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Original Article

Molecular Analysis of Toxigenic *Clostridium difficile* Isolates from Hospital Environment by PCR Ribotyping Method

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Background and Aims: *Clostridium difficile* is an identified cause of antibiotic-associated diarrhea, antibiotic-associated colitis, pseudomembranous colitis and nosocomial diarrhea. The objective of this survey was to determine molecular analysis of toxigenic *Clostridium difficile* isolates from hospital environment in Tehran tertiary medical centers.

Materials and Methods: In this descriptive study, 100 hospital environmental specimens were collected. The specimens were cultured on a selective cycloserine cefoxitin fructose agar, and incubated in anaerobic conditions, at 37°C for 2 days. *Clostridium difficile* isolates were characterized by conventional biochemical tests. Bacterial cytotoxicity was assayed on tissue culture, and also all strains were typed by PCR ribotyping method.

Results: Among toxigenic *Clostridium difficile* isolates, 6 isolates had the same PCR ribotyping patterns, and 11 isolates were typed in four different groups.

Conclusion: Our findings showed that toxigenic *Clostridium difficile* isolates had different PCR ribotyping patterns. Further studies are recommended to evaluate PCR typing of hospital environmental *Clostridium difficile* isolates.

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Introduction

Clostridium difficile is a major spore-forming environmental pathogen that is found in soil, and is nosocomially acquired during outbreaks of diarrhea in hospitalized patients who are immunocompromised or for whom antibiotics are prescribed. estimates are that as many as 300,000 patients suffer from *Clostridium difficile* associated disease each year in the United States [1-3]. Pathogenesis is associated with several toxins produced by toxigenic strains of *Clostridium difficile* [4-7]. Isolation rate of *Clostridium difficile* varies from 90% in specimens of patients with pseudomembranous colitis (PMC) to 20-25% in patients with antibiotic-associated diarrhea (AAD). Major risk factors of nosocomial *Clostridium difficile* -associated diarrhea include advanced age, duration of hospitalization, severity of underlying disease and exposure to antibiotics. This anaerobic bacterium has been identified as the leading cause of nosocomial infectious diarrhea, and can be responsible for large outbreaks [8].

A number of molecular typing methods, such as arbitrarily primed PCR, RFLP, PFGE and PCR ribotyping have been used internationally to study *Clostridium difficile* strains of different origins [9]. Most of these typing methods are suitable for following outbreaks, determining recurrences, studing characterizing endemic strains and tracing the spread of *Clostridium difficile*. PCR ribotyping is a method that is widely used to determine the intraspecies genetic variation of *Clostridium difficile*, in which the 16S and 23S rRNA intergenic spacer region can be amplified using specific primers under stringent amplification conditions [9-12]. The main goal of this study was to determine molecular profile of isolated toxigenic *Clostridium difficile* from hospital environmental specimens in Tehran tertiary medical centers.

Materials and Methods

In this descriptive study 100 specimens of Tehran University of Medical Sciences hospitals (Imam Khomeini hospital, Shariati hospital and Children medical center) were collected.

This project was approved by Ethics Committee for research in Tehran University of Medical Sciences.

The specimens were cultured on selective cycloserine cefoxitin fructose agar (CCFA medium, Bio Merieux, France). Then the cultures were incubated under anaerobic condition (Gas pack, Merk, germany) for 48 hrs at 37°C. The isolates were identified by characteristic morphology and biochemical tests (API20A; Bio Merieux, France) [13-17]. Bacterial cytotoxicity was assayed on Vero cell culture monolayers. A filter-sterilized, 1:10 dilution of stool, and also *Clostridium difficile* isolates broth culture were used to inoculate Vero cell monolayers with and without neutralizing *Clostridium difficile* antitoxin (Tech Lab, Inc, Blacksburg). Tissue cultures were examined at 24 and 48 hrs [18-19].

DNA extraction and PCR-ribotyping

Chromosomal DNA was extracted from colonies of *Clostridium difficile* using the ultraclean soil DNA kit (Mo Bio). The DNA obtained was resuspended in Tris-EDTA buffer and electrophoresed on 1% agarose gel.

