Validation of Loop Mediated Isothermal Amplification Technique (LAMP) to Diagnose *Tuberculosis* (TB) in Sri Lanka.

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Introduction

Large civilian populations living in countries that are affected by complex emergencies such as war or civil unrest, natural disasters, malnutrition and population displacement are burdened with increased mortality and morbidity due to preventable communicable diseases including tuberculosis (TB) (1). Many such calamities occur in developing countries including Sri Lanka, which also bear the burden of TB.

Tuberculosis, caused by *Mycobacterium tuberculosis* (*MTB*), remains one of the deadliest diseases in the world. According to estimations from the World Health Organization (WHO), 9.4 million new cases and 1.8 million deaths occured due to TB in 2008. Southeast Asia counts for more than 33% of all TB patients in the world (2). In Sri Lanka, nearly 17,000 people are estimated to have TB and around 11,000 new cases are being reported annually. Among these newly diagnosed cases 1.4% have been identified to have multidrug resistant-TB (MDR-TB) (2). Further, according to WHO data, the percentage of TB incidence in the country had been increasing over the last decade. Proper TB control programmes; detecting TB positive cases and successfully treating them without increasing the rate of MDR-TB; are therefore a necessity to prevent any future outbreaks of the disease.

A critical obstacle for proper TB control is the lack of early and accurate detection of the disease. Microbiological methods such as microscopy and culture of clinical samples and nucleic acid amplification based methods like PCR are the currently available diagnostic tools for TB (3-5). However, in many developing countries, including Sri Lanka, microscopy and culture are widely used in screening and detecting TB cases, and in many instances microscopy is the only confirmatory test available. However, there are many shortcomings with these traditional methods. In Microscopy, the sensitivity is low and can detect only up to the genus level of the bacterium. Therefore, inaccurate reporting and misdiagnosing can occur. Culture method can specifically identify *Mycobacterium tuberculosis*, however, is very time consuming. Molecular level methods based on PCR technology are also available for more specific diagnosis (4,5); however, complexity of the procedure and the requirement for sophisticated and costly instruments and trained technical support makes it an expensive tool unsuitable for developing countries like Sri Lanka. Partly as a result of this complexity in diagnosis at resource-limited settings, TB control has usually been given less priority among other communicable diseases (6). The situation becomes worse in complex emergency situations. However, if TB is neglected, there is a high risk that it may continue to increase the morbidity and mortality rate, as was previously seen in some African countries (7). Therefore, development of new low cost, less sophisticated, testing methods with high sensitivity and specificity will help greatly improve the early detection and treatment of TB in developing countries, including Sri Lanka.

A recently developed molecular level method called loop mediated isothermal amplification (LAMP) is a promising tool for rapid diagnosis of infectious diseases including TB with minimal infrastructure requirements and technical training, suitable for resource-limited developing countries like Sri Lanka (8-12). Unlike PCR, LAMP is performed at a single constant temperature with no thermal cycling and can be completed within 60-120 minutes. The specificity of the test is high as the reaction uses four primers that are specifically designed to recognize six distinct regions of the target bacterial DNA (10). Furthermore this technique does not require sophisticated equipment or special laboratory facilities. In a nutshell, LAMP is a method, which can be adapted even as a field test to rapidly and accurately diagnose TB at a low cost. Such a technology will particularly benefit the northern and eastern populations, including both civilians and armed forces, of Sri Lankans who are in the rehabilitation phase of postwar communities with challenging infrastructure. Also, it can be used for point-of-care testing at hospitals and primary care facilities, and will help improve TB control of the whole country.

Considering the above concerns, this study was initiated to research the ability of LAMP to diagnose TB in Sri Lanka, at a low cost with minimal resources. Here, the LAMP method was standardized, to suit local settings, for the detection of *Mycobacterium* to the genus level as well as *Mycobacterium tuberculosis* to the species level, using a standard MTB strain H37 Rv. Further, the ability of LAMP to detect TB in already positive cultures of mycobacteria or MTB was also investigated.

Methodology

Study plan

In the first phase of the study, a culture of standard MTB strain H37 Rv was subjected to LAMP assay to optimize the method to suit local settings. Secondly, culture isolates previously collected from patients with suspected TB and later diagnosed to be *Mycobacterium* positive, in the –70C storage at microbiology laboratory of Faculty of medicine, University of Ruhuna and Welisara Chest clinic were subjected to the LAMP assay. Some of these have been well characterized to be MTB by microscopy and culture while the identification of others remains at the genus level.

LAMP assay

Two LAMP reactions were performed. One assay uses universal primers, for *Mycobacterium* genus, that are specific for universally conserved mycobacterium 16S ribosomal DNA sequences. The other LAMP reaction is specific for MTB with primers targeting a gyrB gene sequence (12), which is found only in MTB species.

