DISCRIMINATORY POWER OF MULTIPLEX PCR FOR DETECTION OF MYCOBACTERIAL CO-INFECTION

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The diagnosis of mycobacterial co-infection is one of the pressing public health issues. The study was aimed to determine discriminatory power of multiplex PCR used for species identification when detecting mixed mycobacterial populations. The study involved model samples representing the mixtures of DNA of two mycobacterial species with the ratios of 1 : 1, 1 : 9, 1 : 99, and 1 : 999 and different total DNA concentrations (10^3 gEq/mL to 10^6 gEq/mL). The model samples were assessed using the multiplex PCR-based AmpliTube-RV-Differentiation kit (Syntol LLC; Russia). It has been shown that the kit is capable of detecting the mixtures of mycobacterial species with high discriminatory power. The discriminatory power of real-time PCR used for analysis of the mixture of DNA of two mycobacterial species depended on the total DNA content in the sample and varied between 0.1% for high-rate samples (total DNA concentration 10^6 gEq/mL) and 50% for low-rate samples (total DNA concentration 10^3 gEq/mL) and corresponded to the amount of DNA of the species in the sample of at least 5×10^2 gEq/mL. When the amount of DNA of each species in the mixture was at least 5×10^2 gEq/mL, the results of PCR test for detection of co-infection did not depend on the mucobacterial species contained in the mixture, which should be taken into account when analyzing PCR results.

Keywords: *Mycobacterium tuberculosis* complex, nontuberculous mycobacteria, mycobacterial co-infection, multiplex PCR, mycobacteriosis, tuberculosis

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ДИСКРИМИНИРУЮЩАЯ СПОСОБНОСТЬ МЕТОДА МУЛЬТИПЛЕКСНОЙ ПЦР ПРИ ВЫЯВЛЕНИИ МИКОБАКТЕРИАЛЬНОЙ КОИНФЕКЦИИ

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Диагностика микобактериальной коинфекции — одна из актуальных проблем здравоохранения. Целью исследования было определить дискриминирующую способность метода мультиплексной ПЦР видовой идентификации при выявлении смешанных популяций микобактерий. Исследование выполнено на модельных образцах, представляющих собой смесь ДНК микобактерий двух видов в соотношении 1 : 1, 1 : 9, 1 : 99 и 1 : 999 с разной суммарной концентрацией ДНК (от 10^3 ГЭ/мл до 10^6 ГЭ/мл). Модельные образцы исследовали набором «Амплитуб-РВ-дифференциация» («Синтол»; Россия), основанном на мультиплексной ПЦР. Показано, что набор способен выявлять смеси видов микобактерий с высокой дискриминирующей способностью. Дискриминирующая способность метода ПЦР в режиме реального времени при анализе смеси ДНК двух видов микобактерий зависела от суммарного содержания ДНК в образце и варьировала от 0,1% для высоконагруженных образцов (суммарная концентрация ДНК × 10^6 ГЭ/мл) до 50% для низконагруженных образцов (суммарная концентрация ДНК × 10^3 ГЭ/мл) и соответствовала количеству ДНК вида в смеси не менее 5×10^2 ГЭ/мл. При количестве ДНК каждого вида в смеси не менее 5×10^2 ГЭ/мл результат ПЦР на выявление коинфекции не зависел от вида микобактерий, входящих в смесь, что необходимо учитывать при анализе результатов ПЦР.

Ключевые слова: микобактерии туберкулезного комплекса, нетуберкулезные микобактерии, микобактериальная коинфекция, мультиплексная ПЦР, микобактериоз, туберкулез

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The diseases of mycobacterial etiology, including tuberculosis, constitute a significant challenge to public health. Tuberculosis is the second most common cause of death from infectious diseases [1]. The incidence of tuberculosis in the RF decreases, while the diseases caused by nontuberculous mycobacteria (NTM) become more and more common. The same trend is observed all over the world [2–6]. The researchers believe that the growing rate of mycobacteriosis is associated with ageing of the global population and increasing rate of congenital and acquired immunodeficiency [4, 7–9].

