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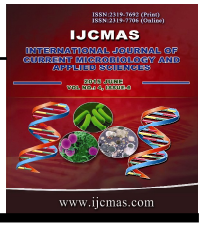

Original Research Article

Detection of *flaA* Virulence Genes *Campylobacter jejuni*, Isolated from Human Faeces and Groundwater Using PCR Method

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A B S T R A C T

Campylobacter jejuni have been recognized as one of major causes of bacterial gastroenteritis (campylobacteriosis) in humans both in developed and developing countries. Incidence of campylobacteriosis in developing countries is estimated to be approximately 1% of the population and the number of cases increased annually in most countries. It was also reported that in Indonesia about 3.6 percent of 21.763 diarrhea cases cause by *Campylobacter jejuni* is higher than that of *Vibrio cholerae* non-O1 and *Salmonella paratyphi* A. The aim of this study was determine *fla*A genes of *Campylobacter jeujuni* isolated from faeces and groundwater to determine whether there is a factor of virulent isolates. Sixty isolates in total were tested in this study, including 44 isolates from human clinical samples (faeces), and 16 from groundwater. Out of these, 35 human faeces and 3 groundwater samples were found positive for *C. jejuni*. The isolates were identified on the basis of morhological, biochemical by vitek and PCR based detection of *16S rRNA* and *flaA* virulence gene, which are reponsible for expression of adherence, invasion, and colonization in *C. jejuni*. The PCR detection showed, 7 isolates positive for *flaA* gene, 6 from faeses and 1 from groundwater. The findings on detection of virulence *flaA genes C. jejuni* from clinical cases of children and groundwater further prove the importance of infection that necessitates the need for proper preventive measures to control the infection in drinking water and household water consumption.

Keywords

Campylobacter jejuni, *flaA* virulance genes, Faeces, Groundwater

Introduction

Campylobacter jejuni have been recognized as one of major causes of bacterial gastroenteritis (campylobacteriosis) in humans both in developed and developing countries. Incidence of campylobacteriosis in developing countries is estimated to be

approximately 1% of the population and the number of cases increase annually in most countries. In developing countries campylobacteriosis is hyperendemic, with a high incidence rate in children around about 40-60% of the population each year (Lodge, 2007; Friedman *et al.,* 2000). It was also reported cases of diarrhea due to Campylobacter infection in some countries, for example in the United States that 2.5 million campylobacteriosis sufferers and 124 sufferers died annually (Hu and Kopecko, 2003). In 1988, it was reported that cases of Campylobacter infection in Denmark was 3,372 cases, as many as 4,382 cases in Spain (Schmidt and Tirado, 2001). Meanwhile, a report issued by the Swedish Institute for the Infection Disease Control in 2008 revealed that in 2007 a total of 7.106 cases of Campylobacter enteritis occurred in Sweden, with 77.45 cases per 100,000 population. While in Indonesia, from 21,763 sufferers diarrhea about 3.6 percent caused by *Campylobacter jejuni* is higher than that of *Vibrio cholerae* non-O1 and *Salmonella paratyphi* A (Tjaniadi *et al.*, 2003).

Campylobacter jejuni infection is caused by eating undercooked poultry, drinking contaminated water and the consumption raw milk (unpasteurized milk). Symptoms of the infection are diarrhea, abdominal pain, fever, headache, nausea and vomiting. Symptoms usually appear 2–5 days after the start of infection and lasts for 3–6 days (WHO, 2009*). Campylobacter jejuni* live commensal on some domestic and wild animals and persistent in the environment (Butzler, 2004). The main transmission pathways to humans is through ingestion or contact with contaminated food, milk or water.

Campylobacter jejuni are asaccharolytic, fastidious bacteria, and this limits the available phenotypic tests by which isolates may be differentiated (Linton *et al.,* 1997). Identification to species level is hindered by variations in methodology and the subjective interpretation of biochemical test results. There are also isolates with atypical phenotypes. For example, the differentiation of *C. jejuni* from *C. coli* relies on the ability of *C. jejuni* to hydrolyze hippurate, but certain atypical *C. jejuni* strains fail to do so, rendering identification based on this single test unreliable. These limitations might in principle be overcome by the use of PCR-based genotypic methods. Furthermore, it would be advantageous to identify campylobacters directly in a fecal or water sample, thereby avoiding the need for culture (Roop *et al.,* 1984; Nicholson and Patton, 1993; Linton *et al.,* 1997)

