Pakistan Veterinary Journal

ISSN: 0253-8318 (PRINT), 2074-7764 (ONLINE)
Accessible at: www.pvj.com.pk

RESEARCH ARTICLE

Occurrence of Co-Infection of *Helicobacter pullorum* and *Campylobacter* spp. in Broiler and Village (Indigenous) Chickens

Soe Soe Wai, A. A. Saleha*, Z. Zunita, L. Hassan and A. Jalila

Faculty of Veterinary Medicine, Universiti Putra Malaysia, 43400 UPM, Serdang, Selangor, Malaysia
*Corresponding author: saleha@vet.upm.edu.my

ARTICLE HISTORY

Received: November 17, 2011
Revised: February 01, 2012
Accepted: March 07, 2012

Key words:
Broiler chickens
Campylobacter spp.
Co-infection
Helicobacter pullorum
Village chickens

ABSTRACT

The reports on prevalence of *Helicobacter pullorum* in broiler chickens are rather limited and lacking in village chickens. This study aimed to determine the occurrence of *H. pullorum* in broiler and village chickens in Selangor, Malaysia and to report the detection of co-infection of *H. pullorum* and *Campylobacter* spp. in these chickens. Village (indigenous) chickens were sampled in five markets and broiler chickens from six farms in different localities. Cecal contents were aseptically obtained from the chickens and subjected to three cultural methods. The isolates were identified by biochemical tests and confirmed using a species-specific PCR assay. *Helicobacter pullorum* were isolated from 25% village chickens and 24.6% broiler chickens, with an overall occurrence of 24.7%. Eleven (50%) of these positive chickens (nine in broiler and two in village chickens) showed co-infection with *Campylobacter* spp.

To Cite This Article: Wai SS, AA Saleha, Z Zunita, L Hassan and A Jalila, 2012. Occurrence of co-infection of *Helicobacter pullorum* and *Campylobacter* spp. in broiler and village (indigenous) chickens. Pak Vet J, 32(4): 503-506.

INTRODUCTION

Chickens are frequently colonized by *Campylobacter* spp., namely *Campylobacter jejuni* and *Campylobacter coli*, which may result in the contamination of chicken meat and products during processing (Corry and Atabay, 2001). It is documented that handling and consumption of contaminated poultry meat and products have been implicated as a primary source of *Campylobacter* infection in man in developed countries, causing acute gastroenteritis and diarrhea (Wingstrand *et al.*, 2006). Most campylobacteriosis are self-limiting; however prolonged cases and severe infections may occur which require antibiotic treatment. Several studies have indicated *C. jejuni* as frequent antecedent pathogen in Guillain-Barre and Fisher Syndromes (Nachamkin *et al.*, 2000). *Helicobacter pullorum* is also reported as another common inhabitant of the caeca and large intestines of broiler chickens (Atabay *et al*., 1998; Gonzalez *et al*., 2008); the organisms have been found in liver and intestinal contents of layer chickens with vibrionic hepatitis (Stanley *et al.*, 1994; Burnens *et al.*, 1996). In man, *H. pullorum* have been isolated from feces and biopsies of human patients with gastroenteritis, chronic liver diseases and inflammatory bowel disease (Burnens *et al*., 1994; Ceelen *et al*., 2005; Gonzalez *et al*., 2008).

Campylobacter spp. and *H. pullorum* are generally considered as commensal in poultry. Corry and Atabay (2001) reported that the colonization of *Campylobacter* spp. and *H. pullorum* in the poultry guts rarely caused disease (avian hepatitis) in these birds.

Many families living in villages and rural areas kept indigenous chickens. These chickens are usually reared in small flocks (5-15 birds per household) under free-range or backyard system. The chickens are free to roam and they scavenge for most of their food and water in the village environment; at time they receive a small supplement of household food scraps. They sleep on trees, in shrubs or some were provided with simple wooden sheds. Village chickens are raised for their eggs and meat, for social, medicinal and traditional values and to supplement family income because their meat and eggs command premium price. The broiler chickens are mainly reared in semi-intensive and intensive commercial farms and given commercial feed and ample supply of drinking water.

