RVC OPEN ACCESS REPOSITORY – COPYRIGHT NOTICE

This is a pre-copyedited, author-produced version of an article accepted for publication in *Clinical Infectious Diseases* following peer review.

The version of record is available online at https://doi.org/10.1093/cid/ciz548.

TITLE: A novel high sensitivity bacteriophage-based assay identifies low level M. tuberculosis bacteraemia in immunocompetent patients with active and incipient TB

AUTHORS: Raman Verma, Benjamin M C Swift, Wade Handley-Hartill, Joanne K Lee, Gerrit Woltmann, Catherine E D Rees, Pranabashis Haldar

JOURNAL TITLE: Clinical Infectious Diseases

PUBLICATION DATE: 22 June 2019 (online)

PUBLISHER: Oxford University Press

DOI: https://doi.org/10.1093/cid/ciz548

RVC Royal Veterinary College University of London

FULL TITLE:

A novel high sensitivity bacteriophage-based assay identifies low level *M. tuberculosis* bacteraemia in immunocompetent patients with active and incipient TB.

Authors:

Raman, Verma. Department of Respiratory Sciences, NIHR Respiratory Biomedical Research Centre, University of Leicester, Leicester, United Kingdom.

Benjamin, MC, Swift. Royal Veterinary College, Hawkshead Campus, Herts, United Kingdom.

Wade, Handley-Hartill. School of Biosciences, University of Nottingham, Sutton Bonington Campus, Leicestershire, United Kingdom.

Joanne, K, Lee. Department of Respiratory Sciences, NIHR Respiratory Biomedical Research Centre, University of Leicester, Leicester, United Kingdom.

Gerrit, Woltmann. Department of Respiratory Medicine, University Hospitals of Leicester NHS Trust, Glenfield Hospital, Leicester, United Kingdom

Catherine, ED, Rees. School of Biosciences, University of Nottingham, Sutton Bonington Campus, Leicestershire, United Kingdom.

Pranabashis, Haldar. Department of Respiratory Sciences, NIHR Respiratory Biomedical Research Centre, University of Leicester, Leicester, United Kingdom.

Keywords: bacteriophage, bacteraemia, human tuberculosis, blood

Running Title: Bacteriophage assay detects TB bacteraemia.

Corresponding author: RV (e-mail: rv55@leicester.ac.uk, Address: NIHR Respiratory Biomedical Research Centre, Glenfield Hospital, Groby Road, Leicester, LE3 9QP, Telephone: 0116 258 3208, Fax: 0116 258 2787).

Alternate corresponding author: PH (e-mail: ph62@leicester.ac.uk, Address, Telephone and Fax: as for RV).

ABSTRACT:

Haematogenous dissemination of *M. tuberculosis* (Mtb) is critical to pathogenesis of progressive tuberculous infection in animal models. Using a novel phage-based blood assay, we report the first concordant evidence in well-characterised immunocompetent human cohorts, demonstrating associations of Mtb bacteraemia with progressive phenotypes of latent infection and active pulmonary TB respectively.

Introduction:

Tuberculosis (TB) is the leading cause of death from an infectious disease [1]. The causative organism, *M. tuberculosis* (Mtb) persists within populations by establishing asymptomatic latent infection (LTBI); a reservoir for future disease. It is estimated that 5-10% of the LTBI population will develop TB prospectively, usually within a 2-year period of acquiring infection [2]. The patho-biological mechanisms underpinning progression and severity of active TB are poorly understood, however animal studies report early haematogenous Mtb dissemination to be an important factor [3].

We previously reported a method to detect mycobacteria using phage combined with DNA amplification (phage-Recombinase Polymerase Amplification) which exploits the efficiency of bacteriophage lysis of mycobacterial cells to relsease DNA for subsequent detection by Nucleic acid amplification tests (NAAT) [4]. Using this method, we found evidence of low-grade *M. bovis* bacteraemia ($<10^2$ cells per ml) in the blood of infected cattle, both with and without manifestations of overt disease.

Here, we report outcomes of a proof-of-concept study applying an adaptation of this method (Actiphage™) to detect human Mtb infection in well-characterised clinical cohorts. Our primary objective was to determine whether Mtb can be detected in the blood of immunocompetent patients with active pulmonary TB (PTB) and recent contacts with LTBI. A secondary objective was to determine the early diagnostic potential of Actiphage™ in PTB.

