Comparison of the Culture and PCR Methods to Determine the Prevalence and Antibiotic Resistance of Helicobacter *pullorum* Isolated from Chicken Thigh Samples in Semnan, Iran

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ABSTRACT

Background: *Helicobacter pullorum* is among the most frequently reported pathogens in poultry. The present study aimed to compare the culture and polymerase chain reaction (PCR) methods to assess the prevalence of *H. pullorum* isolated from chicken tight samples in Semnan, Iran. The antibiotic resistance pattern of the *H. pullorum* isolates was also determined for the first time in Iran.

Methods: In total, 50 chicken thigh samples were collected from the local retail markets in Semnan city during January-September 2019. The samples were examined using the culture method and biochemical tests, and the final confirmation was based on PCR with the 16S rRNA gene. In addition, antibiotic resistance test was performed using the disc-diffusion method.

Results: According to the culture method and biochemical tests, 12 samples (24%) were positive for *H. pullorum*, eight of which (16%) showed *H. pullorum* in PCR as well and resistance against nalidixic acid and ciprofloxacin. On the other hand, the lowest antimicrobial resistance was observed against colistin, chloramphenicol, and fosfomycin.

Conclusion: Our promising findings indicated that PCR based on the 16S rRNA gene may be a valuable and sensitive approach to the detection of H. pullorum in chicken meat.

1. Introduction

Over the past two decades, the poultry industry has been expanding enormously [1]. For the most part, food safety risks are globally triggered by the foods of animal origin due to their high protein content and available vitamins, minerals, and lipids [2]. Chicken is generally considered to be the most common source of meat, and this issue has given rise to various life-threatening, foodborne, and zoonotic diseases, representing a significant healthcare concern [3, 4]. According to statistics, the Iranian poultry industry is the largest in the Middle East and ranked 7th in the world with

the production of approximately two million metric tons of chicken meat each year [5]. Foodborne diseases caused by meat could be transmitted to humans via the consumption of undercooked and poorly processed meat. Therefore, meat may frequently become contaminated during poultry rearing, handling, and slaughtering processes [6]. Among foodborne pathogens such as *Salmonella enterica, Campylobacter spp.*, and Shiga toxin-producing *Escherichia coli, Helicobacter pullorum* has been detected as a novel infectious agent, which belongs to the Helicobacter genus. These enteric pathogens are the most significant causes of mortality and morbidity and cause infectious diarrhea; notably, childhood is considered to be a high-risk period for this type of diarrhea [1, 7]. Therefore, *H. pullorum* should be regarded as a dangerous foodborne pathogen.

H. pullorum is the most frequently reported pathogen in the poultry pertaining to the Enterohepatic helicobacter species. It is a microaerophilic, gram-negative, straight, rod-shaped bacterium with the length and width of 3-4 and 0.3-0.5 micrometers, respectively. This non-spore-forming bacterium is a prevalent inhabitant in the intestinal tract of poultry and is initially detected and isolated from the caeca of asymptomatic poultry, as well as the liver and intestinal contents of laying hens suspected of vibrionic hepatitis [8]. Furthermore, *H. pullorum* has the potential to contribute to gastroenteritis, lower bowel inflammation, and chronic liver diseases in humans [9-11]. *H. pullorum* has been shown to infect raw chicken meat samples and is regarded as a major health hazard to the consumers [7]. Accumulated data have also suggested that the prevalence of *H. pullorum* in poultry flocks is relatively high, and the prevalence rate in poultry (particularly slaughter-age broiler flocks) could reach as high as 100% in the cecum, 47% in the liver, and 23.5% in the meat samples [7, 12, 13]. Notably, the prevalence proportions are largely influenced by the healthy/ill status of poultry and geographical region [14].

Molecular methods such as polymerase chain reaction (PCR) are among the most invaluable and excellent techniques for the rapid detection of various food microorganisms. Owing to its high sensitivity and specificity, PCR is considered to be the leading approach to the recognition of these microorganisms, particularly fastidious pathogens with tough cultures. Given that the factual prevalence of *H. pullorum* has been underestimated due to the multiple common phenotypic characteristics with Campylobacter spp., PCR may be a convenient approach to accurately detect this pathogen in food [15].

