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Phage sensitivity profiles of a nasopharyngeal opportunistic pathogen in *Streptococcus pneumoniae* carrier children with recurrent respiratory infections

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Abstract

Aim. To study the nature of microbiota and estimating the susceptibility to antibiotics and bacteriophages of conditionally pathogenic microflora of the nasopharynx in children-pneumococcal carriers with recurrent respiratory infections.

Methods. Researching microflora was conducted in 182 pneumococcal carriers receiving help in Kazan Scientific and Research Institute of Epidemiology and Microbiology. Microbial identification, testing of susceptibility to antibiotics and bacteriophages was carried out following the regulatory documentation. Bacterial isolates were confirmed by mass spectrometry. The phage titer was determined by the method of agar layers according to Grazia.

Results. Nasopharyngeal *S. pneumoniae* species was presented by *Staphylococcus spp., Moraxella spp., Haemophilus spp., Corynebacterium spp., Klebsiella spp* and *Candida spp.* The antimicrobial resistance profiles of *Streptococcus pneumoniae*: resistant to oxacillin was detected in 20.7% of strains, to erythromycin in 45.9%, to clindamycin in 20%, to trimethoprim-sulfamethoxazole in 18.4%. 19.6% of isolates were multidrug-resistant (MDR, resistant to 3 or more antimicrobial agents). Phage susceptibility test of *S. pneumoniae* showed that 97.2% of isolates were resistant to streptococcal bacteriophage, 75% to pyobacteriophage. All antibiotic-resistant strains remained susceptible to Streptococcus phages. The phage titer of *Klebsiella* in agreement with Grazia method of *Kl. pneumoniae* ranged from 9×10^{-6} to 5×10^{-5} PFU/mL. The ranking results of activities of antistaphylococcal antibiotics (effectiveness descending): fusidic acid > mupirocin > chloramphenicol > cyprofloxacin erythromycin.

Conclusion. Nasopharyngeal microbiota of pneumococci carriers children is represented by a variable polymicrobial association; nasopharyngeal strains are effectively lysed by bacteriophages; mono- and polyvalent bacteriophages can be used as an alternative to antibacterial treatment in Streptococcus pneumoniae carriers children with recurrent respiratory infections.

Keywords: nasopharynx microbiocenosis, pneumococcal carriage, recurrent respiratory infections, antibiotic resistance, bacteriophages.

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Background

Microbiocenosis of the upper respiratory tract as a component of the macroorganism microbiota is actively involved in the training of immunocompetent cells for protection against infection and in the development of the pathological process [1]. When the mucosa is contaminated with opportunistic and pathogenic microflora recognized by PRRs receptors, resident macrophages, NK cells, and dendritic cells are activated. The synthesis of proinflammatory cytokines leads to the migration of neutrophils, monocytes, and other cells of the innate immune system to the focus of inflammation, followed by the activation of their effector mechanisms of pathogen elimination (phagocytosis and oxygen-dependent killing mechanisms) [2].

Innate immune cells are further stimulated by DAMP molecules that accumulate at the inflam-

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mation site due to cellular damage [3]. Moreover, epithelial cells participate in the defense against pathogens by secreting antimicrobial polypeptides (defensins, anionic and cationic peptides, etc.) that destabilize the membranes of foreign organisms [4].

The acquired immune system is responsible for the formation of the mucosal barrier by secreting the IgA-type immunoglobulin. IgA is predominant in the mucous membranes and exhibits pathogen specificity. It is synthesized by dendritic cells that select the bacteria that adhere to the epithelium, and interact with B- and T-cells of the lymphadenoid pharyngeal ring and regional lymph nodes.

However, not all microorganisms are eliminated by the cells of the immune system; some enter into a synergistic relationship with the immune system with the development of tolerance. Although, immunological tolerance to commensals is probably achieved through multiple mechanisms, in the past few years, FOXP3+ regulatory T cells have been considered to play a primary role in this process. Commensals promote the induction of regulatory T cells by the direct perception of microbial products or their metabolites by T cells or dendritic cells that in turn prevents an overreaction of the immune system and the development of inflammation [5].

