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REVIEW ARTICLE

Molecular epidemiology of tuberculosis: methodology and applications

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The resurgence of tuberculosis around the world has renewed interest in understanding the epidemiology and pathogenesis of this disease. A revolutionary advance in the field of tuberculosis research has been the development of molecular techniques that permit identification and tracking of individual strains of *Mycobacterium tuberculosis*. With these techniques, molecular epidemiology has been established as a new discipline that adds another dimension to the classical epidemiology of tuberculosis and has increased our understanding of the transmission dynamics of *M. tuberculosis.* The increased epidemiological knowledge has led to discovery of inadequacies in tuberculosis control programs; this information has helped garner resources for program improvement and has highlighted the need for the continuous surveillance of tuberculosis. Additional genetic methods are being developed based on the knowledge of the genome sequence of *M. tuberculosis.* These simpler and less costly genotyping techniques promise to expand the application of molecular epidemiology to developing nations (where 90% of the disease burden occurs) in support of national tuberculosis programs. Furthermore, these tools permit ever more effective probes into the dynamics of transmission, the population structure, evolution and pathogenesis of *M. tuberculosis*.

Key words: tuberculosis, *Mycobacterium tuberculosis/*pathogenicity/drug effects, molecular epidemiology, genotype, DNA fingerprinting/methods, restriction fragment length polymorphins.

Epidemiología molecular de la tuberculosis: métodos y aplicaciones

La reemergencia de la tuberculosis en el mundo ha despertado el interés en el entendimiento de la epidemiología y patogénesis de esta enfermedad. Un revolucionario avance en este campo de investigación ha sido el desarrollo de técnicas moleculares que permiten identificar y establecer la huella particular de cada cepa de *M. tuberculosis.* Con el uso de estas técnicas, y el establecimiento de la epidemilogia molecular como nueva disciplina se adicionó otra dimensión a la epidemiologia clásica de la tuberculosis y ha incrementado el conocimiento de la dinámica de la transmisión de *M. tuberculosis* dentro de una población. En el proceso han sido identificados problemas en los programas de control, lo cual ha ayudado a obtener recursos para su mejoramineto e implementación. Aún más, se ha resaltado la necesidad de continuar vigilando esta enfermedad. Otras metodologías genotípicas han sido desarrolladas a partir del conocimiento de la secuencia del genoma de *M. tuberculosis*. Estas metodologías genotípicas de fácil implementación y bajo costo se deben aplicar en países en vía de desarrollo, donde existe el 90% de la enfermedad, como apoyo a los programas de control de la tuberculosis. Estas herramientas permitirán conocer la dinámica de transmisión de la tuberculosis, la estructura de la población, la evolución y patogénesis de *M. tuberculosis.*

Palabras clave: tuberculosis, *Mycobacterium tuberculosis/*patogenicidad/efecto de drogas, epidemiología molecular, genotipo, huella de ADN/métodos, polimorfismo de longitud del fragmento de restricción.

Genotyping methods

With the arrival of the genomics era, the ideal method, in principle, to access genetic variability between strains is the comparison of entire individual genome sequences among *Mycobacterium tuberculosis* strains (1). However, assessment of whole genomic variability among strains requires bioinformatics expertise, time, and economical resources. These limitations allow us only to interrogate a fraction of the genome to obtain genotypes with sufficient variability to discriminate among different strains. This approach permits a rapid interpretation of the patterns obtained and is not time consuming. For example, the standardization and application of molecular epidemiology techniques with the insertion sequence IS6110 restriction fragment length polymorphism (RFLP) has brought a new dimension to the study of tuberculosis and with it, a new appreciation of the bacterial ecological complexities within populations that classical epidemiology could not provide. The IS6110 RFLP technique is the most extensively applied and studied method, but is labor intensive and expensive when compared to the newer PCR (polymerase chain reaction) based molecular epidemiology techniques. In addition, the ease of access to the *M. tuberculosis* genome sequence has led to the recognition of more than 30 other repetitive elements, which promise to be useful as typing methods (2). In this review we will briefly discuss the most commonly used genotyping methods.

