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Development of a Panel of Multiplex Real-Time Polymerase Chain Reaction Assays for Simultaneous Detection of Canine Enteric Bacterial Pathogens

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Abstract : A major cause of diarrhea in a dog is an infection with bacteria which include *Salmonella* spp., *Campylobacter* (*C.*) spp., and *Clostridium* (*Cl.*) spp.. It is fastidious to identify these bacteria by the culture. The purpose of this experiment is to devise the method for detecting *Cl. perfringens*, *C. jejuni*, *C. coli*, and *Salmonella* spp. with rapid and high sensitivity. The fecal samples collected from 71 normal and 66 diarrheic dog feces were used to compare the prevalence of the enteric pathogens and to develop a multiplex real-time polymerase chain reaction (PCR) assay for clinical use. Detection of *Cl. perfringens*, *C. coli*, and *C. jejuni* in diarrhea feces was higher than normal feces. A developed multiplex real-time PCR is useful for determining the presence and quantity of pathogen-specific or other unique sequences with in a fecal sample.

Key words **:** PCR, *Clostridium perfringens*, *Campylobacter jejuni*, *Campylobacter coli*, *Salmonella* spp.

Introduction

Common causes of diarrhea in dogs are gastrointestinal infections by bacteria, virus infection, wrong diet, and parasites. The cause of bacterial diarrhea is toxins and bacteria itself (8). The major causes of bacterial diarrhea are *Salmonella* spp., *Shigella* spp., *Escherichia* (*E.*) *coli*, *Campylobacter* (*C.*) spp., *Clostridium* (*Cl.*) spp., and so on. (1). Salmonella is a pathogen for zoonotic disease which is Gram-negative, facultative anaerobic, and motile. It is divided into 6 species in the family Enterobacteriaceae (9,12). Clinical signs of salmonellosis are acute or chronic gastrointestinal illness, fever, malaise, anorexia, vomiting, abdominal pain, watery or mucoid diarrhea, bloody diarrhea, and sepsis in severe cases in dogs. Campylobacter are Gram-negative, microaerophilic and motile bacilli. The most species are nonpathogenic but *C. jejuni* and *C. coli* are pathogenic (8,15). Acute campylobacteriosis can be accompanied by anorexia, intermittent vomiting, watery diarrhea or bloody mucoid diarrhea and fever. Clostridium also cause acute hemorrhagic diarrhea (10,14). These bacterial pathogens can be noticed by fecal culture. The culture condition is depends on a bacteria. *Campylobacter* sp. and *Salmonella* sp. are not growth under the general nutrient agar. *Clostridium* sp. is growth under the anaerobic conditions (10). It is important to detect the causative agent in bacterial diarrhea for the diagnosis and treatment in clinics. In general, bacterial detection performed via culture but it is difficult to detect the bacteria owing to fastidious culture condition. In this study, we reported a multiplex real-time PCR to detect a diarrhea causative bacterial pathogen with high sensitivity and accuracy.

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Materials and Methods

Fecal samples

A total of 137 fecal samples collected from 71 normal and 66 diarrheic feces were used to compare the prevalence of the enteric pathogens in normal and diarrheic canine fecal samples. DNA was extracted using E-sphere Simple NA kit (Phthisis Diagnostics, Daejeon, Korea) as described in the manufacturer's protocols. The extracted DNAs were stored at −20°C until being used in subsequent procedures.

Recombinant vectors

Recombinant vectors for *Cl. perfringens*, *C. jejuni*, *C. coli* and *Salmonella* spp. were constructed. Clinical isolates of each bacteria was amplified using the corresponding PCR primers in Table 1 and cloned into a pUC118 vector of a TA cloning kit (Genotech, Daejeon, Korea) as per the manufacturer's instructions. Heat shock-transformed *E. coli* competent cells were grown on LB plates in the presence of ampicillin. Insertion of the corrected target into the vector was verified by sequencing. Plasmid DNA was purified using a GenoAid™ Plasmid Purification Mini Kit (Genotech, Daejeon, Korea) and the amount of DNA was measured by spectroscopic analysis. Each plasmid was diluted in DEPC-treated distilled deionized water (ddH_2O) using a 10-fold serial dilution technique and used in assessing the analytic sensitivity.

