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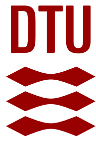

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AniBioThreat

CLASSIFICATION LEVEL: UNCLASSIFIED

TASK **5.1**

ANTHRAX

With the financial support from the Prevention of and
Fight against Crime Programme of the European Union
European Commission – Directorate – General Home Affairs

WP5

TASK 5.1
ANTHRAX

TASK LEADER

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The AniBioThreat project was in 2010 awarded a grant by Directorate General Home Affairs under the programme "Prevention of and Fight Against Crime". One issue stated in the call text in 2009 under this programme was animal bioterrorism threats. The focus of AniBioThreat is therefore based on threats to living animals, animal feed and food of animal origin. As part of this, it is foreseen that the project will enhance international cooperation and promote networking for bridging security with animal and public health.

The objectives are furthermore based upon some of the identified actions in the EU Chemical, Biological, Radiological and Nuclear (CBRN) Action Plan (2009), the recommendations of the CBRN Task Force Report (2009) and especially the work that took place in the Biosubgroup threats to animal, and food and feed for animals (2008), and the Biosubgroup detection and diagnosis (2008, June).

The project is divided into the following six work packages (WPs); WP1 the establishment of a network between law enforcement, forensic institutes, first responders, intelligence, veterinary institutes, public health agencies and universities, WP2 threat assessment, WP3 early warning/ detection, WP4 European Laboratory Response Network for animal bio-terrorism threats, WP5 detection and diagnostics and WP6 dissemination.

SPECIFIC OBJECTIVES OF THE WPS ARE AS FOLLOWS:

- To facilitate effective international cooperation, improve training and establish a network between law enforcement, forensic institutes, first responders, intelligence agencies, veterinary institutes, public health agencies and universities (WP1).
- To improve monitoring and threat assessments (WP2).
- To investigate early warning and rapid alert for animal disease outbreaks caused by criminal acts (WP3).
- To establish a European Laboratory Response Network approach to counter animal bio-terrorism threats (WP4).
- To enhance research and development of detection methods of animal diseases, such as anthrax, botulism and viral diseases caused by criminal acts (WP5).
- To disseminate the outcome of the project to relevant stakeholders through exercises, workshops, publications, and academic courses and to strengthen research through existing EU projects (WP6).

The overall objective of AniBioThreat is to improve the EU's capacity to counter biological animal bioterrorism threats in terms of awareness, prevention and contingency.

CAPACITY AND CAPABILITY

The overall goal of the EU CBRN Action Plan is an all-hazards approach to reduce the threat of damage from CBRN incidents of accidental, natural or intentional origin, including acts of terrorism.

This deliverable has improved EU's capacity and capability to counter biological animal bioterrorism threats in terms of awareness, prevention and contingency in following areas:

- ☐ Education and training capacity and capability
- ■ **Research capability**
- ☐ Risk assessment capability
- ■ **Cooperation/interoperability capability**
- ■ **Surveillance and rapid alert capability**
- ■ **Diagnostic and laboratory response network capacity and capability**
- ■ **Forensic awareness capability**
- ☐ Contingency planning capability
- ☐ Joint exercise capacity
- ☐ Readiness assessment and medical countermeasure capacity
- ☐ Communication and information sharing capability
- ☐ Strategic, tactical and operational decision making capability

ABSTRACT

This report describes the work that was undertaken and results produced on the development of a minimal detection standard for *Bacillus anthracis* in Task 5.1 of the EU Action Grant AniBioThreat. During the project, work was conducted in all parts of the pre-PCR processing chain, including sampling, sample preparation and the PCR step itself, as well as with characterization of isolates. Finally, a culture independent metagenomics approach was evaluated.

Firstly, sampling, and sample preparation methods were evaluated to allow for specific detection of *B. anthracis* in samples from animals, food and environmental samples. Methods for DNA and/or spore extraction from surfaces or complex samples were established that increase the capability to handle an outbreak of *B. anthracis*. The application of forensic sampling strategies was furthermore investigated. This is an important aspect to consider for further work towards a minimal detection standard.

To enable the selection of a PCR assay with optimal selectivity a software, Gegenees, was developed for multiple alignment of genomes and DNA sequences of different lengths. Gegenees was used to make a database with published genomes of *B. anthracis* and closely related species in the *B. cereus* group. *In silico* analysis of all PCR-target sequences reported for identification purposes was conducted using the database in Gegenees to select the assays with highest selectivity. An inter-laboratory evaluation trial, including 6 laboratories and 6 PCR assays, with DNA samples (n = 90) from different *Bacillus* species collected within the task partners´ strain collections was carried out in order to assess different protocols with regard to their specificity, reproducibility and ease of implementation. It was shown that several of the published PCR methods have poor selectivity both *in silico* and *in vitro*, possibly due to that several of the assays were designed before the advent of high-throughput whole genome sequencing.

In addition, work is presented on characterization methods (genotyping) of isolates. A suspension array method for the interrogation of a set of 13 canonical SNP markers (canSNP) that assign the major *B. anthracis* sub-lineages was developed. canSNP scoring is becoming a gold standard to genotype strains within the species. Whole genome sequencing of *B. anthracis* strains was conducted to generate sequence data that

Figure 1. Sampling is an important part of the analysis chain to detect *Bacillus anthracis* and strategies for efficient sampling were therefore included as a part of the work on a minimal detection standard. The picture shows soil and water sampling in an effort to locate the source of the *B. anthracis* spores in the outbreak area in Sweden 2011. Photo provided by SVA.

can be useful when designing future detection and/or typing assays and the use of these data within the project revealed details on how the genome changes during an outbreak.

Finally, a metagenomics based assay was evaluated for screening of unknown bacteria in biological samples where a direct sequencing approach targeting the 16S rDNA gene was evaluated in bottled water and different feed and food samples. The detection level (using *B. cereus* as a model for *B. anthracis*) in bottled water was 10^5-10^6 CFU/L, whereas it was difficult to apply the method on feed and food samples due to e.g. PCR inhibition. This method has the potential to be applied as a screening tool for the analysis of samples where the target is not known beforehand and thus complement more specific and sensitive molecular methods like PCR.

