Comparison of techniques for DNA extraction and agarose gel staining of DNA fragments using samples of *Cryptosporidium*

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ABSTRACT: Differentiating between the *Cryptosporidium* species and their subtypes using only microscopy is impossible. Therefore, molecular tools are indispensable for accurate species and subtype diagnosis. However, if these tools are to be used correctly and accurately, the techniques used must be standardised. In the present study, two molecular techniques for diagnosing *Cryptosporidium* infection in cows were compared to determine the optimal methods. For each technique, we tested two DNA extraction methods, several annealing temperatures for nested PCR reactions targeting the *18S*, *SSU rRNA* (small subunit ribosomal RNA), and the *GP60* (60 kDa glycoprotein) genes, and two types of DNA staining reagents, ethidium bromide and GelRed™. We determined that one of the tested protocols yields a higher purity of extracted DNA. Additionally, optimised temperatures for the nested PCR of the *18S* and *GP60* genes were established. Finally, we determined that the GelRed™ dye was more sensitive than ethidium bromide, and its low toxicity facilitates handling and disposal and reduces environmental contamination.

Keywords: *18S* gene; *GP60* gene; ethidium bromide; GelRed™

Cattle are one of the major hosts of *Cryptosporidium parvum*, a species that includes subtypes capable of infecting humans (Dixon et al. 2011; Meireles et al. 2011; Nazemalhosseini-Mojarad et al. 2011). Due to their high zoonotic potential, the genotyping of this protozoan is mainly performed using nested PCR and DNA sequencing using various DNA extraction methods and several gene targets (Coklin et al. 2010; Diaz et al. 2010; Fayer et al. 2010a,b; Das et al. 2011; Dixon et al. 2011; Meireles et al. 2011; Nazemalhosseini-Mojarad et al. 2011). The *18S* and *GP60* genes are the most commonly analysed genes for *Cryptosporidium* genotyping (Xiao 2010).

Cattle can be infected by several different *Cryptosporidium* species. Four species have been described as being the major infecting species: *C. parvum*, *C. andersoni*, *C. bovis*, and *C. ryanae* (Geurden et al. 2007; Fayer 2010). *C. parvum* is the most concerning due to its zoonotic potential (Diaz et al. 2010). Bovine cryptosporidiosis is highly concerning from both an animal production and a public health perspective (Becher et al. 2004; Adamska et al. 2012).

Differentiating *Cryptosporidium* species and diagnosing *C. parvum* genotypes is impossible using microscopy due to the morphological similarities between the oocysts of the various species and their subtypes (Jex and Gasser 2008; Fayer 2010; Das et al. 2011). Therefore, molecular tools are being increasingly used to detect and differentiate between *Cryptosporidium* species and subtypes (Xiao 2010). Indeed, even if a sample contains only a small amount of *Cryptosporidium* DNA, this genetic material can be amplified thousands of times after extraction (Leng et al. 1996; Morgan and Thompson 1998).

DNA extraction is the first step in the molecular characterisation of a species, and it is an essential step for ensuring the success of the downstream enzymatic reactions (Romano and Brasileiro 1999). Several methods for extracting *Cryptosporidium* DNA are described in the literature. These studies use protocols developed by researchers, commer-

cial kits, or a combination of the two (Becher et al. 2004; Castro-Hermida et al. 2007; Feng et al. 2007; Geurden et al. 2007; Langkjaer et al. 2007; Mendonca et al. 2007; Broglia et al. 2008; Coklin et al. 2009; Keshavarz et al. 2009; Coklin et al. 2010; Diaz et al. 2010; Fayer et al. 2010a,b; Das et al. 2011; Dixon et al. 2011; Meireles et al. 2011; Nazemalhosseini-Mojarad et al. 2011; Adamska et al. 2012).