Antibiotic therapy is essential to the treatment of bacterial infections, while antimicrobial substances could play an indispensable role in the remedy of these pathogens through decreasing the mortality and morbidity of livestock and humans [16]. However, the uncontrolled and excessive use of antimicrobial agents in agriculture, veterinary medicine, and food production has led to the emergence and

dissemination of antimicrobial-resistant bacteria, such as *H. pullorum* [6, 17]. Notably, using antibiotic growth promoters as food additives has been banned in European countries [18]. In many developing countries (including Iran), no plans or bans have been set to prevent the misuse of antibiotics, and many of these countries have been bearing the cumbersome burden of the foodborne diseases caused by drug-resistant pathogens.

The present study aimed to compare the culture method and PCR test based on the 16S rRNA gene in determining the prevalence of *H. pullorum* isolated from chicken thigh samples and also investigate the antibiotic resistance pattern of the isolates to different antibiotic agents for the first time in Iran.

2. Materials and Methods

2.1. Sample Collection

This research was performed during January-September 2019, and the protocol was approved by School of Veterinary Medicine of Semnan University in Semnan, Iran. The minimum sample size was calculated based on the prevalence data of *H. pullorum* in recent studies and using the Cochrane formula, as follows:

$$n = \frac{Z^2\,pq}{d^2}$$

where n is the sample size, Z shows the standard normal deviate corresponding to 5% significance level, p represents the mean prevalence of *H. pullorum* in recent studies, and d is the acceptable sampling error.

By using the formula above and simple random sampling, a total of 50 raw chicken thigh samples were randomly purchased from different local retail markets in Semnan city. Upon purchase, each sample was placed inside a sterile plastic bag, kept in a cool box with ice pads, and transferred to the Food Microbiology Laboratory of Semnan University within a maximum of five hours.

2.2. Bacteriological Method

In order to determine the presence of *H. pullorum* in the raw chicken thigh samples, the standard protocol of ISO10272-1:2006 was employed with some modification [19]. Initially, 25 grams of each sample was aseptically weighed and homogenized in a stomacher (Seward Medical, Norfolk, London, UK) for two minutes with 225 milliliters of Bolton broth (Merck, Darmstadt, Germany) containing Skirrow supplement (Oxoid, Basingstoke, UK) and 5% (v/v) lysed horse blood (Baharafshan, Tehran, Iran). Afterwards, the samples were incubated in microaerophilic conditions using Gas Pack C (Oxoid, Basingstoke, UK) in an anaerobic jar

at the temperature of 37 ± 1ºC for 4-6 hours, followed by 44 ± 4 hours at the temperature of 37.5 ± 1ºC. At the next stage, 100 microliters of an enrichment broth was deposited onto a cellulose acetate membrane filter (0.45 μm; CHM, Barcelona, Spain), which was placed on a Columbia agar plate (Merck, Darmstadt, Germany) supplemented with 5% (v/v) defibrinated sheep blood (Baharafshan, Tehran, Iran). Passive filtration was performed for 15 minutes at room temperature, and after removing the filter, the plates were incubated under the same conditions at the temperature of 37.5 ± 1ºC for 44 ± 4 hours.

2.3. Biochemical Procedures

At this stage, 3-5 Helicobacter-like colonies (small, round, greyish-white) were selected from each plate and sub-cultured on the Columbia agar plate (Merck, Darmstadt, Germany) with 5% (v/v) defibrinated sheep blood (Baharafshan, Tehran, Iran) and incubated under the previously described conditions. Presumptive recognition was carried out via gram staining, microscopic observation (S-shaped, curved rod), catalase and oxidase reactions, nitrate reduction, and urease test. The selected colonies were stored at the temperature of -80ºC in the brain heart infusion broth (Merck, Darmstadt, Germany) with 20% glycerol for the subsequent molecular identification.

2.4. DNA Extraction and PCR Amplification

In this study, PCR was used for the final identification of the *H. pullorum* colonies. Initially, total genomic DNA was prepared from the biochemically suspected colonies using the phenol-chloroform isoamyl alcohol extraction method as previously described [20].

