



Development and clinical evaluation of a new multiplex PCR assay for a simultaneous diagnosis of tuberculous and nontuberculous mycobacteria

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ABSTRACT

Background: The prevalence of non-tuberculous mycobacteria (NTM) has been increasing worldwide in both developed and developing countries. NTM infection is clinically indistinguishable from tuberculosis and therefore poses significant challenges in patient management, especially in patients chronically treated for pulmonary TB. In this study, we evaluated a new highly sensitive Multiplex MTB/NTM assay that can differentiate *M. tuberculosis* complex (MTBC) from all NTM, including the treatable and most common NTM, *M. avium* complex (MAC).

Methods: We developed and optimized a new open- Multiplex MTB/NTM assay with two gene-targets for MTBC (IS6110/senX3-regX3) and two targets for MAC (IS1311/DT1) with samples spiked with stored strains and testing 20 replicates. Patients with presumptive TB and NTM were enrolled at the Respiratory Disease Department of The University Teaching Hospital of Point G, in Mali.

Findings: In the development stage, the new assay showed a high analytic performance with 100% detections of MTBC and MAC at only 5 colony forming units (CFUs). Overall, without the treatment failure cases, the Multiplex assay and the Xpert showed a sensitivity, specificity, PPV and NPV of 83.3% [66.4-92.6], 96.6% [88.6-99.0], 92.5% [82.3-96.5] and 92.2% [82.7-96.5] and the Xpert had values of 96.7% [83.3-99.4], 80.0% [68.2-88.1], 70.7 [55.5-82.3] and 97.9% [89.3-99.6], respectively. The Multiplex assay successfully detected all (5/5) the MAC cases.

Interpretation: Our new Multiplex assay demonstrates better specificity than Xpert for all group studied, in addition to detecting potential NTM cases. The assay could therefore complement the widely used Xpert assay and enhance discrimination of TB and NTM infections.

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1. Introduction

Worldwide, tuberculosis (TB) is the leading single pathogen affecting humans, with deaths greater even than from HIV/AIDS [1].

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The genus *Mycobacterium* includes nearly 200 recognized tuberculous and non-tuberculous mycobacteria (NTM). Some NTM are either strictly or potentially pathogenic, while others are opportunistic or saprophyte non-pathogenic and associated with asymptomatic carriage.[2] The *Mycobacterium tuberculosis* complex (MTBC) species including *Mycobacterium (M.) tuberculosis*, *M. africanum*, *M. bovis*, *M. cannetti*, *M. caprae*, *M. microti*, and *M. pinnipedi* are known to cause all type of TB infections, although substantial differences in virulence

Research in context

Evidence before this study

Tuberculosis (TB) is still a major public health problem in low resource settings. The prevalence of non-tuberculous mycobacteria (NTM) has also been increasing worldwide in both developed and developing countries. NTM infection is clinically indistinguishable from TB and poses significant challenges to patient management. The sputum smear microscopy, a widely used diagnostic test for TB, for more than a century, fails to detect up to half of the TB cases, and is unable to differentiate between mycobacterial species. The World Health Organization recommends use of Xpert MTB/RIF (Xpert) assay; however, Xpert is less sensitive than sputum culture and does not detect NTM infections.

Added value of this study

In this study, we developed and tested a new open-platform Multiplex MTB/NTM assay to simultaneously detect MTB, *Mycobacterium avium* complex (MAC) and other NTM infections. The new assay has demonstrated a complementarity with the Xpert assay, with a sensitivity and specificity of 83.3% [66.4-92.6] and 96.6% [88.6-99.0] for the Multiplex compared with 96.7% [83.3-99.4] and 80% [55.5-82.3] for the Xpert assay. In addition, the Multiplex MTB/NTM assay offers an extra value of detecting MAC and other NTM infections, an option that is not available with the Xpert assay. The Xpert assay, however, has the advantage of providing a rifampin resistance profile. The two assays are therefore highly complementary in patients with TB/NTM-like symptoms.

Implications of all the available evidence

Evidence shows that our new Multiplex MTB/NTM assay has good analytic and clinical performance for detection of TB and NTM infections. This assay complements current diagnostic tools for patients with TB/NTM infection-like symptoms, saves time and resources by its multi-detection nature and, more importantly, by detecting MAC and others NTM infections, will reduce mortality in TB endemic countries and worldwide.

