COLLEGE OF MEDICINE

DEVELOPMENT OF LOOP-MEDIATED ISOTHERMAL AMPLIFICATION (LAMP) FOR DETECTION OF MACROLIDE-RESISTANT GENES IN STREPTOCOCCUS PNEUMONIAE

By

Jacquline Msefula

(BSc Biomedical Sciences)

A Dissertation Submitted to the Department of Pathology, Faculty of Biomedical Sciences and Health Professions in Partial Fulfilment of the Requirements of the Master of Science in Health science (Antimicrobial Stewardship)

December, 2020

Declaration

I, Jacquline Msefula hereby declare that this dissertation is my original work and has not been presented for any other awards at the University of Malawi or any other University.

Name of Candidate Jacquline Msefula

Signature _____

Date 24th May 2021

Certificate of Approval

This dissertation by Jacquline Msefula was approved by the Dissertation Examination Committee:

_____

Professor Fanuel Lampiao

(Chairman, Post Graduate Committee)

_____

Dr. Tonney Nyirenda

(Supervisor)

_____

Dr. Janelisa Musaya

(Co- Supervisor)

_____

Dr. Tonney Nyirenda

(Head of Department)

Declaration of Student Investigator Contribution

Activity	Responsible Individuals
Participant recruitment	**Shared**
Data entry and cleaning	**Sole**
Dissertation database design	**Shared**
Development of protocol	**Shared**
Dissertation literature	**Sole**
Dissertation data analysis	**Sole**
Dissertation report write up	**Sole**

Acknowledgements

Foremost, I would like to express my sincere gratitude to my supervisor Dr Tonney Nyirenda for the continuous support of my Masters Study and research, for his patience, motivation, enthusiasm, and immense knowledge. His guidance helped me in all the time of research and writing of this thesis. I could not have imagined having a better advisor and mentor for my Masters study.

Besides my supervisor, I would like to thank my second supervisor Dr Janelisa Musaya, my protocol advisor Dr Chisomo Msefula, my advisor at Malawi Liverpool Wellcome Trust Todd Swathhart for their encouragement, insightful comments, and hard questions.

I would like to thank James McKena from Belfast University for the advice and support on the development of my protocol and my lab mate at Malawi Liverpool Wellcome trust Comfort Brown for the helpful discussions and working together before deadlines, and for all the fun we have had in the last two years. Also, I thank my friends at College of Medicine for the encouragement and always helping me with my way around the pathology lab.

Last but not the least, I would like to thank my family: my Husband Chisomo Msefula for being my moral and spiritual support throughout my studies, helping me look after our boys Elijah and Ezra while encouraging me every step of the way to be better and My Mother, for giving birth to me at the first place and supporting me spiritually throughout my life.

Abstract

Background: Antibiotic resistance is fast becoming a major health problem, which makes it difficult to treat several infections, including Streptococcus *pneumoniae* infection. Increasing access to antibiotics, presumptive treatment especially in low-resource settings, and self-medication are some of the factors contributing to the rise in antibiotic resistant pathogens. Therefore, a robust surveillance platform for emergence of drug-resistant pathogens is a public health imperative. Diagnostic tools that could help with early detection, monitoring of resistance patterns and determining the prevalence of pathogens and pathogen resistance are urgently needed. In this study we have addressed the latter need by developing a method for the detection of macrolide resistant *Streptococcus pneumoniae* using loop-mediated isothermal amplification (LAMP) assay. The LAMP assay has increased specificity and sensitivity with a reduced time frame of detection.

Rational: There is a growing need to develop diagnostic tools in early detection of antimicrobial resistance and real time surveillance. We aimed to develop a LAMP method for the rapid detection of antimicrobial resistant *S. pneumoniae* that could help in monitoring macrolide resistance in resource limited areas.

Methods: A laboratory method, development study was conducted to develop a LAMP assay for the detection of macrolide resistance genes in *S. pneumoniae* from Blantyre, Malawi. *S. pneumoniae* isolates were obtained from an on-going community-based pneumococcal carriage surveillance project.-Nasopharyngeal swabs were collected from children below the age of five in urban Blantyre. Isolates' resistance profiles including erythromycin and azithromycin resistance were determined using phenotypic methods at Malawi-Liverpool-Wellcome Trust (MLW) laboratories. Whole genome sequencing (WGS) was done at the University of Oxford, United Kingdom. LAMP primers for the detection of macrolide resistance genes *mefA* and *ermB* were designed from two sequenced *S.*

pneumoniae isolates genomes using Premier Biosoft software. The cut-off for amplification and the sensitivity and specificity of the LAMP primers was determined using the Receiver operating characteristic curve (ROC) Antimicrobial resistance phenotyping on culture was used as the gold standard test. Resistance results turn-around time was calculated for both culture and the LAMP assay.

Results: 79 *S. pneumoniae* isolates were analyzed on the LAMP assay and results were compared to conventional culture method. Primers designed to detect azithromycin, *mefA* had sensitivity = 97.1% and specificity = 100%, at 95% confidence interval 0.952 to 1.000; while for erythromycin, *ermB* had sensitivity = 97.1% and specificity = 95.8%, at 95% confidence interval 0.452 to 0.701. LAMP assay amplified both azithromycin and erythromycin resistance genes within 30 minutes of the run. Furthermore, detection time for both *ermB* and *mefA* primers including DNA isolation and LAMP assay took <3 hours versus the conventional culture methods antimicrobial sensitivity testing (AST) which ranges from 24 to 48 hours to generate results.

Conclusion:

The study successfully developed and evaluated the performance of the LAMP assay for *mefA* and *ermB* gene in *S. pneumoniae* isolates. LAMP assay sensitivity and specificity performance for detection of *mefA* was excellent being a classifier of 0.98 the area under the Receiver Operating Curve (ROC). While for *ermB* it was an average classier with 0.57 area under ROC when both compared to conventional AST methods. The turnaround time for LAMP assay from pure isolate was less than 3 hours including nucleic acid isolation and detection compared to culture AST, which ranges from 24 to 48 hours. The study developed LAMP assay that can be used in the detection of bacterial resistance and provide results in timely fashion. Further evaluation of performance of the LAMP assays is recommended.

Table of Contents

List of Tables

List of Figures

List of Abbreviations and Acronyms

AMR	Antimicrobial Resistance
BIP	Backward Inner Primer
B3	Backward primer 5’
CAP	Community-Acquired Pneumonia
COM	College of Medicine
COMREC	College of Medicine Research and Ethics Committee
EPI	Expanded Program on Immunization
F3	Forward primer 3’
FIP	Forward Inner Primer
IPD	Invasive Pneumococcal Disease
LAMP	Loop-Mediated Isothermal Amplification
LB	Loop Backward primer
LF	Loop Forward primer
MIC	Minimum Inhibitory Concentration
MLW	Malawi Liverpool Wellcome Trust
NVT	Non-Vaccine Type
NP	Nasopharyngeal
PCR	Polymerase Chain Reaction
PCVPA	Pneumococcal Carriage in Vulnerable Populations in Africa
STGG	Skim Milk, Glycerol, Glucose and Tryptone Soya Broth
TLR4	Toll-Like Receptors 4
VT	Vaccine Type
GLASS	Global Antimicrobial Resistance Surveillance System
CB	Comfort Brown
JM	Jacquline Msefula

Chapter 1: Introduction and Literature Review

1.1 Disease Epidemiology

Streptococcus pneumoniae (pneumococcus) is a highly infectious facultative anaerobic, Gram-positive, extracellular bacterial pathogen making it the leading cause of morbidity and mortality in the world, with more people dying from it than any other infectious disease. The most vulnerable are the youngest children and the aged, with 1 million children under 5 years of age dying every year with 9% of all deaths occurring in developing countries (1,2).

O'Brian and colleges estimated in their meta-analysis study in 2000, about 14·5 million episodes of serious pneumococcal disease were estimated to occur (3). Pneumococcal disease caused about 826 000 deaths (582 000–926 000) in children aged 1–59 months, of which 91 000 (63 000–102 000) were in HIV-positive and 735 000 (519 000–825 000) in HIV-negative children. Of the deaths in HIV-negative children, over 61% (449 000 [316 000–501 000]) occurred in ten African and Asian countries (3,4). While WHO estimates a range from 1·9 to 2·0 million with an estimated 1 million neonatal deaths are caused by pneumonia, sepsis, and meningitis (5).

Other efforts have been made to estimate global or regional pneumococcal disease burden in young children, the pneumococcal disease burden project of the Sabin Vaccine Institute and the Pan American Health Organization for Latin America and the Caribbean calculated 18 000 pneumococcal deaths in children younger than 5 years, with 327 000

cases of pneumonia and 3900 of meningitis, whereas O'Brian estimated 595 000 (463 000–741 000) cases of pneumonia and 8400 (6000–11 500) of meningitis (1,6). These differences result from different literature review and modelling strategies; for example, the model developed by the Sabin Vaccine Institute and the Pan American Health Organization did not adjust for HIV prevalence or access to care, therefore lowering incidence and fatality rates. Interpretation of estimates is especially challenging for large countries such as China, Indonesia, India, and Nigeria because pneumonia incidence, access to care, and childhood mortality vary substantially within the country (**Fig 1.1**).

In 2000, an estimated 826 000 pneumococcal deaths occurred in children aged less than 5 years old, of whom 91 300 were HIV-positive. The highest mortality rates and case-fatality ratios were in sub-Sahara Africa and south Asia, with an account for 61% (43–79%) of all pneumococcal deaths (pneumococcal deaths in HIV-negative children only). Of the pneumococcal deaths (HIV-positive and HIV-negative), 90% were caused by pneumonia, 7% by meningitis, and 3% by serious non-pneumococcal, non-meningitis clinical syndromes (7).

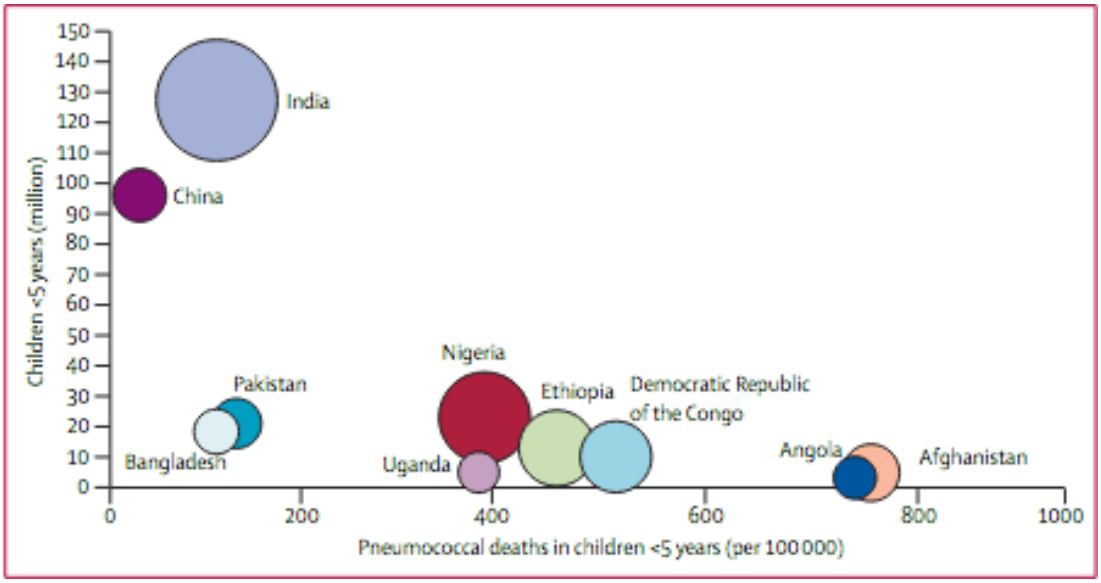

Figure 1.1 Ten countries with the greatest number of pneumococcal deaths in children aged 1–59 months*Bubble size indicates the number of pneumococcal deaths. *Country (number of deaths): India (142 000), Nigeria (86 000), Ethiopia (57 000), Democratic Republic of the Congo (51 000), Afghanistan (31 000), China (30 000), Pakistan (27 000), Bangladesh (21 000), Angola (20 000), and Uganda (19 000) (2)

In Malawi *Streptococcus pneumoniae* is the leading cause of pneumonia, sepsis, bacterial meningitis and death in both children and adults. Malawi is a landlocked nation in southern Africa, located to the south of the traditional meningitis belt and to the north of South Africa, an important economic partner in terms of trade and labor mobility (8,9).

It has a high HIV prevalence, with an estimated 900,000 people living with HIV infection in Malawi in 2007(9). In Blantyre, the general incidence of HIV infection in pediatric inpatients is around 19 percent, with 34 percent in children with bacterial meningitis. Adult inpatient HIV prevalence is over 70 percent, with meningitis cases reaching up to 90 percent (10).

The pneumococcal surface is covered by a polysaccharide capsule that overlays the cell wall comprised of peptidoglycan and teichoic acid. Although the peptidoglycan has the classical Gram-positive structure of *N*-acetylglucosamine, *N*-acetylmuramic acid, and a lysine-

containing stem peptide, the teichoic acid is unusual in containing a ribitol phosphate backbone and covalently attached phosphorylcholine (PCho) (11).

Pneumococcus has over 100 known serotypes and at least 93 different capsular structures/serotypes which have been so far described. Some serotypes are most often present in small children such as 6B, 9V, 14, 19F, and 23F (12). A review of invasive disease isolates and carriage isolates from the same area over the same span of time indicates that virulence varies based on the capsular serotype (13).

Patients infected with serotypes with low invasive disease potential are much more likely to have an underlying disease prior to invasive pneumococcal infection compared to patients infected with serotypes with high invasive disease potential (14). However, the highest mortality rates were reported in patients with lower invasive disease risk serotypes such as type 3, 6B, and 19F (15).

Pneumococcal diseases can cause very minor respiratory mucosal infections such as otitis media and sinusitis or more severe diseases such as pneumonia, septicaemia and meningitis.

Pneumococci naturally takes up DNA from other bacteria and which leads to development of different types of complications or diseases and potentially switch capsular serotype and developing resistance in the process, other methods other than serotyping need to be used to study genetic relationships between clinical pneumococcal isolates (16).

In developing countries, where pneumococcal conjugate vaccine (PCV) coverage is widespread, Invasive pneumococcal disease (IPD) rates have fallen significantly, over a 3-year period IPD rates in children under 5 years caused by vaccine serotypes (i.e. for the PCV13 which has 1,3,4,5,6A,6B,7F,9V,14,19A,19F,18C and 23F) decreased by 78 percent (17). Even though with the introduction of the conjugate vaccines yearly in sub-Saharan Africa alone, about 4 million episodes of pneumonia occur each year resulting in 200,000 deaths. In African and South Asian settings Community acquired Pneumonia (CAP) burden is worsened by the human immunodeficiency virus (HIV) epidemic, malaria and diarrhea (9). In Malawi, HIV-prevalence is 10.3% among those aged 18-40yr old and pneumonia is the common reason for hospitalization and around 75% are HIV-infected (11). Kamng'ona *et al* illustrated this in **Fig 1.1** below by showing a staggering number of individuals of HIV positive status across vaccine and non-vaccine types (18). With ongoing misuse of antibiotics, the situation can be further complicated by antimicrobial resistance, especially when seen to be carried in the non-vaccine types as seen in other countries such as Democratic Republic of Congo (**Figure 1.2**) (19).

