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A multiplex allele specific polymerase chain reaction (MAS-PCR) on the dihydrofolate reductase gene for the detection of *Cryptosporidium parvum* genotypes 1 and 2

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SUMMARY

A multiplex allele specific polymerase chain reaction (MAS-PCR) based on the *Cryptosporidium parvum* dihydrofolate reductase (*dhfr*) gene sequence differentiates genotype 1 ('Human') from 2 ('Cattle') in a 1-step reaction. The MAS-PCR was validated on a panel of 34 microscopically positive *C. parvum* faecal samples of human and animal origin in comparison with 2 published PCR-restriction fragment length polymorphism (RFLP) methods targeting *dhfr* and the oocyst wall protein (*cowp*) genes. A validation panel of 37 negative faecal samples of human and animal origin was also tested in comparison with the *cowp* PCR-RFLP. MAS-PCR was found to be as sensitive for species detection as the most sensitive of the other tests, and detected more mixed genotype infections than the two other tests combined. In addition the MAS-PCR showed equivalent detection sensitivity in comparison with a published nested RFLP targeting the SSU rRNA gene, on a panel of prepared mixed genotype samples. The 1-step reaction is simpler and less expensive to perform than the RFLP methods, while the *C. parvum* specific amplicons and those for genotypes 1 and 2 (575, 357 and 190 bp respectively) can be easily distinguished on agarose gel.

Key words: *Cryptosporidium parvum*, genotyping, dihydrofolate reductase, multiplex polymerase chain reaction.

INTRODUCTION

Cryptosporidium parvum is a protozoan parasite of the intestine, and is the causative agent of cryptosporidiosis in humans and other mammals. In the immunocompetent host the typical watery diarrhoea can be severe, but is self-limiting; in the immunocompromised host it is severe and chronic, and may cause death. The increasing incidence of human cryptosporidiosis attributed to direct or indirect contact with infected animals, has received considerable attention from public health workers and the media in recent years because of large drinking water-associated outbreaks (MacKenzie *et al.* 1994). Studies using molecular typing methods have divided the *C. parvum* population into at least 2 genotypes, using a variety of sequenced genes including *trap* (thrombospondin-related adhesive protein) (Spano *et al.* 1998*a*), *cowp* (*Cryptosporidium* oocyst wall protein) (Spano *et al.* 1997; Pedraza-Dìaz *et al.* 2001*a*), *dhfr* (dihydrofolate reductase) (Vasquez *et al.* 1996; Gibbons *et al.* 1998) and more recently beta-tubulin (Widmer *et al.* 1998; Sulaiman *et al.* 1999*a*; Caccio *et al.* 1999), and Small-Subunit rRNA (SSU-rRNA) gene locus (Xiao *et al.* 1999). Genotype 1 has been found primarily in the human population (although there are single reports in rhesus monkeys and a Dugong (Morgan *et al.* 2000) and successful experimental infections in pigs (Widmer *et al.* 2000) and lambs (Giles *et al.* 2001). Genotype 2 is found in humans and a wide range of mammals. Subdivisions of genotype 1 and genotype 2 have also been described using single and multi-locus microsatellite markers (Spano *et al.* 1998*b*; Aiello *et al.* 1999; Caccio *et al.* 2000; Feng *et al.* 2000). Rarer genotypes from humans, that do not fall within the classification of 1 and 2, have been described which are found in approximately 3% of human isolates (Patel, McLauchlin & Pedraza-Díaz, 1999). It is important to have the capacity to distinguish between genotypes, as the risk of transmission of different genotypes of *C. parvum* from animals to humans has not as yet been formally quantified. Limited data are currently available on the performance of published genotyping PCR's in mixed genotype infections (Morgan *et al.* 1997). In addition most available methods require a 2 or 3-

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step approach, which requires identification of allelic polymorphisms by restriction enzymes or sequencing. The MAS-PCR described here involves 1 step only. A randomly amplified polymorphic DNA (RAPD) direct PCR that differentiates between genotypes has, however, been described utilizing unknown target sequences (Morgan *et al*. 1997) and the ITS1 region (Carraway, Tzipori & Widmer, 1996). Published evaluations of different *C. parvum* genotyping techniques indicate that the PCR-RFLP on the *dhfr* gene (Gibbons *et al*. 1998) and on the SSU rRNA gene are more sensitive than both the direct RAPD-PCR and ITS1 PCR, and more specific than some other published genotyping PCRs (Sulaiman, Xiao & Lal, 1999*b*). Both PCR-RFLPs require a 3-step method, which is possibly time-consuming, expensive and susceptible to cross-contamination. A 1-step system with comparable sensitivity and specificity would minimize these problems.

