Prevalence of Botulinum Neurotoxin C1 and its Corresponding Gene in Environmental Samples from Low and High Risk Avian Botulism Areas

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Summary

Botulinum neurotoxin C1 (BoNt C1) and its corresponding gene were detected in seven aquatic habitats covering a range of low (LR) to high risk (HR) avian botulism outbreak areas during a study period of 10 months. Toxin and gene in sediment and avian faecal samples were analysed before (in situ) and after cultivation (in vitro) by a newly adapted ELISA, the common mouse bioassay and by a recently described nested PCR protocol. BoNt C1 gene fragments were detected in 74% and 83% of all investigated sediment samples by in situ PCR and in vitro PCR, respectively, at comparable frequencies in HR and LR areas. Similar high values were also observed for faecal samples. No BoNt C1 could be detected in the sediment in situ, while 53% and 56% of all cultivated samples contained BoNt C1 as detected in the mouse bioassay and the ELISA, respectively. The percentage of BoNt C1 positive cultivated samples was significantly higher (2-fold) in HR areas than in LR areas. Hence, our data clearly indicate an increased ratio of potentially BoNt C1 producing clostridia to BoNt C1 genes as the frequency or likelihood of botulinum epizootics increases in the environment. In addition, the good correlation between the results from the ELISA and the mouse bioassay for all sediment and faecal samples ($r=0.90$, $p<0.001$, $n=121$) indicates a high potential for the ELISA to reduce/replace the mouse bioassay for the detection of BoNt C1 in environmental samples.

Zusammenfassung: Vorkommen des Botulinum Neurotoxins C1 und des korrespondierenden Gens in Umweltproben von Gebieten mit hoher sowie geringer Wahrscheinlichkeit eines Vogelbotulismus-Ausbruchs

Das Vorkommen von Botulinum Neurotoxin C1 (BoNt C1) sowie des korrespondierenden Toxin-Gens wurde in sieben aquatischen Habitaten (Feuchtgebieten) mit geringer bis hoher Wahrscheinlichkeit für Vogelbotulismus über 10 Monate hinweg gemessen. Das Toxin und sein korrespondierendes Gen wurden im Sediment und in Vogelfäkalien sowohl vor Kultivierung (in situ) als auch nach Kultivierung (in vitro) vergleichend mit einem neu adaptierten ELISA, dem Maus-Bioassay und einem PCR-Ansatz analysiert. Das BoNt C1 Gen-Fragment wurde in 74% bzw. 83% aller untersuchten Sedimentproben durch in situ PCR bzw. in vitro PCR mit vergleichbarer Häufigkeit sowohl in Gebieten mit hoher als auch mit geringer Vogelbotulismus-Wahrscheinlichkeit festgestellt. Ähnlich hohe Prozentsätze ergaben sich auch bei der Analyse der Vogelfäkalien. Kein BoNt C1-Toxin konnte im Sediment in situ nachgewiesen werden, jedoch wurden in 53% bzw. 56% aller kultivierten Proben (in vitro) BoNt C1 im Maus-Bioassay bzw. ELISA detektiert. Im Gesamten war der Anteil an BoNt C1 positiven Proben von Vogelbotulismusgebieten mit hoher Ausbruchswahrscheinlichkeit nach Kultivierung um das 2-fache (signifikant) höher, verglichen mit kultivierten Proben aus Gebieten mit geringer Ausbruchswahrscheinlichkeit. Methodisch wurde eine hohe Korrelation zwischen den Ergebnissen von ELISA und Maustest für die Sediment- und die Vogelfäkalien-Proben festgestellt ($r=0,90$; $p<0,001$; $n=121$). Dies unterstreicht das hohe Potenzial des entwickelten ELISAs als mögliche Reduktions- bzw. Ersatzmethode für den Maus-Bioassay bei der Detektion von BoNt C1 in Umweltproben.

Keywords: avian botulism, botulinum neurotoxin C1, mouse bioassay, ELISA, PCR, environmental samples, toxin expression potential, toxin gene distribution, Clostridium botulinum type C1

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1 Introduction

Avian botulism is one of the most important diseases of wild migratory birds worldwide (Jensen and Price,1987; Locke and Friend, 1987; Rocke et al., 1999). Outbreaks are mainly caused by consumption of food items that contain botulinum neurotoxin type C1 (BoNt C1) (Smith et al., 1982; Mitchell and Rosendal, 1987; Rocke and Samuel, 1993). The toxin is produced by toxigenic clostridia containing a pseudolysogenic tox+ phage encoding for the BoNt C1 gene (Eklund et al., 1987; Fujii et al., 1988). Avian botulism outbreaks primarily occur in wetlands, presumably when favourable conditions allow cell/spore growth/germination and toxin production (Mitchell and Rosendal, 1987; Wobeser et al., 1987; Smith and Sugiyama, 1988; Rocke and Samuel, 1999). While it is an accepted fact that insect larvae feeding on vertebrate carcasses often contain high amounts of BoNt C1 toxin (Duncan and Wayne, 1976; Hubalek and Halouzka, 1991; Wobeser, 1997), there has been a lot of controversy about other wetland associated toxin sources, such as benthic microenvironments (e.g. macro-evertebrates) or the sediment itself (Bell et al., 1955; Haagsma et al., 1972). Currently, there is no consensus on the initial mechanism and necessary ecological conditions that lead to toxin production and epizootics (Marion et al., 1983; Rocke and Brand, 1994; Wobeser, 1997; Rocke et al., 1999; Rocke and Samuel, 1999). The distribution of toxigenic BoNt C1 clostridial spores was not considered to be a limiting factor for avian botulism outbreaks in wetlands, although toxigenic spores have been found more frequently in high risk areas than in low risk areas (Haagsma, 1973; Borland et al., 1977; Smith et al., 1978; Wobeser et al., 1987). More recently, Sandler et al. (1993) found no difference in the prevalence of *C. botulinum* type C1 in marshes with high and low botulism losses and Rocke and Samuel (1999) suggested that chemophysical factors, such as water pH and temperature, play a more important role in determining whether a wetland is at high or low risk for a botulism outbreak. It seems likely that toxin production and subsequent botulism outbreaks are largely controlled by complex interactions of various ecological factors, but current knowledge is inadequate to favour one explanation (Wobeser et al., 1987; Sandler et al., 1993; Sandler et al., 1998).

