

MICROCHIP-BASED RAPID IDENTIFICATION OF BACILLUS ANTHRACIS IN PORTABLE GEL ELECTROPHORESIS DEVICE

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ABSTRACT

In this paper, rapid identification of *Bacillus anthracis* genetic material in a portable microchip device for gel electrophoresis is presented. DNA fragments of virulence genes: cap, pag and sap of *B. anthracis* were separated and detected in a glass microchip within 4 minutes. Developed CCD-based detection setup, utilizing smart image processing, provided sensitive real-time detection of target genes in a nanomolar range.

KEYWORDS: Microchip, Anthrax, Electrophoresis

INTRODUCTION

Bacillus anthracis is one of the most effective bioterrorism agents, due to easy infection by inhalation and simple dissemination of the spores. Although various anthrax identification systems have been developed, their application in field conditions is still limited [1-3]. As a part of emergency preparedness, the mobile laboratory utilizing microchip-based instruments is being developed (Polish Defense & Security Project, 2012-2014). Main target of this project is rapid on-site identification of wide range pathogens, including *Yersinia pestis* (plague), *Variola virus* (smallpox) and discussed here *Bacillus anthracis* (anthrax).

THEORY

Among various methods of anthrax identification, most common are: conventional microbiological cell culture, immunological detection, and nucleic acid based detection. As the first method is most labor-intensive and time-consuming, and the second one is less specific and sensitive, research was focused on PCR/electrophoresis-based identification technique [4,5]. In this method, genetic material is firstly isolated from collected biological sample, and next analyzed utilizing real-time PCR technique. As PCR may be affected by contamination, resulting in false-positive identification, electrophoretic analysis of amplified product is beneficial. Post-PCR electrophoretic analysis provides rapid identification of target genes, corresponding to more- or less-pathogenic variant of anthrax.

EXPERIMENTAL

Anthrax samples were prepared in 3rd biological safety level partnership laboratory (The Military Institute of Hygiene and Epidemiology, Poland). As the major setback in *B. anthracis* detection stems from its similarity to other strains in the genera [1], three target genes were chosen to minimize false-positive identification: capA and capC capsule genes located on the virulence pXO2 plasmid, pagA protective agent genes from pXO1 plasmid and chromosomal sap gene, allowing identification of plasmid-free *B. anthracis*. PCR products (cap: 291 base pairs (bp), pag: 512 bp, sap: 639 bp) were labeled with Cy5 fluorochrome dye utilizing two methods: standard labeling with one Cy5-labeled primer, and more sensitive, utilizing two such primers. Labeled amplicons were prepared in nanomolar concentration range and mixed with loading buffer prior to electrophoresis. Separation was performed utilizing POL-4 electrophoresis gel solution and 1x Tris-Borate-EDTA buffer (A&A Biotechnology).

The analysis was performed in glass microchip for capillary gel electrophoresis, containing sample cross-injector and 27 mm long separation channel (Fig. 1a,b). The chip was fabricated of two borosilicate glass wafers (Schott), 35 mm x 17 mm x 1 mm each. Microfluidic separation channel and cross-injector were wet etched in the bottom layer, utilizing HF : HCl solution and photoresist masking layer. Four via-holes were mechanically drilled in the top layer. Both wafers were cleaned, aligned and directly sealed utilizing thermal bonding process. The chip was mounted in PTFE cassette containing four buffer reservoirs (30 μ l each) and detection window at the ending of separation channel (Fig. 1c). Separation gel was introduced into microfluidic channels using compressed air, and the buffer was injected into reservoirs by a laboratory pipette.