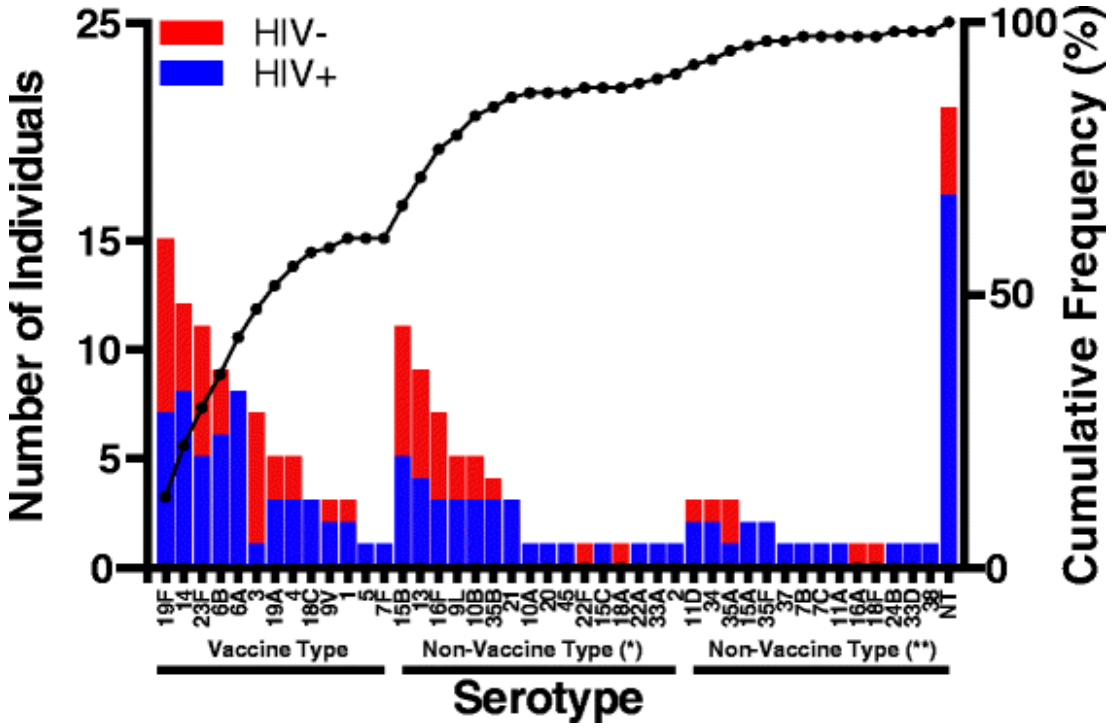

Figure 1.2: Serotype-specific pneumococcal carriage in Malawian children, determined by microarray. A comparison of Serotype-specific pneumococcal carriage in Malawian between persons with and without HIV, determined by microarray (18)

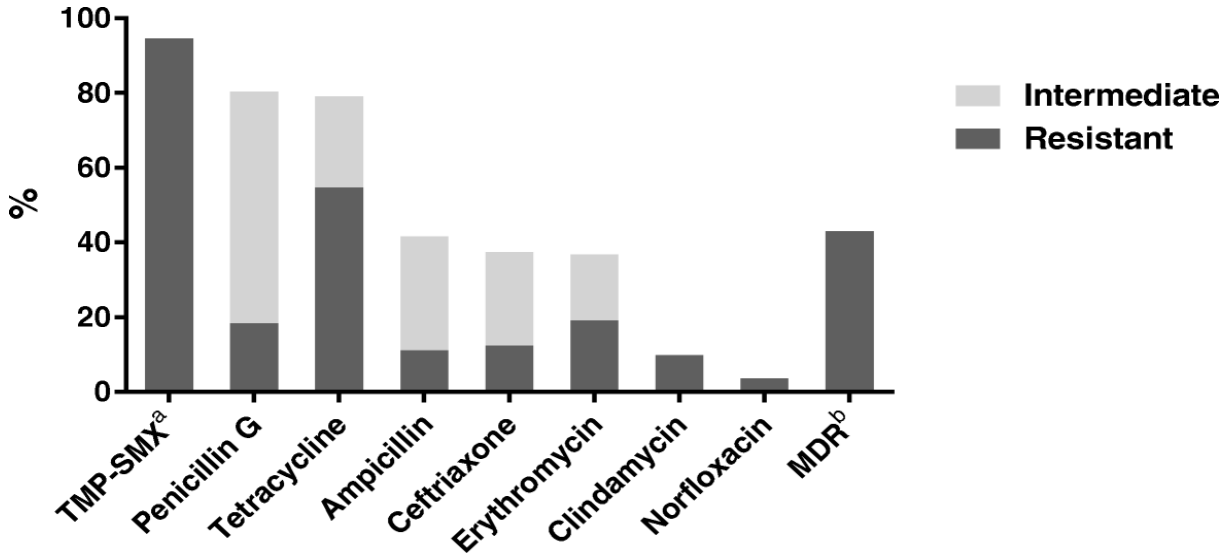

Figure 1.3: The antimicrobial susceptibility pattern from healthy DRC children. The antimicrobial susceptibility pattern determined in Bukavu, DR Congo were 163 pneumococcal strains (Vaccine and non-vaccine types) were isolated and MDR was defined as the pneumococci being non-susceptible to first line antibiotics penicillins and second line antibiotics of macrolides (19).

1.2 Disease Aetiology

1.2.1 Transmission

Pneumococci are common inhabitants of the respiratory tract and may be isolated from the nasopharynx of 5–90% of healthy persons, depending on the population and setting(20). Pneumococcal diseases are known to spread from person to person by droplets/aerosols and nasopharyngeal colonization is a requirement for establishment of pneumococcal disease. Carriage rates increase at about 2–3 years of age and then decrease to <10% in the adult population. However, people with small children at home may have a higher carriage rate.

The bacteria enter the nasal cavity and bind to the nasopharyngeal epithelial cells and can then linger colonized or spread farther to other organs, such as the ears, the sinuses, or through the bronchi to the lungs, and then possibly enter the mucosal barrier to enter the bloodstream and/or cross the blood-brain barrier and cause meningitis (**Fig 1.3** (21,22)) . The nasopharynx has been classified as the main reservoir of *S. pneumoniae*. This is due to the nasopharynx of hosts being colonized without any symptoms. Following colonization, the spreading of the disease depends on carriers coming into close contact with healthy individuals within the community.

The CDC has declared that the main source of *S. pneumoniae* transmission is direct contact with secretions of the respiratory system of a carrier, so breathing in droplets produced by an infected person during coughing or sneezing spreads the bacteria one person to another (23).

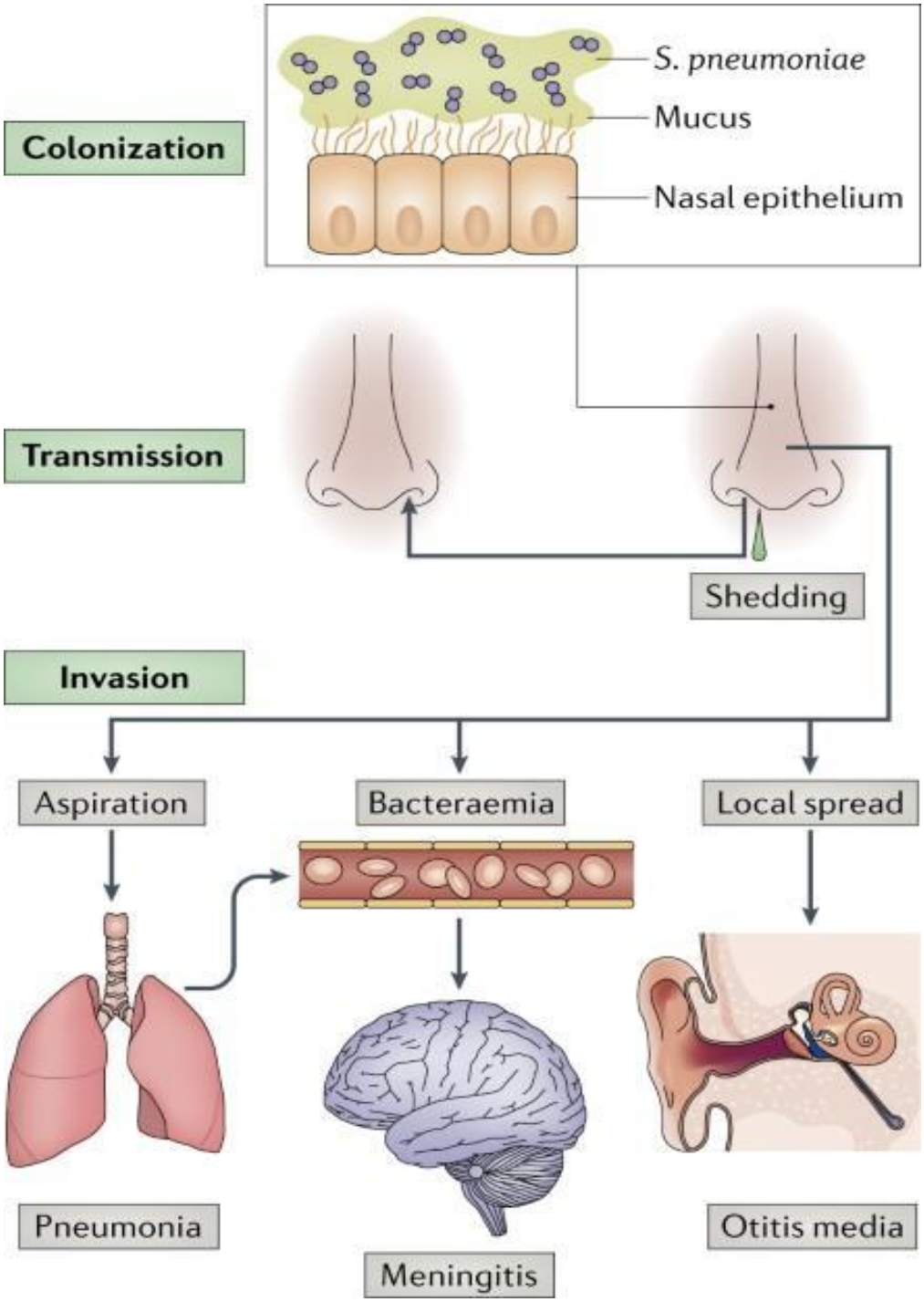

Figure 1.4 Colonization, Transmission and Invasion of *Streptococcus Pneumoniae*. The pneumococci are highly adapted commensals, and their main reservoir on the mucosal surface of the upper airways of carriers enables transmission. They cause severe disease when bacterial and host factors allow them to invade essentially sterile sites, such as the middle ear spaces, lungs, bloodstream and meninges. Transmission, colonization and invasion depend on the remarkable ability of *S. pneumoniae* to evade or take advantage of the host inflammatory and immune responses (12)

1.2.2 Pathogenesis

Pneumococcus secretes a potent cytotoxin, pneumolysin, which is a member of the cholesterol-dependent, pore-forming cytotoxin family. It plays an important role in the respiratory tract, not only by lysing host cells but also by inhibiting the mucociliary beat of respiratory cells and separating epithelial cell tight junctions (24,25).

The pneumolysin also contributes to the etiology of otitis media by cytotoxic effects on sensory hair cells in the middle ear. Pneumolysin stimulates the classical complement system and may be detected by Toll-Like Receptors (TLR4), resulting in an inflammatory response (26).

Pneumolysin has been shown to influence bacterial uptake into human dendritic cells, to inhibit cellular activation and cytokine secretion, and to induce a caspase-dependent apoptosis. A non-functional pneumolysin, such as in some serotype 1 isolates, also influences inflammation induced by the interaction of bacteria and dendritic cells and might affect the development of diseases such as empyema (27).

The most important element in the development of high titer bacteremia is the pneumococcal polysaccharide capsule. Capsule is the key antiphagocytic element and antibody to capsule is opsonic and highly protective. Each of the >90 serotypes have a structurally unique polysaccharide and these vary in their antiphagocytic efficacy. Steric hindrance and negative charge determine the ability of a capsular type to inhibit surface deposition of complement, CRP, mannose-binding proteins, and antibodies that can bridge to receptors on the phagocytes (28,29). The size of capsule also matters because it is a phase changeable characteristic, and encapsulated strains are virtually avirulent.

In terms of disease development, pneumococci with high titers can come into touch with blood–brain barrier vascular endothelial cells. Crossing the tight connections of this specialized endothelium allows bacteria to enter the cerebrospinal fluid and brain parenchyma, resulting in meningitis. Bacterial multiplication is rife in CSF space, therefore there are no natural defenses in this compartment. As a result of the link between the bacterial components and the host responses, brain harm can be clearly observed throughout time. The bacterial elements responsible for the signs and effects of the disease have been mapped by the fractionation of the pneumococcal disease, the specific instillation of parts into the CSF, and inflammation and damage (30). The hallmarks of meningitis are recruitment of leukocytes, blood–brain barrier permeability as measured by increased CSF protein, and vascular dysregulation, leading to increased intracranial pressure. This symptom complex can be recapitulated by instilling only components of the pneumococcal cell wall or toxins in the absence of living bacteria. The significance of this finding is twofold: First, cell wall and toxin interactions that drive inflammation are key to injury and second, antibiotics cause lysis and the release of cell wall components which, in the first few doses, increase CSF inflammation. This provides the rationale for mitigating damage to host cells by decreasing the inflammatory response during antibiotic therapy (31).

Laboratory tests on blood, pleural fluid, joint fluid, or cerebrospinal fluid (CSF) are needed to confirm the diagnosis of invasive disease with antibiotics as the main treatment for invasive *S. pneumoniae* infection.

1.2.3 Disease Prevention and Vaccines

The contagious period for pneumococcal infections varies and may last for as long as the organism is present in the nose and throat. A person can no longer spread *S. pneumoniae* after taking the proper antibiotics for 1-2 days and individuals who have had a *S. pneumoniae* infection can get the infection again.

Frequent handwashing with soap and water (or, when soap and water are not available, the use of alcohol-based hand rubs or gels) can help stop the spread of many viruses and bacteria. Not sharing food, drinks, or eating utensils with other people can also help to stop the spread of the disease. Preventive treatment (e.g., antibiotics) or prophylactic treatment is not required for an infection that can be caused by *S. pneumoniae* (30).

In 2007 the WHO published a list of countries that have included Pneumococcal Conjugate Vaccine (PCV) as part of the routine infant immunization schedule (**Fig 1.4**). The type of pneumococcal vaccine that are given depending on age and health, these are the Pneumococcal conjugate vaccine (PCV) there used to vaccinate children under 2 years old as part of the National Health Vaccine Program me and the second type is Pneumococcal polysacchari de vaccine (PPV) is given to people aged 65 and over and people at high risk because they have long-term health conditions (32).

There are currently two formulations of PCV in the market: 10-valent PCV and 13-valent PCV. With the support of GAVI the Vaccine Alliance many low-income countries have been able to introduce the PCV into their national immunization program.

Vaccines can help to prevent invasive *S. pneumoniae* infections. Pneumococcal conjugate vaccine 13-valent (PCV-13) is recommended for all children starting at two months up to 59 months of age. Pneumococcal polysaccharide vaccine 23-valent (PPSV-23) is used in high-risk individuals two years of age or older. Both PCV-13 and PPSV-23 are recommended for adults; the use of these vaccines is based on lifestyle, health condition, and age (30,32).

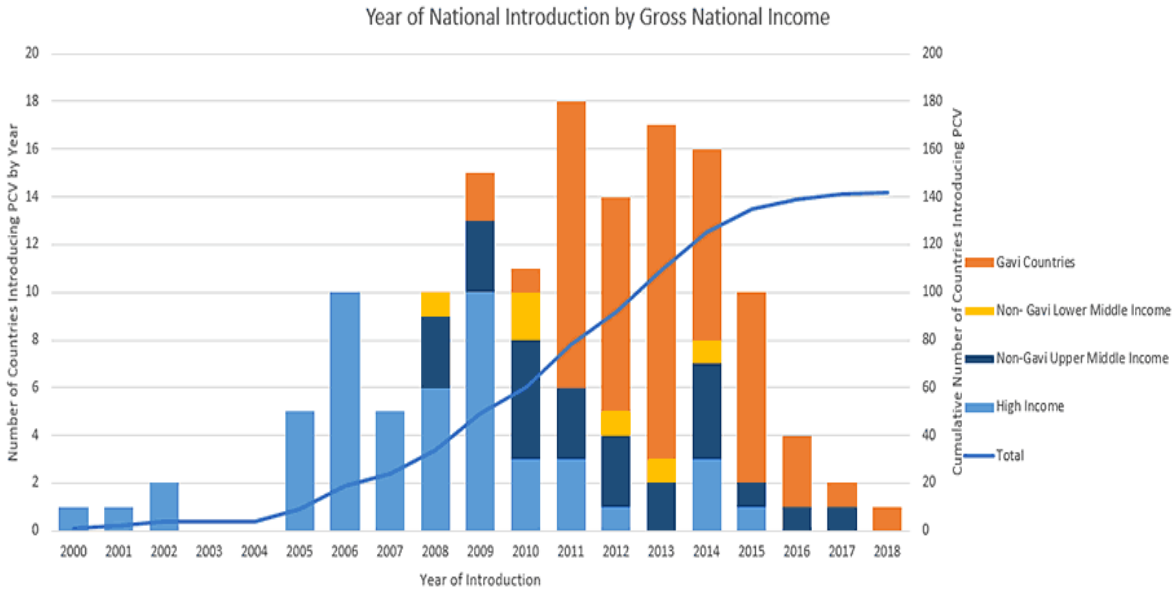

Figure 1.5 GAVI the Vaccine Alliance routine infant immunization schedule
A total of 142 countries have introduced PCV from 2000 through 2018. Of the 73 Gavi-eligible countries, 59 (81%) introduced PCV. Among non-Gavi eligible countries, PCV has been introduced in 6 (50%) of 12 lower middle-income countries, 26 (51%) of 51 upper middle-income countries, and 51 (88%) of 58 high-income countries. Figure courtesy of the International Vaccine Access Center (IVAC) (33).

1.3 Streptococcus Pneumonia and Antibiotic Resistance

Antibiotics are medicines used to kill the bacteria that cause disease. However, bacteria can change or mutate making it possible to resist antibiotics. As seen in *Streptococcus pneumoniae* which has become resistant to one or more antibiotics and resistance can lead to treatment failures, this action according to the Centre of Disease Control and prevention (CDC) is called antibiotic resistance, a bacterial resistance (34). The CDC published from the year 2017 to 2019, the active bacterial core surveillance report of emerging infections

program network of *Streptococcus pneumoniae.* Which has data that shows 30% of their cases of developing resistance to one or more antibiotics, this is referred to as Antimicrobial resistance (AMR). The opposition shown by a microbe to drugs that scientist have created to kill them (34).

The first line of drugs are the β-lactam antibiotics which include penicillin's, cephalosporins, and carbapenems. These compounds inhibit the final steps of peptidoglycan (cell wall) synthesis by binding to high-molecular-weight penicillin-binding proteins (PBPs). These antibiotics have a broad spectrum of activity against Gram-positive and Gram-negative bacteria. β-lactam antibiotics are considered to be time-dependent killers, meaning that increasing concentration significantly above the minimal inhibitory concentration (MIC) does not increase killing (35).

