Molecular Epidemiology of Tuberculosis: Main Findings and Their Application in Spain

E. García-Pachón[a] and J.C. Rodríguez[b]

[a]Sección de Neumología, Hospital General Universitario, Elche, Alicante, Spain.
[b]Sección de Microbiología, Hospital General Universitario, Elche, Alicante, Spain.

Introduction

When repetitive sequences of the genome of *Mycobacterium tuberculosis* were first successfully obtained in 1991,[1,2] it became possible to compare the DNA fingerprints of different isolates and, therefore, distinguish different strains from one another. Differentiation of isolated strains has allowed new information on the epidemiology of the disease to be collected.[3] With this new information, our understanding of how tuberculosis is transmitted has advanced, overturning long-standing preconceptions. Today, we have information on the relative importance of reactivation and recent transmission in areas with differing prevalence and on risk factors, such as the frequency of exogenous reinfection and the frequency of such occurrences in different zones. We also have new data on the infectivity of tuberculosis in situations in which sputum staining gives negative results, and on differences in the transmissibility and other phenotypic characteristics between *M tuberculosis* strains.

This review, which focuses on aspects of clinical interest, first describes the most common methods for molecular characterization of *M tuberculosis* and then summarizes the most novel information gleaned from molecular epidemiology studies, with particular reference to studies done in Spain.

Methods for Molecular Characterization of *M tuberculosis*

For a long time, strains have been characterized by markers based on characteristics expressed by the microorganisms (phage typing, resistance typing, serotyping, and plasmid analysis). These phenotyping techniques are currently being superseded by molecular techniques that analyze the DNA fingerprint. The new approaches are varied—some characterize the degree of similarity and the distribution of these variable elements among the isolates, whereas others study the presence or absence of certain DNA fragments or compare the complete genome of the microorganism.[4,5]

Restriction Fragment Length Polymorphism

The reference method for strain typing is considered to be restriction fragment length polymorphism (RFLP).[6] The technique is based on a standard protocol which analyzes sequence IS*6110*.[7] It is very reproducible and useful for differentiating between epidemiologically related and unrelated isolates, and so has been used as an indicator of recent transmission. The technique is based on counting the number times the IS*6110* restriction fragment is repeated in the genome of the *mycobacterium* (usually between 0 and 25 times). This technique presents certain limitations; for example, if the mycobacterium has less than 6 copies of this restriction fragment other techniques must be used.[8,9] Furthermore, large amounts of DNA are required (with the corresponding need for subculturing which may take several weeks), and the procedure is complex and expensive.[6,10]

Spoligotyping

A method widely used, usually as a complement to RFLP, is spoligotyping because of its relative simplicity, speed, and low cost. Spoligotyping studies the presence or absence of 43 DNA fragments known as spacers. Less DNA is required for this technique than for RFLP and, because expression (of each spacer) is either positive or negative, digital analysis is possible. An international database is available with more than 11 000 standard isolates ("spoligotypes") taken from more than 90 countries.[11] However, spoligotyping will not completely replace RFLP because it provides lower discrimination.[10,12]

This work received financial support from the Escuela Valenciana de Estudios para la Salud (Valencian School of Health Studies).

Correspondence: Dr. E. García-Pachón.
Sección de Neumología. Hospital General Universitario.
03203 Elche. Alicante. España.
E-mail: egpachon@hotmail.com

Manuscript received December 9, 2004. Accepted for publication January 4, 2005.

Variable Number Tandem Repeat

The technique known as variable number tandem repeat (VNTR) detects the number of times that several sequences occur adjacently within the genome of the mycobacterium. The most common approach is mycobacterial interspersed repetitive unit VNTR (MIRU-VNTR), which determines repetitions at 12 loci by polymerase chain reaction. From 2 to 8 alleles are located at each of the 12 loci, generating approximately 20 million possible allele combinations. The discrimination afforded by MIRU-VNTR is better than that of spoligotyping and similar to that of techniques based on RFLP.[13] Given that MIRU-VNTR can be readily automated and the technology is simpler, it may well become the reference method.[6] Moreover, findings can be compared easily with an international database accessible via the Internet.[14]

Amplified Fragment Length Polymorphism

Amplified fragment length polymorphism (AFLP) differs from other methods in that the whole mycobacterial genome is studied, making it potentially more specific than RFLP for confirming recent transmission of a strain.[15] However, this technique, which is based on polymerase chain reaction, is expensive, time-consuming, and requires highly specialized personnel.[16] Furthermore, application of AFLP in molecular epidemiology studies and interpretation of the results are still pending more detailed investigation.

