

Occurrence of *Clostridium difficile* in Raw Bovine, Ovine, Caprine, Camel and Buffalo Milk in Iran

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Summary

This study was conducted to determine the prevalence of *Clostridium difficile* in raw milk in Iran. From January to August 2013, a total of 430 raw milk samples from bovine (n=135), ovine (n=100), caprine (n=80), buffalo (n=49) and camel (n=66) were purchased from randomly selected from 111 dairy farm in Iran and were evaluated for the presence of *C. difficile*. In this study, only 2 of 135 bovine milk samples (1.43%) were contaminated with *C. difficile*. One of the two *C. difficile* strains was positive for *tcdA* and *tcdB* toxin genes that was classified as ribotype 078. Susceptibilities of isolates were determined for 11 antimicrobial drugs using the disk diffusion assay. None of the isolates was resistant to vancomycin, metronidazole, chloramphenicol and tetracycline. To our knowledge, this study is the first report of direct identification of *C. difficile* in bulk milk samples from dairy herds in Iran and the first report of direct identification of *C. difficile* in bulk milk samples from dairy bovine herds.

Keywords: *Clostridium difficile*, Raw milk, Camel, Buffalo, Bovine, Antimicrobial resistance

İran'da Sığır, Koyun, Keçi, Deve ve Manda Çiğ Sütlerinde *Clostridium difficile*'nin Tespiti

Özet

Bu çalışma İran'da çiğ sütlerde *Clostridium difficile*'nin prevalansını belirlemek amacıyla yapılmıştır. Çalışmada, rastgele seçilmiş 111 süt çiftliğinden toplanmış sığır (n=135), koyun (n=100), keçi (n=80), manda (n=49) ve deve (n=66) toplam 430 çiğ süt örneği *C. difficile*'nin varlığını ortaya koymak amacıyla incelendi. Çalışmada, sığır süt örneklerinden 135'inden sadece 2'sinde (1.43%) *C. difficile* kontaminasyonu tespit edildi. İki *C. difficile* suşundan birisi ribotip 078 olarak sınıflandırılan *tcdA* ve *tcdB* toksin genine pozitiflik gösterdi. Disk difüzyon testi kullanılarak 11 antimikrobiyal ilaca karşı izolatların hassasiyetlikleri belirlendi. İzolatların hiçbiri vankomisin, metronidazol, kloramfenikol ve tetrasikline karşı dayanıklı değildi. Bilgimiz dahilinde, bu çalışma İran'da sütçü sürülerden ve sığırlardan elde edilen ham süt örneklerinde *C. difficile*'nin direkt olarak tespit edildiği ilk çalışmadır.

Anahtar sözcükler: *Clostridium difficile*, Çiğ süt, Deve, Manda, Sığır, Antimikrobiyal rezistans

INTRODUCTION

Clostridium difficile is recognised as a nosocomial pathogen associated with antimicrobial drug-associated diarrhoea and pseudomembranous colitis in humans and the infection is believed to be acquired nosocomially [1-3]. The antimicrobial agents most frequently associated with *Clostridium difficile*-associated disease (CDAD) include clindamycin, cephalosporins and ampicillin but almost all antibiotics can cause the disease [1]. *C. difficile* has also been

shown to be an important pathogen causing diarrhoea in humans in communities outside hospital environments [2,3].

The main virulence factors that are currently recognized are two large clostridial toxins, toxin A (*TcdA*, an enterotoxin) and toxin B (*TcdB*, a cytotoxin) [4]. A third, large, unrelated toxin, designated *C. difficile* binary toxin (CDT), can also be produced by some strains [4]. The role of binary toxin



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in disease is currently unclear [3,5], but there is information suggesting that this toxin may be clinically relevant [6].

