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Multiplex real-time PCR for detection of *qac A/B* and *smr* genes in Gram-positive bacteria

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Abstract

Background. Disinfectants are effective means of non-specific prevention of infections associated with the provision of medical care. Violation of disinfectant use regimes leads to the formation of microorganism resistance to them. To monitor the spread of clinically significant microorganisms resistant to disinfectants, the development of methods for their detection, including molecular genetic methods, remains relevant.

The aim of the study was to develop a multiplex real-time PCR for the identification of *qacA/B* and *smr* genes, the determinants of resistance to cationic biocides, in Gram-positive bacteria.

Materials and methods. Conserved regions of *qacA, qacB* and *smr* genes were searched, and primers and probes were designed using BLASTN, GeneRunner, and Multiple Primer Analyzer programs. To evaluate the analytical sensitivity of the multiplex PCR, plasmids pTZ57-*qacA/B*, pTZ57-*smr*, and pTZ57-16S containing *qacA/B*, *smr* and 16S rRNA gene fragments of 197 bp, 127 bp, and 287 bp, respectively, were constructed. The method was tested on clinical isolates of Gram-positive bacteria (n = 30).

Results. A multiplex real-time PCR using TaqMan probes was developed for the detection of *qacA/B* and *smr* genes in Gram-positive bacteria. The 16S rRNA gene was used as an internal amplification control. The sensitivity of the multiplex PCR was 10^3 copies for all genes. Multiplex PCR validation showed that *qacA/B* genes were present in 30%, *smr* genes were present in 10% of the isolates tested. The reproducibility of the results was 100%.

Conclusion. The developed multiplex PCR differs from existing assays by high specificity and short turnaround time, as well as by the presence of an internal amplification control. It can be used for the detection of Gram-positive bacteria potentially resistant to cationic biocides.

Keywords: *disinfectants, resistance, qacA/B, smr, real-time PCR*

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Оригинальное исследование
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Мультиплексная ПЦР в режиме реального времени для выявления генов *qacA/B* и *smr* у грамположительных бактерий

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Аннотация

Актуальность. Дезинфицирующие вещества (ДВ) являются эффективными средствами неспецифической профилактики инфекций, связанных с оказанием медицинской помощи. Нарушение режимов применения ДВ приводит к формированию устойчивости микроорганизмов к ним. Для целей мониторинга распространения клинически значимых микроорганизмов, устойчивых к ДВ, остаётся актуальной разработка методов их выявления, в том числе молекулярно-генетических.

Целью исследования была разработка мультиплексной полимеразной цепной реакции в режиме реального времени (ПЦР-РВ) для выявления у грамположительных бактерий генов *qacA/B* и *smr* — детерминант устойчивости к ДВ из группы катионных поверхностно-активных веществ (КПАВ).

Материалы и методы. Поиск консервативных участков генов *qacA, qacB* и *smr* и разработку праймеров и зондов проводили с помощью программ BLASTN, GeneRunner и Multiple Primer Analyzer. Для оценки аналитической чувствительности мультиплексной ПЦР-РВ были сконструированы плазмиды pTZ57-*qacA/B,* pTZ57-*smr* и pTZ57-16S, содержащие фрагменты генов *qacA/B, smr* и 16S рРНК длиной 197, 127 и 287 п. н. соответственно. Апробацию метода проводили с использованием клинических изолятов грамположительных бактерий (n = 30).

Результаты. Разработана мультиплексная ПЦР-РВ с использованием зондов TaqMan для выявления генов *qacA/B* и *smr* у грамположительных бактерий. В качестве внутреннего контроля амплификации был использован ген 16S рРНК. Чувствительность мультиплексной ПЦР-РВ составила 10^3 копий для всех генов. Апробация мультиплексной ПЦР-РВ показала, что гены *qacA/B* присутствовали у 30% исследованных изолятов, *smr* — у 10%. Воспроизводимость результатов тестирования составила 100%. Специфичность разработанной мультиплексной ПЦР-РВ составила 100%.

