Molecular Epidemiology of Tuberculosis in Denmark in 1992

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The incidence of tuberculosis (TB) is increasing all over the world, including in countries with a high standard of living and good social security. Denmark represents such a region. Furthermore, it is a small country (5 million inhabitants) with a long tradition in TB control, including a centralization of the bacteriological diagnostic facility. The present study was intended to analyze the transmission of *Mycobacterium tuberculosis* in a country in which TB has low endemicity by a combination of conventional epidemiological approaches and DNA fingerprinting techniques, whereby individual bacterial strains can be traced. *M. tuberculosis* isolates from 92% of all new cases of bacteriologically verified TB in Denmark during 1992 were subjected to IS*6110* DNA fingerprinting to visualize the DNA restriction fragment length polymorphism (RFLP) patterns of the isolated strains. The data obtained from the RFLP analyses were interpreted by using demographic data, such as age, sex, ethnicity, and residence, for the patients. The risk factors among the patients for being part of an active chain of transmission, as opposed to demonstrating reactivation of a previously acquired latent infection, were estimated by statistical analyses. The magnitude of TB transmission in 1992 in Denmark was determined, and transmitted infections were shown to comprise at least one quarter of the total number of cases. Almost half of the TB cases involved patients of foreign origin. However, most of these isolates showed unique DNA fingerprint patterns and were rarely part of an active chain of transmission. The major chains of recent transmission were localized to distinct geographical regions in the country. TB is frequent among immigrants, especially from Asia and Africa, but it is apparently readily suspected, diagnosed, and treated by the health care system. Danish patients with pulmonary symptoms are not primarily suspected to have TB and, therefore, play an important role in recent TB transmission in Denmark.

The World Health Organization declared tuberculosis (TB) "a global health emergency" in April 1993 (23). TB is no longer a health problem confined to developing countries: the disease is reemerging in industrialized countries (8, 15). The human immunodeficiency virus epidemic and a general neglect of TB control programs in many countries have facilitated the spread of the causative agent, *Mycobacterium tuberculosis* (4–7). In addition, drug-resistant bacteria have evolved, causing tremendous therapeutic problems (6, 11, 12). Migration across country borders is now more than ever part of our reality; immigrants from politically unstable countries seek a safer life in other parts of the world, and today business and leisure activities take people far from home, with the attendant risk of transfer of infectious, airborne diseases like TB.

It was previously suggested that geographical separation of pools of infective microorganisms like *M. tuberculosis* may lead to the evolution of distinct bacterial clones (13, 24). The level of genetic relatedness of *M. tuberculosis* strains can be visualized by probing the chromosomal DNA of the bacteria with genetic markers in Southern blotting experiments, whereby the restriction fragment length polymorphism (RFLP) patterns of the strains will be exhibited. This technique has already proved to be a powerful tool in strain differentiation and opens the possibility for study of the epidemiology of TB at the clonal level (for a review, see reference 17). Of the five repetitive elements which have so far been discovered in *M. tuberculosis* complex strains, the insertion element IS*6110* proved to be the

most useful marker for epidemiological studies (19, 20, 24). Furthermore, an international consensus regarding a standardized protocol, including recommendations for computer-based storage of these data, which allows comparison and collaboration across country borders has been reached (18). In this study, we present the results from the establishment of a database of DNA fingerprint analyses combined with conventional epidemiological data covering 92% of all new, bacteriologically confirmed cases of TB in Denmark during 1992.