C. difficile also appears to be an important cause of enteric disease in a wide variety of animal species [7-10], suggesting that animals and humans may share a common source [11,12]. In accordance herewith, recent reports show a remarkable overlap between isolates from animals and humans [13]. Food animals are an important source of human enteropathogenic micro-organisms and can be spread to humans through consumption of foods of animal origin. In recent studies *C. difficile* has been isolated from food animals such as poultry, sheep, pigs, goats and cattle [8,14,15].

Moreover, molecular typing of *C. difficile* isolates from calves and humans has shown similarities in PCR ribotypes from the two species including two PCR ribotypes associated with outbreaks of severe disease in humans [16], suggesting that cattle or other animals may be reservoirs of *C. difficile* for humans.

The epidemiology of CDI in Iran is essentially unknown, and to the authors' knowledge, the prevalence rate of *C. difficile* in foodstuff in Iran has never been reported. The aim of this study was to determine the occurrence of *C. difficile* in raw cow, ovine, caprine, buffalo and camel milk in Iran.

MATERIAL and METHODS

Sample Collection

Overall, 111 bovine, ovine, caprine, buffalo and camel herds were randomly selected in Isfahan, Chaharmahal va Bakhtiari, Khuzestan provinces, Iran. From January to August 2013, a total of 135 bovine bulk milk samples from 36 commercial dairy herds, 100 and 80 ovine and caprine bulk milk samples from 31 and 19 sheep and goat breeding farms, herds 49 buffalo bulk milk samples from 13 dairy buffalo herds and 66 camel bulk milk samples from 12 dairy camel were collected. The animals whose milk samples collected for this study were clinically healthy and the milk samples showed normal physical (color, pH, and density) characteristics. The samples were immediately transported to the laboratory in a cooler with ice packs and were processed within 12 h of collection.

Isolation and Identification of *C. difficile*

The samples were processed immediately upon arrival using aseptic techniques. The detection and isolation method used was based on the method described by Rodriguez-Palacios et al. [17] and de Boer et al. [18]. Briefly, 5 mL of each sample was transferred to 20 mL of *C. difficile* broth (CDB), containing *C. difficile* selective supplement (Oxoid SR0173) and 5% (v/v) defibrinated sheep blood. After incubation at 37°C for 10 to 15 days under anaerobic conditions 2 mL of the enrichment was added to 2 mL of 96% ethanol in a centrifuge tube and homogenized for 50

min on a shaker. After centrifugation (3800×g for 10 min), a loopful of material from the sediment was streaked onto *C. difficile* agar base (Oxoid CM0601) supplemented with an antibiotic supplement for the selective isolation of *C. difficile* (Oxoid SR0173) and 7% (v/v) defibrinated sheep blood and the plates were incubated for 48 h at 37°C, under anaerobic conditions. Three colonies per plate were subcultured onto tryptone soya agar (Oxoid CM0131) and tested by standard microbiological and biochemical procedures [8]. Crudely extracted DNA (boiling, 10 min) was used for PCR confirmation (housekeeping *tpi* gene detection), determination of toxin gene (*tcdA*, *tcdB* and *cdtB*), and PCR ribotyping of isolates as performed in previous studies [16,19].

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed by the Kirby-Bauer disc diffusion method using Mueller-Hinton agar (HiMedia Laboratories, Mumbai, India) according to the Clinical Laboratory Standards Institute [20] as has been previously described [8,10]. The antimicrobial agents tested and their corresponding concentrations were as follows: nalidixic acid (30 µg), ciprofloxacin (5 µg), erythromycin (15 µg), tetracycline (30 µg), doxycycline (30 µg), gentamicin (10 µg), metronidazol (5 µg), ampicillin (10 µg), chloramphenicol (30 µg), vancomycin (30 µg), and clindamycin (2 µg). After incubating the inoculated plate for 48 h at 37°C, under anaerobic conditions, the susceptibility of the *C. difficile* to each antimicrobial agent was measured and the results were interpreted in accordance with interpretive criteria provided by CLSI [20].

