microbiology research

MDPI

Article

The Molecular Epidemiology of *Clostridioides difficile* Infection in Central India: A Prospective Observational Cohort Study

Rima Biswas [1], Nick Pinkham [2], Seth T. Walk [2], Qian Wang [2], Shrikant Ambalkar [3], Ashish R. Satav [4], Mark H. Wilcox [5], Rahul Reghunath [6], Kiran Chawla [7], Padmaja A. Shenoy [7], Amit R. Nayak [1], Aliabbas A. Husain [1], Dhananjay V. Raje [1], Rajpal Singh Kashyap [1] and Tanya M. Monaghan [8,9,*]

1 Central India Institute of Medical Sciences, Nagpur 440010, India
2 Department of Microbiology and Cell Biology, Montana State University, Bozeman, MT 59717, USA
3 Department of Clinical Microbiology and Infection, King's Mill Hospital, Sherwood Forest Hospitals NHS Trust, Sutton-in-Ashfield NG17 4JL, UK
4 Meditation, AIDS, Health, Addiction & Nutrition (MAHAN) Trust, C/O Mahatma Gandhi Tribal Hospital, Karmagram, Utavali, Tahsil, Dharni, Amravati 444702, India
5 Leeds Teaching Hospitals NHS Trust and University of Leeds, Leeds LS9 7TF, UK
6 Bionivid Technology Private Limited, Bangalore 560043, India
7 Department of Microbiology, Kasturba Medical College, Manipal, Manipal Academy of Higher Education, Manipal 576104, India
8 NIHR Nottingham Biomedical Research Centre, Nottingham University Hospitals NHS Trust and the University of Nottingham, Nottingham NG5 1PB, UK
9 Nottingham Digestive Diseases Centre, School of Medicine, University of Nottingham, Nottingham NG7 2RD, UK
* Correspondence: tanya.monaghan@nottingham.ac.uk

Abstract: This prospective observational cohort study aimed to establish and compare baseline rates of *Clostridioides difficile* infection (CDI) in community and hospitalized patients in Nagpur and rural Melghat Maharashtra, including adults aged ≥18 years with a diagnosis of diarrhoea as defined as 3 or more loose stools in a 24 h period. All diarrhoeal samples were tested for CDI using the *C. diff* Quik Chek Complete enzyme immunoassay. *C. difficile*-positive stool samples were characterised by toxigenic culture, antimicrobial susceptibility testing and PCR ribotyping. *C. difficile* testing was performed on 1683 patients with acute diarrhoea. A total of 54 patients (3.21%; 95% CI: 2.42–4.17) tested positive for both the GDH antigen and free toxin. The risk factors for CDI included the presence of co-morbidities, antibiotic usage, and immunosuppression. The detected PCR ribotypes included 053-16, 017, 313, 001, 107, and 216. Our findings show that toxigenic *C. difficile* is an important but neglected aetiologic agent of infective diarrhoea in Central India. These results underscore the need to enhance the awareness and testing of patients with diarrhoea in India regarding the presence of toxigenic *C. difficile*, particularly in high-risk individuals with multiple co-morbidities, immunosuppression, and recent or ongoing antibiotic exposure or hospitalization.

Keywords: *Clostridioides difficile*; PCR ribotyping; toxin gene profiling; antibiotic susceptibility testing

Citation: Biswas, R.; Pinkham, N.; Walk, S.T.; Wang, Q.; Ambalkar, S.; Satav, A.R.; Wilcox, M.H.; Reghunath, R.; Chawla, K.; Shenoy, P.A.; et al. The Molecular Epidemiology of *Clostridioides difficile* Infection in Central India: A Prospective Observational Cohort Study. *Microbiol. Res.* **2023**, *14*, 1279–1290. https://doi.org/10.3390/microbiolres14030086

Academic Editor: Luca Grispoldi

Received: 31 July 2023
Revised: 4 September 2023
Accepted: 6 September 2023
Published: 8 September 2023

1. Introduction

Clostridioides difficile (*C. difficile*) is a Gram-positive, spore-forming, anaerobic intestinal bacterium which causes *Clostridioides difficile* infection (CDI) [1]. CDI is a leading cause of healthcare-associated infections (HAIs) in middle- and high-income countries and a global public health threat [2]. The clinical spectrum of CDI ranges from mild diarrhoea to toxic megacolon and death [1]. Our current understanding of disease pathogenesis is that CDI is a multifactorial disease process dictated by pathogenic *C. difficile* toxin production, gut microbial dysbiosis, and altered host inflammatory responses [3]. The increased incidence of primary infection, occurrence of hypertoxigenic ribotypes, and more frequent occurrence of drug resistant, recurrent, and non-hospital CDI underscore the urgent unmet need to

develop new therapeutics to tackle CDI [4]. Independent risk factors for CDI include advanced age, concomitant antibiotic use, gastric acid suppression, chemotherapy, corticosteroids, lymphoma or leukaemia, solid cancer or malignancy, chronic kidney disease, congestive heart disease, diabetes mellitus, chronic obstructive pulmonary disease, diverticular disease, inflammatory bowel disease, gastroesophageal reflux disease, peptic ulcer disease, nasogastric tube feeding, a stay in intensive care, non-surgical gastrointestinal procedures, and hospitalization [5–9].

Despite its prominence in causing antibiotic-associated diarrhoea in nosocomial and community populations in the US and Europe, there are a lack of studies describing the molecular epidemiological burden of CDI in India and other lower–middle-income countries (LMICs) [10–12]. The available data from a small number of Indian studies indicate the prevalence of ribotypes 001, 017, and 106, 045, 126 and 019 [13,14]. Only one genomic study by Singh et al. described the partial sequencing of *C. difficile* isolates [15], and no previous studies have described the phylogenetic relatedness of the strains for their epidemiological significance and the development of public health interventions for CDI. This knowledge gap and limited LMIC-specific surveillance data may be due to a combination of factors including lower prevalence, a lack of testing and awareness, inadequate laboratory facilities for diagnostic testing and culture facilities for obligate anaerobes, and resource limitations [10]. Therefore, diarrhoea is typically treated symptomatically, leading to misdiagnosis, mistreatment, and a possible underestimation of the contribution of *C. difficile* to diarrhoea. Considering the widespread and frequent use of broad-spectrum antimicrobials in India and the substantial challenges of antibiotic stewardship, together with recent economic growth, an ageing population, and the evolving COVID-19 pandemic, it is likely that *C. difficile* is an unrecognised health threat. Thus, the aim of this study was to report on CDI incidence rates for the first time, as well as the antimicrobial susceptibility pattern and the ribotyping characteristics of *C. difficile* strains among adult inpatient and outpatients in rural and urban settings in Central India.