The information of *H. pullorum* in broiler chickens are rather limited and thus far none in village chickens. Therefore, this study was undertaken to determine the presence of *H. pullorum* in village chickens and to report on the co-infection of *H. pullorum* and *Campylobacter* spp. in these chickens.

MATERIALS AND METHODS

Sample collection: A total of 89 chickens were sampled which comprised 32 village and 57 broiler chickens. The village chickens were purchased from five different markets in Klang Valley area, Selangor. The owners brought the chickens to the markets for sale to potential customers. In one of the markets, 10 chickens were purchased and four to seven birds were purchased from each of the other four markets. The chickens were brought to a stall in each market for slaughter and evisceration by the stall workers. The intestinal tract of each bird was removed immediately after evisceration. Then, the caeca were carefully removed from each bird, avoiding cross contamination, from the rest of the intestinal tract, and placed in a sterile petri dish. These dishes were sealed, placed in a cool box packed with ice during transportation to the laboratory and cultured within two to four hours.

The broiler chickens were randomly sampled from six farms located in different localities in Selangor. Ten chickens were sampled from each farm except in one farm in which only seven chickens were sampled. The chickens were brought to a nearby market for slaughter and evisceration. The collection and handling of caeca from each broiler chicken and their transportation to the laboratory was similarly carried out as for village chickens. The samplings were done over a period of six months, from March to August 2010.

Culture and isolation: The cecal contents were obtained aseptically and subjected to three cultural methods to maximize the isolation of *H. pullorum*. These three methods employed were as described by Ceelen *et al*. (2006), noted as Method I, Zanoni *et al*. (2007) as Method II and Miller *et al*. (2006) as Method III. Ceelen *et al*. (2006) initially ran PCR analysis on all samples (pre-screening) and the positive samples cultured to recover *H. pullorum* isolates. In this study, all samples were cultured without pre-screening.

Approximately 2 g of cecal contents were squeezed into 2 ml of 0.85% sterile saline and were mixed using a vortex mixer to obtain a homogenous suspension. An aliquot of 100 µl of each homogenate was then diluted in 400 µl of a sterile mixture containing 25 ml of Bacto-Brain Heart Infusion (BD), 75 ml of inactivated horse serum (Oxoid) and 7.5 g of glucose (Sigma). This sample mixture was also used in Method I and II.

In Method I, each sample mixture was inoculated on Brain Heart Infusion agar (Oxoid) supplemented with 10% horse blood, amphotericin B 20 µg/mL (Sigma) and Vitox (Oxoid) (noted as BHI blood agar). Inoculation was done using the modified filter technique of Steele and McDermott (1984). In brief, 300 µl of each sample mixture was spread on a 47 mm, 0.45 µm pore size sterile membrane filter (Sartorius) which was earlier placed centrally on the agar surface. The agar plates were incubated upright at 37°C for 1 h in 5% CO_2 (1st incubation); the filter was then removed, the filtrate was streaked on the agar surface with a sterile loop and the plates were then incubated under microaerophilic condition at 37°C for a minimum of 72 h (2nd incubation).

In Method II, each sample mixture was inoculated on BBL-Brucella agar (BD) supplemented with 5% defibrinated sheep blood (Oxoid). The modified filter technique of Steele and McDermott (1984) was used as described above and a 47 mm, 0.65 µm pore size sterile membrane filter (Sartorius) was used. Both 1st and 2nd incubation of plates were under microaerophilic condition.

In Method III, each sample of cecal contents in saline solution was directly inoculated on freshly prepared 10% Sheep Blood agar (Oxoid); the modified filter technique of Steele and McDermott (1984) with a 47 mm, 0.45 µm pore size sterile membrane filter (Sartorius) was used for inoculation. Briefly, six drops of the saline mixture were placed on the filter and left to set for 1 h at room temperature. The filter was then removed and the plates were incubated at 42°C for 48 h under anaerobic condition generated by an anaerobic gas pack without palladium catalyst (Oxoid). The reference strains used were *Campylobacter* ATCC 2498 and *H. pullorum* CCUG 33837.