IS6110 RFLP

This insertion sequence is mostly present in the *M. tuberculosis* complex species. The number of copies of IS6110 differs from 0 to over 25 and their insertion position in the *M. tuberculosis* genome is variable between different strains (3). However, IS6110 sequences are non-randomly distributed, suggesting preferential integration regions or insertional hot spots. One of these is an area flanked by a directly repeated (DR) sequence (4). This variability is sufficient to generate polymorphism and serves as the basis for differentiating between strains. This non-random distribution of IS6110 within the genome and the low copy number of some strains depending on geographic region is recognized as a limitation to the discriminative power of this methodology and its broad application (5).

The IS6110 insertion sequence based typing is the gold standard to which other techniques are presently evaluated; this technique has been standardized and is widely used. A variety of factors were recognized for the standardization of *M. tuberculosis* genotyping. For example, the use of a unique restriction enzyme for DNA digestion, and the inclusion of molecular standards to estimate band sizes (6). Consequently this standarization of IS6110 RFLP is now extensively applied in the genotyping investigations of *M. tuberculosis* (7-11). This standardization has made possible the comparison of genotypes performed in different laboratories around the world, allowing the global dispersal of strains to be tracked. For example, an HIV seropositive patient developed primary tuberculosis with a multidrug resistant (MDR) strain in San Francisco. This patient had been hospitalized in Buenos Aires, Argentina, the previous year, during the time of a reported large outbreak of MDR-TB (12). The San Francisco strain was found to have exactly the same IS6110-RFLP pattern and antibiotic resistance profile as the Buenos Aires strains (M. Burgos, unpublished data). This transnational spread demonstrates the geographic extent to which individual strains of *M. tuberculosis* can be disseminated.

Population based molecular epidemiological studies report that most strains have between 8 to 18 copies of the IS6110 insertion element each, a number that is adequate to allow discrimination between the majority of strains. However, there are geographical areas in Asia and Africa where the diversity of IS6110 is considerably reduced (13-15). In San Francisco, out of 1800 patients during a period of 9 years, 1117 (62.2%) had distinct genotypes and 683 (37.9%) were in 171 clusters sharing identical patterns (more than 5 bands). In some parts of Asia and Africa the lack of polymorphism linked with low copy numbers

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limits the epidemiological inferences that can be drawn from the use of this genotypic method. However, other secondary typing methods could help discriminate between strains with few IS6110 copies (16-20).

Understanding the rates at which IS6110 patterns change is important for optimal interpretation of fingerprints in molecular epidemiology studies taking into account that a group of strains may be identical for reasons other than recent transmission, depending on the stability of the marker. For example, if the IS6110 pattern changes too rapidly, the number of false negative epidemiological links will tend to increase and if the marker is too stable the number of false positive links will tend to decrease. Thus, markers that change too rapidly will underestimate the number of true epidemiological links while markers that change too slowly will tend to overestimate the number of true epidemiological links. For instance, Yeh et al. found that the banding patterns of serial isolates collected at least 90 days apart from the same patient, were different in 29% of cases (21). In this study, changes in IS6110 genotypes were confined to strains with 8-14 bands and the rate of change suggested that the number of epidemiological links were underestimating recent transmission. However it is possible that studies involving serial secretors overestimate the rate of change of IS6110 patterns, because replication within a host (growth of the mycobacteria) can provoke IS6110 transposition (10). Nonetheless, serial passage of strains *in vitro*, such as for BCG, has not been associated with such rapid change of RFLP genotypes (22). On the other hand, in the Netherlands, the half-life of change of IS6110 was estimated to be 3.2 years (23). In this study the rate of change of IS6110 supports the use of this genotype in studies of recent transmission of tuberculosis.

PGRS RFLP

The polymorphic GC-rich repetitive sequence (PGRS) is present in multiple chromosomal clusters allowing its use as a second RFLP typing method (19,24,25). This method has a discriminatory power resembling that of IS6110 typing, even in isolates with low copy numbers of IS6110 (26). However, PGRS is comprised of many non-perfect repeats making the RFLP patterns complex and their interpretation difficult. As with IS6110, this method requires viable cultures and large quantities of DNA, which makes it too labor intensive for epidemiological investigations.

Simpler, more rapid, less expensive typing methods than the IS6110 RFLP genotype are necessary in order to study transmission of *M. tuberculosis* in countries with high burden of tuberculosis disease. In consequence faster methods that are PCR based have been developed. In this review we will go over the PCR based genotyping markers most extensively used and those methods that show potential for the study of molecular epidemiology in a global scale.

