EQuATox

Establishment of Quality Assurance for the Detection of Biological Toxins of Potential Bioterrorism Risk

"Biological toxins – ancient molecules posing a current threat"

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SOME BIOLOGICAL TOXINS

RICIN

- Plant poison, obtained from the seeds of *Ricinus communis* or the castor bean plant.
- Can induce cell death by inhibiting protein synthesis.

SAXITOXIN

- Neurotoxic poisons produced naturally by certain types of planktonic algae (dinoflagellates).
- Powerful neurotoxin that binds to the sodium channels in nerve cells blocking the influx of sodium ions, which produces a flaccid paralysis.

STAPHYLOCOCCUS ENTEROTOXIN B

- Produced by the bacterium *Staphylococcus aureus,* which normally present on the skin and in mucous membranes, but can also be found in the environment and in (contaminated) food.
- Deregulates the immune system by stimulating human T-cells, which in turn causes an over-production of cytokines, which causes toxic shock syndrome.

BOTULINUM NEUROTOXINS

- Can cause several forms of the disease botulism, a flaccid paralysis of the muscle leading to death within a few days due to respiratory failure.
- Produced by the anaerobic bacteria *Clostridium botulinum*, *C. butyricum* and *C. baratii*.
- Botulinum neurotoxins are the most toxic molecules known to man.

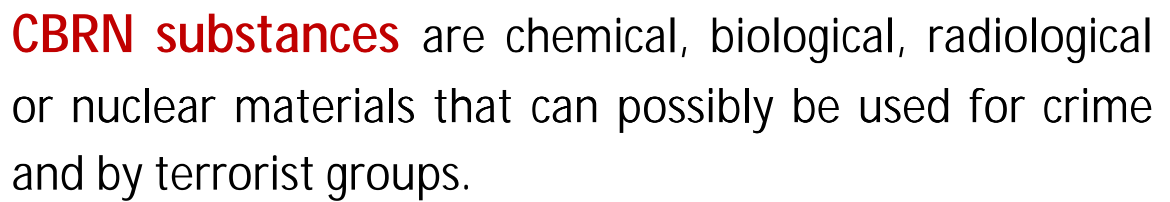
WHO WE ARE

Robert Koch-Institut (RKI) (Coordinator)	Germany	Dr. Brigitte Dorner
European Commission Joint Research Centre (EC-JRC)	Belgium	Dr. Heinz Schimmel
Scientific Institute of Public Health (WIV-ISP)	Belgium	Dr. Joris Van Loco
University of Helsinki, Finnish Institute for Verification of the Chemical Weapons Convention (UH/VERIFIN)	Finnland	Dr. Paula Vanninen
French agency for food, environmental and occupational health safety (ANSES)	France	Dr. Jacques-Antoine Hennekinne
Toxogen GmbH (Toxo)	Germany	Dr. Andreas Rummel
Swedish Defence Research Agency CBRN Defence and Security (FOI)	Sweden	Daniel Jansson
Federal Department of Defence Civil Protection and Sport - Spiez Laboratory (VBS-LS)	Switzerland	Marc-André Avondet
ChemStat (CHS)	Switzerland	Dr. Werner Luginbühl

EQuATox represents a network of nine expert laboratories organizing four large EU-wide proficiency tests for the detection of biological toxins which were conducted in 35 laboratories from 20 countries worldwide.

The research leading to any result within the EQuATox project has received funding from the European Community's Seventh Framework Programme (FP7/2007-2013) under grant agreement no. 285120

WHAT WE DO



CBRN substances are chemical, biological, radiological or nuclear materials that can possibly be used for crime and by terrorist groups.

Biological toxins like **ricin**, **botulinum toxins**, **staphylococcal enterotoxins** and **saxitoxin** could be used for terrorist attacks on the basis of their availability, ease of preparation, high toxicity and/or lack of medical countermeasures.

While different technologies for toxin detection and analysis have been established, hardly any universally agreed "gold standards" are available. Generally, proficiency tests and certified reference materials for the mentioned toxins are lacking.

The **AIM of EQuATox** is to develop common and/or comparable methods, procedures and protocols for the detection, analysis and identification of biological toxins.

OBJECTIVES

Establishment of an EU-wide network focussing on the detection and identification of biological toxins which are at the interface of classical B- and C-agents and are considered to be high priority agents. Toxins in focus were ricin, saxitoxin, SEB and BoNTs

Overview and evaluation of existing methods and diagnostic approaches for screening and identification of selected biological toxins (e.g. spectrometric, immunological, functional, molecular biological approaches) with respect to sensitivity, specificity and their suitability to analyze complex sample matrices

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Generation and characterization of toxin reference materials. The common effort among different European expert laboratories in defining reference materials lays the foundation for certified reference materials which could be worked out in the future. The long-term goal of this activity is to obtain ISO-compliant certified reference materials that enable ISO-accredited proficiency test and hence permit sustainable high-quality detection of biological toxins

Four independent international proficiency tests were conducted to compare diagnostic results attained by different analytical approaches. Both qualitative and quantitative results were evaluated. Results were statistically evaluated in accordance with internationally accepted standards

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Identification of success factors critical for detection of biological toxins at the interface of C- and B-agents. Identification of "best practices" for the analysis of the different biological toxins based on the results obtained in the proficiency tests

Exchange of information and know-how between network partners, including information on protocols, reagents etc. The aim was to network national reference, verification and diagnostic laboratories working on biological toxins in the security, health and food sector

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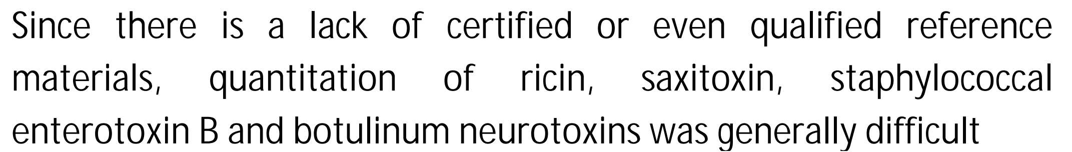
OUTCOME


Since there is a lack of certified or even qualified reference materials, quantitation of ricin, saxitoxin, staphylococcal enterotoxin B and botulinum neurotoxins was generally difficult

Many different technologies were applied by the participating laboratories and from the different approaches conclusions can be drawn on good analytical practices