Proportion of Recent Tuberculosis Transmission

Recent transmission is assumed when several isolates from *M tuberculosis* have identical or similar DNA fingerprints—the so-called cluster cases. On the other hand, isolates with unique DNA fingerprints are considered to indicate reactivation of a previously acquired infection.[5,10] The DNA of *M tuberculosis* undergoes progressive changes over time. The time needed for a DNA fingerprint to change (half-life), according to studies with IS*6110* restriction fragment RFLP, ranges from 2 years to over 30 years.[17-20] Several factors can be assumed to influence the time needed, such as the efficacy of the treatment, the interval between disease onset and treatment, and, probably, the relative predominance of more or less stable strains of the pathogen in each region,[21] as well as whether the disease is latent or active.[20]

Several studies have been published that investigate cases attributable to recent transmission according to the principles outlined above. Interestingly, the proportion of cluster cases can be as high as 40% in the United States[22,23] and Europe,[24,25] even in areas where the incidence of tuberculosis is low, suggesting that recent transmission of *M tuberculosis* could have a greater relative importance than was previously thought. Countries with a high incidence of tuberculosis and an inadequate health care system would be expected to have a high percentage of tuberculosis cases associated with recent transmission. However, the results of studies done to test this have been very variable—the percentage of recent transmission ranges from 30% to 80% in Africa and from 50% to 80% in Asia, but, surprisingly, is only 20% according to other studies.[26-28] This paradoxical finding can be explained by the methodology used, as small studies in areas with high incidences greatly underestimate recent transmission.[29]

In Spain, 9 molecular epidemiology studies of tuberculosis transmission have been published.[30-38] These studies have used RFLP alone or in combination with spoligotyping (Table 1). The percentage corresponding to cluster cases (recent transmission) ranged from 28% in Segovia to 58% on Gran Canaria Island. These percentages might reflect true differences in the disease situation in the different areas, but the characteristics of the studies themselves (such as the study duration and number of cases not included) could also have influenced the results. With the new molecular methods, investigators have also been able to demonstrate the epidemiological connection between outbreaks of multiresistant tuberculosis in Spain.[39]

TABLE
Molecular Epidemiological Studies of Tuberculosis Transmission in Spain*

Author and Year	Study Period, Years	Place	Method	No. of Patients	Percentage of Cluster Cases
Safi et al,[30] 1997	3	Seville	RFLP	175	38
Samper et al,[31] 1998	1	Zaragoza	RFLP	226	39
Íñigo Martínez et al,[32] 2000	2	Madrid	RFLP and spoligotyping	148	42
Solsona et al,[33] 2001	2	Ciutat Vella, Barcelona	RFLP	171	46
Fernández de la Hoz et al,[34] 2001	2	Urban population in south Madrid and prison population	RFLP	231	48
Elizaga et al,[35] 2002	5	Segovia	RFLP and spoligotyping	87	28
Ruiz et al,[36] 2002	7	Elche	RFLP	147	52
Pena et al,[37] 2003	4	Gran Canaria Island	RFLP	409	58
Íñigo Martínez et al,[38] 2003	3	Madrid	RFLP and spoligotyping	233	42

*RFLP indicates restriction fragment length polymorphism.

Many authors prefer to express their results as an incidence of cluster cases per 100 000 inhabitants per year rather than as percentages.[26,40] Thus, for example, the decrease in the incidence of cluster cases in San Francisco between 1991, when it was 10.4 cases per 100 000 inhabitants, and 1997, when it was 3.8 cases per 100 000 inhabitants, was a clear reflection of the decrease in recent transmission over this period.[40]

These findings, in combination with conventional epidemiological methods, can identify, for example, transmission between different ethnic and social groups. According to estimates for the Netherlands, 17% of the cases in the country are due to recent transmission from foreigners,[24] and by 2030, investigators forecast that at least 60% of tuberculosis cases in autochthonous Dutch patients will be due to transmission by immigrants.[41]

Murray and Nardell,[26] however, insist that the cluster cases should be interpreted with caution when estimating the true transmission rate of tuberculosis because case clustering could vary according to the characteristics of the host and the population.[42] For example, in rural areas, similar genotypes may not necessarily be an indication of a recent infection.[43] Several factors in addition to recent transmission of the disease can give rise to similar DNA fingerprints; examples of such factors are the simultaneous reactivation of an infection with the same organism that was acquired long before (temporal coincidence), regional predominance of a bacterial strain that has been present for a long time (endemic strains), a change in the location of the insertion sequence at the same site, and, of course, laboratory errors.[3] The opposite situation is also true: an isolate may be mistaken for an endogenous reactivation when it is actually associated with other undetected cases. A single DNA fingerprint is more likely to be erroneously interpreted as endogenous reactivation in shorter studies (the duration of a study should not be less than 2 years) and in studies that analyze small numbers of cases.[3,44]

Risk Factors for Recent Transmission

Some authors have tried to use case cluster data to determine risk factors for recent transmission. According to a study by Alland et al,[22] younger subjects, those born in the United States of America, Hispanics, and those infected with the human immunodeficiency virus (HIV) were at greater risk. However, there are often discrepancies among different studies, reflecting both the diversity of disease transmission in different communities and methodological shortcomings.[44,45]

The duration of symptoms, which could theoretically be a reflection of risk of transmission, was not associated with cluster size defined by molecular techniques.[46] It is important to point out that although recent transmission is more frequent when patients have positive results from sputum smears, subjects with negative results can be responsible for a relatively large number of transmissions.[47]

In a study which analyzed clinical and socioeconomic factors of tuberculosis in Elche, Spain, age less than 25 years, a high percentage of infections from inner circle contacts, urban residence, use of bronchoscopy to obtain samples, and working in contact with many people were all independent variables predictive of belonging to a case cluster.[48] In an extensive study done on Gran Canaria Island, Spain, the only risk factor associated with belonging to a case cluster was young age.[37]

