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Characterization of *Helicobacter Pylori* and *Escherichia Coli* From Stool of Human and Pet Animals

Faten A. Elkassas[1], Samy A. Khaliel[1], Mohammed Shawky[1]
[1]Animal Health Research Institute, Damanhour branch
[2]Department of Microbiology, Faculty of Veterinary Medicine, Alexandria University

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***Correspondence to:**
norawsoso@yahoo.com

ABSTRACT

The recent isolation of *Helicobacter pylori* from cats and human has potentially important public health implications as well as other bacteria associated with *H.pylori* in stool samples such as *E.coli*. A total of 202 stool samples from human, cats and dogs were examined for detection of *H.pylori* and *E.coli*. For isolation of *H.pylori*, 150 stool samples were cultivated on Dent's medium (modified skirrow's medium) containing selective Dent's antibiotic supplement. while the remaining 52 samples were cultivated on MacConkey, Eosine methylen blue media in attempt to isolate other associated bacteria. Colonies grossly similar to *H.pylori* were tested for catalase, oxidase and urease test. All human samples were tested serologically by using rapid stool antigen test (SAT). PCR assay was performed on bacterial isolates and directly from stool samples by extraction of DNA from stool by boiling method. Positive cultures were subjected to serology testing and PCR. 45samples from humans gave positive results with rapid *H.pylori* stool antigen test (HPSA). Results revealed that *H.pylori* was detected in tested samples from human and cat at a rate of 52.8%, 33.3%, respectively and could not be detected in dog. Also detected serologically at percentage of 84.9% in human stool samples. *H.pylori* could not be detected by PCR, while the other 52 samples were found positive for *Escherichia coli*, isolation at a percentage of 48%. Nine serogroups of *E.coli* (O1, O20, O55, O86, O114, O125, and O146) were detected in 9 samples (3 samples from each species). All serotypes were positive for Iss and Fim genes. From all stool samples subjected for isolation of *E.coli* we obtained 15 isolates which were introduced to PCR. 14 isolates were positive for *E.coli* genus (phoA gene). 9 isolates were used for detection of virulence genes (Iss, Fim, and Tsh genes). 8 isolates were positive for (Iss and Fim) gene. None was positive for (Tsh) gene. We conclude that *H.pylori* is very difficult to culture due to the fastidious nature of the organism and its transport and culture requirements. Also *H.pylori* is the main species of *Helicobacter* present in human. While dogs and cats harbour many gastric *Helicobacter* like organisms (GHLOs) and *E.coli* isolated from human, dogs and cats share the same characters and virulence genes.

1. INTRODUCTION:

Helicobacter spp. is gram-negative, microaerophilic, motile, and curved or spiral bacteria with multiple terminal flagella. They contain large quantities of the enzyme urease, which results in production of ammonia and bicarbonate from urea (Neiger and Simpson (2000).

More than 30 *Helicobacter spp*. have been identified in humans and animals. In addition to the species found in the stomach, others have been identified in the intestine and liver (Fox *et al.* (2001). *H. pylori* are the most common gastric species in humans. It has been shown to be a major cause of gastritis and peptic ulcers as well as to increase the risk of gastric cancer (Uemura *et al*., 2001).

Helicobacter bacteria colonize the gastro intestinal mucosa of humans, domestic animals (such as dog, cat, ferret and poultry) and wild animals (such as the cheetah or monkey) (Abdi *et al*., 2014, and Hong *et al*., 2015). In 1994, the international agency for Research on Cancer (IARC) qualified this bacterium as a class 1 risk for the development of gastric cancer and gastric mucosa associated lymphoid tissue (MALT) lymphoma (Sjunnesson *et al*., 2003, Falsafi *et al*., 2009 and Smith *et al*., 2012). Although *H. pylori* have been identified in research colony cats, (Perkins *et al*., 1996) infection of pet dogs and cats with other species occurs most commonly.

Most *Helicobacter spp*. commonly found in the stomachs of dogs and cats are larger than *H. pylori* (1.5 to 3 μm) (Neiger and Simpson (2000). Large spiral bacteria (4 to 10 μm) identified in the stomachs of dogs were initially called *Gastrospirillum hominis*. They were later

reclassified as *Helicobacter heilmannii* (Lecoindre *et al*., 2000).

