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# A multiplex polymerase chain reaction assay to simultaneously distinguish *Cryptosporidium* species of veterinary and public health concern in cattle

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ABSTRACT

Four species of *Cryptosporidium* are routinely found in cattle: *Cryptosporidium parvum*, *Cryptosporidium bovis*, *Cryptosporidium ryanae*, and *Cryptosporidium andersoni*. It is important to determine the species of *Cryptosporidium* in infected cattle because *C. parvum* is the only serious pathogen for humans as well as cattle. Identification of *Cryptosporidium* species and genotypes currently relies on molecular methods such as polymerase chain reaction (PCR) followed by restriction fragment length polymorphism (RFLP) or gene sequencing. Incorporation of these techniques in a routine veterinary diagnostic laboratory is cost prohibitive. As such, their applications are limited primarily to research and a few public health laboratories. To overcome this problem, a multiplex PCR assay was developed for simultaneously detecting the 4 species of *Cryptosporidium* that commonly infect cattle. This assay specifically identifies *Cryptosporidium* oocysts present in cattle feces, improves the detection of mixed infections, reduces the time and cost relative to current sequencing methods, and further demonstrates the shortcomings of sequencing as the definitive method for identification when analyzing samples containing mixed infections.

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## 1. Introduction

Parasites of the genus *Cryptosporidium* infect humans and animals. Eight species of *Cryptosporidium* and one additional genotype are infectious to cattle (Santín and Trout, 2008) although only four species are found routinely; *Cryptosporidium parvum*, *Cryptosporidium bovis*, *Cryptosporidium andersoni*, and *Cryptosporidium ryanae* (previously known as the deer-like genotype) (Fayer et al., 2006, 2007, 2008; Langkjær et al., 2006; Feng et al., 2007; Brook et al., 2008; Feltus et al., 2008; Santín et al., 2004, 2008). Infections with other *Cryptosporidium* species such as *Cryptosporidium hominis* (Smith et al., 2005; Park et al., 2006; Feng et al., 2007), *Cryptosporidium suis* and *C. suis*-like (Fayer et al., 2006; Geurden et al., 2006, 2007; Langkjær et al., 2006), and *Cryptosporidium felis* (Bornay-Llinares et al., 1999), along with the *Cryptosporidium* pig genotype II (Langkjær et al., 2006), have been reported only rarely. Infection with *Cryptosporidium canis* has been demonstrated under experimentally induced conditions (Fayer et al., 2001) but has not yet been identified in a natural setting.

A relationship exists between the age of the cattle and the species of *Cryptosporidium*. Most *Cryptosporidium* infections in pre-weaned calves are due to *C. parvum* and those in post-weaned calves are due to *C. bovis* and *C.*

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**Table 1**
Number of samples examined and number of samples positive using the different sets of primers.

| *Cryptosporidium* species/genotype identified by PCR/SEQ of a fragment of the SSU rDNA gene | Number of samples examined | Number of positive samples using different sets of primers[a] | | |
|---|---|---|---|---|
| | | 1010/1011 (*C. parvum* and *C. hominis*) | 1005/1008 (*C. bovis* and *C. ryanae*) | 1018/1019 (*C. andersoni*) |
| *C. parvum* | 18 | 18 (*C. parvum*) | 0 | 0 |
| *C. bovis* | 16 | 5 (*C. parvum*) | 16 (15 *C. bovis* and 1 mixed infection with *C. bovis*/*C. ryanae*) | 0 |
| *C. ryanae* | 19 | 9 (*C. parvum*) | 19 (8 *C. ryanae* and 11 mixed infections with *C. bovis*/*C. ryanae*) | 0 |
| *C. andersoni* | 8 | 0 | 3 (2 *C. bovis* and 1 *C. ryanae*) | 8 (*C. andersoni*) |
| *C. canis* | 3 | 0 | 0 | 0 |
| *C. felis* | 5 | 0 | 0 | 0 |
| *C. hominis* | 3 | 3 (*C. hominis*) | 0 | 0 |
| *C. suis* | 1 | 0 | 0 | 0 |
| Pig genotype II | 4 | 0 | 0 | 0 |

[a] In parenthesis: *Cryptosporidium* species identified after sequencing the PCR product.

