

# Physical Pre-Treatment Improves Efficient DNA Extraction and qPCR Sensitivity from *Clostridium Difficile* Spores in Faecal Swine Specimens

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**Abstract** A considerable fraction of the faecal microbiota is spore-forming. Molecular quantification of bacteria may be underestimated if preceded with nucleic acid extraction without special treatment to extract recalcitrant bacterial spores. The objective of this study was to improve the DNA extraction regarding the presence of *Clostridium difficile* spores in faecal swine specimens. Sow faeces were inoculated with spores of *C. difficile* ( $10^6$  CFU), frozen at  $-30$  °C overnight and subjected to DNA extraction. As a preceding step to a standard DNA extraction method (QIAamp DNA stool Mini kit), different physical treatments such as microwave oven heating and repeated bead-beating techniques and a combination of both were applied and compared with each other by means of qPCR. Using a standard DNA extraction method only, *C. difficile* spores were quantified at 4.96 log copy number/200 mg of faeces. A repeated bead-beating at 6 m/s for 10 min followed by a standard DNA extraction resulted in 5.77 log copy number of spores in inoculated faeces. Heating in a microwave oven at 800 W for 1, 3, 5 and 10 min followed by a standard DNA extraction resulted in a gene quantification of up to 4.89 log copy number. A combination of both methods resulted in the bacterial gene quantity of 5.37 log copy number. Pre-treatment with repeated bead-beating led to the highest quantification of bacteria, and therefore it can be applied for more efficient DNA extraction from spores of *C. difficile* in faecal specimens.

**Keywords** DNA extraction · Bead-beating · Spore · *Clostridium difficile*

## Introduction

Cultivation-independent studies are essential in characterisation of microbial communities in different biological environments. The qPCR method has been one of the principal molecular techniques to detect and quantify bacteria of interest in faecal samples [5, 6]. Gut microbial community profiling studies require an optimised DNA extraction protocol ensuring efficient lysis from all bacterial cells and in different life stages. For instance, DNA isolation may be affected by incomplete cell lysis due to the composition and thickness of the bacterial cell wall [9]; the cell wall of Gram-positive bacteria, which contains peptidoglycan layer, is more robust compared to that of Gram-negative bacteria. In addition, a variable fraction of the faecal microbiota is found in the form of spores. Bacteria such as clostridia, bacilli or segmented filamentous bacteria (SFB) among others, form spores which are considered the most challenging regarding nucleic acid extraction. For example, endospores produced by bacilli and clostridia are resistant to common sanitary procedures and harsh environmental conditions such as radiation, heat, high air pressure and chemicals [15], and thus they may also resist disruption by simple homogenisation. Therefore, molecular quantification of spore-forming bacteria may be underestimated if preceded with nucleic acid extraction without special treatment considering the presence of spores. The majority of studies describing recovery of DNA from microbial spores have focused on soil microorganisms [2, 8]. In addition, different techniques aiming at the increase of yield of extracted DNA or RNA,

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detection of certain bacteria, and working-time reduction have been evaluated [3, 13]. Here, we demonstrate the efficient DNA extraction from bacterial spores in faecal specimens. As a model for spore-forming bacteria, we used *Clostridium difficile*. As a preceding step in standard DNA extraction method, we combined different physical treatments such as microwave oven heating and repeated bead-beating techniques.

