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Prevalence of *Helicobacter pullorum* in Egyptian hen's eggs and *in vitro* susceptibility to different antimicrobial agents

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The objective of the study was to investigate the occurrence of *H. pullorum* in chicken hens' eggs and to determine their *in vitro* susceptibility to various antimicrobial agents. A total of 300 fresh commercial chicken eggs of Balady native breed and poultry farms (PF) were collected from both Assiut and Qena Governorates in Egypt. Every five eggs represented one sample (mixed contents of five eggs). The eggshell and eggshell contents were examined for the presence of *Helicobacter species* by conventional methods, next confirmed with PCR. The highest incidence of contamination with *Helicobacter species* was recorded in eggs from Assiut Governorate poultry farms. The obtained results of *Helicobacter pullorum* (*H. pullorum*) isolation revealed that poultry farms egg shells and contents were less contaminated in both Governorates than Balady ones. Additionally, fresh egg contents of poultry farms in both Governorates were free from *H. pullorum*. All 12 isolates that were conventionally classified as carrying *H. pullorum* occurred free from the organism when tested with PCR method by detection of one PCR product on agarose gel that matched the predicted size of 477 bp that corresponding to 16S rRNA region of the gene. *H. pullorum* show *in vitro* susceptibility to almost all tested types of antibiotics except for ampicillin, ceftriaxone, and sulphamethoxazole trimethoprim.

KEY WORDS: eggs / *Helicobacter pullorum* / polymerase chain reaction / antimicrobial susceptibility

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The increasing consumers' awareness of food safety issues has changed the public perception of a "good egg" from shell cleanliness and physical properties to that of microbial integrity. Egg and egg derivatives have been linked to several enteric outbreaks compromising public health. An increasing number of food industries are using pasteurized liquid whole-egg to formulate their products. Liquid whole egg enhances the nutritive value of the end product, and also improves the functional properties required during the production process. Microorganisms can contaminate eggs at different stages, from production through processing to preparation and consumption. Transovarian or "vertical" transmission of microorganisms occurs when eggs are infected during their formation in the hen's ovaries and oviducts. "Horizontal" transmission occurs when eggs are subsequently exposed to a contaminated environment and microorganisms penetrate the shell.

In the last two decades, more than 30 species have been described within the genus *Helicobacter* [On *et al*. 2002]. One of these is *Helicobacter pullorum* (*H. pullorum*) first described by Stanley *et al.* [1994]. It is a gram-negative, motile, slightly curved, non spore-forming oxidase-positive rod, negative for urease production and hippurate hydrolysis. The organism is sensitive to nalidixic acid and mostly catalase positive [On *et al.* 1996]. *H. pullorum* is bile-resistant and requires a microaerobic environment supplemented with H_2 in which growth occurs at 37°C or 42°C [Stanley *et al*. 1994, On *et al.* 1996, Steinbrueckner *et al.* 1997]. *H. pullorum* was originally isolated from the faeces, the intestines and damaged livers of broilers and laying hens [Stanley *et al.* 1994, Burnens *et al.* 1994], and also from the feces of humans [Ceelen *et al.* 2005]. Enterohepatic *Helicobacter* species, including *H. pullorum*, is increasingly recognized as microbial pathogen in humans and animals [On *et al.* 1996, On *et al*. 2002]. *H. pullorum* has been related to enteritis and hepatitis in broiler chickens and laying hens and diarrhea, gastroenteritis, and liver disease in humans [Stanley *et al*. 1994, Burnens *et al.* 1994, Young *et al*. 2000, Ceelen *et al.* 2005]. The organism can be considered a foodborne human pathogen [Atabay *et al.* 1998, Gibson *et al.* 1999, Ceelen *et al.* 2006]. During the last decade, the number of case reports on the presence of *H. pullorum* in samples obtained from human patients suffering from liver and intestinal disease has increased tremendously. In addition, a preliminary study showed that *H. pullorum* was present on 60% of poultry carcasses, pointing to it as a potentially important food- associated human pathogen.

Despite the increasing number of reports stating that *H. pullorum* is a significant food-associated human pathogen, there is a marked lack of information on the actual prevalence of *H. pullorum* infections in hens' eggs performed hitherto. Furthermore, there is almost no data available on the antibiotic sensitivity of the organism.. *H. pullorum* is naturally sensitive to polymyxin B, a phenotypic characteristic distinguishing it from the other *Helicobacter* species [Atabay *et al*. 1998]. Its resistance to cefalotin and cefoperazone has been reported by Stanley *et al*. [1994] and On [1996].Thus far, no susceptibility studies comprising widely used antibiotics with *H. pullorum* strains have been reported.

