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Short Paper

Development of a novel and specialized cultivation method for isolating *Helicobacter pullorum* from chicken meat

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Abstract

Background: It has become established that *Helicobacter pullorum* could be isolated from raw chicken meat. **Aims:** This study was aimed to develop a novel culture method (protocol B) to isolate *H. pullorum* from chicken meat by adding some modifications to the traditional culture method (protocol A), and as a consequence to compare their sensitivity, specificity, and the accuracy of these methods with polymerase chain reaction (PCR) test. **Methods:** 400 chicken meat samples were collected from various retail markets and supermarkets. Each sample was processed by protocol A, protocol B, and PCR test. **Results:** Out of 400 samples, 77 (19.25%), and 163 (40.75%) were culture-positive by protocol A and protocol B, respectively. Using PCR test as a gold standard, 196 (49%) samples were identified as *H. pullorum*. The specificity for both protocols was determined 100%, while the sensitivity of protocol B and protocol A was assessed 83% and 39%, respectively. Also, the higher and lower accuracy belonged to protocol B (92%) and protocol A (70%), respectively. **Conclusion:** The methodology designed herein can provide a suitable, approximately sensitive, specific, and accurate method to cultivate *H. pullorum* from chicken meat.

Key words: Culture method, *Helicobacter pullorum*, PCR, Sensitivity, Specificity

Introduction

The genus *Helicobacter* is composed of more than 35 species that falls into two main categories: Gastric *Helicobacter* (GH), and *Helicobacter* Enterohepatic (EHH) species (Hassan *et al*., 2014; Ochoa *et al*., 2019). *Helicobacter pullorum*, as an EHH species, has been found in the faeces and injurious livers of broilers and laying hens (Stanley *et al*., 1994; Ceelen *et al*., 2005; Javed *et al*., 2019). There have been some significant human illnesses that are linked with this bile resistant bacterium, namely acute gastroenteritis, inflammatory bowel disease, hepatobiliary disease (Ceelen *et al*., 2005; Javed *et al*., 2017; Wafaa and El-ghany, 2020).

It has become established that *H. pullorum* could be isolated from raw chicken meat (Behroo *et al*., 2015; Borges *et al*., 2015; Jebellijavan *et al*., 2020). It has often been said that difficulties in cultivating fastidious microorganisms, in particular *Campylobacter* spp. and *Helicobacter* spp. could be associated with either the peculiar isolation requirements of these pathogens, or the phenotypic concordance between the member species of them (Ceelen and Haesebrouck, 2005). However, there is no sensitive culture method for isolating *H. pullorum* from chicken meat so far.

In a study done by Borges *et al*. (2015), regardless of the very small sample size (17), *H. pullorum* was successfully isolated from meat samples (Borges *et al*., 2015). However, the culture method used in the mentioned study was specified for the isolation of *Campylobacter* species. Furthermore, the low prevalence rate of *H. pullorum* (23.5%) obtained in the mentioned study made us suspect that this culture medium could not be as appropriate and efficient as it should be for isolating *H. pullorum* from meat (Borges *et al*., 2015). Hypothetically, we decided to utilize the enrichment medium which was frequently applied to isolate *H. pullorum* from intestinal contents, comprising brain heart infusion (BHI) broth, inactivated horse serum, and glucose (Manfreda *et al*., 2006; Zanoni *et al*., 2007; Manfreda *et al*., 2011; Zanoni *et al*., 2011; Qumar *et al*., 2017). Actually, we made few modifications to the prior culture method in terms of not only the composition of enrichment medium, but also the time and temperature required for incubation, atmospheric conditions, and the pore size for filtering procedure.

Several techniques have been used for the diagnosis of *H. pullorum* from which polymerase chain reaction (PCR) test has been shown to be highly sensitive, specific, fast, reliable, and an accurate method for detection of *H. pullorum* (Ceelen and Haesebrouck, 2005; Zanoni *et al*., 2007; Zanoni *et al*., 2011; Kahraman

et al., 2013; Jebellijavan *et al*., 2020). However, although the culture-based methods can be labor-intensive, sensitive, inexpensive, and somehow time consuming, they still remain as the routine method used in the diagnostic laboratories (Deshmukh *et al*., 2019). Thus, establishing a sensitive culture method to isolate *H. pullorum* from chicken meat is necessary.

