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Urine lipoarabinomannan as a marker for low-risk of NTM infection in the CF airway

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ABSTRACT

Background: Individuals with Cystic fibrosis (CF) are the most vulnerable population for pulmonary infection with nontuberculous mycobacteria (NTM). Screening, diagnosis, and assessment of treatment response currently depend on traditional culture techniques, but sputum analysis for NTM in CF is challenging, and associated with a low sensitivity. The cell wall lipoarabinomannan (LAM), a lipoglycan found in all mycobacterial species, and has been validated as a biomarker in urine for active *Mycobacterium tuberculosis* infection.

Methods: Urine from a CF cohort ($n = 44$) well-characterized for NTM infection status by airway cultures was analyzed for LAM by gas chromatography/mass spectrometry. All subjects with positive sputum cultures for NTM had varying amounts of LAM in their urine. No LAM was detected in subjects who never had a positive culture (14/45). One individual initially classified as NTM sputum negative subsequently developed NTM disease 657 days after the initial urine LAM testing. Repeat urine LAM testing turned positive, correlating to her positive NTM status. Subjects infected with subspecies of *M. abscessus* had greater LAM quantities than those infected with *M. avium* complex (MAC). There was no correlation with disease activity or treatment status and LAM quantity. A TB Capture ELISA using anti-LAM antibodies demonstrated very poor sensitivity in identifying individuals with positive NTM sputum cultures.

Conclusion: These findings support the conclusion that urine LAM related to NTM infection may be a useful screening test to determine patients at low risk for having a positive NTM sputum culture, as part of a lifetime screening strategy in the CF population.

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1. Introduction

Nontuberculous mycobacteria (NTM) are environmental organisms found in soil and water worldwide that can cause chronic lung infection, usually in the context of lung disease or immunodeficiency. Cystic fibrosis (CF) has been identified as the disease population at greatest risk for CF airway infection [1]. Within the CF population, the vast majority of infections occur as a result of either species within the *M. avium* complex (MAC) or subspecies of *M. abscessus* (MABC). In the largest longitudinal survey to date, 20% of people with CF who had NTM cultures obtained over a 5-year interval had a positive culture for NTM [2]. Among people with CF, detection of NTM in the sputum is of uncertain significance, as often the infection is cleared without treatment, or remains indolent for years [3].

In all aspects of the disease, the lack of sensitive and specific markers of NTM in the airway is a significant barrier to patient care. Currently, culture from the airway is the only method utilized for screening, and the gold standard by which all diagnosis and treatment decisions are made [4]. Limitations of culture often include slow growth (up to 8 weeks), high cost, low sensitivity due

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to required decontamination procedures, and difficulty in obtaining samples in children and non-sputum producers. In addition, a given sputum sample may not reflect the often heterogeneous and compartmentalized nature of NTM in the individual patient, and a positive sputum culture of NTM can lag development of clinical symptoms by up to a year [3]. As a result of the improvements to CF therapy over last two decades and with the growing use of highly effective CFTR modulator therapy, fewer pediatric and even adult patients with CF are able to routinely expectorate sputum. Currently, CFF/ECFS Guidelines recommend annual screening for NTM by sputum [5] but given the limitations outlined above, only 20% of individuals in the CF Patient Registry met this benchmark over a 5-year period, and 21% had no NTM cultures reported over the same interval [2].

Lipoarabinomannan (LAM) is a cell wall lipoglycan found in all mycobacteria species, which is released from metabolically active or degrading cells in the circulation and found in the urine of infected patients. LAM has recently gained attention as a biomarker for active tuberculosis when antigen detection rather than antibody measurement is applied [6-11]. In the setting of tuberculosis, clinical evaluation of LAM by immunological assays are very promising with high specificity and sensitivity. There have been arguments that there was a compromise with specificity for *M. tuberculosis* due to a possibility of infection with NTM [12] and it has not been clearly shown if clinical samples of NTM contain detectable amounts of LAM. Previously, urine LAM was reported to have high specificity (91–99%), but low sensitivity (9–39%,) for pulmonary NTM in the Danish CF population [13].

Whereas approximately 80% of the CF population will not culture NTM over a 5-year interval, we hypothesized that urine LAM would be a useful, non-invasive test to screen for individuals with low risk of having a positive culture. Using a cross-sectional design of well-characterized patients from the Colorado CF Center, we tested the utility of urine LAM over all clinical situations, ranging from fulminant pulmonary disease with both MAC and MABSC to individuals verified culture negative throughout their lifetime. We simultaneously ran two LAM based assays, one assay was an antibody-based immunoassay (TB capture ELISA) and the other was a chemometric assay, both developed in our laboratory for urinary LAM validation in *M. tuberculosis* diagnostics. The latter method involved use of GC/MS and is impartial to use of antibodies ([8, 12]). Herein, we report urine LAM predicted the absence of previous positive NTM sputum cultures in all subjects tested and is a promising marker as part of a two-step screening strategy for the CF population.