2. Methods

2.1. Study Design and Patient Recruitment

A multicentre prospective observational cohort study was undertaken from December 2016 to December 2021 to ascertain the incidence and molecular epidemiology of CDI in urban and rural populations of Central India. The eligibility criteria included age $\geq$ 18 years and patients presenting with diarrhoea, defined as the passage of 3 or more unformed stools over at least 24 h. The urban participants were recruited from the inpatient and outpatient departments of the Central India Institute of Medical Sciences (CIIMS), a tertiary care hospital in Nagpur, Maharashtra, and from other tertiary care multispeciality hospitals in Nagpur city. Rural participants were recruited from primary healthcare centres from villages in Melghat, a tribal belt in Amravati, Maharashtra, and Mahatma Gandhi Tribal Hospital, Melghat. The patient inclusion flowchart is given in Figure 1.

Demographic details were collected by means of a structured patient questionnaire which included age, gender, body mass index (BMI), habitat location (rural/urban), hospitalization status, co-morbidities, history of antibiotics at the time of and within 2 months of recruitment, history of immunosuppression as defined by a history of malignancy or chemotherapy, immunosuppressants including at least 5 mg/day of prednisolone, history of previous enteric infection (all cause), and seasonality. The primary outcome was the proportion of toxigenic *C. difficile* cases amongst urban and rural inpatient and outpatient populations. The secondary outcomes included the proportion of non-toxigenic *C. difficile* cases in the same cohorts, as defined by the *C. diff* Quik Chek Complete assay, PCR ribotype, and the antimicrobial susceptibility of the *C. difficile* isolates. All participants received information about this study and gave their consent. The ethics committees of each participating hospital and rural healthcare centre, in addition to the School of Medicine Research Ethics Committee at the University of Nottingham (REC No.199-1901), approved the study.

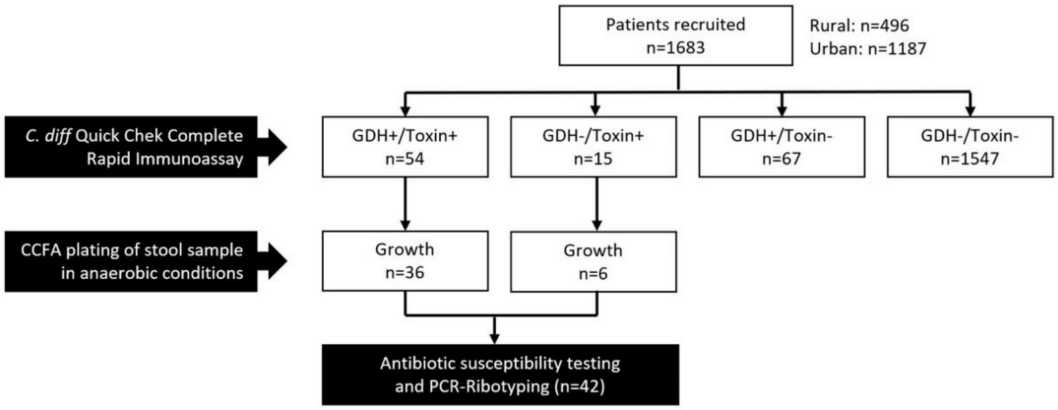

Figure 1. Patient inclusion flowchart.

2.2. Microbiological Analysis

The stool samples were collected in clean, wide-mouthed, disposable containers and were processed immediately. The stool samples were analysed for toxigenic *C. difficile* using the *C. diff* Quik Chek Complete (QCC) rapid membrane immunoassay kit (TechLab, Blacksburg, VA, USA), as previously described [16], which simultaneously detects the presence of glutamate dehydrogenase (GDH) and *C. difficile* toxins A/B in stool samples. Stool samples testing positive for GDH and the toxin or the toxin alone were subsequently cultured for *C. difficile* via a toxigenic culture. *C. difficile* spores were enriched using a pre-treatment with industrialised methylated spirit (IMS, 74 0.P). Briefly, 1.5 mL of liquid stool sample was emulsified in 0.5 mL of 0.9% sodium chloride, and 2 mL of IMS was added to it. The suspension was allowed to incubate for 45–60 min. After this incubation time, 0.1 mL of suspension from the lower faecal layer was plated on cycloserine-cefoxitin-fructose agar (CCFA; Himedia, Mumbai, India) and incubated under anaerobic conditions in a DG250 Don Whitley anaerobic workstation (Yorkshire, UK) for 48–72 h. The positive cultures were further subjected to antibiotic susceptibility testing and PCR ribotyping.

2.3. Minimum Inhibitory Concentration (MIC) Determination

Antimicrobial susceptibility testing was carried out for the positive cultures via Etest for Metronidazole, Clindamycin and Vancomycin using the breakpoints described in the CLSI (https://clsi.org accessed on 3 January 2023) guideline. Briefly, toxin positive isolates were subjected to antimicrobial susceptibility testing against Metronidazole, Clindamycin, and Vancomycin using an antimicrobial gradient diffusion method (Etest, bioMerieux Inc., Marcy L'Etoile, France). The minimum inhibitory concentrations (MICs) were interpreted as susceptible or resistant based on the CLSI guidelines. The MICs were read at the point where the elliptical zone of inhibition intersected the MIC scale after incubation for 24 h. Resistance was defined according to the following breakpoints: Metronidazole $\geq$ 32 mg/L, Clindamycin $\geq$ 8 mg/L, and Vancomycin $\geq$ 4 mg/L.

2.4. Genomic DNA Preparation

DNA isolation from the pure cultures was performed using the Qiagen Blood and Tissue Kit and harvested in microcentrifuge tubes (maximum 2×10^9 cells). In total, 180 µL of lysis buffer ATL was added to the pellet, and the samples were homogenized for 1 min. A total of 20 µL of Proteinase K was added to each sample, and digestion was performed at 56 °C for 1 h. Then, RNase A (4 µL, 100 mg/mL) was added to the sample, and it was incubated for 2 min at room temperature. The DNA was then purified as described in the Qiagen Blood and Tissue protocol. The DNA quality and concentration were quantified using agarose gel electrophoresis and Invitrogen Qubit 4, respectively.

2.5. PCR Ribotyping Analysis of C. difficile Strains

The isolates were further characterised via fluorescent PCR ribotyping using a previously described protocol [17,18]. Ribotyping was performed using a Promega Spectrum Compact CE System and analysed with the publicly available CdiffFragR ribotyping pipeline and database (thewalklab.com/tools, accessed on 4 September 2023), toxin gene profiling was performed (*tcdA*, *tcdB*, *cdtA*, *cdtB*) using PCR, as described in [19], and species identification was carried out using Sanger-style sequencing of the 16S rRNA-encoding gene (primers 8F and 1492R) [20] with online Blastn against the nr database.

