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Research Article

HELICOBACTER PULLORUM PREVALENCE IN PATIENTS WITH GASTROENTERITIS IN HUMANS AND CHICKEN IN THE PROVINCE OF ARDABIL IN 2014

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ABSTRACT
Helicobacter Pullorum is a gram negative, bacillus formed bacteria without spore, which is known as an important nutritional potential pathogen relevant to humans. The purpose of this study was to detect and recognition of Pullorum in patients with gastroenteritis in humans and broiler chicken in the province of Ardabil in 2014. For this purpose 220 samples including 100 diarrhea samples of people with gastroenteritis and 120 samples of broiler chicken with gastroenteritis (40 samples from liver tissue, 40 samples from thigh meat and 40 samples of intestinal swab) were prepared. We used samples prepared by conventional separating methods and additional differentiating and supplementary biochemical tests for recognition this bacteria. We recognized 12 samples out of 120 existing samples positive for the presence of Pullorum, which 6 cases related to human samples (6%) and 6 cases related to broiler chickens (intestinal swab 7.5%, liver 5%, thigh meat 2.5%) were positive for the presence of the bacteria. This amount of prevalence both in humans and chicken indicates that more people will be infected in the future by prevalence of Pullorum. Therefore, extensive studies are essential to unravel the mysteries of life of this potential pathogen, which is the major hurdle for public health.

Keywords*: H. Pullorum, Humans, Broiler Chickens*

INTRODUCTION
Helicobacter genus is a group of fast-growing, gram negative microbial organisms and can be constantly colonized in a variety of mammalian and in some cases may cause clinical disease. In the past two decades, more than 30 species in Helicobacter genus have been reported (On *et al.,* 2002). One of these species is Pullorum which has been described by Stanley *et al.,* for the first time (Stanley *et al.,* 1994). This gram negative bacterium is of the Helicobacter genus in the form of slightly curved bacilli, with 3/4 micron length and approximately 0.4 micron width. It grows at 37 to 42 ° C in microaerophilic conditions and has no spores. The bacterium is able to be transmitted to humans through the food and cause disease. There are reports on the role of this bacterium in developing disease in humans, but there is not enough evidence in this regard (On *et al.,* 1996; Stanley *et al.,* 1994; Steinbrueckne *et al.,* 1997).
Pullorum is one of enter hepatic species [EHS] which was separated at first from the stool, intestine and injured liver of in broiler chickens and laying chickens and human stool. This bacterium causes infection of the intestine and liver in broiler chickens and diarrhea and gastroenteritis in laying chickens, and liver disease in humans (Burnens *et al.,* 1994; Ceelen *et al.,* 2005; Stanley *et al.,* 1994).
Enter pathogenic species of Helicobacter including Pullorum, are increasingly known as microbial pathogens in humans and animals (On *et al.,* 2002; On *et al.,* 1996). This organism can be considered as a pathogen which transmitted from food to humans (Atabay *et al.,* 1998; Ceelen *et al.,* 2006; Gibson *et al.,* 1999). Over the past decade, the number of reported cases of presence of Pullorum, in samples obtained from patients with liver and intestinal diseases has been increased tremendously. In addition, a preliminary study showed that Pullorum is present in 60% of carcasses of chickens, pointed out that it could be considered as an important nutritional potential pathogen relevant to humans (Ceelen *et al.,* 2006). Given that, the chicken is considered as one of the main sources of food and protein supply in the society, in the meantime, food health is very important in provision of public health. One of the issues which should have been paid more attention about the bird is bacterial diseases which in some cases may

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be transmitted in humans in form of zoonosis. Thus, the aim of this study was to recognize this bacterium in humans and chickens as a zoonotic pathogen.

MATERIALS AND METHODS

During the six months with refer to the Imam Khomeini and Alavi hospitals and the health care center of specialized laboratory of center of Ardabil, about 100 samples of diarrhea from referring people with human gastroenteritis and 120 samples of chicken (which consists of 40 intestinal swabs in cecum, 40 samples from liver and 40 samples from thigh of the chickens with diarrhea) were taken from the specialized veterinary clinic and all samples (both human and chicken), were transferred to freeze at -20 ° C.

