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

S. Svraka, E. Kuijper, E. Duizer, D. Bakker, M. Koopmans

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

Article Type: Article

Keywords: viral gastroenteritis; norovirus; Clostridium difficile; nursing homes; the Netherlands; elderly

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Abstract: The coincidental increase in norovirus outbreaks and Clostridium difficile infection (CDI) raised the question if these events could be related, e.g. by enhancing spread through diarrhoeal disease outbreaks. Therefore, we studied the prevalence of C. difficile in outbreaks of viral gastroenteritis in nursing homes for elderly and characterized enzyme-immunoassay (EIA) positive stool samples.

Stool samples from nursing homes 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 neutralization cytotoxicity test, a second EIA and culture. Cultured isolates were tested for the presence of toxin genes, the production of toxins, and characterized by 16S rRNA 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 3 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 emphasizes the need for second confirmation tests to diagnose CDI.

Response to Reviewers: Prof. Dr. Alex van Belkum
Editor-in-Chief, European Journal of Clinical Microbiology & Infectious Diseases

Date: January 18th, 2010.

Subject: Re-submission of the manuscript EJCMIID-D-09-00481

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

Authors: Sanela Svraka, Ed Kuijper, Erwin Duizer, Dennis Bakker, Marion Koopmans

Dear Prof. Dr. Alex Van Belkum,

First of all, we do appreciate you considering the publication of our paper in the European Journal of Clinical Microbiology & Infectious Diseases.

In your reaction you advised us to revise our manuscript and to come up with convincing data on the adequacy of the procedure and resubmit before 23-01-2010. We have gone back to total nucleic acid extracts from the original stool samples, made within 3 days of arrival in the laboratory and stored at -80°C. These extracts were tested by PCR targeting the non-repeat region of the tcdB gene. Only the 2 samples that we identified as positives by VIDAS and cytotoxicity assay were PCR positive. We think this provides convincing evidence that the lack of confirmation of PTAB positivity is not due to storage losses. We have added this new data to the manuscript.

Please find in this letter the response to the questions and points raised by the reviewer. In the revised manuscript we have made the required changes (indicated in red). We hope that our comments meet your expectations and that the manuscript is now acceptable for publication in the European Journal of Clinical Microbiology & Infectious Diseases.

Response to: Clostridium difficile is not associated with outbreaks of viral gastroenteritis in elderly in the Netherlands (EJCMID-D-09-00481Version 1)

All points are addressed consecutively; in normal font is text from the reviewer, in bold font our response.

Yours sincerely,

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Reviewers' comments:

Reviewer #1: This is a well-written manuscript addressing an interesting question of whether there is an association between viral gastroenteritis and C.difficile infections.

Materials and Methods:

-Ideally, the study design would be a matched case-control study. A control group of non-ill patients would have been particularly useful if significant detection of C. difficile was shown to help distinguish from asymptomatic colonization. However, this was not the case and a convenience sample of stools was used.

In our study we strived to assess if the C. difficile infection was related to norovirus infection and if co-infections could contribute to the more serious illness. We decided to study patients from residential institutions because they are a high risk group for diarrheal diseases and rarely studied for the

infections with *C. difficile*, and year 2006 was chosen because of the increased reporting of *C. difficile* infections in hospitals in the Netherlands.

For that purpose we have included the desired control groups of ill patients who were not infected with noroviruses, but with rotaviruses or of unexplained etiologies (material and methods section, page 4, lines 85-100). A matched group of non-ill patients would complete the study, however this was a retrospective study on patients suspected of viral gastroenteritis from residential institutions for elderly. We were not able to obtain stool specimens from this group from the same period.

Furthermore, we should note that we investigated outbreaks of gastroenteritis, meaning that at least 2 patients which were linked in time and space and had two or more episodes of vomiting or diarrhea were reported and tested.

-The methods for viral detection were not described or adequately referenced.

We added the reference for detection of viral pathogens in gastroenteritis outbreaks that is used at our institute, we also briefly described the order of testing, see page 4, lines 88-92. We added "Viral detection as been performed as described previously by Svraka et al. [1], the outbreaks were firstly tested using the assays for noroviruses, all outbreaks that remained negative were further tested using the assays for rotaviruses, adenoviruses, astroviruses, and sapoviruses [1]."