2.6. Novel Ribotype Phylogenetic Analysis

Genomic DNA library preparation was performed using a NEBNext Ultra™ II FS DNA Library Prep kit for Illumina (NEB #E7805S/L, #E6177S/L). Briefly, 500 ng of genomic DNA was used as the starting material, and the manufacturer's protocol for a 200 bp genomic insert was followed. All libraries were quantified using Invitrogen Qubit 4 and the Agilent Technologies Tapestation 2200. The libraries were sequenced on Illumina Novaseq 6000 using S4 reagent kits. The raw reads were aligned to the CD630 reference genome (NCBI RefSeq assembly accession: GCF_000009205.2) using Bowtie2 version 2.4.2, and the resulting alignments were processed with SAMTOOLS Version: 1.16.1. Custom R scripts (available at https://github.com/nvpinkham, accessed on 4 September 2023) were used to extract previously identified core genome loci [21] and MLST genes using local Blastn version 2.12.0+. The MLST loci sequences were queried against the pubmlst database (https://pubmlst.org/ accessed on 6 August 2023) to identify corresponding sequencing types (STs). The genome sequences from isolates representing these STs were obtained from the NCBI (Table S1). Three reference genomes (CD630 = ribotype 012, Mta-79 = ribotype 255, and FDAARGOS_723 = ribotype 027). The most closely related genomes were used to construct a phylogeny based on the core genome loci. Bootstrap values with 100 iterations were generated and labelled at the nodes. The raw sequencing reads were deposited in NCBI's Short Read Archive under the accession number SUB12004371.

2.7. Statistical Analyses

All categorical variables were summarised in terms of frequencies and percentages, and the continuous variable, BMI, was summarised as the mean ± SD. The inpatient and outpatient categorical variables and BMI were compared using Pearson's chi-square test and the *t*-test for independent samples, respectively. All analyses were performed using SPSS version 26.0 (IBM Corp, Armonk, NY, USA) software and the statistical significance was tested at the 5% level.

3. Results

3.1. Incidence of C. difficile in the Study Population and Seasonal Variation

The baseline characteristics of the study population are summarised in Table 1. Out of 1683 recruited patients, 642 (38.1%) were inpatients, while 1041 (61.9%) were outpatients. The age and gender distributions between the two categories showed no significant difference. Compared with outpatients, inpatients were mainly from urban areas ($p \leq 0.001$), had a higher burden of reported co-morbidities, were more likely to be immunosuppressed and used antibiotics ($p < 0.0001$). There were more cases of previous enteric infections in the inpatient category as compared to the outpatient category ($p < 0.0001$).

The distribution of patients according to GDH and toxin test findings and patient characteristics are shown in Table 2. The incidence of both GDH and toxin assay positivity was 3.21% (54/1683; 95% CI: 2.42–4.17), while the occurrence of GDH positivity and toxin negativity was 3.98% (67/1683; 95% CI: 3.1–5.03). The occurrence of GDH negativity and toxin positivity was 0.9% (15/1683; 95% CI: 0.5–1.5%). We also observed a higher incidence of CDI during the monsoon season ($p < 0.0001$). From 1683 samples that were tested using the *C. diff* Quik Chek Complete assay, 69 samples (n = 54 GDH+/Toxin+ and n = 15 GDH-/Toxin+) were selected for toxigenic culture. *C. difficile* growth was seen in 36 GDH+/Toxin+

and 6 GDH-/Toxin+ categories. These 42 *C. difficile* isolates were subsequently subjected to antimicrobial susceptibility testing and PCR ribotyping.

Table 1. Baseline characteristics of patients.

Characteristic	Overall (*n* = 1683)	In-Patients (*n* = 642)	Out-Patients (*n* = 1041)	*p*-Value
Age, years (*n* (%))				
18–40	857 (51)	349 (54)	508 (49)	
41–60	601 (36)	215 (34)	386 (37)	0.082 *
>60	225 (13)	78 (12)	147 (14)	
Gender (*n* (%))				
Male	894 (53)	345 (54)	549 (53)	0.689 *
Female	789 (47)	297 (46)	492 (47)	
Location (*n* (%))				
Rural	496 (30)	160 (25)	336 (32)	0.001 *
Urban	1187 (70)	482 (75)	705(68)	
Presence of co-morbidity (*n* (%))				
Yes	799 (48)	385 (60)	414 (40)	<0.0001 *
No	884 (52)	257 (40)	627 (60)	
Antibiotic usage (*n* (%))				
Yes	588 (35)	450 (70)	138 (13)	<0.0001 *
No	1095 (65)	192 (30)	903 (87)	
Immunosuppression (*n* (%))				
Yes	499 (30)	251 (40)	248 (24)	<0.0001 *
No	1184 (70)	391 (60)	793 (76)	
BMI kg/m^2 (mean ± SD)	19.75 ± 5.05	19.47 ± 5.03	19.91 ± 5.06	0.083 †
Seasons (*n* (%))				
Summer	314 (19)	168 (26)	146 (14)	
Winter	347 (21)	141 (22)	206 (20)	<0.0001 *
Monsoon	1022 (60)	333 (52)	689 (66)	
History of intestinal infection (*n* (%))				
Yes	48 (3)	32 (5)	16 (2)	<0.0001 *
No	1635 (97)	610 (95)	1025 (98)	

* Using Pearson's chi-square test. † Using *t*-test for independent samples.

Table 2. Distribution of patients according to characteristics and outcomes.

Characteristic	GDH+/Toxin+	GDH+/Toxin-	GDH-/Toxin+	GDH-/Toxin-
	(*n* = 54)	(*n* = 67)	(*n* = 15)	(*n* = 1547)
Age, years (*n* (%))				
18 to 40	28 (52)	31 (46)	13 (87)	785 (51)
41 to 60	16 (30)	27 (40)	2 (13)	556 (36)
>60	10 (18)	9 (14)	0	206 (13)
Gender (*n* (%))				
Male	31 (57)	37 (55)	10 (67)	816 (53)
Female	23 (43)	30 (45)	5 (33)	731 (47)
Location (*n* (%))				
Rural	4 (7)	0	1 (7)	491 (32)
Urban	50 (93)	67 (100)	14 (93)	1056 (68)

Table 2. *Cont.*

Characteristic	GDH+/Toxin+	GDH+/Toxin-	GDH-/Toxin+	GDH-/Toxin-
	(*n* = 54)	(*n* = 67)	(*n* = 15)	(*n* = 1547)
Presence of co-morbidity (*n* (%))				
Yes	52 (96)	36 (54)	12 (80)	699 (45)
No	2 (4)	31 (46)	3 (20)	848 (55)
Antibiotic usage (*n* (%))				
Yes	51 (94)	39 (58)	12 (80)	228 (15)
No	3 (6)	28 (42)	3 (20)	1319 (85)
Immunosuppression (*n* (%))				
Yes	34 (63)	34 (51)	8 (53)	423 (27)
No	20 (37)	33 (49)	7 (47)	1124 (73)
BMI kg/m^2 (Mean ± SD)	21.21 ± 4.64	21.02 ±5.13	18.96 ± 5.29	19.65 ± 5.05
Seasons (*n* (%))				
Summer	10 (16)	25 (38)	4 (27)	275 (18)
Winter	3 (5)	5 (7)	2 (13)	337 (22)
Monsoon	41 (79)	37 (55)	9 (60)	935 (60)
History of intestinal infection (*n* (%))				
Yes	8 (15)	16 (24)	0	24 (2)
No	46 (85)	51 (76)	15 (100)	1523 (98)

