Evaluation of the immunodiagnostic potential of a recombinant surface protein domain from *Acanthamoeba castellanii*

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SUMMARY

Acanthamoeba spp. are free-living protists widely distributed in environment, able to cause keratitis, encephalitis and skin lesions in humans and animals. *Acanthamoeba* spp. exist in two forms: an infective trophozoite and a dormant cyst. Several factors contribute to the pathogenesis of *Acanthamoeba* spp. The parasite adhesion to the host cell is the primary step for infection and is mediated by a mannose binding-protein, expressed in the surface and considered the main pathogenicity factor in *Acanthamoeba* spp. So far, there was no evidence of another surface protein of *Acanthamoeba* spp. relevant for host invasion or infection by these organisms. The aims of this study were to identify and characterize an *Acanthamoeba castellanii* surface protein and to evaluate its diagnostic potential. *In silico* predictions of surface proteins allowed to identify the *A. castellanii* calreticulin as a possible surface antigen. The coding sequence of a predicted extracellular domain of *A. castellanii* calreticulin was cloned by *in vivo* homologous recombination and the recombinant polypeptide ($AcCRT_{29–130}$) was produced. Its immunodiagnostic potential was assessed in a recombinant antigen-based ELISA with sera from experimentally infected rats that developed keratitis and encephalitis, and sera from patients with encephalitis. The $AcCRT_{29–130}$ was significantly more recognized by sera from encephalitis infected rats in comparison with the non-infected controls. Human sera from encephalitis patients, however presented no significant response. These results showed the $AcCRT_{29–130}$ potential for *A. castellanii* infection immunodiagnosis in animals, with further studies being required for assessment of its use for human infections.

Key words: *Acanthamoeba castellanii*, calreticulin, recombinant protein, immunodiagnostic, ELISA.

INTRODUCTION

Acanthamoeba species are free-living amoebae (FLA) with worldwide distribution. FLA are found in soil, water and air, and can be readily isolated from these sources (Visvesvara *et al*. 2007). However, these organisms are pathogenic/opportunistic amphizoic protists with potential to infect human and animal hosts (Visvesvara, 2013). *Acanthamoeba* spp. present two stages in their life cycles: a motile, trophic and replicating trophozoite and a resistant cyst stage. Based purely in morphological criteria, as many as 24 species have been included in the genus *Acanthamoeba*. Recently, species identification has relied upon sequencing of the amoeba 18S rRNA genes, and 20 different genotypes (T1–T20) of *Acanthamoeba* spp. have been established, which each genotype exhibits 5% or more of sequence divergence among different genotypes (Corsaro *et al*. 2015). However, the pathogenicity of *Acanthamoeba* spp. could be limited to some genotypes and the majority of the human infections have been associated with the T4 genotype (Khan, 2006).

In recent years, the incidence of infections due to *Acanthamoeba* spp. has shown a remarkable increase. *Acanthamoeba* spp. are the causative agents of a sight-threatening infection of the cornea known as *Acanthamoeba* keratitis (AK) in healthy patients and a fatal disease of the central nervous system (CNS) known as Granulomatous Amebic Encephalitis (GAE) in immunocompromised patients (Baig, 2015; Lorenzo-Morales *et al*. 2015).

Early detection of the infection and treatment are critical to the outcome of the clinical course of AK and GAE infections (Szentmáry *et al*. 2012; Bouheraoua *et al*. 2013; Lorenzo-Morales *et al*. 2013). The diagnosis of AK can often be made by *in vivo* confocal microscopy (IVCM). However, the direct detection of the causative agent in a corneal scrape specimen is the only reliable diagnostic

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method for AK. In case of GAE, magnetic resonance imaging (MRI) or computerized tomography (CT) of the brain show ring-enhancing lesions or low-density abnormalities mimicking a single or multiple space-occupying mass in the cerebral cortex (Schumacher *et al*. 1995; Shirwadkar *et al*. 2006). A significant delay in formulating an appropriate diagnosis before treatment is common, because of lack of suspicion and familiarity with these amoebae by clinicians and pathologists, misdiagnosed as bacterial, viral or fungal keratitis/encephalitis and the absence of rapid-specific immunodiagnostic assays (Martínez *et al*. 1997; Khan *et al*. 2000; Clarke and Niederkorn, 2006). Antibody detection by immunoassays may be a rapid useful diagnostic tool as an adjunct to microscopical diagnosis in detecting these parasites.

