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Expression of *Echinococcus granulosus* recombinant *Eg*Ag5-38 sub-unit and P-29 antigens and evaluation of their diagnostic potential for cystic echinococcosis in buffaloes

MARY NISHA TIGGA[1], AJAYTA RIALCH[2], A ARUN[3], S SAMANTA[4], NAVNEET KAUR[5], R K BAURI[6], Z A GANAIE[7], ANDLEEB AFTAB[8] and O K RAINA[9]

ICAR-Indian Veterinary Research Institute, Izatnagar, Uttar Pradesh 243 122 India

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ABSTRACT

There are no routine, reliable diagnostic methods for detection of *Echinococcus granulosus* infection, the causative agent of cystic echinococcosis in human and domestic animals. Two recombinant proteins *Eg*Ag5-38 sub-unit and P-29 of *E. granulosus* were expressed in prokaryotic expression vectors. The diagnostic potential of these two recombinant proteins was evaluated in the detection of cystic echinococcosis in buffaloes in IgG-ELISA. The *Eg*Ag5-38 sub-unit and P-29 recombinant protein reacted fairly with the hydatid infected buffaloes with *Eg*Ag5-38 sub-unit showing sensitivity and specificity of 67.9 and 78.8%, respectively. However, the recombinant P-29 protein showed a higher sensitivity of 82.1% but a low specificity of 43.9% in the detection of hydatid infection in buffaloes. Cross-reactivity of these recombinant antigens in buffaloes naturally infected with several gastrointestinal trematodes and *Sarcocystis* was studied. The results showed that both these antigens cross-reacted with most of these parasites of buffaloes. Present study is the first report on the evaluation of diagnostic potential of *E. granulosus* recombinant *Eg*Ag5-38 sub-unit and P-29 antigens in buffaloes.

Key words: Buffalo, Diagnosis, *Echinococcus granulosus*, *Eg*Ag5-38 sub-unit, IgG-ELISA, P-29 antigen

Animal and human echinococcosis occurs due to infection by the larval stage of taeniid cestode *Echinococcus*. Hydatid disease is preventable, however, reports indicated increase in its incidence in various regions of the world (Craig *et al*. 2015). The most useful tools to monitor the incidence of the disease in asymptomatic high-risk populations are imaging techniques and serology. Imaging methods are highly sensitive but serology, being less sensitive, can be used as a supplement to these imaging tools (Macpherson *et al*. 2003). There are no routine, reliable methods for diagnosis of the hydatid infection in living animals and the most reliable diagnostic method being thorough detection of cysts during meat inspection or at post-mortem examination. The Indian sub-continent provides ideal conditions for the establishment, propagation and dissemination of hydatidosis in livestock with a low to high prevalence of hydatidosis in different animal hosts being reported by several authors (Kumar *et al*. 2008, Pednekar *et al*. 2009, Gupta *et al*. 2011).

Hydatid cyst fluid is the common source of *E. granulosus* antigens for the immunodiagnosis of CE and its major components are antigen B (AgB) and antigen 5 (Ag5). The diagnostic potential of these two antigens was studied earlier (Barbieri *et al*. 1998, Pagnozzi *et al*. 2014, Pagnozzi *et al*. 2016). AgB and Ag5 are the most studied components of the parasite owing to their high concentration in hydatid cyst fluid and high immunoreactivity (Lorenzo *et al*. 2005, Carmena *et al*. 2006, Diaz *et al*. 2016). Ag5 is a dimeric protein composed of 22 and 38 kDa sub-units linked by a disulphide bridge, with both sub-units bearing an N-glycan modification (Lorenzo *et al*. 2003). Antigen 5 has been regarded as one of the more relevant antigens of *E. granulosus*, and it is widely used in different confirmation techniques in human hydatidosis.