Second line of drugs are the macrolides, which inhibit bacterial protein synthesis by binding to the 23S rRNA component of the 50S ribosomal subunit in bacteria. There are two main mechanisms of macrolide resistance in pneumococcus. One involves target-site (ribosomal) alteration by an enzyme that methylates 23S rRNA, an enzyme that is encoded by the *erm*B (erythromycin-resistance methylase) gene. The resistance phenotype is called MLS_B (macrolide, lincosamide, streptogramin B type) and is responsible for a high level of macrolide resistance. In a low proportion of cases, *erm*B gene variation that modifies the binding site for macrolides and lincosamides confers complete cross-resistance to clindamycin. The second mechanism of resistance involves active efflux pumps encoded by the *mef*E or *mef*A (macrolide efflux) genes. These mutations result in low-level resistance to macrolides but not to the other two agents (36).

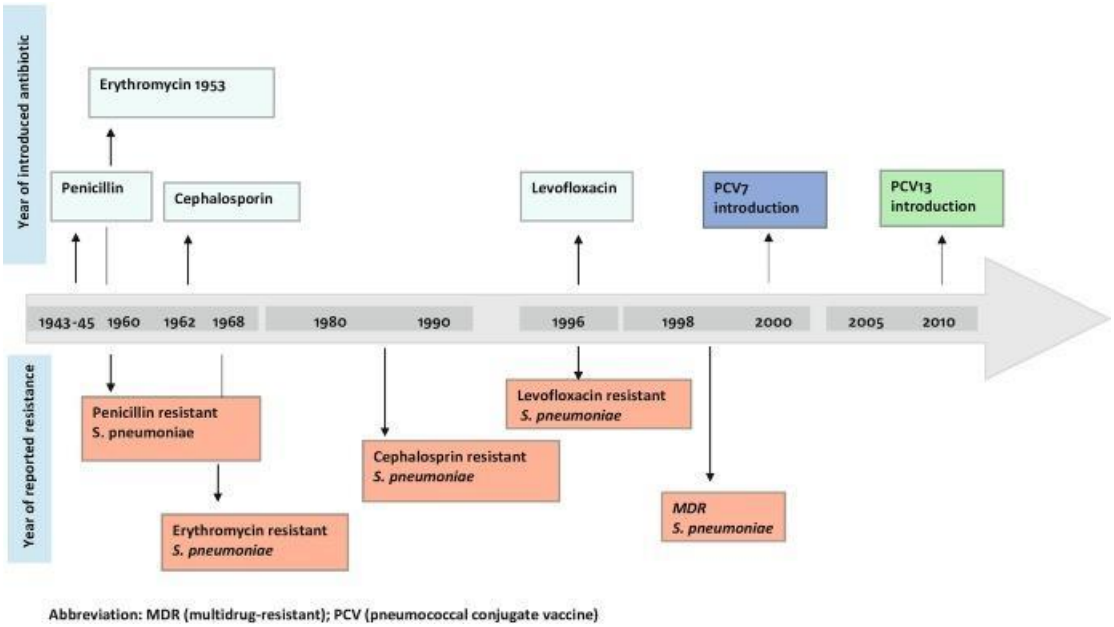

Figure 1.6 Timeline of antibiotics resistance of streptococcus pneumoniae

Erythromycin resistance emerged in the 1968 and multi drug resistance in the late 1998 that also showed a resistance to Azithromycin (12).

According to recommendations by the World Health Organization (WHO), oral amoxicillin is the drug of choice for children with pneumonia, while parenteral ampicillin (or penicillin) together with gentamicin should be used in severe cases. Ceftriaxone is recommended as the second-line treatment in children with severe pneumonia who have failed with the first-line treatment (37).

Chloramphenicol and penicillin have been commonly used in combination for the empirical management of sepsis in Malawi with 99·0% of the *S pneumoniae* isolates still susceptible to this combination despite its wide usage (10).

Development of penicillin resistance in the pneumococcus in the 1980s–1990s shifted antibiotic treatment of suspected pneumococcal upper respiratory infections and pneumonia to macrolides **(Fig 1.5**). Suspected pneumococcal upper respiratory infections

are often treated with macrolide antibiotics such as Azithromycin. Macrolides are increasingly used in the treatment of diseases caused by *S. pneumoniae*. Macrolide resistance in *S. pneumonia* is mediated by three main mechanisms, including; (1) mutations in ribosomal proteins, (2) discharge of antibiotics due to efflux pumps and (3) changes in the structure of the target molecule through methylation of 23s rRNA gene. The genes encoding efflux pumps (*mefA/E*) and methyltransferases enzymes (*ermB*) are carried on transposons, so spreading of resistance genes among the strains is possible.

The prescription of antibiotics is, however, not restricted solely to physicians, and children may be treated by people other than educated health workers and this contributes to the increase in macrolide resistance in *S. pneumoniae* (38,39). Clinical failures of macrolide (azithromycin) treatment of pneumococcal infections have been reported for lower respiratory tract infections (40) and bacteremia (41,42). Widespread macrolide mis-use is a strong selective pressure contributing to the expansion of macrolide-resistant *S. pneumoniae* (38,43).

Therefore, worldwide resistance to macrolides in pneumococcus has increased recently and is associated with the extensive global use of macrolides, principally for community-acquired respiratory tract infections. Thus, rapid detection of *streptococcus pneumoniae* and its resistance is important regarding the initiation of treatment and surveillance.

1.4 Diagnosis

Detection of antimicrobial resistance is done by traditional cultural methods which takes days for results to be detected. The currents methods used in Malawi for detection of antimicrobial resistance include conventional culture of respiratory secretions which may

include sputum, pleural, or bronchoalveolar lavage. These samples are used to diagnose pneumococcal pneumonia and other lower respiratory tract infections; however, the yields are low and the method is time consuming (44). In Malawi, there is lack of relatively affordable molecular methods to help in the detection and surveillance of antibiotic resistance country wide, a situation seen in many developing countries (45).

Data reported to the Global Antimicrobial Resistance Surveillance System (GLASS) is based on detection of phenotypic resistance, in which bacteria are tested for growth in the presence of antimicrobial agents. These methods allow determination of the degree to which an isolated pathogen is resistant to a given antimicrobial, by measuring either the minimum inhibitory concentration (MIC) which is the gold standard, or a zone diameter in disk diffusion testing. These methods provide information for clinical management and for surveillance but not direct information about the mechanism(s) of resistance to the agent. Methods for detecting phenotypic resistance may be generally lengthy (from hours at its fastest to days (46) .

The WHO classified laboratories according to their ability to use molecular methods and in AMR surveillance. These classifications are: a) Type 1, with no prior experience in AMR surveillance or molecular methods; b) Type 2, with prior experience in antimicrobial susceptibility testing (AST), but with little or no prior experience in the use of molecular methods (e.g. a newly established NRL); and c) Type 3, with extensive experience in both AST testing and applying molecular methods in AMR surveillance. The current situation in Malawi for most healthcare centers in the communities, which should be the first point of care, have either Type 1 or Type 2 AMR surveillance system(46,47).

Molecular assay advancements have led to the introduction of new techniques with increased sensitivity and specificity. These molecular approaches to detect and differentiate microorganisms, in contrast to conventional techniques, mostly rely on DNA amplification and are not affected by low microbial loads giving it an advantage over the traditional cultural method (47). Moreover, it is possible to apply molecular procedures to organisms that are slow growing in culture, an advancement which Malawi can possibly adopt to improve on AMR detection methods.

An illustration in **Figure 1.6** shows tests suitable for clinical settings which are marked with a staff symbol, and those suitable for surveillance are marked with a microbe symbol. Molecular tests increase in cost and complexity from left to right and from green to purple along the color gradient. An increasing number of elliptic orbits indicates the three categories of complexity. Overall cost categories (setup and test costs) are indicated by the dollar signs. In the **figure 1.6,** "LAB 1" refers to a laboratory with no previous experience in molecular testing, "LAB 2" to laboratories with some previous experience and "LAB 3" to laboratories with some previous experience.

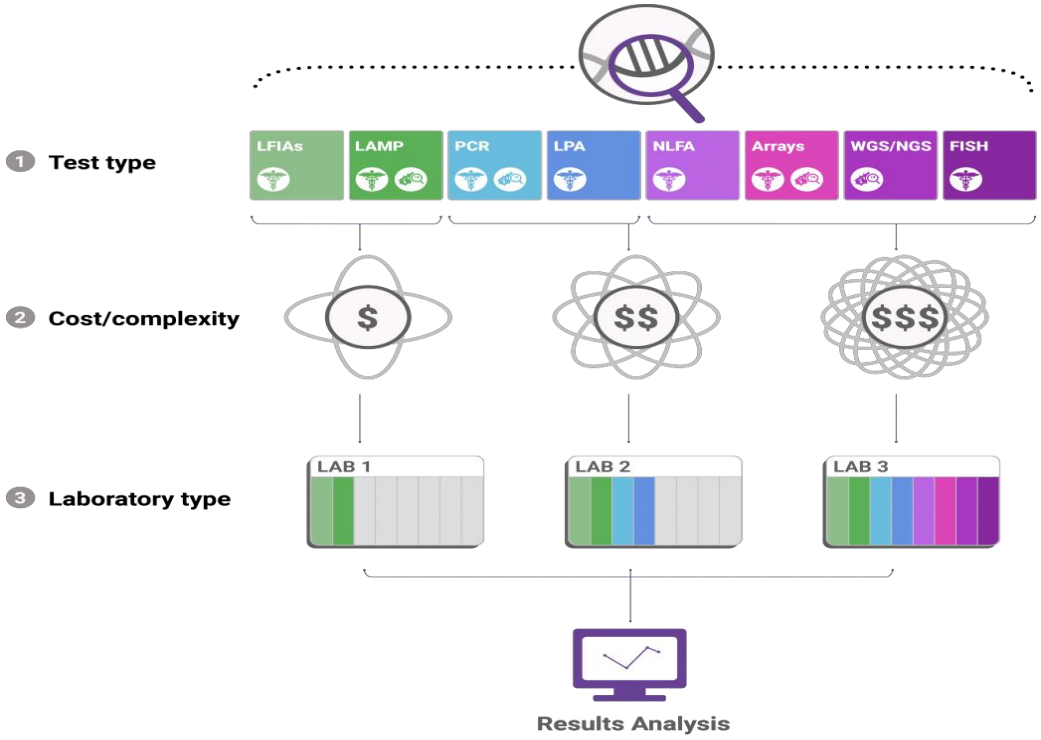

Figure 1.7: GLASS molecular diagnostics tests suitable for laboratories:
The above adopted figure from GLASS shows a clear example of molecular diagnostic test types suitable for laboratories with different capability in routine clinical use and surveillance. LFIA, lateral flow immunoassay; LAMP, loop-mediated isothermal amplification; PCR, polymerase chain reaction; LPA, line probe assay; WGS, whole-genome sequencing; NGS, next-generation sequencing; FISH, fluorescence in situ hybridization (47) .

1.5 Loop-Mediated Isothermal Amplification

Loop-mediated isothermal amplification (LAMP) was developed by Notomi et al. (2000) as a simple and rapid gene amplification technique. The principle of LAMP is based on auto-cycling strand displacement DNA synthesis facilitated by SPN (Streptococcus pneumoniae) DNA polymerase by using a set of four to six primers, including forward inner primer (FIP) and backward inner primer (BIP), outer primers (F3 and B3) and loop primers (LF and LB) the same principle used in this study (48). The loop primers help accelerate the amplification reaction by binding to additional sites that are not accessed by both inner primers (49). This principle has been used when designing the primers used in

this study, the parameters where used in the Premier Biosoft software to come up with the right primer combinations.

The forward inner primer anneals to initiate the first strand synthesis of the target sequence by the SPN DNA polymerase. The forward outer primer anneals to the complementary region F3c, outside of FIP, on the target sequence and displaces the synthesized first strand. The released strand forms a stem-loop structure at the 5′end because of the complementary sequence to the F1 region contained in the FIP primer. This released single strand serves as a template for BIP, similarly to FIP process the B3 primer anneals to its complementary region B3c. Starting from the 3′end of the BIP, synthesis of the complementary DNA starts and reverts the loop structure formed by F1 and F1c regions into a linear structure. A double stranded DNA is produced from the process previously described (48).

After dumbbell-like structure formation, a cyclic reaction is established between the dumbbell-like structure and its complementary product, employing inner primers. The product is made of different size structures consisting of alternately inverted repeats of the target sequence of the same strand (**Figure 2.4)**, producing a cauliflower-like structure(48). When amplification is achieved, LAMP generates large amounts of DNA strands that contain multiple copies of the target DNA. During this reaction magnesium pyrophosphate is produced as a by-product in the form of an insoluble white precipitate which becomes visible when the reaction is terminated (50).

1.6 Detection of LAMP Product

They are several ways of detecting LAMP products. In this study, the real time turbidity was used for conservation if a reaction has taken place or not.

1.6.1 Real-Time Turbidity and Visual observation

In Real-time turbidimetry detection of curves are observed when the pyrophosphate ions are released during the reaction and form a white magnesium phosphate precipitate that can be monitored using a real-time turbidimeter and results are displayed on the assays screen. Initially, turbidity of the final product was used to detect positive amplification of DNA (48,49,51).

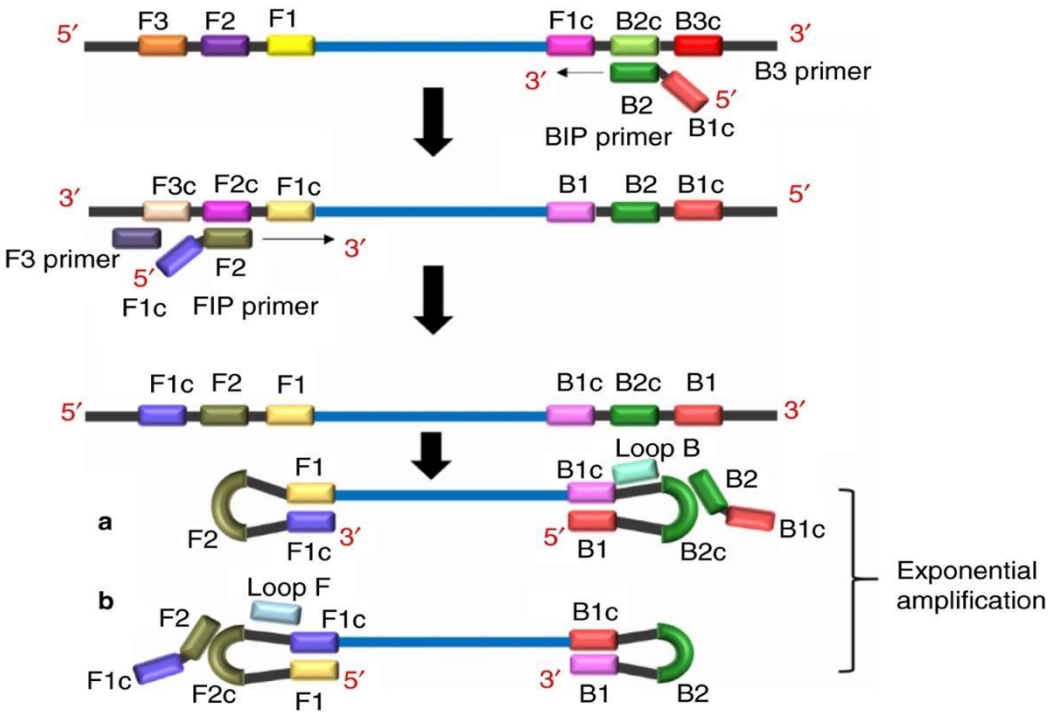

Figure 1.8 **Principle of LAMP method** The formation of the loop structure and dumbbell-like structure formation are formed as show above which leads to the exponential amplification of the DNA skipping the denaturing step which makes the LAMP process shorter compared to the PCR which required the denaturing step (48).

As mention in GLASS, LAMP is categories as a method with low cost of implementation and operation and can be used in a Type 1 lab, a setting mostly found in Malawi (46). LAMP exhibits high specificity and selectivity because of the use of 4 primers recognizing 6 distinct regions on the target base sequence, and can be completed in a short time (1 hour as standard) due to the high amplification efficiency under isothermal conditions without the thermal cycler used in PCR. The detection limit in LAMP is higher than that in conventional PCR, and the result is determined by visual observation (48).

Loop-mediated isothermal amplification (LAMP) rapidly amplifies target DNA with high specificity and efficiency under isothermal conditions over a range of 60-65°C, using a strand displacement reaction (48). The strand displacement reaction has the ability to displace downstream DNA produced during DNA synthesis (New England Biolabs). The method employs a DNA polymerase and a set of four to six specially designed primers that recognize a total of six distinct sites on the target DNA (48,49).

LAMP has shown accurate results in medical sciences for the detection of bacterial pathogens in specimens from humans and animals (52–54).

