A Comparative Analysis of Coprologic Diagnostic Methods for Detection of *Toxoplasma gondii* in Cats

Harold Salant,* Dan T. Spira, and Joseph Hamburger

Department of Parasitology, Hebrew University-Hadassah Medical School, Jerusalem, Israel

Abstract. The relative role of transmission of *Toxoplasma gondii* infection from cats to humans appears to have recently increased in certain areas. Large-scale screening of oocyst shedding in cats cannot rely on microscopy because oocyst identification lacks sensitivity and specificity, or on bioassays, which require test animals and weeks before examination. We compared a sensitive and species-specific coprologic–polymerase chain reaction (copro-PCR) for detection of *T. gondii* infected cats with microscopy and a bioassay. In experimentally infected cats followed over time, microscopy was positive occasionally, and positive copro-PCR and bioassay results were obtained continuously from days 2 to 24 post-infection. The copro-PCR is at least as sensitive and specific as the bioassay and is capable of detecting infective oocysts during cat infection. Therefore, this procedure can be used as the new gold standard for determining potential cat infectivity. Its technologic advantages over the bioassay make it superior for large-scale screening of cats.

INTRODUCTION

Toxoplasma gondii, an obligatory intracellular protozoan parasite, is the cause of toxoplasmosis, a major zoonosis. It can probably infect all warm-blooded animals and humans, which become infected by ingesting tissue cysts from infective undercooked meat or by consuming food or drink contaminated with oocysts excreted by cats, the definitive host for the parasite.¹ Although most persons infected after birth are asymptomatic,² a small percentage develop mild to severe clinical manifestations involving fever, malaise and lymphadenopathy in mild cases,² mental illness³ and ocular disease⁴ in moderate cases, and severe manifestations among infected pregnant women resulting in abortions, stillbirths, or liveborn children with ocular or central nervous system impairment.⁵ Lastly, severe and even fatal disease with pulmonary and multivisceral involvement, possibly from more virulent types of the organism, may occur in postnatally infected persons.⁶ Specifically, toxoplasmosis ranks high on the list of diseases that lead to death in patients with acquired immunodeficiency syndrome (AIDS); approximately 10% of AIDS patients in the United States and up to 30% in Europe are estimated to die from toxoplasmosis.⁷

Serum antibody measurement by using various serologic tests is now commonplace for determining past exposure. Foci of high human seroprevalence exist in Latin America (77% in Brazil),⁸ parts of eastern and central Europe (57.6% in Romania),⁹ the Middle East (63.9% in Iran),¹⁰ parts of southeast Asia (49% in Malaysia),¹¹ and Africa (60% in Côte d'Ivoire).¹² However, a trend towards a lower seroprevalence is observed in many countries in Europe (8.2% in Switzerland)¹³ and in the United States (14% in 1988 and 9% in 2004).¹⁴

Although the authors of a large multicenter European case-control study of toxoplasmosis in pregnant women published in 2000¹⁵ concluded that 30–63% of infections in different centers were attributed to consumption of undercooked or cured meat products and 6–17% were attributed to soil contact, other studies during the same period showed that the seroprevalence of *T. gondii* in meat-producing animals are decreasing considerably in areas with intensive farm management,

where increased measures of biosecurity are undertaken to prevent exposure to cats and their excreta. Thus, seroprevalence decreased from 5.6% to 0.38% in pigs in Europe¹⁶ and from 23.3% to 3% in the same animals in the United States.¹⁴ The decreasing seroprevalence in food animals in these areas is not paralleled by a comparable magnitude of decrease in human seroprevalence, which suggests that the relative role of cats in the epidemiology of human *Toxoplasma* infection is probably of greater importance than is hitherto recognized. This finding is probably caused by feline fecal oocyst contamination of human food and/or water,¹⁷ which could be taking place in addition to direct transmission to people from pet cats. A suitable tool for identifying infective cats should facilitate the evaluation of this matter.

Cats have been shown to excrete *T. gondii* oocysts for a limited and relatively short period when a primary infection takes place.^{1,18} During this period, lasting for approximately 2–3 weeks, millions of oocysts are passed in the stool daily.¹⁹ After a period outside the host, the oocysts sporulate and become infective to mammals and birds.²⁰ Excretion of fewer oocysts occasionally occurs in reinfecting cats.^{1,20}

Historically, infective cats have been identified by microscopy for the detection of the oocysts, approximately 10–13 μ m in diameter, in their feces. Because the sensitivity of this method is low, detection is problematic when small oocyst numbers prevail, as after infection with some strains or after cats are infected by the tachyzoite or oocyst stages of this parasite.^{21,22} Furthermore, oocysts of other coccidian parasites appearing in cat feces, such as those of *Hammondia hammondi*, may not be differentiated microscopically from *T. gondii*.²³ Conversely, bioassay in mice is species specific and sensitive for detection of *T. gondii* oocysts.²⁴ However, bioassay not only introduces a possible biohazard element, but is also expensive, requires infection of animals, facilities for their maintenance, and is time consuming.¹ These features make bioassay unsuitable for large-scale screening. Investigators^{25,26} have experienced some of these problems, specifically caused by the lack of a practical method for differentiation of *T. gondii* oocysts from oocysts of coccidia of similar morphology, when attempting to determine the rate of *T. gondii* oocyst shedding by cats.

