Species-specific amplification by PCR of ribosomal DNA from some equine strongyles

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SUMMARY

The first and second internal transcribed spacer sequences of 28 morphologically-defined species of horse strongyle were characterized, and specific oligonucleotide primers were designed for some species based on the nucleotide differences. Utilizing these primers, a PCR approach was developed for the specific amplification of ribosomal DNA of *Strongylus vulgaris*, *Cyathostomum catinatum*, *Cylicocyclus nassatus*, *Cylicostephanus longibursatus* or *Cylicostephanus goldi*. The method allowed the species-specific amplification of parasite DNA derived from faecal samples and/or copro-cultures, demonstrating the potential of the approach for the diagnosis of equine strongyloidosis. The establishment of this PCR assay also has implications for studying the biology and epidemiology of equine strongyles and anthelmintic resistance using faecal egg count reduction tests.

Key words: horse strongyles, ribosomal DNA, internal transcribed spacers, genetic markers, species identification, diagnosis, PCR.

INTRODUCTION

Infections of horses with parasitic nematodes belonging to the order Strongylida are of major veterinary significance. There are over 60 species of equine strongyloids belonging to 2 subfamilies, the Strongylinae (large strongyles) and the Cyathostominae (small strongyles or cyathostomes) (Popova, 1965; Lichtenfels, 1975; Hartwich, 1986). Traditionally, *Strongylus vulgaris* (subfamily Strongylinae) was considered the most important parasite because of its high prevalence and pathogenic effects (i.e. verminous arteritis) in the horse (Herd, 1990). Over the last 2 decades, the use of modern anthelmintics has significantly reduced the prevalence of *Strongylus* species and the incidence of clinical cases of verminous arteritis (Herd, 1990). However, the excessive use of anthelmintics (particularly benzimidazoles) has had adverse effects, including the development of anthelmintic resistance in cyathostomes, leading to a substantial increase in the prevalence of cyathostome infections and associated clinical cases of larval cyathostomosis (reviewed by Herd, 1990; Klei & French, 1998). Under these circumstances, it has become evident that an integrated strategy is needed for the effective control of strongyloidosis (e.g. Huntington *et al*. 1993).

Accurate *ante mortem* diagnosis of strongyle infections in horses is a central component of control. However, current diagnostic methods are inaccurate and time-consuming to perform. Laboratory procedures involve the flotation of nematode eggs from faeces combined with larval cultures (Boch & Supperer, 1992). Given that the identification of individual eggs to the species or genus level is not possible using morphological parameters, they are cultured to the infective 3rd larval stage to allow their identification using a microscope. This procedure takes 7 days for cyathostomes and 10–14 days for large strongyles and is labour intensive. Even so, it is frequently impossible to identify larvae to genus or species.

Attempts to develop alternative techniques for the diagnosis of strongyle infections of horses have been undertaken (Nichol & Masterson, 1987; Soulé, 1990; Weiland *et al*. 1991), but a reliable system has yet to be developed. The advent of DNA technology has provided useful approaches for the identification of parasites and diagnosis of parasitic infections (Comes *et al*. 1996; McManus & Bowles, 1996; Prichard, 1997). In particular, the polymerase chain reaction (PCR) (Mullis *et al*. 1986; Saiki *et al*. 1988) provides a promising avenue for the specific identification of nematode eggs and larvae, and hence for the diagnosis of infection because of its exquisite sensitivity. In order to establish a PCR-based method for the species-specific identification of parasites or diagnosis of infection, it is of paramount importance to utilize a nucleotide sequence which is unique to a specific parasite. Ribosomal DNA (rDNA) provides useful targets for the definition of species markers in strongylid nematodes (Campbell, Gasser & Chilton, 1995; Chilton, Gasser & Beveridge, 1995; Gasser &

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Hoste, 1995; Hoste *et al*. 1995; Stevenson, Chilton & Gasser 1995; Gasser *et al*. 1996; Newton *et al*. 1997; Romstad *et al*. 1997; Kaye *et al*. 1998) because there is a strong tendency for rDNA to maintain sequence homogeneity within a species (i.e. lack of intraspecific variation) (Elder & Turner, 1995). Also of importance, is that rDNA sequences are repetitive and therefore abundant in individual organisms (i.e. usually hundreds of copies per parasite) (Elder & Turner, 1995), making the development of sensitive diagnostic assays feasible.

DNA studies have shown that the 2 internal transcribed spacers (ITS-1 and ITS-2) of rDNA provide genetic markers for the delineation of some species of horse strongyles (Campbell *et al*. 1995; Hung *et al*. 1996, 1997). Exploiting the sensitivity of PCR, it has been demonstrated that individual eggs and adults of the genus *Strongylus* (subfamily Strongylinae) can be unequivocally identified to species by their ITS-2 sequence (Campbell *et al*. 1995). It has also been shown that PCR-based restriction fragment length polymorphism (RFLP) analysis of the ITS (ITS-1 and ITS-2 plus the intervening 5·8S rRNA gene) allowed the specific identification of another 13 species of equine strongyles (Gasser *et al*. 1996). This indicated that the ITS-1 and ITS-2 may provide genetic markers for any species of equine strongyle, irrespective of developmental stages. In the present study, we extended this work in order to develop a PCR-based technique for the specific amplification of rDNA from *S. vulgaris* and 4 of the most prevalent small strongyles, *Cyathostomum catinatum*, *Cylicostephanus longibursatus*, *Cs. goldi* and *Cylicocyclus nassatus* (see Ogbourne, 1976; Reinemeyer *et al*. 1984; Krecek, Reinecke & Horak, 1989; Antiporda & Eduardo, 1990; Mfitilodze & Hutchinson, 1990; Bucknell, Gasser & Beveridge, 1995; Gawor 1995; Cirak, Hermosilla & Bauer, 1996; Lyons *et al*. 1997). To do this, we determined the ITS-1 and ITS-2 rDNA sequences for 28 morphologically-defined species of horse stongyles and assessed the usefulness of these sequences for their identification. Based on the sequences, we designed oligonucleotide primers to species-specific regions and evaluated their specificity in PCR. Then, we determined the sensitivity of the PCR system and evaluated its capacity to specifically amplify rDNA from copro-culture and faecal samples.

