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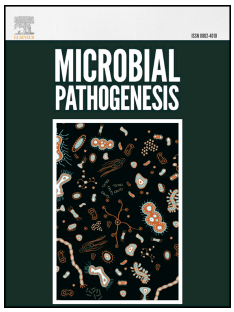

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PII: S0882-4010(20)31009-3

DOI: https://doi.org/10.1016/j.micpath.2020.104643

Reference: YMPAT 104643

To appear in: *Microbial Pathogenesis*

Received Date: 23 August 2020

Revised Date: 10 November 2020

Accepted Date: 12 November 2020

Please cite this article as: Deshmukh AS, Gurupwar R, Mitra P, Aswale K, Shinde S, Chaudhari S, *Toxoplasma gondii* induces robust humoral immune response against cyst wall antigens in chronically infected animals and humans, *Microbial Pathogenesis* (2020), doi: https://doi.org/10.1016/j.micpath.2020.104643.

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

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Keywords

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

ABSTRACT

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

1. Introduction

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

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

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

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

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

Following host immune response, tachyzoites differentiate into the slow-growing bradyzoite stage. Toxoplasma stage differentiation is accompanied with differential expression of several, closely related GPI-anchored surface proteins belonging to the SRS (*S*AG1-*r*elated *s*equence) superfamily [9]. SAG1 is the most abundant SRS antigen expressed in the tachyzoite stage [10]. In oral infection with tissue cysts, bradyzoite stage specific SRS antigens (e.g. SRS9) play a key role in attachment and invasion of intestinal epithelial cells [11,12]. In fact, following ingestion of tissue cyst, SRS antigens expressed by bradyzoites are among the first parasite antigens to be presented to the immune system [11,12]. However, the immunogenicity particularly of the host humoral response against bradyzoite-specific SRS antigens is not known.

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

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

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

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

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

2. Materials and Methods

2.1. Blood and serum samples

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

2.2. Parasite culture

Toxoplasma gondii strains RH (type I), ME49 (type II) and VEG (type III) were maintained in primary human foreskin fibroblasts (HFF, ATCC) cells in Dulbecco's modified Eagle's medium supplemented with 10% FBS, 10 µg/ml Gentamicin and 2mM L-glutamine at 37 °C and 5% CO2 in a humidified incubator. Parasites were purified by filtration through 3.0 µm filters [28].

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 *Nde*I-*Xho*I sites
160 whereas CST1 was cloned into *Nde*I-*Xho*I 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_{600} 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_2PO_4, 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^{2+}-NTA agarose (Qiagen)

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

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

2.5. Toxoplasma gondii native lysate antigen (TLA)

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

2.6. Enzyme-Linked Immunosorbent Assay (ELISA)

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

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

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

2.7. Western blotting

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

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

2.8. Indirect Immunofluorescence assay (IFA)

IFA was performed as described elsewhere [33]. Confluent monolayers of HFF cells were grown on coverslip in 6-well plates and infected with RH strain of *T. gondii* parasites. The infected cells were fixed post 32 to 36 h of infection in 4% paraformaldehyde, followed by permeabilization with 0.35% Triton X-100. The samples were further blocked in 2% BSA and incubated sequentially with primary and secondary antibodies. Goat, sheep and human serum samples were used at 1:50 dilution each. Mouse anti-CST1, mouse anti-SRS9, mouse anti-SAG1 and mouse anti-GRA7 were used at 1:500 dilution each. Alexa Fluor-488 conjugated secondary antibodies [anti-goat IgG (Invitrogen), anti-goat IgM (Invitrogen), anti-sheep IgG (Invitrogen), anti-sheep IgM (Biorad), anti-human IgG (Invitrogen), anti-human IgM (Invitrogen), anti-mouse IgG (Invitrogen)] were used at a dilution of 1:1000. Coverslips were mounted with Vectashield medium (VectorLabs) with 4′,6-Diamidino-2-phenylindole dihydrochloride (DAPI) on glass slide and images were captured with a Leica Confocal microscope with 100× objective. Images were processed using LAS X software.

Bradyzoite induction was performed as described elsewhere [34]. Briefly, filter purified parasites (ME49) were inoculated into a confluent HFF monolayer in a 6-well plate at a multiplicity of infection of 0.5 to 1.0. After 2-h incubation at 37^{o}C in 5% CO_2, the medium was changed to RPMI with 50 mM HEPES (pH 8.2) without $NaHCO_3$, supplemented with 1% FBS and 10 μg/ml Gentamicin, and incubated in a humid 37^{o}C incubator without CO_2 for 5 days. Bradyzoite induction medium was changed every 2 days.

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

2.9. Polymerase chain reaction (PCR)

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

2.10. Statistical analysis

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

3. **Results**

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

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

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

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

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

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

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

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

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

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

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

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

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

3.4. T. gondii positive sera predominantly recognize tachyzoite surface antigens

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

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

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

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

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

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

4. Discussion

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

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

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

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

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

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

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

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

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

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

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

Acknowledgements

This work was supported by the BIRAC-PACE (BT/AIR0470/PACE-15/18) and DST (ECR/2017/002023) to ASD. PM was supported by DST/INSPIRE grant (04/2016/000488). We thank all veterinary doctors, and farmers, students who participated in this study. We also thank NIAB's confocal imaging facility and Mr. Shashikant for his technical support. ASD laboratory members Nikita, Bhavana and Yamini for help with the CST1 antibody and Western blot. KA acknowledges DBT, India for PhD fellowship.

Author statement

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Conflict of interest statement

The authors declare no competing interests.

