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The clinical and environmental spread and diversity of toxigenic *Clostridium difficile* diarrhea in the region of the Middle East

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Abstract

Stool samples of 1822 hospitalized patients with nosocomial diarrhea and 100 environmental samples were collected at three teaching hospitals and PCR amplification of rRNA intergenic spacer regions (ISR) was conducted. Bacterial cytotoxicity was assayed by conducting three assays namely toxigenic culture on vero cells, stool cytotoxin, and enzyme immunoassay. ISR was carried out using two universal primers complementary to conserved regions in the 16S and 23S rRNA genes. It was found that the toxigenic culture, stool cytotoxin and enzyme immunoassay showed close rates of detection of toxigenic *C. difficile*, 124, 121, and 122 /1822 (6.8, 6.64., and 6.7%) respectively. In addition, 32 different ribotypes for toxigenic *C. difficile* were detected, 28 in clinical and 6 in environmental isolates. The predominant ribotypes from the clinical isolates were 13-15, 35.6%, of isolates. Ribotypes were associated with age, location of isolation, and severity of symptoms of clostridial diarrhea ($P<0.05$). Ribotypes 6-9 affected children only. The most common ribotype of *C. difficile* , no. 13, as well as ribotypes 16, 20, and 4 covered almost the whole range of severity of symptoms. Ribotypes 21-27, 1, 3, 6, 7, 9, 11, 14, and 19 caused mild-moderate CDAD symptoms while ribotypes 5, 10 8, 12, 15, 17, and 28 were dominantly of severe symptoms ($P<0.05$). Environmental isolates showed 17% toxigenic strains composed of 4 different ribotypes while ribotypes 5 was shared with clinical isolates. These findings showed that *C. difficile* associated with diarrhea were genetically diverse and linked to environmental strains.

Key words: PCR ribotyping; Antibiotic-associated diarrhea; Hospital environment; Bacterial epidemiology

Introduction

Clostridium difficile is a frequently identified cause of nosocomial gastrointestinal disease [1]. It has proved to be a causative agent in antibiotic-associated diarrhea, antibiotic-associated colitis, and pseudomembranous colitis [2-3]. C. *difficile* is responsible for 15–25% of cases of antibiotic-associated diarrhea and for more than 95% of cases of pseudo-membranous colitis [2]. *C. difficile* associated diarrhea (CDAD) is usually associated with nosocomial acquisition and prior antibiotic therapy; however the immunocompromised state, bowel surgery, bowel stasis, and genetics can also be predisposing factors [4,5,6,7]. CDAD may also occur when no definite risk factors are present [8]. Many strains of *C. difficile* produce two protein exotoxins, A and B, which are thought to be the primary causes of colonic mucosal injury and inflammation [9,10,11]. Toxin A brings about primarily enterotoxic effects, while toxin B is primarily a cytotoxin [9,10,11]. The biological diagnosis of digestive tract infections associated with *C. difficile* is based either on the isolation of the bacterium or on the detection of a specific antigen, glutamate dehydrogenase (GDH), or toxins (A or B) in faecal samples [12]. At present, the reference method is the stool cytotoxin assay, which reveals the presence of toxins in stool samples [12,13,14]. Many various typing methods have been developed to investigate nosocomial outbreaks of *C. difficile*; some are based on phenotype (lysotyping, serogrouping, SDS-PAGE, immunoblotting) and others on genotype (plasmids, REA, ribotyping, PFGE, RAPD) [15,16,17]. A method of PCR-ribo-typing of *Pseudomonas aeruginosa* developed by Kostman et al. [18] was shown to be reproducible, easy to perform, and cost effective. It has been adapted to *C. difficile* by Gurtler [19]. This typing method is based on the presence of several alleles of the rRNA operon on the bacterial chromosome differing by the length of the intergenic spacer region located between the 16S and the 23S rRNA genes [4, 17]. This part of the genome has been shown to be very heterogeneous, in contrast to the rRNA gene themselves, which are highly conserved [4, 17]. *C. difficile* was shown to possess up to 10 more copies of the rRNA genes in its genome, which varied not only between

strains, but also between different copies on the same genome [4]. Currently, a strain known as type BI by REA, North American pulsed-field type 1 (NAP1) by Pulse field gel electrophoresis (PFGE), and 027 by PCR-ribotyping (BI/NAP1/027) is the single most important epidemic strain causing CDAD in North America and Europe [20, 21,22,23]. The emergence of this hypervirulent strain has increased interest in *C. difficile* typing and stimulated the application of newer genotype-based methods such as PCR-ribotyping, amplified fragment length polymorphism (AFLP), multilocus sequence typing (MLST), multilocus variable-number tandem-repeat analysis (MLVA), and surface layer protein A gene sequence typing (slpAST) [24]. In spite of the growing number of studies on CDAD in Western countries, studies on CDAD are limited in the Middle East and Asia where information on the prevalence of *C. difficile* carriage and CDAD is lacking. The present study monitored and compared the ribotypes of 124 clinical isolates originating from patients in three teaching hospitals as well as 17 environmental isolates in order to draw the ribotypic map of *C. difficile* in the region of the Middle East and its association with CDAD attributes.

