Dry-reagent-based PCR as a novel tool for the rapid detection of \textit{Clostridium} spp.

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Improved conventional PCR techniques are required for the rapid on-site detection of human and animal diseases. In this context, a PCR method using dry-stored reagents intended for the detection of \textit{Clostridium} spp. is presented. Basic PCR reagents (BSA, PCR buffer, Mg\textsubscript{Cl\textsubscript{2}} and primers), which were dried on polyolefin matrices, showed stability at ambient temperatures for up to 10 months without any loss of functionality. An outstanding advantage of our amelioration is the elimination of PCR process errors caused by the improper storage and handling of liquid reagents. Moreover, our PCR-based amplification can be performed in less than 30 min, saving time compared with conventional detection methods. Thus, dry-reagent-based PCR is implementable in a suitcase-like modular device for the rapid on-site detection of microbial pathogens such as blackleg of ruminants caused by \textit{Clostridium chauvoei}.

\textbf{INTRODUCTION}

The diagnostic molecular screening of pathogens is well established in modern human and veterinary medicine. Within this context, the detection of \textit{Clostridium chauvoei}, which is the causative agent of blackleg in cattle and sheep, is of great importance (Hatheway, 1990; Quinn \textit{et al.}, 2011). In endemic areas, the endospore-forming bacterium is present in soil and the infection can be transmitted by the oral route during grazing (Timoney \textit{et al.}, 1988). Among infected animals, \textit{C. chauvoei} is associated with a high mortality rate and is invasive if ingested. Symptoms resembling blackleg (e.g. emphysematous swelling and formation of gas gangrene in the heavy muscles) can also be caused by other \textit{Clostridium} spp. (Blaha, 1989). Because of the acute nature of the infection and the associated high mortality rate of ruminants, a rapid, unequivocal diagnosis of \textit{C. chauvoei} is necessary. In recent years, diagnostic tests using PCR have replaced traditional culture-based methods because of their higher speed and sensitivity (Bagge \textit{et al.}, 2009; Garofolo \textit{et al.}, 2011; Halm \textit{et al.}, 2010; Lange \textit{et al.}, 2010; Sasaki \textit{et al.}, 2002). Until now, these tests have been carried out in specialized laboratories. The biopsy material thus has to be transported, which prolongs the analysis time.

Several steps towards portable on-site systems for fast, PCR-based detection have been taken (Seise \textit{et al.}, 2011). Nevertheless, these PCR techniques still require many pipetting steps and highly skilled personnel to set up the assays. Moreover, the liquid reagents have to be transported and stored in a cold chain to ensure their functionality. One important move towards novel on-site PCR devices would be the development of dry-storable reagent mixtures with a long shelf-life. To that end, several approaches using lyophilization devices have been investigated. Such lyophilized reagents have been successfully evaluated in PCR or sequencing tests for the detection of animal as well as human pathogens (Ahlford \textit{et al.}, 2010; Qu \textit{et al.}, 2010; Takekawa \textit{et al.}, 2010, 2011). These dried reagents can be stored vacuum packed at $-20\,^\circ \text{C}$ or at ambient temperatures for up to 6 months without significant loss of their biological activity (Ahlford \textit{et al.}, 2010; Qu \textit{et al.}, 2010). Nevertheless, the addition of protecting/stabilizing reagents such as mannitol, trehalose or polyethylene (PE) glycol is required to maintain the functionality of the dried components (Ahlford \textit{et al.}, 2010; Kim \textit{et al.}, 2009; Qiu \textit{et al.}, 2010; Qu \textit{et al.}, 2010). To further improve on-site PCR diagnostics, the use of inexpensive and easy-to-handle disposables is desirable. To meet this challenge, polyolefins are recommended (Ahn \textit{et al.}, 2004; Koh \textit{et al.}, 2003). Polyolefin surfaces serve as a matrix for the application of PCR reagents, which can be dried \textit{in situ}, and have several benefits, such as batch producibility, biocompatibility and optical transparency (Griebel \textit{et al.}, 2004; Qiu \textit{et al.}, 2010).