MATERIALS AND METHODS

Parasites

Adult strongyles representing 28 species were collected at necropsy from the large intestines of horses, washed extensively in physiological saline and identified according to existing keys and descriptions (Popova, 1965; Lichtenfels, 1975; Hartwich, 1986; Lichtenfels *et al*. 1997) (Table 1). The anterior and posterior ends of each worm were cut off, mounted on a glass slide, cleared in lactophenol and preserved in polyvinyl lactophenol as permanent mounts for reference. Other common parasites of horses, *Trichostrongylus axei*, *Parascaris equorum*, *Gasterophilus intestinalis*, *Draschia megastoma*, *Anoplocephala perfoliata*, *Oxyuris equi* and *Fasciola hepatica*, were also collected, washed extensively and identified morphologically. All samples were frozen at −70 °C prior to DNA isolation.

Coprological samples

Faecal samples were collected from the rectum of 7 horses known to be naturally infected with a range of strongylid nematodes before and 2 weeks after administration of the recommended dosage (7·5 mg/kg) of fenbendazole (Panacur, Hoechst). Third-stage larvae were obtained from copro-cultures (28 °C for 10 days) and mixed (1:1) with 100% ethanol for storage at −20 °C. Faecal samples were also obtained from 3 horses with patent monospecific infection of *S. vulgaris*. The numbers of *S. vulgaris* eggs per gram of faeces were determined by a modified Stoll technique (see Klei & Torbert, 1980). Faecal samples were mixed with an equal volume of 70% ethanol and also stored at −20 °C.

Isolation and purification of DNA

DNA was isolated from individual adult nematodes, copro-cultures, faecal samples and horse musculature by sodium dodecyl-sulphate/proteinase K treatment (Gasser *et al*. 1993). After this treatment, the Wizard™ DNA Clean-Up system (Promega) was used to directly purify DNA from adult nematodes and host musculature. The QIAamp tissue system (Qiagen) was employed to purify DNA from samples derived from faeces. Purification followed the manufacturer's protocols.

Enzymatic amplification

Regions of rDNA were amplified by PCR (Saiki *et al*. 1988) using appropriate primer sets (Fig. 1). PCR reactions (50 μl) were performed in 10 mM Tris-HCl, pH 8·4; 50 mM KCl; 3·0 mM $MgCl_2$; 250 μM of each dATP, dCTP, dGTP and dTTP; 50–100 pmol of each primer (either unlabelled or labelled with γ^{33}P-ATP) and 2 U *Taq* polymerase (Promega) under the following conditions in a thermocycler (Perkin Elmer Cetus): an initial denaturation at 94 °C for 5 min, then 30 cycles of 94 °C, 30 sec (denaturation); 55 °C, 30 sec (annealing); 72 °C, 30 sec (extension), followed by a final extension of 5 min at 72 °C. Usually,

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Table 1. Genomic DNA samples derived from individual adult nematodes used for PCR and sequence analyses

Subfamily/species	Sample codes	Geographical origin*
Cyathostominae		
Cylicocyclus ashworthi	Ccb2x	Benalla, Victoria
	K20	Glasgow, United Kingdom
	K31	Glasgow, United Kingdom
Cc. brevicapsulatus	143–1	Laverton, Victoria
	143–4	Laverton, Victoria
Cc. insignis	Cci1	Werribee, Victoria
	Cci3	Traralgon, Victoria
	Cce1x	Traralgon, Victoria
Cc. leptostomus	Ccl1	Echuca, Victoria
Cc. nassatus	Ccn1	Dandenong, Victoria
	Ccn2	Dandenong, Victoria
	M346	Glasgow, United Kingdom
	Ccb1x	Echuca, Victoria
	Ccb3x	Dandenong, Victoria
Cc. ultrajectinus	1–8	Laverton, Victoria
	1–12	Werribee, Victoria
	1–13	Werribee, Victoria
	1–20	Laverton, Victoria
Cyathostomum catinatum	Cyca1	Echuca, Victoria
	Cyca2	Echuca, Victoria
	Cyca3	Gisborne, Victoria
Cy. coronatum	Cycol	Echuca, Victoria
	6–8	Laverton, Victoria
	J65	Glasgow, United Kingdom
Cy. labiatum	Cyl1	Laverton, Victoria
Cy. labratum	Cylb1	Laverton, Victoria
Cy. pateratum	Cyp1	Echuca, Victoria
Cylicostephanus bidentatus	L103	Glasgow, United Kingdom
	1–19	Laverton, Victoria
Cs. calicatus	Csc1	Echuca, Victoria
Cs. goldi	Csg1	Howlong, New South Wales
	F232	Glasgow, United Kingdom
	Cyco2x	Dandenong, Victoria
Cs. longibursatus	Csl1	Echuca, Victoria
	S351	Glasgow, United Kingdom
Cs. minutus	Csm2	Gisborne, Victoria
Cs. poculatus	Csp2	Howlong, New South Wales
Cylicodontophorus bicoronatus	Cdb1	Laverton, Victoria
	Cdb2	Laverton, Victoria
Cd. mettami	Cdm1	Laverton, Victoria
Poteriostomum imparidentatum	Pi1	Traralgon, Victoria
P. ratzii	Cde1x	Fish Creek, Victoria
	Cde3x	Fish Creek, Victoria
Tridentoinfundibulum gobi	S305	Glasgow, United Kingdom
Strongylinae		
Craterostomum acuticaudatum	F211	Glasgow, United Kingdom
Oesophagodontus robustus	Or1	Werribee, Victoria
Triodontophorus serratus	Ts1	Gisborne, Victoria
Strongylus edentatus	Sed1	Dandenong, Victoria
S. equinus	Seq2	Werribee, Victoria
S. vulgaris	Sv1	Werribee, Victoria

* From Australia unless otherwise stated.

1 μl (5–10 ng) of purified template was added to each PCR reaction. Control samples without DNA were included in each PCR run. Host DNA was subjected to the same amplification procedure as parasite DNA.