PCR based genotyping

Large-scale genotyping of *M. tuberculosis* is not possible because the existing typing methods are labor-intensive, difficult to analyze and their results are often not comparable among laboratories. In addition as mentioned previously, the use of IS6110 and PGRS RFLP are hindered by the need of *M. tuberculosis* cultures. In contrast, PCR based methods do not require bacterial culture and small amounts of DNA can be obtained in some cases directly from clinical specimens (27,28). Significantly, some of these PCR methods can be interpreted in a binary or digital manner, which makes these typing methods very convenient for standardization and user friendly for analysis when making comparisons among databases from different geographical regions(16,29,30). These methods have the potential to expand the application of molecular epidemiology tools in countries with high burden of disease.

Spoligotyping

The spoligotyping method is a PCR technique based on a major polymorphic tandem direct repeat (DR) sequence in the *M. tuberculosis* genome (31). This DR region in *M. tuberculosis* complex strains is composed of multiple direct variant repeats (DVRs), each of which consist of a repetitive 36 base pair element and a short non-repetitive se-

quence (spacer) (32,33). Using a set of primers targeting the direct repeat sequences, it is possible to amplify simultaneously all the region, including the unique non-repetitive sequences, or spacers, located among the direct repeats. The presence or absence of the spacers is then determined by using reverse hybridization. Individual strains are distinguished by the number of spacers absent from a complete spacer set that has been defined by sequencing this region from *M. tuberculosis* strain H37Rv and *Mycobacterium bovis* BCG.

Spoligotyping is widely used due to its low cost, high reproducibility and simplicity (34). This method is also very easy to interpret because of its binary result format and useful when comparing data bases from different geographical regions (17). In addition, atypical mycobacteria do not give a signal with spoligotyping, which indicates the specificity of this technique for the species within the *M. tuberculosis* complex (35). This methodology has been demonstrated to be useful in discriminating between isolates of *M. tuberculosis* with few IS*6110* bands (36-38). Another advantage of this secondary marker is that it is economical and easy to perform. Spoligotyping is a rapid and efficient way of fingerprinting the *Mycobacterium tuberculosis* complex. Spoligotyping can be performed directly on sputum samples, which makes it practical in acute clinical settings (33). These characteristics make spoligotyping a candidate for use in countries with high burden of tuberculosis disease. However, the discriminatory power of this method is lower than IS6110 typing and varies depending on the geographical area of the study (26,39,40).

Variable numbers of tandem repeats

A more promising approach for developing a PCR based typing system is to identify novel polymorphic loci, which are independent of existing techniques such as IS6110 typing. Automated genotyping based on variable-number tandem repeats (VNTR) of genetic elements named mycobacterial interspersed repetitive units (MIRU) is an example of such methods (41-43). MIRUs are a specific class of VNTR, identified at 41 distinctive loci in the genome of *M. tuberculosis*. Each MIRU encompasses strings of short repetitive sequences and the number of repeats varies among strains. PCR amplification across each MIRU generates fragments of different sizes for different strains, and the number of repeats at each locus can be determined. Analysis of a blinded reference set of 90 strains from 38 countries established that this method is 100% reproducible, sensitive, and specific for *M. tuberculosis* complex isolates(26). This performance has not been achieved by any other typing method tested in the same conditions. This type of approach is for the most part adapted to global databases as each typed strain has been assigned a 12 digit number matching the number of repeats at each MIRU locus (43). This coding system makes interlaboratory analysis easy to carry out. In addition, polymorphisms at certain repeat loci reveal the species, whereas other loci can be used for epidemiologic typing. Lately, to take advantage of the convenience of this genotyping methodology, a website became available for the analysis of *M. tuberculosis* MIRU-VNTR genotypes. Thus, unlike other typing systems this methodology now allows for the global molecular epidemiological surveillance of tuberculosis. In principle, this opportunity should also allow us to gain new insights into the transmission and population genetics of tuberculosis in a global scale (44).

Genotyping and the population structure of *M. tuberculosis*

Until recently, most genotyping methods for the study of *M. tuberculosis* were used in defining differences between individual strains in order to establish epidemiological links of recent transmission. Now researchers are proving into the phylogenetic associations amongst distantly related strains with the use of newer genotyping methods. For example Supply *et al.* confirmed the predominant clonal evolution of *M. tuberculosis* with the use of MIRUs (42). The clonal structure of *M. tuberculosis* has not only important implications for molecular epidemiology but also for phylogeny because clonal species are stable in space and time. This stability permits the utilization of molecular epidemiological databases to track the past and present transmission of the multiple strains of tuberculosis. This explains the

renewed interest in defining these phylogenetic relationships and population structure among different genotype families of *M. tuberculosis* (17,45).

