Comparative aspects of laboratory testing for the detection of *Toxoplasma gondii* and its differentiation from *Neospora caninum* as the etiologic agent of ovine abortion

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Abstract. Histologic examination of aborted material is an essential component in the diagnosis of ovine toxoplasmosis. However, the detection of *Toxoplasma gondii* in histologic sections, and its differentiation from the closely related protozoan *Neospora caninum*, is challenging. We developed a chromogenic in situ hybridization (ISH) assay for the identification of *T. gondii* in paraffin-embedded tissue samples. We examined retrospectively the archived placental tissue of 200 sheep abortion submissions for the presence of *T. gondii* by immunohistochemistry (IHC), ISH, and real-time PCR (rtPCR). All placental samples that tested positive for *T. gondii* by rtPCR (9 of 200) were also positive by IHC, with inconclusive IHC staining in an additional 7 rtPCR-negative cases. Further testing for *N. caninum* of all 200 placentas by rtPCR revealed 7 *Neospora*-positive cases. *T. gondii* ISH was positive in 4 of 9 IHC-positive samples and 1 of the 7 *N. caninum* rtPCR-positive samples. Real-time PCR was used as the reference standard for specificity and sensitivity calculations regarding placenta samples. Specificity of ISH and IHC was 99% and 96–100%, respectively. The sensitivity of ISH (44%) was quite low compared to IHC (100%). The exclusive use of ISH for the detection of *T. gondii*, and thus for the diagnosis of ovine toxoplasmosis, was not acceptable. However, combined with rtPCR, both ISH and IHC can be useful detection methods to improve histologic evaluation by visualizing the parasite within tissue sections.

Key words: immunohistochemistry; in situ hybridization; real-time PCR; sheep abortion; toxoplasmosis.

Introduction

Toxoplasma gondii is an obligate intracellular protozoan parasite that infects a broad range of warm-blooded animals, including humans, worldwide.^{13,47} Moreover, it has been recognized as one of the main causes of infectious ovine abortion in numerous countries worldwide.¹³ Clinical ovine toxoplasmosis occurs following primary infection of a pregnant ewe as a result of the ingestion of sporulated oocysts.^{7,32} Depending on the stage of pregnancy at which the ewe becomes infected, early embryonic death and resorption, mummification, abortion, stillbirth, or neonatal death may occur.¹³

To identify *T. gondii* as the causative agent in ovine abortions, histologic evaluation of fetal and placental tissue is an essential component of the pathology examination.³⁴ Therefore it is important to recognize the type of lesion caused by the parasite, given that it may be difficult to observe *T. gondii* stages (i.e., tachyzoites and tissue cysts) in hematoxylin and eosin (H&E)-stained tissue sections.¹³ Characteristic lesions induced by *T. gondii* consist mainly of multifocal necrosis and mineralization with variable, predominantly nonsuppurative inflammation in placental cotyledons as well as multifocal

necrosis and gliosis in the brain of the fetus.^{13,34} However, similar lesions can also be observed in ovine abortions induced by *Neospora caninum*, a closely related cyst-forming apicomplexan parasite, and thus specific techniques are required to differentiate those 2 pathogens.^{5,6,14,31,37}

Chromogenic in situ hybridization (ISH) targets specific nucleotide sequences and allows localization of organisms within microscopic lesions.³⁸ ISH has been used successfully to identify not only tissue stages of *T. gondii*^{35,41} and other protozoa, but also a variety of other pathogenic agents.³⁸ However, to our knowledge, the method has not yet been reported for the detection of *T. gondii* in ovine abortion material.

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Recognized methods for the identification of *T. gondii* in aborted fetuses and placental tissue are immunohistochemistry (IHC) and PCR.^{1,34} IHC is valuable for the visualization of *T. gondii* stages and antigenic residues within ovine tissue sections, even in tissue exhibiting a degree of decomposition, which typically is present in abortion material.⁴⁸ Moreover, conventional and real-time PCR (rtPCR)-based assays have been developed for the detection of *T. gondii* in ovine abortions.^{22,23,29,39,42} Although rtPCR has very high sensitivity and specificity for pathogen detection, in contrast to ISH or IHC, it does not provide information about the distribution of the pathogen in the infected tissue.

