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***Entamoeba histolytica*: a snapshot of current research and methods for genetic analysis**

Laura Morf¹ and Upinder Singh^{1,2,*}

¹Division of Infectious Diseases, Department of Internal Medicine, Stanford University School of Medicine, Stanford, California, United States of America

²Department of Microbiology and Immunology, Stanford University School of Medicine, Stanford, California, United States of America

Abstract

Entamoeba histolytica represents one of the leading causes of parasitic death worldwide. Although identified as the causative agent of amebiasis since 1875, the molecular mechanisms by which the parasite causes disease are still not fully understood. Studying *Entamoeba* reveals insights into a eukaryotic cell that differs in many ways from better-studied model organisms. Thus, much can be learned from this protozoan parasite on evolution, cell biology and RNA biology. In this review we discuss selected research highlights in *Entamoeba* research and focus on the development of molecular biological techniques to study this pathogen. We end by highlighting some of the many questions that remain to be answered in order to fully understand this important human pathogen.

1. Introduction

The protozoan parasite *Entamoeba histolytica* (*E. histolytica*) is a human pathogen and one of the leading parasitic burdens in developing countries, contributing to an estimated 100,000 deaths annually [1]. *E. histolytica* spreads by the fecal-oral route mainly in areas where water sanitation is poor [2]. The parasite has a simple two-stage life cycle. Infection of a human by *E. histolytica* begins by ingestion of the cyst, which is protected from the environment by a highly resistant chitin-containing cell wall [3]. Excystation occurs in the bowel lumen and the trophozoite, the motile and disease-causing form of *Entamoeba*, is released [2]. While most infections are asymptomatic, about 10% of cases result in invasive disease and in rare cases (<1%, mostly adult males) *E. histolytica* also causes extra intestinal diseases, such as liver abscesses [2,4,5]. During the disease causing process in the colon, the trophozoites penetrate the intestinal mucus layer triggering colitis. Invasion involves killing of epithelial cells, neutrophils and lymphocytes by trophozoites. Noninvasive infections can be treated with Paromomycin while Nitroimidazoles are used for treatment of invasive disease [2], however, toxic side effects as well as the need for additional medication often occurs. Interestingly, humans appear to be the only host for *E. histolytica* as thus far no significant environmental or animal reservoirs of the parasite have been detected.

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*Corresponding Author: S-143 Grant Building, 300 Pasteur Drive, Stanford, California, USA, 94305-5107. Phone (650-723-4045); Fax (650-724-3892); usingh@stanford.edu.

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2. What can we learn from studying *E. histolytica*: A selection of *Entamoeba* specific features

Many recent reviews have been published on *E. histolytica*. We have attempted to sum up some of the most exciting recent highlights of *Entamoeba* research; we apologize to those whose work cannot be discussed due to space limitations.

