CHEMISTRY AND TECHNOLOGY OF MEDICINAL COMPOUNDS AND BIOLOGICALLY ACTIVE SUBSTANCES

ХИМИЯ И ТЕХНОЛОГИЯ ЛЕКАРСТВЕННЫХ ПРЕПАРАТОВ И БИОЛОГИЧЕСКИ АКТИВНЫХ СОЕДИНЕНИЙ

ISSN 2686-7575 (Online)

https://doi.org/10.32362/2410-6593-2021-16-3-225-231

UDC 57.083.18

RESEARCH ARTICLE

Specificities of multi-primer polymerase chain reaction optimization for the detection of infectious pneumonia agents in human

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Abstract

Objectives. *The objectives of this work are the development of a multi-primer system based on the polymerase chain reaction (PCR) aimed at the simultaneous detection of six bacterial pathogens that cause human pneumonia and the determination of the parameters important for the optimization of this multi-primer system, including solid-phase PCR systems (biological microarrays).*

Methods. *To determine the optimal parameters of the system, PCR methods were used in monoplex and multiplex formats.*

Results. *Primers for Staphylococcus aureus, Pseudomonas aeruginosa, Haemophilus influenza, Legionella pneumophila, Klebsiella pneumoniae, and Streptococcus pneumoniae detection were designed, and the PCR cycling conditions were optimized. The patterns of primer design for solid-phase PCR were revealed.*

Conclusions. *The developed prototype of a system specifically identifies six clinically significant bacterial pathogens. It could be expanded for the analysis of viral and fungal pathogens and used in clinical diagnostics. A prototype of a system for pathogenic agent detection in the immobilized phase (biological microarray) was created.*

Keywords: *infectious pneumonia, multiplex PCR, biochips, COVID-19*

For citation: Klochikhina E.S., Shershov V.E., Kuznetsova V.E., Lapa S.A., Chudinov A.V. Specificities of multi-primer polymerase chain reaction optimization for the detection of infectious pneumonia agents in human. *Tonk. Khim. Tekhnol. = Fine Chem. Technol.* 2021;16(3):225–231 (Russ., Eng.). https://doi.org/10.32362/2410-6593-2021-16-3-225-231

НАУЧНАЯ СТАТЬЯ

Особенности оптимизации мультипраймерной ПЦР для выявления возбудителей инфекционной пневмонии человека

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Аннотация

Цели. *Разработка мультипраймерной системы на основе полимеразной цепной реакции (ПЦР), направленной на одновременное выявление шести основных бактериальных возбудителей пневмонии человека; выявление параметров и закономерностей, имеющих важное значение для оптимизации мультипраймерной системы, в том числе для разработки систем ПЦР в иммобилизованной фазе (на биологическом микрочипе).*
Методы. *Для определения оптимальных параметров системы использовали методы ПЦР в т.н. «моноплексном» и мультиплексном форматах.*
Результаты. *Сконструированы праймеры, и оптимизирован температурно-временной профиль проведения ПЦР в объеме для выявления Staphylococcus aureus, Pseudomonas aeruginosa, Haemophilus influenza, Legionella pneumophila, Klebsiella pneumoniae и Streptococcus pneumoniae. Выявлены закономерности конструирования праймеров для ПЦР в иммобилизованной фазе.*
Выводы. *Изученные закономерности особенностей оптимизации мультипраймерных систем позволили разработать прототип системы, способной специфично выявлять шесть клинически значимых возбудителей пневмонии человека. Прототип системы может быть расширен для анализа вирусных и грибковых патогенов и применяться в клинической диагностике. Результаты изучения особенностей мультиплексной ПЦР в иммобилизованной фазе привели к созданию прототипа системы для выявления патогенных агентов на биологическом микрочипе.*

Ключевые слова: *инфекционная пневмония, мультиплексная ПЦР, биочипы, COVID-19*

Для цитирования: Клочихина Е.С., Шершов В.Е., Кузнецова В.Е., Лапа С.А., Чудинов А.В. Особенности оптимизации мультипраймерной ПЦР для выявления возбудителей инфекционной пневмонии человека. *Тонкие химические технологии.* 2021;16(3):225–231. https://doi.org/10.32362/2410-6593-2021-16-3-225-231

INTRODUCTION

The term “infectious pneumonia” covers a spectrum of diseases of the respiratory system that differ in etiology and pathogenesis [1]. Accurate and timely identification of the causative agent of the lesion is important for the development of successful treatment regimens [2], as well as for the control of the infection. This has become an urgent problem in the context of the COVID-19 pandemic, when, in addition to a viral disease, patients entering medical institutions are faced with secondary nosocomial infections [3].

