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A New Approach for the Identification and the Diagnosis of *Eimeria media* Parasite of the Rabbit

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CERE, N., HUMBERT, J. F., LICOIS, D., CORVIONE, M., AFANASSIEFF, M., AND CHANTELOUP, N. 1996. A new approach for the identification and the diagnosis of *Eimeria media* parasite of the rabbit. *Experimental Parasitology* **82,** 132–138. In this work, we described a new approach for the isolation of a species-specific probe for the *Eimeria media* parasite of the rabbit based on the use of the random amplified polymorphic DNA (RAPD) technique. A specific fragment of 800 bp of the studied species was isolated after RAPD and then cloned and DIG-radiolabeled. After dot-blotting, we observed that this probe was specific for *E. media.* Sequencing of the 3′ and 5′ ends of this probe enabled the determination of two primers that could be used in a PCR reaction. The amplified product of 750 bp was specific for *E. media.* The use of these primers and of our probe allowed the detection of a very small number of oocysts. With a new protocol of DNA purification, 10 purified oocysts were detected by PCR. The efficiency of the amplification was not changed when two species were mixed. The threshold of detection of oocysts in fecal matter was equal to 30.

INDEX DESCRIPTORS AND ABBREVIATIONS: rabbit; coccidia; *Eimeria media;* identification; diagnosis; species-specific probe; RAPD, random amplified polymorphic DNA; PCR, polymerase chain reaction; PBS, Phosphate-buffered saline.

INTRODUCTION

The coccidia of the genus *Eimeria* are the most common parasites of the rabbit and are responsible for major pathogenicity in their host (Coudert 1989; Licois and Coudert 1982). Ten of the 11 species described are known to infect the intestine of this animal. One is located in the biliary ducts of the liver. The identification of these coccidia is based on the observation of the morphology of the oocysts but several of these species cannot be readily identified. For example, it is not possible to distinguish *E. perforans* from *E. media* and *E. flavescens* from *E. irresidua* only with the form and the measurements (length and breadth) of the oocysts. Then, the differentiation of these species can be difficult and time consuming if the life-cycle (site of development in the intestine, prepatent duration, etc.) or other criteria (clinical signs, etc.) must be studied. New methods are also needed for direct and rapid identification of these parasites.

The RAPD (random amplified polymorphic DNA) method allows the identification of parasites (Cenis 1993; Dias Neto *et al.* 1993; Humbert and Cabaret 1995; Siles-Lucas *et al.* 1993). Three studies (Cere *et al.* 1995; Mac Pherson and Gajadhar 1993; Procunier *et al.* 1993) on *Eimeria* spp. of the domestic fowl, rat, mice, cattle, sheep, and rabbit have demonstrated the efficiency of this technique in differentiating coccidia species. However, this method cannot be used when the species are mixed or in very small quantities in feces. In this case, amplification of specific DNA sequences by PCR and hybridization of these fragments with oligonucleotide probes provide a highly sensitive and specific tool for the detection of coccidia directly from feces.

The first step in the development of this tool was to identify and obtain a species-specific probe. A number of methods have been developed for isolating coccidia species-specific probe but nobody to date has used RAPD markers to isolate one of these species-specific probes in the same way as Barral *et al.* (1993), who discovered a new sex-specific probe of *Schistosoma intercalatum* using a RAPD marker. Second, sequences of the 3′ and 5′ ends of this probe must be determined for the iden-

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tification of a highly selective set of primers for PCR-based detection of this species.

The aim of the present study was to demonstrate that RAPD allows isolation of species-specific probe and to evaluate the ability of the PCR-hybridization assay to detect parasites in feces and to discriminate between species.

Materials and Methods

Parasites and DNA extraction. Sporozoites of each of nine species (*E. coecicola, E. exigua, E. flavescens, E. intestinalis, E. irresidua, E. magna, E. media, E. piriformis, E. vejdovskyi*) and of four strains of *E. media* (France, Balearic Isles, Poland, Guadeloupe in the French West Indies) obtained from 5×10^7 oocytes were placed in 300 μl of extraction buffer (0.1 *M* Tris–HCl, 0.1 *M* NaCl, 50 m*M* EDTA, 1% SDS, 5 mg/ml Proteinase K, pH 8). The tubes were incubated in a water bath at 40°C overnight. DNA was isolated after phenol extraction, ethanol precipitation, cesium chloride density-gradient centrifugation, and a final ethanol precipitation. DNA concentrations were determined with a spectrophotometer.

