Detection of *Clostridium difficile* toxins A and B from Antibiotic- Associated Diarrhea and Colitis in Iraqi children, by ELISA Test

Mona Turkey AL-Mossawei and Luma Yousif Mahdi
Biology Department, College of Science for Women, Baghdad University, Iraq.
E-mail: yousifnawar@yahoo.com
Accepted: 24/2/2015

Summary

This study focuses on diagnosis of *Clostridium difficile* causing Antibiotic – Associated diarrhea and colitis by Elisa method and to detect of *C. difficile* Toxin A and B in stool samples by Elisa test. Two hundred forty (240) stool samples were collected from children suffering from antibiotic-associated diarrhea and Colitis cases at ages from after birth to 15 years old from Baghdad hospitals. Samples were taken during the period of first of June 2013 until the end of April 2014. In addition to that 80 samples from healthy children of the same age and sex as a control group. *Clostridium difficile* Toxin A and B in stool samples were detected. Results of this study indicated that females were more infected than males. Overall positivity was 21.25% in present studied group compared to controls ($P<0.05$). In conclusion, the majority percent from age <1year 15% and this percent decreased with the advance in age.

Keywords: ELISA, *Clostridium difficile*, Antibiotic-Associated diarrhea, colitis.

Introduction

Clostridium difficile infection (CDI) is a hospital-acquired infection with increasing incidence and severity. The most frequently used test to diagnose CDI is an enzyme immunoassay (EIA) for toxins A and B in stool samples (1). Infection with toxin-producing *Clostridium difficile* strains is a common cause of diarrhea. The severity of *C. difficile* infection ranges from mild diarrhea to pseudo membranous colitis and can result in death. A total of 15–25 % of all cases of Antibiotic-Associated Diarrhea result from CDI (2). *Clostridium difficile* is the cause of antibiotic-associated diarrhea increases with the severity of disease, reaching 95–100 % among patients with documented antibiotic-associated colitis. Accurate diagnosis early in the disease course is important to the successful management of CDI (3).

The prevalence and virulence of healthcare-associated *C. difficile* disease has increased, and it is the most common cause of infectious diarrhea in the healthcare setting. While there are multiple tests commercially available for the diagnosis of *C. difficile* infection, enzyme immunoassay (EIA) testing for toxins A and B is most commonly used (4). Two large toxins, TcdA enterotoxin and TcdB cytotoxine (308 kDa and 270 kDa, respectively), are recognized as the main virulence factors of *C. difficile* (5). *C. difficile* carriage rates average 37% for infants (0 – 1) month of age and 30% between (1– 6) months of age, exposure to environments where *C difficile* is present is important (6 and 7). Previous study in Iraq isolated 14%, (diarrhea 8% and healthy 6%) (8), and 13% (9), there are few epidemiological studies involving this pathogen as a cause of diarrhea in children and no detection of *C. difficile* Toxin A and B in stool samples by Elisa test.

Materials and Methods

Two hundred fourty (240) stool samples were collected from children suffering from AAD and Colitis cases at ages from after birth to 15 years old from Baghdad hospitals. Also 80 stool samples were collected from healthy children as a control group. Stool samples were collected into a clean container with no preservative. All stool specimens were stored at 2-8 °C and tested as soon as possible. Ideally, stool specimens were tested within 24 hrs. but specimens stored at 2-8°C for up to 72 hrs. prior to testing. If specimens were not tested within 72 hrs. they were frozen immediately upon receipt at –20 °C or colder.

