A multiplex PCR test to identify four common cattle-adapted *Cryptosporidium* species

SARAH THOMSON[1], ELISABETH A. INNES[1], NICHOLAS N. JONSSON[2] *and* FRANK KATZER[1]*

[1] *Moredun Research Institute, Pentlands Science Park, Bush Loan, Edinburgh EH26 0PZ, UK*
[2] *College of Medical, Veterinary and Life Sciences, University of Glasgow, 464 Bearsden Road, Glasgow G61 1QH, UK*

SUMMARY

Cryptosporidium is a well-known cause of neonatal enteritis in cattle worldwide. Cattle are commonly infected with four different species of *Cryptosporidium* but only one of these, *Cryptosporidium parvum*, is associated with clinical disease. Identification of species in cases of calf scour can give an indication if *Cryptosporidium* is the causative agent or not. In addition, *C. parvum* is a zoonotic species and so has implications for human health, for this reason it is important to identify the species of *Cryptosporidium* infecting cattle particularly where a farm is implicated in an outbreak of cryptosporidiosis in humans. Here a multiplex PCR test, which can identify the four common cattle-adapted *Cryptosporidium* species, including *C. parvum*, has been developed. This test allows quick and accurate detection of *Cryptosporidium* species in cattle fecal samples including mixed infections, which could be missed by the more common method of sequencing the same gene.

Key words: *Cryptosporidium*, cattle, detection, multiplex, PCR.

INTRODUCTION

Cryptosporidiosis is the disease caused by the protozoan parasite *Cryptosporidium*, which affects many different species of mammals, including humans, birds, reptiles, amphibians and fish (Fayer, 2010; Chalmers and Katzer, 2013; Ryan *et al*. 2014). It has been identified as one of the most important causes of bovine neonatal enteritis worldwide (De Graaf *et al*. 1999; Brook *et al*. 2008; Silverlas *et al*. 2009; Wyatt *et al*. 2010).

The disease usually manifests itself as profuse watery diarrhoea, loss of appetite and abdominal pain (Clark and Sears, 1996; Tzipori and Ward, 2002; Klein *et al*. 2008) which lasts approximately 2 weeks with little or no long-term implications on animal health (Klein *et al*. 2008). Most animals will recover but in severe cases complications may arise and death can occur. There are currently over 27 recognized species of *Cryptosporidium*, four of which commonly infect cattle; *Cryptosporidium parvum, Cryptosporidium andersoni, Cryptosporidium bovis* and *Cryptosporidium ryanae* (Chalmers and Katzer, 2013; Ryan *et al*. 2014).

Detection of *Cryptosporidium* in farm livestock is usually done by microscopy (in veterinary diagnostic laboratories) or by molecular detection (research laboratories); of the two methods molecular detection is much more sensitive and allows full speciation (Chalmers and Katzer, 2013). One of the most common genes targeted for PCR detection of *Cryptosporidium* is the 18S rRNA gene, which is located on a multi-copy DNA element meaning that the PCR is more sensitive than those which target a single copy gene. The 18S rRNA gene is also one of the most commonly used genes for species discrimination and there are many 18S rRNA reference sequences available on the National Centre for Biotechnology Information website (www.ncbi.nlm.nih.gov). In order to identify species in *Cryptosporidium*-positive samples, it is necessary to carry out sequencing or PCR–RFLP (polymerase chain reaction followed by restriction fragment length polymorphism). These methods can be expensive, time consuming and it is very difficult to detect mixed infections as often only the most predominant species in a sample is detected (Cama *et al*. 2006). PCR–RFLP can also detect only a limited number of species.

A multiplex PCR test would enable the rapid speciation of a subset of *Cryptosporidium* species and previous attempts have been made to develop such a test (Patel *et al*. 1999; Lindergard *et al*. 2003; Santin and Zarlenga, 2009); the most recent of which can detect the four most commonly found *Cryptosporidium* species in cattle (Santin and Zarlenga, 2009). This test cannot distinguish between *C. bovis* and *C. ryanae* and a further PCR amplification and sequencing would be required to distinguish these species.

In this paper, we describe the development of a multiplex PCR test to identify three cattle-adapted species (*C. andersoni, C. bovis* and *C. ryanae*) plus the zoonotic species *C. parvum* in a nested PCR. It is also possible with this multiplex PCR to detect other *Cryptosporidium* species by sequencing of the

* Corresponding author. Moredun Research Institute, Pentlands Science Park, Bush Loan, Edinburgh, EH26 0PZ, UK. Tel.: +44 (0)31 445 5111. Fax: +44 (0)131 445 6111. E-mail: frank.katzer@moredun.ac.uk

18S rRNA gene, where none of the other species have been detected. This PCR test will be extremely useful in veterinary diagnostics as a tool for accurate diagnosis of scour in neonatal calves and as a research tool to investigate the epidemiology of cryptosporidiosis. The nssm-PCR would allow speciation but would not permit sub-typing, which would be necessary for outbreak investigation. Current diagnostic methods can only confirm that *Cryptosporidium* spp. are present in the feces, but cannot distinguish the disease causing *C. parvum* species from the less virulent *C. andersoni* which has been associated with a reduction in weight-gain and milk yield in adult cattle, *C. bovis* and *C. ryanae*, which have not yet been associated with the clinical disease. The test is already being used to monitor calves on a veterinary school farm to ensure that the calves used for practical classes are free from *C. parvum* to minimize the risk to the students.

