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Identification of Locally Isolated *Clostridium difficile* from Rabbits

Medhat M Taha, Hamed A El-Helw, Elham F. El-Sergany, Hala El Sawy, Yasser A Abdella and Alaa A El-Meneisy

Department of Anaerobic Bacterial Research, Serum and Vaccine Research Institute, Abbassia, Egypt

*Corresponding author`s Email: medhattaha@hotmail.com

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ABSTRACT

Clostridium difficile is one of the most important pathogens causing diarrhea and enteritis in rabbits as it causes pseudomembranous colitis that leads to intestinal damage and deaths. In this study, screening of rabbit farms from different localities in Egypt had shown rabbits suffered from diarrhea and enteritis to detect *Clostridium difficile* by ELISA, it revealed that five out of 50 samples (10%) were positive for it. These samples were further identification by cultivation and culture characters, microscopical examination, agglutination test, pathogenicity test and Polymerase Chain Reaction (PCR) by using specific primers for toxins genes (tcdA and tcdB). The results showing that three out of five isolates were confirmed as *Clostridium difficile* and concluded that these isolates causing pseudomembranous enterocolitis in rabbits and this disease unable to be treated by antibiotics, so it used for preparation of vaccine against the disease in rabbits.

Keywords: *Clostridiunm difficile*, Rabbits, Enteritis

INTRODUCTION

There is a strong and continuing interest in the development of rabbit industry in Egypt. Rabbit industry as one of the small livestock has a unique commercial role in solving the shortage in the meat after poultry industry (Mohamed et al., 2013). To achieve this purpose a special light should be thrown on dangerous rabbit diseases which may affect the industry. Enteritis complex is one of the important causes of disease and economic losses in younger rabbits. Many causes lead to enteritis and can result in health problems for the rabbit (Songer, 1996).

Clostridia species are the cause in the induction of enteritis problem in rabbits with high occurrence percentages (Hong et al., 2017). Different types of Clostridial species found in weaned rabbit farms at Egyptian governorates. Clostridial enterotoxaemia refers to enteritis caused by toxogenic microorganism of the genus Clostridium which characterized by diarrhea and may be sudden death. The main etiological agentsare *Clostridium Perfringens; Clostridium spiroforme; Clostridium Butyricum,*and *Clostridium difficile* (Khalefa et al., 2012; El-Helw et al., 2014)

Clostridium difficile (*C. difficile*) is a gram positive, spore forming,anaerobic, toxin producing bacillus, catalase negative bacterium (Bruce et al., 1999). Pathogenic strains of *C.difficle* produce two potent toxins , enterotoxin A and cytotoxin B, these toxins are the virulence factors of the organism and thereisa global problem which caused by ingestion of vegetative organisms and spores , most likely the latter which survive exposure to gastric acidity and germinate in the colon (Doosti and Mokhtari, 2014**)**. *C. difficile* recognized as a major nosocomial pathogen responsible for antibiotic related diarrhea and is etiological agent of perforation of the colon, pseudomembranous colitis or toxic megacolon and even death in humans (Hung et al., 2012). *C. difficile* also is an important factor for enteric disease in other species; it cause enterocloitis associated with diarrhea in horses specially foals (Taha, 2014), it cause chronic diarrhea in dogs (Andrea et al., 1994), and had been isolated from calves, pigs, rabbits and cats (Rodriguez Palacios et al., 2006). In rabbits, cases of clostridiosis caused by *C. difficile* have been reported from 35 to 55 days of age, in which enteroclitis with liquid caecal content was observed. *C. difficile* was first isolated from faeces of four often healthy newborn infants in 1935; it was named *Bacillus difficilis* because of difficulty in isolating and studying these bacteria. (Hall and O`Toole, 1935)

The aim of this study is to isolate and identify *C. Difficile* and confirm it as a causative bacterial agent of diarrhea from naturally infected rabbits and as a primarily step for preparation of vaccine from locally isolated strain.

MATERIALS AND METHODS

Ethical approval

All procedures performed according to Egyptian ethical standards of the National Research Committee.