As part of the project different networking activities, such as short term study visits, joint experimental work and exchange of strains and DNA material, were included to enable transfer and build-up of knowledge to increase the capability to implement a minimal detection standard for *B. anthracis*.

In conclusion, work presented in this report substantially contributes towards the development and implementation of not only a minimal detection standard for *B. anthracis*, but also with tools and knowledge on characterization/typing and culture independent metagenomics approaches. These contributions will facilitate the future standardization of methods within this field.

DELIVERABLE ACCORDING TO GRANT AGREEMENT

Minimum detection standard on *Bacillus anthracis* (anthrax).

DESCRIPTION OF DELIVERABLE

This deliverable reports the results of the work conducted in Task 5.1 and includes evaluation and development of methods to detect and subtype *B. anthracis* using DNA based methods.

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BRIDGING STATEMENT

Bacillus anthracis is an important pathogenic organism, implicated in both natural and intentional outbreaks. Therefore, fast and accurate diagnosis is crucial in disease outbreak investigations and surveillance, for example by public health authorities. It is important that such methods are internationally recognized and validated. Furthermore, collaboration and the exchange of knowledge is a driving force within the AniBioThreat project. An outcome of this is the development of skills and know-how of scientific staff from partner institutes. Focused and/or longer-term visits between facilities develop stronger bonds between individual partners and institutes.

LINK TO EU CBRN ACTION PLAN

H.24

Based on the outcome of actions [under Goal 2] aimed at establishing validation/certification schemes for CBRN sampling and detection in the EU, the Member States together with the Commission should as far as feasible develop a coherent set of minimum technical detection standards (including within the context of border monitoring) based on scenarios, user requirements and risk and threat assessments while building on existing work (e.g. CEN). When developing such minimum technical standards, adequate engagement of the private sector, where appropriate, should be ensured and forensic requirements for evidence considered.

OTHER RELEVANT ACTIONS

None

CONTRIBUTION TOWARDS OVERALL OBJECTIVE OF ANIBIOTHREAT

Standardized detection and subtyping of *B. anthracis* will improve the response and decision making to identify if the agent is involved in the threat.

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AIMS

The aims of Task 5.1 were:

- To improve methods for detection of anthrax in animal, feed, environmental and food samples.
- To enhance research and development of detection methods of animal diseases, such as anthrax, caused by criminal acts.
- To increase the use of polymorphic sequence markers to characterize European collections of strains and develop a genotyping tool for strain traceability.

BACKGROUND

Risk and threat assessment of anthrax According to Action H24 in the EU CBRN Action Plan it is highlighted to focus on validation/certification schemes for CBRN sampling and detection in the EU, the Member States together with the Commission should as far as feasible develop a coherent set of minimum technical detection standards (including within the context of border monitoring) based on scenarios, user requirements and risk and threat assessments. Before AniBioThreat started another EU-project, namely BIOTRACER (www.biotracer.org), had identified the need to detect *B. anthracis* concerning deliberate spread of in food and feed (1). In AniBioThreat a specific task (task 2.1) has made a thorough risk- and threat assessment of anthrax and other pathogenic organisms, in which anthrax was the most highly risk ranked agent to be used as an agent for deliberate spread in animal, feed and food (2).

Bacillus anthracis *B. anthracis* is a Gram-positive, spore-forming bacterium representing the etiological agent of the acute infectious disease anthrax, a lethal disease of animals and humans (3). Fatality rates can reach up to 100% for untreated inhalational anthrax, and so, prompt diagnosis can have a major impact on the effectiveness of treatment. Since *B. anthracis* is easy to cultivate, the possibility of creating *in vitro* aerosols containing highly stable and resistant spores has made *B. anthracis* a very good weapon for bioterrorism. The major challenges in developing a fool-proof detection system for *B. anthracis* stem from its similarity with other species in the genera, e.g. *B. cereus* and *B. thuringiensis* (4; 5). Nucleic acid-based detection systems for *B. anthracis* rely on the selection of probes and/or primers that are specific for *B. anthracis* and do not cross-react with other species (5).

B. anthracis is one of the few species that has an endospore as a part of its life cycle. This spore form is extremely resilient to heat, drought, starvation, chemicals etc. and can therefore lie dormant in soil or in the environment for several years before given the chance to infect another host. It then goes into a vegetative state and kills

its host whereby bacteria are shed into the environment that transforms into the spore-form again (6). Also regarding bioterrorism, this form of the bacteria is infectious for animals and humans and therefore most likely to be used. For preparedness we must have established methods to isolate and/or detect the spores in complex samples such as feed, soil and other similar matrices. For other pathogens the task of extracting DNA from bacteria in order to detect it with PCR is relatively facile but the characteristics of the *Bacillus*-spore make it hard to crack (5). Enrichment or other kinds of cultivation can be attempted but other spore-formers often outgrow *B. anthracis*.

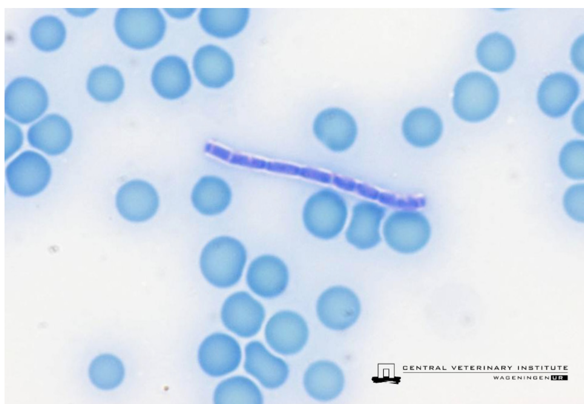

Figure 2. Microscopic picture of a blood smear positive for *B. anthracis*. Photo provided by CVI.

