Detection of cytolethal distending toxin (*cdt*) genes of *Campylobacter Jejuni* and *Coli* in fecal samples of pet birds in Iran

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Abstract:

BACKGROUND: Campylobacteriosis is one of the most common bacterial intestinal disorders of humans in many countries. A wide range of domestic and wild bird species have been identified as natural reservoirs. **OBJECTIVES:** The aim of this study was to determine the *Campylobacter* spp from pet birds and screen the determined Campylobacters for presence of virulence cytolethal distending toxin (*cdt*) genes. **METHODS:** A total of 660 fecal samples from 32 different species of pet birds were taken and examined for detection of *Campylobacter* spp. and were investigated for presence of *cdt* genes. All the samples were collected from clinically healthy birds that were kept in cage, zoological parks, and/ or in zoo of Tehran, the capital of Iran. **RESULTS:** In total, 20 thermophilic Campylobacter were detected from 8 different avian species. From 20 confirmed *Campylobacter* spp., 16 samples (80%) were *C. jejuni* positive and 4 (20%) were *C. coli* in species-specific PCR test. Furthermore, out of 20 detected Campylobacter, 13 (65%) harbored the various subunits of *cdt*A, *cdt*B and *cdt*C genes, and 7 (35%) were negative for all tested *cdt* genes. **CONCLUSIONS:** Our findings indicate that the carriage rate of Campylobacter in different species of cage and/or in zoo birds is high and confirm that *cdt* genes may frequently be present in *campylobacter* spp.

Introduction

Thermophilic Campylobacter has emerged as a leading bacterial cause of foodborne gastroenteritis in humans around the world, with most cases being linked to the handling or consumption of contaminated and undercooked poultry products (Hanninen et al., 1998; Waldenstrom et al., 2005). Campylobacters are not significant pathogens for poultry, but are important to food safety and public health, with *C. jejuni* being responsible for the majority of human campylobacteriosis, followed by *C. coli*, and rarely by *C. lari*. The *C. jejuni* and *C. coli*, adapt well to the bird's intestinal tract as they usually could have prominent colonization with causing no or mild clinical signs in avian host (Incomprehensible) (Blaser, 1997; Newell, 2001). Many animals and a number of domesticated and wild bird species are the most important reservoirs of *Campylobacter* spp. (Avrain et al., 2003). Campylobacters can survive during poultry processing, and the bacteria may spread to uncontaminated poultry products. Contact with feces of infected livestock and pet or wild birds has also been explained as a potential source of Campylobacteriosis (Waldenstrom et al., 2005; Carter et al., 2009; Mullner et al., 2009).

Because of the high rate of Campylobacter infections and the potential severity of those infections, continued research into coloniza-

tion and pathogenicity factors is necessary. A comprehensive review of the colonization factors in chickens was published recently and the list of genes contributing to the infection will probably continue to grow with the advance of genomics and functional genomics (Hermans et al., 2011). Many bacterial factors contribute to the colonization of Campylobacter in poultry, and therefore a number of putative virulence and toxin genes have been studied. The genes include flaA, CiaB, cadF and some other genes which are involved in adhesion and colonization of the host's intestine; however, the specific virulence mechanisms are not fully elucidated (Konkel, 1999; Nuijten et al., 2000; Hermans et al., 2011). Previous reports have indicated that toxicity may be an important virulence factor, with the cytolethal distending toxin (*cdt*) genes being the principal toxicity determinant (Pickett and Whitehouse, 1999; Asakura et al., 2007; Rizal et al., 2010). Nevertheless, different species of *Campylobacter* seem to share *cdt* genes. Cytolethal distending toxin gene cluster consists of three adjacent genes, including *cdt*A, *cdt*B and *cdt*C. The subunit of *cdt*B is recognized as the enzymatically active subunit, and the two heterodimeric subunit *cdt*A and *cdt*C are responsible for the holotoxin binding to cell membrane (Lara-Tejero, and Galan, 2001). *Campylobacter* species Cytotoxin causes DNA lesions, chromatin fragmentation, cytoplasm distension which arrest cell cycle in G2/M transition phase and lead to progressive cellular distension and ultimately cell death (Pickett, and Whitehouse, 1999; Lara-Tejero, and Galàn, 2002; Nesic, and Stebbins, 2005). The aim of this study was to determine *Campylobacter* spp. from pet or companion birds and screen these Campylobacter for presence of various virulence *cdt* subunit genes.