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Screening for detection of *C. difficile* infection in rabbits

Fifty rabbits aged between one to four months were recently dead where suffered from abdominal distension and diarrhoea, obtained from different farms in Egypt. Screening was done by using RIDASCREEN® *C. difficile* toxin A/B kit (R-Biopharm AG, Dermstadt, Germany). This Kit using monoclonal antibodies against *C.difficile* toxins A and B used as screening test for detection of *C. difficile* toxins. Faecal and ceacal samples (approx. 100µl) were aspirated and 1ml RIDASCREEN® *C. difficile* sample dilution buffer were added, samples were homogenized, blend in votex mixer, suspension left stand a short period of time for the coarse stool particles to settle, and clarified supernatant of samples suspension used in the test. The assay was done according to procedure of ELISA Kit manual. The optical density of samples; positive, and negative control were recorded after reading by ELISA reader. Calculation the cut-off for the negative control, an assessment of specimen as positive if extinction rate is more than 10% higher than calculated cut-off value.

Isolation of the causative agent

Positive faecal samples and/or caecal contents from rabbits were collected and alcohol shocked is used **(**Saverio and Pauline, 1980), then pellets were inoculated on *C. difficile*agar base (CM0601, Oxoid LTD, England) with *C. difficile* selective supplement (SR0096E, Oxoid LTD, England) and incubated for 48 hours anaerobically at 37 °C (Saverio and Pauline, 1980). Suspected colonies were harvested and stained with Gram stain. Microscopical examination, culture characters (culture morphology, odour and effect of long wave length ultraviolet) and biochemical tests (catalase and oxidase tests) were carried out (Hafiz and Oakely, 1976**).**

***Clostridium difficile* agglutination test**

Clostridium difficile latex agglutination kit (Oxoid Ltd. DR1107, England) where used for detection of *C. difficile*. A loopful from the suspected colonies, positive control provided (*C.difficile* cell wall antigen) and negative control (0.85 % isotonic saline) were tested using the specific reagent provided (latex particle coated with IgG antibodies specific for *C. difficile* cells wall antigen) on a reaction card, latex particles agglutinate in large visible clumps within two minutes in positive control as well as in positive samples (Kelly et al., 1987).

Pathogenicity test

Selected colonies were suspended in 1% peptone saline, and then was adjusted to 9×10^{10} cells /ml, and inoculated intramuscularly in each of three rabbits aged between 30-40 days. The isolation of organism was done again (Hutton et al., 2014).

Toxin preparation

Thioglycollate broth with 1% glucose was prepared and seeded with *C.difficile* suspected colonies and incubated anaerobically for 72 h, the supernatant collected after centrifugation at 3500 rpm for 30 min then filtered with Seitz filter and the toxognecity test was done by injecting the prepared crude toxin 0.2 ml IV (intravenous) and its three double fold dilution in three mice each (0.2ml /IV /mice) to investigate the minimum lethal dose (MLibby et al., 1982).

Polymerase Chain Reaction

DNA was extracted from the suspected colony of the positive samples transferred into a 0.6-mL microcentrifuge tube containing 100 µl of sterile water and was boiled at 100°C for 10 min. After boiling, the sample was centrifuged at low speed (3000 rpm) to remove cell debris. The supernatant containing the DNA was used for amplification reactions (Perkins et al., 1995). Toxin A and B genes were amplified as described (Stuart et al., 2000) and the sequences of the primers used shown in table 1.

RESULTS

Infected rabbits showed abdominal distension and diarrhea as in figure 1. Specific ELISA kit was used for detection of *Clostridium difficile* toxin A/B for the 50 faecal samples and caecal contents and the obtained results confirm 5 positive samples representing 10% of tested samples, table 2.

Isolation of the positive faecal and caecal samples on specific medium revealed greyish regular smooth colonies (Figure 2) with manure characteristic odour, and the gram stain of the suspected colonies showed gram positive bacilli as in figure 3 revealed. These obtained agreed with authors who stated that *C. difficile* colonies on blood agar were greyish in colour with characteristic distinctive manure odour, and the organism is gram positive rod measuring 0.5X 3 – 6 um (Mohamed et al., 2013). The isolated colonies produce a pale green fluorescence under long wave length ultraviolet as shown in figure 4. Furthermore, ELISA was done using RIDASCREEN® *C. difficile* Toxin A/B kit (R-Biopharm, Germany) as shown in table 2.

