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Genetic Relatedness among *Helicobacter pullorum* Isolates from Broiler Chickens

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Abstract: *Helicobacter pullorum*, an enterohepatic helicobacter with the ability to colonize the intestine and liver of avian species, mice and humans, is increasingly recognized as a food borne zoonotic pathogen. The present study aimed to determine the genetic similarity among *H. pullorum* using pulsed field gel electrophoresis (PFGE) with *Sac*II and *Sma*I restriction endonucleases (REs) digestion. The *H. pullorum* were isolated from broiler chickens in eight farms. Within-farm genetic profiling of isolates showed close relationship (>95%); however genetic diversity was observed between isolates from different farms. Digestion of genome with *Sac*II yielded two clusters comprised 16 pulsotypes, 8-14 DNA bands with a molecular weight ranging from 40 to 400 kb whereas *Sma*I digested genome produced two clusters with 14 pulsotypes, 7 to 13 DNA bands with molecular weight ranging from 40 to 250 kb. The RE *Sac*II showed a higher discriminatory power compared to *Sma*I. In conclusion, within-farm genetic similarity of isolates suggested the same source of clonal origin.

Key words: *Helicobacter pullorum*; chickens; genetic relationship; PFGE

INTRODUCTION

Helicobacter pullorum is a member of enterohepatic helicobacters group, which predominantly colonizes the intestine and hepatobilary system (Fox, 1997). In recent years, *H. pullorum* has emerged as a potential food borne zoonotic pathogen. It has been isolated from caecal contents of broilers, laying hens, turkeys, ostriches, guinea fowls, parrots and psittacine birds (Ceelen *et al*., 2006; Manfreda *et al*., 2011; Nebbia *et al*., 2007). *Helicobacter pullorum* has the capability to cause various degree of gastritis, hepatitis, and intestinal diseases in susceptible humans and animals (Mohamed *et al*., 2010; Varon *et al*., 2009) and has been associated with several human infections such as gastroenteritis, Crohn's disease, chronic liver diseases, hepatocellular carcinoma, acute and chronic cholecystitis (Casswall *et al*., 2010; Rocha *et al*., 2005). Consumption of undercooked chicken meat is considered as a potential route of *Helicobacter* transmission to humans (Wesley, 2001).

The molecular characterization and genetic relatedness scheme is one of the essential tools for epidemiological and evolutionary studies of organisms (Dingle *et al*., 2001). According to Sails *et al*. (2003) pulsed field gel electrophoresis (PFGE) has highest discriminatory power than other molecular typing techniques. Variations among PFGE patterns arise from chromosomal insertions, deletions and recombinations, which increase the discriminatory power of the method and its ability to detect rapidly occurring chromosomal changes (Levesque *et al*., 2008).

Thus far, very few studies have been carried out to assess the genetic relationship among *H. pullorum* isolates using molecular techniques, such as amplified fragment length polymorphism (AFLP) and PFGE (Gibson *et al*., 1999; Manfreda *et al*., 2011; Zanoni *et al*., 2011).

Therefore, the objective of this study was to assess the genetic diversity of *H. pullorum* isolated from broiler chickens using PFGE assay.

MATERIALS AND METHODS

The *H. pullorum* were isolated from broiler chickens in eight farms (farm 5 to farm 12) in a previous study. Two to seven *H. pullorum* isolates were selected as representatives of each farm. PFGE analysis was performed using the procedure as described in PulseNet for *Campylobacter jejuni* (www.cdc.gov/pulsenet); however, slight modifications were made in the selection of restriction enzymes (REs) and electrophoresis conditions which were carried out according to Gibson *et al*. (1999). *Helicobacter pullorum* isolates were harvested by centrifugation, washed in a saline-EDTA solution (0.15M NaCl, 10 mM EDTA, pH 8.0), and re-suspended in Pett IV solution (1M NaCl, 10 mM EDTA, pH 8.0). Each cell suspension was adjusted to an optical density to read in the range of McFarland standard 6-7. To inactivate DNAse activity, formalin

