In silico identification and cloning of novel Rab9 homologue from *Entamoeba histolytica*

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In partial fulfilment of the award of the Degree of

MASTER OF SCIENCE

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CERTIFICATE

This is to certify that the thesis entitled "*In silico* identification and cloning of novel Rab9 homologue from *Entamoeba histolytica*" submitted to Institute of Science, Nirma University, in partial fulfilment for the award of the degree of M.Sc. in Biochemistry/ Biotechnology/ Microbiology, is a record of research work carried out by Ms. Kavya Pandya, Ms. Rashmi Malik, Mr. Swarup Kundu, and Ms. Uditi Raval, under the supervision of Dr. Kuldeep Verma. No part of this thesis has been submitted for any other degree or diploma.

Prof. Sarat Dalai
(Director)

Dr. Kuldeep Verma
(Guide)

Certificate of Plagiarism

It is certified that Masters of Science thesis titled "***In silico* identification and cloning of novel Rab 9 homologue from *Entamoeba histolytica*"** submitted by **Ms. Kavya Pandya(18MBC013), Ms. Rashmi Malik (18MBT043), Mr. Swarup Kundu (18MBC008) and Ms. Uditi Raval (18MMB023)** has been examined by us. We undertake the following:

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INTRODUCTION

1. Introduction:

The pathogenic amoeba *Entamoeba histolytica* is a single-celled enteric pathogen that causes the amoebiasis. As per the world health organisation (WHO) around 50,000 deaths are reported each year(Shirley *et al.*, 2018). The infection is reported worldwide, although the prevalence of the disease mainly occurs in countries with poor hygiene and sanitation.

E. histolytica parasite completes the life cycle(Tanyuksel and Petri, 2003) in two-stage, cysts and trophozoites. Once in the human, amoebic cysts (infective stage of the parasite) enters into the body, it can settle down into the intestine without causing any symptoms and gets converted (known as excysts) into the trophozoites (invasive stage of the parasite). The amoebic trophozoites are highly motile(Aguilar-Rojas *et al.*, 2016) and damage the intestinal gut barriers which enables its entry into the bloodstream.

Tissue damage by amoebic trophozoite requires multi-step processes involving the recognition of the host cell's surface components for adherence and then signal transduction events leading to release of proteolytic substances and other events that lyse the target cells(Ralston and Petri, 2011). For the survival, motility and virulence of the trophozoites in the host heavily depends on efficient membrane trafficking of the parasite(Verma *et al.*, 2018).

Rab GTPases are the master regulator for membrane transport in almost all eukaryotic cells(Zhen and Stenmark, 2015). *E. histolytica* genome encodes more than 100 Rab GTPases (Saito-Nakano *et al.*, 2005)which is significantly higher than the other higher eukaryotes. Some of the amoebic Rab GTPases are characterized in specific virulence pathways such as pinocytosis, phagocytosis and tissue invasion(Verma *et al.*, 2018). Previously, it has been reported that the amoebic lysosomes are important for the trophozoite virulence and localize close to the phagocytic cups during human cell ingestion(Proctor and Gregory, 1972). The amoebic lysosomes are different from other organisms based on morphology, positioning(Cabukusta and Neefjes, 2018) and biochemical composition(Tillack *et al.*, 2007). Rab7 is an important regulator for the biogenesis of lysosomes in higher eukaryotic cells. Interestingly, it has been shown that the amoebic parasite genome encodes nine isoforms(Saito-Nakano *et al.*, 2007) of Rab7 (A-I) which is highest among the other eukaryotic organisms(MacKiewicz and Wyroba, 2009) such as *Arabidopsis thaliana* (8 isoforms), *Lotus japonicus (*4 isoforms*)* and two in *Homo sapiens*. The Rab7 and Rab9 sub-families are closely related to each other. Lombardi et al., in 1993 first demonstrated that the Rab9 is also localized on the late endosomes and mediate the

trafficking of mannose 6 phosphate receptor (M6PR) from late endosomes to the trans-Golgi network(Lombardi *et al.*, 1993). Further, it has been shown that the Rab9 function is important for lysosome biogenesis, recycling of M6PR and delivery of lysosomal hydrolyses (Riederer *et al.*, 1994).