The objective of this study was hence to investigate the occurrence of *H. pullorum* in chicken hens' eggs and to determine its *in vitro* susceptibility to different antimicrobial agents.

Material and methods

Sampling

A total of 300 fresh commercial eggs from a native Balady chicken breed and poultry farms (PF) were investigated. The groups of eggs were collected from city markets (Balady) and poultry farms on the day of laying from both Assiut and Qena Governorates in Egypt, (30 groups each). These included 15 groups from Baldy and 15 groups for poultry farms for each Governorate. Every five eggs constitute one group were placed in a sterile plastic bag and transferred to laboratory with a minimum of delay to be prepared and examined for the concerned organism.

Samples preparation

Eggshells were examined by a surface rinse method described by Moats [1980]. The egg was prepared for evacuation of its content according to Speck [1976].

Enrichment procedure

One ml of rinse solution as well as the homogenized egg contents was transferred to 10 ml of Helicobacter Pylori Special Peptone Broth (HPSPB). It was supplemented with antibiotics (vancomycin –10 mg/L, amphotericin B – 5 mg/L, cefsulodin – 10 mg/L), polymyxin B sulphate (31.000 IU/L) and trimethoprim – 40 mg/ L) and calf serum. The inoculated broth was incubated under a microaerophilic gas mixture (6% O_2, 10% CO_2 and 84% N_2) using an anaerobic jar and gas generating kits (OXOID BR56) at 37°C for 48 h.

Isolation, identification and confirmation of the presence of isolated strains

Loopful of the incubated broth was streaked into plates of Helicobacter Pylori Special Peptone Agar (HPSPA). Inoculated plates were incubated at 37°C for 4 days under the microaerophilic conditions. The suspected colonies were inoculated into slope of the same media for morphological and biochemical tests as estimated by Stanley *et al*. [1994]. The isolates were examined for catalase, oxidase, urease, nitrate reduction and hippurate hydrolysis.

All colonies that were small, greyish-white, haemolytic, gram-negative, slightly curved rods, catalase- and oxidase-positive, urease- and hippurate-negative were selected, purified and used for DNA extraction with the NucleoSpin® Tissue Kit according to manufacturer's recommendations. The bacterial DNA was frozen until used for PCR. The DNA amplification was performed using primers based on the 16S rRNA gene sequence of *H. pullorum* that amplified at 447 base pairs (bp) and was used as described by Stanley *et al.* [1994].

The PCR assay and gel electrophoresis of the PCR products were performed after Baele *et al.* [2004]. DNA extraction and amplification were done in the Department of Microbiology and Immunology, Faculty of Medicine, Assiut University. The PCR was carried out in a programmable thermal cycler using the following cycling parameters: 94°C for 5 min , followed by 35 cycles (94°C for 1 min, 60°C for 1.5 min and 72°C for 1 min) and followed by 72°C for 10 min. The PCR reaction was performed in a mix containing: 5 pmoles of each primer, 40 µM of each dNTP, 3.0 mM of $MgCl_2$, 0.3 U Taq polymerase and 2 µl of the template DNA. The PCR products were visualized using a 2.5% agarose gel containing 0.5 µg of ethidium bromide/ml in relation to DNA mass ladder standard (1000 bp DNA ladder plus MBI FERMENTAS). *H. pullorum* strains were identified in the presence of a band at 477 bp corresponding to 16S rRNA region of the gene (Fig. 1).

Antimicrobial susceptibility testing for *H. pullorum*

All cultures of *H. pullorum* positively identified with PCR (12 strains) were tested for their i*n vitro* sensitivity and resistance patterns to ten different antibiotics by disc diffusion method as described by Chaves *et al.* [1999]. The organism was grown for 24 h in Helicobacter Pylori Special Peptone broth in microaerophilic atmosphere and then was diluted with sterile saline to contain 1.2 million cells/ ml. by comparing its opacity to MacFarland 4 turbidity standard. Muller-Hinton agar plates supplemented with 10% horse blood were inoculated with a sterile cotton swab from the identified H. pullorum suspension.

The discs of ten antibiotics were placed on the surface of Muller-Hinton agar plates using sterile forceps. All plates were incubated for 3 days at 37°C in a microaerophilic atmosphere. The inhibition growth zone diameter was measured in mm with a ruler.