Motility of *Campylobacter* spp. necessitates the production of flagellum, the best characterized virulence determinant of campylobacters. Flagella and flagellar motility are vital to host colonization, virulence in ferret models, secretion, and host-cell invasion (Young *et al.,* 2007; Astorga and Alonso, 2010). The flagella of *C. jejuni* consists of an unsheathed polymer of flagellin subunits, which are encoded by the adjacent *flaA* and *flaB* genes. Both genes are subjected to antigenic and phase variation. Mutants of *flaA*, the primary structural gene for flagella, are unable to colonize chicks and cannot invade human intestinal epithelial cells *in vitro*. Adhesion and invasion are dependent on both motility and flagella expression, as *C. jejuni* mutants with reduced motility show reduced adherence and no invasion. This indicates that, while flagella are involved in adherence, other adhesins are involved in subsequent internalization (van Vliet and Ketley, 2001; Astorga and Alonso, 2010). The aim of this study was to determine *fla*A genes of *C. jejuni* isolated from faeces and groundwater to determine whether there is a factor of virulent isolates.

Material and Method

Bacterial strains and growth conditions

Sixty isolates in total were tested in this study, including 44 isolates from human clinical samples (faeces), and 16 from groundwater. All isolates were incubated on Columbia blood agar base (Oxoid: CM0331B) prepared aseptically with Preston Campylobacter Selective Supplement (Oxoid: SR0117E) at 42 ^{o}C under microaerobic conditions (candle jar) for 48 h.

Preparation of DNA. Bacterial culture was transfered to a 1.5 ml microcentrifuge tube then centrifuged for 5 minutes at 300 x g. The supernatant was discarded then resuspended cells in 200 µl of PBS by pipette. 20 µl of Proteinase K was added then mixed by pipetting and incubated at 60ºC for 5 minutes. **2**00 µl of GSB Buffer was added then mixed by shaking vigorously. Incubated the samples at 60ºC for 5 minutes and inverted the tubes every 2 minutes. Then, 200 µl of absolute ethanol was added to the sample lysate and mixed immediately by shaking vigorously for 10 seconds. If precipitate appeared, broke it up as much as possible with a pipette.

Placed a GD Column in a 2 ml Collection Tube and transfered all of the mixtures (including any insoluble precipitate) to the GD Column. Centrifuged at 14,000 x g for 1 minute. Following centrifugation, if the mixture did not flow through the GD Column membrane, increase the centrifuge time until it passes completely. Discard the 2 ml Collection Tube containing the flow-through then transfer the GD Column to a new 2 ml Collection Tube. Add 400 µl of W1 Buffer to the GD Column. Centrifuge at 14,000 x g for 30 seconds then discard the flow-through. Place the GD Column back in the 2 ml Collection Tube. 600 µl of Wash Buffer (make sure absolute ethanol was added) was added to the GD Column and centrifuged at 14,000 x g for 30 seconds then discarded the flow-through. Placed the GD Column back in the 2 ml Collection Tube and centrifuged again for 3 minutes at 14,000 x g to dry the column matrix. Transfered the dried GD Column to a clean 1.5 ml microcentrifuge tube. Added 100 µl of pre-heated Elution Buffer, TE Buffer into the center of the column matrix. Let stand for at least 3 minutes to allow Elution Buffer, TE Buffer to be completely absorbed. Centrifuged at 14,000 x g for 30 seconds to elute the purified DNA, stored at -20 ^{o}C and used as template DNA.

PCR based confirmation and detection of *vir*ulence genes: The isolates were confirmed by PCR (polymerase chain reaction) based detection of *16SrRNA* using published primers (Morris *et al.,* 2008). The confirmed *C. jejuni* isolates were screened for the presence of pathogenic gene *fla*A. The template DNAs for PCR was extracted by using gSYNC^{TM} DNA extraction kit (Geneaid). The reaction mixture consisted of 4 µl of bacterial lysate (DNA product), 20 µl of master mix green, 0.5 µl of of each forward and reverse PCR primer (16SrRNA and *flaA* gene) to a final volume of 25 µl.