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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.

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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”.

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

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

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

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

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

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

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Keywords

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

ABSTRACT

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

1. Introduction

Toxoplasmosis, caused by *Toxoplasma gondii*, is an intracellular protozoan infection highly prevalent in warm-blooded vertebrate hosts. The disease causes abortions, stillbirth, and neonatal mortality, especially in sheep, goats and swine besides humans, leading to 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

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

Following host immune response, tachyzoites differentiate into the slow-growing bradyzoite stage. Toxoplasma stage differentiation is accompanied with differential expression of several, closely related GPI-anchored surface proteins belonging to the SRS (*S*AG1-*r*elated *s*equence) superfamily [9]. SAG1 is the most abundant SRS antigen expressed in the tachyzoite stage [10]. In oral infection with tissue cysts, bradyzoite stage specific SRS antigens (e.g. SRS9) play a key role in attachment and invasion of intestinal epithelial cells [11,12]. In fact, following ingestion of tissue cyst, SRS antigens expressed by bradyzoites are among the first parasite antigens to be presented to the immune system [11,12]. However, the immunogenicity particularly of the host humoral response against bradyzoite-specific SRS antigens is not known.

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

Several studies indicate that bradyzoite containing cysts are not completely 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

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

2. Materials and Methods

2.1. Blood and serum samples

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

2.2. Parasite culture

Toxoplasma gondii strains RH (type I), ME49 (type II) and VEG (type III) were maintained in primary human foreskin fibroblasts (HFF, ATCC) cells in Dulbecco's modified Eagle's medium supplemented with 10% FBS, 10 µg/ml Gentamicin and 2mM L-glutamine at 37 °C and 5% CO2 in a humidified incubator. Parasites were purified by filtration through 3.0 µm filters [28].

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 *Nde*I-*Xho*I sites
983 whereas CST1 was cloned into *Nde*I-*Xho*I 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_{600} 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_2PO_4, 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^{2+}-NTA agarose (Qiagen)

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

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

2.5. Toxoplasma gondii native lysate antigen (TLA)

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

2.6. Enzyme-Linked Immunosorbent Assay (ELISA)

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

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

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

2.7. Western blotting

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

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

2.8. Indirect Immunofluorescence assay (IFA)

IFA was performed as described elsewhere [33]. Confluent monolayers of HFF cells were grown on coverslip in 6-well plates and infected with RH strain of *T. gondii* parasites. The infected cells were fixed post 32 to 36 h of infection in 4% paraformaldehyde, followed by permeabilization with 0.35% Triton X-100. The samples were further blocked in 2% BSA and incubated sequentially with primary and secondary antibodies. Goat, sheep and human serum samples were used at 1:50 dilution each. Mouse anti-CST1, mouse anti-SRS9, mouse anti-SAG1 and mouse anti-GRA7 were used at 1:500 dilution each. Alexa Fluor-488 conjugated secondary antibodies [anti-goat IgG (Invitrogen), anti-goat IgM (Invitrogen), anti-sheep IgG (Invitrogen), anti-sheep IgM (Biorad), anti-human IgG (Invitrogen), anti-human IgM (Invitrogen), anti-mouse IgG (Invitrogen)] were used at a dilution of 1:1000. Coverslips were mounted with Vectashield medium (VectorLabs) with 4′,6-Diamidino-2-phenylindole dihydrochloride (DAPI) on glass slide and images were captured with a Leica Confocal microscope with 100× objective. Images were processed using LAS X software.

Bradyzoite induction was performed as described elsewhere [34]. Briefly, filter purified parasites (ME49) were inoculated into a confluent HFF monolayer in a 6-well plate at a multiplicity of infection of 0.5 to 1.0. After 2-h incubation at 37^{o}C in 5% CO_2, the medium was changed to RPMI with 50 mM HEPES (pH 8.2) without $NaHCO_3$, supplemented with 1% FBS and 10 μg/ml Gentamicin, and incubated in a humid 37^{o}C incubator without CO_2 for 5 days. Bradyzoite induction medium was changed every 2 days.

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

2.9. Polymerase chain reaction (PCR)

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

2.10. Statistical analysis

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

3. **Results**

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

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

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

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

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

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

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

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

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

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

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

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

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

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

Western blot results revealed that *T. gondii* positive serum samples contain antibodies against a variety of parasite proteins including CST1, SRS9, SAG1, and GRA7. To verify these results, we investigate whether the antibodies against these antigens could also recognize antigens expressed by encysted bradyzoites (cyst stage). To test that, ME49 strain of *T. gondii* was induced for bradyzoite development in tissue culture. *T. gondii* seropositive samples of goat, sheep and human were examined to detect encysted parasites (bradyzoite stage) by IFA. All sera tested against encysted bradyzoites showed two distinct staining patterns *viz* i) cyst wall staining, ii) bradyzoite surface (Fig. 4A-C). Observed immunostaining patterns were compared with reference cyst wall staining of CST1 and bradyzoite surface of SRS9 (Fig. 3A,B) for confirmation. The predominant immunostaining pattern was observed on the cyst wall which is suggestive of what is seen with CST1 antibodies (Fig. 3A). Of the 171 goat serum samples that stained bradyzoites, 155 (90.6%), 4 (2.3%) and 12 (7%) showed the cyst wall, bradyzoite surface, and mixed staining patterns respectively (Fig. 4A) (Table 4). Similarly, of the 34 sheep serum samples, 31 (91.2%), 1 (2.9%), and 2 (5.8%) showed the cyst wall, bradyzoite surface, and mixed staining patterns respectively (Fig. 4B) (Table 4). Likewise, of the 40 human serum samples that stained bradyzoites, 36 (90%), 1 (2%), and 3 (7.5%) showed the cyst wall, bradyzoite surface, and mixed staining patterns respectively (Fig. 4C) (Table 4). Together, based on the encysted bradyzoite IFA results, ~90% *T. gondii* positive serum samples of goat, sheep and humans were shown to have cyst wall staining patterns. In the similar experimental conditions, none