## Materials and Methods

### Preparation of *C. difficile* Spores

Spores of *C. difficile* (donated by Professor Simon Cutting of Royal Holloway University, London, UK) were prepared according to Permponpattana et al. [14] with small modifications. Briefly, *C. difficile* was grown anaerobically on BHIS agar (brain heart infusion agar supplemented with 0.1 % L-cysteine, 5 mg/ml yeast extract and 0.1 % taurocholate as germinant factor) overnight at 37 °C. One fresh single colony from the BHIS plate was inoculated into 10 ml of TGY medium (3 % tryptic soy broth, 2 % glucose, 1 % yeast extract, 0.1 % L-cysteine) and incubated anaerobically at 37 °C overnight. One ml of TGY culture was then subcultured into SMC broth [90 g peptone, 5 g proteose peptone, 1 g (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 1.5 g Tris] containing 0.1 % L-cysteine, incubated overnight at 37 °C and then plated onto SMC agar. After 7 days of anaerobic incubation at 37 °C, the sporulation efficiency was confirmed by phase-contrast microscopy, and spore crops were harvested immediately. Spores were washed five times in ice-cold sterile water (centrifugation 10,000×g for 2 min at room temperature). The resulting pellet was resuspended in 500 µl of 20 % HistoDenz (Sigma-Aldrich Chemie GmbH, Steinheim, Germany), and the suspension was layered onto 1 ml of 50 % HistoDenz and centrifuged for 15 min at 14,000×g, room temperature. The supernatant containing cell debris was removed and the pellet was washed five times with ice-cold sterile water to remove residual HistoDenz. The purity was checked via phase microscopy and the concentration of viable spores was verified on *C. difficile* agar plates (*C. difficile* ChromID agar, Biomerieux, France) and incubated anaerobically, using anaerobic jars with Anaerocult A (Merck KGaA, Darmstadt, Germany), at 37 °C for 48 h. The spore aliquots were stored at 4 °C until use.

### Sample Preparation

Fresh sow faeces (200 mg) were homogenised by mixing, inoculated with 10<sup>6</sup> CFU of *C. difficile* spores (final concentration) and again homogenised. They were then

aliquoted to 2 ml reaction tubes or to FastPrep™ Lysing Matrix E (MP Biomedicals, LLC, Santa Ana, California, USA) tubes for faecal samples and frozen overnight at – 30 °C. All samples were prepared and analysed in triplicate.

### Physical Treatments

The samples were thawed at room temperature for 10 min and subjected to the following four treatments followed by standard DNA extraction using a commercial kit (QIAamp DNA stool Mini kit Qiagen, Hilden, Germany): (1) No treatment control; (2) Repeated bead-beating on a FastPrep-24™ 5G homogeniser (MP Biomedicals, LLC, Santa Ana, California, USA) i.e. 6 m/s, 10 min (with 1 min interval with 30 s bead-beating and 30 s rest); (3) Heating of the inoculated reaction tubes in a microwave oven at 800 W for 1, 3, 5 and 10 min; (4) A combination of microwave oven heating and repeated bead-beating.

### Standard DNA Extraction Method and qPCR Analysis

The DNA from faecal samples was extracted using the QIAamp DNA stool Mini kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Primer sequences included Cdiff-16S-1f (5'-TTGAGCGATTTACTTCCGTAAAGA-3') and Cdiff-16S-1r (5'-CCATCCTGTACTGGCTCACCT-3'). Primers were purchased from MWG Biotech (Straubing, Germany). A Stratagene MX3000p (Stratagene, Amsterdam, The Netherlands) was used for PCR amplification and fluorescent data collection. The master mix consisted of 12.5 µl Brilliant II SYBR Green qPCR Master Mix with Low ROX (Stratagene), 0.5 µl of each primer (10 µM) and 10.5 µl water and 1 µl sample. The amplification programme included an initial denaturation step at 95 °C for 15 min to activate the polymerase. The PCR programme featured an annealing time of 60 s at 58 °C s and a 60 s extension at 72 °C.

The fluorescent products were detected in the last step of each cycle. A melting curve analysis was made after amplification to distinguish the targeted from the non-targeted PCR product. The bacterial gene concentration in each sample was calculated by comparing the C<sub>t</sub> values obtained from standard curves. A standard curve consisted of serial dilutions of a PCR product with known concentrations of copy numbers of the target gene.

### Statistics

All data management and statistical analyses were performed using SPSS version 21.0.0.0 (SPSS Inc., Chicago, IL, USA). Significant differences were calculated by One-way

ANOVA with Tukey Post hoc were applicable. Differences were considered significant at an alpha-level of  $P \leq 0.05$ .

