CHARACTERIZATION OF *CLOSTRIDIUM DIFFICILE* ISOLATED FROM DIARRHEAL PATIENTS IN A TERTIARY-CARE HOSPITAL, KARNATAKA, SOUTH INDIA

Chakraborty Rituparna[1,3], Ballal Mamatha[2,3], Prabhu M Mukhyaprana[4], Hande H Manjunatha[4], Pazhani P Gururaja[5, 6] and Ramamurthy Thandavarayan[5,7]

[1]Department of Microbiology, Melaka Manipal Medical College, Manipal University, Manipal; [2]Department of Microbiology, Kasturba Medical College, Manipal; [3]Enteric Diseases Division, Central Research Laboratory, Manipal University, Manipal; [4]Department of Medicine, Kasturba Hospital, Manipal; [5]Division of Molecular Microbiology, National Institute of Cholera and Enteric Diseases, Kolkata; [6]Center for Drug Discovery and Development, Sathyabama University, Chennai; [7]Center for Human Microbial Ecology, Translational Health Science and Technology Institute, Faridabad, India

Abstract. Increase in *Clostridium difficile* infection in tertiary-care hospitals in Karnataka, South India with a paucity of data on antibiotic susceptibility and genetic characteristics of the pathogen from this region of the country necessitated this study. From April 2012 to December 2014, 480 hospitalized antibiotic-associated diarrhea cases with a history of antibiotic treatment in the previous three weeks were enrolled. Sixteen percent of the samples were positive for *C. difficile* toxins A and B by rapid enzyme immunoassay, anaerobic culture and multiplex PCR. In 40 representative strains, minimum inhibitory concentrations (MICs) determined by E-test revealed that 39 strains were resistant to imipenem and moxifloxacin (MIC > 32 μg/ml), 38 to clindamycin (MIC > 256 μg/ml) and 19 to tetracycline (MIC > 4 μg/ml), while all 40 strains were susceptible to ampicillin (MIC < 2 μg/ml), ampicillin sulbactam (MIC < 8 μg/ml), metronidazole (MIC < 8 μg/ml) and vancomycin group (MIC < 2 μg/ml). Pulsed field gel-electrophoresis (PFGE) of 13 representative strains grouped them into three clusters: cluster A consisting of two strains having > 65% similarity, cluster B of 6 strains with 100% similarity (considered clonal) and 3 strains with > 85% similarity, and cluster C of 2 strains with 50% similarity. Clusters A and C contained unrelated strains having different antibiograms. Periodic monitoring of resistance profiles with epidemiological typing by PFGE should aid in interpretation of emerging drug resistant *C. difficile* clones.

Keywords: *Clostridium difficile*, antibiogram, diarrhea, minimum inhibitory concentration, pulsed field gel-electrophoresis

Correspondence: Prof Mamatha Ballal, Enteric Diseases Division - Incharge, Central Research Laboratory, Kasturba Medical College, Manipal University, Manipal, Karnataka, India 576104.
Tel: +919901466320
E-mail: mamatha.ballal08@gmail.com, mamatha_98@yahoo.com

INTRODUCTION

Clostridium difficile associated diarrhea (CDAD) mainly occurs after hospitalization and administration of broad spectrum antibiotics and is a growing cause of concern in hospitals worldwide (Amidou

et al, 2008). *C. difficile*, a gram-positive spore-forming anaerobe, is implicated in 15-25% of antibiotic-associated diarrhea cases (Bartlett and Gerding, 2008). Rapid and accurate diagnosis of CDAD is important in preventing morbidity and mortality in severe cases. Difficulty in cultivating this nosocomial pathogen may be a reason for the dearth of data on its antibiotic susceptibility and molecular characteristics from Karnataka, South India.

