



Review Article

# Updates in molecules associated with pathogenesis of amoebiasis: A mini review

Devyani Sharma<sup>1</sup>, Upninder Kaur<sup>1</sup>, Rakesh Sehgal<sup>2</sup>

<sup>1</sup>Department of Medical Parasitology, PGIMER, Chandigarh, India, <sup>2</sup>Department of Microbiology, Aarupadai Veedu Medical College and Hospital, Vinayaka Mission's Research Foundation-DU, Puducherry, India.

**\*Corresponding author:**

Dr. Rakesh Sehgal,  
Department of Microbiology,  
Aarupadai Veedu Medical  
College and Hospital, Vinayaka  
Mission's Research Foundation-  
DU, Puducherry, India.

sehgalpgi@gmail.com

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## ABSTRACT

Amoebiasis caused due to the protozoan parasite *Entamoeba histolytica* has been ranked as the third major reason of mortality after Malaria and Schistosomiasis. The pathogen adversely impacts the health of the children and hinders their development and can lead to intestinal and extra-intestinal manifestation, leading to more than 100,000 deaths annually. The most common route of transmission is via faecal oral route, but recent reports also suggest sexual transmission among homosexual men. Recent experiments in *in-vitro*, *in-vivo* and *in-silico* studies have contributed to a better understanding of pathogenesis by filling in the knowledge gaps related to adhesion, invasion, phagocytosis, encystation, trophocytosis and many other processes. Experimental studies evaluating the pathogenic potential of *Entamoeba dispar* also highlights the importance of considering other species under this genus. Despite the recent advances, there is a need for new drug targets due to the lack of a successful vaccine yet. This review therefore summarizes the recent molecules that have been identified and assessed to play a role in various pathogenic mechanisms of the parasite, further contributing to the progression of the disease.

**Keywords:** *Entamoeba histolytica*, pathogenesis, updates, new molecules

## INTRODUCTION

Globally, 50 million people per year are infected with *Entamoeba histolytica*, the etiological agent of amoebiasis.<sup>[1]</sup> It is described as an enteric invasive protozoan pathogen. The World Health Organisation (WHO) has ranked amoebiasis as the third major reason of mortality because of parasitic ailments, accounting for over 100,000 deaths per year, especially in developing countries.<sup>[2]</sup> As it is prevalent in areas of poor sanitation, the cysts of *Entamoeba* are acquired easily by ingesting contaminated food and water.<sup>[3]</sup> The growth of children is also adversely affected due to *E. histolytica*-related diarrhoeal illnesses.<sup>[4]</sup> The National Institute for Allergy and Infectious Diseases (NIAID) classifies *E. histolytica* as a category B biodefense pathogen due to resistance to chlorine, easy spread through food and water, low infection doses, and environmental resistance.<sup>[5]</sup> In about 90% of cases, *Entamoeba* cysts asymptotically colonise the intestinal lumen, leading to non-invasive disease and in another 10% population, trophozoites disrupt the mucosal barrier, invade the lamina propria and result in symptomatic infection.<sup>[6]</sup> The clinical manifestations vary from asymptomatic to severe signs, including extraintestinal abscesses and dysentery. Various organ systems might also be affected, such as respiratory, renal, genitourinary, and peritoneal locations, but among these, the most frequent manifestation is Amoebic Liver Abscess (ALA).<sup>[7]</sup>

The *Entamoeba* genus includes several species in which the intestinal lumen is the habitat of *E. histolytica*, *E. dispar*, *E. moshkovskii*, *E. polecki*, *E. coli* and *E. hartmanni*.

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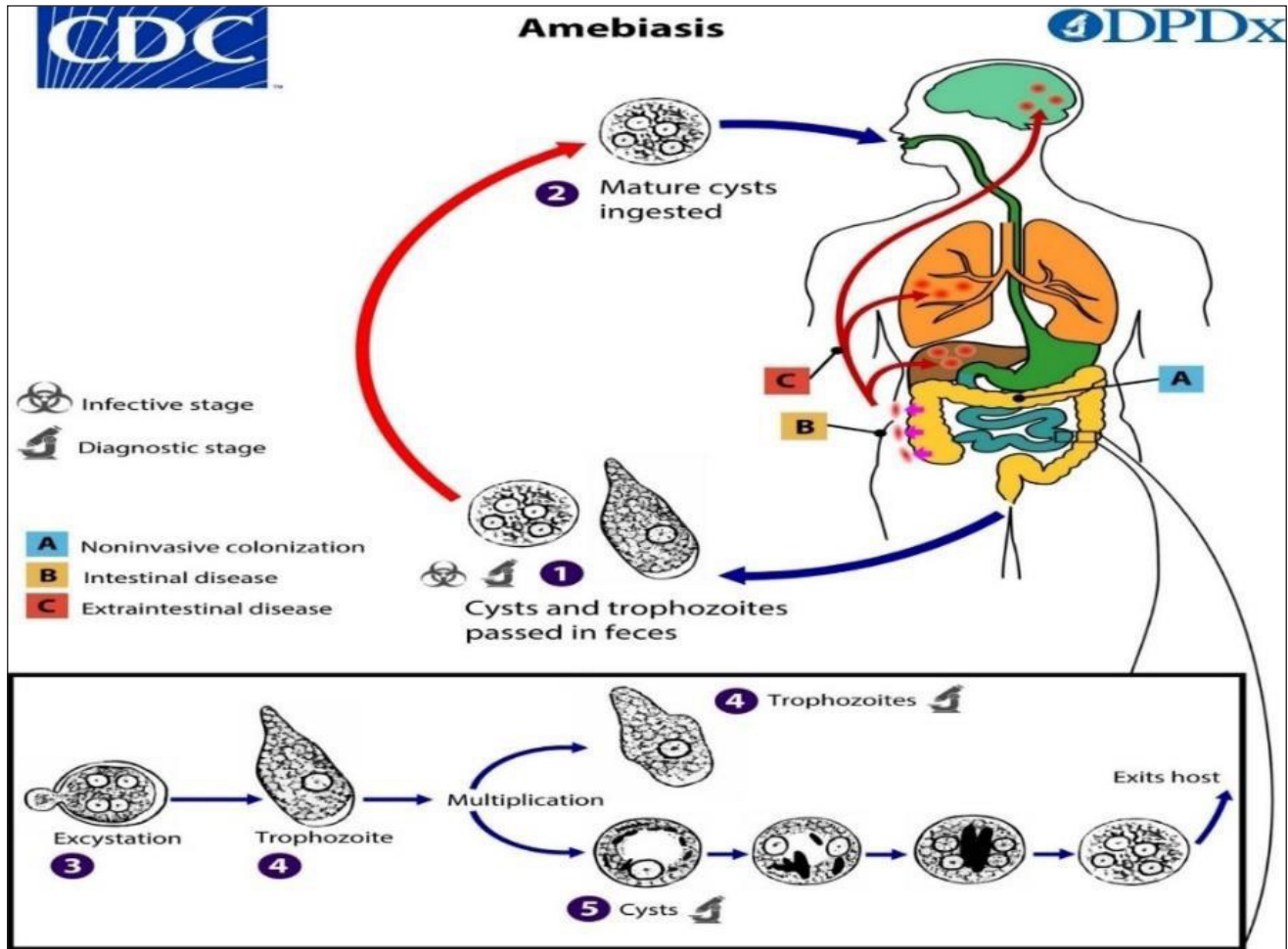