2.1 Host-pathogen interactions

Infection with *E. histolytica* can lead to variable outcomes ranging from asymptomatic colonization, to amebic colitis or even liver abscesses. Clinical studies clearly show that a combination of human genetics, parasite genetics as well as environmental factors play a major role in the variable disease outcomes observed [6]. Highlights of recent publications are two studies on the nutritional hormone leptin and its importance in immunity to *E. histolytica* [7,8]. In a clinical study the investigator's demonstrated that malnutrition, a state where the leptin levels are known to be low, as well as a common genetic polymorphism in the leptin receptor in humans can be the basis for an increased susceptibility to amebiasis [7]. This study is especially important since it is one of the few that demonstrates that host factors are crucial determinants of *Entamoeba* virulence and infection outcome. A second study used genetically engineered mice and revealed that differential susceptibility to amebiasis could be explained at least in part by variable leptin receptor function. This study showed that leptin has a protective role in mucosal resistance to amebic infection and confirmed the initial findings from the clinical study mentioned above [8]. The studies clearly demonstrate that the pathogenic potential of *Entamoeba* is dependent of the host's susceptibility to the parasite, which can be dependent on genetic factors or on environmental factors, such as malnutrition. Additionally, a number of molecular biological studies have revealed detailed insights into *E. histolytica* virulence determinants. A recent publication analyzed the role of a member of the cysteine protease binding family proteins (CPBF8) in phagocytosis and digestion of bacteria ingested by *E. histolytica* [9]. The authors demonstrate that CPBF8 is recruited to the phagosomes during phagocytosis and that it acts as a receptor in binding to and transporting β -hexosaminidase α -subunit and lysozymes to lysosomes/phagosomes. In higher as well as lower eukaryotes lysozymes and hexosaminidases are known to be involved in degradation of ingested bacteria in phagosomes. In accordance, gene knockdown of the CPBF8 protein in *E. histolytica* resulted in reduced ability to digest ingested bacteria and host cells. Knockdown of CPBF8 also resulted in decreased cytopathic activity to mammalian host cells, which was actually the result of the decrease in β -hexosaminidase and lysozymes. The *E. histolytica* genome contains a total of 11 members of cysteine protease binding family proteins and studying each will most likely reveal further insights into *E. histolytica* phagocytosis and virulence. Another recent study analyzed the M8 metalloprotease EhMSP-1 and its potential role in amebic tissue invasion [10]. In *Leishmania major* the well-studied Metalloproteinase MSP is one of the most abundant surface proteins of leishmania promastigotes and is essential for virulence and pathogenesis. Two homologues of the M8 metalloproteases were found in *Entamoeba* species; however, one of them (EhMSP-1) is absent in the non-invasive *E. dispar* suggesting a role of EhMSP-1 in *E. histolytica* invasion. Texeira *et al.* demonstrated that EhMSP-1 localizes to the cell surface and functions in regulation of *E. histolytica* adherence to mammalian host cells. Further, parasites where EhMSP-1 levels were depleted showed decreased motility and decreased tissue monolayer destruction indicating a key role of EhMSP-1 in the amebic invasion process. EhMSP-2 might also be of interest since microarray analysis, which compared gene expression in virulent and avirulent *E. histolytica* trophozoites, detected significant differential expression of EhMSP-2 [11]. Also of great interest is a recent report on a lipid-induced signaling pathway in *E. histolytica*. Genome analysis showed that *E. histolytica* possesses a large number of trans-membrane kinase

(TMK) genes, suggesting that *E. histolytica* has evolved complex signaling pathways to sense its environment. A previous study revealed an important role of the TMK EhTMBKB1-9 in virulence, by demonstrating the involvement of EhTMBKB1-9 in endocytosis and target cell killing [12]. In the recent study these authors demonstrated that EhTMBKB1-9 expression could be induced via a lipid-dependent signaling pathway. While EhTMBKB1-9 levels are low under serum starvation the levels can be reversed by addition of serum. In this study they now show that BSA, instead of serum, could also induce EhTMBKB1-9 expression. The fact that neither fat free BSA nor saturated lipids stimulated EhTMBKB1-9 expression indicated that the lipid compound of BSA acts as a specific activator. Since very little is known on lipid inducible signaling pathways in protozoan parasites this exciting finding may indicate a potential physiological role in *E. histolytica*.

2.2 Mitosomes

E. histolytica belongs to the eukaryotic super group of Amoebozoa, which also includes the human pathogens *Acanthamoeba* and *Balamuthia mandrillaris*. *Entamoeba* was once thought to be a primitive eukaryote and “living fossil”. This hypothesis was based on the proposed absence of mitochondria, which indicated that *E. histolytica* separated from the eukaryotic lineage leading to modern fungi and animals before the endosymbiotic event of a α -proteobacteria that resulted in the presence of mitochondria [13]. The discovery that *E. histolytica* harbors a highly degenerated mitochondrion-related type of organelle called mitosomes [14], proved that *Entamoeba* split after the endosymbiotic event from the eukaryotic tree. *E. histolytica* mitosomes harbor no DNA and were shown to possess a surprisingly minimal protein import pathway of mitochondrial nature [15]. Thus far, Fe-S clusters biosynthesis appears to be the only function of mitosomes [16,17] and has been identified to function in other protozoans such as the *Giardia* mitosomes and of the *Trichomonas* hydrogenosomes [16,18]. A recent study concluded that Fe-S cluster biosynthesis and Fe-S protein-mediated oxygen detoxification are also fundamental functions of *E. histolytica* mitosomes [19]. Interestingly, *Entamoeba* appears to encode a bacterial type rather than a mitochondrial type Fe-S cluster assembly system [20] which was acquired by lateral gene transfer from epsilon-proteo-bacteria and most likely replaced the original mitochondrial endosymbiont Fe-S cluster assembly system [21].

