GEFREASE
GErman FRench Equipment for Analysis and Surveillance of biothreats in the Environment

(2012-2015)

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Abstract – GEFREASE proposes to answer main issues associated with the detection of potential biological warfare agents. On the basis of previous experience gained by the four project partners, we will develop an integrated diagnostic approach combining on-site detection systems (provisional detection) based on immunoassays and confirmed and precise identification methods based on state-of-the-art mass spectrometry. We will target both toxins and microorganisms which all belong to the most powerful agents of biological threats.

1. Introduction

In the past decade, nations have reinforced their domestic plans and networks to handle biothreats, as illustrated through the following French and German examples. Within both countries, the response to a potential biothreat will start with the intervention of first responders (fire brigades and/or police) who will be responsible to secure the scene. In recent years, academic labs and the CBRN industry have proposed technological answers for the identification of biological (B) agents in the field or in laboratories. A range of biosensing technologies based on various principles (nucleic acid hybridisation, affinity sensors, enzymatic sensors…) have been developed ranging from proof-of-principle to more or less advanced Technology Readiness Levels. However, in the case of an intentional release of these agents, experts in France and Germany have identified different technical obstacles and major gaps in these detection technologies which prevent a timely and comprehensive analysis and response.

Although there are various initiatives at state level there is no European accepted / established commercial technology which aims at reliably detecting a broad range of all different relevant biological agents (bacteria, viruses and toxins). In this context, GEFREASE (GErman FRench Equipment for Analysis and Surveillance of biothreats in the Environment) proposes to answer main issues associated with the detection of potential biological warfare agents.

On the basis of previous experience gained by the four project partners, we will develop an integrated diagnostic approach combining **on-site detection systems (provisional detection**) based on immunoassays and **confirmed and precise identification methods** based on state-of-the-art mass spectrometry. We will target both toxins (ricin, botulinum toxin serotypes A, B, C, D, E, F, staphylococcal enterotoxin A and B, abrin) and microorganisms (*Francisella tularensis*, *Bacillus anthracis*, *Yersinia pestis*, *Vibrio cholerae* and *pox virus*) which all belong to the most powerful agents of biological threats. Our technologies will be able to detect and identify the presence of these potential agents in different environmental media (air, environmental and consumable waters, and drinks such as milk), advancing existing technologies available within the consortium which have been developed for clinical microbiology testing so far.

GEFREASE project associates four partners, among them two academic institutions: the CEA (Commissariat à l'Energie Atomique, Atomic Energy Commission, Marcoule), a research body with a technological focus on defence and healthcare technology and the Robert Koch-Institut (RKI, Berlin), the central federal institution responsible for disease control and prevention in Germany which is also as a reference institution for both applied and response-orientated research as well as for the Public Health Sector. Two industrial partners will be associated to the project: Bruker Daltonik (BDAL, Leipzig) which has a long standing experience in development and manufacturing devices and applications for mass spectrometric analysis of biological and chemical substances and Bertin Technologies (Bertin, Montigny-le-Bretonneux) which delivers a complete innovation offer, from technology services to delivery

of high-tech-products in life sciences and biotechnology processes. The CEA will be responsible for the project coordination and management.

By creating a network of experts from France and Germany working in this field, GEFREASE will help to **obtain a sustainability of successful detection technologies**, which have been developed so far at national level within both countries and to close gaps in detection technology in order to improve health and security for citizens in both countries. The combination of leading industrial partners and scientific / federal institutions in the area of B-detection will enable marketable solutions for provisional on-site detection and confirmed lab-based identification with the potential to open an European and world-wide market.

2. Description of workpackages

2.1 General organization

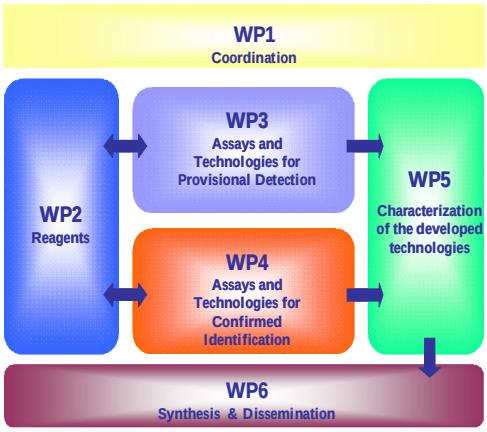

FIG. 1 : Workpackage Organisation

2.2 Details of workpackages

Workpackage 1: Coordination (WP leader: CEA)

The objectives of WP1 are:

- an efficient consortium management and administration of the project (staff and financial issues). Communication with the ANR and BMBF.
- the implementation of the project plan and coordination of work package activities with the goal to generate maximum consortium synergy.
- a continuous quality control, verification of milestones and implementation of means for tracking of progress towards objectives. Time and quality control of reporting activities and deliverables.