Preparation and Cultivation Method

First Stage

Reclamation: For reclamation and enrichment of Pullorum, at first, 10 g of tested sample was inoculated into test tubes containing restored culture medium (7.5 g of glucose, 25 ml of Brain heart infusion broth culture medium and 75 ml of horse serum) and vancomycin supplements (10 mg/l), ceftriaxone (10 mg/l), trimethoprim Sulfametoksazol(40 mg/l), polymixin(31000 units per liter), and amphotericin B (10 mg). After placing the tubes in anaerobic jars in the presence of lighted candles, they were incubated for 72 hours and at 37 ° C. After incubation, all the mediums were examined for the growth of the bacteria and the pipes were selected in which the bacteria had grown and lost their transparency and turbid had been made.

Second Stage

Separation: Then, the samples reclaimed on BHI agar plates containing inactivated horse serum (7%), and antibiotics mentioned in the previous step which were used as supplements, were cultured in a five-line form for separation the bacteria.

The culture mediums were incubated in an atmosphere by placing lighted candles in anaerobic jars for at least 3 days and at 37 ° C. In this stage, after 3 days of incubation, the plates were selected in which typical colonies (very small and grayish white) had grown.

Third Stage

Purification: At this stage, the typical colonies (very small, white to gray), were selected and were cultured for purifying in Mueller Hinton agar medium containing sheep blood and all antibiotics used in the previous steps which were used as supplements in the culture medium in this stage, too. After incubation for three days in conditions of anaerobic jar, bacterial growth was checked again in plates and all plates had grown.

Fourth Stage

To ensure separation of the bacteria, differentiating tests were performed on them including: oxidase, catalase, nitrate reduction**,** hippurate hydrolysis**,** urease, capability of growth at 25, 37, and 42 ° C, growth in the presence of 1% of glycine, 1% of gall and 3.5% of salt and activity of DNase**,** production of SH_2 in TSI(Triple sugar iron agar) and OF test**.**

RESULTS AND DISSCUSSION

Results

In the reclamation stage: 74 samples out of 100 cultured human samples, 28 cases out of 40 swab samples from chicken intestine, 18 cases out of 40 chicken liver samples, and only 13 cases out of 40 cultured chicken thigh samples were positive for bacterial growth. In separation stage: Among 74 human samples of previous stage, after culturing in culture medium, at this stage, only 24 plates were positive for bacterial growth and 9 cases out of 28 swab samples, and 2 plates out of 18 liver cases of previous stage, and only 1 of the thigh cultures were positive for bacterial growth.

Purification stage: In this stage, all the plates of previous stage which were positive for bacterial growth were purified in plates containing Mueller-Hinton agar and all the plates were positive for bacterial growth.

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Differentiating Tests Results

Table 1: Morphology survey and oxidase and catalase tests of suspicious samples of H. Pullorum obtained from human cultured samples plates

Code	Morphology	Gram stain	Oxidase	Catalase
2	Cocci	+	-	-
3	Bacilli	-	+	+
4	Coccobacillus	+	-	-
5	Bacilli	+	+	-
8	Coccobacillus	-	+	+
10	Cocci	+	-	-
11	Bacilli	+	-	-
14	Coccobacillus	+	-	-
15	Cocci	+	-	-
17	Coccobacillus	+	-	-
18	Coccobacillus	+	-	-
19	Coccobacillus	+	-	-
20	Bacilli	+	-	-
22	Coccobacillus	+	-	-
23	Coccobacillus	+	-	-
24	Cocci	+	-	-
25	Coccobacillus	+	-	-
26	Coccobacillus	+	-	-
29	Coccobacillus	-	+	+
56	Coccobacillus	-	+	+
60	Coccobacillus	-	+	+
65	Coccobacillus	-	+	+
72	Coccobacillus	+	+	-
87	Cocci	+	-	-
89	Coccobacillus	-	+	+

Table 2: Morphology survey and oxidase and catalase tests of suspicious samples of H. Pullorum obtained from chicken intestinal swab samples

