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1 **Evaluation of species-specific polyclonal antibodies to detect and differentiate between**

2 ***Neospora caninum* and *Toxoplasma gondii***

3

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17

18 Running head: Species-specific detection of *N. caninum* and *T. gondii*

19

20 **Abstract.** Neosporosis and toxoplasmosis are major causes of abortion in livestock
21 worldwide, leading to substantial economic losses. Detection tools are fundamental to the
22 diagnosis and management of those diseases. Current immunohistochemistry (IHC) tests,
23 using sera raised against whole parasite lysates, have not been able to distinguish between
24 *Toxoplasma gondii* and *Neospora caninum.* We used *T. gondii* and *N. caninum* recombinant
25 proteins, expressed in *Escherichia coli* and purified using insoluble conditions, to produce
26 specific polyclonal rabbit antisera. Our aim was to develop species-specific sera that could be
27 used in IHC on formalin-fixed paraffin-embedded (FFPE) tissue sections to improve the
28 diagnosis of ruminant abortions caused by protozoa. Two polyclonal rabbit sera, raised
29 against recombinant proteins, anti-*Neospora*-rNcSRS2 and anti-*Toxoplasma*-rTgSRS2, had
30 specificity for the parasite they were raised against. We tested the specificity for each
31 polyclonal serum using FFPE tissue sections known to be infected with *T. gondii* and *N.*
32 *caninum*. The anti-*Neospora*-rNcSRS2 serum labelled specifically only *N. caninum*-infected
33 tissue blocks, and the anti-*Toxoplasma*-rTgSRS2 serum was specific to only *T. gondii*-
34 infected tissues. Moreover, tissues from 52 cattle and 19 sheep previously diagnosed by lesion
35 profiles were tested using IHC with our polyclonal sera and PCR. The overall agreement
36 between IHC and PCR was 90.1% for both polyclonal anti-rNcSRS2 and anti-rTgSRS2 sera.
37 The polyclonal anti-sera were specific and allowed visual confirmation of protozoan parasites
38 by IHC, but they were not as sensitive as PCR testing.
39

40 **Keywords**: FFPE; IHC; *Neospora caninum;* polyclonal antibody; polyclonal sera; protozoan
41 detection; recombinant protein; species-specific antibodies; PCR; *Toxoplasma gondii.*
42

43 *Neospora caninum* and *Toxoplasma gondii* are apicomplexan intracellular protozoan
44 parasites.[26] *N. caninum* is known to be a major cause of endemic and epidemic abortions in
45 cattle around the world and has been shown to seriously impact the economic performance of
46 the dairy and beef industries.[26] *N. caninum* is mainly responsible for abortions in cattle, but it
47 has also caused characteristic lesions in small ruminants with clinical signs and lesions similar
48 to those induced by *T. gondii*.[30]

49 Toxoplasmosis is well known for causing abortions in sheep, and *T. gondii* can also be
50 found in other ruminants, including cattle.[3,28] Furthermore, toxoplasmosis is a major zoonosis
51 and, in pregnant women, *T. gondii* can infect the fetus and cause miscarriage or result in
52 congenital toxoplasmosis, which can cause brain damage. In immunocompromised people, *T.*
53 *gondii* infection can be very severe and even life-threatening.[20] *N. caninum* and *T. gondii*
54 cannot be distinguished solely by clinical and microscopic findings, given that they share
55 many common biologic and morphologic similarities.[32] The detection of protozoan parasites
56 is difficult even in tissues from clinically infected animals, because parasites are not always
57 associated with lesions.[10,26] Etiologic detection tests such as immunohistochemistry (IHC) and
58 PCR are required to establish the etiologic cause of abortion and thus permit the design of
59 effective control strategies.

60 IHC can identify one or more immunogenic epitopes, allowing visualization and
61 establishment of the distribution of the pathogen within tissue sections.[35] *N. caninum*
62 polyclonal antiserum raised against whole parasite lysates can cross-react with other cyst-
63 forming parasites, such as *T. gondii* and *Sarcocystis* spp.[27] Antibodies developed using whole
64 parasite lysates often cross-react with other protozoa and hence are unsuitable for
65 distinguishing between these closely related parasites using IHC.[14,17] Various studies have
66 shown that using specific protozoan proteins in their native and recombinant forms results in
67 reduced cross-reactivity with other coccidian parasites.[35]

68 Formalin-fixed paraffin-embedded (FFPE) tissue sections have been used most widely
69 for clinical diagnosis. However, for FFPE tissue sections, it is known that formaldehyde
70 crosslinks proteins, which results in loss of antigenicity as a result of the formation of
71 methylene bridges, making binding of specific antibodies difficult during IHC.[28] Antigen
72 retrieval (AR) methods allow specific recognition of proteins from FFPE tissues by breaking
73 these bridges and exposing the antigenic sites to allow antibodies to bind.[31]