The lack of a cost-effective rapid diagnostic test with high sensitivity and specificity poses a challenge in laboratory diagnosis of *C. difficile*-associated infection. A three-step approach involving a rapid membrane enzyme immunoassay (RMEIA), followed by an enzyme-linked immunosorbent assay (ELISA) for detecting *C. difficile* toxins A and B, and finally by confirmation from stool culture of toxin-positive strains is an ideal procedure. Stool culture is not only highly sensitive and specific but is recommended if antimicrobial susceptibility tests and molecular typing for epidemiological correlations are to be carried out (Issarachaikul *et al*, 2015). Standard strain typing methods include restriction endonuclease analysis (REA), pulsed-field gel electrophoresis (PFGE) and PCR ribotyping with multiloci variable-number tandem-repeat analysis (MLVA), all of which are mostly used in investigations of outbreaks (Janezic and Rupnik, 2010). However, PFGE still reigns supreme as the standard typing method for *C. difficile* in several countries (Huber *et al*, 2013).

The increasing number of toxigenic *C. difficile* isolated from various hospital wards in our tertiary-care hospital, Manipal (as evidenced in our pilot work) prompted us to undertake this study to characterize the phenotypic and genetic profiles of this pathogen isolated from CDAD cases in Manipal, Karnataka, south India. This study also aimed to provide baseline epidemiological and antimicrobial susceptibility data for *C. difficile* isolates which was lacking in this region, emphasizing the need for constant monitoring of the isolates in the light of emerging drug resistance to the conventional drugs used for treating CDI.

MATERIALS AND METHODS

Processing of stool specimens

A total of 480 stool samples obtained during April 2012 and December 2014 from hospitalized (Kasturba Hospital, Manipal) patients, clinically suspected of having antibiotic associated diarrhea (AAD), were studied. Patients enrolled were from 3 to 85 years of age who developed diarrhea (≥ 3 bowel motions for more than 24 hours) and had a history of broad spectrum antibiotic treatment in the preceding 2 to 4 weeks. Children below the age of 2 years were excluded from the study. Stool samples were collected in sterile wide-mouthed leak proof containers and transported to the microbiology laboratory immediately after collection or kept at 4°C for no longer than 10 hours.

C. difficile toxins A and B and glutamate dehydrogenase (GDH) initially were detected using RMEIA (C.DIFF QUICK CHEK COMPLETE Test, TECHLAB, Alere Diagnostics, Waltham, MA), followed by ELISA (Premier *C. difficile* toxins A&B EIA, Meridian Bioscience, Cincinnati, OH) for detecting toxins A and B. Toxin-positive stool specimens were cultured on cycloserine-cefoxitin-fructose agar (CCFA, Oxoid, Hamshire, UK) for 72 hours at 37°C. Putative typical colonies were examined by multiplex PCR for presence of *C. difficile* virulence genes, *tcdA* and *tcdB* and housekeeping triose phosphate isomerase

gene *tpi*. *C. difficile* ATCC 9689 strain was used as control in all experiments.

The study was approved by the Institutional Ethics Committee (Kasturba Hospital, Manipal University, Manipal 1EC 87/2012).

Multiplex PCR

C. difficile colonies grown on CCFA were sub-cultured on Brucella blood agar (Brucella Agar - BD BBL, Franklin Lakes, NJ) and laked horse blood - Oxoid, Hamshire, UK) plates and incubated under an anaerobic condition for 48-72 hours at 37ºC. *C. difficile* culture then was suspended in 0.5 ml of Millipore water, vortexed, boiled for 15 minutes and centrifuged at 2,500*g* for 10 minutes. Supernatant was used directly in the multiplex PCR assay as DNA template. Primers used in the assay are listed in Table 1. PCR mixture consisted of 10X PCR buffer (GENET BIO, Chungnam, Korea), 15 mM $MgCl_2$, 2.5 mM dNTPs, 10 pmol/µl each primer pairs, and 5 U/µl *Taq* DNA polymerase (GENET BIO). Thermocycling conditions (conducted in Applied Biosystems GeneAmp PCR System 9700, Foster City, CA) were as follows: 94°C for 5 minutes; followed by 30 cycles of 94°C for 30 seconds, 60°C for 20 seconds, and 72°C for 30 seconds; then a final step of 72°C for 7 minutes. PCR products were electrophoresed on 2% agarose gels and were stained with ethidium bromide (0.5mg/ml). A 100 bp molecular size marker (New England Biolabs, Ipswich, MA) along with a positive control (*C.difficile* ATCC 9689) and a negative control (*E.coli* ATCC 25922) were run with each gel. Gel pictures were documented using an image processor (Geldoc 2000, BioRad, Hercules, CA). *C.difficile* virulence genes (*tcdA* and *tcdB*) and *tpi* were detected by locating the development of bands corresponding to their base pair sizes of 170 bp, 423 bp and 230 bp, respectively, in comparison to the 100 bp molecular size marker used.

