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Drug Resistance Mechanisms and Molecular Diagnosis Methods for Tuberculosis

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Abstract

Mycobacterium tuberculosis is the main cause of death worldwide. It has been thought that one third of the world population has been infected with *Mycobacterium tuberculosis* (MTB). To assure effective treatment, TB is treated with a combination of anti-TB drugs in the strategy called directly observed treatment short-course (DOTS), which is a treatment regimen that may, lasts from six to eight months. Even though DOTS strategy and effective chemotherapy are used in the past decades, drug-resistant TB is the worldwide problem since the introduction of chemotherapy. Especially, drug resistance is recognized as a worldwide problem after the dramatic outbreaks of Multidrug-resistant *Mycobacterium tuberculosis* (MDR-TB) strains. MDR-TB is a type of drug resistant TB which is developed when MTB strains can withstand at least two potential anti-TB antibiotics, isoniazid and rifampin. Antibiotic resistance can be developed either by natural or acquired mechanisms. Bacterium like *Mycobacterium tuberculosis* can acquire drug resistance by changing their genetic materials which can be targeted for drug resistance diagnosis for early and rapid diagnosis of drug resistant tuberculosis. Since early disease diagnosis can minimize the risk of transmission and improve the patients' survival rate, now a day's molecular techniques have madesignificant progress in the identification of genetic mutations that are related with antibiotic resistance development to offer a rapidly screening of antibiotic resistant *M. tuberculosis*. Such mutation screening methods include DNA sequencing, hybridization, single strand conformation polymorphism and heteroduplex analysis. The diagnosis of drug resistance with molecular techniques help to avoid unnecessary treatments and reduce health complications.

Keywords: Isoniazid; *Mycobacterium tuberculosis*; MDR-TB; Rifampin

Introduction

Tuberculosis is an infectious disease caused by different strains of Mycobacterium, usually *Mycobacterium tuberculosis* [1]. The global fatality rate was found 23 percent and around 1.87 death reported globally due to tuberculosis. The fatality rate exceeded 50 percent in some African countries where human immunodeficiency virus (HIV) is highly prevalent [2]. It is estimated that between 2002 to 2020, approximately 1000 million population will be newly infected, over 150 million individuals will get sick, and 36 million will die due to TB infection, if proper control measures are not taken into consideration [3]. TB is the major public health concern worldwide [4] and it is estimated that one third of the world population is infected with *M. tuberculosis* [5]. Drug resistance is a problem resulted from inadequate chemotherapy, such as treatment with single drug, poor drug quality, inappropriate prescription and lack of adherence to treatment [6]. Drug resistance is said to be present when more than 1% of the colonies are resistant to a specific drug [7]. Drug resistance in TB is emerged due to chromosomal genetic mutation in one or more genes with different mutation frequencies.

Multidrug resistant Tuberculosis (MDR-TB) is defined as disease resulted from infection by *M. tuberculosis* strains which are resistant to at least two first line antibiotics, isoniazid and rifampin [4,7,8]. When *M. tuberculosis* strains develop resistance for these best antibiotics (INH & RIF) and to the most alternative drugs used against MDR-TB, the strains are called extensive drug resistance (XDR) TB [9-11]. The emergence of MDR-TB has complicated the epidemics of both acquired immune deficiency syndrome (AIDS) and tuberculosis [12]. In 2008, about 300,000 new cases of MDR tuberculosis has been occurred [13]. The incidence is worst in developing countries. Most of the health problem associated with TB is occur in developing countries. Over all the TB cases and deaths occurred worldwide, 95% of TB cases and 98% of deaths are estimated to occur in the developing countries [14].

Even though early detection of *M. tuberculosis* infection as soon as possible has a significant impact in the control of the disease expansion and drug resistance development [15]; and drug resistant tuberculosis is in demand of urgent attention to achieve more rapid diagnosis and develop new therapeutic regimens for MDR-TB patients [12]. The information about drug resistant TB is not usually available due to the absence of rapid, affordable and precise diagnosis methods. Conventional methods for the diagnosis of TB are microbiological culture based which require skilled personnel, expensive culture media and highly specialized laboratories; in addition these methods are time consuming [7]. However, scientific understanding of *M. tuberculosis* molecular drug resistance mechanism offers to use genotypic approaches for the diagnosis of drug resistant strains [16]. Polymerase chain reaction (PCR) based sequencing might be useful for the detection and screening of both previously recognized and unrecognized mutations in drug resistant strains [17-19].

