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Isolation and Characterization of *Campylobacter* from Red-Crowned Cranes in China

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Abstract: In this study, 120 cloacal samples were collected from healthy red-crowned cranes in Yancheng National Rare Bird Reservation District of China and identified by API campy test and multiplex PCR. The results shown that 21.67% (26/120) of these samples were detected to be positive in *Campylobacter* infection, of which 15 isolates were identified as *C. jejuni*. Using the transmission electron microscope, we observed the morphologic change of the Bacterial particles with negative staining. *C. jejuni* showing rod-shape and unipolar sheathed flagella. We found that *Campylobacter* infection correlated well with age of birds, which was proved by the findings that significant higher isolation rate was obtained in young red-crowned cranes, compared to adult groups. Meanwhile, antibiotics susceptibility test indicated a middle-level of drug resistance was found in these strains. The presence of flaA (100%), cdtB (100%), racR (100%), dnaJ (96.2%), cdtC (96.2%), cadF (95%), pldA (93%), cdtA (93%), ciaB (90%), ceuE (85%), wlaN (40%) and virB11 (0%). Virulence genes was detected in *C. jejuni* by PCR. The data described here would offer a helpful information for the prevention of *Campylobacter* infection of wild birds and humans.

Key words: Red-crowned cranes, *Campylobacter*, antibiotics, virulence-associated genes

INTRODUCTION

Campylobacter sp. is a gram-negative, thermophilic, obligate microaerophilic bacterium (Humphrey, 2007; Han *et al*., 2007). It is an important epidemic pathogen causing many kinds of diseases including gastroenteritis, abortion, Guillain-Barre Syndrome (GBS), arthritis and Reiter's syndrome in both animals and humans in China (Ge Wei Fang *et al*., 1982; Zhou *et al*., 1988). It localizes widely in the intestine of the wild and domestic animals. Bird is thought as a natural parasitic host for *Campylobacter*. The outbreaks of *Campylobacteriosis* in recent years have raised a major public health concern worldwide (Ronner *et al*., 2004). Two major species, *C. jejuni* and *C. coli*, both could cause diseases via direct contact with pathogen or indirect contact through the contamination of animal food products. Animals such as poultry and dairy cattle may act as asymptomatic reservoirs of *C. jejuni* (Ishihara *et al*., 2004). In addition, investigators found that pig may be a major host for *C.coli*. In recent years, infection of *Campylobacter* was reported extensively in poultry, dairy cattle, pig and experimental monkey in China. However, very few research focused on the investigation of *Campylobacter* infection in wild birds. In the present study, we reported for the first time the isolation and characterization of *Campylobacter* isolated in red-crowned cranes in China. The data presented here would provide useful information for the epidemiological study of *Campylobacter* infection in China.

MATERIALS AND METHODS

Samples collection: A total of 120 faecal samples were collected from healthy red-crowned cranes in China Yancheng National Rare Bird Reservation District and transported in Carry-Blair base within 24 h and assayed timely.

Culture procedures for isolation: Isolates of *Campylobacter* was performed by the following method (Luan *et al*., 2008). Cloacal samples from healthy red-crowned cranes were cultured 18-24 h in *Campylobacter* enrichment broth (Bolton formula, Oxoid) or Hunt enrichment broth and preston broth (HEB, Oxoid) for selective enrichment, then cultured on Modified Skirrow plate for 48 h at 42°C under micro aerobic condition (HEB, Oxoid). Single colony was picked up for further purification and large-scale preparation.

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Multiplex PCR amplification (mPCR): Genomic DNAs were extracted by standard protocol described previously (Niwa *et al*., 2001). The multiplex PCR amplification (mPCR) assay was performed according to a method previously described (Denis *et al*., 1999; Han *et al*., 2007), with slight modifications for identifying *C. jejuni* and *C. coli* used for multiplex PCR were designed to amplify 16S rRNA of *Campylobacter*, mapA of *C. jejuni* and ceuE of *C. coli*. Primers were synthesized (at TakaRa Dalian, China). Amplification reactions' were performed in a 25 μL reaction mixture containing 2 μL of 10× Buffer, 1.5 mM MgCl2, 100 uMdNTPs, 0.6 uM six primers (MD 16S1, MD 16S2, MdmapA1, MdmapA2, MDCeuE1 and MdceuE2) and 2 μL template DNA, 0.2 μL Taq polymerase (TakaRa Dalian, China). PCR products were electrophoresed on agarose gel and pictures were taken by imaging system.