To identify the causative agents of pneumonia, which can be viruses, bacteria, or fungi, possible standard methods can be used for etiological diagnosis, for example, inoculation. This is often inconvenient due to the limitations of working

with biological samples, as well as the lengthy and laborious work involved in cultivation, isolation, and further determination of the pathogenic agents [4, 5]. For these reasons, more accurate and faster identification is often required for correct treatment [6].

Currently, molecular genetic methods of analysis, such as the polymerase chain reaction (PCR) variations, are becoming more widespread. Their application is favorably notable for relative ease, accuracy, and sensitivity of the results, as well as speed [4], which is important for treating diseases with a highly dynamic pathological development process.

This work is devoted to the development and optimization of PCR for the simultaneous detection of six bacterial pathogens of human pneumonia: *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Haemophilus influenza*, *Legionella pneumophila*, *Klebsiella pneumoniae*, and *Streptococcus pneumoniae*. In the described approach, regions of marker genes of pathogens were amplified in one common volume, the resulting products being separated by the electrophoretic method. The lengths of the detected amplified fragments were used to identify the specific pathogen.

Using the developed system as an example, important features for the optimization of multi-primer PCR are described, including those for use in the immobilized phase.

EXPERIMENTAL

Strains

We used the decontaminated genome-wide DNA of bacterial strains from the collection of the State Scientific Center for Applied Microbiology and Biotechnology (Obolensk, Serpukhov, Moscow oblast, Russia). DNA isolation from the cell cultures was completed using the cetyltrimethylammonium bromide method [7].

Primers

Multiple alignment of the genomic target sequences was performed using the ClustalW[1] algorithm. The primers were designed using the Integrated DNA Technologies[2] network resource, and the specificity analysis was performed using the basic local alignment search tool (BLAST)[3] algorithm (National Institutes of Health, USA). The sequences, species specificities, genetic targets, and lengths of the PCR products for all of the primer pairs used are shown below:

S. aureus, ebpS gene, direct ebpS-f (5'-ACTCGACTGAGGATAAAGCGTCT-3'), reverse ebpS-r (5'-CCTCCAAATATCGCTAATGCACC-3'), PCR product length: 283 base pairs (bp), nested reverse R1 (5'-NH_2-CCTCCAAATATCGCTAATGCACC-3'), R2 (5'-NH_2-GGTAACAATACTTTGGCCATGCCACC-3'), nested direct F1(5'-CTGCCGCTTCAAAACCACATGCC-3), F2 (5'-AAAAGGTGGCATGGCCAAAGT-3'), F3 (5'-AGCAAGTAATAGTGCTTCTGCCG-3').

S. pneumonia, cpsB gene, direct cpsB-f1 (5'-TTGATGTAGATGACGGTCCCAAG-3'), reverse cpsB-r1 (5'-TATATCTCTGCGCCATAAGCAAT-3'), PCR product length: 217 bp, nested reverse R3 (5'-NH_2-TATATCTCTGCGCCATAAGCAAT-3'), R4 (5'-NH_2-CGAACCTGAAGAAAGTTTTCTG-3'), R5 (5'-NH_2-GCAATGACTAAATCATCTGCCAC-3'), nested direct F4 (5'-GCGAACCATTGTCTCTACCTCTC-3'), F5 (5'-TCTACCTCTCACCGTCGCAAGGG-3'), F6 (5'-TGGCAGAATCCTACAGGCAGG-3').

L. pneumophila, sidA gene, direct sidA-f (5'-TTCCACTGGTGGGTGGGGTTTTG-3'), reverse sidA-r (5'-TCATGTTGGAGTTCTATGGCACG-3'), PCR product length: 369 bp.

