



Frequency and antibiotic resistance of *Helicobacter pullorum* among exposed and non-exposed population

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Abstract

Background: *Helicobacter pullorum* can infect the intestinal tracts of both humans and avian species. This study aimed to assess the frequency and antibiotic resistance of *H. pullorum* isolated from workers in the poultry slaughterhouses, farms, and markets as exposed population and healthy people who referred to the hospital as non-exposed population by culture method and polymerase chain reaction (PCR) test.

Methods: Two hundred healthy individuals, including 100 individuals from exposed population and 100 from non-exposed population were selected in Semnan. Fresh stool samples were examined by conventional culture method and biochemical tests. PCR test with 16S rRNA gene was employed to confirm the *H. pullorum* isolates. Antibiotic resistance test was done using the disk diffusion method and various antimicrobial agents.

Results: Generally, 17 (17%) samples from exposed population and 12 (12%) samples from non-exposed population were *H. pullorum* positive by culture method and biochemical tests. However, PCR test could confirm 10 (10%) and 7 (7%) samples from exposed and non-exposed populations, respectively. Therefore, the frequency of *H. pullorum* was determined to be 9.5%. Antibiotic resistance test could reveal that most of the isolates were resistant to ciprofloxacin (84.2%), whereas resistance to colistin and fosfomycin was found to be 15.8%.

Conclusion: The present study illustrated that *H. pullorum* can be present among healthy population with the low frequency rate. Moreover, it was indicated that the frequency of this food-borne pathogen is high in the exposed population. Therefore, there is a high demand for good observation for slaughter hygiene and implementation of routine surveillance in the poultry farms and markets.

Keywords: *Helicobacter pullorum*, Frequency, Humans, Poultry, Polymerase chain reaction

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Introduction

Helicobacter pullorum, as an enterohepatic *Helicobacter* species, has been described to be present in the liver, duodenum, and cecum of asymptomatic poultry, as well as in the broiler chickens and laying hens with vibronic hepatitis and enteritis (1,2). It is noteworthy that the prevalence of *H. pullorum* in poultry birds has been reported from different countries, such as Egypt, Malaysia, Belgium, and Italy. Importantly, this emerging zoonotic pathogen can contaminate the carcasses of the poultry and can infect the intestinal tracts of both humans and avian species (3). This bile-resistant bacterium is associated with some human diseases, such as inflammatory bowel disease, gastroenteritis, and chronic liver disease (4). Furthermore, there are several reports from Germany, Sweden, China, and Japan illustrating that *H. pullorum* can trigger gallbladder malignancies (3). *H. pullorum*, as

a non-spore forming bacterium, is detected from patients with gastroenteritis and clinically healthy people (5). Also, the *H. pullorum* DNA is found in colonic biopsies from patients with Crohn's disease (6). It has often been reported that the consumption of raw chicken meat and the cross contamination in the slaughterhouses can be considered as the most important ways of transmitting *H. pullorum* to humans (4,7,8). Therefore, doing the routine surveillance in the poultry slaughterhouses, farms, and markets would be of utmost importance.

It has been proven that this fastidious microorganism needs special growth requirements and suitable culture medium for the ideal recovery (4,6,8-10). Notably, there have been several diagnostic tools for the detection of *H. pullorum*, namely culture method, molecular techniques, and biochemical tests (5,11,12,13). However, it should be noted that the interpretation of the results of biochemical



tests could be difficult, due to phenotypic similarities between member species of the genera *Helicobacter* and *Campylobacter* and specific isolation requirements (5,8). In contrast, the molecular-based techniques, such as polymerase chain reaction (PCR) test have been frequently employed for rapidly detecting *H. pullorum* in several studies all around the world (4,6,7,10,11,13-16). Nevertheless, the PCR technique has not yet been used for the detection of *H. pullorum* in human population in Iran.

There is no doubt that the burden triggered by multidrug-resistant bacteria is one of the most critical health care problems, particularly in developing countries where the health condition is somehow poor and the rate of population growth is rather high. Notably, it is estimated that the antibiotic consumption in Iran is higher than the global standard levels (17). There has been an increase in morbidity and mortality rates caused by using drug-resistant bacteria in both human beings and poultry (7,8). But, there is a lack of detailed studies from Iran or other developing countries about the antibiotic resistance of such drug-resistant bacteria like *H. pullorum* in human population.

To the best of our knowledge, there is extremely limited data about the frequency of *H. pullorum* in healthy individuals, particularly in developing countries like Iran. In addition, the antibiotic resistance pattern of *H. pullorum* in human population has not been thoroughly elucidated in the world.

