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Comparison of four commercial DNA extraction kits for the recovery of *Bacillus* spp. spore DNA from spiked powder samples

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ABSTRACT

Bacillus spp. include human pathogens such as *Bacillus anthracis*, the causative agent of anthrax and a biothreat agent. *Bacillus* spp. form spores that are physically highly resistant and may remain active over sample handling. We tested four commercial DNA extraction kits (QIAamp DNA Mini Kit, RTP Pathogen Kit, ZR Fungal/Bacterial DNA MiniPrep, and genesig Easy DNA/RNA Extraction kit) for sample inactivation and DNA recovery from two powders (icing sugar and potato flour) spiked with *Bacillus thuringiensis* spores. The DNA was analysed using a *B. thuringiensis*-specific real-time PCR assay. The detection limit was 3×10^1 CFU of spiked *B. thuringiensis* spores with the QIAamp DNA Mini, RTP Pathogen, and genesig Easy DNA/RNA Extraction kits, and 3×10^3 CFU with the ZR Fungal/Bacterial DNA MiniPrep kit. The results showed that manual extraction kits are effective and safe for fast and easy DNA extraction from powder samples even in field conditions. Adding a DNA filtration step to the extraction protocol ensures the removal of *Bacillus* spp. spores from DNA samples without affecting sensitivity.

1. Introduction

Rapid and accurate molecular detection and diagnosis of infectious agents is crucial in preparedness for infectious diseases and biothreats (Inglis, 2015; Zasada et al., 2015; Coyne et al., 2004). Inactivation and nucleic acid extraction are often laborious and time-consuming steps needed before DNA analysis. Especially when working in field conditions sample preparation is challenging and a potential cause for inaccurate diagnostic results (Bonini et al., 2002; Panning et al., 2007; Lermen et al., 2014; Wolfel et al., 2015). Safe DNA analysis is challenging if the sample contains pathogenic bacterial spores that have remained active after extraction with a commercial extraction kit (Dauphin and Bowen, 2009; Panning et al., 2007; Schmidt et al., 2011).

Bacillus spp. includes human pathogens such as *Bacillus anthracis*, the causative agent of anthrax (Schmidt et al., 2011). These Gram-positive spore-forming bacteria are found in soil and are mainly associated with animals. Human anthrax spreads usually through infected livestock when handling infected animals (Goel, 2015). *B. anthracis* spores enter the body either through a skin lesion (cutaneous anthrax), lungs (pulmonary anthrax), or gastrointestinal route (gastrointestinal anthrax) (Anderson and Bokor, 2012). Anthrax is a public health concern in countries where agriculture is an important source of income and where widespread vaccination of animals is not practiced (Goel, 2015). Because of the extremely resistant spores and high mortality rates, *B. anthracis* is also a potential biothreat agent (Anderson and Bokor, 2012), and both accidental and deliberate releases have been reported (Jernigan et al., 2001; U.S Department of Defence, 2015) (Jackson et al., 1998). Another spore forming bacteria of the *Bacillus* genus, *Bacillus thuringiensis*, is widely used in pesticides as it has an ability to produce toxins, which are toxic to many insect pests (Arteaga et al., 2014). *B. thuringiensis* is not generally considered a human pathogen (Kaminska et al., 2014), thus it is a suitable simulant for isolation and extraction experiments with *Bacillus* genus spores (Janse et al., 2010b; Carrera et al., 2007).

Manual nucleic acid extraction are in routine use to date (Ip et al., 2015; Dauphin et al., 2011; Dauphin et al., 2010; Whitehouse and Hottel, 2007). Contrary to the previous studies, this study has an emphasis on comparison of DNA extraction kits for the recovery of *Bacillus* spp. spore DNA from spiked powder samples. Furthermore, the compared kits are employing different techniques, including heat treatment, spin column procedures, bead beating, and magnetic beads, and the study focuses on applying these techniques in field.