*ryanae*. *C. andersoni* is most often found in cattle over 1 year of age (Santín et al., 2004, 2008; Fayer et al., 2006, 2007; Langkjær et al., 2006; Feng et al., 2007). It is very important from a public health perspective to identify the species of *Cryptosporidium* present in infected cattle because of the species that commonly infect cattle, only *C. parvum* is considered zoonotic. Identification of *Cryptosporidium* species relies on molecular methods that include polymerase chain reaction (PCR) followed by restriction fragment length polymorphism (RFLP), or gene sequencing. Because most PCR methods that genotype *Cryptosporidium* make use of primers that are conserved among all *Cryptosporidium* species, only the dominant species in a specimen is routinely identified. Thus, infections with multiple species become difficult to diagnose and minor species frequently go undetected (Xiao and Ryan, 2008). This undermines our ability to understand the epidemiology of cryptosporidiosis in cattle.

There have been attempts to develop multiplex PCR tests for differentiating subsets of *Cryptosporidium* species. One such test simultaneously delineates among *C. parvum*, *Cryptosporidium wrairi*, *Cryptosporidium baileyi* and *Cryptosporidium muris* (Patel et al., 1999). Using this assay, *C. parvum* and *C. wrairi* generate identical fragments from the small subunit rDNA (SSU rDNA). As such, a follow-up PCR-RLFP targeting the outer wall protein (COWP) is required to distinguish among them (Patel et al., 1999). Also, a nested multiplex PCR amplifying the heat shock protein 70 was developed for detecting the 4 human *Cryptosporidium* species, *C. parvum*, *C. hominis*, *C. canis*, and *C. felis* (Lindergard et al., 2003); however, no multiplex studies have ever targeted *Cryptosporidium* species commonly found in cattle. Inasmuch as epidemiological studies are frequently directed at cows as a key source for human infections, a multiplex PCR test was developed to rapidly detect and accurately delineate multiple *Cryptosporidium* species in cattle by examining oocyst DNA. This test is based on simultaneously amplifying different sized fragments of the actin gene in the 4 most common species of *Cryptosporidium* found in cattle, followed by amplicon detection on agarose gels thereby eliminating the need to sequence the PCR product.

## 2. Materials and methods

### 2.1. Sources of specimens, oocysts purification, and DNA extraction

Fecal samples containing oocysts of *C. parvum*, *C. bovis*, *C. ryanae*, *C. andersoni*, *C. hominis*, *C. canis*, *C. suis*, *C. felis*, and the *Cryptosporidium* pig genotype II, as defined by sequencing PCR-amplified SSU rDNA fragments, were obtained from infected animals and humans (Table 1). Oocysts were concentrated from feces by CsCl centrifugation as previously described (Fayer et al., 2000; Santín et al., 2004). Total DNA was extracted from each CsCl-cleaned fecal sample using a modification of the DNeasy Tissue Kit (Qiagen, Valencia, California). A total of 50 μl of purified oocysts were suspended in 180 μl of ATL buffer (supplied by the manufacturer) and vortexed. To this suspension, 20 μl of proteinase K (20 mg/ml) was added and the mixture incubated overnight at 55 °C. The next day, 200 μl of AL buffer was added and purification proceeded as per manufacturer's instructions. The nucleic acids were eluted in 100 μl of AE buffer.

### 2.2. Gene amplification and sequencing

Nucleotide sequence analyses of the SSU rDNA followed by alignment with known *Cryptosporidium* sequences were used to validate the identity of the oocysts. A two-step nested PCR protocol was used to amplify a fragment of the SSU rRNA gene of *Cryptosporidium* (~830 bp) using primers and PCR conditions previously described (Xiao et al., 1999; Santín et al., 2004).

