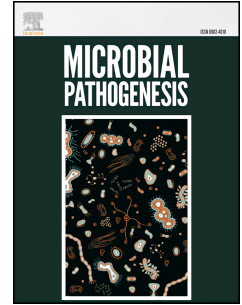


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*Toxoplasma gondii* induces robust humoral immune response against cyst wall antigens in chronically infected animals and humans

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1 ***Toxoplasma gondii* induces robust humoral immune response against cyst wall antigens**  
2 **in chronically infected animals and humans**

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19

20 **Keywords**

21 *Toxoplasma gondii*; bradyzoite; tachyzoite; cyst wall antigens; Humoral immune response

22

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24

25

**26 ABSTRACT**

27 *Toxoplasma gondii* differentiation from proliferating tachyzoites into latent bradyzoites is  
28 central to pathogenesis and transmission. Strong humoral immune response has been reported  
29 against tachyzoite antigens, however, antibody-mediated response towards bradyzoite  
30 antigens is poorly characterized. This work aimed to study the humoral immune response  
31 towards bradyzoite and associated cyst wall antigens particularly CST1. The  
32 immunoreactivity of 404 goats, 88 sheep and 92 human sera to recombinant (CST1 and  
33 SRS9) and native proteins of encysted bradyzoite along with well-established tachyzoite  
34 antigens (SAG1 and GRA7) was determined using ELISA, Western blot and  
35 immunofluorescence analysis (IFA). ELISA results revealed nearly 50% of sera contain *T.*  
36 *gondii* specific antibodies. Results were further validated using Western blot and IFA. *T.*  
37 *gondii* positive sera predominantly recognized the cyst wall besides the known tachyzoite  
38 surface antigens. The presence of CST1 antibodies in seropositive samples were in line with  
39 the staining patterns which were consistent with CST localization. Notably, *T. gondii* IgM-  
40 IgG+ sera recognize the cyst wall whereas IgM+ IgG- sera recognize tachyzoite antigens  
41 indicating acute infection consistent with presence of parasite DNA. The study demonstrates  
42 a strong humoral response against bradyzoite associated cyst wall antigens across naturally  
43 infected animals and humans. CST1 emerged as a key immunomodulatory antigen which  
44 may have direct implications for clinical immunodiagnostics.

45

**46 1. Introduction**

47 Toxoplasmosis, caused by *Toxoplasma gondii*, is an intracellular protozoan infection highly  
48 prevalent in warm-blooded vertebrate hosts. The disease causes abortions, stillbirth, and  
49 neonatal mortality, especially in sheep, goats and swine besides humans, leading to  
50 significant economic losses [1-3]. Humans and animals can become infected by ingestion of

51 food and water contaminated with oocysts or tissue cysts in raw or undercooked meat or  
52 congenitally. Infection not only results in significant reproductive losses in animals, but has  
53 public health implications since consumption of infected meat can facilitate zoonotic  
54 transmission.

55 *Toxoplasma gondii* has a two-stage asexual cycle in warm-blooded animals and a  
56 sexual cycle in Felidae. In the asexual cycle, the two developmental stages are i) tachyzoite:  
57 the rapidly multiplying stage associated with acute infection and ii) bradyzoite: the slowly  
58 multiplying stage associated with chronic infection. The sexual life-cycle takes place  
59 exclusively in enteroepithelial cells of the feline definitive host and results in the excretion of  
60 large numbers of oocysts in the faeces. Animals and humans can be infected with any of the  
61 three major stages.

62 Disease diagnosis is usually based on detection of *T. gondii* specific antibodies.  
63 ELISA is the most preferred serological test used for detection of IgM, IgG and IgA class of  
64 immunoglobulins. Most commercial ELISA kits use Toxoplasma lysate antigen (TLA),  
65 native antigens prepared from tachyzoites grown in mice or *in-vitro* culture. However, in  
66 recent years, many studies have demonstrated that accurate serodiagnosis may also be  
67 achieved using recombinant antigenic proteins of *T. gondii*. Several proteins of the parasite  
68 surface and secretory organelles i.e. microneme, rhoptry and dense granule which are  
69 instrumental for parasite invasion and multiplication have been studied for detection of *T.*  
70 *gondii* specific antibodies [4,5]. A family of surface antigens (SAG) has been demonstrated  
71 to play an important role in host cell invasion and immune modulation [6]. After entering the  
72 host cell, parasite dense granule proteins are secreted into the vacuolar network and the  
73 parasitophorous vacuole (PV) membrane (PVM) [7]. GRA7 is a member of this family that  
74 was earlier identified by serological immunoscreening using infected human sera [8].  
75 Proteins of surface antigen superfamily, microneme, rhoptry and dense granule are potential



76 diagnostic markers and important protective antigens. While these proteins are exposed to the  
77 immune system upon host cell lysis, the dynamics of humoral response is not investigated  
78 completely.

79         Following host immune response, tachyzoites differentiate into the slow-growing  
80 bradyzoite stage. Toxoplasma stage differentiation is accompanied with differential  
81 expression of several, closely related GPI-anchored surface proteins belonging to the SRS  
82 (SAG1-related sequence) superfamily [9]. SAG1 is the most abundant SRS antigen expressed  
83 in the tachyzoite stage [10]. In oral infection with tissue cysts, bradyzoite stage specific SRS  
84 antigens (e.g. SRS9) play a key role in attachment and invasion of intestinal epithelial cells  
85 [11,12]. In fact, following ingestion of tissue cyst, SRS antigens expressed by bradyzoites are  
86 among the first parasite antigens to be presented to the immune system [11,12]. However, the  
87 immunogenicity particularly of the host humoral response against bradyzoite-specific SRS  
88 antigens is not known.

89         Bradyzoite can form tissue cyst in the brain, muscles and visceral organs. Bradyzoite  
90 differentiation and maintenance of tissue cyst are critical for transmission of infection.  
91 During the differentiation process, parasitophorous vacuole membrane (PVM) of tachyzoite  
92 transform into a cyst wall in mature bradyzoite [13,14]. The cyst wall is composed of several  
93 proteins including cyst wall proteins (CST) [15-17], matrix proteins (MAG) [18] and dense  
94 granule proteins [19]. The CST1 is a major cyst wall protein which confers structural  
95 integrity to the cyst wall [15,16]. CST1 among other cyst wall proteins undergo extensive  
96 glycosylation and stain with *Dolichos biflorus* lectin (DBA), which has a high affinity for the  
97 glycosylated structure [16,20,21]. This glycosylation has been proposed to protect cyst wall  
98 proteins from immune responses during chronic infection [22].

99         Several studies indicate that bradyzoite containing cysts are not completely  
100 immunologically silent [23,24]. During the course of infection, intermittent cyst rupture leads

101 to rapid cell-mediated immune response. However, antibody-mediated immune response  
102 against cyst wall antigens is poorly examined. In fact, studies involving experimental  
103 infections have encountered limited humoral responses against bradyzoite as well as cyst wall  
104 antigens. This has led to the widely held notion that there is limited or no humoral response  
105 against bradyzoite containing cyst stage. MAG1 is the only cyst wall matrix protein reported  
106 to produce humoral responses in chronically infected hosts [25,26]. Like MAG1, several cyst  
107 wall proteins can also be potentially immunogenic. In addition, cyst wall and bradyzoite  
108 antigens would also be among the first parasite antigens to be presented to the immune  
109 system following cyst wall rupture [27]. Though, a number of questions remain unanswered  
110 as to the immunogenicity of cyst wall antigens. It is worth mentioning that the diagnostic  
111 usefulness of cyst wall antigens is largely unexplored as most established assays target  
112 tachyzoite stage antigens.

113 In this study, we systematically tested goat, sheep and human sera for anti-*T. gondii*  
114 IgM and IgG specific antibodies in ELISA based on TLA and selected recombinant antigens  
115 (CST1, SRS9, SAG1 and GRA7) of bradyzoite and tachyzoite stages. ELISA results  
116 demonstrated the presence of anti-*T. gondii* antibodies in nearly 50% of animal and human  
117 serum samples. Seropositive samples showed immunoreactivity against intracellularly  
118 replicating tachyzoites and bradyzoites using western blot and immunofluorescence analysis.  
119 The positive sera contain antibodies to a variety of *T. gondii* antigens in addition to tachyzoite  
120 surface antigens. The study also highlights the importance of bradyzoite associated antigens  
121 in eliciting a humoral immune response and their potential utility in serodiagnostic assays.  
122 The study attempted to define how immunostaining patterns vary corresponding to the stage  
123 of infection and a diverse set of parasite antigens may be involved at distinct phases of  
124 pathogenesis. The detection of strong humoral response to cyst wall antigens promises to

125 open new avenues to understanding of *T. gondii* pathogenesis and provides an experimental  
126 basis for devising methodologies to accurately determine the time of infection in hosts.

127

## 128 **2. Materials and Methods**

### 129 *2.1. Blood and serum samples*

130 The use of animal and human serum samples in this study was approved by the Animal and  
131 Human Research Ethics committees of Veterinary College Nagpur (NVC/2019/SC) and  
132 National Institute of Animal Biotechnology (IAEC/NIAB/2019/48/ASD). The written  
133 informed consent was obtained from each human subject. All methods were performed in  
134 accordance with institutional guidelines and regulations ensuring ethically conducted  
135 research. Total 404 goats, 88 sheep and 92 human blood and serum samples were received  
136 from Veterinary College Nagpur, Maharashtra. Serum samples were randomly collected.  
137 Goat and sheep samples were collected only from females. Human blood samples were  
138 collected from veterinary personnel (veterinarians and technicians) and farmers (sheep and  
139 goat). Blood samples were processed for DNA extraction by QIAamp DNA mini kit  
140 (Qiagen). DNA and sera were stored at -20°C until further testing.

141

### 142 *2.2. Parasite culture*

143 *Toxoplasma gondii* strains RH (type I), ME49 (type II) and VEG (type III) were maintained  
144 in primary human foreskin fibroblasts (HFF, ATCC) cells in Dulbecco's modified Eagle's  
145 medium supplemented with 10% FBS, 10 µg/ml Gentamicin and 2mM L-glutamine at 37 °C  
146 and 5% CO<sub>2</sub> in a humidified incubator. Parasites were purified by filtration through 3.0 µm  
147 filters [28].

148

### 149 *2.3. Gene cloning*

150 *T. gondii* RH strain tachyzoites were used to isolate genomic DNA, for PCR amplification  
151 [26]. The primers used to PCR clone are GGAATTCCATATGAAGAAAATAGAGGTTATTCA  
152 and CCCTCGAGTCAAATATCCAGTATTAACGCAGCA for CST1 (98-397 aa);  
153 GGAATTCCATATGGGCGCGGGATCTAGCACT and  
154 CCCTCGAGCAATGAAGCAACAACGAACCC for SRS9 (76-398 aa);  
155 GGAATTCCATATGCCCACTCTTGCGTACTCAC and  
156 CCCTCGAGCGAAGCGTTACCCTGCCA for SAG1 (96-230 aa) and  
157 GGAATTCCATATGGTTGATAGCCTGCGTCCGA and  
158 CCCTCGAGCTGACGTGCATCTTCACCATC for GRA7 (28-236 aa). The amplified DNA  
159 fragments of SRS9, SAG1 and GRA7 were cloned into pET-21a between *NdeI-XhoI* sites  
160 whereas CST1 was cloned into *NdeI-XhoI* sites of pET28a.

161

#### 162 2.4. Expression and purification of recombinant protein and polyclonal antibodies

163 The recombinant proteins: CST1, SRS9, SAG1 and GRA7 were expressed in *Escherichia*  
164 *coli* as C-terminal 6XHis tag and purified as described previously [29]. Briefly, *E. coli* strain  
165 BL21 Rosetta (DE3) (Novagen), transformed with pET-21a- CST1/SRS9/SAG1/GRA7, was  
166 grown in 10 ml LB supplemented with ampicillin (50 µg/ml) and chloramphenicol (34 µg/ml)  
167 overnight at 37°C. Subsequently, 10 ml of the overnight culture was added into 1,000 ml of  
168 LB containing the same antibiotics and incubated at 37°C with vigorous shaking. When the  
169 OD<sub>600</sub> reached 0.4, isopropyl-β-d-thiogalactopyranoside (IPTG) was added to the culture to a  
170 final concentration of 1 mM and the cells were further incubated at 22°C for 16 h. The cells  
171 were then harvested by centrifugation, and the pellets were resuspended in 30 ml of lysis  
172 buffer (50 mM NaH<sub>2</sub>PO<sub>4</sub>, 300 mM NaCl, 10 mM imidazole, 0.1 % Triton X-100 and 0.5mM  
173 PMSF, pH 8.0). After centrifugation, the protein was purified from the supernatant with the  
174 use of a Ni<sup>2+</sup>-NTA agarose (Qiagen)

175 CST1 was purified from the inclusion bodies. The inclusion bodies were solubilized  
176 in 6M GuHCl and the 6xHis tagged CST1 protein was purified over Ni<sup>2+</sup>-NTA agarose under  
177 denaturing condition (Qiagen). The protein was then greatly diluted and refolded (50 mM  
178 Tris-HCl pH 8.5, 9.6 mM NaCl, 0.4 mM KCl, 1mM EDTA, 0.5% Triton X-100, 1mM DTT).

179 Purified recombinant proteins were used to generate specific mouse polyclonal  
180 antibodies. Mouse polyclonal antibodies to recombinant CST1, SRS9, SAG1 and GRA7 were  
181 produced by primary injection in mouse with 30 µg of purified protein in Freund's complete  
182 adjuvant (Sigma) followed by four boosts of 30 µg each in Freund's incomplete adjuvant  
183 (Sigma) at 2-weeks intervals. The antigen emulsion was prepared in total 100 µl volume in a  
184 1:1 ratio with adjuvant. Serum was collected after three weeks of immunization.

185

#### 186 *2.5. Toxoplasma gondii native lysate antigen (TLA)*

187 Toxoplasma lysate antigen (TLA) was prepared as described previously [30]. Freshly  
188 harvested *T. gondii* tachyzoites of RH/ME49/VEG were purified by filtration through 3.0  
189 µm-Nucleopore filter (Sigma). Purified tachyzoites were freeze-thawed in liquid nitrogen  
190 three times followed by sonication to lyse the parasite cells. Further, the parasite lysate was  
191 centrifuged at 12000 g for 30 min at 4°C and supernatant containing *T. gondii* antigens  
192 (TLA) was collected and stored at -80°C. The concentration of protein in the TLA  
193 preparation was determined using BCA Protein Assay Kit (Pierce).

194

#### 195 *2.6. Enzyme-Linked Immunosorbent Assay (ELISA)*

196 ELISA assays were performed as described previously [31]. Nearly 1 µg/ml concentration of  
197 CST1, SRS9, SAG1, GRA7 and TLA were used for coating the plates. Goat, sheep and  
198 human serum samples were used at 1:100 dilution. Bound IgM and IgG of goat, sheep and  
199 human were detected by using horseradish peroxidase (HRP) conjugated antibodies at 1:5000

200 dilution [rabbit anti-goat IgG (Invitrogen) and IgM (Invitrogen), rabbit anti-sheep IgG  
201 (Invitrogen) and IgM (Biorad), and goat anti-human IgG (Invitrogen) and IgM (Invitrogen)].

202 The well validated reference positive and negative sera provided with a commercial  
203 ELISA kits (Abcam, Human IgG-ELISA ab108776; Human IgM ELISA ab108778; and  
204 IDEXX: Sheep and Goat TXT1135T) were used for TLA standardization. The TLA was  
205 standardized for various parameters including its purity, concentration and incubation time  
206 using the standard reference positive and negative serum samples. Subsequently, all serum  
207 samples were analysed and divided into seropositive and seronegative groups in accordance  
208 with the results obtained using the TLA. The cutoff values were determined in both IgG and  
209 IgM ELISAs using 100, 50 and 50 seronegative serum samples of goat, sheep and human  
210 respectively following standard procedure [26,31,32]. For all recombinant antigens, the same  
211 pool of negative sera was used to determine the relative absorbance of each serum sample  
212 and cutoff value. The results were determined for each serum sample by calculating the mean  
213 value of the optical density (OD) reading for duplicate wells. A positive result was defined as  
214 any value higher than the cutoff value plus standard deviations obtained with seronegative  
215 serum groups of goats, sheep and humans.