To maximize specificity of a PCR primer for a single nucleotide polymorphism where the polymorphic nucleotide is located at the 3′ end of the primer in question, one or two bases upstream may be deliberately mismatched (Newton *et al*. 1989).Inevitably this reduces the sensitivity of the detection system. The sequence of the *C. parvum dhfr* allows this approach to be taken to differentiate genotypes 1 and 2 without introducing upstream mismatches since, if the 3′ allele-specific base of the primer is chosen carefully, additional mismatches to the other genotype are naturally present upstream. The MAS-PCR described here is regarded as an improvement on the PCR-RFLP of Gibbons *et al*. (1998) for the detection of genotypes 1 and 2, since it is carried out in 1 step and does not require restriction.

MATERIALS AND METHODS

Sources of C. parvum *samples*

C. parvum isolates (human and animal) used in the studies were collected as positive faecal samples from a number of hospitals and laboratories in England and Denmark. Six samples from this group (after genotyping) were selected for transmission into calves and lambs and the faeces screened by modified Ziehl-Neelsen (mZn) staining for the presence of *C. parvum* and genotyping performed if the *C. parvum* infection was present. A panel of 37 microscopically negative human and animal faecal samples were collected from a number of hospitals, laboratories and farms in S.W. England. Random animal faecal samples, of unknown *C. parvum* status, were also collected from various farms in the Avon and Somerset area. Additional faecal samples of unknown *C. parvum* status from cases of diarrhoea in Egypt and *C. parvum* DNA from human samples that were used in the development of the PCR-RFLP (Gibbons *et al*. 1998) were also obtained.

Modified Ziehl-Neelsen stain (*mZn*)

The staining protocol used was as described by Casemore *et al*. (1984). Briefly, faecal smears of graduated thickness were prepared on slides, heat fixed, stained with carbol fuchsin, counter-stained with aqueous malachite green and twenty fields examined by light microscopy at $\times 400$ magnification. *Cryptosporidium* oocysts stain irregularly red against a dark background, and appear approximately spherical, 4–6 μm in diameter. The number of oocysts visible were counted and scored.

DNA extraction

DNA was extracted from faecal samples previously 'cleaned' by low-speed centrifugation, to remove faecal debris or purified using salt flotation concentration techniques (Webster *et al*. 1996*a*). Total DNA was extracted using the method of Boom *et al*. (1990) modified by McLauchlin *et al*. (1999). The purified DNA eluted in nuclease free water was stored at $+4$ °C. The DNA was not quantified before use in the PCR reactions.

Dihydrofolate reductase PCR-RFLP

The *C. parvum* dihydrofolate reductase (*dhfr*) nucleotide sequence for both genotype 1 (GenBank Accession number U41366) and genotype 2 (GenBank Accession number U41365) has been previously published (Vasquez *et al*. 1996) and a PCR-RFLP published capable of distinguishing between genotype 1 and genotype 2 *C. parvum*, validated by DNA sequencing (Gibbons *et al*. 1998). The 575-bp region in question demonstrates 14 nucleotide differences. This *dhfr* PCR-RFLP was used to genotype selected *C. parvum* isolates from human and animal origin prior to genotyping by MAS-PCR. In addition, the genotype of most isolates was confirmed using a published PCR-RFLP on the *cowp* gene (Spano *et al*. 1997).

MAS-PCR design

The species-specific outer primers were as described by Gibbons *et al*. (1998). Internal genotype-specific primers were designed to match the sequences illustrated in Fig. 1. The 3′ terminal nucleotide of primer 1R was complementary to a single base specific for genotype 1 and included 2 specific upstream single nucleotide polymorphisms, giving a predicted 357-bp amplicon. Primer 2R was designed with the 3′ terminal nucleotide corresponding to a single base specific for genotype 2 and included 2 specific upstream single nucleotide polymorphisms, giving a predicted 190 bp amplicon. Both were antisense to help reduce any spurious results caused by mis-priming.