In a previous report, we described a sensitive and specific polymerase chain reaction (PCR) assay for detection of *T. gondii* DNA in feline feces.²⁷ This coprologic-PCR (copro-PCR) uses primers that amplify a 529-basepair sequence that

*Address correspondence to Harold Salant, Department of Parasitology, Hebrew University-Hadassah Medical School, PO Box 12272, Jerusalem, 91120, Israel. E-mail: tsvis@ekmd.huji.ac.il

is present 200–300 times in the *T. gondii* genome. Although this report described a valid test for *T. gondii* detection, a comparative analysis was still required. The objective of the present study was to determine copro-PCR detection sensitivity, timing, and consistency, and the stage of *T. gondii* detected in comparison with traditionally used coprodetection methods (microscopy and bioassay).

MATERIALS AND METHODS

Animals. *Cats.* Three female laboratory cats (1.5 years of age) were obtained from the Hebrew University Animal Authority and housed in an animal facility under standard conditions. The laboratory cats were used to follow *T. gondii* infection over a period during which different *T. gondii* coprologic detection methods were used (kinetic study) and compared.

Mice. Outbred Sabra female mice (6–8 weeks of age and weight = 20–25 grams) were housed at the Animal Unit, Hadassah Medical School, Hebrew University of Jerusalem. They were used for detecting *T. gondii* in cat feces by bioassay.

Permission for experiments regarding mice and cat infections in Israel was obtained from the Ethics Committee for Animal Experimentation of the Hebrew University of Jerusalem.

Parasites and parasite DNA. Sporulated oocysts of the VEG strain of *T. gondii* were provided by Dr. J. P. Dubey (U.S. Department of Agriculture, Beltsville, MD) and stored in 2% sulfuric acid at 4°C. They were employed to infect mice used as a source of tissue cysts to later infect experimental cats in the kinetic study. They were also employed for spiking negative feces in copro-PCR experiments that used different parasite harvest conditions for differentiating oocysts from non-encysted forms in cat feces.

Fresh tachyzoites derived from mice ascitic effusions were provided by the *Toxoplasma* Laboratory, Ministry of Health, Abu Kabir, Tel Aviv. They were employed for spiking fecal samples in copro-PCR experiments by using different parasite harvest conditions to differentiate oocysts from non-encysted forms in cat feces. They were also used as a DNA source for a *T. gondii*-positive PCR control.

Fecal samples. *Negative samples.* Pooled negative fecal samples for *T. gondii* were collected from laboratory cats kept by Professor V. Svobodova (University of Brno, Brno, Czech Republic) for spiking experiments to differentiate oocysts from non-encysted forms in cat feces. They were taken from cats seronegative for *T. gondii* as determined by two indirect immunofluorescent tests and were free of *Toxoplasma*-like oocysts as determined by light microscopy.

Fecal samples from experimentally infected cats. Fecal samples from each of three separately housed infected laboratory cats used in the kinetic study were collected daily (days 1–35) from their litter boxes after feeding them with mouse tissue cysts. Selected samples collected during this time were examined for *T. gondii* by different coprologic detection methods. Fecal samples from each cat were divided on each day. One gram was stored at 4°C for PCR and the rest (usually 5–10 grams) was used for detecting oocysts by microscopy and bioassay.

Infection of mice and cats. Cats were infected orally with *T. gondii* tissue cysts derived from parasite-containing mice

brains. These mice were infected by gavage two months earlier by using oocysts of the VEG1 strain. Presence of infection in these mice was ascertained by isolating DNA from brain material (DNeasy tissue extraction kit; Qiagen, Valencia, CA), followed by PCR analysis using the 529-basepair fragment amplification. In addition, a modified agglutination test (MAT) as described by Dubey and Desmonts²⁸ was used on serum samples and diluted 1:25 from infected mice whose brains were used for infecting cats in the kinetic experiment. Cats were bled on the day of infection and 35 days later. Their serum samples were tested for antibodies against *T. gondii* at a dilution of 1:25 by using the MAT.²⁸

Coprologic diagnosis of *T. gondii*-infective cats. Flotation of oocysts was used to concentrate oocysts in the upper fluid portion and thereby reduce the amount of potential PCR inhibitors found in feces.²⁹ The flotote was used for coprologic detection of *T. gondii* by microscopy, bioassay, and copro-PCR. Prior to oocyst flotation, fecal samples were washed repeatedly with double-distilled water. Oocyst flotation was carried out according to the procedure of Dubey and Beattie.¹ Sheather's solution (106 grams of glucose, 100 mL of double-distilled water, 0.8 mL of liquid phenol; specific gravity = 1.27) was used for oocyst flotation. One volume of Sheather's solution at least equal to that of the fecal sample was added to each sample, vortexed briefly, and centrifuged for 15 minutes at 1,000 × g. Flotates were further treated as described below to prepare them for each of the coprologic detection assays used.