However, has only 50-80% sensitivity and cannot differentiate between mycobacterial species. While sputum culture (the gold standard) is very sensitive, it is time-consuming, requiring 10-42 days and needs a sophisticated infrastructure that is not typically available in the resource-limited settings where TB is endemic.[13,14] In light of these factors, the World Health Organization (WHO) endorsed use of the Xpert MTB/RIF (Xpert) molecular assay in developing countries. However, while Xpert provide essential information on rifampin resistance, it is less sensitive than TB sputum culture and cannot distinguish NTM infections.[15] Furthermore, the Xpert assay has limited performance for TB smear negatives and paediatric specimens (around 67% sensitivity).[16] Additionally, 57% of TB cases are bacteriologically-confirmed; the remaining are usually treated empirically.[17] These cases include Xpert-negative patients and patients with paucibacillary TB, such as children and people living with HIV (most of whom are sputum smear negatives).[18,19] This study evaluated the performance of a new highly sensitive quantitative polymerase chain reaction (qPCR) assay for simultaneous detection of MTBC and NTM in patients with clinically presumptive pulmonary tuberculosis. We hypothesized that the Multiplex assay may complement the current algorithm of diagnostic of patients with TB/NTM infection-like symptoms.

2. Methods

2.1. Ethics

The study was approved by the Ethics Committee of the University of Sciences, Technics and Technologies of Bamako (USTTB), under the study number 2017/161CE-FMPOS and the Institutional Review Board of Northwestern University (NU) under study STU00206642. Informed consent was obtained from all participants.

2.2. Participants and study design

The Multiplex MTB/NTM assay was first developed at the Center for Innovation in Global Health Technologies (CIGHT) at NU, including primer design, selection and optimization. A clinical feasibility study was conducted at the University Clinical Research Center (UCRC) in Mali from April 2017 to March 2020. Clinically presumptive TB patients with at least 18 years old were from the six referral health centres in Bamako, Mali. Patients were referred to the Respiratory Disease Department at the University Teaching Hospital of Point-G where samples were obtained. Concentrated smear microscopy and sputum culture were performed in the UCRC Biosafety Level-3 (BSL-3) TB Laboratory and, the Multiplex RT-PCR MTB/NTM assay and Xpert assays were performed in UCRC molecular biology Laboratory.

2.3. Multiplex assay development

2.3.1. qPCR MTB/NTM Primers' selection and assay design

Two *M. tuberculosis* target regions that we previously validated and have shown a clinical sensitivity of 95% or more, and a specificity of 100% compared to sputum culture and the Xpert assay.[20,21] These two target regions are preferred because the multi-copy of the Insertion Sequence (*IS*) 6110 and single-copy of target *senX3-regX3* are highly conserved amongst MTBC species, but, more importantly, are not present in NTM species.[20,21] To add the MAC detection panel to the assay, we selected the *IS1311* gene that has also been previously shown to be present in all MAC species except the *M. intracellulare*. [22,23] A second MAC target, *DT1* gene, was added in which *M. intracellulare* (does not have the *IS1311*), *M. avium* subsp. *avium* (serotypes 1, 2 and 3) and *M. avium* subsp. *silvaticuma* are present.[23] The combination of these two gene target regions has been shown to cover and identify all MAC species but none of the MTBC species.[22,23] The Pan-Mycobacterium 16S rRNA gene that is

have been reported between species.[3] Nontuberculous mycobacteria (NTM) represent a major public health threat due to the wide variety of species and the increasing number of people at risk, including the elderly, those with a history of lung disease, and immunocompromised individuals.[4] There are over 140 NTM species reported in the literature, of which 25 species have been strongly associated with NTM diseases.[5] *Mycobacterium avium* complex (MAC) is the most frequently associated with pulmonary disease, causing up to half of NTM cases worldwide.[6-8]

In developing countries where TB is endemic, our group and others have shown that failure to recognize NTM infections commonly leads to misdiagnosis of lung disease presenting with non-specific symptoms like coughs, fever and weight loss.[9,10] This results in inappropriate empiric treatment that is lengthy, expensive, toxic, and may result in the development TB drug resistance.[9,11] In Mali, we recently reported that NTM infections was identified in 18% of clinically classified chronic TB cases.[9,12] We also reported that 12% of all patients in Mali with presumptive TB are in fact infected with NTM.[9] Nearly 73% of the NTM cases, are caused by *Mycobacterium avium* complex (MAC).[9] The same epidemiological pattern has been reported in others TB endemic setting.[7]

Sputum smear microscopy (SSM), the most widely used TB diagnostic tool for more than a century and is relatively easy to perform.

Table 1
Interpretation Criteria of the Multiplex MTB/NTN Assay.

Identification	Criteria					
	IS16110/senX3-regX3	IS1311 +DT1	Pan-Mycobacterium	Extraction control: <i>cotJ</i> C gene	Positive controls MTBC/MAC	Negative control
<i>M. tuberculosis</i> complex	+	-	+	+	+	-
<i>M. avium</i> complex	-	+	+	+	+	-
Other NTM	-	-	+	+	+	-
Negative results	-	-	-	+	+	-

present in all mycobacterial species was included as a third panel to identify Mycobacterium that are not MAC or MTBC. The negative control was composed of the reaction mixture only without any additional nucleic acid. The *cotJ*C gene DNA fragments (Synthetic gBlocks) from Integrated DNA Technologies (IDT, Coralville, IA, USA) was added as the extraction and amplification controls, as previously shown.[20] In addition, a pre-extracted DNA mixture from MTBC and MAC strains also served as the assay reaction positive controls. All the designed primers (at least five for each gene) were crosschecked with related organisms that we had previously stored (clinical isolates), and the best ones in Multiplex format were selected for the final assay design and the analytic performance evaluation.

