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Nontuberculous mycobacterium occurrence in biological material and environmental samples covered by epidemiological surveillance in the Republic of Dagestan

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SUMMARY

An investigation for causes of tuberculosis occurrence and persistence on farms, as well as of continuous presence of tuberculin reactor animals on tuberculosis-free farms impeding allergy diagnosis revealed that the major cause is the persistence of pathogenic and nontuberculous acid-fast mycobacteria in the environment. To determine the occurrence of typical and atypical mycobacteria in samples covered by epidemiological surveillance, 222 biological material samples from cattle, 248 environmental samples (manure, soil, water from different sources, feedstuffs), 44 milk samples from tuberculosis-affected farms, 20 vaginal discharge samples from endometritis-affected cows and 405 sputum samples from tuberculosis-affected humans were tested. Isolation and identification were performed in accordance with the guidelines. Thirty-nine cultures were isolated from the pathological material; of these, 7 (17.9%) were identified as *Mycobacterium bovis* and 32 (82.1%) were identified as atypical mycobacteria. Among nontuberculous mycobacterium cultures, 16 (50.0%) were classified as belonging to group II, 2 (6.2%) – as belonging to group III and 14 (43.8%) – as belonging to group IV according to the Runyon classification. The following species were found to be predominant: group II – *Mycobacterium scrofulaceum* and *Mycobacterium gordonae* (scotochromogenous), group IV – *Mycobacterium smegmatis* and *Mycobacterium fortuitum* (rapidly growing). No mycobacteria were detected in milk samples and vaginal discharge samples from tuberculin reactor cows. From 405 sputum samples from tuberculosis-affected humans, 64 (15.8%) cultures were isolated, of which 55 (85.9%) were classified as *Mycobacterium tuberculosis*, 9 (14.1%) – as *Mycobacterium bovis*. Out of 248 environmental samples tested, mycobacteria were detected in 65 (26.2%) samples, of which 58 (89.2%) were atypical mycobacteria of groups II, III and IV; *Mycobacterium bovis* was isolated from 7 (10.8%) samples (soil and manure). The attempts to isolate *Mycobacterium tuberculosis* failed. The tests demonstrated the wide spread of nontuberculous acid-fast mycobacteria in the environment irrespective of the altitudinal zone. These findings constitute a basis for further monitoring of mycobacterium circulation in the environment in the Republic of Dagestan with a view of optimizing preventive measures.

Keywords: tuberculosis, atypical (nontuberculous) mycobacteria, cattle, allergy diagnosis, environmental objects, biological material, tuberculin PPD for mammals, macroorganism

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Распространение нетуберкулезных микобактерий в объектах эпизоотологического надзора в Республике Дагестан

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РЕЗЮМЕ

При определении причин возникновения и длительного неблагополучия хозяйств по туберкулезу, а также наличия на постоянной основе реагирующих на туберкулин животных в благополучных хозяйствах, способствующих затруднению аллергической диагностики, установлено, что основной является сохранение в объектах внешней среды патогенных и нетуберкулезных кислотоустойчивых форм микобактерий. В целях определения распространенности микобактерий типичных и атипичных форм в объектах эпизоотологического надзора исследовано 222 пробы биологического материала от крупного рогатого скота, 248 проб, отобранных из объектов внешней среды (навоза, почвы, воды из разных источников, кормов), 44 пробы молока из