However, for most of the toxins in the scope of EQuATox, no officially recommended operating procedures are available (e.g. on ricin and BoNT)

Only for one toxin under investigation an official method is available (saxitoxin), but the results indicated that a review of the official method is mandatory to improve performance of the laboratories

Recommended procedures for sample preparation are missing for different matrices taking into account the different physico-chemical properties of high molecular weight and low molecular weight toxins

Based on the coverage of participants among EU-28, no information is available on the preparedness and on technical capabilities of the majority of EU States with respect to biological toxins

NEEDS IDENTIFIED

TECHNICAL SHORTCOMINGS identified in EQuATox that should be addressed in the near future

Capability building on biological toxins of potential bioterrorism risk;
Development of detection and identification criteria for individual detection methods;
Development of recommended operating procedures for different technological approaches;
Closure of gaps in analytical detection capabilities (sensitivity, specificity);
Development of certified reference materials;
Distribution of reagents and methods and exchange of know-how;
Establishment of training capabilities to spread know-how within EU-28;
Regular laboratory exercises with increasing level of difficulty on selected techniques to monitor advancement of technical skills.

Furthermore, RESEARCH is needed

To select methods suitable for forensic investigations, e.g. for the analysis of human specimens or to trace back toxin origin or production method;
To develop sample preparation strategies for different matrices taking into account the different physico-chemical properties of high molecular weight and low molecular weight toxins;
To develop highly sensitive methods replacing the ethically questionable mouse bioassay still performed by many laboratories to detect biological toxins like BoNT or saxitoxin.

Finally, MANAGEMENT NEEDS identified

Consolidation of the EQuATox network followed by extension to ideally all EU States aiming to reach sustainability in the neglected area of biological toxins;
Development of a road-map addressing the response to a potential toxin incident (national scenario versus cross-border event);
Improvement of coordination among national approaches to envision a joint strategy.

EQUATOX IN FIGURES

Who?

9 organizing expert institutions

What?

4 EU-wide proficiency tests for the detection of biological toxins

Where?

35 laboratories in **20** countries

Results

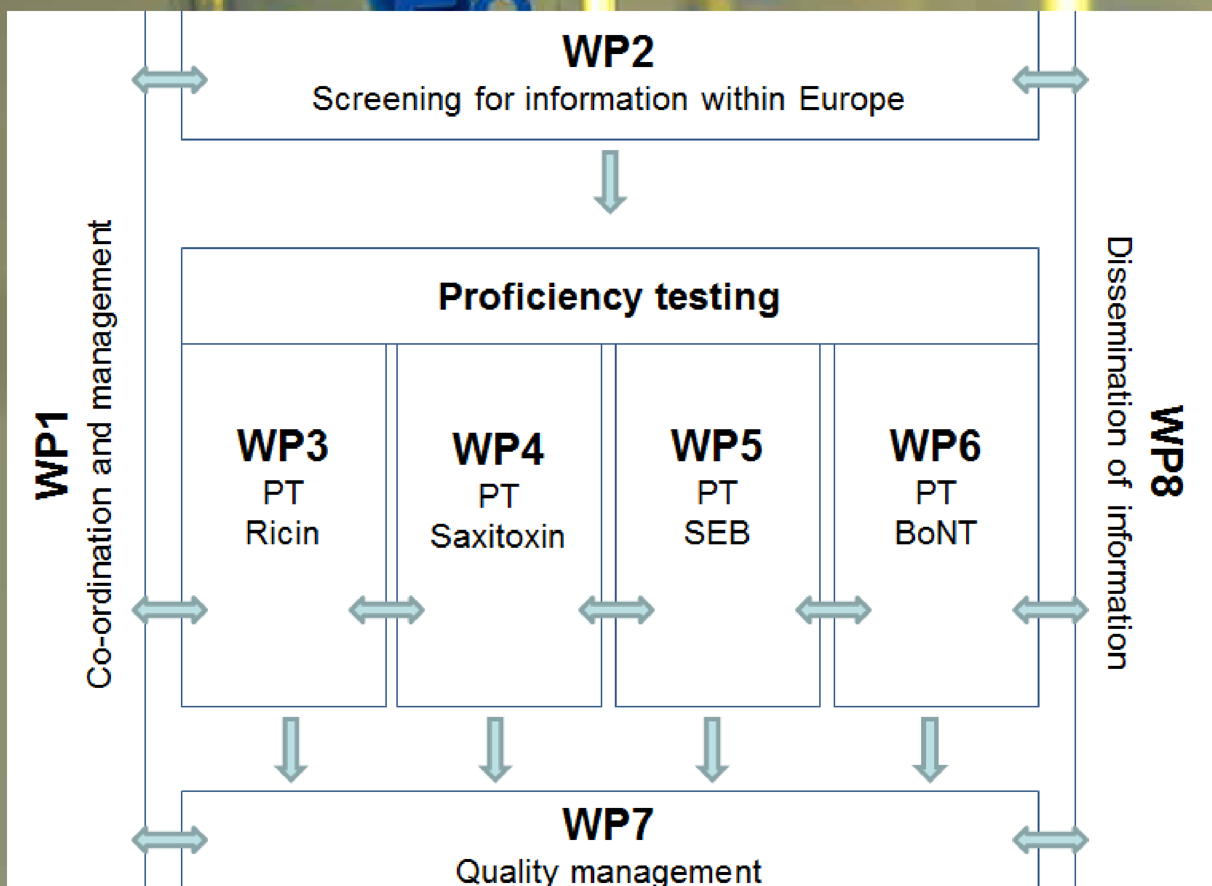
16 presentations of EQuATox activities at international conferences

18 presentations of EQuATox activities to decision makers

6 interactions of EQuATox with EU-projects working on related topics

WORK PACKAGES

The EQuATox project is divided into eight work packages of which the four proficiency tests (WP3-WP6) represent its core



WORK PACKAGE 1

Co-ordination and management

General coordination and management of the project including administrative and financial issues

- Coordination, monitoring and structuring of the EQuATox project aiming at efficient networking and exchange of partners
- Implementing feedback from external partner laboratories as well as Advisory Board and Security Board
- Organization of regular scientific meetings

WORK PACKAGE 2

Screening for information within Europe

Establishment of a network of expert laboratories working on biological toxins

- Collecting a feedback from European and selected non-European expert laboratories on their diagnostic approaches
- Overview and evaluation of existing methods and diagnostic approaches for screening and identification of selected biological toxins

WORK PACKAGE 3 TO 6

Proficiency testing

Organization of large international proficiency tests.

