Advances in Nucleic Acid Detection and Quantification


Nucleic acid detection and quantification in the developing world

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Abstract
Techniques using nucleic acid amplification have not had the same amount of impact on research and clinical diagnosis in the developing world as that observed in the West. This is unsurprising when the costs and infrastructure required to perform nucleic acid amplification are considered. Despite this, nucleic acid amplification is being increasingly used in both research and diagnosis in countries such as Zambia and Tanzania. Scientific research in the developing world is made possible through the support and development of the necessary laboratory infrastructure and the establishment of special transport for the reagents and samples. This has enabled world-leading country-relevant research to be performed by local scientists on subjects ranging from rapid diagnosis of infectious diseases to measuring the RNA gene expression in an immune response. Concomitantly, the challenge presented by the need for tests that are more appropriate for a resource-poor setting has led to a number of newer methodologies for nucleic acid detection, which can be tailored to be performed in the field without the need for training in molecular biology. As nucleic acid amplification techniques become both simpler and cheaper, their impact is likely to play an increasingly crucial role in research and diagnosis in the developing world.

Introduction
The humanitarian and economic costs of infectious diseases can be greatly reduced by accurate diagnosis, enabling prompt treatment. Globally, infectious disease burden is highest in regions with the least developed health-care infrastructures. Of the three major infectious diseases targeted by the MDGs (Millennium Development Goals), rapid point of care tests for malaria and HIV are available and work, but those for TB (tuberculosis) have not performed well [1]. In the absence of these, NAATs (nucleic acid amplification tests) provide rapid methods for TB diagnosis, speciation and drug-susceptibility assessment. In the present article, with specific reference to TB, we outline the current status of NAATs in a developing world setting, sub-Saharan Africa. We also address the immediate hurdles for expansion of this approach and discuss the potential of NAATs to significantly improve point of care infectious disease diagnosis in this region.

TB is caused by the bacterium Mycobacterium tuberculosis, which is predicted to infect >2 billion people worldwide, causing ~1.6 million deaths per annum. Sub-Saharan Africa has the greatest TB burden with 350 people per 100 000 population suffering from active disease of which up to as many as 75% are HIV-positive [2]. This makes the MDG of halving TB mortality by 2015 an extremely difficult task in this region. The most common investigative TB diagnosis tool for much of the world, smear microscopy, is insensitive at best and useless for paucibacillary samples (termed smear-negative TB), a common manifestation in HIV-positive individuals. Culture is far more sensitive, but confirmation of infection by culture may take up to 8 weeks. Furthermore, of the 22 countries

Key words: developing country, developing world, diagnosis, nucleic acid amplification test, sub-Saharan Africa, tuberculosis.

Abbreviations used: LAMP, loop-mediated isothermal amplification; LIPA, line probe assay; MDG, Millennium Development Goal; NAAT, nucleic acid amplification test; NTM, non-tuberculous mycobacterium; TB, tuberculosis; WHO, World Health Organization.

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in the world with the highest disease burden, there are only 17 national reference laboratories with *M. tuberculosis* culturing facilities [3]. It is clear that due to resource limitations at many levels, NAATs are perhaps not the obvious choice for disease diagnosis in developing countries, but the added value provided by these technologies could be huge.

Apart from the increase in speed of diagnosis attainable with NAATs, these techniques can also offer much more than just a yes/no answer for diagnosis in many cases, which is a valuable tool for disease treatment where the infectious agent cannot be cultured (e.g. pneumocystis pneumonia [4]) or differentiated without molecular analysis (e.g. HIV subtyping in positive individuals [5]), and can also enable quantification of infection (e.g. monitoring of treatment in *Trypanosoma cruzi* infections [6]). With increasing research collaborations between countries across the development divide, NAATs are used in the developing world, although the vast majority are tied to answering research questions rather than employed in routine diagnostics. In the present paper, we outline some of the considerations required to implement these techniques in a developing world setting.

**Using NAATs in the developing world**

The ability of any new diagnostic method to provide useful results is inherently linked to the ability to use that test appropriately. In a resource-poor setting, fundamental consideration needs to be given to limiting factors.

Although human resources are often not limiting in developing countries, the skill base necessary to be trained for NAAT frequently is. The people who are most likely to be easily trained in new techniques are those with an existing level of professionalism in the healthcare field: normally nurses, doctors or laboratory staff. These health-care professionals are most likely to be found at the periphery hospital, where they are also likely to have a high workload. It has been estimated that to increase the capacity of smear microscopy globally to reach the increased detection rate necessary for the MDGs, an additional 23,000 laboratory technicians are required globally [7]. Such shortcomings in human resources represent a considerable bottleneck for the introduction of any new diagnostic method.

