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An Analysis upon Molecular Epidemiology of Mycobacterium Tuberculosis to Control TB: A Case Report

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Abstract – It is estimated that one third of the world's population are infected with the bacterium M. tuberculosis and approximately 9.4 million new cases of tuberculosis were diagnosed globally in 2008. Molecular tools, developed over the previous two decades, have allowed further in-depth study of this historic disease. Genotyping M. tuberculosis allows the study of evolutionary relationships and well as the routes of transmission of the organism between hosts. The pairing of genotyping with demographic data allows the analysis of the current trends of disease within a given patient population.

The acquisition of drug resistance-conferring mutations by M. tuberculosis is often presumed to be associated with a fitness cost. Here we investigate the fitness of isolates from two outbreaks involving large numbers of drug resistant strains. The first group of strains was found to be part of the ongoing north London isoniazid resistant outbreak. The data suggests that this outbreak consists of successful, closely related, circulating strains with heterogeneous resistance profiles and mutations and little or no associated fitness cost.

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INTRODUCTION

Tuberculosis (TB), the disease caused by Mycobacterium tuberculosis (MTB) is a major public health problem worldwide especially in developing countries. Despite all the global efforts, TB remains the leading cause of death by a single pathogen. The majority of patients with tuberculosis live in the most populous countries of Asia; India, China, Bangladesh, Indonesia and Pakistan together account for half (48%) of the new cases that arise every year (World Health Organization, 2012). Rapid detection followed by effective therapy and a better understanding of epidemiology and transmission patterns are a prerequisite to control any infectious disease. Despite a 99.9% genomic similarity reported among MTB strains (Garcia-Betancur et al., 2012), it has been possible to detect specific genetic differences with evidence of phylogeographically specific MTB clones (Demay et al., 2012), that has considerably felicitated ongoing studies on transmission dynamics and epidemiology of tuberculosis in conjunction with classical epidemiological approaches.

The discovery of highly repetitive DNA elements on the M. tuberculosis genome as markers for strain identification was soon followed by the description of molecular typing methods based on repetitive elements, namely IS67 70-RFLP, spoligotyping based on the polymorphism of the DR locus and MIRU-VNTRs. Although initially considered a gold-standard, the IS6710-RFLP was later replaced by PCR-based spoligotyping and MIRU-VNTRs due to disadvantages such as requirement of good quality DNA, the presence of strains harboring low/no copy number of IS6110 element in many parts of Asia and certain parts of the world, and difficulties in inter-laboratory comparison of the IS6770-RFLP patterns. Studies using MIRU-VNTR based typing show that the discriminatory power of this method is close to that of \S6110-RFLP for high copy-number strains, and even more discriminatory for low \S6110-copy number isolates.

Only a few studies have been carried out in India to characterize M. tuberculosis genotypes (Varma-Basil et al., 2011); among these the only study from Kerala used IS67 70-RFLP alone, and underlined that this method is not appropriate for the low-copy isolates from this geographical region. In the present study, we aimed to look into the TB molecular epidemiology in Kerala by characterizing the prevalent genotypes of MTB isolated from patients attending TB clinics from different parts of the state, and to compare the genetic relatedness of these stains. We used three different genotyping methods, IS6770-RFLP,

spoligotyping and 15-loci MIRU-VNTRs which include 12 standard MIRU loci, and exact tandem repeats ETR-A, ETR-B, ETR-C. Note that ETR-D & ETR-E were not included since they designate the same regions as MIRU-4 & MIRU-31 (Kremer et al., 2005).

The majority of the 2.23 billion individuals infected with *M. tuberculosis* harbour a latent infection, where the bacteria are contained within the granuloma, and persist for years or decades. Approximately 10% of those with latent infections will progress to clinical (active) disease, although the trigger behind this is undetermined; and this percentage increases substantially with immunodeficiency. Because the disease often has a long incubation period it is typically difficult to properly spot the index case or trace contacts in order to limit further spread of infection using conventional epidemiology alone (Prodinger, 2007). In addition, latent infections which become active may result in increased transmission, and therefore require efficient tests to detect latency. The epidemiology of the disease has further been compounded and modified with the occurrence of TB co-infected with human immunodeficiency virus (HIV), and development of multiple drug resistance. These reasons necessitated the emergence of genotyping where DNA fingerprints of *M. tuberculosis* complex (MTBC) isolates recovered from different patients are analysed to differentiate between exogenous and endogenous infections, identify treatment failures, and detect laboratory cross contamination (Allix-Béguee *et al*., 2008). In addition, these DNA fingerprints can be compared to determine transmission links and control strategies with the aid of classical epidemiology.