Antimicrobial susceptibility testing

Minimum inhibitory concentration (MIC) was determined for 40 representative strains using E-test (bioMerieux-AB, Solna, Sweden) following CLSI guidelines (CLSI, 2007). Brucella agar (BD BBL, Franklin Lakes, Franklin Lakes, NJ) plates supplemented with 5% laked **(**hemolysed**)** horse blood (Oxoid, Hamshire, UK), 1% vitamin K_1 and hemin solution (BD BBL, Franklin Lakes, NJ) were used. Inoculum was adjusted to 1 McFarland following the recommended procedures (CLSI, 2007). Plates were incubated anaerobically for 48-72 hours at 37ºC after which breakpoints were visually noted. Antimicrobial agents tested included ampicillin, ampicillin/sulbactam, clindamycin, imipenem, metronidazole, moxifloxacin, tetracycline, and vancomycin.

PFGE

Genomic DNA was prepared in agarose gel plugs as described by the "PulseNet" protocol of the Centers for Diseases Control (CDC) and Prevention, Atlanta, Georgia, USA with slight modifications (PulseNet, n.d.). In brief, a single colony of *C. difficile* from CCFA was inoculated on sheep blood agar (SBA)(Fitech Biosciences, Bangalore, Karnataka, India) plates to obtain a confluent growth after incubation at 37ºC for 48 hours. From the SBA growth, a cell suspension was made in 5 ml of cell suspension buffer (CSB) (Composition:10 mM Tris HCl, pH 7.2, 20 mM NaCl, 50 mM EDTA, volume adjusted with sterile reagent grade water) adjusted to $A_{600\ nm}$ of 1.5-2. Then 400 µl aliquot of CSB cell suspension was incubated at 37ºC for 10 minutes and 400 µl of molten 1% SeaKem gold agarose (Sigma, St Louis, MI) were added. The temperature

Table 1
Primers used in multiplex PCR assay.

Primer	*Clostridium difficile* gene	Primer sequence (5′-3′)	Amplicon size (bp)	Reference
cdA2 F cdA2 R	toxin A (*tcdA*)	GCTATACGTTATCAAAATAGATTCC ATAACACCATCAATCTCGAAA	170	GenBank acc. no. KC292072
cdB F cdB R	toxin B (*tcdB*)	CAAAGAGCTAAGTGAAACGAGTGA TATTGATGGTGCTGAAAAGAAGTG	423	GenBank acc. no. KC292072
tpi F tpi R	triose phosphate isomerase (*tpi*)	AAAGAAGCTACTAAGGGTACAAA CATAATATTGGGTCTATTCCTAC	230	Lemee *et al* (2004)

Table 2
Minimum inhibitory concentration (MIC) values and antibiogram of 13 representative *Clostridium difficile* strains.