Among the species, pathogenic *Acanthamoeba castellanii* has been used extensively to study the molecular mechanisms of amoeboid locomotion and virulence factors involved in AK and GAE (Pollard and Ostap, 1996; Leher *et al*. 1998; Kennett *et al*. 1999; Kong and Pollard, 2002; Garate *et al*. 2006).

Adherence of trophozoites to corneal epithelial cells followed by injury and invasion of tissue are thought to represent important steps in the establishment of infection. *Acanthamoeba castellanii* binds to mannose containing glycoproteins on the corneal epithelium through a 136-kDa mannose-binding protein on the amoebae surface (Yang *et al*. 1997), but another surface proteins and cellular events governing adherence to the host cell are not completely elucidated (Soto-Arredondo *et al*. 2014). In this study, we identified *in silico* an *A. castellanii* putative calreticulin surface protein (AcCRT) and expressed a protein domain in its recombinant form to assess its potential in the utilization in immunodiagnostic assays for detection of AK and GAE. We tested the immunodiagnostic potential of the AcCRT protein domain by ELISA using sera from infected rats with AK and GAE, and sera from patients with GAE. The results of this study, described for the first time a recombinant domain of the AcCRT that was recognized by sera from infected rats with GAE, but not by sera from patients with GAE, suggesting its immunodiagnostic potential in animal infections.

MATERIALS AND METHODS

Bacterial strains

Escherichia coli BL21 pLysE (Novagen) strain was used to express the recombinant protein domain. The *E. coli* KC8 was used for cloning assays.

In silico analyses of A. castellanii *surface proteins*

Comparative analysis between *Entamoeba histolytica* – the Amebozoa enteropathogen most studied – and *A. castellanii* amino acid sequences of surface proteins were performed using the NCBI/BLASTP tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastp&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome). The *A. castellanii* selected surface protein (see Results Section) was divided in amino acid portions (domains) according AmoebaDB database (http://amoebadb.org/amoeba/) and the translated coding sequences (CDS) of each exon were analyzed using the following software programs: TMHMM Server v. 2·0 (http://www.cbs.dtu.dk/services/TMHMM/) Phobius (http://phobius.sbc.su.se/), HMM-TM (http://bioinformatics.biol.uoa.gr/HMM-TM/), TopPred (http://mobyle.pasteur.fr/cgi-bin/portal.py#forms::toppred) and Predicting Antigenic Peptides (http://imed.med.ucm.es/Tools/antigenic.pl). All programs were used in default parameters. The first three programs predicted the presence of transmembrane (TM) domains, the fourth was used to predict hydropathicity and the last one antigenicity. The parameters for the domain selection were: translated CDS were considered as surface protein when they were predicted as such by at least two of the three TM predicting programs (Siqueira *et al*. 2013), as well as considering the translated CDS with most hydrophilic and antigenic epitopes.

Acanthamoeba castellanii *strain, cultivation and extraction of genomic DNA*

Acanthamoeba castellanii trophozoites of the T4 genotype were obtained from the American Type Culture Collection (ATCC30010) (Clarke *et al*. 2013). Trophozoites were maintained in axenic cultures in peptone-yeast extract-glucose (PYG) medium, as previously described by Schuster (2002); and samples for the genomic DNA extraction were directly taken from these cultures. Four identical and independent cultures with approximately 1×10^6 trophozoites in the exponential growth phase were used for genomic DNA extraction as described by Aljanabi and Martinez (1997); with some modifications. Briefly, the culture was centrifuged, the cellular sediment obtained was homogenized in sterile salt buffer. SDS and proteinase K were added and the samples were incubated at room temperature overnight, after which NaCl was added to each sample. Samples were mixed in vortex at maximum speed, and tubes spun down. The supernatant was transferred to fresh tubes. An equal volume of isopropanol was added to each sample, mixed well and samples were incubated in low temperature. Samples were then centrifuged and the pellet was washed with ethanol, dried and finally resuspended in sterile dH_2O and RNAse was added. The genomic DNA was quantified using Nanodrop 2000 (Thermo Scientific).

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Table 1. Primer sequences used for genomic DNA amplification of *A. castellanii* Neff (ATCC 30010), annealing temperature and amplicon length of the recombinant protein domain CDS

Gene ID	Primers	AT	Amplicon
14923486	**F**:5′ *CCGCGTGGATCTGAATTCCCGGGTC***GACTCGTGGGAGAGCCGAT**3′ **R**:5′ *GCGAGGCAGATCGTCAGTCAGTCA***GTTGTACTTGGTCTCGCCCT**3′	74·6 °C 7·5 °C	304 bp

Gene ID: gene code of *A. castellanii* selected surface protein in the Amoeba DB database. Primers: F, forward; R, reverse. Italic letters correspond to plasmid homologous sequences: pGEX-4T1. The bold letters are the complementary to target CDS.

