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Diagnostics

New assay to diagnose and differentiate between *Mycobacterium tuberculosis* complex and nontuberculous mycobacteria

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ABSTRACT

The purpose of the present study was to create a real-time PCR test system allowing simultaneous detection of nontuberculous mycobacteria (NTM) and *Mycobacterium tuberculosis* complex (MTBC) both in culture and sputum. NTM cultures (18 strains, 18 species), MTBC cultures (16 strains, 2 species) and non-mycobacterial microorganisms from the collection of the Central Research TB Institute (CTRI) were used for the preliminary evaluation of the test system. 301 NTM cultures from patients with mycobacteriosis were used to assess the sensitivity of the developed test system. Clinical respiratory samples (sputum) from 104 patients with mycobacteriosis, 3627 patients with tuberculosis and 118 patients with other lung diseases were used for diagnostic sensitivity and specificity testing. The specificity and sensitivity of the assay for MTBC was found to be 100% both in culture and sputum samples; for NTM, the specificity was 100% in culture and sputum, the sensitivity reached 100% in culture and 73.1% in sputum samples. Positive predictive value (PPV) and negative predictive value (NPV) of the assay for culture were both 100%, for clinical material 100% and 80.8%, respectively. The limit of detection at the probability of detection 95% (LoD95%) was estimated to be 16 cfu/ml for *M. tuberculosis* H37RV and 1200 cfu/ml for *M. avium*.

1. Introduction

Tuberculosis is the ninth leading cause of death worldwide and the leading cause of death from a single infectious agent, ranking above HIV/AIDS [1]. The diagnosis of diseases caused by nontuberculous mycobacteria (NTM) attracts considerable attention [2–4]. NTM can be found in the environment, including water and soil. They can cause opportunistic infections and, among others, chronic pulmonary disease with symptoms similar to tuberculosis (TB) such as chronic cough (with or without sputum production), chest pain and weight loss [5]. Along with that there are a lot of reports of mixed TB-NTM infections especially in countries with high TB burden [5–9]. Such countries also tend to be resource-poor, and the diagnosis of pulmonary TB used there is mostly based on the microscopic detection of acid-fast bacilli (AFB) in sputum samples [7]. Nucleic acid amplification tests such as Xpert MTB/RIF Assay (Cepheid; Sunnyvale, CA) [10] and XtracTB Assay [11] provide a rapid alternative to culture but they do not detect nontuberculous mycobacteria. Mixed infections cannot be ruled out. If MTB-NTM co-infection takes place, NTM might remain undetected and cause failure of drug susceptibility testing. Non-valid results of drug susceptibility testing obtained in the case of mixed culture may in turn lead to the wrong TB treatment regimen chosen. In parallel, undetected clinically significant NTM infection may be treated inefficiently.

In Russia detection and treatment of patients with mycobacteriosis remains a big problem [12,13]. Commercial kits of domestic manufacturers are used to detect MTBC DNA in bacteriological laboratories, however, there are no analogues for rapid detection of NTM and for simultaneous detection of NTM and MTBC. The only available NTM assays in Russia are the kits of Hain Lifescience (Germany), based on the Line Probe Assay technology [14,15]. The technology is time-consuming as the analysis takes at least 5 h, it is labor-intensive and has limitations for direct clinical sample testing [16]. PCR-based assays for detecting NTM have been developed before, but their design requires usage of culture material [17–20]. The first multiplex PCR assays for NTM and MTBC detection and differentiation required restriction analysis [21] and gel electrophoresis [22,23] or used time-consuming

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Table 1
Bacterial strains from CTRI collection used to assess the specificity and sensitivity of the assay for NTM and MTBC DNA.