Premier Toxins A and B Antibody-coated Microwells-Breakaway plastic wells were coated with mouse monoclonal anti-toxin A antibody and polyclonal goat anti-toxin B antibody; Premier Toxins A and B Positive Control – Inactivated toxin A and B in buffered protein solution containing gentamicin and thimerosal (0.02%) as a preservatives; Premier Toxins A and B Samples Diluent/ Negative

Control – Buffered protein solution containing gentamicin and thimerosal (0.02%) as preservatives; Premier 20X Wash Buffer II – Concentrated wash buffer containing thimerosal (0.2%) as a preservative; Premier Toxins A and B Enzyme Conjugate-Polyclonal goat anti-toxin A and antitoxin B antibodies conjugated to horseradish peroxidase in buffered protein solution containing gentamicin and thimerosal (0.02%) as preservatives; Premier Substrate I- Buffered solution containing peroxide and tetramethyl benzidine; Premier Stop Solution I-1M Phosphoric acid; Sample Transfer pipettes; Microwell plate sealers.

Reagent Preparation: Bring the entire kit, including microwell pouch, to room temperature 21-27 °C before use. Return to 2°C immediately after use, Prepare decontamination vessel for discarding reagents and materials, and do not allow microwells to dry out between steps. Reproducibility in any EIA is largely dependent on the consistency and thoroughness with which the microwells are washed carefully follow the recommended wash procedure as outlined in the EIA test procedure. An automated washer may be used, Prepare 1X Wash Buffer as needed. For example: 5.0 mL of 20X Wash Buffer II + 95.0 mL of distilled or deionized wash is sufficient to wash 1 strip. Place in clean squirt bottle. The 1X Wash Buffer can be stored at 21-27 °C for up to 3 months, One Positive and 1 Negative Control well must be included with each run of specimens. Use the Positive Control as provided. Do not dilute, Use EIA plate sealers to cover the assay during incubation steps. Cut to size, then remove paper backing before use.

Specimen Preparation: (1:5 Sample Dilution)**.** Add 200 µL of Sample Diluent to a clean test tube with dropper assembly (or equivalent). Mix stool as thoroughly as possible prior to pipetting for liquid or semi-solid stool: Using the disposable transfer pipettes and measuring to the 1st calibration mark (50 µL), transfer 50 µL of stool into the dilution tube containing Sample Diluent. Rinse the transfer pipette by drawing the stool suspension in and out of the pipette several times. Remove pipette and vortex the suspension thoroughly (15 seconds), and replace pipette in tube. Stool specimens may be centrifuged after dilutions.

Test Procedure: After the pouch has reached 21-27°C, break off the required number of microwells (1 well for each specimen plus 1 positive and 1 negative control well per batch). Place the microwells in the microwell strip holder and record the location of all wells. Unused microwells must be resealed in the pouch immediately. Using the original transfer pipette, draw up diluted stool to the 100 µL calibration point (2nd mark from tip of the pipette) and add to the appropriate well (place pipette tip halfway into well and let sample slowly run down side of well). Add 2 free-falling drops of Positive Control to the appropriate well. Add 100 µL (2nd mark on transfer pipette) of Negative Control (Sample Diluent) to the appropriate well. Add 1 free-falling drop of Enzyme Conjugate (50 µL) to all wells. Mix wells by firmly shaking/swirling the plate for 30 seconds. Cut plate sealer to size and press firmly onto top of microwells to seal. Incubate the plate for 50 minutes at 35-39 °C**.** Carefully, remove the plate sealer and wash wells. Dump plate contents firmly into a biohazard receptacle; Bang the inverted plate on a clean stack of paper towels; Fill all wells with 1X Wash Buffer directing stream of buffer to the sides of the wells to avoid foaming. Immediately proceed to Step 6.d.; Repeat wash cycle (dump, bang on fresh towels, fill) 4 to 6 times (for a total of 5-7 washes). After the last fill, dump and bang plates on fresh towels hard enough to remove as much excess wash buffer as possible but do not allow wells to completely dry at any time. Clean the underside of all wells with a lint free tissue. Add 2 free-falling drops of Substrate I (100 µL) to each well. Firmly shake/swirl the plate for 10-15 seconds then incubate for 10 minutes at 21-27°C. Add 2 free-falling drops of Stop Solution I (100 µL) to all wells. Shake/swirl the plate firmly for 30 seconds to assure complete mixing. After addition of Stop Solution I, wait 2 minutes before reading (Step 11).

