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**FREQUENCY OF FINDING HARD-TO-CULTIVATE PATHOGENIC
MICROORGANISMS CAUSING OF RESPIRATORY TRACT INFECTIONS IN
CHILDREN AND ADULTS AND OPTIMIZATION OF THEIR DETECTION**

Specialization 03.02.03 – Microbiology

SUMMARY

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GENERAL CHARACTERISTICS OF THE WORK

Relevance of the research. Infectious inflammatory diseases of the respiratory system, including nosocomial (or hospital-acquired) pneumonia, are still an acute problem for modern health care. They are the reason for most cases of hospitalization, and they often lead to fatal termination. As official statistics show, in the Russian Federation annually more than 1.5 million people suffer from pneumonia, more than 40.000 die from pneumonia annually, and most cases of fatal termination are men of the employable age (A.G. Chuchalin 2006, 2010; A.I. Sinopalnikov 2010, 2012; G.G. Onischenko, K.B. Ezhlova, Y.V. Dyomina et al., 2013). Respiratory diseases are especially dangerous in closed military bodies where morbidity rate is several times higher than in civilian population (S.D. Zhogolev, P.I. Ogarkov 2003).

Along with traditional etiological pathogens causing respiratory diseases (such as *Streptococcus pneumoniae*, *Haemophilus influenzae*, flu viruses etc.) we come across atypical, hard-to-cultivate pathogens such as *Mycoplasma pneumoniae*, *Chlamydomphila pneumoniae*, *Legionella pneumophila* etc., and herpes viruses such as *Cytomegalovirus* (CMV), *Herpes simplex I/II*. Their role in the development of respiratory infections and the necessity of their indication and identification is reflected in new normative documents including Methodic guideline #3.1.2.3047-13 “Epidemiological Supervision of Community-Acquired Pneumonia (CAP),” Methodic guideline # 4.2.3115-13 “Laboratory Diagnostics of CAP”, Sanitary Rules # 3.1.2.3116-13 “Prevention of CAPs”. As the immune status of the population is deteriorating, the etiological role of these pathogens is growing, causing the respiratory system pathologies (L.A. Vishnyakova 2005, T.A. Karapetyan 2008). According to medical literature, they are responsible for up to 30 per cent of respiratory diseases cases, and the frequency of finding them in different nosologic forms considerably varies in different authors’ works (A.G. Chuchalin 2006; G.G. Onischenko et al., 2013, 2014; Y.V. Dyomina, 2014; B.A. Cunha, 2010; H. Kurz, 2011; G. Lui, 2009).

One should note that the above mentioned “atypical” pathogens are not detected reliably enough by the traditional methods (such as bacteriological, immunological, serologic ones) due to the special requirements to the cultivation conditions, high antigenic variability, etc. This is why, even if time and funds are invested vastly, the etiological factor is not determined in 30 to 70 per cent of cases (A.G. Chuchalin 2008; V. Sao, 2010). This explains why the polymerase chain reaction (PCR), based on the amplification of specific fragments of microorganisms’ genome, is a valuable diagnostic tool that allows for revealing infectious agents in any kind of biological substratum and improves the diagnostics of both acute and chronic respiratory diseases. Usage of the PCR method in clinical practice has been approved and recommended in many normative acts such as Methodological Guidelines # 42.3115-13 “Laboratory Diagnostics of CAPs” and Sanitary Rules # 3.1.2.3116-13 “Prevention of CAPs”. It is important to stress that this method is known for fast response (100-1000 cells or virions), quick results (within 6-8 hours), precision specificity up to 100% (G.A. Shipulin 2014; R. Dumke, 2007).

Degree of elaboration of the research topic. Both Russian and foreign researchers have done much to study and describe the role of hard-to-cultivate microorganisms such as *C. pneumoniae*, *C. psittaci*, *M. pneumoniae*, *L. pneumophila*, *CMV* etc. in the development of respiratory diseases (A.I. Sinopalnikov 2006; I.S. Tartakovsky 2010; A.G. Chuchalin 2010; V.I. Shakhgildyan 2010, and B.A. Cunha, 2010; H. Kurz, 2011; G. Lui, 2009, to name just a few). In the latest decades respiratory tract diseases have been diagnosed with the help of biomolecular methods, mostly different variants of PCR (S.B. Yatsishina 2008; K. Gullsbj, 2008; M. Khanna, 2005; K. Loens, 2008 and others). PCR diagnosticums (diagnostic agents) have been created, certified and produced for detecting pathogens causing respiratory diseases.

However, in the Russian Federation there has never been conducted an investigation of vast contingent of patients in dependence to their age and the form of the respiratory system pathology with a view to PCR-based detection of various forms

of hard-to-cultivate pathogens. Up to now there is no data as to the most informative substratum for such investigations. Usually different biological substrates are being analyzed such as samples of phlegm, throat smears, nasopharynx smears, bronchoalveolar lavage, pleural fluid, biopsy samples (M. Cho, 2012; D.R. Murdoch, 2003), which makes the result interpretation and the final diagnosis hard to fulfill.

Incidence rate in respiratory diseases, a wide spectrum of traditional and “atypical” pathogens, genetic variability of pathogens call for a wide application of unified molecular genetic methods of investigation to laboratory practice for solving diagnostic, clinical and epidemiological problems.

Goal and tasks of the investigation. The goal of this work is to optimize the method of detecting hard-to-cultivate pathogens causing respiratory diseases (*Mycoplasma pneumoniae*, *Chlamydomphila pneumoniae*, *Chlamydomphila psittaci*, *Legionella pneumophila*, *Moraxella catarrhalis*) and herpes viruses and also to estimate the frequency of detecting them in dependence to the patients’ age and the nosologic form of the disease.

According to the stated goal the following tasks were singled out.

1. To determine the most informative biological substratum for the PCR-based detection of hard-to-cultivate pathogens causing inflammatory respiratory diseases.

2. To create an effective and economically justified method of the PCR-based detection of scarce hard-to-cultivate pathogens causing respiratory diseases.

3. To estimate the frequency of revealing *M. pneumoniae*, *C. pneumoniae*, *C. psittaci*, *L. pneumophila*, *M. catarrhalis*, *Cytomegalovirus*, *Herpes simplex I/II* in children of various age groups and in adults (organized and unorganized ones) suffering from different inflammatory respiratory diseases.

4. To elaborate the optimal algorithm of molecular detection of *M. pneumoniae*, *C. pneumoniae*, *C. psittaci*, *L. pneumophila*, *M. catarrhalis*, *Cytomegalovirus*, *Herpes simplex I/II* in patients of different age groups suffering from inflammatory bronchopulmonary diseases.

Academic novelty. A method of PCR-based detection of scarce hard-to-cultivate microorganisms in different biological substrates taken from children and adults with bronchopulmonary pathology has been proposed for the first time (medical novelty patent # 2406088 issued on the 10th of December, 2010, “Method of Detecting Scarce and Hard-to-Cultivate Pathogens Causing Inflammatory Respiratory Diseases (*Cytomegalovirus*, *Chlamydomphila pneumoniae*, *Chlamydomphila psittaci*, *Legionella pneumophila*, *Moraxella catarrhalis*) with the Help of PCR”).

For the first time, a vast inspection of patients of different age groups with various bronchopulmonary infections has been carried out, with PCR-based analysis of their substrates for a wide spectrum of hard-to-cultivate bacterial and viral pathogens.

New knowledge has been achieved as to the frequency of finding individual types of hard-to-cultivate microorganisms in children of different age groups and in adults with various nosologic forms of infectious respiratory diseases.