In some cases, more often in old age and in cases of immunosuppression, the patient can be infected by both Mycobacterium tuberculosis complex (MTBC) and NTM or by several species of nontuberculous mycobacteria [2, 10–12]. The

prevalence of mycobacterial co-infection in Russia is 1.16% of all sputum smear-positive patients. The most common combinations of species in the mixed mycobacterial populations are as follows: *M. tuberculosis* + *M. avium*, *M. tuberculosis* + *M. abscessus*, *M. avium* + *M. intracellulare*, *M. avium* + *M. kansasii*, *M. avium* + *M. abscessus* [2].

In is important to diagnose the cases of mixed mycobacterial infection in time, since undetected co-infection by several mycobacterial species inevitably results in treatment failure. The treatment failure results from the fact, that NTM are resistant to the majority of anti-tuberculosis drugs and have species-specific profiles of antibiotic sensitivity [13–15].

Since tuberculosis and mycobacteriosis have similar clinical and radiographic features, these can be differentiated by identification of species in mycobacterial culture by HPLC, mass spectrometry or molecular genetic methods [16–19]. The advantage of molecular genetic diagnosis of tuberculosis and mycobacteriosis is that these methods ensure analysis of both cultures and clinical diagnostic material. In 2021, the Central Tuberculosis Research Institute together with Syntol LLC developed the multiplex PCR-based AmpliTube-NTM-Differentiation test system ensuring differentiation between MTBC and NTM and detection of 12 NTM species (*M. avium*, *M. intracellulare*, *M. xenopi*, *M. chimaera*, *M. kansasii*, *M. gordonae*, *M. lentiflavum*, *M. paragordonae*, *M. abscessus*, *M. chelonae*, *M. fortuitum*, *M. malmoense*) [20].

Accoding to the purpose, the test system must have a good potential for detection of mycobacterial co-infection, including that caused by several NTM species. Therefore, it seems to be relevant to determine the ability of this kit to detect mixed populations of mycobacteria, depending on the mixture species composition.

The study was aimed to determine discriminatory power of multiplex PCR used for species identification when detecting mixed mycobacterial populations.

METHODS

Research object

The model samples represented DNA of mycobacteria most often identified in the mixed populations mixed at various ratios. The model samples were prepared using DNA extracted from the cultures of the following mycobacterial strains from the mycobacterial culture collection of the microbiology department of the Central Tuberculosis Research Institute: *M. tuberculosis* H37Rv (TMC 102), *M. avium*. (ATCC-35719), *M. intracellulare*. (ATCC-25120), *M. kansasii* (ATCC-12478), *M. abscessus* (ATCC-19977).

Study design

DNA was extracted from the mycobacterial species cultures using the AmpliTube-RV kit (Syntol LLC; Russia). The concentration of DNA of each mycobacterial species was determined by spectrophotometry (Picopet "Picodrop" spectrophotometer; UK) and adjusted for the number of genomic equivalents. Then we prepared serial dilutions of DNA of each mycobacterial species, which were mixed at the ratios of 1 : 1, 1 : 9, 1 : 99, and 1 : 999; the total DNA concentration of the mixtures was 10^6 gEq/mL, 10^5 gEq/mL, 10^4 gEq/mL, and 10^3 gEq/mL. The model samples were assessed using the AmpliTube-NTM-Differentiation kit (Syntol LLC; Russia). Amplification was performed in the CFX96Touch thermal cycler with the optic module (Bio-Rad; USA). Species were identified in accordance with the instructions to the kit based on the presence of fluorescence-enhancement kinetic curves for appropriate signals: kinetic curves in the tube of strip No. 1 reflected accumulation of the amplification products corresponding to specific genome regions of MTBC and/or or genome regions specific for all NTM species, while in the tubes No. 2–4 the fluorescence-enhancement kinetic curves corresponded to the presence of the NTM species the kit was targeted at in the DNA sample (Table 1).

Thus, we assessed samples with various DNA load, each of which contained DNA of two mycobacterial species mixed at different ratios (Table 2). Each model sample variant was assessed in 10 replicates.

