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ORIGINAL PAPER

The detection of *hip*O gene by real-time PCR in thermophilic *Campylobacter* spp. with very weak and negative reaction of hippurate hydrolysis

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Abstract A total of 190 *Campylobacter* spp. isolates, of which 34 gave the result of very weak activity, and 156 gave the negative activity in the test for hippurate hydrolysis were characterized. The genomic DNA was isolated from a fresh culture of each isolate and the real-time PCR, targeting the *hip*O gene, was used to confirm the species distribution of *Campylobacter* isolates. The *hip*O gene was detected in 17 isolates (11%) within the total of 156 negative isolates for hippurate hydrolysis. Out of 34 isolates with very weak activity, 19 isolates (56%) were also found to be positive for *hip*O gene and characterized as *C. jejuni*. The real-time PCR assay used in this study could be employed for more accurate diagnosis of *Campylobacter* infections at species level after the biochemical characterization based on hippuricase activity of the isolates. This could also provide important data for the epidemiology of infections associated with these zoonotic pathogens.

Keywords *Campylobacter* · *hip*O · Real-time PCR

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Introduction

Zoonotic infections in humans, from which the bacteria can be transmitted to humans via food chain, are still serious public health issue. It has been reported that zoonotic pathogens are twice as likely associated with emerging diseases than non-zoonotic pathogens (Taylor et al. 2001). Food-borne zoonotic pathogens such as *Salmonella* and *Campylobacter* are the most common cause of bacterial gastroenteritis in humans and poultry products are the most recognized sources of these infections although the exposure to the pathogens results in prolonged colonization without disease in all domestic livestock (Nielsen et al. 1997; Schlundt et al. 2004; Friedman et al. 2004).

Over the past two decades, several studies from countries all over the world reported that in particular, *C. jejuni* and *C. coli* continue to play a major role in reported cases of bacteria-related food

poisoning (Butzler 2004; Schlundt et al. 2004). These pathogens are also associated with serious complications such as the Guillain-Barré syndrome (GBS) and reactive arthritis (Hughes and Rees 1997; Mead et al. 1999; Hannu et al. 2002). Consequently, the accurate identification of these *Campylobacter* species not only provides important data for surveillance and risk assessment studies but also elucidate the epidemiology of the infections associated with these pathogens.

The identification of bacteria in the clinical microbiology laboratory is performed by conventional culture-based methods require isolating the organism and then determining of the biochemical and morphological profile of the organism. The basis for the difference between *C. jejuni* and *C. coli* is the presence and expression of the N-benzoylglycine amidohydrolase (*hippuricase*, *hip*O) gene only in *C. jejuni*. But, the negative *C. jejuni* isolates for hippurate hydrolysis test have been reported in the literature (Totten et al. 1987; Wainø et al. 2003). Therefore, *C. jejuni* isolates with negative hippurate hydrolysis may be misidentified as *C. coli*. In some reports, the authors used different method including disk method, gas-liquid chromatograph, and PCR to identify the isolates at the species-level and reported some disagreement between the tests used for hippurate activity in the isolates (Nicholson and Patton 1995; Steinhauserova et al. 2001; Wainø et al. 2003). Therefore, the differentiation between *C. jejuni* and *C. coli* is especially delicate, as it is based solely on the hippurate hydrolysis test used routinely in numerous microbiology laboratories, which is accurate only in approximately 90% of cases.

The aim of this study was to determine the presence of *hip*O gene in thermophilic *Campylobacter* spp. that gave very weak or negative reactions in the test for hippurate hydrolysis by real-time PCR since the accurate identification at the species-level provides important data about the epidemiology of and surveillance for *Campylobacter* infections.

Materials and methods

Bacterial isolates and hippurate hydrolysis test

This study was done with thermophilic *Campylobacter* spp. isolated during an epidemiological survey study in broiler poultry. A total of 190 *Campylobacter* spp. isolates, of which 34 gave the result of very weak activity, and 156 gave the negative activity in the test for hippurate hydrolysis were examined. All the isolates were collected from different sources including faecal dropping, caecal, water, and drinking nipple swab samples (Table 1).