2. Materials and methods

2.1. Subjects with CF used to test urine LAM

The study protocol was approved by the Colorado Multiple Institutional Review Board (COMIRB99–113) and the National Jewish Health Institutional Review Board (HS-1234). Some subjects were co-enrolled on the PREDICT (**PR**ospective **E**valuation of NTM **D**isease **I**n **C**ys**T**ic Fibrosis, NCT02073409) and PATIENCE (**P**rospective **A**lgorithm for **T**reatment of **N**TM in **C**ystic Fibrosis, NCT02419989) Trials. All subjects gave written informed consent. The study was conducted in accordance with the Declaration of Helsinki. All subjects were followed by the Colorado CF Center for a minimum of 5 years, with results of at least 5 previous NTM sputum cultures available at time of enrollment.

NTM sputum cultures were obtained at the same clinic visit or hospitalization as the urine sample collection. NTM cultures were tested by the Microbiology Laboratory at Children's Hospital Colorado (CHCO) or Advanced Diagnostic Laboratory at NJH. Specimens were digested/decontaminated using a standard NaOH–NALC procedure, and the sediment used to prepare an auramine smear and inoculate various growth medium including: Lowenstein-Jensen Slant, Middlebrook 7H11 Agar/ Mitchison 7H11 Selective Agar biplate(s) and MGIT (Mycobacteria Growth Indicator Tube) incubated for up to six weeks. Positive AFB culture growth was assessed from solid growth media at 1, 3, and 6 weeks. MGIT specimens were continuously monitored. Upon growth detection genomic DNA was isolated for subsequent PCR amplification at NJH. A targeted segment of the DNA-directed RNA polymerase subunit beta (*rpoB*) gene was performed and a consensus sequence determined, constructed and blasted against a known database to determine molecular identification.

Patients were enrolled either at baseline health or during hospitalization for an acute pulmonary exacerbation. Collection sites include the Colorado Adult Inpatient Service (St. Joseph Hospital), Adult CF Clinic (NJH), Adult Clinical Research Unit (NJH), Pediatric Clinic or Inpatient Service (CHCO). Sample collection occurred between July 2017 and March 2019. Urine samples were collected in sterile containers and frozen within one hour at −20°C and then transferred to an −80 °C freezer. De-identified and NTM status-blinded samples were shipped to Colorado State University (CSU) over dry ice. Frozen samples were thawed to ambient temperature before assays.

2.2. D-Arabinose (D-ara) and tuberculostearic acid (TBSA) analysis by GC/MS

All urine samples were subjected to hydrophobic interaction chromatography (HIC) over Octyl Sepharose (OS)-CL 4B The 40% and 65% n-propanol in 0.1 M NH_4OAc eluents off the HIC column was processed for GC/MS analysis downstream. For D-arabinose (D-ara) estimation, acid hydrolysis (2MTFA) was carried out to release D-ara and 1-(α/β-O- (*R*)−2-octyl)- 2,3,5 tri-O- trifluoroactyl-D-arabinofurano/ pyranoside was synthesized. D-$^{13}C_5$-UL- arabinose (200 ng) was used as an internal standard to compare to the diagnostic four peaks arising due to the formation of α/β anomers of the D-arabinopyranosyl and D-arabinofuranosyl ring conformers during derivatization. The amount of LAM-equivalent was calculated using the previously reported formulation (also see equations in the Additional Files). The D-ara derivatives were then analyzed by GC/MS using MS/MS. The ions *m/z* 420.9 (parent ion) to 192.9 (daughter ion), and *m/z* 425.9 (parent ion) to 197.9 (daughter ion) were monitored respectively for D-ara and D-UL-$^{13}C_5$- arabinose (internal standard, Cambridge Isotope Laboratories Inc.) as reported earlier [12].

For TBSA, the Octyl Sepharose purified LAM from urine was subjected to alkaline hydrolysis and subsequently the corresponding pentafluorobenzyl tuberculostearate derivative was made. D2-palmitic acid was used as the internal standard. The GC/MS analysis of the pentafluorobenzoate ester was carried out using selective ion monitoring program in negative ion chemical ionization mode whereby the characteristic free fatty acyl anion at *m/z* 293.7 was monitored. A comparison between TBSA and D2-palmitic acid (internal standard; 20 ng; *m/z* 257.3) yields the TBSA content in the sample, which was then used to calculate the LAM-equivalent from the formulations in the Additional Information. GC/MS analyses were carried out using a Thermo GC-TSQ8000 Evo Triple Quad GC mass spectrometer. Chromatograms with respective peaks were integrated manually (i.e., peak areas were defined manually and integrated areas were generated by the computer software) for the estimation of total D-ara and TBSA content. The instrument was set to collect data for *m/z* 257.3 in the range of 5 to 19 mins for ISTD and *m/z* 297.3 after 19 mins for TBSA which elutes around 20 mins, to collect sufficient data points for low level mass detection.

