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Evaluation of lateral flow assays for the detection of botulinum neurotoxin type A and their application in laboratory diagnosis of botulism

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Abstract

Four lateral flow assays (LFAs) were evaluated for the detection of purified botulinum neurotoxin A, toxin complex, and unpurified culture supernatant. They included the BioThreat (Tetracore, Rockville, MD), SMART (New Horizons Diagnostics, Columbia, MD), BADD (ADVNT Biotechnologies, Phoenix, AZ), and RAMP (Response Biomedical, Burnaby, BC, Canada) assays. BioThreat and SMART did not detect the purified toxin. The best sensitivity was achieved with the RAMP test (50 ng mL^{-1}). BioThreat and SMART measured as low as 10 ng mL^{-1} of the toxin complex. Specificity data differed among the tests. BADD gave false-positive signals with uninoculated bacterial culture medium. BioThreat and RAMP were further evaluated with clinical sample matrices (serum, gastric, and rectum contents from pigs). Because of matrix effects and a generally low positive response, the assays are unsuitable for the direct detection of the toxin. However, the LFAs can be a helpful tool in screening bacterial cultures for toxigenic *Clostridium botulinum*, if further validated according to the laboratory needs.

Keywords: *Clostridium botulinum* neurotoxin; Immunoassay; Lateral flow test; Clinical samples; Botulism

1. Introduction

Lateral flow assays (LFAs) have gained a growing interest for the detection of a variety of analytes including pathogens and toxins (e.g., Ayong et al., 2005; Delmulle et al., 2005; Mettler et al., 2005). The individual assay layouts are diverse. Some basic features, however, are common to most of the systems, which are commercially available. In general, a ligand (e.g., antibodies) is immobilized on a porous nitrocellulose membrane as a test zone. Antibodies labeled with colloidal gold, dyed latex particles, carbon black, or fluorescing markers are often used as reporters (Lönnberg and Carlsson, 2001; Klewitz et al., 2006). They can either be separately mixed with the sample or are directly bound to the conjugate pad, which is attached to the membrane. In addition, the conjugate pad contains buffer chemicals, blocking reagents, and stabilizers. A sample pad on top of the conjugate pad to prefilter the sample and an absorbent pad to take up the liquid on the opposite side of the membrane complete the assay setup. Once the sample is added, the fluid migrates by capillary action toward the absorbent pad. The analyte/reporter complex binds to the test zone, and excess reagents and fluid are transported to the absorbent pad. In most assays the fluid passes a control zone, where suitable ligands capture excess reporter molecules ensuring that the sample fluid migrated through the length of the test. A typical LFA design is given in Fig. 1. The assay is read out either with the naked eye or with the help of a reader after a defined time.

The underlying principles of the LFA technology are substantially similar to other immunoassays, for example, enzyme-linked immunosorbent assay (ELISA). Major drawbacks compared to the ELISA are the sensitivity, which rarely exceeds the nanogram range, and the limitation to qualitative results in most setups. However, LFAs offer several advantages. They are quick, optimized for point-of-care or on-site testing, and probably most important of all, they can be done by minimally trained personnel. The latter advantages recommend the LFA to test on-site for the deliberate release of bioterrorist agents.

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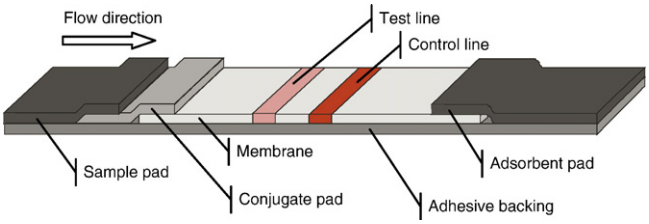

Fig. 1. Schematic view of a typical LFA. A porous membrane is mounted on an adhesive backing. A specific ligand (e.g., antibodies) is immobilized on the test line. On the control line, capture ligands for the reporter molecules are bound. Sample and conjugate pad enable the equal distribution of the liquid sample and serve as a reservoir for the assay reagents. The adsorbent pad takes up excess liquid and ensures sufficient flow-through.

The most poisonous, botulinum neurotoxin (BoNT), poses a major bioweapon threat (Wein and Liu, 2005). *Clostridium botulinum*, an anaerobic spore-former, produces these zinc proteinases (Montecucco and Schiavo, 1993), which are the causative agents of botulism. They inhibit the acetylcholine release at the neuromuscular junction in man and animal leading to an often fatal flaccid muscular paralysis (Böhnel and Gessler, 2005). Currently, 7 distinct neurotoxins are known (A–G), which can be distinguished serologically. However, a recent study revealed that even within the same serotype, differences among the toxins can amount to 31.6% (Smith et al., 2005). BoNT is a 150-kDa di-chain molecule with a 100-kDa heavy chain and a 50-kDa light chain, linked through a disulfide bond. Naturally pH-dependent complexes are formed, which consist of the neurotoxin, nontoxic nonhemagglutinin, and hemagglutinin protein(s). In type A medium (M, ~300 kDa), large (L, ~450 kDa) and large–large (L-L, ~900 kDa) complexes can be found (Johnson, 1999). The nontoxic components in the complex protect the neurotoxin during the gastrointestinal passage and are likely to be involved in the absorption of the toxin into the body (Fujinaga et al., 1997). For *C. botulinum* neurotoxin type A (BoNT/A), a LD_{50} of 1 ng kg^{-1} body mass was reported (Gill, 1982).

