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Short communication

Evaluation of *Echinococcus granulosus* recombinant *Eg*AgB8/1, *Eg*AgB8/2 and EPC1 antigens in the diagnosis of cystic echinococcosis in buffaloes

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ABSTRACT

Three recombinant proteins of *Echinococcus granulosus* including two antigen B sub-units *Eg*AgB8/1 and *Eg*AgB8/2 and *Echinococcus* protoscolex calcium binding protein 1 (EPC1) were expressed in prokaryotic expression vectors. The diagnostic potential of these three recombinant proteins was evaluated in the detection of cystic echinococcosis in buffaloes in IgG-ELISA. The *Eg*AgB8/1 and *Eg*AgB8/2 recombinant proteins reacted fairly with the hydatid infected buffaloes with sensitivity of 75.0% and 78.6%, respectively and specificity of 75.8% while EPC1 recombinant protein showed higher sensitivity (89.3%) but lower specificity (51.5%). Cross-reactivity of these three antigens was assayed with buffalo sera naturally infected with *Explanatum explanatum, Paramphistomum epiclitum, Gastrothylax* spp., *Fasciola gigantica* and *Sarcocystis* spp. *Eg*AgB8/1 and EPC1 antigens cross-reacted with all these sera while *Eg*AgB8/2 showed no cross-reaction with *Sarcocystis* spp. and reacted with some of the *E. explanatum* infected buffalo sera. This study explores the potential of three hydatid antigens *viz.* *Eg*AgB8/1, *Eg*AgB8/2 and EPC1 for their diagnostic potential in buffaloes positive for cystic echinococcosis.

1. Introduction

Cystic echinococcosis (CE) caused by the larval stage of the taeniid cestode *Echinococcus granulosus* is a cosmopolitan, neglected silent cyclozoonoses with estimated 3 billion US $ annual loss to the livestock industry (WHO echinococcosis fact sheet, 2017). Economic losses associated with cystic echinococcosis in India are approx. US $ 212 million where cattle and buffalo industry account for most of the losses (Singh et al., 2014). The accurate assessment of its prevalence is essential for devising control strategies. This involves clinical diagnosis of the disease and epidemiological surveillance of high-risk populations. The clinical diagnosis of CE in human largely depends on imaging scans supplemented with serological tests (Siracusano et al., 2008; Brunetti et al., 2010; Taheri et al., 2013). The most reliable diagnostic method in animals is through the detection of cysts during meat inspection or at post-mortem examination. There are no routine, reliable methods for diagnosis of the hydatid infection in living animals as imaging methods are not practical due to cost factors. In the absence of accurate serological tests infected animal remain in the morbid state, leading to substantial economic losses to the livestock sector.

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 protein components of hydatid cyst fluid, the native and recombinant *Eg*AgB antigens allow for better diagnostic performance (Ortona et al., 2000; Lorenzo et al., 2005; Brunetti et al., 2010). The *Eg*AgB is a thermostable, lipoprotein encoded by a multigene family (Haag et al., 2004) and has been extensively investigated in the immunological diagnosis of hydatid disease in humans (Zhang and McManus, 2006; Craig et al., 2007; Brunetti et al., 2010; McManus et al., 2012; Zhang et al., 2012). However, reports on the development of immunodiagnostic tests for *E. granulosus* infection in domestic ruminants are scanty and the results have not been generally consistent due to poor sensitivity and specificity of the immuno-assays in animals (McManus, 2014). Cattle and buffaloes are predominantly infected with hydatid cysts when compared with other domestic animals in India but fewer reports are available on the development of sero-diagnostic tests for cystic echinococcosis in buffaloes (Samanta et al., 2009; Pan et al.,

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2011; Maity et al., 2014). Therefore, present studies were undertaken to assess the immunodiagnostic potential of three recombinant antigens *Eg*AgB8/1, *Eg*AgB8/2 and EPC1 of *E. granulosus* in buffalo hydatidosis.

2. Materials and methods

2.1. Parasite collection

Hydatid cysts were collected from buffaloes at a local abattoir at Bareilly, U.P, India. These were carefully excised from the infected organs and transported to the laboratory on ice. The host tissues surrounding the cysts were removed and cysts were washed with normal saline. Fertile hydatid cysts were processed for the separation of cyst fluid, protoscolices and germinal membranes. Protoscolices were centrifuged and washed in phosphate buffered saline (PBS) pH 7.2 and processed for the isolation of total RNA.

2.2. cDNA synthesis

Total RNA was isolated from the protoscolices with Trizol reagent (Invitrogen, USA). Briefly, ~0.2×10^4 protoscolices were treated with Trizol reagent (1 ml) 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 revert aid H minus reverse transcriptase enzyme (MBI Fermentas, USA) following standard protocols of cDNA synthesis. The cDNA coding for *Eg*AgB8/1 (accession no: 143813), *Eg*AgB8/2 (accession no: U15001) and EPC1 (accession no: AF481884) target proteins were PCR amplified. The primers were designed at N and C-termini of *Eg*AgB8/1 and EPC1 while *Eg*Ag8/2 primers were designed to amplify the mature peptide (Table 1). The PCR products of three target cDNAs were cloned in p^{DRIVE} cloning vector (Qiagen, Germany) and sequence confirmed for each cDNA.

