Tuberculosis Transmission Based on Molecular Epidemiologic Research

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ABSTRACT

Molecular genotyping techniques developed during the past decade and conventional epidemiological methods have been used synergistically in studies of the transmission and pathogenesis of *Mycobacterium tuberculosis*. Research studies assessing contacts and outbreaks, risk factors for ongoing transmission, and exogenous reinfection with *M. tuberculosis* have advanced with applied molecular epidemiologic techniques. In addition, molecular epidemiologic approaches have enabled scientists to assess the impact of drug resistance on the transmission and pathogenesis of *M. tuberculosis* and to identify strains with broad temporal and spatial distributions. In the near future, the intersection of molecular epidemiology, bacterial population genetics, comparative genomics, immunology, and other disciplines will further our understanding of tuberculosis transmission and pathogenesis, contributing to the development of effective drugs and a vaccine against this important human pathogen.

KEYWORDS: Tuberculosis, transmission, molecular epidemiology, genotyping, *Mycobacterium tuberculosis*

Objectives: Upon completion of this article, the reader should be able to: (1) describe the genotyping methods that are commonly used in molecular epidemiologic studies of tuberculosis; and (2) describe at least four different ways molecular epidemiology has improved our understanding of the transmission of tuberculosis.

Accreditation: The University of Michigan is accredited by the Accreditation Council for Continuing Medical Education to sponsor continuing medical education for physicians.

Credits: The University of Michigan designates this educational activity for a maximum of 1 category 1 credit toward the AMA Physician's Recognition Award.

Interruption in the transmission of *Mycobacterium tuberculosis* is one of the primary goals of tuberculosis control programs. The ability to track specific strains of *M. tuberculosis* as they spread through a population provides opportunities to improve our understanding of the transmission and pathogenesis of tuberculosis, as well as helping us design prevention and control strategies to block further transmission of *M. tuberculosis*. Until recently, the only phenotypic markers that distinguished different strains of *M. tuberculosis* were drug resistance patterns and mycobacterial phage typing. However, the molecular genotyping techniques now available allow us to differentiate isolates of *M. tuberculosis* for the purpose of tracking strains in the community.

Tuberculosis and Other Mycobacterial Infections; Editor in Chief, Joseph P. Lynch, III, M.D.; Guest Editor, Leonid Heifets, M.D., Ph.D., Sc.D. *Seminars in Respiratory and Critical Care Medicine*, volume 25, number 3, 2004. Address for correspondence and reprint requests: Charles L. Daley, M.D., Division of Pulmonary and Critical Care Medicine, University of California, San Francisco, San Francisco General Hospital, Room 5K-1, 1001 Potrero Ave., San Francisco, CA 94110. E-mail: cdaley@itsa.ucsf.edu. [1]Division of Infectious Diseases and Geographic Medicine, Stanford University Medical Center, Stanford, California; [2]Division of Pulmonary and Critical Care Medicine, University of California at San Francisco, San Francisco, California; [3]San Francisco General Hospital, San Francisco, California. 1069-3424,p;2004,25,03,297,306,ftx,en;srm00300x.

Epidemiologic investigations that incorporate genotyping of *M. tuberculosis* have provided novel information about the spread of tubercle bacilli, identified risk factors for transmission and rapid progression to disease, and determined patterns of spatial and temporal distribution of specific strains of *M. tuberculosis*. We review how molecular epidemiology has increased our understanding of the transmission and pathogenesis of *M. tuberculosis*.

GENOTYPING METHODS

Several nucleic acid-based genotyping methods were developed during the past decade that allow us to distinguish between different strains of *M. tuberculosis*. The most widely used method of genotyping is referred to as restriction fragment length polymorphism (RFLP) analysis. Using a standardized protocol for RFLP genotyping of the *M. tuberculosis* complex,[1] this method takes advantage of a specific, well-characterized, repetitive element, insertion sequence 6110 (IS*6110*). Restriction endonucleases cleave the mycobacterial DNA at the sites of specific repetitive sequences, producing DNA restriction fragments of different lengths that can be separated by gel electrophoresis. The DNA restriction fragments are then probed with specific, repetitive, labeled DNA. Only the genomic DNA restriction fragments that are complementary to and hybridize with the probe are visible, resulting in an easily readable band pattern (Fig. 1).