Coprologic diagnosis by microscopy. Fecal samples were examined for sporulated or unsporulated *Toxoplasma* oocysts after flotation. Examination of oocysts was performed by using light microscopy. A wire loop (diameter = 6 mm) was used to touch the surface of the sample flotote after centrifugation, and the sample was examined at 400× magnification.

Coprologic diagnosis by bioassay. Fecal samples from the infected cats were collected at various times, and their fecal Sheather's floats were prepared according to the procedure of Dubey and Beattie.¹ Five milliliters of the stored flotote were washed twice and the pellet was suspended in a final volume of 1 mL of double-distilled water. Approximately 0.2 mL was individually introduced by gavage into each of three mice. Mice were bled before infection and 56 days later at the time they were killed. The serum samples were examined by MAT at a dilution of 1:25. A PCR was also performed on portions of brains from all mice, as described below, to identify infection with *T. gondii*. Identification of *T. gondii* tissue cysts by squash smears of brains was not used because PCR was expected to be more sensitive and objective.^{30,31} Mice were considered infected with *T. gondii* when PCR results were positive for *T. gondii* or when seroconversion occurred.

Coprologic diagnosis by PCR. DNA extraction. After Sheather's flotation of feces, supernatants were carefully removed into new tubes, approximately five volumes of double-distilled water was added to each sample, and the samples were centrifuged at 2,000 × g for 15 minutes. The pellets underwent oocyst disruption in 200 µL of buffer ASL in the QIAamp DNA Stool Mini Kit (Qiagen) at the disruption conditions selected as described above.

For DNA extraction after oocyst disruption, 1.2 mL of buffer ASL for DNA extraction was added according to the manufacturer's instructions with two modifications. Digestion with proteinase was carried out at 60°C for 1 hour instead of

10 minutes, and elution of DNA from spin columns was performed twice instead of once. The final elution volume was 200 μ L per sample. The DNA samples obtained by extraction were stored at -20°C until examined.

***Toxoplasma gondii* PCR.** Polymerase chain reactions containing *T. gondii*-specific primers TOX4 (5'-CGCTGCAGGGAGG AAGACGAAAGTTG-3') and TOX5 (5'-CGCTGCAGA CACAGTGCATCTGGATT-3') were used from the 5' and 3' ends of the 529-basepair repeated sequence,³² respectively. The PCR mixtures contained 0.2 mM of each primer, 100 μ M of dNTPs (Fermentas Inc., Hanover, MD), 60 mM Tris-HCl, pH 9.0, 15 mM $(\text{NH}_4)_2\text{SO}_4$, 2 mM MgCl_2 , and 1 unit of Biotaq (Bioline, Randolph, MA) per reaction. Amplification was performed on a PTC-150 Minicycler Thermocycler (MJ Research Inc., Waltham, MA) with initial denaturation for 7 minutes at 94°C , followed by 35 cycles for 1 minutes at 95°C , 1 minute at 60°C , 1 minute at 72°C , and a final incubation for 10 minutes at 72°C . The PCRs were carried out in 50- μ L mixtures containing 10 μ L of sample extract. The PCR amplification products were subjected to electrophoresis on 1.1% ethidium bromide agarose gels. A molecular mass 100-basepair size standard (New England Biolabs, Beverly, MA) size marker and a positive control containing purified DNA equivalent to 5 *T. gondii* tachyzoites were included. A negative control containing no DNA was also included by adding equivalent volumes of only double-distilled water.

Identification of the *T. gondii* DNA source (parasite stage) from fecal samples. We attempted to establish whether the copro-PCR was detecting parasitic DNA entirely of oocyst origin or non-encysted, intra-epithelial, and free parasitic forms. This distinction is important for determining whether an examined cat is potentially infective.

Given that prior to DNA extraction for the copro-PCR, steps of flotation and mechanical disruption of the oocysts were included; we investigated whether deleting these steps would enable differentiating encysted from non-encysted forms. Thus, the extraction method was modified to exclude the flotation and/or mechanical disruption steps, and the experiment included comparison of a fecal sample from an infected cat (copro-PCR positive) with samples of *T. gondii*-negative feces spiked with encysted forms (oocysts) or with non-encysted forms (tachyzoites) of *T. gondii*.