It is well established that Rab7 (Bucci *et al.*, 2000)and Rab9(Riederer *et al.*, 1994) subfamilies are important for biogenesis of lysosomes and transport of acid hydrolyses into the lysosomal lumen in almostall eukaryotic cells. Interestingly, Rab9 homologue has not beenannotated in Entamoeba genome(Saito-Nakano *et al.*, 2005). There are two isoforms of amoebic Rab7 characterized yet(Saito-Nkano *et al.*, 2007). Amoebic EhRab7A and EhRab7B both are localised into acidic compartments. It has been observed that EhRab7A is targeted to pre-phagosomal vacuoles (PPVs)(Saito-Nakano *et al.*, 2004). The PPVs are transient compartments which are observed upon the ingestion of human erythrocytes. Interestingly, EhRab5 and EhRab7A both are localised on PPV. Further, EhRab7A interaction with Vps26 (a component of the retromer complex) has been elucidated and shown that this interaction is important for the transport of acid hydrolyses in the lysosomal lumen(Nakada-Tsukui *et al.*, 2005). Recently, EhRab7A is identified as an early Rab and important for internalisation of transferrin and low-density lipoprotein (LDL) through giant endocytic vacuoles (GEVs)(Verma *et al.*, 2015). GEVs are steady-state compartment and have morphological similarities with PPVs. Although, GEVs (contain transferrin and LDL) are distinct from phagosomes(Verma *et al.*, 2016). Another isoform EhRab7B is also localised with acidic compartment and important for maturation of transferrin(Verma *et al.*, 2015). Some of the amoebic Rab GTPases are also studied in lysosomal function. EhRab35 is a prime regulator for lysosomal biogenesis(Verma and Datta, 2017). Recently, it has been observed that EhRab21QL mutant increasing number of the lysotracker compartment and suggested to play a role in golgi to lysosome transport(Constantino-Jonapa *et al.*, 2020). The function of amoebic lysosome has been implicated in the virulence of the trophozoite. Although, it is not clear how lysosomal resident proteins/enzymes are transported to amoebic lysosomes. Therefore, we are interested to identify the Rab9 like homologue from *E.histoltyica* genome and cloning of putative amoebic Rab9 into expression vector for investigating the functional role in lysosome mediated virulence of *E. histolytica*.

REVIEW AND LITERATURE

2. Review and Literature

2.1 Rab GTPases:

Rab GTPases are molecular switches, which are controlled by cycling between dormant guanosine diphosphate (GDP) - bound form and operative guanosine triphosphate (GTP) – bound state. GTPases are enacted by administrative proteins such as guanine nucleotide exchange factors (GEFs) which advances GDP separation from GTPases to permit GTP attachment and are deactivated by GTPase – activating proteins (GAPs) to hydrolyse GTP by eliminating gamma phosphate(Cherfils and Zeghouf, 2013; Bos *et al*.,2007) (Figure 1).