Materials and methods

Study site and stool specimens

During the study period (from December 2002 to August 2008), 1822 hospitalized patients with nosocomial diarrhea from three Tehran University hospitals (Emam Khomeini, Shariatei, and Children's Medical Center) in Tehran, Iran, were screened for the presence of *C. difficile* and its toxins. Diarrheal stool samples were selected by laboratory criteria. The selection criteria were: long stay hospitalization (> 5 days), loose, liquid stools (bloody and/or mucoid), lack of other enteric pathogenic bacteria, viruses, ova or parasites. All the Diarrheal stool samples were subjected into multiple testing techniques: isolation and culture of *C. difficile* for bacterial toxgenicity testing (toxigenic culture), stool cytotoxin assay for indicating the presence of toxins in stool, and an enzyme immunoassay system for the specific detection of Tox A and B of *C. difficile* using Tox A/B II kit (TechLab, Inc., Blacksburg, Va) [25,26]. The reason for using three different methods to detect *C. difficile* toxins was for comparing the results among these three methods. It is noteworthy that dealing with human subjects was under Helsinki declaration and the institutional review board had already garnted permission of this project.

Isolation of Clostridium difficile

Selective cycloserine cefoxitin fructose agar (CCFA) medium (Bio Merieux, France) was used on stool samples of 1822 hospitalized patients with nosocomial diarrhea in order to isolate clinical strains of *C. difficile*. Plates were incubated under anaerobic condition for 48h at 37°C. The isolates, in duplicates, were identified as *C. difficile* by characteristic morphology and biochemical test (API20A; Bio Merieux, France). In addition, to determine the distribution of *C. difficile* in hospital environments, 100 environmental samples were taken from several units at Emam Khomeini hospital which was chosen as a representative for other hospitals because this hospital is the largest hospital in the region with nation-wide admission; moreover, the environmental samples of other two hospitals are belived not to vary much as theya relocated at the same city. Environment sites were cultured by using sterile premoistened cotton swabs inoculated into brain heart infusion broth and incubated anaerobically for 48 to 72h at 37°C. The screened environmental surfaces included walls, doorknobs, floors, night tables, bedpans, and washstands. Cultures were then streaked onto CCFA plates, incubated, and purified as described above.

Stool cytotoxin assay

Stool samples (0.5 mL), in duplicates, were added to 0.5 mL of phosphate buffered saline (PBS) at pH 7.4; after the centrifugation (8000 g, 10 min), the supernatant was collected and passed through a 0.4 µm pore filter (14, 17). Before use, the filtrates were diluted 1:10; 1:40 and 1:100. Twenty µL of the 1:10 dilution was inoculated onto previously prepared Vero monolayers in 96-well microtiter plates (Sterilin, UK) with and without the addition of *C. difficile* antitoxin (TechLab, Inc., Blacksburg, Va., USA). 20 µL of the 1:10 and 1:100 dilutions of antitoxin was added to duplicate wells. Plates were covered, incubated at 35°C in 5% CO2, and then examined using an inverted microscope after 24 and 48 h for cytopathic effects characteristic of *C. difficile* toxins. Samples producing cytopathic effects in the well without *C. difficile* antitoxin but not in the *C. difficile* antitoxin-containing well were considered positive for *C. difficile* toxins. A positive *C. difficile* toxin B control (TechLab) was included with each run. Where a cytopathic effect was observed with a 1:10 dilution of faeces and was neutralized by antitoxin, the assay was repeated using higher dilutions (1:40 and 1:100) of faeces (the lower dilution was used for screening and the higher dilution of stool used for the confirmation of the test).