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

3.4. T. gondii positive sera predominantly recognize tachyzoite surface antigens

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

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

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

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

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

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

4. Discussion

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

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

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

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

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

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

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

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

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

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

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

Acknowledgements

This work was supported by the BIRAC-PACE (BT/AIR0470/PACE-15/18) and DST (ECR/2017/002023) to ASD. PM was supported by DST/INSPIRE grant (04/2016/000488). We thank all veterinary doctors, and farmers, students who participated in this study. We also thank NIAB's confocal imaging facility and Mr. Shashikant for his technical support. ASD laboratory members Nikita, Bhavana and Yamini for help with the CST1 antibody and Western blot. KA acknowledges DBT, India for PhD fellowship.

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.

Conflict of interest statement

The authors declare no competing interests.

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Tables

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

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

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

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

Figure Legends

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

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

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

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

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

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

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

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

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 (*n* = 404)	(*n* = 184)			(*n* = 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 (*n* = 88)	(*n* = 38)			(*n* = 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 (*n* = 92)	(*n* = 37)			(*n* = 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 (*n* = 404)	(*n* = 171)			(*n* = 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-.0290	0.290
Sheep (*n* = 88)	(*n* = 34)			(*n* = 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 (*n* = 92)	(*n* = 40)			(*n* = 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 (*n* = 404)	200 (49.50)	29 (7.17%)	16 (3.96)	155 (38.36)	204 (50.49)
Sheep (*n* = 88)	44 (50)	10 (11.36)	6 (6.81)	28 (31.81)	44 (50)
Human (*n* = 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.

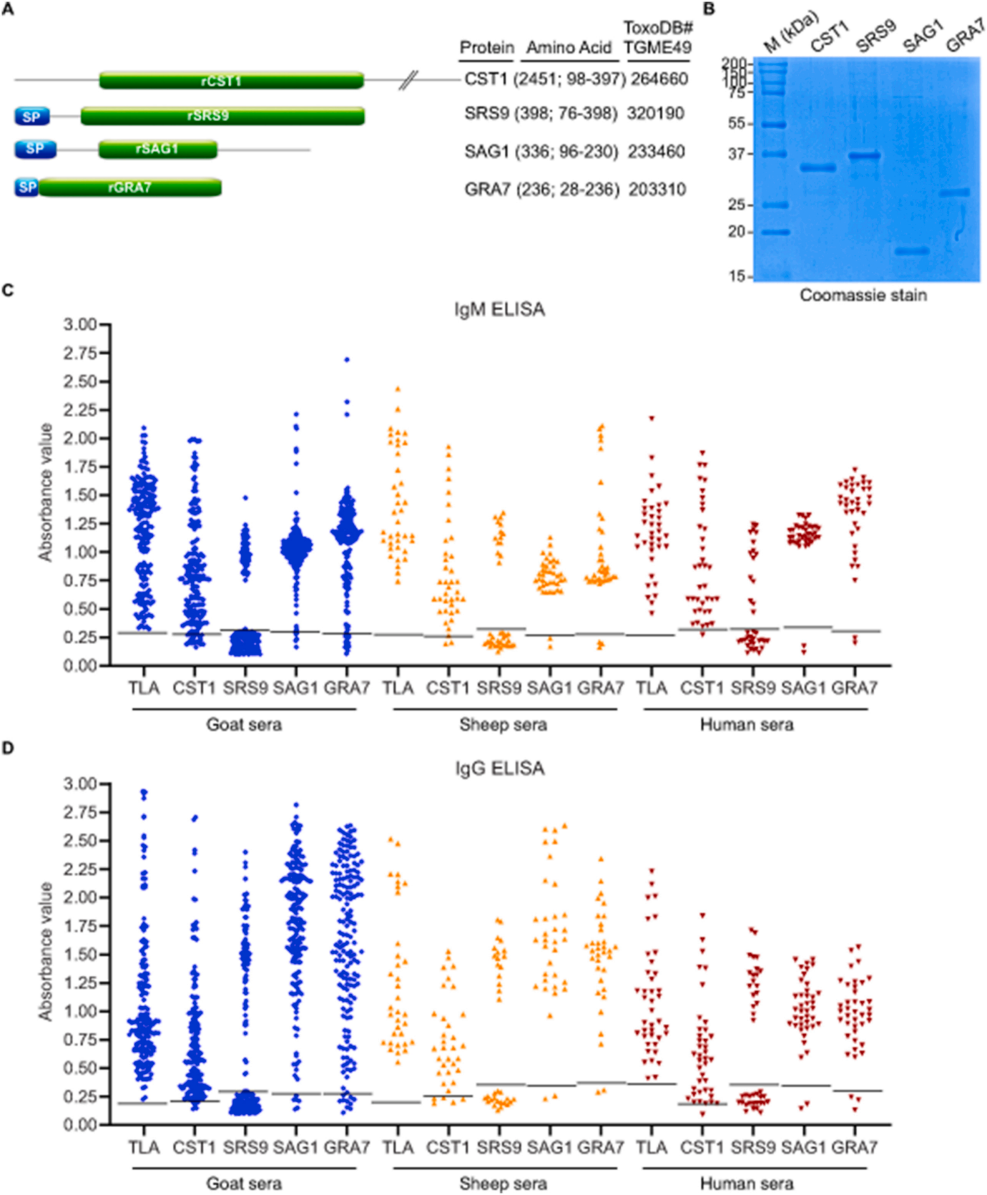
Sera	Bradyzoite- immunostaining patterns			Tachyzoite- immunostaining patterns			
	Cyst wall	Surface	Mixed	Surface	PVM	PV-internal	Mixed
Goat *n* = 171 (%)	155 (90.6)	4 (2.3)	12 (7)	135 (78.9)	30 (17.5)	2 (1.2)	4 (2.3)
Sheep *n* = 34 (%)	31 (91.2)	1 (2.9)	2 (5.8)	27 (79.4)	5 (14.7)	1 (2.9)	1 (2.9)
Human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