The investigation of BoNt C1 production in the environment demands efficient investigation techniques. Traditionally, the presence of BoNt C1 has been based on detection in the mouse bioassay applied to extracts gained from cultivation or from direct extraction (Sandler et al., 1993; Wobeser et al., 1987; Haagsma, 1987). The mouse bioassay still represents the most sensitive standard method of BoNt C1 detection (Hatheway, 1988). As an *in vitro* alternative, enzyme-linked immunosorbent assays (ELISA) have been described for particular types of samples (Notermans and Kozaki, 1987; Thomas, 1991; Rocke et al., 1998). In addition to toxin detection, the prevalence of BoNt C1 encoding clostridial spores and cells has been determined by PCR, either after incubation of the samples in enrichment media (Franciosa et al., 1996; Szabo et al., 1993) or directly from the sediment (Williamson et al., 1999). Both ELISA and PCR are still in the development stage, but nonetheless, the combination of these methods bears great potential for analysing complex biological processes on different functional levels (Wictome et al., 1999; McGrath et al., 2000; Kimura et al., 2001).

The aim of this study was to apply currently available molecular biological techniques (PCR, ELISA) in combination with the traditionally used mouse bioassay to generate a more complete picture of the interrelation between BoNt C1 gene distribution and the potential for BoNt C1 production in the environment. For this purpose we selected an area with 7 adjacent aquatic habitats with low and high avian botulism outbreak risk during the past two decades. Besides investigating the respective sediment areas of the habitats as done previously (Sandler et al., 1998; Sandler et al., 1993), we extended the survey to faeces of aquatic birds in this area, as they can be assumed to be potentially important transfer vectors between widely separated wetlands (Milton, 1992).

The results of this study suggest for the first time a significant difference between the distribution of toxin expressing BoNt C1 clostridia and their corresponding BoNt C1 genes across low and high botulism risk areas.

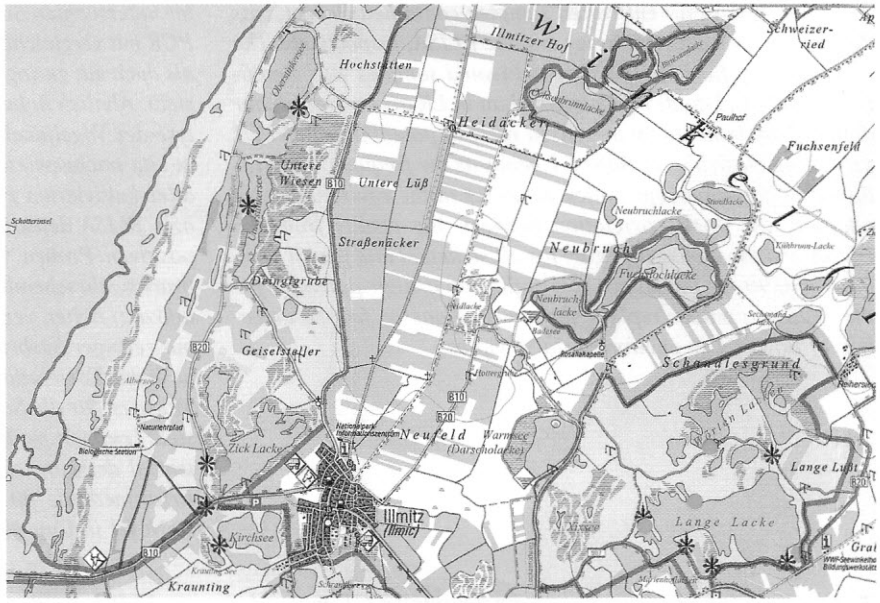

Fig. 1: National park Neusiedler See – Seewinkel and its shallow saltwater pools. Sampling sites are indicated by arrows. Arrows with horizontal lines indicate high risk areas (HR), vertically lined arrows indicate low risk areas (LR).

2 Animals, materials and methods

2.1 Sampling and sampling sites

Samples were collected from seven aquatic habitats located in the Austrian national park Neusiedler See – Seewinkel (Fig. 1), that have shown different risk potentials for botulism outbreaks during the past two decades (Grüll et al., 1987; Grüll et al., 2000). Four high risk and three low risk areas were chosen. High risk areas (HR) were defined as habitats that exhibited an average mortality (20 years observation period) of more than 200 birds per km^2 and year. Low risk areas (LR) were defined as habitats where little or no avian mortality (on average <20 birds per km^2 and year) was recorded. It should be mentioned that no massive epidemics occurred during the study period and animal losses were 42 registered individuals in the LR and 1308 registered individuals in the HR area. The national park is well known for the high number of endangered bird species, which use its shallow saline pools for breeding and resting. Table 1 provides an overview of important sediment characteristics of the high risk and low risk aquatic habitats. A detailed description of the microbial ecology of the investigated pools and the methodology used to determine the parameters in Table 1 can be found elsewhere (Kirschner et al., 2002; Eiler et al., 2003).

