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***Leishmania infantum chagasi*: A genome-based approach to identification of excreted/secreted proteins**

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Abstract

The parasitic protozoan, *Leishmania*, survives in harsh environments within its mammalian and sand fly hosts. Secreted proteins likely play critical roles in the parasite's interactions with its environment. As a preliminary identification of the spectrum of potential excreted/secreted (ES) proteins of *Leishmania infantum chagasi* (*Lic*), a causative agent of visceral leishmaniasis, we used standard algorithms to screen the annotated *L. infantum* genome for genes whose predicted protein products have an N-terminal signal peptide and lack transmembrane domains and membrane anchors. A suite of 181 candidate ES proteins were identified. These included several that were documented in the literature to be released by other *Leishmania* spp. Six candidate ES proteins were selected for further validation of their expression and release by different parasite stages. We found both amastigote-specific and promastigote-specific released proteins. The ES proteins of *Lic* are candidates for future studies of parasite virulence determinants and host protective immunity.

Keywords

Leishmania; genome; secretory pathway; excreted/secreted proteins; Trypanosomatid

INTRODUCTION

Leishmania spp. are vector-borne protozoan parasites that cause a group of diseases prevalent primarily in impoverished populations of 88 nations [1], collectively called leishmaniasis.

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Leishmania are obligate intracellular pathogens residing primarily in mononuclear phagocytes of infected humans and other mammals. Major clinical syndromes include cutaneous, mucosal and visceral leishmaniasis (VL) [1,2]. *L. chagasi*, which is now thought to be the same species as *L. infantum* [3,4] (referred to as *Lic* hereafter), is the most common cause of VL in the New World. VL, the most severe form of leishmaniasis, has an incidence of approximately 500,000 new cases each year, although most cases likely go unreported. VL is characterized by fever, enlarged liver and spleen, anemia and progressive weight loss, and is fatal when left untreated, causing ~57,000 deaths annually. The disease incidence is on the rise due to urbanization and risk of co-infection with HIV [5]. The vast majority of VL is found in Brazil, India, Sudan, Bangladesh and Nepal [6]. Control of these diseases is complicated by difficulty in access to health care, toxicity and expense of treatment regimens, and a lack of a protective vaccine.

Secreted proteins play important roles in the infection process and suppression of host immune systems by both prokaryotic and eukaryotic pathogenic organisms [7,8,9]. Identification of excreted/secreted (ES) proteins of *Leishmania* could provide insight into mechanisms through which the parasite survives the environmental challenges encountered during its digenetic life cycle. These include transmission between the insect vector and the mammal, entry into host tissue and host macrophages, establishment and maintenance of a parasitophorous vacuole within the infected macrophage, acquisition of nutrients from this intracellular location, and modulation of local and systemic host immune factors. Furthermore, it is known that *L. infantum* promastigote culture filtrates elicit a strong immune response that is protective against *L. major* infection in BALB/c mice [10,11], and *L. infantum* ES antigens produce a long lasting and strong protective effect against canine VL [12]. Thus, some ES proteins could also be a source of vaccine antigens that could provide lasting immune protection.

Despite their importance, the proteins secreted from *Leishmania* have not been systematically and comprehensively catalogued. Some ES proteins of *L. donovani* have been identified based on the presence of their enzymatic activity in parasite culture supernatants. These include an acid phosphatase [13], a chitinase [14], a histidine acid phosphatase [15], and a P1/S1 nuclease [16]. Antibodies raised against culture supernatants of *L. major* parasites were used to screen expression libraries to identify ES proteins. This approach yielded proteases, heat shock proteins, spermidine synthase, ubiquitin ligase, ribosomal and a few unknown proteins [17]. Although valid, the approach of examining proteins in extracellular media of cultured parasites is inevitably plagued by the concern that some proteins result from low level parasite lysis, no matter how healthy the culture. Now that three *Leishmania* genomes are available, there is the opportunity to systematically search for ES proteins of leishmania *in silico* and document their expression and release experimentally.

Several of the reported ES proteins bear an N-terminal classical signal peptide indicating that they enter the endoplasmic reticulum (ER)-based secretory pathway. This prompted us to analyze the genome of *L. infantum* to predict its suite of putatively secreted proteins. In this process, we predicted 181 proteins to be secreted from *Lic*. We chose six candidate proteins for further analysis and demonstrate that five of these are indeed present in concentrated culture supernatants of *Lic*. The list included some previously unidentified ES proteins of this parasitic protozoan pathogen. Detailed studies of these and other candidates could shed light on yet undiscovered aspects of *Leishmania* pathogenesis and illuminate possible strategies for disease prevention.

MATERIALS and METHODS

Bioinformatic Analysis

The complete annotated genome of *L. infantum* was downloaded from the Sanger Institute (ftp://ftp.sanger.ac.uk/pub/pathogens/L_infantum/DATASETS/). We used the dataset

released on 4.26.2007. SignalP (<http://www.cbs.dtu.dk/services/SignalP/>), TargetP (<http://www.cbs.dtu.dk/services/TargetP/>), TMHMM (<http://www.cbs.dtu.dk/services/TMHMM-2.0/>) and PHOBIUS (<http://phobius.cgb.ki.se/>) were used to predict the presence of signal peptides, localization in the cell and absence of transmembrane helices respectively. GPI-SOM (<http://gpi.unibe.ch/>) and Big PI Predictor (http://mendel.imp.ac.at/sat/gpi/gpi_server.html) were used to search for GPI anchor sites. Functional domains in candidate proteins were identified using Pfam HMM (<http://pfam.janelia.org/>) and Prosite (<http://ca.expasy.org/tools/scanprosite/>). Searches for homologous proteins were performed by BLAST at NCBI (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>).

Parasites and Bacteria

A Brazilian strain of *L. chagasi* (MHOM/BR/00/1669), originally isolated from a visceral leishmaniasis patient, was used. Parasites were passaged serially through male golden hamsters to maintain virulence. Amastigotes were isolated from infected hamster spleens and were cultivated in Hemoflagellate Modified Minimal Essential Medium (HOMEM) [18] supplemented with 10% fetal calf serum (FCS), at 26°C, pH 7.4, under which conditions they convert into promastigotes. Promastigotes retain their virulence when maintained *in vitro* for up to three weeks after isolation [19]. Amastigotes were cultured in a medium containing 20% FCS at 37°C, 5% CO₂, pH 5.5, as described previously for *Leishmania donovani* [20]. We used a line derived from *Lic*, called LcJ, which cycles in axenic culture between amastigotes at 37°C, pH 5.0 and promastigotes at 26°C, pH 7.4 [21]. Unlike other long-term cultivated *Lic* lines, LcJ parasites continue to retain their infectivity for mice, and to express virulence associated proteins, as long as they are cycled between the life stages every three weeks [21]. All LcJ parasites used in our experiments were three weeks post conversion to ensure complete stage conversion. Metacyclic populations of *Lic* were isolated according to their buoyant density as described previously [22].