Phenotypic identification and biochemical tests: The plates were examined for small, grayish white or translucent colonies which were then picked for cellular morphological examination. Those that showed Gram-negative, slightly curved, slender rods were sub cultured on blood agar plate.

Apart from Gram staining, a wet mount was prepared from a culture and examined to determine the motility of the isolate. The biochemical tests performed were oxidase, catalase, urease and indoxyl acetate hydrolysis tests, following that of Miller *et al*. (2006). *Helicobacter pullorum* and *Campylobacter* spp. are positive to oxidase, catalase, urease tests; however *H. pullorum* is negative to indoxyl acetate hydrolysis test whereas *Campylobacter* spp. are positive to the test.

Genotypic confirmation of isolates: The phenotypic and biochemical tests may result in misidentification and thus confirmation of the suspected isolates was carried out. This was done using a modified **s**pecies-specific PCR method as described by Miller *et al*. (2006) which they modified from that of Stanley *et al*. (1994). The primers used to detect *H. pullorum* were: forward primer 5'-ATGAATGCTAGTTGTTGTGAG-3' and reverse primer 5'-GATTGGCTCCACTTCACA-3' that target a 447 bp fragment. To detect *Campylobacter*, the primers used were genus-specific: forward primer C99-GCGTGGAGGATGACACCT and reverse primer C98-GATTTTACCCCTACACCA that target a 296 bp.

DNA was extracted from suspected colonies using a commercial kit (DNeasy Blood and Tissue Kit, Qiagen). Maxime PCR Premix Kit (iStarTaq, Intron Biotechnology, Korea) was used for PCR assay according to manufacturer's instruction; one µl of DNA preparation was added to a 20 µl (final volume) reaction mixture containing distilled water and 0.25 µM of each primer. The PCR assay was run as follows:

For *H. pullorum*, initial denaturation was conducted at 94°C for 4 min, followed by amplification which was achieved by 30 cycles at 93°C for 1 min, annealing at 65°C for 1 min and extension at 72°C for 1 min. The final elongation step was at 72°C for 5 mins. A 5 µl of amplicone was electrophoresed through a 2% agarose gel containing Tris-Borate-EDTA (TBE) buffer (40 mmol l^{-1}

Tris-acetate, 2 mmol l^{-1} EDTA, pH 7.5) and gel red (3 µl ml^{-1}) in TBE buffer at 75 V, for 80 minutes. The gel was viewed under ultraviolet transillumination. For *Campylobacter* spp., the protocol was similar to *H. pullorum* with the exception of the annealing temperature which was at 55°C.

RESULTS

Occurrence of *H. pullorum* in chickens: Upon initial examination of cultures, three samples were found negative for any growth and colonies from 86 samples were suspected as that of *Helicobacter*. However, on further examination which included morphological identification, biochemical tests and genetic confirmation, 22 samples (24.72%) were confirmed as positive for *H. pullorum* and 11 of these were co-infected with *Campylobacter* spp.

Morphological identification and biochemical tests: The cellular morphology showed Gram negative, slightly curved, slender rods, motile on wet mount, urease negative and oxidase and catalase positive. However, on indoxyl acetate hydrolysis test, only 11 isolates showed negative reaction, indicating they were most likely *H. pullorum*. The reference strain gave the same characteristics. These six *H. pullorum* were isolated from Method III (Table 1). Another 11 isolates produced slight purplish color to indoxyl acetate hydrolysis test which was considered as weak positive and thus suspected as *Campylobacter*-like organisms (CLOs). The remaining isolates gave quite strong reaction, indicating they were most likely *Campylobacter* spp.

Genotypic confirmation of isolates: The species-specific PCR confirmed the 11 isolates, six from village and five from broiler chickens, as *H. pullorum* which together with the reference strain *H. pullorum* CCUG 33837 produced a single band of 447 bp (Figure 1). Two and nine of the CLOs from village and broiler chickens respectively produced two bands with one corresponded to 447 bp and the other to 296 bp, indicating that they were positive for both *H. pullorum* and *Campylobacter* spp. In broiler chickens, one of the eight CLOs isolated from Method II, was not detected by Method III. . Overall, 14 broiler and eight village chickens were positive for *H. pullorum* with nine of the broiler and two of the village chickens were co-infected with *Campylobacter* spp. (Table 1).

