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Original article

Molecular screening of gastric *Helicobacter pullorum* recovered from different avian species in Egypt

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Abstract

Helicobacter pullorum (*H. pullorum*) is a bacterium that colonizes the intestines of poultry and causes gastroenteritis. Because these species are known as human and/or animal pathogens, identification of *H. pullorum* is becoming increasingly necessary. The bacterium has been linked to colitis and hepatitis in humans after being transmitted by infected meat consumption. Misdiagnosis of other enteric zoonotic pathogens such as *Campylobacter* and other *Helicobacter* species makes the diagnosis of *H. pullorum* extremely difficult. This study focused on the molecular detection of *H. pullorum* from the stomach (proventriculus and gizzard) of different avian species as new target organs for detection and transmission between avian species. Proventriculus and gizzards were obtained from 40 freshly dead chickens and resident wild birds (n=40). Diarrhea was found in the farms that were surveyed. DNA was extracted from all collected samples to conduct PCR amplification. The samples were screened for *Helicobacter* genus-specific 16s using C97 and C05 primers. To confirm the existence of *H. pullorum*, the positive samples were sequenced.

H. pullorum was recorded in two out of 40 chicken samples. In addition, *H. pullorum* was recorded in one out of 40 resident wild birds. The 16S rRNA gene sequence for *Helicobacter* genus-specific in poultry and wild birds showed a 100% homology. In conclusion, broiler chickens and resident wild birds are possible reservoirs for *H. pullorum*, according to this report, and possibly act as a source of infection for humans via the food supply.

Key words: *Helicobacter pullorum*, PCR, sequencing, chicken, resident wild birds, zoonoses, Egypt

Introduction

More than 30 species of *Helicobacter* have been identified in the last two decades. *Helicobacter* is a bacterial genus that belongs to the family *Helicobacteriaceae*, order *Campylobacteriales*, and class *Epsilonproteobacteria* (On et al. 2002). *Helicobacter pullorum* is a Gram-negative, microaerophilic rod with monopolar, non-sheathed slightly curved flagella. It was first detected in the ceca and livers of broiler and laying hens, as well as human feces (Stanley et al. 1994). Depending on the site of colonization, these pathogens are divided into two groups: gastric *Helicobacter* species and enterohepatic *Helicobacter* species (EHS). The gastrointestinal tract and, in some cases the biliary tree of their host is colonized preferentially by enterohepatic *Helicobacter* species. These bacteria are classified as human or animal pathogens, with certain species also being zoonotic agents.

The first detection of *H. pullorum* using 16S-rRNA phylogenetic analysis was recorded by Nebbia et al. (2007). This organism is found in farm-raised poultry, such as chickens, turkeys, and Guinea fowl (Nebbia et al. 2007). This species has been associated with diarrhea and enteritis in gastrointestinal patients, as well as hepatitis in chickens (Stanley et al. 1994, Steinbrueckner et al. 1997). *Helicobacter pullorum* was first found in the feces of broilers and laying hens with impaired livers (Burnens et al. 1994, Mladenova-Hristova et al. 2017). Since the most infected birds remain subclinical, *Helicobacter pullorum* prevalence in avian species is poorly recorded (Ceelen et al. 2006).

Wild birds are problematic and may pose an existential threat to poultry farms. Their droppings are messy and may contaminate feed and water. They can also act as vehicles for a variety of diseases and parasites which can invade commercial poultry (Jones 2001, Azevedo et al. 2008). The presence of these birds near the chicken houses in this study was due to the fact that the water baths and feeders had to be put in the open, attracting wild birds due to the abundance of food and water sources available. Due to fecal contamination of drinking water supplies and agricultural crops, wild birds, which are a natural reservoir of enteric bacteria, are frequently mentioned as a potential source of infection for humans and farm animals (Waldenström et al. 2003, Colles et al. 2008)

Helicobacter spp. infection in wild birds has only been recorded in European and American regions (Dewhirst et al. 1994, Seymour et al. 1994, Waldenström et al. 2003, Fox et al. 2006). Enteric bacteria such as *Helicobacter pullorum*, *Helicobacter pametensis*, *Helicobacter canadensis*, *Helicobacter anseris*, and *Helicobacter brantae* are usually found in wild birds

(Whary and Fox 2004, Fox et al. 2006). Two of them (*H. pullorum* and *H. canadensis*) have been linked to human gastroenteritis, indicating that these birds could serve as reservoirs for *Helicobacter* spp. Transmission (Fox et al. 2000, Waldenström et al. 2003, Ceelen et al. 2007). The occurrence of *Helicobacter pullorum* in a diarrheic psittacine bird implies that pet birds may also pose a zoonotic danger (Ceelen et al. 2006). Recently, several published studies provided clear evidence that *H. pullorum*'s pathogenic ability as a cause of human enteritis should not be ignored (Burnens et al. 1994, Steinbrueckner et al. 1997, Ceelen et al. 2007).