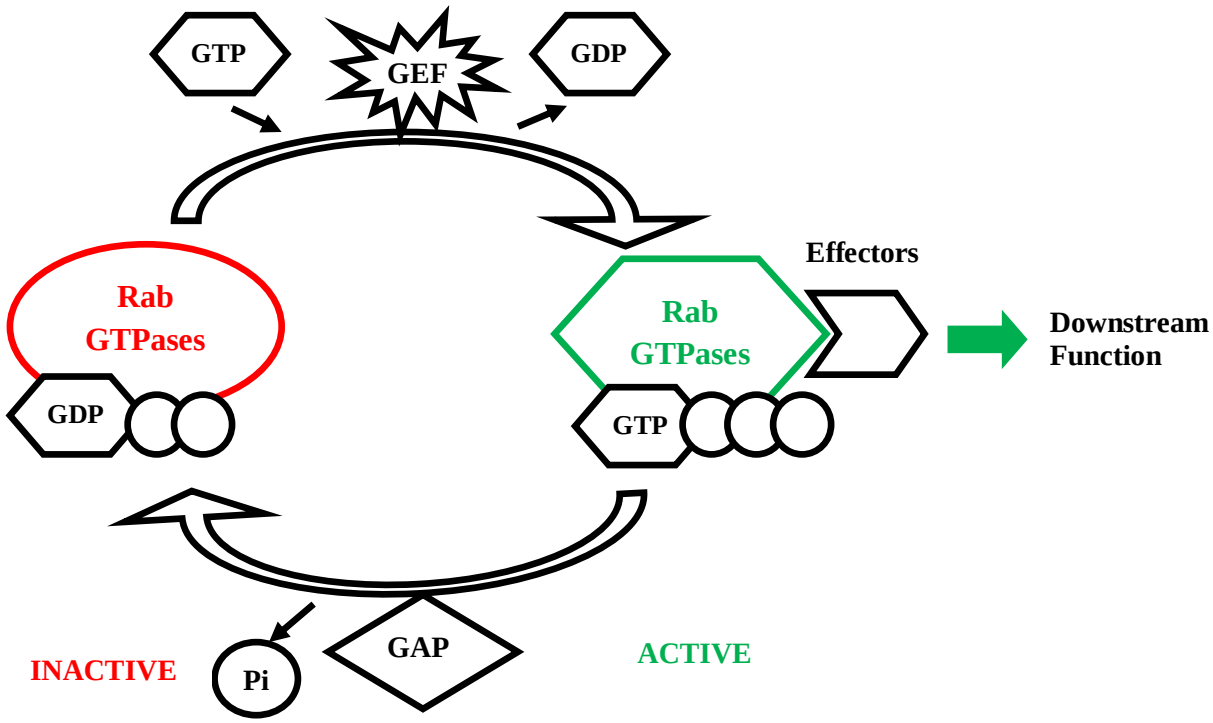

Figure 1RabGTPase cycles between the GDP and GTP bound forms, which possess two dissimilar conformations.

Amino acid arrangement comparisons of these small GTPases from different species demonstrate that they resemble 30–55% (Valencia *et al*., 1991), comprising of a six-stranded β-sheet and five α-helices and has preserved sequence domains for binding of guanine nucleotide (Figure 2).G1 motif(P-loop) consists of GxxxxGKS/T, where x is any amino acid. It binds to β-phosphate of nucleotides andMg^{2+} ion (Walker *et al*., 1982). The G2 motif (Switch-I) has xTx, where x is any amino acid, shows contiguity with the Mg^{2+} ion and GTP's γ-phosphate. G3 motif

(Switch-II) has DxxGq/h and is involved in GTP hydrolysis. Thus, action of Switch-I and Switch-II results in conformational changes and permeates small GTPases to associate with downstream effectors. The G4 motif and G5 motif (G binding) have a conserved sequence N/TKxD and sAK respectively, that makes specific contacts with the guanine base (Rensland *et al*., 1995).

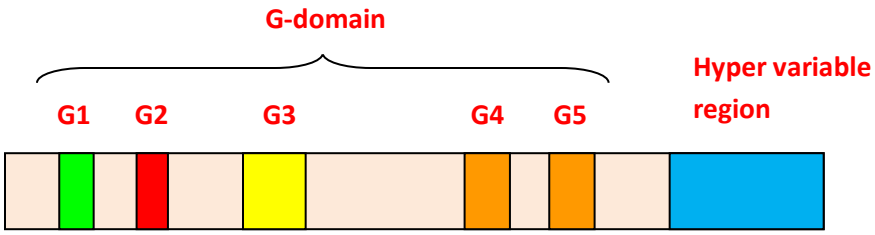

Figure 2. The architecture of GTPases showing G-domains and hypervariable region. Conserved sequence motif is; P-loop, switch I, switch II, G-binding motifare shown as boxes colored green, red, yellow and orange respectively. The hyper variable region, including a polybasic region and a CAAX motif, is highlighted with blue box.