Заключение. Разработанная мультиплексная ПЦР-РВ характеризуется высокой специфичностью и быстротой анализа, а также наличием внутреннего контроля амплификации и может быть использована для выявления грамположительных бактерий, потенциально устойчивых к ДВ из группы КПАВ, при проведении молекулярно-генетических исследований.

Ключевые слова: *дезинфектанты, устойчивость, qacA/B, smr, ПЦР в реальном времени*

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Introduction

The spread of pathogenic bacterial strains resistant to antimicrobials and disinfectants in recent decades is one of the most acute problems of modern public health care. Disinfectants are one of the most effective means of non-specific prevention of infectious diseases and play a leading role in the system of measures to prevent infections associated with the provision of medical care, as well as widely used in catering enterprises, food industry, municipal facilities, educational institutions and households. However, the phenomenon of microbial resistance to disinfectants observed since the 1950s leads to a sharp decrease in the effectiveness of disinfection measures [1], which is associated with the use of ineffective modes of their application, leading to the formation of microbial resistance to disinfectants, as well as cross-resistance to antibiotics due to the presence of common mechanisms of action [2, 3].

Cationic surfactants, which include quaternary ammonium compounds (QACs), guanidine derivatives, and tertiary amines, are the most commonly used surfactants [4, 5]. In 2023, QACs accounted for the largest

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share of disinfectants in the global market[1], and this trend is projected to continue for the next 10 years[2]. According to available data, their share in the Russian market is around 50–70% [6, 7].

The main mechanism of microbial resistance to QACs is their intracellular concentration decrease due to their elimination from the cell by efflux pumps [5, 8], which are united into 6 superfamilies based on structural similarity and peculiarities of functioning:

1) RND (Resistance-Nodulation Division);
2) SMR (Small Multidrug Resistance);
3) MATE (Multidrug And Toxic compound Extrusion);
4) MFS (Major Facilitator Superfamily);
5) ABC (ATP Binding Cassette);
6) PACE (Proteobacterial Antimicrobial Compound Efflux) [9, 10].

Resistance to QACs in Gram-positive bacteria is mainly associated with the efflux pumps QacA, QacB (MFS superfamily), as well as Smr (QacC), QacG, QacH, and QacJ belonging to the SMR superfamily [11, 12]. The prevalence of these efflux pumps among Gram-positive bacteria has been monitored in many countries using molecular genetic methods and has shown that *qacA*, *qacB* and *smr* genes are the most frequent [13–16]. At the same time, information on the distribution of these genes among Russian isolates of Gram-positive bacteria is practically absent. Only one publication presented data on the presence of *qacA* and *qacB* genes in *Staphylococcus aureus* isolates obtained from surface washes in public places in Novosibirsk [17]. Given that *qacA*, *qacB* and *smr* genes have predominantly plasmid localization, they can be widely disseminated by horizontal transfer, leading to the formation of acquired resistance to cationic surfactants in Gram-positive bacteria [12].

The aim of the study was to develop multiplex real-time PCR with fluorescently labeled probes to detect *qacA/B* and *smr* genes in Gram-positive bacteria.

Materials and methods

Bacterial isolates

The study used isolates of Gram-positive bacteria species *Staphylococcus aureus* (*n* = 12), *S. haemolyticus* (*n* = 6), *Enterococcus faecium* (*n* = 6) and *E. faecalis* (*n* = 4), *Streptococcus parasanguinis* (*n* = 1) and *S. epidermidis* (*n* = 1) obtained from medical institutions in Moscow. Isolates were obtained from washes taken from objects in the hospital environment, such as surfaces of ventilators and ultrasound machines, bedside tables, bed rails. Bacteria were cultured for 16 h at 37ºC in GRM-broth (State Research Center for Applied Biotechnology and Microbiology). The species affiliation of isolates was determined using a SMART MS 5020 mass spectrometer (Zhuhai DL Biotech Co., Ltd.).