After extraction, the quantity of the DNA samples was determined by NanoDrop (Thermo Fisher Scientific, Darmstadt, Germany). DNA extraction was amplified for the 16S rRNA gene (primers: forward, 5, ATG AAT GCT AGT TGT TGT CAG 3,; reverse, 5, GAT TGG CTC CAC TTC ACA 3,) with the fragment of 447 bp [8]. PCR amplification was also performed with the total volume of 25 microliters containing 12.5 microliters of 2X PCR master mix (CinnaGene, Tehran, Iran), 1 μM of each primer, 50 nanograms (2 μl) of the template DNA, and 8.5 microliters of deionized distilled water. PCR amplification was carried out using a DNA thermal cycler (Eppendorf, Darmstadt, Germany) at 940C for four minutes, followed by 35 cycles at 940C for one minute, annealing at 580C for two minutes, 720C for 90 seconds, and the final extension at 720C for three minutes. The PCR-amplified products (10 μl) were subjected to electrophoresis in 1.5% agarose gel (Sigma-Aldrich, Darmstadt, Germany) with 100 bp Plus DNA Ladder (Fermentas, Darmstadt, Germany) for fragment size determination.

2.5. Antimicrobial Resistance

Susceptibility to 12 antimicrobial agents was assessed using the disc-diffusion method in accordance with the guidelines of the Clinical Laboratory and Standard Institute (CLSI) using the Mueller-Hinton agar (Merck, Darmstadt, Germany). The antibiotics used in our study (HiMedia, Mumbai, India) were selected randomly from different antibiotic classes, 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). After incubation at the temperature of 41 ± 1 ºC for 48 hours in a microaerophilic atmosphere, the plates were examined for the presence of the inhibition zone.

Based on the guidelines of the CLSI, the diameter of the inhibition zone around each disc was used to categorize the isolate as resistant (R), intermediate (I), and susceptible (S). In order to assess the inhibition zone diameter of ciprofloxacin, erythromycin, and tetracycline, we employed the CLSI zone diameter interpretive standards of infrequently isolated or fastidious bacteria [21]. Due to lack of established breakpoints for *H. pullorum*, the CLSI zone diameter breakpoints of *Enterobacteriaceae spp.* were utilized for the remaining antibiotics [22].

3. Results and Discussion

3.1. Frequency of H. pullorum

Livestock (especially poultry) is considered to be an important repository of diverse pathogens. *H. pullorum* has recently become a great concern as a foodborne human pathogen in terms of public health [7]. Therefore, monitoring the quality of chicken meat is essential to alleviating the load of *H. pullorum* in meat. *H. pullorum* was discovered by Stanley *et al.* (1994) in the early 1990s, and data are still scarce regarding the prevalence and antibiotic resistance of this pathogen in chicken meat in different regions of the world [7, 8]. In fact, extensive research has been focused on cecal contents as caeca is the primary site of *H. pullorum* colonization in poultry. However, research in this regard should be reoriented toward the hazards of *H. pullorum* for the consumers of chicken meat who should be aware of the risks associated with this pathogen.

Out of 50 samples in the present study, 12 cases (24%) resembled *H. pullorum* colonies. The purified colonies were further analyzed by the mentioned biochemical tests, and all the samples were biochemically confirmed. In order to determine the prevalence of *H. pullorum* in the chicken thigh samples, the samples were subjected to the PCR assay by the 16S rRNA gene, and *H. pullorum* was confirmed by PCR in eight samples collected from various regions of Semnan (Figure 1). Therefore, the overall prevalence of

H. pullorum was estimated at 16% in the chicken thigh samples in Semnan. Unfortunately, few comparable cases are available regarding the prevalence of *H. pullorum* in chicken meat in Iran. In fact, the prevalence of *H. pullorum* in chicken thigh meat has been assessed in only one study, in which the pathogen was detected using the culture method and biochemical procedures [23].

In the current research, the prevalence of *H. pullorum* in the chicken thigh meat was calculated at 16%, which is lower than the value reported by Behroo *et al.* (2015) (2.5%). The prevalence in our study could be due to the assessment of frozen samples instead of fresh samples and lack of a membrane filter method [23], which highlights the importance of membrane filter methods in the more effective isolation of *H. pullorum* from chicken meat.