Cloning, expression and purification of the recombinant protein domain

The CDS of the selected surface protein domain was amplified by polymerase chain reaction (PCR) utilizing genomic DNA from *A. castellanii*. The primers were constructed using the program Vector NTI Express Software (Life Technologies) and are demonstrated in Table 1. The amplicons were cloned by *in vivo* homologous recombination as described by Parrish *et al*. (2004) into pGEX-4T1 prokaryotic expression vector (GE Healthcare), linearized by *Eco*R1 and *Bam*H1 restriction enzymes. The recombinant plasmids were transformed by chemical competence in *E. coli* strains BL21 pLysE (Novagen), BL21 RP (Stratagene), BL21 Rosetta (Novagen), BL21 Codon Plus Ril (Stratagene) e BL21 Star (Thermo Scientific). The expression of the selected recombinant surface polypeptide in fusion with glutathione S-transferase (GST) was carried out as described by Smith and Johnson (1988). The bacterial cultures were growth in Circle Growth (MP Biomedicals) and induced with IPTG at final concentration of 0·1 mM shaking during 3–16 h at 37 °C. After induction, the bacterial cultures were submitted to sonication (VC601 Sonic & Material Inc. sonicator) on freeze bath during 6 cycles, 30 s with 1 min gaps in repose, after that, they were centrifuged at 20 000 ***g*** for separating soluble and insoluble fractions. The expression and solubility of the fusion protein was analysed by SDS-PAGE 12%. The fusion protein was purified by affinity chromatography using the Gluthathione-Sepharose resin (GE Healthcare) followed by thrombin (Sigma) cleavage. The purified polypeptide was quantified by Qubit™ (Thermo Scientific). The cleavage efficiency and purified polypeptide recovering were analysed by SDS-PAGE 15%.

In vivo infection models of A. castellanii

The stimulant solution (SS) for infection was prepared from cultures of *A. castellanii* ATCC 30010. The PYG medium containing 1×10^6 trophozoites mL^{-1} was centrifuged at 250 ***g*** for 10 min. The pellet was washed three times with phosphate buffered saline (PBS) and resuspended in 100 μL of PBS. Adult female *Wistar* rats, with a mean age of 3 months, were first immunosuppressed by intraperitoneal injection with dexamethasone (5 mg kg^{-1}) (Capilla *et al*. 2006). For the GAE infection, 10 animals were anesthetized with ketamine hydrochloride (60 mg kg^{-1}) and xylazine hydrochloride (8 mg kg^{-1}) and were inoculated intranasally with 100 μL of the SS. After infection, the animals were followed for 30 days. After this period, blood was collected and the rats were euthanized by barbiturate overdose (100 mg kg^{-1})/lidocaine (10 mg mL^{-1}) (euthanasia solution), intraperitoneal. The parasites were re-isolated from the organ lesions and cultured in plates with non-nutrient agar 1·5% covered with inactivated *E. coli* (ATCC 25922).

For the AK infection model the animal corneas were scratched and challenged with *A. castellanii*. In this case, 10 animals were anaesthetized peritoneally as described earlier. Corneal anesthesia was obtained with topical 0·4% Oxybuprocaine hydrochloride eye drops. After routine disinfection, the corneas of right eyes were scratched three times vertically and three times horizontally with a sterile 30-gauge syringe needle and the stimulating solution, containing 1×10^6 trophozoites mL^{-1} was applied to the scarified corneas. After infection, the animals were observed for 30 days. After this period, blood was collected and the rats were euthanized as described above. The parasites also were re-isolated from the eyes and cultured in plates with non-nutrient agar 1·5% covered with inactivated *E. coli* (ATCC 25922). All of the procedures were previously approved by the Ethics Committee on Animal Use of the Universidade Federal do Rio Grande do Sul with record number 27191 and followed international guidelines for the ethical use of animals in science.