Amplicons were subjected to electrophoresis in 2·5% agarose (80 V) using 65 mM Tris–HCl, 22·5 mM boric acid, 1·25 mM EDTA, pH 9 (TBE) as the buffer. Agarose gels were stained with ethidium bromide and photographed upon transillumination. Amplicons were also subjected to electrophoresis (20 W for 2 h) in neutral 5% polyacrylamide gels (29:1 = acrylamide:*bis*) using TBE. Gels containing radiolabelled amplicons were dried on to blotting

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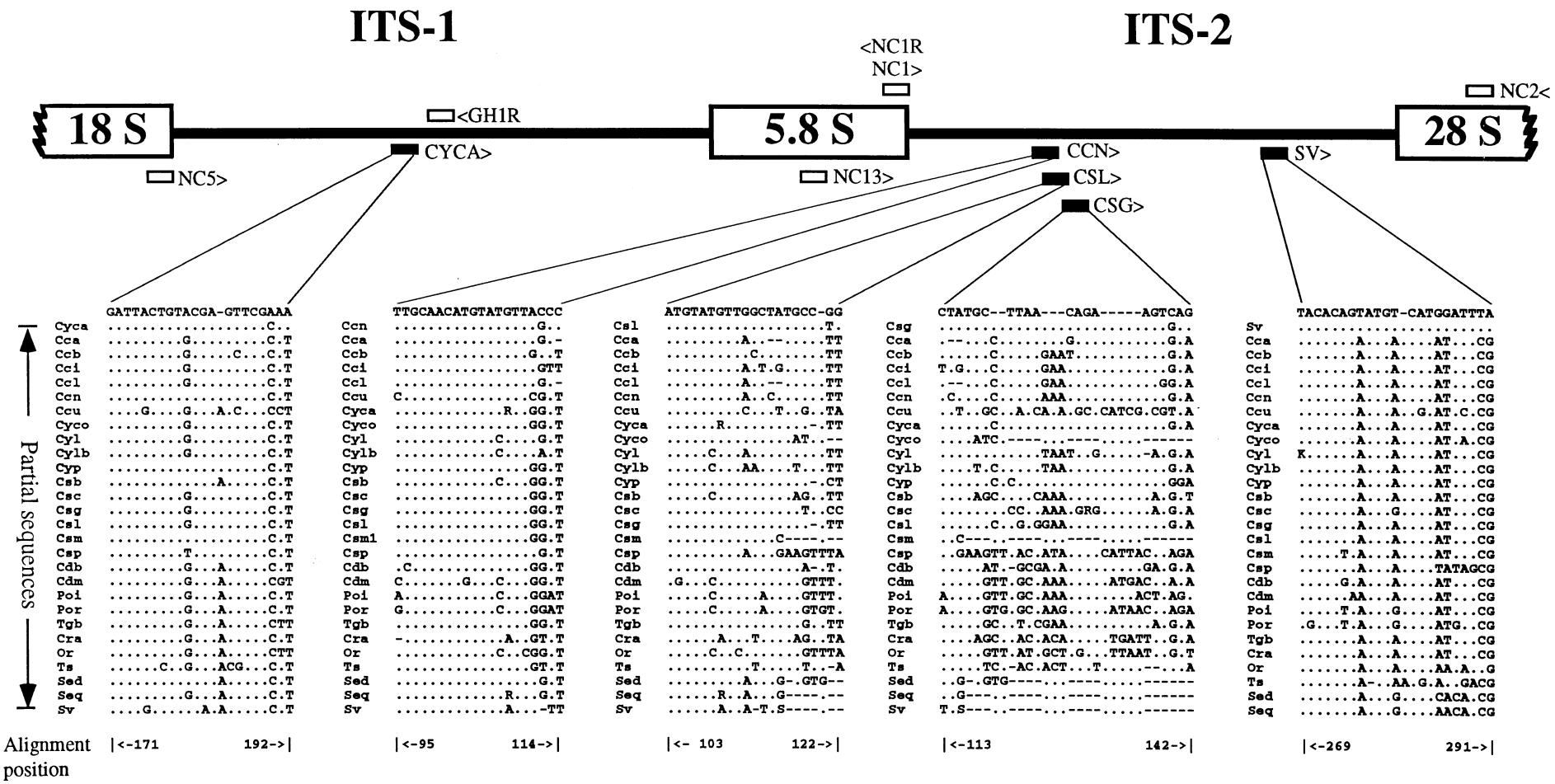

Fig. 1. Schematic representation of rDNA transcriptional unit including the 18S, 5·8S and 28S genes, the first and second internal transcribed spacers (ITS-1 and ITS-2, respectively) showing the positions of the forward (>) and reverse (<) primers (open boxes) used for PCR and sequencing. Under the transcriptional unit are alignments of partial ITS-1 and ITS-2 sequences for *Cylicocyclus ashworthi* (Cca), *Cc. brevicapsulatus* (Ccb), *Cc. insignis* (Cci), *Cc. leptostomus* (Ccl), *Cc. nassatus* (Ccn), *Cc. ultrajectinus* (Ccu), *Cyathostomum catinatum* (Cyca), *Cy. coronatum* (Cyco), *Cy. labiatum* (Cyl), *Cy. labratum* (Cylb), *Cy. pateratum* (Cyp), *Cylicostephanus bidentatus* (Csb), *Cs. calicatus* (Csc), *Cs. goldi* (Csg), *Cs. longibursatus* (Csl), *Cs. minutus* (Csm), *Cs. poculatus* (Csp), *Cylicodontophorus bicoronatus* (Cdb), *Cd. mettami* (Cdm), *Poteriostomum imparidentatum* (Poi), *P. ratzii* (Por), *Tridentoinfundibulum gobi* (Tgb), *Craterostomum acuticaudatum* (Cra), *Oesophagodontus robustus* (Or), *Triodontophorus serratus* (Ts), *Strongylus edentatus* (Sed.), *S. equinus* (Seq) and *S. vulgaris* (Sv), from which specific primers were designed. The sequences of the specific primers (closed boxes) are shown at the top of the sequence alignment. Bases identical to those of the specific primers are represented by a dot (.). Sequences have been deposited in EMBL, GenBank and DDBJ nucleotide sequence databases (see Table 2 for accession numbers).

paper and subjected to autoradiography (RP1 film, Agfa). The size of amplicons was estimated by comparison with a (radio-isotope labelled or unlabelled) ΦX174/*Hae*III marker (Promega).