Epidemiology of Drug Resistant Tuberculosis

Tuberculosis is the leading and deadly communicable disease. Globally, it has been estimated that nine million TB incidences are occurred in 2013; and 20.5% of previously treated and 3.5% of new TB cases were estimated to be MDR-TB [20]. In 2014, 9.6 million TB diseases are estimated to occur worldwide [21]. The incident has been estimated to be 10.4 million TB cases Worldwide in 2015 [22]. A review about drug resistant TB carried out for a long period of time indicated that drug resistance is worldwide problem [1]. Based on drug sensitivity tests conducted on more than 90,000 patients from different countries,

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WHO reported more drug resistant TB cases during 2002-2007 than ever before, which indicates 3.1% MDR-TB cases among new cases and 19% MDR-TB among previous cases [23]. The 2010 global TB report revealed that 8.8 million new TB cases are occurred globally, from which 650,000 are MDR-TB cases and from 95% of deaths occurred in developing countries 1.5 million deaths are associated with TB [24-27]. In 2011 also 12 million (10 million-13 million) TB cases are estimated to occurred worldwide [25]. As a result of such high prevalence of TB and drug resistance development, WHO declared TB as a 'Global Emergency' [18,25,28].

The tuberculosis incidence has been doubled since the early 1980s in Sub-Sahara African countries [29]. It is estimated that more young and middle-aged adults are died globally because of TB than any other infectious disease [18]. In Ethiopia, the result of the survey conducted from late 2011 to early 2012, indicates prevalence of TB is lower than the previously estimated TB prevalence, with most cases occur in adults [25].

The rate of MDR-TB varied considerably in most regions of the world due to differences in the degree of drug misuse, degree of patients studied, and the quality of studies regarding previous treatment and the availability of drug susceptibility test facilities [30]. In countries affected by HIV/AIDS epidemic, like sub-Saharan Africa countries, the rates of TB have dramatically increased [31,32] which is also increasing in Ethiopia as Ethiopia's HIV/AIDS epidemic expands and rose up [33].

According to the 2011 WHO global TB report that sort out the world's 22 high burden countries, Ethiopia ranks seventh with 261/100,000 TB incidence rate; 35/100,000 mortality rate and 394/100,000 all forms of TB prevalence [25]. According to the WHO's Global TB report, Ethiopia had 306.330 TB cases in 2006, with an estimated incidence rate of 379/100.000 cases [34]. Out of all new TB cases occurred in 2007, 1.6% cases are estimated to be MDR-TB cases [33] and Ethiopia ranks fifteenth from the 27 countries that are in highest number of MDR-TB cases [35]. According to the Ministry of health hospital statistical data, TB is the main cause of morbidity, the third cause of hospital admission, and the second cause of death in Ethiopia [36] (Table 1; Figure 1).

Basic Principles of Chemotherapy and Drug Resistance Mechanisms in Tuberculosis

The discovery of streptomycin is marked as the beginning of modern TB chemotherapy. Most of the drugs that are in use today to cure TB are discovered during 1950s and 60s [37]. The first-line anti-TB drugs that are most commonly used now are pyrazinamide, streptomycin, isoniazid, rifampin and ethambutol [18]. For effective treatment of TB, these drugs are delivered to patients in a treatment regimen that lasts 6-8 months by using powerful anti-TB drugs in combination based on the patients' treatment history [38]. Directly observed treatment short-course (DOTS), the treatment strategy that lasts for 6-8 months, is currently the best TB therapy has a cure rate up to 80% [39]. However, the outcomes of treatment are poor when patients infected with MDR-TB are treated with DOTS. Therefore, patients infected with MDR-TB should be treated by DOTS and second-line TB drugs which takes up to two years and is not only costly but also has significant toxicity [37,40].

Bacteria can attain antibiotic resistance through either by natural or acquired mechanisms [41]. The natural antibiotic resistance mechanism of bacteria refers to the situation where a bacterial species is unaffected by an antibiotic due to its fundamental physiological properties [42]. Bacteria have diverse intrinsic antibiotics resistance mechanisms to resist the growth-inhibitory properties of antimicrobial agents. The major natural mechanisms of bacterial resistance to antimicrobial agents include: inactivation of drugs enzymatically; modification of the drug target; reduction of drug permeability; and active efflux of drugs.

