Rapid diagnosis of tuberculosis using ex vivo host biomarkers in sputum

To the Editor:

Tuberculosis continues to be a major public health problem in developing countries [1]. One of the roadblocks in reducing tuberculosis transmission is the lack of accurate laboratory-free diagnostic tests for use at the point of care. If tuberculosis is to be eliminated, we need a robust, low-cost and safe point-of-care diagnostic test, which in turn requires identification of appropriate biomarkers [2]. Rapid tests based on microfluidics (lateral flow tests) hold great promise for tuberculosis diagnostics. They are easy to use, cheap, provide an answer within minutes, do not require specialised equipment and are stable at room temperature, making them ideal for use in high tuberculosis burden, resource-poor settings. To date, however, no such test has been developed for tuberculosis due to lack of sensitivity related to the markers and/or sample type. Development of tests based on host biomarkers requires evaluation of different sample types [3–5] and markers other than interferon (IFN)-γ [5] to provide differential diagnosis of active tuberculosis, latent infection and other respiratory disorders. We have previously shown that a combination of three host factors in pleural fluid resulted in 96% correct classification of tuberculosis among other respiratory diseases (ORD) (including bacterial pneumonia) regardless of HIV status [6]. However, this sample type is not easy to obtain and we therefore wanted to determine if we could use ex vivo sputum, which is noninvasive and easy to obtain in adult pulmonary tuberculosis patients.

Subjects were consecutively recruited from the outpatient clinic and ward at the Medical Research Council Unit, Fajara, the Gambia. All subjects were adults (≥18 years of age) with symptoms suggestive of tuberculosis. Subjects were subsequently classified into two groups: those with culture-confirmed tuberculosis and those with ORD. 75% of the tuberculosis and 50% of the ORD group were positive by the IFN-γ QuantiFERON test (Qiagen, Hilden, Germany). Samples were collected concomitantly from the same patient. Serum was collected using Vacutainers (BD, Franklin Lakes, NJ, USA) following centrifugation and saliva was collected using a passive drool technique. 1 mL of fresh sputum was digested for 15 min at room temperature with 0.1% dithiothreitol. An equal volume of PBS was added, the samples were centrifuged (600 x g for 5 min), and the supernatants were collected and stored at -20°C. Undiluted heparinised blood (450 μL per well) was stimulated with purified protein derivative (PPD) (Statens Serum Institut, Copenhagen, Denmark) or ESAT-6 (6-kDa early secreted antigen)/CFP-10 (10-kDa culture filtrate protein) at a final concentration of 10 μg·mL⁻¹. After 24 h incubation (at 37°C and 5% carbon dioxide), supernatants were harvested and analysed by multiplex cytokine array. Samples were analysed using either a custom 13-plex (stimulated blood) or 27-plex Bio-Plex (serum, saliva and sputum) pre-mixed cytokine/chemokine kits according to the manufacturer’s instructions (Bio-Rad, Nazareth-Eke, Belgium). Levels of cytokines in tuberculosis and non-tuberculosis subjects were analysed using the Mann–Whitney U-test. Logistic regression and receiver operating curve analyses were performed, and adjusted for age and sex. Graphs were generated using GraphPad Prism version 6.0 (Software MacKiev, Boston, MA, USA) and statistical analysis with SPSS version 20 (IBM, Armonk, NY, USA). p-values ≤0.035 were considered significant to account for false-discovery rates.

