Monitoring Antimicrobial Resistance in Children Receiving Azithromycin Using Genetic Methods

Laidlaw Research Project Report

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Introduction

UCL GOS Institute of Child Health and CVD Mali

My Laidlaw research project is part of a collaborative programme with CVD Mali called LAKANA. In Bambara, the native language in the study area, LAKANA means “to protect” or “to be safe”–as the main aim of the trial is to protect children from an early death. LAKANA is testing the effect of azithromycin mass drug administration (MDA) in Mali. It focuses on all-cause child mortality reduction and antimicrobial resistance (AMR) causation. [1]

Mali had the world’s seventh highest national under-five mortality rate (U5MR) in 2020 [1], at 91 deaths per 1000 live births–this remained unchanged in 2024 [2]. The leading causes of these deaths are preventable, including respiratory infections and neonatal conditions [2]. The World Health Organisation (WHO) aims to end all preventable deaths under five years of age, making Mali a highly important target for researching ways to U5MRs–such as azithromycin MDA. [1]

The role of my project is to continue development of a simple, cheap, and fast test to monitor azithromycin resistance that can be used by the team in Mali outside of a laboratory setting (i.e. in the target villages). To do this, I tested samples with our new LAMP assay and qPCR, and compared this to whole genome sequencing and the gold-standard method of disk diffusion assay. comparing the two to each other and to other available methods. I also continued optimising our LAMP assay, ensuring it finishes in a timely manner and results stay consistent after assay completion.

Background of LAKANA

Trachoma, an ocular bacterial infection which causes blindness, is endemic to many low- and middle-income countries. The WHO recommends azithromycin MDA to treat trachoma due to azithromycin’s good safety profile and long duration of action. [3]

The first azithromycin MDA trial was TANA, which aimed to eradicate trachoma in target subkebeles (villages) in Northern Amhara, Ethiopia. There was a marked reduction in trachoma infection, but more surprisingly, a 49.6% reduction in all-cause mortality of 1- to 9-year-old children with mass oral azithromycin administration. [4]

Subsequently, the WHO approved the MORDOR trial to further investigate the potential child mortality impact of azithromycin MDA. MORDOR treated 1-month to 5-year-old children with azithromycin (or placebo) twice annually. It operated in three countries: Niger, Malawi, and Tanzania. Overall all-cause mortality reduction was 13.5%, but varied between countries, at 18.1%, 5.7%, and 3.4% respectively. This suggests there are external factors that impact the efficacy of azithromycin MDA. The highest overall reduction was in 1- to 5-month-old children, at 24.9%. [5]

MORDOR also measured AMR and found that azithromycin MDA increases only resistance to macrolides, the class of antibiotics azithromycin belongs to. This is

promising as resistance to multiple antibiotic classes makes infections much harder to treat with clinically available antibiotics. [5]

After MORDOR's completion, the WHO recognised further azithromycin MDA research for mortality reduction is necessary to determine important details–such as optimal azithromycin dose, dose frequency, and potential downsides (e.g. AMR). This led to the LAKANA trial, which tests both biannual and quarterly azithromycin administration in 1- to 11-month-old children. The age range was chosen due to the largest effect of azithromycin MDA being shown in this group. [1]

Theory

LAMP vs qPCR

The assay we are developing uses loop-mediated isothermal amplification (LAMP). This is an ideal technique for resource-limited settings in comparison to the usual genotypic technique, qPCR. [6] Amplification methods work by creating millions of copies of any target DNA present [6] (in our case azithromycin resistance genes present in *Streptococcus pneumoniae*). A comparison of the two methods is shown below.

LAMP	**qPCR**
4-6 primers	2 primers
Isothermal	Requires multiple temperature stages (denaturation, annealing, extension)
Multiple qualitative ways to detect: • Colour (pink→yellow) • Fluorescence • Turbidity	Quantitative, uses fluorescence to detect change
Takes 20-40 minutes	Takes >1.5 hours
Can use a water bath (or even a hot water bottle)	Requires specialised thermal cycling equipment
Can use unextracted samples in some cases	Requires DNA to be extracted from samples first

Figure 1 A comparison of LAMP and qPCR. Information from [6].