Discriminatory power of the method was determined based on its detectability limit for two mycobacterial species expressed as the smallest proportion of DNA content of one of the mycobacterial species in a sample found in the mixture in all 10 replicates.

RESULTS

The results of model DNA sample assessment by multiplex PCR for identification of mycobacterial species are provided in Table 3. Discriminatory power of multiplex PCR used for identification of species depended on the total DNA concentration in the sample. When the total DNA concentration was high (10^6 gEq/mL), the discriminatory power was 0.1; it was 1% at the concentration of 10^5 gEq/mL, 10% at the concentration of 10^4 gEq/mL, and 50% at the concentration of 10^3 gEq/mL. Such pattern was typical for all the studied mycobacterial species and was discernible when differentiating between MTBC and NTM. In model samples of all kinds this smallest proportion of the species corresponded to the mycobacterial species DNA concentration of at least 5×10^2 gEq/mL.

The probability of finding each species in the proportion one notch below discriminated (hereinafter, pre-discriminated share) varied between various mycobacterial species contained in the mixture (Table 3). MTBC was most likely to be found in the mixture in the pre-discriminated share (1×10^2 gEq/mL), along with *M. avium* and *M. intracellulare* detected as the second species in 7/10–9/10 replicates, depending on the total DNA content. The probability of finding *M. kansasii* in the pre-discriminated share was slightly lower (5/10–6/10), while the lowest probability was reported for *M. abscessus* (2/10–4/10). The same pattern of the probability of finding mycobacteria in

Table 1. The layout of the PCR strip and fluorescence detection channels

№ tube strip	Detection channel			
	FAM	ROX	HEX	Cy5
1	MTBC	NTM	MTBC	–
2	*M. avium*	*M. xenopi*	*M. intracellulare*	*M. chimaera*
3	*M. kansasii*	*M. gordonae*	*M. lentiflavum*	*M. paragordonae*
4	*M. abscessus*	*M. chelonae*	*M. fortuitum*	*M. malmoense*

Table 2. Characteristics of model DNA samples

Ratio	Concentration of DNA of mycobacterial species in the mixture (gEq/mL) with the total DNA load of the sample:			
(species 1: species 2)	10^6 gEq/mL	10^5 gEq/mL	10^4 gEq/mL	10^3 gEq/mL
1 : 1	$5.00 \times 10^5 / 5.00 \times 10^5$	$5.00 \times 10^4 / 5.00 \times 10^4$	$5.00 \times 10^3 / 5.00 \times 10^3$	$5.00 \times 10^2 / 5.00 \times 10^2$
1 : 9	$1.00 \times 10^5 / 9.00 \times 10^5$	$1.00 \times 10^4 / 9.00 \times 10^4$	$1.00 \times 10^3 / 9.00 \times 10^3$	$1.00 \times 10^2 / 9.00 \times 10^2$
1 : 99	$1.00 \times 10^4 / 9.90 \times 10^5$	$1.00 \times 10^3 / 9.90 \times 10^4$	$1.00 \times 10^2 / 9.90 \times 10^3$	$1.00 \times 10^1 / 9.90 \times 10^2$
1 : 999	$1.00 \times 10^3 / 9.99 \times 10^5$	$1.00 \times 10^2 / 9.99 \times 10^4$	$1.00 \times 10^1 / 9.99 \times 10^3$	$1.00 \times 10^0 / 9.99 \times 10^2$
9 : 1	$9.00 \times 10^5 / 1.00 \times 10^5$	$9.00 \times 10^4 / 1.00 \times 10^4$	$9.00 \times 10^3 / 1.00 \times 10^3$	$9.00 \times 10^2 / 1.00 \times 10^2$
99 : 1	$9.90 \times 10^5 / 1.00 \times 10^4$	$9.90 \times 10^4 / 1.00 \times 10^3$	$9.90 \times 10^3 / 1.00 \times 10^2$	$9.90 \times 10^2 / 1.00 \times 10^1$
999 : 1	$9.99 \times 10^5 / 1.00 \times 10^3$	$9.99 \times 10^4 / 1.00 \times 10^2$	$9.99 \times 10^3 / 1.00 \times 10^1$	$9.99 \times 10^2 / 1.00 \times 10^0$

