Development of a Model Ribosomal RNA Hybridization Assay for the Detection of *Sarcocystis* and other Coccidia

Alvin A. Gajadhar, William C. Marquardt and Carol D. Blair

ABSTRACT

Two regions of the primary structure of the small subunit rRNA of *Sarcocystis muris* bradyzoites were compared with nucleotide sequences of *S. gigantea*, *Toxoplasma gondii*, *Plasmodium berghei* and *Mus musculus* and used to design genus- and species-specific probes for the detection and identification of coccidia. Total cellular RNA of purified *S. muris*, *S. cruzi*, *T. gondii* and *Eimeria nieschulzi* and coccidia-infected tissues of mouse, ox, sheep and pig, were assayed using twenty-base oligomers labelled with ^{32}P. Hybridization occurred at temperatures ranging from 21°C to 41°C or 51°C. One probe detected only *S. muris* and another successfully hybridized to several members of coccidia, including *S. muris*, *S. cruzi*, *T. gondii* and *E. nieschulzi*. One ng of total cellular RNA was sufficient to yield detectable hybrids in slot blot assays. The excellent sensitivity suggests that rRNA-based probes are capable of detecting individual parasites, and can assay low levels of coccidial infections not detectable by other methods. The results of this study show that it is possible to customize the specificity of rRNA-based probes for diagnostic, epidemiological or taxonomic purposes.

RÉSUMÉ

Deux régions de la structure primaire de la petite sous-unité rARN de bradyzoïtes de *Sarcocystis muris* ont été comparées à une séquence de nucléotides de *S. gigantea*, *Toxoplasma gondii*, *Plasmodium berghei* et *Mus musculus* et employées pour fabriquer des sondes spécifiques pour la détection et l'identification de coccidies. L'ARN cellulaire total de souches purifiées de *S. muris*, *S. cruzi*, *T. gondii* et *Eimeria nieschulzi* et de tissus infectés de coccidies provenant de souris, de bœufs, de moutons et de porcs ont été analysés en utilisant 20 oligomères de bases marquées au ^{32}P. L'hybridation s'est produite à des températures se situant entre 21–41 °C ou 21–51 °C. Une seule sonde a détecté *S. muris* et une autre a hybridé avec quelques coccidies incluant *S. muris*, *S. cruzi*, *T. gondii* et *E. nieschulzi*. Un ng de l'ARN cellulaire total a été suffisant afin de détecter des hybrides dans un essai de type « slot blot ». L'excellente sensibilité du test suggère que ces sondes ARN sont capables de détecter des parasites sur une base individuelle et peuvent détecter des niveaux d'infection qui ne seraient pas détectables par d'autres méthodes. Les résultats de cette étude montrent qu'il est possible de fabriquer des sondes rARN spécifiques pour des fins diagnostiques, épidémiologiques et taxonomiques. (*Traduit par Dr Pascal Dubreuil*)

INTRODUCTION

Sarcocystis spp. are cyst-forming coccidia that belong to the phylum Apicomplexa. They have obligatory two-host life cycles that follow the predator-prey relationship of the hosts. Definitive hosts become infected by ingesting asexual stages found in the muscles or neural tissues of herbivores and omnivores. The sexual phase of the life cycle occurs in the intestine of meat-eating animals and results in the passing of oocysts which are infective for intermediate hosts. Several species of carnivores may serve as definitive hosts of a *Sarcocystis* sp. but, generally, its intermediate host is restricted to a single species of animal (1). The sporocysts passed in the feces of definitive hosts are similar in structure and cannot be used reliably for species identification. However, the sarcocysts found in the tissues of intermediate hosts have intricate walls and their ultrastructure has been used to name or to identify species (2). Hundreds of species of *Sarcocystis* have been described or named based on their asexual stages (1). However, there is evidence that the ultrastructure of cyst walls is not always a reliable criterion for use in taxonomy (3,4).

Many animals are infected with one or more species of *Sarcocystis* and the infection is common in livestock. Some species of *Sarcocystis* seem to be harmless to the intermediate hosts, while others may contribute to abortion, fever, weight-loss, anorexia, granulomatous myositis and reduced milk production (5). It has been estimated that the infection causes an annual loss of $95 million in the USA's cattle industry (5). Currently, there is no reliable assay for clinical or postmortem diagnosis of sarcocystosis and it is not clear which species of *Sarcocystis* are responsible for carcass condemnation. Although it has been shown that *S. cruzi* may be involved in bovine eosinophilic myositis (6,7), it is likely that this condition is also elicited by other *Sarcocystis* spp. Ultrastructural evidence suggests that in some cases an unknown species of *Sarcocystis* may be involved (8).

