МЕДИЦИНСКИЕ НАУКИ

IMPROVING THE EFFICIENCY OF CHLAMYDIA-MYCOPLASMA INFECTION PATHOGENS DNA DETECTION IN SPUTUM BY USING A NUCLEIC ACIDS EXTRACTION METHOD BASED ON CETYLTRIMETHYLAMMONIUM BROMIDE

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ABSTRACT

To increase the efficiency of detecting DNA of Mycoplasma pneumoniae and Chlamydia pneumoniae in sputum, a variant of extraction has been tested and improved; it is based on the use of CTAB detergent that has the property to bind both polysaccharides and DNA. The combined use of CTAB-detergent, proteinase K, SDS and chloroform-izoamil alcohol mixture purifies nucleic acid preparation from proteins and lipids; therefore the efficiency of chlamydia-mycoplasma DNA identification in sputum increases due to amplification inhibitors decrease.

Keywords: sputum, nucleic acid extraction, Mycoplasma pneumoniae, Chlamydia pneumoniae, CTAB.

Introduction. Sputum examination is one of the leading branches of clinical laboratory diagnostics for respiratory diseases, including infections caused by atypical microorganisms – chlamydia and mycoplasma [1, 7].

The method of polymerase chain reaction (PCR) acquires more and more significance for etiological microbiological diagnostics of respiratory diseases associated with infections caused by hard-to-cultivate microorganisms, like chlamydia and mycoplasma. The basis for PCR-analysis is examination of DNA extracted from biological material. The stage of DNA extraction is one of the key moments of this method; it prepares nucleic acid (NA) to the stage of amplification. The main principles of the NA extraction stage consist in the DNA release and the consequent deletion of PCR inhibitors [2, 3, 5].

Sputum is considered as the most informative biological material for PCR diagnostics of respiratory chlamydiosis and mycoplasmosis. Analytical sensitivity of the PCR-method in detecting DNA of clamidial-mycoplasmal nature greatly depends on the efficiency of DNA extraction from sputum [5].

While choosing a method of NA extraction from sputum, it is necessary to take into account the inhibitor spectrum of this biological material. Methods of NA extraction suitable for one biological material can be totally unsuitable for NA extraction from sputum or result in the product with low DNA concentration, presence of PCR-inhibitors, which can lead to false negative results in detecting DNA of chlamydia and mycoplasma.

Sputum belongs to biological materials with a great amount of amplification inhibitors like mucolytic agents, blood components, polysaccharides. Polysaccharides contained in sputum mucins are responsible for viscosity of sputum and are the main amplification inhibitors, since they are present in sputum in high concentrations. The inhibiting action of polysaccharides is connected to their ability to impede the "work" of DNA-polymerase [3, 6].

Cetyltrimethylammonium bromide (CTAB) is a cationic detergent that purifies preparations from polysaccharide substances and effectively binds DNA. CTAB is widely applied in molecular phytogenetics. Genomic DNA is normally extracted with the use of CTAB extraction buffer, purified with the mixture of phenol and chloroform and precipitated with isopropanol or ethanol. The method of CTAB-based NA extraction is also used for DNA extraction out of respiratory tract secretions, as well as microorganism cultures with pathogenetic importance in the progress of respiratory pathology, such as M. tuberculosis; however, there is no data on the use of CTAB for detecting DNA of chlamydial-mycoplasmial pathogens in sputum [3, 4, 8].

The aim of the research was to develop the most effective way of DNA extraction for Mycoplasma pneumoniae and Chlamydia pneumoniae on the basis of the comparative analysis of three most common clinical-laboratory methods of nucleic acid extraction from sputum – using proteinase K and phenol-chloroform extraction, sorptive extraction with magnetic particles, and CTAB-using method.

