taken into consideration. Figure 6.13 E-H show the percentage of colonised and non-colonised participants in each study with abnormal blood results on Day 4 which were either new or in a worse AE grade than seen at screening. A Grade 1 rise in CRP occurred in one participant in L5, which was considered likely to be due to a number of insect bites rather than Nlac colonisation. Some participants in study B had new or worsening abnormal blood results including anaemia, thrombocytopenia, neutropenia and eosinophilia. None of these abnormalities were assessed as likely to be related to the study procedures.

No safety blood abnormalities were considered to be related to the challenge procedure or colonisation with Nlac.

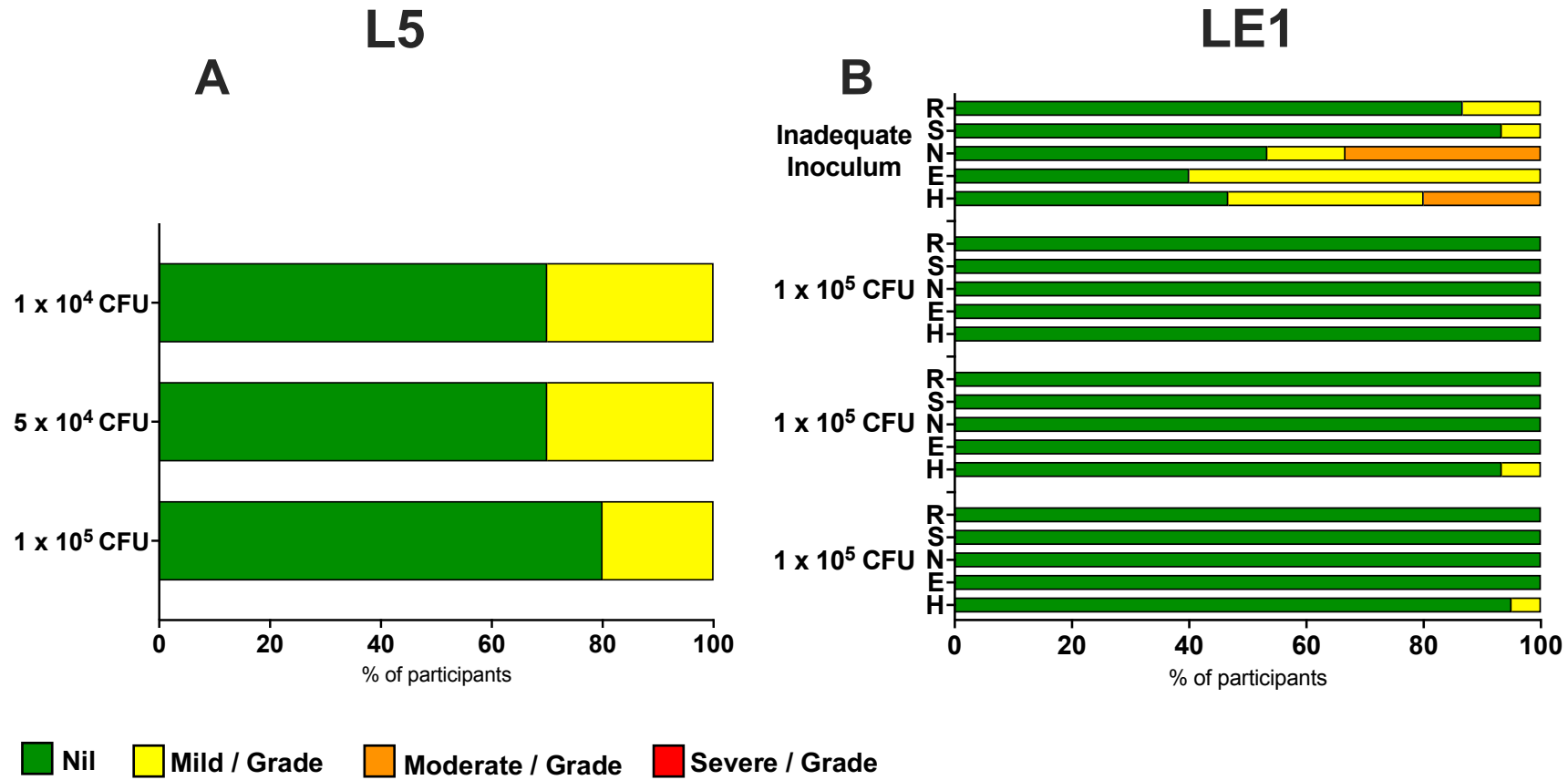


Figure 6.12 Reactogenicity : L5 and LE1

Proportion of each intended dose cohort reporting reactogenicity following inoculation

A: L5 – All reactogenicity was spontaneously reported nasal irritation / stinging

B: LE1 – Solicited reactogenicity symptoms, R = Rhinorrhoea, S = Sneezing, N = Nasal irritation, E = Eye watering, H = Headache

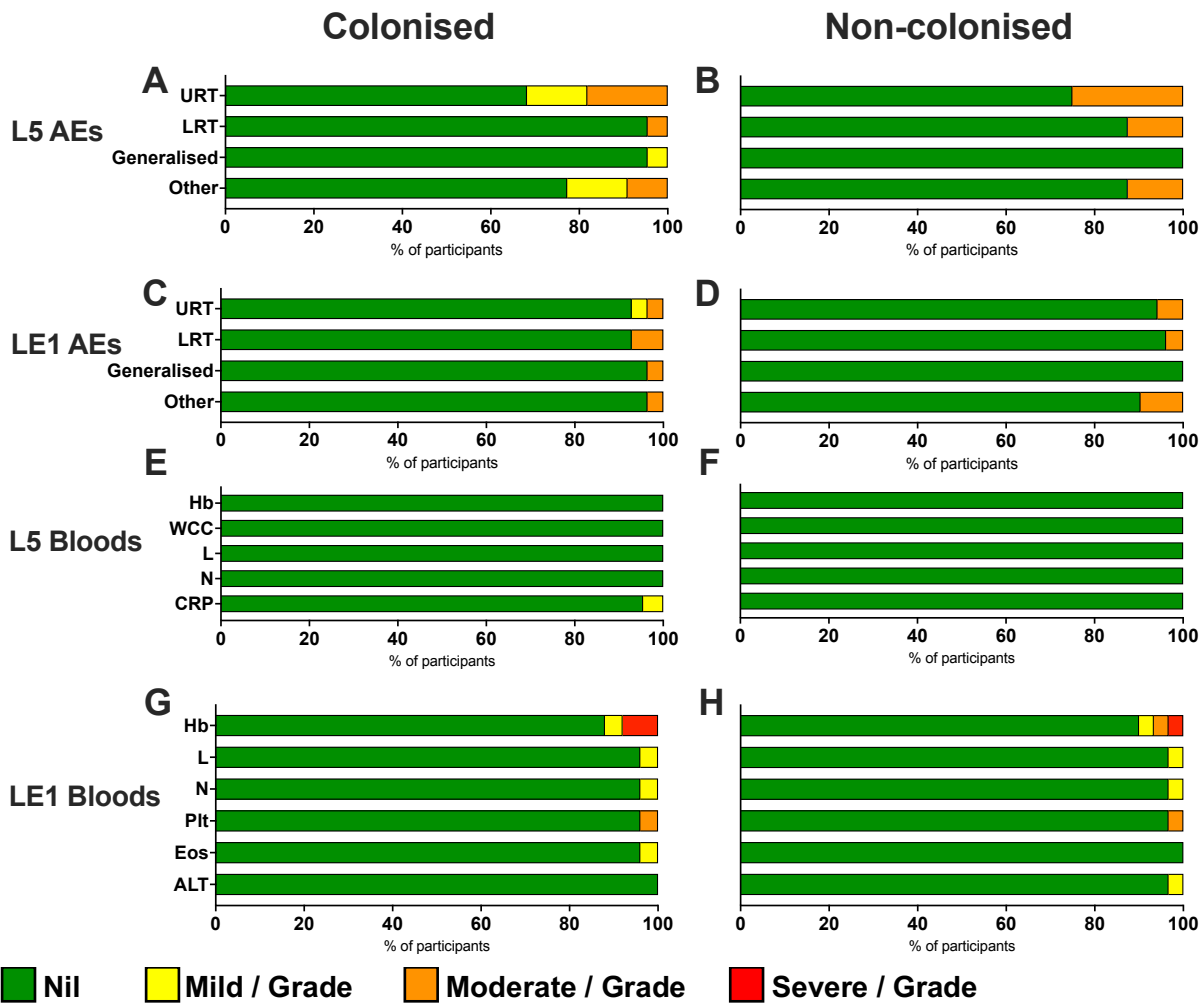


Figure 6.13 Adverse events : L5 and LE1

Percentage of participants reporting any adverse events from Day 0 to Day 28 post challenge Maximum severity reported with comparison of Colonised (A, C, E, G) and Non-colonised (B, D, F, H, J).

