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Clostridium spp. discrimination with a simple bead-based fluorescence assay

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C. chauvoei is the causative agent of blackleg, an endogenous bacterial infection which usually affects cattle and other ruminants. Due to the fact that the symptoms of this severe disease are very similar to the phenotype caused by an infection with *C. septicum*, a reliable differentiation of *C. chauvoei* from other *Clostridium* spp. is mandatory. Traditional microbiological detection methods are time consuming and the proper specification is hampered by the overgrowing tendency of swarming *C. septicum* colonies when both species are in the clinical sample. Thus, there is an urgent need to improve and simplify the specific detection of *C. chauvoei* and *C. septicum*. We report an easy and fast *Clostridium* spp. discrimination method *via* a magnetic bead-based fluorescence assay. To that end, the target DNA was amplified using 16S-23S rDNA spacer region specific primers. These PCR products were employed to generate single-stranded capture probe DNA, which was immobilized on magnetic beads. Functionalized magnetic particles exhibit numerous advantages, like their simple manipulation in combination with a huge binding capacity of biomolecules and make therefore excellent biosensors. In this context, the discrimination between *C. chauvoei* and *C. septicum* was realized by means of hybridization with complementary detection probe DNA. Finally, fluorescence spectroscopy allowed the signal readout. With this approach a precise discrimination between *C. chauvoei*, *C. septicum* and *C. carnis* was accomplished.

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1 Introduction

Clostridium chauvoei (*C. chauvoei*) and *C. septicum* are Gram-positive, rod shaped, anaerobic, endospore forming bacteria. *C. chauvoei* is the causative agent of blackleg which usually infects cattle, sheep, goats and other ruminants. The spores are invasive if they are ingested. It is assumed that they advance to the muscle tissue *via* the blood stream and possibly persist there for years. If the oxygen level decreases, the spores may germinate, multiply and produce toxins.[1] In recent reports it was demonstrated that *C. chauvoei* infects not only ruminants, but also other hosts like humans[2,3] and pets.[4] Prevention vaccination is practiced, but sporadic enzootic outbreaks with high economic losses cannot be avoided.[5] The symptoms of a blackleg infection, like emphysematous swelling, the formation of gas gangrene in the muscles, fever and lameness can also be caused by other *Clostridium* species (*Clostridium* spp.).[6,7] Differentiation of *C. chauvoei* from other *Clostridium* spp. is mandatory to obtain governmental financial support to compensate financial losses due to blackleg in certain counties or districts.[6] Conventional microbiological detection methods are time consuming due to the fact that *C. chauvoei* exhibits slow growth and, furthermore, is often overgrown by swarming *C. septicum* colonies, because both pathogens could be present in the sample.[6,8,9] Recently developed PCR-based identification methods are reliable, fast as well as easy to perform. They target, amongst others, the 16S-23S rDNA spacer region.[8–12]

Magnetic particles are a versatile tool for various bioanalytical applications, like separation of proteins, nucleic acids or even cells.[13–17] Due to their unique properties they allow a convenient and fast isolation of biomolecules from complex surroundings. Especially the very flexible functionalization of magnetic particles by using one of the plethora of coatings proposes their usage for biosensing.[14,18,19] In our assay we combine the convenience of magnetic beads with a DNA-based fluorescence assay[20] to discriminate different *Clostridium* spp. To optimize the hybridization efficacy and thus the output signal intensity, different methods for generating single stranded capture probes immobilized on magnetic beads have been investigated. An easy method to loosen the hydrogen bonds between the complementary DNA bases is heat denaturation at 95 °C. Another approach to obtain single stranded

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DNA is an enzymatic digestion with λ-exonuclease.[21,22] During the PCR one phosphorylated primer is used to produce phosphorylated double-stranded DNA. The exonuclease selectively digests the phosphorylated strand of the double-stranded PCR product from the 5′ to 3′ end and the non-phosphorylated strand remains.[23,24] Further, sodium hydroxide (NaOH) was utilized to induce denaturation of double stranded DNA attached to streptavidin modified magnetic beads.[25,26]

2 Materials and methods

2.1 Bacterial strains and culture conditions

The bacteria type strains *C. chauvoei* (ATCC 10092), *C. septicum* (ATCC 12464) and *C. carnis* (ATCC 25777) were obtained from the American Type Culture Collection (LGC Standards GmbH, Wesel, Germany). They were cultured on agar plates (Nutrient Agar 1 (Sifin, Berlin, Germany)) containing 5% calf-blood under anaerobic conditions.

2.2 Isolation of genomic DNA

The isolation of genomic DNA (gDNA) from bacteria or cattle muscle tissue (25 mg per sample) was performed with the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) according to manufacturer's recommendation.

2.3 DNA amplification

Amplification of the 16S-23S ribosomal (rDNA) intergenic spacer region of *Clostridium* spp. genomic DNA was carried out by using primers[8,9,12] (Eurofins, Ebersberg, Germany) depicted in Table 1. 50 μl PCR reaction volume containing 0.05 U μl^{-1} InnuTaq DNA polymerase (Analytik Jena, Jena, Germany), 0.35 μM dNTPs, 0.5 μM of each primer, 2 mM Mg^{2+}, 1× PCR buffer, 1 μM BSA and 2.5 ng template DNA. The speed amplification profile in the AlphaSpeedCycler (Analytik Jena, Germany) was as follows: initial denaturation 95 °C for 120 s, 35 cycles: 95 °C for 2 s, 55 °C for 2 s and 68 °C for 10 s, and final elongation 68 °C for 120 s.

2.4 PCR product purification

The clean-up of PCR products was performed following the instructions of the innuPREP PCRpure Kit (Analytik Jena, Jena, Germany). The elution of the PCR products was done with an equal volume of water.

Table 1 PCR primers and detection probes used in this study

Name	Sequence 5′ → 3′
Forward primer[a]	GAGAACCTGCGGCTGGATC
Reverse primer[b]	TTCACCATGCGCCCTTTGTAG
C. chauvoei probe[c]	TAAAACAACTTTATTAACAAATGTTA
C. septicum probe[c]	CTGAAAGCGTATGTGAACAG
C. carnis probe[c]	CCTAAGAACGTATGTGAATAG
Clostridium spp. probe[c]	GAGAACCTGCGGCTGGATC

[a] Forward primer is modified w/ 5′-phosphate. [b] Reverse primer is modified w/ 5′-biotin. [c] All detection probes are modified w/ 5′-cyanine 3.

2.5 Agarose gel electrophoresis and semi-quantitative determination of the DNA concentration

PCR products were analyzed by electrophoresis on a 2% agarose gel. For visualization the DNA was stained with GelRed (VWR International GmbH, Darmstadt, Germany) according to the manufacturers' recommendations and an agarose gel image was documented. The concentrations of the PCR products were determined by using the Lane Profile Analysis Tool from AlphaVIEW SA software Version 3.2.2 (Cell Bioscience, Inc.)

2.6 Immobilization of capture probes on magnetic beads

For each reaction 250 μg of streptavidin modified magnetic beads (M-PVA SAV1, PerkinElmer chemagen Technologie GmbH, Germany) were washed twice with 1× PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, pH 7.4)/0.1% BSA buffer. Finally the beads were resuspended in the same buffer and incubated either with the unpurified or processed PCR product.

2.7 Different methods for generating single-stranded capture probes

2.7.1 NaOH denaturation after immobilization of the PCR product on beads. Typically, 50 μl of the PCR product were added to the magnetic beads and incubated for 30 min at room temperature while shaking. After two washing steps with 1× PBS/0.1% BSA, 100 μl of 0.15 M NaOH was added and incubated for 10 min.