Materials and methods. Sputum samples were obtained from 38 children and teenagers with bronchitis and pneumonia of chlamydia-mycoplasma etiology (protracted respiratory disease, no effect from antibiotic therapy for infectious agents of non-chlamydia-mycoplasma nature, detection of antichlamydia and/or antimycoplasma antibodies). To lower the viscosity of sputum, each sample was mixed with the mucolysin reagent in proportion 5:1 (5 parts of mucolysin to 1 part of sputum) and incubated for 30 minutes. The mixture of sputum and mucolysin was centrifuged for 10 min at 7000 g; the precipitate of sputum cellular elements was used to extract DNA in accordance with the procedure for each of the three applied extraction variants: No. 1 – processing with proteinase K and phenol-chloroform extraction, No. 2 – sorptive extraction with magnetic particles, and No. 3 – the CTAB method. Quality and quantity control of purified DNA was conducted on spectrophotometer NanoDrop 1000 (ThermoFisherScientific).

Detection of DNA of Mycoplasma pneumoniae and Chlamydia pneumonia was performed with the

method of multiplex PCR in real time (PCR-PB) using the reagent kit made by OAO Vektor-Best, Russian Federation.

Extraction variant 1 – processing with proteinase K and phenol-chloroform extraction. 400 mcl of lisocyme solution (10 g/l, Sigma) were added to the suspension of cellular sputum elements and were incubated for 1 hour at 37°C. Then 20 mcl of EDTA (50 mM) and 400 mcl of proteinase K solution (10 g/l, Sigma) were added to the mixture and incubated at 60°C for 1 hour. The solution was left overnight at

-20°C. The mixture was divided into 2 parts; to each part 200 mcl of phenol and 200 mcl of chloroform were added. Both of the parts were intensively mixed and centrifuged for 15 min at 10000 g. The upper phase was transferred into a clean tube without touching the lower phase and the interphase; then we added 0,6 of the volume of isopropanol and 1/10 of the volume of sodium acetate to it, mixed thoroughly and left it for 30 minutes at -20°C. DNA was precipitated by centrifuging for 15 minutes at 10000 g. The precipitate was rinsed with 500 mcl of 70% ethanol twice, dried till ethanol completely evaporated and dissolved in 30 mcl of TE buffer.

Extraction variant 2 – sorptive extraction with magnetic particles. DNA extraction was performed using the reagent kit RealBest DNA-extraction 1 (OAO Vektor-Best, Russian Federation).

Extraction variant 3 – the CTAB method. The suspension of sputum cellular elements was incubated at 65°C for 10 minutes with 100 mcl of CTAB/NaCl solution (4.1 g of NaCl, 10 g of CTAB in 100 mcl of sterile water), Sigma. After incubation the extracts were purified with equal volumes of the 92% chloroform/8% isoamyl alcohol mixture in proportion 24:1 with the total volume 700 mcl and then centrifuged for 5 minutes at the maximum speed of 14243 g. The water phase, without touching the middle and lower phases, was transferred into a new tube; DNA was cooled off with cold isopropanol (0,6 of the water phase volume), left for 30 minutes at -20°C to form DNA aggregates, centrifuged for 15 minutes at the maximum speed of 14243 g. The NA precipitate was rinsed with cold (-20^{0}C) 70% ethanol twice for 5 minutes, then dried and dissolved in 30 mcl of TE buffer.

Results and discussion. One of the main criteria for the most effective NA extraction method from sputum for the following PCR analysis is the ability to delete polysaccharides that are present in sputum in large amounts and inhibit amplification.

The concentration values of the extracted genomic DNA, extracted in accordance with the variants 1, 2 and improved 3, amounted to the range from 5,6 to 73,9 ng/mcl.

The median values of the results of three parallel NA extractions through each variant were taken as the DNA amount. The biggest DNA output was demonstrated by the methods of proteinase K and phenol-chloroform extraction and the CTAB method (Figure 1).