Biochemical reaction: Positive samples identified by multiplex PCR were conducted to the tests of oxidase and hippurate hydrolysis. Further identification was performed by API campy system according to the instructions provided by manufactory.

Electron microscope observation with negative staining: In this negative staining is to use heavy metal salts (such as phosphotungstic acid) to spread in the samples containing the online coloring, absorbing dye, the sample after drying, the sample depression Shop a thin layer of heavy metal salts and convex, but not the local deposition of dye, which stained negative effect, resolution up to about 1.5 nm.

Antibiotic resistance test: The disk diffusion assay was performed according to the method described by the National Committee for Clinical Laboratory Standards (NCCLS) (2005). The antimicrobial susceptibility teat disks (Oxoid) used in this study were as follows: amoxicillin (10 μg), ampicillin (10 μg), penicillin G (10 μg), Cefaclor (30 μg), cefotaxime (30 μg), cephalexin (30 μg), cefoperazone (30 μg), streptomycin (10 μg), amikacin (30 μg), gentamicin (10 μg), kanamycin (30 μg), ofloxacin (5 μg), norfloxacin (10 μg), ciprofloxacin (5 μg), levofloxacin (5 μg), nalidixic acid (30 μg), erythromycin (15 μg), azithromycin (15 μg), oxytetracycline (30 μg), tetracycline (30 μg), doxycycline (30 μg) and clindamycin (15 μg). *E. coli* ATCC25922, *S. aureus* ATCC29213 and *C. jejuni* ATCC33291 were used as reference strains. After steaking, the inoculum was dried for 5-10 min and 23 antimicrobial disks were placed onto the surface of the plate. The plate was inverted and incubated at 42°C for 24-48 h under micro aerobic conditions. After incubation, the diameters of the inhibition zone were measured. The zone diameter was interpreted according to NCCLS guidelines for Enterobacteriaceae (National Committee for Clinical Laboratory Standards, 2005).

Virulence genes detected in *C. jejuni*: In this study, flaA, cadF, racR, dnaJ, virB11, ciaB, pldA cdtA, cdtB, cdtC, wlaN and ceuE were selected as pathogenic genes. All twelve sets of primers were described earlier (Kaisar *et al*., 2008). All primers were were synthesized (at TakaRa Dalian, China). All PCR assays were performed according to the procedure described previously (Kaisar *et al*., 2008), with slight modifications: the cycling was as follows: denaturation at 94°C for 40 sec, annealing at a temperature specific for the primer pairs for 1 min and extension at 72°C for 1 min.

RESULTS

Isolation and identification of *C. jejuni*: Twenty of one hundred and twenty samples collected were identified as positive by Agar Diffusion Method and biochemical reaction, the positive rate was 21.67%. Among the 26 positive samples, 15 strains were identified as *C. jejuni* by API Campy System. All 15 strains showed morphological properties and biochemical reactions typical of *Campylobacter* sp. These strains were oxidase and catalase positive and urease negative did not ferment glucose. Correlation of *Campylobacter* infection and age of bird. The positive rate of infection of *Campylobacter* in young red-crowned cranes was significantly higher than that in adult red-crowned cranes ($p<0.01$) indicating that young red-crowned cranes were more sensitive to *Campylobacter* infection compared to adult ones (Table 1).

Table 1: The positive rate of *Campylobacter* in different age of groups

Age group	Number	*Campylobacter* positive rate	*C jejuni* positive rate
Juvenile	60	36.67% (22/60)	21.67% (13/60)
Adult	60	6.67% (4/60)	3.33% (2/60)

