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Entamoeba histolytica acetyl-CoA synthetase: biomarker of acute amoebic liver abscess

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PEER REVIEW

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Comments

This is a well-researched and clearly presented manuscript that will be useful to both fundamental and applied scientists who research on *E. histolytica*, the parasitic protozoan that causes high morbidity and mortality in developing countries endemic for the infection.

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ABSTRACT

Objective: To characterize the *Entamoeba histolytica* (*E. histolytica*) antigen(s) recognized by moribound amoebic liver abscess hamsters.

Methods: Crude soluble antigen of *E. histolytica* was probed with sera of moribund hamsters in 1D- and 2D-Western blot analyses. The antigenic protein was then sent for tandem mass spectrometry analysis. The corresponding gene was cloned and expressed in *Escherichia coli* BL21-AI to produce the recombinant *E. histolytica* ADP-forming acetyl-CoA synthetase (EhACS) protein. A customised ELISA was developed to evaluate the sensitivity and specificity of the recombinant protein.

Results: A ~75 kDa protein band with a pI value of 5.91-6.5 was found to be antigenic; and not detected by sera of hamsters in the control group. Tandem mass spectrometry analysis revealed the protein to be the 77 kDa *E. histolytica* ADP-forming acetyl-CoA synthetase (EhACS). The customised ELISA results revealed 100% sensitivity and 100% specificity when tested against infected (n=31) and control group hamsters (*n*=5) serum samples, respectively.

Conclusions: This finding suggested the significant role of EhACS as a biomarker for moribund hamsters with acute amoebic liver abscess (ALA) infection. It is deemed pertinent that future studies explore the potential roles of EhACS in better understanding the pathogenesis of ALA; and in the development of vaccine and diagnostic tests to control ALA in human populations.

KEYWORDS

Entamoeba histolytica, Amoebic liver abscess, Biomarker, Acetyl-CoA synthetase, Recombinant EhACS, Western blot

1. Introduction

Amoebic liver abscess (ALA) is a fatal manifestation of the enteric protozoan *Entamoeba histolytica* (*E. histolytica*). Human and some non-human primates are the main reservoirs for this protozoan[1]. In order to simulate human ALA to better understand the underlying pathogenesis, mouse, rat and rabbit models have been tested, but only hamster (*Mesocricetus auratus*) and gerbil (*Meriones unguiculatus*) were reported to be susceptible to the trophozoites[2]. The experimental infection routes include direct inoculation of viable, virulence trophozoites into the liver, hepatic portal vein or peritoneal cavity of the animal[3]. ALA infected rodent usually deteriorates to moribund state in approximately a week, which is accompanied by increase malaise, liver tenderness, lethargy and reduce appetite.

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In rodents, antibodies produced against pathogen could be detected as early as 5-7 d, post infection[4]. Hence, the antibodies produced during the acute stage may recognise potentially important biomarker(s) pertinent to the understanding of pathogenesis, vaccine development and diagnosis of ALA. To date, there was no report on the analysis of antibodies produced in either hamster or gerbil post-infected with virulent *E. histolytica* trophozoites.

The objective of this study was to identify and characterize potential *E. histolytica* biomarker(s) recognised by sera of moribund ALA hamsters.

2. Materials and methods

2.1. HM-1:IMSS E. histolytica culture

HM-1:IMSS *E. histolytica* trophozoites were axenically cultured in Diamond TYI-S-33 medium[5]. The trophozoites were continuously passed through the liver of hamsters to maintain their virulence.

2.2. Experimental ALA in hamster and serum collection

The animal experimentation was conducted in accordance with the requirements of Universiti Sains Malaysia Animal Ethics Committee, PPSG/07(A)/044/(2008)(40). A maximum of three 8-10 week old male hamsters were housed in each polycarbonate cage with regular changes of clean woodchips beddings. The animals were provided with alternate 12 h lighting and 12 h darkness, as well as *ad libitum* food pellets and water. ALA was induced by inoculating each hamster with 1×10^6 trophozoites in 0.2 mL phosphate buffered saline via the portal vein[6]. In approximately 6-8 d post-inoculation, the hamster was euthanised with three-time overdose of sodium pentobarbital. Blood was immediately collected via cardiac puncture into a sterile micro-centrifuge tube and allowed to clot. Serum was separated, labelled as 'hamster ALA serum' and kept at -20 °C until used. In the control group, each healthy animal was inoculated with 0.2 mL sterile phosphate buffer saline (PBS) without the trophozoites. The serum collected was labelled as 'hamster control serum' and kept at -20 °C until used. Twenty microlitres of each ALA serum sample were pooled into a microfuge tube, mixed and labelled as 'pooled hamster ALA sera'. Similarly, 'pooled hamster control sera' were pooled from the control serum samples.