Generation and thorough characterization of highly purified reference materials to be used in proficiency test schemes taking into account the intricacies involved in the detection of structurally different high-molecular weight and low-molecular weight toxins:

- Work Package 3 – ricin
- Work Package 4 – saxitoxin
- Work Package 5 – staphylococcal enterotoxin B
- Work Package 6 – botulinum neurotoxins

Statistical analysis of qualitative and quantitative results according to internationally accepted standards;
Identification of good analytical practices and eventual shortcomings in detection technology as a basis for further development.

WORK PACKAGE 7

Quality management

Quality management of the project

- Set criteria for the proficiency tests and evaluation of results with respect to quality assurance measurements

WORK PACKAGE 8

Dissemination of information

Dissemination of project results on biopreparedness in the field of biological toxins

- Raise awareness among decision makers and stakeholders on results obtained
- Distribute knowledge on lessons-learned among the scientific community taking into account the balance between information sharing and confidential issues (dual-use issues)

RICIN

Characterization of ricin and *R. communis* agglutinin reference materials

Ricinus communis intoxications have been known for centuries and were attributed to the toxic protein ricin. On the basis of its availability, toxicity, ease of preparation and the current lack of medical countermeasures ricin gained attention as potential biological warfare agent. While different technologies for ricin analysis have been established, hardly any universally agreed "gold standards" are available. Expert laboratories currently use differently purified in-house materials, making any comparison of accuracy and sensitivity of different methods nearly impossible. Technically challenging is the discrimination of ricin as threat agent from *R. communis* agglutinin, a less toxic, but highly homologous protein also contained in *Ricinus communis*. Here, we established both highly pure ricin and *R. communis* agglutinin reference materials which were extensively characterized by capillary gel electrophoresis, SDS-PAGE, LC-ESI MS and MALDI TOF approaches as well as immunological and functional techniques. The purity reached >97% for ricin and >99% for agglutinin. Different isoforms of ricin as well as agglutinin were unambiguously identified and distinguished by LC-ESI MS/MS. In terms of function, a real-time cytotoxicity assay showed that ricin is approximately 300-fold more toxic than agglutinin. The highly pure ricin and agglutinin reference materials were used to conduct an international proficiency test.

AUTHORS

Sylvia Worbs[1], Martin Skiba[1], Martin Söderström[2], Marja-Leena Rapinoja[2], Reinhard Zeleny[3], Heinz Schimmel[3], Paula Vanninen[2], Sten-Åke Fredriksson[4] and Brigitte G. Dorner[1]*

1 Biological Toxins, Centre for Biological Threats and Special Pathogens, Robert Koch-Institut, Berlin, Germany
2 VERIFIN (Finnish Institute for Verification of the Chemical Weapons Convention), Department of Chemistry, University of Helsinki, Helsinki, Finland
3 European Commission, Joint Research Centre, Institute for Reference Materials and Measurements, Geel, Belgium
4 Department of CBRN Defense and Security, Defense Research Agency (FOI), Umeå, Sweden
* Author to whom correspondence should be addressed; E-Mail: DornerB@rki.de; Tel.: +49 30 18754 2500; Fax: +49 30 1810 754–2501.

An international proficiency test to detect, identify and quantify ricin in complex matrices

While natural intoxications with seeds of *Ricinus communis* have long been known, the toxic protein ricin contained in the seeds is of major concern due to its history of criminal, terrorist and military use. In order to harmonize detection capabilities in expert laboratories an international proficiency test was organized that aimed at identifying good analytical practices (qualitative measurements) and determining a consensus concentration on a highly pure ricin reference material (quantitative measurements). Sample materials included highly pure ricin as well as the related *R. communis* agglutinin spiked into buffer, milk and meat extract; additionally, an organic fertilizer naturally contaminated with *R. communis* shred was investigated in the proficiency test. The qualitative results showed that either a suitable combination of immunological, MS-based and functional approaches or sophisticated MS-based approaches alone successfully allowed to detect and identify ricin in all samples. In terms of quantification, it was possible to determine a consensus concentration for the highly pure ricin reference material. The results provide a basis for further steps in quality assurance and improve biopreparedness in expert laboratories worldwide.

AUTHORS

Sylvia Worbs[1], Martin Skiba[1], Jennifer Bender[1], Reinhard Zeleny[2], Heinz Schimmel[2], Werner Luginbühl[3] and Brigitte G. Dorner[1]*

1 Biological Toxins, Centre for Biological Threats and Special Pathogens, Robert Koch-Institut, Berlin, Germany
2 European Commission, Joint Research Centre, Institute for Reference Materials and Measurements, Geel, Belgium
3 ChemStat, Bern, Switzerland
* Author to whom correspondence should be addressed; E-Mail: DornerB@rki.de;
Tel.: +49 30 18754 2500; Fax: +49 30 1810 754–2501.

Recommended immunological strategies to screen for ricin-containing samples

Ricin, a toxin from the plant *Ricinus communis*, is one of the most toxic biological agents known. Due to its availability, toxicity, ease of production and absence of curative treatments, ricin has been classified by the CDC as category B biological weapon and it is scheduled as a list 1 compound in the Chemical Weapons Convention. An international proficiency test (PT) was conducted to evaluate detection and quantification capabilities of 17 expert laboratories. In this exercise one goal was to analyse the laboratories' capacity to detect and differentiate ricin and the less toxic, but highly homologous protein *Ricinus communis* agglutinin. Four analytical strategies are presented in this paper based on immunological assays (three immunoenzymatic tests and one immunochromatographic test). Using these immunological assays "dangerous" samples containing ricin and/or the *Ricinus communis* agglutinin were successfully identified. Depending on the antibodies used detection and quantification of ricin and *Ricinus communis* agglutinin was successful. The proficiency test for ricin showed that a highly sensitive and precise quantification of ricin and *Ricinus communis* agglutinin is possible using different recommended immunological approaches.