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treatment was given. Aliquot of one milliliter of cell suspension was put in a 1.5 ml microcentrifuge tube and followed by the addition of 100 µL of formaldehyde solution (37-40%); it was incubated at room temperature for 1h. Following incubation, the suspension was centrifuged for 10 min at 13000 x g. The cells were washed three times in one milliliter Pett IV buffer and finally re-suspended in approximately 600 µL of Pett IV buffer. An equal volume of melted 1.5% low-melting-point agarose (Bio-Rad Laboratories) and 18.8 µL of proteinase K (20 mg/ml stock from Merck, Germany) were added to this suspension. The mixture was poured into plug molds and kept at 4°C for 5-7 min. The plugs were then lysed in 5 ml of cell lysis buffer (50 mM Tris, 50 mM EDTA pH 8.0, 1% sarcosine, 0.1 mg of proteinase K/ml) for 25 min at 55°C in a shaker water bath with constant and vigorous agitation. Later, the lysis solution was replaced with TE buffer (10 mM Tris-HCl; 1 mM EDTA [pH 8.0]) containing 1 mM phenylmethylsulfonyl fluoride (PMSF) and kept at room temperature for 2 h. The washing of the plugs were done twice in ultrapure water and four times in TE for 15 to 20 min at 55ºC for every wash. For restriction endonucleases (REs) digestion, 20U of *Sac*II and *Sma*I (Biolab, England) were used for each plug and followed by incubation at room temperature for overnight. Finally, gel electrophoresis was performed in CHEF Mapper XA (Bio-Rad, USA) with the following conditions: total run time 23 h; temperature 14°C; initial switch time 2 s and a final switch time 12 s for *Sac*II digestion while 2 to 10 s for *Sma*I digestion; voltage of 6.6 V/cm and an included angle 120°. The electrophoresed gels were stained with ethidium bromide solution (1 µg/ml) for 15 min, then destained for 1 h, and viewed under a UV transilluminator.

The PFGE patterns were analyzed using the Molecular Analyst Fingerprinting Plus software package (version 4.5; Bio-Rad). Lambda ladder (48.5 concatamers; New England Biolabs) was used as the size standard marker. The TIFF images were normalized by alignment with the appropriate size standard lanes and profiles were compared using the discriminatory index (*D*) (Hunter and Gaston, 1988). Digitalized DNA patterns of isolates were analyzed by the Dice correlation coefficient and represented by unweighted pair group method using arithmetic averages (UPGMA) with 1% optimization and 1.0% tolerance window. Profiles showing ≥ 95% similarity were assigned to the same *Sac*II or *Sma*I PFGE type, assuming that they were closely related (Zanoni *et al*., 2011).

RESULTS

A total of 27 *H. pullorum* isolates from the eight farms were typed using *Sac*II and *Sma*I REs; however, two isolates were untypable by both REs and thus were not included for further analysis. Genome digestion with *Sac*II RE produced a number of recognition sites yielding between 8 to 14 DNA bands. Based on the restriction patterns, *H. pullorum* isolates were assigned into two major clusters comprised 16 pulsotypes (A-P). Seven pulsotypes were shared by two to three isolates while the other nine isolates were unique. Cluster I consisted of isolates from only one farm (farm 11) showing 61.6% similarity however cluster II comprised isolates from seven farms with 69.6% homology (Fig. 1). Isolates from farms 5, 7, 8, 9, 10 and 12 showed within-farm similarity (≥ 95%) while within-farm heterogeneity among isolates was noted from farms 6, 9 and 11 which shared 86%, 88.3% and 61.6% genomes, respectively. The *D* value achieved by PFGE for the 25 isolates was 0.94.

*Sma*I restricted genome produced 7 to 13 DNA bands with a molecular weight ranging from 40 to 250 kb. Taking into account clustering cut off point (≥ 18.7%), two major clusters comprising 14 pulsotypes were produced with 95% similarity. Nine pulsotypes were shared by two to three isolates, however five were unique. Cluster I consisted of two farms (11 and 12) with 44.4% similarity and cluster II comprised of seven farms (5-11) with 52.3% relatedness (Fig. 2). Within-farm relatedness was observed in isolates from all farms. Although two isolates (pulsotype G) from farm 11 were 95.4% similar, other isolates (pulsotypes A and B) from the same farm showed less genetic relatedness (90.2%). The *D* value achieved using PFGE for the 25 isolates was 0.92.