The mode of transmission still unknown, although epidemiologic studies suggest close person to person contact and intra familial spread. There is still uncertainly whether transmission occurs primarily through faecal-oral or gastric-oral route (Covacci *et al*., 1999).

Various tests have been developed to diagnose the infection (Glupczynski 1998). *H.pylori* can be detected by non- invasive and invasive methods, the latter requiring endoscopy. *Helicobacter pylori* can be detected in stool specimens either by culture (difficult due to diverse microorganisms in the stool and fastidious nature of *H. pylori*), *H.pylori* Stool Antigen test (HpSA) (discrepancies also occur from one geographical area to the other) and stool-PCR (with success rates of 25%–100%). Generally, the differences in detection rate of *H. pylori* in stool is due to *H. pylori* degradation in the gastrointestinal tract and/or presence of inhibitors such as complex polysaccharides and also its presence in low concentration in stools (Kabir 2001). Many PCR methods have been developed to detect the organism directly in different clinical specimens. Various authors have reported on the use of stool-PCR for diagnosis of *H. pylori* (Şen *et al*., 2005; Hirai *et al*., 2009; Aktepe *et al*., 2011). The sensitivity with this method in recent times has varied from 21%–65.22% (Aktepe *et a*l., 2011 and Şen *et al*., 2005).

Escherichia coli are a normal inhabitant of the intestinal tract of humans and warm blooded animals. Although usually harmless, various *E. coli* strains have acquired genetic determinants (virulence genes) rendering them pathogenic for both humans and animals. Certain strains of *E. coli* behave as pathogens in dogs and cats, causing gastro intestinal and extra-intestinal diseases) (Beutin, 1999).

Increased serum survival (*iss*) protein, was the first linked to serum resistance and pathogenicity. Iss inhibits the deposition of the membrane attack complex (MAC)(Dziva and Stevens, (2008).The temperature-sensitive hemagglutinin (Tsh) is an autotransporter protein that adheres to red blood cells and also binds to the extracellular matrix proteins. It is primarily responsible for infections that cause agglutination of bird erythrocytes, leading to airsacculitis and colisepticemia (Huja *et al.* (2015). Type 1 fimbriae which encoded by a chromosomally located Fim gene cluster are the most common adhesive organelles of *Escherichia coli*. Fimbriae-mediated adherence, which facilitates colonization and survival in host cells, plays a significant role in pathogenesis (Sokurenko *et al*., 1997).

The aim of the present study was to isolate *H.pylori* and other associated bacteria in stool of human and pet animals as well as detection of some virulence genes of isolated bacteria.

2. MATERIALS AND METHODS:

2.1. Field samples:

A total of 202 stool samples were collected from human (53 samples), dogs (25 samples) and cats (72 samples) either healthy or suffering from intestinal disorders from El-Behera governorate during 2014-2016.

2.2. Isolation and biochemical identification of H.pylori and other associated bacteria:

For isolation of *H.pylori*, 150 collected samples were inoculated into sealed sterilized tubes containing 2-3 ml of Stuart,s transport medium then inoculated into modified skirrow's medium containing selective antibiotic supplement(Vancomycine, Trimethoprime, Cefsulodine and Amphotericin B).plates incubated at 37^{o}c for (5-7) days under microaerophilic conditions using microaerophilic gas generating kits(Campygen 2.5 L, oxoid) and examined after (3-5) days for any growth .incubation period may be extended to10 days **(**Boyanova, (2003). Stool samples were tested serologically by using rapid *H.pylori* stool antigen test (HPSA) for detection of *H.pylori* antigen in stool. The remaining 52 samples were inoculated into tryptic soya broth and incubated at 37^{o}C for18 hrs then streaked into nutrient agar, Eosine Methylene agar (EMB), and MacConkey agar and incubated at 37^{o}C for (24-48) hrs. Suspected colonies from different media were picked up and subjected to morphological and biochemical identification (Quinn *et al.,* 2011).

2.3. Serotyping of E.coli isolates:

Nine out of 14 *E.coli* isolates were sent to Animal Health Research Institute, Cairo for serotypes differentiation using available polyvalent and monovalent *E.coli* antisera.

