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Isolation and identification of *Clostridium difficile* from cases of diarrhea in young farm animals, and the determination of antimicrobial susceptibility

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Abstract: *Clostridium difficile* was isolated for the first time in 1935 from fecal samples of infants, although it was not until 1978 that its pathogenicity started to be considered, when it was shown to cause antibiotic-associated diarrhea and pseudomembranous colitis. In this study, it was aimed to determine the virulence and antibiotic resistance profiles of *C. dificile* in young ruminants with diarrhea and chickens fed on the farm. A total of 200 fecal samples (50 from calves, 50 from lambs and 50 from kid goats with neonatal diarrhea, as well as 50 cloacal swab samples taken from chickens) were taken and analyzed. *C. difficile* was isolated from 58 of the fecal samples (29.0%), being isolated from 35 of the fecal samples taken from calves (70.0%), 15 from lambs (30.0%), seven from kid goats (14.0%) and one from chickens (2.0%), and of these, 28 isolates were found to have toxigenic characteristics (48.2%) following species identification and toxin characterization. In the following stage, antimicrobial susceptibility tests were performed for a total of 24 toxigenic strains using the microbroth dilution method, and the toxigenic isolates were found to be resistant to ampicillin, cefoxitin, clindamycin, penicillin and tetracycline. The study identified the presence of toxigenic *C. difficile* in diarrhea cases in neonatal calves and lambs for the first time in our country.

Key words: *Clostridium difficile,* antimicrobial susceptibility test, Erzurum, neonatal, Farm animal, calf

1. Introduction

Clostridium difficile was isolated for the first time in 1935 from fecal samples taken from infants by Hall and O'Toole [1]. From the time of the first discovery of the agent right up until 1978, *C. difficile* was not considered pathogenic, but was then found to cause antibiotic-associated diarrhea and pseudomembranous colitis an infection of the colon that usually leads to necrosis and death. There has been a recent global increase in human cases of *C. difficile* associated disease (CDAD) [1].

C. difficile is a gram-positive, spore-forming, rod-shaped, obligate anaerobe bacterium that is generally motile in a liquid culture and has peritrichous flagella [2]. The vegetative form of the bacterium is sensitive to oxygen, being obligate anaerobe, while the spore form is resistant to drying, heat, disinfectants and physical agents [2,3]. The spores of the agent can maintain their viability in the environment and on surfaces [2]. It can synthesize three toxins, namely toxin A, toxin B and binary toxin (CDT), all of which play a role in the pathogenesis of *C. difficile* infections [1,4].

It has been isolated from many animal species, including sheep, pigs, chickens, goats, cattle, etc. [5], and the strains isolated from farm animals and humans are similar [6]. Farm animals are a significant source of human infections, being considered reservoirs of the disease [5,6]. While the risk of infection is high in humans of advanced age, the disease forms observed in farm animals usually appear early in life [7].

C. difficile is also a significant infection source in calves [8], being observed in the form of enterocolitis in the small and large intestine. The experimental inoculation of *C. difficile* toxins into the intestines of calves was found to cause tissue damage and neutrophil infiltration [9].

Given the threat to both animal and human health, it was considered necessary to carry out studies of farm animals to gain a better understanding of this disease, given that they are considered as reservoirs. Accordingly, the intention in the present study was to detect *C. difficile* in calves, lambs, goat kids and farm-raised chickens with diarrhea; to carry out a toxigenic characterization of these isolates; and to perform antibiogram tests on the toxigenic isolates to help in the selection of the most appropriate antibiotic.

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2. Materials and methods

2.1. Collection of fecal samples

A total of 200 fecal animal samples – 50 from calves, 50 from lambs and 50 from kid goats, all of which were aged 0–28 days and had diarrhea, as well as 50 cloacal swab samples from chickens – were collected from the Turkish provinces of Ağrı, Artvin, Erzincan, Erzurum, Gümüşhane and Iğdır between March and June of 2015, and taken into 50 mL sterile screw-cap containers**.**

2.2. Culture

Each fecal sample that was brought to the laboratory was incubated with absolute ethanol for 45 min at a ratio of 1:1 to induce "alcohol shock". A 1 mL sample of each was taken and inoculated into a *Clostridium difficile* mannitol taurocholate broth, and the inoculated media were incubated for 24 h at 37 °C under anaerobic conditions. Then, 100 µL from each liquid medium was taken and inoculated into a *Clostridium difficile* agar, and the inoculated media were then incubated for 48 h at 37 °C under anaerobic conditions [1].

After incubation, colonies of gram-positive rod-shaped bacteria with a typical colony morphology were passaged in the media [1]. The jars containing the passaged cultures were opened following 48 h of incubation, the pure colonies were inspected and 2–3 colonies of bacteria within the media that were considered to be positive were stored at –20 °C.

