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Short Communication

Pathogenicity testing and antimicrobial susceptibility of *Helicobacter pullorum* isolates from chicken origin

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Abstract This work aimed to study the pathogenicity and to investigate the antimicrobial susceptibility and resistance patterns of *Helicobacter pullorum* (*H. pullorum*). The minimum inhibitory concentration (MIC) value of ciprofloxacin, ampicillin, gentamycin, erythromycin, colistin sulfate and tetracycline was determined for eight different *H. pullorum* isolates. *H. pullorum* resulted into 33.3% mortality of infected chickens with signs of diarrhea, stunted growth and poor conversion rate in survivors. All experimentally infected embryonated chicken eggs showed embryonic mortalities within 48-h post yolk sac inoculation. *H. pullorum* was re-isolated from cecum, liver, yolk sac and air-sacs of all dead and sacrificed infected chickens. *H. pullorum* was also re-isolated from dead embryos, embryonic membranes and fluids of infected embryonated chicken eggs. Polymerase Chain Reaction (PCR) assay was used to detect *H. pullorum* in experimentally infected chickens and embryonated chicken eggs. All tested *H. pullorum* isolates were resistant to ciprofloxacin, gentamycin and erythromycin, while 7 out of 8 isolates were resistant to tetracycline. All isolates were susceptible to colistin sulfate and ampicillin.

1. Introduction

Helicobacter species is a group of taxonomically related Gram-negative, microaerophilic bacteria, some of which are pathogenic and known to colonize the gastrointestinal and biliary tracts of many animal species. These pathogens are generally separated into two groups, gastric and enterohepatic, based on their preferred site of colonization [19]. During the last decade, Enterohepatic Helicobacter Species (EHS) have gained recognition in the field of emerging infectious pathogens [7]. Infection with this group of micro-organisms is generally characterized by colonization of the distal gastrointestinal tract and, in selected cases, the biliary tree. As reported for the gastric pathogen *Helicobacter pylori*, gastrointestinal colonization

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by EHS can be associated with chronic inflammation and neoplasia [8,10,22].

Helicobacter pullorum is an EHS, which was first isolated by Stanley et al. [19] from the feces of diarrheic humans and the intestinal contents and livers of chickens. The organism is suspected to cause vibrionic hepatitis in chickens. Infection with this organism is most often associated with farm raised birds, including chickens, Turkeys and Guinea fowl [16,19]. In one report, *H. pullorum* was isolated from human feces three months following the patients' initial presentation with diarrhea [20]. In another case, *H. pullorum* was isolated from the feces of a male with diarrhea and elevated liver enzymes [3]. *H. pullorum* has also been identified by PCR in humans with inflammatory bowel disease, hepatitis, cholecystitis and hepatocellular carcinoma [4,9,18,21]. A recent report identified an association between EHS and Crohn's disease, with *H. pullorum* being one of the most prevalent EHS identified [13].

Despite the frequent occurrence of *H. pullorum* in chickens and its possible association with hepatoenteric disease, the interactions of *H. pullorum* with its natural host have not yet been studied [6]. In addition, there is little information in the literature about *H. pullorum* antibiotic resistance [5].

The aim of the present work is to study the pathogenicity of *H. pullorum* by experimental infection of one day-old chicks and embryonated chicken eggs and to investigate the antimicrobial susceptibility and resistance patterns of the organism by minimum inhibitory concentration technique.

2. Materials and methods

2.1. Experimental infection in one-day old chicks

2.1.1. Chickens and ***H. pullorum*** isolates

Fifty-two, one-day old chicks were kept separately and fed on antibiotic free ration in cleaned and disinfected isolation units. All chicks were examined clinically. Pooled cloacal swabs were collected from examined chicks and isolation trials of the pathogen were done with special reference to *H. pullorum* to ensure their freedom of infections.