2.3. Preparation of crude soluble antigen (CSA)

Ten million *E. histolytica* trophozoites were mixed with 500 μL complete Lysis-M buffer added with protease inhibitor cocktail (Roche, Germany) and 20 μL of 0.5 mol/L iodoacetamide (Sigma, USA). The mixture was sonicated (Branson, UK) at 10% amplitude for three cycles of 1 min sonication with 0.5 seconds pulse-on and 0.5 seconds pulse-off. The lysate was then centrifuged at 10000 × g (9020 r/min) for 10 min at 4 °C to collect the CSA supernatant.

2.4. 1D-PAGE and Western blot analysis

Twenty micrograms of CSA per well were separated by 9% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and electro-blotted (Bio-Rad, USA) onto a 0.45 μm-pore-size nitrocellulose membrane. The membrane was blocked for 1 h at room temperature with 5% skimmed milk. Subsequently, the membrane was cut into multiple strips and each strip was incubated with Tris-buffered saline (TBS) control, pooled hamster control sera, individual hamster ALA serum samples, or pooled hamster ALA sera at dilution of 1:50 in TBS containing 0.1% Tween 20 (TBS-T) for 2 h at RT. After washing with TBS-T for 3×5 min, the strips were incubated with monoclonal mouse anti-hamster IgG-HRP (Sigma, USA) at a dilution of 1: 4000 in TBS for 1 h at RT. The signals were developed using enhanced chemiluminescence (ECL) blotting reagent (Roche Diagnostics, Germany) and captured using X-ray film.

2.5. 2D-PAGE and Western blot analysis

The proteins of *E. histolytica* CSA were first fractionated based on their isoelectric points (pI) using 3100 OFFGEL Fractionator (Agilent Technologies, Germany). Immobiline™ Dry Strip (GE Healthcare, UK) with linear pH range 3-10 in a 12-well setup was used. The fractionation was performed according to the manual provided by the manufacturer. Twenty microlitres of a fractionated protein sample were mixed with 5 μL of 5X sample buffer without boiling, and subsequently separated by 9% SDS-PAGE. Western blot analysis was performed on the 12 protein fractions using pooled hamster ALA sera and pooled hamster control sera to identify the antigenic fraction(s). For confirmation of the antigenic fraction, the Western blot experiment was repeated on the fraction using individual hamster ALA serum samples.

2.6. Mass spectrometry analysis and protein identification

The antigenic protein band was sent to the Australian Proteomic Service for tandem mass spectrometry (MS/MS) analysis. At the proteomic facility, the protein sample was digested with trypsin and the peptides were extracted and analysed by electro-spray ionisation mass spectrometry using the Ultimate 3000 nano high performance liquid chromatography system [Dionex] coupled to a 4000 Q TRAP mass spectrometer [Applied Biosystems]. The mass-spectrometry analysis was performed with two different gel slice samples to ensure reproducibility. According to peptides sequence BLAST via Mascot, *E. histolytica* acetyl-CoA synthetase (UniProt accession number: Q9NAT4) was identified.

2.7. Cloning and expression of recombinant acetyl-CoA synthetase

Genomic DNA was isolated from 3×10^6 *E. histolytica* trophozoites using QIAamp DNA mini kit (QIAGEN GmbH, Germany). PCR primers targeting the amplification of *E. histolytica* acetyl-CoA synthetase (EhACS) gene from the genomic DNA were F-5′-GGA ATT CCA TAT GAT GCA ATT TGA GCC ACT and R-5′-CCG CTC GAG TTA TGG TTG GAT GAC GA. The PCR product (2 142 bp) was cloned into the pET-14b vector and confirmed via sequencing. Recombinant EhACS (rEhACS), was then propagated in *Escherichia coli* XL 1-blue and expressed in *Escherichia coli* BL21-AI. During expression, 1 mL of overnight starter culture was inoculated into 50 mL Luria-Bertani broth supplemented with 50 μg/mL ampicillin. At OD_{600} 0.6-0.8, over-expression of rEhACS protein was induced with 0.2% (w/v) L-arabinose at 32 °C, overnight. The recombinant protein was purified using HisPur™ Ni-NTA Resin (Fisher Scientific, USA), according to the manufacturer's protocol, and the concentration was estimated using the Bradford method.

