Genotypic detection of antimicrobial resistance in *Streptococcus pneumoniae* isolates using multiple PCR methods

Laidlaw research project report

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Introduction

This six-week Laidlaw research project on monitoring antimicrobial resistance (AMR) is part of the LAKANA trial, a collaborative program with CVD Mali that involves the mass drug administration (MDA) of azithromycin, an antibiotic, to 1- to 11-month-old children in Mali, West Africa, aimed at reducing the high mortality rate of children. In Mali, the under-five mortality rate (i.e., the probability that a child would die before reaching the age of 5 years, expressed per 1,000 live births) reached 93.8 per 1,000 live births in 2022, making it the 9th highest worldwide [1]. The leading causes of these under-five deaths include neonatal conditions, lower respiratory infections, diarrhoea, and malaria [2]. Ending all preventable deaths under five years of age is one of the targets mentioned in Sustainable Development Goal (SDG) 3: *Good Health and Wellbeing* [3]. Hence, to reduce the under-five mortality rate, the WHO recommends the MDA strategy [4]. The first azithromycin MDA trial established was the trachoma control program, aimed at eradicating trachoma, a bacterial disease that causes blindness. Azithromycin was chosen as the treatment drug due to its safety profile, long duration of action, and relatively low-cost production [5]. The trial was successful in multiple countries, and it was further discovered that the trial seemed to reduce the under-five mortality rate [4]. Following this, various clinical trials on azithromycin MDA were conducted to test whether azithromycin MDA can indeed lower child mortality. This includes the MORDOR trial in Malawi, Niger, and Tanzania [6] and the AVENIR trial in Niger [7], which had proven to be effective in lowering child mortality.

The WHO emphasised that further research on azithromycin MDA is necessary [8]. This includes determining the optimal dose, intervention frequency, and potential harms, especially AMR [8]. Hence, the LAKANA study aims to investigate these. The primary objective of this study is to determine the impact of different annual rounds of azithromycin intervention to 1- to 11-month-old infants on mortality rates. Based on this, a cluster-randomised, double-blinded, and placebo-controlled trial was designed [8]. Selected participating villages (clusters) are located within three of Mali's non-urban regions: Kayes, Kita, and Koulikoro [8]. The participating children are divided into three groups: control (4 doses of placebo a year), 4-dose azithromycin (4 doses of azithromycin a year), and 2-dose azithromycin (2 doses of azithromycin and 2 doses of placebo a year) [8]. The trial lasts 3 years, with an initial village recruitment and allocation visit, 8 MDA visits, one close-out visit, and one follow-up visit (i.e., 11 visits in total) [8].

Furthermore, one of the secondary objectives of the LAKANA study is to determine the impact of different annual rounds of azithromycin intervention to 1- to 11-month old infants on the prevalence of AMR in the population. Despite the benefit of promoting survival, the MDA of antibiotics poses the risk of AMR developing in bacteria. Previously, the emergence of resistant *S. pneumoniae* strains was reported following azithromycin interventions [9]. AMR is a condition when microbes, such as bacteria, are no longer sensitive to antimicrobial agents such as antibiotics. This is a major global health concern, as the effectiveness of certain antibiotics in treating bacterial infections would decrease. Furthermore, the discovery of new antibiotics for *S. pneumoniae* is currently in stagnant progress [10]. Hence, monitoring the prevalence of AMR as the trial proceeds is necessary. We need to ensure that this potential issue does not outweigh the advantages of MDA. If AMR persists and no front-line antibiotic treatments continue to work effectively, a person's life may be at risk in the case of a serious bacterial infection.

In this study, to monitor AMR, *Streptococcus pneumoniae* and *Escherichia coli* isolates are used. This is because these two bacteria are commensal in the human body and hence can be indicators of AMR in the population due to horizontal gene transfer [11]. Specifically, *S. pneumoniae* colonises the nasopharynx and *E. coli* colonises the gut. AMR samples are collected at MDA visit 1 (for baseline), MDA visit 5 (1 year post-treatment), visit 9 (2-year timepoint and after treatment cessation), and at visit 11 (1 year after treatment cessation) [8]. The *S. pneumoniae* and *E. coli* samples are obtained from nasopharyngeal swabs and rectal swabs, respectively. Two child groups are sampled: 4- to 14-month-old (treated group) and 49- to 59-month-old (contact group) to test if AMR potentially spreads from younger (treated) children to older ones. All the isolates were phenotypically tested beforehand by CVD Mali for resistance against multiple antibiotics using disk diffusion assays and E-tests. Disk diffusion assay involves placing antibiotic-impregnated disks on a plate of agar inoculated with bacteria. Following incubation and bacterial growth, the inhibition zones around the disks are recorded. Meanwhile, E-test involves placing a strip with a gradient of antibiotic concentrations onto a plate of agar inoculated with bacteria. Following incubation, the minimum inhibitory concentration (MIC), i.e., the lowest concentration of antibiotic that prevents the visible growth of bacteria, is recorded.

The Laidlaw research project focused on genotypically detecting AMR in *S. pneumoniae* isolates. Genotype is the genetic makeup of an organism, whereas phenotype is the physical characteristics manifested from the genotype. It is essential to also test genotypic AMR along with phenotypic AMR. While phenotypic testing reveals the nature of the bacteria in the presence of antibiotics, genotypic testing shows the specific genes and modes of action. In this project, we are genotypically testing resistance against azithromycin (class: macrolide) and penicillin (class: β-lactam), as these two classes are the major anti-pneumococcus agents [10]. This is done by employing polymerase chain reaction (PCR) methods. In this project, 3 types of PCR are used: quantitative PCR (qPCR) and colourimetric loop-mediated isothermal amplification (LAMP) assays to analyse the extracted samples, and droplet digital PCR (ddPCR) as a reference to estimate and compare the sensitivity of the other 2 PCR methods. We extracted the DNA of each isolate and targeted 4 genes in total: *lytA* (an autolysin gene specific to *S. pneumoniae*), *ermB*, *mefA* (most common resistance genes against macrolide in *S. pneumoniae*), and *pbp2b* (susceptibility gene to penicillin in *S. pneumoniae*). In addition, we are also testing whether the LAMP assays work on non-extracted samples.

The main aim of this project is to compare the obtained genotypic data with the respective phenotypic data to see if they match and whether genotypic analysis can be used to predict resistance patterns. We hypothesised that there would be some discrepancies between the genotype and phenotype of each sample (i.e., a phenotypically sensitive sample might be genotypically resistant, and vice versa). Additionally, we aimed to compare the sensitivity (i.e., the limit of detection) of qPCR and LAMP. Since qPCR is the current gold standard for diagnosis, we hypothesised that qPCR is more sensitive than LAMP.

We are especially interested in finding out the accuracy of the colourimetric LAMP assay. This is because the LAMP assay provides many advantages over other PCR methods, such as its ability for rapid detection and its relative simplicity, as the reaction only requires one temperature and, in some cases, does not require the extraction of DNA [12, 13]. Moreover, in this case, the result, which would be a colour change, is observable by the naked eye without requiring additional procedures. If the LAMP assay is proven sensitive and specific enough, including with non-extracted samples, this method would be suitable for use in clinical trials in resource-limited settings [12, 13], including the LAKANA trial in Mali.