Toxigenic culture

For toxigenic culture, 3-6 colonies of *C. difficile* were inoculated into Brain Heart Infusion Broth (Oxoid, UK) in an anaerobic chamber and incubated for 5-7 days at 37°C [11]. Broths were centrifuged for 10 min at 2500 g and the supernatant was collected and passed through a 0.2 µm pore filter). 100 µL of culture filtrates which were prepared by serial 10-fold dilution in Eagle minimal essential medium (Gibco) supplemented with 2% faetal bovine serum (Sigma, USA) were inoculated, in duplicates, onto previously prepared Vero monolayers in 96-well microtiter plates, with and without the addition of *C. difficile* antitoxin (TechLab, Inc., Blacksburg, Va., USA) [11]. Tissue cultures were examined after 24 and 48 h. Characteristic cytopathic effect (CPE) in more than 50% of cells across the cell sheet that was neutralized by antitoxin (TechLab) was interpreted as a positive result.

Enzyme immunoassay (C. difficile Tox A/B ,TechLab)

The faecal samples, in duplicates, were tested directly for Toxins A and B according to the manufacturer's recommended procedures (TechLab, Inc., Blacksburg, Va., USA). This kit is designed to simultaneously detect C.difficile toxins A and B in stool samples. Briefly, C.difficile toxins were extracted from the stool samples and immobilized at the designated detection zones of panels. Then, conjugated antitoxin antibodies and substrates, which were provided with the kits, were added to the

Table 1. Results of culture of organisms , toxigenic culture, stool cytotoxin assay and enzyme immunoassay

Results	Culture	Toxigenic culture*	Stool cytotoxin assay	Enzyme immunoassay
Positive	178	124	121	122
Negative	1644	1698	1701	1699
Total	1822	1822	1822	1822
Sensitivity (%)	-	-	97.5	98.4
Specificity (%)	-	-	100	100
Predictability of positives (%)	-	-	100	100
Predictability of negatives (%)	-	-	99.82	99.88

*: It was considered as golden standard on which percentage of sensitivity, specificity, and predictabilities were calculated

appropriate panels. Presence of toxins was indicated by the development of a color bar in the appropriate detection zone. Positive controls for QC-toxinA/B were included in the panels. A test was considered valid if a color bar appeared in the appropriate zone.

DNA extraction

Chromosomal DNA was extracted from colonies of *C. difficile* by using the UltraClean soil DNA kit (Cambio, UK) according to the manufacturer's instructions. The DNA obtained was resuspended in Tris-EDTA buffer (Sigma, USA) and electrophoresed on a 1% agarose gel to determine its integrity before being stored at -20°C until required. The gels were stained with ethidium bromide (Sigma, USA) and a single band was observed at the desired position on ultraviolet light transilluminator (Vilber Lourmat, Cedex, France).

PCR-ribotyping

PCR amplification of the intergenic spacer region (ISR) was carried out by using two universal primers complementary to conserved regions in the 16S and 23S rRNA genes, as recommended previously [22]. The forward primer sequence is located at nucleotide positions 1477 to 1493 on the 16S rRNA gene of *C. difficile* strain 630 (region 4, 5'-GGC TGG ATC ACC TCC TT-3') while the reverse primer sequence is located at nucleotide positions 21 to 41 on the 23S rRNA gene of *C. difficile* strain 630 (region 5, 5' -TAG TGC CAA GGC ATC CGC CCT-3') [27]. All reagents of PCR reaction were from Promega Inc., USA. DNA templates were amplified, in duplicates, in a total reaction volume of 50 µl containing 2.5 U of AmpliTaq Gold thermostable polymerase, 50 pmol of each primer, 200 mM of each deoxynucleotide, 1.5 mM $MgCl_2$, 10 mM Tris-HCl (pH 8.3), and 50mM KCl. Amplification was carried out in a GeneAmp 2400 thermal cycler (Applied Biosystems, USA) with initial denaturation at 94°C for 10 min, followed by 30 cycles according to the following program: 94°C for 1 min, 54°C for 1 min, 72°C for 2 min, and a final extension of 10 min at 72°C to complete partial polymerizations. Wells of negative control were made of the same reaction mixture but devoid of primers while the positive control wells were composed of reference strain ATCC 43593. The resulting amplification products were analyzed on a 2% agarose gel, stained with ethidium bromide (Sigma, USA), and viewed on a UV transilluminator (Vilber Lourmat, Cedex, France).

