

# We are IntechOpen, the world's leading publisher of Open Access books Built by scientists, for scientists

5,000

Open access books available

125,000

International authors and editors

140M

Downloads

Our authors are among the

154

Countries delivered to

TOP 1%

most cited scientists

12.2%

Contributors from top 500 universities



WEB OF SCIENCE™

Selection of our books indexed in the Book Citation Index  
in Web of Science™ Core Collection (BKCI)

Interested in publishing with us?  
Contact [book.department@intechopen.com](mailto:book.department@intechopen.com)

Numbers displayed above are based on latest data collected.  
For more information visit [www.intechopen.com](http://www.intechopen.com)



# Current Methods for Detecting the Presence of Botulinum Neurotoxins in Food and Other Biological Samples

Luisa W. Cheng<sup>1</sup>, Kirkwood M. Land<sup>2</sup> and Larry H. Stanker<sup>1</sup>  
*Foodborne Contaminants Research Unit, Western Regional Research Center,*  
<sup>1</sup>*Agricultural Research Service, U.S. Department of Agriculture, Albany, CA,*  
<sup>2</sup>*Department of Biological Sciences, University of the Pacific, Stockton, CA,*  
USA

## 1. Introduction

Botulinum neurotoxins (BoNTs) are some of the most lethal human bacterial toxins and the causative agent of botulism (Arnon et al., 2001; Simpson, 2004). The usual routes of intoxication for BoNTs are oral ingestion of clostridial spores or pre-formed toxin, manifested as infant, foodborne and adult onset botulism. An increasingly common route of intoxication is associated with intravenous drug use resulting in wound botulism. BoNTs are also classified as Select Agents and have been used as agents of bioterrorism (Arnon et al., 2001; Bigalke and Rummel, 2005). Potential methods for toxin exposure include intentional contamination of the food and drink supply, or by aerosol spread, leading to inhalational botulism.

Usually, an identification of botulism is made through clinical manifestations and diagnosis, with subsequent confirmation by laboratory identification of clostridial spores or toxin in foods, environmental or clinical samples (CDC, 1998; Lindström and Korkeala, 2006; Solomon and Lilly, 2001). The speed of recovery from botulism increases with the timely administration of antitoxin or medical interventions (Arnon et al., 2001; Simpson, 2004). Thus, sensitive and rapid toxin detection and diagnostic methods are critical for improved recovery time, as well as, facilitate the epidemiologic study of outbreaks.

Due to the potential for bioterrorism use, much effort and resources have been dedicated to the development of detection methods, treatment, and prevention of botulism. A multitude of assay formats have been developed over many years, with in some cases, reported sensitivities at the attomolar level (Grate et al., 2010). Many assays were designed for use in the validation of toxin production, for commercial purposes, or for high-throughput screening methods to identify therapeutics that inhibit toxin function. These highly sensitive methods usually detect highly purified BoNT samples and are used in research type applications. Many such assays are not usable for the detection of BoNT contamination in food or other complex samples. This chapter focuses on the diagnostic methods for toxin detection and the challenges encountered while adapting analytical methods for the detection of BoNTs in foods and other biological and environmental samples.

The biology and mechanisms of action of BoNTs are described in a previous chapter in this book, and readers should refer to the botulinum neurotoxins chapter by Webb, Roxas-Duncan and Smith, for more background reading. This chapter will briefly describe the properties of BoNTs as they relate to detection methods and will compare and contrast currently used methods for food and biological sample analyses and methods in development. For detailed analyses and descriptions of detection assays please also refer to excellent reviews by (Grate et al., 2010; Lindström and Korkeala, 2006; Scarlatos et al., 2005; Sharma and Whiting, 2005). We apologize to others not named here due to space constraints.

## 2. Overview of botulinum neurotoxin structure and function as they relate to the development of diagnostic tools

A single gram of BoNT released and subsequently inhaled can lead to the deaths of more than one million people (Arnon et al., 2001; Hill et al., 2007). BoNTs are produced by the ubiquitously distributed, gram-positive, strictly anaerobic, spore-forming bacteria *Clostridium botulinum*, *C. barati*, *C. butyricum* and *C. argentinense*. To date, seven different botulinum serotypes, indicated by letters A through G, have been identified. Serotypes A, B, E, and F have been associated with human disease (Table I).

Type of botulism	Average number of cases per year	Percent of total	BoNT Serotypes
Infant	85	66	A, B, E, F
Wound	24	19	A, B
Foodborne	19	15	A, B, E, F
Adult	0.4	0.3	A, B, F
Unknown	1.4	1	A, B, F

Table 1. Survey of U.S. human botulism cases from 2001-2009 as reported by the U.S. Centers for Disease Control and Prevention (CSTE).

BoNT serotypes can differ from each other by 34-64% at the amino acid level (Garcia-Rodriguez et al., 2011; Hill et al., 2007; Jacobson et al., 2011; Smith et al., 2007; Smith et al., 2005). Genetic variation within each serotype is sometimes significant. And 32 toxin subtypes with amino acid level differences of 2.6-32% have been identified thus far, with more likely to be identified in the future. Serotype and subtype diversity may impact antibody and molecular-based assay designs.

BoNT is synthesized as an ~150 kDa protein, also called the holotoxin, that is subsequently processed by a clostridial trypsin-like protease into two polypeptides linked by a single disulfide bond; and are thus similar to other known bacterial A-B dimeric toxins (Oguma, Fujinaga, and Inoue, 1995; Singh, 2000). The ~100 kDa fragment, known as the heavy chain (HC), facilitates toxin binding to specific host cell receptors and later, translocation of the toxin from vesicles into the cell cytosol. The ~50 kDa fragment, known as the light chain (LC), contains the enzymatic domain. The LC fragment is often used for the development of activity-based assays. HC and LC specific antibodies have been developed for toxin neutralization and toxin detection immunoassays.

BoNT holotoxin is secreted from bacteria in association with other non-toxic proteins, called neurotoxin associated proteins or NAPs, forming large protein complexes of 500-900 kDa. These large protein complexes are referred to as progenitor toxins or simply as BoNT complex (Inoue et al., 1996). Complexed BoNTs are significantly more toxic in oral intoxications (Cheng et al., 2008; Ohishi, Sugii, and Sakaguchi, 1977) than holotoxins. NAPs are thought to protect the holotoxin from gastric digestion as well as help holotoxins cross the intestinal barrier (Fujinaga et al., 2009; Niwa et al., 2007; Simpson et al., 2004). Toxin complexes that survive the gastric challenge translocate across the epithelial cell barrier (transcytosis) gaining access into the bloodstream, where the holotoxin is released. BoNT complexes are the forms that will most likely be found in natural intoxication and bioterrorism cases. Thus, the detection of toxin when associated with NAPs or the use of NAPs as detection targets in foodborne intoxications is a consideration in the design of new assays.

