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Confirmation of anthrax occurrence using real-time PCR

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By application of the real-time PCR we manage to confirm the diagnoses and occurrence of a disease, which is already rare in the territory of our country – the cutaneous form of anthrax. Using this method we have proven the presence of DNA of *Bacillus anthracis* in the biological sample (swab from ulcer), where other detection methods have failed. There were confirmed the presence on plasmids localized *capB* and *pagA* genes, which are responsible for virulence of this microbe. This method of detection of pathogens seems to be very rapid, simple, and specific method. In the case of adequate technical laboratory equipment it may become a very suitable and important supporter in explanation and confirmation of the occurrence of severe bacterial infections. Biologia, Bratislava, **59:** 803—807, 2004; ISSN 0006-3088. (Biologia). ISSN 1335-6399 (Biologia. Section Cellular and Molecular Biology).

Key words: anthrax, real-time PCR, anthrax after more than 20 years.

Introduction

The application of knowledge of bacterial genetics is no longer a new issue and the domain of the research and development centers, but they are increasingly applied in practice. Using the molecular biology, through binding of suitable genes, is possible to construct recombinant molecules of DNA and these, for example, we can then use in diagnostics (Bednář et al., 1996). One of such a method is so-called polymerase chain reaction (PCR). In this method, if in the reaction only a small portion of double-stranded DNA occurs (which can be, e.g., some part of the agents), it will multiply several times enabling thus its detection (Bednář et al., 1996; Žúrek, 2003). Recently, even a real-time PCR was developed (Graham & Sabelnikov, 2004). This, compared to the conventional PCR, has several advantages: (i) compared to conventional PCR, preparation, pretreatment of the sample and processing of reaction are simplified and require far less time (time required for detection is shortened from 8 to 3 hours); (ii) the reaction runs in a closed system, which significantly reduces the possibility of any contamination of analytical sample; (iii) it has a broad spectrum of application, such as the possibility to detect of mutations and deletions, analysis of gene expression

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in the molecular oncology, and others; and (iv) the possibilities to detect of pathogens, which are impossible or difficult to cultivate, or they occur in such a small number that they cannot be detected by other methods (REISCHEL et al., 2002; ŽÚREK, 2003).

And this was the case, when it was necessary to confirm the severe diagnosis – the cutaneous form of anthrax, when the patient was already treated with many antibiotics and the cultural confirmation was not, and could not be successful.

Material and methods

Principle

Basically, real-time PCR is a combination of PCR with fluorescent probe detection in the closed reaction tube (capillary). As soon as the fluorescent labeled probes find in the examined sample the homologue segment of DNA typical for certain types of bacteria, they bind to it, and so come to the fluorescent resonant energy transfer (FRET) and to the emission of red light, which can be detected by the LightCycler PCR instrument. The fluorescence increases with the growing number of formed amplicons (they grow with each PCR cycle) and this can be seen by the growing of fluorescent curves. In the case that the pathogenic DNA is not present in the examined sample, it will not be amplified during the PCR. Therefore the fluorescent probes cannot bind to the DNA and there is not emission of positive fluorescent radiation. The fluorescence neither will grow, nor the measurement will be positive (ŽÚREK, 2003). The process of the reaction is monitored on the PC screen. The positivity or negativity of the testing sample is determined on the basis of increasing the fluorescent curves and on the melting curve analysis. The melting peak basically means the temperature, where the fluorescence intensity is the highest. For each amplified segment of DNA the melting peak is specific and depends on the length of the amplified fragment of this nucleic acid (Roche Diagnostics GmbH, 1999). In the case of *Bacillus anthracis* DNA presence the melting peaks are 67.0±2.5 °C and 61.5±2.5 °C for *capB* gene and *pagA* gene, respectively (Roche Diagnostics, 2001).

Subject

P.J., a 37-year old man from small village, during demolishing an old stall, which was in no use for more than 40 years and where the previous endemic cases of anthrax were reported (ŠIMKO et al., 2003), hurt its forefinger on his left hand. Three days later he has begun feel ill and got high temperature (39.0–39.5 °C). On the site of cut a cutaneous pimple appeared. Then, around of central papule a ring of vesicles developed there. The central papule ulcerated and dried to form the eschar. The lesion was c. 2.4 cm in diameter, surrounded by oedema. General practitioner prescribed him wide spectrum antibiotic – oral ciprofloxacin, but temperature did not decrease. His left axillary lymphnode got swollen to 6–7 cm in diameter and was painful