2.3 RNAi

In a recent study we described the presence of an endogenous small RNA pathway in *E. histolytica* [22,23,24]. Three populations of sRNAs were detected (27nt, 22nt, and 16nt) with the 27nt being of highest abundance. The sRNA populations changed in abundance in response to different environmental stresses (heat stress, oxygen stress) (personal communication, H. Zhang and U. Singh). This finding indicates that sRNA populations might have distinct biological roles and current efforts are ongoing to address this hypothesis. A number of genes of the RNAi pathway were identified in the *Entamoeba* genome including three putative Argonaute protein-encoding genes with conserved Piwi and PAZ domains, and two genes with RdRP domains [22]. Sequencing of the small RNAs (sRNA) that associate with the *E. histolytica* Argonaute 2–2 gene revealed that the small RNAs are 27nt in size, map antisense (AS) to genes, harbor 5'-polyphosphate termini (indicating a dicer independent biosynthesis pathway), and can mediate robust gene silencing [22,23]. The structural features of the 5' termini of the small RNAs are characteristics shared with the secondary 22nt small RNAs in *C. elegans*, which are derived by an RNA dependent RNA Polymerase (RdRP) dependent pathway [25,26]. The *C. elegans* 5'- polyphosphate secondary AS sRNAs are thought to be generated in the context of a larger sRNA pathway with the precedent generation of the Dicer derived short interfering RNA (siRNA) harboring the typical 5' monophosphate termini [27]. Despite the fact that additional sRNA populations have been identified in *E. histolytica* [22], thus far no

population could be clearly identified with 5' monophosphate termini; this might be due low abundance of the other (16nt, 22nt) sRNA populations. Also, despite extensive effort to characterize the only protein with a conserved RNaseIII domain (EHI_068740) encoded by the *E. histolytica* genome, to date no Dicer homologue has been identified, suggesting that the fully functional RNAi pathway in *Entamoeba* may function without a conserved dicer enzyme.

3. Working with *E. histolytica*

We have learned a great deal on eukaryotes from the study of model organisms. However, it remains a challenge to adapt this knowledge to non-model organisms, such as the human pathogen *E. histolytica*. While molecular biology techniques to study model organism have rapidly developed, the study of non-model organisms often lags behind. Here we would like to summarize recently developed techniques for *Entamoeba* research as well as the hurdles that we are currently trying to overcome.

3.1 Strains and basic genetic tools

Three *Entamoeba* species are most prevalent in human infection, the pathogenic *E. histolytica*, *E. moshkovskii*, which has been reported to be capable of causing human disease, and the nonpathogenic *E. dispar* [28]. The genome of *E. histolytica* HM-1:IMSS is fully sequenced and annotated on the Amoeba database (<http://eupathdb.org>). Transcriptional expression data are available for a number of strains and among them are two strains that are thus far predominantly used for molecular analyses, the virulent *E. histolytica* HM-1:IMSS and the non-virulent *E. histolytica* Rahman [29,30,31]. The HM-1:IMSS genome consists of approximately 21MB of DNA encoding an estimated 8,200 predicted genes of which about 46% are assigned to a predicted protein function and an AT content of as high as 75% [32]. The comparison of the transcriptomes by whole genome microarrays of virulent and non-virulent *Entamoeba* strains has been a productive avenue of investigation for the identification of novel, as well as for detailed analysis of known, virulence determinants in *E. histolytica* [33,34]. In addition, microarray data of various stress conditions and trophozoites isolated from clinical samples are available [31,35]. Recent efforts to perform RNA sequencing of HM-1:IMSS and Rahman are underway (personal communication, Nancy Guillen) and will enable a more detailed comparison of gene expression between the virulent and non-virulent strains. Additionally, there has been increased focus on developing molecular tools in *Entamoeba invadens*, a species infecting reptiles. The advantage of working with *E. invadens* lies in the ability to induce *in vitro* encystation and excystation, procedures that are currently not possible in *E. histolytica* (Table 1). During stage differentiation the parasite undergoes a complete change in morphology with the cyst wall formation occurring in approximately 48h. Although *E. invadens* is a very useful tool to study stage development, the molecular approaches in this system were lacking. However, recent establishment of transient and stable transfection of *E. invadens* has been achieved using an electroporation based transfection method [36,37,38]. Furthermore, transcriptome analysis of encystation is underway using microarray technology and will reveal great insights into the *Entamoeba* life cycle (personal communication, T. Nozaki). Additionally, RNA sequencing of the developmental cascade is also underway; the data will be valuable to define developmentally regulated genes as well as the transcriptional start and stop sites of genes, define introns and possible stage-specific alternative splicing (personal communication, G. Ehrenkaufer, N. Hall, and U. Singh).