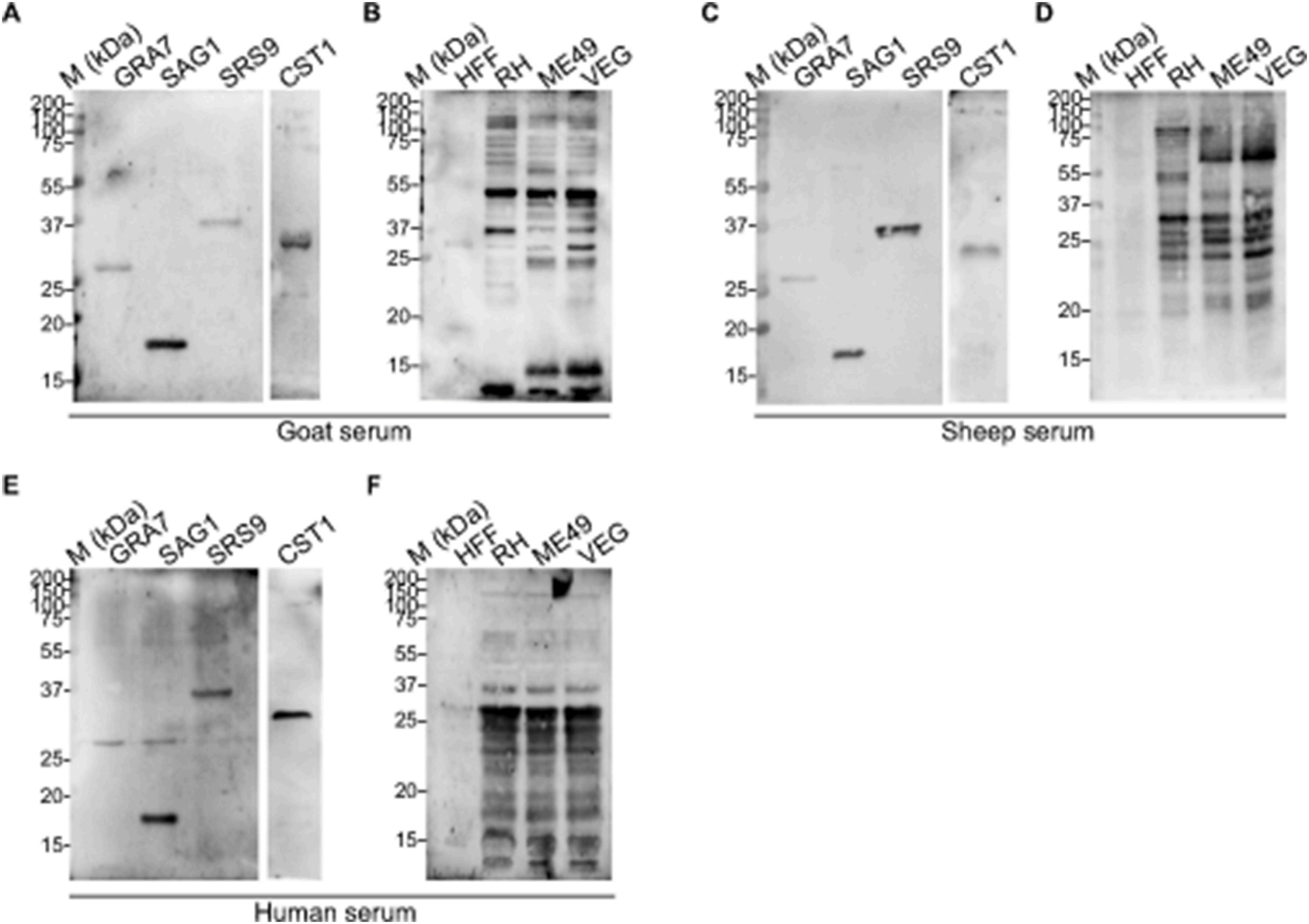
Highlights

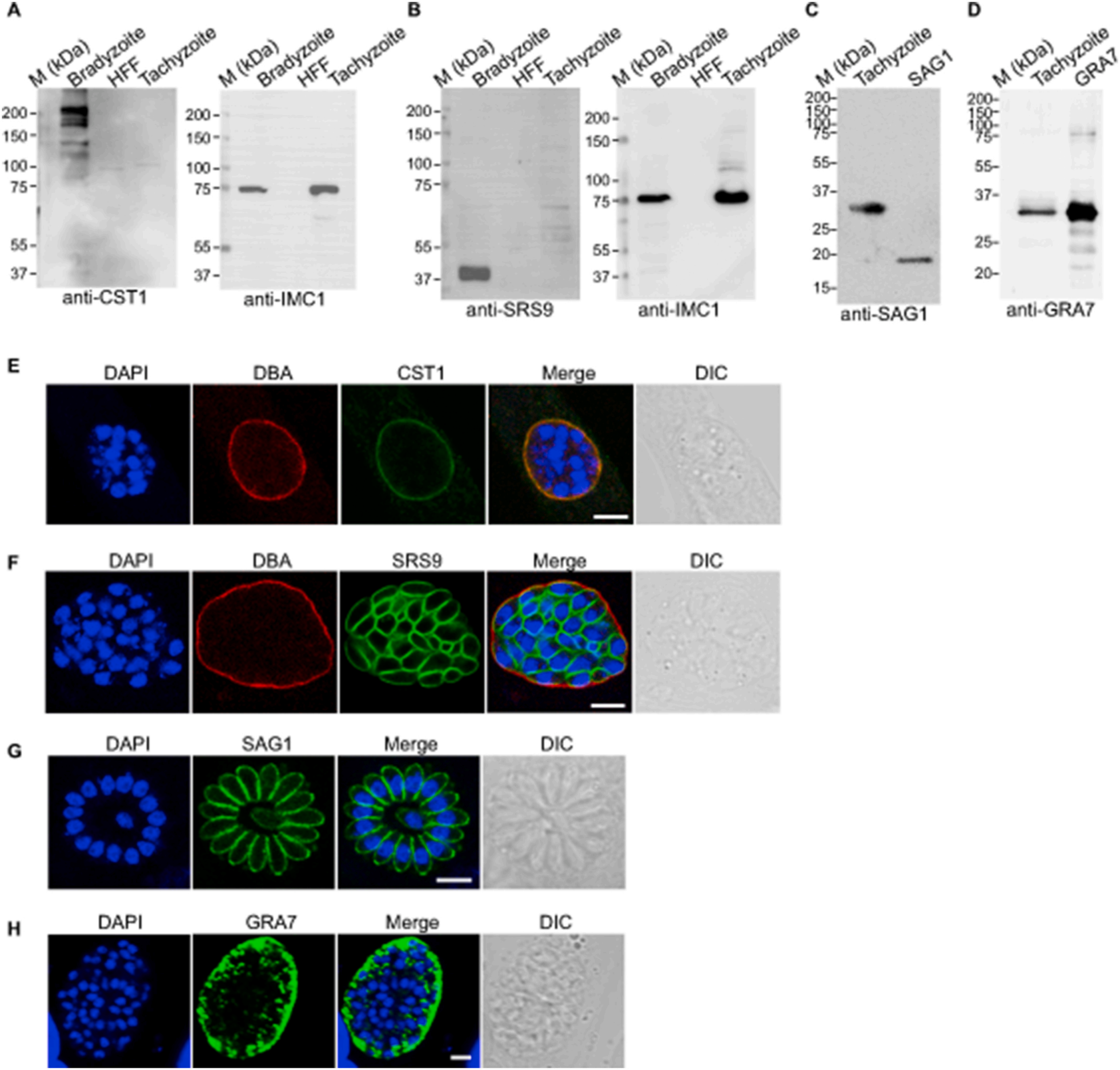
- Encysted bradyzoite represents a key developmental stage of Toxoplasma infection
- Humoral response against bradyzoite associated cyst antigen remains to be examined
- *Toxoplasma gondii* IgG+ IgM- sera recognized cyst wall antigens
- Robust humoral response observed against cyst wall antigen CST1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

