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RESEARCH ARTICLE

Isolation and molecular identification of thermophilic *Campylobacter* species from mallard (*Anas platyrhynchos*)

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Yaban ördeklerinden (*Anas platyrhynchos*) termofilik *Campylobacter* türlerinin izolasyonu ve moleküler tanımlanmasının değerlendirilmesi

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Öz

Amaç: Bu çalışmada, yaban ördeklerinde insan ve hayvan sağlığını olumsuz yönde etkileyen *Campylobacter* türlerinin araştırılması amaçlandı.

Gereç ve Yöntem: Yaban ördeklerinden toplanan 110 dışkı örneği kültürel ve moleküler yöntemlerle incelendi. Ön zenginleştirme amacıyla örnekler Preston Campylobacter Enrichment Broth'a ekildi ve inkübe edildi. İnkübasyondan sonra ön zenginleştirme kültürden Preston Campylobacter Selective Agar'a ekim yapıldı ve 42°C 24-48 saat inkübasyona bırakıldı. Üremenin olduğu kültürler önce koloni morfolojisi ve sonra mikroskobik görünüm açısından değerlendirildi. Şüpheli koloniler değerlendirildikten sonra multiplex PCR ile incelendi.

Bulgular: Toplam 110 dışkı örneğinin 10'unda (%9,1) *Campylobacter* spp. bulundu. Yapılan Multiplex PCR ile 10 izolatın *Campylobacter* jejuni olduğu teyit edilmiştir.

Öneri: Bu çalışma ile, yaban ördeklerinden *Campylobacter* spp. varlığı belirlendi ve yaban ördeklerinin *Campylobacter* türleri için önemli bir rezervuar olabileceği kanaatine varıldı. Ayrıca, Kars bölgesi kuşların göç güzergahında olduğu için göçmen kuşlarının potansiyel taşıyıcı olabilecekleri göz önünde tutulmalıdır.

Anahtar kelimeler: Termofilik *Campylobacter*, izolasyon, multiplex-PCR, yaban ördeği.

Abstract

Aim: The aim of this study was to investigate the Camplyobacter species which adversely affect human and animal health in mallard.

Materials and Methods: 110 stool samples were collected from the mallard and examined by the cultural and molecular method. For pre-enrichment step, samples were inoculated with Preston Campylobacter Enrichment Broth. At the end of the incubation, pre-enriched culture was inoculated on Preston Campylobacter Selective Agar and the plates were incubated for 48-72 hours at 42°C. The cultures in which the growth was observed were first evaluated for the colony morphology and then for microscopic appearance. Suspected colonies were examined with multiplex PCR.

Results: In this study, *Campylobacter* spp. was found in 10 (9.1%) of the 110 stool specimens. All 10 isolates were typed as *Campylobacter jejuni* by multiplex PCR.

Conclusion: In this study, *Campylobacter* was detected in mallards and it was concluded that mallard may be an important reservoir for *Campylobacter* species. Besides, since Kars area is on the migration route of birds, it is important to examine the migratory birds and to determine the infections which birds have the potential.

Keywords: Thermophilic *Campylobacter*, isolation, multiplex-PCR, mallard

Introduction

Campylobacter is one of the major causes of human gastroenteritis in worldwide. *Campylobacter jejuni, Campylobacter coli, Campylobacter lari,* which are found in the genus *Campylobacter* and termed as thermophilic, are usually found in the gastrointestinal tract in domestic and wild animals and cause infections (Mohan 2015). It has also been identified in feces and products of both domestic and wild birds (Aarestrup and Engberg 2001, Colles et al 2011). In a report of European Union Team the *Campylobacter* species level have been identified among human cases as 93.4% *C. jejuni*, 2.3% *C. coli* and 0.22% *C. lari* (Eurosurveillance 2010). Thermophilic *Campylobacter* species can also be found commensally and asymptomatically in some poultry such as adult chickens, ducks and turkeys, and that makes it difficult to detect bacteria and poses a potential public health hazard (Keller et al 2014).