In addition to the extraction protocol, one must choose the best dye for staining agarose gels containing the DNA from the PCR. Many substances exist for this purpose including ethidium bromide, SYBR Gold, SYBR Green, GoldView, GeneFinder, GoldStar, GelGreen, and GelRed™ (Huang and Fu 2005; Huang et al. 2010). Despite its disadvantages, such as high toxicity, mutagenic ability (Huang and Fu 2005), and difficult environmental decontamination, ethidium bromide is still the most commonly used dye for visualizing *Cryptosporidium* spp. DNA fragments (Geurden et al. 2007; Broglia et al. 2008; Coklin et al. 2009; Fayer et al. 2010a; Das et al. 2011).

GelRed™ is a red fluorescing nucleic acid dye that is visible under the same ultraviolet light wavelength used to visualise ethidium bromide (Huang et al. 2010). GelRed™ is a member of a new generation of dyes, has chemical characteristics designed to minimise potential interactions with nucleic acids in living cells, is thought to have low toxicity, and is not very mutagenic. In addition to these characteristics, it is also advantageous because it has low environmental contamination levels (Huang et al. 2010; manufacturer's manual: htpp//www.biotium.com). Studies using GelRed™ are rare, and no study has reported using it to stain *Cryptosporidium* spp. DNA fragments in agarose gels.

The aims of this study were to compare two DNA extraction methods in calf faecal samples that were positive for the presence of *Cryptosporidium* spp. oocysts by microscopy and to compare two staining methods on the DNA fragments obtained from these samples, using ideal conditions for amplifying *Cryptosporidium* spp. DNA fragments from the *18S* and *GP60* genes.

MATERIAL AND METHODS

Sample collection, processing, and microscopic diagnosis. Faecal samples were obtained from three naturally infected dairy calves. The samples were considered to be positive for *Cryptosporidium* spp. if oocysts were observed under a light microscope using both bright field and phase contrast. The selected samples had between three and 15 *Cryptosporidium* oocysts per visual field.

DNA extraction.Two different extraction protocols were used for each sample to compare extraction efficiency. The first protocol (Extraction Protocol I) used a commercially available Qiagen kit (QIAamp DNA Stool Mini Kit) (Geurden et al. 2007; Langkjaer et al. 2007; Coklin et al. 2009; Das et al. 2011; Adamska et al. 2012), and the manufacturer's instructions were followed, with a few modifications.

The two incubation periods were performed at 95 °C. During the longer of the two incubations (10 min), the protocol was slightly modified to optimise the extraction of *Cryptosporidium* spp. oocyst DNA. Instead of using a water bath, as prescribed in the original protocol, a temperature-controlled shaker was used at 1000 × rpm to aid in the sample homogenisation process. The samples were eluted in 100 µl of AE buffer (supplied by the manufacturer) after DNA extraction instead of the 200 µl volume suggested in the instruction manual (Fayer et al. 2010a).

The second protocol (Extraction Protocol II) was developed and described by Huber et al. (2007).

Quality of the extracted DNA. After completing the extraction protocols (I and II), 10 µl of each sample was run on a 1% agarose gel (100 V for 40 min) using an electrophoresis system (Mupid-ex UMini-gel Basic System/MEX-002). To measure the integrity of the extracted DNA, the DNA was stained using either GelRed™ (10×) or ethidium bromide (5 µg/ml). The DNA was then visualized under ultraviolet light, and the gels were analysed using a gel imager (Bio-Rad – Gel Doc™ EQ). The DNA quality was also measured using a spectrophotometer (Thermo – Nanodrop 2000) by calculating the 260/280 absorbance ratio of each sample.

Agarose gel staining methods. Two staining methods, ethidium bromide and GelRed™, were used to visualise the *Cryptosporidium* spp. DNA in the agarose gels. The ethidium bromide stock and working solutions used in the present study were 5 mg/ml and 5 µg/ml, respectively. The GelRed™ stock solution was used at a 10 000× concentration, and the working solution was used at a 10× concentration.

A minor change was made to the GelRed™ manufacturer's protocol in the present study. The stain was mixed with both the samples and the sample buffer and then loaded onto the agarose gel. For

every 5 µl of DNA, 2 µl of GelRed™ and 1 µl of 6× loading buffer (type III) were added (Sambrook and Russel 2001).

