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Research Article

Studies on Virulence Factors of Locally Isolated *Clostridium difficile* from Horses

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ABSTRACT

Clostridium difficile is a confirmed pathogen in a wide variety of mammals; it causes enterocolitis associated with diarrhea in horses which may lead to necrohaemorrhagic enteroclitis with high mortality rate. In this study, the previously locally isolated strain of Clostridium difficile from horses was obtained and cultivated on the specific growth medium, then several media were used to determine the best media that could be used for production of the toxins A and B of Clostridium difficile and comparing the results using ELISA we found that thioglycolate medium and peptone medium with addition of 1% glucose give best results in toxin production. By performing multiplex PCR for identification of tcdA, tcdB, cdtA and cdtB genes for toxin A, toxin B, binary toxin (enzymatic and binding components) respectively revealed that presence of genes tcdA (602 bp) and tcdB (399 bp) while absence of genes cdtA and cdtB in the isolate. However lacking genes of binary toxin, we could conclude that the locally isolated strain Clostridium difficile from horses is toxogenic strain that could be used for production of vaccine against Clostridium difficile infection in horse.

Key words: Virulence Factors, *Clostridium difficile,* Clostridium difficile

INTRODUCTION

Clostridium difficile is a gram positive, anaerobic spore forming bacillus commonly associated with diarrhea and colitis in humans and other mammals. This organism was first isolated from feces of clinically healthy human babies and was originally named Bacillus difficilis because of its morphology and the difficulties encountered cultivating it. (McCollum and Rodriguez, 2012)

The first time *C.difficile* was isolated from mature horses was in a study of Potamic horse fever, then *C.difficile* was associated with typhlocolitis in an adult horse that was treated with antibiotics, however it was isolated from untreated diarrheic horses (Diab *et al*., 2013). *C.difficile* has been shown to be associated with diarrhea in foals during several outbreaks. *Clostridium difficile* causes acute enteroclitis in horses, infection usually develops while the horses are being treated with antibiotics and may begin several days after the treatment is withdrawn (Baverud, 2002).

The severity of *Clostridium difficile* infection can vary from mild to fulminant colitis with a high mortality rate, diarrhea may range from loose pasty to watery faeces accompanied by abdominal pain, fever, diminished appetite, septic shock and even sudden death (Baverud *et al*., 1997).

For the development of *Clostridium difficile* enteric disease, exposure to a toxigenic strain of *C.difficile* is required (Buggy *et al.,* 1983) and it can survive for prolonged periods of time in spore form which is relatively resistant to most disinfectants, making *Clostridium difficile* hardly environmental contaminant.

By antibiotic treatment there is an initial disruption of the normal colonic bacterial flora, allowing *C.difficile* from endogenous or exogenous origins to establish itself in the colon and proliferate. If the isolate is toxigenic, toxins A and B are produced simultaneously in most cases.

These protein toxins bind to specific receptors on the luminal aspect of the colonic epithelium and are then, by receptor mediated endocytosis, transported into cytoplasm. *C.difficile* toxins produce mucosal injury in the colon as a result of damage to the cytoskeleton and inhibition of the functioning of tight junctions.

The Primary virulence factors of *Clostridium difficile* are the two major toxins, toxin A(*TcdA*) and toxin B (*TcdB*). Toxin A has a molecular weight of 308 kDa and toxin B is 269 KDa. Furthermore, these two toxins share genetic characteristics, making it likely that one appeared first in evolutionary time and that the other has resulted from a gene duplication event (Von Eichel-Streiber *et al.,* 1992). Divergent evolution has led to some differences between TcdA and TcdB. The C-terminal portion of *TcdA*

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and *TcdB* mediates toxin binding to enterocytes. Toxins gain access to the cytoplasm and their enzymatic portions, which have monoglucosyl transferase activity and catalyze glucosylation, inactivate small regulatory proteins of the eukaryotic actin cytoskeleton (Artiushin *et al*., 2012) Loss of these proteins, The Rho-GTPases leads to disorganization of the cytoskeleton and cell death.

Some strains of *Clostridium difficile* may also produce an ADP-ribosylating binary toxin that is made up of two components; CDTa is an enzymatic component and CDTb mediates entry of CDTa into target cells; binding component (Davies *et al*., 2011).

This study aimed to study the virulence factors of the *Clostridium difficile* that has been previously locally isolated from horses. Firstly, toxins A and B of *Clostridium difficile* were prepared on different growth media to determine the most productive medium for toxin, then, ELISA was carried out, and polymerase chain reaction for the genes tcdA(toxin A), tcdB(toxin B), and binary toxin genes (CDTa and CDTb) was done using the specific primers to use this locally isolated strain laterally in vaccine development against acute enterocolitis in horses.

MATERIALS AND METHODS

Sample

Locally isolated strain of Clostridium difficile (Taha, 2014) was kindly obtained from Anaerobic Dept., Serum and Vaccine Research Inst., Cairo.