2.7.2 λ-exonuclease digestion before immobilization of the PCR product on beads. The 25 μl reaction mixture contained 10 μl purified PCR product, 2.5 μl exonuclease buffer and 0.6 μl (25 U μl^{-1}) λ-exonuclease (Roboklon Berlin, Germany). Incubation was performed at 37 °C for 10 min followed by a 5 min heat inactivation of the enzyme at 95 °C. The reaction volume was set to 150 μl, added to the magnetic beads and incubated for 30 min at room temperature while shaking.

2.7.3 Heat denaturation before immobilization of the PCR product on beads. 50 μl of PCR product were heat denatured at 95 °C for 5 min and immediately cooled on ice to avoid reassociation of complementary DNA strands. After the addition to magnetic beads an incubation period of 30 min followed.

2.8 On-bead hybridization with single-stranded detection probes

Before hybridization with specific detection probes (Table 1), the magnetic beads harboring immobilized capture DNA were washed three times with 1× PBS/0.1% BSA. Finally, the beads were resuspended in buffer supplemented with 0.1 nmol Cy3-modified probe DNA and incubated for 30 min at room temperature.

2.9 Fluorescence measurements

Fluorescence signals were measured with a JASCO spectrofluorometer type FP-6200 (excitation 513 nm, emission 558 nm).

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3 Results and discussion

Our aim was to develop an easy-to-perform and cost-efficient assay that allows the robust discrimination of different *Clostridium* spp. To that goal a combination of magnetic beads, the generation of single-stranded capture probes and a fluorescence-based detection was investigated.

3.1 Amplification of a target sequence in the 16S-23S rDNA intergenic spacer region of seven *Clostridium* spp.

Due to the huge binding capacity and simple handling of magnetic beads as an enabling tool for analytics, we decided to use them for our assays. The beads have a streptavidin coating, so we performed the PCR with biotinylated reverse primers enabling the immobilization on the particles. The target DNA isolated from *C. carnis*, *C. chauvoei* and *C. septicum* was amplified using primers described by Sasaki *et al.* and analyzed on an agarose gel (Fig. 1). These primers allow the amplification of the 16S-23S rDNA spacer region of *C. carnis*, *C. chauvoei, C. septicum, C. perfringens, C. novyi* and *C. haemolyticum*.[8,9] According to the literature, up to three PCR products were detectable when using the primer set (Fig. 1 and 6a).[8,9] Moreover, with this primer pair and varying amounts of *C. chauvoei* template DNA (29 nanograms to 29 attograms) an amplification was possible when 2.9 picogram template DNA was present in the PCR reaction (data not shown). A calculation of the corresponding colony forming units (CFU) revealed that isolated genomic DNA from at least 1000 CFU of *C. chauvoei* is enough to generate sufficient amounts of amplicons by PCR. Nevertheless, further optimization of PCR conditions could improve the sensitivity.

3.2 Improvement of on-bead hybridization by generating single-stranded capture probes

We implemented streptavidin coated magnetic particles in our assay to exploit the strong interaction between bead immobilized streptavidin and biotin.[27] The latter one is covalently coupled to the PCR products by using biotinylated primers.[26] We examined three different methods to generate single-stranded capture probes from a PCR product to optimize the on-bead hybridization.[21] Depending on the approach the PCR product was bound to the beads before (NaOH) or after (enzymatic, heat) denaturation (Fig. 2). For the denaturation with NaOH, the double-stranded PCR product (Fig. 2, lane 1) was immobilized on the magnetic beads. It was reported in the literature that the streptavidin biotin interaction is stable as long as the denaturation time does not exceed 15 min and the NaOH concentration is not higher than 0.15 M.[28,29] We designed our experimental assays according to these recommendations. After binding the double-stranded PCR product to the magnetic beads the supernatant showed no band at all, indicating an effective binding of the capture probe on the bead's surface (Fig. 2, lane 2). Applying sodium hydroxide on-bead denaturation the dissociated complementary DNA strand was detectable in the supernatant (Fig. 2, lane 3). A weak band which represents the released double-stranded PCR product was also detectable. Thus, applying on-bead denaturation the strong interaction between streptavidin and biotin of a small fraction immobilized double-stranded DNA can be broken.[30,31] The generation of single-stranded capture probes by enzymatic digestion with λ-exonuclease was also effective prior to the immobilization on the magnetic particles (Fig. 2, lane 4). The stronger band represents biotinylated single stranded DNA that migrates faster in the electrophoresis.[21] The weak band above is a small amount of undigested double stranded PCR product. The digestion has to be carried out before binding the PCR product to the magnetic beads, since the streptavidin–biotin interaction would be destroyed during heat inactivation of the enzyme. The effective binding of the biotinylated capture DNA to the beads was demonstrated with this approach (Fig. 2, lane 5).