Resistance to metronidazole (5 µg) was determined by an inhibitory zone of ≤ 16 mm [Chaves *et al.* 1999]. However, for the remaining antibiotics the zone diameter (mm) of resistance was accepted as recommended by National Committee of Clinical Laboratory Standards (M31-A2) [2002], *i.e.* for nalidixic acid 30 µg, for erythromycin 15 µg, for ampicillin 10 µg, and for ceftriaxone 30 µg – ≤13 mm. For gentamycin 10 µg, for lincomycin 10 µg and for tobramycin 10 µg – ≤12 mm. For sulphamethoxazole trimethoprim 2.5µg – ≤10 mm and for ciprofloxacin 5µg – ≤15 mm.

Results and discussion

The idea of the study dealing with the prevalence of *H. pullorum* in chicken hens' eggs is coming from the fact that the organism was originally isolated from the faeces of broilers and laying hens [Burnens *et al.* 1994, Stanley *et al*. 1994]. In addition, a preliminary study showed its presence on 60% of poultry carcasses pointing to it as to a potentially important food-associated human pathogen.

Table 1. Prevalence of *Helicobacter species* in egg samples examined

Source of samples	No. of samples examined	Positive samples	
		balady breed	poultry farms
		No. (%)	No. (%)
Assiut Governorate	30	6 (20)	15 (50)
Qena Governorate	30	5 (16.6)	3 (10)
Total	60	11 (18.3)	18 (30)

Table 2. Prevalence of *Helicobacter pullorum* in egg samples examined

Source of samples	No. of samples examined	Positive samples			
		egg shell		egg content	
		balady breed	poultry farms	balady breed	poultry farms
		No. (%)	No. (%)	No. (%)	No. (%)
Assiut Governorate	30	4 (13.3)	2 (6.6)	2 (6.6)	0 (0.0)
Qena Governorate	30	2 (6.6)	1 (3.3)	1 (3.3)	0 (0.0)
Total	60	6 (10)	3 (5)	3 (5)	0 (0.0)

Table 1 shows that 18.3% of Balady and 30% of poultry farm hens' eggs were contaminated with *Helicobacter species*. The highest incidence of contamination was recorded in Assiut Governorate poultry farms (50%).

As shown in Table 2 the shells contamination in Balady hens' eggs in both Governorates was almost two times higher than in those coming from poultry farm. This indicates that in the latter, efficient hygienic measures were applied leading to minimized shell contamination. Moreover, shells contamination in both Balady and poultry farm (13.3% and 6.6%) eggs in Assiut exceeded almost twice that found in Qena Governorate (6.6% and 3.3% respectively). This confirms the opinion of Burnens *et al*. [1994] and Stanley *et al* [1994] that the contamination came from faeces of hens.. Fresh contents of eggs from poultry farms of both Governorates were free from contamination whereas contamination in the marketable egg (Balady breed) contents in Assiut was almost twice as high (6.6%) as in Qena Governorate (3.3%).

Using PCR method on the isolated colonies that were grown in broth culture is more specific and less time-consuming than working directly on egg samples [Schmitz *et al*. 1997]. It has developed several characteristics to survive in an unquestionably hostile ecological niche. In addition, Helicobacter culture *in vitro* is very demanding and special conditions are to be maintained. Table 3 and Figure 1 indicate that conventional methods led to identification of 29 bacterial isolates as *H.*

Table 3. PCR results of the isolated *Helicobacter pullorum*

Bacteria strain	No. of isolates	PCR			
		positive		negative	
		No.	%	No.	%
H. pullorum	12	12	100	0	0.00
Other *H. species*	17	0	0.00	17	100
Total	29	12	41.37	17	58.62