Department of Biology (Gajadhar, Marquardt) and Department of Microbiology (Blair), Colorado State University, Fort Collins, Colorado 80523.
Present address of Dr. A.A. Gajadhar: Health of Animals Laboratory, Agriculture Canada, 116 Veterinary Road, Saskatoon, Saskatchewan S7N 2R3.

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In this paper we report the design, construction, and evaluation of *Sarcocystis* genus-specific and *Sarcocystis* species-specific probes using *S. muris* as a model. The small subunit ribosomal RNA (SSU-rRNA) sequences of organisms contain regions that are highly or semiconserved, or hypervariable (9). Because sequences of several apicomplexan SSU-rRNAs are known, it has been suggested that it is possible to design probes with various specificities (10). In order to test this hypothesis we used the primary sequence of the SSU-rRNA of *S. muris* (11) to design genus- and species-specific probes for coccidia.

MATERIALS AND METHODS

PARASITES

Sarcocystis muris was maintained in the laboratory by cyclic passages between CF-1 mice and SPF cats. Mice were orally inoculated with sporocysts, and mouse muscle containing infective sarcocysts was fed to cats. Sporocysts were obtained from the intestinal scrapings of cats by a digestion and centrifugation method (12). Asexual stages (bradyzoites) of *S. cruzi* were obtained from skeletal muscles of normal beef carcasses made available at a local abattoir (Monfort of Colorado Inc., Greeley, Colorado).

Bradyzoites were removed from tissues by a digestion-centrifugation method that was similar to standard techniques (13). Tissues were chopped into small pieces, homogenized in a blender, and incubated in a digestive solution (0.6% pepsin, 0.8% NaCl, 0.8% HCl) for 30–60 min at 37°C with agitation. Four layers of gauze were used to remove coarse material and the filtrate was washed twice by centrifugation in a saline solution (0.15 M NaCl, pH 7.0). The sediment was resuspended in Percoll by mixing two parts of isotonic Percoll stock solution and one part of saline-suspended sediment. The mixture was centrifuged in 15-mL conical tubes in a swing-out rotor at 1160 *g* for 20 min at room temperature. When zoites were present they were concentrated in the sediment or suspended in a band. The organisms were removed, washed twice in saline and stored in PBS at −70°C until used.

Unsporulated oocysts of *Eimeria nieschulzi* were harvested from the feces of experimentally infected rats, using a standard sugar solution flotation procedure (14). The oocysts were counted in a McMaster chamber and stored in a 2.5% potassium dichromate solution at 4°C until used. Tachyzoites of *Toxoplasma gondii*, RH strain, were obtained from the peritoneal cavity of experimentally infected mice and cleaned by several centrifugation washes in phosphate buffered saline. The number of tachyzoites was estimated, using a hemocytometer, then stored in PBS at −70°C until used.

PREPARATION OF RNA

Total cellular RNA was extracted from isolated parasites and from infected and uninfected tissues of mice, ox, sheep and swine. We used a single-step RNA extraction procedure employing guanidine isothiocyanate-phenol-chloroform (15). Samples processed included one gram of minced tissue or approximately 10^6, 10^7, or 10^8 purified parasites suspended in approximately ten volumes of a denaturing solution (4 M guanidine isothiocyanate, 0.75 M sodium citrate, pH 7.0, 1% sarkosyl, 0.1 M 2-mercaptoethanol). Prior to RNA extraction procedure the suspension was homogenized in a motorized glass-teflon tissue grinder for 5–10 min in an ice bath. Five μL aliquots of the extracted RNA sample were removed for reading of absorbance at 260 nm and 280 nm in a spectrophotometer.

Samples of RNA at concentrations of 10, 25 or 100 ng/μL were denatured in formaldehyde. Three volumes of equal parts of 37% formaldehyde and 20× SSC (1× SSC is 0.15 M NaCl, 15 mM Na citrate, pH 7.0) were mixed with one volume of RNA suspension and incubated for 15 min at 65°C. DNA samples of a cloned SSU-rRNA gene of *S. muris* (11) were denatured by mixing ten volumes of DNA with one volume of 3 M NaOH and incubating for 1 h at 65°C. After the mixture was cooled to room temperature, sufficient 20× SSC was added to bring the final concentration to 6×. Using 10× SSC as a diluent and a multiwell slot-blot apparatus (Schleicher and Schuell, Keene, New Hampshire) various amounts of RNA or DNA were applied to Nytran nylon membranes that were prewet with 6× SSC. The membranes were then baked *in vacuo* for 2 h at 80°C. Prepared membranes were kept at 4°C until they were used in hybridization assays.

