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ARTICLE

Clostridium difficile is not associated with outbreaks of viral gastroenteritis in the elderly in the Netherlands

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Abstract The coincidental increase in norovirus outbreaks and *Clostridium difficile* infection (CDI) raised the question of whether these events could be related, e.g. by enhancing spread by diarrhoeal disease outbreaks. Therefore, we studied the prevalence of *C. difficile* in outbreaks of viral gastroenteritis in nursing homes for the elderly and characterised enzyme immunoassay (EIA)-positive stool samples. Stool samples from nursing home residents (n=752) in 137 outbreaks of viral aetiology were investigated by EIA for the presence of *C. difficile* toxins. Positive samples were further tested by a cell neutralisation cytotoxicity test, a second EIA and culture. Cultured isolates were tested for the presence of toxin genes, the production of toxins and characterised by 16S rRNA polymerase chain reaction (PCR) and sequencing. Twenty-four samples (3.2%) tested positive in the EIA. Of these 24 positive samples, only two were positive by cytotoxicity and three by a second EIA. Bacterial culture of 21 available stool samples yielded a toxinogenic *C. difficile* PCR ribotype 001 in one patient sample only. In conclusion, we found no evidence in this retrospective study for an association between viral gastroenteritis outbreaks and *C. difficile*. The high rate of false-positive EIA samples emphasises the need for second confirmation tests to diagnose CDI.

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Introduction

Outbreaks of acute gastroenteritis (AGE) of suspected viral aetiology are reported to the National Institute for Public Health and the Environment (RIVM) as part of the ongoing viral gastroenteritis outbreak surveillance system. Although these outbreaks are selected based on criteria thought to be highly specific for viral gastroenteritis outbreaks, and despite extensive testing, the cause of approximately 12% of outbreaks of AGE remains unknown [1]. Of the outbreaks in which a viral pathogen was detected, the vast majority is associated with noroviruses (NoV).

Since 2002, the epidemiology of NoV appears to have changed, with the introduction of a novel lineage of the dominant genogroup (G) II.4 viruses. This introduction coincided with increased levels of reporting of NoV outbreaks across Europe and a noticeable peak in mortality in the elderly, which coincides with the NoV seasonal peak [2]. Simultaneously, Canada, the USA and, later, the United Kingdom (UK), the Netherlands, Belgium, France and Austria reported the emergence of *Clostridium difficile* ribotype 027, which appeared to have increased virulence compared to other *C. difficile* strains [3, 4]. In the Netherlands, this ribotype was detected in 26 hospitals and ten nursing homes by May 2008 [5, 6]. Antibiotic use has been associated with an increased risk of infection with *C. difficile* and is responsible for 15 to 25% of all cases of antibiotic-associated diarrhoea in hospitals [7]. Outbreaks and cases outside hospitals are less frequently recognised

but have been described, sometimes without the typical risk factors [8, 9]. *C. difficile* outbreaks and cases in nursing homes have been studied less systematically and, although *C. difficile* type 027 has been found in at least ten nursing homes in the Netherlands, detailed information about these infections is absent [6].

The coincidental increase in norovirus GII.4 outbreaks and the spread of *C. difficile* ribotype 027 raised the question of whether these events could be related [2, 10]. In theory, mixed infections of NoV and *C. difficile* ribotype 027 could increase the severity of the disease. Furthermore, norovirus-induced episodes of vomiting and diarrhoea might trigger or exacerbate *C. difficile* diarrhoea, resulting in increased spread of *C. difficile* due to multiple episodes of vomiting and diarrhoea, as has been suggested in some recent publications [11–13]. Therefore, we decided to investigate a possible association of norovirus and rotavirus outbreaks with *C. difficile* infections (CDIs), particularly with respect to *C. difficile* polymerase chain reaction (PCR) ribotype 027.

Materials and methods

Sample collection

In total, we tested 752 faecal samples from 137 outbreaks of gastroenteritis (number of stool samples varying from 2 to 25 per outbreak). All outbreaks occurred in 2006 in nursing homes and were selected because the *C. difficile* ribotype 027 problem emerged in that period in the Netherlands and these samples provided a snapshot of what is present within the elderly population. Viral detection has been performed as described previously by Svraka et al. [1]. The outbreaks were first tested using the assays for NoV. Then, all outbreaks that remained negative were further tested using the assays for rotaviruses, adenoviruses, astroviruses and sapoviruses [1]. Of these, 109 were confirmed norovirus outbreaks (644 stool samples), 14 outbreaks were confirmed rotavirus outbreaks (70 samples) and 14 outbreaks (38 samples) were of unexplained aetiology, reflecting the typical aetiology of such outbreaks. The outbreaks were reported throughout the year, but with a peak in winter, as part of our ongoing surveillance for viral gastroenteritis outbreaks, which explains the high number of NoV outbreaks in this study [1]. Undiluted stool samples had been stored at 4°C, as recommended for viral gastroenteritis diarrhoea, before testing for the presence of *C. difficile*.

