Development of a Linear After The Exponential PCR assay for the efficient diagnosis of Multi-Drug Resistant Tuberculosis

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Abstract

Multi-Drug Resistant Tuberculosis is an emerging threat to the global control of tuberculosis. MDR-TB is the product of single-nucleotide polymorphisms on the genome sequence of drug-susceptible TB, which render it resistant to at least rifampin (RIF) and isoniazid (INH), the two first-line drugs typically used to treat TB. Resistance to RIF is a reliable indicator of multi-drug resistance. Disease hotspots have been determined to be primarily in South Asia, specifically in China, India and the Russian Federation. The disease is a growing cause for concern in Sub-Saharan Africa, home to 60% of all HIV affected individuals worldwide, as HIV infection is a primary risk factor of MDR-TB and it has a 90% mortality rate among HIV patients. Currently, the most rapid methods of MDR-TB detection use symmetric polymerase chain reaction to amplify relevant gene sequences to detect mutations by use of fluorescent molecular beacons. These assays, while successful at detecting MDR-TB, are not as accurate or informative. In addition, there is currently no method for rapid and reliable testing for MDR-TB in the field. This thesis provides a quick, reliable asymmetric PCR assay for the detection of MDR-TB using Linear-After-The-Exponential-PCR (LATE-PCR). This assay is sensitive enough detect single nucleotide changes that give rise to drug resistance in as low as 10 copies of MDR-TB, even in the presence of 10,000,000 human genomes, and is designed to be used either in laboratory settings or in the field using the Bio-Seeq, a portable PCR machine designed by Smiths Detection.

Introduction

Bacterial History, Morphology and Life Cycle

Mycobacterium Tuberculosis (MTB) is the etiologic agent responsible for causing Tuberculosis (TB), the most lethal infectious disease for humans worldwide [Cole et al, 1998]. Humans are the only reservoir for this pathogen [Todar, 2009]. The tubercle bacillus was first discovered by Robert Koch in 1882. Four variants of the bacillus exist, M.tuberculosis, M.bovis, M.africanum and M.microti [Daniel, 2006]. The latter three are all pathogenic to animals [Gordon et al, 2009]. It is thought that an early progenitor of the M.tuberculosis complex (MTBC), containing all four variants, arose from a soil bacterium 3 million years ago in East Africa [Daniel, 2006]. A novel
fifth member of the MTBC was identified in 1969, M.canetti [Vincent, 2002]. All of the MTBC members have 99.9% sequence similarity, but can be distinguished on the basis of synonymous single-nucleotide polymorphisms (sSNPs) [Smith, 2003]. Initially, archaeological evidence of human infection by M.bovis led to the hypothesis that infection occurred from consumption of contaminated milk, and M.Bovis could be an evolutionary pre-cursor of MTB [Cole et al, 1998]. This was later disproved by sSNP analysis, which suggested that both strains evolved independently at the same time, possibly from a common precursor related to M.canetti [Smith, 2003].

M.tuberculosis is classified under the phylum Actinobacteria, genus Mycobacterium, of the family Mycobacteriaceae. It is a small, non-motile, aerobic, rod-like bacillus about 2-4μm in length and 0.2-0.5μm in diameter [Todar, 2009]. It is Gram positive, with a complex cell envelope consisting of a massive core of peptidoglycan, beyond which is an additional layer rich in unusual lipids such as mycolic acids, lipoarabinomannan, arabinogalactan, phenolthiocerol and mycoleric acid [Cole et al, 1998]. The lipids influence such factors of the bacterium as longevity, inflammatory host-response and pathogenicity [Cole et al, 1998]. The hydrophobicity of the lipids and the overall thickness of the cell wall provide protection against noxious molecules, but hinder export of bacterial products, [Champlon et al, 2007]. Consequently the bacterium developed an additional protein secretion system in addition to the Sec A and Tat pathways already present, the ESX-1 secretion system [Abdallah et al, 2007]. Also, the impermeability of the cell wall contributes to a unique pattern of dye staining: the high content of mycolic acid ensures poor dye absorption but high retention [Todar, 2009]. Gram staining is faint, thereby requiring the use of the Ziehl-Neelson staining procedure to characterize the pathogen [Davies, 2007]. Addition of acid prevents decolorization, and the bacterium is termed acid-fast [Davies, 2007]. The Middlebrook and Lowenstein-Jensen media are used to grow MTB cultures. Visual colonies are small and buff-colored and typically appear in 4-6 weeks.

