RESEARCH ARTICLE

Sensitivity of *Campylobacter jejuni* Virulence Genes flaA,mapA and hipO by Polymerase Chain Reaction

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Abstract

Campylobacter jejuni is more frequently associated with zoonotic bacterial cause of foodborne gastroenteritis in humans. Effective diagnosis of *C. jejuni* infection in various matrix like foods of animal origin, environment and human is crucial to maintain public health. Since molecular diagnosis of *C. jejuni* carries several advantages over conventional microbial culture techniques, in this study, the limit of detection for the selected virulence genes of *C. jejuni* (*flaA,mapA and hipO*) were evaluated to know the suitability of them to be used as molecular diagnostic targets for early detection of *C. jejuni* by in- house designed primers by conventional PCR. Analytical sensitivity of these genes were assessed in terms of Colony Forming Units (10^3 CFU/ mL, 10^2 CFU/ mL, 10^1 CFU/ mL, 10^0 CFU/ mL) and DNA concentration (100 ng,10 ng, 1 ng, 100 pg, 10 pg and 1 pg) of *C jejuni* cultures. Results revealed that the primers targeting *flaA* have superior limit of detection as 10 CFU and 1pg of *C. jejuni* DNA. Whereas *mapA* and *hipO* have 10 CFU; 10 pg DNA and 100 CFU; 10 ng DNA detection limit respectively. Gene *mapA* had comparable detection limits in terms of CFU with *flaA* but found to be less sensitive with respect to DNA concentration but *hipO* has 10^2 fold lesser limit of detection.

Key words: Analytical sensitivity, *C.jejuni*, *flaA*, PCR.

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Introduction

Globally, Campylobacteriosis is recognised as major food borne bacterial infection that cause intestinal health problems in humans. In general, higher incidence of clinical Campylobacteriosis is reported in young children (below 5 years) and elders (over 75 years) (Kaakoush *et al.,* 2015). Among 26 *Campylobacter* spp. recognized, *C. jejuni* is considered as the most pathogenic causative agent of human Campylobacteriosis (Kaakoush *et al.,* 2015). Ninety percent of human Campylobacter illness are caused primarily by *C.jejuni* (Lin, 2009). *C. jejuni* infections are self limiting but often leads to poor weight gain, accelerated effects of malnutrition and neuromuscular paralysis Guilain Barre syndrome (Attia *et al.,* 2016). However, poultry especially chicken remain as the major reservoir of food borne zoonotic campylobacteriosis worldwide (Young *et al.,* 2007).

In India, epidemiological studies of zoonotic Campylobacter infections are scarce due to poor surveillance and monitoring system. In addition, lack of epidemiological data is often linked to the difficulties related to time consuming conventional microbiological isolation techniques of this fastidious organism. In the recent years, molecular detection techniques like PCR are widely employed in surveillance network of food borne infections (Young *et al.,* 2007). Rapid detection and quantification of *C. jejuni* in different settings from poultry farm, poultry slaughter house, poultry meat products and also among human is crucial to develop an efficient epidemiological tracking and have proper understanding of *C.jejuni* transmission (Bessede *et al.,* 2011) .

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Whole genome sequencing of *C. jejuni* have demonstrated various virulence and toxin genes among these some are exclusively specific to *C. jejuni*. Of these virulence genes *flaA* responsible for effective colonization of gastro intestinal

tract, *mapA* an outer membrane protein that helps to escapes phagocytosis and *hipO* hippurtae hydrolysis, a signature biochemical feature of *C. jejuni* attracts various research interest (Steele *et al.,* 2006). However, comparative assessment of these genes in terms of sensitivity will help to develop quick and reliable diagnostic assays. With this backdrop the present work was designed to study the analytical sensitivity (limits of detection) and specificity of the above three genes which are pathogenic and highly conserved for *C.jejuni*.

Materials and Methods

Standard poultry origin *C. jejuni* isolate was procured from the Food borne pathogen repository maintained at Division of Veterinary Public Health, Indian Veterinary Research Institute (IVRI), Bareilly, India. Standard culture was used as positive reference throughout the PCR standardization after its molecular confirmation as per Linton *et al.,* (1996). Reference culture was grown in mCCDA broth and mCCDA agar with recommended supplements (Himedia) at 42°C at microaerophilic condition for overnight. The genomic DNA was isolated using GenElute™ bacterial genomic DNA extraction kit (Sigma-Aldrich) according to manufacturer's instructions.