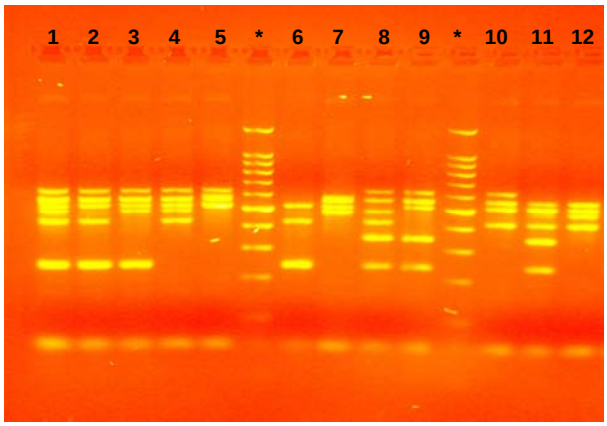

Fig 1. PCR ribotypes of *C.difficile* isolates. Lane *, 100 bp ladder; Lanes 1-12 , PCR ribotypes 1-12

Statistical analysis

Data of the current study were processed using statistical software SPSS version 12.0.01 and MS Excel 2007. The categorized qualitative data were analyzed by using Chi square test with Yate's correction and Fisher's exact test when needed. Pairwise analyses of data were conducted by using Tukey test. P values less than 0.05 were considered significant.

Results

During December 2002 to August 2008, 1822 diarrheal stool samples were studied; 973 of patients were males (53.4%) and 849 (46.6%) were females. And diarrheal stool samples were taken from 855 patients (46.9%) in Emam Khomeini Hospital, 662 patients (36.3%) in Children' s Medical Center, and 305 patients (16.8%) in Shariatei Hospital. Most of patients involved in this study were hospitalized in the Internal Medicine, Pediatrics and Surgery wards and intensive care units of the earlier mentioned three hospitals. In vitro toxin testing of isolates revealed that isolated *C. difficile* from clinical and environmental samples were either of toxigenic or non-toxigenic nature. During the study period, 178 (9.8%) *C. difficile* isolates were isolated from 1822 diarrheal faecal samples; however 54 (30.3%) isolates were nontoxigenic by toxigenic culture method and 124 out of 178 (69.7%) were

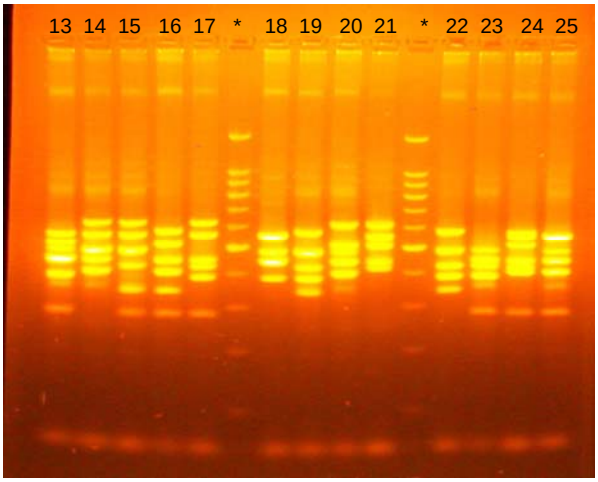

Fig 2. PCR ribotypes of *C.difficile* isolates. Lane *, 100 bp ladder; PCR ribotypes 13-25.

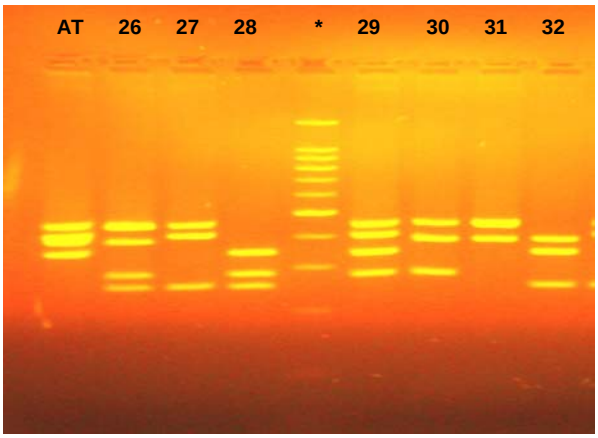

Fig 3. PCR ribotypes of *C.difficile* isolates. Lane *, 100 bp ladder; Lane AT, ATCC 43593; PCR ribotypes 26-32