The mouse bioassay is still the method of choice to detect and quantify the biologically active BoNTs in clinical, food, and environmental samples. This method, however, takes 3–4 days, is expensive, and does not comply with our modern understanding of animal welfare.

Various immunoassays including LFAs for the detection of BoNT were developed as alternative in vitro methods. Some of the LFAs have become commercially available, but for environmental samples only. They are mainly intended to be used for the detection of BoNT in biodefense and biosecurity.

In this study we evaluated 4 commercially available detection kits. The assays were tested with respect to sensitivity and specificity using purified BoNT, BoNT complex, and BoNT-positive culture supernatants. Based on these initial data, 2 systems were selected for further evaluation with spiked clinical sample matrices.

2. Materials and methods

All reagents and chemicals were purchased from Merck, Darmstadt, Germany, if nothing else is stated.

2.1. Lateral flow assays and reader hardware

The LFAs used in this study were immunoassay test kits supplied by New Horizons Diagnostics (Columbia, MD), ADVNT Biotechnologies (Phoenix, AZ), Tetracore (Rockville, MD), and Response Biomedical (Burnaby, BC, Canada). All test kits are intended for use in the field for performing rapid detection of BoNT from environmental samples.

Both Tetracore and Response Biomedical offer a reader for the measurement of their test strips. The Guardian reader for Tetracore LFAs reports a quantitative result on the display, whereas the reader from Response Biomedical enables qualitative readouts only. To compare the test strips from Tetracore, New Horizons, and ADVNT, each test strip was read out additionally with Matest reader 200 (Matest Systemtechnik, Mössingen, Germany). This reader consists

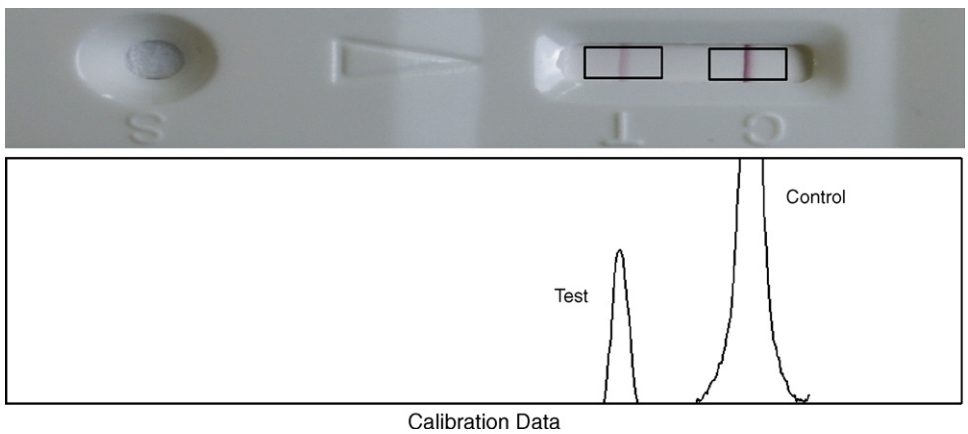

Fig. 2. Matest readout. A digital camera in the Matest reader 200 captures an image of the test strip. The intensities of the signals of the test and control lines are calculated and displayed in a graph on the screen using the Matest software, which allows a quantitative analysis of the peak areas of the test and control lines.

of a digital camera, which takes an image of the test strip (Fig. 2). The peak area of the test line was divided by the peak area of the control allowing a quantitative readout.

2.2. Mouse bioassay

The mouse bioassay followed the AOAC (1979) and German DIN (1992) guidelines in general. NMRI mice (Harlan Winkelmann, Borchen, Germany) weighing 18–25 g were used. The sample was diluted 10-fold in gelatine phosphate buffer (GPB: 50 mmol L^{-1} phosphate-buffered saline, 0.2 % gelatine, pH 6.2) to keep the number of animals at a minimum. Of each dilution, 0.5 mL was injected intraperitoneally into 2 mice. The animals were observed and clinical signs or death reported over a period of 4 days.

The toxicity is given in minimal lethal doses for mice per milliliter (MLD mL^{-1}), which corresponds to the reciprocal value of the highest dilution at which both mice developed typical clinical signs and died.

