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Short communication

Assessment of the *Campylobacter jejuni* and *C. coli* in broiler chicken ceca by conventional culture and loop-mediated isothermal amplification method

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ABSTRACT

In a two-year survey of the 24 Japanese broiler chicken flocks at 9 farms from 2013 to 2014, *C. jejuni*/*C. coli* prevalence was assessed in a total of 131 slaughtered broiler chicken cecal samples by conventional culture methods and loop-mediated isothermal amplification (LAMP) assay. While 93 samples were *C. jejuni*/*C. coli*-negative, 38 (29.0%) showed *Campylobacter* loads of between 6.4 and 9.0 log CFU/g of ceca in conventional culture methods. The performance of LAMP assay was 100% accurate in terms of diagnostic sensitivity (38/38), specificity (93/93). Furthermore, LAMP assay enabled direct screening of *C. jejuni* and *C. coli* in cecal samples from broiler chicken chickens as rapid and cost-effective detection within 90 min and less than 1 US dollar, which can help monitor release of *Campylobacter*-contaminated chicken into the food chain, thereby reducing the incidence and public health risk of campylobacteriosis. Seasonal changes in *C. jejuni* and *C. coli* prevalence in broiler chicken ceca were significantly correlated with the frequency of food poisoning incidents caused by these bacteria in Japan.

1. Introduction

The rising concern of consumers for food safety, in addition to the continuous public health impacts of foodborne campylobacteriosis, particularly from chicken, has prompted many governments and food safety agencies to target the prevention and/or control of this pathogen. Food safety agencies have reported that globally, *Campylobacter* is the most common bacterial cause of human gastroenteritis and a small percentage of patients with acute *Campylobacter* infection experience long-term and potentially serious sequelae (WHO, 2013, p. 57). The two most important species in food-borne infections in humans are *Campylobacter jejuni* and *C. coli*, and a major route of human infection is the handling and consumption of poultry meat (Jacobs-Reitsma, Bolder, & Mulder, 1994; Yamazaki et al.,2009, 2016).

Broiler chicken intestine, especially the cecum, has a high carriage rate of *Campylobacter*, and previous investigators have reported rates reaching up to 100% of total broiler chicken cecal samples tested (Berrang, Buhr, & Cason, 2000; Jacobs-Reitsma et al.1994). In addition, Rudi, et al. (2004) reported that *Campylobacter* content was higher in cecal samples than in fecal samples. If the cecum is handled improperly during processing and ruptures, it can release a large quantity of *Campylobacter* onto the carcass, potentially cross-contaminating numerous other broiler chicken carcasses. Although quantitative analyses of ceca have been reported to evaluate contamination risk to carcasses (Berndtson,

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Danielsson-Tham, & Engvall, 1996; Rudi et al., 2004), little is known about Japanese broiler chicken flocks. With respect to human infection, seasonal changes in *Campylobacter* prevalence in broiler chicken fecal and cecal samples have been reported (Kapperud et al., 1993; Llarena, Huneau, Hakkinen, & Hanninen, 2015).

One effective means of assisting the control and prevention of human campylobacteriosis is the rapid detection of the pathogen. There is a growing need for the standardization and validation of rapid and sensitive laboratory methods for specific and cost-effective detection and identification of zoonotic microorganisms. Presently, *Campylobacter* strains are still isolated and identified using conventional time-consuming culture methods. Loop-mediated isothermal amplification (LAMP) is a rapid and highly specific gene amplification assay (Notomi et al., 2000). In our previous studies, a LAMP assay was successfully used for the direct detection of *C. jejuni* and *C. coli* in naturally contaminated human stool samples (Yamazaki et al., 2008) and in enrichment broth cultures of the retailed chicken meat products (Yamazaki et al., 2009).

In the present study, we carried out quantitative isolation of *C. jejuni* and *C. coli* by using conventional culture methods, following to first employment of LAMP for the direct detection of *C. jejuni* and *C. coli* in broiler chicken cecal samples. Furthermore, the relationship between seasonal changes in *Campylobacter* prevalence in broiler chicken ceca and food poisoning frequency was statistically analysed.

2. Material and methods

2.1. Broiler chickens' selection and sample collection

Cecal samples were collected from 24 broiler chicken flocks belonging to 11 broiler chicken houses in nine farms in Kyushu, Japan, between August 2013 and December 2014. No samples were obtained between February and May 2014 due to farmers' preventative measures against highly pathogenic avian influenza (HPAI). Of the 24 flocks, 22 were sampled twice at approximately week 4 (days 20–38) and week 6 (days 36–49); the remaining two flocks were sampled at days 36 and 42. Three live broiler chickens from each population were randomly chosen and sent to our laboratory at the University of Miyazaki, where they were killed humanely in accordance with the University of Miyazaki guidelines for animal experiments (H19-02-22). Ceca were retrieved under aseptic conditions to obtain their contents. A total of 131 cecal samples were obtained.