Methods:

The study prospectively recruited HIV sero-negative adult patients (aged ≥18 years) into 4 groups (Table 1): i. Active PTB – positive Xpert-Ultra (Cepheid Inc) or Mtb culture from respiratory tract samples with supporting clinical and radiological disease characteristics; ii. LTBI – asymptomatic recent PTB contacts identified at contact tracing, with a positive QuantiFERON-TB Gold Plus (QFT; Qiagen Inc) and normal chest X-ray (CXR); iii. Non-TB illness control group – referred with suspected PTB but diagnosed with a non-TB illness and concomitant microbiological exclusion of Mtb; and iv. Healthy control group – asymptomatic and QFT negative with no history of previous TB contact.

All participants provided blood samples for Actiphage™ testing on recruitment and received 12-month prospective clinical follow-up. PTB patients were sampled before commencing anti-tuberculous treatment. In addition, the LTBI group had QFT and Actiphage™ testing repeated after 8-12 weeks to capture IGRA seroconversion events. Participants were retained if they were QFT-positive at the second time-point and declined anti-tuberculous chemoprophylaxis. Clinical and laboratory teams were blinded to the outcome of Actiphage™ testing and the study groups from which samples originated, respectively, until the end of the study. Ethics approval was provided by the regional Research and Ethics Committee (REC 15/EM/0109) and all participants provided written informed consent at enrolment.

For the Actiphage™ test (PBD Biotech Ltd), blood (5 ml) was collected into sodium heparin tubes (Sarstedt) and stored at room temperature until processing. PBMCs were isolated from

2 ml aliquots by: i. Ficoll-Paque Plus (GE Healthcare) using leucosep tubes (Sigma) and ii. Hetasep (Stem Cell Technologies) according to the manufacturer's instructions. PBMCs from 1 ml of blood were resuspended in 200 µl of Actiphage™ media and samples then transferred to Actiphage™ Rapid Tubes and bacteriophage D29 added (20 µl; ~10^7 pfu) [5]. The samples were incubated for 3.5 h at 37°C, centrifuged (13,000 x g; 3 min, room temperature) and the flow-through containing the released mycobacterial DNA was further concentrated (Zymo DNA Clean and Concentrator-5) and Mtb DNA detected by PCR amplification of IS*6110* [6].

Results:

Sixty-six participants were recruited (Table 1). Of the 15 participants with PTB, one had evidence of miliary disease, with a single cerebral tuberculoma. For the remainder, there were no radiological or clinical features of multi-organ involvement. Of the 18 LTBI participants, one had QFT seroconversion with the rest persistently QFT positive. All had normal CXR reported by a thoracic radiologist. All five participants of the control group with non-TB respiratory illness had PTB excluded with bronchoscopy and were treated effectively with antibiotics for community acquired pneumonia.

Eleven of the 15 PTB cohort (73%), including the miliary disease case, and 3 of 18 LTBI contacts gave a positive Actiphage™ test (Table 1). The remaining participants with LTBI and all participants of both control groups gave a negative Actiphage™ test result. The quantitative QFT values and clinical/ CXR characteristics were no different at baseline between Actiphage™-positive and Actiphage™-negative LTBI participants.

In the PTB cohort, Actiphage™-positive results were associated with sputum smear positivity, higher baseline CRP and shorter times to mycobacterial culture (Table 1). In the LTBI group, Actiphage™ results were concordant at both time-points and of the three Actiphage™-positive LTBI participants, two developed active, culture-positive PTB after 7 months. Whole genome sequence analysis of the Mtb isolates from these cases confirmed origin from their respective

index cases (data not shown). The third Actiphage™-positive LTBI contact had QFT seroconversion but did not develop TB. After 12-months follow up, no Actiphage™-negative participants had developed TB.

As a clinical diagnostic in symptomatic patients with suspected PTB, Actiphage™ testing had a sensitivity and specificity (95 % CI) of 73.3 % (48.1–89.1) and 100 % (56.6–100), respectively. When applied to the whole cohort at baseline, specificity (95% CI) for detecting PTB was 94.2% (84.1–98.4) with no change in sensitivity.

Discussion:

This study used phage-based DNA extraction (Actiphage™) combined with PCR to detect low levels of viable Mtb in blood. We demonstrate, for the first time, detection of Mtb in the blood of a majority of immunocompetent patients with active PTB and in addition, a proportion of recent TB contacts with LTBI that associated with progression to active TB. Our observations provide novel pathological insights of human Mtb infection and support further development of Actiphage™ as a blood-based TB diagnostic.