Strains belonging to genus *Mycobacterium*				Strains not belonging to genus *Mycobacterium*	
NTM strains		MTBC strains			
1	*M.avium,* CTRI-NTM-3	1	*M.bovis* Bovinus An-1, CTRI-40	1	*Achromobacter xylosoxidans*
2	*M.abscessus spp. abscessus,* 15-0131	2	*M.bovis* Bovinus An-2, CTRI-41	2	*Bukholderia cenocepacia*
3	*M.abscessus spp. chelonae*, CTRI-NTM-9	3	*M.bovis* Bovinus An-5, CTRI-42	3	*Candida ablicans*
4	*M.fortuitum spp. peregrinum,* CTRI-NTM-206	4	*M.bovis* Bovinus An-6, CTRI-43	4	*Corynebacterium* sp.
5	*M.fortuitum spp. fortuitum*, CTRI-NTM-318	5	*M.bovis* Vallee, CTRI-38	5	*Gordonia polyisoprenivorans*
6	*M.gastri,* CTRI-NTM-6	6	*M.bovis* Bovinus 8, M-12	6	*Nocardia farcinica*
7	*M.gordonae,* 14-8773	7	*M.bovis* BCG_1878, CTRI-45	7	*Klebsiella pneumoniae*
8	*M.intracellulare,* CTRI-NTM-29	8	*M.bovis* BCG 361, GISK	8	*Moraxella catarrhalis*
9	*M.interjectum,* CTRI-143	9	*M.bovis* BCG 367, GISK	9	*Proteus vulgaris*
10	*M.kansasii,* CTRI-NTM-7	10	*M.bovis* BCG 368, GISK	10	*Pseudomonas aeruginosa*
11	*M.malmoense,* CTRI-NTM-256	11	*M.bovis* BCG GFP	11	*Rhodococcus bronchialis*
12	*M.mucogenicum,* CTRI-NTM-378	12	*M.tuberculosis* W186*, R-101*	12	*Staphylococcus aureus*
13	*M.nonchromogenicum,* CTRI-NTM-65	13	*M.tuberculosis* AI73*, R-485*	13	*Staphylococcus epidermidis*
14	*M.scrofulaceum,* CTRI-NTM-53	14	*M.tuberculosis* AI60*, R-434*	14	*Streptococcus pneumoniae*
15	*M.smegmatis,* 14-0313	15	*M.tuberculosis* W221*, R-477*		
16	*M.simiae,* CTRI-NTM-155	16	*M.tuberculosis* BJ27*, R-487*		
17	*M.szulgai,* CTRI-NTM-164				
18	*M.xenopi*, CTRI-NTM-14				

melting curve analysis [17–20]. There are several real-time PCR tests allowing simultaneous detection of NTM and MTBC DNA, however, they have rather low sensitivity for both MTBC and NTM [24] or there is no specific probe for NTM detection so it is impossible to detect MTBC/NTM co-infection [24–26]. Our goal was to create a real-time PCR-based test system allowing simultaneous one-tube detection of NTM and MTBC both in culture and in clinical samples.

2. Materials and methods

2.1. Mycobacterial strains

NTM, MTBC and non-mycobacterial microorganism cultures from the collection of the Microbiology department of the Central TB Research Institute (CTRI) were used at the first stage of the assay standardization and evaluation (Table 1). The collection strains have been isolated from patients with suspected tuberculosis, with complications after BCG vaccination, or with suspected mycobacteriosis.

At the second stage of the assay evaluation specificity and sensitivity were assessed using 301 NTM cultures isolated from 301 respiratory samples from CTRI patients with suspected pulmonary tuberculosis and 500 MTBC cultures from 500 respiratory samples from CTRI patients with tuberculosis. The isolates were cultured in the Bactec MGIT 960. Ziehl-Neelsen staining, blood agar culture and immunochromatographic test (MPT64TB Ag Kit, Standard Diagnostics, Korea) were done for primary identification of all cultures studied. Mycobacterium species were identified using the Hain Lifescience GenoTypeCM/AS and GenoType MTBC reagent kits. The experiment scheme is presented in Fig. 1.

2.2. Clinical samples

Clinical samples were obtained from patients undergoing treatment in the CTRI. The material from the patients was collected for routine laboratory tests (BACTEC MGIT 960 liquid culture, luminescent microscopy, PCR detection of MBTC DNA, NTM species identification) according to the regulations establishing TB clinical procedures in Russia (orders of the Russian Federation Ministry of health No. 951 dated December 29, 2014 and No. 932n dated November 15, 2012). Sputum from 104 patients with confirmed diagnosis of mycobacteriosis, 3627 patients with confirmed diagnosis of pulmonary tuberculosis and 118 patients with other lung diseases (54 with Chronic obstructive pulmonary disease (COPD), 31 with bronchiectasis, 33 with pneumonia) were used for this study. Each sputum sample was decontaminated with *N*-acetyl-L-cysteine–sodium hydroxide (NALC-NaOH) and divided into two parts. One part was used for smear microscopy and cultured in BACTEC MGIT 960, the other part was deposited for storage at −70 °C. When the first part had flagged positive on MGIT, DNA was isolated from the second part and real-time PCR was performed. The experiment scheme is shown in Fig. 1.

