Abstract

Clostridium difficile infection (CDI) is an important cause of hospital diarrhea. Conventional CDI testing by enzyme immunoassays is fast yet shows poor sensitivity and specificity, whereas the ‘gold standard’ toxigenic culture is laborious and time consuming. Molecular tests for CDI are fast and highly sensitive and specific. Our laboratory has implemented a molecular screening procedure that allows for rapid detection of CDI directly in stool samples. It is based on the isolation of highly pure DNA extracts from feces using the MagNA Pure 96 System, followed by detection of the C. difficile toxins tcdA and tcdB using real-time PCR on the LightCycler® 480 System. The easy-to-use pretreatment of feces combined with nucleic acid isolation on the MagNA Pure 96 System resulted in only 0.5% of inhibited samples. The positivity rate for CDI detection increased from 7.3% using conventional methods to 13.7% using real-time PCR. Nearly all PCR positive samples were confirmed by toxigenic culture, demonstrating the high specificity of this molecular routine screening test.

With the MagNA Pure 96 System, handling steps were minimized and a high throughput and standardization were gained, causing less complexity, user interference and costs.
The Radboud University Nijmegen Medical Center (RUNMC) is one of eight university medical centers in The Netherlands. The principle tasks are patient care, research and medical education. The RUNMC has close to 1,000 beds and more than 30,000 clinical admissions annually. The department of Medical Microbiology of the RUNMC consists of the sections Bacteriology, Virology, Parasitology, Mycology and Molecular Diagnostics. The Molecular Diagnostics section provides for the detection of a wide range of microorganisms, covering the entire field of medical microbiology. In addition to commercial CE-IVD marked diagnostic tests, in-house or home-brew tests constitute an important part of the routine molecular diagnostics of infectious diseases. Increasing sample numbers and increasing diversity in the diagnostic pallet demand for efficient and automated extraction platforms, combined with flexible nucleic acid amplification systems. For this purpose, we have implemented the combination of the MagNA Pure 96 System and Roche LightCycler® 480 Real Time PCR Instruments in our laboratory for most of the molecular routine use procedures.

Clostridium difficile infection (CDI) is the major cause of hospital diarrhea. The laboratory analytics of CDI is based on the demonstration of toxin A/B directly in stool samples or in culture after isolation of the pathogen. The direct cytotoxicity test has been the gold standard in laboratory diagnosis for many years, but more recently toxigenic culture has been used for this purpose [1]. As these methods are both labor-intensive and have a long time-to-result, enzyme immunoassays (EIAs) are now used widely in routine laboratories [2]. However, the performance of these EIAs have been described to be poor, with sensitivity and specificity ranging from 60% to 99% and 70% to 100%, respectively [3,4]. Real-time PCR detection of C. difficile toxins is fast and was shown to be both sensitive and specific [4, 5]. To improve CDI diagnostics, our laboratory replaced the traditional routine tests used to detect toxin-producing C. difficile (i.e., enzyme immunoassay and toxigenic culture) by molecular detection, starting January 2011. To this end, stool samples are processed to yield 10% (w/v) fecal suspensions which after an easy-to-use pretreatment procedure are directly processed using the MagNA Pure 96 System. Real-time PCR targeting both C. difficile toxins A and B (tcdA and tcdB) is performed using the LightCycler® 480 Instrument to detect the presence or absence of CDI.

3 Materials and Methods

Sample material
For clinical validation of the real-time PCR procedure, 45 culture positive stool samples were studied retrospectively. In addition, 150 consecutive stool samples requested for CDI diagnostics during a 2 month period were analyzed prospectively. Samples were from individual, hospitalized subjects, and were the first sample of each subject [6]. Subsequently, a total of 1,254 stool specimens were screened for the presence of C. difficile toxin genes by real-time PCR in the routine setting from January 2011 until December 2011.

Nucleic Acid Isolation on the MagNA Pure 96 System
Stool specimens were collected in plastic containers without additives. Upon arrival in the laboratory, 10% (w/v) fecal suspensions were prepared by adding (dependent on the consistency) approximately 100 mg or 100 µl stool sample to 1 ml of sterile PBS. The remainder of the stool specimens was stored at +2 to +8°C for subsequent culture in case of a positive PCR result. Alternatively, the S.T.A.R. buffer (Roche Diagnostics) can be used for transport and storage of fecal samples. This might be advantageous for some applications; however, in our hands the use of S.T.A.R. buffer as compared to PBS did not improve results in terms of yield or PCR inhibition.

