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Isolation and Antibiotic Resistance of *Helicobacter pullorum* from Chicken Wings

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ABSTRACT

Introduction: *Helicobacter pullorum* has recently emerged as a significant foodborne pathogen. The present study aimed to isolate and determine the antibiotic resistance of *H. pullorum* from fresh chicken wing using the culture method and a molecular technique in Semnan, Iran.

Methods: A total of 60 fresh chicken wings samples were purchased from various local retail markets in different regions of Semnan and processed using the culture method. For initial confirmation, biochemical tests were applied. Suspected colonies were subjected to polymerase chain reaction (PCR) by the *16S rRNA* gene. The antibiotic resistance of the isolates was also assessed using the disk-diffusion method.

Results: Among 60 samples, 27 (45%) were *H. pullorum*-positive based on the culture method and biochemical tests. However, the PCR test indicated 18 samples (30%) to be positive for *H. pullorum*. In the antibiogram, the highest and lowest resistance rates were observed against ciprofloxacin and fosfomycin, respectively.

Conclusion: This was the first report in Iran to clearly illustrate that *H. pullorum* could be found in fresh chicken wings at a moderate level. In addition, the antibiotic resistance of the *H. pullorum* isolates was confirmed, and the PCR test based on the *16S rRNA* gene was considered a reliable and sensitive technique for the detection of this pathogen. However, further investigation is required to explore the life cycle of this novel foodborne pathogen in the other regions of Iran.

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Introduction

It is generally believed that the public consumption of poultry meat (particularly chicken meat) is higher compared to the consumption of red meat. The most significant reasons for this belief could be the high protein content, available vitamins and minerals, low fat, and lower market prices of chicken meat compared to red meat (1-3). Furthermore, it is estimated that the Iranian poultry industry is the largest in the Middle East with producing approximately two million metric tons of chicken meat each year (4). Therefore, evaluating the frequency of foodborne pathogens and monitoring human food sources for the improvement of food safety are of utmost importance.

Helicobacter pullorum is of the genus *Helicobacter* and has been recently emerged as an important foodborne pathogen (5-8). Notably, genus *Helicobacter* could be divided into two main groups of gastric helicobacter and enterohepatic helicobacter (9). *H. pullorum* is considered to be the most commonly studied foodborne pathogen in the latter, especially in developing countries (10-12). This zoonotic bacterium is a gram-negative, slender, non-spore-forming microaerophilic agent, which was first isolated from the caeca of asymptomatic poultry, as well as the liver and intestinal contents of laying hens with vibrionic hepatitis (10, 13, 14).

According to the literature, *H. pullorum* is an emerging pathogen that could be isolated from raw or undercooked chicken meat (5, 7, 15), as well as table eggs (16), house flies, house floors (17), and contaminated water (18). More importantly, this bacterium is detected in patients with gastroenteritis and even healthy individuals (11, 19). Moreover, various avian

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species could be a source of *H. pullorum* infection; such examples are turkeys, laying hens, parrots, psittacines, and guinea fowls (4, 8, 20-22). However, data are scarce regarding the incidence of *H. pullorum* infection in chicken meat products, such as chicken wings.

Several studies have indicated that *H. pullorum* is a fastidious microorganism, and its culture requires special isolation media and proper temperatures (7, 8, 15). Some techniques could be used for the detection of *H. pullorum* in different samples, including the culture method, biochemical tests, and molecular methods (7, 23-25). Undoubtedly, culture-based methods are time-consuming and labor-intensive. In contrast, polymerase chain reaction (PCR) is considered to be a reliable method for the detection of *H. pullorum* owing to its sensitivity, specificity, reliability, and cost-effectiveness (1, 2, 8, 26).

Globally, antibiotic therapy is a challenging issue in the treatment of infectious diseases (6). Antibiotic agents are also used in livestock for various purposes, such as growth promotion and therapeutic use. However, the inappropriate use and misuse of antimicrobials have led to the emergence of resistant bacteria such as *H. pullorum* (6, 27, 28). In order to set up a useful surveillance plan to monitor foodborne pathogens, it is essential to recognize antibiotic resistance patterns. To date, no studies have been focused on the antibiotic resistance of *H. pullorum* in chicken wings.