Figure 1: Life cycle of *Entamoeba histolytica* (<https://www.cdc.gov/parasites/amebiasis/pathogen.html>)

The only species concerned with pathogenesis is *Entamoeba histolytica*, while others are considered non-pathogenic to humans.<sup>[8]</sup> Recent findings have demonstrated that patients with gastrointestinal symptoms are infected with *E. dispar*. But there is no conclusive proof between the existence of the organism and the host symptoms.<sup>[9]</sup> The pathogenic potential of *E. dispar* is still not clear. Hence, the objective of this review is to emphasise and offer a summary of the most recent molecules that have been identified to participate in the pathogenesis of *E. histolytica*. The progression of a disease, commonly referred to as pathogenesis, comprises five key phases: Exposure, adhesion, invasion, infection and transmission. The phases of exposure and transmission are most clearly understood when studying the parasite's life cycle.

### LIFE CYCLE

*E. histolytica*'s life cycle is described as monogenetic, as it requires only a single host, the humans. No secondary host is required, but some mammals have known to be with it

infected by it.<sup>[10]</sup> Infection is caused by the intake of mature cysts, which are quadrinucleate [Figure 1]. These are mainly acquired through infected eatables, water or hands. The excystation takes place in the ileum and there is no effect of gastric juice on the chitinous cyst layer.<sup>[7]</sup> The cysts further transform into trophozoites by the action of trypsin, which disrupts the cyst wall. The quadric-nucleated amoeba emerges and proliferates further; eight amebula are then produced on division. They bind to the mucin layer of the intestine and continue to accumulate chromatid-like glycogen and black rod granules as their reserve food. It further forms a resistant cyst wall, completing the process of encystation, which occurs in the intestine lumen. The appearance of four nuclei marks the end of the development of cysts. An analysis on parasite *Entamoeba invadens*, infecting reptiles revealed that the encystation is initiated by the removal of galactose-binding lectin present on the surface membrane of pathogen.<sup>[11]</sup> On completion of the process, trophozoites present in loose stools (diarrhoea) and cyst in formed stools both are passed in the faeces. The cysts withstand the harsh environmental

conditions for months due to their resistant walls. The trophozoites are destroyed in the gastric environment and also in the stool when they leave the body.<sup>[12]</sup> The asymptomatic colonisation can be described on the basis of the above, which accounts for 90% of infections. But the trophozoites colonise the intestine in the rest 10–20% of infections, which is done by the attachment of trophozoites to the large intestine's mucin glycoprotein by a Gal/GalNAc-specific lectin.<sup>[12]</sup> These then obtain their nutrition and undergo binary fission. Neutrophils react to trophozoite intrusion and are accountable for colon disruption. Further invasion of the mucosal layer of the ileum by trophozoites gives them access to the bloodstream, which provides them a pathway to spread to the liver, lungs and other locations, resulting in various clinical forms. From these sites, the trophozoites or cysts cannot leave the host.<sup>[13]</sup>

**Transmission:** The primary routes of infection, despite the investments in better sanitation infrastructure, are the waterborne and foodborne transmission. Raw and undercooked food consumption increases the risk of foodborne transmission.<sup>[14]</sup> If food handlers do not ensure good sanitation, they can also lead to contamination. Flies and cockroaches further help in spreading the infection.<sup>[15]</sup> The use of biosolids as fertilisers often spreads bacteria through cross-contamination with animal waste, soiled implements, irrigation of polluted water, or sewage.<sup>[16]</sup> Oral–anal sexual practices among male homosexual men also lead to transmission.<sup>[17]</sup>

**Reservoirs:** Mostly humans and non-human primates are infected by *Entamoeba*. Till date, no well-defined zoonotic reservoir has been described except humans. The cysts on ingestion start dividing in the ileum, from where they reach the colon, and the epithelial lining is thereafter targeted by them.<sup>[15]</sup> The infection is further spread by cyst excreter. In other animals like cats, dogs, pigs, the infection is not frequent as they do not excrete cysts in their faeces. Furthermore, cysts are not able to survive outside the bodies. There were also no recorded vectors.<sup>[15]</sup>

**Incubation period:** It lasts from two weeks to three weeks, but this can further vary.<sup>[18]</sup> Around 4 to 10% of patients carrying *E. histolytica* experience clinical illness in 12 months. Even years after exposure, ALA can occur, which may further lead to the suppression of immune response.<sup>[6]</sup>

**Communicability Time:** People who are infected keep excreting the cysts in a recurrent way for years if they do not seek any treatment. The chances of transmission are poor in cases of acute amoebic dysentery since there are no cysts in dysenteric faeces and the fragile nature of trophozoites does not allow them to survive for a longer period of time. Also, after several years, patients with chronic infection will transfer the pathogen onto others via the excretion of cysts.<sup>[18]</sup>

## RECENT ADVANCES IN THE PATHOGENESIS

Many studies involving various *in vitro*, *in vivo* methodologies, *in silico* investigations and docking analyses were conducted in the past year to analyse the functions of numerous molecules involved in the pathogenesis of *Entamoeba histolytica*. These have contributed to a better understanding of pathogenesis by elucidating various mechanisms that were previously unknown. The following sections highlight the newly identified compounds and their potential roles in *Entamoeba histolytica* pathogenesis adhesion, infection and invasion [Figures 2 and 3]. In the life cycle, the stages of exposure and transmission have already been defined.