The standardized method of IS*6110* RFLP genotyping has several disadvantages; it is a slow, labor intensive, and technically demanding technique. Because it requires relatively large amounts of high-quality DNA from each strain of *M. tuberculosis*, this genotyping technique can only be done on cultures of *M. tuberculosis*. Computer software and technical support are required to compare, analyze, and interpret large numbers of IS*6110* RFLP band patterns. Finally, it has relatively poor discriminatory power for isolates with < 6 copies of IS*6110* and should be supplemented by analyses with other methods such as polymorphic guanine-cytosine-rich sequence (PGRS) genotyping or spoligotyping.[2] The patterns that PGRS genotyping generates are difficult to interpret and are less discriminatory than IS*6110*-based RFLP genotyping. Spoligotyping is more rapid and easier to perform, but is also less discriminatory than IS*6110*-based RFLP genotyping.

Spoligotyping is a polymerase chain reaction (PCR)-based method that interrogates a small direct repeat (DR) sequence with 36 base pair (bp) repeats interspersed with short, unique, nonrepetitive sequences.[3] All of the unique, nonrepetitive sequences, or "spacers," between the direct repeats can be amplified simultaneously using one set of primers. Strains are differentiated by the number and position of the spacers that are missing from the complete spacer set (Fig. 2). Spoligotyping has at least two advantages over IS*6110*-based genotyping: (1) smaller amounts of DNA are needed so the procedure can be performed on clinical samples or on strains of *M. tuberculosis* shortly after inoculation into liquid culture, and (2) the spoligotyping results can be expressed in a digital format.[4] Spoligotyping can be used either as a secondary genotyping method or as a primary genotyping method, followed by another genotyping method with greater discriminatory power.[5,6]

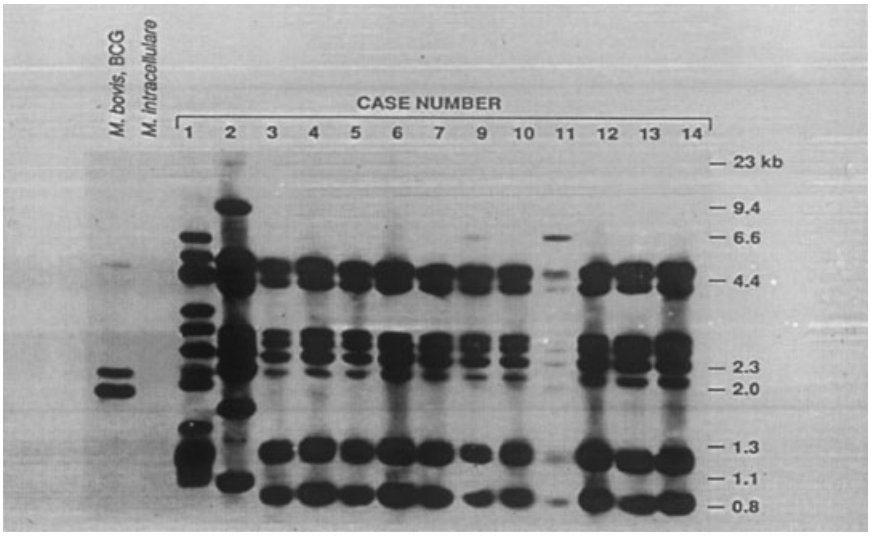

Figure 1 Restriction fragment length polymorphism (RFLP) patterns of *M. tuberculosis* isolates obtained from an outbreak of tuberculosis in a human immunodeficiency virus (HIV) congregate living site in San Francisco. The first two lanes are the RFLP patterns of *M. bovis*, bacille Calmette-Guérin, and *M. intracellulare*. Cases 1 and 2 were receiving antituberculosis therapy when they entered the facility. Tuberculosis developed in the remaining patients while they lived in the facility. Note the shift of the band in the upper part of lane 11. (Adapted from Daley et al.[24])