The culturing of fastidious microorganisms such as *H. pullorum* has long been a challenging issue for scientists, and they have had considerable difficulty culturing this pathogen due to the use of inefficient culture media [7].

Therefore, progress in the accurate constitution of *H. pullorum* has been impeded by this issue. To address this issue, Borges *et al.* (2015) successfully used the method previously described for the isolation of *H. pullorum* from poultry cecal samples, by which H. pullorum could be isolated from four cases (23.5%) out of 17 chicken meat samples in the center and south of Portugal. The higher isolation rate in the mentioned study compared to our study could be attributed to the small sample size and regional/geographical differences more importantly [7].

With regard to the culture method and PCR test in the present study, all the *H. pullorum*-like colonies (n=12) were confirmed by the biochemical tests, which were gram-negative, catalase- and oxidase-positive, and urease-negative and could reduce nitrate as well. Among the 12 samples, *H. pullorum* could not be detected in four samples by the 16S rRNA gene. Due to the phenotypic similarity between microorganisms (e.g., *H. pullorum*) with other species, the use of biochemical tests may result in misdiagnosis [6].

For instance, the urease test is a biochemical test for the differentiation of *H. pullorum* from *Campylobacter spp.*, which might increase false positive and false negative tests results due to the emergence of some urease negative *Campylobacter spp.*, such as *C. lari* [24]. Furthermore, H. canadensis is a urease-negative species, and urease test may not be appropriate to differentiate *H. pullorum* from this species [25]. Therefore, it could be inferred that PCR is highly accurate for the final confirmation of *H. pullorum* in chicken meat samples owing to its sensitivity and reliability.

The isolation and identification of *H. pullorum* have customarily been performed through selective cultures and biochemical tests [13], while these methods are rather costly and time-consuming. On the other hand, the PCR assay has become widely accepted as a rapid and cost-effective approach to the detection of these fastidious microorganisms [15]. Surprisingly, there is no empirical evidence supporting the use of PCR for the detection of *H. pullorum* in chicken meat in Iran. Our groundbreaking research could detect eight samples of thigh chicken meat by the 16S rRNA gene. In another study by Gonzalez *et al.* (2008), it was revealed that the novel real-time PCR assay, which was first designed in the mentioned study, has significantly higher sensitivity against conventional PCR assay with 16s rRNA gene to detect *H.pullorum* [15].

Unexpectedly, not only did we detect *H. pullorum* in the chicken meat samples, we also observed that the conventional PCR method was rather sensitive and reliable, laying emphasis on the use of PCR for the detection of this bacterium in chicken meat. Several studies have also demonstrated the superiority of PCR over culture methods [13, 26-28]. In a study conducted by Jamshidi *et al.* (2014) in Iran, 100 cecal samples of poultry were evaluated, and 85 samples (85%) were reported to be positive for *H. pullorum*, of which 41 cases (41%) yielded the 16S rRNA gene of *H. pullorum* [27]. Therefore, it could be inferred that PCR based on the 16S rRNA gene is the optimal technique for the ide notification of *H. pullorum* in meat samples.

3.2. Antibiotic Resistance Test

According to the information in Table 1, all the samples harbored resistance against nalidixic acid and ciprofloxacin. Furthermore, most of the samples were resistant to tetracycline (87.5%), followed by doxycycline (75%), clarithromycin (62.5%), gentamicin and ampicillin (37.5%), and erythromycin and neomycin (25%). The lowest antimicrobial resistance was observed against colistin, chloramphenicol and fosfomycin (12.5%). Table 2 shows the antimicrobial resistance profile and multiple antibiotic resistance index.