Assessment of the immunodiagnostic potential of the recombinant protein domain

Serum total IgG detection were performed by indirect ELISA, as described by Virginio *et al*. (2003) with modifications in the dilution of sera and secondary antibodies. Briefly, microtitration plates (Maxisorp, Nunc) were coated with 0·4 μg $well^{-1}$ of selected recombinant polypeptide diluted in 0·1 M carbonate/bicarbonate buffer (pH 9·6). Three AK infected rat serum samples, 8 rat serum samples infected with

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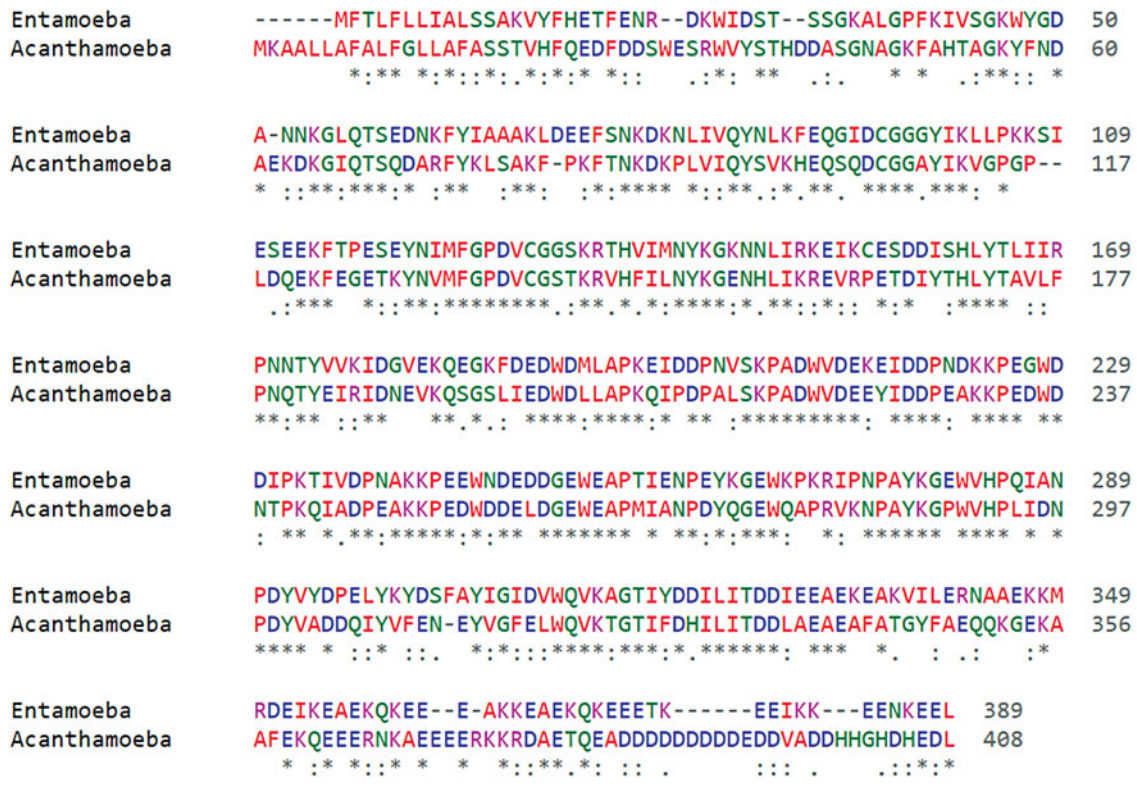

Fig. 1. BLAST alignment of the calreticulin (CRT) protein coding sequences between *Entamoeba histolytica* and *Acanthamoeba castellanii* using Clustal Omega. (*) – indicates positions which have a single, fully conserved residue; (:) and (.) – indicate conservation between groups of strongly and weakly similar amino acids properties, respectively. The colours in the sequences represent physicochemical properties.

GAE and 4 human serum samples infected with GAE were diluted in Blotto (PBS containing 5% milk powder) at 1:1000 in case of infected rat serum samples and 1:2000 in case of infected human serum samples, and all of them tested in duplicate. Positive and negative sera and conjugate controls were included in each plate. Goat anti-rat IgG conjugated to horseradish peroxidase (Sigma) and Goat anti-human IgG (whole molecule) were used as secondary antibodies, diluted in Blotto 5% at 1:1000 and 1:30 000, respectively. OPD (Sigma)/H_2O_2 in citrate-phosphate buffer were used during 15 min for reaction developing. Afterwards, the reactions were interrupted for adding H_2SO_4 1N and absorbance were measured to 492 nm (A_{492}) in ELISA reader (BIORAD). *Acanthamoeba castellanii* total protein extract was used as positive control of the ELISA in the assays with infected human serum samples for verify the serum titres as well as reject any mistake during the assays with the recombinant polypeptide. The infected human serum samples with GAE were gently granted by Govinda S. Visvesvara/Centers for Disease Control and Prevention (CDC).

