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SHORT COMMUNICATION

Fast Identification of *Yersinia pestis*, *Bacillus anthracis* and *Francisella tularensis* Based on Conventional PCR

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Abstract

Rapid and accurate diagnostic tools for detection and identification of *Y. pestis, B. anthracis* and *F. tularensis* are essential for timely initial appropriate treatment of exposed individuals, which will be critical to their survival, as well as for reduction of the public health impact and the spread of the disease. The paper presents application of fast polymerases and fast dry electrophoresis in conventional PCR as an alternative for real-time PCR application for detection and identification of the above pathogens. The proposed method takes less than 50 min. to obtain final results of the tests and is cheaper than real-time PCR.

Key words: *B. anthracis, F. tularensis, Y. pestis*, detection, PCR

Yersinia pestis, *Bacillus anthracis* and *Francisella tularensis* are etiological agents of plague, anthrax and tularemia, respectively – severe diseases in humans and animals. Although rare in majority of developed countries, the three pathogens still exist in nature, especially in endemic areas, and cause diseases. For example, many parts of Europe, Asia, Africa, Australia, and North, Central, and South America are regarded as anthrax endemic areas (Gasper and Watson, 2001). Plague is endemic in many natural foci of Asia, Africa, and the Americas (Riehm *et al.*, 2011). Whereas the most virulent *F. tularensis* subspecies are found mainly in North America (Vogler *et al.*, 2009). Development of mass tourism to endemic regions of diseases caused by *Y. pestis, B. anthracis* and *F. tularensis* can result in transmission of the diseases to countries regarded as free from these pathogens. Moreover, the pathogens are listed as a category A bio-threat agents according to the Centers for Disease Control and Prevention (CDC) of USA. The category A agent is an organism that poses a risk to national security because it can be easily disseminated or transmitted from person to person, results in high mortality rates, has the potential for public health impact, might cause public panic and social disruption and requires special action for public health preparedness (http://emergency.cdc.gov/agent/agentlist-category.asp).

Rapid and accurate diagnostic tools for detection of these pathogens are essential for timely initial appropriate treatment of exposed individuals which will be critical to their survival as well as for reduction of the public health impact and the spread of the disease. PCR has been regarded as an accurate diagnostic tool for detection and identification of *Y. pestis, B. anthracis* and *F. tularensis*. Application of real-time PCR technology has made this tool very rapid. However, the real-time PCR equipment is not always accessible in local laboratories in many countries, especially in endemic areas which are often located in the developing countries.

In this paper we present a possibility of significant time reduction necessary for *Y. pestis, B. anthracis* and *F. tularensis* identification by use of rapid polymerases and rapid dry electrophoresis in conventional PCR assay.

In this study five fast polymerases were tested: Phire Hot Start II DNA Polymerase (Finnzymes), PyroStart Fast PCR Master Mix (Fermentas), AmpliTaq Gold Fast PCR Master Mix (Applied Biosystems), SapphireAmp Fast PCR Master Mix (Takara), Qiagen Fast Cycling PCR (Qiagen). PCR conditions were followed according manufacturers' instructions (Table I). The primers for species and subspecies identification were used as described ealier for *Y. pestis* by Zhou D *et al.* (2004), for *B. anthracis* by Jackson PJ *et al.* (1998), for *F. tularensis* by Johansson A *et al.* (2000), Tomaso H *et al.* (2007) and Barns SM *et al.* (2005). DNA samples isolated from the following strains were used: *B. anthracis* BL1 and BL6, *Y. pestis* NCTC00570 and 03–1506, *F. tularensis* spp. *tularensis* Schu S4, *F. tularensis* spp. *holarctica* A104-15,

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Table I
PCR conditions applied for tested polymerases and duration of thermocycling.

	Phire Hot Start II DNA Polymerase (Finnzymes)	PyroStart Fast PCR Master Mix (Fermentas)	AmpliTaq Gold Fast PCR Master Mix (Applied Biosystems)	Sapphire Amp Fast PCR Master Mix (Takara)	Qiagen Fast Cycling PCR (Qiagen)
Initial denaturation	30 s	1 min	10 min	1 min	5 min
Denaturation	5 s	1 s	3 s	5 s	5 s
Anealing	5 s	5 s	3 s	5 s	5 s
Extension	10 s	25 s	15 s	10 s	20 s
Final extension	1 min	10 s	10 s	–	1 min
Theoretical minimal time of 30 cycles	11 min 30 s	16 min 40 s	20 min 40 s	11 min	21 min
Thermocycling duration with Bio-Rad C1000 Thermal Cycler	39 min	42 min	47 min	36 min	47 min
Thermocycling duration with Eppendorf Mastercycler	63 min	66 min	72 min	65 min	72 min

F. tularensis spp. *novicida* Ft26. The reactions were conducted comparatively in Eppendorf Mastercycler and in Bio-Rad C1000 Thermal Cycler. Dry electrophoresis of the products was conducted using E-Gel Base System (Invitrogen). The maximal time of electrophoresis was 10 minutes.