2.3. C. botulinum neurotoxins

The purified BoNT/A as well as the neurotoxin complex were purchased from Metabiologics (Madison, WI). The protein concentrations were determined using the BCA protein assay kit (Pierce Biotechnology, Rockville, IL) according to the manufacturer's instructions.

2.4. Culture supernatants

C. botulinum type A (6 strains) as well as types B–G (1 strain of each) were grown anaerobically in RCM (Oxoid, Wesel, Germany) for 4 days at 37 °C. Bacterial cultures were centrifuged at 4000 × *g* for 5 min. Subsequently, the supernatants were filtered with sterile 0.2-μm filters (Sartorius, Göttingen, Germany). Aliquots of the supernatants were stored at −80 °C until use. The toxicities of the culture supernatants were determined in the mouse bioassay. More information on the cultures used in this study is given in Table 1.

Table 1
Origin of *C. botulinum* strains

Type	Nomenclature (used in this study)	MLD mL^{-1}	Physiological group	Source	Original strain identification
A	1028	10^2	I	NCTC	7272
A	2267	10^5	I	CECT	551
A	2277	10^5	I	CCUG	7968
A	2292	10^5	I	NZRCC	4997
A	2295	10^3	I	NZRCC	35KA29
A	2298	10^6	I	OPU	62A
B	2269	10^5	I	CECT	4610
C	2300	10^4	III	OPU	003-9
D	2142	10^4	III	IP	1873D
E	2271	10^4	II	CECT	4611
F	1033	10^3	I	NCTC	10281
G	1073	10^4	IV	ATCC	27322

NCTC = National Collection of Type Cultures, London, UK; CECT = Colleccción Española de Cultivos Tipo, Valencia, Spain; CCUG = Culture Collection, University of Göteborg, Sweden; NZRCC = New Zealand Reference Culture Collection, Porirua, New Zealand; OPU = Department of Veterinary Science, Osaka Prefecture University, Osaka, Japan; IP = Institute Pasteur, Paris, France; ATCC = American Type Culture Collection, Rockville, MD.

2.5. Sample preparation for sensitivity and specificity testings

The purified BoNT as well as the BoNT complex and the culture supernatants were always diluted in sample buffer supplied with each test kit. The concentrations of the purified BoNT and the BoNT complex ranged from 0 to 1000 ng mL^{-1}. The toxicity of the diluted culture supernatants varied from 1 to 5 × 10^5 MLD mL^{-1}.

2.6. Spiked clinical samples

Serum samples as well as samples from gastric and rectum contents were taken from 5 pigs at the local abattoir as a surrogate for human samples. They were stored at −80 °C.

Four grams of gastric and rectum contents (4 mL serum) were spiked with 1 mL BoNT/A culture supernatant (2298, 10^6 MLD mL^{-1}). Nonspiked samples were prepared by mixing 4 g of gastric or rectum contents with 10 mL GPB (4 mL serum with 1 mL GPB). Incubation reference controls (RCs) contained 1 mL BoNT/A culture supernatant diluted with 14 mL GPB (for serum RCs 4 mL GPB) only. Samples and controls were incubated at room temperature (23 °C) for 1 h. After incubation, samples from gastric and rectum contents were mixed with 10 mL GPB and incubated again on a horizontal shaker (100 min^{-1}) at 4 °C overnight. This should guarantee a complete sorption of the toxin to the sample matrix. To remove solid particles, these suspensions were centrifuged at 1800 × *g* for 10 min. The supernatant was removed and aliquots were centrifuged at 16,400 × *g* for 10 min again. The supernatants were filtered with sterile 0.45-μm followed by 0.2-μm filters (Sartorius).

Shortly before starting each measurement, positive controls (PCs) were prepared according to the protocol for the RCs, but without further incubation.

Nonspiked GPB served as negative control.

2.7. Test procedures

All test procedures followed each manufacturer's instructions. Measurements were done in triplicate. Cutoffs were determined for each test system according to the following formula: cutoff = mean value of negative controls + 3 × SD.

With the Tetracore and Response Biomedical readers the cutoff is given by the system.