H. pullorum isolates (broth cultures were adjusted to 10^{11} colony-forming units (CFU)/ml) biochemically identified and confirmed by PCR at the Laboratory of Poultry Diseases Diagnosis, Faculty of Veterinary Medicine, Assiut University [11] using the method described by Stanley et al. [19]. The standard plate count method technique [2], with slight modification was used to adjust the number of *H. pullorum* per milliliter in the inoculated brain heart infusion (BHI) broth.

2.1.2. Experimental design

Fifty-two, one-day old chicks were randomly divided into two groups; first group was thirty-nine chicks, were inoculated with *H. pullorum* isolates via gavages (forced feeding). Each chick received a 200 μl of BHI broth containing 10^{11} CFU of *H. pullorum* organism/ml [19]. The second group was thirteen chicks, were inoculated with sterile BHI broth via gavages and kept as a non-infected negative control. All infected and control chicks were observed daily for clinical signs. By the end of experiment (40th day of age), survived infected and control chickens were sacrificed and subjected for necropsy and bacteriological examination.

2.2. Experimental infection in embryonated chicken eggs

2.2.1. Embryonated chicken eggs and *H. pullorum* isolates

Seventy, six-day-old embryonated chicken eggs were used. Five randomly selected different *H. pullorum* isolates (broth cultures were adjusted to 10^{10} CFU/ml) previously identified [11].

2.2.2. Experimental design

Ten embryonated chicken eggs were randomly selected from the total number for bacteriological isolation with special attention for *H. pullorum* to ensure their freedom of pathogenic infections. The embryonated chicken eggs were classified into six groups; each one contained ten embryonated chicken eggs. Five groups were inoculated with five different *H. pullorum* isolates via yolk sac route of inoculation. Each embryonated chicken egg was inoculated with 0.2 ml of BHI broth culture containing 10^{10} CFU of *H. pullorum* organism/ml. The 6th group was inoculated with sterile brain heart infusion broth via yolk sac and kept as a negative control group. All infected and control embryonated chicken eggs were incubated at a temperature 37 °C and humidity 70% with manual turning twice per daily. All infected and control embryonated chicken eggs were daily observed by candling for embryonic mortality.

2.3. Isolation of *H. pullorum*

2.3.1. Sampling

Necropsy of dead/sacrificed, infected and control chickens was done and tissue samples of cecum, liver, yolk sac and air-sacs were collected for bacteriological isolation. In addition, dead embryos, embryonic fluids and sacs were collected from embryonated chicken eggs.

2.3.2. Isolation

Samples were inoculated into brain heart infusion (BHI) broth containing 10% sterile inactivated horse serum and Skirrow's supplement (Oxoid LDT, Biolife, Sydney, Australia) then incubated in a microaerophilic condition (5% H_2, 5% CO_2, 5% O_2, and 85% N_2) in CampyPak II anaerobic system jar with CampyPak gas generating system envelopes (BBL Becton Dickinson Microbiology Systems, Sparks, USA) or in CO_2 incubator with the same gases in same proportions at 42 °C for 24–48 h. Sub-culturing was carried out on (BHI) agar plates enriched with 5–10% sheep blood and containing Skirrow's supplement and incubation at 42 °C for 48 h under a microaerophilic atmosphere. The cultured plates were examined for typical *H. pullorum* colonies.

2.4. PCR analysis and gel electrophoresis

DNA was extracted from randomly selected *H. pullorum* colonies retrieved from infected chickens and embryonated chicken eggs, using QIAamp DNA mini extraction kit (Qiagen, Germany) according to the manufacturer's instructions. Species identification was confirmed using the *H. pullorum* species-specific 16S rRNA gene PCR assay [19]. In brief, the primer sequences were: 5-ATG AAT GCT AGT TGT TGT CAG-3 (forward) and 5-GAT TGG CTC CAC CAC TTC ACA-3 (reverse) (Bioneer incorporation Daejaon 306-220, Korea). The parameters for all reactions were described in the following