216

## 217 2.7. Western blotting

218 Filter purified parasites (RH/ME49/VEG) were washed in PBS, and pelleted by  
219 centrifugation. The parasite cell pellet was lysed in SDS sample buffer with a final  
220 concentration of  $2 \times 10^7$  tachyzoites/ml. HFF cells were trypsinised, centrifuged, washed with  
221 PBS, and lysed in SDS sample buffer at a final concentration of  $4 \times 10^6$  cells/ml. Separated  
222 proteins (Twenty  $\mu$ l of each lysate or 500 ng/ $\mu$ l of recombinant CST1/SRS9/SAG1/GRA7  
223 protein) were electrotransferred to PVDF membrane (1 hr, 120mA/cm gel<sup>2</sup>). After blocking  
224 with 5% fat-free milk in PBS, the membrane was incubated for 1h at room temperature with

225 serum samples diluted in PBS (Goat/sheep/human 1:100 and CST1/SRS9/SAG1/GST1  
226 1:1000), washed and incubated with HRP-conjugated secondary antibodies diluted at 1:5000  
227 (details of antibodies are mentioned in the ELISA method). The proteins on these PVDF  
228 membranes were visualized with super Enhanced Chemiluminescent Substrate (Pierce).

229

### 230 2.8. Indirect Immunofluorescence assay (IFA)

231 IFA was performed as described elsewhere [33]. Confluent monolayers of HFF cells were  
232 grown on coverslip in 6-well plates and infected with RH strain of *T. gondii* parasites. The  
233 infected cells were fixed post 32 to 36 h of infection in 4% paraformaldehyde, followed by  
234 permeabilization with 0.35% Triton X-100. The samples were further blocked in 2% BSA  
235 and incubated sequentially with primary and secondary antibodies. Goat, sheep and human  
236 serum samples were used at 1:50 dilution each. Mouse anti-CST1, mouse anti-SRS9, mouse  
237 anti-SAG1 and mouse anti-GRA7 were used at 1:500 dilution each. Alexa Fluor-488  
238 conjugated secondary antibodies [anti-goat IgG (Invitrogen), anti-goat IgM (Invitrogen), anti-  
239 sheep IgG (Invitrogen), anti-sheep IgM (Biorad), anti-human IgG (Invitrogen), anti-human  
240 IgM (Invitrogen), anti-mouse IgG (Invitrogen)] were used at a dilution of 1:1000. Coverslips  
241 were mounted with Vectashield medium (VectorLabs) with 4',6-Diamidino-2-phenylindole  
242 dihydrochloride (DAPI) on glass slide and images were captured with a Leica Confocal  
243 microscope with 100× objective. Images were processed using LAS X software.

244 Bradyzoite induction was performed as described elsewhere [34]. Briefly, filter  
245 purified parasites (ME49) were inoculated into a confluent HFF monolayer in a 6-well plate  
246 at a multiplicity of infection of 0.5 to 1.0. After 2-h incubation at 37°C in 5% CO<sub>2</sub>, the  
247 medium was changed to RPMI with 50 mM HEPES (pH 8.2) without NaHCO<sub>3</sub>,  
248 supplemented with 1% FBS and 10 µg/ml Gentamicin, and incubated in a humid 37°C  
249 incubator without CO<sub>2</sub> for 5 days. Bradyzoite induction medium was changed every 2 days.

250 Bradyzoites IFA were performed as for tachyzoites. Rhodamine labeled- *Dolichus biflorus*  
251 Agglutinin (DBA, VectorLabs), a lectin that recognizes cyst wall proteins was used (conc. 10  
252 µg/ml) as a bradyzoite marker. Serum samples were marked positive for anti-*T. gondii*  
253 antibodies if specific signals were obtained from the parasite structures. CST1 antibody was  
254 used to label the cyst wall as well as bradyzoite marker.

255

### 256 2.9. Polymerase chain reaction (PCR)

257 The triplex PCR assay was used to screen blood samples for *T. gondii* DNA [35]. For PCR  
258 amplification three targets namely B1 gene [36], 529 bp repetitive element [37] and ITS-1  
259 [38] were used. The primers were used to amplify a 747 bp fragment of the B1 gene were  
260 TCGCAGTACACCAGGAGTTG and CACTCCATCTCTCGTCTTCT, 234 bp fragment of  
261 the ITS-1 were ACACGTCCTTATTCTTTATTAACCA and  
262 ATCCCAACAGAGACACGAATT and 182 bp fragment of the 529 bp were  
263 TGTGCTTGGAGCCACAGAAG and GCAGCCAAGCCGGAAACAT respectively.  
264 Following cycle conditions were used: 95°C, 5 min followed by 35 cycles of 95°C, 30 s;  
265 59°C, 30 s, and 72°C, 30 s.

266

### 267 2.10. Statistical analysis

268 Statistical analysis of the ELISA results was performed using GraphPad Prism software and  
269 analysed by unpaired student's t test.

270

271

## 272 3. Results



273 *3.1. Reactivity of IgM and IgG antibodies from goat, sheep and human sera with TLA and*  
274 *recombinant antigens (CST1, SRS9, SAG1, and GAR7) in ELISA*

275 To investigate the presence of humoral immune response against cyst wall and bradyzoite  
276 antigens, we tested the immunoreactivities of CST1 (major cyst wall antigen), SRS9  
277 (bradyzoite surface antigen) and compared with SAG1 (tachyzoite surface antigen) and  
278 GRA7 (PV-associated antigen) in ELISA using animal and human sera. In order to evaluate  
279 antigenicity of recombinant antigens, CST1 (98-397 aa), SRS9 (76-398 aa), SAG1 (96-230  
280 aa) and GRA7 (28-236) were bacterially expressed (Fig. 1A) and purified using C-terminal  
281 (N-terminal in case of CST1) histidine tags (Fig 1B). In case of SRS9, GRA7 and SAG1,  
282 signal peptide sequence was avoided for recombinant protein generation. SAG1 protein was  
283 further truncated in order to get good quality protein. In case of CST1, only N-terminus was  
284 used to make the recombinant proteins as this domain was sufficient enough to elicit a strong  
285 immune response [16] and was devoid of SRS domain to avoid the cross reactivity with other  
286 SRS domain containing proteins. Except CST1 all other proteins SRS9, SAG1, and GRA7  
287 were expressed as soluble proteins with estimated molecular masses of ~32kDa, ~37 kDa,  
288 ~18 kDa, and ~23 kDa respectively (Fig. 1B).

289 Five separate IgM and IgG ELISAs were developed using standardized TLA, CST1,  
290 SRS9, SAG1, and GRA7 as a coating antigen to determine the seroprevalence and evaluate  
291 the potential of each of these antigens for the serodiagnosis of toxoplasmosis in goat, sheep  
292 and human. A total 404 of goat, 88 sheep and 92 human serum samples were examined. All  
293 serum samples were divided into seropositive and seronegative groups in accordance with the  
294 results obtained using the TLA. Seronegative samples of goat, sheep and human were used to  
295 determine the cutoff values. A positive result was defined as any value higher than the cutoff  
296 value plus standard deviations obtained with seronegative serum groups of goats, sheep and  
297 humans in respective ELISAs.

298 In case of 404 goat serum samples in IgM ELISA (Fig. 1C), the CST1, SRS9, SAG1,  
299 GRA7, and TLA reacted with 167 (41.3%), 62 (15.3%), 178 (44%), 170 (42%), and 184  
300 (45.5%) of the positive sera respectively (Table 1), whereas in IgG ELISA (Fig. 1D), the  
301 CST1, SRS9, SAG1, GRA7, and TLA reacted with 165 (40.8%), 77 (19 %), 165 (40.8%),  
302 160 (39.6%), and 171 (42.3%) of the positive sera respectively (Table 2). In case of 88 sheep  
303 serum samples in IgM ELISA (Fig. 1C), the CST1, SRS9, SAG1, GRA7, and TLA reacted  
304 with 35 (39.7%), 14 (15.9%), 36 (40.9%), 35 (39.7%), and 38 (43.1%) of the positive sera  
305 respectively (Table 1), whereas in IgG ELISA (Fig. 1D), the CST1, SRS9, SAG1, GRA7, and  
306 TLA reacted with 29 (32.9%), 16 (18.1%), 32 (36.3%), 32 (36.3%), and 34 (38.6%) of the  
307 positive sera respectively (Table 2). In case of 92 human serum samples in IgM ELISA (Fig.  
308 1C), the CST1, SRS9, SAG1, GRA7, and TLA reacted with 36 (39.1%), 15 (16.3%), 35  
309 (38%), 35 (38%), and 37 (40.2%) of the positive sera respectively (Table 1), whereas in IgG  
310 ELISA (Fig. 1D), the CST1, SRS9, SAG1, GRA7, and TLA reacted with 35 (38%), 19  
311 (20.6%), 38 (41.3%), 37 (40.2%), and 40 (43.4%) of the positive sera respectively (Table 2).  
312 Overall, based on both the ELISA results, the percentage of seropositivity obtained using  
313 CST1, SAG1 and GRA7 was comparable to that of TLA. However, for SRS9 the number of  
314 reactive serum samples observed were lower with respect to CST1, SAG1, GRA7 and TLA.

315 The sensitivity of both IgM and IgG ELISAs calculated from all positive goat, sheep  
316 and human serum samples tested, were different for the individual antigens (Table 1,2). In  
317 both the ELISAs irrespective of species, higher sensitivity was observed for CST1 (IgM: 90.8  
318 to 97.3% and IgG: 85.3 to 96.5%), SAG1 (IgM: 94.6 to 96.7% and IgG: 94.1 to 96.5%) and  
319 GRA7 (IgM: 92.1 to 94.6% and IgG: 92.5 to 94.1%), whereas lowest sensitivity was noticed  
320 for SRS9 (IgM: 33.7 to 40.5% and IgG: 45 to 47.5%) compared to TLA. In the same  
321 experiment, slightly lower specificity was observed for CST1 (IgM: 92.8 to 97.3% and IgG:

322 94.3 to 96.2%) SAG1 (IgM: 94.6 to 100% and IgG: 100%), GRA7 (IgM: 96.4 to 100% and  
323 IgG: 97.9 to 100%) and SRS9 (IgM: 92.8 to 98% and IgG: 96.3 to 100%) compared to TLA.

324 Together, based on the TLA-ELISA results, 49.5% goat, 50% sheep, and 54.34%  
325 human sera contain *T. gondii* specific IgM-positive/ IgG-positive (IgM+ IgG+) antibodies  
326 (Table 3); 7.17% goat, 11.36% sheep, and 10.86% human sera contain *T. gondii* specific  
327 IgM-positive/ IgG-negative (IgM+ IgG-) antibodies (Table 3); 3.96% goat, 6.81% sheep, and  
328 14.13% human sera contain *T. gondii* specific IgM-negative/ IgG-positive (IgM- IgG+)  
329 antibodies (Table 3).

330 Statistical analysis confirmed a high sensitivity of ELISA with CST1 for serum  
331 samples from IgM- IgG+ and IgM+ IgG+ as compared to IgM+ IgG-. The results obtained  
332 for CST1 were found to be statistically significant ( $P < 0.001$ ).

333

### 334 3.2. *T. gondii* positive sera recognize both recombinant and native parasite proteins

335 To further characterize the IgG positive sera (Table 3), Western blot analysis was performed  
336 using recombinant CST1, SRS9, SAG1, and GRA7 proteins. All serum samples (irrespective  
337 of species) recognized CST1, SRS9, SAG1, and GRA7 recombinant proteins. In each  
338 species, nearly 70% serum samples showed immunoreactivity to recombinant CST1, SRS9,  
339 SAG1 and GRA7 proteins either singly or in combinations whereas the remaining 30% serum  
340 samples recognized all the four recombinant proteins (Fig. 2A,C,E). Further, the same set of  
341 serum samples were used to determine the immunoreactivity against *T. gondii* native  
342 proteins. To test that, parasite lysates of all three strains RH (type I), ME49 (type II), and  
343 VEG (type III) of *T. gondii* were used in Western blot analysis. All sera that recognized  
344 recombinant proteins (Fig. 2A,C,E), also recognized different native proteins in the parasite  
345 lysates of all the three strains (Fig. 2B,D,F), suggesting presence of antibodies against a  
346 variety of parasite proteins in naturally acquired *T. gondii* infection. These serum samples did

347 not recognize any prominent protein band in the HFF host cell lysate indicating presence of  
348 specific antibodies against parasite proteins (Fig. 2B,D,F). In the similar experimental  
349 conditions, none of the ELISA negative sera showed immunoreactivity towards recombinant  
350 or native parasite proteins (Supplementary Fig. 1A-C). Many *T. gondii* antigens show high  
351 sequence homology with antigens of *Neospora caninum* (closest phylogenetic relative to *T.*  
352 *gondii*). The possibility of immunoreactivity due to cross-reacting antibodies specific against  
353 *Neospora caninum* (IDEXX) was tested using a similar experiment. Neither recombinant  
354 antigens nor native parasite proteins showed any cross reactivity with *Neospora caninum*  
355 antibodies (Supplementary Fig. 1D). These results are in accordance with previous findings  
356 [39,40].

357 Further, we determined the localization of CST1, SRS9, SAG1, and GRA7 proteins in  
358 the parasite using specific antibodies against these proteins. Purified recombinant proteins  
359 (Fig. 1B) were used to generate specific mouse polyclonal antibodies. Antisera obtained  
360 against CST1 and SRS9 recognized the native protein in the bradyzoite stage lysate of the  
361 parasite at the expected molecular weight ~250 kDa and ~37 kDa respectively (Fig. 3A, B).  
362 Neither antibody showed any cross reactivity with tachyzoite nor host cell (HFF) proteins  
363 confirming their expression exclusively in the bradyzoite stage of the parasite. SAG1 and  
364 GRA7 antisera recognized the recombinant proteins (SAG1 ~18 kDa and GRA7 ~23 kDa)  
365 and confirmed their native expression (SAG1 ~30 kDa and GRA7 ~23 kDa) (Fig. 3C,D). The  
366 specific antibodies were utilized to determine the localization of CST1, SRS9, SAG1, and  
367 GRA7 proteins in the parasite using immunofluorescence assay (IFA). As expected, antisera  
368 of CST1, SRS9, SAG1, and GRA7 stained cyst wall (encysted bradyzoite stage) (Fig. 3A),  
369 bradyzoite surface (encysted bradyzoite stage) (Fig. 3B), tachyzoite surface (Fig. 3C), and  
370 parasitophorous vacuole membrane (tachyzoite stage) (Fig. 3D) respectively. *Dolichos*

371 *biflorus* Agglutinin (DBA) which binds to the cyst wall was used as encysted bradyzoite  
372 stage marker [16].

373

374 *3.3. T. gondii positive sera predominantly recognize cyst wall antigens in encysted bradyzoite*

375 Western blot results revealed that *T. gondii* positive serum samples contain antibodies against

376 a variety of parasite proteins including CST1, SRS9, SAG1, and GRA7. To verify these

377 results, we investigate whether the antibodies against these antigens could also recognize

378 antigens expressed by encysted bradyzoites (cyst stage). To test that, ME49 strain of *T.*

379 *gondii* was induced for bradyzoite development in tissue culture. *T. gondii* seropositive

380 samples of goat, sheep and human were examined to detect encysted parasites (bradyzoite

381 stage) by IFA. All sera tested against encysted bradyzoites showed two distinct staining

382 patterns *viz* i) cyst wall staining, ii) bradyzoite surface (Fig. 4A-C). Observed

383 immunostaining patterns were compared with reference cyst wall staining of CST1 and

384 bradyzoite surface of SRS9 (Fig. 3A,B) for confirmation. The predominant immunostaining

385 pattern was observed on the cyst wall which is suggestive of what is seen with CST1

386 antibodies (Fig. 3A). Of the 171 goat serum samples that stained bradyzoites, 155 (90.6%), 4

387 (2.3%) and 12 (7%) showed the cyst wall, bradyzoite surface, and mixed staining patterns

388 respectively (Fig. 4A) (Table 4). Similarly, of the 34 sheep serum samples, 31 (91.2%), 1

389 (2.9%), and 2 (5.8%) showed the cyst wall, bradyzoite surface, and mixed staining patterns

390 respectively (Fig. 4B) (Table 4). Likewise, of the 40 human serum samples that stained

391 bradyzoites, 36 (90%), 1 (2%), and 3 (7.5%) showed the cyst wall, bradyzoite surface, and

392 mixed staining patterns respectively (Fig. 4C) (Table 4). Together, based on the encysted

393 bradyzoite IFA results, ~90% *T. gondii* positive serum samples of goat, sheep and humans

394 were shown to have cyst wall staining patterns. In the similar experimental conditions, none

395 of the ELISA and Western blot negative sera showed immunoreactivity towards encysted  
396 bradyzoites tested using IFA (Supplementary Fig. 2A-C).