All the tested polymerases amplified the genetic markers in sizes ranging from 136 bp to 1200 bp efficiently (as an example results for *F. tularensis* are presented in Fig. 1). The duration of 30 cycles of PCR varied from 63 min to 72 min on Eppendorff Mastercycler and from 36 min to 47 min on Bio-Rad C1000 Thermal Cycler (Table I). As it was supposed based on the theoretically calculated minimal duration of thermocycling the fastest polymerase was SapphireAmp Fast PCR Master Mix (Takara). The blue dyes and a material for increasing the specific gravity of the solution contained in the master mix did not influence the electrophoresis results although the manufacturer of the E-Gel base system warns that loading buffer with tracking dye may mask DNA bands. Almost as fast as SapphireAmp Fast PCR Master Mix was Phire Hot Start II DNA Polymerase (Finnzymes). Preparation of a reaction mixture with Phire Hot Start II DNA Polymerase takes a little more time as it was the only polymerase tested that was not available as "ready to use" mix containing all necessary reagents except primers. The disadvantage of PyroStart Fast PCR Master Mix was that the DNA bands were getting blurred during dry electrophoresis. It was probably caused by high concentration of salts in the PCR buffer. This effect can be overcome by dilution of the PCR products before loading onto the gel.

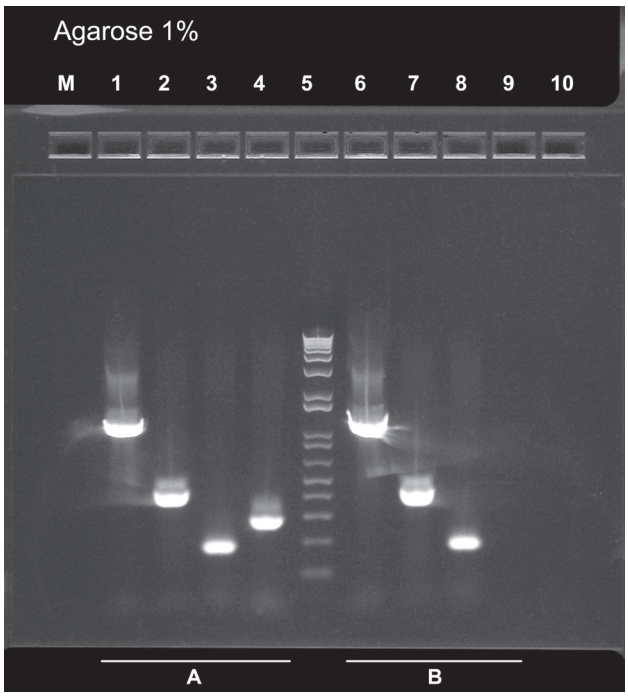

Fig. 1. PCR products obtained for *F. tularensis* ssp. *novicida* (A) and *F. tularensis* ssp. *holarctica* (B) after 7 minutes of dry elecrophoresis.

Lines: 1 and 6–16S rRNA marker (1200 bp), 2 and 7 – *tul* marker (428 bp), 3 and 8 – FtC marker (170 bp), 4 and 9 – *pdpD* marker (285 bp in *F. tularensis* ssp. *novicida*, negative in *F. tularensis* ssp. *holarctica*), 5 – E-Gel 1 kb Plus DNA Ladder.

Speed of thermocycling reaction depends not only on the amount of time spent for incubation at each temperature step but also from the time taken to reach the incubation temperature (the ramp time) what is a characteristic of a PCR device. Comparison of two termocyclers conducted in these studies revealed possibility of up to 43% of reduction of PCR duration depending on the thermocycler used. Matero *et al.* (2011) also revealed significant differences in duration of thermocycling comparing two real-time PCR instruments. As real-time PCR is regarded as faster than conventional PCR, we reviewed literature to compare real-time PCR duration with different instruments used for *B. anthracis* detection. The comparison concerned only duration

Table II
Comparison of real-time PCR duration with different instruments used for *B. anthracis* detection.

Detected markers	Instrument (manufacturer)	Thermocycling duration	References
pagA, capB	ABI 7300/7500 (Applied Biosystems)	100 min	(Matero *et al.*, 2011)
pagA, capB	RAZOR (Idaho Technology Inc.)	40 min	(Matero *et al.*, 2011)
plcR, gyrA	LightCycler 480 (Roche Diagnostics)	60 min	(Derzelle *et al.*, 2011)
pagA, capB	LightCycler (Roche Applied Science)	60 min	(Bell *et al.*, 2002)
pagA	Smart Cycler (Cepheid)	55 min	(Selvapandiyan *et al.*, 2005)
pagA, capB	Rotor-Gene 6000 (Corbett Life Science)	52 min	(unpublished data)

