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SHORT COMMUNICATION

A Comparison between Hippurate Hydrolysis and Multiplex PCR for Differentiating *C. Coli* and *C. Jejuni*

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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 *C. coli* and *C. jejuni*. To achieve this, hippurate hydrolysis test was compared with a 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 between both methods. Thus, the hippurate hydrolysis test is useful 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

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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 ^{o}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 metabolism (Velayudhan and Kelly, 2002) but obtain their energy from amino

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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). ESFA (2005) report also indicated that the number of campylobacteriosis cases per year have surpassed salmonellosis in the EU. In most developing countries including Africa reliable data on foodborne illness cases is unavailable (Adzitey and Nurul, 2010). 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. *Campylobacter jejuni* is responsible for about 90 % of all campylobacter infections, and most of the rest are caused by *C. coli* (ESFA, 2005). *Campylobacter 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 micro-organisms 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 coloured is formed. Hippurate hydrolysis has been successfully used to identify group B streptococci (Hwang and Ederer, 1975; Merge, 1983). In recent years, several PCR-based techniques have been mentioned or described which are more specific, accurate and sensitive than phenotypic methods for distinguishing *Campylobacter* species (ESFA 2005; Didelot and Falush, 2007; Ridley *et al.,* 2008; Adzitey and Nurul, 2010). 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 (Aartstrup *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 or multiplex Polymerase Chain Reaction (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 hydrolyze hippurate was checked using a modification of the method of Hwang and Ederer (1975). One hundred µl of 1 % (w/v) sodium hippurate solution were

dispensed into each well of a microtitre plate. A loopful (1 µl) of *Campylobacter* growth from a culture grown on modified cefaperazone charcoal deoxycholate agar (mCCDA) microaerobically for 48 h at 41.5 °C was added, agitating 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, well mixed and incubation 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.

DNA templates were prepared by adding a 10 µl loopful of *Campylobacter* culture (previously tested for hippurate hydrolysis) 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) 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), 2.75 µl nuclease free water, 0.75 µl of 50 mM magnesium chloride (Qiagen) 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.

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

A comparison of the 18 isolates tested for hippurate hydrolysis and multiplex Polymerase Chain Reaction (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 looks like on a microtitre plate. Deep purples colour indicates *C. jejuni's* whilst medium, light or no colour changes are *C. coli's.*

Hydrolysis of sodium hippurate by *C. jejuni* produces a deep purple colour, while *C. coli* strains produce medium or no colour change. Multiplex PCR (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 (Figure 2).

One isolate was identified as *C. coli* (medium purple) by a negative hippurate test but was *C. jejuni* by the mPCR (Figure 2, lane 2). The samples may contain 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 oppressed 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 *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 speciate 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.

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 multiplex PCR 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 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).

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REFERENCES

Aarestrup F M Nielsen E M Madsen M and Engberg J. (1997). Antimicrobial susceptibility patterns of thermophilic *Campylobacter* spp. from human, pigs, cattle and broilers in Denmark. *Antimicrobial Agents and Chemotherapy* 41: 2244-2250.

Adzitey F and Nurul H. (2010). Campylobacter in poultry: incidences and possible control measures. *Research Journal of Microbiology* 6: 182-192.

Bang D D Wedderkopp A Pedersen K and Madsen M. (2002). Rapid PCR using nested primers of the 16S rRNA and hippuricase (hip O) genes to detect *Campylobacter jejuni* and *Campylobacter coli* in environmental samples. *Molecular and Cellular Probes* 16: 359-369.

Burnett T A Hornitzky M A Kuhnert P and Djordjevic S P. (2002). Speciating *Campylobacter jejuni* and *Campylobacter coli* isolates from poultry and humans using six PCR-based assays. *FEMS Microbiology Letters* 216: 201-209.

Corry J E L Atabay H I Forsythe S J and Mansfield L P. (2003). Culture media for the isolation of campylobacters, helicobacters and arcobacters. In: Handbook of Culture Media for Food Microbiology Corry, J.E.L., G.D.W. Curtis, and R.M. Baird, (Eds.), Second Edition. *Elsevier Science, Amsterdam,* pp. 271-315.

Didelot X and Falush D. (2007). Inference of bacterial microevolution using multilocus sequence data. *Genetics* 175: 1251-1266.

Dingle K E Colles FM Falush D and Maiden M C. (2005). Sequence typing and comparison of population biology of *Campylobacter coli* and *Campylobacter jejuni. Journal of Clinical Microbiology* 43: 340-347.

European Food Safety Authority (EFSA). **(**2005). Scientific Report of the Scientific Panel on Biological Hazards on the request from the Commission related to Campylobacter in animals and foodstuffs. *Annex to the EFSA Journal* 173: 1-105.

Hwang M N and Ederer G M. (1975). Rapid hippurate hydrolysis method for presumptive identification of group B streptococci. *Journal of Clinical Microbiolology* 1: 114-115.

Klena J D Parker C T Knibb K Ibbitt J C Devane P M Horn S T Miller W G and Konkel M E. (2004). Differentiation of *Campylobacter coli, Campylobacter jejuni, Campylobacter lari,* and *Campylobacter upsaliensis* by a multiplex PCR developed from the nucleotide sequence of the lipid A gene *lpxA. Journal of Clinical Microbiology* 42: 5549-5557.

Mead P S Slutsker L Dietz V McCaig L F Bresee J S Shapiro C Griffin P M and Tauxe R V. (1999). Food-related illness and death in the United States. *Emerging Infectious Diseases* 5: 607-625.