DESIGN AND CONSTRUCTION OF PROBES

The SSU-rRNA sequences of *S. muris* (11), *S. gigantea*, *T. gondii*-RH strain (16), *P. berghei*-A gene (17) and *Mus musculus* (18) were compared to determine if there were regions unique for *S. muris*, or homologous for members of the genus *Sarcocystis* but different from sequences in the mouse and *Plasmodium*. Two 20-base regions were selected to represent a *S. muris*-specific and a *Sarcocystis* genus-specific sequence and were chemically synthesized for use as probes. Beta-cyanoethyl phosphoramidites were used to synthesize the oligomers which were provided by Macromolecular Resources, Department of Biochemistry, Colorado State University, Fort Collins, Colorado.

The enzyme T4 polynucleotide kinase was used to radioactively end-label the probes in an exchange reaction. By following the manufacturer's instructions, the 5′ Terminus Labelling System (BRL, Gaithersburg, Maryland) was used to transfer [^{32}P] phosphate from 10 μCi/μL [gamma-^{32}P] ATP (Amersham, Arlington Heights, Illinois) to the 5′ends of the oligonucleotides. The labelled probes were purified using column chromatography (Bio-Spin 6, Bio-Rad Laboratories, Richmond, California).

HYBRIDIZATION PROCEDURE

Various conditions were used to carry out hybridization reactions with the *Sarcocystis* genus-specific and *S. muris*-specific probes. Methods reported previously were followed in the design of protocols used in this study (19,20).

Nytran nylon membranes with affixed nucleic acids were prehybridized in a polyethylene heat-sealable bag and incubated for at least 20 min at 37°C or 42°C. The membranes were immersed in 10 mL of prehybridization buffer (6× SSPE, 0.1% SDS, 10× Denhardt's reagent, 50 μg/mL tRNA and 50 μg/mL salmon sperm DNA). Following prehybridization the buffer was replaced with 10 mL of hybridization buffer consisting of

TABLE 1. Highest yields obtained from extractions of total RNA of various coccidia (*Sarcocystis*, *Toxoplasma* and *Eimeria*) and uninfected and infected tissues

	RNA yield per		
	10^6 organisms	(1 organism)	1 gram tissue
S. muris bradyzoites	7.4 μg	7.4 pg	
S. cruzi bradyzoites	8.0 μg	8.0 pg	
T. gondii tachyzoites	2.6 μg[a]	2.6 pg	
S. muris sporozoites	0 μg	0 pg	
E. nieschulzi oocysts (unsporulated)	4.0 μg	4.0 pg	
S. muris-infected muscle[b] (8.3×10^6 bradyzoites/g)[c]			1104 μg
S. cruzi-infected heart[d] (2.1×10^6 bradyzoites/g)[c]			164 μg
S. cruzi-infected esophagus[d] (3.0×10^6 bradyzoites/g)[c]			104 μg
Coccidia[e]-infected sheep liver[d] (5.0×10 zoites/g)[c]			40 μg
Uninfected[c] ox liver[d]			1284 μg
Uninfected[c] swine heart[d]			680 μg
Uninfected[c] sheep liver[d]			24 μg

[a]Five other extractions of *T. gondii* tachyzoites yielded no RNA
[b]Experimentally infected
[c]Determined by pepsin-HCl digest
[d]Normal meat (approved for human consumption)
[e]Zoites appeared similar to tachyzoites of *T. gondii*

