Scientific Report

Isolation and identification of *Helicobacter pullorum* from caecal content of broiler chickens in Mashhad, Iran

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Summary

The presence of *Helicobacter pullorum* in intestinal tract of broiler chickens may be a potential risk for human health. In this study, a total of 100 caecal samples of broilers carcasses from 20 flocks at a poultry abattoir in Mashhad suburb were tested for the presence of *H. pullorum* using modified conventional culture method by combination of culturing on *Brucella* sheep blood agar and a filtering technique. Suspected colonies were determined as *H. pullorum* using polymerase chain reaction (PCR) by amplifying a 447 bp fragment of the 16SrRNA gene of this bacteria. 41% of caecal content samples and samples from 12 broiler flocks (60%) were determined as positive for the presence of *H. pullorum*. This is the first report of *H. pullorum* in Iranian poultry flocks. The results showed high prevalence of this bacterium in broiler chickens in this area of Iran. It seems using combination of conventional culture method and PCR assay based on amplification from conserved genes allows reliable detection and identification of *H. pullorum*.

Key words: *Helicobacter pullorum*, Broiler, PCR

Introduction

Since the identification of *Helicobacter pylori* in 1984, some more *Helicobacter* species have been described (Solnick and Schauer, 2001). Most of these species appear to be hosted by one or more animals and enzootic infection is evident in most geographic regions (Solnick, 2003).

Whereas the *H. pylori* is well characterized as a severe gastric pathogen, the pathogenic potential of the other species within the genus is not known (Tee *et al*., 2001).

Helicobacter pullorum was classified as a new species of *Helicobacter* by Stanley *et al*. (1994) on the basis of 16SrRNA sequence data. This organism has been isolated from the livers and intestinal contents of laying hens with vibrionic hepatitis and also from the caeca of broiler chickens (Stanley *et al*., 1994; Atabay *et al*., 1998). In human, the organism has been isolated from gastroenteritis, diarrhea, and liver and gall bladder diseases (Stanley *et al*., 1994). It has been suggested that *H. pullorum* may play a role in Crohn's disease (Bohr *et al*., 2002). The genomic DNA has also been detected in the livers from patients with primary sclerosing cholangitis, cirrhosis and hepatocellular carcinoma (Rocha *et al*., 2005), although it was unclear if the organism had a causal role in these infections (Gibson *et al*., 1999).

It appears that the number of *H. pullorum* infections in human has most probably been underestimated because of the phenotypic similarities between the genera *Helicobacter* and *Campylobacter* on one hand and the specific isolation requirements of *H. pullorum* on the other hand (Atabay *et al*., 1998; Gibson *et al*., 1999). Therefore, a significant number of patients with diarrhea may have been misdiagnosed in the past (Atabay *et al*., 1998).

Poultry carcasses can be contaminated by *H. pullorum* during slaughtering (Atabay *et al*., 1998). The preferred colonization site is the caecum wherein the bacterium shows close association with the surface epithelium (Ceelen *et al*., 2006).

There is no superior method for isolation of all *Helicobacter* species, therefore a combination of phenotypic and genotypic methodologies has been recommended for identifying the organism (On *et al*., 1996; On *et al*., 2005).

The aim of this study was to investigate the occurrence of *H. pullorum* in caecal contents of broiler chickens, collected from a slaughterhouse in Mashhad, Iran, employing combination of a modified conventional culture method and a PCR assay.

Materials and Methods

Sampling

During the summer of 2011, a total of 100 samples were randomly collected from gastrointestinal tract of

broiler chickens, belonging to 20 broiler flocks (5 samples from each flock), after the evisceration stage in the processing line at a commercial broiler slaughtering facility in Mashhad. The complete intestinal tract from each bird was obtained and packed into a separate sterile plastic bag. The samples were brought to the laboratory on ice and examined within 4 h after sampling.

Conventional culture method

In the laboratory, the caeca were aseptically severed and their surfaces were decontaminated using ethyl alcohol. Approximately 5 g of caecal contents were squeezed into 5 ml of sterile saline and vortexed to obtain a homogenous suspension. An aliquot of 200 µl of each sample was diluted in 400 µl of a sterile mixture containing 7.5 g glucose, 25 mL brain heart infusion broth (Merck, Germany), and 75 mL sterile inactivated horse serum, and then homogenized (Ceelen *et al*., 2006).

Samples were then inoculated on *Brucella* agar (Merck, Germany) supplemented with 5% sheep blood using the modified filter technique of Steele and McDermott (1984). Briefly, a sterile cellulose acetate membrane filter (0.45 µm) was applied with a sterile pair of tweezers directly onto the surface of the agar.