Commercial serological kits mostly use hydatid cyst fluid as target antigen but its composition is variable from cyst to cyst (Pagnozzi *et al*. 2014, Ahn *et al*. 2015) and as a consequence, sensitivity and specificity are very heterogeneous across tests. Ahn *et al*. (2015) reported that Ag5 is immuno-reactive in every stage of the developing

Present address: [1,6]Touring Veterinary Officer (marynisha28 @gmail.com, drrkbouri@gmail.com), Department of Animal Husbandry, Jharkhand. [2]Assistant Professor (ajaytarialch @gmail.com), Department of Veterinary Parasitology, DGCNCOVAS, CSKHPKV, Palampur. [3]Veterinary Surgeon (drarun925@gmail.com), Animal Husbandry Department, Kerala. [4,9]Principal Scientist (samntaivri @gmail.com, rainaok @rediffmail.com). [5]Research Associate (navneetcharu2253 @gmail.com), Animal Nutrition Division. [7]Ph.D Scholar (zamirali5366@gmail.com), Department of Veterinary Parasitology, SKUAST, Srinagar. [8]Ph.D Scholar (andleebaftab17 @gmail.com), SHUATS, Allahabad.

cyst and can be a better antigen for detecting antibodies elicited against different stages of the developing cyst. Pagnozzi *et al*. (2014) reported that Ag5 is a sensitive antigen for detection of CE in human. Therefore, diagnosis of hydatidosis in livestock warrants evaluation of Ag5 sub-units and optimization of ELISA for its higher sensitivity. Another potential antigen (P-29) of *E. granulosus* was characterized by Gonzalez *et al.* (2000). The protein P-29 is a potential serological marker for post-treatment monitoring of CE in humans (Ben Nouir *et al*. 2009, Boubaker *et al*. 2014) but has not been tested in animals for its diagnostic potential. The present investigation was, therefore, aimed at evaluating *E. granulosus Eg*Ag5-38 sub-unit and P-29 protein in the sensitive and specific detection of hydatidosis in buffaloes.

MATERIALS AND METHODS

Cloning of Eg*AG5-38 sub-unit and P29 cDNAs:* Protoscolices were retrieved from a fertile hydatid cyst from buffalo liver at a local abattoir and total RNA was isolated from these protoscolices using Trizol reagent (Rialch *et al.* 2017). Briefly, ~0.3 $\times 10^4$ protoscolices were treated with 1 ml Trizol reagent (Invitrogen, USA) and manually homogenized with a micropestle in a sterile 2.0 ml centrifuge tube with several cycles of freeze-thawing at –80°C. Total RNA was isolated from the lysed protoscolices following standard RNA isolation protocol and was transcribed 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 two target proteins was PCR amplified with gene specific primers (Table 1). The PCR products were cloned in p^{DRIVE} cloning vector (Qiagen, Germany) and sequence confirmed for each cDNA.

Expression of the recombinant Eg*Ag5-38 sub-unit and P-29 protein:* The cDNAs coding for Eg*Ag5-38* sub-unit and P-29 proteins were PCR amplified with primers designed with suitable restriction enzyme sites and expressed in prokaryotic expression vector $p^{PROEXHT-b}$ (Table 1). The open reading frame of each cDNA was cloned in frame with the vector histidine fusion tag and sequenced. Expression of the recombinant *Eg*Ag5-38 sub-unit and P-29 proteins in *Escherichia coli* BL21 (DE3) cells was carried out by transformation of the bacterial cells with the recombinant plasmids and induced with Isopropyl thio-â-D galactopyranoside (IPTG) at 1mM concentration at 37°C. Recombinant *Eg*Ag5-38 sub-unit protein was purified from the *E. coli* BL21 cells following a mild denaturation protocol of urea lysis. Bacterial cells expressing the recombinant protein were lysed in 8 M urea buffer (pH 8.0), supplemented with 10 mM imidazole and 10 mM β-mercaptoethanol for 2 h at room temperature. The protein was purified to complete homogeneity with Ni-NTA affinity chromatography by binding the histidine tagged recombinant protein in the bacterial cell lysate to the Ni-NTA resin (Qiagen, Germany) for 2 h at room temperature. The affinity column was washed with wash buffer supplemented with 10 mM imidazole at pH 6.5 and elution of the recombinant protein carried out with elution buffer at pH 4.2.

Recombinant P-29 antigen was purified using 6 M guanidine hydrochloride as a strong denaturant. The IPTG induced *E. coli* cells were lysed in the lysis buffer (pH 8.0) containing 6 M guanidine hydrochloride and supplemented with 10 mM imidazole and 10 mM β-mercaptoethanol for 2 h at room temperature. The wash buffer (pH 6.7) was supplemented with 10 mM imidazole and recombinant protein was eluted with elution buffer 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 6 M guanidine hydrochloride or 8 M urea as protein denaturant.