Oocysts (0, 100, and 200) were spiked into 200 μ L of negative feline feces. Tachyzoites (0, 100, and 500) were also added into negative feline feces. A fecal sample from an infected, copro-PCR-positive cat was taken from the kinetic study. A PCR was carried out for detecting *T. gondii* DNA and cat DNA as a control for indicating presence of cat epithelial cells.

Specifically, PCR for cat-specific DNA was performed to establish whether DNA from feline intestinal epithelial cells was retrieved from feces by applying harvest conditions with prior flotation before further routine extraction was conducted. It was important to establish whether intracellular *T. gondii* from sloughed infected feline epithelial cells in the feces would possibly be detected in other encysted amplified parasitic DNA. Specific primers for domestic cat DNA (*Felis catus*) based on mitochondrial DNA cytochrome b gene sequences, FC3-forward (5'-GATGAAACTTCGGCTCCCTT-3') and FC-5R-reverse (5'-CCGTCCCACATGTATGTACAGG-3') were used on positive *T. gondii* fecal extracts in reactions described.³³ Fragments of 212 basepairs were expected on examined gels.

RESULTS

Experimental infection of cats and examination of their feces collected daily for 35 days post-infection comprised the basis for the comparison of PCR with microscopy and bioassay for identification of potentially infective cats. Cats were infected by feeding with brains of seropositive mice that were also shown to be infected with *T. gondii* by PCR. Serum samples from three cats tested prior to infection by tissue cysts from brain material and 35 days thereafter by using MAT at a serum dilution of 1:25 showed seroconversion in all samples.

The results of the comparative examination of fecal samples from the experimentally infected cats examined by microscopy, bioassay, and copro-PCR are shown in Table 1. Samples were examined continuously from day 1 through day 5 post-infection and subsequently at time intervals of 1, 2, 3, or 6 days. After copronegativity was detected twice by all tests with fecal samples collected at days 25 and 28 post-infection, one additional test was carried out on day 35. This testing sequence enabled detection of oocyst shedding from day 2 through day 24 post-infection.

Although microscopy identified oocyst shedding by cats only occasionally (*T. gondii*-like oocysts were detected in only 8 of 42 samples tested during days 5–22 post-infection), results of bioassay and copro-PCR closely corresponded in detection consistency. In this regard and in the more sensitive detection by copro-PCR (where all three cats were positive in most cases than in the bioassay), copro-PCR was more sensitive and consistent than bioassay because it detected 27 (90%) of 30 experimentally infected cat fecal samples compared with 19 (70.4%) of 27 identified by bioassay (Table 1). Although these findings provide a comparative evaluation of the coprologic diagnostic tests used in terms of sensitivity and consistency, more tests are required to establish these findings by using different strains of *T. gondii*.

Positive PCR results for *T. gondii* were found in at least one of three mice brains on all but one (day 22 post-infection) groups tested. As shown in Table 1, after day 24 post-infection, all PCR results on brain extractions (brain copro-PCR) and results of serologic tests for *T. gondii* in mice were negative.

TABLE 1
Comparison of PCR, bioassay, and microscopy after flotation of feces from *Toxoplasma gondii* infection of uninfected cats (three cats/test/day)*

Days post-infection	Copro-PCR	Bioassay		Microscopic
		Brain PCR	Serologic†	
1	–	–	–	–
2	+++	+++	+++	+++
3	+++	+++	+	–
4	++	ND	ND	–
5	+++	+++	++	++
11	+++	+	+	++
12	+++	+	+	–
15	+++	+++	+++	–
21	+++	++	++	–
22	+++	–	+	+
24	+	+	++	–
25	–	–	–	–
28	–	–	–	–
35	–	–	–	–

* Copro-PCR = coprologic-polymerase chain reaction; – = all cats were negative; + = one cat was positive; ND = not done.

† Number of cats that showed seroconversion for at least one of three mice fed oocysts by gavage.

A good correspondence was found between positive *T. gondii* IgG serologic results and positive brain PCR results in the tested mice.

Presence of oocysts in cat fecal material can be considered to indicate infectivity if one considers that oocysts sporulate as soon as one day after being shed and that oocysts have great environmental resilience for long periods.¹ Their presence can be determined directly by microscopy (albeit without species specificity) and by bioassay after treating them with sulfuric acid, in which infectivity is demonstrated by definition of a bioassay. In the case of copro-PCR, asserting the presence of oocysts as a source of a positive result was required, given the known presence of sloughed parasitized epithelial cells or free non-encysted forms.³⁴ This test was conducted by applying different DNA harvest conditions and using unencysted forms as a control.