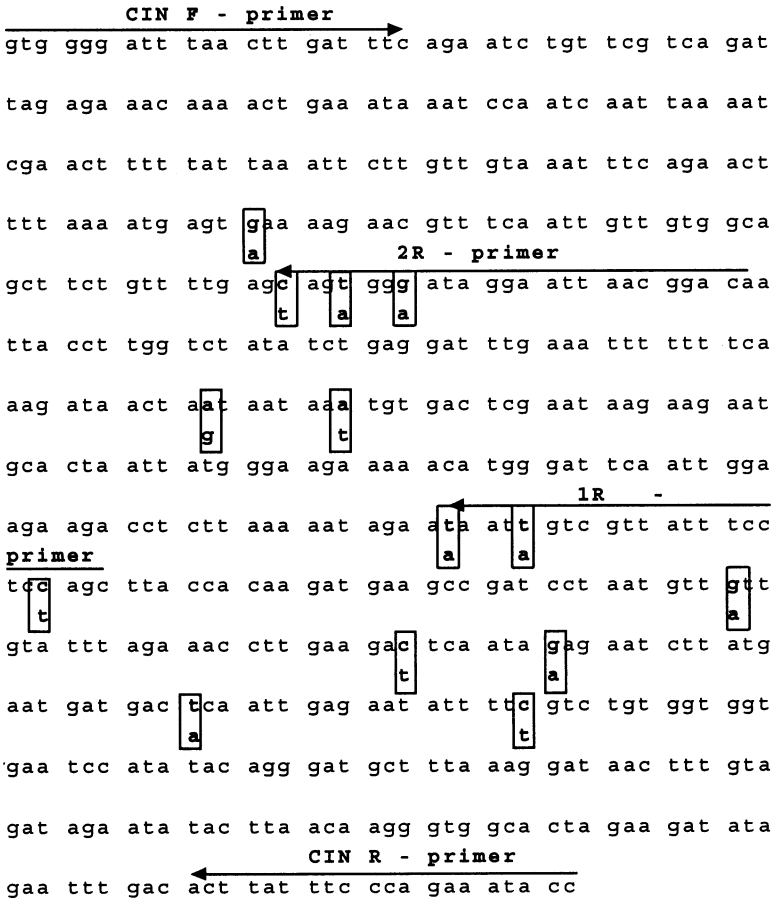

Fig. 1. The sequence of a 575 bp region of the genotype 1 *Cryptosporidium parvum dhfr* gene. The genotype 2 allele differences are indicated in the boxes. Outer primers (CINF/CINR) are previously published *C. parvum* specific sequences. The internal primers (1R/2R) are genotype specific primers.

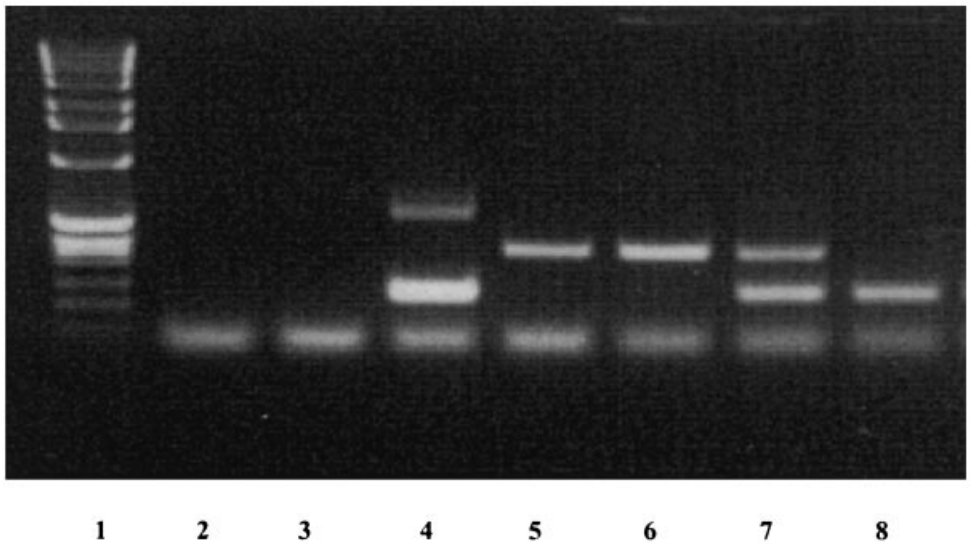

Fig. 2. Photograph of the MAS-PCR from a genotype 1, a genotype 2 and mixed genotype *Cryptosporidium parvum* isolates, resolved on a 2% agarose gel and visualized with 10 μg/ml ethidium bromide. Lane 1, 1 kb molecular weight marker; lane 2, negative control; lane 3, negative control; lane 4, genotype 2 *C. parvum* showing 575 bp *C. parvum*-specific band; lane 5, genotype 1 *C. parvum*; lane 6, genotype 1 *C. parvum*; lane 7, mixed genotype 1 and 2 *C. parvum*; lane 8, genotype 2 *C. parvum*.