MATERIALS AND METHODS

Design of Cryptosporidium species-specific primers

Alignments of multiple 18S rRNA gene sequences (between 50 and 100 for each species) for *Cryptosporidium* species (*C. parvum, C. bovis, C. ryanae, C. andersoni, C. ubiquitum, C. hominis, C. muris, C. xiaoi* and *C. suis*) were made using the BioEdit Software Program (www.mbio.ncsu.edu/bioedit/bioedit.html) to identify representative sequences for each species. Clustal W (www.ebi.ac.uk/Tools/msa/clustalw2) alignments of the representative sequences were made and regions for species-specific primers were identified which would produce amplicons of different sizes for each species when used with the 18S rRNA reverse primer (AL3032) (Xiao *et al*. 2000). The locations of the primers used are shown in Fig. 1; The outer primers are the same as those described by Xiao *et al*. 2000 (AL1691/AL1687) as are the first set of inner primers (AL1598 and AL3032) these amplify a 840 bp genus-specific fragment, the second inner primer set (CaF and AL3032) amplify a 625 bp fragment from *C. andersoni*; the third set (CrF and AL3032) amplify a 415 bp fragment from *C. ryanae* the forward primer in this set contains a degenerate base (R) where the *C. ryanae* sequence contains either an 'A' or a 'G'; the fourth set (CphF and AL3032) amplify a 305 bp fragment from *C. parvum, C. hominis* and *C. suis*, which can be later distinguished with an additional PCR if desired and the final set of primers (CbF and AL3032) amplify a 241 bp fragment from *C. bovis*. The primer sequences are shown in Table 1.

Fecal sample collection and DNA extraction

Fecal samples ($n = 498$) were collected from 190 calves and 308 adult cattle from 21 beef farms as part of another study. These samples were collected from calves aged 7–14 days regardless of clinical signs ($n = 116$), all calves suffering from diarrhoea regardless of age ($n = 74$), adult cattle pre- and post-calving ($n = 177$ and 120, respectively) and other adult cows ($n = 11$). Samples were collected by the farmers and sent to the Moredun Research Institute by their veterinary practices.

DNA was extracted from the calf feces processing with a QIAamp DNA Stool Minikit (Qiagen) according to the manufacturer's instructions except that 10× freeze–thaw cycles in liquid nitrogen were carried out after adding 200 μg sample to lysis buffer (provided in the kit). Adult feces were subjected to a sedimentation step prior to DNA extraction. The resultant pellet was added to lysis buffer and subjected to 10× freeze–thaw cycles in liquid nitrogen followed by processing by QIAamp DNA Stool Minikit (Qiagen) according to the manufacturer's instructions.

To identify *Cryptosporidium*-positive samples 18S nested PCR was carried out as described by Xiao *et al*. 2000. This work was carried out as part of another study at the Moredun Research Institute. Microscopic examination of the feces was not carried out for any of the samples.

Testing of individual species-specific primers

First round reactions were carried out as described by Xiao *et al*. 2000 using DNA from known positive samples for each of the four species. Secondary reactions were carried out to test each individual species-specific forward primer with the reverse 18S rRNA primer (AL3032). First round PCR products were diluted with 50 μL dH$_2$O and 1 μL of the dilution used as a template in a reaction containing 2·5 μL 10× PCR buffer (45 mM Tris–HCL pH 8·8, 11 mM (NH$_4$)$_2$SO$_4$, 4·5 mM MgCl$_2$, 4·4 μM EDTA, 113 μg mL^{-1} BSA, 1 mM of each of the four deoxyribonucleotide triphosphates) (Katzer *et al*. 2014), 0·5 unit BioTaq (Bioline, UK) and 10 μM forward and reverse primers. The final volume of each reaction was made up to 25 μL using dH$_2$O.

Cycling conditions were 3 min at 94 °C, followed by 35 cycles of 45 s at 94 °C, 45 s on a temperature gradient of 49–59 °C and 1 min at 72 °C. The final extension was 7 min at 72 °C. Second round PCR products were visualized following electrophoresis on a 1·5% agarose gel stained with GelRed™ (Biotium, UK) on an AlphaImager 2200.