-The major limitation of this study is the length of time between collection of stools and testing for *Cdiff* (up to 16 months). It is difficult to interpret the low detection of *Cdiff* in this cohort because there is no literature demonstrating that *Cdiff* toxins are stable for this long under these conditions. While the authors compared stools stored for 5 months at 4 deg vs -20 deg, the ideal comparison would have been stools stored at 4 deg for this duration of time vs. stools processed within 48 hours.

We agree that the ideal comparison would have been comparison of stools that are processed within 48 hours and those stored at 4 degrees, and that this meets clostridium criteria, however in this study we tested stool specimens sent for norovirus detection, and storage conditions for samples suspected of norovirus infections are set on 4°C. After performing diagnostic assays for viral pathogens causing gastroenteritis, we tested these samples for presence of *C. difficile*. Because of this we decided to split a cohort of obtained specimens, store them at 4°C and -20°C and to compare the results. Storage at 4°C did not lead to lower number of positive samples, moreover we detected three positive samples in stead of two that were found when stored at -20°C. To our knowledge clinical laboratories freeze stool samples prior to PTAB testing to prevent false negative results. In our study we are dealing with the false positive results obtained using the EIA, which, to our knowledge, has never been published. So, we were expecting that the sensitivity would be lower, but we did not expect that the specificity would change. Furthermore, Borriello et al. (Evaluation of a Commercial enzyme immunoassay kit for the detection of *C. difficile* toxin A, Eur. J. Clin. Microbiol. Infect. Dis Vol. 11, 1992) and J. Freeman and M.H. Wilcox (The effects of storage conditions on viability of *C. difficile* vegetative cells and spores and toxin activity in human feces, J Clin Path 2003 (56): 126-128) described that the storage for respectively 44 and 56 days at 4°C had no discernible effect on *C. difficile* cytotoxin.

Given the concerns raised in your review, we decided to do an additional experiment. We retracted stored total nucleic acids extracted from the fecal samples on the day (up to three days) of arrival of the stool samples which were included in our study and tested them by a diagnostic real time PCR for *Clostridium difficile*. We used a real-time PCR as described by van den Berg et al. (van den Berg, R. J., Kuijper, E. J., van Coppenraet, L. E. & Claas, E. C. J. (2006). Rapid diagnosis of toxinogenic *Clostridium difficile* in faecal samples with internally controlled real-time PCR. Clin Microbiol Infect 12, 184-186.) We tested 154 fecal specimens (20% of all samples tested in EIA) that were isolated within three days of arrival, only two of these samples were positive (table I samples 13/8 and 23/18), these samples were found positive in used assays. All other samples were negative.

Therefore, we believe that data we present are true and have added this data to the manuscripts as follows:

to sections material and methods (Lines 158-165 we added

"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 EIA) fecal samples within three 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 TcdB gene as described by van den Berg et al. [14, 20]."), results (lines 218-222 we added

"Evaluation of the approach of Clostridium detection

Of the 154 total nucleic acids that had been isolated from stool samples within three days of the arrival, the two samples that were cytotoxic and VIDAS positive were positive by tcdB real-time PCR assay (Table I samples 13/8 and 23/18). All other samples were negative."), and discussion (lines 248-252 we added "Furthermore, testing of original total nucleic acids isolated from fecal samples within three 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 degradation of C. difficile toxins [23, 24].")

Results:

-Again, it is difficult to interpret the results because the toxin may have degraded under the storage conditions for such a prolonged period of time.

See comment mentioned above. We did not find any samples positive in the real-time PCR that were negative when EIA was used, therefore we strongly believe that data we present are true.

In result section lines 218-222 we added

"Evaluation of the approach of Clostridium detection

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

-Can the authors explain why a number of the cultured bacteria had a positive VIDAS test when the stools were negative for Cdiff by VIDAS?

Positive VIDAS test of the cultured bacteria while stools are negative for C. difficile can be explained by used cut off values. We applied same cut off values of VIDAS for in vitro bacterial toxin production as for detection in stool samples (as stated in material and methods section, page 7 line 153-158) while the cut off values probably need to have other (probably higher) cut off value. Moreover, cut off values as presented by the manufacturer are evaluated for the stool specimens and not for cultured bacteria.

Discussion:

-The last concluding sentence probably needs to be rephrased because the limitations of the methods are so substantial. The title also needs to be revised because it is misleading since the data are so difficult to interpret.