Molecular methods and microbial forensics

When developing and standardising PCR based detection methods it is essential to take the whole analysis chain into account, i.e. sampling, sample preparation and the use of an optimal PCR chemistry (7). A sample preparation technique that yields high amounts of DNA can for example result in bad performance of the PCR due to co-extraction of PCR inhibitory substances. Moreover, different sampling materials have been shown to influence the PCR performance negatively (7).

Forensic work during a bacterial disease outbreak, e.g., anthrax, which has a potentially antagonistic source, will most likely focus around the genetic material of the agent (i.e., the genome). The ability to quickly determine whether the agent originates from a laboratory or has a natural source is of great importance. For this purpose, we need rapid standardized methods for subtyping and/or whole genome sequencing of the pathogen. Furthermore, the forensic aspect needs to be considered when designing and implementing such techniques (8).

B. anthracis is a highly clonal species with extremely high genetic homology. Due to the lack of diversity, microbiological forensics increasingly rely on molecular markers, such as polymorphisms in DNA sequence (notably single nucleotide polymorphisms (SNPs)), to obtain reliable information regarding the source of a suspicious strain (9). Genome-wide SNP analyses have proven effective to differentiate strains and distinguish between intentional and naturally acquired infections (10).

Molecular methods are often optimized for the detection of a single or a very limited number of organisms, e.g. *B. anthracis*. In the case of an unclear or unknown contamination laborious and time consuming steps are needed to run assays for all possible targets. Furthermore, in the case of mutations in the primer and/or probe binding sites, variants of the targets might not be detectable. Hence, there is a need for a culture independent technique for a broad screening of targets.

Metagenomics has been mentioned as such a technique where the entire microbial gene pool of e.g. food, feed, water or environmental samples is examined. The metagenomics-based approach identifies those biomarkers that are actually present in the sample and makes it possible to match them to the microflora that is present (11). Metagenomics based methods

can be divided into two main groups: (i) whole genome based and (ii) target specific approaches (reviewed by (12; 13)). A commonly used marker in the target specific approach is the 16S rDNA gene. This gene is universally conserved, and has a high degree of diversity in bacteria (14). Direct sequencing is one example of a target based metagenomics based approach where the entire microflora is sequenced without prior cultivation (15).

Need for international collaboration Finally, to enable efficient development, validation, standardization and implementation of novel DNA-based detection and characterization methods collaboration and the exchange of knowledge between institutes and countries is essential. Examples of approaches to achieve extended networking and exchange of knowledge are to support focused and/or longer-term visits between facilities to develop stronger bonds between individual partners and institutes.

METHODOLOGY

This section descibes the methods that were used to achieve the goals in Task 5.1 and are divided into numbered sections, each representing a subtask. The same numbering is used in the section Results and Discussion below.

1. Genomics and bioinformatics tools

For developing highly specific molecular diagnostics a large reference database of genetic information is needed to make an optimal assessment of possible cross reactions with other species/subspecies. The same requirement is true for subtyping of an outbreak isolate – the more strains that can be compared to the isolate, the more information can be deducted from the comparison. Since the amount of available genomes (i.e., the complete DNA in a strain) is growing exponentially the computing power needed to analyse them is also growing rapidly. Better solutions for handling of the genomes and comparing them to each other are therefore needed. In the case of an antagonistic release of a pathogen, a lot of information can be acquired from the genetic code of the pathogen, e.g., to reveal if the strain comes from a laboratory.
In these cases speed is of the essence and the genomic workflow must then be as rapid as possible to assist forensic investigations. The goal of this subtask was to develop software that could rapidly compare several hundred microbial genomes at once and thus increase the preparedness capabilities regarding natural outbreaks and antagonistic releases of pathogens.

2. *In silico* evaluation of PCR assays

Several anthrax PCR detection assays have been described in literature (16). Detection of the highly conserved virulence plasmids pXO1 and pXO2 is relatively straightforward, yet there is no consensus about the optimal method for the detection of chromosomal DNA from *B. anthracis*. In fact, multiple assays have been described, each claiming superior detection capability, including selectivity. However, to our knowledge, systematic comparisons of these methods have not been made. To resolve this, a literature review was conducted to gather all the publically available PCR-based diagnostic assays for *B. anthracis*. All primers and probes, i.e. DNA target sequences, were collected in a database (16).

A specific PCR primer/probe should get a perfect alignment to the intended target genomes, which in this case is all the *B. anthracis* genomes, and a poor hit against other *Bacillus* spp. genomes which is hereafter referred to as the background genomes. The software tool, called Gegenees (17), developed in subtask 1 is primarily developed for whole genome comparisons but it has been complemented with a ‘primer-mapping’-feature. It allows many short sequences to be aligned to a

large amount of whole genomes. The sequences gathered in the literature review were aligned by Gegenees to all the >130 genomes of the *Bacillus* genus, containing both target anthrax genomes and background genomes. The primers/probes were then sorted for specificity and the best (in theory) PCR assays were selected for further *in vitro* studies (see subtask 3).

3. Interlaboratory *in vitro* evaluation of PCR assays

The analytical specificity of the primers and probes selected in subtask 2 was evaluated using 1) a panel *B. anthracis* reference strains collected from the participants and other international strain collections, 2) closely related strains from the *B. cereus* family and 3) a panel of non-related bacteria. The top range of chromosomal assays (Table 1) from the literature review and *In silico* evaluation was tested and compared for specificity. Six assays were selected, including the OIE recommended assay (18).

An interlaboratory comparison between 5 laboratories (CVI, DTU, SVA, ANSES and RIVM) of the 6 selected assays was performed. Genomic DNA from the 90 exchanged *Bacillus* strains were from the same stocks. Oligonucleotides and thermocycling programs were as published, but all 5 participating laboratories used the oligonucleotide provider, amplification kit and qPCR amplification platform of their choice. Further details on the study design can be found in (16).

