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SHORT COMMUNICATION

A Comparison between Hippurate Hydrolysis and Multiplex PCR for Differentiating *Campylobacter coli* and *Campylobacter jejuni*

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Abstrak: Pengenalpastian spesies penting untuk tujuan epidemiologikal, klinikal dan perubatan. Tujuan kajian ini adalah untuk mengetahui sama ada ujian hidrolisis hippurate berkesan untuk membezakan antara *Campylobacter coli* dan *Campylobacter jejuni.* Untuk mencapai ini, ujian hidrolis hippurate dibandingkan dengan *multiplex Polymerase Chain Reaction* (mPCR) untuk mengkaji kebolehan membezakan spesies *C. coli* dan *C. jejuni*. Lapan belas strain *Campylobacter* daripada sampel haiwan ternakan digunakan. Keputusan 17 daripada 18 strain menunjukkan keberkesanan menggunakan kedua-dua jenis ujian. Maka ujian hidrolisis hippurate berkesan untuk membezakan *C. jejuni* daripada *C. coli,* walaupun kadang-kadang terdapat strain *C. jejuni* yang diidentifikasi secara salah sebagai *C. coli.*

Kata kunci: *C. coli*, *C. jejuni*, Hidrolisis Hippurate, mPCR

Abstract: Species identification is important for epidemiological, clinical and treatment purposes. The aim of this study was to find out whether hippurate hydrolysis is a reliable test for differentiating between *Campylobacter coli* and *Campylobacter jejuni*. To achieve this, hippurate hydrolysis test was compared with multiplex Polymerase Chain Reaction (mPCR) for their ability to speciate *C. coli* and *C. jejuni*. Eighteen *Campylobacter* strains from poultry samples were used for this study. The results from 17 of the 18 strains were in agreement with both methods. Thus, the hippurate hydrolysis test can be used for distinguishing *C. jejuni* from *C. coli* although occasionally some strains of *C. jejuni* may be mis-identified as *C. coli*.

Keywords: *C. coli*, *C. jejuni*, Hippurate Hydrolysis, mPCR

Campylobacters are Gram-negative, nonspore-forming, oxidase and catalase positive, curved spiral or rod shaped bacteria, that are microaerophilic in nature and unable to grow at 25°C (Corry *et al*. 2003). They are also motile, with either uni- or bi-polar flagella, 0.2–0.5 mm wide and 0.5–8 mm long (Corry *et al.* 2003; Moore *et al.* 2005). *Campylobacters* cannot ferment carbohydrate because they do not have the enzyme phosphofructokinase which is engaged in energy

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metabolism (Velayudhan & Kelly 2002) but obtain their energy from amino acids and/or tricarboxylic acid cycle intermediates (Vandamme 2000; EFSA 2005).

Campylobacters have been reported to be the most common cause of foodborne bacterial enteritis (Mead *et al.* 1999). The European Food Safety Authority (EFSA 2005) report also indicated that the number of campylobacteriosis cases per year have surpassed salmonellosis in the European Union (EU) nations. In most developing countries including Africa reliable data on foodborne illness cases is unavailable (Adzitey & Nurul 2011). The genus *Campylobacter* is made up of 17 species (On 2001) of which *C. jejuni* and *C. coli* are the most important in terms of food safety. *C. jejuni* is responsible for about 90% of all campylobacter infections, and most of the rest are caused by *C. coli* (EFSA 2005). *C. jejuni* infection can lead to serious autoimmune diseases such as Guillain-Barré syndrome and reactive arthritis.

Hippurate hydrolysis relies on the ability of the enzyme called hippurate hydrolase produced by microorganisms to hydrolyse sodium hippurate to benzoic acid and glycine. This test does not require the microorganisms to grow, but instead it detects the presence of already formed enzyme by testing for glycine, one of the end products of the hydrolysis. If glycine is present a blue or deep purple colour is formed. Hippurate hydrolysis has been successfully used to identify group B streptococci (Hwang & Ederer 1975; Mugg 1983). In recent years, several Polymerase Chain Reaction (PCR)-based techniques have been mentioned or described which are more specific, accurate and sensitive than phenotypic methods for distinguishing *Campylobacter* species (EFSA 2005; Didelot & Falush 2007; Ridley *et al.* 2008; Adzitey & Nurul 2011). However, most *Campylobacter* isolates from human cases or from poultry are either *C. jejuni* or *C. coli*, and it is important for clinical and treatment purposes to be able to distinguish between them by means of a simple and economical test.

