MMSL
Military Medical
Science Letters

Mil. Med. Sci. Lett. (Voj. Zdrav. Listy) 2011, vol. 80(4), p. 159-168
ISSN 0372-7025
DOI: 10.31482/mmsl.2011.022

ORIGINAL ARTICLE

INTERLABORATORY COMPARATIVE TESTS OF BIOLOGICAL LABORATORIES OF THE NATO ARMIES

Libor Pisa[1,2]✉, Radoslav Krupka[1], Veronika Formankova[1], Vera Neubauerova[1], Jiri Dresler[1,2], Martin Hubalek[2]

[1] Central Military Health Institute, Prague, Czech Republic
[2] Institute of Molecular Pathology, Faculty of Military Health Sciences, University of Defense, Hradec Králové, Czech Republic

Received 31st October 2011.
Revised 26th November 2011.
Published 9th December 2011.

Summary

One of the key requirements of the biodefense system of the Czech Armed Forces is a capability to identify the biological warfare agents (BWA). In this regard the Central Military Health Institute that is responsible for the biodefense in the Czech Armed Forces took part in 10th Annual international comparative exercise of the NATO (North Atlantic Treaty Organization) military biological laboratories. The aim of this test was the identification of *Bacillus anthracis* in unknown samples which were contaminated by different disinfectants. Real-time polymerase chain reaction (PCR) was chosen as optimal method for this exercise because of a robustness, speed and flexibility of this method. Due to the presence of a disinfectant the identification procedure could only be conducted after including an additional step to sample preparation. Tandem mass spectrometry was selected as a confirmatory method for the exercise. Our test result was in full agreement with the exercise design. This exercise confirmed that method of provisional identification deployable in mobile component of biodefense system of the Czech Armed Forces is sensitive and robust for use in the field conditions. The tandem mass spectrometry analysis confirmed PCR results and verified the valuable confirmatory role of mass spectrometry in the identification of biological agents.

Key words: Bacillus anthracis strain Sterne; lightcycler R.A.P.I.D.; GeneXpert system; Tandem mass spectrometry

INTRODUCTION

The Czech Republic Ministry of Defense (MoD) strategy in protection against weapons of mass destruction [1] determines the basic principles of the biodefense system of the Czech Armed Forces. One of the key requirements of this system is a capability to identify selected biological agents. This capability is also a part of fulfilment of the Force Goals of the Czech Armed Forces. Respecting the above mentioned requirement, the Central Military Health Institute that is responsible for the biological defense in the Czech Armed Forces, took part in 10th Annual international comparative exercise of the NATO military biological laboratories in 2010.

This exercise was arranged by the NATO Sampling and Identification of Biological, Chemical and Radiological Agents (SIBCRA) subgroup, which is

✉ Central Military Health Institute, U vojenské nemocnice 1200, 169 02 Prague, Czech Republic
l.pisa@email.cz
+420 973208180
+420 224315222

one of the working groups of NATO Joint Capability Group on Chemical, Biological, Radiological and Nuclear Defense. The area of responsibility of the SIBCRA subgroup is the provision of advice and guidance to personnel charged with sampling and identification of biological, chemical and radiological agents. The focus of the 2010 exercise was to assess the efficiency of identification methods for samples that might contain *Bacillus anthracis* strain Sterne and were contaminated by different disinfectant.

Bacillus anthracis is very virulent biological agent that possesses high risk and can be easily abused as a biological warfare agent or for the bioterroristic act. *Bacillus anthracis* is the etiological agent of anthrax and its virulence is determined by presence of two plasmids, pX01 and pX02, which are essential for toxicity [2]. Plasmid pX01 contains the three toxin genes coding edema factor (*cya*), lethal factor (*lef*) and protective antigen gene A (*pagA*). The capsular protein gene A, B and C (*capA, capB and capC*) are located on plasmid pX02.

The evidence of these plasmids in *Bacillus anthracis* strains is an attribute that can be used for DNA identification screening and initial detection of virulent strains. Chromosomal genes, e.g. DNA gyrase A gene (*gyrA*) and RNA polymerase beta subunit gene (*rpoβ*), in general, are not as sensitive as the plasmid-borne gene analytical assays due to the presence of more than one copy of the pXO1 and pX02 plasmid per *Bacillus anthracis* chromosome in the bacterial cell [3-7]. On the other hand, during the recent years, *Bacillus cereus* strains that contain the pX01 plasmid were discovered and strains with both pX01 and pX02 have been isolated from great apes in Africa [2]. Therefore, the presence of pX01 and pX02 no longer principally separates *Bacillus anthracis* from other Bacilli and the chromosomal based analysis remains important [8]. However, the presence of the toxin and capsule encoding plasmids in Bacillus cereus alone is insufficient to render the strain as virulent as *Bacillus anthracis* [9].