All procedures with animals were approved by the institutional Animal Care and Use Committees at the University of Iowa and the Iowa City VA Medical Center.

E. coli strains were routinely cultured in Luria Bertani broth at 37°C. Antibiotics were used at the following concentrations (µg/ml): ampicillin, 100; kanamycin, 50; carbenicillin, 100; chloramphenicol, 30.

Cloning and recombinant protein expression

Genes of interest were amplified by polymerase chain reaction (PCR) from *L. chagasi* genomic DNA as per standard protocols [23]. Oligonucleotide primers were purchased from Integrated DNA Technologies (Coralville, IA). Full-length genes including signal peptides were cloned into either pET28a (Novagen, Gibbstown, NJ) or pDEST17 (Invitrogen, Carlsbad, CA) vector, in frame with an N-terminal His₆ tag, for expression in *E. coli*. Clones were verified for sequence accuracy and proper fusion to the His tag. DNA sequencing was done at the University of Iowa DNA core facility with the Automated DNA sequencer, Model 3730 (Applied Biosystems). DNA sequences were analyzed with FinchTV software and BLASTN and BLASTP [24] available through the *L. infantum* GeneDB (<http://www.genedb.org/genedb/linfantum/>). Plasmid constructs were introduced into *E. coli* strains BL21(DE3) (for pET28a) or BL21-A1 (for pDEST17) by transformation. Expression was induced by the addition of either IPTG or arabinose to the cultures, respectively. Proteins were separated by SDS-PAGE and visualized by staining with Coomassie Brilliant Blue per standard protocols [23]. The presence of the N-terminal His tag was confirmed on immunoblots using an anti-Penta His antibody (Molecular Probes, Eugene, OR) and a peroxidase conjugated

goat anti mouse secondary antibody (BioRad, Richmond, CA). Immunoblots were developed by an enhanced chemiluminescence kit (Denville Scientific, Metuchen, NJ).

Antibody generation and immunoblots

Antiserum to recombinant parasite proteins was raised in rats as per approved protocols at Cocalico Biologicals (Reamstown, PA). Slices containing the recombinant proteins were excised from polyacrylamide gels and used for immunization. Sera prior to and after immunizations were analyzed against *E. coli* – expressed recombinant protein for recognition specificity by standard immunoblots [23]. Parasite samples were analyzed using 1:2000 dilution of the primary antibodies and 1:10,000 dilution of a peroxidase-conjugated goat anti rat secondary antibody (Invitrogen, Carlsbad, CA), unless otherwise mentioned.

Parasite lysates and supernatants

For total cell lysates, three week old LcJ promastigotes and amastigotes were harvested by centrifugation, followed by lysis in standard SDS-PAGE sample buffer containing either DTT or β ME or both, as the reducing agent. For supernatants, stationary phase LcJ promastigotes, or LcJ amastigotes were suspended for 24 hrs in serum-free BSA-free medium, and then centrifuged (1300 \times g, 10 minutes) to remove parasites. The resulting supernatant was carefully removed and passed through a 0.22 μ m filter. Centrifugal concentration cells (Millipore, Bedford, MA) with a molecular weight cutoff of 10 kD were used to concentrate supernatants 50-fold. Samples were reduced by boiling in SDS-PAGE sample buffer prior to electrophoresis. Proteins from 1×10^7 parasites were used in immunoblots for detection of ES protein in cell lysates, whereas concentrated supernatants from 5×10^7 parasites were compared for the presence of a parasite protein in the extracellular medium. Similar numbers of parasites were also used for immunoblots containing log, stationary and metacyclic stage *Lic* promastigotes. Immunoblots shown are representative of results obtained from 2–3 independent sets of samples.

Visceral Leishmaniasis patient serum

Serum was obtained from patients with symptomatic visceral leishmaniasis in Natal, Brazil. Disease was documented by characteristic symptoms, *i.e.*, parasites viewed in bone marrow smears, positive serology and response to therapy. All subjects or their legal guardian signed an informed consent. The protocol was approved by the Universidade Federal do Rio Grande do Norte Ethical committee (CEP-UFRN 172–06, CONEP 13745, CAAE 0130.0.051.069–06), the NIH, and the University of Iowa IRB.

Immunoblots were performed using 1:5000 dilution of the pooled VL serum in 5% milk and 1:10,000 dilution of a peroxidase-conjugated goat anti-human secondary antibody, as per standard protocols.

Confocal Microscopy

5×10^7 parasites were stained with carboxyfluoresceinsuccinimidyl ester (CFSE) as described previously [25], and allowed to adhere to 12 mm coverslips that were pretreated with poly-L-lysine (60 min). Parasites were fixed with 4% paraformaldehyde for 30 min and permeabilized with 0.2% Triton X-100 for 7 min. The coverslips were blocked with 5% Normal Goat Serum (NGS)/PBS for 60 min and incubated with 1:20 primary antibody against parasite ES protein of choice for 60 min. This was followed by 60 min incubation with 1:200 Alexa fluor 568 (red) conjugated goat anti-rat IgG. All incubations were done at room temperature. After rinsing with PBS, coverslips were mounted with Vectashield H-1000 (Vector labs, Burlingame, CA) and examined on a BioRad Radiance 2100MP Multiphoton/Confocal Microscope. Images were captured using the Laser Sharp 2000 software and analyzed using ImageJ software. All

microscopic studies were performed at the University of Iowa Central Microscopy Research Facility.

Post-imaging analysis

Over 20 confocal micrographs of promastigotes per condition were analyzed for their fluorescence specificity. Promastigote images were divided in half, and the mean gray values (MGV) of the anterior and posterior sections were calculated. In order to determine whether antisera detection was stronger in the anterior versus the posterior end, the following ratio was calculated: (Red MGV of the anterior section / red MGV + green MGV of the anterior section) : (red MGV of the posterior section / red MGV + green MGV of the posterior section). MGV were calculated using ImageJ software, and statistical analyses were performed on GraphPad Prism 5.