M.tuberculosis has a long generation period of 12 to 18 hours as compared to other bacteria such as E.coli, which divide once every twenty minutes.

**Epidemiology**

As of 1993, TB was declared a global emergency by the World Health Organization, with 8 million new cases and more than 2 million deaths attributed to the disease each year. Poor adherence to treatment regimen by infected patients as well as inefficient control programmes led to the emergence of multidrug-resistant tuberculosis (MDR-TB), defined by resistance to at least rifampicin and isoniazid, the two first line drugs used to treat the disease. MDR-TB is already at critical levels in certain regions of the world, and as such, implementation of proper control measures is of vital importance to hinder further advancement of the disease.
Although the current definition only includes resistance to isoniazid and rifampin, the TB bacterium has the ability to acquire resistance to all of the drugs used against it. This necessitates the use of combination therapy to prevent or decrease the likelihood of drug resistance. The typical treatment regimen for previously untreated individuals affected with drug susceptible TB is a two month course of isoniazid, rifampin, ethambutol, and pyrazinamide. For MDR-TB however, serious difficulties in treatment arise due to resistance to the two first line drugs, rifampin and isoniazid, and as such, individualized treatment schedules based upon patient medication history and drug susceptibility tests (DSTs) are recommended. The exact number of second-line drugs in an MDR-TB treatment regimen is unknown. Also, the lower potency of second-line drugs requires high-end dosing. Typically, MDR-TB is treated with a combination of ethambutol, pyrazinamide, kanamycin, ciprofloxacin and ethionamide, depending upon DST results.

Drug resistant TB has existed from as early as 1944, when streptomycin was first used to treat the disease. In the 1960s, The British Medical Research Council conducted clinical trials using isoniazid, streptomycin and para-aminosalicylic (PAS) to treat TB, and soon after, cases of resistance emerged. Between 3% to 13% prevalence of resistance to one of the drugs were reported from Finland, Great Britain, Japan, Australia, Italy, Canada, France and the United States. Among the developing countries, prevalence in Hong-Kong, Kenya and India was 20%, 14.7% and 22% respectively. With the introduction of rifampin in the late 1960s, cases of drug resistance in developed countries showed considerable decline, such that TB control programmes and even surveillance of MDR-TB were discontinued. The current global situation of MDR-TB is the product of this negligence.

Much of the epidemiological research of MDR-TB has been conducted by the World Health Organization (WHO): A 1994 survey reported MDR-TB hotspots to be in Estonia, Latvia, Tomsk in Russia, the Oblasts of Ivanovo and the Henan and Zhejiang Provinces in China. As of February 2008, the World Health Organization (WHO) estimated 424,000 new cases of MDR-TB occur each year. This accounts for 5% of the 9 million new TB cases reported in 2008 and sees an increase from the 3.2% (273,000) MDR-TB cases that arose from the 8.4 million total number of new TB cases in 2000. The 2008 study found the highest incidence of MDR-TB to be in Azerbaijan, with 22.3% of all TB cases being MDR-TB. Overall, the WHO concluded that MDR-TB was recorded at the highest rates ever, with 19.4% in Moldova, 16% in Donetsk, Ukraine, 15% in Tomsk Oblast, Russia and 14.8% in Tashkent, Uzbekistan. In 2005, it was found, also by the WHO, that incidence of MDR-TB in three countries, India, China and the Russian Federation, cumulatively contributed to 62% of all MDR-TB cases. Other hotspots were determined to be in South Africa, South and Latin America, the Middle East, different parts of Asia, including Thailand, Myanmar, Vietnam, the Philippines and Korea.