Fecal suspensions were homogenized by vortexing for 1 minute, left to rest for 10 minutes at room temperature (+15 to +25°C) and again vortexed for 1 minute. Suspensions were centrifuged for 30 s at 1,000 x g, and 195 µl of the supernatant was spiked with 5 µl of the isolation control Phocine Herpes Virus (PhHV) [7], and used for DNA isolation on the MagNA Pure 96 System. DNA was isolated using the MagNA Pure 96 DNA and Viral NA Small Volume Kit (Roche Diagnostics) and the Viral NA Plasma SV protocol. This isolation procedure is used routinely in our setting, and by using the same isolation protocol for all different types of clinical samples, we are able to combine all routine samples that need to be processed on one day in one MagNA Pure 96 run.

The input volume was 200 µl and the elution volume was set at 50 µl. Each isolation run was controlled using a negative control sample (195 µl PBS).
**3 Materials and Methods continued**

**Real-Time PCR Amplification and Detection**

A multiplex real-time PCR assay was developed, in which the tcdA and tcdB *C. difficile* toxin genes were detected together with the gB polymerase gene of phocine herpesvirus (PhHV-1), which was included as internal control. Primers and TaqMan® probes for tcdB and PhHV detection were as described ([5, 7], respectively). For tcdA detection, primers were as described [8], whereas the anti-sense molecular beacon [8] was replaced by a sense TaqMan® probe (5’-CTACTAgAggAAgAgATTCAAAATC-CTCA-3’). As it is not clinically significant to discriminate between tcdA, tcdB or tcdA/tcdB positive specimens, both tcdA and tcdB probes are 6FAM labeled. PhHV was detected using a LightCycler® Red 610 labeled FRET probe. All primers and probes were from TIB Molbiol, Berlin.

**4 Results**

**Validation**

Validation of the real-time PCR procedure for CDI routine testing was performed retrospectively on 45 stool samples that were tested positive by toxigenic culture, and prospectively on 150 consecutive stool samples requested for CDI diagnostics [6]. All 45 toxigenic culture positive samples were positive by real-time PCR. Of the 150 prospective samples, 18 (12.0%) tested positive by real-time PCR, of which 17 were positive by toxigenic culture. This discrepancy is most likely the result of the higher sensitivity of the PCR compared to toxigenic culture.

All PCR negative samples were negative by toxigenic culture. Compared to the toxigenic culture gold standard, sensitivity and specificity of the real time PCR test were 100% and 99.2%, respectively [6].

**Routine use**

From January 2011 until December 2011, a total of 1,254 stool specimens were screened for the presence of *C. difficile* toxins by real-time PCR. No discrimination between tcdA, tcdB or tcdA/tcdB positive specimens was made, as both detection probes were 6-FAM labeled. Routinely, samples were collected and pretreated during day 1, DNA isolation using the MagNA Pure 96 System and real-time PCR were performed on day 2, and results were made available that same day.

Real-time PCR mixes (50 µl) consisted of 25 µl of 2x LightCycler® 480 Probes Master (Roche), 0.5 µM of each primer and 0.1 µM of each probe, and 5 µl of extracted DNA. Real-time PCR was performed using the Roche LightCycler® 480 System using the following conditions: 10 min denaturing and hot-start at +95°C, followed by 50 cycles of: 10 s at +95°C and 60 s at +60°C. All real-time PCR runs were controlled by using one negative PCR control sample (5 µl of PCR grade H2O), and two positive control samples of purified plasmid preparations containing tcdA and PhHV or tcdB and PhHV PCR products, respectively.

For data analysis with the LightCycler® 480 Software, the AbsQuant/2nd Derivative Maximum analysis method was used.

To monitor for PCR inhibition, samples were spiked with PhHV isolation control. The mean crossing point (Cp) value for PhHV of 100 processed stools was 34.65, with a standard deviation (SD) of 1.19. Based on these data, samples were regarded as inhibited when the Cp value of the PhHV PCR was > 37 (i.e., the mean + 2x SD). Forty samples (3.2%) showed PCR inhibition in their initial PCR runs. Of these inhibited samples, stool suspensions were diluted 1:5 and extraction and real-time PCR were repeated. Six specimens (0.5%) remained inhibited after retesting and were reported as ‘not interpretable due to PCR inhibition’.

A total of 172 stool specimens (13.7%) tested positive for *C. difficile* toxins by real-time PCR. An example of the results that were gained using the LightCycler® 480 System are shown in Figure 1. In the year 2010, the positivity rate was 7.3% using enzyme immunoassay and toxigenic culture for confirmation, which represents an almost 90% increase as the result of PCR-based screening. Figure 2 shows the number of subject samples and individual subject tested and the number and percentage of positive samples and subjects in the years 2010 and 2011. Two real-time PCR positive samples were not available for culture. Of the remaining positive samples, all but one were confirmed by culture.
4 Results continued

**Figure 1:** LightCycler® 480 results of a typical *C. difficile*/PhHV duplex real-time PCR run. Amplification plots are shown of *tcdA*/*tcdB* (6FAM-label, panel A) and PhHV (LCred610-label, panel B). Two positive controls (*tcdA* and PhHV, and *tcdB* and PhHV), and two negative controls (one isolation and one PCR control) are included in each run. Panel A shows *tcdA*/*tcdB* amplification curves of the two positive controls and two positive routine samples. In panel B, all samples except the two negative controls show amplification of PhHV.