Thus, this pioneering research aimed to investigate the frequency of *H. pullorum* isolated from workers in the poultry slaughterhouses, farms, and markets as exposed population, and healthy people who referred to the hospital in Semnan for doing routine biochemical tests as non-exposed population using culture method and PCR test with 16S rRNA gene, as well as to ascertain the antibiotic resistance pattern of the *H. pullorum* isolates.

Materials and Methods

Study design

This cross-sectional study was conducted among healthy individuals who referred to the hospital for doing routine biochemical tests, such as FBS (fasting blood sugar) and cholesterol test as non-exposed population, and workers from poultry slaughterhouses, farms, and markets as exposed population from January to September 2019 in Semnan province under the approval of the Ethics Committee of Semnan University (Ethical code: 127). Individuals who had a history of gastroenteritis and diarrhea, as well as people who had received antibiotic treatment a month prior were excluded from the study. The written consent form was obtained from all willing participants.

Sample procedure

An easy sampling method was used to select individuals.

In general, a total of 200 healthy individuals, comprising 100 individuals from exposed population and 100 from non-exposed population, were randomly selected in Semnan. Fresh stool sample was taken from each individual, and then, put in the sterile stool container. Each fecal sample was immediately transferred at 4°C to the microbiology laboratory of Semnan University in the Cary-Blair medium (Merck, Germany).

Bacteriological analysis

Upon arrival, approximately 5 g of each fecal sample was squeezed into 5 mL of sterile saline and was shaken using a vortex mixer (Eppendorf, Germany) to obtain a homogenous suspension. An aliquot of 200 µL of each sample was diluted in 400 µL of a sterile mixture containing 25 mL of Brain Heart Infusion (BHI) broth (Merck, Germany), 75 mL of inactivated horse serum (Baharafshan, Iran), and 7.5 g of glucose (Sigma Aldrich, Germany). Each sample was inoculated on Brucella agar (Merck, Germany) supplemented with 5% sheep blood using modified filter technique of Steele and McDermott (18). Briefly, a sterile cellulose acetate membrane filter with 47 mm and 0.45 µm pore size (Sartorius, Germany) was placed on the surface of the agar using a sterile pair of tweezers. After absorbing the filter, 300 µL of each diluted sample was spread in the middle of the filter. Then, the plate was incubated upright at 37°C for 1 hour in a microaerobic atmosphere (10% CO₂, 5% O₂, and 85% N₂). After incubation, the filter was removed and the agar surface was streaked with a sterile loop. The plate was then incubated again under the same conditions as described above for a week and examined daily for growth (16).

Biochemical activities

For presumptive identification, three to five *H. pullorum*-like colonies (small, round, and greyish-white) (Figure 1) were sub-cultured onto Brucella agar plate (Merck, Germany). Colonies that were gram negative, gently curved, and slender-rod bacterial (Figure 2), suggestive of *H. pullorum*, were further screened through biochemical tests, including catalase, oxidase, and urease. Final confirmation was based on the PCR test with 16S rRNA gene, as described below.

DNA extraction

Genomic DNA was extracted from pure culture of the colonies which were gram negative, catalase and oxidase positive, and urease negative using phenol-chloroform-isoamyl alcohol method (19). The quality of the extracted DNA was assessed using a NanoDrop spectrophotometer (Eppendorf, Germany).

Polymerase chain reaction test

The PCR test was performed to confirm the *H. pullorum* isolates from stool samples with specific primers (forward,

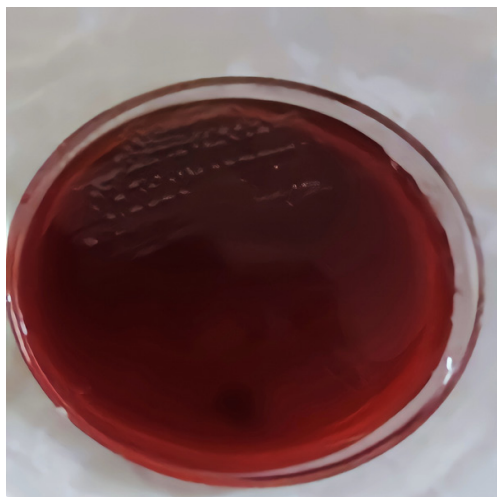


Figure 1. The typical colonies of *H. pullorum* isolates among healthy individuals on Brucella agar plate.