A and B: L5 Reported adverse events: Colonised (A) and Non-colonised (B) participants

C and D: LE1 Reported adverse events: Colonised (A) and Non-colonised (B) participants

(A-D categorised as URT = Upper respiratory tract symptoms, LRT = Lower respiratory tract symptoms, Generalised = Systemic illness or Other)

E and F: L5 Abnormal blood results

G and H: LE1 Abnormal blood results

(Hb = Haemoglobin, WCC = White cell count, L = Lymphocyte count, N = Neutrophil count, CRP = C-reactive protein, Plt = Platelets, Eos = Eosinophil count, ALT = Alanine aminotransferase).

6.3.13 Immunogenicity

Nlac and Nmen specific IgG titres at Day 28 post challenge were compared to baseline for those participants who were successfully colonised with Nlac, and those who were not.

Individual participant trends in titre are shown in Figure 6.14 with comparison between Day 0 and Day 28 titres for each participant. In both studies, Nlac and Nmen IgG titres were higher for colonised participants at Day 28 than at baseline. Individual participants with a fold-change ≥ 2 are highlighted in blue (Nlac) or red (Nmen). 52.3% of Nlac colonised participants (11 of 21) in L5 and 24% (6 of 25) in LE1 had at least a two-fold increase in Nlac IgG, and 42.9% (9 of 21) in L5 and 20.0% (5 of 25) in LE1 had at least a two-fold increase in Nmen IgG.

In contrast, participants who were not Nlac colonised did not have a significant rise in Nlac or Nmen IgG titres in either study. One non-colonised participant in LE1 (7%) had a 2-fold rise in Nlac IgG, and a 1.5-fold increase in Nmen IgG. This may have been a result of undetected or transient colonisation with Nlac or another *Neisseria* species.

The median fold changes of Nlac and Nmen IgG titres among colonised and non-colonised participants are shown in Table 6.10.

	L5		LE1	
	Colonised	Non-colonised	Colonised	Non-colonised
Nlac IgG FC Median (IQR)	2.2 (1.4 – 4.2)	1.0 (0.74 – 1.0)	1.3 (1.0 – 1.9)	1.0 (0.9 – 1.2)
Nmen IgG Median (IQR)	1.4 (1.2 – 3.7)	1.0 (0.8 – 1.0)	1.3 (1.0 – 1.7)	1.0 (0.8 – 1.3)

Table 6.10 Nlac and Nmen IgG fold change

Nlac and Nmen IgG fold change from baseline to Day 28, median and interquartile range shown for colonised and non-colonised participants in each study

Summary data for all colonised and non-colonised participants are shown in Figure 6.15. This figure compares the absolute change in titre from Day 0 to Day 28, for colonised and non-colonised participants. There was a significant difference in absolute titre change for both Nlac and Nmen IgG for both studies, with colonised participants having a greater positive titre change in both studies.

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Comparing the Nlac and Nmen specific responses seen in the two studies, the absolute and relative (fold change) increase in titres appears to be higher in L5 than in LE1. This reaches statistical significance for Nlac IgG but not for Nmen IgG. This comparison is shown in Figure 6.16.

Two participants in L5 and four in LE1 were known to be Nmen carriers at baseline. These participants were enrolled as Nmen carriage was not an exclusion criterion for either of these studies and are included in the above analyses. However, Nmen carriage could potentially have a confounding effect on the induction of Nmen or Nlac humoral immune responses. As such, a post-hoc analysis was also carried out excluding those participants who had been Nmen carriers at baseline. The exclusion of these participants minimally altered the p values for each analysis but did not impact the statistical significance or overall findings. These p values are summarised in Table 6.11.

There was a significant correlation between titres of IgG specific to Nlac and Nmen, both at baseline, and in absolute and relative change in titres from baseline to Day 28 post challenge. This is likely to be due to orthologous proteins shared by both species resulting in IgG which is cross-reactive. Figure 6.17 shows these positive correlations, with all challenged-per-protocol participants from both studies included in each figure regardless of colonisation status. These correlations were significant overall, for each study, and for either colonised or non-colonised participants. Table 6.12 shows the Spearman correlation co-efficient and p value for each of these comparisons.

