Molecular characterization and toxin-typing of *Clostridium difficile* isolates of dogs and pigs from Assam and Mizoram of North East India

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Clostridium difficile **with its virulence factors A and B toxins cause Pseudomembranous colitis. Bacterium was isolated from 57 dog and 41 pig diarrheic faecal samples in cycloserinecefaxitin fructose agar media and molecular detection was done by amplification of *gluD* gene (755 bp). Variability of toxin genes in positive isolates was tested by multiplex PCR. Detection of binary toxin genes (*cdt*A and *cdt*B) was also done. Results showed 33.67% positivity with 18 and 15 from dog and pig respectively, from which 10 and 5 were toxigenic and 11 pig isolates exhibited binary toxin. PCR-RFLP demonstrated toxinotype 0 in all A^+B^+ isolates.**

Keywords: Diarrhoea, NE India, Pseudomembranous colitis, toxin-typing, virulence.

CLOSTRIDIUM DIFFICILE are a group of anaerobic, spore-forming, Gram-positive bacteria. It causes Pseudomembranous colitis (PMC) leading to infectious diarrhoea called *C. difficile* associated diarrhoea (CDAD). Presence of *C. difficile* in environment, soil and water and occurrence in food leads to a serious concern for human and animal health in India in general and North East (NE) India in particular. The bacterium was discovered in 1935 and was regarded as faecal flora in new-born[1]. It was in 1978 that this bacterium was recognized as a human pathogen[2–4] and a causal agent of antibiotic associated PMC. The pathogenic *C. difficile* produces two major types of toxins. The enterotoxin (toxin A) is responsible for changes in cellular activities by modifying host cell GTPase proteins leading to fluid secretion in the intestinal loops[5]. The other toxin is cytotoxin (toxin B), which causes cell damage. The genes encoding these *tcd*A and *tcd*B toxins are located in 19.6 kb pathogenicity locus (*PaLoc*) of the chromosome of *C. difficile*. The variations in *PaLoc* sequence are utilized to detect toxin-types of different *C. difficile* strains[6]. Binary toxin (CDT) of certain strains consists of two separate toxin components, CDTa and CDTb, encoded by two separate genes, *cdtA* and *cdtB* and is recorded to be located on outer side of the *PaLoc* region[7]. Various diagnostic methods are proposed for detection of *C. diffile* in faecal sample of animals and birds. The most preferred one is the stool culture in a well-defined selective media and molecular detection thus avoiding error and mis-diagnosis of *C. difficile* infection. Two common methods adapted to type toxigenic strains of bacterium are toxinotyping and PCR ribotyping. Although several studies have been made worldwide, and in India regarding certain aspects of *C. difficile*, there is a vast requirement of proper surveillance and development of sensitive molecular detection and toxinotyping methods of the bacterium. Reports and detection techniques from NE India on *C. difficile* are very scanty. It is mostly due to the lack of information and detail study on CDAD in humans and animals. However, some human cases of CDAD have been reported from certain hospitals from a few parts of the country, but no information could be traced from the available literature regarding prevalence and molecular characterization of the organism in different animal species, both apparently healthy and clinically affected[8–11]. In order to bridge this gap, the present study was aimed at studying the isolation and molecular characterization of the organism and its toxin typing in diarrheic dogs and pigs from this region of India.

Materials and methods

Collection of sample

Ninety eight faecal samples of diarrhoeic dogs (57) and pigs (41) were collected from the states of Assam and Mizoram in North East India. The details of sample collection are shown in Table 1. Among the antibiotic

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Table 1. Number of samples collected from Assam and Mizoram for *Clostridium difficile* isolation

Sources	Nature of faecal samples	Treatment with antibiotic	Assam	Mizoram	Number of samples
Dog	Diarrheic	Treated	21	12	33
		Non-treated	19	5	24
	Total				57
Pig	Diarrheic	Treated	24	6	30
		Non-treated	6	5	11
	Total				41
	Grand total				98

Table 2. List of primer sequence and amplicon size

Name of primer	Details of sequence (5′–3′)	Gene	Size of amplicon (bp)
gluD-F	GGAAAAGATGTAAATGTCTTCGAGATG	*gluD*	755
gluD-R	CTGATTTACACCATTCAGCCATAGC		
tcdA-F	AGATTCCTATATTTACATGACAATAT	*tcdA*	369
tcdA-R	TATCAGGCATAAAGTAATATACTTT		
tcdB-F	GGAAAAGAGAATGGTTTTATTAA	*tcdB*	160
tcdB-R	ATCTTTAGTTATAACTTTGACATCTTT		
tpi-F	AAAGAAGCTACTAAGGGTACAAA	*tpi*	230
tpi-R	CATAATATTGGGTCTATTCCTAC		
cdtA-F	TGAACCTGGAAAAGGTGATG	*cdtA*	376
cdtA-R	AGGATTATTTACTGGACCATTTG		
cdtB-F	CTTAATGCAAGTAAATACTGAG	*cdtB*	510
cdtB-R	AACGGATCTCTTGCTTCAGTC		

treated diarrhoeic dogs, 21 were collected from the Teaching Veterinary Clinics of Assam Agricultural University, Khanapara while the remaining 12 faecal samples were collected from Mizoram. From untreated dog samples, 19 and 5 were of Assam and Mizoram respectively. Similarly, among pig faecal samples, 24 were collected from Assam and 6 from Mizoram undergoing antibiotic treatment, while the remaining 6 and 5 samples from Assam and Mizoram respectively, were collected from unorganized pig farms without antibiotic treatment. Most commonly used antibiotic for the treatment of diarrhoea in dogs and pigs was recorded to be ciprofloxacin. Faecal samples were collected in sterile screw capped container for processing to isolate *C. difficile*.