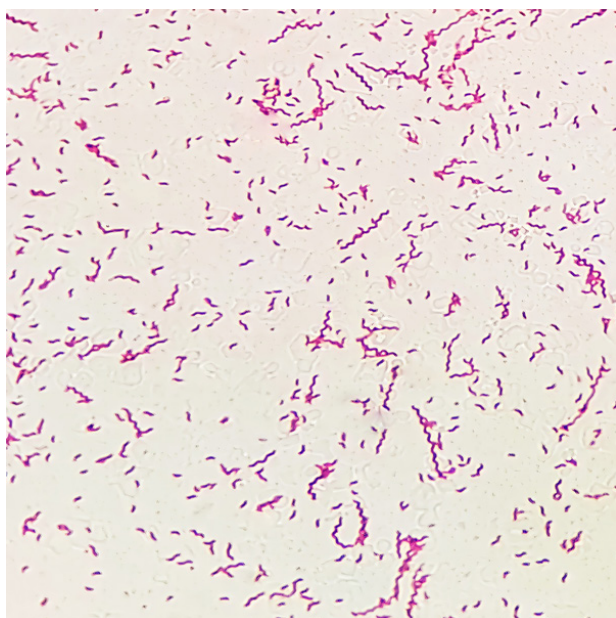


Figure 2. Gram staining of *H. pullorum* isolated from healthy people (gently curved and slender rod bacterial).

5' ATG AAT GCT AGT TGT TGT CAG 3'; reverse, 5' GAT TGG CTC CAC TTC ACA 3') (Bioneer, Korea) amplifying a 447 bp fragment of the 16S rRNA gene (11). The PCR mixture was conducted on the final volume of 25 μ L containing 12.5 μ L of 2X Master Mix (CinnaGene, Iran), 1 μ L of each forward and reverse primer, 50 ng (2 μ L) of template DNA, and 8.5 μ L of distilled water. The PCR amplification was done using DNA thermal cycler (Eppendorf, Germany) with an 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, 72°C for 90 seconds with final extension at 72°C for 3 minutes. The PCR products (10 μ L) were run on 1.5% agarose gel (Sigma-Aldrich, Germany) using gel electrophoresis (Padidehnojen, Iran) and visualized using Gel Documentation System. In the

present study, *H. pullorum* (ATCC 51864) and sterile distilled water were used as the positive and negative controls, respectively.

Antibiotic resistance testing

The antibiotic resistance patterns of *H. pullorum* isolates were assessed using the Kirby-Bauer disk diffusion method on Mueller-Hinton agar plates (Merck, Germany) according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (20). Briefly, two to three *H. pullorum* colonies sub-cultured on Brucella agar plate (Merck, Germany) were picked using a sterile Pasteur loop and emulsified in sterile normal saline. The turbidity of the suspension was adjusted approximately to 0.5 McFarland. The bacteria were spread on Mueller-Hinton agar plate (Merck, Germany) using a sterile cotton swab. Then, the antibiotic disks were placed on the surface of plate and each plate was incubated at 37°C for 48 hours at microaerobic conditions as described earlier. The *H. pullorum* isolates were tested using 12 antibiotics (HiMedia, India) of different classes commonly used to treat animal and human bacterial infections, as shown in Table 1. Inhibition zone diameter was measured and interpreted according to the standard recommendations of the CLSI as Resistant (R), Intermediate (I), and Susceptible (S).

Statistical analysis

Data were transferred to Microsoft Excel spreadsheet (Microsoft Corporation, Redmond, WA, USA) for statistical analysis using SPSS version 24. The data were analyzed by Chi-square and Fisher's Exact tests. $P < 0.05$ was statistically considered significant.

Results

In general, out of 200 fecal samples, 27 (13.5%) were positive for *H. pullorum* using culture method, of which

Table 1. Antibiotic resistance of the *H. pullorum* isolates from exposed and non-exposed populations in Semnan, Iran (n = 19)

Antibiotic	Susceptible (S)	Intermediate (I)	Resistant (R)	% Of resistant isolates
Ciprofloxacin	0	3	16	84.2
Clarithromycin	0	5	14	73.7
Nalidixic acid	3	5	11	57.9
Erythromycin	0	11	8	42.1
Chloramphenicol	3	8	8	42.1
Tetracycline	3	8	8	42.1
Doxycycline	0	14	5	26.3
Gentamycin	3	11	5	26.3
Ampicillin	11	3	5	26.3
Colistin	5	11	3	15.8
Fosfomycin	5	11	3	15.8
Neomycin	11	8	0	0