2.3. DNA isolation

DNA isolation from cultures and clinical samples was performed on a robotic station for molecular genetic analysis developed by Syntol JSC (Syntol, Russia) using M-Sorb-Tube-Automat kit (Syntol, Russia). For DNA isolation 0.5 ml of flagged positive BACTEC MGIT 960 culture or 0.5 ml of sputum decontaminated with NALC-NaOH were used. First, the sample was mixed with 0.5 ml of inactivating reagent supplied with the M-Sorb-Tube-Automat kit. Next, 1 ml of the mixture obtained was subjected to the DNA isolation using magnetic sorbent according to the manufacturer's instruction. The volume of extracted DNA sample was 0.12 ml, and 20 μL of that was used for each PCR.

2.4. Primer and probe design

For the development of the assay, the reagent kit Amplitub-RV-Scrin (Syntol, Russia) for rapid detection of *M. tuberculosis* complex by multiplex real-time PCR (registration certificate FSR 2010/07635 from May 6, 2010) was extended with the manufacturer's consent. In the Amplitub-RV-Scrin kit, the detection of MTBC DNA is carried out using two fluorescence channels, FAM is used for detection of *IS6110* multicopy insertion sequence and ROX for detection of the single-copy MTBC gene *regX*. The amplification of the internal positive control DNA is detected in the HEX channel. The positive control used in the assay is plasmid DNA unrelated to the assay sequences. To assess the amplification of NTM DNA, the Cy5 channel was added into the system. To differentiate NTMs from MBTC and other phylogenetically related bacteria, we analyzed a number of conservative protein sequences found by pairwise comparison of NTM genomes presented in the NCBI database at the beginning of our study using the online version of Artemis software (//www.webact.org/WebACT/generate). The selected conservative protein sequences were further analyzed using NCBI protein-protein blast for all NTM and MBTC strains and related bacteria (*Corynebacterium*, *Rhodococcus*, *Nocardia*). For protein sequences that were conserved within the NTM group, the corresponding nucleotide