Isolation and identification

Collected samples were subjected to isolation of *C. difficile* on Cycloserine Cefaxitin Fructose Agar (CCFA) medium as per earlier described protocol[12] with slight modification. Faecal samples, weighing approximately one gram, were pre-enriched by inoculating into CCF Broth (Hi-Media Lab, Mumbai) and incubated anaerobically for 12 days at 37°C. The pre-nourished cultures were subjected to ethanol treatment by mixing 500 µl of culture with equal amount of absolute ethanol for 45 min. After centrifugation of the mixtures for 15 min at 12,000 rpm, a loop full of pellet was inoculated in CCF Agar (Hi-Media Lab, Mumbai) plates with 5% sheep blood (v/v). These were incubated anaerobically for 48–72 h at 37°C. Colonies with its typical morphology, cell morphology and staining characteristics suggestive of *C. difficile* was further sub-cultured for purification in CCFA or brain heart infusion (BHI) agar plates with 5% sheep blood.

Molecular confirmation and characterization of the C. difficile isolates

All the suspected isolates were confirmed as *C. difficle* by molecular identification, targeting the marker *gluD* (GDH protein) gene, by simplex PCR. The confirmed isolates were further characterized with respect to the virulence associated genes, viz. *tcd*A, *tcd*B and binary toxins (*cdt*A and *cdt*B), along with *tpi*, the house keeping gene. The primer sequence and amplicon size are shown in Table 2.

Total DNA was extracted as described earlier[13]. Briefly, total DNA was extracted from each bacterial culture by mixing 3–4 pure colonies directly into sterile miliQ water. Then after 10 min of boiling, the suspension was cooled down immediately on ice for 20 min (hot cold

Table 3. List of primer sequence and restriction enzymes with amplicon size

Primer name	Sequence (5′–3′)	Gene	Amplicon size (kb)	Enzyme
A1C	GGAGGTTTTTATGTCTTTAATATCTAAAGA	*A1*	3.1	*Nco*I
A2N	CCCTCTGTTATTGTAGGTAGTACATTTA			
A2C	TAAATGTACTACCTACAATAACAGAGGG	*A2*	2.0	*Hae*III
A3N	CTTGTATATAAATCAGGTGCTATCAATA			
A3C	TATTGATAGCACCTGATTTATATACAAG	*A3*	3.1	*Eco*RI
A4N	TTATCAAACATATATTTTAGCCATATATC			
B1C	AGAAAATTTTATGAGTTTAGTTAATAGAAA	*B1*	3.1	*Hinc*II/*Acc*I
B2N	CAGATAATGTAGGAAGTAAGTCTATAG			
B2C	ATAGACTTACTTCCTACATTATCTGAA	*B2*	2.0	*Rsa*I
B3N	CATCTGTATAAATATTTGGTGAAATTAC			
B3C	AATTTCACCAAATATTTATACAGATG	*B3*	2.0	*Hind*III/*Rsa*I
B4N	ATTTAACATATTTTTATCTATTCA			
Lok 3	TTTACCAGAAAAAGTAGCTTTAA	*PL2*	2.0	*Nsi*I
Primex B	ACATATTCATCTTCTTGAGTACGA			

lysis). Centrifuging it for 10 min at 12,000 rpm the supernatant containing DNA was collected and the concentration of extracted DNA was assessed by Nano drop spectrophotometer (Thermo Scientific, USA). DNA was extracted from a toxigenic strain of *C. difficile* (NICED 511160) and a non-toxigenic strain (ATCC 43593) which served as controls during PCR assay. The specific forward and reverse primers used during the study were commercially synthetized from Metabion International AG (Deutschland).

Detection of the glutamate dehydrogenase (gluD) gene

The GDH associated *gluD* gene was detected by standard protocol of simplex PCR[14] with slight modifications. PCR was reacted 25 µl reaction volume of 12.5 µl of 2× DreamTaq Green PCR master mix (Fermentas, Germany), 0.5 µl of each primer (10 pmol each) and making the final volume with nuclease free water. Reaction mixture was then exposed to a thermal cycler (Applied Biosystems, USA) initially denaturing at 94°C for 4 min, followed by 35 cycles of denaturation at the same temperature for 30 s. Annealing was carried out for 1 min at 56°C and extending to 72°C for 1 min and then finally to 10 min. The products amplified were then analysed in 1.5% agarose gel (Amresco) and in gel documentation system (Kodak, Bio Step, Germany).