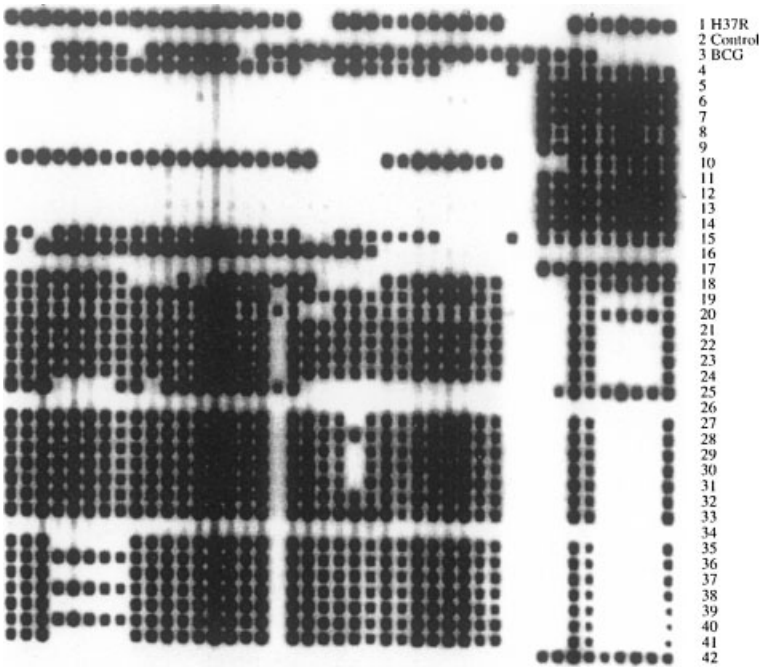

Figure 2 Hybridization patterns (spoligotypes) of amplified mycobacterial DNA of *M. tuberculosis* isolates and *M. bovis*, bacille Calmette-Guérin (BCG). Lane 1 is the reference strain, H37R. Lane 2 is a negative control and Lane 3 is *M. bovis* BCG. The black dots represent hybridization signals and the spaces represent lack of hybridization. Note that the spoligotype patterns of the strains in lanes 5–9, 11–14, 17, and 42 are characteristic of the Beijing genotype group. (Courtesy of Roxanne Aga.)

One of the most promising PCR-based methods is a high-resolution genotyping technique that characterizes the number and size of the variable number tandem repeats (VNTR) in each of 12 independent mycobacterial interspersed repetitive units (MIRUs).[7,8] Relative to IS*6110* RFLP genotyping, MIRU-VNTR profiling is appropriate for strains, regardless of their IS*6110* RFLP copy number, can be automated for large-scale genotyping, and permits rapid comparison of results from independent laboratories using a binary data classification system. The MIRU-VNTR method also reduces the number of isolates that are falsely clustered by IS*6110* RFLP and spoligotyping, allowing more focused contact investigations.[9] This method has been adopted by the Centers for Disease Control and Prevention as the primary genotyping method for the national genotyping surveillance system.

METHODOLOGICAL ISSUES INTERPRETING IS*6110* RFLP GENOTYPING

To interpret the results of genotyping, we assume that epidemiologically related strains will have the same genotype pattern, and epidemiologically unrelated strains will have different patterns. Clustering has often been equated with recent or ongoing transmission, and the factors associated with clustering have been sought as a means to identify and target subpopulations with substantial ongoing transmission. By contrast, patients whose isolates of *M. tuberculosis* have genotype patterns that do not match any other isolates in the community are considered to be unique and likely represent disease caused by reactivation of a latent tuberculosis infection (LTBI). Molecular techniques enable us to distinguish tuberculosis due to recent or ongoing infection versus reactivation of LTBI and to estimate the proportion of ongoing tuberculosis transmission in a community.

However, in some instances, strains may be identical for reasons other than recent transmission and there is not always an epidemiological link between patients whose isolates have identical genotype patterns. Studies in areas with high or low tuberculosis incidence rates have demonstrated that clustered cases often have no discernible contact or other epidemiological links among themselves, even in relatively stable populations.[10,11] In addition, the amount of transmission represented by genotypic clustering will depend on the sampling strategy and duration of the study.[12,13] Undersampling can bias the estimates of the proportion of tuberculosis cases that were likely caused by recent or ongoing transmission and it can bias the estimates of the risk factors associated with clustering. Biases may also be introduced if a molecular epidemiologic study does not cover an adequate time period. Two population-based cohort studies that have been implemented for more than 10 years in San Francisco[14] and The Netherlands[15] reported that the percentage of clustered strains was high during the first 2 years and declined thereafter. Clustering based on < 2 years of sampling is unlikely to identify the source and secondary cases in a chain of transmission and will likely underestimate the amount of ongoing transmission.