In addition to MIRUs, deletion analysis by microarrays is, for the most part, an attractive approach for studying the phylogeny of *M. tuberculosis* strains because in theory it could provide information about the evolution and pathogenetic potential of a strain phenotype (1,46). The goal is to employ the collection of typing techniques now available to identify individual strains or clonal groupings of strains with specific phenotypic characteristics, such as transmissibility and antigenicity, or resistance to antimicrobial agents. These strains can then be subjected to genome wide analysis, using techniques as microarrays for expression profiling or detection of genomic deletions, to determine the genetic basis of these important phenotypes. This multidisciplinary approach could lead to important advances in our understanding of the pathogenesis of human tuberculosis as well as of mechanisms of drug resistance. For example, by using a microarray, the genome of a strain can be assessed to determine if deletions have occurred relative to a referenced strain. Since these deletions rarely occur independently at exactly the same chromosomal locus, they can be seen as unique and irreversible genetic events (47). Furthermore, the number and distribution of these deletions can provide a genomic framework, for constructing phylogenetic associations and to carry out molecular epidemiology studies (48).

These genotypic techniques have been compared extensively in terms of its sensitivity, easy to use and cost (49). Table 1 summarizes some of the most important characteristics of these techniques.

Molecular epidemiology and transmission dynamics

Over the past decade, molecular epidemiology research has been very useful in understanding outbreaks and transmission dynamics of *M. tuberculosis* within different settings. These studies are based upon the assertion that patients infected with strains showing identical fingerprints, termed 'clustered cases', are the result of recent transmission whereas those infected with a unique strain are the result of reactivation of strains ac-

Table 1. Comparison of genotypic methods for *M. tuberculosis.*

Method	Repeat sequence target	Copy number	Polymorphism	Quantity DNA	Time	Output	Discrimination compared with IS6110 RFLP	Limitation	Advantage
RFLP IS6110	IS6110	0-25	High	Well grown pure culture	weeks	Gel pattern	Similar	High cost	Gold standard
RFLP PGRS	PGRS	26-30	High	Well grown pure culture	weeks	Gel pattern	Similar	High cost	None
Spoligotyping	DR-cluster	43	High	Few grow pure culture	<1 week	Digital code	Lower	Less discriminative	Low cost Easy to use
VNTR-MIRU	MIRU	41	High in 12 MIRU of interest	Few grow pure culture	<1 week	Digital code	Diverse	None	Easy to use Very discriminative

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quired in the remote past (8). However, this interpretation largely depends on the study question, the population under study and the genotyping methods employed. Thus genotyping methods that are stable could be very useful in global tracking and evolutionary studies of tuberculosis, while those that change within years could be used in the study of outbreaks. So far, most molecular epidemiology studies have investigated sporadic outbreaks, cross contamination and the associated risk factors for recent transmission of the cases involved. Another purpose of these studies has been to use molecular epidemiological studies to help differentiate between reactivation of tuberculosis and recent transmission. In this part of the review we will discuss the importance of molecular epidemiology in the context of tuberculosis control.

The impact of HIV on tuberculosis

In addition to the above studies, molecular epidemiology studies have demonstrated the explosive potential for tuberculosis to progress to disease and spread amongst HIV-infected persons (50). In one study, epidemiological surveillance in a tuberculosis control program detected twelve cases of tuberculosis in a residential facility for persons with HIV disease. Analysis of isolates by IS6110 RFLP demonstrated that newly acquired tuberculosis infection in HIV-infected patients spread readily and progressed to tuberculosis disease within 3 months of exposure. This study demonstrated the tremendous impact HIV has on the pathogenesis and the transmission of *M. tuberculosis*.

