Research Article

Performance of Different Laboratory Methods for Detection of *Clostridium difficile* in Animal Samples

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Abstract | *Clostridium difficile* is a well-known enteric pathogen causing antibiotic-associated diarrhea and pseudomembranous colitis among humans. Lately, *C. difficile* has emerged to cause enteric problems in food producing animals, horses and household pets particularly young ones. This study was conducted to investigate the performance of different laboratory diagnostic methods for *Clostridium difficile* in veterinary field. For this purpose, ninety fecal samples collected from diarrheic sheep, goats and chickens, were examined for the detection of *C. difficile* using three laboratory methods: direct Polymerase chain reaction (PCR) on DNA extracted from fecal samples, conventional culture followed by molecular confirmation of isolates and glutamate dehydrogenase (GDH) ELISA on feces. The detection rates of *C. difficile* were 45.6%, 16.7% and 8.9% by direct PCR, conventional culture followed by molecular confirmation of isolates and GDH-ELISA, respectively. Direct PCR yielded the highest detection rate, however, false negative results were recorded in 3 samples being positive by culture method, whereas, all GDH-ELISA positive samples were also positive by the other techniques. In conclusion, Direct PCR on DNA extracted from fecal samples of animals showed the highest detection rate nevertheless false negative results cannot be ruled out.

Keywords | *Clostridium difficile*, Laboratory diagnosis, Animals

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INTRODUCTION

Clostridium difficile is a Gram-positive, spore forming, strictly anaerobic, rod shaped bacilli that causes pseudomembranous colitis and antibiotic associated diarrhea in humans, known as *Clostridium difficile* infection (CDI) (Kachrimanidou et al., 2019). *Clostridium difficile* was isolated for the first time in 1935 from stool of newly born infant (Hall and O'toole, 1935). Afterwards, CDI was known as a nosocomial infection due to its high prevalence in hospitalized patients. However, this infection rapidly finds its way toward the population outside healthcare facilities and community acquired *Clostridium difficile* infection was emerged which may be owed the empirical use of antibiotics (Limbago et al., 2009; Gupta and Khanna, 2014).

On the other hand, In 1960, *C. difficile* was firstly described among mammals and birds through a biological investigation in Antarctica (McBee, 1960). Thereafter, *C. difficile* has become a causative agent of eneteric problems and diarrhea in many animal species, including

food animals (pigs, cattle, sheep, goats), horses and pets (Rodriguez et al., 2012; Wei et al., 2019).

Moreover, *C. difficile* infection (CDI) has been considered an important cause of neonatal enteritis in some food animals and sometimes mortality. As such, CDI among animals has gained the attention of researchers, epidemiologists and veterinarians to better understand the epidemiology and diagnosis of such infections among animals (Moono et al., 2016).

It is noteworthy that there are different laboratory tests available for detection of *C. difficile* in human stool samples which mainly classified into three categories. First, tests that target conventional isolation and identification of bacteria from the stool. Second, tests that identify some bacterial products by enzyme immunoassay, such products as glutamate dehydrogenase (GDH) which is the common antigen on *C. difficile* strains and bacterial exotoxins as A and B toxins. Third, tests that detect *C. difficile* genes in stool by molecular methods as PCR (Sushil, 2018).

Unfortunately, most available diagnostic tests have been adjusted for human samples with suboptimal performance on animal samples (Knight et al., 2014). Accordingly, in the veterinary medicine, much remains unknown about the appropriate laboratory diagnostic tool to diagnose CDI among animals. Therefore, this study was carried out in order to investigate the performance of different diagnostic methods when testing animal samples to detect *C. difficile* and thereby better diagnosis of CDI in veterinary medicine.

MATERIALS AND METHODS

Animals and sampling

A total of 90 fecal samples collected from the recta of diarrheic sheep, goats and the large intestine of poultry carcasses (with necrotic enteritis lesion). The fecal samples were collected in sterile cups and then transferred to the laboratory in ice box with minimum delay to be investigated for the presence of *C. difficile* using three different laboratory diagnostic methods: direct PCR on DNA extracted from fecal samples; bacteriological isolation and identification with molecular confirmation; detection of *C. difficile* in feces by enzyme-linked immunosorbant assay against glutamate dehydrogenase (GDH ELISA). Accordingly, each sample was divided into three portions; 2 of them were stored at -20 ˚C till precessing for direct PCR on DNA extracted from fecal samples and detection of *C. difficile* in feces by enzyme-linked immunosorbant assay against glutamate dehydrogenase (GDH ELISA) whereas, the third portion was directly processed for bacteriological isolation and identification with molecular confirmation of *C. difficile*.