The participating labs have been asked to confirm the presence or the absence of *Bacillus anthracis* strain Sterne. The *Bacillus anthracis* strain Sterne contains only the pX01 plasmid encodes the toxins, but lacks plasmid pX02 and therefore lacks the capsule [2]. Each participating laboratory chose suitable analytical methods for the detection of strain Sterne.

In case of analysis by the biodefense laboratory of the Central Military Health Institute, the identification method was selected on the base of two aspects. First, select only those identification methods that are suitable for use in field conditions and that are deployable as a mobile component of biodefense system of the Czech Armed Forces. These methods have to be robust, simple (easy-to-operate) and able to be operated also by personnel with limited training. At the same time, these methods have to be fast enough, so the results can be available on time relevant basis for correct decision making and for possibility to adopt appropriate measure. The second aspect had to take into account that the samples were inactivated (gamma irradiation), thus only methods that do not require viable particles can be applied.

Polymerase chain reaction is considered to be the basic method in molecular biology for detection, identification and hierarchical DNA typing of biological agents [10]. Actually, there are a number of methods based on PCR principle that are used for detection, characterization and individualization of so called "microbial DNA signature" specific to biological agents [5].

Real-time PCR was chosen as optimal method for this exercise due to the above mentioned reasons [11]. Real-time PCR is fast measuring and quantification of PCR product, which combine classical PCR with detection of amplification product in the same test tube, whereby the risk of contamination is minimized and in addition it makes possible to quantify initial quantity of nucleic acid in samples. Next advantage is robustness, speed and flexibility of this method. The detection of PCR products is performed by detecting of fluorescence signal [12].

Intentional dispersion of anthrax spores by postal letters after 11th September 2001 has started development of real-time PCR procedures for fast detection of highly hazardous biological agents and the methods were described in a number of publications: *Bacillus anthracis, Yersinia pestis, Francisella tularensis, Brucella spp., Bordetella spp., Burkholderia* spp. [13-23].

Figure 1. Lightcycler R.A.P.I.D. (Idaho Technology Inc., Salt Lake City, UT, USA). Source: www.idahotech.com

The Czech Armed Forces are for this purpose, equipped by two different real-time PCR systems: (i) complete product solution for rapid identification, based on field-deployable lightcycler R.A.P.I.D. (Ruggedized Advanced Pathogen Identification Device) (Idaho Technology Inc., Salt Lake City, UT, USA), which can be complete with home-made identification kit (Figure 1) and (ii) automated GeneXpert system (Cepheid, Sunnyvale, CA, USA) (Figure 2).

Figure 2. GeneXpert system (Cepheid, Sunnyvale, CA, USA). Source: www.cepheid.com

For military application, Idaho Technology Inc. offers complete product solution for rapid identification, which contains sample purification kits, freeze-dried reagents and identification device lightcycler R.A.P.I.D. with Idaho Technology's Ready Response Kit. IT 1-2-3™ Sample Purification Kits for extraction and purification of DNA and RNA enables extraction of DNA or RNA from pathogens found in food, water, blood and other sources (clinical or environmental samples). Freeze-dried ready-to-use reagents for the detection and identification of selected biothreat agents, contain all of the ingredients necessary for real-time PCR (primers, probes, enzymes and buffers) in a simple, convenient format and they can be stored at room temperature. The R.A.P.I.D. is a portable real-time PCR system designed to identify biological agents [3, 7, 16, and 18].

The Cepheid GeneXpert is a four-site, self-contained device integrating automated sample processing and real-time PCR detection of infectious agents. All steps required for identification of bacterial and viral threat agents in various biological specimens, including sample preparation, amplification and detection, are combined within a single instrument that provides results in approximately 30-40 min [24]. The GeneXpert is designed to utilize self-contained sample preparation cartridges that eliminate complex manual procedures as well as amplicon or agent contamination and PCR inhibitors. With the incorporation of internal positive control for PCR (IPC) and sample processing control (SPC), the GeneXpert is a rapid and reliable system for detecting specific organism. IPC monitors PCR inhibitors that may cause false negative reactions and SPC verifies whether sample processing within the cartridges worked properly [24].