Our objective was to determine whether ISH is a practical test for the detection of *T. gondii* in histologic sections of placental tissue from field cases of ovine abortion. We compared ISH results to those obtained by standard methods (histopathology, IHC, and rtPCR) with respect to applicability, as well as diagnostic sensitivity and specificity, using rtPCR as the reference method. We also took into consideration *N. caninum* as an important differential causative agent in cases of ovine abortion.

Materials and methods

Ovine abortions

We performed our study retrospectively on formalin-fixed, paraffin-embedded (FFPE) tissue samples from ovine abortions, stillbirths, or neonatal deaths submitted for autopsy to the Department of Veterinary Pathology of the Bavarian Health and Food Safety Authority (Erlangen, Germany) between 2003 and 2018. We examined 200 abortions in which FFPE placental tissue had at least one cotyledon, which yielded 151 abortion submissions with at least 1 corresponding fetus (e.g., twins or triplets), and 49 submissions of fetal membranes without fetus from 107 flocks in Bavaria (Germany). Each submission was considered a separate case.

Each case was subjected to a routine pathology examination at the time of submission, including macroscopic description and documentation of fetal crown-rump length. Furthermore, laboratory tests for the detection of abortifacient pathogens were conducted. Bacterial cultures of placenta and, if available, liver, kidney, lung, and stomach content, were performed in every case. For the detection of *Chlamydia* spp. and *Coxiella* spp., Stamp stains of placental smears (187 of 200), partly complemented by a rapid immunoassay for the detection of *Chlamydia* spp. antigen (123 of 200), and/or rtPCR (*Chlamydia* spp.: 35 of 200; *Coxiella* spp.: 47 of 200) were utilized. PCR was further used for the detection of viral agents (Schmallenberg virus, bluetongue virus, pestivirus) in 21 cases exhibiting pathologic changes indicating infection with those viruses. In cases exhibiting protozoal-like lesions, PCR or IHC were carried out for the detection of *N. caninum* or *T. gondii*, respectively.

All placental specimens were initially examined by IHC and ISH for the presence of *T. gondii* without respect to the diagnosis or histopathologic findings recorded in the autopsy report. Real-time PCR was performed afterward to verify IHC and ISH results. In *T. gondii*-positive cases, tissue sections of all available fetal organs were investigated histologically and examined by IHC and ISH for the presence of *T. gondii* tissue stages. Moreover, the placental samples were examined by rtPCR for the presence of *N. caninum*, and available fetal organs were further examined histologically in positive cases.

Histopathology

Tissue samples of placenta, and if available, fetal brain, lung, heart, skeletal muscle, and liver, were collected during autopsy, fixed in 10% neutral-buffered formalin, processed routinely, and sections stained with H&E for histologic examination.

IHC

IHC was performed on deparaffinized and rehydrated 3- μ m tissue sections using a rabbit anti-*T. gondii* polyclonal antibody (BioGenex) in 1:1,000 dilution, incubated overnight. The primary polyclonal antibody that we used had been utilized in previous studies for the detection of *T. gondii* on FFPE placenta sections.^{27,45,46} The staining was carried out at room temperature, and washing steps between incubation times were performed with a Tris-based washing buffer (DCS Diagnostics). Before labeling, antigen retrieval was performed (Protease XXIV; BioGenex) for 10 min and, to prevent background staining, the slides were incubated with a blocking reagent (DCS Diagnostics) for 8 min. Bound primary antibody was detected with an anti-rabbit/anti-mouse alkaline phosphatase-conjugated polymer detection system (PermaRed chromogen, SuperVision Red 2 AP kit; DCS Diagnostics). Finally, the slides were counterstained with Mayer hematoxylin for examination by light microscopy.

ISH

Probe design. Two oligonucleotide probes targeting the 18S and the 28S ribosomal RNA (rRNA) for the detection of *T. gondii* were designed based on homology studies on all available GenBank sequences of both *T. gondii* rRNA genes. The following sequences complementary to regions of complete homology among *T. gondii* were selected as probe sequences: 5'-CCACACAATGAAGTGTGGAGAAATCCAGAAGG-3' (18S rRNA) and 5'-ACAAGTCAACAGCTCGGAAAGAGCAGTTG-3' (28S rRNA). Both probe sequences were further submitted to BLAST analysis (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) to check for homologies with other closely related cyst-forming coccidia and to exclude unintentional cross-reactivity with other organisms.