The restriction patterns of two REs were compared and it was observed that *Sac*II showed a higher discriminatory power, D = 0.94 (16 pulsotypes) compared to *Sma*I, D = 0.92 (14 pulsotypes). The range of similarity of restriction patterns was 41.0-88.3% for *Sac*II and 18.7-88.8% for *Sma*I. Moreover, *Sac*II digestion of four isolates from farm 11 showed four distinct pulsotypes (A, B, C and D), while *Sma*I showed three pulsotypes (A, B and G). Similarly, *Sac*II restricted the DNA at 8-14 sites whereas 7-13 patterns were generated using *Sma*I.

DISCUSSION

The untypability of *H. pullorum* isolates by PFGE using *Sac*II and *Sma*I has also been reported previously by Gibson *et al*. (1999), Manfreda *et al*. (2011) and Zanoni *et al*. (2011). The lack of bands probably occurred due to the absence of restriction sites or restriction site methylation (Oyarzabal *et al*., 2008). Microrestriction of genome with at least two enzymes is required to prevent misinterpretation of strain affinities (Gibson *et al*., 1999; Arcangioli *et al*., 2011). It was reported that 19 isolates with identical *Sma*I profiles displayed 15 different profiles when digested with another enzyme, *Kpn*I (Lindmark *et al*., 2004). Such findings emphasize the need to use a second enzyme when the relatedness between isolates is determined.