2.8. Evaluation of rEhACS via indirect ELISA

The rEhACS identified as a potential biomarker in hamster experimentation was evaluated using hamster ALA serum samples in an indirect-ELISA format. The customised rEhACS-ELISA was optimized and performed based on modifications of the method described by Reen[7]. Each well of the flat-bottom microtiter plate (NUNC, Denmark) was coated with 100 μL of 25 μg/μL of the recombinant protein in 0.1 mol/L carbonate buffer, pH 9.6, overnight at 4 °C in a humid box. Each well was then blocked with 200 μL blocking reagent (Roche, Germany) for 1 h. Then, 100 μL of each hamster serum sample, followed by 100 μL of monoclonal mouse anti-hamster IgG-HRP (Sigma, USA) were incubated in each well for 1 h at serum dilutions of 1:100 and 1:1 000 in PBS, respectively. Each cycle of washing (3× 5 min) was performed using 200 μL per well of phosphate-buffer saline (PBS) containing 0.05% Tween 20. After a final incubation with 100 μL TMB substrate per well for 15 min in the dark, 100 μL of 1 N H_2SO_4 was added as the stop solution. The absorbances were read at 450 nm using a Multiskan FC Microplate Reader (Fisher Scientific, USA). The cut-off value for the indirect ELISA was then determined based on the mean optical density (OD) readings plus 2 standard deviations (SD) obtained for the five hamster control serum samples.

3. Results

3.1. 1D-Western blot analysis of CSA probed with hamster serum samples

The antigenic profile of CSA probed with the hamster ALA serum samples revealed 5 antigenic bands, *i.e.* ~100 kDa, ~75 kDa, ~65 kDa, ~50 kDa and ~45 kDa, which did not react with the controls. Interestingly, only the ~75 kDa protein band was detected by all the hamster ALA serum samples (Figure 1).

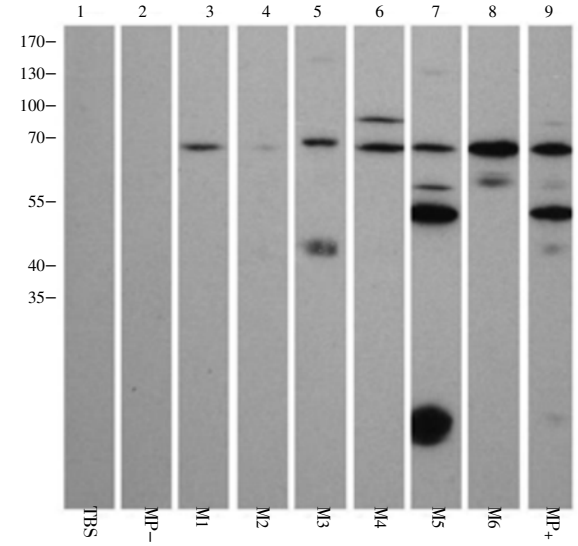

Figure 1. Western blot analysis of CSA probed with hamster serum samples (Representive data).
MP_+: Pooled hamster ALA sera; MP-: Pooled hamster healthy sera; M1-M6 represent individual hamster ALA serum samples.