The target for BoNT holotoxin is the peripheral cholinergic nerve ending, resulting in flaccid paralysis (Simpson, 2004). Specific receptors for the toxin HC of BoNTs have been identified; BoNT/A binds to glycoprotein SV2; serotype F binds SV2 and gangliosides; and serotype G binds synaptotagmin I and II. Toxin binding to nerve cells is followed by receptor-mediated endocytosis and subsequent translocation of the LC (directed by the translocation domain of the HC) into the cytoplasm. The LC of different serotypes targets different SNARE proteins. BoNT serotypes A, C and E target SNAP-25, and serotypes B, D, F, and G target VAMP2, while serotype C targets syntaxin (Hakami et al., 2010). Different SNARE targets of BoNTs have been used to develop *in vitro* assays for toxin activity. Peptides with fluorescent labels and quencher molecules have been designed and used in various forms of enzymatic activity assays.

### 3. Challenges to the development of detection assays for botulinum neurotoxins

The development of robust and sensitive detection assays for BoNTs requires consideration of at least six factors explored in detail below.

#### 3.1 Sensitivity

Assay sensitivity is not a simple criterion to define and is determined in part by the specific application. For example, the human lethal dose (LD) for oral intoxication is estimated at 1 µg/kg or about 70 µg for a 70 kg adult (Arnon et al., 2001; Scarlatos et al., 2005). Assays designed for evaluating food must detect at least this amount in a typical portion. Since portions vary widely between individual foods, assay sensitivity requirements may vary with specific matrixes. Foods that typically have large portion sizes would require assays with lower detection limits. Furthermore the dose to cause illness but not death might be lower. Our experience with BoNT exposure in rodents is that a level 10-fold lower than the minimal lethal dose falls into this latter category. Thus, a dose level 10-fold lower than the LD in humans, 7 µg, translates into an assay sensitivity of 70 ng/mL if a serving is typically 100 mL. A 10-fold threshold lowers the sensitivity to 7 ng/mL. In contrast, detection levels for tests used in sera or other clinical matrices should be as sensitive as possible to account for low toxin levels. For example, in oral mouse toxicity studies, only a small portion of the

ingested BoNT actually survives the harsh conditions in the gut to reach the bloodstream (Cheng and Henderson, 2011; Cheng et al., 2008). The lethal toxin intravenous dose varies between 20-200 ng in an adult human with approximately 5 liters of blood (Arnon et al., 2001). Taking into account natural degradation, and clearance of toxin in sera, the assay sensitivity for diagnostic evaluation must be in the low to sub-pg/ml range.

### 3.2 Specificity

There are currently seven known serotypes of BoNTs, and 32 known subtypes. New subtypes are expected to be identified in the future. Amino acid sequence differences can vary as much as 70% among serotypes (Hill et al., 2007; Smith et al., 2005). This level of genetic diversity and variation can prove challenging for both molecular and antibody-based diagnostic methods. False negative results could be obtained if a gene or protein structure of the toxin differ from what established oligonucleotides/PCR primers or antibodies can recognize. At the very least, assay performance needs to be established on as many toxin sero- and subtypes as practical. Reagents generated for detection assays should ideally recognize all known subtypes of each serotype.

### 3.3 Matrix effects

In almost all scenarios, BoNT samples to be tested would be found in a wide variety of matrices of food, clinical (serum, sputum, feces, etc) or environmental samples (dust, soil, water, etc). Yet, most assay methods are designed, tested and optimized in buffer conditions and thus the sensitivity or application in complex matrices may be diminished. Complex matrices may contain many challenging conditions such as high fat, high protein or salt content, low or high pH; the presence of other active proteases could also interfere with detection sensitivity, increase background signal, and give false positive or negative signals. Methods to alleviate matrix interference range from simple sample dilution, pH rebalancing, addition of protease inhibitors, to specific affinity binding steps prior to detection. Extensive analysis of different matrices will be necessary to evaluate assay sensitivity and determine the best methods to circumvent matrix effects on assay performance.

### 3.4 Activity

The potent toxicity associated with BoNTs is attributed to their enzymatic properties. The differentiation of active versus inactive forms of the toxin is needed for proper risk assessment and should be an important consideration in assay design. An active BoNT has many roles, it must be able to bind host cell receptors, translocate across membranes and finally reach the host cell cytosol and cleave its target protein. Few assays can measure all aspects of toxin function. Immunoassays (IA) can generally detect both active and inactive toxin and may give false positive results even when no active toxin is present. However, positive results from IA requiring the presence of both HC and LC are predictive of active toxin (Stanker et al, 2008). Assays measuring endopeptidase activities of BoNTs are available but are not as sensitive and amenable to use in complex matrices. Genomic methods, while sensitive, detect the presence of toxin genes but not that of toxin. Depending on the diagnostic needs, a combination of methods may have to be used to get a full activity profile of the toxin.

### 3.5 Ease of use

For the widest application of an assay, it must be user-friendly and allow for a timely diagnosis. Furthermore, assays need to be validated in multiple laboratories, use equipment or tools that are readily available, and require minimal training to execute. Ideally, the assay should also be field deployable.

### 3.6 Cost

The cost of an assay in terms of reagent or equipment availability can be an important factor on how widely an assay is used and deployed.

## 4. Current diagnostic methods of toxin detection

The current “gold standard” for detection of BoNTs is the mouse bioassay. Despite many attempts and much research to replace the use of animals, it is still the best assay to model all aspects of BoNT intoxication: binding, translocation and enzymatic activity (Grate et al., 2010). In attempts to replace the mouse bioassay and improve assay time and sensitivity, both *in vitro* and *in vivo* systems have been developed for the detection of BoNTs. The development of a robust detection assay for BoNT requires that the assay meet as many of the six challenges mentioned above as possible. This section of the chapter will mainly focus on assays that can be used in food and biological samples for the detection of BoNTs.