A few factors that have been determined to be associated with multi-drug resistance are HIV infection, alcoholism, diabetes mellitus, as well as the presence of certain alleles HLA-DRB1 and DQB1 (both human genes). According to the WHO, TB patients living with HIV are twice
as likely to be multi-drug resistant than TB patients without HIV. MDR and XDR (extreme drug resistant TB), are extremely lethal in HIV patients and contribute to over 90% mortality rates. In Sub-Saharan Africa, HIV is rapidly fuelling the spread of MDR-TB. As a means of combating the surge in MDR-TB, the WHO introduced the DOTs plus (Directly Observed Treatment, Short-Course), strategy, an extension of the original DOTs strategy established in 1993 for TB. The main goals defined by DOTs are appropriate detection and registration of affected individuals, provision of standardized multiple drug treatment and supply, as well as evaluation of patient and cohorts to monitor cure and overall programme performance. Thus far, 182 countries have adopted this method of TB treatment, with 17.1 million patients treated and DOTs implemented in areas inhabited by 77% of the global population. Although much success has been achieved, success is quality dependent, and inefficient implementation does not yield benefits. Treatment of a single case of MDR-TB is 50 times more expensive to a drug-susceptible case, presenting a substantial total cost for resource-limited countries. With more than 40,000 are fatal cases out of the 490,000 cases of MDR-TB arising yearly, there is a desperate need for quick, efficient and cost-effective detection of MDR-TB.

**Virulence and Pathogenesis**

*M. tuberculosis* does not code for any toxins. Instead, the novel ESX-1 secretion system secretes the proteins ESAT-6 (Early Secreted Antigen Target 6kDaA) and CFP-10 (Culture Filtrate Protein, 10kDa), [Champion et al, 2007]. The proteins are transcribed by genes on the RD1 (Region of Difference 1) locus, and are key virulence factors for MTB [Simeone et al, 2008]. A second factor implicated of virulence is the PGL (phenolic glycolipid) molecule derived from the cell wall. The outermost layer of the mycobacterial cell envelope contains the lipids phthiocerol dimycocerosates (DIM or PDIM). They are not covalently bound to the cell wall, and this allows the DIM/PDIMs to interact with host cells. In mice, they have been shown to promote multiplication and persistence of MTB in the lungs after three weeks of infection. Some strains of MTB produce the DIM/PDIM lipids linked to a tri-glycolated phenol ring (derivates of p-hydroxybenzoic acid or HBAD), to form molecules called phenolic glycolipids. However, other strains of MTB fail to synthesize the final PGL molecule and can only synthesize the pre-cursor, HBAD. These strains lack a 7 basepair sequence of the polyketide synthase gene (pks 1-15), which would normally aid in the formation of p-hydroxyphenylalkanoic acid by elongation of p-hydroxybenzoic acid with malonyl CoA. The PGL molecules have been seen to promote hypervirulence in strains of MTB. Finally, the formation of serpentine cords by certain MTB cultures is an indication of hypervirulence of those strains.

Tuberculosis spreads through droplet transmission. MTB present in airborne droplet nuclei 1-5μm in diameter enter the alveolar passages [Smith, 2003, Frieden et al, 2003]. This results in formation of the initial lesion, at the site of bacterial infection [Davies, 2007]. Host inflammatory
molecules such as prostaglandins and histamine are stimulated, resulting in an influx of macrophages and neutrophils in a surge of tissue fluid and local swelling [Orme, 2007]. The pathogen enters into macrophages, [Frieden et al, 2003] or type II pneumocytes (these are more numerous in alveoli, and MTB can grow in them ex vivo) [Smith, 2003]. Colonization of macrophage establishes infection, but disease will only develop if bacterium is able to replicate in host cell [Orme, 2007]. Bacterial replication is slow but continuous, and spreads to the hilar lymph nodes via the lymphatic system [Friedman et al, 2003], and thus leading to infection.

**Immune Evasion and Immune Response**

Infection only develops after MTB penetrates the lung interstitium and establishes colonization in macrophages [Orme, 2007]. The bacterium successfully evades immune response by the action of virulence factors ESAT-6 and CFP-10, which break down host cell membranes and jump from macrophages elongated along the alveolar membrane, into lung tissue [Orme, 2003].

