

Evaluation of Diagnostic Potential of *Echinococcus granulosus* Recombinant *EgAgB8/1*, *EgAgB8/2* and EPC1 Antigens for Cystic Echinococcosis in Goats

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Echinococcus granulosus recombinant proteins including two antigen B sub-units *EgAgB8/1* and *EgAgB8/2* and *Echinococcus* protoscolex calcium binding protein (EPC1) were expressed in prokaryotic expression vectors. The diagnostic potential of these three recombinant proteins was evaluated in the detection of cystic echinococcosis in goats in IgG-ELISA. The *EgAgB8/1* and *EgAgB8/2* recombinant proteins reacted fairly with the hydatid infected goats with sensitivity of 66.7% and 80.0% and specificity of 71.3% and 73.3%, respectively while EPC1 recombinant protein showed lower sensitivity (60%) but comparable specificity (72.3%). Cross-reactivity of these three antigens with goat gastro-intestinal strongyle nematodes and *Taenia hydatigena* under field conditions was studied. Results showed that *EgAgB8/1*, *EgAgB8/2* and EPC1 antigens cross-reacted with most of the parasites in the goat host.

Keywords: *Echinococcus granulosus*; goat; Antigen B8 sub-units; EPC1; Diagnosis; IgG-ELISA; Cross-reactivity.

Cystic echinococcosis (CE) is a cyclozoonosis caused by the larval stage of the taeniid cestode *Echinococcus granulosus*. It is a serious public health concern and in India the economic losses of ~ US \$ 212 million associated with cystic echinococcosis in livestock are high¹. Infection of the intermediate host occurs by incidental ingestion of the oncosphere that develops in the liver and lungs to hydatid fluid filled cyst with protoscolices and daughter cysts². Early detection of the infection significantly reduces morbidity and mortality associated with CE. However, early detection of hydatidosis in animals and man has been a challenge. The diagnosis of CE largely depends on imaging scans and serological tests. Diagnosis of CE in animals

by imaging methods is not feasible in the developing countries due to cost factors and in the absence of accurate serological tests hydatid infected animals remain in the morbid state. Hydatid cyst fluid has been used most frequently as a source of *E. granulosus* antigens and its components have been comprehensively investigated for their applicability in serological tests. Among several proteins of hydatid cyst fluid, antigen 5 (*EgAg5*) and antigen B (*EgAgB*) are known to be potent diagnostic antigens but the native and recombinant *EgAgB* antigens allow for better diagnostic performance²⁻⁴. *EgAgB* is a thermostable, lipoprotein encoded by a multigene family⁵. The literature describing the immunological diagnosis of hydatid disease in humans is extensive [2, 6-9] but research efforts towards the development of immunodiagnostic tests for *E. granulosus* infection in domestic ruminants are limited and the results have not been generally consistent due to poor sensitivity and specificity

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of the immuno-assays in animals¹⁰. The only reliable method of diagnosing hydatidosis in animals is by detection of cysts at necropsy. Therefore, present studies were undertaken to express three recombinant antigens *EgAgB8/1*, *EgAgB8/2* and EPC1 known to be potent diagnostic antigens in human cystic echinococcosis and to assess their immunodiagnostic potential for cystic echinococcosis in goats.

MATERIALS AND METHODS

Three recombinant proteins of *E. granulosus* including two *EgAgB* sub-units *EgAgB8/1* and *EgAgB8/2* and *Echinococcus* protoscolex calcium binding protein (EPC1) were expressed in the prokaryotic expression vectors. Total RNA was isolated from the protoscolices retrieved from a fertile hydatid cyst from buffalo liver at a local abattoir using Trizol reagent (Invitrogen, USA). Briefly, $\sim 0.2 \times 10^4$ protoscolices were treated with Trizol reagent (1ml) and manually homogenized with a micropestle in a sterile 2.0 ml microcentrifuge tube. The lysed protoscolices were freeze-thawed at -80°C for multiple cycles together with manual homogenization to completely lyse the parasites. Total RNA was isolated from the lysed protoscolices following standard RNA isolation protocol (Invitrogen, USA). The RNA was converted to single stranded cDNA using oligo-dT primer and reverse transcriptase enzyme (MBI Fermentas, USA) following standard protocols of cDNA synthesis. The cDNA coding for each of the above three target proteins was PCR amplified with gene specific primers (Table 1). The PCR products of three target cDNAs were cloned in p^{DRIVE} cloning vector (Qiagen, Germany) and sequence confirmed for each cDNA.