2.3. Capture ELISA

The polystyrene microplate (Corning Costar) was coated with 100ul of a capture antibody at 10ug/mL concentration in PBS and incubated at 4 °C overnight. Urine control samples were spiked with known amount of LAM at different concentrations and incubated at 4 °C overnight to allow for the complexation of LAM and protein/s. Clinical as well as control samples were pretreated with Proteinase K and the supernatant used for ELISA. After overnight incubation, the antibody coated plates and the LAM samples were brought to RT and the plates were blocked for 1 hr at RT. The plates were washed with the wash buffer (200ul x 10) and the control and the clinical samples were added to the appropriate wells (100ul) and incubated. Following a second wash, the plates were incubated with the biotinylated detection antibody at a final concentration of 250 ng/mL in wash buffer. Biotinylation of the antibody was carried out using EZ-Link Sulfo NHS-LC Biotin (Thermofisher Scientific) following the kit protocol and the labeled antibody was desalted on Zeba spin desalting columns, 7 K MWCO (Thermofisher Scientific) as per the kit protocol. Following a third wash, 100ul of 1:200 dilution of Streptavidin-Horseradish Peroxidase (HRP) (R & D Systems) was added to the plates and incubated for 25 min. After the final wash, 100ul Ultra TMB- ELISA chromogenic substrate (Thermofisher Scientific) was added to the plates and incubated for 30 min. Reaction was stopped by addition of Sulphuric acid (Fisher Scientific) and the absorbance was read at 450 nm. All the controls were run in triplicates and reported as the mean ± standard deviation. The samples were run in duplicates and plotted against the standard curve generated by spiking the urine from a healthy volunteer with known amounts of LAM and serially diluted. Limits of blank (LoB) and limits of detection (LoD) were generated from the standard curve using CLSI standard

2.4. LAM and mAbs used for standardization of immunoassay

The LAM used in this study was isolated and purified from *M. tuberculosis* CDC1551 and H37Rv, *M. avium* 2285 rough *in vitro* cultures. The LepLAM was purified from *M. leprae* whole cells isolated from infected armadillo spleen and liver. LAM from all the above mentioned sources was prepared in our laboratory at CSU as described in Mycobacterial Protocols, IInd ED [14]. Characterization and purification of all mAbs has been described previously ([15, 16]).

3. Results

3.1. History of NTM infection and disease in the study cohort

Forty-five urine samples were analyzed from 44 patients with well-documented NTM culture status. Patients were representative of the CF population in general, ranging in age from 11 to 63 years (mean=24.6 years), with 16 men and 28 women. Thirty-one subjects had a history of a positive NTM sputum culture (Table 1) (11 MABSC, 20 MAC) ranging from 0 to 2385 days prior to urine collection. Sixteen subjects had a positive NTM sputum culture at the same clinic visit or hospitalization as their urine collection, and 1 was smear positive. Among the 31 patients with a history of positive NTM cultures, 7 had been treated and were culture negative for > 1 year and were classified as having “cleared” their sputum, while the remaining 24 were culture positive within the last year, or had never received NTM treatment. Fourteen subjects had a history of infection with more than one NTM species or complex (**Table 1**). All patients were co-infected with at least one other typical CF pathogen, including *Pseudomonas aeruginosa, Staphylococcus aureus*, or *Aspergillus* species.

In order to better understand the predictive value of a negative urine LAM result, the NTM sputum culture history was examined in detail for the fourteen subjects classified as never having a positive NTM sputum culture. In total, these subjects had 407 negative NTM sputum cultures over a mean interval of 2.75 years (range 0 to 9.9 years) (**Fig. 1**). As enrollment in this study occurred over a two-year period, follow-up culture results were also available for some subjects after urine LAM analysis. Subjects had follow-up sputum cultures, with a mean of 10 (range 1 to 27) cultures per subject and a median follow-up time of 12.75 months (**Fig. 1**). Over the course of trial enrollment one of the subjects (BP011) initially classified as negative subsequently developed NTM disease with 3 positive cultures for *M. avium* occurring 657 days after the initial urine LAM testing. Repeat urine LAM testing on this subject determined that her urine LAM assay turned positive at the time of her positive NTM cultures, supporting the ability of this assay to detect a conversion from sputum culture negative to positive. The subject met criteria for NTM disease and was started on antibiotic treatment with conversion of her cultures to negative (**Fig. 1**).

3.2. Correlation of urine LAM with NTM phenotype and species

All subjects with a known previous positive sputum culture for NTM had detectable urine LAM through GC/MS analysis of either D-ara or TBSA (**Table 1**). Among patients whose current or most recent infection was a subspecies of *M. abscessus*, the quantity of both TBSA and D-ara were significantly greater than those infected with MAC ($p = 0.0073$ and $p = 0.03$ for TBSA and D-ara, respectively) (**Fig. 2**). There was no clear correlation between quantity of detected LAM and culture status, as subjects who had positive cultures within a year of urine collection had a similar range of LAM detected as subjects who had cleared their sputum culture for >1 year. Representative GC/MS chromatograms showing the absence and/or presence of urinary LAM related to NTM-negativity and/or positivity is in **Fig. 3**. Chromatograms of all samples are included in Appendix A.