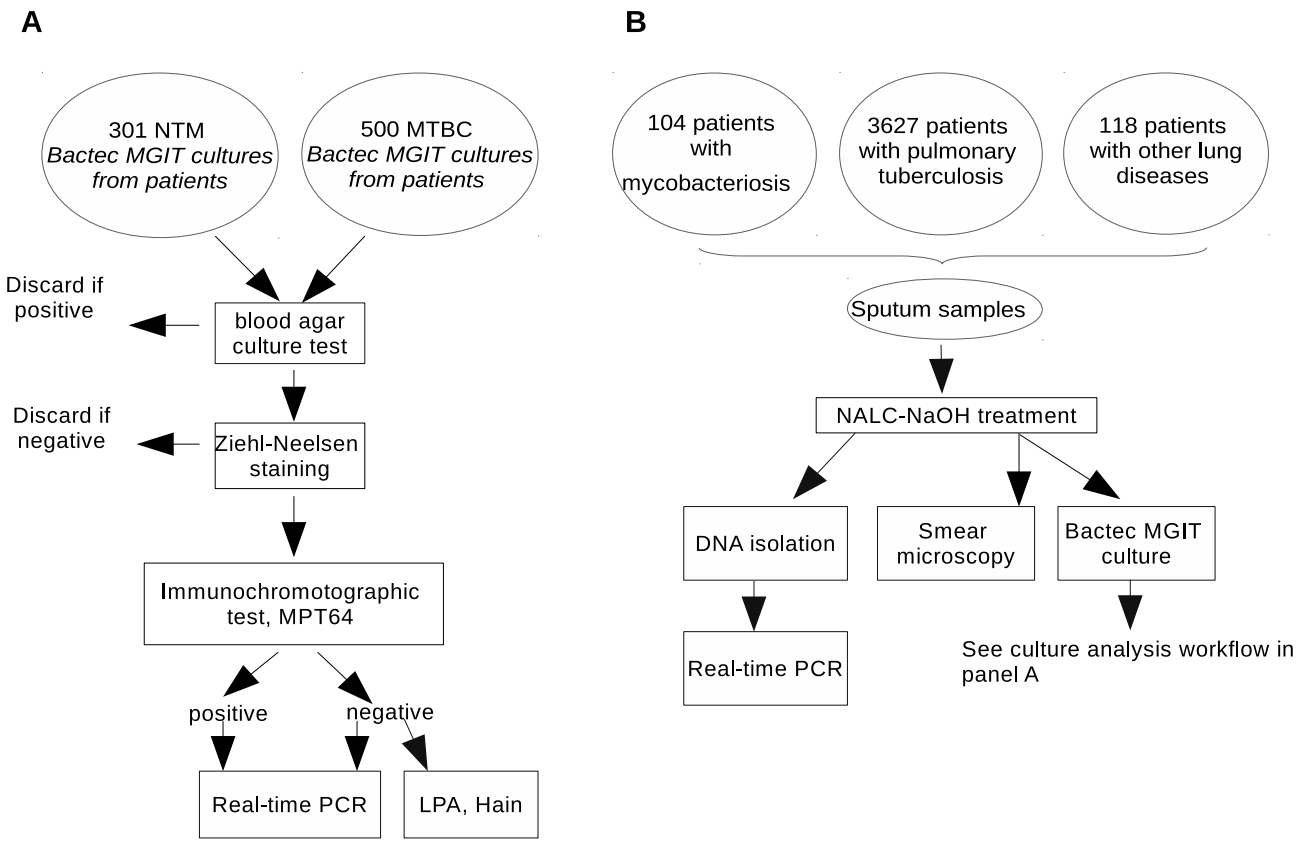

Fig. 1. Workflow of the assay evaluation. A - Evaluation of the assay analytical sensitivity and specificity on culture samples. B - Evaluation of the assay sensitivity and specificity on sputum samples.

sequences were found and aligned. The conservative region of the *meth* gene, which most efficiently differentiates NTMs and MBTC, was chosen for the assay, however, this gene is not present in *M. xenopi, M. kansasii, M. malmoense*, for which *tuf* gene was selected. A fragment of *tuf* gene was previously used as phylogenetic marker and target for species identification within the genus *Mycobacterium* using classical PCR and subsequent RFLP [27]. We analyzed the gene sequence and selected a region suitable for identifying species that do not contain the conservative region of the *meth* gene. Using the Muscle algorithm in Ugene UniPro [28] software, complete nucleotide sequences of the genes *meth* (for *M. abscessus spp. abscessus, M. abscessus spp. chelonae, M. avium subs. avium, M. avium sbs. paratuberculosis, M. intracellulare, M. smegmatis, M. phlei, M. marinum, M. neoaurum, M. parascrofulaceum, M. simiae)* and *tuf* (for *M. gastri*, *M. kansasii, M. malmoense, M. xenopi)* from the NCBI RefSeq database were aligned. Based on the resulting alignments, primers were selected for direct sequencing of *meth* (MeSy902_F 5′-CCTGCCCAACGCCTTCGGCGA-3' – forward primer; MeSy1420_R 5′-GAGATCGAGTTGACGATCGGCTTGCC-3' - reverse primer) and *tuf* (Tuf_f_153 5′-CTCGTCGAGATGGAGGTCC-3' - forward primer; Tuf_r_640 5′-ATGTAAACCTGGCCCTCGAA-3' - reverse primer) gene regions of NTM strains from the collection of CTRI (Table 1). The obtained sequences of 18 strains of NTM from the CTRI collection were added to the alignment of complete nucleotide sequences of these genes from GenBank produced before. Based on the obtained alignments, primers and TaqMan probes were selected, allowing for specific amplification of *meth* and *tuf* gene fragments. The selection of primers and probes was carried out in the Primer3 software [29]. To ensure the simultaneous detection of NTM and MTBC in a single tube, the selected primers and probes were optimized to work in multiplex with the reaction mixture of the Amplitub-RV-Scrin kit.

2.5. Real-time PCR

Each PCR was performed in a total volume of 25 μL containing 60 pmol of multiplexed primer and probe mix, 5 μL of concentrated 5XPCR master mix (Syntol, Russia), 10 units of DNA polymerase (Syntol, Russia) and 20 μL of extracted DNA sample. The reactions were performed in CFX-96 real-time PCR instrument (Bio-Rad, USA). The PCR program included a 95 °C activation step for 15 min followed by 45 cycles consisting of 15 s at 95 °C, 45 s at 63 °C and measurement of the fluorescence.

