Isolation and toxin gene detection of *Clostridium* (*Clostridioides*) *difficile* from traditional and commercial quail farms and packed quail meat for market supply

**Short title: Genotyping of *C. difficile* in quails**

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Abstract

*Clostridium (Clostridioides) difficile* is a Gram-positive anaerobic rod shaped bacterium and the main cause of nosocomial diarrhea in humans. In recent years, *C. difficile* transmission from environmental reservoirs (e.g. food) to humans became a major focus of research.

The study’s aim was to investigate the prevalence and corresponding toxin genes of *C. difficile* in fecal samples and meat of quails. 30 samples of packed quail meat in Mashhad, Iran and 500 fecal samples (pooled to n=5) were collected on quail farms in the Northeastern Khorasan region for further investigation. Of 100 pooled quail fecal samples 10% showed cultural growth of *C. difficile*. In meat samples two of 30 specimens (7%) showed cultural growth. Of fecal samples in six of ten isolates toxin genes were present (*tcdB* and *tcdA*) while four isolates harbored no toxin genes. However, in meat isolates no toxin genes were present. Mutations in the *tcdC* gene were not detected indicating that “hypervirulent” strains such as RT027 and RT078 were not present.

The data suggest that quail and quail products might hold potential for spread of *C. difficile*. 
Main article

Clostridium (Clostridioides) difficile is a spore-forming, anaerobic, Gram-positive anaerobic rod shaped bacterium, which has been identified as a bacterial pathogen in both humans and animals (Lawson et al., 2016). It has been implicated as the cause of enteric disease in a broad variety of animal species including foals, piglets, adult horses and rabbits (Levett, 1986).

In addition, some studies have recently raised the importance of wild animals as a reservoir of C. difficile for humans and domestic animals (al Saif and Brazier, 1996; Baverud, 2002; Borriello et al., 1983; Lefebvre et al., 2006; Songer and Anderson, 2006).

Antibiotic treatment seems to be the main factor of disease development since C. difficile can thrive when the normal gut flora is disrupted (Rupnik et al., 2009). The pathogen’s main virulence factor are toxin A and B (corresponding genes: tcdA and tcdB) (Gerding et al., 2014). Additionally some strains might express a third toxin termed binary toxin (CDT) which is preferentially detected in epidemic isolates (Gerding et al., 2014). C. difficile infection can be diagnosed by a variety of assays including toxigenic culture, toxin detection and toxin gene PCR (Crobach et al., 2016). Transcription of tcdA and tcdB, being located on the pathogenicity locus (PaLoc), is controlled by two regulators, TcdR (gene: tcdR) and TcdC (gene: tcdC). TcdR is an alternative sigma factor that positively regulates transcription of tcdA and tcdB (Belanger et al., 2003) while TcdC negatively regulates TcdR (Dupuy et al., 2008). Of note, several studies have reported tcdC does not influence toxin production (Curry et al., 2007; Samie et al., 2008; Stare et al., 2007). However, tcdC mutations in presumably “hypervirulent” strains such as ribotype 027 (RT027) and RT078 are typical findings (Persson et al., 2011; Wolff et al., 2009). The latter genotype is furthermore detected in animals probably holding a zoonotic potential (Knetsch et al., 2014).
Concerning studies targeting poultry, the main research focus was on the prevalence on chickens, ostriches and turkeys while for smaller bird animals such as quails data are scarce (Abdel-Ghil et al., 2018; Songer, 2004).

In order to assess the impact of *C. difficile* in this poultry subgroup small traditional and larger commercial quail farms and packed quail meat were investigated for presence of *C. difficile*. The primary aim of this study was to determine the prevalence of *C. difficile* in quails and quail meat including further characterization using toxin gene detection and sequencing of mutations being indicative for “hypervirulent” strains (RT027 and RT078 respectively).

100 pooled quail fecal samples (500 individual samples) were obtained randomly from 10 quail farms (four traditional and six commercial farms). 20 and 80 pooled samples stemmed from traditional and commercial farms respectively. Additionally 30 packs of quail meat (each pack consisting out of five carcasses) were purchased from six different shopping centers in Mashhad, Iran. All samples stemmed from the Khorasan region of Iran consisting of the provinces North, South and Razavi Khorasan (population >6 million) which have been allocated from December 2013 to April 2014.