2.2.2. Results interpretation

A test was considered positive for MTBC if it was positive for *IS16110/senX3-regX3* genes, Pan-Mycobacterium, and for the positive controls (extraction/tube and assay controls), but negative for the negative control and MAC (Table 1). A test was considered positive for MAC if it was positive for *IS1311/DT1* genes and positive for the Pan-Mycobacterium and positive controls, but negative for the negative control and MTBC. A test was considered positive for the “other NTM” panel if it was negative for MTBC, MAC and the negative control, but positive for Pan-Mycobacterium and positive controls. A negative test result was one negative for all the targets except the positive controls only. Any other combination of positive and negative results was considered inconclusive (Table 1).

2.2.3. Analytic evaluation of the multiplex assay

This evaluation was conducted at CIGHT in Evanston using a Qiagen Rotor-Gene Q qPCR instrument. Stored uninfected sputum samples were spiked with mycobacterial DNA for MTB, MAC and other NTM, and were used to assess the designed primers and optimize the Multiplex assay using different concentrations corresponding to 50 CFUs/mL, 20 CFUs/mL, 10 CFUs/mL, 5 CFUs/mL and 1 CFU/mL in order to determine the limits of detection (Table 2). Twenty (20) samples were used for each dilution to determine the analytical performance and the detection limits of the assay. Further optimizations were performed in Mali on the Applied Biosystems™ 7500 Fast Dx Real-Time PCR Instrument using clinical isolates that were molecularly confirmed by Accuprobe™ (GenProbe Inc, San Diego, CA, USA) prior to the start of the clinical feasibility evaluation. The fully

Table 2
Analytic Performance and Limits of Detection of the New Multiplex MTB/NTM Assay.

CFU/mL	Detection of MTBC and MAC		
	IS16110 + senX3-regX3 for MTBC	IS1311 + DT1 for MAC	Pan-Mycobacterium for all Mycobacteria
50	20/20	20/20	20/20
20	20/20	20/20	15/20
10	20/20	20/20	12/20
5	20/20	20/20	0/20
1	0/20	0/20	0/20

MTBC: *Mycobacterium tuberculosis* complex, MAC: *Mycobacterium avium* complex

characterized isolates included *M. tuberculosis* H37Rv, *M. avium*, *M. scrofulaceum*, *M. smegmatis*, *M. intracellulare*, *M. fortuitum*, *M. goodii*, *M. kansasii*, *M. bovis*, *M. chelonae*, *M. palustre*, *M. simiae*, *M. cubicae*.

2.4. Clinical Feasibility Study: Data Collection and Management

After signing an IRB-approved informed consent form, new clinically TB presumptive patients which are 18 years old and more. To be enrolled patient can be chronically and empirically treated for TB with for more than six months with first-line drugs, and clinically presumptive NTM patients, were enrolled in the study. The control subjects were recruited from the same health care centres as tested patients and 5 mL sputum was collected from each as for the other groups. These controls were symptomatic patients presenting with presumptive TB but ended up being sputum culture negative. Two sputum samples and blood samples for HIV testing were obtained from each participant. A standardized questionnaire was used to collect participant's socio-demographic and clinical information. All data including laboratory results were recorded into a secure web-based data management platform, REDCap.

2.5. Statistics

The data were exported to and analysed with Stata 17® and Epi info version 7.2. The sensitivity, specificity, VPP, and VPN and their confidence intervals were determined using Wilson Score. The study was designed to be a “clinical feasibility study” and the early diagnostic performance between the new Multiplex and the Xpert MTB/RIF was measured. The full clinical evaluation study is expected to be next. The patients that were missing data from the multiplex, Xpert MTB/RIF or sputum culture for contamination or indeterminate results (6 cases) were not considered for data analysis.

2.6. Sample size determination

This was a pilot early clinical feasibility study to evaluate the potential performance of the new designed assay. As such, a sample size calculation was not needed, but most feasibility studies range between 10 and 300 participants.[24] In this particular study, a total of 190 participants were enrolled in 4 disease groups and a control group (Table 3). This first step of development will allow an improved next version that will move to a full automation and a formal clinical validation study with bigger sample size in different study settings.

2.7. TB Sputum smear microscopy, mycobacterial culture and identification

Part of each sputum samples received was decontaminated using the modified Petroff's method with N-acetyl-L-cysteine–sodium hydroxide (NALC-NaOH) by mixing 5 mL of sputum with an equal volume of NALC- NaOH in a 50 ml round bottom processing tube, which was then vortexed periodically for 15 minutes. This mixture was neutralized with a phosphate buffer (pH 6.8) to a maximum total volume of 50 ml and centrifuged for 8000 g for 10 minutes. The

Table 3
Clinical and Sociodemographic Characteristics of the Study Participants.