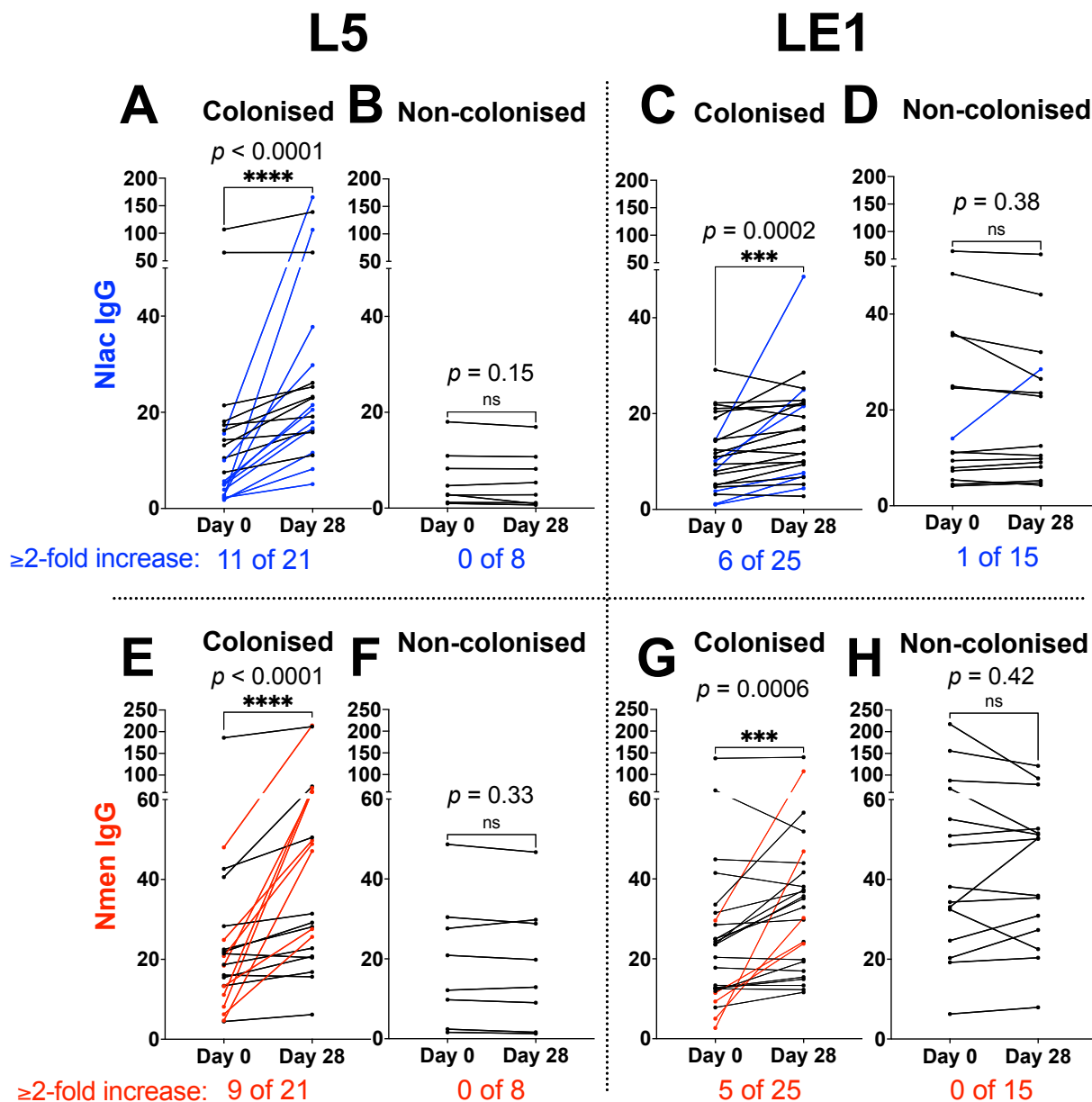


Figure 6.14 Niac and Nmen IgG trends : L5 and LE1

Niac and Nmen IgG titre – trend from Day 0 with Day 28 post challenge shown for individual participants, p values derived from Wilcoxon signed ranks test. A-D Niac IgG, A: L5 colonised participants, B: L5 non-colonised participants, blue lines indicate individual participants with ≥ 2 -fold increase from baseline. E-H Nmen IgG, E: L5 colonised participants, F: L5 non-colonised participants, G: LE1 colonised participants, H: LE1 non-colonised participants, red lines indicate individual participants with ≥ 2 -fold increase from baseline.

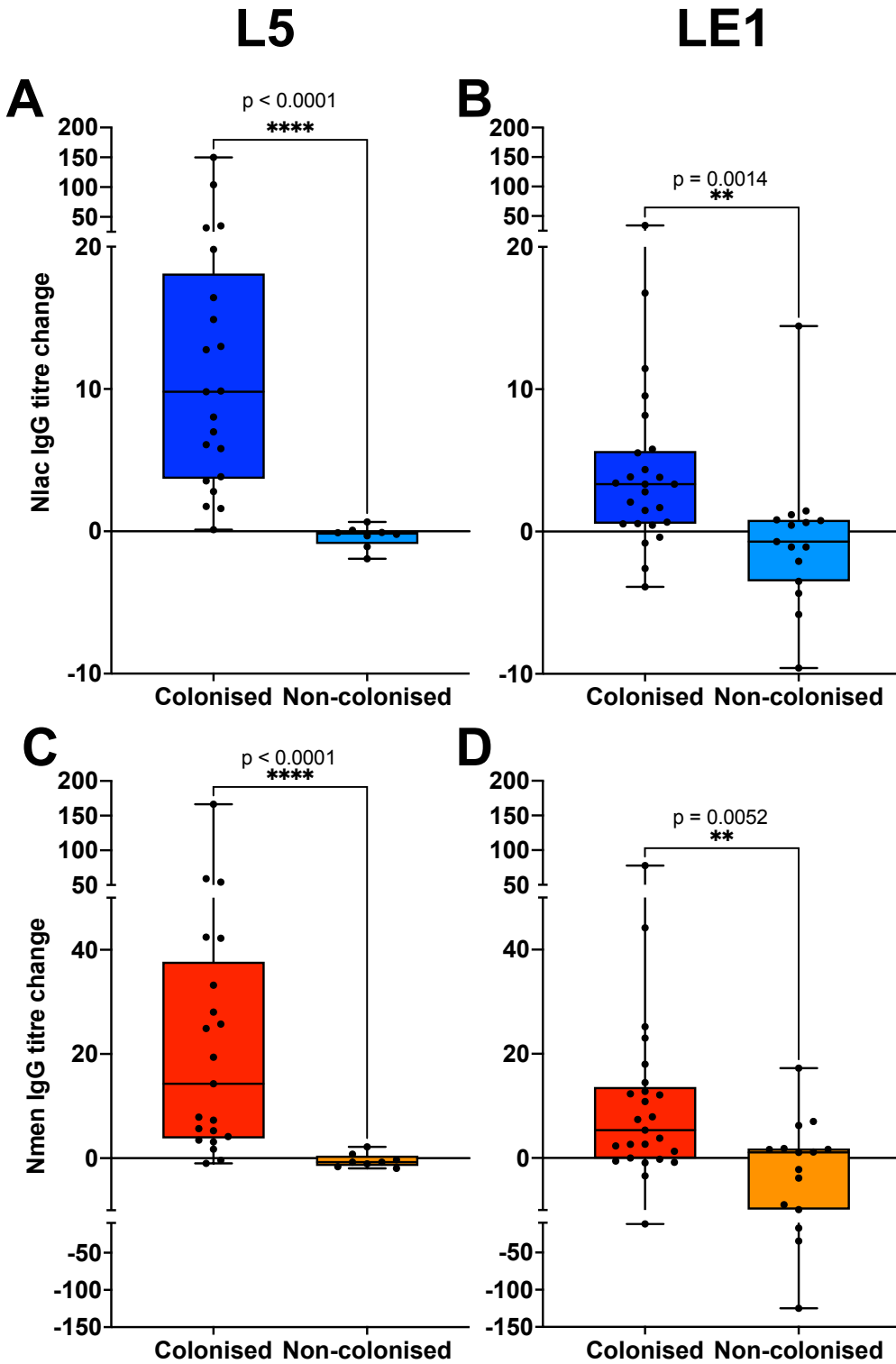


Figure 6.15 Niac and Nmen IgG change from baseline

Absolute change in individual participant Niac (blue) and Nmen (red) IgG titre from Day 0 to Day 28 comparing colonised and non-colonised participants, individual values, median, IQR and range shown

A = L5 Niac, B = LE1 Niac, C = L5 Nmen, D = LE1 Nmen

p values derived from Mann Whitney test.