DNA sequencing and calculation of sequence differences

PCR products amplified from parasite DNA with primers NC5 (forward: 5′-GTAGGTGAACCTGCGGAAGGATCATT-3′) and NC2 (reverse: 5′-TTAGTTTCTTTTCCTCCGCT-3′) (see Fig. 1) were column purified (Wizard™ PCR-Prep, Promega) and subjected to manual cycle-sequencing (Gasser *et al*. 1993) using the *fmol*™ kit (Promega). Primers NC5, GH1R (reverse: 5′-CATCAAGTCTAAAGCTAGC-3′) and NC1R (reverse: 5′-AACAACCCTGAACCAGACGT-3′) were used to sequence ITS-1, and primers NC13 (forward: 5′-ATCGATGAAGAACGCAGC-3′) and NC2 were used to sequence the ITS-2. Cycling conditions were the same as for primary amplification, except that annealing temperatures of 42, 55 or 60 °C were employed. Sequencing products were separated in LongRanger™ gels (FMC BioProducts, USA) and subjected to autoradiography.

Sequences were read manually. The 5′ and 3′ ends of the ITS-1 and ITS-2 were determined by comparison with the sequences from other species of strongylid nematodes (Campbell *et al*. 1995; Chilton *et al*. 1995; Hoste *et al*. 1995; Stevenson *et al*. 1995; Stevenson, Gasser & Chilton, 1996; Hung *et al*. 1996, 1997). Sequences were aligned manually to maximize the overall similarity. Pairwise sequence comparisons were performed for all the species using computer program AssemblyLIGN™ (Kodak; version 1.0.7). The level of sequence difference (D) was calculated using the formula $D = N/L$, where N is the number of alignment positions at which the 2 taxa do not have a base in common, and L is the total number of the alignment positions over which the 2 taxa are compared.

Primer design

The general criteria for primer design followed those of Sharrocks (1994). Primers to specific regions within the ITS-1 or ITS-2 were designed based on the principles for allele-specific amplification, which utilized the refractoriness of primers with 3′ end mismatch(es) in PCR to achieve selective amplification (e.g. Matsunaga, Tomita & Tagami, 1995; Rhodes *et al*. 1997). *S. vulgaris* and 4 species of cyathostomes (*Cy. catinatum*, *Cs. longibursatus*, *Cc. nassatus* and *Cs. goldi*) were chosen for the design of PCR primers. These species were chosen because of their high prevalence and/or high intensity of infection (cyathostomes) (Ogbourne, 1976; Bucknell *et al*. 1995; Lyons *et al*. 1997) or their high pathogenicity (*S. vulgaris*) (Ogbourne & Duncan, 1985).

RESULTS

Characteristics of the ITS-1 and ITS-2 sequences

The characteristics and accession numbers for the ITS-1 and ITS-2 rDNA sequences of 28 species of horse strongyles are shown in Table 2. The lengths of the ITS-1 sequences ranged from 366 to 394 bp, whereas the ITS-2 ranged from 216 to 352 bp. The ITS-2 sequences of 3 species of *Strongylus*, *Cylicostephanus minutus* and *Cyathostomum coronatum* were ~100 bp shorter compared with the other 23 species. For all 28 species, the G+C content was higher in ITS-1 (41·2–49·1%) than in the ITS-2 (30·9–44·3%). The ITS-1 and ITS-2 sequences were aligned over 425 and 385 positions, respectively. There was 50·6% similarity among all of the ITS-1 sequences, whereas the degree of similarity among the ITS-2 sequences was significantly lower (22·9%). Pairwise comparison revealed differences in sequence ranging from 0·6 to 23·7% in the ITS-1 and from 1·3 to 56·3% in the ITS-2. The extent of intraspecific or intraindividual variation in the sequences was low (0–0·3%) compared with the magnitude of interspecific differences.

Design of specific primers

For *S. vulgaris*, the primer SV was designed across alignment positions 269–291 (Fig. 1). This primer sequence had 2 mismatches with all species at the last 2 bases (except for *O. robustus* with only 1 mismatch) at the 3′ end (TA:CG) and additional 2 mismatches at the 6th and 7th bases from the 3′ end (GG:AT) with most species (see Fig. 1). Given that nucleotide differences in the ITS-1 and ITS-2 were distributed across the alignment for most species of cyathostome, primers usually had to be designed to the target sequence based on single nucleotide differences. The strategy exploited the introduction of intentional mismatches into primers to reduce PCR amplification efficiency from heterologous sequences (see Matsunaga *et al*. 1995; Hessner, McFarland & Endean, 1996; Lampel, Keasler & Hanes, 1996; Rhodes *et al*. 1997). The primer CYCA was designed based on a unique base (A) at alignment position 192 (Fig. 1) in the ITS-1 sequence of *Cy. catinatum*. A mismatch (C > A) was deliberately introduced to the primer sequence at alignment position 190 (Fig. 1). Thus, primer CYCA had 1 internal mismatch with the ITS-2 of *Cy. catinatum*, but at least 2 mismatches (including a A:G primer/template mismatch) within the last 4 bases with all other sequences. Similarly, for *Cc. nassatus*, the primer CCN was designed based on a unique C at the alignment position 114 of

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Table 2. Lengths, G + C contents and accession numbers for ITS-1 and ITS-2 sequences of 28 species of equine strongyles