### 4.1 Mouse bioassays

The mouse bioassay is still one of the most sensitive and robust methods to detect BoNTs (Schantz and Kautter, 1978; Solomon and Lilly, 2001). The mouse bioassay measures BoNT in minimal lethal dose (MLD) units, which is the lowest dose at which all tested mice die. Mice are usually injected intraperitoneally with 0.5 ml of BoNT sample in a dilution series, and then monitored over several days for signs of intoxication and death (CDC, 1998; CFSAN, 2001). Signs of intoxication include: ruffled fur, wasp-waist (Figure 1), labored-breathing, paralysis and death. Signs of intoxication can appear from a few hours post-injection to a few days depending on the dose and type of BoNT. When enough sample is available, the identity of the unknown BoNT can simultaneously be tested by the addition of neutralizing antibodies against each of the serotypes (A-G). The serotype is identified by the antibody that protects their respective mice from death. The mouse bioassay sensitivity is in the range of 20-30 pg for BoNT/A and 10-20 pg/ml for BoNT/B (Ferreira et al., 2004; Wictome et al., 1999).

While the mouse bioassay has high sensitivity, can detect different serotypes and subtypes, measures different aspects of active toxin, and is amenable to use in different matrices, it has many drawbacks. These include: long assay times, requires specialized animal facilities, trained staff, and the use of animals (with death used as an endpoint). There is also substantial variation of results observed among different research laboratories.

Alternative refined animal assays that do not use death as an endpoint such as the mouse phrenic nerve hemi-diaphragm assay have been evaluated (Rasetti-Escargueil et al., 2009). Although they may be sensitive and faster than the use of whole animals, these assays require use of sophisticated equipment and training, and are not amenable for use with

large samplings of complex matrices (Grate et al., 2010). A recently developed *in vivo* assay using the toe-spread reflex model was tested for the detection of BoNT in buffer, serum and milk samples (Wilder-Kofie et al., 2011). This new assay can provide results more quickly than standard mouse bioassays. The robustness of this assay and how easily staff can be trained to perform this assay have yet to be determined.



Fig. 1. Mouse bioassay. Mice were intraperitoneally treated with BoNT/A (right mouse) or phosphate buffered saline (left mouse). The intoxicated mouse shows a typical wasp-waist phenotype.

## 4.2 Nucleic acid based methods of detecting *C. botulinum* in food matrices and other biological samples

### 4.2.1 Polymerase chain reaction

The use of the polymerase chain reaction (PCR) to identify the presence of *C. botulinum* DNA was originally used to detect the presence of bacterial spores. The method could detect the presence of as few as  $10^2$  spores per reaction mixture for serotypes A, E and F and only 10 spores per reaction mixture for BoNT/B. To enhance sensitivity, Lindström and colleagues developed an enrichment method that could detect as few as  $10^{-2}$  spores/gram of sample for serotypes A, B and F and  $10^{-1}$  spores/gram of sample for BoNT/E (Lindström et al., 2001). However, one critical drawback of this method is that enrichment often requires 5 days. Furthermore, the applicability of the assay for detection of food contamination was diminished by the observation that beef could interfere with the sensitivity of the assay. Also, if contamination were to occur with the actual toxin, and not cells, this traditional PCR method would not be useful.

### 4.2.2 Multiplex polymerase chain reaction

It is highly desirable to analyze unknowns for multiple targets, such as different pathogens and/or associated gene products of those pathogens. This approach, known as Multiplex technology, is conceptually simple for PCR based assays. Different sets of PCR primers, each

one highly specific for a gene of interest can be easily generated, allowing for the amplification of multiple targets in one reaction tube. One such multiplex method was able to discriminate among BoNT serotypes A, B, E, and F, corroborating mouse bioassay results (De Medici et al., 2009). Furthermore, Peck and colleagues developed a culture enrichment methods that when coupled with multiplex PCR, can identify strains of *C. botulinum* that are non-proteolytic (BoNT serotypes B, E, and F) (Peck et al., 2010). Importantly, this method was robust and reasonably rapid for use with food samples contaminated with *C. botulinum*.

Recently, Fach and colleagues have adapted the use of the GeneDisc Cyclor (GeneSystems PCR Technology) to amplify *C. botulinum* genes encoding BoNT serotypes A, B, E, and F on different microchambers (Fach et al., 2011). This technology allows the simultaneous amplification of multiple targets along with a number of different internal controls. A number of different toxin-producing clostridia and non-toxin producing bacteria that were isolated from different food, clinical, and environmental samples and results were compared with those obtained from the mouse bioassay. Notably, all of the botulinum genes were detected correctly and no cross-reactivity was observed with either non-toxin producing bacteria or with *C. botulinum* serotypes C, D, and G. Four European laboratories evaluated this technology, examining 77 toxin producing clostridia as well as 10 food and clinical samples. In all cases, this GeneDisc Cyclor was specific and reliable for identifying *C. botulinum* serotypes A, B, E, and F; and was also useful for screening naturally contaminated food and fecal samples.

#### 4.2.3 Real-time polymerase chain reaction

Real-time or quantitative PCR is useful in studies of gene expression; specifically differential expression of genes under different environmental conditions or for comparative studies among different organisms. For detection of clostridia, real-time PCR methods that examine expression of the NTNH (non-toxic, non-hemagglutinin) genes have been developed, as well as methods to study toxin gene expression in *C. botulinum* serotypes A, B, E, and F (Fach et al., 2009). In that study, twenty-nine different strains of toxin-producing *C. botulinum* were screened, and compared with expression profiles from non-toxin producing clostridia as controls. This assay has a sensitivity of 100-fg/1000 fg total DNA in the PCR tube (equivalent to approximately 25-250 genomes). Converting this DNA concentration to its equivalent in cells/ml, suggested a detection limit of approximately  $10^3$  to  $10^4$  cells/mL. Following a 48-hour enrichment under anaerobic conditions, these investigators reported the detection of *C. botulinum* serotype A in a naturally contaminated sample of *foie gras* suspected in a botulism outbreak. Recently, pentaplex methods have been developed to simultaneously identify and discriminate among larger numbers of different serotypes using a wider array of different genes (Kirchner et al., 2010). This technology should prove to be efficient and cost-effective.

#### 4.2.4 DNA microarrays

Microarray technology for toxin identification of contaminated food has not been widely used. This may be due to the challenge in isolating high-quality RNA samples from clostridia in food matrices. A recent oligonucleotide microarray with 62 different sequences based on known strain variable regions in the genome of *C. botulinum* strain ATCC 3502 was constructed and used to differentiate different *C. botulinum* type A strains (Raphael et al.,

2010). Regions corresponding to BoNT genes of various serotypes, and other markers components, and other markers were observed. Further development of microarray based assay approaches may provide a means to rapidly identify toxin-producing strains.