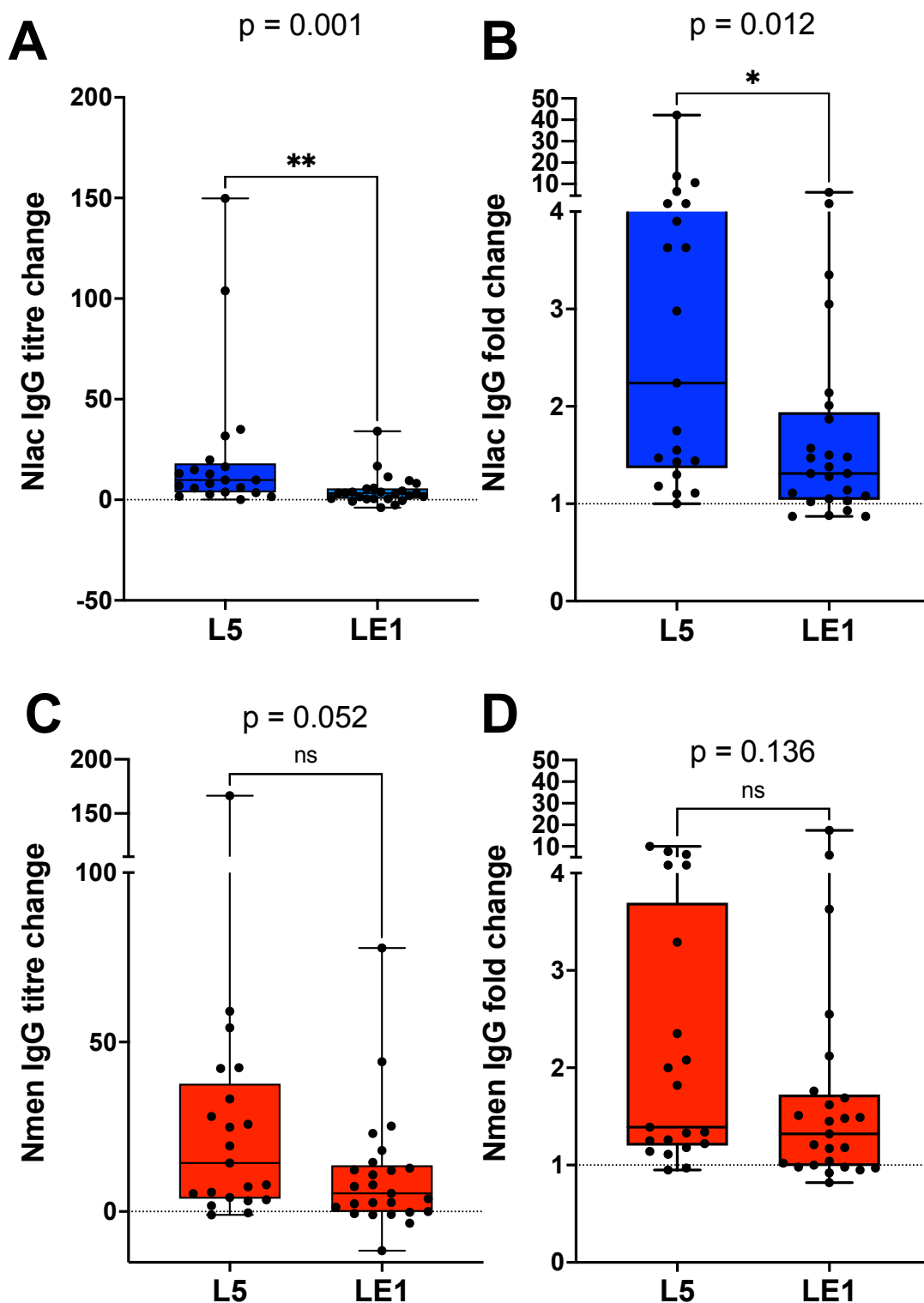


Figure 6.16 Comparison of immunogenicity : L5 vs LE1

Change in individual participant Niac (blue) and Nmen (red) IgG titre from Day 0 to Day 28 for participants colonised with Niac comparing L5 and LE1. Individual values, median, IQR and range shown.

A+C: Absolute titre change from baseline

B+D: Fold change from baseline

Dotted lines indicate no change from baseline

p values derived from Mann Whitney test.

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Study / Colonisation status	IgG	Comparison	Corresponding figure	Statistical test	p (including Nmen carriers)	p (excluding Nmen carriers)	Statistically significant?
L5 Colonised	Nlac	D0 vs D28	6.14 A	Wilcoxon signed ranks	<0.0001	< 0.0001	Y
L5 Non-colonised	Nlac	D0 vs D28	6.14 B	Wilcoxon signed ranks	0.15	0.22	N
LE1 Colonised	Nlac	D0 vs D28	6.14 C	Wilcoxon signed ranks	0.0002	0.0003	Y
LE1 Non-colonised	Nlac	D0 vs D28	6.14 D	Wilcoxon signed ranks	0.38	0.46	N
L5 Colonised	Nmen	D0 vs D28	6.14 E	Wilcoxon signed ranks	<0.0001	<0.0001	Y
L5 Non-colonised	Nmen	D0 vs D28	6.14 F	Wilcoxon signed ranks	0.33	0.5	N
LE1 Colonised	Nmen	D0 vs D28	6.14 G	Wilcoxon signed ranks	0.0006	0.0008	Y
LE1 Non-colonised	Nmen	D0 vs D28	6.14 H	Wilcoxon signed ranks	0.42	0.68	N
L5	Nlac	Absolute titre change Colonised vs non-colonised	6.15 A	Mann Whitney	<0.0001	<0.0001	Y
LE1	Nlac	Absolute titre change Colonised vs non-colonised	6.15 B	Mann Whitney	0.0014	0.0046	Y
L5	Nmen	Absolute titre change Colonised vs non-colonised	6.15 C	Mann Whitney	<0.0001	<0.0001	Y
LE1	Nmen	Absolute titre change Colonised vs non-colonised	6.15 D	Mann Whitney	0.0052	0.0164	Y
Colonised	Nlac	Absolute titre change L5 vs LE1	6.16 A	Mann Whitney	0.001	0.009	Y
Colonised	Nlac	Fold change L5 vs LE1	6.16 B	Mann Whitney	0.012	0.010	Y
Colonised	Nmen	Absolute titre change L5 vs LE1	6.16 C	Mann Whitney	0.052	0.054	N
Colonised	Nmen	Fold change L5 vs LE1	6.16 D	Mann Whitney	0.136	0.106	N

Table 6.11 Statistical impact of excluding Nmen carriers

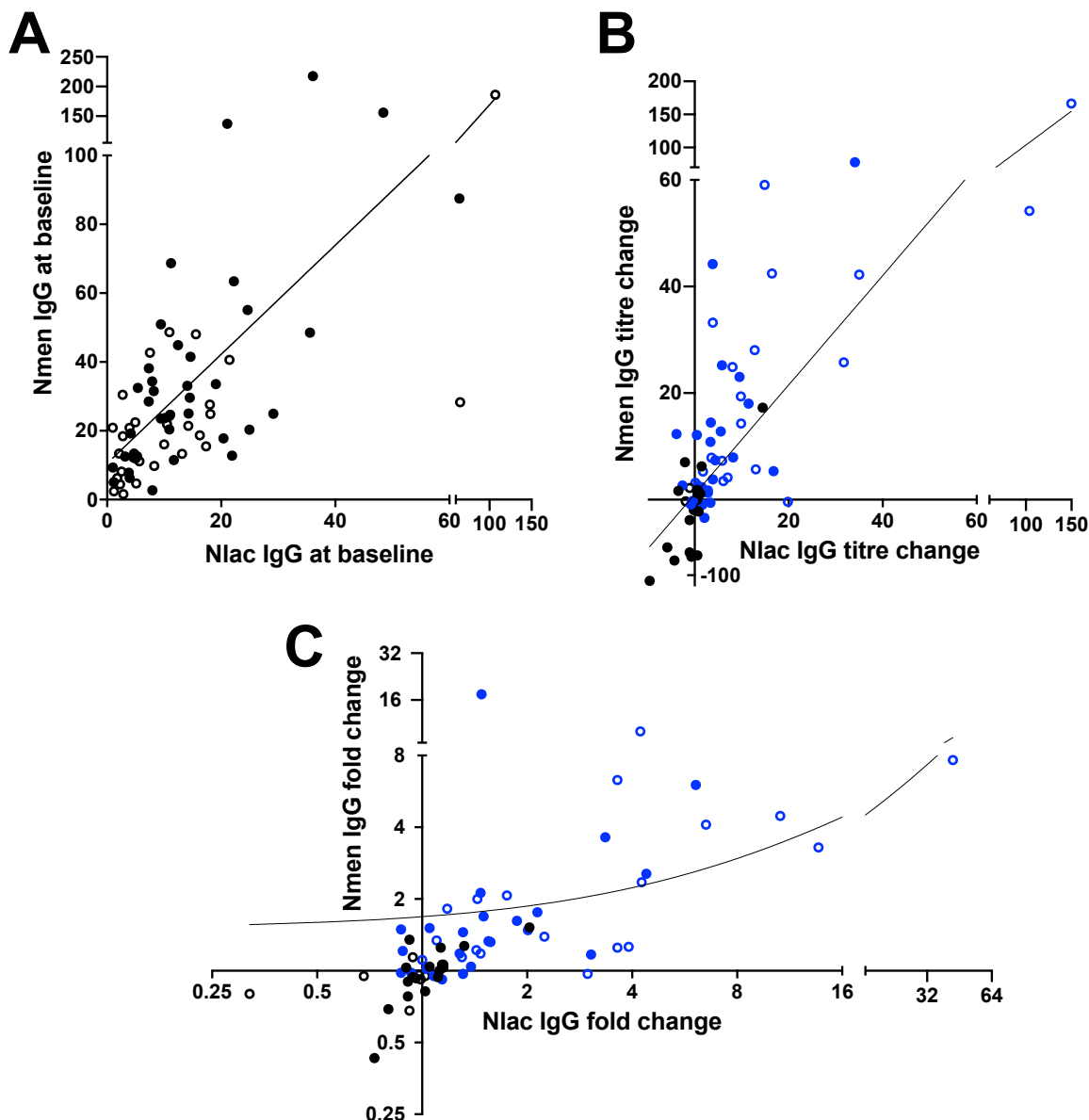


Figure 6.17 Relationship between Nlac and Nmen IgG

Simple linear regression analysis of Nlac and Nmen IgG results

A: Individual participant Nlac vs Nmen IgG titres at baseline

B: Individual participant absolute change in Nlac vs Nmen titre from Day 0 to Day 28