Molecular characterization of C. difficile isolates with respect to certain virulence associated genes

All the *C. difficile* isolates were subjected to molecular characterization by targeting the genes associated with triose phosphate isomerase housekeeping gene (*tpi*) and certain virulence associated genes, viz. *tcd*A, *tcd*B, *cdtA* and *cdtB*.

Multiplex PCR approach was carried out for detection of genes associated with toxin A (*tcd*A) and B (*tcd*B) and triose phosphate isomerase (*tpi)* in *C. difficile* isolates as per protocol[15] described earlier with slight modifications. PCR reaction was conducted in PCR master mix (Fermentas, Germany) with an additional 0.5 µl of $MgCl_2$ (25 mM), 0.5 µl of each primer (10 pmol), 3 µl of DNA template and nuclease-free water (to make up the required volume). The PCR reaction mixtures were subjected to initial denaturation of 95°C for 5 min with 5 cycles of denaturation (95°C) for 30 s, annealing (60°C) for 30 s and extension (72°C) for 30 s. It was followed by another 40 cycles of annealing at 55°C for 30 s and extension at 72°C for 30 s and a final extension at 72°C for 10 min.

Genes associated with binary toxin (*cdt*A and *cdt*B) in the isolates were detected by multiplex PCR assay as described earlier[16] with slight modifications. PCR reaction was conducted in 25 µl reaction mixture (Fermentas, Germany), 1 µl of each primer pairs (10 pmol concentration each), 3 µl of DNA template and nuclease free water up to the required volume. The PCR mixtures were subjected to a thermo-cycler with initial denaturation (95°C) for 4 min, followed by 30 cycles of denaturation (94°C) for 45 s, annealing (52°C) for 1 min and extension (72°C) for 80 s.

All PCR products were run in 1.5% agarose gel (Amresco) and visualized in Gel documentation system (Kodak, Biostep, Germany).

Toxinotyping of C. difficile isolates

The isolated *C. difficile* were subjected to toxinotyping on the basis of PCR-RFLP pattern. The PCR RFLP was carried out as per earlier described protocol[6,17]. Isolates were subjected to amplification of all fragments of toxin A (A1, A2, A3) and toxin B (B1, B2, B3), using specific primers as shown in Table 3. PCR reaction was

conducted in 25 µl reaction volume. The reaction mixture was made with PCR master mix (Fermentas, Germany), 1 µl of each primer pairs (10 pmol each), 3 µl of template DNA and nuclease-free water. The cycling conditions for A1, A2 and A3 was with initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation (94°C for 1 min), annealing (58°C for 1 min), extension (72°C for 6 min) and final extension at 72°C for 10 min. The thermal cycling conditions for B1 and B2 were initial denaturation at 93°C for 3 min with 10 cycles of 62°C for 8 min and 93°C for 6 min, and 20 cycles of 58°C for 8 min, 93°C for 3 s and 47°C for 10 min of final extension. The cycling conditions for B3 were with initial denaturation at 93°C for 3 min, followed by 30 denaturation cycles of 52°C for 1 min, annealing of 72°C for 1 min and extension to 93°C for 1 min and the reaction was stopped by incubation at 72°C for 10 min.

The undigested amplicons in the PCR products were checked and visualized on 1.5% agarose. Polymorphisms of restriction site were studied, with respect to the cutting sites of the amplicons by a set of restriction enzymes, viz. *Nco*I (*A1*), *Hae*III (*A2*), *Eco*RI (*A3*), *Hin*cII and *Acc*I (*B1*), *Rsa*I (*B2*), *Hin*dIII/*Rsa*I (*B3*) and *Nsi*I (*PL2*). All the digested products were visualized on 1.5% agarose. Interpretation was made by comparing the findings with published patterns of restriction from http://www.mf.uni-mb.si/Mikro/tox/.

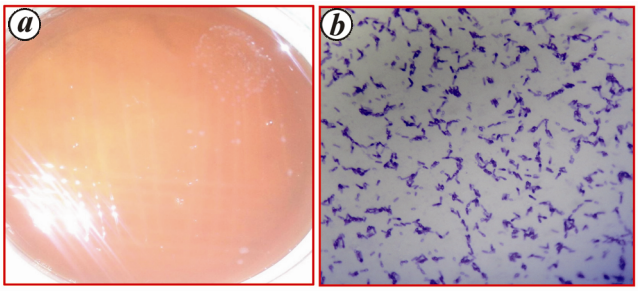

Figure 1. ***a***, CCFA culture plate showing pure colony of *C. difficile*. ***b***, Gram's stain showing *C. difficile* cells.

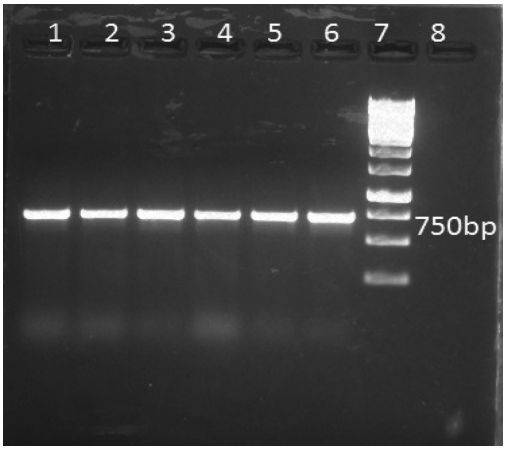

Figure 2. Amplification of *glu*D gene (755 bp) of *C. difficile*. Lanes 1–3, PCR products of *C. difficile* from dogs. Lanes 4–6, PCR products of *C. difficile* from pigs. Lane 7, DNA ladder (1 kb, Thermo Scientific). Lane 8, Negative template control.