Expression of the recombinant *EgAgB8* sub-units and EPC1 protein

The cDNAs coding for *EgAgB8/1*, *EgAgB8/2* and EPC1 proteins were PCR amplified with primers designed with suitable restriction enzyme sites and expressed in three different prokaryotic expression vectors (Table 1).

EgAgB8/1 protein was expressed in p^{Rham} vector in frame with solubility enhancing sumo-fusion protein (ExpressoTM Rhamnose cloning and expression kit; Lucigen, USA). *E. cloni*® 10G chemically competent cells were transformed with

the recombinant p^{Rham} vector and a moderate level of the recombinant fusion protein was generated on induction of the culture with 0.2% L-rhamnose for 8 h at 37°C . The bacterial cells were disrupted with 6 M guanidine hydrochloride in the lysis buffer (pH 8.0) supplemented with 18 mM imidazole and 10 mM β -mercaptoethanol, followed by sonication of the cell lysate with 3 bursts of 30 sec each at 10 micron amplitude. The protein was purified to complete homogeneity using Ni-NTA affinity chromatography. The recombinant protein was bound to Ni-NTA resin (Qiagen, Germany) at room temperature for 2 h with constant shaking. Affinity column was washed with wash buffer (pH 6.0) supplemented with 18 mM imidazole and protein eluted with elution buffer (pH 4.2).

Expression of the *EgAgB8/2* recombinant protein was carried out in p^{ET32a (+)} vector in *Escherichia coli* BL21 (DE3). The cDNA was PCR amplified and cloned in the expression vector in frame with the vector fusion tag. An optimum level of expression of the *EgAgB8/2* recombinant protein was achieved at 6 h post-1mM IPTG induction at 37°C . The recombinant protein was purified by lysis of the bacterial cells in lysis buffer (pH 8.0) containing 8 M urea and supplemented with 12 mM imidazole and 10 mM β -mercaptoethanol for 2 h at RT. The cell lysate was then sonicated on ice for 5 cycles of 30 sec each at 5 micron amplitude. The recombinant protein was bound to Ni-NTA resin at room temperature for 2 h with constant shaking. The affinity column was washed with wash buffer (pH 6.5) supplemented with 12 mM imidazole and recombinant protein eluted with elution buffer at pH 4.2.

The EPC1 target protein was expressed in p^{PROEXHT-b} vector, with a higher level of expression of the recombinant protein achieved at 8 h post-IPTG induction at 37°C . *E. coli* BL21 (DE3) cells induced with 1mM IPTG were disrupted in lysis buffer (pH 8.0) containing 6 M guanidine hydrochloride and supplemented with 16 mM imidazole at room temperature for 2 h and the recombinant protein was purified by Ni-NTA affinity chromatography. The wash buffer (pH 5.9) was supplemented with 16 mM imidazole and recombinant protein was eluted at pH 4.2. The composition of the lysis, wash and elution buffers used in the purification steps of each recombinant protein was 10 mM tris and 100 mM potassium

dihydrogen phosphate containing 6M guanidine hydrochloride or 8M urea as protein denaturant.

The purified recombinant fusion proteins *EgAgB8/1*, *EgAgB8/2* and EPC1 resolved at 25, 29 and 10.5 kDa, respectively in the SDS-PAGE.

