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Original Research

Detection and Identification of *Helicobacter pullorum* in Poultry Species in Upper Egypt

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Abstract

This work aimed to detect, identify and study the epidemiology of *Helicobacter pullorum* in avian species in Upper Egypt. A total of 1800 samples, including cloacal swabs, cecal swabs and livers were collected from chickens, turkeys and ducks in five different governorates in Upper Egypt. Using conventional phenotypic methods for isolation and identification, *Helicobacter pullorum* could be identified only from chickens with percentage 39.33% and no presence of the organism in turkeys and ducks. Sixteen randomly selected phenotypically identified *Helicobacter pullorum* isolates were confirmed using PCR assay based on 16S rRNA gene. In conclusion, conventional phenotypic methods for detection and differentiation of *Helicobacter pullorum* are often hampered by many limitations, while molecular methods, and PCR, in particular, have marked an important step forward in bacterial diagnostics and can provide a sensitive and rapid alternative method for detection and identification and highlights the potential of PCR technology in routine detection and identification of pathogens.

Keywords: Helicobacter pullorum; Detection; Identification; Molecular; Poultry

Introduction

The bacterial genus Helicobacter belongs to the family Helicobacteriaceae, order Campylobacterales, and class Epsilonproteobacteria, was created in 1989 and currently comprises 23 validly published species (On *et al*., 1996). The genus Helicobacter nowadays includes at least 26 formally named species, with additional novel species in the process of being characterized (Fox, 1997; Whary and Fox, 2006). The genus Helicobacter is generally separated into two groups; gastric Helicobacter species and enterohepatic Helicobacter species (EHS) depending on the preferred site of colonization. Enterohepatic Helicobacter species preferentially colonizes the gastrointestinal tract and, in some cases, the biliary tree of their host (Stanley *et al*., 1994).

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Helicobacter pullorum (*H. pullorum*), an EHS, was identified as a novel species of this genus in 1994 by Stanley *et al*. (1994) based on 16S-rRNA phylogenetic analysis. *Helicobacter pullorum* is a Gram negative, microaerophilic, slightly curved rod with monopolar, non-sheathed flagella. It was originally cultured from the caeca and livers of broiler and laying hens, and the faeces of humans (Stanley *et al*., 1994). *Helicobacter pullorum* prevalence in avian species is poorly documented. Although most infected birds remain subclinical (Stanley *et al*., 1994; Atabay *et al*., 1998; Ceelen *et al*., 2006a), *H. pullorum* infection has been linked to vibrionic hepatitis and enteritis in chickens (Stanley *et al*., 1994; Atabay *et al*., 1998). Several reports published in recent years indicate some strong evidence that the pathogenic potential of *H. pullorum* as a cause of enteritis in humans should not be neglected (Burnens *et al*., 1994; Steinbrueckner *et al*., 1997; Ceelen *et al*., 2005). In Italy, *H. pullorum* has been isolated from, or identified

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by PCR, in a high percentage of broiler chickens, laying hens and Guinea fowl (Nebbia *et al*., 2007). *Helicobacter pullorum* was detected at 100% prevalence in broilers and laying hens from 15 different farms (Zanoni *et al*., 2007), as well as in a majority of turkeys slaughterhouse (Zanoni *et al*., 2011). In Belgium, 110 broilers from 11 flocks were colonized with *H. pullorum* in the caecum and colon, and to a lesser degree in the liver and jejunum (Ceelen *et al*., 2006b). *Helicobacter pullorum* has also been isolated from a diarrheic psittacine bird (Ceelen *et al*., 2006a), suggesting that pet birds may be a zoonotic risk.

The true prevalence of *H. pullorum* in poultry and in human disease may be underestimated due to misidentification, since this species shares many phenotypic similarities with Campylobacter species and accurate tests to distinguish among them are lacking (Steinbrueckner *et al*., 1997; Andersson *et al*., 2002). The aim of the present study was to detect, identify and study the epidemiology of *H. pullorum* organism in different avian species in Upper Egypt using phenotypic and genotypic techniques of detection and isolation.