A new medical technology has been developed, “Complex PCR Based Inspection of Patients with Infection-Allergic Respiratory Diseases” (license for using FS # 2009/084 issued on the 21st of April, 2009, the Ministry of Health and Social Security of the Russian Federation) that allows for reducing the cost price of investigation and raising the effectiveness of detecting infectious agents.

Scientific and practical value of the work. New information has been gathered concerning the frequency of finding hard-to-cultivate microorganisms during bronchopulmonary diseases, which can help to investigate the aetiopathogenesis and the development of the infection process during respiratory diseases.

It has been shown that the frequency of detecting hard-to-cultivate microorganisms, pathogens causing respiratory diseases, depends on the type of the pathogen, the contingent and the age of the people inspected, the nosologic form of the disease.

The suggested method of PCR-based detection of hard-to-cultivate pathogens causing respiratory diseases allows for raising the effectiveness and the reliability of the

diagnostic investigation, shortening its duration and lowering the cost by 3.8 to 7.6 times.

It has been proved that the identification of the “atypical” microorganisms while supporting the process of treatment is conducive to efficient adjustment of antibacterial and immunomodulatory therapy, which allows for shortening the period of a patient’s hospitalization.

The developed method of detecting hard-to-cultivate pathogens causing respiratory diseases, defended by patent # 2406088, has been instilled in the diagnostic work of several medical institutions of Nizhny Novgorod and Nizhny Novgorod region.

The results of the investigation have been used in developing the medical technology “Complex PCR Based Inspection of Patients with Infection-Allergic Respiratory Diseases” (license for using FS # 2009/084 21st of April, 2009, the Ministry of Health and Social Security of the Russian Federation), Methodological Guidelines “PCR-Based Detection of Scarce Hard-to-Cultivate Pathogens Causing Inflammatory Respiratory Diseases” (Methodological guidelines 4.2.0060-12, 2012), Methodological Guidelines “Algorithm of Detecting a Wide Spectrum of Pathogens Causing Inflammatory Respiratory Diseases” (recommended for usage on the regional level by the letter from the Federal Directorate for Consumer Rights and Health Protection # 01/8010-12-26, the 18th of July, 2012).

The theses submitted for defense.

1. The technique of PCR-based detection of scarce hard-to-cultivate microorganisms, based on using the method of “double minipools”, allows for making a diagnostically and economically justified screening of patients with various types of respiratory pathology, for it lessens the cost and the duration of diagnostic investigation.

2. The frequency of detecting hard-to-cultivate infection agents causing respiratory diseases is determined by the nosologic form of the respiratory disease, the contingent and the age of the investigated, the specific peculiarities of the pathogen.

3. Speaking of hard-to-cultivate microorganisms, the domineering pathogen in the etiological structure of the respiratory disease is *M.pneumoniae* for children, *M.pneumoniae* and *C.pneumoniae* for adults.

Reliability and validity of the results of the investigation are proved by the sufficient volume of the analyzed groups sampling, an ample number of examinations carried out (samples of different clinical substrates from 1841 patients with bronchopulmonary pathology and 449 persons from screening groups; all in all, 31 843 PCR-based investigations have been conducted), also by the usage of certified molecular-genetic, immunologic methods that are known for their fast response and specificity.

Approbation of the work. The thesis was approved at the joint meeting of the Academic Council and Interlaboratory Scientific Seminar at the Federal Budget Science Institution “Nizhny Novgorod Scientific Research Institute of Epidemiology and Microbiology named after Academician I.N. Blokhina” (Record #8, the 30th of October, 2014).

The results of the work have been presented and discussed at the VII Russian Congress of Infectiologists “New Technologies in Diagnosing and Treating Infectious Diseases”, Nizhny Novgorod, 2006; at the Second All-Russian Forum “The Health of the Nation is the Ground for Russia’s Well-Being”, Moscow, 2006; the scientific conference dedicated to the 85th anniversary of Academician of the Russian Academy of Science I.N. Blokhina, Nizhny Novgorod, 2006; the IV All-Russia academic and research conference “Molecular Diagnostics”, Moscow, 2007; the Jubilee All-Russia academic and research conference dedicated to the 90th anniversary of the Nizhny Novgorod Scientific Research Institute of Epidemiology and Microbiology named after Academician I.N. Blokhina, Nizhny Novgorod, 2009; the academic and research conference for young scientists “Innovative Technologies in Antiepidemic Protection of

Population”, Nizhny Novgorod, 2011; the V Annual All-Russia Congress on Infectious Diseases, Moscow, 2013.

Connection of the work with scientific programs and the author’s personal contribution to the investigation. The investigation was held within the framework of sectorial programs “Scientific Aspects of Securing Sanitary and Epidemiological Well-Being in the Russian Federation for 2006-2010” (section of the research effort “Study of Distribution and the Peculiarities of Circulation of Atypical and Hard-to-Cultivate Pathogens Causing Inflammatory and Infection-Allergic Respiratory Diseases” (state registration number 01200612239)) and “Scientific Research and Projects to Ensure Sanitary and Epidemiological Well-Being and Reduction of Infections Rate in the Russian Federation” in 2011-2015 (requirements specification dated the 11th of January, 2011, “Study of Persistence of Herpes Viruses (I, II, III, IV, V, VI, VII types) and Bacteria *Mycoplasma* in Children with the Help of Molecular-Genetic Methods. Working out the Algorithm of Monitoring Active Replication of Herpes Viruses in Children of Different Age Groups” (state registration number 01201175712)). One of the units of the work was developed within the limits of the Russian Ministry of Defense’s task “Study of the Peculiarities of the Epidemiology and Clinical Course of CAP in Military Personnel at the Present Stage” (2003-2007).

The author’s personal contribution to the results stated in the thesis consists in performing molecular-genetic investigation, the theoretic generalization of the results, and the statistical treatment of the data. The competitor together with her co-authors has suggested a new medical technology (license for using the medical technology FS # 2009/084), methodological guidelines 4.2.0060-12, methodological guidelines “Algorithm of Detecting a Wide Spectrum of Pathogens Causing Inflammatory Respiratory Diseases” (letter from the Federal Directorate for Consumer Rights and Health Protection # №01/8010-12-26 dated the 18th of July, 2012). The results the author has come up with are the basis for acquiring medical patent # 2406088.

Published papers. 16 papers were published based on the thesis, out of which: 5 in the reviewed editions, 8 in collected conference and congress reports, 1 a medical patent, 1 a medical technology, 1 methodological guidelines.

The volume and structure of the thesis. The thesis contains 132 pages of printed text and consists of an Introduction, the analysis of the sources, 6 chapters of independent research, Conclusion, practical recommendations, prospects and directions for further work, a list of the abbreviations used, and a reference list (bibliography). The thesis contains 13 illustrations and 8 tables. The reference list includes 200 references, out of which 20 are Russian and 180 are foreign publications.