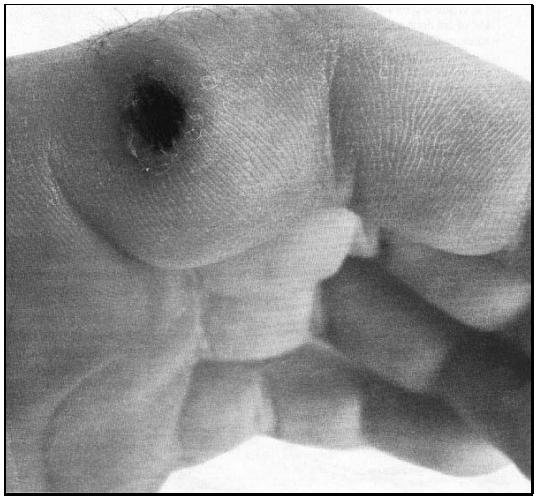

Fig. 1. The characteristic eschar of cutaneous anthrax on the patient's left forefinger after the 5th day of high temperature. Photo by author I. SCHRÉTER.

in contact. He felt increasingly worse. By the sixth day the lesion became a thick black, eschar firmly adherent to the underlying tissues (Fig. 1). At the Septic Station of the Faculty of Medicine (P. J. Šafárik University in Košice), ciprofloxacin and ampicillin with sulbactam (Unasyn) was administrated him. Because of patient's state had declining all the time and there was suspicion of cutaneous anthrax, he was admitted to the Department of Infections Diseases of the Faculty of Medicine (P. J. Šafárik University in Košice). Only here the materials for microbiological and PCR examination were taken. Parentheral administrated amoxicillin, ciprofloxacin, Procain-Penicillin G and Paralen (Paracetamol) resulted in improvement of patient's state, as well as local finding. After his 13 days stay at the hospital, he was discharged into walk-in cure. Here the peroral cure with ciprofloxacin took 47 days. The whole treatment with antibiotics took 60 days.

Samples

The examined biological materials (serum and swabs) were taken from the ulcer on the patient's left forefinger on the 14th day after beginning of antibiotic therapy. Materials were delivered to the laboratory by a special courier.

Culture

For the isolation and detection of *B. anthracis* from pathological materials selective and non-selective media were used. These were PLET Agar Base from Hi-Media; Blood agar base No. 2 and 4, Imuna with 30 000 U/L Colimycin and 5% of defibrinated sheep blood; MacConkey agar, Imuna; for multiplication Nutrient broth No. 2, Imuna. Cultivation was formed under standard conditions.

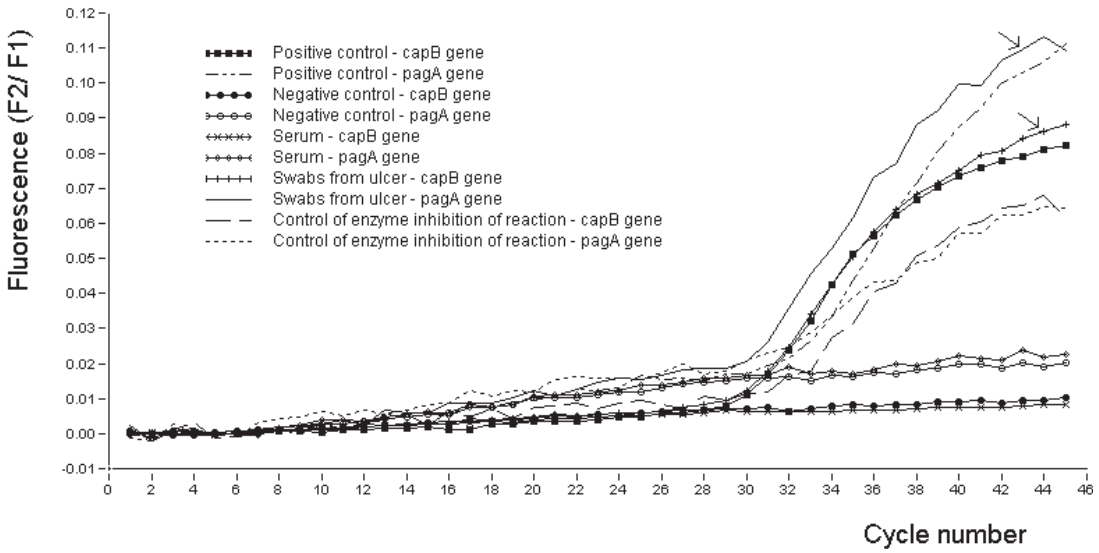

Fig. 2. Analysis of PCR. With growing number of cycles during amplification, values of fluorescence at the examined sample of ulcer grow (arrows), what suggests presence of *capB* and *pagA* genes of *Bacillus anthracis*. Curves of serum sample and negative control are linear, what proves absence of antrax genes.