2.4. Molecular detection and genotyping of bacterial isolates by PCR:

DNA for *H.pylori* and *E.coli* was extracted by the boiling lysis method as described by Sambrook and Russell (2001). All of the primers used in the present study are listed in Table (1). The amplification reactions were performed in a total volume of 25µl containing12.5µlof Emerald Amp GT PCR master mix (2x premix), 1µl of each primer (20pmol), 4.5µl of nuclease free water and 6µlof DNA template and incase of multiplex PCR was performed in a total volume of 40µl containing 20µl of Emerald Amp GT PCR master mix (2x premix), 2 µl of each primer (20pmol), 4µl of nuclease free water and 12µl of DNA template .The mixture of different PCR reactions was subjected to 35cycles

of amplification (table 2). The amplified products were electrophoresed through a 1.5% agarose gel and then visualized by ethidium bromide staining.

Table (1): Oligonucleotide primers used for molecular detection and genotyping of bacterial isolates:

Isolated Bacteria	Target gene	Primer used	Primer sequence (5'-3')	Amplified product (bp)	Reference
H.pylori	*16S rRNA*	*HP1-F*	CTGGAGAGACTAAGCCCTCC	110 bp	Mapstone *et al*., 1993
		HP2-R	ATTACTGACGCTGATTGTGC		
E.coli	PhoA	*phoA-F*	CGATTCTGGAAATGGCAAAAG	720bp	Hu *et al*., 2011
		phoA-R	CGTGATCAGCGGTGACTATGAC		
	Iss	*Iss-F*	ATGTTATTTTCTGCCGCTCTG	266bp	Yaguchi *et al*., 2007
		Iss-R	CTATTGTGAGCAATATACCC		
	Fim H	*Fim-F*	TGCAGAACGGATAAGCCGTGG	508	(Tiba *et al*., 2008)
		Fim-R	GCAGTCACCTGCCCTCCGGTA		
	Tsh	*Tsh-F*	GGTGGTGCACTGGAGTGG	620	(Provence and Curtiss, 1994)
		Tsh-R	AGTCCAGCGTGATAGTGG		

Table (2): Thermo cycling conditions for different PCR reactions applied in the current study:

Gene	Intial denaturation	Denaturation	Annealing	Extension	No. of cycles	Final extension
PhoA	94˚C 5 min.	94˚C 30 sec.	58˚C 45 sec	72˚C 45 sec	35	72˚C 10 min
Iss	94˚C 5 min.	94˚C 30 sec.	54˚C 45 sec	72˚C 45 sec	35	72˚C 10 min.
Tsh	94˚C 5 min.	94˚C 1min.	55˚C 1min.	72˚C 2 min.	30	72˚C 10 min.
FimH	95˚C 2 min.	94˚C 30 sec.	58˚C 30 sec.	72˚C 1min.	33	72˚C 7 min.

Table (3): Results of detection of *H.pylori* from human and pets

Species	Total sample	No. of isolates	%
Human	**53**	**28**	**52.83**
Cat	**72**	**24**	**33.33**
Dog	**25**	**0**	**0**

3. RESULT AND DISCUSSION

This study planned to identify *H.pylori* and other associated bacteria causing intestinal disorders in human, dog and cat as well as detection of some virulence genes of isolated bacteria.

The results of detection of *H.pylori* from humans and pets revealed that *H.pylori* was detected in stool samples of human and cat at percentage of 52.8%, 33.3%, respectively (table, 3). These results are higher than those obtained by Al-Sulami *et al.* (2008) who reported that the isolation rate of *H.pylori* using Columbia blood agar medium was 44.1% from human patients with gastric ulcer. He attributed this low rate of isolation to the presence of bacterial contamination of the medium as in the present study where proteus contamination of the medium was very high, this contamination may occur during obtaining, transporting and prepairing of defibrinated sheep blood added to the medium.

Also *H.pylori* was detected in stool of cat by culture at percentage of 33.3%. This result is nearly similar to that obtained by smith *et al.*, (2012) who analysed 97 stool samples and found that 38(39.2%) were positive for *Helicobacter* species.

On the other hand *H.pylori* could not be isolated from dogs. This result is similar to result of Jankowiski *et al.*, (2016) who reported that *Helicobacter Heilmanni* was found to be the most common species (73.9%) of gastric helicobacter species in dogs while *H.felis, H.pylori* and *H.bizzozeroni* were not detected in any of the samples. Also the high incidence of *Helicobacter heilmannii* in dogs in Poland was confirmed by Kubiak (2006), who carried out a study on 20 healthy and 137 sick dogs and found *Helicobacter heilmannii* in 95% and 83.2% of the dogs, respectively.