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Table 1. Sequence of the primers used for polymerase chain reaction of the isolates designed for *Clostridium difficile*

Gene target	Primer Name	Sequence (5`-3`)	Amplicon size (bp)
tcd A	F-YT-28	GCATGATAAGGCAACTTCAGTGG	602
	R-YT-29	GAGTAAGTTCCTCCTGCTCCATCAA	
tcd B	F-YT-17	GGTGGAGCTGCTTCATTGGAGAG	399
	R-YT-18	GTGTAACCTACTTTCATAACACCA	

Table 2. Optical Density readings at 450 nm wave length five positive samples from the fifty infected rabbits *Clostridium difficile*, 2018 in Egypt

Samples	Mean of O.D.(Absorbance)
1	0.465±
2	0.785±
3	0.692±
4	0.474±
5	0.545
Negative control	0.128±
Positive control	1.242±
Cut-off (negative control+0.15)	0.278

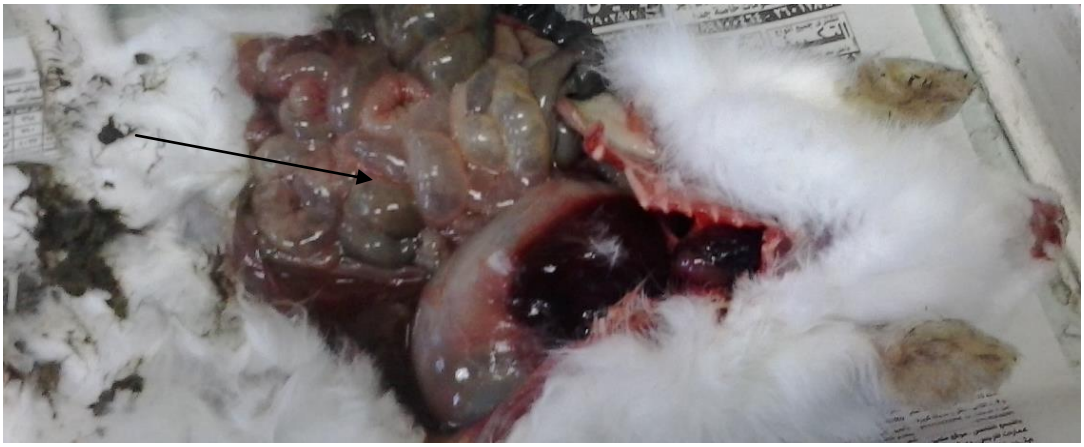

Figure 1. Abdominal distension in rabbits infected with *Clostridium difficile*

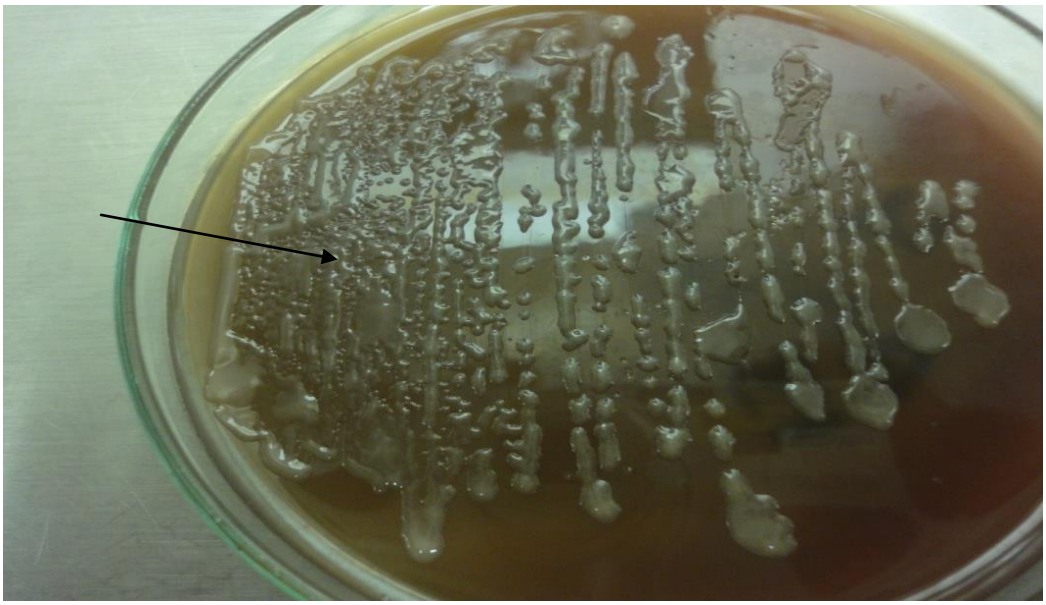

Figure 2. Suspected colonies of *Clostridium difficile* after cultivation on the specific medium

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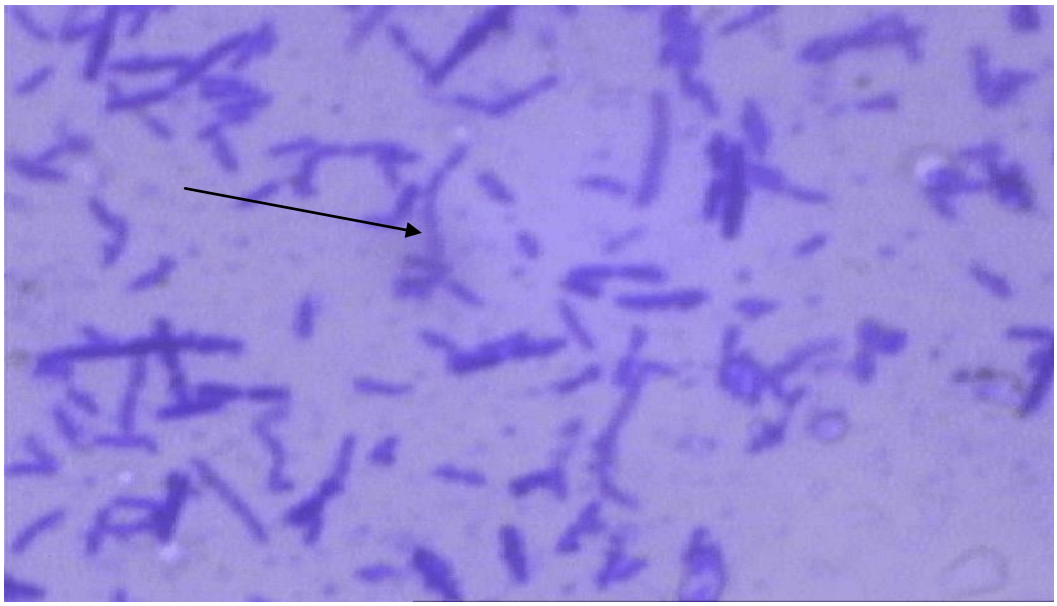