Isolation of DNA from small numbers of oocysts. Recently, Stucki *et al.* (1993) have proposed a new method of isolating DNA from small numbers of oocysts based on the modification of the hot phenol method used for isolating RNA. Good results were obtained with this technique but the use of hot phenol in routine clinical diagnosis poses the problem of toxic vapors in the laboratory. Several other methods were tested for isolating DNA from small numbers of oocysts. The best results were obtained with the following protocol. Fifty percent of sodium hypochlorite (12% available chlorine) was mixed with the solution of purified oocysts and then incubated overnight at 4°C. After centrifugation (10 min, 4°C, 2500*g*,) the resulting pellet was resuspended in 200 μl of PBS 1× buffer (NaCl, 1.4 *M;* KCl, 0.03 *M;* KH_2PO_4, 0.01 *M;* Na_2HPO_4, 0.08 *M*) and 200 μl of proteinase K (10 mg/ml) and incubated for 2 hr at 50°C. After centrifugation (10 min, 4°C, 2500*g*) and washing in PBS 1×, the pellet was resuspended in a solution of 200 μl of PBS, 200 μl of Ham's F10 nutrient mixture (Sigma), and 10 μl of 2*M* L-cysteine (Sigma). This solution was also incubated for 2.5 hr at 39°C in satured CO_2 atmosphere. For *E. media* (not for other species), sporocysts were always liberated from the oocysts by crushing with a Potter-Elvejehm homogenizer. The sporozoites were obtained after incubation in an excystation medium (PBS 1×, 0.33% bile salt (Sigma), 0.13% Trypsin (Sigma)) for 3 min. After centrifugation (10 min, 4°C, 9000*g*), the resulting pellet of sporozoites was resuspended in 20 μl of DNA purification kit. The best results were obtained with the GeneFizz purification kit (Eurobio). The purification was performed according to the manufacturer's instructions (Eurobio) with the exception that the mixture was centrifuged (1 min, 12,000*g*) at the end of the incubation. Ten microliters of the PCR-ready DNA was then removed from the supernatant directly to the PCR reaction.

To test the ability of our PCR assay to detect small number of oocysts in fecal matter, we have performed twofold serial dilutions of oocysts in one rabbit feces (150 mg). For the extraction of DNA, fecal matter was first incubated with the solution of sodium hypochlorite and then filtered on different sieves (250 to 50 μm). The last sieve (50 μm) was then washed and the solution was recovered in a 30-ml tube. After three centrifugations (10 min, 4°C, 2500*g*) and washing in PBS 1×, the last pellet was resuspended in 200 μl of PBS 1× buffer, 200 μl of proteinase K (10 mg/ml) and incubated for 2 hr at 50°C. The rest of the protocol was as described above.

RAPD reaction. In a previous study (Cere *et al.* 1995), a genetic polymorphism was estimated in rabbit coccidia using 11 primers by RAPD. With each primer, the profiles obtained were varied markedly with the species. There were numerous species-specific markers. For the RAPD reaction, about 10 ng of DNA were used as template. The reaction mixture (25 μl) contained 1× *Taq* polymerase buffer, 1.5 m*M* $MgCl_2$, deoxynucleotide triphosphate at the concentration of 80 μmoles each, 0.5 unit of *Taq* DNA polymerase (Appligene), and 0.5 pmole of primer G2 (5′-GGCACTGAGG-3′, Kit G, Operon Technologies, Alameda, CA, U.S.A.). The PCR cycle was carried out for 40 sec at 92°C, 30 sec at 35°C, and 90 sec at 72°C for a total of 35 cycles followed by one extensive polymerization reaction (10 min at 72°C). The PCR were performed in a MJ Research thermal cycler. Controls (without DNA) were always negative with an overall cycle number of 35.

Analysis of RAPD products. Twenty microliters of the amplified reaction was electrophoresed in a 1.5% agarose gel (Appligene) in TBE buffer (pH 8) for 4 hr at a constant voltage (100 V). Products were stained with ethidium bromide and photographed (type 665 polaroid film). The negatives were analyzed and the molecular sizes of the fragments were determined using a DNA molecular weight marker Raoul (Appligene) and a Bio-Image system (Millipore).