MATERIALS AND METHODS

Bacterial isolates. The strains of *M. tuberculosis* analyzed in this study were collected at the Mycobacteria Department at Statens Seruminstitut in Copenhagen, Denmark. This laboratory serves as a central diagnostic service laboratory for all of Denmark, including Greenland and the Faroe Islands. A total of 270 new, bacteriologically verified TB cases were registered in Denmark during 1992. From these, 245 isolates of *M. tuberculosis* were available for DNA fingerprinting. The species identification of the isolates was based on standard microbiological tests and was confirmed with a DNA-RNA hybridization technique (Accu Probe; GenProbe Inc., San Diego, Calif.). All the isolates were examined for their susceptibility to ethambutol, isoniazid, streptomycin, rifampin, and pyrazinamide by using a radiometric culture system (BACTEC; Becton Dickinson and Company, Sparks, Md.). Primary resistance to isoniazid was observed in 5% of the isolates. Resistance to all other drugs was less than 5%. One strain isolated from a Danish patient who had been travelling in Nepal was resistant to all of the above-mentioned drugs. Discrimination between *M. tuberculosis*, *Mycobacterium bovis* and *M. bovis* BCG isolates was based on sensitivity to cycloserine and thiopheno-2-carboxylic acid hydrazide (10).

RFLP analyses. The chromosomal DNA of the isolates was prepared essentially as described previously (21). The RFLP analyses included the IS*6110* probing and the direct repeat reprobing. The IS*6110* probe was a 245-bp, PCR-amplified and agarose electrophoresis-purified DNA probe directed against the right arm of the IS*6110* (20). The direct repeat probe was a 36-bp synthetic oligonucleotide directed against the directly repeated sequences of 36 bp which are clustered in one region of the genome of *M. tuberculosis* and interspersed with nonrepetitive sequences of 36 to 41 bp (14). The probes were nonradioac-

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tively labelled with an enhanced chemiluminescence kit (ECL; Amersham International plc, Little Chalfont, United Kingdom). DNA was digested with restriction endonuclease PvuII. Southern blotting experiments were performed as described previously (24). To facilitate the computer-assisted comparison of the fingerprints, we used a mixture of PvuII-digested supercoiled DNA ladder and HaeIII-digested φX174 DNA as an internal marker, as recommended previously (18, 21). The PvuII-digested chromosomal DNA of M. tuberculosis Mt14323 (18) was used as a reference in each analysis.

The fingerprint patterns of the isolates were compared both by computer-assisted analyses using Bioimage Whole Band Analyzer, Version 3 (Millipore Corporation, Ann Arbor, Mich.), as described previously (24), and by visual examination.

Patient information. The filing of the demographic data of the patients in conjunction with the results of the laboratory analyses and of the clinical diagnoses was approved by the Danish Ministry of Health (kt.j.nr.93/0763-9) as required by Danish legislation. The ethical aspects of this study, i.e., the cases in which patients were contacted directly and asked about possible contacts with other TB patients and travel activities, were evaluated and approved by the ethics committee of Copenhagen [J.nr.(KF)01-503/93].

The distinction between foreigners, or immigrants, and inhabitants of Danish origin was primarily based on the names of the patients. Patients with typical Danish names were not further questioned about their origin. Patients with names which are not commonly used in Denmark or with a personal registration number indicating that they were applying for residency in Denmark were directly questioned about their origin. This group also included second-generation immigrants.

Statistical analyses. For the comparison of fractions, Fisher's exact test was used. For the 245 patients, four explanatory variables (sex, age, residence, and origin) were used in a logistic regression model to predict the probability of a TB patient being part of an active chain of transmission. Each variable was dichotomous, i.e., female versus male; age, <60 versus ≥ 60 years; urban versus provincial residence; and Danish versus foreign origin. The reduction from four to three explanatory variables was tested with a likelihood ratio test. Adjusted odds ratios with 95% confidence intervals were calculated from the estimated parameters. The model fit was acceptable when judged both by a likelihood ratio and by the standardized residuals. The summed square of the $2 \times 2 \times 2$ standardized residuals was 6.8, giving a P value of roughly 0.15 in a chi-square distribution with 4 degrees of freedom. The statistical methods used are described in reference 9.

RESULTS

Patient characteristics. The 270 new, bacteriologically verified TB cases in Denmark in 1992 involved 155 patients of Danish origin (57.4%), 9 immigrants from Greenland (3.3%), and 104 immigrants from 27 foreign countries (38.5%). The origin of the remaining two patients (0.8%), who had non-Danish names, could not be traced. About half of the patients in the immigrant group originated from Pakistan ($n = 23$), Somalia ($n = 22$), and Vietnam ($n = 11$). The age distribution of the Danish and the foreign TB patients is shown in Fig. 1. About 70% of the patients were between 20 and 60 years of age.