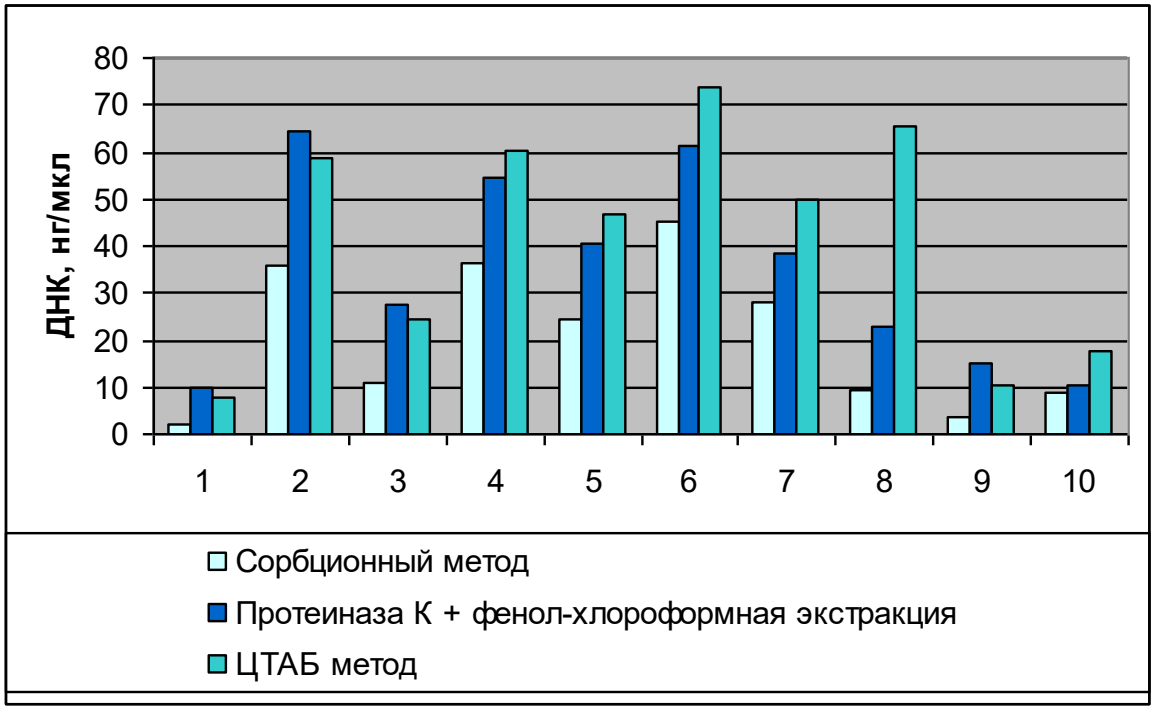

Figure 1.The concentration of DNA extracted from sputum samples through different methods.

To evaluate the DNA preparation purity, the optic density of DNA solutions was measured in TE buffer at wavelengths 260, 280 and 235 nm, which correspond to the maximums of absorption of DNA solutions, proteins and polysaccharides correspondingly. The value of A260/280 for pure DNA is 1,8-1,9, the value A260/235 – 2,2-2,3 (Table 1).

Table 1

THE RELATIONS OF ABSORPTIONS OF DNA SAMPLES AT DIFFERENT EXTRACTION VARIANTS

Variant of NA extraction	A260/280	A260/235
Extraction 1	1,78±0,05	1,85±0,07
Extraction 2	1,72±0,06	1,62±0,07
Extraction 3	1,86±0,05	2,22±0,06

While comparing the improved CTAB method with the method of sorptive extraction (extraction variant 2), the presence of statistically significant differences is shown (Student's test, p = 0,03).

At the next stage we conducted molecular-genetic tests of Mycoplasma pneumoniae and Chlamydia pneumoniae in sputum that underwent the stage of sample preparation using different extraction methods (Table 2).

Table 2

THE DETECTION RATE OF DNA OF MYCOPLASMA PNEUMONIAE AND CHLAMYDIA PNEUMONIAE

Detection rate (%)	Extraction 1	Extraction 2	Extraction 3
DNA Mycoplasma pneumoniae	31,58±5,27	23,68±4,64	42,10±5,95
DNA Chlamydia pneumoniae	15,79±3,85	7,89±2,77	18,42±4,14

It was established that the detection rate of DNA of Mycoplasma pneumoniae and Chlamydia pneumoniae in sputum depends on the NA extraction method.

Conclusion. Application of classical NA extraction methods from sputum can lead to obtaining false results due to insufficient deletion of PCR inhibitors. Application of the CTAB method together with proteinase K and SDS helps to increase the efficiency of PCR DNA detection while detecting DNA of the microorganisms that have particular significance in respiratory pathology development.

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