C: Individual participant fold change in Nlac vs Nmen titre from Day 0 to Day 28

Open circles = L5 participants, closed circles = LE1 participants

B+C only blue circles = Nlac colonised participants, black circles = Non colonised participant

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Study / studies	Colonisation status	Comparison	Corresponding figure	Spearman r	95% CI	p
L5 and LE1	Colonised and Non-colonised	Baseline Nlac vs Nmen titres	6.17A all data points	0.647	0.478-0.768	<0.0001
L5	Colonised and Non-colonised	Baseline Nlac vs Nmen titres	6.17A open circles only	0.599	0.288-0.796	0.0006
LE1	Colonised and Non-colonised	Baseline Nlac vs Nmen titres	6.17A closed circles only	0.653	0.421-0.805	<0.0001
L5 and LE1	Colonised and Non-colonised	Nlac vs Nmen absolute titre change from Day 0 to Day 28	6.17B all data points	0.734	0.598-0.830	<0.0001
L5 and LE1	Colonised	Nlac vs Nmen absolute titre change from Day 0 to Day 28	6.17B Blue circles only	0.670	0.464-0.807	<0.0001
L5 and LE1	Non-colonised	Nlac vs Nmen absolute titre change from Day 0 to Day 28	6.17B Black circles only	0.660	0.355-0.838	0.0002
L5	Colonised and Non-colonised	Nlac vs Nmen absolute titre change from Day 0 to Day 28	6.17B open circles only	0.801	0.608-0.905	<0.0001
LE1	Colonised and Non-colonised	Nlac vs Nmen absolute titre change from Day 0 to Day 28	6.17B closed circles only	0.658	0.429-0.808	<0.0001
L5 and LE1	Colonised and Non-colonised	Nlac vs Nmen titre fold change from Day 0 to Day 28	6.17C all data points	0.757	0.630-0.845	<0.0001
L5 and LE1	Colonised	Nlac vs Nmen titre fold change from Day 0 to Day 28	6.17C Blue circles only	0.669	0.462-0.806	<0.0001
L5 and LE1	Non-colonised	Nlac vs Nmen titre fold change from Day 0 to Day 28	6.17C Black circles only	0.658	0.325-0.845	0.0006
L5	Colonised and Non-colonised	Nlac vs Nmen titre fold change from Day 0 to Day 28	6.17C open circles only	0.839	0.677-0.924	<0.0001
LE1	Colonised and Non-colonised	Nlac vs Nmen titre fold change from Day 0 to Day 28	6.17C closed circles only	0.701	0.491-0.834	<0.0001

Table 6.12 Correlation between Nlac and Nmen IgG

Comparison between Nlac and Nmen IgG titres, absolute titre change and fold-change. Spearman correlation used for all comparisons

6.4 Discussion

L5 and LE1 were designed to optimise our Nlac CHIM by using a lyophilised strain of Nlac (LyoNlac) and to compare the reliability, safety, optimal dose, colonisation fraction and duration, and immunogenicity with that seen when using inocula prepared from frozen stocks of Nlac.

The ability of lyophilised bacteria to induce immunogenic nasopharyngeal colonisation has previously been demonstrated with the live attenuated *Bordetella pertussis* candidate vaccine BPZE1. This vaccine has been developed into a lyophilised product, shown to be microbiologically stable at ambient temperatures over 2 years (171). When administered nasally, the vaccine strain colonises the nasopharynx and induces specific immune responses (168, 172). However, carriage of BPZE1 with is only transient with no ongoing colonisation detected by Day 29 post administration (172). A lyophilised strain of *Shigella sonnei* has also been used to induce disease in an enteric model of controlled human infection (173). To our knowledge, lyophilised bacteria have not previously been used to induce prolonged colonisation in a respiratory tract controlled human infection model, and the impact of lyophilisation on bacterial upper respiratory tract colonisation dynamics and immunogenicity are unknown.

In L5, nasal challenge with LyoNlac safely induced nasopharyngeal colonisation in a very similar way to that achieved with Nlac prepared from frozen stocks. The standard inoculum dose identified was the same as that used from frozen stocks, with no reduction in colonisation fraction apparent. The duration of colonisation was also similar to that seen in previous Nlac CHIM studies, in which ongoing colonisation was detected at 24 weeks in 65% of previously colonised volunteers (123) or at 26 weeks in 49% of colonised volunteers (124). In L5, 79% of previously colonised participants who attended the Day 168 (24 week) visit remained colonised following inoculation with LyoNlac. Of those participants who attended the 18 month – 2 year visit only 15% of previously colonised participants remained colonised, however no data are available to compare this to carriage following inoculation with Nlac prepared from frozen stocks.

The immunogenicity of colonisation also appeared to be comparable to frozen Nlac stocks, with a median Nlac IgG fold change of 2.2 by Day 28 which was very similar to that seen in a previous Nlac CHIM study (123). A rise in cross-reactive Nmen IgG was also seen. This demonstrates that lyophilisation of Nlac does not appear to impede its ability to colonise the nasopharynx or to induce a specific humoral immune response, in a population of healthy UK adult volunteers.

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LE1 was designed to translate the LyoNlac CHIM to a population in the meningitis belt. The protective effect of Nlac colonisation could potentially be of great relevance but the feasibility and acceptability of such an intervention, plus the ability to induce Nlac colonisation in that population, was unknown.

Undertaking a complex CHIM study in such a different setting and population, and during a pandemic, brought many challenges and difficulties. The intention had been to be far more “hands on” in the conduct of the study, but due to COVID restrictions, this became impossible and so the whole study was supervised remotely. There were significant difficulties and errors, probably at least in part due to this. However, once these errors had been recognised, investigated and mitigated, the remainder of the study ran smoothly and successfully. Recruitment and retention of volunteers was superior to that seen in Southampton, the collaboration between different investigators and institutions provided valuable different insights and experiences into the study design and conduct, and it was shown that translation of the model to such a different setting was indeed acceptable and logistically possible.

Nasal inoculation with LyoNlac did safely induce long-standing and immunogenic colonisation in a similar way to in the UK. However, there were some notable differences. The standard inoculum dose identified was substantially higher at 10^7 CFU compared to 10^5 CFU in the UK. Of note, in L5, this dose was identified by achieving the desired colonisation fraction of approximately 80%, whereas in LE1 it was identified by reaching the maximum permitted dose, without achieving the intended colonisation fraction. The colonisation fraction did not significantly increase with increasing dose, suggesting that the maximum possible colonisation fraction within this population may be lower than in the UK, and that a dose of 10^5 CFU may be appropriate for future studies. Duration of colonisation also appeared to be shorter in LE1. A larger sample size would be required to definitively demonstrate these differences. While the pattern of immunological response to Nlac colonisation was similar in the two settings, the response appeared more pronounced in the UK, particularly for Nlac specific IgG.

Possible reasons for these observed differences include variation in the upper respiratory tract microbiome, the presence or absence of competing commensal organisms and differences in population health and nutrition. Additionally, differences in prior exposure to Nmen and pre-existing immunity are likely to be important factors. There are notable behavioural differences between the two settings which may impact the risk of transmission within households, for example in Mali the average household size is much

larger than the UK, with multiple generations living together and often sharing a single drinking vessel (personal communication and observation).