Optimization of Gene Target Specific Polymerase Chain Reaction

Primer pairs specific for each of *C. jejuni flaA, mapA and hipO* genes were designed using DNASTAR Laser gene and synthesized at Synergy Genentech CO, India. (Table 1) for three different Polymerase Chain Reactions. Each PCR reaction was carried out in 25 µL reaction mixture containing 2.5 µL of 10X PCR buffer (Sigma Aldrich), 1.5 µL of 25 mM MgCl2, 0.5 µL of 200 µM each primer, 1 µL of dNTP (0.1 mM each) 5 units of polymerase enzyme, 1 µL of template DNA and 18 µL of nuclease free water. DNA from standard *C. jejuni* was used as positive control to standardize the PCR protocols. PCR reaction was carried out as mentioned in Table 2. The PCR products were analyzed by electrophoresis (45 min at 75 V) in 1.5% agarose gel and stained with ethidium bromide.

Analytical Ssensitivity and Specificity

Pure colonies of standard *C. jejuni* was adjusted to an optical density (OD) 1.0 at a wavelength of 600 nm and its corresponding colony forming units (CFU) was determined by limiting dilution plating. Broth with optical OD of 1.0 had 10^4 CFU /mL. From the 10^4 CFU/ mL of broth, serial dilutions were made in 4 tubes each containing 10^3 CFU/ mL, 10^2 CFU/ mL, 10^1 CFU/ mL, 10^0 CFU/ mL respectively. Then DNA was extracted from each dilution using bacterial genomic DNA extraction kit (Sigma-Aldrich) and DNA from each dilution was subjected to PCR individually, and the lowest concentration of colony forming units that showed a visible band in PCR was taken as the analytical sensitivity of gene targets in terms of CFU. Similarly eight lowering dilutions were prepared by 10 fold serial dilution of *C.jejuni* DNA (quantified by nanodrop) using Nuclease free water, such that 1 mL of each dilution containing 100 ng,10 ng, 1 ng, 100 pg, 10 pg and 1 pg, aliquotes were prepared. Stock and working solutions were stored at -20°C and 4°C respectively. The lowest concentration of DNA that showed a visible band in PCR was taken as the analytical sensitivity of gene targets. Specificity of the gene targets were assessed with the DNA of *Staphylococcus aureus*, *Salmonella Enterica, Escherichia coli, Listeria monocytogenes* and *Campylobacter coli* by PCR.

Results and Discussion

Optimization of Gene Target Specific PCR

PCR is largely dependent on the choice of the primers, reaction conditions, number of cycles and the presence of inhibitors etc., Of these, the identification of suitable gene target is the most critical one. Primer pairs targeting the selected gene targets *flaA, mapA and hipO* of *C. jejuni* successfully amplified the gene targets after extensive optimisation with DNA isolated from *C. jejuni* reference cultures and specific amplicons were formed (Fig. 1). The optimised conditions for each primer pair is provided in Table 2**.**

In fact, *flaA* gene responsible for production synthesis of flagellin, the main component of flagella is the deciding role in colonizing chicks and invading human intestinal epithelial cells in vitro (Astorga and Alonso, 2010; Baldvinsson et al., 2014). The 5′ and 3′ ends of flagellin gene (*flaA*) is highly conserved in the different *C. jejuni* species and hence utilization of primer sequence from these region guarantees the high specificity of the PCR assay (Wegmuller *et al.,* 1993;

Table 1: Primer sequences designed in the study

Organism	Primer	Primer sequence (5′ - 3′)	Product size (bp)
C. jejuni	*flaA-F*	TCTGCTAAGGCTCCAAGT	367
	flaA-R	CTCAAGCGGCTCAAGATG	
	mapA-F	CTTGCTTGGTACGGATTG	429
	mapA-R	CTTGTGAAAGTCCTGGTG	
	hipO-F	TCCGAAGAAGCCATCATC	136
	hipO-R	GTGGTGCTAAGGCAATGA	

Vashin *et al.,* 2012). Attempts to study the virulence gene profile of *C. jejuni* virulent strains isolated from human and poultry revealed the presence of *flaA gene* in all the isolates (Datta *et al.,* 2003; Rossler *et al.,* 2020).

Analytical Sensitivity and Specificity of Gene Targets in *C. jejuni* Detection

The detection limit of *flaA* and *mapA* primer pairs were found to be 10^1CFU, whereas *hipO* gene specific primer pairs yielded visible amplicons at 10^2 CFU dilution only. In case of DNA concentration studies, *flaA* gene specific primer pair detected up to 1 pg, whereas *mapA* specific primer pairs has the detection limit upto 10 pg. The h*ipO* gene, detection limit was found to be 100 CFU and 1 ng DNA. Of all the 3 genes (*flaA, hipO and mapA*) *flaA* and *mapA* genes showed analytical sensitivity equivalent to 10 CFU while *hipO* was found to be at least 10 fold lower in sensitivity (100 CFU). When the analytical sensitivity was analysed using DNA concentration, it was found that *flaA* gene (1 pg) PCR was slightly (10 fold) more sensitive than the *mapA* gene (10 pg). (Table 3,4). Hence it may be recommended that the *flaA* gene is an ideal gene target for detection of *C. jejuni* using the primers and PCR cyclic conditions employed in this study. The high sensitivity of PCR assay for detection of *C. jejuni* from pure culture was in agreement with observation of Persson and Olsen (2005) who detected 10 – 100 cells per reaction. The gene targets were specific in amplification of *C. jejuni* since no amplification occurred with DNA of other 5 organism (*E.coli*, *Staphylococcus*, *Listeria*, *Salmonella* and *C. coli*) tested.

