



Quantitative detection of *Clostridium difficile* in hospital environmental samples by real-time polymerase chain reaction

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Summary *C. difficile*-associated diarrhoea occurs commonly in hospitals and is a significant cause of morbidity and mortality. Hospital surfaces are often contaminated with nosocomial pathogens and may be responsible for cross-transmission, especially if hardy Gram-positive and spore-forming organisms are involved. The aim of this study was to quantify *C. difficile* in the hospital environment near *C. difficile*-positive and -negative patients using a quantitative real-time polymerase chain reaction. A total of 531 samples was collected from the clinical environment and classified into three groups according to patient and ward status for *C. difficile*. As expected, there were significantly higher counts of *C. difficile* on the floor and in the near environment of *C. difficile* patients. However, a significant correlation was found between *C. difficile* counts on the floor and on the hands of patients and health-care workers (HCWs) in wards without evidence of *C. difficile*. This suggests that asymptomatic carriage among patients and HCWs can also contribute towards *C. difficile* transmission in hospitals. In conclusion, *C. difficile* can be transmitted via personal contact or via contaminated areas of the hospital environment.

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Introduction

Clostridium difficile is considered an important aetiological agent of antibiotic-associated diarrhoea (CDAD) and plays an important role in hospital-acquired diarrhoea.^{1–4} The main routes of transmission causing spread of the bacteria among hospitalised patients are the faecal–oral route or aerosols.^{1,5,6} Infected persons with acute diarrhoea can excrete 10⁷ to 10⁹ micro-organisms per gram of faeces leading to heavy contamination of the environment with spores. Asymptomatic patients can be carriers and contribute to environmental contamination and may be responsible for some endemic disease in hospitals.^{7,8}

C. difficile spores are a major concern for nosocomial infections. Spores can persist in dust or on surfaces for months and be transmitted to other patients or to healthcare workers (HCWs). Studies have shown that the occurrence of *C. difficile* spores may range between 10% and 15% in areas with infected patients.⁷ The spores cannot be destroyed by usual detergents and survive hand disinfection with alcoholic preparations.⁹ Therefore, it is important to establish effective infection control strategies and provide HCW training to avoid the spread of the pathogen.

Several studies have investigated environmental contamination around patients with diarrhoea and *C. difficile* using polymerase chain reaction (PCR).^{10–13} However, to the best of our knowledge this is the first study to report the development of a real-time PCR method to verify *C. difficile* contamination in a large hospital setting. The objective of the present study was to compare *C. difficile* counts on environmental surfaces potentially contaminated by *C. difficile* spores with areas which supposedly should be free of contamination.

Methods

The study was designed as an observational study. The main outcome measure was the overall occurrence of *C. difficile* in the environment around *C. difficile*-positive patients and *C. difficile*-negative patients.

Sample collection and preparation

The study was conducted at the University Hospital of Philipps-University, Marburg, Germany. This is a large regional hospital with 1300 beds and all major specialities. In total 531 environmental samples were collected and classified into three

groups according to patient and ward status for *C. difficile*:

- 1 *C. difficile* positive, ward positive: samples were obtained from the environment of hospitalised patients with *C. difficile* diarrhoea. The faecal samples were confirmed toxin positive by the Institute of Medical Microbiology using an enzyme immunoassay test (Ridascreen® *Clostridium difficile* Toxin A/B, R-Biopharm Rhône Ltd, St Didier au Mont d'or, France).
- 2 *C. difficile* negative, ward positive: samples were obtained from the environment of hospitalised patients negative for *C. difficile* and with formed stools, but present on the same ward with *C. difficile*-positive patients.
- 3 *C. difficile* negative, ward negative: samples were obtained from the environment of hospitalised patients not carrying *C. difficile* and from a ward with no *C. difficile*-positive patients for at least six months.

The environmental samples were collected using flocked swabs (eSwab®, Copan, Brescia, Italy) from different surfaces, including the hands of patients and HCWs, toilets, beds and other surfaces in the periphery of *C. difficile*-positive and -negative patients. Before sampling, the flocked swabs were wiped with sterile physiological saline. For most of the surfaces an area of about 10 × 10 cm was estimated and samples were collected by the swab method. For door handles the entire surface was swabbed. Samples from the palm of the hand were collected from patients and (the dominant hand of) the HCWs. The samples were divided into five different categories: the living environment, which included the two categories of patient and the hands of the HCWs; the near environment (<40 inches) around the patient (including the bed, bed linen, bedside table); the distant environment (>40 inches) around the patient (including furniture, door handles, sink taps and toilet and the floor).