Generally, in natural antibiotic resistance mechanism, the resistance is developed due to the fact that microorganisms do not posses target sites for the drugs naturally; or naturally they have low permeability for the drugs because of the chemical nature differences between the drug and the microbial membrane [43]. The natural drug resistance development depends on the hydrophilicity of the antibiotics and mediated by cell composition, biofilm formation, or by enzymatic inactivation [44,45]. The role of efflux mechanisms have also been recognized as an important factor for natural drug resistance development in mycobacterium [46]. In addition to natural drug resistance mechanism, pathogenic bacteria including *M. tuberculosis* are also able to acquire resistance to a particular drug in different ways.

Acquired drug resistance reflects the ability of the bacterium to

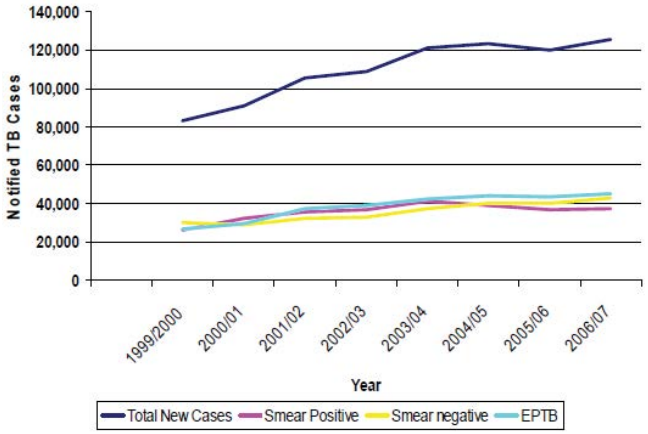

Figure 1: Notified cases of TB in the last eight years in Ethiopia, 1992-1999 EC [36].

Year (GC)	Total New Cases	Smear positive	%	Smear negative	%	EPTB	%	Case Notification Rate per 10^5 Population	
								Smear Positive	All Forms
1999/00	83334	26459	32	30333	36	26542	31	42	131
2000/01	90729	32423	36	28994	32	29312	32	50	139
2001/02	105250	35915	34	32197	31	37138	35	53	157
2002/03	108488	37014	34	32656	30	38818	36	54	157
2003/04	121026	41430	34	37119	31	42477	35	59	173
2004/05	123090	38800	31	40269	33	44021	36	53	169
2005/06	120163	36674	31	40234	33	43255	36	49	160
2006/07	126809	38040	30	43500	34	45269	36	49	164

Table 1: An overview of TB case reports in Ethiopia, 1999-2007 (1992-1999E.C) [36].

resist a drug which was once was effective *in vivo* due to either genetic mutation or horizontal gene transfer [47]. However, horizontal gene transfer assisted drug resistance is rare in *M. tuberculosis* [18]. Rather, all *M. tuberculosis* known acquired drug resistances are mediated by chromosomal mutations caused by selective pressure of antibiotics [48]; because the presence of an antimicrobial agent favors the multiplication of a mutant organism.

Molecular Diagnosis of Drug Resistant *M. tuberculosis*

Early diagnosis and treatment of drug resistant tuberculosis is important not only from the patient's perspective but also for the community at large to control the transmission of the disease [30,49]. However, the absence of effective and affordable rapid diagnostic techniques for drug sensitivity hampered the diagnosis of MDR-TB and XDR-TB.

Even though DOTS services is expanded in Ethiopia for prevention and control activities, case detection rate for smear positive TB, was estimated to be low and almost constant in the past 10 years (Figure 2).

Phenotypic and molecular diagnosis approaches have been explored to develop rapid, reliable and accurate methods for drug resistant *M. tuberculosis* detection [51]. Rapid and early identification of MDR-TB patients is crucial to provide early and appropriate treatment, to increase the patients' survival rate, minimize disease transmission risk and protect the progression of MDR-TB to XDR-TB [52].

However, developing convenient methods for drug resistant *M. tuberculosis* rapid detection are still in progress. Anti-Mycobacterium susceptibility testing still depends on culture methods which last up to 6 weeks to obtain bacterial growth and another 2-4 weeks for drug susceptibility pattern [16]. Such traditional Mycobacterium identification methods mainly depend on growth characteristics and certain biochemical tests which are slow, tedious and inconclusive [53]. However, currently new molecular biology techniques have made significant progress for the identification of genetic mutations that are related with antibiotic resistance development to offer a rapidly screening of antibiotic resistant *M. tuberculosis* isolates [53]. Such molecular mutation screening and detection methods include probe based hybridization methods, PCR-RFLP, DNA sequencing, single strand conformation polymorphism, heteroduplex analysis, molecular beacons and ARMS-PCR [30,49].