Both stains were visualized using an ultraviolet transilluminator using a wavelength range of 548–630 nm and a specific filter that was capable of visualizing DNA fragments using either ethidium bromide or GelRed™.

Positive and negative controls for the PCR and nested PCR reactions. A positive *Cryptosporidium parvum* control that was previously sequenced and submitted to GenBank (accession number DQ885333) was used to standardise the PCR and nested PCR reactions (Huber et al. 2007). Each PCR and nested PCR reaction contained a positive *C. parvum* control at a concentration of 17 ng/µl. To establish the detection limit of the positive control in both reactions, five serial dilutions were made using ultrapure nuclease-free water (Promega). The concentrated sample and its dilutions were amplified, purified, and then quantified on a spectrophotometer (Thermo – Nanodrop 2000).

Standardisation of the PCR and nested PCR reactions for the 18S gene. The PCR reactions for the *SSU rRNA* gene or the *18S* gene used the following primers to obtain a 1325 base pair (bp) amplicon: 18SF: 5'-TTC TAG AGC TAA TAC ATG CG-3' (*forward*) and 18SR: 5'-CCC ATT TCC TTC GAA ACA GGA-3' (*reverse*) (Xiao et al. 1999; Fayer et al. 2010a). The nested PCR for the same target used the following primers, resulting in an approximately 830 bp amplicon: 18SNF: 5'-GGA AGG GTT GTA TTT ATT AGA TAA AG-3' (*forward*) and 18SNR: 5'-AAG GAG TAA GGA ACA ACC TCC A-3' (*reverse*) (Xiao et al. 1999; Fayer et al. 2010a).

Each PCR reaction contained 4mM $MgCl_2$ (Invitrogen), 0.2µM of each primer (18SF and 18SR – Invitrogen), 1 × Taq buffer (Invitrogen), 200µM of each deoxyribonucleotide (dNTPs – Invitrogen), 1.0 IU Platinum Taq Polymerase (Invitrogen), and template DNA. The total volume of each PCR was brought to 25 µl using ultrapure nuclease-free water (Promega). The nested PCR used the same conditions as the PCR; however, the $MgCl_2$ concentration was decreased to 2mM, and 0.2µM of the 18SNF and 18SNR primers were used.

The thermal cycle profile of the nested PCR had an initial temperature of 94 °C for 3 min, followed by a total of 35 cycles using a DNA denaturation step at 94 °C for 45 s, an oligonucleotide hybridisation step using a temperature gradient from 55 °C to 60 °C for 45 s, and a product extension step at 72 °C for 1 min. At the end of the 35 cycles, a final extension step was performed at 72 °C for 7 min.

After all of the cycles were completed, the samples were removed from the thermocycler, and 5 µl of each reaction was visualised by electrophoresis using a 1% agarose gel (100 V for 40 min) stained with ethidium bromide (5 µg/ml) or GelRed™ (10×). A 6× loading buffer (type III) (Sambrook and Russel 2001) and a 1 Kb Plus DNA Ladder (Invitrogen) were used. The agarose gels were observed under ultraviolet light, analysed, and photographed.

Standardisation of the PCR and nested PCR reactions for the *GP60* gene. The positive control for the *GP60* PCR reaction was the same as that used for the *18S* gene with the additional nested-PCR reaction, but with *GP60* as the target gene. To obtain a 950 bp amplicon, the following primers were used for the PCR: AL3531: 5'-ATA GTC TCC GCT GTA TTC-3' (*forward*) and AL3534: 5'-GCA GAG GAA CCA GCA TC-3' (*reverse*) (Peng et al. 2003). For the nested PCR, the primers were AL3532: 5'-TCC GCT GTA TTC TCA GCC-3' (*forward*) and AL3533: 5'-GAG ATA TAT CTT GGT GCG-3' (*reverse*), which targeted *GP60*, and they generated an approximately 550 bp amplicon (Peng et al. 2003).

