

Discussion

Molecular epidemiology of *Mycobacterium tuberculosis* and its relevance to the surveillance and control of TB: an e-debate

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1. First question: Give in a few lines your definition of ‘Molecular Epidemiology’ and your opinion about what it is good for in general (not only for TB)

1.1. Response from LT

In the last 20 years, Molecular Epidemiology has become a major field of research, which corresponds to the integration of molecular approaches into the conventional epidemiologic studies. This scientific domain implies several disciplines, comprising medicine, molecular biology, epidemiology and biostatistics.

Molecular epidemiology can be defined as a science that permits to understand the transmission, the pathogenesis and the etiology of a disease in a human populations.

Based on this definition, the use of molecular epidemiology to study the infectious diseases has mainly permitted to or will permit to:

- estimate the association of risk factors with the transmission of the disease;
- detect and confirm outbreaks in institutional settings;
- screen strains of public health importance;
- distinguish between reactivation and recent infection (case of TB);
- track global spread of pathogens;
- understand the virulence and resistance mechanisms of different strains;
- improve the knowledge on transmission dynamics and dissemination pathways of infectious diseases;

- and the last but not the least, to develop strategies for the treatment and the prevention of the disease.

1.2. Response from BK

As correctly described by Loubna, molecular epidemiology has opened many pathways of investigation and in regard to tuberculosis, a number of central dogmas have been challenged. This includes the finding that exogenous infections occur in both HIV+ and HIV– tuberculosis patients, and that the percentage of recent transmission in a given population is higher than previously expected. The molecular tools have also provided precise markers to distinguish between primary and acquired drug resistance in a given population. In regard to virulence and pathogenesis studies, molecular epidemiology proves to be directive in identifying clones associated with a given phenotype—from drug resistance to toxin production.

It should be stressed that the information derived from molecular epidemiological investigations is always dependent on the sampling and that a non-bias, representative collection is critical to each study.

1.3. Response from CC

The molecular epidemiology is the application of molecular biology techniques to the study of the distribution and determinants of an infection and communicable diseases. For the clinical microbiologist the notion of epidemiologic problem is most often related to a nosocomial infection which is usually caused by bacteria. To verify the existence of a nosocomial infection it is necessary to establish that all cases are caused by the same bacteria. That is the role of the

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microbiologist. When epidemiologic typing of the bacterial strains is performed, phenotypic techniques are most often insufficient for discriminating among the isolates. The use of molecular techniques gives most of the time the answer about whether strains are epidemiologically related or not.

1.4. Comment from MT

About Loubna's excellent response: please remember that the interest of molecular epidemiology is not limited to human infections, and includes pathogens of animals and plants. This makes it possible, in the domain of human medicine, to clarify the epidemiology of human diseases that have an animal reservoir, such as Chagas disease (caused by the parasitic protozoan *Trypanosoma cruzi*, that can infect any mammal) or infections by *Mycobacterium bovis*.

2. Second question: In the field of tuberculosis, have you heard about, or can you think about—a case where a result from a molecular epidemiology survey has led, or could lead, to a practical decision in terms of public health?

2.1. Response from BK

Over the last decade the genotyping of *M. tuberculosis* has provided evidence based data to evaluate transmission, distinguishing relapse from exogenous reinfection, and in identifying cases of laboratory cross-contamination. The fact that patients are subjected to incorrect medical decisions based on a false laboratory result is a serious public health problem. We and others have shown that the use of routine monitoring of the mycobacteriology laboratory and the use of rapid techniques, such as spoligotyping, has a positive impact identifying these cases.

In addition to laboratory cross-contamination, there are unique settings where genotyping *M. tuberculosis* has had an impact on medical care. Decisions on using pyrazinamide in a treatment regimen has been problematic, as phenotypic susceptibility analysis is inaccurate and provides no clinical guidance. Sequence analysis of the *pncA* gene and the high correlation between mutations in *pncA* and resistance provides a laboratory basis to change treatment decisions.