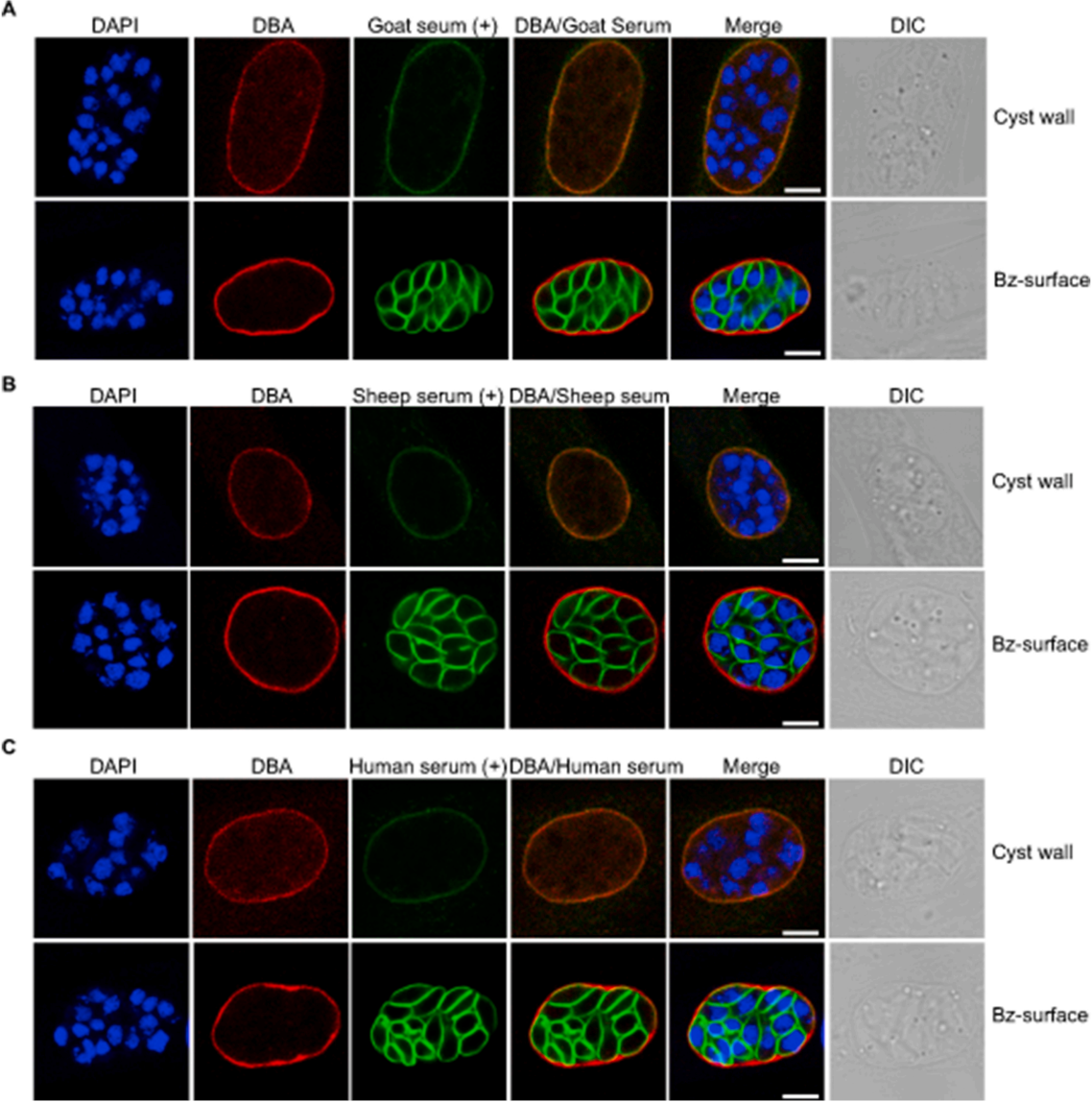
Author statement

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.


A
Protein
Amino Acid
ToxoDB# TGME49
rCST1
CST1 (2451; 98-397) 264660
SP
rSRS9
SRS9 (398; 76-398) 320190
SP
rSAG1
SAG1 (336; 96-230) 233460
SP
rGRA7
GRA7 (236; 28-236) 203310
B
M (kDa)
CST1
SRS9
SAG1
GRA7
200
100
75
55
37
25
20
15
Coomassie stain
C
IgM ELISA
Absorbance value
3.00
2.75
2.50
2.25
2.00
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
TLA CST1 SRS9 SAG1 GRA7 TLA CST1 SRS9 SAG1 GRA7 TLA CST1 SRS9 SAG1 GRA7
Goat sera
Sheep sera
Human sera
D
IgG ELISA
Absorbance value
3.00
2.75
2.50
2.25
2.00
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
TLA CST1 SRS9 SAG1 GRA7 TLA CST1 SRS9 SAG1 GRA7 TLA CST1 SRS9 SAG1 GRA7
Goat sera
Sheep sera
Human sera


A
M (kDa)
GRA7
SAG1
SRS9
CST1
200
150
100
75
55
37
25
20
15
B
M (kDa)
HFF
RH
ME49
VEG
200
150
100
75
55
37
25
20
15
Goat serum
C
M (kDa)
GRA7
SAG1
SRS9
CST1
200
150
100
75
55
37
25
20
15
D
M (kDa)
HFF
RH
ME49
VEG
200
150
100
75
55
37
25
20
15
Sheep serum
E
M (kDa)
GRA7
SAG1
SRS9
CST1
200
150
100
75
55
37
25
20
15
F
M (kDa)
HFF
RH
ME49
VEG
200
150
100
75
55
37
25
20
15
Human serum


A
M (kDa)
Bradyzoite
HFF
Tachyzoite
200
150
100
75
55
37
anti-CST1
anti-IMC1
B
anti-SRS9
anti-IMC1
C
Tachyzoite
SAG1
25
20
15
anti-SAG1
D
GRA7
anti-GRA7
E
DAPI
DBA
CST1
Merge
DIC
F
SRS9
G
SAG1
H
GRA7