Field samples

Primers were tested on a panel of the only available DNA samples, which included known positive samples for *C. parvum, C. andersoni, C. bovis* and *C. ryanae* from naturally infected cattle aged from 1 day old to adult animals ($n = 128$). Results from

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                    10         20         30         40         50
              ....|....| ....|....| ....|....| ....|....| ....|....|
C. parvum     GGAAGGGTTG TATTTATTAG ATAAAGAACC AATATAATT- -GGTGACTCA
C. andersoni  .......... .......... ..........  ... ...GAGC..- -.....T...
C. bovis      .......... .......... ..........  ... .G...TT..- -.........
C. ryanae     .......... .......... ..........  ... ...T.TT..T T.........
C. hominis    .......... .......... ..........  ... ..........- -.........
C. suis       ---------- ---------- ----------  --- ---------- ----------
C. ubiquitum  ---------- ---------- ----------  --- ---------- ----------
C. xiaoi      ---------- ----..... ..........  ... ....TT..- -.........
C. muris      .......... .......... ..........  ... ...GAGC..- -.....T...

                    60         70         80         90        100
              ....|....| ....|....| ....|....| ....|....| ....|....|
C. parvum     TAATAACTTT ACGGATCACA TTAA--ATGT GACATATCAT TCAAGTTTCT
C. andersoni  .......... .......G.. .CTCTG...C .......... ..........
C. bovis      .......... .......... ..----.... .......... ..........
C. ryanae     .......... .......... C.----.... .......... ..........
C. hominis    .......... .......... A.T.--.... .......... ..........
C. suis       .......... .......... ..TTTA.... .......... ..........
C. ubiquitum  ---------- ---------- ---------- ---------- ----------
C. xiaoi      .......... .......... ..----.... .......... ..........
C. muris      .......... .......G.. .CTCTG...C .......... ..........

                   110        120        130        140        150
              ....|....| ....|....| ....|....| ....|....| ....|....|
C. parvum     GACCTATCAG CTTTAGACGG TAGGGTATTG GCCTACCGTG GCAATGACGG
C. andersoni  .......... .......... .......... .......... ..T.......
C. bovis      .......... .......... .......... .......... ..T.......
C. ryanae     .......... .......... .......... .......... ..T.......
C. hominis    .......... .......... .......... .......... ..........
C. suis       .......... .......... .......... .......... ..........
C. ubiquitum  ---------- -......... .......... .......... ..........
C. xiaoi      .......... .......... .......... .......... ..T.......
C. muris      .......... .......... .......... .......... ..T.......

                   160        170        180        190        200
              ....|....| ....|....| ....|....| ....|....| ....|....|
C. parvum     GTAACGGGGA ATTAGGGTTC GATTCCGGAG AGGGAGCCTG AGAAACGGCT
C. andersoni  .......... .......... .......... .......... ..........
C. bovis      .......... .......... .......... .......... ..........
C. ryanae     .......... .......... .......... .......... ..........
C. hominis    .......... .......... .......... .......... ..........
C. suis       .......... .......... .......... .......... ..........
C. ubiquitum  .......... .......... .......... .......... ..........
C. xiaoi      .......... .......... .......... .......... ..........
C. muris      .......... .......... .......... .......... ..........