4. Development of methods to isolate spores and/or DNA

At SVA, methods were developed for detection of *B. anthracis* spores in soil samples and these were applied during the 2011 Swedish outbreak of anthrax (*Figures 1 and 3*). To investigate the spread of the spores, different kinds of samples, including soil, sediment and water, were analyzed. Follow up studies were performed, of which one will reveal the rate at which detection probability decreases over time in an outdoor area.

Figure 3. Digging and soil/water sampling in an effort to locate the source of the *B. anthracis* spores in the outbreak area in Sweden 2011. Photo provided by SVA.

Furthermore, methods for detection of DNA from *B. anthracis* in complex samples were established and complemented with methods for isolating the live bacteria. Isolating the strain enables follow-up studies such as whole genome sequencing or subtyping. The spore isolation study also evaluated the usage of a semi-automated surface sampling machine called Mvac (www.m-vac.com) that could maximize the chance of retrieving spores from surfaces (*Figure 8*). This method was coupled with the development of an immunomagnetic separation assay that isolates and extracts anthrax spores from the matrix and the surrounding bacterial flora. To see if the Mvac

could isolate cells and/or DNA from softer materials, a study was carried out in collaboration with SKL, where the method was applied on different kinds of fabric as sample material.

At DTU, different commercial DNA extraction kits have been evaluated to find the optimal DNA extraction protocol for the metagenomics based direct sequencing technique. DNA extractions were carried out on food and feed samples, artificially contaminated with *B. cereus* spores at different contamination levels.

5. Development of a suspension microarray for anthrax genotyping

The interrogation of a set of 13 canonical SNP markers that assign the major *B. anthracis* sublineages is increasingly becoming a gold standard method for *B. anthracis* genotyping. ANSES has developed a suspension array method for the rapid and high-throughput canSNPs analysis and differentiation of strains within the *B. anthracis* species using the Luminex multiplex detection platform (19).

A MLPA-based method (Multiplex Ligation-dependent Probe Amplification) using the xTAG® beads technology was used for SNP scoring (20). It comprises three steps: detection and discrimination of each SNP (DNA hybridization and ligation), signal amplification (PCR), capture onto the microsphere array and flow cytometry analysis. Thirteen duplex assays targeting both alleles of each canSNP marker in a 26-plex method were designed.

To assess the robustness of the developed tool and improve the use of these polymorphic sequence markers to characterize European collections of *B. anthracis* strains, an interlaboratory ring trial was conducted including the Task 5.1 partners (SVA, RIVM, CVI, DTU and ANSES). A panel of DNAs exchanged between the AniBioThreat European laboratories network is in the process of being analyzed.

6. Whole-genome sequencing for characterization of isolates

During an outbreak, the responsible pathogen is often characterized by different subtyping methods to reveal how the outbreak strain is related to other strains. For this purpose, analysis methods targeting certain genomic areas or single nucleotides polymorphisms are often used. Specific PCRs can also indicate the presence of known virulence traits and antibiotic resistance genes. However, with the second generation sequencers being available at low costs and having short turn-around times (21) the outbreak pathogens can now be characterized at a much higher resolution. The whole genome sequencing is not a targeted method that looks for certain pre-defined targets but instead shows all the genomic information that can lead to understanding certain behaviour of a pathogen.

Single-nucleotide-polymorphisms (SNPs) can be used to reveal molecular epidemiology during an outbreak as well as finding the primary source of the disease. When comparing the SNPs from all animal isolates to isolates from environmental sources it can, e.g., be concluded if origin of the isolate comes from the disease source in the ground or if it is a secondary contamination from a sick/deceased animal.

7. Metagenomics

In this study, the direct 16S rDNA targeted sequencing approach (15) was evaluated as a diagnostic tool for detection of *B. cereus* artificially inoculated in bottled water without prior cultivation. *B. cereus* was used as a model for *B. anthracis* because it is possible to work with *B. cereus* in a BSL2 laboratory. Two strains that had previously been found to resemble *B. anthracis* were selected (22). Bottled water was purchased from local grocery stores. Spectra generated from the sequencing of the samples were compared using multivariate statistical methods, including

principle component analysis (PCA) and multivariate curve resolution (MCR).

8. Study visits, interlaboratory training and meetings

Within Task 5.1 several study visits and interlaboratory training were conducted to enable transfer of technologies and/or skills and to develop closer collaboration between partner organizations. Details on these activities are described next.

MedVetNet Association Short Term Mission, RIVM and DTU visit to SVA Representatives from ANSES, DTU and RIVM visited SVA in Uppsala, Sweden. The aims of this study tour were to:

- Learn about *Bacillus* spore isolation and how to isolate spores from complex samples.
- Exchange knowledge regarding the differences between “glove box-labs” and “suit-labs” within a high containment facility.
- Learn how Next Generation Sequencing (NGS) could be applied for preparedness applications.

Figure 4. One of the visiting scientists is learning how to use a class III biosafety cabinet during the study visit at SVA. Photo provided by SVA.

SVA study visit to ANSES, France One person from SVA visited ANSES facilities for Biosafety Level 3 (BSL3) organisms in Maisons Alfort, France. The aim of the visit was to learn from each other regarding *B. anthracis* detection and typing as well as laboratory practices and safety precautions for BSL3 organisms. The participant was given access to the BSL3 laboratory and given a tour by the head of the laboratory. This was followed by actual lab work to fully learn about the safety precautions and how work on *B. anthracis* is performed at ANSES. There were also seminars and discussions about typing and detection methods and ideas for collaborations were discussed.

SVA and SKL study visits on forensics and sampling
SVA and SKL started a small study to evaluate the use of the Mvac-system for forensic DNA samples. The machine has been tested for anthrax spore sampling but without any regards to forensic use. One person from SKL visited SVA for one day where the machine was evaluated whether it could be used for SKL’s purposes. The results seemed promising so one person from SVA then visited SKL for a full day of laboratory work where the machine was thoroughly tested using different kinds of spiked fabric as samples.