DISCUSSION

The data available on the occurrence of *H. pullorum* were found only in broiler chickens. The number of studies on the prevalence of *H. pullorum* in broiler chickens, showed the rates ranging from 4% in Switzerland (Burnen *et al*., 1996), 13.5% in Australia (Miller *et al*., 2006), 33.6% in Belgium (Ceelen *et al*., 2006) to 78.3% in Czech Republic (Svobodova and Borilova, 2003) and 100% in Italy (Zanoni *et al*., 2007). Manfreda *et al*. (2006) reported 96.9 and 66.7% of intensive and extensive broiler farms respectively in Italy were *H. pullorum* positive and also showed high occurrence of *H. pullorum* in cecal contents of broilers reared in intensive farms (84.1%) compared to those in extensive farms (57.8%). Campylobacter are reported as ubiquitous in the environment and could be readily carried into the chicken houses. A number of factors have been reported in the colonization of the organisms in chickens; among these risk factors are inadequately cleaned and disinfected houses and facilities, presence of pests such as rats, birds, insects (flies, beetles) and pets (dogs, cats), contaminated water supplies and boots and clothes of farm workers (Newell and Fearnley, 2003). There is no information on the risk factors associated with *H. pullorum* except for the possible role of water in the transmission (Azevedo *et al.*, 2008).

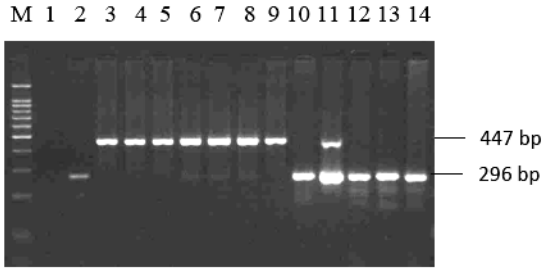

Fig. 1: Species-and genus-specific PCR to detect presence of *Helicobacter pullorum* and *Campylobacter* spp. in chickens. M: 100bp ladder; lane 1-*E coli* ATCC 25922 (negative control); lane 2-*C. jejuni* ATCC 24928 (*Campylobacter*- ; lane 3-*H. pullorum* CCUG 33837; lanes 4 to 9-*H. pullorum* isolates; lane 11-mixed isolate of *H. pullorum* and *Campylobacter* spp.; lanes 10, 12 to 14-*Campylobacter* spp. isolates.

The present study showed the occurrence of *H. pullorum* in both village and broiler chickens and 50% showed mixed infection with *Campylobacter* spp. The co-infection of *Helicobacter* sp. and *Campylobacter* sp. has been reported in cats (Shen *et al.*, 2001), that is, 64 or 28% of the 227 cats examined were initially diagnosed as positive for CLOs; of the 51 isolates obtained, PCR assay identified 92% positive for *Campylobacter* spp., 41% positive for *Helicobacter* spp. while 33% were mixed cultures of *Campylobacter* and *Helicobacter* organisms.

Ceelen *et al*. (2006) subjected a large number of samples consisted of liver, caecum, jejunum and colon to PCR assay and then subjected those positive to cultural method; 33.6% of the cecal samples showed presence of *H. pullorum* by using PCR and sixteen isolates were obtained. Zanoni *et al*. (2007) isolated *H. pullorum* from chicken cecal contents and upon using PCR assay, found all were positive for *H. pullorum*.

