Jun 01, 2024

Urine Lipoarabinomannan as a Biomarker for Mycobacterium tuberculosis and non-tuberculous mycobacterial infections

DOI
dx.doi.org/10.17504/protocols.io.3byl497o8go5/v1

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OPEN ACCESS

DOI: dx.doi.org/10.17504/protocols.io.3byl497o8go5/v1

Protocol Citation: Jordan Manzer, Ibrahim Aboellail, Jerry Nick, Delphi Chatterjee, Anita G Amin 2024. Urine Lipoarabinomannan as a Biomarker for Mycobacterium tuberculosis and non-tuberculous mycobacterial infections. **protocols.io**
https://dx.doi.org/10.17504/protocols.io.3byl497o8go5/v1

Manuscript citation:
- https://doi.org/10.1371/journal.pone.0144088
- DOI: **10.1016/j.tube.2018.06.004**
- DOI: **10.1016/j.jcf.2020.06.016**
- DOI: **10.1038/s41598-021-82445-4**
- https://doi.org/10.1371/journal.pone.0275838

Protocol status: Working
We use this protocol and it's working

Created: April 18, 2024

Last Modified: June 01, 2024

Protocol Integer ID: 98441

Keywords: Lipoarabinomannan, Cystic Fibrosis, M. abscessus, Cystic Fibrosis, M. abscessus, M. avium complex, NTM LAM, TB Diagnostics

Funders Acknowledgement:
Delphi Chatterjee
Grant ID: RO1 AI132680
Jerry Nick & Delphi Chatterjee
Grant ID: NICK17K0
Jerry Nick & Delphi Chatterjee
Grant ID: NICK18P0
Jerry Nick & Delphi Chatterjee
Grant ID: NICK20Y2-SVC
Jerry Nick & Delphi Chatterjee
Grant ID: NICK20Y2-OUT

Abstract

Lipoarabinomannan (LAM) is a unique cell wall component containing a fatty acid region with a large mannan core attached to poly-arabinan chains, sometimes followed by mannose capping in Actinomyces species. The hydrophobic region made of tuberculostearic acid (TBSA) and two palmitic acid chains linked to a phosphoinositol, anchors LAM to the mycobacterial cell wall. As a major constituent of all mycobacteria, including *Mycobacterium tuberculosis* (M.tb), LAM has been shown to be a useful diagnostic biomarker in the identification of MTB infection in HIV positive (HIV+) patients. The size of LAM makes it difficult to analyze on GC-MS when using complex matrices such as urine, therefore chemical processing and derivatization is required. Two methods have been developed to quantify urine-LAM amounts, one by measuring D-arabinose and the other TBSA. Both components are unique and not metabolized through basic pathways in mammalian cells and can therefore serve as LAM surrogates. Using either chemical assay, urine-LAM can be detected in low quantities with sensitive GC-MS, providing a diagnostic tool for clinical assessment of infection. These methods are robust and have been applied to a significant number of TB and non-tuberculous mycobacterial (NTM) urine samples. These protocols provide additional sensitivity beyond what is required for assays of MTB in HIV+ patients, allowing for identification of both M.tb and NTM infections in immunocompetent hosts.

Materials

KIMAX glass 13 x 100 mm test tubes with phenolic screw PTFE lined caps
Benchtop centrifuge
Vacuum Concentrator
Heating block/s
Vortex
10 mL BIORAD Poly-Prep® columns
Indicator paper pH 1.0-12.0
Nitrogen evaporator
Ice bucket
Chemical fume hood
$5^3/_4$in glass Pasteur Pipettes
12 x 32 mm Clear GC vials
Teflon GC vial caps with TS Septa
0.25 mL deactivated glass inserts for GC vials

All reagents should be highest purity ACS grade
Hexane
Chloroform
200 proof absolute Ethanol
Autoclaved milli-Q water
Octyl Sepharose™ CL-4B (GE Lifesciences)
Ammonium Acetate (Sigma-Aldrich)
n-Propanol
Palmitic acid 2,2-D_2 (Sigma-Aldrich)
Sodium Hydroxide
Sulfuric Acid
28% Ammonium Hydroxide (Sigma-Aldrich)
Acetonitrile (Sigma-Aldrich)
N-Ethyldiisopropylamine (Sigma-Aldrich)
2,3,4,5,6 Pentafluorobenzyl bromide (Sigma-Aldrich)
Trifluoroacetic acid (Sigma-Aldrich)
Trifluoroacetic anhydride (Sigma-Aldrich)
(R)- (2) Octanol (Sigma-Aldrich)
D-(UL-$^{13}C_5$)-Arabinose (Cambridge Isotope Laboratories Inc.)