The main advantage of LAMP shown in figure 1 is that isothermal amplification means less complex and cheaper equipment can be used, reducing the time required. [6]

Qualitative detection can be an advantage as it requires less training, but it does also introduce more subjectivity into detection. For our LAMP assay, a positive result is a colour change from pink to yellow. This is due to the pH indicator in the reaction

mixture changing colour due to the acidic nature of the millions of target DNA copies made. [6]

Disk Diffusion Assays and Whole Genome Sequencing

Disk diffusion assays are a phenotypic method used to test if a bacterial strain is susceptible to an antibiotic. Discs are soaked in antibiotic and placed on a lawn of the bacteria. The plate is then incubated, and the area of the dead zone around the antibiotic disk is proportional to how effective the antibiotic is. This is useful as sometimes bacteria have the genes conferring antibiotic resistance but do not express them, leading to a phenotypically negative result. Also, if a bacterium has an unknown resistance mechanism, it would be genotypically negative but phenotypically positive [7].

Whole genome sequencing (WGS) is another genotypic method used to find known azithromycin resistance mechanisms–and can be used to find previously unknown resistance mechanisms if a sample is phenotypically resistant but doesn't have any known resistance mechanisms. [7]

Therefore, we compared the results of our LAMP assay with qPCR, disk diffusion assay and WGS to see how specific and sensitive our LAMP assay was. Ideally, the results of LAMP and qPCR would be the same for each sample tested as qPCR is the standard alternative method.

Our LAMP assay

Streptococcus pneumoniae

We detect azithromycin resistance in *Streptococcus pneumoniae* in our assay. This is for three main reasons:

1. *S. pneumoniae* is a nasopharyngeal organism. This means that sample can be collected with a simple nasopharyngeal swab. [8]

2. *S. pneumoniae* is common and generally commensal, but can also be infectious, particularly in young children. This means that most people have *S. pneumoniae* in their nasopharynx, so AMR present in *S. pneumoniae* is a good indicator of population AMR prevalence. [8]

3. *S. pneumoniae* can be used to test for resistance to both macrolides and β-lactams. This is because *S. pneumoniae* is a Gram-positive bacterium, which are more susceptible to β-lactams than Gram-negative bacteria. β-lactams are usually the antibiotic of choice with *S. pneumoniae* infections, so we use qPCR to look for resistance to both β-lactams and macrolides.

Azithromycin

Azithromycin is a macrolide used to treat a broad spectrum of diseases, including trachoma. [8] The structure of azithromycin is shown in Figure 2 below, with the box around its macrocyclic lactone ring.

Azithromycin binds to the 23S rRNA of the 50S bacterial ribosome subunit. The macrocyclic lactone ring fits across the exit (E) site of the ribosome, as shown in Figure 3 below. During translation, an aminoacyl-tRNA enters the aminoacyl (A) site, then transfers to the peptidyl (P) site, where it transfers the amino acid it is carrying to the growing peptide chain at the A site. Under normal conditions, the tRNA then moves to the E site and leaves the ribosome. However, when azithromycin is present the E site is blocked, so the tRNA cannot move from the P site to the E site, and cannot exit the ribosome–thereby halting protein production. [8]

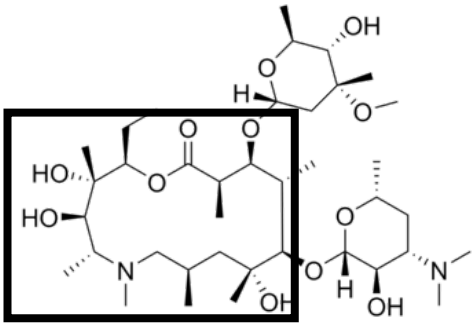

Figure 2 The structure of azithromycin

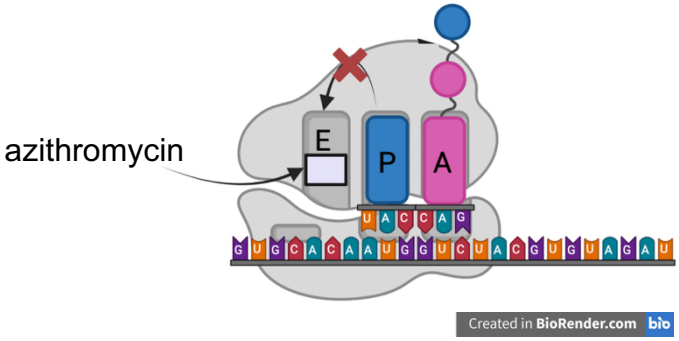

Figure 3 Diagram of azithromycin's mechanism of action

Resistance Mechanisms

There are three main mechanisms of macrolide (including azithromycin) resistance:

1. mefA encodes an efflux pump (i.e. macrolide pumped out of bacterium on entry) [8]
2. mefE also encodes an efflux pump [8]
3. ermB methylates a single adenine base on the 23S rRNA, making azithromycin and other macrolides unable to bind and block the E site [8]

There is also mel, which encodes an efflux pump. This is much rarer than the three above so it is not included in our LAMP assay as it is less relevant to population-wide AMR. [8]

Pbp2b is a resistance mechanism to penicillin (and other β-lactams) which works by changing the structure of penicillin binding proteins in the bacterial cell wall. [8] We test for this in qPCR when looking for multi-drug resistance. Due to the constraints of our current project we don't test for this in LAMP, but it is a possible future improvement of the assay.