Theory

S. pneumoniae is a Gram-positive, lancet-shaped, alpha-hemolytic bacterium capable of colonising the nasopharynx and causing pneumococcal diseases such as pneumonia [14]. To date, more than 90 *S. pneumoniae* serotypes have been identified based on their capsular polysaccharides [14]. There are several virulence factors through which *S. pneumoniae* causes infections in humans. These include the polysaccharide capsule, pili, and various pneumococcal proteins, such as pneumolysin, autolysin, and pneumococcal surface protein A [14, 15]. They have different mechanisms by which they interfere with the host's immune system.

Autolysins are enzymes produced by Gram-positive bacteria that break down peptidoglycan in their cell walls, causing autolysis (self-degradation). Bacteria undergo autolysis to release components such as toxins, enabling the bacteria to colonise host cells and cause infection. An example of autolysin is lytic amidase, which is present in all *S. pneumoniae* strains and is said to be specific to them [16]. Lytic amidase is coded by a gene called *lytA*. Hence, *lytA* becomes a reference gene for identifying the presence of *S. pneumoniae* [16]. To confirm that our isolates are indeed *S. pneumoniae*, which is our bacteria of interest, we tried to amplify and detect the *lytA* gene in the PCR experiments.

Moreover, we tried to detect resistance against azithromycin and penicillin in the *S. pneumoniae* isolates. These antibiotics belong to the macrolide and β-lactam classes, respectively, which are the two major ones for treating pneumococcal infections [10]. Macrolide works against bacteria by binding to the 23S rRNA component of the 50S ribosomal subunit in bacteria, inhibiting bacterial protein synthesis [5, 10]. Meanwhile, β-lactam works by binding to penicillin-binding proteins (PBPs) in bacteria, inhibiting the final steps of peptidoglycan synthesis in bacterial cell walls [10].

AMR in *S. pneumoniae* can develop due to several mechanisms, each caused by a distinct gene. For macrolide resistance, the two most common resistance genes are *mefA* and *ermB*. The *mefA* gene codes for active efflux pumps capable of pumping out macrolides, whereas the *ermB* gene codes for a methylase enzyme that methylates the 23S rRNA, altering the ribosomes and making the bacteria resistant to the action of macrolides [10]. Hence, the presence of *mefA* and/or *ermB* indicates resistance against macrolides, while the absence of both may indicate susceptibility. Meanwhile, β-lactam resistance arises due to the absence of the *pbp2b* gene. The *pbp2b* gene codes for PBP (penicillin-binding protein), which accounts for the affinity of the bacterial cell walls for β-lactams [10]. Hence, the presence of *pbp2b* indicates susceptibility to β-lactams, while its absence may indicate resistance.

To amplify and detect the mentioned genes, polymerase chain reaction (PCR) techniques are used. In general, all PCR techniques involve the repeated replication of a certain segment of DNA, depending on the primers. However, different PCR methods have different principles behind DNA synthesis and signal detection. *Table 1* summarises the features of the three PCR methods used in this project.

	qPCR [17]	LAMP assay [18]	ddPCR [19]
Distinct principles	Uses a probe containing a dye capable of emitting fluorescence as DNA synthesis proceeds. The fluorescence intensity is measured.	Uses a set of 4-6 primers. A loop structure is formed in the newly synthesised DNA. The reaction happens at a constant temperature.	Involves the partition of DNA into individual droplets. Each droplet is analysed to determine the fraction of positive droplets in the original sample.
Output	Real-time amplification curves	Observable colour change (for positive result)	Absolute copy number of DNA per µl
DNA quantification	Relative quantification	Qualitative; no quantification	Absolute quantification
Advantages	A highly established technique that can be used for various purposes (besides diagnostics)	Less time-consuming; less resources and expertise needed	Higher sensitivity and precision; does not need a standard curve
Disadvantages	Requires a standard curve	Greater possibility of cross-contamination; more difficult to design primers	More expensive, requires multiple specialised equipment

Table 1: Comparison of 3 different PCR methods: qPCR, LAMP assay, and ddPCR.

Materials and methods

S. pneumoniae isolates

In this project, we tested a total of 24 *S. pneumoniae* isolates, some of which are phenotypically resistant to erythromycin (macrolide) and/or oxacillin (β-lactam). These isolates have unique identifier codes (child IDs) assigned by CVD Mali and sample IDs assigned by us. The 24 isolates were equally divided into 2 groups (12 isolates in each group), with the 2 groups cultured and extracted separately on different days.

Controls

For the positive controls of the PCR, four lab strains of *S. pneumoniae* from BEI resources were used: NR-51849 (strain GA17457, wild-type), NR-51851 (strain OREP4, wild-type), NR-19118 (strain GA17457, *mefA* positive), and NR-19160 (strain GA47179, *ermB* positive). Additionally, *pbp2b*-positive DNA from Great Ormond Street Hospital (GOSH) was used. For the negative controls, nuclease-free water from Severn Biotech Ltd. and *E. coli* DNA extract were used.

1. Bacterial culturing

S. pneumoniae isolates previously enriched in glycerol stock and frozen at -80°C were streaked in Columbia Blood Agar (CBA) plates from Oxoid Ltd. in a zigzag manner until the whole plate was filled to maximise the number of colonies that grew. The plates were then incubated at 37°C in 5% CO_2 for approximately 16 hours. CBA plates contain heme, which is required for *S. pneumoniae* growth. The morphologies and colony sizes were analysed after incubation. Furthermore, the extent of alpha-haemolysis by *S. pneumoniae* in the blood agar can be seen through the resulting brownish-green colour of the agar after incubation. The darker the colour, the greater the degree of hemolysis.

2. DNA extraction and quantification

Following incubation, the bacterial DNA was extracted from the culture using the QIAmp DNA Mini Kit from QIAGEN. We tested two different extraction protocols for the 2 isolate groups: (1) using 100 µl AE buffer, 50 µl mutanolysin, and 50 µl lysozyme; and (2) using 147 µl TE buffer, 29.5 µl mutanolysin, and 23.5 µl hyaluronidase. The colonies on each plate were swept using a plastic loop and directly suspended in the buffer and enzyme mixture by twisting the loop back and forth. The mixture is then incubated at 37°C for 1 hour. We then continued following the manufacturer's instructions for extraction. Once extracted, the amount of DNA in each sample was quantified using the Qubit dsDNA High Sensitivity (HS) Quantification Assay Kit and the Qubit 2.0 Fluorometer from Invitrogen. The amount of DNA extract was recorded as a concentration in ng/µl.

3. Automated electrophoresis

To reveal the purity and integrity of the extracted DNA and re-check the DNA concentrations, we performed automated electrophoresis on selected samples from each extraction group using the Agilent 4200 TapeStation System and Genomic DNA ScreenTape.

4. Quantitative PCR (qPCR)

To amplify and detect the presence of *lytA*, *ermB*, *mefA*, and *pbp2b* genes in the extracted DNA, qPCR experiments were performed on 96-well plates, adapting a protocol developed by GOSH. Each sample was tested in two duplexes: (1) *lytA* and *pbp2b* duplex, and (2) *ermB* and *mefA* duplex. We used a QuantiTect Multiplex PCR Kit and probes and primer pairs from Merck (*Table 2*) to make the reaction mix. For each reaction, we used 12.5 µl master mix, 0.5 µl of each probe, 0.5 µl of each forward primer, 0.5 µl of each reverse primer, and water to make up a total volume of 23 µl. To each well, we added 23 µl reaction mix and 2 µl sample, making a total reaction volume of 25 µl. Prior to testing the 24 extracts, we made standard curves with a dilution series of lab strains. Next, two runs of qPCR were done, one with a 10^{-2}

sample dilution and one with a 10^{-1} sample dilution. We used *E. coli* DNA as an extraction control and nuclease-free water as a no-template control. The qPCR was cycled as follows: initial enzyme activation at 95°C for 15 minutes, followed by 45 cycles of denaturation at 95°C for 5 seconds, and annealing + extension at 58°C for 30 seconds.