The oligonucleotide primers used are *C. jejuni 16SrRNA* forward: 5'-TCC TAC GGG AGG CAG CAG T-3' reverse: 5'-GGA CTA CCA GGG TAT CTA ATC CTG TT-3' and *flaA* gene forward: 5'-CTC CGC AGC AGA TGA TGC TT-3', reverse: 5'-CCA TGG CAT AAG AGC CAC TTT GAG C-3'. The positive control was a local isolate of *Campylobacter jejuni* and *Campylobacter* gBlocks^{TM} (Integrated DNA Technology). The reaction mixture was amplified in a T100-thermal cycler (Bio-Rad). The following PCR conditions were used: heat denaturation at 95°C for 14 min,

35 cycles with denaturation at 94°C for 15 s, annealing at 50°C for 1 min, extension at 72°C for 1 min, and a final extension at 72 °C for 10 min. The PCR product was separated by electrophoresis in 2 % agarose gel at 100 V for 40 min in Tris-acetate buffer, visualized by ethidium bromide staining, illuminated by UV-transilluminator and documented by a gel documentation apparatus (Bio-Rad). A 100 bp DNA ladder was used as a size reference for PCR assay.

Result and Discussion

In conventional culture method, numbers of 60 samples from human faeces and groundwater were analyzed by enrichment, selective plating and biochemical tests from suspected colonies and examining them for morphology, motility, gram staining and analyzed by automatic machine Vitek® 2 BioMereux with NH card. Base on vitek analyzed, a total of 59 (98,6 %) samples were determined as unidentified organisms and only one (1,4 %) were determined as C. *jejuni* (low detection) 38 (63.33%) out of total 60 samples screened, show the presence of *C. jejuni*. All the isolates are confirmed by PCR based detection of 16S rRNA gene (Fig. 1–4). Because of strong conservation of regions in the rRNA genes (5S, 16S and 23S rRNA), these genes are suitable targets for PCR based identification of *Campylobacter* (Rizal *et al.,* 2010). The results indicate that 79.55% of the human faeces samples and 18.75% of groundwater samples are positive for *C. jejuni*. The human isolates are from patients suffering from diarhea and below the age of 5 years. The varying isolation rates of *C. jejuni* from human and chicken faecal samples have been recorded in different studies (Nadeau *et al.,* 2002, Baserisalehi *et al.,* 2005). Eyigor *et al.* (1999) also emphasized that the isolation rates may vary, depending on the year, sampling procedure, isolation methodology, and whether the sample is fresh or frozen. In this study, examined culture isolates were stored in incubators 42^{o}C for a long time so it is difficult to detected with conventional culturing or vitek. This is because *C. jejuni* are very sensitive to extreme environmental stress such as drought, high levels of oxygen, and others (van Vliet and Ketley, 2001; WHO, 2011).

The findings for the virulence *flaA* genes are summarized in table 1. Not all the positive *C. jejuni* isolates are from human faeces and groundwater have *fla*A genes (Fig. 5 and 6). the positive *C.jejuni* isolates from human faces have seven *flaA* genes, while on the positive from groundwater have one *flaA* gene. The findings are in agreement with earlier observations regarding the presence of *fla*A genes in *C. jejuni* species isolated from human as well as chicken (Konkel *et al.,* 1999, Dorrell *et al.,* 2001, Bang *et al.,* 2003, Datta *et al.,* 2003, Rozynek *et al.,* 2005). The products of these genes are responsible for the expression of adherence and colonization (Nuijten *et al.,* 2000; Ziprin *et al.,* 2001).

The prevalence of *fla*A gene in both human and groundwater isolates indicates pathogenic potential since the *fla*A gene plays an important role in *Campylobacter* pathogenesis. The flagella has a three pronged approach to colonisation and adhesion in that it imparts motility, is a glycosylated adhesin and serves as the export apparatus for some virulence proteins, including the Cia (Konkel *et al.,* 2004) and *flaC* (Song *et al.,* 2004) which are both involved in invasion.

Waterborne outbreaks of Campylobacter occur occasionally and are normally associated with faecal contamination of the water source from agricultural waste run-off, bird droppings or sewage outflow (Cook and Bolster, 2006).

