Isolation and Identification of *Clostridium difficile* from Antibiotic- Associated Diarrhea and Colitis in Iraqi children, using API20A Anaerobic system.

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Abstract

Clostridium difficile were isolated by 51 (21.25%) from a total of 240 stool sample collected from children having diarrhea and colitis at ages from after birth to 15 years old from Baghdad hospitals, who were under treatment with broad spectrum antibiotics, and Sample were taken during the period of first of June 2013 till the end of april 2014. Also 80 stool samples were collected from healthy ,non diarrhea children as controls. Morphological and API 20A tests were used for the identification of this bacterium to evaluate the performance of the API 20A anaerobe system in definitively identifying strains of *C. difficile*. . This study shows that females were more infected than males .Overall positivity was (21.25%)in this study group compared to controls ($P<0.05$). The isolation percent from age <5years was (18.75%) , while it in age>5years was(2. 5 %) . It was found that this percent decreased with age get older.

Key words: API20A KIT , *Clostridium difficile*, Antibiotic-Associated Diarrhea(AAD), Colitis.

Introduction

Clostridium difficile is the most common cause of Antibiotic-Associated Diarrhea and Colitis, usually occurring after exposure to antibiotics[1]. During past few years,

C. difficile infection has become more frequent, more severe, more refractory to standard treatment, and more likely to relapse [2]. *C. difficile* remains the most important cause of healthcare-associated diarrhea and is increasingly important as a community pathogen. The strains of *C. difficile*

with hyper virulent have been reported for after the use of nearly all systemic antibacterial agents worldwide[3] .

The decreased effectiveness of metronidazole relative to vancomycin in the treatment of *C.difficile* has been demonstrated[4]. The pathogenic effects of *C. difficile* are mucosal damage to the colon that is caused by the production of toxin A (308 kDa) and/or toxin B (270 kDa) [5] . Many infants are colonized by toxigenic or non -toxigenic strains during the first two years of their life. A study was sparked by the finding of stool positive for *C. difficile* whose mortality was linked to sudden infant death syndrome , showed significantly greater colonization in newborns fed on formula 71% than in breast-fed infants 7%. [6 , 7]. *C. difficile* is a spore-forming , obligate anaerobic, Gram positive bacillus and is usually acquired either from the environment or via the fecal- oral route [8]. *C. difficile* is a bacterium of ubiquitous nature whose spore producing ability renders it highly resistant to environmental and food production associated stresses[9]. CDI has become an increasingly common infection and has shown an increase in severity over the past several years[10]. Since not all strains of *C. difficile* form toxins and approx. 2 % healthy adults and up to50% of children under the age of 2 years may be parasitized with *C. difficile*. [11]. Previous study in Iraq isolated 14%,diarrhea8%,and healthy 6%[12] ,and due to the importance of detection of *C.difficile* infection especially in children under 5 years, and because of rare of previous study in Iraq of this bacteria ,suggested to do this study.

Material &Methods

Sample:

335 stool sample were collected from children suffering from Antibiotic-Associated Diarrhea(AAD) and Colitis, after exposure to antibiotics ,cases at ages from after birth to 15 years old in Baghdad hospitals, and 240 samples of them was chosen from non-fungus and non-parasite fesses . Sample were taken during the period of first of June 2013 till the end of April 2014. Also80 stool samples were collected from healthy ,non diarrhea children as control .

Media:

C. difficile agar base : for the isolation of *C. difficile* when used with supplements .(SIGMA-ALDRICH,USA) .CCFA (Cycloseren-Cefoxitin-Fructose Agar.(As a selective media).And 7%horse blood[13].

Technique for Alcohol Shock Treatment[13, 14].:

Mix equal parts of industrial absolute alcohol and the fecal specimen. Homogenize using a vortex mixer. Leave at room temperature for 1 hr. Inoculate on to *C. Difficile* Selective Agar and Incubate at 37ºC in a conventional anaerobic gas jar. The use of the Oxoid Anaerobic Jar with Anaerogen (AN0025,OXOID,UK)gas back Kit and observe after 24-48 hrs in anaerobic conditions .

Gram stain reagent and Malachite Green: For morphological study.

Identification was performed by the fermentation of glucose, lactose, maltose and sucrose, in peptone yeast extract (PY) broth, and indole production; gelatin, esculin and starch hydrolysis ,and motility[15] .To further confirmation utilized API 20A kits (bio Mérieux).

