Totally drug-resistant tuberculosis strains: evidence of adaptation at the cellular level

To the Editors:

In the Eastern Mediterranean Region (EMRO), there are no accurate estimates of numbers of patients with multidrug-resistant (MDR)/extensively drug-resistant (XDR) tuberculosis (TB). In 2006, we documented the existence and transmission of XDR-TB among patients with MDR-TB [1]. In a further study, we isolated more dangerous forms of bacilli, named totally drug-resistant (TDR) [2]. This group of strains showed *in vitro* resistance to all first- and second-line drugs tested (*i.e.* aminoglycosides, cyclic polypeptides, fluoroquinolones, thioamides, serine analogues and salicylic acid derivatives). TDR-TB patients remained smear- and culture-positive after 18 months' median treatment with second-line drugs. Even changing the treatment to co-amoxyclav (625 mg per 8 h) or clarithromycin (1,000 $mg \cdot day^{-1}$), along with a high dose of isoniazid (15 $mg \cdot kg^{-1}$) led to no improvement [2]. In the present study, transmission electron microscopy (TEM) was used to evaluate the differences between such deadly bacilli and MDR or susceptible ones at the ultrastructural level. Furthermore, to evaluate the genotyping patterns, the strains were subjected to spoligotyping and variable number of tandem repeats (VNTR) analysis.

Susceptibility testing against first- and second-line drugs was performed using the proportional method on Löwenstein–Jensen media [3].

For spoligotyping and VNTR analysis, five isolates from each group of strains (*i.e.*, susceptible, MDR- and TDR-TB) were included in this study. The spoligotyping and VNTR analysis were performed on extracted DNA using standard protocols [4, 5].

For TEM analysis, a loop of Löwenstein–Jensen culture media was inoculated into Middlebrook 7H9 broth (Difco, Franklin Lakes, NJ, USA) supplemented with 0.2% glycerol and 10% Middlebrook OADC enrichment (Difco). Cells at an optical density (OD) of 0.6 at 600 nm were used for further experiments. These cells were first centrifuged at $800 \times g$ for 5 min, then the supernatant was adjusted to an OD of 580 nm, corresponding to 6.3×10^7 colony-forming units of *Mycobacterium tuberculosis* per mL. 10 μL of this supernatant were subjected to acid-fast bacilli and Gram staining. Nontuberculous mycobacterial contamination was check by culturing 100 μL of supernatant on 7H11 agar plates. Niacin production, spoligotyping and IS6110–PCR were performed on isolated colonies from each plate. Thereafter, ultrathin sections for TEM analysis were prepared from fixed and solidified bacterial cells [6].

The identified spoligotypes were as follows: Haarlem I (n=3; 20%); Beijing (n=2; 13%); EAI (n=4; 26 %); CAS (n=4; 26%); T (n=1; 6.6%); and U (n=1; 6.6%) super families. The VNTR profiles of spoligotype strains were different in each superfamily.

In the TEM analysis, three different cell populations were observed at the exponential phase of TDR tubercle bacilli: 1) ordinary (80–70%); 2) round or oval-shaped (15–20%); and 3) extraordinarily thick cell wall bacilli (21–26 nm) that resembled features of stationary or anaerobic dormant bacilli (5–7%) (fig. 1). These adapted forms were detected in all TDR isolates, irrespective of their superfamilies or genotypes patterns. The adapted forms of TDR tubercle bacilli were not detected in susceptible or MDR strains. Under the TEM, marked differences were observed in the thickness of cell walls: 20.2 ± 1.5 nm and 17.1 ± 1.03 nm for the TDR- and MDR-TB bacilli, respectively and 15.6 ± 1.3 nm for the susceptible isolates ($p<0.05$).

While the problem of XDR-TB cases remains unresolved in much of the world, here we report on a more dangerous form of the disease, which we call TDR-TB [2]. In these strains, 30% of the total *M. tuberculosis* populations transformed into adapted forms, producing either round or oval shaped bacilli (15–20%), or having an extraordinarily thick cell wall (5–7%) (fig. 1). These forms were not found among the susceptible or MDR population. At present, we have not demonstrated the transmission of TB disease by these round or oval-shaped bacilli, but the possibility of transmission cannot be ignored [7]. The TDR-TB cells with an extraordinarily thick cell wall (26 nm) resembled stationary or dormant-phase bacilli. Such cells were not found in longitudinal and cross-sectional studies at different levels of MDR and susceptible strains, although it has been reported that these cells (1–3%) are present in normal population of *M. tuberculosis* [8]. Under normal circumstances, stationary cells are viable but non-dividing [8]. The TDR stationary bacilli in the process of division may represent the dominant population of TDR tubercle bacilli in the near future.

The next important issue is: what drugs can really penetrate these cells, which have cells walls about 21–26 nm thick? As such the tuberculosis chemotherapy is very different from that of other bacterial infections. *Mycobacterium* has different populations of bacilli which can survive in an aerobic environment, or low-PH conditions (the conditions necessary for pyrazinamide activity) and microaerophilic conditions [9]. Each of anti-TB drugs has a major role in dealing with one of these populations [9]. However, there are still persistent bacterial populations that are not killed by any available TB drugs [10]. Clinically, we have experienced such cases in our TB wards. These patients did not respond to any standardised treatment and remained smear- and culture-positive after prolonged therapy with second-line drugs. Therefore, the problem of TDR-TB should be taken very seriously and this is an issue requiring urgent attention from the global scientific community.