Sample collection took place in biweekly to monthly intervals from May 2000 to January 2001. Approximately 35 g of the top 10 cm of sediment, including the oxic (0-1 cm) and the anoxic sediment layer (1-10 cm), as well as 3 to 5 samples of fresh avian faeces (visually sediment free), were collected at 10 different locations at each sampling site. Both the sediment and faecal samples were then pooled in one sterile 200 ml centrifugation tube (Nalgene, Vienna, Austria) per location. 75 mixed invertebrate samples (fly larvae) were collected from the intestinal tracts of bird carcasses (n=173) with sterile forceps. All samples were stored at 4 °C and processed within 24 h of collection. After homogenisation with a potter, sediments and faecal samples were processed as described below, according to the scheme presented in Figure. 2.

2.2 *In situ* isolation and purification of DNA from sediment and faecal samples

Briefly, 0.25 g sediment or avian faeces were extracted using MOBIO Ultra-Clean Soil-DNA-Isolation-Kits (Prolabo, Paris, France), according to the manufacturer's instructions. For cell and phage lysis the bead beating process was performed with a Vortex genie 2 (Scientific Industries, Vienna) at full speed for 10

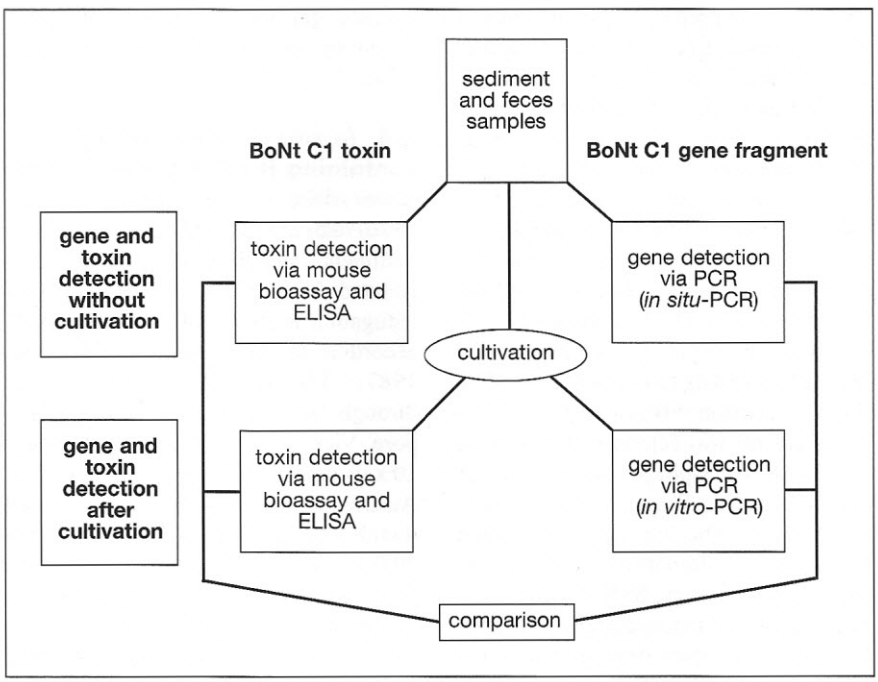

Fig. 2: Sample processing. Samples were analysed for BoNt C1 toxicity via mouse bioassay and immunostick ELISA as well as for the occurrence of BoNt C1 gene fragments via PCR with and without cultivation, respectively.

Tab. 1: Sediment characteristics of high and low risk botulism areas considering seven shallow saltwater pools in the national park Neusiedler See – Seewinkel, Austria.
Parameters were measured biweekly at representative locations in the year 2000 (n = 36); values represent median and range in parenthesis.

		pH	WC (%FM) [a]	OM (%DM) [a]	BN (10^9 ml^{-1}) [a]	MZB (Ind. m^{-2}) [a]
HR [b]	aerobic	9.6 (8.4-11.0)	60.3 (31.7-77.5)	6.0 (2.7-13.3)	4.0 (1.3-9.9)	710 (20 - 4400)
	anaerobic	8.0 (7.0-10.0)	38.3 (19.1-60.8)	4.3 (1.0-11.1)	2.3 (0.5-7.3)	n.d.
LR [b]	aerobic	9.4 (8.2-10.3)	47.2 (25.8-82.1)	3.2 (0.8-11.0)	2.3 (0.9-7.4)	120 (0 - 505)
	anaerobic	8.6 (7.1-10.2)	25.7 (13.9-59.2)	1.5 (0.7-5.1)	1.0 (0.4-2.4)	n.d.

[a] WC: water content; FM: fresh mass; OM: organic matter; DM: dry mass; BN: bacterial numbers; MZB: macrozoobenthic organisms
[b] HR: high risk area; LR: low risk area

min at 4 °C. Quality and quantity of the extracted bacterial DNA was tested with Low DNA Ladder standards (Life Technologies, Vienna, Austria) by 1.2% agarose gel electrophoresis and visualised by ethidium bromide staining. The method allows DNA extraction from vegetative bacteria and bacteriophages, while spores remain intact. Cell suspensions for seeding experiments were prepared by incubation of the *C. botulinum* strain 468 type C in oxygen free cooked-meat medium. Cells were counted with a Neubauer haemocytometer (Hecht Assistent, Vienna, Austria). Aliquots were prepared and stored at -80 °C. Spore suspensions were prepared by inoculation of *C. botulinum* strain 468 type C spores after incubation at 75 °C for 20 min in sporulation medium according to Segner et al. (1971). After 5 days incubation at 30 °C the spores, remaining cells and debris were harvested by centrifugation. After washing 3 times with double-distilled H_2O, the preparation was sonicated several times and the remaining cell debris including DNA fragments was digested by trypsin, lysozyme and DNase I according to Grecz et al. (1962). Phage extraction efficiency from different sediments was determined by spiking with DNA free λ-phage suspensions (donation from Udo Blaesi, Vienna Biocenter, Austria). The λ-phage was chosen because of its similarities in DNA content and morphology. BoNt C1 free sediments and avian faeces comparable to the HR and LR samples were spiked with a serial dilution of cells, spores, and λ-phages, respectively. DNA extraction was performed immediately as described above, the yield of the DNA was observed by gel electrophoresis and visualised by ethidium bromide staining.