Statistical analysis

Standard deviation and parametric Student's *t* distribution set to 95% confidence interval were performed for analysing the response levels of antibodies detected against the recombinant polypeptide between the different groups of infected sera. Differences were considered to be significant if *P*-values $\leqslant 0{\cdot}05$. Statistical software packages in PRISM Version 6 (GraphPad Software In., La Jolla CA) were used.

RESULTS

In silico analyses of A. castellanii *surface proteins*

Among the different surface proteins analysed *in silico* for comparison between *E. histolytica* and *A. castellanii*, calreticulin from *A. castellanii* Neff strain (ATCC 30010) (AcCRT) with protein code XP_004349632·1 in the AmoebaDB and NCBI databases, and with 14923486 of Genbank access number exhibited 83% of query coverage and 55% of identity in the BLAST tool compared with calreticulin from *E. histolytica* (EhCRT) with protein code XP_655241·1 in the AmoebaDB and NCBI databases, and with 3409565 of Genbank access number (Fig. 1). The TM merged results showed that AcCRT has a small intracytoplasmic region at the beginning of the protein sequence indicating a possible signal peptide segment. There is a TM region located from the amino acid 5 until amino acid 23. The most part of the protein is extracellular.

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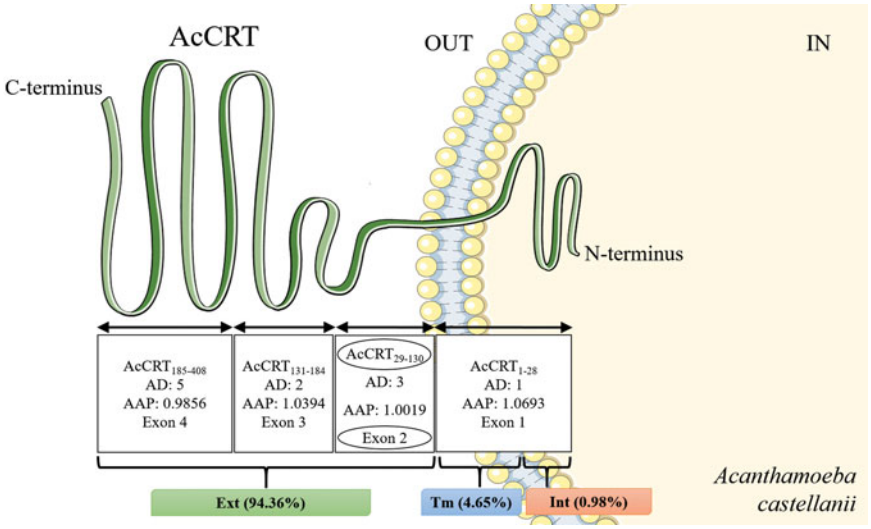

Fig. 2. Domains representation of the calreticulin from *Acanthamoeba castellanii* (AcCRT) correlating with its 4 exons. The scheme depicts the predicted AcCRT with its amino end (N-terminus) inside of the amoeba and the carboxyl (C-terminus) outside. AcCRT is divided in extracellular (Ext), transmembrane (Tm) and intracellular (Int) regions, comprising 94·36, 4·65 and 0·98% of the amino acids sequences, respectively. The exon number two (circulated) encodes the domain between the amino acids (aa) 29 to 130 ($AcCRT_{29–130}$). This domain is located extracellularly, and exhibits three Antigenic Determinants (AD) with an Average Antigenic Propensity (AAP) of 1·0019. The other values from the different exons also are showed. All antigenicity values were obtained in the online site http://imed.med.ucm.es/Tools/antigenic.pl.

Table 2. Molecular characteristics of $AcCRT_{29–130}$ exon number two

Target exon	Amino acids and nucleotides length	Molecular weight	Amino acids	CDS length
$AcCRT_{29–130}$	101 amino acids 305 nucleotides/ base pairs	11·415 kDa	dswesrwvysthddasgnagkfah-tagkyfndaekdkgiqtsqdarfykl-sakfpkftnkdkplviqysv-kheqsqdcggayikvgpgpldqek-fegetky	5′GACTCGTGGGAGAGCCG ATGGGTGTACTCCACCCAC GATGATGCCTCGGGCAACG CTGGCAAGTTCGCGCACAC CGCCGGCAAGTACTTCAAC GACGCTGAGAAGGACAAG GGCATCCAGACCTCGCAGG ATGCCAGGTTCTACAAGCT GTCGGCCAAGTTCCCGAAG TTCACCAACAAGGACAAGC CCCTCGTGATCCAGTACTC CGTCAAGCACGAGCAGTCT CAGGATTGCGGTGGTGCC TACATCAAGGTCGGCCCTG GCCCTCTTGACCAGGAGAA GTTCGAGGGCGAGACCAAG TACAA3′

The CDS of the AcCRT protein has 3 introns and 4 exons according to the AmoebaDB database. All amino acidic sequences codified by 4 exons were analysed considering that we established working experimentally with *A. castellanii* genomic DNA. The exon number two, which encodes the protein portion between amino acid number 29 to amino acid number 130 ($AcCRT_{29–130}$), showed antigenic, hydrophilic and extracellular amino acidic sequences (Fig. 2). The molecular characteristics of $AcCRT_{29–130}$ are described in Table 2.