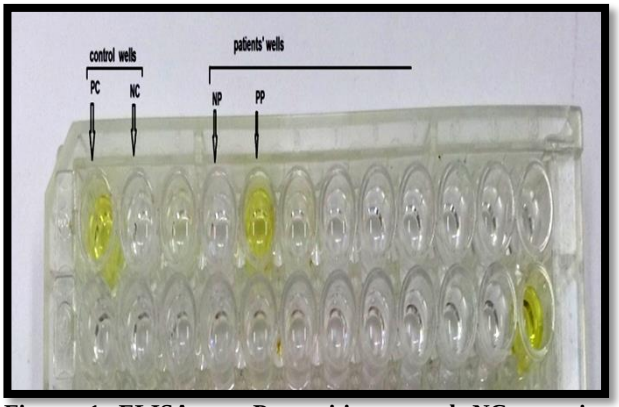
Initial color of positive reaction is blue, which changes to yellow upon addition of Stop Solution I. Observe reactions: Visual Determination – Read within 15 minutes after adding Stop Solution I; Spectrophotometric Determination – Zero EIA reader on air. Wipe the underside of wells with a lint free tissue. Read absorbance at 450 nm or 450/630 nm

within 30 minutes of adding Stop Solution I. (10 and 11).

Results and Discussion

Visual reading (Fig. 1), negative= colorless to faint (barely visible) yellow, Positive= definite yellow color. Spectrophotometric Single Wavelength (450 nm). Negative= OD450 < 0.150, Positive= OD450 ≥ 0.150.

A positive result indicates the presence of *C. difficile* toxin A and/or B. A negative result indicates the absence of toxins A and B, or that the level of toxin is below that which could be detected by the assay. Sensitivity and specificity compared to the reference cytotoxin method were 94.7 and 97.3 %, respectively. Fifty one samples (21.25%) were positive for *C. difficile*, maximum positive cases were in ≤ 5 years age group (18.75%), while it form (2.5 %) in age group > 5 years (Table, 1).



Figure, 1: ELISA test .Pc, positive control .NC, negative control. NP, negative patient. PP, positive patient.

Table, 1: Distribution of toxin A and/or B according to age groups human samples.

Age (group) years	Patient		Control		Chi-square-χ^2
	Sample No.	Positive No. (%)	Sample No.	Positive No. (%)	
≥ 5	216	45 (18.75)	60	12 (15.00)	0.849 NS
6-10	15	6 (2.50)	10	0 (0.00)	0.851 NS
11-15	9	0 (0.00)	10	0 (0.00)	0.00 NS
Total	240	51 (21.25)	80	12 (15.00)	2.077 NS
Chi-square-χ^2	----	6.71 **	----	5.724 *	----

* (P<0.05), ** (P<0.01), NS: Non-significant.

Percentage of positivity of *C. difficile* in study as compared to control group, was not statistically (P<0.05) significant. The isolation from age <1year was 15%, (Table, 2), and this decreased with age. As compared with control, there was no statistically (P<0.05) significant. This study shows that females were more infected than males. Overall positivity was 21.25% in this study group compared to controls (P<0.05), (Table, 3).

Table, 2: Distribution of toxin A and/or B according to age groups <5 years.

Age (group) years	Patient		Control		Chi-square-χ^2
	Sample No.	Positive No. (%)	Sample No.	Positive No. (%)	
> 1	147	36 (15.00)	20	11 (13.75)	0.637 NS
1-2	48	6 (2.50)	20	1 (1.25)	0.422 NS
2-3	9	2 (0.83)	10	0 (0.00)	0.048 NS
3-4	6	1 (0.42)	5	0 (0.00)	0.009 NS
4-5	6	0 (0.00)	5	0 (0.00)	0.00 NS
Total	216	45 (18.75)	60	12 (15.00)	0.804 NS
Chi-square-χ^2	----	6.057 **	----	4.739 *	----

* (P<0.05), ** (P<0.01), NS: Non-significant.

