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Real-time PCR for *Clostridium botulinum* type C neurotoxin (BoNTC) gene, also covering a chimeric C/D sequence—Application on outbreaks of botulism in poultry

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ABSTRACT

In recent years, botulism type C has become a serious problem in poultry flocks in Sweden. A real-time PCR assay for *Clostridium botulinum* (*C. botulinum*) type C neurotoxin (BoNTC) gene was developed as an alternative to the mouse bioassay for detection and identification of *C. botulinum* type C. The complete method consists of an optimized enrichment protocol followed by automated DNA extraction prior to real-time PCR. The sensitivity of the PCR assay was determined with purified DNA to approximately 50 copies per PCR reaction. The specificity of the PCR assay was evaluated on a panel of about thirty relevant bacteria and on samples of caecum from birds collected in connection with botulism outbreaks on Swedish poultry farms. The PCR assay also covers a previously reported chimeric C/D sequence of the gene. Caecum samples from the outbreaks were positive by real-time PCR. Some of these samples were also examined with a set of conventional PCR methods, to distinguish the gene for the chimeric form from the conserved type C gene. Interestingly, the caecum samples were found to be positive for the chimeric C/D sequence. This is the first study in Europe demonstrating the chimeric C/D sequence. When the toxin gene in two of the samples was sequenced, it was closely identical (99–100%) with several previously reported C/D chimeric sequences. DNA extraction and the real-time PCR assay were both performed in a 96-well format, facilitating for future large-scale detection in outbreak situations and prevalence studies.

1. Introduction

Botulism is a neurotoxic disease that has become a serious problem in Swedish poultry flocks during recent years (Blomqvist et al., 2007) and has also been reported in waterfowl in Sweden (Neimanis et al., 2007). The causative organism is *C. botulinum*, a universal anaerobic spore-forming bacterium present in soil and aquatic environments. It produces botulinum neurotoxin (BoNT), which occurs in eight different types, A–G. In most cases of botulism in Swedish poultry and wild birds, the toxin has been found to be neutralised by types C and D antitoxins. Japanese studies have shown that the neurotoxin gene in isolates from cases of avian botulism comprises two-thirds of the BoNTC gene and one-third of the BoNTD gene (Takeda et al., 2005). The genes for types C and D toxins are carried by bacteriophages (Sakaguchi et al., 2005), which can be lost during subcultivation and sporulation.

In botulism, the intoxication is usually caused by ingestion of preformed toxin or less commonly after ingestion of spores. Inability to detect preformed toxin in the environment of poultry units has been reported in most of outbreaks, including those in Sweden. It has therefore been suggested that the intoxication results from proliferation of *C. botulinum* in the intestine of affected birds, as in human infant botulism (Quinn et al., 1994).

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Confirmation of botulism requires the demonstration of BoNT in the serum of suspected animals (Quinn et al., 1994). Further, the traditional method for detection and identification of the organism *C. botulinum* includes enrichment of anaerobic culture, followed by testing for type-specific toxin. The current reference method for toxin detection is the mouse bioassay, an expensive and time-consuming method, requiring the use of laboratory animals (NMKL, 1991). We describe here the development of a real-time PCR for the BoNTC gene, also covering a chimeric C/D sequence, to be used for detection in outbreak situations and prevalence studies.

2. Materials and methods

2.1. Animals and samples

Caecum samples from 15 poultry collected at the time of botulism outbreaks on three different Swedish poultry farms (A–C) was used in the study (Table 1). The farms were located in three different counties with no transference of animals between them. Samples from farms A and C were taken at the farm from euthanized birds. Samples from farm B were taken at slaughter. The caecum samples were stored at −70 °C until used. The clinical signs seen were elevated mortality, difficulties in eating and drinking, incoordination, ruffled feathers, flaccid paralysis of legs, neck and eyelids and finally paralysis of the breathing muscles causing respiratory failure. Caecum samples from healthy birds on farms without any indications of botulism were taken at slaughter and used as negative controls.