Participant Characteristics	Control n= 47	Naive n=30	HIV/TB n=36	Rx Failure n= 70	NTM n= 7	TOTAL N= 190
Gender						
Male n (%)	32 (23.5)	23 (16.9)	19 (14.0)	57 (41.9)	5 (3.7)	136 (71.6)
Female n (%)	15 (27.8)	7 (13.0)	17 (31.5)	13 (24.1)	2 (3.7)	54 (28.4)
HIV Status						
Positive n (%)	8 (11.9)	14 (20.9)	33 (49.3)	11 (16.4)	1 (1.5)	67 (35.3)
Negative n (%)	39 (31.7)	16 (13.0)	3 (2.4)	59 (48.0)	6 (4.9)	123 (64.8)
Age						
< 35 n (%)	15 (22.4)	15 (22.4)	11 (16.4)	26 (38.8)	0 (0.0)	67 (35.3)
35 to 55 n (%)	29 (28.7)	11 (10.9)	21 (20.8)	33 (32.7)	7 (6.9)	101 (53.2)
>56 n (%)	9 (40.9)	4 (18.2)	4 (18.2)	5 (22.7)	0 (0.0)	22 (11.5)
Marital status						
Married n (%)	34 (24.4)	26 (17.9)	32 (22.1)	47 (32.4)	6 (4.1)	145 (76.3)
Single n (%)	11 (28.9)	3 (7.9)	3 (7.9)	21 (55.3)	0 (0.0)	38 (20.0)
Others n (%)	2 (28.6)	1 (14.3)	1 (14.3)	2 (28.6)	1 (14.3)	7 (3.7)
Smoking						
Yes n (%)	4 (11.1)	5 (13.9)	3 (8.3)	24 (66.7)	0 (0.0)	36 (18.9)
No n (%)	43 (27.9)	25 (16.2)	33 (21.4)	46 (29.9)	7 (4.5)	154 (81.1)
Sputum Culture n (%)						
Positive n (%)	0 (0.0)	17 (56.7)	8 (22.2)	23 (32.9)	7 (100)	55 (28.9)
Negative n (%)	42 (89.4)	11 (36.7)	28 (77.8)	45 (64.3)	0 (0.0)	126 (66.3)
Contaminated n (%)	5 (10.6)	2 (6.6)	0 (0.0)	1 (2.8)	0 (0.0)	8 (4.8)

supernatant was discarded aseptically. The pellet was used for Acid Fast Bacilli (AFB) smears after staining with Auramine/Rhodamine and re-suspension of the pellet in 2 mL solution of the phosphate buffer; 0.5 mL of the suspension was used for the BACTEC™ Mycobacteria Growth Indicator Tube (MGIT) liquid medium inoculation and 0.1 mL of the suspension was used for the solid media 7H11 Middlebrook inoculation under sterile conditions. The inoculated MGIT-960 tubes were loaded in the BACTEC™ 960 system, and continuously monitored for positivity during over a 42-day period. The 7H11 solid media were also incubated at 37°C for 6 weeks and checked every week for bacterial growth. Positive results for the BACTEC™ MGIT-960 tubes and colonies on 7H11 Middlebrook agar were confirmed by the presence of AFB on microscopic examination of a smears made from the cultures. Positive AFB culture was identified phenotypically and confirmed by the TB Capilia test, a validated immune-chromatographic test for the MTB complex members' identification.[25] If an AFB positive culture was found to be negative for the Capilia test, it was considered to be a possible case of NTM infection and was investigated further using the GeneProbe Accuprobe molecular confirmatory assay (GenProbe Inc, San Diego, CA, USA) for NTM identification. All laboratory members performing the control tests were blinded to the PCR results and vice-versa.

2.8. Method of the multiplex MTB/NTM assay

2.8.1. Specific capture DNA extraction

Our new specific capture DNA extraction method included several steps as previously described.[20,21] gBlocks of the *cotJc* gene were used as the process control. Ten microliters of extraction control (DNA of *CotJc* gene) and 200 μL of proteinase K were added to 1 mL of sputum in a 1.8 mL microtube containing air dried 2% SDS (Thinning tube). This solution was incubated and shaken at 55°C in a shaking heat bloc for eight minutes. The temperature was then raised to 100°C for 10 minutes. The entire volume of the solution was then transferred into a Binding tube containing 5 μL of capture probes, sodium salt and magnesium chloride. The capture probes are made of MTBC, MAC and Pan Mycobacteria-specific biotinylated oligonucleotides, which were added to the sputum. These oligonucleotides allow a streptavidin-coated paramagnetic particles (PMP) specific capture. The suspension was vortexed, incubating and shaken at 60°C for 20 minutes in the heat shaker bloc at 1,500 rpm. Twenty microliters of

the PMP suspension were then added to each binding tube and mixed with a rotating tube holder for 10 minutes. This process links/captures the DNA to the magnetic beads. The mixture is placed in a magnetic rack in order for the captured DNA to remain linked to the magnetic beads and separated from the supernatant, which is then discarded. After repeating this step three times, the DNA is then separated from the magnetic beads by first adding 20 μL of Buffer solution. That solution is incubated in shaking heat bloc at 70°C for 3 minutes. The eluted DNA containing solution was placed in a clean and DNase free microtube for the Multiplex Assay. The extracted samples were used, immediately, for PCR reaction or stored at -20°C for later use.