The low incidence of these bacteria in stool was confirmed by Ekman *et al*., 2013, who did not find any gastric *Helicobacter spp.* but did find enterohepatic *Helicobacter* species in stool samples. On the other hand, Hong *et al*., 2015, who identified gastric *Helicobacter spp*. DNA in the stool and sections of the gastric mucosa, indicated that 100% of the species found at the two sites concurred.

However, that study was limited by the small sample size.

The results of detection of *H. pylori* antigen from human stool indicated that 45 out of 53 (84.9 %) of examined samples were positive for *H.pylori* antigen (table, 4). These results agree with Alborzi *et al.* (2006) who reported *H. pylori* antigen positivity of 82.0% and Jafar *et al.* (2013) who recorded an antigen positivity of 64.2%. On the other hand, lower percentages were recorded by Naous *et al.* (2007) who reported stool antigen positivity of 21% and Mishra *et al.* (2008) who reported stool antigen positivity of 42.8%.

On the other hand *H.pylori* antigen could not be detected among cat and dog stool. These results agree with Hong *et al.* (2016) who examined stool specimens from cats by SD Bioline *H. pylori* Stool Ag kit detection device and the result was negative(one red line).

Bacteriological examination of *E.coli*:

The results presented in table (6) revealed that *E.coli* was isolated from human, dog and cat at percentage of 77.3, 75, and 79.2, respectively. These results are nearly similar to that detected by **Alnahass *et al.*, (2016)** who confirmed *E.coli* biochemically in 70% of isolates.

During the current study, 9 serotypes of *E.coli* were identified as O1, O114, O146, O55, O20, O86 and O125.the serotype O125and O146 nearly similar to the serotype obtained by Abd El Tawab *et al.*, (2014)

Also nearly similar the serotype obtained by Weber *et al.*, (1990) who reported that *E. coli* belonging to some of the classical human EPEC serogroups (026, 044, 055,086, 0111, 0114, 0119, 0125, 0126, 0127, 0128, 0142 and 0158) were occasionally isolated from diarrhoeic cats and dogs.

Amplification of *E.coli* (phoA) coding gene by using PCR: Fifteen isolates of biochemically identified *E.coli* were randomly studied for detection of phoA gene using PCR technique. The specificity of the primers was confirmed by positive amplification of fragment with the extracted DNA of the bacterial isolate as shown in (figure1).Out of 15 tested isolates, 14 isolates (93.33%) were positive for the phoA gene (figure1). The PCR assay yielded amplified products of 720 bp specific for (phoA) gene.

Table (4): Results of detection of *H.pylori* antigen in stool of human and pets

Species	Total sample	No. of positive samples	%
Human	53	45	84.9
Cat	16	0	0
Dog	9	0	0

Table (6): Results of isolation of *E.coli* from human and pets stool:

Species	No. of examined samples	No. of isolates	%
Cat	24	19	79.2
Dog	16	12	75
Human	22	17	77.3

Table (7): Results of serotyping of *E. coli* isolated from humans and pets.

Isolate No.	Species	Polyvalent antisera	Monovalent antisera
1	Cat	2	O146
2		5	O20
3		2	O125
4	Dog	2	O55
5		3	O114
6		1	O1
7	Human	1	O86
8		5	O20
9		1	O1

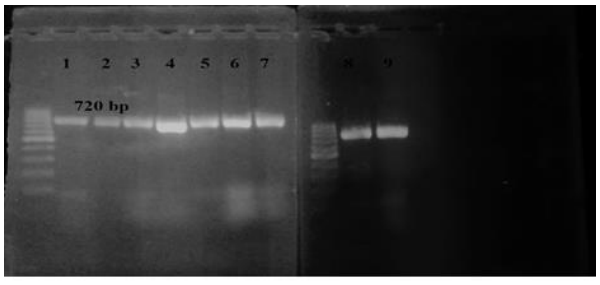

Figure (1): Agarose gel electrophoresis of the amplified phoA gene of *E.coli* isolated from humans and pets and stained with ethidium bromide**.** Lanes: 1, 2, 3, 4, 5, 6, 7, 8 and 9 were positive for phoA gene. The PCR assay yielded amplified products of 720 bp specific for (phoA) gene.