TRANSMISSION OF TUBERCULOSIS

Molecular genotyping has revolutionized our ability to track strains of *M. tuberculosis* as they spread through a community and has provided insights into the

transmission of *M. tuberculosis*. Following are examples of the way genotyping has improved our understanding of the transmission and pathogenesis of tuberculosis.

Contact and Outbreak Investigation

Conventional tuberculosis contact investigations use the "stone-in-the-pond" or concentric circle approach to collect information and to screen household contacts, coworkers, and increasingly distant contacts for tuberculosis infection and disease.[16] However, the concentric circle method may not be adequate in many out-of-household settings and particularly mobile populations. In low-incidence areas such as San Francisco[17] and Amsterdam,[18] a relatively small proportion (5–10%) of the cases that had identical IS*6110*-based genotyping patterns were actually identified and named as a contact by the source case. Unsuspected transmission of tuberculosis occurs and is not easily detected by conventional contact tracing investigations. In a 5-year, population-based study in The Netherlands, contact investigations of persons in five of the largest clusters identified epidemiological links between them based on time, place, and risk factors.[15] However, tuberculosis transmission also occurred through only short-term, casual contact that was not easily identified in routine contact investigations.

Even when the essential elements of tuberculosis control are in place, ongoing transmission of *M. tuberculosis* will continue to produce cases unless patients are diagnosed early and all contacts are identified. For example, in a population-based molecular epidemiological study in an urban community in the San Francisco Bay area, 75 (33%) of 221 cases in this community had the same strain of *M. tuberculosis*.[19] Thirty-nine (53%) of the 73 patients developed tuberculosis because they were not identified as contacts of source case-patients; 20 case-patients (27%) developed tuberculosis because of delayed diagnosis of their sources; and 13 case-patients (18%) developed tuberculosis because of problems associated with the evaluation or treatment of contacts; and one case-patient (1%) developed tuberculosis because of delays identifying the person as a contact.

Some populations, such as the urban homeless, present unique challenges for conventional contact investigations. Contact tracing in the community can be ineffective in tuberculosis outbreaks if patients do not live in stable settings and either do not know or are unwilling to reveal the names and whereabouts of contacts. However, studies that incorporate genotyping are able to provide information about the chains of transmission in these groups.[20,21] A prospective study of tuberculosis transmission in Los Angeles, California, identified 162 patients who had culture-positive tuberculosis and interviewed the patients to identify their contacts and whereabouts.[22] Patients whose isolates had identical or closely related genotyping patterns were considered clustered, and the degree of homelessness and having used daytime services at three shelters were independently associated with clustering. Traditional contact investigations did not reliably identify patients infected with the same strain of *M. tuberculosis:* only two of the 96 clustered cases named others in the cluster as contacts. This study demonstrated that locations where the homeless congregate are important sites of tuberculosis transmission.

Additional studies support the idea that specific locations can be associated with recent or ongoing tuberculosis transmission. In a 30-month prospective, citywide study of all tuberculosis cases in Baltimore, Maryland, using traditional contact investigations and IS*6110*-based genotyping, 46% (84/182) of initial isolates were clustered and 32% (58/182) of the cases were considered to have tuberculosis that was recently transmitted.[23] Only 24% (20/84) of clustered cases had an identifiable epidemiologic link of recent contact with an infectious tuberculosis patient. Using geographic information system (GIS) data, the 20 clustered cases with epidemiologic links in geographic areas of the city with low socioeconomic status and high drug use were spatially aggregated. Such studies suggest that location-based control efforts may be more effective in some populations than traditional concentric circle-based contact tracing for early identification of cases.