To conduct the multiplex assay, a nested PCR was required. First, a single set of degenerate primers was used to amplify a fragment of the actin gene from homogeneous preparations of the 4 most common species of *Cryptosporidium* that infect cattle; *C. parvum*, *C. bovis*, *C. ryanae*, and *C. andersoni*. These primers (forward 5′-ATGRGWGAAGAAGWARYWCAAGC and reverse 5′-AGAARCAYTTTCTGTGKACAAT) produced a single PCR product approximately 1095 bp in length (Sulaiman et al., 2002). The primary PCR mixture contained 1× PCR buffer, 3 mM $MgCl_2$, 0.2 mM dNTP, 2.5 U Taq (Qbiogene, Irvine, CA), 2.5 μl

```
C. parvum      360TGGTATGGTCAAGGCAGGTGTTGCAGGTGATGACGCTCCAAGATGTGTTTTCCCATCGATCGTTGGTCGCCCAAAAATGCCAGGT
C. andersoni     G.................AT....T........T.....TC.C...............A..T..AC.AAA.A....GG........T
C. bovis                ..A.........A.A..T.....C..T.....TC.......A..T.....A..A..A.....T.............A
C. ryanae               ..A..........A..T.....C........TC.T.....A..T..G..A..A..A.....T.....G.....G..A

C. parvum      GTTATGGTAGGTATGGACCAAAAGGACTGTTATGTTGGTGATGAAGCTCAATCAAAGAGAGGTATTTTGACTTTGAAATACCCAA
C. andersoni   A................T..G..AA...................G..A..G..C..A.........C.A.....A..G..T....
C. bovis       ........T.........................A.....C..G....................A.....A........T....
C. ryanae      ........T........T................A........G....................A.....AC....G.......

C. parvum      TTGAGCATGGTATTGTTACAAACTGGGAAGATATGGAGAAGATCTGGCATCATACTTTCTATAATGAATTACGTGTTGCTCCTGA
C. andersoni   ....A................T....................A.....C.....A..T..C........G.....A..C.....
C. bovis       .......Y....................G........A.....A...........A..T..C........G.....C.....G..
C. ryanae      .......C..C................G.............A...........A.....C.....G..G.............

C. parvum      GGAACACCCAGTATTATTGACTGAAGCACCAATGAACCCAAAGGTAAATCGTGAAAGAATGACTCAAATAATGTTTGAAACATTC
C. andersoni   A.....T.....T.....A..A.....T........T.....A.C...C....................T...........T...
C. bovis       A..G..T........G.....A..G..C........T.........................A.....T........G.....T
C. ryanae      ...G..T..T..T........A.....C........T.....A...........G..G.....G....................

C. parvum      AATGTACCAGCAATGTATGTTAATATTCAGGCTGTCTTATCATTGTATGCCTCTGGTCGTACAACTGGTATTGTTTTAGATAGTG
C. andersoni   ........C...........A.....A..A.....TC.G..T........A.....A.....T.....A.....AC.T.......
C. bovis       ..............................A.....T..G..T...........A...........A........A..........
C. ryanae      ..C.....T.......................A..T..G..T.....C.....C...........A........A..........

C. parvum      GTGATGGTGTATCACATACTGTTCCAATCTATGAAGGTTATGCTATTCCACATGCTATTATGAGATTAGATCTAGCTGGACGTGA
C. andersoni   ....................A.....T..T.....G.........C....T.....C.......................T.....
C. bovis       .A........T..G..C..C........T..............CC....T.....A........G..G...T.G.....T.....
C. ryanae      ..........T.....C...........T..C.....C......C....T.....A..............CT.G..C..T..C..

C. parvum      TTTAACTGATTTCTTGATGAAGATTCTCCACGACCGTGGTTACAGCTTTACAACAACAGCTGAGAGAGAAATCGTAAGAGATATT
C. andersoni   .C.......C.ATC.A.....A.....A..T..T.................T.....T.....A........A..T.........
C. bovis       C..G..A..C...C.C.....A...T.A..T..T.....C.......................A........A..G..G.....C
C. ryanae      ...G..C..C...C.T........A..T..T..T.....C.....T..C........G..A..A..G.....A..G........C

C. parvum      AAGGAAAAGCTCTGCTACATTGCTTTAGATTACGAGGAAGAAATGAAGAAATCTCAAGAATCTTCTGAATTAGAGAAGACATATG
C. andersoni   .........T.A..T.............G..T..A..T...T.A.....GG.C..G............A.T.....A.......