1.6.2 Advantages and Limitations of Lamp

In this study we consider the LAMP assay method because unlike other nucleic acid amplification techniques, LAMP has the advantage of greater simplicity with good amplification efficiency, which can be greatly suitable for the current setting of Type 1 labs in Malawi (55) . It also offers a lower cost in running the assay compared to the quantitative Polymerase chain reaction, at the same time it does not require a thermocycler and amplification can be readily achieved at a single temperature in a shorter amount of

time compared to PCR, which promises lesser time in detection of microorganism and potential for further implementation in a field setting (48,56). In addition, gel electrophoresis to visualize the amplified DNA is not required, for the LAMP amplification can be visualized using intercalator dyes including PicoGreen, SYBR Green I and ethidium bromide and metal (e.g. magnesium) indicator dyes such as Calcein and HNB (55) while in this study we use the metal (magnesium) indicator method to visualize detection.

Despite its simplicity the LAMP assay is less versatile than the PCR, the most well-established nucleic acid amplification technique. LAMP is useful primarily as a diagnostic or detection technique, but is not useful for cloning or many other molecular biology applications enabled by PCR. Because LAMP uses 4 (or 6) primers targeting 6 (or 8) regions within a fairly small segment of the genome, and because primer design is subject to numerous constraints, it is difficult to design primer sets for LAMP and Multiplexing approaches for LAMP are less developed than for PCR. Despite the addition of SYBR green dye to view LAMP in real-time, late amplification, primer-dimer amplification may contribute to a false positive signal. Although different mitigation strategies have been proposed for false-positive results in assays based on this method, nonspecific amplification due to various factors including the absence of temperature gating mechanisms is one of the major limitations of Loop-mediated isothermal amplification. LAMP is still at an experimental stage of implementation in Malawi. A few ongoing studies have been set up at the Queen Elizabeth Centre hospital in Blantyre, evaluating the feasibility of method, specificity and sensitivity of the assay on the detection of Group B Streptococcus. In the current study we tend to looking into the ability of the LAMP assay to correctly detect antimicrobial resistance only in the *S. Pneumoniae* organism

(specificity), and the ability for that assay to correct distinguish the positives from negatives (sensitivity).

1.7 Problem Statement

The development of resistance to first line antibiotics (penicillins) and the alternative readily available therapeutic options such as the macrolides emphasizes the urgency for continued surveillance of antimicrobial resistance in every part of the world. However, in low resource settings such as Malawi. The current methods for detection of antimicrobial resistance are expensive and not easily scalable. Also, the current antimicrobial sensitivity methods are lengthy and require a complex level of expertise. There is a need for a diagnostic method that cannot only give results in a reduced amount of time but also requires minimal expertise to operate. A new technique that can be implemented even in rural settings is required. Molecular methods for detection of *S. pneumoniae* resistance such as LAMP assay have been considered and may provide a solution that is cheap and easy to use in both clinical and public health settings.

1.8 Main Objective

A. Board Objective

To develop the loop-mediated isothermal amplification (LAMP) method for detection of macrolide resistance among pneumococcal isolates from NP swabs.

B. Specific Objectives

1. To design loop primers against *ermB* and *mefA* macrolide resistant genes.
2. To test designed primers against the macrolide resistant *Streptococcus pneumoniae* isolates.
3. To determine the specificity and sensitivity of detection of *ermB* and *mefA* primers in comparison to cultural methods.
4. To assess the detection time of the *ermB* and *mefA* primers macrolide resistant *Streptococcus pneumoniae* in comparison to cultural methods.

Chapter 2: Methodology

2.1 Study Design

A laboratory method development study was conducted to develop LAMP assay test for detection of SPN resistance genes on stored isolates obtained from a previous study. SPN sample were collected from otherwise healthy, HIV-uninfected, vaccinated children under the age of 5 who were sampled nasopharyngeal swabs (NPS). The samples were processed and recorded of their phenotypic resistant profile of azithromycin and erythromycin and the SPN isolates were stored in STGG (skim milk, glycerol, glucose and trytone soya broth) at -80 °C freezer for further investigation (**Figure 2.2**). The Isolates where sent to Oxford for Whole Genome Sequencing and the results were sent back to MLW which were kept in a laptop that was password protected (**Figure 2.3**). To further assist in specification and sensitivity other isolates from other microorganisms where used in this study, *Escherichia coli,* S. *Typhi, Staphylococcus aureus* and *Group B Streptococcus* (**Table 2.1**).

Table 2.1*These were taken from the MLW Core microbiology lab, they bought from Public Health of England., ^Previously confirmed GBS isolate from another study

Escherichia coli ATCC 35668	*Commercial.
S. *Typhi*	*Commercial.
Staphylococcus aureus NCTC 6571	*Commercial.
Group B Streptococcus	^In house positive.
Group B Streptococcus NCTC 8181	*Commercial.

2.2 Sample Size

2.2.1 Using the Quantitative research sampling size calculator[1] https://blog.

Using the PCVPA study data base a population size of 1,400 SPN isolates were randomly selected using STATA's gsample command. To determine sample size, we used parameters of a standard deviation of 0.5, margin error of 10% and confidence interval of 95%. These parameters were used in the online sample size calculator, giving us a sample size of 90. We used the gsample command to generate a list of 90 samples from the 1,400 to work with.

$$S = (z^2 (d(1 - d))/ e^2) / 1 + (z^2 (d(1 - d)) / e^2)$$

S *= sample size |* ***P*** *= population size |* ***z*** *= z-score |* ***e*** *= margin of error |* ***d*** *= standard deviation*

[1] **https://blog.flexmr.net/sample-size-calculator**

$S = (0.95^2 (0.5(1-0.5))/0.1^2) / 1+ (0.95^2 (0.5(1-0.5))/0.1^2)$ S= 90

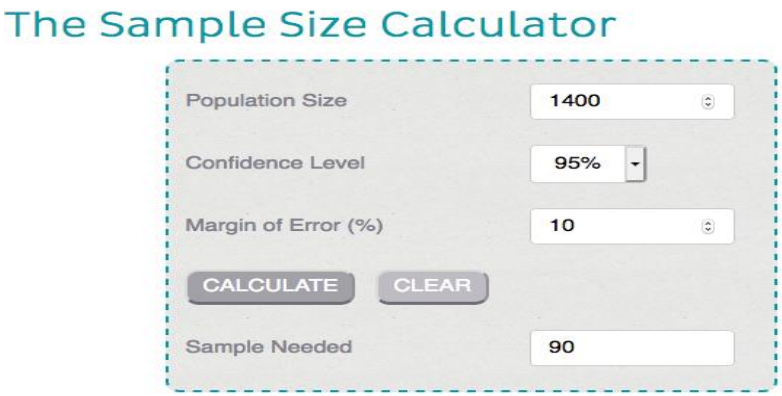

Figure 2.1 An illustration of how the sample size calculator looks when calculating the sample size needed [2] . A sample size 84 SPN bacterial isolates was obtained. Isolates were identified using laboratory ID number only and picked from the existing macrolide resistance profile in LIMS. The bacterial isolates were re-grown, DNA extracted and tested for using LAMP at the Pathology Department at the College of Medicine.

[2] https://blog.flexmr.net/sample-size-calculator.

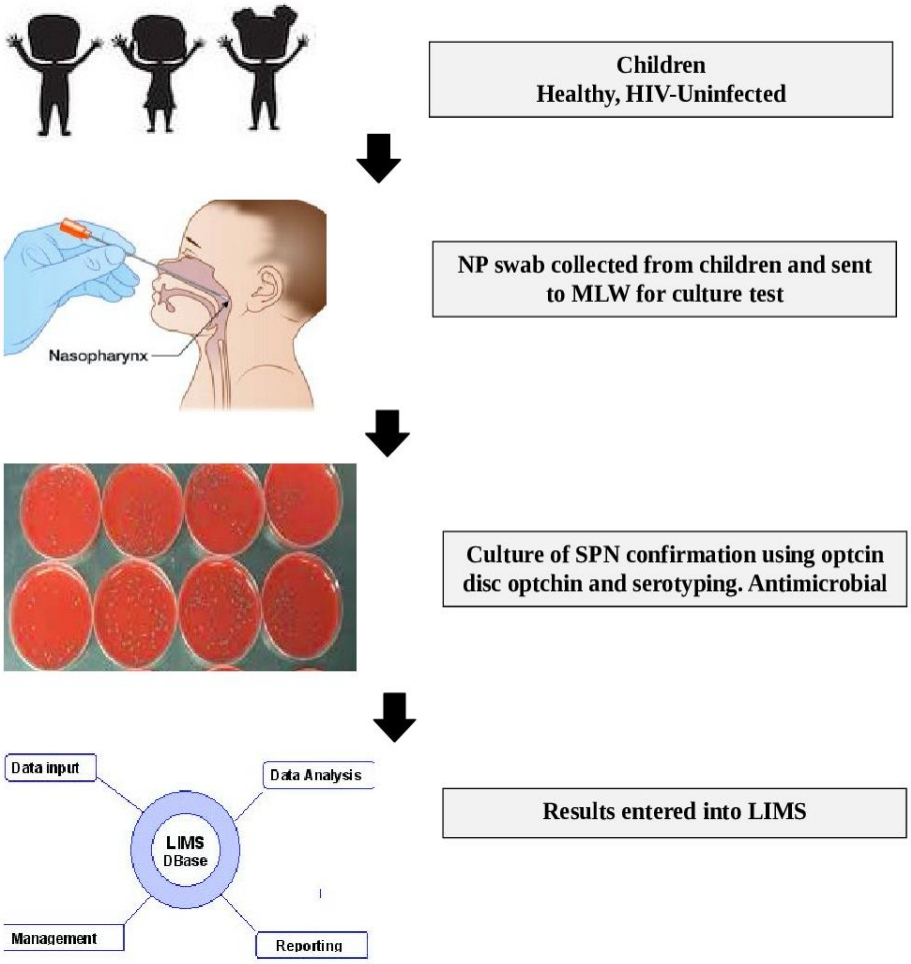

Figure 2.2: Sample Collection and processing. In the figure above the nasopharyngeal swabs collected from children in Blantyre in the comfort of their homes or schools and samples were sent to Malawi Liverpool Welcome Trust Laboratory for processing. Grown on selective media, sheep blood agar with gentamicin and an optochin disc to confirm for SPN and results recorded into LIMS, which were later extracted and used for the current study.

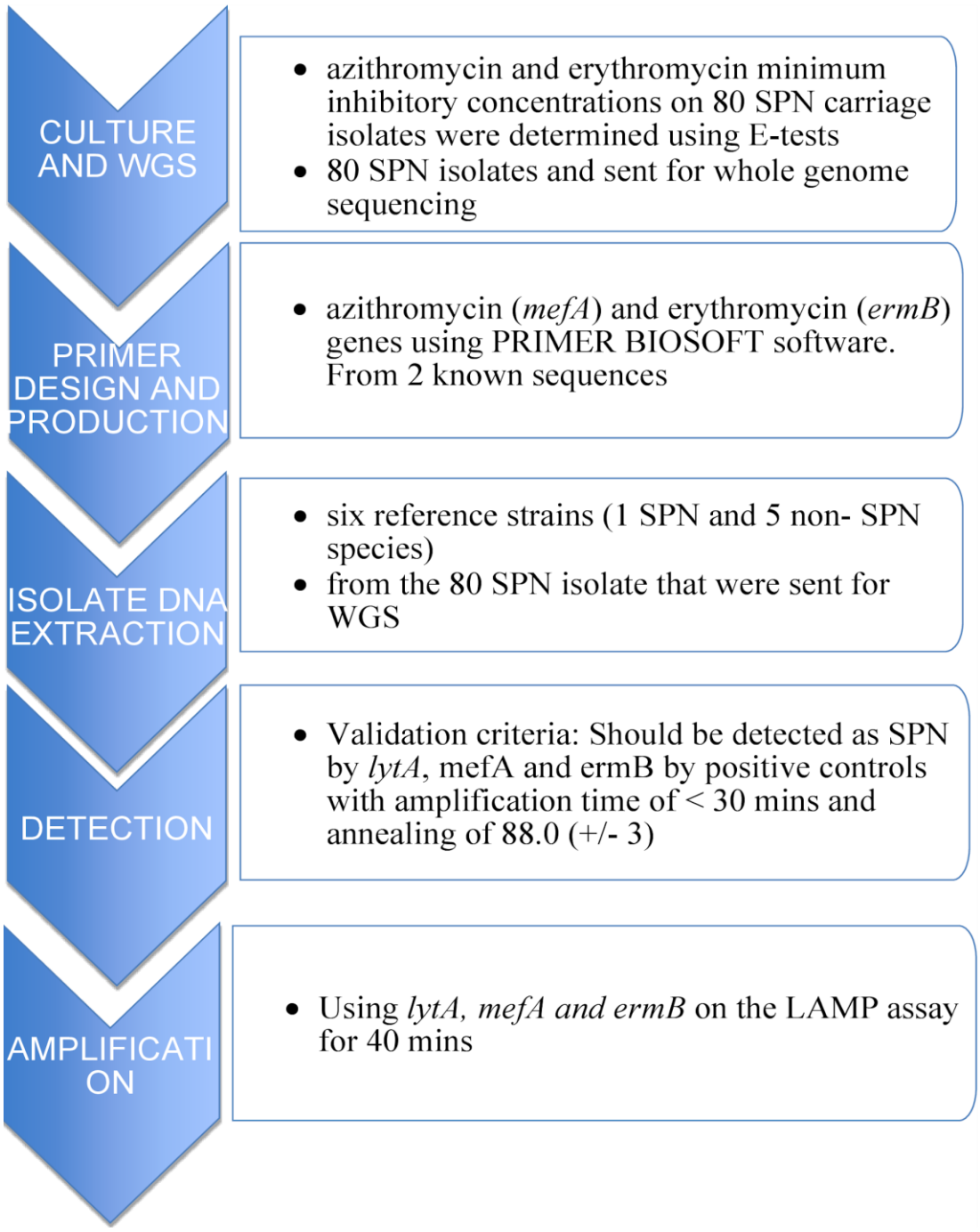

Figure 2.3: Project methodological approaches. In the figure above, the flow charts show the project methodological approaches, started from the cultural methods which took place at Malawi Liverpool Wellcome Trust and Whole genome sequencing done at Oxford University of the 80 *Streptococcus pneumoniae* samples. Primer designing took place at the College of Medicine which later led into isolate preparation and extraction of DNA and amplification of the DNA on the LAMP assay.

2.3 Properties and Design of Lamp Reagents

LAMP reagents vary in some aspects to one of the techniques more widely used, conventional PCR, for the detection of *S. Pneumoniae*. In this study for the detection of *S. pneumoniae* to confirm we are working with the right organism we adopted already designed primer set from work done by Kim and colleges from a paper published in 2012 (57). To design the macrolide resistant of *S. pneumoniae*, like in other studies we used the concept of six primers which provide a greater level of target specificity than the one achieved with two primers normally used in PCR (48) as well as the use of a DNA polymerase with strand displacement activity (Bst DNA polymerase) under isothermal conditions and lower temperature (e.g. 60-65°C). Bst DNA polymerase contains the $5' \rightarrow 3'$ polymerase activity, but lacks $5' \rightarrow 3'$ exonuclease activity (New England Biolabs, Beverly, MA). The enzyme has a heat-resistant property and a strand- displacement type DNA polymerase activity, which synthesizes a new DNA strand while separates the hydrogen bond of the double stranded template DNA. Since the strand- displacement DNA polymerase does not require dissociation of double-stranded DNA, the DNA can be synthesized at a constant temperature.

As seen in other studies the optimum temperature for SPN DNA polymerase ranges from 50-65°C, which facilitates primer annealing and enhance tolerance to inhibitors typically found in diagnostic samples; an advantage compared to other polymerases. However, inhibition above 65°C and inefficient incorporation of dNTPs has been reported (58,59), that is why in this study we opted to using 62°C for the runs as used in other studies to be the optimal temperature for the detection of SPN, of which the SPN primer used in this study was adopted from (Tanner, New England Biolabs, Inc.). The DNA polymerase is

supplied with Thermopol buffer (20 mM Tris-HCl, 10 mM (NH4)2SO4, 10 mM KCl, 2 mM MgSO4, 0.1% Triton®-X-100, pH 8.8 @ 25°C) that ensures polymerase activity. The concentration of dNTPs in the PCR reaction mixture also plays an important role in achieving the desired results. Slight deviation from the required concentration has led to dramatic increase of mis incorporation levels of dNTPs (60). In most of the publications about LAMP, dNTPs concentration is very high (1.4 mM) compared to conventional PCR (50).

The presence of magnesium source (MgSO4) in conventional PCR is required for polymerase activity, primer annealing, strand dissociation and product specificity. Previous reports on conventional PCR have demonstrated that optimum concentrations of MgSO4 should contain 0.5 to 2.5 mM over the total dNTPs concentration (Innis and Gelfand, 1990). Previous LAMP procedures concentrations up to 2.5 mM have been reported (61). In LAMP assays, magnesium concentration can also interfere with dye reactions (62). Previous studies have reported that the addition of enhancers such as Betaine improves yield and specificity in GC rich regions and decreases the production of undesired products (63–65) due to the DNA regions rich in GC concentration which sometimes can be problematic due to inefficient separation of DNA strands or the formation of secondary structures.

2.4 Sample Preparation and DNA Extraction

2.4.1 Isolate Preparation

Following WHO recommendations on *Streptococcus pneumoniae* sample processing, preparation and enrichment (pre-treatment) (66) on the first day SBG plates were brought to room temperature and were divided into two and labelled with study ID number.