RESULTS

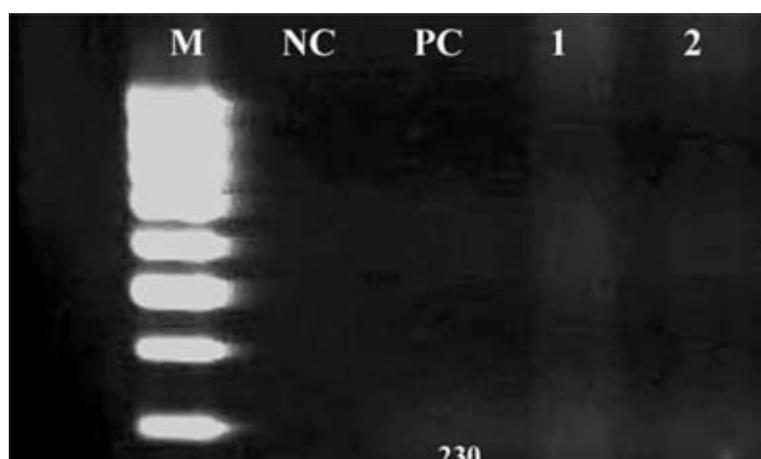
In the present study, a total of 430 bulk milk samples from 111 dairy bovine, ovine, caprine, buffalo and camel herds in Isfahan, Chaharmahal va Bakhtiari and Khuzestan provinces of Iran were tested for *C. difficile*. In this study, 2 of 135 (1.48%) bovine milk samples were positive (Table 1). The positive samples were from 1 of 36 (2.78%) commercial dairy herds (Fig. 1). One of the *C. difficile* isolated was positive for *tcdA* and *tcdB* toxin genes and was classified as ribotype 078.

All 100 ovine bulk milk samples from 31 sheep breeding farms, 80 caprine bulk milk samples from 19 goat breeding farms, 49 buffalo bulk milk samples from 13 buffalo breeding farms and 66 camel bulk milk samples from 12 camel breeding farms were negative.

In this study, the antimicrobial resistance pattern of two *C. difficile* isolates was tested to 11 antimicrobial agents (Table 2). All two isolates were resistant to clindomycin, gentamycin and nalidixic acid. No resistance to chloramphenicol, metronidazole, tetracycline and vancomycin was observed.

Table 1. Prevalence of *Clostridium difficile* detected in bovine, ovine, caprine, camel and buffalo milk samples**Table 1.** Sığır, koyun, keçi, deve ve manda süt örneklerinde tespit edilen *Clostridium difficile* prevalansı

Meat Sample	No. of Samples	No. of <i>C. difficile</i> -Positive Samples	No. of Isolates Positive for Toxins			Ribotype 078
			tcdA	tcdB	cdtB	
Bovine	135	2 (1.48%)	1	1	-	1
Ovine	100	0 (0.0%)	-	-	-	-
Caprine	80	0 (0.0%)	-	-	-	-
Buffalo	49	0 (0.0%)	-	-	-	-
Camel	66	0 (0.0%)	-	-	-	-
Total	430	2 (0.47%)	1	1	-	1

**Fig 1.** Electropherogram of the amplification products of the polymerase chain reaction (PCR) assay. M, 100 bp DNA ladder; lane 1, negative control (NC); lane 2, positive control (PC); lanes 3 and 4, *Clostridium difficile* positive milk samples**Şekil 1.** Zincirleme Polimeraz Reaksiyonunda amplifikasyon ürünlerinin elektroferogramı. M, 100 bp DNA merdiveni; sütun 1, negatif kontrol (NC); sütun 2, pozitif kontrol (PC); sütun 3 ve 4, *Clostridium difficile* pozitif süt örnekleri**Table 2.** Antimicrobial resistance of two *Clostridium difficile* isolated from raw milk**Table 2.** Çiğ sütlerden izole edilen 2 *Clostridium difficile*'nin antimikrobiyal rezistansı