Genotyping has been particularly useful identifying otherwise unsuspected and undetected transmission in the community. By identifying tuberculosis patients whose isolates have identical strains of *M. tuberculosis*, genotyping can infer epidemiologic links or connections between individuals and can highlight locations or settings where tuberculosis transmission is occurring. Molecular epidemiologic techniques have confirmed suspected and unsuspected tuberculosis transmission in places such as residential care facilities (see Fig. 1),[24] bars,[25–27] crack houses,[28] sites of illegal floating card games,[29] schools,[30,31] hospitals,[32,33] and jails and prisons.[34–36] Tuberculosis transmission has also been demonstrated among groups such as church choirs,[37] interstate transgender social networks,[38] and renal transplant patients,[39] and from patient to health care providers[40] and from health care providers to patients.[41,42]

Some quite unusual sources of tuberculosis transmission have been confirmed with molecular epidemiologic methods. For example, processing contaminated medical waste resulted in transmission of *M. tuberculosis* to at least one medical waste treatment facility worker.[43] Genotyping was also used to document unsuspected bronchoscopy-related transmission and the cross-contamination of patients.[44,45] Without the availability of genotyping, it would have been very difficult to confirm that transmission had occurred in such settings.

Potential Infectiousness of Patients

Molecular epidemiology studies have confirmed the variation in infectivity that exists between patients with tuberculosis. For example, a single patient with smear-positive pulmonary tuberculosis was directly or indirectly responsible for 6% of the tuberculosis cases in San Francisco during a 2-year period, including those in the human immunodeficiency virus (HIV) residential care facility.[24] In another report, IS*6110* RFLP analysis showed that a single homeless tuberculosis patient with highly infectious pulmonary tuberculosis who was a regular patron of a neighborhood bar likely infected 42% (41/97) of the contacts who were regular customers and employees of the bar and caused disease in 14 (34%) of them. Among 12 patients whose isolates of *M. tuberculosis* were available, all had identical IS*6110* RFLP band patterns.[25]

Studies have also demonstrated that patients with sputum smears that are negative for acid-fast bacilli but culture-positive for *M. tuberculosis* can transmit infection to others in the community, although they are less infectious than smear-positive patients. Behr and colleagues[45a] reported that patients with smear-negative culture-positive pulmonary tuberculosis were likely responsible for 17% of cases in San Francisco. Despite receiving a full course of antimicrobial therapy, these patients served as a significant source of infection in the community. What is unknown, however, is the potential role of smear-negative pulmonary tuberculosis patients as a source of infection in a different setting if they remained undetected and untreated. This finding has important implications for control measures that can decrease transmission. More recently, investigators in San Francisco reported that patients with pleural tuberculosis with negative sputum cultures are very unlikely to generate secondary cases of tuberculosis.[45b] The potential for transmitting tuberculosis should be considered with all suspect pulmonary tuberculosis patients particularly in settings and environments that facilitate transmission, such as shelters, hospices, health care facilities, prisons, and other institutional or crowded settings. Although international guidelines for the diagnosis and treatment of tuberculosis prioritize the detection and treatment of sputum smear-positive patients, timely diagnosis and treatment of sputum smear-negative culture-positive cases should be considered when resources permit, as it has been outlined in the World Health Organization document addressing the expanded directly observed therapy short-course (DOTS) framework strategy.[45c]

Community Epidemiology and Risk Factors for Clustering

Tuberculosis develops by rapid progression from a recently acquired infection, reactivation of LTBI, or occasionally from exogenous reinfection. Most molecular epidemiology studies have assumed that the proportion of clustered isolates in a population estimates the amount of recent or ongoing transmission of *M. tuberculosis*. The number and proportion of tuberculosis cases that are in clusters varies from study to study (Table 1). The frequency of clustering ranges from 17% in low incidence areas such as Vancouver, British Columbia,[46] and 34 to 46% in urban areas in the United States[17,24] and western Europe.[15,47] Among gold miners in South Africa, 50% of tuberculosis patients were in clusters[11] and in Botswana 42% of the cases were clustered.[48] However, it is difficult to compare studies because they varied in several important ways such as the population studied, the proportion of all tuberculosis cases studied, the duration of the study, the definition of clustering, and the method of secondary genotyping used.