H. influenza, fucK gene, direct fucK-f (5'-TGCTCACTCAACGCTTAACTGGT-3'), reverse fucK-r (5'-TTCTGGGCTAATGGTGTACGTAA-3'), PCR product length: 193 bp.

P. aeruginosa, oprL gene, direct oprL-f (5'-GCGTGCGATCACCACCTTCTACT-3'), reverse oprL-r (5'-TTCTTCAGCTCGACGCGACGGTT-3'), PCR product length: 321 bp.

K. pneumonia, rmpA gene, direct rmpA-f (5'-ATCAATAGCAATTAAGCACAAAA-3'), reverse rmpA-r (5'-TCATAATCACACCCTTTAGGATA-3'), PCR product length: 177 bp.

Multiplex PCR

The reaction mixture (30 μL) contained 1.5 units of Taq polymerase (*Thermo Scientific*, United States) in the buffer produced by the same company, dNTP at a concentration of 200 μM each, five pairs of specific primers, and a genome-wide bacterial template (or a mixture of bacterial DNA). The reaction was carried out in a MiniCycler DNA amplifier (*MJResearch*, USA) under the following conditions: 95°C for 5 min (initial denaturation), 30 cycles of 20 s at 95°C, 30 s at 66°C, and 30 s at 72°C; the final incubation was 5 min at 72°C. Gradient PCR and determination of the system sensitivity using real-time PCR were performed in an IQ5 amplifier (*Bio-Rad*, USA). The PCR products were separated in 4% agarose gel and colored with ethidium bromide. The lengths of the amplification products in ultraviolet light were used to determine the type of analyzed DNA.

[1] Clustal: Multiple Sequence Alignment. URL: www.clustal.org (accessed December 15, 2020).
[2] Integrated DNA Technologies. URL: www.idtdna.com (accessed December 17, 2020).
[3] Basic Local Alignment Search Tool. URL: https://blast.ncbi.nlm.nih.gov/Blast.cgi (accessed December 15, 2020)

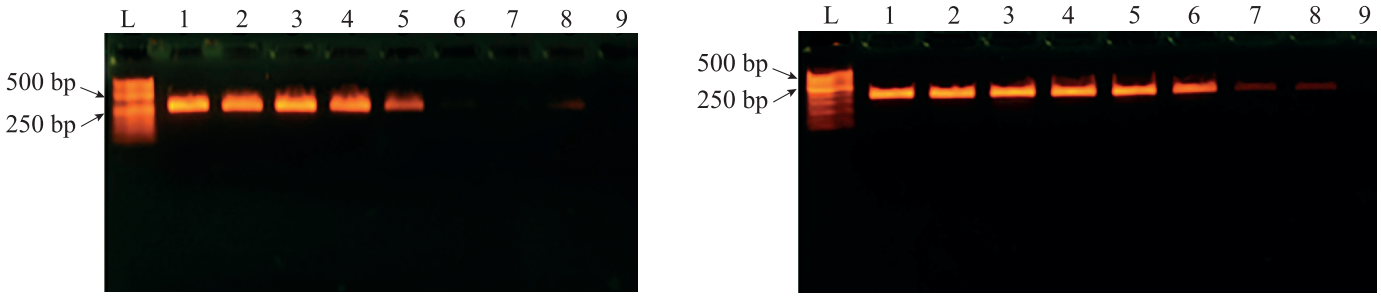

Fig. 1. Electropherograms of PCR products of gradient PCR. L – GeneRuler 50bp length marker (*Thermo Scientific*, USA). 4% agarose gel, coloring with ethidium bromide.
(a) *S. aureus*. Primers annealing temperatures: Well 1: 65.0°C, Well 2: 65.6°C, Well 3: 66.5°C, Well 4: 67.7°C, Well 5: 69.5°C, Well 6: 70.8°C, Well 7: 71.7°C, Well 8: 72.0°C, Well 9: negative control;
(b) *L. pneumophila*. Primers annealing temperatures: Well 1: 57.0°C, Well 2: 58.2°C, Well 3: 60.0°C, Well 4: 62.7°C, Well 5: 66.5°C, Well 6: 69.3°C, Well 7: 71.0°C, Well 8: 72.0°C, Well 9: negative control.