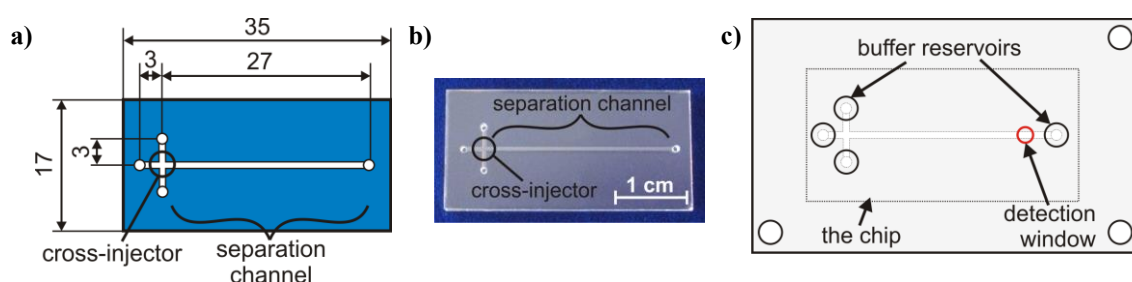


Figure 1: Microchip for gel electrophoresis: a) scheme, b) glass chip at a glance, c) scheme of the PTFE cassette

The chip was inserted into the docking station, which is an opto-electro-mechanical system with temperature stabilization (and gradient, if necessary) of the chip in a range $5\pm 50^{\circ}\text{C}$, high voltage generation & control, as well as LIF & CCD detection setup [6]. The station is controlled by a miniature single board computer with dedicated software and operated by external touchscreen. The instrument is small and portable; it provides automatic DNA analysis and real-time fluorimetric signal processing (Fig. 2).

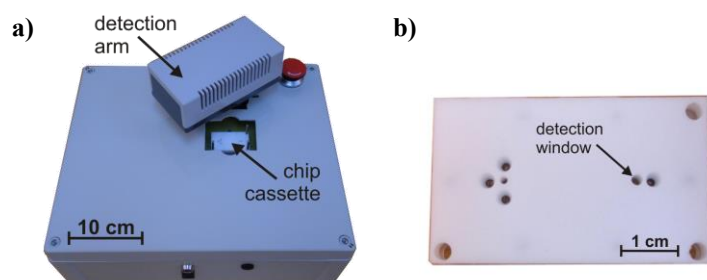


Figure 2: Portable microchip instrument for electrophoretic DNA analysis: a) docking station, b) chip cassette

Miniaturization of the instrument required application of simple but sensitive detection system. We have developed CCD-based detection setup utilizing smart image processing (SIP): instead of using whole matrix area as one detector, we are utilizing multiple single-pixel detectors in the area of interest. In comparison to standard detection method with single element photodetector, applied here SIP technique provides considerably higher sensitivity (Fig. 3). Application of microchip electrophoresis with smart detection method allowed for separation and identification of 20 bp to 500 bp DNA fragments with efficiency up to 7 million of theoretical plates per meter, so far.

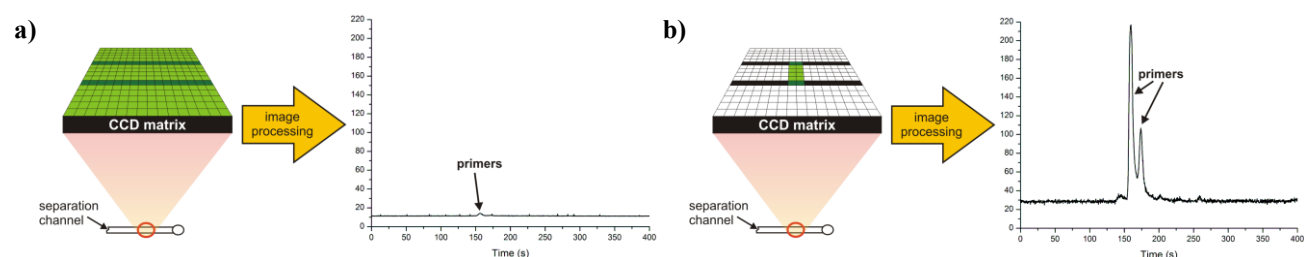


Figure 3: Standard (a) and smart (b) LIF-based detection methods utilizing CCD matrix and their influence on gained sensitivity: results for two Cy5-labeled primers separated in equal conditions (active image processing area of the CCD matrix is marked in green)

RESULTS AND DISCUSSION

Analysis results of *Bacillus anthracis* virulence fragments cap, pag and sap labeled with two methods are compared in Figure 4. In both cases, amplicons in concentration range from 25 to 40 nM were successfully separated and detected within 4 minutes. Average separation efficiency, measured in theoretical plates per meter, for according amplicons was as follows: 1.2 mln, 2.9 mln and 2.9 mln. The first peak at each electropherogram corresponds to residuals of Cy5 primer.