397

#### 398 *3.4. T. gondii positive sera predominantly recognize tachyzoite surface antigens*

399 Like encysted bradyzoites, we questioned whether the same set of serum samples that  
400 recognized cyst wall and bradyzoite antigens could also recognize diverse antigens of  
401 tachyzoites. To test that we performed IFA with the same set of serum samples against  
402 intracellular tachyzoites of RH stain parasites grown in HFF cells. All sera tested against  
403 intracellular tachyzoites showed three distinct staining patterns viz i) parasite  
404 surface/membrane staining, ii) parasitophorous vacuole membrane (PVM) staining and iii)  
405 parasitophorous vacuole internal (PV-internal) (Fig. 5A-C). Observed immunostaining  
406 patterns were compared with reference tachyzoite surface staining of SAG1, and PVM  
407 staining of GRA7 (Fig. 3C,D) for confirmation. The predominant immunostaining pattern  
408 was observed on parasite membrane, which is suggestive of what is seen with SAG  
409 antibodies (Fig. 3C). Of the 171 goat serum samples that stained intracellular tachyzoites, 135  
410 (78.9%), 30 (17.5%), 2 (1.2%), and 4 (2.3%) showed parasite surface, PVM, PV internal and  
411 mixed staining patterns respectively (Fig. 5A) (Table 4). Similarly, Of the 34 sheep serum  
412 samples tested, 27 (79.4%), 5 (14.7%), 1 (2.9%), and 1 (2.9%) showed parasite surface,  
413 PVM, PV internal and mixed staining pattern respectively (Fig. 5B) (Table 4). Likewise, of  
414 the 40 human serum samples that stained intracellular tachyzoites, 32 (80%), 6 (15%), 1  
415 (2.5%), and 1 (2.5%) showed parasite surface, PVM, PV internal and mixed staining patterns  
416 respectively (Fig. 5C) (Table 4). Together, based on the intracellular tachyzoites IFA results,  
417 ~80% *T. gondii* positive serum samples of goat, sheep and humans were shown tachyzoite  
418 surface staining pattern whereas 15-18% showed staining on PVM. In the similar  
419 experimental conditions, none of the ELISA and Western blot negative sera showed

420 immunoreactivity towards intracellular tachyzoites tested using IFA (Supplementary Fig. 3A-  
421 C).

422

### 423 3.5. *T. gondii* IgM+ IgG- sera recognize tachyzoite surface antigens only

424 In earlier experiments, we showed that IgM+ IgG+ *T. gondii* sera recognize both tachyzoite,  
425 and encysted bradyzoite antigens suggesting presence of humoral immune response against  
426 acute and chronic infections. In *T. gondii* infection, IgM level can persist for several years,  
427 therefore, a chronic *T. gondii* infection can be erroneously categorized as an acute infection.  
428 For these reasons, we selected only IgM+ IgG- *T. gondii* sera (Table 3) and tested whether  
429 these sera recognize tachyzoite or bradyzoite or both the stages. We used *T. gondii* IgM+  
430 IgG- sera from each species (29 goat, 10 sheep and 10 human; Table 3) and performed IFA  
431 against intracellularly replicating tachyzoites and *in-vitro* induced bradyzoites. IgM-IFA  
432 showed specific reactivity towards intracellular tachyzoites and not the bradyzoites. The  
433 immunofluorescence patterns by IgM staining in the tachyzoite stage were similar to that  
434 observed for IgG staining in earlier experiments (Fig. 4A-C). Unlike earlier observed distinct  
435 immunostaining patterns for intracellular tachyzoites here we observed only tachyzoite  
436 surface staining for all the sera tested (Fig. 6A-C). The immunostaining pattern was observed  
437 on parasite membrane, which is suggestive of what is seen with SAG antibodies (Fig. 3C).  
438 However, none of the serum samples which were IgM+ IgG- showed detectable  
439 immunofluorescence against *in-vitro* induced bradyzoites. To test further, we performed  
440 CST1 Western blot analysis with the same set of sera, none of the serum samples detected  
441 recombinant CST1 protein (data not shown), consistent with an acute infection or initial  
442 antibody response directed against tachyzoites.

443

### 444 3.6. *T. gondii* IgM+ IgG- goat, sheep and human show active infection



445 Serological diagnosis of toxoplasmosis provides high sensitivity, but specificity varies  
446 depending on the test used. Following acute infection, *T. gondii* IgM antibodies may persist  
447 for many months or even years. These complicate the correct interpretation of a positive *T.*  
448 *gondii* IgM result. Therefore, the detection of *T. gondii* DNA using PCR is a useful  
449 laboratory tool particularly during early acute infections. PCR based assay that targeted the  
450 B1 gene, ITS1 and repetitive region 529 bp could detect 10 parasites in the presence of  
451 100,000 human leukocytes [36]. Earlier study has demonstrated triplex PCR containing  
452 primer sets for all three abovementioned regions using different body fluids and DNA  
453 extracted from the organs of animals infected with *T. gondii* [35]. Accordingly, the triplex  
454 PCR was used to detect *T. gondii* specific DNA in peripheral blood mono-nuclear cells  
455 (PBMCs) of IgM+ IgG- 29 goat, 10 sheep and 10 human (Table 3, Fig. 6A-C). We observed  
456 that 25 (86.2%) of 29 goat, 9 (90%) of 10 sheep and 9 (90%) of 10 human samples were  
457 found to be positive for *T. gondii* specific DNA. For representative purpose one positive  
458 sample from IgM+ IgG- goat, sheep and human were shown (Fig. 7). RH strain DNA and  
459 IgM- IgG- goat sample were used as positive and negative controls respectively. This result  
460 suggests that *T. gondii* IgM+ IgG- individuals may carry an active infection. Hence, the  
461 combination of both the tests may help to improve the sensitivity of early stage toxoplasma  
462 detection.

463

#### 464 **4. Discussion**

465 The study focused on understanding the humoral immune responses against recombinant and  
466 native antigens of encysted bradyzoite and tachyzoite stages of *T. gondii*. To this aim, we  
467 demonstrated that robust humoral immune response is generated against cyst wall antigens  
468 which is comparable to the immune response generated against tachyzoite surface antigens in  
469 *T. gondii* infected animals and humans. The immunoreactivity of sera to recombinant and



470 native proteins of encysted bradyzoite and tachyzoite origins was established using ELISA,  
471 Western blot and immunofluorescence analysis. The recombinant proteins represent key  
472 immunodominant antigens of cyst wall, CST1; bradyzoite surface, SRS9; tachyzoite surface,  
473 SAG1; and tachyzoite PVM, GRA7. Based on IgM and IgG ELISA results nearly 50% serum  
474 samples of goat, sheep and human were found positive for *T. gondii* specific antibodies.

475         Recombinant CST1, SAG1 and GRA7 proteins have a greater diagnostic performance  
476 than SRS9 recombinant protein. Notably, we found a meagre antibody response against  
477 bradyzoite surface antigen, SRS9, than to cyst wall antigen, CST1. This limited humoral  
478 response towards bradyzoite surface antigens compared with cyst wall antigens could be  
479 because cyst wall antigens are more exposed to immune cells upon cyst wall rupture during  
480 reactivation of infection as well as oral infection. While the usefulness of recombinant SAG1  
481 and GRA7 in diagnosis of human and animal toxoplasmosis have been previously reported  
482 [41-44], the utility of cyst wall antigen like CST1 and others requires to be thoroughly  
483 explored. The present study reinforces the idea that bacterial recombinant antigens offer  
484 many advantages in the *Toxoplasma* diagnosis as they allow better standardization of the  
485 tests and reduce the costs of production. Accordingly, more comprehensive understanding of  
486 antigens expressed at different stages of *T. gondii* is needed. However, it is evident from IFA  
487 and western blot results that by incorporating additional parasite antigens efficacy of  
488 serological diagnosis could be significantly improved. This study offers robust evidence to  
489 support the incorporation of additional parasite antigens expressed across different  
490 developmental stages to the current repertoire of antigens to improve detection of parasite  
491 specific antibodies in both animals and humans.

492         We observed nearly 40% of serum samples irrespective of species are IgM+ positive.  
493 Most of these samples were also IgG+ and only 29% in goats and 10% each in sheep and  
494 humans were IgG- (Table 3). This is not unusual with respect to the *T. gondii* infection as

495 IgM level can persist for several months to years after an acute infection [45]. Therefore, only  
496 IgM positive test is not a good marker of an acute infection. This observation is consistent  
497 with our IFA results where *T. gondii* IgM+ IgG+ sera are able to recognize both tachyzoite  
498 and bradyzoite stage antigens whereas IgM+ IgG- sera do not recognize bradyzoite antigens  
499 but recognize only tachyzoite antigens. These results suggest that IgM testing in different  
500 assays like ELISA and IFA can be used for detection of acute *T. gondii* infection. In fact,  
501 PCR in addition to IgM-ELISA or IgM-IFA is a good method to determine an acute infection.  
502 With the use of triplex PCR, we observed 86 to 90% of *T. gondii* IgM+ IgG- animals and  
503 humans were positive for parasite DNA. These results suggest that most of the IgM+ IgG-  
504 individuals are suffering from an acute infection. Therefore, the combination of both the tests  
505 may help to improve the sensitivity of early stage toxoplasma detection more than either PCR  
506 or IgM-ELISA alone.

507         The tachyzoite to bradyzoite stage conversion might take place early during infection.  
508 However, it should be noted that bradyzoite and cyst proteins are released from ingested  
509 parasites within the gastrointestinal tract during primary infection. Thus, the host immune  
510 response against cyst wall and bradyzoite antigens could originate from this first exposure.  
511 With the advancement in bradyzoite biology, now we know that cysts are dynamic structures;  
512 they regularly break down or rupture host cells [3,46]. When tissue cyst rupture, they elicit a  
513 strong inflammatory response in chronically infected hosts. In line with this, we also  
514 observed the presence of anti-*T. gondii* IgG antibodies against bradyzoite antigens in animal  
515 and human sera. Though the antigens from tachyzoite and bradyzoite shared a fair level of  
516 homology, we observed a diverse immunoreactivity pattern in IFA against both the stages of  
517 the parasite indicating the presence of humoral response towards stage specific antigens. This  
518 is consistent with recent studies which detected bradyzoite specific antibodies from *T. gondii*  
519 positive human sera and chronically infected mice [25,47]. On similar lines, bradyzoite

520 specific humoral response in human sera was demonstrated recently by detecting a variety of  
521 immunostaining patterns for bradyzoites [48]. Therefore, the onset of anti-bradyzoite  
522 antibody response and the role of humoral response in controlling encysted latent infection  
523 merit further investigation.

524 It is established that cell-mediated immunity plays an important role in the host  
525 resistance to *T. gondii* infection [49], but this study has highlighted the importance of the  
526 humoral response to this intracellular pathogen which is consistent with other reports [50,51].  
527 Our study further demonstrates that specific cyst wall and bradyzoite antigens may contribute  
528 to the stimulation of humoral immunity against *T. gondii* infection in animals and humans,  
529 supporting the hypothesis that a combination of bradyzoite and tachyzoite antigens should be  
530 used for development of serological tests. Also, it is important to determine if the detection of  
531 anti-bradyzoite antibodies can serve as a valuable tool to distinguish between acute and  
532 chronic infection and merits further investigation.

533 The present study showed that 50% of veterinary personnel contain *T. gondii* specific  
534 antibodies and this percentage did appear to be high. As per our knowledge, this is the first  
535 study of its kind conducted in India. However, more samples need to be screened to support  
536 this observation. Similar study was undertaken in Malaysia, in which nearly 20%  
537 seroprevalence was reported among people having close contact with animals [52]. Based on  
538 these results, primary screening of Toxoplasma infection should be particularly initiated in  
539 high titer seropositive individuals like veterinary personnel, pet owners and women. This  
540 program not only help to reduce the Toxoplasmosis incidence in high risk groups but also  
541 enables us to identify the potential risk factors of infection.

542 In summary, the present study provides strong evidence for the presence of humoral  
543 immune response towards cyst wall antigens in naturally acquired *T. gondii* infections.  
544 However, it is not known whether the presence of antibodies to bradyzoite antigens has a

545 protective effect in *T. gondii* infected animals or humans and needs to be keenly looked at.  
546 This study broadens our understanding of humoral dynamics and add to the repertoire of  
547 immunomodulatory antigens of *T. gondii*. Moreover, it provides an experimental basis for not  
548 defining and designing diagnostic and therapeutic approaches for its clinical management.  
549 Further prospective studies to examine immunoreactivity towards other cyst wall antigens are  
550 warranted.

551

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559

### 560 **Author statement**

561 Abhijit S. Deshmukh: Conceptualization, Data curation, Funding acquisition, Methodology,  
562 Investigation and Writing- Original draft preparation. Rajkumar Gurupwar: Resources and  
563 investigation. Pallabi Mitra: Investigation, Writing- Original draft preparation. Kalyani  
564 Aswale: Investigation and Formal analysis. Shilpshri Shinde: Resources and investigation.  
565 Sandeep Chaudhari: Resources and investigation.

566

### 567 **Conflict of interest statement**

568 The authors declare no competing interests.

569

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748 **Tables**

749 **Table 1.** Comparison of the immunoreactivities of recombinant CST1, SRS9, SAG1, and  
750 GRA7 proteins and TLA in the IgM ELISA using pools of goat, sheep and human sera.

751

752 **Table 2.** Comparison of the immunoreactivities of recombinant CST1, SRS9, SAG1, and  
753 GRA7 proteins and TLA in the IgG ELISA using pools of goat, sheep and human sera.

754

755 **Table 3.** TLA-ELISA (IgM and IgG) results using pools of goat, sheep and human sera.

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757 **Table 4.** Different immunofluorescence patterns detected for encysted bradyzoites and  
758 intracellular tachyzoites by positive serum for anti-*Toxoplasma gondii* antibodies.

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773 **Figure Legends**

774 **Fig. 1.** Comparison of immunoreactivities of the recombinant proteins and TLA in IgM and  
 775 IgG ELISA using goat, sheep and human sera. (A) Schematic diagram of full length CST1,  
 776 SRS9, SAG1 and GRA7. Gene details including ToxoDB accession number, SP: signal  
 777 peptide region and recombinant protein region are shown. (B) SDS-PAGE profiles of the  
 778 purified recombinant proteins: CST1, SRS9, SAG1, and GRA7. (C) Comparison of the  
 779 immunoreactivities of CST1, SRS9, SAG1, GRA7, and TLA in the IgM ELISA using sera  
 780 samples of goat, sheep and human. (D) Comparison of the immunoreactivities of CST1,  
 781 SRS9, SAG1, GRA7, and TLA in the IgG ELISA using sera samples of goat, sheep and  
 782 human. The horizontal lines represent the cutoff values.

783  
 784 **Fig. 2.** Western blotting analyses against recombinant proteins and HFF infected RH or  
 785 ME49 or VEG strain of *Toxoplasma gondii* tachyzoites. Representative results of Western  
 786 blotting analyses against recombinant proteins and *T. gondii* strains (RH, ME49 and VEG)  
 787 were tested with anti-*T. gondii* goat (A-B), sheep (C-D) and human (E-F) serum samples.