Hybridization-Based Disease Diagnosis Techniques

The probe based hybridization bacterial identification method is one of the most successful molecular disease diagnosis method [54]. This diagnosis method is easy to perform and it uses well defined oligonucleotide probes based on the information about specific gene sequence of clinically relevant mycobacterium strains [55]. In this method, the PCR product of genes, known to confer drug resistance, are hybridized to an allele-specific probe which is complementary to either to the wild type or to the mutant sequence of the gene. The hybridized molecules can then be detected by different methods which can be enhanced by radioisotopes, chemiluminescence, alkaline phosphatase or other detection systems [49,56]. In hybridized based disease detection method, known oligonucleotide molecules are immobilized at known locations on membrane and used to hybridize under strictly controlled conditions with PCR product [57]. The possibility of a random hybridization event between a specifically-designed probe and lack of available automation are probably the greatest practical limitation to this technique [58]. Line probe assay is successful in detection of gene mutation responsible for drug resistance with high sensitivity [59,60].

PCR-Single Stranded Conformation Polymorphism Analysis (SSCP)

PCR-SSCP is simple and rapid molecular technique that can be used to determine the presence or the absence of mutations in specific region of DNA based on the migration pattern of DNA in a gel [61,62]. This method has a high level of accuracy for the detection of drug resistance with good utility as a rapid screening tool, especially in settings with high rates of MDR-TB [63]. In this molecular method, nucleotide sequence small changes might result in differences of the secondary structure as well as DNA mobility which can be detected on a non-denaturing polyacrylamide gel [64]. In PCR-SSCP, target region of a gene is amplified by PCR and the product is denatured into two single-stranded molecules and subjected to non-denaturing polyacrylamide gel electrophoresis. Under non denaturing conditions, the single-stranded DNA (ssDNA) molecule has a secondary structure which can be determined by the nucleotide sequence, buffer conditions, and temperature [61]. However, PCR-SSCP analysis has been found to be technically demanding and not sufficiently sensitive [49]. There are reports about the presence of silent mutations. However, such mutations which are not responsible for drug resistance development, the detection of silent mutation by PCR-SSCP method may lead to false positive result [65]. In PCR-SSCP method the amplicon can be contaminated because of the extensive post-PCR manipulation and cannot be practical for other antibiotics because need to screen larger DNA regions and more than one region for one antibiotic [49]. To

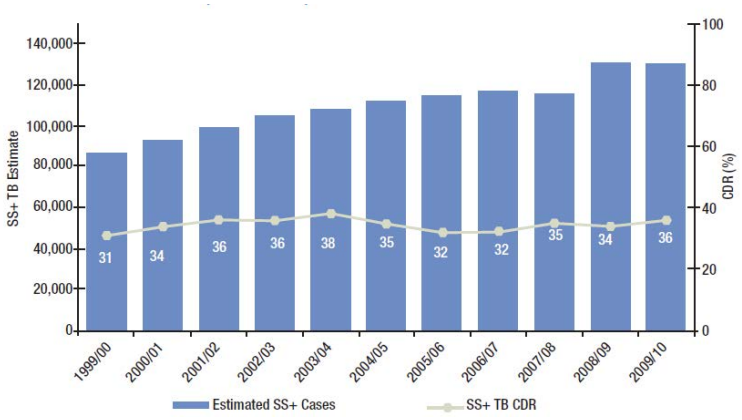

Figure 2: Trend of WHO SS+TB Estimate versus Case Detection Rate (CDR) in Ethiopia-FMoH 1999/00-2009/10 (1992-2002 EC) [50].

date PCR-SSCP is in application to identify *M. tuberculosis* genetic mutations associated with drug resistance against frontline drugs like, rifampin and isoniazid [64].

Amplification Refractory Mutation System (ARMS)-PCR

ARMS-PCR is a well established technique which is used for the detection of any point mutation [49]. ARMS- PCR is also defined as allelic specific PCR which is simple, rapid and reliable PCR amplification method, and easy to interpret the results [66]. Allelic specific PCR has significant single nucleotide polymorphism discriminatory potential [67]. The mutation detection procedure is inexpensive and requires only standard PCR and electrophoresis equipment [68].