Interlaboratory training and participation in courses Several of the WP partners participated in courses organized within the framework of the project including courses in:

- DNA Amplification Technology (Lund University, Sweden)
- Diagnostic preparedness in an outbreak situation (SLU, Sweden)
- Rapid Detection, Enumeration and Characterization of Foodborne Pathogens (DTU, Denmark)

Figure 5. Group picture taken during one of the WP5 meetings. Photo provided by SVA.

Meetings WP meetings as well as meetings in connection with the AniBioThreat Annual meetings were held during the course of the project to discuss the progress of the work within the project and to plan further work.

9. Publications

During the project several scientific publications were written and submitted or are planned to be submitted to international journals with peer-review and/or as book chapters.

RESULTS AND DISCUSSION

This section contains the analysis and interpretation of the results presented using the same numbering as in the previous section.

1. Genomics and bioinformatics tools

The genomic comparison methodology has been developed and intensively evaluated. To make the method more accessible to researchers the whole workflow was implemented into a graphical user-interface. The software, called Gegenees, uses a multithreaded BLAST control engine that optimizes memory and processor usage, thus being able to compare several hundred microbial genomes on a normal desktop workstation. After the comparison has been performed by the computer, the genomes are put in a phylogenomic context to each other, i.e., how they are related to each other. The results can also be mined for information depending on the study and when new genomes are available they can be complemented to the existing comparison thus saving precious time during an outbreak situation. Shared genomic signatures for a certain user-defined group of genomes can be found which are suitable as markers for detection of this group of genomes, for instance by designing a PCR within the signature region.

The study was published in the journal PLoS ONE (17) and the software can be found at www.gegenees.org where it is continuously updated and support documentation can be found.

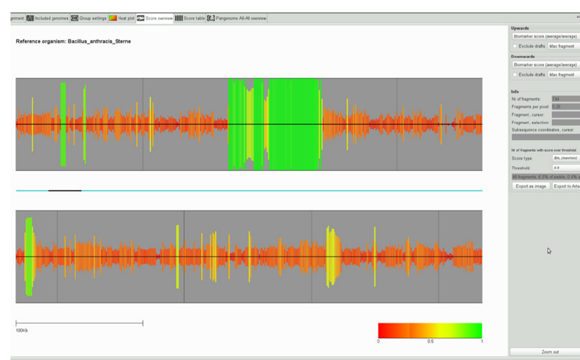

Figure 6. Screenshot of a biomarker score overview in the Gegenees software. The green areas are genomic areas where the *B. anthracis* genomes share a unique feature that can be used as a specific biomarker. Red areas represent areas shared with other *Bacillus* species.

2. *In silico* evaluation of PCR assays

Out of the ~20 chromosomal PCR assays analyzed with Gegenees, four stood out as highly specific for detection of the anthrax chromosome (Table 1). This means that they produced perfect hits toward all sequenced *B. anthracis* genomes and poor hits towards all other sequenced *Bacillus* spp. genomes. Three of them were probe-based which is a criterion to be included in a robust laboratory workflow and were therefore chosen for the *in vitro* study. A fourth assay that produced some undesired cross reactions was also identified and included in the *in vitro* study as proof-of-principle since the cross reactions seen *in silico* will theoretically also be present *in vitro*.

Assay #	No of *In silico* cross reactions	Used for *in vitro* ring-trial	*In vitro* cross reactions
1	0	Yes	No
2	0	Yes	No
3	0	Yes	No
4	2	No	ND
5	1-19*	No	ND
6	1-20*	No	ND
7	2-20*	No	ND
8	2	No	ND
9	2	No	ND
10	2-40*	No	ND
11	4-6*	No	ND
12	6	No	ND
13	8-22*	No	ND
14	0-15*	Yes	Several
15	0-40*	Yes	Several
16	15	Yes	Several
17	20	No	ND
18	20	No	ND
19	16-40*	No	ND
20	5-50*	No	ND

**depending on primer/probe analyzed, ND: Not Done*

Table 1. Summary of the *in silico* and *in vitro* evaluation. Results from 20 of the 35 assays are shown. The remaining assays all produced In silico cross-reactions. Information about the assays can be found in reference (16).

A surprisingly high amount of the PCR assays (~88 %) were found not specific for anthrax and this is mostly because not much was known about the genetically close-neighbouring strains up until around 2009 when sequencing a genome became a more trivial task. With this in mind, there were still several recently published PCRs that were very unspecific so the need for a thorough PCR marker study was apparently well motivated.

3. Interlaboratory *in vitro* evaluation of PCR assays

Six assays were selected, each targeting a different chromosomal marker for *B. anthracis* detection (*Table 1*). The primers and probes of three of the selected assays scored best in the evaluation using the Gegenees tool. The three other assays scored lower and served as controls. A non-related sequence was developed for inclusion as internal amplification control in all assays.

The ring trial showed that the three assays that scored best in the Gegenees evaluation were able to detect all *B. anthracis* strains, without producing false-positive results from closely related *Bacillus* strains (*Figure 7*). In contrast, two of the older assays produced a considerable number of false positive and negative results, whereas the current assay reported by WHO guidelines also yielded false-negative results for a few strains. A manuscript is in preparation describing the detailed outcome of this study (16).

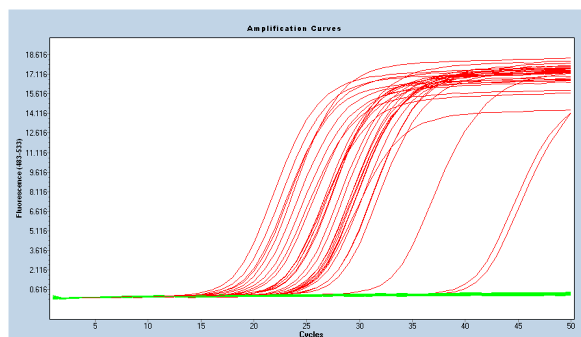

Figure 7. Real-time PCR results from one of the participating laboratories for one of the best performing assays. Amplification was achieved for all tested *B. anthracis* strains (red curves), whereas none of the non-target strains (other closely related *Bacillus* strains) generated amplification during PCR (green curves). Picture provided by RIVM.