неблагополучных по туберкулезу хозяйств, 20 проб влагалищных выделений больных эндометритами коров и 405 проб мокроты больных туберкулезом людей. Выделение и идентификацию проводили в соответствии с рекомендациями. Из патматериала удалось выделить 39 культур, из которых 7 (17,9%) идентифицированы как *Mycobacterium bovis* и 32 (82,1%) – как атипичные. Из числа нетуберкулезных микобактерий 16 (50,0%) отнесены к группе II, 2 (6,2%) – к группе III и 14 (43,8%) – к группе IV по классификации Раньона. Установлено доминирующее значение видов из группы II – *Mycobacterium scrofulaceum* и *Mycobacterium gordonae* (скотохромогенные), группы IV – *Mycobacterium smegmatis* и *Mycobacterium fortuitum* (быстрорастущие). В пробах молока и влагалищных выделений от реагировавших на туберкулин коров микобактерии не обнаружили. Из 405 проб мокроты больных туберкулезом людей удалось изолировать 64 (15,8%) культуры, из которых 55 (85,9%) отнесены к *Mycobacterium tuberculosis*, 9 (14,1%) – к *Mycobacterium bovis*. В 65 (26,2%) образцах из объектов внешней среды из 248 исследованных обнаружены микобактерии, 58 (89,2%) из которых составляли атипичные виды II, III и IV групп, в 7 (10,8%) случаях из почвенных проб и навоза выделены *Mycobacterium bovis*. Изолировать *Mycobacterium tuberculosis* не удалось. Исследования показали широкое распространение нетуберкулезных кислотоустойчивых форм в объектах внешней среды, независимо от вертикальной зональности. Полученные данные представляют базовую основу для дальнейшего динамического слежения за циркуляцией микобактерий в природе в условиях Республики Дагестан в целях оптимизации профилактических мероприятий.

Ключевые слова: туберкулез, атипичные (нетуберкулезные) микобактерии, крупный рогатый скот, аллергическая диагностика, объекты внешней среды, биоматериал, ППД-туберкулин для млекопитающих, макроорганизм

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INTRODUCTION

The issue of tuberculosis prevention has become particularly relevant, given that large numbers of animals are kept in relatively small areas with deficient insolation. Such conditions, together with intensified performance, lead to decreased body resistance and facilitate the airborne transmission of tuberculosis agent [1–3].

The improvement of diagnosis system and the development of new reliable differential diagnosis tools, involving, *inter alia*, the investigation of the role of atypical acid-fast mycobacteria in macroorganism sensitization to tuberculin PPD for mammals, are important for enhancing the effectiveness of measures to combat bovine tuberculosis [4–7].

The wide spread of atypical acid-fast mycobacteria in the environment has rendered intradermal tuberculin test with PPD for mammals less indicative and significantly complicated the diagnosis of tuberculosis [8]. The number of reports on the isolation of nontuberculous mycobacteria from animals and humans has grown with the improvement of mycobacterium culture isolation methods and the deepening of relevant knowledge [9–11]. According to the abundant literature data, atypical mycobacteria are isolated from tuberculin reactor animals in 44.6% of cases, from non-reactors – in 48.8%. However, the question of what mycobacterium species can sensitize animals to tuberculin and under what conditions has not been fully elucidated; many researchers are of the view that the ecological relationships of such species have not been adequately investigated [12, 13].

The interest in this group of mycobacteria is attributed to their ability to sensitize a microorganism to tuberculin without causing any lesions associated with tuberculosis [14, 15]. In view of this, in the absence of pathological manifestations characteristic of tuberculosis, bacteriological tests are carried out; based on the test results, the diagnosis is either confirmed or excluded. It should be added that laboratory diagnosis methods are time-consuming and require the use of highly efficient nutrient media to obtain the most accurate result [7, 16]. Bioassay, a basic laboratory method used to differentiate between specific sensitization and non-specific one (caused by atypical mycobacteria), is time-consuming (up to 3 months or more). The analysis of literature shows that bioassay has low specificity for differentiation of most atypical mycobacterium species [17, 18].

It is known that not all atypical mycobacteria can sensitize animals to tuberculin. Therefore, the issues of mycobacterium culture isolation from materials collected from animals and identification thereof should be studied inseparably from detection of allergic reactions and tuberculosis-specific postmortem lesions [19]. The issues of human-animal interface and transmissibility, as well as the possibility of human and animal mycobacterium cross-circulation remain under-researched [20]. There are reports that, in some cases, atypical mycobacteria were found to be the etiological agents of different diseases in humans [21, 22]. Most researchers reject their pathogenicity for cattle and believe that such mycobacteria only cause sensitization to tuberculin [23, 24]. It is also important that some atypical mycobacteria can cause mastitis in cows and lymphadenitis in pigs [11, 25].