Domestic and wild birds, livestock, farms and the areas in where these farms are located, as well as river and still water form an ecological environment for *Campylobacter* species, and this environment has the potential to become contaminant, especially with the feces of wild birds (Padungton and Kaneene 2003). Therefore, environmental contamination is the most basic source of infection for animals (Corry and Atabay 2001).

Wild birds are natural reservoirs of *Campylobacter* species and have been identified as potential reservoir of infections in human, animal and domestic poultry. Although the prevalence of *Campylobacter* spp. has been determined in humans and various domestic poultry, information about poultry is limited. Understanding of the epidemiology and ecology of *Campylobacter* spp. in wild poultry is necessary to understand the transport of *Campylobacter* species in humans and domestic animal (Kwon et al 2017).

Determining the presence of *Campylobacter* spp. in the mallard is especially important for human and animal health. Because these migratory animals have the risk of infecting other vulnerable animals and people in that contaminating of the area and the water sources they use during migration. The aim of this study is to investigate fecal samples obtained from mallard and to investigate the carriage of thermophilic *Campylobacter* species in these animals. In this context, it was aimed to identify and distinguish these three pathogens by polymerase chain reaction (PCR) using specific primers for *C. jejuni, C. coli and C. lari species.*

Material and Methods

Sample collection

Fresh faecal samples were collected from sampling areas where the mallard rested while for the starlings nesting areas. Fresh stool specimens was collected from mallard living near the Kars River in February-March, 2017 and it was evaluated for the presence of *Campylobacter* spp. The experiment was carried out with the approval of the Local Ethical Committee in Kars (KAÜ-HADYEK/2019-043)

Bacterial isolation and phenotypic identification

In this study, 110 stool samples were examined by cultural method. Stool sample taken by sterile swabs was transported to the laboratory in Carry-Blair Transport Medium in cold chain conditions. For pre-enrichment step, samples were inoculated with Preston Campylobacter Enrichment Broth containing 7% defibrinated horse blood and Preston Campylobacter selective supplement (SR117, OXOID) and incubated in microaerobic conditions (Anaerocult® C, Sigma) at 42ºC for 48 hours. At the end of the incubation, 100 µl of the pre-enriched culture was inoculated on Preston Campylobacter Selective Agar and the plates were incubated for 48-72 hours at 42°C. The cultures in which the growth was observed were first evaluated for the colony morphology and then for microscopic appearance (Skirrow 1980, Vandamme 1992). Suspect colony for *Campylobacter* spp. was purified by passaging to Blood agar plates (CM271, Oxoid). The purified colonies were subjected to the tests such as oxidase, catalase and hippurate hydrolysis and then transferred to Brucella broth containing 20% glycerol and stored at -20 °C for subsequent molecular typing.

DNA extraction and multiplex PCR

The classical phenol-chloroform extraction method (Sambrook and Russell 2001) was used for DNA extraction from the isolates and then multiplex PCR technique was applied on (Wang et al 2002). The primer sets targeting the *23S rRNA* gene of *Campylobacter* spp., the *hip*O gene of *C. jejuni,* the *glyA* gene of *C. coli* and *C. lari* were used with the exception of the specific amplified products as 650, 323, 126 and 251, respectively (Table 1). Both genus and specific PCR was conducted in a single reaction.

The Multiplex PCR reaction was prepared in a total volume of 25 µl. For this, 10 µl of Taq PCR Master Mix (Qiagen), 1 µl of each primer pair at 20 pmol concentrations, 4 µl of DNase free water (Qiagen) and 3 µl of template DNA were used. DNA amplification was carried out in a thermocycler using an initial denaturation step at 95°C for 6 min followed by 30 cycles of amplification (denaturation at 95°C for 0.5 min, annealing at 59°C for 0.5 min, and extension at 72°C for 0.5 min), and was finalized with an extension at 72°C for 7 min. The PCR reaction is accompanied by the *Campylobacter* reference strains and the amplified products were visualised by 1.5% agarose jel electrophoresis and the images were photographed under UV transilluminator (UVP, CA 91786, U.S.A.).