5’-3’ sequences of probes and primers

	lytA	*ermB*	*mefA*	*pbp2b*
Probe	[6FAM]TTTGCCGAAAACGCTTGATACAGGG[BHQ1]	[Cyanine3]AAGTCTCGATTCAGCAATTGCTTAAG[BHQ2]	[HEX]CCGTAGCATTGGAACAGCTTTTC[BHQ1]	[JOE]CACAGCGGTCCA AGCTCT[BHQ-1]
Forward primer	ACGCAATCTAGCAGATGAAGC	CTTGGATATTCACCGAACAC	TATGGAGCTACCTGTCTGGA	ATTCTTGGTATACTCAGGCT
Reverse primer	TGTTTGGTTGGTTATTCGTGC	TTGGTTTAGGATGAAAGCAT	GGTACTAAAAGTGGCGTAACC	[MOD]GGTTTGGACCATATAGGTATT

🟪 Fluorescent dye 🟥 Quencher

Table 2: The 5’-3’ sequences of the probes and primer pairs designed for the qPCR experiments.

5. Loop-mediated isothermal amplification (LAMP) assay

The second PCR method we tested was the colourimetric LAMP assay, using the New England Biolab WarmStart LAMP protocol. We first performed the assay on extracted isolates. Three genes were targeted: *lytA*, *ermB*, and *mefA*. Upon the amplification and quantity increase of DNA, a colour change from dark pink to yellow would be observed due to a decrease in pH. We used a 2x WarmStart Colorimetric LAMP Master Mix from New England Biolabs and primer sets from Merck (*Table 3*) to make the reaction mix. The three primer sets were used in singleplexes. Prior to testing the extracted samples, we optimised the assay using lab strains (NR-19118 and NR-19160) to determine the optimum temperature and sample volume for the reactions. We found that these were 65°C and 1 µl in a total reaction volume of 25 µl, respectively. The assays were run for one hour, but we checked for any colour change every 10 minutes. Next, we performed the optimised assay on the DNA extracts. This was done in two runs: one with a 10^{-2} dilution and one undiluted. Nuclease-free water was used as the no-template control.

5’-3’ sequences of primers

	lytA	*ermB*	*mefA*
F3 (forward outer primer)	ACAGGCTGGAAGAAAATCGC	AAGTGGTTTTTGAAAGCCA	CATTAATCACTAGTGCCATCC
B3 (backward outer primer)	GCCATCTGGCTCTACTGTGA	GGGTAAGTTTTATTAAGACACTGTT	ACAGGTAGCTCCATACAGAA
FIP (forward inner primer)	TGGCGCCTTCTTTAGCGTCTAACAACGAAGAAGGTGCCATGA	CGGTGAATATCCAAGGTACGCTGCGTCTGACATCTATCTGA	GTCCCAAAATCGCATAGGGTAAAAATTACCTTACAGAAAAAACAGGATC
BIP (backward inner primer)	TGCCTTTATCCAGTCAGCGGACGGCTTGTCTGCCAGTGTTC	AACACTAGGGTTGCTCTTGCAGTTTAGGATGAAAGCATTCCG	GCTAGTGGATCGTCATGATAGGAAGAACAATAGCAAGCACTGC
LF (loop forward primer)	CTTGTACTTGACCCAGCCTGTC	-	TGAAGCCATAGACAAGACCATCG
LB (loop backward primer)	CAGGCTGGTACTACCTCAAACCA	CGATTCAGCAATTGCTTAAGCTGC	GTGCCGATTTAATTATCGCAGCAG

Table 3: The 5’-3’ sequences of the primer sets designed for the LAMP experiments.

The assay was also performed on selected non-extracted *S. pneumoniae* isolates in PCR tubes. The selected isolates were freshly cultured on CBA plates the previous day to grow colonies. For each primer set, two methods were tested: (1) directly suspending the colonies in a 24 µl reaction mix in PCR tubes, and (2) inactivating the colonies in 20 µl of PBS buffer by heating them at 95°C for 10 minutes and taking 1 µl for the PCR tube. We attempted to sweep a single colony from the CBA plate for each reaction. All the PCR tubes were then incubated in the PCR machine at 65°C for one hour. Any colour change was checked every 10 minutes.

6. Droplet digital PCR (ddPCR) for sensitivity determination

We performed a QX200 droplet digital PCR system from Bio-Rad with a dilution series of lab strains (NR-19118 and NR-19160) to determine the absolute copies of DNA per µl for each dilution. These can later be used to estimate and compare the sensitivity of qPCR and LAMP. The following dilution series was used: 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, and 10^{-8}. Two genes were targeted: *lytA* and *ermB*. We used ddPCR Supermix for Probes (no dUTP) from Bio-Rad and the same probes and primer pairs used in qPCR (*Table 2*). A total reaction volume of 22 µl was used in each well. Each reaction was done in duplicate, i.e., two copies of the same reaction.

Results

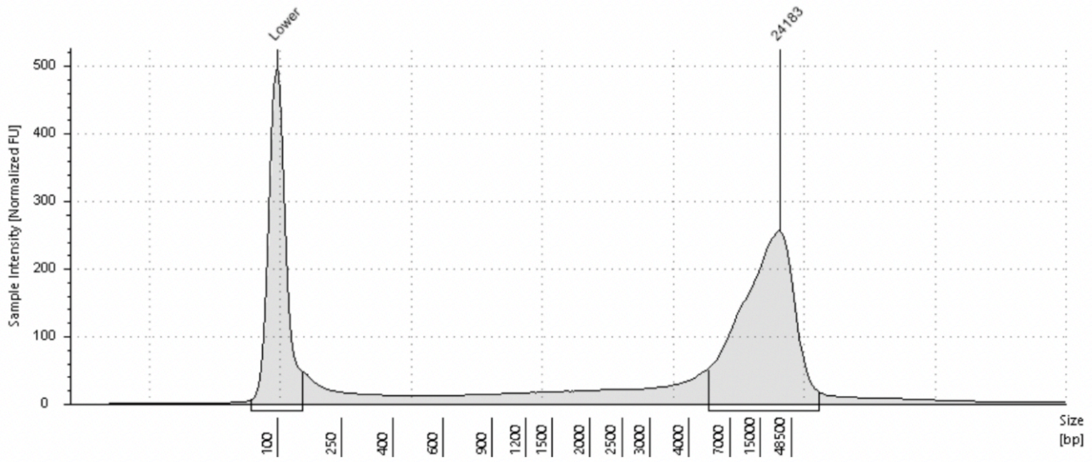
1. Bacterial culture

Following incubation, the bacterial colonies that grew on the CBA plates had relatively small sizes, with diameters of approximately 0.5–1 mm. The morphologies were mainly greyish, flattened colonies with depressed centres, surrounded by brownish-green zones of alpha-hemolysis. Various extents of alpha-haemolysis occurred, as shown by the resulting colour of the agar. Some plates were seen to have mixed cultures, as different morphologies, such as white, larger colonies with raised centres, were observed. This suggests that more than one isolate may be present in the respective samples, or contamination may have occurred.