Cloning, expression and purification of the $AcCRT_{29–130}$ recombinant protein domain

Oligonucleotides for PCR amplifications (see Table 1) were designed based on exon number two of AcCRTprotein. The amplified CDS of $AcCRT_{29–130}$ was cloned into bacterial expression vector pGEX-4T1 in the *E. coli* strain KC8. All recombinant plasmids were sequenced using the Dyenamic ET Dye Terminator Cycle Sequencing kit for MegaBace DNA Analysis Systems (GE

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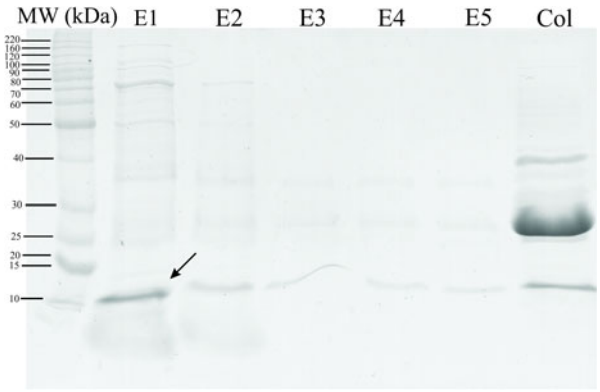

Fig. 3. Elution pattern of AcCRT$_{29–130}$ from Glutatione Sepharose resin. Purified AcCRT$_{29–130}$ polypeptide band is indicated with an arrow (11·4 kDa). E1–E5: Five continuous elutions of the obtained purified polypeptide. Col: resin column sample showing part of the AcCRT$_{29–130}$-GST fusion protein (37 kDa) and only GST tag protein expression (26 kDa). Lane MW, molecular weight.

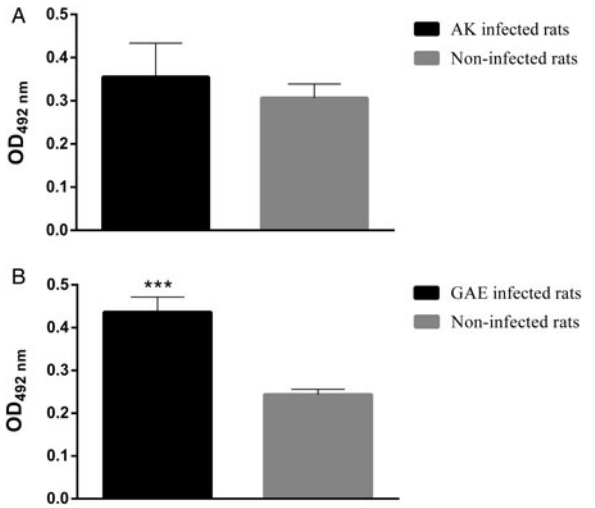

Fig. 4. Total IgG responses in sera of infected rats with *A. castellannii* against AcCRT$_{29–130}$ determined by indirect ELISA. Microtiter plates were coated with 0·4 μg well^{-1} of AcCRT$_{29–130}$ purified recombinant polypeptide. Rat sera were collected at 30 days of infection with *Acanthamoeba* keratitis (AK) (A) and granulomatous amebic encephalitis (GAE) (B) and were diluted 1:1000. The data represent the mean optical density at 492 nm (OD492) (±S.D.) from three rats in the AK group and eight rats in the GAE group. The asterisks indicate significant differences of antibody responses (***$P \leqslant 0{\cdot}0009$), compared with negative controls (non-infected rats).

Healthcare). There were significant differences in the expression of recombinant plasmids (pGEX-4T1/AcCRT$_{29–130}$) using the different *E. coli* expression strains. The BL21 pLysE (DE3) strain expressed more fusion protein compared with the other strains (data not shown). AcCRT$_{29–130}$ purification was evaluated by SDS-PAGE 15% as shown in Fig. 3 in lane E1. The calculated 37 kDa molecular mass of the fusion recombinant protein AcCRT$_{29–130}$-GST comprise the GST tag (26 kDa) plus purified polypeptide (11·4 kDa). The purified AcCRT$_{29–130}$ polypeptide was recovered with yields of 3·64 mg L^{-1} of culture.