Materials and Methods

Sample collection: A total of 660 fecal or cloaca swab samples were collected from different species of the companion birds. The samples were taken from January 2013 to June 2013, from the birds that were referred to the clinic of the faculty of veterinary medicine, university of Tehran, or birds that were kept in some zoological or wild life parks in Tehran, the capital of Iran.

Campylobacter isolation: First, the swabs samples were immediately streaked on Campylobacter Charcoal Differential Agar (CCDA) plate as direct culture (Funbacmedia, 23122, Tehran, Iran). Then, these swabs were numbered and kept in normal saline serum for DNA extraction. The plate was incubated in micro-aerobic incubator with 8% of CO2) at 42°C for 24-48 hour. Colonies suspected as being *Campylobacter* species were examined for cell-morphology by optical microscope. Single colonies were picked and characterized to species level by their oxidase and catalase reactions and their ability to hydrolyze hippurate and indoxyl acetate according to standard procedures (On and Holmes, 1992). Isolates were stored with glycerol 15% at -70°C until required for further investigations (Bang et al., 2001).

DNA extraction: A- DNA was extracted from isolates, and a loop full of Campylobacter colonies from the CCDA was transferred and suspended in 300 µL of ultrapure water then heated at 100 °C for 10 min and centrifuged for 10 min at 12000 rpm. The supernatant fluid was used for DNA extraction and frozen at –20 °C until needed further use.

B- DNA was also extracted from swabs samples that Campylobacters could not be isolated by classic culture-based procedures. Therefore, normal saline of each tube (pooled swabs) was centrifuged. Sediment pellet was placed into microcentrifuge tube for DNA extraction by using the DNeasy Tissue kits (Qiagen GmbH, Hiden, Germany), according to the manufacturer's instructions.

Identification of Campylobacter species using multiplex -PCR: For species identifi-

cation, PCR was performed initially with the universal Campylobacter 16s rRNA primer (Linton et al., 1996). All PCR positive samples were then subjected to a second PCR for differentiation of *C. jejuni* from *C. coli* (Table 1). For CampF/CampR primer (Sinaclon, Tehran, Iran), the reaction was performed in a 25 µl solution, containing 5.0 ng DNA, 0.1 µM each of CampF and CampR primers, 2 units of Taq DNA polymerase (Sinaclon, Tehran, Iran), 0.2 mM of each dNTP, 10 mM Tris-HCl and 2.5 mM MgCl2. The amplification reactions were carried out using a Perkin Elmer 9600 thermo cycler with the following program: one cycle of 10 min at 95°C, 35 cycles of 30 s at 95°C, 1.5 min at 59°C, 1 min at 72°C and a final extension step of 10 min at 72°C. For Campylobacter species used 0.2 µM of MDmapA1/MDmapA2 and COL3/ MDCOL2 primers. The amplification reactions were carried out using a Perkin Elmer 9600 thermo cycler with the same program described above. For the visualization of PCR products, 10 µl aliquots were subjected to electrophoresis in 1.5% Agarose gel, stained with ethidium bromide for 1.5 hour at 100 V and viewed under UV light (Fig. 1).

Detection of *cdt* genes from Campylobacter isolates by multiplex PCR: The primers and multiplex PCR were used to detect the *cdt* genes of *C. jejuni* and *C. coli* are summarized in table 2 (Asakura et al., 2008; Findik et al., 2011). The PCR protocol of Findik et al. (2011) was modified as described below. Our result proves that the concentration of the primer sets (Sinaclon, Tehran, Iran) in the multiplex PCR reactions was appropriate, 40 µl of PCR reaction mix contained 0.2 mM dNTP mix and 1.0 U of Ex Taq DNA polymerase. PCR products were analyzed by 1.5% agarose gel electrophoresis. Bands were visualized and images were captured.

