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Occurrence of Toxigenic *Clostridium difficile* among Diarrheic Sheep and Goats in Rural Settings: Public Health Concern

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ABSTRACT

Clostridium difficile is a global pathogen with great public health concern. This study was conducted to inquire into the presence of *C. difficile* amongst diarrheic sheep and goats in rural environments. Fecal samples were collected from 60 diarrheic animals (36 sheep and 24 goats). Samples were cultivated for isolation of *C. difficile* using selective medium while suspected colonies were confirmed by both serological and molecular techniques. Afterwards, toxigenic *C. difficile* isolates were recognized using PCR after finding *tcdA* and *tcdB* genes encoding toxin A and toxin B, respectively. The overall incidence of *C. difficile* was 20% (12/60) whereas the prevalence rates were 19.4% and 20.8% for sheep and goats, respectively. Only 3/12 isolates were toxigenic, two isolates (one from sheep and another from a goat) were *tcdA*-positive, but only one isolate from sheep was *tcdB*-positive. The phylogenetic analysis of the obtained *tcdA* gene sequence from sheep revealed that this sequence was grouped in the same clade with those isolated from beef, pig and human case. In conclusion, the current study highlights the occurrence of toxigenic *C. difficile* strains among diarrheic sheep and goats, a matter which has a high concern in both veterinary medicine and public health.

Key words: *C. difficile*, Sheep, Goats, Rural settings

INTRODUCTION

Clostridium difficile was initially isolated in 1935 from stool of clinically healthy new-born (Hall and O'toole 1935). However, *C. difficile* was thought to be a harmless commensal in human guts, it may stand behind severe cases of diarrhea in some people (Weese 2020). *C. difficile* is a Gram-positive, strictly anaerobic, spore forming, rod shaped bacterium (Troiano et al. 2015). The long-standing antibiotics usage in humans and animals depletes the normal gut microflora allowing *C. difficile* to colonize the intestine and induce disease (Thitaram et al. 2016).

Generally, the virulence of *C. difficile* is referred to its ability to produce toxins. Two major exotoxins, toxin A (enterotoxin) and toxin B (cytotoxin) encoded by the tcdA and tcdB genes, respectively, are responsible for the intestinal damage and the clinical symptoms induced by *C. difficile*. Furthermore, some *C. difficile* strains have dual toxin (ADP-ribosyltransferase) encoded by cdtA and cdtB genes (Hampikyan et al. 2018).

The epidemiology and transmission of *C. difficile*, generally for community-associated infections, are not completely understood. Recently, several studies confirmed the isolation of *C. difficile* from diseased and apparently healthy food animals such as cattle, sheep and pigs (Rodriguez et al. 2013). However, there have been no confirmed cases of any foodborne illness caused by *C. difficile*, its occurrence in the intestinal tracts of livestock highlights the significant role of animals in the contamination of food products, environment and thereby *C. difficile* finds its way to human guts. Moreover, direct transmission of *C. difficile* from animals to humans through

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fecal oral route cannot be ruled out. Therefore, attention to such animals should be paid because they may be potential reservoirs for *C. difficile* infections to humans (Candel-Pérez et al. 2019).

Sheep and goats are important components of rural environment elsewhere; they are either grazing animals passing long distances every day or common farm animals. They are reared for meat and milk productions and usually kept in a close association with humans in such settings (Ghoneim et al. 2010). Accordingly, such circumstances give a lot of opportunity to sheep and goats to pass different pathogens to humans. However, there is a paucity of studies on the occurrence of *C. difficile* among small ruminants whilst, the contribution of *C. difficile* as a cause of diarrhea among sheep and goats is poorly understood (Weese 2020).

Therefore, this work is aimed to study the occurrence of *C. difficile* in diarrheic sheep and goat in rural settings and to detect the toxigenic strains of *C. difficile* among the examined animals in order to provide more knowledge to better combat such pathogen in veterinary medicine, a matter which may have a great public health impact in such settings.

MATERIALS AND METHODS

Collection of Samples

All procedures of samples collection were compatible with guidelines approved by the Ethics of Animal Experiments Committee, Faculty of Veterinary Medicine, Cairo University, Egypt.