Materials and methods

Samples tested

During the period, since March 2008 until February 2009, samples were collected from different poultry species (Chickens, turkeys and ducks). Samples were collected from different breeds (native and foreign breeds). Different ages were subjected for study, ranging from day-old chicks to fifty-two-weeks old birds. Sampling process was done from birds suffering from enteritis as well as from apparently healthy birds. Samples were obtained from poultry farms at different governorates in Upper Egypt. A total of 1800 samples were collected, including, 600 cloacal swabs (300 from chickens, 150 from turkeys and 150 from ducks), 600 cecal swabs (300 from chickens, 150 from turkeys and 150 from ducks) and 600 liver samples (300 from chickens, 150 from turkeys and 150 from ducks). Samples were collected into sterile tubes containing brain heart infusion (BHI) broth and were transferred immediately to the laboratory in an icebox, where they subjected to bacteriological examination with special reference for *Helicobacter pullorum*.

Isolation

Samples were inoculated into BHI broth containing 10% sterile inactivated horse serum and Skirrow's supplement then incubated in microaerophilic condition (5% H_2, 5% CO_2, 5% O_2, and 85% N_2) in CampyPak II anaerobic system jar with CampyPak gas generating system envelopes or in CO_2 incubator with the same gases in same proportions at 37°C-42°C for 24–8 hours. Sub-culturing was carried out on BHI agar plates enriched with 5-10 % sheep blood and containing Skirrow's supplement and incubation at 37°C-42°C for 48 hours under a microaerophilic atmosphere. The growth was examined for typical *Helicobacter pullorum* colonies.

Phenotypic identification

To confirm the presence of *Helicobacter pullorum* on suspected isolates, gram stained films, motility test, biochemical reactions, including catalase production, oxidase production, urease production, nitrate reduction, hippurate hydrolysis, H_2S production on triple sugar iron (TSI) and nalidixic acid / cephalothin resistance / sensitivity tests and growth profile, including growth with 1% glycine, growth with 3.5% NaCl, growth on MacConkey's agar, growth at 25°C, growth at 37°C, growth at aerobic atmosphere and growth at anaerobic atmosphere were used.

Molecular identification by PCR of 16S-rRNA gene

Total genomic DNA was extracted from 16 randomly selected phenotypically identified *H. pullorum* isolates using QIAamp DNA mini extraction kit (Qiagen, Germany) according to the manufacturer's instructions. The DNA concentration was determined by UV spectrophotometer (Beckman DU 640, CA, USA) and adjusted to being 50 ng/µl. Three microliters (150 ng) of each template were used for the PCR. Species identification was confirmed using the *H. pullorum* species-specific 16S rRNA gene PCR assay (Stanley *et al*., 1994). In brief, the primer sequences were: 5-ATG AAT GCT AGT TGT TGT CAG-3 (forward) and 5-GAT TGG CTC CAC CAC TTC ACA-3 (reverse) (Bioneer incorporation Daejaon 306-220, Korea). The parameters for all reactions were described in the following profile; initial denaturation at 94°C for 3 minutes followed by 35 cycles of denaturation at

94°C for 45 seconds, annealing at 56°C for 30 seconds and extension for 1.5 minutes at 72°C. The final extension took 10 minutes at 72°C. The PCR product (448bp) was seen by electrophoresis in a 1.5% agarose gel stained with ethidium bromide for visualization performed in a horizontal gel chamber plate. The running buffer was 0.5X TBE (Tris borate EDTA (pH 8.3). The 1 kb plus DNA ladder was used as a reference standard molecular weight marker.

Results

Incidence of Helicobacter pullorum

As shown in Table 1 and Fig. 1, *H. pullorum* was isolated only from chicken, while no isolation was revealed from turkeys and ducks. The highest incidence of isolation was from cecal swabs followed by liver samples then cloacal swabs as 64% (192 out of 300), 47% (141 out of 300) and 7% (21 out of 300), respectively. Generally, it was observed that there was higher incidence rate obtained from samples collected from birds suffer from enteritis (44.4%) than that collected from apparently healthy birds (36.6%). It was observed that the rate of incidence of *H. pullorum* was higher in native breeds (44.1%) than foreign breeds (36.1%).

Phenotypic identification of Helicobacter pullorum isolates

Helicobacter pullorum suspected colonies were observed among 354 isolates of 900 samples (39.33%). *Helicobacter pullorum* suspected growth on BHI broth supplemented with horse serum, and Skirrow's supplement appeared in the form of turbidity (turbid broth). *Helicobacter pullorum* sus-

Table 1. Incidence of Helicobacter pullorum from chickens, turkey and ducks in Governorates of Upper Egypt