The aim of this research work was to investigate the occurrence of gastric *H. pullorum* infections in domestic and free-living wild birds using direct molecular identification, and to study the disease transmission to other avian species.

Materials and Methods

Ethical approval

Samples were collected from freshly dead birds and humanely slaughtered resident wild birds. Sampling was carried out in full compliance with the recommendations of the Faculty of Veterinary Medicine, Cairo University, Egypt guidelines.

Samples

Samples were collected from 40 freshly dead birds obtained from commercial broiler and layer farms (20 from each) as well as 40 tissue samples from free-living resident wild birds (crows, cattle egret, laughing dove, and domestic pigeon) after being captured by traps, and humanely slaughtered and eviscerated. Samples were collected from the Qalyubia governorate, Egypt. Each sample was deposited in a separate waterproof plastic bag, labeled, and transported to the laboratory using a dry icebox for further examination.

Postmortem examination

The birds were examined and dissected in the laboratory to collect the proventriculus and gizzard from each sampled bird. The proventriculus and gizzard were then stored in sterile vials at -20°C until DNA extraction.

DNA extraction

A QIAamp tissue kit (Qiagen, Hombrechtikon, ZH, Switzerland) was used to extract DNAs, with some changes to the manufacturer's instructions (Elhariri et al. 2018).



Fig. 1. postmortem examination of proventriculus showing congested inflamed mucosa with pinpoint hemorrhages (proventriculitis).

Table 1. Occurrence of *H. pullorum* in examined birds in Qalyubia governorate, Egypt.

Source of samples	Species	No. of examined samples	Positive samples
Chickens	Broiler	40	2/40
	Crows (<i>Corvus Cornix</i>)	11	0
	Cattle Egret (<i>Bubulcus Ibis</i>)	9	0
Wild birds	Laughing Dove (<i>Spilopelia Senegalensis</i>)	10	0
	domestic pigeons (<i>Columba domestica</i>)	10	1/10
Total		80	3/80

Molecular identification of *Helicobacter* genus-specific by PCR targeting 16S rRNA gene

A PCR assay targeting the *Helicobacter* genus-specific 16S rRNA gene was performed using C97; GCTATGACGGGTATCC and C05; ACTTCACCCC AGTCGCTG primers with an amplicon size of 1200 base pairs (Hamza et al. 2018).

The temperature profile was as follows: 30 s at 95°C, 30 s at 55 C, and 30 s at 72°C. The final extension cycle was 72°C for 5 min. The PCR products were analyzed on a 1.5% agarose electrophoresis gel stained with ethidium bromide.

Sequencing and nucleotide sequence analysis

To confirm the existence of *H. pullorum*, sequencing of *Helicobacter*-specific 16S rRNA PCR positive samples was carried out. The PCR products were purified using a Qiaquick purification kit (Qiagen) and were sequenced using a Big Dye Terminator V3.1 sequencing kit (Applied Biosystems, Waltham, MA, USA). The obtained nucleotide sequences were compared with those in the Public Database using the NCBI-BLAST server and were confirmed as *H. pullorum*. The sequences have been deposited in the GenBank database under

accession numbers OK569893 and OK570022 for poultry and wild birds, respectively.

H. pullorum sequences were retrieved and put into BioEdit 7.0.1.4 for multiple alignments with the BioEdit Clustal W tool. The neighbor-joining strategy was used in phylogenetic analysis using MEGA software version X. 1000 resamplings were used in the Bootstrap analysis.

Results

On the examined farms, the poultry were suffering from diarrhea, with mortalities ranging from 2-5% on the examined commercial broilers farms and from 3-7% on layer farms.