3.2. Antimicrobial Susceptibility

The minimum inhibitory concentration (MIC) results showed that all 42 isolates were sensitive to Metronidazole (MIC < 8 µg/mL) and Vancomycin (MIC < 4 µg/mL), with resistance to Clindamycin (MIC > 8 µg/mL) observed for 16 of the 42 isolates analysed. Please see Table 3.

Table 3. Antimicrobial susceptibility profile of *C. difficile* isolates (*n* = 42) recovered from stool samples of the study subjects.

Antibiotic	Sensitive to Antibiotics	Resistant to Antibiotics	Range on E-test (in µg/mL)	Susceptible (in µg/mL)	Intermediate (in µg/mL)	Resistant (in µg/mL)	MIC 50 (µg/mL)	MIC90 (µg/mL)
Metronidazole	42 (100%)	-	0.016–256	≤8	16	≥32	0.25	5
Clindamycin	26 (46.6%)	16 (53.3%)	0.016–256	≤2	4	≥8	256	256
Vancomycin	42 (100%)	-	0.016–256	≤4	-	≥4	1.5	2

3.3. PCR Ribotyping Analysis of C. difficile Strains

In total, 42 *C. difficile* isolates were PCR ribotyped from 42 adult inpatients with a mean age of 44.1 years (SD 19.7 years), of which 41/42 were from urban areas. Of these, 27/42 were immunosuppressed and all presented with a fever and abdominal pain with 1 to 4 days of diarrhoea. Six patients had a previous history of *C. difficile* infection, and the hospital duration ranged from one to seven days (Table S1). Most of the isolates (*n* = 25, 59.5%) belonged to the following six ribotypes: 053–163 (10 isolates, 23.8%), 017 (8 isolates, 19%), 313 (3 isolates, 7.1%), 001 (2 isolates, 4.8%), 107 (1 isolate, 2.4%), and 216 (1 isolate, 2.4%). The remaining 17 isolates (40.5%) did not match to any ribotype in our database and represented at least eight different ribotypes, designated A–H, based on individual chromatograms (Figure S1). Toxin gene profiling (*tcdA/B*, and *cdtA/B*) and Sanger-style sequencing of the 16S rRNA-encoding gene (8F/1492R primers; Table S2 and Figure S2) were performed on these isolates. No toxin genes were present in the isolates representing ribotypes A, B, and C, and 16S reads from these isolates were more like non-*Clostridioides*

species, so we concluded that these isolates were not bone fide *C. difficile*. The representative ribotype D isolates were confirmed to be *C. difficile* according to Sanger 16S sequencing but were non-toxigenic. After removing ribotypes confirmed as non-*C. difficile*, we identified 12 potential novel ribotypes (Figure 2).

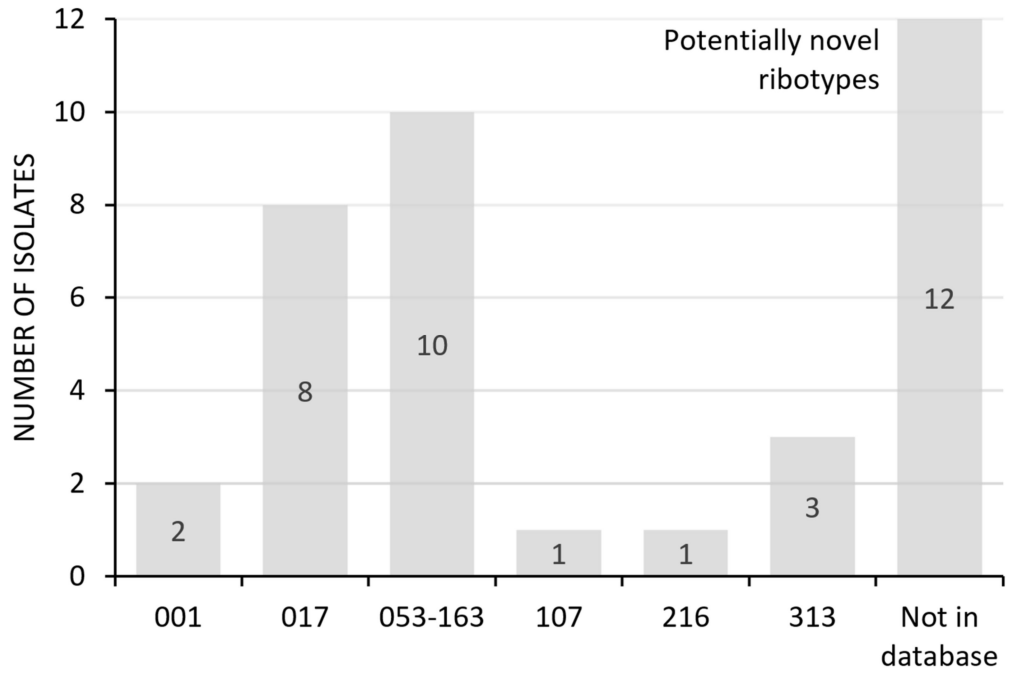

Figure 2. Ribotype frequency per Central India area, 2016–2021. In a total of 42 samples, 12 samples are potential novel ribotypes.

3.4. Novel Ribotype Phylogeny

Genome sequencing was conducted on isolates that did not match ribotypes in the Walk Lab ribotyping database. Phylogenetic analyses were conducted using both multilocus sequence typing (MLST) loci [22] and core genome loci [21] to identify those that were closely related. Reference strains representing commonly observed ribotypes (012, 027, 017) were included for comparison. Both analyses suggested that the unmatched isolates were closely related to previously sequenced isolates (Supplementary Figures S3 and S4), indicating that the novel *C. difficile* genetic diversity represented in these isolates is minor.