RESULTS AND DISCUSSION

Genetic targets were selected for the six most important causative agents of pneumonia, and primers were designed for multiplex PCR [8–16].

We were guided by the general requirements when designing the primer sequences: species specificity and intraspecific conservatism of the selected regions of the genetic targets. The primer annealing sites were manually selected. Additionally, the need to obtain different lengths of PCR products for the convenient subsequent detection of pathogens by electrophoretic separation was taken into account. The physicochemical characteristics were determined for each oligonucleotide sequence; a BLAST analysis was then performed and examined for the formation of dimers, hairpins, and other secondary structures.

The initial amplification conditions for subsequent optimization were chosen as follows: denaturation at 95°C and 30 cycles of 20 s at 95°C, 30 s at 57°C, and 30 s at 72°C. For the pathogens *S. aureus* and *L. pneumophila*, gradient "monoplex" PCRs were performed. The results revealed that the annealing temperature of the latter was increased to 66°C (Fig. 1).

In a new temperature–time cycle, monoplex PCR variants were carried out for the remaining four pathogens. The primer effects were checked, comparing the lengths of the PCR products with the theoretical ones. Then, in the mode of a mixture of the DNA templates (Fig. 2) of several pathogens, their specificity and ability to detect only their pathogens without providing false-positive results were confirmed.

The result of differential detection of a pathogen's DNA in a sample by multiplex PCR is shown in Fig. 3.

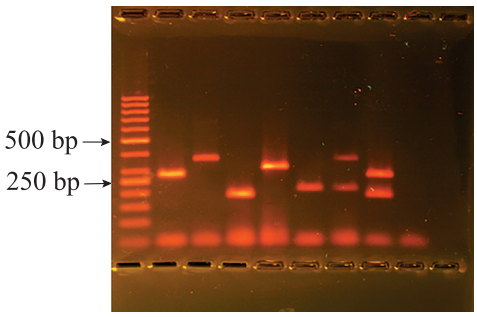

Fig. 2. Electrophoretic separation of PCR products by multiplex PCR with a mixture of primers.
(1) GeneRuler 50bp length marker (*Thermo Sientific*, USA), (2) *S. aureus*, (3) *L. pneumophila*, (4) *H. influenzae*, (5) *P. aeruginosa*, (6) *S. pneumoniae*, (7) *L. pneumophila* + *S pneumoniae*, (8) *S. pneumoniae* + *H. Influenzae* + *S. aureus*.
The PCR products were separated in 4% agarose gel and colored with ethidium bromide.

In the cases of *S. aureus* and *S. pneumoniae*, specific primers were designed for subsequent immobilization on a biochip incorporating fluorescently labeled nucleotides into the immobilized growing chain. The above principles were also used in this work. The high sensitivity of the primers designed for immobilization was confirmed by volumetric PCR undertaken under the conditions of the previously found optimal temperature–time profile. The results of the monoplex PCRs in volume for *S. aureus* and *S. pneumoniae* are shown in Figs. 4 and 5. It can be seen from the pherograms that the lengths of the PCR products obtained using various combinations of primers were in good agreement with the theoretical ones.

When analyzing the pherograms, primers showing insufficient sensitivity or specificity were re-analyzed