Thanks and regards. The author expresses her sincere gratitude to her scientific adviser, Professor E.I. Yefimov, Doctor of Medicine, for the attentive appraisal of the work, the fruitful discussion of the results and comprehensive help; and also to Associate Professor V.N. Mazepa, Doctor of Biology, under whose supervision the research which is the basis for this work was launched and carried out. In particular the author would like to thank Associate Professor N.F. Brusnigina, Candidate of Medical Science, the head of the laboratory of metagenomics and molecular indication of pathogens, for her priceless help in creating the thesis and for her support, and also all the researchers of the laboratory, for their help in performing the experiments. The author expresses her gratitude to Professor G.I. Grigorieva, Doctor of Biology, deputy director for scientific work of the Federal Budget Science Institution “Nizhny Novgorod Scientific Research Institute of Epidemiology and Microbiology named after Academician I.N. Blokhina”, for her valuable ideas; to I.S. Dobrotina, Candidate of Medical Science, Associate Professor of the Chair of Hospital Therapy named after V.G. Vogralik of the State Budget Higher Educational Institution “Nizhny Novgorod State Medical Academy” for scientific consultations; to Professor V.Y. Talaev, Doctor of Medicine, for joint investigations; and also to the researchers of the laboratories of cellular immunology and immunochemistry of the Federal Budget Science Institution

“Nizhny Novgorod Scientific Research Institute of Epidemiology and Microbiology named after Academician I.N. Blokhina”. The author is grateful to all the employees of medical establishments and institutions who have taken part in conducting this investigation and applying its results.

CONTENTS OF WORK

MATERIALS AND METHODS

Object of study

The investigation was conducted from 2005 till 2013 and involved a checkup of children of different ages and adults, organized as well as unorganized ones. 1227 children in Nizhny Novgorod and Nizhny Novgorod region, from 15 days old to 16 years old, were examined; the patients had been previously diagnosed clinically and with X-ray as having CAP, acute bronchitis, bronchial asthma, and/or acute respiratory disease (ARD)/ acute respiratory viral infection (ARVI). The group of organized adults consisted in 469 patients of the 5th Branch of Military Clinical Hospital #1586 of the Russian Ministry of Defense, the military personnel who had been previously diagnosed clinically and with X-ray as having CAP and/or acute bronchitis, and 145 unorganized adults, a heterogeneous group of patients from 25 to 60 years of age, undergoing either outpatient or in-patient treatment in hospitals of Nizhny Novgorod, diagnosed as having CAP and/or acute bronchitis. The screening group consisted of 127 practically healthy children of different age groups, 322 practically healthy adults, out of which 270 were (organized) recruits and 52 were (unorganized) people undergoing clinical examination. All the people from the screening group had no clinical symptoms of inflammatory respiratory diseases at the moment of examination nor within the previous month.

The analyzed material was phlegm, oropharynx smears, blood, bronchoalveolar lavage; for infants younger than one year old – saliva, for healthy ones – oropharynx smears. The material was collected in accordance with methodological guidelines 4.2.2039-05 “Technique of collecting and transporting biomaterials to microbiological laboratories” in procedure units of hospitals and polyclinics.

Molecular-genetic methods

DNA was isolated out of biological substrates with the help of “DNK-sorb B” (“DNA-sorb B”) set of reagents. Samples of phlegm were first preconditioned with reagent “Mukolizin” (by the Federal Budget Institution of Science “Central Research Institute of Epidemiology” of The Federal Service on Customers’ Rights Protection and Human Well-being Surveillance). To isolate DNA out of a mix of substrates, different types of biological substrates from one patient were mixed in a disposable test-tube 1.5 ml. in equal quantities so that the volume of the mix came up to 100 microliters.

M. pneumoniae, *Cytomegalovirus*, *Herpes simplex I/II* were PCR-detected on test systems “AmpliSense” produced by the Federal Budget Institution of Science “Central Research Institute of Epidemiology” of The Federal Service on Customers' Rights Protection and Human Well-being Surveillance, according to the manual. Amplification was done on devices “Tertsic MS-2” (DNA technology, Moscow) or “My Cyclor” (Bio-Rad, the USA).

C. pneumoniae, *C. psittaci*, *L. pneumophila*, *M. catarrhalis* were PCR-detected with reagents sets “GenPak DNA PCR test” produced by LLC “Isogen” (Moscow) according to the manual. Amplification was done on devices “Tertsic MS-2”.

The sensitivity of test systems “AmpliSense” and “GenPak DNA PCR test” is no less than $1 \times 10^3 - 5 \times 10^3$ bacterial cells or DNA-comprising viral parts per milliliter of clinical sample.

Amplification products were detected by horizontal electrophoresis in 1.8% agarous gel with subsequent registration of the results with the help of ultraviolet transilluminator (“Biocom”, Russia) on the image processing system “Biotest-1” (by the Federal Budget Institution of Science “Central Research Institute of Epidemiology” of The Federal Service on Customers’ Rights Protection and Human Well-being Surveillance, Moscow).

Immunological investigation

The immunological investigation was carried out jointly with the laboratories of biochemistry and cellular immunology of the Federal Budget Science Institution “Nizhny Novgorod Scientific Research Institute of Epidemiology and Microbiology” named after Academician I.N. Blokhina, with the laboratories’ heads respectively V.V. Nemov, Candidate of Science in Biology, and Professor V.Y. Talaev, Doctor of Medicine. 119 military people were examined who had been previously diagnosed as having CAP (clinically and with X-ray), and different types of hard-to-cultivate pathogens causing respiratory diseases and herpes viruses were detected in their tests. The screening groups consisted of practically healthy adult donors (50 people) and healthy men of call-up age (50 people). The sick patients had their blood taken and tested thrice.

The objects of study were indices of humoral immunity (the level of total immunoglobulins, lactoferrin, secretory (exocrine) immunoglobulin A, the level of circulating immune complexes). Immune-enzyme analysis and precipitation analysis were used, with the help of test-systems by the closed (joint-stock) company “Vector Best”.

Cellular immunity was studied by measuring the distribution of cytokines in patients’ blood serum with the help of enzyme immunoassay, defining the functional state of T-cells and the quantity of interferon-producing T-cells after polyclonal activation of lymphocytes by estimating the intracellular cytokines with the help of laser flow cytometry.

Statistic methods

The data was processed with Microsoft Office (Excel) programs, the statistic program package Statz, Statistica 6.0. The reliability of the differences was stated by the generally accepted method of counting the standard error of mean (**m**) and indices of relevancy and probability (**t**).

RESULTS OF INVESTIGATION AND THEIR DISCUSSION

In order to determine the most informative biological substratum (or their mix) for defining *M. pneumoniae*, *C. pneumoniae*, *C. psittaci*, *L. pneumophila*, *M. catarrhalis*, *CMV*, *HSV I/II*, the author analyzed the results of detecting these pathogens in samples from 176 organized adult patients with CAP. Both samples of a single type substratum and their mixtures in different combinations were examined. Also samples of bronchoalveolar lavage from 19 military people were analyzed separately.

It was stated that most often pathogens causing the infections were found in phlegm: in 52.8 ± 3.8 % cases (Figure 1). In oropharynx smears the pathogens were found positively (validly) less often: in 41.9 ± 3.7 % cases ($p < 0.05$). In blood samples, in single instances herpes viruses were found, and no pathogens causing bacterial infections were detected in blood.