## ADHESION

Adhesion plays a key function in virulence and pathogenesis. It describes the motility, colonisation, nutrient uptake and tissue damage caused to the host. Rhomboid intramembrane proteases, like lectins, govern parasite–host interactions. Welter *et al.* investigated the link between rhomboid proteases and their effect on the lectin subunit submembrane position. They found that by lowering the activity of rhomboid protease *EhROM1* using various ways, a shift in three Gal/GalNAc subunits was found on the cell membrane, including an escalation in the molecular weight of Hgl and Lgl, thereby shedding light on the molecular pathways that control parasite–host adhesion.<sup>[19]</sup> On the other hand, Rab GTPases are implicated in the cytolytic effect of *E. histolytica* as they interact with Gal/GalNAc lectins, which are further essential for the attachment to the intestinal cells as well as the production of virulence factors *EhCP* Cysteine proteinase. Batra *et al.* investigated *EhRabX10*, a pseudoRab GTPase, using physicochemical techniques and various *in silico* methods. They classified it as a pseudoRab GTPase having four non-conserved G-motifs, clearing the path for more research into the nucleotide identification, binding, and hydrolysis mechanisms.<sup>[20]</sup> Rab small GTPases also play a role in the cytoskeleton and signalling control. In the aetiology of *E. histolytica*, membrane trafficking, and cytoskeletal regulation are critical. The authors of another study characterised Rab7 in order to better understand its function. The cells in which *EhRab7D* was overexpressed, there was a reduction in erythrophagocytosis, trogocytosis of mammalian cells, further acidification and maturation of the phagosome. Transcriptional gene suppression of *EhRab7D* gene resulted in phagocytosis/trogocytosis and phagosome maturation phenotypes that were opposite. The findings show that *EhRab7D* has two opposing functions: a repressive role in phagocytosis/trogocytosis and lysosome/phagosome/trogosome formation, and a stimulatory involvement in adherence and motility, probably through interactions with specific effectors.

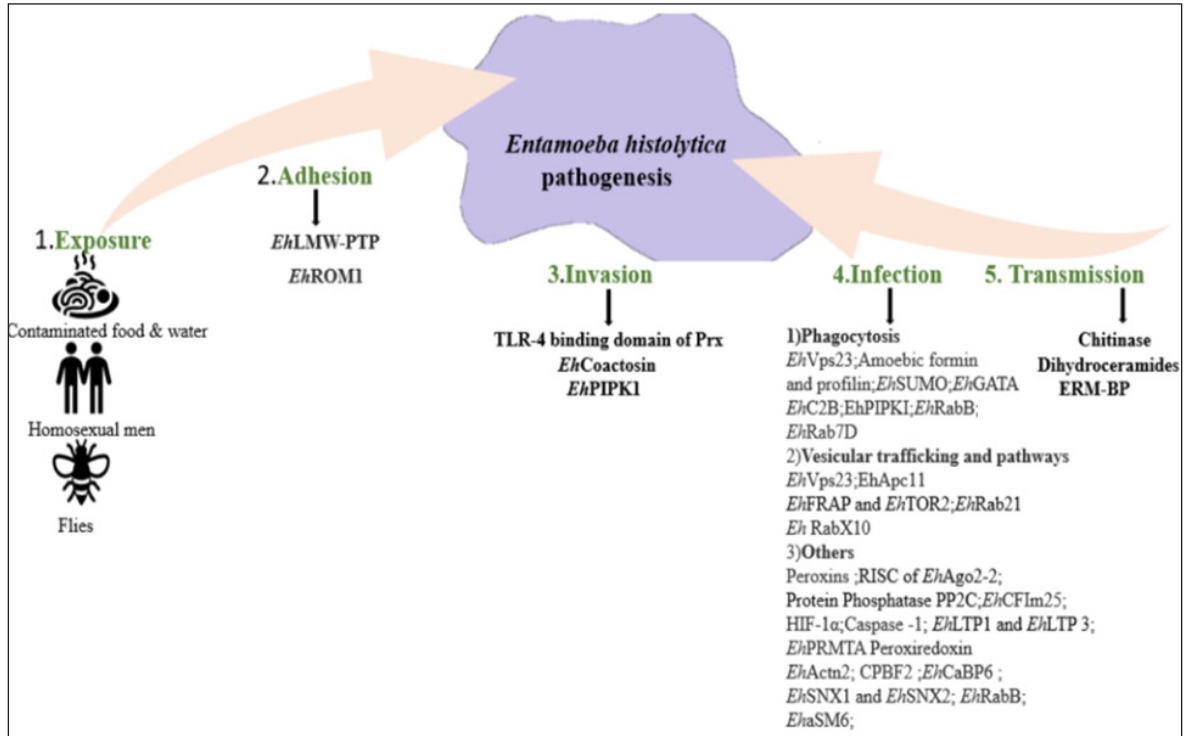


Figure 2: Molecules involved in different stages of (Made with MS powerpoint 2019).

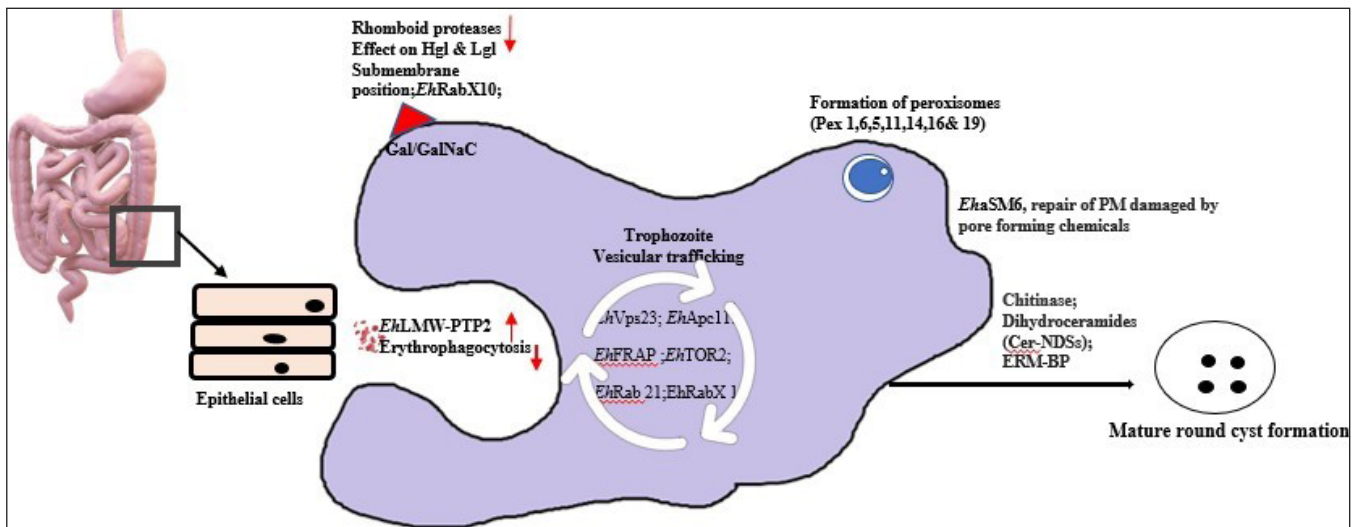


Figure 3: Diagrammatic representation of the main molecules involved (Made with MS powerpoint 2019).