The postmortem examination revealed the presence of mild enteritis with watery yellow color intestinal content with the presence of undigested feed particles. The proventriculus in most of the examined birds was inflamed with the presence of congested mucosa with pinpoint hemorrhages as seen in Fig. 1. The percentage of occurrence of *H. pullorum* in broilers is summarized in Table 1. The *Helicobacter 16SrRNA* gene was detected in the DNA of two out of 40 chicken tissues

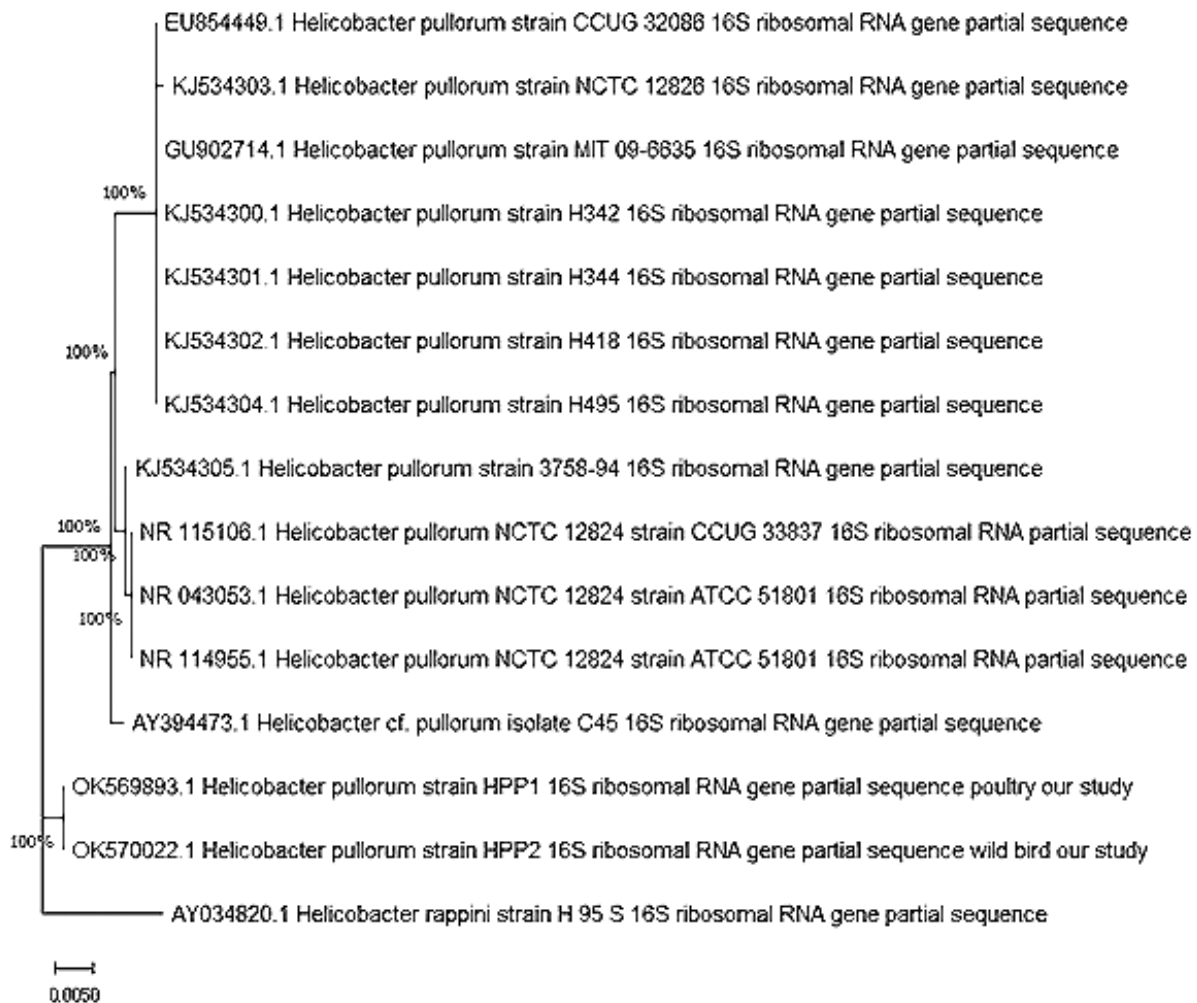


Fig. 2. The Evolutionary history was inferred using the Neighbor-Joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) was performed. This analysis involved 15 nucleotide sequences. Evolutionary analyses were conducted in MEGA X.

and these positive samples were from the gizzard and proventriculus. The *Helicobacter 16SrRNA* gene was detected in one out of 40 resident wild birds. The sequence analysis for the *Helicobacter* genus-specific 16S rRNA gene in poultry and the wild bird showed a 100% homology (Fig. 2).