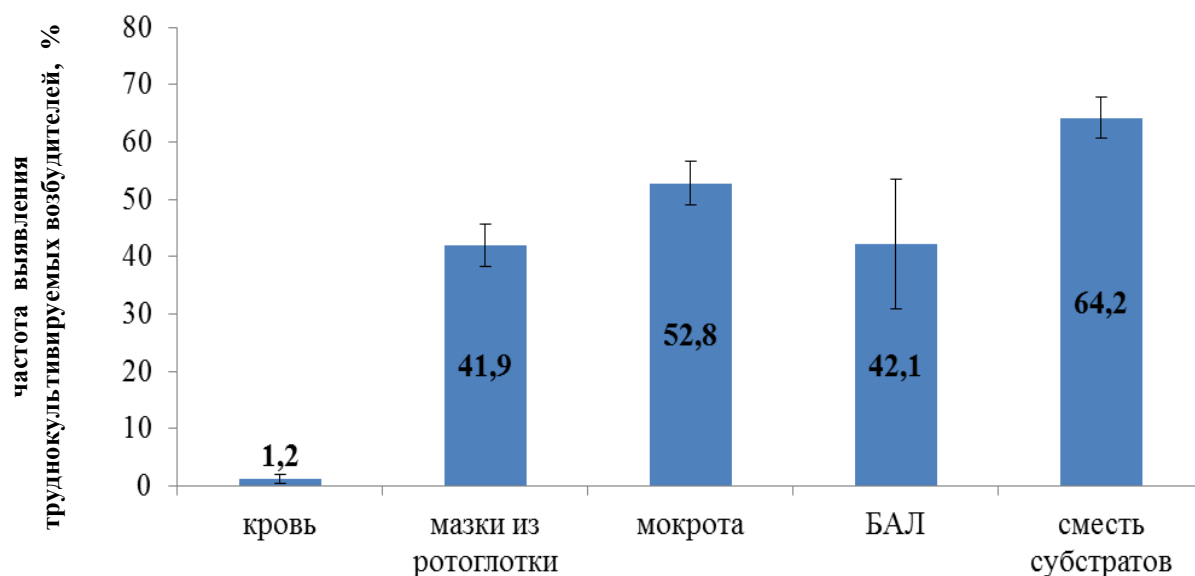


Figure 1. Contamination of different biological substrates with hard-to-cultivate pathogens causing respiratory diseases.

1.2 – blood

41.9 – oropharynx smears

52.8 – phlegm

42.1 – bronchoalveolar lavage

64.2 – a mixture of substrates

It was established that in the mixture of phlegm and oropharynx smear the percentage of detected pathogens was positively (validly) higher and came up to 64.2 ± 3.6 % ($p < 0.05$). In bronchoalveolar lavage samples ($n=19$) hard-to-cultivate pathogens were found in 42.1 % of cases.

Consequently, it was established that investigating a mixture of several clinical substrates (phlegm, oropharynx smear, bronchoalveolar lavage) for a spectrum of the sought pathogens lowers the possibility of false-negative results by 18-35 % in comparison with analyzing only one type of substratum.

Based on the data from literary sources and the results of the author's own independent research, a spectrum of pathogens causing respiratory diseases was defined; the frequency of finding them, as a rule, was not higher than 5%: for adults these are *C. psittaci*, *C. pneumoniae*, *L. pneumophila*, *M. catarrhalis*, *CMV*; for children *C. pneumoniae*, *C. psittaci*, *L. pneumophila*, *M. catarrhalis*. PCR-based analysis for every kind of biological substratum from one and the same patient for 4 to 5 infectious agents according to the traditional scheme appears to be a very expensive method of investigation. To solve this problem the author suggests a new medical technology FS # 2009/084, based on medical patent # 2406088. The essence of this method is detection of scarce pathogens (appearing in less than 5 % cases) in a DNA pool isolated from a substrates mixture (phlegm, smears from tunica mucosa of mouth, bronchoalveolar fluid) from five patients.

In order to check the sensitivity of the “double minipools” method, comparative investigation of 50 DNA samples was carried out. The DNA was isolated from a substrates mixture with the known content as to infectious agents. PCR-based analysis of the samples as “double minipools” and also individual PCR-based analysis for every

single sample were performed. In order to get the infectious agent's DNA maximally diluted, every pool consisted of one positive and four negative samples. The results acquired by the "double minipools" method and the results acquired by the individual sample analysis coincided in 100 % cases. It was shown that the dilution while pooling five samples has no influence on the effectiveness of revealing an infectious agent. The analysis of a substrates mixture took 1.5-2 times less time than a comprehensive analysis of a single substratum for every infectious agent.

To substantiate the economic effect of using the "double minipools" method, the author carried out a PCR-based research of samples from 100 adult patients with CAP for four infections pathogens: *CMV*, *C. psittaci*, *L. pneumophila*, *M. catarrhalis* by the comprehensive method and by the "double minipool" method. 20 minipools of the substrates from five people each were formed, and a mixture of 2 substrates from every patient was analyzed.

The "double minipools" method required 105 PCR-based analyses. Yet a comprehensive study of every substratum from each patient for four infections pathogens would require 800 PCR-based analyses; a study of a substrates mixture without pooling would require 400 PCR-based analyses. Accordingly, the usage of the "double minipools" method allows to lower the cost of investigation by 3.8–7.6 times.

The investigation made it possible to estimate the frequency of detecting *M. pneumoniae*, *C. pneumoniae*, *C. psittaci*, *L. pneumophila*, *M. catarrhalis*, *CMV*, *HSV I/II* in patients suffering from inflammatory respiratory diseases.

In Nizhny Novgorod region, bacterial hard-to-cultivate pathogens in infants under one year of age suffering from respiratory diseases were detected extremely seldom. Only two cases of detecting such pathogens were registered: *M. Pneumoniae* in a 11-month-old child suffering from acute bronchitis and *C. psittaci* in a 2-month-old child suffering from pleuropneumonia.

M. pneumoniae appeared to be the most frequent pathogen for children from 1 to 16 years of age having a respiratory pathology; the frequency of detecting it varied significantly (from 3.6 to 50%) depending on the nosologic form and the age of the

examined patients (Figure 2). The highest value of this characteristics (50.5 ± 4.7 %) was positively registered in children from 7 to 16 years of age suffering from CAP ($p < 0.001$).

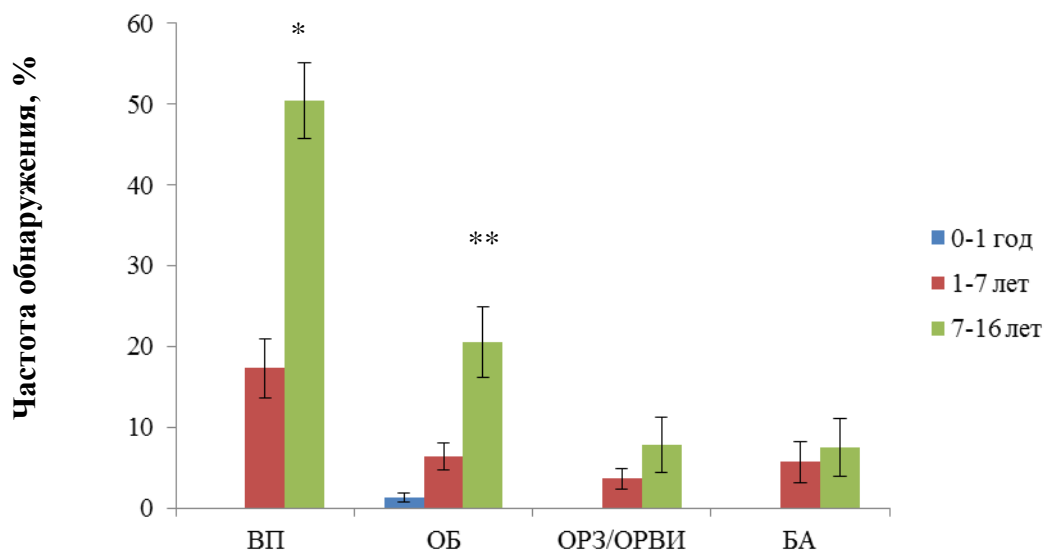


Figure 2. Frequency of detecting *M. pneumoniae* in children of different age groups with respiratory diseases.