There are also rare cases where the genotype of an *M. tuberculosis* strain impacts on the treatment regimen. As an example, the New York City multidrug-resistant (MDR) outbreak clone, strain W, which has caused disease in over 500 patients, is always resistant "first-line" therapy and its identification by IS6110 analysis provides strong evidence to alter therapy to second-line agents. The development of a rapid PCR-based multiplex method to rapidly identify this primary resistant clone was developed at the CDC with the aim of controlling the spread of this serious MDR clone.

It is clear that over the last decade, the integration of molecular techniques in the mycobacteriology laboratory

has had a major impact on the control and treatment of tuberculosis.

2.2. Response from CC

The case of cross-contamination of clinical samples during the sampling or culture procedure is possible and more and more frequently reported (Bauer et al., 1997; Gascoyne-Binzi et al., 2001; Ramose et al., 1999). Thanks to molecular epidemiology study of the *Mycobacterium tuberculosis* isolates these incidents can be identify.

The source of cross-contamination can be the sampling material (contaminated bronchoscope) or laboratory contamination of cultures. In the first case, the material has not been sterilized properly (error in the decontamination procedure). In the second case, patient's culture is positive for *M. tuberculosis* but there is not a high clinical suspicion for disease; the culture contamination involves positive clinical samples processed concurrently with negative samples.

These errors can lead to unnecessary visits of falsely diagnosed patients to medical consultants and unnecessary long-term antimicrobial treatment. In terms of public health, mycobacteriology laboratories should institute strict procedures to prevent and identify cross-contamination episodes. Careful documentation of positive specimens, together with close liaison between clinical and laboratory staff involved in culturing and molecular characterization, is necessary to identify such incidents effectively.

2.3. Response from LT

Besides the case of the cross-contamination raised by Dr. Carrière and Dr. Kreiswirth, molecular epidemiology allowed also to identify and to understand nosocomial infections, exogenous reinfection, and to distinguish recent infection from reactivation of latent infection. The last case cited seems to me very fundamental to establish decisions in terms of public health.

Active tuberculosis may result from the reactivation of latent infection or from recent infection. The molecular distinction between these two cases led to determine the risks for TB transmission in the communities, and to strengthen the control of this disease in function of the epidemic situation identified.

In spite of these advances, the situation in developing countries like Morocco, my country of origin, is worrying. Despite the recommendations of WHO (DOTS strategy), the incidence of TB is still very high, and the problem of HIV and multidrug-resistance promotes this increasing in such communities. The future challenge for molecular epidemiology is to provide better understanding of the transmission dynamics of tuberculosis in these countries and to stimulate the implementation of control measures on a more global scale.

3. Third question: Here is a list of markers classically used in molecular epidemiology. Please expose your opinion about their qualities and drawbacks for MTB molecular typing and their suitability for internet-operated surveillance networks: Multilocus Enzyme Electrophoresis (MLEE); Random Primed Amplified Polymorphic DNA; Pulse Field Gel Electrophoresis (PFGE); Spoligotyping; IS6110 Restriction Fragment Length Polymorphism (RFLP) typing; Multilocus Sequence Typing (MLST)

3.1. Response from BK

M. tuberculosis has a monomorphic genome, similar to the limited polymorphism observed in isolates of *Bacillus anthracis* or *Yersinia pestis*. The comparative analyses of the available genomes and the comparative DNA sequence studies revealed limited synonymous mutations; a finding that led to the hypothesis that *M. tuberculosis* is a “young” pathogen. As a result of the limited diversity, MLEE and MLST do not provide a high level of discrimination and are not the method of choice to differentiate *M. tuberculosis* isolates.

In considering genotyping methods to sub-speciate bacteria the IS6110 system is by far the most well accepted standardized method and performed on a global scale. Currently there are more than 60,000 isolates that have been genotyped with IS6110 and this data is comparable from laboratory to laboratory. IS6110 is clearly the present gold standard with the understanding that it has well known limitations—strains with six or less copies of IS6110 should be re-evaluated using an unrelated method such as spoligotyping, MIRUs or Southern hybridization with PGRS.