10 g of each homogenized fecal sample was incubated for 30 min in room temperature with equal volume of 96% ethanol stock. Cultural sampling was carried out under anaerobic conditions on Columbia agar (Merck, Darmstadt, Germany) for 72 h. *C. difficile* isolates were identified based on the characteristic traits including colony morphology, positive Gram staining appearance with typical spore formation and characteristic odor and using molecular confirmation by species-specific PCR (16S rRNA).
Multiplex PCR was performed to detect the toxin genes tcdA, tcdB, cdtAB and C. difficile 16S ribosomal DNA as described previously (Persson et al., 2008). Briefly, genomic DNA was extracted from a single colony using an extraction kit (Bioneer, Daejeon, South Korea) according to the manufacturer’s instructions. The multiplex PCR was carried out for the detection of target genes. Amplification reactions were prepared in a 50 μl reaction volume containing 25 μl Master Mix and 25 μl including primer mixture (Persson et al., 2008), template and double distilled water. Amplification was programmed in a thermocycler (Techne TC 3000, Staffordshire, Great Britain) as follows: 94°C for 10 min followed by 35 cycles of 94°C for 50 sec, 54°C for 40 sec, 72°C for 50 sec and a final extension at 72°C for 3 min.

Furthermore tcdC was analyzed as described previously (Antikainen et al., 2009). Briefly, amplification reactions were prepared in a 25 μl reaction volume containing 12.5 μl MasterMix, 5 μl template DNA, 1 μl (10pm/μl) from each of forward and reverse primers and 5.5 μl deionized water. PCR was initiated with a denaturation step at 94°C for 5 min followed by 36 cycles at 98°C for 10 sec, 60°C for 20 sec, 72°C for 20 sec and a final extension at 72°C for 10 min. The amplified products were detected on ethidium bromide stained 1.5% agarose gel (Cinnagen, Tehran, Iran) after electrophoresis and ultraviolet illumination. PCR reagents were provided by Ampliqon (Odense, Denmark) except for the DNA molecular weight marker 100 bp originating from Dena Zist Asia (Mashhad, Iran).

Of 100 pooled quail samples 10% showed cultural growth of C. difficile as confirmed by morphological traits and PCR. Concerning traditional and commercial quail farms samples were positive in 5/20 (25%) and 5/80 (6%) of cases respectively. In six of ten acquired fecal isolates, both toxin genes (tcdB and tcdA respectively) could be identified while in the remaining four strains no toxin genes could be detected. In commercial farms, two toxigenic and three non-
toxigenic isolates were present while in traditional farms four toxigenic and one non-toxigenic isolates were detected.

In meat samples, *C. difficile* prevalence was similar with 7% (2/30). Within the two meat isolates however, no toxin genes were found. None of the isolates in both sample sets showed mutations in the *tcdC* gene indicating that “hypervirulent” strains in particular RT027 and RT078 were absent being in line with the lack of *cdtAB* in all strains.

Carrier rates in birds may vary greatly ranging from 0-62% (Abdel-Gliil et al., 2018). In poultry products such as meat *C. difficile* could be isolated on up to 15% of samples in the past posing a potential risk for humans (Varshney et al., 2014). Several RTs being frequently encountered in human disease could be isolated in poultry in recent studies. This includes RT001 (Abdel-Gliil et al., 2018; Indra et al., 2009), RT002 (Hussain et al., 2016), RT014 (Hussain et al., 2016), RT027 (Varshney et al., 2014), RT039 (Abdel-Gliil et al., 2018) and RT078 (Varshney et al., 2014; Weese et al., 2010).

Of note RT001 (Azimirad et al., 2017; Kouhsari et al., 2019), RT014/020 [both RTs correspond to sequence type 2 in the respective study (Shoaei et al., 2019)], RT039 (Kouhsari et al., 2019) and RT078 (Jalali et al., 2012) have been detected in the Iranian population. However, most studies focused predominately on chicken, ostriches and turkeys and data for other poultry such as quails are scarce. Breeding of quails for meat and egg production is of special agricultural interest in many countries besides Iran (Nasar et al., 2016; Saka et al., 2018). This emphasizes a proper risk assessment concerning *C. difficile*.

The carrier rate of toxigenic *C. difficile* in fecal specimens was 6% while in meat products only non-toxigenic strains could be detected. Furthermore no isolates with *tcdC* mutations indicative
for “hypervirulent” strains could be found. Of note, non-toxigenic strains made up 50% of all
*Clostridium difficile* isolates. Due to the isolation of toxigenic *C. difficile* strains in quail feces, this bird
species might also hold a potential for *C. difficile* transmission. However, in quail meat no
toxigenic isolates could be detected. Concerning the fact that “hypervirulent” strains were absent,
it should be taken into account that this finding is in line most studies targeting humans in Iran
except for RT078 in one study (Jalali et al., 2012) as they seem to be of minor importance in Iran
(Azimirad et al., 2017; Kouhsari et al., 2019; Shoaei et al., 2019).

For a better assessment of the pathogen’s role in disease development in quails clinical studies in
these animals are needed in the future. The main limitation of the study is the non-availability of
further processing to acquire further molecular data (e.g. through ribotyping). These data are
however, necessary for the comparison of the acquired isolates to global circulating strains and
to those, which cause disease in the Iranian human population.

In conclusion, quails may nevertheless pose a reservoir for zoonotic *C. difficile* transmission.

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