Among the diseases that have threatened the health and lives of people and animals in the past century, tuberculosis (TB) has played a signifi cant role. In Croatia, bovine TB occurrence has decreased as a result of an ongoing control program against the disease in the past decades. Despite the effort, it has not yet been eradicated completely.

Bovine TB represents a major problem in terms of epidemiology, especially in countries where certain wild animal species, e.g. badgers (*Meles meles*), possums (*Trichosurus vulpecura*) and wild boars (*Sus scrofa*), represent environmental reservoirs and a source of infection for domestic animals and humans (NARANJO et al., 2008). In Europe, the infection in cattle is most frequently caused by *Mycobacterium (M.) bovis* and *M. caprae* (CVETNIC et al., 2006). Apart from cattle, both species may also affect other mammals. In humans, they are causative agents of zoonotic TB, i.e. TB transmitted from animals to humans. Zoonotic TB is most frequently caused by *M. bovis*, although *M. caprae* infection has also been recorded frequently in recent years (BONIOTTI et al., 2009). In Croatia, *M. caprae* infection in humans was fi rst described in 2006 (CVETNIĆ et al., 2007).

Albeit rare, *M. tuberculosis* infection may occur in cattle. *M. tuberculosis* does not appear to have an indigenous animal host or reservoir and the infected animals most probably represent accidental hosts. Humans suffering from active TB are strongly believed to represent the main source of *M. tuberculosis* in animals, including cattle (STEELE, 1980; THOEN et al., 1981).

DESCRIPTION OF *M. TUBERCULOSIS*

M. tuberculosis is assigned to the genus *Mycobacterium*, family Mycobacteriaceae, order Actinomycetales, class Actinomycetes. The majority of mycobacteria exist as saprophytes but small groups are pathogenic. The genus comprises of 71 identified species which are split on the basis of growth rate into two major groups: the fast growers and the slow grower species. The fast growing bacteria produce visible colonies within seven days under appropriate conditions. They include several species such as *Mycobacterium fortuitum* and *Mycobacterium abscessus* that have limited pathogenic effects and produce atypical clinical signs in humans and animals. In contrast, the slow growers, comprising MTBC, *Mycobacterium avium*, *Mycobacterium leprae* and *Mycobacterium kansasii*, elicit more pathogenicity in humans and animals, and cause chronic diseases.

M. tuberculosis has a rod-shaped appearance with a width ranging from 0.2 µm to 0.6 µm and a length ranging from 1 µm to 10 µm (Godreuil *et al*., 2007). It is non-motile, non-capsulated Gram-positive aerobic or facultative anaerobic bacterium. The genus shares genetic similarity and phenotypic characteristics with other genera including; *Nocardia*, *Corynebacterium* and *Actinomycetes*. Mycobacteria show marked tropism to lung tissues probably due to the presence of abundant oxygen. They are also facultative intracellular pathogens infecting macrophages, where they reside and subsequently disseminate to different parts of the body (van Soolingen *et al*., 2001). *M. tuberculosis* has the ability to synthesise most of nutrients essential for its survival including amino-acids, vitamins and enzyme co-factors. The cell wall of the organism is a unique structure mainly composed of mycolic acid and peptidoglycan, which give the genus its acid fast characteristic, allowing mycobacteria to be visualised microscopically using Ziehl-Nielsen stained sputum smears in which the organisms will be seen as red rods embedded in a blue background of the counter stain.

MYCOBACTERIUM TUBERCULOSIS

Trends in tuberculosis notifications and the contribution of HIV coinfection - Tuberculosis, that is, active clinical disease following M. *tuberculosis* infection, remains one of the most common fatal infectious diseases of man. In the last ten years the

reversal of the steady decline in tuberculosis seen in the developed and parts of the developing world has been a matter of great concern. The World Health Organisation (WHO) estimated that in 1990 there were eight million new cases of tuberculosis and nearly three million deaths; notifications rose by 24.6% compared to the averages of the mid 1980s. HIV infection is the most important risk factor for tuberculosis. The relative risk of developing tuberculosis following infection with M. *tuberculosis* is 170.3 for patients with AIDS, compared to 30 for those with silicosis and 11.9 for patients on immunosuppressive treatment (A. Pozniak, personal communication). Although its role varies from one part of the world to another, HIV is partly responsible for recent changes in tuberculosis epidemiology.