Preparation of C.difficile toxins

Locally isolated *C.difficile* was cultivated on *Clostridium difficile*Agar base (CM0601, Oxoid LTD, England) with *Clostridium difficile* selective supplement (SR0096E, Oxoid LTD, England) and incubated for 48 hrs anaerobically. *Clostridium difficile* colonies were harvested on Peptone, Thioglycollate broth, cooked meat medium, brain heart infusion medium and on same media with addition of 1% glucose for each, then incubated 72 hrs. anaerobically (levettt, 1985). The supernatant was then obtained after centrifugation at 3500 rpm for 30 min then Seitz -filtered and the crude toxin precipitated from the cultures by slow addition of equal volume of 70 ammonium sulphatesolution. Finally, the precipitate was dialysed against distilled water for 48 hrs. and dissolved in 1/20 of original volume in sterile saline (Hafiz and Oakley, 1975).

ELISA for C.difficile

Toxins A and B of Clostridium difficile can be specifically determined using RIDASCREEN® *Clostridium difficile* Toxin A/B kit (R-Biopharm AG, Dermstadt, Germany), which is an immuneassay depends on monoclonal antibodies of toxins A and B in sandwich type method, were these monoclonal antibodies are attached to the well surface of the microwell plate. The prepared toxin (from each medium) and the positive and negative controls were placed into the wells of microplate with the biotinylated antitoxin A/B antibodies as conjugate 1. then the plate incubated for 60 min and the wells washed using the washing buffer (phosphate buffered NaCl solution), and the streptavidin polyperoxidase (Conjugate 2) was added in each well, then the plate reincubated for 30 min and washed for 3 times and the substrate (Hydrogen peroxide/TMB) was added and finally the reaction stopped using 1N sulpheric acid after 15 min. the plate was read at 450 nm .

PCR for identification of tcdA, tcdB, cdtA and cdtB genes

C.difficile was cultivated on the toxin production media for 48 hrs and DNA was extracted by boiling for 10 min then centrifugation to remove cell debris, the supernatant collected and stored at -20c. A 4 plex PCR were designed as in Table 1.

Table 1: Primers for identification of C.difficile tcdA, tcdB, cdtA and cdtB genes

Gene target	Primer name	Sequence (5`-3`)
tcdA	tcdA-F	GCATGATAAGGCAACTTCAGTGG
	tcdA-R	GAGTAAGTTCCTCCTGCTCCATCAA
tcdB	tcdB-F	GGTGGAGCTGCTTCATTGGAGAG
	tcdB-R	GTGTAACCTACTTTCATAACACCA
cdtA	cdtA-F	GGAAGCACTATATTAAAGCAGAAGC
	cdtB-R	TCTGGGTTAGGATTATTTACTGGAC
cdtB	cdtB-F	AAAGTTGATGTCTGATTGGGAAG
	cdtB-R	TTTGTTGTTGGTGTCACTTTGTA

The multiplex PCR was run in final reaction volumes of 50 ul containing 2ul genomic DNA, 200uM each of dATP, dCTP, dGTP and dTTP, 50 pmol of each primer, 6mM MgCl2, 50 ml mMKCl, 10mM Tris HCl (pH 8.5) and 5 unit of Taq DNA polymerase. Reaction were initiated at 95C for 5 min followed by 40 cycles of 94C for 1 min, 59C for 1 min.72C for 1 min and finally 72C for 7min. detection of PCR products were detected in 1.5% ethidium bromide stained agarose gel electrophoresis. Constant voltage of 84 V for 25 min was used for products separation (Start *et al.*, 2000; Doosti and Mokhtari Farsani, 2014).

RESULTSAND DISCUSSION

Clostridium difficile is an anaerobic spore-forming bacterium that is mainly associated with colitis in horses, but severe small intestinal lesions have been reported in naturally and experimentally infected foals. This organism has also been isolated from the small intestine of horses and ponies with various small intestinal disorders. *Clostridium difficile* associated enteritis also occur in humans, and there have been increasing numbers of *C. difficile* associated ileitis reports in recent years (Aorryo *et al*., 2017).

Most common disinfectants don't work on *C. difficile* spores making *C. difficile* an inflexible environmental contaminant. Sources of infection include but are not limited to horses or foals infected with *C. difficile*. Transmission occurs by the oral-fecal route by ingestion of *C. difficile* spores from infected horses, possibly other animals, human beings, or contaminated environment. Adult horses or foals are susceptible to CDI and the horses develop symptoms of CDI either sporadically or as outbreaks.

The CDI in horses ranges from 5 to 63% and this huge variability maybe partially caused by the study designs, diagnostic methods, animal age, and sample collection variation, etc. (Zhang *et al*., 2014).