Discussion

Recently, many studies have shown the importance of the *H. pullorum* infection and described it as a zoonotic disease. *H. pullorum* naturally infects many poultry birds, some rodents, and humans. Gastroenteritis in farm-raised birds, including chicken, turkey, and guinea fowl has been associated with *H. Pullorum* infection. The infection has been linked to vibronic hepatitis lesions in chickens (Burnens et al. 1994, Stanley et al. 1994) and diarrhea in humans (Ceelen et al. 2007). *H. pullorum* prevalence in avian species is poorly documented, and the most infected birds

remain subclinical (Stanley et al. 1994, Atabay et al. 1998, Ceelen et al. 2006).

In the present study, despite the small sample size, our results highlight the presence of *H. pullorum* infection on broiler farms, and our result agrees with Wai et al. (2019) who noticed that *H. pullorum* was prevalent on chicken farms. Previous studies showed high occurrences of *H. pullorum* in chickens ranging from 60% in the UK (Atabay et al. 1998) to 78.3% in the Czech Republic (Svobodova and Boribova 2003) and 100% in Italy (Zanoni et al. 2007). However, some studies showed low-to-moderate isolation rates which ranged from 4% in Switzerland (Burnens et al. 1994) to 13.5% in Australia (Miller et al. 2006), 33.6% in Belgium (Ceelen et al. 2006) and 39.3% in Egypt (Hassan et al. 2014). The variation in prevalence percentage may be attributed to the different geographical distribution, differences in ambient temperature, intensive farming that may encourage the spreading of germs and oro-fecal transmission, unfavorable factors such

as stress, drug therapy, bad litter, bad hygiene, and unbalanced diet.

The prevalence of *H. pullorum* in the raw meat, cecum, and liver of poultry from different countries has been previously reported; this prevalence could possibly pose a high health risk to humans via its transmission through food, in the same way as *Campylobacter* species (Zanoni et al. 2007, Manfreda et al. 2011). However, the prevalence and molecular characteristics of *H. pullorum* from chicken gastric samples (gizzard and proventriculus) have not been studied in greater detail, including the genomic and epidemiological attributes.

The occurrence of *Helicobacter* spp. infection in wild birds was isolated for the first time in 1994 from house sparrows, gulls, and terns in an estuarine environment in Massachusetts, USA (Seymour et al. 1994), and was reported in wild geese in Sweden (Waldenström et al. 2003), even though their occurrence is low.

In our study, *H. pullorum* was detected in one out of 40 resident wild birds and this positive sample come from a domestic pigeon (*Columba domestica*). This result was in contrast to Robino et al. 2010 who reported that domestic pigeons, crows, sparrows, and mallards were all negative for *Campylobacter* and *Helicobacter* spp, whereas urban Rock Pigeons (*Columba livia*) and Passeriformes were (0-8%) (Seymour et al. 1994, Robino et al. 2010). Other studies have percentages of 15-40% in wild aquatic birds such as geese (*Branta* spp.), Common Terns (*Sterna hirundo*), and gulls spp. (Dewhirst et al. 1994, Fox et al. 2006, Waldenström et al. 2007). Wild birds have also been reported to carry *Campylobacter* and *H. canadensis*, a probable zoonotic pathogen and closely related to *H. pullorum* (Shen et al. 2014).

PCR analysis was used to detect *H. pullorum* from the tissues of infected chickens. Many microbiological studies depend mainly on molecular methods instead of traditional isolation protocols (Elhariri et al. 2017, Elhariri et al. 2018, Elhelw et al. 2020). The confident identification of *H. pullorum* is necessary to avoid its misdiagnosis with *Campylobacter* spp. They share a common habitat within the cecum and large intestine of chickens (Corry and Atabay 2001).

In our study, PCR assay and sequencing of *Helicobacter*-specific 16S rRNA were used to detect *H. pullorum* from tissues of chickens and wild birds. The results of this study are in accordance with Ceelen et al. (2006), who reported the specificity of the protocol and its ability to discriminate between closely related species.

Based on the sequencing of the *Helicobacter*-specific 16S rRNA gene, one hundred percent identity was reported between the poultry and the wild bird (Fig. 2).

This confirmed hypothesis that disease transmission to other species is very strong. More studies are necessary to establish the role of *H. pullorum* as a potentially emerging pathogen or commensals in wild birds. Since these birds live in a habitat that is different from poultry, it might be reasonable to presume that different housing systems may result in different levels of exposure to *Helicobacter* spp.