Specificity of primers

The specificity of primers 1R and 2R was checked using BLASTN by searching published sequences on the EMBL databases. A limited experimental study was also performed to confirm absence of reaction using DNA from taxonomically related protozoa including *Eimeria tenella*, *Eimeria maxima*,

Toxoplasma gondii, *Cyclospora cayetanensis* and *Plasmodium falciparum*, as template. In addition the published PCR-RFLP methods for the *dhfr*, *cowp* and SSU rRNA genes were used to confirm the MAS-PCR genotyping results.

MAS-PCR amplification

The PCR amplification was performed in one tube, each 50 μl reaction volume contained 40 pmoles of sense primer (CINF 5′GTG GGG ATT TAA CTT GAT TT3′) and 20 pmoles of each anti-sense primer (CINR 5′GGT ATT TCT GGG AAA TAA GT3′, 1R 5′GCT GGA GGA AAT AAC GAC AAT TA3′, 2R 5′TGT CCG TTA ATT CCT ATT CCT CTA3′) (Oswell DNA Service), 47 μl of Megamix Blue ready PCR mix (Microzone) and 1 μl of DNA template.

The amplifications were performed in a Biometra Trioblock thermocycler, first for 1 cycle at 94 °C for 5 min; then 35 cycles at 94 °C for 45 sec, 50 °C for 1 min, 72 °C for 3 min; then 1 cycle at 72 °C for 10 min and held at 4 °C. PCR products were resolved on 2 % agarose gel (Bio-Rad ultra pure DNA grade) or 10 % TBE PAGE pre-cast gels (Bio-Rad), stained with 10 μg/ml ethidium bromide (Sigma), photographed and saved as a TIFF file for documentation (Fig. 2).

Analysis of results : sensitivity and specificity

The diagnostic parameters of sensitivity and specificity for MAS-PCR were calculated as follows, on the basis of, initially, microscopy as a comparison, and subsequently the combined result from *dhfr* PCR-RFLP and *cowp* PCR-RFLP. Sensitivity: percentage of MAS-PCR test results that agreed with the positive comparative result. $(TP/TP+FN) \times 100$. Specificity: percentage for negative test results agreeing with the negative comparative result $(TN/TN+FP) \times 100$ (where TP = true positives; FN = false negatives; TN = true negatives; FP = false positives).

Mixed genotype isolates

Five *C. parvum* isolates of genotype 1, and 4 isolates of genotype 2 were selected, by PCR analysis on the *dhfr*, *cowp* and SSU rRNA genes, as pure genotype isolates. The oocysts were purified by salt flotation and counted using a Fuchs Rosenthal counting chamber (Webster *et al.* 1996*b*). Each isolate was diluted to a 50000 oocysts per ml (50 oocysts/μl) concentration. Varying quantities, between zero and 50000 oocysts, of genotype 1 to genotype 2 were prepared, using different combinations of purified isolates (Table 4). DNA was extracted as previously described and assessed by MAS-PCR and SSU-RNA nested RFLP (Xiao *et al.* 1999).

RESULTS

Specificity of primers

The BLASTN search for both internal primers 1R and 2R revealed a 100 % segment pair match with *C. parvum* bifunctional dihydrofolate reductase-thymidylate synthase (*dhfr-ts*) gene (GenBank accession numbers U41365 and U41366) as expected. No other close matches were detected in the total GenBank repository. The DNA from taxonomically related protozoa including *Eimeria tenella*, *Eimeria maxima*, *Toxoplasma gondii*, *Cyclospora cayetanensis* and *Plasmodium falciparum* did not produce any amplified product in the optimized MAS-PCR test.

Comparison of MAS-PCR genotyping results with the *dhfr* and *cowp* PCR-RFLPs showed complete correlation, where a result was obtained, taking either *cowp* or *dhfr* PCR-RFLP positivity as indicative with the exception of mixed genotype infections. The MAS-PCR detected 2 mixed genotype infections whereas the *cowp* PCR-RFLP detected one and the *dhfr* PCR-RFLP detected none. One of 2 mixed genotype infections detected by the MAS-PCR was confirmed by the *cowp* PCR-RFLP (Tables 1 and 2).