PCR amplification of the intergenic spacer region (ISR) was carried out using two universal primers complementary to conserved regions in the 16S and 23S rRNA genes. The forward and reverse primer sequences are located at nucleotide positions 1477 to 1493 on the 16S rRNA gene of *Clostridium difficile* strain 630 (region 4, 5'-GGC TGG ATC ACC TCC TT-3' and region 5, 5' -TAG TGC CAA GGC ATC CGC CCT-3' complementary to positions 21 to 41 on the 23S rRNA gene), respectively [20]. DNA templates were amplified in a total reaction volume of 50 µl containing 2.5 U of AmpliTaq Gold thermostable polymerase (Roche company, USA), 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) with 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. The resulting amplification products analyzed on a 2% agarose gel, stained with ethidium bromide, and viewed on a UV transilluminator [21].

Results

Out of the total hospital environmental specimens, 17 toxigenic *Clostridium difficile* were isolated. The PCR ribotypes consisted of patterns comprising 2-10 bands, with the size of the bands varying from 250-630 bp. Among of toxigenic *Clostridium difficile* isolates, 6 isolates had the same PCR-ribotyping patterns, and 11 isolates were typed in four different groups (Table 1).

Table 1. Specification of toxigenic *Clostridium difficile* isolates from 100 hospital environmental specimens on the basis of PCR ribotyping patterns

Toxigenic *Clostridium difficile* isolates	**PCR ribotyping patterns**			
	Number	**Percentage**	**Similare**	**Different**
Positive	17	17	6	11
Negative	83	83	-	

Discussion

Over the past 20 years, toxigenic *Clostridium difficile* appeared to be the main cause of nosocomial diarrhea and hospital outbreaks. *Clostridium difficile* accounts for 15 to 25% of cases of antibiotic-associated diarrhea and 95% of cases of pseudomembranous colitis [22, 23]. A number of molecular methods have

been developed to investigate nosocomial outbreaks of *Clostridium difficile*. Among these typing methods, PCR amplification of rRNA intergenic spacer regions (PCR ribotyping) is a discriminatory, easy to perform, cost effective and reproducible typing method [24,25]. This increase in studies coincided with the emergence of a new hypervirulent PCR ribotype 027, which produces larger amounts of toxins A and B, due to an 18–base pair fragment and a deletion at 117 of tcdC toxin regulator gene. Soon after these reports, other publications confirmed the presence of this new emerging strain in the USA, England, Scotland, Ireland, Belgium, France, Austria, Switzerland, Denmark, Poland, Netherlands and Canada [26-28]. According to previous studies, the distribution of dominant ribotypes in the Budapest region were; 14(29%), 2(19·4%) and 18(12·9%), in England ribotype 106 [29-31]. During a very recent European survey among thirty-eight hospitals in fourteen different countries, 322 toxigenic strains of *Clostridium difficile* were tested using PCR ribotyping. Sixty-six different PCR ribotypes were characterized. Among them, 12 PCR ribotypes (001, 002, 012, 014, 017, 020, 027, 048, 077, 078, 126, 168) accounted for 65.5% of the strains. Type 002 was found in 6% (19 of 322 isolates) and type 012 in 4% (13 of 322 isolates) [32]. Our study showed that 6 out of 17 hospital environmental isolates had the same PCR ribotyping patterns, and 11 isolates were typed in four different groups. Contaminated environmental surfaces and health care personnel hand carriage are as important sources for *Clostridium difficile* transmission in hospitals [33]. Several studies have documented the presence of *Clostridium difficile* spores in areas occupied by infected patients, but these have been over short time periods, and evidence of bacterial acquisition from exposure to contaminated environmental sources is scarce. Several factors including antibiotic prescribing practice, patient type and cleaning efficiency may have influenced either incidence of *Clostridium difficile*-associated dirrhea or environmental contamination [34-38].

Conclusion

The results of this study showed that hospital environmental toxigenic *Clostridium difficile* isolates had different PCR-ribotyping patterns. Further studies to evaluate PCR-typing are suggested.

Conflict of Interest

The authors declare that they have no conflicts of interest.

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