Rab proteins consist of a peculiar GTPase domains which includes two 'switch regions' which binds to γ phosphate of GTP and manifests extensive conformational change in its active and inactive form (Stroupe and Brunger, 2000). High affinity binding of nucleotide and its hydrolysis requires presence of Mg^{2+}, which acts as a cofactor. Guanine nucleotide interaction mainly involves few specific residues present in the G motifs which are highly conserved in GTPases. The P-loop consists of a motif which includes GxxxxGK(S/T), ensures contact of phosphate. Threonine is an important residue of switch-I domain which binds to the γ phosphate as well as is coordinated with Mg^{2+} ion in the functional form. While another motif DxxGQ in switch-II includes Aspartate, which interacts with water molecules and stabilizesMg^{2+} ion, a glycine residue that contacts the γ phosphate and a catalytic residue Glutamine which is responsible for activation of intrinsic GTPase activity (Gabe Lee*et al*., 2009).Fundamental feature which makes eukaryotic cells different from prokaryotes is having membrane trafficking between the organelles by vesiculartubular carriers. Post translational modification of Rab enables its binding to the membrane by geranylgeranyl groups, these modifications are mostly observed at one or two carboxy-terminal Cysteine residues (Stenmark, 2009).Rabs have a central role in ensuring that the cargo is delivered to the correct target and these includes various key events such as

vesicle budding, removal of coats, fusion and motility mediated by the enlistment of various effector proteins, such as sorting connectors, tethering factors, phosphatases, kinases, and motor proteins and exchange of signals between different Rabs ensures regulation of trafficking (Stenmark, 2009).

Study carried out in 1990 using MDCK Rab homologs showed that Rabs show a peculiar intracellular localization pattern among their members. It was observed that Rab2 has association with a structure which has features of an intermediary compartment between the ER and Golgi. Whereas Rab5 is positioned on plasma membrane and early endosomes, while late endosomes show localization of Rab7 (Chavrier *et al*., 1990).

As different Rabs have specific localization similarly they are involved in specific function such as Rab5 is involved in cargo sequestration as it participates in construction of clathrin coat at plasma membrane and forendocytosis of transferrin receptors via clathrin, Rab5 along with Rab34 is involved in macropinocytosis while with Rab22 and rab14 is involved in development of early phagosomes. Maturation as well as combination of late endosomes and phagosomes with lysosome is mediated by Rab7. Trafficking from late endosome to Trans Golgi Network is regulated by Rab9 for example it is involved in receptor recycling. Recoveryof mannose-6-phosphate receptors (M6PRs) from late endosomes to the trans-Golgi network (TGN) is mediated by Rab9.Other than Rab9 two more Rabs are associated with recycling endosomes namely Rab11 and Rab35(Stenmark, 2009).

2.2 Rab7 GTPasein*E. histolytica*:

The genome of *E.histolytica*codes for exceptionally high number of Rabs i.e. 91 Rab which is highest among all the organisms till date, and this gives us an instinct that it might be involved in the survival of this parasite in strainous conditions as well as in virulence. The characteristic of this parasite is similar to that of a professional phagocytic cell and engulfs the host cell via phagocytosis or trogocytosis. This suggests that for phagocytic behavior of the cell it requires constant supply of virulence factors such as MMPs, Cysteine proteases, Aspartate proteases to the membrane and transport of cargo to amoebic lysosomes for degradation (Verma *et al*., 2018).

Rab GTPases grasps more attention in understanding Entamoeba histolytica biology. Lysosomes harbors various degradative enzymes which are involved in degradation of cargo, and Rab7 is observed to be the key player in lysosome biogenesis as reported in 2001(Bucci *et al.*, 2000). Most of the eukaryotes codes for only one Rab7 while a shift in paradigm is observed in case of this parasite which codes for 9 isotypes of Rab7 which is again highest among all the organisms starting from *Saccharomyces cerevisiae* (codes for only 1 Rab7), *Arabidopsis thaliana* (4 isotypes) human (only 1 Rab7). To study the significance of these many numbers of isotypes, a study was carried out in 2007 for two isotypes namely EhRab7A and EhRab7B. The localization study revealed localization of EhRab7B on acidic vacuoles which contains lysosomal proteins such as cysteine proteases and amoebapore-A. While EhRab7A colocalizes partially with lysosomal proteins. Now to check the effect of these two proteins expression study was carried out which showed that over-expression of EhRab7A and EhRab7B boosts lysosome/late endosome acidification while expression of mutant of EhRab7B (i.e. EhRab7B-GTP) resulted into decreased late endosome and lysosome acidification, mis-sorting of lysosomal proteins and disturbed formation of pre-phagosomal vacuoles which is similar to dominant negative phenotype. While EhRab7A mutant showed no effect on intracellular or cysteine protease activity(Nakano *et al*., 2007).