The occurrence of *Helicobacter pullorum* in poultry and wild birds implies that birds may pose a zoonotic danger. *H. pullorum* can colonize broiler chickens and be excreted in their feces until the age of slaughter (Ceelen et al. 2007, Borges et al. 2015). *H. pullorum* can contaminate the carcasses of poultry and is considered a food-borne pathogen (Mohamed et al. 2010). Overall, it may be expected that the prevalence of *H. pullorum* is generally underestimated (Waino et al. 2003).

Conclusion

In conclusion, *Helicobacter pullorum* is a bacterium with zoonotic potential and it is assumed that poultry may be the vehicle for human infections. The epidemiological role of wild birds in the spread of enteric *Helicobacter* is still unclear and further studies are necessary to establish the importance of wild populations as *Helicobacter* carriers. PCR is a rapid, accurate method for the detection of *H. pullorum*.

References

- Atabay HI, Corry JE, On SL (1998) Identification of unusual *Campylobacter*-like isolates from poultry products as *Helicobacter pullorum*. *J. Appl. Microbiol.* 84: 1017-1024.
- Azevedo NF, Almeida C, Fernandes I, Cerqueira L, Dias S, Keevil CW, Vieira MJ (2008) Survival of gastric and enterohepatic *Helicobacter* spp. in water: implications for transmission. *Appl Environ Microbiol* 74: 1805-1811.
- Borges V, Santos A, Correia CB, Saraiva M, Ménard A, Vieira L, Sampaio DA, Pinheiro M, Gomes JP, Oleastro M (2015) *Helicobacter pullorum* isolated from fresh chicken meat: antibiotic resistance and genomic traits of an emerging foodborne pathogen. *Appl Environ Microbiol* 81: 8155-8163.
- Burnens A, Stanley J, Morgenstern R, Nicolet J (1994) Gastroenteritis associated with *Helicobacter pullorum*. *Lancet* 344: 1569-1570.
- Ceelen LM, Decostere A, Chiers K, Ducatelle R, Maes D, Haesebrouck F (2007) Pathogenesis of *Helicobacter pullorum* infections in broilers. *Int J Food Microbiol* 116: 207-213.
- Ceelen LM, Decostere A, Van den Bulck K, On SL, Baele M, Ducatelle R, Haesebrouck F (2006) *Helicobacter pullorum* in chickens, Belgium. *Emerg Infect Dis* 12: 263-267
- Colles FM, Dingle KE, Cody AJ, Maiden MC (2008) Com-