PFGE and Random Primed Amplified Polymorphic DNA does not provide any advantage to the current use of IS6110 and spoligotyping. PFGE analysis has not been well established for *M. tuberculosis*, standardization and analysis between laboratories is difficult and there are questions whether the procedure needs to be run in a biosafety cabinet. As with all PCR-based method, standardization of the Random Primed Amplified Polymorphic DNA has never been shown to be reproducible within and among laboratories and regardless, it will not provide the discrimination that is found with IS6110 and spoligotyping.

It is likely that SNP analysis will be the eventual method to genotype *M. tuberculosis* and replace IS6110 and spoligotyping. This will be an objective approach that will ultimately database arrays of genetic alterations in numerous target loci and each strain will be defined by the collective presence or absence of mutations at these sites.

3.2. Comment from MT on BK's response to the third question

Barry: (i) It is important to make clear that the monomorphism of MTB genome concerns only housekeeping genes and should be rather termed—“limited polymorphism”.

(ii) About RAPD: its reputation of non-reproducibility is not justified. In experienced hands, its reproducibility is fair. I agree that it is more a research tool than a tool for routine identification. However, its power in terms of population genetics (by allowing multilocus analysis) and phylogenetic analysis (by identifying various species- and strain-specific (“synapomorphic”) characters) is much higher than of IS6110 RFLP. By its power in elucidating *M. tuberculosis* population structure and that of many other pathogens, and in reliably delimiting many species, its contribution to molecular epidemiology has been indirectly considerable.

3.3. Comment from BK on that comment

Michel: The polymorphism is not only in housekeeping genes but in most genes analyzed. The Musser lab has done extensive comparative sequencing on more than 50 genes and even third position codon changes are rare. The genome data on the clinical isolates supports this finding.

It is also clear that the SNP approach will be much more informative than RAPD analyses and provide a more objective approach for both genotyping and evolutionary studies.

3.4. MT

Barry: Musser's hypothesis has been recently challenged by Fleischmann et al. (2002), who found an extensive polymorphism in MTB based on SNP analysis. Please note that this perfectly fits RAPD results of our group (Loubna Tazi and Anne-Laure Bañuls).

3.5. Response from CC to third question

The IS6110 RFLP typing is the most widely applied molecular typing method for *M. tuberculosis*. To facilitate the interlaboratory comparability of this method, all aspects of the procedure have been standardized and it is considered to be the reference method for *M. tuberculosis* typing. However, this technique suffers from significant drawbacks, it is laborious, requiring many technical steps and several micrograms of chromosomal DNA, this implies a culture delay of a few weeks and requires viable organisms. It is an expensive method and sophisticated computer software is required to analyze the patterns in an accurate way. On the other hand, it has been possible to establish international databases of RFLP patterns from different geographical area. Thanks to that database it is possible to trace the source of infection for multidrug-resistant strains and it permitted to know that the Beijing genotype has a high impact on the tuberculosis epidemic in Asia and USSR republics.

Recently, PCR-based methods have been developed. One of them, spoligotyping is a method detecting 43 known spacer sequences which intersperse the DRs in the genomic DR region of *M. tuberculosis* complex strain. It is easy, robust, highly reproducible and rapid. Moreover the

results can be read as a digital code suitable for use in a computer-assisted analysis and, therefore, for internet surveillance network. Another advantage of spoligotyping is that it can be used simultaneously for the detection and typing of the *M. tuberculosis* complex bacteria in one assay. It could be interesting to study Beijing epidemic since these strains harbour a typical spoligotype pattern (reaction with the last nine spacers in the panel of 43).

However, spoligotyping has shown a discriminatory ability lower than of IS6110 RFLP analysis with the exception of that for *M. tuberculosis* isolates with low copy numbers.

