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Report from Round Robin 2006; SIBA 7th laboratory exercise

Gunnar Skogan, Janet Martha Blatny and Jaran Strand Olsen

Forsvarets forskningsinstitutt (FFI)

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English summary

The NATO group SIBCRA (Sampling and Identification of Biological Chemical and Radiological Agents) arranged a training exercise with respect to identification of biological agents in March 2006. This is an annual exercise and was arranged for the first time in 1999. The object of this year's exercise was to identify the presence, if any, of biological agents in various complex environmental samples. The challenge of the 7th SIBA exercise was to identify different concentrations of *Bacillus anthracis*, *Burkholderia pseudomallei*, *Francisella tularensis* or the Vaccinia virus in soil samples. The results proved that FFI was able to identify all agents using real-time PCR. However, the concentration levels of the biological agents were not determined.

Sammendrag

Nato-gruppen SIBCRA (Sampling and Identification of Biological Chemical and Radiological Agents) arrangerte i mars 2006 en interlaboratorieøvelse for Nato og PfP land (Partnership for Peace). Laboratorieøvelsen er årlig, og ble første gang arrangert i 1999. Hensikten er å kunne analysere ukjente prøver med innhold av ulike biologiske agens. Den sjuende SIBA laboratorieøvelsen bestod i å identifisere ulike konsentrasjoner av *Bacillus anthracis*, *Burkholderia pseudomallei*, *Francisella tularensis* eller Vaccinia virus i jordprøver. Resultatene fra denne øvelsen viste at FFI klarte å identifisere alle biologiske agensene i de tilsendte prøvene ved bruk av real-time PCR.

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1 Introduction

The NATO group SIBCRA (Sampling and Identification of Biological Chemical and Radiological Agents) arranges annually an interlaboratory exercise for NATO member states and PfP countries (Partnership for Peace). The first exercise was held in 1999, where the samples consisted of inactivated biological agents in a clean solution (i.e. no analytical interferrents). Since then, the complexity of the samples has increased. Today, the participating laboratories must be able to analyse complex environmental samples containing a wide range of concentrations of organic and inorganic compounds mixed with various concentrations of the target agents.

The 7th SIBA laboratory exercise was held in March 2006. The objective of this exercise was to analyse 14 soil samples spiked with, if any, one or two of the inactivated biological agents *Bacillus anthracis*, *Burkholderia pseudomallei*, *Francisella tularensis*, or Vaccinia virus. The concentration range of bacterial cells and virus particles, was 10^4 - 10^7 cfu/gram soil and 10^5 - 10^8 pfu/gram soil, respectively. Each sample contained 2–3 gram soil. The laboratories may choose any method for analysis, but the analysis time is limited to ten days. FFI has participated in every interlaboratory exercise arranged by the NATO group SIBCRA since it initiated in 1999.

FFI has established several real-time PCR assays for various biological agents [6]. Also a method for identifying *Vibrio cholerae* has been established [7].

2 Materials and methods

DNA from all samples was isolated prior to analysis. Real-time polymerase chain reaction (PCR) was chosen for analysis [6] using both general and agent specific primers and probes (table 3.1 and 3.2). At first, general real-time PCR, using the *16S rDNA* gene as a target was performed to investigate any presence of bacterial cells in the samples. After this screening, the samples were analysed by using agent species specific primers. The amplified products were verified using melting point analysis and electrophoresis. The chemicals used in this work is shown in table 3.3.

2.1 Description of samples

The samples were spiked with various biological agents and prepared at Dugway Proving Ground, USA, where the bacterial cells (*Bacillus anthracis*, *Burkholderia pseudomallei*, *Francisella tularensis*) and virus particles (Vaccinia) were certified killed by cobalt irradiation. 14 soil samples containing unknown concentrations of the agents arrived at our laboratory March 2nd 2006. The samples were stored on ice at the time of arrival.

FFI received the following samples: sample 118, 123, 160, 199, 219, 226, 233, 241, 242, 275, 300, 302, 327 and 351.

2.2 Sample preparation and DNA isolation

The soil samples were mixed by vortexing to ensure homogeneity. 0.5 gram of soil sample was transferred to a microcentrifuge tube. One ml TE buffer (TRIS-EDTA, pH=8) containing 100 μg/ml polyadenylic acid was added to each sample. The suspension was then vortexed until the soil was dispersed.