2.7.1. RAMP®, Response Biomedical (RAMP)

Response Biomedical markets the RAMP Bot Tox Test system, which is an immunochromatographic test for the detection of BoNT/A only. In contrast to the 3 other systems, which are described subsequently, the antibodies of the RAMP system are conjugated with fluorescent-dyed latex particles instead of gold nanoparticles. The direct readout of the signals is not possible. Response Biomedical offers a portable fluorescence-based reader, which measures fluorescence emitted by the complexes bound in the

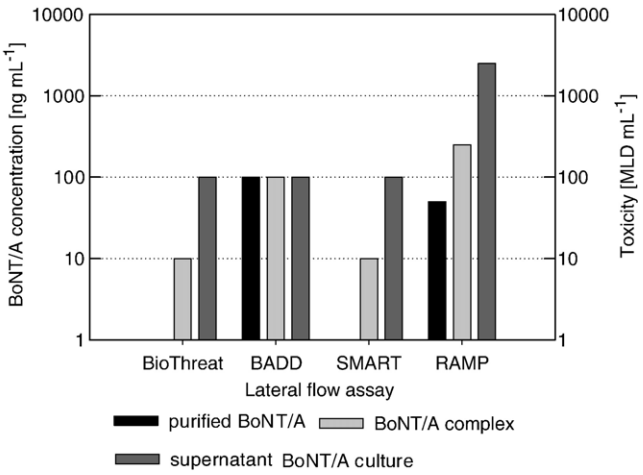

Fig. 3. Detection limit of each test system for purified BoNT/A and BoNT/A complex (ng mL^{-1}), as well as for BoNT/A culture supernatant (MLD mL^{-1}).

detection zone (test line) and internal control zone (control line), and then calculates a ratio between these 2 signals. The kit pouches contain a test cartridge as well as a single assay tip, which is coated with fluorescent-dyed latex particles. Ten microliters of the liquid sample were diluted with 150 μL sample buffer. Following the instructions, which were given on the display of the reader, 70 μL of the sample were fully dispensed into the sample well of the test cartridge. The test cartridge was inserted immediately into the reader, which began timing the test development process after recording the bar code of the test cartridge. Within approximately 15 min, the test was complete. The reader scanned the test cartridge, performed data analysis, and reported the qualitative result on the display.

2.7.2. BioThreat Alert™ Test Strip, Tetracore (BioThreat)

The BioThreat Alert test strip is a hand-held test kit for the detection of BoNT/A and BoNT/B. Samples were diluted in sample buffer. One hundred fifty microliters of each sample dilution were dispensed into the round sample port of the test cartridge. After an incubation of 15 min, the colored lines appeared and the results were read out with the Guardian reader provided from Tetracore. In additional testings, strips were read out with the Matest 200.

2.7.3. BADD™ Test Strip, ADVNT Biotechnologies (BADD)

The BADD botulinum detection device is supplied for the detection of BoNT/A. After the samples had been diluted in the provided buffer, 125 μL of the diluted samples were dispensed into the sample well of the cassette. To get quantitative results, test strips were measured with the Matest 200 after 15 min.

2.7.4. SMART™-II, New Horizons (SMART)

New Horizons designed an LFA to detect BoNT/A from environmental samples. The collection kits include the lateral flow test device, plastic droppers as well as buffers. The samples were diluted in buffer, and 100 μL of liquid sample were dispensed into the sample well of the lateral flow device. After 3 min, 2 falling drops of buffer from the dropper bottle were added. The results were read out after 15 min by observing the color of the control and test lines. In addition, test strips were measured with Matest 200.

3. Results

3.1. Sensitivity

In the first experiments, the detection limits and cross-reactions of the different test systems were obtained. Detection limits of all test strips were determined for the purified BoNT/A, their complexes, as well as BoNT/A culture supernatants.

Fig. 3 shows the detection limits for the different test systems. Purified BoNT type A could be detected with BADD and RAMP only. The best detection limit was found to be 50 ng mL^{-1} of purified BoNT/A for the RAMP assay. In contrast, purified BoNT/A was not detected neither with SMART nor with BioThreat. However, both tests gave positive results with 10 ng mL^{-1} BoNT/A complex having the best sensitivities compared to BADD (100 ng mL^{-1}) and RAMP (250 ng mL^{-1}).

To assess the detection limit for BoNT/A in culture supernatants, tests were done with supernatant filtrates ranging from 1 to 5×10^5 MLD mL^{-1}. The detection limit for BioThreat, SMART, and BADD was found to be 100 MLD mL^{-1} for BoNT/A in culture supernatants, whereas RAMP detected 2500 MLD mL^{-1} only.