788  
 789 **Fig. 3.** WB and IFA using bradyzoite and tachyzoite stage specific markers. (A,B)  
 790 Expression of CST1 and SRS9 in bradyzoite stage parasite was analysed by Western blotting  
 791 with anti-CST1 and anti-SRS9 antibodies. Specific antibodies recognized native protein  
 792 (CST1 ~250 kDa and SRS9 ~37 kDa). *T. gondii* inner membrane complex 1 (IMC1), was  
 793 used as a loading control for both tachyzoite and bradyzoite lysates. (C,D) Expression of  
 794 SAG1 and GRA7 in tachyzoite stage parasite was analysed by Western blotting with anti-  
 795 SGA1 and anti-GRA7 antibodies. Specific antibodies recognized recombinant (SAG1 ~18  
 796 kDa and GRA7 ~23 kDa) and native protein (SAG1 ~30 kDa and GRA7 ~23 kDa). (E) anti-  
 797 CST1 antibodies stained “cyst wall”. (F) anti-SRS9 antibodies stained “bradyzoite surface”.

798 (G) anti-SAG1 antibodies stained “tachyzoite surface” and (H) anti-GRA7 predominantly  
799 stained “parasitophorous vacuole”. DIC: Differential interference contrast.

800

801 **Fig. 4.** IFA with *Toxoplasma gondii* positive sera exhibit two prominent immunofluorescence  
802 patterns viz cyst wall and parasite surface in the bradyzoites. (A-C) Representative images of  
803 each staining pattern using goat (A), sheep (B) and human (C) are shown. Scale bar = 5  $\mu$ m.

804

805 **Fig. 5.** IFA with *Toxoplasma gondii* positive sera exhibit three prominent  
806 immunofluorescence patterns viz parasite surface, PVM and PV - internal in the intracellular  
807 tachyzoites. (A-C) Representative images of each staining pattern using goat (A), sheep (B)  
808 and human (C) are shown. Scale bar = 5  $\mu$ m.

809

810 **Fig. 6.** IFA with *Toxoplasma gondii* IgM+ IgG- sera recognize only tachyzoites and not  
811 bradyzoites. (A-C) Representative images of each staining pattern using serum samples of  
812 goat (A), sheep (B) and human (C) are shown. Scale bar = 5  $\mu$ m.

813

814 **Fig. 7.** Agarose gel electrophoresis of triplex PCR products viz B1 gene, ITS1 and 529  
815 regions. Representative PCR amplification using DNA samples from IgM+ IgG- goat, sheep  
816 and human. *T. gondii* RH strain DNA and IgM- IgG- goat DNA were used as positive and  
817 negative controls respectively.

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824 ***Toxoplasma gondii* induces robust humoral immune response against cyst wall antigens**  
825 **in chronically infected animals and humans**

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843 **Keywords**

844 *Toxoplasma gondii*; bradyzoite; tachyzoite; cyst wall antigens; Humoral immune response

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849 **ABSTRACT**

850 *Toxoplasma gondii* differentiation from proliferating tachyzoites into latent bradyzoites is  
851 central to pathogenesis and transmission. Strong humoral immune response has been reported  
852 against tachyzoite antigens, however, antibody-mediated response towards bradyzoite  
853 antigens is poorly characterized. This work aimed to study the humoral immune response  
854 towards bradyzoite and associated cyst wall antigens particularly CST1. The  
855 immunoreactivity of 404 goats, 88 sheep and 92 human sera to recombinant (CST1 and  
856 SRS9) and native proteins of encysted bradyzoite along with well-established tachyzoite  
857 antigens (SAG1 and GRA7) was determined using ELISA, Western blot and  
858 immunofluorescence analysis (IFA). ELISA results revealed nearly 50% of sera contain *T.*  
859 *gondii* specific antibodies. Results were further validated using Western blot and IFA. *T.*  
860 *gondii* positive sera predominantly recognized the cyst wall besides the known tachyzoite  
861 surface antigens. The presence of CST1 antibodies in seropositive samples were in line with  
862 the staining patterns which were consistent with CST localization. Notably, *T. gondii* IgM-  
863 IgG+ sera recognize the cyst wall whereas IgM+ IgG- sera recognize tachyzoite antigens  
864 indicating acute infection consistent with presence of parasite DNA. The study demonstrates  
865 a strong humoral response against bradyzoite associated cyst wall antigens across naturally  
866 infected animals and humans. CST1 emerged as a key immunomodulatory antigen which  
867 may have direct implications for clinical immunodiagnostics.

868

869 **1. Introduction**

870 Toxoplasmosis, caused by *Toxoplasma gondii*, is an intracellular protozoan infection highly  
871 prevalent in warm-blooded vertebrate hosts. The disease causes abortions, stillbirth, and  
872 neonatal mortality, especially in sheep, goats and swine besides humans, leading to  
873 significant economic losses [1-3]. Humans and animals can become infected by ingestion of

874 food and water contaminated with oocysts or tissue cysts in raw or undercooked meat or  
875 congenitally. Infection not only results in significant reproductive losses in animals, but has  
876 public health implications since consumption of infected meat can facilitate zoonotic  
877 transmission.

878 *Toxoplasma gondii* has a two-stage asexual cycle in warm-blooded animals and a  
879 sexual cycle in Felidae. In the asexual cycle, the two developmental stages are i) tachyzoite:  
880 the rapidly multiplying stage associated with acute infection and ii) bradyzoite: the slowly  
881 multiplying stage associated with chronic infection. The sexual life-cycle takes place  
882 exclusively in enteroepithelial cells of the feline definitive host and results in the excretion of  
883 large numbers of oocysts in the faeces. Animals and humans can be infected with any of the  
884 three major stages.

885 Disease diagnosis is usually based on detection of *T. gondii* specific antibodies.  
886 ELISA is the most preferred serological test used for detection of IgM, IgG and IgA class of  
887 immunoglobulins. Most commercial ELISA kits use Toxoplasma lysate antigen (TLA),  
888 native antigens prepared from tachyzoites grown in mice or *in-vitro* culture. However, in  
889 recent years, many studies have demonstrated that accurate serodiagnosis may also be  
890 achieved using recombinant antigenic proteins of *T. gondii*. Several proteins of the parasite  
891 surface and secretory organelles i.e. microneme, rhoptry and dense granule which are  
892 instrumental for parasite invasion and multiplication have been studied for detection of *T.*  
893 *gondii* specific antibodies [4,5]. A family of surface antigens (SAG) has been demonstrated  
894 to play an important role in host cell invasion and immune modulation [6]. After entering the  
895 host cell, parasite dense granule proteins are secreted into the vacuolar network and the  
896 parasitophorous vacuole (PV) membrane (PVM) [7]. GRA7 is a member of this family that  
897 was earlier identified by serological immunoscreening using infected human sera [8].  
898 Proteins of surface antigen superfamily, microneme, rhoptry and dense granule are potential

899 diagnostic markers and important protective antigens. While these proteins are exposed to the  
900 immune system upon host cell lysis, the dynamics of humoral response is not investigated  
901 completely.

902         Following host immune response, tachyzoites differentiate into the slow-growing  
903 bradyzoite stage. Toxoplasma stage differentiation is accompanied with differential  
904 expression of several, closely related GPI-anchored surface proteins belonging to the SRS  
905 (SAG1-related sequence) superfamily [9]. SAG1 is the most abundant SRS antigen expressed  
906 in the tachyzoite stage [10]. In oral infection with tissue cysts, bradyzoite stage specific SRS  
907 antigens (e.g. SRS9) play a key role in attachment and invasion of intestinal epithelial cells  
908 [11,12]. In fact, following ingestion of tissue cyst, SRS antigens expressed by bradyzoites are  
909 among the first parasite antigens to be presented to the immune system [11,12]. However, the  
910 immunogenicity particularly of the host humoral response against bradyzoite-specific SRS  
911 antigens is not known.

912         Bradyzoite can form tissue cyst in the brain, muscles and visceral organs. Bradyzoite  
913 differentiation and maintenance of tissue cyst are critical for transmission of infection.  
914 During the differentiation process, parasitophorous vacuole membrane (PVM) of tachyzoite  
915 transform into a cyst wall in mature bradyzoite [13,14]. The cyst wall is composed of several  
916 proteins including cyst wall proteins (CST) [15-17], matrix proteins (MAG) [18] and dense  
917 granule proteins [19]. The CST1 is a major cyst wall protein which confers structural  
918 integrity to the cyst wall [15,16]. CST1 among other cyst wall proteins undergo extensive  
919 glycosylation and stain with *Dolichos biflorus* lectin (DBA), which has a high affinity for the  
920 glycosylated structure [16,20,21]. This glycosylation has been proposed to protect cyst wall  
921 proteins from immune responses during chronic infection [22].

922         Several studies indicate that bradyzoite containing cysts are not completely  
923 immunologically silent [23,24]. During the course of infection, intermittent cyst rupture leads

924 to rapid cell-mediated immune response. However, antibody-mediated immune response  
925 against cyst wall antigens is poorly examined. In fact, studies involving experimental  
926 infections have encountered limited humoral responses against bradyzoite as well as cyst wall  
927 antigens. This has led to the widely held notion that there is limited or no humoral response  
928 against bradyzoite containing cyst stage. MAG1 is the only cyst wall matrix protein reported  
929 to produce humoral responses in chronically infected hosts [25,26]. Like MAG1, several cyst  
930 wall proteins can also be potentially immunogenic. In addition, cyst wall and bradyzoite  
931 antigens would also be among the first parasite antigens to be presented to the immune  
932 system following cyst wall rupture [27]. Though, a number of questions remain unanswered  
933 as to the immunogenicity of cyst wall antigens. It is worth mentioning that the diagnostic  
934 usefulness of cyst wall antigens is largely unexplored as most established assays target  
935 tachyzoite stage antigens.

936 In this study, we systematically tested goat, sheep and human sera for anti-*T. gondii*  
937 IgM and IgG specific antibodies in ELISA based on TLA and selected recombinant antigens  
938 (CST1, SRS9, SAG1 and GRA7) of bradyzoite and tachyzoite stages. ELISA results  
939 demonstrated the presence of anti-*T. gondii* antibodies in nearly 50% of animal and human  
940 serum samples. Seropositive samples showed immunoreactivity against intracellularly  
941 replicating tachyzoites and bradyzoites using western blot and immunofluorescence analysis.  
942 The positive sera contain antibodies to a variety of *T. gondii* antigens in addition to tachyzoite  
943 surface antigens. The study also highlights the importance of bradyzoite associated antigens  
944 in eliciting a humoral immune response and their potential utility in serodiagnostic assays.  
945 The study attempted to define how immunostaining patterns vary corresponding to the stage  
946 of infection and a diverse set of parasite antigens may be involved at distinct phases of  
947 pathogenesis. The detection of strong humoral response to cyst wall antigens promises to



948 open new avenues to understanding of *T. gondii* pathogenesis and provides an experimental  
949 basis for devising methodologies to accurately determine the time of infection in hosts.

950

## 951 **2. Materials and Methods**

### 952 *2.1. Blood and serum samples*

953 The use of animal and human serum samples in this study was approved by the Animal and  
954 Human Research Ethics committees of Veterinary College Nagpur (NVC/2019/SC) and  
955 National Institute of Animal Biotechnology (IAEC/NIAB/2019/48/ASD). The written  
956 informed consent was obtained from each human subject. All methods were performed in  
957 accordance with institutional guidelines and regulations ensuring ethically conducted  
958 research. Total 404 goats, 88 sheep and 92 human blood and serum samples were received  
959 from Veterinary College Nagpur, Maharashtra. Serum samples were randomly collected.  
960 Goat and sheep samples were collected only from females. Human blood samples were  
961 collected from veterinary personnel (veterinarians and technicians) and farmers (sheep and  
962 goat). Blood samples were processed for DNA extraction by QIAamp DNA mini kit  
963 (Qiagen). DNA and sera were stored at -20°C until further testing.

964

### 965 *2.2. Parasite culture*

966 *Toxoplasma gondii* strains RH (type I), ME49 (type II) and VEG (type III) were maintained  
967 in primary human foreskin fibroblasts (HFF, ATCC) cells in Dulbecco's modified Eagle's  
968 medium supplemented with 10% FBS, 10 µg/ml Gentamicin and 2mM L-glutamine at 37 °C  
969 and 5% CO<sub>2</sub> in a humidified incubator. Parasites were purified by filtration through 3.0 µm  
970 filters [28].

971

### 972 *2.3. Gene cloning*

973 *T. gondii* RH strain tachyzoites were used to isolate genomic DNA, for PCR amplification  
974 [26]. The primers used to PCR clone are GGAATTCCATATGAAGAAAATAGAGGTTATTCA  
975 and CCCTCGAGTCAAATATCCAGTATTAACGCAGCA for CST1 (98-397 aa);  
976 GGAATTCCATATGGGCGCGGGATCTAGCACT and  
977 CCCTCGAGCAATGAAGCAACAACGAACCC for SRS9 (76-398 aa);  
978 GGAATTCCATATGCCCACTCTTGCGTACTCAC and  
979 CCCTCGAGCGAAGCGTTACCCTGCCA for SAG1 (96-230 aa) and  
980 GGAATTCCATATGGTTGATAGCCTGCGTCCGA and  
981 CCCTCGAGCTGACGTGCATCTTCACCATC for GRA7 (28-236 aa). The amplified DNA  
982 fragments of SRS9, SAG1 and GRA7 were cloned into pET-21a between *NdeI-XhoI* sites  
983 whereas CST1 was cloned into *NdeI-XhoI* sites of pET28a.

984

#### 985 2.4. Expression and purification of recombinant protein and polyclonal antibodies

986 The recombinant proteins: CST1, SRS9, SAG1 and GRA7 were expressed in *Escherichia*  
987 *coli* as C-terminal 6XHis tag and purified as described previously [29]. Briefly, *E. coli* strain  
988 BL21 Rosetta (DE3) (Novagen), transformed with pET-21a- CST1/SRS9/SAG1/GRA7, was  
989 grown in 10 ml LB supplemented with ampicillin (50 µg/ml) and chloramphenicol (34 µg/ml)  
990 overnight at 37°C. Subsequently, 10 ml of the overnight culture was added into 1,000 ml of  
991 LB containing the same antibiotics and incubated at 37°C with vigorous shaking. When the  
992 OD<sub>600</sub> reached 0.4, isopropyl-β-d-thiogalactopyranoside (IPTG) was added to the culture to a  
993 final concentration of 1 mM and the cells were further incubated at 22°C for 16 h. The cells  
994 were then harvested by centrifugation, and the pellets were resuspended in 30 ml of lysis  
995 buffer (50 mM NaH<sub>2</sub>PO<sub>4</sub>, 300 mM NaCl, 10 mM imidazole, 0.1 % Triton X-100 and 0.5mM  
996 PMSF, pH 8.0). After centrifugation, the protein was purified from the supernatant with the  
997 use of a Ni<sup>2+</sup>-NTA agarose (Qiagen)

998 CST1 was purified from the inclusion bodies. The inclusion bodies were solubilized  
999 in 6M GuHCl and the 6xHis tagged CST1 protein was purified over Ni<sup>2+</sup>-NTA agarose under  
1000 denaturing condition (Qiagen). The protein was then greatly diluted and refolded (50 mM  
1001 Tris-HCl pH 8.5, 9.6 mM NaCl, 0.4 mM KCl, 1mM EDTA, 0.5% Triton X-100, 1mM DTT).

1002 Purified recombinant proteins were used to generate specific mouse polyclonal  
1003 antibodies. Mouse polyclonal antibodies to recombinant CST1, SRS9, SAG1 and GRA7 were  
1004 produced by primary injection in mouse with 30 µg of purified protein in Freund's complete  
1005 adjuvant (Sigma) followed by four boosts of 30 µg each in Freund's incomplete adjuvant  
1006 (Sigma) at 2-weeks intervals. The antigen emulsion was prepared in total 100 µl volume in a  
1007 1:1 ratio with adjuvant. Serum was collected after three weeks of immunization.