74 Similarly for PCR analysis, when fresh tissues are not available, FFPE tissue samples
75 can be used for the identification of parasite DNA.[2,33] However, the extraction of DNA from
76 FFPE samples remains challenging given that various fixatives, including formaldehyde,
77 cause cross-linkage. The cross-linkage of DNA by formaldehyde can cause fragmentation,
78 strand breaks, and chemical modifications, which can inhibit PCR.[31] Fixation decreases PCR
79 sensitivity, thus making PCR amplification of large- and high-molecular-weight DNA
80 fragments considerably more difficult.[13,22] Nevertheless, DNA can be successfully purified,
81 and the amplification of fragments of <500 bp via PCR is possible.[25] Various PCR assays
82 have been described that are able to detect *N. caninum, T. gondii*, and *Sarcocystis* spp.,
83 targeting multicopy rDNA, containing the 18S ribosomal RNA gene and the internal
84 transcribed spacer (ITS1) region, from frozen tissue samples.[11] There are several
85 commercialized protocols that allow the amplification of protozoan parasite DNA from FFPE
86 tissue samples.[6,34]

87 Our aim was to produce species-specific antibodies, raised against recombinant
88 proteins of *N. caninum* and *T. gondii*, which could then be used to detect and characterize
89 protozoan parasites in diagnostic and research studies investigating cases of abortion in
90 ruminants. We also aimed to evaluate the diagnostic sensitivity and specificity of the species-
91 specific antisera in IHC in direct comparison to PCR results conducted on DNA extracted

92 from matching FFPE tissue sections in which we detected protozoan parasites and determined
93 their species.

94 Materials and methods

95 Recombinant protein production and testing

96 *N. caninum (NcSRS2)* and *T. gondii* (*TgSRS2*) surface antigen genes were selected and used to
97 produce recombinant proteins. The chosen regions were polymorphic and amplified using
98 gene-specific primers (Table 1). DNA samples, extracted from *N. caninum* (Nc1) and *T.*
99 *gondii* (M4), were used as positive controls, and negative water was included as negative
100 controls in all PCRs. Positive amplicons were sequenced using the MWG sequencing service
101 (Table 1). DNA sequences were analyzed using BLAST to confirm the species and to
102 determine sequence identity.

103 Each PCR reaction contained 2 µL of 10x custom PCR mix, 13.8 µL of water, and 2
104 µL of sample DNA using PCR conditions described previously,[4,24] with the exception of an
105 annealing temperature of 60 °C for 1 min. Positive PCR amplicons were purified and
106 cloned.[24] Plasmids were verified by DNA sequencing, and DNA inserts were digested,
107 purified, and ligated into pre-digested expression vector pQE-30/31 (QIAexpressionist;
108 Qiagen) using appropriate restriction enzymes (Table 1). Expression constructs were verified
109 by sequencing and used to express the recombinant proteins in *Escherichia coli* M15 (pREP4)
110 according to the manufacturer's instructions. The proteins were purified under denaturing
111 conditions (HisPur Ni-NTA spin columns; Thermo Fisher) according to the manufacturer's
112 instructions, except for using 6 wash steps with 2 resin-bed volumes of denaturing stock wash
113 buffers: 20 mM Na NaH_2PO_4, 300 mM NaCl, 25 mM imidazole, 8 M urea and 20 mM
114 NaH_2PO_4, 300 mM NaCl, 50 mM imidazole, 8 M urea. Recombinant proteins were dialyzed
115 into 2 M urea (Slide-A-LyzerTM G2 dialysis cassette; Thermo Fisher) and concentrated

116 (Vivaspin 6 at 10,000 MWCO; GE Healthcare) for 15 min. Protein concentrations were
117 determined (Pierce BCA protein assay kit; Thermo Fisher).

118 Western blots were performed to identify reactivity and cross-reactivity among the *N.*
119 *caninum* and *T. gondii* recombinant proteins with positive and negative bovine antisera (Table
120 2).[35] Briefly, a recombinant protein concentration of 3 ng/mL was used. We used 10 µL of
121 positive controls of *N. caninum* water-soluble antigen fraction (NAF; 25 µg/mL) and T.
122 gondii water-soluble antigen fraction (TAF; 27 µg/mL). The proteins were transferred to a
123 nitrocellulose blotting membrane (Amersham Protran premium 0.45 µm, GE Healthcare;
124 XCell IITM blot module, Invitrogen) according to the manufacturers' instructions and
125 incubated overnight at 4°C in transfer buffer (1x Novex Tris-glycine; Thermo Fisher).
126 Membranes were incubated in 4% non-fat dried milk (Marvel) for 1 h with primary antisera
127 (Table 2). Membranes were washed 3 times for 5 min after each incubation with a wash
128 buffer (0.05% Tween 80, 500 mM NaCl, 1xPBS). The secondary conjugate was incubated for
129 1 h (Table 2). Protein bands were detected (SuperSignal west pica chemiluminescent substrate
130 kit; Thermo Fisher) and visualized (ImageQuant Las-4000 multi-mode imager; GE).

131 **Production of rabbit polyclonal sera**

132 Our study was carried out in strict accordance with the Animals (Scientific Procedures) Act
133 1986 and in compliance with all UK Home Office Inspectorate regulations under
134 PPL70/8627.