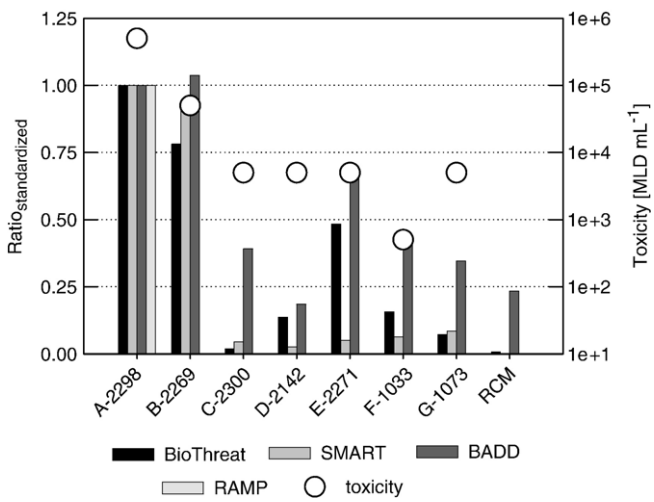

Fig. 4. Results of the specificity tests for all assays. All LFAs used in this study are marketed for the detection of BoNT/A. Results of signal intensities were measured with Matest reader 200 and are given as ratios (ratio = peak $area_{test\ line}$/peak $area_{control\ line}$). For the comparison of cross-reactions of the test systems, ratios of BoNT types B–G as well as RCM were standardized using following formula: $ratio_{standardized} = ratio_{measured}/ratio_{BoNT/A}$, where $ratio_{measured}$ is the ratio of the BoNT types B–G and RCM measurements, and $ratio_{BoNT/A}$ is the ratio measured with BoNT/A (reference). The toxicity data refer to the results of the mouse bioassay and are given as minimal lethal doses for mice (MLD mL^{-1}).

3.2. Specificity

The cross-reactions for each test system are illustrated in Fig. 4. In general, red-colored test and control lines of BioThreat were weaker than those of SMART and BADD.

According to the manufacturer, BioThreat Alert test strip allows for the detection of BoNT types A and B. It was slightly less sensitive for type B, followed by type E, F, D, and G in descending order. Type C was below the assay cutoff.

SMART was almost equally sensitive for types A and B, all other types were close to the cutoff.

BADD detected type B with the same sensitivity as type A. Positive results were also obtained with type E, F, C, G, and D (with decreased signal intensities). RCM gave a false-positive result.

The RAMP assay detected type A only.

With all assays the readouts did not correlate with the toxicities of the culture supernatants.

3.3. Spiked clinical samples

RAMP and BioThreat were used for the evaluation with spiked clinical sample matrices: RAMP was able to detect the purified BoNT as well as the toxin complex and showed no matrix effects with RCM. BioThreat had the best sensitivity for the toxin complex. Both systems delivered quantitative data, because Response Biomedical provided new lot cards allowing for quantitative readouts.

The results with spiked clinical sample matrices and the controls are summarized in Fig. 5. No false-positive signals were obtained for the nonspiked samples from the stomach and the rectum with RAMP. Regarding the spiked rectum samples, BoNT was not detected in 4 samples. In gastric contents, BoNT/A was measured in 2 samples (P-2, P-3). However, false-negative signals were measured for the RC, whereas the PC prepared shortly before measurement gave a