API 20A KIT(bio Merieux ,Inc.USA). System for the identification of anaerobes. The API 20 A system enables 21 tests to be carried out quickly and easily for the biochemical identification of anaerobes(table5). Other tests such as colonial and microscopic morphology, Gram stain, Malachite Green. Should be performed and the results used to confirm or complete the identification.[16].

Statistical Analysis

The Statistical Analysis System- SAS [17] was used to effect of different factors in study parameters. Chi-square test was used to significant compare between percentage in this study.

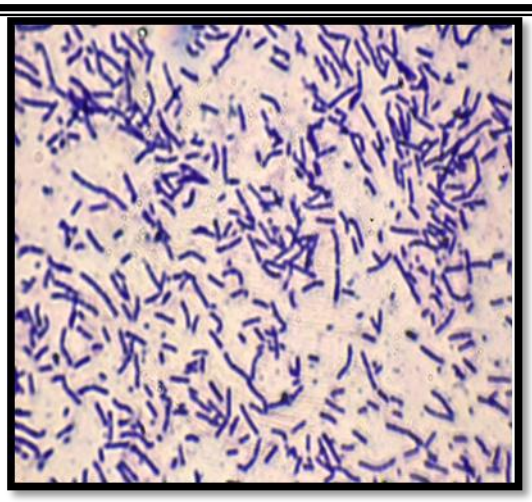
Results and Discussion:

Isolation and Identification:

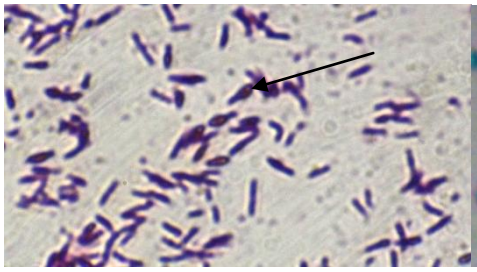
After 48 hrs *C. difficile* colonies grow circular, raised, opaque grey-white, sometimes with irregular borders, and(2-4)mm in diameter. In addition ,under UV light they produce a characteristic fluorescence. but with no signs of hemolysis. Well-grown cultures have a characteristic "horse-stable-like" odour. [18]. (fig:1).

Gram stain: positive bacillus cell shape, Arrangement: single or chains(2-6)cell sub terminal endo spore forming(fig2,3,4). API 20A strips used for identification of *C. difficile* . After 48 hrs of incubation all the isolates was confirmed.(table:4)and (fig5).

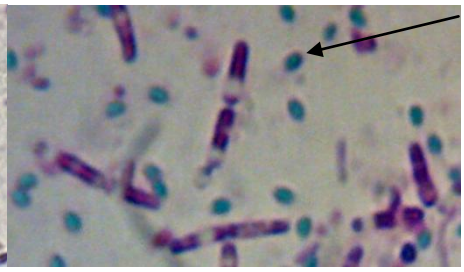
Figure(1). Typical *C .difficile* colonies on CCFA containing blood.



Figure(2). Gram positive (1000x)



Figure(3):spore forming bacteria.1000x



Figure(4):Malachite green stain spore.1000x

Figure(5):API 20A strip used for identification of *C. difficile* . After 48 hrs of incubation all the isolates was confirmed.

Table (4). Api20A identification results of 51 isolate.

Test	results	No. positive	No. negative
IND	--	0	51
URE	--	0	51
GLU	+	51	0
MAN	±	5	1
LAC	--	0	51
SAC	--	0	51
MAL	--	0	51
SAL	±	5	46
XYL	--	0	51
ARA	--	0	51
GEL	+	51	0
ESC	+	51	0
GLY	--	0	51
CEL	±	2	49
MNE	±	50	1
MLZ	+	51	0
RAF	--	0	51
SOR	±	1	50
RHA	±	1	50
TRE	±	1	50
CAT	--	0	51
SPOR	+	51	0
GRAM	+	51	0
COCC	--	0	51

C. difficile were isolated by 51 (21.25)% from a total of 240 stool samples collected from children having diarrhea. The isolation percent from age≤5 years was18.75% while it in age >5was2.5%(table:1)and(fig:1) , and it was found that this percent decreased with age, it formed higher isolation percentage in ages<5years. As a compare with controle, the difference was non statistically significant ($P< 0.05$).

Table 1. Distribution of study sample according to age groups

Age groups 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.					