Bird species	age	breed	Clinical signs*	Cloacal swabs			Cecal swabs			livers			Total isolates	%
				No	isolates	%	No	isolate	%	No	isolate	%		
Chickens (300)	Young	Native	H	.25	1	4	.25	20	80	.25	11	44	354	39.33
			D	15	2	13	15	10	75	15	9	60		
		Foreign	H	40	2	5	40	22	55	40	17	42		
			D	20	2	10	20	12	60	20	10	50		
	Semi-mature	Native	H	25	2	8	25	18	72	25	10	40		
			D	15	1	7	15	12	80	15	10	75		
		Foreign	H	40	2	5	40	20	50	40	18	45		
			D	20	2	10	20	14	70	20	9	45		
	Adult	Native	H	25	1	4	25	19	76	25	11	44		
			D	15	2	13	15	11	73	15	9	60		
		Foreign	H	40	2	5	40	21	52	40	17	42		
			D	20	2	10	20	13	65	20	10	50		
Turkeys (150)	Young	Native	H	40	0	0	40	0	0	40	0	0	0	0
			D	10	0	0	10	0	0	10	0	0		
	Semi-mature	Native	H	40	0	0	40	0	0	40	0	0		
			D	10	0	0	10	0	0	10	0	0		
	Adult	Native	H	40	0	0	40	0	0	40	0	0		
			D	10	0	0	10	0	0	10	0	0		
Ducks (150)	Young	Native	H	40	0	0	40	0	0	40	0	0	0	0
			D	10	0	0	10	0	0	10	0	0		
	Semi-mature	Native	H	40	0	0	40	0	0	40	0	0		
			D	10	0	0	10	0	0	10	0	0		
	Adult	Native	H	40	0	0	40	0	0	40	0	0		
			D	10	0	0	10	0	0	10	0	0		

*H =apparently healthy birds, D= Diseased birds with enteritis

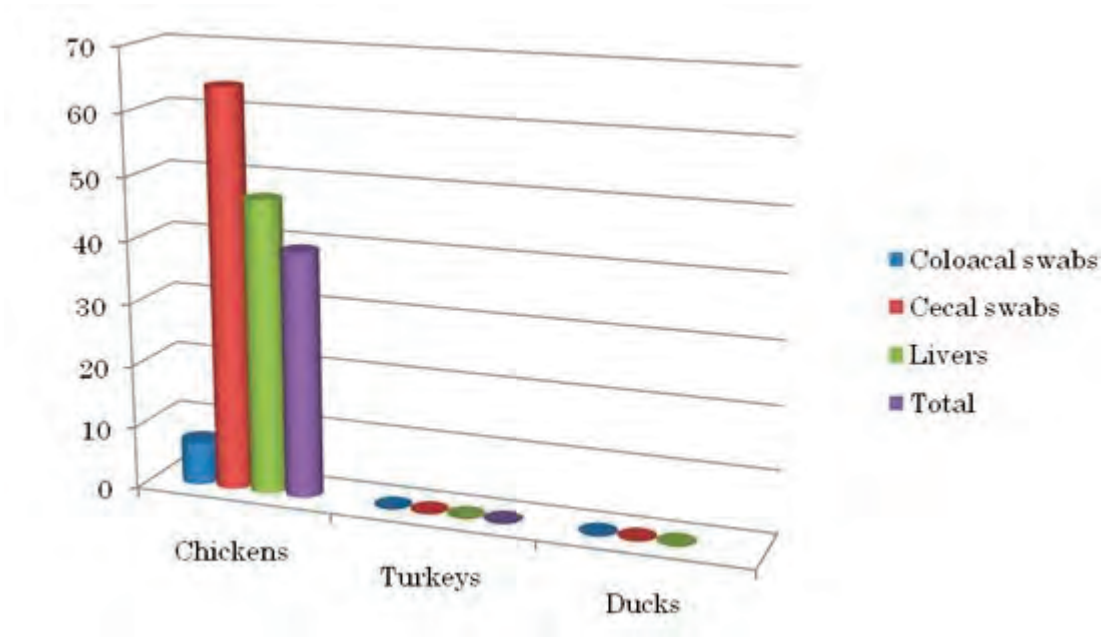

Fig. 1. Incidence percentage of H. pullorum in chickens, turkeys and ducks in Governorates of Upper Egypt

pected colonies on BHI blood agar shown as pinpoint to one mm in diameter, grayish white, non pigmented, smooth, translucent and alpha-hemolytic at 48-72 hours. *Helicobacter pullorum* suspected colonies were observed and subjected to gram staining and revealed that cells were gram negative slightly curved short rods. *Helicobacter pullorum* suspected strains were phenotypically identified for confirmation based on their reaction and growth profile. Results were illustrated in Table 2.