C. bovis       ........A..T.....T..C.....G..C..T..A..G.........G.....G........A...A.T..A..........
C. ryanae      .....G.....T........C..C..G..C..T..A..G.........G.....G..G.....A...A.T.....A.....C.

C. parvum      AACTACCAGATGGCCATGTAATTACTGTAGGTAGTGAAAGATTCAGATGTCCTGAGGCTTTATTCCAACCAGGTTTTTAGGTAA
C. andersoni   ..T..........TA....T..............C..G.....T........A..A..AC.T.........AA.C.A........
C. bovis       ..T....T.....A.........M....G..A.....G.....T.....C..A..A.....G.....G..T.....A..G.....
C. ryanae      .GT.G..T.....A.....G.....C..G..A..C..G..............A..A..C..G........G..C..A........

C. parvum      AGAAGCTGTCGGTATTGGTGAAACTACATTCCAATCTATCATGAAATGTGATCTTGATATCCGTAAAGACCTTTATGCCAATATT
C. andersoni   G.......CT...G.............T...........T..................C........G..TT.G.....A...G..
C. bovis       ...G.....T...........G..C..T...........T.....G..C...........T.....G..T..C..C..A..C...
C. ryanae      .........T...........G.....T.....G.....T.....G..T.....C...........G..T..C.....A..C...

C. parvum      GTTTTATCTGGAGGTACAACAATGTATCCAGGTATTGGAGAAAGAATGACAAAAGAGCTCACTTCTTTGGCTCCATCAACAATGA
C. andersoni   ..CC.T.....T.....T...........T..AC....T..G........T.....A..T....GC..A.....T..T..T....
C. bovis       .....G.....T.....G..............C.....T..G..............A..T.....A..A..A..T..T.......
C. ryanae      ........C..T.........................C..G..............A..T..C..A.....A..T..C.......

C. parvum      AGATCAAGGTTGTTGCACCTCCTGAGCGTAAATACTCTGTATGGATTGGTGGTTCCATCTTATCTTCTTTATCTACTTTCCAACA
C. andersoni   ....T..A........C.........A.A.....T..A...........A..A.....A.....A.......G...G.....G..
C. bovis       ....T..A.....C..C..A..A........G..T..C..T..............A..T........GC.C..A..G........
C. ryanae      ....T.....G..C..C..A.....A..C.....T..C..T..............A..T..G.....GC.C..A..A........

C. parvum      AATGTGGATCACTAAGGAAGAG1407
C. andersoni   G........T_.A.....G..A
C. bovis       ............A.........
C. ryanae      ............A
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**Fig. 1.** Locations of primers used for nested multiplex PCR. Sequences above are derived from the following accession numbers: M86241 (*C. parvum*), AF382352 (*C. andersoni*), AY741307 (*C. bovis*), and EU410345 (*C. ryanae*). Nucleotide numbering is based upon the complete nucleotide sequence of the *C. parvum* actin gene. Locations of the forward and reverse primers corresponding to each species are underlined and highlighted in bold. Primer sets were defined as 1010/1011 (*C. parvum* and *C. hominis*); 1005/1008 (*C. bovis* and *C. ryanae*) and; 1018/1019 (*C. andersoni*). The first bold primer for each species is designated as the forward primer, and all reverse primers are the reverse complement of those shown above. "Dots" indicate conserved nucleotides based upon the *C. parvum* sequence. First round PCR was performed with forward and reverse degenerate primers as defined in Section 2. These primers bind at positions 322 and 1429, respectively, and are not shown in the sequence above.

BSA (0.1 g/10 ml), 1 μM of each primer, and 5 μl of the template DNA in a 50 μl reaction. A total of 35 cycles, each consisting of 94 °C for 45 s, 50 °C for 45 s, and 72 °C for 1 min, was performed; an initial pre-heat step at 94 °C for 5 min and a final extension step at 72 °C for 10 min were also included.