The capability of *E. histolytica* to outlive inside the liver and during liver abscess formation is fulfilled by a strong adaptive reaction which needs the specific control of a number of amoeba proteins. Utilizing differential display polymerase chain reaction (DD-PCR), they compared RNA expression between *E. histolytica* developed beneath normal culture conditions and trophozoites separated from liver abscesses of contaminated gerbils. In total 12 genes were recognized among which seven were observed to be overexpressed and five were downregulated during abscess formation. To further identify whether these genes are specific for abscess formation or they are the change in expression is due to heat stress in abscess derived tissue revealed that genes encoding ribosomal proteins (S30, L37A), GTP-binding protein Rab7D,ferredoxin 2, and cyclophilin are upregulated. Which means Rab7D upregulation is observed during liver abscess which suggests its role in pathogenesis (Bruchhaus *et al*., 2002).

To understand role of Rab GTPases in phagocytosis a study was carried out in which different phases of phagocytosis of type-1 *E. coli* were examined using laser scanning confocal

microscopy. The results of this experiment signify the presence of EhRab7A on phagosomes as well as its involvement in pre and post phases of phagocytosis of type-1 *E. coli* (Verma *et al.*, 2016).

Cytolysis of tissues involves cysteine proteases. A report shows that cysteine protease transport in this parasite is directed through particular interaction of EhRab7A with a retromer like complex (Nozaki *et al.*,2005).

For survival of this parasite in human system, it has acquired various ways to sequester various nutrients; one of the most important is iron. To look over the function of Rab GTPases in intracellular transport of human holo-transferrin a structured study was carried out which revealed that EhRab7A and EhRab5 are essential for development of Giant endocytic vacuoles plus regulation of intracellular trafficking of transferring during early phase. While EhRab7B is linked to degradation of transferrin cargo in amoebic lysosomal compartments which shows its involvement in late phase of endocytosis. These results show contrast with mammalian system where early phases are regulated by Rab5 while late phases show involvement of Rab7 (Verma *et al*., 2015).

2.3 Rab9:

Most organelles which are involved in endocytic and secretory pathway harbors distinct Rabs on the surface which indirectly gives a hint that Rabs ensure the high efficiency of vesicular targeting events. In 1990, Rab9 was first recognized in a screening for SEC4 and YPT1 protein related cDNA clones, full length protein having an identity of 54% to Rab7 and 39% to YPT1 protein (Chavrier *et al*., 1990).Rab9 is interesting because it harbors seven locales that are essentially distinctive in conformation from other Rab proteins(Chen *et al*., 2004).Localization study of Rab9 revealed its presence on late endosome. To further understand the role of Rab9 in MPR transport from late endosome to TGN, anti-Rab9 antibodies were used which showed that blocking of Rab9 inhibits the transfer from late endosomes to TGN. This suggests that Rab9 is required in late endosome to TGN transport. Later to check the impact of Rab9 expression on transport, Rab9 was overexpressed in BHK cells which showed that overexpression of Rab9 triggers the overall transport reaction by about 2.5 times. While invitro prenylation is also a prerequisite for activation of Rab9, prenylated Rab9 is stimulates the transport of MPR from late

endosome to TGN but truncated Rab9 (which lacks prenylation on C-terminus) failed to stimulate the transport (Lombardi *et al*., 1993).

Lysosomal enzymes which are newly synthesized are transported to prelysosomes via Mannose-6-phosphate receptors (MPRs) in TGN and later MPRs are recycled. To analyze role of Rab9 in recycling of MPRs dominant negative mutant i.e. Rab9 S21N and it was observed that same Rab9 mutant expressing cells (CHO cells) shows inhibition in MPR recycling. This suggests importance of Rab9 in recycling of MPRs in vitro. While it also showed that cells expressing Rab9 S21N has decreased efficiency of lysosomal enzyme sorting. Other than this it is also reported that Rab9 S21N enables compensatory lysosome biogenesis (Riederer *et al*., 1994).