Usually, ARMS-PCR is a multiplex reaction in which three or more primers are used for the simultaneously amplification of the same region. In this method, one of the primers should be specific for the mutant allele and will work with a common primer during amplification. The other primer will work with the same common primer to generate an amplified fragment which is larger than the fragment from the mutant allele primer, which is amplification internal control. An amplification product should always be present in the larger internal control amplified fragment; if this is the case then the absence or presence of the smaller product will indicate the presence or absence of a mutant allele. This technique has successfully been used for the detection of mutations associated with rifampin resistance in *M. tuberculosis* even if it needs optimization [49]. However, stringency of ARMS-PCR is difficult to be stably adjusted, especially in multiplex mutation detection, and the number of primers in a single PCR reaction is limited [68].

Real-Time PCR (RT-PCR)

Polymerase chain reaction has completely revolutionized nucleic acids detection and characterization methods [69]. Because of high sensitivity and specificity for the detection of the *Mycobacterium tuberculosis* complex, RT-PCR is commonly used to diagnose TB [70]. Real time PCR works based on the principle of simultaneous amplification of different DNA targets and fluorimetric detection by labeled probes. This method is preferable to the speed and lower cross-contamination problems [71]. This method has also been proposed for rapid detection of genetic mutations associated with drug resistance in *M. tuberculosis* [72]. The real-time PCR has unique features; such as high sensitivity, specificity, speed and lower risk of contamination [73]. However, it requires expensive equipment, reagents, and skilled personnel [72].

Polymerase Chain Reaction based DNA Sequencing

This technique often used to study the genetic mechanisms of drug resistance and detection of both previously recognized and unrecognized mutations [18]. This method involves amplification of the genetic mutations associated with drug resistance and subsequent sequencing of the amplified product to determine the presence or absence of specific mutations. Sequencing is the most accurate and reliable method for mutation detection and it is used as the gold standard technique. PCR based sequencing allows detection of both previously recognized and unrecognized mutations [61,74]. However, due to the need to perform several sequencing reactions, this technique is not readily applicable for routine identification of drug resistance associated genetic mutations [17-19]. This method is also costly and requires expertise, which make it unpractical for use in routine laboratories, especially in developing countries, where simple, cost effective drug susceptibility testing is needed [4,49]. Hence, the *M. tuberculosis* genes of wild type and clinical isolates can be analyzed by aligning the nucleotide sequences of the genes and their deduced amino acid sequences [75]. Though detection of mutation by DNA sequencing is gold standard, it cannot be used as mutation screening method due to cost and technical complexity of the method [76].

Conclusion

Even though different efforts are made to control tuberculosis, the disease persists with serious implications for human health because of the emergence of different types of drug resistance *Mycobacterium tuberculosis* strains such as; MDR-TB and XDR-TB. Majority of the drug resistant strains develop resistance because of the *Mycobacterium tuberculosis* genetic mutation of some genes. When such genetic mutations associated with drug resistance are detected with molecular techniques in the suspected patients, screening and diagnosis of drug resistant *M. tuberculosis* would be rapid and accurate. In addition to screening and diagnosing patients accurately and rapidly, disease diagnosis by molecular approaches also has crucial influence on TB clinical management. Disease diagnosis with nucleic acid amplification, nucleic acid hybridization and electrophoresis methods provide more rapid and accurate diagnosis. Thus, the rapid diagnosis of tuberculosis by molecular assays can be a considerable advantage to patient management. Because, the rapid diagnosis and appropriate treatment can lead to fewer health complications; reduce the number of hospitalized patients and avoid unnecessary treatments. However, all the new molecular diagnosis techniques must be cheap, robust and should be easily accessible for poor countries. This is due to the fact that the majority of the TB cases and drug resistant TB epidemics are occurred in countries with low infrastructures; poor trained experts, low capital per income and poorly advanced technology.

Conflict of Interest

I hereby confirm that the disclosure made above are complete and correct to the best of my information and belief. I shall not be participating in the discussion and decision making of this matter. I agree that if I become aware of any information that might indicate that this disclosure is inaccurate or that I have not complied with the conflict of interest policy, I will notify the board chair or vice-chair immediately.

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