Assessment of the immunodiagnostic potential of the AcCRT$_{29–130}$ using human and rat sera infected with Acanthamoeba keratitis *and Granulomatous Amebic Encephalitis*

The AcCRT$_{29–130}$ recombinant polypeptide was tested in anti-IgG indirect ELISA with sera from eight rats infected with GAE and three rats infected with AK. The non-infected rat sera were used as negative controls. The three rat serum samples infected with AK produced low levels of total IgG against AcCRT$_{29–130}$ ($P = 0{\cdot}1502$) (Fig. 4A), contrary to the significantly higher levels of total IgG from rat sera infected with GAE ($P \leqslant 0.0009$) (Fig. 4B) both compared with non-infected controls. Four human serum samples infected with GAE were also tested but did not produce detectable antibodies (total IgG) against AcCRT$_{29–130}$ and *A. castellanii* total protein extract (data not shown), suggesting loss of specific antibody titres due to long-term storage or transport problems.

DISCUSSION

The CRT, a calcium (Ca^{+2})-binding protein plays a variety of important roles in the regulation of key cellular functions. The identification of CRT protein homologues in endoplasmic reticulum (ER) or cellular surface of various parasites (Ferreira *et al*. 2004) suggests that this protein could have many conserved roles like parasite/host interactions, phagocytosis, and modulation of host immune response (Mendlovic, 2010). The role of CRT in host/parasite interactions has recently become an important area of research. CRT genes from a number of parasites (*Trypanosoma, Leishmania, Entamoeba, Onchocerca, Schistosoma* and *Haemonchus*) have been cloned and sequenced, revealing approximately 50% of identity with CRT human gene; (Suchitra and Joshi, 2005; González *et al*. 2011). In human trypanosomiasis, the role of TcCRT as an immune evasion mechanism is easily understood because CRT is located on the surface of the trypomastigote in blood during the acute phase of infection and accessible for binding to C1q (Ximénez *et al*. 2014). In *E. histolytica*, the EhCRT induces an important immunogenic response in the human host. More than 90% of patients with amoebic liver abscesses develop high levels of serum antibodies against EhCRT (González *et al*. 2002).

Comparative *in silico* analyses between CRT sequences demonstrated a mean identity of amino acid sequences (55%) between *E. histolytica* and *A. castellanii*. The *in silico* predictions revealed the

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region corresponding to the AcCRT$_{29–130}$ domain is in the outside of the cell, suggesting the presence of an immunodominant epitope in this portion of the protein supported by information obtained with Predicting Antigenic program.

Recently, the presence of CRT was reported in cytoplasmic membranes of *E. histolytica* and *Entamoeba dispar* trophozoites previously activated with Jurkat cells (Vaithilingam *et al*. 2012) or concanavalin A (Girard-Misguich *et al*. 2008). It was demonstrated that trophozoites activated by human PBMC also show the presence of CRT in the surface membrane. *In vivo* experiments of amoebic liver abscess in hamsters showed CRT also on the surface of trophozoites. The immunohistochemical assays on trophozoites using monospecific antibodies against recombinant CRT showed that EhCRT is located in the cytoplasmic membrane (Ximénez *et al*. 2014).

Despite its localization, described primarily in ER of other parasites (González *et al*. 2002; Ferreira *et al*. 2004), the CRT can have various isoforms and is found in many sites inside or outside the cell (Coppolino and Dedhar, 1998; Michalak *et al*. 2009), suggesting moonlight protein functions during the pathogenesis as already reported for other parasites (Karkowska-Kuleta and Kozik, 2014).

Early diagnosis has always been a priority to determine the appropriate treatment and prevent fatalities in amoebic infections. In addition, no more than ever, advances in diagnostics can help prevent transmission and provide active surveillance (Ricciardi and Ndao, 2015). Several factors have contributed to make diagnosis of *E. histolytica* infection difficult, including the occurrence of asymptomatic carriers and the existence of a morphologically identical, non-pathogenic amoeba: *E. dispar* (Laughlin and Temesvari, 2005). *Acanthamoeba castellanii* infection diagnostic is not the exception. Clinical suspicion is the first and most vital step in managing *A. castellanii*. A detailed clinical history will usually reveal risk factors, either contact lens wear in western countries or trauma and water exposure in the developing world. Traditionally, identification of AK infection includes microscopic visualization-identification, culture and histological examination and molecular techniques as PCR (Lorenzo-Morales *et al*. 2015). In GAE the CT and MRI show single or multiple pace-occupying lesions in the brain (Visvesvara, 2013), but these assays are labour-intensive, insensitive and may not be useful in the differentiation of pathogen and non-pathogens genotypes. Despite the development of microscopic and molecular-based approaches, there is an evident need for the development of immunoassays as a rapid and early diagnostic tool.