Unfortunately, it is impossible to predict when a particular commensal microorganism will overcome the tolerance of the immune system and become more pathogenic. The nasopharyngeal microbiota is an extremely dynamic system, wherein the microorganisms continuously interact with the immune system of the macroorganism and other microorganisms that colonize this biotope; such as *Streptococcus pneumonia, Moraxella catarrhalis, Hemophilus influenza, Staphylococcus spp., Prevotella spp., Veillonella*, etc. [6].

Preschool children suffering from recurrent respiratory infections often have a joint colonization of opportunistic and pathogenic microorganisms in the nasopharyngeal microbiome [7]. *S. pneumoniae* is one of the key microorganisms responsible for mucosal respiratory and invasive infections [8]. Carrying *S. pneumoniae* is a characteristic feature of frequently ill children; the most frequently observed joint colonization is that of *S. pneumoniae* with *Staphylococcus aureus, Haemophilus influenza, Moraxella catarrhalis, and Klebsiella pneumoniae.* Simultaneous colonization by several pathogens leads to the summation of the virulent potential that increases the risk of repeated respiratory infections. Moreover, frequent use of antibiotics provokes the formation of antimicrobial-resistant strains.

Vaccination is not only the most effective measure for reducing the incidence of invasive pneumococcal infections, but also an important tool for reducing the level of antibiotic resistance of pneumococci, since vaccine serotypes are most resistant to chemotherapy [9, 10].

One of the methods for eradicating pathogenic and opportunistic microflora, in particular *S. pneumoniae*, is by using virulent bacteriophages with a wide range of lytic activity. Bacteriophages should be chosen based on the in vitro sensitivity of the pathogen to them. Bacteriophages used for phage therapy and phagoprophylaxis, produced by different manufacturers, differ in the composition of the phage cocktail. For enhancing the efficacy, the phages in agents are periodically changed.

The efficiency of the phage preparations depends on the matching of the series of the agent tested in the laboratory with the series of the agent prescribed to the patient. Another consideration is the development of a strain-specific antiphage immune response upon repeated use of bacteriophages.

The aim of our research was to study the nature of the opportunistic nasopharyngeal microflora of the children carrying *S. pneumoniae* with recurrent respiratory infections and to evaluate their sensitivity profile to antibiotics and bacteriophages. The study was approved by the local ethics Committee of the Kazan Research Institute of Epidemiology and Microbiology (report No. 2 dated 09.09.2019).

Material and methods

The study included 182 children with recurrent respiratory infections who had received medical advice at the Kazan Research Institute of Epidemiology and Microbiology, and had previously been found to carry *S. pneumonia*. There were 96 children under 2 years of age, 47 under 3–4 years of age, and 39 children under 5–7 years of age.

Seeding of the biomaterial was performed on agar (Columbia, Bio-Rad, USA) with the addition of 3% of the red blood cell mass, mannitol-salt yolk agar, chocolate agar containing 10 micrograms/ml of nicotinamide adenine dinucleotide that was added to the nutrient medium and precooled to 50°C –60°C, Endo, and Saburo. Identification of microorganisms was performed in accordance with the regulatory documentation [11]. The selected cultures were confirmed using the MALDI-TOF mass spectrometer MALDI Biotyper (Bruker Daltonics, Bremen, Germany).

The antimicrobial sensitivity profile was evaluated and the results were interpreted according to the clinical recommendations in a paper by EUCAST (2018) titled, "Determination of microbial sensitivity to antimicrobial drugs."

Sensitivity to bacteriophages was determined using a screening method (spot test). The study

included bacteriophages produced by NPO Microgen: Klebsiella polyvalent purified bacteriophage (Ufa) and piobacteriophage polyvalent "Sextafag" (Perm). The Grazia method was used to determine the bacteriophage titer per unit volume [counting the number of negative spots formed, PFU/ml[1] (plaque-forming unit)].

Results

The study of the nasopharyngeal microbiota of preschool children with recurrent respiratory infections showed that *Streptococcus pneumoniae* was isolated in 28.5% of cases in the monoculture with a high degree of colonization [10^4–10^6 CFU/ml[1] (colony-forming unit)]. *Staphylococcus aureus* was seen in association with pneumococcus and prevailed in children over 5 years of age (46.1%) with a high degree of colonization (10^5–10^6 CFU/ml). *Moraxella catarrhalis* (39.3%) and *Hemophilus influenzae* (36.8%) were also present with less frequent co-colonization with *Moraxella nonliquefaciens* (23%) and *Corynebacterium pseudodiphtheriticum* (21.2%). Bacterial associations of *S. pneumoniae* with other pathogens such as *K. pneumoniae* (9.3%) and *Candida fungi* (5.5%) were detected.