2.2. Bacterial strains

Two *Campylobacter* standard strains capable of producing human campylobacteriosis were used to determine the detection limit of the LAMP assay: *C. jejuni* subsp. *jejuni* JCM2013 = ATCC29428 and *C. coli* JCM2529^{T} = ATCC33559 ^{T}. These were isolated from the diarrheic stool of a child, and pig feces, respectively.

2.3. Isolation of C. jejuni and C. coli from broiler chicken cecal samples

Campylobacter cells from cecal samples were isolated both by direct plating and by conventional culturing with selective enrichment followed by plating. For direct plating, serial 10-fold dilutions of the cecal samples in phosphate-buffered saline (PBS, pH 7.2) were prepared. Then, 100 μl of each cecal dilution between 10^{-1} to 10^{-6} was inoculated onto mCCDA agar (Oxoid Ltd, Basingstoke, Hampshire, England) (maximum enumerating number is 9.5 log CFU/g of ceca) and incubated under microaerobic conditions (approximately 8% O_2, 7%CO_2, and 85% N_2) at 42 °C for 44–48 h. For selective enrichment the protocol described in our previous study (Yamazaki et al. 2009). Shortly, the remaining cecal samples (left-over of the direct plating) were firstly enriched on 9 ml of Preston enrichment broth (Oxoid) supplemented with 5% (v/v) lysed horse blood, and the samples were then incubated at 42 °C for 20–24 h under microaerobic conditions. Secondly, an approximately 10 μl loopful of each Preston enrichment broth cultures were stroked onto Butzler and mCCDA using a disposable bacteria inoculating loop, and then, incubated at 42 °C for 44–48 h microaerobically.

LAMP assays and multi locus sequence typing (MLST) analysis were used to identify 161 isolated *Campylobacter*-like colonies from a randomly chosen three to five typical *Campylobacter*-like colonies in all 38 isolation positive samples as either *C. jejuni* or *C. coli* (Yamazaki et al., 2016).

2.4. Extraction of template DNA from broiler chicken cecal samples with a three-step centrifugation protocol

DNA extraction followed the procedure in our previous report (Yamazaki et al., 2008; 2009) with slight modification. After mixing using a delta mixer (Se-08; Taitec Co., Ltd., Kyoto, Japan), 0.5 ml of 10% cecal content prepared by using PBS (pH 7.2), the homogenate was transferred to a new, 1.5 ml microcentrifuge tube, followed by centrifugation at 900 × *g* (3300 rpm) for 1 min The supernatant was transferred to a new 1.5-ml microcentrifuge tube followed by centrifugation at 10,000 × *g* (10,800 rpm) for 5 min. Supernatant was discarded and the pellets were resuspended in 100 μl of NaOH (25 mmol/L), followed by mixing. The mixture was then heated at 95–100 °C for 10 min on a heat block. Then, 8 μl of Tris-HCl buffer (1 mol/L, pH 7.5) was added to neutralize the solution. The neutralized solution was centrifuged at 20,000 × *g* for 5 min at 4 °C. Supernatant was transferred to a new sterile tube and stored at −80 °C until use. 1 μl of extracted DNA was used as template DNA in the LAMP assay.

2.5. LAMP assay

The primer sequences of the LAMP assays were performed as previously described (Yamazaki et al., 2008, 2009), and the cost-effective homemade reagents were used according to the composition in the previous study (Yamazaki et al., 2014). Each LAMP reaction was incubated at 65 °C for 60 min, followed by 80 °C for 5 min to complete the reaction, and then assessed using a real-time turbidimeter (Loopamp EXIA; Teramecs, Kyoto, Japan). A duplex LAMP assay using a mixture of the two primer sets was also performed to effectively screen the two pathogens under the same conditions.

2.6. Determination of analytical sensitivity of the LAMP assay with spiked chicken broiler chicken cecal samples

Analytical sensitivity of the LAMP assay for detection of *C. jejuni* and *C. coli* in spiked chicken boiler cecal samples was determined as previously described (Yamazaki et al., 2008, 2009) with slight modification. The two *Campylobacter* reference strains were freshly prepared on blood agar (Oxoid) with overnight cultivation (16–18 h). One loopful (10 μl-diameter) of colonies was inoculated into PBS (pH 7.2). Then, serial 10-fold dilutions of *C. jejuni* and *C. coli* were separately prepared in PBS, then 100 μl of each dilution was mixed thoroughly with a mixture of ten *Campylobacter*-negative chicken cecal samples in a 15-mL polypropylene tube using delta mixer (Se-08; Taitec), then dispensed over 1.5 ml sterile micro-centrifuge tubes. Using the three-step centrifugation protocol