profile; initial denaturation at 94 °C for 3 min followed by 35 cycles of denaturation at 94 °C for 45 s, annealing at 56 °C for 30 s and extension for 1.5 min at 72 °C. The final extension took 10 min at 72 °C. The PCR product (448 bp) was seen by electrophoresis in a 1.5% agarose gel stained with ethidium bromide (Sigma–aldrich, Missouri, USA) for visualization performed in a horizontal gel chamber plate. The running buffer was 0.5× TBE (Tris borate EDTA (pH 8.3). The 1 kb plus DNA ladder (Invitrogen, California, U.S.A.) was used as a reference standard molecular weight marker. A gel documentation system (Biometra, Goettingen, Germany) with a digital camera was used for image capturing.

2.5. Determination of antimicrobial susceptibility and resistance patterns of *H. pullorum* using MIC

2.5.1. *H. pullorum* isolates and antimicrobial agents

Eight randomly selected different *H. pullorum* isolates (broth cultures were adjusted to 10^6 CFU/ml) previously identified [11]. The standard plate count method technique [2], with slight modification was used to adjust the number of *H. pullorum* per milliliter in the inoculated BHI broth.

The MIC value of ciprofloxacin, ampicillin, gentamycin, erythromycin, colistin sulfate and tetracycline was determined. All antimicrobial agents were purchased from Sigma (Missouri, USA), except for ciprofloxacin, which was obtained from Bayer AG (Leverkusen, Germany).

2.5.2. Antimicrobial susceptibility testing

It was carried out by broth micro-dilution method using micro-titer plates [12]. The antibiotic concentrations ranged from 0.25 to 256 μg/ml. Since there are no break-points currently available for *H. pullorum*, we tentatively used Enterobacteriaceae break-points as described by the Clinical and Laboratory Standard Institute (CLSI, formerly NCCLS) for *Campylobacter jejuni* and related species [15].

3. Results

3.1. Clinical signs, mortalities and necropsy findings

A total 13 out of 39 chickens were infected via gavages by *H. pullorum* isolates died from 4th to 9th -day post infection as summarized in Table 1 and Fig. 1. Chickens died at 4th-day post infection did not show any clinical signs while signs of loss of appetite, depression, ruffled feathers, anorexia and yellowish-white diarrhea were observed in chickens found dead from other days post infection. Necropsy of dead chickens revealed distended abdomen, unabsorbed yolk sac with severe congestion and dark yellow to brown contents, enlarged and congested liver with streaks of hemorrhage, distended ceca with frothy yellowish exudates, mild fibrinous pericarditis and air-sacculitis, in some cases hydropericardium, perihepatitis and yellowish gelatinous exudates might be present in abdominal cavity. Survivor chickens were retarded in growth with a poor conversion rate. They expressed symptoms of weakness, depression, loss of appetite, diarrhea and mild respiratory signs. Necropsy of scarified survivors showed emaciated carcasses with prominent keel bone, enlarged, friable and hemorrhagic liver, mild fibrinous pericarditis and air-sacculitis, congested intestine with small patches of hemorrhage and ulcers on the intestinal mucosa, distended ceca with frothy exudates, and some chickens developed ascites; some chickens retained unabsorbed yolk sac, and pneumonia might be found in some cases. No mortalities, clinical signs or necropsy findings were observed in chickens of the negative control group.

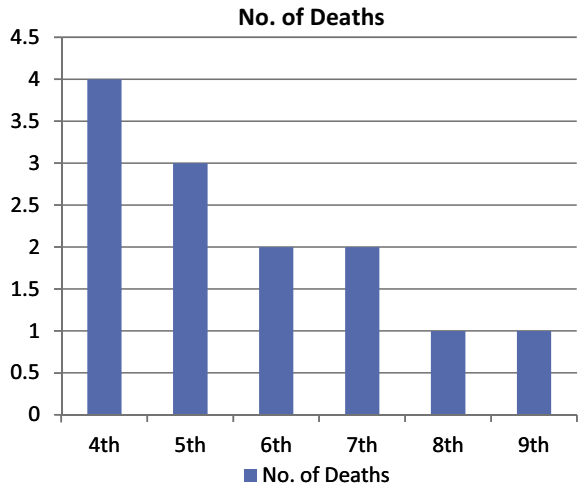

Fig. 1 Showing number of deaths of experimentally infected chicks with *H. pullorum* infection per day post infection.