DNA polymorphism of M. tuberculosis complex strains. As shown in Table 1, 186 different DNA fingerprint patterns were observed in the 245 isolates analyzed. Of the 186 patterns, 17 were shared by two or more isolates, whereas the remaining 169 patterns (69%) were observed only once each in this investigation. The number of IS6110 copies per isolate ranged from 1 to 24 (Fig. 2). One isolate was repeatedly found not to carry any IS6110 element. Differences in copy number of the IS6110 element between strains isolated from patients of foreign origin and strains obtained from Danish patients were observed. About 16% of the isolates from patients of foreign origin were found to be low-copy-number strains (carrying from one to five copies of the IS6110 element), whereas only 3% of the strains isolated from the Danish patients belonged to this group. Fifteen percent of the isolates from the foreign patients in contrast to only 5% of the isolates from Danish patients were found to carry more than 15 copies of IS6110. The majority of the investigated isolates from Danish patients (92%) carried from 6 to 15 copies of the IS6110 element.

Both strains carrying a low number of IS6110 copies and strains carrying more than 15 copies of the element were al-
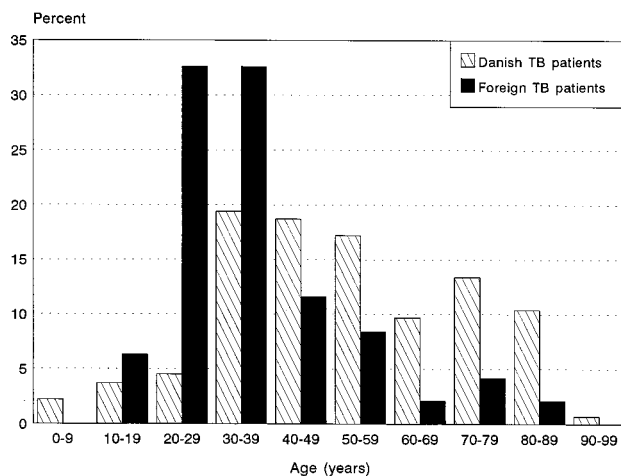

FIG. 1. Age distribution of Danish and foreign TB patients with newly bacteriologically verified TB in Denmark in 1992.

most exclusively found in samples obtained from immigrants originating from Asia and from Africa, i.e., from Vietnam, Somalia, and Pakistan. Four different single-band patterns, caused by four different chromosomal locations of the IS6110 element, were seen in 15 isolates. The most prevalent single-band pattern, shared by seven isolates of M. tuberculosis, involved six patients from Vietnam and one patient from Pakistan. Reprobing of these strains with the direct repeat probe revealed that these strains were not identical. One of the four single-band patterns was found in two isolates, obtained from two patients of Danish origin, which were identified as M. bovis. The remaining two single-band patterns were seen in the isolates obtained from patients from Somalia and Pakistan. The isolate without any IS6110 element in the chromosome was isolated from a patient originating from Iran. No correlation between the DNA fingerprint patterns of the isolates and their drug susceptibility was observed.

Risk factors predisposing for being part of an active chain of TB transmission. It is generally accepted that M. tuberculosis

TABLE 1. Number and size of RFLP patterns of M. tuberculosis isolates among 245 newly diagnosed TB patients in Denmark during 1992[a]

Size of pattern (no. of isolates)	No. of patterns	Total no. of isolates
19	1	19
12	1	12
8	1	8
7	1[b]	7
4	1[b]	4
3	2	6
2	10[c]	20
1	169	169
All	186	245

[a] The possibility of laboratory cross-contamination can be eliminated because all the matching strains which share identical RFLP patterns were isolated on different days, and each of the patients infected with identical clones of M. tuberculosis had at least two cultures positive on both on Löwenstein-Jensen medium and BACTEC.