They also postulated a concept in which three *EhRab7* isoforms were involved in phagocytosis/trogocytosis in a sequential manner.<sup>[21]</sup>

*EhLMW-PTPs* genes from *E. histolytica* encode for low molecular weight tyrosine. Phosphatases are found both in trophozoites and cysts but the role of these phosphatases in the parasite's pathogenicity has yet to be determined.<sup>[22]</sup> Differential expression of the LMW-PTPs at the mRNA

and protein levels in an asynchronous trophozoite culture was also evaluated further, discovering that trophozoites overexpressing *EhLMW-PTP2* had lesser fibronectin adherence and a decreased cytopathic impact, possibly due to a reduction in phagocytic cups, exhibiting less erythrophagocytosis. These findings suggest that the parasite's *EhLMW-PTPs* regulate the parasite's proliferation, adhesion and phagocytosis mechanisms, which are all important in the parasite's pathogenicity<sup>[22]</sup> [Table 1].

**Table 1:** Molecules having a possible role in adhesion.

Molecule identified	Possible role in pathogenesis	Reference
<i>Eh</i> LMW-PTPs	Proliferation, adhesion and phagocytosis	[22]
<i>Eh</i> ROM1	Sub-membrane distribution of Gal/GalNAc lectin subparts	[19]

## INVASION

When trophozoites encounter host cells, they may increase the expression of peroxiredoxins Prx as well as their secretion, implying that they have cell-to-cell contact-independent effects on macrophages. The C-terminal of peroxidoxin is accountable for triggering the NLRP3 inflammasome in macrophages, revealing parasites' unique outside-in method for reducing NF- $\kappa$ B-dependent pro-inflammatory responses by activating caspase-1 via NLRP3 and degrading cullin-1/5 from macrophages.<sup>[23]</sup> But during amoebic dysentery, trophozoites *invade* the colon wall and migrate through tissue layers that are rich in the extracellular matrix. The parasite's capacity to move through tissue layers is a crucial need for invasion. Hasan *et al.* examined protein phosphorylation variations along with previously found hyperadherent *E. histolytica* cell line with impaired movement. They also discovered that *Eh*Coactosin, an actin-binding protein that had never been recognised to be phosphorylated, is actually one of the differently phosphorylated proteins in *E. histolytica*. It is also responsible for the regulation of cell membrane dynamics and motility and together with other proteins, it was found to be differently phosphorylated, laying the framework for finding kinases and phosphatases which modulate tissue invasiveness.<sup>[24]</sup> Sharma *et al.*, in their study, characterised *E. histolytica* type I PIPK *Eh*PIPKI. The type I and III PIPK families have homologs, while type II PIPK was missing, according to the data obtained by computational analyses. Therefore, they concluded that the parasite contains a single *Eh*PIPKI gene with unusual posttranslational processing features, including the existence of a domain that was repetitive and showed substrate selectivity that was absent from other PIPK enzymes<sup>[25]</sup> [Table 2].

## INFECTION AND OTHER CELLULAR PROCESSES

Peroxisomes were earlier thought to be absent in *E. histolytica*, but in the research conducted by Verner *et al.* they provided the first evidence of peroxisomes being present in the parasite. They identified seven proteins accountable for peroxisome formation, which were Pex1,6,5,11,14,16, and 19 providing an insight into new possible mechanisms of pathogenesis.<sup>[26]</sup>

**Table 2:** Molecules having a possible role in Invasion.

Molecule identified	Possible role in pathogenesis	Reference
Novel TLR4-Binding Domain of Peroxiredoxin	Activates the NLRP3 Inflammasome in macrophages	[23]
<i>Eh</i> Coactosin	Coactosin phosphorylation controls the cell membrane dynamics and amoebic movement	[24]
<i>Eh</i> PIPKI	Posttranslational processing and signalling mechanisms involved in invasion and pathogenesis	[25]

The *E. histolytica* ESCRT-I complex is involved in vesicular trafficking and phagocytosis, and it also coordinates with other molecules that have a role in a similar process. Galindo *et al.* found that on knocking down the *Eh*vps23 gene it leads to a reduction in the frequency of phagocytosis and the vesicular structures which participate in vesicular trafficking. Antiviral defence, chromatin & transposon silencing, and other processes related to gene silencing are all controlled by RNA interference in parasites. There is not enough information about *Entamoeba* RNA-induced silencing complex RISC.<sup>[27]</sup> Zhang *et al.* studied the RISC of *Eh*Ago2-2, *Entamoeba*'s highly expressed Argonaute protein, and revealed 43 protein constituents with a diverse range of functional activity. Two proteins with nucleosome assembly protein NAP domains were discovered to be novel core members of amoebic RISC, having never been detected before in other RNAi systems. This finding suggests that RNAi regulation may affect a variety of cellular functions in *E. histolytica*.<sup>[28]</sup> Protein phosphatase PP2C have been reported in *E. histolytica*, *E. invadens* and *E. dispar*. The molecule's expression, activity and ultrastructural localisation changed between the cyst and trophozoite stages of *E. invadens*' life cycle. Because the serine/threonine protein's phosphorylation and dephosphorylation regulate different activities of host-parasite interactions, the *Entamoeba* PP2C found in this study might have a role in invasion, intracellular replication and the trophozoite encystation procedure.<sup>[29]</sup> Silencing of polyadenylation factor *Eh*CFIm25 in *E. histolytica* altered trophozoite growth, mortality, and virulence, implying that *Eh*CFIm25 could also be a new biochemical target.<sup>[30]</sup> Modulated proteins are mostly connected to glycolysis, carbon metabolism, cytoskeleton dynamics, parasite virulence, gene expression, and protein changes, according to the STRING analysis, functional GO-term annotations, and pathway analyses KEGG performed by the authors. More research is needed to corroborate the theories that have emerged from this proteomic study in order to provide a complete picture of the molecular pathways involved. Anaphase-promoting

complex cyclosome is an E3 ubiquitin ligase that uses the ubiquitin-proteasome system to synchronise chromosomal segregation and anaphase advancement. *EhApc11* from *E. histolytica*, a homologue of Apc11 in *Homo sapiens*, was chosen for thorough *in silico* research and molecular approaches to elucidate its structural and functional characteristics. *EhApc11* is a powerful antigenic extracellular protein that is hydrophilic and thermostable. *EhApc11* can have a possible relation with a variety of proteins involved in the cell cycle pathway, as well as with *EhApc10*. It's crucial since human Apc10 has been linked to the regulation of the Apc2-Apc11 interaction.<sup>[31]</sup>

Endocytic mechanisms, such as phagocytosis, are critical for the protist parasite *E. histolytica*'s survival in the human gut. By combining a variety of actin-binding and regulating proteins, the actin cytoskeleton plays a part in the creation of pseudopods and phagosomal vesicles. Formin and profilin are two actin-binding proteins that are involved in modulating the endocytic processes by regulating actin cytoskeleton dynamics. Bharadwaj *et al.* found formin and profilin to be involved in phagocytosis and hypothesised that these two proteins interact and that the sequential recruitment at the site is needed for successful phagocytosis completion. Downregulation of formin expression results in the lack of profilin at the phagocytosis site, whereas profilin downregulation has no effect on formin localisation.<sup>[32]</sup> Pérez-Hernández *et al.* found that Gal lectin colocalises with c-Met in cocultures of *E. histolytica* trophozoites and HepG2, indicating that the two molecules interact and that contact with c-Met is required for cytotoxicity. TOR kinases regulate cell growth and proliferation through a variety of signalling pathways. *EhFRAP* and *EhTOR2* are two genes in *E. histolytica* that code for TOR-like proteins. Structural resemblances are found with TOR kinases in case of TOR amoebic homologs. Both of them have the ability to form TORC complexes and take part in protein-protein interaction networks that contribute to pathogenesis, according to the findings.<sup>[33]</sup> The structure-function connections of *EhFRAP* and *EhTOR* have never been characterised *in silico* before.<sup>[34]</sup>

