DISCUSSION PAPERS, LECTURES, NEW TRENDS IN MEDICAL SCIENCE

ISOLATION AND WHOLE GENOME SEQUENCING OF A LIPOPHILIC ANAEROBIC BACTERIUM, A REPRESENTATIVE OF THE SPECIES COMPLEX *CORYNEBACTERIUM TUBERCULOSTEARICUM*, FROM A TUBERCULOSIS FOCUS

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ABSTRACT

Background. *The study of the lower respiratory tract microbiome has been actively developed in recent years with the help of whole genome sequencing (WGS) methods. Due to this, it became clear that the nature of the lungs microbiota is very different from other microbial communities inhabiting the human body. One of the important directions in the study of pathological lungs biocenosis is the study of the role of the satellite microbiota of the tuberculosis focus.*
The aim of the work. *To isolate and characterize oxygen-tolerant anaerobes from the necrotic contents of tuberculomas.*
Materials and methods. *Biopsy material from 5 patients with pulmonary tuberculosis was obtained during a planned surgical treatment of tuberculoma. A pure culture was isolated from one sample during anaerobic cultivation. Lipase activity of strain was determined by plating on brain heart infusion agar (HIMEDIA, India) supplemented with 0.1 % Tween-80 and 10 mM of $CaCl_2$. Antibiotic susceptibility was determined by RAPMYCO u SLOWMYCO of TREK Diagnostic Systems (Thermo Fisher Scientific, USA). DNA from the sediment of the broth culture was isolated by the CTAB chloroform method. Whole genome sequencing was performed on a DNBSeq-G400 NGS sequencer by Genomed (Russia).*
Results. *Based on WGS results and phylogenetic analysis, the strain was identified as Corynebacterium kefirresidentii. The strain was characterized by high lipase activity and resistance only to Isoniazid, Ethionamide and Trimethoprim/Sulfamethoxazolin.*
Conclusion. *The isolation of a lipophilic anaerobic representative of the Corynebacterium tuberculostearicum species complex from a tuberculous focus indicates a possible role of the non-tuberculous microbiota in the liquefaction of caseous necrosis. We assumed that in some cases, favorable conditions are created inside the tuberculous focus for the development of satellite anaerobic lipophilic microbiota.*

Key words: *microbiome of tuberculosis focus, tuberculoma, WGS, Corynebacterium kefirresidentii*

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ВЫДЕЛЕНИЕ И ПОЛНОГЕНОМНОЕ СЕКВЕНИРОВАНИЕ ЛИПОФИЛЬНОЙ АНАЭРОБНОЙ БАКТЕРИИ, ПРЕДСТАВИТЕЛЯ ВИДОВОГО КОМПЛЕКСА *CORYNEBACTERIUM TUBERCULOSTEARICUM*, ИЗ ТУБЕРКУЛЁЗНОГО ОЧАГА

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РЕЗЮМЕ

Обоснование. *Исследование микробиома нижних дыхательных путей активно развивается последние несколько лет за счёт применения методов полногеномного секвенирования (WGS, whole genome sequencing). Благодаря этому стало понятно, что природа микробиоты лёгких сильно отличается от других микробных сообществ, населяющих тело человека. Одним из важных направлений исследования патологических биоценозов в лёгких является изучение роли сателлитной микробиоты туберкулёзного очага.*
Цель работы. *Выделение и характеристика толерантных к кислороду анаэробов из некротического содержимого туберкулом.*
Материалы и методы. *Биопсийный материал от 5 больных туберкулёзом лёгких был получен в процессе плановой операции по иссечению туберкулом. Из одного образца при анаэробном культивировании была выделена чистая культура. Липазную активность штамма определяли посевом на сердечно-мозговой агар (HIMEDIA, Индия) с добавлением 0,1 % Tween-80 и 10 мМ $CaCl_2$. Чувствительность к антибиотикам определялась в RAPMYCO и SLOWMYCO TREK Diagnostic Systems (Thermo Fisher Scientific, США). ДНК из осадка бульонной культуры выделяли CTAB-хлороформным методом. Полногеномное секвенирование осуществлено на NGS-секвенаторе DNBSeq-G400 компанией «Геномед» (Россия).*
Результаты. *По результатам WGS и по данным филогенетического анализа штамм был идентифицирован как Corynebacterium kefirresidentii. Штамм характеризовался высокой липазной активностью и устойчивостью только к изониазиду, этионамиду и триметоприму/сульфаметоксазолу.*
Заключение. *Выделение из туберкулёзного очага липофильного анаэробного представителя видового комплекса Corynebacterium tuberculostearicum свидетельствует о возможной роли нетуберкулёзной микробиоты в процессах разжижения казеозного некроза. Нами выдвигается гипотеза о том, что внутри туберкулёзного очага в некоторых случаях создаются благоприятные условия для развития вторичной анаэробной липофильной микробиоты.*