Isolates were removed from -80°C freezers and placed in an ice bucket. Three beads were transferred onto SBG plates, optochin discs placed onto plates and plates incubated for 18-24 hours in CO^2 incubator at 35°C. Original isolates were returned to archive -80°C freezer.

On the second day, a second set of SBG plates were removed from the fridge and brought to room temperature. Plates were divided into 3 and labelled with study ID. The previous days SBG plates were removed from incubator, read and results recorded into study book. (Either confirmatory *Streptococcus pneumoniae* or not). Those with confirmatory *Streptococcus pneumoniae* were transferred onto the second SBG plate. With reference to sample data on resistance profile, an azithromycin or erythromycin disc was used and placed onto the plate corresponding to lab number. Plates were then placed into a CO^2 incubator for 18-24 hours at 35°C. On the third day, plates were removed from incubator, read and recorded. Confirmatory azithromycin or erythromycin resistance or not, with Zone of Inhibition. If the plate had resistance, growth was taken from the plate and stored unto cryobeads and Todd Hewit broth labelled tube with study ID and date. Isolates were then stored into -80 degrees Celsius for later use of extraction and running on the LAMP assay.

2.4.3 Pre-Treatment

To increase DNA concentration during the extraction procedure, the isolates went through pre-treatment stage due to SPN cell wall that makes it difficult to breakdown.

Inoculated Todd Hewit broth was removed from -80 °C freezer and placed on bench to thaw. 400 µl lysis solution was pre-aliquoted into labelled Eppendorf with study sample ID consisting of 10 U l −1 ACH in 10 mmol l −1 Tris/HCl pH 8.0 and 1 mmol l −1 EDTA (Sigma-Aldrich) and 30 µl of the TH broth was added into pre-liquated lysis solution (66). Eppendorf's were then incubated at 'room temperature' (22–24 °C) for 5 min before boiling at 95 °C for 5 min in a dedicated heat block to simultaneously deactivate lysis solution and denature any target DNA for LAMP analysis. Placing directly on ice to cool the samples, the lysed supernatant was stored at 4 °C.

2.4.4 DNA Extraction

DNA extraction was performed using a DNA extraction kit (Qiagen ltd UK, www.qiagen.com), based on the manufacturer's instructions (**Figure 2.5**).

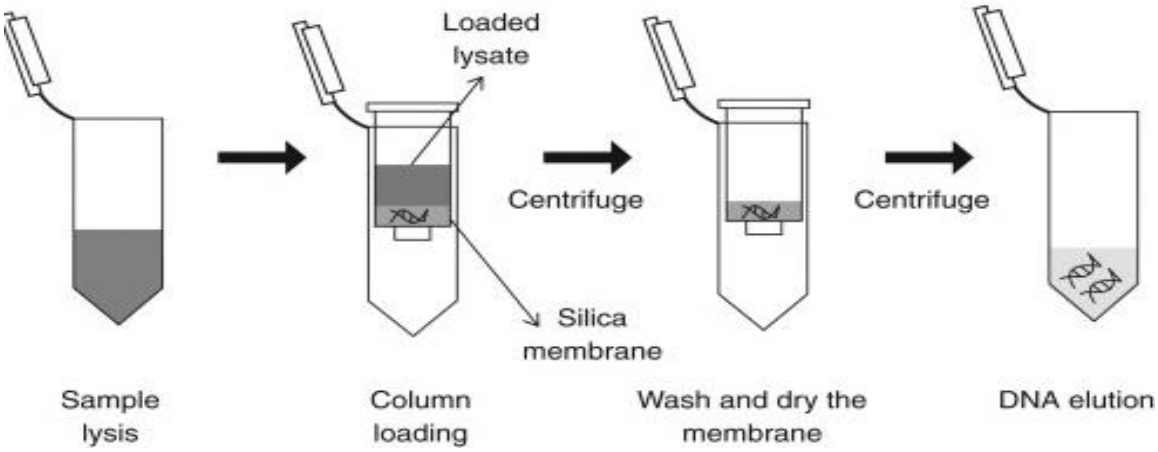

Figure 2.4: DNA extraction. Showing the membrane method of DNA extraction which is widely used in most laboratories. A NP swab sample is lysed and filtered through a membrane which is washed and DNA elute is collected**.** Confirmed SPN plate was sweeped with a loop and growth is transfer to a 1.5ul eppendoff for enzymic lyse stage. The lyste is then extracted by membrane method giving a DNA elusion at the end which is use in the LAMP assay for DNA detection.

2.5 LAMP Reaction

The overview of flow of the setup of the LAMP assay starting with the extracted DNA is illustrated in the **Figure 2.6** while the LAMP reaction mixture was prepared using a total volume of 25 µl (**Table2.1**) containing 2.5 µL of 10X Bsm Buffer, 20 mM Tris-HCl (pH 8.8 at 25°C), 10 mM KCl, 10 mM (NH4)2SO4, 5 mM MgSO4, 0.1% Tween 20 (Thermol Scientific-Fermentas, Vilnius, Lithuania), and 2.5 µL of DNA template. Using the Gene II LAMP machine, the profile was set to 62°C amplification for 40 minutes and 80 -93 °C of annealing 0.05C/s.

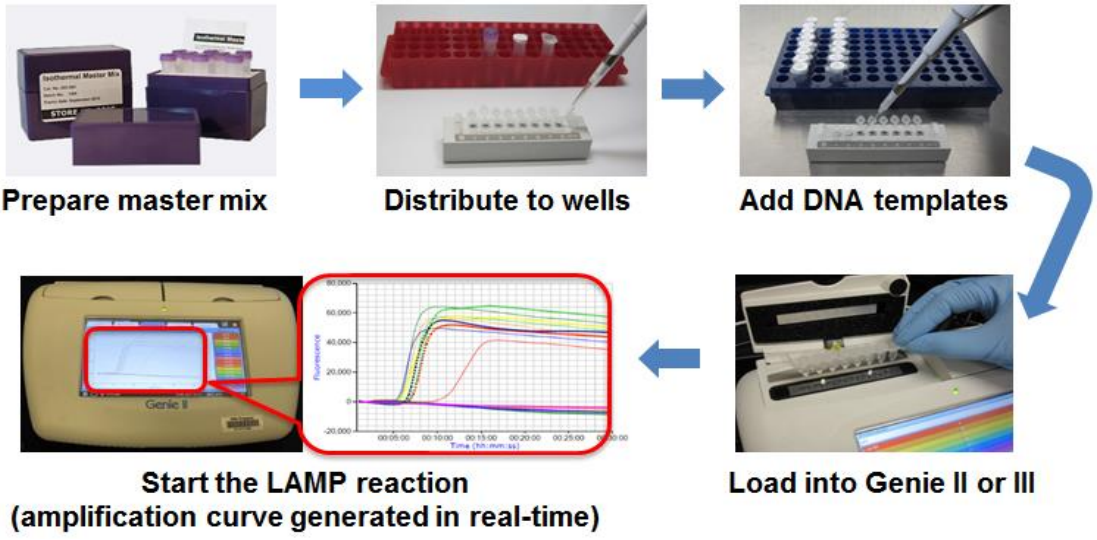

Figure 2.5: LAMP assay bench procedure. Where the master mix that contains the primers and DNA elute are pipetted into wells (specific eppendoffs) and then loaded into the LAMP machine which has already been set to the right parameters. The final elution has a combination of the master mix and the extracted DNA which is loaded into the LAMP assay and the assay gives amplification curves in real time. The results are later on recorded and analyzed in excel.

Table 2.2: **Components of LAMP reaction mixture**
The LAMP assay requires 2000 microliters of the master mix, 250 microliters of primer mix and 2.5 microliters from each DNA template for the procedure.

Component	**Working concentration**	**Final concentration**	**Volume per sample**	**Volume in Master mix for 100 samples**
ISO- DR001 isothermal master mix	N/A	1x	20ul	2000
Primer mix	100uM	F3- 2uM B3- 2uM FIP- 8uM BIP- 8uM LF- 4uM LB- 4uM	2.5ul	250
DNA template	N/A	N/A	2.5ul	N/A

2.6 Data Collection and Analysis

Quantitative data was collected from the LAMP assay i.e. amplification time, detection of presence of resistance which were used.

To determine the relation between the results from the data to the results generated from the LAMP assay, a chi-square test was used. Comparing the culture results to the LAMP output, using EXCEL and SPSS software (IBM SPSS Statistic 22) to compliment and review the results and to determine the specificity and sensitivity of the LAMP assay. In both platforms the chisq option is used on the statistics subcommand of the crosstabs command to obtain the test statistic and its associated p-value (significance).

2.7 Ethics and Limitations

Ethical clearance was sought from College of Medicine Research Ethics Committee (COMREC) under approval P.01/19/2568. Letters of support and/or permission were sort from Head of Pathology Department at College of Medicine and from the mother study PCVPA COMREC approval number P.02/15/1677. All isolates used in this study had an identification number, no names of patients were used, and all data is password-protected.

Chapter 3: Results

3.1 LAMP Primer Design and Parameters

As described in chapter two the first objective of the study was to be achieved by designing LAMP primers based on two whole genome sequences of *S. pneumoniae* isolated from colonized, otherwise healthy children in the community. A set of six primers targeting *ermB* gene (**Fig 3.1**) encoding resistance to erythromycin and another set of six primers targeting *mefA* (**Fig3.2**) encoding resistance to azithromycin were generated. The melting temperature of the primers ranged from 64 to 66ºC; GC content ranged from 47 to 65%; primer length ranged from 18 to 25 bases (**Table 3.2**). The primer sequences were checked for complementarity of start and end of sequence to avoid formation of primer dimers during LAMP assay amplification. As shown in **Table 3.2**, none of the designed primers had potential for formation of dimers. All other primer parameters suitable for optimal target amplification for the designed primers are shown in the spreadsheet in **Appendix Table 5.2**.

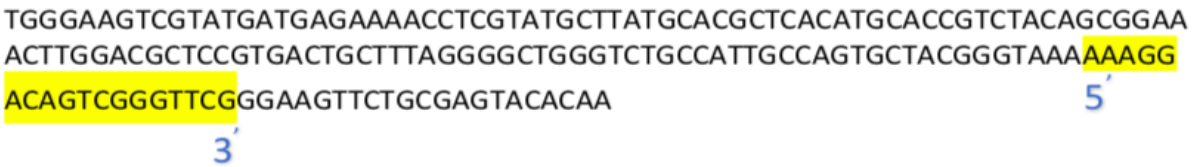

Figure 3.1 Best product amplicon of *ermB* gene. Above the highlighted Loop primer section used to design the primers from sequence definition WTCHG_585651_257101.fasta with sequence length of 2,087,488. The amplicon has a length of 167, Tm (Temperature for free energy calculation) of 73.8, GC% of 52.7 and Rating of 89.4.

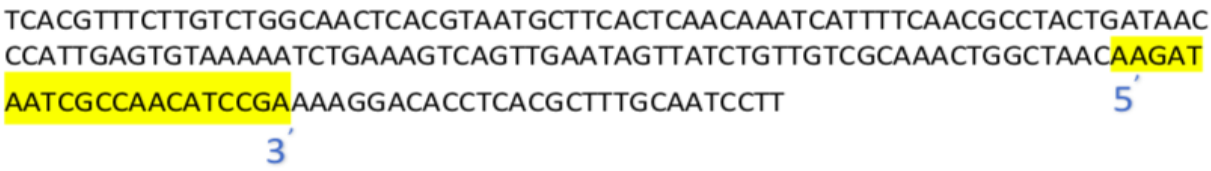

Figure 3.2 Best product amplicon of *mefA* gene. Above the highlighted Loop primer section used to design the primers from sequence definition WTCHG_585651_252148.fasta with sequence length 2,197,469. The amplicon has a length of 180, Tm of 69.1, GC% of 40.6 and Rating of 86.3.

Table 3.1 Primer sets of *lytA*, *ermB* and *mefA*. For a normal sequence (45 %< GC<60%) entered as a default setting. the target sequences are AT rich (GC content < 45%) or GC rich sequences (GC content > 60%), then the primers are designed with the Tm, Length, and GC content set as FIP and BIP (Forward
Inner Primer and Backward Inner Primer), F3 and B3 (Forward outer primer and Backward outer primer) and; LF and LB (Loop Forward primer and Loop Backward primer) generated from PrimerBiosoft.

Name		Sequence (5' to 3')	Tm	GC%	Length
lytA					
FIP		CCGCCAGTGATAATCCGCTTCACACTCAACTGGGAATCCG C	-	-	-
BIP		TCTCGCACATTGTTGGGAACGGCCAGGCACCATTATCAAC AGG	-	-	-
F3		GCGTGCAACC ATATAGGCAA	-	-	-
B3		AGCATTCCAA CCGCC	-	-	-
LP		TGCATCATGCAGGTAGGA	-	-	-
ermB					
FIP (F1c + F2)	TGGGAAGTCGTATG ATGAGA	GCTGTAGACGGTGCATGTGATGGGAAGTCGTATGATGAGA	60-64	45-55	40
	GCTGTAGACGGTGC ATGTGA				
BIP (B1c + B2)	TTGTGTACTCGCAG AACTTC	GTCTGCCATTGCCAGTGCTATTGTGTACTCGCAGAACTTC	60-65	45-55	40
	GTCTGCCATTGCCAG TGCTA				
F3		AGCTGGGCGATTTAATGAG	60	47.4	19
B3		AGCTACTGTACCGATGGTT	60.1	47.4	19
LF		GCGTGCATAAGCATACGAG	61.5	52.6	19
LB		AAAGGACAGTCGGGTTCG	61.7	55.6	18
mefA					
FIP (F1c+F2)	TGGGTTATCAGTAGG CGTTGAA	TGGGTTATCAGTAGGCGTTGAATCACGTTTCTTGTCTGGC	60-63.8	45.5-50	40
	TCACGTTTCTTGTCT GGC				
BIP (B1c+B2)	TCTGTTGTCGCAAA CTGGCT	TCTGTTGTCGCAAACTGGCTAAGGATTGCAAAGCGTGA	59.9-65	45.5-50	38
	AAGGATTGCAAAGC GTGA				
F3		CTGAATCGCTCATGTCCATAA	60.2	42.9	21
B3		GTGCTATGGAAGACGAGC	60	55.6	18
LF		TGAGTGAAGCATTACGTGAGT	61.7	42.9	21
LB		AAGATAATCGCCAACATCCGA	62	42.9	21

3.1.1 Designed Primer Set Specificity in Online Microbial Sequence Databases

In silico specificity of the primers were determined by searching the primers against nucleotide sequences in GenBank. These sequences in the database included *S. pneumoniae* with and without *ermB* or *mefA* and other also sequences from other loci in *S. pneumoniae* genomes. In silico specificity was also determined against other microorganisms other than pneumococcus. All the designed primers were highly specific for *ermB* and *mefA* targets. The predictive value of the primer sets correctly identifying the resistance gene sequences (*mefA* and *ermB*) ranged from a minimum of 97.9% against a number varied microbial sequences in the database.

3.1.2 Optimized Primer Conditions Determined for Target Amplification

As described in methodology chapter two section 2.8, concentrations of the newly designed primers for target amplifications were investigated using the DNA from two *S. pneumoniae* whose sequences were the templates for primer design. All the primers sets were able to amplify the target on LAMP at a final working concentration of 2uM and amplification temperature of 62C, within a maximum duration of 40 minutes set on the LAMP machine. Annealing temperature ranged from 84 to 90^{o}C.

To confirm we were working with *S. pneumoniae* isolates the *lytA* primers where tested against all 84 isolates, which gave us a 100% detection of the isolates conforming the identification of *S. pneumoniae.*

Then we tested for *mefA* detection, running the primer against the referral isolate *mefA_55* that used to design the primer that was our positive control and *mef_53* with known azithromycin resistance and NEG being a nuclease free water. Under 14 minutes that

machine showed amplification of the positive control and 10 minutes later amplification of *mef_53* (**Figure3.3**). As for the NEG, n amplification was seen until later after 60 minutes, which is a true negative as the cut-off point of the assay design was 30 minutes. This clearly gave us results that shows that the primers design of *mefA* were able to detect the presence of azithromycin resistance.

As for the primer design of *ermB*, we replicated the procedure and tested the *ermB* primers against the erythromycin refer gene *ermB_88* which was our positive control, an isolate *ermB_65* and nuclease free water as our negative. Amplification results of the positive control were seen at 12 minutes followed by *ermB_66* 5 minutes later, with no detection of the NEG (**Figure 3.4**). In this case results clearly showing the amplification and detection of erythromycin resistance by the designed primers of *ermB*.

