In conclusion, the IP-10 test performs with equal sensitivity to the QFT-IT in a TB/HIV-endemic setting. Combining the tests significantly improves sensitivity, even in HIV-positive patients. The IP-10 test seems less affected by a low CD4 cell count than the QFT-IT. Further approaches for improvement of TB diagnosis are needed, especially in TB/HIV-endemic settings, and the IP-10 biomarker represents a promising example.

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New insight into extremely drug-resistant tuberculosis: using atomic force microscopy

To the Editors:

We have recently documented the emergence of new forms of resistant tuberculosis (TB) bacilli (totally drug resistant (TDR)-TB or extremely drug-resistant (XXDR)-TB strains) among multidrug-resistant (MDR)-TB patients [1]. XXDR-TB defines any case of TB with resistance to all first- and second-line anti-TB drugs whose smears and cultures remain positive despite prolonged therapy [1–3]. At the cellular level of XXDR-TB strains, adaptation was observed and evaluated using transmission electron microscopy (TEM) [4, 5]. In the exponential phase, three different cell populations were clearly distinguished: one displayed an ordinary pattern (70–80%), one exhibited a round or oval shape (15–20%), and the third displayed an extraordinarily thick cell wall (21–26 nm) with features similar to stationary or anaerobic dormant bacilli (5–7%) [3, 4]. These adapted forms were detected in all XXDR-TB isolates, irrespective of their super families or genotype patterns. We tried to evaluate the different cell population of XXDR-TB strains in comparison to susceptible cells using atomic force microcsopy (AFM). To achieve this goal, we included sputum and culture positive specimens of the same

