Differentially culturable tubercule bacilli are generated during nonpulmonary tuberculosis infection

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*Mycobacterium tuberculosis* (*Mtb*) is a major global cause of death from infectious disease (1). Rarely contagious, extra-pulmonary tuberculosis (EPTB) is neglected in TB control strategies. Nonetheless, EPTB contributes significantly to the burden of disease, is frequently difficult to diagnose and inflicts significant morbidity (2).

Following the establishment of infection in the host, it is thought heterogeneous populations develop, distinguishable by specific growth requirements (3-5), metabolic features (6, 7), and differential susceptibility to antimicrobial agents (3, 8). The application of limiting dilution method to enumerate *Mtb* in liquid media has previously revealed that human TB sputum was dominated by *Mtb* populations which could not be detected by standard methods but required addition of culture supernatant (CS) obtained from actively growing *Mtb* or recombinant Rpf (3, 4). The importance of Rpf in recovery of these bacteria was further supported by control experiments with Rpf-depleted or Rpf-inactivated culture supernatants (3, 9). However, a recently published study demonstrated the higher complexity of mycobacterial populations in sputum including bacilli growing only in 7H9 supplemented with CS or Rpf-deficient CS, those growing in 7H9 medium only (non-plateable) and growing on standard 7H10 agar plates (plateable) *Mtb* bacilli, collectively designated as Differentially Culturable Tubercule Bacteria (DCTB) (4, 5). Furthermore, it was proposed that relative proportions of these populations depended on patient CD4+ T-cell counts (4) and it is unknown whether other host factors may hold influence. DCTB can be generated *in vitro* by application of combined stresses (10) or treatment with antimicrobial agents (11).
There is accumulating experimental evidence to suggest the presence of DCTB has ramifications for clinical and treatment outcomes. CS-and Rpf-dependent *Mtb* bacilli were apparently enriched in treated patents and showed high tolerance to killing by antimicrobials including rifampicin (3), isoniazid and streptomycin (8). Furthermore, the eradication of CS-dependent *Mtb* prevented relapse in a Cornell mouse model (12). Finally, the application of recombinant Rpf or Rpf-containing CS improved time-to-positivity in a substantial number of sputum samples and often enhanced bacterial recovery (4, 13). While three-independent studies demonstrated the presence of Rpf-or CS-dependent bacilli in sputum samples (3, 4, 13), it remains unclear whether similar bacilli are commonly generated in EPTB. An understanding of DCTB populations in EPTB has important implications for diagnosis and treatment. Therefore, we examined a broad range of extra-pulmonary samples to answer: 1. Are CS-dependent bacilli present in EPTB? 2. Can we identify host factors which may modulate CS-dependent populations?

We used previously developed methodology and assessed the number of *Mtb* bacilli recoverable on agar plates (CFU), in liquid 7H9 medium (MPN_7H9) or in 7H9 medium supplemented with CS (MPN_CS) (3). The study was approved by the National Research Ethics Service Committee East Midlands Leicester (07/Q2501/58).

41 patients were recruited before the onset of chemotherapy, 18 were culture positive for *Mtb*, one patient having two separate positive samples (see Figure 1). Most patients were from Indian subcontinent backgrounds (83%) and the remainder Black African (17%); 67% were male; 17% were infected with Human Immunodeficiency Virus (HIV); 82% were vitamin D deficient (cut off <30nmol/L). Samples were obtained from
varying anatomical locations including colonic tissue, vertebral bone, abscess pus and lymph nodes and Ziehl-Neelsen stained within 24 hours after collection. No bacilli were detected using this protocol in any samples before cultivation, which fits with the typically paucibacillary nature of EPTB disease (14).

We detected substantial differences in DCTB counts and relative DCTB proportions with bacillary counts ranging over $\log_{10} 0.69-6.7$ for MPN_CS, over $\log_{10} 0.69-4.5$ for MPN_7H9 and over $\log_{10} 1-3.9$ for CFU counts (Fig.1). In most samples (11/19) the MPN_7H9 counts were lower than MPN_CS and CFU counts. In 52% (10/19) samples CS-dependent *Mtb* were dominating populations, including 3 samples which produced *Mtb* cultures only with CS-supplementation. Interestingly in 6 out of 19 samples (32%) there was no significant difference between CFU and MPN_CS counts (taking in consideration limit of confidence) and in two samples plateable *Mtb* exceeded those grown in liquid media.