Isolation of DNA from the sample
We have isolated the bacterial DNA using the High Pure PCR Template Kit (Roche Diagnostics, 2002) according to the manufacturer's manual. We have used 5 μL of isolated bacterial DNA for each of the reaction.

PCR
PCR was performed using the LightCycler PCR instrument, LighCycler software version 3.5.3 and the LightCycler – *Bacillus anthracis* Detection Kit (Roche Diagnostics, 2001), all from Roche Diagnostics. The Kit also contained primers, probes, TaqDNA polymerase, nucleotides, positive controls of *capB* and *pagA* genes. The presence of *capB* and *pagA* genes was proven in special capillaries under identical reaction conditions, together with positive and negative controls. The reaction has ran in the following steps: Denaturing: 10 min at 95 °C. Amplification was done in 45 cycles. For each cycle there were the following conditions: 10 s 95 °C (denaturing), 15 s 55 °C (annealing – application of primers and probes), 12 s 72 °C (extension of primers – DNA synthesis).

Evaluation of results
Culture was evaluated under growing conditions, morphology and microscopy of bacteria. *Bacillus anthracis*-like bacteria grew neither on non-selective nor selective media.

During the amplification with growing number of cycles the fluorescence increased, what suggested presence of *capB* and *pagA* genes of *B. anthracis* in the examined sample (Fig. 2). The analysis of the reaction was done on the basis of the melting curve, which was obtained from the gradual temperature increase (from 52.0 to 83.0 °C) in the cycle chamber with simultaneous measuring the fluorescence after the completion of amplification (Fig. 3). The peak values in the examined sample were 67.0 $\pm$ 2.5 °C and 61.5 $\pm$ 2.5 °C for *capB* gene and *pagA* gene, respectively.

Control of reaction
At the same time and under the same conditions, the controls of procedure of PCR were inspected using the positive control of *capB* gene, positive control of *pagA* gene, negative control of *capB* gene, negative control of *pagA* gene, and the exclusion of possible enzyme inhibition of reaction by examined sample (reference positive sample + examined sample).

Results and discussion

Cultural examination of both biological samples (pus and serum) was negative.

Based on the analyses of melting curves and increasing of fluorescent curves at real-time PCR, neither the presence of *capB* nor *pagA* genes in serum was confirmed. Contrary, the presence of *capB* and *pagA* genes was confirmed in the pus (Figs 2,3), which proves the presence of pX01 and pX02 plasmids.

Although cultural methods in microbiology are considered to be the standard methods for identification and confirmation of *B. anthracis* in biological materials as well as in materials coming from the environment, these methods are work

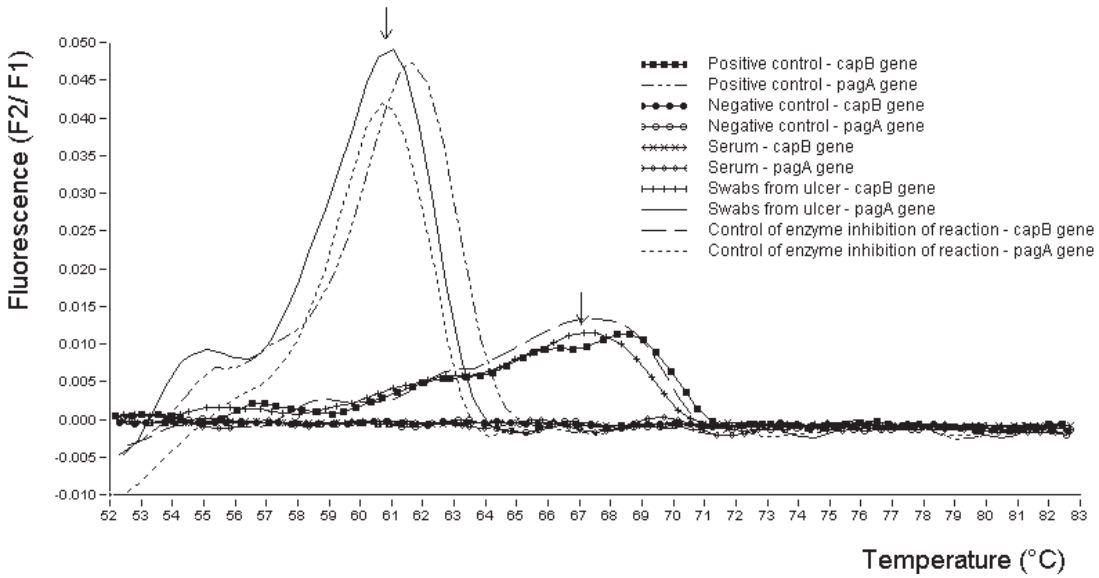

Fig. 3. Melting curves. By gradual increasing of temperature (52.0–83.0 °C) in the cycle chamber and simultaneous measuring of fluorescence, after the completion of amplification, the melting curves were obtained, where peak values are 61.5 ± 2.5 °C and 67.0 ± 2.5 °C in the examinated ulcer (arrows) and positive control samples, what proves presence of *capB* and *pagA* genes of *Bacillus anthracis*. In the serum and negative control sample melting peaks are missing and melting curves are linear, what eliminates the presence of anthrax genes there.