Risk of Recent Transmission in Patients With HIV Infection

Patients with HIV infection are known to be at high risk of contracting tuberculosis, although current antiretroviral treatments may help to lower the incidence of the disease in this population group.[49] Different molecular epidemiological studies have found HIV infection to be the biggest risk factor for recent transmission. Although most studies indicate that HIV infection is a strong risk factor,[22,23,36,40,50] not all studies have confirmed this finding.[31,51] Variable social factors among these patients (such as use of intravenous drugs or, in particular, imprisonment) in a given country may influence the risk of transmission.[32,34,52]

Exogenous Reinfection

One of the long-standing debates about tuberculosis transmission was the true importance of exogenous reinfection. For a long time, it was thought that around 90% of the cases of tuberculosis in developed countries were due to reactivation of a previously acquired infection.[53] Nevertheless, the occurrence of exogenous reinfection had already been demonstrated with conventional epidemiological methods and phenotyping (for resistance) of the mycobacteria.[54-56] It clearly used to be difficult to establish whether a case of tuberculosis was due to activation of latent disease or to a new exogenous infection, but our knowledge of this aspect has been improved by molecular epidemiological data.

In a region with a high incidence of tuberculosis, investigators analyzed 16 cases of patients with disease recurrence after correctly completing treatment and identified strains other than those responsible for the initial disease in 12 of them.[57] Reinfection is also important in countries with a lower incidence of tuberculosis.[58,59] In Spain, Caminero et al[59] performed genotyping of *M tuberculosis* isolated from the 18 patients with positive tuberculosis cultures taken at least 12 months apart and found that 44% of these cultures had mycobacteria with different genotypes, indicating exogenous reinfection. An extensive study of recurrence in cases of tuberculosis in Madrid found that recurrence in 14 of the 43 patients analyzed (33%) in a period of 12 years was due to exogenous reinfection.[60] In contrast, in countries with a low incidence of tuberculosis, such as the United States of America or Canada, the proportion of reinfections appears lower.[61]

Tuberculosis Control Programs

With molecular epidemiological studies, investigators have been able to detect outbreaks of related tuberculosis cases which would have been missed by conventional epidemiology.[23,62-64] This has improved contact investigations for tuberculosis and one study was even able to identify 3 times as many contacts for each tuberculosis case.[40] The application of a capture-recapture method in Spain that combined conventional and molecular epidemiology greatly improved the detection of related cases in recent transmission.[65]

In addition to contact investigations, information from molecular epidemiology studies is useful for assessing the results of disease control programs. As mentioned earlier, between 1991 and 1997, the number of cluster cases in San Francisco decreased, suggesting that the approaches for controlling the disease reduced the spread of tuberculosis.[40]

The epidemiological analyses done in some countries to guide the implementation of specific policies to combat tuberculosis is an obvious example of the need for this type of study. It is important to determine whether the active disease among the population in a given area is due to recent infection or reactivation of latent disease. If the number of cases of recent infection turns out to be significant, case finding and treatment should be stepped up, but if latent infection is the main cause, then measures should be focused more on preventing reactivation.[66]

Doubts remain about the methods for genotyping (selection of ideal method with reproducible information at a reasonable cost) and interpretation of the findings, but application of these techniques in normal practices of prevention and control of tuberculosis is currently considered justified.[67] Reference centers seem particularly useful, as they allow studies that extend beyond the limits of a given health area.[68-70]

Risk of Transmission of Tuberculosis to Health Care Workers

In a recent review, Seidler et al[71] found only 2 molecular epidemiological studies that separately calculated the percentage of tuberculosis cases among health care workers and nonhealth care workers corresponding to cluster cases.[72,73] A study in New York that included 142 tuberculosis cases found that 65% of infected health care workers and 41% of infected patients had a clustering pattern.[72] The authors concluded that many of the cases among health care workers could be considered as occupational infection. In contrast, another study in Amsterdam, the Netherlands, concluded that health care workers had a lower chance of belonging to a cluster, although the authors could not explain this finding.[73]

In Spain, Tudó et al[74] used RFLP to analyze patients admitted to hospital with tuberculosis in search of a possible undetected nosocomial transmission of the disease. They found no cluster cases resulting from transmission by these patients in the hospital. Likewise, the authors reported no cases of transmission to patients who shared a room with tuberculosis patients. These findings suggest that the risk of infection is very low. However, other Spanish studies have also reported an increased accumulated incidence of tuberculosis among health care workers,[75] suggesting that there is a risk due to occupational exposure.

All these findings underline the need to perform prospective studies that combine methods of conventional and molecular epidemiology to establish the true risk of tuberculosis transmission in health care workers.[71]

Transmission of Different Strains of *M tuberculosis*

It used to be thought that the different strains of *M tuberculosis* had a similar virulence,[6] but molecular studies have been able to confirm the existence of strains with specific and characteristic phenotypes. Several of the strains identified have been associated with epidemic outbreaks in different regions and at different times and it might be assumed that these strains are more virulent or easily transmitted (superspreaders).