We then applied a capture ELISA that was developed for the purpose of TB diagnosis [8]. We selected three antibodies (CHCS9–08, CS-35 and A194–01) that worked well with the *M. tuberculosis* clinical urine samples in our hands [8]. Prior to running the CF clinical samples, we tested the three antibodies individually against serial dilution of LAM from *M. tuberculosis, M. leprae* and *M. avium. M. avium* LAM shares many common structural features with TBLAM [17], and the species are more closely related to each other than with *M. abscessus*. The antibodies were selected based on the following evaluation in our laboratory (**Fig. 4**). Each antibody had significantly lower affinity towards NTMLAM compared to LepLAM, or TBLAM, despite having selected antibodies from our collection that have broad epitope binding affinity.

All the forty-five NTM urine samples that had previously been analyzed by GC/MS were tested in an ELISA format following pretreatment with Pro-K. LAM was detected in only two of the samples from NTM positive subjects, and none of the NTM negative subjects by immunoassay irrespective of any combination of antibodies used (**Supporting Information Fig S6**).

4. Discussion

In a CF cohort well characterized for NTM status, we were able to demonstrate complete correlation between the presence of LAM in the urine and a history of NTM recovered from sputum samples (**Table 1**). In these patients, NTM burden ranged from smear and sputum culture positive at the time of urine culture to an individual whose last positive culture was 6.5 years prior. Based on their most recent positive culture, subjects were classified as having either MABSC or MAC, but 9 individuals had a history of >1 NTM

Table 1
GC/MS based quantification of urine LAM markers TBSA and D-ara.

Subject	Culture Status[1]	Days since last pos. Cx	Current or most recent NTM ssp	Current or most recent complex	ID of other NTM species[2]	LAM (TBSA)[3]	LAM (D-Ara)[4]
BP-001	Never pos.	N/A[5]	N/A	none	N/A	ND[6]	ND
BP-002	Never pos.	N/A	N/A	none	N/A	ND	ND
BP-011	Never pos.	N/A	N/A	none	N/A	ND	ND
BP-202	Never pos.	N/A	N/A	none	N/A	ND	ND
BP-204	Never pos.	N/A	N/A	none	N/A	ND	ND
BP-209	Never pos.	N/A	N/A	none	N/A	ND	ND
BP-210	Never pos.	N/A	N/A	none	N/A	ND	ND
BP-308	Never pos.	N/A	N/A	none	N/A	ND	ND
BP-309	Never pos.	N/A	N/A	none	N/A	ND	ND
BP-310	Never pos.	N/A	N/A	none	N/A	ND	ND
BP 501	Never pos.	N/A	N/A	none	N/A	ND	ND
BP502	Never pos.	N/A	N/A	none	N/A	ND	ND
BP 503	Never pos.	N/A	N/A	none	N/A	ND	ND
BP 505	Never pos.	N/A	N/A	none	N/A	ND	ND
BP-208	Cleared	2385	MAC (unspecif)	MAC	none	21.0	16.3
BP-008	Cleared	1095	*M. abscessus*	MABSC	*M. avium*	37.8	19
TCT-008	Cleared	813	*M. abscessus*	MABSC	None	4.8	na[7]
BP-301	Cleared	634	*M. avium*	MAC	*M. abscessus, M. gordonae*	3.2	5.6
BP-311	Cleared	601	*M intracellulare*	MAC	none	18.8	16.8
BP-403	Negative	469	*M.chimaera*	MAC	none	7.8	11.0
BP-305	Cleared	447	*M. avium*	MAC	*M. avium*	12.8	8.6
BP-004	Cleared	391	*M. intracellulare*	MAC	*M. abscessus*	15.7	13
TCT-003	Negative	236	*M. avium*	MAC	*M. triplex M. montefiorence*	4.4	na
BP-009	Positive	91	*M. intracellu-lare/yongonense*	MAC	*M. massiliense*	5	4.8
BP-304	Positive	88	*M. avium*	MAC	*M. abscessus, M gordonae*	9.3	11.1
BP-406	Negative	88	*M. chimaera*	MAC	*M.avium*	15.4	12.6
BP-404	Negative	78	*M. avium*	MAC	none	10.3	11.3
BP-003	Positive	52	*M. abscessus*	MABSC	none	5.2	5.6
BP-201	Positive	23	*M. massiliense*	MABSC	none	35	29
BP-303	Positive	8	*M. massiliense*	MABSC	none	12.2	19.1
BP-306	Positive	6	*M intracellulare*	MAC	*M avium, M. yongonense, M. abscessus*	16.5	21.3
BP-203	Positive	4	*M. abscessus*	MABSC	none	18.5	14.4
BP-011	Positive[8]	4	*M. avium*	MAC	none	5.1	na
BP-405	Positive	2	*M. abscessus*	MABSC	none	8.2	9.2
BP-307	Positive	1	*M. avium*	MAC	*M. massiliense, M. chimaera*	16.9.	14.5
BP-401	Positive	1	*M. intracellulare*	MAC	none	8.0	8.6
BP-005	Positive	0	*M. intracellulare*	MAC	none	9.6	6.2
BP-006	Positive	0	*M. abscessus*	MABSC	*M. avium*	27.2	19.4
BP-007	Positive	0	*M. avium*	MAC	*M. intracellulare*	5	3.4
BP-010	Positive[9]	0	*M. abscessus*	MABSC	none	27.7	31.2
BP-205	Positive	0	*M. abscessus*	MABSC	none	19.6	15.7
BP-206	Positive	0	*M. abscessus*	MABSC	none	20.8	14.5
BP-207	Positive	0	*M. intracellu-lare/yongonense*	MAC	none	13.7	20.3
BP-302	Positive	0	*M. avium*	MAC	*M. massiliense*	9.9	11.6
BP-402	Positive	0	*M.chimaera*	MAC	*M. abscessus*	13.6	na