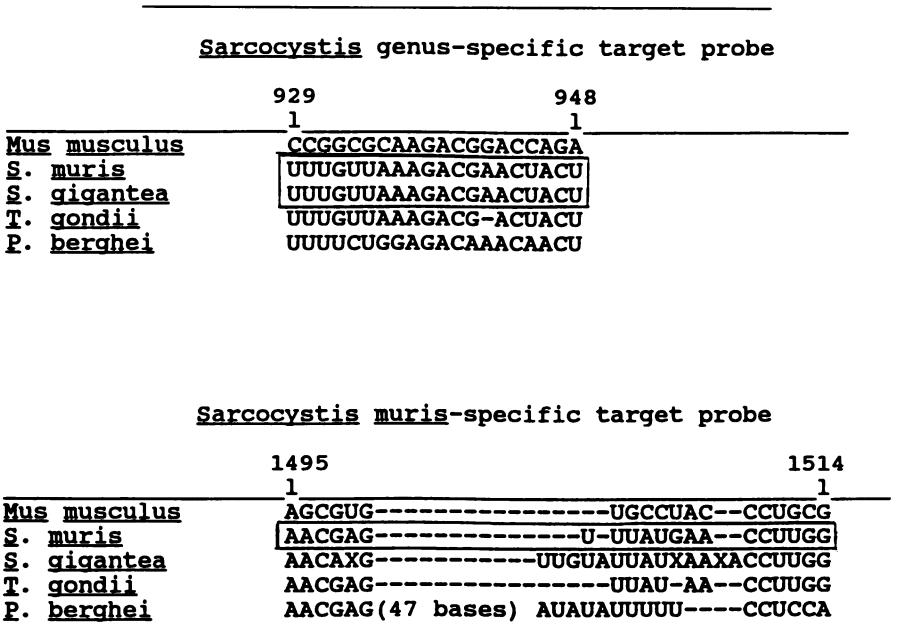

Fig. 1. Comparison of nucleotide sequences of small subunit rRNA (boxed areas) used in *Sarcocystis* genus-specific and *S. muris*-specific probes with corresponding regions from a host and other apicomplexans.

6× SSPE, 0.1% SDS, and approximately 0.5×10^6 CPM of labelled probes. Hybridization reactions were carried out overnight at 51°C, 47°C, 37°C or at room temperature (21°C). Membranes were then washed twice in a solution of 6× SSPE (0.18 M NaCl, 10 mM $NaPO_4$, pH 7.7, 1 mM EDTA) and 0.1% SDS for 10 min each at room temperature. The last wash was repeated at the hybridization temperature before a final 10 min wash in 6× SSPE alone was carried out at room temperature.

The wet membranes were immediately wrapped in plastic wrap, and exposed to X-ray film at −70°C overnight, using intensifying screens. In a few instances, the membranes were treated to remove the labelled probes in order to reuse the target nucleic acids on the filter in subsequent assays. The wet blots were washed for 2 h at 65°C in a solution consisting of 5 mM Tris-HCl (pH 8.0), 0.2 mM EDTA, 0.05% pyrophosphate and 0.1× Denhardt's reagent (Schleicher and Schuell, Keene, New Hampshire).

RESULTS

EXTRACTED RNA

The 260/280 nm absorbance readings of extracted RNA were consistently between 1.7 and 2.0, most often closer to 2.0. Yields of RNA of various parasites and uninfected and infected tissues are presented in Table I.

PROBES

The target sequence of the SSU-rRNA for *Sarcocystis* genus-specific probes was from nucleotide positions 929-948, and bases 1495-1514 were used for *S. muris*-specific probes. Figure 1 indicates the suitability of the regions for genus- or species-specific hybridization assays.

The complementary sequence to each target region was deduced and used to synthesize 20-mers. The *Sarcocystis* genus-specific sequence consisted of 70% A + U bases, and 60% A + U were present in the *S. muris*-specific sequence. The longest stretches of the same base were three adenine and three uracil residues. Using the formula $2(A + T) + 4(G + C)$ as a guide (21), the T_m of the hybrids formed by the genus- and species-specific probes were estimated to be 52°C and 56°C and the optimum hybridization temperatures were 47°C and 51°C, respectively.

HYBRIDIZATIONS

Autoradiograms showing the results of hybridization assays are presented in Figs. 2 and 3 and a summary of the specificities of the two probes is given in Table II.

A wide range of temperature conditions yielded similar hybridization results. The hybridization reactions were successful whether prehybridization was carried out at 42°C or 37°C. Specific and detectable hybridization of purified parasites using the genus-specific probe occurred at room temperature, 37°C and 47°C (Figs. 2 and 3). The optimal temperature for

achieving high sensitivity while maintaining species or genus specificity was 51°C for the *S. muris* probe (Fig. 2a). The efficiency of the assays appeared to be unaffected whether 1× or 6× SSPE were used.

The DNA oligonucleotide probes annealed with DNA as well as RNA targets. However, weaker signals were obtained from DNA-DNA hybridizations than from DNA-RNA hybrids when the amounts of probes and targets were constant (Figs. 2b, c). The smallest amounts of RNA and DNA detected by either probe were 1 ng and 5 ng, respectively (Figs. 2a, b). The *S. muris*-specific probes specifically hybridized only to nucleic acids of *S. muris*, and even at low stringencies there was no evidence of annealing with other species (Table II).

The genus-specific probes hybridized successfully with RNAs of *S. muris*, *S. cruzi*, and *E. nieschulzi* (Figs. 2b, c, and 3b). Occasionally, faint bands were present on the *T. gondii* blots. Both probes were also able to detect target RNAs in infected muscles but the genus-specific probes displayed greater sensitivity (Figs. 3a, b). Despite this high sensitivity the faint signals from "uninfected" tissues indicate some lack of specificity in the genus-specific probe (Fig. 3b).