Results

In this study, Campylobacter was isolated from 13 out of 660 individual swab samples collected from 32 different avian species. All isolates were oxidase and catalase positive; this finding indicates that they were thermophilic campylobacters. Based on microbiological and biochemical investigations, e.g. hippurate hydrolysis studies on these 13 isolates, 10 isolates were *C. jejuni* and 3 isolates were *C. coli*. All these findings were confirmed by PCR assay (Fig. 1). From swab samples that campylobacter had not been isolated by culture-based procedures, 7 campylobacter spp. were identified by PCR test and 6 of them were *C. jejuni* and 1 was *C. coli*.

In total, from 20 confirmed *Campylobacter* spp., identified by PCR test, 16 samples (80 %) were *C. jejuni* positive and 4 (20 %) were *C. coli* positive. (Table 3).

PCR method was used not only for confirmation of genus and species but also for detecting *cdt* genes using three different primer sets in two different combinations (Table 2, Figs. 2 and 3). In this study, from the 16 detected *C. jejuni*, 5 (31%) had *cdt* ABC, 2 (13%) had *cdt* AB, 4 (25%) had *cdt* BC genes, and 5 (31%) had non *cdt* present. Furthermore, out of 4 *Campylobacter coli* detected, 1 had *cdt* ABC genes, 1 had *cdt* AB, and 2 had no *cdt* genes (Table 3).

Discussion

Campylobacter spp. is a zoonotic bacteria and is recognized as important enteric pathogens in humans. A wide range of domestic and wild animal species, including birds, have been identified as natural reservoirs of Campylobacter in industrialized as well as in developing countries, and they could be a source of contamination for human beings (Jeffrey et al., 2001). The role of feral and domestic birds in the transmission of organisms to poultry and especially to humans has been well documented by many authors (Jeffrey et al., 2001; Waldenstrom et al., 2002; Abulreesh et

Table 1. Primers used for identification genus and species of *Campylobacter* by polymerase chain and multiplex polymerase chain reaction.

Target	Primer Name	Sequence (5'-3')	PCR Product Size	Specificity	References
16S rRNA	CampF CampR	AGTCTTGGCAGTAATGCACCTAACG ATATGCCATTGTAGCACGTGTGTCG	408	Genus	Wangroongsarb et al, 2011
MapA	MDmapA1 MDmapA2	CTATTTTATTTTTGAGTGCTTGTG GCTTTATTTGCCATTTGTTTTATTA	589	*C. jejuni*	Stucki et al, 1995
CeuE	COL3 MDCOL2	AATTGAAAATTGCTCCAACTATG TGATTTTATTATTTGTAGCAGCG	462	*C. coli*	Gonzalez et al, 1997

Table 2. Characteristics of PCR primers used for *cdt* genes in the study. (*= *C.jejuni* ; **= *C.coli)*.

Primer	Sequence (5'–3')	Target	PCR condition			Amplicon (bp)
			Denaturing	Annealing	Extension	
*Cj-*Cdt*AU2	AGGACTTGAACCTACTTTTC	Cj-*cdt*A	94 ˚C, 30s	55 ˚C, 30s	72 ˚C, 30s	631
Cj-*Cdt*AR2	AGGTGGAGTAGTTAAAAACC					
Cj-*Cdt*BU5	ATCTTTTAACCTTGCTTTTGC	Cj-*cdt*B	94 ˚C, 30s	56 ˚C, 30s	72 ˚C, 30s	714
Cj-*Cdt*BR6	GCAAGCATTAAAATCGCAGC					
Cj-CDTC-F	ACTCCTACTGGAGATTTGAAAG	Cj-*cdt*C	94 ˚C, 60s	57 ˚C, 60s	72 ˚C, 60s	339
Cj-CDTC-R	CACAGCTGAAGTTGTTGTTGTTGGC					
**Cc-*Cdt*AU1	ATTGCCAAGGCTAAAATCTC	Cc-*cdt*A	94 ˚C, 30s	55 ˚C, 30s	72 ˚C, 30s	329
Cc-*Cdt*AR1	GATAAAGTCTCCAAAACTGC					
Cc-*Cdt*BU5	TTTAATGTATTATTTGCCGC	Cc-B	94 ˚C, 30s	56 ˚C, 30s	72 ˚C, 30s	413
Cc-*Cdt*BR5	TCATTGCCTATGCGTATG					
Cc-*Cdt*CU1	TAGGGATATGCACGCAAAAG	Cc-*cdt*C	94 ˚C, 30s	55 ˚C, 30s	72 ˚C, 30s	313
Cc-*Cdt*CR1	GCTTAATACAGTTACGATAG					

Table 3. Molecular confirmation and the occurrence of *cdt* gene subunits in 20 *Campylobacter* species detected in different birds.