It is undisputed that, due to high resistance to various physical and chemical factors owing to the high lipid substance content in the bacterial cell, pathogenic forms

of mycobacteria are widely spread in nature and have extensive contact with the microorganism [26–29].

According to the numerous reports, nontuberculous acid-fast mycobacterium circulation monitoring results indicate that such mycobacteria are well-established in the environment and currently represent the major cause of cattle sensitization to tuberculin PPD for mammals [30–35].

Despite multiple papers concerning the occurrence of these taxons in nature and their relationships with macroorganisms, many aspects of this issue require further investigation.

The study was aimed at the determination of mycobacterium occurrence in biological material samples from animals and humans, as well as environmental samples in the Republic of Dagestan in relation to the altitudinal zonality and species composition.

MATERIALS AND METHODS

For testing, 222 biological material samples from cattle were used. Besides, 248 environmental samples such as manure, soil from livestock facilities and pastures, water from different sources, feedstuffs (straw, silage, haylage, mixed grasses) were collected. In addition, 44 milk samples, 20 vaginal swab samples from endometritis-affected cows and 405 sputum samples from tuberculosis-affected humans were tested.

Before inoculation, homogenized biological materials were treated with a mixture of a 3% sodium lauryl sulfate solution and a 3% sodium hydroxide solution, human sputum samples were treated with a 0.5% chlorhexidine bigluconate solution.

The isolation of cultures was carried out using egg and saline media most commonly applied under laboratory conditions and demonstrating different growth intensity and rate (the Löwenstein – Jensen, Petragnani, Sauton, Finn-II media). Differentiation between the human tuberculosis agent and other mycobacteria was performed based on cultural and morphological, as well as biochemical properties. In some cases, mycobacteria were typed with bioassay in guinea pigs and rabbits through animal inoculation with the test material suspension prepared with a sterile saline solution.

The identification of the isolated cultures was carried out using conventional methods in accordance with GOST 26072-89 "Agricultural animals and poultry. Methods of laboratory diagnostics of tuberculosis" (COMECON Standard 3457-81) and GOST 27318-87 "Agricultural animals. Methods of identification of non-typical mycobacteria" (COMECON Standard 5627-86).

TEST RESULTS

During the bacteriological tests of 222 samples from tuberculin reactor animals, 39 cultures were isolated, of which 7 (17.9%) were identified as *Mycobacterium bovis* and 32 (82.1%) – as atypical mycobacteria. Among 32 nontuberculous mycobacterium cultures, 16 (50.0%) were classified as belonging to group II, 2 (6.2%) – as

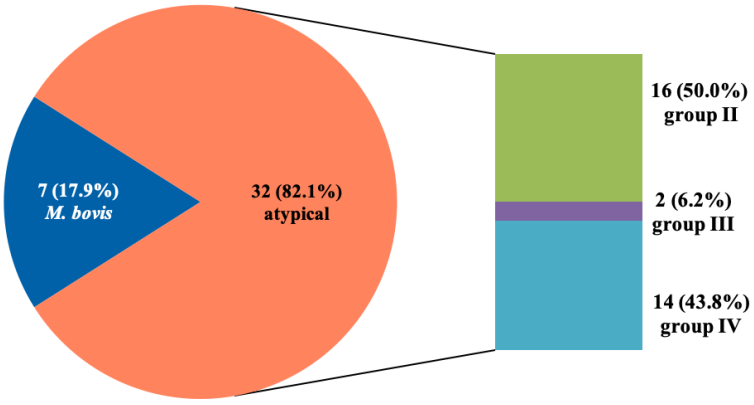

Fig. 1. Mycobacteria isolated from biological material samples from cattle

Table 1
Diversity of mycobacteria isolated from biological material samples from animals and humans