Carriage studies within the meningitis belt have shown an overall Nmen carriage prevalence of approximately 5%, (170, 174) i.e. lower than the adult carriage prevalence reported in European studies (73). However, substantial variation is seen over time and place, with much higher carriage prevalence and rapid acquisition of carriage seen in outbreak settings (170, 175) but shorter duration of carriage episodes at 3-4 months (170) in comparison to 5-6 months as seen in Europe (38). This might suggest that individuals in the meningitis belt are exposed to more frequent but shorter episodes of Nmen carriage than those in the UK, potentially providing regular boosting of the immune response with varying strains.

Comparing baseline immunity in L5 and LE1, baseline titres of Nmen IgG were higher in Mali, with a non-significant trend towards higher Nlac IgG titres. However, in LE1, baseline Nmen specific IgG was significantly higher in those who did not subsequently become colonised, than in those who did. This might suggest that those individuals who do not become colonised with Nlac may also be those who are already more protected from meningococcal colonisation and therefore disease.

Further aims of these studies were to demonstrate that the preparation of a dose of inoculum from LyoNlac is simple, accurate, reproducible, and well tolerated by the participants. Unfortunately, some of the difficulties encountered during the conduct of these studies directly impacted dose accuracy and tolerability. The dose preparation steps were intended to be simple and straight forward, but clearly errors were made, namely the use of an incorrect concentration of PBS in LE1. This resulted in an inaccurate and unreliable dose for the first 15 participants. However, once those participants are excluded, then the accuracy of the remainder of inoculum doses were generally superior to that seen with inocula prepared from frozen stocks of Nlac.

The PBS concentration error, and the use of water as a diluent in L5, resulted in higher than anticipated reactogenicity. However, once the correct concentration of an appropriate and isotonic diluent was used, the inoculum was very well tolerated.

Further LyoNlac CHIM studies will need to ensure that inoculum preparation training is robust to avoid future similar errors. For use on a larger scale, single use vials of inoculum could be supplied with the correct volume of the correct diluent to ensure that preparation steps are accurate.

The sample sizes for these studies were deliberately small as they were chosen to assess the ability of LyoNlac to colonise at different doses in the two populations and to demonstrate immunogenicity. This does mean that we have been unable to demonstrate any impact on meningococcal acquisition or carriage as has previously been shown in the UK using Nlac prepared from frozen stocks (124). Demonstration of such an impact on carriage would require a far bigger sample size, with a non-challenged control group and ideally regular sampling over a longer period of time. A larger sample size may also have allowed more robust conclusions to be made about apparent differences observed between the two populations.

The immunological endpoints for these studies were limited. Again, this was a deliberate decision to demonstrate equivalence with frozen stocks of Nlac. The immunological endpoint of most interest would be the induction of a protective level of anti-meningococcal SBA. However, as this has not been demonstrated using frozen stocks of Nlac in the UK (123), it is unlikely that this would be achieved with LyoNlac and would not therefore have been an efficient use of clinical samples and funding. Further immunological assays such as Nlac and Nmen specific B_{PLAS} and B_{MEM} , along with assessment of mucosal responses would have been very interesting, but again would likely have required increased sample size and funding and so were not felt to be appropriate for these small pilot studies.

In conclusion we have demonstrated that LyoNlac can be used safely and effectively, providing a resolution to the limitations of Nlac CHI using frozen stocks. This allows it to be transferable to a population within the meningitis belt with long term refrigerated storage and transport beyond a cold chain making the model logistically possible. Further work will build on this model to further assess the immunogenicity of Nlac colonisation, the impact on meningococcal carriage, and the potential for prevention of meningococcal disease in the meningitis belt.

Chapter 7 Discussion

7.1 Overview of aims and findings

7.1.1 The Nlac controlled human infection model

The studies described in this thesis sit within the Nlac CHIM research programme, which aims to investigate and exploit the observed relationship between Nlac and Nmen, in order to develop improved strategies for protection from meningococcal disease. This over-arching aim may be achieved indirectly, with the model facilitating further study of the relationship between the two organisms, the immunological response to colonisation and to the expression of specific antigens, and the evaluation of potential correlates of protection from colonisation. The model may also be developed further to provide protection directly, using a GM Nlac strain as a form of bacterial medicine to interrupt or prevent meningococcal colonisation.

The four studies described have each been designed to address specific research questions and aims. The initial steps were to transfer the previously described Nlac CHIM (123, 124) to the University of Southampton and optimise it for further use, including assessing the impact of an increased inoculum dose. An important research question for L3 was to investigate the impact of duration of colonisation on immunogenicity. In L4, the aim was to safely induce nasopharyngeal colonisation with a GM Nlac strain, expressing the meningococcal antigen NadA, with the purpose of inducing a NadA specific immune response and potentially an improved cross-reactive Nmen specific immune response.

The model was further optimised in L5 with the use of a lyophilised inoculum (LyoNlac). This was necessary to make the model transferable to a lower resourced setting, such as a population within the meningitis belt, where protective strategies to combat meningococcal disease would have particular relevance. The aim of L5 was to identify the standard inoculum dose of LyoNlac required to induce colonisation, and to assess the safety and immunogenicity of that colonisation. Lastly, LE1 transferred the model to a research centre in Mali, a setting within the meningitis belt. The standard inoculum dose, safety and immunogenicity were also assessed in this population and compared to that seen in a UK population.

7.1.2 Optimisation of the model

Optimisation of the Nlac CHIM was achieved by increasing the inoculum dose to 10^5 CFU, which was shown to be safe, well tolerated and to achieve a colonisation fraction of 0.86 by Day 7 post challenge, higher than seen at the previous dose of 10^4 CFU where the colonisation fraction was 0.34-0.59 (123, 124). This increased colonisation fraction will reduce the sample size required for future studies and therefore increases the efficiency of the model. Any future clinical intervention based on colonisation with Nlac will require a high colonisation fraction in order to produce a meaningful clinical impact.

A single dose of Ciprofloxacin was shown to be highly effective in rapidly clearing colonisation. This important finding improves the scientific and ethical robustness of the model, as a reliable method of terminating carriage is desirable for any CHI study (128) and may be considered necessary for future GM Nlac strains. Methods were also developed to assess the shedding of Nlac during colonisation.

7.1.3 The impact of duration of colonisation

L3 demonstrated a relationship between duration of colonisation and immunogenicity, with participants colonised for 14 days demonstrating a specific humoral immune response, but with no such response detected in those in whom colonisation was limited to 4 days. The threshold duration to induce an immune response, and a comparison with longer periods of colonisation were not investigated. Duration of colonisation is linked to duration of response, because in L3, in which colonisation was terminated at Day 14 post challenge, there was a trend towards a decrease in Nlac and Nmen IgG titres from Day 14 to Day 42. In contrast, in L4 in which colonisation was allowed to continue, the serological response to GM Nlac colonisation was sustained to Day 90 post challenge. The maximum fold changes in Nlac IgG titres were higher for participants colonised in L4 (2.82 and 3.04) and L5 (2.2) than that seen in participants colonised for a maximum of 14 days in L3 (1.79) suggesting that sustained colonisation beyond Day 14 may further improve the immune response. However, as these studies are not directly comparable in terms of nature and concentration of inoculum and post-inoculation sampling periods, it is not possible to draw robust conclusions from this observation.

7.1.4 Controlled human infection with GM Nlac

L4 was a ground-breaking study in which two GM Nlac strains, one of which expressed a heterologous antigen, were used as challenge agents. Colonisation with GM Nlac expressing the meningococcal antigen NadA was shown to be safe, both for participants and for the wider population. Participants were admitted for close observation for 4.5 days, and subsequently followed up on outpatient basis with infection control procedures

and regular assessment of shedding and onward transmission in place. This study design was both acceptable to the relevant regulatory and ethical bodies, and effective for the control of the GM strains, with no shedding or transmission to bed-sharing contacts detected. The successful conduct of this study has laid a foundation for the safe and robust design of future studies with GM challenge strains.

