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# Characterisation and antibiotic susceptibility profile of *Clostridioides (Clostridium) difficile* isolated from chicken carcasses

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## Abstract

**Introduction:** *Clostridioides (Clostridium) difficile* is a Gram+, anaerobic, spore-forming, rod-shaped bacterium that can produce toxins, and it is mainly because its virulence is attributed. The objective of this study was to evaluate the presence of *C. difficile* and hyper virulent ribotypes in chicken carcasses and the antibiotic susceptibility of isolated strains. **Material and Methods:** *C. difficile* was isolated from chicken carcasses by microbiological methods, its ribotypes were identified by means of PCR, the toxin production ability was defined by ELISA, and the susceptibility of the isolates to selected antibiotics was determined by minimum inhibitory concentration evaluator strips. **Results:** The bacterium was isolated from 69 out of 185 (37.3%) examined chicken carcass samples, and six out of the 69 (8.7%) isolates were identified as ribotype 027. All isolates were susceptible to amoxicillin-clavulanic acid (100.0%), vancomycin (97.1%), metronidazole (88.4%), and tetracycline (95.7%), whereas they were resistant to cefotaxime (97.1%) and imipenem (89.9%). **Conclusion:** The results of this study demonstrate the presence of toxigenic *C. difficile* isolates such as ribotype 027 (one of the most common causes of *C. difficile* infection in humans) in chicken carcasses. Although there is no case for stating that *C. difficile* is a food-borne pathogen, the presence of *C. difficile* in chicken may be considered to be a potential risk to consumers.

**Keywords:** chicken, *C. difficile*, ribotype, antibiotic susceptibility, toxin.

## Introduction

*Clostridioides (Clostridium) difficile* is a Gram+, anaerobic, spore-forming, rod-shaped bacterium which can colonise the entire intestinal tract of humans and various animal species (19, 29). The most frequent predisposing risk factor for *C. difficile* infection (CDI) in humans and animals is long-duration antibiotic usage that results in the destruction of regular intestinal microflora. As a result, *C. difficile* can multiply throughout the intestines and lead to gastrointestinal symptoms that vary but usually include mild to serious diarrhoea. Deaths can even be seen in some critical cases (6, 22, 28).

The *tcdA* and *tcdB* genes of *C. difficile* encode the production of its toxins, which are A (enterotoxin) and B (cytotoxin), and some strains have *cdtA/B* genes which encode binary toxin production (adenosine diphosphate-ribosyltransferase). The virulence of this bacterium is mostly related to the existence of these toxins. Certain *C. difficile* ribotypes have increased toxin generation and efficient sporulation characteristics, which makes them hypervirulent, and in this subset, human pathogenic ribotypes like RT027 and RT078 are at the forefront and known as the cause of human CDI (10, 20, 24).

*C. difficile* can be found in the environment (soil and water), poultry, slaughter animals, seafoods, meat products, vegetables, and ready-to-eat food varieties. Recently, prevalence studies on *C. difficile* and its human pathogenic ribotypes in chicken carcasses have drawn attention to these animals as a presumptive source of contamination with this bacterium for humans (10, 17, 20, 21).

The objective of this study conducted in the Marmara Region of Turkey was to quantify the presence

of *C. difficile* in whole chicken carcasses, to identify *C. difficile* strains, to analyse ribotype diversity including RT027 and RT078 by PCR, to gauge the toxin production ability by ELISA, and to determine the susceptibility of the isolates to the antibiotics that are most widely used for the treatment of *C. difficile* infection.

## Material and Methods

**Samples and sampling technique.** A total of 185 whole chicken carcasses were obtained from butchers (at least 15 different establishments in each city) located in nine different cities in the Marmara Region of Turkey (population: over 25,000,000; surface area: 67,000 $km^2$). The samples were collected once a month from each of the different cities and were promptly taken to the laboratories of Istanbul University-Cerrahpasa in an insulated icebox, and the analyses were started on the same day (in less than 24 h).

The sampling technique was performed by using the whole carcass rinsing method as per the US Department of Agriculture Food Safety and Inspection Service directive (30). For this purpose, the chicken carcass samples were picked up by the legs and placed in a sterile sample bag, 400 mL of buffered peptone water was added, and the carcass was rinsed for approximately 1 min and inverted about 30 times in the process.