Collection of buffalo sera: Buffaloes (244) were screened for hydatid infection by carcass examination of liver, lungs and spleen at their slaughter at a local abattoir in Bareilly, Uttar Pradesh. Sera were retrieved from these animals and screened for anti-hydatid antibodies by IgG-ELISA with two recombinant antigens. Sera were also collected from buffaloes naturally infected with other parasites for their cross-reactivity studies. Negative control sera for IgG-ELISA were collected from healthy buffalo calves maintained at Indian Veterinary Research Institute, Izatnagar. All experiments on buffaloes 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 *Eg*Ag5-38 sub-unit

Table 1. Primer sequences designed for PCR amplification of two target genes coding for *Eg*Ag5-38s and P-29 proteins

Gene	Primer name	Primer length	Primer sequence (5' → 3')	Amplicon size
*Eg*Ag5-38s	Ag5-38-S-FOR	21 bp	ATT CTT GCT GGA AAA AGC GCA	890 bp
	Ag5-38-REV	22 bp	TAG ACT GCG TAG CGG TTG ATC C	
P-29	P-29-FOR	22 bp	ATG TCC GGA TTT GAC GTT ACT A	717 bp
	P-29-REV	24 bp	CTA CTC GCC CAG CAT CAT ACT GCA	
*Eg*Ag5-38s	Ag5-38s FOR-EX	31 bp	*CCA TGG ATC C*AT TCT TGC TGG AAA AAG CGC A	910 bp
	Ag5-38s REV-EX	32 bp	*ACC TGA AGC TT*A GAC TGC GTA GCG GTT GAT CC	
P-29	P29-FOR-EX	33 bp	*CCA TGG GAT CC*A TGT CCG GAT TTG ACG TTA CTA	738 bp
	P29-REV-EX	34 bp	*GCT TTC TAG A*CT ACT CGC CCA GCA TCA TAC TGC A	

*Italic sequences indicate restriction enzyme sites incorporated in the primer sequences for cloning in respective expression vectors.

and P-29 antigens coated on each well of the 96-well microtitre plate was optimized to 1.0 µg/ml and 2.0 µg/ml of coating buffer, respectively. The optimal dilutions of serum samples and anti-bovine IgG-HRP conjugate (Sigma Chemicals, USA) used in the assay were 1:100 and 1:200 and 1:6000 and 1:12000, respectively for these two 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*. (1999). The area under the ROC curve (AUC) was the parameter used to define the antigen's discriminatory values between ELISA positive and negative animals (Fig. 3).

RESULTS AND DISCUSSION

An optimum level of expression of the recombinant *Eg*Ag5-38 sub-unit at 5 h post-IPTG induction at 37°C and a higher level of expression of the recombinant P29 protein at 6 h of IPTG induction at 37°C were achieved. The purified recombinant fusion proteins *Eg*Ag5-38s and P-29 resolved at 35 and 29 kDa, respectively in the SDS-PAGE (Fig.1 a, b).

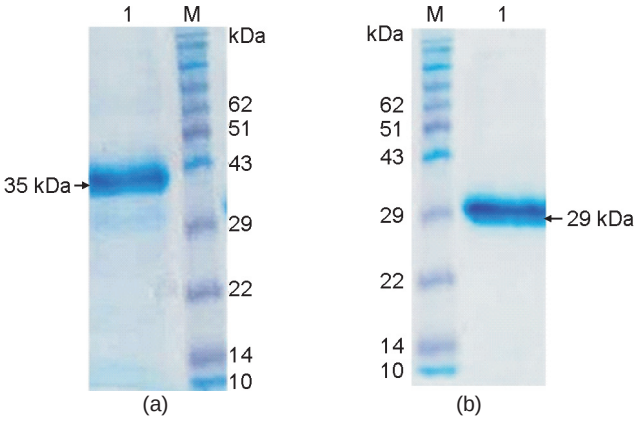

Fig.1. SDS-PAGE (15%) showing Ni-NTA affinity purified *E. granulosus* recombinant antigen 5-38 sub-unit (a) & P-29 (b). Lane M, Pre-stained protein molecular weight marker. Lane 1, Purified recombinant Ag5-38 sub-unit (35 kDa) and P-29 (29 kDa) fusion proteins.