The samples were centrifuged for 2 sec at 2000*g* in a Witz microcentrifuge. The supernatant was transferred to a 2 ml microcentrifuge tube with screw cap, containing 0.5 gram zirconia beads of 0.5 mm and 0.1 mm diameter. The soil pellet was then washed in 750 µl TE-buffer and centrifuged. The supernatant was transferred to a 2 ml microcentrifuge tube, homogenized in a Mini-BeadBeater-8™ from BioSpec Products Inc. for 1 min at 3200 rpm and centrifuged at 12000*g* for 1 min. The supernatant was transferred to a sterile 15 ml tube. 100% ethanol and 3M sodium acetate, pH=5.2, was added (2.5x and 0.1x the sample volume, respectively). The samples were inverted several times to mix the contents and incubated at -20 ˚C for 15 min. The samples were centrifuged at 6000*g* for 45 min, 4 °C, the supernatant was decanted and the pellet was dried for 1 min (tube placed upside down on a clean paper towel). 2 ml prechilled 70% ethanol was added to the pellet and the tube was gently inverted before centrifugation at 6000*g* for 15 min. The pellet was dried until all ethanol evaporated. The DNA pellet was dissolved in 100 ml sterile, DNase free water. The pellet was gently vortexed and incubated for 10 min on ice.

DNA was further isolated using the NucliSens Isolation Reagents (bioMerieux, France), according to the manufactures procedures.

2.3 Primers and probes

The primers and hybridisation probes are listed in table 3.1 and 3.2, respectively. Primers were synthesized by Eurogentec (Belgium) or Invitrogen (UK), and probes were synthesized by Tib Molbiol (Germany).

Table 3.1 Primers used in the 7th SIBA interlaboratory exercise.

Primer	Agent	Target	Length (bp)	Sequence	Ref.
Gram 16S-F	Bacteria	*16S rDNA*	187	5’- tac ggg agg cag cag t -3’	[1]
Gram 16S-2	Bacteria	*16S rDNA*	187	5’- tat tac cgc ggc tgc t -3’	[1]
BA813-1	*B. anthracis*	*BA813*	152	5’- tta att cac ttg caa ctg atg gg -3’	[2]
BA813-2	*B. anthracis*	*BA813*	152	5’- aac gat agc tcc tac att tgg ag -3’	[2]
BAlef-f*	*B. anthracis*	*lef*	156	5’- gca gat tcc tat tga gcc aaa -3’	[6]
BAlef-r	*B. anthracis*	*lef*	156	5’- gaa tca cga ata tca att tgt agc -3’	[6]
Tul-1	*F. tularensis*	*17 kDa lipoprotein*	300	5’- tat caa tcg cag gtt tag -3’	[3]
Tul-2	*F. tularensis*	*17 kDa lipoprotein*	300	5’- tcg ttc ttc tca gca tac tta g -3’	[3]
FT17k-1	*F. tularensis*	*17 kDa lipoprotein*	153	5’- gca agc tgc tgc tgt atc ta -3’	[6]
FT17k-2	*F. tularensis*	*17 kDa lipoprotein*	153	5’- tgg cac tta gaa cct tct gg -3’	[6]
BP16S-f	*B. pseudomallei*	*16S rDNA*	565	5’- ttc tgg cta ata ccc gga gt -3’	[4]
BP16S-r	*B. pseudomallei*	*16S rDNA*	565	5’- gcc caa ctc tca tcg ggc -3’	[4]
VACha-1	Vaccinia	*hemaglutinin*	273	5’- atg caa ctc tat cat gta a -3’	[5]
VACha-2	Vaccinia	*hemaglutinin*	273	5’- cat aat cta ctt tat cag tg -3’	[5]

Table 3.2 Hybridisation probes used in the 7th SIBA interlaboratory exercise.

Agent	Designation	Sequence	Ref
Bacteria	Gram 16S iFL	5'- tat tac cgc ggc tgc tg -3'	[1]
Gram-negative bacteria	Gram-LC640	5'- ccg cag aat aag cac cgg cta act ccg t -3'	[1]
Gram-positive bacteria	Gram+LC705	5'- cct aac cag aaa gcc acg gct aac tac gtg -3'	[1]
B. anthracis	Ba813-FL	5'- ata gaa cct ggc att aaa aga ctc att ga -3'	[6]
B. anthracis	Ba813-LC640	5'- aac tcg tta atg ctt caa att ctg tgt tt -3'	[6]

Table 3.3 Chemicals used in the 7th SIBA interlaboratory exercise.