7.1.5 The impact of NadA expression

Challenge with the GM Nlac strains was successful with no apparent impact of either the transformation itself, or the insertion of *nadA*, on the ability of Nlac to colonise the nasopharynx, or to induce an Nlac-specific immune response comparable to that seen following colonisation with wild-type Nlac. While not directly measured *in vivo*, the immunological results suggest that NadA was successfully expressed during the period of nasopharyngeal colonisation, as an antigen-specific immune response was demonstrated. Colonisation with the NadA expressing strain resulted in seroconversion to NadA and an increase in sNadA specific B_{PLAS} and B_{MEM} cells whereas no NadA specific responses were seen in the control group. These results show that it is possible to induce a specific immune response to a bespoke antigen, expressed by a recombinant organism during nasopharyngeal carriage. This model has the potential to be modified further to facilitate the analysis of the immunological response to the expression of other antigens of interest, either different meningococcal antigens, or antigens from different organisms.

The impact of NadA expression on the cross-reactive Nmen immunity was assessed, using the internationally accepted correlate of protection of an anti-meningococcal SBA titre ≥ 4 . A protective level of anti-meningococcal SBA was induced in 71.4% of previously unprotected participants colonised with the NadA expressing strain in comparison to 44.4% of previously unprotected participants colonised with the control strain. This encouraging finding does suggest that the addition of NadA may have improved cross-reactive immunity but the difference between the two groups was not significant. This was partly due to the small sample size, and partly due to the use of a wild-type equivalent Nlac strain as the control, which may in itself have induced cross-reactive SBA in a proportion of participants. The use of GM Nlac as an intervention to induce protection from meningococcal disease has therefore not been demonstrated, but further investigation with a study designed and powered for this endpoint is justified.

7.1.6 Lyophilisation

Storage of Nlac as a lyophilised product (LyoNlac) has been demonstrated to be stable, with improved accuracy in dosing once the study was properly established, and no reduction seen in the ability of the bacteria to colonise the nasopharynx or to induce a

specific humoral immune response. The colonisation fraction and duration, immunogenicity and safety profile of L5 were comparable to that seen with frozen stocks of Nlac.

7.1.7 Translation of the model to the meningitis belt

The optimised Nlac CHIM was successfully translated to a research centre within the meningitis belt. The study was feasible for the local study team and acceptable to the local population. Nasal administration of LyoNlac was well tolerated and resulted in safe, longstanding, immunogenic colonisation in a similar way to that seen in the UK. Comparing the two populations, there was a trend towards a lower colonisation fraction, and as a result a higher standard inoculum dose in LE1 in comparison to L5, with a shorter duration of colonisation. Baseline titres of Nmen IgG were higher in LE1 than L5, with a trend towards higher baseline Nlac IgG. While Nlac colonisation was still immunogenic, the increase in Nlac IgG was higher in L5 than in LE1, with a trend to the same for Nmen IgG.

7.2 Comparison to other controlled human infection models

A wide variety of CHIMs have been developed, including models of both disease and asymptomatic carriage, those using viral, bacterial and parasitic organisms, and with administration of the challenge agent via the skin, gastro-intestinal and genito-urinary tracts, and the respiratory tract (126).

The majority of respiratory CHIMs involve viral agents administered via the nasal route such as influenza, RSV, Rhinovirus and SARS-CoV-2. Bacterial respiratory tract CHIMs include *Streptococcus pyogenes*, in which the agent is administered directly onto the pharynx with a swab (176), and two other models using nasal administration; the *Streptococcus pneumoniae* (Spn) and *Bordetella pertussis* (Bp) models. These two models therefore provide the best comparison for the Nlac CHIM.

7.2.1 The Experimental Human Pneumococcal Carriage programme

Controlled human infection with *S. pneumoniae* (Spn) was first conducted in 2001 in the United States (177, 178) and has subsequently been further developed in Liverpool as the Experimental Human Pneumococcal Carriage (EHPC) programme (179). To date approximately 1500 healthy adult volunteers have been challenged (180) to address questions such as correlates of protection from, and immunological responses to Spn carriage (136), vaccine efficacy (181), the relationship between Spn colonisation and viral co-infection (182), influenza vaccination (183) and the upper respiratory tract microbiome (184).

The EHPC, like the Nlac CHIM, aims to induce asymptomatic nasopharyngeal colonisation. However, the key difference is the use of the pathogen of interest itself (Spn), in contrast to the use of a commensal organism closely related to the pathogen of interest (Nlac and Nmen). While the safety profile of the EHPC is reassuring (180), the potential for causing disease remains, whereas in the Nlac CHIM the risk of disease is negligible. Consideration has been given to a potentially cross-protective relationship between Spn and the commensal *Streptococcus mitis* (Sm). This has been investigated *in vitro*, in animal models and using sera from EHPC participants, but not yet as a Sm CHIM (185-187).

The colonisation fraction seen following Spn challenge is dose-dependent, ranging from 0.1-0.6 as the dose increases from 10,000 to 60,000 CFU/naris. Further increase of the inoculum dose does not appear to substantially increase the colonisation fraction (136). This is lower than seen in our Nlac CHIM UK-based studies where a comparable dose achieved a colonisation of 0.8-1.0. However, it is similar to that seen in LE1, where a dose of 100,000 CFU achieved a colonisation fraction of 0.6 with minimal increase seen with further increases in inoculum dose. This apparent plateau of colonisation fraction may be due to baseline immunity as a result of the “boosting” effect of natural carriage episodes (136).

Similarly to the Nlac CHIM, experimental colonisation with Spn has been shown to induce a specific humoral immune response, with an increase in serum IgG seen to both capsular polysaccharide (CPS) and to Spn-specific proteins. However, in contrast to Nlac, a transient increase in serum IgG to some Spn-proteins has also been demonstrated in response to Spn challenge without colonisation (136) as well as more sustained increase in mucosal IgG to Spn-proteins (188). In the Nlac model, it appears that microbiologically-proven infection (not just inoculation) is essential for generating an immune response.

Assessment of B-cell mediated immunity in relation to Spn challenge demonstrated that higher baseline B_{MEM} with specificity to Spn CPS was associated with protection from colonisation. In those participants who were challenged but not colonised, a reduction in B_{MEM} but transient increase in B_{PLAS} with specificity to Spn CPS was seen, suggesting that the pre-existing B_{MEM} cells differentiated into B_{PLAS} cells resulting in clearance of Spn and protection from colonisation. A non-specific trend to increased B_{PLAS} was seen in colonised participants (169). In L4 a transient but significant increase in Nlac specific B_{PLAS} and more sustained increase in B_{MEM} was seen in colonised participants. No data is available for challenged but non-colonised participants for L4 due to the high colonisation fraction.

Episodes of Spn carriage protect against subsequent homologous challenge (136) but not heterologous challenge (169). The protective effect of an episode of Nlac carriage has not yet been investigated in the Nlac CHIM.

The EHPC model has been translated to Malawi, a setting where pneumococcal carriage and disease is known to be higher than in the UK. A randomised controlled vaccine-challenge study with PCV-13 followed by Spn challenge was undertaken. The colonisation fraction in the placebo arm was 0.29 at 80,000 CFU/naris and 0.35 at 160,000 CFU/naris, in comparison to approximately 0.6 seen in the UK at 80,000 CFU/naris. A protective effect of the vaccine was seen but with less efficacy than is seen in the UK (189). These Africa-UK differences are parallel to the relatively lower colonisation fraction and immunogenicity following Nlac challenge seen in LE1 in comparison to L5. Although the two settings are distinct (Malawi in South-East Africa for the Spn vaccine-challenge versus Mali in West Africa for LE1), as two low income countries in the same global region, it is reasonable to consider that there may be common environmental, health and epidemiological factors influencing these immunological differences.