Samples were analysed blindly as to the sample origin.

Quantification of *C. difficile* using real-time PCR

Genomic DNA extracted from pure cultures from different bacterial species was used to confirm the specificity of primers and probes in the real-time PCR assay. The bacterial species were: *Bacillus cereus* (HIM 365-7); *Bacillus subtilis* (HIM 367-7); *C. difficile* (clinical isolate); *Clostridium histolyticum* [Medical Culture Collection Marburg (MCCM)

02471]; *Clostridium paraputrificum* (HIM 542-8); *Clostridium perfringens* (HIM 473-4); *Clostridium tetani* (HIM 216-1); *Enterococcus faecalis* (ATCC 29212); *Enterococcus faecium* (ATCC 6657); *Escherichia coli* (ATCC 25922); *Proteus mirabilis* (ATCC 14153); *Proteus vulgaris* (clinical isolate); *Staphylococcus aureus* (ATCC 29213); *Streptococcus pyogenes* (clinical isolate). *Clostridium difficile* agar with 7% sheep blood and Schaedler agar plates with vitamin K₁ and 5% sheep blood (Becton Dickinson, Heidelberg, Germany) were used to culture *C. difficile*. The other *Clostridium* species were cultivated on CDC Anaerobe agar with 5% sheep blood and Schaedler agar plates with vitamin K₁ and 5% sheep blood (Becton Dickinson, Heidelberg, Germany). For the recovery of all the other reference strains, Schaedler agar plates with vitamin K₁ and 5% sheep blood and MacConkey selective agar plates (Becton Dickinson) were used. Anaerobic and aerobic bacteria were cultured at 37 °C under anaerobic or aerobic conditions for 24–72 h respectively.

Genomic DNA from pure bacterial cultures was extracted with the DNeasy tissue kit (Qiagen, Hilden, Germany).

The hospital environmental samples were first treated in an ultrasonic bath to disrupt the coating of the bacterial spores. The DNA from these samples was extracted using the phenol-chloroform method.

The sequences of the *C. difficile*-specific primers were described elsewhere.¹⁴ The probe was based on species-specific highly conserved regions from the 16S rRNA gene sequences, which were retrieved using the program Entrez (<http://www.ncbi.nlm.nih.gov>) from the National Center for Biotechnology Information databases. *Clostridium difficile* primers and probe were based on species-specific highly conserved regions from the 16S rRNA gene sequences.¹⁴ For real-time PCR, primers forward (5'-TTGAGCGATTTACTTCGGTAAAGA-3') and reverse (5'-CCATCCTGTAAGGCTCACCT-3') were used to amplify 157 bp of the *C. difficile* 16S rRNA gene. A specific 6-carboxyfluorescein-labelled Taqman probe (TaqMan Probe CGGCGGACGGTGAGTAACG, this study) was used as an internal probe.

Samples were assayed in duplicate in a 25 µL reaction mixture containing 2.5 µL of template DNA, 12.5 µL of Ready Mix (Sigma, Munich, Germany), 2.5 µL of MgCl₂, 0.5 µM of forward primer and reverse primer (MWG, Munich, Germany), and 0.15 µM of the probe (Eurogentec, Liège, Belgium). Amplification and detection were performed with an ABI Prism 7700 sequence detection system (Applied Biosystems, Foster City, CA, USA) by using

the manufacturer's standard protocols. The cycling conditions used were as follows: 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min each. To determine the sensitivity of the real-time PCR assay, a standard curve was achieved by using serial 10-fold dilutions from 10² to 10⁸ cfu/mL of previously quantified bacteria lysate of a *C. difficile* pure culture and subjected to real-time PCR. Standard and clinical samples were run in duplicate and averaged for calculation of the bacterial load.

Statistical methods

Bacterial samples were averaged at the patient level and the averages were used in the analysis. Means and standard errors were calculated and reported. Comparisons of the estimates were carried out using the Wald test and the chosen level of statistical significance was 5%.

Pairwise Spearman correlations for all surfaces studied were estimated and reported. Additionally, a linear regression model was used to estimate the effect of HCWs' and patients' hand counts of *C. difficile* on different surfaces. Unadjusted estimates of the association were calculated by including each factor on the regression model, and adjusted estimates were obtained after including both factors. Bacterial counts were rank-transformed to improve model fitting. Beta coefficient and standard errors are reported.