135 The pre-immune sera from 14 rabbits (Orgyen Antibodies), were tested to rule out
136 nonspecific reactivity with *N. caninum* or *T. gondii*. Western blot analysis was performed
137 using 10 µL of rNcSRS2 and rTgSRS2 recombinant proteins (3 ng/mL) and NAF/TAF (22.5
138 µg/mL; Table 2). IHC was carried out using the rabbit pre-immune sera on FFPE positive
139 control blocks from the pathology archive at the Moredun Research Institute (Midlothian,
140 Scotland, UK), which were previously confirmed to be *N. caninum*-positive (4 bovine brain

141 and heart tissue blocks; 2 *T. gondii*-positive ovine brain tissue blocks). The presence of
142 protozoan parasites had been confirmed previously by lesion profile and the presence of
143 parasite on H&E staining and IHC.

144 Four-µm thick paraffin-embedded tissue sections were cut and placed onto glass slides
145 (SuperFrost Plus; Thermo Scientific). Tissues were dewaxed in xylene for 20 min and
146 rehydrated through graded ethanol. The endogenous peroxidase was quenched by immersion
147 of the slides in 3% hydrogen peroxide in methanol solution for 20 min. No antigen retrieval
148 method was used for testing rabbit pre-immune sera. Slides were assembled with cover plates
149 in sequenza racks and washed 3 times with Tris-buffer saline (TBS; 1M Tris HCl, 5 M NaCl,
150 pH 7.6) until the wash buffer was fully drained following each incubation. Tissues were first
151 blocked with 25% normal goat serum in TBS. Slides were next incubated with rabbit pre-
152 immune sera overnight at 4°C. Antigen-antibody reactions were detected using a horseradish
153 peroxidase-labelled polymer. The slides were treated with peroxidase substrate solution
154 (AEC; Vector) following the manufacturer's instructions, and counterstained with Mayer
155 hematoxylin and Scot tap water substitute (STWS) for 2 min each. Slides were mounted with
156 a coverslip (ImmunoHistoMount, Sigma-Aldrich; permanent Consul Mount, Shandon).

157 Polyclonal sera were produced by Orgyen Antibodies by using rNcSRS2 and
158 rTgSRS2 to immunize rabbits whose pre-immune sera had no reactivity with protozoan
159 parasites by western blots and IHC. New Zealand white rabbits were immunized 4 times
160 (weeks 1, 4, 8, 12) with 150 µg per dose of each recombinant protein. Per injection, 0.25 mL
161 of recombinant proteins at stock concentrations of 600 µg/mL were emulsified with Freund
162 adjuvant and administered into each rabbit. Test bleeds were taken during weeks 5 and 9.
163 Rabbits were exsanguinated at week 13.

164 **Standardization and specificity of rabbit polyclonal sera**

165 Preliminary unpublished studies, conducted at the Moredun Research Institute, demonstrated
166 that polyclonal sera raised against whole tachyzoite lysates were cross-reactive with *N.*
167 *caninum, T. gondii*, and *Sarcocystis* spp. by IHC. The reactivity of each polyclonal antiserum
168 raised against the recombinant proteins was evaluated by IHC on FFPE tissues using the same
169 method as described above under specific conditions. To establish optimal working
170 conditions, dilutions (1:100, 1:200, 1:500, 1:1,000, 1:2,000, 1:3,000, 1:4,000, 1:6,000,
171 1:8,000, 1:10,000) were tested for each antiserum. Different antigen retrieval methods were
172 used to 'de-mask' the modification of antigen targets caused by fixation. The optimal antigen
173 retrieval methods were evaluated by using: no antigen retrieval treatment, heat-induced
174 epitope antigen retrieval (HIER; citrate buffer, 10 mM citric acid, at pH 6.0 in a pre-heated
175 autoclave at 121°C for 10 min) and proteolytic enzyme-induced epitope antigen retrieval
176 (PIER; 0.1% protease, *Streptomyces griseus*, [Sigma-Aldrich] in TBS at 37°C for 10 min).
177 The method with the highest labelling intensity and optimal working conditions was the PIER
178 method and a dilution of 1:6,000 for the serum raised against rSRS2 from *N. caninum,* and
179 serum raised against rSRS2 from *T. gondii* at a dilution of 1:2,000 (Table 3).
180 Cross-reactivity of rabbit anti-rTgSRS2 and rabbit anti-rNcSRS2 were tested using
181 western blot and IHC analysis. Western blots were performed as described above with the
182 exception of using the polyclonal sera (Table 2). IHC analyses were performed using the
183 PIER method, on 2 positive *Neospora* (canine brain), 2 positive *Toxoplasma* (1 murine liver
184 and feline brain), and 2 positive *Sarcocystis* (ovine heart) control tissue sections. Rabbit pre-
185 immune sera were used as negative antibody controls.
186 **Protozoan detection on archived FFPE tissue blocks by IHC and PCR**
187 The polyclonal anti-sera (anti-rNcSRS2, anti-rTgSRS2) were tested using the optimized
188 conditions (Table 3) on a selection of FFPE tissue blocks obtained from the Moredun
189 pathology and surveillance archive. These tissue blocks were from 71 animals (52 cattle, 19

190 sheep) and included fetal brain, placenta, and skeletal muscle with lesions compatible with
191 protozoan infection. One block per animal was selected and one 4-µm thick paraffin-
192 embedded tissue section was cut and used per antiserum.