TABLE II. Summary of hybridization assays of various coccidial organisms (*Sarcocystis*, *Toxoplasma* and *Eimeria*) and rDNA using *Sarcocystis* genus-specific and *S. muris*-specific probes

Total RNA tested	Probes	
	S. muris	*Sarcocystis*
S. muris clone of SSU-rRNA gene(DNA)	+/+[a]	+/+[a]
S. muris bradyzoites	+/+	+/+
S. muris-infected mouse muscle	+/+	+/+
Mouse muscle	−/−	−/+
S. cruzi bradyzoites	−/−	+/+
S. cruzi-infected bovine heart	−/−	−/+
S. cruzi-infected bovine esophagus	−/−	−/+
Bovine liver	−/−	−/+
T. gondii tachyzoites	−/−	+−/+−
E. nieschulzi unsporulated oocysts	−/−	+/+
Sheep diaphragm	−/−	−/+
Sheep liver	−/−	−/+
Pig heart	−/−	−/+

[a]The first result is for conditions of high stringency and the second is for low stringency

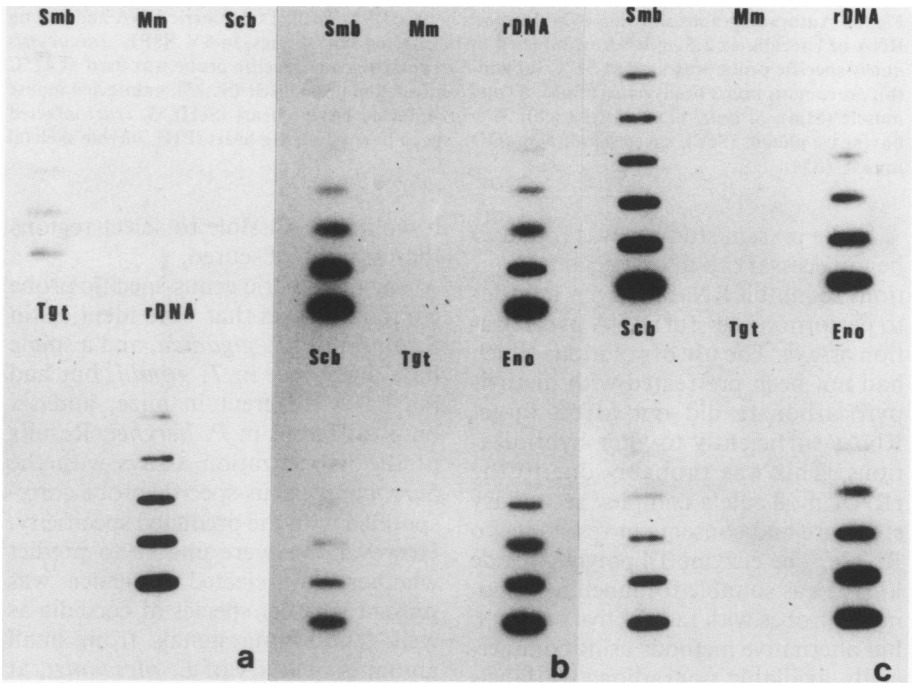

Fig. 2. Autoradiographs depicting hybridizations between ^{32}P-labelled oligomeric DNA and rRNA of coccidia, in 6× SSPE. The amounts of cellular RNA blotted were approximately 1, 5, 12.5, 25, 50, 100 and 200 ng, from top to bottom. Faint bands seen on x-ray films may not appear in this reproduction. A *Sarcocystis muris*-specific probe was used at 51°C (a) and a *Sarcocystis* genus-specific probe was used at 47°C (b) or at room temperature (c). *Sarcocystis muris* bradyzoites (Smb), *S. cruzi* bradyzoites (Scb), *Toxoplasma gondii* tachyzoites (Tgt), *Eimeria nieschulzi* oocysts (Eno), cloned small subunit rRNA gene (rDNA), uninfected mouse muscle (Mm).

DISCUSSION

Traditional methods of microscopy, tissue digestion, immunoassay and infection by transmission have serious limitations in the diagnosis of sarcocystosis. Structural criteria are unreliable, immunoassays lack specificity, and experimentally determining the appropriate hosts is costly and time consuming (5). Methods for detection of nucleic acids appear to provide suitable alternatives. Although successful probes for the detection of DNA have been developed, it has been estimated that detection of rRNA can be several orders of magnitude more sensitive (10). This is due primarily to the fact that rRNA represents approximately 90% of a cell's total RNA, about 30% of which is SSU-rRNA, whereas highly repetitive DNA sequences make up less than 1% of total DNA.