2.6. Data analysis

Data was collected and analyzed using Microsoft Excel and Microsoft Access software. The evaluation of positive predictive value (PPV) and negative predictive value (NPV) (Bayesian statistics) for cultures and clinical material was performed. The limit of detection value for the probability of detection 95% (LoD95%) was estimated using log-logistic model applied to the probability of detection (PoD) data from supplementary Table S1 [30].

3. Results

We have identified loci that allow differentiating NTM from MTBC and non-specific flora which are localized within the genes *meth* and *tuf*. Primers and probes specific for NTM DNA were selected that allow differentiating NTM from MTBC and other microorganisms in DNA isolated from the clinical samples. In combination with the reaction mixture of the Amplitub-RV-Scrin reagent kit, they provide simultaneous detection of *M. tuberculosis* complex DNA and non-tuberculous mycobacteria DNA in a single tube. The sequences of primers and probes are presented in supplementary Table S2. The final alignment of

the hybridization sites of the selected primers and probes within *tuf* and *meth* genes of the NTM species under study are shown in the supplementary Figs. S1, S2.

3.1. Activity of NTM-specific primers within the reaction mix of Amplitub-RV-Scrin

A series of 6 dilutions of *M. avium*, *M. kansasii*, *M. malmoense* and *M. xenopi* DNA from the CTRI collection (Table 1) containing 10^5, 10^4, 10^3, 10^2, 20, and 10 genome equivalents was prepared to evaluate the effectiveness of primers specific for NTM DNA, in the presence of other reaction mix components of the Amplitub-RV-Scrin kit. These NTM species were selected for the analysis because they represent 4 main groups, according to the alignment of the nucleotide sequences of the *meth* and *tuf* gene fragments at the priming sites (supplementary Fig. S1, S2). The number of genome equivalents N in the original DNA samples taken for the preparation of dilution series was calculated by the formula:

$$N = \frac{C[g/L] \times V[L] \times N_A}{M_w[Da] \times L[kb] \times 10^3}$$

where C [g/L] is the concentration of dsDNA measured with a Quantus fluorometer (Promega, USA); V [L] is the volume in which dsDNA is dissolved; N_A is the Avogadro number; M_W [Da] is the average molecular weight of the base pair (649 Da); L [kb] is the length of a genome. The genome length values presented in the KEGG database [31] were used. Each concentration was tested by PCR in 3 replicates. The PCR characteristics for NTM-specific primers with the DNA of 4 different NTMs were calculated automatically using the CFX Manager Software version 3.0. The results of the analysis are presented in Table 2.

Correlation coefficient (R^2) for all the reactions studied was 0.997–0.998 (Table 3), while the values $R^2 > 0.980$ are recommended to maintain amplification efficiency across different starting template copy numbers [32].

To evaluate the mutual influence of the amplification of specific NTM and MTB genome regions, DNA samples of four NTM species with different combinations of *meth* and *tuf* gene sequence variants (*M.avium*, *M. kansasii*, *M. malmoense*, *M. xenopi*) and MTB DNA were amplified in a single tube with the designed reaction mix. DNA of each NTM species was added to the tube with the reaction mix together with *M. tuberculosis* H37Rv DNA in the following ratios (in the genome equivalents): 10^6:10^6; 10^3:10^6; 10^2:10^6; 10^6:10, respectively. In parallel, the same amounts of each NTM and *M. tuberculosis* H37Rv DNA were added to the reaction mix separately. The amplification was carried out in a CFX96-Touch instrument (Bio-Rad, USA) in two replicates.

The difference in the average amplification threshold cycles ΔCt (channels FAM, ROX, Cy5) between mixed samples and single DNA samples of MTB and 4 types of NTM is presented in Table 3. The difference in the threshold cycles was determined by the formula ΔCt = Ct (NTM + MTB) - Ct (MB), where NTM + MTB is a mixed sample of one NTM plus MTB, MB is a sample into which single DNA of either NTM or MTB was added.

Table 3
The interference of the NTM and MTB DNA amplifications in a single-tube reaction.