Fecal samples were collected from 60 animals (36 sheep and 24 goats) showing diarrhea. Samples were gathered from the recta of animals during clinical examination and were put in germ-free cups to be transported in icebox as early as possible to the laboratory for bacteriological examination.

Isolation and Identification of *Clostridium difficile*

About one gram of each fecal sample was pre-enriched in 5 ml brain, heart infusion (BHI) broth (Oxoid, Basingstoke, UK) and incubated for 10 days at 37°C in anaerobic jars. After the enrichment step, 500µL of cultured broth was treated with an equal volume of absolute ethanol for 30min (Romano et al. 2012). Then after, a loopful is plated on Clostridium difficile specific media (LIOFILCHEM, Italy) with *Clostridium difficile* supplement (D-cycloserine 125mg, cefoxitin 4mg and 5% defibrinated sheep blood as manufacturer mentioned. The plates were then incubated anaerobically at 37°C for 48hrs. Suspected colonies with characteristic morphology and odor were examined by Gram's strain films and were subjected to latex agglutination test using *Clostridium difficile* latex kit, (Liofilchem, Italy) according to the manufacturer's guidelines for further identification (Cheong et al. 2017).

Molecular Confirmation of *Clostridium difficile* and Identification of Toxigenic Strains

All suspected *C. difficile* isolates were verified by PCR for molecular confirmation. The bacterial genomic DNA was extracted from the obtained isolates using the G-spin genomic DNA extraction kit (iNtRON Biotechnology, Korea), the extraction procedure was done according to manufacturer's guidelines. The extracted DNA was stored at -80°C till use.

Molecular Confirmation of *Clostridium difficile*

DNAs from the obtained isolates were enrolled in PCR to detect 16SrRNA gene of *C. difficile* using set of specific primers as described earlier (Zhang et al. 2016). The reaction was performed using PreMix Kit with high purity pfu DNA polymerase with low error rate (Maxime™ PCR PreMix (i-pfu) iNtRON Biotechnology, Korea). The PCR cycling conditions were initial denaturation for 1 min at 95°C, then 40 cycles of denaturation (95°C for 20s), annealing (44°C for 20s) and extension (72°C for 30s), followed by final extension step at 72°C for 7min. The PCR products were entered electrophoresis step to identify the specific band at 270 bp (Fig. 1).

Detection of Toxigenic *C. difficile strains*

The toxigenic strains of *C. difficile* were identified by PCR using specific primer sets targeting tcdA and tcdB genes (Cohen et al. 2000). PCRs were performed with the following thermal profile: Initial denaturation at 95°C for 3min, then 40 cycles of denaturation 95°C for 20s, annealing at 53℃ for 25s; 49℃ for 20s for tcdA and tcdB genes, respectively and extension at 72°C for 1min and 45s for tcdA and tcdB genes respectively, whereas final extension step at 72°C for 7min. Amplicons were enrolled in gel electrophoresis and specific bands were noted at 602 bp and 399 bp for tcdA and tcdB genes, respectively.

Sequencing Step

Two PCR products (one for tcdA gene and another for tcdB gene) were enrolled in gene sequencing. The amplicons were purified using a Qiaquik purification kit (Qiagen, Germany) after that whereas the sequencing was conducted using Big Dye Terminator V3.1 Cycle sequencing Kit (Applied Biosystems). The obtained sequences were compared with those available at GenBank through BLAST analysis.

GenBank Accession Numbers

The obtained sequences from the current study for tcdA and tcdB genes were deposited in the GenBank under the following accession numbers: MT497381 and MT497382 for tcdA and tcdB genes, respectively.

Phylogenetic Analysis of the Obtained *tcdA* Gene Sequence

After BLAST analysis, the obtained tcdA gene sequence in the current study of sheep isolate was aligned against the most similar ones from humans and animal origin. The alignment was done using BioEdit software version (7.0.9) whereas the phylogenetic tree was constructed through neighbor-joining method using Mega7 software version 7.0.26 (Fig. 2).