One example is the *M tuberculosis* family denominated Beijing/W. The Beijing genotype strain has been implicated in large-scale transmissions in the United States of America, Asia, Eastern Europe, and the Russian Federation.[76] This strain is widely distributed, especially in Asia, where it is predominant, but also in Europe, the United States, South America, Caribbean countries, and Africa.[5] Spoligotyping has shown that strain W, detected in the 1990s in the United States and responsible for outbreaks of multiresistant disease, belongs to an evolutionary branch of strains of the Beijing genotype, and so the Beijing/W family was established.[10] The predominance of Beijing strains in many regions might indicate that they have a selective advantage over other strains.[10,77] On the island of Gran Canaria, Spain, this strain was shown to have spread rapidly from a single patient to account for 21.7% of isolates of *M tuberculosis* within 4 years.[77] The Beijing strain has often, though not always, been associated with drug-resistance in a number of studies.[78] These strains of *M tuberculosis* have a greater ability to replicate in human macrophages and this could be the main propagation mechanism.[79] However, apparent differences in virulence of the mycobacterium could also be due to variations in immunogenicity, transmissibility, growth rates, or characteristics of the exposed population.[26,80]

Transmission of Drug-Resistant Strains of *M tuberculosis*

The transmission rate of drug-resistant strains is often assumed to be lower than that of other strains. Several studies have observed a negative correlation between case clusters and resistance to tuberculosis

drugs.[51,81] However, not all findings are consistent—some studies describe outbreaks that include many cases of multiresistant tuberculosis.[82,83] Indeed, in a study in Norway, infection with a resistant strain was independently associated with forming part of a cluster (recent transmission).[45] A high risk of transmission of resistant stains of the Beijing/W family has also been reported.[22,84]

Murray and Nardell[26] propose several possibilities to explain why clusters of tuberculosis resistant to one or more drugs could be smaller than the clusters of susceptible patients. For example, patients with multiresistant tuberculosis might have worse access to health care systems in many countries, they might have fewer social contacts, or they might have been exposed to the pathogen more often.

Other Uses of Genotyping

A large study in London found that only 0.93% of false positives in tuberculosis cultures were due to cross-contamination in the laboratory,[85] whereas some studies indicate that up to 3% of the cultures of tuberculosis are false positives, particularly with negative sample staining and single culture growth.[85,86] In such cases, and when clinical suspicion does not support a diagnosis of tuberculosis, typing of isolates could provide information that avoids unnecessary treatments.[6,86] If the genotype of the strain of doubtful clinical significance corresponds with that of another strain handled at the same time in the laboratory, it is likely that contamination has occurred.

Another possible use is in patients who are receiving or who have received tuberculosis treatment and for whom resistances appear in new cultures. Genotyping could distinguish between exogenous reinfection and a strain that has developed resistance. In the latter instance, the treatment compliance of the patient, the possibility of malabsorption of the drugs, or pharmacological interactions would have to be investigated.[6] Poor treatment compliance has been shown to be a cause of spread of the disease.[87]

In clinical studies of tuberculosis drugs, it is important (and now possible) to determine whether therapeutic failures are due, in fact, to new infections or not.[6]

Genotyping also allows comparison of families of *M tuberculosis* in international databases already available or in development,[4,88] and so it will be possible to detect and control large outbreaks that might not be suitably identified in local studies.

Conclusions and Outlook

The molecular epidemiology of tuberculosis has provided novel and often surprising information on the transmission of the disease. Of course, this method should be used as a complement to conventional epidemiology and not as a replacement. It is important to know which strains predominate in the community and how the cases attributable to recent transmission change over time as well as to determine why transmission occurs and which social groups require the most care. Growing immigration, which brings new strains of *M tuberculosis,* will challenge our epidemiological surveillance system and molecular studies are going to be essential. To meet this challenge, long-term studies are necessary in Spain and reference centers should probably be set up to coordinate this information.

Automated techniques are now available to analyze the results with reference to international databases at a reasonable cost. In a few years, our approach to tuberculosis will be influenced by this new information. The application of molecular epidemiology in combination with conventional epidemiological methods is a reasonable aim for the coming years in developed countries. To achieve this, close collaboration of pulmonologists, epidemiologists, and microbiologists will be necessary.