NETosis is a neutrophil process that includes a series of processes from pathogen recognition through the release of DNA containing antimicrobial proteins, as well as the primary creation of NADPH oxidase dependent or independent reactive oxygen species. Earlier, the authors observed that NETosis activated by *E. histolytica* trophozoites is independent of neutrophil NADPH oxidase activity but is dependent on parasite survival, with no ROS source detected. These findings imply that *E. histolytica*-induced NETosis requires ROS produced by trophozoites and processed by extracellular MPO during interaction with neutrophils.<sup>[35]</sup> SUMOylation has yet to be investigated in *E. histolytica*. Its importance

in phagocytosis, along with the role played by *E. histolytica* SUMO gene and its product were studied.<sup>[36]</sup> It was found to have an elongated N-terminus, which distinguishes it from SUMO and ubiquitin, according to the findings. The GG residues at the C-terminus and the KXE/D binding motif, both of which have a role to play in target protein interaction, were also shown. Phagocytic channels had sumoylated proteins present around the swallowed RBC's. The association of *EhSUMO* with *EhADH* was predicted by docking analysis. The immunoprecipitation and immunofluorescence techniques showed that it was higher during phagocytosis; however, in the *EhVps32*, which is an ESCRT-III complex protein, the *EhSUMO* association seemed stronger under basal conditions. Lipid transfer proteins are essential for distinct organelle distribution of lipids and cellular lipid homeostasis.<sup>[36]</sup> Das *et al.* discovered a new role for LTPs in parasitic protist *E. histolytica* phagocytosis, trogocytosis, pinocytosis, biosynthetic secretion, pinosome recycling and motility. They demonstrated that two LTPs with StAR-related lipid transfer START domains *EhLTP1* and *EhLTP3* are active in various biological processes in an LTP-specific manner. The discoveries deliver new proposals for LTPs that are important for understanding the pathophysiology of parasitic protist-caused illnesses.<sup>[37]</sup>

Protein arginine methylation controls a variety of cellular processes like epigenetics, splicing, translation and stress response. Protein arginine methyltransferases PRMTs catalyse this posttranslational alteration, which are categorised into four types based on the molecules they produce. *E. histolytica* possesses four PRMTs related to type I, out of which three are comparable to PRMT1, but the fourth doesn't display enough homology to be placed in any known PRMT family, hence it is referred to as an atypical PRMT *EhPRMTA*. The native enzyme changed its expression level after heat shock and erythrophagocytosis, and the recombinant *EhPRMTA* showed catalytic activity on commercial histones in an experimental set-up. Furthermore, knocking down *EhPRMTA* increased cell development and phagocytosis but decreased the cell migration and survival of trophozoites exposed to heat shock, implying the role of protein in regulating these activities. As a result, the findings imply that this methyltransferase is involved in cellular activities associated with virulence and cell survival<sup>[38]</sup>. The presence of GATA transcription factor in *E. histolytica* and its role as regulator of phagocytosis-related genes was investigated further.<sup>[27]</sup> In those trophozoites where *EhGATA* was expressed more, the amount of *Ehadh* and *Ehvps32* mRNAs was altered, indicating the role of *EhGATA* in their transcription. The morphology was also altered with trophozoites depicting more spherical shape, an increase in the level of proliferation, and a decrease in the level of

erythrophagocytosis. These findings therefore, shed light on *EhGATA*'s activity as a non-canonical transcription factor that regulates the genes involved in phagocytosis.<sup>[27]</sup>

Peroxiredoxin, a key antioxidant enzyme for parasite survival during the invasion phase, was found to trigger autophagy in macrophages in a study.<sup>[23]</sup> In order to establish infection within the host, pathogens must hijack the autophagy system, which is a crucial element of innate immunity. In RAW264.7 cells and mice, autophagosomes were observed in macrophages treated with recombinant Prx for 24 hours. Immunofluorescence and immunoblotting techniques were used to detect this. RAW264. Seven macrophages developed cytotoxicity due to Prx when the time period was increased to 48 hours, which was attributable to autophagy-dependent cell death. According to RNA interference studies, Prx stimulated autophagy via the TRIF pathway TLR4-TIR domain containing adaptor-inducing interferon. The key functional area for autophagy activation was the 100-amino-acid C-terminal part of Prx. These data points to *E. histolytica* Prx as a possible cause of autophagy and cytotoxicity in macrophages, indicating a potential pathogenic mechanism for a parasite.<sup>[23]</sup> *E. histolytica* needs a lot of iron to survive, therefore, when it invades, it utilises iron proteins from the host. Iron is delivered to all the cells with the help of transferrin, which is a plasma iron-binding protein. By attaching to an amoebic TfR *EhTfR*, iron-loaded Tf holoTf in humans can stimulate amoebae proliferation *in vitro*, and amoebae endocytose it inside clathrin-coated vesicles. In this study, it was discovered that *EhTfR* phosphorylation is essential for *E. histolytica* to endocytose human holoTf. Human holoTf could be destroyed by cysteine proteases for nutritional purposes once this complex is endocytosed. With the involvement of phosphoinositide 3-kinase PI-3 K and Ca<sup>2+</sup>, holoTf endocytosis activates the mitogen-activated protein kinases MAPKs and focal adhesion kinase FAK pathways, which stimulate cell proliferation. The study of these proteins and their signalling pathways could aid in the development of future treatments.<sup>[39]</sup> In several eukaryotic pathogens, second messenger signalling regulates a remarkable variety of activities. The cAMP signal transduction pathway of *E. histolytica* was studied using the existing genetic data available. In its genome, it has three heptahelical proteins with varying G-protein-coupled receptor domains, heterotrimeric G-proteins, soluble adenylyl cyclase, cyclase-associated protein and the enzyme carbonic anhydrase. They noticed variations in intracellular cAMP levels as well as a deficiency in the parasite *E. histolytica*'s rate of phagocytosis of red blood cells when they used particular inhibitors against key identified targets. The scientists believe that characterising some of these previously unknown critical signalling determinants

will lead to a paradigm shift in our knowledge of this organism's pathogenicity.<sup>[40]</sup>