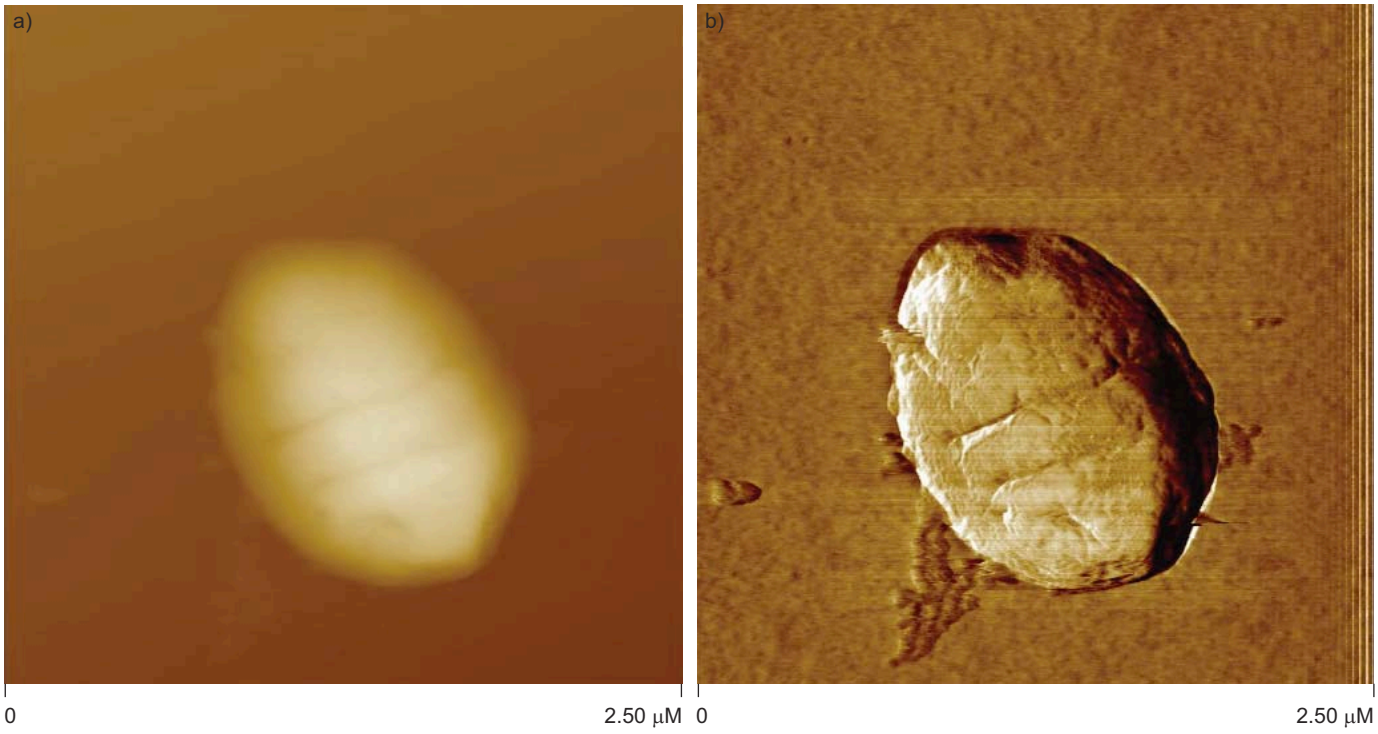

FIGURE 1. In the exponential phase of growth, 15–20% of extremely drug-resistant tuberculosis (XXDR-TB) bacilli has an oval or rounded shape and is 300–900 nm in dimension. The average surface roughness of a rod-shaped bacilli was 13.7±1.5 nm in comparison to oval XXDR-TB cells which was 10±0.8 nm on 200×200 nm.

patients in our study. AFM images were recorded in contact mode using an optical lever microscope equipped with a liquid cell (Nanoscope IV Multimode AFM; Veeco Metrology Group, Santa Barbara, CA, USA) [5]. To image mycobacteria by AFM, the cells were immobilised by mechanical trapping onto isopore polycarbonate membrane (Millipore Corporation, Billerica, MA, USA), with pore size similar to the cell size. After filtering a concentrated cell suspension, the filter was gently rinsed with deionised water, carefully cut (1×1 cm) and attached to a steel sample puck (Veeco Metrology Group) using a small piece of adhesive tape. The mounted sample was transferred into the AFM liquid cell. Images were recorded in both height and deflection modes; using oxide-sharpened microfabricated Si3N4 cantilevers (microlevers; Veeco Metrology Group) with spring constant of 0.01 nm^{-1} [6, 7]. Through AFM observations, we confirmed the existence of oval shaped TB cells among XXDR-TB populations (fig. 1). All XXDR-TB bacilli showed prominent ultrastructural alterations (characteristic concentric striations and increased surface roughness) in comparison to susceptible bacilli. The average surface roughness of all adapted XXDR cells (14.8±1.4 nm on 200×200 nm height images) was higher than susceptible strains (8±2.5 nm on 200×200 nm height image) and the differences were statistically significant (table 1). Although not statistically significant, the adapted XXDR rod-shaped bacilli with thicker cell wall (~20–26 nm) revealed a greater surface roughness (13.7±2.1 nm) than oval cells (10±0.8 nm) (fig. 2). We also noted that our AFM images did not show differences in ultrastructure of the cells isolated from sputum compared to the cell taken from Lowenstein–Jensen culture media. The observed striations and surface roughness in XXDR-TB bacilli were attributed to a direct effect of drugs and were co-related to the mode of arabinogalactan and protein assemblies in the cell wall envelope [8]. Our overall observations were similar to studies by ALTSEENS *et al.* [8] and VERBELEN *et al.* [6], who reported that *in vitro* treatment of native *Mycobacterium bovis* bacilli Calmette–Guérin with first-line drugs induces the striations and surface roughness. These studies suggest that the efficiency of a given therapy may progressively change with the erosion of the envelope, which may not be in a positive manner. Additionally, in 2–3% of XXDR-TB bacilli,

TABLE 1 Average surface roughness in susceptible and extremely drug-resistant tuberculosis (XXDR-TB) cells

Type of *Mycobacterium tuberculosis* bacilli	Cell wall thickness	Length	Average surface roughness on 200×200 nm
Susceptible bacilli	15.6±1.3 nm	1.8 to 3 μm	8±2.5 nm
XXDR-TB bacilli: rod like	20.2±1.5 nm	1.8 to 3 μm	10±0.8 nm
XXDR-TB bacilli: round	19.3±1.8 nm	0.3 to 0.9 μm	12.2±1.3 nm
Resistant rod-like bacilli: thick cell wall	21. 8±6.2 nm	1.5 to 2 μm	13.7±2.1 nm

Data are presented as the sum of 15–20 steel sample packs that were observed under atomic force microscopy.