The thermal cycling parameters used for the *GP60* nested PCR included an initial temperature of 95 °C for 3 min, followed by 35 cycles of a DNA denaturation step at 94 °C for 45 s, an oligonucleotide hybridisation step using a temperature gradient from 50 °C to 60 °C for 45 s, and an extension step at 72 °C for 1 min. At the end of the 35 cycles, a final extension step at 72 °C for 10 min was performed.

The protocols described above for the *SSU rRNA* gene amplification reactions were also used to visualise the DNA fragments obtained from the PCR and nested PCRs. Because the PCR amplification of the *GP60* gene using the primers described by Peng et al. (2003) was not satisfactory, we used those reported by Sulaiman et al. (2005) (i.e., AL3531 and AL3533 in the first PCR reaction and AL3532 and LX0029 in the nested PCR reaction (5'-CGA ACC ACA TTA CAA ATG AAG T-3' – *reverse*)), which resulted in an approximately 400 bp product. The thermal cycling parameters described above were used in this reaction. All primer pairs used in the *GP60* reactions were acquired from Invitrogen.

Purification and quantification. The nested PCR products were purified using the Wizard® SV Gel and PCR Clean-up System (Promega). The purified products were quantified using a spectrophotometer (Thermo – Nanodrop 2000).

RESULTS

Sample extractions

Both of the DNA extraction methods (I and II) used in this study were successful in breaking down the oocyst cell walls and collecting the DNA of the *Cryptosporidium* spp. obtained from calf faecal samples. The quality of the extracted DNA was evaluated in two different manners. First, the 260/280 absorbance ratio was calculated by quantifying the sample using spectrophotometry. Second, the amount of DNA degradation was measured using agarose gel electrophoresis. The extracted DNA concentrations and the absorbance ratios are displayed in Table 1.

The DNA from Extraction Protocol I was not observed in the agarose gel. Degradation of the extracted material was observed in the DNA collected using Extraction Protocol II.

Table 1. Quantification and absorbance ratios of the DNA extracted from *Cryptosporidium* spp. oocysts obtained from calf fecal samples

Sample	Extraction protocol	Nucleic acid concentration (ng/µl)	260/280 ratio
1	I	14.1	1.8
2		4.9	2.0
3		8.3	1.8
1'	II	35.9	1.9
2'		13.7	1.8
3'		100.2	1.3

Temperature gradient

***18S* target gene**. All of the samples containing *Cryptosporidium* DNA were extracted using Extraction Protocol I and amplified by both PCR and nested PCR using a temperature gradient. Based on the visualisation of the amplified products in the agarose gel, the ideal annealing temperature for the PCR was determined to be 58 °C, and the ideal annealing temperature for the nested PCR was found to be 59 °C.

***GP60* target gene**. In the *GP60* PCR and nested PCRs, the positive control and its first two dilutions were used as samples. Tests using the primer pairs described by Peng et al. (2003) did not have the expected efficiency in the conditions used in our laboratory. Therefore, we used the primers described by Sulaiman et al. (2005), which showed better results under our laboratory conditions. The optimal annealing temperature for the PCR and nested PCRs using the Sulaiman et al. (2005) primers was 56.8 °C.