and time consuming (Bell et al., 2002). The real-time PCR method, which we have used for detection of *B. anthracis* in biological materials from the patient suspicious for the occurrence of the cutaneous form of anthrax, seems to be very useful either to confirm or to refuse the presumptive diagnose.

We were not able to confirm the occurrence of *B. anthracis* in the observed samples using standard cultural methods. This is quite logic since the samples delivered to the laboratory were taken from the patient on the 14th day of treatment with various antibiotics (ciprofloxacine, ampicillin with sulbactam, amoxicillin and Prokain Penicillin G). The anthrax bacilli could not resist this selection pressure of aggressive and broad-spectral antibiotics and they were killed. Contrary, using the real-time PCR, we were able to prove the presence of *capB* and *pagA* genes in the pus sample from the ulcer on the patient's left hand, whereby we have proven the presence of pX01 and pX02 plasmids. The pX01 plasmid is responsible for the formation of toxin and plasmid pX02 for the formation of capsule. If the organism is virulent, it has to contain both of these plasmids (Bell et al., 2002; Dragon et al., 2001). Since we have proven their presence in the pus, we have indirectly proven, that the primarily present microbe *B. anthracis* was virulent. Namely, not all isolated strains from biological materials or from the environment must be virulent. By passaging the strains in the laboratory during their cultivation, or due to various environmental factors, the bacterial cell may spontaneously loose one or even both of the mentioned plasmids and thus become avirulent and from the point of view of disease not interesting. Therefore the PCR methods may help to differentiate between the occurrence of virulent, avirulent or a vaccine strain (Bell et al., 2002).

Another great advantage of the PCR methods is the fact, that due to the DNA amplification, they are able to detect the presence of even one live or dead cell, or even a part of live or dead cell of the agents (1 DNA molecule), which make them very sensitive and specific methods (Makino & Cheun, 2003). This means that really a small amount of the examined sample is enough for the detection of agents. In our case, to prove both virulence genes, we have managed with only 10 μL of the sample.

This method also significantly reduces the time required for determination of final result. In cultural methods, the time required for processing and evaluation of results usually requires 24–48 hours. Using the real-time PCR method, the duration of a measurement is nearly 2.25 hours

(DNA isolation 1.25 h, amplification 50 min, analysis of the result 10 min). That means that within more-or-less 3 hours we are able to confirm the presence of the pathogenic DNA in the examined sample. This fact is especially significant in relation to therapy and in the introduction of efficient epidemiological and safety precautions.

However, PCR methods cannot completely replace the traditional cultural methods (BELL et al., 2002). In the antibiotic treatment of anthrax it is often necessary to determine the actual qualitative or quantitative sensitivity of the bacteria against the anti-bacterial agents, or to detect the immunological response of the host against the agents. Due to this fact, the cultural methods cannot be replaced by the PCR methods completely.

The diagnosis of anthrax in humans is quite difficult, because the initial symptoms of the disease are not specific and are similar to various diseases (MAKINO et al., 2001; IRMAC et al., 2003; SCHRÉTER et al., 2004). The diagnosis based on cultural methods may take several days. The real-time PCR offers a rapid, flexible, and mainly efficient possibility for rapid detection of presence of *B. anthracis* in biological and environmental samples. In Slovakia, anthrax has not occurred recently. The last recorded case was in 1983. That was also the cutaneous form of anthrax, which occurred in the animal nurse at a farm. The anthrax, which we have diagnosed and confirmed using real-time PCR method, has thus occurred after more than 20 years. Due to the facts that (i) the anthrax spores in soil keep their vitality for decades; (ii) the sick patient was working in the demolished site where used to be a livestock stall in the past; and (iii) in the district, where this case has occurred, this disease has occurred in more than 100 farms in the past until 1965 (DRAGON et al., 2001; ŠIMKO et al., 2003) – the professionals were not too much surprised with this case, even if it really is a rare case concerning time.

Conclusion

In conclusion it is possible to point out that the real-time PCR method and the LightCycler system are suitable assistants for both the clinical and the epidemiological practice. The laboratories equipped with these techniques and possibilities really posses a useful and efficient tool for the prevention and therapy of such severe infection disease, as the anthrax really is.

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