AUTHORS

Stéphanie Simon[1,†], Sylvia Worbs[2,†], Marc-André Avondet[3,†], Dobryan Tracz[4,†], Julie Dano[1], Hervé Volland[1], Brigitte G. Dorner[2] and Cindi Corbett[4,*]

1 CEA Saclay, Institute of Biology and Technologies of Saclay, Laboratory for immunoanalytical researches, Gif sur Yvette, France
2 Biological Toxins, Centre for Biological Threats and Special Pathogens, Robert Koch-Institut, Berlin, Germany
3 Federal Department of Defence, Civil Protection and Sport - SPIEZ LABORATORY, Spiez, Switzerland
4 Bacteriology & Enteric Diseases Division National Microbiology Laboratory, Public Health Agency of Canada, Winnipeg, Canada
† These authors contributed equally to this work.
* Author to whom correspondence should be addressed; E-Mail: Cindi_corbett@phac-aspc.gc.ca
Tel.: +1-204-784-5929; Fax: +1-204-789-5009.

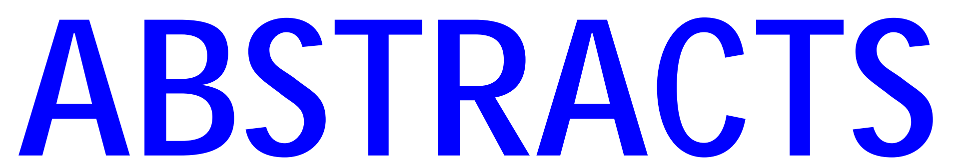
Recommended MS-based strategies to identify ricin-containing samples

Ricin is a toxic protein produced by *Ricinus communis* together with a related protein known as *R. communis* agglutinin (RCA). Mass spectrometric (MS) assays have the capacity to unambiguously identify ricin and detect ricin's activity in samples with complex matrices. These qualitative and quantitative assays enable detection and differentiation of ricin from the less toxic RCA through determination of the amino acid sequence of the protein in question and active ricin can be monitored by MS as the release of adenine from the depurination of a nucleic acid substrate. In this work, we describe the application of MS-based methods to detect, differentiate, and quantify ricin and RCA in nine blinded samples supplied as part of the EQuATox proficiency test. Overall, MS-based assays successfully identified all samples containing ricin or RCA with the exception of the lowest concentration standard (0.414 ng/mL). In fact, mass spectrometry was the most successful method for differentiation of ricin and RCA based on amino acid determination. Mass spectrometric methods were also successful at ranking the functional activities of the samples, successfully yielding semi-quantitative results. These results indicate that MS-based assays are excellent techniques to detect, differentiate, and quantify ricin and RCA in complex matrices.

AUTHORS

Suzanne R. Kalb[1,†], David M. Schieltz[1,†], François Becher[2,†], Crister Åstot[3,†], Sten-Åke Fredriksson[3,†] and John R. Barr[1,*]

1 Centers for Disease Control and Prevention, Atlanta, USA;
2 Service de Pharmacologie et d'Immunoanalyse, Institut de Biologie et de Technologies de Saclay (iBiTec-S), Commissariat à l'Énergie Atomique et aux Énergies Alternatives (CEA), Gif-sur-Yvette, France;
3 Department of CBRN Defense and Security, Defense Research Agency (FOI), Umeå, Sweden
† These authors contributed equally to this work.
* Author to whom correspondence should be addressed; E-Mail: jbarr@cdc.gov;
Tel.: +1-770-488-7848; Fax: +1-770-488-0509.



SAXITOXIN

Results of saxitoxin proficiency test including characterization of reference material and stability studies

A saxitoxin proficiency test (PT) was organized as part of the EQuATox project (Establishment of Quality Assurance for the Detection of Biological Toxins of Potential Bioterrorism Risk), under the 7th European Union Framework Programme for Research. The aim of this PT was to provide an evaluation of both existing methods and the European laboratories′ capabilities for analysis of saxitoxin and some of its analogues in real samples. Homogenized mussel material and algal dinoflagellate cell material containing paralytic shellfish poisoning (PSP) toxins were produced as reference sample matrices. The reference material was characterized using various analytical methods. Acidified algal extract samples at two concentration levels were prepared from a bulk culture of PSP producing species *Alexandrium ostenfeldii*. The homogeneity and stability of the prepared proficiency test samples were studied and found to be fit-for-purpose. Thereafter, eight saxitoxin PT samples of four sample types were sent to ten participating laboratories from eight countries. The PT offered the participating laboratories the possibility to assess their performance regarding the qualitative and quantitative detection of PSP toxins. Various analytical techniques such as official AOAC (Association of Official Analytical Chemists) methods, immunoassays, and liquid chromatography–mass spectrometry were used.

AUTHORS

Kirsi Harju[1,*], Marja-Leena Rapinoja[1], Marc-André Avondet[2], Werner Arnold[2], Martin Schär[2], Werner Luginbühl[3], Anke Kremp[4], Sanna Suikkanen[4], Harri Kankaanpää[4], Stephen Burrell[5], Martin Söderström[1] and Paula Vanninen[1]

1 VERIFIN (Finnish Institute for Verification of the Chemical Weapons Convention), Department of Chemistry, University of Helsinki, Helsinki, Finland;
2 Federal Department of Defence, Civil Protection and Sports, SPIEZ LABORATORY, Spiez, Switzerland;
3 ChemStat, Bern, Switzerland;
4 Finnish Environment Institute, Marine Research Centre, Helsinki, Finland;
5 Marine Institute, Marine Environment and Food Safety Services, Galway, Ireland;
* Author to whom correspondence should be addressed; E-Mail: kirsi.harju@helsinki.fi;
Tel.: +358-50-448-6561; Fax: +358-2-941-504-37.

LC-MS/MS method for the identification and quantification of saxitoxin and some selected PSP analogues in mussel samples

A new method was developed for analysis of saxitoxin and some selected paralytic shellfish poisoning (PSP) analogues in mussel sample with liquid chromatography–tandem mass spectrometry (LC–MS/MS). Sample extraction and purification methods of mussel matrix were optimized for LC-MS/MS analysis. The identification of PSP toxins was based on product ion spectra and retention times. Certified reference standards from the National Research Council, Canada were used to identify and quantify the PSP analogs present in the naturally contaminated mussel samples. The calibration samples were prepared by spiking certified reference standards in toxic mussel extracts. The developed method was applied in the analysis of the homogenized mussel samples in the proficiency test within the EQuATox project (Establishment of Quality Assurance for the Detection of Biological Toxins of Potential Bioterrorism Risk) under the 7th European Union Framework Programme for Research. The results obtained with the developed LC–MS/MS method were within the z-score of ±1 when compared to the results measured with official AOAC (Association of Official Analytical Chemists) method 2005.06, pre-column oxidation high-performance liquid chromatographic with fluorescence detection.