Table 1 The distribution of *Campylobacter* spp. gave the results of very weak activity and negative activity according to the hippurate hydrolysis test versus the source of isolation

Samples	No. of isolates with very weak reaction	No. of isolates with negative reaction
Faecal dropping	29	96
Cecum	–	33
Water	3	23
Drinking nipple swab	2	4
Total	34	156

Briefly, thermophilic *Campylobacter* spp. were isolated from faecal dropping and caecal samples using a direct plating method. All samples were homogenized and cultured on modified Charcoal Cefoperazone Deoxycholate Agar (mCCDA) (CM739, Oxoid) with selective supplement (SR155, Oxoid) (El-Shibiny et al. 2005; On and Holmes 1992). For water and drinking nipple swab samples, it was applied the isolation procedure including an enrichment step with Hunt enrichment broth and then the enrichment cultures were subcultured to mCCDA (Hunt 1992). All plates were incubated under microaerophilic conditions for 48 h at 42°C. Small, curved, catalase and oxidase-positive Gram negative bacilli were presumed to be *Campylobacter* spp.

To identify the *Campylobacter* species, standard biochemical tests including indoxyl acetate hydrolysis, H_2S production in TSI, and susceptibility to cephalothin were done and classified as described previously (Blaser et al. 1983; Skirrow and Benjamin 1980). For hippurate hydrolysis test, the method described by Morris et al. (1985) was used with minor modification. A loopful of bacterial colonies was collected and suspended in 0.4 ml of 1% sodium hippurate solution. After 2 h at 37°C in a water bath, 0.2 ml ninhydrin reagent (3.5% w/v ninhydrin an a 1:1 acetone and butanol mixture) was added slowly in each tube on the top of the hippurate solution. Further incubation at 37°C was carried out for 10 min for colour development. A positive test was recorded as a

dark purple colour, is indicating the presence of glycine that resulted from the hydrolysis of the hippurate. The appearance of a pale purple colour was noted as a very weak activity for hippurate hydrolysis. Colourless tubes were considered as negative for hippurate hydrolysis.

DNA isolation from *Campylobacter* isolates and real-time PCR

Campylobacter genomic DNAs were extracted from bacterial suspensions of the isolates using the QIAamp DNA mini kit (Qiagen) as described by the manufacturer. One hundred microliters of elution buffer was used to resuspend the DNA. The genomic DNAs were stored at −20°C until use as template in real-time PCR.

The *hip*O gene was amplified with primers HIP400F (5′-GAA GAG GGT TTG GGT GGT-3′) and HIP1134R (5′-AGC TAG CTT CGC ATA ATA ACT TG-3′) that were synthesized by TibMolBiol (Berlin, Germany) (Hani and Chan 1995; Linton et al. 1997). For real-time PCR, each reaction tube contained 4 µl of LightCycler FastStart Master SYBR Green I (Roche), 2 µl of primer set with 0.5 mM final concentration for each primer, 9 µl of PCR-grade water, and 5 µl of template DNA in a total of 20 µl PCR mixture. The reaction protocol for *hip*O was as fallows: an initial FastStart Taq DNA polymerase activation phase at 95°C for 10 min; a 35 cycle amplification phase consisting of a 95°C denaturation segment for 0 s, a 59°C annealing segment for 5 s, and a 72°C extention segment for 30 s. After completion of the amplification process, the reaction mixture was denaturated 95°C for 0 s, held at 55°C for 15 s, and then slowly heated to 95°C for 0 s at a ramp rate of 0.1°C per s. At the end of the cycles, a cooling step at 40°C for 30 s was performed for each reaction.

All runs were included one negative DNA control consisting of PCR-grade water and one positive control consisting of the genomic DNA of *C. jejuni* NCTC 11638.

Results

The isolates were divided into two distinct groups depending on absence or very weak activity for hippuricase by phenotypic method. Each test was repeated two times at least and then the results were recorded. Of the all isolates, 34 (18%) were referred as isolates with very weak reaction while the remaining (156; 82%) shown no reaction for hippuricase, and were identified as *C. coli*.

Figure 1 illustrates the melting curve analysis of real-time PCR-amplified product that is in size 735-bp using the *hip*O primer set for the differentiation of the isolates at species level. Melting curve analysis of the amplicon from *C. jejuni* exhibited Tm of 81 ± 1°C while there was no peak for *C. coli* isolate and negative control.