2.3 Isolation and purification of DNA from sediment and faecal samples after cultivation

0.25 g of sediment and avian faeces were incubated at 37 °C in 10 ml anaerobic cooked meat medium (CMM) consisting of 1.5% casein peptone (Merck, Vienna, Austria), 0.5% dibasic potassium phosphate (Sigma, Vienna, Austria), 0.5% yeast extract (Difco, Vienna, Austria), 0.05% L-cysteine-HCl (Merck, Vienna, Austria), 0.3% glucose (Merck, Vienna, Austria), 1.25 g cooked meat (Difco, Vienna, Austria), and 100 µl vitamin K-hemin solution (Bacton & Dickinson, Vienna, Austria). After 24 h, 1 ml of the overnight culture was transferred to a new CMM cultivation tube and incubated again for 3 days at 37 °C. A volume of 1 ml of the supernatant was used for the isolation of DNA with InstaGene Matrix (BioRad, Vienna, Austria) according to the manufacturer's instructions (Zechmeister et al., 2002). Quality and quantity of the extracted DNA was tested with Low DNA Ladder standards (Life Technologies, Vienna, Austria) by 1.2% agarose gel electrophoresis and visualised by staining with ethidium bromide.

2.4 *In situ* detection of toxin containing fluids from sediment (interstitial water) and invertebrate samples

Sediment interstitial water (n=77) was isolated from 300 g sediment by centrifugation at 1500 x g for 10 min at 4 °C, according to Haagsma et al. (Haagsma, 1987). The supernatant was filtered through 0.22 µm syringe filters (Millipore, Vienna, Austria) and concentrated 10 x using dialysis tubes (Sigma, Vienna, Austria) with a 12 kDa molecular cut off membrane with polyethylene glycol 20.000 (Merck, Vienna, Austria) under gentle agitation at 4 °C. The optimal conditions for the concentration as well as for the extraction process were evaluated at the beginning of the study using a standard dilution series of purified BoNt C1 from *C. botulinum* 468 and toxin detection by mouse bioassay (n=77) and ELISA (n=21). For determination of the extraction efficiency, toxin-containing solutions were incubated with homogenised toxin free sediments under defined conditions and isolated as described above. Extraction efficiency was 10% to 15% (n = 5). The concentration process by dialysis was evaluated by serial dilution and concentration experiments. The dialysis process could concentrate the extracted toxin 8.6-9.4 fold (n=5). Thus the final concentration was between 0.7 fold and 1.2 fold of the original concentration of the sediment. The resulting overall detection limit of toxin extraction in our investigated sediments was 5 MLD_{50} per ml sediment. No fluid could be extracted from faecal samples because of the low liquid content. For toxin extraction from invertebrate larvae, insects were homogenised in gelatine phosphate buffer and refrigerated overnight at 4 °C (Duncan and Wayne, 1976; Hubalek and Halouzka, 1991). The extract was centrifuged and the supernatant was made aseptic by filtration through a 0.22 µm syringe filter (Millipore, Vienna, Austria). The presence of toxin in the solutions was determined by mouse bioassay only (see below).

2.5 Detection of toxin-containing fluids from sediment and faecal samples after cultivation

For detection of toxin after cultivation, the supernatant from each enrichment culture (described above) was made aseptic by filtration and tested by mouse bioassay and immunostick ELISA.

2.6 Detection of BoNt C1 gene fragments

PCR assays (50 µl reaction mixtures) were performed with a DNA thermal cycler (MWG Primus 25, Munich, Germany) using DNA extracted from both uncultivated and cultivated samples (*In situ*-PCR and *In vitro*-PCR, respectively)(c.f. Fig. 2). The amplification reaction mixtures (reaction volume 100 µl) contained 1 x PCR buffer (50 mM KCl, 10 mM Tris-HCl; pH 8.3), 3 mM $MgCl_2$, 0.2 mM of each desoxynucleoside triphosphate, 0.6 µM of each primer, 1 U of Hi-Expand-PCR-polymerase (Roche, Germany), 40 µg BSA (Roche, Germany), and 2 µl of DNA template in a 0.5 ml thin-walled, polypropylene PCR tube (MWG, Munich, Germany) without a layer of oil. The primer pairs ToxC-625, ToxC-1049R and ToxC-625, ToxC-850R were used for the initial and nested PCR approach, respectively, as described by Williamson, et al. (Williamson et al., 1999). The reaction was heated to 80 °C for 5 min prior to the addition of the desoxynucleoside triphosphates, the second primer, and Hi-Expand-PCR-polymerase. For the initial PCR an amplifica-

tion profile of 95 °C for 60 sec, 56 °C for 30 sec and 72 °C for 90 sec was performed in touch-down modus (-0.1 °C of the annealing temperature/cycle) for 30 cycles, followed by one cycle at 72 °C for 10 min. The nested PCR was performed using 3 µl of the initial PCR product. The amplification profile was similar to the initial approach, but the cycle number was reduced to 15 in touch-down modus (-0.2°C of the annealing temperature/cycle). Initially, the specificity of the applied PCR protocol was determined with different BoNt toxigenic and non-toxigenic clostridial strains, namely NCTC 7272 Type A, NCTC 7273 Type B, Type E Beluga (provided by A. Binder, Institute of Medical Service, Muenster, Germany), 468 Type C (provided by M. Popoff, Pasteur Institute, Paris, France), 2257 Type C, 2300 Type C, 2145 Type C, 2142 Type D, 2301 Type D (provided by F. Gessler, Georg-August-University of Goettingen, Department of Animal Hygiene in the Tropics, Germany).