### Results and Discussion

Numerous protocols are currently available for the extraction of DNA from bacterial cells. They include enzymatic, chemical or thermal lysis, mechanical disruption by bead-beating, sonication or DNA-binding magnetic beads [6, 7, 11]. Efficient DNA extraction from bacteria at all life stages (vegetative cells, spores) is crucial for a proper characterisation using sensitive molecular methods.

In most endospore-forming bacteria, a number of layers including cortex, coat and exosporium protect the spore core and nucleic acids from harsh environmental conditions. Low water content, high peptidoglycan, dipicolinic acid and mineral content or the presence of silicon among others contribute to heat and ultraviolet resistance [12], and possibly also increase physical durability of spores compared to vegetative cells. Nakanishi and colleagues [10] demonstrated that the hardness of several *Bacillus* spores was significantly firmer than the corresponding vegetative cells. In addition, the hardness of bacterial spores correlated with heat or ultraviolet resistance.

Our study demonstrates that an improved DNA extraction from bacterial spores in faecal specimens can be achieved by a physical pre-treatment. As a model of a spore-forming bacterium, we used *C. difficile* ribotype 078 strain. We compared repeated bead-beating techniques, microwave oven heating and a combination of both methods to find the most sensitive way for DNA extraction from spores.

### Standard DNA Extraction

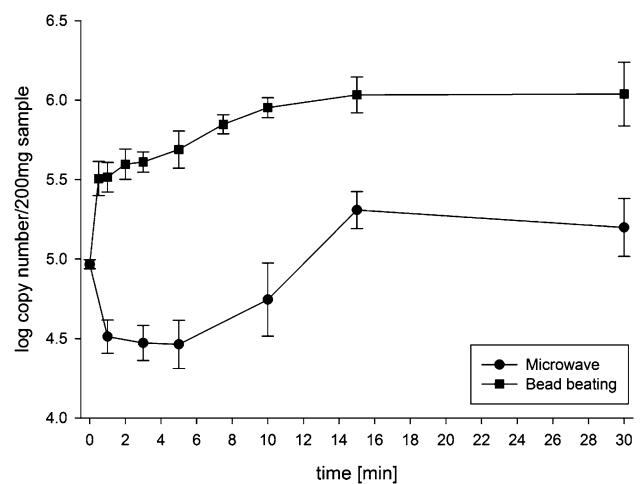
When DNA was extracted directly using a standard extraction method (QIAamp DNA stool Mini kit), we could detect 4.96 log copy numbers/200 mg of *C. difficile* spores. Compared to the inoculum of  $10^6$  cells per sample, the recovery was thus 9.1 %. The QIAamp DNA stool Mini kit is a common DNA extraction method in many laboratories. However, the homogenisation in a lysis buffer may not be sufficient for total DNA release from spores due to low mechanical powers generated during homogenisation. The manufacturer does not provide any specific information regarding DNA extraction from bacterial spores. In addition, published works lack data on bacterial spore extraction methods. The lack of such reports is surprising, especially considering a high number of studies using molecular methods and focusing on gut microbiota of which spores make up a significant fraction.

### Bead-beating Pre-Treatment

An alternative to a simple lab vortex mixer is the use of an automatic homogeniser which provides a much higher mechanical power. A study by Frostegård and colleagues [4] demonstrated that grinding increases extracellular DNA yield compared with the yield obtained without any lysis treatment in soil specimens. For the optimal sample lysis, the manufacturer of the FastPrep™ device recommends a bead-beating of the faecal samples for 40 s at a speed of 6 m/sec. We increased the number of bead-beating cycles to up to 30 cycles for a total of 30 min to cover a large range of cycles (Fig. 1). Using this method, we were able to extract the spore DNA at a significantly higher level (5.51 log copy number/200 mg of sample) already after 30 s of bead-beating, as compared to the standard DNA extraction. The recovery was further increased by repeated bead-beating cycles, showing a maximum of 5.95 log copy numbers/sample after 15 cycles. This led to a recovery of 89.1 %. No significant differences were found between 10, 15 and 30 cycles. Thus, we concluded that 10 bead-beating cycles (5.77 log copy numbers/200 mg of sample) were optimal for an efficient extraction of *C. difficile* spores from the faecal matrix.