of thermocycling, excluding time of DNA extraction. The comparison (Table II) allowed to draw a conclusion that application of fast polymerases and rapid dry elecrophoresis in conventional PCR enables to obtain results in time similar as in real-rime PCR. The minimal time necessary for obtaining results of identification of the pathogens was less than 50 min. It is worth to underline that use of conventional PCR with fast enzymes and rapid dry electrophoresis is cheaper than use of real-time PCR in terms of equipment as well as reagents, what means that it could be more accessible to local laboratories. Our calculation revealed that the costs of analysis of one sample using fast conventional PCR and dry elecrophoresis varied from 0.95 euro to 3.15 euro depending on the fast polymerase used, whereas costs of analysis of one sample using real-time PCR are 2.50–12 euro. Even more significant are differences in costs of equipment. The costs of a conventional thermocycler together with equipment for dry electrophoresis are 3900–7450 euro. The costs of real-time PCR platform are 16450–47700 euro. Moreover, the fast PCR assay together with rapid dry electrophoresis might be used in the field as the instruments are small, light and portable.

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Literature

Barns S.M., C.C. Grow, R.T. Okinaka, P. Keim and C.R. Kuske. 2005. Detection of diverse new Francisella-like bacteria in environmental samples. *Appl. Environ. Microbiol.* 71: 5494–5500.

Bell C.A., J.R. Uhl, T.L. Hadfield, J.C. David, R.F. Meyer, T.F. Smith and F.R. Cockerill III. 2002. Detection of *Bacillus anthracis* DNA by LightCycler PCR. *J. Clin. Microbiol.* 40: 2897–2902.

Derzelle S., C. Mendy, S. Laroche and N. Madani. 2011. Use of high-resolution melting and melting temperature-shift assay for specific detection and identification of *Bacillus anthracis* based on single nucleotide discrimination. *J. Microbiol. Methods* 87: 195–201.

Gasper P.W. and R.P. Watson. 2001. Plague and yersiniosis, pp. 313–329. In: Williams E.S. and I.K. Barker (eds). Infectious diseases of wild mammals. 3rd ed. Iowa State University Press, Ames, Iowa.

Jackson P.J., M.E. Hugh-Jones, D.M. Adair, G. Green, K.K. Hill, C.R. Kuske, L.M. Grinberg, F.A. Abramova and P. Keim. 1998. PCR analysis of tissue samples from the 1979 Sverdlovsk anthrax victims: The presence of multiple Bacillus anthracis strains in different victims. *PNAS* 95: 1224–1229.

Johansson A., A. Ibrahim, I. Goransson, U. Eriksson, D. Gurycova, J.E. Clarridge III and A. Sjostedt. 2000. Evaluation of PCR-based methods for discrimination of Francisella species and subspecies and development of a specific PCR that distinguishes the two major subspecies of Francisella tularensis. *J. Clin. Microbiol.* 38: 4180–4185.

Matero P., H. Hemmila, H. Tomaso, H. Piiparinen, K. Rantakokko-Jalava, L. Nuotio and S. Nikkari. 2011. Rapid field detection assay for *Bacillus anthracis, Brucella spp., Francisella tularensis* and *Yersinia pestis*. *Clin. Microbiol. Infect.* 17: 34–43.

Riehm J.M., L. Rahalison, H.C. Schulz, B. Thoma, M. Pfeffer, L.M. Razanakoto, S. Al Dahouk, H. Neubauer and H. Tomaso. 2011. Detection of *Yersinia pestis* using real-time PCR in patients with suspected bubonic plague. *Mol. Cell. Probes* 25: 8–12.

Selvapandiyan A., A. Stabler, N.A. Ansari, S. Kerby, J. Riemenschneider, P. Salotra, R. Duncan and H.L. Nakhasi. 2005. A novel semiquantitative fluorescence-based multiplex polymerase chain reaction assay for rapid simultaneous detection of bacterial and parasitic pathogens from blood. *J. Mol. Diagn.* 7: 489–494.

Tomaso H., H.C. Scholz, H. Neubauer, S. Al Dahouk, E. Seibold, O. Landt, M. Forsman and W.D. Splettstoesser. 2007. Real-time PCR using hybridization probes for the rapid and specific identification of Francisella tularensis subspecies tularensis. *Mol. Cell. Probes* 21: 12–16.

Vogler A.J., D. Birdsell, L.B. Price, J.R. Bowers, S.M. Beckstrom-Sternberg, R.K. Auerbach, J.S. Beckstrom-Sternberg, A. Johansson, A. Clare, J.L. Buchhagen, J.M. Petersen, T. Pearson, J. Vaissaire, J.P. Dempsey, P. Foxall, D.M. Engelthaler, D.M. Wagner and P. Keim. 2009. Phylogeography of *Francisella tularensis*: global expansion of a highly fit clone. *J. Bacteriol.* 191: 2474–2484.

Zhou D., Y. Han, E. Dai, D. Pei, Y. Song, J. Zhai, Z. Du, J. Wang, Z. Guo and R. Yang. 2004. Identification of signature genes for rapid and specific characterization of *Yersinia pestis*. *Microbiol. Immunol.* 48: 263–269.