AUTHORS

Kirsi Harju[1,*], Marja-Leena Rapinoja[1], Marc-André Avondet[2], Werner Arnold[2], Martin Schär[2], Stephen Burrell[3], Werner Luginbühl[4] and Paula Vanninen[1]

1 VERIFIN (Finnish Institute for Verification of the Chemical Weapons Convention), Department of Chemistry, University of Helsinki, Helsinki, Finland;
2 Federal Department of Defence, Civil Protection and Sports , SPIEZ LABORATORY, Spiez, Switzerland;
3 Marine Institute, Marine Environment and Food Safety Services, Galway, Ireland;
4 ChemStat, Bern, Switzerland
* Author to whom correspondence should be addressed; E-Mail: kirsi.harju@helsinki.fi;
Tel.: +358-50-448-6561; Fax: +358-2-941-504-37.

STAPHYLOCOCCAL ENTEROTOXIN B

Organization of the first proficiency testing to evaluate the ability of European Union laboratories to detect staphylococcal enterotoxins type B (SEB) in buffer and milk

The aim of the EQuATox program is to evaluate the capability of European Union member states and associated countries to properly detect selected biological toxins which could be used as potential bioweapons. For this purpose, the European Union Reference Laboratory for coagulase positive staphylococci, as work package leader in this program, organized the first proficiency testing trial dedicated to staphylococcal enterotoxin B (SEB) detection in milk and buffer solutions. This paper describes the overall organization of the proficiency testing trial according to the EN ISO 17043 requirements. Characterization of the SEB stock solution was performed by various methods including amino acid analysis used to define assigned value. Two milk and two buffer samples at various levels of SEB were prepared. Moreover, in order to evaluate sensitivity and specificity of the methods used by participants one blank and one milk SEA spiked sample were also prepared. Finally, homogeneity and stability studies were carried out according to the EN ISO 13528 using both screening and quantitative ELISA based methods. Results demonstrated that the prepared samples were sufficiently homogeneous and stable to evaluate the methods used by participants.

AUTHORS

Mélanie Rodriguez[1,†], Yacine Nia[1,†], Sabine Herbin[1], Frédéric Auvray[1] and Jacques-Antoine Hennekinne[1,*]

1 French agency for Food, Environmental and Occupational Health Safety (Anses) – Food safety laboratory of Maisons-Alfort, Maisons-Alfort, France

† These authors contributed equally to this work.

* Author to whom correspondence should be addressed; E-Mail: jacques-antoine.hennekinne@anses.fr Tel.: +33 1 49 77 26 24; Fax: +33 1 49 77 26 50.

Characterization scheme using ELISA based methods to detect and quantify SEB in foods and in buffer solutions

The main objective of the EQuATox project was to obtain an overview of the methods used in the EU-28 to detect selected biological toxins in order to identify good analytical strategies and to highlight potential gaps in detection technology. For this purpose, the European Union Reference Laboratory for coagulase positive staphylococci, as work package leader in this program, organized the first proficiency testing trial dedicated to staphylococcal enterotoxin B (SEB) detection in food matrices (milk) and buffer solution. 18 laboratories took part and received six samples including four milk samples and two buffer solutions. These samples were spiked or not with various levels of SEB or with other SEs types in order to evaluate sensitivity and specificity of the methods used by participants. As presented in the previous paper, the samples prepared were proved to be homogeneous and stable. On the basis of the results obtained, it was possible to evaluate the performance of the methods currently in use by the participants: immunological and/or mass spectrometry-based methods. Among the immunological methods used, this paper focuses on the performance of ELISA-based tools in order to detect SEB and to propose a step-by-step characterization scheme using both qualitative and quantitative methods.

AUTHORS

Mélanie Rodriguez[1], Frédéric Auvray[1], Uwe Fiebig[2], Marc-André Avondet[3] and Jacques-Antoine Hennekinne[1*]

1 French agency for Food, Environmental and Occupational Health Safety (Anses) – Food safety laboratory of Maisons-Alfort, Maisons-Alfort, France

2 Biological Toxins (ZBS3), Center for Biological Threats and Special Pathogens, Robert Koch-Institut, Berlin, Germany

3 Federal Department of Defence, Civil Protection and Sport - SPIEZ LABORATORY, Spiez, Switzerland

* Author to whom correspondence should be addressed; E-Mail: jacques-antoine.hennekinne@anses.fr
Tel.: +33 1 49 77 26 24; Fax: +33 1 49 77 26 50.

Application of LC-MS/MS MRM for the discrimination and quantification of staphylococcal enterotoxins in milk

Staphylococcus aureus is the primary aetiological agent of food intoxications in Europe and can cause gastro-enteritis through production of various staphylococcal enterotoxins (SEs) in foods. Due to the stability, the ease of production and dissemination, some SEs have also been studied as potential agents for bioterrorism. The EQuATox project evaluates the readiness of the laboratories to detect natural toxins, like this one that can be used as potential bioweapon. Therefore, specific and accurate analytical tools for detection and quantification of SEs are required. Online liquid chromatography electrospray ionisation tandem mass spectrometry (LC-ESI-MS/MS) based on multiple reaction monitoring (MRM) was used to detect and quantify two types of SEs (A and B) spiked in milk and buffer solution. SE extraction and concentration was performed according to the EU-RL for CPS European Screening Method. Trypsin digests were screened for the presence of SEs using selected proteotypic heavy-labelled peptides as internal standards. SEA and SEB were successfully detected in milk samples using LC-MS/MS in MRM mode. The selected SE peptides were proteotypic for each toxin allowing the discrimination of SEA and SEB in a single run. The detection limit of SEA and SEB was approximately 8 and 4 ppb, respectively.

AUTHORS

Mirjana Andjelkovic[1,*], Varvara Tsilia[1], Andreja Rajkovic[2], Koen De Cremer[2], Sandra Cosijns[1] and Joris Van Loco[1]

1 Scientific Institute of Public Health (WIV-ISP), Food, Medicine and Consumer Safety, Brussels, Belgium

2 Ghent University (UGent), Laboratory of Food Microbiology and Food Preservation, Ghent, Belgium

* Author to whom correspondence should be addressed; E-Mail: Mirjana.andjelkovic@wiv-isp.be; Tel.: +32-2-642-5200; Fax: +32-2-642 5691.