It has been reported that *H. pullorum* and *Campylobacter* spp. share a common habitat within the caeca and large intestine of chickens (Atabay *et al*., 1998; Manfreda *et al*., 2006; Zanoni *et al*., 2007). The colonies on culture were observed as similar for both organisms causing difficulty in selecting suspected colonies of *H. pullorum*. Atabay *et al.* (1998) reported that *H. pullorum* can be easily misidentified as *Campylobacter* species (in particular *C. coli* and *C. lari*) because they shared several key phenotypic traits. According to Miller *et al*. (2006), *H. pullorum* showed slow-jerky-tumbling motility whereas *Campylobacter* showed darting, corkscrew movement, however only an experienced observer is able to observe the difference; thus the use of indoxyl acetate

Table 1: Detection of *H. pullorum* and *Campylobacter* spp. in village (indigenous) and broiler chickens

Methods	*H. pullorum* only		*Campylobacter* spp. only #		*H. pullorum* and *Campylobacter* spp.	
	Village chickens	Broiler chickens	Village chickens	Broiler chickens	Village chickens	Broiler chickens
Method I	0	0	21	49	2*	3
Method II	0	0	20	44	2*	8**
Method III	6	5	13	44	2*	8

* The same two samples were detected by the three methods; ** *Helicobacter pullorum* in one of the samples was not detected by Method III; # On subcultures, three isolates each from Methods I and II and two from Method III did not grow (possibly could have entered into viable but nonculturable form). The remaining isolates were not *Campylobacter* spp.

hydrolysis test as a screening test was suggested because *H. pullorum* are negative and *Campylobacter* spp. are positive to the test. Since *H. pullorum* are commonly being mistaken as *Campylobacter* spp. (Miller *et al.*, 2006), a reliable method is therefore needed to confirm their presence which currently the *H. pullorum* - specific PCR assay of Stanley *et al.* (1994) and Miller *et al.* (2006) has serve the purpose. Shen *et al.* (2001) suggested for laboratories to incorporate PCR-based assays using *Helicobacter* and *Campylobacter* genus- and species- specific primers for accurate diagnosis of infections with these bacteria.

This study also showed a high occurrence of *Campylobacter* spp. in village and broiler chickens. Previous studies had shown the prevalence of *Campylobacter* infection in indigenous chickens in Malaysia ranged from 8.0 to 81.9% (Saleha, 2003). Indigenous chickens are free to roam the household environment; thus those chickens that are *Campylobacter*-infected could contaminate the environment by their indiscriminate feces shedding. Marquis *et al.* (1990) reported that children in families that kept chickens were 12 times more likely to contract *Campylobacter* enteritis compared to households without chickens. Contaminated birds' droppings can be brought into the household, such as by footwear and flies (Messens *et al*., 2009).

The presence of *H. pullorum* and *Campylobacter* spp. in chickens could lead to their presence on the carcasses. Studies have shown the common presence of *Campylobacter* in chicken meat and chicken products, however, few studies were reported on *H. pullorum* on chicken products. Atabay *et al*. (1998) found *H. pullorum* in 60% (9/15) of chicken carcasses from a poultry abattoir and Gonzalez *et al.* (2008), using real-time PCR assay, detected *H. pullorum*-like organisms in 70% (7/10) of chicken carcasses and 10% (1/10) of chicken burger sold in retail outlets in Valencia, Spain. Hence, the presence of these organisms in chickens and chicken meat and products is of public health concern.

Acknowledgment: The authors thank Ministry of Science, Technology and Innovations (MOSTI) for financial support through Science fund Project (No. 05-01-04-SF0502) and Puan Fauziah for her kind assistance.

REFERENCES

Atabay HI, JE Corry and SLW On, 1998. Identification of unusual *Campylobacter*-like isolates from poultry products as *Helicobacter pullorum*. J Appl Microbiol, 84: 1017-1024.

Azevedo NF, C Almeida, I Fernandes, L Cerqueira, S Dias, CW Keevil and MJ Vieira, 2008. Survival of gastric and enterohepatic *Helicobacter* spp. in water: implications for transmission. Appl Environ Microbiol, 74: 1805-1811.

Burnens AP, J Stanley, R Morgenstern and J Nicolet, 1994. Gastroenteritis associated with *Helicobacter pullorum*. Lancet, 344: 1569-1570.