Safety warnings

All the procedures described in the protocol using patient urine samples should be performed in a biosafety level 2 laboratory with the proper personal protective equipment. All steps involving organic solvents must be performed in a chemical fume hood, and borosilicate glassware, glass pasteur pipets or glass capillaries should be used for dispensing the organic solvents and performing the chemical derivatization steps.

Ethics statement

Sample Collection

De-identified urine samples used for testing were collected at National Jewish Health (NJH), Denver, Colorado, from people with Cystic Fibrosis (pwCF). PwCF are the population with the highest reported risk for NTM lung infections. Samples were obtained as part of the PAINLESS Trial, a prospective, single-center, nonrandomized observational study. The study is approved by the Biomedical Research Alliance of New York (BRANY) review board (20-08-402-528) and is registered in ClinicalTrials.gov (NCT04579211). We comply with the Declaration of Helsinki and Good Clinical Practice guidelines. Samples were also obtained in studies of the treatment of *M. abscessus* with bacteriophage, and from pwCF with well-classified NTM disease status.

Archived and de-identified urine samples were also obtained from Foundation for Innovative New Diagnostics (FIND), Geneva, and Laboratorio Socios En Salud Sucursal, Peru. The samples were collected from patients with symptoms of pulmonary tuberculosis presenting prior to the initiation of treatment to clinics in Vietnam, South Africa and Peru. All human urine specimens were collected from adult participants and symptomatic children in whom TB was ruled out by a pediatric pulmonologist on the basis of negative bacteriological (i.e; smear and culture) results from sputum or gastric aspirate; chest X-ray, and tuberculin skin testing, with written informed consent. For controls, urine was collected from a healthy volunteer, from a TB non-endemic region, and used in standardizing the protocol by spiking with laboratory purified M.tb or *Mycobacterium abscessus* (M.ab) LAM. The collection of urine samples conformed to the Declaration of Helsinki and was approved by the local IRB and Ethics Committee of NJH, FIND and Colorado State University.

Before start

1. Use only glass tubes with phenolic screw caps with PTFE lining for all chemical derivatization reactions. The glass tubes should be rinsed with sterile MilliQ water and then cooked in a 400^{o}F Kiln oven to get rid of all impurities (to reduce background on GC-MS) before use.
2. Only glass pipettes, glass pasteur pipets and glass capillaries must be used for dispensing the chemicals.
3. All drying steps should be done in Nitrogen evaporator and make sure the samples are completely dry with no visible mositure on the inner walls of the tubes before proceeding to next step.
4. Controls are prepared using urine from a healthy volunteer (TB non endemic area) and are spiked with M. tb CDC-1551 LAM.

Urinary LAM Tuberculostearic acid Analysis

1 Hexane Extraction (Removes exogenous proteins and lipids from urine)

1.1 Using 1 mL of urine in a KIMAX 13 x 100mm glass tube with teflon screw cap, 1 mL of Hexane is added.

1.2 Vortex thoroughly for 20 seconds, two times.

1.3 Centrifuge at 3,500 rpm for 5 minutes.
1.3.1 If bubbly interphase exists, then repeat centrifugation for 5 to 10 minutes to achieve complete bilayer separation.
1.3.2 If the aqueous layer appears cloudy, or bubbly interphase persists repeat extraction with another 1 mL of Hexane.

1.4 Remove the upper Hexane layer and discard.
1.4.1 If there is precipitation at the bottom of the test tube, transfer the lower aqueous layer to a new tube, discard precipitate.

1.5 Dry the aqueous layer in a vacuum concentrator.
1.5.1 Hexane extraction is required only for urine samples obtained from pwCF for the NTM LAM analysis. For urine samples collected from TB suspects, Hexane extraction is not required, instead, 0.5 mL to 0.75mL of urine sample is transferred to a KIMAX 13 x 100mm tube and vacuum dried.

2 Hydrophobic Interaction Chromatography (Purify and concentrate LAM)

2.1 Clean 10 mL BIORAD Poly-Prep® column with two rinses of milli-Q water and one rinse of 200 proof ethanol, followed by additional two rinses with milli-Q water.