When the filter was totally absorbed on the agar, 300 µl of the mixture was placed in the middle of the filter. The inoculated plates were incubated upright at 37°C for 1 h in a microaerobic atmosphere. After at least 1 h of incubation the filter was removed with a sterile pair of tweezers and the filtrate was streaked on the agar with a loop. Incubation was conducted in microaerobic conditions (10% CO_2, 5% O_2, and 85% N_2) at 37°C for a week and examined daily for growth. Approximately five small, grayish-white colonies of Gram-negative, gently curved, slender rod bacteria were pooled and sub-cultured from each plate on sheep blood agar.

DNA extraction

Suspected colonies which were subcutured on sheep blood agar were collected and suspended in 500 µl of sterile, deionized distilled water and heated in a boiling water bath for 10 min. The samples were cooled immediately on ice for 5-10 min and centrifuged at 13,000 × g for 5 min. The supernatants were used as DNA template for PCR assay.

The PCR assay

A PCR assay amplifying a 447 bp fragment of the 16SrRNA of *H. pullorum* was used (Stanley *et al*., 1994). To confirm the isolates identity, the reaction mixture consisted of 2.5 µl of bacterial lysate, 2.5 µl of 10 × BSA buffer (1 ml of 10 × contained 500 µl of 1 M Tris-HCl, pH = 8.5, 200 µl of 1 M KCl, 30 µl of 1 M $MgCl_2$, 5 mg of BSA and 270 µl of deionized water), 2.4 µl of 10 × dNTP mixture (2.5 mM of each dNTP), 0.7 µl of each primer (100 pmoles/µl), 0.2 µl of *Taq* DNA polymerase (5 U/µl) and deionized water to a final volume of 25 µl. According to Stanley *et al*. (1994), the sequences of used primers were as follow: 5′ ATG AAT GCT AGT TGT TGT CAG 3′ and 5′ GAT TGG CTC CAC TTC ACA 3′. The conditions used for the amplifications were as follow: an initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 90 s, elongation at 72°C for 90 s, and a final elongation at 72°C for 5 min. The PCR products were separated by electrophoresis in 1.5% agarose gel at 100 V for 40 min in Tris-acetate buffer, visualized by ethedium bromide staining, illuminated by UV-transilluminator and documented by a gel documentation apparatus. A 100 bp DNA ladder (fermentas) was used as a size reference for PCR assay.

For positive control, the first positive sample in PCR assay with a molecular weight of 447 bp was purified and DNA sequence analysis of the amplicon was performed. By the use of BLAST software, the sequence was compared to published data for *H. pullorum* 16SrRNA sequences in GenBank (accession No. AY631956, L36143, and L36144). Confirmed isolate was used as positive control through the experiment and sterile distilled water was used as negative control.

Individual and herd level prevalence and 95% confidence interval (CI) for *H. pullorum* was calculated.

Results

In total 100 samples from caecal contents of poultry carcasses, representing 20 broiler flocks were analysed by conventional culture method using a modified filter technique. In 85 out of 100 samples suspected colonies appeared on sheep blood agar.

In PCR assay using specific primers of the 16SrRNA gene of *H. pullorum*, the expected amplicon of 447 bp was produced in 41 suspected colonies, representing 41% of all 100 samples examined (Fig. 1). In total, 12 flocks out of 20 flocks, representing 60% were confirmed to be carrier of (or contaminated by) *H. pollorum*.

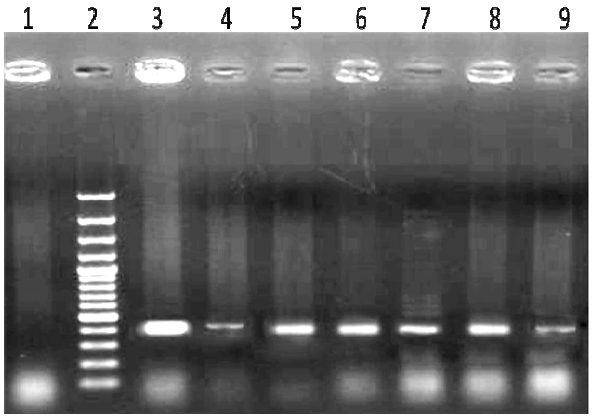

Fig. 1: Detection of *H. pullorum* in samples of caecal content of broilers carcasses by PCR assay, amplifying 447 bp segment of 16SrRNA gene. Lane 1: Negative control, Lane 2: 100 plus bp markers, Lane 3: Positive control and Lanes 4-9: Positive samples