Approvals

Studies using Brazilian human sera were approved by the following Institutional Review Boards: the University of Iowa, the Universidade Federal do Rio Grande do Norte, the NIH and the Brazilian government. The Brazilian IRB is registered with the NIH.

RESULTS AND DISCUSSION

Bioinformatic identification of ES proteins

The genomes of four *Leishmania* species, *i.e.*, *L. infantum* (identical to *Lic*, the focus of this study), *L. major*, *L. braziliensis* and *L. mexicana* have been sequenced and made publicly available [26,27]. We analyzed the annotated genes of the *Lic* genome for amino-acid sequence characteristics that predict ES proteins and identified a suite of these predicted proteins. The secretory pathway algorithms applied herein contrast to targeting signals reported to direct proteins toward organelles of the kinetoplastids. For instance, targeting to glycosomes in *T. brucei* requires only a tripeptide in the C-terminus, whereas targeting to glycosomal membranes requires a 15-mer internal binding site for the import protein PEX19 and a transmembrane domain [28,29]. Targeting of proteins for synthesis in the endoplasmic reticulum requires a signal sequence, whereas targeting for retention in the ER was shown to require the C-terminal tetrapeptide MDDL [30].

As a first step, we screened the entire annotated genome using the TargetP [31] algorithm, which predicts the subcellular localization of eukaryotic proteins. This yielded a subset of proteins that were predicted to possess a secretory pathway signal peptide and would transit through the ER. Next, this set was analyzed for the presence of a signal peptide using the SignalP algorithms [32]. We chose only those genes that fulfilled the criteria in both the algorithms that are used by the SignalP program. These candidates were further screened for transmembrane domains using TMHMM and PHOBIUS [33] programs. We included all proteins that were predicted by at least one program to have no transmembrane-like domain other than the signal peptide. The resultant set consisted of 222 candidates from *Lic* that are predicted to enter the secretory pathway, have signal peptides and lack any transmembrane domains. GPI anchors are commonly found in *Leishmania* proteins that are localized on the surface of the parasite. Therefore, we also confirmed the absence of GPI anchors in these proteins using the algorithms big-PI Predictor and GPI-SOM [34,35].

This approach yielded 181 proteins, 115 of which were hypothetical proteins and 66 of which were known or homologues of known proteins. These 66 proteins included candidates that are involved in processes such as sugar and lipid metabolism and oxidoreduction, as well as housekeeping proteins, degradative enzymes, chaperones and others (Table 1S). Whereas several of the hypothetical proteins also possessed domains suggestive of their possible

functions, the majority did not (Table 2S). The approach was validated by the finding of a number of proteins already reported as *Leishmania* spp. secreted proteins (e.g., acid phosphatase [13], chitinase [14], histidine acid phosphatase [15], P1/S1 nuclease [16], dihydrolipoamide acetyltransferase [17], and protein disulphide isomerase [17]).

Based on their annotated identities, we selected six ES candidates that might influence the host environment and/or cause protective immunity for further characterization. Of these, P1/S1 nuclease, dihydrolipoamide acetyltransferase (DHA) and protein disulphide isomerase (PDI) were chosen because they are known to be secreted by other *Leishmania* species and would serve as “positive controls”, in addition to their possible roles in promoting virulence. Another candidate, the peptidyl-prolyl cis-trans isomerase MIP (macrophage infectivity potentiator), was chosen because of its similarity to other known parasite virulence factors. The remaining two were selected on the basis of their possible relevance to host pathways believed to be involved in the infection process and in host recognition. These candidates are listed in Table 1.

Expression/Secretion analysis of candidate ES proteins in LcJ parasites

Lic is a dimorphic organism with a flagellated, extracellular promastigote form and an aflagellate, obligate intracellular amastigote form. Whereas the promastigote develops through several intermediates to the infectious metacyclic form within the gut of the sand fly, the amastigote is the only form within infected mammals. We hypothesized that ES proteins are likely to be expressed by each of these life stages, but the expressed proteins may not necessarily be the same in both life stages. Since there are currently no algorithms to predict the probability of expression in different life stages, we investigated the possible presence of ES proteins in cell lysates and culture supernatants of both promastigotes and amastigotes of *Lic*. Towards this purpose, we used the stage-converting LcJ line that was derived from *Lic*. Axenic LcJ amastigotes have been previously demonstrated to be similar to true amastigotes based on their expression of the amastigote-specific A2mRNA [36], the absence of the promastigote-specific ARL3A transcript [37], the absence of lipophosphoglycan (LPG) in amastigote lysates [21], and the release of MSP at similar levels as wild type amastigotes [21].

Immunoblots revealed a wide variation in the stage-specific expression and secretion patterns of the candidate ES proteins (Fig. 1 and Fig. 2). There were several differences between the predicted and observed sizes of some protein candidates (Table 1). These differences in size could be due to modifications occurring in the parasite, since all the recombinant *E. coli* proteins all displayed their expected sizes (Table 3S). Identical immunoblots of cell lysates were probed with α -tubulin as a loading control. Immunoblots of concentrated culture supernatants were probed with polyclonal antisera for P36 and for MSP (also called GP63). P36 is an internal protein of *Lic* and thereby served as a negative control to rule out cell lysis [19]. Major surface protein (MSP), a protein known to be released from *Lic*, served as a positive control known secreted protein [21]. As expected, only MSP (Fig. 1 and Fig. 2) but not P36 was observed in the supernatants of all samples.

These expression patterns could provide clues to the roles of these proteins in different stages of the parasite's life cycle. ES1, which encodes a serine carboxypeptidase/peptidase, was expressed and secreted predominantly by the amastigote form of LcJ (Fig. 1). Closely related serine proteases have been identified as secreted virulence factors in *L. amazonensis* [38], *Trypanosoma cruzi* [39], *Plasmodium* spp. [40], *Toxoplasma gondii* [41] and parasitic helminths [42,43], that can digest host membrane proteins, collagen, fibrinogen, gelatin and other proteins and thereby facilitate host tissue invasion and parasite dissemination [44]. Unlike the *L. amazonensis* serine protease expressed by promastigotes, our ES1 candidate was expressed and secreted exclusively by the LcJ amastigote stage. ES2 is a hypothetical protein that contains a flavodoxin domain, and was expressed equally by both LcJ promastigotes and

amastigotes. Whereas both forms secreted the protein, amastigotes secreted significantly greater quantities than promastigotes (Fig. 1). Since *Leishmania* are susceptible to killing by reactive oxidant species generated during phagocytosis by macrophages [45], it is possible that the secreted ES2 is part of the protection mechanism adopted by promastigotes to reduce oxidant-mediated killing.