Tandem mass spectrometry was selected as a confirmatory method for the exercise. Since it is based on identification of different type of biomolecules (proteins), this approach is suitable as a complementary tool to methods based on analysis of DNA in the workflow of identification of biological agents. The method relies on the presence of bacterial (in this case *Bacillus anthracis*) proteins in the sample. The proteins are digested by protease trypsin and the resulting peptides are subjected to fragmentation inside of the mass spectrometer. The resulting tandem mass spectrum is specific for the analyzed peptide, thus it can be used for the identification of the protein and also the organism that carries the gene, coding the protein on the chromosome or on the plasmid. This promising technique has already been used

for microorganism identification and there is an ongoing research in this field [25-27]. In this exercise, we used MALDI-TOF/TOF (Matrix-assisted laser desorption/ionization – time of flight) instrument to analyze the samples.

MATERIALS AND METHODS

Samples

These tests were prepared by experts from U. S. Army Test & Evaluation Center in Dugway Proving Ground (Dugway Proving Ground, Utah, U.S.A.). The test panel consisted of twelve unknown cotton swabs each, in the leak-proof tube. The samples likely have come in contact with decontamination reagents (disinfectants) that may interfere with the analysis [13]. It was clear neither which of the swab was contaminated by *Bacillus anthracis* strain Sterne nor what disinfectant was present.

All chemicals used were of minimum analytical grade and were used as received without any further purification. Chemicals and kits were obtained from different vendors (East Port Praha s.r.o., Czech Republic; Merck s.r.o., Czech Republic; Rescue Technical and Training Institute, s.r.o., Czech Republic and Sigma-Aldrich, Czech Republic).

PCR sample preparation

The bacterial content was extracted from the swab by shaking in 400 µl of 0,001% Tween 20 (Sigma-Aldrich Inc., St. Louis, MO, USA) in PBS for a period of 60 minutes and subsequently centrifuged. After that, all samples were tested for PCR inhibitors by adding known DNA and performed PCR to check for the expected product. Test of inhibition proved significant presence of inhibitors in sample 511. The purification of DNA was performed using IT 1-2-3 SWIPE Sample Purification Kit (Idaho Technology Inc., Salt Lake City, UT, USA). The procedure in this kit employs following steps: mechanical disruption of the cells by bead-beating, DNA/RNA binding and concentration on a filter, washing and removing of contaminants including inhibitors and elution of DNA/RNA from the filter. Because of the inhibitors in 511, this sample was later treated by phenol/chloroform extraction to better separate the DNA/RNA from the interfering chemicals [28].

Real-time PCR

Three different primer sets were applied for the PCR reaction. First, the freeze - dried reagent B. anthracis Target 1 (Idaho Technology Inc., Salt Lake City, UT, USA) was employed for the PCR reaction. The PCR kit targets chromosomal gene *rpoβ* (RNA polymerase beta subunit) of *Bacillus anthracis*. The analysis was performed also with the home-made PCR kit, targeting again the *rpoβ* gene to test the effectiveness of this kit. DNA purified by IT 1-2-3 kit for all samples was used, with the exception of sample 511 (phenol/chloroform purification). Third employed method for the analysis was the Cepheid GeneXpert system. In that case, the target of the real-time PCR was protective antigen gene A (pagA) located on pX01 [24].

Sample preparation for Mass spectrometry

For the proteomic sample preparation (reduction, alkylation and digestion), FASP (filter aided proteome preparation) method [29] on 10kDa filter (Amicon Ultra 0.5, Ultracel10 Membrane, Millipore, Billerica, MA, USA), was used. And finally, the samples were desalted on PepClean, Pierce C-18 Spin Columns, Thermo Fisher Scientific, Rockford, IL, USA), dried in vacuum and solubilized in 5% acetonitrile/ 0,1% formic acid (Sigma-Aldrich Inc., St. Louis, MO, USA) so that the final solution should contain 1mg of proteins (as measured BCA - Bicinchoninic acid) per ml [30].

Tandem mass spectrometry

An aliquot was mixed in 1:1 ratio with the matrix solution (5 mg/ml of α-cyano-4-hydroxycinnamic acid in 50% acetonitrile, 0,1% trifluoroacetic acid) and spotted on a target MALDI plate in triplicates. The mass spectra were recorded on ABI 4800 MALDI-TOF/TOF mass analyzer (Applied Biosystems, Foster City, CA, USA). The peptide mass fingerprints were recorded in the reflectron mode in the *m/z* range 800-4000 Da and calibrated internally using trypsin autolysis peptides as markers. The fragmentation analysis of eight most intensive peaks was performed without applying CID (collision-induced dissociation). The acquired data were evaluated using GPS Explorer software v.3.6 (Applied Biosystems, Foster City, CA, USA) integrating the Mascot search algorithm and ProteinPilot 2.0 software (Applied Biosystems, Foster City, CA, USA) employing the Paragon algorithm. Datasets were searched against FASTA

file comprising all *Bacillus anthracis* protein sequences available on 5th May 2010. Protein score confidence intervals greater than 95 were significant ($p<0.05$).