2.2 Add 2 mL of 5% n-propanol and 1 ml of room temperature Octyl Sepharose™ CL-4B to the clean column.
2.2.1 For elution from Octyl Sepharose™ CL-4B, a gradient of n-propanol (5%, 15%, 40% and 65%) in 0.1 M ammonium acetate is used.
2.2.2 Columns should never be allowed to dry out, add 5% or 15% n-propanol when needed to avoid dry columns before moving to elution steps (2.10 -2.14).

2.3 Allow the Octyl Sepharose™ CL-4B to settle in the column for 10 minutes and then pack the column by centrifugation for 10 minutes at 3,500 rpm.

2.4 Add 0.1 mL of 5% n-propanol to the vacuum dried aqueous layer (step 5) and centrifuge at 3,500 rpm for 30 seconds.

2.5 Remove the luer end fitting with snap off tip from the bottom of the columns and allow them to drip into a reservoir.

2.6 Before all the liquid completely drains add 2 mL of 5% n-propanol.

2.7 Repeat another 2 mL addition of 5% n-propanol before liquid drains.

2.8 Using a pasteur pipet, add the 0.1mL from the sample tube to the column.

2.9 Repeat with another 0.1mL 5% n-propanol addition to the sample tube and centrifuge at 3,500 rpm for 30 seconds.

2.10 Transfer the final 0.1mL to the column using a pasteur pipet.

2.11 Add 2 mL of 5% n-propanol to the columns and allow to drip into reservoir.

2.12 Repeat with another 2 mL addition of 5% n-propanol.

2.13 Before the liquid completely drains out of the column add 2 mL of 15% n-propanol.

2.14 Repeat another 2 mL addition of 15% n-propanol.

2.15 Once the liquid completely drains, cap and cover the columns, remove the reservoir and replace with a fresh clean 13 x 100mm glass tube.

2.16 Add 2 mL of 40% n-propanol to the column and collect eluent in the glass tube.

2.17 Before the liquid drains add 2 mL of 65 % n-propanol.

2.18 Once both 40% and 65% n-propanol have been completely eluted into the glass tube, vortex to mix and dry in vacuum concentrator.

3 Saponification, Acidification and Neutralization

3.1 Add 5 ng of Palmitic Acid 2,2-D_2 (Internal Standard) to each test tube.

3.1.1 Palmitic acid can be resuspended in Hexane and stored in -20^{o}C.

3.2 Add 0.2mL of 0.25 N sodium hydroxide and incubate at 80^{o}C for 1 hour.

3.3 Allow tubes to cool to room temperature and centrifuge 3,500 rpm for 30 seconds.

3.4 Add 0.5mL milli-Q water.

3.5 Titrate to a pH of 2 (pH paper) with dropwise addition of 3:1 (milli-Q water : sulfuric acid).

3.6 Add 0.5mL chloroform to the tube and vortex for 20 seconds 2 times.

3.7 Centrifuge at 3,500 rpm for 5 minutes.

3.8 Remove the upper, aqueous layer and discard.

3.9 Dry the lower chloroform layer under nitrogen flow.

3.10 Once completely dry add 0.15mL of ammonium hydroxide and gently vortex.

3.11 Dry under nitrogen flow in chemical fume hood.

4 Esterification

4.1 In a chemical fume hood, add 0.1mL of acetonitrile, 0.07mL ethyldi-isopropylamine, and 0.02mL 2,3,4,5,6 pentafluorobenzyl bromide.

4.2 Centrifuge at 3,500 rpm for 20 seconds to bring each solution to bottom of the test tube ensuring proper concentration.

4.3 Incubate at room temperature for 30 minutes.

4.4 Dry under nitrogen flow.

5 GC-MS analysis

5.1 Resuspend the dried sample in 0.1 mL of Hexane.

5.2 Using a pasteur pipet, transfer the sample to a GC vial.

5.3 Inject on GC-MS in negative chemical ionization with a low polarity J&W VF-5ms fused silica capillary GC column.

5.4 GC Oven settings: oven temperature was held at 50°C for 1 min and programmed at 20°C / min to 150°C and then programmed at 10°C / min to 310°C. (MS transfer line temperature: 315^{O}C, Ion source temperature: 250^{O}C).