ABSTRACTS BOTULINUM NEUROTOXINS

Generation and characterization of six recombinant botulinum neurotoxins as reference material for an international proficiency test organized by the EQuATox consortium

The detection and identification of botulinum neurotoxins (BoNT) is complex due to the existence of seven serotypes and currently 40 subtypes. No certified reference materials are presently available and no inter-laboratory exercises focusing on the detection of BoNTs spiked into real sample materials have previously been performed, making a comparison of analytical results obtained in different laboratories impossible. Here, six BoNT/A-F prototypes have been successfully produced by recombinant techniques, facilitating handling as well as improving purity, yield, reproducibility and biosafety. The materials were thoroughly characterized with respect to purity, identity, protein concentration, catalytic and biological activity. All six BoNT were quantitatively nicked into active di-chain toxins linked by a disulphide bridge. Their biological activities were quantified *ex vivo* by the mouse phrenic nerve hemidiaphragm assay. BoNT/A, B, E and F were unambiguously identified by means of immunological and mass spectrometric methods and the specific activities were assigned by LD50 mouse bioassay. For human pathogenic BoNT/A, B and E, the catalytic activity and the precise protein concentration were determined by endopep-MS and amino acid analysis, respectively. In conclusion, highly pure recombinant BoNT reference materials were produced, thoroughly characterized and employed in a worldwide BoNT proficiency test.

AUTHORS

Jasmin Weisemann[1], Nadja Krez[1], Uwe Fiebig[2], Sylvia Worbs[2], Martin Skiba[2], Martin B. Dorner[2], Tomas Bergström[3], Amalia Muñoz[4], Ingrid Zegers[4], Youssef Fikri[5], Christian Müller[6], Stephen Jenkinson[6], Marc-André Avondet[6], Laurence Delbrassinne[5], Reinhard Zeleny[4], Heinz Schimmel[4], Crister Astot[3], Brigitte G. Dorner[2] and Andreas Rummel[1*]

1 toxogen GmbH, Hannover, Germany
2 Biological Toxins, Centre for Biological Threats and Special Pathogens, Robert Koch-Institut, Berlin, Germany
3 Department of CBRN Defense and Security, Defense Research Agency (FOI), Umeå, Sweden
4 European Commission Joint Research Centre, Institute for Reference Materials and Measurements (EC-JRC-IRMM), Geel, Belgium
5 Scientific Institute of Public Health (WIV-ISP), Brussels, Belgium
6 Federal Department of Defence Civil Protection and Sport - SPIEZ LABORATORY (VBS-LS), Spiez, Switzerland
* Author to whom correspondence should be addressed; E-Mail: rummel@toxogen.de;
Tel.: +49-511-642132-84 ; Fax: +49-511-642132-77.

Qualitative and quantitative detection of BoNT from complex matrices: results of the first international proficiency test

In the framework of the EU project EQuATox a first international proficiency test (PT) on the detection and quantification of BoNT was conducted. Sample materials included BoNT serotypes A, B and E spiked into buffer, milk, meat extract and serum. A variety of methods was applied by the participants combining different principles of detection, identification and quantification. Based on qualitative assays, 95% of all results reported were correct. Successful strategies for BoNT detection were based on a combination of complementary immunological, MS-based and functional methods or on suitable functional *in vivo* / *in vitro* approaches (mouse bioassay, hemidiaphrama assay, Endopep-MS assay). Quantification of BoNT/A, BoNT/B and BoNT/E was performed by 48% of participating laboratories. It turned out that precise quantification of BoNT was difficult resulting in a substantial scatter of quantitative data. This was especially true for results obtained by the mouse bioassay which is currently seen as "gold standard" for BoNT detection. The results clearly demonstrate the urgent need of certified BoNT reference materials and the development of methods replacing animal testing. In this context, the BoNT PT provided the valuable information that both the Endopep-MS assay and the hemidiaphrama assay delivered quantitative results superior to the mouse bioassay.

AUTHORS

Sylvia Worbs[1], Uwe Fiebig[1], Reinhard Zeleny[2], Heinz Schimmel[2], Andreas Rummel[3], Werner Luginbühl[4] and Brigitte G. Dorner[1,*]

1 Robert Koch-Institut, Center for Biological Threats and Special Pathogens, Biological Toxins, Berlin, Germany
2 European Commission, Joint Research Centre, Institute for Reference Materials and Measurements, Geel, Belgium
3 Toxogen GmbH, Hannover, Germany
4 ChemStat, Bern, Switzerland
* Author to whom correspondence should be addressed; E-Mail: dornerb@rki.de;
Tel.: +49-30-18754-2500; Fax: +49-30-1810754-2501.

Recommended immunological strategies to screen for botulinum neurotoxin-containing samples

Botulinum neurotoxins (BoNTs) cause the life-threatening neurological illness botulism in humans and animals and are divided into seven serotypes (BoNT/A–G) of which serotypes A, B, E, and F cause the disease in humans. The BoNTs are classified as "category A" bioterrorism threat agents and are relevant in the context of the Biological Weapons Convention. An international proficiency test (PT) was conducted to evaluate detection, quantification and discrimination capabilities of 23 expert laboratories from the health, food and security areas. Here we describe immunological strategies which proved to be successful within this proficiency test (PT) for the detection and quantification of BoNT/A, B and E serotypes taking into account the restricted sample volume distributed (1 mL). In order to analyse the samples qualitatively and quantitatively, one strategy was based on sensitive immunoenzymatic assays, limiting the amount of sample used and on immunochromatographic assays for fast qualitative and quantitative analyses. In an alternative approach, a bead-based suspension array (Luminex technology) was used for screening purposes followed by conventional sandwich-ELISA for quantification. The results provide guidance for further steps in quality assurance and highlight problems to address in the future.