Subfamily/species	Length		G + C content		Accession numbers	
	ITS-1 (bp)	ITS-2 (bp)	ITS-1 (%)	ITS-2 (%)	ITS-1	ITS-2
Cyathostominae						
Cylicocyclus ashworthi	366	315	46·1–46·4	40·0–40·6	Y08586	Y08586
Cc. brevicapsulatus	370	320	47·3	41·2	AJ004848	AJ004835
Cc. insignis	369	321	47·4–47·7	39·5–39·8	Y08588	Y08588
Cc. leptostomus	366	318	45·6–45·9	39·9	AJ004849	Y08587
Cc. nassatus	370	320	47·0–47·6	40·3	Y08585	Y08585
Cc. ultrajectinus	387	352	46·5	41·8	AJ004850	AJ004836
Cyathostomum catinatum	368	316	47·3	39·2–39·9	AJ004851	Y08619
Cy. coronatum	369	228	47·7	37·7	AJ004852	AJ004837
Cy. labiatum	369	319	48·5	38·2–39·2	AJ004853	Y08584
Cy. labratum	367	320	48·5	41·3	AJ004854	AJ004838
Cy. pateratum	369	316	47·2–47·4	40·2	AJ004855	Y08583
Cylicostephanus bidentatus	370	320	46·8–47·0	40·0	AJ228237	AJ004839
Cs. calicatus	368	320	47·8	40·6–40·9	AJ228238	AJ004840
Cs. goldi	369	316	47·7–48·0	40·5	AJ228239	A004841
Cs. longibursatus	369	320	47·7	40·3–40·6	AJ228240	AJ004842
Cs. minutus	370	216	48·6	40·7	AJ228241	AJ004843
Cs. poculatus	374	337	44·4	38·9	AJ228242	AJ004844
Cylicodontophorus bicoronatus	371	318	48·0	41·5	AJ228243	AJ004845
Cd. mettami	373	325	46·7	41·8	AJ228244	AJ004846
Poteriostomum imparidentatum	369	325	45·5	40·0	AJ228245	Y08590
P. ratzii	371	332	47·7	44·3	AJ228246	Y08589
Tridentoinfundibulum gobi	373	320	47·8	41·2	AJ228247	AJ004847
Strongylinae						
Craterostomum acuticaudatum	367	331	46·6–46·9	38·1–38·7	AJ228254	AJ228236
Oesophagodontus robustus	368	329	45·9	40·7–41·3	AJ228253	Y08592
Triodontophorus serratus	371	333	49·1	42·6	AJ228252	Y08591
Strongylus edentatus	394	235*	41·4	38·3*	AJ228249	X77808
S. equinus	385	229*	42·6	35·8–36·2*	AJ228250	X77807
S. vulgaris	386	217*	41·2–42·0	30·9–31·3*	AJ228251	X77863

* Data from Campbell *et al*. (1995).

the ITS-2 (Fig. 1). An intentional mismatch (G > C) was introduced at position 102. Thus, primer CCN had a single internal mismatch with *Cc. nassatus* sequence but at least 2 mismatches (including a C:C primer/template mismatch for all species, except for *Cc. brevicapsulatus*) within the last 4 bases with the other species. A different primer design strategy was used for *Cs. longibursatus* and *Cs. goldi*, because there was no unique base for these 2 species in the alignment of the ITS-1 or ITS-2 for all species. For *Cs. longibursatus*, the primer CSL was designed to the ITS-2 across alignment positions 103–122 (see Fig. 1). Although the 3′ terminal base G was shared with *P. imparidentatum*, *P. ratzii*, *Cd. bicoronatus* and *Cd. mettami*, the 4th and 5th bases from the 3′ end of the 4 species mismatched with primer CSL. By introducing an intentional mismatch (T > G) at position 121 in the primer sequence, it had a single internal mismatch with the *Cs. longibursatus* sequence and at least 2 mismatches within the last 4 bases with the sequences of all other species (Fig. 1). For *Cs. goldi*, the primer CSG was designed across alignment positions 113–142 of the ITS-2 (Fig. 1). Although the 3′ terminal base G was shared with the *P. imparidentatum* sequence, with the introduction of an intentional mismatch (G > C) at position 140 in the primer sequence, the primer CSG had a single internal mismatch with the *Cs. goldi* sequence and 2 mismatches within the last 6 bases with the sequences of all other species (Fig. 1).

Evaluating the specificity and sensitivity of primers in PCR

The 5 (radio-isotope labelled) primer sets CSL-NC2, CYCA-NC2, CCN-NC2, CSG-NC2 and SV-NC2 were then assessed for their species-specificity in PCR. Amplicons were subjected to agarose gel electrophoresis and detected by autoradiography. The 5 primer sets were individually tested in PCR using DNA from 26 of the 28 species of strongyles and the primer set NC1-NC2 was used to control for amplification efficiency from individual DNA samples (Fig. 2). For all primer sets, specific amplification was achieved from the appropriate species, and individual amplicons were of the