Colonies in 100 ul of culture were lysed by incubating in a boiling water bath, for 10min, and the lysate was used for LAMP reaction. The heated sample (11ul) was added to the reaction tube in which the reaction mix (14ul), including Bst DNA polymerase (20u), DNA primers (universal or MTB specific) (1.6uM each inner primer, 0.2 uM each outer primer and 0.8uM each loop primer), had been reconstituted with the amplification buffer. Amplification was carried out at constant 65°C temperature for 45 min. The reaction was terminated by heating in a boiling water bath for 2 min to inactivate the polymerase. LAMP amplified product in the reaction tube was directly detected

visually by adding 1ul of 1/10 diluted original SYBR green I DNA stain to the tube and observing the colour of the solution by naked eye or under UV light. The solution turned green in the presence of a LAMP amplified product (a positive test) while it remained orange in the absence of amplification (a negative test). In the negative control, Bst DNA polymerase was omitted from the reaction mix. A schematic representation of the complete procedure is illustrated in Figure 1.

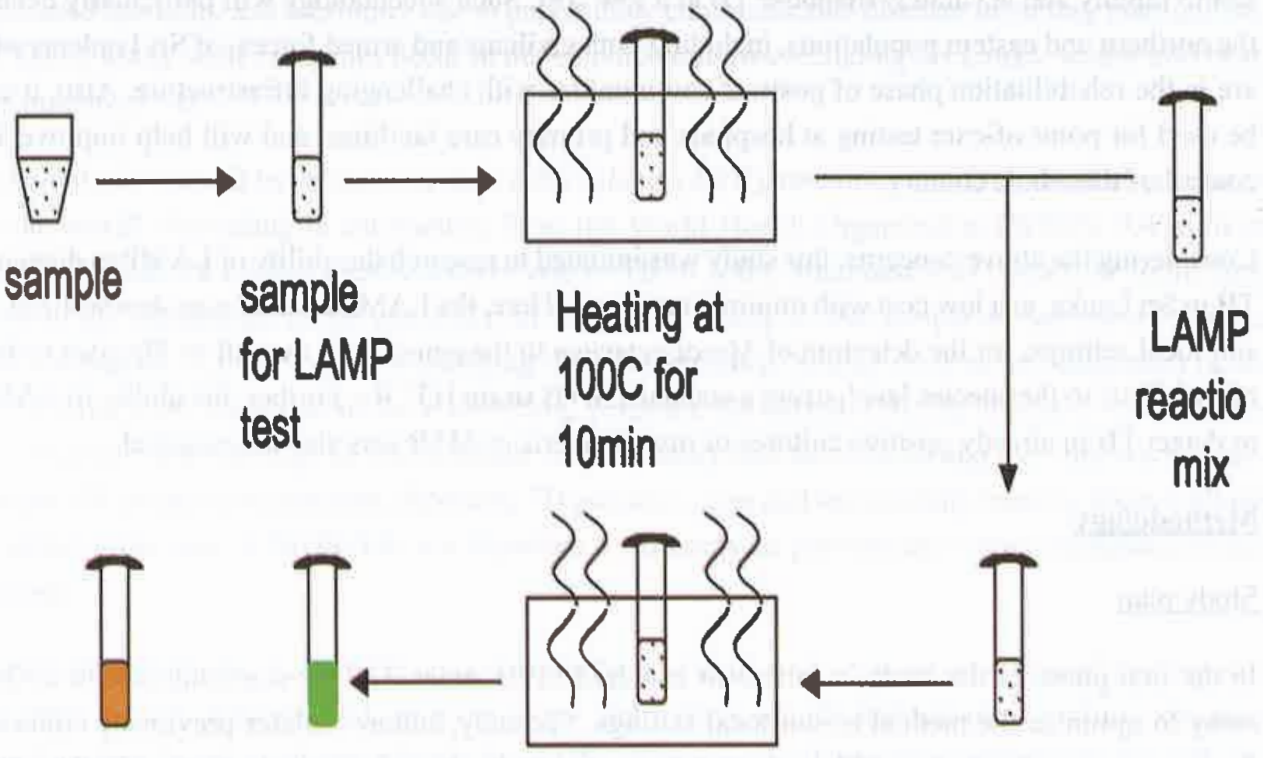

Figure 1: Schematic representation of LAMP assay procedure.

Results and Discussion

Two LAMP assays were performed with the standard MTB strain H37 Rv either with universal primers or MTB specific primers. In both tests the final reaction turned green upon addition of ZYBR green. The colour development could be visualized both by the naked eye and under UV light. Figure 2 illustrates the results of an above experiment conducted with universal primers. The same was observed in the LAMP test with MTB specific primers.