**Isolation of *C. difficile* from samples.** A 50 mL volume of rinsate was collected immediately and mixed with 50 mL of *C. difficile* moxalactam norfloxacin (CDMN) broth with 0.1% taurocholate (Biological Reference Preparation, European Pharmacopoeia (EP) Reference Standard, Sigma-Aldrich, St Louis, MO, USA) (15). Then, the mixture was incubated at 37°C for 10 days under anaerobic conditions using an Anaerogen Kit (SR0173, Oxoid, Basingstoke, UK). After alcohol shocking, the sediment was spread on *C. difficile* selective agar (CM0601+ CDMN supplement SR 0173 + 5% defibrinated horse blood, Oxoid), and then, the Petri dishes with the agar were incubated for 48–72 h at 37°C under anaerobic conditions (10). Colonies with a greyish ground glass appearance and a horse manure odour were classified as suspected colonies, and further analyses were carried out on them such as Gram staining and a latex agglutination test according to the manufacturer's manual (DR1107A *C. difficile* test kit, Oxoid). Before PCR analysis, a pure culture of *C. difficile* was isolated on tryptic soy agar (CM0131, Oxoid) including 5.0% defibrinated horse blood and incubated anaerobically at 37°C for 48–72 h.

**Genomic DNA preparation.** For amplification process, a loopful of colony which had been cultivated in blood agar was diluted in 1 mL of sterile saline solution (0.85%) and boiled for 10 min. Then, extracted DNA was stored at −20°C.

**Molecular confirmation of isolates and detection of toxin-producing genes.** The *C. difficile*-specific triose phosphate isomerase (tpi) gene and *tcdA* and *tcdB* toxin-producing genes were detected by PCR. For this purpose, the primers listed in Table 1 were used according to Lemee *et al.* (14) with minor modifications. The PCRs were performed on a CG 1-96 Palm-Cycler (Genetix Biotech Asia, New Delhi, India) in a final volume of 25 µL containing: 5 µL of DNA template, 10% (v/v) glycerol, 1 µM of each primer (except for *tpi*-F and *tpi*-R, of which there was 0.5 µM), 200 µM of each deoxynucleoside triphosphate, and 0.5 U of *Taq* DNA polymerase in a 1× amplification buffer (10 mM Tris-HCl, pH 8.3) (Thermo Fisher Scientific, Waltham, MA, USA).

The PCR mixtures were denatured at 95°C for 3 min and then, a touchdown step was applied at 95°C for 30 s. An annealing step for 30 s at temperatures decreasing from 65°C to 55°C in the first 11 cycles and a final extension step at 72°C for 30 s were performed (in total 40 cycles). Binary toxin genes (*cdtA* and *cdtB)* were determined by means of a multiplex PCR as described by Stubbs *et al.* (27) (Table 1). The PCRs were performed in a final volume of 50 µL containing: 10 µL of DNA template, 0.15 µM of each primer, 1.5 mM of $MgCl_2$, 1U of Taq polymerase, and 200 µM of each deoxynucleoside triphosphate in a 1× amplification buffer (10 mM Tris-HCl (pH 8.3) and 50 mM KCl) (Thermo Fisher Scientific). The mixtures were put through 30 cycles of a denaturation step at 94°C for 45 s, an annealing step at 52°C for 1 min, and a final extension step at 72°C for 80 s. For the electrophoresis process, 1.5% agarose gel with the addition of ethidium bromide was used and for gel screening, a UV transilluminator provided imaging with the Dolphin-Doc analysing system (Wealtec, Sparks, NV, USA).

**Table 1.** Primer sequence list used in the study

| Gene | Primers | Amplicon size | Reference |
|---|---|---|---|
| *tpi* | F: 5′-AAAGAAGCTACTAAGGGTACAAA-3′<br>R: 5′-CATAATATTGGGTCTATTCCTAC-3′ | 230 bp | 15 |
| *tcdA* | F: 5′-AGATTCCTATATTTACATGACAATAT-3′<br>R: 5′-GTATCAGGCATAAAGTAATATACTTT-3′ | 369 bp | 15 |
| *tcdB* | F: 5′-GGAAAAGAGAATGGTTTTATTAA-3′<br>R: 5′-ATCTTTAGTTATAACTTTGACATCTTT-3′ | 160 bp | 15 |
| *cdtA* | F: 5′-TGAACCTGGAAAAGGTGATG-3′<br>R: 5′-AGGATTATTTACTGGACCATTTG-3′ | 353 bp | 28 |
| *cdtB* | F: 5′-CTTATTGCAAGTAAATACTGAGAGTACTATATC-3′<br>R: 5′-ACCGGATCTCTTGCTTCAGTC-3′ | 490 bp | 28 |