We compared the safety and efficiency of four commercial DNA extraction kits (QIAamp DNA Mini Kit, RTP Pathogen Kit, ZR Fungal/Bacterial DNA MiniPrep, and genesig Easy DNA/RNA Extraction kit). Swift and simple methods that can be used in field conditions were preferred. The ability of the kits to inactivate *B. thuringiensis* spores and the amount of extracted DNA from spiked powder samples (icing sugar and potato flour) were evaluated. The performance of the kits was evaluated

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based on the processing time, DNA yield and purity, the need for additional laboratory equipment (e.g. heat-block, vortex), the elimination of potential PCR inhibitors, and the sensitivity of subsequent real-time PCR analysis. Also centrifugal filter units (Millipore Ultrafree-MC Centrifugal Filter Devices) were tested for the removal of spores from DNA samples.

2. Materials and methods

2.1. Spiking and preparation of powder samples.

Icing sugar and potato flour were spiked with an insecticidal product (TUREX 50 WP, Certis, Columbia, MD, USA), containing *B. thuringiensis* ssp. *kurstaki-aizaway* strain GC-91 spores. A spiking stock of TUREX powder (4 g) and nuclease-free water (NFW) (Sigma-Aldrich, St. Louis, MO, USA) (100 ml) was prepared. The suspension was mixed by vortexing and divided into 1 ml aliquots and immediately stored at −70 °C. Three aliquots were thawed and diluted ten-fold with NFW to determine the colony forming unit (CFU) count of the spiking stock. 100 μl of dilutions from 10^{-6} to 10^{-12} were plated on two parallel lysogeny broth (LB) (Bertani, 2004) plates and incubated at 37 °C for 21 h. CFU count was calculated based on the number of visible colonies. Spiking was performed as follows: One aliquot of spiking stock (4% TUREX suspension) was freshly thawed and serially diluted 100-fold with NFW. Next, one part of spiking stock dilutions (10^{-2}, 10^{-4}, or 10^{-6}) were added as duplicates to four parts of 10% powder suspensions.

To control the homogeneity of the powder samples, they were handled as liquid suspensions. Thus, powder samples were prepared freshly by mixing 1 g of icing sugar or potato flour with 10 ml of NFW. Six parallel powder samples were spiked with TUREX dilutions as described above. In addition, three control samples were included in each DNA extraction assay: undiluted spiking stock as positive control and unspiked powder suspension as well as NFW as negative controls. All DNA extraction assays were performed with a total sample volume of 200 μl.

2.2. DNA extraction methods

DNA was extracted from a sample volume of 200 μl according to manufacturers' protocols for difficult-to-lyse or Gram-positive bacteria, if available. Elution volume was 100 μl. DNA samples were stored at −70 °C. When using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany), DNA was extracted according to manufacturer's protocol "Isolation of genomic DNA from Gram-positive bacteria". With RTP Pathogen Kit (Stratec, Birkenfeld, Germany), the samples were prepared according to manufacturer's protocol, thus adjusted from 200 μl sample volume to a total volume of 400 μl using NFW, and DNA was extracted following the protocol "Extraction of DNA from bacterial pellets" for Gram-positive bacteria. DNA extraction using the ZR Fungal/Bacterial DNA MiniPrep (Zymo Research, Irvine, CA, USA) kit was performed according to the manufacturer's protocol. No beta-mercaptoethanol was added to the Fungal/Bacterial DNA Binding Buffer. For the Genesig Easy DNA/RNA Extraction kit (Primerdesign, Rownhams, Southampton, UK), the samples were prepared according to manufacturer's protocol for faeces/soil. DNA was extracted following the detailed protocol and using the Primerdesign Magnetic Rack.

2.3. Real-time PCR analysis

10-fold serial dilutions were made of extracted DNA ranging from 10^{-1} to 10^{-4} dilutions. All analyses were run as duplicates and amplification of the targeted template area in both parallel reactions was qualified as a positive result. *B. thuringiensis* ssp. *kurstaki-aizaway* strain GC-91 DNA was included in each PCR run as external positive control and no-template-control as negative control.