Direct PCR on DNA extracted from fecal samples

DNA was extracted from fecal samples using QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany), the extraction was done according to the manufacturer's instructions. DNA was stored at -80˚C, until processing. Five microliters of extracted DNA was directly used for amplification using specific primers targeting 16SrRNA gene with a 270 bp fragment length (Zhang et al., 2016). PCR was performed on a Veriti 96-Well Thermal Cycler (Applied Biosystems) in a final volume of 20 µl containing 1 µl of each primer, 3 µl DNA template, and 15 µl nuclease free water, all put on dried and aliquoted PreMix pfu master mix (Maxime™ PCR PreMix (i-pfu) iNtRON Biotechnology, Korea). The PCR thermal profile was initial denaturation for 1 min at 95˚C; a total of 40 cycles of denaturation (95˚C for 20s), annealing (44 for ˚C 20s) and extension (72˚C for 30s); final extension (72˚C for 7 min). The PCR products were resolved by electrophoresis on a 1% agarose gel stained with ethidium bromide.

Bacteriological isolation, identification and molecular confirmation

About one gram of each fecal sample was pre-enriched in 5 ml brain heart infusion broth (Oxoid, Basingstoke, UK) and anaerobically enriched for 10 days at 37˚C in anaerobic jar. Then enriched broth was treated with an equal volume of absolute ethanol for 30 min, to eliminate vegetative cells while spores were recovered (Romano et al., 2012). Then, a loopful of broth sediment was streaked on *C. difficile* selective agar (*Clostridium difficile* Agar Base Liofilchem, Italy) supplemented with *Clostridium difficile* supplement (Liofilchem, Italy) and 5% defibrinated sheep blood as recommended by the manufacturer (Hampikyan et al., 2018). The plates were incubated anaerobically at 37˚C for 48h. A 48-h solid culture of *C. difficile* on selective agar media exhibiting flat, grayish, non-hemolytic colonies with irregular edges were picked up for further identification by Gram staining (Martínez-meléndez et al., 2017) then by latex agglutination test (Rodriguez et al., 2012; Spigaglia et al., 2015) according to the manufacturer's instructions (*Clostridium difficile* Latex Kit, Liofilchem, Italy). Colonies were finally confirmed by PCR on extracted DNA from bacterial colonies using the G-spin genomic DNA extraction kit (iNtRON Biotechnology, Korea) as the manufacturer's instructions. PCRs were carried out using specific primers targeting 16S rRNA gene of *Clostridium difficile* as aforementioned above.

Detection of *C. difficile* by GDH ELISA

The collected samples were subjected to the GDH test using commercial enzyme immunoassay EDI™ Fecal *C. difficile* GDH ELISA (Epitope Diagnostics, Inc., San Diego, CA 92121,USA). The procedure of the test was carried out according to the manufacturer's directions.

Table 1: Isolation and identification of *C. difficile* from examined animal samples.

Total no. of samples	Growth on medium		Latex agg. positive		Confirmed PCR positive	
	No. positive	%	No. positive	%	No. positive	%
90	24	26.7	20	22.2	15	16.7

Table 2: The detection rates of C. difficile using different diagnostic tests.

Total no. of samples	Direct PCR on fecal samples		Isolation and identification followed by PCR confirmation		GDH ELISA	
	No. positive	%	No. positive	%	No. positive	%
90	41	45.6	15	16.7	8	8.9

RESULTS AND DISCUSSION

Twenty-four out of 90 samples yielded bacterial growth on *Clostridium difficile* specific medium with the colonial morphology of *C. difficile*. Gram's stain films of the suspected colonies revealed Gram-positive bacilli. Of these 24, only 20 samples were recorded positive using latex agglutination test while 15 samples (16.7%) 15/90 were confirmed by PCR as *C. difficile* (Table 1). Furthermore, The detection rate of *C. difficile* by direct PCR on extracted DNA from fecal sample method was 41/90 (45.6%) samples, whereas eight out of 90 samples (8.9%) were positive by GDH ELISA (Table 2). The distribution of positive samples obtained by each test was displayed in Table 3.

Table 3: Distribution of positive samples obtained by different tests.