Figure 8. The MVac System sampling device uses a liquid to facilitate the extraction of microbes from a surface. The liquid is sprayed onto the surface and the extract is collected by the machine using a vacuum. Photo provided by SVA.

4. Spore and/or DNA isolation

At SVA, methods were developed for detection of *B. anthracis* spores in soil- and environmental samples and these were applied during the 2011 Swedish outbreak of anthrax (*Figures 1 and 3*). To investigate the spread of the spores, soil-, sediment- and water samples were analyzed. The methods were robust and the results aided decision makers in how to handle the area of the outbreak. Follow up studies are being performed, including revealing the rate at which detection probability decreases in the soil. We have also isolated spores from the soil environmental samples so that the strain is available for further studies.

Furthermore, the Mvac system has been evaluated for spore isolation on different surfaces and has shown good preliminary results. The results will be published during 2014. The collaboration with SKL gave good results for isolating cells/DNA from fabric and the forensic application of the Mvac could be further developed.

At DTU, the direct sequencing method was evaluated for the use as a fast identification method for a potentially unknown pathogen in food and feed samples, using *B. cereus* as a model for *B. anthracis*. Five commercial DNA extraction kits were evaluated for the extraction of *B. cereus* from four food and feed sample types. The results demonstrate that the performance of the kits varied to a large extent. The PCA could not clearly distinguish between the two *B. cereus* strains and based on the sequences it was only possible to classify five samples as belonging to the class of Bacilli (23).

The results show that the method could not provide reliable conclusions on the ability to distinguish between strains or detect *B. cereus* in the food and feed samples. For the method to be useful in detection of bacteria in food and feed samples, additional optimization and validation are therefore needed. Results using the same method for bottled water are presented in subtask 7.

5. Development of a suspension microarray for anthrax genotyping

A fast multiplex nucleic acid assay adapted to the Luminex detection platform was developed to simultaneously detect multiple genetic markers such as canonical single nucleotide polymorphisms (canSNPs). This tool allows a first characterization of a strain, determining its phylogenetic sub-lineage and putative origin. In Europe, the population structure of *B. anthracis* is largely diversified with three sub-lineages more largely distributed (A. Br.008/009, B.Br.CNEVA, and A.Br.001/002 canSNP groups). Minor sub-groups include A.Br.005/006, A.Br.Aust94,

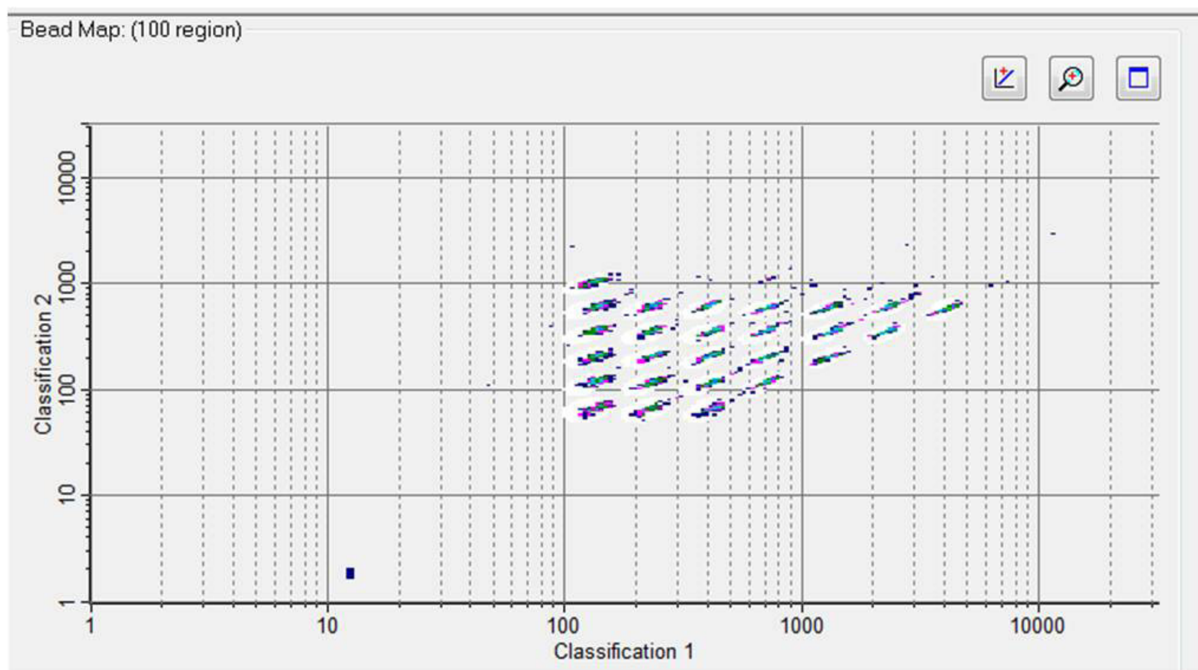

Figure 9. Classification of Luminex beads according to their colour, each white circle representing a specific bead region.

A.Br.Vollum, B.Br001/002, A.Br.Ames and A. Br.003/004.

The 26-plex assay currently designed was successfully applied for the interrogation of 13 canSNPs against a representative panel of 70 strains isolated in Europe (affiliated to 10 of the 13 major *B. anthracis* sub-lineages) (24). The effectiveness and robustness of the tool was further validated in an interlaboratory ring trial performed by the five Task 5.1 laboratories (*Figure 9*). The developed method is efficient, fast and user-friendly. It can easily be improved by the inclusion of additional SNP markers.