Collection of goat sera

Goats (n=116) were screened for hydatid infection at necropsy at the local abattoir. Out of the 116 animals examined at necropsy, 15 were positive for hydatid cysts in the liver or lungs and remaining 101 were negative for hydatid cysts. Sera were retrieved from these animals and screened for anti-hydatid antibodies by IgG-ELISA with the above three recombinant antigens. Sera were also collected from goats infected with other parasites for their cross-reactivity studies. Sera collected

from healthy goats, maintained at Indian Veterinary Research Institute, Izatnagar, were used as negative control in the subsequent immuno-assays. All experiments on goats were conducted as per the guidelines of the Institute Animal Ethics Committee.

Enzyme Linked Immunosorbent Assay

Checker board titrations were done to optimize the concentration of each antigen. The amount of recombinant *EgAgB8/1*, *EgAgB8/2* and EPC1 antigens coated on each well of the 96-well microtitre plate was optimized to 1.0 µg/ml, 2.0 µg/ml and 2.0 µg/ml of coating buffer, respectively. The optimal dilutions of serum samples and anti-goat IgG-HRP conjugate (Sigma Chemicals, USA) used in the assay were 1:100-1:200 and 1:6000-

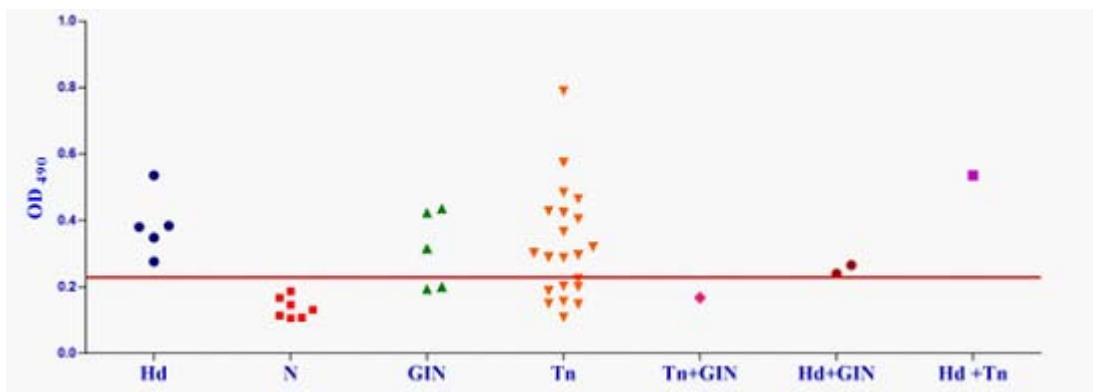
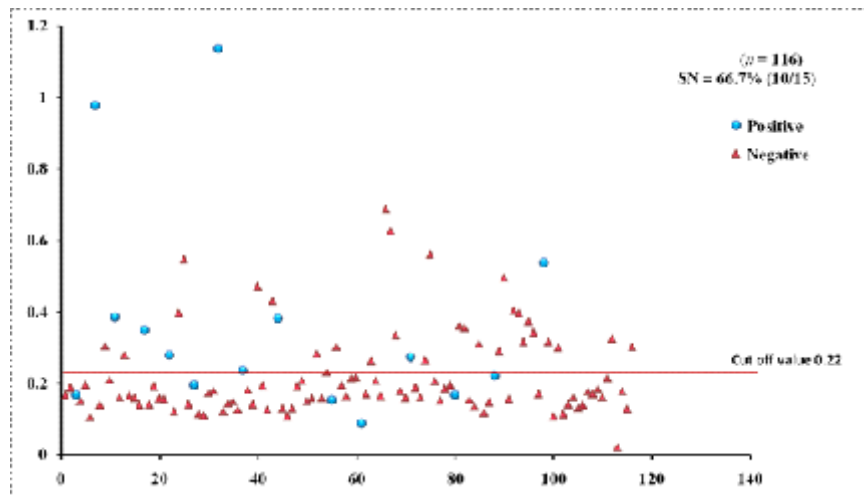