Chemicals	Manufacturer
Primers	Invitrogen, Eurogentec
Probes	Tib Molbiol
DNA 500 Reagents & Supplies	Agilent Technologies
LightCycler FastStart DNA Master^{PLUS} SYBR Green I	Roche Diagnostics
LightCycler FastStart DNA Master^{PLUS} HybProbe	Roche Diagnostics
Sodium acetate, pH 5,2	Merck
Ethanol	Arcus
TE-buffer, pH 8,0	Merck

2.4 Real-time PCR amplification

Real-time PCR was performed in a total volume of 20 µl, using the LightCycler^{TM} and the FastStart DNA Master^{PLUS} SYBR Green I reaction mix. When hybridisation probes were used, the FastStart DNA Master^{PLUS} HybProbe reaction mix was utilized. The samples were analysed for presence of biological agents using a primerset amplifying the bacterial *16S rDNA* gene. In order to differentiate between Gram-negative and Gram-positive bacteria in the samples, a hybridisation probe was used together with the *16S rDNA* primer set. Hybridisation probes were added to the reaction mix to a final concentration of 0,2 µM. Specific primers were used to identify *B. anthracis, B. pseudomallei, F. tularensis* and Vaccinia virus (table 3.1). Prior to 45 cycles of PCR, an initial step of 10 min at 95 °C was required to activate the Taq DNA polymerase. The extension step was carried out at 72 °C. The optimized PCR conditions for all agents are shown in table 3.4. The Master^{PLUS} kits contains an optimized concentration of Mg^{2+}, further addition of Mg^{2+} was not necessary.

Melting point analysis was performed using the LightCycler^{TM}. In addition, 1 µl of the PCR products was analysed by electrophoresis using the DNA 500 LabChip Kit and the 2100 Bioanalyzer instrument (Agilent Technologies).

Table 3.4 Optimized real-time PCR conditions for identification using the primers in table 3.1.

Microorganism	Primer set	Primer concentration	Annealing temperature	Amplification cycle program[a]	No. Cycles
B. anthracis	BA813-1/2	1.0 uM	58 ºC	0 – 2 – 10 (sec)	45
B. anthracis	BAlef-f*/r	1.0 uM	58 ºC	0 – 2 – 10 (sec)	45
F. tularensis	Tul-1/2	1.0 uM	60 ºC	0 – 5 – 15 (sec)	45
F. tularensis	FT17k-1/2	1.0 uM	60 ºC	0 – 2 – 10 (sec)	45
B. pseudomallei	BP16S-1/2	1.0 uM	58 ºC	0 – 5 – 20 (sec)	45
Vaccinia	VACha-1/2	1.0 uM	58 ºC	0 – 5 – 15 (sec)	45
Bacteria	Gram 16S-F/2	1.0 uM	52 ºC	0 – 5 – 15 (sec)	45

[a] The amplification cycle program is noted as the seconds used for denaturation, annealing and extention , respectively.

3 Results

14 soil samples containing four different irradiated biological agents, provided by Dugway Proving Ground USA, were analysed using real-time PCR. FFI received the samples March 2nd 2006 and the analysis started March 7th 2006. The final results were obtained March 14th 2006. Figure 3.1 shows an example of a real-time PCR analysis of *F. tularensis*, using the Tul-1/Tul-2 primerset. Figure 3.2 shows the corresponding electrophoresis analysis of the amplified products (Tul-1/2) on an Agilent Bioanalyzer, using the DNA 1000 Assay.

The results achieved are presented in table 3.6. These results were reported to the organizer of the exercise, Dr. Bruce Harper (Dugway Proving Ground), who confirmed and verified that all results obtained were correct. The results from the other participating countries were be presented at the next SIBCRA meeting in Canada, 14-16 April 2007.

Table 3.5 Real-time PCR analysis of the biological agents in soil samples produced by Dugway Proving Ground . Concentrations are given by Dr. Bruce Harper.

