Using of PCR assay for identification of Helicobacter Species in hens' eggs

Karima Galal Abdel Hameed*[1] and Wallaa Farouk Amin[2]

1. Department of Food Hygiene and Control, Faculty of Veterinary Medicine, South Valley University, Qena, Egypt, 2. Department of Food Hygiene and Control, Faculty of Veterinary Medicine, Assiut University, Assiut, Egypt
*Corresponding author e-mail: karima_galal2004@yahoo.com

Abstract

The prevalence of Helicobacter Species in hens' eggs sold in Assiut and Qena cities, Egypt was determined. A total of 300 hens' eggs were collected from poultry farms and farmers, houses from both cities in which every 5 eggs were represented as one egg pooled sample. Each of egg shell and content was subjected to procedures of isolation of Helicobacter Species followed by PCR assay for the 16S rRNA gene for identification. The obtained results revealed that *H. pylori* recorded as the highest percentage of contamination (23.33%) followed by *H. pullorum* (20%). Contamination of egg shells (18.3%) and contents (11.6%) in farmer's houses was higher than poultry farm ones, concluding that egg shell was more subjected to contamination with Helicobacter Species than egg content. All *H. pylori* and *H. pullorum* isolates were confirmed by PCR by detection of one PCR product on agarose gel at 109 bp in case of H. pylori and at 477 bp in case of *H. pullorum* that corresponding to 16S rRNA region of the gene, confirming the specificity of PCR assay for identification of both strains. Regarding the in vitro susceptibility testing, it was found that both strains were sensitive to almost antibiotic used except for ceftriaxone and Sulphamethoxazole +Trimethoprim.
Key words: Eggs, Helicobacter spp., Antimicrobial susceptibility, PCR

Introduction

More than 30 species have been described within the genus Helicobacter. Members of the genus Helicobacter are micro aerobic, spiral motile rod by flagella (Josenhans et al., 2000). Helicobacters colonize the gastrointestinal tract of humans and animals causing gastrointestinal disease (Fox, 2002). The genus divided into gastric and enterohepatic Helicobacter species (EHS). All gastric Helicobacter species have strong urease activity and manage to survive gastric acidity by expressing urease at high level (Sachs et al., 2003). *Helicobacter pylori* considered the most prevalent species of Helicobacter genus. In humans, *H. pylori* is associated with peptic ulcer, malignant lymphoma, and gastric cancer (Uemura et al., 2001). In animals, Helicobacter spp. cause ulcerative gastritis, hepatitis and can lead to tumors in chronic infections (Fox, 2002). EHS do not colonize the gastric mucosa, but do have characteristics of physiology in common with the gastric H. species. EHS, including *H. pullorum* has been linked with enteritis and hepatitis in laying hens and diarrhea, gastroenteritis, and liver disease in humans (Ceelen et al., 2005). Moreover, *H. pullorum* considered a food borne human pathogen (Ceelen et al., 2006).

Therefore, the discovery of Helicobacter species has sparked an interest in exploring the pathogenic potential of these organisms in food especially in eggs because eggs are considered as an essential food element for growth and maintenance of human health. In addition to its high nutrient contents, low caloric value and ease of digestibility. However the nutrients that make eggs a high-quality food for human are also a good growth medium for bacteria (Frazier and Westhoff, 1986). PCR has become an important tool to identify Helicobacter species as a fastidious organism by direct amplification of 16S rRNA. The prevalence of resistance to antibiotics among bacteria isolated from eggs has been emphasizing the potential to cause therapeutic problems in consumers (Adesiyun et al., 2006).

Therefore the present study was then performed to detect Helicobacter species in hen's eggs by using PCR assay and to determine the in vitro susceptibility to different antimicrobial agents.

Materials and methods

The present study was carried out during the period between May, 2008 and December, 2009 in the department of Food Hygiene and Control, Faculty of

Veterinary Medicine Assiut University.

a) Samples collection and preparation:

A total of 300 commercial hen's eggs were collected from poultry farms and farmer's houses in Assiut and Qena cities, Egypt. Every 5 eggs constitute one group, so there are 60 groups representing poultry farms (30 groups) and farmer's houses (30 groups) in both cities. Each group was placed in a sterile plastic bag and transferred to laboratory to be prepared and examined for the Helicobacter species. Egg shells were tested by a surface rinse method as described by Moats, (1980). The egg was prepared for evacuation of its content according to Speck, (1976).

b) Culture technique:

Isolation of Helicobacter species was adopted as described by Stevenson et al., (2000). Enrichment procedure was done using Helicobacter Pylori Special Peptone Broth (HPSPB) which was supplemented with antibiotics and calf serum, and incubated under a microaerophilic gas mixture using an anaerobic jar and gas generating kits (Oxoid BR56) at 37°C for 48 hours. From each enrichment culture, a loopful streaked into plates of Helicobacter Pylori Special Peptone Agar (HPSPA) and incubated at 37°C for 4 days under microaerophilic conditions. All colonies that were small, greyish-white, Gram-negative, slightly curved rod were differentiated biochemically according to Zenner (1999) as follow, urease positive, hippurate and nitrate negative were classified as *H. pylori*. Whereas those were catalase and oxidase positive, urease and hippurate negative were classified as *H. pullorum.*

c) DNA extraction and PCR amplification of Helicobacter species:

Bacterial DNA was extracted following grown up in the HPSPB, one millilitre of inoculated HPSPB was centrifuged at 8000 g for 5 minutes. The NucleoSpin® Tissue Kit (Germany) was used to obtain bacterial DNA, according to the manufacturer recommendations and stored at -20°C until use.

DNA amplification: PCR were carried out in 50 µl reaction volumes containing 10 mM Tris- HCl (pH 8.0), 15 mM MgCl2, 1 mM each of the four dNTP´s (dATP, dCTP, dGTP, dTTP), (5 U/µl) of Ampli Taq Gold® polymerase (Applied Biosystems, Germany), 5 µl template DNA and 50 PM of each of the oligonucleotide primers. The primer sequences were used is described in Table..

PCR was conducted in the programmable thermal cycler (Germany) with the following cycling parameters: an initial denaturation at 94°C for 5 min, followed by 36 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 1.5 min then elongation at 72°C for 1 min, and final elongation at 72°C for 10 min. 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 Sigma).

d) Antimicrobial susceptibility testing for both *H. pylori* and *H.pullorum:*

All *H. pylori* and *H. pullorum* (26 strains) that were positive by PCR were tested for their Invitro sensitivity and resistance patterns to 10 different antibiotics (Oxoid) by disc diffusion method as described by (Chaves et al.,1999).

Results and Discussion

Results are described in Tables. More than 50% of the world's population harbor *H. pylori* in their upper gastrointestinal tract. Moreover, a preliminary study showed that *H. pullorum* was present on 60 % of poultry carcasses pointing to *H. pullorum* as a potentially important food associated human pathogen. So the present study was carried out to investigate the prevalence of Helicobacter species in hen's eggs. Table 1 show that *H. pylori* and *H. pullorum* were the most frequently isolated species in the examined egg groups, samples.

As presented in Table 2 Farmer's houses egg shells and contents show higher contamination in both cities than poultry farm ones. It was found that egg shells were contaminated by 34.9% while egg contents were contaminated by 13.2%, concluding that egg shell was more subjected to contamination with Helicobacter Species than egg content. This indicates the hygienic measures that applied in the farm and its role in minimizing contamination.

In Assiut city it was found that *H. pylori* was isolated from all examined egg shells and only from farmer's houses egg contents. Whereas in Qena city it was isolated only from poultry farm egg shells. Concerning H. pullorum it was detected in all examined egg shells and only in farmer's houses egg contents in both cities. The prevalence of H. pullorum was almost twice in Assiut than in Qena city. Poultry farm egg oth *H.*

Table. Primer Sequence

Species	Primer sequences		References
H. pylori	Sense primer	CTG GAG AGA CTA AGC CCT CC	
	Antisense primer	ATT ACT GAC GCT GAT TGT GC	Mapstone et al., (1993)
H. pullorum	Sense primer	ATG AAT GCT AGT TGT TGT CAG	
	Antisense primer	GAT TGG CTC CAC TTC ACA	Stanley et al., (1994)

Table-1. Prevalence of Helicobacter species in the examined egg groups, samples

Egg samples	Isolated strains							
	H. pylori		*H. pullorum*		*H. cinaedi*		*H. felis*	
	No./ 60	%	No./ 60	%	No./ 60	%	No./ 60	%
Egg shell	9	15	9	15	2	3.3	1	1.6
Egg content	5	8.3	3	5	-	-	-	-
Total	14	23.3	12	20	2	3.3	1	1.6

Table-2. Incidence of Helicobacter species in the examined eggs shell and content in Assiut and Qena cities