[b] Single-band patterns, which were excluded from the analysis of relative risk for being part of an active chain of transmission.

[c] Two of these 10 patterns were single-band patterns and were, therefore, also excluded from the analysis of relative risk for being part of an active chain of transmission.
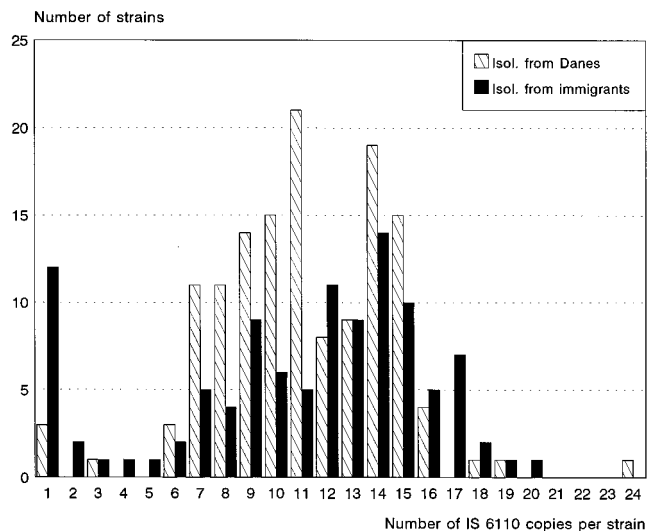
FIG. 2. Numbers of IS6110 elements in *M. tuberculosis* isolates from Danish patients and in *M. tuberculosis* isolates from foreign patients.

TABLE 2. Estimation of risk for belonging to an active chain of TB transmission

Risk factor	No. of patients (%)	% of patients with identical RFLP patterns
Sex[a]		
Male	153 (60.0)	37.3
Female	102 (40.0)	22.6
Age (yr)		
<60	199 (77.4)	34.2
≥60	58 (22.6)	20.7
Origin of patient population[b]		
Denmark	134 (52.3)	36.6
Foreign country	95 (37.1)	12.6
Greenland	27 (10.6)	70.4
Residence[a]		
City[c]	147 (57.7)	30.0
Province	81 (31.8)	21.0
Greenland	27 (10.5)	70.4

[a] Excluding two patients for whom data regarding sex and residence were not available.

[b] Excluding one patient for whom information concerning origin was not available.

[c] Category includes cities with more than 60,000 inhabitants.

isolates with identical DNA fingerprint patterns, especially when they consist of multiple IS6110 hybridizing bands, are epidemiologically closely related (17). However, it was also concluded from previous studies that a clonal relation cannot with any high degree of likelihood be inferred to exist between isolates of *M. tuberculosis* possessing only one copy of IS6110 (20, 26). The limited observations from the present investigation also support this conclusion. Accordingly, the 15 *M. tuberculosis* isolates with only one copy of IS6110 were excluded from the analyses of risk factors of importance for being part of an active chain of TB transmission. The data for samples from Greenland for which the RFLP patterns were described previously (24) were included because they represent a part of this cross-sectional study. Consequently, the analysis was based on 257 patients (230 from Denmark and 27 from Greenland).

TB patients from Greenland were much more likely to be part of an active, infectious chain of TB transmission than any other patient subpopulation in this study. Although the incidence of TB in the Danish population is lower than in the countries in which the disease is highly endemic from which the immigrants came, it was observed that Danish TB patients had a higher risk of belonging to a chain of transmission compared with the foreign TB patient group (Table 2).

The multivariate logistic regression analysis of risk factors (Table 3) showed only a moderate adjustment of estimates and revealed significant differences between younger and older patients. The risk appeared, furthermore, to be higher for patients living in big cities than for patients living in provincial regions and also higher for male than for female patients. The highest odds ratio obtained from the analysis, 6.1, was for the origin of the patients (Danish versus foreign origin).