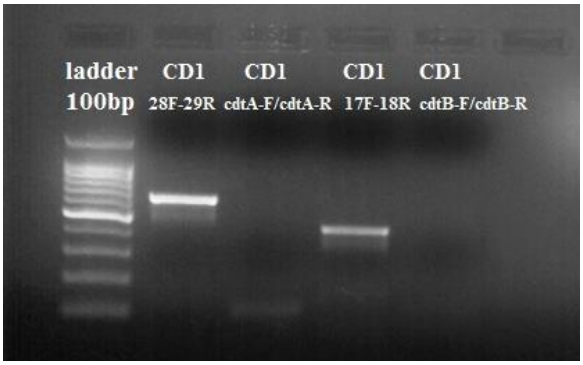

Fig. 1: PCR of the isolated Clostridium difficile. Lane (1) : Mol. marker 100-1000 bp , lane (2) tcdA , lane (3) cdtA , lane (4) tcdB, and lane (5) cdtB.

Table 2: ELISA for comparison of different toxin production media for *C.difficile*

Type of media	O.D. at 490 by ELISA
Cooked meat	0.354
Cooked meat + glucose 1%	0.472
Brain heart infusion	0.322
Brain heart infusion + glucose 1%	0.534
Thioglycolate	0.706
Thioglycolate + glucose 1%	0.819
Peptone	0.432
Peptone + glucose 1%	0.802
Negative control	0.064
Positive control	1.414
Cut off (negative control +0.15)	0.214

The two major risk factors for development of Clostridium difficile associated diahrrea are antibiotic treatment and hospitalization (Madewell *et al*., 1995). Antibiotics have adverse effect on the distribution and number of bacteria and protozoa in the cecum and colon by disrupting the balance of protective commensal organisms which can permit the overgrowth of pathogenic species. Clostridium difficile toxins include toxin A, toxin B and a binary toxin. Toxin A (enterotoxin) and B (cytotoxin) (308 kDa and 270 kDa respectively are encoded by two separate genes tcdA and tcdB. The binary toxin is encoded by the genes cdtA and cdtB. *C.difficile* toxins directly affect the colon epithelial cells and immune cells are forced to produce chemokines and cytokines.

Previously (Taha, 2014), twenty faecal samples and seven rectal swabs from foals suffering from diarrhea, were collected and cultured onto Cefoxitin Fructose Agar (CFA) as specific growth medium. Isolates from twenty-one (77.7%) of these samples gave the characteristic microscopical examination and colony morphology as well as they gave negative results with catalase and oxidase tests also they gave fluorescence after exposure to long wave length ultraviolet light. Specific agglutination kit was used for confirmation, revealed nineteen isolates (70.4%) gave positive for agglutination, The A and B crude toxins were prepared from the isolates, toxogencity test in mice was done for the prepared toxins as preliminary test for toxogencity, nine (33.3%) isolates found to be lethal to mice, then, SDS-PAGE was done to the fifth isolate (as it gave the highest MLD, indicating its the most toxogenic isolate) where the toxins gave bands at molecular masses of 308 and 270 kDa, which resemble that of toxins A and B of *Clostridium difficile* respectively.

This Clostridium difficile that was previously locally isolated from horses (9) was cultivated on the specific agar base (CM0601, Oxoid LTD, England) with *Clostridium difficile* selective supplement (SR0096E, Oxoid LTD, England) and incubated for 48 hrs anaerobically. The toxins were prepared by cultivation the colonies on different media, and ELISA was carried out to determine the most medium that could be used laterally for toxin production. as shown in Table 2. According to the results in Table 2, thioglycolate medium with addition of 1% glucose or peptone with 1% glucose showing best results for toxin production of Clostridium difficile, however brain heart infusion medium and cooked meat medium with addition of glucose 1% could be also used for toxin production, these results agreed to what previously stated (Oakely and Warrack, 1951; Borriello and Pauline, 1981; David *et al*., 1988).

For more confirmation, PCR for identification of tcdA, tcdB, cdtA and cdtB genes from the locally isolated Clostridium difficile from horses was done, which revealed single band at 602 bp for tcdA gene and at 399 bp for tcdB while no bands formed with cdtA and cdtB primers as in Figure 1.

These results proved that the isolate includes the genes tcdA and tcdB, the genes for toxin A and toxin B respectively, while the genes cdtA and cdtB for the enzymatic and binding components of the binary toxin not found in this isolate. These results agreed with many pervious results (Person *et al*., 2008; Dale *et al*., 2014) which stated that pathogenesis has been linked to the production of toxins, toxin A (tcdA) and toxin B (tcdB) as they are considered these two toxins as the main virulence factors of Clostridium difficile although the binary toxins frequently observed in Clostridium difficile strains associated with increases severity of the infection.

Finally, we could concluded that the previously isolated strain of *Clostridium difficile* from horses is toxogenic as it contains genes tcdA and tcdB and it produces toxins A and B which are the main virulence factors for *Clostridium difficile*. Consequently, we could use this strain laterally for production of vaccine against antibiotics induced enterocolitis infection in horses.

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