The effect of various DNA harvest conditions on *T. gondii* copro-PCR positivity of known encysted or non-encysted forms, in parallel with an unknown sample, is shown in Table 2. Positive copro-PCR results were obtained in oocyst-spiked feces only after flotation and mechanical disruption were performed prior to further extraction. When tachyzoites were spiked into negative feces, copro-PCR results were positive whether or not mechanical forces applied, but only if flotation was not applied prior to the DNA extraction process. This finding indicates that copro-PCR-positive results for *T. gondii* were not detected for extracts of the upper portion when flotation was applied in tachyzoite-spiked feces. When extractions were performed on fecal samples from infected cats (five samples) on days in which cats had positive copro-PCR results for *T. gondii*, extraction required flotation and mechanical disruption of oocysts to give positive results for parasite DNA.

Cat-specific primers amplified cat-specific DNA in all feline fecal samples after fecal flotation. Therefore, the presence of cat DNA, which suggests the presence of gut epithelial cells in fecal samples, is irrelevant to positivity of copro-PCR. The method stands out as one which is suitable for differentially identifying oocysts.

DISCUSSION

If one considers the comparative sensitivity of coprodiagnostic methods used for identification of cats infected with *T. gondii*, the sensitivity of the copro-PCR is superior to the sensitivity of microscopic detection of oocysts as reported by other investigators: 1,000 oocysts per gram of feces detected by Dubey JP (unpublished data) and 250 oocysts per gram of feces detected by Rothe and others.³⁵ Microscopic detection of oocysts can at best identify oocysts as *Toxoplasma*-like.³⁶ In the present study, in which mice were infected with mouse brain tissue containing the VEG strain of *T. gondii*, it was not

possible to consistently detect *T. gondii* oocysts by microscopy compared with copro-PCR or bioassay. Clearly, detection sensitivity by microscopy is low and varies depending on those performing the test. The copro-PCR, by the nature of the technology, is highly reproducible and objective, as suggested by results obtained when multiple samples were examined in this study and in other spiking experiments.²⁷

By comparison, the bioassay for testing mice, although potentially able to detect one oocyst per infective dose^{24,37} is cumbersome, expensive, and usually requires six weeks before tissue cysts can be observed.²⁷ Therefore, it cannot be considered suitable for large-scale screening. Because all animals used in the bioassay may not all be infected,²⁴ a number of mice would be necessary for each result. Conversely, the copro-PCR can be considered more suitable for large-scale screening because of ease in handling of a large number of samples and the short time required for obtaining results with high detection sensitivity, specificity, and reproducibility.

PCR has been shown to be more sensitive for detection of other organisms when compared to other methods.³¹ Although infected cats usually shed oocysts for approximately 2–3 weeks,^{1,20} it was possible to obtain positive bioassay and copro-PCR results from fecal samples of experimentally infected cats for as long as 22 days post-infection (Table 1). After infection by meat containing *T. gondii* tissue cysts, most cats will shed oocysts between 3–10 days post-exposure.²⁷ It was possible to detect *T. gondii*-specific DNA in feline fecal extractions as early as two days post-exposure after feeding *T. gondii*-infected mouse brain material to cats. Mice that were infected orally with fecal float material showed positive PCR results for brain extractions or seroconverted consistently on or after two days post-infection of cats (Table 1). These findings were observed until 24 days post-infection, after which time mice showed no evidence of infection with *T. gondii* (Table 1). Positive PCR results for brain tissue of bioassayed mice corresponded with their positive *T. gondii* IgG serologic results as far as identification of at least one infected cat per day examined. Copro-PCR results correlated with bioassay results and the copro-PCR was more sensitive (Table 1). The copro-PCR method was far superior to microscopy by all criteria tested.

Feces of cats infected with *T. gondii* may contain infected enteric epithelial cells³⁴ that are periodically shed, and these cells are present in the intestinal fecal contents because enteric sexual forms of *Toxoplasma* exist before oocysts are shed and persist in intestines of cats for several months after infection.³⁸ Infected cells would float in the Sheather's solution, as was shown in experiments on copro-PCR extracts using cat-specific primers (Table 2). Thus, these cells would be available for further extraction in the test protocol used. However, in the experiment in which different DNA harvesting conditions were applied to determine *T. gondii* copro-PCR positivity, 529-basepair PCR results specific for *T. gondii* were not seen when mechanical vortexing and freezing-thawing steps were not applied to the samples in the test protocol (Table 2), a critical stage of the initial disruption of oocysts prior to routine DNA extraction. Thus, the DNA extracted was not from sloughed intracellular parasites within the stool but from oocysts. Therefore, the high sensitivity of the copro-PCR cannot be caused by infected epithelial cells. Also, unencysted forms (free bradyzoites, sporozoites, and tachyzoites) would not have been isolated in the pre-extraction preparation because it was observed that DNA from tachyzoites spiked

TABLE 2
Effect of DNA isolation conditions on detection of *Toxoplasma gondii* by coprologic-polymerase chain reaction*

Flotation/ disruption	<i>T. gondii</i> oocyst spiked feces	<i>T. gondii</i> tachyzoite spiked feces	<i>T. gondii</i> infected cat feces	Is cat DNA present? (cat ribosomal DNA primers used) ³⁵
+/+	+	–	+	+
+/-	–	–	–	+
-/+	–	+	ND	ND
-/-	–	+	ND	ND

* ND = not done.