In this research, the ATCC 9689 *C. difficile* strain was used as the positive control for the *tcdA* and *tcdB* genes, the BAA 1870 strain was the equivalent for the *cdtA*, and *cdtB* genes, both of these references could serve as the positive control for the *tpi* gene, and Milli-Q water served as the negative control (Merck, Darmstadt, Germany).

**PCR – ribotyping.** The 16S–23S intergenic spacer regions of *C. difficile* isolates were amplified according to Bidet *et al*. (3), and capillary electrophoresis was carried out by means of an ABI 310 Genetic Analyser using performance-optimised polymer 4 and GeneScan 1200 LIZ size standard (all Applied Biosystems, Carlsbad, CA, USA), with a 36 cm array length and provision of default fragment analysis. The WEBRIBO database was used for ribotype determination after Gene Mapper v4.9 (Applied Biosystems) software processing (12).

**Detection of *C. difficile* toxin A and B production.** A Ridascreen ELISA kit (C0801, R-Biopharm AG, Darmstadt, Germany) was used for the detection of toxin production. A loopful of colony cultured on blood agar and confirmed as *C. difficile* was diluted in 1 mL of sample dilution buffer and centrifuged at 2,500 × g for 5 min. After centrifugation, the supernatant was used for the detection of toxin presence according to the manufacturer's protocol.

**Antibiotic susceptibility test.** The antibiotic susceptibility of *C. difficile* isolates was examined by minimum inhibitory concentration evaluator strips (Oxoid) according to the supplied instructions. The breakpoint values for imipenem, cefotaxime, amoxicillin-clavulanic acid, tetracycline, clindamycin, ampicillin, and metronidazole were taken from the CLSI (5), and for vancomycin the values derived from EUCAST (8).

## Results

A total of 185 chicken carcasses were analysed for the presence of the *tpi* gene, which is specific for *C. difficile* by PCR, and the gene was found in 69 (37.3%) isolates. According to the PCR ribotyping results, 6/69 (8.7%) isolates were determined as RT027, whereas the other hypervirulent human pathogenic strain, RT078, could not be detected in any chicken carcass samples.

When the antibiotic sensitivity of isolates was evaluated, it was determined that 69 (100%) isolates were susceptible to amoxicillin-clavulanic acid, 61 (88.4%) to metronidazole, 66 (95.7%) to tetracycline, and 67 (97.1%) to vancomycin. On the other hand, 62 (89.9%) and 67 (97.1%) out of 69 chicken carcass isolates were resistant to imipenem and cefotaxime, respectively (Table 2). The susceptibility profiles of *C. difficile* isolates obtained from chicken carcasses are shown in Table 3.

**Table 2.** Susceptibility profiles of *C. difficile* isolates from chicken carcasses

| n | Susceptibility | AMP (%) | AMC (%) | DA (%) | IMP (%) | MTZ (%) | TE (%) | VA (%) | CTX (%) |
|---|---|---|---|---|---|---|---|---|---|
| 69 | Susceptible | 48 (69.6) | 69 (100) | 39 (56.5) | 6 (8.7) | 61 (88.4) | 66 (95.7) | 67 (97.1) | 2 (2.9) |
| | Intermediate | 19 (27.5) | 0 (0) | 14 (20.3) | 1 (1.4) | 0 (0) | 1 (1.4) | 0 (0) | 0 (0) |
| | Resistant | 2 (2.9) | 0 (0) | 16 (23.2) | 62 (89.9) | 8 (11.6) | 2 (2.9) | 2 (2.9) | 67 (97.1) |

AMP – ampicillin; AMC – amoxicillin-clavulanic acid; DA – clindamycin; IMP – imipenem; MTZ – metronidazole; TE – tetracycline; VA – vancomycin; CTX – cefotaxime; n –number of samples