Correct differentiation of *C. coli* from *C. jejuni* is important particularly in the treatment of human illness, because the antibiotics employed will depend on the causative species. For example, erythromycin is commonly used to treat gastrointestinal infections caused by *C. jejuni*, while *C. coli* is more likely to be resistant to this antibiotic, rendering treatment ineffective if *C. coli* is the causative agent (Aarestrup *et al.* 1997). It is also important to differentiate between these two pathogenic species because *C. jejuni* infection is an important predisposal factor in the development of Guillian-Barré syndrome, as well as reactive arthritis and Reiter's syndrome whereas *C. coli* is less strongly associated with these sequelae (Smith 1995). This paper compares the results of distinguishing *C. jejuni* and *C. coli* using the hippurate hydrolysis test and multiplex PCR (mPCR).

The study was conducted in the laboratory of the School of Veterinary Medicine, University of Bristol (UK) using 18 campylobacter isolates originating from carcasses, faeces and caecal contents taken from different poultry flocks and various poultry processing plants.The ability of the *Campylobacter* strains to hydrolyse hippurate was checked using a modification of the method of Hwang and Ederer (1975). 100 µl of 1% (w/v) sodium hippurate solution were dispensed

into each well of a microtitre plate. A loopful (1 µl) of *Campylobacter* culture grown on modified cefaperazone charcoal deoxycholate agar (mCCDA) microaerobically for 48 h at 41.5°C was added, agitated with the loop to produce a suspension, and covered with cling film for incubation aerobically for 4 h at 37°C. After incubation, 50 µl of 3.5% (w/v) ninhydrin solution was added to each suspension, mixed well and incubation was continued at 37°C for 30 min before the results were checked. A deep purple (not medium or light purple) positive reaction was developed by all *C. jejuni* strains. Figure 1 shows a typical example of colour changes observed for the hippurate test.

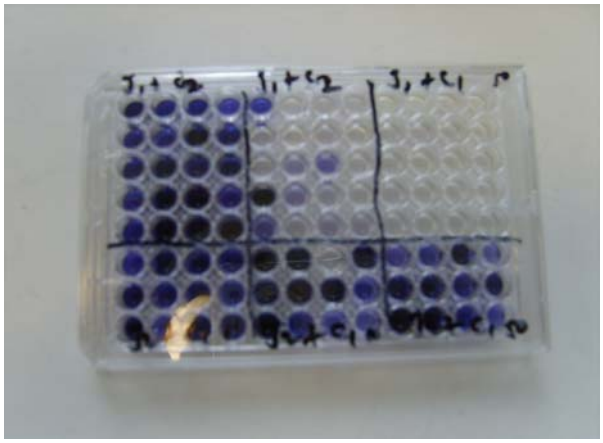

Figure 1: Representative results of hippurate hydrolysis test carried out on microtitre plate.

Note: Deep purple colour indicates *C. jejuni* while medium or no colour change indicates *C. coli.*

DNA templates were prepared by adding 10 µl loopful of *Campylobacter* culture from mCCDA plate to 500 µl peptone buffered saline (PBS) contained in an eppendorf tube and heated to 100°C for 10 min using a heating block. Primers were made to a concentration of 100 pmol according to manufacturer's (MWG Operon, Eberberg, Germany), instructions. They were then diluted to a working concentration of 10 µM by adding 10 µl of concentrated (100 pmol) primer stocks into 90 µl nuclease free water. The primers used for the detection of *C. jejuni* and *C. coli* strains are listed in Table 1. The PCR mixture for one reaction contained 0.5 µl of each primer (10 µM concentration), 12.5 µl hotstart *taq* mastermix (Qiagen, West Sussex, UK), 2.75 µl nuclease free water, 0.75 µl of 50 mM magnesium chloride (Qiagen, West Sussex, UK) and 5 µl template DNA. The temperature cycling was performed at 95°C for 15 min, 30 cycles of denaturing at 94°C for 1 min, annealing at 60°C for 1 min and extension at 72°C for 1 min, with a final extension time of 72°C for 10 min.