toxin producers which was confirmed by one or all of the three toxin-detection methods, namely enzyme immunoassay for detection of *C. difficile* toxins A and B, toxigenic culture, and stool cytotoxin assay on Vero cells. Comparison between the results of these technique methods showed that all the three techniques are of close detection rate (Table 1) and there was no significant difference among them (P>0.05) rendering all of these techniques are equally reliable for the detection of *C. difficile* toxins. Clinicians had requested *C. difficile* toxin testing for only 290 of the 1822 diarrheal stool samples and only 32 (11%) of these samples were toxin positive. Of the 1532 remaining samples, 92 (6%) were toxin-positive by one or all of the three toxin-detection methods. Although, the rate of *C. difficile* toxin positive cases ordered by clinicians was significantly higher than that ordered merely by our research team (P<0.01), it is apparent that 6% of CDAD cases are still missed by clinicians which cannot be properly diagnosed. Among environmental samples, 24 (24%) *C. difficile* isolates were isolated; 17 (17%) isolates were toxigenic and 7(7%) isolates were nontoxigenic. It was found that the positive toxgenicity of *C. difficile* in diarrheal stools was not associated with the various age groups of CDAD patients (P>0.05). Moreover, it was found that the positive toxgenicity of *C. difficile* diarrheal stool samples was not associated with the sex of patients (P>0.05), 69 (55.6%) males and 55 (44.4%) females patients. One hundred and twenty four isolates of toxigenic *C. difficile* from symptomatic patients in three teaching hospitals in Tehran (including 74 isolates from Emam Khomeini Hospital, 32 isolates from Children's Medical Center, and 18 from Shariatei Hospital) and 17 environment isolates from Emam Khomeini hospital were typed by PCR amplification of rRNA intergenic spacer regions (PCR ribotyping). The PCR ribotypes consisted of patterns comprising 2-10 bands, with the size of the bands varying from 250-630 bp (Fig. 1, 2, and 3). A total of 28 different ribotypes was detected among the clinical isolates. The predominant ribotypes from the clinical isolates were ribotypes 13, 14, and 15, which accounted for 35.6% of all isolates. It was found that the distribution of *C. difficile* ribotypes was different among the three studied hospitals (P<0.05) indicating that *C. difficile* bacteria possess gentotypic variants even in the same geographical region. Ribotypes 1-5 were isolated only from hospitalized patients at Emmam Khomeini hospital, three clinical isolates from internal diseases department at Emmam Khomeini hospital. Ribotypes 6-9 were detected only at Children Medical Center and ribotypes 10-12 were restricted at Shariati hospital. Ribotypes 13-17 were five distinct clones that were circulating in all three hospitals. The ribotypes 18-21 were common in Emmam Khomeini and Shariati hospitals, 22-25 in Emmam Khomeini hospital and Children Medical Center and ribotypes 26-28 among Shariati hospital and Children Medical Center (Fig. 4). Interestingly, age of CDAD patients was found significantly associated with *C. difficile* ribotypes (P<0.05). Ribotypes 6-9 were found only in children patients as seen in Fig. 4. The majority of the most common ribotype, no. 13, was at age 5-30 years (P<0.05) (Fig.5). The age group 5-30 years lacks the following ribotypes: 10-12, 22-28, 1, 3, and 15-16. However, there was no specific ribotype found for this age group. For age group 31-55 years, it possessed exclusively the following ribotypes: 12, 24, 26, and 27; moreover, this age group possessed ribotypes, no. 13, 16, 22, and 23, more than in the older age group, >55 years, (P<0.05). For age group >55 years (elderly patients), it possessed exclusively ribotypes no. 10, 11, and 28 and possessed ribotypes, no. 1, 2,14, and 20, more than other age groups (P<0.05) (Fig. 5). Regarding severity of CDAD, it was found to be different among the different ribotypes of *C. difficile* (P<0.05). The most common ribotype of *C. difficile* , no. 13, as well as ribotypes 16, 20, and 4 covered almost the whole range of severity of symptoms (Fig. 6). Ribotypes 21-27, 1, 3, 6, 7, 9, 11, 14, and 19 caused mild-moderate CDAD symptoms while ribotypes 5 and 10 led to only severe symptoms of CDAD and ribotypes 8, 12, 15, 17, and 28 were dominantly of severe symptoms (P<0.05) (Fig. 6). For environmental isolates, 6 isolates had the same PCR-ribotyping patterns, no. 5, and these isolates were all derived exclusively from gastrointestinal ward. Other environmental isolates (11 isolates) were typed as ribotypes 29, 30, 31, and 32 which were distributed similarly in five different locations, namely infection ward, internal ward, surgery ward, ICU and CCU (P>0.05).