Diagnostic assays used for the screening of samples

The screening of 752 outbreak specimens for the presence of *C. difficile* was done using the PTAB enzyme immunoassay (EIA, Premier *C. difficile* Toxin A&B, Meridian Diagnostics, Inc., Cincinnati, OH, USA) on all stool samples. Assay was performed as described by the manufacturer. The stool samples had been stored at 4°C for periods longer than 3–4 days, which is in accordance with recommendations for the diagnostics of viral gastroenteritis, but not in accordance to the manufacturer's recommendations, before testing for the presence of *C. difficile*. Consequently, we validated the PTAB EIA assay for use in samples that were stored longer at 4°C by the parallel testing of 96 stool samples that were aliquoted directly after receipt and stored for five months at −20 and 4°C.

The PTAB EIA was interpreted visually (within 15 min) and by spectrophotometer (within 30 min), as described by the manufacturer; 376 samples were read both visually and on a Labsystems Multiskan RC spectrophotometer plate reader (Thermo Fisher Scientific, Breda, the Netherlands) using dual wavelengths of 450 and 450/630 nm. The samples were described as positive if optical density (OD) values of 0.100 or more were measured at a wavelength of 450/630 nm and/or 0.150 or more at a wavelength of 450 nm, as described by the manufacturer. There was 100% concordance between reading visually and by using the spectrophotometer plate reader. The remaining 376 samples were read visually only.

Confirmatory testing on PTAB-positive samples

Stool samples found positive using the PTAB EIA assay were cultured and tested using a neutralisation cytotoxicity assay and the automated immunoanalyzer VIDAS® *C. difficile* A & B (bioMérieux) [14].

For culture, stool samples found positive in the PTAB EIA assay were treated with absolute ethanol (96%) prior to inoculation onto Columbia agar containing colistin and nalidixic acid, *C. difficile*-selective agar with cefoxitin, amphotericin B and cycloserine (CLO; bioMérieux) and blood agar. CLO medium was also used to inoculate faecal samples without pre-treatment with ethanol. Inoculated faecal samples were incubated in an anaerobic environment at 37°C for 2 days.

Clostridium-suspected colonies were tested further using PCR assays for *C. difficile*-specific GluD targeting the GDH gene [15], *C. difficile* ribotyping [16, 17], enterotoxin A (tcdA) [5], cytotoxin B (tcdB) [18] and 16S rRNA PCR [19]. For this, DNA was isolated from suspected colonies by a QIAamp DNA isolation column (Qiagen, Holden, Germany) according to the manufacturer's recommendations, including 10 min incubation at 55°C with proteinase K. The final volume of the DNA extracts was 200 μL.

The neutralisation cytotoxicity assay was performed using Vero cells in a 24-well format. Faecal samples were diluted in 1:4 Eagle's minimum essential medium (EMEM)

containing 5% foetal bovine serum and centrifuged for 10 min at 3,000 rpm. The supernatant was filtered through a 0.45-µm-pore filter, 150 µL supernatant was mixed with 150 µl EMEM and neutralisation of the cytotoxic effect was performed using 150 µL faecal supernatant and 150 µl of 1:25 dilution of specific *C. difficile* antitoxin (Techlab, Blacksburg, VA, USA). These two mixtures were incubated for 30 min at room temperature; subsequently, 200 µl of each mix was inoculated on Vero cells. The cytotoxic effect was evaluated daily for 3 days microscopically.

Stool samples positive in the PTAB EIA were also tested using the VIDAS assay. Stool samples were centrifuged for 10 min at maximum speed (14,000 rpm) and 300 µl of supernatant was used, according to the manufacturer's instructions. Samples with OD value lower than 0.13 were described as negative, OD values between 0.13 and 0.37 were equivocal and OD values higher than 0.37 were described as positive.