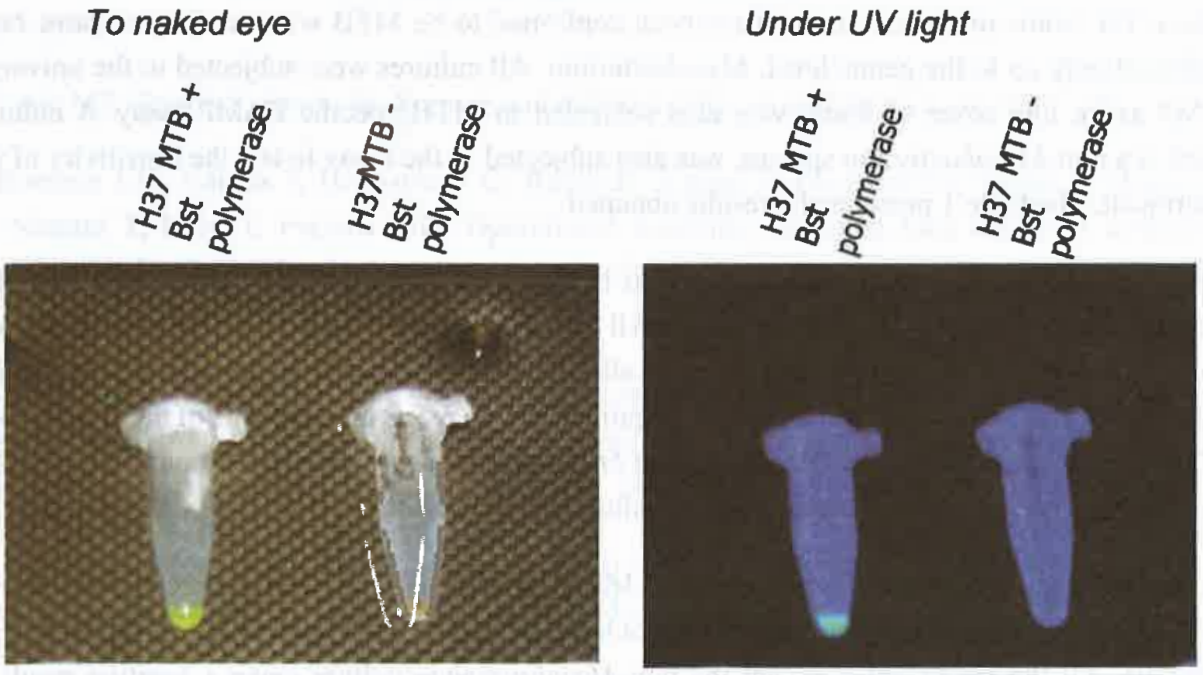

Figure 2: LAMP assay of MTB strain H37.

MTB strain H37 was subjected to LAMP assay with universal primers. In the negative control, Bst polymerase was omitted from the reaction mix. The colour of the final reaction mix after addition of ZYBR green has been visualized by the naked eye and under the UV light.

Table 1: Results for the lamp assay tested with universal primers and MTB specific primers for previous ly isolated cultutes and the Standard MTB culture.

Number of Samples	LAMP Assay		Result		Inference	
	With universal *primers*	**With *MTB specific primers***	**With universal *primers***	**With *MTB specific primers***	**With culture technique**	**With LAMP technique**
6	tested	tested	+ve	-ve	mycobacteria	mycobacteria
1	tested	tested	+ve	+ve	mycobacteria	*M. Tuberculosis*
6	tested	not-tested	+ve	?	*M . Tuberculosis*	myco bacteria
1	tested	not-tested	-ve	?	Non-mycobacteria	Non-mycobacteria

LAMP experiments were also conducted with some cultures prepared from samples suspected to have TB. Some of these cultures have been confirmed to be MTB whereas the rest have been confirmed only up to the genus level, Mycobacterium. All cultures were subjected to the universal LAMP assay, and some of them were also subjected to MTB specific LAMP assay. A culture, which is a non-*Mycobacterium* species, was also subjected to the assay to test the sensitivity of the experiment. The table 1 presents the results obtained.

Seven cultures, which have been confirmed to belong to *Mycobacterium* genus, were subjected to both universal and MTB specific assays. All cultures were positive with the universal assay confirming that they are mycobacteria. Out of all, one sample was also positive for MTB specific LAMP assay and the other six cultures were negative. This suggests that only one of those cultures is MTB, whereas the rest are mycobacteria but not *Mycobacterium tuberculosis*. To confirm this result, these cultures need be further tested with the culture technique.

Another seven cultures, one of which is a non-*Mycobacterium* culture, were subjected to universal LAMP assay. All the other six cultures in this lot have been confirmed to be MTB, using the culture technique. All the six samples except the non-*Mycobacterium* culture, gave a positive result to universal LAMP assay correctly identifying them as Mycobacterium. To check the ability of LAMP assay to identify MTB to the species level, LAMP assays need be repeated on these cultures with MTB specific primers.

In conclusion, it can be said that the standardization of the technique, to suit the local settings, was successfully completed with the H37 MTB standard culture subjected to the assay with both universal and MTB specific primers. Further, the LAMP assay with universal primers successfully identifies *Mycobacterium* at genus level. The assay also effectively discriminate non-*Mycobacterium* cultures. The ability of the LAMP test to specifically identify MTB, with specific primers needs more experiments, which are currently in progress.

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