Emergence of Potential Superbug *Mycobacterium Tuberculosis,* Lessons from New Delhi Mutant-1 Bacterial Strains

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ABSTRACT

Recent reports have shown that certain bacterial strains attain the New Delhi Metallo-beta-lactamase-1 (NDM-1) enzyme and become resistant to a broad range of antibiotics. Similarly, more dangerous "superbugs" of multi-drug resistant (MDR) and extensive drug resistant (XDR) *Mycobacterium tuberculosis* strains are gradually emerging through rapid genetic mutation caused by prescription non-compliance or unsupervised indiscriminate use of anti-tubercular drugs or other antibiotics. *Mycobacterium tuberculosis* cases have been reported in highly susceptible population groups including the aboriginal communities of US and Canada. In Canada alone, the total number of reported tuberculosis cases has decreased over the past decade. However, there is a steady increase in HIV cases in certain communities including the aboriginal communities. Reintroduction of MDR/XDR strains of tuberculosis is possible in these susceptible communities, which in turn may pose serious public health situation. MDR/XDR strains of tuberculosis are virtually untreatable using current anti-tubercular medication protocols. Thus, MDR/XDR tuberculosis presents a grave global public health threat. The unpredictable genetic mechanism involved in generating MDR/XDR resistant strains of *Mycobacterium tuberculosis* may pose greater challenges in developing appropriate treatment strategies. In this article, we briefly review potential genetic mechanism of emerging NDM-1 bacterial strains and draw a rationale parallel to the underlying genetic mechanism of MDR/XDR *Mycobacterium tuberculosis* strain development.

Key words: Tuberculosis, multidrug resistant, class I integron, NDM-1

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Introduction

The term "superbug" is often used to a specific strain of microbes that have undergone gradual changes within its genome conferring them multi drug resistance. [1-3] The emergence of multidrug resistant *Escherichia coli,* [4] *Staphyloccocus aureus,* [5] *Clostridium difficile,* [6, 7] *Streptococccus pneumoniae* [8] and *Klebsiella pneumoniae* [9] is documented. Recently, New Delhi Metallo-beta-lactamase-1 (NDM-1) enzyme has come to the attention of epidemiologist and infectious disease control experts. Kumarasamy *et al* (2010) have demonstrated the prevalence of NDM-1 in multidrug-resistant *Enterobacteriaceae* in India, Pakistan and the UK. The NDM-1 gene encodes for the beta lactamase enzyme (carbapenemases) generating multi-drug resistant (MDR) strains of *Klebsiella pneumoniae* which were clonally diverse and highly resistant to all antibiotics except tigecycline and colistin. [10] MDR/XDR tuberculosis (MDR/XDR-TB) poses a grave global health risk overwhelming the healthcare system of major developed and developing countries. The emergence of MDR strains is partly due to misuse of anti-infectives and rapid migration of infected individuals between continents, which allow for random changes within the microbial genome and developing new mechanisms to generate resistant strains. Similarly, we report potential emergence of mysterious 'superbug' strain of multi-drug resistant (MDR) and extensive drug resistant (XDR) *Mycobacterium tuberculosis*, and critically review the possible molecular mechanism involved in generation of these MDR/XDR strains in context with NDM-1 strains.

Etiology, prevalence and pathophysiology of multi-drug resistant strains of tuberculosis

About 10 years ago, the World Health Organization (WHO) had projected that about 1.7 billion people, which is about one third of world's population may carry the tubercle bacillus, [11] and each year there were 8 million new cases leading to 3 million deaths. [12] These figures were grave indication about the rapidly growing tuberculosis cases in the world. In addition, there were 489,139 reported cases of multi-drug resistance tuberculosis, which is roughly about 4.8% of the total number of estimated cases of tuberculosis during 2006 in 185 countries. China and India together have an estimated 240,680 cases of MDR tuberculosis which account for 50% of all MDR-TB worldwide estimated cases. [13] Over the last decade alone, AIDS pandemic have increased the incidences of multidrug resistant tuberculosis in several regions in Asia alone. [12, 13] Similarly the incidences of multi-drug resistant tuberculosis among AIDS patients have been reported in Africa. [13] In the last 10 years, there has been an increase in migration of people, which is attributed to the geopolitical and ethnic conflicts in Africa, Latin America, Afghanistan, and Pakistan. These rapid migrations have provided conditions whereby communicable diseases such as TB could rapidly spread and may undergo rapid genetic mutations. Thus, XDR/MDR tuberculosis is a ticking time-bomb that is about to overwhelm the public health care systems and current global measures to treat, control and reverse tuberculosis incidences in both developed and developing countries.