[1] Culture Status: Never pos. represents a minimum of 5 NTM cultures over the 5 years previous to collection, as well as no known history of a previous NTM culture, Cleared represents NTM sputum cultures negative for > 1 year, Positive represents NTM sputum cultures positive within the last year. Negative represents sputum cultures that were previously positive but now negative, either with less than a year of treatment or spontaneously.
[2] ID of other NTM species: Identification of NTM in subjects with a lifetime history of > 1 species or subspecies prior to most recent culture.
[3] LAM (TBSA): Urine LAM TBSA quantity as ng/mL.
[4] LAM (D-ara): Urine LAM D-ara quantity as ng/mL.
[5] N/A: Not Applicable.
[6] ND: Not Detected.
[7] na: Not available due to insufficient quantity of sample.
[8] Subject tested twice, first classified as never positive, and then retest at the time of positive sputum cultures for MAC.
[9] AFB smear positive on date of urine collection.

species, subspecies or complex. The quantity of urinary LAM correlated generally with identification of subspecies of *M. abscessus* or MAC in the most recent sputum sample, with higher quantities associated with *M. abscessus* (Fig. 2). There was no correlation between urine LAM quantity and traditional markers of disease activity or bacterial burden in this small cohort, including having negative cultures for >1 year, the consensus endpoint for treatment [5].

The lack of correlation between urine LAM and NTM infection status is not unexpected. Although NTM diagnosis and nearly all therapeutic decisions in the CF population depend on sputum culture results, it is well-recognized that sputum cultures are neither

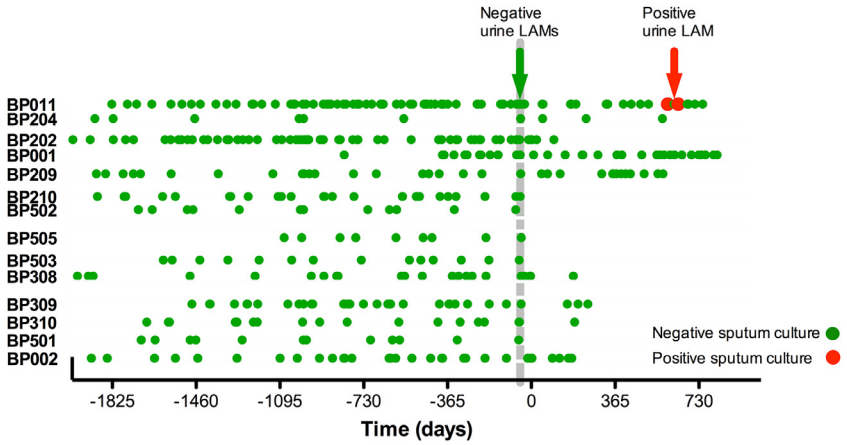

Fig. 1. Urine LAM analysis of subjects with negative NTM sputum cultures. Subjects ($n = 14$) with no evidence for NTM airway infection with >5 cultures, and no history by chart review. Urine LAM assay collected at day 0 correlated completely with previous negative sputum culture for all subjects. Subject BP011 (top row) developed NTM disease 1.8 years following initial urine LAM (red circles). A urine LAM analysis at that time changed to positive (red arrow). The subject was initiated on treatment for *M. avium*, with subsequent conversion to negative sputum cultures (green circles).