Results and conclusions

Ninety eight diarrheic faecal samples of dogs and pigs from different parts of Assam and Mizoram were examined and studied.

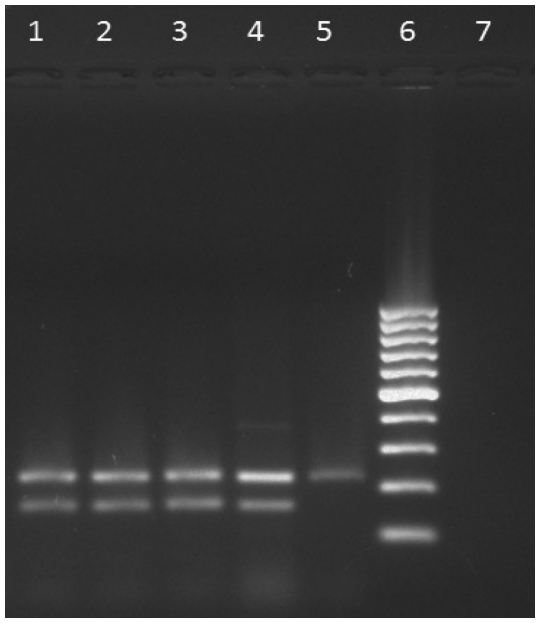

Figure 3. Amplification of *tpi* (230 bp), *tox*A (369 bp), *tox*B (160 bp) genes of *C. difficile*. Lanes 1–3, PCR products showing positive for *tpi* and *tox*B genes. Lane 4, PCR product showing positive for *tpi*, *tox*A and *tox*B genes. Lane 5, PCR product showing *tpi* gene. Lane 6, DNA ladder (100 bp, Thermo Scientific). Lane 7, Negative template control.

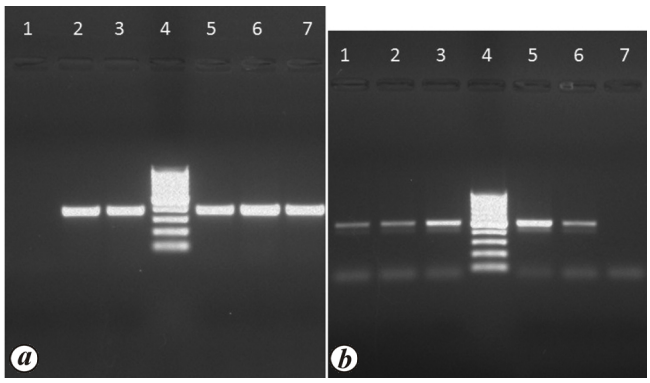

Figure 4. ***a***, Amplification of *cdt*A (376 bp) gene of *C. difficile*. Lane 1, Negative template control. Lanes 2, 3, 5–7, PCR products showing positive for *cdt*A gene. Lane 4, DNA ladder (100 bp, Thermo Scientific). ***b***, Amplification of *cdt*B (510 bp) gene of *C. difficile*. Lanes 1–3, 5, 6, PCR products showing positive for *cdt*B gene. Lane 4, DNA ladder (100 bp, Thermo Scientific). Lane 5, Negative template control.

Table 4. Number of samples tested and positive for *Clostridium difficile* from different sources

Sources	Nature of faecal samples	Treatment with antibiotic	Number of samples	Positive samples	Per cent positivity
Dog	Diarrheic	Treated	33	12	
		Non-treated	24	6	
	Total		57	18	31.57
Pig	Diarrheic	Treated	30	11	
		Non-treated	11	4	
	Total		41	15	36.5
	Grand total		98	33	33.67

Table 5. Characterization of the *Clostridium difficile* isolates and toxin typing

		Molecular characterization					
		No. of isolates positive for					
Species	Number of positive samples	*gluD* and *tpi* gene	*toxA* gene (A + B–)	*toxB* gene (A – B+)	Both *toxA* and *toxB* gene (A + B+)	Binary toxin (*cdtA* and *cdtB*) gene	Toxino type
Dog	18	18	0	0	10	0	Type 0
Pig	15	15	0	2	3	11	Type 0

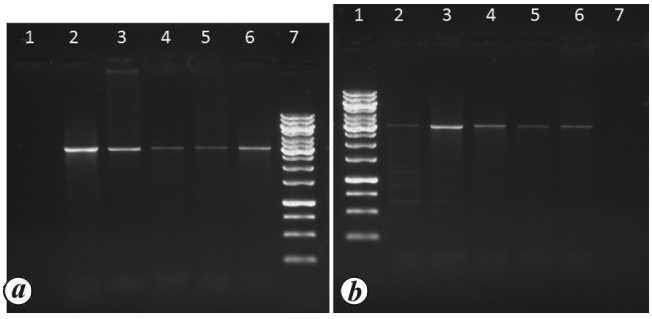

Figure 5. ***a***, Amplification of A1 (3.1 kb) fragment of toxin A of *C. difficile*. Lane 1, Negative control. Lanes 2–6, PCR products showing amplified A1 fragment. Lane 7, DNA ladder (1 kb, Thermo Scientific). ***b***, Amplification of A3 (3.1 kb) fragment of toxin A of *C. difficile*. Lane 1, DNA ladder (1 kb, Thermo Scientific). Lanes 2–6, PCR products showing amplified A3 fragment. Lane 7, Negative template control.