                   210        220        230        240        250
              ....|....| ....|....| ....|....| ....|....| ....|....|
C. parvum     ACCACATCTA AGGAAGGCAG CAGGCGCGCA AATTACCCAA TCCTAATACA
C. andersoni  .......... .......... .......... .......... ....G.C...
C. bovis      .......... .......... .......... .......... ..........
C. ryanae     .......... .......... .......... .......... ..........
C. hominis    .......... .......... .......... .......... ..........
C. suis       .......... .......... .......... .......... ..........
C. ubiquitum  .......... .......... .......... .......... ..........
C. xiaoi      .......... .......... .......... .......... ..........
C. muris      .......... .......... .......... .......... ....G.C...
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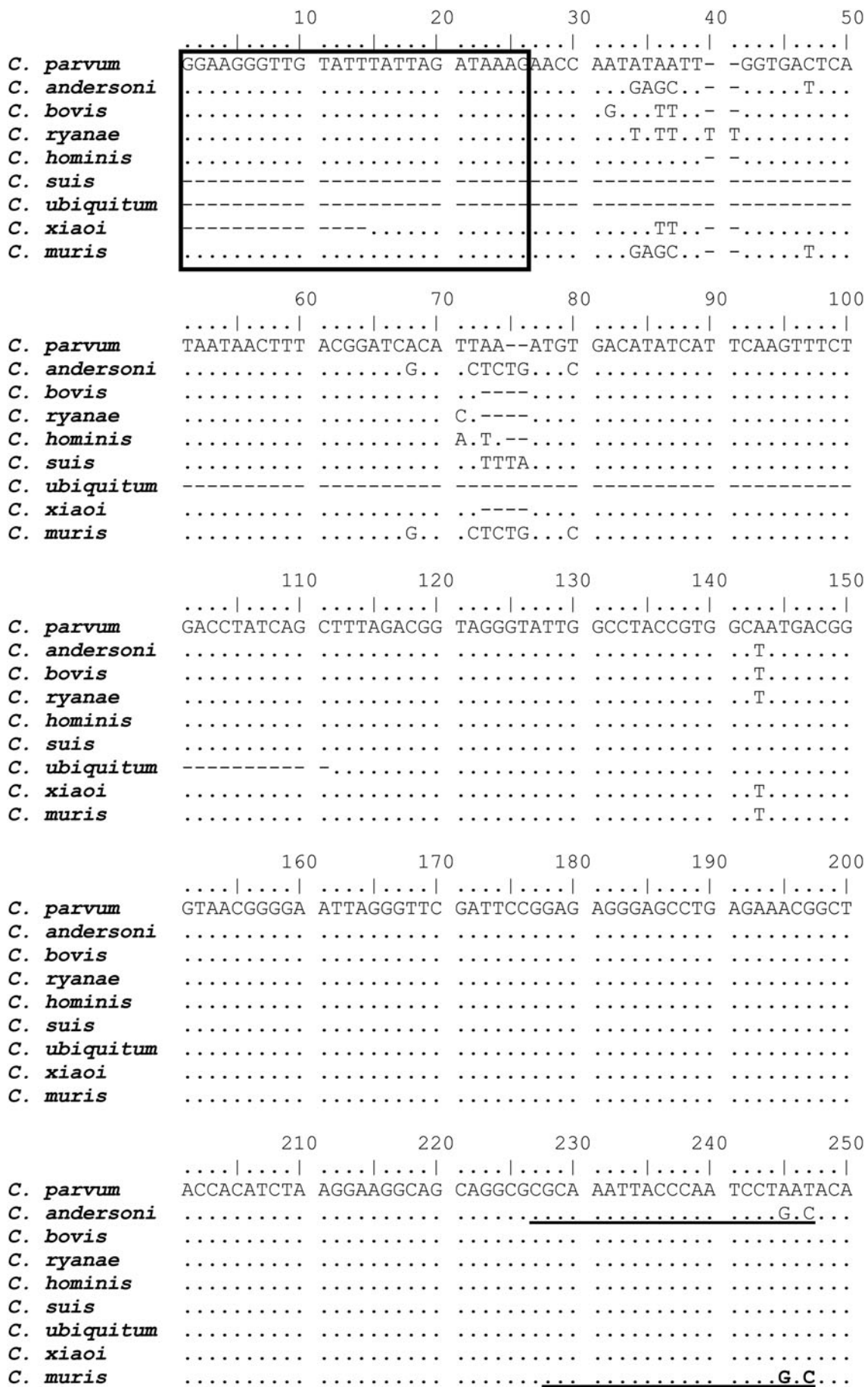
Fig. 1. Positions of species-specific primers on the 18S rRNA gene plus the location of the 18S rRNA internal primers.

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                    260        270        280        290        300
             ....|....| ....|....| ....|....| ....|....| ....|....|
C. parvum    GGGAGGTAGT GACAAGAAAT AACAATACAG GACTT-TT-T GGTTTTGTAA
C. andersoni .......... .......... .......... .G.C.-AA-C ...C......
C. bovis     .......... .......... .......... A..C.-.A-C ..........
C. ryanae    .......... .......... .......... AG.C.-.A-C ..........
C. hominis   .......... .......... .......... .....-..-. ..........
C. suis      .......... .......... .......... .....T..-. A.........
C. ubiquitum .......... .......... .......... ....TAAA. A.........
C. xiaoi     .......... .......... .......... A..C.-.A-C ..........
C. muris     .......... .......... .......... .G.C.-AA-C ...C......

                    310        320        330        340        350
             ....|....| ....|....| ....|....| ....|....| ....|....|
C. parvum    TTGGAATGAG TTAAGTATAA ACCCCTTTAC AAGTATCAAT TGGAGGGCAA
C. andersoni .......... .G........ .......... G......... ..........
C. bovis     .......... .......... .......A.. .......... ..........
C. ryanae    .......... .......... .......A.. .......... ..........
C. hominis   .......... .......... .......... .......... ..........
C. suis      .......... .......... .......C. .......... ..........
C. ubiquitum .......... .......... .......... .......... ..........
C. xiaoi     .......... .......... .......A.. .......... ..........
C. muris     .......... .G........ .......... G......... ..........

                    360        370        380        390        400
             ....|....| ....|....| ....|....| ....|....| ....|....|
C. parvum    GTCTGGTGCC AGCAGCCGCG GTAATTCCAG CTCCAATAGC GTATATTAAA
C. andersoni .......... .......... .......... .......... ..........
C. bovis     .......... .......... .......... .......... ..........
C. ryanae    .......... .......... .......... .......... ..........
C. hominis   .......... .......... .......... .......... ..........
C. suis      .......... .......... .......... .......... ..........
C. ubiquitum .......... .......... .......... .......... ..........
C. xiaoi     .......... .......... .......... .......... ..........
C. muris     .......... .......... .......... .......... ..........