Individual level prevalence of *H. pullorum* was 41%

(95% CI: 31.4%-50.6%) and heard level prevalence of *H. pullorum* was 60% (95% CI: 38.6%-81.4%)

Discussion

In this study, *H. pullorum* was detected in 60% of broiler flocks and 41% of broiler carcasses. Our results point out a high presence, although it may not represent a true prevalence of *H. pullorum* in broiler farms of the region under investigation. Higher prevalence of *H. pullorum* in broiler flocks in Italy (100%) and France (100%) has been reported (Pilon *et al*., 2005; Zanoni *et al*., 2007). Atabay *et al*. (1998) reported the occurrence of *H. pullorum* in 60% of poultry carcasses in the UK and more recently Ceelen *et al*. (2006), reported the presence of *H. pullorum* in 33.6% of cecal content of broiler carcasses and in 64% of broiler flocks from Belgium. In Egypt *H. pullorum* was identified in 39.33% of chickens caecal droppings tested (Moemen *et al*., 2010). At present, it is well-known that the recovery of *H. pullorum* can be more optimized by examining the freshest possible samples (On *et al*., 2005). In this study the whole gastrointestinal tract of slaughtered broiler chickens were collected and brought to the laboratory on ice and examined within 4 h after sampling. Unlike some other reports (Atabay and Corry, 1997), in our study samples were processed freshly. For a better recovery of *H. pullorum*, it has also been recommended to employ non-selective methods or of selective media without polymyxin B (Atabay *et al*., 1998). Moreover, it has been proposed that the best strategy for identifying the organism is the use of a combination of different phenotypic and genotypic methods (On *et al*., 1996). In this study for primary isolation of bacterial colonies, samples were inoculated on a non selective media supplemented with 5% sheep blood in combination with modified filter technique of Steele and McDermott (1984).

The pore size of sterile cellulose acetate membrane filter which was used in this study was 0.45 µm. It has also been recommended that a 0.65 µm filter be used instead of the 0.45 µm filter used in this study (Zanoni *et al*., 2007), although it is logical to speculate that this practice will result in the growth of more colonies on sheep blood *Brucella* agar, but it seems the selectivity of the method will decrease.

Furthermore, Atabay and Corry (1997) and Ceelen *et al*. (2006) who incubated the plates in an aerobic atmosphere before removing the filter, a microaerobic atmosphere aiming to keep the microorganisms more viable and motile was used in this investigation.

Microaerobic atmosphere with and without hydrogen has been used for isolation of *H. pullorum* (Zanoni *et al*., 2007). In this study, microaerobic atmosphere was used without inclusion of the hydrogen. As a result of this practice, the hydrogen dependent strains may have been missed. In this study, the samples were obtained from the caecum of apparently healthy broiler chicken carcasses from flocks holding a health approval for slaughter. It should be noted that the pathogenesis of *H. pullorum* in poultry is not well elucidated. Chickens experimentally infected with *H. pullorum* showed slight gross and histologic cecal lesions, although no clinical manifestation was noted (Stanley *et al*., 1994; Ceelen *et al*., 2007). It was also shown that the preferred colonization site for the organism was the caecum (Ceelen *et al*., 2007), therefore the evisceration in the processing plant may lead to carcass contamination. Several authors have suggested that *H. pullorum* has zoonotic potential and might be involved in GI pathology and hepatopathy in humans (Ceelen *et al*., 2005), but it is not clear whether *H. pullorum* in humans is acquired by consumption of undercooked poultry meat, as is the case with *Campylobacter jejuni* (Humphrey *et al*., 2007; Verhoeff-Bakkenes *et al*., 2008). By the way, retail raw poultry meats and other poultry products may constitute vehicles for human *H. pullorum* infections through consumption of the contaminated carcasses (Antolin *et al*., 2001). In this study we used PCR method for final confirmation of suspected colonies, using primers, designed by Stanley *et al*. (1994) that target a 447 bp fragment of the *H. pullorum* 16SrDNA gene. Although in general, a concordance between different detection techniques for *H. pullorum* has been reported (Ceelen *et al*., 2007), a combination of conventional culture and PCR assay seems to increase the chance of *H. pullorum* identification. Concerning health monitoring, PCR may be helpful in detecting this pathogen not only in intestinal tissue but also in broiler chicken cecal droppings (Ceelen *et al*., 2006).

In conclusion, the results of this experiment demonstrate the presence of *H. pullorum* in caecum content of broiler chicken at a high prevalence, possessing a potential risk for human population. To our knowledge, this is the first report of *H. pullorum* in Iranian poultry flocks. The results may be considered as a base in larger scale studies for estimating the true prevalence.

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