The recent increase in the incidence of tuberculosis (TB) in certain parts of the world and the emergence of multi-drug resistant (MDR) strains, has urged the need for a rapid diagnostic tools. [14] The delayed identification and susceptibility testing of drug resistant *Mycobacterium* and failure to appropriately isolate TB patients has led to rapid transmission of MDR *Mycobacterium tuberculosis*. Moreover, the constant movement of people between endemic to non-endemic regions could provide opportunities for an outbreak of MDR/XDR tuberculosis into the general population. Thus; the multidrug resistance and antibiotic drug abuse, through prescription non-compliance, eventually generate extensive drug resistant (XDR) strains. [15] Interestingly, prescription non-compliance alone may not be a primary reason for developing multi-drug resistance as 1 % of tuberculosis patients with perfect adherence have shown to develop MDR-tuberculosis. This may be attributed to the potency of the anti-tubercular drug and their pharmacokinetic variability. [16]

According to Public Health Agency of Canada, the total number of reported tuberculosis cases in Canada has decreased over the past decade; however, there is a steady increase in HIV cases in the prairies provinces especially among the Canadian aboriginal communities. [17] A few reports have shown that the HIV positive individuals are more prone to extensive drug resistant (XDR-TB). Coincidentally, the XDR-TB is virtually untreatable with currently available anti-tubercular medications. Multidrug resistant (MDR) and/or XDR tuberculosis presents a grave global public health threat, particularly in high HIV prevalent communities in the developed and developing nations such as Canada, USA, Europe, Russia, India, China, Mexico, Brazil and Africa. [18, 19] The identification of resistance profile and molecular characterization of MDR/XDR *Mycobacterium tuberculosis* will provide insights to scientific community in developing treatment strategies or preventative vaccines, which possibly could provide an avenue for successful eradication of TB. [14, 20]

The emergence of other multi-drug resistant (MDR) strains such as NDM-1 has provided us with a wealth of information about underlying genetic mechanisms leading to acquiring drug resistance. In the subsequent section, we will briefly discuss the potential emergence of MDR/XDR strains in context with NDM-1 mutant strain development.

The Crisis of NDM-1 mutant strain development

NDM-1 was first detected in a *Klebsiella pneumoniae* isolated from a Swedish patient of Indian origin in 2008. [21] It was later detected in several other bacterial strains in India, Pakistan, United Kingdom, United States, Europe and Canada. [22, 23] The most common bacteria that make this enzyme are Gram negative such as *Escherichia coli* and *Klebsiella pneumoniae*, but the gene for NDM-1 can spread from one strain of bacterium to another by horizontal gene transfer. The newly described NDM-1 enzymes are found on mobile genetic elements, and able to confer resistance to all available β-lactam antibiotics. [24] These mobile genetic elements are DNA strands usually plasmids or transposons which typically carry genes that confer antibiotic resistances and able to transfer from one bacterium to another. Nucleotide and protein based studies have identified the gene encoding for NDM-1 as ^{bla}*NDM-1* gene which was found on a 140 kb plasmid isolated from an *Escherichia coli* strain found in the Swedish patient's feces. [21] They reported that this gene translated a 28 kDa monomeric protein corresponding to NDM-1. In addition, PCR analysis of the isolates also detected Class 1 integron, (*intI* and *qacE*Δ*1*/*sul*) of 4.8 kb, a unique set of genes previously reported from Asia. A study have shown that arr-2 gene, a rifampicin resistance gene, is located on a gene cassette within a class I integron, *intI* gene [25] in antibiotic resistant *Pseudomonas aeruginosa* suggesting an uncanny genetic mechanism that may potentially link to the mechanism of MDR-TB strain development. The precise mechanisms that link antibiotic resistant *Mycobacterium tuberculosis strains* to mutations in specific regions of the Class 1 integron gene cassette is briefly discussed below.

Interestingly, the carbapenem class of antimicrobials, which comprises imipenem, meropenem, ertapenem and doripenem, are often considered the last resort for the safe and effective treatment of infections caused by multidrug resistant gram negative bacteria. However, resistance to carbapenems are reported to occur through several mechanisms, including the production of carbapenemases, the enzymes that rapidly metabolise carbapenams. The fatality rate of bacteremias caused by carbapenemase producing *Klebsiella* species is reportedly as high as 50%. [26, 27] Unfortunately, there is very limited clinical experience regarding the treatment of patients infected with carbapenemase producing *Enterobacteriaceae.* [28] Only two classes of drugs, polymyxins and glycylcyclines have shown to have good *in vitro* activity against NDM-1 producers. [29] This may further lead us to reconsider the potential role of gene mutations in Class 1 integrons that may be involved in developing multi-drug resistance or even extremely drug resistant bacterial strains.