TABLE 3
Summary of comparison of coprodection tests for *Toxoplasma gondii**

Characteristic	Copro-PCR	Bioassay	Microscopy
TRCP*	2–24	2–24	2, 5, 11, 22
Fecal samples, no. positive/no. tested (%)	27/30 (90)	19/27 (70.4)	8/30 (27)
Coprologic positivity per days tested, (%) within maximum positivity (range)	10/10 (100)	9/9 (100)	4/10 (40)
No. positive fecal samples/days tested within TRCP (average/day out of 3 tested)	27/10 (2.7)	19/9 (2.1)	8/10 (0.8)
Specificity	Species specific ²⁷	Species specific ¹	<i>T. gondii</i> -like ^{1,23}
Sensitivity	1–2 oocysts/0.2 gram of feces ²⁷	1 oocyst ³⁷	250 oocysts ³⁵
Parasitic stage detected	Sporulated/unsporulated oocyst (Table 2)	Sporulated oocyst ¹	Sporulated/unsporulated oocyst
Time required before diagnosis/ test	6–7 hours	6–8 weeks	0.5 hours

* Copro-PCR = coprologic–polymerase chain reaction; TRCP = time range of copropositivity, i.e., day post-infection started to day post-infection ended.

into parasite-negative feces did not produce positive PCR results when the complete test protocol was applied (Table 2). Specifically, PCR signals were not detected in tachyzoite-spiked fecal samples if flotation in Sheather's solution was applied (Table 2).

It can be concluded from these experiments that copro-PCR results were specific for oocysts and not for other non-encysted parasitic forms. This finding indicates that the copro-PCR is suitable for identification of infective cats. This detection was previously possible only by bioassay, which detects infective forms. The difference between these two tests in detecting infective cats lies in the last step preceding the bioassay (but not necessarily in PCR performed on fresh fecal material), namely sporulation of oocysts. Essential differences in the three coprodection methods available for *T. gondii* are shown in Table 3.

Detecting infection in bioassayed mice was successful when PCR was used with brain extracts. Positive serologic results for *T. gondii* and identification of tissue cysts in unstained impression smears are used routinely to detect *T. gondii* infection in mice by bioassay. Microscopic examination of tissue sections has been shown to be less sensitive than PCR, even for sections that have been stained.³⁰ Thus, we recommend that PCR testing of brain extracts be used for identification of infected bioassayed animals, in addition serologic tests, especially by less experienced personnel.

Because oocyst ingestion in animals and humans may induce more severe disease than that derived from infected meat,^{27,39} development of a sensitive and specific copromolecular assay to detect infective cats is important and integral for epidemiologic studies and for assessing potential danger of shedding cats for pet owners. This sensitive, specific, and robust assay reduces the misclassification of potentially infective cats and the requirement for specialized personnel for epidemiologic surveys. Furthermore, it may be used for the assessment of drugs used to prevent oocyst shedding by *T. gondii*-infected cats⁴⁰ or for monitoring the effectiveness of potential vaccines that would limit oocyst shedding in cats.⁴¹

The results of the present study demonstrate the sensitivity, consistency, and infective-stage specificity of the copro-PCR. When combined with the previously demonstrated test sensitivity and specificity,²⁷ it can be concluded that the copro-PCR is at least as sensitive, consistent, and infective stage-specific as the bioassay. Therefore, we propose that the copro-PCR be used as the new gold standard for determining potential cat infectivity.

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Authors' address: Harold Salant, Dan T. Spira, and Joseph Hamburger, Department of Parasitology, Hebrew University-Hadassah Medical School, PO Box 12272, Jerusalem, 91120, Israel, E-mail: tsvis@ekmd.huji.ac.il.