This pioneering study was initiated to develop a specialized cultivation method for the maximum retrieval of *H. pullorum* from chicken meat by adding some beneficial modifications to the prior culture method, and to compare these with PCR test based on *16S rRNA* gene, as a gold standard, in terms of their sensitivity, specificity, accuracy, and predictive value of positive and negative results.

Materials and Methods

Sample collection

A total of 400 samples of raw chicken meat (a mixture of chicken thigh and breast) were randomly collected from various retail markets and supermarkets in different regions of Semnan city. Each meat sample was aseptically collected and transferred at a temperature of 4°C to the Food Microbiology Laboratory of Semnan University under the approval of the Ethics Committee of Semnan University of Veterinary Medicine, Semnan, Iran (project No.: 2019/127).

Bacterial isolates

At first, each chicken meat sample was cut in half and then subjected to two different enrichment media. For protocol A, 25 g of fresh meat was cultured according to the study by Borges *et al*. (2015).

For protocol B, 25 g of meat was homogenized in a stomacher (Seward, Norfolk, UK) with enriched medium containing 75 ml of inactivated horse serum (Baharafshan, Iran), 25 ml of BHI broth (Merck, Germany), and 7.5 g of glucose (Merck, Germany). The samples were incubated at 37 ± 1°C for 4 to 6 h, followed by 24 ± 2 h at 41.5 ± 1°C, under microaerophilic circumstances without hydrogen. After incubation, 100 µL of enrichment broth was deposited onto a 0.45 µm cellulose filter membrane (Sartorius, Germany) placed onto a Colombia agar (Merck, Germany) plate supplemented with 5% sheep blood (Baharafshan, Iran). The plates were then incubated for 1 h at 37°C under microaerophilic atmosphere without hydrogen. Afterwards, the filter was removed and plates were incubated again under the same circumstances for 44 ± 4 h at 41.5 ± 1°C.

The suspected colonies (small, round, greyish-white) (Fig. 1) from each protocol were selected and tested by gram stain, and oxidase test and then sub-cultured onto a blood agar (Merck, Germany) with 5% sheep blood (Baharafshan, Iran) (Borges *et al*., 2015).

DNA extraction

Total genomic DNA was extracted from all the chicken meat samples using phenol chloroform isoamyl alcohol method (Bello *et al*., 2001). After extraction, the quantity and quality of DNA samples were determined using nano-drop spectrophotometer (Thermo Fisher Scientific, USA).

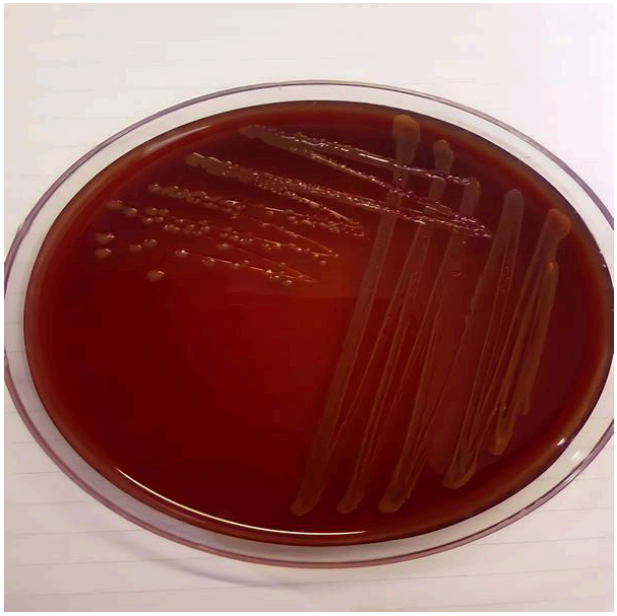

Fig. 1: Colonies of *H. pullorum* isolated from chicken meat on Colombia agar with 5% sheep blood

PCR amplification

In order to detect *16S rRNA* gene of *H. pullorum* from each sample, the specific primers and procedure (forward, 5´ ATG AAT GCT AGT TGT TGT CAG 3´; reverse, 5´ GAT TGG CTC CAC TTC ACA 3´) (Bioneer, Korea) targeting 447 bp fragment were employed, as previously described (Stanley *et al*., 1994). In the current study, *H. pullorum* (ATCC 51864) and sterile distilled water were used as positive and negative controls, respectively. The PCR amplified products (10 µL) were subjected to electrophoresis in 1.5% agarose (Sigma-Aldrich, Germany) gel with 100 bp Plus DNA Ladder (Fermentas, Germany) for amplicon determination.