5.5 Using Selective Ion Monitoring (SIM)

5.5.1 TBSA ester derivative anion at *m/z* 297.3 with a peak at 19.6-19.9 minutes retention time.

5.5.2 D_2 Palmitic acid internal standard anion at *m/z* 257.3 at 17.7-18.1 minutes retention time.

6

$$T_0 = \frac{Area\ m/z\ 297.3}{Area\ m/z\ 257.3}\ X\ 5\ ng$$

The approximate molecular weight of full-length LAM is ~17.3 kDa

The mass contribution of TBSA is 298/17300 = 0.0172 or 1.72 %.

$$L_1 = \frac{T_0}{0.0172\ X\ n}\ ng/mL$$

T_0 - Is the mass of TBSA in the tube.

L_1 – is the concentration of LAM per mL of urine.

n = volume of urine used in mL

Quantification of LAM based on TBSA using the above equation

A. shows a flow chart depicting the steps taken in chemical processing of TBSA from urinary LAM to generate 2,3,4,5,6 pentafluorobenzyl tuberculostearic ester. **B.** shows a GC-MS chromatogram of LAM spiked urine (positive control) from a healthy volunteer compared to a blank urine (negative control).

Urinary LAM D-Arabinose Analysis

7 **Hexane Extraction (Removes exogenous proteins and lipids from urine)**

7.1 Perform Hexane extraction same as shown in TBSA protocol, steps 1.1 - 1.5.

8 **Hydrophobic Interaction Chromatography (Purify and concentrate LAM)**

8.1 Purify and concentrate LAM using steps 2.1 - 2.18 (the same as stated for TBSA).

9 **Hydrolysis**

9.1 Add 200 ng of D-(UL-$^{13}C_5$)-Arabinose internal standard to each tube.

9.2 In chemical fume hood, add 0.2mL of freshly made 2 M Trifluoroacetic Acid (TFA).
9.2.1 2M TFA should be made fresh and stored on ice before use.

9.3 Screw Teflon® caps on tightly and incubate at 120^{o}C for 2 hours.

9.4 Dry under nitrogen flow.

10 **Octanolysis**

10.1 In chemical fume hood, add 0.1 mL of (R)-(2) octanol and 0.02 mL concentrated TFA to each tube.

10.2 Incubate at 120^{o}C overnight (16 – 18 hours)

10.3 Dry under nitrogen flow.
10.3.1 Make sure to dry the sample completely before proceeding to the next step. The drying takes longer than normal.

11 **Acetylation**

11.1 In a chemical fume hood, add 0.1 mL of chilled acetonitrile.

11.1.1 Acetonitrile must be chilled on ice prior to addition.

11.2 Add 0.01mL concentrated TFA.

11.3 Add 0.2 mL of trifluoroacetic anhydride.

11.4 Incubate at 55^{O}C for 20 minutes.

11.5 Dry under nitrogen flow.

11.5.1 Acetylated samples can be stored at -20^{O}C and are stable for about a week.

12 GC-MS analysis

12.1 Resuspend samples in 0.1 mL chloroform.

12.2 Using a pasteur pipet, transfer the sample to a GC vial.

12.3 Inject on GC-MS in electron ionization with Mid Polarity Phenomenex® ZB-5HT Zebron™ Inferno™.

12.4 GC oven settings: oven temperature was held at 50°C for 1 min and programmed at 20°C/min to 150°C and then programmed at 2.5°C/min to 215°C. (MS transfer line temperature: 275^{O}C, Ion source temperature: 300^{O}C).

12.5 Using selective ion monitoring, look for 4 peaks between 10 minutes to 12 minutes retention time.

12.5.1 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).