The cloning and expression of recombinant protein domains of *A. castellanii* are an important alternative for the production of antigens with potential for the immunodiagnosis of AK and GAE. Here, the ability of infected rat and human sera to recognize *A. castellanii* recombinant AcCRT$_{29–130}$ was determined by indirect ELISA. Four positive GAE infected human sera were tested against AcCRT$_{29–130}$ and there was not significant total IgG antibody detection compared with the highly humoral immune response detected in rats infected with GAE. Factors like sampling age (obtained in 1989), transportation and maintenance of GAE infected human sera can alter the reactivity by loss of titres. These results are supported by ELISA where the infected human sera were tested against total protein extract from *A. castellanii*. There was not significant antibody detection in these sera.

Equally important, has been shown that a secretory protease from the pathogenic *Acanthamoeba* spp. degrades IgA, IgG and IgM. The degradation of host's defense-oriented or regulatory proteins by *A. castellanii* proteinase suggested that the enzyme might be an important virulence factor in the pathogenesis of GAE infections (Na *et al*. 2002). Even though there was not significant immune response of CRT against the available GAE infected human sera samples, CRT has been recognized in other human and parasitic infections. Humans with trypanosomiasis produce antibodies against the TcCRT (Aguillón *et al*. 1997; Marcelain *et al*. 2000). Interestingly, parasite CRT is considered to be responsible for inducing autoimmune pathologies in infections caused by *Onchocerca* and *Trypanosoma* (Rokeach *et al*. 1994).

In contrast, the analysis of the antibody responses in GAE infected rats revealed that AcCRT$_{29–130}$ recombinant polypeptide was strongly recognized for these sera compared with AK infected rat samples. It is known that due to the rarity of the disease and symptoms common to other pathogens causing CNS infections, the diagnosis of GAE is problematic and requires very high suspicion, one of the main reasons why it is difficult to obtain human infected serum samples. The demonstration of high levels of *Acanthamoeba*-specific antibodies in patient's sera may provide a useful and straightforward method to clarify suspect and get a faster diagnostic of GAE.

To the best of our knowledge, we have demonstrated for the very first time in an animal model that AcCRT$_{29–130}$ recombinant polypeptide was strongly recognized by sera of the rats infected with GAE. Evaluating protein portions as antigenic determinants represent the key features responsible for crucial events such as the ability to be specifically targeted by antibodies and to induce effective immune response. Pioneer studies in protein folding (Atassi, 1975, 1978) suggest that protein surfaces contain a limited number of exclusive sites that can be immunogenic although some have a greater

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potential to stimulate the immune system so at present raised the question about what are the protein (or portions) traits that could define a strong epitope. Many parameters have been proposed to correlate with immunogenicity, such as hydrophilicity (Hopp and Woods, 1981), accessible surface area (Novotný *et al*. 1986) and protrusion from the protein surface (Thornton *et al*. 1986). In a recent study (Virginio *et al*. 2014) was showed that the expression of hydrophilic portions from recombinant proteins also helped in antigen purification by avoiding solubility problems, one of the reasons we decided express $AcCRT_{29–130}$ as specific recombinant protein domain.

In conclusion, our results indicated the identification of a *A.castellanii* CRT, which was predicted as a surface protein, and the recombinant form of a predicted extracellular domain, $AcCRT_{29–130}$, was strongly recognized in sera of rats infected with GAE. Therefore, these findings suggest a diagnostic immunoassay in animal model contributing to veterinary area in revealing new potential therapeutic targets of GAE. On the other hand, it is important to highlight that if infected human samples were obtained in an acceptable number, the presence of anti-CRT IgG antibodies could be assessed in the future, as a mean to detect recent infections. However, given the high complexity of immune response to *A. castellanii* and the high possibilities of detecting antibodies in healthy human patients, the assay proposed here, aimed at detecting the immunodiagnostic potential of $AcCRT_{29–130}$ by ELISA may represent an important complement to diagnose these diseases. Further, research and production of new recombinant proteins/domains as antigens could open new ways for an accurate diagnosis of *A. castellanii* infections.

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