For the nested PCR, three sets of specific primers were designed based upon multiple sequence alignment of the actin genes from each species. The locations of the primers used for the multiplex are shown in Fig. 1; the first primer set 1010 (5′-AATCGTGAAAGAATGACTCAAATA) 1011 (5′-CTGGTAGTTCATATGTCTTCTCTAA) amplifies a 400 bp fragment from *C. parvum*; and the second primer set 1018 (5′-GCTGTGTTTTCCCATCAATTGTACGA) and 1019 (5′-GCATACAAAGACAGAACAGCTTGTATATTT) amplifies a 350 bp fragment of *C. andersoni*; and the third primer set 1005 (5′-GAAGTCTCAGGAATCTTCAGAAATT) and 1008 (5′-TAATCTTCATTGTAGAAGGTGCT**T**AT) amplifies a 300 bp fragment from both *C. bovis* and *C. ryanae*; The third base (bold) from the 3′ end of primer 1008 was deliberately changed from "A" to "T" to destabilize non-specific primer binding to DNA from species other than *C. bovis* and *C. ryanae*. The nested PCR mixture contained 1× PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTP, 2.5 U Taq (Qbiogene, Irvine, CA), 2.5 μl BSA (0.1 g/10 ml), 1 μM of each primer, and 2 μl of the primary PCR reaction in a 50 μl reaction. A total of 40 cycles, each consisting of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 2 min, was performed; an initial pre-heat step at 95 °C for 2 min and a final extension step at 72 °C for 7 min were also included.

PCR products were separated on 2% NuSieve GTC agarose gels (FMC BioProducts, Rockland, ME) and visualized after ethidium bromide staining. All PCR products were purified using Exo-SAP-IT™ (USB Corporation, Cleveland, OH), and sequenced in both directions on an ABI3100 Genetic Analyzer (Applied Biosystems, Foster City, CA) using the actin nested PCR primers and Big Dye™ Terminator v3.1 chemistries (Applied Biosystems, Foster City, CA). Sequence chromatograms of each strand were aligned and examined with Lasergene software (DNASTAR, Inc., Madison, WI).

### 2.3. Experimental design

The amplification specificities of the PCR primers putatively identified as species specific were validated on DNAs from known homogeneous samples of *C. parvum*, *C. bovis*/*C. ryanae*, and *C. andersoni* both individually and in all possible DNA combinations to mimic naturally derived samples. The specificity of the primers was validated by sequencing all PCR amplicons generated during the experiment to corroborate that the correct *Cryptosporidium* species was being amplified and that no non-specific amplification was taking place (data not shown). Comparable amounts of DNA from each species were included in all mixtures prior to diluting to 6 μl. Concentrations of DNA could not be spectrophotometrically quantitated for parasite material because of host and environmental DNAs which routinely contaminate the samples. As such, PCR using parasite-conserved rDNA primers was used to normalize the amounts of each parasite DNA prior to mixing. To normalize the DNA concentrations, serial dilutions of DNA from each species were PCR amplified under identical conditions where the chosen cycle number was within the linear range of the amplification curve for all dilutions. PCR products were separated by agarose gel electrophoresis and stained with ethidium bromide. The gels were scanned and band signal intensities compared. Stock DNA samples were diluted to approximate the same DNA level in each sample (species). The above experiment was then repeated after the DNAs were diluted to validate the normalized stock DNA samples. DNA trial samples were prepared as follow: samples 1–3 contained 2 μl of *C. parvum*, *C. bovis*, and *C. andersoni*, respectively, plus 4 μl of $dH_2O$ to obtain a final volume of 6 μl; sample 4 contained 2 μl each of *C. parvum* and *C. bovis*, plus 2 μl of water to obtain a final volume of 6 μl; sample 5 contained 2 μl each of *C. parvum* and *C. andersoni*, plus 2 μl of water to; sample 6 contained 2 μl each of *C. bovis* and *C. andersoni*, plus 2 μl of water to obtain a final volume of 6 μl; and sample 7 contained 2 μl each of *C. parvum*, *C. bovis*, and *C. andersoni* to obtain a final volume of 6 μl. All 6 μl samples were subjected to the primary PCR followed by the nested multiplex PCR.