Samples, genome equivalents per PCR			PCR results		
			ΔCt Mean,	ΔCt Mean,	ΔCt Mean,
NTM		Cy5 (NTM)	FAM (MTB)	ROX (MTB)	
M.avium	10^6	10	0,09	0,33	−0,4
	10^6	10^6	1,33	0,28	0,9
	10^3	10^6	1,2	0,15	0,15
	10^2	10^6	1,7	0,1	0,46
M.kansasii	10^6	10	−0,12	−0,2	−0,4
	10^6	10^6	1,77	0,05	0,98
	10^3	10^6	1,38	0,14	0,09
	10^2	10^6	1,6	0,19	0,15
M.malmoense	10^6	10	0,31	0,7	0
	10^6	10^6	0,15	−0,09	0,9
	10^3	10^6	0,4	0,06	0
	10^2	10^6	−0,2	0,15	0,2
M.xenopi	10^6	10	−0,1	0,6	−0,6
	10^6	10^6	1,36	0,14	1,1
	10^3	10^6	2	0,06	0,1
	10^2	10^6	1,9	0,2	0,1

The addition of primers and probes specific for NTM to the reaction mixture did not affect the activity of primers specific for MTB. For all the mixed samples containing NTM and MTB DNA, the positive results were obtained in the FAM, ROX and Cy5 channels. ΔCt for FAM and ROX channels between samples containing 10^6 genome equivalents of NTM DNA mixed with 10 genome equivalents of MTB DNA and samples containing only 10 genome equivalents of MTB were less than or equal to 0,7. The efficiency of NTM-specific reaction in the mixed samples was lower than in the samples containing only NTM DNA. Amplification threshold cycles in the Cy5 channel for mixed samples containing the highest amount of MTB DNA and samples containing only NTM DNA varied for different NTM groups. For all NTM species except *M. malmoense*, ΔCt was the highest for samples containing small amount of NTM DNA (10^2-10^3 genome equivalents) and a large amount of MTB DNA (10^6 genome equivalents). There was no ΔCt difference between the mixed samples and the samples containing only *M. malmoense* DNA (Table 3). Apparently, this is explained by the greater specificity of the amplification reaction for *M. malmoense* DNA compared to the other NTM groups due to the reverse primer unique for this species. For the other NTM species tested, the corresponding values ranged from 1.7 cycles (*M.avium*) to 2.0 cycles (*M.xenopi*) (Table 3).

Thus, the possibility of detecting a minimum of 10^2 genome equivalents of NTM DNA in the presence of high MTB DNA concentrations in the same sample and a minimum of 10 genome

Table 2
Characteristics of PCR for NTM-specific primers, in the presence of other reaction mix components of the Amplitub-RV-Scrin kit.

Starting quantity, genome equivalents per PCR	*M.avium*		*M.kansasii*		*M.xenopi*		*M.malmoense*	
	Mean Ct	SD	Mean Ct	SD	Mean Ct	SD	Mean Ct	SD
10^5	22,09	0,07	22,83	0,18	22,33	0,12	22,49	0,16
10^4	25,56	0,11	26,08	0,03	25,82	0,16	25,69	0,25
10^3	28,93	0,23	29,6	0,17	29,39	0,09	29,08	0,14
100	32,13	0,05	32,97	0,44	32,7	0,24	32,3	0,25
20	34,08	0,23	34,9	0,13	34,98	0,34	34,7	0,49
10	35,15	0,12	35,75	0,52	35,56	0,26	35,84	0,47
E, % (amplification efficiency)	103,5		102,5		98,8		99,8	
R^2 (correlation coefficient)	0,998		0997		0,997		0997	
Slope	−3241		−3264		−3351		−3327	
y-intercept	39,069		39,981		39,237		39,058	

Table 4
Results of NTM/MTBC DNA detection by PCR in samples of NTM cultures obtained from patients with suspected pulmonary tuberculosis.

Species identification by Genotype CM/AS kit	No. of cultures	PCR results for NTM/MTBC	
		Positive in Cy5 channel (NTM DNA detected)	Positive in FAM channel (MTBC DNA detected)
M.avium + M.tuberculosis	1	1	1
M.abscessus + M.tuberculosis	3	3	3
M.avium	107	107	0
M.intracellulare	38	38	0
M.gordonae	54	54	0
M.kansasii	23	23	0
M.xenopi	14	14	0
M.mucogenicum	1	1	0
M.scrofulaceum	1	1	0
M.peregrinum	2	2	0
M.fortuitum	25	25	0
M.abscessus	10	10	0
M.chelonae	8	8	0
M.lentiflavum	14	14	0
Total NTM	301	301	4
M.tuberculosis	494	0	494
M.bovis Bovinus	3	0	3
M.bovis BCG	3	0	3
Total MTBC	500	0	500

equivalents of MTB DNA in the presence of high NTM DNA concentrations was confirmed (see examples of *M. avium* and *M. tuberculosis* assay in supplementary Figure S3).