### **4.3 Antibodies as detection tools for BoNT contamination in food**

High-affinity monoclonal antibodies (mAbs) that specifically bind individual or multiple BoNT serotypes (and subtypes) have been generated using either mouse hybridoma technology or yeast affinity maturation methods (Grate et al., 2010). These antibodies have been used extensively in traditional ELISA, bead-based, immuno-PCR, microarray assays or in sample preparation before use in a detection assay. Several such assays used in food and biological matrices are highlighted below.

#### **4.3.1 ELISA and ELISA-based methods of detection**

ELISA is a widely used detection assay format that uses anti-BoNT capture and detector antibodies usually in a sandwich type format. The read-out for the assay can be colorimetric, luminescence or other formats. Most older generation BoNT immunoassays are about 10 times less sensitive than the mouse bioassay (Ferreira et al., 2004; Scarlatos et al., 2005; Sharma and Whiting, 2005). Although not as sensitive, ELISA based methods are relatively fast, inexpensive and simple. They are also less subject to matrix effects. Sharma and colleagues designed an amplified enzyme-linked immunosorbent assay (ELISA) for detecting toxins in food matrices (Sharma et al., 2006). Specifically, toxins for serotypes A, B, E, and F could be detected in liquids, solid, and semisolid food. Assay performance in a range of foods include broccoli, orange juice, bottled water, cola soft drinks, vanilla extract, oregano, potato salad, apple juice, meats, and dairy items were evaluated. Assay sensitivity varied for each botulinum complex serotype, and were reported as 60 pg/ml for BoNT/A, 176 pg/ml for BoNT/B, 163 pg/ml for BoNT/E, and 117 pg/ml for BoNT/F. The tests readily detected 2 ng/ml of serotypes A, B, E, and F in a variety of the foods tested.

Recently, traditional format sandwich ELISA assays using highly sensitive mAbs against BoNT/A and BoNT/B have detected as low as 5 pg/mL and 25 pg/mL BoNT/A, in buffer and in a milk matrix, respectively (Stanker et al., 2008); and 100 fg and 39 pg/ml of BoNT/B in the buffer and milk matrix, respectively (Scotcher, Cheng, and Stanker, 2010). These mAbs were also used in electrochemiluminescence ELISA type assays using a Meso Scale Discovery (MSD) instrument. Detection sensitivities for BoNT/A using the MSD instrument were similar to traditional ELISAs in the buffer system but offered marked improvement in detection limits and reduction in backgrounds in liquid food matrices (Cheng and Stanker, unpublished results). The higher sensitivity and less time required for these new ELISA assays make them great alternatives or complements for the mouse bioassay.

#### **4.3.2 Multiplex antibody-based detection systems**

The multiplex technology has been applied to the development of methods to analyze multiple epitopes on a single antigen or multiple targets in a single sample. This approach uses multiple mAbs as well as polyclonal antibodies to reduce false-positive and false-negative results. The Luminex xMAP technology utilizes microsphere beads conjugated with antibodies. The antibody-bead complexes detect multiple epitopes in single sample; for

instance, this technology was used to detect abrin, ricin, botulinum toxins, and staphylococcal enterotoxins in spiked food samples (Garber, Venkateswaran, and O'Brien, 2010). The study used paramagnetic beads instead of non-magnetic polystyrene beads to help in the analysis of food matrices, such as chipotle mustard, which contain large amounts of particulate matter.

#### **4.3.3 Affinity immunochromatography column-based methods**

Accurate and sensitive detection of contaminated food and other biological samples in the field is critical. To this end, Brunt and colleagues (Brunt, Webb, and Peck, 2010) have developed a number of rapid affinity immunochromatography column (AICC) assays for the detection of BoNT serotypes A, B, E, and F in food matrices. These authors reported a detection limit for BoNT/A of 0.5 ng, two fold more sensitive than earlier reported lateral flow methods. For serotypes B, E, and F, the minimum detection limit ranged from 5 ng to 50 ng. Although not as sensitive as ELISA or mouse bioassays, immunochromatographic methods generally are rapid assays, requiring only 15 to 30 minutes to complete, do not require enrichment steps, making them highly amenable to use in the field.

#### **4.3.4 Lateral flow technology**

The application of lateral flow methods for detecting toxins has led to the development of a number of kits for sensitive and rapid testing. The principle here is that capture antibodies are printed on nitrocellulose membranes. Detection antibodies are labeled with materials that can be visualized (eg., colloidal gold, or colored latex beads) The sample is added to a reagent pad containing labelled detection antibodies that bind toxin, wick across the membrane where toxin is retained, thus concentrating the labelled detection antibody. A positive reaction leads to a colorimetric change that is usually detected as a line. These assays are generally qualitative, and determine the presence or absence of toxin. Sharma and coworkers tested different commercial lateral flow devices (such as the Bot-Tox-BTA kit) for their capacities to detect toxin in food samples (Sharma et al., 2005). They were able to detect as little as 10 ng/ml of BoNT serotypes A and B and 20 ng/ml of BoNT/E in a variety of liquids such as milk products, soft drinks, and fruit juices. Results by Stanker (unpublished) show sensitivity of 0.5 and 1 ng/ml for BoNT/A in buffer and milk, respectively, in lateral flow devices using sensitive mAbs described in the ELISA section above (Stanker et al., 2008). Although simple lateral flow tests have poorer sensitivities compared to other methods, they produced rapid results, require no additional reagents or equipment, are easily interpreted, and have many applications. They can be useful for the quick screening of samples where the presence of BoNT may be more abundant.

#### **4.3.5 Immuno-polymerase chain reaction (I-PCR)**

An innovative approach for toxin detection combines antibodies with the amplification power of PCR in an assay called immuno-PCR (I-PCR). Here, instead of a secondary antibody conjugated to the detection enzyme, template DNA is conjugated to the antibody; and upon binding of antigen by the antibody, an indirect test for the presence of the BoNT is carried out using PCR. Chao et al. described a sensitive I-PCR method (femtogram amounts,  $10^{-15}$  grams) for detection of BoNT/A. These investigators also

compared standard ELISA as well as sandwich ELISA methods with the sensitivity of the I-PCR method. Both ELISA methods were sensitive for toxin detection down to 50 fg, and the I-PCR method was between  $10^3$  to  $10^5$  times more sensitive (Chao et al., 2004; Wu et al., 2001). For more background on the basic principles of I-PCR, the reader is referred to Niemeyer and colleagues (Niemeyer, Adler, and Wacker, 2005). The use of I-PCR for highly sensitive detection of BoNT in food matrices or other biological backgrounds has yet to be developed.