Detection of hydatid infection in buffaloes: Necropsy examination of the 244 buffaloes at the slaughter showed 112 animals positive for hydatid cysts. The *Eg*Ag5-38 sub-unit antigen showed sero-reactivity with 76 out of 112 necropsy positive animals with OD_{492} above the cut-off value of 0.34. But 36 out of 112 necropsy confirmed positive animals were sero-negative in this IgG-ELISA leading to the sensitivity of 67.9%. However, IgG-ELISA with this antigen detected 28 out of 132 (21.2%) necropsy negative animals for hydatid cyst as sero-positive (Fig. 2a). With antigen P-29, 92 out of 112 necropsy confirmed positive animals were ELISA positive with OD_{492} values above the cut-off (0.27). This antigen missed 20 out of 112 necropsy positive animals and showed them sero-negative, thereby depicting the sensitivity of 82.1% (Fig. 2b). But this antigen detected 74 out of 132 (56.0%) necropsy negative animals as sero-positive. The comparative sensitivity, specificity, positive and negative predictive values of the two assays showed that *Eg*Ag5-38 sub-unit antigen had lower sensitivity but higher specificity than P-29 antigen (Table 2).

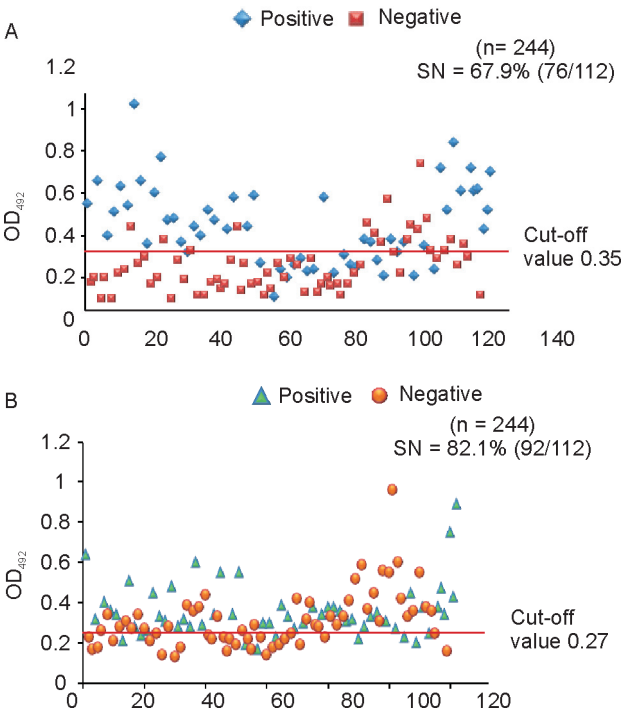

Fig. 2a. IgG-ELISA showing immuno-reactivity of buffalo sera with recombinant Ag5-38 sub-unit. b. IgG-ELISA showing immuno-reactivity of buffalo sera with recombinant P-29 antigen.

Studies on the cross-reactivity of each recombinant antigen were conducted with buffalo sera positive for *Paramphistomum epiclitum, Gastrothylax* spp., *Explanatum explanatum, Sarcocystis* spp. and with the sera from animals with mixed infections of *E. explanatum* and *Fasciola gigantica*, *E.explanatum* and rumen amphistomes, hydatid and *E.explanatum*, hydatid and *Sarcocystis* spp. and hydatid, *E. explanatum* and rumen amphistomes. The *Eg*Ag5-38 sub-unit antigen showed immuno-reactivity with sera of

Table 2. Comparative analysis of the sero-diagnostic potential of two recombinant antigens in IgG-ELISA in buffalo

Antigen	AUC	Cut-off	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Accuracy (%)
Ag5-38s	0.806	0.342	67.9	78.8	73.07	74.28	73.77
P-29	0.636	0.269	82.1	43.9	55.42	74.35	61.47