Another PCR-based method, the MIRU–VNTRs has been developed. It is based on the variability in copy numbers of tandem repeats of 40–100 bp in length at 12 different intergenic regions of the *M. tuberculosis* complex genome. The discriminatory power of the 12 MIRU–VNTR regions is much higher than that of spoligotyping and close of IS6110 RFLP for typing of *M. tuberculosis*. The results can be read as digital code and this opens the way to the construction of databases. The simple numerical format of the data generated should allow laboratories in different parts of the world to compare their local isolates with those found elsewhere by submitting their MIRU–VNTR data to a central database that can be created on a web site.

To my knowledge, at the present time, three databases are available for internet-operated surveillance network of *M. tuberculosis*: the RFLP patterns, the spoligotype patterns and the MIRU–VNTR pattern.

3.6. Response from LT to third question

The most widely used genotyping method for MTB isolates is the Restriction Fragment Length Polymorphism-based on IS6110. This technique is considered as the gold standard method for the study of the epidemiology of tuberculosis, and its standardization has permitted to establish databases allowing the comparison of the molecular typing results between laboratories. However, this technique requires a high amount of DNA (1 µg), and its protocol comprises several steps contrary to PCR methods. It depends also on the number of IS6110 copies, and for isolates with six or less copies, the use of a secondary marker (spoligotyping, MIRU, PGRS . . .) is indispensable for a better discrimination between strains. Moreover, RFLP-IS6110 data cannot be used for population genetics study. Indeed for an haploid organism as *M. tuberculosis*, the tests for studying the population structure are mainly based on the study of linkage disequilibrium (non-random association of genotypes occurring at different loci). And this technique does not reveal the variability of independent genetic loci and then cannot be used to analyze linkage disequilibrium.

Several other genotyping methods based on PCR, have been developed. The spoligotyping, for example, has shown its potentialities to type *M. tuberculosis* strains, and to identify W-Beijing Families. Besides its use as a molecular marker for genotyping MTB isolates, the spoligotyping per-

mits also to differentiate between members of *M. tuberculosis* complex.

This technique is rapid and highly reproducible. Moreover, like RFLP-IS6110, a database for internet-operated surveillance networks is available. Despite all these properties, the spoligotyping remains less discriminative than RFLP-IS6110, and as RFLP-IS6110, it cannot be used for population genetics study.

Few studies have used Pulsed Field Gel Electrophoresis (PFGE) for molecular typing of MTB strains. This technique seems especially laborious, but PFGE analyses provide more discrimination among isolates with fewer than five copies of IS6110 and less clustering in isolates with five or more copies. Moreover, the application of PFGE with four independent enzymes can be used for population genetics.

Concerning Random Amplified Polymorphism DNA (RAPD), its application on molecular epidemiology of *M. tuberculosis* has been limited because of problems of standardization of this method between laboratories. RAPD is used in routine in our lab to type several microorganisms, and the selection of primers seem to be fundamental, since only the primers that give reproducible and legible patterns are used. Once the problem of reproducibility resolved, RAPD presents some properties: it is a rapid technique and it requires few ADN (20 ng). Moreover, it is a multilocus marker, and it can be used for population genetics study. RAPD is also a generalist marker, so it permits the comparison of the genetic diversity among several microorganisms.

As underlined by Dr. Kreiswirth, several studies have shown that *M. tuberculosis* genome presents a limited genetic diversity (Sreevatsan et al., 1997). As this negligible polymorphism concerns the structural genes, the use of MLEE and MLST is not informative for the differentiation between MTB isolates.

Finally, as Dr. Kreiswirth and Dr. Carrière, I would like to speak about the new molecular marker, MIRU–VNTR. These markers are specific for MTB complex and they are useful for molecular epidemiology studies of tuberculosis. A database comprising MIRU data for different MTB populations is also available. This molecular approach based on PCR is very reproducible, rapid, and shows a high power of discrimination in comparison with spoligotyping. Moreover, this technique is a multilocus marker, thus, it can be used for the study of MTB population structure.