#### **4.4 Activity based assays for detecting food contamination**

Rapidly distinguishing between the presence and absence of active versus inactive toxin is critical for intervention. Since BoNTs are zinc metalloproteases, enzyme-substrate assays have been developed using knowledge of the human targets for these enzymes. Activity assays range from mixing toxin with recombinant versions of host targets (such as SNAP-25) and then using immunoblotting to detect cleavage of those substrates, to measuring fluorescence emitted from cleavage of fluorogenic peptide substrates. One such peptide, called SNAPtide, used in an assay with a reverse phase HPLC with a fluorescence detector, can detect as low as 5 pg/mL of BoNT/A in skim milk (Christian, Suryadi, and Shine, 2010). Other peptide substrates: VAMPtide and SYNTAXtide, useful for their cognate BoNTs have been developed. The levels of substrate cleavage correlate well with toxin activity.

Other investigators have looked for other indications of substrate cleavage by BoNTs. For instance, Nuss and colleagues generated antibodies that specifically recognize the full-length version of human SNAP25 and not the cleaved form (Nuss et al, 2010). Use of this antibody to confirm the absence of toxin activity (by detecting only the intact, full length substrate) might be useful to confirm the absence of bioactive forms of the toxin.

Other activity-based approaches have used physical methods such as surface plasmon resonance to detect cleavage of substrates. For instance, Ferraci and colleagues have demonstrated that cleavage of the BoNT/B substrate VAMP2, a membrane SNARE protein associated with synaptic vesicles, can be measured using real-time surface plasmon resonance; vesicle capture is detected by specific antibodies coupled to microchips (Ferracci et al., 2010). This assay is functional in low ionic strength buffers and stable over a wide range of pH values (5.5-9.0). Cleavage of VAMP2 was detected within 10 minutes with 2 pM of native BoNT/B holotoxin. Contamination of liquid food products such as carrot juice, apple juice, and milk with low picomolar amounts of BoNT/B toxin is revealed within 3 hours. BoNT/B activity was detected in sera samples from botulism patients but not in healthy patients or in patients with other neurological diseases.

##### **4.4.1 Cell-based assays and their possible use in detecting food contamination**

Cell-based assays measure BoNT receptor binding, translocation and enzymatic activity and can be viable alternatives to the mouse bioassay. A number of different neuronal and non-neuronal derived cell lines have been generated for use in BoNT assays. These include: rat spinal cord cells (Pellett et al., 2007); chick embryo neuronal cells (Stahl et al., 2007); neuroblastoma cells N2A (Eubanks et al., 2007); and BE(2)-M17 cells (Hale et al., 2011). The read-out for most of the cell-based assays for detection of BoNT/A is the cleavage of SNAP-25. Antibodies for SNAP-25 allow immunoblot detection of cleavage products, specifically detecting a decrease in size of endogenous SNAP-25 protein.

Investigators continue to examine different parameters in order to develop a more robust cell-based assay. The U.S. Food and Drug Administration recently approved a cell-based assay developed by Allergan for use as possible replacement of the mouse bioassay. Details of the assay have yet to be published. Cell-based assays may yet prove valuable for toxin detection in food.

#### **4.5 Combining assay methods to increase detection sensitivity**

Detection methods can exploit the power of sensitive antibodies for enrichment or sample preparation, as well as the signal amplification ability of enzymatic assays. Two recent approaches are highlighted below.

##### **4.5.1 ALISSA (assay with a large immunosorbent surface area)**

The ALISSA utilizes a two-step approach; first, an antibody-mediated step concentrates toxin onto a large bead surface. Captured toxin molecules are then subjected to a SNAPtide protease assay (Bagramyan et al., 2008). When compared to other established methods for toxin detection in food matrices, the ALISSA assay can detect toxin concentrations as low as 50 fg/mL, more sensitive than the mouse bioassay or either immunoassay or SNAPtide assay alone. The use of this method to evaluate a number of different food matrices suggests that it may be useful in food contamination studies.

##### **4.5.2 ENDOPEP-MS: antibodies, activity assays, and mass spectrometry**

The ENDOPEP-MS method uses antibodies to concentrate and extract BoNT serotypes A, B, E, and F from test samples. The concentrated toxins are then subjected to an endopeptidase activity-based assay to generate target cleavage products. Finally, mass spectrometry is used to identify cleavage target products (Kalb et al., 2005; Kalb et al., 2006). This approach has been successful in identifying BoNT serotypes A, B, E, and F in a variety of food and clinical sample matrices with sub-mouse bioassay sensitivities. To advance this technique even further, a single, high-affinity mAb (4E17.1) that can simultaneously identify BoNT serotypes A, B, E and F has been developed (Kalb et al., 2010). The use of this mAb reduced assay time while maintaining assay sensitivity. The use of mass spectrometry can give fast and definitive results. With the future development of low cost equipment, this method may be more readily available to investigators.

## **5. Conclusion**

Detection of BoNT presents a unique set of challenges. The high toxicity of BoNT requires detection methods capable of toxin measurement in the low to sub pg/mL range. PCR methods can readily detect the presence of low levels of *C. botulinum* DNA but do not detect the presence or absence of the toxin. In many cases, such as blood samples, only toxin may be present. Current methods for toxin detection rely on 1) the gold standard mouse bioassay, or 2) *in vitro* tests such as molecular tests, immunoassays and/or activity-based assays. Recent improvements in the generation of high-affinity mAbs have resulted in immunoassays with sensitivities equal to or lower than the mouse bioassay. However these tests generally do not distinguish active from inactive toxin. Activity-based assays can detect

active toxin but generally have poorer detection limits than immunoassays. New assays must also be carefully validated in individual food matrices or for as many toxin subtypes as possible, in order to establish assay performance standards. With refinement of the methods described above, the prospect of an assay that is sensitive, cost effective, and fast could be possible for use in food or other biological samples. Furthermore, these new strategies for assay development can easily be extended to other toxins and pathogens.

## 6. Acknowledgment

Due to space constraints, the authors apologize for not being able to devote enough attention to many worthy assay methods. We thank our colleagues for insightful comments and discussions. Thomas D. Henderson II for help with background research. KML was funded by the Department of Biological Sciences and the Office of Grants and Sponsored Programs, the University of the Pacific. LWC and LHS were funded by the United States Department of Agriculture, Agriculture Research Service, CRIS project 5325-42000-048-00D, the National Institute of Allergy And Infectious Diseases Service Grant U54 AI065359, and DHS agreement 40768. The USDA is an equal opportunity provider and employer.