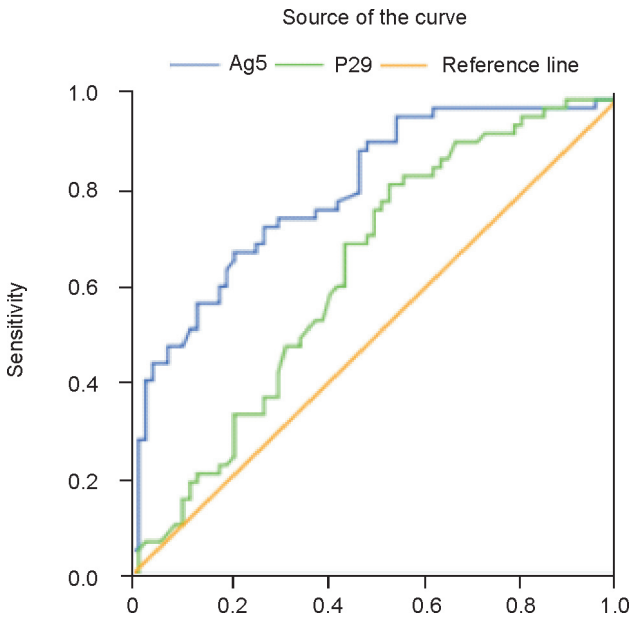

Fig. 3. Receiver-operating-characteristic (ROC) curves used to determine the cut-off values for two recombinant antigens Ag5-38 sub-unit and P-29 tested in IgG-ELISA against sera from hydatid infected and healthy buffaloes.

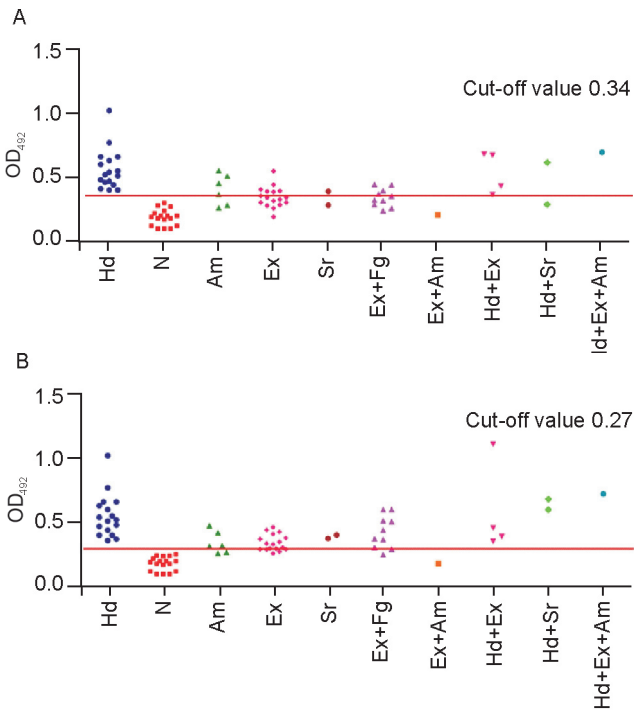

Fig. 4. IgG-ELISA of buffalo sera showing cross-reactions of recombinant antigens Ag5 (a) and P29 (b) with various buffalo parasites. (Hd-Hydatid positive sera, N-healthy buffalo sera (negative control), Am-rumen amphistomes, Ex-*Explanatum explanatum*, Sr-*Sarcocystis* spp., Fg-*Fasciola gigantica* positive buffalo sera).

buffaloes infected with rumen amphistomes (4/6), *E. explanatum* (7/17) and *Sarcocystis* spp. (1/2) with OD_{492} above the cut-off. Also 4 out of 10 sera from buffaloes with a mixed infection of *E. explanatum* and *F. gigantica* reacted in this ELISA while 0 out of 1 serum sample from buffalo with *E. explanatum* and rumen amphistome infection showed sero-reactivity. Animals with mixed infections of hydatid and *E. explanatum* (4/4), hydatid and *Sarcocystis* spp. (1/2), and hydatid, *E. explanatum* and rumen amphistomes (1/1) showed positive reactivity in the assay (Fig. 4a).