RESULTS AND DISCUSSION

Real-time PCR results

The first test results (commercial *rpoβ* targeting kit) on the lightcycler R.A.P.I.D showed that samples 389, 428 and 511 (extracted by IT 1-2-3 Kit) were negative for the presence of *Bacillus anthracis* the others were positive. Using the home-made kit the results of the sample 511 (additionally treated by phenol/chloroform extraction) proved the presence of the *Bacillus anthracis* DNA. All other samples gave the same result as with the commercial kit. Comparing the Ct values (the cycle number at which the fluorescence crosses the threshold line) proves that the home-made kit reaches similar identification efficiency as the commercial kit. However, there is a difference in the storage stability at room temperature, where the commercial kit outperforms the home-made kit.

The GeneXpert confirmed the same results – all samples except samples 389 and 428 were positive. All results are summarized in Table 1.

Sample number15	IT reagents	„home-made“ kit	Gene Xpert	Result
45	POS	POS	POS	**POS**
94	POS	POS	POS	**POS**
114	POS	POS	POS	**POS**
177	POS	POS	POS	**POS**
236	POS	POS	POS	**POS**
389	NEG	NEG	NEG	**NEG**
428	NEG	NEG	NEG	**NEG**
511	NEG	POS*	POS	**POS**
617	POS	POS	POS	**POS**
745	POS	POS	POS	**POS**
863	POS	POS	POS	**POS**
925	POS	POS	POS	**POS**

Table 1. PCR based test results. * Sample 511 was treated by phenol/chloroform extraction.

It is evident, that without testing samples for PCR inhibitors followed by treatment of sample 511 by phenol/chloroform extraction or using the GeneXpert, we could not identify *Bacillus anthracis* in the sample either. It is possible to deal with the inhibitors of PCR reaction either as selected procedure in extra reaction (test of inhibition), and then using an alternative method of DNA extraction and purification or by insertion of an internal amplification control (IAC). An IAC is a non-target DNA sequence present in the same sample reaction tube which is co-amplified simultaneously with the target DNA. The inclusion of an IAC prevents false-negative results due to the inhibition of the polymerase enzyme or as well the malfunction of the thermal cycler [24, 31]. That manner is however more demanding on a design of PCR reaction [32-34]. Other option is the dilution of samples. For many situations, dilution of inhibited samples provides a rapid way of permitting amplification. This dilution exploits the sensitivity of PCR by reducing the concentration of inhibitors relative to target PCR.

Sample Number	Protein name/ NCBI Reference Sequence	Peptide sequence
45	Small, acid-soluble spore protein B/YP_030791	ANGSVGGEITKR
94	Small, acid-soluble spore protein B/YP_030791	ANGSVGGEITKR
		IAQEFGVQLGADATAR
		YEIAQEFGVQLGADATAR
	Elongation factor Tu/YP_026394	GITISTAHVEYETETR
	Hypothetical protein BAS3634/ YP_029886	SVLDNFDQWK
		GLDGGAVSDMAFR
		VGDYLANEVEAR
114	Small, acid-soluble spore protein B/ YP_030791	ANGSVGGEITKR
	Hypothetical protein BAS3634/ YP_029886	GLDGGAVSDMAFR
		VGDYLANEVEAR
		SFLGER
		SVLDNFDQWK
177	Elongation factor Tu/ YP_026394	GITISTAHVEYETETR
	Hypothetical protein BAS3634/ YP_029886	SVLDNFDQWK
		GLDGGAVSDMAFR
		VGDYLANEVEAR
236	Small, acid-soluble spore protein B/YP_030791	IAQEFGVQLGADATAR
		ANGSVGGEITKR
		YEIAQEFGVQLGADATAR
		LVSLAEQQLGGFQK
	Hypothetical protein BAS3634/YP_029886	SVLDNFDQWK
		GLDGGAVSDMAFR
		VGDYLANEVEAR
389		
428		
511	Small, acid-soluble spore protein B/YP_030791	YEIAQEFGVQLGADATAR
		ANGSVGGEITKR
		LVSLAEQQLGGFQK
		YEIAQEFGVQLGADATSR
617	Small, acid-soluble spore protein B/YP_030791	ANGSVGGEITKR
745	Elongation factor Tu/ YP_026394	GITISTAHVEYETETR
	Hypothetical protein BAS3634/ YP_029886	SVLDNFDQWK
		GLDGGAVSDMAFR
		VGDYLANEVEAR
863	Hypothetical protein BAS3634 /YP_029886.1	GLDGGAVSDMAFR
		VGDYLANEVEAR
925	Hypothetical protein BAS3634 /YP_029886.1	VGDYLANEVEAR