Code	Morphology	Gram stain	Oxidase	Catalase
4	Coccobacillus	-	+	+
7	Coccobacillus	-	+	+
10	Coccobacillus	-	+	+
12	Bacilli	-	+	+
16	Cocci	+	+	-
17	Cocci	+	+	-
18	Cocci	+	-	-
26	Coccobacillus	-	+	+
31	Coccobacillus	-	+	+

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Table 3: Morphology survey and oxidase and catalase tests of suspicious samples of H. Pullorum obtained from chicken liver samples

Code	Morphology	Gram stain	Oxidase	Catalase
13	Coccobacillus	-	+	+
14	Bacilli	-	+	+

Table 4: Morphology survey and oxidase and catalase tests of suspicious samples of H. Pullorum obtained from chicken thigh samples

Code	Morphology	Gram stain	Oxidase	Catalase
29	Bacilli	-	+	+

Negative gram stains of bacilli and coccobacillus cell shape, with positive oxidase and positive catalase were considered as positive samples in terms of H. Pullorum infection and further differentiating and supplementary tests were performed on them. In this case, according to tables, 6 cases of human diarrhea samples, 5 cases of chicken intestinal swab samples, 2 cases of chicken liver samples, and only one case of chicken thigh meat are positive which the results of differentiating tests are as follows:

Table 5: Differentiating, and supplementary tests performed on human diarrhea samples

Code	Nitrate reduction	Hippurate hydrolysis	Salt 3.5%	Bayl	Urease	Glycine %1	TSI-H_2	OF	DNASE
8	+	-	-	+	-	-	-	f	-
29	+	-	-	+	-	-	-	f	-
56	+	-	-	+	-	-	-	f	-
60	+	-	-	+	-	-	-	f	-
65	+	-	-	+	-	-	-	f	-
89	+	-	-	+	-	-	-	f	-

Table 6: Differentiating, and supplementary tests performed on chicken intestinal swab samples

Code	Nitrate reduction	Hippurate hydrolysis	Salt 3.5%	Bayl	Urease	Glycine %1	TSI-H_2	OF	DNASE
4	-	-	+	+	-	+	-	[2]o	-
7	+	-	-	+	-	-	-	[3]f	-
10	+	-	-	+	-	-	-	f	-
12	+	-	-	+	-	-	-	f	-
26	-	-	+	+	+	+	-	o	-
31	-	-	+	+	-	+	-	[4]u	-

[2] - *Oxidative*
[3] - *Fermentative*
[4] – *Unreactive*

Table 7: Differentiating, and supplementary tests performed on chicken liver samples

Code	Nitrate reduction	Hippurate hydrolysis	Salt 3.5%	Bayl	Urease	Glycine %1	TSI-H_2	OF	DNASE
13	+	-	-	+	-	-	-	f	-
14	+	-	-	+	-	-	-	f	-

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Table 8: Differentiating, and supplementary tests performed on chicken thigh meat samples

Code	Nitrate reduction	Hippurate hydrolysis	Salt 3.5%	Bayl	Urease	Glycine %1	TSI-H_2	OF	DNASE
29	+	-	-	+	-	-	-	f	-

Nevertheless, we can say that all the samples in Table 5, i.e. 6 human samples, 3 samples of chicken intestinal swab, 2 samples of chicken liver, and 1 sample of chicken meat are positive for the presence of H. Pullorum. If we want to express the results in terms of percentage, we can display them in a diagram as follows:

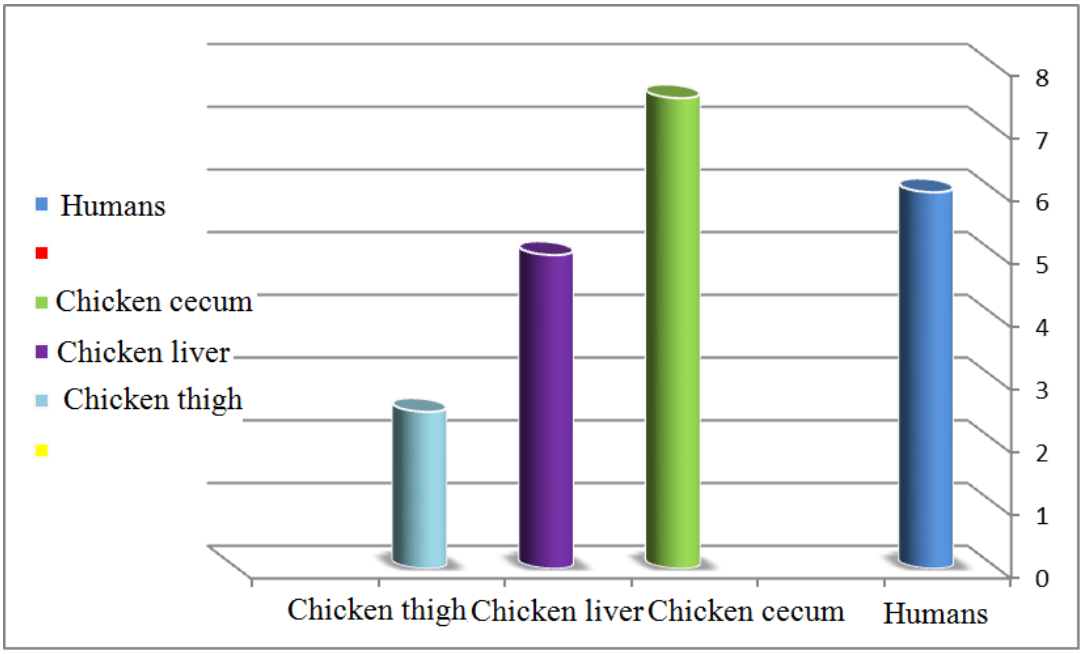

Figure 1: The percentage expression of H.Pullorum in humans and chicken in province of Ardabil

Conclusion

Pullorum is an intestinal pathogen with strong ability to occupy the poultry and human's liver and intestinal tract end (Atabay *et al.*, 1998; Fox *et al.*, 1998; Stanley *et al.*, 1994). According to the information obtained by some researchers, this organism is related to vibrionic hepatitis and diarrhea in chicken, and diarrhea and vomiting, liver and gallbladder disease in humans (Fox *et al.*, 1998; Fox, 1997; Stanley *et al.*, 1994).

It was also reported that Pullorum may have an important role in Crohn's disease (Andersson *et al.*, 2002; Bohr *et al.*, 2002). Several reports were published in recent years about the incidence and prevalence of Pullorum from cecum samples; Burnens *et al.*, achieved a rate of about 4% for prevalence after sampling the contents of the cecum of 150 healthy broiler chickens and they also separated 9 cases out of 18 layer cecum with vibrionic hepatitis (Burnens *et al.*, 1996).

Atabay and Corry, identified H.Pullorum successfully from the similar organism of Campylobacter, i.e, they reported 9 of 15 frozen cecum (60%), and 9 of 15 fresh carcasses(60%), which were collected from two different farms (Atabay and Corry, 1997). Zanoni *et al.*, (2010) investigated high prevalence of H. Pullorum in poultry cecum samples, so that all laying chickens and broiler chickens of the studied farms (100%) were positive for the presence of this bacterium (Ahmed *et al.*, 2014).

Also, Ceelen *et al.*, (2006) reported prevalence of Pullorum infection in Belgium broiler chickens about 33.6% of the cecum and 4.6% of the liver samples in their study (Ceelen *et al.*, 2006). Ahmed *et al.*, (2014) conducted a research to identify and study the epidemiology of Pullorum in birds species in Upper

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Egypt and obtained the most separation of the bacteria from the cecum and then from the liver of broiler chickens (Ahmed *et al.*, 2014).

By reflection and comparison the conducted studies, all suggest that although the prevalence and incidence of Pullorum varies in different climates, this bacterium has the most colonization in the cecum of broiler chickens. The results of the present study indicate that Pullorum in 7.5% of the samples is cecum, 5% liver, and 2.5% thigh meat of broiler chickens collected from the clinic of poultry disease diagnosis using culture method. Although the percentage obtained from the cecum samples in this study, indicates high percentage rather than some studies including Burnens *et al.,* (1996) study, but expresses a rather low prevalence in comparison with other researchers' studies, which may be involved with different factors, such as race, age, ecologic conditions and type of chicken feeding and the method used in separation of this bacterium could be effective. With this description, this study indicates a high incidence of Pullorum in the cecum, too.