Table 1: Primers used for the identification of *C. jejuni* and *C. coli* by mPCR.

Species	Target gene	Reaction direction	Sequence (5'-3')	Reference
C. jejuni	*lpxA*	forward	ACA ACT TGG TGA CGA TGT TGT A	Klena *et al.* (2004)
		reverse lpxARKK2m	CAA TCA TGD GCD ATA TGA SAA TAH GCC AT	
	hipO	forward	ACT GCA AAA TTA GTG GCG	Bang *et al.* (2002)
		reverse	GAG CTT TTA GCA AAC CTT CC	
C. coli	*lpxA*	forward	AGA CAA ATA AGA GAG AAT CAG	Klena *et al.* (2004)
		reverse lpxARKK2m	CAA TCA TGD GCD ATA TGA SAA TAH GCC AT	
	glyA	forward	TCA AGG CGT TTA TGC TGC AC	Dingle *et al.* (2005)
		reverse	CCA TCA CTT ACA AGC TTA TAC	

The restriction fragments were separated by gel electrophoresis, on a 2% agarose gel prepared by adding 4 g agarose (Sigma-Aldrich, Dorset, UK) to 200 ml tris acetate EDTA (1xTAE) buffer (Sigma, Dorset, UK) containing 1 µg ml^{-1} ethidium bromide (Sigma, Dorset, UK), and visualised on an ultra violet transilluminator (UVP BioDoc-It™ imaging-system, Cambridge, UK). Hyperladder IV (Bioline, London, UK) was used as the molecular weight marker and band positions were determined by eye using the molecular weight marker.

Comparison of the 18 isolates tested for hippurate hydrolysis and mPCR assay demonstrated that the same species result was obtained for 17 isolates. Figure 1 shows a representative sample of how hippurate positive (*C. jejuni*) and hippurate negative (*C. coli*) *Campylobacter* species look on a microtitre plate. Deep purple coloured well indicates *C. jejuni* whilst medium, light or no colour changes is *C. coli.* A comparison between hippurate hydrolysis and mPCR for the identification of *C. jejuni* and *C. coli* is depicted in Table 2.

Hydrolysis of sodium hippurate by *C. jejuni* produces a deep purple colour, while *C. coli* strains produce medium or no colour change. mPCR amplification of the DNA from *C. jejuni* yielded two bands of approximately 331 and 800 bp, while amplification of *C. coli* DNA yielded bands of 391 and 600 bp. No amplification products were obtained from PCR analysis of the negative control (Fig. 2).

One isolate was identified as *C. coli* (medium purple) by a negative hippurate test but was identified as *C. jejuni* by the mPCR (Fig. 2, lane 2). The samples might have contained both *C. coli* and *C. jejuni* or contained a hippurase hydrolysis-negative *C. jejuni* strain (personal communication with Dr. Frieda Jørgenson). This could happen if the gene was present but not expressed. Rönner *et al.* (2004) reported that 5% of human *Campylobacter* isolates and 10% of chicken isolates were hippurase negative (presumptive *C. coli* isolates) but were actually *C. jejuni*. Similarly, Burnett *et al.* (2002) reported that the hippurate hydrolysis test was particularly unreliable since 28 of 29 *hipO* negative isolates, mostly of poultry origin were positive in this biochemical test. The PCR method

offers more accurate results for species identification since the hippurate test could yield misleading reactions. This experiment agrees that hippurate hydrolysis test can be used to differentiate between *C. jejuni* and *C. coli* especially in areas where molecular equipments are unavailable.

A missing *hipO* band was observed with one *C. jejuni* strain (band 17). This is most likely because the DNA from the strain did not bind to the primers (as the strain was able to hydrolyse hippurate). To investigate this, the PCR has to be repeated using primers designed for a different region of the *hipO* gene (personal communication with Dr. Frieda Jørgenson). Slater and Owen (1997) reported that occasionally a typical strain of *C. jejuni* (less than 1%) may not produce *hipO* product due to the base pair substitution/deletion in the annealing site of these primers.