the mixture in the pre-discriminated share was reported when analyzing the results of differentiating between MTBC and NTM: *M. avium* as NTM in the pre-discriminated share were found in the mixture with *M. tuberculosis* in 8/10–9/10 replicates, with M. abscessus in 4/10–5/10 replicates. When the proportion of mycobacterial species was two notches below discriminated (which corresponded to 1.00×10^1 gEq/mL), the results of PCR test for this species were negative, and only one dominant species was reported for the mixture.

Thus, it can be concluded that multiplex PCR allows one to detect the mixture of mycobacterial species, if the concentration of DNA of each species is at least 5×10^2 gEq/mL. When mycobacterial DNA concentration is lower (1×10^2 gEq/mL), sensitivity of the kit used for detection of mixed mycobacterial populations depends on the species composition: the probability of finding MTBC, *M. avium*, and *M. intracellulare* in the mixture is higher, than the probability of finding *M. kansasii* and especially *M. abscessus*.

DISCUSSION

MTBC and NTM cause human diseases characterized by almost the same clinical and radiographic features [21]. However, the treatment regimens for patients differ radically depending on the causative agent. That is why the need for differential diagnosis of the diseases caused by MTBC and NTM is enshrined in legislation [22, 23]. However, the existing legislation does not take into account the fact that the same patient can be infected with several mycobacterial species and does not define the value of the methods for etiological diagnosis of mycobacterial co-infection.

The molecular genetic methods allowing one to determine the pathogen within 24 h are especially useful for diagnosis of the diseases caused by mycobacteria, in contrast to the culture-based tests, the results of which can be obtained no earlier than in three weeks [24]. There is quite a lot of domestic PCR tests allowing one to detect MTBC and/or differentiate between species of *M. tuberculosis* complex [25]. There are only two kits for detection of NTM registered in the RF: the MTB-Test produced by TestGen (Russia) for detection of MTBC or NTM and the AmpliTube-NTM-Differentiation kit produced by Syntol LLC (Russia) used in our study, which enables both differentiation between MTBC and NTM and identification of NTM species. Therefore, AmpliTube-NTM-Differentiation is the only domestic kit registered in the RF that enables express identification of the species of pathogens causing mycobacterial infections. That is why our study was aimed to estimate the ability of this test to detect mycobacterial co-infection. For that, the model samples representing DNA of various mycobacterial species mixed at various ratios were prepared. It has been shown that the AmpliTube-NTM-Differentiation kit allows one to detect mycobacterial co-infection, if the concentration of DNA of NTM species in the mixture is at least 5×10^2 gEq/mL, of between 0.1 and 50% of species in the mixture depending on the total DNA concentration in the sample.

There is a lack of studies assessing the diagnostic value of molecular genetic methods for detection of mixed infection. Only one study was focused on assessing the GeneXpert methods (Cepheid; USA) and multilocus sequence analysis used for detection of mixed mycobacterial cultures [26]. The authors have shown that GeneXpert can identify MTBC mixed with various NTM species in the proportion of 1% (in the reported study, the detection limit for the species in the mixture is 3000 CFU/mL).

Comparison of discriminatory power of the GeneXpert method and the real-time PCR used in our study has shown that discriminatory power of multiplex PCR used to detect MTBC in the mixed populations is higher that that reported for GeneXpert (5×10^2 gEq/mL vs. 3×10^3 CFU/mL, respectively). Furthermore, the GeneXpert system detects MTBC only and is unable to detect mixtures of various mycobacteria. In cases of MTBC and NTM co-infection, the GeneXpert test results demonstrate the presence of MTBC only, while in cases when there is a mixture of NTM, the GeneXpert test result is negative.