2. DNA concentration, purity and integrity

Overall, high DNA concentrations in the extracts were measured by Qubit, with a few exceptions. For some extracts, the TapeStation revealed similar concentrations to Qubit, but for others, the concentrations detected in TapeStation were significantly lower than those in Qubit. This implies that impurities may be present, which were misidentified by the Qubit as DNA. In addition, the graphs and the DNA integrity number (DIN) shown on the TapeStation (*Figures 1.1 and 1.2*) revealed that some DNA shearing has occurred. If a minimum level of shearing occurred, we would expect a high molecular peak at around 20000–30000 base pairs. Moreover, the closer the DIN to 10, the higher the quality of the DNA.

C1: S66



Sample Table

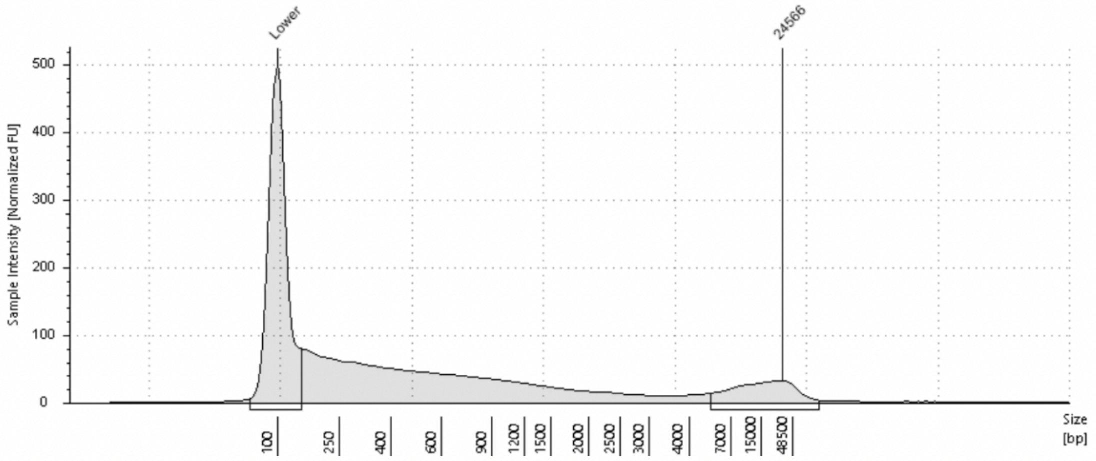
Well	DIN	Conc. [ng/µl]	Sample Description	Alert	Observations
C1	6.9	21.2	S66	⚠	Caution! Expired ScreenTape device

Peak Table

Size [bp]	Calibrated Conc. [ng/µl]	Assigned Conc. [ng/µl]	% Integrated Area	From [bp]	To [bp]	Peak Comment	Observations
100	8.50	8.50	-	68	144		Lower Marker
24183	13.7	-	99.41	5205	>60000		
-	-	-	-	-	-		Sample Well

Figure 1.1: The TapeStation result for one of the samples extracted using mutanolysin and lysozyme. The DNA extract has a concentration of 21.2 ng/µl. This concentration is similar to that revealed by Qubit, which is 22 ng/µl. This implies that the DNA extract is relatively pure. The peak at 100 base pairs is the lower marker. The wide-shaped curve around the peak of 24183 base pairs, and a DNA integrity number (DIN) of 6.9, show that some shearing has occurred.

D1: T2



Sample Table

Well	DIN	Conc. [ng/µl]	Sample Description	Alert	Observations
D1	1.0	13.4	T2	⚠	Caution! Expired ScreenTape device

Peak Table

Size [bp]	Calibrated Conc. [ng/µl]	Assigned Conc. [ng/µl]	% Integrated Area	From [bp]	To [bp]	Peak Comment	Observations
100	8.50	8.50	-	66	141		Lower Marker
24566	1.94	-	100.00	5305	>60000		

Figure 1.2: The TapeStation result for one of the samples extracted using mutanolysin and hyaluronidase. The DNA extract has a concentration of 13.4 ng/µl. This concentration is much lower than that revealed by Qubit, which is 73.2 ng/µl. This indicates that impurities may be present in the DNA extract. The peak at 100 base pairs is the lower marker. The low, indistinct peak at 24566, and a DIN of 1.0, indicate that we may have a low quantity of DNA. They could also mean that a lot of shearing has occurred.

3. qPCR

The qPCR results are shown as graphs displaying the progress of gene amplification. For each channel, a threshold is set (indicated by the straight horizontal line in the graph), and the Cq value (i.e., the cycle number at which the fluorescence rises above the threshold) of each isolate is recorded.

a. First run: 100-fold diluted DNA extracts

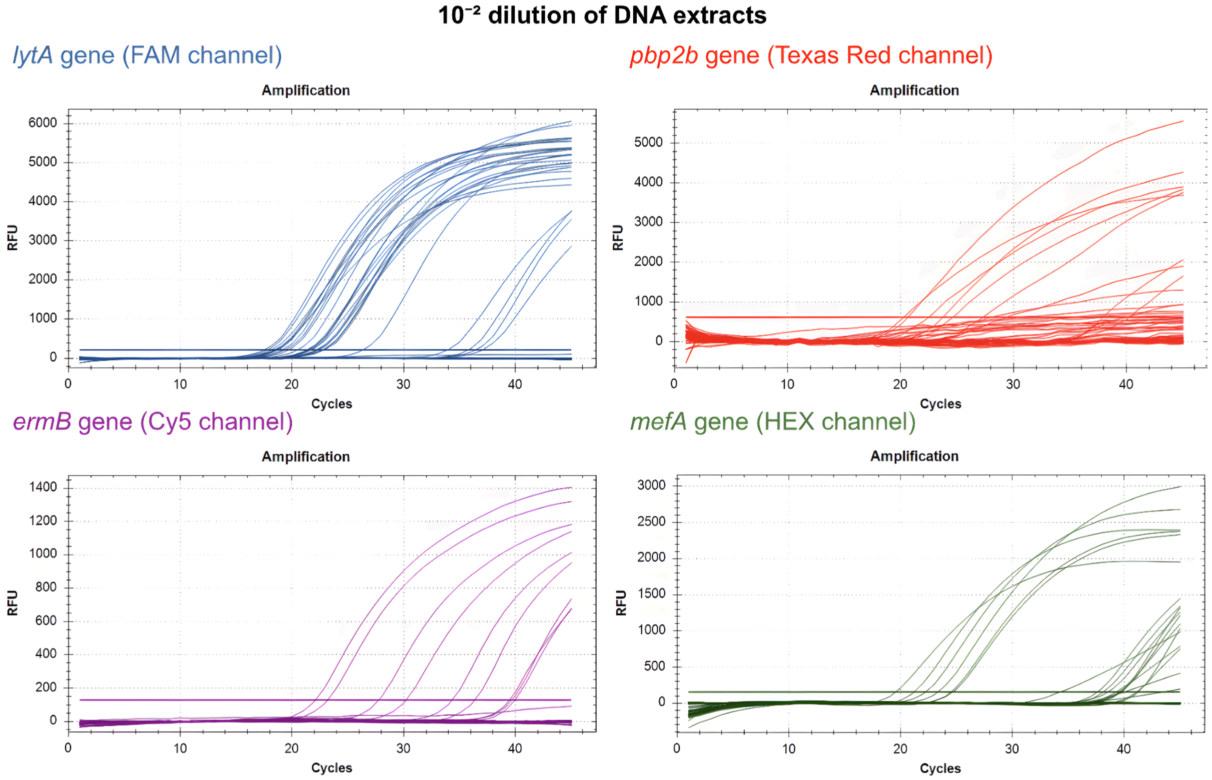


Figure 2.1: The amplification graphs for all 24 samples with a 10^{-2} dilution over 45 cycles. Positive and negative controls were also included. Four genes were targeted: *lytA* (blue), *pbp2b* (red), *ermB* (purple), and *mefA* (green). The fluorescence of the *lytA*, *pbp2b*, *ermB*, and *mefA* gene probes are detected in the FAM, Texas Red, Cy5, and HEX channels, respectively. The rise of the RFU value above the threshold may indicate the presence of a certain gene.