193 For PCR analysis, two 20-µm thick FFPE tissue sections were cut from the same
194 blocks to confirm the presence of DNA from protozoan parasites. DNA was extracted
195 (QIAamp DNA FFPE tissue kit, 56404; Qiagen) according to the manufacturer's instructions,
196 with the following modifications. Sections were de-paraffinized with xylene and rehydrated in
197 100% ethanol. Approximately 300 µL of buffer ATL and 30 µL proteinase K (both supplied
198 within the QIAamp DNA FFPE tissue kit) were used. Samples were lysed overnight at 56°C
199 in a water bath. To increase DNA concentrations, columns were incubated for 5 min with 50
200 µL ATE buffer (QIAamp DNA FFPE tissue kit) before centrifugation. The nucleic acid
201 concentration and purity were determined (Nanodrop 1000 spectrophotometer; Thermo
202 Fisher). Parasite DNA was detected using nested PCRs, and reaction conditions for the first-
203 and second-round PCR were carried out as described previously (Table 4),[24] with the
204 exception of the annealing temperature of 58°C for the 18S and 55°C for the ITS1 PCR assays
205 in the second-round PCR assay. The first-round PCR assay used external primers that
206 recognized apicomplexan parasites, including *N. caninum*, *T. gondii, Sarcocystis* spp., and
207 *Hammondia* spp. (*H. hammondi*, *H. heydorni*, *H triffitae*), and the second-round PCR assay
208 included the genus- and species-specific primers to distinguish among *N. caninum, T. gondii,*
209 and *Sarcocystis* spp. DNA samples were tested in triplicate by PCR. *N. caninum*-and *T.*
210 *gondii*-positive DNA samples were included as positive controls, and water was included as a
211 negative control in the PCRs. Positive amplicons were sequenced (DNA sequencing service;
212 MWG) using internal primers (NTS-18S-F1 and R1; Table 4). Sequences were analyzed
213 using seqMan Pro and BLAST searches to determine the species identified against published
214 sequences.

215 **Data analysis**

216 We analyzed our data with R system for statistical computing (v.4.0.2). The proportion of
217 positive samples for each test and parasite species were calculated, including 95% CIs based
218 on a binomial probability distribution.[8] In the absence of a gold standard, agreement statistics
219 between the IHC and PCR test results were computed based on combined data from both
220 ovine and bovine species. Namely, overall percent agreement, and associated 95% CI, along
221 with Cohen kappa coefficient.[21] This latter measure ranges in [0; 1], with 0 meaning that the
222 agreement is not better than random, and 1 meaning perfect agreement between tests.
223 Statistical significance was concluded at the 5% significance level ($p \leq 0.05$).

224 **Results**

225 **Development, purification and testing of recombinant proteins in western blots**

226 Amplicons for the polymorphic regions of the *NcSRS2* (1,057 bp) and *TgSRS2* (540 bp) were
227 generated and cloned into pQE expression vectors. Sequencing the expression vectors
228 confirmed the presence of the desired open-reading frames, and >99% sequence identity to
229 the published *NcSRS2* and *TgSRS2* sequences encoding predicted amino acid sequences of
230 354 for rNcSRS2 and 180 amino acids for rTgSRS2. All recombinant proteins were
231 successfully purified, resulting in a rNcSRS2 protein with a predicted size of 37.2 kDa and a
232 rTgSRS2 protein with a predicted size of 19.2 kDa. In both cases, the purified proteins were
233 slightly larger on the SDS-PAGE gels, and for rTgSRS2, larger bands of about twice the size
234 are visible, which represents protein dimers, and multimers that are even larger (Suppl. Figs.
235 1A, 1B).

236 Western blot results revealed that *Neospora*-positive bovine sera reacted with the
237 rNcSRS2 and NAF; no reaction was seen with rTgSRS2 and TAF (Suppl. Fig. 2A). No
238 reactions were observed with recombinant proteins or TAF with *Neospora*-negative bovine
239 sera; a very weak reaction was seen with NAF (Suppl. Fig. 2B). Furthermore, western blot

240 results revealed that the *Toxoplasma*-positive bovine sera reacted with the rTgSRS2 and TAF;
241 no reaction was seen with rNcSRS2 and NAF (Suppl. Fig. 2C). No reactions were observed
242 with recombinant proteins and NAF with *Toxoplasma*-negative bovine sera (Suppl. Fig. 2D).
243 However, a slight reaction with TAF was observed with *Toxoplasma*-negative bovine sera
244 (Suppl. Fig. 2D).

245 **Selection of rabbits for immunization**

246 Both the western blots and IHC were performed to test the reactivity of the rabbit pre-immune
247 sera with NAF/TAF and recombinant proteins. For the western blot analysis, 4 of 14 pre-
248 immune sera reacted with recombinant proteins rNcSRS2 and rTgSRS2, and none of the sera
249 reacted with NAF/TAF. The pre-immune sera screen via IHC showed strong background and
250 nonspecific binding of 11 of 14 sera with *N. caninum* and *T. gondii.* Based on these combined
251 results, 3 rabbits had unreactive sera, from which 2 were used for antibody production.