Extracellular vesicles, EVs, which are released by cells to move biomolecules to the outside environment, are vital in cell-cell interaction via cargo transfer. Sharma *et al.* recognised and characterised these vesicles from *Entamoeba*. Exosome marker proteins, such as proteins involved in the development of these vesicles, cell signalling, metabolism and cytoskeletal proteins, were prominent in EV proteome, as analysed via mass spectrometry. EVs were also discovered to selectively package small RNAs, which comprised a subset of the cellular antisense small RNA population previously identified in *Entamoeba* RNA interference RNAi pathway proteins, including Argonaute. Surprisingly, they discovered that amoebic EVs affected parasite intercellular communication and encystation efficiency. EVs from encysting parasites aided encystation in other parasites, whereas EVs from metabolically active trophozoites hampered it. These findings show that parasite releases these vesicles that are alike in both the size and structure of exosomes identified earlier from other parasites and also, EVs have a specified protein and short RNA payload, playing a role in parasite intercellular communication and growth dynamics.<sup>[41]</sup>

Pinotsis *et al.* undertook a study to describe the control of *E. histolytica* actinin 2 *EhActn2* by Ca<sup>2+</sup>-Calcium. Ca<sup>2+</sup>-free and Ca<sup>2+</sup>-bound *EhActn2* crystal structures revealed a calmodulin-like domain, which was discovered to be located within the rod domain. Various integrative investigations have demonstrated that the *EhActn 2* calmodulin has a high affinity for calcium ions. The concentration of magnesium ions further plays a role in this. Calcium ion binding increases the rigidity of multidomain proteins; after that, interdomain cross-talk limits the conformational flexibility of F-actin-binding domains, impeding F-actin bundling. *In vivo* research conducted by the same group revealed that *EhActn2* is involved in the development of phagocytic cups.<sup>[42]</sup> Transporting receptors control the appropriate targeting and release of lysosomal hydrolases. CPBFs are also present in *E. histolytica* which attach to a wide variety of cargos, like cysteine proteases and glycosidases, which are involved in disease progression. Research on CPBF2, a protein implicated in cell motility and extracellular matrix invasion, was carried out. Surprisingly, these functions of CPBF were unrelated to its cargo that is alpha-amylase. This is the first proof that a putative hydrolase receptor is involved in parasitic protozoa cell motility and invasion.<sup>[43]</sup>

Various biological functions like cell polarity, gene transcription, microtubule dynamics and cell cycle are regulated by GTPases that function as molecular switches. Verma *et al.* identified an *E. histolytica* Ca<sup>2+</sup>-binding protein

*EhCaBP6* as a new GTPase. They discovered the active location for GTP hydrolysis within *EhCaBP6*'s C-terminal domain, and also found that GTP binding causes *EhCaBP6* to change conformation. The discovery of this novel and atypical Ca<sup>2+</sup>-dependent GTPase is critical for understanding *E. histolytica*'s peculiar cell cycle. Rab21 is a GTPase that has been discovered in a wide range of eukaryotic cells.<sup>[44]</sup>

The findings of a study conducted by Constantino-Jonapa *et al.* suggest that Rab 21 is involved in maintaining the structure of the Golgi apparatus, a vesicular trafficking organelle. Furthermore, proteins like Rab1A and Sec24, which were found to be connected to *EhRab21* in this work, have a twofold function: first, they maintain the organelle structure, and second, they transport COPII vesicles from the endoplasmic reticulum to the Golgi apparatus. Lysosome development was also influenced by *EhRab21*. Using hamsters *Mesocricetus auratus* as an ALA formation model, the role of *EhRab21* in the pathogenesis of amoebiasis was confirmed. The overexpression of *EhRab21Q64L*, a type of positive dominant mutant protein, resulted in a reduction in the number of liver abscesses.<sup>[45]</sup> Phosphatidylinositol phosphates PIPs play a vital role in a variety of cellular processes. PI3-phosphate, PI3P, is important for phagosome/trogosome biogenesis because it recruits PI3P effectors. Watanabe *et al.* used a novel technique to discover the proteins PI3P, which were dependently recruited to phagosomes because no known PI3P downstream effectors are preserved in *E. histolytica*. *EhSNX1* and *EhSNX2*, the two putative SNXs, were discovered in the genome. *EhSNX2* was recruited to trogosomes while *EhSNX1* was attracted to the trogocytic cup and tunnel-like shaped structures, as seen via live and immunofluorescence imaging. Silencing the *EhSNX2* gene increased trogocytosis, implying that it is a trogocytosis inhibitor. Many cellular functions in the amoebic cell need the remodelling of the actin cytoskeleton in response to external inputs. *EhC2B*, a C2 domain-containing protein, was shown to be an actin regulator in this study.<sup>[46]</sup> The findings of Tripathi *et al.* suggested that *EhC2B* works as a molecular bridge that promotes membrane deformation in a calcium-dependent way throughout the evolution of the phagocytic cup via its actin nucleation activity. *Entamoeba*'s virulence and survival are linked to the parasite's motility and phagocytosis. These are regulated through actin-dependent mechanisms.<sup>[47]</sup> During phagocytosis, the scientists looked at the involvement of PtdIns4,5P2 and the enzyme that produces *EhPIPKI*. *EhPIPKI* was found to be enriched at various phases of phagocytosis, from onset to cup closure, in immunofluorescence and live cell pictures. However, after phagosomes were squeezed away from the membrane, the enzyme was no longer present. The development of phagocytic cups was reduced, and the frequency of erythrocyte engulfment was inhibited when a dominant

negative mutant was upregulated. Furthermore, with the exception of PIPKs from other animals, *EhPIPKI* specifically binds to F and G-actin. A GFP-tagged PH-domain from PLC, which exclusively binds PtdIns4,5P2 in trophozoites, indicated that the enzyme's product, PtdIns4,5P2, follows the same distribution pattern during phagocytosis. In conclusion, *EhPIPKI* controls actin dynamics to regulate phagocytosis initiation.<sup>[25]</sup>

Mobility and phagocytosis are critical elements in *E. histolytica*'s colonisation and infiltration of tissues. The functional molecules required during phagocytosis are transported to their correct cellular location with the help of *EhRab* proteins. During phagocytosis, the interaction of *EhRabB* with *EhCPADH* and actin was investigated. By preventing actin polymerisation, which was achieved by Latrunculin A, the scientists evaluated the role of the actin cytoskeleton. The mobility of *EhRabB* and *EhCPADH*, and the rate of phagocytosis were impacted. The association of this molecule with *EhCPADH* was demonstrated in mutant trophozoites with the *EhRabB* gene being silenced, reinforcing the involvement of actin during erythrophagocytosis.<sup>[48]</sup> Gronebery *et al.* found that via IL-6, HIF-1 $\alpha$  modifies the immune response, resulting in different disease susceptibilities in males and females.<sup>[49]</sup> While fighting off the host's defences, *E. histolytica* damages its plasma membrane, PM, resulting in viability loss. However, it is unknown if the trophozoites are able to repair the harm. The following research looked at six genes that code for aSMases and were transcribed in the HM1:IMSS strain. In basal growth circumstances, the *EhaSM6* gene was shown to be the most transcribed and also created a functional protein. The findings reveal that the aSMase6 enzyme aids in the repair of pore-forming molecules and damaged plasma membranes, thereby preventing the loss of cell integrity. Therefore, when confronted with the host's lytic defence systems, this unique system may hence act further [Table 3].<sup>[50]</sup>