Antimicrobial agent	Sensitive	Intermediate	Resistant
Ampicillin	0 (0.0%)	1 (50.0%)	1 (50.0%)
Chloramphenicol	2 (0.0%)	0 (0.0%)	0 (0.0%)
Ciprofloxacin	1 (50.0%)	1 (50.0%)	0 (0.0%)
Clindamycin	0 (0.0%)	0 (0.0%)	2 (100%)
Doxycycline	1 (50.0%)	1 (50.0%)	0 (0.0%)
Erythromycin	1 (50.0%)	1 (50.0%)	0 (0.0%)
Gentamicin	0 (0.0%)	0 (0.0%)	2 (100%)
Metronidazole	2 (100%)	0 (0.0%)	0 (0.0%)
Nalidixic acid	0 (0.0%)	0 (0.0%)	2 (100%)
Tetracycline	2 (100%)	0 (0.0%)	0 (0.0%)
Vancomycin	2 (100%)	0 (0.0%)	0 (0.0%)

DISCUSSION

Recent reports indicate that a large proportion of CDAD are not linked to recent antibiotic therapy, older age, significant comorbidity or previous hospitalization. Possible community sources for CDAD include animals and food, and therefore a surveillance study on the prevalence of *C. difficile* in MILK was performed. In total, only 2 of 430

raw milk samples (0.47%) were found to be contaminated with *C. difficile*. The positive samples were from 2 of 135 (1.48%) bovine milk samples. This result is similar to a recent report in Austria that showed, all 50 raw bulk milk samples were negative for *C. difficile* [21]. The results of this study show that raw milk is not an important source for *C. difficile* infection. Nevertheless, it could also indicate that the extent of *C. difficile* contamination is below the detection limit of the method used. In a recently published Swedish study, the total spore count in milk varied between 10^2 and 2×10^2 spores per litre of raw milk [22]. Since the mass of spores originated from aerobic spore formers or clostridia species other than *C. difficile*, the negative results could also be explained by the very low number that was likely to be present, or even suboptimal culture conditions.

The source of *C. difficile* in food products is unclear. Contamination of milk might be due to *C. difficile* residing in the gastro-intestinal tract of animals, but could also originate from the hands of personnel during milking, milk processing equipment. The prolonged survival of *C. difficile* spores in the environment increases the possibilities for contamination of animals and foods.

One of the *C. difficile* isolated in this study that were positive for *tcdA* and *tcdB* toxin genes was classified as ribotype 078. Similar results were reported in other studies [7,23]. Ribotype 078 has been associated with hyper-virulent properties [13], a toxin regulatory gene, and which is

often associated with food animals [24]. It is also increasingly associated with community-onset *C. difficile* infection [25]. Contamination of food with this ribotype suggests a possible human health concern. Ribotype 078 has been reported to be the predominant type in food animals such as cattle and pigs [9,24], and other food sources [3,25].

None of the isolates was resistant to vancomycin, metronidazole, chloramphenicol and tetracycline. Vancomycin and metronidazole, are the most commonly used to treat *C. difficile* diarrhoea. The isolates were resistant or intermediately resistant to clindomycin, gentamycin, nalidixic acid ciprofloxacin, erythromycin, and ampicillin. These results are comparable to those reported by other investigators [15,21,26]. The results of antimicrobial resistance found in this study are correlated with antibiotics usage to treat infections in food animals in Iran.

To our knowledge, the present study is the first report of direct identification of *C. difficile* in bulk milk samples from dairy bovine in Iran. Although no extensive prevalence study was undertaken, the results of this study indicate that clinically healthy cow can be sources of *C. difficile* infection. The present results also suggest that the bulk tank milk, which is easy and inexpensive to collect, could be used to assess, on a larger scale at a low cost, the efficiency of control schemes aimed at controlling and/or preventing *C. difficile* shedding in dairy herds.

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