MAS-PCR

The MAS-PCR identified 26·5 % (9 samples/from the total of 34 samples) isolates as genotype 1, 64·7 % (22/34) genotype 2, 5·9 % (2/34) mixed genotype 1 and 2 isolates and 2·9 % (1/34) as negative. The *dhfr* PCR-RFLP identified 32·4 % (11/34) of samples as genotype 1, 64·7 % (22/34) as genotype 2, 0 % (0/34) as mixed genotype (samples identified as mixed genotype by the MAS-PCR were shown to be genotype 1 only) and 2·9 % (1/34) were negative (Fig. 3).

The *cowp* PCR identified 23·5 % (8/34) of samples as genotype 1, 50 % (17/34) as genotype 2, 2·9 % (1/34) as mixed genotype, 11·8 % (4/34) were negative and the remaining 11·8 % (4/34) were not tested due to an insufficient amount of sample. The *cowp* PCR-RFLP confirmed 1 of the MAS-PCR mixed genotype 1 and 2 samples (sample 254).

One isolate was negative in all the genotyping PCR's, despite having a mZn staining count of 4+. The DNA extraction was repeated and the results remained negative. Another PCR technique capable of amplifying from a repeat sequence in the *C. parvum* genome was also used (results not shown) to assess the quality of the template DNA (Riley, Samadpour & Krieger, 1991). It did not amplify any product. The DNA was also spiked with DNA from another isolate shown to amplify product (results not shown), again no product was amplified.

Both the MAS-PCR and *cowp* PCR detected 1 human faecal sample from the panel of micro-

Table 1. mZn and genotyping results from *Cryptosporidium parvum* positive human and animal faecal samples

(ITT, Insufficient to test. mZn score: 1+ = 1/few parasite per slide ($< 10^5$), 2+ = 1 parasite per field ($< 10^6$), 3+ = 2–5 parasites per field ($< 2{\cdot}5 \times 10^6$); 4+ = 5–10 parasites per field ($< 5 \times 10^6$), 5+ = more than 10 parasites per field ($> 5 \times 10^6$); 1 = *C. parvum* genotype 1, 2 = genotype 2, 1/2 = mixed genotypes 1 and 2; neg = negative (no amplified product); bov1/1 = human isolate passaged in calf number 1, bov1/2 = human isolate passaged in calf number 2, ov1/1 = human isolate passaged in lamb number 1, ov1/2 = human isolate passaged in lamb number 2.)

Sample information			Test results			
Sample ID	Host origin	Geographical location	mZn score	*dhfr* MAS-PCR	*dhfr* PCR-RFLP	*cowp* PCR-RFLP
254	Human	Kent, UK	2+	1/2 mix	1	1/2 mix
263	Human	Kent, UK	2+	2	2	Neg
T8	Human	Taunton, UK	1+	1	1	1
T10	Human	Taunton, UK	2+	1	1	1
T12	Human	Taunton, K	2+	2	2	2
T14	Human	Taunton, UK	3+	2	2	2
H27	Human	UK	DNA only	2	2	ITT
H1C8	Human	UK	DNA only	1	1	ITT
B8	Human	Bristol, UK	3+	1	1	1
B11	Human	Bristol, UK	2+	1	1	1
30	Bovine	Denmark	DNA only	2	2	ITT
253	Bovine	Denmark	DNA only	2	2	ITT
E1	Human	Egypt	DNA only	1	1	1
E2	Human	Egypt	DNA only	1	1	1
E3	Human	Egypt	DNA only	1	1	1
E4	Human	Egypt	DNA only	1	1	1
L1	Bovine	Bristol, UK	1+	2	2	2
L2	Bovine	Bristol, UK	4+	2	2	2
L3	Bovine	Bristol, UK	2+	2	2	2
L7	Bovine	Bristol, UK	5+	2	2	2
T13	Human	Taunton, UK	4+	Neg	Neg	Neg

Table 2. mZn and genotyping results from original samples and their transmission experiments into calves and lambs

(N.A., Not applicable. mZn score: 1+ = one/few parasite per slide ($< 10^5$), 2+ = one parasite per field ($< 10^6$), 3+ = 2–5 parasites per field ($< 2{\cdot}5 \times 10^6$); 4+ = 5–10 parasites per field ($< 5 \times 10^6$), 5+ = more than 10 parasites per field ($> 5 \times 10^6$); 1 = *C. parvum* genotype 1, 2 = genotype 2, 1/2 = mixed genotypes 1 and 2; neg = negative (no amplified product); bov1/1 = human isolate passaged in calf number 1, bov1/2 = human isolate passaged in calf number 2, ov1/1 = human isolate passaged in lamb number 1, ov1/2 = human isolate passaged in lamb number 2.)