There was no nucleotide mismatch to the corresponding 18S rRNA gene sequence of *Hammondia hammondi* and 1 mismatch to the 18S rRNA gene sequence of *N. caninum* and *Hammondia heydorni*; there were 1, 2 and 3 mismatches to the corresponding 28S rRNA gene sequence of *H. hammondi*, *H. heydorni*, and *N. caninum*, respectively. Therefore, cross-hybridization could not be ruled out completely by in silico analysis. With other closely related protozoa, such as *Besnoitia besnoiti*, there were 5 or more mismatches to corresponding gene sequences, which was considered to be an adequate difference to prevent cross-hybridization. Moreover, both *Besnoitia* spp. and *Hammondia* spp. are not considered to be relevant causes of disease or abortion in sheep.^{16,21} Thus, we evaluated the specificity of both ISH probes in situ only for *N. caninum* as a potential differential etiology. We evaluated the specificity of those ISH probes on canine brain tissue containing *N. caninum* tissue cysts; no cross-reactivity was found. The sensitivity of the oligonucleotide probes was validated in situ by staining PCR- and IHC-positive tissue sections of a squirrel monkey liver and lung containing numerous developmental stages of *T. gondii*. To achieve a staining intensity comparable to IHC, a combination of both ISH probes was used.

ISH. ISH was performed based on a protocol described previously,⁹ with slight modifications. Except for the hybridization step, the staining was carried out at room temperature. Briefly, 3- μ m FFPE sections were deparaffinized and rehydrated. Proteolytic treatment was performed (Protease XXIV; BioGenex) for 10 min. The slides were rinsed with distilled water, dehydrated in ethanol, and air-dried. Hybridization mixture (100 μ L) composed of 50 μ L of deionized formamide (AppliChem), 20 μ L of 20 $\times$ standard sodium citrate (SSC), 11 μ L of distilled water, 10 μ L of dextran sulfate (50%, w/v; Carl Roth), 5 μ L of herring sperm DNA (50 μ g/mL; Invitrogen), 2 μ L of Denhardt solution (50 $\times$; AppliChem), and 1 μ L of each of the two 3'-end digoxigenin-labeled *T. gondii* probes (Eurofins Genomics) at a concentration of 1 μ g/ μ L, was applied on the slides and covered with a coverslip. The slides were incubated at 95°C for 6 min and then placed in an incubator at 40°C overnight for hybridization. On the second day, the sections were stringently washed sequentially in 2 $\times$ SSC, 1 $\times$ SSC, and 0.1 $\times$ SSC (10 min each). Hybridized probes were detected by incubating the slides with anti-digoxigenin-alkaline phosphatase Fab fragments (1:100; Roche Diagnostics) for 60 min. After washing in Tris-buffered saline (TBS; pH 7.5), the binding of Fab fragments was visualized by using the color substrates BCIP (5-bromo-4-chloro-3-indolyl phosphate) and NBT (4-nitro blue tetrazolium chloride; Roche Diagnostics), for 60 min in the dark. Levamisole was added to avoid nonspecific alkaline phosphatase activity. The staining reaction was stopped by placing the slides in Tris-EDTA buffer (pH 8.0) for 10 min. Finally, the slides were counterstained with Mayer hematoxylin and mounted under coverslips using an aqueous

mounting medium (Aquatex; Merck) for examination by light microscopy.

Tissue of a squirrel monkey liver containing numerous parasite tachyzoites that tested positive for *T. gondii* by rtPCR was used as a positive control for IHC and ISH.

DNA extraction and rtPCR

Four-to-eight sections, 5- μ m thick, from the FFPE placental tissue of each case were placed separately into 1.5-mL tubes. To avoid DNA contamination during collection, the microtome was cleaned and decontaminated (Sagrotan Schimmelfrei; Reckitt Benckiser),²⁰ and microtomic blades were changed between cases. Following the manufacturer's instructions, the FFPE sections were deparaffinized with xylol and ethanol. DNA was isolated (DNeasy blood and tissue kit; Qiagen). To increase the yield, purified DNA was eluted with 100 μ L of elution buffer. *T. gondii*-negative ruminant FFPE tissue samples were included as sample processing controls in each extraction run.

The extracted DNA was amplified (ID Gene *Toxoplasma gondii* duplex; IDvet) targeting the *B1* gene for the detection of *T. gondii*. PCR was performed in a total volume of 13 μ L with 8 μ L of master mix and 5 μ L of DNA. A thermocycler (LightCycler 96; Roche Diagnostics) was used with the following cycling conditions: 95°C for 10 min, and 40 cycles of a 2-step PCR consisting of 95°C for 15 s and 60°C for 60 s. Each amplification run included *T. gondii* DNA as positive control template and water as negative control template.