b. Second run: 10-fold diluted DNA extracts

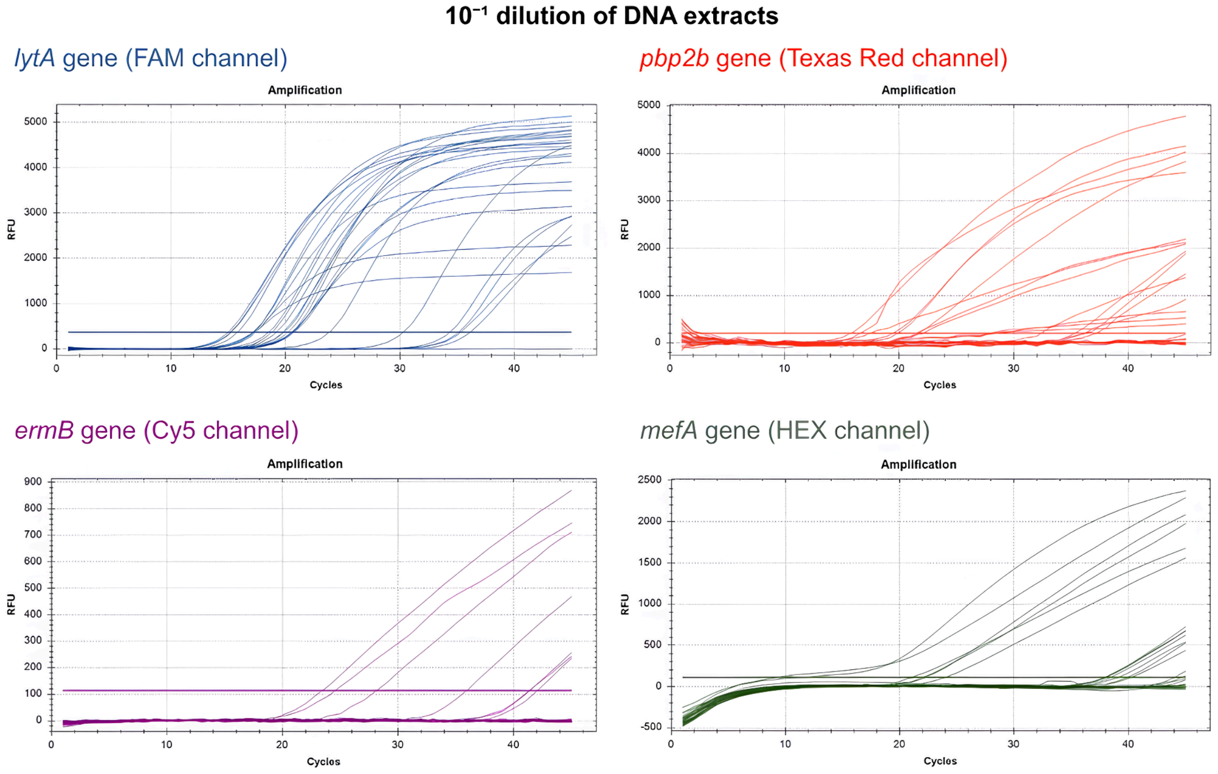


Figure 2.2: The amplification graphs for all 24 samples with a 10^{-1} dilution over 45 cycles. Positive and negative controls were also included. For each gene, some noticeable differences between the graphs from the first and second runs can be observed (i.e., the overall shapes of the graphs and the number of positive samples).

4. LAMP assay

The results of the LAMP assays are shown as colours (*Figures 3.1, 3.2, 3.3, and 3.4*). Yellow indicates a positive result, while pink indicates a negative result. The colour changes can already be observed clearly after 25–30 minutes.

I. Optimisation

Before testing our 24 samples, we aimed to determine the optimum conditions for the LAMP assay. This includes the primer sets, temperature, and sample volume.

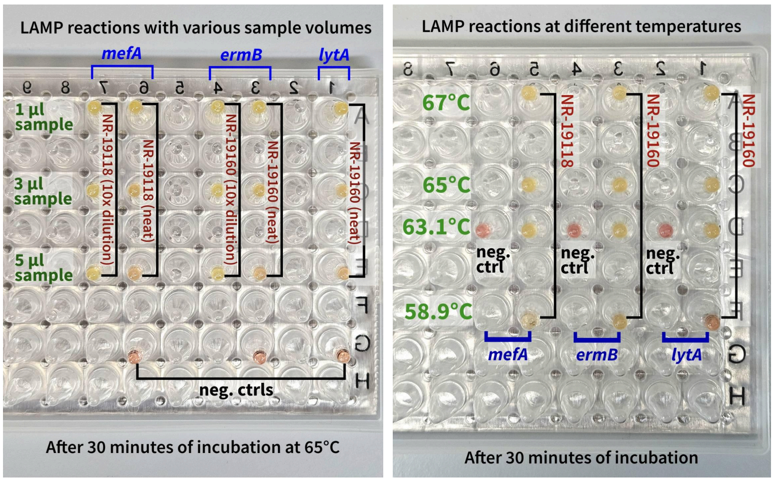


Figure 3.1: Optimisation of the LAMP assay. We first set a temperature gradient (left picture) on the PCR machine to determine the optimum temperature for the assay. It was revealed that the assay worked best at 65°C. Next, we set the assay temperature to 65°C and used different sample volumes (right picture) to find out the optimum sample volume for the assay. It was revealed that this was 1 µl.

II. Samples

a. First run: 100-fold diluted DNA extracts

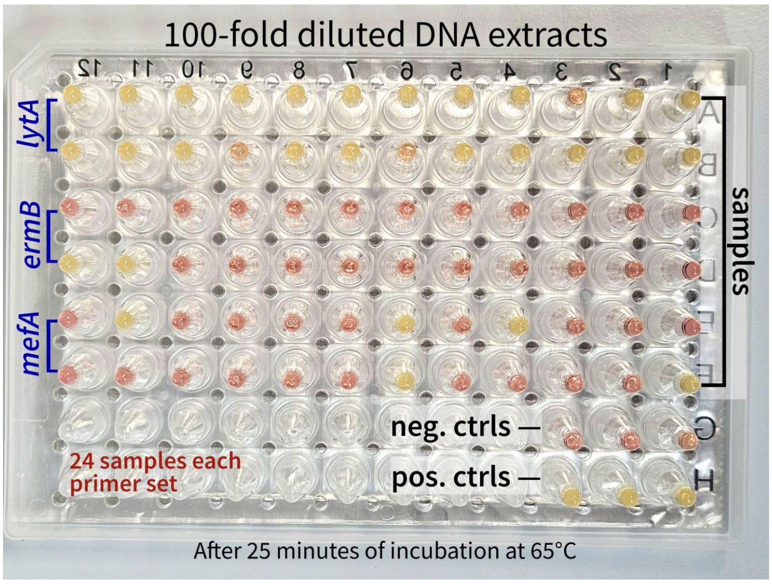


Figure 3.2: The LAMP assay result for all 24 extracts with a 10^{-2} dilution after 25 minutes of incubation at 65°C. From the picture, we can deduce that for the *lytA* primer set (rows A and B), all of the samples are positive, although 3 of them turned yellow quite late. For the *ermB* primer set (rows C and D), 2 of the samples are positive, and for the *mefA* primer set (rows E and F), 5 of the samples are positive. The negative controls, which are nuclease-free water (row G), all stayed negative, and the positive controls, which are lab strains (row H), all turned positive, indicating that the assay worked well.