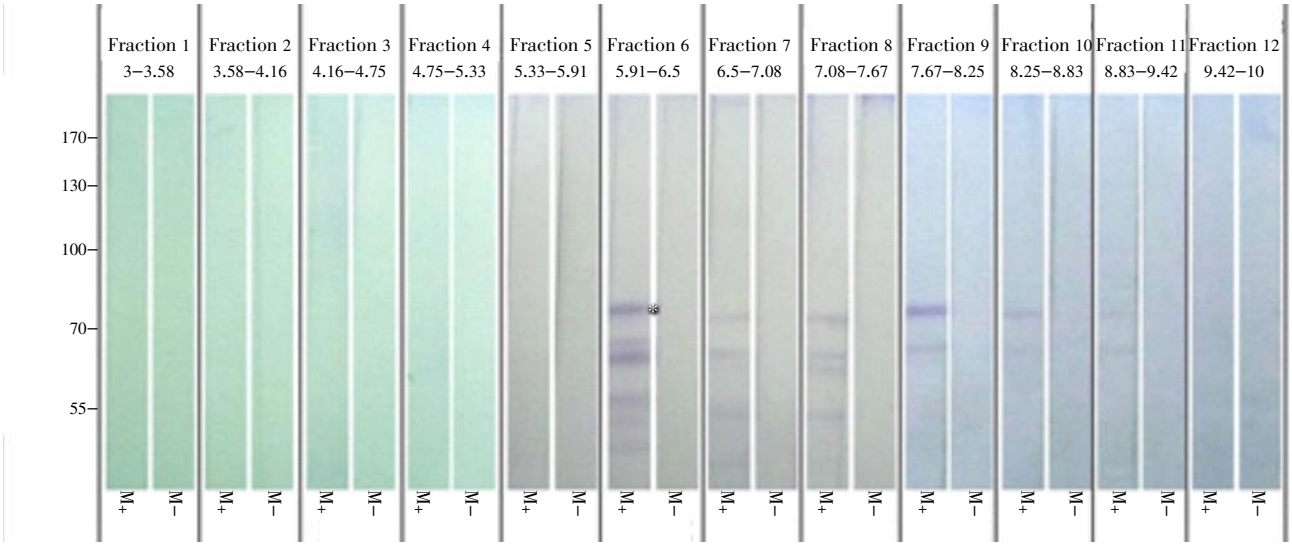

Figure 2. Western blot analysis of fractionated CSA probed with hamster pooled control and ALA serum samples.
*: Position of ~75 kDa protein band; M_+: Pooled hamster ALA sera; M-: Pooled hamster healthy sera.

3.2. 2D protein separation and identification

CSA was fractionated into 12 fractions according to the pI values of its proteins. Western blot analysis of the fractions revealed that the expected ~75 kDa protein was located mostly in Fraction 6 with a pI range of 5.91-6.5 (Figure 2). Further Western blot analysis on this protein fraction indicated that the ~75 kDa protein was recognized by all the individual hamster ALA serum samples (Figure 3). The targeted protein band was excised from SDS-PAGE gel and sent for MS/MS analysis. Mass spectrometric analysis identified the ~75 kDa antigenic protein as: tr|C4LUV9|acetyl-CoA synthetase, putative Tax_Id=5759 [*E. histolytica*]. The protein and peptide scores were 294 and 1456 respectively, with sequence coverage of 44%. In the peptide report, there were 7 of 48 peptides which were above the cut-off ion score of 60 which indicated identity or extensive homology (P<0.05).

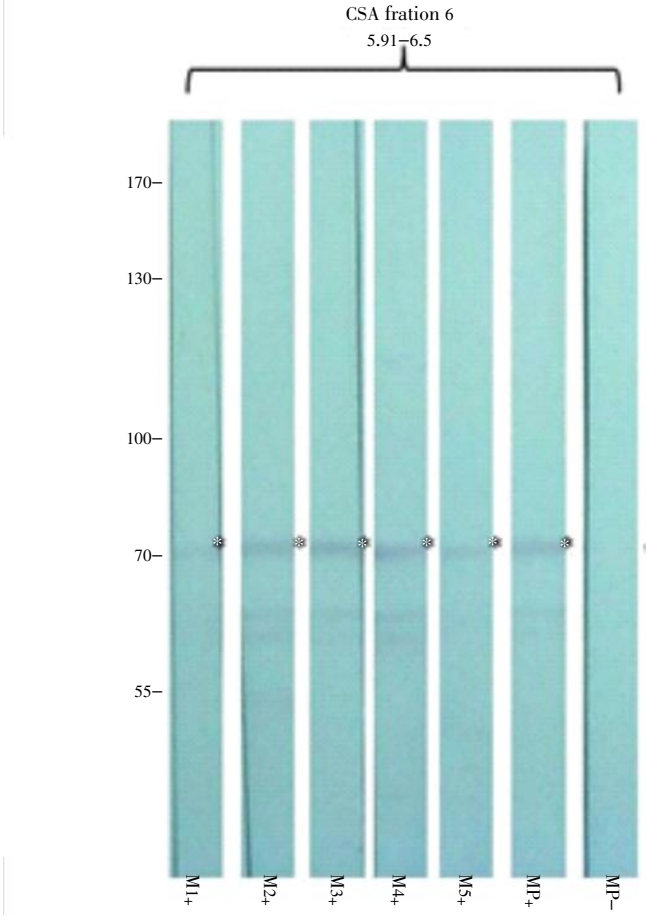

Figure 3. 2D-Western blot analysis of CSA probed with individual hamster ALA serum samples.

*: Position of the ~75kDa protein band; $M1_+$ to $M5_+$: Individual hamster ALA serum samples; MP_+: Pooled hamster ALA sera; M-: Pooled hamster control sera.

3.3. Examination rEhACS antigenicity via ELISA

In the customized rEhACS-ELISA, a cut-off value of 0.1573 was determined based on mean OD+2SD of the five hamster control serum samples. The ELISA revealed 100% sensitivity and specificity when tested with serum samples from moribund hamster with ALA (n=31) and the control group (n=5), respectively (Figure 4).

