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Single-tube nested PCR assay for the detection of avian botulism in cecal contents of chickens

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ABSTRACT

This paper describes a novel diagnostic method for the detection of avian botulism caused by *Clostridium botulinum* type C and C/D, using single-tube nested PCR assay. This assay was developed to overcome the disadvantages of bioassays used in experiments with mice. Three primer pairs including an antisense primer were designed to target the N-terminal of the toxin gene from *C. botulinum* types C and C/D. The specificity of the PCR assay was confirmed by using 33 bacterial strains and chicken cecal contents from farms that experienced botulism outbreaks. The detection limit for purified DNA was 1.1 fg/μl, and for bacterial spores was 4.3 spores/200 mg of cecal contents. While checking for specificity of the PCR assay, the reactions with the templates form *C. botulinum* type C and C/D which were tested became positive, but the rest of the reactions turned negative. However, the results for all clinical samples (n = 8) were positive. The PCR assay results for cecal samples obtained from 300 healthy chickens (150 Korean native chickens and 150 broilers) were all negative. This assay is rapid and straightforward and evades ethical issues associated with mouse bioassay. Moreover, it is more economical than real-time PCR.

1. Introduction

Botulism is caused by the botulinum neurotoxin (BoNT) produced by *Clostridium botulinum*. The toxin inhibits the release of the neurotransmitter acetylcholine, causing paralysis [1]. Based on the serological properties of the toxins they produce, *C. botulinum* strains are divided into toxin types A to G [1]. Further, there are two mosaic strains, C/D and D/C based on the chimeric toxins they produce [2,3].

Although the mouse bioassay has been considered the "gold standard" for the diagnosis of botulism for years, it has major drawbacks: it is time consuming, requires an animal facility, and has issues regarding animal ethics [4–6]. To circumvent these issues and allow for speedy, sensitive, and specific high-throughput screening, we developed an alternative diagnostic method that uses single-tube nested PCR (STNP).

Although nested PCR can improve assay sensitivity, there are two main disadvantages: The protocol is more complex than conventional PCR and the risk of cross-contamination is considerably high. To overcome these problems, Michael et al. used antisense oligonucleotides to develop a single-tube nested PCR (STNP) method [7].

In Korea, Avian botulism have done harm to poultry farm. Although its occurrence is not frequent, its mortality is high (24.5%–58.3%) and the pathogen is hard to eradicate [8].

In this study, we describe the development of a STNP for the detection of avian botulism. Avian botulism is most commonly caused by *C. botulinum* type C or C/D [7–9], thus, we designed our assay to target the BoNT gene of this bacterial subgroups. We demonstrate how to (i) design and carry out the STNP assay, (ii) check the specificity and limit of detection (LOD), (iii) use the assay for clinical samples and (iv) investigate disease prevalence using the STNP technique.

2. Materials and methods

2.1. Bacterial strains

To determine the limit of detection (LOD), we isolated *C. botulinum* type C/D (strain KVCC 1400025) from chicken livers.

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Strains used for assessing the specificity of the STNP assay are listed in Table 1.

2.2. Media and enrichment

One colony of the *C. botulinum* strain KVCC 1400025 to determine the LOD, or 200 mg cecal content, to examine disease prevalence, were inoculated in 9 ml pre-reduced tryptose–peptone–glucose–yeast extract (TPGY) broth – 5% Tryptone (Difco, MI, USA),0.5% Proteose Peptone (Difco, MI, USA), 0.4% glucose, 2% yeast extract (Difco, MI, USA) and 0.1% starch (Sigma Aldrich, MO, USA) – supplemented with 0.1% L-cysteine HCl and 0.14% NaHCO3 (Sigma Aldrich, MO, USA), and heated at 70 °C for 15 min [10]. The samples were incubated at 37 °C under an anaerobic atmosphere using a GasPak™ EZ Anaerobe Container system (BD, ML, USA) in the Gaspak™ EZ container (BD, ML, USA) for 24 h. For cultivation and isolation of other bacteria, sheep blood agar (Kisanbio, Seoul, Korea) was used. *Clostridium* spp. were incubated under anaerobic conditions. *Campylobacter* spp. were incubated under micro-aerobic conditions using a GasPak™ EZ Campy Container system (BD, ML, USA) in the Gaspak™ EZ container (BD, ML, USA). All other bacteria were incubated under aerobic conditions. All bacterial species were incubated at 37 °C for 24 h.

2.3. Primer design

The primers used in this study are shown in Table 2. Three pairs of primers; outer, inner, and antisense, targeting the N-terminal part of both *C. botulinum* type C and C/D toxin genes, were designed. Designing was based on the consensus nucleotide sequences of the *C. botulinum* type C/D (*AB037166*, *AB745659*, *CP002411*) and type C BoNT genes (*X72793*, *AB061780*, *AB745658*). Primer specificity was examined *in silico* by carrying out BLAST search on GenBank. Primers were designed using the CLC Main Workbench software (CLC BIO, Aarhus, Denmark). T_m was estimated for each primer pair with reaction parameters set at 2.5 mM of $MgCl_2$ and 600 nM of primer.

Table 1
Bacterial strains used to evaluate the specificity of the PCR assay.

Gram staining	Spore formationc	Genus	Species	Type	Strain
G (+)[a]	+[c]	*Clostiridium*	*botulinum*	A	CVI[e] 61884
				B	QIA[f] 1-031-CBB-CO-044
				C	CVI CKIII-u
				D	CVI 16878
				C/D	BKT[g] 15925
					KVCC[h] BA1400020
					KVCC BA1400021
					KVCC BA1400022
					KVCC BA1400023
					KVCC BA1400024
					KVCC BA1400025
					D77[i]
					D35[j]
					Q547[k]
					Q587[l]
					Q621[m]
					Q668[n]
			novyi	A	ATCC[o] 17861
			difficile		ATCC 9689
			perfringens	A	KVCC BA1100001
					NCTC[p] 8359
				B	KVCC BA1100002
				C	KVCC BA1100003
				D	KVCC BA1100004
				E	KVCC BA1100005
		Bacillus	*subtilis*		ATCC 6633
			cereus		ATCC 11778
	–[d]	*Enterococcus*	*faecalis*		ATCC 29212
G (–)[b]	–	*Salmonella*	*enteritidis*		ATCC 13076
			typhimurium		ATCC 14028
		Escherichia	*coli*		ATCC 25922
		Campylobacter	*jejuni*		ATCC 33560
			coli		ATCC 43484

[a] G (+), Gram positive.
[b] G (–), Gram negative.
[c] +, spore forming.
[d] –, non-spore forming.
[e] CVI strains from the Central Veterinary Institute, Netherlands.
[f] QIA strain from Animal and plant quarantine agency, Korea.
[g] BKT strain form the Swedish National veterinary Institute.
[h] KVCC Korea Veterinary Culture Collection.
[i] D77 was isolated from a quail's liver in 2013.
[j] D35 was isolated from a layer's liver in 2014.
[k] Q547 was isolated from a Korean native chicken's liver in 2014.
[l] Q587 was isolated from a Korean native chicken's liver in 2014.
[m] Q621 was isolated from a pheasant's liver in 2014.
[n] Q668 was isolated from a mallard duck's cecum in 2014.
[o] ATCC American Type Culture Collection.
[p] NTCC National Collection of Type Cultures.