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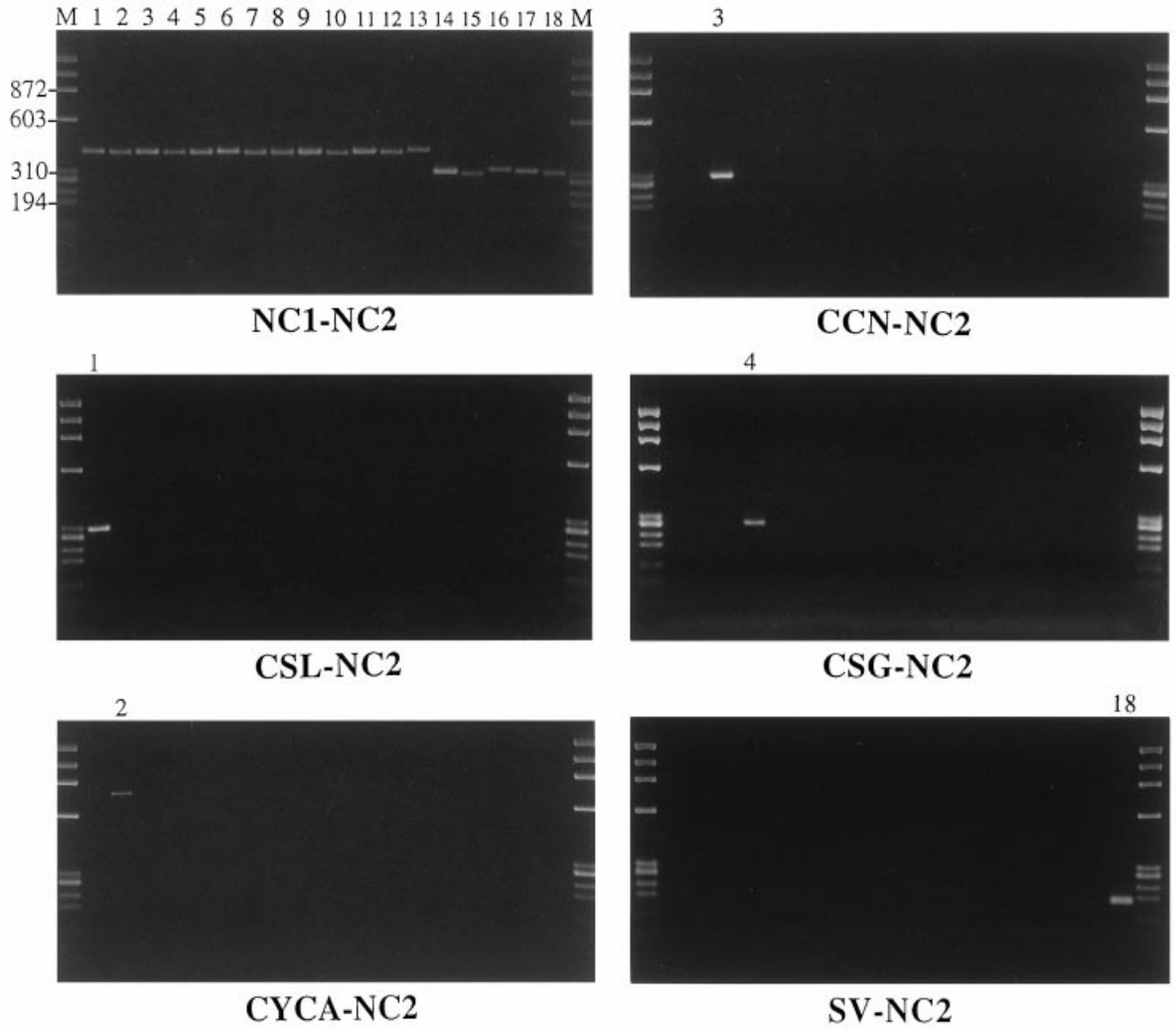

Fig. 2. Representative agarose gels demonstrating the specificity of the primer sets CSL-NC2, CYCA-NC2, CSL-NC2, CSG-NC2 and SV-NC2 in the PCR. Primer set NC1-NC2 was used to simultaneously assess the amplification efficiency from each DNA sample. Genomic DNA samples from *Cylicostephanus longibursatus*, *Cyathostomum catinatum*, *Cylicocyclus nassatus*, *Cs. goldi*, *Cc. brevicapsulatus*, *Cc. insignis*, *Cc. leptostomus*, *Cy. labiatum*, *Cy. labratum*, *Cy. pateratum*, *Poteriostomum imparidentatum*, *Cylicodontophorus bicoronatus*, *Triodontophorus serratus*, *Cy. coronatum*, *Cs. minutus*, *Strongylus edentatus*, *S. equinus* and *S. vulgaris* (lanes 1–18, respectively) were individually amplified by PCR using the 5 primer sets. Samples representing 7 non-strongyloid species (*Trichostrongylus axei*, *Parascaris equorum*, *Gasterophilus intestinalis*, *Draschia megastoma*, *Anoplocephala perfoliata*, *Oxyuris equi* and *Fasciola hepatica*) and no-DNA controls were run on a separate gel, and no products were detected either upon ethidium bromide staining or autoradiography (not shown). M represents the *Φ*X174/*Hae*III size marker (bp).

expected sizes (Fig. 2). Sequence analysis of the specific amplicons using primer NC2 confirmed that the 5 specific products were derived from the appropriate rDNA region. The specificity of the primer sets was also assessed in PCR by cycling with heterologous genomic DNA from *T. axei*, *P. equorum*, *G. intestinalis*, *D. megastoma*, *A. perfoliata*, *O. equi*, horse muscular tissue and a 'parasite-free' faecal sample. In no case were PCR products detected in those control samples upon autoradiographic exposure of agarose gels (not shown). Finally, the 'sensitivity' of the PCR using different primer sets was determined by amplification from serial titrations of homologous DNA. The lowest amount of DNA detectable on agarose gels after amplification was 1 pg for primer sets CYCA-NC2, CCN-NC2 and SV-NC2, 100 pg for CSL-NC2 and 1 ng for CSG-NC2.

Having assessed the specificity of the primer sets in PCR, we then established an electrophoretic procedure that allowed the analysis of multiple specific amplicons in 1 lane. As a consequence of the size differences between the 5 specific amplicons, they could be pooled and subjected to electrophoresis in 1 lane of a neutral polyacrylamide gel (Fig. 3). For some genomic samples of *S. vulgaris* tested with primer set SV-NC2, an apparently non-specific product was amplified which was of a similar size to the specific CCN-NC2 amplicon (see Fig. 2). Therefore, the samples amplified with the primer set

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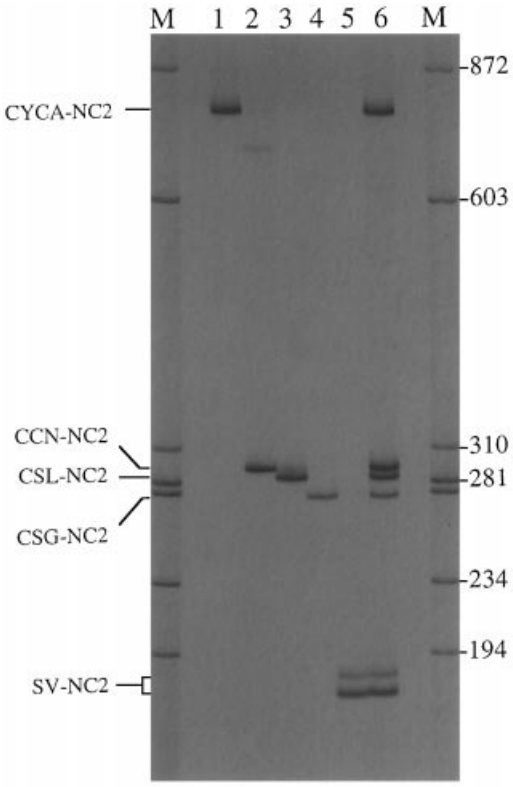