b. Second run: undiluted DNA extracts (selected samples)

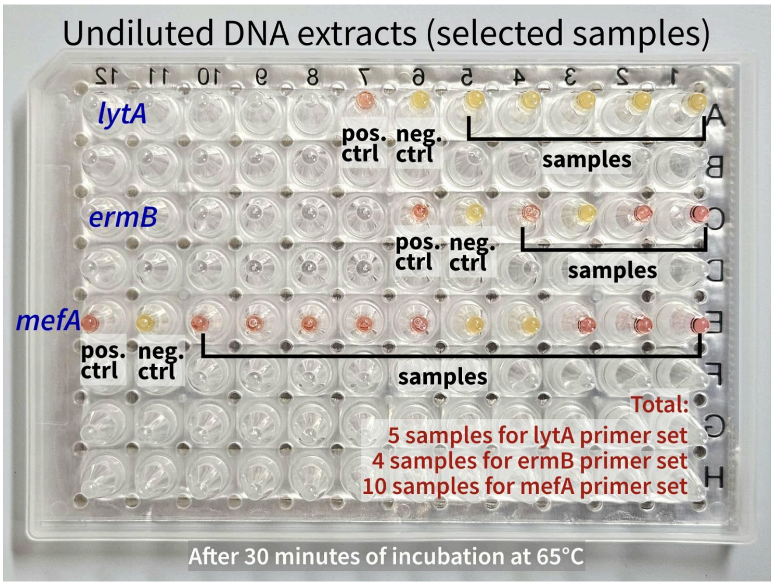


Figure 3.3: The LAMP assay result for selected DNA extracts, undiluted. We selected extracts that we considered ambiguous after comparing the result of the first LAMP experiment to that of qPCR with the same dilution. For the *lytA* primer set (row A), all 5 samples turned positive. For the *ermB* primer set (row C), 3 out of 4 samples turned positive. For the *mefA* primer set (row E), 2 out of 10 samples turned positive. Some of the positive results observed here were previously negative in the LAMP with 100-fold dilution. This suggests that the genes might be present but at low levels. All the positive controls turned positive and all the negative controls stayed negative, indicating that the assay worked well.

c. Third run: unextracted samples (selected samples)

In clinical trials, it may be preferable to carry out the assay on unextracted samples rather than extracted samples since it is faster and requires fewer resources. Hence, we are testing whether the assay works well on unextracted samples.

It was observed that the actual results (*Figure 3.4*) align with the expected results, showing that the LAMP assay worked well for unextracted samples in this case.

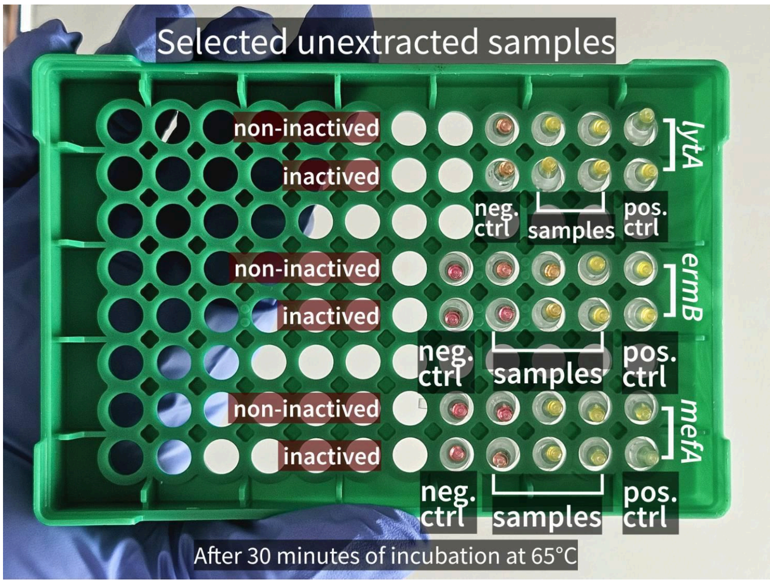


Figure 3.4: The LAMP assay result for selected unextracted samples. For each primer set, two methods were tested: non-inactivated and inactivated (in PBS and heat). From the picture, it is evident that the two methods give very similar outcomes. For the *lytA* primer set (rows A and B), we tested two samples, all of which were supposed to give positive results. For the *ermB* (rows D and E) and *mefA* (rows G and H) primer sets, we tested three samples in each set, two of which were expected to be positive and one which was expected to be negative, based on the previous qPCR and LAMP results. It can be seen that the actual results align with the expected results. Moreover, all the positive controls turned positive, and all the negative controls stayed negative.

5. Digital droplet PCR (ddPCR)

The purpose of ddPCR was to obtain the absolute copy numbers of DNA for known sample dilutions. It was observed that generally, dilution by a factor of 10 also reduced the number of DNA copies/µl by roughly a factor of 10, as expected. However, exceptions can be found for the last two dilutions: 10^{-7} and 10^{-8}. This is likely because the number of DNA copies was too low, leading to the detection inaccuracy. For a pair of duplicates, the numbers of DNA copies/µl were similar, indicating no pipetting errors. We averaged the copies/µl for each duplicate pair and obtained the results as shown in *Table 4*. These results will later be used for estimating qPCR and LAMP sensitivity.

Target	Sample	Dilution	Avg. copies/µl	Target	Sample	Dilution	Avg. copies/µl
mefA	NR-19118 (lab strain)	10^{-2}	49797	*lytA*	NR-19160 (lab strain)	10^{-2}	22035
		10^{-3}	5504.9			10^{-3}	2360.0
		10^{-4}	588.12			10^{-4}	258.40
		10^{-5}	58.179			10^{-5}	28.592
		10^{-6}	7.9166			10^{-6}	4.5117
		10^{-7}	0.9032			10^{-7}	0
		10^{-8}	0.2996			10^{-8}	0

Table 4: The result of the ddPCR with serial dilution of lab strains. The copies/ul shown are the averages from the duplicates.

qPCR and LAMP assay results comparison

Overall, for the tested 24 samples, the two methods show great consistency in the results. Only one sample was observed to be qPCR-positive but LAMP-negative for the ermB gene (*Table 5*).

***lytA* gene**	qPCR positive	qPCR negative	***ermB* gene**	qPCR positive	qPCR negative	***mefA* gene**	qPCR positive	qPCR negative
LAMP positive	24	0	LAMP positive	2	0	LAMP positive	5	0
LAMP negative	0	0	LAMP negative	1	21	LAMP negative	0	19

Table 5: Comparison of the qPCR and LAMP assay results for the same sample dilution of 10^{-2}.