3.2. The assay sensitivity and specificity evaluation on DNA isolated from cultures

The initial evaluation of the assay on 18 NTM cultures, 5 M. *tuberculosis* cultures, 5 M. *bovis* BCG cultures, and 6 M. *bovis* Bovinus cultures from the CTRI collection showed that the developed test system successfully identifies 18 species of NTM and also two species belonging to the *M. tuberculosis* complex: *Mycobacterium tuberculosis* and *Mycobacterium bovis*. The system evaluation on 14 strains of microorganisms not belonging to the genus *Mycobacterium* demonstrated 100% specificity of the developed test system for NTM and MTBC DNA.

The second stage of the assay evaluation was performed using 301 cultures of NTM and 500 cultures of MTB produced from clinical samples. The experiment scheme is presented in Fig. 1A. The cultures analyzed belonged to 12 species of NTM and 2 species of MTBC (Table 4).

The developed test system for simultaneous detection of NTM/MTBC showed 100% sensitivity and specificity for NTM/MTBC DNA. One sample containing a mixture of *M. tuberculosis* and *M. avium* and 3 samples containing a mixture of *M. tuberculosis* and *M. abscessus* were positive in both FAM (MTBC) and Cy5 (NTM) channel.

3.3. The assay sensitivity and specificity evaluation on DNA isolated from clinical samples

To evaluate diagnostic specificity and sensitivity of real-time PCR test we analyzed sputum specimens from 3627 patients with a verified diagnosis of pulmonary tuberculosis confirmed by the results of the liquid culture (BACTEC MGIT 960), 104 patients with confirmed diagnosis mycobacteriosis in which NTM were detected in BACTEC twice or more times, and 118 patients with other lung diseases: 54 patients with COPD, 31 patient with bronchiectasis, 33 patients with pneumonia. The experiment scheme is presented in Fig. 1B. The results of the evaluation in comparison with Bactec culture and luminescent microscopy are presented in Table 5.

None of the 118 patients with COPD, bronchiectasis or pneumonia were positive for the NTM or MTBC DNA by the real-time PCR. All the 3627 samples obtained from patients with tuberculosis confirmed by positive liquid culture results were MTBC positive and NTM negative by real-time PCR, while the luminescent microscopy revealed AFB in 2152 out of 3627 samples (58.6%). Seventy six (73%) out of 104 patients with confirmed mycobacteriosis were NTM positive and MTBC negative by real-time PCR, 63 (60,5%) of them were AFB-positive by luminescent microscopy. The smear microscopy results for samples not detected by the NTM PCR assay are provided in the supplementary Table S3. Two sputum samples with a positive microscopy result and a negative PCR result were obtained from two patients diagnosed with cystic fibrosis and bronchoectatic disease. A negative PCR result for the detection of MBTC/NTM for a sample with high bacterial load (3 + in luminescent microscopy) may be associated with the co-infection caused by acid-fast bacilli not belonging to the genus *Mycobacterium*, for instance *Corynebacterium* sp. Such bacteria look the same as mycobacteria in the fluorescent staining, which leads to false-positive test results or an increase in smear score. Representatives of the *Corynebacterium* family (as well as those of the genus *Mycobacterium*) are often detected in patients with cystic fibrosis and in elderly patients with bronchiectasis [33–35]. Thus, the assay showed 100% specificity for the detection of MTBC and NTM DNA in the sputum. Sensitivity of the test system for MTBC and NTM was 100% and 73,1% respectively. The PPV and NPV of the real-time PCR test were 100% and 80.8% respectively.

3.4. Evaluation of the assay LoD

To estimate the limit of detection of the strain *M. tuberculosis* H37Rv, 1 ml spiked sputum specimens containing 20, 10, 5 and 2 cfu/ml of MTB H37Rv were prepared, and 20 replicates of each concentration were assayed. To estimate the limit of detection of the *M. avium* (CTRI-NTM-3, Table 1 in the paper) 1 ml spiked sputum specimens containing 10,000, 1000, 500, 300, 150 cfu/ml of *M. avium* were prepared. Eight, 16, and 20 replicates of samples containing 10,000, 1000, and 150–500 cfu/ml respectively were assayed. The LoD95% for *M. tuberculosis* H37RV was estimated to be 16 cfu/ml in the *IS6110* assay; 58 cfu/ml in the *regX* assay, and 16 cfu/ml in the combined *IS6110* and *RegX* assay. The LoD95% for *M. avium* was estimated to be 1200 cfu/ml. More data on the LoD analysis is presented in the supplementary Table S2.