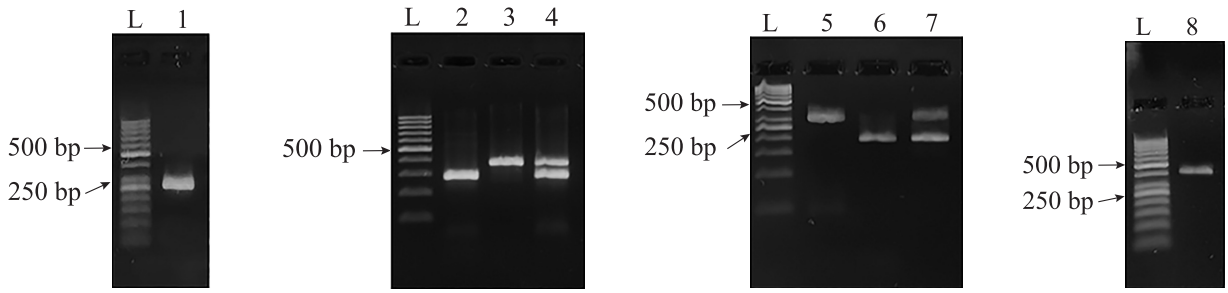

Fig. 3. Determination of the pathogen DNA by electrophoretic separation of PCR products. L – GeneRuler 50bp length marker (*Thermo Scientific*, USA), (1) *S. pneumoniae*, (2) *S. aureus*, (3) *L. pneumophila*, (4) *S. aureus* + *L. pneumophila*, (5) *H. influenzae*, (6) *P. aeruginosa*, (7) *H. influenzae* + *P. aeruginosa*, (8) *K. pneumoniae*. The PCR products were separated in 4% agarose gel and colored with ethidium bromide.

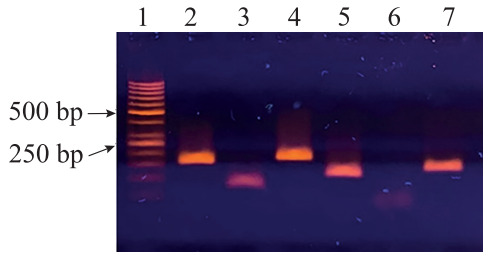

Fig. 4. Electrophoretic separation of *S. aureus* PCR products. (1) GeneRuler 50bp length marker (*Thermo Sientific*, USA), (2) R1 + F1 (163 base pairs (bp)), (3) R1 + F2 (78 bp), (4) R1 + F3 (180 bp), (5) R2 + F1 (115 bp), (6) R2 + F2 (30 bp), (7) R2 + F3 (132 bp). The letters "R" and "F" indicate the numerical indexes used to designate various primers. The theoretical length of the corresponding PCR product is indicated in parentheses.

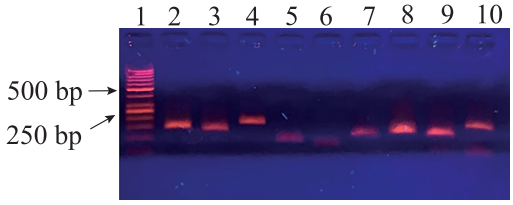

Fig. 5. Electrophoretic separation of PCR products of *S. pneumoniae.* (1) GeneRuler 50bp length marker (*Thermo Sientific*, USA); (2) R3 + F4 (144 bp); (3) R3 + F5 (131 bp); (4) R3 + F6 (169 bp); (5) R4 + F4 (87 bp); (6) R4 + F5 (74 bp); (7) R4 + F6 (112 bp); (8) R5 + F4 (126 bp); (9) R5 + F5 (113 bp); (10) R5 + F6 (151 bp). The letters "R" and "F" indicate the numerical indexes used to designate various primers. The theoretical length of the corresponding PCR product is indicated in parentheses.

for the presence of secondary structures (both intramolecular and intermolecular). If correction by "shifting" the primer along the complementary strand or varying its length or melting temperature to change the structures formed was impossible, the primer was replaced with one that was newly designed.

CONCLUSIONS

PCR for the simultaneous detection of six clinically important bacterial pathogens of human pneumonia was designed and optimized.

Currently, the design of primers for immobilization is being undertaken, and a test system based on biochips is being developed. In the future, the system could have an expanded range of diagnosed pathogenic agents of infectious pneumonia and could be used in accelerated clinical diagnostics.

Acknowledgments

The study was supported by the Russian Science Foundation grant No. 20-14-00287.

Authors' contribution

E.S. Klochikhina – research, preparation of the manuscript.

V.E. Shershov – synthesis of fluorescently labeled dNTPs.

V.E. Kuznetsova – synthesis of fluorescently labeled dNTPs.

S.A. Lapa – planning experiments, editing the manuscript.

A.V. Chudinov – academic advising.

The authors declare no conflicts of interest.

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The article was submitted: March 23, 2021; approved after reviewing: April 11, 2021; accepted for publication: May 28, 2021.

Translated from Russian into English by M. Povorin
Edited for English language and spelling by Enago, an editing brand of Crimson Interactive Inc.