Moore J E Corcoran D Dooley J S G Fanning S Lucey B Matsuda M McDowell D A Mégraud F Millar B C O'Mahony R. O'Riordan L O'Rourke M Rao J R Rooney P J Sails A and Whyte P. (2005). Review Article, Campylobacter. *Veterinary Research* 36: 351-382.

Mugg P. (1983). A Rapid Hippurate Hydrolysis Test for the Presumptive Identification of Group B Streptococci. *Pathology* 15: 251 – 252.

On S L W. (2001). Taxonomy of Campylobacter, Arcobacter, Helicobacter and related bacteria: current status, future prospects and immediate concerns. *Journal of Applied Microbiology* 90: 1S–15S.

Ridley A M Allen V M Sharma M Harris J A and Newell D G. (2008). Real-time PCR approach for detection of environmental sources of campylobacter strains colonizing broiler flocks. *Applied and Environmental Microbiology* 74: 2492-2504.

Smith J L. (1995). Arthritis, Gullain-Barré syndrome and other sequelae of *Campylobacter jejuni* enteritis. *Journal of Food Protection* 58: 1153-1170.

Slater E R and Owen R J. (1997). Restriction fragment length polymorphism analysis shows that the hippuricase gene of *Campylobacter jejuni* is highly conserved. *Letters in Applied Microbiolology* 25: 274-278.

Rönner A C Engvall E O Andersson L and Kaijser B. (2004). Species identification by genotyping and determination of antibiotic resistance in *Campylobacter jejuni* and *Campylobacter coli* from humans and chickens in Sweden. *International Journal of Food Microbiolology* 96: 173-179.

Vandamme P. (2000). Microbiology of campylobacter infections: taxonomy of the family Campylobacteraceae. In: *Campylobacter* Nachamkin, I. and M.J. Blaser, (Eds.), Washington, DC., *ASM Press*, pp: 3-26.

Velayudhan J and Kelly D J (2002). Analysis of gluconeogenic and anaplerotic enzymes in *Campylobacter jejuni*: an essential role for phosphoenolpyruvate carboxykinase. *Microbiology* 148: 685–694.

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

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.* (2004)
		Reverse	CCA TCA CTT ACA AGC TTA TAC	

Table 2: Comparison between hippurate hydrolysis and multiplex-PCR for the identification of *C. jejuni* and *C. Coli*

Campylobacter isolate used	Colour	Hippurate results	Lanes	Multiplex-PCR 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*

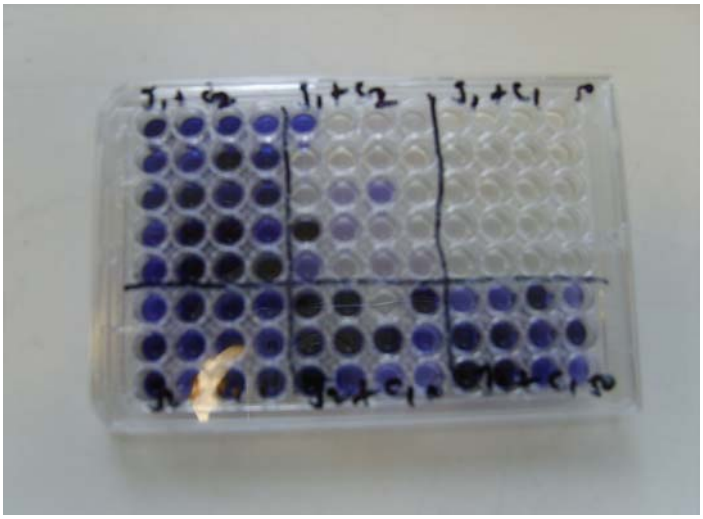

Figure 1: Results of hippurate hydrolysis test carried out on microtitre plate. Deep purple colour indicates *C. jejuni* while medium or no colour change indicates *C. coli.*

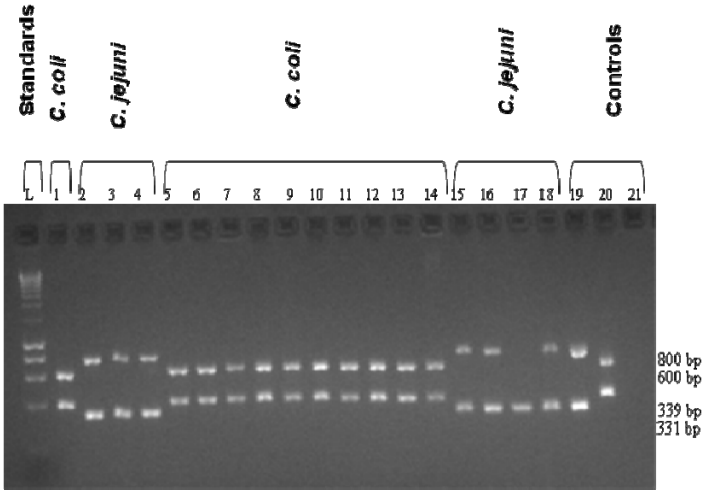

Figure 2: A representative multiplex PCR assay showing results for 18 *Campylobacter* isolates from poultry-related samples. Lane 1, *C. coli*; lanes 2-4, *C. jejuni*; lanes, 5-14, *C. coli*; lanes, 15-18, *C. jejuni* (lane 17, has a missing *hipO* band*)*, lane 19, *C. jejuni* (11168) positive control; lane 20, *C. coli* (RM28) negative control and lane 21, negative control. Lanes 1-2, were medium purple (*C. coli*); lanes 3-4, were deep purple (*C. jejuni*); lanes 5-14, were medium purple (*C. coli*) and lanes 15-18, were deep purple (*C. jejuni*) by hippurate hydrolysis test.