## 7. References

- Arnon, S. S., Schechter, R., Inglesby, T. V., Henderson, D. A., Bartlett, J. G., Ascher, M. S., Eitzen, E., Fine, A. D., Hauer, J., Layton, M., Lillibridge, S., Osterholm, M. T., O'Toole, T., Parker, G., Perl, T. M., Russell, P. K., Swerdlow, D. L., and Tonat, K. (2001). Botulinum toxin as a biological weapon: medical and public health management. *Jama* 285(8), 1059-70.
- Bagramyan, K., Barash, J. R., Arnon, S. S., and Kalkum, M. (2008). Attomolar detection of botulinum toxin type A in complex biological matrices. *PLoS ONE* 3(4), e2041.
- Bigalke, H., and Rummel, A. (2005). Medical aspects of toxin weapons. *Toxicology* 214(3), 210-20.
- Brunt, J., Webb, M. D., and Peck, M. W. (2010). Rapid affinity immunochromatography column-based tests for sensitive detection of *Clostridium botulinum* neurotoxins and *Escherichia coli* O157. *Appl Environ Microbiol* 76(13), 4143-50.
- CDC (1998). Center for Disease Control and Prevention. Botulism in the United States (1899-1996). Handbook for epidemiologists, clinicians and laboratory workers. U.S. Department of Health and Human Services, CDC. Atlanta, Ga.
- CFSAN (2001). Center for Food Safety and Applied Nutrition. Bacteriological analytical manual (BAM). U.S. Food and Drug Administration, Washington, D.C.
- CSTE Council of State and Territorial Epidemiologists. 2001-2009. Botulism Surveillance Summary. [http://www.cdc.gov/national-surveillance/botulism\\_surveillance.html](http://www.cdc.gov/national-surveillance/botulism_surveillance.html)
- Chao, H. Y., Wang, Y. C., Tang, S. S., and Liu, H. W. (2004). A highly sensitive immunopolymerase chain reaction assay for *Clostridium botulinum* neurotoxin type A. *Toxicon* 43(1), 27-34.
- Cheng, L. W., and Henderson, T. D., 2nd (2011). Comparison of oral toxicological properties of botulinum neurotoxin serotypes A and B. *Toxicon* 58(1), 62-7.
- Cheng, L. W., Onisko, B., Johnson, E. A., Reader, J. R., Griffey, S. M., Larson, A. E., Tepp, W. H., Stanker, L. H., Brandon, D. L., and Carter, J. M. (2008). Effects of purification on the bioavailability of botulinum neurotoxin type A. *Toxicology* 249(2-3), 123-9.

- Christian, T., Suryadi, K., and Shine, N. (2010). Ultra Sensitive HPLC Detection Assay for Botulinum Neurotoxin Type A. *Presented at the 47th Annual Interagency Botulinum Research Coordinating Committee Meeting, November 2010 in Atlanta, GA.*
- De Medici, D., Anniballi, F., Wyatt, G. M., Lindström, M., Messelhauser, U., Aldus, C. F., Delibato, E., Korkeala, H., Peck, M. W., and Fenicia, L. (2009). Multiplex PCR for detection of botulinum neurotoxin-producing clostridia in clinical, food, and environmental samples. *Appl Environ Microbiol* 75(20), 6457-61.
- Eubanks, L. M., Hixon, M. S., Jin, W., Hong, S., Clancy, C. M., Tepp, W. H., Baldwin, M. R., Malizio, C. J., Goodnough, M. C., Barbieri, J. T., Johnson, E. A., Boger, D. L., Dickerson, T. J., and Janda, K. D. (2007). An in vitro and in vivo disconnect uncovered through high-throughput identification of botulinum neurotoxin A antagonists. *Proc Natl Acad Sci U S A* 104(8), 2602-7.
- Fach, P., Fenicia, L., Knutsson, R., Wielinga, P. R., Anniballi, F., Delibato, E., Auricchio, B., Woudstra, C., Agren, J., Segerman, B., de Medici, D., and van Rotterdam, B. J. (2011). An innovative molecular detection tool for tracking and tracing *Clostridium botulinum* types A, B, E, F and other botulinum neurotoxin producing Clostridia based on the GeneDisc cycler. *Int J Food Microbiol* 145 Suppl 1, S145-51.
- Fach, P., Micheau, P., Mazuet, C., Perelle, S., and Popoff, M. (2009). Development of real-time PCR tests for detecting botulinum neurotoxins A, B, E, F producing *Clostridium botulinum*, *Clostridium baratii* and *Clostridium butyricum*. *J Appl Microbiol* 107(2), 465-73.
- Ferracci, G., Marconi, S., Mazuet, C., Jover, E., Blanchard, M. P., Seagar, M., Popoff, M., and Leveque, C. (2010). A label-free biosensor assay for botulinum neurotoxin B in food and human serum. *Anal Biochem* 410(2), 281-8.
- Ferreira, J. L., Eliasberg, S. J., Edmonds, P., and Harrison, M. A. (2004). Comparison of the mouse bioassay and enzyme-linked immunosorbent assay procedures for the detection of type A botulinum toxin in food. *J Food Prot* 67(1), 203-6.
- Fujinaga, Y., Matsumura, T., Jin, Y., Takegahara, Y., and Sugawara, Y. (2009). A novel function of botulinum toxin-associated proteins: HA proteins disrupt intestinal epithelial barrier to increase toxin absorption. *Toxicon* 54(5), 583-6.
- Garber, E. A., Venkateswaran, K. V., and O'Brien, T. W. (2010). Simultaneous multiplex detection and confirmation of the proteinaceous toxins abrin, ricin, botulinum toxins, and Staphylococcus enterotoxins A, B, and C in food. *J Agric Food Chem* 58(11), 6600-7.
- Garcia-Rodriguez, C., Geren, I. N., Lou, J., Conrad, F., Forsyth, C., Wen, W., Chakraborti, S., Zao, H., Manzanarez, G., Smith, T. J., Brown, J., Tepp, W. H., Liu, N., Wijesuriya, S., Tomic, M. T., Johnson, E. A., Smith, L. A., and Marks, J. D. (2011). Neutralizing human monoclonal antibodies binding multiple serotypes of botulinum neurotoxin. *Protein Eng Des Sel* 24(3), 321-31.
- Grate, J. W., Ozanich, R. M., Jr., Warner, M. G., Marks, J. D., and Bruckner-Lea, C. J. (2010). Advances in assays and analytical approaches for botulinum-toxin detection. *Trends in Analytical Chemistry* 29(10), 1137-1156.
- Hakami, R. M., Ruthel, G., Stahl, A. M., and Bavari, S. (2010). Gaining ground: assays for therapeutics against botulinum neurotoxin. *Trends Microbiol* 18(4), 164-72.
- Hale, M., Oyler, G., Swaminathan, S., and Ahmed, S. A. (2011). Basic tetrapeptides as potent intracellular inhibitors of type A botulinum neurotoxin protease activity. *J Biol Chem* 286(3), 1802-11.