Isolation, radiolabeling, and hybridization of RAPD marker. RAPD-specific marker obtained with primer G2 was isolated from agarose gel by electroelution and purified with a phenol extraction and an ethanol precipitation. Two methods were tested:

—First, a new RAPD was performed with the same primer, followed by the isolation and purification of the fragment. This fragment was labeled by random priming with Digoxigenin (DIG, Boehringer),

—Second, the specific marker was cloned into pCR Vector (Invitrogen) and DIG labeled by random priming.

These two labeled markers (noncloned and cloned) were then used as a probe in dot-blot or in Southern blot. In the first case, 2.0 μg of genomic DNA of the nine species were dotted onto a nylon membrane (Boehringer) and in the second, after separation of RAPD or PCR products, the agarose gels were blotted on nylon membranes. Hybridization (16 hr

at 42°C) and detection of the bands with CSPD were performed with the digoxigenin kit according to the manufacturer's instructions (Boehringer Mannheim Biochemica).

Sequencing and PCR conditions. The nucleotide sequences of the 3′ and 5′ ends of our cloned probe were determined using DNA Sequencing Kit version 2.0 (Sequenase, United State Biochemical) on two different clones. Sequences were analyzed with the FastA program (Pearson and Lipman 1988) of the Genetics Computer Groups (GCG) package. Two oligonucleotide sequences located at the ends of the probe were then selected and the two primers corresponding to these sequences were synthesized. Reaction mixture (25μl) contained 1× *Taq* polymerase buffer, 1.5 m*M* $MgCl_2$, deoxynucleotide triphosphate at the concentration of 80 μmoles each, 1 unit of *Taq* DNA polymerase (Appligene), and 0.5 pmole of each primer. PCR was achieved using MJ Research thermal cycler with following cycle profile: 94°C for 40 sec, 61°C for 30 sec, and 72°C for 90 sec for a total of 35 cycles. After electrophoresis, agarose gels were stained with ethidium bromide and blotted on Nylon membranes (Boerhinger). Hybridization with the two labeled markers was performed 16 hr at 42°C.

RESULTS

Isolation of a species-specific fragment. In the RAPD performed with the G2 primer (Fig. 1), we chose the 800-bp species marker for *E. media* for further analyses, because this fragment (1) is well amplified, (2) has an appropriate size for cloning, and (3) was present in the four strains of *E. media* tested. This marker was isolated and labeled with the two methods previously described. Results of the Southern blot are demonstrated in Fig. 2. With the first method (noncloned marker), nonspecific hybridizations were observed (1 fragment of 500 bp) with several of the tested species and at least 10 bands were observed in the profile of *E. media*. With the second method (cloned marker), only one band of the expected size (800 bp) was observed in *E. media* profile. To test the species specificity of the cloned marker, this marker was hybridized to dot-blots of DNA from the nine studied species. Only *E. media* was recognized by this probe (Data not shown).

Sequencing of the 5′ and 3′ ends of this fragment was performed to determine two primers for PCR amplification. The nucleotide sequence of these fragments are shown in Fig. 3. No significant homologies were found by comparison of these sequences with the GenBank nucleotide sequence data base. Two primers were determined:

Em1: 5′-AAAGCAGCGTGGCTCGCCTG-3′
Em2: 5′-GGCACTGAGGGGAGCAAGTG-3′

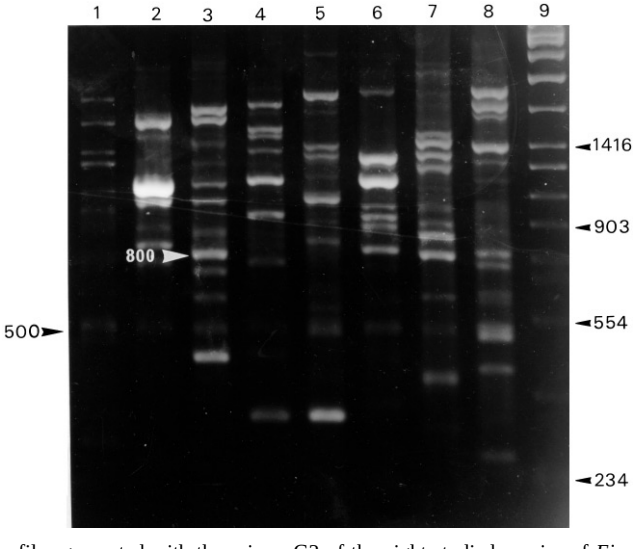

FIG. 1. RAPD profiles generated with the primer G2 of the eight studied species of *Eimeria* parasite of the rabbit. Species-specific fragment (800 bp) of *E. media* and common fragment (500 bp) to all the species are shown with arrows. Lane 1, *Eimeria coecicola;* 2, *E. exigua;* 3, *E. media;* 4, *E. flavescens;* 5, *E. vejdovskyi;* 6, *E. piriformis;* 7, *E. magna;* 8, *E. intestinalis;* 9, size marker Raoul (Appligene).