The presence of *hip*O gene was determined in 17 *Campylobacter* spp. (11%) within 156 isolates with negative reaction by hippurate hydrolysis. Therefore, these isolates identified as *C. coli* by

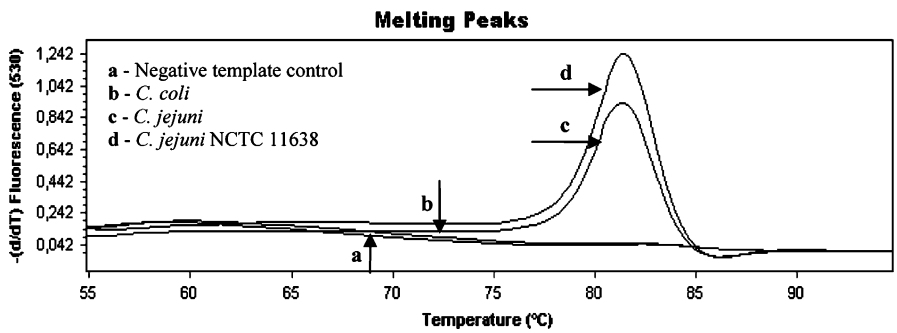

Fig. 1 The melting peaks generated with HIP400F and HIP1134R primers in *C. jejuni* amplicons at the end of run of real-time PCR produced a Tm of 81 ± 1°C. (a) Negative template control; PCR-grade water instead of template was added to reaction. (b) The isolate with negative activity by hippurate test and the Tm was not produced as expected. (c) The isolate with negative activity by hippurate test and the isolate gave a positive Tm for *hip*O gene. (d) *C. jejuni* NCTC 11638, which is known to have *hip*O gene, was used as positive control

Table 2 The presence of *hip*O in thermophilic *Campylobacter* spp. gave the results of very weak activity and negative activity according to the hippurate hydrolysis test

Source of isolates	*Campylobacter* spp. with very weak activity ($n = 34$)		*Campylobacter* spp. with negative activity ($n = 156$)	
	*hip*O+	*hip*O−	*hip*O+	*hip*O−
Faecal dropping	19	10	17	79
Cecum	–	–	–	33
Water	–	3	–	23
Drinking nipple swab	–	2	–	4

phenotypic methods before were characterized as *C. jejuni*. Of 34 isolates with very weak reaction by hippurate hydrolysis, nineteen (56%) isolates were found to be positive for *hip*O gene and were identified as *C. jejuni*. Interestingly, all isolates identified as *C. jejuni* based on the presence of *hip*O by real-time PCR were isolated from faecal dropping samples (Table 2). We have also tested the presence of *hip*O gene in *C. jejuni* isolates with positive reaction for hippurate test by real-time PCR, and found that all of the *C. jejuni* isolates gave positive Tm for *hip*O gene (data was not shown).

Discussion

In the present study, a total of 190 *Campylobacter* isolates that were hippuricase-ambiguous and -negative by phenotypic method were studied. The genomic DNA was isolated from a fresh culture of each isolate and the PCR primer set, HIP400F and HIP1134R, specific to the *hip*O gene of *C. jejuni* was used to confirm the species distribution of *Campylobacter* isolates. Out of 34 isolates with very weak activity, 19 isolates (56%) were found to be positive for *hip*O gene and characterized as *C. jejuni*. One hundred fifty-six isolates with negative activity by hippuricase test were already identified as *C. coli*. Out of 156 isolates, 17 (11%) were found to be positive for *hip*O gene by real-time PCR. Denis et al. (1999) and Wainø et al. (2003) reported that the prevalence of hippurate-negative *C. jejuni* represented 7% and 13.4% respectively, of *C. jejuni* strains obtained from chickens. Rönner et al. (2004) reported that 5% of the human isolates and 10% of the chicken isolates of *C. jejuni* were found "false negative" by PCR/REA assay. The present study support the reports carried out in the previous studies on the presence of sodium-hippurate negative *C. jejuni* and the prevalence was generally concordant with the similar studies carried out in other countries. Interestingly, 36 false-negative isolates were obtained from faecal dropping samples. This result suggests that *hip*O gene may not be expressed or weakly expressed in faecal drooping samples which were characterized with high-level of background flora.

Rautelin et al. (1999) reported that when bacteria were harvested from blood agar, hippurate test was more often positive than when bacteria were collected from blood-free agar. Bacterial suspension of each isolate used in the present study for hippurate test was prepared from the bacterial colonies grown on the agar supplemented without blood and each test was repeated two times at least. In addition to, it was reported that inadequate buffering of the reaction mixture or low inoculum size could lead to false negative results (On and Holmes 1991; On 1996; Gorkiewicz et al. 2003). There is no consensus on the pH value at which the hippurate medium is buffered (Hwang and Ederer 1975; Kotsis and Adám 1987; Moore and Murphy 2000). Since a loopful of bacteria was used for hippurate hydrolysis test, the effect of inoculum size should not interfere with the results.