Once validated on control samples, the PCR assay was used to examine 61 DNA samples obtained from naturally infected cattle. The results obtained directly from agarose gels and from sequencing each band of the multiplex PCR were compared to infection data obtained by benchmark sequencing the SSU rDNA of each environmentally derived sample followed by alignment with published sequences (Table 1). In addition, 16 samples containing DNA from 4 other *Cryptosporidium* species (*C. canis*, *C. felis*, *C. hominis*, *C. suis)* and one genotype (pig genotype II) not routinely found in cattle were tested to validate primer specificity (Table 1).

To determine the sensitivity of the methods used to detect *Cryptosporidium* oocysts in the present study, *Cryptosporidium* negative bovine feces were obtained. Six replicate 15 g fecal specimens were each spiked with oocysts of *C. parvum* at the rate of 10, 100, and 1000 oocysts/g and subjected to the same methods of concentration and molecular detection as described above.

## 3. Results

In controlled experiments, normalized amounts of DNA from each species were mixed in all possible combinations to validate the multiplex test and the choice of primers (Fig. 1). The expected size amplicons were generated from each pure *Cryptosporidium* species DNA (lanes 1–3) as well as all possible combinations of these DNAs (lanes 4–7) (Fig. 2). No primer cross-reactivity was observed among the cattle species tested.

In the 61 DNA samples obtained from naturally infected cattle, the targeted amplicons for all 4 cattle species (*C. parvum*, *C. andersoni*, *C. bovis*, or *C. ryanae*) were observed and in various combinations. The presence of *C. parvum* and *C. andersoni* resulted in amplicon sizes of 400 bp and 350 bp, respectively. The presence of *C. bovis* or *C. ryanae* resulted in a single amplicon of 300 bp. Also, an amplicon of 400 bp was obtained when the *C. parvum* primer set

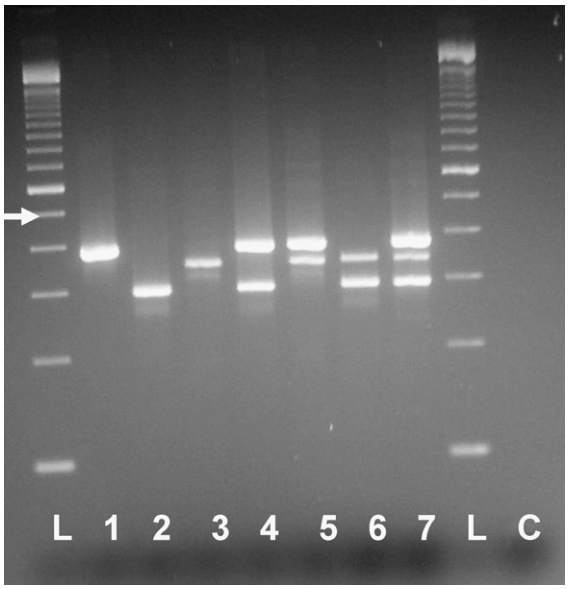


**Fig. 2.** Multiplex PCR amplification of *C. parvum*, *C. bovis*, and *C. andersoni* observed on 2% agarose gels stained with ethidium bromide. Lane L, 100-bp DNA ladder; lane 1, *C. parvum*; lane 2, *C. bovis*; lane 3, *C. andersoni*; lane 4, *C. parvum* and *C. bovis*; lane 5, *C. parvum* and *C. andersoni*; lane 6, *C. bovis* and *C. andersoni*; lane 7, *C. parvum*, *C. bovis*, and *C. andersoni*; lane C, negative control. The white arrow indicates the 500 bp marker of the 100 bp DNA ladder.

1010/1011 was used to amplify 3 DNA samples identified previously as *C. hominis* by SSU rDNA sequencing. All 3 primer sets failed to amplify DNA from *C. canis*, *C. felis*, *C. suis*, and the *Cryptosporidium* pig genotype II.

All PCR products were sequenced for confirmation. In every case, the *Cryptosporidium* species identified by the multiplex PCR assay corresponded with the species defined by SSU rRNA gene sequencing; however, the multiplex PCR also identified mixed infections of *C. parvum*/*C. bovis*, *C. parvum*/*C. ryanae*, *C. andersoni*/*C. bovis*, and *C. andersoni*/*C. ryanae* in 5, 9, 2, and 1 of the samples examined, respectively (Table 1). These mixed infections were missed by SSU rDNA sequencing (Table 1). Furthermore, the 12 mixed infections containing both *C. bovis* and *C. ryanae* were confirmed by multiple peaks in key locations in the sequence chromatograms (i.e., the presence of two nucleotides at the same position) of the actin gene fragment (data not shown).