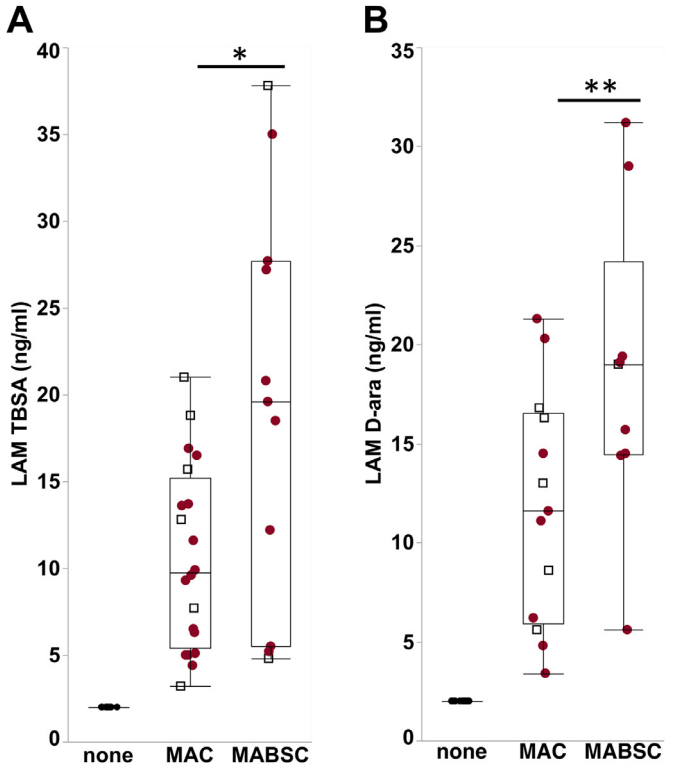

Fig. 2. Quantity of urine LAM in persons with CF culture positive for MABSC and MAC. Panel A: TBSA LAM (ng/ml) and **Panel B**: D-ara LAM (ng/ml) detected by GC/MS for subjects whose most recent sputum NTM culture was identified as MAC or MABSC. Mean TBSA and D-ara quantities were greater in subjects whose most recent sputum NTM species was within MABSC ($^{*}p = 0.007$ and $^{**}p = 0.03$ respectively, by one-way ANOVA). Individuals who have cleared their sputum cultures for > 1 year are depicted with open squares, while subjects who have had a positive NTM culture within a year are depicted with a red circle. Box and whiskers plot represent median, 25th-75th quartile, median and range.

sensitive or specific for any aspect of the infection. Evidence of clinical decline attributed to NTM disease can be detected in the year prior to a first positive NTM culture, but the presence of a positive culture does not by itself serve as an indication for treatment. The nearly universal occurrence of bacterial co-pathogens in the CF sputum results in the need for decontamination procedures, which significantly reduce NTM viability and can be the source of false negative results. Likewise, a single sputum sample may not be representative of infection in all areas of the lung, especially if the NTM is localized within the cavity or in a segment with atelectasis, collapse or extensive mucous plugging.

Detection of LAM in urine has not been extensively studied in the context of NTM or CF. We found two relevant studies in the literature, one describing GC/MS as a technique for fingerprinting of twenty metabolites/lipids that would distinguish NTM from *M. tuberculosis*, which requires early culture of bacteria from sputum samples followed by chemometric analyses [20] and a pilot study in people with CF using the commercially available DETERMINE TB LAM Ag test [13]. Qvist et al. compared the urine LAM antigen test results to sputum NTM culture results over the past year and found the quantity of detected urine LAM antigen in NTM positive CF patients was quite low compared to patients with mTB. Using the threshold recommended for diagnosis of TB, the urine LAM antigen test had a high specificity (99%), but very low sensitivity (9%) for identifying patients with positive NTM sputum cultures in this CF population. When they lowered the threshold to the lowest detectable quantity of LAM, the sensitivity of the test improved to 39%, but they concluded that the test kit they studied was not suitable for diagnostic use in the CF population [13], which was supported by our result from capture ELISA.

The detection of LAM in urine by GC/MS is complex as normal urine can contain both stereoisomeric forms D- & L-arabinose (from nutrients and food), which complicate the detection of D-arabinose (D-Ara) that is specific to LAM. Through our NIH-supported Tuberculosis Diagnosis program we have developed a sample handling protocol whereby LAM was separated from these endogenous neutral polysaccharides by way of hydrophobic interaction chromatography followed by chiral glycosylation (with R-2-octanol) which efficiently distinguished D-and L-stereoisomers of arabinose. This protocol also served the purpose of releasing LAM from its protein complex and to distinguish L- from D- arabinose present in other arabinose containing neutral polysaccharide(s). An internal standard was then added (uniformly ^{13}C-labeled D-Ara, 200 ng) to allow efficient quantification of LAM D-Ara. In addition to estimating D-Ara, a TBSA that is present on the anchor region of LAM-detection method in urine has also been developed. The derivatization is performed on the LAM fractions eluted off the Octyl Sepharose columns with the rationale that the fractions that contain D-Ara must also have TBSA, pointing to the presence of intact LAM.