Table 2. Results from tandem mass spectrometry. The table shows the identified peptides within the sample and corresponding proteins with NCBI Reference Sequence numbers. The peptide sequences were identified in Bacillus anthracis str. Sterne, Taxonomy ID: 260799

Mass spectrometry results

The tandem mass spectrometry analysis confirmed PCR results. The peptide sequences from identified proteins are shown in Table 2. All samples but samples number 389 and 428 produced fragmentation spectra that match at least one peptide corresponding to the *Bacillus anthracis* strain Sterne protein sequence. There were 3 proteins identified in the samples overall: see Table 2. The representative interpretation of MS/MS fragmentation spectrum of ANGSVGGEITKR peptide from Small, acid-soluble spore protein B is depicted on Figure 3.

Residue	b	y
A	72.0444	**1171.6066**
N[Dea]	187.0713	1100.5695
G	**244.0928**	985.5425
S[Dhy]	**313.1143**	**928.5211**
V	**412.1827**	859.4996
G	**469.2041**	**760.4312**
G	**526.2256**	703.4097
E	**655.2682**	646.3883
I	768.3523	**517.3457**
T	869.3999	**404.2616**
K	997.4949	**303.2139**
R	1153.5960	**175.1190**

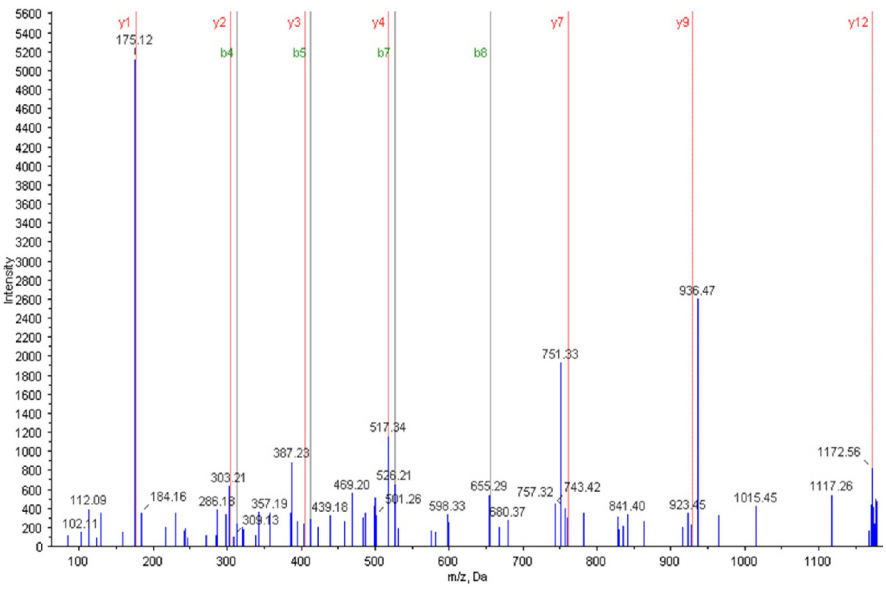

Figure 3. Representative spectrum and its interpretation using ProteinPilot 2.0 software for peptide ANGSVGGEITKR from Small, acid-soluble spore protein B. The bolded values represents the y (numbered bottom- up) and b (numbered top-down) fragment series positive peaks.

The tandem mass spectrometry was selected as a confirmatory method for SIBCRA exercise. All PCR positive samples contained Bacillus anthracis proteins and negative PCR samples were negative also by mass spectrometry. Thus the tandem mass spectrometry analysis fully confirmed PCR results. The digestion of the proteins by trypsin and process of mass spectrometry ionization may be inhibited by certain compounds. The procedure of the sample preparation for mass spectrometry was therefore chosen to be robust enough to eliminate possible interfering chemicals [27]. The proper sample preparation proved to be a key element of successful identification strategy for both PCR and Mass Spectrometry approach.

Overall

In total, fifteen laboratories from twelve countries (Austria, Belgium, Czech Republic, Denmark, Finland, Germany, Norway, Poland, Spain, Sweden, Switzerland and United States) took part in 10th Annual international comparative exercise of the NATO military biological laboratories in 2010. Nine of the participating laboratories, including the laboratory of the Central Military Health Institute, achieved full agreement with the exercise design (Table 3). The most frequent mistake of other laboratories was false negative result because of the presence of inhibitor (low pH phenolic disinfectant) within the sample 511 (Table 4), thus confirming that phenolic substances are very strong inhibitors of the polymerase chain reaction [10, 28].