Results

Compared with *C. difficile*-negative patients and wards, the environment of *C. difficile*-positive patients had significantly higher counts of bacteria from the floor and the near environment (Table I). No significant differences were observed between study groups on other surfaces, although the environment of *C. difficile*-positive patients had consistently higher counts of bacteria. Interestingly, no differences in *C. difficile* counts were observed on the hands of HCWs working in either a *C. difficile*-positive ward or *C. difficile*-negative ward nor on the hands of *C. difficile*-positive and -negative patients.

C. difficile counts on different surfaces were highly correlated (Table II). Highly significant correlations ($r \geq 0.7$) were observed between *C. difficile* counts on the floor and on patients' hands, HCWs' hands and distant environment. Counts of *C. difficile* on HCWs' hands were also highly associated with the bacterial counts on near environments. *C. difficile* counts on patients' hands

Table I Comparison of mean *C. difficile* counts in different surfaces in the hospital environment quantified by real-time polymerase chain reaction

Ward status	Patient status	Floor		Patients' hands		HCWs' hands		Near environment (≤40 inches)		Distant environment (>40 inches)	
		Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Cd+	Cd+	9117.3 ^a	3701.6	5535.0 ^a	2537.7	1549.7 ^a	693.0	2768.4 ^a	1153.7	2825.7 ^a	1293.1
Cd+	Cd−	843.1 ^a	319.4	2158.4 ^a	777.4	663.1 ^a	250.7	661.2 ^a	204.5	4133.4 ^a	2213.6
Cd−	Cd−	1028.1 ^a	362.0	1811.0 ^a	740.8	215.6 ^a	98.8	321.6 ^a	96.2	639.6 ^a	247.1

HCWs, healthcare workers; Cd, *C. difficile*.

^a Means followed by the same superscript letter are not significantly different ($P > 0.05$).

were also strongly correlated ($r \geq 0.6$ and $r < 0.7$) with HCWs' hands and distant environment counts.

HCWs' and patients' hand counts of *C. difficile* were significantly associated with bacterial counts on the floor, near and distant environments (Table III). After adjusting for patients' hand counts of *C. difficile*, HCWs' hands remained significantly associated with all surfaces studied. By contrast, patients' hand counts of *C. difficile* were significantly associated with bacterial counts on the floor and distant environment, but not with near environment.

Discussion

C. difficile was quantified using a real-time PCR technique developed to monitor the micro-organism in a hospital setting. Samples were analysed according to patient and ward *C. difficile*-positive status on five different environmental surfaces. Our results show that despite routine surface disinfection, *C. difficile*-positive patients' surroundings were more likely to be contaminated than those of *C. difficile*-negative patients. Moreover, it seems that HCWs may play an important role in spreading the bacteria in the hospital environment.

Hand hygiene has been proposed as a very important factor for preventing nosocomial spread

of pathogens in the hospital setting and community.^{15,16} In this study, samples collected from the hands of patients and HCWs showed *C. difficile* loads ranging from 6.8×10^1 to 5.3×10^4 . *Clostridium difficile*-symptomatic patients can excrete large numbers of organisms and/or spores and can be found in the environment of these patients.¹⁷ The spores of *C. difficile* can spread among patients and hospital staff by direct contact, and HCWs caring for patients with CDAD have also been found with *C. difficile* on their hands.^{1,3} Hand hygiene is the mainstay of preventing the transmission of nosocomial pathogens and should be reinforced in hospitals as a simple preventive strategy to reduce disease transmission rates.¹⁸ In the case of *C. difficile*, usual hand hygiene procedures should be changed, as intensive hand washing with soap and water is an important step in hand disinfection, i.e. quantitative reduction of spores can be achieved, in contrast to an alcohol-based disinfection, which is not effective against spores.

In addition to contamination of hands, environmental contamination is considered an important factor in hospital-acquired infections with several bacteria being able to survive for months on inanimate surfaces.^{3,19,20} Hospital standard environmental control programmes include daily

Table II Pairwise correlations of *C. difficile* counts between different surfaces

	Floor	Patients' hands	HCWs' hands	Near environment (≤40 inches)	Distant environment (>40 inches)
Floor	1.00				
Patients' hands	0.75 ^{**a}	1.00			
HCWs' hands	0.72 ^{**a}	0.60 ^{**b}	1.00		
Near environment (≤40 inches)	0.68 ^{**b}	0.47 ^{**}	0.73 ^{**a}	1.00	
Distant environment (>40 inches)	0.71 ^{**a}	0.65 ^{**b}	0.58 ^{**}	0.57 ^{**}	1.00

HCWs, healthcare workers.