Bacterial isolates cultured from EIA-positive faeces samples were also investigated for the production of tcdA and tcdB by VIDAS and EIA. Bacteria were cultured in liquid brain heart infusion (BHI) bouillon medium for 4 days. Subsequently, the BHI cultures were centrifuged at 3,000 rpm for 10 min and the supernatant was further investigated. The same cut-off values of EIA and VIDAS were used for in vitro bacterial toxin production as for toxin detection in faeces samples.

Evaluation of the approach of *Clostridium* detection

Optimal sample storage conditions differ for viral testing and bacterial testing, and this raised the question whether *Clostridium* EIA test results could be explained by suboptimal use of the assay. Therefore, we validated this approach by testing original total nucleic acids isolated from 154 (20% of all samples tested in the EIA) faecal samples within 3 days of arrival from the stool samples which were included in our study. These extracts had been stored at −80°C. We used a real-time PCR for the tcdB gene, as described by van den Berg et al. [14, 20]

Results

Testing of stool samples using the PTAB EIA

For the validation of the use of the PTAB EIA on our sample set, we used 96 stool samples that were stored for 5 months at −20 and at 4°C. Of the samples stored at −20°C, two were positive, and of the samples stored at 4°C, three were positive, including the ones that were positive after storage at −20°C.

In total, 24 (3.2%) of 752 stool samples from outbreaks of acute gastroenteritis in nursing homes in 2006 tested positive in the PTAB EIA assay (Table 1). Of the 24 PTAB EIA-positive stool samples, 22 were from 17 norovirus outbreaks, one was from a rotavirus outbreak and the other one was from an outbreak of unexplained aetiology.

Confirmatory testing of PTAB-positive stool samples

Specialised stool culture for *Clostridium* spp. (Table 1) was performed on 24 stool samples. For three stool samples, culture was negative, and in 21 stool samples, *Clostridium*-suspected colonies were found. Subsequently, neutralisation cytotoxicity assay and VIDAS assay were performed on 21 stool specimens of which sufficient amounts of material were available. This yielded two positive samples both by neutralisation cytotoxicity assay and VIDAS, and an additional weakly positive result using the VIDAS assay (Table 1).

Analysis of bacterial isolates from the stool samples

From the 21 *Clostridium*-suspected colonies, which were grown on the CLO medium plates, DNA was extracted and analysed by 16S DNA sequencing and further using a range of assays for the characterisation of toxin-producing *C. difficile* (Table 1). 16S DNA sequencing revealed *Lactobacilli* in six stool samples (37.5%) and different *Clostridium* species in 15 samples (62.5%): *C. difficile*, *C. disporicum*, *C. perfringens*, *C. sordellii*, *C. boltei*, *C. butyricum*, *C. baratii*, *C. subterminale* and *C. bifermentans* (Table 1). Using the GluD, ribotyping PCR, tcdA and tcdB assays, one sample was positive and identified as *C. difficile*. One isolate of *C. disporicum* was weakly positive for tcdA and one isolate of *C. subterminale* for tcdB.

One *Clostridium* species from a faecal sample (13/8, Table 1) tested positive by EIA, cytotoxicity and VIDAS was identified as *C. sordellii*. This isolate was negative for tcdA and tcdB and did not produce a cytotoxin.

The results of the in vitro bacterial toxin production measured by cytotoxicity, PTAB and VIDAS revealed only strongly positive results for one sample that was identified as *C. difficile*. Using cut-off values of PTAB and VIDAS as applied on stool samples, (weakly) positive reactions were obtained for 14 samples using PTAB and six samples using the VIDAS assay. However, none of these isolates produced cytotoxins.

Epidemiological data on *C. difficile*- and *C. sordellii*-positive samples

Only one stool sample and the corresponding bacterial isolate were positive in all assays and proven to be *C. difficile* type 001 (23/18, Table 1). This sample was from an outbreak of unexplained aetiology that occurred in November 2006, with eight cases being reported. All eight samples were tested

Table 1 Results on stool samples and characterisation of the isolates from the stool samples. These results are obtained for stool samples positive in the PTAB EIA and tested further using neutralisation cytotoxicity, VIDAS and culture assays. OD values of the VIDAS assay are given in the brackets. Results on the isolates cultured from the stool samples were by polymerase chain reaction (PCR) assays for GluD, ribotyping, tcdA, tcdB, and 16S rRNA and sequencing. Additionally, grown cultures were tested using neutralisation cytotoxicity assay, PTAB EIA and VIDAS. OD values of VIDAS and PTAB EIA (measured at 450 nm) are given in the brackets