Ключевые слова: *микробиом туберкулёзного очага, туберкулома, WGS, Corynebacterium kefirresidentii*

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The nature of the microbiota of the lower respiratory tract is very different from other microbial communities of the human body, for example, the gut microbiota and even the microbiota of the upper respiratory tract (URT), which are significantly more colonized by bacteria compared to the lower respiratory tract (LRT). In general, the microbiota of healthy lungs is characterized by low biomass and dynamic diversity [1]. Oligotrophic living conditions are created in the respiratory tract for microorganisms in comparison with the rich environment of the gastrointestinal tract. URTs are colonized by bacterial, viral and fungal communities, which are the main source of microbiota for the lower parts of the lungs. In healthy people, the lung microbiota seems to consist mainly of transient microorganisms, and its composition is determined by the balance between microbial immigration and elimination [2]. Despite the transitivity of the URT microbiota, it is possible that its composition has a protective effect against pathogens, preventing their penetration into the lower parts of the lungs [2]. The expediency of maintaining low bacterial contamination in the deep parts of the lungs is determined by the need to ensure effective gas exchange in the alveoli [1]. Therefore, the deep sections of the lungs have hundreds and thousands of times lower bacterial load than the URT. The microbial biomass is only 10^3–10^5 colony-forming units per 1 g of mammalian lung tissue (CFU/g) [3] or approximately 2.2×10^3 bacterial genomes per 1 cm^2 of the surface of human lungs [4]. For comparison, the lower parts of the human gastrointestinal tract are inhabited by 10^{11}–10^{12} CFU/g of tissue [5]. The microbiota of the URT of an adult is dominated by various representatives of the genera *Prevotella*, *Veillonella*, *Streptococcus*, *Leptotrichia*, *Rothia*, *Neisseria*, *Haemophilus*, *Moraxella*, *Staphylococcus*, *Corynebacterium*, *Fusobacterium*, etc. [6]. Our previous studies [7] indicate that the microbiota of the tuberculosis focus is divided into at least 2 types of communities: 1) mycobacterial caseoma (tuberculoma), in which more than 70 % of the genomes belong to *Mycobacterium tuberculosis*; 2) a polybacterial community in which the concentration of *Mycobacterium tuberculosis* varies from 0 to 30 %. According to our data, clinical strains of *Mycobacterium tuberculosis* are mostly unable to form biofilms [8], at the same time, in an *in vitro* experiment, the causative agent of tuberculosis significantly increased its number in the composition of a polymicrobial biofilm [9]. In other words, the study of polymicrobial communities of caseous contents can shed light on the microbial component of pathological processes occurring in the center of a tuberculous focus.

Granuloma (tuberculoma) was recognized as the leading pathology in pulmonary tuberculosis for more than 100 years ago [10]. The modern definition of pulmonary tuberculoma (caseoma) is a volumetric caseous – necrotic formation separated from the adjacent tissue by a capsule [11]. In the framework of this study, we postulate the hypothesis that the formation of the microbiota of a tuberculous focus occurs due to instability, compartmentalization and the transient nature of microbial communities in the lungs. It should be borne in mind that the conditions created by the patient's immune system in a tuberculous focus, capsule formation and curd necrotization of the contents with a predominance of lipids [12] should selectively stimulate the development of anaerobic microorganisms capable of multiplying due to the utilization of lipids. *Mycobacterium tuberculosis* is unable to reproduce under these conditions and survives in most cases in a dormant state [10]. Based on the above, the **aim of this study** was to isolate and characterize oxygen-tolerant anaerobes from the necrotic contents of tuberculosis.

MATERIALS AND METHODS

The study was approved by the Ethics Committee of the «Scientific Centre for Family Health and Human Reproduction Problems» (Protocol No. 4 dated November 16, 2020).