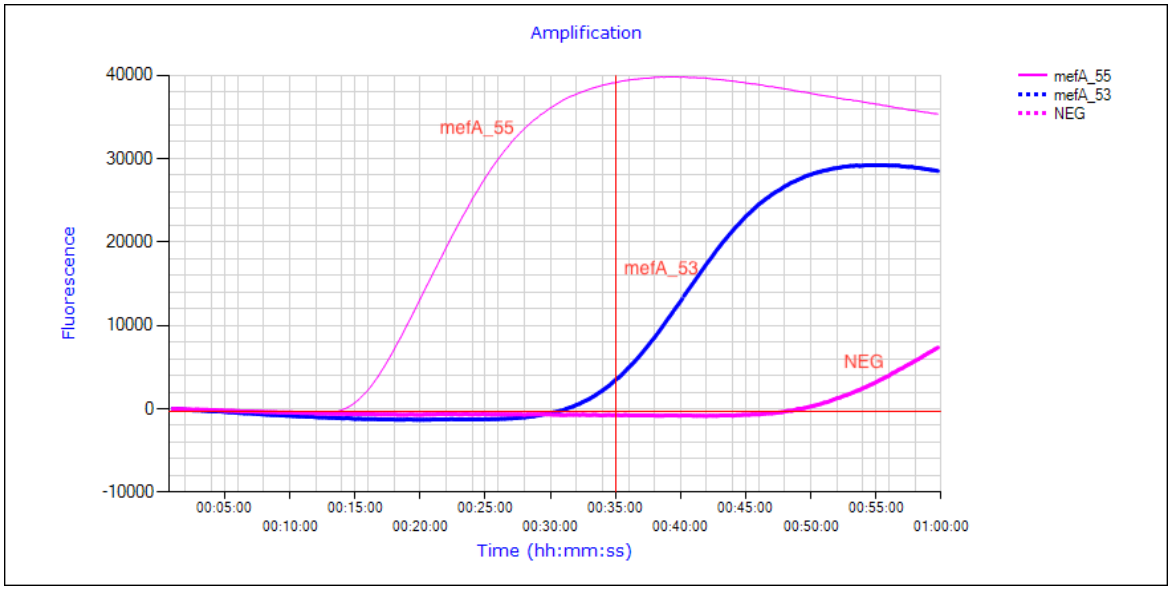

Figure 3.3 The amplification Tab of *mefA* detection.
Run 3.9 of *mefA* and *ermB* primers where the positive control *mefA55* real time turbidity curve peaks at 14 minutes, before the sample *mef53* and NEG control.

3.2 Duration of Detection of Observed Target Amplification in the LAMP Assay

As stated in chapter two section 2.6, DNA was extracted from 79 S. pneumoniae strains, two Group B Streptococci strains, one *Salmonella Typhi*, and one *Escherichia coli strain*. The extracted DNA was run on the LAMP assay run using the designed primers sets and the optimized run conditions. Out of the 79 *S. pneumoniae* strains 15 had *ermB* gene only, 29 had *mefA* gene and 5 had both *mefA* and *ermB* genes, as confirmed on whole genome sequencing that was conducted as part of mentioned pneumococcal carriage study in Blantyre, COMREC number P.02/15/1677. The remaining 30 *S. pneumoniae* strains did not have either *mefA* or *ermB* genes, as also confirmed by whole genome sequencing from the aforementioned carriage study.

The LAMP assay detected the *mefA* and *ermB* genes in all the strains known to harbor the gene as determined from the available whole genome DNA sequences. However, the duration of detection among the *S. pneumoniae* strains was varied. The minimum duration time for detection of *mefA* was 8 minutes and the maximum 19 minutes. For the *ermB* resistance gene, the minimum time to observed amplification in the LAMP assay was 11 minutes and the maximum was 40 minutes (**Fig 3.5**). Amplification of the target sequence was not observed for the 30 strains that were known not to harbor either *mefA* or *ermB* resistance genes. As illustrated in **Figure 3.6** below amplification was not observed in all non-S. pneumoniae bacterial strains depicting specificity.

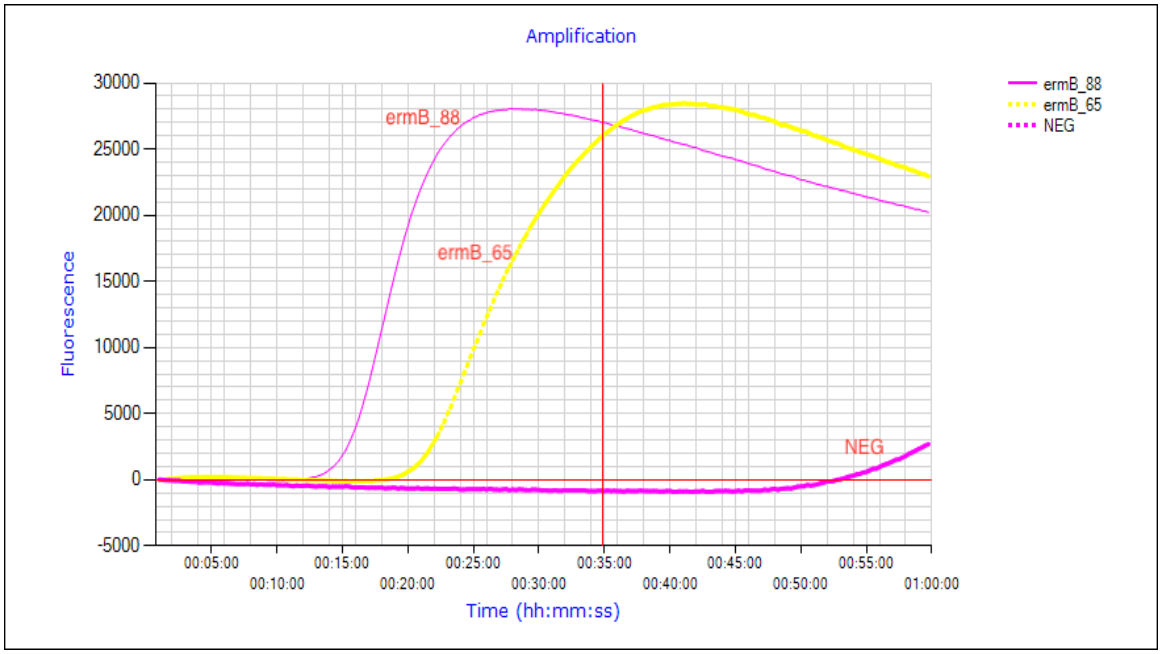

Figure 3.4 The amplification of *ermB* detection.
Run 3.4 of *mefA* and *ermB* primers where the positive control *ermB88* real time turbidity curve peaks at 12 minutes, before the sample erm*B65* and NEG control.

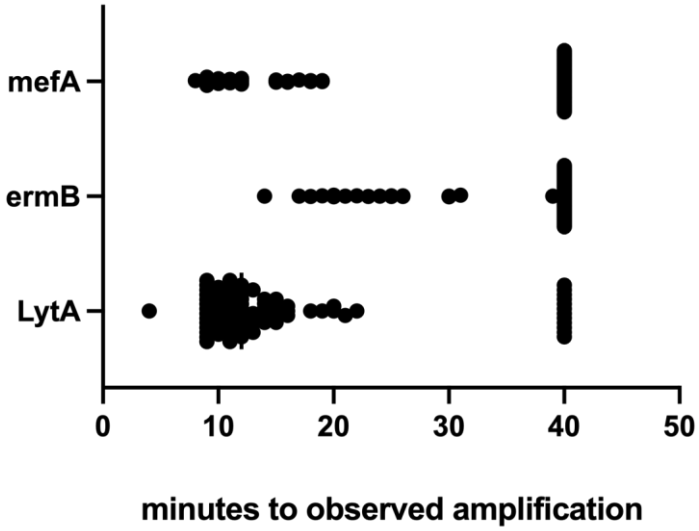

Figure 3.5 Duration of detection of *lytA*, *ermB* and *mefA*. The detection of isolates by the *lytA* primers happens as early as 5 minutes into the experiment, as seen in the scatter graph the *lytA* detections are seen closely together showing the effectives of the primers. While for the *ermB* primers detection occurrence is spread out until 40 minutes, this can show that the primers have a decreased sensitivity. Lastly for the *mefA* primers the detection is similar to that of *lytA* where the isolates are clustered and detection is happening as early as 8 minutes into the experiment, showing a higher sensitivity than the *ermB* primers.

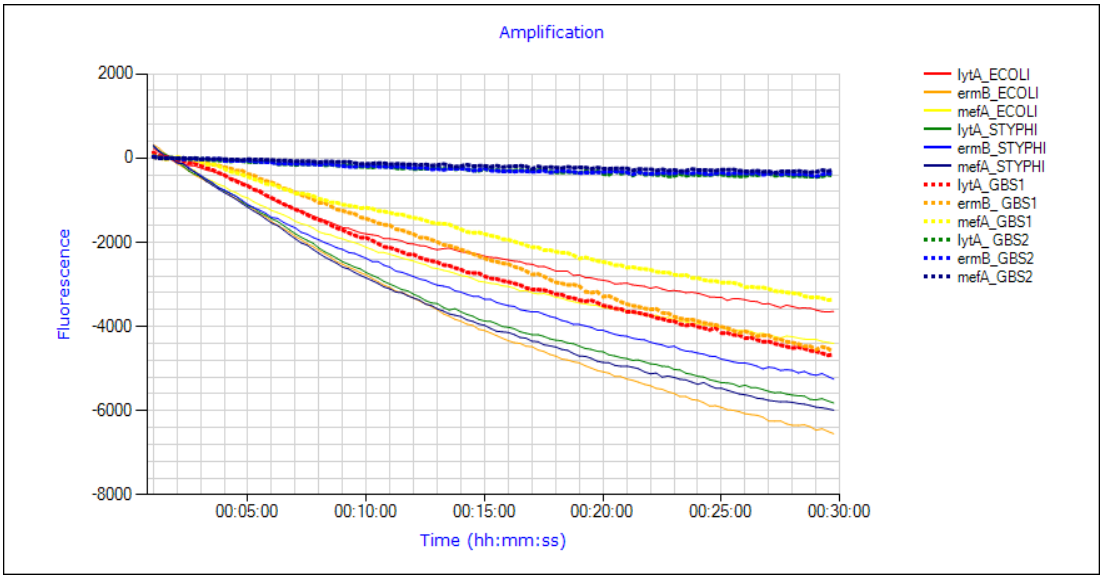

Figure 3.6 Detection specificity of LAMP primers non-SPN. The LAMP assay run and gave results of testing of the *lytA* primers on non SPN species. *Escherichia coli (*ECOLI), *Staphylococcus aureus (*NS), S. *Typhi (*STYPHI) and *Group B Streptococcus* (GBS) which showed no amplification.

3.3 Confirmation of Erythromycin and Azithromycin Resistance or Susceptibility: Culture Vs Whole Genome Sequencing Vs LAMP Assay

As stated in chapter two, section 2.5, a previously published pan *S. pneumoniae* primer *lytA* was used to verify the bacteria under investigation to be *S. pneumoniae*. DNA templates from the 79 bacteria labelled as *S. pneumoniae* were subjected to a LAMP assay run using *lytA* primer and amplification was observed in all the samples confirming that the bacterial strains under investigation are truly *S. pneumoniae*. There was no amplification for *S. Typhi*, *Group B Streptococci*, *E. coli* and *Staphylococcus spp* DNA templates.

All 79 confirmed S. pneumoniae strains and the two group B streptococci strains were cultured and assessed for sensitivity to erythromycin and azithromycin. Expression of *mefA* gene encoding azithromycin resistance and expression of *ermB* gene encoding erythromycin resistance was confirmed by culture in the 15 strains with *ermB* gene only, 29 strains with *mefA* gene only and 5 strains with both *ermB* and *mefA* gene. The remaining 30 *S.pneumoniae* without *ermB* or *mefA* gene were susceptible to both erythromycin and

azithromycin. The two *Group B streptococci* strains were also susceptible to both erythromycin and azithromycin (**Appendix Table 5.1**)

3.4 Specificity and Sensitivity of the LAMP Assay and Cut-Off

As described in section 3.1.2 above late amplification of *mefA* gene was observed at approximately 19 minutes, for *ermB* gene at 40 minutes in the LAMP assay runs that were conducted. A cut-off point to call a positive result for both primer sets designed in this study was therefore set at 30 minutes, before which a sigmoid curve of amplification has been observed. Sensitivity and specificity of the designed LAMP assay were calculated using the Receiver operating characteristic curve (ROC) and area under the curve was equal to 1.0 depicting excellent discrimination. (**Figure 3.8A and 3.8B**). Using the ROC curve sensitivity and specificity for the respective primer sets was maximized at a cut-off of 30 minutes LAMP amplification duration (**Figure 3.7**) for both *mefA* and *ermB* gene targets (*mefA*: sensitivity = 97.1% and specificity = 100%, at 95% confidence interval 0.952 to 1.000; *ermB*: sensitivity = 97.1% and specificity = 95.8%, at 95% confidence interval 0.452 to 0.701) (**Table 3.3 and 3.4**)

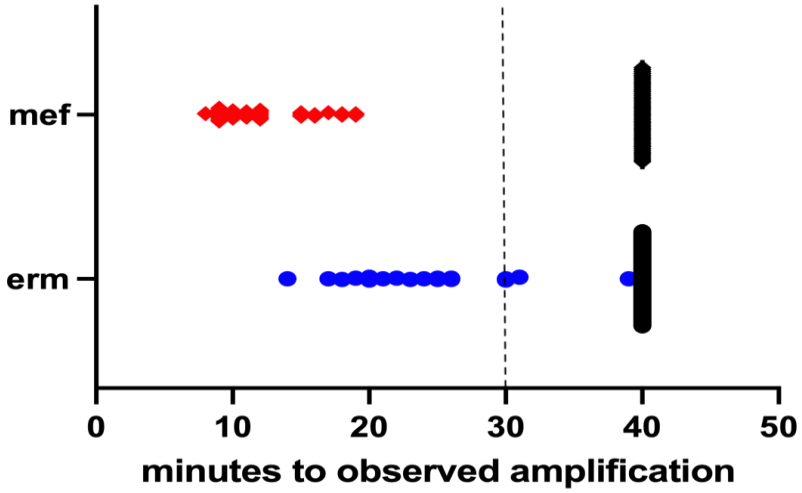

Figure 3.7 Determination of Cut-off point. The cut-off point was determined to be at the 30 minutes to accommodate most of the detection that has occurred giving off the most sensitivity and specificity.

A. *mefA* primers **B. *ermB* primers**

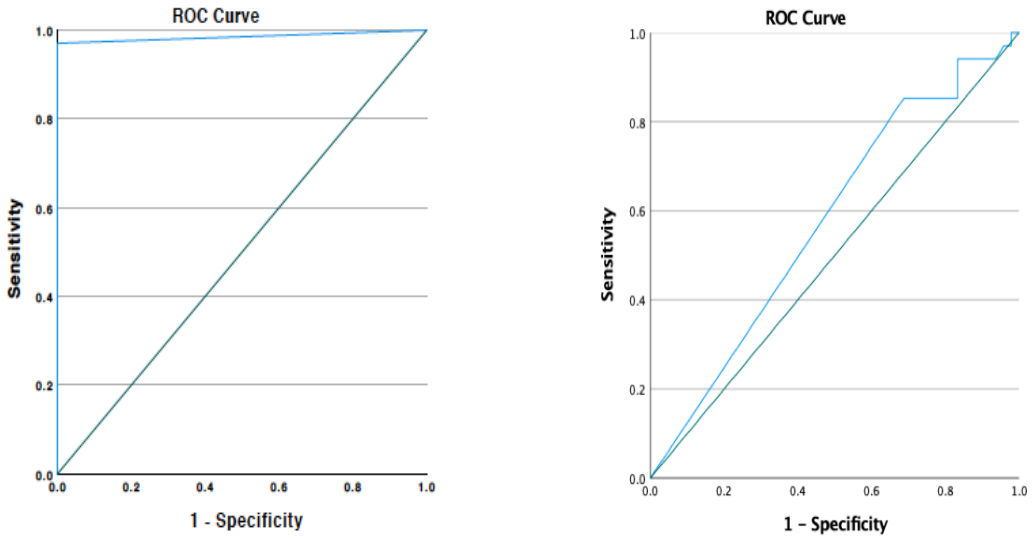

Figure 3.8 (A and B) Receiver operating characteristic curve (ROC) for *mefA* and *ermB*. The area under the curve for *mefA* was 0.98 and *ermB* was 0.56. The Area under the curve value lies between 0.5 to 1 where 0.5 denotes for a bad classifier and 1 denotes an excellent classifier. As for the primers in this study *mefA* if an excellent classifier and *ermB* is a good classifier but requires further testing to improve.

Table 3.2 A and B: Culture vs LAMP for the Detection of Streptococcus Pneumoniae Using the *ermB* Primer

The run had 15/20 true positives, no false positives and 65 true negatives which gave a sensitivity of 97.1 % and Specificity of 95.8% at 30.50 minutes, with confidence interval of 45-70. The area under the graph was 0.57 which is close to denoting the primers to be a bad classifier despite the assay being able to detect the resistance past the 30-minute mark and gave false negative. The spreading out of detection further away from the ideal 30 minutes is a concern of the primers being specific to the target and a question of developing of primer dimers far along into the experiment.

A.

Coordinates of the Curve

Test Result Variable(s): ermB

Positive if Less Than or Equal To[a]	Sensitivity	1 – Specificity
-1.00	.000	.000
7.00	.853	.688
15.50	.853	.708
17.50	.853	.729
18.50	.853	.750
19.50	.853	.771
20.50	.853	.833
21.50	.882	.833
22.50	.912	.833
23.50	.941	.833
24.50	.941	.854
25.50	.941	.896
28.00	.941	.938
30.50	.971	.958
35.00	.971	.979
39.50	1.000	.979
41.00	1.000	1.000

B.