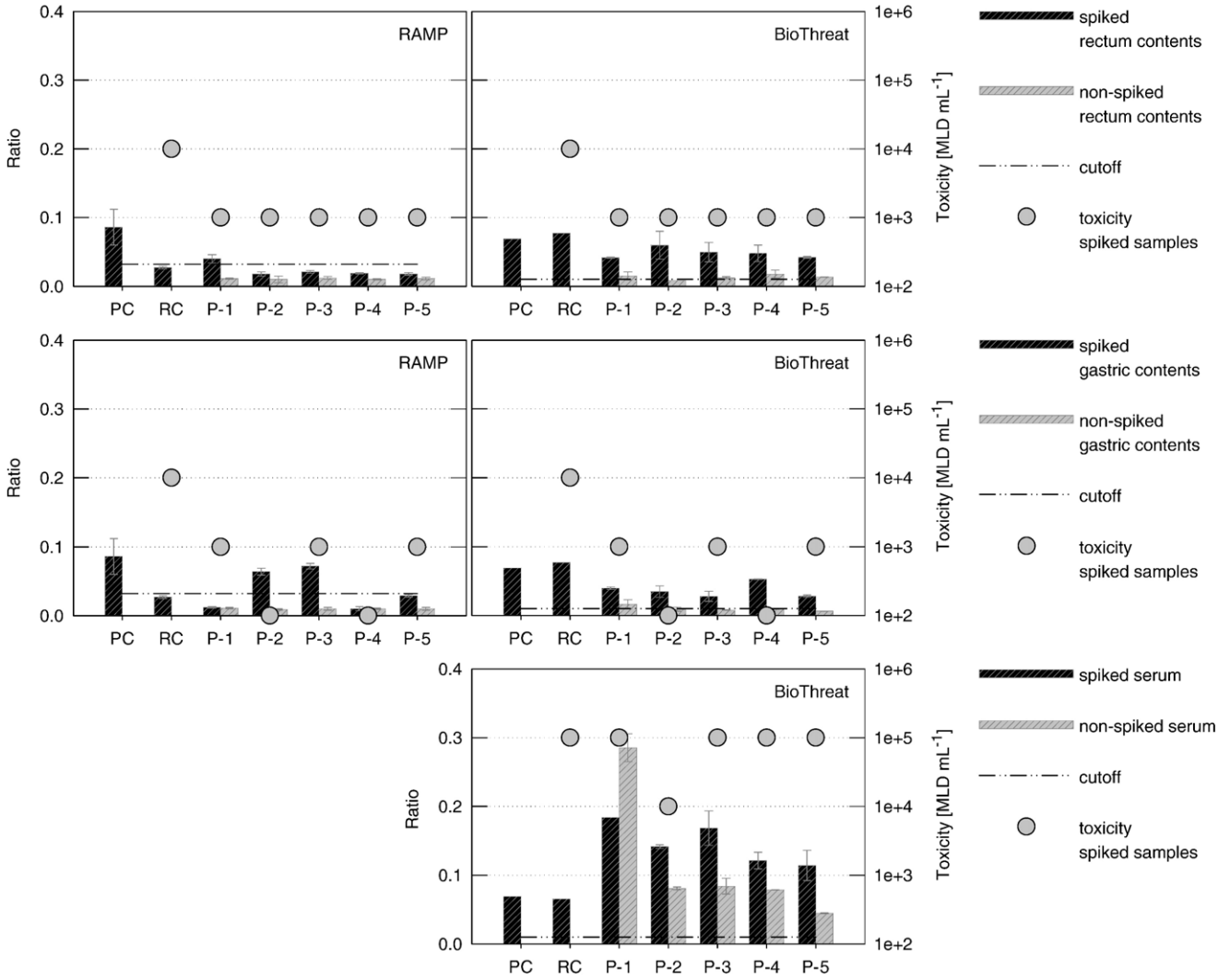

Fig. 5. Detection of BoNT/A in spiked and nonspiked porcine samples of serum, gastric, and rectum contents using RAMP, BioThreat, and the mouse bioassay. The ratios measured for the PC, the incubation RC, and for the spiked and nonspiked gastric and rectum contents as well as for spiked and nonspiked serum samples of 5 pigs (P-1, P-2, P-3, P-4, P-5) are given for RAMP and BioThreat. Results of the mouse bioassays are displayed as toxicity in minimal lethal doses for mice (MLD mL^{-1}).

positive signal. Therefore, spiked serum samples were investigated with BioThreat only.

BioThreat gave false-positive signals for the nonspiked gastric and rectum contents in a few samples. BoNT/A could be measured in spiked gastric contents as well as in spiked rectum contents. Regarding the serum samples, false-positive results were obtained for all nonspiked serum samples. The nonspiked control even exceeded the ratio for the corresponding spiked sample in one measurement.

In the mouse bioassay the toxicity of the RC was higher than the toxicity of spiked gastric and rectum contents. With the spiked serum samples, however, the toxicity was identical with the toxicity of the RC in 4 of the samples. The toxicities of the spiked samples did not correlate with the ratios of the LFA results.

4. Discussion

The mouse bioassay is still considered the gold standard for the detection of BoNTs rather on historical than on scientific grounds. Modern requirements of thorough assay validation have never been fulfilled with this assay. Therefore, the urgent need for alternative testing methods is not only generated by ethical, but by scientific reasons as well. During the last decades a considerable progress toward refining and/or replacing the mouse bioassay was made. These new approaches were recently summarized by Lindström and Korkeala (2006). Tests measuring the biological activity such as the paralysis assay or the hemidiaphragm assay refined the animal test. In vitro activity methods have been developed, which quantify the biological activity of the light chain of the toxin molecule (Ekong et al., 1997; Boyer et al., 2005). Dong et al. (2004) developed a method that detects the toxin activity in real time in living cells.

Most newly developed methods are immunoassays. They include ELISAs with signal amplification systems (Doellgast et al., 1993; Ferreira et al., 2003), Immuno-PCR (Chao et al., 2004), immunoaffinity column assay (Gessler et al., 2005), and lateral flow devices (Ahn-Yoon et al. 2004; Chiao and Shyu, 2004; Klewitz et al., 2006). LFAs are superior, when rapid results are needed, especially when no sophisticated laboratory infrastructure is available. Therefore, several companies have developed this technology to detect biothreat agents on site. The assays have become available for toxins, bacteria, and viruses.

In this study we evaluated 4 commercially available systems for BoNT detection initially with purified BoNT and BoNT complex. Two LFAs (BioThreat and SMART) were unable to detect the purified BoNT even with the highest concentration tested (1000 ng mL^{-1}). Since both systems detected the BoNT complex, antibodies in the tests are likely to be directed toward nontoxic nonhemagglutinin or hemagglutinin proteins of the BoNT complex. As to whether this has to be considered as a major drawback of the assays for the detection of BoNT as a biothreat agent depends on bioterrorism risk analyses: false-negative results would be obtained only if purified BoNTs were deliberately released and not the complex. However, this feature of the assay makes it susceptible for cross-reactivity with non-botulinum metabolites because the nonneurotoxic components of the BoNT complex bear many similarities with other proteins (Mancheño et al., 2005). The detection limit for purified BoNT/A achieved with BADD (100 ng mL^{-1}) and RAMP (50 ng mL^{-1}) corresponded to the data supplied by the manufacturers.