Figure 3. Gram stain of the suspected colonies from the specific agar medium

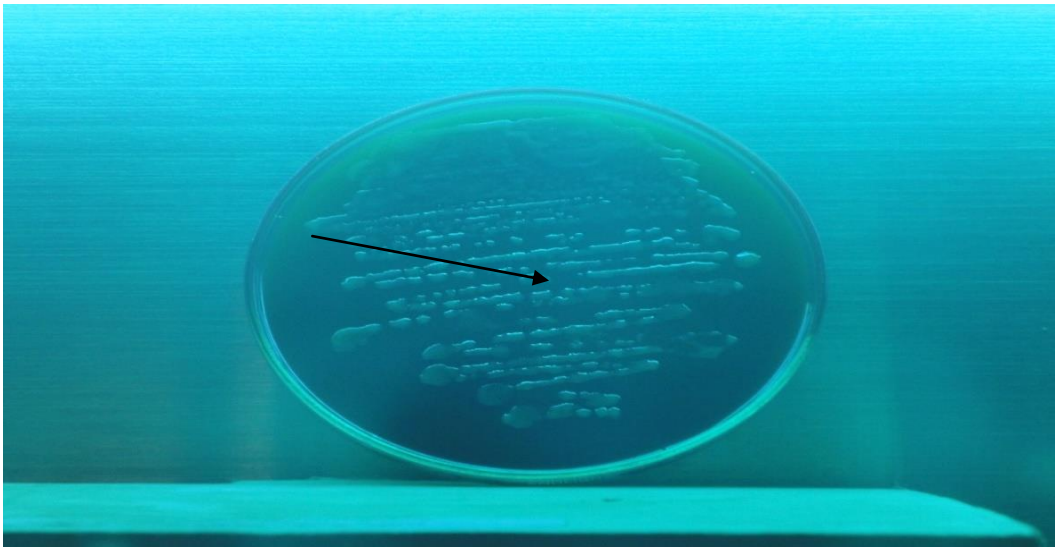

Figure 4. Pale green fluorescence colonies under long wave

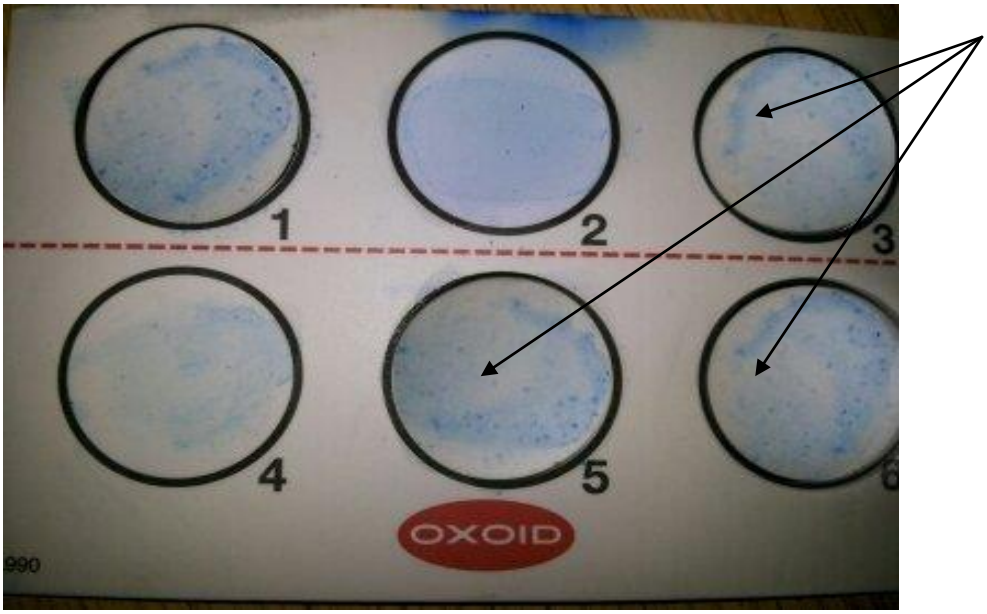

Figure 5. Latex agglutination test using the isolated *Clostridium difficile*. 1: positive control, 2: negative control. 3, 5, 6: positive samples and 4: negative samples

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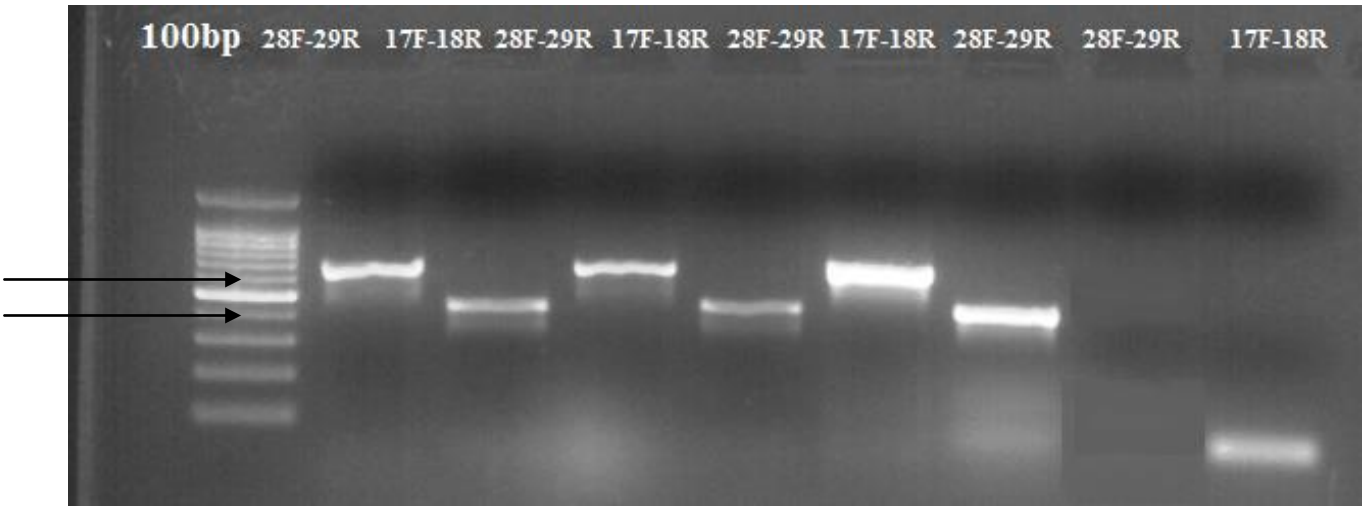