Strain ID	MIC (μg/ml)								
	AM	AB	VA	MZ	TC	CH	IP	MX	Antibiogram[a]
RC2	1.0	0.25	1.0	0.25	0.032	0.19	> 32	> 32	IP, MX
RC3	1.5	0.25	0.25	0.19	4	> 256	> 32	> 32	CH, IP, MX
RC4	1.0	0.25	0.19	0.19	4	> 256	> 32	> 32	CH, IP, MX
RC5	1.5	0.023	0.25	0.047	2	> 256	> 32	> 32	CH, IP, MX
RC6	1.0	0.19	1.5	0.38	8	> 256	0.094	> 32	TC, MX
RC7	0.5	0.19	0.38	0.094	0.75	> 256	> 32	> 32	CH, IP, MX
RC8	2.0	1.5	0.19	0.032	2	> 256	> 32	> 32	CH, IP, MX
RC10	1.0	0.25	0.25	0.19	3	> 256	> 32	> 32	CH, IP, MX
RC13	1.0	0.25	0.38	0.125	13	> 256	> 32	> 32	TC, CH, IP, MX
RC14	0.75	0.38	0.25	0.25	4	> 256	> 32	> 32	CH, IP, MX
RC15	1.5	0.38	0.38	0.38	3	> 256	> 32	> 32	CH, IP, MX
RC17	0.25	0.19	0.19	0.064	3	> 256	> 32	> 32	CH, IP, MX
RC21	1.0	0.25	0.38	0.5	2	> 256	8	0.5	CH, IP, MX

[a]Based on CLSI M11-A7 guidelines. AM, ampicillin; AB, ampicillin-sulbactam; IP, imipenem; MZ, metronidazole; MX, moxifloxacin; TC, tetracycline; VA, vancomycin.

of the molten agarose was maintained at 55-60°C. The mixture was dispensed into wells of a reusable plug mold (10 well reusable plug mold, BioRad, Hercules, CA) and the plugs were allowed to solidify for 10 minutes at room temperature. Solidified plugs were incubated in 1 mg/ml lysozyme solution at 37°C for at least 2 hours, then rinsed twice with sterile reagent grade water and incubated with freshly prepared 1 mg/ml proteinase K (Invitrogen) overnight at 50°C. Plugs were washed once with 1.0X washing buffer (20 mM Tris-HCl pH 8 containing 50 mM EDTA), followed by a second wash with washing buffer containing 1 mM phenyl methyl sulphonyl fluoride (PMSF). This was followed by 2 more washes with

Table 3
Clostridium difficile isolates grouped by pulsed field gel-electrophoresis.

Cluster	Strain	Year of Isolation	Age (years) of patient	Sex of patient	Ward
A	RC 2	September 2013	81	Female	Medicine
	RC 10	August 2013	65	Female	Nephrology
B	RC 4	January 2014	41	Male	Neurosurgery
	RC 5		65	Female	
	RC 7		43	Male	
	RC 8	February 2014	55	Female	
	RC 14		60	Male	
	RC 15	April 2013	71	Male	
	RC 3	December 2013	47	Male	
	RC 17	January 2013	20	Male	
	RC 21	April 2013	55	Male	Orthopedic
C	RC 6	February 2014	65	Male	
	RC 13	September 2013	65	Male	Medicine

0.1X washing buffer at room temperature. Plugs were incubated with 1 ml of 1.0X restriction enzyme buffer at room temperature for 1 hour, then treated with 50 U *Sma*I (New England Biolabs, Ipswich, MA) in 0.6 ml of restriction enzyme buffer overnight at 37ºC. PFGE was performed with a CHEF-Mapper (BioRad, Hercules, CA) at 6V/cm for 18 hours at 14ºC at an angle of 120ºC (initial switch time, 6.76 seconds; final switch time, 38.35 seconds). Thiourea (200 μM) was included in the electrophoresis buffer to prevent degradation of DNA. Gels were stained with ethidium bromide and visualized under UV light. Gel images were normalized with reference to peaks of *Salmonella enterica* serotype Braenderup H9812 size standard and analyzed using BioNumerics software version 5.0 (Applied Maths, Austin, TX).