Based on the additional data provided here, we think that our conclusions are valid. Therefore we have not modified the title.

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

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7 4 **Sanela Svraka¹, Ed Kuijper², Erwin Duizer¹, Dennis Bakker², Marion**
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31 14 Running title: *C. difficile* and viral gastroenteritis outbreaks are not associated

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27 **Abstract**

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3 28 The coincidental increase in norovirus outbreaks and *Clostridium difficile*
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5 29 infection (CDI) raised the question if these events could be related, e.g. by enhancing
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7 30 spread through by diarrhoeal disease outbreaks. Therefore, we studied the prevalence
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9 31 of *C. difficile* in outbreaks of viral gastroenteritis in nursing homes for elderly and
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12 32 characterized enzyme-immunoassay (EIA) positive stool samples.
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14 33 Stool samples from nursing homes residents (n=752) in 137 outbreaks
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17 34 of viral aetiology were investigated by EIA for the presence of *C. difficile*
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19 35 toxins. Positive samples were further tested by a cell neutralization cytotoxicity
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24 37 toxin genes, the production of toxins, and characterized by 16S rRNA PCR and
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41 44 association between viral gastroenteritis outbreaks and *C. difficile*. The high rate of
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43 45 false positive EIA samples emphasizes the need for second confirmation tests to
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45 46 diagnose CDI.
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48 47 Key words: viral gastroenteritis, norovirus, *Clostridium difficile*, nursing
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50 Introduction

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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.

Since 2002, the epidemiology of noroviruses (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 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 10 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 recognized 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 at least in 10 nursing homes in the Netherlands, detailed information about these infections is absent [6].

74 The coincidental increase in norovirus GII.4 outbreaks and spread of *C.*
75 *difficile* ribotype 027 raised the question if these events could be related [2, 10]. In
76 theory, mixed infections of noroviruses and *Clostridium difficile* 027 could increase
77 the severity of the disease. Furthermore, norovirus induced episodes of vomiting and
78 diarrhoea might trigger or exacerbate *C. difficile* diarrhoea resulting in increased
79 spread of *C. difficile* due to multiple episodes of vomiting and diarrhoea as has been
80 suggested in some recent publications [11-13]. Therefore, we decided to investigate a
81 possible association of norovirus and rotavirus outbreaks with *C. difficile* infections,
82 particularly with respect to *C. difficile* PCR ribotype 027.

83

84 **Material and methods**

85 *Sample collection*

86 In total, we tested 752 faecal samples from 137 outbreaks of gastroenteritis
87 (number of stool samples varying from 2 to 25 per outbreak). All outbreaks occurred
88 in 2006 in nursing homes and were selected because the *Clostridium difficile* 027
89 problem emerged in that period in the Netherlands and these samples provided a
90 snapshot of what is present within elderly population. **Viral detection as been**
91 **performed as described previously by Svraka et al. [1], the outbreaks were firstly**
92 **tested using the assays for noroviruses, all outbreaks that remained negative were**
93 **further tested using the assays for rotaviruses, adenoviruses, astroviruses, and**
94 **sapoviruses [1].** Of these, 109 were confirmed norovirus outbreaks (644 stool
95 samples), 14 outbreaks were confirmed rotavirus outbreaks (70 samples), and 14
96 outbreaks (38 samples) were of unexplained aetiology, reflecting the typical aetiology
97 of such outbreaks. The outbreaks were reported throughout the year, but with a peak
98 in winter as part of our ongoing surveillance for viral gastroenteritis outbreaks, which

1 99 explains the high number of NoV outbreaks in this study [1]. Undiluted stool samples
2 100 had been stored at 4°C, as recommended for viral gastroenteritis diarrhoea, before
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4 101 testing for presence of *C. difficile*.

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7 102 *Diagnostic assays used for screening of samples*

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9 103 Screening of 752 outbreak specimens for the presence of *Clostridium difficile*
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11 104 was done using PTAB enzyme immunoassay (EIA, Premier *C. difficile* Toxin A&B,
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13 105 Meridian Diagnostics, Inc., Cincinnati, Ohio) on all stool samples. Assay was
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15 106 performed as described by manufacturer. Stool samples had been stored at 4°C for
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17 107 periods longer than 3-4 days, which is in accordance with recommendations for
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19 108 diagnostics of viral gastroenteritis, but not in accordance to the manufacturer's
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21 109 recommendations, before testing for presence of *C. difficile*. Consequently, we
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23 110 validated the PTAB EIA assay for use in samples that were stored longer at 4°C by
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25 111 parallel testing of 96 stool samples that were aliquoted directly after receipt and stored
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27 112 for five months at -20°C and 4°C.