6. Whole-genome sequencing for characterization of isolates

During the anthrax outbreak in 2011 in Sweden, strain isolations were made from animal samples throughout the outbreak and also from environmental samples. These isolates were sequenced using an Illumina MiSeq at SVA and the whole genomes could be analyzed for discriminatory nucleotides. This kind of information can indicate the source of the disease. Using sequencing data, we could see how *B. anthracis* mutated during the outbreak. We sequenced RNA to correlate expression with the mutations seen in the DNA and much was learned about how the bacterium combats antibiotics. We could also, definitively, answer the question as to how *B. anthracis* turns on its otherwise inactivated resistance to beta-lactam antibiotics (25).

The methodology developed in this subtask is easily transferred to a new outbreak investigation and the preparedness for genomic analysis during a bacterial outbreak has been raised. The results will be submitted for publication during 2013 (25).

In a project separate from the AniBioThreat project, whole genome sequencing and comparative study of a hundred of French strains have been performed by ANSES to discover new genetic features specific for the *B. anthracis* sub-lineages present in France. Up to now,

five diagnostic SNP markers targeting the major B.Br.CNEVA sub-lineage have been identified. These new markers (and some additional ones) will be included to the suspension microarray developed in a near future.

As a consequence of this work, Europe will possess the tool and required know-how to react rapidly when a strain identity needs to be established.

7. Metagenomics

The direct sequencing method was evaluated for the use as a fast identification method for a potentially unknown pathogen in bottled water samples, using *B. cereus* as a model for *B. anthracis*. Results showed a detection level of 10^5-10^6 CFU/L. Furthermore, it was possible to separate the two *B. cereus* strains by the PCA plot (*Figure 10*). A linear correlation between the artificial contamination level and the relative amount of the *Bacillus* artificial contaminant in the metagenome was observed where a relative amount value above 0.5 confirmed presence of *Bacillus*. The background flora in the bottled water was found to vary between the water types that were included in the study. This method has the potential to be adapted to other biological matrices and bacterial pathogens for fast screening of unknown bacterial threats in outbreak situations. A manuscript presenting results from this study has been accepted for publication (26).

8. Study visits and interlaboratory training

All the conducted study tours and interlaboratory training helped to increase the capability to develop methods.

MedVetNet Association Short Term Mission, RIVM and DTU visit to SVA Collaboration and the exchange of knowledge is a driving force within the AniBioThreat project. An outcome of this is the development of skills and know-how of scientific staff from partner institutes. Focused and/or longer term visits between facilities develop stronger bonds between individual partners and institutes.

SVA study visit to ANSES, France The study visit and lab training was very fruitful and both the visitor and hosts learned a lot. We identified that ANSES and SVA work on a lot of similar areas and these could form the basis of future collaborations.

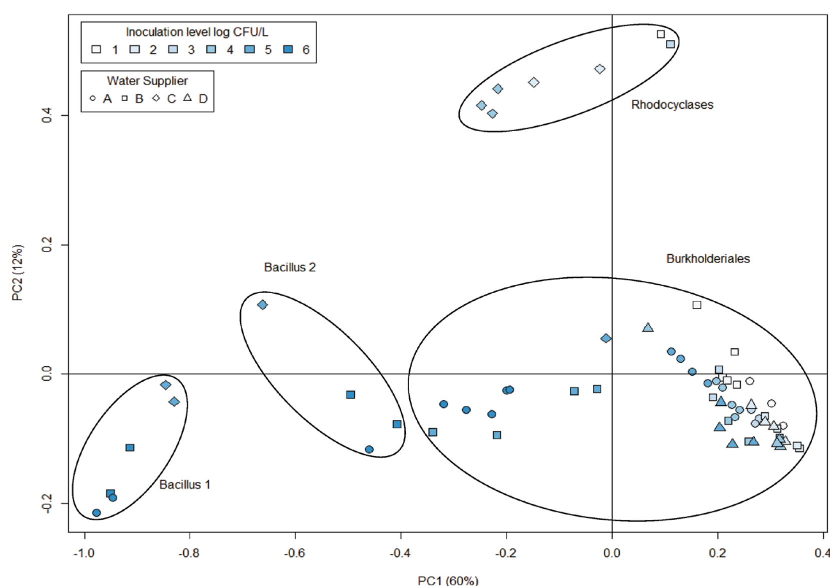

Figure 10. Principle component analysis (PCA) score plot. The principle component 1 (PC1) was plotted against PC2. The colouring of samples represents the level of artificial inoculation and symbols the water supplier. The percentage shown on each axis represents the portion of variance explained by that principal component (PC). Circles represent the identified bacterial flora of the samples. Circles around data points represent the four main groups that were visually identified from the PCA plot with the main order/genus that was identified in each group written next to the circle. *Bacillus* 1 and *Bacillus* 2 are two separate groups where Bacillales was identified as the most frequently order found in these samples. Figure re-printed from (26), with permission from Mary Ann Liebert, Inc.

SVA and SKL study visits on forensics and sampling

It can be difficult to extract cells/DNA from fabric using a tape or a swab and the vacuuming effect of the Mvac could be useful for these applications.

Different kinds of samples were analyzed and compared with other methods of fabric sample extraction. The results were promising and will form the basis for follow-up studies and will most likely be published in a scientific journal. Methods for sampling developed at this visit will be of use for the sampling of anthrax-spores and of course other pathogens.

9. Publications

A number of papers in international journals with peer-review (9; 12; 16; 17; 23-26), book chapters (7; 27-29) and a PhD thesis (30) were published or are planned to be published as a result of the work in Task 5.1 during the AniBioThreat project. Details on the publications can be found in the References section below.

CONCLUSION

This project has contributed to a European network of people working on anthrax, thus facilitating collaboration and exchange of knowledge.

The primary outcome of this task is a validated PCR-detection method that is in line with the minimum detection standard criterion in action H.24 (31). The risk of using unspecific diagnostic markers, that can produce ambigous results, has been reduced as a result of the work performed by the project partners. For other laboratories in the EU, data will be made available that enlightens the need to make use of sequenced genomic data to improve diagnostic markers, regardless of pathogen.