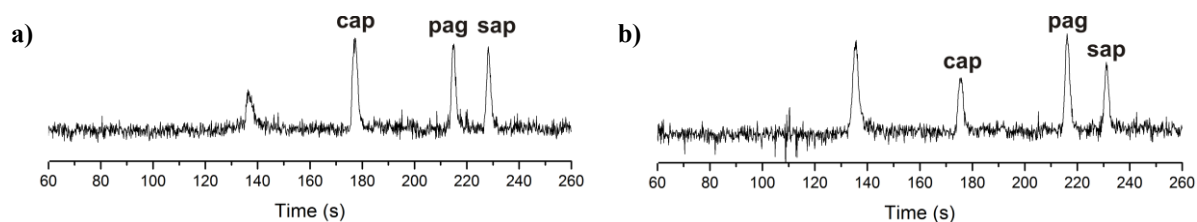


Figure 4: Results of separation and detection of *B. anthracis* virulence genes (DNA fragments): cap (291 bp), pag (512 bp), sap (639 bp) labeled utilizing one (a) and two (b) Cy5-primers; concentrations of cap, pag and sap were as follows: 40 nM, 40 nM and 40 nM (a), 25 nM, 40 nM and 33 nM (b); higher primer concentration for second labeling method is visible (first peak)

The influence of smart image processing method on detection limit is presented in Figure 5. The sample of virulence DNA fragments (approx. 280 nM each) labeled with two Cy5 primers was analyzed in the same conditions, utilizing standard CCD detection (Fig. 5a) and SIP technique (Fig. 5b). It is clear that in comparison to single element photodetector, SIP technique provides much higher sensitivity. The disadvantage of this technique is higher disturbance of fluorescence signal baseline, resulting from addition of noise from each single-pixel detector. However, smoothing (filtering) of the output signal, as well as active cooling of CCD matrix may improve signal to noise ratio.

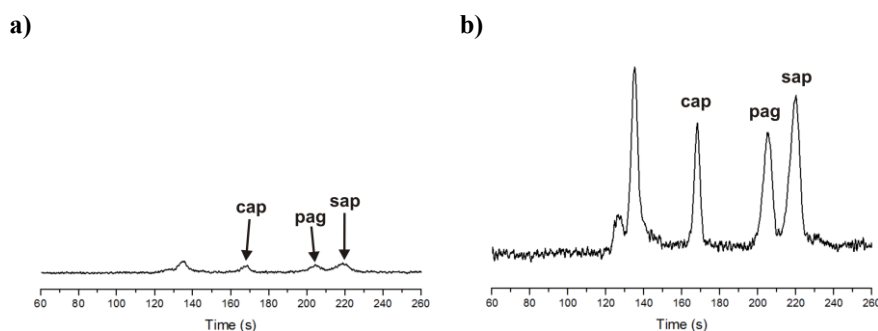


Figure 5: Results of separation and detection of *cap*, *pag*, *sap* DNA fragments labeled with double Cy5 primers, utilizing standard single-element photodetector (a) and smart image processing detection (b); pictures in the same scale

CONCLUSION

Developed instrument for microchip-based electrophoretic analysis provides rapid identification of *Bacillus anthracis* virulence genes. Amplicons of nanomolar concentration range were successfully separated and detected within 4 minutes. Applied smart signal processing technique allows for highly sensitive DNA analysis, utilizing simple and miniature detection setup. Analysis results confirmed applicability of the device for fast post-PCR product analysis of pathogens, as well as testing of primers.

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