



Prevalence and Antibiotic Resistance of *Helicobacter pullorum* Isolates in Poultry From Semnan Province, Iran

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Abstract

Background: *Helicobacter pullorum* predominantly colonizes the gut of apparently healthy chickens and the livers and intestinal contents of hens with enteritis and vibronic hepatitis.

Objective: The aim of this study was to assess the prevalence and antibiotic resistance of *Helicobacter pullorum* in broiler chickens, laying hens, and turkeys in Semnan province.

Materials and Methods: A total of 300 samples were collected from 60 poultry farms in Semnan province, including 240 cecal samples from 48 broiler farms, 30 fecal samples from 6 laying hen farms, and 30 cecal samples from 6 turkey farms. Each sample was analyzed by conventional culture method and biochemical tests. The suspected colonies were subjected to polymerase chain reaction (PCR) using 16S rRNA gene. Antibiotic resistance of the confirmed colonies was determined using disk diffusion method.

Results: Of 300 samples, 85 (28.3%) samples obtained from 36 (60%) poultry farms were positive for *H. pullorum*. Of these samples, 72 (30%) were from 30 (62.5%) broiler farms, 4 (13.3%) were from 2 (33.3%) laying hen farms, and 9 (30%) were from 4 (66.7%) turkey farms. Moreover, resistance to ciprofloxacin was observed in all of the *H. pullorum* isolates.

Conclusion: This study demonstrated the moderate prevalence of *H. pullorum* in poultry in Semnan province for the first time, while the prevalence of this pathogen in laying hen and turkey has not been determined in Iran. In addition, this study could reveal the antibiotic resistance profile of *H. pullorum* as the first report in Iran. Therefore, more studies are needed to focus on the prevalence and antibiotic resistance of *H. pullorum* in poultry in other regions of Iran.

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Background

The genus *Helicobacter* is divided into two major categories, including gastric *Helicobacter* (GH) and enterohepatic *Helicobacter* (EHH) species.¹ *Helicobacter pullorum* has been and is continuing to be a serious health challenge.² This well-known enterohepatic species, first defined as a novel species by Stanley et al on the basis of 16s rRNA phylogenetic analysis. It is a curved rod-shaped organism with unsheathed flagella which can grow easily at 37°C and/or 42°C with sufficient H₂ in the microaerophilic environment.^{3,4}

In poultry, *H. pullorum* predominantly colonizes the gut of apparently healthy chickens and the livers and intestinal contents of hens with enteritis and vibronic hepatitis.⁴ Isolating this pathogen from raw or undercooked broiler chicken meat has also generally led to the idea that chicken meat may possibly be taken into account as a significant source of *H. pullorum* infection.² This non-pylori *Helicobacter* species, frequently detected in the cecum

and on the poultry carcasses at the slaughterhouses, could be considered an emerging foodborne human pathogen, posing a great risk to human health. It is noteworthy that this contamination can occur during the poultry rearing, handling, and slaughtering processes.⁵ In people, *H. pullorum* has been shown to be involved in several digestive disorders, namely inflammatory bowel disease, gastroenteritis, and chronic liver disease.² This bacillus has also been occasionally detected in feces obtained from patients with gastroenteritis and clinically healthy people.³

It is important to keep in mind the fact that *H. pullorum* is a fastidious microorganism and its culture has imposed some difficulties on the researches. It can be inferred that polymerase chain reaction (PCR), as an alternative approach, has gained a proven superiority over culture-based methods in terms of its efficiency, accuracy, and sensitivity for rapidly identifying this pathogen from cecal and meat samples of the poultry.^{2,6} Worldwide,

there have been several studies regarding the prevalence of *H. pullorum* in poultry. In fact, the prevalence of this Gram-negative bacterium has varied considerably from 4% to 100%.⁷ On the contrary, relatively few studies have been carried out in relation to the prevalence of *H. pullorum* in Iran,⁸⁻¹⁰ where the poultry industry has become an important economic activity in the Middle East.¹¹ Moreover, the antibiotic resistance of *H. pullorum* has not yet been investigated in this country. Accordingly, determination of the true prevalence and antibiotic resistance of this pathogen in this area are highly needed.

Based on what mentioned above, this study has attempted to ascertain the prevalence and antibiotic resistance of *H. pullorum* isolated from cecal contents of broiler chickens, laying hens, and turkeys in Semnan province.