4. Discussion

The results of NTM and MTBC culture analyses demonstrated 100% sensitivity and specificity of the assay for MTBC and NTM detection. Direct sputum analysis with the real-time PCR showed 100% diagnostic sensitivity and specificity for MTBC DNA and 73.1% sensitivity and 100% specificity for NTM DNA. The sensitivity of the assay for NTM and MTBC DNA is higher than the sensitivity of luminescent microscopy. Unlike luminescent microscopy, our method allows direct specific detection and differentiation between NTM and MTBC, while luminescent microscopy provides only AFB detection and is not specific for *Mycobacterium* species. There are some more real-time PCR tests providing detection of NTM or NTM/MTBC in one-tube reaction, however, they do not allow detection of NTM/MTBC co-infection due to primer and probe design [5,24–26,36]. The problem arises from the fact that two reactions in such assays detect *Mycobacterium* genus-specific marker in one channel plus MBTC in the other one; as a result TB infection produces the same assay outcome as co-infection. Misdiagnosing cases of co-infection as TB will cause the failure of drug susceptibility testing and will lead to inadequate treatment regimen [7,9,37]. Our assay allows simultaneous NTM and MTBC detection. In the present study we found 4 patients with NTM/MTBC co-infection (1 patient had *M. tuberculosis/M.avium*, 3 patients had *M. tuberculosis/M.abscessus*).

Table 5
Detection of NTM/MTBC DNA in the sputum from patients with nonspecific lung diseases and tuberculosis.

Samples in total		BACTEC MGIT 960 culture		Luminescent microscopy	PCR detection of NTM/MTBC DNA	
		MTBC detected, number (% of total)	NTM detected, number (% of total)	AFB detected, number (% of total	MTBC DNA detected, number (% of total)	NTM DNA detected, number (% of total)
Tuberculosis	3627	3627 (100)	0 (0)	2152 (58.6%)	3627 (100)	0 (0)
Mycobacteriosis	104	0 (0)	104 (100%)	63 (60.5%)	0 (0)	76 (73.1%)
COPD	54	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Bronchiectasis	31	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Pneumonia	33	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)

The limit of detection of the assay for MTBC in sputum is 16 cfu/ml (estimated on H37Rv strain) which is comparable with 5 cfu/ml LoD for MTB in the XtracTB Assay (estimated on H37Ra strain) [11] while LoD of the world market leader Xpert MTB/RIF Assay (Cepheid; USA) is 131 cfu/ml [10]. The LoD95% for *M. avium* was estimated to be 1200 cfu/ml while LoD of Genotype CM assay by Hain Lifescience (Germany) is $1.65{\cdot}10^5$ CFU/ml for culture samples (estimated on *M. bovis* BCG) [38].

5. Conclusion

We have developed the assay for the simultaneous one-tube detection of NTM and MTBC DNA in cultures and clinical samples. The assay was tested on the DNA of 2 species of MTBC (*M.tuberculosis* and *M. bovis*) and 18 species of NTM: *M. fortuitum, M. peregrinum, M. gastri, M. intracellulare, M. scrofulaceum, M. smegmatis, M. avium, M. abscessus, M. chelonae, M. gordonae, M. interjectum, M. kansasii, M. malmoense, M. mucogenicum, M. nonchromogenicum, M. simiae, M. szulgai, M. xenopi*. The possibility of detecting at least 10^2 genome equivalents of NTM DNA and 10 genome equivalents of MTB DNA in the mixed MTB/NTM culture samples was confirmed. The assay allows for specific detection and differentiation between NTM and MTBC, as well as detection of NTM/MTBC co-infection. The clinical sensitivity of the assay for MTB is comparable with XtracTB Assay [11] being higher than the sensitivity of Xpert MTB/RIF Assay [10]. The clinical sensitivity of the assay for NTM will be improved in the further development.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.tube.2018.10.004.

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