- Hill, K. K., Smith, T. J., Helma, C. H., Ticknor, L. O., Foley, B. T., Svensson, R. T., Brown, J. L., Johnson, E. A., Smith, L. A., Okinaka, R. T., Jackson, P. J., and Marks, J. D. (2007). Genetic diversity among Botulinum Neurotoxin-producing clostridial strains. *J Bacteriol* 189(3), 818-32.
- Inoue, K., Fujinaga, Y., Watanabe, T., Ohyama, T., Takeshi, K., Moriishi, K., Nakajima, H., Inoue, K., and Oguma, K. (1996). Molecular composition of *Clostridium botulinum* type A progenitor toxins. *Infect Immun* 64(5), 1589-94.
- Jacobson, M. J., Lin, G., Tepp, W., Dupuy, J., Stenmark, P., Stevens, R. C., and Johnson, E. A. (2011). Purification, modeling, and analysis of botulinum neurotoxin subtype A5 (BoNT/A5) from *Clostridium botulinum* strain A661222. *Appl Environ Microbiol* 77(12), 4217-22.
- Kalb, S. R., Garcia-Rodriguez, C., Lou, J., Baudys, J., Smith, T. J., Marks, J. D., Smith, L. A., Pirkle, J. L., and Barr, J. R. (2010). Extraction of BoNT/A, /B, /E, and /F with a single, high affinity monoclonal antibody for detection of botulinum neurotoxin by Endopep-MS. *PLoS One* 5(8), e12237.
- Kalb, S. R., Goodnough, M. C., Malizio, C. J., Pirkle, J. L., and Barr, J. R. (2005). Detection of botulinum neurotoxin A in a spiked milk sample with subtype identification through toxin proteomics. *Anal Chem* 77(19), 6140-6.
- Kalb, S. R., Moura, H., Boyer, A. E., McWilliams, L. G., Pirkle, J. L., and Barr, J. R. (2006). The use of Endopep-MS for the detection of botulinum toxins A, B, E, and F in serum and stool samples. *Anal Biochem* 351(1), 84-92.
- Kirchner, S., Kramer, K. M., Schulze, M., Pauly, D., Jacob, D., Gessler, F., Nitsche, A., Dorner, B. G., and Dorner, M. B. (2010). Pentaplexed quantitative real-time PCR assay for the simultaneous detection and quantification of botulinum neurotoxin-producing clostridia in food and clinical samples. *Appl Environ Microbiol* 76(13), 4387-95.
- Lindström, M., Keto, R., Markkula, A., Nevas, M., Hielm, S., and Korkeala, H. (2001). Multiplex PCR assay for detection and identification of *Clostridium botulinum* types A, B, E, and F in food and fecal material. *Appl Environ Microbiol* 67(12), 5694-9.
- Lindström, M., and Korkeala, H. (2006). Laboratory diagnostics of botulism. *Clin Microbiol Rev* 19(2), 298-314.
- Niemeyer, C. M., Adler, M., and Wacker, R. (2005). Immuno-PCR: high sensitivity detection of proteins by nucleic acid amplification. *Trends Biotechnol* 23(4), 208-16.
- Niwa, K., Koyama, K., Inoue, S., Suzuki, T., Hasegawa, K., Watanabe, T., Ikeda, T., and Ohyama, T. (2007). Role of nontoxic components of serotype D botulinum toxin complex in permeation through a Caco-2 cell monolayer, a model for intestinal epithelium. *FEMS Immunol Med Microbiol* 49(3), 346-52.
- Nuss, J. E., Wanner, L. M., Tressler, L. E., and Bavari, S. (2010). The osmolyte trimethylamine N-oxide (TMAO) increases the proteolytic activity of botulinum neurotoxin light chains A, B, and E: implications for enhancing analytical assay sensitivity. *J Biomol Screen* 15(8), 928-36.
- Oguma, K., Fujinaga, Y., and Inoue, K. (1995). Structure and function of *Clostridium botulinum* toxins. *Microbiol Immunol* 39(3), 161-8.
- Ohishi, I., Sugii, S., and Sakaguchi, G. (1977). Oral toxicities of *Clostridium botulinum* toxins in response to molecular size. *Infect Immun* 16(1), 107-9.