Materials and Methods

Sample Collection

From January to September 2019, a total of 300 samples from 60 poultry farms were tested in Semnan province. On average, 5 samples were obtained from each farm tested and all of the animals were healthy and conventionally bred. Specifically, 240 cecal samples were from 48 broiler farms, 30 cecal samples were from 6 turkey farms, and 30 fecal samples were from 6 laying hen farms (Table 1). The cecal samples of broiler chickens and turkeys were collected from various slaughterhouses located in Semnan, but fecal samples of laying hens were taken from live birds. All of the samples were submitted to the Food Microbiology Laboratory of Semnan University in a cooler with ice packs within 5 hours after sampling. In the slaughterhouse, the complete intestinal tract was obtained immediately after evisceration, packed into a separate sterile plastic bag, and transported to the lab in less than 5 hours. To avoid cross-contamination, protective suits, disposable gloves, and shoes were worn during sampling.

Isolation and Identification

In the laboratory, the ceca samples were aseptically collected and their surfaces were washed with phosphate-buffered saline to minimize contamination. Then, 200 mg of each fecal or cecal sample was homogenized in 400 μ L of enriched medium containing 7.5 g of D-(+)-Glucose (Sigma-Aldrich, Germany), 2.5 mL of brain heart infusion (BHI) broth (Merck, Germany), and 75 mL of inactivated horse serum (Bahar Afshan, Iran). After homogenization, the modified filter technique of Steele and McDermott was used.¹² Briefly, a sterile cellulose acetate membrane filter with a diameter of 47 mm and pore size of 0.45 μ m (Sartorius, Germany) was applied onto the surface of Brucella agar plate (Merck, Germany) supplemented with 5% defibrinated sheep blood (Bahar Afshan, Iran) and 5 mg/L of Skirrow's medium (Oxoid, UK). After absorption of the filter in the agar, 10 drops (300 μ L) of the homogenized sample was placed on the

top of the filter, each drop in a separate location on the filter, and then plates were incubated for 1 h at 37°C under microaerobic atmosphere with hydrogen (6% O₂, 7% CO₂, 7% H₂, 80% N₂) generated by Gas pack jar system (Anaerocult C gas pack) (Oxoid, UK). After incubation, the filter was removed and plates were incubated again under the same conditions as described above for a week and examined daily for growth. Afterwards, 5 typical colonies (small, greyish-white) were selected from each plate and sub-cultured in the Brucella agar plates. The selected colonies were tested by Gram stain, microscopic morphology, oxidase and catalase reactions, and urease activity. The urease test was used as a phenotypic test to distinguish between *Campylobacter* spp. and *H. pullorum*. The suspected colonies which were gram-negative, spiral shaped, oxidase and catalase positive, and urease negative were subjected to PCR assay for final confirmation.

DNA Extraction and PCR Amplification

Total genomic DNA of suspected *H. pullorum* colonies was extracted using phenol chloroform isoamyl alcohol method as described earlier.¹³ The quality of the extracted DNA was evaluated using NanoDrop spectrophotometer (Eppendorf, Germany). In fact, the NanoDrop spectrophotometer could evaluate the purity of DNA by assessing the ratio of absorbance at 260 nm and 280 nm and the ratio higher than 1.8 was accepted as pure for the extracted DNA. The extracted DNA was amplified using the specific primers for 16S rRNA gene (forward, 5' ATG AAT GCT AGT TGT TGT CAG 3'; reverse, 5' GAT TGG CTC CAC TTC ACA 3') (Bioneer, Korea). These primers can amplify an approximately 447 bp fragment as described by Stanley et al.⁴ PCR amplification was done in a final volume of 25 μ L containing 50 ng of template DNA, 1 μ mol of each primer, 2 μ mol of MgCl₂, 5 μ L of 10 PCR buffer, 200 μ M of dNTPs, and 1 unit of Taq DNA polymerase (Takapouzist, Iran). The samples were amplified in a thermocycler (Eppendorf, Germany) under the following condition: initial denaturation at 94°C for 4 minutes, followed by 35 cycles of 94°C for 1 minute, annealing at 58°C for 2 minutes, extension at 72°C for 90 seconds, and final extension at 72°C for 3 minutes. The PCR products (10 μ L) were subjected to electrophoresis in 1.5% agarose gel (Sigma-Aldrich, Germany) with 100 bp Plus DNA Ladder (Fermentas, Germany) for fragment size determination. After electrophoresis, the gel was stained with ethidium bromide and images were taken by a UV transilluminator.