Our results indicate that a considerable amount of RNA can be extracted from most coccidial organisms. The reason for the lack of consistent yields from *T. gondii* is not clear. Similar poor yields of RNA from *T. gondii* have been reported (22–24). A more efficient method of extracting RNA may be required in order to use RNA-based probes for the detection of *T. gondii*. Also, the reason for failure to obtain RNA from sporocysts of *S. muris* is not known. Perhaps this is typical in the dormant phase of a coccidian life cycle. Studies show that there is limited turnover and synthesis of RNA during sporulation of *E. tenella* oocysts (25). It is possible that little or no RNA is synthesized in fully sporulated oocysts and RNase may degrade existing molecules. The sporocysts of *S. muris* used in this study were stored for six months or more at 4°C and RNA may have been degraded.

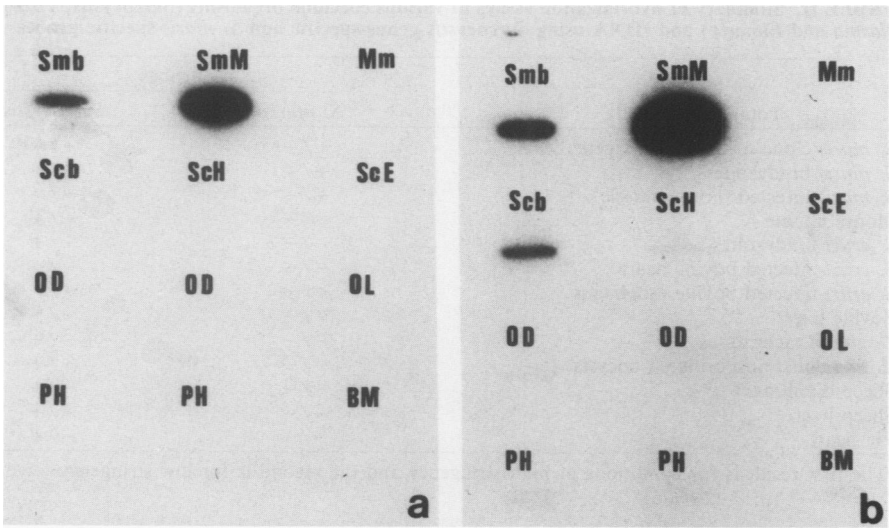

Fig. 3. Autoradiographs showing hybridizations between ^{32}P-labelled oligomeric DNA and 100 ng RNA of coccidia or 2.5 μg RNA of infected or uninfected host tissues, in 6× SSPE. *Sarcocystis muris*-specific probe was used at 51°C (a) and *Sarcocystis* genus-specific probe was used at 47°C (b). *Sarcocystis muris* bradyzoites (Smb), *S. muris*-infected mouse muscle (SmM), uninfected mouse muscle (Mm), *S. cruzi* bradyzoites (Scb), *S. cruzi*-infected bovine heart (ScH), *S. cruzi*-infected bovine esophagus (ScE), sheep diaphragm (OD), sheep liver (OL), pig heart (PH), bovine skeletal muscle (BM).

In the present study, it was found to be unnecessary to take special precautions to inhibit RNase activity in order to perform successful rRNA hybridization assays. The use of solutions which had not been pretreated with diethylpyrocarbonate did not affect target RNAs sufficiently to alter hybridizations. This was probably due to the rRNA molecule's complex secondary structure and consequent resistance to RNase. The enzyme T4 polynucleotide kinase was suitable for labelling oligomeric probes with radioactive markers, but alternative methods using commercially available nonradioactive labels may have to be employed.

This study confirms other reports of the use of probes for SSU-rRNA as a powerful and versatile identification assay (10,26–29). The complete SSU-rRNA requences of only three apicomplexans (*S. muris*, *P. berghei* and *P. falciparum*) were available for comparison. Availability of sequences from other apicomplexans was limited to three short regions in *S. gigantea* and *T. gondii* (16). Thus the search for genus- and species-specific probes was limited to these relatively small regions of the SSU-rRNA, so that it is possible that other regions of the molecule may have been more suitable for our purposes. Furthermore, by elucidating the secondary structure of the molecule it would be possible to select regions that are not obscured.

The oligomeric genus-specific probe contained bases that were identical in *S. muris* and *S. gigantea*, and a single base difference in *T. gondii*, but had ten bases different in mice, and six bases different in *P. berghei*. Results of the hybridization assays with the *Sarcocystis* genus-specific probe corresponded with the predicted specificity. However, we were unable to predict whether the selected sequence was present in other species of coccidia as well. The strong signals from small amounts of RNA of *E. nieschulzi*, at both high and low stringencies, suggest that this region may have a high degree of homology with other coccidia. It may be possible to use this probe for a broader coccidia-specific assay, but further tests are necessary. The lack of consistent hybridization signals when *T. gondii* was assayed is probably related to the difficulty of extracting nucleic acids from this organism. Although unlikely, it is also possible that signals from *T. gondii* was absent because of an unstable hybrid due to bond strains created by the absence of a nucleotide within the target sequence. The occasional weak signal from "uninfected" tissues may be due to nonspecific binding. Alternatively, it is possible that these tissues contained an unknown latent form of coccidia. Latent forms of parasites are known to occur in other apicomplexans such as *Plasmodium* spp. (30). The extremely low level of infection in the esophagus assayed was refleced in faint hybridization signals.