No. of samples	Isolation and identification followed by PCR	Direct PCR on fecal samples	GDH ELISA
8	+	+	+
4	+	+	-
3	+	-	-
29	-	+	-

C. difficile is a common pathogen that is associated with diarrhea in humans and different animal species, including small ruminants, pigs, horses and poultry. Many laboratory techniques are used for diagnosis of CDI. However, the pros and cons of each technique make it hard to select the optimal one (Tenover et al., 2011). The conventional culture method is of low cost but it is time consuming and depends upon viable bacteria. Other diagnostic methods likewise, molecular techniques and ELISA yielded rapid results however, its high cost (Vaishnavi, 2014).

The results of the present study revealed that the detection rate of *C. difficile* by direct PCR on fecal samples, conventional anaerobic cultivation with molecular confirmation and GDH ELISA were 45.6%, 16.7% and 8.9 % respectively. Notably, The highest detection rate (45.6%) was obtained by the direct PCR on fecal samples. Such high detection rate may be attributed to the high sensitivity of the used primers which have been recorded to detect as few as 10 cells of *C. difficile* in a total of 10^6 *E.coli* bacterial cells, with 100-fold increased sensitivity over the conventional bacterial cultivation methods (Gumerlock et al., 1991). Seriuosly, inspite of the highest detection rate was obtained by direct PCR method on fecal samples, this test failed to detect 3 samples while there were positive by conventional culture method, accordingly these samples are false negative. Similarly, Monteiro et al. (2013) recorded 6 PCR-negative samples but culture-positive and attributed it to the presence of PCR inhibitors during DNA extraction from fecal samples. On the other hand, the conventional cultivation and identification on samples revealed a detection rate 16.7% which is lower that rate of direct PCR, probably because of dead bacteria in fecal sample or very low live bacterial count as live bacteria of at least 1000 cfu/gram of feces are needed for successful recovery on the specific media (Gumerlock et al., 1991). Our anaerobic cultivation was firstly pre-enriched in suitable broth as brain heart infusion broth as it considered more efficient pre-enrichment broth than the other commonly used broths, such as Cooked Meat broth, to promote better growth of *C. difficile* as mentioned by (Connor et al., 2018) then plating on specific media supplemented with *Clostridium difficile* supplement which inhibit the growth of most Gram-negative and Gram-positive bacteria, without affecting the growth of *C. difficile*. Latex agglutination test is used for further identification as a more accurate and cheaper than biochemical tests which are expensive and time consuming (El-Leboudy et al., 2014). Only 20 samples from the 24 samples which showed growth were latex agglutination positive, indicating that some other bacteria could efficiently grow on *C. difficile* selective agar with similar colonial morphology. Similarly, Limbago et al. (2012) and Rodriguez et al. (2016) recovered other clostridia species especially *C. sporogenes* on *C. difficile* selective medium emphasizing that it is not totally selective. Interestingly, the 20 latex agglutination-positive samples included only 15 confirmed PCR-positive isolates and 5 false positive. These results indicate that latex agglutination test may give false positive results, probably

owed to a shared antigen between *C. difficile* and other clostridia like *C. sordellii* and *C. bifermentans* (Bowman et al., 1986). Therefore, we recommended that latex test must be followed by PCR confirmation.

In the present study, GDH ELISA revealed the lowest detection rate 8.9% such low dection rate was concides with that proposed by Miyajima et al. (2011) who recorded only one out of 149 fecal samples positive by GDH ELISA and they attributed that to the levels of GDH antigen in other samples were under the minimal detection threshold for this test. However, in the current study all GDH-ELISA-positive samples were also positive by both direct fecal PCR and culture methods indicating that this GDH-ELISA is highly specific.

CONCLUSION AND RECOMMENDATIONS

For detection of *C. difficile* in animal fecal samples, it is recommended that conventional isolation to be followed by molecular confirmation, whereas direct PCR on DNA extracted from feces yielded the highest detection rate after simple and rapid handling procedure however, false negative results cannot be ruled out.

AUTHOR'S CONTRIBUTION

Ahmed Samir, Khaled A. Abdel-Moein and Wafaa A. Osman: Idea, study design and writing manuscript. Mohamed Fathy, Tarek Mosallam: Sample collection. Mohamed Fathy, Hossam Mahmoud, Amani Hafez and Tarek Mosallam: Bacteriology techniques. Ahmed Erfan, A. Prince: Molecular work.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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