A
DAPI
DBA
Goat seum (+)
DBA/Goat Serum
Merge
DIC
Cyst wall
Bz-surface
B
DAPI
DBA
Sheep serum (+)
DBA/Sheep seum
Merge
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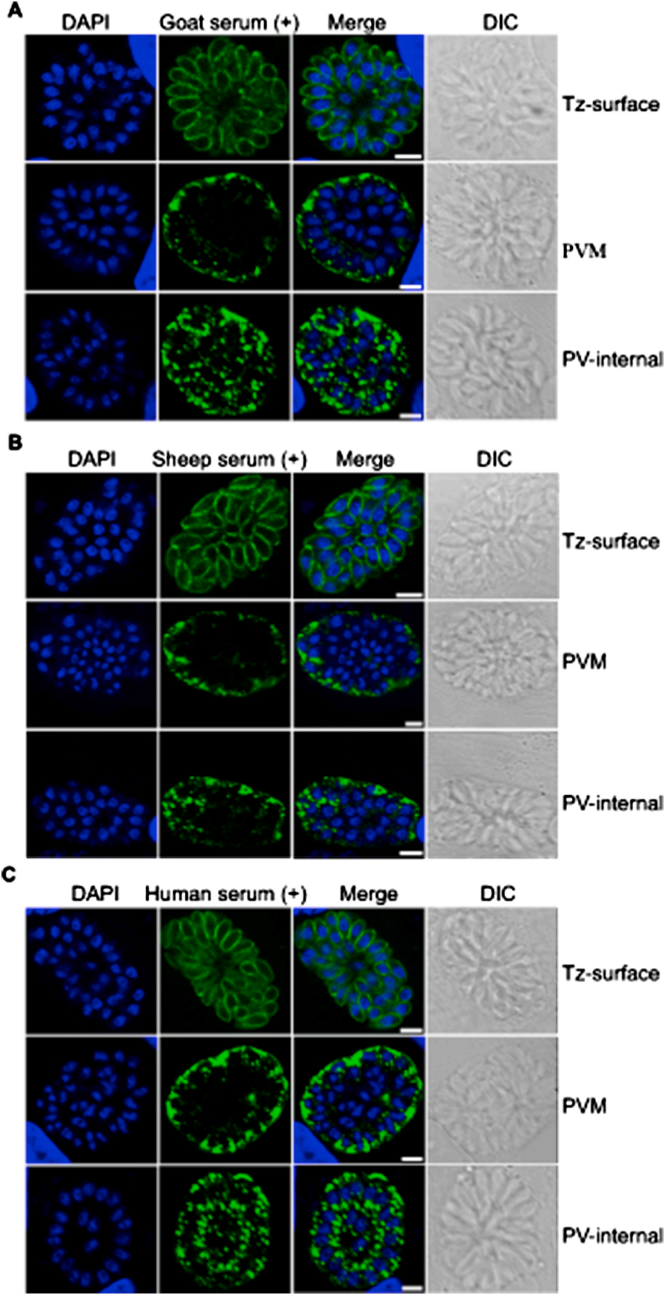