The *in situ* sensitivity of the whole direct detection approach (extraction, purification, PCR) was assayed by *C. botulinum* type C1 free sediment inoculation with defined numbers of BoNt C1 cells according to Williamson et al. (1999) and was in the range of 50 vegetative cells per 0.25 g sediment. In the case of the λ-phage we could detect 500 phages per 0.25 g sediment as revealed by spiking experiments. Primer Lambda 1 (5'–TCAGCCAAACGTCTCTTC-3') was located between bp 37.226 and 37.245, and primer Lambda 2 (5'-AAGAGCAGCTTGAGGACG-3') was located between bp 37.913-37.894 within the C1 repressor region of the λ-genome. PCR mixture and amplification cycles were as described above, but the annealing temperature was 56 °C instead of 55 °C.

Negative and positive controls were included in each PCR. The PCR products were separated by 1% agarose gel electrophoresis (Life Technologies, Vienna, Austria) in TAE buffer. The resulting bands were stained by adding ethidium bromide (Sigma, Vienna, Austria) at a final concentration of 1 µg/ml to the molten gel. DNA fragments were visualised under a UV illuminator (302 nm) (Spectroline model TR-302 transilluminator).

2.7 Toxin detection

The immunostick ELISAs were prepared and used for toxin detection of cultivated and representative uncultivated samples as described by Rocke et al. (1998) with the following modifications: Immunosticks were coated with affinity-purified rabbit BoNt C1 antitoxin (5 µg/ml) on the bottom half. Antibody-coated immunosticks were incubated with 1 ml sterile filtered supernatant of cultivated samples or with concentrated interstitial sediment water for 24 h at 4 °C under gentle agitation. Affinity-purified horse BoNt C1 antitoxin (CDC, Atlanta, Georgia) diluted to 10 µg/ml in milk diluent was added for 1.5 h at 37 °C under gentle agitation. After 3 washing steps peroxidase labelled goat anti-horse antibody (1.0 mg/ml; Kirkegaard & Perry) was added in milk diluent for 1.5 h at 37 °C under gentle agitation. TMB peroxidase (Kirkegaard & Perry) treatment was applied to the immunosticks. Positive reactions were indicated by purple coloration only at the rabbit antibody-coated bottom half of the immunostick. The specificity was tested using stock cultures of several strains of *Clostridium botulinum* types C and D as well as of strains of *Clostridium botulinum types* A, B, E described above and with semipurified type C botulinum toxin (Wako Chemicals USA, Inc., Richmond, Virginia, USA). The sensitivity of the ELISA was 0.12 ng toxin per ml, using defined amounts of the type C botulinum toxin (Wako Chemicals, USA) as a standard. No cross-reaction between peroxidase labelled goat anti-horse antibody and rabbit BoNt C1 antitoxin was visible (data not shown). No cross-reactions between type C1 and type D strains were observed.

Mouse bioassays were performed by inoculating culture filtrates into 6-week-old Swiss/ICR mice (Charles River, Germany), according to the general methods described by Quortrup and Sudheimer (1943). Culture supernatants or interstitial sediment water concentrates were filtered through a 0.22 µm syringe filter and mixed 5:1 with Hanks medium (15,000 units penicillin G, 1,000 units mycostatin, 5% glycerin, 15 mg streptomycin-sulphate, 200 µg gentamycine in 1 l Hanks balanced salt solution; pH 7.6) to inhibit bacterial growth. Two mice were required for each test sample. One was inoculated intraperitoneally with 0.2 ml type C Clostridium botulinum antitoxin produced in horses (Center of Disease Control, Atlanta, USA). After 30 minutes, both the antibody-protected and a non-protected mouse were inoculated intraperitoneally with 0.5 ml of the test sample. The mice were observed for 5 days post inoculation for signs of botulism, such as hind-limp paralysis, wasp waist, laboured breathing, and death.

2.8 Statistical analyses

All statistical analyses were performed with SPSS 10.0 for Mac. Spearman rank correlations were regarded as significant at a significance level of $p \leq 0.05$. Mann-Whitney U-test was used to compare high and low risk areas.

3 Results

3.1 *In situ* BoNt C1 gene, protein and toxicity detection in samples

In 74% of all investigated sediment samples the BoNt C1 gene fragment could be detected by *in situ* PCR. Similar values were observed for faecal samples (Tab. 2). No BoNt C1 *in situ* toxicity could be detected by the mouse bioassay in any of the analysed sediment samples throughout the investigation period (Tab. 2). The negative mouse bioassays were further corroborated by the negative ELISAs of a representative set of samples (Tab. 2), suggesting that no detectable amount of toxin was produced in the sediment itself during the period of investigation. In contrast, 21 out of 75 maggot samples from bird carcasses (28%) collected in the study area contained easily detectable amounts of BoNt C1 toxicity (by mouse bioassay) and protein (by ELISA) in the mixed invertebrate larvae extracts. All of the BoNt C1 positive maggots were found in HR (70 bird carcasses with maggots), but not in LR (5 carcasses with maggots).

3.2 BoNt C1 gene, protein and toxicity detection in samples after cultivation

The BoNt C1 gene fragment could be detected in 83% of the total analysed samples by the *in vitro* PCR, which was about 10% higher as compared to the non-cultivated approach in all types of samples (Tab. 2). This showed that cultivation increased the average percentage of detectable BoNt C1 gene fragments. Only a portion (53%) of the cultivated samples produced enzymatically active BoNt C1 toxin as detected in the mouse bioassay. With the immunostick ELISA, 56% of all samples were BoNt C1 positive (Tab. 2). *In vitro* PCR resulted in more positive samples for the BoNt C1 gene fragment in enriched cultures as compared to protein and toxicity detection by immunostick ELISA and the mouse bioassay (83% *versus* 53% and 56%; Tab. 2). The same trend described for sediment samples was also observed in the avian faecal samples, although the frequency of positive BoNt C1 production during cultivation was lower (Tab. 2).