Understanding the molecular basis of drug-resistance in TB, drawing parallels to NDM-1 mutant generation

The molecular basis of drug resistance of *Mycobacterium tuberculosis* is drawing close

parallel to that of NDM-1 mutants because of chromosomal mutations which confers resistance. Moreover, this mutation can not only introduce resistance against two or more drugs, but this probability is multiplicative. [30] Thus; the genetic and molecular mechanisms of acquisition of drug resistance by *Mycobacterium tuberculosis* are concomitantly providing reasons for developing various molecular and gene based strategies for rapid detection of the type and degree of resistance. [14, 31]

In contrast to NDM-1 bacterial strains, the mechanism of resistance in *Mycobacterium tuberculosis* is versatile and more complicated. However, the common link between the two strains is the complex class I integron multiple gene mutations that are involved. In *Mycobacterium tuberculosis*, the antibiotic resistance can be developed against more than 15 drugs though limited drug resistance can be observed in the NDM-1 strains. The superbug *Mycobacterium tuberculosis* is different from NDM-1 in another way by its distinctive molecular features and morphological structure. Its virulence, epidemiology and patho-physiology are more complicated and probably comparatively hard to cure. In this section, we will briefly review the known mechanisms of drug resistance in TB.

Rifampin/Rifampicin resistant *Mycobacterium tuberculosis*: Rifampin/Rifampicin is a first-line antitubercular drug that has highly effective bactericidal action against *Mycobacterium tuberculosis*. Several studies including our group have reported incidences of Rifampin resistant *Mycobacterium* in clinical isolates. [32-35] Interestingly, the 96 % of the *Mycobacterium tuberculosis* clinical isolates screened were found to have in 81 bp core region of rpoB gene, which encodes for the beta subunit of RNA polymerase. [36] Missense mutations in codons 513, 526, or 531 resulted in high level Rifampin resistance; whereas amino acid changes at position 514 or 533 usually resulted in low levels of Rifampin resistance. The molecular mechanism of resistance in 4% of Rifampin resistant tuberculosis isolates that lacked 81-bp rifampin resistance determining region (RRDR) changes is unknown. [35] Our group has found that about 90% of rifampin-resistant clinical isolates in the South Asia were also resistant to ethambutol and isoniazid. [34, 37] This may lead us to hypothesize that rifampin resistance could be mediated through a surrogate genetic marker, such as Class I integron that have been found to confer multidrug resistance in other bacterial strains, indicating that second and third line anti-tubercular drugs to which these isolates may be susceptible would also be rendered useless by subsequent anti-tubercular therapy. However, several investigators have also reported that many INH resistant clinical isolates may have small deletions or insertion mutations.[37-39] These type of mutations leading to INH resistance have also been identified in different gene targets including *KatgG, inhA,* and *ahpC*, as well as mutations in different gene combinations such as *KatG-inh*A and *KatG-ahp*C. [35, 40, 41] In addition, the amino acid replacements in the NADH binding site of *InhA* resulted in INH resistance by preventing the inhibition of mycolic acid biosynthesis. However, studies have shown that the mutations in the *KatG* or *inhA* did not account for all INH resistant strains since 15-25% INH resistant clinical isolates had both wild-type *KatG* and *inhA* genes. In spite of the wealth of information available through current gene based techniques the exact mechanism of INH resistance in some bacterial strains is yet to be characterized.

Ethionamide resistant *Mycobacterium tuberculosis*: Ethionamide is a second line antitubercular drug that may inhibit mycolic acid biosynthesis in *Mycobacterium tuberculosis*. Studies have shown that for certain strains, low level of INH resistance is correlated with co-acquisition of ethionamide resistance, suggesting that INH and ethionamide may share a common molecular target and most likely through the *mab-inhA* genes. [42, 43]

Streptomycin resistant *Mycobacterium tuberculosis*: Streptomycin is another first-line antitubercular drug that binds to 16S rRNA of the 30S subunit of the bacterial ribosome, thus interfering with the binding of formyl-methionyl-tRNA to 30S subnit. This leads to the inhibition of the protein synthesis in the *Mycobacterium tuberculosis*. Mutations associated with streptomycin resistance in tuberculosis have been identified in the 16S rRNA gene (*rrs*) and *rpsL* gene. [44, 45] In contrast to other bacteria that have multiple copies of rRNA genes,

Mycobacterium tuberculosis complex members have only one copy. Therefore, single nucleoside changes can potentially produce potent antibiotic resistance. Mutations in the *rrs* are clustered in two regions around nucleotides 530 and 951. The 530 loop 16S rRNA is highly conserved and is located adjacent to the 915 region in secondary structure models. The majority of mutations producing streptomycin resistance occur in *rpsL* gene that encodes for the ribosomal protein S12. The primary structure of proteinS12 is well conserved among the mycobacteria, even those such as *M. avium, M. gordonae and M.* szulgai that are naturally resistant to streptomycin. [45] The most common mutation observed in *M. tuberculosis* is at the codon 43. Mutations may also occur in codon 88. About 65-75% of streptomycin resistant isolates also had resistance-associated changes in *rpsL* or *rrs* genes (44). This suggests that failure to identify resistance-associated variations in these genes in 25-35% of organisms may indicate that other molecular mechanisms of streptomycin resistance may also exist.

