Superconvective PCR: using a new, rapid real-time instrument to detect few DNA copies in tricky samples

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SUMMARY

qPCR analysis of DNA prepared from faecal samples is often compromised by the presence of inhibiting substances that reduce the sensitivity of the assay. Inhibition can be reduced by taking less sample but in situations with both inhibiting substances and a low number of target molecules, taking less sample will result in more false negative results. Another way to reduce the influence of inhibiting substances is to simply increase the PCR reaction volume. Here we report on using a new instrument, QuanTyper™-48, that runs reaction volumes from 20 to 200 µl to improve speed as well as detection sensitivity in samples with high levels of inhibition. We can show that large-volume, superconvective PCR overcomes the negative effects of PCR inhibition and thus avoids false negative results that are common with DNA prepared from faecal samples.

BACKGROUND

DNA prepared from bovine faecal samples is often tricky to analyse because of the concentration of inhibitors such as bile salts and polysaccharides (ref 1). PCR analysis is particularly difficult when trying to detect few target molecules, in a background of DNA originating from host cells, food and enteric bacteria. A common strategy for dealing with inhibition in routine diagnostics is to dilute and re-test the sample. Less sample and small reaction volumes, however, limit detection sensitivity. Therefore, if sensitivity needs to be high, other strategies must to be considered.

SuperConvection (ref 2), i.e. thermal ramping under elevated g-force, is useful for rapid and sensitive real-time PCR (ref 3). Here we report on using a new instrument, QuanTyper-48, to improve speed as well as detection sensitivity in samples with high levels of inhibition. We started out by testing the hypothesis that an increased PCR reaction volume could alleviate the effects of inhibition, using a model system where we amplified a human DNA sequence in the presence of an inhibitory DNA preparation from bovine faeces.

Mycobacterium avium subspecies *paratuberculosis (MAP)* is the cause of Johne's disease in cattle, where faeces is the clinical sample used for *MAP* detection. *MAP* is a hardy, difficult to culture and slow growing bacteria (may require as long as 16 weeks of culture for detection). Nevertheless, culture is considered the 'gold standard' for *MAP* detection. Thus, a sensitive and robust real-time PCR assay for *MAP* detection would be step forward for veterinary diagnostics. Aiming at such a step, we analysed 65 bovine faecal blind samples for the presence of *MAP*.

RESULTS

Figure 1 shows the result of a proof of principle study where we are able show how DNA amplification in a 25 µl PCR reaction, that was totally inhibited by 10 and 20% (v/v), respectively, of a DNA preparation from bovine faeces, could be gradually rescued by increasing the total PCR reaction volume up to 200 µl. The template amount , 4 gene copies, was kept constant.

Figures 2 and 3 show results from a panel of 65 bovine faecal blind samples analysed for the presence of MAP. We analysed all samples in duplicates in 20 µl using an in-house designed *MAP*-specific *LightUp* probe. All samples that appeared negative in 20 µl PCR reactions, plus those that were positive only in one out of two, were re-tested in 200 µl using the same sample amount or 5 times more sample, i.e. 40 µl. A total of 8 *negative* samples turned *positive* when analyzed in 200 µl (see examples in Figure 2). Two out of those were positive only when using 40 µl sample (see examples in Figure 3).

After having done the analyses, we were subsequently informed about the outcome of liquid cultures being grown in parallel. In comparison to liquid culture, we scored all 45 positive samples correctly but mis-classified three samples as false positives. One of them was positive only in one out of two when analysed in 20 µl but for obscure reasons negative in 200 µl. Another of the false positives was positive in only one out two duplicates in 200 µl indicative of a very low titer.

Regardless of the extraction method, we believe we have evidence saying that large-volume, superconvective PCR overcomes the negative effects of PCR inhibition and thus avoids false negative results that are common to DNA prepared from faecal samples.

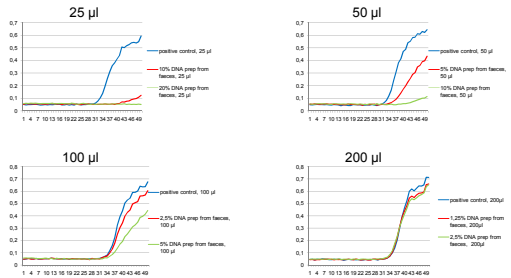

Figure 1. A 25 µl PCR reaction was totally inhibited by 10 and 20% (v/v), respectively, of a DNA preparation from bovine faeces. DNA amplification was gradually rescued by increasing the total PCR reaction volume.