**Table 3.** The distribution of determined ribotypes in terms of antibiotic susceptibility

| RTs | n | AMP | | | AMC | | | DA | | | IMP | | | MTZ | | | TE | | | VA | | | CTX | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | S | I | R | S | I | R | S | I | R | S | I | R | S | I | R | S | I | R | S | I | R | S | I | R |
| RT027 | 6 | 4 | 2 | - | 6 | - | - | 4 | 1 | 1 | - | - | 6 | 6 | - | - | 6 | - | - | 6 | - | - | - | - | 6 |
| RT087 | 4 | 3 | 1 | - | 4 | - | - | 3 | 1 | - | - | - | 4 | 4 | - | - | 4 | - | - | 4 | - | - | - | - | 4 |
| RT470 | 4 | 2 | 2 | - | 4 | - | - | 4 | - | - | - | - | 4 | 4 | - | - | 4 | - | - | 4 | - | - | - | - | 4 |
| RT085 | 4 | 4 | - | - | 4 | - | - | 3 | 1 | - | - | - | 4 | 4 | - | - | 4 | - | - | 4 | - | - | - | - | 4 |
| RT456 | 2 | 2 | - | - | 2 | - | - | 2 | - | - | - | - | 2 | 2 | - | - | 2 | - | - | 2 | - | - | - | - | 2 |
| RT020 | 2 | 2 | - | - | 2 | - | - | 1 | 1 | - | - | - | 2 | 2 | - | - | 2 | - | - | 2 | - | - | - | - | 2 |
| RT010 | 1 | 1 | - | - | 1 | - | - | 1 | - | - | - | - | 1 | 1 | - | - | 1 | - | - | 1 | - | - | - | - | 1 |
| RT003 | 1 | 1 | - | - | 1 | - | - | 1 | - | - | - | - | 1 | 1 | - | - | 1 | - | - | 1 | - | - | - | - | 1 |
| ML027 | 6 | 3 | 3 | - | 6 | - | - | 3 | 2 | 1 | - | - | 6 | 6 | - | - | 6 | - | - | 6 | - | - | - | - | 6 |
| NR | 39 | 26 | 11 | 2 | 39 | - | - | 17 | 8 | 14 | 6 | 1 | 32 | 31 | - | 8 | 36 | 1 | 2 | 37 | - | 2 | 2 | - | 37 |
| TOTAL | 69 | 48 | 19 | 2 | 69 | - | - | 39 | 14 | 16 | 6 | 1 | 62 | 61 | - | 8 | 66 | 1 | 2 | 67 | - | 2 | 2 | - | 67 |

AMP – ampicillin; AMC – amoxicillin-clavulanic acid; DA – clindamycin; IMP – imipenem; MTZ – metronidazole; TE – tetracycline; VA – vancomycin; CTX – cefotaxim; n – ribotype number; ML – most likely; S – susceptible; I – intermediate; R – resistant; RTs – ribotypes

**Table 4.** The distribution of the virulence genes and ribotypes of *C. difficile* isolates (n = 69)

| Toxigenic genes | | | Samples (%) | Ribotypes ($n^r$) |
|---|---|---|---|---|
| $tcdA^+$ | $tcdB^+$ | $cdtA/B^+$ | 17 (24.6) | 027 (6), 003 (1), ML#027 (6), NR* (4) |
| $tcdA^+$ | $tcdB^+$ | $cdtA/B^-$ | 14 (20.3) | 087 (4), 020 (2), NR (8) |
| $tcdA^+$ | $tcdB^-$ | $cdtA/B^+$ | 1 (1.5) | NR (1) |
| $tcdA^+$ | $tcdB^-$ | $cdtA/B^-$ | 3 (4.4) | NR (3) |
| $tcdA^-$ | $tcdB^+$ | $cdtA/B^+$ | 13 (18.8) | NR (13) |
| $tcdA^-$ | $tcdB^+$ | $cdtA/B^-$ | 11 (15.9) | 470 (4), 456 (2), NR (5) |
| $tcdA^-$ | $tcdB^-$ | $cdtA/B^+$ | 0 (0) | ND** |
| $tcdA^-$ | $tcdB^-$ | $cdtA/B^-$ | 10 (14.5) | 010 (1), 085 (4), NR (5) |

NR* – new ribotype; ND** – not detected; ML# – most likely; $n^r$ – number of ribotypes

In this study, the toxin genes of *C. difficile* isolates were detected by PCR and these, the *tcdA, tcdB*, and *cdtA/B* genes, were determined in 35 (50.7%), 55 (79.7%), and 31 (44.9%) out of 69 chicken isolates, respectively.