Table 1. Primer sequences used in the multiplex PCR assay and the expected sizes of the products (Wanget al 2002)

Primer	Sequence (5_–3_)	GenBank accession no	Target gene	Size (in bp)
23SF 23SR	TATACCGGTAAGGAGTGCTGGAG ATCAATTAACCTTCGAGCACCG	Z29326	*Campylobater* spp. 23S rRNA	650
CJF CJR	ACTTCTTTATTGCTTGCTGC GCCACAACAAGTAAAGAAGC	Z36940	*C. jejuni hipO*	323
CCF CCR	GTAAAACCAAAGCTTATCGTG TCCAGCAATGTGTGCAATG	AF136494	*C. coli glyA*	1226
CLF CLR	TAGAGAGATAGCAAAAGAGA TACACATAATAATCCCACCC	AF136495	*C. lari glyA*	251

Results

Isolation results

The thermophilic *Campylobacter* were isolated showing microscopic characteristics such as small size, pinpoint morphology, non-hemolytic, and Gram-negative "gull-wing" shaped bacilli. Suspected isolates were subjected to tests such as catalase, oxidase and hippurate hydrolysis which yielded totally positive reaction. Thus, thermophilic *Campylobacter* was isolated in 10 (9.1%) of the 110 stool samples which were examined by cultural method

PCR results

All 10 isolates, which were phenotypically characterized as *Campylobacter* spp., were future identified as *C. jejuni* by using species-specific multiplex PCR (Fig. 1).

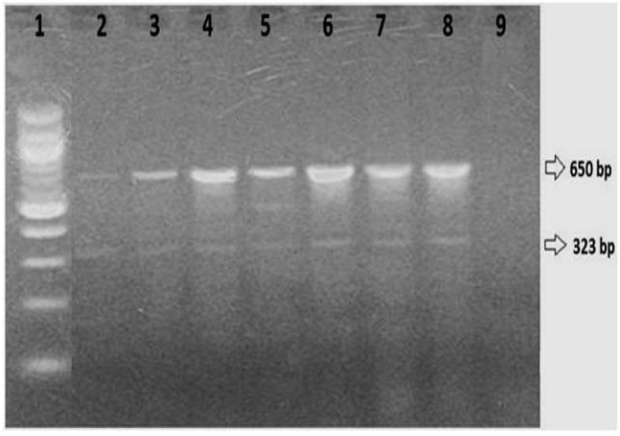

Figure 1. Gel electrophoresis image of PCR which includes both genus and species specific amplified product in a single gel. 1: DNA marker (Gene ruler 100 bp DNA Ladder, Fermentas); 2-7: Positive samples; 8: Positive control for *C. jejuni;* 9: Negative control (ddH2O)

Discussion

Generally domestic and wild birds are referred to as natural reservoirs of *Campylobacter* spp. Especially the wild birds are known as reservoirs which play roles in spread of infection to poultry, farm animals and humans (Şeker et al 2007). In this context migratory birds are thought to be responsible for the extensity of some important pathogens in large geographical areas and are reported to contribute infection cycle acting as host. Environment and environmental waters are also considered as potential sources of pathogens through the faecal contamination caused by domestic and wild mammals, poultry and humans (Colles et al 2008).