3.3 Comparison of samples from low and high botulism risk areas

To compare the detection of BoNt C1 by mouse test and ELISA between samples from HR and LR, only cultivated samples were considered, because *in situ* samples were all negative. The percentage of samples tested positive by mouse test and ELISA was significantly higher (about 2-fold) in four HR than in the three LR sediments (Tab. 2; Mann-Whitney U-test; both p=0.03). For faecal samples, higher percentages of positive samples were again observed in HR, although the differences were not statistically significant (Tab. 2; Mann-Whitney U-test; p=0.11 and 0.37, respectively). The percentage of detectable BoNt C1 gene fragments in cultivated sediment and faecal samples was not different between HR and LR for *in vitro* PCR (Tab. 2, Mann-Whitney U-test; p=0.48 and 0.86, respectively) or for *in situ* PCR (Tab. 2, Mann-Whitney U-test; p=0.59 and 0.11, respectively). Thus, the results indicated an about 2-fold higher BoNt C1 toxin expression of cultivated samples from HR compared to LR, although percentages of BoNt C1 gene fragments could be detected at comparable frequencies in both areas.

Table 3 shows all possible combinations of the results of the mouse bioassay, the two PCR approaches and their frequencies in LR and HR areas. The combinations numbered 1, 2, and 3 were observed more frequently for the HR (50x) than for the LR (15x). If positive gene detection was observed by *in situ* PCR and/or *in vitro* PCR, the probability of a positive mouse bioassay was 3.3x higher for the HR than for the LR. On the other hand, the combinations numbered 4, 5 and 6 were observed more frequently in the LR (31x) than in the HR (17x), showing a 1.8x higher probability of a negative mouse bioassay for LR than for HR, although the BoNt C1 gene fragment could be detected by *in situ* PCR and/or *in vitro* PCR. Combination number 7 with neither gene fragment nor *in vitro* toxicity detection was observed slightly more often in LR than in HR, whereas combination 8 was never observed.

3.4 Comparison of detection techniques for BoNt C1

For cultivated samples from HR areas, ELISA results were highly correlated with results of the mouse bioassay (Tab. 4; r =0.90; p<0.001) with 98% of all samples showing corresponding results. Only one sample yielded a positive result in the ELISA but was negative in the mouse bioassay. No sample yielded a positive result for BoNt C1 in the mouse bioassay when the ELISA was negative. Both PCR approaches resulted in a very low correlation with the BoNt C1 mouse bioassay. Only the *in situ* PCR approach was statistically significantly correlated (Tab. 4), with 80% of all samples showing corresponding results with the mouse bioassay. 7% (*in situ* PCR) and 9% (*in vitro* PCR) of the samples yielded posi-

Tab. 2: Positive detection of BoNt C1 and corresponding gene fragments in environmental samples.

sample type	total number of samples	mouse bioassay[a] cultivated		non cultivated		ELISA[a] cultivated		non cultivated		PCR[a] cultivated *in vitro*[a] PCR		non cultivated *in situ*[a] PCR	
		n[b]	p[b]	n	p	n	p	n	p	n	p	n	p
total samples	121	64	53%	0[c]	%	68	56%	0[d]	0%	100	83%	90	74%
HR[a] sediments	46	40	87%	0	0%	41	89%	0[e]	0%	39	85%	37	80%
LR[a] sediments	31	12	39%	0	0%	13	42%	0[f]	0%	25	81%	23	74%
HR[a] faeces	24	10	42%	n.d.	n.d.	11	46%	n.d.	n.d.	20	83 %	16	67%
LR[a] faeces	20	3	15%	n.d.	n.d.	4	20%	n.d.	n.d.	16	80 %	15	75%

[a] ELISA: immunostick ELISA; PCR: nested PCR approach; *in vitro* - PCR: PCR of samples after cultivation; *in situ* - PCR: PCR of uncultivated samples; HR: high risk area; LR: low risk area
[b] n: number of positive samples; p: percentage of total number
[c] n = 77
[d] n = 21
[e] n = 12
[f] n = 9

tive results when the mouse bioassay was negative. A significant positive correlation was observed between the *in situ* PCR approach and ELISA, with 83% of all samples showing corresponding results, whereas no correlation was found with the *in vitro* PCR approach (Tab. 4).

For sediment samples from LR areas and avian faeces from both HR and LR areas, the ELISA was also highly correlated with the mouse bioassay (r=0.94; p£0.001 for LR sediments, r =0.92; p£0.001 for HR faeces and r=0.49; p£0.03 for LR faeces), with 97%, 96% and 85% corresponding results, respectively. No correspondence of the mouse bioassay and the ELISA was found with either PCR approach for these samples. Positive results were obtained in 45% (LR sediments), 40% to 44% (HR faecal samples) and 60% to 70% (LR faecal samples) of these samples with both PCR methods, when the ELISA and mouse bioassay were negative.

4 Discussion

4.1 Methodological aspects of toxin detection

The high correlation of the immunostick ELISA with the mouse bioassay found in this study suggests this *in vitro* assay as a practical alternative for the detection of BoNt C1 in environmental samples after cultivation. Up to now, except for a smaller set of samples from the same environment (Zechmeister et al., 2002), this method has only been applied to non-cultivated samples like blood sera (Rocke et al., 1998). Our results further indicate its potential use for the *in situ* detection of BoNt C1 in environmental samples where increased amounts of toxin may occur (e.g. fly larvae). We observed a slight tendency to a higher number of positive results (5 in total) compared to the mouse bioassay. This may be explained by binding of non or low functional toxins with a slightly different protein structure caused by single base mutations within the toxigenic gene (Oguma et al., 1984; Gregory et al., 1996), by protein denaturation, or by the co-occurrence of related strains with lower toxicity (Lee and Riemann, 1970). The ELISA detects epitope structures of the BoNt C1, either active or inactive, in contrast to the mouse bioassay, which detects only active toxin.