The sequence of the species-specific oligomeric probes was identical in *S. muris* but heterologous at most positions in other species examined. The sequence in *T. gondii* shared the largest number of bases (18 of 20) with this species-specific probe. This two-base mismatch was sufficient to allow hybridization to RNA and DNA of stages of *S. muris* alone. The annealing of the probes to samples of cloned SSU-rRNA genes indicated that rRNA-based probes will hybridize to genomic ribosomal DNA and will detect all phases of a life cycle, even when the RNA may not be expressed. This may be possible in apicomplexans that have complex life cycles and distinct rRNA genes that are expressed in some stages and not in others (31,32).

This study demonstrates that nucleic acid probes for rRNA may be used to detect and identify parasites in tissues. Because of the high sensitivity of the genus-specific probe it should be possible to estimate the level of infection in a sample. Since a single, related apicomplexan (*Plasmodium falciparum* trophozoite) contains approximately 0.2–1 pg of RNA (33), a single sarcocyst containing many thousands of bradyzoites should contain μg amounts of RNA. Because our results show that 1 ng of RNA can be detected, it is likely that probes for rRNA can detect a single parasite in an extract of RNA. Whenever there is a paucity of target molecules the sensitivity of the assay may be enhanced by the use of PCR methods. Useful and practical application of this probe may also be possible *in situ* hybridization assays. Nevertheless, the present method is relatively rapid, sensitive and specific, and may be adapted for field applications. The use of nonradioactive labels will make it environmentally safe and eliminate the need for specially trained laboratory personnel. Also, by determining the sequences of several species it should be possible to identify probes with varied specificities and improved sensitivity for parasites that are of veterinary and medical importance.