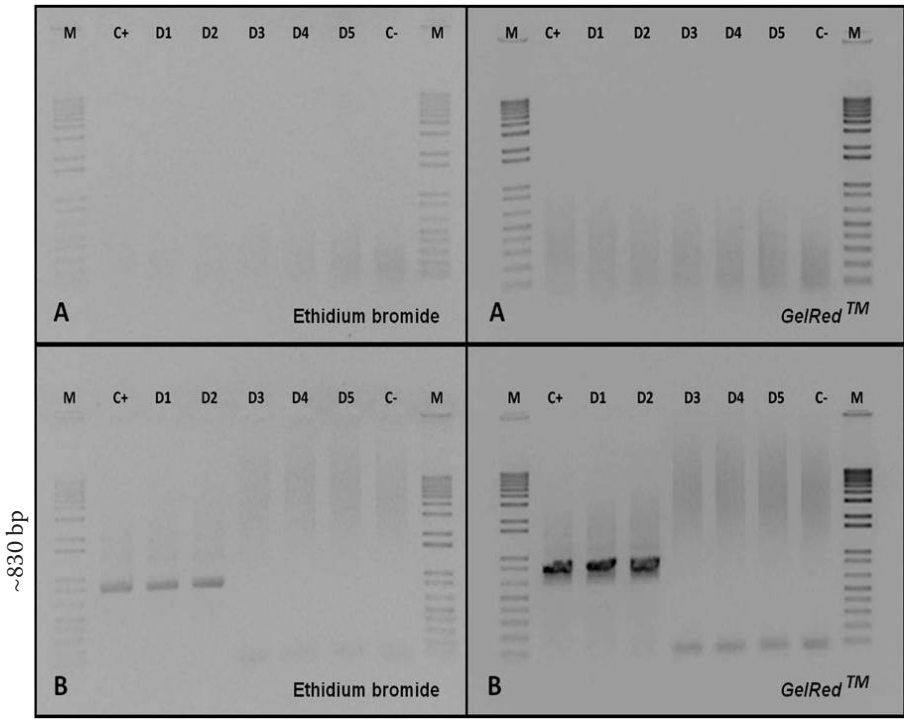

Figure 1. Positive and negative controls for the *18S* PCR and nested-PCR analyzed on a 1% agarose gel stained with ethidium bromide and GelRed™. The UV exposure time was approximately 0.9 seconds. A = initial PCR reaction; B = nested PCR reaction; L = ladder; C^+ = concentrated positive control; D1 = dilution 1 = D2 = dilution 2; D3 = dilution 3; D4 = dilution 4; D5 = dilution 5; C^- = negative control