TABLE 1

CHARACTERISTICS OF THE EXAMINED SAMPLES

Sample #	Sex	Year of birth	CT diagnosis	Fraction, size of CT focus (mm)	Calcination
2201	F	1995	Tuberculoma of the left lower lobe with destruction	S6; up to 18	Yes
2202	F	1979	Tuberculoma of the upper lobe of the left lung in the insemination phase	S1; up to 21	Yes
2203	M	1993	Tuberculoma of the upper lobe of the right lung in the insemination phase	S1,2; up to 30	Yes
2204	F	1986	Tuberculoma of the lower lobe of the right lung in the insemination phase	S6; up to 19	No
2208	F	1984	Tuberculoma of the lower lobe of the right lung in the insemination phase	S6; up to 43	No

Note. CT – computed tomography.

Biopsy material from 5 patients with pulmonary tuberculosis was obtained during a surgical treatment of tuberculoma at the Irkutsk Regional Clinical Tuberculosis Hospital in 2022 (Table 1).

The caseous contents of the foci were cut out of the surgical biopsy with a sterile disposable scalpel in the bacteriological laboratory of a tuberculosis hospital and transfered to in 5 ml of LB broth under sterile vaseline oil in a volume of 1–2 g per tube. After 2 weeks, 0.1 ml of LB-broth was sieved onto LB-agar. The incubation of the cups was carried out in an anaerostat with Anaerogaz gas-generating packages (INKO, Russia). Isolated colonies were sown in 5 ml of LB broth under sterile vaseline oil to accumulate biomass. Antibiotic sensitivity was determined using TREK Diagnostic Systems (Thermo Fisher Scientific, USA) test systems: RAPMYCO for fast-growing mycobacteria and SLOWMYCO for slow–growing mycobacteria, according to the manufacturer's protocol. The assessment of resistance or sensitivity depending on the minimum inhibitory concentrations (MIC) was carried out in accordance with international recommendations [13]. Brain Heart Infusion Broth (HIMEDIA, India) was used as a broth for culture breeding, incubation was performed at 37 °C for three days in an anaerostat with Anaerogaz gas generating packages (INKO, Russia).

Lipase activity was determined by plating on Brain Heart Infusion agar (HIMEDIA, India) supplemented with 0.1 % Tween-80 and 10 mM of $CaCl_2$ (final concentrations). After incubation at 37 °C for three days in an anaerostat with Anaerogaz gas generating packages (INKO, Russia), the cups were incubated for a day at 4 °C. The presence of exogenous lipase activity was assessed by the formation of halo insoluble calcium salts of free fatty acids in the agar thickness around the colonies. DNA from the sediment of the broth culture was isolated by the CTAB chloroform method, as described earlier [14]. Whole genome sequencing was performed on a DNBSeq-G400 NGS sequencer by Genomed (Russia). The primary genome sequences are located at the National Center for Biotechnology Information of the USA (NCBI), project PRJNA971334.

The assembly of genomic readings into scaffolds was carried out using the Spades v. 3.11.1 software [15]. Genomic annotation and identification of the genes encoding *16S* rRNA and *23S* rRNA of the studied strain 2204 in the assembly was carried out using the SqueezeMeta software package [16]. The search for the nearest strains and species of bacteria with decoded complete genomes was carried out using the Type (Strain) Genome Server[1] using the algorithm of «genome wide hybridization» of strains *in silico* [17]. Cassettes of *16S* rRNA and *23S* rRNA genes were extracted from the complete genomes of identified closely related strains (*Corynebacterium kefirresidentii SB, Corynebacterium tuberculostearicum DSM, Corynebacterium curieae c8Ua, Corynebacterium yonathiae c21Ua, Corynebacterium marquesiae c19Ua)* according to information from the NCBI annotation. The *16S* rRNA and *23S* rRNA gene sequences were aligned in the MAFFT v. 7 program [18] and used to calculate genetic distances and determine the species of strain 2204 by the phylogenetic method (ML method; IQTREE v. 2 program [19] with an assessment of tree topology supports by the «ultrafast bootstrap» method – 1000 replicas). 99 % was considered the threshold of species identity for the complete *16S* rRNA gene [20].

[1] https://tygs.dsmz.de

RESULTS

Isolation and microbiological characteristics of the culture

The search for literary sources that can help in the development of methods for cultivating the satellite microbiota of the tuberculosis focus, both in Russian and in English language literature, has not led to any significant results. For example, the PubMed search for keywords «caseum + tuberculosis + cultivation + microbiota» returned a zero result. A search for «caseum + tuberculosis + cultivation» returned 20 publications, but none of them related to this topic. Based on this, it was decided to produce anaerobic cultivation under the most standardized conditions – LB-broth under vaseline oil.