Table, 3: Distribution of toxin A and/or B according to sex.

Age (group)	Patient		Control		Chi-square-χ^2
	Sample No. (%)	Positive No. (%)	Sample No. (%)	Positive No. (%)	
Male	102 (42.50)	21 (8.75)	36 (45.00)	5 (6.25)	0.144 NS
Female	138 (57.20)	30 (12.60)	44 (55.00)	7 (8.75)	0.208 NS
Total	240 (100)	51 (21.25)	80 (100)	12 (15.00)	0.963 NS
Chi-square-χ^2	5.612 *	1.82 NS	4.853 *	1.579 NS	----

* (P<0.05), NS: Non-significant.

This result was almost similar to that in the study by (12), who reported 13.6 % positivity, but lower than that reported by (13) 28.8%. 2.7 % isolation of *C. difficile* from control group (14), but other authors (15) all of the control stool samples were negative for toxins A/B by ELISA, and males 53% were more infected than female 46%. Our results are similar to (16) who said that females were more infected than males. This result agrees with (17) who reported 18.2% positivity. The organism has been recovered from the hands of hospital personnel, baby baths, oxi meters, electronic thermometers, and hospital floors. Breastfed infants have lower carriage rates than do formula-fed infants 14 -30 %, respectively. At

6 to 12 months of age, approximately 14% of children are colonized with *C. difficile*, and by 3 years of age, the rate is similar to that of non-hospitalized adults 0-3%. Recognized risk factors for older children acquiring CDI included antimicrobial therapy (6 and 7). The diagnosis of *C difficile* disease is based on the presence of diarrhea and of *C difficile* toxins in a diarrheal stool specimen. In conclusion, the most common testing method used today for *C. difficile* toxins is the commercially available enzyme immunoassay (EIA), which detects toxins A and/or B.