Fig 1a & b. IgG-ELISA showing immuno-reactivity (a) and cross reactivity (b) of the goat sera with recombinant antigen *EgAgB8/1* (Hd -Hydatid positive sera, N- Healthy goat sera (Negative control), GIN – Gastro intestinal nematodes, Tn-*Taenia hydatigena* positive sera)

1:12000, respectively for different antigens. Receiver Operating Characteristic (ROC) curve analysis was performed to determine the cut-off value for each recombinant antigen. Levels of sensitivity were plotted against the levels of one minus specificity at each cut-off point on a ROC curve. Cut-off values were selected that gave the highest sum of sensitivity (%) and specificity (%), as described by Amagai *et al*¹¹. The area under the ROC curve (AUC) was the parameter used to define the antigen's discriminatory values between ELISA positive and negative animals.

RESULTS

The *EgAgB8/1* antigen showed positive reactivity with 10/15 necropsy positive animals with

OD₄₉₂ ranging between 0.22-1.18 above the cut-off value 0.22. However, 5/15 necropsy confirmed positive animals were negative with *EgAgB8/1* ELISA leading to the sensitivity of 66.7%. The IgG-ELISA with this antigen showed 29/101 (28.7%) necropsy negative animals for hydatid cyst as sero-positive (Fig 1a). With antigen *EgAgB8/2*, 12/15 necropsy confirmed positive animals were ELISA positive with OD₄₉₂ values above the cut-off (0.29), thereby depicting the sensitivity of 80.0%. The IgG-ELISA with this antigen showed 27/101 (26.7%) necropsy negative animals as ELISA positive (Fig 2a). The EPC1 based ELISA carried out on the same number of sera had a lower sensitivity of 60% with 9/15 necropsy confirmed positive animals showing A₄₉₂ above the cut-off value (0.29) and 6 were sero-negative with A₄₉₂

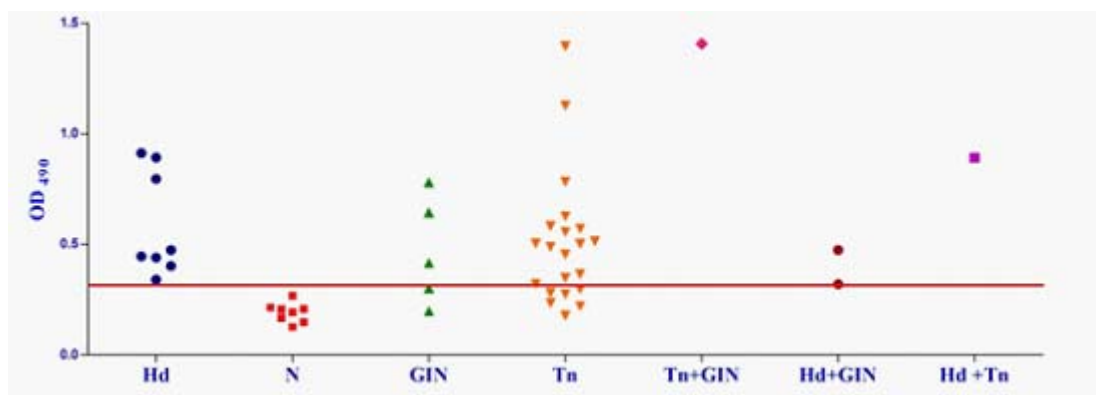
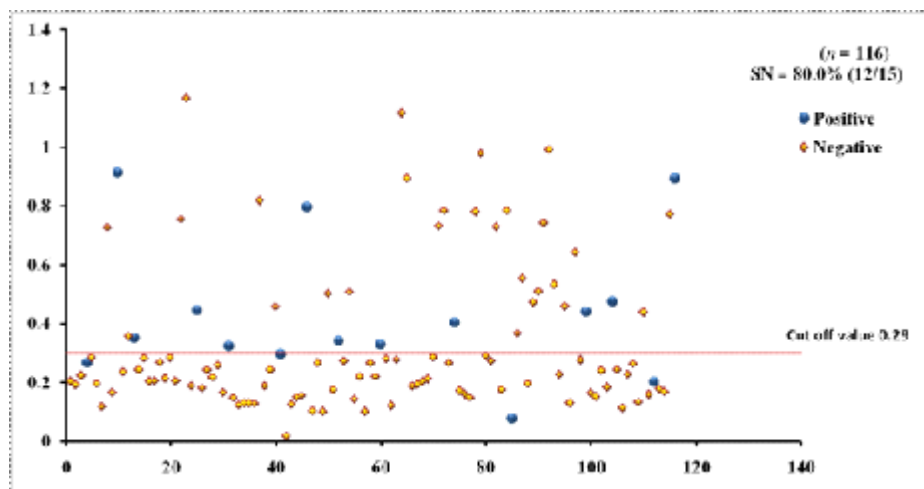