Laboratory	Technique use by laboratory	% Correct	False (+)	False (-)
1	PCR	100	0	1
2	PCR	80	2	0
3	PCR & Tandem MS	100	0	0
4	PCR & EM	100	0	0
5	PCR	100	0	0
6	Immunoassay & PCR	90	0	1
7	PCR, ELISA & IF	100	0	0
8	PCR	90	0	1
9	PCR	100	0	0
10	PCR	100	0	0
11	PCR	90	0	1
12	PCR	100	0	0
13	PCR	80	1	1
14	PCR	90	0	1
15	PCR	100	0	0

Table 3. Summary of results – 2010 NATO SIBCRA Exercise. In total fifteen laboratories from twelve countries (Austria, Belgium, Czech Republic, Denmark, Finland, Germany, Norway, Poland, Spain, Sweden, Switzerland and United States) took part in the exercise. PCR – Polymerase Chain Reaction, EM – Electron Microscopy, ELISA – Enzyme-Linked ImmunoSorbent Assay, Tandem MS – Tandem Mass spectrometry, IF - Immunofluorescence

Sample number	BA Sterne	Sample preparation	Decontamination reagent type	Vendor
45	positive	Swab from spores treated with BacDown Detergent	quaternary ammonium detergent	Fisher
94	positive	Swab from spores treated with 70% Ethanol	ethanol, reagent grade, denatured	Sigma-Aldrich
114	positive	Swab from spores treated with CiDecon Detergent	phenolic disinfectant	Fisher
177	positive	Swab from spores treated with BacDown Foam Hand Sanitizer	quaternary ammonium detergent	Fisher
236	positive	Swab from spores treated with Conflikt Detergent	quaternary ammonium detergent	Fisher
389	negative	Blank sample – no spores or decontamination reagent	-	-
428	negative	Blank sample – no spores or decontamination reagent	-	-
511	positive	Swab from spores treated with LopHen Detergent	low pH phenolic disinfectant	Fisher
617	positive	Swab from spores treated with Sporgon Sporicidal	peracetic acid-based disinfecting solution	Fisher
745	positive	Dried spores milled with fluidizer	-	-
863	positive	wab of spores – no decontamination	-	-
925	positive	Swab from spores treated with 10% Bleach	hypochlorite	Fox Packaging

Table 4. Answer key lists a decontamination reagent type in each sample.

CONCLUSION

The biodefense laboratory of the Central Military Health Institute took part for the first time in international comparative exercise of the NATO military biological laboratories and employed up-to-date techniques based on analysis of DNA and proteins for this testing. The combination of provisional identification by real-time PCR and confirmatory identification by tandem mass spectrometry proved to be useful. The orthogonal nature of the techniques ensures that the agent is truly identified in the sample as whole organism and not only as extracted DNA or proteins. Above all, the combination of the techniques lowers the probability of false negative results.

The real-time PCR system in available format is easily deployable as part of mobile component of biodefense system of the Czech Armed Forces. The method is sufficiently sensitive and robust for use in the field conditions. The result of the tandem mass spectrometry analysis confirmed PCR results and verified the valuable confirmatory role of mass spectrometry in the identification of biological agents. The role is however, so far possible only in the reach-back laboratory. The combination of techniques can be applied onto the identification of other bacterial pathogens, and thus could be part of general algorithm applied to possible real scenarios in military settings.

ACKNOWLEDGEMENTS

We acknowledge the financial support The Ministry of Defense, Czech Republic (Research Program OVUOFVZ200901 „Biodefense“).