Furthermore, studies have demonstrated that most tuberculosis outbreaks associated with HIV infection occur in hospitals, prisons and longterm residential facilities in general (51-54). However these outbreaks in some geographical regions are now impacting the population in general. In South Africa, a middle-income country, for example, sentinel surveys attributed 44% of the total tuberculosis cases observed to coinfection with HIV. More alarming is the prediction made by a simulation model based partly on molecular epidemiology data which anticipates that HIV will increase the frequency and severity of tuberculosis outbreaks beyond what is already expected (55). Additional molecular epidemiology studies could help quantitate the actual tuberculosis burden attributed to HIV infection in areas where these two pandemics converge.

Transmission of drug resistance

Molecular epidemiology has also highlighted the epidemic spread of drug-resistant strains among hospitalized patients (12,56-59). Studies conducted on patients hospitalized in New York City revealed that more than one third of patients with multidrug-resistance had clustered RFLP patterns, suggesting recent transmission of *M. tuberculosis* (60). At the peak of the tuberculosis epidemic in the United States, New York City accounted for almost one fourth of all cases of MDR-TB during a 43-month period. Most of these patients were infected with HIV and were found to have acquired MDR-TB while hospitalized (59). Lessons learned from these studies have led to more rigorous adherence to infection control policies in hospital settings by National Tuberculosis Control Programs. As can be inferred from the description above, this is particularly important in settings where there are many HIV-infected persons. In New York City measures intended to control and prevent tuberculosis were associated with a 52% decrease in the number of patients with MDR-TB (61). In this study the DOTS strategy and hospital infection control policies that included the physical separation of HIV infected patients from potential tuberculosis cases has led to dramatic reductions in tuberculosis rates and has drastically curtailed the development of drug resistance.

Transmission and tuberculosis control

The percentage of patients that developed *M. tuberculosis* disease as a result of recent transmission in developed countries was subject to speculation before the introduction of molecular epidemiology. The development of molecular epidemiology tools considerably advanced our understanding of this subject. Population based studies in different developed countries demonstrated that tuberculosis resulting from recent transmission was higher than the estimated 10%

predicted by traditional epidemiological studies. In these countries, 25% to 45% of new cases of tuberculosis were demonstrated to be the result of recent transmission (8,62,63). A common denominator of risk factors was found to be associated with recent transmission: lower socioeconomic status and HIV coinfection. It was demonstrated that ongoing transmission contributed to the disease burden at much higher rates than previously thought and helped gather resources to strengthen tuberculosis control. These studies also showed the importance of control efforts aimed at groups who are at high risk of transmitting or acquiring tuberculosis disease.

In contrast to the studies mentioned above, the percentage of clustering of tuberculosis isolates found in Norway and Switzerland was low, 16% and 17.5% respectively (64,65). In essence this low level of transmission confirmed that these countries had effective tuberculosis control measures in place. In San Francisco, for example, the rates of clustered tuberculosis cases decreased significantly both overall and among persons in high-risk groups after a period during which tuberculosis control measures were intensified (66). These studies demonstrate that molecular epidemiology studies can be used as a tool to access and monitor the performance of tuberculosis control programs by targeting interventions in groups at risk that disproportionately contribute to transmission (67).

However, to date there are still limits to the widespread use of genotyping to guide tuberculosis control. These limitations are usually related to the lack of infrastructure, the complexity of the techniques used, time required to interpret results and cost. These limitations could soon be overcome with advances in the field of PCR base genotyping techniques. Moreover, there are now some reference laboratories in developing countries with genotyping capabilities that want to support the tuberculosis control program with these new technologies (68). In theory these techniques could allow the widespread use of molecular epidemiology as a control measure in the activities of national tuberculosis programs in developing countries.

Contact investigations

In the hope of discovering additional cases, local tuberculosis control programs in industrialized countries often evaluate individuals who have been in contact with infectious tuberculosis patients. The basic notion of this policy is that contacts are likely to have been infected by the infectious case and thus carry the same strain as the index case. This hypothesis was tested in a study of index and contact cases with genotyping markers (69). In this study Behr *et al.* compared the DNA fingerprints of indexes and corresponding contacts with tuberculosis, and found that 30% of the contacts had different fingerprints. The contacts were probably infected in the distant past by other strain, from a third person, and happened to reactivate their disease during the time the study was performed. This complexity in transmission links is also seen in broad population based molecular epidemiology studies. For example, RFLP and contact investigation in different settings demonstrated real epidemiological links in only 5% to 10% of cases (8,64,70). Also, contrary to the dogma that tuberculosis is usually acquired following prolonged exposure to an infectious case, tracking strains in the community demonstrates that transmission can occurr after only transient contact (71).