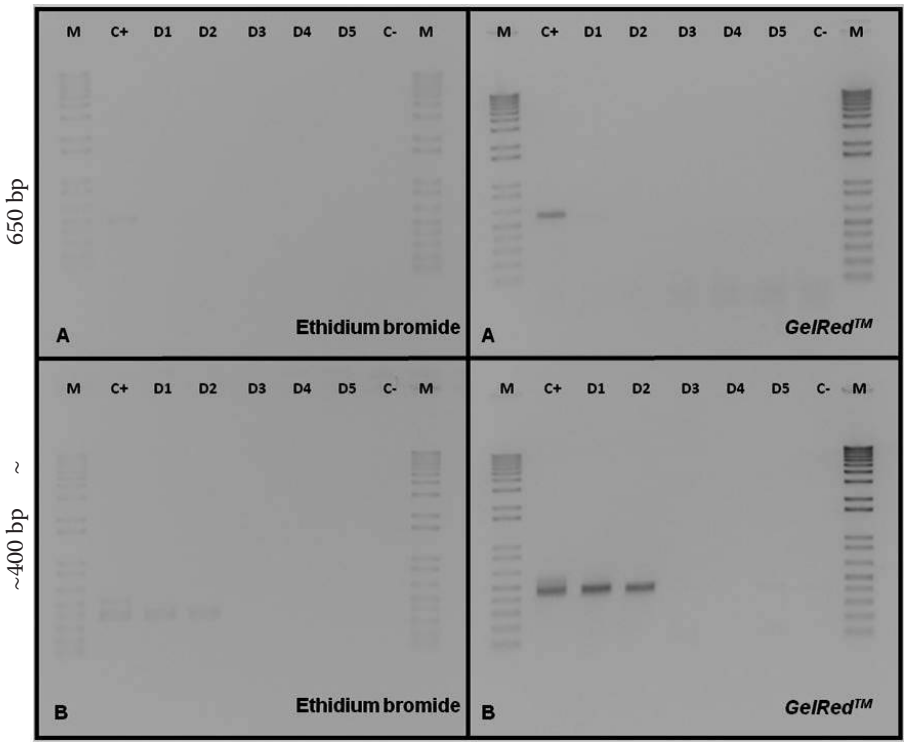

Figure 2. Positive and negative controls for the *GP60* PCR and nested-PCR analyzed on a 1% agarose gel stained with ethidium bromide and GelRed™. The UV exposure time was approximately 0.9 s. A = initial PCR reaction; B = nested PCR reaction; L = ladder; C^+ = concentrated positive control; D1 = dilution 1 = D2 = dilution 2; D3 = dilution 3; D4 = dilution 4; D5 = dilution 5; C^- = negative control

PCR and nested PCR of the positive control – *18S* and *GP60* gene targets

From the *Cryptosporidium parvum* purified DNA, we performed agarose gel analyses of the *18S* (Figure 1) and *GP60* (Figure 2) PCR products from the positive control, the template dilutions, and the negative control. The optimal temperatures determined for the gradient PCR and nested PCRs were used to measure the limit of detection of the positive controls.

DISCUSSION

DNA extraction

Obtaining high-quality genetic material from a given DNA extraction method is essential for successful PCR amplification (Chiari et al. 2009). Samples must contain minimal amounts of impurities to prevent inhibition of the enzymatic reactions or interference with the gel migration patterns (Romano and Brasileiro 1999; Adamska et al. 2012). Therefore, the higher the purity of the extracted samples, the better the results of the nested PCR. Choosing the best extraction protocol can be based on two parameters: (1) the integrity of the DNA when analysed using an agarose gel; and (2) the 260/280 absorbance ratio as measured using spectrophotometry. A 260/280 ratio between 1.8 and 2.0 is indicative of pure DNA, while a ratio below 1.8 indicates contamination by proteins and a ratio above 2.0 indicates phenol contamination (Romano and Brasileiro 1999). In the present study, the choice of extraction protocols for subsequent amplification via nested PCR considered both the integrity of the DNA when analysed using agarose gel electrophoresis and the absorbance ratio, as measured using a Nanodrop spectrophotometer.

The DNA collected from all samples using Extraction Protocol I had low DNA yields; however, the total yield was sufficient for DNA amplification, and the material had an ideal 260/280 absorption ratio. This did not occur with any of the samples when Extraction Protocol II was used. Thus, Extraction Protocol I was selected for the nested PCR experiments.

When we evaluated the integrity of the DNA using agarose gel electrophoresis, we observed degradation in the samples extracted using Protocol II (i.e., they did not have a defined DNA band). The degree of DNA degradation in the samples obtained from Extraction Protocol I could not be determined by gel electrophoresis, which was likely due to the low DNA yields. The reduced quantity of DNA may be because the kit purifies the samples during the extraction. This effect was also observed in other

studies where the same commercial kit was used to purify the DNA obtained from the extraction (Sulaiman et al. 2005; Feng, et al. 2007).

In addition to the technical factors, another factor that influenced the choice to use Extraction Protocol I was the time required to complete the procedure. This extraction method requires fewer steps to obtain the final product, thus decreasing the probability of errors occurring during the procedure. Therefore, Protocol I is a faster and more practical technique than Extraction Protocol II. Protocol I is also less expensive than Protocol II.

Temperature gradient

After optimisation using an annealing temperature gradient, the two primer pairs used for the *18S* gene target effectively identified samples from the *Cryptosporidium* genus. These primers have a theoretical annealing temperature; however, the annealing temperature can vary depending on the laboratory conditions, such as the reagents and equipment used. Therefore, we generally found it difficult to reproduce the results of other authors.

The use of a temperature gradient allows measurement of the behaviour of samples at various temperatures, thus allowing the choice of the best temperature. Low annealing temperatures can produce non-specific products. This problem is solved using higher temperatures; however, the amount of product obtained from the PCR is also reduced (Halfeld-Vieira et al. 2001).