Primers

Primers are short strands of DNA used in amplification techniques to recognise the target DNA and allow DNA polymerase to make copies. [6] In LAMP, each primer set is made up of 4-6 primers. [6] Our LAMP assay uses three primer sets:

1. AlytA: tests for presence of *Streptococcus pneumoniae* [9]
2. mef: tests for presence of the mefA or mefE efflux pump [8]
3. AermB: tests for presence of the 23S rRNA adenine methylation [8]

The A prefix denotes a different set of primers testing for the same thing (i.e. lytA primers and ermB primers also exist). We did not find ermB primers to be effective for our assay, but AermB primers are. LytA primers were found not to be effective by Celine Tedja, a Laidlaw scholar from last year, and so AlytA primers were used instead. Due to the precise way in which primer sets must work for amplification to be successful, primer sets cannot be tweaked and if they are nonfunctional a whole new set needs to be developed.

In LAMP, primer sets tend to have 1-2 loop primers (as part of the overall 4-6). These are optional but significantly speed up the target DNA amplification. [6] For our AermB set, we often tested with and without loop primers to get primer sets to work at the same time.

Results

Xu Ngai Sui, a Laidlaw scholar from Hong Kong University, also worked under Dr. Alber's supervision this summer and these tables (excluding figure 8b and 8c) are a collation of both our work.

How does LAMP compare to qPCR, phenotypic methods, and whole genome sequencing?

Comparison of LAMP with qPCR

		AlytA - LAMP	
		Positive	Negative
qPCR	Positive	91	5
	Negative	13	3

Specificity = 95%
Sensitivity = 19%

Figure 4a Comparison of LAMP AlytA results with qPCR

		mef - LAMP	
		Positive	Negative
qPCR	Positive	3	4
	Negative	9	94

Specificity = 43%
Sensitivity = 91%

Figure 4b Comparison of LAMP mef results with qPCR

		AermB - LAMP	
		Positive	Negative
qPCR	Positive	5	1
	Negative	3	97

Specificity = 83%
Sensitivity = 97%

Figure 4c Comparison of LAMP AermB results with qPCR

Figure 4 Tables comparing each of the LAMP primer sets with qPCR results, giving specificity and sensitivity measurements

Comparison of LAMP and qPCR with WGS

		mef - LAMP	
		Positive	Negative
WGS	Positive	3	1
	Negative	7	84

Specificity = 75%
Sensitivity = 92%

Figure 5a Comparison of LAMP mef results with WGS

		AermB - LAMP	
		Positive	Negative
WGS	Positive	3	3
	Negative	5	86

Specificity = 50%
Sensitivity = 95%

Figure 5b Comparison of LAMP AermB results with WGS

		WGS	
		Positive	Negative
qPCR	Positive	4	4
	Negative	4	46

Specificity = 50%
Sensitivity = 92%

Figure 5c Comparison of WGS results with qPCR

Figure 5 Tables comparing each of the LAMP mefA and AermB sets with WGS, and qPCR with WGS

Comparison of phenotypic testing with LAMP, qPCR, and WGS

		LAMP	
		Positive	Negative
Pheno	Positive	7	9
	Negative	16	80

Specificity = 44%
Sensitivity = 84%

Figure 6a Comparison of LAMP results with phenotypic testing

		qPCR	
		Positive	Negative
Pheno	Positive	6	7
	Negative	5	90

Specificity = 46%
Sensitivity = 95%

Figure 6b Comparison of qPCR results with phenotypic testing

		WGS	
		Positive	Negative
Pheno	Positive	8	10
	Negative	3	87

Specificity = 44%
Sensitivity = 97%

Figure 6c Comparison of WGS results with phenotypic testing

Figure 6 Tables comparing each of LAMP, qPCR, and WGS with phenotypic testing

How do we optimise our LAMP assay for AlytA, mef, and AermB?

Our aim was for all three primer sets to work at the same time. Using the same conditions for all three is ideal as it makes the assay very simple, and makes errors due to experimental conditions less likely.