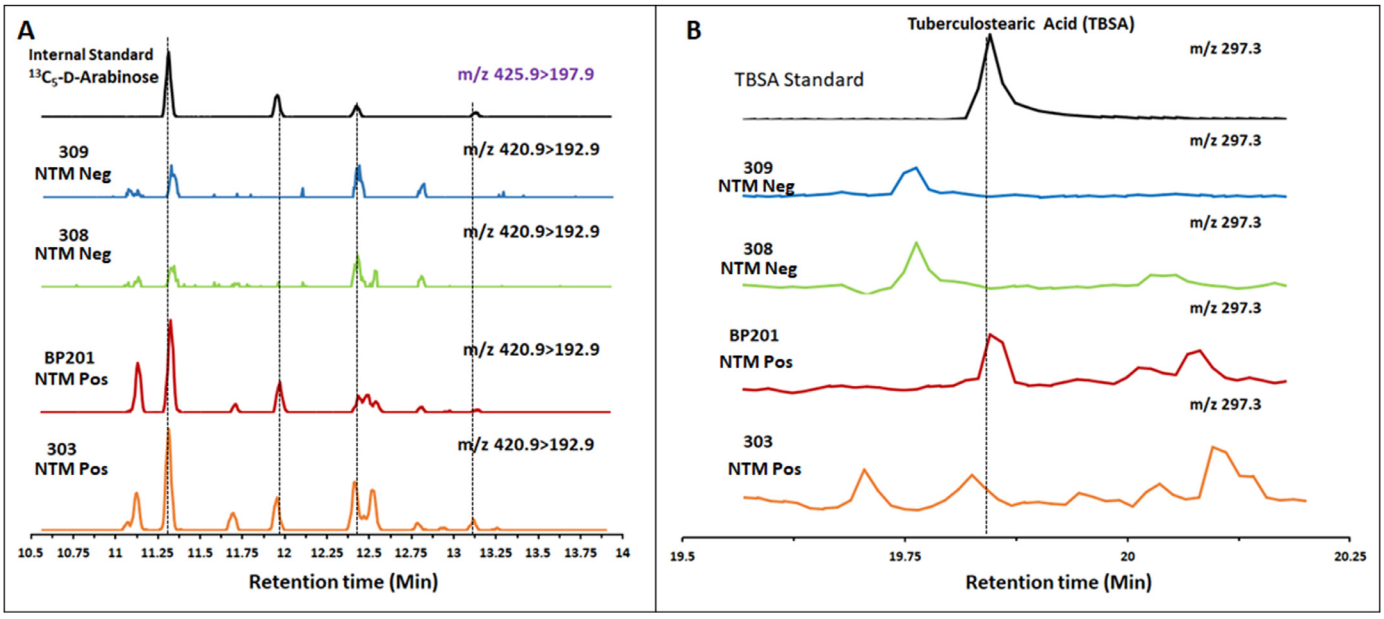

Fig. 3. Representative GC/MS chromatograms showing the absence and/or presence of urinary LAM related to NTM-negativity and/or positivity. A) D-Arabinose (D-ara) MS/MS method monitoring *m/z* 420.9-*m/z* 192.9): Four characteristic peaks of Internal Standard ($^{13}C_5$-D-Arabinose; top panel); Sequentially (top to bottom), 309 and 308 NTM negative, D-ara negative; BP201 and 303 are positive, LAM positive, NTM positive. B) Tuberculostearic acid (TBSA) Single ion monitoring (SIM) at *m/z* 297.3: TBSA standard (C:19; top panel); Sequentially, 309 and 308 NTM negative, TBSA negative; BP201 and 303 are TBSA positive, LAM positive, NTM positive.