Rapid detection of pathogenic organisms that cause food-borne illness is needed to ensure for food safety. Polymerase chain reaction-based assays present powerful alternatives with high sensitivity and specificity for immediate identification of specific microorganisms, especially food-borne pathogens. In contrast to conventional PCR, real-time PCR-based assays, targeting specific genetic markers, has important advantages including its decreased risk of cross-contamination and potential to amplify the DNA and/or mRNA with low quantities. There are several methods to detect *C. jejuni* and *C. coli* in poultry samples by PCR-based methods, even several real-time PCR assays for detecting *C. jejuni* have been reported (Rudi et al. 2004; Yang et al. 2003) but these methods has stricted advantages in terms of epidemiological investigations such as serotyping, biotyping, and antimicrobial resistance patterns without conventional culture-based methods. In fact, the detection of *hip*O gene is more reliable than biochemical test for hippuricase activity (Linton et al. 1997; Rautelin et al. 1999). Here we describe

a real-time PCR analysis for rapid and sensitive detection of *hip*O gene in thermophilic *Campylobacter* spp. Results show that real-time PCR, which is reliable and cost-effective, could be used for the identification of thermophilic *Campylobacter* spp.

In summary, the prevalence of hippurate-negative *C. jejuni* in the current study was 11%. It was also found that 56% of the isolates with very weak activity for hippuricase gave positive results for the presence of *hip*O gene. Although the phenotypic characterization has successful for almost 90% of the isolates, molecular methods such as real-time PCR should be employed for more accurate diagnosis of *Campylobacter* infections at species level, especially in the isolates with very-weak activity and negative activity by hippurate test.

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References

Blaser MJ, Taylor DN, Feldman RA (1983) Epidemiology of *Campylobacter jejuni* infections. Epidemiol Rev 5:157–176

Butzler JP (2004) *Campylobacter* from obscurity to celebrity. Clin Microbiol Infect 10:868–876. doi:10.1111/j.1469-0691.2004.00983.x

Denis M, Soumet C, Rivaol K, Ermel G, Blivet D, Salvat G et al (1999) Development of a m-PCR assay for simultaneous identification of *Campylobacter jejuni* and *C. coli*. Lett Appl Microbiol 29:406–410. doi:10.1046/j.1472-765X.1999.00658.x

El-Shibiny A, Connerton PL, Connerton IF (2005) Enumeration and diversity of campylobacters and bacteriophages isolated during the rearing cycles of free-range and organic chickens. Appl Environ Microbiol 71:1259–1266. doi:10.1128/AEM.71.3.1259-1266.2005

Friedman CR, Hoekstra RM, Samuel M et al (2004) Risk factors for sporadic *Campylobacter* infection in the United States: a case–control study in FoodNet sites. Clin Infect Dis 38(Suppl 3):285–296. doi:10.1086/381598

Gorkiewicz G, Feierl G, Schober C, Dieber F, Kofer J, Zechner R et al (2003) Species-specific identification of campylobacters by partial 16S rRNA gene squencing. J Clin Microbiol 41:2537–2546. doi:10.1128/JCM.41.6.2537-2546.2003

Hani EK, Chan VL (1995) Expression and characterization of *Campylobacter jejuni* benzoglycine aminohydrolase (hippuricase) gene in *Escherichia coli*. J Bacteriol 177: 2396–2402

Hannu T, Mattila L, Rautelin H, Pelkonen P, Lahdenne P, Siitonen A et al (2002) Campylobacter-triggered reactive arthritis: a population-based study. Rheumatology 41: 312–318. doi:10.1093/rheumatology/41.3.312

Hughes RA, Rees JH (1997) Clinical and epidemiologic features of Guillain-Barre syndrome. J Infect Dis 176:92–98. doi:10.1086/513793

Hunt JM (1992) Campylobacter. In: Food and drug administration bacteriological analytical manual, 7th edn. Association of Official Analytical Chemists International, Arlington, VA, pp 77–94

Hwang M, Ederer GM (1975) Rapid hippurate hydrolysis method for the presumptive identification of group B streptococci. J Clin Microbiol 1:114–115