The distribution of the toxin genes and the number of ribotypes which were detected in chicken carcass isolates are shown in Table 4. Seventeen (24.6%) isolates had all three toxin genes (six were RT027 and one was RT003). In contrast, 10 (14.5%) isolates did not include any *tcdA*, *tcdB*, or *cdtA/B* genes.

ELISA was used for the detection of *C. difficile* toxins A and B. A total of 47 out of 69 (68.1%) chicken isolates had the toxin production attribute, whereas no toxin production was observed in 22 out of 69 (31.9%) isolates.

## Discussion

The results of this research are further proof of the presence of *C. difficile* in chicken carcasses. There are also a number of studies from different countries confirming the detection of this organism in poultry and poultry-originated products. In a study performed by de Boer *et al*. (6), *C. difficile* was found in 7 out of 257 (2.7%) chicken carcass samples. In another piece of research conducted in Canada, Weese *et al.* (32) isolated the organism from 12.8% (26/203) of chicken carcasses (thigh, wing, and leg). Guran and Ilhak (9) conducted similar research in which they obtained 310 chicken samples from supermarkets and butcher's shops located in the eastern part of Turkey and found that 25 (8.1%) of them were contaminated with the bacterium. From Indra *et al.* (13), in Austria came research results in which C. *difficile* was noted in three out of 59 (5.1%) broiler chicken samples. Our results are higher than those seen in these studies. Contrary to this trend, in the USA, Mooyottu *et al.* (18) reported that they could not detect any *C. difficile* strains in 100 chicken wing samples. Limbago *et al*. (15) found similar findings: the researchers could not determine the bacterium in 614 minced turkey or 259 chicken breast samples obtained from retail markets. Likewise, Ersöz and Coşansu (7) from Turkey could not ascertain any *C. difficile* presence in 27 chicken breast samples.

The existence of the bacterium not only in chicken carcasses but also in chicken faeces was detected and reported by other researchers (25, 26, 33). The persistence of *C. difficile* and its spores in the environment (in soil and water), ineffective hygiene in rearing operations, and deficient manufacturing practices such as unhygienic slaughterhouse conditions (insufficient cleaning, sub-structural deficiencies, *etc*.), an unsuitable plucking process, the contamination of carcasses with faeces because of careless evisceration or contact with the floor, improper chilling processes, unhygienic storage conditions, poor personnel and equipment hygiene (contaminated hands, clothes, knives, *etc.*), and inattentive and improper disposal of animal remnants and extraneous matter are some important factors fostering *C. difficile* presence in poultry carcasses (10, 21, 31).

Recently, *C. difficile* isolates detected in poultry carcasses have shown similarities with some strains like RT027 and RT078, which are related to CDI outbreaks in humans. In this context, Varshney *et al.* (31) examined 76 minced turkey meat samples and found *C. difficile* in 11 (14.5%) of them (including one RT027 and two RT078 isolates). In another study, Weese *et al*. (32) found *C. difficile* in 26 out of 203 (12.8%) chicken samples, and all isolates were R078. In contrast to this, RT078 could not be detected in the present study; however, RT027 was found in 6 out of 69 (8.7%) examined chicken samples. As a counterpoint, neither RT027 nor RT078 could be isolated in chicken meat samples by a number of researchers (1, 6, 31). In various studies, *C. difficile* and its hypervirulent ribotypes were reported in poultry carcasses with different rates of prevalence. Guran and Ilhak (9) and Rodriguez-Palacios *et al*. (23) reported that the prevalence of *C. difficile* was generally higher in winter than in other seasons. In another study, Lund and Peck (16) reported that the isolation rates of *C. difficile* were relatively low (4.3%) in Europe, whereas they were higher (44.0%) in North America, and they indicated that one of the reasons for

this difference may be the different natures of each continent's enrichment, isolation, and identification methods. Blanco *et al*. (4) stated that the procedure used to isolate *C. difficile* can have a significant impact on prevalence data for this organism. Zidaric *et al.* (33) reported that *C. difficile* colonisation in chickens was probably established within the first two weeks post-hatching and subsequently decreases with age. In the light of this data, dissimilar characteristics of the sampled animals (age, breed, *etc*.), geographical and seasonal differences, the use of distinct sampling techniques (material, sampling amount, *etc*.), and the adoption of different isolation methods can be the explanation of the differences in prevalence rates of *C. difficile* in chickens (7, 10, 31).