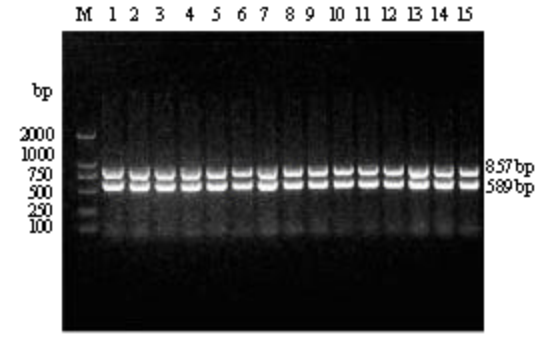
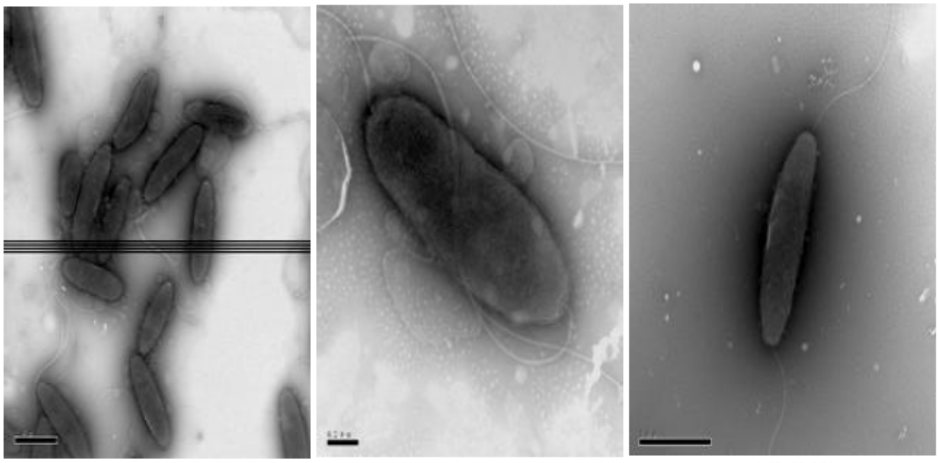


Fig. 1: Multiplex PCR detection. M: DNA Marker; Lane 1: 15: samples detected



(a) X 13,500 (b) X 46,000 (c) X 23,000

Fig. 2: Detailed morphology of examples of *C. jejuni* species

Table 2: Results of antibiotic resistance tests of *Campylobacter* strains

Antibiotics (%)	R	I	S
Amoxicillin	0 (0)	0 (0)	26 (100)
Ampicillin	1 (3.85)	1 (3.85)	24 (92.30)
Penicillin G	0 (0)	2 (7.70)	24 (92.30)
Cefaclor	0 (0)	0 (0)	26 (100)
Cefotaxime	0 (0)	0 (0)	26 (100)
Cephalexin	2 (7.70)	3 (11.55)	21 (80.75)
Cefoperazone	2 (7.70)	1 (3.85)	23 (88.45)
Streptomycin	0 (0)	0 (0)	26 (100)
Amikacin	0 (0)	0 (0)	26 (100)
Gentamicin	0 (0)	0 (0)	26 (100)
Kanamycin	0 (0)	0 (0)	26 (100)
Ofloxacin	0 (0)	0 (0)	26 (100)
Norfloxacin	0 (0)	0 (0)	26 (100)
Ciprofloxacin	0 (0)	0 (0)	26 (100)
Levofloxacin	0 (0)	0 (0)	26 (100)
Nalidixic acid	0 (0)	0 (0)	26 (100)
Co-trimoxazole	25 (96.15)	0 (0)	1 (3.85)
Erythromycin	0 (0)	0 (0)	26 (100)
Azithromycin	0 (0)	0 (0)	26 (100)
Oxytetracycline	0 (0)	0 (0)	26 (100)
Tetracycline	0 (0)	1 (3.85)	25 (96.15)
Doxycycline	0 (0)	0 (0)	26 (100)
Clindamycin	0 (0)	0 (0)	26 (100)

R: Resistance; I: Intermediate, S: Susceptible; N = 26 *C. jejuni* isolates

Multiplex PCR: Multiplex PCR identification of the 26 positive strains indicated that 15 out of the 26 strains were identified as *C. jejuni*, as specific bands in length of 857 and 589 bp were detected, respectively (Fig. 1). Single *Campylobacter* specific 857 bp band was found in the other 11 samples, indicating these remaining 11 strains were classified as *Campylobacter* species.