Workpackage 2: Reagents (WP leader: RKI)

The objective of WP 2 is to close gaps in detection tools by generating novel monoclonal and polyclonal antibodies (mAb, pAb) against potential biothreat bacteria, viruses and toxins enabling highly specific and sensitive detection of the target structures. The tools will be shared within the consortium. In close collaboration RKI and CEA will exchange existing and novel reagents and know-how in order to effectively approach the task, to find synergisms and to prevent unnecessary duplication of work

Workpackage 3: Assays and technologies for provisional detection (WP leader: Bruker)

WP3 aims to establish provisional detection methods for potential biothreat toxins and pathogens on the pTD platform and on the KIM platform based on antibodies developed within the consortium (RKI and CEA). Immobilization of toxin- as well as bacteria-specific antibodies onto electrical biochips and integration of a multiplex assay into the automated detection platforms pTD and KIM, respectively, will be the main tasks of this work package. RKI will establish and validate a new pTD biochip for detection of five relevant toxins, whereas BDAL will optimize and validate a new biochip for detection of five BWA-relevant bacteria.

Workpackage 4: Assays and technologies for MS-based confirmed identification (WP leader: CEA)

The objective of this work package is to develop the MS-based technologies and associated sample preparation. The strategy involves sample concentration using antibodies targeted against toxins, whole organisms or part of organisms (peptides belonging to specific proteins of each organism). The antibodies will be immobilized on magnetic beads or onto packed columns. Additionally sample preparation tools (microorganism's lysis, denaturation and enzymatic digestion) will be developed in order to provide an integrated system.

Workpackage 5: Characterization of the developed technologies (WP leader: RKI)

WP5 will focus on characterizing the developed on-site detection and verification methods for provisional detection and confirmed identification of biothreat pathogens and toxins. Within this WP, the perspective of end users from France and Germany will be implemented in order to make sure that the developed technologies are

in line with the needs of first responders and enable sound crisis communication and management.

Workpackage 6: Synthesis and Dissemination (WP leader: CEA)

The projects results will be described and analyzed in the final report. The partners will define potential improvements of the process and device, which could be implemented subsequently to the GEFREASE project.

The consortium takes a positive view of disseminating the results through public channels. Academic partners will prefer peer-reviewed publications in high quality journals and contributions to international conferences as major avenues for disseminating of results.

Exploitation of the product and technology will be fostered by a clear strategy valorisation and by performing a preliminary market analysis in order to identify promising market sectors, possibly already during the project life time.

3. Technologies

The CEA and RKI worked in different programs developed by the French Ministry of Defence in the field of the detection of bioterrorist agents. These activities rely on a strong expertise and capacity on in-house polyclonal and monoclonal antibody production, production of recombinant antigens, mass spectrometry (using either electro spray or MALDI ionization) and bioinformatics [1-6]. A typical example is one of our published assay for ricin which combines immunocapture and both functional detection and mass spectrometry targeting specific peptides (Fig 2). CEA teams are localized in a strongly secure area at Marcoule integrating a Biological Safety Level 3 laboratory (BSL3).

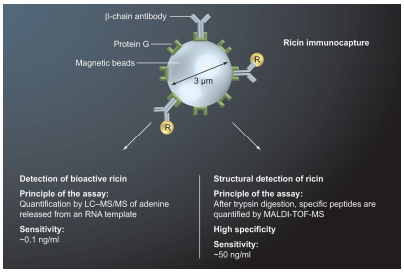

FIG. 2 : Workpackge Organization

The manipulation of microorganisms are performed in the Biosafety Laboratory level 3 (BSL3) at Marcoule (France). (Fig. 3)

FIG. 3 : BSL 3 at the CEA/Marcoule

In the previous ten years, Bertin has developed innovative sample preparation technologies and now offers through its Lab Equipment Division lysis and homogenization solutions (Precellys family, Fig. 4) and air-sampling devices (Coriolis family) for both laboratories and field application. Precellys tissue homogenizers are dedicated to the grinding, lysis and homogenization of biological samples, through a beat beating technology. A 8-figure multi-directional motion gives a high energy level to the beads that grind up to 24 2ml-samples together in few seconds. Bertin provides also dedicated kits and consumables, especially a proprietary 7 ml tube to grind samples up to 5 g, and a cooling module (Cryolys) to maintain low temperatures while grinding.

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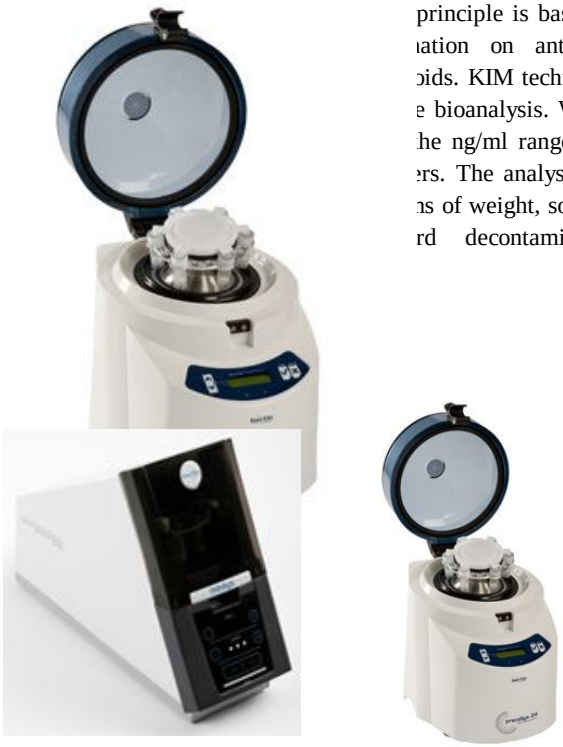