4. Fourth question: In your own TB molecular epidemiology approach, what is the specific contribution of evolutionary genetics concepts (population genetics/linkage disequilibrium analysis; phylogenetic analysis), if any?

4.1. Response from LT

My Ph.D. work concerns the study of the molecular epidemiology of tuberculosis in Casablanca, the economic

capital of Morocco, my country of origin. Up to now, few genetic studies have been done on Moroccan MTB isolates. Thus, this work represents the first evolutionary genetics study performed in Morocco.

Two molecular techniques (RAPD and MIRU) have been used in this study in order to analyze the population structure of the Moroccan population and to understand the dynamics of TB transmission in this country. The results obtained with the two markers have shown a significant linkage disequilibrium, which represents evidence for clonal population structure. Moreover, the population genetics tests with phylogenetic studies have permitted to better understand TB burden in Morocco, and particularly in Casablanca. As an example from these results, tuberculosis seems to have an ancient origin and an endemic situation in this country.

4.2. Response from CC

The data provided by the molecular epidemiology approach of the variability among natural population of *M. tuberculosis* contributes to understanding their evolution and pathogenesis. The most important, but difficult to define parameter of a genotyping system is the time period over which the degree of relatedness demonstrated is informative. For example, it has been related that the evolution rate of MIRU–VNTRs could be slightly slower than that of IS6110 RFLP (Mazars et al., 2001). These observations suggested that MIRU–VNTRs typing could be more appropriate than IS6110 RFLP for long-term epidemiological studies. In terms of phylogeny, the genetic relationship among *M. tuberculosis* isolates can be analyzed on dendrograms obtained with a software-assisted analysis of patterns. This can be done with the RFLP patterns as well as with the spoligotyping and MIRU–VNTR patterns.

I would like to point out that, as we develop more and more refined molecular methods for analysis of the bacterial genome do not we risk 1 day to consider as “epidemiologically unrelated” drug-resistant bacteria which might only be variants of the same clonal type?

For that purpose the molecular epidemiologist will need to interface with clinicians, epidemiologists and computer scientists to study for example an epidemic problem and more generally to interpret the results he obtained in term of evolution and phylogeny.

4.3. Response from BK

The molecular epidemiology of tuberculosis is a young science that has grown with the advances in secondary genotyping methods and with the understanding that strain collections must be representative of a given population. Interpreting the molecular data and the clustering of strains is complicated by fact tuberculosis is predominantly a chronic reactive disease, that is most cases are reactive over a life-

time and that human migration facilitates dispersion of the pathogen (via both dormant and active infection).

Evolutionary genetic concepts have greatly enhanced and in some instances augmented molecular epidemiological approaches. Much of our molecular epidemiology is based on clonal theory, that is sister cells that share in common traits of the parent. Furthermore, population genetics would suggest that certain medically relevant traits are non-randomly distributed along clonal or phylogenetic lines. That is, some lineages of tuberculosis may possess specific traits that enable successful host colonization (by evading the host immune response), pathogenesis, and efficient transmission (given by a clone-specific reproductive number). These concepts, in conjunction with molecular biology, microbiology and epidemiology were used in an (ongoing) investigation of large phylogenetic groups of *M. tuberculosis* strains as outlined below.

In the early 1990s, my laboratory genetically characterized and monitored the spread of the highly drug-resistant W strain which caused repeated “institutional” outbreaks in both prisons and hospitals in New York (Bifani et al., 1996). The fact that nearly 85% of the infected population were HIV positive led to the early progression of disease and the blossoming of case clusters. More than 500 W strain cases have been identified in New York during the last decade and the isolates are commonly resistant to INH, RIF, STP, EMB, PZA. The molecular analysis of these infecting isolates revealed that the IS6110 pattern has evolved during the strains transmission pathway among a New York population, and we have now identified nine closely related hybridization patterns. Although their IS6110 pattern vary, these strains are confidently linked to the W strain outbreak on the basis of the identical array of DNA sequence mutations in the numerous drug-resistant gene targets, including *rpsL*, *katG*, *rpoB*, *embB* and *pncA*.