                    410        420        430        440        450
             ....|....| ....|....| ....|....| ....|....| ....|....|
C. parvum    GTTGTTGCAG TTAAAAAGCT CGTAGTTGGA TTTCTGTTAA TAATTTATAT
C. andersoni .......... .......... .......... .......GT AT.A..T...
C. bovis     .......... .......... .......AAT C........- --....T...
C. ryanae    .......... .......... .......AAT ........- --....T...
C. hominis   .......... .......... .......... .......... ..........
C. suis      .......... .......... ...T...... .......... ..........
C. ubiquitum .......... .......... .......... .......... ..........
C. xiaoi     .......... .......... .......AAT C........- --....T...
C. muris     .......... .......... .......... .......GT AT.A.CT-..

                    460        470        480        490        500
             ....|....| ....|....| ....|....| ....|....| ....|....|
C. parvum    AAAATATTTT GA-TGAATAT TTAT-ATAAT ATTAACATAA TTCATATTAC
C. andersoni .--.....AC C--AAGG..A ....T...T. ..C.....CC ...C.....T
C. bovis     .T-...A.A. C--ACG.... ....-..... .......... ..........
C. ryanae    .T-.C.A.GC T--ACGG... ....-..... .......... ..........
C. hominis   .......... ..-....... ....-..... .......... ..........
C. suis      .T........ T----..... ....-..... .......... ..........
C. ubiquitum .T........ ATT--..... ....-...G. .......... ..........
C. xiaoi     .T-.C.A.A. C--ACG.... ....-..... .......... ..........
C. muris     .--.....AC T--AAGG... A...T...T. ..C.....CC ...C.....T
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Fig. 1. Continued

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                        510        520        530        540        550
              ....|....| ....|....| ....|....| ....|....| ....|....|
C. parvum     TATATATTTT ---AGTATAT GAAATTTTAC TTTGAGAAAA TTAGAGTGCT
C. andersoni  AT.-CTAAA. ----A....G .......... .......... ..........
C. bovis      .T.T..G--- -----..... ....C..... .......... ..........
C. ryanae     .T.T..G--- -----..... ....C..... .......... ..........
C. hominis    ...T.T.... TTT....... .......... .......... ..........
C. suis       ....AT.... ATT....... .......... .......... ..........
C. ubiquitum  ....--.... AT-....... .......... .......... ..........
C. xiaoi      .T.T..G--- -----..... ....C..... .......... ..........
C. muris      AT.TCTAAA. ----A....G ....C..... .......... ..........

                        560        570        580        590        600
              ....|....| ....|....| ....|....| ....|....| ....|....|
C. parvum     TAAAGCAGGC ATATGCCTTG AATACTCCAG CATGGAATAA TAT-TAAAGA
C. andersoni  .......... .AC....... .......... .......... ..AG...G..
C. bovis      .......... TAT....... .......... .......... ...-...G..
C. ryanae     .......... TAT....... .......... .......... ...-...G..
C. hominis    .......... .......... .......... .......... ...-......
C. suis       .......... .......... .......... .......... ...-A.....
C. ubiquitum  .......... ..TA...... .......... .......... ...-A.....
C. xiaoi      .......... TAT....... .......... .......... ...-...G..
C. muris      .......... .AC....... .......... .......... ..AG...G..

                        610        620        630        640        650
              ....|....| ....|....| ....|....| ....|....| ....|....|
C. parvum     TTTTTATCTT TCTTATTGGT TCTAAGATAA GAATAATGAT TAATAGGGAC
C. andersoni  C....G.... .......... ....G..C.. A.G.....G. ..........
C. bovis      .......TC. .......... ....GA.... A....G.... ..........
C. ryanae     .......TC. .......... ....GA.... A......... ..........
C. hominis    .......... .T........ .......... .......... ..........
C. suis       .......... .T........ .......... A......... ..........
C. ubiquitum  .......... .T........ .......... A......... ..........
C. xiaoi      .......TC. .......... ....GA.... A....G.... .......A..
C. muris      C....G.... .......... ....G..C.. A.G.....G. ..........

                        660        670        680        690        700
              ....|....| ....|....| ....|....| ....|....| ....|....|
C. parvum     AGTTGGGGGC ATTTGTATTT AACAGTCAGA GGTGAAATTC TTAGATTTGT
C. andersoni  .......... ...C...... .....C.... .......... ..........
C. bovis      .......... .......... .......... .......... ..........
C. ryanae     .......... .......... .......... .......... ..........
C. hominis    .......... .......... .......... .......... ..........
C. suis       .......... .......... .......... .......... ..........
C. ubiquitum  .......... .......... .......... .......... ..........
C. xiaoi      .......... .......... .......... .......... ..........
C. muris      .......... ...C...... .....C.... .......... ..........