Lic ES3 is annotated as a dihydrolipoamide acetyltransferase with attachment sites for both lipoic acid and biotin. Also known as E2, this protein is an integral part of the pyruvate dehydrogenase complex in mitochondria. Its homolog in *L. major* has been previously reported to be secreted [17]. Our immunoblots suggested that ES3 is expressed equally by both life forms, but is secreted only by amastigotes (Fig. 1).

Our next candidate, ES4, is a protein disulphide isomerase that was previously reported to be a virulence factor [46] secreted by *L. major* [17]. We observed that LcJ ES4 was expressed and secreted only by the promastigote form (Fig. 2). Most PDIs are ER residents that are involved in protein folding [47], but *Lic* ES4 and its *L. major* homolog *LmPDI* are released despite having an ER retention-like signal (EEDL) [17]. *LmPDI* activity has been shown to serve an essential function in promastigotes [46], and *Lic* ES4 expression progressively increased as promastigotes developed from less virulent (log phase) to more virulent (metacyclic) forms. These observations could signify a possible role of *Lci* ES4 in metacyclic promastigotes.

We also analyzed a homolog of a Class I secretory nuclease that was previously reported as a secreted nuclease of *L. donovani* [16]. *Lic* ES5 encodes a P1/S1 nuclease that can hydrolyze all nucleic acids. Unlike its counterpart in *L. donovani*, which is expressed in both life stages, *Lic* ES5 was expressed and secreted only by LcJ amastigotes (Fig. 2). Since the *Leishmania* spp. parasites are purine auxotrophs, secreted nucleases may hydrolyze host-derived nucleic acids and support growth of the parasite in a nutritionally-restricted environment [48]. A change in Mr on SDS-PAGE could additionally suggest that secreted *Lic* ES5 is post-translationally modified.

Our final candidate, *Lic* ES6, is a homolog of a peptidyl prolyl cis-trans isomerase initially identified in *Legionella pneumophila* and named macrophage infectivity potentiator (Mip) for its role in macrophage infection [49]. Mip is a secreted protein reported to contribute to the virulence of the parasitic protozoan *Trypanosoma cruzi* [50], and is expressed by several other bacterial pathogens [51,52]. Our immunoblots showed that ES6 is expressed only by LcJ promastigotes but not amastigotes, and is not present in concentrated culture supernatants of either LcJ promastigotes or amastigotes suggesting that it was not secreted by *Leishmania* species (Fig. 2). Consistent with these observations, Mip homologs in several bacterial pathogens are surface-localized [53,54] despite the presence of a signal peptide. Nonetheless, it supports the assumption that *in silico* prediction of secretion requires experimental validation of the same.

Differences between the predicted sizes of proteins and observed sizes on immunoblots suggested post-translational processing (Figs. 1 and 2). Comparing secreted versus parasite cell-associated ES1 and ES4, and metacyclic versus logarithmic cell-bound ES6, revealed the proteins migrated further into gels indicating processing (e.g., proteolytic cleavage). In contrast, secreted ES3 and ES5 migrated more slowly than cell-bound forms, suggesting different processing events had occurred (e.g., glycosylation). Both the cell-bound and the extracellular ES2 protein seemed to have undergone processing, as the observed size (~65kD) was smaller than its predicted size (90kD). Similarly, cell bound ES4 protein was likely modified, since its observed relative migration (~90kD) was higher than the predicted size of 53kD.

Expression in metacyclics

Metacyclogenesis is a developmental process that occurs within the gut of the sand fly, the insect vector of *Leishmania* spp., during which the less infective procyclic promastigotes transform into highly infective metacyclic promastigotes that are ready for inoculation into a host [55]. Metacyclogenesis is accompanied by changes in morphology and increased expression of several leishmania virulence factors (e.g., MSP and LPG) [56,57]. Metacyclic *Lic* promastigotes can be isolated from day 7–9 cultures using a ficoll step gradient method [22]. Since metacyclic organisms are poised for infecting the mammalian host, we hypothesized that some ES antigens that are expressed by amastigotes might also be expressed by metacyclics.

Analysis of our selected ES proteins revealed that three of the six candidates are expressed in metacyclic *Lic* parasites. These were DHA (ES3), the protein disulphide isomerase (ES4) and the non-secreted protein Mip (ES6). Of these, ES3 was the only one that was also expressed in amastigotes. Although ES3 was equally expressed in all the *Lic* stages (LcJ promastigotes, LcJ amastigotes, *Lic* logarithmic and stationary promastigotes), it was secreted extracellularly only by amastigotes (Fig. 1). ES4 and ES6 were expressed by all promastigotes (log, stationary, metacyclic) but not by amastigotes. The progressive increased expression of ES4 in log, stationary and metacyclic *Lic* promastigotes suggested the protein was associated with promastigotes virulence. ES6, our only candidate protein that was not secreted, exhibited two reactive bands in logarithmic promastigotes that corresponded to the expected protein sizes before and after the cleavage of the signal peptide. ES6 expression was reduced in the stationary and metacyclic stages of *Lic*, and in these stages the presumed mature ES6 [lower relative mass (M_r)] protein dominated (Fig. 2).

Amongst the remaining candidates, ES1 and ES5 were expressed and secreted only by the LcJ amastigotes but not promastigotes. Both these candidates were also expressed in logarithmic phase but not stationary or metacyclic wild type *Lic* promastigotes (Figs. 1 and 2). The final candidate, ES2, was expressed and secreted by both promastigotes and amastigotes according to LcJ blots, whereas blots of wild type *Lic* revealed that ES2 was not expressed by metacyclic promastigotes. The highest M_r band in the ES2 immunoblot was also recognized by pre-immune serum blots, and thus was thought to be non-specific (Fig. 1). Overall there was a rough correlation between ES protein expression in the amastigote stage and logarithmic-growth promastigotes. Not surprisingly, immunoblots suggest that stationary parasite preparations were a mixture of logarithmic-type and metacyclic promastigotes.