REFERENCES

1. van Soolingen D, Hermans PW, de Haas PE, Soll DR, van Embden JD. Occurrence and stability of insertion sequences in *Mycobacterium tuberculosis* complex strains: evaluation of an insertion sequence-dependent DNA polymorphism as a tool in the epidemiology of tuberculosis. J Clin Microbiol. 1991;29:2578-86.
2. Cave MD, Eisenach KD, McDermott PF, Bates JH, Crawford JT. IS6110 conservation of sequence in the *Mycobacterium tuberculosis* complex and its utilization in DNA fingerprinting. Moll Cell Prob. 1991;5:73-80.
3. Glynn JR, Bauer J, de Boer AS, Borgdorff MW, Fine PE, Godfrey-Faussett P, et al. Interpreting DNA fingerprint clusters of *Mycobacterium tuberculosis*. European Concerted Action on Molecular Epidemiology and Control of Tuberculosis. Int J Tuberc Lung Dis. 1999;3:1055-60.
4. Kanduma E, McHugh TD, Gillespie SH. Molecular methods for *Mycobacterium tuberculosis* strain typing: a user's guide. J Appl Microbiol. 2003;94:781-91.
5. Nguyen LN, Gilbert GL, Marks GB. Molecular epidemiology of tuberculosis and recent developments in understanding the epidemiology of tuberculosis. Respirology. 2004;9:313-9.
6. Barnes PF, Cave MD. Molecular epidemiology of tuberculosis. N Engl J Med. 2003;349:1149-56.
7. van Embden JDA, Cave MD, Crawford JT, Dale JW, Eisenach KD, Gicquel B, et al. Strain identification of *Mycobacterium tuberculosis* by DNA fingerprinting: recommendations for a standardized methodology. J Clin Microbiol. 1993;31:406-9.
8. Soini H, Pan X, Teeter L, Musser JM, Graviss EA. Transmission dynamics and molecular characterization of *Mycobacterium tuberculosis* isolates with low copy numbers of IS6110. J Clin Microbiol. 2001;39:217-21.
9. Ruiz M, Rodríguez JC, Samper S, Royo G. Caracterización genómica de cepas de *Mycobacterium tuberculosis* con menos de 5 copias de IS6110: utilidad de amplified-fragment length polymorphism (AFLP), variable number tandem repeat (VTNR) y spoligotyping. Med Clin (Barc). 2005;124:75-6.
10. van Soolingen D. Molecular epidemiology of tuberculosis and other mycobacterial infections: main methodologies and achievements. J Intern Med. 2001;249:1-26.
11. Filliol I, Driscoll JR, van Soolingen D, Kreiswirth BN, Kremer K, Valetudie G, et al. Global distribution of *Mycobacterium tuberculosis* spoligotypes. Emerg Infect Dis. 2002;8:1347-9.
12. Dale JW, Brittain D, Cataldi AA, Cousins D, Crawford JT, Driscoll JJ, et al. Spacer oligonucleotide typing of bacteria of the *Mycobacterium tuberculosis* complex: recommendations for standardised nomenclature. Int J Lung Dis. 2001;5:216-9.