4.2 Methodological aspects of DNA detection

Although *in situ* PCR vs. *in vitro* PCR gave different information on the occurrence of BoNt C1 gene targets, as proven by correlation analysis (e.g. Tab. 4), more than two thirds of the pooled samples
revealed corresponding results (Tab. 3). Whereas the applied *in situ* PCR targeted BoNt C1 genes in vegetative cells, tox^+ phages or naked DNA in the sediment, the *in vitro* PCR additionally covered targets resulting from cell/spore growth/ germination after cultivation. The high number of corresponding cases suggests a sufficient amount of BoNt C1 gene targets present in the sediment that can be detected whether or not cultivation is used. Nonetheless, in 18% of the analysed samples, only the *in vitro* PCR detection (Tab. 3) yielded positive results, suggesting that growth of cells/spores during cultivation led to detectable target concentrations and thus increased sensitivity. Whether initial BoNt C1 targets in the sediment could not be detected because initial targets were simply below sensitivity and/or only pronounced spore concentrations were available, remains open. In future, fractionated DNA extraction distinguishing spores, phages and vegetative cells could solve this question. In contrast, 14 cases occurred in which only *in situ* PCR could detect BoNt C1 targets. These cases were likely caused either by target degradation during cultivation, out-dilution effects of, and/or growth-multiplication of sequences not amenable to the primer sequences used. However, for a more detailed investigation of such scenarios in future, comparative sequence type analysis of recov-

Tab. 3: Possible combinations of the results of the mouse bioassay and the *in vitro*-PCR and *in situ*-PCR approaches, and observed frequencies in low risk areas (LR) and high risk areas (HR).

no.	mouse bioassay	*in vitro*-PCR	*in situ*-PCR	LR n (%)	HR n (%)
1	+	+	+	9 (7)	34 (28)
2	+	+	-	4 (3)	11 (9)
3	+	-	+	2 (2)	5 (4)
4	-	+	+	24 (20)	10 (8)
5	-	+	-	3 (3)	4 (3)
6	-	-	+	4 (3)	3 (3)
7	-	-	-	5 (4)	3 (3)
8	+	-	-	0 (0)	0 (0)

Tab. 4: Spearman rank correlations of cultivated samples (n = 70) of high risk areas.

	mouse bioassay		ELISA[a]		*in vitro* - PCR[a]	
	r[b]	p[b]	r	p	r	p
ELISA[a]	0.902	0.000				
in vitro - PCR[a]	0.195	0.193	0.046	0.759		
in situ - PCR[a]	0.297	0.045	0.356	0.015	-0.056	0.710

[a] ELISA: immunostick ELISA; *in vitro* - PCR: nested PCR of DNA extracts of sediments and faeces after cultivation; *in situ* - PCR: nested PCR of DNA extracts of uncultivated sediments and faeces

[b] r: Spearman rank correlation coefficient; p: level of significance

ered PCR amplicons from *in situ* versus cultivation approaches would be helpful, as demonstrated already for other microorganisms (Farnleitner et al., 2000). In addition, the detection sensitivity of *in situ* PCR could be increased by methodical improvements such as primer and fluorescent probe combinations with real time PCR detection (Kimura et al., 2001; Stubner, 2002). Nonetheless it is well known that inhibitory substances, crude DNA, or reduced extraction efficiencies lead to a low PCR performance/sensitivity when soil or sediment with high organic matter content or salinity (Szabo et al., 1993; Winthingerode et al., 1997) or faecal samples (Franciosa et al., 1996) are analysed. This sets some inherent sensitivity limits on PCR based direct detection approaches.

It is notable, that when both PCR results were negative (combination 8, Tab. 3), toxin was not detected in any case. On the other hand, if the combined BoNt C1 PCR results (*in vitro* PCR and *in situ* PCR) had been used to monitor the BoNt C1 production potential in the considered environmental samples (i.e. BoNt C1 production during CMM cultivation), this would have led to a significant overestimation of the BoNt C1 expression potential, which is indicated by the lower number of positive toxicity tests and ELISAs (Tab. 2; Tab. 3). Thus the qualitative BoNt C1 PCR approach cannot be recommended to monitor/infer functional BoNt C1 expression during CMM cultivation by replacing the mouse bioassay or the ELISA approach. It may be that competition among bacterial species during cultivation results in poor growth of *C. botulinum*, preventing toxin production in the cultured sample. Thus, the lower percentage of *in vitro* toxin expression potential in comparison with the PCR results may be underestimations of the toxin expression potential in the field. Nevertheless, as demonstrated in this study, a sensitive combination of *in vitro* PCR, *in situ* PCR and ELISA/mouse bioassay leads to a more complete picture of the molecular situation of BoNt C1 toxigenesis in a given habitat (c.f. ecological implications). Future PCR applications will have to evaluate the significance of quantitative data on BoNt C1 gene abundance (Kimura et al., 2001) as well as BoNt C1 amplicon sequence information (Muyzer et al., 1996).

4.3 Ecological implications

This study experimentally indicates that BoNt C1 genes are quite abundant and ubiquitous in wetlands inhabited by bird populations, whilst BoNt C1 toxin expressing clostridia are more frequently encountered in habitats where avian botulism epidemics have been observed in previous years. A concentration effect of toxigenic BoNt C1 clostridia cells/spores in wetlands with documented losses due to botulism has been reported by several authors (Haagsma, 1973; Smith et al., 1978; Wobeser et al., 1987). Only Sandler et al. (1998), in a more recent study, did not find a difference in the prevalence of toxigenic BoNt C1 clostridia in marshes with high and low losses to avian botulism. However the authors mentioned that avian botulism epizootics had occurred in all investigated marshes in the longer past. Up to now, except for the primary method-focused work on the *in situ* detection of BoNt C1 fragments in the environment (Farnleitner et al., 2000; Witcome et al., 1999), nothing has been reported on the relationship between BoNt C1 gene abundance and the occurrence of BoNt C1 expressing clostridia.