BoNT complex was detected by all assays. RAMP showed a considerable shift in sensitivity compared to the purified BoNT/A. The 250 ng mL^{-1} BoNT/A complex was found to be the detection limit. An explanation for the decrease in sensitivity may be the structure of the toxin complex. The molecular mass of the complex is about 3 times as much as the mass of BoNT itself. If the antibodies in the assay are specific to sites of the heavy or light chain of the toxin, the number of epitopes they can bind to does not increase in the toxin complex.

All assays were tested with culture supernatants of BoNT/A to G for 2 reasons. First, specificity data for the LFAs should be generated. Second, it should be assessed, if the assays can be used to detect BoNT in culture supernatants. Laboratory diagnostics in environmental as well as in samples from botulism cases focus on the detection of BoNT as a direct method, but also on the presence of toxigenic *C. botulinum* in the sample as an indirect approach. One of the common methods to check for *C. botulinum* is based on culturing the sample in liquid media and subsequent detection of BoNT in the culture supernatant by the mouse bioassay (Kautter and Solomon, 1977; Lindström and Korkeala, 2006).

The 4 assays gave positive results with the supernatants of the toxin types they are marketed for. The positive testing of the heterologous culture supernatants varied with the assay and the toxin type. In general, cross-reactions were seen in all assays with all toxin types except for RAMP, which was specific for BoNT/A. The cross-reactions could be explained by structural similarities of the toxins (Singh and DasGupta, 1989; Smith et al., 2005) and have been previously reported for LFAs (Sharma et al., 2005). Standard bacteriological medium components interfered with the BADD assay only: a false-positive signal was recorded for RCM. This renders the assay unsuitable for the testing of culture material. The remaining tests have the potential to be applied as a screening tool for putative *C. botulinum* cultures if carefully validated for the tests they are used for. SMART can be used for type A or B, and BioThreat for type A, B, or E. RAMP is limited to the detection of highly toxigenic type A cultures due to its lower sensitivity for the complex.

Serum, gastric contents, and feces are usually available for the laboratory diagnosis of botulism (Lindström and Korkeala, 2006). Spiked gastric and rectum contents were used as surrogates. The signal-to-noise ratios as well as the absolute signal intensities were low, although high toxin

concentrations were added to the samples. False-positive signals were obtained with the nonspiked matrix in the Bio-Threat assay. Therefore, both tested systems are unsuitable for a preliminary screening of conventional sample matrices.

To conclude, 4 LFA systems were tested for the detection of BoNT. Two of the assays did not detect the purified toxin. All assays measured the purified BoNT/A complex and the BoNT/A complex in the culture supernatant. The systems differed in sensitivity and specificity. LFAs are quick and easy to perform. They can be used for an initial screening in the biodefense-related field. With clinical samples the assays are limited to the testing of bacterial cultures, where they can speed up the isolation of *C. botulinum* and contribute to a confirmatory diagnosis.

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References

Ahn-Yoon S, DeCory TR, Durst RA (2004) Ganglioside–liposome immunoassay for the detection of botulinum toxin. *Anal Bioanal Chem* 378:68–75.

AOAC Official Method 977.26 (1979) *Clostridium botulinum* and its toxins in foods.

Ayong LS, Tume CB, Wembe FE, Simo G, Asonganyi T, Lando G, Ngu JL (2005) Development and evaluation of an antigen detection dipstick assay for the diagnosis of human onchocerciasis. *Trop Med Int Health* 10:228–233.

Böhnel H, Gessler F (2005) Botulinum toxins—cause of botulism and systemic diseases? *Vet Res Comm* 29:313–345.

Boyer AE, Moura H, Woolfitt AR, Kalb SR, McWilliams LG, Paviopoulos A, Schmidt JG, Ashley DL, Barr JR (2005) From the mouse to the mass spectrometer: detection and differentiation of the endoproteinase activities of botulinum neurotoxins A–G by mass spectrometry. *Anal Chem* 77:3916–3924.

Chao HY, Wang YC, Tang SS, Liu HW (2004) A highly sensitive immuno-polymerase chain reaction assay for *Clostridium botulinum* neurotoxin type A. *Toxicon* 43:27–34.

Chiao DJ, Shyu RH (2004) Colloidal gold-based immunochromatographic assay for detection of botulinum neurotoxin type B. *J Chromatogr B* 809:37–41.