For the detection of *N. caninum*, samples were analyzed by rtPCR as described previously.¹⁰ Primers and probe were synthesized commercially (Eurofins Genomics). The reaction mixture (25 μ L) contained 5 μ L of extracted DNA, 800 nM of each primer, 100 nM of TaqMan probe, and 12.5 μ L of commercial master mix (QuantiTect probe PCR kit; Qiagen). The amplification program included an initial activation step at 95°C for 15 min, followed by 45 cycles of denaturation at 94°C for 15 s, and annealing at 60°C for 60 s.

Statistical analysis

Statistical analysis was performed using the computing environment R (v.3.5.1; <https://www.r-project.org>). Sensitivity, specificity, and predictive values, including their 95% CIs, were calculated for IHC and ISH using rtPCR as the reference method. The agreement between IHC and PCR and between ISH and PCR was evaluated using the Cohen kappa statistic.⁴⁹

Results

Detected pathogens associated with ovine abortions

A potential abortion-inducing infectious agent was identified in 123 of 200 (61.5%) cases (Table 1). Among them, 8

Table 1. Detected abortifacient agents in ovine abortions, 2003–2018.

Detected abortifacients	No. of positive cases*	Positive cases (%)
Infectious†	123 (200)‡	61.5
Bacterial	113 (200)	56.5
<i>Chlamydia</i> spp.	84	42
<i>Coxiella</i> spp.	8	4
<i>Campylobacter</i> spp.	5	2.5
<i>Listeria</i> spp.	2	1
<i>Salmonella</i> spp.	1	0.5
Other bacteria	13	6.5
Viral	2 (21)§	9.5
Parasitic	16 (200)	8
<i>Toxoplasma gondii</i>	9	4.5
<i>Neospora caninum</i>	7	3.5
Unknown‡	77 (200)	38.5

* Numbers in parentheses are the number of cases examined.

† Cases with an etiologic diagnosis.

‡ Eight cases with multiple pathogens identified.

§ Only 18 of these cases were tested for Schmallenberg virus (SBV), 4 for pestivirus, and 2 for bluetongue virus. Infection with SBV was detected in 2 cases.

‡ Cases in which no specific abortion-inducing infectious agent was detected; includes 36 cases with placentitis, indicating an infectious cause.

exhibited double infections. There was no evidence of immunosuppression in those cases. In one case, coinfection by *Chlamydia* spp. and *T. gondii*, and in 2 cases, coinfection by *Chlamydia* spp. and *N. caninum*, was present. In the other 5 cases, coinfection by 2 different bacterial agents was present.

Examination of ovine abortions with special emphasis on *T. gondii*

Positive results were obtained in 10 of 200 examined placental samples with at least 1 of the utilized methods for the detection of *T. gondii* (Table 2). However, infection with *T. gondii* was confirmed in only 9 of those 10 cases by the reference method (rtPCR), whereas the last remaining case tested positive for *N. caninum* by rtPCR.

According to the autopsy reports, 1 of 9 *T. gondii*-positive submissions exhibited characteristic gross lesions consisting of small, yellow-white foci of necrosis within placental cotyledons. None of the corresponding fetuses had any specific macroscopic alteration.

Histologically, variable multifocal necrotizing placentitis with some areas of dystrophic mineralization was observed in the cotyledons of all 9 *T. gondii*-positive placentas (Fig. 1A, B). Characteristic protozoal lesions (multifocal necrosis and gliosis; Fig. 1C) were found in the brains of 6 of 8 corresponding fetuses. In other fetal tissue samples (heart, lung, liver, and skeletal muscle), no inflammatory changes indicating a protozoal infection were observed

Table 2. Comparison of immunohistochemistry (IHC), in situ hybridization (ISH), and real-time PCR (rtPCR) for the detection of *Toxoplasma gondii* in ovine placental tissue.

Case	Placenta		
	IHC	ISH	rtPCR
1	+	+	+
2	+	+	+
3	+	+	+
4	+	+	+
5	+	–	+
6	+	–	+
7	+	–	+
8	+	–	+
9	+	–	+
10*	–	+	–
11–17	±	–	–

+ = positive test result; – = negative test result; ± = inconclusive test result.