Immunolocalization in parasites

We used confocal microscopy to localize two candidate proteins (ES1 and ES3) with different expression patterns in amastigotes versus promastigotes. LcJ promastigotes or amastigotes were labeled with CFSE, fixed on cover slips and probed with pre-immune or post-immune sera. Control confocal studies with pre-immune sera showed only background staining was present. The Alexafluor-conjugated secondary antibody showed only background staining.

Confocal micrographs (Fig. 3 and Fig. 4) mirrored the data obtained with immunoblots. ES1 immunostaining above background staining with pre-immune serum was observed only in LcJ amastigotes but not promastigotes, whereas ES3 staining above background was seen in both life forms. ES3 appeared to localize to an intense focus of fluorescence near the flagellar pocket of LcJ promastigotes, consistent with its secretion. However, post-imaging comparison of the mean gray values of the pixels in the anterior versus the posterior end of the promastigote revealed that there was no significant focal accumulation of the protein at either end.

The pattern of ES3 staining was reminiscent of that of ER staining, with obvious clearance of the nucleus. Antiserum to the *Lic* heat shock protein 70 (HSP70), a constitutively expressed cytoplasmic protein, was used as the positive control for this study. In a pattern that was distinctly different from that of ES3, HSP70 showed a strong and uniform staining of the entire parasite cytoplasm. Some LcJ amastigotes showed punctuate staining with both ES1 and ES3. However, due to the small size of LcJ amastigotes, it is difficult to attribute the staining to specific sub-cellular compartments.

Recognition by pooled VL serum

We analyzed the ability of serum from VL patients to recognize recombinant ES antigens synthesized in *E. coli*. Of the six ES proteins analyzed, we demonstrate that at least two candidates, ES3 and ES5 were recognized by VL patient serum (Fig. 5). Although this does not indicate that the proteins are secreted in vivo during human infection, they do indicate that the proteins are exposed to the human immune system. ES4, which was not recognized by patient serum, served as a negative control. Importantly, both ES3 and ES5 are released extracellularly by the amastigote stage (Fig. 1 and Fig. 2), the parasite form residing intracellularly in an infected mammalian host, leading us to speculate that the ES proteins that are secreted exclusively by the promastigotes (e.g., ES4) might be released only within the sand fly but not the human host.

This study describes an initial attempt to define the secretome of *L. chagasi infantum* according to the sequence characteristics of proteins in the genome. We were able to verify experimentally that indeed five of our six candidate proteins are secreted by one or several forms of the pathogen. Furthermore, several proteins predicted by these algorithms to be secreted were already documented in the literature to be secreted/excreted, further validating the approach.

Despite the above validations, published analyses of the promastigote or “secretome” additionally include a variety of proteins that lack secretory sequence features, suggesting these protozoa possess additional pathways for protein release. Amongst eukaryotic cells, the *Leishmania* spp. and other trypanosomatid protozoa are unique in their possession of a dense subpellicular network of microtubules, preventing conventional pinocytosis or endocytosis as occurs in other eukaryotic cells. Instead these protozoa have a flagellar pocket, a deep invagination near the anterior site of emerging flagellum, which is the sole site of cellular endocytosis and exocytosis. Considering that the flagellar pocket constitutes less than 5% of the surface area of the cell, protein trafficking through this site occurs at a very high rate. Furthermore, clathrin coated pits and vesicles originating at the flagellar pocket membrane are essential for turnover of the variant surface protein (VSG) in *Trypanosoma brucei*, including both endocytosis and exocytosis [58,59]. Sorting of membrane-bound proteins within endocytic and exocytic vesicles may involve selective inclusion or exclusion of membrane lipids or anchors including cholesterol, sphingomyelin and GPI anchored proteins [58,59]. In addition to VSG, other GPI anchored membrane proteins have clearly been found released to the external environment [e.g., GP63/MSP of promastigotes [19,60]]. Some proteins exported to the cell surface lack a predicted signal sequence [e.g. gene B protein of *L. major*; [61]]. The spatial relation between clathrin vesicle-mediated transport and proteins predicted to exit the cell via the secretory pathway is not known. These unpredicted pathways of protein trafficking indicate that there is much to be learned about protein trafficking in the Trypanosomatid protozoa.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Abbreviations

ES	excreted/secreted
Lic	<i>Leishmania infantum chagasi</i>

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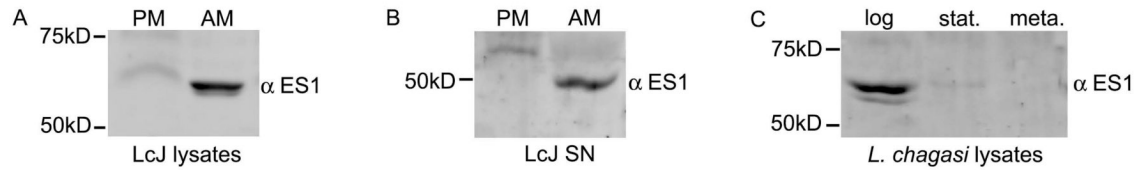
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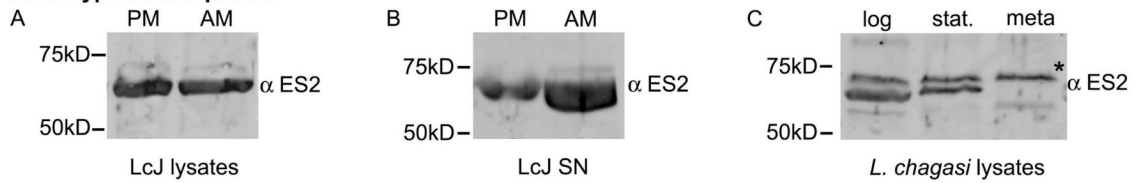
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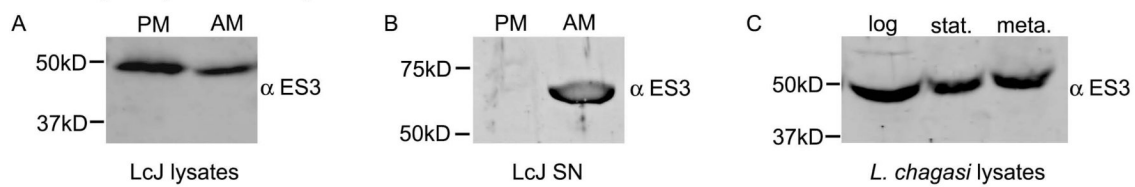
ES1: Serine Carboxypeptidase