2.3. Identification and detection of toxin genes with PCR

The boiling method was used for the extraction of DNA from the culture-positive colonies of *C. difficile* isolates. Triose phosphate isomerase (*tpi*) was used for the species identification of *C. difficile*, while primers specific to the *tcdA* and *tcdB* genes were used to detect the presence of toxin A and toxin B. A multiplex polymerase chain reaction was then performed in line with the approach described by Lemee et al. [10]. The method described by Person et al. [11] was used to identify the presence of the *C. difficile* binary toxin in the isolated samples, and the presence of *cdtA* and *cdtB* genes, which are responsible for the production of binary toxin, was investigated. After the PCR, amplicons were subjected to 1.5% agarose gel electrophoresis, after which, the gel was inspected in a transilluminator.

2.4. Antimicrobial susceptibility test

A Sensititre Anaerobic Susceptibility Testing Kit (Trek Diagnostic Systems, Thermo Fisher Scientific,Waltham, MA, USA) was used to test for the antimicrobial susceptibility of the toxigenic *C. difficile* strains, with all analyses carried out in accordance with the kit protocol. The results of the antimicrobial susceptibility test were compared with EUCAST and CLSI standard MIC values [12,13].

3. Results

3.1. Culture results

C. difficile was isolated from 70.0% of the fecal samples taken from calves (35), 30.0% of the fecal samples taken from lambs (15), 14.0% of the fecal samples taken from kid goats (7) and 2.0% of the fecal samples taken from chickens (1). Overall, the agent was isolated from 29.0% of the fecal samples (58) in the study (Table 1).

3.2. Identification and detection of toxin genes with PCR

A polymerase chain reaction was used for the molecular identification of the strains identified using the bacteriological culture method. Among the strains isolated from the fecal samples of calves, lambs, kid goats and chickens, a total of 58 isolates with suspected *C. difficile* were identified as such from the *tpi* gene. Following the species identification, 28 of the isolates (48.2%) were found to have toxigenic characteristics (Figure 1).

3.3. Antimicrobial susceptibility test

The microbroth dilution method was used to determine the antimicrobial susceptibility of the 28 isolates with toxigenic characteristics. The antibiotic concentrations of the well in which the last bacterial growth was identified was accepted as the minimum inhibitory concentration (MIC). During the analyses, contamination was detected on the antibiogram microplates of four strains, and these

Table 1. *C. difficile* isolation in the samples and its toxigenic characteristics.

	Total sample	Positive sample (%)	Toxigenic isolate			
			Toxigenic positive (%)	A+B+CDT+	A–B+CDT–	A–B–CDT+
Calf	50	35 (70)	22 (44)	10	12	0
Lamb	50	15 (30)	6 (12)	0	1	5
Kid goat	50	7 (14)	0 (0)	0	0	0
Chicken	50	1 (2)	0 (0)	0	0	0
Total	200	58 (29)	28 (14)	0	0	0

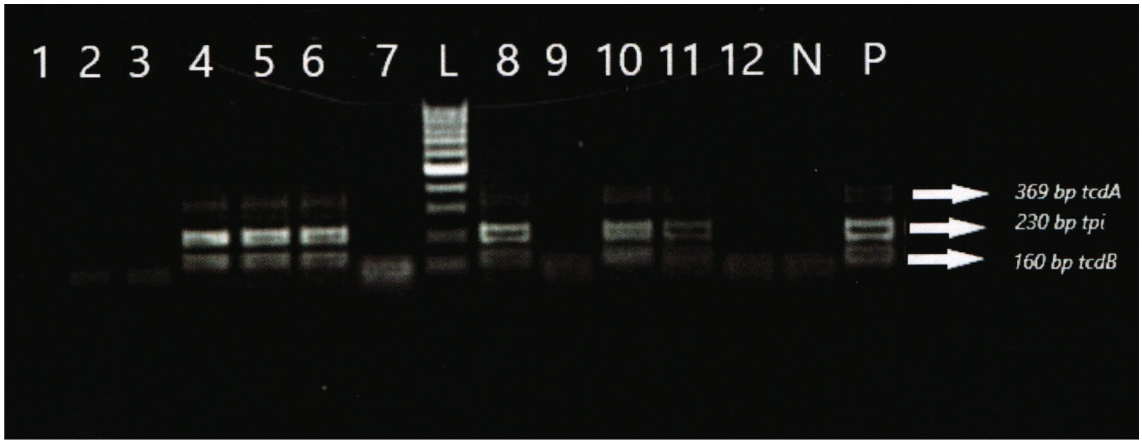

Figure 1. Identification and toxin characterization of *C. difficile* (L: DNA Ladder 100–1000 bp).

were excluded from the assessment. The MIC50 and MIC90 values of the strains that were subjected to antimicrobial susceptibility tests, as well as the susceptibility-resistance percentages, are presented in Table 2.