Tuberculosis in patients with HIV infection - Persons infected with HIV are at increased risk of tuberculosis in two ways. Reactivation of latent tuberculous infection becomes more likely as the number of functioning CD4 lymphocytes is progressively depleted as a result of HIV. For example, in a prospective study of intravenous drug users in N,ew York whose tuberculin sensitivity (an indicator of previous infection with *M. tuberculosis)* and HIV status were known, it was shown that the rate of tuberculosis in the HIV-seropositive cohort was 7.9 per 100 person years in those who were initially tuberculin positive compared to 0.3 per 100 person years in those who were tuberculin negative. The rate of conversion from negative to positive tuberculin sensitivity was similar for both HIV positive and negative subjects during the study. A similar relationship between incidence rate of tuberculosis and tuberculin skin test reactivity in Italian HIV infected patients was reported by Antonucci *et al.* (1995), who also demonstrated increasing incidence of tuberculosis with declining CD4 lymphocyte count. Secondly, because of impaired cell-mediated immunity, HIV patients who have not previously been infected are more likely to develop active primary tuberculosis if exposed to tubercle bacilli. Thus, in an outbreak of tuberculosis in a housing facility for HIV patients in San Francisco 15 out of 30 residents exposed to a source were infected and in 11 of these (37 %) infection rapidly progressed to active disease. By comparison, none of the staff members exposed to the same source developed active tuberculosis although some had positive tuberculin tests.

Typing methods for *Mycobacterium tuberculosis* - The study of tuberculosis epidemiology has been greatly enhanced in recent years by the development of molecular methods of typing *M. tuberculosis* which are capable of typing nearly all strains and of reproducibly distinguishing between them with great discriminative power. Such methods allow outbreaks of tuberculosis to be traced precisely and on a larger scale may be applied to cases in populations in order to monitor patterns of disease transmission, for example the relative contributions of exogenous infection and reactivation.

METHODOLOGY

Bacterial strains and molecular typing - This study included a total of 168 Mycobacterium tuberculosis isolates from as many patients attending different public and private TB clinics in Kerala, India during 1998-2005. The isolates in this study was obtained from a repository of M. tuberculosis strains isolated from a single sputum specimen of patients sent for drug susceptibility testing. The sputum specimens were sent for routine diagnosis on doctor's advice and written informed consent from the patients was not required for this purpose. The details of isolation, culture and drug sensitivity studies were published earlier (Joseph et al, 2009). The use of isolates for molecular analysis performed retrospectively was approved by the Bio-Safety and Human Ethics Committees of the Institutional Review Board (IRB). Care was taken throughout to maintain the confidentiality of patient's personal details. The multiplex polymerase chain reaction (PCR) analysis developed in-house (Anilkumar et al., 2012) was used for the characterization of the strains. The drug susceptibility testing of the isolates was done against four first-line drugs by Alamar Blue (AccuMed International, Cincinnati, OH, USA) dye reduction assay, as described earlier (Joseph et al., 2009; Kumar RA, 2001). DNA was isolated both from the M. tuberculosis H37Rv type strain and the clinical isolates as reported (van Soolingen et al., 2001), followed by molecular typing using published procedures: IS6770-RFLP (van Embden et al., 1993), spoligotyping (Kamerbeek et al., 1997), 12-loci MIRUs (Cowan et al., 2002) and ETR-A, -B and -C (Frothingham and Meeker-O'Connell, 1998).

Computer-assisted genotype analysis and comparison with databases - Spoligotype patterns as octal codes and 12-loci MIRU patterns were entered in the SITVIT2 proprietary database of the Institut Pasteur de la Guadeloupe, which is an updated in-house version of the publicly released SpolDB4 (Brudey et al., 2006) and SITVITWEB (Demay et al., 2012) databases. At the time of this analysis, the SITVIT2 database contained genetic patterns of about 86,000 MTB clinical isolates from 160 countries of origin. In this database, Spoligotype International Type (SIT) and MIRU International Type (MIT) designate identical patterns shared by 2 or more patient isolates, whereas "orphan" designates patterns reported for a single isolate that does not correspond to any of the 86,000 strains recorded in the repository of the SITVIT2 database. The BioNumerics software Version 3.5 (Applied Maths NV, Sint-Martens-Latem, Belgium) was used to build

minimum spanning trees (MST) based on spoligotyping and/or MIRU data obtained in this investigation. MST is an undirected network in which all of the isolates are linked together with the fewest possible linkages between nearest neighbors.