* Case tested positive for *Neospora caninum* by rtPCR.

(Table 3). Furthermore, no tissue cysts or tachyzoites were seen in any H&E-stained tissue sections.

All 9 placental samples that tested positive for *T. gondii* by rtPCR were also positive by IHC (Table 2). Positive immunohistochemical labeling detected a large amount of *T. gondii* antigen, identifiable by distinct, pink-red staining, primarily associated with areas of focal necrosis within the placental cotyledons (Fig. 1D). Tachyzoite-like, round-to-oval structures, were detected singly or in clusters. In intact organisms, especially in tachyzoite clusters, a fine granular structure could be observed. In the more well-preserved tissue sections, tachyzoites were located intracellularly in trophoblast cells in areas with largely intact placental structure (Fig. 1E). In several of the 200 abortion cases, nonspecific background staining was detected, especially in degenerate or in peripheral areas of the IHC-stained placental sections, and an interpretation could not be made with certainty, leaving 7 cases questionable by IHC (Table 2). None of these cases were positive by rtPCR for either *T. gondii* or *N. caninum*. Single-to-multiple *T. gondii* tissue cysts were detected by IHC in lung tissue of 1 fetus and heart tissue of 2 infected fetuses (Table 3).

Four of 9 placental samples that tested positive for *T. gondii* by rtPCR and IHC were also positive by ISH (Table 2). Moreover, one positive ISH result was obtained in a placental sample that was neither detected by *T. gondii* rtPCR nor by *T. gondii* IHC staining, but was found to be positive for *N. caninum* by rtPCR. Positive hybridization was characterized by dark-brown to purple staining of tachyzoite-like structures, singly or in clusters. Stained organisms were rarely associated with necrotic foci, but rather could be observed in mostly well-preserved areas within the placental cotyledon, especially in the trophoblast layer (Fig. 1F). Nonspecific

Figure 1. Histologic sections of aborted ovine fetuses and placental tissue infected with *Toxoplasma gondii*. **A.** Severe multifocal necrotizing placentitis in a cotyledon with some central dystrophic mineralization in the necrotic foci. H&E. **B.** Small focus of necrosis next to largely well-preserved ovine placental tissue. H&E. **C.** Necrotic focus surrounded by gliosis in the cerebral white matter of an aborted ovine fetus. H&E. **D.** Immunostained *T. gondii* antigen (red) associated with a necrotic focus in the ovine placenta. **E.** Detection of individual *T. gondii* tachyzoites and tachyzoite clusters (red) in the trophoblast layer of ovine placenta by immunohistochemistry. **F.** Detection of individual *T. gondii* tachyzoites and tachyzoite clusters (dark-brown) in the trophoblast layer of ovine placenta by chromogenic in situ hybridization.

Table 3. Results of histologic examination, immunohistochemistry (IHC), and in situ hybridization (ISH) in *Toxoplasma gondii*-positive ovine fetuses.

Case	Brain			Heart			Lung			Liver			Skeletal muscle		
	H&E	IHC	ISH	H&E	IHC	ISH	H&E	IHC	ISH	H&E	IHC	ISH	H&E	IHC	ISH
1	+	–	–	ND	ND	ND	–	–	–	ND	ND	ND	–	–	–
2	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–
3	+	–	–	–	+	+	–	–	–	–	–	–	–	–	–
4	+	–	–	–	–	+	–	–	–	ND	ND	ND	–	–	–
5	–	–	–	–	–	–	–	+	–	ND	ND	ND	–	–	–
6	ND	ND	ND	ND	ND	ND	–	–	–	–	–	–	ND	ND	ND
7	–	–	–	–	–	–	–	–	–	ND	ND	ND	ND	ND	ND
8	+	–	–	–	+	–	–	–	–	ND	ND	ND	–	–	–
9	+	–	–	–	–	–	–	–	–	–	–	+	ND	ND	ND

H&E = hematoxylin and eosin (+ = presence of protozoal-associated lesions; – = absence of protozoal-associated lesions); ND = not done and/or tissue not available. IHC, ISH: + = visualization of *T. gondii* tissue cysts without corresponding inflammation; – = negative.

background staining in placental tissue was almost absent using ISH. Single-to-multiple *T. gondii* tissue cysts were detected by ISH in the liver tissue of 1 fetus and heart tissue of 2 infected fetuses (Table 3).