Electron microscope observation: Detailed morphology of examples of *C. jejuni* sp. (Fig. 2) showing S-shape and unipolar sheathed flagella (X 13,500) showing rod-shape with periplasmic fibrils entwining the bacterium and enlarged cell body with multiple bipolar flagella (X 46,000; bar = 2 μm) showing fusiform cell body encircled by tightly wound piroplasmic fibers and multiple sheathed flagella (X 23,000; bar = 1 μm).

Antimicrobial susceptibility: The drug-resistance susceptibility test of the 15 samples shown that these isolated strains were completely susceptible to the antibiotics used in this study, such as Amoxicillin, Penicillin G, Cefaclor, Cephalothin, Streptomycin, Amikacin, Gentamicin, Knamycin, Chloramphenicol, Ofloxacin, Norfloxacin, Ciprofloxacin, Levofloxacin, Nalidixic Acid, Erythromycin, Azithromycin, Oxytetracycline, Doxycycline, Tetracycline. More than 70% of these strains were sensitive to Ampicillin, Cephalexin and Cefoperazole and were resistant to some frequently used medicines such as co-trimoxazole 96.15% (Table 2).

Results of virulence gene test by PCR: The amplicons of five pathogenic genes (flaA, cdtB and racR) were detected in all of the *C. jejuni* isolates tested. All *C. jejuni* strains except one (D-21) were positive for dnaJ and cdtC genes. The prevalences of the cadF, pldA, cdtA, ciaB, ceuE and walN genes were 95, 93, 93, 90, 85 and 40%, respectively. The $virB_{11}$ gene was not detected in any of the strains.

DISCUSSION

Campylobacter localizes in the intestinal mucosa infected birds including chicken, duck, quail, ostrich and also wild birds (Newell *et al*., 2000, 2003). Huadong National Rare Bird Reservation District of China is the rest hub for many kinds of wild migrating birds. Hundreds kinds of migrating birds such as wild ducks live in the reservation district for months along with the National Rare Birds some kinds of domestic including

red-crowned cranes, while in the same area, poultries were also hosted, which may result in the infection and transmission of *Campylobacter* among various species. Therefore, it is important to monitor the *Campylobacter* infection in wild birds such as red-crowned cranes in this reservation district. For this purpose, in this study, we sought to investigate for the first time the state of *Campylobacter* infections in red-crowned cranes. We found that *Campylobacter* infection rate in red-crowned cranes is lower compared to that in other kind of birds such as chicken, duck and monkey. However, it should be noted that the inadequate samples used in this study may affect the final evaluation of results. In addition, we also detected the infection of *Campylobacter* in reindeer and no infection was found. We suggest continuing investigation in this area in different season needs to be performed in order to get more reliable data. Importantly, in this study *Campylobacter* was isolated in health red-crowned cranes individuals, which indicated that red-crowned cranes may be an ideal reservoir for *Campylobacter* to survive in this area. The correlation between the *Campylobacter* incidence and the age of the infected bird in this study was consistent with the data obtained in humans (Nylen *et al*., 2002).

Previous research has shown that the biochemical methods do not have a 100% specificity (Denis *et al*., 1999). In the study, fifteen isolates of *C. jejuni* were identified and characterized by growth properties, API campy system, transmission electron microscope characterization and multiplex PCR. For the rest of 11 strains, no distinct biological properties were observed, more identifications such as sequencing may however, be needed. Virulence genes was detected in *C. jejuni* by PCR. A similar observation was obtained by Kaisar *et al*. (2008). The $virB_{11}$ gene was detected in none of strains. Furthermore, the characterization on the pathogenicity of these isolates is now under way, we expect to determine, whether these strains could cause disease in humans, all of these studies would provide useful information for the development of novel diagnostic methods in human public health.

Drug-resistance rate of these *Campylobacter* isolated from feces of the red-crowned cranes was lower than that from human and poultry (Ronner *et al*., 2004), one explanation for that may be that rare use of medications in the cranes in this area, which would result in the higher susceptibility to most drugs, such as aminoglycoside, macrolides and other regular antibiotics. However, we found that part of these isolates were resistant to cephalosporins and Co-trimoxazole, demonstrating that selection of these antibiotics would be an idea choice in treating *Campylobacter* infection. It should pay more attention to the surveillance of antimicrobial resistance in *Campylobacter* sp.

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