The proportion and rate of clustering can be used as indicators to assess the performance of tuberculosis control programs. In an evaluation of tuberculosis transmission over a 7-year period in San Francisco, the number and proportion of clustered tuberculosis cases declined, particularly among the U.S.-born population. This was attributed to the implementation of targeted tuberculosis prevention and control programs such as screening high-risk populations and implementation of DOT to ensure high cure rates.[49] A recent study in New York City showed that as tuberculosis case rates fell from recent high levels, the proportion of tuberculosis cases caused by recent transmission dropped from 63.2% in 1993 to 31.4% in 1999.[50] Tuberculosis was unlikely to result from recent transmission in persons born outside the United States. By contrast, an 8-year study in Greenland showed that the annual incidence of tuberculosis doubled from 1990 to 1997 and the percentage of culture positive tuberculosis cases in RFLP clusters increased to 85%, reflecting microepidemics among adults and young children in small, isolated settlements.[51]

Conventional epidemiological methods can be used in combination with molecular genotyping techniques to identify the risk factors associated with recent infection and rapid progression to disease (see Table 1). In studies in low incidence areas, young age, being in an ethnic minority group, homelessness, and substance abuse were associated with recent infection.[17,18,23,52] In a recent study in New York City, birth outside the United States, age ≥ 60 years, and diagnosis after 1993 were factors independently associated with having a unique strain, whereas homelessness was associated with clustering or recent transmission. Tuberculosis among foreign-born persons was more likely to result from recent transmission among those who were HIV-infected and more likely to result from the reactivation of LTBI among those who were not infected with HIV.[53] These data suggest that tuberculosis prevention and control strategies need to be targeted to the large number

Table 1 Frequency of Clustering and Risk Factors for Clustering in Selected Population-Based Studies

Study and Location	Study Population	*N*	Ever Clustered (%)	Risk Factors for Clustering
Low/Moderate Incidence Areas				
Small et al, 1994[17] San Francisco, CA	Community-based	473	40	• Acquired immunodeficiency syndrome • U.S.-born
Bishai et al, 1998[23] Baltimore, MD	Community-based	182	46	• Intravenous drug use
van Soolingen et al, 1999[15] The Netherlands	Country-based	4266	46	• Male gender • Urban residence • Dutch and Surinamese nationality • Long-term residence in Netherlands
Hernandez-Garduño et al, 2002[46] Vancouver, BC	Community-based	793	17	• Canadian-born aboriginals • Canadian-born nonaboriginals • Injection drug users
Diel et al, 2002[47] Hamburg, Germany	Community-based	423	34	• Alcohol abuse • History of contact tracing • Unemployment
High Incidence Areas				
Godfrey-Faussett et al, 2000[11] South Africa	Gold miners	419	50	• Treatment failure • Time spent working in mines
Lockman et al, 2001[48] Botswana	Community-based	301	42	• Imprisonment

of foreign-born persons in New York City who have latent tuberculosis infection.

There are few population-based studies from high-incidence areas. In a study of South African gold miners, tuberculosis patients who had failed treatment at entry to the study were more likely to be in clusters (adjusted OR = 3.41), and patients with multi-drug-resistant tuberculosis (MDR-TB) were more likely to have failed tuberculosis treatment but less likely to be clustered than those with a drug-susceptible strain (OR = 0.27).[11] HIV seropositivity, although common (53.6%), was not associated with clustering. Apparently, persistently infectious individuals who had previously failed treatment were responsible for one third of the tuberculosis cases in this population.

Exogenous Reinfection with *M. tuberculosis*

Molecular genotyping can determine whether a patient with a recurrent episode of tuberculosis has a relapse with the previous strain of *M. tuberculosis* or exogenous reinfection with a new strain. Several studies reported that exogenous reinfection can occur in both immunocompromised and immunocompetent persons.[53–55] In Cape Town, South Africa, where there is a high incidence of tuberculosis and ongoing transmission, 16 of 698 patients had more than one episode of tuberculosis, 75% of whom (12/16) had pairs of isolates of *M. tuberculosis* with different IS*6110*-based genotyping patterns.[56] Episodes of tuberculosis reinfection in areas with a low incidence of tuberculosis, such as Switzerland[57] and The Netherlands,[58] are uncommon compared with those in high to moderate incidence regions.[59–63]

Some cases of suspected exogenous reinfection may be due to initial infections that include more than one strain. Multiple infections were demonstrated in a patient in San Francisco,[64] in two patients who worked in a medical-waste processing plant in Washington State,[65] and among prisoners in Spain.[66] These observations indicate that simultaneous infections with multiple strains of *M. tuberculosis* occur in immunocompetent hosts and may be responsible for conflicting drug-susceptibility results[67] or episodes of relapse caused by exogenous reinfection.