RESULTS AND DISCUSSION

Out of 60 examined animals 12 produced *C. difficile* in their feces with a total isolation rate 20%. Whilst, the prevalence rates among diarrheic sheep and goats were 19.4% and 20.8% respectively. The isolation rate among

diarrheic sheep and goats under 3 months' age was higher than that among animals with 3-6 months of age, whereas none of the examined animals above 6 months yielded positive results Table 1. Furthermore, out of 12 *C. difficile* isolates, 3 were positive for tcdA or tcdB genes giving a percentage 25%, two isolates (one from sheep and another from a goat) were tcdA-positive but only one isolate from sheep was tcdB-positive.

Table 1: Distribution of *C. difficile* among examined animals with different age range

Animal age	Animals examined	Isolation rate	
		No. of positive	%
2 weeks- 3 months	37	9	15
3 months- 6 months	18	3	5
>6 months	5	0	0
Total	60	12	20

C. difficile is a common enteric pathogen causing diarrhea in people and animals, especially those kept on long antibiotic course. Several studies have studied the epidemiology of *C. difficile* in people and animals, however, the data about the occurrence of *C. difficile* in small ruminants especially sheep and goat is scanty (Weese 2020).

In this study, we investigated the presence of *C. difficile* among diarrheic sheep and goats in rural setting. The overall incidence of *C. difficile* among the examined animals was 20% with prevalence rates 19.4% and 20.8% among sheep and goats, respectively. Such results were higher than those obtained by Avberšek et al. (2014) in Slovenia who recorded isolation rates (based on cultivation and molecular results) 5.7% and 9.2% among sheep and goats, respectively. Moreover, the results of the current study are higher than those reported by (Esfandiari et al. 2015) in Iran who found that prevalence rates in fecal samples were 2% (1/50) and 8% (2/25) in sheep and goats, respectively. Also, Romano et al. (2012) isolated *C. difficile* from 7.5% (3/40) of goats irrespective to age, a result which is lower than that reported here.

On the other hand, the prevalence rate reported in the current study for the examined sheep was nearly similar to that reported earlier (Ismael et al. 2019) in Egypt who identified *C. difficile* in 14.29% (4/28) among the examined diseased sheep. However, on the contrary to our results they could not detect *C. difficile* among examined goats even among the diseased ones.

Importantly, the highest isolation rate was appeared in young animals (15%) below 3 months' age, whereas none of examined adult ones (more than 6 months) yielded positive result. Such results were comparable with those obtained by (Knight and Riley 2013) who referred to the detection rate in in lambs (6.5%) was higher than that in sheep (0.6%) and augmented by (Avberšek et al. 2014) who mentioned that none of the examined adult sheep/goats were positive for *C. difficile*.

The high occurrence of *C. difficile* reported in the current study may be attributed to that the vast majority of the examined animals were lambs and kids which are more susceptible to colonization of *C. difficile* because of the relatively ill-developed microflora (Hammitt et al. 2008; Kachrimanidou et al. 2019).

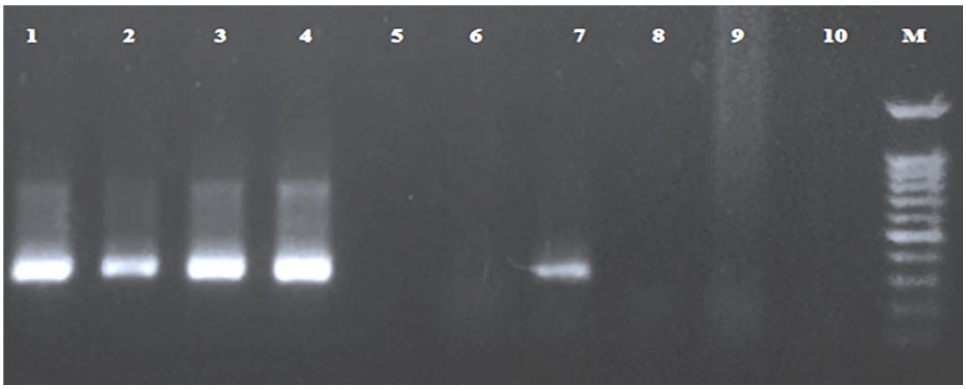

Fig. 1: Molecular detection of *Clostridium difficile* 16SrRNA gene: Lanes 1,2,3,4,7 positive samples with specific bands at 270bp; lanes 5,6,8,9 negative samples; lane 10 negative control (nuclease free water), lane M, DNA ladder 100bp.