As a result of incubation for 2 weeks, pronounced growth in a liquid LB medium was detected in one of the five tubes in the form of turbidity of the entire thickness and sediment on the surface of the biopsy. Sieving 0.1 ml of all five broth cultures on LB-agar followed by incubation in an anaerostat in only one case gave visible growth on Petri dishes.

FIG. 1.
Lipase activity of strain 2204. A halo is observed around the bacterial growth, indicating a high lipase activity of the isolated strain.

TABLE 2

TESTING OF THE SUSCEPTIBILITY OF STRAIN 2204 TO ANTITUBERCULOSIS AND ANTIMYCOBACTERIAL DRUGS WITH DETERMINATION OF MINIMUM INHIBITORY CONCENTRATIONS

No.	Antibiotics	MIC (μg/ml)	Result
1	Clarithromycin	0.06	Susceptibility
2	Rifabutin	0.25	Susceptibility
3	Ethambutol	4.0	Susceptibility
4	**Isoniazid**	**> 8**	**Resistance**
5	Moxyflixacin	0.12	Susceptibility
6	Rifampin	0.12	Susceptibility
7	**Trimethoprim/Sulfamethoxazolin**	**4/76**	**Resistance**
8	Amikacin	1.0	Susceptibility
9	Linezolid	1.0	Susceptibility
10	Ciprofloxacin	0.12	Susceptibility
11	Streptomycin	4	Susceptibility
12	Doxycicline	0.25	Susceptibility
13	**Ethionamide**	**> 20**	**Resistance**
14	Cefoxitin	4.0	Susceptibility
15	Tigecyclin	0.25	Susceptibility*
16	Imipenem	2.0	Susceptibility
17	Cefepime	1.0	Susceptibility
18	Amoxicillin/Clavulanic acid	2.0/1.0	Susceptibility
19	Ceftriaxon	4.0	Susceptibility
20	Minociclin	1.0	Susceptibility
21	Tobramicin	1.0	Susceptibility

Note. * – expected result (there are no international standards for necessary and sufficient MIC).

As can be seen from Figure 1 and Table 2, the isolated strain 2204 was characterized by high lipase activity and a relatively small spectrum of resistance to first-line antituberculosis and antimicobacterial drugs.

Whole genome sequencing and species identification of strain 2204

The primary short reads were reorganized into 6 scaffolds with a total length of 2,428,638 base pairs, among which 4 gene cassettes of the ribosomal operon *16S* rRNA – ITS – *23S* rRNA were found. Analysis using the algorithm of «genome-wide hybridization» of strains *in silico* for strain 2204 identified 5 of the closest reference genomes of the NCBI database: *Corynebacterium kefirresidentii SB; Corynebacterium tuberculostearicum DSM; Corynebacterium curieae c8Ua; Corynebacterium yonathiae c21Ua; Corynebacterium marquesiae c19Ua*. The genome length of strain 2204 corresponded to the genomes of closely related strains (lengths from 2,348,605 to 2,830,499 base pairs). The completeness of decoding the genome of strain 2204 by the presence of all necessary single-copy genes was 99.49 %. The *16S* rRNA and *23S* rRNA gene sequences of all the above genomes were used for phylogenetic identification of strain 2204. Fig. 2 shows the resulting phylogenetic tree with the above-mentioned nucleotide sequences. The shaded bush contains the tested nucleotide sequence of strain 2204. The calculation of the number of nucleotide substitutions relative to the nearest taxa showed more than 99 % identity of the strains (0.004 % substitutions). According to the strictest criteria to date,