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29 113 The PTAB EIA was interpreted visually (within 15 minutes) and by
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31 114 spectrophotometer (within 30 minutes), as described by the manufacturer; 376
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33 115 samples were read both visually and on a Labsystems Multiskan RC
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35 116 spectrophotometer plate reader (Thermo Scientific, Breda, the Netherlands) using dual
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37 117 wavelengths of 450 nm and 450/630 nm. The samples were described as positive if
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39 118 optical density (OD) values of 0.100 or more were measured at wavelength of
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41 119 450/630 nm and/or 0.150 or more at wavelength of 450 nm, as described by
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43 120 manufacturer. There was 100% concordance between reading visually and by
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45 121 spectrophotometer plate reader. The remaining 376 samples were read visually only.

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47 122 *Confirmatory testing on PTAB positive samples*
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123 Stool samples found positive using PTAB EIA assay were cultured, and tested
124 using neutralization cytotoxicity assay, and automated immunoanalyzer VIDAS®
125 *Clostridium difficile* A & B (BioMerieux) [14].

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

132 *Clostridium* suspected colonies were tested further using PCR assays for *C. difficile*-
133 specific GluD targeting GDH gene [15], *C. difficile*-ribotyping [16, 17], enterotoxin A
134 (tcdA) [5], cytotoxin B (tcdB) [18], and 16S rRNA PCR [19]. For this, DNA was
135 isolated from suspected colonies by QiaAMP DNA isolation column (Qiagen,
136 Holden, Germany) according to manufacturers recommendations, including 10
137 minutes incubation at 55°C with proteinase K. Final volume of the DNA extracts was
138 200 µL.

139 The neutralization cytotoxicity assay was performed using Vero cells in a 24-
140 well format. Faecal samples were diluted in 1:4 Eagle's minimum essential medium
141 (EMEM) containing 5% fetal bovine serum and centrifuged for 10 minutes at 3.000
142 rpm. The supernatant was filtered through a 0.45 µm pore-filter, 150 µL supernatant
143 was mixed with 150 µl EMEM and neutralization of the cytotoxic effect was
144 performed using 150 µL faecal supernatant and 150 µl of 1:25 dilution of specific *C.*
145 *difficile* antitoxin (Techlab, Blacksburg, USA). These two mixtures were incubated
146 for 30 minutes at room temperature; subsequently 200 µl of each mix was inoculated

147 on Vero cells. The cytotoxic effect was evaluated daily for three days

148 microscopically.

149 Stool samples positive in PTAB EIA were also tested using VIDAS assay.

150 Stool samples were centrifuged for 10 minutes at maximum speed (14.000 rpm) and

151 300 µl of supernatant was used, according to manufacturer's instructions. Samples

152 with OD value lower than 0.13 were described as negative, OD values between 0.13

153 and 0.37 were equivocal, and OD values higher than 0.37 were described as positive.

154 Bacterial isolates cultured from EIA positive faeces samples were also

155 investigated for the production of TcdA and TcdB by VIDAS and EIA. Bacteria were

156 cultured in liquid brain heart infusion (BHI) bouillon medium for four days.

157 Subsequently, the BHI cultures were centrifuged at 3.000 rpm for 10 minutes and

158 supernatant was further investigated. Same cut-off values of EIA and VIDAS were

159 used for in vitro bacterial toxin production as for toxin detection in faeces samples.

160 *Evaluation of the approach of Clostridium detection*

161 *Optimal sample storage conditions differ for viral testing and bacterial testing, and*

162 *this raised the question whether Clostridium EIA test results could be explained by*

163 *suboptimal use of the assay. Therefore we validated this approach by testing original*

164 *total nucleic acids isolated from 154 (20% of all samples tested in EIA) fecal samples*

165 *within three days of arrival from the stool samples which were included in our study.*

166 *These extracts had been stored at -80°C. We used a real-time PCR for TcdB gene as*

167 *described by van den Berg et al. [14, 20]*

168

169 **Results**

170 *Testing of stool samples using PTAB EIA*

171 For the validation of the use of PTAB EIA on our sample set, we used 96 stool
172 samples that were stored for five months at -20°C and at 4°C. Of the samples stored at
173 -20°C two were positive and of the samples stored at 4°C three were positive:
174 including the ones that were positive after storage at -20°C.