\textbf{METHODS}

Preparation of dried PCR reagents on polyolefin surfaces. The polyolefins polypropylene (PP) and PE were supplied by Nowofol or
Bacterial strains and culture conditions. The bacteria type strains *C. chauvoei* (ATCC 10092) and *Clostridium septicum* (ATCC 12464) were obtained from the American Type Culture Collection (ATCC). The bacteria were cultured on agar plates (Nutrient Agar 1; Sifin) containing 5 % calf blood under anaerobic conditions.

Isolation of genomic DNA. Isolation of genomic DNA was performed according to the DNA-extraction protocol for Gram-positive bacteria of the DNeasy Blood & Tissue kit (Qiagen).

PCR with liquid-stored PCR reagents. Amplification of DNA sequences enabling the detection of *Clostridium* spp. by PCR was carried out using primers for the 16S–23S rDNA intergenic spacer region (16-23F and 16-23R) (Sasaki et al., 2000, 2001) and the 23S rDNA region (ma-f and ma-R) (Friedrich-Loeffler-Institut). Primers were purchased from Eurofins MWG Operon (16-23F: 5’-GAGAACCTGCGGCTGGATC-3’; ma-F: GGTTAGTCGGGACCTAAG-3’; ma-R: 5’-CTGTGTCGTTTGGGTAC-3’). The PCR volume was 23 µl, containing 1 µM BSA, 1 x PCR buffer, 2 mM MgCl₂, 0.5 µM forward and reverse primer, 0.35 µM dNTPs, 0.05 U InnuTaq DNA polymerase (AJ Innuscreen) µl⁻¹ and 1 ng genomic DNA of *C. chauvoei* or *C. septicum* µl⁻¹.

With the rapid mode of the AlphaSC thermo cycler (Analytik Jena), PCRs were completed in less than 30 min according to the following cycle profile: initial denaturation at 95 °C for 120 s; 35 cycles, each consisting of denaturation at 95 °C for 2 s, primer annealing at 55 °C for 2 s and extension at 68 °C for 10 s; and one final extension step at 68 °C for 120 s. The amplification products were held at 4 °C until they were further processed.

PCR with dry-stored PCR reagents. After 0.5, 1, 3, 7 and 10 months, the dried reagents of polyolefin foils were reconstituted by adding nuclease-free deionized water. A rehydration time of 1 min allowed solubilization of the dried reagents. PCR was supplemented with the remaining reagents, including 0.05 U InnuTaq DNA polymerase µl⁻¹ and 1 ng genomic DNA µl⁻¹, and was performed as described above.

Agarose gel electrophoresis and determination of PCR product concentration. The PCR products were analysed by electrophoresis on 2 % (w/v) agarose gels. The pUC19-Marker ‘ready-to-use’ was supplied by Carl Roth and the Quick-Load PCR Marker was supplied by New England BioLabs. For visualization, the gels were stained with ethidium bromide (0.5 µg ml⁻¹) and documented with the RED Personal Gel Imaging System [ProteinSimple (formerly Cell Biosciences)]. The concentrations of the PCR products were determined using the lane profile analysis tool from AlphaView SA software, version 3.2.2 (ProteinSimple).

RESULTS

Until now, no validated on-site technique for the rapid detection of *Clostridium* spp. has been available. To close this gap, we tested different approaches concerning the applicability of dry-reagent-based PCR. In our experimental setup, polyolefin surfaces (i.e. PP or PE) were evaluated as storing matrices for PCR reagent mixtures of BSA, PCR buffer, MgCl₂, primer and dNTPs. Single reagents or various mixtures of reagents were placed dropwise onto PP or PE foils, dried and stored at ambient temperatures for up to 10 months (Fig. 1).

First, we analysed the functionality of the original liquid PCR reagents in a conventional PCR setup (Fig. 2a). The PCR was completed in less than 30 min employing the rapid PCR mode of the AlphaSC thermo cycler. As described in the literature, three PCR products were detectable when using the 16S–23S rDNA-specific primer set for the amplification of *C. chauvoei* DNA (Sasaki et al., 2000).