4. Discussion

C. difficile is a bacterium that has been threatening human health in many countries around the world since 1978, leading also to economic losses, and is resistant to many antimicrobial agents. Treating an infection with antibiotics can lead to an infection in the intestine, particularly in the colon, due to the resistance of the agent to antibiotics, and the infection becomes more severe as antibiotic use continues [1,14]. In the present study we sought to isolate and identify *C. difficile* in the fecal samples of calves, lambs and kid goats with diarrhea, and in the cloacal swab samples of adult chickens raised on the same farm as the mentioned animals, to carry out a toxigenic characterization, and to investigate the antimicrobial susceptibility of toxin-producing isolates. Consequently, the presence of toxigenic *C. difficile* wasidentified in young farm animals.

Previous studies of calves have reported *C. difficile* isolation rates in the 1.7%–61.0% range [6,15–22], which is consistent with the results of the present study,in which the isolation rate of *C. difficile* was recorded as 70.0%, although in the earlier studies reporting on the isolation of *C. difficile* in the fecal samples of calves, the positivity rates were found differ from those of the present study, which may be attributed to such factors as the conditions under which the samples were stored, the ages of animals sampled, the prior use of antibiotics, the sampling season, the isolation method used, etc. [18,19,22].

No study was identified investigating the role of *C. difficile* in the etiology of diarrhea in lambs or kid goats. Being considered a zoonotic disease, studies of small ruminants have targeted the detection of shedding of the agent for human health. Previous studies have reported isolation rates of *C. difficile* in small ruminants of 6.5%–7.7% in lambs [23,24], 10.1% in kid goats [23], 0%–18.2% in sheep [17,18,24–26] and 0%–7.5% in goats [17,25,27]. In the present study, *C. difficile* was isolated and identified in 30.0% of the fecal samples of neonatal lambs with diarrhea, and isolated and identified in 14.0% of the fecal samples of neonatal kid goats with diarrhea. The highest *C. difficile* shedding rate was noted between the ages of 0 and 16 days [23]. The early isolation rates in lambs and kid goats with diarrhea in the present study were found to be higher than those reported by Avbersek et al. [23] and Knight and Riley [24], which was attributed to the greater bacterial shedding in neonatal animals than in adults. It has been further suggested that the different results between studies may be due to such factors as dietary changes, husbandry conditions, flock density, etc. [24].

C. difficile isolation rates of 1.6%–62.2% have been reported in fecal samples taken from chickens [7,17,18,26]. In the present study, *C. difficile* was identified in 2.0% of the 50 cloacal swab samples taken from adult chickens raised on large or small ruminant farms, and the isolation rate in chickens was found to concur with similar studies [18,26,28]. The different isolation rates reported by different studies may be attributed to geographical differences, temporal differences in terms of the prevalence of *C. difficile*, the difference in the ages of animals, etc. [18]. Hussain et al. [17] isolated *C. difficile* in broilers raised on commercial farms, but did not isolate the agent from free-range chickens. In their study of *C. difficile* shedding in chickens, Zidaric et al. [7] reported that the presence of bacteria in feces decreased with age.

In the present study, *C. difficile* was isolated from 70.0% (35/50) of the fecal samples of calves, and 44.0% of the strains were toxigenic, compared to the 12.0% that were toxigenic in the fecal samples of lambs. In similar studies, toxin-producing *C. difficile* was reported to be isolated

Table 2. Antimicrobial susceptibility test results.

C. difficile (n = 24)							
	MIC						
	MIC50	MIC90	Range		S %	I %	R %
SAM	0.5/0.25	1/0.5	0.5/0.25–4/2		100 (≤8/4)	0 (16/8)	0 (≥32/16)
AMC	0.5/0.25	4/2	0.5/0.25–4/2		100 (≤4/2)	0 (8/4)	0 (≥16/8)
AMP	1	16	0.5 – >16		25 (≤0.5)	25 (1)	50 (≥2)
CTT	4	32	4–32		83.4 (≤16)	16.6 (32)	0 (≥64)
FOX	>32	>32	32 – >32		0 (≤16)	8.3 (32)	91.7 (≥64)
CHL	4	8	2–16		91.7 (≤8)	8.3 (16)	0 (≥32)
CLI	4	>8	4 – >8		0 (≤2)	16.6 (4)	83.4 (≥8)
IPM	0.5	2	0.12–2		100 (≤4)	0 (8)	0 (≥16)
MEM	2	8	0.5–8		83.4 (≤4)	16.6 (8)	0 (≥16)
MTZ	0.5	1	0.5–1		100 (≤8)	0 (16)	0 (≥32)
MEZ	8	8	4–16		100 (≤32)	0 (64)	0 (≥128)
PEN	2	4	0.5–4		8.3 (≤0.5)	33.3 (1)	58.3 (≥2)
PIP	8	16	4–16		100 (≤32)	0 (64)	0 (≥128)
TZP	8/4	16/4	0.5/4–16/4		100 (≤32/4)	0 (64/4)	0 (≥128/4)
TET	8	>8	0.25 – >8		41.6 (≤4)	41.6 (8)	16.6 (≥16)