Transmission in immigrants

The impact of foreign-born individuals on the epidemiology of tuberculosis is seen as an important public health issue in developed countries. The reason is that a large proportion of tuberculosis cases are diagnosed among immigrants. In Canada, 80% of the tuberculosis reported in Montreal and Vancouver occurred among immigrants (72,73). Moreover between 1986 and 1997 the number of tuberculosis cases diagnosed in foreign-born persons in the United States increased by more than 50%, raising the question of whether or not immigrants do contribute to active transmission of tuberculosis to the native population. In regards to this question in San Francisco, only one out of 43 cases among immigrants resulted in two secondary cases of tuberculosis infection among the US born population (74). Interestingly, in the study, one-

fifth of the Mexican born patients acquired their tuberculosis in San Francisco from US born cases. Another study from the same area showed that immigrants developed tuberculosis at a much higher rate due to reactivation, than US born cases. In this study, native born individual developed tuberculosis mostly from recent transmission (75). In the Netherlands and Norway most recent transmission occurs among foreign-born individuals of the same nationality and not between immigrants and the native community (76,77). In Vancouver, the risk of belonging to a cluster was higher among immigrants cases than among Canadian born cases.

Several studies from Europe, Canada, and the United States showed that the greatest risk for developing tuberculosis in immigrants occurs in the first 3 to 5 years upon arrival in the host country (78,79). One possible reason for these observations is that recent immigrants usually encounter poorer social and economical conditions on arrival in the host country compared to the conditions they left behind in their native country. These poor socioeconomic conditions are thought to result in reactivation of latent tuberculosis and in some cases active transmission. One could make a case that in addition to strengthening tuberculosis control strategies, improving socioeconomic conditions among recent immigrants could help to decrease the rates of tuberculosis. Further awareness of the transmission dynamics and risk factors of tuberculosis among group of immigrants will permit priorizing strategies to further decrease tuberculosis infection.

Reinfection and reactivation

The percentage of reinfection versus recurrence of tuberculosis has important public health implications. It was not until molecular fingerprinting techniques became available that the exogenous reinfection phenomenon was convincingly demonstrated to occur (80). This topic is of extreme importance in developing countries with high incidence of tuberculosis because exogenous reinfection can make a significant contribution to the overall burden of tuberculosis. Consequently establishing the rates of reinfection in different settings is imperative for predicting the effects of control strategies, such as directly observed therapy (DOTS) and treatment of latent infection on the course of the current tuberculosis epidemic. For example, it is interesting to speculate the impact DOTS will have on different scenarios of rates of reinfection. If DOTS rapidly shuts off transmission, and reinfection is rare, the epidemic will die off slowly because cases will continue to appear only through reactivation. On the other hand, if reactivation is uncommon, the epidemic will decline relatively quickly as DOTS prevents new cases by stopping transmission and reinfection (10). Unfortunately, today there are only few molecular epidemiology studies that provide data on reinfection rates and most of them do not provide the methodological design required to estimate the incidence on recurrence due to reinfection (81).

In high incidence settings, particularly those with high rates of HIV coinfection, it is anticipated that reinfection plays an important role. This was apparently the case in a study in Africa where up to 75% of patients had different fingerprints in their initial and second episode of disease (82). However, in another study in the same country only 2% of cases were found to be due to reinfection (83). In a population based study in Spain in a setting with good tuberculosis control measures, the authors found that 44% of cases were the result of exogenous reinfection (84). Larger population based studies are needed to determine reinfection rates in high incidence countries and to shed light on important questions of vaccine development, tuberculosis control and chemoprophylaxis. Apart from high incidence areas the problem of recurrence of tuberculosis due to reinfection is not likely to have important implications for tuberculosis control programs.

Insights into the pathogenesis of tuberculosis

The impact of resistant on the pathogenesis of M. tuberculosis

Experimental models suggest that antibiotic resistance imposes a biological cost on bacterial fitness (85). For example, it is thought that the mechanism by which *M. tuberculosis* develops

resistance (genetic alterations mostly by mutations in the genome) incurs a fitness cost on the microorganism. This hypothesis is supported by in vitro studies, which show that *katG* (the gene that activates isoniazid into its active form) is required for optimal survival of *M. tuberculosis* in animal models (86).