Sample information				Test results			
Sample ID	Host origin	Transmission host	Geographical location	mZn score	*dhfr* MAS-PCR	*dhfr* PCR-RFLP	*cowp* PCR-RFLP
314	Human	N.A.	Kent, UK	4+	2	2	2
314/ov1/1		ovine		3+	2	2	2
314/bov1/1		bovine		2+	2	2	2
314/bov1/2		bovine		4+	2	2	2
T11	Human	N.A.	Taunton, UK	4+	1/2 mix	1	Neg
T11ov1/1		ovine		4+	2	2	Neg
T11bov1/1		bovine		5+	2	2	2
B9	Human	N.A.	Bristol, UK	5+	2	2	2
B9ov1/1		ovine		5+	2	2	2
B9ov1/2		ovine		5+	2	2	2
B10	Human	N.A.	Bristol, UK	5+	2	2	2
B10 ov1/2		ovine		5+	2	2	2
B10 bov 1/1		bovine		5+	2	2	2

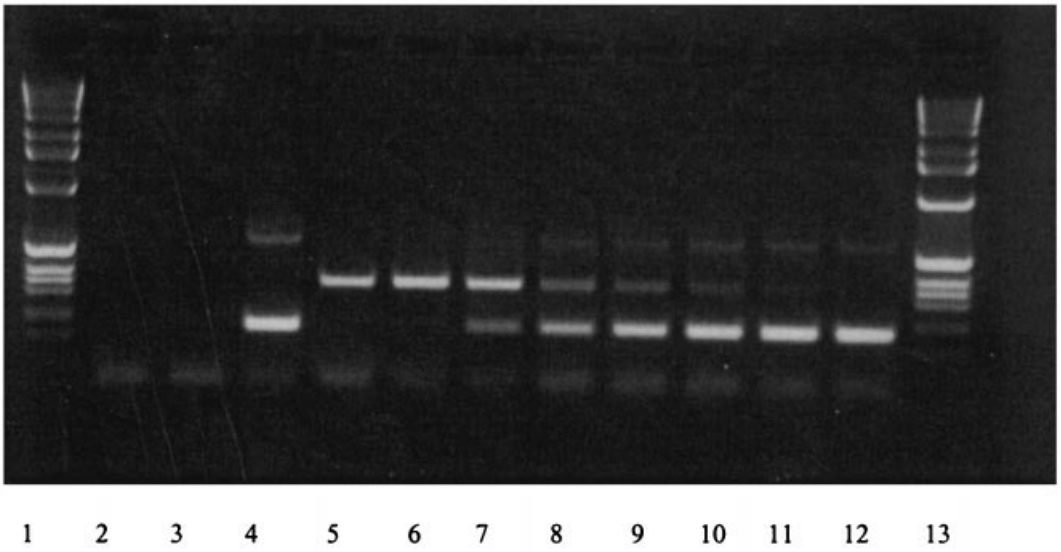

Fig. 3. Photograph of the MAS-PCR on isolate combination S0018 (genotype 1) and S0011 (genotype 2), see Table 4, demonstrating the detection of mixed genotypes in a single reaction. Resolved on a 2% agarose gel and visualized with 10 μg/ml ethidium bromide. Lane 1, 1 kb marker (Gibco); lane 2, negative; lane 3, negative; lane 4, genotype 2 positive control; lane 5, genotype 1 positive control; lane 6, isolate (a) genotype 1; lane 7, isolate (b) mixed genotype; lane 8, isolate (c) mixed genotype; lane 9, isolate (d) mixed genotype; lane 10, isolate (e) mixed genotype; lane 11, isolate (f) mixed genotype; lane 12, isolate (g) genotype 2; lane 13, 1 kb marker (Gibco).

Table 3. Analysis of the sensitivity and specificity of the MAS-PCR in comparison with microscopy and published PCR-RFLP techniques

	Microscopy (mZn) either genotype	*dhfr/cowp* PCR-RFLP either genotype	*dhfr/cowp* PCR-RFLP genotype 1	*dhfr/cowp* PCR-RFLP genotype 2
Sensitivity (%)	96·1	100	100	100
95% C.L.*	87–103	99–101	99–101	99–101
Specificity (%)	97·3	100	100	100
95% C.L.*	92–103	99–101	99–101	99–101
Total positive	26	34	11	24
Total negative	37	37	60	47
TP	25	34	11	24
TN	36	37	60	47
FP	1	0	0	0
FN	1	0	0	0
Total	63	71	71	71

* Confidence limits.

scopically negative samples as a genotype 2. The remaining 36 samples did not amplify any product with either PCR.