2.8.2. PCR Reaction

The PCR amplifications were performed as previously described.[20,21] The final optimization for the PCR reaction solution included adding 10% of glycerol, 1M of Bicine-KOH (pH= 8.0), 135mM of Potassium acetate, 20mM of Magnesium chloride, and 30mM Tris (pH=8.0) to 15ul per reaction with ultrapure water before adding 10ul of DNA sample for a total of 25ul. The two MTBC target genes are detected on FAM dye, the two MAC targets on HEX dye, the Pan-Mycobacterium target on TEX 615, and the *cotJc* extraction control on Cy5 dye (Supplementary Table 1). These dyes are on most qPCR instruments and were chosen for broad applicability of the assay).

2.9. Role of funding source

The funders had no role in study design, data collection, data analyses, interpretation, or writing of the manuscript.

3. Results

3.1. Analytic performance of the multiplex MTB/NTM

To determine the analytic performance of the newly designed Multiplex assay and its limit of detection, we selected the best performing dual-primers from a list of four primers for MTBC, MAC and Pan-Mycobacteria gene targets (Table 2). Each of the dual primers were tested on twenty (20) replicates of spiked sputum samples at each of the following concentrations: 50, 20, 10, 5 and 1 CFUs/mL. All tested 20 samples were successfully detected (100%) by the Multiplex

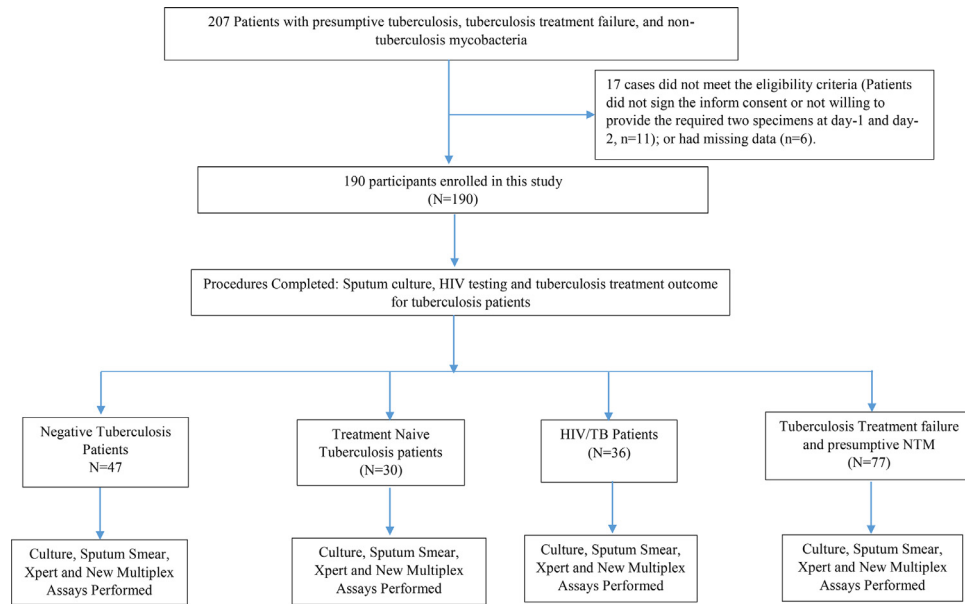


Fig. 1. Study Participants' Flow Chart.

assay down to 5 CFUs/mL for both MTBC and MAC spiked samples. The mycobacterial strains that were not MTBC or MAC were all detected by the Pan-Mycobacteria panel only at 50 CFUs/mL (20/20), 75% (15/20) at 20 CFUs/mL and 60% (12/20) at 10 CFUs/mL (Table 2). The new assay was successfully confirmed to detect all previously stored strains in Mali before beginning the clinical feasibility evaluation (Supplementary Table 2).

3.2. Clinical feasibility evaluation

3.2.1. Participants

In order to conduct a clinical feasibility evaluation for the Multiplex MTB/NTM assay, a total of one hundred and ninety (190) participants with presumptive TB and/or NTM infections were enrolled (Fig. 1). Of the participants, 71.58% (136/190) were males and 28.42% (54/190) females, and 28.9% (55/190) were culture Positive and 66.3% (126/190) were culture Negative (Table 3). Participants average age was 41.5±13.8 years with a mean weight of 58.1±16.3 kg; 76.3%

(145/190) of participants were married, 20% single, and 3.7% (7/190) other status (Table 3).