252 **Optimization of rabbit polyclonal sera**

253 IHC titration showed that rabbit anti-rNcSRS2 and rabbit anti-rTgSRS2 had the highest
254 signal:background ratio at a dilution of 1:6,000 for *N. caninum* and 1:2,000 for *T. gondii*. In
255 the absence of a retrieval method, parasites had very faint labelling for both polyclonal sera
256 (Suppl. Figs. 3A, 3D). Parasites had positive labelling when treated with HIER, but histologic
257 quality was altered (Suppl. Figs. 3B, 3E). Slides treated with PIER had the best labelling of
258 the protozoan parasites (Suppl. Figs. 3C, 3F). No labelling was observed with the rabbit pre-
259 immune sera.

260 **Rabbit polyclonal antisera specificity**

261 Western blot results revealed that rabbit anti-rNcSRS2 reacted with the rNcSRS2 and the
262 NAF, and did not react with the TAF or rTgSRS2 (Suppl. Fig. 4A). Rabbit anti-rTgSRS2 had
263 strong reactivity with rTgSRS2, and no reactivity with NAF, TAF, or rNcSRS2 (Suppl. Fig.

264 4B). Antisera raised against rNcSRS2 and rTgSRS2 reacted with dimers and multimers of the
265 purified recombinant proteins that they were raised against (Suppl. Fig. 4).
266 The rabbit anti-rNcSRS2 serum had specific labelling of *N. caninum* parasites using
267 IHC (Fig. 1A), but no specific labelling of *T. gondii* (Fig. 1B). The rabbit anti- rTgSRS2
268 serum had specific labelling of *T. gondii* parasites using IHC (Fig. 1C), but no specific
269 labelling of *N. caninum* (Fig. 1D). No labelling was observed on the *Sarcocystis* tissue control
270 (data not shown).
271 **Protozoan detection on archived FFPE tissue blocks by IHC and PCR**
272 A total of 71 ruminant samples were tested by IHC and PCR in parallel to detect infections by
273 protozoan parasites (Suppl. Fig. 5). IHC using rabbit anti-rNcSRS2 serum showed that 10 of
274 71 (14.1%, 95% CI: 7.0–24.4%) ruminant tissue samples were positive (Fig. 2). A total of 10
275 of 52 (19.2%, 95% CI: 9.6-32.5%) bovine samples had positive labelling for *N. caninum* (Fig.
276 2). No ovine samples were positive for *N. caninum* (Fig. 2).
277 PCR results revealed that 13 of 71 (18.3%, 95% CI: 10.1-29.3%) ruminant samples
278 were positive for *N. caninum*; 13 of 52 cattle samples (25.0%, 95% CI: 14.0-38.9%) were
279 positive for *N. caninum* (Fig. 2). No ovine samples were positive by PCR for *N. caninum*
280 (Fig. 2).
281 IHC using rabbit anti-rTgSRS2 serum showed labelling in 3 of 71 ruminant tissue
282 samples (4.2%, 95% CI: 0.9-11.9%) (Fig. 2); 3 of 19 (15.8%, 95% CI: 3.4-39.6%) ovine
283 samples showed positive labelling for *T. gondii*, yet no bovine samples were positive for *T.*
284 *gondii* (Fig. 2).
285 Using *T. gondii* specific PCR, 10 of 71 (14.1%, 95% CI: 7.0-24.4%) ruminant samples
286 were positive, of which 3 of 52 (5.8%, 95% CI: 1.2-15.9%) were cattle samples and 7 of 19
287 (36.8%, 95% CI: 16.3-61.6%) were ovine samples (Fig. 2).

288 PCR analysis revealed that 8 of 71 (11.3%, 95% CI: 5.0-21.0%) ruminant samples
289 were positive (at least one of the triplicate PCRs) for *Sarcocystis* spp.,1 of 52 (1.9%, 95% CI:
290 0.1-10.3%) cattle samples was positive for *S. cruzi*, and 7 of 19 ovine samples (36.8%, 95%
291 CI: 16.3-61.6%) were positive for *Sarcocystis* species (Fig. 2). *Sarcocystis tenella* was
292 detected in 3 animals and *S. gigantea* in 4 according to BLAST results with >99% sequence
293 identity (*S. tenella* L24383; *S. gigantea* L24384). One ovine placenta sample was positive for
294 both *S. tenella* (by PCR) and *T. gondii* (by PCR and IHC), and one bovine brain sample was
295 positive for both S. *cruzi* (by PCR) and *N. caninum* (by PCR and IHC; Suppl. Fig. 5).
296 When assessing all ruminant samples for *N. caninum,* 80% of IHC positive samples
297 were also positive by PCR, whereas 8.19% of samples positive by PCR were negative by
298 IHC. The overall agreement (including negative results) for *N. caninum* was 90.1% (95% CI:
299 82.0-94.8%), with a kappa statistic of 0.64 ($p < 0.001$). In the case of *T. gondii*, all IHC
300 positive samples were also positive by PCR, whereas 10.3%of samples positive by PCR were
301 negative by IHC. The overall percent agreement for *T. gondii* was 90.1% (95% CI: 84.4-
302 93.9%), with the kappa of 0.42 ($p < 0.001$).