ВП – community-acquired pneumonia (CAP)

ОБ – acute bronchitis

ОРЗ/ОРВИ – acute respiratory disease (ARD) / acute respiratory viral infection (ARVI)

БА – bronchial asthma

BLUE – 0-1 year of age

RED – 1-7 years of age

GREEN – 7-16 years of age

Note: * – the difference from the group of children 1-7 years of age is relevant and valid with $p < 0.001$;

** – the difference from the group of children 1-7 years of age is relevant and valid with $p < 0.01$.

M. pneumoniae also prevailed in children with acute bronchitis, ARD / ARVI, bronchial asthma in age groups 1-7 y.o. and 7-16 y.o. In children suffering from acute bronchitis the frequency of detecting it came to 6.4 % and 20.5 % respectively ($p < 0.01$); for those suffering from ARD / ARVI 3.6 % and 7.8 %; for those suffering from bronchial asthma – 5.7 % and 7.5 % respectively.

Other bacterial pathogens (*C. pneumoniae*, *C. psittaci*, *M. catarrhalis*) in children with bronchopulmonary pathology were detected quite seldom. *C. pneumoniae* was detected in samples from children of 7-16 years of age suffering from CAP in 3.6 ± 1.8 % cases. In children suffering from acute bronchitis the pathogen was found in single instances in age groups of 1-7 years of age and 7-16 years of age.

The highest result for the frequency of detecting *C. psittaci* (4.0 ± 1.4 % in 1-7 years of age group and 3.6 ± 2.0 % in 7-16 years of age group) was achieved for children suffering from acute bronchitis; during other nosologic forms of respiratory diseases this microorganism was found but in single instances.

M. catarrhalis was detected but in single instances and only in children suffering from bronchitis in 1-7 years of age group and in 7-16 years of age group.

L. pneumophila was not found in any form of pathology in any children's age group.

In the screening group hard-to-cultivate bacterial microorganisms were practically not found. This data shows that a wide circulation of “atypical” bacterial pathogens is not characteristic of Nizhny Novgorod region.

The results of the investigation showed a high frequency of DNA replication for herpes viruses in children with any nosologic form. For example, *CMV* was found in children suffering from CAP in 34.9 ± 2.6 % cases, in children suffering from acute bronchitis in 51.9 ± 2.6 % cases, in children suffering from ARD / ARVI in 49.0 ± 2.5 % cases, and in children suffering from bronchial asthma in 40.0 ± 4.1 % cases (Figure 3). In healthy children *CMV* was detected less often by 2.5-3.5 times ($p < 0.001$).

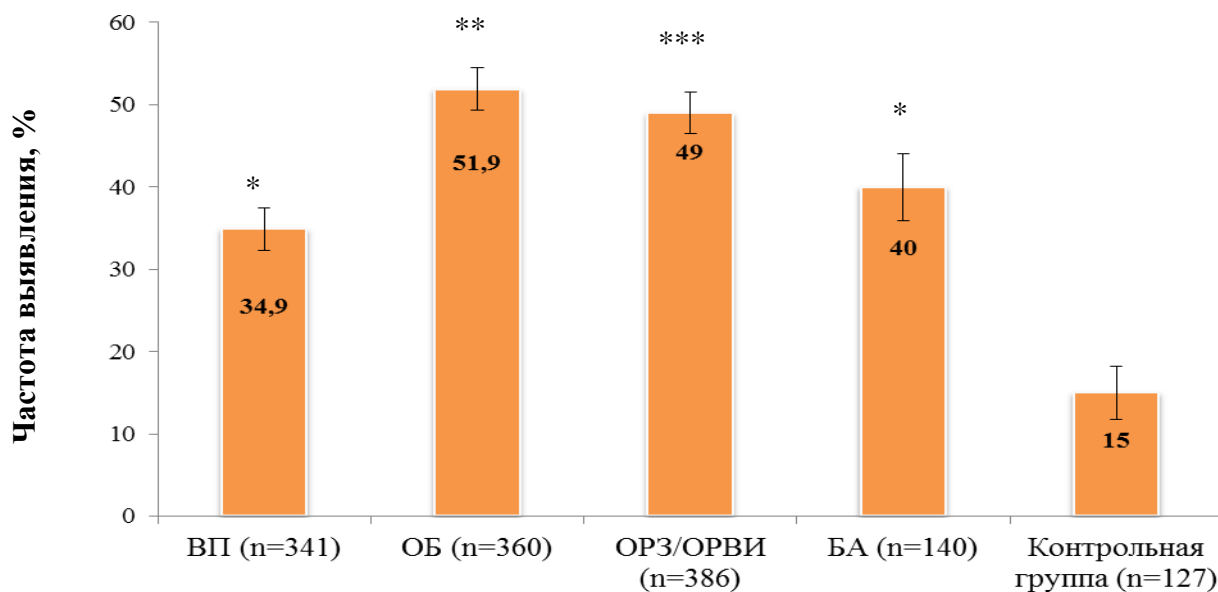


Figure 3. Frequency of finding *CMV* in children of different age groups with inflammatory respiratory diseases.

ВП – community-acquired pneumonia (CAP)

ОБ – acute bronchitis

ОРЗ/ОРВИ – acute respiratory disease (ARD) / acute respiratory viral infection (ARVI)

БА – bronchial asthma

Контрольная группа – screening group

Note: * – the difference from the screening group is relevant and valid with $p < 0.001$;

** – the differences are relevant and valid between the group of children suffering from acute bronchitis and groups of children with bronchial asthma ($p < 0.05$), CAP, screening group ($p < 0.001$);

*** – the differences are relevant and valid between the group of children suffering from ARD / ARVI and groups of children suffering from CAP, screening group ($p < 0.001$).

The indices of detecting *CMV* were high enough in infants under one year of age, they came up to 26.2 ± 3.9 % in children suffering from CAP; 44.0 ± 5.7 % in children suffering from acute bronchitis; 56.2 ± 5.8 % in children suffering from ARD / ARVI (Figure 4).

Most often *CMV* appeared to be detected in children from 1 to 7 years of age. In children suffering from CAP the virus was found in 56.7 ± 4.9 % cases; in children suffering from acute bronchitis in 64.4 ± 3.4 % cases; in children suffering from ARD / ARVI in 51.8 ± 3.2 % cases; in children suffering from bronchial asthma in 50.6 ± 5.4 % cases ($p < 0.001$). Only in children under 1 year of age and between 1 and 7 years of age suffering from ARD / ARVI the difference in the frequency of detecting *CMV* appears unreliable (not valid).

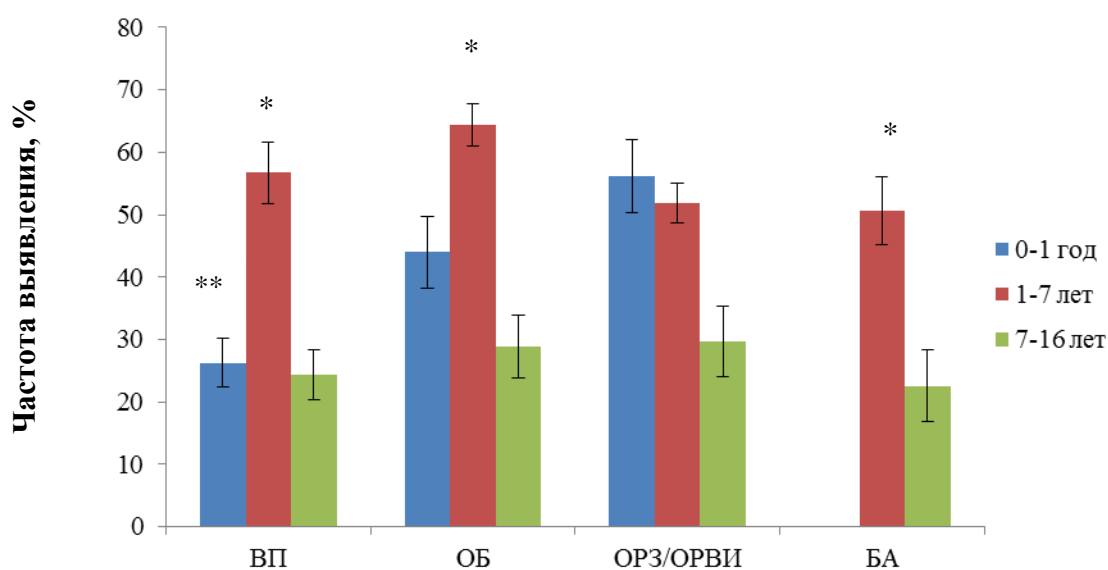


Figure 4. Frequency of detecting the *CMV* DNA replication in children of different age groups having various respiratory diseases.