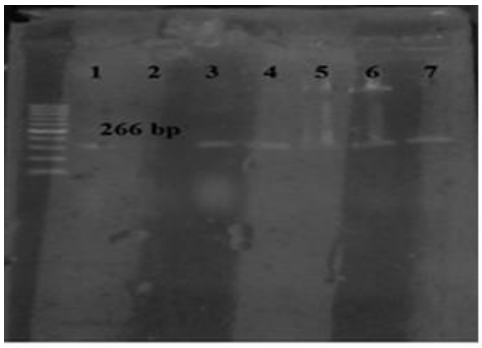

Figure (2): Agarose gel electrophoresis of the amplified Iss gene of *E.coli* isolated from humans and pets and stained with ethidium bromide**.** Lanes: 1, 3, 4, 5, 6 and 7 was positive result for Iss gene.

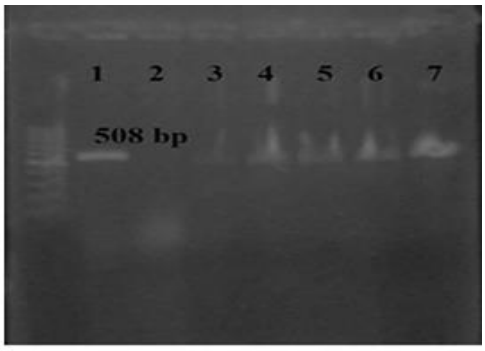

Figure (3): Agarose gel electrophoresis of the amplified Fim gene of E.coli isolated from humans and pets and stained with ethidium bromide. Lanes 1, 3, 4, 5, 6, and 7 were positive result for Fim gene. Lane (2): negative result for Fim gene.

Table (8): Results of molecular detection of virulence genes of *E.coli*

Species	No. of examined isolates	Virulence genes					
		Iss		*Fim*		*Tsh*	
		No. of positive	%	No. of positive	%	No. of positive	%
Human	**3**	**3**	**100**	**3**	**100**	**0**	**0**
Dog	**3**	**3**	**100**	**3**	**100**	**0**	**0**
Cat	**3**	**2**	**66.7**	**2**	**66.7**	**0**	**0**

The obtained results of amplification of phoA gene showed that *E.coli* was isolated at a rate of 93.33% .This result is higher than that obtained by Chen *et al.* (2003) who isolated hemolytic *E.coli* from feces of healthy cats and 21% of feces of healthy bitches. Also higher than Beutin *et al.* (1993) who reported that *E.coli* was found in fecal samples from healthy cattle (21.1%), sheep (66.6%), goats (56.1%), pigs (7.5%), cats 13.8% and dogs 4.8%.

The results of molecular detection of virulence genes of *E.coli* revealed that Iss and Fim gene was detected in 100% of *E.coli* isolates from human and dog and in 66.7% from cat isolates while Tsh gene was not detected in any isolate from all species (table, 8). In contrast to our results for Tsh gene, McPeake *et al.* (2005) demonstrated that occurrence of Tsh gene in *E. coli* isolates from healthy birds is 93.3%; But, Delicato *et al.* (2002) identified only 4% of fecal isolates positive for Tsh. Also Maurer *et al.* (1998) detected Tsh in 46% of clinical isolates and showed the absence of this gene in all commensal *E. coli*. Also this result disagrees with the result obtained by (Alnahass et al., 2016) who detected Tsh gene in 50% of isolates from intestinal samples from chicken.

The FimH genes that encode type 1 fimbriae were detected in 100% of examined isolates of dog and human, this result is similar to that indicated by Ghanbarpour *et al.*, (2011) who reported 96.4% of fecal isolates positive for FimH gene and higher than results obtained by Eftekharian *et al.*, (2016) who detected Fim H gene in almost 50% of isolates. Also this result is higher than that reported by Kaczmarek *et al.*, (2012) who reported that approximately 80 % of all *E. coli* strains of fecal origin are able to produce type 1 fimbriae, which are encoded by the chromosomal Fim operon.

On the other hand Iss gene was detected in 100% of isolates of dog and human and 66.7% of isolates of cat. This result agree with the result mentioned by (Alnahass *et al.*, 2016) who recorded that PCR amplification of Iss gene of *E.coli* was 90%.and also agree with results obtained by Cunha *et al.*, (2014) who detected Iss gene in 93% of strains.

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