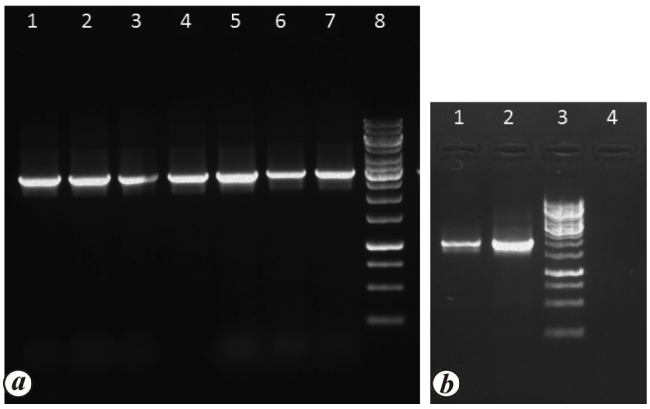

Figure 6. ***a***, Amplification of B1 (3.1 kb) fragment of toxin A of *C. difficile*. Lanes 1–7, PCR products showing amplified B1 fragment. Lane 8, DNA ladder (1 kb, Thermo Scientific). ***b***, Amplification of B2 (2 kb) fragment of toxin B of *C. difficile*. Lanes 1, 2, PCR products showing amplified B2 fragment. Lane 4, Negative template control.

Isolation and detection of gluD gene

From 98 samples, 71 were suspected to be positive for *C. difficile* morphologically, out of which, 33 were confirmed by molecular detection of *gluD* gene. CCFA plate and Gram's stain result is shown in Figure 1. Thus, positivity was found to be 33.67%. In case of dogs, 18, while in pigs, 15 were found to be positive. Thus the positivity for each species was found to be 31.57% and 36.5% respectively. Among the positive dog samples, 12 were antibiotic treated and 6 were without treatment, while among the positive pig samples 11 were antibiotic treated and 4 were untreated. The results are shown in Table 4 and the gel photograph yielding *glu*D gene of 755 bp is shown in Figure 2.

Detection of tpi, tcdA and tcdB by multiplex PCR assay

All the 33 isolates were found to harbour the housekeeping gene, *tpi*, of size 230 bp (Figure 3). Variability pattern was observed among the isolates with respect to the *tcd*A and *tcd*B genes, detected either alone or in combination (Figure 3). Ten of the 18 dog isolates were toxigenic, bearing *tcd*A and *tcd*B genes (tox A^+B^+), while the rest 8 isolates could not reveal their presence. Among the 15 pig isolates, only 5 were toxigenic with detection of *tcd*B alone in 2 isolates and 3 isolates bearing *tcd*A and *tcd*B in combination as shown in Table 5.

Detection of binary toxins (cdtA and cdtB)

All the 18 dog isolates were negative for binary toxin components, while 11 pig isolates exhibited presence of

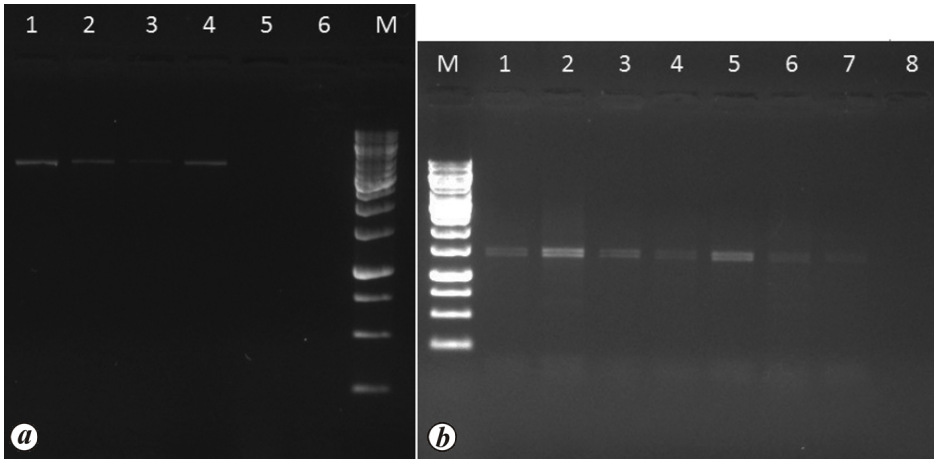

Figure 7. ***a***, Restriction pattern of *Nco*I with A1 PCR fragments. Lanes 1–4, Restriction pattern of A1 PCR fragments. Lane 5, Negative template control. Lane M, DNA ladder (1 kb, Thermo Scientific). ***b***, Restriction pattern of *Eco*RI with A3 PCR fragment. Lanes 1–7, Restriction pattern of A3 PCR fragments. Lane 8, Negative template control. Lane M, DNA ladder (1 kb, Thermo Scientific).

the components, *cdt*A (Figure 4 *a*) and *ctd*B (Figure 4 *b*) of binary toxin as shown in Table 5.