Secondary outcomes involve the development of freely available software that can be used to improve diagnostic markers and also to characterize the outbreak pathogen. A genotyping method based on diagnostic DNA signatures (canSNP markers) has also been developed. Precise genotyping of *B. anthracis* isolates enables tracking of natural anthrax cases in Europe or forensic investigations of malevolent acts. As a consequence of this project, task 5.1 partner laboratories will possess the required know-how to react rapidly when a strain identity needs to be established.

The other studies performed has given us capabilities to extract DNA/bacteria from complex samples for analysis. This greatly increases the likelihood to be able to handle an outbreak as efficiently as possible. As seen in the anthrax outbreak in Sweden in 2011, the use of these new methods and techniques facilitates the handling of the outbreak and makes work more efficient in such situations.

In conclusion, task 5.1 has delivered a minimum detection standard for PCR-based detection of *B. anthracis* together with several other methods that are not yet the minimum standard but the first steps towards using these latest technologies. This work will therefore significantly contribute to improve minimum standards for detection of *B. anthracis* within EU.

FUTURE OUTLOOK AND RECOMMENDATIONS

Based on the work performed in this task we would like to recommend the following actions to be taken to improve EU´s capacity to detect and trace *B. anthracis* in the future:

1. Diagnostic methods for *B. anthracis* detection should be validated and standardized to allow harmonization between laboratories.
2. Next generation sequencing technologies are powerful tools for the typing of any pathogen. By interrogating nearly every base of a genome, individualizing genetic markers can be reliably discovered, thus facilitating high-resolution strain tracking. Extensive

typing data set including autochthonous but also worldwide isolates are required for comparison so that accurate hypotheses can be made about isolate origins. Therefore, a whole-genome sequencing approach which focuses on identifying novel, strain-specific informative SNPs could be recommended for future work within this field of research.

3. Forensic aspects of bioterrorism diagnostic methods, within the field of microbial forensics, were identified as another research subject that needs future work.

PUBLICATIONS FROM TASK 5.1

Peer-review:

- Derzelle S, Thierry S. Genetic diversity of *B. anthracis* in Europe: Involvement of genotyping methods in forensic and epidemiologic investigations. Biosecurity and Bioterrorism: Biodefense Strategy, Practice, and Science 2013;(in press). (9)
- Karlsson O, Hansen F, Knutsson R, Löfström C, Granberg F, Berg M. Metagenomic detection methods within biopreparedness outbreak scenarios. Biosecurity and Bioterrorism: Biodefense Strategy, Practice, and Science 2013;(in press). (12)
- Ågren J, Hamidjaja R, Hansen T, Ruuls R, Thierry S, Vigre H, et al. *In silico* and *in vitro* evaluation of PCR-based assays for the detection of *Bacillus anthracis* chromosomal signature sequences. Virulence (submitted) 2013. (16)
- Ågren J, Sundström A, Hafström T, Segerman B. Gegenees: fragmented alignment of multiple genomes for determining phylogenomic distances and genetic signatures unique for specified target groups. PLoS One 2012;7(6):e39107. (17)
- Hansen T, Löfström C. Evaluation of a direct 16S rDNA sequencing approach for screening of *Bacillus cereus* in feed and food. In preparation 2013. (23)
- Thierry S, Girault G, Derzelle S. Multiplexed SNP genotyping of *Bacillus anthracis* by Luminex xMAP suspension array. Journal of Microbiological Methods (submitted) 2013. (24)
- Ågren J, Segerman B, et al. Whole genome sequencing enables molecular epidemiology of *Bacillus anthracis* isolates during an outbreak in Sweden where several isolates developed antibiotic resistance. In preparation 2013. (25)
- Hansen T, Skånseng B, Hoorfar J, Löfström C. Evaluation of direct 16S rDNA sequencing as a metagenomics based approach for screening of bacteria in bottled water. Biosecurity and Bioterrorism: Biodefense Strategy, Practice, and Science 2013;(in press). (26)

Book chapters:

- Hedman J, Lövenklev M, Wolffs P, Löfström C, Knutsson R, Rådström P. Pre-PCR processing strategies. In: Nolan T, Bustin SA, editors. PCR Technologies: Current innovations, 3rd Ed. Boca Raton: CRC Press; 2013. p. 3-17. (7)
- Knutsson R, Båverud V, Elvander M, Olsson Engvall E, Eliasson K, Sternberg Lewerin S. Managing and learning from an anthrax outbreak in a Swedish beef cattle herd. In: Hoorfar J, editor. Case studies in food safety and authenticity. Lessons from real-life situations. Cornwall, UK: Woodhead Publishing Limited; 2012. p. 151-60. (27)
- Josefsen MH, Löfström C, Hansen T, Reynisson E, Hoorfar J. Instrumentation and fluorescent chemistries used in qPCR. In: Filion M, editor. Quantitative real-time PCR in applied microbiology.Norfolk, UK: Caister Academic Press; 2012. p. 27-53. (28)
- Thierry S, Derzelle S. Multiplexed genotyping of *Bacillus anthracis* by Luminex xMAP suspension array. In: Cunha MV, Inacio J, editors. Methods in Molecular Biology, Molecular diagnosis in veterinary laboratory practice. Humana Press (in preparation); 2014. (29)

PhD thesis:

- Hansen T. Molecular diagnostics of foodborne pathogens National Food Institute, Technical University of Denmark. ISBN: 978-87-92763-86-0; 2013. (30).

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AniBioThreat

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AniBioThreat

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BRIDGING SECURITY, SAFETY AND RESEARCH

The aim of the project AniBioThreat
is to improve the EU's capacity to counter
biological animal bioterrorism threats
in terms of awareness, prevention and contingency.

The project will contribute to create
a safer and more secure world.
To succeed, we need to carry on a borderless dialogue.

AniBioThreat builds bridges across boundaries
dividing countries, competencies, and disciplines.

In our work, we strive to be **C**ollaborative,
Learning, **E**fficient, and **A**lert,
to be a **R**obust organization.
Keep it **CLEAR**!

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