The development of numerous genotyping methods, including spoligotyping and IS6110 insertion site mapping has identified additional molecular markers to link the W strain to a very large phylogenetic lineage that is found in principal genetic group 1. Specifically, a deletion in the DR region and an IS6110 insertion in the origin of replication group geographically disparate isolates to this phylogenetic lineage (Kurepina et al., 1998). The current TB literature repeatedly shows that members of this lineage, called the W-Beijing strain family, successfully spread and cause disease in the human population. Strain 210, a member of the W-Beijing strain family and one that caused multiple outbreaks in western states in the US is being sequenced at TIGR and its genome will add to the data to unravel the genetic basis for the success of strains in this lineage (Bifani et al., 2002).

The molecular epidemiology of the W-Beijing strain family in human populations indicate it has been successful, we now need to incorporate this information in other biological systems such as in making decisions about strains in animal studies and in the choice of strains for vaccine challenges. Therefore, the application or interpretation of evolu-

tionary concepts in describing the molecular epidemiology (or disease burden) should be done with caution. Evolutionary clock-speeds at certain genetic loci and with specific genetic backgrounds are not well-understood or characterized therefore, a multidisciplinary approach is recommended.

It should be emphasized that this data has been synthesized over a decade and its growth is truly the result of a collective group of laboratories around the world that have shared their genotyping data through the internet, at meetings, and in collaborative studies. The result is that tuberculosis is the model disease to study principals in bacterial molecular epidemiology.

4.4. MT

This ends this very fruitful e-debate on a hot topic. I thank you all three very much for participating in it.

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Loubna Tazi is a Moroccan PhD student at the Genetics of Infectious Diseases Laboratory (UMR CNRS-IRD 9926) at the “Institut de Recherche pour le Développement” (IRD), Montpellier, France. Her thesis focuses on molecular epidemiology of tuberculosis in Morocco, her country of origin. This work is under the supervision of Dr. Anne-Laure Bañuls and Dr. Michel Tibayrenc.

Since TB represents a major public health in her country, she is interested in studying the genetic diversity of *M. tuberculosis* isolates circulating in the economic capital of Morocco, Casablanca. This work will lead to determine the population structure of *M. tuberculosis* and the dynamics of its transmission in this high incidence community.

Loubna Tazi has participated to the students roundtable at the MEEGID VI Congress in Paris, France (July 2002), and she has received the award of best communication by a scientist from a developing country.



Barry Kreiswirth is American. He was trained as a molecular biologist under the direction of Dr. Richard Novick at the Public Health Research Institute. As a student, the focus of his research was the cloning and characterization of the staphylococcal toxic shock syndrome toxin-1. The use of molecular probes to identify the iatrogenic spread of TSST-1 from a neurosurgeon to four patients led to his interest in molecular epidemiology and the tracking of nosocomial infections.

The focus of his laboratory is to develop the use of objective and rapid molecular targets to control the spread of both nosocomial infections, especially methicillin resistant *Staphylococcus aureus*, and the global spread of *M. tuberculosis*. In this regard they have used DNA sequence comparison of variable number tandem repeat sequences in the *S. aureus* protein A gene as a model to rapidly identify and track this pathogen in both the hospital and the community. Their current goal is to develop a battery of DNA sequence targets that will provide an objective approach to build large relational databases to control the spread of bacterial pathogens.



Christian Carrière, born in Montpellier, France, is French. He is head of the bacteriology service of the Montpellier Hospital. His lab has an activity of clinical diagnosis in bacteriology and particularly in mycobacteriology. Besides his activity of clinical microbiologist he has performed research on molecular epidemiology of nosocomial infections and has worked in the determination of antibiotic susceptibilities of *M. tuberculosis* strains by using luciferase reporter mycobacteriophages.