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

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181 *Confirmatory testing of PTAB positive stool samples*

182 Specialized stool culture for *Clostridium* spp. (Table 1) was performed on 24
183 stool samples. For three stool samples culture was negative, and in 21 stool samples
184 *Clostridium* suspected colonies were found. Subsequently, neutralization cytotoxicity
185 assay, and VIDAS assay were performed on 21 stool specimens of whom sufficient
186 amounts of material were available. This yielded two positive samples both by
187 neutralization cytotoxicity assay and VIDAS, and an additional weak positive using
188 VIDAS assay (Table 1).

189

190 *Analysing of bacterial isolates from the stool samples*

191 From the 21 *Clostridium* suspected colonies, which were grown on the CLO
192 medium plates, DNA was extracted and analysed by 16S DNA sequencing and further
193 using a range of assays for characterization of toxin producing *Clostridium difficile*
194 (Table 1). 16S DNA sequencing revealed *Lactobacilli* in six stool samples (37.5%),
195 and different *Clostridium* species in 15 samples (62.5%): *C. difficile*, *C. disporicum*,

196 *C. perfringens*, *C. sordellii*, *C. boltei*, *C. butyricum*, *C. barati*, *C. subterminale* and *C.*
197 *bifermentans* (Table 1). Using the GluD, ribotyping PCR, tcdA and tcdB assays, one
198 sample was positive, and identified as *C. difficile*. One isolate of *C. dispersicum* was
199 weak positive for tcdA and one isolate of *C. subterminale* for tcdB.

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

203 The results of in-vitro bacterial toxin production measured by cytotoxicity,
204 PTAB and VIDAS revealed only strong positive results for one sample that was
205 identified as *C. difficile*. Using cut-off values of PTAB and VIDAS as applied on
206 stool samples, (weak) positive reactions were obtained for 14 samples using PTAB
207 and 6 samples using VIDAS assay. However, none of these isolates produced
208 cytotoxins.

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

210 Only one stool sample and the corresponding bacterial isolate were positive in
211 all assays and proven to be *C. difficile* type 001 (23/18, Table 1). This sample was
212 from an outbreak of unexplained aetiology that occurred in November 2006, with
213 eight cases reported. All eight samples were tested using PTAB EIA, however only
214 one was positive and tested further in other assays.

215 The *C. sordellii* positive stool sample (13/8, Table 1) was from a norovirus
216 outbreak that affected four persons from a residential institution in an endemic region
217 for *C. difficile* in November 2006. The faeces samples of 3 other patients were tested
218 for CDI, but were negative.

219 *Evaluation of the approach of Clostridium detection*

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

225

226 Discussion

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

236 However, our study was performed on outbreaks in nursing homes, while
237 other studies describe hospital outbreaks where *C. difficile* infections are more
238 common [3-5]. Wilcox and co-workers have shown that *C. difficile* infection rates are
239 higher in closed hospital units which are affected by viral gastroenteritis than in open
240 units where no viral gastroenteritis was detected [11]. Results were not confirmed
241 using *C. difficile* specific assays, and our data suggest that false positivity may explain
242 previously noted association between norovirus and *Clostridium*. We were unable to
243 confirm the presence of *C. difficile* in all but one PTAB EIA positive stool. This is not
244 explained by inability to culture, because other *Clostridium* species were isolated
245 from 63.3% of all reactive samples.

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

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

260 Bignardi et al. noted that if a large number of stool specimens are submitted
261 for testing of prevalence of *C. difficile* during norovirus outbreaks, it is likely that the
262 number of false positive results will increase [12]. The *Clostridium* species, such as *C.*
263 *disporicum*, *C. perfringens*, *C. sordellii*, *C. boltei*, *C. butyricum*, *C. barati*, *C.*
264 *subterminale* and *C. bifermentans*, found in this study are bacteria that are commonly
265 found in the gut. These bacterial isolates did react weakly positive, using the cut-off
266 values for the faecal material, by *C. difficile* PTAB EIA confirming the specificity
267 problem on stool samples stored for a long period at 4°C [26-31]. The Premier *C.*
268 *difficile* Toxin A & B EIA assay has a positive predictive value (PPV) of 87.4%
269 according to manufacturer's assessment when tested according to manufacturer's
270 recommendations. A prospective multicenter study using the cytotoxicity assay as a

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271 gold standard, revealed a PPV value of 50.9% and specificity of 94.3%, indicating
272 that this assay is not highly specific when used for broader testing [14]. A recently
273 performed meta-analysis by the European Study group of *Clostridium difficile*
274 confirmed the PTAB to have an unacceptable low PPV of 50% at a prevalence rate of
275 5% [32].