^{**} $P < 0.01$.

^a $r \geq 0.70$.

^b $r \geq 0.60$ and $r < 0.70$.

Table III Crude and adjusted effect of healthcare workers' (HCWs') and patients' hands *C. difficile* counts on bacteria counts on other surfaces

	Floor $\beta \pm SE$	Near environment $\beta \pm SE$	Distant environment $\beta \pm SE$
Unadjusted estimates			
HCWs' hands	0.80 \pm 0.15**	1.06 \pm 0.17**	0.84 \pm 0.21**
Patients' hands	0.62 \pm 0.10**	0.53 \pm 0.15**	0.74 \pm 0.13**
Adjusted estimates			
HCWs' hands	0.44 \pm 0.19*	1.02 \pm 0.21**	0.40 \pm 0.22*
Patients' hands	0.39 \pm 0.15*	0.02 \pm 0.17	0.60 \pm 0.18**

* $P < 0.05$; ** $P < 0.01$.

cleaning and disinfection of patient and staff rooms and lounge areas with non-aldehydic formulations (Terralin protect, Schuelke, Norderstedt, Germany). However, in isolated cases or outbreaks of *C. difficile* infection an aldehydic preparation (Buraton 10F, Schuelke, Norderstedt, Germany) is used for disinfection of the *C. difficile*-positive environment. It is well known that spores are difficult to remove from the environment and that disinfecting surfaces with commonly used detergent-based cleaners is not effective in removing *C. difficile* spores.²¹ In this study, samples were collected before any intervention to change disinfection against *C. difficile*, showing that standard disinfection procedures are not sufficient to prevent cross-contamination. The longer the *C. difficile* persists on a surface, the longer it can be a source for transmission.

In addition, the dissemination of *C. difficile* through different geographical locations in the hospital highlights the complexity of the multiple routes of transmission. In this study samples were also obtained from the environment of *C. difficile*-negative patients on wards with known *C. difficile* patients and from wards where *C. difficile* patients had not been for at least six months. Although the numbers of *C. difficile* in these samples were lower in comparison to areas with *C. difficile*-positive patients, it is clear that the inanimate surroundings as well as patients and HCWs play a role in the transmission of *C. difficile*. Established proven infection control strategies for *C. difficile* include isolation or cohorting infectious patients, the wearing of aprons, gloves and even facemasks, with a strict emphasis on intensive hand washing with soap and water to remove spores. The bacterium has been detected on the hands of the clinical staff caring for *C. difficile*-negative persons, which illustrates the importance of correct hand washing, in addition to hand disinfection with an alcoholic preparation.¹⁹ Medical equipment should also be patient specific;

especially thermometers which can become heavily contaminated after rectal use.

The present study used real-time PCR methodology based on the 16S rRNA gene to assess *C. difficile* counts on different environmental surfaces. The advantage of this culture-independent method is the absolute quantification of the level of bacterial contamination present on the environment surfaces. Culturable bacterial counts in the hospital environment can underestimate the problem because of the culture conditions required for this organism. However, the molecular approach used in this study also has its own limitations. First, the toxinogenic profile of the environmental samples was not characterised; second, the clonal relationship could not be determined using a 16S rRNA-based real-time PCR. In this case we can assume that cross-contamination between the patients, hands of HCWs and the hospital environment took place, although this assumption cannot be proven. Another limitation of using molecular-based methods is the inability to differentiate between viable versus non-viable DNA in the tested samples. This may be an important confounding factor in explaining possible routes of cross-contamination.

In conclusion, *C. difficile* can be detected in higher numbers in the hospital environment of *C. difficile* patients, while asymptomatic carriers may have the potential to contribute to *C. difficile* transmission. In addition, patients and HCWs may have an important role in spreading the bacteria in the hospital environment. The use of real-time PCR for detection of toxinogenic and non-toxinogenic *C. difficile* strains would offer benefits for examining the hospital environment as it can rapidly and specifically detect this micro-organism.

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Conflict of interest statement

None declared.