SAM: ampicillin-sulbactam, AMC: amoxicillin-clavulanic acid, AMP: ampicillin, CTT: cefotetan, FOX: cefoxitin, CHL: chloramphenicol, CLI: clindamycin, IPM: imipenem, MEM: meropenem, MTZ: metronidazole, MEZ:mezlocillin, PEN: penicillin, PIP: piperacillin, TZP: piperacillin/tazobactam, TET: tetracycline, S: susceptible, I: intermediate, R: resistant.

from 10.2%–42.7% of the fecal samples of calves, and from 1.0%–18.2% of the fecal samples of lambs, concurring with the results of other studies [18,21,24,26]. In similar studies reporting on a toxin analysis of *C. difficile* isolates taken from the fecal samples of calves, lambs and kid goats, the isolation of *C. difficile,* which can produce A–B–CDT+, A+B+CDT–, A+B+CDT+, A–B+CDT–, A–B+CDT+ or A+B–CDT+ toxins, was reported [6,15,16,18–22,24]. In the present study, A+B+CDT+ and A–B+CDT– toxins were found during toxin production analyses of *C. difficile* isolates from the fecal samples of calves, while *C. difficile* isolates from the fecal samples of lambs were found to be strains that could produce A–B+CDT– and A–B–CDT+ toxins. The results of the present study were considered to be consistent with those of similar studies.

The toxigenic isolates were found in the present study to be resistant to ampicillin, cefoxitin, clindamycin, penicillin and tetracycline, while no resistance was found against the other antimicrobial agents analyzed. In this study of *C. difficile* isolates taken from farm animals, resistance to penicillin was identified in 58.3% of the total 24 analyzed isolates. In studies investigating the effect of penicillin on *C. difficile*, a resistance of between 38.5% and 40.0% was reported [23,29]. Resistance to antibiotic ampicillin from among the beta-lactams was found to be 50.4% in toxigenic *C. difficile* isolates. The resistance to ampicillin in *C. difficile* isolates from farm animals was found to be between 6.8% and 20.8% in similar studies [23,29,30]. In the present study, 83.4% of the toxin-producing *C. difficile* isolates from calves and lambs were found to be resistant to clindamycin, while in other studies, resistance to clindamycin was found in the range of 10.0%–90.9% [23,29,30]. A total of 16.6% of the isolates obtained within the present study were demonstrated to be resistant to the antibiotic tetracycline, which has a bacteriostatic effect, while resistance to tetracycline was reported in the range of 1.9%–93.0% in earlier studies [15,16,29,30]. Some 91.7% of the isolates were found to be resistant to antibiotic cefoxitin, which is another member of the cephalosporin family that was analyzed within the study. Resistance was reported between 97.9% and 100% to cefoxitin among the *C. difficile* isolates from farm animals [23,29]. The resistance profiles of toxin-producing *C. difficile* are very important in the identification of treatment protocols.

In isolates from young animals, no resistance to metronidazole, which is a substance that can be used in

the treatment of human *C. difficile* infections prior to vancomycin, was identified. It has been reported that the resistance of *C. difficile* to metronidazole is increasing. In similar studies, the resistance to metronidazole has been reported as between 0% and 24.9% [23,29,30], and thislow resistance to metronidazole in *C. difficile* isolates can be considered an important finding for the treatment of human infections.

This study, which is the first to analyze toxin-producing *C. difficile* strains isolated from fecal samples taken from neonatal calves, lambs and kid goats with diarrhea, and from chickens raised on the same farm, identified the presence of toxigenic *C. difficile* strains in the fecal samples of calves and lambs. *C. difficile* is considered pathogenic in humans, pigs and horses around the world, and has been reported by many researchers to play a role in the etiology of calf diarrhea. The isolation of pathogenic strains from neonatal diarrhea cases supports the suggestion that there are agents that play a role in the etiology of this disease. According to the findings of studies carried out in this regard, it can be concluded that *C. difficile* should be taken into account when making a microbiological analysis of diarrhea in neonates, particularly in cases that respond poorly to antibiotic treatment, and to identify whether the strains are toxigenic when the agent is isolated.

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