Concerns about the use of antibiotics in poultry have gradually increased in recent years. Although a number of countries have prohibited their use, still different antibiotics are used in the poultry industry to promote growth, to treat sick animals, and to prevent diseases. Therefore, concern about antibiotic resistance developing in *C. difficile* in poultry would seem to be founded. On the other hand, some antibiotics such as vancomycin and metronidazole are the medicines of first resort for the treatment of CDI and CDI-related diarrhoea in humans. Several research publications demonstrated that the majority of the isolated *C. difficile* strains from various foods are resistant to imipenem and cefotaxime but susceptible to amoxicillin, ampicillin, tetracycline, metronidazole, and vancomycin (2, 10, 11, 20, 28, 29). Simango and Mwakurudza (26) reported that all isolated *C. difficile* strains from chicken samples were found susceptible to vancomycin, metronidazole and tetracycline, despite them all being resistant to cefotaxime. As shown in Table 2, our findings parallel these results. Our investigation demonstrated that the isolates were susceptible to amoxicillin, tetracycline, vancomycin, and metronidazole at rates of 100.0%, 95.7%, 97.1%, and 88.4%, respectively. The cefotaxime and imipenem resistance rates in chicken carcass samples were found to be 97.1% and 89.9%, respectively.

Toxin production from the genes with this purpose can be regarded as the primary virulence factor of *C. difficile*; however, the presence of toxin genes does not mean that they have toxin production capacity. In this research, toxin production was detected in 47 out of 69 (68.1%) chicken carcass isolates, whereas toxin production could not be detected in the other 22 (31.9%) examined samples. In the study performed by Guran and Ilhak (9), it was reported that five out of 25 *C. difficile* strains isolated from chicken parts had toxin A, and eight of 25 isolates had toxin B, whereas the isolates did not contain any binary toxin. In a similar study, Simango and Mwakurudza (26) determined *C. difficile* in 29 out of 100 (29.0%) chickens, and they reported that 26 (89.7%) of these isolates had the toxin production attribute. In Iran, Rahimi *et al*. (20) analysed 368 ready-to-eat food products, among which the organism was found in only five samples, and they detected that three out of five (60.0%) strains produced toxins A and B.

In conclusion, the results of this study conducted in Turkey reveal the presence of *C. difficile* isolates in chicken carcasses. Although the significance of foods contaminated with *C. difficile* in human infection is still unclear, chicken carcasses can be a presumptive *C. difficile* contamination route for humans, and in consequence, chicken and chicken products can be considered one of the probable transmission pathways for humans and a potential risk for consumers.

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## References

1. Abdel-Glil M.Y., Thomas P., Schmoock G., Abou-El-Azm K., Wieler L.H., Neubauer H., Seyboldt C.: Presence of *Clostridium difficile* in poultry and poultry meat in Egypt. Anaerobe 2018, 51, 21–25, doi: 10.1016/j.anaerobe.2018.03.009.
2. Bakri M.: Prevalence of *Clostridium difficile* in raw cow, sheep, and goat meat in Jazan, Saudi Arabia. Saudi J Biol Sci 2018, 25, 783–785, doi: 10.1016/j.sjbs.2016.07.002.
3. Bidet P., Barbut F., Lalande V., Burghoffer B., Petit J.C.: Development of a new PCR-ribotyping method for *Clostridium difficile* based on ribosomal RNA gene sequencing. FEMS Microbiol Lett 1999, 175, 261–266, doi: 10.1111/j.1574-6968.1999.tb13629.x.
4. Blanco J.L., Álvarez-Pérez S., García M.E.: Is the prevalence of *Clostridium difficile* in animals underestimated? Vet J 2013, 197, 694–698, doi: 10.1016/j.tvjl.2013.03.053.
5. Clinical and Laboratory Standards Institute: Performance Standards for Antimicrobial Susceptibility Testing, CLSI Document M100. Clinical and Laboratory Standards Institute, Wayne, 2018.
6. De Boer E., Zwartkruis-Nahuis A., Heuvelink A.E., Hurmanus C., Kuijper E.J.: Prevalance of *Clostridium difficile* in retailed meat in the Netherlands. Int J Food Microbiol 2011, 144, 561–564, doi: 10.1016/j.ijfoodmicro.2010.11.007.
7. Ersöz Ş.Ş., Coşansu S.: Prevalence of *Clostridium difficile* isolated from beef and chicken meat products in Turkey. Korean J Food Sci Anim Resour 2018, 38, 759–767, doi: 10.5851/kosfa.2018.e14.
8. European Committee for Antimicrobial Susceptibility Testing (EUCAST): EUCAST Clinical Breakpoint Table v. 9.0, 2019, p. 72. https://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Breakpoint_tables/v_9.0_Breakpoint_Tables.pdf.
9. Guran H.S., Ilhak O.I.: *Clostridium difficile* in retail chicken meat parts and liver in the Eastern Region of Turkey. J Verbr Lebensm 2015, 10, 359–364, doi: 10.1007/s00003-015-0950-z.
10. Hampikyan H., Bingol E.B., Muratoglu K., Akkaya E., Cetin O., Colak H.: The prevalence of *Clostridium difficile* in cattle and sheep carcasses and the antibiotic susceptibility of isolates. Meat Sci 2018, 139, 120–124, doi: 10.1016/j.meatsci.2018.01.020.