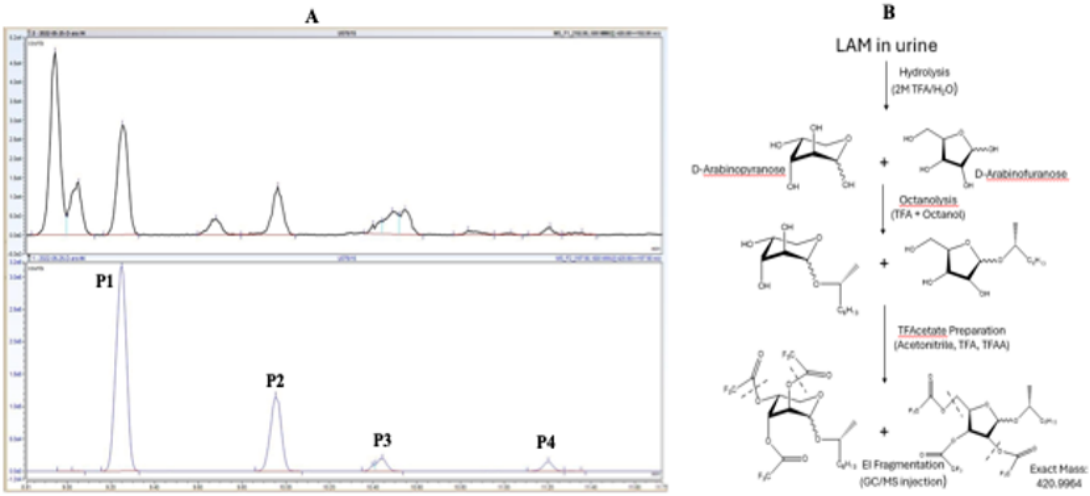
13 Urinary LAM based on D-arabinose can be calculated using the equation below

$$A0 = \frac{1}{4}\left(\frac{P1}{I1} + \frac{P2}{I2} + \frac{P3}{I3} + \frac{P4}{I4}\right) \times 200\ ng$$

$$L1 = \frac{A0}{0.6(n)}\ ng/L$$

A0 - is the amount of arabinose in the tube.
P1 - is the area under the 1st D-arabinose peak (P2 the 2nd peak, P3 the 3rd, and P4 the 4th).
I1 - is the area under the 1st internal standard peak (I2 the 2nd peak, I3 the 3rd and I4 the 4th)
L1 - is the amount of LAM per mL.
n - is the amount of sample used (Typically 0.75 mL to 0.5 mL).

Using the equation above, urinary LAM can be quantified based on D-arabinose.



A. Shows an image of a GC-MS chromatogram with peak ratio for the four different stereoisomeric forms of 2,3,5-trifluoroacetyl-1-(R-2-octyl)-arabinosyl glycosides. D-(UL-$^{13}C_5$)-Arabinose (200ng, lower panel) is used as an internal standard (*m/z* 425.9)to compare to the diagnostic four peaks (P1,P2, P3 and P4) arising due to the formation of α/β anomers of the D-arabinopyranosyl and D-arabinofuranosyl ring conformations during derivatization. **B.** Schematic showing the different steps in D-arabinose derivatization from urinary LAM.

Advantages

14 The quantification of of D-arabinose and TBSA is emerging as a valuable tool for the detection of non-tuberculous mycobacterial (NTM) infections, particularly in immunocompromised patients. This non-invasive test offers several advantages over traditional diagnostic methods, which often require respiratory samples and complex laboratory processing. Urine LAM testing may facilitate earlier diagnosis, and although it has been extensively utilized in diagnosing

tuberculosis, its application for NTM infections is still being explored. These methods appear to have great promise in assessment of NTM infection in at-risk populations, especially when the disease burden is low such as in CF. To date these assays have been used successfully to identify pwCF with a history of both *M. avium* complex or *M. abscessus* infection, as well as a marker of treatment response of *M. abscessus* to phage. The use of these assays is currently being tested in a cohort of pwCF without a history of NTM positive cultures (ClinicalTrials.gov: NCT04579211), in order to determine the utility of urine LAM as a screening tool for NTM in this high risk population. These assays are also being validated in well-defined disease states using samples banked from the CFF-sponsored PREDICT Trial: Prospective Evaluation of NTM Disease in CF (NCT02073409) and the Prospective Algorithm for Treatment of NTM in Cystic Fibrosis Trial (PATIENCE: NCT02419989). The capacity of these assays to track response to treatment linked to novel therapeutics is being tested in the FDA-sponsored Phase 1b, Multi-center Study of IV Gallium Nitrate in Patients with Cystic Fibrosis who are Colonized with Nontuberculous Mycobacteria (ABATE Trial: NCT04294043) and the CFF-sponsored PrOSpecTive STandardized Assessment of MycobacterioPhage (POSTSTAMP) Trial (NCT06262282). Together, these propspective clinical trials will provide a clear assessment of the performance characteristics of this urine LAM assay in different stages of infection, disease and treatment response in the CF population. These results will likely be applicable to other forms of lung disease at-risk for NTM, as well as the potential to detect other presentations of NTM infection.

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