FIG. 4 : Sample preparation: Precellys Dual and Minilys

FIG. 5 : Kim Anlayzer

FIG. 6 : MALDI-TOF MS device (microflex) for MALDI Biotyper analysis

MALDI-TOF MS based identification of bacteria – the MALDI Biotyper of Bruker – is a popular system in clinical diagnostics, environmental and taxonomic research as well as in food-processing quality control. Starting with a single bacterial colony, centrifuged portion of a liquid culture or a small amount of white powder (around 10^5 bacterial cells are sufficient), the sample can be smeared directly onto a MALDI target and overlaid with matrix. The MS analysis and evaluation of spectra is then realized within few minutes. In case of critical samples standard short extraction protocols are available. Identification of unknown bacteria is accomplished via pattern matching through comparison of the generated peak lists with a large library containing thousands of spectra information of various species and genera. The MALDI Biotyper software incorporates all functionalities for automatic processing mass spectra as well as for identification and classification.

Generally, the MALDI Biotyper approach is a very robust identification method, because it is insensitive against mutations or cultivation conditions. The remarkable reproducibility of the methodology is based on measurement of constantly expressed high-abundant proteins, such as ribosomal. The MALDI Biotyper emerges to a new standard method replacing conventional biochemical methods, especially in the field of clinical diagnostics. Several central laboratories (e.g. Synlab, Dynacare, Labor Limbach, DSMZ) select Bruker's MALDI Biotyper for rapid microbial identification and implemented the system in their diagnostic procedure.

Bruker has taken over the electrical biochip technology pTD for rapid on-site detection of toxins. The pTD detection principle is based on an enzyme-linked immunosorbent assay (ELISA) procedure. Capture antibodies immobilized on gold electrodes facilitate the specific binding of corresponding toxins. In the next step, biotinylated detection antibodies are incubated and bind only to the affinity complexes. Binding of reporter enzyme to detector antibodies brings the reporter enzyme in vicinity of particular electrodes and the enzyme substrate is hydrolyzed to an electrochemically active product. The current correlates to the amount of target molecules captured by the antibodies. The very sensitive detection range (ng/mL to pg/mL-range) is owed to two facts: First, the high turnover of enzymatic reaction contributes to the signal amplification and second, a redox recycling of product, built into the experimental procedure, provides a second signal amplification. The portable Toxin Detector (pTD) allows the rapid, specific and sensitive identification of currently five toxins as described above (BoNT/A, BoNT/B, BoNT/F; SEB and ricin) within less 20 min using a fully automated process.

FIG. 7 : Bruker pTD

4. Initial results

The kick-off meeting was held in June 2012 in Marcoule and second meeting was held in December 2012 in Leipzig.

Production, purification and control of monoclonal antibodies are under progress: at the CEA:

- FTC 100R rat IgG
- FTC101R rat IgG
- FTC129Z mouse IgG2b
- FTC139R rat IgM

The first results concern the obtention of antibodies to *Francisella tularensis* and the development of immuncocncentration strategy for detection by MALDI-TOF. The following picture show a typical spectra of *F tularensis* which will be used as control for the testing of bacteria concentration by antibodies fixed on magnetic beads. This is also the basis for identification of specific biomarkers.

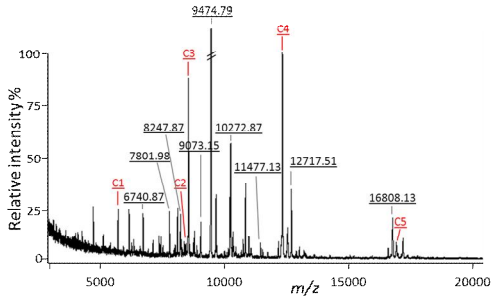

FIG. 8 : Maldi profile of *F tularensis* proteins

❑ .

The CEA Marcoule completed the building of the BSL3 laboratory (total cost 950 k€) (see Fig. 3 and 9) in July 2012 and the building was in operation a the end of October 2012. A significative part of the project will be performed in this new installation.

FIG. 9 : View of the BSL3 laboratory

In parallel, we initiate the :

- ❑ Production of batches of alive and heat-killed *Francisella tularensis holarctica*
- ❑ Production and purification of inactivated (with β-propiolactone) *Vaccinia virus* and immunization

Bertin has initiated the development of the Kim immunoassay platform for ricine and SEB enterotoxin.

The Robert Koch-Institute has started the development of antibodies for abrin, botulinum toxins and SEA enterotoxin.

Bruker Daltronik has started the development of digestion strategies and analysis by mass spectrometry for the ricin toxin.

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