References

1. Abhishek, D.; Vinay, P.; Preethi, P.; Gati, A. and David, D. K. R. (2011). Repeat stool testing to diagnose *Clostridium difficile* infection using enzyme immunoassay does not increase diagnostic yield. Clinical Gastroenterology and Hepatology, 9: 665–669.
2. Luis, A. (2013). Laboratory tests for diagnosis of *Clostridium difficile* infection: Past, present, and future. Enferm Infecc Microbiol Clin., 31(2): 65–67.
3. Christina, M. S. MD.; Lawrence, J. B. MD. and David G. B. MD. (2013). Guidelines for Diagnosis, Treatment, and Prevention of *Clostridium difficile* Infections. Am. J. Gastroenterol., 108: 478 – 498.
4. John, G. B. and Dale, N. C. (2008). Clinical Recognition and Diagnosis of *Clostridium difficile* Infection. Clin. Infect. Dis., 46:12-18.
5. Lissa, S. T.; Yaw B. O.; Julian G. H. and Dianqing S. (2014). Progress in the Discovery of Treatments for *C. difficile* Infection: A Clinical and Medicinal Chemistry Review. Current Topics in Medicinal Chemistry, 14(1): 152-175.
6. Jangi, S. and Lamont, J. T. (2010). Asymptomatic colonization by *Clostridium difficile* in infants: implications for disease in later life. J Pediatr Gastroenterol Nutr., 51(1): 2–7. pmid: 20512057.
7. Gordon, E.; Schutze, M. D.; Rodney, E. and Willoughby, MD. (2013). *Clostridium difficile* Infection in Infants and Children. Pediatrics 131(1): 196 -200.
8. Awanes, A. S. (1997). Isolation and Identification of *Clostridium difficile* from children in Baghdad. M.Sc. thesis, College of science, University of Baghdad.
9. Ghanim, H. M. (2003). Isolation, Identification and Toxin production From *Clostridium difficile* in patients with diarrhea and colitis. Ph.D. Thesis, College of Science, Baghdad University. Pp: 121.
10. Bartlett, J. G. (1990). *Clostridium difficile*: Clinical consideration. Rev. Infect. Dis., 12: 243-251.
11. Bartlett, J. G.; Chang, T. M.; Gurwith, M.; Gorbach, S. L. and Onderdonk, A. B. (1978). Antibiotic associated pseudomembranous colitis due to toxin producing Clostridia N Engl. J. Med., 298: 531-534.
12. Dhawan, B.; Chaudhry, R. and Sharma N. (1999). Incidence of Clostridium difficile infection: a prospective study in an Indian hospital. J Hosp Infect., 43: 275-80.
13. Sultana, Q.; Chaudhry, N. A.; Munir, M.; Anwar, M. S. and Tayyab, M. (2000). Diagnosis of *C. difficile* antibiotic associated diarrhoea culture versus toxin assay. J. Pak. Med. Assoc., 50: 246-249.
14. Dutta, P.; Niyogi, S. K.; Mitra, U.; Rasaidy, R.; Bhattacharya, M. K.; and Chakraborty, S. et al. (1994). *Clostridium difficile* in antibiotic associated pediatric diarrhea. Indian Pediatr., 31: 121- 126.
15. Gogate, A.; De, A.; Nanivadekar, R.; Mathur, M.; Saraswathi, K.; Jog, A. and Kulkarni, M.V. (2005). Diagnostic role of stool culture and toxin detection in antibiotic associated diarrhoea due to *Clostridium difficile* in children. Indian J. Med. Res., 122: 518-524.
16. Norakhoda, S.; Mohammad, H. S. and Mohammad R. G. (2010). The Incidence of Nosocomial Toxigenic Clostridium difficile Associated Diarrhea in Tehran Tertiary Medical Centers. Acta Medica Iranica; 48(5): 320-325.
17. Victor, R. C. M.; Viviane, N.; Sydney, M. F. and Mario, J. A. C. (2014). Genes Encoding Toxin of *Clostridium difficile* in Children with and without Diarrhea. Hindawi Publishing Corporation Scientifica. Volume 2014, Article ID 594014, 4 pages. http://dx.doi.org/10.1155/2014/594014

تشخيص بكتيريا *Clostridium difficile* من اطفال عراقيين يعانون من الاسهال والتهاب القولون المصاحب للمضادات الحيوية باستعمال تقنية ELISA

منى تركي الموسوي و لمى يوسف مهدي
قسم علوم الحياة، كلية العلوم للبنات، جامعة بغداد، العراق.
E-mail: yousifnawar@yahoo.com

الخلاصة

هدفت الدراسة الحالية إلى تشخيص بكتريا *Clostridium difficile* المسببة للإسهال والتهاب القولون المصاحب للمضادات الحيوية باستعمال تقنية الألايزا وذلك بالكشف عن وجود السموم بنوعيها أ و ب والتي تنتجها هذه البكتريا. وقد خضعت 240 عينة براز والتي أخذت من أطفال يعانون من الإسهال للفحص وباعمار من بعد الولادة ولغاية 15 سنة، فضلاً عن 80 عينة من اطفال اصحاء كمجموعة سيطرة. جُمعت العينات من المدّة مابين الاول من حزيران 2013 ولغاية نهاية نيسان 2014. أثبتت النتائج وجود سموم أ وب لجرثومة الكلوستريديم وأن نسبة إصابة الإناث بتلك الجرثومة أعلى بقليل من الذكور، ولم تظهر فروق معنوية بين المرضى والسيطرة. واظهرت النتائج أن (21.25%) من العينات موجبة ويمكن أن نستنتج أن أعلى نسبة استيطان كانت في الاعمار أقل من سنة اذ بلغت 15%. وأن نسبة وجودها قلت بزيادة العمر.

الكلمات المفتاحية: تقنية الألايزا، كلوستريديم، الإسهال المصاحب للمضادات الحيوية، إلتهاب القولون.