Genotypic identification of the isolated Helicobacter pullorum isolates

In this study, only 16 randomly selected isolates of *Helicobacter pullorum*, which were identified previously based on phenotypic reactions, were investigated for the identification of 16S-rRNA gene using polymerase chain reaction (PCR). Results elucidated positive amplification of 16S rRNA gene 448 bp (Fig. 2) from all the tested isolates.

Discussion

Helicobacter pullorum could be isolated from the feces of asymptomatic chickens and hens with hepatitis (Burnens *et al*., 1994; Steinbrueckner *et al*., 1997). Several reports published in recent years about the incidence and prevalence of *H. pullorum* from cecal samples; Burnens *et al*. 1996 found an incidence rate of 4% upon sampling the caecal contents of 150 healthy broiler chickens and isolated *H. pullorum* from 9 out of 18 caeca of laying hens affected by vibrionic hepatitis. Atabay and Corry, (1997) isolated Campylobacter-like organisms, successfully identified as *H. pullorum*, from 9 out of 15 frozen caeca (60%), and from 9 out of 15 fresh carcasses (60%) collected from two different farms. Zanoni *et al*. (2007) found a high prevalence of *H. pullorum* in cecal samples of poultry: 100% of the laying hen and broiler chicken farms as well as 100% of the 60 birds examined were positive. Regarding to isolation rate of *H. pullorum* in liver samples, Ceelen *et al*. (2006a) carried out a study on the gastrointestinal tract and liver tissue of 110 broiler chickens coming from 11 different flocks. Using PCR for *H. pullorum*, they found samples belonging to 7 out of the 11 flocks tested to be positive for *H. pullorum*. In particular, using the caecal tissue, they found 37 out of 110 birds to be positive. The present study introduces some of the epidemiological aspects of *H. pullorum*. Our results revealed that *H. pullorum* was isolated only from chicken (39.33%), while no isolation was revealed from turkeys and ducks. The highest incidence of isolation was from cecal swabs (64%) followed by liver samples (47%) then cloacal swabs (7%). Birds suffering from enteritis showed a higher incidence rate of *H. pullorum* incidence than those apparently healthy birds, 44.4% and 36.6% respectively. It was observed that the rate of incidence of *H. pullorum* was higher in native breeds (44.1%) than foreign breeds (36.1%).

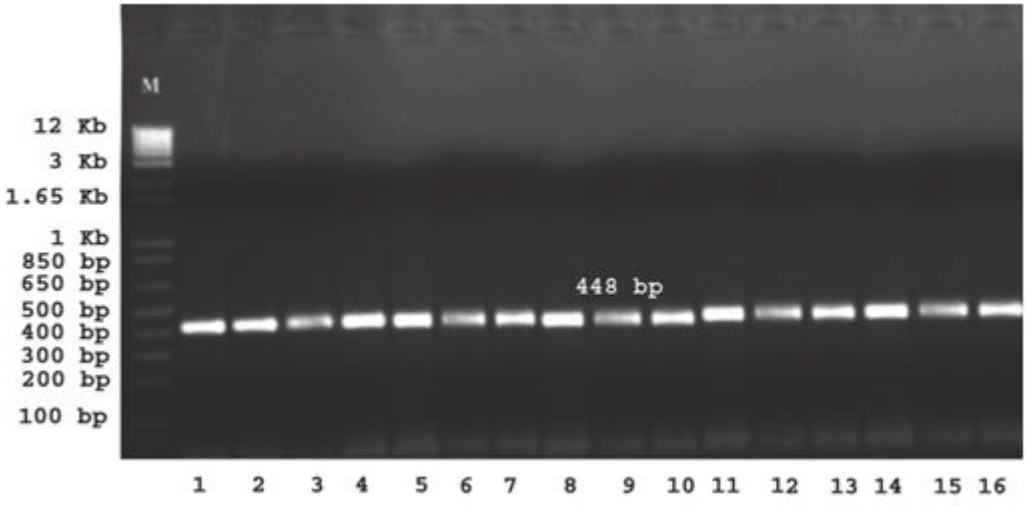

Fig. 2. Result of PCR of H. pullorum isolates: PCR positive reaction from Helicobacter pullorum isolates to 16S-rRNA gene. Lane M: 1 Kb plus DNA ladder.