4. Discussion

This is the first detailed and largest *C. difficile* molecular epidemiology study to be conducted in Central India. We report a relatively low prevalence of toxigenic *C. difficile* cases compared with other generally smaller studies in India, where the prevalence was found to range widely from 1.2% [23] to 29% [24]. Comparatively, the *C. difficile* prevalence rates observed in Central India are also lower than those reported in a systematic review and meta-analysis assessing the prevalence of *C. difficile*-associated diarrhoea in developing countries, which was 15% (95% CI 13–17%), including community and hospitalized patients within four regions: the Africa–Middle East region, developing Asia, Latin America, and China [25]. In another earlier meta-analysis by Borren et al., the pooled prevalence rate of CDI in Asia was 14.8% (95% CI 12.9–16.7%) among all the patients tested (37,663) and 16.4% among hospitalized patients with diarrhoea, with a higher prevalence in East Asia (19.5%) compared with South Asia (10.5%) or the Middle East (11.1%). On estimate, there were 5.3 episodes of CDI per 10,000 patient days, similar to the rates reported for Europe and North America [26].

With recent advancements in genotyping methods, it has become possible to characterize strains according to the preferred method of PCR-ribotyping (PCR-RT), which is based on the heterogeneity of the ribosomal intergenic spacer [27]. In the current study,

PCR-ribotyping analyses revealed that most of the analysed isolates belonged to ribotypes 053-163, 017, 313, 001, 107, and 216. These results compare favourably with those of Vaishnavi et al., who also reported the PCR ribotypes 001 and 017 alongside 106 in toxigenic isolates, as well as 009 and 010 for the non-toxigenic isolates in India [14]. Collins et al. similarly reported RTs 001 and 017 in Asia, as well as the toxinogenic ribotypes 002, 014, and 018 [28]. In studies from Tehran, a high prevalence of the *C. difficile* ribotypes 001 and 026 was reported [29]. Similarly, a study from Cape Town in South Africa showed a predominance of the ribotype 017 strains [30]. Comparatively, a recent five-year, pan-European, longitudinal surveillance study of *C. difficile* ribotype prevalence reported the most commonly detected isolate to be RT027, which represented a mean prevalence of 11.4% [31]. The most prevalent PCR ribotypes found in Texas from 2011 to 2018 were 027 (17.5%), 014-020 (16.1), 106 (11.6%), and 002 (9.1%). This study also reported a 2.6% prevalence of RT053-163 as well as the emergence of a novel ribotype 255 strain [32]. Interestingly, RT027 and RT078 are not commonly reported in lower–middle-income countries such as India or Africa [12].

We found that most of the diarrhoeal cases presented during the Indian monsoon period (between April and September), which coincides with torrential rainfall; this was also the case for the detection of toxigenic *C. difficile.* An increased incidence of gastrointestinal infections, including *C. difficile*, as well as an increased risk of other disease outbreaks, such as hepatitis E and leptospirosis, have been reported after flood events, particularly in areas with poor hygiene and displaced populations [33,34]. Furthermore, epidemiological studies have documented a seasonal variation in the frequency of CDI, yet the mechanisms responsible for this variability remain poorly understood. A systematic review reported similar CDI seasonal patterns in Northern and Southern Hemisphere countries characterized by CDI peaking in spring and being lowest during the summer/autumn months [35]. Exposure to antibiotics, which typically peak in consumption during the months of August and September in India [36], as well as environmental reservoirs or sources of *C. difficile* such as food, air, water, and animals, may also play an important role in human infections [34]. In this regard, although there are no published studies confirming the foodborne transmission of *C. difficile*, at least one study by Keisam at el. has reported a high prevalence of bacterial pathogens with both toxigenic and pathogenic potential, including *C. difficile* in traditional fermented foods in the northeast region of India using MiSeq amplicon sequencing [37]. Toxigenic *C. difficile* has also been detected in animals in India, including domestic dogs, cattle, pigs, and poultry [38].

In terms of antimicrobial susceptibility patterns, we detected *C. difficile* resistance to Clindamycin in half of the isolates tested, whereas all the isolates were sensitive to Metronidazole and Clindamycin. Although new variants of *C. difficile* have emerged with a reduced susceptibility to the first-line antimicrobials Metronidazole and Vancomycin, high levels of resistance to Clindamycin, Ciprofloxacin, Moxifloxacin, Erythromycin, and Imipenem have been reported, especially among ribotypes 027 and 078 in Northern America, Europe, and Asia [39].

The high resistance of *C. difficile* isolates to Clindamycin could be because of selective pressure following the extensive use of Clindamycin in first-line treatment regimens for treating infections of the skin, abdomen, bone, heart, and soft tissues in India. Clindamycin is the preferred agent due to its excellent pharmacokinetic properties, availability in both intravenous and oral formulations with 90% oral bioavailability, cheaper cost, good tissue penetration, and accumulation in deep abscesses [40].

In terms of potential risk factors, our findings match those from similar studies which indicate that the presence of co-morbidities, antibiotic usage, and immunosuppression seem to be associated with the development of toxigenic CDI [2,5].

Our study does have some limitations. In this study, we tested stool samples from adult patients alone for *C. difficile*. As such, future studies will need to consider testing children in resource-limited settings. We did not perform hospital environmental sampling for *C. difficile*. Due to a lack of clinically trained personnel involved in this study, we were

unable to obtain granular details on patient co-morbidities or their dietary patterns. Due to the impact of the pandemic and late acquisition of an anaerobic cabinet, we were not able to culture all the relevant positive stool samples in this cohort. For the same reasons, we were unable to re-culture samples suggestive of mixed cultures or characterise the *C. difficile* isolates through whole-genome sequencing. We also did not explore the potential mechanisms for antimicrobial resistance.

Despite the widespread use of antimicrobials in India, we postulate that dietary factors may have a protective role in mediating colonization resistance against CDI. In Indian society, many people enjoy a diet which is rich in fibre, yoghurt, buttermilk, and turmeric, a spice used in curry which has been used for centuries to help decrease the rate of gastrointestinal infections. Turmeric extract and its active ingredient, curcumin, can inhibit the growth of various toxigenic strains of *C. difficile* [41], and in another study, curcumin was more effective than fidaxomicin in inhibiting *C. difficile* toxin production and showed no negative effective on the gut microbiota [42]. Indian cuisine is also high in carbohydrates, and indeed, studies have reported the protective effects of high-carbohydrate, low-protein diets in antibiotic-induced CDI mouse models [43,44].

Interestingly, in our previous studies, we showed that the Central Indian gut microbiota composition varies principally according geography rather than diarrhoeal or *C. difficile* toxin status or, indeed, BMI, where patients with CDI on antibiotics carried antimicrobial resistance genes to virtually every antibiotic class [16,45]. It is likely that geographical location is one of the strongest explanatory factors of human gut microbiota variation due to prominent differences in diet and eating habits across geographic regions [45–47]. Importantly, food patterns vary from region to region in India, which may have accounted for the lower incidence rates of CDI in Central India compared to those seen in other parts of India [48,49].