Type of sample	Number of samples	Number of isolated cultures	Type of mycobacteria					
			M. tuberculosis	*M. bovis*	Runyon's group			
					I	II	III	IV
Biological material from animals	222	39	–	7 (17.9%)	–	16 (50.0%)	2 (6.2%)	14 (43.8%)
Milk	44	–	–	–	–	–	–	–
Vaginal discharge	20	–	–	–	–	–	–	–
Sputum from humans	405	64 (15.8%)	55 (85.9%)	9 (14.1%)	–	–	–	–
Total	691	103	55	16	–	16	2	14

Table 2
Diversity of mycobacteria isolated from environmental samples

Type of sample	Number of samples	Number of isolated cultures	Type of mycobacteria					
			M. tuberculosis	*M. bovis*	Runyon's group			
					I	II	III	IV
Pasture soil	29	12	–	4	–	5	3	–
Farm soil	17	3	–	–	–	3	–	–
Stagnant water	22	2	–	–	–	–	–	2
Artesian water	26	–	–	–	–	–	–	–
River water	24	–	–	–	–	–	–	–
Mixed grass hay	30	6	–	–	–	5	1	–
Straw	20	4	–	–	–	1	3	–
Haylage	21	8	–	–	–	6	1	1
Silage	25	3	–	–	–	2	1	–
Manure	16	15	–	3	–	8	3	1
Samples from facilities	18	12	–	–	–	3	9	–
Total	248	65	–	7	–	33	21	4

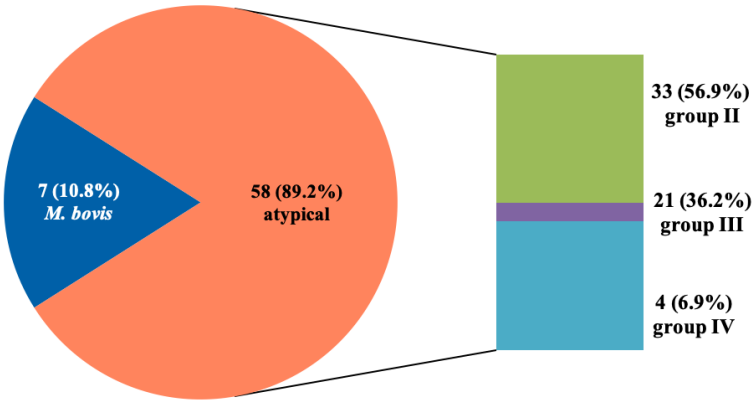

Fig. 2. Mycobacteria isolated from environmental samples

belonging to group III and 14 (43.8%) – as belonging to group IV (Fig. 1) according to the Runyon classification.

The analysis of isolated culture differentiation data allowed for identification of the predominant associations of atypical mycobacteria inhabiting the animal body. These were the combinations of *Mycobacterium scrofulaceum* and *Mycobacterium gordonae* (group II), *Mycobacterium smegmatis* and *Mycobacterium fortuitum* (group IV) according to the Runyon classification (Table 1).

No mycobacteria were detected in 44 milk samples from tuberculin reactor cows from tuberculosis-affected farms and in 20 vaginal discharge samples from endometritis-affected cows with positive tuberculin test results.

During the tests of 405 sputum samples from tuberculosis-affected humans using the Löwenstein – Jensen medium, 64 (15.8%) mycobacterium cultures were isolated, of which 55 (85.9%) were identified as *Mycobacterium tuberculosis* and 9 (14.1%) – as *Mycobacterium bovis*.

During the tests of 248 environmental samples, mycobacteria were detected in 65 (26.2%) of them; among these, 58 (89.2%) were classified as belonging to groups II, III and IV according to the Runyon classification. Bovine mycobacterium culture was isolated in 7 (10.8%) cases, the attempts to isolate *Mycobacterium tuberculosis* failed. The results are presented in Table 2 and Figure 2.