PTAB-positive samples/outbreak number	Viral diagnosis	Cytotoxicity assay	VIDAS	Culture	16S rRNA PCR and sequencing	GluD and ribotyping PCR	tcdA PCR	tcdB PCR	Assays on cultured bacteria		
									Cytotoxicity assay	PTAB EIA	VIDAS
1/1	Norovirus (II.4 2006b)	−	− [0.02]	+	*Lactobacillus casei*	−	−	−	−	− [0.121]	− [0.03]
2/2	Norovirus (II.4 2004)	ND	ND	+	*Clostridium disporicum*	−	−	−	−	+ [0.324]	+ [0.44]
3/2	Norovirus (II.4 2004)	−	− [0.02]	+	*Clostridium perfringens*/ *Clostridium disporicum*	−	−	−	−	+ [0.470]	+ [1.12]
4/2	Norovirus (II.4 2004)	−	− [0.03]	+	*Clostridium disporicum*	−	+	−	−	+ [0.427]	+ [0.62]
5/2	Norovirus (II.4 2004)	−	− [0.10]	+	*Clostridium disporicum*	−	−	−	−	+ [0.342]	+ [0.61]
6/2	Norovirus (II.4 2004)	−	− [0.03]	+	*Clostridium subterminale*	−	−	+	−	+ [0.287]	− [0.02]
7/2	Norovirus (II.4 2004)	−	− [0.01]	−	ND	ND	ND	ND	ND	ND	ND
8/3	Norovirus (II.4 2004)	ND	ND	+	*Clostridium boltei*	−	−	−	−	+ [0.440]	+ [0.48]
9/4	Norovirus (II.4 2004)	−	− [0.03]	+	*Clostridium butyricum*	−	−	−	−	+ [0.179]	− [0.02]
10/5	Norovirus (II.4 2006b)	−	− [0.13]	+	*Clostridium bifermentans*	−	−	−	−	+ [0.384]	+/− [0.29]
11/6	Norovirus (II.4 2006b)	−	− [0.01]	−	ND	ND	ND	ND	ND	ND	ND
12/7	Norovirus (II.4 2006b)	−	− [0.08]	+	*Clostridium disporicum*	−	−	−	−	+ [0.208]	− [0.02]
13/8	Norovirus (II.4 2006a)	+	+ [2.32]	+	*Clostridium sordellii*	−	−	−	−	+ [0.178]	− [0.02]
14/9	Norovirus (II.4 2006a)	−	− [0.04]	+	*Clostridium baratii*	−	−	−	−	+ [0.190]	− [0.09]
15/10	Norovirus (II.4 2006b)	−	− [0.03]	+	*Lactobacillus*	−	−	−	−	− [0.069]	− [0.04]
16/11	Norovirus (II.4 2006a)	−	− [0.01]	+	*Lactobacillus*	−	−	−	−	− [0.100]	− [0.05]
17/12	Norovirus (II.4 2004)	−	− [0.07]	+	*Clostridium perfringensfringes*	−	−	−	−	+ [0.165]	− [0.04]
18/13	Norovirus (I.2)	−	− [0.01]	+	*Lactobacillus paracasei*	−	−	−	−	− [0.120]	− [0.02]
19/14	Norovirus (II.4 2006a)	−	− [0001]	+	*Clostridium disporicum*	−	−	−	−	+ [0.169]	− [0.05]
20/15	Norovirus (II.4 2004)	−	+ [0055]	+	*Lactobacillus paracasei*	−	−	−	−	− [0.125]	− [0.04]
21/16	Norovirus (II.4 2006a)	−	− [0001]	−	ND	ND	ND	ND	ND	ND	ND
22/17	Norovirus (II.4 2006a)	−	− [0.04]	+	*Lactobacillus casei*	−	−	−	−	− [0.069]	− [0.03]
23/18	Unexplained aetiology	+	+ [6.49]	+	*Clostridium difficile* type 001	+	+	+	+	+ [3.137]	+ [4.24]
24/19	Rotavirus	ND	ND	+	*Clostridium disporicum*	−	−	−	−	+ [0.196]	− [0.07]

−: negative

+: positive

ND: not done

OD: optical density

VIDAS cut-off OD values: negative <0.13, equivocal ≥ 0.13 to <0.37, positive ≥ 0.37

PTAB EIA: Premier *C. difficile* Toxin A&B enzyme immunoassay

PTAB cut-off OD values: negative < 0.150, positive >0.150

GluD: glutamate dehydrogenase gene

tcdA: enterotoxin A gene

tcdB: cytotoxin B gene

using the PTAB EIA; however, only one was positive and tested further in other assays.