In the Iranian food industry and unlike other developing countries, the consumption of chicken wings is highly common due to its chemical composition and the fact that it could be distributed in various packages in several retails and supermarkets. Although *H. pullorum* is a foodborne and life-threatening pathogen and may pose significant health risks to humans, no data have been documented regarding the detection of this pathogen in chicken wings in Iran or other regions of the world.

The present study aimed to isolate and determine the antibiotic resistance of *H. pullorum* in chicken wings using the culture method and PCR based on the *16S rRNA* gene in Semnan, Iran.

Materials and Methods

Sample Collection

In total, 60 fresh chicken wings were randomly purchased from different regions of Semnan. Each sample was placed in an ice bag and transferred to the food microbiology laboratory of Semnan University.

Isolation of H. pullorum

We used a specialized culture method that had been previously designed by our research team to isolate *H. pullorum* from chicken meat (7). In brief, 25 grams of the chicken wing samples was aseptically weighed and homogenized in a stomacher (Seward, Norfolk, UK) with an enrichment medium containing 75 milliliters of inactivated horse serum (Baharafshan, Iran), 25 milliliters of brain heart infusion broth (Merck, Germany), and 7.5 grams of glucose (Merck, Germany). Each sample was incubated at the temperature of 37±1ºC for 4-6 hours, followed by the temperature of 41.5±1ºC for 24±2 hours, under microaerophilic conditions without hydrogen. After incubation, 100 microliters of the enrichment broth were deposited onto a cellulose filter membrane (0.45 µm; Sartorius, Germany) and placed on a Columbia agar plate (Merck, Germany), supplemented with 5% sheep blood (Baharafshan, Iran). At the next stage, each plate was incubated for one hour at the temperature of 37^0C under microaerophilic conditions without hydrogen. Afterwards, the filter was removed, and the plates were incubated again under the same circumstances for 44±4 hours at the temperature of 41.5±1ºC (7).

Biochemical Activity

Helicobacter-like colonies (small, round, greyish-white) were selected from each plate and sub-cultured on the Columbia agar (Merck, Germany), supplemented with 5% sheep blood (Baharafshan, Iran) under the same conditions as mentioned earlier. Gram staining, microscopic observation, catalase and oxidase tests, a urease test, and nitrate reduction were employed for the initial confirmation of *H. pullorum* (1).

Genomic DNA Extraction

Genomic DNA extraction from the *H. pullorum* isolates was performed using the phenol-chloroform isoamyl alcohol method (29). The quality and quantity of the extracted DNA were assessed using a NanoDrop spectrophotometer (Eppendorf, Germany).

PCR Amplification

For the amplification of the *16S rRNA* gene of the *H. pullorum* isolates, we applied specific primers (forward: 5، ATG AAT GCT AGT TGT TGT CAG 3, reverse: 5، GAT TGG CTC CAC TTC ACA 3،;

Bioneer, Korea), which targeted the 447 bp fragment of the gene (7). The PCR mixture was prepared in 25-microliter mixtures containing 12.5 microliters of a 2X master mix (CinnaGene, Iran), one microliter of each forward and reverse primer, 50 nanograms (2 µl) of template DNA, and 8.5 microliters of distilled water. PCR amplification was performed using a DNA thermal cycler (Eppendorf, Germany) with an initial denaturation at 94^0C for four minutes, followed by 35 cycles at 94^0C for one minute, and annealing at 58^0C for two minutes and 72^0C for 90 seconds, with the final extension at 72^0C for three minutes. At the next stage, the PCR products (10 µl) were run on 1.5% agarose gel (Sigma-Aldrich, Germany) via gel electrophoresis (Padidehnojen, Iran) and visualized using a gel documentation system. In this study, *H. pullorum* ATCC 51864 and sterile distilled water were used as the positive and negative controls, respectively.