## TRANSMISSION

Cysts are the only way for amoebiasis to spread. Lipids are most likely among the metabolites that play a role in encystation. However, there hasn't been a full lipid analysis published because the majority of the important lipid metabolic pathways are unknown. By using lipidomics, Mi-ichi *et al.* discovered dihydroceramides Cer-NDSs with very long N-acyl chains, C26 to C30, as critical metabolites for encystation. They also discovered that these Cer-NDSs are required for the parasite to display dormancy as it repels substances and avoids water loss by generating membrane impermeability. As a result, *Entamoeba*'s parasitic existence depends on ceramide metabolism. During cyst formation, the parasites create a wall structure surrounding it to confer



**Table 3:** List of molecules identified with a possible role in the cellular processes related to pathogenesis.

Molecule identified	Possible role in pathogenesis	Reference
Peroxisins Pex1, 6, 5, 11, 14, 16 & 19	Proteins involved in the formation of peroxisomes	[26]
<i>EhVps23</i>	ESCRT-I component involved in vesicular trafficking and phagocytosis	[51]
RISC of <i>EhAgo2-2</i>	There were 43 protein hits identified with activities ranging from transcription, translation to sRNA loading and other cellular functions	[28]
Protein Phosphatase PP2C	Possible role in invasion, intracellular replication and encystation	[29]
<i>EhCFIm25</i>	Trophozoite proliferation, death and pathogenicity	[30]
<i>EhApc11</i>	Interacts with other proteins <i>EhApc10</i> which are involved in cell cycle pathway	[31]
Amoebic formin and profilin	Phagocytosis	[32]
<i>EhFRAP</i> and <i>EhTOR2</i>	Targets for targeted inhibition in <i>E. histolytica</i> , resulting in the disruption of TOR-associated signalling cascades	[34]
<i>EhSUMO</i>	Phagocytosis	[36]
<i>EhLTP1</i> and <i>EhLTP 3</i>	Endocytosis, exocytosis and motility	[37]
<i>EhPRMTA</i>	Proliferation of cells, heat shock response and regulation of virulence <i>in vitro</i>	[38]
<i>EhGATA</i>	Regulation of genes associated with phagocytosis	[27]
Peroxioredoxin	Activates autophagy and cytotoxic effects in macrophages	[52]
<i>EhActn2</i>	Phagocytic cup formation	[42]
CPBF2	Motility and invasion of the parasite	[43]
<i>EhCaBP6</i>	Cell proliferation	[53,44]
<i>EhC2B</i>	Phagocytosis of RBCs through actin nucleation	[47]
<i>EhRab 21</i>	Actin cytoskeleton regulation, lysosomal biogenesis and Golgi structuration	[45]
<i>EhSNX1</i> and <i>EhSNX2</i>	Trogocytosis	[46]
<i>EhPIP1</i>	By modulating actin dynamics, it controls the initiation of phagocytosis	[25]
<i>EhRabB</i>	During phagocytosis, it mobilises the EhCPADH complex via the actin cytoskeleton	[48]
<i>EhaSM6</i>	To avoid losing cell integrity, supports the repair of PMs that have been damaged by pore-forming chemicals	[50]
<i>EhRab7D</i>	Phagocytosis, trogocytosis and cytoskeletal reorganisation	[21]
<i>Eh RabX10</i> , a pseudoRab GTPase	Participation in the Rab-Sec signalling cascade involved in vesicular pathway in later stages	[20]

resistance. Chitin along with other proteins, like chitinase, makes up the cyst wall. To better understand the role of chitinase in cyst development, the authors also screened a library of 400 chemicals for a new chitinase inhibitor.<sup>[54,55]</sup> They demonstrated that inhibiting chitinase induces the creation of aberrant cyst walls, with the formation of ‘pot-like structure’, using the inhibitor. As a result, the number of mature cysts produced decreased. *Entamoeba* must consequently create mature cysts in order to transmit to a new host, which necessitates the use of chitinase. Cysts survive in harsh environments and are further spread by contaminated food and water, being responsible for disease transmission from one host to another.<sup>[54-55]</sup> Manna *et al.* found a novel transcription factor called Encystation Regulatory Motif-Binding Protein, or ERM-BP. It plays an important part in encystation and responds to NAD<sup>+</sup>. When a mutation in the ERM-BP-DBM DNA binding domain and the ERM-BP-C198A Nicotinamidase domain resulted in protein mislocalisation, the efficiency of encystation was lowered. The importance of ERM-BP in stress response was also underlined in this study. The NAD<sup>+</sup> responsive transcription factor is implicated in both development and the response to heat shock<sup>[56]</sup> [Table 4].

## IMMUNOPATHOGENESIS

The infection associated with *E. histolytica* induces an immune response in the host’s body. Reactive oxygen species are known to play both protective as well as destructive role in *E. histolytica*. Therefore, the parasite has oxidative stress defense mechanisms due to the excessive ROS production. L-cysteine, flavodiiron proteins, peroxiredoxin and trichostatin A are the antioxidants that contribute to both the virulence as well as the defense mechanism. Cell death also occurs by the processes of apoptosis and necrosis, resulting in damage to the host.<sup>[35]</sup>

Begum *et al.* showed that inducing the production of an alarmin molecule, HMGB1, during *Entamoeba* infection has a dual purpose. It functions as both a chemoattractant and a pro-inflammatory cytokine. In the parasite, this chemical plays a critical role in shaping the pro-inflammatory environment. The mechanism by which *E. histolytica* binds to macrophages at the intercellular interface in disease pathogenesis and produces severe pro-inflammatory responses is unknown. The parasite overtakes the autophagy mechanism in macrophages to cause a malfunctioning in the pro-inflammatory responses. On the direct interaction of live trophozoites