Cities	Egg shell				Egg content			
	Poultry farm		Farmer's houses		Poultry farm		Farmer's houses	
	No./30	%	No./30	%	No./30	%	No./30	%
Assiut	6	20	9	30	-	-	6	20
Qena	4	13.3	2	6.6	1	3.3	1	3.3
Total (60)	10	16.6	11	18.3	1	1.6	7	11.6

Table-3. Prevalence of *Helicobacter pylori* and *H. pullorum* in the examined eggs shell and content in Assiut and Qena cities

Cities	species	Egg shell				Egg content				Total (60)	
		Poultry farm		Farmer's houses		Poultry farm		Farmer's houses			
		No./30	%	No./30	%	No./30	%	No./30	%	No./60	%
Assiut	H. pylori	2	6.6	7	23.3	-	-	4	13.3	13	21.6
	H. pullorum	4	13.3	2	6.6	-	-	2	6.6	8	13.3
Qena	H. pylori	1	3.3	-	-	-	-	-	-	1	1.66
	H. pullorum	2	6.6	1	3.3	-	-	1	3.3	4	6.66
	Total (60)	9	15	10	16.6	-	-	7	11.6	26	43.33

Table-4. Specificity of PCR method for *H. pylori* and *H. pullorum* isolates

Bacterial strain	No. of isolates	PCR			
		Positive		Negative	
		No.	%	No.	%
H. pylori	14	14	100	-	-
H. pullorum	12	12	100	-	-
Other H. species	3	-	-	3	100
Total	29	26	89.6	3	10.3

contents in both cities were free from contamination by both *H. pylori* and *H. pullorum* (Table 3). The obtained results revealed the degree of contamination and public health hazard in the surroundings contacting eggs until reaching the markets and consequently the consumers.

The results in Table 4 indicated the specificity of the PCR assay for identification of *H. pylori* and *H. pullorum* that previously identified by conventional methods. More attention was paid to both strains due to their higher prevalence. So all *H. pylori* and *H. pullorum* isolates were confirmed by PCR by detection of one PCR product on agarose gel that confirm the actual size of 109 bp and 477 bp for *H. pylori* and *H. pullorum* respectively, that corresponding to 16S rRNA region of the gene. The correlation coefficient between conventional and PCR assay was 0.98 and was significant at $P \leq 0.001$. It means exact agreement was found between both conventional and PCR assay for identification of both strains. Stanley et al., (1994), and Ceelen et al., (2006) obtained the same specificity of the protocol and its ability to discriminate between closely related species.

This study constitutes part of in vitro d 6 % and 28

Table-5. **Antibiotic sensitivity and resistance for *H. pylori* and *H. pullorum* isolates**

Antibiotics	Sensitive		Resistant	
	H. pylori	***H. pullorum***	***H. pylori***	***H. pullorum***
Nalidixic acid	±	±	±	±
Erythromycin	+	+		
Metronidazole		+	+	
Ampicillin	+			+
Gentamicin	+	+		
Ceftriaxone			+	+
Lincomycin	+	+		
Sulphamethoxazole & trimethoprim			+	+
Ciprofloxacin	+	+		
Tobramycin	+	+		

susceptibility testing of both *H. pylori* and *H. pullorum* against various antimicrobial agents that frequently used in human patients with gastrointestinal disease and poultry. Nalidixic acid was included to clarify the alleged susceptibility of Helicobacter species against this antibiotic. As recorded in Table 5 it is clear that both strains show susceptibility to almost all tested types of antibiotics except for Ceftriaxone and Sulphamethoxazole trimethoprim, they show great resistance. Similar findings reported by Ceelen et al. (2005). Concerning nalidixic acid, it is difficult to draw clear-cut conclusions about susceptibility versus resistance of the tested Helicobacter strains. Hitherto, for Helicobacter species, no internationally accepted criteria for susceptibility testing are available. Part of the explanation probably lies herein that the specific growth requirements and the fastidious nature of Helicobacter make it difficult for establishing standardized determination procedures. Different resistance percentages exhibited by both *H. pylori* and *H. pullorum* to nalidixic acid were encountered by several research groups. 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 among the tested strains in a study of Melito et al., (2000). Thus far, no susceptibility studies comprising widely used antibiotics with *H. pullorum* strains have been reported. Contradictory results were seen between both strains regarding sensitivity or resistance toward Ampicillin and Metronidazole. This can be due to extend of infection, the immune status of individuals and the biochemical and genetic bases of microbial resistance (Vuldhuyzen et al., 1998).

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