DNA extraction

DNA was isolated from bacterial culture using ExtractDNA Blood & Cells DNA extraction kit (Evrogen) according to the manufacturer's recommendations and stored at –20ºC. The concentration of DNA samples was determined using a NanoDrop 2000C spectrophotometer (ThermoFS).

Development of primers and probes

The nucleotide sequences of the efflux pump genes *qacA*, *qacB*, *smr* and 16S rRNA of Gram-positive bacteria were taken from the GenBank database[3] and were analyzed using the BLASTN program[4]. Primers and probes were designed based on conserved gene regions using the GeneRunner v. 62.2.55 Beta[5] and Multiple Primer Analyzer[6] programs.

Multiplex real-time PCR testing

Multiplex real-time PCR was performed in a volume of 25 µl in 96-well PCR plates using the CFX96 Real-Time System thermocycler (Bio-Rad Laboratories, Inc.). The reaction mixture included 5× buffer and 2.5 U of Taq polymerase (Lytech LLC), primers (0.5 µM each), TaqMan probes (0.25 µM each; **Table**), and DNA sample (5 ng). TaqMan probes were synthesized by "DNA Synthesis LLC", primers were synthesized by Eurogen. Reaction profile: 95ºC — 2 min, 95ºC — 15 s, 56ºC — 20 s (36 cycles), 72ºC — 30 s.

Determination of analytical sensitivity of multiplex real-time PCR

To evaluate the analytical sensitivity of the method, plasmids pTZ57-*qacA/B*, pTZ57-*smr* and pTZ57-16S containing fragments of *qacA/B*, *smr* and 16S rRNA genes with lengths of 197, 127 and 287 bp, respectively, were constructed. Cloning was performed using the InsTAclone PCR Cloning Kit (ThermoFS) according to the manufacturer's recommendations. Plasmid concentrations were assessed using the Spectra Q BR kit (Sesana LLC) and the Qubit fluorimeter (ThermoFS). Sequencing of the obtained plasmids was per-

[1] Global antiseptics and disinfectants market size, share, trends & growth forecast report — segmented by type (alcohol and aldehyde, phenols and derivatives, biguanides and amides, quaternary ammonium compounds, iodine compounds and others), end user (domestic user and institutional user) and region — industry forecast from 2024 to 2029. URL: https://www.marketdataforecast.com/market-reports/antiseptics-and-disinfectants-market (date of access 23.10.2024).

[2] Global antiseptics and disinfectants market size to exceed USD 79.25 Billion by 2033 | CAGR of 10.67%. URL: https://finance.yahoo.com/news/global-antiseptics-disinfectants-market-size-160000859.html (date of access 23.10.2024).

[3] URL: https://www.ncbi.nlm.nih.gov/genbank

[4] URL: https://blast.ncbi.nlm.nih.gov/Blast.cgi

[5] URL: http://www.generunner.net

[6] URL: https://www.thermofisher.com

Nucleotide sequences of the primers and probes

Genes	Nucleotide sequence (5'–3')	Size, bp
qacA/B	qacA/B-D: 5'-CTGGCTTATACCTATTACCTA-3' qacA/B-R: 5'-TCCAACTAAAATTAATGCTAAAG -3' qacA/B-Pb: 5'-HEX- CGATTTGGACCGAAAATAGTGTTAC-BHQ1	197
smr	smr-F: AGTAAAACAATGCAACACCTAC-3' smr-R: ATACTATAGTTATTAGATTTATTTG-3' smr-Pb: 5'-FAM-TTAGTCTTAACAACCGTAGTCTCAAT-BHQ1	127
16S rRNA	16S-D: 5'-CAGCAGCCGCGGTAATAC-3' Bakt_805R: 5'-GACTACHVGGGTATCTAATCC-3' 16S-Pb: Cy5-5'-TGTAGCGGTGAAATGCG- BHQ2'	287

formed by the Sanger method. The samples of plasmids pTZ57-*qacA/B*, pTZ57-*smr* and pTZ57-16S with the concentration of 10^1-10^6 copies in 1 μl were obtained by tenfold dilutions and used as a template for real-time PCR. The results were analyzed using the CFX96 Real-Time System software (Bio-Rad Laboratories, Inc.).