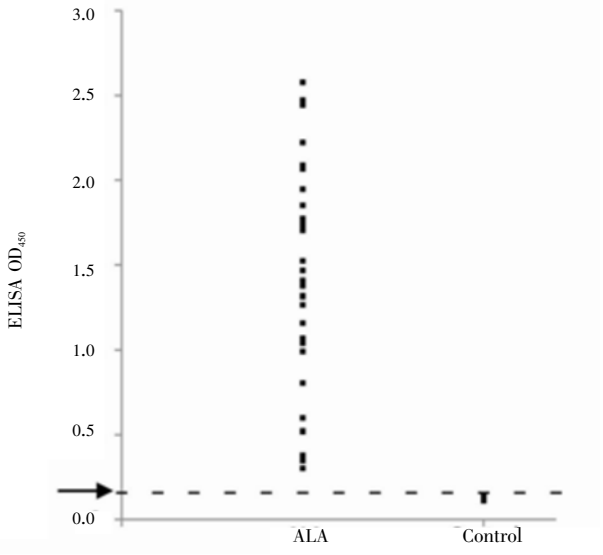

Figure 4. OD_{450} distribution of hamster ALA and control serum samples in rEhACS-ELISA.

4. Discussion

Virulent strain of HM-1:IMSS of *E. histolytica* was maintained by routinely passing the trophozoites through hamster livers twice a month[6]. In this study, serum samples obtained from the euthanized animals were found to possess sufficient antibodies for immunoblot analysis. In the Western blot analyses, all the ALA hamster sera, but not the healthy control sera, were found to recognise the 77 kDa *E. histolytica* ADP-forming ACS. This is therefore a potential diagnostic marker during the acute and terminal stage of the infection. The detectable antibody level produced during this relatively short period of 7 d was in accordance with a previous report that revealed rodent antibodies were generally first detected between 5-7 d post-infection[4].

In experimentally induced hamster ALA, there was massive death of trophozoites during the first few hours post-infection[6]. It is highly probable that *E. histolytica* antigens, including EhACS, released from these dead amoebas triggered the host humoral responses during this early stage of infection. It was also pointed out that the 12 h post-infection period was the critical stage for successful invasion by the amoebas into the hamster liver, where the lowest number of trophozoites was observed in the infected liver[6]. Possibly the live amoebas continued to secrete or excrete EhACS that boosted the production of antibody, or antibody production was due to release of the highly immunogenic antigen during subsequent death of trophozoites.

In mitochondrial organisms, acetyl-CoA is the fuel for tricarboxylic acid cycle but this pathway is absent from the amitochondriate *E. histolytica*. The amoeba ADP-forming converts acetyl-CoA to acetate by hydrolysis to generate ATP from ADP and Pi[8]. However, it does not appear to synthesise ATP from AMP and Ppi[9]. Interestingly, it is also involved in degradation of amino acids by accepting propionyl-CoA as a

substrate to generate ATP[8,10]. Although the functions of EhACS in *E. histolytica* metabolism are relatively well reported, its potential roles in pathogenesis, vaccine and diagnosis are basically unexplored[11-13].

In conclusion, this study has successfully identified EhACS as a biomarker for ALA in moribund hamsters. The potential role(s) of EhACS in elucidating pathogenesis, developing vaccine and improving diagnosis of human amoebiasis should be further studied.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

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Comments

Background

ALA is a fatal manifestation of *E. histolytica*. To better understand the pathogenesis of human ALA, hamster (*Mesocricetus auratus*) has been used to maintain the virulence of *E. histolytica* in liver of hamster. This study attempted to identify and characterize potential biomarker(s) of *E. histolytica* recognized by antibodies of hamsters with acute ALA.

Research frontiers

This is the first study that utilized a 5-7 d post-infected hamster sera in Western blotting to reveal *E. histolytica* acetyl-CoA synthetase as a potentially important biomarker in acute ALA infection.

Related reports

The *E. histolytica* acetyl-CoA synthetase recombinant protein was subsequently expressed and utilized in an indirect ELISA format to show that it was highly specific and sensitive in the detection of hamster antibodies during acute stage of ALA infection. There are no other reports and this is a new finding.

Innovations and breakthroughs

The innovative aspect of this paper is the utilization of 5-7 d sera obtained from hamsters with acute ALA to perform Western blotting to identify the biomarker. In comparison, other studies normally focused on the infected liver.

Applications

The potential roles of EhACS in elucidating pathogenesis, developing vaccine and improving diagnosis of human amoebiasis should be further studied. In addition, it would be interesting to ascertain the potential of rEhACS in determining antibodies from human with ALA.

Peer review

This is a well-researched and clearly presented manuscript that will be useful to both fundamental and applied scientists who research on *E. histolytica*, the parasitic protozoan that causes high morbidity and mortality in developing countries endemic for the infection.

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