Fig 2a & b. IgG-ELISA showing immuno-reactivity (a) and cross reactivity (b) of the goat sera with recombinant antigen *EgAgB8/2* (Hd -Hydatid positive sera, N- Healthy goat sera (Negative control), GIN – Gastro intestinal nematodes, Tn-*Taenia hydatigena* positive sera)

below cut-off. A number of necropsy negative animals (26/101) were sero-positive (25.7%) with this antigen with A_{492} above the cut-off (Fig 3a). The comparative sensitivity, specificity, positive and negative predictive values of the three assays showed that *EgAgB8/2* antigen has higher sensitivity and specificity than the other two antigens (Table 2).

Studies on the cross-reactivity of each recombinant antigen were conducted with goat sera positive for gastrointestinal (GI) strongyle nematodes and *Taenia hydatigena*. The *EgAgB8/1* antigen showed immuno-reactivity with sera of 3/5 GI nematode infected goats and 14/21 *T. hydatigena* positive goats with OD_{492} above cut

off (0.22). However, serum of single goat with a mixed infection of *T. hydatigena* and GI nematodes showed OD_{492} below negative cut-off. All the goats with a mixed infection of hydatid, *T. hydatigena* and GI nematodes reacted with the antigen *EgAgB8/1*(Fig 1b).

The *EgAgB8/2* antigen showed reactivity with 4/5 sera positive for GI nematodes and 16/21 *T. hydatigena* positive sera with OD_{492} above cut off (0.29). The serum of a single goat with mixed infection of *T. hydatigena* and GI nematodes showed OD_{492} above negative cut-off. Goats with a mixed infection of hydatid, *T. hydatigena* and GI nematodes also reacted with the antigen *EgAgB8/2* (Fig 2b).

