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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 ($\geqslant$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 serum 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 ($600 \times g$ 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 $\mu g \cdot mL^{-1}$. 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 $\leqslant 0.035$ 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 ($p=0.0313$, $p=0.0040$ 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).

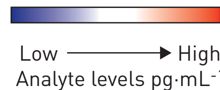
We next compared cytokine levels in *ex vivo* sputum from tuberculosis and non-tuberculosis (ORD) subjects (fig. 1c). Interestingly, we found no difference in pro-inflammatory cytokines (*i.e.* TNF-α, IFN-γ and IP10) but significantly lower levels of IL-10 ($p=0.004$), IL-13 ($p=0.003$) and IL-15 ($p=0.022$) 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) $pg \cdot mL^{-1}$ and 2.2 (0–325) $pg \cdot mL^{-1}$, 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* sputum were significantly higher than levels measured following *in vitro* antigen-stimulated blood cultures, thus reducing time to diagnosis. Sputum is routinely used for microbiological detection of *Mycobacteriun tuberculosis* and is easily obtainable, making it an ideal sample type for development of a lateral flow test for tuberculosis. Interestingly, no difference in Th1 cytokine levels were observed between tuberculosis and non-tuberculosis subjects using *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].

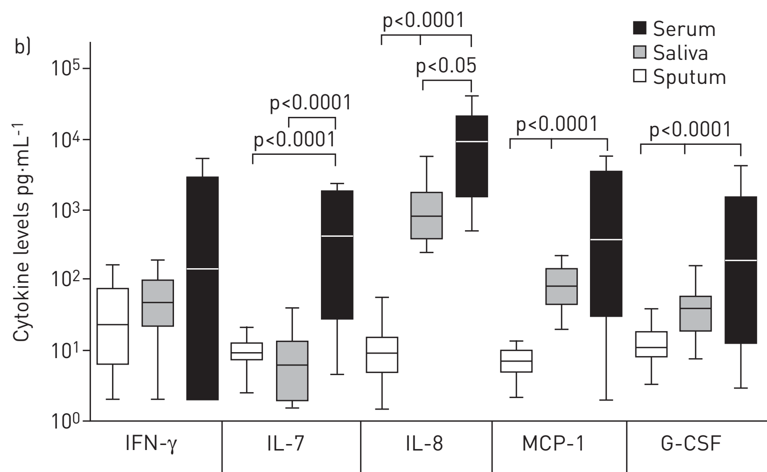
We only analysed subjects with culture-confirmed tuberculosis and, of these, only three were smear negative (14%), so it is difficult at this stage to determine sensitivity in smear-negative subjects. However, 96% correct classification of tuberculosis using a combination of FGF, IL-13 and IFN-γ from sputum is significantly higher than results reported from current blood-, breath- or urine-based tests [4, 7]. Thus, our

a)

	Control			ESAT-6/CFP-10			PPD		
	TB	ORD	p-value	TB	ORD	p-value	TB	ORD	p-value
CD40L	2949	1728	0.089	3171	1869	0.0063	3647	1946	0.0089
EGF	50	46	NS	57	39	NS	54	34	NS
MIP-1β	136	95	NS	3463	4740	NS	2566	4251	0.0351
TGF-α	6	3	0.0020	23	4	<0.0001	30	10	<0.0001
VEGF	81	3	NS	34	13	NS	76	3	NS
IFN-γ	6	3	0.0313	204	120	NS	443	600	0.0313
IL-10	3	3	NS	34	59	NS	314	90	0.0034
IL-2	3	3	NS	21	25	NS	205	514	0.0040
IP10	4353	1127	0.0005	10 000	10 000	NS	10 000	10 000	NS
MCP-1	1065	1221	NS	7838	9908	NS	9378	10 000	NS
TNF-α	6	3	0.016	163	133	NS	103	116	NS



b)



c)

	IL-1β	IL-1ra	IL-4	IL-6	IL-7	IL-8	IL-10	IL-12p70	IL-13	FGF	G-CSF	IFN-γ	IP10	MCP-1	MIP-1β	PDGF	TNF-α	VEGF
TB	2499	3458	39	41	423	9288	7	268	25	287	189	144	5064	373	702	44	11	11
ORD	1577	11092	7	98	357	14629	210	802	155	2	1805	659	9164	1186	473	150	64	64
p-value	NS	0.0005	NS	NS	NS	NS	0.0004	NS	0.0003	0.0071	0.0041	NS	NS	NS	NS	NS	NS	NS

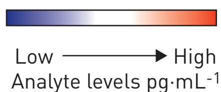


FIGURE 1 a) Heat map of cytokine profiles following overnight stimulation of whole blood with tuberculosis antigens. Median values are presented. b) Comparison of cytokine levels in serum, saliva and sputum from tuberculosis patients (n=25, n=20 and n=23, respectively). Note that values shown are not adjusted for dilution of the sputum cytokines during digestion (four-fold). Boxes indicate interquartile ranges, horizontal lines indicate medians and whiskers indicate 5–95% range. Data were anlysed using the Kruskal–Wallis test followed by Dunn's multiple comparison. p-values ⩽0.035 were considered significant and are indicated. c) Heat map of cytokine levels in *ex vivo* sputum. Median values are presented for subjects with tuberculosis (n=23) and those with other respiratory disorders (ORD) (n=29). Data were anlysed using a Mann–Whitney U-test. p-values ⩽0.035 were considered significant and are indicated. Control: no antigen; ESAT-6: 6-kDa early secreted antigen; CFP-10: 10-kDa culture filtrate protein; PPD: purified protein derivative; TB: culture-confirmed tuberculosis; CD40L: CD40 ligand; EGF: epidermal growth factor; MIP: macrophage inflammatory protein; TGF: transforming growth factor; VEGF: vascular endothelial growth factor; IFN: interferon; IL: interleukin; IP10: 10-kDa IFN-γ-inducible protein; MCP: monocyte chemoattractant protein; TNF: tumour necrosis factor; NS: nonsignificant; G-CSF: granulocyte colony-stimulating factor; IL-1Ra: IL-1 receptor antagonist; FGF: fibroblast growth factor; PDGF: platelet-derived growth factor.

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 http://ow.ly/tsx2S

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Serial testing using interferon-γ release assays in nursing students in India

To the Editor:

We have previously shown that Indian healthcare workers have higher prevalence of latent tuberculosis infection (LTBI) and are at increased risk for new infection [1–4]. Interferon-γ release assays (IGRAs) have been introduced as an alternative to the tuberculin skin test (TST) for diagnosing LTBI in healthcare workers and other high-risk groups. They have logistical advantages over the TST and will not cross-react with the bacille Calmette Guérin vaccine. IGRAs are now being widely used for screening healthcare workers [5], yet recent reports indicate that switching from TST to IGRAs for the serial testing of healthcare workers may result in increased rates of test conversions and reversions [3, 6–8]. Most of these studies are from low tuberculosis (TB) incidence settings, with limited opportunity for nosocomial TB exposure; as a result, the increased conversion rates are considered false-positive test conversions, making it difficult for clinicians to