Figure 6. Polymerase chain reaction for the isolates of *Clostridium difficile* for tcdA and tcdB genes lane 1 :molecular markers 100-1000, lanes 2,4,6,8: for the four isolates with tcd A gene , lanes 3,5,7,9: the four isolates with gene tcd B, 2018

DISCUSSION

Clostridium difficile is a confirmed pathogen in a wide variety of mammals, but the incidence of disease varies greatly in relation to host species, age, environmental density of spores, administration of antibiotics, and possibly of other factors. Lesions vary as well, in severity and distribution within individuals, and in some instances, age groups, of a given species. The cecum and colon are principally affected in most species, but foals and rabbits develop severe jejunal lesions (Keel and Songer, 2006).

C. difficile is usually a harmless environmental bacterium and the normal intestinal microflora has to be disturbed before *C. difficile* can become established and produce its toxins. There is an initial disruption of the normal colonic bacterial floraby antibiotic treatment, allowing *C. difficile* from endogenous or exogenous origins to express itself in the colon then proliferate and produce toxins A and B simultaneously, these protein toxins bind to specific receptors on the luminal aspect of colonic epithelium transported to the cytoplasm by receptor mediated endocytosis. Thus, *C. difficile* toxins cause themucosal injury in the colon as a result damage to the cytoskeleton and inhibition of the functioning of tight junctions and cause fluid secretion, inflammation and mucosal damage, which by its turn lead to diarrhea and psudomembranous colitis.

The isolates gave catalase negative using hydrogen peroxide (as no bubbles of liberated oxygen formed)and oxidase negative when tetramethyl-p-phenylendiamne added where no change in colour occurred , these results agreed with those who reported that *C. difficile* is catalase and oxidase negative (Perkins et al., 1995). *C. difficile* latex agglutination test for the colonies revealed 3 positive samples out from five samples, in comparing to the positive and negative controls provided as shown in figure 5. ELISA done revealed three positive sample as recorded in table 2. Toxin A/B was prepared from one of the positive colonies and inoculated IV in two mice each, the four mice (two of the toxins as it is and two with double fold dilution. of the toxin) were all dead within 48 hours which revealed that the isolated colony is toxogenic.

PCR results using tcdA and tcdB genes primers as showed in figure 6, revealed that bands at 602 bp and 399 bp respectively for three positive samples, while other sample was negative for both genes ,that agreed completely with **(**Stuart et al., 2000) who detected four positive samples using PCR for the genes of toxins A and B from 10 samples. Also authors stated that 62% of samples revealed positive samples for *C.difficile* using multiplex PCR and they stated that tcdA and tcdB genes confirmed that isolate is toxogenic (Leond et al., 2000).

CONCLUSION

All the results obtained from present study indicated that *C. difficile* which have been isolated is toxogenic and could be used for production of vaccine against pseudomembranous colitis in rabbits which in turn help the rabbit industry by providing the specific vaccine.

DECLARATIONS

Author`s contribution

TMM and EHA isolate *Clostridium difficile* from infected rabbits and perform ELISA, EFE contributes agglutination test HES and YAA perform PCR for the isolated *C. difficile* and AAE provide the infected rabbits from the farms.

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Competing interests

All authors have no conflict of interest.

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