65°C

Time (minutes)	AlytA	mef	AermB with loop	AermB without loop
20	✗	✗	✓	✓
30	✓	✓	✗	✓
40	✓	✓	✗	✗

Figure 7a Table showing performance of primer sets at 65°C with red (✗) being poor results and green (✓) being good results

63°C

Time (minutes)	AlytA	mef	AermB with loop	AermB without loop
20	✗	✓	✓	✗
30	✗	✗	✓	✓
40	✗	✗	✓	✓

Figure 7b Table showing performance of primer sets at 63°C with red (✗) being poor results and green (✓) being good results

As AlytA was not working at all at 63°C, I tested five different temperatures: 59°C, 61°C, 63°C, 65°C, 67°C. The only temperature that worked well for all three sets (61°C) is shown below.

61°C

Time (minutes)	AlytA	mef	AermB with loop
20	X	X	X
30	X	X	X
40	✓	✓	✓

Figure 7c Table showing performance of primer sets at 61°C with red (X) being poor results and green (✓) being good results

Discussion

Comparison of LAMP with qPCR, phenotypic methods, and whole genome sequencing

Figure 4

Comparison of figure 4a, 4b, and 4c shows our LAMP assay and qPCR are generally comparable, except for a couple of surprisingly low statistics.

A specificity of 43% is shown in figure 4b, looking at mef LAMP versus qPCR. This is very low but may be partially due to the low number of positive results, meaning any negative results on LAMP that are positive on qPCR have a large statistical effect. Further testing and comparison should be carried out to see if this changes.

A sensitivity of 19% is shown in figure 4a, looking at AlytA LAMP versus qPCR. This is worryingly low but suggests issues with the AlytA primer set rather than the LAMP assay as a technique. Optimisation of the LAMP assay and testing occurred concurrently, so these results may have been skewed by subjectivity of interpretation pertaining to intermediate results. Now that promising conditions for all 3 primer sets have been found (61°C for 40 minutes), these samples should be retested and statistics recalculated.

Figure 5

Comparison of figure 5a, 5b, and 5c shows our LAMP assay performs similarly to qPCR in comparison with WGS (AlytA is not included as bacterial species is not tested for by WGS). This is promising, as it shows our LAMP assay is comparable to qPCR, which is the standard technique that we are developing our assay against. It also suggests issues shown in figure 4 are due to incomplete assay optimisation rather than an inherent disadvantage that LAMP is conferring in comparison to qPCR.

Figure 6

Comparison of figure 6a, 6b, and 6c shows LAMP, qPCR, and WGS to have similar results in comparison to phenotypic testing, whilst a slightly reduced sensitivity is present in LAMP compared to qPCR and WGS. When comparing genotypic and phenotypic techniques, all resistance types in the genotypic technique are bucketed

together, as phenotypic testing cannot differentiate between resistance mechanisms. [7]

It is promising that overall our LAMP assay performs similarly to other genotypic methods, and suggests issues shown in figure 4 are due to incomplete assay optimisation rather than unsuitability of the technique for detecting azithromycin resistance. Disk diffusion assays, a phenotypic method, are known to be more accurate in detecting expression of AMR, but this is significantly more resource and training-intensive, so is not suitable outside of a laboratory environment, unlike LAMP. [7]

Optimisation of LAMP assay for AlytA, mefA, and AermB

A poor result denoted by red means many intermediate results, which due to the qualitative nature of detection, are unreadable. A good result denoted by green denotes very few/no intermediate results.

As shown in figure 7a, AermB doesn't work at the same time as mefA and AlytA at 65°C, and even while detection is possible after 30 minutes, such a short window for checking results (<10 minutes), isn't feasible in realistic testing settings.

Xu Ngai Sui, who also worked on the project in the beginning, found AermB worked well at 63°C but, as shown in figure 7b, when I tested with the other primer sets it was clear it was not a feasible temperature.

As shown in figure 7c, 61°C is a promising condition as all 3 primer sets begin to work at the same time (40 minutes), but further testing needs to be done to determine if these results are accurate and would stay consistent if read late.

Further Research

Further research avenues for this project include:

1. Comparison of intermediate results with phenotypic results and qPCR, are any intermediates positive, as was the case with a SARS-CoV-2 LAMP assay [10]
2. Re-test qPCR samples to fix *lytA* primer issues
3. Further testing of 61°C to analyse consistency of results after >40 minutes
4. Testing of unextracted samples using nasopharyngeal swabs and comparison with other techniques as not needing to extract the DNA would make the testing process even faster and cheaper
5. Testing of other bacteria common in the nasopharynx to check for lack of cross reaction (i.e. false positives) – this has already been done for *Streptococcus pyogenes* (group A strep)

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