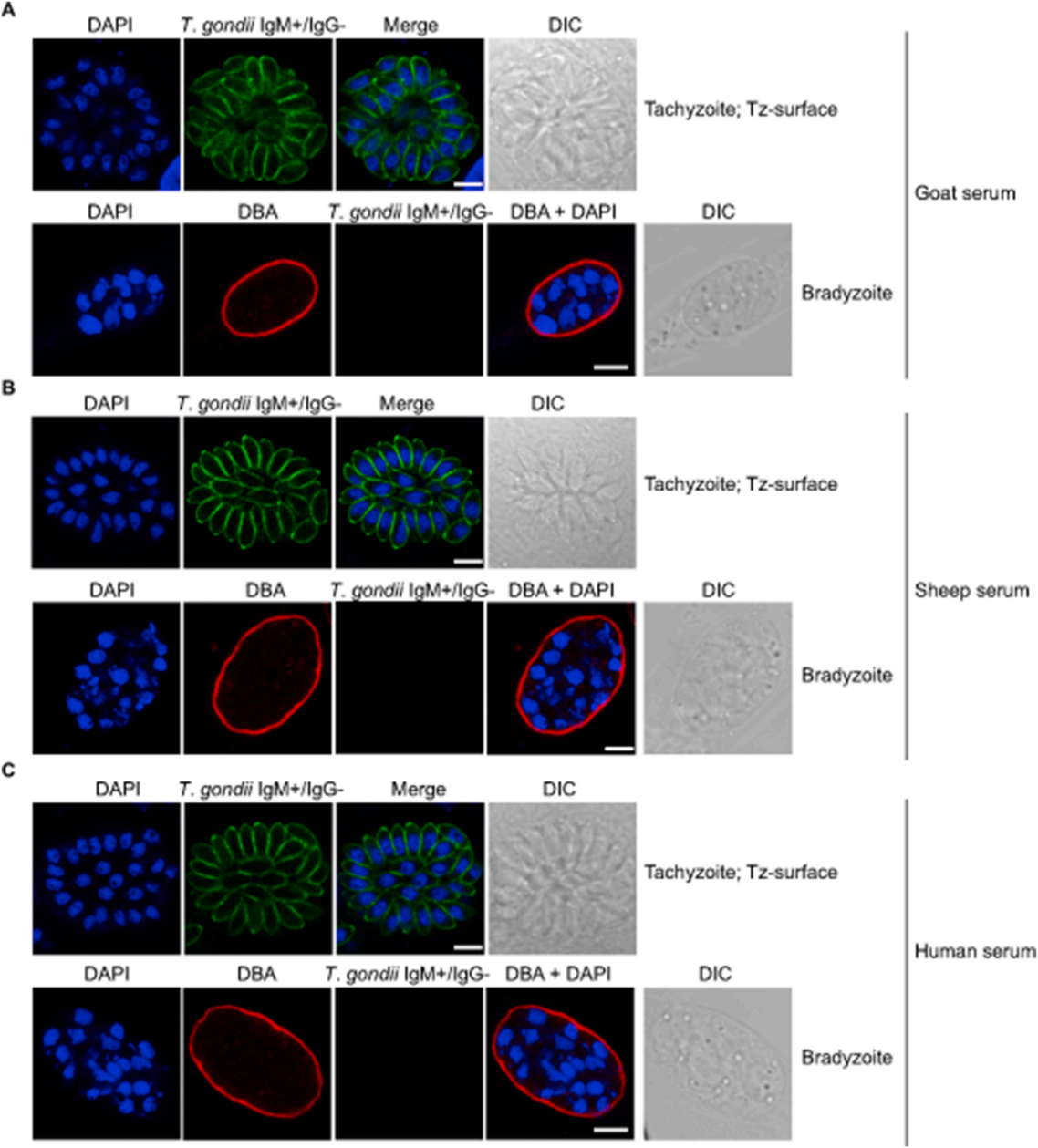
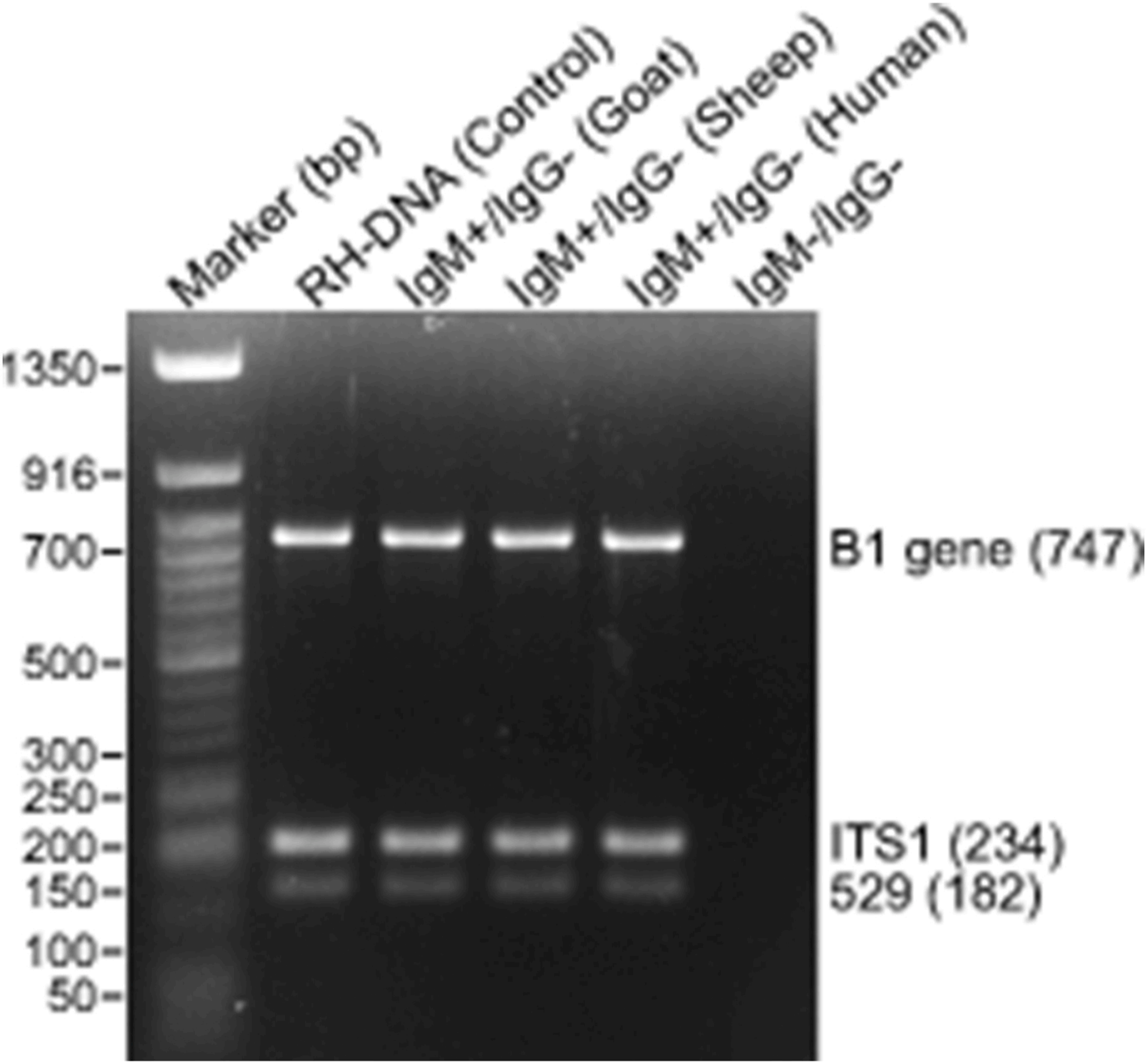
Cyst wall
Bz-surface
C
DAPI
DBA
Human serum (+)
DBA/Human serum
Merge
DIC
Cyst wall
Bz-surface