Primers and probes

Sequences of primers and probes designed and used in the

Table 1. Oligonucleotide sequences of primers and probes for a multiplex real-time PCR

Target pathogen	Target gene	5'dye	3'dye	Primer sequence	Product Size (bp)
Campylobacter jejuni	OFR-C	FAM	TAMRA	F:TTGGTATGGCTATAGGAACTCTTATAGCT R:CACACCTGAAGTATGAAGTGGTCTAAGT P:TGGCATATCCTAATTTAAATTATTTACCAGGAC	115
Campylobacter coli	*ceu*E	HEX	TAMRA	F:AAGCTCTTATTGTTCTAACCAATTCTAACA R:TCATCCACAGCATTGATTCCTAA P:TTGGACCTCAATCTCGCTTTGGAATCATT	102
Clostridium perfringens	16S rDNA	FAM	TAMRA	F:CGCATAACGTTGAAAGATGG R:CCTTGGTAGGCCGTTACCC P:TCATCATTCAACCAAAGGAGCAATCC	105
Salmonella tyhimum, enteritidis	ttrBCA	HEX	BHQ1	F:CTCACCAGGAGATTACAACATGG R:AGCTCAGACCAAAAGTGACCATC P:CACCGACGGCGAGACCGACTTT	95

qPCR panel are summarized in Table 1. The panel was designed as two sets of qPCR assay consisting of PCR A (*C. jejuni* and *C. coli*) and PCR B (*Cl. perfringens* and *Salmonella* spp.). To minimize interference among reporting dyes for the probe, each assay comprised dyes in a combination of FAM and HEX.

PCR conditions

The panel was optimized using a QuantiTect® multiplex PCR kit (Qiagen, Valencia, CA, USA) by following the manufacturer's recommended protocols in a reaction volume of 20 μl. The PCR amplification was performed on Eco™ Real-time PCR system (Illumina, San Diego, CA, USA). Each reaction contained 0.4 μM of each primer, 0.2 μM of each probe, and 2 μl of template. The cycling condition was as follows: a 15 min initial activation step at 95°C, and 45 cycles of 60 sec at 94°C and 90 sec at 60°C.

Results

Prevalence of the enteric pathogens

Enteric pathogens were detected in 18/71 (25.4%) of fecal samples collected from normal dogs, whereas 42/66 (63.6%) from dogs with diarrhea. The detection frequency of each type of sample was *Cl. perfringens* (9/71, 12.7%), and *C. coli* (9/17, 12.7%) for normal feces, and *Cl. perfringens* (27/66, 40.9%), *C. coli* (14/66, 21.2%), and *C. jejuni* (1/66, 1.5%) for diarrheic feces (Table 2). None of samples were simultaneously detected more than two pathogens.

Table 2. Prevalence of the enteric pathogens in normal and diarrheic feces

Type of sample	Isolated bacteria	Frequency (%)
Normal feces (n = 71)	*Clostridium perfringens*	9 (12.6)
	Campylobacter coli	9 (12.6)
	Total	18 (25.4)
Diarrheic feces (n = 66)	*Clostridium perfringens*	27 (40.9)
	Campylobacter coli	14 (21.2)
	Campylobacter jejuni	1 (1.5)
	Total	42 (63.6)

Analytic sensitivity of the panel

The analytic sensitivity (i.e., detection limit) of the qPCR panel was estimated using serially-diluted recombinant vectors with known copy number per 1 ml. The estimated detection limit (molecules/ml) of the panel for each target bacteria was: 3.7 for *Cl. perfringens*, 3.7 for *C. jejuni*, 3.7 for *C. coli*, and 3.7 for *Salmonella* spp. Standard curves generated by the assay using 10-fold serial dilutions of each target genes