REFERENCES

- Dubey JP, Beattie CP, 1988. *Toxoplasmosis of Animals and Man*. Boca Raton, FL: CRC Press, 1–220.
- Montoya JG, Liesenfeld O, 2004. Toxoplasmosis. *Lancet* 363: 1965–1976.
- Brown AS, Schaefer CA, Quesenberry CP Jr, Liu L, Babulas VP, Susser ES, 2005. Maternal exposure to toxoplasmosis and risk of schizophrenia in adult offspring. *Am J Psychiatry* 162: 767–773.
- Holland GN, 2003. Ocular toxoplasmosis: a global reassessment. Part 1: epidemiology and course of disease. *Am J Ophthalmol* 136: 973–988.
- Remington JS, McLeod R, Thulliez P, Desmonts G, 2006. *Toxoplasmosis*. Remington JS, Klein JO, Wilson CB, Baker CJ, eds. *Infectious Diseases of the Fetus and Newborn Infant*. Philadelphia, PA: Elsevier Saunders, 947–1091.
- Carne B, Bissuel F, Ajzenberg D, Bouyane R, Aznar C, Demar M, Bichat S, Louvel D, Bourbigot AM, Peneau C, Neron P, Dardé ML, 2002. Severe acquired toxoplasmosis in immunocompetent adult patients in French Guiana. *J Clin Microbiol* 40: 4037–4044.
- Luft BJ, Remington JS, 1992. Toxoplasmic encephalitis in AIDS. *Clin Infect Dis* 15: 211–222.
- Porto AM, Amorim MM, Coelho IC, Santos LC, 2008. Serologic profile of toxoplasmosis in pregnant women attended at a teaching-hospital in Recife. *Rev Assoc Med Bras* 54: 242–248.
- Olariu TR, Cretu O, Darabus GH, Marincu I, Jurovits O, Erdelean V, Tirnea L, Neghina R, Iacobiciu I, Petrescu C, Koreck A, 2008. Screening for *Toxoplasma gondii* antibodies among women of childbearing age, in Timis County, Romania. Abstracts, 13th International Congress of Infectious Diseases, Kuala Lumpur, Malaysia, June 2008, Abstract 489.
- Youssefi MR, Sefidgar AA, Mostafazadeh A, Mahdavi Omran S, 2007. Serologic evaluation of toxoplasmosis in matromonial women in Babol, Iran. *Pak J Biol Sci* 10: 1550–1552.
- Nissapatorn V, Noor Azmi MA, Cho SM, Fong MY, Init I, Roheia M, Khairul Anuar A, Quek KF, Latt HM, 2003. Toxoplasmosis: prevalence and risk factors. *J Obstet Gynaecol* 23: 618–624.
- Adou-Bryn KD, Ouhon J, Nemer J, Yapo CG, Assoumou A, 2004. Serological survey of acquired toxoplasmosis in women of