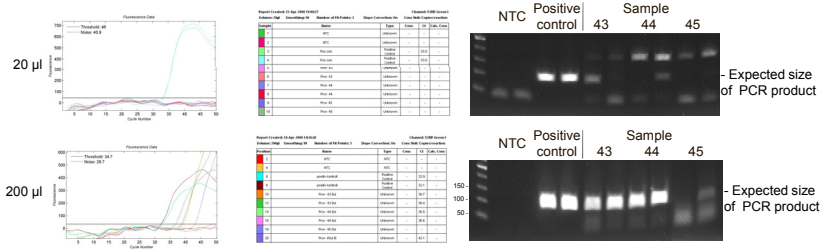

Figure 2. Samples that appeared negative in 20 µl were re-analysed in 200 µl reaction volume to dilute inhibitors. Interestingly, despite weak amplification in some of the 20 µl PCR reactions (lanes 6 and 9, upper gel (possibly the correct PCR products)), binding of and signalling from the 'LightUp' probe, that was used for detection, was also inhibited. 10 µl of each PCR reaction was separated by 2% agarose gel electrophoresis.

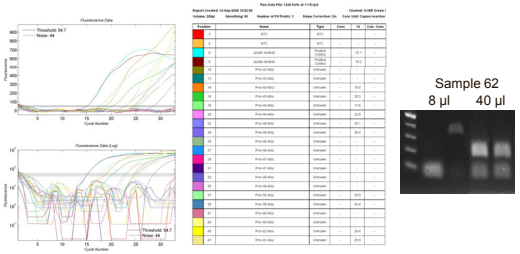

*Figure 3. Samples that appeared negative in 20 µl were also re-analysed in 200 µl using **40 µl sample** (i.e. 20% of the total DNA preparation). Ct values were markedly lower using this DNA amount. Agarose gel (right panel) shows sample 62, amplified either using 8 or 40 µl sample. 10 µl PCR reaction was separated by 2% agarose gel electrophoresis.*

METHODS, FIGURE 1

Two DNA preparations were used for the experiments presented in Figure 1. One bovine faeces DNA preparation was used as a background in qPCR reactions and one standard human DNA preparation was used as target for the amplification. DNA was prepared from bovine faeces using the QIAamp® DNA Stool Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Initial amounts of faeces were 100 mg for each preparation. The faeces were collected from a single dropping from a healthy animal. DNA was eluted in 100 µL. This preparation, having a DNA concentration of 15 ng/µL, was used as a standard DNA/inhibitor background in the experiment. The bovine DNA to human DNA ratios were 2750:1 for the 10% sample and 5500:1 for the 20% sample. The PCR amplified a 115 bp fragment of the corticotropin releasing hormone receptor 2 gene in human genomic DNA in 25, 50, 100, and 200 µl, respectively, with the following composition: 1X PCR reaction buffer, 2,5 mM MgCl2, 0,04 U/µl of Platinum Taq Polymerase, 0,125x SYBR Green I (all from Invitrogen, Carlsbad, CA), 0,2 µM each of forward and reverse primers (Biomers GmbH, Ulm, Germany), 200 µM dNTPs (GE Healthcare, Uppsala, Sweden). PCR temperature profile: 95 ºC for 90 s, followed by 50 cycles of 95 ºC for 0 s, 58 ºC for 3 s (5 s for 100 and 200 µl) and 72 ºC for 9 s. In each PCR reaction, regardless of volume, 4 copies of human DNA were used as target template.

METHODS, FIGURE 2 &3

1 g each of 65 bovine faecal blind samples was used for DNA preparation essentially as described by Herthnek, 2006 (ref 5). DNA was eluted in 200 µL whereof 8 (Figure 2) and 40 µl (Figure 3), respectively, was used for qPCR analysis. The PCR amplified a fragment of the IS900 sequence and was run with the following composition in 20 and 200 µl: 1X KOD Hot Start DNA Polymerase buffer, 1,5 mM $MgSO_4$, 0,01 U/µl of KOD Hot Start DNA Polymerase, 200 µM dNTPs (all from Novagen/Merck), 0,5 µM each of forward and reverse primer (Biomers GmbH, Ulm, Germany), 0,35 µM *MAP* specific *LightUp* probe (LightUp Technologies, Huddinge, Sweden). PCR temperature profile: 95 ºC for 120 s, followed by 50 cycles of 96 ºC for 0 s, 58 ° C for 6 s and 74 ºC for 3 s. Total PCR run times were 20 and and 40 minutes, respectively for 20 and 200 µl reactions. Noise and threshold levels were set automatically by the AlphaHelix 'QT-analysis' software.

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