17 (17%) belonged to the exposed population and 10 (10%) belonged to the non-exposed population. The biochemical tests could reveal that all the culture positive samples were gram negative, catalase and oxidase positive, and urease negative. In other words, the frequency of *H. pullorum* by biochemical tests was determined to be 13.5%. On the contrary, out of 27 biochemically suspected samples, 12 (12%) from exposed population and 7 (7%) from non-exposed population were found to be positive for *H. pullorum* using the PCR test (Figure 3). Hence, the frequency of *H. pullorum* among the mentioned populations was assessed 9.5% by PCR test, as shown in Figure 4. Considering the statistical analysis, there was no significant difference in the frequency of *H. pullorum* between the exposed and non-exposed populations.

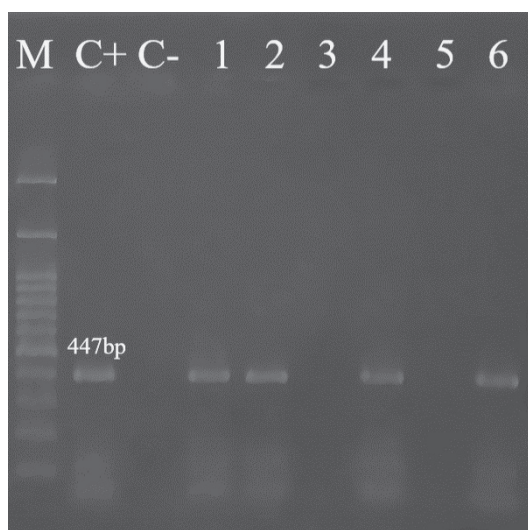


Figure 3. PCR gel electrophoresis results for the detection of *H. pullorum* isolated from healthy people in the present study. M: DNA size marker (100 bp DNA ladder), C+: Positive control, C-: Negative control, Lanes 1, 2, 4, and 6: Positive samples of *H. pullorum*.

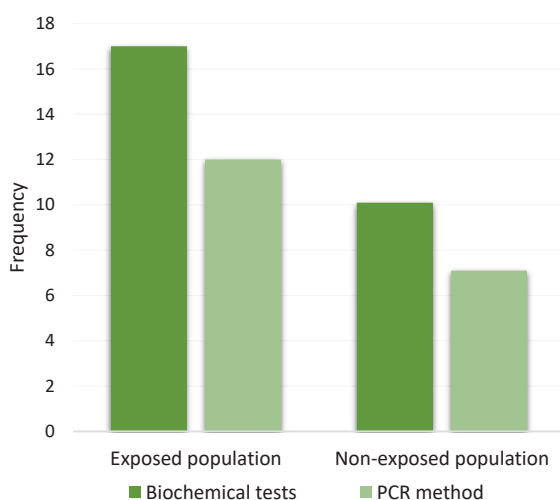


Figure 4. The frequency of *H. pullorum* isolated from exposed and non-exposed populations using biochemical tests and PCR method.

Given the results of the antibiotic resistance test (Table 1), 16 (84.2%) isolates were resistant to ciprofloxacin, as the highest resistance rate. In addition, resistance to clarithromycin and nalidixic acid was determined to be 73.7% and 57.9%, respectively. In addition, the intermediate resistance was observed against erythromycin, chloramphenicol, and tetracycline by 42.1% and against doxycycline, gentamycin, and ampicillin by 26.3%. The low frequency of resistance (15.8%) was also against colistin and fosfomycin. However, none of the isolates was resistant to neomycin.

Discussion

Although it is assumed that poultry workers can be considered as healthy carriers for *H. pullorum* (4,7,16,21,22), there are very few studies concerning the frequency and antibiotic resistance pattern of this food-borne pathogen among human population (5,23). According to the study conducted by Behroo et al in Ardebil, it was revealed that among 100 people with gastroenteritis, 6 (6%) persons were positive for *H. pullorum*. But the difference in prevalence rate between the present study and the mentioned study could be due to the use of various isolation methods for isolating *H. pullorum* from fecal samples. Besides, using frozen clinical samples could result in the reduction of cultivability of the samples (23). In contrast, the present study could successfully utilize the conventional culture method with the help of membrane filter technique, which was previously described to isolate this pathogen from poultry samples (4,7,12,14). Inopportunely, in the study by Behroo et al, the biochemical tests were used to confirm the *H. pullorum* isolates (23). Obviously, based on the results of the present study (Figure 4), 5 samples from exposed population and 3 samples from non-exposed population were not detected by the PCR test, although these samples were biochemically positive. One of the most significant reasons for this observation could be that there may be some other urease negative *Helicobacter* spp. (24) or even *Campylobacter* spp. (25), which can increase the false positive of the results of biochemical tests. From this finding, it can be inferred that the best technique for the final confirmation of *H. pullorum* in human population is the PCR test by 16S rRNA gene.