Molecular epidemiology studies carried out in the Netherlands, Mexico and South Africa in which less clustering was observed among drug-resistant strains, suggest that these strains do not spread at the same rate as drug susceptible strains (71,83,87). In a more in-depth molecular epidemiology analysis of clustering over a decade in San Francisco, the authors assessed the relative pathogenicity of drug-resistant and drug-susceptible strains, in a human population by tracking the strains of *M. tuberculosis* as they spread in the community (11). In this study strains that were resistant to isoniazid either alone or in combination with other drugs, were found to have a significantly reduced relative pathogenicity when compared to susceptible phenotypes. The fitness cost observed in this study diminished the epidemiological impact of isoniazid and MDR-TB resistant phenotypes in this population. However the resistant organism's diminished capacity to cause disease might be offset by delay or inappropriate treatment because of longer potential duration of infectiousness. Protracted infectiousness occurs not infrequently in most developing countries where drug resistance is not detected or treated effectively. Thus before extrapolating the findings of this study to other settings it will be important to identify the molecular basis of the attenuation of resistant mutants and to determine whether similar findings are occurring under different epidemiological conditions.

The Beijing genotype and virulence of M. tuberculosis

A distinct family of *M. tuberculosis* strains was associated with more than 350 cases in New York City and at one point accounted for 25% of all MDR-TB cases in the United States (88). These strains were later demonstrated to belong to a branch of a distinct family and were named the 'Beijing genotype' because of their high prevalence in the Beijing area of China. The Beijing IS6110 RFLP pattern is not unique and is often difficult to distinguish from other genotypes. On the other hand, the spoligotype of the Beijing family is very much distinctive and straightforward to identify. In almost every country where it has been studied, the Beijing phenotype has been found. For example, with the use of spoligotyping, the Beijing strains have been associated with transmission in Asia, Europe (specially, Eastern Europe) and the Americas (89-95). Its presence has been detected throughout South East Asia and Hong Kong, and in one area of study the Beijing genotype family accounted for 70% of all isolates (96).

In the Canary Islands, this genotype was apparently initially introduced by an immigrant from Africa and over a period of 4 years this genotype became the most predominant strain after its introduction (97). In some areas of the world, the Beijing genotype has been associated with cases of tuberculosis in young individuals and febrile illness associated to treatment (98). The association of this genotype with a younger age group is recognized as an indication of ongoing transmission. Also, reports have found a significant correlation between the Beijing genotype and transmission of drug resistance (89,94,99). The global dissemination and apparent transmissibility in association with drug resistance of this genotype has alerted to the possibility that these epidemiological characteristics of this genotype are a reflection of an intrinsic biological property unique to this family. In fact, recently, Rad *et al.* (100) demonstrated the association of alterations in DNA repair genes (mutator genes) to the Beijing genotype. Mutator genes are presumed to allow the accumulation of mutations in drug resistant target genes at higher rates allowing for the development of resistance. Another example of molecular mechanisms possibly involved in virulence of drug resistant strains is the regulation of genes mediated by IS6110. This insertion sequence appears to be associated with the upregulation of genes implicated in virulence of Beijing strains and other clinical strains that have caused MDR-TB outbreaks (101,102). Further studies with this family

of strains will reveal the molecular mechanisms involved in virulence, if in fact these strains are more virulent than others, and their impact on the worldwide tuberculosis epidemic. Future molecular epidemiologic studies may be able to use these findings to specifically identify virulent strains; this would allow to individualize control efforts based on the epidemiological, environmental, host and bacterial characteristics.

Conclusion

Molecular epidemiology has added a new dimension to the classical epidemiology of tuberculosis and greatly improved our understanding of the transmission dynamics of *M. tuberculosis* within different populations. New genotyping methodologies are emerging from genetic manipulation of bacteria and the complete genome sequence of *M. tuberculosis* that are providing us with novel tools for probing further into the pathogenesis and epidemiology of tuberculosis. With these new tools a ground-breaking role for molecular epidemiology is emerging that studies transmissibility in terms of environment, host and bacterial characteristics. However, the remaining challenge is to understand the transmission dynamics of tuberculosis with molecular epidemiology techniques in those countries with the highest burden of disease. The application of molecular epidemiology in support of national tuberculosis control programs in high incidence countries could unleash the true potential of these methodologies as a research tool.

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