Pyrazinamide resistant *Mycobacterium tuberculosis*: Pyrazinamide (PZA) is a structural analogue of nicotinamide that is used as a first line antitubercular drug. PZA kills semi-dormant tubercle bacilli under acidic conditions. It is believed that in the acidic environment of phagolysosomes the tubercle bacilli produce pyrazinamidase, an enzyme that converts PZA to pyrazinoic acid, the active derivative of this compound. To define the molecular mechanism of PZA resistance the *Mycobacterium tuberculosis pncA* gene encoding pyrazinamidase has been sequenced. The results provided evidences that *pncA* mutations conferred PZA resistance in these bacterial strains. While the DNA sequencing of PZA resistance clinical isolates identified mutations at codons, 63, 138, 141, and 162. In contrast, the susceptible organisms had wild type sequences. Lack of *pncA* mutations in 28% of PZA resistant isolates suggested the existence of at least one additional gene participating in resistance. A remarkably wide array of *pncA* mutations resulting in structural changes in the *PncA* has been identified in greater than 70% of drug resistant isolates. It is presumed that these structural changes detrimentally change enzyme function, thereby altering conversion of PZA to its bioactive form. [34, 46]

Ethambutol resistant *Mycobacterium tuberculosis*: Ethambutol is another important bactericidal first line antitubercular drug. This agent has been proposed to be an arabinose analog; the specific target is likely to be an arabinosyl transferase, presumably a functionally important site. As mentioned earlier, our group have found that about 90% of rifampin-resistant clinical isolates in South Asia also showed resistance to ethambutol. [35] To understand the mechanism of resistance to ethambutol, a two gene locus (*embAB*) that encodes arabinosyl transfer has been established. Automated sequencing of these regions in clinical isolates discovered that 69% of ethambutol resistant isolates had an amino acid substitution in *EmbB* that was not found in ethambutol susceptible strains. [47] The great majority (98%) of strains had mutations in codon 306; however, mutations were also identified in 3 additional codon 285, 330, and 630. These mutations were also uniquely represented among ethambutol resistant organisms. The data are consistent with the idea that specific amino acid substitutions in *EmbB* detrimentally affect the interaction between Ethambutol, a putative anabinose analogue and *EmbB* likely to be an arabinosyl transferase. *EmbB* mutations are associated with Ethambutol resistance in roughly 70% of Ethambutol isolates of *Mycobacterium tuberculosis*. The cause of Ethambutol resistance in many organisms lacking mutations in ethambutol resistance determining region (ERDR) of *EmbB* is unknown. [47]

Interestingly, several literatures agree that the probability of *Mycobacterium tuberculosis* to undergo multiple mutations is quite high. This may confer specific mycobacterial strains a degree of multi-drug resistance and thus prove to be unmanageable with current treatment protocols. Moreover, monitoring MDR/XDR resistant TB among the population has its limitations, which could provide a window of opportunity of the MDR/XDR strains to rapidly spread within the population group that are susceptible to TB.

Conclusion

No doubt the NDM-1 mutant superbugs are big challenges to clinicians and public health

professionals but the MDR and XDR *tuberculosis* may prove to be even more mysterious and complicated in many ways due to the intricate and baffling nature of antibiotic resistant gene elements involved. Thus, it may become hard to control MDR or XDR-TB if introduced into susceptible population group. The emergence of NDM-1 mutant strain and the nature of strain development is probably a tip of an iceberg, which may suggest an intricate and complex nature of gene mutations involving class I integron draws close parallels to multi-drug resistant TB strain development. An understanding of the genetic mechanism involved in NDM-1 strain development may also provide us with the opportunity to understand the ways to defend against potential superbug development. With ever mobile global population, the spread of MDR/XDR-TB cannot be monitored or controlled, hence through this report we would like to bring awareness among the clinicians, public health professionals, and policy makers about the emergence of MDR/XDR-TB. To reconsider the current treatment protocols for infectious disease containment, and develop rapid detection methods using interdisciplinary approaches to defend against it.

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Conflict of Interest

The authors declare no conflict of interest

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