3.2 Phenotypic assays

With humans the only host infected with *E. histolytica* there will always be the challenge of finding a suitable model organism to study infection and the immune response to infection

in vivo. Many animal models were tested including dogs and kittens, rats, gerbils and pigs [39]. A recent study used baboons with the goal to establish a suitable primate model to study *in vivo* *E. histolytica* intestinal infection and colitis [40]. In this study the authors show that vaccinating baboons with a Gal-lectin-based intranasal synthetic peptide vaccine was highly efficacious in preventing *E. histolytica* infection and colitis indicating its use as a potential *E. histolytica* vaccine. Even though important progress in understanding host-parasite interaction from animal models has been made, none of the models mimic the whole cycle of human disease. Easier to perform are the various *ex-vivo* assays available (Figure 1). *Entamoeba* parasites can be assessed for their ability to perform phagocytosis, adherence, migration, cytotoxicity and invasion as used in many studies, including the recent study analyzing EhMSP-1 [10] or the role of Rhomboid proteases in host parasite interactions [41]. In addition, the *ex-vivo* human colon model (colonic explants) offers a promising tool to study both sides of the host-parasite interaction. In this assay the virulence potential of parasites as well as the effect of parasite penetration of the mucus layer and the inflammatory response of the host cell to parasite interaction can be measured [42]. A recent study used in addition to the human colon explant an *in vitro* 3D matrix of FITC-containing fibrillar collagen to study the early stages of amebic infection, in particular parasite migration and invasion [43]. Using the 3D matrix of FITC-containing fibrillar collagen they show that *E. histolytica*'s ability to remodel and cleave collagen is mainly due to its cysteine protease activity and that cysteine proteases are required for migration of the parasite through a three-dimensional collagen scaffold. Moving to the colonic explant model and state of the art imaging techniques they then visualized how *Entamoeba* migrates along the collagen network and disorganizes it before invading the matrix. Based on their data the authors present a model on *E. histolytica*'s invasion route and interstitial collagen network degradation during intestinal amebiasis. The model suggests that the contact of trophozoites with colonocytes provokes a lysis/apoptotic reaction, induced by a specific cysteine protease (CP-A5), enabling the trophozoites to disseminate into tissue. Therefore the authors propose a strong interplay between *E. histolytica*'s invasion capacity and the induction of a host inflammatory response. Taken together, the assays to evaluate *E. histolytica*'s invasion and virulence potential have become highly powerful.

3.3 Methods for gene silencing

Gene knockout is one of the most powerful tools to study the specific function of proteins in any organism. Unfortunately, gene knockouts by homologous recombination are impossible due to the polyploid nature of *Entamoeba*. To compensate, several methods have been established using RNAi based methods for gene knockdown in *E. histolytica* (Figure 1). Using a typical long dsRNA hairpin expression approach well known from model organisms as *C. elegans* and *Drosophila melanogaster*, a 340–790bp long region of a gene was placed head to head on both sides of an unrelated DNA linker (approximately 300bp) for stable expression of a ds-hairpin RNA [44]. This approach was successful for down regulation of a number of amebic genes (Diaphanous, *KlpA1*, *Klp2*, *Klp3*, *Klp4*, *Klp5*) [45,46]. Unfortunately, the gene knockdown efficiency varied between approximately 40% – 80% and furthermore loss of gene silencing despite presence of the hairpin expressing plasmid was observed [47]. Also, thus far no data are available that this dsRNA is indeed processed into siRNA leaving us in the dark about the mechanism behind this method of gene silencing. A similar approach was taken with expression of short hairpin RNA (shRNA) [48]. In contrast to mammalian cell lines where 22nt length shRNA is often used for silencing, in *E. histolytica* a 25–29bp hairpin stem resulted in the most efficient silencing. This is not too surprising given the observation that *E. histolytica* 27nt sRNAs are potent gene silencers [22]. However, as in the dsRNA-based approach, great variation in silencing efficiency was observed and the method appeared sadly not to be applicable for all genes tested. Another study describes gene knock down by sRNA soaking or bacterially expressed