11. Harvey R.B., Norman K.N., Andrews K., Hume M.E., Scanlan C.M., Hardin M.D., Scott H.M.: *Clostridium difficile* in retail meat and processing plants in Texas. J Vet Diagn Invest 2011, 23, 807–811, doi: 10.1177/1040638711407893.
12. Indra A., Huhulescu S., Schneeweis M., Hasenberger P., Kernbichler S., Fiedler A., Wewalka A., Allerberger F., Kuijper E.J.: Characterization of *Clostridium difficile* isolates using capillary gel electrophoresis-based PCR ribotyping. J Med Microbiol 2008, 57, 1377–1382, doi: 10.1099/jmm.0.47714-0.
13. Indra A., Lassnig H., Baliko N., Much P., Fiedler A., Huhulescu S., Allerberger F.: *C. difficile*: a new zoonotic agent? Wien Klin Wochenschr 2009, 121, 91–95, doi: 10.1007/s00508-008-1127-x.
14. Lemee L., Dhalliun A., Testelin S., Mattrat M.A., Maillard K., Lemeland J.F., Pons J.L.: Multiplex PCR targeting tpi (triose phosphate isomerase), *tcdA* (toxin A), and *tcdB* (toxin B) genes for toxigenic culture of *Clostridium difficile*. J Clin Microbiol 2004, 42, 5710–5714, doi: 10.1128/jcm.42.12.5710-5714.2004.
15. Limbago B., Thompson A.D., Greene S.A., MacCannell D., MacGowan C.E., Jolbitado B., Hardin H.D., Estes S.R., Weese J.S., Songer J.G., Gould L.H.: Development of a consensus method for culture of *Clostridium difficile* from meat and its use in a survey of U.S. retail meats. Food Microbiol 2012, 32, 448–451, doi: 10.1016/j.fm.2012.08.005.
16. Lund B.M., Peck M.W.: A possible route for foodborne transmission of *Clostridium difficile*? Foodborne Pathog Dis 2015, 12, 177–182.
17. Metcalf D.S., Costa M.C., Dew W.M.V., Weese J.S.: *Clostridium difficile* in vegetables. Canada Lett Appl Microbiol 2010, 51, 600–602, doi: 10.1111/j.1472-765X.2010.02933.x.
18. Mooyottu S., Flock G., Kollanoor-Johny A., Upadhyaya I., Jayarao B., Venkitanarayanan K.: Characterization of a multidrug resistant *C. difficile* meat isolate. Int J Food Microbiol 2015, 192, 111–116, doi: 10.1016/j.ijfoodmicro.2014.10.002.
19. Pelaez T., Alcalá L., Blanco J.L., Álvarez-Pérez S., Marín M., Martín-López A., Catalán P., Reigadas E., García M.E., Bouza E.: Characterization of swine isolates of *Clostridium difficile* in Spain: A potential source of epidemic multidrug resistant strains? Anaerobe 2013, 22, 45–49, doi: 10.1016/j.anaerobe.2013.05.009.
20. Rahimi E., Afzali Z.S., Baghbadorani Z.T.: *Clostridium difficile* in ready-to-eat foods in Isfahan and Shahrekord, Iran. Asian Pac J Trop Biomed 2015, 5, 128–131, doi: 10.1016/S2221-1691(15) 30156-8.
21. Rodriguez C., Avesani V., Van Broeck J., Taminiau B., Delmée M., Daube G.: Presence of *Clostridium difficile* in pigs and cattle intestinal contents and carcass contamination at the slaughterhouse in Belgium. Int J Food Microbiol 2013, 166, 256–262, doi: 10.1016/j.ijfoodmicro.2013.07.017.
22. Rodriguez C., Taminiau B., Van Broeck J., Avesani V., Delmée M., Daube G.: *Clostridium difficile* in young farm animals and slaughter animals in Belgium. Anaerobe 2012, 18, 621–625, doi: 10.1016/j.anaerobe.2012.09.008.