7.2.2 *Bordetella pertussis* CHIM

The *Bordetella pertussis* CHIM has been developed in Southampton. As with the Spn CHIM, this was designed as a model of asymptomatic carriage, but has the potential to cause disease as the challenge agent is a wild-type virulent organism (190). The initial dose-ranging study was therefore conducted on an inpatient basis. The colonisation fraction is dose-dependent, reaching 0.8 at a dose of 100,000 CFU in participants selected for low baseline anti-PT IgG (191). This is comparable to the colonisation fraction seen in unselected adults challenged with Nlac in the UK. Serum IgG to Bp specific antigens increased among colonised, but not non-colonised participants, and a trend to increased Bp-specific B_{PLAS} was seen in colonised but not non-colonised participants. Shedding of Bp was assessed using a range of techniques, including those used in the Nlac CHIM. As in the Nlac CHIM, no shedding of Bp was detected from colonised but asymptomatic participants (191). Further studies using this model are underway, investigating potential correlates of protection from colonisation, the protective effect of an episode of colonisation on homologous rechallenge and the efficacy of novel Bp vaccines in protection from colonisation (unpublished data).

BPZE1 is a nasally administered live attenuated Bp candidate vaccine. Studies involving administration of BPZE1 have been designed as CTIMP vaccine studies, but they could also be considered to be controlled human infection studies as they aim to induce nasopharyngeal colonisation with the vaccine strain. Following nasal administration of a liquid formulation containing BPZE1, asymptomatic nasopharyngeal colonisation with this strain was achieved in a proportion of participants that was dose-dependent at lower doses from 10^5 to 10^7 CFU (142) but with a plateau in the colonisation fraction at about 0.8 seen with further dose increases to 10^8 and 10^9 CFU (167). This is a similar pattern to that seen with both the Spn CHIM in the UK and the Nlac CHIM in Mali (LE1), although with a higher final inoculum dose. However in contrast, colonisation with BPZE1 is transient, with spontaneous clearance before Day 28 in almost all participants, and by Day 50 in all participants (167). Despite this shorter duration, colonisation is immunogenic with induction of Bp specific systemic and mucosal humoral responses (167, 168). Colonisation with BPZE1 has also been shown to be protective against rechallenge (172). A lyophilised preparation of BPZE1 has been shown to be stable, and to induce immunogenic colonisation in a similar way to the original liquid formulation (171, 172). This development mirrors the successful modification of our Nlac CHIM to use lyophilisation as a reliable and stable method of inoculum storage and preparation.

7.3 Strengths and limitations

The Nlac CHIM is a safe and reliable model allowing investigation of the immunogenicity and potential protection against Nmen colonisation and disease induced by nasopharyngeal colonisation with Nlac, and by the expression of bespoke antigens during that colonisation.

A notable strength of this model is the reassuring safety profile, both in theory, as Nlac is a non-virulent commensal in contrast to many other controlled human infection models, and also as demonstrated in the clinical studies to date. However, while Nlac is closely related to Nmen, and can be manipulated to express meningococcal antigens, there are limitations to the questions that this model can reliably answer. For example, a future use of this model could be to demonstrate protection from colonisation with Nlac expressing Nmen antigens, as a proxy for Nmen. While such data would be valuable, such a study would not be as robust as one involving challenge with Nmen itself. However, an Nmen CHIM would be unlikely to be considered ethically appropriate due to the higher risk involved.

The high colonisation fraction achieved in this model, particularly in the UK, means that it is a very efficient model, optimising the sample size and therefore time and resources

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required to investigate specific research questions. Even the lower colonisation fraction (per unit of dose) demonstrated in LE1 is comparable to that seen in the Spn CHIM.

The ability to clear carriage quickly and efficiently is another strength of the model. However, a single dose of Ciprofloxacin has been used for this clearance, and this may no longer be appropriate considering updated MHRA guidance following concerns about the safety profile of Ciprofloxacin (192). Other antibiotics such as Rifampicin or Azithromycin are likely to be effective in eradicating the inoculum strain of Nlac (103, 193), but this will need to be demonstrated in a future study.

Specific strengths and limitations of each study described in this thesis have already been discussed in each study specific chapter, but there are some common limitations. In each study the sample size was limited to that required to answer the primary research question. However, this did mean they were insufficiently powered to robustly investigate some of the secondary endpoints, for example the impact of NadA expression on colonisation fraction and duration, and on cross-reactive Nmen immunogenicity. In addition, there was not a non-challenged or sham challenged control group for any of the studies as this was not required for the primary objectives, but could have provided valuable additional data. For example the induction of anti-meningococcal serum bactericidal activity by GM Nlac colonisation with NadA expression in comparison to a sham-challenged control group may have reached statistical significance.

The immunological endpoints assessed for each study were limited to systemic humoral immunogenicity (L3, L5, LE1) plus B_{MEM} , B_{PLAS} and anti-meningococcal SBA (L4). Additional important endpoints to consider would be local mucosal immune responses and T-cell responses. Demonstrating an impact on Nmen colonisation and/or disease would also have been ideal, but this would have required a far greater sample size.

These study design decisions were made on the basis of the resources and time available, prioritising the primary objectives for each study. However, resolutions for these limitations will be considered in the design of future Nlac CHI studies.

7.4 Future direction of the research programme

Further development of the Nlac CHI research programme is planned, with some studies already funded and at the pre-clinical and study design stage, and others planned for further grant and fellowship applications.

In the immediate future, preparations will be completed for two GM Nlac studies involving challenge with strains expressing different meningococcal antigens. The impact of colonisation with these strains on immunogenicity, including anti-meningococcal SBA and mucosal endpoints, as well as on protection from homologous rechallenge, will be investigated. A larger sample size and a sham-challenged control group will be included in the study design.

An impact of Nlac colonisation upon Nmen carriage has previously been demonstrated in the UK (124). Future studies could investigate this impact in a population within the meningitis belt, either as a long-term carriage study, or as an intervention during a meningococcal outbreak within a defined region, with endpoints of both carriage and disease rates in a specific geographical area in comparison to a similar but discrete area. Such a study would require significant advance planning and resources, and the ability to deliver the study very rapidly upon an outbreak being detected. This is therefore a very ambitious but potentially hugely valuable future project. A similar study has previously been successfully conducted using antibiotic prophylaxis (92) showing that such a study design is feasible. The use of either wild-type or GM Nlac as an inoculum in such a study will need to be considered. The results of the future GM Nlac studies mentioned above, plus further assessment of the acceptability, feasibility and colonisation fraction within the target population will need to be taken into consideration.

Recently it has been demonstrated that deployment of OMV-containing meningococcal vaccines (4CMenB and MenNZ) in adults is associated with a reduction in gonococcal disease with a vaccine effectiveness of around 30% (194, 195). As Nlac also has reasonably close genetic homology to *N. gonorrhoea* (115, 196), and as it lacks the immunodominant antigen PorA (197), there is a possibility that exposure to Nlac in a mucosal compartment in adults could generate a similar protective response. There is a shared mucosal immune axis involving the gut and respiratory tracts (198) and it is likely that this is shared also by the upper respiratory tract. Therefore, future work might explore the potential for nasopharyngeal induced colonisation by Nlac to induce cervicovaginal immune responses and to determine whether these could be cross-protective against *N. gonorrhoea*.

7.5 Conclusion

This thesis describes the development of the Nlac CHIM from the original two studies (123, 124) to the current model, and then to future planned studies. Each of the four studies described have progressed the overall programme and answered specific research questions including the optimal dose, consideration of the duration of carriage, demonstration of the immunogenic expression of a heterologous antigen of interest, optimisation of the storage and preparation methodology, and successful translation into a relevant setting and population for future studies. These four studies provide a robust foundation for further development of the Nlac CHIM towards the overall aim of achieving a clinically meaningful impact.

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