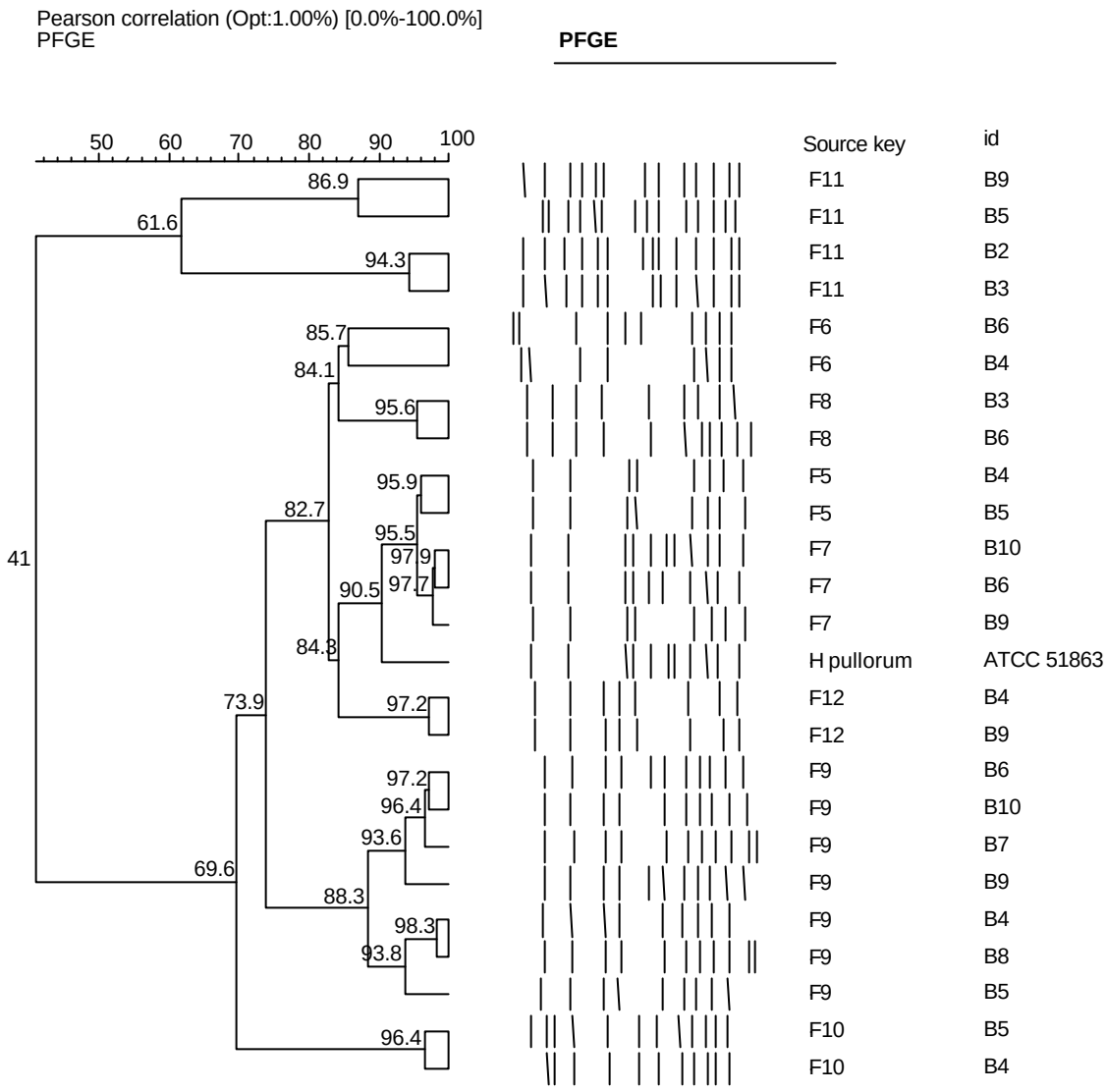

Fig. 1: Dendrogram resulting from the analysis of *H. pullorum* PFGE profiles digested with *Sac*II

Restriction endonucleases, *Sac*II and *Sma*I, have been used widely for microrestriction of genomes of *Campylobacter* species, and some species of *Helicobacter* such as *H. mustelae, H. hepaticus* and *H. pullorum* (Gibson *et al*., 1999; Wassenaar *et al*., 1998; Zanoni *et al*., 2011). Using *Sac*II, 8-14 DNA fragments with molecular weight ranging from 40 to 400 kb were generated while *Sma*I cut the genome into 7-13 fragments with molecular weight ranging from 40 to 250 kb. Other studies have revealed variable number of DNA fragments with molecular weight using *Sac*II RE. Manfreda *et al*. (2011) reported the number of bands ranged between 11 and 21 with sizes approximately 48 to 437 kbp and Zanoni *et al*. (2011) reported between 5 and 17 DNA bands with a molecular weight up to approximately 570 kb.

In assessing the level of genetic similarity among *H. pullorum* isolates, some strains showed indistinguishable profiles within-farm, while other strains were closely related. Zanoni *et al*. (2011) reported isolates from only two turkey farms showed within-farm genetic relatedness whereas the other seven turkey farms were colonized with different genotypes. Close relatedness among the isolates of healthy broiler chickens from the same flock and the highly conserved polymorphisms indicated they were most probably derived from a single clonal line (Gibson *et al*., 1999).

High level of genetic diversity was observed in the isolates from different farms. These results allow prediction of the flock contamination by genetically different isolates. High genetic diversity was also