23. Rodriguez-Palacios A., Reid-Smith R.J., Staempfli H.R., Daignault D., Janecko N., Avery B.P., Martin H., Thompson A.D., McDonald L.C., Limbago B., Weese J.C.: Possible seasonality of *Clostridium difficile* in retail meat, Canada. Emerg Infect Dis 2009, 15, 802–805.
24. Romano V., Albanese F., Dumontet S., Krovacek K., Petrini O., Pasquale V.: Prevalence and genotypic characterization of *Clostridium difficile* from ruminants in Switzerland. Zoonoses Public Hlth 2012, 59, 545–548, doi: 10.1111/j.1863-2378.2012.01540.x.
25. Simango C.: Prevalence of *Clostridium difficile* in the environment in a rural community in Zimbabwe. Trans R Soc Trop Med Hyg 2006, 100, 1146–1150, doi: 10.1016/j.trstmh.2006.01.009.
26. Simango C., Mwakurudza S.: *Clostridium difficile* in broiler chickens sold at market places in Zimbabwe and their antimicrobial susceptibility. Int J Food Microbiol 2008, 124, 268–270, doi: 10.1016/j.ijfoodmicro.2008.03.020.
27. Stubbs S., Rupnik M., Gibert M., Brazier J., Duerden B., Popoff M.: Production of actin-specific ADP-ribosyltransferase (binary toxin) by strains of *Clostridium difficile*. FEMS Microbiol Lett 2000, 186, 307–312, doi: 10.1111/j.1574-6968.2000.tb09122.x.
28. Thitaram S.N., Frank J.F., Siragusa G.R., Bailey J.S., Dargatz D.A., Lombart J.E., Haley C.A., Lyon S.A., Fedorka-Cray P.J.: Antimicrobial susceptibility of *Clostridium difficile* isolated from food animals on farms. Int J Food Microbiol 2016, 227, 1–5. doi: 10.1016/j.ijfoodmicro.2016.03.017.
29. Troiano T., Harmanus C., Sanders I.M.J.G., Pasquale V., Dumontet S., Capuano F., Romano V., Kuijper E.J.: Toxigenic *Clostridium difficile* PCR ribotypes in edible marine bivalve molluscs in Italy. Int J Food Microbiol 2015, 208, 30–34, doi: 10.1016/j.ijfoodmicro.2015.05.002.
30. United States Department of Agriculture Food Safety and Inspection Service: *Salmonella* and *Campylobacter* verification program for raw meat and poultry products, Directive 10,250.1. U.S. Department of Agriculture Food Safety and Inspection Service, Washington, 2013.
31. Varshney J.B., Very K.J., Williams J.L., Hegarty J.P., Stewart D.B., Lumadue J., Venkitanarayanan K., Jayarao B.M.: Characterization of *Clostridium difficile* isolates from human fecal samples and retail meat from Pennsylvania. Foodborne Pathog Dis 2014, 11, 822–829, doi: 10.1089/fpd.2014.1790.
32. Weese J.S., Reid-Smith R.J., Avery B.P., Rousseau J.: Detection and characterization of *Clostridium difficile* in retail chicken. Lett Appl Microbiol 2010, 50, 362–365, doi: 10.1111/j.1472-765X.2010.02802.x.
33. Zidaric V., Zemljic M., Janezic S., Kocuvan A., Rupnik M.: High diversity of *Clostridium difficile* genotypes isolated from a single poultry farm producing replacement laying hens. Anaerobe 2008, 14, 325–327, doi: 10.1016/j.anaerobe.2008.10.001.