Epidemiological investigation of isolates with identical RFLP patterns. Epidemiological investigations were concentrated on the three most prevalent patterns listed in Table 1. The most prevalent pattern involving 19 isolates was identified as being related to a chain of TB transmission between Greenland and Denmark which was recently reported (24). The second most prevalent pattern was traced to a group of patients living in Jutland, centered mostly in the southern (provincial) part (Fig. 3). The third most prevalent pattern was found to be spread in Copenhagen and to Odense (a large city) and a provincial location in southern Jutland (Fig. 3). None of the

patients belonging to these two chains of transmission were of foreign origin. Questionnaires were mailed to 29 patients, all of whom belonged to an active chain of TB transmission as defined by our criteria. Approximately half of the patients responded, and we could conclude that none of the patients were aware of where they had contracted the disease nor did they believe that they had spread the disease to any other persons.

DISCUSSION

Denmark is a northern European country with 5 million inhabitants, of which 1 million are living in the capital, Copenhagen. The social security system is mainly public and is financed through taxes. As in the other Scandinavian and northern European countries, the general standard of living has steadily improved during the entire postwar era, up until the 1980s, when a general economical stagnation was observed in Denmark as in many other industrialized countries. The incidence of TB closely reflects, and is inversely associated with, this tendency. The annual number of notified cases of TB in 1985 was as low as 275, but it is increasing and will during 1994 exceed 400 cases, according to the National Surveillance System, Department of Epidemiology, Statens Seruminstitut. Denmark is, with regard to TB epidemiology, in a unique position because the bacteriological diagnostic service facility is centralized at one location, Statens Seruminstitut, where the

TABLE 3. Multivariate analysis of risk factors for belonging to an active chain of TB transmission

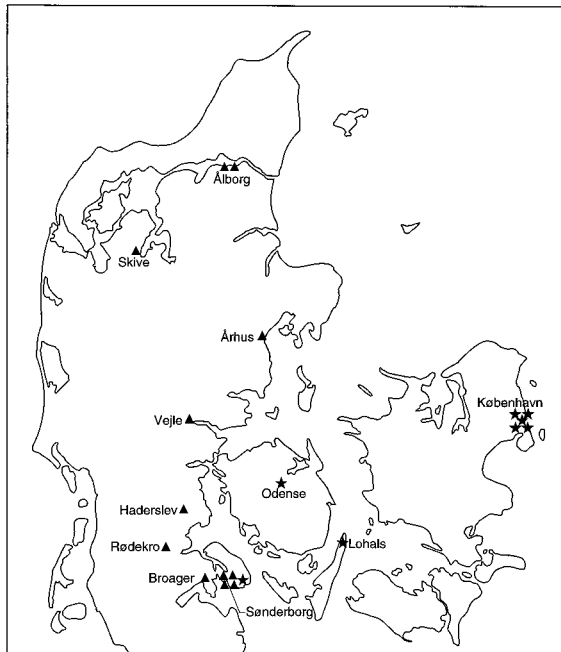| Risk factor	Groups compared (odds ratio)	95% Confidence interval	P value
Sex	Male/female (2.0)	1.0–4.0	0.04
Age	<60 yr/≥60 yr (3.1)	1.3–7.3	0.002
Origin	Danish/immigrant (6.1)	2.8–13.2	0.0001
Residence	City/province (2.1)	1.0–4.3	0.003

FIG. 3. Geographical map of Denmark showing the locations of two prevalent RFLP patterns which involved 12 (triangle) and 8 (star) isolates of *M. tuberculosis.* The peninsula is Jutland.

National Surveillance System is also situated. The number of notified TB cases is very similar to the number of bacteriologically verified TB cases. In other words, the clinicians in Denmark do not normally initiate anti-TB treatment unless the TB diagnosis is bacteriologically verified.