Data analysis

Similarity analysis was performed with Dice's coefficient and clustering was performed by means of unweighted pair group mean association (UPGMA) (Tenovar *et al*, 1995; Janezic and Rupnik, 2010). A dendrogram showing the hierarchical representation of the level of linkage among the isolates was drawn to demonstrate their degree of clonal relatedness. Antimicrobial resistance profiles of the 13 randomly selected isolates from 2013 to 2014 was used to better understand the epidemiology of the prevalent strains by analyzing the dendrogram generated by PFGE. Information regarding age, sex, and other pertinent details of all the patients were noted.

RESULTS

C. difficile toxins A and B were detected in 78/480 (16%) stool samples from hospitalized patients with diarrhea using both RMEIA and ELISA (data not shown). The results were confirmed by stool culture and by detecting the presence of the virulence associated genes *tcdA* (encoding toxin A) and *tcdB* (encoding toxin B) in all the isolated strains by multiplex PCR

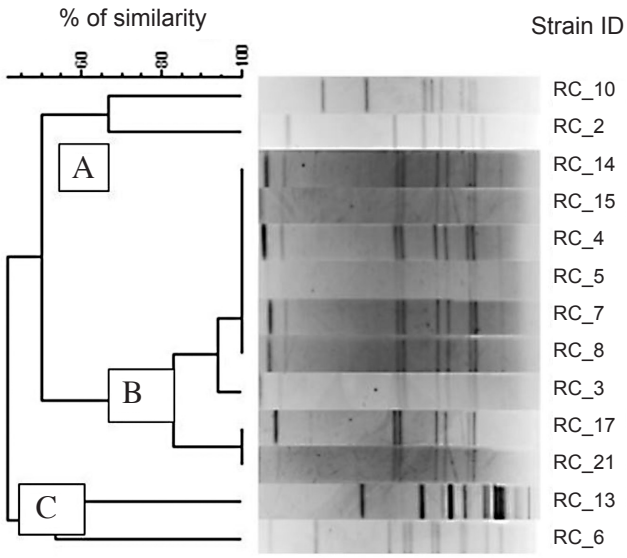

Fig 1–Pulsed field gel-electrophoresis profiles and dendrogram of 13 representative *Clostridium difficile* isolates. Plugs were treated with 50 U *Sma*I (New England Biolabs, Beverly, MA). PFGE was performed with a CHEF-Mapper (BioRad, Hercules, CA) at 6V/cm for 18 hours at 14ºC at an angle of 120º (initial switch time, 6.76 seconds; final switch time, 38.35 seconds). Gel images were normalized with reference to peaks of *Salmonella enterica* serotype Braenderup H9812 size standard and analyzed using BioNumerics software version 5.0 (Applied Maths, Austin, TX). Similarity analysis was performed with Dice's coefficient and clustering was performed by means of unweighted pair group mean association (UPGMA).

(data not shown).

All 40 tested strains exhibited susceptibility to ampicillin (MIC ≤ 2 μg/ml), ampicillin-sulbactam (MIC ≤ 8 μg/ml), metronidazole (MIC ≤ 8 μg/ml), and vancomycin (MIC ≤ 2 μg/ml) (Table 2). Resistance to moxifloxacin and imipenem (MIC > 32 μg/ml each) was detected in 39 strains, clindamycin resistance (MIC > 256 μg/ml) in 38 strains and tetracycline resistance (MIC > 4 μg/ml) in 19 strains.

PFGE analysis of 13 representative strains revealed 3 clusters: A, B and C. (Fig 1). Cluster A contained 2 strains (RC 2 and RC 10) showing > 65% similarity, cluster B 6 strains (RC 4, 5, 7, 8, 14 and 15) showing 100% similarity and were considered clonal with a subset of 3 strains (RC 3, RC 17 and RC 21) with > 85% similarity, and cluster C 2 strains (RC 6 and RC 13) showing 50% similarity. Clusters A and C consisted of unrelated strains and their resistance profile was different from the rest (Table 2). All 6 clonal strains of cluster B and the subset of 3 strains had the same antibiogram (Table 3). The two strains in clusters A and C, each had a different antiobiogram.