REFERENCES

1. The Czech Republic Ministry of Defense strategy in protection against weapons of mass destruction, **2007**, The Czech Ministry of Defence
2. Kolsto, A.B.; Tourasse, N.J., Okstad, O.A. What sets Bacillus anthracis apart from other Bacillus species? *Annu. Rev. Microbiol.* **2009**, 63, 451-76
3. Ellerbrok, H.; Nattermann, H.; Ozel, M.; Beutin, L.; Appel, B., Pauli, G. Rapid and sensitive identification of pathogenic and apathogenic Bacillus anthracis by real-time PCR. *FEMS Microbiol. Lett.* **2002**, 214, 51-9
4. Hurtle, W.; Bode, E.; Kulesh, D.A.; Kaplan, R.S.; Garrison, J.; Bridge, D.; House, M.; Frye, M.S.; Loveless, B., Norwood, D. Detection of the Bacillus anthracis gyrA gene by using a minor groove binder probe. *J. Clin. Microbiol.* **2004**, 42, 179-85
5. Jones, S.W.; Dobson, M.E.; Francesconi, S.C.; Schoske, R., Crawford, R. DNA assays for detection, identification, and individualization of select agent microorganisms. *Croat. Med. J.* **2005**, 46, 522-9
6. Ko, K.S.; Kim, J.M.; Kim, J.W.; Jung, B.Y.; Kim, W.; Kim, I.J., Kook, Y.H. Identification of Bacillus anthracis by rpoB sequence analysis and multiplex PCR. *J. Clin. Microbiol.* **2003**, 41, 2908-14
7. Qi, Y.; Patra, G.; Liang, X.; Williams, L.E.; Rose, S.; Redkar, R.J., DelVecchio, V.G. Utilization of the rpoB gene as a specific chromosomal marker for real-time PCR detection of Bacillus anthracis. *Appl. Environ. Microbiol.* **2001**, 67, 3720-7
8. Bode, E.; Hurtle, W., Norwood, D. Real-time PCR assay for a unique chromosomal sequence of Bacillus anthracis. *J. Clin. Microbiol.* **2004**, 42, 5825-31
9. Wilson, M.K.; Vergis, J.M.; Alem, F.; Palmer, J.R.; Keane-Myers, A.M.; Brahmbhatt, T.N.; Ventura, C.L., O'Brien, A.D. Bacillus cereus G9241 Makes Anthrax Toxin and Capsule like Highly Virulent B. anthracis Ames but Behaves like Attenuated Toxigenic Nonencapsulated B. anthracis Sterne in Rabbits and Mice. *Infect. Immun.* 79, 3012-9
10. Mullis, K.B., Faloona, F.A. Specific synthesis of DNA in vitro via a polymerase-catalyzed chain reaction. *Methods. Enzymol.* **1987**, 155, 335-50
11. Deepak, S.; Kottapalli, K.; Rakwal, R.; Oros, G.; Rangappa, K.; Iwahashi, H.; Masuo, Y., Agrawal, G. Real-Time PCR: Revolutionizing Detection and Expression Analysis of Genes. *Curr. Genomics*. **2007**, 8, 234-51
12. Šmarda, J.; Doškař, J.; Pantůček, R.; Růžičková, V., Koptíková, J. Methods of molecular biology, **2008**, Masaryk University, Brno, p. 125 - 175
13. Espy, M.J.; Uhl, J.R.; Sloan, L.M.; Buckwalter, S.P.; Jones, M.F.; Vetter, E.A.; Yao, J.D.; Wengenack, N.L.; Rosenblatt, J.E.; Cockerill, F.R., 3rd, Smith, T.F. Real-time PCR in clinical microbiology: applications for routine laboratory testing. *Clin. Microbio.l Rev.* **2006,** 19, 165-256
14. Fujita, O.; Tatsumi, M.; Tanabayashi, K., Yamada, A. Development of a real-time PCR assay for detection and quantification of Francisella tularensis. *Jpn. J. Infect. Dis*. **2006**, 59, 46-51