Table.1 Detection of genes Campylobacter by PCR

Source	No. of samples	Gene deteced – no. of positive isolate (% positive)	
		16S rRNA	***flaA***
Human faeces	44	35 (79.54)	7 (20)
Groundwater	16	3 (18.75)	1 (33.33)
Total	60	38 (63.33)	8 (21.05)

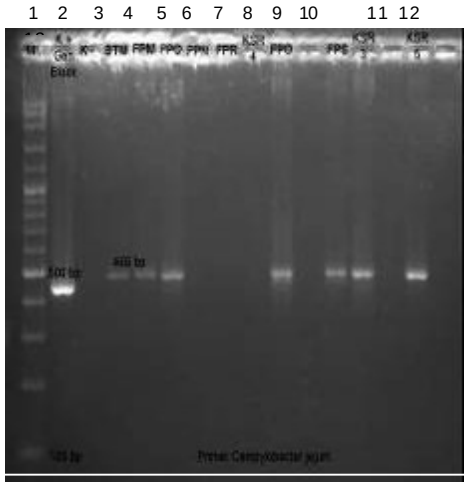

Fig.1. PCR detection of 16S rRNA gene (466 bp) of *C. jejuni*. Lane 1 = Marker, Lane 2 = + control (gBlock), Lane 3 = neg. control, Lane 4 – 13 test isolates

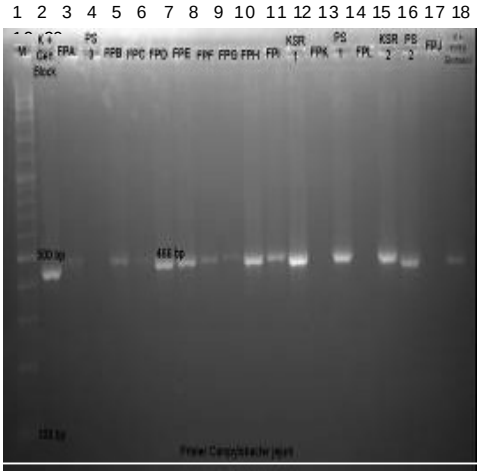

Fig.2. PCR detection of 16S rRNA gene (466 bp) of *C. jejuni*. Lane 1 = Marker, 2 = + control (gBlock), 3-19 = test isolates, 20 = + control (local strain).

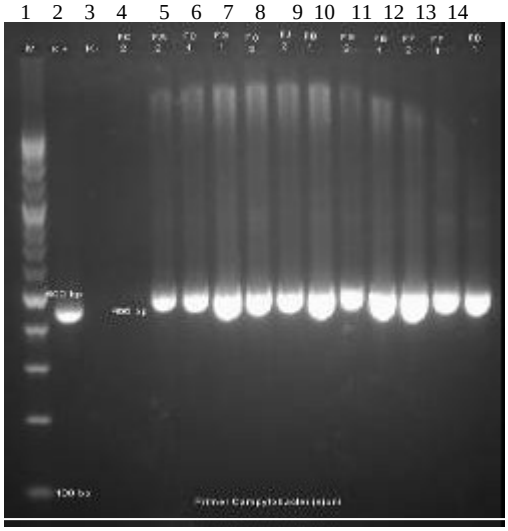

Fig.3. PCR detection of 16S rRNA gene (466 bp) of *C. jejuni*. Lane 1 = Marker, Lane 2 = + control (gBlock), Lane 3 = neg. control, Lane 4 – 15 test isolates

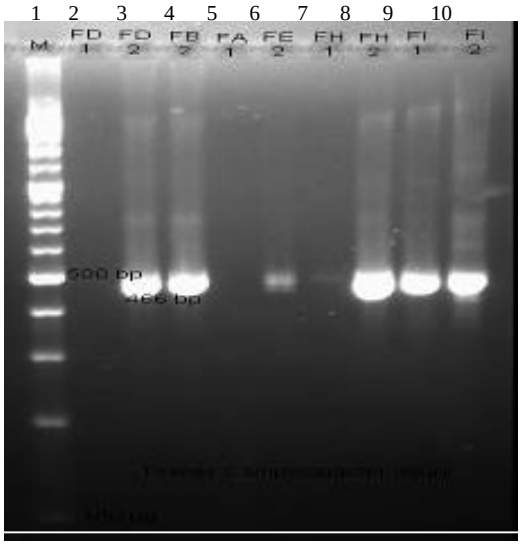

Fig.4. PCR detection of 16S rRNA gene (466 bp) of *C. jejuni*. Lane 1 = Marker, Lane 2 = + control (gBlock), Lane 3 = neg. control, Lane 4 – 13 test isolates