Delmulle BS, De Saeger SMDG, Sibanda L, Barna-Vetro I, Van Peteghem CH (2005) Development of an immunoassay-based lateral flow dipstick for the rapid detection of Aflatoxin B1 in pig feed. *J Agric Food Chem* 53:3364–3368.

DIN 10102 (1992) Mikrobiologische Untersuchung von Fleisch und Fleischerzeugnissen. Nachweis von *Clostridium botulinum* und Botulinum-Toxin. Berlin: Beuth.

Doellgast GJ, Triscott MX, Beard GA, Bottoms JD, Cheng T, Roh BH, Roman MG, Hall PA, Brown JE (1993) Sensitive enzyme-linked immunosorbent assay for detection of *Clostridium botulinum* neurotoxins A, B, and E using signal amplification via enzyme-linked coagulation assay. *J Clin Microbiol* 31:2402–2409.

Dong M, Tepp WH, Johnson EA, Chapman ER (2004) Using fluorescent sensors to detect botulinum neurotoxin activity in vitro and in living cells. *PNAS* 101:14701–14706.

Ekong TAN, Feavers IM, Sesardic D (1997) Recombinant SNAP-25 is an effective substrate for *Clostridium botulinum* type A toxin endopeptidase activity in vitro. *Microbiology* 143:3337–3347.

Ferreira JL, Maslanka S, Johnson E, Goodnough M (2003) Detection of botulinal neurotoxins A, B, E, and F by amplified enzyme-linked immunosorbent assay: collaborative study. *J AOAC Int* 86:314–331.

Fujinaga Y, Inoue K, Watanabe S, Yokota K, Hirai Y, Nagamachi E, Oguma K (1997) The haemagglutinin of *Clostridium botulinum* type C progenitor toxin plays an essential role in binding of toxin to the epithelial cells of guinea pig small intestine, leading to the efficient absorption of the toxin. *Microbiology* 143:3841–3847.

Gessler F, Hampe K, Böhnel H (2005) Sensitive detection of botulinum neurotoxins type C and D with an immunochromatographic column test. *Appl Environ Microbiol* 71:7897–7903.

Gill DM (1982) Bacterial toxins: a table of lethal amounts. *Microbiol Rev* 46:86–96.

Johnson EA (1999) Clostridial toxins as therapeutic agents: benefits of nature's most toxic proteins. *Annu Rev Microbiol* 53:551–575.

Kautter DA, Solomon HM (1977) Collaborative study of a method for the detection of *Clostridium botulinum* and its toxins on foods. *J AOAC Int* 60:541–545.

Klewitz T, Gessler F, Beer H, Pflanz K, Scheper T (2006) Immunochromatographic assay for determination of botulinum neurotoxin type D. *Sensor Actuat B* 113:582–589.

Lindström M, Korkeala H (2006) Laboratory diagnostics of botulism. *Clin Microbiol Rev* 19:298–314.

Lönnberg M, Carlsson J (2001) Quantitative detection in the attomole range for immunochromatographic tests by means of a flatbed scanner. *Anal Biochem* 293:224–231.

Mancheño JM, Tateno H, Goldstein IJ, Martinez-Ripoll M, Hermoso JA (2005) Structural analysis of the *Laetiporus sulphureus* hemolytic pore-forming lectin in complex with sugars. *J Biol Chem* 280:17251–17259.

Mettler M, Grimm F, Capelli G, Camp H, Deplazes P (2005) Evaluation of enzyme-linked immunosorbent assays, an immunofluorescent-antibody test, and two rapid tests (immunochromatographic-dipstick and gel tests) for serological diagnosis of symptomatic and asymptomatic *Leishmania* infections in dogs. *J Clin Microbiol* 43:5515–5519.

Montecucco C, Schiavo G (1993) Tetanus and botulism neurotoxins: a new group of zinc proteases. *Trends Biochem Sci* 18:324–327.

Sharma SK, Eblen BS, Bull RL, Burr DH, Whiting RC (2005) Evaluation of lateral-flow *Clostridium botulinum* neurotoxin detection kits for food analysis. *Appl Environ Microbiol* 71:3935–3941.

Singh BR, DasGupta BR (1989) Molecular topography and secondary structure comparisons of botulinum neurotoxin types A, B and E. *Mol Cell Biochem* 86:87–95.

Smith TJ, Lou J, Geren IN, Forsyth CM, Tsai R, LaPorte SL, Tepp WH, Bradshaw M, Johnson EA, Smith LA, Marks JD (2005) Sequence variation within botulinum neurotoxin serotypes impacts antibody binding and neutralization. *Inf Immun* 73:5450–5457.

Wein LM, Liu Y (2005) Analyzing a bioterror attack on the food supply: the case of botulinum toxin in milk. *PNAS* 102:9984–9989.