- Peck, M. W., Plowman, J., Aldus, C. F., Wyatt, G. M., Izurieta, W. P., Stringer, S. C., and Barker, G. C. (2010). Development and application of a new method for specific and sensitive enumeration of spores of nonproteolytic *Clostridium botulinum* types B, E, and F in foods and food materials. *Appl Environ Microbiol* 76(19), 6607-14.
- Pellett, S., Tepp, W. H., Clancy, C. M., Borodic, G. E., and Johnson, E. A. (2007). A neuronal cell-based botulinum neurotoxin assay for highly sensitive and specific detection of neutralizing serum antibodies. *FEBS Lett* 581(25), 4803-8.
- Raphael, B. H., Joseph, L. A., McCroskey, L. M., Luquez, C., and Maslanka, S. E. (2010). Detection and differentiation of *Clostridium botulinum* type A strains using a focused DNA microarray. *Mol Cell Probes* 24(3), 146-53.
- Rasetti-Escargueil, C., Jones, R. G., Liu, Y., and Sesardic, D. (2009). Measurement of botulinum types A, B and E neurotoxicity using the phrenic nerve-hemidiaphragm: improved precision with in-bred mice. *Toxicon* 53(5), 503-11.
- Scarlato, A., Welt, B. A., Cooper, B. Y., Archer, D., DeMarse, T., and Chau, K. V. (2005). Methods for detecting botulinum toxin with applicability to screening foods against biological terrorist attacks. *Journal of Food Science* 70(8), 121-130.
- Schantz, E. J., and Kautter, D. A. (1978). Standardized assay for *Clostridium botulinum* toxins. *Journal of the AOAC* 61(1), 96-99.
- Scotcher, M. C., Cheng, L. W., and Stanker, L. H. (2010). Detection of botulinum neurotoxin serotype B at sub mouse LD(50) levels by a sandwich immunoassay and its application to toxin detection in milk. *PLoS One* 5(6), e11047.
- Sharma, S. K., Eblen, B. S., Bull, R. L., Burr, D. H., and Whiting, R. C. (2005). Evaluation of lateral-flow *Clostridium botulinum* neurotoxin detection kits for food analysis. *Appl Environ Microbiol* 71(7), 3935-41.
- Sharma, S. K., Ferreira, J. L., Eblen, B. S., and Whiting, R. C. (2006). Detection of type A, B, E, and F *Clostridium botulinum* neurotoxins in foods by using an amplified enzyme-linked immunosorbent assay with digoxigenin-labeled antibodies. *Appl Environ Microbiol* 72(2), 1231-8.
- Sharma, S. K., and Whiting, R. C. (2005). Methods for detection of *Clostridium botulinum* toxin in foods. *J Food Prot* 68(6), 1256-63.
- Simpson, L. L. (2004). Identification of the major steps in botulinum toxin action. *Annu Rev Pharmacol Toxicol* 44, 167-93.
- Simpson, L. L., Maksymowych, A. B., Park, J. B., and Bora, R. S. (2004). The role of the interchain disulfide bond in governing the pharmacological actions of botulinum toxin. *J Pharmacol Exp Ther* 308(3), 857-64.
- Singh, B. R. (2000). Intimate details of the most poisonous poison. *Nat Struct Biol* 7(8), 617-9.
- Smith, T. J., Hill, K. K., Foley, B. T., Detter, J. C., Munk, A. C., Bruce, D. C., Doggett, N. A., Smith, L. A., Marks, J. D., Xie, G., and Brettin, T. S. (2007). Analysis of the neurotoxin complex genes in *Clostridium botulinum* A1-A4 and B1 strains: BoNT/A3, /Ba4 and /B1 clusters are located within plasmids. *PLoS One* 2(12), e1271.
- Smith, T. J., Lou, J., Geren, I. N., Forsyth, C. M., Tsai, R., Laporte, S. L., Tepp, W. H., Bradshaw, M., Johnson, E. A., Smith, L. A., and Marks, J. D. (2005). Sequence variation within botulinum neurotoxin serotypes impacts antibody binding and neutralization. *Infect Immun* 73(9), 5450-7.
- Solomon, H. M., and Lilly, T. J. (2001). Chapter 17. *Clostridium botulinum*. *Bacteriological Analytical Manual, 8th Edition, Revision A, 1998*.

- Stahl, A. M., Ruthel, G., Torres-Melendez, E., Kenny, T. A., Panchal, R. G., and Bavari, S. (2007). Primary cultures of embryonic chicken neurons for sensitive cell-based assay of botulinum neurotoxin: implications for therapeutic discovery. *J Biomol Screen* 12(3), 370-7.
- Stanker, L. H., Merrill, P., Scotcher, M. C., and Cheng, L. W. (2008). Development and partial characterization of high-affinity monoclonal antibodies for botulinum toxin type A and their use in analysis of milk by sandwich ELISA. *J Immunol Methods* 336(1), 1-8.
- Wictome, M., Newton, K., Jameson, K., Hallis, B., Dunnigan, P., Mackay, E., Clarke, S., Taylor, R., Gaze, J., Foster, K., and Shone, C. (1999). Development of an in vitro bioassay for *Clostridium botulinum* type B neurotoxin in foods that is more sensitive than the mouse bioassay. *Appl Environ Microbiol* 65(9), 3787-92.
- Wilder-Kofie, T. D., Luquez, C., Adler, M., Dykes, J. K., Coleman, J. D., and Maslanka, S. E. (2011). An alternative in vivo method to refine the mouse bioassay for botulinum toxin detection. *Comp Med* 61(3), 235-42.
- Wu, H. C., Huang, Y. L., Lai, S. C., Huang, Y. Y., and Shaio, M. F. (2001). Detection of *Clostridium botulinum* neurotoxin type A using immuno-PCR. *Lett Appl Microbiol* 32(5), 321-5.

IntechOpen



## **Bioterrorism**

Edited by Dr. Stephen Morse

ISBN 978-953-51-0205-2

Hard cover, 192 pages

**Publisher** InTech

**Published online** 28, March, 2012

**Published in print edition** March, 2012

This book consists of nine chapters, written by international authorities, discussing various aspects of bioterrorism preparedness and response. Five of the chapters are agent-specific and highlight the pathogenesis, prevention and treatment, and the potential of specific organisms (*Rickettsia* and *Yersinia pestis*) or toxins (ricin, botulinum neurotoxins, and staphylococcal enterotoxins) to be used for nefarious purposes. Four chapters discuss different aspects of detecting and responding to a bioterrorism attack. These include methods for spatio-temporal disease surveillance, international laboratory response strategies, detection of botulinum neurotoxins in food and other matrices, and the use of physical methods (ie Raman spectroscopy) to detect spores.

### **How to reference**

In order to correctly reference this scholarly work, feel free to copy and paste the following:

Luisa W. Cheng, Kirkwood M. Land and Larry H. Stanker (2012). Current Methods for Detecting the Presence of Botulinum Neurotoxins in Food and Other Biological Samples, *Bioterrorism*, Dr. Stephen Morse (Ed.), ISBN: 978-953-51-0205-2, InTech, Available from: <http://www.intechopen.com/books/bioterrorism/botulinum-neurotoxins>

**INTECH**  
open science | open minds

### **InTech Europe**

University Campus STeP Ri  
Slavka Krautzeka 83/A  
51000 Rijeka, Croatia  
Phone: +385 (51) 770 447  
Fax: +385 (51) 686 166  
[www.intechopen.com](http://www.intechopen.com)

### **InTech China**

Unit 405, Office Block, Hotel Equatorial Shanghai  
No.65, Yan An Road (West), Shanghai, 200040, China  
中国上海市延安西路65号上海国际贵都大饭店办公楼405单元  
Phone: +86-21-62489820  
Fax: +86-21-62489821

© 2012 The Author(s). Licensee IntechOpen. This is an open access article distributed under the terms of the [Creative Commons Attribution 3.0 License](#), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

IntechOpen

IntechOpen