Toxinotyping

Based on the PCR RFLP patterns of A1, A2, A3 (Figure 5 *a*, *b*) fragments of toxin A and B1, B2, B3 (Figure 6 *a*, *b*) fragments of toxin B, all the 10 dog isolates were positive for both A and B toxins and identified as toxinotype 0. Similarly fragmentation pattern in the 3 pig isolates also indicated to be of toxinotype 0 as shown in Table 4 (Figures 7 *a*, *b* and 8 *a*, *b*).

C. difficile isolated from different animal species has been implicated in enteric diseases[18–20]. In India, although few reports are available on *C. difficile* infection (CDI) in man[8,9,11], no reports are available on any aspect of *C. difficile* from animals and birds. In the present study, a total number of 18 isolates were recovered from 57 dog samples, out of which 12 were from treated and 6 from untreated diarrheic samples (Table 3). So far, most studies suggest isolation of *C. difficile* in dogs in veterinary hospitals[21]. Out of 18 isolates, 10 (55.5%) were found to carry toxin A and B genes in combination (A^+B^+), 12 (44.4%) were non-toxigenic (Table 4). This is quite close to previous report where 37.9% to 83.3% *C. difficile* isolates from dogs were found to be toxigenic[22–27]. However, no dog isolate was found to be binary toxin positive, which is quite rare[28]. In the case of pigs, out of 41 samples, 15 isolates were recovered as *C. difficile* (11 from treated and 4 from non-treated) yielding a positivity of 36.5% (Table 3). Recent studies suggest that currently *C. difficile* is an important uncontrolled cause of neonatal diarrhoea in pigs[29]. However, this cannot be concluded from our study as age was not considered, but the present study does correlate with a previous finding where isolation rates of *C. difficile* was found to range between 0% and 64% (ref. 30). Out of 15 isolates, 2 carried only *tcd*B gene (A^-B^+) and 3 carried both genes (A^+B^+), thus giving a toxigenic rate of 33.33%. Again, 11 isolates (73.33%) were positive for *cdt*A and *cdt*B binary toxins. Recent study revealed that 85% isolates were toxin genes *tcd*A and *tcd*B positive and only 1.1% isolates as A^-B^+ and *cdt*B positive[28]. However, the present study relates to the finding where 81% and 87% of isolates revealed *cdt*A and *cdt*B respectively[22]. The discrepancy regarding absence and presence of binary toxin in dogs and pigs respectively, may have some genetic base which may require further research as in case of dog isolates, absence of binary toxin is reported to be quite rare[26]. In the present study, toxinotype of A^+B^+ isolates was revealed to be 0 when compared to the reference findings[6] by matching the RFLP patterns. However, some previous findings reported toxinotype V in majority of A^+B^+ isolates[31]. The present finding is similar to that of a recent study, which observed that the *C. difficile* isolates from animals including dogs belonged to toxinotype 0 (ref. 32).

Summary

It can be concluded that there is presence of infection of *C. difficile* in Assam and Mizoram of NE India irrespective of dog and pig species and it is a serious threat to antibiotic treated animals, since it causes antibiotic associated diarrhoea. Additionally, in this study the same toxinotype (type 0) was revealed, which indicates that genetic relatedness exists between *C. difficile* strains circulating in this area regardless of these two animal species. Thus, CDAD causing *C. difficile* is an emerging infectious pathogen in the global scenario and this study