Burnens AP, J Stanley and J Nicolet, 1996. Possible association of *Helicobacter pullorum* with lesions of vibrionic hepatitis in poultry. In: *Campylobacters, Helicobacters* and Related Organisms. (Newell DG, Ketley, JM, Feldman RA, eds), Plenum Press, New York, pp: 291-294.

Ceelen LM, A Decostere, K Van den Bulck, SLW On, M Baele, R Ducatelle, and F Haesebrouck, 2006. *Helicobacter pullorum* in chickens, Belgium. Emerg Infect Dis, 12: 263-267.

Ceelen LM, A Decostere, K Chirers, R Ducatelle, D Maes and F Haesebrouck, 2005. Pathogenesis of *Helicobacter Pullorum* infections in broilers. Int J Food Microbiol, 116: 207-213.

Corry JEL and HI Atabay, 2001. Poultry as a source of campylobacter and related organisms. J Appl Microbiol, 90: 96S-114S.

Gonzalez A, P Piqueres, Y Moreno, I Canigral, RJ Owen, J Hernandez and MA Ferrus, 2008. A novel real-time PCR assay for the detection of *Helicobacter pullorum*-like organisms in chicken products. Int Microbiol, 11: 203-208.

Manfreda G, M Rossi, B Sanguinetti, R Gavioli, P Lazito, AD Cerare and RG Zanoni, 2006. Prevalence of *Helicobacter pullorum* in broiler chickens reared in intensive and extensive farms. World's Poultry Science Association- Proc (2006). XII European Poultry Conference, Verona, Italy, 10-14 September 2006. http://www.wpsa.com/proceedings/wpsa_proceeding.htm.-6k (15.3.08)

Messens W, L Herman, L De Zutter and M Heyndrick, 2009. Multiple typing for the epidemiological study of contamination of broilers with thermotolerant *Campylobacter*. Vet Microbiol, 138: 120-131.

Miller KA, LL Blackall, JK Miflin, JM Templeton and PJ Blackall, 2006. Detection of *Helicabacter pullorum* in meat chicken in Australia. Aust Vet J, 84: 95-97.

Nachamkin I, BM Allos and TW Ho, 2000. *Campylobacter jejuni* infection and the association with Guillain-Barre Syndrome. In: *Campylobacter*, 2nd Ed (Nachamkin I and and Blaser MJ, eds) ASM Press, Washington, USA.

Newell DG and C Fearnley, 2003. Sources of *Campylobacter* colonization in broiler chickens. Appl Environ Microbiol, 69: 4343-4351.

Saleha AA, 2003. Overview of *Campylobacter* in poutry, other animals and in meat in reference to Malaysia. J Vet Malaysia, 15: 1 -6.

Shen Z, Y Feng, FE Dewhirst and JG Fox, 2001. Coinfection of enteric *Helicobacter* spp and *Campylobacter* spp. in cats. J Clin Microbiol, 39: 2166-2172.

Stanley J, D Linton, AP Burnens, FE Dewhirst, SLW On, A Porter, RJ Owen and M Costas, 1994. *Helicobacter pullorum* sp. nov. - genotype and phenotype of new species isolated from poultry and from human patients with gastroenteritis. Microbiology, 140: 3441-3449.

Steele TW and SN McDermott, 1984. The use of membrane filters applied directly to the surface of agar plates for the isolation of *Campylobacter jejuni* from feces. Pathology, 16: 263-265.

Svobodova I and G Borilova, 2003. Incidence of *Helicobacter pullorum* and *Campylobacter* spp. in healthy broilers in the Czech Republic. Zoonoses Publ Health, 54 (suppl 1): 75.

Wingstrand A, J Neimann, J Engberg, EM Nielsen, P Gerner-Smidt, HC Wegener and K Molbak, 2006. Fresh chicken as main risk factor for campylobacteriosis, Denmark. Emerg Infect Dis, 12: 280-285.

Zanoni RG, M Rossi, D Giacomucci, V Sanguinetti and G Manfreda G, 2007. Occurrence and antibiotic susceptibility of *Helicobacter pullorum* from broiler chickens and commercial laying hens in Italy. Int J Food Microbiol, 116: 168-173.