Overall, the diagnostic performance of the Multiplex MTB/NTM assay was lower than the sputum culture but comparable to the Xpert assay for the different patient groups (TB culture negative control group, TB/HIV positive group, treatment failures and TB positive naive to treatment). The Xpert assay had greater sensitivity, but the Multiplex assay demonstrated higher specificity for all groups evaluated.

3.2.2. Multiplex assay performance for tuberculosis

The multiplex assay was able to detect *M. tuberculosis* at a Ct value of 23 for tuberculosis and 23-24 for MAC with ABI 7500 PCR Instrument (Supplementary Table 2). Overall, this assay had a sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of 80.4% [66.8-89.3], 85.7% [77.7-91.1], 71.1% [57.7-81.6] and 90.9% [83.6-95.1] and the Xpert had value of 93.4% [82.85-97.7], 61.9% [52.3-70.6], 51.8% [41.2-62.2] and 95.5% [87.8-98.4],

Table 4
Multiplex and Xpert MTB/RIF diagnostic performances relative to sputum culture.

		Multiplex RT-PCR	Xpert MTB-RIF	
All participants (N=190)	Sensitivity (%) [CI]	80.4 [66.8-89.3]	93.4 [82.85-97.7]	
	Specificity (%) [CI]	85.7 [77.7-91.1]	61.9 [52.3-70.6]	
	PPV (%) [CI]	71.1 [57.7-81.6]	51.8 [41.2-62.2]	
	NPV (%) [CI]	90.9 [83.6-95.1]	95.5 [87.8-98.4]	
	TB Patient Naive to treatment (N=30)	Sensitivity (%) [CI]	82.4 [58.9-93.8]	94.1 [73.0-98.9]
TB Patient Naive to treatment (N=30)	Specificity (%) [CI]	90.9 [62.2-98.3]	81.8 [52.3-94.8]	
	PPV (%) [CI]	93.3 [70.1-98.8]	88.9 [67.2-96.9]	
	NPV (%) [CI]	76.9 [49.7-91.8]	90.0 [59.5-98.2]	
	HIV Patient (N=36)	Sensitivity (%) [CI]	71.4 [52.9-84.7]	96.4 [82.2-99.3]
	HIV Patient (N=36)	Specificity (%) [CI]	96.2 [81.1-99.3]	76.9 [57.9-88.9]
PPV (%) [CI]		95.2 [77.3-99.1]	81.8 [65.6-91.3]	
NPV (%) [CI]		75.8 [58.9-87.1]	95.2 [77.3-99.1]	
Treatment failure, retreatment and NTM suspected patients (N=77)		Sensitivity (%) [CI]	75.0 [50.5-89.8]	87.5 [63.9-96.5]
		Specificity (%) [CI]	71.1 [56.6-82.2]	37.7 [25.1-52.3]
	PPV (%) [CI]	48.0 [30.0-66.5]	33.3 [21.0-48.4]	
	NPV (%) [CI]	88.8 [74.6-95.5]	89.4 [68.6-97.0]	
	All (without treatment failure participants) (N=113)	Sensitivity (%) [CI]	83.3 [66.4-92.6]	96.7 [83.3-99.4]
Specificity (%) [CI]		96.6 [88.6-99.0]	80.0 [68.2-88.1]	
PPV (%) [CI]		92.5 [82.3-96.5]	70.7 [55.5-82.3]	
NPV (%) [CI]		92.2 [82.7-96.5]	97.9 [89.3-99.6]	

CI: confidence Interval; PPV: Positive Predictive Value; NPV: Negative Predictive Value

Table 5
Clinical Performance of the Multiplex MTB/MTN in the Detection of *Mycobacterium avium* complex using Wilson confidence interval.

Sputum Culture + Accuprobe MAC	Multiplex MTB/NTM		
	Positive	Negative	Total
Positive (n)	5	0	5
Negative (n)	0	185	185
Sensitivity % (CI)	100 [56•6-100]		
Specificity % (CI)	100 [97•9-100]		
Positive Predictive Value % (CI)	100 [56•6-100]		
Negative Predictive Value % (CI)	1000 [98•0-100]		

Mycobacterium avium complex (MAC)

respectively (Table 4). The specificity calculation did not include the MAC cases detected. These results are impacted by the inclusion of treatment failure cases, for which molecular assays such as the Xpert assay are known to not perform well, yielding high rates of false positives, due to residual bacterial nucleic acid materials remaining in the lung and sputum samples after treatment.[26] While the bacteria will not grow on agar plates, they may yield a positive molecular assay. The rationale of having the treatment failure group was to increase the likelihood of detecting NTM cases.[9] When excluding treatment failure cases, the Multiplex assay had a Sensitivity, Specificity, PPV and NPV of 83.3% [66.4-92.6], 96.6% [88.6-99.0], 92.5% [82.3-96.5] and 92.2% [82.7-96.5] and the Xpert had values of 96.7% [83.3-99.4], 80.0% [68.2-88.1], 70.7 [55.5-82.3] and 97.9% [89.3-99.6], respectively (Table 4).