303 **Discussion**

304 Our specific polyclonal sera for *N. caninum* and *T. gondii* did not cross-react with the other
305 pathogen and did not label *Sarcocystis* spp., demonstrating that specific polyclonal antisera
306 can be applied to IHC testing of FFPE tissue sections for support of species-specific diagnosis
307 of *T. gondii*- or *N. caninum*-induced abortions. Previous tests that used antibodies raised
308 against whole parasite lysates (i.e., tachyzoites) have reported cross-reactivity in IHC for *N.*
309 *caninum* and *T. gondii.*[14,27] Recombinant proteins have provided an alternative method for
310 producing more specific antibodies.[5,36] We chose the *TgSRS2* and *NcSRS2* antigen genes for
311 recombinant protein production because there is minimal amino acid sequence identity
312 between the 2 regions chosen, making it more likely that they do not share B-cell epitopes and

313 that antisera raised against these recombinant proteins would not cross-react with the other
314 species. The *SRS2* gene of *N. caninum* and its homologue in the closely related *T. gondii*
315 encode proteins that have only 43% identity, which made these good candidates for the
316 development of species-specific antisera.[9,29] Our results are supported by findings of other
317 studies, in which recombinant NcSRS2 surface antigens were not recognized by *T. gondii*
318 immune cat, cattle, and mouse sera.[7,18] It was demonstrated[18] that, even though TgSRS2 is a
319 homologue of NcSRS2 and shares structural similarities, the levels of amino acid sequence
320 identity of TgSRS2 and NcSRS2 was not sufficient to elicit a cross-reactive antibody
321 response between the antigens. Our results indicate that the recombinant proteins generated
322 did not have any cross-reactivity between bovine positive *N. caninum* and *T. gondii* sera and
323 were therefore a good target for the development of polyclonal antibodies. However, it was
324 surprising that negative control cattle sera reacted weakly with NAF or TAF. These sera
325 originated from experimental studies conducted at the Moredun Research Institute and were
326 thought to be uninfected with *N. caninum* and *T. gondii*.[35]
327 The development of polyclonal sera was initiated by screening the rabbit pre-immune
328 sera to choose the best candidate for immunization with recombinant proteins. The screening
329 of pre-immune sera revealed that only 3 of 14 rabbits could be suitable for polyclonal serum
330 production given that they did not react with *N. caninum* or *T. gondii* by either western blot or
331 IHC analyses. The positive rabbits may have had antibodies against other biologic material
332 that either recognized the parasite epitopes directly, or nonspecifically cross-reacted with
333 parasite epitopes. Rabbits are known to produce large amounts of nonspecific antibodies,
334 creating high background signals during IHC analysis.[23] Many environmental factors can
335 encourage immune responses prior to immunization against various pathogens (i.e., bacteria,
336 fungi, viruses).[36] An ELISA study[23] showed that 53% of the rabbit pre-immune sera had
337 positive reactions with 10 or more bacterial cultures. These and our results suggest that the

338 presence of cross-reactive antibodies in pre-immune sera against microorganisms such as
339 bacteria and protozoa is an important common problem that should be considered when using
340 rabbits to develop specific antisera.[23]

341 Our results from the rabbit polyclonal antisera, tested for their specificity by western
342 blot analyses, showed that the rabbit anti-rNcSRS2 serum only reacted with NAF and
343 rNcSRS2. This indicates that the serum specifically recognizes *N. caninum,* making it a good
344 candidate for IHC, given that no reactivity with rTgSRS2 or TAF was seen. The rabbit anti-
345 rTgSRS2 serum reacted only with rTgSRS2, indicating that the polyclonal serum is specific to
346 the *T. gondii* protein. However, the lack of reactivity with TAF suggests that the rTgSRS2
347 may not have displayed native epitopes, as a result of the refolding of the recombinant protein
348 during dialysis and following immunization. Further tests could be conducted to see if these
349 polyclonal antibodies would also make good candidates for other serologic assays, such as
350 ELISAs or indirect IFAs. A study has shown that it is important that proteins used are
351 homogeneous, correctly folded, and presented so that the critical epitopes are accessible.[12]
352 Each recombinant protein expressed in our study was insoluble and, even though refolding
353 procedures were used, some proteins may have not been able to reproduce the properties of
354 the native protein.[9] Protein oxidation, aggregation, and degradation are known issues, which
355 can severely affect the outcome of antibody generation, and therefore it was important to test
356 functionality and specificity of the polyclonal sera using more than one assay (i.e., western
357 blots, IHC).[12]

358 We further evaluated each polyclonal serum using archived tissues from ruminants
359 (sheep and cattle), and the results demonstrated that the polyclonal antiserum could be used
360 for the specific detection of *N. caninum* and *T. gondii* in FFPE tissue from naturally occurring
361 protozoan cases. The level of agreement between IHC and PCR results were high for both *T.*
362 *gondii* and *N. caninum* (overall agreement 90.1%). In all cases, the agreement was statistically

363 significant (kappa of 0.42-0.64), suggesting that both tests can be used for the detection of *N.*
364 *caninum* and *T. gondii*. However, it is worth remembering that although PCR analysis is more
365 sensitive, it can only detect genomic DNA whereas IHC can detect the parasite within the
366 tissue samples providing useful information on pathogen location and distribution and
367 allowing visualization of the host tissue response. Findings that some samples were positive
368 by one method and not the other may be the result of the impossibility to test the exact same
369 sections by PCR and IHC, hence giving variable results of cases being positive by one test but
370 negative by the other and vice versa. In general, protozoan parasites are unevenly distributed
371 in tissue samples, making it generally more difficult to obtain matching results unless multiple
372 FFPE scrolls are used with each test.[1]