In summary, our data show that *C. difficile* is a clinically relevant pathogen in India which should be considered in the routine diagnostic workup of diarrhoeal cases. As stated by one group of researchers based in Wuhan, China, "not testing for CDI does not mean the CDI does not exist" [50]. Future efforts should concentrate on enhancing laboratory capacity and sample testing for *C. difficile*, as well as strengthening antimicrobial stewardship, to meet the rising burden of antimicrobial resistance in India and other LMICs. If resource limitations are the main barrier to testing, then less expensive diagnostic tests should be developed and made available in low-resource settings [51]. Targeted surveillance for CDI which includes strain typing and antibiotic susceptibility testing should be undertaken on a national level to monitor rates of infection, the emergence of epidemic strains, and the development of antibiotic-resistant strains. The improvement of awareness of *C. difficile* among healthcare providers can be achieved through the development of LMIC-specific *C. difficile* prevention guidelines and/or international recommendations from the World Health Organisation that are specific to *C. difficile*. It is conceivable that increasing awareness of this pathogen could potentially act as a powerful deterrent to the indiscriminate prescription of antibiotics, which, in turn, could reduce the global burden of antimicrobial resistance.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/microbiolres14030086/s1, Figure S1. Ribosomal DNA fragment profiles of eight different potential novel ribotypes (From A–H); Figure S2. Toxin gene PCR amplification.; Figure S3. MLST gene phylogeny; Figure S4. Core genome phylogeny; Table S1. Reference strain metadata; Table S2. Antibiotics.

Author Contributions: T.M.M. conceived and designed the study with the co-lead in India, R.S.K., T.M.M., R.S.K. and S.T.W. take responsibility for the integrity of the data and the accuracy of the data analysis. D.V.R. led the statistical analyses and compiled the results of the univariate and multivariate analyses with assistance from T.M.M. and R.S.K., R.B. undertook all the *C. difficile* diagnostic assays and culture work. N.P., S.T.W. and Q.W. were responsible for the PCR ribotyping and genomic characterisation studies. T.M.M., R.S.K. and S.T.W. drafted the paper and S.A., A.R.S., M.H.W., R.R., K.C., P.A.S., A.R.N., A.A.H. and R.B. provided support in the data analysis and writing of the final draft of the manuscript, as well as additional expert oversight. All authors have read and agreed to the published version of the manuscript.

Funding: This study was funded by a University of Nottingham Anne McLaren Fellowship to TM, supplemented by the National Institute for Health Research (NIHR) Nottingham Digestive Diseases Biomedical Research Centre based at the Nottingham University Hospital NHS Trust and the University of Nottingham. Research funding for the C. difficile diagnostic tests was provided by Mark Wilcox, University of Leeds. The funders were not involved in the study design, writing of the manuscript, or the decision to submit it for publication.

Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki, and approved by the Institutional Review Board (or Ethics Committee) of University of Nottingham (ethics reference number 199–1901, 28 February 2019) and the Research Ethics Committee of the Central India Institute of Medical Sciences (reference number 28-2-18).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: Raw sequencing reads have been deposited to NCBI's Short Read Archive under accession number SUB12004371. Other data is available upon request.

Acknowledgments: We are grateful to the participants who made this research possible. We thank Lokendra Singh, Central India Institute of Medical Sciences (CIIMS), for granting the study approval.

Conflicts of Interest: MHW received the following: consulting fees from AiCuris, Bayer, Crestone, Da Volterra, Deinove, EnteroBiotix, the European Tissue Symposium, Ferring, GSK, Menarini, Merck, Nestlé, Paion, Paratek, Pfizer, Phico Therapeutics, Qpex Biopharma, Seres, Surface Skins, Summit, Tillotts, Vaxxilon/Idorsia and Vedanta; lecture fees from GSK, Merck, Pfizer, Seres & Tillotts; and grant support from Almirall, Da Volterra, EnteroBiotix, GSK, Merck, MicroPharm, Nabriva, Paratek, Pfizer, Seres, Summit, The European Tissue Symposium and Tillotts. All other authors declare no conflicts of interest.

References

1. Martin, J.S.; Monaghan, T.M.; Wilcox, M.H. *Clostridium difficile* infection: Epidemiology, diagnosis and understanding transmission. *Nat. Rev. Gastroenterolo. Hepatol.* **2016**, *13*, 206–216. [CrossRef] [PubMed]
2. Finn, E.; Andersson, F.L.; Madin-Warburton, M. Burden of *Clostridioides difficile* infection (CDI)—A systematic review of the epidemiology of primary and recurrent CDI. *BMC Infect. Dis.* **2021**, *21*, 456. [CrossRef] [PubMed]
3. Monaghan, T.M. New perspectives in *Clostridium difficile* disease pathogenesis. *Infect. Dis. Clin. North Am.* **2015**, *29*, 1–11. [CrossRef]
4. Monaghan, T.M.; Seekatz, A.M.; Mullish, B.H.; Moore-Gillon, C.; Dawson, L.F.; Ahmed, A.; Kao, D.; Chan, W.C. *Clostridioides difficile*: Innovations in target discovery and potential for therapeutic success. *Expert Opin. Ther. Targets* **2021**, *25*, 949–963. [CrossRef] [PubMed]
5. Eze, P.; Balsells, E.; Kyaw, M.H.; Nair, H. Risk factors for *Clostridium difficile* infections—An overview of the evidence base and challenges in data synthesis. *J. Glob. Health* **2017**, *7*, 010417. [CrossRef] [PubMed]
6. Furuya-Kanamori, L.; Stone, J.C.; Clark, J.; McKenzie, S.J.; Yakob, L.; Paterson, D.L.; Riley, T.V.; Doi, S.A.R.; Clements, A.C. Comorbiditoes, Exposure to Medications, and the Risk of Community-Acquired *Clostridium difficile* Infection: A systematic review and meta-analysis. *Infect. Control. Hosp. Epidemiol.* **2015**, *36*, 132–141. [CrossRef] [PubMed]
7. Furuya-Kanamori, L.; Wangdi, K.; Yakob, L.; McKenzie, S.J.; Doi, S.A.R.; Clark, J.; Paterson, D.L.; Riley, T.V.; Clements, A.C.A. 25-Hydroxyvitamin D Concentrations and *Clostridium difficile* Infection: A Meta-Analysis. *JPEN J. Parenter. Enteral Nutr.* **2017**, *41*, 890–895. [CrossRef]
8. Bignardi, G.E. Risk factors for *Clostridium difficile* infection. *J. Hosp. Infect.* **1998**, *40*, 1–15. [CrossRef]
9. Vardakas, K.Z.; Konstantelias, A.A.; Loizidid, G.; Rafailidis, P.I.; Falagas, M.E. Risk factors for development of *Clostridium difficile* infection due to BI/NAP1/027 strain: A meta-analysis. *Int. J. Infect. Dis.* **2012**, *16*, e768–e773. [CrossRef]
10. Monaghan, T.M.; Biswas, R.; Satav, A.; Ambalkar, S.; Kashyap, R.S. *Clostridioides difficile* epidemiology in India. *Anaerobe* **2022**, *74*, 102517. [CrossRef]