A
DAPI
Goat serum (+)
Merge
DIC
Tz-surface
PVM
PV-internal
B
DAPI
Sheep serum (+)
Merge
DIC
Tz-surface
PVM
PV-internal
C
DAPI
Human serum (+)
Merge
DIC
Tz-surface
PVM
PV-internal


A
DAPI
T. gondii IgM+/IgG-
Merge
DIC
Tachyzoite; Tz-surface
DAPI
DBA
T. gondii IgM+/IgG-
DBA + DAPI
DIC
Bradyzoite
Goat serum
B
DAPI
T. gondii IgM+/IgG-
Merge
DIC
Tachyzoite; Tz-surface
DAPI
DBA
T. gondii IgM+/IgG-
DBA + DAPI
DIC
Bradyzoite
Sheep serum
C
DAPI
T. gondii IgM+/IgG-
Merge
DIC
Tachyzoite; Tz-surface
DAPI
DBA
T. gondii IgM+/IgG-
DBA + DAPI
DIC
Bradyzoite
Human serum


Marker (bp)
RH-DNA (Control)
IgM+/IgG- (Goat)
IgM+/IgG- (Sheep)
IgM+/IgG- (Human)
IgM-/IgG-
1350
916
700
500
300
250
200
150
100
50
B1 gene (747)
ITS1 (234)
529 (182)

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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