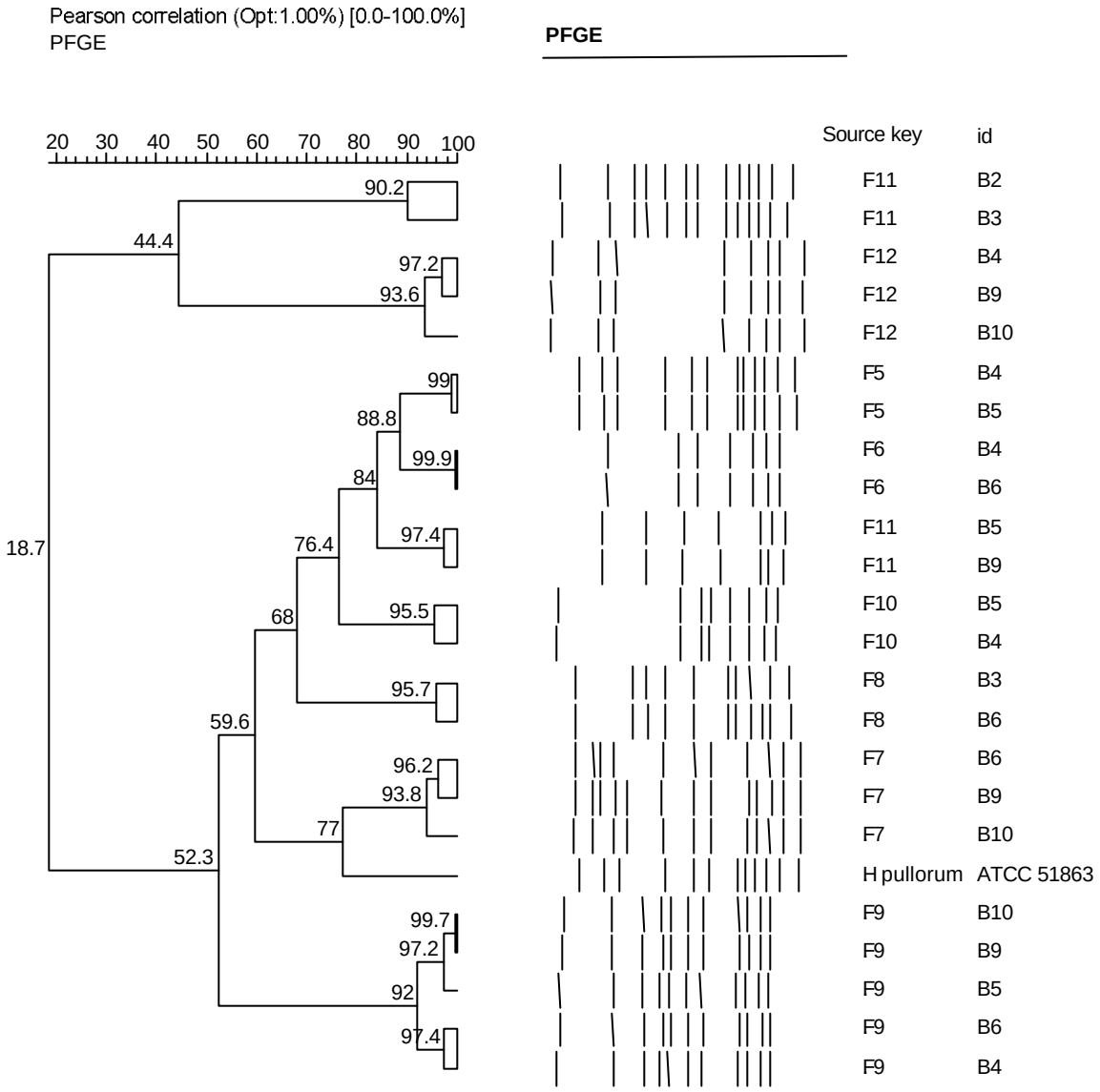

Fig. 2: Dendrogram resulting from the analysis of *H. pullorum* PFGE profiles digested with *Sma*I

observed by Gibson *et al*. (1999) who tested 13 human and seven poultry *H. pullorum* strains. The results are also in agreement with previous reports on genetic diversity of *H. pullorum*, such as that by Manfreda *et al*. (2011) and Miller *et al*. (2006). The present data on *H. pullorum* indicated that this species is more diverse than *H. mustalae* (Taylor *et al*., 1994) and *H. hepaticus* (Saunders *et al*., 1997), but similar to *H. felis* (Jalava *et al*., 1999) and *H. pylori* (Taylor *et al*., 1992). The possible cause of such genetic diversity could be the high degree of genomic recombination among a limited number of parent genomes (Hume *et al*., 2001). Bacteria with smaller genomes undergo genomic rearrangement readily due to environmental stresses (Wassenaar *et al*., 1998). Various factors such as type and number of REs used, technician analyzing the band patterns, software used, and the criteria set to differentiate between related and unrelated strains may also influence the outcomes of PFGE (Tenover *et al*., 1995).