The microflora of the mucous membranes of children free from *S. pneumoniae* was represented mainly by the coagulase-negative staphylococci (*S. epidermidis, S. capitis,* and *S. hominis*).

We used the *Shannon diversity index* (1,7) to assess the species diversity by accounting for the most significant and rare bacterial species. It showed an increase in the role of transient microflora; including, gram-negative bacteria of the genus *Pseudomonas aeruginosa*, *Klebsiella pneumoniae, Enterobacter spp.*

Antibiotic sensitivity testing of *Streptococcus pneumoniae* revealed resistance to oxacillin, erythromycin, and clindamycin in 20.7% (n = 48), 45.9% (n = 113), and 20% (n = 48) of the strains, respectively. Phenotypic resistance to co-trimoxazole (sulfamethoxazole + trimethoprim) was detected in 18.4% (n = 45) of the strains. Multiresistant strains (resistance to three or more antibiotics) were seen in 19.6% of the isolates (n = 48); while, 14 strains were resistant to 3 groups of antimicrobial agents (oxacillin + erythromycin + co-trimoxazole). Multiresistance to the combination of oxacillin + erythromycin + clindamycin was seen in 34 isolates.

Bacteriophage sensitivity of the selected cultures of *S. pneumoniae* showed that 97.2% of strains were sensitive to the streptococcal phage, 75% were sensitive to eubacteria, and all the resistant strains remained sensitive to the streptococcal phage. As is known, streptococcal bacteriophage has the ability to specifically lyse Streptococcus bacteria; while, piobacteriophage causes a specific lysis of the following bacteria: *Streptococcus, Enterococcus, Staphylococcus, enteropathogenic Escherichia coli, Pseudomonas aeruginosa, Proteus mirabilis, Proteus vulgaris, Klebsiella oxytoca*, and *Klebsiella pneumoniae.* Both these agents can be used as a part of the complex therapy for the diseases of the ear, throat, nose, upper respiratory tract, and lungs.

Further, the role of nasopharyngeal carriage of *Staphylococcus* aureus and *Klebsiella pneumoniae* together with *Streptococcus pneumoniae* in the development of recurrent infections of the respiratory tract is not excluded. *Staphylococcus aureus and Klebsiella pneumoniae* are ESKAPE (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa*, and *Enterobacter* spp. capricola) pathogens that have a pronounced tendency to develop resistance compared with other microorgansims of the upper respiratory tract. This is especially unfavorable for children who regularly receive antimicrobial therapy for frequent respiratory infections.

Considering this hypothesis, the antibiotic and phage sensitivity to the most commonly used antibacterial agents was evaluated. Evaluation of the resistance profile of the nasopharyngeal strains of *Klebsiella pneumoniae* (n = 23) showed that furazolidone and gentamicin were the most active anti-Klebsiella agents (84.8% of sensitive isolates). The proportion of isolates sensitive to ciprofloxacin, ceftriaxone and cefixim, chloramphenicol, and tetracycline were 78.3%, 78.3%, 71.7%, and 69.6% of strains, respectively; while 14% were polyresistant strains (n = 9).

The spot test used for the determination of the lytic activity of bacteriophages revealed elimination of 39.1% and 45.7% of the isolates for Klebsiella polyvalent (against Klebsiella pneumonia) and polyvalent "sextafag" (against eubacteria), respectively.

The Grazia method (n = 8) was used to quantify the lytic activity of bacteriophages in cultures with moderate sensitivity (++). This method allowed us to record the number of negative colonies (sterile spots) formed in the agarized nutrient medium as a result of incubation of the bacteriophage suspension during its cultivation with the tested isolates. The results were taken into account by counting the BOE sown with the highest dilutions (Fig. 1).

When negative colonies were detected in the cup sector the culture was evaluated as sensitive.

