Simultaneous Infection with Two Drug-Susceptible\n*Mycobacterium tuberculosis* Strains in an\nImmunocompetent Host

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An important assumption for DNA fingerprinting of *Mycobacterium tuberculosis* is that patients are infected with only one strain at a time. Nonetheless, we demonstrate a case of simultaneous infection with two drug-susceptible strains of *M. tuberculosis* in an immunocompetent patient by IS6110 restriction fragment length polymorphism and spoligotyping. Epidemiological data prove the patient’s involvement in two independent clusters. Thus, double infections should be suspected with fingerprints showing divergent band intensities.

Molecular methods have turned out to be valuable for investigating the transmission of tuberculosis (TB). For DNA fingerprinting of *Mycobacterium tuberculosis*, a protocol based on the restriction fragment length polymorphism (RFLP) generated by IS6110 is regarded as the “gold standard” (5, 11), provided that more than five copies of IS6110 are present in the genome of a given strain (12). With the exception of the mixed-linker PCR (2), most other methods, including spoligotyping (4), have less discriminatory power (5). They complement IS6110-RFLP, e.g., when nonviable or low-copy-number strains are tested. The assumption that an individual patient is infected with only one strain of *M. tuberculosis* at a given time is meaningful for the interpretation of DNA fingerprinting results. Over time, however, a given strain may be replaced or become masked by another strain: DNA fingerprinting of sequential isolates demonstrated that drug-resistant strains can replace drug-susceptible strains as a result of reinfection (3, 7, 8) or of reactivation under treatment (10). Furthermore, two different strains have been isolated from different anatomical sites in one human immunodeficiency virus-infected individual at the same time (1). However, the simultaneous isolation of drug-susceptible strains from an immunocompetent person has not been clearly demonstrated so far. Here, we demonstrate such a case of double infection by DNA fingerprinting. Both strains found in this patient caused clusters in the patient’s hometown, and epidemiological data confirmed that the patient belonged to both clusters.

**Cluster analysis.** Primary *M. tuberculosis* isolates from Western Austria have been fingerprinted since 1994, by using the standardized IS6110-RFLP technique and spoligotyping. In a Tyrolean rural region, two TB clusters had been caused by two different drug-sensitive strains, strain P and strain Oe (Fig. 1). The more rapidly appearing cluster (first seven cases within 10 months) was caused by the newly introduced strain P. Different strains with the same distinct spoligotype (see Fig. 2) are very prevalent in the Caribbean (9) and were also observed in an outbreak of multidrug-resistant TB in Buenos Aires, Argentina (6). By patient interviewing (to which all patients gave their informed consent), the likely source case for this outbreak (patient 1) was identified. His *M. tuberculosis* isolate had not been viable for performing IS6110-RFLP and could be tested only by spoligotyping. Transmissions from patient 1 to several adolescents were associated with pub X. Two nosocomial infections of other patients occurred in the course of patient 1’s admission to hospitals A and B, respectively.

The second cluster caused by strain Oe consisted of four patients (b to e) and very probably (by epidemiological evidence) included patient a, who was diagnosed before fingerprinting was set up. Patients a, b, and e together frequented pub Y for years. Contacts with patients c and d could not be proven, although they lived in the same town.

**A case of double infection.** Patient 7/f, a bartender in pub Y, became exposed to strain Oe (by patients b and e) and to strain P (by patient 1, who frequented pub Y in addition to his favorite, pub X). Nevertheless, he was not immediately recognized as indeed belonging to both clusters. In the IS6110-RFLP analysis, multiple faint but clear-cut bands were initially regarded as being caused by incomplete DNA digestion, especially as the stronger bands clearly formed the strain P fingerprint. The possibility that the faint bands belonged to strain Oe arose rather in a moment of inspiration and was not suggested by the fingerprinting software (the 7/f isolate had only 73 and 55% similarity [Dice index] to strains P and Oe, respectively). The relative band intensities suggested a mixture of strains P and Oe at a ratio of 2:1 to 3:1 (Fig. 2, top and middle). To test this possibility, we prepared single colonies on Middlebrook 7H10 agar plates by a subculture from the original Lowenstein-Jensen tube. DNA from 28 colonies was obtained by picking colonies with a pipette tip, suspending it in 200 μl of 10 mM Tris–1 mM EDTA (pH 8.0), and boiling it for 10 min. Ten microliters thereof served as DNA template in a 25-μl spoligotyping-PCR mixture (4). Twenty colonies were typed as strain P, six were typed as strain Oe, and two showed an additive pattern (probably due to nonclonal colonies). The same mixed pattern was obtained from DNA extracted from the initial patient 7/f Lowenstein-Jensen culture for RFLP purposes (Fig. 2, bottom). This result corroborated the suspicion of a mixed infection and the about-threefold abundance of strain P. A subculture of the original isolate yielded the same RFLP (data not shown), making DNA contamination during fingerprinting procedures an unlikely cause for the patterns observed. Furthermore, isolates or samples belonging to either outbreak strain had not been handled during the culti-

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observations have been frequently made with sequential isolates (separated by at least 90 days) from the same patient (14).

In summary, patient 7/f, an immunocompetent individual, was productively infected with two different, fully drug-susceptible strains of M. tuberculosis at the time of diagnosis. His infection occurred as a consequence of occupational exposures to two different chains of active transmission. As RFLP patterns with distinct faint and strong bands are not very uncommon, we conclude that the possibility of a double infection should be considered more often. This conclusion is underlined by a report published while the manuscript was under review, describing a case of simultaneous infection with two strains detected by employing IS6110-RFLP and phage typing (13).

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