Fig. 3. Assessing the feasibility of using neutral polyacrylamide gels to separate 5 specific amplicons in 1 lane. Lanes 1–5 represent amplicons produced individually from genomic DNA of *Cyathostomum catinatum*, *Cylicocyclus nassatus*, *Cylicostephanus longibursatus*, *Cs. goldi* and *Strongylus vulgaris* by PCR using primer sets CYCA-NC2, CCN-NC2, CSL-NC2, CSG-NC2 and SV-NC2, respectively. Lane 6 shows the separation of individual specific bands from a pool of the 5 amplicons. M represents the *Φ*X174/*Hae*III size marker (bp).

SV-NC2 were run in separate lanes. The sensitivity of autoradiographic detection on polyacrylamide gels was determined to be the same as for agarose gels (results not shown), except that amplification with primer set SV-NC2 could detect as little as 0·1 pg of *S. vulgaris* DNA.

Specific amplification from copro-cultures and faecal samples

Fourteen copro-culture and 3 faecal samples were used to establish the ability of the PCR, using specific primer sets, to amplify DNA from *S. vulgaris*, *Cy. catinatum*, *Cs. longibursatus*, *Cc. nassatus* or *Cs. goldi*. DNA extracted from copro-culture samples was amplified separately using 5 primer sets, including appropriate positive and negative controls. The amplicons produced using each primer set were run separately on a polyacrylamide gel (data not shown), and then the amplicons produced individually using the 4 cyathostome specific primer sets were pooled and run

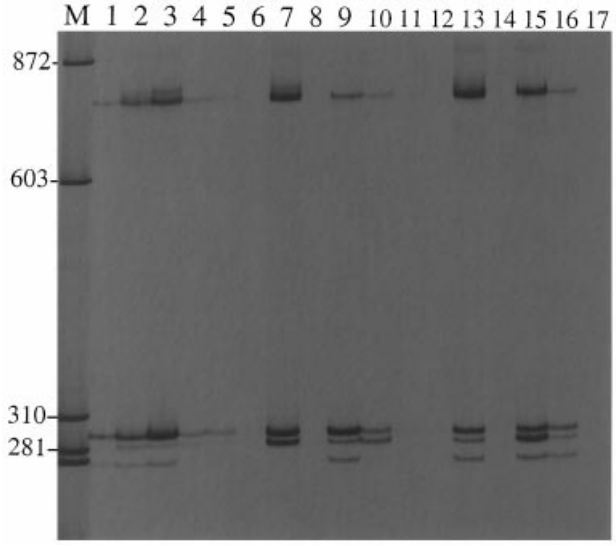

Fig. 4. Electrophoretic separation on a polyacrylamide gel of specific amplicons produced from DNA derived from 7 different copro-culture samples using primer sets CYCA-NC2, CCN-NC2, CSL-NC2 and CSG-NC2. Lanes 1, 3, 5, 7, 9, 11 and 13 represent samples collected prior to the treatment of horses with a therapeutic dose of fenbendazole. Lanes 2, 4, 6, 8, 10, 12 and 14 represent the corresponding post-treatment samples. Lanes 15 and 16 represent appropriate positive controls at higher (1 ng/μl) and lower DNA concentrations (end-point of sensitivity), respectively. Lane 17 represents a no-DNA control. M represents the *Φ*X174/*Hae*III size marker (bp).

together (Fig. 4). The level of detectability for the 4 species in the pooled amplicons was found to be the same as when individual samples were run separately. Using appropriate primer sets, DNA of *Cc. nassatus*, *Cy. catinatum*, *Cs. longibursatus* and *Cs. goldi* was amplified from 7, 7, 6 and 4 of the 7 samples prior to fenbendazole treatment, respectively. DNA of *Cy. catinatum*, *Cc. nassatus*, *Cs. longibursatus* and *Cs. goldi* was amplified from 3, 3, 3 and 1 of the 7 post-treatment samples, respectively (Fig. 4). DNA of *S. vulgaris* was amplified by PCR with primer set SV-NC2 in one pre-treatment sample (not detected by microscopical examination) but was not amplified from any of the post-treatment samples.

For 3 faecal samples representing horses with monospecific *S. vulgaris* infection, the DNA extracted from each sample could be amplified using SV-NC2 primer set (Fig. 5). Sequence analysis of the specific amplicon using primer NC2 confirmed that the specific PCR product was derived from the appropriate rDNA region. A faecal sample from a helminth-free foal was also included in the same amplification run, but no amplicon could be detected using ethidium bromide staining or upon autoradiography. To investigate that the negative result was not due to the inhibition of PCR, genomic DNA extracted from an adult worm of *S. vulgaris* was

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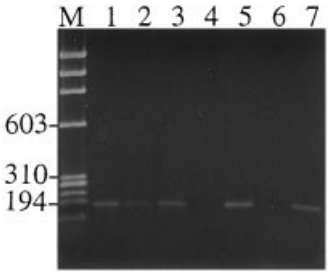

Fig. 5. PCR amplification (using primer set SV-NC2) of DNA derived from faecal samples from 3 different horses with monospecific *Strongylus vulgaris* infection. Lanes 1–3 represent DNA from samples with 72, 34 and 23 eggs/g faeces, respectively. Lanes 4–7 represent a no-DNA control, a positive control, a DNA sample derived from parasite-free faeces, and a genomic DNA sample derived from a 'parasite-free' faecal sample spiked with DNA from an adult *S. vulgaris* worm, respectively. M represents the *Φ*X174/*Hae*III size marker (bp) on the agarose gel.

spiked with the parasite-free sample and subjected to the same amplification procedure. The detection of an amplicon of the expected size in the spiked sample (lane 7; Fig. 5) demonstrated that the DNA from the parasite-free sample did not inhibit the PCR reaction. DNA extracted from larval samples which failed to be amplified with any of the 5 primer sets was also checked for inhibition, and no inhibition was found in any of the samples (not shown).