ВП – community-acquired pneumonia (CAP)

ОБ – acute bronchitis

ОРЗ/ОРВИ – acute respiratory disease (ARD) / acute respiratory viral infection (ARVI)

БА – bronchial asthma

BLUE – 0-1 year of age

RED – 1-7 years of age

GREEN – 7-16 years of age

Note: * – the differences between the group of children under 1 and in group 7-16 are relevant and valid with $p < 0.001$;

**– the differences between the group of children under 1 with CAP and groups of children under 1 with acute bronchitis and ARD /ARVI ($p<0.01$ and $p<0.001$, respectively).

For the group of children from 7 to 16 the indices of detecting *CMV* were validly lower and came up to 24.3 ± 4.0 % with CAP; 28.9 ± 5.0 % with acute bronchitis; 29.7 ± 5.7 % with ARD / ARVI and $22.6\pm 5.7\%$ with bronchial asthma ($p<0.001$).

The indices of detecting *HSV I/II* in children suffering from CAP (6.7 ± 1.4 %) and those suffering from ARD / ARVI (5.2 ± 1.1 %) were higher than in the screening group ($p<0.01$ and $p<0.05$, respectively). There has been found no valid difference in the frequency of detecting *HSV I/II* in healthy children and children with bronchial asthma and those with acute bronchitis.

HSV I/II was more often detected in children from 7 to 16 years of age in all nosologic forms: if they had CAP – in 10.8 ± 2.9 % cases, if they had acute bronchitis – in 9.6 ± 3.2 %, if they had ARD / ARVI – in 7.8 ± 3.6 % cases, if they had bronchial asthma – in 3.8 ± 2.6 % cases (Figure 5).

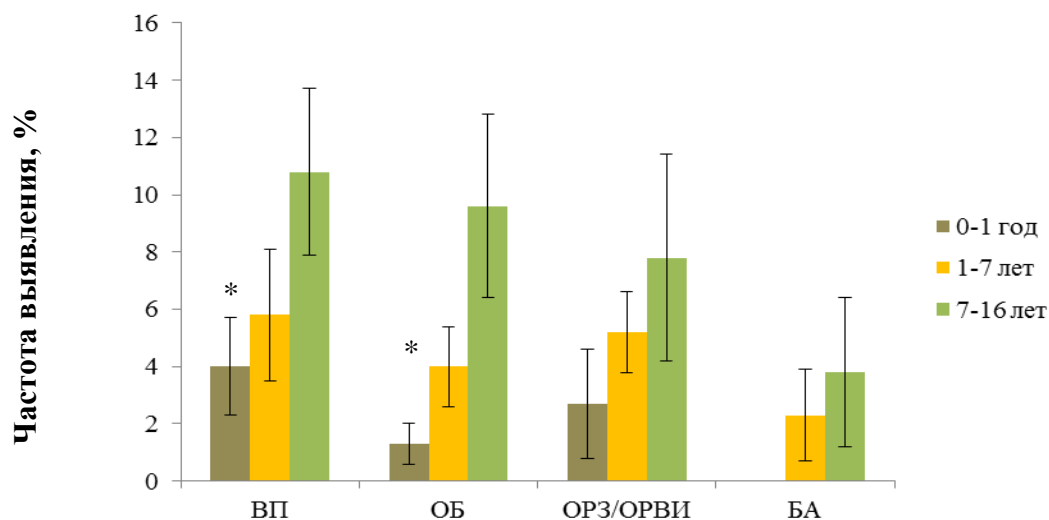


Figure 5. Frequency of detecting *HSV I/II* DNA in children of different age groups having various forms of respiratory diseases.

ВП – community-acquired pneumonia (CAP)

ОБ – acute bronchitis

ОРЗ/ОРВИ – acute respiratory disease (ARD) / acute respiratory viral infection (ARVI)

БА – bronchial asthma

BROWN – 0-1 years of age

YELLOW – 1-7 years of age

GREEN – 7-16 years of age

Note: * – the difference with the group of children from 7 to 16 is relevant and valid with $p < 0.05$.

Since the majority of the examined adults, both unorganized and organized, were diagnosed with CAP, and the number of patients having acute bronchitis was minor, the author found it expedient to analyze them as one group, especially because the frequency of detecting the pathogens causing these two types of infections showed no statistically relevant difference. In Table 1 one can find the data on the frequency of detecting pathogens causing various infections in adults suffering from inflammatory respiratory diseases.

Table 1.

Frequency of Detecting Hard-to-Cultivate Pathogens in Adults ($M \pm m(\%)$).

Pathogen	organized		unorganized	
	patients (n=469)	screening (n=270)	patients (n=145)	screening (n=52)
<i>M. pneumoniae</i>	9.8±1.4	0	2.8±1.4	0
<i>C. pneumoniae</i>	10.4±1.4	0	1.4±1.0	0
<i>C. psittaci</i>	0.2±0.2	0	0	0
<i>L. pneumophila</i>	0.6±0.4	0	0	0
<i>M. catarrhalis</i>	1.7±0.6	1.1±0.6	2.1±1.2	1.9±1.9
CMV	5.1±1.0	4.1±1.2	3.5±1.5	3.8±2.7
HSV I/II	15.4±1.6	3.3±1.0	10.3±2.5	1.9±1.9

As one can infer from Table 1, *M. pneumoniae* and *C. pneumoniae* in organized adult patients were found in 9.8 % and 10.4 % cases respectively, whereas in the screening group these pathogens were not detected.

The frequency of detecting these pathogens in the group of unorganized adults was validly lower and came up to 2.8 % and 1.4 % respectively ($p < 0.001$). In both groups of patients the frequency of detecting *HSV I/II* was high as compared to the screening groups ($p < 0.01$). With the military personnel the index was 15.4 %, with unorganized adults 10.3 %, and statistically these two indices did not differ. *L. pneumophila* was detected only in samples from three military people. *C. psittaci* was found but in one military person who had CAP. It was not found in unorganized adult patients. The frequency of detecting *M. catarrhalis* in organized and unorganized patients showed no statistical difference and came up to 1.7 % and 2.1 % respectively.

The immunologic investigation showed that the called-up military people who suffered from CAP had “immunologic deficiency”. For example, estimation of the level of cytokines in the patients’ blood serum indicates relative weakness of the cellular immune reaction when CAP developed. At the moment of hospitalization, i.e. at the peak of the inflammatory process development, the patients’ tests displayed no significant growth of cytokines produced by T helper cells of the first type, proinflammatory regulators of interferon- γ and tumor necrosis factor- α (Table 2). The concentration of such cytokines exceeded the average for healthy people but by 1.3 and 1.6 times, respectively, at that the maximal concentration of cytokines in 98 % of the patients did not exceed 40 pcg/ml and 4 pcg/ml, respectively. The interferon- α concentration in those suffering from CAP did not validly exceed the average for healthy people, either. The mean concentration was 7.4 pcg/ml, and 65.5 % patients’ tests showed no interferon- α in blood serum.