3.2.3. Multiplex assay performance for tuberculosis naive to treatment

The performance of the Multiplex assay for this group had a sensitivity, specificity PPV, NPV of 82.4%, 90.9%, 93.3% and 76.9%) and the Xpert had a value of 94.1%, 81.8%, 88.9%, 90.0%, respectively (Table 4).

3.2.4. Multiplex assay performance for tuberculosis and HIV co-infected

Similar trends were seen in this group with better sensitivity for Xpert but increased specificity with the Multiplex assay, with a sensitivity and specificity of 71.4% and 96.2% for the Multiplex and 96.4 and 76.9 for the Xpert, respectively (Table 4).

3.2.5. Multiplex assay performance for tuberculosis treatment failure cases

The rationale for including this group, although historically performing less accurately under molecular assays, was to increase the likelihood of finding NTM cases given that the Multiplex has a panel for NTM detections. As expected, the sensitivity and specificity for this group were worse for both the Multiplex and Xpert at 75.0% and 71.1%, and 87.5% and 37.7%, respectively (Table 4).

4.2.6. Multiplex assay performance for nontuberculous mycobacterial infections

Of the 190 participants, there were seven presumptive NTM infection cases, but five (5) were confirmed to be positive by sputum culture. All five (5) were MAC cases (Table 5). These cases were all confirmed indirectly (post sputum culture) by Accuprobe assay. We should note that all five cases fulfilled the American Thoracic Society (ATS)'s definition of MAC disease, with MAC detected in at least two sputum samples collected at different times, a presence of TB/NTM infection-like respiratory symptoms (fever, cough, weight lost, night sweats) and *M. tuberculosis* not detected in the sputum samples.[27] The Multiplex assay in combination with the Xpert assay, could serve as a complement to the current diagnostic algorithm for patients with TB/NTM infection-like symptoms. For the pan-mycobacteria panel and "Other-NTM", the clinical context is part of the diagnostic process, as patients have to fulfil the American Thoracic Society (ATS) diagnostic criteria for NTM disease described above.[27]

4. Discussion

This new Multiplex molecular assay for TB, MAC and other NTM infections can significantly improve the management of patients presenting with TB/NTM infection-like symptoms. This is particularly important in the growing number of settings where these infections co-exist and are continuing to increase both in developing countries traditionally with endemic TB and in developed countries where MAC infections dominate among TB/NTM-like cases. Our Multiplex assay showed a great analytic and clinical performance similar to the Xpert assay, in addition to providing an option for simultaneous detection of mycobacterial infections other than TB. Some NTM stored samples previously identified were tested on the new primers and 100% concordance was found. This multiplex assay has shown a great analytical performance (5 CFUs/mL for TB and MAC and 20 CFUs/mL for other NTMs). The analytical performance for other NTMs (other than MAC) is lower at a detection limit of 20, but it is still at least as good as the analytic performance reported for the Xpert assay for tuberculosis (15.6 to 112.6 CFUs/mL).[28,29] The overall sensitivity, specificity, PPV and NPV of the Multiplex in cases without treatment failure were 83.3%, 96.6%, 92.5% and 92.2%, while the Xpert had value of 96.7%, 80.0%, 70.7% and 97.9%, respectively for the same patients compared to sputum culture (Table 4). The Multiplex assay with the Xpert assay, may be used as a complement to the current algorithm of diagnosis of patients with TB/NTM infection-like symptoms. The Multiplex and Xpert did not perform well in this study, because in big part of the inclusion of the treated group, in which residual DNA during and after treatment could lead to false positives.

Globally, NTM is not routinely diagnosed despite the availability of treatment, including the most prevalent, MAC infection. MAC is the leading cause of pulmonary NTM infections in both patients with or without HIV infection.[7,30] NTM infections are associated with substantial morbidity and mortality across the world.[31] While NTM pulmonary or systemic disease have been well characterized in developed countries,[32] it is largely undescribed and unappreciated in developing countries with a high incidence of both tuberculosis and HIV. This may be due to detection bias, because symptoms of NTM are often indistinguishable from TB and there is an absence in low and middle-income countries of appropriate diagnostic tools for NTM infections. A high proportion of TB patients are still empirically treated without any bacteriological confirmation.[17] SSM, in TB endemic settings, is often the only biological test available for TB.[13] It has a high detection limit [10,000 colony forming units per millilitre (CFU/mL)] [33] and very poor sensitivity, identifying only 50-80% of all TB cases, particularly in settings where NTM is common.[9,34] Patients are generally presumed of potentially having NTM disease only after failure of toxic and lengthy TB treatment.[35]

Immunocompromised patients, including those with HIV infection, have worse outcomes for both TB and NTM. NTM and TB infections are often associated with immunocompromised patients (i.e., HIV infection); however, NTM infections are increasing in both prevalence and incidence in immunocompetent patients as well. [31,36,17,37,38] The ability to discriminate NTM from TB infection is thus of paramount importance in immunocompromised patients,[36] and highlights the need for new diagnostic tools to simultaneously detect the two types of mycobacterial infections.