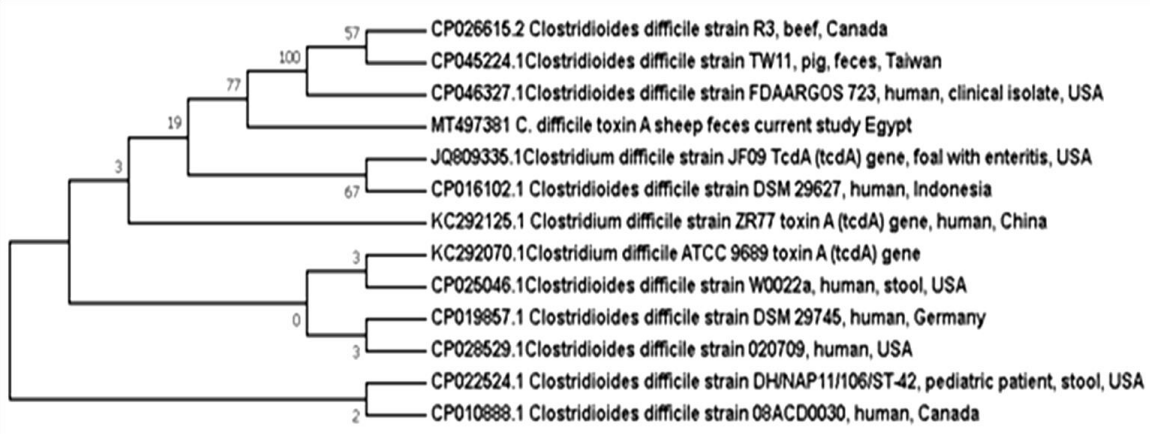

Fig. 2: Phylogenetic bootstrap consensus tree demonstrates the evolutionary history of the obtained *tcdA* gene sequence and the selected sequences retrieved from Genbank after BLAST analysis. The tree was constructed through neighbor-joining approach using Mega 7 software.

Strikingly, out of 12 PCR-confirmed isolates, only 3 *C. difficile* isolates were shown to be toxigenic; 2 carried tcdA gene and 1 carried tcdB gene giving a percentage 25% of the isolates. Such percentage was lower than those reported elsewhere (Knight and Riley 2013); 93.3% (14/15) of the obtained *C. difficile* isolates from sheep and lambs were positive for tcdA and tcdB (A+ B+), and the remaining isolate was A- B+, (Romano et al. 2012); 70% (7/10) for tcdA, and tcdB (A+B+).

The occurrence of toxigenic *C. difficile* strains among diarrheic sheep and goats points out to the possible role of *C. difficile* as a causative agent of diarrhea in such animals. Accordingly, the shedding of *C. difficile* from such animals may contaminate the carcass and abattoir environment which possesses a zoonotic risk for humans.

The phylogenetic analysis of the obtained tcdA gene sequence from sheep revealed that, this sequence was clustered in the same clade with those isolated from beef, pigs from Canada and Taiwan respectively. Moreover, the obtained sequence showed high genetic relatedness to that obtained from human clinical isolate from USA to indicate the public health implication of such toxigenic strain and hence the probable role of sheep to be a reservoir for toxigenic *C. difficile* strains in rural settings.

Conclusion

The present study provides further knowledge about the occurrence of toxigenic *C. difficile* among diarrheic sheep and goats to underscore the potential role which may be played by such animals in the epidemiology of toxigenic *C. difficile* in rural settings where human-animal interface.

Author's Contribution

Khaled A. Abdel-Moein and Ahmed Samir put the idea and the protocol of work; Mohamed Fathy and Adel M. Elkattan collected the samples. Wafaa A. Osman supplied the media and kits with practical supervision; Alaa A. Elgabaly and Mohamed Fathy performed the isolation and identification step; Abdelbary Prince and Ahmed M. Erfan applayed the molecular identification, sequencing step and phylogenetic analysis; Mohamed Fathy and Khaled A. Abdel-Moein wrote the manuscript; Khaled A. Abdel-Moein, and Ahmed Samir revised the manuscript.

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