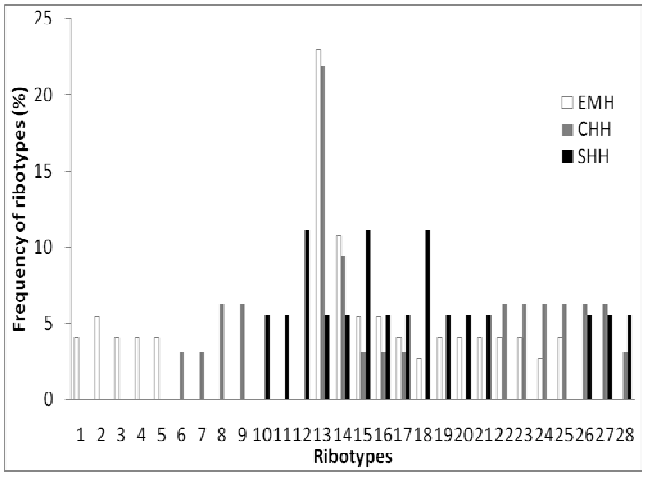

Fig4. Frequency of ribotypes distribution of *C. difficile* isolates, in term of percentage, among three source locations: EMH (Emam Khomeini Hospital), CHH (Children hospital, and SHH (Shariati Hospital).

Discussion

The current study revealed some important points of consideration; the CDAD patiens' age and sex was not different from that of other diarrheal patients. This referred to a notion that age has no role in increasing or decreasing CDAD. Moreover, the 124 toxigenic *C. difficile* patients were not associated with certain age group rendering age of minimal effect on the incidence of CDAD. Similar findings were found elsewhere in the world [30] confirming the minor role of age in CDAD. On the other hand, the three used toxin detection assays were shown to be of very close efficacy marking using one of them is credible enough for confirming CDAD diagnosis. The current study showed high percentage of toxigenic strains of *C. difficile* , 69.7% of the whole isolated bacteria and 9.8% of the whole 1822 diarrheal patients. This indicated that toxigenic *C. difficile* is widely distributed in the region of the Middle East classifying *C. difficile* as a quite health-endangering bacteria. An interesting finding, 6% of cases of diarrheal stool that were not suspected by physicians as CDAD were shown, by toxin assays conducted in the current study, as CDAD. Taken CDAD can be of serious consequences, misdiagnosis of CDAD and the failure to detect *C. difficile* bacteria along with their toxins can sometimes be so hazardous. Therefore, epidemiological studies, particularly these using precise molecular assays, on *C. difficle* seem to be essential for giving clear image on the diversity and spread of this bacterium along with the associated CDAD. Molecular typing methods are generally regarded as superior to phenotypic methods in terms of the stability of marker expression and providing greater levels of typeability. A number of molecular methods have been developed to investigate nosocomial outbreaks of *C. difficile*. Among these typing methods, PCR amplification of rRNA intergenic spacer regions (PCR ribotyping) of *C. difficile* is a discriminatory, easy to perform, cost effective and reproducible typing method [19]. Intergenic spacer regions may differ in size by less than 10 nucleotides in *C. difficile;* in these conditions, the smaller the DNA fragments generated by PCR-ribotyping, the more easily they are separated on an agarose gel matrix which lacks resolution to differentiate between two high molecular mass DNAs differing in size by a few nucleotides [19].

In the current study, ribotyping showed a great sensitivity for detecting the fine genetic diversity of *C. difficile* bacteria. We have discovered 32 different ribotypes of *C. difficile* scattered in three hospitals. The results of ribotyping in the current study agree with these obtained by Collier et al. [30] who analyzed representative isolates of the 10 serogroups of *C. difficile* composed of 39 clinical isolates (30 toxigenic and 9 nontoxigenic) by using two previously described arbitrary-primer PCR (AP-PCR) molecular typing methodologies (AP-PG05 and AP-ARB11) and PCR ribotyping. PCR ribotyping identified 8 unique groups. In addition, results obtained by PCR methods were compared with typing data generated by pulsed-field gel electrophoresis (PFGE); PCR ribotyping and PFGE were found to be in agreement for 83% of isolates typeable by both techniques while AP-PG05 was in agreement with PFGE for only 60% and AP-ARB11 only 44%. Therefore, it was indicated that PCR ribotyping is of more discriminatory approach than AP-PCR for typing *C. difficile*. Hence, the present study confirmed even further the high sensitivity of ribotyping in revealing 32 ribotypes just in three hospitals in closely related regions. Another study done in the region of the Middle East, Rotimi et al. [31], typed 95 isolates of *C. difficile* from symptomatic and asymptomatic patients and 18 from their environment in the intensive-therapy units (ITUs) of four teaching hospitals in Kuwait by PCR amplification of rRNA intergenic spacer regions (PCR ribotyping). Similar to our study, a total of 32 different ribotypes was detected among the clinical isolates. In our study, the most predominant ribotype was PCR ribotype 13, which accounted for 20.2% of clinical isolates and ribotypes 13, 14, and 15 collectively accounted for 35.6% of all isolates. Ribotype no. 13, to a lesser extent 14 and 15, were found in all three hospitals, covered almost all age groups and caused full range of symptoms severity making them as the most wide spread and important strains. A striking finding, ribotypes distribution of *C. difficile* was associated significantly with the the source hospitals. This indicated that *C. difficile* bacteria possess genotypic variants even in the same geographical region which in turn provides evidence on the high diversity of this bacterium that might impose growing difficulties on the antimicrobial treatment of *C. difficile*. In addition, ribotypes of *C. difficile* were found to be significantly associated with age. There were many ribotypes which were seen only in certain age group; for example' ribotypes 6-9 were seen only in children hospital; ribotypes 10, 11, and 28 were seen only in elderly group (>55 years old) and so on. The reason behind such predilection of different ribotypes to certain age groups has not been understood yet. However, this points out to the role of the genetic make up of *C. difficile* in the host-microbe relationship which might governs the success of CDAD in different age groups of human subjects. As far as our knowledge, no previous study correlated age of CDAD patients with the *C. difficile* ribotypes. Thus, there is a need to explore the bases of age association with *C. difficile* genetic variation more specifically. Most interestingly, ribotypes of *C. difficile* were remarkably associated with the severity of symptoms of CDAD. This striking finding can open the door to pinpoint which ribotype is more dangerous and which source or geographical region imposes higher risk to the population. Some ribotypes, 21-27, 1, 3, 6, 7, 9, 11, 14, and 19, caused only mild-moderate CDAD symptoms while ribotypes 5 and 10 led