Based on *D* value calculation, *Sac*II showed a higher discriminatory power than *Sma*I. *Sac*II restriction patterns showed more similarity among isolates compared to *Sma*I digestion patterns. Low discriminatory potential of *Sma*I has also been reported (Hanninen *et al*., 1998). Rivas *et al*. (2004) observed low discriminatory power of *Sma*I in comparison to *Eag*I and *Sac*II when *Arcobacter* genome was digested. Similarly, in another study conducted to type *C. jejuni* with two REs, *Kpn*I restriction showed better discrimination than the *Sma*I analysis (Michaud *et al*., 2001).

To conclude, this is the first characterization study of *H. pullorum* isolates from broilers in Malaysia. The results showed within-farm genetic similarity which suggested the same source of bacterial origin. It also showed that *Sac*II is the enzyme of choice for molecular epidemiology study of *H. pullorum*. Further study on broilers in farms in other parts of Malaysia is suggested to understand the distribution and possible route of spread and clonal similarity of *H. pullorum*.

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REFERENCES

Arcangioli, M.A., H. Aslan, F. Tardy, F. Poumarat and D. Le Grand, 2011. The use of pulsed-field gel electrophoresis to investigate the epidemiology of Mycoplasma bovis in French calf feedlots. Vet. J., 192: 96-100.

Casswall, T.H., A. Németh, I. Nilsson, T. Wadström and O.H. Nilsson, 2010. *Helicobacter* species DNA in liver and gastric tissues in children and adolescents with chronic liver disease. Scand. J. Gastroentero., 45: 160 - 167.

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

Dingle, K.E., F.M. Colles, D.R.A. Wareing, R. Ure, A.J. Fox, F.E. Bolton, H.J. Bootsma, R.J.L. Willems, R. Urwin and M.C.J. Maiden, 2001. Multilocus sequence typing system for *Campylobacter jejuni*. J. Clin. Microbiol., 39: 14-23.

Fox, J.G., 1997. The expanding genus of *Helicobacter*: pathogenic and zoonotic potential. Semin. Gastrointes. Dis., 8: 124-141.

Gibson, J.R., M.A. Ferrus, D. Woodward, J. Xerry and R.D. Owen, 1999. Genetic diversity in *Helicobacter pullorum* from human and poultry sources identified by an amplified fragment length electrophoresis. J. Appl. Microbiol., 87: 602-610.

Hänninen, M.-L., S. Pajarre, M-L. Klossner and H. Rautelin, 1998. Typing of *Campylobacter jejuni* isolates in Finland by pulsed-field gel electrophoresis. J. Clin. Microbiol., 36: 1787-1789.

Hume, M.E., R.B. Harvey, L.H. Stanker, R.E. Droleskey, T.E. Poole and H.B. Zhang, 2001. Genotypic variation among *Arcobacter* isolates from farrow-to-finish swine facility. J. Food Prot., 64: 645-651.

Hunter, P.R. and M.A. Gaston, 1988. Numerical index of the discriminatory ability of typing systems: an application of Simpson's index of diversity. J. Clin. Microbiol., 26: 2465-2466.

Jalava, K., M.C. De Ungria, J. O'Rourke, A. Lee, U. Hirvi and M.L. Hänninen, 1999. Characterization of *Helicobacter felis* by pulsed-?eld gel electrophoresis, plasmid pro?ling and ribotyping. Helicobacter, 4: 17-27.

Levesque, S., E. Frost, R.D. Arbeit and S. Michaud, 2008. Multilocus sequence typing of Campylobacter jejuni isolates from humans, chickens, raw milk, and environmental water in Quebec, Canada. J. Clin. Microbiol., 46: 3404 - 3411.

Lindmark, H., B. Harbom, L. Thebo, L. Andersson, G. Hedin, B. Osterman, T. Lindberg, Y. Andersson, A. Westoo and E.O. Engvall, 2004. Genetic characterization and antibiotic resistance of Campylobacter jejuni isolated from meats, water, and humans in Sweden. J. Clin. Microbiol., 42: 700-706.

Manfreda, G., A. Parisi, A. Lucchi, R.G. Zanoni and A. De Cesare, 2011. Prevalence of *Helicobacter pullorum* in conventional, organic, and free-range broilers and typing of isolates. J. Appl. Environ. Microbiol., 77: 479-484.