                        710        720        730        740        750
              ....|....| ....|....| ....|....| ....|....| ....|....|
C. parvum     TAAAGACAAA CTAATGCGAA AGCATTTGCC AAGGATGTTT TCATTAATCA
C. andersoni  .......G.. ...C...... .......... .......... ..........
C. bovis      .......... ...C...... .......... .......... ..........
C. ryanae     .......... ...C...... .......... .......... ..........
C. hominis    .......... .......... .......... .......... ..........
C. suis       .......... ...G...... .......... .......... ..........
C. ubiquitum  .......... ...G...... .......... .......... ..........
C. xiaoi      .......... ...C...... .......... .......... ..........
C. muris      .......G.. ...C...... .......... .......... ..........
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Fig. 1. Continued

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                    760        770        780        790        800
             ....|....| ....|....| ....|....| ....|....| ....|....|
C. parvum    AGAACGAAAG TTAGGGGATC GAAGACGATC AGATACCGTC GTAGTCTTAA
C. andersoni .......... .......... .......... .......... ..........
C. bovis     .......... .......... .......... .......... ..........
C. ryanae    .......... .......... .......... .......... ..........
C. hominis   .......... .......... .......... .......... ..........
C. suis      .......... .......... .......... .......... .......----
C. ubiquitum .......... .......... .......... ........- ----------
C. xiaoi     .......... .......... .......... .......... ..........
C. muris     .......... .......... .......... .......... ..........