Several authors (Xiao et al. 1999; Huber et al. 2007; Castro-Hermida et al. 2009; Khan et al. 2010) described 55 °C as the optimal annealing temperature for the *18S* primers used in both the PCR and nested PCR reactions. However, under the conditions used in our laboratory, this temperature was not very efficient because it allowed non-specific annealing during the PCR, which was observed using agarose gel electrophoresis.

Using higher temperatures in the PCR and nested PCR reactions solved the non-specific annealing problems in the *Cryptosporidium* spp. samples. Higher temperatures were also used by Feltus et al. (2006), Fayer et al. (2006, 2010a), and Coklin et al. (2010); however, these authors used a temperature that was one degree higher for the PCR reaction and one degree lower for the nested PCR than the temperatures used in the present study. We used an annealing temperature of 58 °C in the PCR to inhibit non-specific annealing without greatly decreasing the amount of product. We used a temperature of 59 °C in the nested PCR to increase the specificity of the reaction. We used the same procedure (i.e., performing a temperature gradient for each of the DNA amplification reactions) for the *GP60* gene target.

An annealing temperature of 50 °C, which was used in the studies conducted by Peng et al. (2003) and Sulaiman et al. (2005), was not optimal for the laboratory conditions described in the present study. The use of this annealing temperature produced a DNA quantity that was barely visible in the agarose gel. We determined that increasing the temperature, described before, in 6.8 °C improved the primer annealing, as determined by agarose gel electrophoresis.

DNA visualisation

Staining DNA with ethidium bromide after gel electrophoresis is widely used to visualise DNA fragments from *Cryptosporidium* spp. (Diaz et al. 2010; Fayer et al. 2010a,b; Khan et al. 2010; Das et al. 2011; Nazemalhosseini-Mojarad et al. 2011). In the present study, gel comparisons were used to identify the detection limits for the *18S* and *GP60* positive controls. We observed that staining with GelRed™ was superior to ethidium bromide for determining the presence of DNA and its fragments because this stain was more sensitive than ethidium bromide.

Few studies have been performed to demonstrate the advantages of using GelRed™ versus other staining methods; however, Huang et al. (2010) emphasizes its low cost, the absence of changes in DNA fragment mobility, its long shelf-life, improved DNA visualisation using a UV transilluminator, and its low toxicity.

Using GelRed™ at a 10× concentration instead of the 100× used by Huang et al. (2010) may be of interest economically because it further reduces staining cost without affecting the dye's sensitivity. This was observed in the present study, where using 10× GelRed™ effectively stained the DNA fragments of *Cryptosporidium* spp. in the agarose gels.

Huang et al. (2010) noted that using GelRed™ at 100× did not change the mobility of the DNA during agarose gel electrophoresis; however, this was not confirmed in the present study. Analysis of the DNA fragments produced by nested PCR determined that the amplicons from PCRs generally produce a linear pattern, while those produced

by nested PCR do not behave in the same manner. Several factors can affect the migratory behaviour of DNA fragments, including DNA fragment size, agarose gel concentration, voltage, and the electrophoresis buffer. The manufacturer's manual (htpp// www.biotium.com) also states that because the GelRed™ molecule is 10-fold larger than the ethidium bromide molecule, it integrates with higher affinity for this nucleic acid, and thus, a greater number of molecules bind to the DNA. This can often lead to slower migration, especially for DNA fragments larger than 400 bp.

Several advantages of using Extraction Protocol I (Qiagen kit) were observed in the present study, including purity of the resulting genetic material, the speed of the procedure, and the reduced cost of obtaining DNA. By using a temperature gradient for the nested PCR reactions, it was possible to determine the optimal temperatures for amplifying *Cryptosporidium* DNA, thereby improving the quality of the DNA amplicons obtained from the nested PCRs of both the *18S* and *GP60* genes.

The improved performance of the GelRed™ stain compared to ethidium bromide was mainly due to its higher sensitivity for visualising DNA fragments and its low toxicity, which facilitates its handling and disposal and reduces environmental contamination.

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