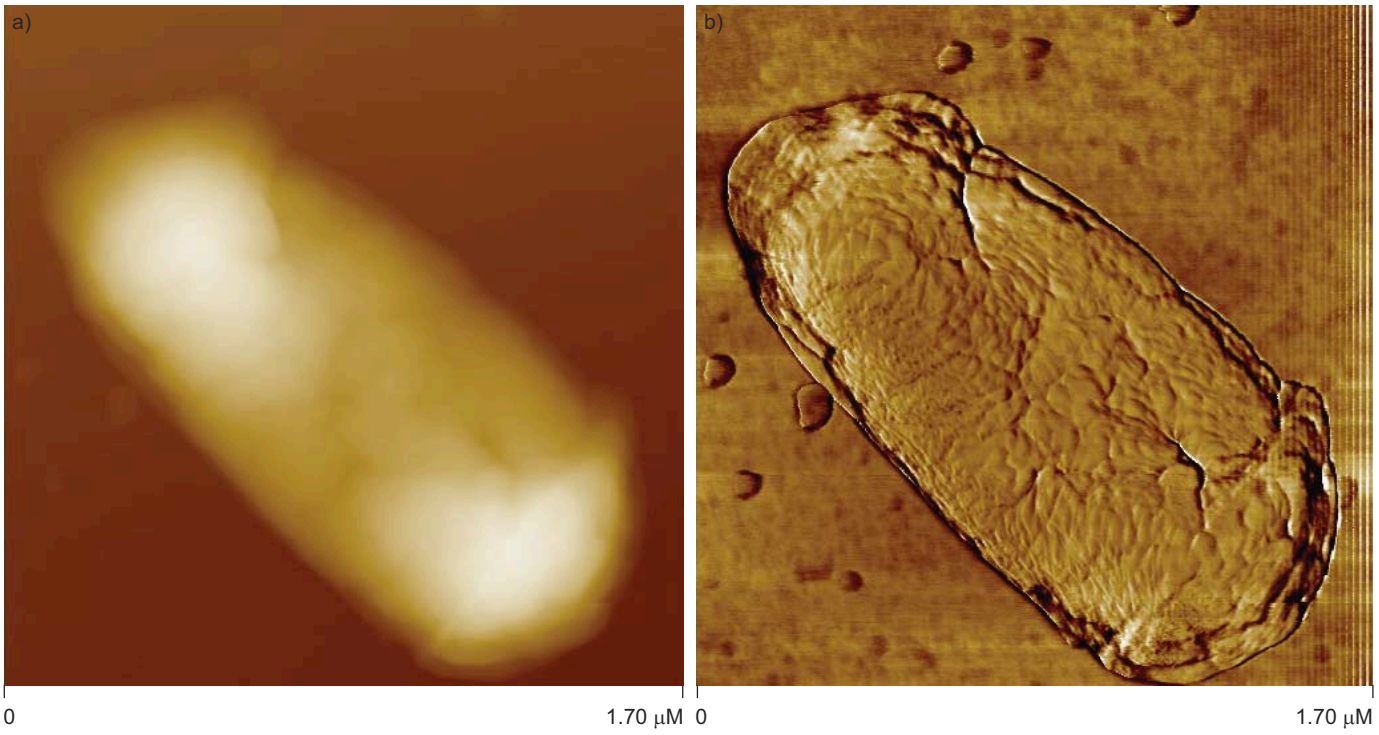

FIGURE 2. The average surface roughness of all adapted extremely drug-resistant tuberculosis cells was higher (14.8±1.9 nm) than susceptible cells (8±2.5 nm) and the differences were statistically significant.