                    810        820        830        840
             ....|....| ....|....| ....|....| ....|....| ....|
C. parvum    CCATAAACTA TGCCAACTAG AGAT TGGAGG TTGTTCCTTA CTCCT
C. andersoni .......... ....G..... .... ...... .......... .....
C. bovis     .......... .......... .... ...... .......... .....
C. ryanae    .......... .......... .... ...... .......... .....
C. hominis   .......... .......... .... ...... .......... .....
C. suis      ---------- ---------- ---- ------ ---------- -----
C. ubiquitum ---------- ---------- ---- ------ ---------- -----
C. xiaoi     .......... .......... .... ...... .......... ...--
C. muris     .......... ....G..... .... ...... .......... .....
```
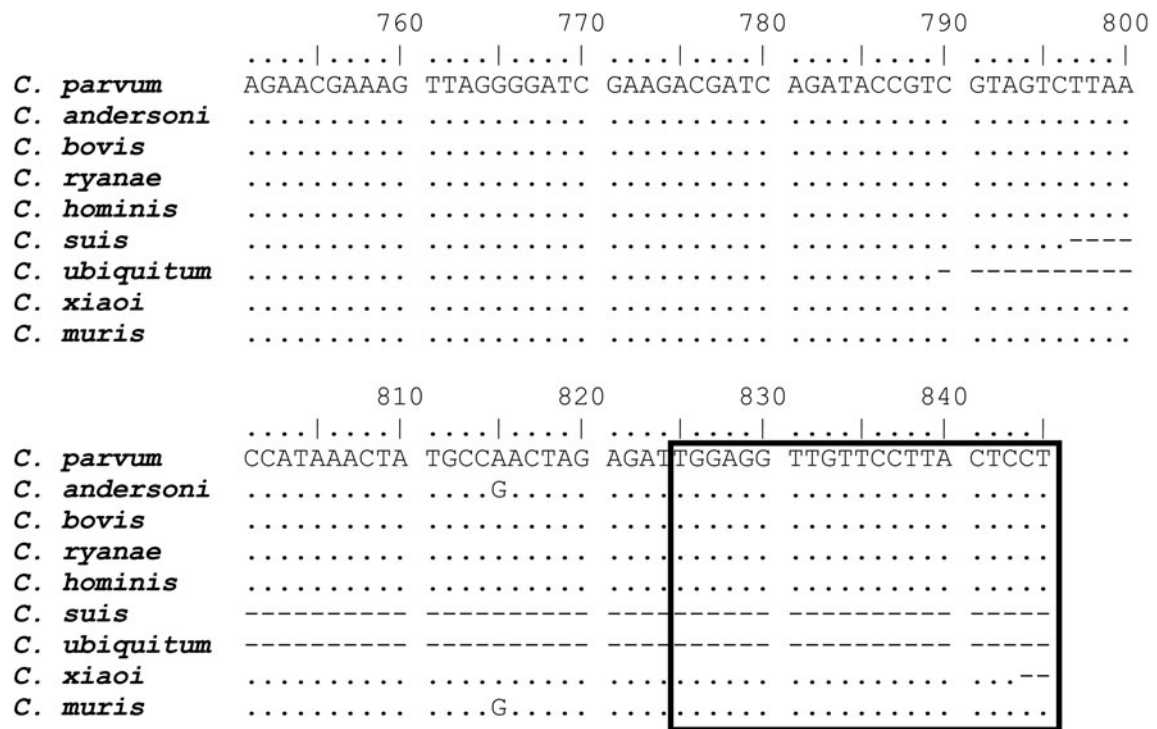
Fig. 1. Continued

the nssm-PCR were compared with sequence data for all of these samples.

Sequence analysis

To confirm the results of the nssm-PCR when testing field samples, sequence analysis of the 18S rRNA amplicon was carried out. Sequencing of PCR products was carried out by GATC (GATC, Germany) using their 'Light-run' service. Roughly 5 μL of purified PCR product was sent at a concentration of between 20 and 80 ng μL with 5 μL of the appropriate primer at a concentration of 5 pM μL. In some circumstances, instead of the light-run service, unpurified PCR products were sent to GATC from sequencing using their 'Supremerun96' sequencing option which includes PCR purification. With this service 20 μL PCR product was sent with a separate 1·5 mL tube containing 50 μL of the appropriate primer at a concentration of 10 pM μL. Sequences were analysed using BioEdit Sequence Alignment Software (www.mbio. ncsu.edu/bioedit/bioedit.html) and Chromas Lite (www.technelysium.com).

Sensitivity

The sensitivity of the nssm-PCR assay was assessed as part of another study (Wells B, Thomson S, Innes E.A and Katzer F Development of a sensitive

Table 1. Primer sequences, size of amplicon and species detected with each primer pair

Primer pair	Sequence 5′–3′	Fragment size (bp)	Species detected
AL1687 (EF) AL1691 (ER)	TTCTAGAGCTAATACATGCG CCCATTTCTTCGAAACAGGA	1370	Genus-specific external
AL1598 (IF) AL3032 (IR)	GAAGGGGTTGTATTTATTAGATAAAG AAGGAGTAAGGAACAACCTCCA	840	Genus-specific internal
CaF AL3032 (IR)	GCAAATTACCCAATCCTGAC AAGGAGTAAGGAACAACCTCCA	625	*C. andersoni*
CrF AL3032 (IR)	TGTTAATTTTTATATACAAT**R**[a]CTACGG AAGGAGTAAGGAACAACCTCCA	415	*C. ryanae*
CphF AL3032 (IR)	AGAGTGCTTAAAGCAGGCATA AAGGAGTAAGGAACAACCTCCA	305	*C. parvum*
CbF AL3032 (IR)	CTTCTTATTCCTTCTAGAATAAAAATG AAGGAGTAAGGAACAACCTCCA	241	*C. bovis*

[a] The degenerate base in the *C. ryanae* primer is shown in bold and underlined.

method to extract *Cryptosporidium* oocysts from adult cattle fecal samples – submitted). Fecal samples were spiked with *C. parvum* oocysts, subjected to different concentration steps and DNA extracted. The DNA was then amplified using the nssm-PCR.

RESULTS

Primer design

The primer designed for *C. parvum* will also amplify *C. hominis* and *C. suis*, the *C. andersoni* primer will also amplify *C. muris* and the *C. bovis* primer will amplify *C. xiaoi* as the 18S region of these species are very similar (Fig. 1). *Cryptosporidium suis* and *C. hominis* have only been found very rarely in cattle (Smith *et al.* 2005; Fayer *et al.* 2006) and *C. muris* and *C. xiaoi* have yet not been identified in cattle.

Cryptosporidium-*specific 18S rRNA PCR*

All 498 samples were tested for the presence of *Cryptosporidium* parasite DNA using the 18S genus-specific PCR, 4·13% ($n = 13$) of the samples from adult cattle and 60·5% ($n = 115$) of the samples from calves tested positive for *Cryptosporidium*. These samples were used to test the nssm-PCR.

Individual species PCR

Amplicons of the expected size for each species (*C. andersoni, C. ryanae, C. bovis* and *C. parvum*) were obtained when tested in a single species PCR with the species-specific forward primer and the 18S rRNA reverse primer. DNA was obtained from samples (confirmed by sequencing) from calves

aged <1 week (*C. parvum* DNA), calves aged 3 months (*C. bovis* and *C. ryanae* DNA) and an adult cow (*C. andersoni* DNA).

Nested species-specific multiplex PCR

Amplicons of the expected size for each of the four species (*C. andersoni, C. ryanae, C. parvum* and *C. bovis* – individually and mixed combinations) were obtained using the nssm-PCR assay (Fig. 2) from a variety of cattle samples collected from calves aged 7–14 days regardless of clinical signs ($n = 116$), all calves suffering from diarrhoea regardless of age ($n = 74$), adult cattle pre- and post-calving ($n = 177$ and $n = 120$, respectively) and other adult cows ($n = 11$). The expected amplicons for all four cattle species [*C. parvum* ($n = 92$), *C. andersoni* ($n = 1$), *C. ryanae* (only in mixed infections) and *C. bovis* ($n = 13$)] were observed as were various combinations of mixed infections (Fig. 2).