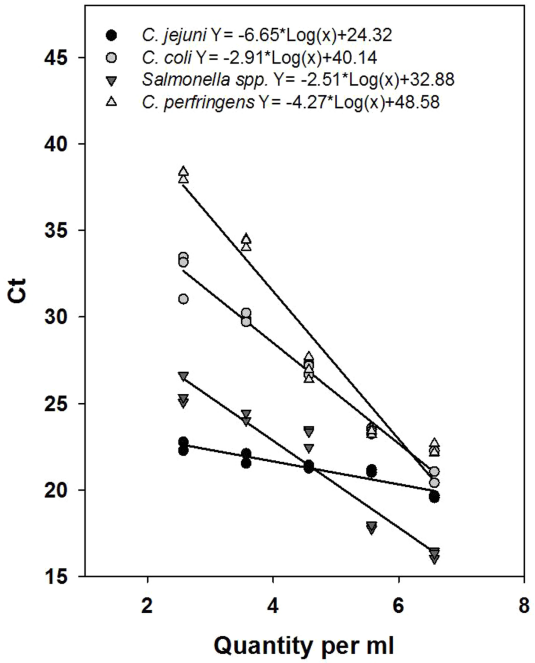

Fig 1. Standard curves of the qPCR panel. A series of 10-fold dilutions of recombinant vectors was prepared and used for assessing analytic sensitivity of the assay. The Y-axis indicates the Ct value of targets and each regression line was constructed based on triplicate measurement.

showed correlation coefficients (R_2) ranging from 0.885 to 0.974 and slopes of 2.51-6.65 (Fig 1), indicating acceptable linearity of the PCR reaction.

Discussion

Salmonella spp. and *Campylobacter* spp. are not cultured under the general nutrient medium (5). Incubation time is 24-48 hours and specificity of Salmonella culture is different depending on the type of medium (9). Clostridium can be incubated at 37°C in 5% CO_2 humidified atmosphere (5). Selective media has been effective for the isolation of *C. jejuni* and *C. coli*. The best temperature is 42°C and 48h incubation (2,3,7).

In this study, bacteria which are difficult to culture in the general nutrient media were detected in real-time PCR. Detection of *Cl. perfringens*, *C. coli*, *C. jejuni* in diarrhea feces was higher than normal feces. This proves that it have a high relationship between diarrhea and bacterial pathogens. *Cl. perfringens* have four of these toxin are classify this microorganism into five toxinotypes A, B, C, D and E. These toxins are correlated with gastrointestinal disease (6,11). *Cl. perfringens* toxin tests are required to distinguish a direct impact on the gastrointestinal infection. Previously reported, it was found that 58% of healthy dogs and 97% of diarrheic dogs shed detectable levels of *Campylobacter* spp. with *C. coli* and *C. jejuni* (4,13). *Campylobacter* spp. could be detected in feces from both the healthy and diarrheic dogs. However, in immunocompromised or febrile animals or hemorrhagic diarrhea, antimicrobial treatment may be needed (10,16).

Because it is difficult to detect bacteria that require demanding culture conditions and take a long time to be cultured and are difficult to be identified, it would be easy to detect the bacteria by qPCR for DNA extracted directly from feces. The molecular diagnosis of bacterial pathogen in patient with diarrhea is considered suitable for applying bacteria that culture sensitivity is low or costly and taking a long time to culture.

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개의 장내 병원균의 동시 검출을 위한 다중 실시간 중합효소연쇄반응분석 패널개발

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요 약 : 개에서 설사를 일으키는 주요 원인이 되는 장내 병원균으로 *Salmonella* spp., *Shigella* spp., *Campylobacter* spp., *Clostridium* spp. 가 있다. 이들 세균은 배양으로 검출이 어렵다. 본 실험에서는 *Salmonella* spp., *C. coli, C. jejuni,* 그리고 *Cl. perfringens* 를 신속하고 민감하게 검출할 수 있는 방법을 고안하였다. 정상견 71마리와 설사증상이 있는 66마리에서 수집한 분변 시료에서 장내 병원균의 유병률을 알아보고자 하였다. 장내 병원균은 실시간 중합효소연쇄 반응 분석을 이용하여 검출하였다. 설사변에서 *Salmonella* spp., *C. coli*, *C. jejuni*, *Cl.* Perfringens 는 정상변보다 검출률이 높았다. 개발한 다중실시간 중합효소연쇄반응은 분변시료의 병원균 존재 및 양 또는 기타 고유 서열을 확인하는데 유용하였다.

주요어 : 중합효소연쇄반응, *Cl. perfringens*, *C. jejuni*, *C. coli*, *Salmonella* spp.