15. Hoffmaster, A.R.; Fitzgerald, C.C.; Ribot, E.; Mayer, L.W., Popovic, T. Molecular subtyping of Bacillus anthracis and the 2001 bioterrorism-associated anthrax outbreak, United States. *Emerg. Infect. Dis.* **2002**, 8, 1111-6
16. Christensen, D.R.; Hartman, L.J.; Loveless, B.M.; Frye, M.S.; Shipley, M.A.; Bridge, D.L.; Richards, M.J.; Kaplan, R.S.; Garrison, J.; Baldwin, C.D.; Kulesh, D.A., Norwood, D.A. Detection of biological threat agents by real-time PCR: comparison of assay performance on the R.A.P.I.D., the LightCycler, and the Smart Cycler platforms. *Clin. Chem.* **2006**, 52, 141-5
17. Loiez, C.; Herwegh, S.; Wallet, F.; Armand, S.; Guinet, F., Courcol, R.J. Detection of Yersinia pestis in sputum by real-time PCR. *J. Clin. Microbiol.* **2003**, 41, 4873-5
18. McAvin, J.C.; McConathy, M.A.; Rohrer, A.J.; Huff, W.B.; Barnes, W.J., Lohman, K.L. A real-time fluorescence polymerase chain reaction assay for the identification of Yersinia pestis using a field-deployable thermocycler. *Mil. Med.* **2003**, 168, 852-5
19. McAvin, J.C.; Morton, M.M.; Roudabush, R.M.; Atchley, D.H., Hickman, J.R. Identification of Francisella tularensis using real-time fluorescence polymerase chain reaction. *Mil. Med.* **2004**, 169, 330-3
20. Newby, D.T.; Hadfield, T.L., Roberto, F.F. Real-time PCR detection of Brucella abortus: a comparative study of SYBR green I, 5'-exonuclease, and hybridization probe assays. *Appl. Environ. Microbiol.* **2003**, 69, 4753-9
21. Redkar, R.; Rose, S.; Bricker, B., DelVecchio, V. Real-time detection of Brucella abortus, Brucella melitensis and Brucella suis. *Mol. Cell. Probes.* **2001**, 15, 43-52
22. U'Ren, J.M.; Van Ert, M.N.; Schupp, J.M.; Easterday, W.R.; Simonson, T.S.; Okinaka, R.T.; Pearson, T., Keim, P. Use of a real-time PCR TaqMan assay for rapid identification and differentiation of Burkholderia pseudomallei and Burkholderia mallei. *J. Clin. Microbiol.* **2005**, 43, 5771-4
23. Versage, J.L.; Severin, D.D.; Chu, M.C., Petersen, J.M. Development of a multitarget real-time TaqMan PCR assay for enhanced detection of Francisella tularensis in complex specimens. *J. Clin. Microbiol.* **2003**, 41, 5492-9
24. Ulrich, M.P.; Christensen, D.R.; Coyne, S.R.; Craw, P.D.; Henchal, E.A.; Sakai, S.H.; Swenson, D.; Tholath, J.; Tsai, J.; Weir, A.F., Norwood, D.A. Evaluation of the Cepheid GeneXpert system for detecting Bacillus anthracis. *J. Appl. Microbiol.* **2006**, 100, 1011-6
25. Byers, H.L.; Campbell, J.; van Ulsen, P.; Tommassen, J.; Ward, M.A.; Schulz-Knappe, P.; Prinz, T., Kuhn, K. Candidate verification of iron-regulated Neisseria meningitidis proteins using isotopic versions of tandem mass tags (TMT) and single reaction monitoring. *J. Proteomics.* **2009**, 73, 231-9
26. Jabbour, R.E.; Deshpande, S.V.; Stanford, M.F.; Wick, C.H.; Zulich, A.W., Snyder, A.P. A protein processing filter method for bacterial identification by mass spectrometry-based proteomics. *J. Proteome. Res.* **2011**, 10, 907-12
27. Jabbour, R.E.; Deshpande, S.V.; Wade, M.M.; Stanford, M.F.; Wick, C.H.; Zulich, A.W.; Skowronski, E.W., Snyder, A.P. Double-blind characterization of non-genome-sequenced bacteria by mass spectrometry-based proteomics. *Appl. Environ. Microbiol.* **2010**, 76, 3637-44
28. Wilson, I.G. Inhibition and facilitation of nucleic acid amplification. *Appl. Environ. Microbiol.* **1997**, 63, 3741-51
29. Wisniewski, J.R.; Zougman, A.; Nagaraj, N., Mann, M. Universal sample preparation method for proteome analysis. *Nat. Methods.* **2009**, 6, 359-62
30. Smith, P.K.; Krohn, R.I.; Hermanson, G.T.; Mallia, A.K.; Gartner, F.H.; Provenzano, M.D.; Fujimoto, E.K.; Goeke, N.M.; Olson, B.J., Klenk, D.C. Measurement of protein using bicinchoninic acid. *Anal. Biochem.* **1985**, 150, 76-85
31. Hoorfar, J.; Cook, N.; Malorny, B.; Wagner, M.; De Medici, D.; Abdulmawjood, A., Fach, P. Making internal amplification control mandatory for diagnostic PCR. *J. Clin. Microbiol*. **2003**, 41, 5835
32. Abdulmawjood, A.; Roth, S., Bulte, M. Two methods for construction of internal amplification controls for the detection of Escherichia coli O157 by polymerase chain reaction. *Mol. Cell. Probes*. **2002**, 16, 335-9
33. Hartman, L.J.; Coyne, S.R., Norwood, D.A. Development of a novel internal positive control for Taqman based assays. *Mol. Cell. Probes*. **2005**, 19, 51-9
34. Hoorfar, J.; Malorny, B.; Abdulmawjood, A.; Cook, N.; Wagner, M., Fach, P. Practical considerations in design of internal amplification controls for diagnostic PCR assays. *J. Clin. Microbiol*. **2004**, 42, 1863-8