13. Mazars E, Lesjean S, Banuls AL, Gilbert M, Vincent V, Gicquel B, et al. High-resolution minisatellite-based typing as a portable approach to global analysis of *Mycobacterium tuberculosis* molecular epidemiology. Proc Natl Acad Sci USA. 2001;98: 1901-6.
14. Supply P, Lesjean S, Savine E, Kremer K, van Soolingen D, Locht C. Automated high-throughput genotyping for study of global epidemiology of *Mycobacterium tuberculosis* based on mycobacterial interspersed repetitive units. J Clin Microbiol. 2001;39:3563-71.
15. Ruiz M, Rodríguez JC, Rodríguez-Valera F, Royo G. Amplified fragment length polymorphism as a complement to IS16110 restriction fragment length polymorphism analysis for molecular typing of *Mycobacterium tuberculosis*. J Clin Microbiol. 2003; 41:4820-2.
16. Fernández Cuenca F. Aplicación de técnicas de PCR en la epidemiología molecular de las enfermedades infecciosas. Enferm Infecc Microbiol Clin. 2004;22:355-60.
17. Yeh RW, Ponce de León A, Agasino CB, Hahn JA, Daley CL, Hopewell CP, et al. Stability of *Mycobacterium tuberculosis* DNA genotypes. J Infect Dis. 1998;177:1107-11.
18. de Boer AS, Borgdorff MW, de Haas PE, Nagelkerke NJ, van Embden JD, van Soolingen D. Analysis of rate of change of IS6110 RFLP patterns of *Mycobacterium tuberculosis* based on serial patient isolates. J Infect Dis. 1999;180:1238-44.
19. Warren RM, van der Spuy GD, Richardson M, Beyers N, Borgdorff MW, Behr MA, et al. Calculation of the stability of the IS6110 banding pattern in patients with persistent *Mycobacterium tuberculosis* disease. J Clin Microbiol. 2002;40:1705-8.
20. Lillebaek T, Dirksen A, Vynnycky E, Baess I, Thomsen VO, Andersen AB. Stability of DNA patterns as evidence of *Mycobacterium tuberculosis* reactivation occurring decades after the initial infection. J Infect Dis. 2003;188:1032-9.
21. Siedler A, Nienhaus A, Diel R. The transmission of tuberculosis in the light of new molecular biological approaches. Occup Environ Med. 2004;61:96-102.
22. Alland D, Kalkut GE, Moss AR, McAdam RA, Hahn JA, Bosworth W, et al. Transmission of tuberculosis in New York City. An analysis by DNA fingerprinting and conventional epidemiologic methods. N Engl J Med. 1994;330:1710-6.
23. Small PM, Hopewell PC, Singh SP, Paz A, Parsonnet J, Ruston DC, et al. The epidemiology of tuberculosis in San Francisco. A population-based study using conventional and molecular methods in the 1990s. N Engl J Med. 1994;330:1703-9.
24. Borgdorff MW, Nagelkerke N, van Soolingen D, de Hass PE, Veen J, van Embden JD. Analysis of tuberculosis transmission between nationalities in the Netherlands in the period 1993-1995 using DNA fingerprinting. Am J Epidemiol. 1998;147:187-95.
25. Pfyffer GE, Strassle A, Rose N, Wirth R, Brandli O, Shang H, et al. Transmission of tuberculosis in the metropolitan area of Zurich: a 3 year survey based on DNA fingerprinting. Eur Respir J. 1998;11:804-8.
26. Murray M, Nardell E. Molecular epidemiology of tuberculosis. Achievements and challenges to current knowledge. Bull World Health Organ. 2002;80:477-82.
27. Pineda-García L, Ferrera A, Hoffner SE. DNA fingerprinting of *Mycobacterium tuberculosis* strains from patients with tuberculsis in Honduras. J Clin Microbiol. 1997;35:2393-7.
28. Doroudchi M, Kremer K, Basiri EA, Kadivar MR, van Soolingen D, Ghaderi AA, et al. IS6110-RFLP and spoligotyping of *Mycobacterium tuberculosis* isolates in Iran. Scan J Infect Dis. 2000;32:663-8.
29. Glynn JR, Vynnycky E, Fine PE. Influence of sampling on estimates of clustering and recent transmission of *Mycobacterium tuberculosis* derived from DNA fingerprinting techniques. Am J Epidemiol. 1999;149:366-71.
30. Safi H, Aznar J, Palomares JC. Molecular epidemiology of *Mycobacterium tuberculosis* strains isolated during a 3-year period (1993-1995) in Seville, Spain. J Clin Microbiol. 1997;35: 2472-6.
31. Samper S, Iglesias MJ, Rabanaque MJ, Lezcano MA, Vitoria LA, Rubio MC, et al. The molecular epidemiology of tuberculosis in Zaragoza, Spain: a retrospective epidemiological study in 1993. Int J Tuberc Lung Dis. 1998;2:281-7.
32. Íñigo Martínez J, Chaves Sánchez F, Arce Arnáez A, Alonso Sanz M, Palenque Mataix E, Jaén Herreros F, et al. Transmisión reciente de tuberculosis en Madrid: utilidad de las técnicas moleculares. Med Clin (Barc). 2000;115:241-5.