Approbation and validation of the method

Multiplex real-time PCR validation was performed using DNA isolated from Gram-positive bacterial isolates (n = 30). To assess the variability of PCR threshold cycle (Cq) values, each sample was tested in 3-fold repetition and the mean Cq values, standard deviations (SD) and coefficient of variability (Cv, %) were calculated. The obtained amplicons of *qacA/B* and *smr* genes were analyzed by electrophoretic separation in 1.5% agarose gel and sequenced by the Sanger method. To validate multiplex real-time PCR, the previously described PCR methods with electrophoretic detection of *qacA/B* [18] and *smr* gene fragments [19] were used.

Results

For multiplex real-time PCR, primers and TaqMan probes complementary to highly conserved regions of *qacA*, *qacB* and *smr* genes were designed (Table), which were identified on the basis of multiple alignment of all full-length nucleotide sequences of these genes available in GenBank, EMBL[7] and DDBJ[8] databases. We analyzed 302 nucleotide sequences of the *qacA/B* genes and 220 sequences of the *smr* gene. Analysis of *qacA* and *qacA/B* genes showed that they differed by 8 nucleotides, so common primers and probe were designed for them.

In PCR testing, the use of an internal amplification control is important to exclude false negatives due to missing or an insufficient amount of DNA in the reaction mixture for detection. The 16S rRNA gene was used as an endogenous internal amplification control in the developed multiplex real-time PCR. The 16S-D primer and probe were designed based on the analysis of 5000 16S rRNA sequences (Table). The universal primer Bakt_805R, developed earlier, was used as a reverse primer [20].

Using the primers developed by us, we searched for isolates of Gram-positive bacteria (n = 30) containing *qacA/B* and *smr* genes. The amplicons obtained were analyzed by electrophoretic separation in 1.5% agarose gel. The amplicon lengths of the *qacA/B* and *smr* genes were as expected (Table; **Fig. 1**). The specificity of the primers was confirmed by sequencing the amplicons using the Sanger method.

To evaluate the analytical sensitivity of multiplex real-time PCR, plasmids pTZ57-*qacA/B*, pTZ57-*smr* and pTZ57-16S containing the obtained amplicons of *qacA/B*, *smr* and 16S rRNA genes were constructed. Using tenfold dilutions, their samples with concentrations ranging from 10^1 to 10^6 copies in 1 μL were obtained and used as a template for the developed multiplex real-time PCR. The sensitivity of multiplex real-time PCR amounted to 10^3 copies for all genes. The amplification efficiency of *qacA/B*, *smr*, and 16S rRNA genes was 95.1, 91.3 and 101.8%, respectively (**Fig. 2**).

The developed multiplex real-time PCR was tested using 30 clinical isolates of Gram-positive bacteria obtained from medical institutions of Moscow. Testing was performed in 3 repetitions. It was found that 30% of isolates (6 isolates of *S. haemolyticus* and 1 isolate each of *E. faecium*, *E. faecalis* and *S. parasanguinis*) had *qacA/B* genes, and 10% of isolates (2 isolates of *S. haemolyticus* and an isolate of *S. epidermidis*) contained the *smr* gene. Both *qacA/B* and *smr* were found in 2 *S. haemolyticus* isolates. Reproducibility of test results for all samples was 100%, and the coefficient of variability of Cq values ranged from 3.4 to 6.2%. The results of multiplex real-time PCR completely matched both the data of monoplex PCR assays with electrophoresis detection and the results of Sanger sequencing of amplicons. PCR methods with electrophoretic detection of *qacA/B* and *smr* genes, previously proposed by K.H. Lin et al. [18] and N. Noguchi et al. [19], respectively, were used for comparison with the developed multiplex real-time PCR. The results obtained using these methods were also in complete agreement. Thus, the specificity of our developed multiplex real-time PCR with probes labeled with fluorescent dyes was 100%.