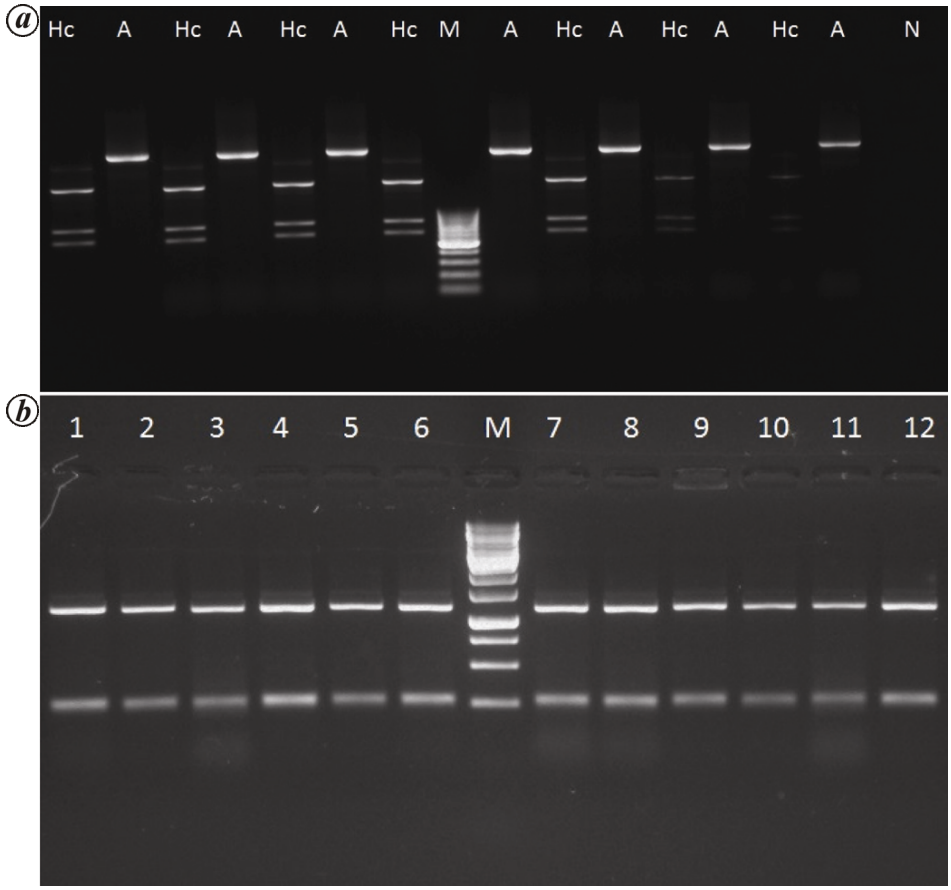

Figure 8. ***a***, Restriction pattern of *Hin*cII and *Acc*I with B1 PCR fragments. Lanes 1–7 and 9–15: Restriction pattern of B1 PCR fragments. Lane N, Negative template control. Lane M, DNA ladder (1 kb, Thermo Scientific). ***b***, Restriction pattern of *Rsa*I with B2 PCR fragments. Lanes 1–6 and 7–12, Restriction pattern of B2 PCR fragments. Lane M, DNA ladder (1 kb, Thermo Scientific).

may pave the way in understanding its pathogenicity and evolve suitable control measures.

Conflict of interest: Authors declare no conflict of interest with anyone with respect to present paper.

1. Hall, I. C. and O'Toole, E., Intestinal flora in new-born infants with a description of a new pathogenic anaerobe, *Bacillus difficilis*. *Am. J. Dis. Child*, 1935, **49**, 390–402.
2. Bartlett, J. G., Chang, T. W., Gurwith, M., Gorbach, S. L. and Onderdonk, A. B., Antibiotic-associated pseudomembranous colitis due to toxin-producing clostridia. *N. Engl. J. Med*., 1978, **298**, 531–534.
3. George, R. H. *et al.*, Identification of *Clostridium difficile* as a cause of pseudomembranous colitis. *Br. Med. J*., 1978, **1**, 695.
4. Larson, H. E., Price, A. B., Honour, P. and Borriello, S. P., *Clostridium difficile* and the aetiology of pseudomembranous colitis. *Lancet*, 1978, **1**, 1063–1066.
5. Libby, J. M., Jortner, B. S. and Wilkins, T. D., Effects of the two toxins of *Clostridium difficile* in antibiotic-associated cecitis in hamsters. *Infect. Immun*., 1982, **36**, 822–829.
6. Rupnik, M., Avesani, V., Janc, M., Von Eichel-Streiber, C. and Delmee, M., A novel toxinotyping scheme and correlation of toxinotypes with serogroups of *Clostridium difficile* isolates. *J. Clin. Microbiol*., 1998, **36**, 2240–2247.
7. Rupnik, M., Widmer, A., Zimmermann, O., Eckert, C. and Barbut, F., *Clostridium difficile* toxinotype V, ribotype 078, in animals and humans. *J. Clin. Microbiol*., 2008, **46**, 2146.
8. Niyogi, S. K. *et al.*, Prevalence of *Clostridium difficile* in hospitalised patients with acute diarrhoea in Calcutta. *J. Diarrhoeal. Dis. Res*., 1991, **9**, 16–19.
9. Dhawan, B., Chaudhry, R. and Sharma, N., Incidence of *Clostridium difficile* infection: a prospective study in an Indian hospital. *J. Hosp. Infect*., 1999, **43**, 275–280.
10. Chaudhry, R., Joshy, L., Kumar, L. and Dhawan, B., Changing pattern of *Clostridium difficile* associated diarrhoea in a tertiary care hospital: a 5 year retrospective study. *Indian J. Med. Res*., 2008, **127**, 377–382.
11. Vaishnavi, C. and Singh, M., Preliminary investigation of environmental prevalence of *Clostridium difficile* affecting inpatients in a north Indian hospital. *Indian J. Med. Microbiol*., 2012, **30**, 89–92.
12. Delmee, M., Van, B. J., Simon, A., Janssens, M. and Avesani, V., Laboratory diagnosis of *Clostridium difficile*-associated diarhoea; a plea for culture. *J. Med. Microbiol*., 2005, **54**, 187–191.
13. Cohen, Stuart, H., Tang, Y. J. and Silva Joseph Jr, Analysis of the Pathogenecity locus of *Clostridium difficile* strains. *J. Infect. Dis.*, 2000, **18**, 659–663.
14. Zheng, L. *et al*., Multicenter evaluation of a new screening test that detects *Clostridium difficile* in fecal specimens. *J. Clin. Microbiol*., 2004, **42**, 3837–3840.