#### 1009 2.5. *Toxoplasma gondii* native lysate antigen (TLA)

1010 *Toxoplasma* lysate antigen (TLA) was prepared as described previously [30]. Freshly  
1011 harvested *T. gondii* tachyzoites of RH/ME49/VEG were purified by filtration through 3.0  
1012 µm-Nucleopore filter (Sigma). Purified tachyzoites were freeze-thawed in liquid nitrogen  
1013 three times followed by sonication to lyse the parasite cells. Further, the parasite lysate was  
1014 centrifuged at 12000 g for 30 min at 4°C and supernatant containing *T. gondii* antigens  
1015 (TLA) was collected and stored at -80°C. The concentration of protein in the TLA  
1016 preparation was determined using BCA Protein Assay Kit (Pierce).

#### 1018 2.6. Enzyme-Linked Immunosorbent Assay (ELISA)

1019 ELISA assays were performed as described previously [31]. Nearly 1 µg/ml concentration of  
1020 CST1, SRS9, SAG1, GRA7 and TLA were used for coating the plates. Goat, sheep and  
1021 human serum samples were used at 1:100 dilution. Bound IgM and IgG of goat, sheep and  
1022 human were detected by using horseradish peroxidase (HRP) conjugated antibodies at 1:5000

1023 dilution [rabbit anti-goat IgG (Invitrogen) and IgM (Invitrogen), rabbit anti-sheep IgG  
1024 (Invitrogen) and IgM (Biorad), and goat anti-human IgG (Invitrogen) and IgM (Invitrogen)].

1025 The well validated reference positive and negative sera provided with a commercial  
1026 ELISA kits (Abcam, Human IgG-ELISA ab108776; Human IgM ELISA ab108778; and  
1027 IDEXX: Sheep and Goat TXT1135T) were used for TLA standardization. The TLA was  
1028 standardized for various parameters including its purity, concentration and incubation time  
1029 using the standard reference positive and negative serum samples. Subsequently, all serum  
1030 samples were analysed and divided into seropositive and seronegative groups in accordance  
1031 with the results obtained using the TLA. The cutoff values were determined in both IgG and  
1032 IgM ELISAs using 100, 50 and 50 seronegative serum samples of goat, sheep and human  
1033 respectively following standard procedure [26,31,32]. For all recombinant antigens, the same  
1034 pool of negative sera was used to determine the relative absorbance of each serum sample  
1035 and cutoff value. The results were determined for each serum sample by calculating the mean  
1036 value of the optical density (OD) reading for duplicate wells. A positive result was defined as  
1037 any value higher than the cutoff value plus standard deviations obtained with seronegative  
1038 serum groups of goats, sheep and humans.

1039

## 1040 2.7. Western blotting

1041 Filter purified parasites (RH/ME49/VEG) were washed in PBS, and pelleted by  
1042 centrifugation. The parasite cell pellet was lysed in SDS sample buffer with a final  
1043 concentration of  $2 \times 10^7$  tachyzoites/ml. HFF cells were trypsinised, centrifuged, washed with  
1044 PBS, and lysed in SDS sample buffer at a final concentration of  $4 \times 10^6$  cells/ml. Separated  
1045 proteins (Twenty  $\mu$ l of each lysate or 500 ng/ $\mu$ l of recombinant CST1/SRS9/SAG1/GRA7  
1046 protein) were electrotransferred to PVDF membrane (1 hr, 120mA/cm gel<sup>2</sup>). After blocking  
1047 with 5% fat-free milk in PBS, the membrane was incubated for 1h at room temperature with

1048 serum samples diluted in PBS (Goat/sheep/human 1:100 and CST1/SRS9/SAG1/GST1  
1049 1:1000), washed and incubated with HRP-conjugated secondary antibodies diluted at 1:5000  
1050 (details of antibodies are mentioned in the ELISA method). The proteins on these PVDF  
1051 membranes were visualized with super Enhanced Chemiluminescent Substrate (Pierce).

1052

### 1053 2.8. Indirect Immunofluorescence assay (IFA)

1054 IFA was performed as described elsewhere [33]. Confluent monolayers of HFF cells were  
1055 grown on coverslip in 6-well plates and infected with RH strain of *T. gondii* parasites. The  
1056 infected cells were fixed post 32 to 36 h of infection in 4% paraformaldehyde, followed by  
1057 permeabilization with 0.35% Triton X-100. The samples were further blocked in 2% BSA  
1058 and incubated sequentially with primary and secondary antibodies. Goat, sheep and human  
1059 serum samples were used at 1:50 dilution each. Mouse anti-CST1, mouse anti-SRS9, mouse  
1060 anti-SAG1 and mouse anti-GRA7 were used at 1:500 dilution each. Alexa Fluor-488  
1061 conjugated secondary antibodies [anti-goat IgG (Invitrogen), anti-goat IgM (Invitrogen), anti-  
1062 sheep IgG (Invitrogen), anti-sheep IgM (Biorad), anti-human IgG (Invitrogen), anti-human  
1063 IgM (Invitrogen), anti-mouse IgG (Invitrogen)] were used at a dilution of 1:1000. Coverslips  
1064 were mounted with Vectashield medium (VectorLabs) with 4',6-Diamidino-2-phenylindole  
1065 dihydrochloride (DAPI) on glass slide and images were captured with a Leica Confocal  
1066 microscope with 100× objective. Images were processed using LAS X software.

1067 Bradyzoite induction was performed as described elsewhere [34]. Briefly, filter  
1068 purified parasites (ME49) were inoculated into a confluent HFF monolayer in a 6-well plate  
1069 at a multiplicity of infection of 0.5 to 1.0. After 2-h incubation at 37°C in 5% CO<sub>2</sub>, the  
1070 medium was changed to RPMI with 50 mM HEPES (pH 8.2) without NaHCO<sub>3</sub>,  
1071 supplemented with 1% FBS and 10 µg/ml Gentamicin, and incubated in a humid 37°C  
1072 incubator without CO<sub>2</sub> for 5 days. Bradyzoite induction medium was changed every 2 days.

1073 Bradyzoites IFA were performed as for tachyzoites. Rhodamine labeled- *Dolichus biflorus*  
1074 Agglutinin (DBA, VectorLabs), a lectin that recognizes cyst wall proteins was used (conc. 10  
1075 µg/ml) as a bradyzoite marker. Serum samples were marked positive for anti-*T. gondii*  
1076 antibodies if specific signals were obtained from the parasite structures. CST1 antibody was  
1077 used to label the cyst wall as well as bradyzoite marker.

1078

### 1079 2.9. Polymerase chain reaction (PCR)

1080 The triplex PCR assay was used to screen blood samples for *T. gondii* DNA [35]. For PCR  
1081 amplification three targets namely B1 gene [36], 529 bp repetitive element [37] and ITS-1  
1082 [38] were used. The primers were used to amplify a 747 bp fragment of the B1 gene were  
1083 TCGCAGTACACCAGGAGTTG and CACTCCATCTCTCGTCTTCT, 234 bp fragment of  
1084 the ITS-1 were ACACGTCCTTATTCTTTATTAACCA and  
1085 ATCCCAACAGAGACACGAATT and 182 bp fragment of the 529 bp were  
1086 TGTGCTTGGAGCCACAGAAG and GCAGCCAAGCCGGAAACAT respectively.  
1087 Following cycle conditions were used: 95°C, 5 min followed by 35 cycles of 95°C, 30 s;  
1088 59°C, 30 s, and 72°C, 30 s.

1089

### 1090 2.10. Statistical analysis

1091 Statistical analysis of the ELISA results was performed using GraphPad Prism software and  
1092 analysed by unpaired student's t test.

1093

1094

## 1095 3. Results

1096 *3.1. Reactivity of IgM and IgG antibodies from goat, sheep and human sera with TLA and*  
1097 *recombinant antigens (CST1, SRS9, SAG1, and GAR7) in ELISA*

1098 To investigate the presence of humoral immune response against cyst wall and bradyzoite  
1099 antigens, we tested the immunoreactivities of CST1 (major cyst wall antigen), SRS9  
1100 (bradyzoite surface antigen) and compared with SAG1 (tachyzoite surface antigen) and  
1101 GRA7 (PV-associated antigen) in ELISA using animal and human sera. In order to evaluate  
1102 antigenicity of recombinant antigens, CST1 (98-397 aa), SRS9 (76-398 aa), SAG1 (96-230  
1103 aa) and GRA7 (28-236) were bacterially expressed (Fig. 1A) and purified using C-terminal  
1104 (N-terminal in case of CST1) histidine tags (Fig 1B). In case of SRS9, GRA7 and SAG1,  
1105 signal peptide sequence was avoided for recombinant protein generation. SAG1 protein was  
1106 further truncated in order to get good quality protein. In case of CST1, only N-terminus was  
1107 used to make the recombinant proteins as this domain was sufficient enough to elicit a strong  
1108 immune response [16] and was devoid of SRS domain to avoid the cross reactivity with other  
1109 SRS domain containing proteins. Except CST1 all other proteins SRS9, SAG1, and GRA7  
1110 were expressed as soluble proteins with estimated molecular masses of ~32kDa, ~37 kDa,  
1111 ~18 kDa, and ~23 kDa respectively (Fig. 1B).

1112 Five separate IgM and IgG ELISAs were developed using standardized TLA, CST1,  
1113 SRS9, SAG1, and GRA7 as a coating antigen to determine the seroprevalence and evaluate  
1114 the potential of each of these antigens for the serodiagnosis of toxoplasmosis in goat, sheep  
1115 and human. A total 404 of goat, 88 sheep and 92 human serum samples were examined. All  
1116 serum samples were divided into seropositive and seronegative groups in accordance with the  
1117 results obtained using the TLA. Seronegative samples of goat, sheep and human were used to  
1118 determine the cutoff values. A positive result was defined as any value higher than the cutoff  
1119 value plus standard deviations obtained with seronegative serum groups of goats, sheep and  
1120 humans in respective ELISAs.

1121 In case of 404 goat serum samples in IgM ELISA (Fig. 1C), the CST1, SRS9, SAG1,  
1122 GRA7, and TLA reacted with 167 (41.3%), 62 (15.3%), 178 (44%), 170 (42%), and 184  
1123 (45.5%) of the positive sera respectively (Table 1), whereas in IgG ELISA (Fig. 1D), the  
1124 CST1, SRS9, SAG1, GRA7, and TLA reacted with 165 (40.8%), 77 (19%), 165 (40.8%),  
1125 160 (39.6%), and 171 (42.3%) of the positive sera respectively (Table 2). In case of 88 sheep  
1126 serum samples in IgM ELISA (Fig. 1C), the CST1, SRS9, SAG1, GRA7, and TLA reacted  
1127 with 35 (39.7%), 14 (15.9%), 36 (40.9%), 35 (39.7%), and 38 (43.1%) of the positive sera  
1128 respectively (Table 1), whereas in IgG ELISA (Fig. 1D), the CST1, SRS9, SAG1, GRA7, and  
1129 TLA reacted with 29 (32.9%), 16 (18.1%), 32 (36.3%), 32 (36.3%), and 34 (38.6%) of the  
1130 positive sera respectively (Table 2). In case of 92 human serum samples in IgM ELISA (Fig.  
1131 1C), the CST1, SRS9, SAG1, GRA7, and TLA reacted with 36 (39.1%), 15 (16.3%), 35  
1132 (38%), 35 (38%), and 37 (40.2%) of the positive sera respectively (Table 1), whereas in IgG  
1133 ELISA (Fig. 1D), the CST1, SRS9, SAG1, GRA7, and TLA reacted with 35 (38%), 19  
1134 (20.6%), 38 (41.3%), 37 (40.2%), and 40 (43.4%) of the positive sera respectively (Table 2).  
1135 Overall, based on both the ELISA results, the percentage of seropositivity obtained using  
1136 CST1, SAG1 and GRA7 was comparable to that of TLA. However, for SRS9 the number of  
1137 reactive serum samples observed were lower with respect to CST1, SAG1, GRA7 and TLA.

1138 The sensitivity of both IgM and IgG ELISAs calculated from all positive goat, sheep  
1139 and human serum samples tested, were different for the individual antigens (Table 1,2). In  
1140 both the ELISAs irrespective of species, higher sensitivity was observed for CST1 (IgM: 90.8  
1141 to 97.3% and IgG: 85.3 to 96.5%), SAG1 (IgM: 94.6 to 96.7% and IgG: 94.1 to 96.5%) and  
1142 GRA7 (IgM: 92.1 to 94.6% and IgG: 92.5 to 94.1%), whereas lowest sensitivity was noticed  
1143 for SRS9 (IgM: 33.7 to 40.5% and IgG: 45 to 47.5%) compared to TLA. In the same  
1144 experiment, slightly lower specificity was observed for CST1 (IgM: 92.8 to 97.3% and IgG:



1145 94.3 to 96.2%) SAG1 (IgM: 94.6 to 100% and IgG: 100%), GRA7 (IgM: 96.4 to 100% and  
1146 IgG: 97.9 to 100%) and SRS9 (IgM: 92.8 to 98% and IgG: 96.3 to 100%) compared to TLA.

1147 Together, based on the TLA-ELISA results, 49.5% goat, 50% sheep, and 54.34%  
1148 human sera contain *T. gondii* specific IgM-positive/ IgG-positive (IgM+ IgG+) antibodies  
1149 (Table 3); 7.17% goat, 11.36% sheep, and 10.86% human sera contain *T. gondii* specific  
1150 IgM-positive/ IgG-negative (IgM+ IgG-) antibodies (Table 3); 3.96% goat, 6.81% sheep, and  
1151 14.13% human sera contain *T. gondii* specific IgM-negative/ IgG-positive (IgM- IgG+)  
1152 antibodies (Table 3).

1153 Statistical analysis confirmed a high sensitivity of ELISA with CST1 for serum  
1154 samples from IgM- IgG+ and IgM+ IgG+ as compared to IgM+ IgG-. The results obtained  
1155 for CST1 were found to be statistically significant ( $P < 0.001$ ).

1156

### 1157 3.2. *T. gondii* positive sera recognize both recombinant and native parasite proteins

1158 To further characterize the IgG positive sera (Table 3), Western blot analysis was performed  
1159 using recombinant CST1, SRS9, SAG1, and GRA7 proteins. All serum samples (irrespective  
1160 of species) recognized CST1, SRS9, SAG1, and GRA7 recombinant proteins. In each  
1161 species, nearly 70% serum samples showed immunoreactivity to recombinant CST1, SRS9,  
1162 SAG1 and GRA7 proteins either singly or in combinations whereas the remaining 30% serum  
1163 samples recognized all the four recombinant proteins (Fig. 2A,C,E). Further, the same set of  
1164 serum samples were used to determine the immunoreactivity against *T. gondii* native  
1165 proteins. To test that, parasite lysates of all three strains RH (type I), ME49 (type II), and  
1166 VEG (type III) of *T. gondii* were used in Western blot analysis. All sera that recognized  
1167 recombinant proteins (Fig. 2A,C,E), also recognized different native proteins in the parasite  
1168 lysates of all the three strains (Fig. 2B,D,F), suggesting presence of antibodies against a  
1169 variety of parasite proteins in naturally acquired *T. gondii* infection. These serum samples did

1170 not recognize any prominent protein band in the HFF host cell lysate indicating presence of  
1171 specific antibodies against parasite proteins (Fig. 2B,D,F). In the similar experimental  
1172 conditions, none of the ELISA negative sera showed immunoreactivity towards recombinant  
1173 or native parasite proteins (Supplementary Fig. 1A-C). Many *T. gondii* antigens show high  
1174 sequence homology with antigens of *Neospora caninum* (closest phylogenetic relative to *T.*  
1175 *gondii*). The possibility of immunoreactivity due to cross-reacting antibodies specific against  
1176 *Neospora caninum* (IDEXX) was tested using a similar experiment. Neither recombinant  
1177 antigens nor native parasite proteins showed any cross reactivity with *Neospora caninum*  
1178 antibodies (Supplementary Fig. 1D). These results are in accordance with previous findings  
1179 [39,40].