T. gondii DNA was identified in 9 of 200 ovine placental samples by rtPCR (Table 2). Further, the presence of *T. gondii* was confirmed by rtPCR by an external laboratory (CVUA, Karlsruhe, Germany) in these cases. All 9 *T. gondii*-positive samples were negative for *N. caninum* by PCR. For IHC, the sensitivity was 100% (95% CI: 66–100%), and the specificity was 96–100% (95% CI: 93–99% and 98–100%). For ISH, the sensitivity and specificity were 44% (95% CI: 14–79%) and 99% (95% CI: 97–100%), respectively (Table 4).

PCR detection of *N. caninum* in ovine abortions

To confirm the specificity of the detection of *T. gondii* by rtPCR and to exclude cross-reactivity of the utilized primary antibody for IHC and ISH probes with *N. caninum*, all placental samples were further examined for the presence of genome of *N. caninum* by PCR. *N. caninum* DNA was detected by rtPCR in 7 of 200 placental samples (Table 1). According to the autopsy reports, one of these cases had small, yellow-white foci of necrosis within placental cotyledons. Microscopically, multifocal necrotizing placentitis was observed in the cotyledons of all but one placental sample. In 3 cases, fetal brain tissue was available and suitable for further examination, each with characteristic protozoal lesions (Fig. 2A). Furthermore, multifocal, mixed inflammatory cell myocarditis was found in 2 corresponding fetuses (Fig. 2B). IHC staining for the detection of *T. gondii* was negative in every *N. caninum*-positive placenta. As noted above, a positive result was obtained by ISH in one of these placental samples.

Table 4. Sensitivity, specificity, predictive values, and kappa values of immunohistochemistry (IHC) and in situ hybridization (ISH) for the detection of *Toxoplasma gondii* in placental tissue using real-time PCR (rtPCR) as reference method.

	IHC	ISH
Sensitivity (%)	100 (66–100)	44 (14–79)
Specificity (%)	96 (93–99)*–100 (98–100)†	99 (97–100)
PPV (%)	56 (30–80)*–100 (66–100)†	80 (28–99)
NPV (%)	100 (98–100)	97 (94–99)
Kappa value	0.70*–1†	0.56

NPV = negative predictive value; PPV = positive predictive value. Numbers in parentheses are 95% confidence intervals.

* On the assumption that all cases with inconclusive IHC staining were assessed incorrectly as “positive” (referring to rtPCR as reference standard).

† On the assumption that all cases with inconclusive IHC staining were assessed correctly as “negative” (referring to rtPCR as reference standard).

Discussion

We detected no *T. gondii* stages in any H&E-stained tissue sections, neither in fetal membranes nor in fetal organs. Similar results were described previously; *T. gondii* was recognized only infrequently in H&E-stained tissue sections of both field outbreaks of toxoplasmic abortion and experimentally infected ovine fetuses.^{24,48} These findings might be explained by uneven distribution and poor detectability of *T. gondii* organisms in fetal tissue, particularly in distinguishing between tachyzoites and cell debris in placental tissue.¹³ Therefore, the focus of histologic examination should be the detection of characteristic tissue lesions for the diagnosis of *T. gondii*-induced abortion.¹³ However, in accordance with previous studies,^{5,6,14,31,37} lesions in ovine fetuses and fetal membranes induced by *T. gondii* were quite similar to those caused by *N. caninum*. Multifocal necrotizing placentitis compatible with a protozoal infection was present in the placental cotyledons of almost every one of our protozoan-associated abortion cases (15 of 16). Characteristic

Figure 2. Aborted ovine fetuses infected with *Neospora caninum*. **A.** Focal gliosis in the cerebral gray matter (cortex) of an aborted ovine fetus. H&E. **B.** Mild multifocal myocarditis in an aborted ovine fetus. H&E.

lesions, marked as focal-to-multifocal necrosis and/or gliosis, were observed most frequently in available brain tissue of fetuses associated with *T. gondii* (6 of 8) and *N. caninum* (3 of 3). Although protozoan-associated lesions in fetal organs (excluding brain) were described more frequently in fetuses infected with *T. gondii*,³⁷ we observed multifocal myocarditis exclusively in the heart tissue of 2 fetuses infected with *N. caninum*. These findings support the need for a method that is able to differentiate between the 2 protozoan parasites.