	Campylobacter	Occurrence of *cdt* gene types in thermophilic Campylobacter															
Birds	Number (%)	*C. jejuni*								*C. coli*							
		ABC	AB	AC	BC	A	B	C	non	ABC	AB	AC	BC	A	B	C	non
Mynah	6 (30%)	4	1	-	-	-	-	-	1	-	-	-	-	-	-	-	-
Pigeon	2 (10%)	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1
Duck	3 (15%)	-	-	-	2	-	-	-	1	-	-	-	-	-	-	-	-
Goose	3 (15%)	-	-	-	2	-	-	-	-	-	-	-	-	-	-	-	1
Swan	1 (5%)	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Canary	1 (5%)	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-
Partridge	2 (10%)	-	-	-	-	-	-	-	-	1	1	-	-	-	-	-	-
Finch	2 (10%)	-	-	-	-	-	-	-	2	-	-	-	-	-	-	-	-
Total	20/660 (3 %)	5 (25%)	2 (10%)	0	4 (20%)	0	0	0	5 (25%)	1 (5%)	1 (5%)	0	0	0	0	0	2 (10%)

al., 2007). Many studies have been conducted in Europe, the United States, North America, and developing countries which reported Campylobacter positive poultry flocks ranging from 3% to 97% (Newell and Wagenaar, 2000; Newell and Fearnley, 2003). In Iran, Ansari-Lari et al. (2011) studied the prevalence of Campylobacter infection and showed that 76% of broiler flocks were positive for thermophilic *Campylobacter* spp. The contamination of turkey carcasses by *Campylobacter* spp. at slaughterhouses of Iran was evaluated by us-

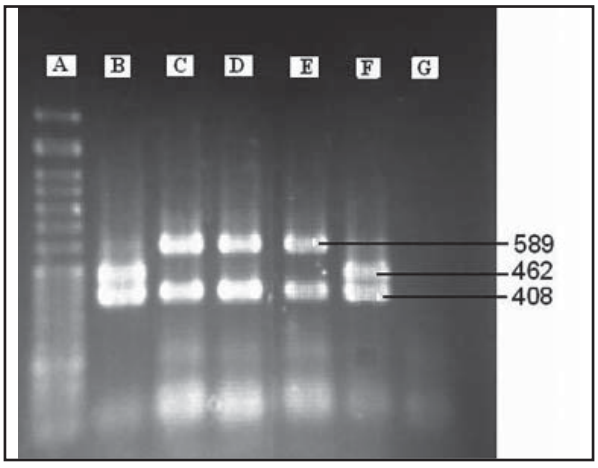

Figure 1. Multiplex polymerase chain reaction for detection of Campylobacter. A: Gene Ruler, B: Sampled *C. coli*, C & D: Sampled *C. jejuni*, E: Positive control of *C. jejuni*(589 bp), F: Positive control of *C. coli* (462 bp) and G: Negative control.

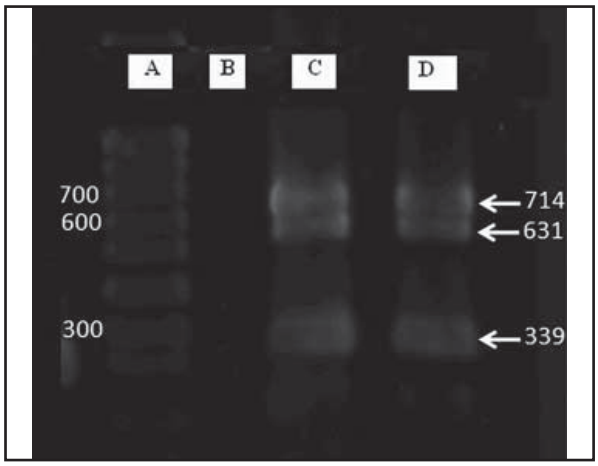

Figure 2. Multiplex polymerase chain reaction for detection of *cdt* genes of *C. jejuni*. *Cdt*A: 631 bp, *cdt*B: 714 bp, *cdt* C: 339. A: Gene Ruler, B: Negative control C: Positive control of *C. jejuni* and D: Sampled *C. jejuni*.

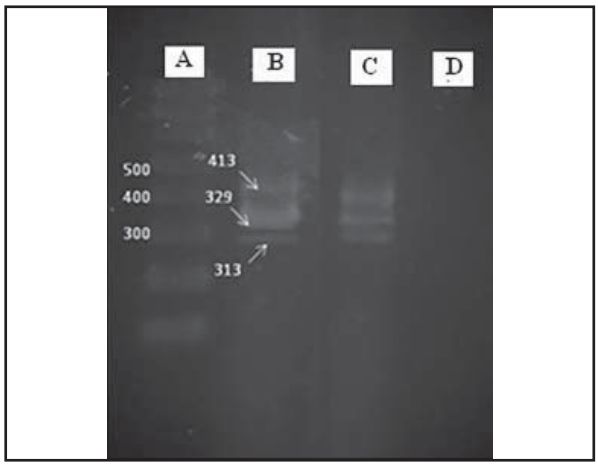

Figure 3. Amplification of *cdt* A, *cdt* B and *cdt* C genes of *C. coli*, using multiplex PCR. *Cdt*A: 329 bp, *cdt*B: 413 bp, *cdt*C: 313 bp. A: Gene Ruler, B: Positive control of *C. coli*, C: Sampled *C. coli* and D: Negative control.

ing PCR method, and the results revealed 62% infection rate in commercial turkeys (Rahimi et al., 2010). A recent study by Mirzaie et al. (2011) in Iran indicated that 55 % of samples taken from turkeys and 22 % samples taken quails flocks were positive for *Campylobacter* spp., respectively.

The percentage of positive cloacal or faecal samples of thermophilic campylobacters in diverse wild birds from a wide range of geographical locations has been reviewed by Abulreesh et al. (2007). In this report, the prevalence of *Campylobacter* spp. of waterfowl were 11% in UK, 42% in USA, 44% in Taiwan, and 5% in Sweden, respectively; while, in feral pigeons (Columba livia), the percentage was 26% in Spain, 14% in Croatia, and 4% in USA. Other reports indicate that the isolation rates of house sparrow was 33% in Chile, starling was 3%, and blackbird was 6% in Sweden; while, in France, *Campylobacter jejuni* was isolated from 106 of 200 (53%) in domestic pigeon feces samples (Megraud, 1987).

Generally, the carriage rate of Campylobacter in domestic poultry was found to be much higher than that of wild birds (Yogasundram et al., 1989; Shane, 1992). This finding is probably due to the high bird density in commercial poultry houses, which facilitates the spread of Campylobacter between birds. In our study, the rate of thermophilic Campylobacter isolation in pet or companion birds was much lower (Table 3) compared to those reports in wild birds and was more obvious when compared with the prevalence of Campylobacter in industrial poultry of Iran. The use of antibiotics for disease control, which were implemented in cage condition, may have positive effects on lowering infection rate of these birds with Campylobacter. However, this method is not recommended for the prevention strategy due to emergence of antimicrobial resistance and public health concerns for humans.