1180 Further, we determined the localization of CST1, SRS9, SAG1, and GRA7 proteins in  
1181 the parasite using specific antibodies against these proteins. Purified recombinant proteins  
1182 (Fig. 1B) were used to generate specific mouse polyclonal antibodies. Antisera obtained  
1183 against CST1 and SRS9 recognized the native protein in the bradyzoite stage lysate of the  
1184 parasite at the expected molecular weight ~250 kDa and ~37 kDa respectively (Fig. 3A, B).  
1185 Neither antibody showed any cross reactivity with tachyzoite nor host cell (HFF) proteins  
1186 confirming their expression exclusively in the bradyzoite stage of the parasite. SAG1 and  
1187 GRA7 antisera recognized the recombinant proteins (SAG1 ~18 kDa and GRA7 ~23 kDa)  
1188 and confirmed their native expression (SAG1 ~30 kDa and GRA7 ~23 kDa) (Fig. 3C,D). The  
1189 specific antibodies were utilized to determine the localization of CST1, SRS9, SAG1, and  
1190 GRA7 proteins in the parasite using immunofluorescence assay (IFA). As expected, antisera  
1191 of CST1, SRS9, SAG1, and GRA7 stained cyst wall (encysted bradyzoite stage) (Fig. 3A),  
1192 bradyzoite surface (encysted bradyzoite stage) (Fig. 3B), tachyzoite surface (Fig. 3C), and  
1193 parasitophorous vacuole membrane (tachyzoite stage) (Fig. 3D) respectively. *Dolichos*

1194 *biflorus* Agglutinin (DBA) which binds to the cyst wall was used as encysted bradyzoite  
1195 stage marker [16].

1196

1197 *3.3. T. gondii positive sera predominantly recognize cyst wall antigens in encysted bradyzoite*

1198 Western blot results revealed that *T. gondii* positive serum samples contain antibodies against

1199 a variety of parasite proteins including CST1, SRS9, SAG1, and GRA7. To verify these

1200 results, we investigate whether the antibodies against these antigens could also recognize

1201 antigens expressed by encysted bradyzoites (cyst stage). To test that, ME49 strain of *T.*

1202 *gondii* was induced for bradyzoite development in tissue culture. *T. gondii* seropositive

1203 samples of goat, sheep and human were examined to detect encysted parasites (bradyzoite

1204 stage) by IFA. All sera tested against encysted bradyzoites showed two distinct staining

1205 patterns *viz* i) cyst wall staining, ii) bradyzoite surface (Fig. 4A-C). Observed

1206 immunostaining patterns were compared with reference cyst wall staining of CST1 and

1207 bradyzoite surface of SRS9 (Fig. 3A,B) for confirmation. The predominant immunostaining

1208 pattern was observed on the cyst wall which is suggestive of what is seen with CST1

1209 antibodies (Fig. 3A). Of the 171 goat serum samples that stained bradyzoites, 155 (90.6%), 4

1210 (2.3%) and 12 (7%) showed the cyst wall, bradyzoite surface, and mixed staining patterns

1211 respectively (Fig. 4A) (Table 4). Similarly, of the 34 sheep serum samples, 31 (91.2%), 1

1212 (2.9%), and 2 (5.8%) showed the cyst wall, bradyzoite surface, and mixed staining patterns

1213 respectively (Fig. 4B) (Table 4). Likewise, of the 40 human serum samples that stained

1214 bradyzoites, 36 (90%), 1 (2%), and 3 (7.5%) showed the cyst wall, bradyzoite surface, and

1215 mixed staining patterns respectively (Fig. 4C) (Table 4). Together, based on the encysted

1216 bradyzoite IFA results, ~90% *T. gondii* positive serum samples of goat, sheep and humans

1217 were shown to have cyst wall staining patterns. In the similar experimental conditions, none

1218 of the ELISA and Western blot negative sera showed immunoreactivity towards encysted  
1219 bradyzoites tested using IFA (Supplementary Fig. 2A-C).

1220

#### 1221 *3.4. T. gondii positive sera predominantly recognize tachyzoite surface antigens*

1222 Like encysted bradyzoites, we questioned whether the same set of serum samples that  
1223 recognized cyst wall and bradyzoite antigens could also recognize diverse antigens of  
1224 tachyzoites. To test that we performed IFA with the same set of serum samples against  
1225 intracellular tachyzoites of RH stain parasites grown in HFF cells. All sera tested against  
1226 intracellular tachyzoites showed three distinct staining patterns *viz* i) parasite  
1227 surface/membrane staining, ii) parasitophorous vacuole membrane (PVM) staining and iii)  
1228 parasitophorous vacuole internal (PV-internal) (Fig. 5A-C). Observed immunostaining  
1229 patterns were compared with reference tachyzoite surface staining of SAG1, and PVM  
1230 staining of GRA7 (Fig. 3C,D) for confirmation. The predominant immunostaining pattern  
1231 was observed on parasite membrane, which is suggestive of what is seen with SAG  
1232 antibodies (Fig. 3C). Of the 171 goat serum samples that stained intracellular tachyzoites, 135  
1233 (78.9%), 30 (17.5%), 2 (1.2%), and 4 (2.3%) showed parasite surface, PVM, PV internal and  
1234 mixed staining patterns respectively (Fig. 5A) (Table 4). Similarly, Of the 34 sheep serum  
1235 samples tested, 27 (79.4%), 5 (14.7%), 1 (2.9%), and 1 (2.9%) showed parasite surface,  
1236 PVM, PV internal and mixed staining pattern respectively (Fig. 5B) (Table 4). Likewise, of  
1237 the 40 human serum samples that stained intracellular tachyzoites, 32 (80%), 6 (15%), 1  
1238 (2.5%), and 1 (2.5%) showed parasite surface, PVM, PV internal and mixed staining patterns  
1239 respectively (Fig. 5C) (Table 4). Together, based on the intracellular tachyzoites IFA results,  
1240 ~80% *T. gondii* positive serum samples of goat, sheep and humans were shown tachyzoite  
1241 surface staining pattern whereas 15-18% showed staining on PVM. In the similar  
1242 experimental conditions, none of the ELISA and Western blot negative sera showed

1243 immunoreactivity towards intracellular tachyzoites tested using IFA (Supplementary Fig. 3A-  
1244 C).

1245

### 1246 3.5. *T. gondii* IgM+ IgG- sera recognize tachyzoite surface antigens only

1247 In earlier experiments, we showed that IgM+ IgG+ *T. gondii* sera recognize both tachyzoite,  
1248 and encysted bradyzoite antigens suggesting presence of humoral immune response against  
1249 acute and chronic infections. In *T. gondii* infection, IgM level can persist for several years,  
1250 therefore, a chronic *T. gondii* infection can be erroneously categorized as an acute infection.  
1251 For these reasons, we selected only IgM+ IgG- *T. gondii* sera (Table 3) and tested whether  
1252 these sera recognize tachyzoite or bradyzoite or both the stages. We used *T. gondii* IgM+  
1253 IgG- sera from each species (29 goat, 10 sheep and 10 human; Table 3) and performed IFA  
1254 against intracellularly replicating tachyzoites and *in-vitro* induced bradyzoites. IgM-IFA  
1255 showed specific reactivity towards intracellular tachyzoites and not the bradyzoites. The  
1256 immunofluorescence patterns by IgM staining in the tachyzoite stage were similar to that  
1257 observed for IgG staining in earlier experiments (Fig. 4A-C). Unlike earlier observed distinct  
1258 immunostaining patterns for intracellular tachyzoites here we observed only tachyzoite  
1259 surface staining for all the sera tested (Fig. 6A-C). The immunostaining pattern was observed  
1260 on parasite membrane, which is suggestive of what is seen with SAG antibodies (Fig. 3C).  
1261 However, none of the serum samples which were IgM+ IgG- showed detectable  
1262 immunofluorescence against *in-vitro* induced bradyzoites. To test further, we performed  
1263 CST1 Western blot analysis with the same set of sera, none of the serum samples detected  
1264 recombinant CST1 protein (data not shown), consistent with an acute infection or initial  
1265 antibody response directed against tachyzoites.

1266

### 1267 3.6. *T. gondii* IgM+ IgG- goat, sheep and human show active infection

1268 Serological diagnosis of toxoplasmosis provides high sensitivity, but specificity varies  
1269 depending on the test used. Following acute infection, *T. gondii* IgM antibodies may persist  
1270 for many months or even years. These complicate the correct interpretation of a positive *T.*  
1271 *gondii* IgM result. Therefore, the detection of *T. gondii* DNA using PCR is a useful  
1272 laboratory tool particularly during early acute infections. PCR based assay that targeted the  
1273 B1 gene, ITS1 and repetitive region 529 bp could detect 10 parasites in the presence of  
1274 100,000 human leukocytes [36]. Earlier study has demonstrated triplex PCR containing  
1275 primer sets for all three abovementioned regions using different body fluids and DNA  
1276 extracted from the organs of animals infected with *T. gondii* [35]. Accordingly, the triplex  
1277 PCR was used to detect *T. gondii* specific DNA in peripheral blood mono-nuclear cells  
1278 (PBMCs) of IgM+ IgG- 29 goat, 10 sheep and 10 human (Table 3, Fig. 6A-C). We observed  
1279 that 25 (86.2%) of 29 goat, 9 (90%) of 10 sheep and 9 (90%) of 10 human samples were  
1280 found to be positive for *T. gondii* specific DNA. For representative purpose one positive  
1281 sample from IgM+ IgG- goat, sheep and human were shown (Fig. 7). RH strain DNA and  
1282 IgM- IgG- goat sample were used as positive and negative controls respectively. This result  
1283 suggests that *T. gondii* IgM+ IgG- individuals may carry an active infection. Hence, the  
1284 combination of both the tests may help to improve the sensitivity of early stage toxoplasma  
1285 detection.

1286

#### 1287 **4. Discussion**

1288 The study focused on understanding the humoral immune responses against recombinant and  
1289 native antigens of encysted bradyzoite and tachyzoite stages of *T. gondii*. To this aim, we  
1290 demonstrated that robust humoral immune response is generated against cyst wall antigens  
1291 which is comparable to the immune response generated against tachyzoite surface antigens in  
1292 *T. gondii* infected animals and humans. The immunoreactivity of sera to recombinant and

1293 native proteins of encysted bradyzoite and tachyzoite origins was established using ELISA,  
1294 Western blot and immunofluorescence analysis. The recombinant proteins represent key  
1295 immunodominant antigens of cyst wall, CST1; bradyzoite surface, SRS9; tachyzoite surface,  
1296 SAG1; and tachyzoite PVM, GRA7. Based on IgM and IgG ELISA results nearly 50% serum  
1297 samples of goat, sheep and human were found positive for *T. gondii* specific antibodies.

1298         Recombinant CST1, SAG1 and GRA7 proteins have a greater diagnostic performance  
1299 than SRS9 recombinant protein. Notably, we found a meagre antibody response against  
1300 bradyzoite surface antigen, SRS9, than to cyst wall antigen, CST1. This limited humoral  
1301 response towards bradyzoite surface antigens compared with cyst wall antigens could be  
1302 because cyst wall antigens are more exposed to immune cells upon cyst wall rupture during  
1303 reactivation of infection as well as oral infection. While the usefulness of recombinant SAG1  
1304 and GRA7 in diagnosis of human and animal toxoplasmosis have been previously reported  
1305 [41-44], the utility of cyst wall antigen like CST1 and others requires to be thoroughly  
1306 explored. The present study reinforces the idea that bacterial recombinant antigens offer  
1307 many advantages in the *Toxoplasma* diagnosis as they allow better standardization of the  
1308 tests and reduce the costs of production. Accordingly, more comprehensive understanding of  
1309 antigens expressed at different stages of *T. gondii* is needed. However, it is evident from IFA  
1310 and western blot results that by incorporating additional parasite antigens efficacy of  
1311 serological diagnosis could be significantly improved. This study offers robust evidence to  
1312 support the incorporation of additional parasite antigens expressed across different  
1313 developmental stages to the current repertoire of antigens to improve detection of parasite  
1314 specific antibodies in both animals and humans.

1315         We observed nearly 40% of serum samples irrespective of species are IgM+ positive.  
1316 Most of these samples were also IgG+ and only 29% in goats and 10% each in sheep and  
1317 humans were IgG- (Table 3). This is not unusual with respect to the *T. gondii* infection as



1318 IgM level can persist for several months to years after an acute infection [45]. Therefore, only  
1319 IgM positive test is not a good marker of an acute infection. This observation is consistent  
1320 with our IFA results where *T. gondii* IgM+ IgG+ sera are able to recognize both tachyzoite  
1321 and bradyzoite stage antigens whereas IgM+ IgG- sera do not recognize bradyzoite antigens  
1322 but recognize only tachyzoite antigens. These results suggest that IgM testing in different  
1323 assays like ELISA and IFA can be used for detection of acute *T. gondii* infection. In fact,  
1324 PCR in addition to IgM-ELISA or IgM-IFA is a good method to determine an acute infection.  
1325 With the use of triplex PCR, we observed 86 to 90% of *T. gondii* IgM+ IgG- animals and  
1326 humans were positive for parasite DNA. These results suggest that most of the IgM+ IgG-  
1327 individuals are suffering from an acute infection. Therefore, the combination of both the tests  
1328 may help to improve the sensitivity of early stage toxoplasma detection more than either PCR  
1329 or IgM-ELISA alone.

1330         The tachyzoite to bradyzoite stage conversion might take place early during infection.  
1331 However, it should be noted that bradyzoite and cyst proteins are released from ingested  
1332 parasites within the gastrointestinal tract during primary infection. Thus, the host immune  
1333 response against cyst wall and bradyzoite antigens could originate from this first exposure.  
1334 With the advancement in bradyzoite biology, now we know that cysts are dynamic structures;  
1335 they regularly break down or rupture host cells [3,46]. When tissue cyst rupture, they elicit a  
1336 strong inflammatory response in chronically infected hosts. In line with this, we also  
1337 observed the presence of anti-*T. gondii* IgG antibodies against bradyzoite antigens in animal  
1338 and human sera. Though the antigens from tachyzoite and bradyzoite shared a fair level of  
1339 homology, we observed a diverse immunoreactivity pattern in IFA against both the stages of  
1340 the parasite indicating the presence of humoral response towards stage specific antigens. This  
1341 is consistent with recent studies which detected bradyzoite specific antibodies from *T. gondii*  
1342 positive human sera and chronically infected mice [25,47]. On similar lines, bradyzoite



1343 specific humoral response in human sera was demonstrated recently by detecting a variety of  
1344 immunostaining patterns for bradyzoites [48]. Therefore, the onset of anti-bradyzoite  
1345 antibody response and the role of humoral response in controlling encysted latent infection  
1346 merit further investigation.

1347         It is established that cell-mediated immunity plays an important role in the host  
1348 resistance to *T. gondii* infection [49], but this study has highlighted the importance of the  
1349 humoral response to this intracellular pathogen which is consistent with other reports [50,51].  
1350 Our study further demonstrates that specific cyst wall and bradyzoite antigens may contribute  
1351 to the stimulation of humoral immunity against *T. gondii* infection in animals and humans,  
1352 supporting the hypothesis that a combination of bradyzoite and tachyzoite antigens should be  
1353 used for development of serological tests. Also, it is important to determine if the detection of  
1354 anti-bradyzoite antibodies can serve as a valuable tool to distinguish between acute and  
1355 chronic infection and merits further investigation.

1356         The present study showed that 50% of veterinary personnel contain *T. gondii* specific  
1357 antibodies and this percentage did appear to be high. As per our knowledge, this is the first  
1358 study of its kind conducted in India. However, more samples need to be screened to support  
1359 this observation. Similar study was undertaken in Malaysia, in which nearly 20%  
1360 seroprevalence was reported among people having close contact with animals [52]. Based on  
1361 these results, primary screening of Toxoplasma infection should be particularly initiated in  
1362 high titer seropositive individuals like veterinary personnel, pet owners and women. This  
1363 program not only help to reduce the Toxoplasmosis incidence in high risk groups but also  
1364 enables us to identify the potential risk factors of infection.