The P-29 recombinant antigen showed reactivity with 4 out of 6 sera positive for rumen amphistomes and 15 out of 17 *E. explanatum* positive sera with OD_{492} above cut-off. However, when two buffalo sera with infection of S*arcocystis* spp. were screened with this antigen, cross-reactivity was observed with both the sera with OD_{492} above cut-off. Eight out of ten buffaloes with a mixed infection of *E. explanatum* and *F. gigantica* reacted with the recombinant antigen and one serum sample from *E. explanatum* and rumen amphistome infected buffalo did not react in this ELISA with OD_{492} below the cut-off. All the four animals with mixed infection of hydatid and *E. explanatum*, two hydatid and *Sarcocystis* spp. infected animals and single animal with hydatid, *E. explanatum* and rumen amphistome infection showed positive reactivity in the assay (Fig. 4b).

The recombinant Ag5-38 sub-unit antigen showed sensitivity of 67.9% and specificity of 78.8%, respectively. However, P-29 antigen showed a higher sensitivity (82.1%) but lower specificity (43.9%). The recombinant Ag5-38 sub-unit antigen cross-reacted with the sera of buffaloes infected with parasites including *Fasciola gigantica, Explanatum explanatum,* rumen amphistomes and *Sarcocystis* spp. Likewise, P-29 that has been reported as potential antigen in the immunodiagnosis of human hydatidosis (Shi *et al.* 2009, Ben Nouir *et al.* 2009, Boubaker *et al.* 2014) also cross-reacted with the above parasites in the buffalo host. These results indicate that the above proteins are conserved between *E. granulosus*, trematodes and *Sarcocystis* spp. investigated in the present study and share cross-reacting epitopes. However, studies on the genome analysis of these parasites of buffalo will determine the cross-reactive epitopes present in these antigens. This cross-reactivity can thus be minimized by avoiding these epitopes while expressing the recombinant proteins or by designing synthetic peptides. The immunoglobulin IgG sub-classes have not been evaluated in the diagnosis of CE in buffaloes using different recombinant antigens that may enable for better sensitivity and specificity of these antigens in buffaloes.

Least research efforts have been made towards the development of immunodiagnostic tests for *E. granulosus* infection in domestic ruminants and the results have been generally disappointing and often contradictory (McManus 2014). An accurate sero-diagnostic test development for the detection of CE in livestock would represent a major advance in the control of the disease in endemic regions. However, the development of a sensitive and specific sero-diagnostic test has being hindered by antigenic cross-reactivity of several taeniid cestodes and other parasites (Lightowlers and Gottstein 1995). Furthermore, natural

intermediate host animals produce very poor antibody responses to infection compared with the relatively high levels of specific antibody evident in human infection (Lightowlers and Gottstein 1995). Kittelberger *et al*. (2002) observed that sheep are capable of mounting a specific immunological response during natural *E. granulosus* infection but that the response does not lead to raised serum antibody levels in many animals or they are not maintained throughout the course of the infection. Similar to antigen B, cross-reactivity of antigen 5 with antigens of other cestodes has been a recurrent challenge (McManus 2014). Immunodiagnosis of CE investigated in different hosts including pigs, cattle, goats, buffaloes and camels have not shown consistent results due to false-positive reactions and the weak serological response generated in the infected animals (Lightowlers and Gottstein 1995, Ibrahem *et al*. 2002, Golassa *et al*. 2011). Ahn *et al*. (2015) reported that immunoreactivity of Ag5 was detected in every stage of the hydatid cyst development (CE1-CE5) indicating a better sensitivity of Ag5 than AgB. Recently, Pagnozzi *et al*. (2016) while developing a robust chromatographic technique for enriching the Ag5 antigen from the hydatid cyst reported this antigen as highly sensitive and specific in the detection of human CE. Therefore, studying the components of the Ag5 for their relevance in the serological diagnosis of CE in the livestock has become necessary.

In the present study, the recombinant Ag5-38 sub-unit and P-29 proteins showed a fair degree of sensitivity but cross-reacted with the buffalo parasites. These results provide first information on the cross-reactivity of the two potent CE diagnostic molecules, viz. *Eg*Ag5-38 sub-unit and P-29 of *E. granulosus* with the helminth parasites of buffalo.

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