Cell-mediated immunity typically presents after 2-8 weeks of infection in most affected individuals [Friedman et al, 2003]. Neutrophils and macrophages are both attracted to the inflammatory sites of the infected lung, however, neutrophils are short-living cells and their role is unclear [Orme, 2007]. The toll-like-receptors (TLRs), specifically TLR-2 receptors, present on the surfaces of macrophages are activated by cell components of the bacterium, such as lipoteichoic acid, peptidoglycan, etc. [Crevel et al, 2002]. T cell lymphocytes are activated by activated macrophages [Friedman et al, 2003]. Several important cytokines, such as interleukins 12, 18, 17 and 23 are also released by activated macrophages, which stimulate CD4+ lymphocytes to release interferon gamma (IFNγ) [Friedman et al, 2003, Orme, 2007]. Although the bacterium inhibits IFNγ transcriptional activity, its secretion leads to the release of tumor necrosis factor alpha (TNFa) [Friedman et al, 2003]. T lymphocytes and macrophages form granulomatous focal lesions, which effectively prevent further spread of bacteria [Smith, 2003]. TNFa plays a key role in granuloma formation, induction of macrophage activation, and immunoregulation [Crevel et al, 2002]. T-lymphocyte response is antigen specific is influenced by the major histocompability complex [Friedman et al, 2003].

**Genome Sequence**

So far, the genomes of six species of mycobacteria have been sequenced, including two strains of M.tuberculosis, the laboratory strain H37Rv, and the clinical strain CDC1551 [Arcus et al, 2006]. As a species, MTB exhibits very little sequence diversity, with a 99.9% sequence
similarity between all MTBC strain sequences [Fleischmann et al, 2002, Smith, 2003]. The genome is thought to be highly conserved as a result of lack of horizontal gene transfer (LGT) between species, owing to the organisms’ solitary lifestyles within their hosts [Coros, 2008]. Transposable elements and drug resistant phenotypes give rise to most of the genetic variability between strains of MTB [Fleischmann et al, 2002]. Specifically, restriction fragment length polymorphism (RFLP) analysis of two classes of repetitive DNA elements, insertion sequence elements (IS), predominantly the IS6110, and small repetitive DNAs 3 to 36 bp long, are used to determine the molecular epidemiology of MTB [Sajduda et al, 2004, Soolingen et al, 2005]. The best characterized strain is the laboratory strain H37Rv, sequenced in 1998, containing 4,411,529 base pairs, and approximately 4000 genes that account for more than 91% of its potential coding capacity [Cole, 2002, Cole et al, 1998]. 50 genes are known to code for RNA species whereas 3924 genes are protein coding [Cole, 1999]. The genome is extremely G+C rich, 65.6% overall and as high as 80% in some areas. This contributes to an abundance of basic amino acids in the sequence [Cole, 2002], and dispels the presence of horizontally transferred pathogenicity islands of atypical base composition in the genome [Cole et al, 1998]. Two large protein families, PE and PPE, both exceptionally glycine-rich, have been identified in the proteome. The PPE contains a high proportion of asparagine, an amino acid that is rare elsewhere in the proteome [Cole, 1999].

Multi-drug resistance in TB is resistance to at least rifampin (RIF) and isoniazid (INH). Nine separate genes have been isolated and implicated in drug resistance: rpoB for rifampin, katG, aphC and kasA for isoniazid, rpsL and rrs for streptomycin (STR), emb for ethambutol and pncA for pyrazinamide resistance. Multi-drug resistance is the product of simultaneous mutations in a combination of these genes [Van Rie et al, 2000].

**LATE-PCR Assay**

The novelty of LATE-PCR lies in the generation of single stranded product, thus allowing for probe hybridization of product within a closed tube, and eliminating the risk of contamination. LATE-PCR allows for the production of single-stranded DNA from as little as a single target copy [Pierce et al, 2007]. This is ideal for the detection of low copy numbers of bacterium, and would ensure quick, positive results from infected patients of MDR-TB. This is of course of vital importance as early treatment is the only means of preventing fatality.

The purpose of this thesis is the development of a novel diagnostic assay for MDR-TB using the highly reliable asymmetric LATE-PCR, to be used in the field. Primers and probes were designed for the rpoB gene sequence, which is highly conserved, and a reliable indicator of multi-drug resistance in TB. An 89-bp region of the rpoB gene known to be highly mutable in MDR-TB is amplified and probed. PCR testing in the field will be possible by use of the Bio-Seeq, a portable machine created by Smiths Detection. The development of this assay will provide a specific and highly reliable method for on-site diagnosis of MDR-TB.
References:


