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Rapid Determination of Sexually Transmitted Infections by Real-time Polymerase Chain Reaction Using Microchip Analyzer

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ABSTRACT

Development of non expensive and time-saving techniques based on the polymerase chain reaction (PCR) is of great importance for modern diagnostics. We considered a new approach for PCR determination of a variety of sexually transmitted infections using microchip analyzer "AriaDNA", which had been tested using clinical samples in several medical institutions of St. Petersburg (Russia). The use of microchips containing lyophilized PCR reagents allows reducing significantly time of analysis and the number of manipulations thus preventing possible sample contamination.

Keywords: Polymerase Chain Reaction; Sexually Transmitted Infections; Microchip

1. Introduction

Sexually transmitted diseases (STDs) are common for modern society. According to the World Health Organization, 448 million new cases of different sexually transmitted infections (STIs) are registered each year. Although the number of laboratories to perform STIs analyzes increases significantly, it is challenge of modern diagnostics to develop new approaches for rapid screening of samples. The development of molecular biology methods has allowed significant progress in diagnostics of STDs. A polymerase chain reaction (PCR) method is usually used to detect and identify pathogens (e.g. pathogenic microorganisms, fungi and viruses). Compared to cultural methods of analysis, PCR has many advantages and allows to solve many diagnostic tasks quickly and accurately [1]. Currently, there is a wide range of commercial kits designed to detect a variety of STIs by real time PCR (rt-PCR). PCR system's miniaturization is one of the attractive directions for medical device development, which decrease analysis time, consumption of expensive reagents and improve the sensitivity. In the last decade many devices that use microchip technology were extensively investigated [2]. We propose a new approach to miniaturize rt-PCR system, which allows to simplify automation, reduce analysis time and the number of manual operations. Thus, in the designed system, all necessary reagents for PCR are lyophilized in microreactor cells of the microchip.

2. Development of Microchips with Lyophilized PCR Reagents for STI Determination

2.1. The Lyophilization of PCR Reagents

A microchip consisted of a silicon plate with 30 cells (Lumex, Russia) has been used as a substrate for lyophilization (see **Figure 1**).

To stabilize dried PCR reagents and to avoid degradation of polymerase or other components of PCR mixture we have previously developed a stabilizer solution [3]. Using of this solution prevents aging of reagents and allows to achieve the PCR efficiency for lyophilized reagents similar to that of liquid PCR mixture.

A solution containing stabilizers, dNTPs, Taq-polymerase, and primers corresponding to STI's DNA was put into each microreactor. As a positive control (K^+), several microreactors included the synthetic plasmid DNA with insertion of particular bacteria sequence, while as a negative control (K^-) Deionized water was used. It should be noted that particular microchip may include different quantities and combinations of microreactors for identifying different STIs, depending on the problem being solved.

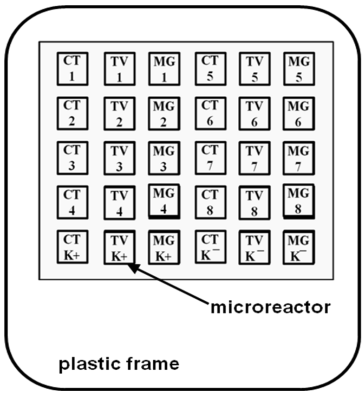

Figure 1. Microchip for CT, TV and MG identification in eight samples (each digits represent different samples). Microchip size:35 x 35 mm, microreactor size 1.6 x 1.6 mm

We have designed eight test systems for *Trichomonas vaginalis (TV), Candida albicans (Ca), Mycoplasma genitalium (MG), Mycoplasma hominis (MH), Chlamydia trachomatis (CT), Ureaplasma spp (Ur.spp), Neisseria gonorrhoeae (NG),* and *Herpes simplex virus 1/2 (HSV)* identification. **Figure 1** shows one of the developed layout of microchip for *CT, TV* and *MG* identification. In this example, on a single microchip eight samples can be analyzed for three infections.

From previous studies the microchips with lyophilized PCR reagents can be transported and stored at ambient temperature, due to stabilizing additives that preserve the activity of heat-sensitive PCR components [3]. One of the advantages of this microchip system is reducing the number of manual operations during the preparation of amplification mixture, which significantly reduces the possibility of false-positive results. For example, one sample can be pipetted into three cells and results for different infections can be obtained at once (see **Figure 1**).

2.2. PCR Instrumentation

Microchip analyzer "AriaDNA" (Lumex, Russia) carries out the thermal cycling using Peltier element and detection of PCR products in microreactors in real time using a dual-channel fluorescence detector. DNA analysis of selected STIs was carried out in the microchip using a cycling protocol consisted of melting stage 120 s at 94 ˚C and 45 two-step cycles of 1 s at 94 ˚C, and 30 s at 60 ˚C. A sealing liquid has been used to prevent an evaporation of samples from cells.

Detection of PCR product performed using two channels: FAM (for internal control) and ROX (for STIs). Analysis of the results obtained was performed using the software “Microchip analyzer "AriaDNA". A threshold cycle (Ct) was determined according to second derivative’s maximum method [4].

3. Results

The specificity is extremely important for diagnostic test systems. Specificity can be achieved by accurate selection of a DNA fragment to be amplified with the correct primers and probes selection.

The specificity of each developed test systems have been estimated against remaining seven non-specific STI’s and the following frequently occurring microorganisms: *Candida glabrata; Candida krusei;; Neisseria flava; Neisseria subflava; Neisseria sicca; Neisseria mucosa; Treponema pallidum.* Samples of those microorganisms with DNA concentration of $1 \cdot 10^5$ genome-equivalents per 1 ml did not yield non-specific products and confirmed high specificity of the reagent kits.

In order to evaluate the sensitivity of the microchip rt-PCR system, a variety of DNA samples, extracted from the scrapping material of urogenital system, urea samples and human prostate secretion has been verified. Isolation and purification of DNA was performed using a kit of reagents for DNA extraction "DNA-Sorb-AM" (ILS, Russia) in accordance with manufacturer's instructions. As a result, it was found that the analytical sensitivity of the proposed system was $1 \cdot 10^3$ genom-equivalent per 1 ml confirmed by control samples provided by ILS, Russia. For all eight test systems the efficiency of PCR was within 95-100% for lyophilized microchips.

To evaluate a storage time of developed system microchips were stored under identical temperature conditions (t = 24 – 26 °C) in a dry dark place. The efficiency of PCR for microchips stored for 5 month decreased from 100% to 97%, which is satisfactory for practical applications.

Optimal PCR analysis time was obtained to be 33 min for 45 cycles that is 2-3 times faster than conventional test-tube PCR analysis. It is important that we have reduced the consumption of PCR reagents by 20 times compared to that of test-tube PCR analysis. A number of pipetting steps for lyophilized microchip is decreased by 3 times compared to liquid PCR reagents.

Experimental evaluation of our analytical system with lyophilized microchips was carried out in several clinical institutions in St. Petersburg, Russia. Clinical material was taken according to the procedure provided in the [5]. DNA extraction was carried out using a DNA extraction kit "DNA-Sorb-AM" (ILS, Russia) according to manufacturer's instructions. Each sample in microchip was analyzed three times. Isolated DNA was also analyzed by reference method (rt-PCR and PCR with gel-electrophoresis). All reference analyzes were performed in certified laboratories..

For statistical estimation of false positive results we have determined diagnostic specificity of developed test systems. For MH, Ca, MG, TV and HSV the value of the diagnostic specificity was 100%. For CT, Ur.spp, NG diagnostic specificity was 99%, 92.4% and 93.8% respectively. It should be noted that false positive results were observed in only one of three repetitions in each case.

Figure 2 illustrates diagnostic sensitivity for all eight test systems. The deviation from 100% value can be explained by two different factors. It should be noted that discordant samples were observed mainly when Ct in test-tube rt-PCR (reference method) was larger than 35. This limitation can be overcome by increasing microreactor volume.

Another factor could be resulted from inhibition of the reaction. Several samples showed the PCR inhibition monitored by internal control that would lead to re-extraction of DNA from the sample with re-analyze followed. To decrease the number of re-analyzed samples we plan to optimize the applied methods of sample preparation for obtaining higher purity of DNA solution that do not include compounds which can inhibit PCR.

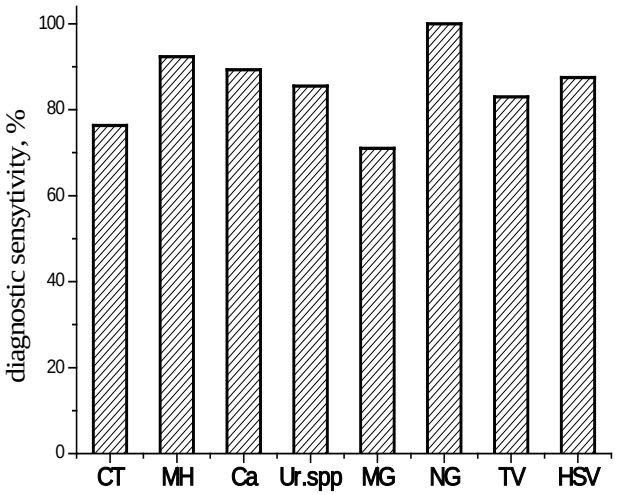

Figure 2. Diagnostic sensitivity for designed test systems.

4. Acknowledgements

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