The median age of patients in the study group was 52 years (inter-quartile range of 24). Thirty-five (45%) patients diagnosed with CDAD were from neurosurgery ward, 22 (28%) from general medicine ward, 9 (12%) from nephrology ward, 8 (10%) from gastroenterology ward, and 4 (5%) from orthopedic ward. Four *C. difficile* toxin-positive patients died: two had acute pseudomembranous colitis, one urosepsis and chronic renal failure, and one sepsis and multiorgan dysfunction. Among five patients who followed-up, all were males in the age range of 42 to 45 years and had been primarily admitted to the neurosurgery ward and undergone surgery. Four of the five patients initially had developed mild to moderate diarrhea and were treated with two weeks of oral therapy with 500 mg of metronidazole thrice daily for ten days. Only one patient with severe illness

initially received 500 mg of metronidazole thrice daily for three days but since his condition had not improved much he was given 200 mg of oral vancomycin for five days which was then reduced to 100 mg for another five days. All the five patients were culture- and ELISA/RMEIA-negative for *C.difficile* toxins on follow-up after treatment.

DISCUSSION

Patients most vulnerable to CDI are those who are on long-term antibiotic therapies for complicated infections (Planche *et al*, 2008). Increasing prevalence of CDAD demands accurate diagnosis, which is essential to control its spread and define its epidemiology. Laboratory diagnosis of CDAD is generally not performed routinely in most of the developing countries (Garcia *et al*, 2007). This may be due to poor infrastructure for cultivating this fastidious, slow growing anaerobe, cost involved and lack of expertise required for identification of *C. difficile* for toxin testing.

Variations in sensitivity and specificity of ELISA used for rapid detection of *C. difficile* toxins (Planche *et al*, 2008) necessitate multiple tests for confirmation of *C. difficile* infection (ASM, 2010). In our study, we used both ELISA and RMEIA for detecting *C. difficile* toxins. RMEIA was used as a screening test for *C. difficile* glutamate dehydrogenase and toxins A and B, and positive results were then confirmed by culturing on CCFA followed by multiplex PCR. Results obtained using immunological and PCR techniques were in agreement in detection of toxigenic *C. difficile*. Applying this detection protocol should help in reducing the number of false positive or false negative samples. Stool culture provides isolated strains for their further characterization by molecular typing methods, which provide useful information for epidemiological analysis, especially during nosocomial outbreaks.

C. difficile typing methods should have good reproducibility and high discriminatory power. Although PCR ribotyping has been increasingly used as a simple and affordable typing method, routine laboratories cannot employ this technique unless correct reference strains are available for assigning the ribotypes (Huber *et al*, 2013). In such situations, although PFGE requires specialized equipment and is a longer procedure, it provides an efficient molecular typing method. It is important to note that even though our work represented only a small sampling group, PFGE was able to group the 13 strains into three clusters and reveal their genetic relatedness. The 4 unrelated strains as evidenced from the dendrogram had different antibiograms. It is worth noting that the 6 clonal strains were isolated from the same hospital ward and isolated from April 2013 onwards.

In this study, samples were obtained from diarrheagenic patients who were treated with antibiotics (amikacin, cefoperazone-sulbactam, cefotaxime, ciprofloxacin, and piperacillin-tazobactam) for different infections/comorbidities. We suggest that the use of these antibiotics for more than two weeks led to CD diarrhea (confirmed through lab diagnosis) in majority of these cases. These antibiotics are important risk factors for CDI (Bartlett and Gerding, 2008). However, since the resistance profile of the clinical isolates was not known, the panel, of antibiotics, including clindamycin, imipenem and moxifloxacin were selected for MIC determination following CLSI guidelines for anaerobic bacilli (CLSI, 2007). We demonstrated that the PFGE cluster analy-

sis of the clinical isolates could be correlated with the *in vitro* resistance towards clindamycin, imipenem and moxifloxacin. Although these antimicrobials were not prescribed for the patients, through *in vitro* testing, an epidemiological characterization of the corresponding clinical isolates could be established. Moreover, though clinically we agree that there may be discrepancy between *in vitro* and clinical findings, however, that discrepancy is very small (MacGowan, 2008). In addition, 92% of the strains were from patients in the advanced age group, an important risk factor for CDAD. (Bartlett and Gerding, 2008)