DISCUSSION

In this study, sequence analysis demonstrated that each of the 28 species of horse strongyles examined could be differentiated from one another by their ITS-1 and ITS-2 rDNA sequences. The magnitude of sequence differences in the ITS-1 (0·6–23·7 %) and ITS-2 (1·3–56·3 %) among the 28 species was greater than the within-species sequence variation (0–0·3 %) for 6 species (*Cc. ashworthi*, *Cc. nassatus*, *Cc. ultrajectinus*, *Cs. goldi*, *Cy. catinatum* and *Cc. insignis*) where multiple geographical isolates were available. This demonstrated that the ITS-1 and ITS-2 sequences are useful for the specific identification of equine strongyles. The ITS-1 and ITS-2 sequence data for 28 species provided the basis with which to design specific primers SV, CYCA, CSL, CCN and CSG to *S. vulgaris*, *Cy. catinatum*, *Cs. longibursatus*, *Cc. nassatus* and *Cs. goldi*, respectively, for use in PCR. The evaluation of the 5 primer sets (SV-NC2, CYCA-NC2, CSL-NC2, CCN-NC2 and CSG-NC2) individually in PCR (using control DNA samples from a range of heterologous strongyle species, a range of common parasites from horses, host and faecal control samples) demonstrated that they achieved species-specific amplification of the appropriate rDNA region from genomic DNA. Also, specific and effective amplification could be achieved from as little as 1 pg–1 ng of DNA, depending on primer set used, and the introduction of intentional mismatches did not appear to affect significantly the amplification efficiency. This is in accordance with the findings of Kwok *et al.* (1990). These results indicate clearly the usefulness of the intentional-mismatch-strategy of primer design for the selective amplification of sequences that differ by a single nucleotide.

PCR using specific primers together with primer NC2 achieved amplification of the appropriate species from copro-culture samples. DNA of *Cy. catinatum*, *Cc. nassatus* and/or *Cs. longibursatus* larvae were detected by PCR in 6–7 of 7 samples prior to treatment of horses with a therapeutic dose of fenbendazole. After treatment, 3 of 7 post-treatment samples tested still contained DNA of those species, which suggested that fenbendazole treatment was ineffective at the dosage used or that the species present were resistant to the anthelmintic. *S. vulgaris* DNA was detected by PCR in 1 pre-treatment sample but not by copro-culture/microscopy, indicating that the SV-NC2 PCR was more sensitive than the traditional method of copro-culture. After treatment, no *S. vulgaris* DNA was detected, indicating that the parasite was removed effectively by fenbendazole treatment. Importantly, no inhibition of PCR amplification was detected for any of the larval or faecal samples tested, which was most likely due to the capacity of the QIAamp tissue purification kit to remove PCR inhibitors from the samples. A recent study has demonstrated the effectiveness of this system to remove PCR inhibitors from faecal samples (Monteiro *et al.* 1997), which is also supported by unpublished findings by other workers (J. Verweij, personal communication). Nonetheless, further work is required to critically test inhibition of PCR by faecal contaminants using a large number of samples.

The ability to effectively amplify DNA of any of the 5 species from larval culture samples and DNA of *S. vulgaris* from faecal samples by specific PCR has important implications for the diagnosis of infection and for studying the population biology and epidemiology of small strongyles. Moreover, this molecular approach may be useful for studies on the prevalence of anthelmintic resistance in conjunction with egg count reduction testing (Ihler, 1995; Ihler & Bjørn, 1996; Borgsteede, Dvojnos & Kharchenko, 1997). It may also be useful for the differentiation of larvae from pasture samples and the specific detection of strongyle DNA or eggs in faecal samples. In the present study, we also demonstrated that SV-NC2 PCR amplified specifically *S. vulgaris* DNA directly from QIAamp column-purified faecal DNA (from mono-specifically-infected horses) and that the assay could

detect DNA in samples with as few as 23 eggs/g faeces. This information indicates clearly the potential of specific PCR for the diagnosis of patent strongyle infections. If the sensitivity of the PCR with the other 4 primer sets is inadequate for the amplification from faecal samples, then a nested or semi-nested PCR approach could be exploited to achieve increased sensitivity. We have recently demonstrated the effectiveness of a semi-nested PCR approach for the specific diagnosis of *Oesophagostomum bifurcum* infection in humans and the specific detection of less than 1 pg of *O. bifurcum* DNA from soil samples (unpublished). Another possible application of specific PCR could be in the diagnosis of cyathostomosis by amplification of parasite-specific ITS rDNA from faeces and/or biopsy samples from the large intestine. Moreover, the specific PCR system could be used as a tool to investigate the distribution of small strongyle larvae in the large intestinal wall and the pre-patent period of individual species, which has important implications for studying their biology.

Detection of the specific PCR products relied on their resolution by neutral polyacrylamide gel electrophoresis. For the 4 species of cyathostome, amplicons could be accurately separated in 1 lane of a gel, whereas this was not possible on agarose gels as a consequence of small size differences among some of the amplicons (e.g. CCN-NC2 *vs.* CSL-NC2). A similar sensitivity of detection was achieved using polyacrylamide compared with agarose gels when double the volume of sample was loaded. In spite of the usefulness of the present system, improvements could be made to the assay format. For example, it would be worthwhile to assess an enzyme-based system for the detection of amplicons in a 96-well microtitration plate format, similar to that described by Gale *et al.* (1996), although currently this would be associated with a high cost per test sample. With such a modification, it would be possible to achieve a high sample through-put. A possibility to reduce the work-load and expense could be achieved by establishing a multiplex-PCR strategy (e.g. Edward & Gibbs, 1996), whereby a range of primers, including the ones assessed herein, are incorporated into 1 PCR reaction to achieve specific amplification with primer NC2. This warrants future investigation.

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