An accurate diagnosis and thus the distinction between these 2 agents is especially important for both epidemiologic and prophylactic reasons; only *T. gondii* is considered to be of zoonotic importance.^{15,47} The parasites have different definitive hosts,^{15,47} which is important regarding effective preventive measures. Moreover, a commercial vaccine is available only for ovine toxoplasmosis.⁴

According to a study of experimentally infected fetuses, IHC is sensitive and specific for the detection of *T. gondii* in ovine fetuses and fetal membranes.⁴⁸ In a study from Germany, IHC appeared even more sensitive than conventional PCR for the detection of *T. gondii* in abortions of sheep.⁴² We detected *T. gondii* antigen most consistently in placental cotyledons of infected fetuses, and thus found a sensitivity of 100% (95% CI: 66–100%) by IHC. In line with previous results, large amounts of *T. gondii* antigen were usually present, predominantly within the necrotic foci of placental cotyledons.^{46,48} This may be explained by the fact that *T. gondii* initially settles and multiplies in placentomes of the gravid uterus before spreading to the fetus.^{3,24} Using rtPCR for the quantification of *T. gondii* in ovine maternal and fetal tissues from experimentally infected ewes, placenta has been demonstrated to be positive from the earliest time post-infection and contains the highest load of *T. gondii* genome.²³ This might explain why placental tissue is superior to fetal tissue

for the detection of *T. gondii* by IHC, as demonstrated in our study. In contrast to placental tissue, *T. gondii* was detected only sporadically in fetal heart (2 of 7) and lung tissue (1 of 9). No developmental stages of *T. gondii* were visualized in brain tissue by IHC.

Despite the high sensitivity of IHC (100%; 95% CI: 66–100%), specificity (96–100%; 95% CI: 93–99% and 98–100%) appeared lower compared to rtPCR, which was used as the reference method. Given the common nonspecific background staining in placental tissue, microscopic evaluation was hampered in several cases and thus 7 of 200 examined cases could not be assessed as positive or negative. A variety of factors may affect IHC staining, including technical aspects, such as processing of tissue specimens, the utilized detection system, or the interaction of primary antibody.² Furthermore, the type of tissue investigated may have a great influence. Diffuse nonspecific background staining, as well as staining of plant debris and placental pigments, have been described previously in IHC-stained ovine placenta sections screened for *Coxiella burnetii*.¹¹ Another reason for background staining in IHC is necrosis of the investigated tissue.⁸ Thus, the distinction between nonspecific necrosis-associated staining and staining truly induced by antigen of degenerate *T. gondii* organisms was challenging in our study. This fact is a relevant disadvantage for IHC screening of placental tissue for the detection of *T. gondii* given that aborted placental tissue is marked by autolysis and necrotic alterations in many cases.

Contrary to previous investigations using another commercial anti-*T. gondii* polyclonal antibody,⁴³ we observed no cross-reactivity with tissue stages of *N. caninum* in cotyledons of *N. caninum*-positive placental samples. This implies species specificity of the anti-*T. gondii* primary antibody utilized in our study.

To our knowledge, evaluation of ISH has not been reported previously on the tissue of aborted ovine fetuses for the detection of *T. gondii*. Because placental tissue appears well suited for the identification of the protozoan parasite by IHC, we tested its use for ISH. Yet, a true positive result was gained in less than half (4 of 9) of the abortion cases infected with *T. gondii* by ISH. This resulted in substantially lower sensitivity of ISH (44%; 95% CI: 14–79%) compared to IHC (100%; 95% CI: 66–100%). Given that extensive tissue necrosis was supposed to lead to reduced or even absent ISH signal in previous investigations,^{26,36} the necrotic foci in the placental cotyledons may be the most reasonable explanation for false-negative results obtained by ISH. Necrotic tissue might interfere with nucleic acid hybridization and might inhibit the development of a visible signal.²⁶ Furthermore, tissue degradation, which frequently occurs in placental tissue, might cause a release of endogenous nucleases, destroying nucleic acid and thus preventing hybridization with the oligonucleotide probes.²⁶ However, antigen (e.g., of the parasite surface) may remain detectable by IHC. False-negative ISH results might also be explained by the fact that only viable, and therefore intact, organisms could be detected by the ISH method that we used, as a result of declining levels of rRNA after cell death.^{35,51} Therefore, it is advisable to fix tissue specimens as soon as possible to prevent further post-mortem decay.