Statistical analysis

The sensitivity, specificity, accuracy, and predictive value of positive and negative results of each test were determined by means of PCR test as gold standard (Amjad, 2020) and using the formulae according to the study by Molla Kazemiha *et al*. (2014).

Results

In general, 77 (19.25%) samples were positive using protocol A, and 163 (40.75%) samples were positive by protocol B. The PCR test could detect 196 positive samples (Fig. 2). Therefore, the accurate prevalence of *H. pullorum* in chicken meat samples in Semnan city was reported 49%. As seen in the Table 1, the specificity of both protocols was determined 100%, while the sensitivity of protocol B and protocol A was assessed

Table 1: Comparison of the statistical parameters related to each protocol

Protocol	Sensitivity	Specificity	Accuracy	Predictive value of positive results	Predictive value of negative results
A	39	100	70	100	63
B	83	100	92	100	86

83% and 39%, respectively. In addition, the higher and lower accuracy belonged to protocol B (92%) and protocol A (70%), respectively. The predictive value of positive results was estimated as 100% for both protocols, while the predictive value of negative results was calculated 86% for protocol B, and 63% for protocol A.

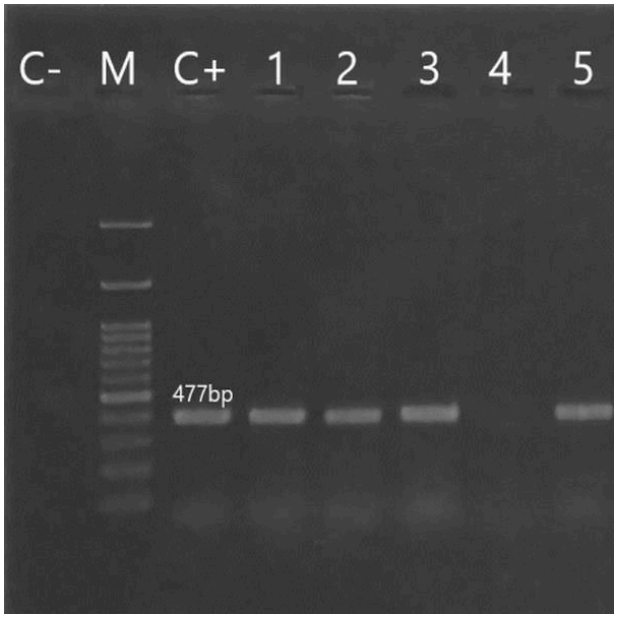

Fig. 2: Representative image of agarose gel electrophoresis showing amplification of *16S rRNA* gene of some isolates. M: 100 bp standard marker. Lane C+: Positive control, Lane C-: Negative control, Lanes 1-3 and 5: Positive samples of *H. pullorum* using of PCR assay, and Lane 4: Negative sample of *H. pullorum*

Discussion

The culture-based methods still remain the most widely used methods in diagnostic laboratories. Notably, regarding the low prevalence of *H. pullorum* reported by the study of Borges *et al*. (2015) in comparison with other investigations (Gonzalez *et al*., 2008; Mohamed *et al*., 2010), the applied method seemed to be less sensitive and specific for culturing *H. pullorum* from chicken meat (Borges *et al*., 2015).

Here, we developed a specialized cultivation method, which is more sensitive, less time consuming, and cost-effective to isolate *H. pullorum* from chicken meat. To measure the effectiveness of culture method designed here (protocol B), we have compared this protocol with the method previously described (protocol A). With regard to the results of the present study, protocol B allowed a significantly higher isolation rate, compared to protocol A. We could recover *H. pullorum* in 77 (19.25%) by protocol A, and in 163 (40.75%) by protocol B, out of 400 chicken meat samples. Furthermore, around half (49%, 196/400) of the samples were found positive for *H. pullorum* using PCR assay (more accurate prevalence).