Antimicrobial Susceptibility Testing

The Kirby-Bauer disk-diffusion method was used along with the Mueller-Hinton agar (Merck, Germany) to evaluate the antibiotic resistance of the *H. pullorum* isolates in accordance with the guidelines of the Clinical and Laboratory Standards Institute (CLSI) (30). The *H. pullorum* isolates were tested using 12 antibiotics of various classes (HiMedia, India), including nalidixic acid (30 µg), ciprofloxacin (5 µg), gentamicin (10 µg), neomycin (10 µg), tetracycline (15 µg), doxycycline (30 µg), colistin (10 µg), ampicillin (10 µg), chloramphenicol (30 µg), erythromycin (15 µg), clarithromycin (15 µg), and fosfomycin (200 µg). The obtained results were interpreted as resistant (R), intermediate (I), and susceptible (S) based on the interpretive criteria provided by the CLSI.

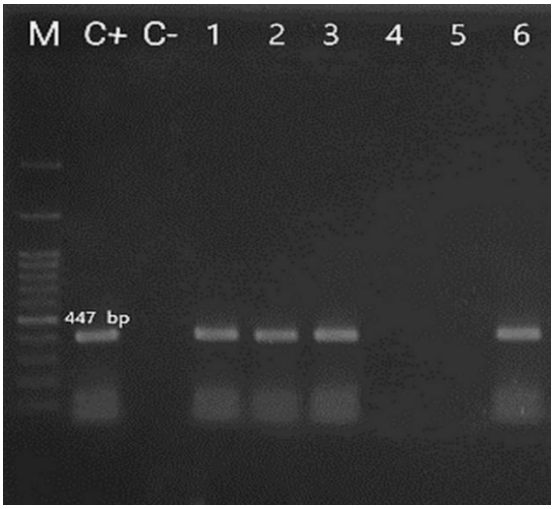

Figure 1. Amplified PCR products of 16S rRNA gene of *H.pullorum* isolated from chicken wing. M: 100 bp standard marker. Lane C+: Positive control. Lane C-: Negative control. Lanes 1, 2, 3, and 6: positive samples for *H.pullorum*. Lanes 4 and 5: Negative samples.

Results

Among 60 chicken wings, 27 samples (45%) were positive for *H. pullorum* based on the culture method. According to the biochemical tests, the culture-positive samples were gram-negative, catalase- and oxidase-positive, and urease-negative, and nitrate reduction was also observed in these samples. Out of 27 biochemically suspected samples, 18 cases (30%) were positive for *H. pullorum* based on the PCR test with the *16S rRNA* gene (Figure 1). Therefore, the frequency of *H. pullorum* isolated from the chicken wings in Semnan was estimated at 30%.

According to the antibiogram results (Table 1), the highest resistance rate was observed against ciprofloxacin. Moreover, resistance against tetracycline, gentamicin, and clarithromycin was similar (frequency rate: 16). Furthermore, moderate antibiotic resistance was observed against doxycycline, neomycin, and erythromycin (frequency rate: 14), and the lowest resistance rate was observed against fosfomycin.

Table 1. Antibiotic resistance frequency of *H.pullorum* isolated from chicken wing in Semnan (n: 18).

Antibiotic	Susceptible (S)	Intermediate (I)	Resistant (R)	% of resistant isolates
Fosfomycin	16	0	2	11.1
Chloramphenicol	11	1	6	33.3
Colistin	11	1	6	33.3
Ampicillin	8	4	6	33.3
Erythromycin	6	2	10	55.6
Neomycin	6	2	10	55.6
Doxycycline	4	4	10	55.6
Clarithromycin	2	2	14	77.8
Gentamycin	2	2	14	77.8
Tetracycline	2	2	14	77.8
Nalidixicacid	0	2	16	88.9
Ciprofloxacin	0	0	18	100

Discussion

Today, poultry meat (especially chicken meat products) is considered to be the most important source of protein and minerals in developing countries (1, 2), and this notion may increase the risk of foodborne diseases and threaten public health. Therefore, determining the level of these agents is crucial for food safety (5). Among various foodborne pathogens, *H. pullorum* is considered to be an emerging zoonotic pathogen of the genus *Helicobacter* (7). This microaerophilic bacterium could cause several disorders in humans, such as chronic liver disease, acute gastroenteritis, inflammatory bowel disease, and hepatobiliary disease (4, 7). The present study aimed to isolate and determine the antibiotic resistance of *H. pullorum* in fresh chicken wings using the culture method and PCR test with the *16S rRNA* gene in Semnan, Iran.