Antimicrobial Susceptibility Testing

One colony from each *H. pullorum* positive sample was chosen for susceptibility tests using a Kirby-Bauer disc diffusion method on Mueller-Hinton agar (Merck, Germany) as recommended by Clinical Laboratory Standards Institute (CLSI). According to the study done by Muntari in 2019, several classes of antibiotics

(HiMedia, India) were applied, which were commonly used to treat infections in people and animals, including Quinolone (nalidixic acid 30 µg), fluoroquinolone (ciprofloxacin 5 µg), aminoglycoside (gentamycin 10 µg and neomycin 10 µg), tetracycline (tetracycline 15 µg and doxycycline 30 µg), polymyxin (colistin 10 µg), B-lactams (ampicillin 10 µg), phenicol (chloramphenicol 30 µg), macrolide (erythromycin 15 µg and clarithromycin 15 µg), and fosfomycin (200 µg).¹⁴ After incubation at 37°C for 72 hours in a microaerophilic atmosphere as described before, the susceptibility of the *H. pullorum* was measured to each antimicrobial agent and the results were interpreted according to the Clinical Laboratory Standards Institute (CLSI) as Susceptible (S), Intermediate (I), and Resistant (R).

Results

Prevalence of *Helicobacter pullorum*

A total of 300 cecal and fecal samples taken from three different sources, including broiler chickens, laying hens, and turkeys were examined using the culture method and biochemical tests and final confirmation was carried out using PCR assay. Of 300 samples, 85 colonies (28.3%) of *H. pullorum* were isolated by the culture method and biochemical tests. Of these samples, 72/240 (30%) were from broiler chickens, 4/30 (13.3%) were from laying hens, and 9/30 (30%) were from turkeys. The results of the culture method and biochemical tests were corroborated by means of PCR assay using 16s rRNA gene primers as shown in Figure 1. Additionally, of the 60 poultry farms included in this study, 30 (62.5%) farms of broiler chickens, 2 (33.3%) farms of laying hens, and 4 (66.7%) farms of turkeys were positive for *H. pullorum* with an overall prevalence rate of 60% (36/60). The results of the prevalence of *H. pullorum* isolated from poultry in

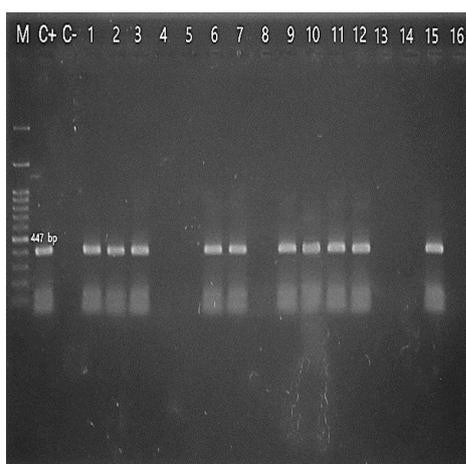


Figure 1. Amplification of 16S rRNA Gene of Some *H. pullorum* Isolates in 1.5% Agarose Gel.

M: 100 bp plus ladder. Lane C+: Positive control, Lane C-: Negative control, Lanes 1-3, 6, 7, 9-12, and 15: Positive samples of *H. pullorum*, Lanes 4, 5, 8, 13, 14, and 16: Negative samples of *H. pullorum*

Semnan province are shown in Table 1.

Antibiotic Resistance of the *Helicobacter pullorum* Isolate

The antibiotic resistance profile of 85 *H. pullorum* isolates from different sources is shown in Table 2. Considering the results of the antibiogram, resistance to ciprofloxacin (76.5%) was common among all the sources. Relatively high rates of resistance were observed against nalidixic acid (58.8%), gentamycin (51.8%), erythromycin (48.2%), chloramphenicol (41.2%), tetracycline (35.3%), neomycin (32.9%), doxycycline (31.8%), and ampicillin (30.1%). In addition, low resistance rates were also observed against colistin (27.1%) and clarithromycin (25.9%). Finally, the lowest antibiotic resistance was observed against fosfomycin (1.2%).