Denmark is comparable in size to only a region or a county of other large countries and is, therefore, well suited as a site for assessing the technique of DNA-based molecular epidemiology. In this study, we collected bacterial isolates from nearly all of the newly diagnosed TB patients during 1992, submitted the samples to RFLP analyses, applying the IS*6110* element as a probe, and combined the results with available epidemiological information. These analyses provided an overview of the molecular epidemiology of TB in Denmark in 1992 and enabled us to define the magnitude of TB transmission in the country during a certain period of time and to localize the active chains of transmission. Similarly, we examined the impact of TB imported by immigrants.

It was recently suggested that the majority of the cases of TB in developed countries result from the reactivation of previously acquired bacteria (2). However, the present study, in accordance with two recent studies conducted in San Francisco and in New York (1, 16), suggested that recent transmission certainly also exists in industrialized countries. The estimated risk for being part of an active chain of TB transmission was 26.5% among TB patients in Denmark in 1992 and was observed to be 40% in San Francisco and in New York. Human immunodeficiency virus infection contributed significantly to the pool of infectious sources in the two studies mentioned above. It is by law prohibited in Denmark to get access to information on a person's human immunodeficiency virus status. However, anonymous data from 1992 reported in total only seven cases of AIDS-related TB (3). The possibility that some of the patients in the groups of patients infected with the same clones of mycobacteria were also infected with human immunodeficiency virus cannot be excluded.

According to previous experience, epidemiologically related isolates have identical IS*6110*-associated DNA fingerprint patterns and, in contrast, epidemiologically nonrelated strains show unique patterns, with the exception of single-copy strains (16, 22, 24). The statistical analysis of the relative risk for strains to have identical RFLP patterns, which may be translated as the risk of belonging to an active chain of transmission, rated the inhabitants of Greenland to have the highest risk for being infected from other patients with active TB compared with the other groups included in the study population.

Statistically, at the 5% level, parameters including sex, age, and the residence of the patient were also found to be significant, independent risk factors for being part of a transmission chain. In general, male TB patients, under age 60 and living in a big city were at a higher risk for being newly infected by other TB patients than, e.g., female TB patients older than 60 years and living in the countryside, who were more likely to have reactivated an old TB lesion.

It is often an emotional matter of public debate whether infectious diseases are introduced into a society because of the increased migration across country borders. Comparison of Danish TB patients with TB patients of foreign origin revealed that a majority of the imported isolates exhibited unique RFLP patterns. This observation may reflect the fact that TB is more readily diagnosed by the medical care system when clinical symptoms are presented by patients from a country where the disease is highly endemic. Symptoms like persistent cough and subfebrility presented by a patient from Denmark, where smoking is still a big health problem, may in most cases be explained by chronic bronchitis or, ultimately, a malignant disease. The possible delay in diagnosis and treatment of TB in Danish patients compared with that for foreign patients might have facilitated the disease transmission in the Danish population.

A clear correlation between occurrence of a low number of IS*6110* elements per chromosome and classification into the Asian subgroup of *M. tuberculosis* strains was observed in a previous study in Tanzania (25). In another study, one third of the *M. tuberculosis* strains from Asia were found to contain only a single copy of the IS*6110* element (20). It was found in the present study that 81% (17 of 21) of the isolates carrying a low number of IS*6110* copies were obtained from patients originating from Asia or Africa. Fifty-three percent of the single-copy strains were obtained from immigrants from Asia, and 20% were obtained from immigrants from Africa. In addition, two thirds of the isolates carrying more than 15 copies of the IS*6110* element were obtained from immigrants from Somalia and Pakistan. These observations support the hypothesis of the existence of certain genotypes of *M. tuberculosis* in a given geographic region.

The data obtained during this study will form the basis of a database of DNA fingerprint data and epidemiological information concerning all bacteriologically verified cases of TB in Denmark from 1992 onward. We hope in the future thereby to extend the observations of this preliminary study for which a rather arbitrary time limit of one year was introduced. It is our goal to provide a practical tool for clinicians and public health authorities in the tracing of potential sources of infection. A detailed TB surveillance in a country which is manageably sized and in which TB has low endemicity like Denmark will hopefully contribute to a more thorough understanding of TB transmission and thereby ultimately have an impact on TB control.

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