The earlier reported discriminatory power of the sequencing method [26] depended on the mycobacterial species contained in the mixture. The mixture of MTBC with *M. intracellulare*, *M. kansasii*, *M. abscessus*, and *M. fortuitum* was determined as two species by the sequencing method, when the share of one of two species was at least 1% (3×10^3 CFU/mL). When the mixture of MTBC and *M. avium* was assessed by the sequencing method, the presence of *M. avium* in the mixture was determined, if its proportion was at least 10% (3×10^4 CFU/mL); when the proportion of *M. avium* was lower than 10%, MTBC only was determined [26]. Therefore, discriminatory power of multiplex PCR is higher, than that of the sequencing method, for the species *M. avium*, *M. intracellulare*, *M. kansasii*, *M. abscessus*. We have not determined discriminatory power of multiplex PCR for the mixture of MTBC + *M. fortuitum*, since, according to our data, the *M. fortuitum* species is not a common cause of mycobacterial diseases and is extremely rare in the mixed populations isolated from patients [2]. The advantage of multiplex PCR over sequencing when used for detection of mycobacterial co-infection is that DNA isolated from mycobacterial cultures is used for sequencing, while DNA isolated directly from the diagnostic material is used for multiplex PCR, which speeds up data acquisition and makes the results independent from the species-specific mycobacterial culture features.

Table 3. Results of testing of model samples of the DNA mixture of two mycobacterial species by multiplex PCR

Combination of species (species 1 : species 2)	Ratio	Number of positive PCR results among the tested DNA samples by target detection channels with the total DNA concentration			
		10^6 gEq/mL	10^5 gEq/mL	10^4 gEq/mL	10^3 gEq/mL
M. tub. : M. avi.	1 : 1	MIX –10/10	MIX – 10/10	MIX – 10/10	MIX – 10/10
	1 : 9	MIX –10/10	MIX – 10/10	MIX – 10/10	MIX – 9/10; *M. avi.* – 1/10
	1 : 99	MIX –10/10	MIX – 10/10	MIX – 8/10; *M. avi.* – 2/10	*M. avi.* – 10/10
	1 : 999	MIX –10/10	MIX – 9/10; *M. avi.* – 1/10	*M. avi.* – 10/10	*M. avi.* – 10/10
	9 : 1	MIX –10/10	MIX –10/10	MIX – 10/10	MIX – 7/10; МБТК – 3/10
	99 : 1	MIX –10/10	MIX –10/10	MIX – 7/10; МБТК – 3/10	МБТК – 10/10
	999 : 1	MIX –10/10	MIX – 8/10; МБТК – 2/10	МБТК – 10/10	МБТК – 10/10
M. tub. : M. abs.	1 : 1	MIX – 10/10	MIX – 10/10	MIX – 10/10	MIX – 10/10
	1 : 9	MIX – 10/10	MIX – 10/10	MIX – 10/10	MIX – 8/10; *M. abs.* – 2/10
	1 : 99	MIX – 10/10	MIX – 10/10	MIX – 9/10; *M. abs.* – 1/10	*M. abs.* – 10/10
	1 : 999	MIX – 10/10	MIX – 8/10; *M. abs.* – 2/10	*M. abs.* – 10/10	*M. abs.* – 10/10
	9 : 1	MIX – 10/10	MIX –10/10	MIX – 10/10	MIX – 3/10 МБТК – 7/10
	99 : 1	MIX – 10/10	MIX –10/10	MIX – 3/10; МБТК – 7/10	МБТК – 10/10
	999 : 1	MIX – 10/10	MIX – 4/10; МБТК – 6/10	МБТК – 10/10	МБТК – 10/10
M. avi. : M. int.	1 : 1	MIX –10/10	MIX – 10/10	MIX –10/10	MIX – 10/10
	1 : 9	MIX –10/10	MIX – 10/10	MIX –10/10	MIX – 7/10; *M. int.* – 3/10
	1 : 99	MIX –10/10	MIX – 10/10	MIX – 8/10; *M. int.* – 2/10	*M.int.* – 100%
	1 : 999	MIX –10/10	MIX – 9/10; *M. int.* – 1/10	*M. int.* – 10/10	*M.int.* – 100%
	9 : 01	MIX –10/10	MIX – 10/10	MIX –10/10	MIX – 9/10; *M. avi.* – 1/10
	99 : 1	MIX –10/10	MIX – 10/10	MIX – 8/10; *M. avi.* – 2/10	*M. avi.* – 10/10
	999 : 1	MIX –10/10	MIX – 8/10; *M. avi.* – 2/10	*M. avi.* – 10/10	*M. avi.* – 10/10
M. avi. : M. kans.	1 : 1	MIX –10/10	MIX – 10/10	MIX –10/10	MIX – 10/10
	1 : 9	MIX –10/10	MIX – 10/10	MIX –10/10	MIX – 8/10; *M. kans.* – 2/10
	1 : 99	MIX –10/10	MIX – 10/10	MIX – 9/10; *M. kans.* – 1/10	*M. kans.* – 10/10
	1 : 999	MIX –10/10	MIX – 8/10; *M. kans.* – 2/10	*M. kans.* – 10/10	*M. kans.* – 10/10
	9 : 1	MIX –10/10	MIX – 10/10	MIX –10/10	MIX – 5/10; *M. avi.* – 5/10
	99 : 1	MIX –10/10	MIX – 10/10	MIX – 5/10; *M. avi.* – 5/10	*M. avi.* – 10/10
	999 : 1	MIX –10/10	MIX – 6/10; *M. avi.* – 4/10	*M. avi.* – 10/10	*M. avi.* – 10/10
M. avi. : M. abs.	1 : 1	MIX –10/10	MIX – 10/10	MIX –10/10	MIX – 10/10
	1 : 9	MIX –10/10	MIX – 10/10	MIX –10/10	MIX – 9/10; *M. abs.* – 1/10
	1 : 99	MIX –10/10	MIX – 10/10	MIX – 8/10; *M. abs.* – 2/10	*M. abs.* – 100%
	1 : 999	MIX –10/10	MIX – 9/10; *M. abs.* – 1/10	*M. abs.* – 10/10	*M. abs.* – 100%
	9 : 1	MIX –10/10	MIX – 10/10	MIX –10/10	MIX – 2/10; *M. avi.* – 8/10
	99 : 1	MIX –10/10	MIX – 10/10	MIX – 4/10; *M. avi.* – 6/10	*M. avi.* – 10/10
	999 : 1	MIX –10/10	MIX – 3/10; *M. avi.* – 7/10	*M. avi.* – 10/10	*M. avi.* – 10/10