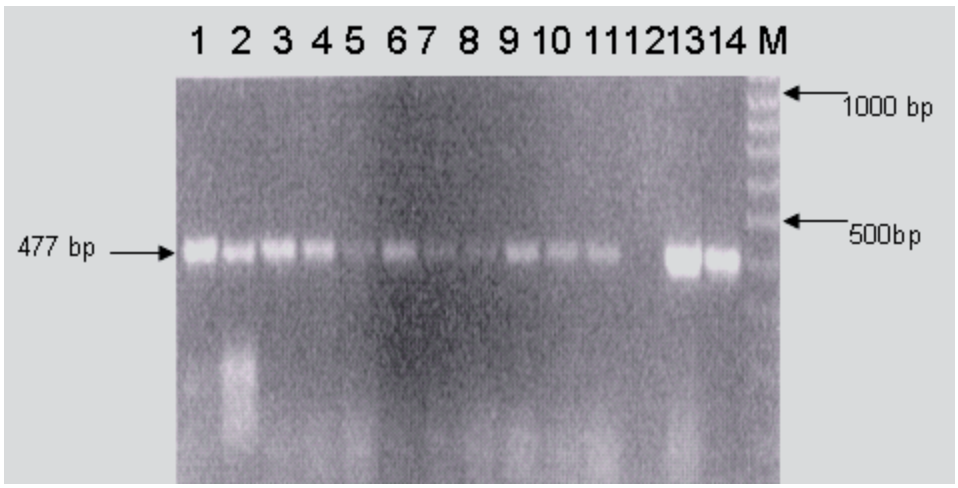

Fig.1. PCR products for *H. pullorum*. Line M – molecular size marker 1000 base pair (bp). Line 12 – negative control. Line 1, 2, 3, 4, 6, 9, 10, 11, 13, 14 – positive samples for *H. pullorum* (477 bp). Line 5, 7 and 8 – negative samples.

species, 12 as *H. pullorum* and 17 as other *H. species* based on biochemical tests. All 12 isolates were confirmed as *H. pullorum* with PCR by detection of one PCR product on agarose gel that matched the predicted size and confirmed the actual size of 477 bp that corresponded to 16S rRNA region of the gene. The remaining 17 isolates were classified as other *H. species* indicated by absence of bands on agarose gel. The correlation coefficient between conventional and PCR method was 0.98 ($P \leq 0.001$) indicating the exact accordance between the two identification methods. The results of this study are in accordance with those of Stanley *et al*. [1994] and Ceelen *et al.* [2006] who obtained the same specificity of the protocol and its ability to discriminate between closely related species.

This study constitutes a part of *in vitro* susceptibility testing of *H. pullorum* against various antimicrobial agents. There are no guidelines approved by the National Committee for Clinical Laboratory Standards (NCCLS) for methods determining antibiotic susceptibility of enterohepatic *Helicobacter* species. Furthermore, disk

diffusion testing is simple, economical, and very often used. However, the selection of these antimicrobials was based on current recommendations of the NCCLS (M31-A2) [2002]. This list was completed with other antibiotics that are fairly frequently used for human patients with gastrointestinal disease and for poultry. Nalidixic acid was included in the tests to clarify the alleged susceptibility of *H. pullorum* against this antibiotic.

Table 4. Antibiotic sensitivity test for *Helicobacter pullorum* by disc diffusion method

Antibiotic	Sensitive	Resistant
Nalidixic acid	±	±
Erythromycin	+	
Metronidazole	+	
Ampicillin		+
Gentamycin	+	
Ceftriaxone		+
Lincomycin	+	
Sulphamethoxazole trimethoprim		+
Ciprofloxacin	+	
Tobramycin	+	

As given in Table 4, *H. pullorum* showed susceptibility to almost all tested types of antibiotics except for ampicillin and ceftriaxone, being simultaneously highly resistant to sulphamethoxazole trimethoprim. Similar relationships were reported by Ceelen *et al.* [2005]. For nalidixic acid, it is difficult to draw clear-cut conclusions about susceptibility *versus* resistance of the tested *H. pullorum* strains. Hitherto, for *Helicobacter species*, no internationally accepted criteria are available for susceptibility testing. Part of the explanation probably lies herein that the specific growth requirements and the fastidious nature of *Helicobacter* make establishing the standard determination procedures difficult

Several research groups encountered for different resistance percentages exhibited by *H. pullorum* to nalidixic acid. On [1996] and Atabay *et al.* [1998] reported 6% and 28% *in vitro* resistance, respectively, while antimicrobial susceptibility assays showed 55% resistance to this antimicrobial agent in a study of Melito *et al.* [2000].

Due to lack of information in the literature on antibacterial susceptibility concerning *H. pullorum*, the results of the present study were compared to data of NCCLS (M31-A2) [2002]. but using minimum inhibitory concentration method (MIC) Ceelen *et al.* [2005]. Furthermore, for *Helicobacter species*, no internationally accepted criteria for susceptibility testing are available. Thus far, no susceptibility studies comprising widely used antibiotics with *H. pullorum* strains have been reported.

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