Giannatale *et al*., (2019) studied the level of contamination of thermo-tolerant Campylobacter spp in chicken and meat samples and found that most of meat samples are contaminated with load of 10 CFU/g by the enumeration method. Hence, assay with detection limit of 10 CFU can only be employed in active surveillance of *C.jejuni* thereby unravel the actual sources of infection to understand the transmission pattern of *C. jejuni*. This study results are impressive in filling the gap by providing highly sensitive *flaA* gene based PCR to detect 10 CFU level.

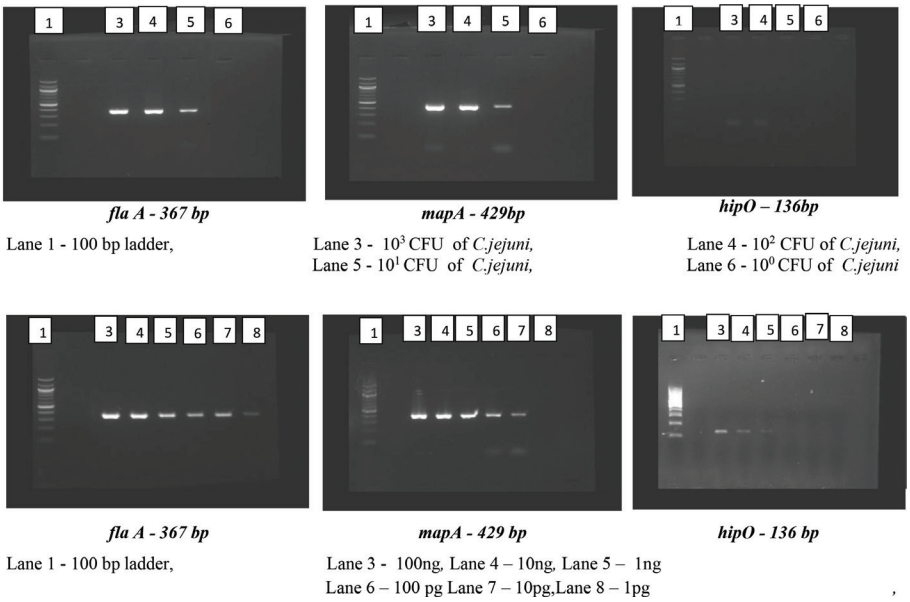

Fig 1: Analytical sensitivity of *C.jejuni* gene targets by lowering dilutions of CFU and DNA

Table 2: Optimized cyclic conditions for the primers employed in the study

Primer/Gene target		Initial Denaturation	Denaturation	Annealing	Extension	No of cycles	Final extension
Organism	**Target**						
C. jejuni	*flaA*	94°C – 5 min	94°C -1 min	54°C-45 s	72°C-1 min	34	72°C -10 min
	mapA	94°C – 5 min	94°C – 1 min	54°C-45 s	72°C-1 min	34	72°C -10 min
	hipO	94°C – 5 min	95°C -1 min	60°C-1 min	72°C-30 s	34	72°C -7 min

Table 3: Analytical sensitivity of *C. jejuni* specific gene targets in terms of Colony Forming Units

Organism	Gene target	Colony Forming Units			
		10^3CFU	**10^2CFU**	**10^1CFU**	**10^0CFU**
C. jejuni	*flaA*	+	+	+	-
	mapA	+	+	+	-
	hipO	**+ weak**	**+ weak**	-	-

Table 4**:** Analytical sensitivity of *C. jejuni* specific gene targets in terms of DNA concentration

Organism	Gene target	DNA Concentration					
		100 ng	10 ng	1ng	100 pg	10 pg	1 pg
C. jejuni	*hipO*	+	+	+weak	-	-	-
	flaA	+	+	+	+	+	+weak
	mapA	+	+	+	+	+ weak	-

Conclusions

Employing virulence gene *flaA* with high sensitivity of 10CFU and 1pg in regular diagnostic assay not only ensures progress in rapid reliable detection of important zoonotic pathogen *Campylobacter jejuni* but also build hopes to decipher its sources of contamination and zoonotic transmission in food chain. However, assays developed with the virulence gene needs extensive validation with clinical samples to confirm its practical feasibility.

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