Quantitative distribution of isolated tuberculous cultures showed that such mycobacteria were isolated from pasture soil and manure samples only. Nontuberculous mycobacteria were detected in maize silage samples collected from the pit silo at the bovine tuberculosis-free dairy complex SPK "Dylym" in the Kazbekovsky Raion (submountain zone), even with direct microscopy, and this is indicative of their survivability and possible replication under the technological conditions of maize silage fermentation. Subsequently, a more detailed analysis found an association between permanent reactions to tuberculin PPD for mammals on this farm and continuous (based on the laboratory test results for a number of years) circulation of atypical mycobacteria in the environment.

The tests showed that nontuberculous mycobacteria are detected in samples collected on farms, whether tuberculosis-affected or not, irrespective of the altitudinal zone. In particular, *Mycobacterium smegmatis* and *Mycobacterium phlei*, the representatives of group IV of atypical mycobacteria, were isolated from manure samples and leftover feed samples collected from the feed bunks on the farms SPK im. Chapayeva (mountain zone) and KFKh "Rassvet" (submountain zone). *Mycobacterium scrofulaceum* (group II) was isolated from samples collected at the dairy complex SPK "Khamamatyurtovsky" (flatland zone) and those collected in the area adjacent to the farm SPK "Turchidag" (mountain zone).

The number of mycobacterium detections in the soil samples from the flatland zone pastures is higher than that in samples from the mountain zone. For example, no bacteria were isolated from samples collected in some pasture units of the farms SPK "Turchidag" and SPK im. Chapayeva (mountain zone); however, they were detected practically in all samples from the flatland zone pastures.

Also, no mycobacteria were detected in the mountain river and artesian well water samples. A group IV representative (*Mycobacterium fortuitum*) was isolated from water samples from the stagnant water bodies located near the area adjacent to the farm SPK "Rassvet" and the tuberculosis-affected dairy complex SPK "Tersky" (the Kizlyar zone of distant pastures) located in the flatland zone.

DISCUSSION AND CONCLUSIONS

The analysis of data from the study clearly reveals that atypical mycobacteria of groups II, III and IV (according to the Runyon classification) can be the major cause of macroorganism sensitization to tuberculin PPD for mammals. The predominance of group IV bacteria in soil, manure and stagnant water samples allows the conclusion that they are the typical obligate representatives of nontuberculous mycobacteria that have steadily established themselves in the environment of the Republic of Dagestan and shape the gastrointestinal mycobacterial landscape in cattle. Our findings are consistent with those of P. S. Guseynova et al., S. I. Dzhupina, M. Ridell, as well as E. Stackebrandt and B. M. Goebel from the determination of major causes of cattle sensitization to tuberculin for mammals [30–32, 35].

At the same time, in some cases during testing, no atypical mycobacteria were detected in the test samples. We believe that this is due to the imperfection of laboratory diagnosis, nontuberculous bacterium transition to a nonculturable state, various transformations and changes in the genetic structure. This fact is very important, since numerous studies, including those of recent years, show that atypical mycobacteria are isolated from bedding material and environmental samples, but they are not detected in the biological material samples from tuberculin reactor animals, and vice versa [17, 18, 22].

There is therefore a need to use specific tests for each particular typical and atypical mycobacterium species to enable the characterization of their isolation, cultivation, typing, as well as mycobacterium-like microorganisms' ability to sensitize a macroorganism to tuberculin.

Enhanced laboratory diagnosis and monitoring of nontuberculous mycobacterium circulation in the environment will allow for rapid response and interpretation of allergy test results for timely implementation of veterinary and sanitary measures.

Thus, the presented data show that atypical mycobacteria are widely spread in the environment. Bovine tuberculosis agents are isolated from the biological material samples from tuberculin reactor animals, environmental samples and soil samples from areas adjacent to tuberculosis-affected farms, as well as sputum samples from tuberculosis-affected humans. Further work will be needed to monitor the circulation of all types of mycobacteria in all physical and climatic zones of the Republic of Dagestan and to ensure control over the implementation of the veterinary and sanitary measures aimed to prevent mycobacterium spread in the natural reservoirs.

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