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

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

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392 Table I. Results on stool samples and characterization of the isolates from the stool samples. These results are obtained for stool samples positive
 393 in PTAB EIA and tested further using neutralization cytotoxicity, VIDAS, and culture assays. OD values of VIDAS assay are given between
 394 brackets. Results on the isolates cultured from the stool samples by PCR assays for GluD, ribotyping, tcdA, tcdB, and 16S rRNA and
 395 sequencing. Additionally, grown cultures were tested using neutralization cytotoxicity assay, PTAB EIA, and VIDAS. OD values of VIDAS and
 396 PTAB EIA (measured at 450 nm) are given between 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]	+	<i>Lactobacillus casei</i>	-	-	-	-	- [0.121]	- [0.03]
2/2	Norovirus (II.4 2004)	ND	ND	+	<i>Clostridium disporicum</i>	-	-	-	-	+ [0.324]	+ [0.44]
3/2	Norovirus (II.4 2004)	-	- [0,02]	+	<i>Clostridium perfringens/Clostridium disporicum</i>	-	-	-	-	+ [0.470]	+ [1.12]
4/2	Norovirus (II.4 2004)	-	- [0,03]	+	<i>Clostridium disporicum</i>	-	+	-	-	+ [0.427]	+ [0.62]
5/2	Norovirus (II.4 2004)	-	- [0,10]	+	<i>Clostridium disporicum</i>	-	-	-	-	+ [0.342]	+ [0.61]
6/2	Norovirus (II.4 2004)	-	- [0,03]	+	<i>Clostridium subterminale</i>	-	-	+	-	+ [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	+	<i>Clostridium boltei</i>	-	-	-	-	+ [0.440]	+ [0.48]
9/4	Norovirus (II.4 2004)	-	- [0,03]	+	<i>Clostridium butyricum</i>	-	-	-	-	+ [0.179]	- [0.02]
10/5	Norovirus (II.4 2004)	-	- [0,13]	+	<i>Clostridium bifementans</i>	-	-	-	-	+ [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]	+	<i>Clostridium disporicum</i>	-	-	-	-	+ [0.208]	- [0.02]
13/8	Norovirus (II.4 2006a)	+	+ [2,32]	+	<i>Clostridium sordellii</i>	-	-	-	-	+ [0.178]	- [0.02]
14/9	Norovirus (II.4 2006a)	-	- [0,04]	+	<i>Clostridium barati</i>	-	-	-	-	+ [0.190]	- [0.09]
15/10	Norovirus (II.4 2006b)	-	- [0,03]	+	<i>Lactobacillus</i>	-	-	-	-	- [0.069]	- [0.04]
16/11	Norovirus (II.4 2006a)	-	- [0,01]	+	<i>Lactobacillus</i>	-	-	-	-	- [0.100]	- [0.05]
17/12	Norovirus (II.4 2004)	-	- [0,07]	+	<i>Clostridium perfringensfringes</i>	-	-	-	-	+ [0.165]	- [0.04]

18/13	Norovirus (I.2)	-	- [0,01]	+	<i>Lactobacillus paracasei</i>	-	-	-	-	- [0.120]	- [0.02]
19/14	Norovirus (II.4 2006a)	-	- [0,01]	+	<i>Clostridium disporicum</i>	-	-	-	-	+ [0.169]	- [0.05]
20/15	Norovirus (II.4 2004)	-	+ [0,55]	+	<i>Lactobacillus paracasei</i>	-	-	-	-	- [0.125]	- [0.04]
21/16	Norovirus (II.4 2006a)	-	- [0,01]	-	ND	ND	ND	ND	ND	ND	ND
22/17	Norovirus (II.4 2006a)	-	- [0,04]	+	<i>Lactobacillus casei</i>	-	-	-	-	- [0.069]	- [0.03]
23/18	Unexplained etiology	+	+ [6.49]	+	<i>Clostridium difficile type 001</i>	+	+	+	+	+ [3.137]	+ [4.24]
24/19	Rotavirus	ND	ND	+	<i>Clostridium disporicum</i>	-	-	-	-	+ [0.196]	- [0.07]