All embryonated chicken eggs of the six experimentally infected groups showed embryonic mortalities 48-h post yolk sac inoculation, while no embryonic mortalities in the negative control group.

3.2. Isolation of *H. pullorum*

Randomly selected chicks for clinical and bacteriological examination were free from any infectious pathogen, including *H. pullorum*. Also, trails for isolation of pathogens from randomly selected embryonated chicken eggs were negative for *H. pullorum* and other pathogens. *H. pullorum* was re-isolated from cecum, liver, yolk sac and air-sacs of all infected dead chickens and scarified survivors. No *H. pullorum* was isolated from control chickens. *H. pullorum* was re-isolated from dead embryos, embryonic fluids and sacs. No *H. pullorum* was isolated from the control group of embryonated chicken eggs.

Table 1 Showing results of experimental infection of one-day old chicks with *H. pullorum* isolates.

Group	Inoculated agent	No. of chicks	Rout of inoculation	No. of deaths/day post infection						Total no. of deaths	No. of survivors	Mortality percentage (%)
				4th day	5th day	6th day	7th day	8th day	9th day			
Experimental	*H. pullorum* isolates	39	Oral	4	3	2	2	1	1	13	26	33.3
Control	Sterile BHI broth	13	Oral	–	–	–	–	–	–	–	13	00.0

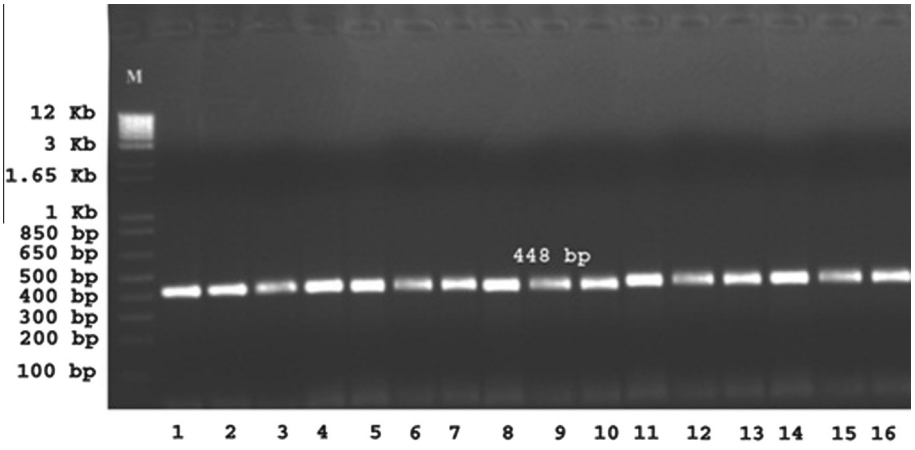

Fig. 2 PCR positive reaction from *H. pullorum* isolates to 16S-rRNA gene. Lane M: 1 Kb plus DNA ladder.

3.3. PCR analysis and gel electrophoresis

The results of PCR analysis of suspected *H. pullorum* isolated from experimentally infected chickens, and embryonated chicken eggs are shown in Fig. 2. Results revealed the appearance of 448 bp bands denoting the positive amplification of 16SrRNA for all tested isolates.

3.4. Antimicrobial susceptibility testing

Determination of antibacterial susceptibility and resistance patterns of *H. pullorum* using MIC for six antibiotics; ciprofloxacin, gentamycin, colistin, tetracycline, erythromycin and ampicillin against different eight *H. pullorum* isolates resulted in that all isolates were resistant to ciprofloxacin, gentamycin and erythromycin, while 7 out of 8 isolates were resistant to tetracycline. All isolates were susceptible to colistin sulfate and ampicillin, as summarized in Table 2 and Fig. 3.