1365         In summary, the present study provides strong evidence for the presence of humoral  
1366 immune response towards cyst wall antigens in naturally acquired *T. gondii* infections.  
1367 However, it is not known whether the presence of antibodies to bradyzoite antigens has a

1368 protective effect in *T. gondii* infected animals or humans and needs to be keenly looked at.  
1369 This study broadens our understanding of humoral dynamics and add to the repertoire of  
1370 immunomodulatory antigens of *T. gondii*. Moreover, it provides an experimental basis for not  
1371 defining and designing diagnostic and therapeutic approaches for its clinical management.  
1372 Further prospective studies to examine immunoreactivity towards other cyst wall antigens are  
1373 warranted.

1374

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1382

### 1383 **Author statement**

1384 Abhijit S. Deshmukh: Conceptualization, Data curation, Funding acquisition, Methodology,  
1385 Investigation and Writing- Original draft preparation. Rajkumar Gurupwar: Resources and  
1386 investigation. Pallabi Mitra: Investigation, Writing- Original draft preparation. Kalyani  
1387 Aswale: Investigation and Formal analysis. Shilpshri Shinde: Resources and investigation.  
1388 Sandeep Chaudhari: Resources and investigation.

1389

### 1390 **Conflict of interest statement**

1391 The authors declare no competing interests.

1392

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1571 **Tables**

1572 **Table 1.** Comparison of the immunoreactivities of recombinant CST1, SRS9, SAG1, and  
1573 GRA7 proteins and TLA in the IgM ELISA using pools of goat, sheep and human sera.

1574

1575 **Table 2.** Comparison of the immunoreactivities of recombinant CST1, SRS9, SAG1, and  
1576 GRA7 proteins and TLA in the IgG ELISA using pools of goat, sheep and human sera.

1577

1578 **Table 3.** TLA-ELISA (IgM and IgG) results using pools of goat, sheep and human sera.

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1580 **Table 4.** Different immunofluorescence patterns detected for encysted bradyzoites and  
1581 intracellular tachyzoites by positive serum for anti-*Toxoplasma gondii* antibodies.

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1596 **Figure Legends**

1597 **Fig. 1.** Comparison of immunoreactivities of the recombinant proteins and TLA in IgM and  
 1598 IgG ELISA using goat, sheep and human sera. (A) Schematic diagram of full length CST1,  
 1599 SRS9, SAG1 and GRA7. Gene details including ToxoDB accession number, SP: signal  
 1600 peptide region and recombinant protein region are shown. (B) SDS-PAGE profiles of the  
 1601 purified recombinant proteins: CST1, SRS9, SAG1, and GRA7. (C) Comparison of the  
 1602 immunoreactivities of CST1, SRS9, SAG1, GRA7, and TLA in the IgM ELISA using sera  
 1603 samples of goat, sheep and human. (D) Comparison of the immunoreactivities of CST1,  
 1604 SRS9, SAG1, GRA7, and TLA in the IgG ELISA using sera samples of goat, sheep and  
 1605 human. The horizontal lines represent the cutoff values.

1606  
 1607 **Fig. 2.** Western blotting analyses against recombinant proteins and HFF infected RH or  
 1608 ME49 or VEG strain of *Toxoplasma gondii* tachyzoites. Representative results of Western  
 1609 blotting analyses against recombinant proteins and *T. gondii* strains (RH, ME49 and VEG)  
 1610 were tested with anti-*T. gondii* goat (A-B), sheep (C-D) and human (E-F) serum samples.

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 1612 **Fig. 3.** WB and IFA using bradyzoite and tachyzoite stage specific markers. (A,B)  
 1613 Expression of CST1 and SRS9 in bradyzoite stage parasite was analysed by Western blotting  
 1614 with anti-CST1 and anti-SRS9 antibodies. Specific antibodies recognized native protein  
 1615 (CST1 ~250 kDa and SRS9 ~37 kDa). *T. gondii* inner membrane complex 1 (IMC1), was  
 1616 used as a loading control for both tachyzoite and bradyzoite lysates. (C,D) Expression of  
 1617 SAG1 and GRA7 in tachyzoite stage parasite was analysed by Western blotting with anti-  
 1618 SGA1 and anti-GRA7 antibodies. Specific antibodies recognized recombinant (SAG1 ~18  
 1619 kDa and GRA7 ~23 kDa) and native protein (SAG1 ~30 kDa and GRA7 ~23 kDa). (E) anti-  
 1620 CST1 antibodies stained “cyst wall”. (F) anti-SRS9 antibodies stained “bradyzoite surface”.



1621 (G) anti-SAG1 antibodies stained “tachyzoite surface” and (H) anti-GRA7 predominantly  
1622 stained “parasitophorous vacuole”. DIC: Differential interference contrast.

1623

1624 **Fig. 4.** IFA with *Toxoplasma gondii* positive sera exhibit two prominent immunofluorescence  
1625 patterns viz cyst wall and parasite surface in the bradyzoites. (A-C) Representative images of  
1626 each staining pattern using goat (A), sheep (B) and human (C) are shown. Scale bar = 5  $\mu$ m.

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1628 **Fig. 5.** IFA with *Toxoplasma gondii* positive sera exhibit three prominent  
1629 immunofluorescence patterns viz parasite surface, PVM and PV - internal in the intracellular  
1630 tachyzoites. (A-C) Representative images of each staining pattern using goat (A), sheep (B)  
1631 and human (C) are shown. Scale bar = 5  $\mu$ m.

1632

1633 **Fig. 6.** IFA with *Toxoplasma gondii* IgM+ IgG- sera recognize only tachyzoites and not  
1634 bradyzoites. (A-C) Representative images of each staining pattern using serum samples of  
1635 goat (A), sheep (B) and human (C) are shown. Scale bar = 5  $\mu$ m.

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1637 **Fig. 7.** Agarose gel electrophoresis of triplex PCR products viz B1 gene, ITS1 and 529  
1638 regions. Representative PCR amplification using DNA samples from IgM+ IgG- goat, sheep  
1639 and human. *T. gondii* RH strain DNA and IgM- IgG- goat DNA were used as positive and  
1640 negative controls respectively.

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**Table 1.** Comparison of the immunoreactivities of CST1, SRS9, SAG1, GRA7, and TLA in the IgM ELISA using pools of goat, sheep and human sera.

Antigen	Pool of seropositive sera			Pool of seronegative sera			Cutoff value
	No. (%) of positive sera	Mean absorbance value	Ranges absorbance value	No. (%) of positive sera	Mean absorbance value	Ranges absorbance value	
Goat ( <i>n</i> = 404)	( <i>n</i> = 184)			( <i>n</i> = 220)			
TLA	184 (100)	1.188	0.324-2.091	0	0.224	0.090-0.320	0.320
CST1	167 (90.8)	0.816	0.163-1.990	6 (2.7)	0.210	0.115-0.310	0.310
SRS9	62 (33.7)	0.477	0.100-1.476	6 (2.7)	0.202	0.112-0.330	0.330
SAG1	178 (96.7)	1.015	0.165-2.211	0	0.206	0.114-0.310	0.310
GRA7	170 (92.4)	1.072	0.106-2.692	8 (3.6)	0.202	0.112-0.308	0.308
Sheep ( <i>n</i> = 88)	( <i>n</i> = 38)			( <i>n</i> = 50)			
TLA	38 (100)	1.431	0.742-2.439	0	0.168	0.09-0.2900	0.294
CST1	35 (92.1)	0.812	0.193-1.927	2 (4)	0.220	0.150-0.350	0.350
SRS9	14 (36.8)	0.566	0.125-1.346	1 (2)	0.209	0.114-0.344	0.344
SAG1	36 (94.7)	0.775	0.170-2.113	2 (4)	0.200	0.125-0.291	0.291
GRA7	35 (92.1)	0.992	0.162-2.110	0	0.189	0.112-0.287	0.287
Human ( <i>n</i> = 92)	( <i>n</i> = 37)			( <i>n</i> = 55)			
TLA	37 (100)	1.172	0.458-2.173	0	0.191	0.089-0.290	0.297
CST1	36 (97.3)	0.889	0.273-1.870	4 (7.2)	0.230	0.140-0.340	0.340
SRS9	15 (40.5)	0.504	0.111-1.241	4 (7.2)	0.233	0.113-0.389	0.389
SAG1	35 (94.6)	1.111	0.117-1.325	3 (5.4)	0.201	0.107-0.339	0.339
GRA7	35 (94.6)	1.317	0.201-1.723	0	0.223	0.125-0.320	0.320

**Table 2.** Comparison of the immunoreactivities of CST1, SRS9, SAG1, GRA7, and TLA in the IgG ELISA using pools of goat, sheep and human sera.

Antigen	Pool of seropositive sera			Pool of seronegative sera			Cutoff value
	No. (%) of positive sera	Mean absorbance value	Ranges absorbance value	No. (%) of positive sera	Mean absorbance value	Ranges absorbance value	
Goat ( <i>n</i> = 404)	<i>(n</i> = 171)			<i>(n</i> = 233)			
TLA	171 (100)	1.077	0.226-2.936	0	0.140	0.900-0.200	0.203
CST1	165 (96.5)	0.733	0.141-2.707	9 (3.8)	0.296	0.173-0.381	0.381
SRS9	77 (45)	0.781	0.101-2.400	3 (1.2)	0.219	0.100-0.300	0.300
SAG1	165 (96.5)	1.759	0.138-2.816	0	0.190	0.100-0.286	0.286
GRA7	160 (93.6)	1.595	0.109-2.635	5 (2.1)	0.180	0.100-0.290	0.290
Sheep ( <i>n</i> = 88)	<i>(n</i> = 34)			<i>(n</i> = 54)			
TLA	34 (100)	1.266	0.553-2.516	0	0.149	0.930-0.200	0.200
CST1	29 (85.3)	0.725	0.190-1.528	3 (5.5)	0.275	0.184-0.295	0.295
SRS9	16 (47)	0.808	0.130-1.805	2 (3.7)	0.233	0.116-0.364	0.364
SAG1	32 (94.1)	1.633	0.232-2.634	0	0.214	0.108-0.350	0.350
GRA7	32 (94.1)	1.465	0.292-2.342	0	0.217	0.110-0.375	0.375
Human ( <i>n</i> = 92)	<i>(n</i> = 40)			<i>(n</i> = 52)			
TLA	40 (100)	1.094	0.409-2.230	0	0.253	0.113-0.370	0.373
CST1	35 (87.5)	0.645	0.094-1.839	3 (5.7)	0.250	0.190-0.374	0.374
SRS9	19 (47.5)	0.722	0.110-1.714	0	0.214	0.122-0.351	0.351
SAG1	38 (95)	1.006	0.145-1.455	0	0.233	0.116-0.364	0.364
GRA7	37 (92.5)	0.961	0.134-1.564	0	0.211	0.112-0.308	0.308

**Table 3.** TLA-ELISA (IgM and IgG) results using pools of goat, sheep and human sera.

Sera	Seropositive No (%)	IgM+ IgG- No (%)	IgM- IgG+ No (%)	IgM+ IgG+ No (%)	Seronegative No (%)
Goat ( <i>n</i> = 404)	200 (49.50)	29 (7.17%)	16 (3.96)	155 (38.36)	204 (50.49)
Sheep ( <i>n</i> = 88)	44 (50)	10 (11.36)	6 (6.81)	28 (31.81)	44 (50)
Human ( <i>n</i> = 92)	50 (54.34)	10 (10.86)	13 (14.13)	27 (29.34)	42 (45.65)

**Table 4.** Different immunofluorescence patterns detected for encysted bradyzoites and intracellular tachyzoites by positive serum for anti-*Toxoplasma gondii* antibodies.

Sera	Bradyzoite- immunostaining patterns			Tachyzoite- immunostaining patterns			
	Cyst wall	Surface	Mixed	Surface	PVM	PV-internal	Mixed
Goat <i>n</i> = 171 (%)	155 (90.6)	4 (2.3)	12 (7)	135 (78.9)	30 (17.5)	2 (1.2)	4 (2.3)
Sheep <i>n</i> = 34 (%)	31 (91.2)	1 (2.9)	2 (5.8)	27 (79.4)	5 (14.7)	1 (2.9)	1 (2.9)
Human <i>n</i> = 40 (%)	36 (90)	1 (2.5)	3 (7.5)	32 (80)	6 (15)	1 (2.5)	1 (2.5)

### Highlights

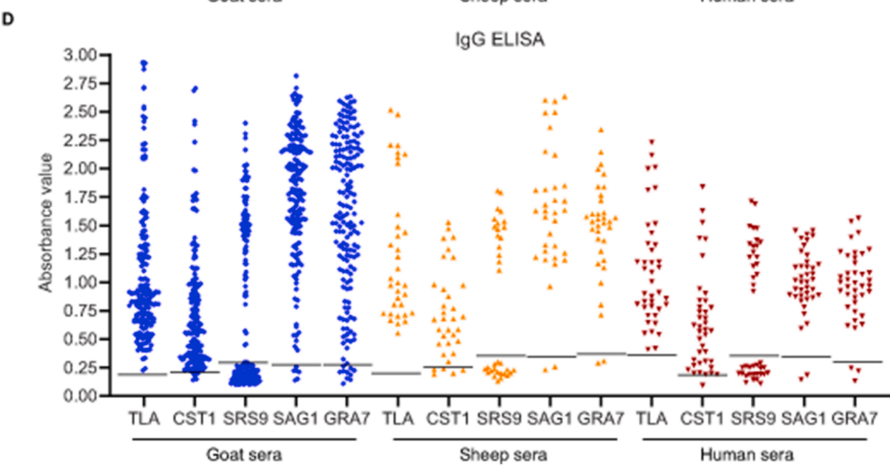
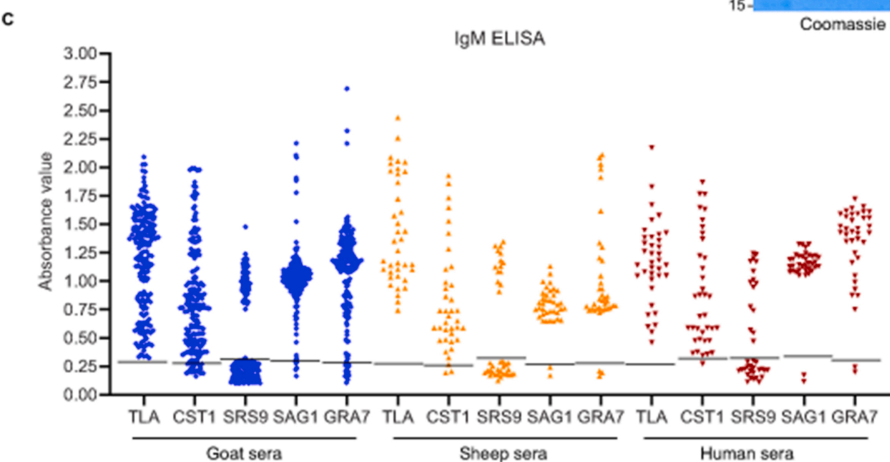
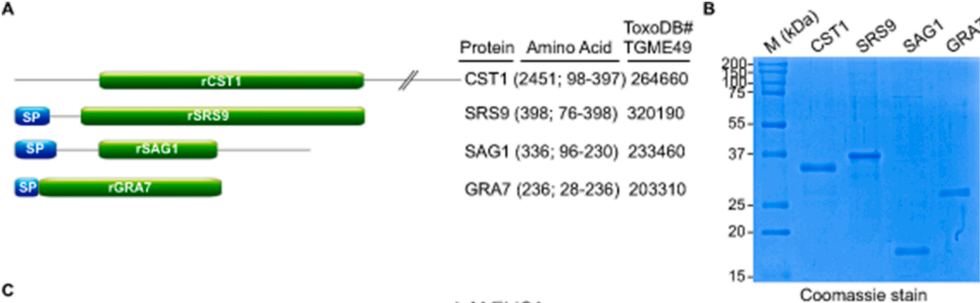
- Encysted bradyzoite represents a key developmental stage of *Toxoplasma* infection
- Humoral response against bradyzoite associated cyst antigen remains to be examined
- *Toxoplasma gondii* IgG+ IgM- sera recognized cyst wall antigens
- Robust humoral response observed against cyst wall antigen CST1
- CST1 emerged as a key immunodominant antigen with diagnostic potential