33. Solsona J, Cayla JA, Verdú E, Estrada MP, García S, Roca D, et al. Molecular and conventional epidemiology of tuberculosis in an inner city district. Int J Tuberc Lung Dis. 2001;5:724-31.
34. Fernández de la Hoz K, Íñigo J, Fernández Martín JI, Arce A, Alonso Sanz M, Gómez Pintado P, et al. The influence of HIV infection and imprisonment on dissemination of *Mycobacterium tuberculosis* in a large Spanish city. Int J Tuberc Lung Dis. 2001; 5:696-702.
35. Elizaga J, Carrero P, Íñigo J, Chaves F. Transmisión reciente de la tuberculosis en un área con baja incidencia: estudio epidemiológico y molecular. Med Clin (Barc). 2002;118:645-9.
36. Ruiz M, Rodríguez JC, Navarro JF, Samper S, Martín C, Royo G. Molecular epidemiology of tuberculosis in Elche, Spain: a 7-year study. J Med Microbiol. 2002;51:273-7.
37. Pena MJ, Caminero JA, Campos Herrero MI, Rodríguez Gallego JC, García Laorden ML, Cabrera P, et al. Epidemiology of tuberculosis on Gran Canaria: a 4-year population study using traditional and molecular approaches. Thorax. 2003;58:618-22.
38. Íñigo Martínez J, Arce Arnáez A, Chaves Sánchez F, Palenque Mataix E, Burgoa Arenales M. Patrones de transmisión de la tuberculosis en un área sanitaria de Madrid. Rev Esp Salud Pública. 2003;77:541-51.
39. Perfecto B, Sánchez JR, González AI, López I, Dorronsoro I. Brote de tuberculosis multiresistente. An Sist Sanit Navar. 2000;23:257-63.
40. Jasmer RM, Hahn JA, Small PM, Daley CL, Behr MA, Moss AR, et al. A molecular epidemiologic analysis of tuberculosis trends in San Francisco. Ann Intern Med. 1999;130:971-8.
41. van Wolleswinkel BJ, Nagelkerke NJ, Broekmans JF, Borgdorff MW. The impact of immigration on the elimination of tuberculosis in The Netherlands: a model based approach. Int J Tuberc Lung Dis. 2002;6:130-6.
42. Murray M. Determinants of cluster distribution in the molecular epidemiology of tuberculosis. Proc Natl Acad Sci USA. 2002;99:1538-43.
43. Braden CR, Templeton GL, Cave MD, Valway S, Onorato IM, Castro KG, et al. Interpretation of restriction fragment length polymorphism analysis of *Mycobacterium tuberculosis* isolates from a state with a large rural population. J Infect Dis. 1997;175: 1446-52.
44. Murray M, Alland DA. Methodological problems in the molecular epidemiology of tuberculosis. Am J Epidemiol. 2002;155:565-71.
45. Heldal E, Dahle UR, Sandven P, Caugant DA, Brattaas N, Waaler HT, et al. Risk factors for recent transmission of *Mycobacterium tuberculosis*. Eur Respir J. 2003;22:637-42.
46. Giordano TP, Soini H, Teeter LD, Adams GJ, Musser JM, Graviss EA. Relating the size of molecularly defined clusters of tuberculosis to the duration of symptoms. Clin Infect Dis. 2004; 38:10-6.
47. Hernández-Garduno E, Cook V, Kunimoto D, Elwood RK, Black WA, FitzGerald JM. Transmission of tuberculosis from smear negative patients: a molecular epidemiology study. Thorax. 2004;59: 286-90.
48. Ruiz M, Navarro JF, Rodríguez JC, Larrosa JA, Royo G. Effect of clinical and socio-economic factors on the rate of clustering of *Mycobacterium tuberculosis* clinical isolates in Elche (Spain). Epidemiol Infect. 2003;131:1077-83.
49. Calpe JL, Chiner E, Martínez Pardo J, Calpe A, Armero V. Impact of the human immunodeficiency virus on the epidemiology of tuberculosis in area 15 of the Valencian community in Spain. Int J Tuberc Lung Dis. 2004;8:1204-12.
50. Camarena JJ, Artero A, Nogueira JM, Navarro JC, Olmos A, Blanquer R. Tuberculosis en pacientes con sida: aportación del análisis de los fragmentos de restricción de longitud polimórfica de aislados de *Mycobacterium tuberculosis*. Med Clin (Barc). 1998;111:721-4.
51. van Soolingen D, Borgdorff MW, de Haas PE, Sebek MM, Veen J, Dessens M, et al. Molecular epidemiology of tuberculosis in The Netherlands: a nationwide study from 1993 through 1997. J Infect Dis. 1999;18:726-36.
52. McLaughlin SI, Spradling P, Drociuk D, Ridzon R, Pozsik CJ, Onorato I. Extensive transmission of *Mycobacterium tuberculosis* among congregated, HIV-infected prison inmates in South Carolina, United States. Int J Tuberc Lung Dis. 2003;7:665-72.