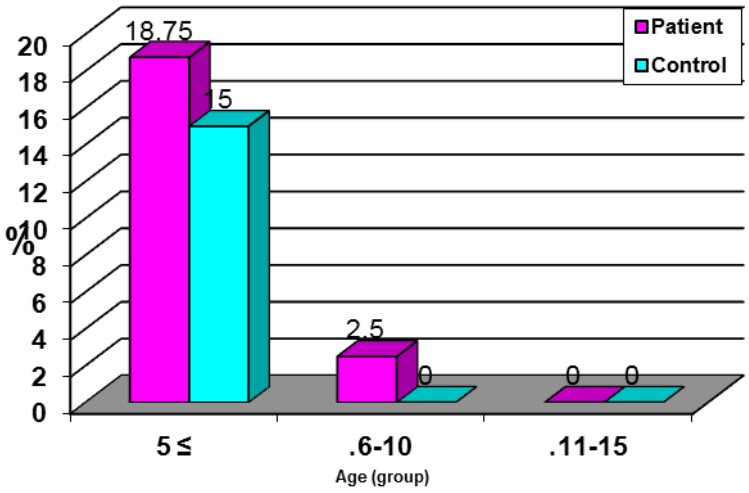


Figure 1. Distribution of sample study according to age group (%)

The isolation percent from age <1year was 15%(table:2)and(fig:2), while in age>1year was 6.25 %.(table:1, 2). As a compare with controle, the difference was non statistically significant ($P< 0.05$).

Table 2. Distribution of study samples according to age groups<5years.

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.					

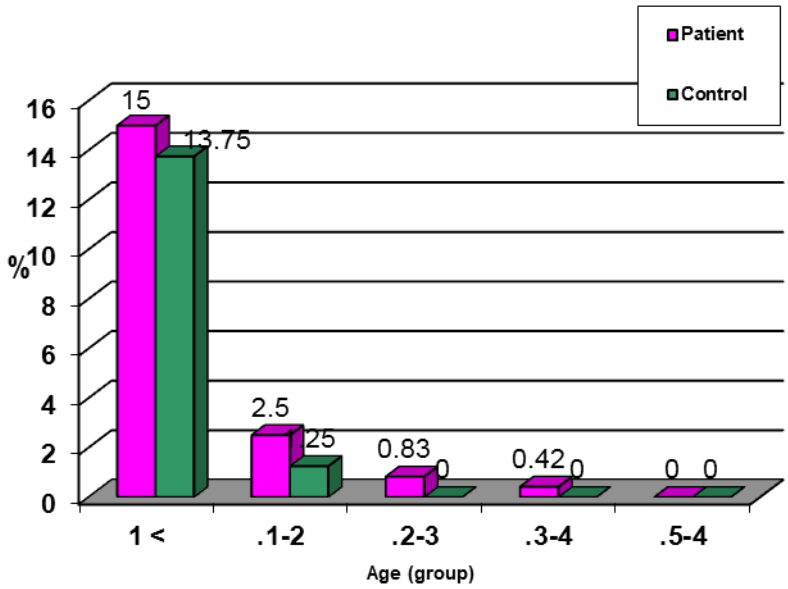


Figure 2. Distribution of sample study according to age group (%)

It was found that this percent decreased with age get older. This study shows that females were more infected than males (table:3)and(fig:3) .

Table 3. Distribution of sample study 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.					

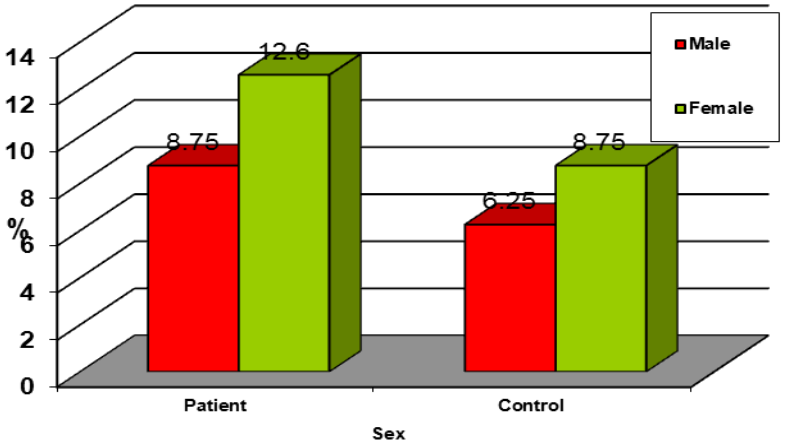


Figure 3. Distribution of sample study according to sex (%)

This result agrees with previous studies in Iraq [19] who isolated 13%,in age<1year10%and in age>1year 3% of *C. difficile* from children suffering from diarrhea on medication with antibiotic , while in healthy children 12%.And with another study who isolation percent was 17 %under 2 years[20].