Recent evidence supports a transitional state of human Mtb infection, termed “incipient TB” – characterised by clinical latency but associated with increased risk of active TB progression and a host blood transcriptional profile that overlaps with disease [7, 8]. Mechanistic studies of early Mtb infection in animal and zebrafish models propose the importance of haematogenous seeding in new granuloma formation associated with disease progression [3, 9, 10]. This study presents the first concordant evidence in humans to support these observations. We identified 3 recent PTB contacts with detectable Mtb in blood that persisted over 3 months and preceded QFT seroconversion in one participant, supporting the view that following inhalation, translocation of Mtb to blood is governed by innate immunity. Two participants from this subgroup progressed to TB after 7 months, with neither showing clinical or radiological evidence of active TB at their interim 3-month follow-up. Our data suggests that

incipient disease associates with, and may be identified by, detecting Mtb in blood during early infection. Although our numbers were small, the proportion with detectable Mtb in blood (16%) is comparable to the proportion we have previously reported with a TB-like host transcriptional profile in blood [8]. This association, together with a detailed characterisation of TB progression risk, requires further study.

The evidence for Mtb bacteraemia during active PTB is unclear. Previous studies applying culture and NAAT to blood samples from patients with active TB have been disappointing [11], with one study reporting Mtb detectable in the blood of only 21% of HIV positive patients with severe miliary disease using Xpert MTB/RIF [12]. The relative inaccessibility of intracellular Mtb DNA within circulating PBMCs probably contributes to the poor sensitivity of existing methods. Our approach overcomes this by using efficient phage-mediated lysis of Mtb released from PBMCs to then allow sensitive detection by NAAT. Our results detected circulating Mtb in the majority of active PTB participants with clinical and radiological features of single organ disease, suggesting a low grade bacteraemia, below the sensitivity threshold of existing techniques. Bacteraemia was associated with greater disease extent, characterised by higher bacillary burden, pulmonary cavitation and elevated levels of systemic inflammation. In contrast, failure to detect Mtb in the blood of four PTB participants with clinically milder disease expression suggests a distinct phenotype of earlier disease characterised by effective immune-mediated retention of Mtb in the lung, with any circulating bacteria present at levels below the sensitivity threshold of the assay. Together, our data indicates the pathogenesis of PTB to associate with a detectable spike in haematogenous Mtb dissemination during two phases of Mtb infection – the first during early infection before control has been established, reflecting a failure of innate immunity, and later during disease progression, when bacillary replication overcomes adaptive immunity. The additional prognostic value of Actiphage^{TM} in the phenotypic characterisation and clinical outcome of Mtb infection requires further study, including developing methods for quantifying the bacterial load.

Although preliminary, this study demonstrates early promise with Actiphage™ as a blood-based diagnostic tool for infectious PTB to improve earlier diagnosis in patients unable to expectorate sputum, and requires investigation in other cohorts with an unmet need including extra-pulmonary, paediatric and miliary TB. Developments in the assay to simplify sample preparation and molecular detection are required to enhance accessibility for low-resource settings; but the advantages of a rapid, low-cost assay that offers a microbiological diagnosis in the absence of sputum, are clear.

Financial Support:

WH-H received a Doctoral Training Partnership studentship from the Biotechnology and Biological Sciences Research Council. Reagents were supplied by PBD Biotech Ltd (Suffolk, UK).

Conflict of Interests:

The authors declare that the Actiphage™ technology described in this paper is currently in the process of being patented (PCT/GB2014/052970). CEDR and BMCS are directors of, and hold stock in, PBD Biotech Ltd.

Acknowledgements:

We thank all the patients for sample contribution. We acknowledge the NIHR Leicester Biomedical Research Centre for their support of the study at Leicester. The views expressed are those of the author(s) and not necessarily those of the NHS, NIHR or the Department of Health.