AUTHORS

Stéphanie Simon[1,*,†], Uwe Fiebig[2,†], Yvonne Liu[3], Rob Tierney[3], Julie Dano[1], Sylvia Worbs[2], Tanja Schreiber[2], Marie-Claire Nevers[1], Hervé Volland[1,†], Dorothea Sesardic[3] and Martin B. Dorner[2,†]

1 CEA Saclay, Institute of Biology and Technologies of Saclay, Laboratory for immunoanalytical researches, Gif sur Yvette, France

2 Biological Toxins, Centre for Biological Threats and Special Pathogens, Robert Koch-Institut, Berlin, Germany

3 Division of Bacteriology, National Institute for Biological Standards and Control, South Mimms, Potters Bar, United Kingdom

† These authors contributed equally to this work.

* Author to whom correspondence should be addressed; E-Mail: Stephanie.SIMON@cea.fr
Tel.: +33-169-087704: Fax: +33-169-085907

Recommended MS-based strategies to identify BoNT-containing samples

Botulinum neurotoxins (BoNTs) cause the disease called botulism, which can be lethal. BoNTs are proteins secreted by some species of clostridia and are known to cause paralysis by interfering with nerve impulse transmission. Although the human lethal dose of BoNT is not known, it is estimated to be between 0.1 µg to 70 µg, so it is important to enable detection of small amounts of this toxin. Our laboratory previously reported on the development of Endopep-MS, a mass-spectrometric based endopeptidase method to detect, differentiate, and quantify BoNT immunoaffinity purified from complex matrices. In this work, we describe the application of Endopep-MS to the analysis of thirteen blinded samples supplied as part of the EQuATox proficiency test. This method successfully identified the presence or absence of BoNT in all thirteen samples and was able to successfully differentiate the serotype of BoNT present in the samples, which included matrices such as buffer, milk, meat extract, and serum. Furthermore, the method yielded quantitative results which had z-scores in the range of -3 to +3 for quantification of BoNT/A containing samples. These results indicate that Endopep-MS is an excellent technique for detection, differentiation, and quantification of BoNT in complex matrices.

AUTHORS

Suzanne R. Kalb[1] and John R. Barr[1,*]

1 Centers for Disease Control and Prevention, Atlanta, USA

* Author to whom correspondence should be addressed; E-Mail: jbarr@cdc.gov
Tel.: +1-770-488-7848; Fax: +1-770-488-0509

Botulinum neurotoxins: qualitative and quantitative analysis using the mouse phrenic nerve hemidiaphragm assay (MPN)