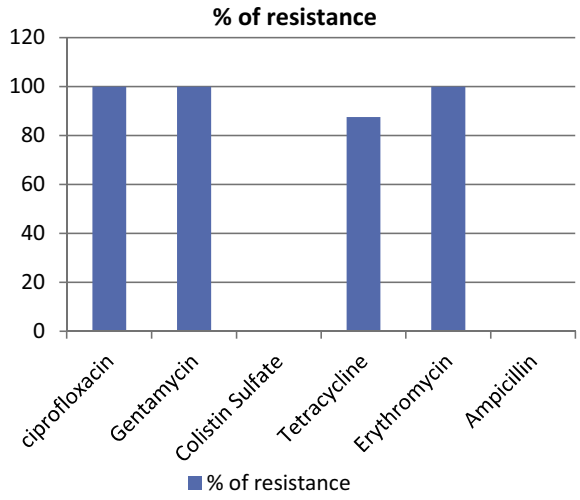

Fig. 3 Showing percentage of resistant isolates of *H. pullorum* for each antibacterial drug used in the experiment.

4. Discussion

H. pullorum is an enterohepatic pathogen with a powerful ability to colonize the distal intestinal tract, liver of poultry and human beings. This species has been associated with diarrhea in gastrointestinal patients and enteritis and hepatitis in chickens [1,9,19,20].

Ceelen et al. [6], who were the first to study the pathogenicity of *H. pullorum*, and they concluded that *H. pullorum* can colonize broiler chickens and additionally is excreted in their feces until the age of slaughter. The preferred colonization site is the cecum wherein the bacterium shows close association with the surface epithelium. Experimentally infected chickens did not reveal overt clinical signs, although mild lesions in the ceca were present. This study is only a first step in the investigation of the interaction of *H. pullorum* with its chicken host and stipulates further research [6]. The pathogenicity of chicken *H. pullorum* isolates in this work was evaluated by oral inoculation of one day-old chicks, our results revealed 33.3% mortality with signs of diarrhea, retardation of growth and

Table 2 Showing results of susceptibility of 8 chicken isolates of *H. pullorum* to antimicrobial agents.

Antibiotic	No. of isolates with MIC (μg/ml) of:											Break-point of drug resistance	No. of resistant isolates	% of resistant isolates (%)
	0.25	0.5	1	2	4	8	16	32	64	128	256			
Ciprofloxacin	–	–	–	–	–	2	1	5	–	–	–	⩾4	8	100
Gentamycin	–	–	–	–	–	–	–	4	4	–	–	⩾16	8	100
Colistin	–	–	–	–	–	–	4	4	–	–	–	⩾32	–	0
Tetracycline	–	–	–	–	–	–	1	4	3	–	–	⩾16	7	87.5
Erythromycin	–	–	–	–	–	–	2	2	4	–	–	⩾8	8	100
Ampicillin	–	–	–	–	–	2	–	6	–	–	–	⩾32	–	0

poor conversion rate in survivors. The differences in results of mortalities, clinical signs and autopsy findings may be attributed to many factors, like the difference in the age at which infection performed, dose of inoculation and period of experimental infection.

The present study was the first one that evaluated the pathogenicity of *H. pullorum* using the embryonated chicken eggs as an experimental model, and it is found that all infected embryonated chicken eggs showed embryonic mortalities within 48-h post yolk sac inoculation. This means that there is no variation in the pathogenicity of different *H. pullorum* chicken isolates.

H. pullorum was re-isolated from cecum and colon of experimental chickens with different bacteriological titrations, but it could not be isolated from liver tissue of experimentally infected chickens [6]. In the current study, *H. pullorum* was re-isolated from cecum, liver, yolk sac and air-sacs of all experimental dead and sacrificed chickens. *H. pullorum* was also re-isolated from dead embryos as well as from embryonic membranes and embryonic fluids. The differences in results may be due to using different methods of isolation and age of infection.