53. Burgos MV, Pym AS. Molecular epidemiology of tuberculosis. Eur Respir J. 2002;20 Suppl 36:54-65.
54. Nardell E, McInnis B, Thomas B, Weidhaas S. Exogenous reinfection with tuberculosis in a shelter for the homeless. N Engl J Med. 1986;315:1570-4.
55. Small PM, Shafer RW, Hopewell PC, Singh SP, Murphy MJ, Desmond E, et al. Exogenous reinfection with multidrug-resistant *Mycobacterium tuberculosis* in patients with advanced HIV infection. N Engl J Med. 1993;328:1137-44.
56. Shafer RW, Singh SP, Larkin C, Small PM. Exogenous reinfection with multidrug-resistant *Mycobacterium tuberculosis* in an immunocompetent patient. Tuber Lung Dis. 1995;76:575-7.
57. van Rie A, Warren R, Richardson M, Victor TC, Gie RP, Enarson DA, et al. Exogenous reinfection as a cause of recurrent tuberculosis after curative treatment. N Engl J Med. 1999;341:1174-9.
58. Bandera A, Gori A, Catozzi L, Degli Esposti A, Marchetti G, Molteni C, et al. Molecular epidemiology study of exogenous reinfection in an area with a low incidence of tuberculosis. J Clin Microbiol. 2000;39:2213-8.
59. Caminero JA, Pena MJ, Campos-Herrero MI, Rodríguez JC, Afonso O, Martín C, et al. Exogenous reinfection with tuberculosis on a European island with a moderate incidence of disease. Am J Respir Crit Care Med. 2001;163:717-20.
60. García de Viedma D, Marín M, Hernangómez S, Díaz M, Ruiz Serrano MJ, Alcalá E. Tuberculosis recurrences: reinfection plays a role in a population whose clinical/epidemiological characteristics do not favor reinfection. Arch Intern Med. 2002;162:1873-9.
61. Jasmer RM, Bozeman L, Schwartzman K, Cave MD, Saukkonen JJ, Metchock B, et al. Recurrent tuberculosis in the United States and Canada: relapse or reinfection? Am J Respir Crit Care Med. 2004;170:1360-6.
62. Weis SE, Pogoda JM, Yang Z, Cave MD, Wallace C, Kelley M, et al. Transmission dynamics of tuberculosis in Tarrant County, Texas. Am J Respir Crit Care Med. 2002;166:36-42.
63. McNabb SJ, Kammerer JS, Hickey AC, Braden CR, Shang N, Rosenblum LS, et al. Added epidemiologic value to tuberculosis prevention control of the investigation of clustered genotypes of *Mycobacterium tuberculosis* isolates. Am J Epidemiol. 2004;160: 589-97.
64. van Deutekom H, Hoijng SP, de Haas PE, Langendam MW, Horsman A, van Soolingen D, et al. Clustered tuberculosis cases: do they represent recent transmission and can they be detected earlier? Am J Respir Crit Care Med. 2004;169:806-10.
65. Íñigo J, Arce A, Martín-Moreno JM, Herruzo R, Palanque E, Chaves F. Recent transmission of tuberculosis in Madrid: application of capture-recapture analysis to conventional and molecular epidemiology. Int J Epidemiol. 2003;32:763-9.
66. Geng E, Kreiswirth B, Driver C, Li J, Burzybski J, dellaLatta P, et al. Changes in the transmission of tuberculosis in New York City from 1990 to 1999. N Engl J Med. 2002;346:1453-8.
67. McNabb SJ, Braden CR, Navin TR. DNA fingerprinting of *Mycobacterium tuberculosis*: lessons learned and implications in the future. Emerg Infect Dis. 2002;8:1314-9.
68. Bauer J, Kok-Jensen A, Faurschou P, Thuesen J, Taurdorf E, Andersen AB. A prospective evaluation of the clinical value of nation-wide DNA fingerprinting of tuberculosis isolates in Denmark. Int J Tuberc Lung Dis. 2000;4:295-9.
69. Crawford JT, Braden CR, Schable BA, Onorato IM. National Tuberculosis Genotyping and Surveillance Network: design and methods. Emerg Infect Dis. 2002;8:1192-6.
70. Lambregts van Weezenbeek CS, Sebek MM, van Gerben PJ, de Vries G, Verver S, Kalisvaart NA, et al. Tuberculosis contact investigation and DNA fingerprinting surveillance in The Netherlands: 6 years' experience with nation-wide cluster feedback and cluster monitoring. Int J Tuberc Lung Dis. 2003;12 Suppl 3:46370.
71. Seidler A, Nienhaus A, Diel R. The transmission of tuberculosis in the light of new molecular biological approaches. Occup Environ Med. 2004;61:96-201.
72. Sepkowitz KA, Friedman CR, Hafner A, Kwork D, Manoach S, Floris M, et al. Tuberculosis among urban health care workers: a study using restriction fragment length polymorphism typing. Clin Infec Dis. 1995;21:1098-101.
73. van Deutekom H, Gerritsen JJ, van Soolingen D, van Ameijden EJ, van Embden JD, Coutinho RA. A molecular epidemiological approach to studying the transmission of tuberculosis in Amsterdam. Clin Infect Dis. 1997;25:1071-7.
74. Tudó G, González J, Gatell JM, Caylà JA, Martínez E, García A, et al. Detection of unsuspected cases of nosocomial transmission of tuberculosis by use of a molecular typing method. Clin Infect Dis. 2001;33:453-9.
75. Casas X, Ruiz-Manzano J, Casas I, Andreo F, Sanz J, Rodríguez N, et al. Tuberculosis en personal sanitario de un hospital general. Med Clin (Barc). 2004;122:741-3.
76. Bifani PJ, Mathema B, Kurepina NE, Kreiswirth BN. Global dissemination of the *Mycobacterium tuberculosis* W-Beijing family strains. Trends Microbiol. 2002;10:45-52.
77. Caminero JA, Pena MJ, Campos-Herrero MI, Rodríguez JC, García I, Cabrera P, et al. Epidemiological evidence of spread of a *Mycobacterium tuberculosis* strain of the Beijing genotype on Gran Canaria Island. Am J Respir Crit Care Med. 2001;164:1165-70.
78. Glynn JR, Whiteley J, Bifani PJ, Kremer K, van Soolingen D. Worldwide occurrence of Beijing/W strains of *Mycobacterium tuberculosis*: a systematic review. Emerg Infect Dis. 2002;8:843-9.
79. Zhang M, Gong J, Yang Z, Samten B, Cave MD, Barnes PF. Enhanced capacity of a widespread strain of *Mycobacterium tuberculosis* to grow in human macrophages. J Infect Dis. 1999; 179:1213-7.
80. Manca C, Tsenova L, Barry CE, Bergtold A, Freeman S, Haslett PA, et al. *Mycobacterium tuberculosis* CDC1551 induces a more vigorous host response in vivo and in vitro, but is not more virulent than other clinical isolates. J Immunol. 1999;162:6740-6.
81. García-García ML, Ponce de León A, Jiménez-Corona ME, Jiménez-Corona A, Palacios-Martínez M, Balandrano-Campos S, et al. Clinical consequences and transmissibility of tuberculosis in souhern Mexico. Arch Intern Med. 2000;160:630-6.
82. Frieden TR, Sherman LF, Maw KL, Fujiwara PI, Crawford JT, Nivin B, et al. A multi-institutional outbreak of highly drug-resistant tuberculosis: epidemiology and clinical outcomes. JAMA. 1996;276:1229-35.
83. Kruuner A, Hoffner SE, Sillastu H, Danilovits M, Levina K, Svenson SB, et al. Spread of drug-resistant pulmonary tuberculosis in Estonia. J Clin Microbiol. 2001;39:3339-45.
84. Bifani PJ, Mathema B, Liu Z, Moghazeh SL, Shopsin B, Tempalski B, et al. Identification of a W variant outbreak of *Mycobacterium tuberculosis* via population-based molecular epidemiology. JAMA. 1999;282:2321-7.
85. Ruddy M, McHugh TD, Dale JW, Banerjee D, Maguire H, Wilson P, et al. Estimation of the rate of unrecognized cross-contamination with *Mycobacterium tuberculosis* in London microbiology laboratories. J Clin Microbiol. 2002;40:4100-4.
86. Burman WJ, Reves RR. Review of false-positive cultures for *Mycobacterium tuberculosis* and recommendations for avoid unnecessary treatment. Clin Infect Dis. 2000;31:1390-5.
87. O'Brien JK, Sandman LA, Kreiswirth BN, Rom WN, Schluger NW. DNA fingerprinting from *Mycobacterium tuberculosis* isolates of patients confined for therapy noncompliance show frequent clustering. Chest. 1997;112:387-92.
88. Majeed AA, Ahmed N, Rao KR, Ghousunnissa S, Kauser F, Bose B, et al. AmpliBase MT(TM): a *Mycobacterium tuberculosis* diversity knowledge base. Bioinformatics. 2004;20:989-92.