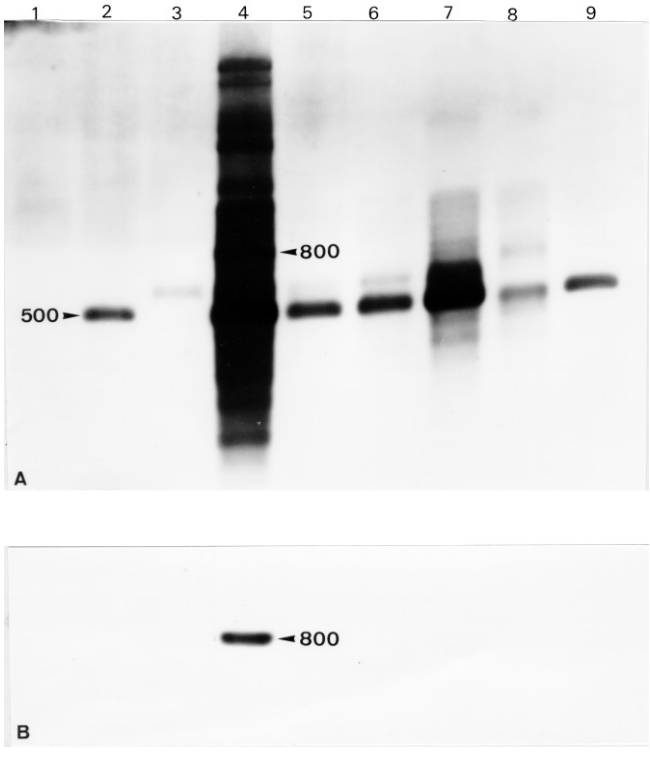

FIG. 2. Autoradiography of the RAPD products shown in Fig. 1 analyzed by Southern blotting onto nylon membranes and hybridization with DIG-labeled probes obtained with (A) the first method (noncloned fragment) or (B) the second method (cloned fragment). Lane 1, negative control; 2, *E. coecicola;* 3, *E. exigua;* 4, *E. media;* 5, *E. flavescens;* 6, *E. vejdovskyi;* 7, *E. magna;* 8, *E. piriformis;* 9, *E. intestinalis.*

Specificity and sensitivity of the PCR for the detection of E. media. Using these primers, DNA from all the studied species were subjected to PCR amplification (Fig. 4). One major fragment of about 750 bp and one minor of 1500 bp were only observed in the four strains of *E. media.* Hybridization of these PCR products with the two labeled probes (cloned and

A

Em1 →

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1    ACGGAACTTT GCGCA***AAAGC AGCGTGGCTC GCCTC***GTCTG ATGCATAGAC 50
51   GGGGAGATCA TCAAGATACG CAATGACTCC CTCCCCGATG TATGGGAAGA 100
101  CCATGTGAGT CATTACGGCC TGAAACTTAT CAGGAGCGGC TTGCAAACCG 150
151  AACGTGCATC ACTTTGAACT CATCCTGGCC CATGCAAGGT ACGAAATGCA 200
201  GTCTGTACTG ATCT                                       214
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B

Em2 →

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1    TT***GGCACTGA GGGGAGCAAG TG***GCCTGCCC GGCGCTATCG GACACGACCC 50
51   CCCCTCCTAC AGCAAGTTCG AGGTATGGCT GACGGGAGAC GGGAAGCTCT 100
101  GTCCCACGCT AATCCGGCAA ACTCTGGATG GGTTCAAGCA CCTCTTTCGT 150
151  GACAAATTGC CTCCTGGATT ACCTCCAGCT CGAGTTATAG ACCATA     196
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FIG. 3. DNA sequence of the 3′ and 5′ ends of *E. media*-specific probe. The position of the two chosen primers (Em1 and Em2) is indicated with arrows. (A) 5′ end; (B) 3′ end.

