Research Note

Detection of Sarcocystis Parasites in Retail Beef: A Regional Survey Combining Histological and Genetic Detection Methods

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ABSTRACT

Sarcocystis spp. are parasitic protists acquired when undercooked, cyst-laden meat is consumed. While both Sarcocystis hominis and S. cruzi encyst in beef, only S. hominis is pathogenic to humans. In this study, we used histological methods and novel molecular techniques to determine the regional prevalence and identity of Sarcocystis spp. in retail beef. Of 110 samples, 60 supported amplification of parasite rRNA by PCR. All 41 sequenced representatives were identified as S. cruzi. To compare detection methods, 48 samples were then examined in parallel by histology and PCR, and 16 and 26 samples, respectively, were positive. Five samples positive by initial histologic sections were not amplified by PCR. Fifteen PCR-positive samples did not contain sarcocysts on initial histologic section, but additional sections from these samples revealed sarcocysts in an additional 12 samples. When combined, histology with additional sections and PCR detected 31 positive specimens of the 48 total specimens. We found no evidence of human pathogen S. hominis and confirm that cattle pathogen S. cruzi is highly prevalent in this regional sample. PCR assays may increase the detection sensitivity of Sarcocystis spp. and contribute diagnostic precision.

Diverse microbes occur in food animals, and protecting veterinary and public health requires an ability to discriminate those which pose appreciable health risks from those that do not. Among these microbes are the coccidia, which comprise an order of protistan parasites in the phylum Apicomplexa. There exist some 200 named coccidian species that encyst in the muscles of herbivores and are transmitted to carnivores (including humans) upon ingestion (3, 4, 6). The coccidian Sarcocystis hominis has been implicated as a human pathogen in situations where consumption of undercooked beef is common, and it can cause an array of symptoms in humans, including acute diarrhea, abdominal pain, distension, eosinophilia, anemia, fatigue, dizziness, fever, chills, vomiting, and respiratory distress (1, 6, 7). It is not known how this infection affects an immunosuppressed host.

The prevalence of this zoonotic parasite is difficult to assess in locales where the practice of eating uncooked meat is uncommon, such as the United States. Furthermore, the nearly ubiquitous occurrence of a genetically and morphologically related parasite, Sarcocystis cruzi, renders it difficult to ascertain the true geographical limits of S. hominis. Neither S. cruzi, which employs dogs as definitive hosts, nor S. hirsuta, which employs cats, poses an appreciable public health risk (6).

To date, human sarcocystosis has not been described from domestic exposure in the United States, and S. hominis has not been identified in domestic retail beef. Identification of this pathogenic species in the United States would have important public health implications, and studies to assay for its presence are needed.

In tissue such as beef, the muscle stage of Sarcocystis spp. can be differentiated from cysts of other coccidian parasites, such as Toxoplasma gondii, by light microscopy alone (8, 12). However, definitive identification of Sarcocystis to the species level cannot be performed by basic histology and requires electron microscopy (13, 14) or molecular analysis. While electron microscopy is not suited for large epidemiologic studies, molecular methods offer a viable means for screening, detection, and/or identification and have improved the sensitivity and specificity of parasite detection from environmental sources. Therefore, the goals of this study were to compare histology and PCR as means for detecting Sarcocystis spp. in retail beef and to use novel molecular methods to test the assumption that any parasites detected would correspond to a species infectious to dogs (i.e., S. cruzi) but not to people.

MATERIALS AND METHODS

Beef samples. One hundred ten beef samples (representing 110 animals) were purchased from national grocery chain stores
located in Vermont and a Vermont food cooperative during a 9-month period (October 2004 to June 2005). These stores sold beef from steers raised in the western United States, Vermont, New York, or Uruguay. Samples were purchased from several stores on separate days. Given the large quantity of beef sold at each store daily, this sampling technique virtually ensured that each sample represented a separate animal. Of the 110 samples, 100 were conventional U.S.-raised beef, comprising 83 samples from the western United States, 5 samples from New York, and 10 samples from Vermont. In addition, 12 samples of organic beef from Uruguayan-raised steers were purchased from the Vermont food cooperative. All samples were sirloin cuts representing beef from the posterior loin region of the animal.

**Histology.** From each sample, a single, roughly rectangular section was obtained for histological processing. Sections had an average area of 2.0 cm² and ranged in size from 1.0 to 4.0 cm². Sections were fixed in 10% neutral buffered formalin and processed routinely for light microscopic examination. Following paraffin embedding, a single section, 5 µm in thickness (one section per case), was stained with hematoxylin and eosin (H&E). Slides were screened for the presence of sarcocysts at ×100 the original magnification and then further analyzed at ×400 the original magnification. The presence of other pathologic features, including inflammation and its association with the sarcocysts, was noted for each case.