The close prevalence of the bacteria in the liver (5%), compared to the results obtained by Ceelen *et al.,* (2006) (4.6%), and Ahmed *et al.,* (2014) showed that the liver is a secondary good place to cecum for colonization of this bacterium in poultry. It means that according to the studies conducted by researchers, all suggest that the liver can be the place of accommodation and proliferation of this bacterium in both poultry and humans, and with regard to the role of the liver in maintaining the health of both of them and Pullorum being zoonotic, there is a large warning in endangering human health in particular. Detection of this bacterium in thigh meat in the present study may describe the development of the bacteria to other tissues and make them infected. The tissues in which no study suggested the presence of Pullorum, except González *et al.,* (2008), in order to detect and identifying infection rate in chicken products by H. Pullorum from 3 types of meat products, they recognized 99% genetic match with H. Pullorum (Gonzalez *et al.,* 2008). According to studies, the results of this study also suggest that most H. Pullorum infection existed in the intestine and especially in cecum and later in the liver and finally the lowest colonization existed in chicken thigh in the present study.

Also, in this study, the bacteria were detected in diarrheal samples of 6% of people with gastroenteritis. In this regard, Ceelen *et al.,* (2005), studied patients with gastroenteritis and also healthy individuals, and the presence of Pullorum were respectively 4.3% and 4% (Ceelen *et al.,* 2005).

This close percentage in both groups may be indicating the possible relationship between Pullorum and gastrointestinal and liver diseases. In fact, one can indicate that Pullorum may be replaced as normal flora in human intestine. Also, its presence in stool samples may be due to the proliferation of these organisms in the intestine, acquired through inactive contaminated food.

However, few conducted studies have shown that Pullorum is related to gastroenteritis, and consequently is related to diarrhea and gallbladder and liver diseases in humans (Burnens *et al.,* 1994; Fox *et al.,* 1998; Steinbrueckner *et al.,* 1997). Observation of DNA of Pullorum in the stools of patients with gastrointestinal diseases and healthy individuals does not mean that these microorganisms are pathogenic (Ceelen *et al.,* 2005).

In fact, predisposing factors which are unknown so far may change some strains of Pullorum from harmless inhabitants of the gastrointestinal tract to factors causing clinical disease. This hypothesis may be completed with possible existing of strains with different very dangerous virulences that cause diarrhea disease (Ceelen *et al.,* 2005). Several studies on the development of hepatitis, liver cancer and inflammatory bowel disease in mice infected with Helicobacter Hepaticus indicate that the genetic basis for the disease caused by infection of Helicobacter is of great importance (Ward *et al.,* 1996a; Ward *et al.,* 1994).

Consequently, Ceelen *et al.,* (2005) report was the first detailed report on the prevalence of Pullorum in both patients with gastrointestinal diseases and healthy human, by proving that H. Pullorum is fairly regularly present in the stool of persons belonging to both groups (Ceelen *et al.,* 2005). Recognition of this bacterium in approximately 6% of patients with gastroenteritis in the present study requires more attention to control and prevent its prevalence; by taking into account microbial organisms can exchange genetic information in different ways.

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Since the bacteria can infect poultry carcasses at slaughter, and can be considered as a foodborne human pathogen (Dunn *et al.*,1997; Fox *et al.*,1994). Partial raw chicken and other poultry products may be transmission device of infection by the bacteria to humans by contamination of the carcass, as has already been reported for Campylobacter and arcobacter species (Euzéby, 2000; Fox *et al.*, 1994; Steele and Dermott, 1984). Despite the increasing number of reports suggesting that Pullorum is a major pathogen of human in relation to food, in general, there are little information about the prevalence of this species in human beings, and that so far understanding the molecular basis of colonization and virulence of Pullorum was weak, and therefore, extensive studies are essential to unravel the mysteries of life of this potential pathogen, which is the major hurdle for public health.

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