The genotyping results showed different VNTR profiles among identified superfamilies of *M. tuberculosis* (Haarlem, Beijing, EAI, CAS, and T). The obtained patterns ruled out recent transmission among the studied cases. TEM analysis showed no ultrastructural differences among identified superfamilies. Therefore, changes at the ultrastructural level of resistant

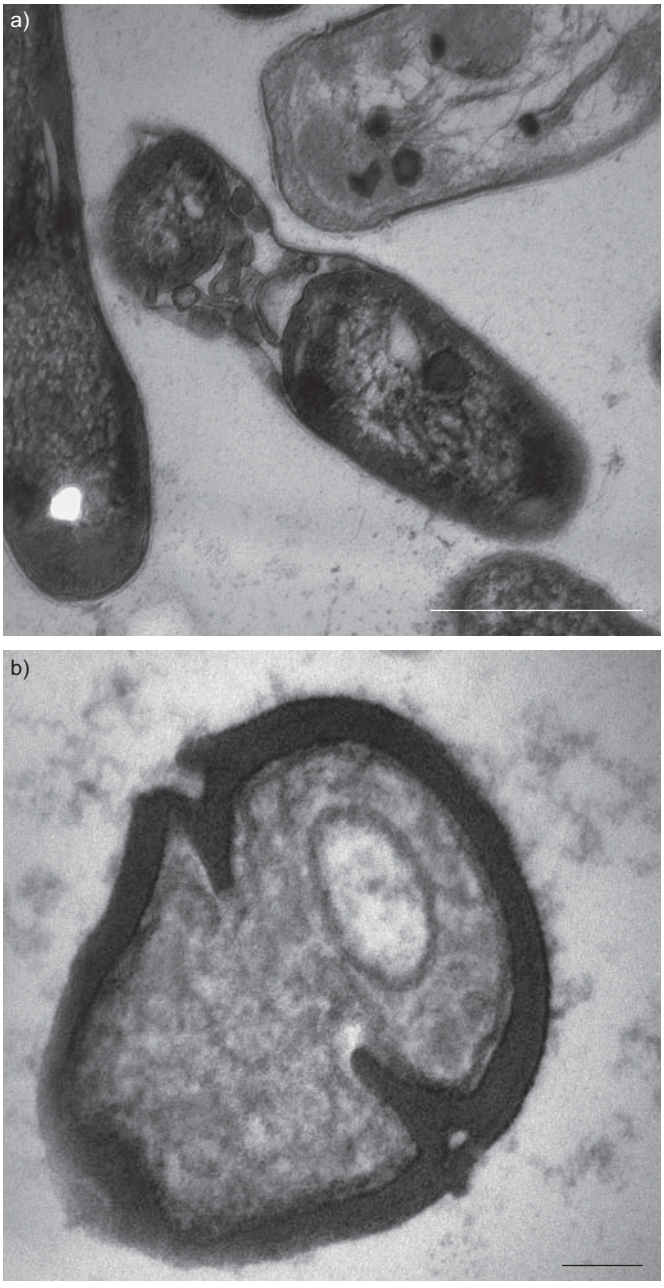

FIGURE 1. a) Totally drug-resistant (TDR)-tuberculosis (TB) bacilli with oval or round bodies inside them. Scale bar=500 nm. b) TDR-TB bacilli with stationary or anaerobic dormant bacilli in dividing stage. The increased size of the cell wall is very clear. Scale bar=100 nm.

strains may occur progressively and this is always preventable by prompt and reliable laboratory detection of drug-resistant TB cases.

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REFERENCES

1 Masjedi MR, Farnia P, Sorooch S, *et al.* Extensively drug-resistant tuberculosis: 2 years of surveillance in Iran. *Clinical Infect Dis* 2006; 43: 841–847.
2 Velayati AA, Masjedi MR, Farnia P, *et al.* Emergence of new forms of totally drug-resistant tuberculosis bacilli: super extensively drug-resistant tuberculosis or totally drug-resistant strains in Iran. *Chest* 2009; 136: 420–425.
3 World Health Organization (WHO). Guidelines for drug susceptibility testing for second line anti tuberculosis drugs for DOTs-plus WHO/CDC/TB/2001.288. Geneva, World Health Organization, 2001.
4 van Embden JD, Cave MD, Crawford JT, *et al.* Strain identification of *Mycobacterium tuberculosis* by DNA fingerprinting: recommendations for a standardized methodology. *J Clin Microbiol* 1993; 31: 2406–2409.
5 Frothingham R, Meeker-O'Connell WA. Genetic diversity in the *Mycobacterium tuberculosis* complex based on variable numbers of tandem DNA repeats. *Microbiology* 1998; 144: 1189–1196.
6 Toda T, Takeya K, Koike M, *et al.* Electron microscopy of ultrathin sections of *Mycobacterium*. Fine structure of the cells grown *in-vitro* and *in-vivo*. *Proc Japan Acad* 1960; 36: 372–375.
7 Farnia P, Masjedi MR, Varahram M, *et al.* The recent-transmission of *Mycobacterium tuberculosis* strains among Iranian and Afghan relapse cases: a DNA-fingerprinting using RFLP and spoligotyping. *BMC Infect Dis* 2008; 8: 109–116.
8 Cunningham AF, Spreadbury CL. Mycobacterial stationary phase induced by low oxygen tension: cell wall thickening and localization of the 16-kilodalton α-crystallin homolog. *J Bacteriol* 1998; 180: 801–808.
9 Mitchison DA. The action of antituberculosis drugs in short-course chemotherapy. *Tubercle* 1985; 66: 219–225.
10 Velayati AA, Farnia P, Masjedi MR, *et al.* Differences in cell wall thickness between resistant and non resistant of *Mycobacterium tuberculosis*; using transmission electron microcopy. *J Chemotherapy* 2009; 55: 303–307.

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