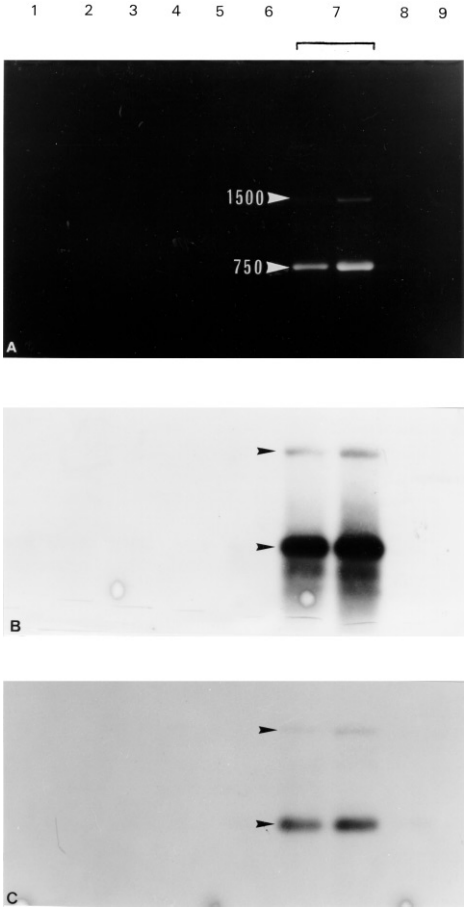

FIG. 4. Ethidium bromide staining (A) of PCR amplification with primers Em1 and Em2 of DNA from the eight studied species of *Eimeria* from rabbit and autoradiography of the PCR products analyzed by Southern blotting onto nylon membranes and hybridization with DIG-labeled probes obtained with (B) the first method (noncloned fragment) or (C) the second method (cloned fragment). Two bands of 750 and 1500 bp are only visualized in the two strains of *E. media.* Lane 1, negative control; 2, *E. intestinalis;* 3, *E. magna;* 4, *E. flavescens;* 5, *E. vejdovskyi;* 6, *E. piriformis;* 7, two strains of *E. media;* 8, *E. exigua;* 9, *E. coecicola.*

noncloned RAPD fragments) confirmed the specificity of this PCR (Fig. 4) since only the two fragments previously described (750 and 1500 pb) were visualized in the four strains of *E. media.*

The sensitivity limit of the PCR-amplified DNA assay for detection of small quantities of *E. media* was first estimated on serial 10-fold dilutions of DNA. The detection limit after agarose gel electrophoresis (three repetitions of the experiment) was about 100 and 10 pg after hybridization of this gel with the labeled probe (data not shown). Second, amplifications were performed on serial dilutions of purified oocysts (Fig. 5A). From 4 to 10 sporulated oocysts (three repetitions of the experiment) were detected when isolation of DNA was performed with the GeneFizz purification kit (Eurobio). When oocysts of two species are mixed (*E. media* + *E. intestinalis*), no differences were observed in the efficiency of the amplification of the species-specific fragment of *E. media* (Fig. 5B) even when oocysts of *E. media* constituted less than 1% of the total. Third, one experiment was also conducted to establish the sensitivity of the PCR on serial dilutions of oocysts in rabbit feces (Fig. 5C) and to verify that no inhibitor of the PCR amplification accompanied DNA directly extracted from feces. The threshold of sensitivity of our PCR assay was 30 sporulated oocysts per rabbit feces. This threshold of sensitivity was independent from the quantities of fecal matter (30 oocysts were also detected when they were placed in 2 to 10 fecal pellets).

DISCUSSION

In this study we have developed a new approach for the construction of a coccidia diagnostic tool. This tool is based on the isolation and the DIG-labeling of a species-specific probe after a RAPD and determination of primers in this probe for a PCR which is more specific than RAPD. The advantages of this approach in comparison with other methods to isolating species-specific probes are as follows: the RAPD very quickly provides a great number of species markers as we have observed in another study (Cere *et al.* 1995) and it is very easy and quick to verify that these markers are well conserved in different strains of the studied species.