**Figure 2:** Comparison of CDI test results from routine testing in the years 2010 (EIA and toxigenic culture) and 2011 (PCR). Bars represent the absolute numbers of subject samples and individual subjects as well as the numbers of positive samples and positive subjects. Lines represent the percentages of positive samples and subjects.
We show in this note that the MagNA Pure 96 System is suitable for use in DNA isolation from stool specimens in routine testing. The combination of a simple pretreatment protocol together with the MagNA Pure 96 DNA and Viral NA Small Volume Kit and the Viral NA Plasma SV protocol shows excellent performance, resulting in PCR inhibition in only 3.2% of all samples in the initial run and 0.5% upon retesting of 1:5 diluted stool suspensions of the inhibited samples. This percentage is low compared to what is shown in other reports concerning PCR inhibition of stool samples, where PCR inhibition is found in 1.8% up to 15% of the samples tested [9 and references therein].

Minimization of handling steps with high throughput
This isolation protocol does not require additional substances or specific buffers, cycles of freezing and thawing, nor prolonged off-board incubation in lysis buffer to avoid PCR inhibition, and is therefore both user-friendly and suitable for high-throughput screening of stool samples.

Real-time PCR method increased find-rate
Implementation of this real-time PCR approach into routine use has increased both the sensitivity and specificity of C. difficile detection. Using real-time PCR, sample positivity increased by almost 90% compared to traditional testing using enzyme immunoassay. Nearly all PCR positive samples were confirmed by toxigenic culture.

Conventional CDI testing by enzyme immunoassay is fast yet shows poor sensitivity and specificity, whereas the ‘gold standard’ toxigenic culture is laborious and time consuming.

Application reduces complexity and cost
The LightCycler® 480 System offers the possibility to detect both C. difficile and the DNA isolation control PhHV in one multiplex reaction, reducing the costs of this molecular test. In addition, combining this test in one single multiwell plate with other molecular tests allows for efficient use of automated platforms, offering the possibility to perform a variety of molecular analytics tests on small numbers of samples per test, in a flexible and efficient manner. This is an absolute requirement in our university hospital setting.

5 Discussion
Real-time PCR method increased find-rate
Implementation of this real-time PCR approach into routine use has increased both the sensitivity and specificity of C. difficile detection. Using real-time PCR, sample positivity increased by almost 90% compared to traditional testing using enzyme immunoassay. Nearly all PCR positive samples were confirmed by toxigenic culture.

Conventional CDI testing by enzyme immunoassay is fast yet shows poor sensitivity and specificity, whereas the ‘gold standard’ toxigenic culture is laborious and time consuming.

Application reduces complexity and cost
The LightCycler® 480 System offers the possibility to detect both C. difficile and the DNA isolation control PhHV in one multiplex reaction, reducing the costs of this molecular test. In addition, combining this test in one single multiwell plate with other molecular tests allows for efficient use of automated platforms, offering the possibility to perform a variety of molecular analytics tests on small numbers of samples per test, in a flexible and efficient manner. This is an absolute requirement in our university hospital setting.

6 References
(6) de Jong E, de Jong AS, Bartels CJM, van der Rijt-van den Biggelaar C, Melchers WJG and Sturm PDJ. Clinical and Laboratory Evaluation of a Real-Time PCR for Clostridium difficile Toxin A and B genes. EJCMID 2011; conditionally accepted.
7 Important Note

- Data included in this article are the sole responsibility of the authors that have published them. These authors are responsible for following the respective local regulations for assay setup and validation.
- Regarding the specific assay(s) described, Roche was neither involved in establishing the experimental conditions nor in defining the criteria for the performance of the specific assays. Roche therefore cannot take any responsibility for performance or interpretation of results obtained for the biological target parameter(s) described by the authors or other users using a similar experimental approach.
- Potential users are informed to be aware of and in accordance with local regulations for assay validation and the scope of use for the involved Roche products, and to ensure that their use is valid in the countries where the experiments are performed.
- The MagNA Pure 96 System (Cat. Nr. 05 195 322 001) and LightCycler® 480 Real-Time PCR System are not intended for *in vitro* diagnostic use in the U.S.

For life science research only. Not for use in diagnostic procedures.

NOTICE: Product may be subject to certain use restrictions. Before using these product please refer to the Online Technical Support page (http://technical-support.roche.com) and search under the product number or the product name, whether this product is subject to a license disclaimer containing use restrictions.

TAQMAN, LC, LIGHTCYCLER and MAGNA PURE are trademarks of Roche.