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 if these events could be related, e.g. by enhancing spread through by 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 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-09-00481Version 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 diarreal 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 4ºC. 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. Infct. 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 4ºC 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.

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

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Running title: *C. difficile* and viral gastroenteritis outbreaks are not associated

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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 by 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.

Key words: viral gastroenteritis, norovirus, *Clostridium difficile*, nursing homes, the Netherlands, elderly

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.

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].

The coincidental increase in norovirus GII.4 outbreaks and spread of *C. difficile* ribotype 027 raised the question if these events could be related [2, 10]. In theory, mixed infections of noroviruses and *Clostridium difficile* 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, particularly with respect to *C. difficile* PCR ribotype 027.

Material 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 *Clostridium difficile* 027 problem emerged in that period in the Netherlands and these samples provided a snapshot of what is present within elderly population. 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]. 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 4ºC, as recommended for viral gastroenteritis diarrhoea, before testing for presence of *C. difficile.*

Diagnostic assays used for screening of samples

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

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

Confirmatory testing on PTAB positive samples

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

For culture, stool samples found positive in 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; bioMerieux), 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 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 QiaAMP DNA isolation column (Qiagen, Holden, Germany) according to manufacturers recommendations, including 10 minutes incubation at 55°C with proteinase K. Final volume of the DNA extracts was 200 µL.

The neutralization 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% fetal bovine serum and centrifuged for 10 minutes 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 neutralization 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, USA). These two mixtures were incubated for 30 minutes at room temperature; subsequently 200 µl of each mix was inoculated

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

Stool samples positive in PTAB EIA were also tested using VIDAS assay. Stool samples were centrifuged for 10 minutes at maximum speed (14.000 rpm) and 300 µl of supernatant was used, according to 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 four days. Subsequently, the BHI cultures were centrifuged at 3.000 rpm for 10 minutes and supernatant was further investigated. 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 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

Testing of stool samples using PTAB EIA

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

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

Specialized 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, neutralization cytotoxicity assay, and VIDAS assay were performed on 21 stool specimens of whom sufficient amounts of material were available. This yielded two positive samples both by neutralization cytotoxicity assay and VIDAS, and an additional weak positive using VIDAS assay (Table 1).

Analysing 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 characterization of toxin producing *Clostridium 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. barati*, *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 weak positive for tcdA and one isolate of *C. subterminal*e for tcdB.

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

The results of in-vitro bacterial toxin production measured by cytotoxicity, PTAB and VIDAS revealed only strong positive results for one sample that was identified as *C. difficile.* Using cut-off values of PTAB and VIDAS as applied on stool samples, (weak) positive reactions were obtained for 14 samples using PTAB and 6 samples using 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 reported. All eight samples were tested using 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 3 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 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.

Discussion

In this study we investigated whether the emergence of a successful norovirus strain [10] could coincides 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 *C. difficile* infections are more common [3-5]. Wilcox and co-workers have shown that *C. difficile* infection rates are higher in closed hospital units which are affected by viral gastroenteritis than in open units where no viral gastroenteritis was detected [11]. Results were not confirmed using *C. difficile* specific assays, and our data suggest that false positivity may explain previously noted association 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 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 4ºC with a maximum of 16 months. This is in accordance with recommendations for diagnostics of viral gastroenteritis, but 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, 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 [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 *C. difficile* infections 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 testing of prevalence of *C. difficile* during norovirus outbreaks, it is likely that the number of false positive results will increase [12]. The *Clostridium* species, such as *C. disporicum, C. perfringens, C. sordellii, C. boltei, C. butyricum, C. barati, C. subterminale* and *C. bifermentans,* found in this study 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 Premier *C. difficile* Toxin A & B EIA assay has a positive predictive value (PPV) of 87.4% according to manufacturer's assessment when tested according to manufacturer's recommendations. A prospective multicenter study using the cytotoxicity assay as a

gold standard, revealed a PPV value of 50.9% and 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 of *Clostridium difficile* confirmed the PTAB to have an unacceptable 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 weak positive results just nearby 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 neutralization cytotoxicity assay, which is 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 spread of norovirus and *Clostridium difficile*. Previous reports that suggested this association may be explained by false positive PTAB tests.

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Table I. Results on stool samples and characterization of the isolates from the stool samples. These results are obtained for stool samples positive in PTAB EIA and tested further using neutralization cytotoxicity, VIDAS, and culture assays. OD values of VIDAS assay are given between brackets. Results on the isolates cultured from the stool samples by PCR assays for GluD, ribotyping, tcdA, tcdB, and 16S rRNA and sequencing. Additionally, grown cultures were tested using neutralization cytotoxicity assay, PTAB EIA, and VIDAS. OD values of VIDAS and 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]	+	*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 2004)	-	- [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 barati*	-	-	-	-	+ [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)	-	- [0,01]	+	*Clostridium disporicum*	-	-	-	-	+ [0.169]	- [0.05]
20/15	Norovirus (II.4 2004)	-	+ [0,55]	+	*Lactobacillus paracasei*	-	-	-	-	- [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]	+	*Lactobacillus casei*	-	-	-	-	- [0.069]	- [0.03]
23/18	Unexplained etiology	+	+ [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

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-09-00481Version 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 diarreal 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 4ºC. 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. Infct. 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 4ºC 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.