Michaud, S., R.D. Arbeit and C. Gaudreau, 2001. Molecular strain typing of Campylobacter jejuni by pulsed-field gel electrophoresis in a single day. Can. J. Microbiol., 47: 667-669.

Miller, K.A., L.L. Blackall, J.K. Miflin, J.M. Templeton and P.J. Blackall, 2006. Detection of *Helicabacter pullorum* in meat chicken in Australia. Aust. Vet. J., 84: 95-97.

Mohamed, M.A., R.S. Ibrahim, A.S. Moustafa and M. El-Refaie, 2010. *Helicobacter pullorum* among poultry in Assiut-Egypt: genetic characterization, virulence and MIC. Int. J. Poult. Sc., 9: 521-526.

Nebbia, P., C. Tramuta, M. Ortoffi, E. Bert, S. Cerruti and P. Robino, 2007. Identification of enteric *Helicobacter* in avian species. Schweiz Arch Tierheilkd, 149: 403-407.

Oyarzabal, O.A., S. Backert, L.L. Williams, A.J. Lastovica, R.S. Miller, S.J. Pierce, S.L. Vieira and F. Rebollo-Carrato, 2008. Molecular typing, serotyping and cytotoxicity testing of *Campylobacter jejuni* strains isolated from commercial broilers in Puerto Rico. J. Appl. Microbiol., 105: 800-812.

Rivas, L., N. Fegan and P. Vanderlinde, 2004. Isolation and characterization of *Arcobacter butzleri* from meat. Int. J. Food Microbiol., 91: 31-41.

Rocha, M., P. Avenaud, A. Menard, B. Le Bail, C. Balabaud, P. Bioulac-Sage, Q.D.M. de Magalhaes and F. Megraud, 2005. Association of *Helicobacter* species with hepatitis C cirrhosis with or without hepatocellular carcinoma. Gut, 54: 396-401.

Sails, A.D., B. Swaminathan and P.I. Fields, 2003. Utility of multilocus sequence typing as an epidemiological tool for investigation of outbreaks of gastroenteritis caused by Campylobacter jejuni. J. Clin. Microbiol., 41: 4733-4739.

Saunders, K.E., K.J. McGovern and J.G. Fox, 1997. Use of pulsed-field gel electrophoresis to determine genomic diversity in strains of Helicobacter hepaticus from geographically distant locations. J. Clin. Microbiol., 35: 2859-2863.

Taylor, D.E., N. Chang, N.S. Taylor and J.G. Fox, 1994. Genome conservation in Helicobacter *mustelae* as determined by pulsed-field gel electrophoresis. FEMS Microbiol.,118: 31-36.

Taylor, D.E., M. Eaton, N. Chang and M. Salama, 1992. Construction of a *Helicobacter pylori* genome map and demonstration of diversity at the genome level. J. Bacteriol., 174: 6800-6806.

Tenover, F.C., R.D. Arbeit, R.V. Goering, P.A. Mickelsen, B.E. Murray, D.H. Persing and B. Swaminathan, 1995. Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. J. Clin. Microbiol., 33: 2233-2239.

Varon, C., A. Duriez, P. Lehours, A. Menard, S. Laye, F. Zerbib, F. Megraud and D. Laharie, 2009. Study of *Helicobacter pullorum* proin?ammatory properties on human epithelial cells *in vitro*. Gut, 58: 629-635.

Wassenaar, T.M., B. Geilhausen and D.G. Newell, 1998. Evidence of genomic instability in *Campylobacter jejuni* isolated from poultry. J. Appl. Environ. Microbiol., 64: 1816-1821.

Wesley, I.V., 2001. *Arcobacter* and *Helicobacter*. In: Labbe, G., Garcin, S., (Eds.), Guide to Foodborne Pathogens. New York: A John Wiley and Sons, Inc., pp: 23-34.

Zanoni, R.G., S. Piva, M. Rossi, F.R. Pasquali, A. Lucchi, A. De Cesare and G. Manfreda, 2011. Occurrence of *Helicobacter pullorum* in turkeys. Vet. Microbiol., 149: 492-496.