Sample no.	**Agents**	**Concentration**[a]
118	Vaccinia	$2x10^7$
123	*B. pseudomallei*	$2x10^7$
160	*B. anthracis*	$2x10^4$
199	*F. tularensis*	$2x10^7$
219	*F. tularensis*	$2x10^4$
226	Vaccinia	$2x10^6$
233	*F. tularensis*	$2x10^5$
241	Blank (PBS buffer)	-
242	*F. tularensis and B. pseudomallei*	$2x10^6$ and $2x10^6$
275	*B. pseudomallei*	$2x10^5$
300	*B. anthracis*	$2x10^5$
302	*B. anthracis*	$2x10^7$
327	*B. pseudomallei*	$2x10^4$
351	Vaccinia	$2x10^5$

[a] Concentrations are given as cfu (colony forming units) for bacteria, and pfu (plaque forming units) per gram soil.

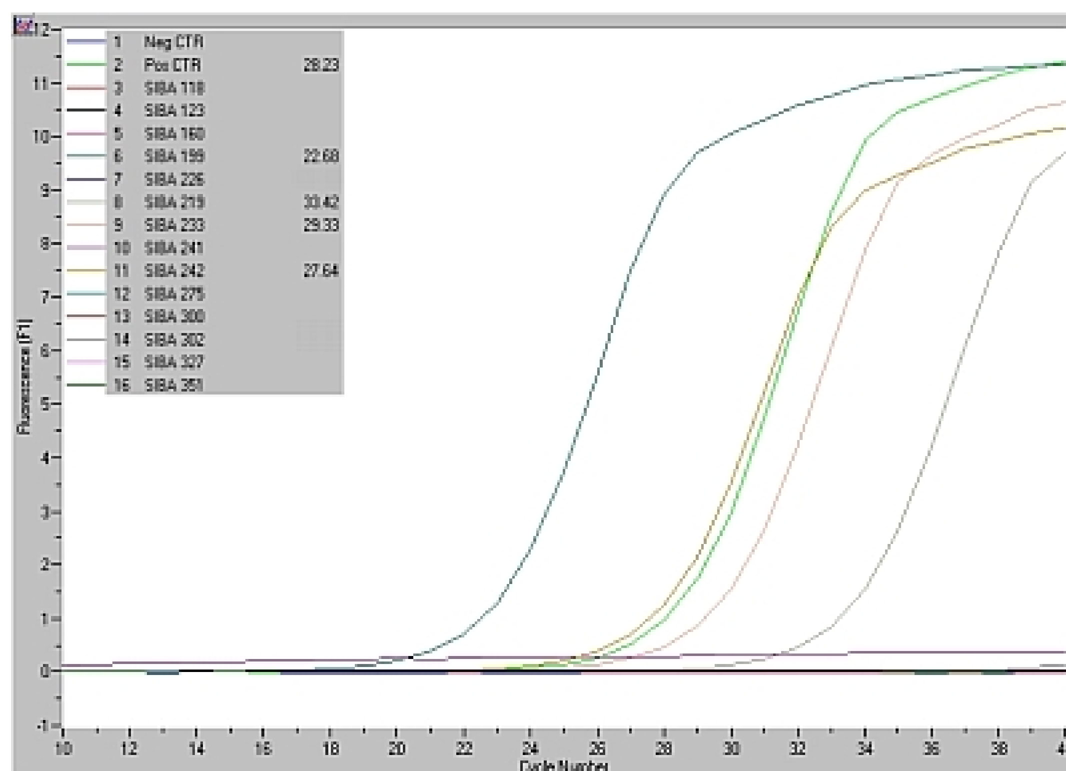

Figure 3.1 Real-time PCR analysis of a sample containing F. tularensis, using the Tul-1/Tul-2 primerset.