373 The chances of labelling and detecting *N. caninum* or *T. gondii* using a single tissue
374 block and single section can be low compared to using multiples and thicker sections, and the
375 true number of positives of these protozoan parasites in ruminants could be underestimated. In
376 our study, the percentage of negative results could be explained by only testing one random
377 section from one block with each antiserum, resulting in false-negative results for the animal.
378 It has been shown[1] that certain areas of the ovine brain had a higher density of *T. gondii*
379 parasites than others, and that protozoan tissue cysts and antigen were recorded more
380 frequently in the forebrain and midbrain. Hence, for diagnostic purposes, consideration should
381 be given to the use of multiple sections per block and several blocks per animal. Moreover,
382 blocks in which the protozoan parasites are most likely to occur should be tested more
383 frequently to decrease false-negative results. The limited number of positive IHC results
384 compared to PCR results can be explained by the size of the sample for IHC being a 4-µm
385 section from a single block (and not always from the same tissue block) compared to 20 µm
386 for PCR, decreasing the chance of parasite detection considerably given that parasite
387 distribution is not homogeneous.[27] Moreover, tissue morphology changes considerably with

388 each section cut, and parasites seen in one section might be absent in the following section,

389 especially as these protozoan parasites can be smaller than 4 µm.

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Table 1. Cloning details for the generation of recombinant proteins from *Neospora caninum* and *Toxoplasma gondii.*

Accession (database)	Name	Restriction enzyme	Primer with restriction sites (5'-3')	Vector	Expected protein, aa	Protein molecular weight, kDa	Expected coding sequence, bp
TGME49_233480 (ToxoDB)	Tg-SRS2	SpHI PstI	gcatgcGTTGGTTGCAGGCACAACAC ctgcagAGAATCGGATCCTGCCAAACC	PQE-30	180	19	540
NCLIV_033250 (NCBI)	Nc-SRS2	KpnI XmaI	ggtaccGGTGTCGGGTGCGCCGTTCAAG cccgggTCAGTACGCAAAGATTGCCGTTGC	pQE-31	354	37	1057

Small nucleotides represent the restriction enzyme sites; capitalized nucleotides represent the actual primer.

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489

Table 2. Western blot conditions, including serum dilutions, to confirm specificity of recombinant proteins and of sera raised against recombinant *Neospora caninum* and *Toxoplasma gondii* proteins.

1° antisera	Primary sera/ antisera dilution	Secondary conjugate	Conjugate dilution	Origins
Neospora +ve/-ve bovine serum	1:200	Rabbit anti-bovine HRP (Thermo Fisher)	1:1,000	Moredun Research Institute
Toxoplasma +ve/-ve bovine serum	1:200	Rabbit anti-bovine HRP (Thermo Fisher)	1:1,000	
Rabbit pre-immune sera (1-14)	1:200	Goat anti-rabbit HRP (Thermo Fisher)	1:1,000	This study
Polyclonal rabbit anti-*Neospora* (rNcSRS2)	1:6,000	Goat anti-rabbit HRP (Thermo Fisher)	1:1,000	This study
Polyclonal rabbit anti-*Toxoplasma* (rTgSRS2)	1:2,000	Goat anti-rabbit HRP (Thermo Fisher)	1:1,000	This study

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Table 3. IHC conditions, including serum dilutions, and retrieval treatment to detect *Neospora caninum* and *Toxoplasma gondii* in FFPE tissues.

Protozoan parasite target	Primary antibody	Dilution	Retrieval treatment	Secondary conjugate	Developed from
Neospora caninum	Rabbit anti-*Neospora*-rNcSRS2	1:6,000	PIER	Dako Envision goat anti-rabbit HRP	Recombinant proteins
Toxoplasma gondii	Rabbit anti-*Toxoplasma*-rTgSRS2	1:2,000	PIER	Dako Envision goat anti-rabbit HRP	Recombinant proteins

493

Table 4. Sequences of primers used for the detection of *Neospora*, *Toxoplasma* and *Sarcocystis* spp. DNA from 18S rDNA and the ITS1 region.

Region		Primer	Name	Primer	Amplicon size (bps)	Temperature (C°)	Species specificity	Reference
18S	External	Forward	NTS-18S-F1	5′- GCC ATG CAT GTC TAA GTA TAA G -′3	~470	56	*N. caninum, T. gondii, B. besnoiti, Sarcocystis* spp., *Hammondia* spp,	23
		Reverse	NTS-18S-R1	5′- CCT ATC ATT CCA ATC ACT AGA AAT -′3				
	Internal	Forward	NTS-18S-F2	5'- GGA TAA CCG TGG TAA TTC TAT G -'3	~230	58	*N. caninum, T. gondii, B. besnoiti, Sarcocystis* spp., *Hammondia* spp.	23
		Reverse	NTS-18S-R2	5'- TCC CCG TTA CCC GTC AC -'3				
ITS1	External	Forward	NTH-ITS1-F	5'- CATGAGYTTGYATCTCTCT -'3	~355/330	56	*N. caninum, T. gondii, Hammondia spp.*	This study
		Reverse	NTH-ITS1-R	5'- TTTAGKAAGYAATCTGAAAGC -'3				
	Internal	Forward	Neo-NP1	5'- TAC TAC TCC CTG TGA GTT G -'3	~249	55	*N. caninum*	19
		Reverse	Neo-NP2	5'- TCT CTT CCC TCA AAC GCT -'3				
		Forward	Toxo-NP1	5'- GTG ATA GTA TCG AAA GGT AT -'3	~227		*T. gondii*	6
		Reverse	Toxo-NP2	5'- ACT CTC TCT CAA ATG TTC CT -'3				