ES2: Hypothetical protein



ES3: Dihydrolipoamide acetyltransferase



Controls

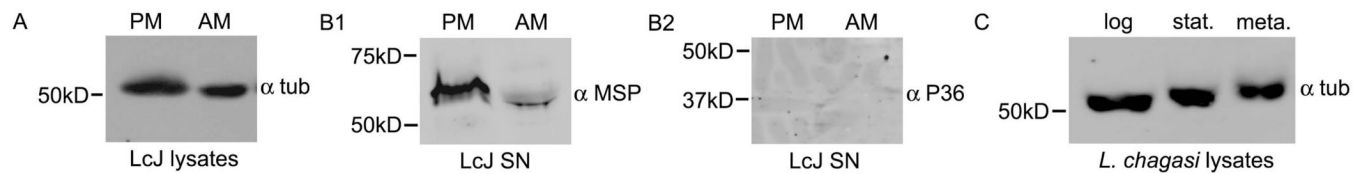
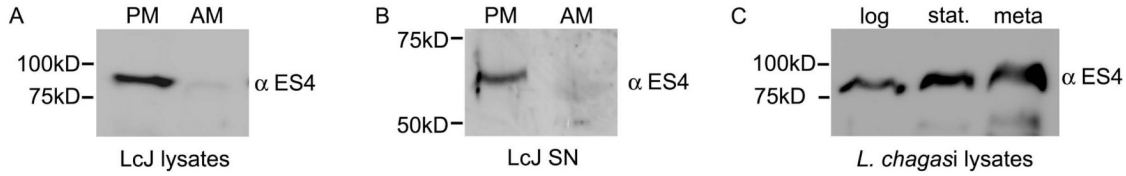
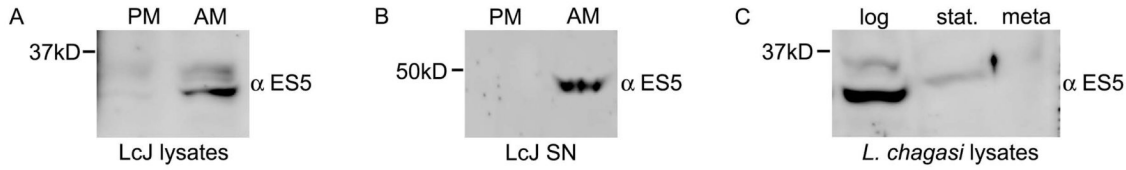


Fig. 1. Expression and secretion analysis of candidate proteins ES1, 2, and 3. (A) Total protein extracted from 1×10^7 LcJ promastigotes (PM) or amastigotes (AM), or (B) concentrated supernatants derived from 5×10^7 PM or AM were separated by SDS-PAGE and probed with 1:2000 primary ES protein-specific antisera. After treatment with secondary antibody, immunoblots were visualized by enhanced chemiluminescence. (C) Total lysates of log, stationary or metacyclic stage *Lic* promastigotes were similarly analyzed with immunoblots. Identical blots were also probed with antibody to α -tubulin as a control for equal loading of cell lysates. Immunoblots of supernatant samples were probed for the presence of MSP and absence of P36 to ensure that no significant cell lysis occurred during sample preparation. “*” marks a non-specific band on the ES2 immunoblot that is also present in blots probed with pre-immune serum.

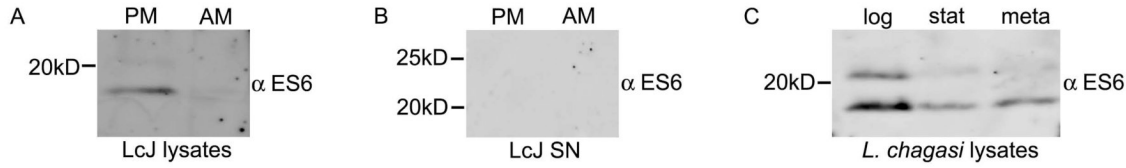
ES4: Protein disulphide isomerase



ES5: P1/S1 nuclease



ES6: Peptidyl prolyl isomerase



Controls

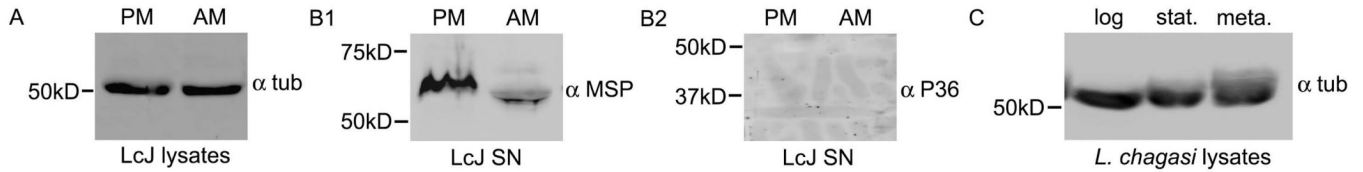


Fig. 2. Expression and secretion analysis of candidate proteins ES 4, 5, and 6. (A) Total protein extracted from 1×10^7 LcJ promastigotes (PM) or amastigotes (AM), or (B) concentrated supernatants derived from 5×10^7 PM or AM were separated by SDS-PAGE and probed with 1:2000 primary ES protein-specific antisera. After treatment with secondary antibody, immunoblots were visualized by enhanced chemiluminescence. (C) Total lysates derived from log, stationary or metacyclic stage *Lic* parasites were similarly analyzed with immunoblots. Immunoblots of supernatant samples were probed for the presence of MSP and absence of P36 to ensure that no significant cell lysis occurred during sample preparation.