RESULTS -

Genotyping profiles of the M. tuberculosis strains and discriminatory power of typing schemes - Present study included 168 isolates of Mycobacterium tuberculosis from a patient group that consisted of 129 males and 39 females whose age ranged from 18 to 78 years. IS6770-RFLP was done on 156 isolates and majority (71.15%) of the isolates had low copy numbers of IS6770 insertion < 6 copies). Zero copy isolates and single copy isolates represented 10.89 % and 44.87% of the strains respectively.

The spoligotyping analysis was done by assigning SIT numbers and genotypic clade designations by comparing with the SITVIT2 database, and the results obtained on orphan patterns (n=17, 10.11%) and shared-types (51 SITs containing n=151, or 89.9% of the isolates). Among the 51 SITs recorded, 42 SITs (containing 136 isolates, 80.95%) matched a preexisting SIT in the SITVIT2 database, whereas 9 SITs (containing 15 isolates, 8.92%) were newly-created either within the present study or after a match with an orphan in the database. Lastly, 21 patterns corresponded to clustered isolates in this study (n=121 or 72%, 2 to 48 strains per cluster) as opposed to 30 unique patterns.

The largest cluster consisting of 48 strains, (28.6% of all isolates), corresponded to SIT 11 which is the prototype of the ancestral EAI3-IND genotypic lineage in the SpolDB4 and SITVITWEB databases with a high phylogeographical specificity for India (Brudey et al., 2006; Demay et al., 2012).

If strains (n=168) were classified based on lineages (and not SITs/sublineages alone), 64.28% (108/168) of the isolates belonged to the EAI lineage in spoligotyping based classification of Brudey et al. (2006), which corresponds to the Indo-Oceanic lineage defined by Gagneux and Small (2007). Out of the 168 isolates, a majority belonged to EAI3-IND (60/168 or 35.71%), followed by sublineages EA11-SOM (21/168), EAI5 (22/168), EAI6-BGD1 (3/168), EAI-MDG (1/168) and EAI7-BGD2 (1/168), all of which are known to be ancestral M. tuberculosis strains harboring the TbD1 region and belong to the Principal Genetic Group 1 (PGG1) based on katG-gyrA polymorphism (Sreevatsan et al., 1997).

As opposed to these “ancestral” PGG1/TbD1+ strains, “modern” PGG1/TbD1-negative strains belonging to the CAS1 -Delhi and Beijing lineages was sparsely represented (4.16% and 2.4%). There were also minor representations from other clades belonging to the “evolutionary recent” PGG2/3-TbD1-negative M. tuberculosis strains lacking spoligotyping spacers 33 to 36 (Rastogi and Sola, 2007) belonging to a large LSP-based group called as “Euro American Lineage” (Gagneux et al., 2007). In our study, it was limited to spoligotyping based sublineages T1 (n=5), and one strain each of T3, LAM9, H3 and H1 among the 168 strains. Lastly, 2 strains belonging to Manul spoligotype pattern were also recorded as SIT100 and a newly-created SIT3358.

The efficiency of MIRU-VNTR and spoligotyping methods together was evaluated in discriminating the clinical isolates. A composite data analysis was done giving equal weightage to spoligotyping and MIRU-VNTRs. A dendrogram was generated using BioNumerics Version 3.5. It showed 141 patterns and the largest cluster had 16 isolates. Among these 141 patterns, 130 patterns were unique (results not shown). Thus, it was observed that combined together, spoligotyping and MIRU-VNTR are more efficient in discriminating the isolates with an HGDI of 0.9904.

CONCLUSION

In conclusion, genotyping *M. tuberculosis* plays a pivotal role in the holistic approach to understanding tuberculosis. The relationship between genotype and phenotype is complicated, but the identification of outbreaks and over-represented lineages of strains can further add to the understanding of the evolutionary success of the organism. Prospective, real-time genotyping can enable the monitoring and control of disease as well as its prevention. Bridging the gaps between the laboratory and clinical practice is essential for the eradication of this global pathogen.

MIRU-VNTR typing proved to be useful tool for the epidemiological investigation of the fi rst documented case of *M. tuberculosis* infection in cattle in Croatia. Though rare, the possibility of an anthropozoonotic transmission of *M. tuberculosis* infection should not be overlooked. In regions where bovine TB and human TB still coexist, a detailed microbiological investigation of specimens of tuberculin-positive animals should always be performed in order to discriminate between *M. tuberculosis* and *M. bovis* infections.

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