While many studies (Keller 2011, Mohan 2015, Kwon 2017) have been conducted on the prevalence of *Campylobacter* in wild poultry in different countries, studies in our country are very limited. In a study conducted by Pacha et al. (1988), *Campylobacter* spp. carriage has been reported in wild migratory waterbirds as 81% in sandhill crane (Grus canadensis tabida), 73% in ducks (Aythya collaris, Anas carolinensis, Aythya Americana, and Anas platyrhynchos) and 5% in Canada geese (Branta canadensis) and all were identified as *C. jejuni.* In a similar study, Mohan et al. (2015) investigated the *Campylobacter* spp. in wild birds such as duck, starling, goose and pied stilt and reported the prevalence of *Campylobacter* spp. as 33% in feces. Kwon et al. (2017) found 15.3% prevalence rate of *Campylobacter* spp. in a study conducted on wild ducks in South Korea. In a similar study reported by Keller and Shriver (2014) in USA, stool samples collected from three wild bird species (Anatidae, Scolopacidae, and Laridae) were examined to determine the prevalence of thermophilic *Campylobacter* species. *Campylobacter* spp. was found in 9.2% of the 781 stool samples collected from all species. The prevalence of *C. jejuni* was 8.1%, while the prevalence of *C. coli* and *C. lari* was 1.4% and 0.3%, respectively. Keller et al. (2011) investigated *Campylobacter* spp. in the stool samples collected from 333 wild birds, consisting of 32 species belong to 10 families. As the result of their study, *Campylobac-*

ter spp. was isolated from 24 (7.2%) samples. They reported that the highest isolation rate was obtained as *C. jejuni* from crow and gull species.

Despite the limited studies conducted in Turkey, the data obtained are noteworthy. In a study carried out by Arıkoğlu and Aydın (2000) in Kars region, 270 healthy and domestic animals (50 sheep, 8 dog, 50 cattle, 58 goose, 10 seagull, 10 crow, 15 duck, 32 chicken, 5 horse, 12 calves, 10 pigeon and 10 lambs) were examined for the presence of *C. jejuni* and it was isolated from 186 (68.8%) of samples. The isolation rate was determined as 100% in seagull and 10% in pigeon cloacal swab samples. In a study conducted in Ankara, Şeker et al. (2007) evaluated stools samples of cage birds for thermophilic *Campylobacter* species and found 11% (14/130) positivity. 10 of the isolates were recovered from the canaries with a distribution as 7 (70%) *C. jejuni* and 3 (30%) *C. coli* whereas 4 isolates were obtained from budgerigars with the following order as 3 (75%) *C. jejuni* and 1 (25%) *C. coli.* In the present study, 110 stool samples ontained from the wild ducks living on the edge of Kars river were evaluated and *Campylobacter* spp. were isolated in 9.1% (10/110) of samples. The isolation rate of this study was found to be lower than these reported previously (Pacha et al 1988, Arıkoğlu and Aydın 2000, Şeker et al 2007, Mohan 2015, Kwon et al 2017). As things stand, it is thought that the differences in prevalence rate may be due to the different geographical characteristics of the regions in where the studies are conducted or because of the different wild poultry species included in the studies. Additionally, since the thermophilic *Campylobacter* carriage may vary among the poultry species, it is thought that the lesser number of samples together with the sampling periods may effect compared to the other studies. The isolation rate obtained from the current study was due to be different of bird species because of higher than similar studies conducted by Keller et al. (2011). However, it is thought that the proximity of animals to water sources may also be effective. Additionally, it is anticipated that the living space of birds (cage birds) can affect the isolation rates.

Considering the identified thermophilic *Campylobacter* species, it is seen that *C. jejuni* was found as a single species in the present study as it seen in other studies too (Arıkoğlu and Aydın, 2000, Keller and Shriver 2014). In this study, as the reasons were not determined exactly for *C. coli* and *C. lari* species, it is thought that the using various animal species in the other studies (Keller and Shriver 2014), therefore carrier rates of this different species may change and also the general health conditions of animals will influence as well as body conditions. In addition to the ecological environment, it is estimated that poultry species can play the efficient role to be the reservoir for several agents and their transport to other domestic and wild animal when considering the environment and host adaptation characteristics of *Campylobacter* spp.

Conclusion

Wild birds are reported to be important sources of *Campylobacter* infections in humans and domestic animals. In this study, it is important as evidence of *Campylobacter* spp. in mallards. Another important role of migratory birds, they can cause contaminated of another area by contaminated water in emigrated area. Therefore, it should be noted that the identification of *Campylobacter* spp. carriage in these animals indicates that migratory birds namely as reservoirs play an important role in the spread of agent due to relations with other animals or humans.

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