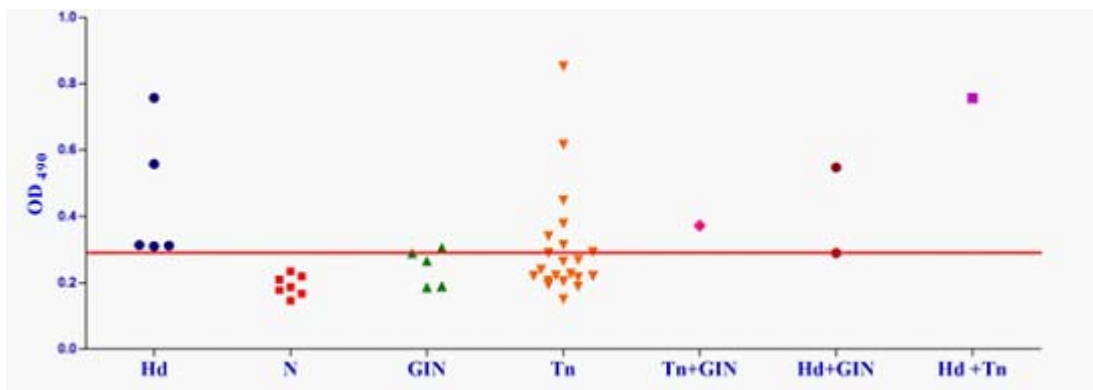
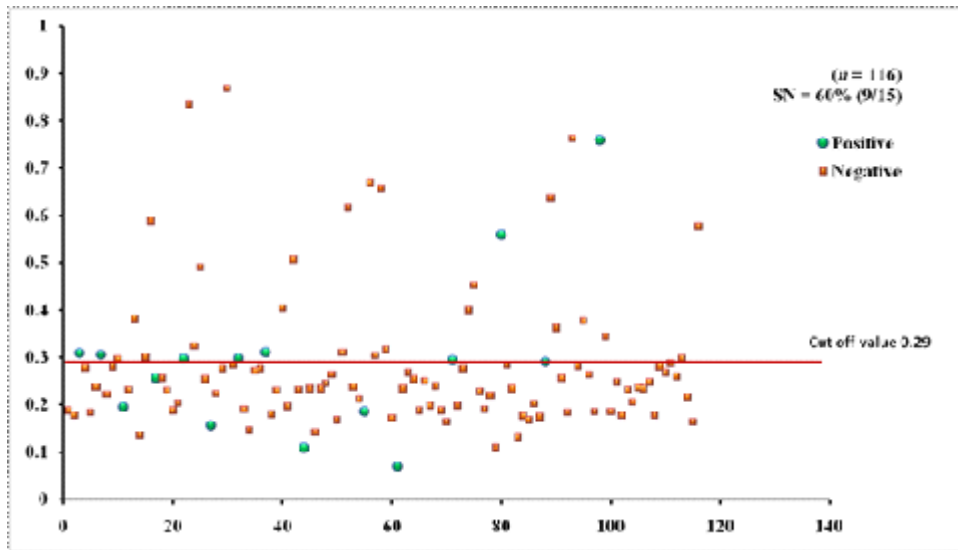


Fig 3a & b. IgG-ELISA showing immuno-reactivity (a) and cross reactivity (b) of the goat sera with recombinant antigen EPC1 (Hd - Hydatid positive sera, N- Healthy goat sera (Negative control), GIN - Gastro intestinal nematodes, Tn-*Taenia hydatigena* positive sera)

The EPC1 antigen showed sero-reactivity with 2/5 GI nematodes, 8/21 *T. hydatigena* infected goats with OD₄₉₂ above cut-off (0.29). The serum of the goat with a mixed infection of *T. hydatigena* and GI nematodes reacted with this antigen with OD₄₉₂ above negative cut-off. Goats with a mixed infection of hydatid, *T. hydatigena* and GI nematodes also reacted with the antigen EPC1 in IgG ELISA (Fig 3b).

DISCUSSION

Two sub-units of *E. granulosus* AgB viz. *EgAgB8/1* and *EgAgB8/2* along with EPC1 were expressed as recombinant proteins and evaluated in the present investigation for the detection of CE

in goats. The two antigen B sub-units *EgAgB8/1* and *EgAgB8/2* showed a sensitivity of 66.7% and 80% and specificity of 71.3% and 73.3%, respectively. However, EPC1 antigen showed a lower sensitivity (60%) but comparable specificity (72.3%). The three recombinant antigens were found to cross-react with the sera of goats infected with gastrointestinal strongyle nematodes and *Taenia hydatigena* equally. Likewise, EPC1 that has shown a potential in the immunodiagnosis of human hydatidosis¹²⁻¹⁴ also cross-reacted with the above parasites in goat host. The results indicate that these proteins share cross-reacting epitopes between the taeniid cestodes and strongyle nematodes. IgG4 sub-class antibody has been shown to reduce the cross-reactivity of *EgAgB8/*

Table 1. Primer sequences designed for PCR amplification (A) and cloning of three cDNAs coding for the recombinant antigens in expression vectors (B). *Sequences with restriction enzyme sites are indicated in bold and sequences for homologous recombination with SUMO / pRham vector sequence are indicated in bold with italics (B).