When combining heat denaturation with streptavidin modified magnetic beads the immobilization of already single stranded capture probes is mandatory. The interaction between streptavidin and biotin is permanently destroyed at temperatures higher than 70 °C.[26,32] In order to preserve a high yield of single-stranded DNA on the beads, the denatured DNA was rapidly cooled on ice. Nevertheless, some renaturation occurs and a double-stranded capture probe appears (Fig. 2, lane 6). In order to demonstrate the hybridization improvement due to

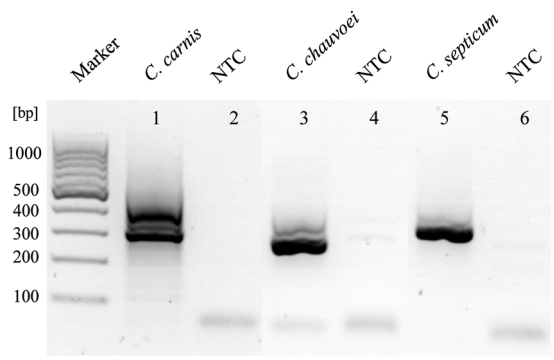

Fig. 1 Image from analytical agarose gel electrophoresis to monitor the successful amplification of various *Clostridium* spp. target DNA (NTC = non-template control).

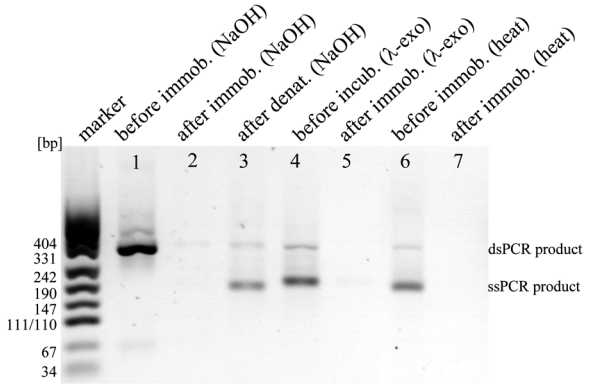

Fig. 2 Analytical agarose gel image of the successful generation of the ssPCR product by NaOH, enzymatic (λ-exo) and heat denaturation and the immobilization of these probes on magnetic beads.

already particle-immobilized single-stranded capture probes, the interaction with complementary fluorescence labeled detection probes was performed as described in the Materials and methods section. The fluorescence data for hybridization with *C. chauvoei* specific detection probes concomitant with a side-by-side comparison of the denaturation methods are shown in Fig. 3. The magnetic particles in the buffer solution scatter the excitation light in every direction, so that a weak signal is also detectable in the case that no DNA is present (Fig. 3, w/o). Roughly the same fluorescence signal intensity was measurable when double-stranded DNA was immobilized on the magnetic particles (data not shown). The direct comparison of three DNA denaturation methods to produce single stranded capture DNA on the beads points out that NaOH is slightly more effective than the application of λ-exonuclease or heat (Fig. 3). In general, all methods are suitable for the bead-based fluorescence detection of seven *Clostridium* spp. Due to the fact, that chemical denaturation of DNA with sodium hydroxide is cheap and easy to perform, we decided to apply this reagent for all future experiments. We want to point out that the enzymatic digestion is also a quite effective method but has some drawbacks. Firstly, the PCR product had to be purified in order to obtain sufficient amounts of single stranded DNA. Secondly, the λ-exonuclease is expensive and requires proper storage conditions to maintain optimal enzymatic performance.