Of the 6 fecal specimens each spiked at 10, 100, and 1000 *C. parvum* oocysts per gram, 66%, 100%, and 100% were found positive by the multiplex PCR, respectively (data not shown).

## 4. Discussion

For identification of *Cryptosporidium* species and genotypes, SSU rRNA gene sequencing is the most commonly used method. This technique as well as PCR-RFLP are very sensitive and can detect a wide range of *Cryptosporidium* species and genotypes (Xiao and Ryan, 2008); however, these methods have not been found useful in detecting mixed infections because the dominant species or genotype in the specimen becomes preferentially amplified by the PCR when only genus-specific primers are used (Cama et al., 2006). To develop the multiplex PCR assay, the actin gene was targeted because it provided more sequence variation for primer design.

The multiplex PCR assay developed herein generated the predicted 3 different sized amplicons for *C. parvum*, *C. andersoni*, and *C. bovis*/*C. ryanae* thereby identifying the most common species of *Cryptosporidium* that infect cattle. Furthermore, the multiplex PCR assay was able to detect mixed infections that were missed by conventional sequencing of the SSU rRNA gene. Preliminary data on assay sensitivity suggest that DNA extracted from 10 oocysts in feces was insufficient to generate consistent detection. However, 100 oocysts in feces reproducibly gave positive results by multiplex PCR. Detection efficiency in the range of 10–100 oocysts was not determined. These data confirm the results of others advocating the inadequacies of sequencing only (Cama et al., 2006), and suggest that mixed infections in cattle may be more prevalent than originally believed.

It is crucial to differentiate among *Cryptosporidium* species infecting cattle because only *C. parvum* is zoonotic where *C. bovis*, *C. andersoni*, and *C. ryanae* are cattle adapted (Lindsay et al., 2000; Fayer et al., 2005, 2008; Santín and Trout, 2008). Results presented here indicate that 44% (14/32) of the *C. parvum*-infected animals were missed by conventional SSU rDNA sequencing. Thus, the multiplex PCR is a better method for differentiating between zoonotic and non-zoonotic species of *Cryptosporidium* of cattle and is critical for determining the risk to public health. Furthermore, the amplification of *C. hominis* using the *C. parvum* primer set 1010/1011 can be considered an added advantage because the presence of the 400 bp amplicon will indicate that one of the two most common species responsible for human cryptosporidiosis, *C. parvum* and *C. hominis*, is present in a particular sample.

With regard to animal health, *C. parvum* infection causes diarrhea in young calves (Fayer et al., 1998; de Graaf et al., 1999) and *C. andersoni* infection causes reduced milk production in mature cows (Esteban and Anderson, 1995); however, neither disease nor changes in host physiology have been linked to animals infected with *C. bovis* or *C. ryanae* (Fayer et al., 2005, 2008). As such, there is no practical need to differentiate between these species at this time. In conclusion, the advantages of the multiplex PCR test described in this study are its ability to: (1) identify mixed infections which are critical to understanding cryptosporidiosis epidemiology; (2) differentiate the zoonotic pathogen *C. parvum* from the other 3 common species of *Cryptosporidium* in cattle without further analysis, and; (3) identify the presence of *C. parvum* as a minor component of *Cryptosporidium*-infected animals that is often missed using conventional sequencing methodologies.

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