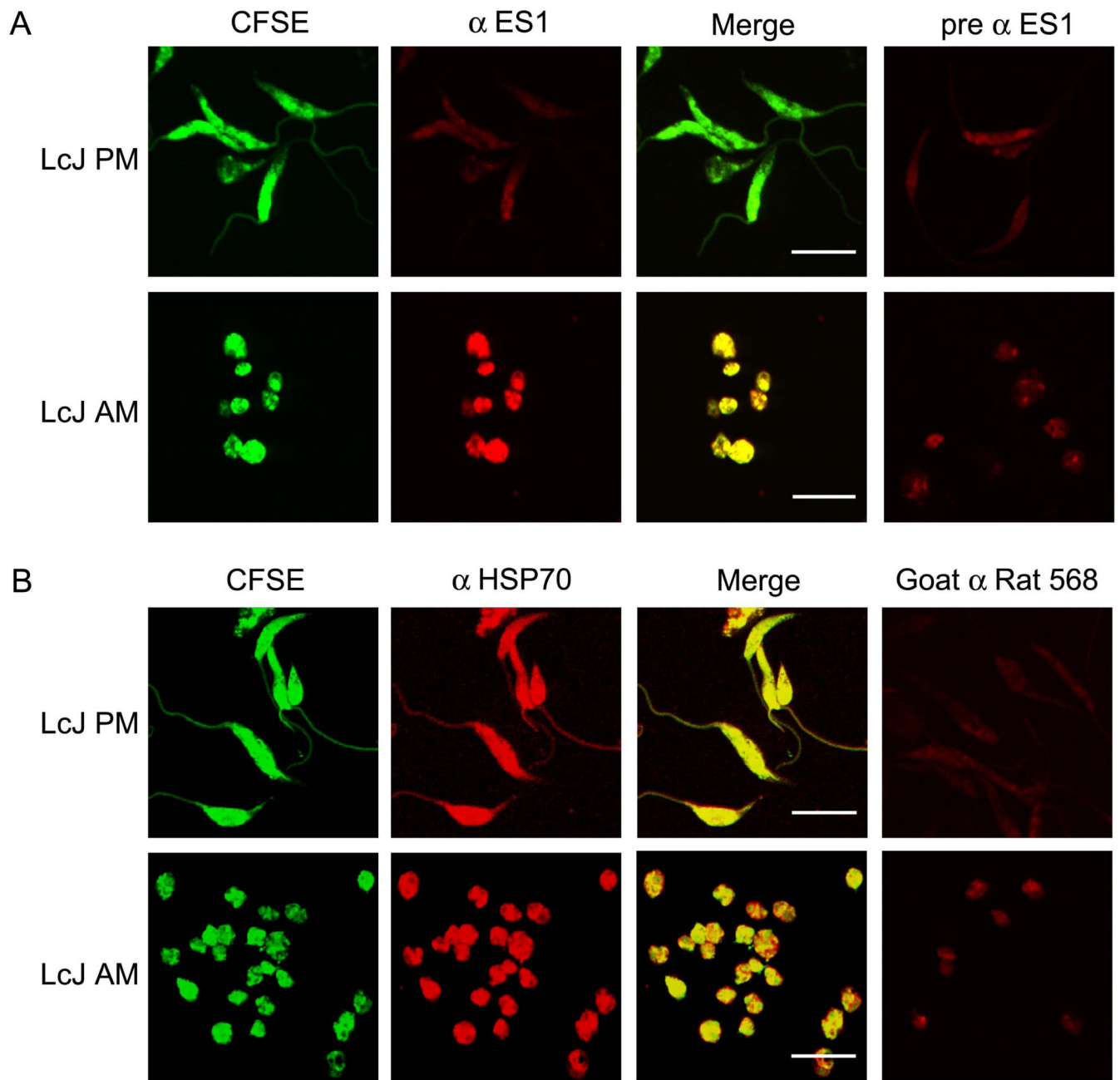


Fig. 3. Immunohistochemical localization of ES1. (A) 5×10^6 CFSE-stained LcJ promastigotes or amastigotes were fixed on 12mm coverslips and permeabilized. Coverslips were probed with primary antiserum to ES1 (α -ES1) followed by Alexafluor 568-conjugated secondary antibody, and visualized by confocal microscopy. (B) Control studies included parasites probed with α -HSP70, which is expressed by both forms of the parasite. No signals were observed in parasite samples probed with α -ES1 pre-immune serum (pre α ES1) or with secondary antibody only (Goat α Rat 568). Scale bar represents 10 microns in all panels.