Area Under the Curve

Test Result Variable(s): ermB

Area	Std. Error[a]	Asymptotic Sig.[b]	Asymptotic 95% Confidence Interval	
			Lower Bound	Upper Bound
.577	.064	.239	.452	.701

Table 3.3 A and B: Culture vs LAMP for the Detection of Streptococcus Pneumoniae Using the *mefA* Primer

The run had 43/48 true positives with no false positives, no false positives with 32 true negatives which gave a sensitivity of 97 % and Specificity of 100% in 29.50 minutes with a confidence interval of 95 to 100. In this run gave true positive in less than 30 minutes and the area under the curve is 0.985 which shows these primers are an excellent classifier.

A.

Coordinates of the Curve

Test Result Variable(s): mefA_LAMPmin

Positive if Less Than or Equal To[a]	Sensitivity	1 – Specificity
7.00	.000	.000
8.50	.029	.000
9.50	.235	.000
10.50	.382	.000
11.50	.500	.000
13.50	.676	.000
15.50	.765	.000
16.50	.824	.000
17.50	.853	.000
18.50	.912	.000
29.50	.971	.000
41.00	1.000	1.000

B.

Area Under the Curve

Test Result Variable(s): mefA_LAMPmin

Area	Std. Error[a]	Asymptotic Sig.[b]	Asymptotic 95% Confidence Interval	
			Lower Bound	Upper Bound
.985	.017	.000	.952	1.000

Chapter 4: DISCUSSION

4.1 Introduction

Streptococcus pneumoniae remains a major cause of morbidity and mortality in Malawi and sub-Saharan Africa (10). Management of pneumococcal infections is hampered by emergence of multidrug resistance and scarcity of diagnostic laboratories necessary to confirm clinical diagnosis. Previously, the susceptibility of *S. pneumoniae* to the commonly available penicillin was highly predictable but resistance has emerged even to alternative classes of antimicrobials Laboratory (47) facilities to monitor antimicrobial resistance is therefore a high priority to inform antimicrobial stewardship strategies. However, laboratory facilities are not always available in low resource settings, and in places where laboratory facilities are available the techniques employed to grow the organisms and assess antimicrobial susceptibility are expensive and turn-around for results is long (47). This study has developed loop mediated isothermal amplification (LAMP) primer sets for the identification of macrolide resistance. This study also demonstrates that the developed LAMP assay produces results of Malawian *S. pneumoniae* susceptibility and resistance to macrolides within a short period compared to culture methods. The LAMP assay primers detecting erythromycin and azithromycin resistance genes showed similar sensitivity and specificity comparable to phenotypic expression of resistance using the culture method. This chapter discusses the processes of generating the primers and outcomes of the evaluation that was done on the selected *S. pneumoniae* isolated in carriage in asymptomatic Malawian children.

4.2 LAMP Primer Design

LAMP primers are more complex than PCR primers to design, requiring six primers for a single target gene (48). The six primers allowed for increased sensitivity compared to using other molecular tests such as PCR which utilizes only two primers (57). In this study the primer sets were designed using Premier Biosoft software which simplified the designing process. There are other online primer designing tools such as the most widely used Primer-Explorer tool which is free but does not design loop primers concurrently, requiring second serial primer design execution (47). The primer designer used in this study generated loop primers concurrently and allowed for automatic interpretation of primer blast search results as mentioned by Notomi and colleges in their study. There was no need for exploring other primer design tools because Premier Biosoft provided a set of primers that amplified the targets in control strains on first attempt during the optimization process. The output from Premier Biosoft included five sets of six primers providing a choice for the primer set with the best parameters. However, if the financial and time resources were available it would have been stringent to test all five primer sets on actual LAMP runs to choose the best option, based on real time performance.

4.3 Detection of Antimicrobial Resistance

The two generated primer sets respectively amplified macrolide resistance genes *mefA* and *ermB*, each encoding azithromycin and erythromycin resistance. The performance was comparable between the two primer sets in terms of sensitivity and specificity. However, the range of detection was wider for the erythromycin (*ermB*) resistance gene than for the azithromycin resistance gene (*mefA*). Consequently, sensitivity of *mefA* primers was determined to be higher than for *ermB* primers. This was not seen in other studies done by Seki et al. and Nagamine et al. It should be noted that the same DNA template, hence

concentration, and isothermal cycling conditions were used in the study for both *mefA* and *ermB* primers. Based on the performance of the primers in the LAMP assay it will be necessary to conduct further optimization of the *ermB* primers to identify a suitable temperature for amplification to increase the sensitivity of the LAMP assay for erythromycin resistance.

Although there were differences in the sensitivity of the macrolide resistance primer sets both *mefA* and *ermB* genes were highly specific for the targets of amplification. None of the primer sets amplified DNA of any of the non-Streptococcus pneumoniae bacteria, indicating that it is possible to use these primers directly in a clinical specimen which will likely be contaminated with other bacterial genera, and still identify the intended target.

4.4 Culture Vs LAMP Assay (Specificity and Sensitivity)

Both culture and LAMP assays detected erythromycin and azithromycin resistance. The difference is that culture detected phenotypic expression which is useful in management of a patient and LAMP assay detected presence of the genes of resistance. Application of the developed LAMP assay on Clinical specimens would be appropriate, because the primers were designed to overlap *S. pneumoniae* core DNA sequence and the genes of macrolide resistance sequence. Therefore, the chances that the primers are detecting *mefA* and *ermB* primers in other bacterial species are minimized. The advantage of the developed LAMP assay over culture was the reduced time of detection within 30 minutes. Culture is a longer process of detection taking at least 24 hours when sub culturing from an isolate from storage (47). If culture is used right from the clinical specimen, then the time to detection of antimicrobial resistance increases to at least four days. Using the ROC curve analysis

on the LAMP assay developed in this study a cut-off that maximized both sensitivity and specificity of the primer sets was set at 30 minutes, for both *ermB* and *mefA* primers.

Chapter 5: Conclusion and Recommendations

The aim of this study was to develop LAMP primers for the detection of azithromycin and erythromycin resistance in *S. pneumoniae* and this was achieved. The primers were able to detect resistance in all isolates in less than 30 minutes except for 3 isolates where the erythromycin resistance gene *ermB* was not amplified. Despite the three false negative results observed for the gene *ermB* the designed primer sets demonstrated high specificity and sensitivity. The primers in this study did perform as seen in other studies done by Notomi et al., but assay is still at the developmental stage and require further evaluation. Therefore, the LAMP assay shows promise to offers a viable alternative for the detection of macrolide resistance especially in low resource countries such as Malawi and other countries in sub-Saharan Africa.

This study laid down a platform for further research and development of this cheap and easily scalable LAMP assay for the detection of macrolide resistance in *S. pneumoniae*. It is recommended that further evaluation of the developed LAMP assay be conducted focusing on the following aspects: ascertain the limit of detection of the primers in terms of the least number of DNA copies, which will require DNA quantification to be done; also determine the diagnostic potential of the primers on various clinical specimens including blood, sputum, and throat swabs; comparing performance of the LAMP assay to published real-time PCR assays; evaluation of the LAMP assay on a larger sample than the 79 carriage isolates in this study.

References

1. O'Brien KL, Wolfson LJ, Watt JP, Henkle E, Deloria-Knoll M, McCall N, et al. Burden of disease caused by Streptococcus pneumoniae in children younger than 5 years: global estimates. The Lancet [Internet]. 2009;374(9693):893–902. Available from: http://dx.doi.org/10.1016/S0140-6736(09)61204-6
2. Oza S, Lawn JE, Hogan DR, Mathers C, Cousens SN. Neonatal cause-of-death estimates for the early and late neonatal periods for 194 countries: 2000–2013. Bulletin of the World Health Organization. 2015;93(1):19–28.
3. Williams BG, Gouws E, Boschi-Pinto C, Bryce J, Dye C. Estimates of world-wide distribution of child deaths from acute respiratory infections. Lancet Infectious Diseases. 2002;2(1):25–32.
4. Progress in introduction of pneumococcal conjugate vaccine--worldwide, 2000-2008. MMWR Morbidity and mortality weekly report. 2008 Oct;57(42):1148–51.
5. World Health Organization. WHO recommendations on the diagnosis of HIV infection in infants. 2010;1–56.
6. Brooks WA, Breiman RF, Goswami D, Hossain A, Alam K, Saha SK, et al. Invasive pneumococcal disease burden and implications for vaccine policy in urban Bangladesh. The American Journal of Tropical Medicine and Hygiene. 2007 Nov;77(5):795–801.
7. Russell F, Sanderson C, Temple B. Global review of the distribution of pneumococcal disease by age and region. WHO Pneumococcal Epidemiology Report 31102011 v3 [Internet]. 2011;1–76. Available from:

https://www.who.int/immunization/sage/6_Russel_review_age_specific_epidemiology_PCV_schedules_session_nov11.pdf

8. Everett DB, Mukaka M, Denis B, Gordon SB, Carrol ED, van Oosterhout JJ, et al. Ten years of surveillance for invasive streptococcus pneumoniae during the era of antiretroviral scale-up and cotrimoxazole prophylaxis in Malawi. PLOS One [Internet]. 2011 Mar 15;6(3):e17765. Available from: https://doi.org/10.1371/journal.pone.0017765
9. Aston SJ, Ho A, Jary H, Huwa J, Mitchell T, Ibitoye S, et al. Etiology and isk Factors for mortality in an adult community-acquired pneumonia cohort in Malawi. American Journal of Respiratory and Critical Care Medicine. 2019 Aug 1;200(3):359–69.
10. Cornick JE, Everett DB, Broughton C, Denis BB, Banda DL, Carrol ED, et al. Invasive Streptococcus pneumoniae in children, Malawi, 2004-2006. Emerging Infectious Diseases. 2011 Jun;17(6):1107–9.
11. Bean B, Tomasz A. Choline metabolism in pneumococci. Journal of Bacteriology. 1977 Apr;130(1):571–4.
12. Weiser JN, Ferreira DM, Paton JC. Streptococcus pneumoniae: transmission, colonization and invasion. Nature Reviews Microbiology. 2018 Jun;16(6):355–67.
13. Brueggemann AB, Peto TEA, Crook DW, Butler JC, Kristinsson KG, Spratt BG. Temporal and geographic stability of the serogroup-specific invasive disease potential of Streptococcus pneumoniae in children. The Journal of Infectious Diseases. 2004 Oct;190(7):1203–11.
14. Sjöström K, Spindler C, Ortqvist A, Kalin M, Sandgren A, Kühlmann-Berenzon S, et al. Clonal and capsular types decide whether pneumococci will act as a primary or opportunistic pathogen. Clinical Infectious Diseases: an official publication of the Infectious Diseases Society of America. 2006 Feb;42(4):451–9.

15. Sonego M, Pellegrin MC, Becker G, Lazzerini M. Risk factors for mortality from Acute Lower Respiratory Infections (ALRI) in children under five years of age in low and middle-income countries: a systematic review and meta-analysis of observational studies. PLOS One [Internet]. 2015 Jan 30;10(1):e0116380. Available from: https://doi.org/10.1371/journal.pone.0116380

16. Dagerhamn J, Blomberg C, Browall S, Sjöström K, Morfeldt E, Henriques-Normark B. Determination of accessory gene patterns predicts the same relatedness among strains of Streptococcus pneumoniae as sequencing of housekeeping genes does and represents a novel approach in molecular epidemiology. Journal of Clinical Microbiology. 2008 Mar;46(3):863–8.

17. Walker CLF, Perin J, Katz J, Tielsch JM, Black RE. Diarrhea as a risk factor for acute lower respiratory tract infections among young children in low income settings. Journal of Global Health. 2013;3(1).

18. Kamng'ona AW, Hinds J, Bar-Zeev N, Gould KA, Chaguza C, Msefula C, et al. High multiple carriage and emergence of Streptococcus pneumoniae vaccine serotype variants in Malawian children. BMC Infectious Diseases [Internet]. 2015;15(1):234. Available from: https://doi.org/10.1186/s12879-015-0980-2

19. Birindwa AM, Emgård M, Nordén R, Samuelsson E, Geravandi S, Gonzales-Siles L, et al. High rate of antibiotic resistance among pneumococci carried by healthy children in the eastern part of the Democratic Republic of the Congo. BMC Pediatrics [Internet]. 2018;18(1):361. Available from: https://doi.org/10.1186/s12887-018-1332-3

20. Zar HJ, Jeena P, Argent A, Gie R, Madhi SA, McNally L, et al. Diagnosis and management of community-acquired pneumonia in childhood: South African Thoracic Society guidelines. South African Medical Journal. 2005;95(12 II):977–90.

21. Kaijalainen T. Identification of Streptococcus pneumoniae. International Journal of Circumpolar Health. 2006; 65:459–460.

22. Henriques-Normark B, Tuomanen EI. The pneumococcus: epidemiology, microbiology, and pathogenesis. Cold Spring Harbor Perspectives in Medicine [Internet]. 2013 Jul 1;3(7):a010215. Available from: https://pubmed.ncbi.nlm.nih.gov/23818515

23. Lowth M, Mb MA, Frcgp B. Pneumococcal disease in children. 2015:45(10).

24. Berry AM, Yother J, Briles DE, Hansman D, Paton JC. Reduced virulence of a defined pneumolysin-negative mutant of Streptococcus pneumoniae. Infection and Immunity. 1989 Jul;57(7):2037–42.

25. Kadioglu A, Taylor S, Iannelli F, Pozzi G, Mitchell TJ, Andrew PW. Upper and lower respiratory tract infection by Streptococcus pneumoniae is affected by pneumolysin deficiency and differences in capsule type. Infection and Immunity. 2002 Jun;70(6):2886–90.

26. Malley R, Henneke P, Morse SC, Cieslewicz MJ, Lipsitch M, Thompson CM, et al. Recognition of pneumolysin by Toll-like receptor 4 confers resistance to pneumococcal infection. Proceedings of the National Academy of Sciences of the United States of America. 2003 Feb;100(4):1966–71.

27. WHO report on Surveillance of Antibiotic Consumption implementation [Internet]. Geneva: WHO; 2018. p.128. Available from: http://apps.who.int/iris%0Ahttps://apps.who.int/iris/bitstream/handle/10665/277359/9789241514880-eng.pdf

28. Briles DE, Crain MJ, Gray BM, Forman C, Yother J. Strong association between capsular type and virulence for mice among human isolates of Streptococcus pneumoniae. Infection and Immunity. 1992 Jan;60(1):111–6.

29. Austrian R. Pneumococcus: the first one hundred years. Reviews of Infectious Diseases. 1981;3(2):183–9.

30. Schroeder MR, Stephens DS. Macrolide Resistance in Streptococcus pneumoniae. Frontiers in Cellular and Infection Microbiology. 2016;6:98.

31. Tuomanen E, Liu H, Hengstler B, Zak O, Tomasz A. The induction of meningeal inflammation by components of the pneumococcal cell wall. The Journal of Infectious diseases. 1985 May;151(5):859–68.

32. Ledwith M. Pneumococcal conjugate vaccine. Current Opinion in Pediatrics. 2001;13(1):70–4.

33. Johns Hopkins Bloomberg school of Public Health. VIEW-hub Report: Global vaccine introduction and implementation. International Vaccine Access Center (IVAC) 2021 March. Available from: https://view-hub.org/map/?set=countries-with-impact-evaluations&group=impact-studies&category=pcv

34. US Department of Health and Human Services, CDC. Antibiotic resistance threats in the United States. Centers for Disease Control and Prevention [Internet]. 2019;1–113. Available from: https://www.cdc.gov/drugresistance/biggest_threats.html

35. Cillóniz C, Garcia-Vidal C, Ceccato A, Torres A, Shortridge VD, Doern G v, et al. Antimicrobial resistance among Streptococcus pneumoniae. Clinical infectious diseases: an official publication of the Infectious Diseases Society of America. 2018 Nov;29(5):1186–8.

36. Owens RCJ, Shorr AF. Rational dosing of antimicrobial agents: pharmacokinetic and pharmacodynamic strategies. American journal of health-system pharmacy: AJHP: official journal of the American Society of Health-System Pharmacists. 2009 Jun;66(12 Suppl 4):S23-30.

37. Koornhof HJ, Jacobs MR. Resistant pneumococcal infections. Comprehensive Therapy. 1979;5(5):69–74.

38. Bergman M, Huikko S, Huovinen P, Paakkari P, Seppälä H. Macrolide and azithromycin use are linked to increased macrolide resistance in Streptococcus pneumoniae. Antimicrobial Agents and Chemotherapy. 2006 Nov;50(11):3646–50.

39. Malhotra-Kumar S, Lammens C, Piessens J, Goossens H. Multiplex PCR for simultaneous detection of macrolide and tetracycline resistance determinants in streptococci. Antimicrobial Agents and Chemotherapy [Internet]. 2005 Nov;49(11):4798–800. Available from: https://pubmed.ncbi.nlm.nih.gov/16251336

40. Klugman KP. Bacteriological evidence of antibiotic failure in pneumococcal lower respiratory tract infections. The European Respiratory Journal Supplement. 2002 Jul;36:3s–8s.