Table 2.

Level of Cytokines During Inflammation in
Blood Serum of Patients Suffering from Community-Acquired Pneumonia

Cytokines	Concentration of cytokines (pcg/ml) in blood serum	
	Patients with pneumonia (n=100)	Healthy donors (n=50)
Interferon- α	4.41 \pm 1.03	4.31 \pm 1.05
Interferon $-\gamma$	32.31 \pm 4.25	24.8 \pm 6.3
Tumor necrosis factor $-\alpha$	3.62 \pm 0.13	3.02 \pm 0.31
Interleukin-8	52.14 \pm 7.83*	25.9 \pm 4.67
Interleukin 1 receptor antagonist	1039.57 \pm 123.8*	232.45 \pm 54.1

Note. * – the difference from the average of the screening group of healthy donors is relevant and valid with $p < 0.05$.

The average concentration of interleukin-8 in military patients suffering from CAP validly exceeded the concentration of this cytokine for healthy donors. However, its concentration in samples from the majority of the examined patients with pneumonia (58.9 %) did not exceed 30 pcg/ml which is the top of the norm for interleukin-8 in healthy adults.

The most informative parameters appeared to be the ones showing the functional state of the immune system cells, particularly T-lymphocytes. The author studied the capability of T-lymphocytes to produce interferon- γ , not by estimating the number of T-cells producing this kind of cytokine during the illness but by analyzing the potential capability of the immune system to fulfill this function. It was shown that the number of T-lymphocytes that can produce interferon- γ in the examined military patients

hospitalized with CAP was considerably lower than in the screening group of practically healthy donors.

The average number of T-cells that produce interferon when they get activated was for patients with pneumonia, at the beginning of their disease period, $16.24 \pm 2.15\%$, and for healthy donors this index came up to $29.25 \pm 3.5\%$ ($p < 0.001$). The fact that this functional deficiency was detected means it would be sensible to take some action in order to support the immune system, to compensate for the deficiency of the produced interferons during pneumonia. In this light, it is not expedient to use inductors of interferon production when treating CAP, and it is more appropriate to use substitutive therapy with interferon preparations. It is shown that the analysis of the circulating immune complexes levels has high prognostic value for patients with CAP. The average level of high-molecular circulating immune complexes in sick people's samples came up to 68 ± 13.5 standard units, the level of low-molecular circulating immune complexes was 149 ± 13 standard units. The ratio between low-molecular circulating immune complexes and high-molecular circulating immune complexes (the so-called relative coefficient) varies within the range of 0.3-23.4, which can testify to a high diagnostic value of this index during inflammatory destructive processes in the lungs. When the disease progressed and its clinical course was aggravating, the number of circulating immune complexes was lowering, and the relative coefficient of the circulating immune complexes was growing. For example, for groups with a light clinical course of pneumonia the level of high-molecular circulating immune complexes came up to 360 standard units, and the lowest index of the relative coefficient of the circulating immune complexes was 0.34. For those with a more serious state, the level came up to 120 standard units, and the lowest index of the relative coefficient of the circulating immune complexes was 1.1. The most serious clinical course of CAP was accompanied with a paradoxical effect of the decline in the circulating immune complexes level, which is explained by the decline in the immunocompetence of the humoral immunity unit. The level of the circulating immune complexes was the lowest in this group (74 standard

units), and the lowest index of the relative coefficient of the circulating immune complexes was 2.03.

The analysis of the frequency of detecting *M. pneumoniae*, *C. pneumoniae*, *C. psittaci*, *L. pneumophila*, *M. catarrhalis*, *CMV*, *HSV I/II* allowed to elaborate an algorithm of PCR-based detection of these pathogens for an initial screening in children and adults suffering from respiratory diseases. The investigation is based on analyzing phlegm, bronchoalveolar lavage, oropharynx smears; for infants under one year of age one can use saliva. The first step is to isolate DNA out of a mixture of various biological substrates from one and the same patient. The substrates are mixed in equal proportions so that the total volume comes up to 100 microliters, which is the recommended volume for isolating DNA with the help of reagents set “DNK-sorb B” (“DNA-sorb B”) (by the Federal Budget Institution of Science “Central Research Institute of Epidemiology” of The Federal Service on Customers’ Rights Protection and Human Well-being Surveillance). Further, PCR-based investigation is carried out in order to detect pathogens that tend to appear more often than in 5% of samples: for infants under 1 year of age *CMV*; for children from 1 to 7 *M.pneumoniae*, *CMV*, *HSV I/II*; for children from 7 to 16 *M.pneumoniae*, *CMV*, *HSV I/II* (Figure 4); for unorganized adults *HSV I/II*. For detecting *CMV*, *HSV I/II*, *M. pneumoniae* with the help of PCR, test-systems of “AmpliSense” series (by the Federal Budget Institution of Science “Central Research Institute of Epidemiology” of The Federal Service on Customers' Rights Protection and Human Well-being Surveillance) were used, according to the manual. For PCR-based detection of *C. pneumoniae*, *C. psittaci*, *L. pneumophila*, *M. catarrhalis*, “GenPak DNA PCR test” reagents sets were used, produced by LLC “Isogen” (Moscow). Amplification was done on devices “Tertsic MS-2” (DNA technology, Moscow) or “My Cyclor” (Bio-Rad, the USA).

For detecting scarce pathogens causing inflammatory respiratory diseases with the help of PCR, a minipool of DNA preparations is formed, the preparations being isolated out of a mixture of substrates from five (four, three) patients. The DNA preparations are mixed in equal proportions so that the mixture volume for one PCR-

based analysis comes up to 10 microliters (2 microliters from 5 people, or 2.5 microliters from 4 people, or 3.3 microliters from 3 people). Amplification products were detected by horizontal electrophoresis in 1.8% agarous gel. If one or several pathogens are found in the minipool, every component of the pool gets analyzed. The same test-systems and electrophoresis are used as methods of detecting the PCR products. An example of this algorithm is shown in Figure 6.