In the current research, 60 chicken wings were aseptically purchased from local retail markets in various districts of Semnan and examined using the culture method, biochemical tests, and PCR. In total, 27 samples (45%) samples were positive for *H. pullorum* based on the culture method and biochemical tests. Among 27 samples, 18 cases (30%) were also corroborated as *H. pullorum* based on the PCR test with the *16S rRNA* gene. Given the lack of similar studies in this regard in Iran or other countries, the moderate frequency rate obtained in our research could be attributed to various reasons. First, *H. pullorum* has been reported to be present in fresh chicken meat products (e.g., liver, thighs, and breasts) (1, 2, 7). Cross-contamination during handling and/or cooking could lead to the transmission of the bacterium to other surfaces. For instance, contamination with intestinal contents may be observed in chicken feathers and skin during the slaughtering process. Another important reason in this regard could be incomplete scalding or post-scalding contamination (31). In the present study, the rate of contamination with *H. pullorum* was estimated at 30% in the chicken wing samples in Semnan.

The main reason for selecting chicken wing samples in the current research was the fact that chicken wings are delicious and popular and contain large amounts of protein and minerals. Moreover, any part of chicken meat (e.g., thighs or wings) could become contaminated with *H. pullorum* during the rearing, handling, and slaughtering processes (i.e., cross-contamination) (4, 6). Furthermore, several studies have demonstrated that this pathogen could be isolated and detected in chicken breast (7), chicken thigh (1, 7, 24), and chicken liver (2, 12, 24, 25). To the best of our knowledge, no studies have involved the isolation and identification of this bacterium from chicken wings. Therefore, the results of the present study cannot be fully supported, and our study should be considered a pioneering research, indicating that fresh chicken wings may be contaminated with *H. pullorum*.

As mentioned earlier, *H. pullorum* is a fastidious pathogen, and its culturing requires special isolation media, atmospheric conditions, filtering procedures, and proper temperatures (7, 8). In the present study, biochemical tests were used for the initial confirmation of the isolates, including a urease test, catalase and oxidase tests, and nitrate reduction. Surprisingly, the PCR test could not corroborate nine isolates although these samples were biochemically positive. The urease test was utilized to differentiate *H. pullorum* from campylobacter species. Notably,

there may be urease-negative *Helicobacter* spp. (e.g., *H. canadensis* (32) or *Campylobacter* spp. (e.g., *C. lari*) (33), which could increase the false positive of the results and lead to misdiagnosis. As such, the present study explicitly illustrated that the PCR test based on the *16S rRNA* gene could be highly sensitive, specific, and reliable for the detection of *H. pullorum* in chicken wing.

One of the most critically important aspects of the present study was evaluating the antibiotic resistance pattern of *H. pullorum* isolated from the chicken wings as the first report published in this regard. The antibiotic resistance of this pathogen has not yet been investigated in chicken wings. According to the antibiogram results, the highest and lowest antibiotic resistance were observed against ciprofloxacin and fosfomycin (frequency rate: 18 and 2, respectively). The second highest resistance rate was observed against nalidixcic acid (frequency rate: 16) (Table 1). Interestingly, similar findings were observed in our previous studies on chicken thigh and liver (1, 2). Furthermore, the study conducted by our team in 2020 on broiler chicken, laying hens, and turkeys yielded the same results in terms of antibiotic resistance (4). Therefore, it could be inferred that fosfomycin is an effective antibiotic in the treatment of *H. pullorum* infection in the study area.

Conclusion

According to the results, *H. pullorum* was detected in the fresh chicken wing samples with a moderate frequency rate. Moreover, the antibiotic resistance of this pathogen in the chicken wings was investigated for the first time in Iran, and fosfomycin was observed to be the most effective antibiotic for the treatment of *H. pullorum* infection. The PCR test based on the *16S rRNA* gene would also be an absolutely sensitive and reliable technique for the detection of this pathogen in chicken wing samples. However, further investigation is required to explore the life cycle of this novel foodborne pathogen in the other regions of Iran.

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Conflicts of Interest

None declared.

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