DNA was detected with *B. thuringiensis*–specific primers and probe targeting the *cry* gene, as described earlier (Matero et al., 2011). The amplification mixture in a total volume of 10 μl or 20 μl contained DyNAmo ColorFlash qPCR Mix (Thermo Scientific, Waltham, MA, USA), 0.3 μM of forward primer, 0.9 μM of reverse primer, 0.25 μM of probe, 0.01 U/μl AmpErase Uracil N-Glycosylase (Applied Biosystems, Carlsbad, CA, USA) for inhibition of PCR carryover contamination, the TaqMan Exo IPC Mix and Exo IPC DNA (Applied Biosystems) as internal positive control assay, template, and NFW. PCR reactions were performed using PikoReal Real-Time PCR System (Thermo Scientific). Reaction conditions were 2 min at 50 °C, 7 min at 95 °C, followed by 45 cycles of 5 s at 95 °C and 30 s at 60 °C.

2.4. Measuring DNA concentration and purity

The NanoDrop™ One Spectrophotometer (Thermo Scientific, Software version 1.1.0) was used for measuring of DNA concentration and purity after DNA extraction. The related elution buffer was used as a blank and DNA measured from two parallel 1 μl samples per eluate.

2.5. Evaluation of bacterial spore inactivation or removal

B. thuringiensis spore inactivation was studied by incubating 10% of the extracted DNA sample volume (i.e. 10 μl) originating from positive extraction controls on three parallel nutrient-rich LB plates at 37 °C for 14 days. Positive extraction control DNA was chosen for plating as the original sample (undiluted spiking stock) contained the highest spore amount compared to the other samples, and thus it would challenge the kits' inactivation capacity the most. Freshly thawed *B. thuringiensis* spiking stock served as a positive and elution buffers from each kit as negative plating controls.

2.6. DNA filtration

Centrifugal filtering was used for removal of spores from the DNA samples originating from the positive extraction controls. 40 μl of the extracted DNA was filtered with Millipore Ultrafree-MC Centrifugal Filter Devices with a pore size of 0.1 μm (Merck, Kenilworth, NJ, USA) by centrifugation at 12.000 × *g* for 4 min. Freshly thawed *B. thuringiensis* spiking stock and DNA elution buffer in question were used as controls. The removal of the spores was verified by culturing as described above.

3. Results

A simplified workflow from sample preparation to DNA extraction and subsequent analyses is presented in Fig. 1.

Depending on the kit, the processing time of nine samples varied from 65 min to 155 min and DNA yield from 17 to 78 ng/μl (Table 1). Potato flour caused clogging of the RTP Pathogen Kit's spin filters and reduced the total volume of eluted DNA from 100 μl to approximately 45 μl (data not shown). Additional laboratory equipment was needed with all four kits (Table 1).

The kits' efficiency to extract spore DNA from spiked powder samples was determined with a *B. thuringiensis*–specific real-time PCR assay. The detection limit was 3×10^1 CFU of spiked spores with the QIAamp DNA Mini, RTP Pathogen and genesig Easy DNA/RNA Extraction kits, and 3×10^3 CFU with the ZR Fungal/Bacterial DNA MiniPrep kit (Fig. 2). Internal positive control (IPC) assay was included in all real-time PCR reactions for detection of possible PCR inhibitors. No signs of PCR inhibitors were noticed.

To evaluate the sample inactivation efficiency of the extraction protocols, DNA aliquots originating from positive extraction controls were incubated on LB plates. DNA extracted with the genesig Easy DNA/RNA Extraction kit showed bacterial growth after 18 h of incubation (Table 1). DNA extracted using the other three kits did not show any bacterial growth in 14 days, after which the test was terminated. DNA