Our study disagreement with another study was isolated 5.5%of children under 5 years and no healthy children harbored this microorganisms[21] .And disagreement with [22] was isolated 3% <15 years, also females were infected more than males [22] . This is an indicator that CDI has become an increasingly common infection and has shown an increase in severity over the past several years. Our goal with this study is that physicians will be more aware of the seriousness of AAD and be vigilant about quick diagnosis and appropriate treatment.

C. difficile is the cause of approximately(25–30)% of all cases of (AAD) [23]. The growth of infection may be caused by multiple factors including inappropriate antibiotic usage, poor standards of environmental cleanliness, changes in infection control practices, large outbreaks of *C. difficile* infection in hospitals, alteration of circulating strains of *C. difficile*, and spread of hyper virulent strains. [23].

Detection of high-risk populations could be helpful for prompt diagnosis and consequent treatment of patients suffering from *C. difficile* infection. Current treatments for *C. difficile* infection consist of supportive care, discontinuing the unnecessary antibiotic, and specific antimicrobial therapy.[24].

The purpose of this report is to evaluate the performance of the API 20A anaerobe system in definitively identifying strains of *C. difficile*. ,and to further confirmation utilized API 20A kits (bioMérieux).[25].

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Table:5 READING TABLE API20A

TESTS	ACTIVE INGREDIENTS	REACTIONS/ENZYMES	RESULTS	
			NEGATIVE	POSITIVE
IND	L-tryptophane	INDole formation	XYL - mix / 2-3 min Yellow	+EHR / 5 min Red
URE	urea	UREase	yellow-orange	red
GLU MAN LAC SAC MAL SAL XYL ARA	D-glucose D-mannitol D-lactose (bovine origin) D-saccharose (sucrose) D-maltose salicin D-xylose L-arabinose	acidification (GLUcose) acidification (MANnitol) acidification (LACtose) acidification (SACcharose) acidification (MALtose) acidification (SALicin) acidification (XYLose) acidification (ARAbinose)	BCP purple	BCP Yellow/yellow-green
GEL	gelatin (bovine origin)	hydrolysis (protease) (GELatin)	no diffusion of pigment (1)	diffusion of black pigment (1)
ESC	esculin ferric citrate	hydrolysis (ß-glucosidase) (ESCulin)	yellow (2) in UV (365 nm fluorescence	brown-black (2) nm no fluorescence
GLY CEL MNE MLZ RAF SOR RHA TRE	glycerol D-cellobiose D-mannose D-melezitose D-raffinose D-sorbitol L-rhamnose D-trehalose	acidification (GLYcerol) acidification (CELlobiose) acidification (ManNosE) acidification (MeLeZitose) acidification (RAFfinose) acidification (SORbitol) acidification (RHAmnose) acidification (TREhalose)	BCP purpule	BCP Yellow/yellow-green
CAT		CATalase	After 30 min in air No bubble	H_2O_2 in a positive tube bubble
SPOR		spores	absent	present
GRAM		Gram reaction	pink	violet
COCC		morphology	rod	coccus

عزل وتشخيص جرثومة *Clostridium difficile* من أطفال عراقيين يعانون الإسهال والتهاب القولون والمصاحب للمضادات الحيوية بواسطةAPI20A anaerobic system

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الخلاصة:

تضمنت الدراسة عزل وتشخيص *Clostridium difficile*من الأطفال المصابين بحالات الإسهال والتهاب القولون الناجمين عن تناول المضادات الحيوية وبأعمار من بعد الولادة ولغاية 15 سنة.تم جمع العينات من الفترة مابين الاول من حزيران 2013 ولغاية الاول من نيسان 2014 .اضافة الى جمع 80 عينة من اطفال اصحاء كمجموعة سيطرة.اعتمدت الاختبارات المظهرية واستخدام نظام API20A في التشخيص وتعزيز الاختبارات الكيمياحيوية. بينت النتائج أن نسبة عزلها من عينات الإسهال عند الاطفال البالغة 240 عينة كان (21.25%).اوضحت الدراسة ان نسبة اصابة الاناث اعلى بقليل من الذكور,وعدم ظهور فروق معنوية بين المرضى والسيطرة.وعند دراسة تأثير العمر على نسبة عزلها تبين ان النسبة تقل مع زيادة العمر, إذ بلغت أعلى نسبة عزل(18.75%) عند الفئة العمرية الاقل من 5سنوات ,بينما عزلت بنسبة (2.5%)بعمر أكثر من 5سنوات.