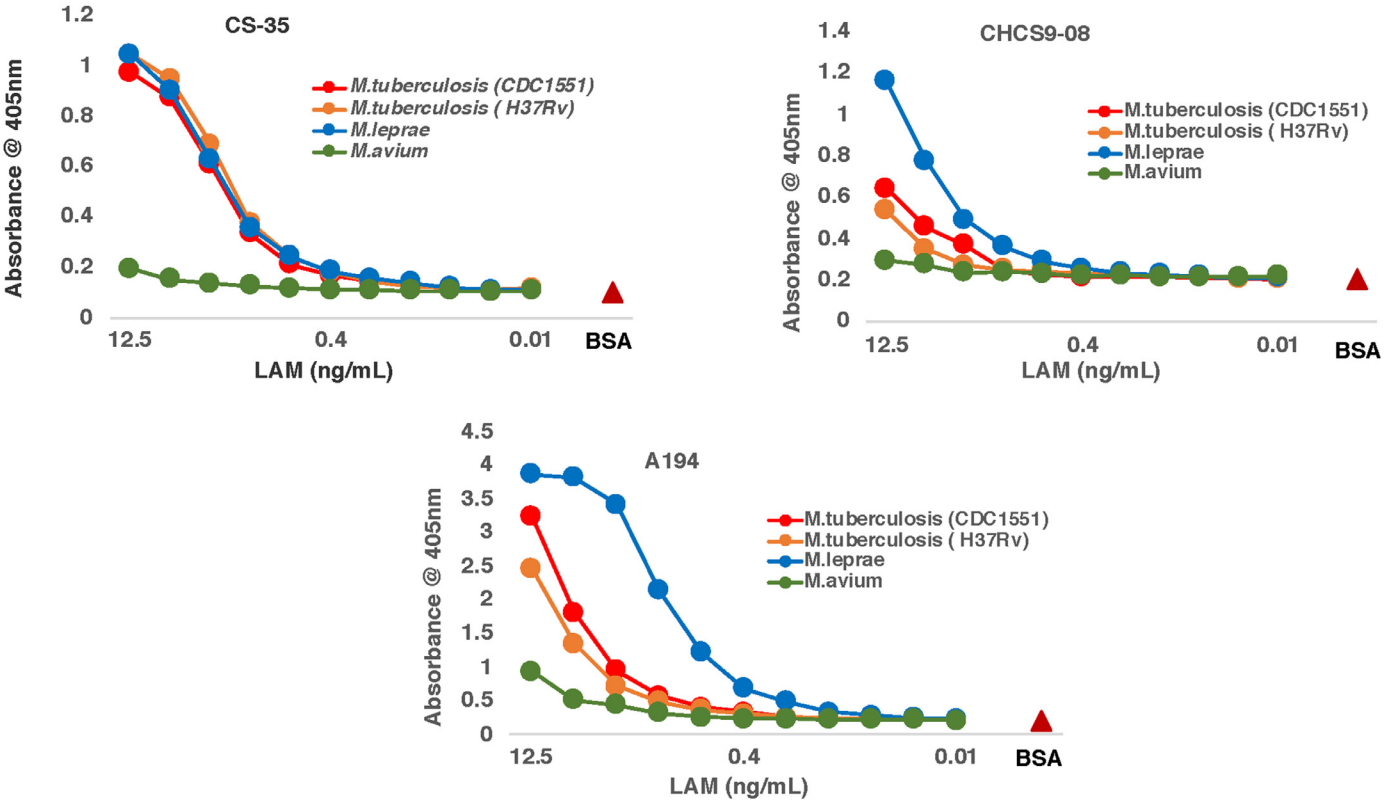

Fig. 4. Direct ELISA for selection of antibody for NTMLAM analysis in clinical samples. LAM samples were prepared and purified as described [14] except LepLAM was purified from *M. leprae* cells isolated from infected armadillo liver and spleen [18]. A194–01 is a human monoclonal derived from a tuberculosis patient against TBLAM and CS35 and CHCS9–08 were raised against *M. leprae* whole cells. Reactivity and specificity of the mAbs have been described in our previous published work ([8, 19]).

The results of this pilot study indicate that urine LAM analysis by GC/MS could be a useful, non-invasive screening tool to identify CF patients for which NTM sputum cultures are not needed. The great majority of people with CF will not develop NTM infection, and in the absence of clinical suspicion, a negative urine LAM result could potentially replace sputum cultures as a method of annual screening. In particular, children are at low risk for NTM infection, and are also often unable to expectorate sputum. Going forward, the widespread use of highly effective CFTR modulator therapy will significantly reduce the proportion of people with CF who are capable of expectorating sputum at baseline health, while at the same time increasing the life expectancy of the population. Identification of non-invasive, culture-independent markers for NTM infection has been identified as a research priority for the CF population . We propose urine LAM could be part of a lifetime screening strategy for the diagnosis of NTM, and that a larger, prospective longitudinal clinical trial is warranted to validate these results and to establish the durability of the signal.

Author contributions

PD participated in GC/MS analyses, data interpretation, and manuscript preparation, AA ran all the ELISA assays, analyzed data and helped with the manuscript, BG performed the initial statistical analyses, DC developed the concept, data analysis and manuscript preparation; JAN participated in study design, data analysis and interpretation, and manuscript preparation. SLM and MTS participated in study regulation, data interpretation, and manuscript preparation. SMC, KRP, and MCJ participated in study enrollment, sample collection and preparation, and manuscript preparation.

Declaration of Competing Interest

Authors have no competing interests.

CRediT authorship contribution statement

Prithwiraj De: Investigation, Methodology, Formal analysis. **Anita G. Amin:** Formal analysis, Investigation, Methodology, Writing - review & editing. **Stacey L. Martiniano:** Project administration, Validation. **Silvia M. Caceres:** Data curation, Project administration. **Katie R. Poch:** Data curation, Project administration. **Marion C. Jones:** Data curation, Project administration. **Milene T. Saavedra:** Project administration, Validation. **Jerry A. Nick:** Conceptualization, Funding acquisition, Validation, Writing - review & editing. **Delphi Chatterjee:** Conceptualization, Funding acquisition, Project administration, Writing - original draft.

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Supplementary materials

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