Intact ISH-stained organisms were found predominantly in relatively normal areas of placental cotyledons and fetal organs. These results suggest that our ISH procedure may be an efficient tool for the detection of *T. gondii* in well-preserved and unaltered tissue specimens. This, of course, limits its use in the diagnosis of ovine toxoplasmosis.

One of the most significant advantages of ISH is its high specificity, which has been demonstrated to be 100% in previous studies investigating fungi, viral agents, and protozoa other than *T. gondii*.^{25,26,33,40} In our study, the specificity of ISH was 99% (95% CI: 97–100%). Although contrary to IHC, nonspecific background staining was nearly absent in ISH-stained tissue sections, ISH revealed a false-positive result when utilized on the placental cotyledons of 1 of 7 *N. caninum*-positive cases. The false-positive result implies cross-hybridization of at least 1 of the 2 oligonucleotide probes. This is explainable given the high homology of the 18S rRNA gene sequences of *T. gondii* and *N. caninum*,^{18,28,30} and therefore this oligonucleotide probe shows only a single nucleotide variation. Hence, a second probe, targeting the 28S rRNA sequence—still highly similar¹⁹—with a maximum of 3 mismatches, was additionally chosen for the possible differentiation of both species. However, the validation process showed that only the combination of both probes provided sufficient signal intensity.

Although it is possible that the high homology of both rRNA sequences causes cross-reactivity, we chose this locus because rRNA is supposedly the only target present in sufficient abundance to warrant effective ISH staining of protozoal

cells.¹² Nevertheless, verifying the specificity of both probes in situ by staining brain tissue infected with *N. caninum* has shown no cross-hybridization. In those sections, mainly tissue cysts of *N. caninum* were detected, which typically have decelerated replication. As previously hypothesized for slowly replicating stages of other protozoa, protein synthesis takes place to a lesser extent in those persistent stages and thus rRNA content is lower, resulting in a weaker or even absent signal.⁴⁴ Further investigations of *N. caninum*-positive tissue samples containing a high amount of fast-replicating stages (tachyzoites) are necessary to definitively determine whether the ISH procedure established in our study can distinguish *T. gondii* from *N. caninum*.

According to our results, both IHC and ISH can be useful additional techniques to improve the detection of *T. gondii* by visualizing developmental stages in tissue sections. However, because of its low sensitivity, ISH should be used in combination with other detection methods. Although the specificity of both ISH probes was not clarified, our results suggest that our ISH procedure might be inappropriate for the differentiation between *T. gondii* and *N. caninum*. Nevertheless, the morphologic evaluation of ISH-stained tissue sections is less challenging compared to IHC given the absence of background staining. Real-time PCR is considered a very sensitive and suitable tool for the detection of *T. gondii* in ovine abortion material, especially in placental cotyledons.²³ Furthermore, the rtPCR method that we used was very specific with regard to the distinction between *T. gondii* and *N. caninum*. Moreover, using commercial PCR test kits, rtPCR is comparatively simple and fast to implement in any PCR laboratory. However, the use of a highly sensitive test such as PCR to determine infectious agents as the cause of small ruminant abortion does not distinguish between infection and disease causation.²⁷ *T. gondii* DNA has been detected in placental tissue of congenitally infected but apparently healthy live-born lambs by PCR.^{17,50} Despite the detection of *T. gondii* in abortion cases, the causative agent might remain uncertain, especially if another abortifacient pathogen is identified. Similar to previous investigations,^{27,45} our study revealed one double infection with *Chlamydia* spp. and *T. gondii*. Therefore, a positive PCR test should be interpreted in conjunction with other testing as well as gross and histologic examination. Factors influencing PCR interpretation include history and supportive lesions as well as identification and exclusion of other pathogens using bacterial culture and smears, viral PCR testing, and IHC.

Acknowledgments

We thank Dana Schleinitz, Helmut Puchta, and Thomas Neuß for their excellent technical advice and assistance regarding histologic and molecular methods. Furthermore, we highly appreciate the support of Dr. Monir Majzoub-Altweck, Dr. Elisabeth Kemter, and Gudrun Goldmann of the Ludwig-Maximilians-Universität, Munich, Germany with the establishment of the ISH technique utilized in our study.

Declaration of conflicting interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The authors received no financial support for the research, authorship, and/or publication of this article.

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