**DNA extraction and PCR.** Molecular analysis was performed with 0.05 to 0.25 g of tissue (average, 0.08 g) taken immediately adjacent to the corresponding histologic section. DNA extraction was performed using Qiagen DNAeasy (Valencia, Calif.) commercially available kits according to the manufacturer's instructions, yielding 0.4 to 71.6 ng of DNA per µl (average, 19.9 ng/µl). This was followed by PCR amplification of rRNA motifs conserved among *Sarcocystis* species but distinct from vertebrate, fungal, and bacterial homologues. Each 20-µl reaction volume contained 2 to 2.5 µl of template, 0.5 U of Platinum High Fidelity *Taq* Polymerase (Invitrogen), 0.2 mM deoxynucleoside triphosphates, and 10 pmol of each primer. An ~1,100-bp portion of the 18S rRNA gene was amplified using primers 18SIF (GGATAACCGTGGATAATTCAGT) and 18S 11R (TCCTATGTCTGGACCTGGTAG) (5) using a 3-min denaturation at 94°C, followed by 35 cycles of 94°C for 30 s, 53°C for 45 s, and 72°C for 90 s, and a final extension of 72°C for 10 min. Each assay included uninfected human liver and kidney specimens as negative controls and *Besnoitia darlingi* and *Sarcocystis neurona* templates as positive controls.

Prior to direct cycle sequencing, 4 µl of PCR product was treated with 1.6 µl of EXO-SAP-IT (USB Corp.) and incubated at 37°C for 5 min and then at 80°C for 15 min. To these were added 2 µl of Big Dye Terminator Version 3.1 (Applied Biosystems), 2 µl of 2.5× Big Dye terminator buffer, and 1 µl (3.2 pmol) of either the forward or reverse amplification primer. After cycle sequencing according to manufacturer recommendations, each reaction mixture was eluted through a column (Edge Biosystems) to remove excess primers and dinucleoside triphosphates prior to electrophoresis using an ABI 3100 automated sequencer.

**Additional histology sections.** Following PCR, three additional histologic sections (5 µm thick) were obtained from each case for which light microscopy had not previously identified sarcocysts but in which PCR had detected parasite DNA. These sections were each taken at approximately 100-µm intervals from the previous section, while cutting deeper into the paraffin block. Additional sectioning was not performed on samples which were negative by both PCR and histology.

**RESULTS**

**Histology.** Examination of the initial tissue section from each case by light microscopy revealed sarcocysts in 44 (40%) of 110 samples. The sarcocysts measured 0.4 mm in maximum length, and their counts averaged 1.4/cm² of beef in positive samples (median, 1.0), with a range of 0.3 to 6.2/cm². Scattered foci of mild to moderate lymphocytic inflammation were identified in 42 samples (38%), 19 of which also contained sarcocysts by light microscopy. One of these samples contained eosinophils as well as lymphocytes. None of the sarcocysts were directly associated with an inflammatory response. Four samples contained numerous surface collections of bacteria without an associated host inflammatory response. There were no appreciable differences in the histologic findings, including number of sarcocysts, between Uruguayan and U.S. beef samples. Examples of histologic findings are shown in Figures 1 through 4.

PCR. Amplification of parasite rRNA was achieved in 60 (54.5%) of the 110 samples. In all samples, positive and negative controls gave appropriate results. Each of 41 samples randomly chosen for sequencing, including U.S. and Uruguayan samples, corresponded perfectly to one another and to other *S. cruzi* exemplars previously deposited in GenBank (AF176932 and AF176934) and not to any previously submitted representatives of *S. hominis* (e.g., AF176942-5) or other congeners (10).

**Method comparison.** We compared the sensitivity of the histologic technique and that of PCR in two ways. First, we considered the first 48 samples of U.S. beef. Of these, light microscopic examination identified sarcocysts in the first section in 16 samples (33%). By contrast, *Sarcocystis* rRNA was detected by PCR in 26 samples (54%). In 17 instances, infection was detected by neither assay. Five positive samples identified by a single histologic section were not amplified by PCR, whereas 15 samples detected by PCR did not contain identifiable cysts on the initial tissue section.