Kotsis IH, Adám M (1987) Paper-disc method for *campylobacter* hippurate-hydrolysis test. A note. Acta Microbiol Hung 34:173–177

Linton D, Lawson AJ, Owen RJ, Stanley J (1997) PCR detection, identification to species level, and fingerprinting of *Campylobacter jejuni* and *Campylobacter coli* direct from diarrheic samples. J Clin Microbiol 35:2568–2572

Mead PS, Slutsher L, Dietz V, McCaig LF, Bresse JS, Shapiro C et al (1999) Food-related illness and death in the United States. Emerg Infect Dis 5:840–842

Moore JE, Murphy PG (2000) Hippurate hydrolysis and speciation of thermophilic *Campylobacter* spp. Br J Biomed Sci 57:180–181

Morris GK, el Sherbeeny MR, Patton CM, Kodaka H, Lombard GL, Edmans P et al (1985) Comparison of four hippurate hydrolysis methods for identification of thermophilic *Campylobacter* spp. J Clin Microbiol 22:714–718

Nicholson MA, Patton CM (1995) Evaluation of disk method for hippurate hydrolysis by *Campylobacter* species. J Clin Microbiol 33:1341–1343

Nielsen EM, Engberg J, Madsen M (1997) Distribution of serotypes of *Campylobacter jejuni* and *C. coli* from Danish patients, poultry, cattle and swine. FEMS Immunol Med Microbiol 19:47–56

On SLW (1996) Identification methods for campylobacters, helicobacters, and related organisms. Clin Microbiol Rev 9:405–422

On SLW, Holmes B (1991) Effect of inoculum size on the phenotypic characterization of *Campylobacter* species. J Clin Microbiol 29:923–926

On SLW, Holmes B (1992) Assessment of enzyme detection tests useful in identification of campylobacteria. J Clin Microbiol 30:746–749

Rautelin H, Jusufovic J, Hänninen M-L (1999) Identification of hippurate-negative thermophilic campylobacters. Diagn Microbiol Infect 35:9–12. doi:10.1016/S0732-8893(99)00057-7

Rönner A-C, Engvall EO, Andersson L, Kaijser B (2004) Species identification by genotyping and determination of antibiotic resistance in *Campylobacter jejuni* and *Campylobacter coli* from humans and chickens in Sweden. Int J Food Microbiol 96:173–179. doi:10.1016/j.ijfoodmicro.2004.03.017

Rudi K, Høidal HK, Katla T, Johansen BK, Nordal J, Jakobsen KS (2004) Direct real-time PCR quantification of *Campylobacter jejuni* in chicken fecal and cecal samples by integrated cell concentration and DNA purification. Appl Environ Microbiol 70:790–797. doi:10.1128/AEM.70.2.790-797.2004

Schlundt J, Toyofuku H, Jansen J, Herbst SA (2004) Emerging food-borne zoonoses. Rev Sci Tech 23:513–533

Skirrow MB, Benjamin J (1980) Differentiation of enteropathogenic campylobacter. J Clin Pathol 33:1122. doi:10.1136/jcp.33.11.1122

Steinhauserova I, Češkova J, Fojtikova K, Obrovska I (2001) Identification of thermophilic *Campylobacter* spp. by phenotypic and molecular methods. J Appl Microbiol 90: 470–475. doi:10.1046/j.1365-2672.2001.01267.x

Taylor LH, Latham SM, Woolhouse ME (2001) Risk factors for human disease emergence. Philos Trans R Soc B 29: 983–989

Totten PA, Patton CM, Tenover FC, Barrett TJ, Stamm WE, Steigerwalt AG et al (1987) Prevalance and characterization of hippurate-negative *Campylobacter jejuni* in King County, Washington. J Clin Microbiol 25:1747–1752

Wainø M, Bang DD, Lund M, Nordentoft S, Andersen JS, Pedersen K et al (2003) Identification of campylobacteria isolated from Danish broilers by phenotypic tests and species-specific PCR assays. J Appl Microbiol 95:649–655. doi:10.1046/j.1365-2672.2003.01996.x

Yang C, Jiang Y, Huang K, Zhu C, Yin Y (2003) Application of real-time PCR for quantitative detection of *Campylobacter jejuni* in poultry, milk and environmental water. FEMS Immunol Med Microbiol 38:265–271. doi:10.1016/S0928-8244(03)00168-8