Table 2. Biochemical reactions and growth profile of suspected H. pullorum isolates

Test/characteristic	Result	%
Catalase production	328/354	92.6
Oxidase production	354/354	100
Urease production	0/354	0
Nitrate reduction	354/354	100
Hippurate hydrolysis	0/354	0
H_2S on TSI	308/354	87
Resistance to naladixic acid	25/354	7
Resistance to cephalothin	354/354	100
Growth with 1% glycin	0/354	0
Growth with NaCl	0/354	0
Growth on MacConkey agar	0/354	0
Growth at 25°C	0/354	0
Growth at 37°C	354/354	100
Growth at 42°C	354/354	100
Growth at aerobic atmosphere	0/354	0
Growth at microaerophilic atmosphere	354/354	100
Growth at anaerobic atmosphere	354/354	100

Concerning the incidence of *H. pullorum* in turkey and ducks, we could not isolate the organism, although Zanoni *et al*. (2011) could isolate it from turkey; this is might be due to different isolation methods used. The higher incidence rate of *H. pullorum* in caecal samples of chicken than cloacal swabs and livers could be attributed to the normal presence of the micro-organism in the gastrointestinal tract.

Helicobacter pullorum is fastidious in growth and require temperature ranges from 37°C to 42°C and microaerophilic atmosphere typically containing $CO_2/N_2/O_2/H_2$ (5 : 88 : 5: 2), the latter enhances, and is sometimes essential for helicobacterial growth and requires incubation period 2-3 days. In the current study, we obtained re-

sults similar to those reported by other investigators (Goodwin *et al*., 1989; Taneera *et al*., 2002; Pilon *et al*., 2005; Ceelen *et al*., 2006a; Zanoni *et al*., 2007).

Helicobacter pullorum is gram negative, slightly curved short rods and motile. The isolated strains gave pinpoint to one mm in diameter, grayish white, nonpigmented, smooth, translucent and alpha-hemolytic colonies, the same were reported by Ceelen *et al*. (2006a) and Zanoni *et al*. (2007).

Helicobacter organisms bear a close resemblance to certain Campylobacter species (notably *Campylobacter lari*), with which it also shows similarities in host range and disease associations (Goodwin *et al*., 1989). In the present study, some biochemical reactions and growth profile were used to distinguish between *H. pullorum* and Campylobacter species (*C. jejuni*, *C. coli* and *C. lari*); they were H2S production on TSI [*H. pullorum* (+ve), *C. jejuni* and *C. lari* (-ve) and C. coli (variable)], growth on MacConkey [*H. pullorum* (-ve), Campylobacter species (+ve)], growth with 1% glycine [*H. pullorum* (-ve), Campylobacter species (+ve)] and resistance to nalidixic acid and cephalothin [*H. pullorum* (resistant, sensitive), *C. jejuni, C. coli* (sensitive, resistant) and *C. lari* (resistant, resistant).

It is worth mentioning that, the use of conventional bacteriological tests for identification and differentiation of *Helicobacter pullorum* are often hampered by many limitations. In addition, these bacteria are fastidious, asaccharolytic and possess few distinguishing biochemical characteristics. Thus, there is a great need for simple methods for detection and reliable differentiation of *Helicobacter pullorum* especially from Campylobacter species. Due to aforementioned limitations, molecular methods, and PCR, in particular, have marked an important step forward in bacterial diagnostics. In species identification gene sequences of 16S-rRNA was used frequently by many authors (Stanley *et al*., 1994; Pilon *et al*., 2005; Ceelen *et al*., 2006a; Zanoni *et al*., 2007). We used a definitive, reliable and easy molecular method for identification of *Helicobacter pullorum*, which is based upon PCR of the 16S-rRNA. We used this method due to its broad ability to detect and identify members of ε-group of Protobacterieae (genera Campylobacter, Helicobacter and Arcobacter), which also colonize bird's intestinal tract. In this study only 16 randomly selected isolates of *Helicobacter pullorum*, which were identified previously based on phenotypic reactions, were investigated for identification of 16S-rRNA gene using PCR. Results elucidated positive amplification of 16S-rRNA gene 448 bp from all the tested isolates, the matter which means that all tested isolates belonged to *Helicobacter pullorum*.

Conclusion

Conventional phenotypic methods for detection and differentiation of *Helicobacter pullorum* are often hampered by many limitations, while molecular methods and PCR in particular, have marked an important step forward in bacterial diagnostics and can provide a sensitive and rapid alternative method for detection and identification and highlights the potential of PCR technology in routine detection and identification of pathogens.

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