Treatments and outcomes may differ from one NTM species to another.[36] However, there is no available treatment for many species, but fortunately, MAC, the most frequent NTM disease, is treatable with two or three antimicrobials for at least 12 months, including macrolides (clarithromycin or azithromycin), ethambutol, and rifamycins (rifampin or rifabutin).[36] Physicians, in low resource settings, often initiate presumptive treatment, which may be toxic and time consuming, using precious resources that could have cured another patient for a more efficacious diagnostic test. Identifying

these patients and ensuring appropriate treatment is critical in order to combat TB drug resistance and effectively treat those with NTM infection or TB. The NTM prevalence is not negligible in TB/NTM infection like patients and is increasing worldwide. In Mali and many other TB endemic countries, significant overlap exists between NTM diseases and TB.[2,3] In a recent study in Bamako, Mali, we found that 12% of presumptive TB cases were in fact infected by NTM, and, of those, 73% were infected by MAC.[9] Our most recent data from Mali also confirmed a 12% prevalence of NTM in all TB suspects (new and previously treated) seen at the major TB Referral Center at the Point-G Hospital of Bamako.[12] Similar data have been reported from Kenya, Burkina Faso and Nigeria.[39-41] Although it is not understood why MAC and other NTM diseases have been increasing worldwide including traditionally TB endemic setting,[7] environmental changes, aging, HIV/AIDS and use of immunosuppressors are known to be associated factors.[7,36]

The Multiplex had a better specificity than Xpert for all TB infection tested groups, although the Xpert had greater sensitivity. The improved specificity of the Multiplex is clinically relevant, as reflected in a better PPV. The new version of Xpert, the Xpert Ultra, has a 5% improvement compared to the old version but at the cost of the assay specificity.[26,42] Moreover, the new assay can detect and differentiate TB from NTM infection, while the Xpert includes drug resistance option. Thus, the new assay is not meant to replace the Xpert assay, but rather can serve as a complement by providing an additional fast and effective option, depending on geography, epidemiology and the patient's individual clinical context.[10] This will save resources as in the current practice, NTM patients often get tested for TB first (and sometimes even treated unnecessarily with TB drugs). The sputum culture for TB takes at least 8 weeks for negative samples before being tested for NTM. We found during the field clinical evaluation a slight decrease in the performance of the assay. An automated version of the assay in the next phase of development will ease the assay procedure and may help in performance consistency.

This study has some limitations. The assay is still at this point an open-system, usable on many PCR platforms but not yet automated to warranty consistency in performance. In addition, the project was not a clinical validation study but rather one of clinical feasibility to evaluate the early performance of the assay. Weaknesses of the assay from this early study will be recorded and corrected in the next phase of development. The Multiplex assay seems to have a great performance for MAC detection, but only five MAC cases were included in this study. Future clinical studies should include more MAC and NTM cases from different settings. Nevertheless, based on these encouraging results, the Multiplex assay should be adapted to a scalable, quick (less than 30 minutes) and easy point of care (POC) automated system that should be tested in various clinical and geographical settings in a multi-centric study. Many potential options are available for automation such as making cartridges for the assay for Cepheid's GeneXpert instrument or BD MAX™ PCR instruments. Nevertheless, this project has laid the framework and provided the rationale and justification for bigger trials of the assay, particularly in areas where TB and NTM disease are both prevalent.

This novel multiplex MTB/NTM assay has a great analytic and clinical performance for TB and NTM infections and will complement the current diagnostic algorithm and tools for patients with TB/NTM infection like symptoms. The employment of this multiplex assay will save time and resources by testing for more than one analyte, and more importantly, it has the potential to save lives worldwide, wherever TB and NTM coexist.

Contributors

YSS, MAB, BD, SMM, BT, RLM, JLH, FS, OK, MT, MG, BB, SD, IBD, DD, BPPD, IC, IK, ED, KO, BK, MM designed the study; MAB, YSS, MM, SMM set up and optimized the assay; FS, OK, MT, MG, BB, SD, IBD,

DD, BPPD, IC, IK, ED, KO and BK enrolled, conducted the lab assays, collected and entered the data; YSS, IC, BK, AK and MM analysed the data; YSS, OK and FS verified the underlying data; YSS, BB, OK, IC, BPP, MM, SD drafted the manuscript; MM, CJA, BD, GT, YT, SD, BT, JLH, RLM, SD, SMM edited the manuscript. All authors read and approved the final version of the manuscript.

Declaration of Competing Interest

The authors have no conflicts of interest to report.

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Data sharing statement

All data and code used for producing the results are freely available for others upon request. Request can be sent to these emails: mamoudou.maiga@northwestern.edu and/or sadio@icermali.org.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ebiom.2021.103527.

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