Journal Pre-proof

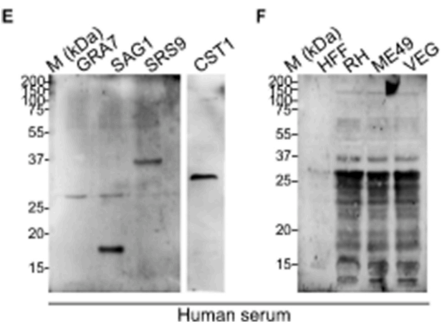
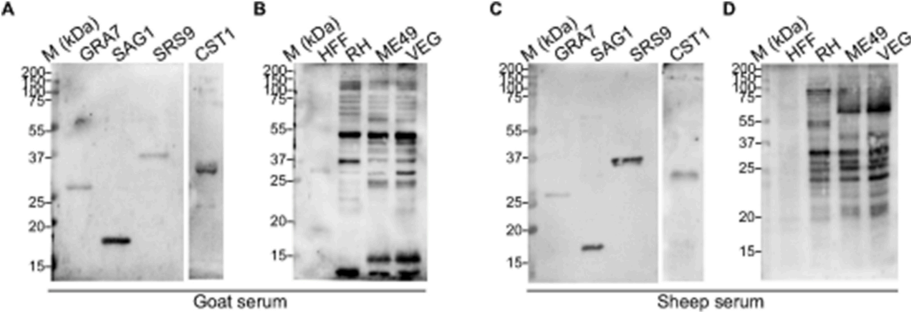
**Author statement**

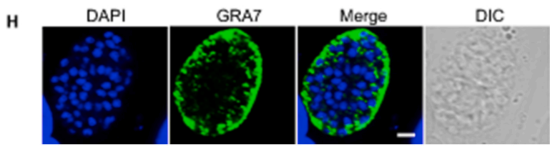
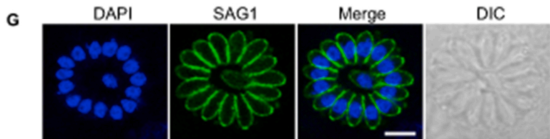
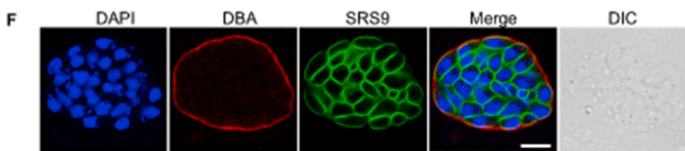
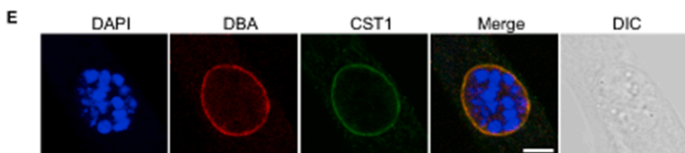
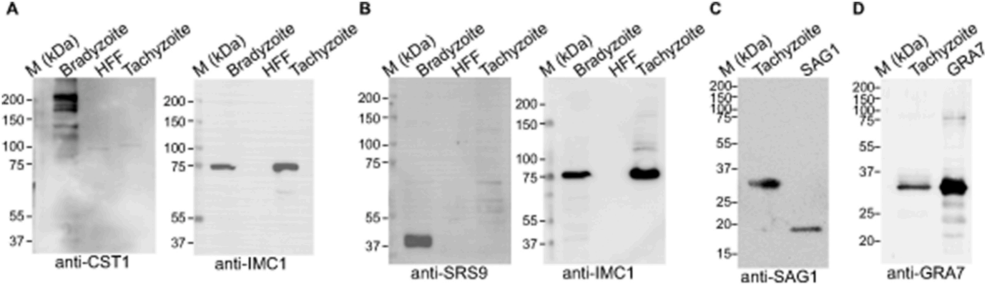
Abhijit S. Deshmukh: Conceptualization, Data curation, Funding acquisition, Methodology, Investigation and Writing- Original draft preparation. Rajkumar Gurupwar: Resources and investigation. Pallabi Mitra: Investigation, Writing- Original draft preparation. Kalyani Aswale: Investigation and Formal analysis. Shilpshri Shinde: Resources and investigation. Sandeep Chaudhari: Resources and investigation.

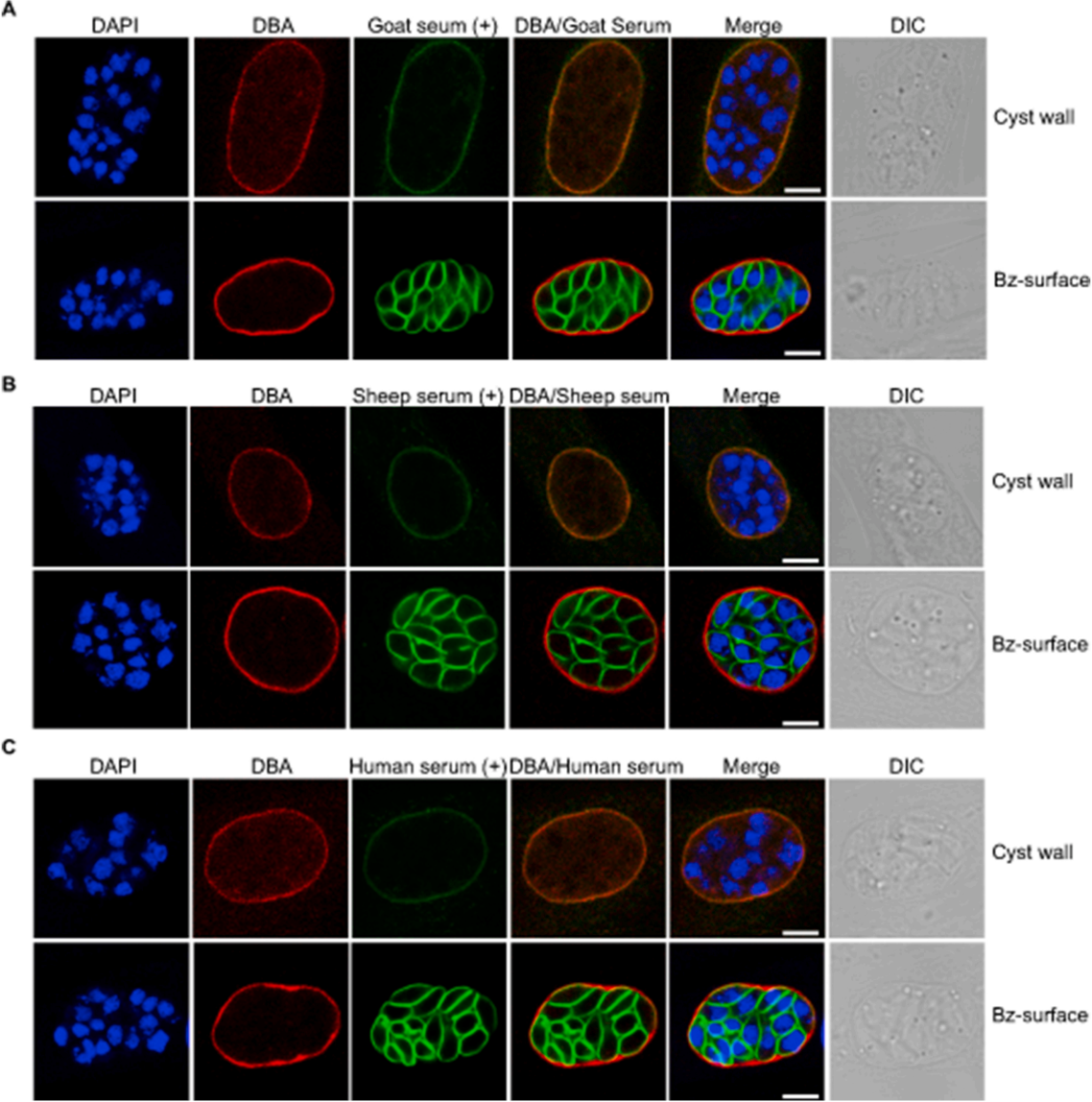
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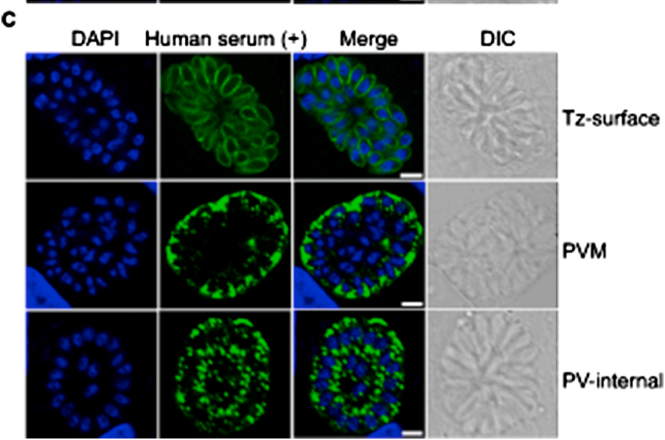
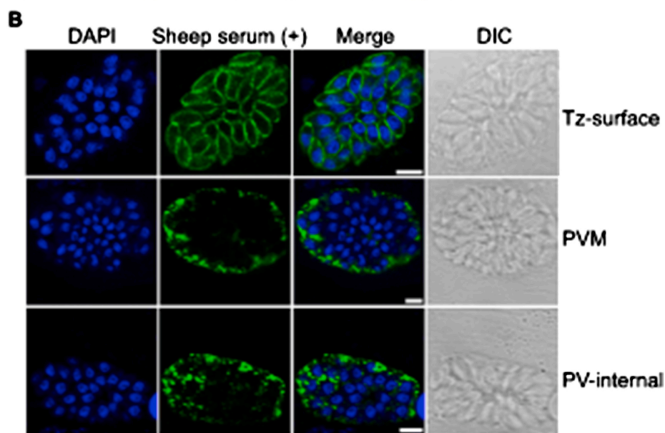
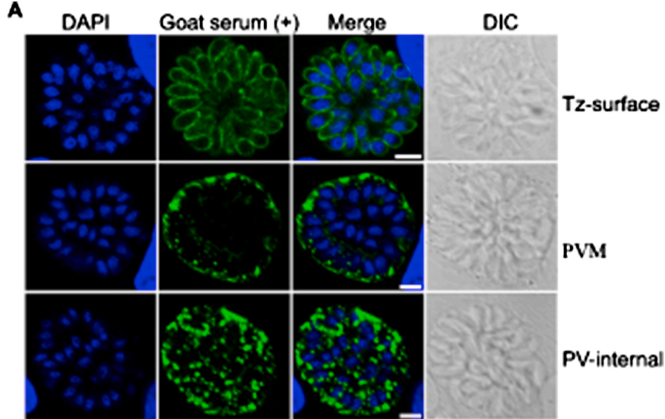


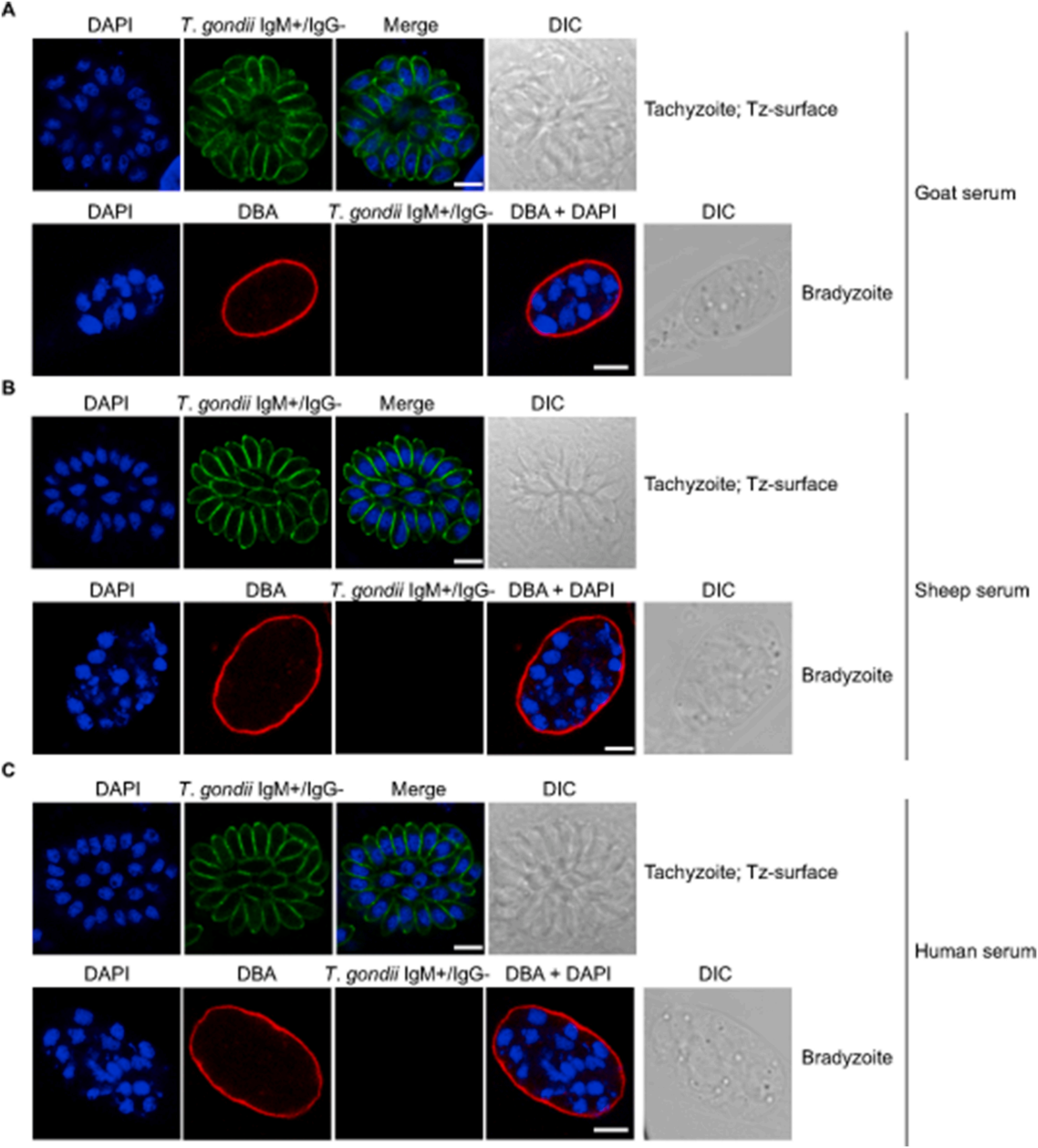




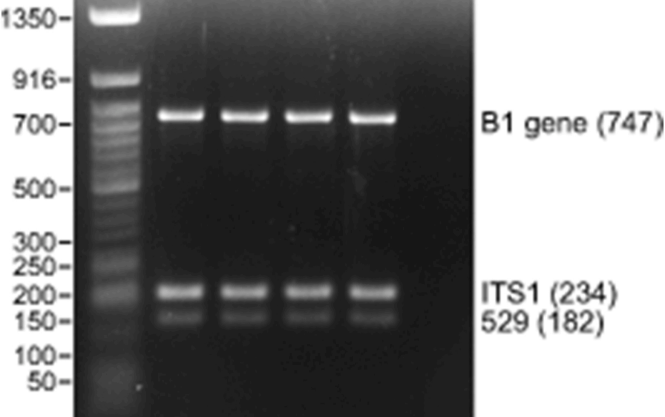








Marker (bp)  
RH-DNA (Control)  
IgM+/IgG- (Goat)  
IgM+/IgG- (Sheep)  
IgM+/IgG- (Human)  
IgM-/IgG-



### Declaration of interests

✓ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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