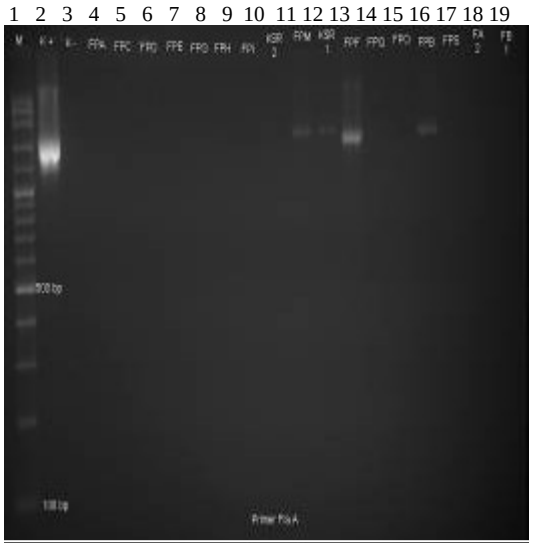

Fig.5. PCR detection of *flaA* gene (1558p) of *C. jejuni*. Lane 1 = Marker, Lane 2 = + control, Lane 3 = neg. control, Lane 12, 13, 14 and 17 *flaA genes C. jejuni*

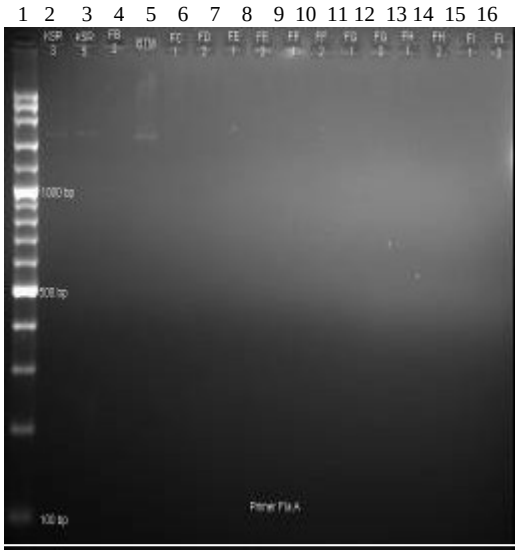

Fig.6. PCR detection of *flaA* gene (1558 bp) of *C. jejuni*. Lane 1 = Marker, Lane 2, 3, 5 *flaA genes C. jejuni*

Contaminated groundwater may serve as a source of inoculation of drinking waters with *C. jejuni* or the introduction of the organism into livestock populations where groundwater is used as a source of drinking water or houshold water consumption and irrigation (Pearson *et al.,* 1993; Stanley *et al.,* 1998; Hanninen *et al.,* 2003).

The survival of *C. jejuni* in groundwater is not surprising as the environmental conditions (e.g. low redox potential, low temperatures, lack of UV exposure and desiccation) often favour its maintenance (Stanley *et al.,* 1998; Jones, 2001). Detection of *C. jejuni* in groundwater suggests that this organism does exhibit significant vertical movement in the environment (Stanley and Jones, 2003), possibly owing to electrostatic repulsion of the bacteria and negatively charged soil particles (Bolster *et al.,* 2006).

Finding virulence *flaA* genes *C. jejuni* in groundwater samples indicate the public health hazard due to this emerging water borne organism in the region. The isolates show a wide variation for the presence of pathogenic genes; however, presence of virulence genes revealed the pathogenic potential of the isolates. The findings on detection of virulence *flaA genes C. jejuni* from clinical cases of children further prove the importance of infection that necessitates the need for proper preventive measures to control the infection in drinking water and household water consumption. Transmission of Campylobacter infection to human via the consumption of drinking water has been reported in numerous outbreak (Revez *et al.,* 2014). The infective dose of *C.jejuni* cells is very small and it has been estimated that as few as 500 cells could cause human illness (Lodge, 2007; Khanzadi, *et al.,* 2010). This means even very smaal number of virulence *C. jejuni* cells, present a potensial health hazad.

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