- parison of *Campylobacter* populations in wild geese with those in starlings and free-range poultry on the same farm. *Appl Environ Microbiol* 74: 3583-3590.
- Corry JE, Atabay HI (2001) Poultry as a source of *Campylobacter* and related organisms. *J Appl Microbiol* 90: 96-114.
- Dewhirst FE, Seymour C, Fraser GJ, Paster BJ, Fox JG (1994) Phylogeny of *Helicobacter* Isolates from Bird and Swine Feces and Description of *Helicobacter pametensis* sp. nov. *Int J Syst Bacteriol* 44: 553-560.
- Elhariri M, Elhelw R, Hamza D, El-Mahallawy HS (2017) Serologic evidence and risk factors for *Helicobacter pylori* infection in animals and humans. *J Infect Dev Ctries* 11: 414-419.
- Elhariri M, Hamza D, Elhelw R, Hamza E (2018) Occurrence of *cagA+* *vacA* *s1a* *m1* *il* *il* *Helicobacter pylori* in farm animals in Egypt and ability to survive in experimentally contaminated UHT milk. *Sci Rep* 8: 1-13.
- Elhelw R, Elhariri M, Ragab E, Kadry M, Hamza D (2020) Dog as Potential Source of *Helicobacter Pylori* in Egypt: Public Health Significance. *World* 10: 446-450.
- Fox JG, Chien CC, Dewhirst FE, Paster BJ, Shen Z, Melito PL, Woodward DL, Rodgers FG (2000) *Helicobacter canadensis* sp. nov. isolated from humans with diarrhea as an example of an emerging pathogen. *J Clin Microbiol* 38: 2546-259.
- Fox JG, Taylor NS, Howe S, Tidd M, Xu S, Paster BJ, Dewhirst FE (2006) *Helicobacter anseris* sp. nov. and *Helicobacter brantae* sp. nov., isolated from feces of resident Canada geese in the greater Boston area. *Appl Environ Microbiol* 72: 4633-4637.
- Hamza D, Elhelw R, Elhariri M, Ragab E (2018) Genotyping and antimicrobial resistance patterns of *Helicobacter pylori* in human and dogs associated with A2142G and A2143G point mutations in clarithromycin resistance. *Microb Pathog* 123: 330-338.
- Hassan AK, Shahata MA, Refaie EM, Ibrahim RS (2014) Detection and identification of *Helicobacter pullorum* in poultry species in upper Egypt. *J Advanced Vet Res* 4: 42-48.
- Jones K (2001) *Campylobacters* in water, sewage and the environment. *J Appl Microbiol* 90: 68S-79S.
- Manfreda G, Parisi A, Lucchi A, Zanoni RG, De Cesare A (2011) Prevalence of *Helicobacter pullorum* in conventional, organic, and free-range broilers and typing of isolates. *Appl Environ Microbiol* 77: 479-784.
- Miller KA, Blackall LL, Mifflin JK, Templeton JM, Blackall PJ (2006) Detection of *Helicobacter pullorum* in meat chickens in Australia. *Aust Vet J* 84: 95-97.
- Mohamed M, Ibrahim R, Shahata M and El-Refai E (2010) *Helicobacter pullorum* among poultry in Assiut-Egypt: genetic characterization, virulence and MIC. *Int J Poult Sci* 9: 521-526.
- Mladenova-Hristova I, Grekova O, Patel A (2017) Zoonotic potential of *Helicobacter* spp. *J Microbiol Immunol Infect* 50: 265-269.
- Nebbia P, Tramuta C, Ortoffi M, Bert E, Cerruti Sola S, Robino P (2007) Identification of enteric *Helicobacter* in avian species. *Schweiz Arch Tierheilkd* 149: 403-407.
- On SL, Hynes S, Wadström T (2002) Extragastric *Helicobacter* species. *Helicobacter* 7: 63-67.
- Robino P, Tomassone L, Tramuta C, Rodo M, Giammarino M, Vaschetti G, Nebbia P (2010) Prevalence of *Campylobacter jejuni*, *Campylobacter coli* and enteric *Helicobacter* in domestic and free living birds in North-Western Italy. *Schweiz Arch Tierheilkd* 152: 425-431.
- Shen Z, Sheh A, Young SK, Abouelliel A, Ward DV, Earl AM, Fox JG (2014) Draft genome sequences of six enterohepatic *Helicobacter* species isolated from humans and one from rhesus macaques. *Genome Announc* 2: e00857-14.
- Seymour C, Lewis RG, Kim M, Gagnon DF, Fox JG, Dewhirst FE, Paster BJ (1994) Isolation of *Helicobacter* strains from wild bird and swine feces. *Appl Environ Microbiol* 60: 1025-1028.
- Stanley J, Linton D, Burnens AP, Dewhirst FE, On SL, Porter A, Owen RJ, Costas M (1994) *Helicobacter pullorum* sp. nov.-genotype and phenotype of a new species isolated from poultry and from human patients with gastroenteritis. *Microbiology* 140: 3441-3449.
- Steinbrueckner B, Haerter G, Pelz K, Weiner S, Rump JA, Deissler W, Bereswill S, Kist M (1997) Isolation of *Helicobacter pullorum* from patients with enteritis. *Scand J Infect Dis* 29: 315-318.
- Svobodova I, and Boribova G (2003) Incidence of *Helicobacter pullorum* and *Campylobacter* spp. In healthy broilers in the Czech republic. *Zoo Public Health* 54: 54-75.
- Wainø M, Bang DD, Lund M, Nordentoft S, Andersen JS, Pedersen K, Madsen M (2003) Identification of *campylobacteria* isolated from Danish broilers by phenotypic tests and species-specific PCR assays. *J Appl Microbiol* 95: 649-655.
- Wai SS, Abdul Aziz S, Bitrus AA, Zunita Z, Abu J (2019) *Helicobacter pullorum* in broiler chickens and the farm environment: A one health approach. *Int J One Heal* 5: 20-25.
- Waldenström J, On SL, Ottvall R, Hasselquist D, Harrington CS, Olsen B (2003) Avian reservoirs and zoonotic potential of the emerging human pathogen *Helicobacter canadensis*. *Appl Environ Microbiol* 69: 7523-7526.
- Waldenström J, On SL, Ottvall R, Hasselquist D, Olsen B (2007) Species diversity of *campylobacteria* in a wild bird community in Sweden. *Journal of applied microbiology* 102: 424-432.
- Whary MT, Fox JG (2004) Natural and experimental *Helicobacter* infections. *Comp Med* 54: 128-158.
- Zanoni RG, Rossi M, Giacomucci D, Sanguinetti V, 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.