2.3. Expression of the recombinant EgAgB8 sub-units and EPC1 protein

The cDNAs coding for *Eg*AgB8/1, *Eg*AgB8/2 and EPC1 proteins were expressed in prokaryotic expression vectors after their PCR amplification with primers designed with suitable restriction enzyme sites (Table 1). The *Eg*AgB8/1 protein was expressed in p^{Rham} vector in frame with solubility enhancing SUMO-fusion protein (Expresso™ Rhamnose cloning and expression kit; Lucigen, USA). *E. cloni*® 10G chemically competent cells were transformed with the recombinant p^{Rham} vector and 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 tris-phosphate buffer (pH 8.0) supplemented with 18 mM imidazole and 10 mM β-mercaptoethanol, followed by sonication of the cell lysate at 10 micron amplitude for 5 cycles of 30 sec each. The protein was purified to complete homogeneity using Ni-NTA affinity chromatography. Briefly, the recombinant protein was allowed to bind 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 recombinant protein eluted with elution buffer at pH 4.2.

Expression of the *Eg*AgB8/2 recombinant protein was carried out in $p^{ET32a(+)}$ vector in *Escherichia coli* BL21 (DE3).The cDNA was cloned in the expression vector in frame with the vector fusion tag. An optimum level of expression of the *Eg*AgB8/2 recombinant protein was achieved at 6 h post–1 mM 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 room temperature. The cell lysate was sonicated on ice for 5 cycles of 30 sec each at 5 micron amplitude and binding of the recombinant protein to Ni-NTA resin was carried out 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 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 1 mM IPTG were disrupted in lysis buffer (pH 8.0) containing 6 M guanidine hydrochloride and 16 mM imidazole for 2 h at room temperature and the recombinant protein bound to Ni-NTA affinity resin as described for other two proteins. The wash buffer (pH 5.9) was supplemented with 16 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 sodium dihydrogen phosphate containing either 6 M guanidine hydrochloride or 8 M urea as protein denaturants. The three recombinant proteins were dialyzed in a step gradient of tris-phosphate buffer from 8.0 M to 0.0 M urea and finally against PBS (pH 7.2).

The purified recombinant fusion proteins *Eg*AgB8/1, *Eg*AgB8/2 and EPC1 resolved at 25, 29 and 10.5 kDa, respectively in the SDS-PAGE (Fig. 1).

2.4. Collection of buffalo sera

Buffaloes (n = 244) were randomly screened for hydatid infection at necropsy at the local abattoir. Animals that were visually positive for hydatid cysts were considered confirmed positive for calculating true positive in the Enzyme Linked Immunosorbent Assay. Out of the 244

Table 1
Primer sequences for PCR amplification of cDNAs coding for three recombinant antigens (A) and for their cloning in expression vectors (B). Bold and underlined sequences indicate restriction enzyme sites for *Nco* I, *Hind* III, *BamH*I and *Xho*I, respectively and nucleotide sequences (bold in italics) are homologous to sequences coding for SUMO protein in p^{RHAM} vector, respectively.

	Gene	Primer name	Primer length	Primer Sequence (5′ → 3′)	Amplicon size
A	*Eg*AgB8/1	B8/1-FOR	24 bp	ATGCTTCTCGCTCTGGCT CTCGTC	247 bp
		B8/1-REV	24 bp	CTATTCACCTTCAGCAAT CAACCC	
	*Eg*AgB8/2	B8/2-FOR	21 bp	AAAGATGAGCCAAAAGCA CAC	250 bp
		B8/2-REV	24 bp	TTACTTTGAATCATCATC TTTTTC	
	EPC1	EPC-FOR	21 bp	TGCGTTTGTCGTTCCTGC CGT	231 bp
		EPC-REV	22 bp	TTAGAAGAGAGCCATTAA CTCA	
B	*Eg*AgB8/1	B8/1-FOR-SUM	39 bp	***CGCGAACAGATTGGAGGT***ATGCTTCTCGCTCTGGCT CTC	281 bp
		B8/1-REV-SUM	40 bp	***GTGGCGGCCGCTCTATTATTC***ACCTTCAGCAATCAA CCCT	
	*Eg*AgB8/2	B8/2-FOR-EX	33 bp	**GGATCCATGG**GCAAAGAT GAGCCAAAAGCACAC	270 bp
		B8/2-REV-EX	32bp	**CTCGAAGCTT**ACTTTGAA TCATCATCTTTTTC	
	EPC1	EPC-FOR-EX	31 bp	**CCATGGATCC**TGCGTTTG TCGTTCCTGCCGT	251 bp
		EPC-REV-EX	32 bp	**CGAGTCTAGA**TTAGAAGA GAGCCATTAACTCA	