11. Brajerova, M.; Kikova, J.; Krutova, M. *Clostridium difficile* epidemiology in the Middle and Far East. *Anaerobe* **2022**, *74*, 102542. [CrossRef] [PubMed]
12. Kullin, B.; Abratt, V.R.; Reid, S.J.; Riley, T.V. *Clostridioides difficile* infection in Africa: A narrative review. *Anaerobe* **2022**, *74*, 102549. [CrossRef] [PubMed]
13. Hussain, I.; Borah, P.; Sharma, R.K.; Rajkhowa, S.; Rupnik, M.; Saikia, D.P.; Hasin, D.; Hussain, I.; Deka, N.; Barkalita, L.; et al. Molecular characteristics of *Clostridium difficile* isolates from human and animals in the Northeastern region of India. *Mol. Cell. Probes* **2016**, *30*, 306–311. [CrossRef] [PubMed]
14. Vaishnavi, C.; Singh, M.; Mahmood, S.; Kochhar, R. Prevalence, and molecular types of *Clostridium difficile* isolates from faecal specimens of patients in a tertiary care centre. *J. Med. Microbiol.* **2015**, *64*, 1297–1304. [CrossRef] [PubMed]
15. Singh, M.; Vaishnavi, C.; Mahmood, S.; Kochhar, R. Toxinotyping and Sequencing of *Clostridium difficile* Isolates from Patients in a Tertiary Care Hospital of Northern India. *Front. Med.* **2017**, *4*, 33. [CrossRef] [PubMed]
16. Monaghan, T.M.; Sloan, T.J.; Stockdale, S.R.; Blanchard, A.M.; Emes, R.D.; Wilcox, M.; Biswas, R.; Nashine, R.; Manke, S.; Gandhi, J.; et al. Metagenomics reveals impact of geography and acute diarrheal disease on the Central Indian human gut microbiome. *Gut Microbes* **2020**, *12*, 1752605. [CrossRef] [PubMed]
17. Walk, S.T.; Micic, D.; Jain, R.; Lo, E.S.; Liu, E.W.; Almassalha, L.M.; Ewing, S.A.; Ring, C.; Galecki, A.T.; Rogers, M.A.M.; et al. *Clostridium difficile* ribotype does not predict severe infection. *Clin. Infect. Dis.* **2012**, *55*, 1661–1668. [CrossRef]
18. Martinson, J.N.V.; Broadway, S.; Lohman, E.; Johnson, C.; Alam, M.J.; Khaleduzzaman, M.; Garey, K.W.; Schlackman, J.; Young, V.B.; Santhosh, K.; et al. Evaluation of portability and cost of a fluorescent PCR ribotyping protocol for *Clostridium difficile* epidemiology. *J. Clin. Microbiol.* **2015**, *53*, 1192–1197. [CrossRef]
19. Persson, S.; Torpdahl, M.; Olsen, K.E.P. New multiplex PCR method for the detection of *Clostridium difficile* toxin A (*tcdA*) and toxin B (*tcdB*) and the binary toxin (*cdtA/cdtB*) genes applied to a Danish strain collection. *Clin. Microbiol. Infect.* **2008**, *14*, 1057–1064. [CrossRef]
20. Schmidt, T.M.; Relman, D.A. Phylogenetic identification of uncultured pathogens using ribosomal RNA sequences. *Methods Enzymol.* **1994**, *235*, 205–222. [CrossRef]
21. He, M.; Sebaihia, M.; Lawley, T.D.; Stabler, R.A.; Dawson, L.F.; Martin, M.J.; Holt, K.E.; Seth-Smith, H.M.; Quail, M.A.; Rance, R.; et al. Evolutionary dynamics of *Clostridium difficile* over short and long time scales. *Proc. Natl. Acad. Sci. USA* **2010**, *107*, 7527–7532. [CrossRef] [PubMed]
22. Jolley, K.A.; Bray, J.E.; Maiden, M.C.J. Open-access bacterial population genomics: BIGSdb software, the PubMLST.org website and their applications. *Wellcome Open Res.* **2018**, *3*, 124. [CrossRef] [PubMed]
23. Kumar, N.; Ekka, M.; Raghunandan, P.; Ranjan, S.; Sinha, S.; Sharma, S.K.; Chaudhry, R.; Sharma, N.; Ahmad, H.; Samantaray, J.C.; et al. *Clostridium difficile* infections in HIV-positive patients with diarrhoea. *Natl. Med. J. India* **2014**, *27*, 138–140. [PubMed]
24. Abuderman, A.A.; Mateen, A.; Syed, R.; Aloahd, M.S. Molecular characterization of *Clostridium difficile* isolated from carriage and association of its pathogenicity to prevalent toxin genes. *Microb. Pathog.* **2018**, *120*, 1–7. [CrossRef] [PubMed]
25. Curcio, D.; Cane, A.; Fernandez, F.; Correa, J. *Clostridium difficile*-associated diarrhea in developing countries: A systematic review and meta-analysis. *Infect. Dis. Ther.* **2019**, *8*, 87–103. [CrossRef] [PubMed]
26. Borren, N.Z.; Ghadermarzi, S.; Hutfless, S.; Ananthakrishnan, A.N. The emergence of *Clostridium difficile* infection in Asia: A systematic review and meta-analysis of incidence and impact. *PLoS ONE* **2017**, *12*, e0176797. [CrossRef] [PubMed]
27. Azimirad, M.; Krutova, M.; Nyc, O.; Hasani, Z.; Afrisham, L.; Alebouyeh, M.; Zali, M.R. Molecular typing of *Clostridium difficile* isolates cultured from patient stool samples and gastroenterological medical devices in a single Iranian hospital. *Anaerobe* **2017**, *47*, 125–128. [CrossRef] [PubMed]
28. Collins, J.; Auchtung, J.M. Control of *Clostridium difficile* Infection by Defined Microbial Communities. *Microbiol. Spectr.* **2017**, *5*, 10. [CrossRef]
29. Baghani, A.; Mesdaghinia, A.; Kuijper, E.J.; Aliramezani, A.; Talebi, M.; Douraghi, M. High prevalence of *Clostridiodes diffiicle* PCR ribotypes 001 and 126 in Iran. *Sci. Rep.* **2020**, *10*, 4658. [CrossRef]
30. Kullin, B.; Brock, T.; Rajabally, N.; Anwar, F.; Vedantam, G.; Reid, S.; Abratt, V. Characterisation of *Clostridium difficile* strains isolated from Groote Schuur Hospital, Cape Town, South Africa. *Eur. J. Clin. Microbiol. Infect. Dis.* **2016**, *35*, 1709–1718. [CrossRef]
31. Freeman, J.; Vernon, J.; Pilling, S.; Morris, K.; Nicolson, S.; Shearman, S.; Clark, E.; Palacios-Fabrega, J.A.; Wilcox, M.; The Pan-European Longitudinal Surveillance of Antibiotic Resistance among Prevalent Clostridium difficile Ribotypes' Study Group. Five-year Pan-European, longitudinal surveillance of Clostridium difficile ribotype prevalence and antimicrobial resistance: The extended ClosER study. *Eur. J. Clin. Microbiol. Infect. Dis.* **2020**, *39*, 169–177. [CrossRef] [PubMed]
32. Gonzales-Luna, A.J.; Carlson, T.J.; Dotson, K.M.; Poblete, K.; Costa, G.; Miranda, J.; Lancaster, C.; Walk, S.T.; Tupy, S.; Begum, K.; et al. PCR ribotypes of *Clostridioides difficile* across Texas from 2011 to 2018 including emergence of ribotype 255. *Emerg. Microbes Infect.* **2020**, *9*, 341–347. [CrossRef] [PubMed]
33. Alderman, K.; Turner, L.R.; Tong, S. Floods and human health: A systematic review. *Environ. Int.* **2012**, *47*, 37–47. [CrossRef] [PubMed]
34. Lin, C.J.; Wade, T.J.; Hilborn, E.D. Flooding and *Clostridium difficile* Infection. A Case-Crossover Analysis. *Int. J. Environ. Res. Public Health* **2015**, *12*, 6948–6964. [CrossRef] [PubMed]
35. Furuya-Kanamori, L.; McKenzie, S.J.; Yakob, L.; Clark, J.; Paterson, D.L.; Riley, T.V.; Clements, A.C. *Clostridium difficile* Infection Seasonality: Patterns across Hemispheres and Continents—A Systematic Review. *PLoS ONE* **2015**, *10*, e0120730. [CrossRef]