Occasionally an additional non-specific band appeared with the amplicon for *C. parvum* but of a different size to the species-specific bands. The results of the nssm-PCR were compared with sequence data for the 18S rRNA gene of these samples. In all cases, the single species of *Cryptosporidium* identified by nssm-PCR matched the species identified by sequencing. In addition, the nssm-PCR identified mixed infections in 14 of the samples examined. The mixed infections included; *C. bovis, C. parvum* and *C. ryanae* ($n = 1$), *C. ryanae* and *C. bovis* ($n = 5$), *C. parvum* and *C. bovis* ($n = 1$), *C. parvum* and *C. ryanae* ($n = 1$) and *C. andersoni* and *C. ryanae* ($n = 6$). These mixed infections were not identified by sequencing. Eight of the samples failed to amplify using the nssm-PCR.

Sensitivity

The sensitivity of the nssm-PCR when tested on fecal samples spiked with *C. parvum* oocysts was 5 opg (oocysts per gram of feces) (Table 2).

DISCUSSION

In this paper, we describe the development of a multiplex PCR test, which allows the discrimination
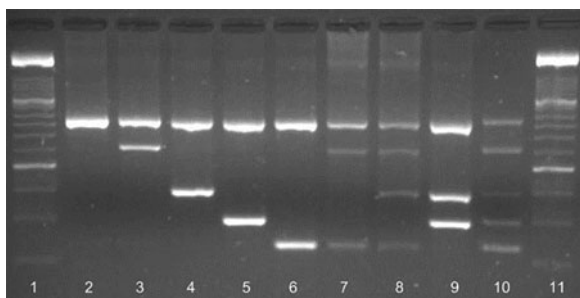

Fig. 2. Amplification of *Cryptosporidium* spp. on a 2% agarose gel stained with GelRedTM (Biotum, UK). Lane 1, 100 bp marker XIV (Roche); lane 2, 18S rRNA genus-specific amplicon (840 bp); lane 3, *C. andersoni* (625 bp); lane 4, *C. ryanae* (415 bp); lane 5, *C. parvum* (305 bp); lane 6, *C. bovis* (241 bp); lane 7, *C. andersoni* and *C. bovis*; lane 8, *C. andersoni, C. ryanae* and *C. bovis*; lane 9, *C. ryanae* and *C. parvum*; lane 10, nssm-PCR molecular marker; lane 11, 100 bp marker XIV (Roche). Amplicons in lanes 2–6 were produced using cloned DNA and lanes 7–9 were from naturally infected cattle.

Table 2. Sensitivity of nssm-PCR when tested on DNA extracted from feces spiked with 0, 5, 10, 100 or 1000 *C. parvum* oocysts per gram

0	5	10	100	1000
−	++	+++	+++	+++

−, negative; +, positive 1/3 PCRs; ++, positive 2/3 PCRs; +++, positive 3/3 PCRs.

of four of the most commonly detected *Cryptosporidium* species of cattle (one of which poses a risk to human health). The test allows the differentiation of these species by generating distinct amplicons of the 18S rRNA gene: *C. andersoni* (625 bp), *C. ryanae* (415 bp), *C. parvum* (305 bp) and *C. bovis* (241 bp) in addition to a *Cryptosporidium* genus-specific amplicon (840 bp). Currently, the most popular method of species identification in *Cryptosporidium*-positive samples is gene sequencing of the 18S rRNA gene. This method is very sensitive and can detect a large range of *Cryptosporidium* species and genotypes, but is limited when mixed infections occur as it has been shown that in a mixed sample either the dominant *Cryptosporidium* species is preferentially amplified by PCR meaning that only one species will be identifiable by sequencing or the sequence is unreadable (Santin *et al*. 2004; Cama *et al*. 2006; Silverlas *et al*. 2010). PCR amplification followed by sequence analysis is also more costly and time-consuming than multiplex PCR. The multiplex PCR described here allows the detection of mixed infections of up to four different common cattle species of *Cryptosporidium* and is an excellent method for detecting underlying infections of zoonotic *C. parvum* in calves which may have higher burdens of the less pathogenic *Cryptosporidium* species. This has very important implications for human health and the new test has also proven useful when determining which calves are most suitable for use in calf handing classes at a local veterinary school to reduce the risk of *Cryptosporidium* infection to students. It has also been used to identify the species of *Cryptosporidium* present in feces from adult cattle and their calves as part of a whole catchment study in the North East of Scotland (Wells *et al*. 2015).

In addition to the easy and quick visual differentiation of the four common cattle species other species of *Cryptosporidium* may be identified by sequencing of the 18S rRNA gene when a fragment responding to the *Cryptosporidium* genus-specific amplicon (840 bp) is amplified with no species-specific amplicons. At present, using this method, it is not possible to distinguish between *C. parvum* and *C. hominis* however, as *C. hominis* has rarely been detected in cattle (Smith *et al*. 2005; Park *et al*. 2006) there is, at this stage, no real need to distinguish between these two species.

The advantages of the PCR protocol described here over other methods used for species discrimination (PCR–RFLP, sequencing) are: (1) the ability to detect mixed infections, which may be missed by other methods; (2) the ability to identify other *Cryptosporidium* species by further sequencing when none of the 'cattle species' are detected as other species of *Cryptosporidium* may be a risk to other animals or humans; (3) the ability to test large numbers of samples quickly and easily; and

(4) the ability to detect subclinical infections of the zoonotic species *C. parvum* in animals shedding high numbers of the non-zoonotic species.

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