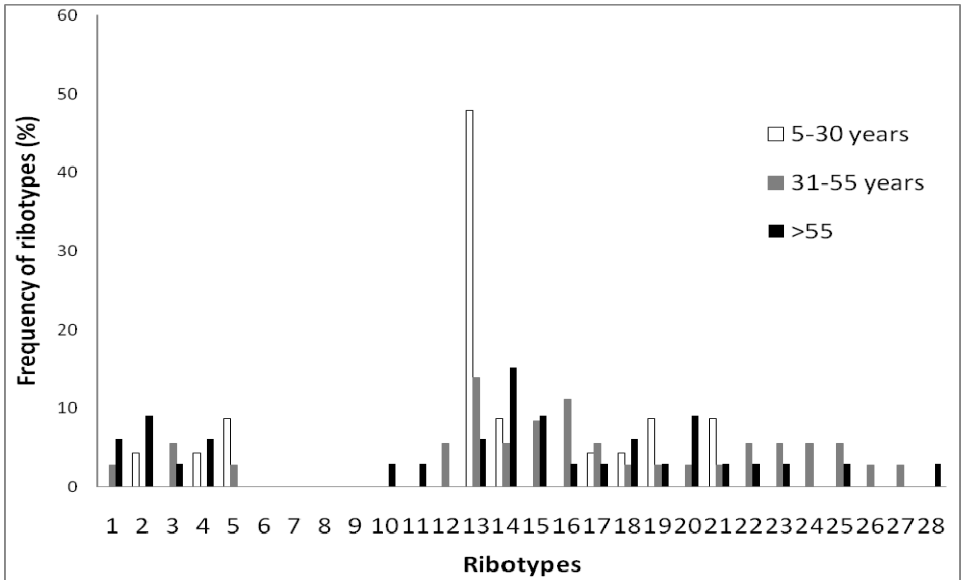

Fig 5. Frequency of ribotypes distribution of *C. difficile* isolates, in term of percentage, among three age groups: young (5-30 years), middle (31-55), and elderly (>55 years)