[7] URL: https://www.embl.org
[8] URL: https://www.ddbj.nig.ac.jp

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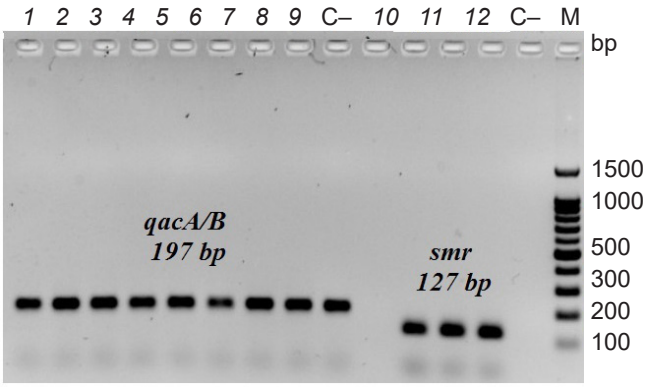

Fig. 1. Results of the amplification of *qacA/B* and *smr* genes.
1–9 — *qacA/B*-positive isolates; *10–12* — *smr*-positive isolates.
C– — negative control. M — DNA length marker.

Discussion

Detection of resistance genes by PCR methods is a widespread and available procedure for their monitoring. Analysis of literature sources has shown that several methods for detection of *qacA/B* and *smr* genes based on PCR with electrophoretic detection of results [9, 14, 21–23] and real-time PCR using intercalating DNA dyes [18, 24] have been proposed to date, but these methods have a number of disadvantages related to the duration of testing and reliability of results. The PCR method with electrophoretic detection requires gel electrophoresis, which increases the duration of the assay compared to real-time PCR and makes it laborious and inconvenient for testing a large number of clinical isolates. PCR using intercalating dyes allows real-time detection of amplicons, which reduces the duration of testing compared to the previous method, but may give false-positive results because intercalating dyes bind to all double-stranded DNA, including primer dimers and possible nonspecific PCR products, which requires careful selection of primers and observance of PCR conditions, including the reagents used [25].

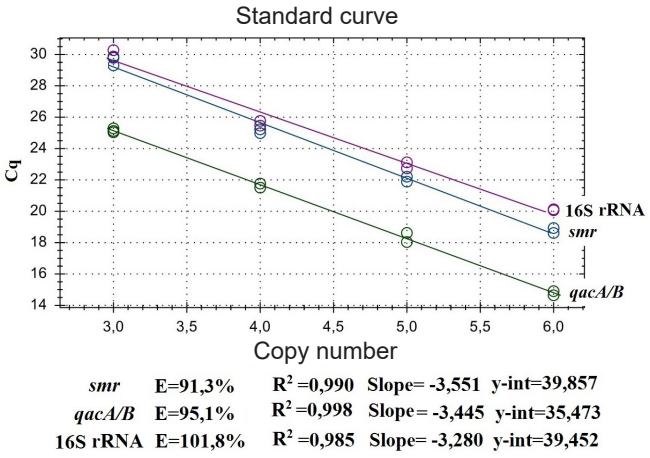

Fig. 2. Results of multiplex PCR sensitivity evaluation for detection of *qacA/B* and *smr* genes in gram-positive bacteria.

A multiplex real-time PCR method has been developed, which lacks the disadvantages of the above-mentioned methods due to the use of TaqMan probes labeled with fluorescent dyes, as well as the presence of internal amplification control.

Conclusion

A multiplex real-time PCR with probes labeled with fluorescent dyes for detection of *qacA/B* and *smr* genes of Gram-positive bacteria. It differs from the existing methods in its simplicity and rapidity of analysis, as well as by the presence of internal amplification control, which eliminates false-negative test results. The developed multiplex real-time PCR can be used to monitor the prevalence of *qacA/B* and *smr* genes to detect Gram-positive bacteria potentially resistant to cationic biocides. However, it should be taken into account that all molecular genetic assays are diagnostic tools and do not exclude the necessity to confirm the resistance phenotype by microbiological methods.

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