Genotype and phenotype comparisons

Using the PCR data we obtained and the disk diffusion assay data from CVD Mali, we made several graphs visualising the comparisons between the genotypes and phenotypes for resistance against macrolide and β-lactam.

a. Macrolide resistance (*ermB* and *mefA* genes)

For the phenotypic data, we used the disk diffusion assay results for erythromycin, which belongs to the macrolide class. For the genotypic data, we used the qPCR and LAMP results for *ermB* and *mefA*. We used the EUCAST criteria to mark the borderline between phenotypic resistance and sensitivity. For erythromycin, a zone of inhibition of less than 22 mm around the disk indicates phenotypic resistance.

From the graph (*Figure 4.1*), it can be observed that 3 out of 11 (27.3%) phenotypically resistant isolates are genotypically sensitive, and 2 out of 13 (15.4%) phenotypically sensitive isolates are genotypically resistant.

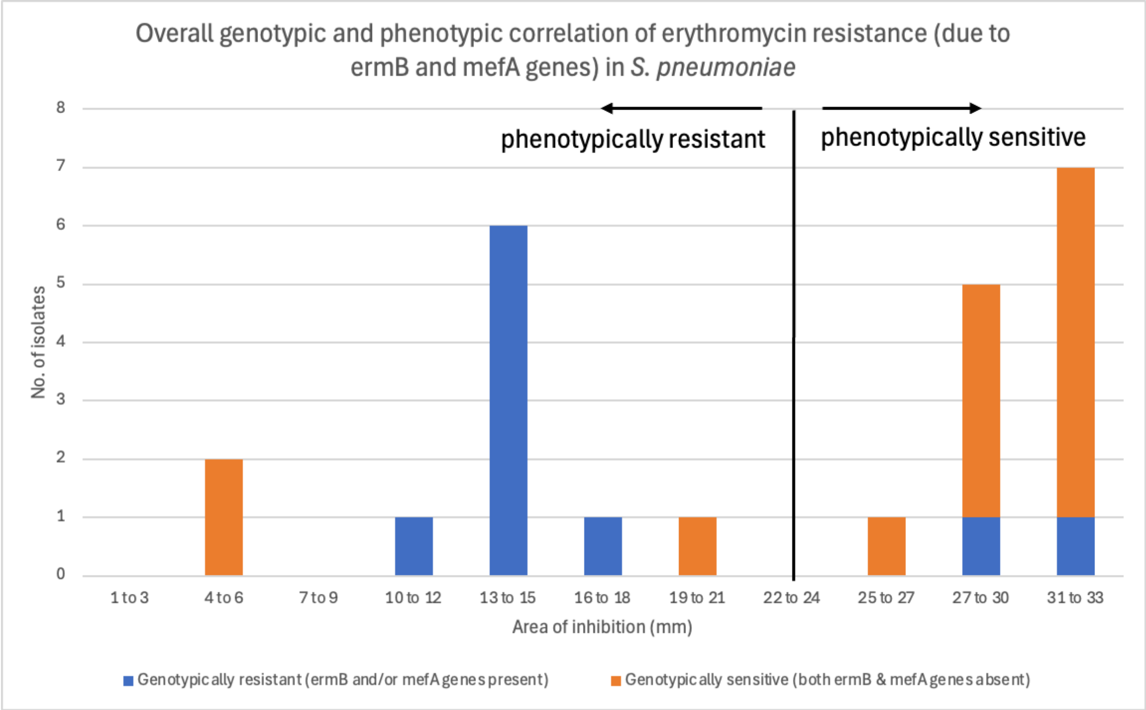


Figure 4.1: A graph showing the genotypic and phenotypic correlation of erythromycin resistance. If an isolate contains one or both the *ermB* and *mefA* genes, it is considered genotypically resistant. If an isolate has an area of inhibition of less than 22 mm in the disk diffusion assay, it is considered phenotypically resistant. If the genotypic and phenotypic data are fully consistent, we would expect the bars to the left of the borderline to all be blue, whereas those to the right of the borderline would all be orange.

b. β-lactam resistance (*pbp2b* gene)

For the phenotypic data, we used the disk diffusion assay results for oxacillin, which belongs to the β-lactam class. For the genotypic data, we used the qPCR results for *pbp2b*. We used the EUCAST criteria to mark the borderline between phenotypic resistance and sensitivity. For oxacillin, a zone of inhibition of less than 20 mm around the disk indicates phenotypic resistance.

From the graph (*Figure 4.2*), it can be observed that 10 out of 21 (47.6%) phenotypically resistant isolates are genotypically sensitive, and 2 out of 3 (66.7%) phenotypically sensitive isolates are genotypically resistant.

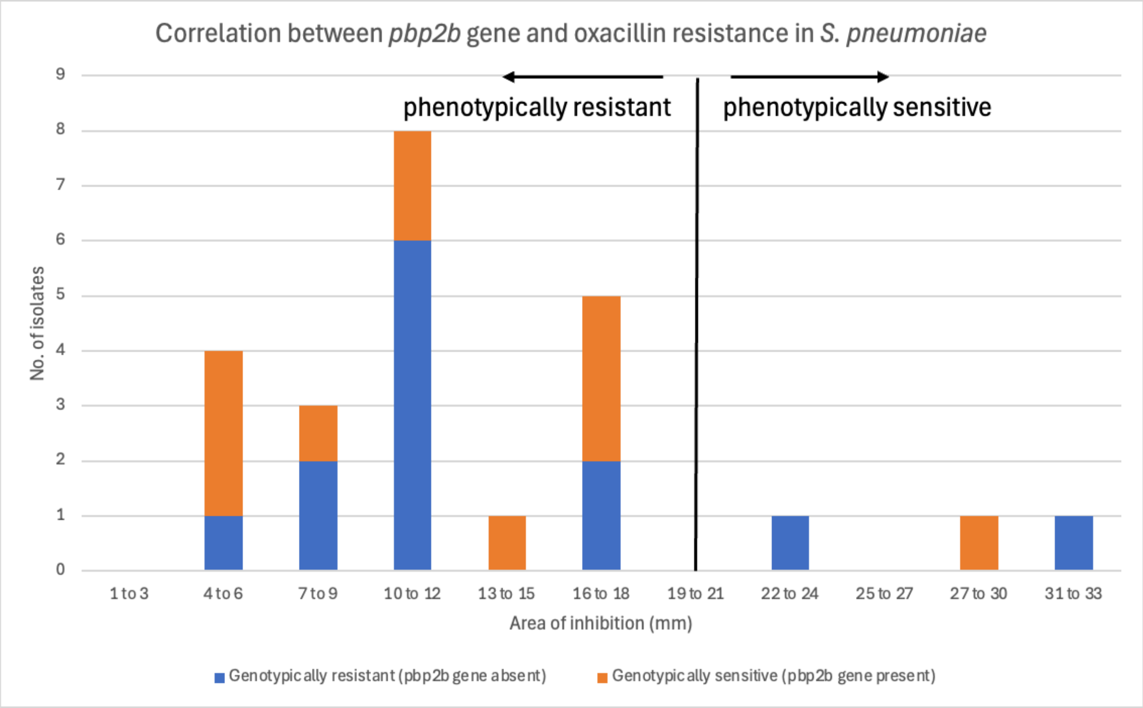


Figure 4.2: A graph showing the genotypic and phenotypic correlation of oxacillin resistance. If an isolate is *pbp2b*-negative, it is considered genotypically resistant. If an isolate has an area of inhibition of less than 20 mm in the disk diffusion assay, it is considered phenotypically resistant. If the genotypic and phenotypic data fully match, we would expect the bars to the left of the borderline to all be blue, whereas those to the right of the borderline would all be orange.