398 -: negative

399 +: positive

400 ND: not done

401 OD: Optical Density

402 VIDAS cut off OD values: Negative <0.13 Equivocal >= 0.13 to <0.37 Positive >= 0.37

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

404 PTAB cut off OD values: Negative < 0.150 Positive >0.150

405 GluD: glutamate dehydrogenase gene

406 tcdA: enterotoxin A gene

407 tcdB: cytotoxin B gene

408

Prof. Dr. Alex van Belkum
Editor-in-Chief, European Journal of Clinical Microbiology & Infectious Diseases

Date: January 18th, 2010.
Subject: Re-submission of the manuscript EJCMID-D-09-00481
Title: Clostridium difficile is not associated with outbreaks of viral gastroenteritis in elderly in the Netherlands

Authors: Sanela Svraka, Ed Kuijper, Erwin Duizer, Dennis Bakker, Marion Koopmans

Dear Prof. Dr. Alex Van Belkum,

First of all, we do appreciate you considering the publication of our paper in the European Journal of Clinical Microbiology & Infectious Diseases.

In your reaction you advised us to revise our manuscript and to come up with convincing data on the adequacy of the procedure and resubmit before 23-01-2010. We have gone back to total nucleic acid extracts from the original stool samples, made within 3 days of arrival in the laboratory and stored at -80°C. These extracts were tested by PCR targeting the non-repeat region of the tcdB gene. Only the 2 samples that we identified as positives by VIDAS and cytotoxicity assay were PCR positive. We think this provides convincing evidence that the lack of confirmation of PTAB positivity is not due to storage losses. We have added this new data to the manuscript.

Please find in this letter the response to the questions and points raised by the reviewer. In the revised manuscript we have made the required changes (indicated in red). We hope that our comments meet your expectations and that the manuscript is now acceptable for publication in the European Journal of Clinical Microbiology & Infectious Diseases.

Response to: Clostridium difficile is not associated with outbreaks of viral gastroenteritis in elderly in the Netherlands (EJCMID-D-09-00481 Version 1)

All points are addressed consecutively; in normal font is text from the reviewer, in **bold** font our response.

Yours sincerely,

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Reviewers' comments:

Reviewer #1: This is a well-written manuscript addressing an interesting question of whether there is an association between viral gastroenteritis and C.difficile infections.

Materials and Methods:

-Ideally, the study design would be a matched case-control study. A control group of non-ill patients would have been particularly useful if significant detection of C. difficile was shown to help distinguish from asymptomatic colonization. However, this was not the case and a convenience sample of stools was used.

In our study we strived to assess if the C. difficile infection was related to norovirus infection and if co-infections could contribute to the more serious illness. We decided to study patients from residential institutions because they are a high risk group for diarrheal diseases and rarely studied for the infections with C. difficile, and year 2006 was chosen because of the increased reporting of C. difficile infections in hospitals in the Netherlands.

For that purpose we have included the desired control groups of ill patients who were not infected with noroviruses, but with rotaviruses or of unexplained etiologies (material and methods section, page 4, lines 85-100). A matched group of non-ill patients would complete the study, however this was a retrospective study on patients suspected of viral gastroenteritis from residential institutions for elderly. We were not able to obtain stool specimens from this group from the same period. Furthermore, we should note that we investigated outbreaks of gastroenteritis, meaning that at least 2 patients which were linked in time and space and had two or more episodes of vomiting or diarrhea were reported and tested.

-The methods for viral detection were not described or adequately referenced.

We added the reference for detection of viral pathogens in gastroenteritis outbreaks that is used at our institute, we also briefly described the order of testing, see page 4, lines 88-92. We added “Viral detection as been performed as described previously by Svraka et al. [1], the outbreaks were firstly tested using the assays for noroviruses, all outbreaks that remained negative were further tested using the assays for rotaviruses, adenoviruses, astroviruses, and sapoviruses [1].”