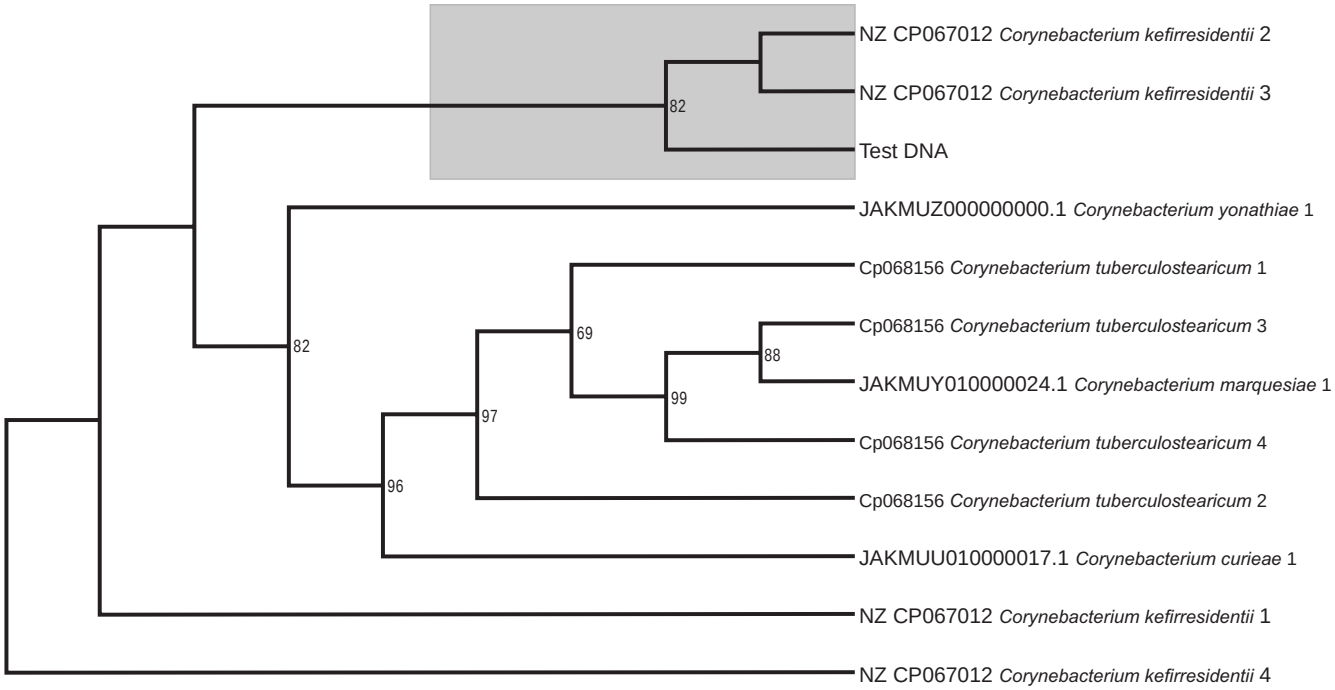

FIG. 2.

Phylogenetic relationships of strain 2204 with reference species. At the bottom of the nodes there are the bootstrap support values for branching. The shaded bush contains the tested nucleotide sequence of strain 2204

99 % should be considered the threshold of species identity for the complete *16S* rRNA gene [20]. Thus, the isolated strain can be uniquely identified as a species of *Corynebacterium kefirresidentii*.

DISCUSSION

The *Corynebacterium* genus of gram-positive rod-shaped bacteria belongs to the large family Corynebacteriaceae, was first proposed by Lehmann and Neumann in 1896 and currently has 177 species, some of which are of medical, veterinary or biotechnological interest [21]. Species of this genus are widespread and potentially pathogenic – primarily *C. diphtheria*. Corynebacteria are the dominant member of the human skin microbiota. Bacteria of the *Corynebacterium* genus account for 30 % of the total number of bacterial inhabitants of human skin [22]. The most common skin-dwelling species are represented by lipophilic *Corynebacterium tuberculostearicum*, *Corynebacterium kefirresidentii* and *Corynebacterium aurimucosum* type E, which form a narrow species complex [23]. It is of interest to note that for the first time, *C. tuberculostearicum* was isolated from focal skin lesions in leprosy [23]. An important feature of this group of species is the lack of the ability to biosynthesize *de novo* fatty acids [24], while the ability to produce specific mycolic acids, partly similar to those produced by mycobacteria, is observed. The peculiarities of lipid metabolism may explain drug resistance to isoniazid and ethionamide (Table 2), since this group of corynebacteria lacks the *InhA* gene [24, 25]. In other words, resistance to two anti–tuberculosis drugs – isoniazid and ethionamide – in this group of corynebacteria is constitutive.

CONCLUSION

The isolation of a lipophilic anaerobic *Corynebacterium tuberculostearicum* narrow species complex from a tuberculous focus, together with the results of our previous studies [7], indicates a possible role of the non-tuberculous microbiota in the liquefaction of caseous necrosis. This is important for understanding the pathological mechanisms of the formation of tuberculosis foci in the late stages of infection. We hypothesize that the formation of the microbiota of a tuberculous focus occurs due to instability, compartmentalization and the transient nature of microbial communities in the lungs. However, the conditions inside the tuberculous focus create favorable situation for the development of a secondary anaerobic lipophilic microbiota. Apparently, representatives of the species complex *C. tuberculostearicum* can play a negative role in pathological processes inside anaerobic tuberculoma, possibly provoking processes in the liquefaction of caseous masses.

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Conflict of interest

The authors of this article declare the absence of a conflict of interest.

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