Our next goal was to elucidate the functionality of our dry PCR reagent mixtures on polyolefin mini-matrices. For long-term storage monitoring, the pre-dispensed and dried PCR mixtures, containing BSA, PCR buffer, MgCl₂, primer and dNTPs, were reconstituted after 0.5, 1, 3, 7 or 10 months and applied in PCR as described in Methods. As depicted in a representative agarose gel image, illustrating 1 day storage of PCR reagents on PP foil, the amplification of *C. septicum* DNA was successfully performed with various reagent combinations (Fig. 2b). No product was detectable when the dry PCR mixture contained dNTPs (Fig. 2b, lane 3). When dNTPs are dried together with other PCR components on polyolefin foils, slight pH alterations during the drying process and/or the lack of additives can lead to nucleotide instability. This is in accordance with reports of dNTP stability only in a narrow...
pH range and loss of functionality when no additive was present during the drying process (Bajaj-Elliott & Hauer, 2001).

Dried PCR reagent mixtures (BSA, PCR buffer, MgCl₂, primer 16-23F/R) on PP and PE foils were stored for up to 10 months at ambient temperatures, reconstituted and applied in PCRs to amplify C. chauvoei DNA. As shown in Fig. 3, PP and PE matrices were usable as dry supplies of the PCR reagent mixtures. Successful amplification could be performed following prolonged storage at ambient temperatures. Nevertheless, a decrease in sensitivity was detectable with longer storage.

**DISCUSSION**

There is an urgent need for an on-site detection system for *Clostridium* spp. to cope with severe diseases such as *C. chauvoei*-caused blackleg, a gangrenous infection in cattle and sheep (Hatheway, 1990; Quinn et al., 2011). We have designed a PCR test with dry reagent mixtures on polyolefin matrices for *Clostridium* spp. detection; these matrices were stable at ambient temperatures over a long period of time. Using this system, PCR assay variability caused by improper transportation or storage of reagents could be significantly reduced. In addition, there is no requirement for the addition of stabilizers or protectors to the drying mixtures, and expensive drying devices are not needed. The drying procedure is fast and can be performed in a basic laboratory using a fume hood. Moreover, the pre-dried PCR reagent mixtures can be reconstituted 'just in time', minimizing contamination risk and pipetting errors, which can occur during the addition of various liquid components as for a conventional PCR assay. Due to our findings that dNTP-containing dry PCR mixtures were not functional, the drying of pre-mixed BSA, PCR buffer, MgCl₂ and specific primer on mini-polyolefin foils is recommended. Today, dNTPs and *Taq* DNA polymerase are commercially available as dried reagents that are stable at ambient temperatures. Their storage together with the foil-dried PCR reagent mixture in one reaction tube and the subsequent addition of nuclease-free water and target DNA could allow priming of the PCR. Such ambient-temperature-stable pre-loaded PCR tubes could omit the necessity for dNTP as well as enzyme storage at −20 °C. Nevertheless, future experiments should be conducted to unravel the putative combination of our pre-mixed dried PCR reagents with dry dNTPs/*Taq* DNA polymerase for the detection of *Clostridium* spp. In addition, a side-by-side comparison of dried PCR reagents applied in standard and real-time PCR should be performed.

A future development could allow the 'on-board' storage of dry PCR reagents by means of separate stocking areas for PCR components on inexpensive disposables such as...
polylefin foils integrated into a portable, suitcase-like detection device. After reconstitution, the liquid PCR mixture contained all the reagents required for successful amplification, which may facilitate the rapid diagnosis of animal disease directly on farms (Knabbe et al., 2009). Our PCR schedule allows the fast amplification of Clostridium spp. DNA in less than 30 min. A further detailed specification of the bacterial pathogen in a hybridization module is desirable (Schüler et al., 2009; Seise et al., 2011). For example, the rapid detection and confirmation of blackleg caused by C. chauvoei in a herd could provide the opportunity for immediate countermeasures to prevent this severe disease from spreading. Early diagnosis would also facilitate the treatment of animals at risk or in the early stages of disease with the appropriate doses of antibiotics. Furthermore, a positive diagnosis would help to identify grazing land and feeding stuffs contaminated with C. chauvoei spore, and therefore limit economic loses of productive livestock.

To summarize, this is the first demonstration of Clostridium spp. detection with dry-reagent-based PCR.

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