The isolated *Mtb* strains were acid fast positive and diverse with 8 (42%) of isolates belonging to the Delhi/CAS lineage, 4 (21%) East African-Indian, 2 (11%) Haarlem, 1 (5%) Beijing, 1 Turkish, 1 S-type and 1 H37Rv-like as determined by MIRU-VNTR typing (15). We found no relationship between MPN_CS counts and strain lineage ($p=0.45$; Kruskal-Wallis test). Examination of host factors elucidated a significant correlation ($p=0.04$) between the host peripheral lymphocyte counts (PLC) and MPN_CS bacillary counts. However, additional experiments are required since multivariate regression analysis to adjust for confounding was precluded by the sample size. No other host parameters including HIV, diabetes mellitus or vitamin D status were significantly associated with DCTB. Furthermore, there was also no correlation between
MPN_CS counts and C-reactive protein levels, neutrophil or monocytes counts or the monocyte: lymphocyte ratio. All patients responded appropriately to anti-tuberculous therapy and by the end of June 2017, 83% of patients were treatment complete according to WHO definitions (16), while treatment outcome for 17% of patients could not be evaluated since they returned to home countries.

CS-dependent DCTB were isolated from a wide range of anatomically and physiologically distinct sites (Fig. 1) indicating that CS-dependent and Rpf-dependent bacilli are not exclusive to sputum. Rather, their formation is an important manifestation of Mtb and host interactions as previously demonstrated in a mouse model (17). Whether DCTB population distributions do differ between pulmonary and extrapulmonary disease is unknown. We identified CS-dependent DCTB populations in only 52% of EPTB samples, considerably less than detected in sputum studies (3, 4) although the absence of a comparator group prevents clarification whether a true difference exists.

The significant positive correlation demonstrated between the PLC and MPN_CS bacillary counts supports the contention that the cellular host immune response is a major factor determining population proportions of CS-dependent bacilli (4, 17). Differences in the effectiveness of individual host immune responses and consequently mycobacterial stress may explain the interhost differences in CS-dependent DCTB populations. We examined certain host factors such as vitamin D deficiency and diabetes mellitus but they did not influence CS-dependency in our study. Whilst this pilot study was underpowered for multivariate analysis, these factors may impact DCTB populations and should be examined in future studies. At this stage, we also cannot
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Figure legend

Figure 1. Mycobacterial subpopulations present in extrapulmonary tuberculosis samples. The error bars indicate the 95% confidence intervals for the MPN values. Due to small sample volumes and the requirement for invasive sampling, only one CFU assay technical replicate per sample was possible. Volumes of liquid samples were in range of 1-10 ml. The LN sample sites (from left to right) are left axillary LN; left axillary LN; intrathoracic lymph node (ILN) obtained via mediastinoscopy; ILN station 7; ILN station 4R; ILN station 4R. The MPN assay limit of detection was 5 cells /ml. ¶ The CFU assay limit was 10 CFU/ml. * Samples with dominating CS-dependent populations. † Two samples obtained from the same patient separated by 10 days. Checked bars – samples recovered only with CS supplementation. Abbreviations: Bx – biopsy; LN – lymph node; MPN_CS – MPN with supplementary culture supernatant; MPN_7H9- MPN with standard 7H9 media.
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For Review Only

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**References**


TABLE 1. THE CORRELATION OF HOST FACTORS TO THE MPN_CS BACTERIAL COUNTS

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<th>P-value</th>
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<td>Age (year) 35 (29-47)</td>
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<tr>
<td>Weight (kg) 64.5 (60-83)</td>
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<td>Hemoglobin (g/dl) 11.9 (11.2-13.2)</td>
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<td>Neutrophils (x10^9 cells/L) 4.4 (3.4-5.9)</td>
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<tr>
<td>Lymphocytes (x10^9 cells/L) 1.5 (1.0-2.0)</td>
<td><strong>0.04</strong></td>
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<tr>
<td>Monocytes (x10^9 cells/L) 0.5 (0.4-0.6)</td>
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</tr>
<tr>
<td>Monocyte: Lymphocyte ratio 0.35 (0.28-0.45)</td>
<td>0.11</td>
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<tr>
<td>Creatinine (µmol/l) 58 (51-62)</td>
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<tr>
<td>C-reactive protein (mg/L) 87 (29-118)</td>
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<tr>
<td>25-OH Vitamin D₃ (nmol/L) 14 (14-14)</td>
<td>0.83</td>
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Sociodemographic and clinical data acquisition was concurrent with microbiological sampling. Median value for MPN_CS log_{10} was 2.9 (2.5-3.6). * Interquartile range indicated in brackets. Missing data: a=4; b=1.
**Figure legend**

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