15. Lemee, L., Dhalluin, A., Pestel-Caron, M., Lemeland, J. F. and Pons, J. L., Multilocus sequence typing analysis of human and animal *Clostridium difficile* isolates of various toxigenic types. *J. Clin. Microbiol.*, 2004, **42**, 2609–2617.
16. Stubbs, S., Rupnik, M., Gibert, M., Brazier, J., Duerden, B. and Popoff, M., Production of actin-specific ADP-ribosyltransferase (binary toxin) by strains of *Clostridium difficile*. *FEMS Microbiol. Lett.*, 2000, **186**, 307–312.
17. Rupnik, M., Brazier, J. S., Duerden, B. I., Grabnar, M. and Stubbs, S. L., Comparison of toxinotyping and PCR ribotyping of *Clostridium difficile* strains and description of novel toxinotypes. *Microbiology*, 2001, **147**, 439–447.
18. Porter, M. C., Reggiardo, C., Bueschel, D. M., Keel, M. K. and Songer, J. G., Association of *Clostridium difficile* with bovine neonatal diarrhea. In Proc. 45th Ann. Mtg. Amer. Assoc. Vet. Lab. Diagn., St. Louis, MO, USA, 2002.
19. Songer, J. G. and Anderson, M. A., *Clostridium difficile*: an important pathogen of food animals. *Anaerobe*, 2006, **12**, 1–4.
20. Hammitt, M. C. *et al.*, A possible role for *Clostridium difficile* in the etiology of calf enteritis. *Vet. Microbiol.*, 2008, **127**, 343–352.
21. Schneeberg, A., Rupnik, M., Neubauer, H. and Seyboldt, C., Prevalence and distribution of *Clostridium difficile* PCR ribotypes in cats and dogs from animal shelters in Thuringia, Germany. *Anaerobe*, 2012, **18**, 484–488.
22. Struble, A. L., Tang, Y. J., Kass, P. H., Gumerlock, P. H., Madewell, B. R. and Silva Jr, J., Fecal shedding of *Clostridium difficile* in dogs: a period prevalence survey in a veterinary medical teaching hospital. *J. Vet. Diagn. Invest.*, 1994, **6**, 342–347.
23. Weese, J. S., Reid-Smith, R. J., Avery, B. P. and Rousseau, J., Detection and characterization of *Clostridium difficile* in retail chicken. *Lett. Appl. Microbiol.*, 2010, **50**, 362–365.
24. Marks, S. L., Kather, E. J., Kass, P. H. and Melli, A. C., Genotypic and phenotypic characterization of *Clostridium perfringens* and *Clostridium difficile* in diarrheic and healthy dogs. *J. Vet. Intern. Med.*, 2002, **15**, 533–540.
25. Koene, M. G. J. *et al.*, *Clostridium difficile* in Dutch animals: their presence, characteristics and similarities with human isolates. *Clin. Microbiol. Infect.*, 2012, **18**, 778–784.
26. Silva, R. O. S. *et al.*, Detection of toxins A/B and isolation of *Clostridium difficile* and *Clostridium perfringens* from dogs in Minas Gerais, Brazil. *Brazilian J. Microbiol.*, 2013, **44**, 133–137.
27. Lefebvre, S. L., Waltner-Toews, D., Peregrine, A. S., Reid-Smith, R., Hodge, L., Arroyo, L. G. and Weese, J. S., Prevalence of zoonotic agents in dogs visiting hospitalized people in Ontario: implications for infection control. *J. Hosp. Infect.*, 2006, **62**, 458–466.
28. Fry, P. R., Thakur, S., Abley, M. and Gebreyesa, W. A., Antimicrobial resistance, toxinotype, and genotypic profiling of *Clostridium difficile* isolates of swine origin. *J. Clin. Microbiol.*, 2012, **50**, 2366–2372.
29. Schwan, C. *et al.*, *Clostridium difficile* toxin CDT induces formation of microtubule-based protrusions and increases adherence of bacteria. *PLoS Pathog.*, 2009, **5**, e1000626.
30. Alvarez-Perez, S., Blanco, J. L., Bouza, E., Alba, P., Gibert, X., Maldonado, J. and Garcia, M. E., Prevalence of *Clostridium difficile* in diarrhoeic and non-diarrhoeic piglets. *Vet. Microbiol.,* 2009, **137**, 302–305.
31. Baker, A. A., Davis, E., Rehberger, T. and Rosener, D., Prevalence and diversity of toxigenic *Clostridium perfringens* and *Clostridium difficile* among swine herds in the midwest. *Appl. Environ. Microbiol.*, 2010, **76**, 2961–2967.
32. Janezic, S. *et al.*, International *Clostridium difficile* animal strain collection and large diversity of animal associated strains. *BMC Microbiol.*, 2014, **14**, 173.

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