[1]PFU — plaque-forming unit

[2]CFU — colony-forming unit

Table 1. Lytic activity of Klebsiella phage in relation to the nasopharyngeal strains of *Klebsiella pneumoniae*

The number of the strain	The causative agent	Spot test	Klebsiella polyvalent bacteriophage purified	
			Titer on the Grazia, BFU/ml (according to dilutions)	
269	K. pneumoniae	++	47×10^{-5}	3×10^{-6}
135	K. pneumoniae	++	67×10^{-5}	7×10^{-6}
145	K. pneumoniae	+	5×10^{-5}	1×10^{-6}
151	K. pneumoniae	+/–	5×10^{-5}	0×10^{-6}
285	K. pneumoniae	+	46×10^{-5}	4×10^{-6}
282	K. pneumoniae	++	94×10^{-5}	9×10^{-6}
347	K. pneumoniae	++	87×10^{-5}	9×10^{-6}
621	K. pneumoniae	+	18×10^{-5}	1×10^{-6}

Fig. 1. The Number of negative colonies in 1 ml of bacteriophage in various dilutions

The following results were obtained: against the background of uniform growth *of Klebsiella pneumoniae*, zones of complete absence of growth (complete lysis) were visible. Each plaque was formed as a result of the action of a single phage particle. The titer of the tested bacteriophage (the number of active phage particles) varied from 9×10^{-6} to 5×10^{-5} BFU/ml (Table 1).

Determination of the antimicrobial resistance profile of nasopharyngeal *Staphylococcus aureus* strains (n = 113) showed the following results: all strains were sensitive to fusidic acid, sensitivity to mupirocin was 75.2%. 71.6% of strains were sensitive to chloramphenicol, 64.6% to fluoroquinolones (ciprofloxacin), and 61.6% of *Staphylococcus aureus* isolates were sensitive to macrolides (erythromycin). Results of ranking of antistaphylococcal activity of chemotherapy agents against nasopharyngeal *Staphylococcus aureus* in the order of decreasing effectiveness: fusidic acid > mupirocin >chloramphenicol >ciprofloxacin > erythromycin.

Phage preparations showed a high antibacterial activity: staphylococcal bacteriophage and piobacteriophage had a lytic effect against 71.6% and 63.7% of isolates, respectively. It should be noted that bacteriophages also lysed Staphylococcus aureus strains with multiple antimicrobial resistance.

Discussion

Thus, a variable community of opportunistic and pathogenic microorganisms represents the microbiocenosis of the nasopharynx of children carrying pneumococci. Analysis of the carrier pathogens with invasive properties is necessary for the development of methods for the prevention of diseases of the respiratory tract due to the associative nature of the nasopharyngeal microbiota. The results of the study showed that strains of opportunistic bacteria that colonized the nasopharynx of children with recurrent infections were quite effectively lyzed by bacteriophage preparations; therefore, bacteriophages may be used as alternative antibacterial agents for phagotherapy and phagoprophylaxis. The advantage of combined use of chemotherapeutics and bacteriophages is that the bacteria do not have common mechanisms of resistance to antimicrobial agents and bacteriophages. Hence, they can be used for successful eradication of antibiotic-resistant pathogens.

Researches aimed at studying the nasopharyngeal carriers of these pathogens and their re-

lationship will allow for targeted prevention and treatment of infectious processes. Due consideration must be given to the regional characteristics, in particular, caused by the associations and monocultures of the bacteria.

Conclusions

1. Microbiocenosis of nasopharynx of children carrying pneumococci is represented by a variable polymicrobial association.

2. Nasopharyngeal strains are efficiently lysed by bacteriophages. Thus, mono- and polyvalent bacteriophages may be used as alternatives to antibacterial treatments in children carrying *Streptococcus pneumoniae* with recurrent respiratory infections.

3. It is essential to monitor the resistance of nasopharyngeal bacteria to bacteriophages to update the phage composition.

Contribution of authors. L.T.B. the head of the work, participated in the systematization of material and analyzed the data; O.F.T. participated in the systematization of material and did the research; T.A.CH. participated in the collection of material and did the research; N.S.K. did the research; K.N.S. did the research; and G.SH.I. analyzed the data.

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Conflict of interest. The authors have no conflicts of interest to declare.

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