All 13 test strains were resistant to clindamycin, imipenem and moxifloxacin. Though resistance to the commonly used drugs for treating *C. difficile* diarrhea is not a concern yet, it is noteworthy that *C. difficile* is naturally resistant to many antibiotics used for treating other infections (CDC, 2013). Resistance observed in the 13 strains to the three drugs mentioned earlier could be multifactorial as observed in other reports (Freeman *et al*, 2015). It could be due to acquisition of mobile genetic elements or increased use of the antibiotic as in case of the fluoroquinolones and carbapenems, etc. Massive use of fluoroquinolones has been attributable not only to resistance developing to this group of drugs but it has also been linked to the emergence of *C. difficile* ribotype 027, a trait which was not observed in historic strains of the same ribotype (Spigaglia, 2016). *C. difficile* is known to be historically resistant to clindamycin but imipenem resistance though observed in our study and in other reports (Freeman *et al*, 2015) is not well documented in *C. difficile*.

Metronidazole is currently used as the drug of choice for treating *C. difficile* diarrhea, probably due to its low cost and concerns associated with the spread of vancomycin-resistant nosocomial bacteria (Chia *et al*, 2013). As clinical isolates are generally sensitive to metronidazole and vancomycin, most laboratories do not perform susceptibility testing of this nosocomial pathogen. Nonetheless, many findings indicate the emerging problem of metronidazole resistance (Baines *et al*, 2008; Peláez *et al*, 2008; Huang *et al*, 2010; Spigaglia *et al*, 2011), which necessitate periodic monitoring of *C. difficile* antibiogram along with detection of resistance gene markers. Such information will not only help in controlling the spread of drug resistance *C. difficile* clones but may also help in the modification of treatment regime thereby preventing morbidity associated with CDAD.

In conclusion, gram-positive spore forming anaerobe *C. difficile* has emerged as a global threat in the hospitals as well as in the community. We were able to isolate this pathogen from hospitalized patients on long term antibiotic treatment for other comorbidities. PFGE allowed cluster analysis and antibiogram gave important information regarding the characteristics of *C. difficile* prevalent providing preliminary information on the epidemiology of *C. difficile* in a hospital setting in this part of India.

ACKNOWLEDGEMENTS

The study was supported by a grant from the Vision Group on Science & Technology, KFIST Level 1, Government of Karnataka, India. The authors thank Dr Asha Kamath, Associate Professor, Department of Community Medicine, Kasturba Medical College, Manipal University, Manipal for her input and advice.

REFERENCES

Amerian Society for Microbiology (ASM). A practical guidance document for the laboratory detection of toxigenic *Clostridium difficile*. New York: ASM, 2010. [Cited 2014 May 15]. Available from: http://www.alere.com/content/dam/alere/docs/guidelines/2010_ASM_guidelines_dated_9.21.10.pdf

Amidou S, CL Obi, Jason F, *et al*. PCR detection of *Clostridium difficile* triose phosphate isomerase (*tpi*), toxin A (*tcd*A), toxin B (*tcdB*), binary toxin (*cdt*A, *cdt*B), and *tcd*C genes in Vhembe District, South Africa. *Am J Trop Med Hyg* 2008; 78: 577-85.

Baines SD, O'Connor R, Freeman J, *et al*. Emergence of reduced susceptibility to metronidazole in *Clostridium difficile*. *J Antimicrob Chemother* 2008; 62: 1046–52.

Bartlett JG, Gerding DN. Clinical recognition and diagnosis of *Clostridium difficile* infection. *Clin Infect Dis* 2008; 46 (suppl i): 12-8.