In another comparable study in Belgium, the prevalence of *H. pullorum* among 522 human patients with gastroenteritis and 100 clinically healthy people was determined to be 4.3% and 4%, respectively using PCR test and culture method (5). This similar prevalence rate in the study by Ceelen et al could show that *H. pullorum* can be present not only in people with gastroenteritis, but also in clinically healthy individuals (5). This finding is consistent with the results of the present study where there was no statistically significant difference between the exposed and non-exposed populations. There have been several critical reasons being associated with this

similar prevalence. First and foremost, clinically healthy people, especially those who are exposed to poultry and poultry products can be considered as healthy carriers for *H. pullorum*. Secondly, this pathogen may be one of the normal human intestinal microbiota. Another reason for this observation could be that some host factors or even ethnicity and regional factors may play a plausible role in this regard. Though, it is important to remember that *H. pullorum* is a food-borne pathogen and can trigger some human illnesses. Accordingly, due to the lack of reports about the frequency of *H. pullorum* among healthy persons, the present study can be considered as a comprehensive study in this regard.

One of the most interesting aspects of the present study is determining the antibiotic resistance pattern of *H. pullorum* among human population. It is thought that the use of novel antibiotics is indispensable for controlling the outbreak of new infectious diseases (26). The reason why the disk diffusion method was used in this study could be that this method is cost-effective, less time consuming, and user friendly in clinical practice (27). Moreover, there have been several reports demonstrating the usefulness of this method for analyzing the phenotypic resistance of the microaerophilic bacteria like *Helicobacter* or *Campylobacter* in humans and poultry throughout the world (7,25-30). However, given that there are very few studies about the antibiogram of *H. pullorum* among people with gastroenteritis, the results of the present study could not be fully supported (31,32). Findings from the present study demonstrated that most of the *H. pullorum* isolates (84.2%) exhibited high resistance against ciprofloxacin. This result is consistent with the findings of a study performed in Spain where all of the *H. pullorum* isolates were resistant to tetracycline, ciprofloxacin, and levofloxacin (32). In the present study, the resistance to clarithromycin was observed in 14 isolates (73.7%). Similar to the present research, in a study done by Shen et al, draft genome sequence of *H. pullorum* isolated from humans showed that this pathogen is resistant to ciprofloxacin and clarithromycin (33). Hence, it can be concluded that the observed resistance to ciprofloxacin could be due to easy access and overuse of this antibiotic in this part of Iran. In the study by Bascuñana et al, all the *H. pullorum* isolates were sensitive to chloramphenicol, while most of them were sensitive to erythromycin and gentamycin (32). However, the low resistance rates (15.8%) in the present study were observed against colistin and fosfomycin. Moreover, none of the isolates was also resistant to neomycin (Table 1). Some possible explanations for this finding could be the limited availability, rare use, or even high cost of these antibiotics in the study area, meaning that the mentioned antimicrobial agents can be effectively utilized for the treatment of *H. pullorum* infection. Therefore, it is recommended that more attention should be paid to the antibiotic resistance pattern of *H. pullorum* in human population in other regions.

Conclusion

According to the results of the present study, *H. pullorum* can be present among healthy population with the low frequency rate. This finding suggests that healthy individuals, particularly those who are exposed to poultry workers may constitute a reservoir and carrier for this pathogen, and consequently, can transmit this pathogen to other people. Furthermore, this study revealed that the most effective antibiotics for the treatment of *H. pullorum* infection would be neomycin, colistin, and fosfomycin. Another significant point of this study is that the PCR technique is considered as the best confirmatory test for detecting *H. pullorum* from fecal samples. And finally, further studies are needed to investigate the frequency and antibiotic resistance pattern of *H. pullorum* in human population in other regions.

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Ethical issues

This research article was approved by the Ethics Committee of Semnan University (Ethical code: 127). The written consent form was obtained from all willing participants. The authors certify that all data collected during the study are as stated in the manuscript, and no data from the study has been or will be published elsewhere separately.

Competing interests

The authors declare that there is no conflict of interests.

Authors' contributions

All authors participated equally in the data collection, analysis and interpretation, as well as laboratory operations. All authors critically reviewed, refined, and approved the manuscript.

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