According to the estimated *in situ* PCR sensitivity in this study, a number of at least 200 BoNt C1 gene targets per gram sediment or faeces were present in 80% and 74% of HR and LR samples (Tab. 2). As demonstrated in this study, the abundance of BoNt C1 genes in the environment does not necessarily reflect the prevalence of BoNtC1 toxigenic clostridia able to express active BoNt C1. In principle, the detected BoNt 1 gene could have been present in the sediment as free or adsorbed DNA fragments, as DNA in tox^+ phages and as a gene in clostridial populations. Furthermore, portions of the detected BoNt C1 DNA could have been partially hydrolysed. In addition, BoNt C1 clostridial spores could not be detected in by the selected *in situ* PCR this study, thus anticipated BoNt C1 gene targets should be even higher in the investigated environment and the interpretations can be considered conservative.

On a molecular basis, successful neurotoxin production depends on several factors and mechanisms, i.e. the availability of BoNt C1 genes, their transcription of mRNA products, successful protein translation, as well as post-translational toxin activation (Minton, 1995; Johnson and Bradshaw, 2001). Successful toxin expression requires a tox^+ phage carrying the BoNt C1 gene in a pseudolysogenic state in the respective clostridial cell. Disturbance of the relationship between the pseudolysogenic phage and the cell results in a loss of BoNt C1 toxicity (Eklund et al., 1987; Hariharan and Mitchell, 1976; Eklund et al., 1989). On the other hand, aneurotoxigenic strains can be converted to neurotoxigenic strains (BoNt C1 producers) by infection with specific bacteriophages as shown in laboratory experiments, by Eklund et al. (1987). The extent of bacterial conversion in nature is unknown, but an increase in conversion events in toxigenic BoNt populations in a wetland may be related to the occurrence of epizootics (Eklund et al., 1987). Furthermore, like the faeces of the birds reflected the BoNt C1 gene and toxigenic cell distribution of the environment (Tab. 2), birds could act as important transmission vectors of functional BoNt C1 expression units and/or parts of them (Lamanna, 1987).

From a tox^+ phage's ecological point of view, it does not seem surprising, that an environment (as indicated by the detected frequencies in this study, Tab. 2) harbours more BoNt C1 genes than corresponding functional BoNt C1 expression units. It is well known that several lysogenic phages replicate inside the cell and become lytic if the environment turns unfavourable (Hariharan and Mitchell, 1976; Eklund et al., 1987). So called "burst sizes" of phages are known at the level of 1000 to 100,000 virus particles per lysed bacterium, thus increasing phage DNA abundance (Gold, 1950) in the surrounding environment. Hence, the ratio of BoNt C1 gene abundance to toxigenic BoNt C1 clostridia abundance (BG/BC) should increase as the frequen-

cy or likelihood of epizootics decreases. This theoretical prediction is in agreement with the data from the investigated area, indicating an about 2-fold significantly increased averaged BoNt C1 toxin expression ability of cultivated samples from HR compared to LR, although percentages of BoNt C1 gene fragments could be detected at comparable frequencies across the whole area (Tab. 2).

Considering possible *in situ* BoNt C1 sources for bird populations during the investigation period, only insect larvae (maggots) feeding on bird carcasses could be found as relevant environmental toxin compartments within the considered habitats. No BoNt C1 was detectable in the sediments, including sediment water and macrozoobenthos, although the samples underwent a 10-fold concentration by dialysis of the extracts. Thus, in this study, maggots feeding on carcasses can be suspected to be dominant locations in which massive growth of toxigenic BoNt C1 clostridia with subsequent BoNt C1 production occurred. Hence, the surrounding environment (i.e. HR area) could have been contaminated by toxigenic BoNt C1 clostridia from carcasses, resulting in a decreased BG/BC ratio (see above). The detected sources of BoNt C1 toxin in this study are in agreement with a wealth of literature available supporting the hypothesis that carcasses/maggots are the main locations of the *in situ* BoNt C1 toxigenesis in wetlands (Wobeser, 1997). However, during this study no massive outbreak occurred and losses of birds resembled an endemic occurrence of botulism, which was recently suggested to be a common form of avian botulism between epidemic events (Reed and Rocke, 1992; Wobeser, 1997). Our results should thus not be extrapolated to situations in which epidemics occur/have occurred. It should be mentioned that an extended two year study at the same locations revealed possible trace amounts of BoNt C1 in ≤10% of the sediment pore water as well as macrozoobenthos samples (Farnleitner et al., 2003). These concentrations were at the detection limit of the mouse bioassay (no mice died although they showed symptoms of botulism) and the exposure effect and risk assessment revealed no relevance for bird intoxications during the investigated period (Farnleitner et al., 2003).

In conclusion, the present study suggests that BoNt C1 genes are abundant and ubiquitously distributed in wetlands inhabited by bird populations, whereas the ratio of BoNt C1 gene to toxigenic clostridia decreases as the likelihood of favourable conditions for the growth and maintenance of BoNt C1 toxigenic clostridia increases. Future investigations should be undertaken to test whether this prediction is applicable on a global scale and equally for outbreak and non-outbreak situations or whether compartments other than carcasses provide suitable environments (e.g. sediment – microenvironments) to maintain a pseudolysogenic phage – cell relationship. The good correlation between the results from the ELISA and the mouse bioassay for all sediment and faecal samples ($r = 0.90$, $p < 0.001$, $n = 121$), indicates the high potential of the used ELISA to reduce/replace the mouse bioassay for detection of BoNt C1 in environmental samples.

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