Discussion

Although there have been numerous investigations concerning the prevalence of *H. pullorum* in poultry throughout the world, the fastidious nature of this pathogen has imposed some impediments on the researches.² In spite of the emerging character and pathogenic role of *H. pullorum* against human, there are few attempts to assess the prevalence of this bacterium in poultry in Iran.⁸⁻¹⁰ Moreover, what makes the assessment of this bacterium more significant in this region is that there is no formal study emphasizing the antibiotic resistance of *H. pullorum*. As a consequence, conducting a comprehensive study on the prevalence and antibiotic resistance of *H. pullorum* in poultry in Iran would be absolutely necessary. In general, a total of 300 samples were collected from 60 poultry farms in Semnan province, including 240 cecal samples from 48 broiler farms, 30 fecal samples from 6 laying hen farms, and 30 cecal samples from 6 turkey farms. Of 300 samples, 85 (28.3%) samples were *H. pullorum* obtained from 36 (60%) farms using the culture method and the final confirmation was carried out by PCR. The overall prevalence of *H. pullorum* in poultry in Semnan province was therefore calculated to be 28.3%.

Considering broiler chicken, 72 (30%) cecal sample from 30 (62.5%) broiler farms were positive for *H. pullorum*. The result of this part of the experiment could be compared with other studies in Iran. One similar study was conducted by Jamshidi et al in Mashhad, where 41 (41%) cecal sample from 12 (60%) broiler farms were positive for *H. pullorum*.¹⁰ This difference in prevalence rate between two studies may be due to the inclusion of several slaughterhouses in the current study in comparison with one slaughterhouse applied in the study conducted by Jamshidi et al; however, the culture method used was approximately the same in the two studies.¹⁰ In another study conducted in Ardabil in Iran, Behroo et al examined 40 cecal samples of chickens, of which 3 (7.5%) samples were confirmed as *H. pullorum* by culture method and biochemical tests.⁹ The use of frozen clinical

Table 1. Prevalence of *Helicobacter pullorum* Isolated from Poultry in Semnan Province

Source	Number		Number of positive samples (%)	
	Farm	Sample	Farm	Sample
Broiler	48	240	30 (62.5%)	72 (30%)
Laying hen	6	30	2 (33.3%)	4 (13.3%)
Turkey	6	30	4 (66.7%)	9 (30%)
Total	60	300	36 (60%)	85 (28.3%)

Table 2. Antimicrobial Resistant Profiles of *Helicobacter pullorum* Isolated from Poultry in Semnan Province

Antibiotics	Broiler (n=72)			Laying hen (n=4)			Turkey (n=9)			Total Number of Resistant Isolates (%)
	S	I	R	S	I	R	S	I	R	
Ciprofloxacin	0	18	54	0	1	3	0	1	8	65 (76.5%)
Nalidixic acid	0	30	42	0	2	2	0	3	6	50 (58.8%)
Gentamicin	9	21	42	1	3	0	1	6	2	44 (51.8%)
Erythromycin	8	31	33	0	3	1	0	2	7	41 (48.2%)
Chloramphenicol	14	26	32	2	1	1	1	6	2	35 (41.2%)
Tetracycline	13	34	25	3	0	1	3	2	4	30 (35.3%)
Neomycin	17	30	25	0	2	2	4	4	1	28 (32.9%)
Doxycycline	18	33	21	1	2	1	0	4	5	27 (31.8%)
Ampicillin	15	34	23	2	2	0	2	4	3	26 (30.1%)
Colistin	21	32	19	1	1	2	1	6	2	23 (27.1%)
Clarithromycin	12	40	20	3	1	0	2	5	2	22 (25.9%)
Fosfomycin	43	29	0	2	1	1	6	3	0	1 (1.2%)

S: Susceptible, I: Intermediate, R: Resistance.

samples as well as inappropriate culture method could be some possible explanations for the low prevalence rate observed in the study by Behroo et al.⁹ It has often been suggested that freshly processed samples would improve the identification of *H. pullorum* from different sources.¹⁰ There are some comparable studies regarding the prevalence of *H. pullorum* in chickens all around the globe. In contrast to the present study, the high prevalence rates of *H. pullorum* were reported to be 64% (192/300) in different regions of Assiut province in Egypt,¹⁵ 78.47% (164/209) in northern and central part of Italy,¹⁶ 81.8% (203/248) in different regions of Italy,¹⁷ and 100% (24/24) in the north of Italy,¹⁸ respectively. In agreement with the present study, the moderate prevalence of *H. pullorum* was reported as 32.29% (31/96) from Marmara region of Turkey.¹⁹