494

495 **Figure 1.** Immunohistochemistry with anti-rNcSRS2 or anti-rTgSRS2 antisera on *N.*
496 *caninum*- or *T. gondii*-positive control tissues. **A.** *N. caninum* control tissue (dog brain) with
497 anti-rNcSRS2 antiserum; positive labelling of tachyzoites (arrowhead) and pseudocyst
498 (circle). **B.** Anti-rNcSRS2 antiserum did not label on *T. gondii* control tissue (mouse liver). **C.**
499 Anti-rTgSRS2 antiserum on *T. gondii* control tissue (mouse liver sequential section to image
500 B); positive labelling of tachyzoites (arrowheads) and pseudocyst (circle). **D.** Anti-rTgSRS2
501 antiserum did not label *N. caninum* control tissue (dog brain) negative pseudocyst (circle).
502 **Figure 2.** Summary of positive *N. caninum* and *T. gondii* results from the IHC and PCR
503 tests, and *Sarcocystis* spp. results from the PCR tests, both for ovine and bovine samples
504 separately and for all ruminants combined. The percentage positive column shows the
505 percentage of positive cases and the lower confidence limits (LCL) and upper confidence
506 limits (UCL) columns show the estimated LCLs and UCLs of a 95% CI for this percentage
507 based on a binomial probability distribution.
508
509 **Supplemental Figure 1.** SDS-PAGE gels of proteins purified using 8 M urea. **A.**
510 Purification of rNcSRS2. **B.** Purification of rTgSRS2. The sizes of the molecular weight
511 markers are shown in kDa on the left-hand side of the gel. E = elution (E1-E3) of the
512 recombinant protein from the columns; FT = column flow-through; W = washes (W1-W6).
513 **Supplemental Figure 2**. Western blot analysis of reactivity and cross-reactivity of bovine
514 sera with recombinant *Neospora caninum* and *Toxoplasma gondii* proteins. **A.** *Neospora*-
515 positive bovine serum. **B.** *Neospora*-negative bovine serum. C. *Toxoplasma*-positive bovine
516 serum. D. *Toxoplasma*-negative bovine serum. The sizes of the molecular weight markers are
517 shown in kDa on the left-hand side of each blot. NAF = *Neospora* antigen fraction; TAF =
518 *Toxoplasma* antigen fraction; 1 = rTgSRS2; 2 = rNcSRS2.

519 **Supplemental Figure 3.** Immunohistochemistry using polyclonal sera (dilution 1:2,000)
520 on positive control tissues, with or without citrate (HIER) or pronase (PIER) retrieval
521 methods. **A**. Faint sparce labelling with rabbit anti-rNcSRS2 on *Neospora*-positive control
522 tissue (dog brain), no retrieval. **B**. Labelling with rabbit anti-rNcSRS2 on *Neospora*-positive
523 control tissue (dog brain), HIER. **C**. Strong clear labelling with rabbit anti-rNcSRS2 on
524 *Neospora*-positive control tissue (dog brain), PIER. **D**. Faint labelling with rabbit anti-
525 rTgSRS2 on *Toxoplasma*-positive control tissue (cat brain), no retrieval. **E**: Labelling with
526 rabbit anti-rTgSRS2 on *Toxoplasma*-positive control tissue (cat brain), HIER. **F**: Strong and
527 clear labelling with rabbit anti-rTgSRS2 on *Toxoplasma*- positive control tissue (cat brain),
528 PIER.

529 **Supplemental Figure 4.** Western blot analysis to test reactivity and specificity of the
530 polyclonal rabbit serum raised against recombinant *Neospora caninum* and *Toxoplasma*
531 *gondii* proteins. **A**. Serum raised against rNcSRS2. **B**. Serum raised against rTgSRS2. The
532 sizes of the molecular weight markers are shown in kDa on the left-hand side of each blot.
533 NAF = *Neospora* antigen fraction; TAF = *Toxoplasma* antigen fraction; 1 = rNcSRS2; 2 =
534 rTgSRS2.

535 **Supplemental Figure 5.** Results from FFPE samples selected from the Moredun Research
536 Institute Pathology Archive, marked as having a histologic diagnosis of protozoal infection.
537 The samples were tested by IHC, using polyclonal rabbit serum raised against recombinant
538 *Neospora caninum* and *Toxoplasma gondii* proteins, and PCR in parallel to compare
539 effectiveness of detections of protozoan parasites. NA = not available; NL = not labelled.