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REFERENCES

1. **LEVINE ND.** The taxonomy of *Sarcocystis* (Protozoa, Apicomplexa) species. J Parasitol 1986; 72: 372-382.
2. **MEHLHORN H, HEYDORN AO.** The Sarcosporidia (Protozoa, Sporozoa): Life structure and fine structure. Adv Parasitol 1987; 16: 43-72.
3. **KIRKPATRICK CE, DUBEY JP, GOLDSCHMIDT MH, SAIK JE, SCHMIDT JA.** *Sarcocystis* sp. in muscles of domestic cats. Vet Parasitol 1986; 23: 88-90.
4. **MUNDAY BL, OBENDORF DL.** Morphology of *Sarcocystis gigantea* in experimentally-infected sheep. Vet Parasitol 1984; 16: 193-199.
5. **FAYER R, DUBEY JP.** Bovine sarcocystosis. Compendium 1986; 8: F130-F142.
6. **JENSEN R, ALEXANDER AF, DAHLGREN RR, JOLLEY WR, MARQUARDT WC, FLACK DE, BENNETT BW, COX MF, HARRIS CW, HOFFMANN GA, TROUTMAN RS, HOFF RL, JONES RL, COLLINS JK, HAMAR DW, CRAVANS RL.** Eosinophilic myositis, and muscular sarcocystosis in the carcasses of slaughtered cattle and lambs. Am J Vet Res 1986; 47: 587-593.
7. **GAJADHAR AA, MARQUARDT WC.** Ultrastructural and transmission evidence of *Sarcocystis cruzi* associated with eosinophilic myositis in cattle. Can J Vet Res 1992; 56: 41-46.
8. **GAJADHAR AA, YATES WDG, ALLEN JR.** Association of eosinophilic myositis with an unusual species of *Sarcocystis* in a beef cow. Can J Vet Res 1987; 51: 373-378.
9. **SOGIN ML, GUNDERSON JH.** Structural diversity of eukaryoyic small subunit ribosomal RNAs. Evolutionary implications. Ann NY Acad Sci 1987; 503: 125-139.
10. **LAL AA, CHANGKASIRI S, HOLLINGDALE MR, McCUTCHAN TM.** Ribosomal RNA-based diagnosis of *Plasmodium falciparum* malaria. Mol Biochem Parasitol 1989; 36: 67-72.
11. **GAJADHAR AA, MARQUARDT WC, HALL R, GUNDERSON J, ARIZTIA-CARMONA E, SOGIN ML.** Evolutionary relationships among Apicomplexans, Dinoflagellates and ciliates: ribosomal RNA sequences of *Sarcocystis muris*, *Theileria annulata*, and *Crypthecodinium cohnii*. Mol Biochem Parasitol 1991; 45: 147-154.
12. **BOX ED, SMITH JH.** The intermediate host spectrum in a *Sarcocystis* species of birds. J Parasitol 1982; 68: 668-673.
13. **FAROOQUI AA, ADAMS DD, HANSON WL, PRESTWOOD AK.** Studies on the enzymes of *Sarcocystis suicanis*: purification and characterization of an acid phosphate. J Parasitol 1987; 73: 681-688.
14. **SLOSS MW.** Veterinary Clinical Parasitology. 4th ed. Ames, Iowa: Iowa State University Press, 1970.
15. **CHOMCZYNSKI P, SACCHI N.** Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 1987; 162: 156-159.
16. **JOHNSON AM, MURRAY PJ, ILLANA S, BAVERSTOCK J.** Rapid nucleotide sequence analysis of the small subunit ribosomal RNA of *Toxoplasma gondii*: evolutionary implications for the apicomplexa. Mol Biochem Parasitol 1987; 25: 239-246.
17. **GUNDERSON JG, McCUTCHAN TF, SOGIN ML.** Sequence of the small subunit ribosomal RNA gene expressed in the bloodstream stages of *Plasmodium berghei*: evolutionary implications. J Protozool 1986; 33: 525-529.
18. **RAYNAL F, MICHOT B, BACHELLERIE JP.** Complete nucleotide sequence of mouse 18s rRNA gene: comparison with other available homologs. FEBS Lett 1986; 167: 263.
19. **SAMBROOK J, FRITSCH EF, MANIATIS T.** Molecular Cloning. A Laboratory Manual. Second Edition. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press, 1989.
20. **WAHL GM, BERGER SL, KIMMEL AR.** Molecular hybridization of immobilized nucleic acids: theoretical concepts and practical considerations. Methods Enzymol 1987; 152: 399-407.
21. **WALLACE RB, MIYADA CG.** Oligonucleotide probes for the screening of recombinant DNA libraries. Methods Enzymol 1987; 152: 432-442.
22. **JOHNSON AM, McDONALD PJ, ILLANA S.** Characterization and in vitro translation of *Toxoplasma gondii* ribonucleic acid. Mol Biochem Parasitol 1986; 18: 313-320.
23. **HYDE JE, GOMAN M, HALL R, OSLAND A, HOPE IA, LANGSLEY G, ZOLG JW, SCAIFE JC.** Characterization and translation studies of messenger RNA from the human malaria parasite *Plasmodium falciparum* and construction of a cDNA library. Mol Biochem Parasitol 1984; 10: 209-285.
24. **DASILVEIRA JF, MERCEREAU-PUIJALON O.** *Plasmodium chabaudi* antigens synthesized in an mRNA-dependent cell-free translation system. Mol Biochem Parasitol 1989; 7: 159-172.
25. **WANG CC.** Biochemistry and physiology of coccidia. In: Long PL, ed. The Biology of the Coccidia. Baltimore University Park Press, 1982: 167-228.
26. **GOBEL UB, GEISER A, STANBRIDGE EJ.** Oligonucleotide probes complementary to variable regions of ribosomal RNA discriminate between *Mycoplasma* species. J Gen Microbiol 1987; 133: 1969-1974.
27. **CAZENAVE A, CHEYROU A, BLOUIN P, JOHNSON AM, BEGUERET J.** Use of polymerase chain reaction to detect *Toxoplasma*. J Clin Pathol 1992; 44: 1037.
28. **DELONG EF, WICKHAM GS, PACE NR.** Phylogenetic stains: ribosomal RNA-based probes for the identification of single cells. Science 1989; 243: 1360-1363.
29. **WATERS AP, McCUTCHAN TM.** Rapid, sensitive diagnosis of malaria based on ribosomal RNA. Lancet 1989, 1: 1343-1346.
30. **MARKUS MB.** Possible support for the sporozoite hypothesis of relapse and latency in malaria. Trans R Soc Trop Med Hyg 1976; 70: 535.
31. **GUNDERSON JH, SOGIN ML, WOLLETT G, HOLLINGDALE M, DE LA CRUZ VF, WATERS AP, McCUTCHAN TF.** Structurally distinct, stage-specific ribosomes occur in *Plasmodium*. Science 1987; 238: 933-937.
32. **WATERS AP, SYIN C, McCUTCHAN TF.** Developmental regulation of stage specific ribosome populations in *Plasmodium*. Nature 1989; 342: 438-440.
33. **BAHR GF.** Quantitative cytochemistry of malaria infected erythrocytes. Milit Med 1969; 134: 1013-1025.