To the best of our knowledge, there is no formal report concerning the prevalence of *H. pullorum* in laying hens and turkeys in Iran. Considering the laying hens, 4 (13.3%) fecal samples obtained from 2 (33.3%) laying hen farms were positive for *H. pullorum*. There are few studies demonstrating the prevalence of *H. pullorum* in laying hens worldwide.^{4,18,20} The result of the current study is inconsistent with the study conducted by Zanoni et al who indicated that 100% (36/36) of cecal samples of laying hens were *H. pullorum* positive.¹⁸ Considering the

turkeys, 9 (30%) cecal samples obtained from 4 (66.7%) turkey farms were positive for *H. pullorum*. A considerable point to be noted is that there is just one successful report exhibiting the prevalence of *H. pullorum* in Turkey throughout the world.²¹ Zanoni et al in their study of 55 cecal samples collected from 11 turkey farms, found that 100% of samples were *H. pullorum* positive.²¹ Notably, the discrepancies observed in prevalence rate among the mentioned studies could be attributed to some important factors, namely geographic region, sampling procedure, and method applied for determination. Accordingly, this research could show the true prevalence of *H. pullorum* in poultry in Semnan province.

As far as we know, there is no comprehensive report on the antibiotic resistance of *H. pullorum* in Iran. Besides, it is noticeable that studies demonstrating the antibiotic resistance of this foodborne pathogen in poultry are rare all around the world. In the current study, approximately three quarters (76.5%) of the isolates were resistant to ciprofloxacin. Additionally, in broiler chicken, the highest antibiotic resistance was observed against ciprofloxacin, nalidixic acid and/or gentamycin, and erythromycin with frequency rates of 54, 42, and 33, respectively. Almost the same results were obtained by Mohamed et al in a study conducted in Egypt.¹⁵ In laying hens, resistance to ciprofloxacin (3/4 isolates), nalidixic acid,

neomycin, and colistin (2/4 isolates) was reported to be high. Similarly, a previous study carried out on broiler chicken and laying hen in Italy reported a high resistance against erythromycin and ciprofloxacin.¹⁸ In turkeys, resistance to ciprofloxacin, erythromycin, and nalidixic acid was observed in 8, 7, and 6 isolates of *H. pullorum*, respectively, as observed in the study conducted by Zanoni et al in Italy.²¹ Due to the uncontrolled use of antibiotics in poultry industry, knowing the antibiotic resistance profile of the zoonotic pathogens, particularly *H. pullorum*, is absolutely essential in developing countries such as Iran where there is no study regarding the antibiotic resistance profile of this pathogen. Given the high resistance of *H. pullorum* isolates against ciprofloxacin in the present study, this antibiotic could not be effective in treating *H. pullorum* infection in this region. Conversely, the lowest antibiotic resistance was observed against fosfomycin in both broiler chickens and turkeys. Likewise, the lowest resistance to clarithromycin and tetracycline was observed in laying hens. Based on what mentioned above, it can be understood that fosfomycin, clarithromycin, and tetracycline can be utilized for preventing *H. pullorum* infection in poultry in this region.

Arguably, it has been proven that using phenotypic methods, e.g., culture method and biochemical tests along with genotypic methods, e.g., PCR assay using 16s rRNA gene primers may be beneficial in recognizing *H. pullorum* from intestinal contents of poultry,^{10,15} as shown in the present study. Indeed, in line with earlier publications, the present study could explicitly demonstrate that the results of phenotypic method can be in agreement with the results of genotypic methods.¹⁵⁻¹⁷

Conclusion

In conclusion, this comprehensive study primarily illustrated the true prevalence of *H. pullorum* in poultry in Semnan province for the first time, meaning that this foodborne pathogen can be present in this area, and as a consequence, can put the public health at risk. Further, the antibiotic resistance profile of all the *H. pullorum* isolates was determined in the current study, as the first report in Iran. Therefore, clinicians should take these results into account as an efficient and helpful measure to prevent and reduce the risk of *H. pullorum* infection. However, further studies are required to validate or refute these findings and determine the prevalence and antibiotic resistance of this bacterium in other parts of Iran.

Authors' Contributions

HA: Investigation, performing laboratory operations and writing original draft; SHEC: Conceptualization, methodology, reviewing, and editing; AJJ: Methodology, reviewing, and editing.

Ethical Approval

This research was conducted in accordance with the protocol approved by the Semnan University, Faculty of Veterinary Medicine, Semnan, Iran.

Conflict of Interest Disclosures

The authors declare that they have no conflict of interests.

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