The minimal requirements to perform successful NAATs include correct storage and preparation of the sample before analysis, amplification and detection of the amplified product. All these steps require electricity from a generated source: for refrigeration, preparation (e.g. centrifugation) and incubation. Modifications to conventional methodology are slowly reducing the reliance on electricity from a generated source, by removing the need for refrigeration and using battery-operated equipment, but a NAAT suitable for TB diagnosis in the developing world remains at least 4 years in the future (http://www.finddiagnostics.org/activities/tb/tb_pipeline.shtml) with most of the NAATs being developed for the peripheral laboratory level or above [8]. Perhaps the closest to application for TB diagnosis is the LAMP (loop-mediated isothermal amplification) approach [9] for which sensitive assays have been developed for a number of infectious diseases prevalent in the developing world [10–15]. Thus developments of such a platform could significantly improve disease diagnosis for a number of diseases at once. The current LAMP approach to *M. tuberculosis* DNA detection removes the requirement for expensive thermocyclers and can be assessed by eye, but still requires constant incubation at an elevated temperature (63°C) [16,17].

For NAATs, a reliable, controlled electrical supply is currently essential for correct assay execution and accurate results. Where electrical infrastructure is basic, power surges and unexpected cuts can cause considerable damage to equipment, waste valuable samples, reagents and time, making the protection of a UPS (universal power supply) for each piece of equipment essential. The application of NAATs for TB diagnosis has the added advantage that they require the same biosafety risk as microscopy. A laboratory performing *M. tuberculosis* culture requires BSL3 (Biosafety Level 3) containment and thus a greater infrastructure requirement than for an NAAT.

In addition to local infrastructure and resources, performing NAAT in the developing world requires establishing supply chains for consumables. This can prove challenging, not only because orders can take much longer to arrive, but also because many of the necessary reagents must be kept below freezing; consequently, a cold chain must be set up, which can be complex to maintain [18]. This can add considerably to the costs of the reagents/test. Where research laboratories are few and far between, economies of scale are not possible. For routine diagnosis using an NAAT, the cost savings in terms of speed of diagnosis must be balanced against the costs of shipping reagents and consumables in such a volume as to use them efficiently and cost effectively. Experimentation with approaches that simplify transport, e.g. freeze-dried reagents [19], will be a welcome contribution to assist in performing such techniques in the developing world.

**Are TB NAATs appropriate for the developing world?**

It is well established that effective disease treatment can have considerable economic advantage, as shown by the implementation of highly active anti-retroviral treatment for AIDS [20]. Where the existing diagnostics are affordable, sensitive and quantitative, such as microscopical determination of *Plasmodium falciparum* malaria in a highly endemic region, the economic benefits of a move to NAAT-based technologies for diagnosis are debatable [21]. However, for TB, where sputum smear microscopy is estimated to be able to only detect at best 60% of active pulmonary infections [22], a more expensive NAAT could still remain economically viable.

In addition, the complex picture of TB in HIV-positive individuals can be impossible to differentiate in a timely manner without NAAT technology. HIV-infected individuals are more likely to be infected with NTMs (non-tuberculous mycobacteria), which are indistinguishable by the newer liquid culture methods and smear microscopy, but require a different treatment regimen [23]. Commercially
available LIPAs (line probe assays) have been used for exactly this purpose in sub-Saharan Africa [24]. Although the direct determination of M. tuberculosis complex using sputum is possible, clinical use of the LIPA-based speciation of mycobacteria remains at present an adjunct to culture and therefore restricted to the referral laboratory setting.

NTMs can be confused with drug-resistant M. tuberculosis, as neither is treatable by standard TB regimens. It is likely that the prevalence of drug-resistant TB is severely underestimated in sub-Saharan Africa and that this will increase due to incomplete drug adherence and the inability to perform conventional drug susceptibility testing to identify and manage the problem [25]. Without rapid identification of drug resistance, the continued use of conventional therapy serves to perpetuate the problem: first, the patient will not get better; secondly, the strain they are infected with may develop further resistance to other drugs within the treatment regimen (which comprises four drugs at once); and thirdly, the patient is at risk of passing the drug-resistant strain on to another person. The emergence of an epidemic of drug-resistant TB would transfer physicians into a pre-antibiotic era. NAATs provide the only feasible method by which this problem can be managed fast enough to impact on clinical management and infection control. As such, the WHO (World Health Organization) now advocates the use of the LIPA as the method of choice for identification of drug-resistant mycobacteria (http://www.who.int/tb/features_archive/policy_statement.pdf).