**Table 4:** Molecules having a possible role in transmission Cyst formation.

Molecule identified	Possible role in pathogenesis	Reference
Chitinase	Formation of a mature round cyst	[54]
Dihydroceramides Cer-NDSs	By switching membrane characteristics, the Cer-NDS lipid subclass plays a critical role in <i>Entamoeba</i> cell development and morphogenesis	[55]
Encystation Regulatory Motif-Binding Protein ERM-BP	Encystation, its role in development, and how it responds to heat shock	[56]

with macrophages, it triggered caspase-6, which resulted in the fast proteolytic disintegration of the protein complex associated with autophagy ATG16L1 as it wasn't dependent on the stimulation of NLRP3 inflammasome, along with caspase-3/8, implying that the ATG16L1 protein complex has a role to monitor the modifying pro-inflammatory response in amoebiasis. It has been hypothesised that androgens are a factor behind the increased risk of developing monocyte-mediated pathologies.<sup>[57]</sup> Supporting this hypothesis Sellau *et al.* found that in castrated males which were suffering from hepatic amoebiasis, an elevation in the frequency of TNF/CXCL-1 producing classical monocytes was observed. Due to its similarities to trogocytosis in other organisms, *Entamoeba* is known to kill host cells in a similar manner.<sup>[58]</sup> Miller *et al.* showed that *E. histolytica* obtains and presents human cell membrane proteins via trogocytosis, resulting in protection from human serum lysis. The findings are the initial ones to show the display of human cell membrane proteins by amoebae, implying that membrane protein attainment and presentation are a common aspect of trogocytosis. These findings have substantial ramifications for *E. histolytica*'s interactions with the immune system, as well as also revealing a unique pathogen immune evasion technique<sup>[59]</sup> [Table 5].

**Table 5:** List of molecules identified with a possible role in the cellular processes related to immunopathogenesis

Molecule identified	Possible role in pathogenesis	Reference
HIF-1 $\alpha$	During hepatic amoebiasis, it regulates sex-specific Th17/Treg responses	[49]
Caspase-1	Cullin, which inhibits NF-kappaB-dependent signalling in macrophages, is degraded by Caspase-1 pathway	[60]

### Co-infection

Coinfection with virulent organisms, particularly enteropathogenic bacteria, is one of the reasons that have been suggested to contribute to the change of the usual intestinal microbiota, immunological modulation and, as a result, enhancing the virulence of the parasite. The interactions of *Entamoeba* with enteropathogenic *Escherichia coli* bring

about a change in the virulence factors, thereby modifying the innate immune responses of the host which are linked to disease progression.<sup>[61]</sup>

### ASYMPTOMATIC INFECTION

Yanagawa *et al.* in their study provided an insight into the pathophysiology of asymptomatic infections where the infection sites were restricted to the proximal colon where trophozoites were present, but the cystic forms were frequently found in faeces. Asymptomatic invasive cases had a more homogeneous gut microbiome composition than symptomatic invasive cases, with a higher abundance of *Ruminococcaceae*, *Coriobacteriaceae* and *Clostridiaceae* and a lower abundance of *Streptococcaceae*.<sup>[62]</sup>

### *Entamoeba dispar*

It infects about 12% of the global population and was formerly categorised as non-invasive.<sup>[2]</sup> In a study by da Silva *et al.* ACFN strain was found to be the most pathogenic. The second pathogenic strain was MCR. The findings showed that *E. dispar* trophozoites possess the capability of phagoerythrocytosis, but in lesser proportions than *E. histolytica*. Pathophysiology was characterised by large areas of necrosis and well-developed chronic granulomatous inflammation.<sup>[63]</sup> KERP1 has been recognised as a virulence factor, which is expressed on the cell surface. It is a protein rich in glutamic acid and lysine. KERP1 was earlier not assumed to be present in *E. dispar* but Weedall *et al.*, 2020 in their study found the gene to be present in *E. dispar* and *E. moshkovskii*. Infection with *E. histolytica* is generally linked with neutrophil infiltration.<sup>[64]</sup> The presence of trophozoites activates the neutrophil extracellular traps. However, there is no evidence that non-pathogenic *E. dispar* can cause NET formation. While looking into the potential of *E. dispar* to cause NET formation or not, *E. histolytica*-induced NET was significant for capturing and killing amoebas; *E. dispar*, on the other hand, did not cause NET formation, neutrophil degranulation or the formation of reactive oxygen species under any circumstances. These findings demonstrate that only live pathogenic *E. histolytica* stimulates neutrophils to make NET, and that the parasite is recognised by a carbohydrate with an axial HO- group at the fourth carbon.<sup>[65]</sup>

## COVID-19 AND AMOEBIASIS

Although no studies have looked at the part played by viruses in promoting *E. histolytica* infection, SARS-CoV2 infection is identified as a factor that can cause the intestinal homeostasis to be dysregulated. Severe inflammation is the outcome of this invasion/infection as immune cells respond to it. In case of COVID-19-infected patients, intestinal bacterial diversity is known to decline. The microbes opportunistic in nature, like *Streptococcus*, *Rothia*, *Veillonella*, and certain fungi like *Aspergillus* and *Candida* spp. depict an increase in their relative abundance, whereas that of useful symbionts is reduced.<sup>[66,67]</sup> Invading viruses can both govern and regulate the ecosystem of commensal bacteria, facilitating either stimulatory or suppressive actions. Corticosteroids, which are commonly used to treat severe COVID-19, may have a part in this process by altering gut flora and favouring parasite infection. Although the mechanisms by which asymptomatic infection and dysenteric colitis progress into an ALA are unknown, these undiscovered pathways could provide an insight into anti-amoebic and anti-SARS-CoV-2 responses. SARS-CoV2 may influence the cytokine storm. The consequences of this are inflammation along with tissue destruction. Firstly, it compromises the effectiveness of the serum complement system, secondly, the secretion of IFN- $\gamma$  is inhibited, and lastly, the composition of gut microbiome is also altered. These factors may, therefore, contribute to or enhance the development of the extraintestinal form.<sup>[66,67]</sup> COVID-19 can exacerbate parasitic infections, which are already present, but recent findings suggest that it may also serve a preventive effect, mediated via the generation of Interferon- $\gamma$ .<sup>[68]</sup>

## CONCLUSION

The socioeconomic settings in which *E. histolytica* thrive remains a big problem in developing countries. Although awareness regarding hygiene practices has resulted in people paying a certain degree of attention, there is still a long way to go. Vaccines have yet to be developed. Furthermore, resistance to currently used medications has been discovered, underlining the need for novel treatment techniques to be developed, and recent discoveries of novel pathogenic mechanisms have opened the road for it. Understanding the biology of the parasite will need the identification of new molecules and their likely roles in various mechanisms such as invasion, adhesion and infection. These new results may be useful in deciphering the pathophysiology of various other protist infections. *E. dispar*'s pathogenic potential has also been underlined in new research. Therefore, further studies are required for a deeper insight into its pathology. These breakthroughs also establish the groundwork for further research to be done in the future.

## Ethical approval

The Institutional Review Board approval is not required.

## Declaration of patient consent

Patient's consent not required as there are no patients in this study

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## Conflicts of interest

Dr. Rakesh Sehgal is on the Editorial Board of the Journal

## Use of artificial intelligence (AI)-assisted technology for manuscript preparation

The authors confirm that there was no use of artificial intelligence (AI)-assisted technology for assisting in the writing or editing of the manuscript and no images were manipulated using AI.

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