Gene	Primer name	Primer length	Primer Sequence (5' à 3')	Amplicon size
A	<i>EgAgB8/1</i> B8/1-FOR	24 bp	ATG CTT CTC GCT CTG GCT CTC GTC	247 bp
	B8/1-REV	24 bp	CTA TTC ACC TTC AGC AAT CAA CCC	
	<i>EgAgB8/2</i> B8/2-FOR	21 bp	AAA GAT GAG CCA AAA GCA CAC	250 bp
	B8/2-REV	24 bp	TTA CTT TGA ATC ATC ATC TTT TTC	
EPC1	EPC-FOR	21 bp	TGC GTT TGT CGT TCC TGC CGT	231 bp
	EPC-REV	22 bp	TTA GAA GAG AGC CAT TAA CTC A	
B	<i>EgAgB8/1</i> B8/1-FOR-SUM	39 bp	<i>CGC GAA CAG ATT GGA GGT</i> ATG CTT	281 bp
			CTC GCT CTG GCT CTC	
	B8/1-REV-SUM	40 bp	<i>GTG GCG GCC GCT CTA TTA</i> TTC ACC	
			TTC AGC AAT CAA CCC T	
	<i>EgAgB8/2</i> B8/2-FOR-EX	33 bp	GGA TCC ATG GGC AAA GAT GAG	270 bp
			CCA AAA GCA CAC	
	B8/2-REV-EX	32bp	CTC GAA GCT TAC TTT GAA TCA	
			TCA TCT TTT TC	
EPC1	EPC-FOR-EX	31 bp	CCA TGG ATC CTG CGT TTG TCG	251 bp
			TTC CTG CCG T	
	EPC-REV-EX	32 bp	CGA GTC TAG ATT AGA AGA GAG	
			CCA TTA ACT CA	

Table 2. Showing sensitivity, specificity and accuracy of IgG-ELISA with three recombinant antigens based on ROC analysis

Antigen	AUC	Cut-off	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Accuracy (%)
<i>EgAgB8/1</i>	0.690	0.22	66.7	71.3	25.6	93.5	70.6
<i>EgAgB8/2</i>	0.740	0.29	80	73.3	30.7	96.1	74.1
EPC1	0.558	0.29	60	72.3	25.7	92.5	72.4

2 in human patients^{15, 16} but no such study has been carried out in goats. Therefore, immunoassays using IgG4 isotype of the antibody could be evaluated for better sensitivity and specificity of these antigens in goats. Peptide based ELISA for improving the sensitivity and specificity of these antigens may also be tested.

Immunodiagnosis of CE in different hosts like pig, cattle, goat, buffalo and camel has been investigated but specific and sensitive diagnosis has been compromised due to false positive reactions and weak serological response generated in infected animals¹⁷⁻¹⁹. Serological cross-reactivity of the hydatid cyst antigens with taeniid cestodes including *Taenia hydatigena* and *T. ovis* has hindered the accurate serological diagnosis of hydatid infection in sheep¹⁷. Furthermore, natural intermediate host animals produce very poor antibody responses to infection compared with the relatively high levels of specific antibody evident in human infection¹⁷. Different groups have reported different findings on using the same antigenic component in diagnostic assays, with some authors reporting low antibody response in sheep to antigen B^{20, 21} while others showing reasonably high and specific antibody reactivity against antigen B in livestock^{18, 22-26}. Cross-reactivity of antigen B (similar to antigen 5) with other helminth parasites has been a recurrent challenge to the immunodiagnostic potential of these antigens¹⁰.

The cross-reactivity of the three recombinant antigens with other helminths in goats detected in the present study compromises the specificity of the test. However, these antigens can be used in the sero-surveillance of the goat flocks for cystic echinococcosis in the endemic regions of the country. The present results provide first information on the cross-reactivity of the three potent human CE diagnostic molecules EgAgB8/1, EgAgB8/2 and EPC1 of *E. granulosus* with the helminth parasites of goat. This investigation was carried out on a smaller number of goats but screening of larger sample size and determining the cross-reactivity with more species of helminth parasitizing goats is needed for validating the utility of these three antigens in the sero-diagnosis of cystic echinococcosis in goats.

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