References:

1. Organisation WH. Global Tuberculosis Report. **2018**.
2. Behr MA, Edelstein PH, Ramakrishnan L. Revisiting the timetable of tuberculosis. BMJ **2018**; 362: k2738.
3. Davis JM, Ramakrishnan L. The role of the granuloma in expansion and dissemination of early tuberculous infection. Cell **2009**; 136(1): 37-49.
4. Swift BM, Convery TW, Rees CE. Evidence of Mycobacterium tuberculosis complex bacteraemia in intradermal skin test positive cattle detected using phage-RPA. Virulence **2016**; 7(7): 779-88.
5. Froman S, Will DW, Bogen E. Bacteriophage active against virulent Mycobacterium tuberculosis. I. Isolation and activity. Am J Public Health Nations Health **1954**; 44(10): 1326-33.
6. Hellyer TJ, DesJardin LE, Hehman GL, Cave MD, Eisenach KD. Quantitative Analysis of mRNA as a Marker for Viability of *Mycobacterium tuberculosis*. J Clin Mircobiol **1999**; 37(2): 290-5
7. Cobelens F, Kik S, Esmail H, Cirillo DM, Lienhardt C, Matteelli A. From latent to patent: rethinking prediction of tuberculosis. The Lancet Respiratory Medicine **2017**; 5(4): 243-4.
8. Singhania A, Verma R, Graham CM, et al. A modular transcriptional signature identifies phenotypic heterogeneity of human tuberculosis infection. Nat Commun **2018**; 9(1): 2308.
9. Cadena AM, Flynn JL, Fortune SM. The Importance of First Impressions: Early Events in Mycobacterium tuberculosis Infection Influence Outcome. MBio **2016**; 7(2): e00342-16.
10. Polena H, Boudou F, Tilleul S, et al. Mycobacterium tuberculosis exploits the formation of new blood vessels for its dissemination. Sci Rep **2016**; 6: 33162.

11. Shenai S, Amisano D, Ronacher K, et al. Exploring alternative biomaterials for diagnosis of pulmonary tuberculosis in HIV-negative patients by use of the GeneXpert MTB/RIF assay. J Clin Microbiol **2013**; 51(12): 4161-6.

12. Feasey NA, Banada PP, Howson W, et al. Evaluation of Xpert MTB/RIF for detection of tuberculosis from blood samples of HIV-infected adults confirms Mycobacterium tuberculosis bacteremia as an indicator of poor prognosis. J Clin Microbiol **2013**; 51(7): 2311-6.

		Active Pulmonary TB (N=15)		Non-TB Acute Respiratory Illness (N=5)	Pulmonary TB Contacts With LTBI (N=18)		Healthy Controls: No LTBI (N=28)
Actiphage™ Result		Positive (n=11)	Negative (n=4)	All Negative	Positive (n=3)	Negative (n=15)	All Negative
Male Gender (%)		5 (45.5)	2 (50)	2 (40)	1 (33.3)	10 (55.6)	11 (39.3)
Age (years; mean ±SD)		31.5 (±13.9)	38.8 (±13.5)	50 (±21.7)	25.3 (±6.4)	54.7 (±12.3)	38.9 (±14.6)
UK Born (%)		3 (27.2)	1 (25)	2 (40)	1 (33.3)	5 (33.3)	10 (35.7)
BCG Vaccination	Yes (%)[$]	4 (36.4)	2 (50)	2 (40)	2 (66.7)	7 (63.6)	12 (50)
	Unknown (%)	0	0	0	0	4 (26.7)	4 (14.3)
BMI (kg/m^2; mean ±SD)		19.9 (±3.6)	20.9 (±3.0)	25.7 (±5.3)	21.9 (±2.0)	26.2 (±6.9)	27.1 (±8.2)
TB Disease Characteristics	Smear Positive	7	0	0	0	N/A	N/A
	Smear Negative	4	4	0	2	N/A	N/A
	Xpert-Ultra Grade	Medium - High	Very Low - Low	All Negative	Medium*	N/A	N/A
	CRP (median, IQR)	63 (36 to 65)	41 (27 to 45.5)	84 (45 to 110)	5 (5 to 5)[^]	10 (5 to 13.75)	5 (5 to 10)
	Days to Positive Culture (median, IQR)	15 (10.5 to 22)	21 (21 to 21)	1 blood culture (*S. aureus*) 1 sputum culture (*M. avium*, 6 days)	26 (23.5 to 28.5)*	N/A	N/A

Table 1. Demographic and clinical characteristics of all subjects in this study. (BCG, Bacillus Calmette-Guérin; TB, Tuberculosis; BMI, Body Mass Index; CRP, C-Reactive Protein; LTBI, Latent TB Infection; *S. aureus*, Staphylococcus aureus; *M. avium*, Mycobacterium avium). * Data presented are at the time of presentation with TB in two contacts, [^] CRP values refer to data collected at baseline, consistent with the data for the other groups, [$] Percentages calculated from the subgroup for which BCG status was known.