Note: NTM — nontuberculous mycobacteria; MTBC — *Mycobacterium tuberculosis* complex; *M. tub*. — *M. tuberculosis*; *M. avi.* — *M. avium*; *M. int.* — *M. intracellulare*; *M. kans.* — *M. kansasii; M. abs*. — *M. abscessus*; MIX — model sample is recognized as the mixture of mycobacterial species; cells with the rate of detecting mycobacterial species in the pre-discriminated share are highlighted in gray.

CONCLUSIONS

The diagnostic value of multiplex PCR used for detection of mixed mycobacterial populations was studied. It was shown that the multiplex PCR-based AmpliTube-NTM-Differentiation kit was capable of detecting mycobacterial mixtures with high discriminatory power. The discriminatory power of real-time PCR used for analysis of the mixture of DNA of two mycobacterial species depended on the total DNA content in the sample and varied between 0.1% for high-rate samples (total DNA concentration 10^6 gEq/mL) and 50% for low-rate samples (total DNA concentration 10^3 gEq/mL), it corresponded to the amount of DNA of species in the mixture of at least 5×10^2 gEq/mL. When the amount of DNA of each species in the mixture was at least 5×10^2 gEq/mL, the results of PCR test for co-infection did not depend on the species of mycobacteria contained in the mixture. When the amount of DNA of mycbacterial species in the mixture was below 5×10^2 gEq/mL, the PCR results depended on the mycobacterial species: the probability of detecting *M. avium* and *M. abscessus* in the MTBC mixture was higher, than the probability of detecting *M. kansasii*; the lowest probability of being detected in the pre-discriminated share is reported for *M. abscessus*, which should be considered when performing the analysis of PCR results.

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