Initially, we decided to use the fresh chicken meat because freezing process may reduce the cultivability of fastidious microorganisms (El-Shibiny *et al*., 2009). The Bolton broth was used to enrich the meat samples in protocol A, as was employed by Borges *et al*. (2015). The low prevalence rate observed in this procedure could be attributed to the composition of Bolton broth containing three antibiotics: cefoperazone, vancomycin, and trimethoprim. Extended-spectrum beta-lactamase (ESBL)-producing *Escherichia coli*, which is resistant to cephalosporins, can grow on the Bolton broth (Kim *et al*., 2019). Furthermore, the expenditure of Bolton broth is in effect high and makes the isolation procedure more expensive.

In the current study, we appointed the enrichment medium including BHI broth, inactivated horse serum, and glucose for the *H. pullorum* growth; because this composition leads to distributing a greater quantity of inoculum on the membrane as well as reducing the swarming of *Campylobacter* spp. on the agar media (Zanoni *et al*., 2007). Fortunately, the considerable jump produced in isolation rate from 19.25% to 40.75% confirmed that this medium is more suitable for better recovery of *H. pullorum* from chicken meat.

It has been suggested that a pre-enrichment step at 37°C in the isolation procedure would improve the recovery of the bacteria from foods (Bojanic *et al*., 2019). Accordingly, we applied this step to better isolate *H. pullorum* from chicken meat in our protocol for 4-6 h, as Borges *et al*. (2015) did. The poultry body requires a high rate of temperature (Tilmanne *et al*., 2019). Thus, we decided to incubate each sample at high temperature (42°C) in the microaerophilic atmosphere with the time reduced to 24 h. According to the study by Ochoa *et al*. (2019), the majority of EHH do not necessarily require the atmospheric hydrogen for growth (Ochoa *et al*., 2019). Besides, the presence of hydrogen can lead to improving the recovery rate of some other enteric pathogens (Bojanic *et al*., 2019). As a result, we resolved this problem by providing the microaerophilic conditions without hydrogen supplement.

In the next set of our experiment, all the enriched samples from each protocol were transferred to the selective agar plates with the use of filter membrane. Undoubtedly, the Colombia agar supplemented with sheep blood is one of the best media to isolate fastidious microorganisms, such as *Campylobacter* spp. or EHH (Ochoa *et al*., 2019; Tilmanne *et al*., 2019). Owing to the relatively high price as well as the suppressive effects

that antimicrobials have on a wide range of species (Bojanic *et al*., 2019), we would prefer to use the membrane filter instead of antibiotic supplement. In disagreement with the study by Borges *et al*. (2015), that exerted the 0.65 µm pore size cellulose acetate filter (Borges *et al*., 2015), we utilized the 0.45 µm pore size filter, presumably due to the fact that 0.65 µm filter can increase the level of contaminated bacteria (Tilmanne *et al*., 2019). In addition, some publications have accentuated the usefulness and practicality of this pore size filter to isolate *H. pullorum* from poultry cecal contents (Jamshidi *et al*., 2014; Wai *et al*., 2019; Jebellijavan *et al*., 2020). Also, retaining the agar plates in a microaerophilic atmosphere before the removal of the filter could lead to increasing the motility and viability of microorganisms (Zanoni *et al*., 2007), as we did this step in protocol B.

In the present study, all the chicken meat samples were subjected to the PCR test. Worldwide, the utility and feasibility of the PCR assay for the detection of *H. pullorum* has been acknowledged by many researchers (Manfreda *et al*., 2006; Zanoni *et al*., 2007; Manfreda *et al*., 2011; Zanoni *et al*., 2011; Qumar *et al*., 2017; Jebellijavan *et al*., 2020). However, considering the results of the current study, the sensitivity, accuracy, and predictive value of negative results obtained by protocol A (39%, 70%, and 63%, respectively) were improved by use of protocol B as 83%, 92%, and 86%, respectively (Table 1).

From this finding, it can be concluded that laboratories without molecular instruments, the culture method designed herein could be ideally replaced for the isolation and detection of *H. pullorum* from chicken meat.

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Conflict of interest

The authors declare that there are no conflicting interests related to this manuscript.

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