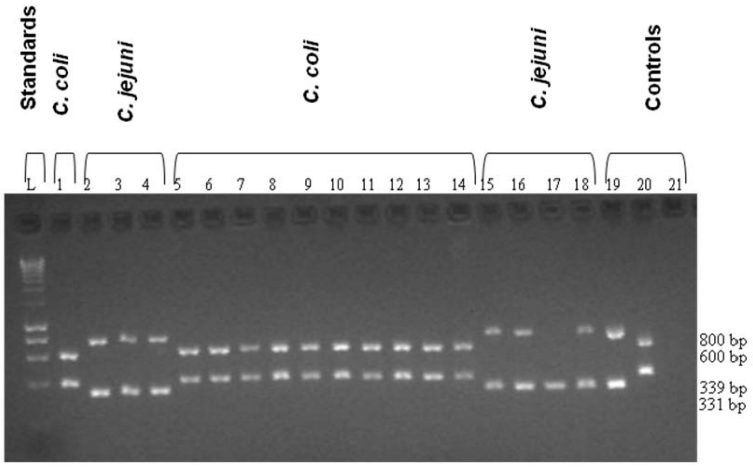

Figure 2: A representative mPCR assay showing results for 18 *Campylobacter* isolates from poultry-related samples. Lane 1, *C. coli*; lanes 2–4, *C. jejuni*; lanes 5–14, *C. coli*; lanes 15–18, *C. jejuni* (lane 17, has a missing *hipO* band*)*; lane 19, *C. jejuni* (11168) positive control; lane 20, *C. coli* (RM28) negative control; lane 21, negative control.

Notes: Lanes 1–2, were medium purple (*C. coli*); lanes 3–4, were deep purple (*C. jejuni*); lanes 5–14, were medium purple (*C. coli*); 15–18, were deep purple (*C. jejuni*) by hippurate hydrolysis test.

The results reported in this study confirm that the hippurate hydrolysis test is useful for distinguishing *C. jejuni* from *C. coli* although additional verification using methods such as mPCR is very useful. Sodium hippurate hydrolysis reagents are easily available in bacteriolological laboratories in Africa and other developing countries, and can easily be used to differentiate or identify hippurase positive and negative *Campylobacters*. Additionally, as *C. jejuni* (subsp. *jejuni* and subsp. *doylei*) is the only *Campylobacte*r species positive for hippurase, this test is also a quick method for identifying any *Campylobacter* isolate as *C. jejuni*; assuming that it has been correctly identified as a *Campylobacter*. Presumptive identification of *Campylobacter* species is best determined from oxidase reaction (positive) and from typical spiral morphology

on microscopic examination of a fresh culture, together with the inability to grow in aerobic atmosphere, so that if hippurase positive, it is almost certainly *C. jejuni*, as no other *Campylobacter* or *Arcobacter* species is hippurase positive (Corry *et al.* 2003).

Table 2: Comparison between hippurate hydrolysis and mPCR for the identification of *C. jejuni* and *C. coli.*

Campylobacter isolate used	Colour	Hippurate results	Lane	mPCR results
C. coli	medium	*C. coli*	1	*C. coli*
C. jejuni	medium	*C. coli*	2	*C. jejuni*
C. jejuni	deep purple	*C. jejuni*	3	*C. jejuni*
C. jejuni	deep purple	*C. jejuni*	4	*C. jejuni*
C. coli	medium	*C. coli*	5	*C. coli*
C. coli	medium	*C. coli*	6	*C. coli*
C. coli	medium	*C. coli*	7	*C. coli*
C. coli	medium	*C. coli*	8	*C. coli*
C. coli	medium	*C. coli*	9	*C. coli*
C. coli	medium	*C. coli*	10	*C. coli*
C. coli	medium	*C. coli*	11	*C. coli*
C. coli	medium	*C. coli*	12	*C. coli*
C. coli	medium	*C. coli*	13	*C. coli*
C. coli	medium	*C. coli*	14	*C. coli*
C. jejuni	deep purple	*C. jejuni*	15	*C. jejuni*
C. jejuni	deep purple	*C. jejuni*	16	*C. jejuni*
C. jejuni	deep purple	*C. jejuni*	17	*C. jejuni*
C. jejuni	deep purple	*C. jejuni*	18	*C. jejuni*

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