3.3 Discrimination of *C. chauvoei*, *C. septicum* and *C. carnis* with a simple bead-based fluorescence assay

The accurate detection of *C. chauvoei* and *C. septicum* is essential for the differential diagnosis of blackleg. Moreover, there is an urgent need for assay simplification without the loss of sensitivity and specificity.[33] The assay (Fig. 4) presented within this work fulfills all the needs for a convenient and robust confirmation tool. The target DNA from *C. chauvoei*, *C. carnis* and *C. septicum* was amplified by PCR and bound to streptavidin modified magnetic beads. Subsequently the NaOH denaturation was performed to generate single-stranded capture probes as well as hybridization with specific Cy3-labeled detection probes. The detection probe for the internal control will hybridize with PCR products from all three species due to their sequence redundancy. Fig. 5 illustrates the results of three independent experiments for each specification experiment. All three *Clostridium* spp. were significantly distinguishable. Depending on the presence of target DNA in the assay, only the specific complementary detection probe hybridized (Fig. 5). The fluorescence signals for the relevant probes and the internal controls were always higher than those of the blank as well as the non-matched capture DNA from other *Clostridium* spp.

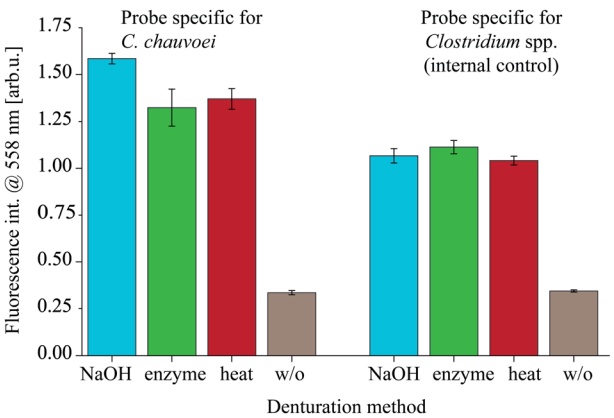

Fig. 3 Comparison of three different denaturation methods (NaOH, enzyme, and heat) for the improvement of on-bead hybridization (w/o = without DNA). The diagram represents data from three independent experiments using the *C. chauvoei* PCR product for generating single-stranded DNA. The successful on-bead hybridization was visualized *via* the hybridization with a Cy3-labeled complementary detection probe. The functionality of the assay was monitored by implementation of an internal control that represents *Clostridium* spp.

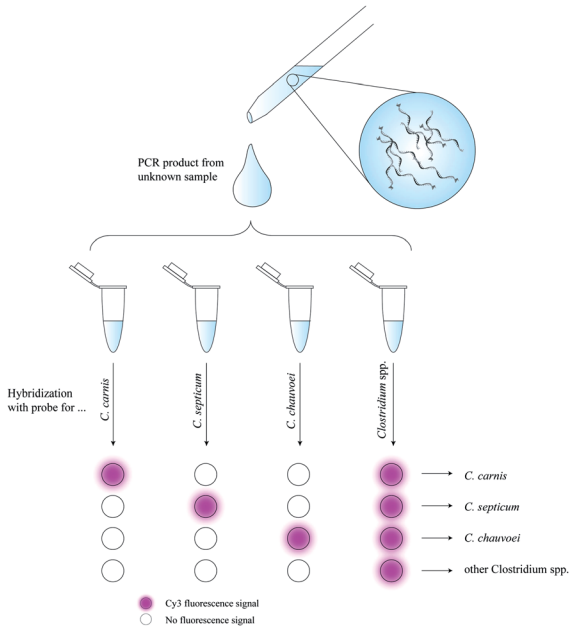

Fig. 4 Scheme illustrating the general procedure and signal interpretation for the fluorescence-based discrimination between different *Clostridium* spp.