Centers for Disease Control and Prevention. Antibiotic resistance threats in the United States. Atlanta: CDC, 2013. [Cited 2014 May 15]. Available from: URL: http://www.cdc.gov/drugresistance/pdf/ar-threats-2013-508.pdf

Chia JH, Lai HC, Su LH, Kuo AJ, Wu TL. Molecular epidemiology of *Clostridium difficile* at a medical center in Taiwan: persistence of genetically clustering of A^-B^+ isolates and increase of A^+B^+ isolates. *PLOS One* 2013; 8: 1-8, e75471.

Clinical and Laboratory Standards Institute (CLSI). Methods for antimicrobial susceptibility testing of anaerobic bacteria; approved standard. M11-A7. Wayne: CLSI, 2007; 27 (2).

Freeman J, Vernon J, Morris K, *et al*. Pan-European longitudinal surveillance of antibiotic resistance among prevalent *Clostridium difficile* ribotypes. *Clin Microbiol Infect* 2015; 21: 248.e9-248.e16.

Garcia C, Samalvides F, Vidal M, Gotuzzo E, Dupont HL. Epidemiology of *Clostridium difficile*-associated diarrhea in a Peruvian tertiary care hospital. *Am J Trop Med Hyg* 2007; 77: 802-5.

Huang H, Weintraub A, Fang H, Wu W, Zhang Y, Nord CE. Antimicrobial susceptibility and heteroresistance in Chinese *Clostridium difficile* strains. *Anaerobe* 2010; 16: 633-5.

Huber CA, Foster NF, Riley TV, Paterson DL. Challenges for standardization of *Clostridium difficile* typing methods. *J Clin Microbiol* 2013; 51: 2810-14.

Issarachaikul R, Khantipong M, Sawatpanich A, Suankratay C. Prospective evaluation of a novel two-step protocol for screening of *Clostridium difficile* infection in hospitalized adult patients. *Southeast Asian J Trop Med Public Health* 2015; 46: 1037-48.

Janezic S, Rupnik M. Molecular typing methods for *Clostridium difficile*: pulsed-field gel electrophoresis and PCR ribotyping. *Methods Mol Biol* 2010; 646: 55-65.

Lemee L, Dhalluin A, Testilin S, *et al*. Multiplex PCR targeting *tpi* (triose phosphate isomerase), *tcdA* (toxin A) and *tcdB* genes for toxigenic culture of clostridium difficile. *J Clin Microbiol* 2004; 42: 5710-14.

MacGowan AP on behalf of the BSAC Working Parties on Resistance Surveillance. Clinical implications of antimicrobial resistance for therapy. *J Antimicrob Chemother* 2008; 62 (suppl 2): ii105-14.

Peláez T, Cercenado E, Alcalá L, *et al*. Metronidazole resistance in *Clostridium difficile* is heterogeneous. *J Clin Microbiol* 2008; 46: 3028-32.

Planche T, Aghaizu A, Holliman R, *et al*. Diagnosis of *Clostridium difficile* infection by toxin detection kits: a systematic review. *Lancet Infect Dis* 2008; 8: 776-84.

PulseNet, Centre for Disease Control and Prevention (CDC). One-day (24-26 h) standardized laboratory protocol for molecular subtyping of *Campylobacter jejuni* by pulsed field gel-electrophoresis (PFGE).

Atlanta: CDC, n.d. [Cited 2014 Mar 1]. Available from: URL: www.cdc.gov/pulsenet/protocols/campy_protocol.pdf

Spigaglia P, Barbanti F, Mastrantonio P. Multidrug resistance in European *Clostridium difficile* clinical isolates. *J Antimicrob Chemother* 2011; 66: 2227-34.

Spigaglia P. Recent advances in the understanding of antibiotic resistance in *Clostridium difficile* infection.*Ther Adv Infect Dis* 2016; 3: 23-42.

Tenovar FC, Arbeit RD, Goering RV, *et al*. Interpreting chromosomal DNA restriction patterns produced by pulsed field gel-electrophoresis: criteria for bacterial strain typing. *J Clin Microbiol* 1995; 33: 2233-9.