Impact of Drug-Resistance on Transmission and Pathogenesis

Molecular epidemiologic studies have reported that patients with drug-resistant strains were less likely to be in clusters, inferring that drug-resistant strains could be less predisposed to being transmitted or to cause active tuberculosis.[11,15,68] A recent study by Burgos and colleagues[69] reported that the number of secondary cases generated by isoniazid-resistant cases of tuberculosis was significantly less than drug susceptible cases. This difference in the generation of secondary cases was noted regardless of HIV status and place of birth. The results of the genotyping studies are consistent with animal studies, which have shown that isoniazid-resistant strains caused significantly less disease in guinea pigs than drug-susceptible strains.[70] Mutations or deletions within the *katG* gene of isoniazid-resistant strains

of *M. tuberculosis* have been associated with a decrease in the pathogenicity in animal models.[71]

There are populations in which drug resistance is neither detected nor treated effectively, and where the longer duration of infectiousness for patients with drug-resistant organisms treated with standard regimens might offset the bacterium's diminished capacity to cause secondary cases.[69] In areas with high prevalence rates of HIV, the increased host susceptibility, even to strains with diminished virulence, may offset bacterial differences. Because poor tuberculosis control and underlying HIV infection are common in many areas, drug resistance may disseminate locally despite the diminished propensity of drug-resistant strains to cause disease.

Geographical Distribution and Dissemination of *M. tuberculosis*

Population-based data from the San Francisco Bay area suggest that *M. tuberculosis* does not rapidly transmit and spread across geographic boundaries and tuberculosis control programs should focus on transmission within well-defined areas.[72] However, some strains of *M. tuberculosis* are widely dispersed both geographically and temporally, suggesting that the strains are either more transmissible or they are more likely than other strains to cause disease. The Beijing family of strains, for example, has been detected in high proportions among strains in China,[73] other parts of Asia,[74] the former Russian Federation,[75] Estonia,[76] Europe,[77–79] and South Africa[80] and has been associated with large outbreaks, febrile responses,[81] treatment failure and relapse,[82] and drug resistance.[83] The "W strain," a multi-drug-resistant strain of *M. tuberculosis* that caused many cases of tuberculosis among patients and health care workers in nosocomial outbreaks and other institutional settings in New York City[84–87] is a member of the Beijing family.[88] It is unclear why the Beijing family strains are so widely disseminated.[89] Perhaps the Beijing genotype was introduced into multiple locations before other strains and had more time to spread. One study reported that mutations are present in putative mutator genes in the Beijing genotype and not in other strains.[90] It is possible that the Beijing genotype has a selective advantage and is more readily aerosolized, can establish infection more effectively, or can progress more rapidly from infection to disease.[4,91]

THE FUTURE OF MOLECULAR EPIDEMIOLOGY

Molecular genotyping, in combination with conventional epidemiologic investigations, has contributed greatly to our understanding of the transmission and pathogenesis of tuberculosis. The development of real-time amplification-based genotyping techniques should improve our ability to rapidly define a genotype and to do effective, timely contact and outbreak investigations.[92]

In the near future, molecular epidemiology will help us determine whether the observed genotypic variations in *M. tuberculosis* are associated with significant phenotypes and are important in the pathogenesis of tuberculosis. For example, some IS*6110* transpositions or mutations may alter gene expression and directly facilitate reactivation of a latent tuberculosis infection or confer another selective advantage. Some genotypes may be predisposed to survive aerosolization, whereas others may be better able to evade the host immune system and cause rapid progression to disease. As our current molecular epidemiologic approaches intersect with developments in mycobacterial population genetics, comparative genomics, immunology, and other disciplines, a variety of genotyping techniques will help distinguish between different strains with specific phenotypic characteristics such as transmissibility, pathogenicity, or resistance to antimicrobial agents.

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