2.2. Mouse bioassay for detection of BoNT

The method was performed according to the Nordic Committee on Food Analysis (NMKL, 1991). Blood samples were collected from 10 poultry with clinical signs of botulism at the time of botulism outbreaks on farms A–C, and centrifuged. All sera from each farm were pooled to one sample and investigated. Adult white mice weighing approximately 20 g were used. For each test, two mice were injected intraperitoneally with 0.5 ml of serum and observed for up to 4 days for signs of botulism. Mice were euthanized if signs of botulism developed. Simultaneously, controls consisting of 0.5 ml of boiled sera (from each pooled sample) were injected into two mice respectively, to determine whether the toxic agent was thermolabile. The strength of the toxin was determined by diluting the sera with 0.9% NaCl (1:10, 1:100 and 1:1000) and injecting 0.5 ml of the dilutions into the mice. Identification was made by neutralisation with specific antitoxins of the various types of *C. botulinum* (A–E). Antitoxins (A, B, D and E) were obtained from the National Institute for Biological Standards and Controls, Hertfordshire, UK and type C antitoxin was developed at the National Veterinary Institute, Uppsala, Sweden from *C. botulinum* strain Stockholm. A mixture of 0.1 ml of antitoxin and 1 ml of the toxic sera of the lowest lethal dilution was allowed to stand at room temperature for 1 h before injection. Each animal was injected with 0.5 ml of the mixture and the testing was performed in duplicate for each mixture. Simultaneously, sera without antitoxin were injected into two other mice. The testing was approved by the Swedish Ethical Committee on Animal Experiments.

2.3. Enrichment

One gram of caecum sample was added to 9 ml pre-reduced tryptose–peptone–glucose–yeast extract (TPGY) medium (5% Tryptone [Difco], 0.5% Proteose Peptone [Difco], 0.4% glucose, 2% Yeast Extract [Oxoid], 0.1% starch [Merck] and 0.1% sodiumthioglycolate [Merck]), heat treated for 10 min at 70 °C and incubated under anaerobic conditions at 30 °C for 72 h. The enrichment cultures were stored at −70 °C for subsequent use.

2.4. DNA preparation

DNA was extracted from 150 to 200 μl enrichment culture using MagattractDNA mini M48 kit (Qiagen, Hilden, Germany) based on magnetic beads, in the Magnatrix automated nucleic acid extraction machine (NorDiag, Oslo, Norway). Elution was effected in 75 μl of AE Buffer (Qiagen) and the samples were stored at −20 °C. For comparison, a limited number of samples were also extracted in the vacuum filter based 6100 Nucleic Acid PrepStation (Applied Biosystems, Foster City, USA). To 150 μl enrichment culture, 150 μl proteinase K (20 mg/ml, Applied Biosystems) and 85 μl BloodPrep PK Digestion Buffer (Applied Biosystems) was added and incubated at 58 °C for 10 min. After addition of 500 μl BloodPrep DNA Purification Solution (Applied Biosystems), the sample was centrifuged (9000 × *g*, 5 min) and the supernatant was processed in the 6100 Nucleic Acid PrepStation, according to instructions from the manufacturer.

Table 1
Information on botulism outbreaks in Swedish poultry.

Farm	Type of holding (age of birds at sampling of caecum)	Year of botulism outbreak	Clinical signs of botulism	Neutralised with *C. botulinum* antitoxin by mouse bioassay[a]	No. of animals[b] positive for chimeric BoNTC/D gene (no. of tested animals)[c]
A	Broiler breeding farm (54 weeks)	2004	Yes	C and D	5 (5)
B	Broiler farm (35 days)	2006	Yes	C and D	6 (6)
C	Broiler farm (31 days)	2007	Yes	C and D	4 (4)

[a] Sera were analysed.
[b] Caecum samples were investigated.
[c] PCR according to Takeda et al. (2005), in order to distinguish the C/D chimeric BoNT gene from the conserved genes of BoNTC and BoNTD.