In the present study, the *H. pullorum* isolates exhibited six antibiotic resistant patterns within the range of 0.083-0.666. To the best of our knowledge, no parallel cases have been reported with our findings regarding the antibiotic resistance of *H. pullorum* in Iran.
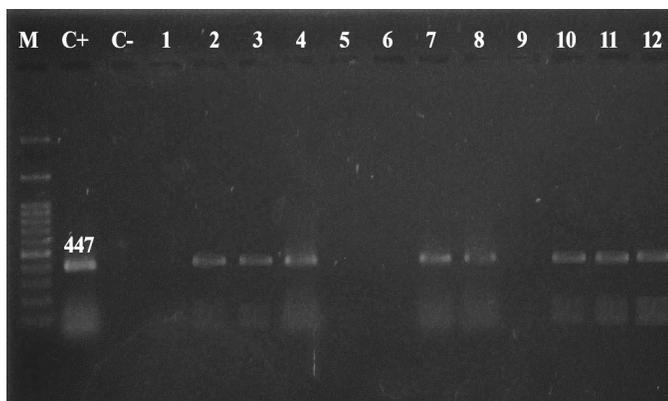

Figure1: Electerophoretic analysis (1.5% agarose gel) of amplified fragments (447bp) for H.pullorum isolated from chicken thigh samples. M: DNA ladder (100bp), C+: Positive control, C-: Negative control, Lanes 2-4, 7-8, and 10-12 positive samples of *H.pullorum*.

Table 1: Antimicrobial resistance pattern of *H.pullorum* isolated from chicken thigh samples (n: 8)

Antibiotic	Resistant		Intermediate		Susceptible	
	N	%	N	%	N	%
Fosfomycin	1	12.5	0	0	7	87.5
Colistin	1	12.5	0	0	7	87.5
Chloramphenicol	1	12.5	3	37.5	4	50
Neomycin	2	25	4	50	2	25
Erythromycin	2	25	4	50	2	25
Ampicillin	3	37.5	3	37.5	2	25
Gentamycin	3	37.5	3	37.5	2	25
Clarithromycin	5	62.5	2	25	1	12.5
Doxycycline	6	75	1	12.5	1	12.5
Tetracycline	7	87.5	1	12.5	0	0
Ciprofloxacin	8	100	0	0	0	0
Nalidixicacid	8	100	0	0	0	0

Table 2: Antibiotic resistance profile and Multiple Antibiotic Resistance (MAR) index of *H.pullorum* isolated from chicken thigh samples

Number	Pattern	MAR index
1	CIP	0.083
2	CIP, NA	0.166
3	CIP, NA, TE	0.25
4	CIP, NA, TE,	0.25
5	CIP, NA, TE, DO, CTM	0.416
6	CIP, NA, TE, DO, CTM,	0.416
7	CIP, NA, TE, DO, CTM, G	0.50
8	CIP, NA, TE, DO, CTM, G, AMP, E	0.666

Since only one study has investigated the antibiotic resistance of *H. pullorum* isolated from chicken meat so far, the extensive comparison of our findings was not possible [7].

According to the current research, the highest resistance rate was observed against ciprofloxacin (100%), which is consistent with the study by Borges *et al.* (2015), in which *H. pullorum* was isolated from chicken meat. In addition, the resistance rate against tetracycline was observed to be high in the mentioned study and our research [7]. Our findings also indicated that all the *H. pullorum* isolates were resistant to at least one antimicrobial agent, which confirmed the uncontrolled and excessive use of antibiotics in the poultry industry. The results of the present study provide novel and reliable data on the antibiotic resistance of *H. pullorum* in chicken thigh samples in Iran.

4. Conclusion

Despite the decreased prevalence rate of *H. pullorum* infection in this study, we should not lose sight of the fact that *H. pullorum* is a foodborne pathogen that could be transmitted to humans via chicken thigh meat consumption, which highlights the need to adopt fundamental strategies for the monitoring of the chicken meat industry. Furthermore, our findings could clearly illustrate that PCR based on the 16S rRNA gene is a highly sensitive, accurate, and reliable test compared to the culture method in the detection of *H. pullorum*. Notably, we identified the antibiotic resistance patterns of the *H. pullorum* isolates of chicken meat for the first time in Iran.

Authors' Contributions

A.J.J., H.E., H.S., and H.A., designed the study and conducted the experiments; H.A., analyzed the data and drafted the manuscript; A.J.J., revised and edited the manuscript. All the authors read and approved the final manuscript.

Conflicts of Interest

The Authors declare that there is no conflict of interest.

Acknowledgements

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