Analysis of results : sensitivity and specificity

Comparisons of the sensitivity and specificity of the MAS-PCR using both microscopy and the *dhfr* RFLP or the *cowp* RFLP was calculated and 95% confidence limits determined (Table 3). The MAS-PCR showed 96% sensitivity and 97% specificity against microscopy and 100% specificity and sensitivity against the PCR-RFLP tests in detecting *C. parvum* DNA in the faecal samples. The calculated error (95% limits) was small as the sample size was 63 for microscopy and 71 for PCR. In detection of genotype 1, again sensitivity and specificity were 100% (in this case samples where genotype 2 alone was seen in the PCR-RFLP, were regarded as negative). Similarly, in the detection of genotype 2, samples with genotype 1 alone were regarded as negative. The sensitivity and specificity were both 100%, with a sample size of 71 and confidence limits of 5%.

Mixed genotype isolates

The MAS-PCR detected all ratios of mixed isolates in 3 out of the total 4 experiments, one experiment did not detect genotype 1 until it was

Table 4. Genotyping results of a mixed genotype panel as determined by MAS-PCR and SSU rRNA-PCR

Isolate ID	Number of oocysts per ml		MAS-PCR	SSU rRNA PCR
	Isolate S/0018 genotype 1	Isolate S/0011 genotype 2		
a	0	50000	2	2
b	5000	45000	1/2 mix	1/2mix
c	15000	35000	1/2 mix	1/2 mix
d	25000	25000	1/2 mix	1/2 mix
e	35000	15000	1/2 mix	1/2 mix
f	45000	5000	1/2 mix	1/2 mix
g	50000	0	1	1
	S/0023 genotype 1	S/0008 genotype 2		
h	0	50000	2	2
i	5000	45000	2	2
j	15000	35000	2	1/2 mix
k	25000	25000	1/2 mix	1/2 mix
l	35000	15000	1/2 mix	1/2 mix
m	45000	5000	1/2 mix	1/2 mix
n	50000	0	1	1
	S/0041 genotype 1	S/0017 genotype 2		
o	0	50000	2	2
p	5000	45000	1/2 mix	1/2 mix
q	15000	35000	1/2 mix	1/2 mix
r	25000	25000	1/2 mix	1/2 mix
s	35000	15000	1/2 mix	1/2 mix
t	45000	5000	1/2 mix	1/2 mix
u	50000	0	1	Negative
	S/0066 genotype 1	S/0011 genotype 2		
v	0	50000	2	2
w	5000	45000	1/2 mix	1/2 mix
x	15000	35000	1/2 mix	1/2 mix
y	25000	25000	1/2 mix	1/2 mix
z	35000	15000	1/2 mix	1/2 mix
Aa	45000	5000	1/2 mix	1
Bb	50000	0	1	1

mixed in equal quantity with the genotype 2. The SSU-RNA PCR detected all ratios of mixed isolates in 2/4 experiments, one mixed isolate was shown to be a single genotype in 2 samples from the 4 experiments (Table 4).

DISCUSSION

This study describes the design and evaluation of a 1-step PCR for the determination of *C. parvum* genotypes from DNA extracted directly from faeces and from purified *C. parvum* oocysts. The MAS-PCR can be performed with or without inclusion of the outer *C. parvum* specific primer (CINR). The decision to include CINR was made as when the primer was omitted there was slightly reduced sensitivity since 1 sample (263) was negative. Although omission of the CINR primer produced results with a clearer resolution in some of the samples (data not shown). The increased sensitivity when the CINR primer was included is most likely due to extra template production for the genotype specific internal primers, as the *C. parvum* specific region that is amplified flanks the regions amplified by the internal primers. The decrease in resolution of the amplified product in some samples may be due to the presence of too much template generated by the *C. parvum* specific primers, as the DNA used for template was not quantified before addition.

Analysis of the MAS-PCR in comparison to published PCR-RFLP techniques, on the *dhfr* and *cowp* genes, showed 100% sensitivity and specificity for the detection of *C. parvum* genotypes 1 and 2. An increased sensitivity and specificity was indicated in the detection of mixed genotype samples, mainly with the *dhfr* RFLP as it utilizes the same region of gene sequence. The 2 samples 254 and T11, which

showed mixed genotype infections with the MAS-PCR, were deemed genotype 1 only with the *dhfr* RFLP and 1 sample (254) was confirmed as a mixed genotype by *cowp* RFLP. The increased sensitivity of PCR utilizing the *dhfr* gene over the *cowp* gene in this study, confirms the results published in a comparison of different genotyping techniques (Sulaiman *et al*. 1999*b*).