The *C. sordellii*-positive stool sample (13/8, Table 1) was from a norovirus outbreak that affected four persons from a residential institution in an endemic region for *C. difficile* in November 2006. The faeces samples of the three other patients were tested for CDI, but were negative.

Evaluation of the approach of *Clostridium* detection

Of the 154 total nucleic acids that had been isolated from stool samples within 3 days of their arrival, the two samples that were cytotoxic and VIDAS-positive were positive by tcdB real-time PCR assay (Table 1 samples 13/8 and 23/18). All other samples were negative.

Discussion

In this study, we investigated whether the emergence of a successful norovirus strain [10] could coincide with the spread of *C. difficile* and whether the emergence of *C. difficile* could explain some unresolved outbreaks of acute gastroenteritis in nursing homes, as has been postulated. This was initiated by publications suggesting that such an association may exist [11–13, 21]. We did not find any evidence for such an association in our retrospective study. *C. difficile* PCR ribotype 001 was found in a single patient in one gastroenteritis outbreak of unexplained aetiology in a nursing home. This type is one of the most frequently circulating and detected types in the Netherlands.

However, our study was performed on outbreaks in nursing homes, while other studies describe hospital outbreaks, where CDIs are more common [3–5]. Wilcox and Fawley have shown that CDI rates are higher in closed hospital units which are affected by viral gastroenteritis than in open units where no viral gastroenteritis was detected [11]. Their results were not confirmed using *C. difficile*-specific assays, and our data suggest that false-positivity may explain previously noted associations between norovirus and *Clostridium*. We were unable to confirm the presence of *C. difficile* in all but one PTAB EIA-positive stool. This is not explained by their inability to culture, because other *Clostridium* species were isolated from 63.3% of all reactive samples.

Our study does have some limitations: first, stool samples had been sent by regular mail and stored at 4°C with a maximum of 16 months. This is in accordance with recommendations for the diagnostics of viral gastroenteritis, but is unusual for CDI tests. We did, however, find that the sensitivity of the PTAB EIA was not affected by storage of 5 months at 4°C relative to storage at −20°C. Furthermore, the testing of original total nucleic acids isolated from faecal samples within 3 days of arrival yielded the same positives as the combination of cytotoxicity test and VIDAS. Therefore, we conclude that our findings are not due to the degradation of *C. difficile* toxins [22, 23].

In addition, this was a retrospective study and stool samples were selected from patients with a suspected viral gastroenteritis [24]. Criteria for viral gastroenteritis differ from those for *Clostridium* gastroenteritis, since symptoms for CDIs are highly variable, ranging from mild diarrhoea to life-threatening colitis, and including watery diarrhoea [25].

Bignardi et al. noted that, if a large number of stool specimens are submitted for the testing of the prevalence of *C. difficile* during norovirus outbreaks, it is likely that the number of false-positive results will increase [12]. The *Clostridium* species found in this study, such as *C. disporicum*, *C. perfringens*, *C. sordellii*, *C. boltei*, *C. butyricum*, *C. baratii*, *C. subterminale* and *C. bifermentans*, are bacteria that are commonly found in the gut. These bacterial isolates did react weakly positive, using the cut-off values for the faecal material, by *C. difficile* PTAB EIA, confirming the specificity problem on stool samples stored for a long period at 4°C [26–31]. The PTAB EIA has a positive predictive value (PPV) of 87.4% according to the manufacturer's assessment when tested according to the manufacturer's recommendations. A prospective multicentre study using the cytotoxicity assay as a gold standard revealed a PPV value of 50.9% and a specificity of 94.3%, indicating that this assay is not highly specific when used for broader testing [14]. A recently performed meta-analysis by the European Study Group on *Clostridium difficile* (ESGCD) confirmed the PTAB to have an unacceptably low PPV of 50% at a prevalence rate of 5% [32].

A possible explanation for the high rate of false-positive PTAB results could be that suboptimal sample storage influenced the tests with weakly positive results just near to the cut-off values. Unfortunately, we were unable to test this hypothesis by a comparison of OD values of the false-positive samples with OD values of CDI-confirmed samples. Nonetheless, we recommend additional confirmatory testing, preferably a cell neutralisation cytotoxicity assay, which is the reference testing method for detection, or a molecular detection test including typing, specifically when samples are not sent in and stored according to *C. difficile* diagnostic criteria.

In summary, we found no evidence for an association between the spread of norovirus and *C. difficile*. Previous reports that suggested this association may be explained by false-positive PTAB tests.

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