Discussion

Interpreting the results of the PCR experiments

To determine the presence of a certain gene in a sample, we analysed and compared all the qPCR and LAMP assay results. Theoretically, for qPCR, each dilution of 10 folds will decrease the Cq value by ~3.3 cycles. This pattern was observed for most samples in our two qPCR runs. For a certain sample and either the *lytA*/*mefA*/*ermB* gene, if amplification signals are detected in both qPCR runs, and the LAMP assay gives a positive result, the sample is most likely positive for the gene. If no amplification signal is detected in one or both of the qPCR runs, and the LAMP assay gives a negative result, the sample is most likely negative for the gene. However, an unusual case can be seen for the *lytA* gene (*Figures 2.1 and 2.2*). For two samples, no *lytA* gene amplification signal was detected in the first qPCR run (100-fold sample dilution), but it was detected early in the second qPCR run (10-fold sample dilution). The LAMP assays for these (*Figures 3.2 and 3.3*) also showed positive results. A possible reason for this is pipetting mistakes that may have occurred when preparing the first qPCR plate, giving inaccurate results. It could also be due to a mixed culture, in which a low amount of *S. pneumoniae* is present. Hence, for these two samples, we consider them to be *lytA*-present.

For the *pbp2b* gene, we adopted a different method to determine its presence in a sample, since we did not do the LAMP on the *pbp2b* gene. We used a criteria developed by the Great Ormond Street Hospital (GOSH), which is as follows: A penicillin-susceptible result is *pbp2b* PCR positive with a Cq value of no more than 3 cycles greater ($\Delta Cq \leq 3$) than that for the *lytA* PCR run simultaneously, while a reduced penicillin susceptibility (i.e., penicillin resistance) result is *pbp2b* PCR negative [20]. In this case, all the positive results for *pbp2b* fulfill the mentioned criteria, indicating the presence of the *pbp2b* gene.

Based on our interpretation, we also discovered that overall, there is no strong correlation between DNA concentrations, colony morphologies, and how quickly the fluorescence rises above the threshold when a sample is positive for a gene.

Genotype and phenotype comparisons

Genotypic-phenotypic discrepancies are evident in the comparison graphs (*Figures 4.1 and 4.2*). For erythromycin, 27.3% of the phenotypically resistant isolates were genotypically sensitive (P+G-), and 15.4% of the phenotypically sensitive isolates were genotypically resistant (P-G+). Meanwhile, for oxacillin, 47.6% of phenotypically resistant isolates were genotypically sensitive (P+G-), and 66.7% of the phenotypically sensitive isolates were genotypically resistant (P-G+). A possible explanation for P+G- is that there might be another gene causing the resistance, which was not targeted in the PCR experiments. It could also be that the bacterial plasmid carrying the resistance gene had been lost after the phenotypic testing [21]. Meanwhile, P-G+ is likely caused by a mutation in the gene, inactivating it and preventing it from being expressed [22]. It could also be due to a mutation in other components of the operon, e.g., in the promoter, making it non-functional. However, it is worth noting that only 24 out of 351 available samples were tested, so this discrepancies analysis is not a valid representation of the whole sampled population.

PCR sensitivity comparison

Using the ddPCR results, we estimated and compared the sensitivity of the two PCR methods employed in sample analysis: qPCR and LAMP. We marked the lowest dilution fold that each of these PCR methods can go until no more or very little gene amplification is detected. This dilution fold corresponds to a certain number of DNA copies/µl, as revealed by ddPCR. We found that LAMP is capable of detecting as little as ~50 DNA copies per µl, whereas qPCR is about 8x more sensitive than LAMP (~6 DNA copies per µl). Despite being less than that of qPCR, the sensitivity of LAMP might still be considered adequate, as we observed positive results on samples that are expected to be so based on the qPCR results.

Challenges and suggestions for improvements

Several challenges were encountered during the lab experiments and the data analysis. As mentioned earlier, some pipetting mistakes might have happened when setting up the qPCR plate. These can lead to false positives or false negatives. To minimise the possibility of misinterpretation due to pipetting mistakes, we can perform the PCR experiments in duplicates of triplicates. This would allow us to spot any false positives or false negatives caused by pipetting mistakes.

Another problem is determining the maximum Cq value where a sample is considered positive for a gene in qPCR. If the Cq value is too high, we cannot be confidently sure that it implies a positive result. Hence, as a solution, we performed ddPCR, which would allow us to calculate an endpoint. Moreover, for this reason, it is also helpful to do the qPCR experiments in duplicates or triplicates to see if the high Cq values detected are just false negatives.

Further steps

In accordance with the main aim of this project, we would need to conduct genotypic testing on the remaining samples to establish a more accurate genotypic-phenotypic comparison. Statistical analysis can then be conducted to determine if the discrepancies between the genotypes and phenotypes are significant.

Furthermore, DNA sequencing can be performed on ambiguous samples, and the sequences can be compared against available databases to confirm the presence of a gene of interest and whether or not any mutation has occurred. Mutation, when it causes a gene to be inactivated, might be one of the reasons behind genotypic-phenotypic discrepancy, specifically G+P-.

In addition, as mentioned earlier, mixed cultures were observed in some plates, possibly indicating contamination of the *S. pneumoniae* isolates with other bacterial strain(s). Hence, to see if other bacteria might be present in the mixed cultures, MALDI-TOF mass spectrometry can be performed. This gives us the mass-to-charge ratio of the components present in a sample and their relative intensity. Based on the result, we can deduce the bacterial strains that are present, given that we know the masses of different bacteria. Alternatively, whole genome sequencing can be done to show if we have a mixed culture. Furthermore, it shows whether a certain resistance gene is present and whether there are potentially other resistance mechanisms that we are not detecting.

Finally, to determine the suitability of the LAMP assay for implementation in clinical settings, further tests on sensitivity and specificity are required. To obtain a more precise sensitivity value (i.e., a more precise limit of detection in no. of DNA copies/µl), we can do the PCR experiments in a shorter range of dilution series. For example, based on our previous results, this could be $1x10^{-5}$, $8x10^{-6}$, $4x10^{-6}$, $2x10^{-6}$, and $1x10^{-6}$. Meanwhile, to determine specificity, we will need to test the LAMP primer sets on several other bacterial species. If specific, we would expect them to give negative results.

Conclusion

Based on the data obtained in this research project, we can say that the first hypothesis, stating that there would be some discrepancies between the genotype and phenotype of each sample, is accepted. It is evident that several mismatches between the genotypes and phenotypes for AMR occurred in some samples. However, to be more confident in this, we would need to repeat the PCR experiments and redo the phenotypic testing on the regrown isolates. Meanwhile, the second hypothesis, stating that qPCR is more sensitive than LAMP, is accepted. The sensitivity analysis shows that for the same dilution, qPCR is capable of detecting a lower number of DNA copies/µl than LAMP.

Since this research project is part of a bigger study, the findings here would also be useful in deciding the next steps to be taken and which specific methods are preferred. This could be choosing the DNA extraction protocol, choosing the suitable sample dilution for the qPCR and LAMP, etc.

Lastly, the satisfactory results of the LAMP assays in this project show that it has great potential to be implemented in clinical trials. The relative convenience and simplicity of the LAMP assay compared to other PCR methods are a major plus, especially for use in places with limited resources. Hence, further research and development on the technique would be worth investing in.

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