We then examined three additional histology sections on these 15 samples, revealing sarcocysts in an additional 12, for a total of 28 positive samples by histology. Putting the results in mutually exclusive categories resulted in 14 samples that were positive by neither PCR nor a single histologic section, 11 that were positive by both PCR and a single histologic section, 12 that were positive by PCR and three deeper histologic sections, 6 that were positive by PCR but not by histology despite three deeper sections, and 5 that were positive by a single histologic section, but not PCR (48 total cases). Most commonly, additional sarcocysts were apparent in the first of the three additional tissue sections (cut approximately 100 µm from the original tissue section).

**DISCUSSION**

*Sarcocystis* spp. are unicellular coccidian protozoan parasites with a worldwide geographic distribution (1, 6,
Although, to this date, there have been no reported cases of human sarcocystosis originating in the United States, the ease of international travel and globalization of the world's food supply exposes the population to a growing number of foodborne parasites (9). Clearly, an awareness of both domestic and global parasitic pathogens is warranted.

We examined 110 samples of beef with the goal of determining the prevalence and identity of Sarcocystis spp. in beef available to persons living in the United States, specifically in Vermont. Although we confirmed that S. cruzi is widely prevalent, we found no evidence of zoonotic S. hominis in 41 sequenced samples. This supports the belief that S. hominis infrequently, if ever, occurs in U.S. retail beef. Because our primers target portions of the 18S rRNA gene that are highly conserved among coccidia, they should be capable of amplifying any species of Sarcocystis occurring in such samples. If the summed prevalence of such congeners were 10%, we would have a 99% chance of observing at least one organism in a random sample of 41 sequenced individuals. Similarly, we have 88% power to detect at least one organism if their summed population prevalence is only 5%, but only 34% power to detect one organism if their population prevalence is less than 1%. Thus, our sample is adequate to rule out the possibility that other species of Sarcocystis occur frequently in the beef available for retail sale in our sampled region, but this cannot exclude the possibility of their rare occurrence.

The most sensitive known means to verify the presence of S. cruzi in beef specifically is by feeding it to this parasite's natural definitive host, the dog. Parasite sporocysts have been excreted by nearly every dog fed beef samples...
DETECTION OF SARCOCYSTIS PARASITES IN RETAIL BEEF

originating in the United States, suggesting near-universal bovine infection (2). So if the true prevalence of S. cruzi in the U.S. beef supply was assumed to be 95%, then examining a single histologic section detected 42% of such infections, PCR detected 57%, and combining the methods detected 75%. Although evidently less sensitive, these methods are far more economical and feasible than feeding beef samples to laboratory dogs. When PCR and histology are used in combination, they can be expected to detect three-fourths of all S. cruzi infections in beef.

If used separately, molecular and histologic methods each offer unique advantages. For example, PCR may allow for greater detection of Sarcocystis spp. in beef than would a single histology section. In our comparison of 48 cases, PCR detected infection in 10 more samples than did one round of histology; an increase of almost 21%. Given our sampling effort, we can be 90% confident that PCR increases the detection sensitivity by between 4.0 and 37.6%, and we can be 80% confident that PCR increases the sensitivity by between 7.1 and 34.5%. This is likely due in part to the larger volume of beef that may be sampled by a single PCR assay (approximately 2 mm³ in our study) than what comprises a standard 2-cm², 0.5-μm-thick histologic section. This suggestion is supported by our observation that sarcocysts occur in histological sections adjacent to sections in which no such sarcocysts are visible and explains why additional histologic sections detected many more positive samples. Another advantage of molecular amplification is that it allows for subsequent sequencing of isolated RNA and thus provides unambiguous identification to the species level, which is not possible by morphology alone.

In contrast to PCR, histologic processing and examination are comparatively inexpensive and easy to perform. Furthermore, the examination of additional tissue sections is often routine practice in pathology laboratories and may substantially increase the detection sensitivity for sarcocysts. Histologic examination also enables parasite quantification and identifies pathologic findings not assayed by PCR, such as other meatborne parasites and inflammation. We found it interesting that lymphocytic inflammation was present in 37% of our samples, including a single case with intermixed eosinophils. The cause of this inflammation is unclear; however, sarcocysts were not associated with the inflammatory cells in any of the samples. Numerous bacteria (coci and bacilli) were also identified in four samples but lacked an accompanying immune response, implying that contamination rather than true ante-mortem infection was the source.

Despite the encouraging lack of demonstrable human pathogenic Sarcocystis spp. in both the U.S. and Uruguayan samples, it is important to understand that other pathogens, primarily bacteria, may cause significant human illness when undercooked or improperly stored beef is consumed. Furthermore, other domestic or imported beef not sampled in this study may present an undefined risk of harboring S. hominis. Therefore, we recommend continued beef surveillance and adequate cooking and storage of all meat products.

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REFERENCES