Our assay was further challenged with real blackleg samples from cattle with confirmed infection of *C. chauvoei*. Genomic DNA was extracted from muscle tissue samples of three different animals and a PCR reaction was performed to specifically amplify the 16S-23S rDNA spacer region. The agarose gel picture displayed in Fig. 6a shows that the pathogen DNA could be successfully amplified for all three samples. The PCR products were further analyzed with the bead-based hybridization assay. In all three cases the assay outcome indicates an infection with *C. chauvoei*. In Fig. 6b the fluorescence intensities of one of the samples are displayed as representative examples. The signal of the probe specific for *Clostridium* spp. shows a high fluorescence intensity confirming that the hybridization assay was carried out correctly. Moreover, the signal intensity of *C. carnis* and *C. septicum* specific probes cannot be distinguished from the negative control, while the probe for *C. chauvoei* shows a significant signal that indicates an infection.

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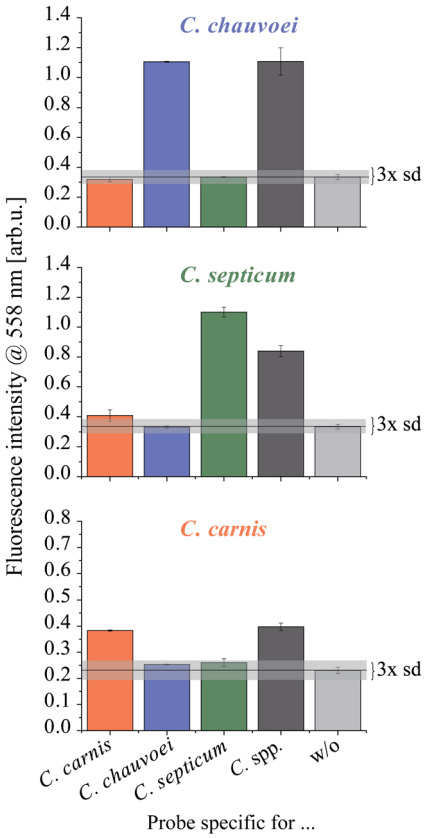

Fig. 5 Application of different *Clostridium* spp. showing the specificity of the on-bead fluorescence detection. *C. chauvoei* (top), *C. septicum* (middle) and *C. carnis* (bottom) as the target DNA color-coded with their specific complementary detection probe (sd – standard deviation).

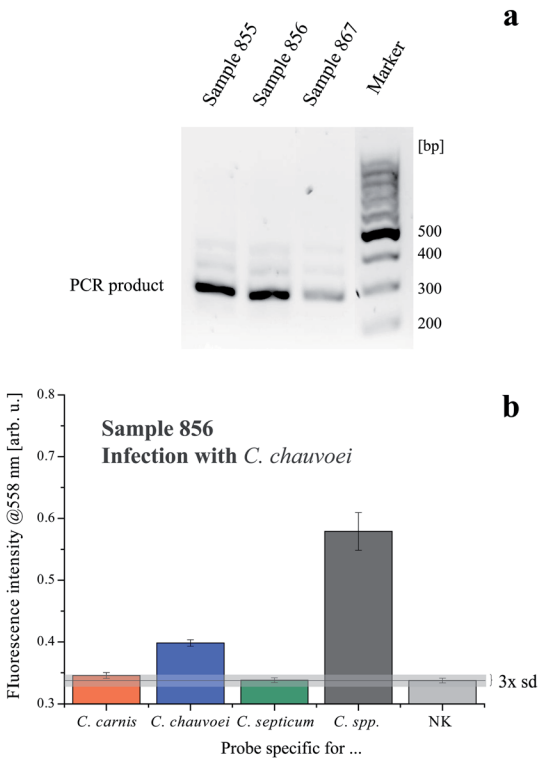

Fig. 6 (a) Analytical agarose gel to confirm the successful amplification of *C. chauvoei* DNA from three muscle tissue samples of blackleg-infected cattle. (b) Exemplarily the fluorescence intensities of the bead-based hybridization assay for sample 856 are displayed.