Following overnight whole-blood stimulation, 10-kDa IFN-γ-inducible protein (IP10) and monocyte chemoattractant protein (MCP)-1 levels were high in both groups following all stimulations, while transforming growth factor (TGF)-α, epidermal growth factor and vascular endothelial growth factor (VEGF) levels were low (fig. 1a). IP10, CD40 ligand (CD40L), TGF-α, tumour necrosis factor (TNF)-α and IFN-γ were all significantly higher in subjects with confirmed tuberculosis compared with ORD in unstimulated samples (p=0.0005, p=0.0089, p=0.0020, p=0.0016 and p=0.0313, respectively). Following background subtraction, the main differences were observed in the PPD-stimulated samples, with higher levels of CD40L, IL-10 and TGF-α in tuberculosis compared with non-tuberculosis subjects (p=0.0089, p=0.0034 and p<0.0001, respectively) but lower levels of IFN-γ, interleukin IL)-2 and macrophage inflammatory protein (MIP)-1β (p=0.0313, p=0.040 and p=0.035, respectively). Using logistic regression analysis, the best classification was achieved following PPD stimulation with a combination of CD40L, TGF-α and IL-10, giving 89% correct classification of tuberculosis or ORD. Digested sputum showed surprisingly high levels of cytokines ex vivo compared with both saliva and serum (fig. 1b). Levels of IL-4, IL-5, IL-10, IL-13, IL-7, IL-8, IL-12(p70) and MIP-1β were all significantly higher in sputum compared with both saliva
and serum (illustrated in fig. 1b by IL-7 and IL-8), while IL-1β, IL-17, granulocyte colony-stimulating factor (G-CSF), granulocyte–macrophage colony-stimulating factor, MCP-1 and TNF-α were significantly higher in both saliva and sputum compared with serum (illustrated in fig. 1b by G-CSF and MCP-1). IL-6 was the only cytokine that was lower in saliva compared with both serum (p<0.01) and sputum (p<0.0001), with no
difference between serum and sputum (data not shown), and no difference in IFN-γ levels seen between the three sample types (fig. 1b).

We next compared cytokine levels in *ex vivo* sputum from tuberculosis and non-tuberculosis (ORD) subjects (fig. 1c). Interestingly, we found no difference in pro-inflammatory cytokines (*i.e.* TNF-α, IFN-γ and IP10) but significantly lower levels of IL-10 (*p*=0.004), IL-13 (*p*=0.003) and IL-15 (*p*=0.022) were found in sputum from tuberculosis compared with non-tuberculosis subjects (fig. 1c). Additionally, the innate cytokines IL-1 receptor antagonist, G-CSF and VEGF were all significantly lower (*p*=0.005, *p*=0.004 and *p*=0.030, respectively), while fibroblast growth factor (FGF) was significantly higher in tuberculosis compared with non-tuberculosis subjects (median (interquartile range) 287 (40–764) pg·mL⁻¹ and 2.2 (0–325) pg·mL⁻¹, respectively; *p*=0.007) (fig. 1c). Levels of FGF alone gave 74% correct classification of tuberculosis (sensitivity (95% CI) 78% (56–93%) and specificity 67% (47–83%)). Logistic regression (with age and sex adjustment) revealed a combination of IL-13, FGF and IFN-γ resulted in 96% correct classification of tuberculosis (sensitivity 85% and specificity 96%).

Alongside high sensitivity and specificity, one of the main criteria for development of a lateral-flow based point-of-care test for tuberculosis is time to results [7]. We found levels of host biomarkers in *ex vivo* sample types. This may be due to the level of latent *M. tuberculosis* infection in the non-tuberculosis group (50% were positive by Quantiferon test). Conversely, the Th2 cytokines IL-10 and IL-13 were both significantly lower in tuberculosis compared with non-tuberculosis sputa, indicating a bias towards Th1 responses in subjects with tuberculosis. In a previous study in Brazil, levels of IFN-γ in sputum were shown to equate with treatment response [8] but we did not assess that in the present study. G-CSF is required for neutrophil recruitment and was found to be significantly lower in sputum from tuberculosis compared with non-tuberculosis subjects in our study. This is interesting, as neutrophils are a major component of the protective immune response to tuberculosis [9] and G-CSF administration has been shown to increase response to tuberculosis therapy [10]. While most factors were lower in tuberculosis compared with non-tuberculosis samples, FGF was significantly higher. Interestingly, *M. tuberculosis*-infected fibroblasts lose their capacity for antigen presentation, suggesting that *M. tuberculosis* may evade T-helper immune surveillance by infecting fibroblasts, thereby resulting in bacterial persistence [11].

We only analysed subjects with culture-confirmed tuberculosis and, of these, only three were smear negative (14%), so it is difficult at this stage to determine sensitivity in smear-negative subjects. However, 96% correct classification of tuberculosis using a combination of FGF, IL-13 and IFN-γ from sputum is significantly higher than results reported from current blood-, breath- or urine-based tests [4, 7]. Thus, our findings hold promise for future development of a rapid lateral flow-based diagnostic test for tuberculosis that is applicable for use in resource-limited settings. Tuberculosis elimination will never be reached in developing countries without a synergistic approach including development of better diagnostics that are fast and affordable [2, 7, 12].

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**Sputum host biomarkers provide accurate diagnosis of tuberculosis and may be suitable for a rapid point-of-care test**

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References