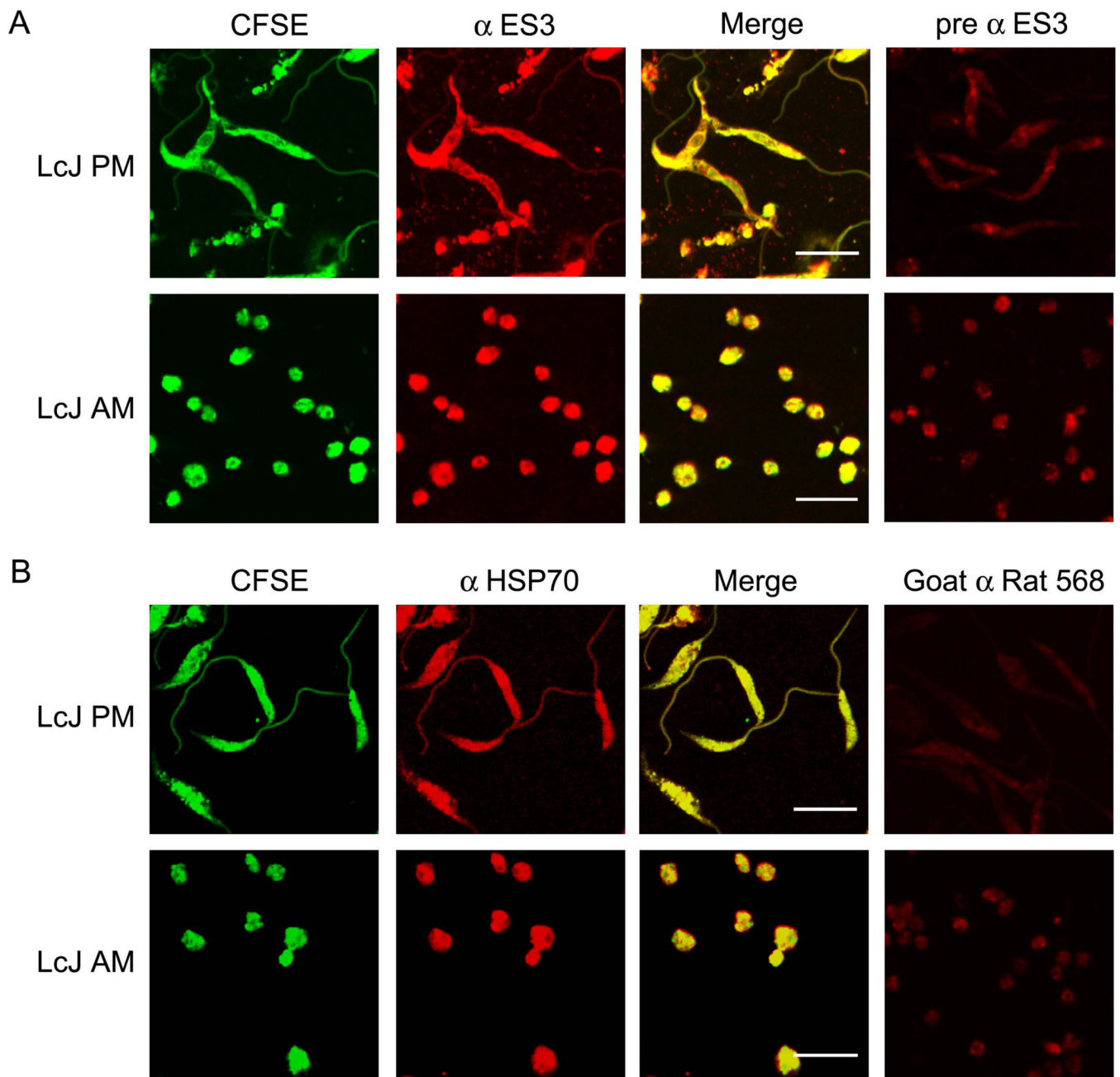


Fig. 4. Confocal micrographs of ES3 expression in LcJ parasites. (A) 5×10^6 LcJ promastigotes or amastigotes were stained with CFSE, fixed on 12mm coverslips and permeabilized. Samples were probed with antiserum to ES3 (α -ES3) followed by Alexafluor 568-conjugated secondary antibody, and visualized by confocal microscopy as in Figure 3. (B) Control coverslips were incubated with α -HSP70. No colocalization was observed in samples probed with either α -ES3 pre-immune serum (pre α ES3) or with Alexafluor 568-conjugated secondary antibody only (Goat α Rat 568). Scale bar represents 10 microns in all panels.

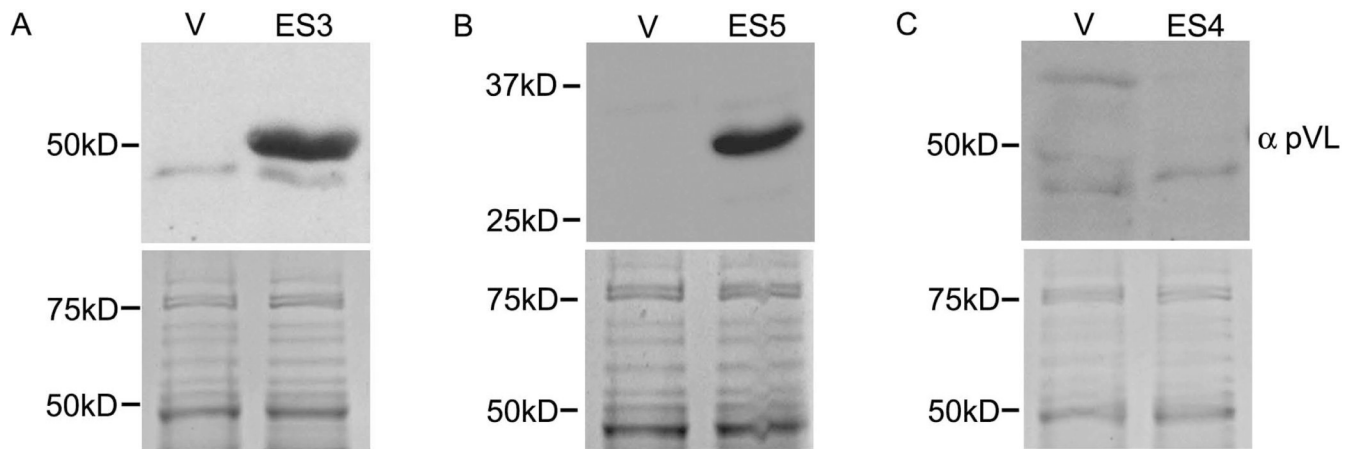


Fig. 5.

Recognition of ES proteins by pooled VL serum. Total protein obtained from *E. coli* strains expressing recombinant (A) ES3, (B) ES5 and (C) ES4 protein were subject to immunoblotting with pooled VL patient serum. Protein derived from *E. coli* strains harboring the empty expression vector (V) but treated to similar induction as the recombinant strain was used as control. Both recombinant ES3 and ES5 were strongly recognized by VL serum, suggesting their possible secretion during *in vivo* infection. ES4 is representative of the ES proteins that were not recognized. The lower panels show representative coomassie brilliant blue stain of the gels.

Table 1

Candidate excreted/secreted proteins of *L. chagasi infantum*.

Candidate	ID ^a	Gene	TP ^b	TMHMM		SP ^c	TM ^d	PHOBIUS		Mass (kDa)	
				Loc	SP ^e			Topology	SP ^f	Topology	Predicted
ES1	LinJ18.V3.0450	Serine carboxypeptidase	S	Y	1	i7-29o	0	Y	n5-17c29/30o	51/48	58/50
ES2	LinJ05.V3.0750	Flavodoxin domain hypothetical protein	S	Y	0	o	0	Y	n3-21c26/27o	90/87	~60/70
ES3	LinJ21.V3.0610	Dihydroipoamide acetyltransferase	S	Y	0	o	0	Y	n5-12c23/24o	43.5/41	50/60
ES4	LinJ36.V3.7280	Protein disulphide isomerase	S	Y	1	i5-24o	0	Y	n5-16c20/21o	53/50	~90/60
ES5	LinJ30.V3.1520	PI/S1 nuclease	S	Y	0	o	0	Y	n9-19c24/25o	35/32	30/45
ES6	LinJ19.V3.0920	Mip PPIase	S	Y	1	o10-32i	0	Y	n10-24c28/29o	22/19	22,18/na

^aGene ID from *L. infantum* GeneDB.^bPrediction of cellular location of protein by TargetP program; "S" indicating entry into secretory pathway.^cPresence of signal peptide by SignalP program.^dPrediction of transmembrane domain (TM) by TMHMM program. TMHMM considers the signal peptide as a TM.^ePrediction of TM by PHOBIUS program.^fPrediction of signal peptide by PHOBIUS program.^gPredicted molecular mass in the cell or secreted into the medium