After isolation of the chosen species-specific RAPD marker, it appeared that it was necessary to clone this marker for improvement of the specificity of the probe since isolation on gel and purification of the RAPD marker was not

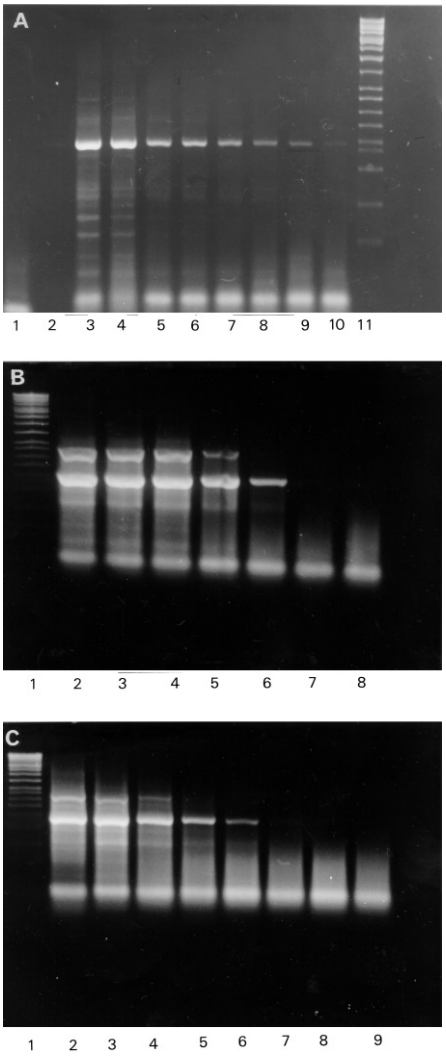

FIG. 5. Ethidium bromide staining of PCR amplification with primers Em1 and Em2 of (A) DNA from twofold serial dilutions of purified oocysts (Lane 1, negative control; 3, 500 oocysts; 4, 250 oocysts; 5, 125 oocysts; 6, 62 oocysts; 7, 31 oocysts; 8, 16 oocysts; 9, 8 oocysts; 10, 4 oocysts; 11, size marker Raoul (Appligene)), (B) DNA from mixed oocysts of *E. media* and *E. intestinalis* (Lane 1, size marker; 2, 1000 oocysts of *E. media;* 3, 1000 oocysts of *E. media* + 100 oocysts of *E. intestinalis;* 4, 1000 oocysts of *E. media* + 1000 oocysts of *E. intestinalis;* 5, 100 oocysts of *E. media* + 1000 oocysts of *E. intestinalis;* 6, 10 oocysts of *E. media* + 1000 oocysts of *E. intestinalis;* 7, 1000 oocysts of *E. intestinalis;* 8, negative control), and (C) DNA from twofold serial dilutions of oocysts in fecal matter (Lane 1, size marker; 2, 1000 oocysts of *E. media;* 3, 500 oocysts; 4, 250 oocysts; 5, 125 oocysts; 6, 63 oocysts; 7, 31 oocysts; 8, 15 oocysts; 9, negative control).

sufficient to obtain just the chosen fragment. We do not have any ready explanation for the excessive hybridization observed with the noncloned fragment. It is possible that another fragment, highly reiterated and of the same size (about 800 bp) comigrated with our marker. This phenomenon has been observed previously in other study (Smith *et al.* 1994).

The determination of two primers after sequencing of the 3′ and 5′ ends of our probe, allows the amplification by PCR, of a specific fragment of 750 bp. This product seems to be conserved in *E. media* species since it was amplified in all the strains tested originating from different countries (France, Balearic Isles, Poland, Guadeloupe in the French West Indies). We have observed the presence of a second fragment of 1500 bp that also hybridizes with the probe. This fragment probably fits with a repeat sequence since its length is twice that of the other 750-bp fragment. The presence of this fragment does not invalidate this technique.

The results on the sensitivity of our PCR assay for detection of purified oocysts are comparable with those of Stucki *et al.* (1993) who detected two oocysts of *E. tenella*, Webster *et al.* (1993) who detected 20 oocysts of *Cryptosporidium parvum*, and those obtained on other Protozoa (see review of Weiss (1995)). For oocysts in fecal matter, any other results on coccidia are available in the literature. With our method, only 30 oocysts in 0.15 to 1.5 g of fecal matter (1 to 10 fecal pellets) are sufficient to detect the presence of coccidiosis in rabbits. This result is better than those obtained with other traditional diagnostic tools (microscopy) which detect 50 to 100 oocysts per gram of fecal matter (Coudert *et al.* 1995).

This work should be extended to the other species of *Eimeria* which are pathogenic in the rabbit in order to establish of a set of suitable tools for the diagnosis and the identification of these parasites.

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