PCR analysis was used to detect *H. pullorum* from liver, jejunum, cecum and colon of experimentally infected chickens [6]. In our study, PCR assay was used to detect *H. pullorum* from *H. pullorum* colonies retrieved from experimentally infected chickens and embryonated chicken eggs.

Despite the increasing number of reports emphasizing the significance *of H. pullorum*, hardly any data about the antibiotic sensitivity of *H. pullorum* are available in the literature [5]. They mentioned that different resistance percentages exhibited by *H. pullorum* to nalidixic acid were encountered by several research groups. On et al. [17] and Atabay et al. [1] reported 6% and 28% *in vitro* resistance respectively, while antimicrobial susceptibility assays showed 55% resistance to this antimicrobial agent among the tested strains in a study of Melito et al. [14]. Thus far, no susceptibility studies comprising widely used antibiotics with *H. pullorum* strains have been reported [5]. It was reported that *H. pullorum* is resistant to cephalothin and cefoperazone [17,19]. *H. pullorum* is naturally sensitive to polymyxin B, which is a phenotypic characteristic distinguishing this species from the other *Helicobacter* species [1]. Zanoni et al. [23] concluded that all the tested isolates of *H. pullorum* were resistant to cephalothin and all but one susceptible to nalidixic acid [23]. The results of the present study proved that *H. pullorum* isolates were resistant to most antibacterial drugs used in that study. It could be inferred that ampicillin and/or colistin sulfate is the drugs of choice that can help in prevention and control of *H. pullorum* infection in chickens. Variation in results of determination of antimicrobial susceptibility and resistance patterns of *H. pullorum* may be due to several factors, from which; method used for determination, types of antimicrobial drugs used, types and doses of prophylactic antimicrobial drugs used in poultry farms.

In conclusion, *H. pullorum* resulted into 33.3% mortality with signs of diarrhea, stunted growth and poor conversion rate in survivors. All experimentally infected embryonated chicken eggs showed embryonic mortalities within 48-h post yolk sac inoculation; this means that there is no variation in the pathogenicity of *H. pullorum* isolates. *H. pullorum* was re-isolated from cecum, liver, yolk sac and air-sacs of all dead with sacrificed chickens with dead embryos, embryonic membranes and fluids. PCR assay was used to detect *H. pullorum* from experimentally infected chickens and embryonated chicken eggs. All tested isolates of *H. pullorum* were resistant to ciprofloxacin, gentamycin and erythromycin, while 7 out of 8 isolates were resistant to tetracycline. All isolates were susceptible to colistin sulfate and ampicillin.

We certify that we handled the chickens and chicken embryonated eggs during our experimental work in accordance with The Code of Ethics of the World Medical Association for experiments.