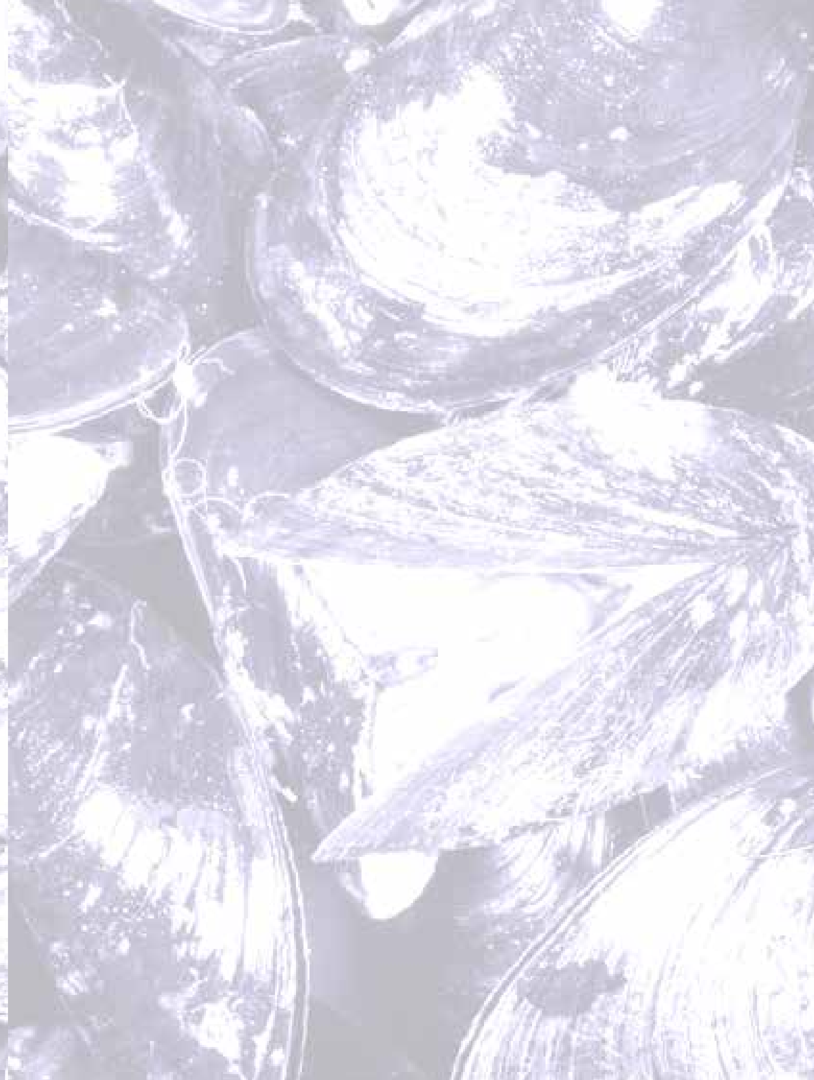
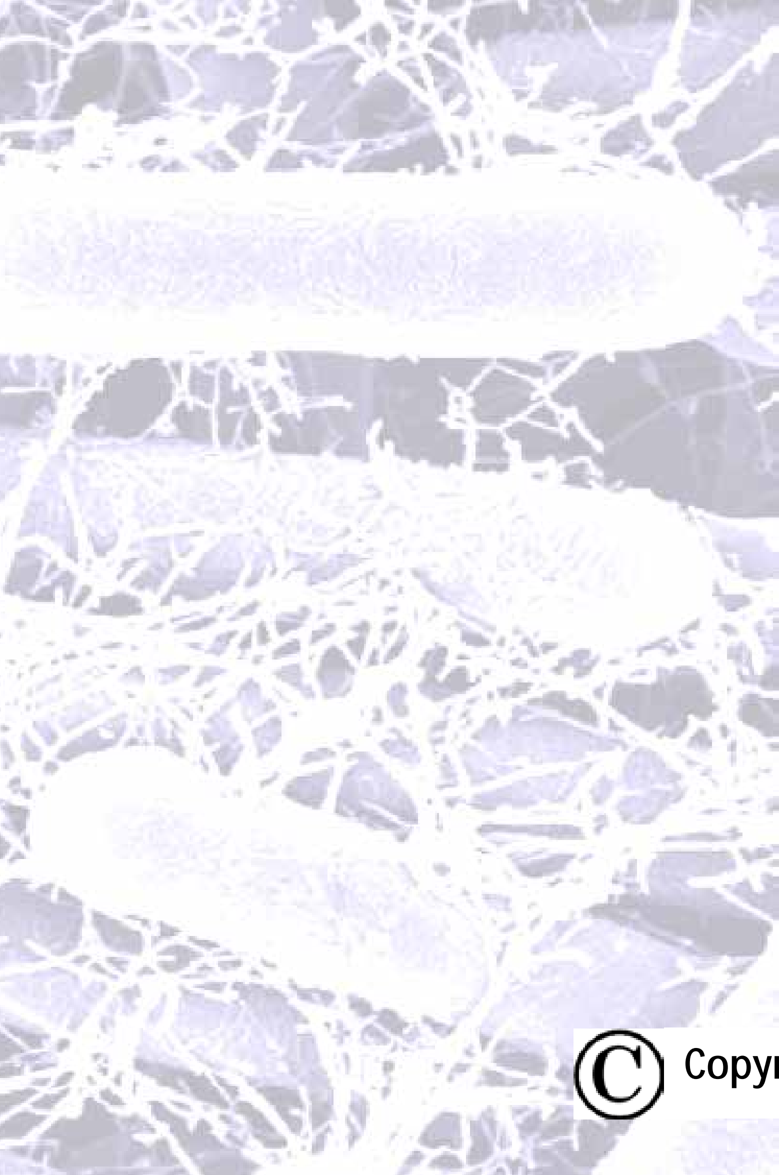
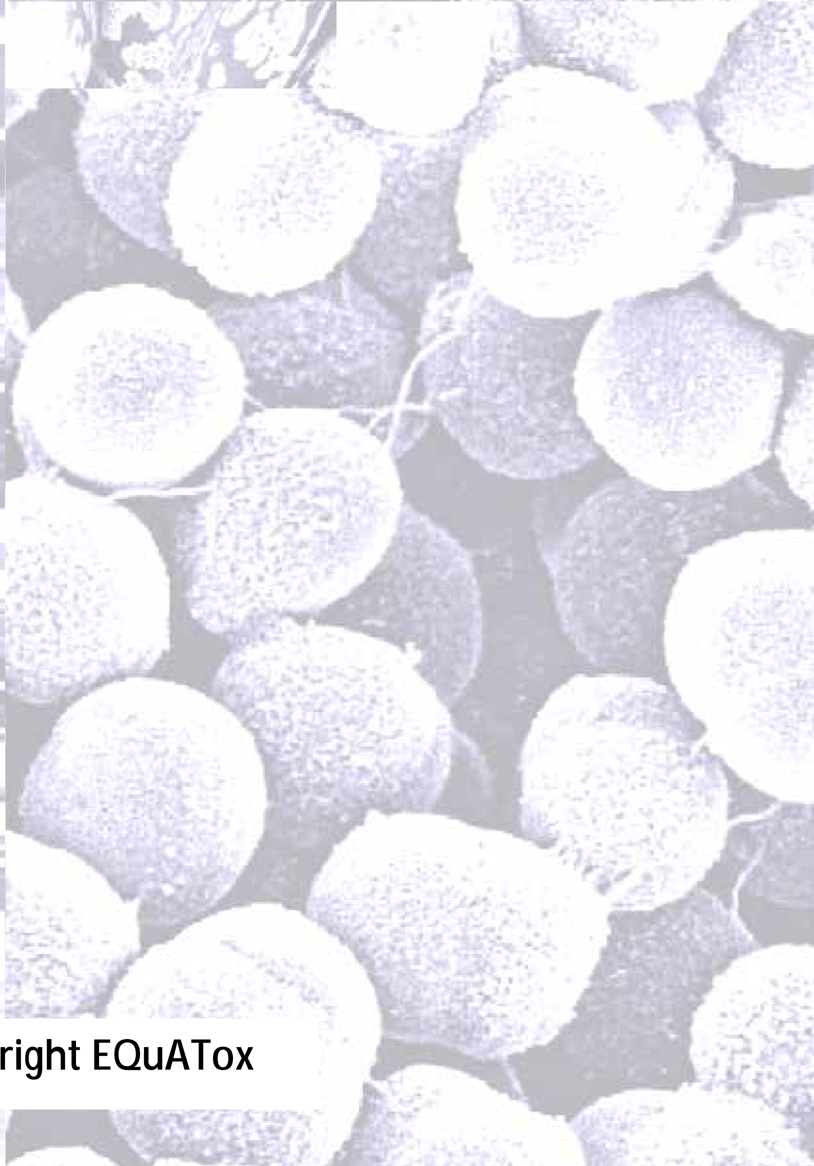
The easiest method for detection of botulinum neurotoxin (BoNT) is represented by the mouse bioassay that determines the survival rate of the animals. Since the endpoint of the test is the paralysis of the respiratory muscle, a truncated version of the test employs the isolated *N. phrenicus*-hemidiaphragm tissue, called MPN. BoNT causes a dose dependent decrease of the contraction amplitude of the indirectly stimulated muscle named Time to Paralysis (TTP). All delivered 13 BoNT PT samples were serially diluted to a bath concentration resulting in a TTP within the range of calibration curves constructed with BoNT/A, B and E standards. For serotype characterization the diluted samples were pre-incubated with polyclonal anti-BoNT/A, B or E antitoxin or a combination of each. All 13 samples were qualitatively correctly identified thereby delivering superior result compared to single in vitro methods. Having characterized the BoNT serotype, the final bath concentrations were calculated using the calibration curves and then multiplied by the respective dilution factor to achieve the sample concentration. Depending on the source of the BoNT standards used the quantitation of ten BoNT/A containing samples delivered a mean z-score of 7 and of three BoNT/B or BoNT/E containing samples z-scores <2.

AUTHORS

Hans Bigalke[1,*] and Andreas Rummel[1]

1 Toxogen GmbH, Hannover, Germany

* Author to whom correspondence should be addressed; E-Mail: bigalke@toxogen.de
Tel.: +49 (0) 511 642132 (ext. 83); Fax: (ext. 77).

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