One of the main characterized Campylobacter pathogenic markers is the *cdt* gene

which may be intimately involved in successful enterocyte colonization processes and in the ability of the organism to cause inflammatory diarrhea in humans (Nuijten et al., 2000; Pasquali et al., 2011). In the present study, three *cdt* genes were followed and with regard to the PCR test, out of 20 *C. jejuni* and *C. coli* detected in the current study, 6 (30%) possessed all three toxin gene subunits. Three (15%) of the detected Campylobacter had both *cdt*A and *cdt*B markers and 4 of them showed both the *cdt*B and *cdt*C markers. However, 7 (35%), out of 20 Campylobactesrs tested, did not have any detectable *cdt* subunits. Until now, there is no information concerning the prevalence of the *cdt*A, *cdt*B, and *cdt*C genes published on Campylobacter isolates from pet birds and on the isolates of Iran either. However, we recently studied the virulence markers of *cdt* in industrial poultry (broiler chickens, turkeys, and quail). In this study, regardless of avian sample type, only 66.4% of all *C. jejuni* isolates and 43.6% *C. coli* isolates had all three types of *cdt* genes (A, B and C) and the rest of the isolates showed one or two subunits of *cdt* genes. The percentage of toxin-positive Campylobacter examined by other authors was different (Lara-Tejero and Galàn, 2001,2002; Lutful Kabir et al., 2011). These differences illustrates that the variations of subunits *cdt* genes have a wide range and depend on the samples from birds and also the geographical locations. The complete role of *cdt* in campylobacteriosis is unknown, nevertheless, Lee et al. (2003) reported that *cdt*A and *cdt*C subunits have roles in binding to the host cell, and the *cdt*B subunit has a nuclease activity. Abuoun et al. (2005) reported that the levels of toxin expressed might be strain dependent, since some strains have no detectable *cdt* activity. Additionally, these authors explained that *cdt*-negative strains were also obtained from patients having symptoms of campylobacteriosis (Abuoun et al., 2005). Based on this information, it could be suggested that other pathogenicity genes e.g. flaA, cadF or factors such as invasion might also be important for campylobacter pathogenesis and survival within epithelial cells (Hickey et al., 2005).

This is the first molecular investigation on campylobacter spp. in pet birds, especially with the presence of *cdt* genes in *Campylobacter* spp. The results obtained here reveal that many companion birds carry campylobacter that could be recognized as reservoir and potential for human pathogens; therefore, handling these birds could be a risk for public health in society. Additionally, our results confirm that *cdt* genes may be frequently present in *Campylobacter* spp.; however, the role of those in campylobacteriosis is still unclear. Further study is needed to improve the understanding of the role of CDT in the pathogenesis of campylobacteriosis

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شناسایی ژن‌های حدت *cdt* کمپیلوباکترهای ژژونی و کلی از نمونه‌های مدفوعی پرندگان خانگی (زینتی) ایران

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چکیده

زمینه مطالعه: کمپیلوباکترها در بسیاری از موارد سبب آنتریت انسانی می‌شوند. تعداد زیادی از پرندگان اهلی و وحشی مخزن کمپیلوباکترها بوده و دارای ژن‌های حدت *cdt* می‌باشند. **هدف:** در این مطالعه ابتدا کمپیلوباکترهای ژژونی و کلی در پرندگان خانگی شناسایی و سپس حضور ژن‌های *cdt* در آنها مورد مطالعه قرار گرفت. **روش کار:** تعداد ۶۶۰ نمونه سواب مدفوعی، از ۳۲ گونه مختلف پرنده خانگی یا زینتی که به کلینیک دانشکده دامپزشکی مراجعه یا در قفس‌های پارک‌ها و باغ وحش تهران نگهداری می‌شدند تهیه گردید. با استفاده از روش باکتریولوژیک و PCR ابتدا کمپیلوباکترها شناسایی و سپس ژن‌های *cdt* آنها مطالعه گردید. ضمناً محصولات PCR برای سکانس ژن ارسال گردید. **نتایج:** تعداد ۲۰ نمونه کمپیلوباکتر شامل ۱۶ نمونه ژژونی (۸۰٪) و ۴ نمونه کلی (۲۰٪) از ۸ گونه مختلف پرنده خانگی جدا و یا با روش PCR شناسایی و تأیید گردید. در ۲۰ نمونه مثبت، تعداد ۱۳ نمونه (۶۵٪) حداقل دارای یک یا چند ژن حدت *cdt* بودند و ۷ نمونه از آنها هیچ یک از ژن‌های *cdt* را در مطالعه مولکولی نشان ندادند. نتایج اولیه سکانس ژن‌های حدت موید این بود که قرابت بالایی بین ژن‌های حدت این باکتری‌ها با ژن‌های حاصل از جدایه‌های انسانی و طیور صنعتی وجود دارد. **نتیجه‌گیری نهایی:** مطالعه حاضر نشان داد که پرندگان خانگی آلوده به کمپیلوباکتر بوده و حاوی ژن‌های حدت هستند و می‌توانند بعنوان مخزن باکتری محسوب و سبب آلودگی سایر پرندگان و انسان‌ها بشوند.

واژه‌های کلیدی: کمپیلوباکتر گرمادوست، ژن حدت، پرندگان زینتی

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