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References

[1] Atabay HI, Corry JEL, On SLW. Identification of unusual *Campylobacter*-like isolates from poultry products as *Helicobacter pullorum*. J Appl Microbiol 1998;84:1017–24.
[2] Black JG. Microbiology: principles and applications. 3rd ed. Upper Saddle River (NJ): Prentice Hall; 1996, p. 140–4.
[3] Burnens AP, Stanley J, Morgenstern R, Nicolet J. Gastroenteritis associated with *Helicobacter pullorum*. Lancet 1994;344:1569–70.
[4] Castera L, Pedeboscq A, Rocha M, et al. Relationship between the severity of hepatitis C virus-related liver disease and the presence of *Helicobacter* species in the liver: a prospective study. World J Gastroenterol 2006;12:7278–84.
[5] Ceelen LM, Decostere A, Devriese LA, Ducatelle R, Haesebrouck F. *In vitro* susceptibility of *Helicobacter pullorum* strains to different antimicrobial agents. Microb Drug Resist 2005;11(2):122–6.
[6] Ceelen LM, Decostere A, Chiers K, et al. Pathogenesis of *Helicobacter pullorum* infections in broilers. Int J Food Microbiol 2007;116:207–13.
[7] Fox JG. The non-*H. pylori* helicobacters: their expanding role in gastrointestinal and systemic diseases. Gut 2002;50:273–83.
[8] Fox JG, Yan L, Shames B, et al. Persistent hepatitis and enterocolitis in germfree mice infected with *Helicobacter hepaticus*. Infect Immun 1996;64:3673–81.
[9] Fox JG, Dewhirst FE, Shen Z, et al. Hepatic *Helicobacter species* identified in bile and gallbladder tissue from Chileans with chronic cholecystitis. Gastroenterology 1998;114:755–63.
[10] Garcıa A, Ihrig MM, Fry RC, et al. Genetic susceptibility to chronic hepatitis is inherited codominantly in *Helicobacter hepaticus*-infected AB6F1 and B6AF1 hybrid male mice, and progression to hepatocellular carcinoma is linked to hepatic expression of lipogenic genes and immune function-associated networks. Infect Immun 2008;76:1866–76.
[11] Hassan AK, Shahata MA, Refaie EM, Ibrahim RS. Preliminary studies on *Helicobacter pullorum* infection in domesticated birds, MVSc Thesis 2009; Faculty of Veterinary Medicine, Assiut University, Egypt.
[12] Jennifer MA. Determination of minimum inhibitory concentrations. J Antimicrob Chemother 2001;48(Suppl. S1):5–16.
[13] Laharie D, Asencio C, Asselineau J, et al. Association between entero-hepatic *Helicobacter* species and Crohn's disease: a

prospective cross-sectional study. Aliment Pharmacol Ther 2009;30:283–93.
[14] Melito PL, Woodward DL, Bernad KA, et al. Differentiation of clinical *Helicobacter pullorum* isolates from related *Helicobacter* and *Campylobacter* species. Helicobacter 2000;5(3):142–7.
[15] National Committee for Clinical Laboratory Standards (NCCLS). Performance standards for antimicrobial disk and dilution susceptibility tests for bacteria isolated from animals, 2nd ed. NCCLS, Wayne, Approved Standard M31-A2, 2002: 80.
[16] Nebbia P, Tramuta C, Ortoffi M, Bert E, Cerruti Sola S, Robino P. Identification of enteric *Helicobacter* in avian species. Schweiz Arch Tierheilkd 2007;149:403–7.
[17] On SLW, Holmes B, Sackin MJ. A probability matrix for the identification of campylobacters, helicobacters and allied taxa. J Appl Bacteriol 1996;81:425–32.
[18] Rocha M, Avenaud P, Menard A, et al. Association of *Helicobacter* species with hepatitis C cirrhosis with or without hepatocellular carcinoma. Gut 2005;54:396–401.
[19] Stanley LD, Burnens AP, Dewhirst FE, et al. *Helicobacter pullorum* sp. nov. – genotype and phenotype of a new species isolated from poultry and from human patients with gastroenteritis. Microbiology 1994;140:3441–9.
[20] Steinbrueckner B, Hearter G, Pelz K, et al. Isolation of *Helicobacter pullorum* from patients with enteritis. Scand J Infect Dis 1997;29:315–8.
[21] Veijola L, Nilsson I, Halme L, et al. Detection of *Helicobacter* species in chronic liver disease and chronic inflammatory bowel disease. Ann Med 2007;39:554–60.
[22] Ward JM, Fox JG, Anver MR, et al. Chronic active hepatitis and associated liver tumors in mice caused by a persistent bacterial infection with a novel *Helicobacter* species. J Natl Cancer Inst 1994;86:1222–7.
[23] Zanoni RG, Rossi M, Giacomucci D, Sanguinetti V, Manfreda G. Occurrence and antibiotic susceptibility of *Helicobacter pullorum* from broiler chickens and commercial laying hens in Italy. Int J Food Microbiol 2007;116:168–73.