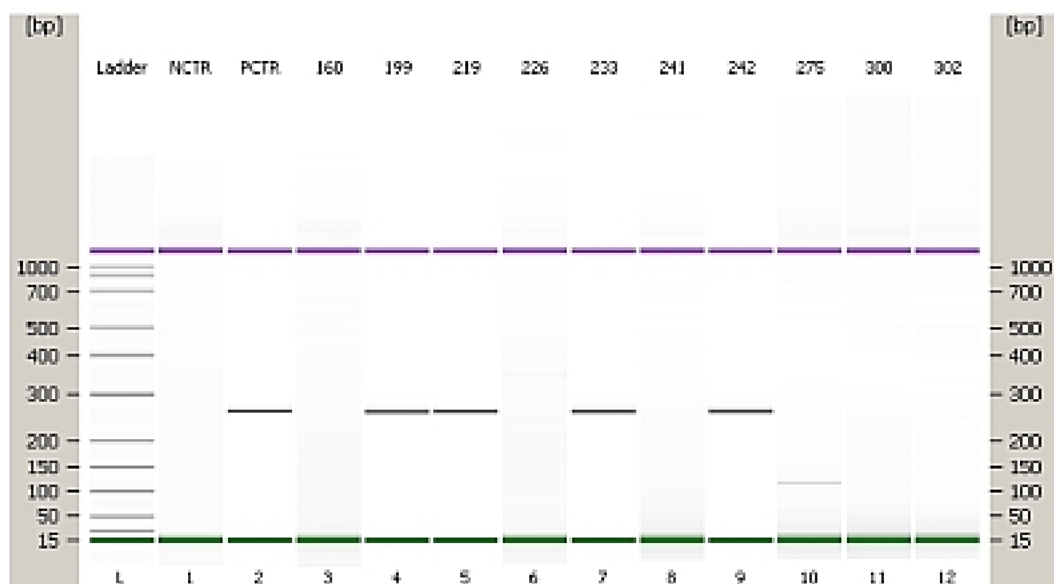

Figure 3.2 Analysis of the amplified products obtained by real-time PCR of a sample containing F. tularensis, using the Tul-1/2 primerset, using the Agilent Bioanalyzer DNA 1000 Assay.

FFI did not identify the concentration levels of the biological agents in the samples. However, the relative concentration levels were determined. By using real-time PCR we were able to distinguish those samples containing a high level of *B. anthracis* ($1x10^7$) compared to the samples with a lower concentration of *B. anthracis* ($1x\ 10^4$). Similar was found for *F. tularensis, B. pseudomallei* and Vaccinia virus (table 3.5).

4 Conclusion

FFI was able to identify all four biological agents, *B. anthracis, B. pseudomallei, F. tularensis* and Vaccinia virus, using real-time PCR, in various concentrations in 14 soil samples provided by Dugway Proving Ground in the 7th SIBA annual exercise.

References

[1] Klaschik, S., Lehmann, L. E., Raadts, A., Book, M., Hoeft, A., Stuber, F. Real-time PCR for detection and differentiation of gram-positive and gram-negative bacteria. *Journal of Clinical Microbiology,* **41** (6), 2799-2799.

[2] Patra, G., Sylvestre, P., Ramisse, V., Therasse, J., Guesdon, J. L. (1996). Isolation of a specific chromosomic DNA sequence of Bacillus anthracis and its possible use in diagnosis, *FEMS Immunology and Medical Microbiology*, **15**, (4), 223-231.

[3] Shaw et al (1998). Rapid Detection of Tularemia with Real-time PCR. *Infections in Medicine* **15,** (5), 326-330.

[4] Tomaso H., Pitt .T L., Landt O., Al Dahouk S, Scholz H. C., Reisinger E. C., Sprague L. D., Rathmann I., Neubauer H. (2005). Rapid presumptive identification of Burkholderia pseudomallei with real-time PCR assays using fluorescent hybridization probes. *Molecular and Cellular Probes*, **19,**(1), 9-20.

[5] Ropp, S. L., Jin, Q., Knight, J. C., Massung, R. F., Esposito, J. J. (1995). PCR strategy for identification and differentiation of smallpox and other orthopoxvirus, *Journal of clinical microbiology*, **33**, (8), 2069-2076.

[6] Fykse, E. M., Olsen, J. S., Skogan, G. (2004). Identification of biological agents using real-time PCR. FFI/RAPPORT – 2004/04247.

[7] Fykse, E. M., Skogan, G., Davies, W., Olsen, J. S., Blatny J. M. (2007). Detection of Vibrio cholerae by Real-Time Nucleic Acid Sequence-Based Amplification. *Applied and environmental microbiology AEM. American Society for Microbiology*, **73**, (5), 1457-1466.