**Geographical considerations**

For infectious disease NAATs developed for the Europe and North American markets to be useful in the developing world, they must be appropriate for the local disease genotype. In NAAT HIV diagnoses, viral load can be considerably underestimated if applying commercially available HIV tests developed for Europe and North America in developing world settings [26,27]. A number of in-house approaches to enable identification and quantification of developing world subtypes have been produced, which at present all rely on real-time quantitative PCR platforms [5]. There is some debate as to whether newer diagnostic NAAT would be better for the developing world if they are developed commercially or as in-house tests [28]. Certainly the former tends to require less technical expertise; there is usually commercial assistance in test interpretation, training and troubleshooting. This comes at increased cost and there can be a dependence on one supplier, but commercial tests offer a simpler approach when infrastructure and expertise are limiting. However, it is worth noting that almost all current TB diagnosis is performed by non-commercially driven microscopy; furthermore, whether a diagnostic NAAT is commercial or in-house, it will require quality control.

The ability to detect the appropriate strain of the infectious agent for the disease setting has been discussed above, yet for a disease such as TB, consideration needs to be given to the clinical manifestation. Certain populations may be more likely to develop different presentations of TB [29] and the occurrence of paucibacillary disease is far more common in resource-poor settings where HIV prevalence is high [30]. Well-established commercial NAATs such as the Roche Amplicor and LCx M TB test from Abbott have limited sensitivity when smear-negative pulmonary specimens are used [31,32]. In-house-developed NAATs have proved utility over culture in the diagnosis of TB meningitis [33], a common manifestation in children in countries with high disease burden. Our own work to diagnose TB by targeting the M. tuberculosis trans-renal DNA present in the patient's urine (http://ec.europa.eu/research/health/poverty-diseases/projects/159_en.htm) could have considerable application for the disseminated TB form, which is common in HIV-positive people and in those from whom it is difficult to obtain sputum, such as children.

**Flexible platforms**

By far the most important piece of equipment for the diagnosis of many infectious diseases is the light microscope. As well as enabling accurate and fast diagnosis, it is not limited to one disease or clinical specimen. In many ways, the thermocycler shares the microscope’s versatility for disease diagnosis with the specificity being produced by the primers and/or specific oligonucleotide probes. However, without continued care in upkeep and an understanding of how to use the machine, even the best laboratory equipment can become obsolete. Even if the equipment itself remains fully functional, the absence of the right consumables can again render it useless. Where microscopy has another major advantage is that it is already established. Although the expertise required to troubleshoot a NAAT result is no more taxing than a microscopy result, the lack of familiarity represents a considerable hurdle.

As molecular tools evolve, so the essential laboratory equipment necessary to complete the task will too. It is vital that a NAAT transferred to the resource-poor setting is able to keep pace with developments in protocol and application. Both the LIPA- and LAMP-based NAATs for TB assessment are able to build on existing technology normally available in a research laboratory, such as a conventional PCR machine. The self-contained automated system-based NAATs such as Cepheid’s GeneXpert do reduce the likelihood of contamination and the need for training, but constitute a large single investment for a platform limited to only a few infectious diseases. The specific consumables required for such systems make them reliant on the commercial supply and expensive when compared with in-house methods [34]. However, if such platforms are able to reduce the user variation associated with NAAT diagnosis of diseases such as TB [35] while maintaining efficacy, their impact could be considerable.

**Usable results**

The proposed benefits through improved diagnosis of TB using NAATs in the developing world are totally dependent on the availability of appropriate treatment of disease. The promotion of the treatment by the WHO and the Stop TB
Partnership has made considerable advancement towards correctly administered and monitored therapy; however, case detection in sub-Saharan Africa is likely to be much lower than the global 59% reported in 2006 [36]. The current indications of a reduction in disease mortality and morbidity in both malaria and HIV are due to an integrated approach to diagnosis, treatment and monitoring. Simplified NAATs, such as LAMP, to enable improved diagnosis of TB could still have a huge impact on disease diagnosis if integrated with a functioning referral system and combined with HIV diagnosis and treatment [37,38].

Conclusion

Improved disease diagnosis in the developing world can be achieved through the application of the appropriate NAAT for the setting and disease. Considerable attention to quality assurance of reagents and equipment and quality control for diagnostic measures need to be ensured if NAAT-based TB diagnosis is to realize its full potential. The future of disease diagnosis is not reliant on stepwise increments in development; top-down and bottom-up approaches are possibly equally appropriate. What matters is that a quick, reliable diagnosis that can lead to prompt and appropriate treatment is possible. NAATs can and are used in resource-poor settings and their use is likely to increase in the next 5 years, providing a valuable contribution to the research, diagnosis and management of TB.

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