41. Mandell LA, Peterson LR, Wise R, Hooper D, Low DE, Schaad UB, et al. The battle against emerging antibiotic resistance: Should fluoroquinolones be used to treat children? Clinical Infectious Diseases [Internet]. 2002 Sep 15;35(6):721–7. Available from: https://doi.org/10.1086/341900

42. Iannini PB, Paladino JA, Lavin B, Singer ME, Schentag JJ. A case series of macrolide treatment failures in community acquired pneumonia. Journal of Chemotherapy [Internet]. 2007 Oct 1;19(5):536–45. Available from: https://doi.org/10.1179/joc.2007.19.5.536

43. Grimwood K. Childhood Pneumonia screener: a concept. Pneumonia [Internet]. 2015;6(1):i–ii. Available from: https://doi.org/10.1007/BF03371463

44. Naucler P, Galanis I, Morfeldt E, Darenberg J, Örtqvist Å, Henriques-Normark B. Comparison of the impact of Pneumococcal conjugate vaccine 10 or Pneumococcal conjugate vaccine 13 on invasive Pneumococcal disease in equivalent

populations. Clinical Infectious Diseases: an official publication of the Infectious Diseases Society of America. 2017 Nov;65(11):1780–9.

45. Burnham CAD, Leeds J, Nordmann P, O'Grady J, Patel J. Diagnosing antimicrobial resistance. Nature Reviews Microbiology [Internet]. 2017;15(11):697–703. Available from: http://dx.doi.org/10.1038/nrmicro.2017.103

46. Organization WH. Global Antimicrobial Resistance Surveillance System (GLASS): molecular methods for antimicrobial resistance (AMR) diagnostics to enhance the Global Antimicrobial Resistance Surveillance System [Internet]. Geneva: World Health Organization; Available from: https://apps.who.int/iris/handle/10665/310993

47. Assembly SWH. Global Action Plan on antimicrobial resistance. Microbe Magazine. 2015;10(9):354–5.

48. Notomi T, Okayama H, Masubuchi H, Yonekawa T, Watanabe K, Amino N, et al. Loop-mediated isothermal amplification of DNA. Nucleic Acids Research [Internet]. 2000 Jun 15;28(12):E63–E63. Available from: https://pubmed.ncbi.nlm.nih.gov/10871386

49. Nagamine K, Hase T, Notomi T. Accelerated reaction by loop-mediated isothermal amplification using loop primers. Molecular and cellular probes. 2002 Jun;16(3):223–9.

50. Mori Y, Nagamine K, Tomita N, Notomi T. Detection of loop-mediated isothermal amplification reaction by turbidity derived from magnesium pyrophosphate formation. Biochemical and biophysical research communications. 2001 Nov;289(1):150–4.

51. Mori F, Pecorari L, Pantano S, Rossi ME, Pucci N, de Martino M, et al. Azithromycin anaphylaxis in children. International Journal of Immunopathology and Pharmacology. 2014;

52. Iwamoto T, Sonobe T, Hayashi K. Loop-mediated isothermal amplification for direct detection of Mycobacterium tuberculosis complex, M. avium, and M. intracellulare in sputum samples. Journal of Clinical Microbiology. 2003 Jun;41(6):2616–22.

53. Dukes JP, King DP, Alexandersen S. Novel reverse transcription loop-mediated isothermal amplification for rapid detection of foot-and-mouth disease virus. Archives of virology. 2006 Jun;151(6):1093–106.

54. Wastling SL, Picozzi K, Kakembo ASL, Welburn SC. LAMP for Human African Trypanosomiasis: a comparative study of detection formats. PLOS Neglected Tropical Diseases [Internet]. 2010 Nov 2;4(11):e865. Available from: https://doi.org/10.1371/journal.pntd.0000865

55. Parida M, Sannarangaiah S, Dash PK, Rao PVL, Morita K. Loop mediated isothermal amplification (LAMP): a new generation of innovative gene amplification technique; perspectives in clinical diagnosis of infectious diseases. Reviews in medical virology. 2008;18(6):407–21.

56. Tomlinson J. Potential of LAMP for detection of plant pathogens. Cab Reviews: Perspectives in Agriculture, Veterinary Science, Nutrition and Natural Resources. 2010 Jan 1;3.

57. Kim DW, Kilgore PE, Kim EJ, Kim SA, Anh DD, Dong BQ, et al. The enhanced pneumococcal LAMP Assay: a clinical tool for the diagnosis of meningitis due to Streptococcus pneumoniae. PLOS One [Internet]. 2012 Aug 10;7(8):e42954-. Available from: https://doi.org/10.1371/journal.pone.0042954

58. Seki M, Kilgore PE, Kim EJ, Ohnishi M, Hayakawa S, Kim DW. Loop-mediated isothermal amplification methods for diagnosis of bacterial meningitis. Frontiers in pediatrics [Internet]. 2018 Mar 12;6:57. Available from: https://pubmed.ncbi.nlm.nih.gov/29594087

59. Lee D, Kim EJ, Kilgore PE, Kim SA, Takahashi H, Ohnishi M, et al. Clinical evaluation of a Loop-Mediated Isothermal Amplification (LAMP) assay for rapid detection of Neisseria meningitides in cerebrospinal fluid. PLOS One [Internet]. 2015 Apr 8;10(4):e0122922. Available from: https://doi.org/10.1371/journal.pone.0122922

60. Schultze M., McPherson M. J. and Møller S. G. Polymerize chain reaction Oxford: BIOS Scientific Publishers. 2000. pp. 288

61. Ravindran A, Levy J, Pierson E, Gross DC. Development of a loop-mediated isothermal amplification procedure as a sensitive and rapid method for detection of "candidatus Liberibacter solanacearum" in potatoes and Psyllids. Phytopathology. 2012 Sep;102(9):899–907.

62. Goto M, Honda E, Ogura A, Nomoto A, Hanaki K-I. Colorimetric detection of loop-mediated isothermal amplification reaction by using hydroxy naphthol blue. BioTechniques. 2009 Mar;46(3):167–72.

63. Thomac C. The polymerase chain reaction. In: Bryant JBT-M (Ed). Molecular biology [Internet]. Academic Press; 1993. p. 117–40. Available from: https://www.sciencedirect.com/science/article/pii/B9780124610200500123

64. Henke W, Herdel K, Jung K, Schnorr D, Loening SA. Betaine improves the PCR amplification of GC-rich DNA sequences. Nucleic Acids Research. 1997 Oct;25(19):3957–8.

65. Haddar CH, Joly J, Carricajo A, Verhoeven PO, Grattard F, Mory O, et al. Strategy using a new antigenic test for rapid diagnosis of Streptococcus pneumoniae infection in respiratory samples from children consulting at hospital. BMC Microbiology. 2020;20(1):1–9.

66. Boelsen LK, Dunne EM, Gould KA, Ratu FT, Vidal JE, Russell FM, et al. The challenges of using oropharyngeal samples to measure pneumococcal carriage in adults. mSphere. 2020;5(4):1–15.

Appendices

Appendix 1: Table 5.1 Whole genome sequencing, Culture, LAMP detection and LAMP time of detection raw data results table

ID	SPN_WGS	ermB_WGS	mefA_WGS	culture_erythr	culture_azithrd	culture_lyt	lytA_LAMP	ermB_LAMP	mefA_LAMP	lytA_LAMP(min	ermB_LAMP(mi	mefA_LAMP(min)
BVY12E	1	1	1	1	1	1	1	1	1	11	21	10
BVY12F	1	1	1	1	1	1	1	1	1	11	23	11
BVY1FS	1	1	1	1	1	1	1	1	1	12	22	12
BVX148	1	1	1	1	1	1	1	1	1	15	39	19
BVY74L_2	1	1	1	1	1	1	1	1	1	14	30	15
BVX12X	1	1	0	1	0	1	1	1	0	11	20	40
BVY1AF	1	1	0	1	0	1	1	1	0	14	40	40
BVY1BU	1	1	0	1	0	1	1	1	0	12	25	40
BVX18D	1	1	0	1	0	1	1	1	0	10	24	40
BVX1RB	1	1	0	1	0	1	1	1	0	16	25	40
BVX19I	1	1	0	1	0	1	1	1	0	15	26	40
BVY53B	1	1	0	1	0	1	1	1	0	13	30	40
BVX31I	1	1	0	1	0	1	1	1	0	10	18	40
BVX3CS	1	1	0	1	0	1	1	1	0	16	26	40
BVX31S	1	1	0	1	0	1	1	1	0	11	20	40
BVY55C	1	1	0	1	0	1	1	1	0	10	14	40
BVX320	1	1	0	1	0	1	1	1	0	18	20	40
BVY6NI	1	1	0	1	0	1	1	1	0	11	17	40
BVY554	1	1	0	1	0	1	1	1	0	10	19	40
BVY782	1	1	0	1	0	1	1	1	0	13	31	40
BVY15N	1	0	1	0	1	1	1	0	1	14	0	17
BVY1CT	1	0	1	0	1	1	1	0	1	9	0	9
BVY194	1	0	1	0	1	1	1	0	1	9	0	16
BVY1C3	1	0	1	0	1	1	1	0	1	11	0	11
BVY1BC	1	0	1	0	1	1	1	0	1	14	0	8
BVY1DR	1	0	1	0	1	1	1	0	1	12	0	9
BVX13B	1	0	1	0	1	1	1	0	1	9	0	12
BVY188	1	0	1	0	1	1	1	0	1	10	0	10
BVY1JK	1	0	1	0	1	1	1	0	1	11	0	12
BVY1RK	1	0	1	0	1	1	1	0	1	10	0	15
BVY1SS	1	0	1	0	1	1	1	0	1	11	0	19
BVY1P6	1	0	1	0	1	1	1	0	0	11	0	12
BVY1SD	1	0	1	0	1	1	1	0	1	10	0	18
BVX187	1	0	1	0	1	1	1	0	1	9	0	16
BVY1QA	1	0	1	0	1	1	1	0	1	9	0	12
BVY27L	1	0	1	0	1	1	1	0	1	9	0	15
BVY2LV	1	0	1	0	1	1	1	0	1	9	0	9
BVY24S	1	0	1	0	1	1	1	0	1	11	0	10
BVY2K0	1	0	1	0	1	1	1	0	1	9	0	12
BVY26D	1	0	1	0	1	1	1	0	1	9	0	9
BVY5CS	1	0	1	0	1	1	1	0	1	11	0	9
BVY4XZ	1	0	1	0	1	1	1	0	1	9	0	11
BVY1PT	1	0	1	0	1	1	1	0	1	10	0	40
BVY2DJ	1	0	1	0	1	1	1	0	1	15	0	10
BVY572	1	0	1	0	1	1	0	0	1	0	0	18
BVY4UH	1	0	1	0	1	1	1	0	1	9	0	9
BVY5UK	1	0	1	0	1	1	1	0	1	11	0	10
BVY77D	1	0	1	0	1	1	1	0	1	9	0	11
BVY6VQ	1	0	1	0	1	1	1	0	1	10	0	9
BVY1AP	1	0	0	0	0	1	1	0	0	12	0	40
BVY13F	1	0	0	0	0	1	1	0	0	11	0	40
BVY1BV	1	0	0	0	0	1	1	0	0	15	0	40
BVY1FW	1	0	0	0	0	1	1	0	0	9	0	40
BVY1HJ	1	0	0	0	0	1	1	0	0	10	0	40
BVY1CD	1	0	0	0	0	1	1	0	0	20	0	40
BVY1DZ	1	0	0	0	0	1	1	0	0	12	0	40
BVX13I	1	0	0	0	0	1	1	0	0	21	0	40
BVY1CX	1	0	0	0	0	1	1	0	0	9	0	40
BVY13E	1	0	0	0	0	1	1	0	0	13	0	40
BVY21K	1	0	0	0	0	1	0	0	0	0	0	40
BVY50T	1	0	0	0	0	1	1	0	0	15	0	40
BVY7I9	1	0	0	0	0	1	1	0	0	12	0	40
BVY722	1	0	0	0	0	1	1	0	0	12	0	40
BVY7DL	1	0	0	0	0	1	1	0	0	4	0	40
BVY7FS	1	0	0	0	0	1	1	0	0	13	0	40
BVY8AQ	1	0	0	0	0	1	0	0	0	0	0	40
BVY7K5	1	0	0	0	0	1	1	0	0	20	0	40
BVY583	1	0	0	0	0	1	1	0	0	15	0	40
BVY5CM	1	0	0	0	0	1	1	0	0	22	0	40
BVY4UK	1	0	0	0	0	1	0	0	0	0	0	40
BVY5U6	1	0	0	0	0	1	1	0	0	19	0	40
BVY5TB	1	0	0	0	0	1	0	0	0	0	0	40
BVY59F	1	0	0	0	0	1	1	0	0	16	0	40
BVY2HS	1	0	0	0	0	1	1	0	0	12	0	40
BVY1WJ	1	0	0	0	0	1	0	0	0	0	0	40
BVY29F	1	0	0	0	0	1	0	0	0	0	0	40
BVY28T	1	0	0	0	0	1	1	0	0	14	0	40
BVY27H	1	0	0	0	0	1	1	0	0	11	0	40
BVY27I	1	0	0	0	0	1	1	0	0	14	0	40
Spn	1	0	0	0	0	0	1	0	0	10	0	40
gbs1	0	0	0	0	0	0	0	0	0	0	0	40
gbs2	0	0	0	0	0	0	0	0	0	0	0	40

Appendix 2: Table 5.2 Raw data from Premier Biosoft of the 6-primer set of *ermB* and *mefA streptococcus pneumoniae* resistance

Accession Number	Sequence Definition	Sequence Length	Name	Quality	Primer	Concentration	Position	Length	Tm	GC%	Rating	GC Clamp	3'End dG	Self Dimer dG(Internal)	Hairpin dG(Internal)
*000001_ermB	WTCHG_585651_257101.fasta	2,087,448	F3		AGCTGGGCGATTTAATGAG	4.5	2,015,072	19	60	47.4	86.2	1	-0.2	-0.9	0
			B3		AGCTACTGTACCGATGGTT	4.9	2,015,370	19	60.1	47.4	92	2	-1.1	-0.9	0
			FIP(F1c+F2)		GCTGTAGACGGTGCATGTGATGGGAAGTCGTATGATGAGA	2.2		40							
			BIP(B1c+B2)		GTCTGCCATTGCCAGTGCTATTGTGTACTCGCAGAACTTC	2.4		40							
			LoopF		GCGTGCATAAGCATACGAG	4.5	2,015,168	19	61.5	52.6	87.5	2	-1.4	-1.1	-0.3
			LoopB		AAAGGACAGTCGGGTTCG	4.8	2,015,254	18	61.7	55.6	85.8	2	-0.5	0	0
			F2		TGGGAAGTCGTATGATGAGA	4.2	2,015,126	20	60		86.7	1	-1.9	0	0
			F1c		GCTGTAGACGGTGCATGTGA	4.5	2,015,188	20	64.9	55	91.6	1	-1	-1.1	0
			B2		TTGTGTACTCGCAGAACTTC	4.8	2,015,292	20	60.2	45	92.7	1	-0.3	0	0
			B1c		GTCTGCCATTGCCAGTGCTA	4.9	2,015,226	20	65.2	55	92.2	2	-0.7	0	0
			Product	Best				167	73.8	52.7	89.4				
	Amplicon	TGGGAAGTCGTATGATGAGAAAACCTCGTATGCTTATGCACGCTCACATGCACCGTCTACAGCGGAAACTTGGACGCTCCGTGACTGCTTTAGGGGCTGGGTCTGCCATTGCCAGTGCTACGGGTAAAAAAGGACAGTCGGGTTCGGGAAGTTCTGCGAGTACACAA														
*000003_mefA	WTCHG_585651_252148.fasta	2,197,469	F3		CTGAATCGCTCATGTCCATAA	4.4	992,164	21	60.2	42.9	92.4	1	-0.3	0	0
			B3		GTGCTATGGAAGACGAGC	4.8	992,427	18	60	55.6	92.9	2	-2	0	0
			FIP(F1c+F2)		TGGGTTATCAGTAGGCGTTGAATCACGTTTCTTGTCTGGC	2.4		40							
			BIP(B1c+B2)		TCTGTTGTCGCAAACTGGCTAAGGATTGCAAAGCGTGA	2.3		38							
			LoopF		TGAGTGAAGCATTACGTGAGT	4.1	992,244	21	61.7	42.9	91.1	1	-0.9	-1	0
			LoopB		AAGATAATCGCCAACATCCGA	4.1	992,336	21	62	42.9	93.3	3	0.1	0	0
			F2		TCACGTTTCTTGTCTGGC	5.8	992,205	18	60	50	86	3	-1.5	-1	0
			F1c		TGGGTTATCAGTAGGCGTTGAA	4	992,276	22	63.8	45.5	76	1	-0.1	0	0
			B2		AAGGATTGCAAAGCGTGA	4.5	992,384	18	59.9	44.4	84.8	2	-1.5	-1.8	0
			B1c		TCTGTTGTCGCAAACTGGCT	4.8	992,313	20	65	50	86.7	3	-0.9	0	0
			Product	Best				180	69.1	40.6	86.3				
	Amplicon	TCACGTTTCTTGTCTGGCAACTCACGTAATGCTTCACTCAACAAATCATTTTCAACGCCTACTGATAACCCATTGAGTGTAAAAATCTGAAAGTCAGTTGAATAGTTATCTGTTGTCGCAAACTGGCTAACAAGATAATCGCCAACATCCGAAAAGGACACCTCACGCTTTGCAATCCTT														