3.4 Limit of detection for the bead-based fluorescence assay

For determining the detection limit of the assay the amount of the PCR product in the reaction volume was varied between 0.32 nmol and 64 nmol. As can be seen in Fig. 7 the smallest amount of DNA does not allow significant discrimination from the blank signal. With regard to the standard deviations of the blank sample the limit of detection for *C. chauvoei* was 0.5 nmol PCR product. Overall the assay format allows highly specific and reliable detection as well as discrimination of *C. chauvoei*, *C. septicum* and *C. carnis*. In Fig. 4 the principle of the simple magnetic bead-based assay is depicted. After isolation of genomic DNA from a biopsy and its subsequent amplification with primers for a phylogenetic relevant target region of *Clostridium* spp., the PCR product is divided into four tubes.

Then the PCR product is immobilized *via* a biotin–streptavidin interaction on the surface of the magnetic beads. The generation of a single stranded capture probe is achieved by using NaOH treatment. Subsequently one of the four different dye-labeled detection probes is added per vial which enables very specific hybridization. If there is only a signal for the internal control and no signal for the three addressed *Clostridium* spp., an infection with the pathogen is present but further discrimination needs a follow-up assay (Fig. 4).

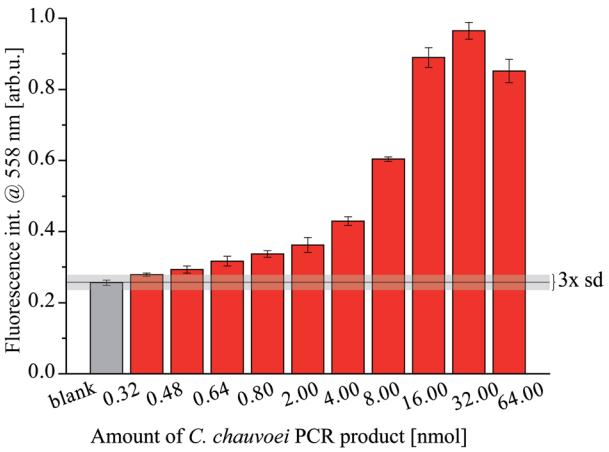

Fig. 7 Determination of the detection limit for the bead-based fluorescence assay (sd – standard deviation).

4 Conclusion

A simple magnetic bead-based fluorescence assay for the reliable discrimination between blackleg causative *C. chauvoei* and *C. septicum* has been introduced. The assay design includes the verification of different methods of generating single-stranded capture DNA. While it has been shown that all the three approaches yield suitable amounts of single-stranded DNA, the

denaturation with sodium hydroxide was proven to be the method of choice. This is due to the low cost of the reagent, the uncomplicated storage at ambient temperatures and the ease of use. It was demonstrated that the discrimination between at least three different *Clostridium* spp., namely *C. chauvoei*, *C. septicum* and *C. carnis* is possible with high specificity as well as sensitivity. The capability of our assay was further confirmed by successfully detecting *C. chauvoei* in muscle tissue samples of blackleg-infected cattle.

The introduced magnetic bead-based assay for *Clostridium* spp. detection has great potential for on-site applications. Magnetic beads are well known as binding platform for the extraction, concentration and separation of DNA. Thus fast portable extraction devices with easy separation by simple magnets are published.[34,35] Additionally simple and off-lab assays are described utilizing magnetic beads as detectable elements within magnetic readout techniques[36] or as sample carriers.[37] Combining and integrating these portable magnetic bead-based devices, the duration of an entire assay from DNA extraction to signal readout can be as low as two hours. Another important feature of our proposed method is the very low demand concerning instrumentation. The rapidness and sensitivity of chemoluminescence[38] or fluorescence reactions could be detected with small portable spectrometers.[39,40] Therefore parallel analysis of numerous samples is possible without much effort in the field.

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