-The major limitation of this study is the length of time between collection of stools and testing for Cdiff (up to 16 months). It is difficult to interpret the low detection of Cdiff in this cohort because there is no literature demonstrating that Cdiff toxins are stable for this long under these conditions. While the authors compared stools stored for 5 months at 4 deg vs -20 deg, the ideal comparison would have been stools stored at 4 deg for this duration of time vs. stools processed within 48 hours.

We agree that the ideal comparison would have been comparison of stools that are processed within 48 hours and those stored at 4 degrees, and that this meets clostridium criteria, however in this study we tested stool specimens sent for norovirus detection, and storage conditions for samples suspected of norovirus infections are set on 4°C. After performing diagnostic assays for viral pathogens causing gastroenteritis, we tested these samples for presence of *C. difficile*. Because of this we decided to split a cohort of obtained specimens, store them at 4°C and -20°C and to compare the results. Storage at 4°C did not lead to lower number of positive samples, moreover we detected three positive samples in stead of two that were found when stored at -20°C. To our knowledge clinical laboratories freeze stool samples prior to PTAB testing to prevent false negative results. In our study we are dealing with the false positive results obtained using the EIA, which, to our knowledge, has never been published. So, we were expecting that the sensitivity would be lower, but we did not expect that the specificity would change. Furthermore, Borriello et al. (Evaluation of a Commercial enzyme immunoassay kit for the detection of *C. difficile* toxin A, Eur. J. Clin. Microbiol. Infect. Dis Vol. 11, 1992) and J. Freeman and M.H. Wilcox (The effects of storage conditions on viability of *C. difficile* vegetative cells and spores and toxin activity in human feces, J Clin Path 2003 (56): 126-128) described that the storage for respectively 44 and 56 days at 4°C had no discernible effect on *C. difficile* cytotoxin.

Given the concerns raised in your review, we decided to do an additional experiment. We retracted stored total nucleic acids extracted from the fecal samples on the day (up to three days) of arrival of the stool samples which were included in our study and tested them by a diagnostic real time PCR for *Clostridium difficile*. We used a real-time PCR as described by van den Berg et al. (van den Berg, R. J., Kuijper, E. J., van Coppenraet, L. E. & Claas, E. C. J. (2006). Rapid diagnosis of toxinogenic *Clostridium difficile* in faecal samples with internally controlled real-time PCR. Clin Microbiol Infect 12, 184–186.) We tested 154 fecal specimens (20% of all samples tested in EIA) that were isolated within three days of arrival, only two of these samples were positive (table I samples 13/8 and 23/18), these samples were found positive in used assays. All other samples were negative.

Therefore, we believe that data we present are true and have added this data to the manuscripts as follows:

to sections material and methods (Lines 158-165 we added

“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 EIA) fecal samples within three 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 TcdB gene as described by van den Berg et al. [14, 20].”), results (lines 218-222 we added

“Evaluation of the approach of Clostridium detection

Of the 154 total nucleic acids that had been isolated from stool samples within three days of the arrival, the two samples that were cytotoxic and VIDAS positive were positive by tcdB real-time PCR assay (Table I samples 13/8 and 23/18). All other samples were negative.”), and discussion (lines 248-252 we added “Furthermore, testing of original total nucleic acids isolated from fecal samples within three 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 degradation of *C. difficile* toxins [23, 24].”)

Results:

-Again, it is difficult to interpret the results because the toxin may have degraded under the storage conditions for such a prolonged period of time.

See comment mentioned above. We did not find any samples positive in the real-time PCR that were negative when EIA was used, therefore we strongly believe that data we present are true.

In result section lines 218-222 we added

“Evaluation of the approach of Clostridium detection

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

-Can the authors explain why a number of the cultured bacteria had a positive VIDAS test when the stools were negative for Cdiff by VIDAS?

Positive VIDAS test of the cultured bacteria while stools are negative for *C. difficile* can be explained by used cut off values. We applied same cut off values of VIDAS for in vitro bacterial toxin production as for detection in stool samples (as stated in material and methods section, page 7 line 153-158) while the cut off values probably need to have other (probably higher) cut off value. Moreover, cut off values as presented by the manufacturer are evaluated for the stool specimens and not for cultured bacteria.

Discussion:

-The last concluding sentence probably needs to be rephrased because the limitations of the methods are so substantial. The title also needs to be revised because it is misleading since the data are so difficult to interpret.

Based on the additional data provided here, we think that our conclusions are valid. Therefore we have not modified the title.