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References

- Gerding DN, Johnson S, Peterson LR, Mulligan ME, Silva Jr J. *Clostridium difficile*-associated diarrhea and colitis. *Infect Control Hosp Epidemiol* 1995;16:459–477.
- Poutanen SM, Simor AE. *Clostridium difficile*-associated diarrhea in adults. *Can Med Assoc J* 2004;171:51–58.
- McFarland LV, Mulligan ME, Kwork RY, Stamm WE. Nosocomial acquisition of *Clostridium difficile* infection. *N Engl J Med* 1989;320:204–210.
- Johnson S, Clabots CR, Linn FV, Olson MM, Peterson LR, Gerding DN. Nosocomial *Clostridium difficile* colonisation and disease. *Lancet* 1990;336:97–100.
- Clabots CR, Johnson S, Olson MM, Peterson LR, Gerding DN. Acquisition of *Clostridium difficile* by hospitalized patients: evidence for colonized new admissions as a source of infection. *J Infect Dis* 1992;16:561–567.
- Shim JK, Johnson S, Samore MH, Bliss DZ, Gerding DN. Primary symptomless colonisation by *Clostridium difficile* and decreased risk of subsequent diarrhoea. *Lancet* 1998;351:633–666.
- Widmer A, Pittet D. *Clostridium difficile*: Epidemiologie und präventive Massnahmen. Swiss-NOSO, Nosokomiale Infektionen und Spitalhygiene: Aktuelle Aspekte. Oktober 1995, Band 2, Nummer 3.
- Riggs MM, Sethi AK, Zabarsky TF, Eckstein EC, Jump RL, Donskey CJ. Asymptomatic carriers are a potential source for transmission of epidemic and nonepidemic *Clostridium difficile* strains among long-term care facility residents. *Clin Infect Dis* 2007;45:992–998.
- Gordin FM, Schultz ME, Huber RA, Gill JA. Reduction in nosocomial transmission of drug-resistant bacteria after introduction of an alcohol-based handrub. *Infect Control Hosp Epidemiol* 2005;26:650–653.
- Van den Berg RJ, Bruijnesteijn van Coppenraet LS, Gerritsen HJ, *et al.* Prospective multicenter evaluation of a new immunoassay and real-time PCR for rapid diagnosis of *Clostridium difficile*-associated diarrhoea in hospitalised patients. *J Clin Microbiol* 2005;43:5338–5340.
- Bélanger SD, Boissinot M, Clairoux N, Picard FJ, Bergeron MG. Rapid detection of *Clostridium difficile* in feces by real-time PCR. *J Clin Microbiol* 2003;41:730–734.
- Tonooka T, Sakata S, Kitahara M, *et al.* Detection and quantification of four species of the genus *Clostridium difficile* in infant feces. *Microbiol Immunol* 2005;49:987–992.
- Penders J, Vink C, Driessen C, London N, Thijs C, Stobberingh EE. Quantification of *Bifidobacterium* spp., *Escherichia coli* and *Clostridium difficile* in faecal samples of breast-fed and formula-fed infants by real-time PCR. *FEMS Microbiol Lett* 2005;243:141–147.
- Rinttilä T, Kassinen A, Malinen E, Krogius L, Palva A. Development of an extensive set of 16S rDNA-targeted primers for quantification of pathogenic and indigenous bacteria in faecal samples by real-time PCR. *J Appl Microbiol* 2004;97:1166–1177.
- Larson E. A causal link between hand washing and risk of infection? Examination of the evidence. *Infect Control Hosp Epidemiol* 1998;9:28–36.
- Nystrom B. Impact of hand washing on mortality in intensive care: examination of the evidence. *Infect Control Hosp Epidemiol* 1994;15:435–436.
- Kaatz GW, Gitlin SD, Schaberg DR, *et al.* Acquisition of *Clostridium difficile* from the hospital environment. *Am J Epidemiol* 1988;127:1289–1294.
- Saloojee H, Steenhoff A. The health professional's role in preventing nosocomial infections. *Postgrad Med J* 2001;77:16–19.
- Samore MH, Venkataraman L, DeGirolami PC, Arbeit RD, Karchmer AW. Clinical and molecular epidemiology of sporadic and clustered cases of nosocomial *Clostridium difficile* diarrhoea. *Am J Med* 1996;100:32–40.
- Kramer A, Schwebke I, Kampf G. How long do nosocomial pathogens persist on inanimate surfaces? A systematic review. *BMC Infect Dis* 2006;16:130.
- Wilcox MH, Fawley WN, Wigglesworth N, *et al.* Comparison of the effect of detergent versus hypochlorite cleaning on environmental contamination and incidence of *Clostridium difficile* infection. *J Hosp Infect* 2003;54:109–114.