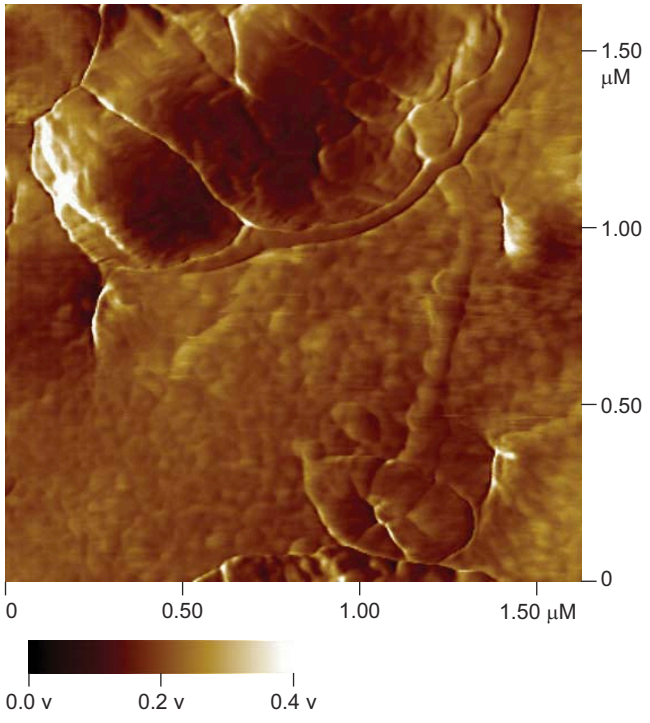

FIGURE 3. Of the extremely drug-resistant tuberculosis (XXDR-TB) bacilli, 2–3% had elaborated long, tubular extension of the cell envelope (length: 890 nm to 1 μm; diameter: 70–75 nm), with tapering towards the end. Tubular protrusion had low surface roughness (2.5±1.2 nm) in comparison to both the XXDR-TB and susceptible tuberculosis cell envelope.

elaboration of long, tubular extensions of the cell envelope (length: 890 nm to 1 μm; diameter: 70–75 nm), which tapered towards one end, have also been observed (fig. 3). These appendages had very low surface roughness (2.5±1.2 nm) in comparison to both XXDR-TB and susceptible-TB cell envelopes. Therefore, we hypothesised that these structures were synthesised by the incorporation of new envelope materials that may comprise inner and outer membranes, peptidoglycan and periplasmic space. For the first time, these tubular extensions have been seen in *Mycobacterium tuberculosis* and, at present, we do not know whether they are functional under genetic or environment controls. In our study, the investigated TB strains were isolated from XXDR-TB patients. All patients were subjected to second-line anti-TB drugs for 18–24 months with the following protocols: ofloxacin (400–800 mg·day^{-1}); cycloserine (750–1,000 mg·day^{-1}); prothionamide (750–1,000 mg·day^{-1}); and amikacin (15 mg·kg^{-1}·day^{-1} for 5 days per week, maximum 1 g·day^{-1}). Therefore, further investigations are warranted in order to determine whether these morphological changes are induced by the combination therapy or certain specific anti-TB drugs. Finally, our observations have raised three questions. First, could Mycobacteria produce tubular structure on environmental constraints (effect of different drugs and/or low availability of nutrients)? Secondly, are persistent Mycobacteria also capable of producing such appendages? Thirdly, and most importantly, are such passages important for uptake of drugs inside the bacilli? In conclusion, XXDR-TB is a more serious and complex clinical concern than previously appreciated. Thus, urgent strategies are required to speed up novel anti-TB drugs toward resistant bacilli. It is important to understand the possibility of disease transmission through oval shape XXDR-TB bacilli. The size of these bacilli (300–900 nm) is smaller than rod-shaped bacilli (1.5–3 μm) and

could easily escape attack from the body's immune system. Therefore, if this could happen, then this is an issue requiring urgent attention from the global scientific community.

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