### Microwave Pre-Treatment

The microwave oven thermal shock has already been shown to aid in extraction of nucleic acids from bacteria such as *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Saccharomyces cerevisiae* in activated sludge, soil and sediments [13], *S. aureus* and bacilli in pure cultures [1]. In our study, the pre-treatment in a microwave oven at 800 W



**Fig. 1** Impact of time on microwave or bead-beating pre-treatments on the recovery of *C. difficile* in faecal samples (log copy number *C. difficile* 16S rDNA/200 mg). Comparison to DNA extraction without pre-treatment (4.96 log copy number/200 mg)

**Table 1** Comparison of different physical treatments on DNA quantification efficiency from *C. difficile* spores in faecal specimens [log copy number *C. difficile* 16S rDNA/200 mg]

Treatment	Log copy number	Percent recovery
Standard	4.96 ( $\pm$ 0.28) <sup>a</sup>	9.1
Microwave <sup>1</sup>	4.89 ( $\pm$ 0.53) <sup>a</sup>	7.7
Bead-beating <sup>2</sup>	5.95 ( $\pm$ 0.26) <sup>b</sup>	58.8
Combination <sup>3</sup>	5.37 ( $\pm$ 0.01) <sup>a, b</sup>	23.4

<sup>1</sup> 15 minutes microwave

<sup>2</sup> 15 cycles

<sup>3</sup> most efficient treatment for microwave and bead-beating

Different superscripts letters denote significant differences within a column (One-way ANOVA, Tukey Post Hoc,  $P \leq 0.05$ )

for up to 30 min followed by the standard DNA extraction resulted in gene quantification of up to 5.30 log copy numbers/200 mg after 15 min (Fig. 1). However, compared to a standard DNA extraction, microwave-treated samples showed a drastic decrease from 1 to 10 min. The reasons for this severe reduction in spore DNA recovery remain unknown. It may be speculated that the microwave treatment modifies the physical structure of the complex faecal matrix. Contrary to normal heating, microwave energy heats particles from the inside. This may lead to a different physical structure than by external heat, which is applied during the subsequent lysis step of disrupted spores. Intact spores, apparently extractable with the standard DNA extraction method, could be protected by adhesion to a modified matrix at shorter heating times, while prolonged incubation disrupts the matrix itself and leads to higher DNA recoveries. Nevertheless, even 15 min microwave treatment yielded only 7.7 % recovery of inoculated *C. difficile* spore DNA (Table 1).

### Combination Pre-Treatment

A combination of microwave oven heating for 15 min, followed by repeated bead-beating for 10 min resulted in a recovery of 5.37 log copy number/200 mg (23.4 % recovery) (Table 1). Surprisingly, the recovery of DNA from spores using this technique was lower compared to a treatment where only a repeated bead-beating was used. Again, the physical structure of the heterogeneous faecal matrix rich in residual undigested feed, enriched in plant cell walls, may have been affected by a microwave heating, leading to a lower DNA detection by qPCR. Less likely, microwave oven heating may have also disrupted the spores releasing DNA, while the following repeated bead-beating fragmented the free nucleic acids resulting in lower numbers of gene copies as detected by qPCR method. An

overview of the comparison of different physical treatments on DNA quantification efficiency from *C. difficile* spores in faecal specimens is shown in Table 1.

### Conclusions

Taken together, the results presented in this study demonstrate that pre-treatment with the repeated bead-beating compared to microwave oven heating, standard DNA extraction or a combination of bead-beating and microwave, facilitates efficient DNA extraction and leads to an increased quantification of bacteria in faecal specimens. Therefore, we conclude that the pre-treatment with repeated bead-beating is highly efficient in nucleic acid release from bacterial spores and should be used in addition to a standard DNA extraction method.

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### Compliance with Ethical Standards

**Conflict of Interest** The authors declare no conflict of interest to the present study.

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