- childbearing age in Yopougon (Abidjan, Côte d'Ivoire). *Bull Soc Pathol Exot* 97: 245–348.
13. Zufferey J, Di Mito C, Auckenthaler R, 2007. Evaluation of the new Vidia toxoplasmosis IgG and IgM assays in women of childbearing age. *Clin Microbiol Infect* 13 (Suppl. 1): 124.
 14. Dubey JP, Jones JL, 2008. *Toxoplasma gondii* infection in humans and animals in the United States. *Int J Parasitol* 38: 1257–1278.
 15. Cook AJ, Gilbert RE, Buffolano W, Zufferey J, Petersen E, Jenum PA, Foulon W, Semprini AE, Dunn DT, 2000. Sources of *Toxoplasma* infection in pregnant women: European multi-centre case-control study. European Research Network on Congenital Toxoplasmosis. *BMJ* 321: 127–128.
 16. Tenter AM, Heckeroth AR, Weiss LM, 2000. *Toxoplasma gondii*: from animals to humans. *Int J Parasitol* 30: 1217–1258.
 17. Conrad PA, Miller MA, Kreuder C, James ER, Mazet J, Dabritz H, Jessup DA, Gulland F, Grigg ME, 2005. Transmission of *Toxoplasma*: clues from the study of sea otters as sentinels of *Toxoplasma gondii* flow into the marine environment. *Int J Parasitol* 35: 1155–1168.
 18. Dubey JP, 1976. Reshedding of *Toxoplasma* oocysts by chronically infected cats. *Nature* 262: 213–214.
 19. Davis SW, Dubey JP, 1995. Mediation of immunity to *Toxoplasma gondii* oocyst shedding in cats. *J Parasitol* 81: 882–886.
 20. Dubey JP, 1986. Toxoplasmosis in cats: a review. *Feline Pract* 16: 12–45.
 21. Dubey JP, 1995. Duration of immunity to shedding of *Toxoplasma gondii* oocysts by cats. *J Parasitol* 81: 410–415.
 22. Dubey JP, Frenkel JK, 1976. Feline toxoplasmosis from acutely infected mice and the development of *Toxoplasma* cysts. *J Protozool* 23: 537–546.
 23. Schares G, Herrmann DC, Beckert A, Schares S, Hosseinienejad M, Pantchev N, Globokar Vrhovec M, Conraths FJ, 2008. Characterization of a repetitive DNA fragment in *Hammondia hammondi* and its utility for the specific differentiation of *H. hammondi* from *Toxoplasma gondii* by PCR. *Mol Cell Probes* 22: 244–251.
 24. Dubey JP, 1996. Infectivity and pathogenicity of *Toxoplasma gondii* oocysts for cats. *J Parasitol* 82: 957–961.
 25. Dabritz HA, Miller MA, Atwill ER, Gardner IA, Leutenegger CM, Melli AC, Conrad PA, 2007. Detection of *Toxoplasma gondii*-like oocysts in cat feces and estimates of the environmental oocyst burden. *J Am Vet Med Assoc* 231: 1676–1684.
 26. Schares G, Vrhovec MG, Pantchev N, Herrmann DC, Conraths FJ, 2008. Occurrence of *Toxoplasma gondii* and *Hammondia hammondi* oocysts in the faeces of cats from Germany and other European countries. *Vet Parasitol* 152: 34–45.
 27. Salant H, Markovics A, Spira DT, Hamburger J, 2007. The development of a molecular approach for coprodiagnosis of *Toxoplasma gondii*. *Vet Parasitol* 146: 214–220.
 28. Dubey JP, Desmonts G, 1987. Serological responses of equids fed *Toxoplasma gondii* oocysts. *Equine Vet J* 19: 337–339.
 29. Wolffs P, Knutsson R, Norling B, Rådström P, 2004. Rapid quantification of *Yersinia enterocolitica* in pork samples by a novel sample preparation method, flotation, prior to real-time PCR. *J Clin Microbiol* 42: 1042–1047.
 30. Garcia JL, Gennari SM, Machado RZ, Navarro IT, 2006. *Toxoplasma gondii*: detection by mouse bioassay, histopathology, and polymerase chain reaction in tissues from experimentally infected pigs. *Exp Parasitol* 113: 267–271.
 31. James GS, Sintchenko VG, Dickeson DJ, Gilbert GL, 1996. Comparison of cell culture, mouse inoculation, and PCR for detection of *Toxoplasma gondii*: effects of storage conditions on sensitivity. *J Clin Microbiol* 34: 1572–1575.
 32. Homan WL, Vercammen M, De Brackeleer J, Verschueren H, 2000. Identification of a 200- to 300-fold repetitive 529 bp DNA fragment in *Toxoplasma gondii*, and its use for diagnostic and quantitative PCR. *Int J Parasitol* 30: 69–75.
 33. Kwose W, Masuda R, Tatana M, 2005. Feral DNA analysis for identifying species and sex of sympatric carnivores: a non invasive method for conservation on the Tsushima Islands, Japan. *J Hered* 96: 688–697.
 34. Feng X, Milhausen M, 1999. Localization of *Toxoplasma gondii* in feline intestinal tissue using PCR. *J Parasitol* 85: 1041–1046.
 35. Rothe J, McDonald PJ, Johnson AM, 1985. Detection of *Toxoplasma* cysts and oocysts in an urban environment in a developed country. *Pathology* 17: 497–499.
 36. Pena HF, Soares RM, Amaku M, Dubey JP, Gennari SM, 1996. *Toxoplasma gondii* infection in cats from Sao Paulo State, Brazil: seroprevalence, oocyst shedding, isolation in mice, and biologic and molecular characterization. *Res Vet Sci* 81: 58–67.
 37. Dubey JP, 1997. Tissue cyst tropism in *Toxoplasma gondii*: a comparison of tissue cyst formation in organs of cats, and rodents fed oocysts. *Parasitology* 115: 15–20.
 38. Dubey JP, 1977. Persistence of *Toxoplasma gondii* in the tissues of chronically infected cats. *J Parasitol* 63: 156–157.
 39. de Moura L, Bahia-Oliveira LM, Wada MY, Jones JL, Tuboi SH, Carmo EH, Ramalho WM, Camargo NJ, Trevisan R, Graça RM, da Silva AJ, Moura I, Dubey JP, Garrett DO, 2006. Waterborne toxoplasmosis, Brazil, from field to gene. *Emerg Infect Dis* 12: 326–329.
 40. Malmasi A, Mosallanejad B, Mohebbi M, Sharifian Fard M, Taheri M, 2009. Prevention of shedding and re-shedding of *Toxoplasma gondii* oocysts in experimentally infected cats treated with oral clindamycin: a preliminary study. *Zoonoses Public Health* 56: 102–104.
 41. Mateus-Pinilla NE, Dubey JP, Choromanski L, Weigel RM, 1999. A field trial of the effectiveness of a feline *Toxoplasma gondii* vaccine in reducing *T. gondii* exposure for swine. *J Parasitol* 85: 855–860.