sRNA feeding [49,50]. Although both approaches look highly promising, unfortunately, the knockdown efficiency appears to vary in both techniques. Additionally, little is known about a possible mechanism for how these sRNAs could reach their target gene and mediate gene silencing. A different and currently most commonly used approach for gene silencing in *E. histolytica* is the G3 strain [51,52,53]. In this approach the amoebapore (*ap-a*) gene was incidentally silenced by episomal expression of a SINE element located upstream of the endogenous *ap-a* gene [51]. Amoebapore genes are virulence factors involved in pore formation and lysis of ingested bacteria [54]. However, single gene knockdown cannot be achieved with this method since in the G3 strain, the amoebapore A gene and well as related genes (amoebapore B and Saplip), which have sequence homology to amoebapore A are also silenced. Using the G3 strain and the regulatory region that silenced amoebapore A, a number of other unrelated genes have been successfully silenced. A detailed analysis revealed that the *ap-a* gene silencing was related to nucleosome compaction and decreased levels of histone (H3K4) methylation and was maintained despite the removal of the trigger plasmid [55]. Recent data from our laboratory further showed that antisense sRNAs to the silenced *ap-a* gene are generated, associate with the Argonaut protein and are located in the nucleus [23]. These data clearly indicate that the gene silencing in the G3 strain is linked to the endogenous RNAi pathway.

4. Outlook

In this review we have given an overview of recent progress that has been made in studying the enteric pathogen *E. histolytica*. We are excited about recent successes in gene silencing in *E. histolytica* and especially hope that a more detailed understanding of the RNAi pathway will help to improve the methods currently available as well as to establish new methods. In addition, advances in technology have allowed the sequencing of entire genomes as well as RNA sequencing and data from these approaches will undoubtedly open new avenues of investigation. Significant inroads have been made on characterizing *E. histolytica* biology and improved technologies hold promise for continued progress in the future.

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Highlights

- *Entamoeba histolytica* is an important human pathogen
- Recent important research findings are summarized in this article
- A review of cell biological and molecular tools available is reviewed

Figure 1. Schematic of gene knock down techniques as well as phenotypic assays performed in *E. histolytica*

Several RNAi based gene knockdown techniques have been established in *E. histolytica* such as feeding of bacteria expressing dsRNA to a gene of interest, stem-loop expression of dsRNA as well as soaking of the parasites in dsRNA. Alternatively the G3 cell line, in which gene silencing occurs based on an RNAi based mechanism, can be used to achieve gene knockdown. Evaluation of the “gene knockdown” cell lines can be performed using a wide variety of assays to evaluate for fitness (migration assay, host cell adhesion assay, motility assay) and pathogenic potential (phagocytosis assay, human colonic explant model, cytotoxicity assay, host cell monolayer destruction).

Table 1
Summary of most common strains used in the laboratory and established techniques

The three *E. histolytica* cell lines HM-1:IMSS (virulent), Rahman (non virulent), 200:NIH (virulent) are the most commonly used for studies of amebic pathogenicity. A number of techniques such as transient and stable transfection as well as transcriptional analyses have been developed. In HM-1:IMSS gene knockdown techniques are also available. In *E. invadens*, the induction of *in vitro* encystation and excystation is possible and transient and stable transfection were recently established. In addition, transcriptional analyses of *E. invadens* stage differentiation are pending. (N/A = not available)

Technique	<i>E. histolytica</i>			<i>E. invadens</i>
	HM-1:IMSS (virulent)	Rahman (non-virulent)	200:NIH (virulent)	<i>E. invadens</i>
Transient transfection	✓	✓	✓	✓
Stable transfection	✓	✓	?	✓
Gene knock down	✓	?	?	?
<i>In vitro</i> stage differentiation	N/A	N/A	N/A	✓
Microarray data	✓	✓	✓	pending
sRNA library	✓	✓	N/A	N/A
RNA sequencing	pending	pending	N/A	pending