36. Farooqui, H.H.; Selvaraj, S.; Mehta, A.; Heymann, D.L. Community level antibiotic utilization in India and its comparison vis-à-vis European countries: Evidence from pharmaceutical sales data. *PLoS ONE* **2014**, *13*, e0204805. [CrossRef] [PubMed]
37. Keisam, S.; Tuikhar, N.; Ahmed, G.; Jeyaram, K. Toxigenic and pathogenic potential of enteric bacterial pathogens prevalent in the traditional fermented foods marketed in rhe Northeast regions of India. *Int. J. Food Microbiol.* **2019**, *296*, 21–30. [CrossRef] [PubMed]
38. Hussain, I.; Sharma, R.K.; Borah, P.; Rajkhowa, S.; Hussain, I.; Barkalita, L.M.; Hasin, D.; Choudhury, M.; Rupnik, M.; Deka, N.K.; et al. Isolation and characterization of *Clostridium difficile* from pet dogs in Assam, India. *Anaerobe* **2015**, *36*, 9–13. [CrossRef]
39. Tenover, F.C.; Tickler, I.; Persing, D.H. Antimicrobial-resistant strains of *Clostridium difficile* from North America. *Antimicrob. Agents Chemother.* **2012**, *56*, 2929–2932. [CrossRef]
40. Majhi, S.; Dash, M.; Mohapatra, D.; Mohapatra, A.; Chayani, N. Detection of inducible and constitutive isolates in a tertiary care hospital, Eastern India. *Avicenna J. Med.* **2016**, *6*, 75–80. [CrossRef]
41. Rattan, P.; Yang, N. Inhibiting Hospital Associated Infection of Toxigenic *Clostridium difficile* Using Natural Spice—Tumeric (Curcumin). *Am. J. Gastroenterol.* **2020**, *105*, pS122.
42. Mody, D.; Athamneh, A.I.M.; Seleem, M.N. Curcumin: A natural derivative with antibacterial activity against *Clostridium difficile*. *J. Glob. Antimicrob. Resist.* **2020**, *21*, 154–161. [CrossRef] [PubMed]
43. Mefferd, C.C.; Bhute, S.S.; Phan, J.R.; Villarama, J.V.; Do, D.M.; Alarcia, S.; Abel-Santos, E.; Hedlund, B.P. A High-Fat/High-Protein, Atkins-Type Diet Exacerbates *Clostridioides* (*Clostridium*) *difficile* Infection in Mice, whereas a High-Carbohydrate Diet Protects. *mSystems* **2020**, *5*, e00765-19. [CrossRef] [PubMed]
44. Hryckowian, A.J.; Van Treuren, W.; Smits, S.A.; Davis, N.M.; Gardner, J.O.; Bouley, D.M.; Sonnenburg, J.L. Microbiota-accessible carbohydrates suppress *Clostridium difficile* infection in a murine model. *Nat. Microbiol.* **2018**, *3*, 662–669. [CrossRef] [PubMed]
45. Monaghan, T.M.; Biswas, R.N.; Nashine, R.R.; Joshi, S.S.; Mullish, B.H.; Seekatz, A.M.; Blanco, J.M.; McDonald, J.A.K.; Marchesi, J.R.; Yau, T.O.; et al. Multiomics profiling reveals signatures of dysmetabolism in urban populations in India. *Microorganisms* **2021**, *9*, 1485. [CrossRef] [PubMed]
46. Gaulke, C.A.; Sharpton, T.J. The influence of ethnicity and geography on human gut microbiome composition. *Nat. Med.* **2018**, *24*, 1495–1496. [CrossRef] [PubMed]
47. Senghor, B.; Sokhna, C.; Ruimy, R.; Lagier, J.C. Gut microbiota diversity according to dietary habits and geographical provenance. *Hum. Microbiome J.* **2018**, *7–8*, 1–9. [CrossRef]
48. Green, R.; Milner, J.; Joy, E.J.M.; Agrwal, S.; Dangour, A.D. Dietary patterns in India: A systematic review. *Br. J. Nutr.* **2016**, *116*, 142–148. [CrossRef]
49. Tak, M.; Shankar, B.; Kadiyala, S. Dietary Transition in India: Temporal and Regional Trends, 1993 to 2012. *Food Nutr. Bull.* **2019**, *40*, 254–270. [CrossRef]
50. Galaydick, J.; Xu, Y.; Sun, L.; Landon, E.; Weber, S.G.; Sun, D.; Zhou, J.; Sherer, R. Seek and you shall find: Prevalence of *Clostridium difficile* in Wuhan, China. *Am. J. Infect. Control.* **2015**, *43*, 301–302. [CrossRef]
51. Roldan, G.A.; Cui, A.X.; Pollock, N.R. Assessing the Burden of *Clostridium difficile* Infection in Low-and Middle-Income Countries. *J. Clin. Microbiol.* **2018**, *56*, e01747-17. [CrossRef]