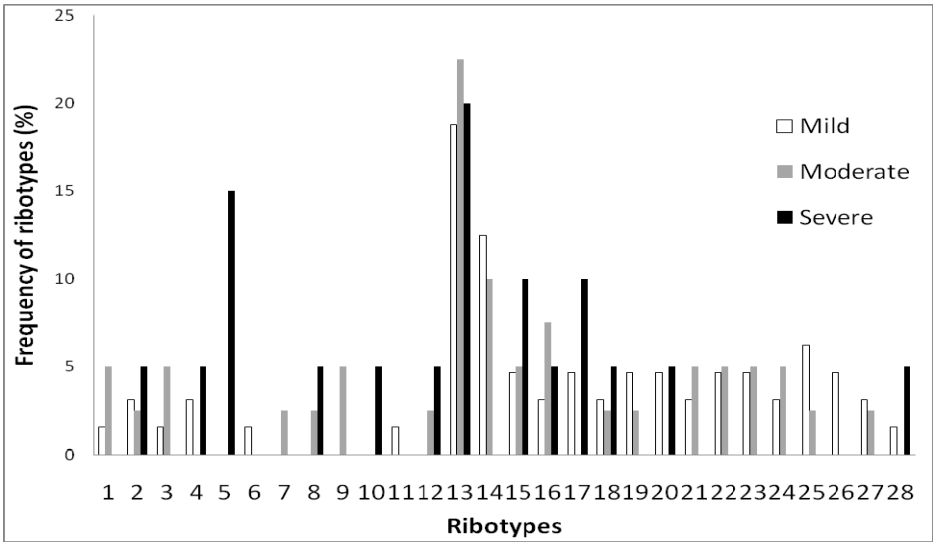

Fig 6. Frequency of ribotypes distribution of *C. difficile* isolates, in term of percentage, among three groups of symptoms severity of CDAD: mild, moderate, and severe groups.

to only severe symptoms of CDAD and ribotypes 8, 12, 15, 17, and 28 were dominantly of severe symptoms. Collectively, most common ribotype, no. 13, covered mainly patients of age less than 55 years and caused wide spectrum of symptoms severity. On the other hand, ribotypes affected only children, no. 6-9, were found mainly of mild-moderate symptoms except for ribotype 8 which caused severe symptoms. By the contrary, ribotype 5 caused severe symptoms of CDAD in patients of age ≤ 55 years while ribotype 10 and 28 caused severe symptoms in elderly patients (>55 years). This provided us with invaluable network of information on the connection between *C. difficile* genotype and patients' age and severity of symptoms altogether. For example, ribotypes 10 and 28 can be classified as extremely dangerous as they affect only elderly patients and cause only severe symptoms; alike, ribotype 8 is very dangerous too as it affects young children only and cause severe symptoms as well. In a study by Alcides et al. [29] in Brazil, isolates of *C. difficile* from faecal stool were analyzed by PCR ribotyping. They found that certain ribotypes were mostly associated with severely symptomatic rather than asymptomatic or mild symptomatic children. Consequently, these observations reveal a fact that the detected ribotypes of *C. difficile* in the Middle East or in other parts of the world have different virulent factors which in turn lead to a wide range of CDAD symptoms at different zones of age. This network of associations needs to be confirmed by other studies in different geographical regions in order to use this information for formulating proper preventive and therapeutic measures against *C. difficile* infections. On the other hand, ribotypes of

environmental isolates were different from these of clinical origin. The current study showed that many infections of CDAD were of patients-patients transmission and separate of the environmental line of transmission. Nevertheless, ribotype no. 5 was found common between environmental and clinical isolates. Given that ribotype no. 5 causes exclusively severe CDAD symptoms in young and middle age patients, this ribotype shall be addressed of higher hazard for environmentally-acquired infection of *C. difficile* leading to severe CDAD. Therefore, contaminated environmental surfaces with *C. difficile* ribotype 5 might be as important source for *C. difficile* transmission inside hospitals. It has been shown that contaminated environmental surfaces and health care personnel hand carriage are important sources for *C. difficile* transmission in hospitals [32]. Nevertheless, which method of transmission is the most dominant, the environmental or patients's cross infection, it is still under controversy. Previous studies systematically examined the relationship between environmental contamination and CDAD; they documented marked environmental contamination and transmission to personnel and patient contacts by an endemic *C. difficile* strain over a 6-month period [33, 34]. One study concluded that disparate strains responsible for causing disease were more likely to have originated from an environmental source than from cross-infection from patient to patient [34]. Elsewhere, a cluster of CDAD on a surgical unit was associated with an identical strain found in the environment [35]. Conversely, Cohen et al. found no evidence to suggest environmental acquisition of *C. difficile* [36]. Attempts to determine whether infected patients or contaminated environments are the prime source for cross-infection by *C. difficile* had limited success and therefore it was not possible to determine whether patients with CDAD preceded a rise in ward contamination or vice versa, but several factors including antibiotic prescribing practice, patient type and cleaning efficiency, may have influenced either incidence of CDAD or environmental contamination.

Conclusions

Taken together, rapid and sensitive diagnostic tests for laboratory confirmation of CDAD are important in the current health care environment in order to choose an effective antibiotic treatment and to take adequate measures to control nosocomial spread. Moreover, different genotypic variants of *C. difficile* are associated with different geographical locations, age groups, and levels of severity of symptoms providing evidence for the importance of ribotyping as a sensitive mean of studying the genotypic diversity of *C. difficile* and it is role in morbidity of CDAD.

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