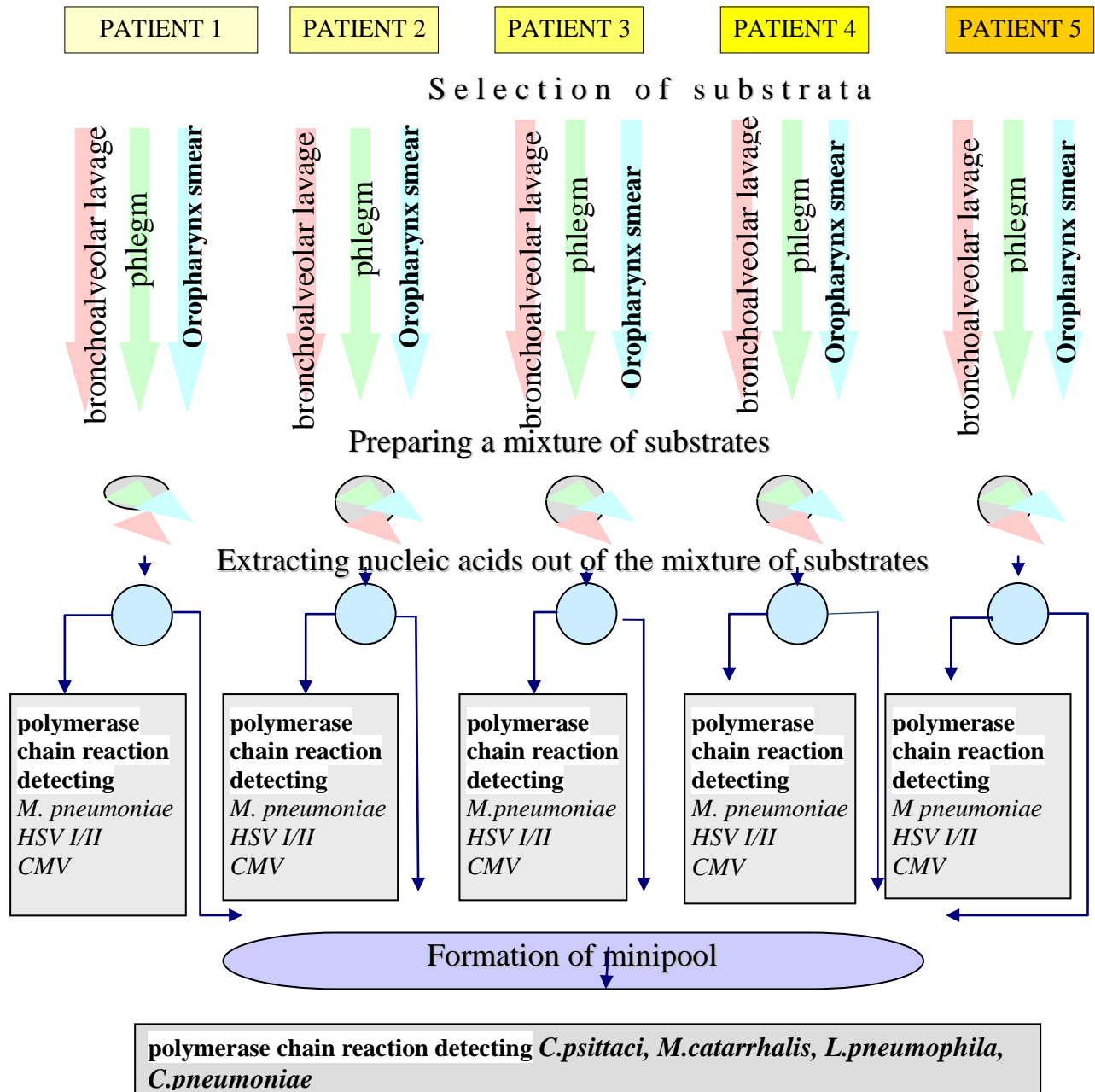


Figure 6. Algorithm of detecting hard-to-cultivate pathogens causing respiratory diseases in children from 1 to 6 years of age with the help of PCR.

This method has been instilled in the work of the laboratory for metagenomics and indicating pathogens and Volga-Vyatka Regional Academic and Research Center for Indication, Identification and Taxonomy of Microorganisms and Organization of Anti-Epidemic work in Extreme Conditions of the Federal Budget Science Institution “Nizhny Novgorod Scientific Research Institute of Epidemiology and Microbiology named after Academician I.N. Blokhina” of the Federal Directorate for Consumer Rights and Health Protection.

The method of “double minipools” is effective in detecting the following infectious agents: for infants under 1 year of age: *M.pneumoniae*, *HSV I/II* *munos*, *C.psittaci*, *C.pneumoniae*, *L.pneumophila*, *M.catarrhalis*; for children from 1 to 7 years of age: *C.pneumoniae*, *C.psittaci*, *L.pneumophila*, *M.catarrhalis*; for children from 7 to 16: *C.pneumoniae*, *C.psittaci*, *L.pneumophila*, *M.catarrhalis*; for unorganized adults: *M.pneumoniae*, *C.pneumoniae*, *C. psittaci*, *L. pneumophila*, *M. catarrhalis*, *CMV*; for organized adults: *C.psittaci*, *L.pneumophila*, *M.catarrhalis*.

CONCLUSION

The experiments that have been conducted give the author the opportunity to formulate the following conclusions.

1. The author has optimized the method of detecting hard-to-cultivate microorganisms, i.e. pathogens causing respiratory diseases, with the help of PCR. The method is based on pooling mixtures of substrates from 3 to 5 patients (“double minipools”) and allows to make a low-costing screening of patients suffering from different types of respiratory pathology and to cut the time of diagnostic investigation by 1.5-2 times.
2. It is proved that the most informative substrates for PCR-based detection of hard-to-cultivate microorganisms causing respiratory infections are phlegm (the infectious agent gets detected in 52.8 % samples) and smears from the posterior pharyngeal wall (41.9 % results are positive). Examining a mixture of these substrates allows to raise the frequency of detecting pathogens by 18-35%.

3. The author registers a high frequency of detecting *M. pneumoniae* in children from 7 to 16 years of age suffering from CAP and acute bronchitis (50.5% and 20.5% respectively). At the same time, she registers a low frequency of detecting *C. psittaci*, *L. pneumophila*, *M. catarrhalis* in Nizhny Novgorod region for children and adults (both organized and unorganized) with bronchopulmonary pathologies.
4. The investigation results display an active replication of herpes viruses in children of different ages. The highest frequency of detecting *CMV* (50.6% – 64.4%) have been registered in samples taken from children from 1 month old to 7 years old, *HSV I/II* (7.8% – 10.8%) – in children from 7 to 16 notwithstanding the nosologic form of the prevailing disease.
5. It is revealed that for adults in organized collectives the leading types of hard-to-cultivate pathogens causing respiratory diseases are *M. pneumoniae* (9.8%) and *C. pneumoniae* (10.4%). For unorganized adults these pathogens appear less often: *M. pneumoniae* – 2.8%, *C. pneumoniae* – 1.4%. The frequency of detecting *HSV I/II* was high for both organized and unorganized adults and came up to 15.4% and 10.3%, respectively, which validly exceeds this index in the screening group of practically healthy people.

Practical Recommendations

1. For epidemiological supervision of patients suffering from CAP and for elaboration of the medical and preventive measures it is sensible to take into consideration the properties of hard-to-cultivate infectious agents' distribution peculiar to the specific region.
2. Since some types of hard-to-cultivate microorganisms associated with respiratory diseases are widespread, it is necessary to give patients a complex PCR-based examination.
3. The suggested algorithm of PCR-based detection of hard-to-cultivate pathogens causing respiratory diseases is recommended for a low-costing screening of patients

suffering from different types of respiratory pathology as it allows to cut the cost and the length of the diagnostic investigation.

4. Since the examined military personnel with CAP displayed deficiencies in the immune system work, it is advisable for such patients to have a full checkup of the immune system and, if necessary, to undergo a course of treatment correcting the immunity and compensating for the interferon production deficiency.

Prospects and Directions for Further Work in this Field

Several prospects are to be named. The first task is monitoring of the distribution of hard-to-cultivate pathogens causing respiratory diseases, on the basis of the suggested algorithm with the help of PCR. The second task is to broaden the specter of infectious agents that can be analyzed on the basis of the “double minipools” method in molecular diagnostics for respiratory diseases. The third prospect is studying the genetic variability and antibiotic resistance of hard-to-cultivate infectious agents on the basis of the highly productive sequencing technology that allows for analyzing the circulating populations of pathogens causing respiratory diseases, in more detail.

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ABBREVIATION LIST

CAP – community-acquired pneumonia

PCR – polymerase chain reaction

ARD – acute respiratory disease

ARVI – acute respiratory viral infection

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