A 100% agreement between the MAS-PCR and the *cowp* RFLP on the *C. parvum*-negative panel in conjunction with the results from the *C. parvum*-positive samples validates the specificity of the primers. The sample, which was negative by microscopy, gave a genotype 2 result with both MAS and *cowp* PCRs. This is not unexpected as *C. parvum* is an ubiquitous organism and the mZn has a detection level of approximately 20000 oocysts per gram of faeces (Webster *et al*. 1996*b*). The *dhfr* and *cowp* PCR-RFLPs have demonstrated a detection limit of between 1 and 10 oocysts respectively (Sulaiman *et al*. 1999*b*).

Due to the mZn *C. parvum*-positive sample (T13) which did not give a positive PCR result with any of the tests used, the sensitivity of all the PCR tests used was less than 100% against microscopy. The lower specificity (97·3%) is accounted for by a so-called false positive appearing in samples where microscopy had not detected a subsequently PCR confirmed infection.

The comparison of the MAS-PCR with a published nested RFLP targeting the SSU rRNA gene, on a panel of prepared mixed genotype samples showed an equivalent sensitivity in detection of both genotypes by the MAS-PCR. The SSU rRNA PCR has been evaluated as one of the more sensitive techniques with a detection level down to 1 oocyst (Suliaman *et al*. 1999*b*). This study shows that both the MAS-PCR and SSU rRNA PCR can detect 5 oocysts per reaction of either genotype, in the presence of 45 oocysts of the other genotype. The MAS-PCR detected the same number of the mixed genotype isolates than the SSU rRNA PCR again indicating comparable sensitivity. Detection of a known mixed genotype isolate as a single genotype occurred in both the MAS-PCR and SSU rRNA PCR. This may be due to preferential amplification by the primers of one genotype over the other, unknown inhibitory factors hindering the amplification of one genotype more than the other or to the individual PCR reaction conditions and/or primer specificity. For these reasons an under-reporting of mixed genotype samples may occur when only 1 PCR target is examined. Isolate (T13), which was shown microscopically to contain *C. parvum* oocysts, yielded no amplified product in either *dhfr* or *cowp* PCR-RFLP and protozoan-specific PCRs, even when spiked with *C. parvum* DNA that previously amplified product. This suggests that the sample may have contained PCR reaction inhibitory factors, rather than a genotype difference within the isolate itself, although amplification of product by the protozoan specific PCR would have been expected. With recent evidence of unusual *Cryptosporidium* species recovered from human faeces within the UK, however, the latter cannot be discounted (Pedraza-Dìaz, Amar & McLauchlin, 2000; Pedraza-Dìaz *et al*. 2001*b*).

Most PCR-based genotyping assays available for *C. parvum* require the additional step of restriction enzyme digestion. The MAS-PCR utilizes the genotype allele changes within the *dhfr* gene, but requires no restriction enzymes to differentiate the two genotypes. To improve the detection sensitivity, PCR assays using multicopy rRNA genes have been developed (Morgan & Thompson, 1998). However, associated problems with heterogeneity of the rDNA transcription units have also been reported (Le Blancq *et al*. 1997). The use of the single gene copy *dhfr* sequence would not be affected by such heterogeneity, as only 1 genotype-specific profile is possible for each isolate, due to complete allelic dimorphism at the *dhfr* locus (Gibbons & Awad-El-Kariem, 1999). Thus when both genotypes are detected in an infection, it is possible to be confident in that it is a true mixed genotype 1 and 2. This 'one-step' approach has advantages over PCR-RFLP methods; with mixed genotype infections the RFLP requires enough amplified DNA from both genotypes to enable visualization upon an agarose gel after dilution with the reagents required to perform the digestion, the MAS-PCR product is run directly on an agarose or TBE-PAGE gel with no dilution; it eliminates the need for nested PCR reactions and the associated problems with contamination (Morgan & Thompson, 1998) and the precautions required (Gibbons & Awad-El-Kariem 1999), without compromising sensitivity.

It is important to develop species discriminatory techniques in conjunction with multilocus genotyping and subtyping (fingerprinting) to characterize individual isolates fully, to aid epidemiological studies, outbreak tracing and in validation of phylogenetic studies.

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