As it is already known that MPRs are involved in transport of lysosomal enzymes to endosomes and then return back to Golgi. Studies were carried out to know the effectors of Rab9 which revealed two important effectors i.e. (1) TIP47(tail-interacting protein of 47 kDa) and (2) p40 (a 40-kDa protein). TIP47 binds to MPR and is involved in recycling of MPR to Golgi both in vivo and invitro. This TIP47 is directly associated with active Rab9 and it has been observed that Rab9 increments the liking of TIP47 for its cargo. This showed another role of Rab9 in selection of cargo (Carroll *et al*., 2001). While Rab9 too encourages vesicular transport by matching with its related Rabeffector p40. This 40-kD protein (p40) that binds Rab9–GTP is very potent transport factor which significantly stimulates mannose-6-phosphate receptor transport (Diaz *et al*., 1997).

Other than lysosomes, Rab9 is also shown to be associated with melanosome which is a type of lysosome-related organelle. To understand its role further study was carried out using mice melanocytes which revealed that which has shown that Rab9A is related with melanosomes and knockdown of this protein resulted into decreased pigmentation of melanosome due to inaccurate transport of melanosomal proteins to lysosomes (Mahanty *et al*., 2016).In mammalian cells, Rab9 interacts with TIP47, a vesicle cargo selection protein, that binds to the cytoplasmic tail of the HIV-1 envelope glycoprotein subunit gp41. So, Rab9 is distinguished to be a key cellular component for replication. Thus, inhibition of Rab9 function can be useful in controlling HIV-1, Ebola, Marburg, and measles. This concept and mechanism of Rab9 can to discover a novel antiviral drug (Chen *et al*., 2004).

OBJECTIVES

3. OBJECTIVES:

1. *In silico* identification of Rab9 in *Entamoeba histolytica* genome.
2. Cloning of putative Rab9 from *Entamoeba histolytica* genome in amoebic expression vector.

MATERIALS AND METHODS

4.1 Materials

1. Amplification of gene using Polymerase chain Reaction (PCR):

2X Phusion Master Mix

Forward Primer

Reverse Primer

Template (cDNA)

MQ water

2. Agarose Gel Electrophoresis:

1X TBE (Tris base, Boric acid, 0.5M EDTA, double distilled water)

1% Agarose gel (0.7g of Agarose, 70 ml of 1X TBE buffer)

Nucleic-acid stain, ETBR

DNA sample

6X gel loading dye

1 Kbp DNA Ladder

3. Restriction Digestion:

10X Cut smart buffer

SmaI [Catalogue #R0141S]

XhoI [Catalogue #R0146S]

DNA sample

MQ water

4. Cleanup:

QIAGEN Gel extraction kit(Cat #28704)

MQ water

5. Plasmid Isolation:

QIAGEN mini prep kit

MQ water

6. Digestion of pEhTetEx-HA:

10x Cut smart buffer

SmaI [Catalogue #R0141S]

XhoI [Catalogue #R0146S]

pEhTetEx-HA (vector)

7.Dephosphorylation:

Digested pEhTetEx-HA

10X Cut smart buffer

CIP (Alkaline Phosphatase)

MQ water

8. Ligation of Rab9 into pEhTetEx-HA vector:

T4 DNA ligase buffer (10X)

Vector (pEhTetEx-HA)

Insert (Digested)

T4 DNA ligase

MQ water

9. Preparation of competent cells using $CaCl_2$ method:

E.coli DH5α

LB agar plates (LB broth, Agar-Agar, Double-distilled water)

LB broth

$MgCl_2$-$CaCl_2$ solution (80mM $MgCl_2.6H_20$ + 20mM $CaCl_2$)

0.1M $CaCl_2$ solution

10. Transformation of ligated product:

Competent cells

Ligated product (Plasmid)

Ice

SOC/LB broth

LB Agar plates (LB broth, Agar-Agar, double-distilled water)

Ampicillin

11. Plasmid Extraction from Transformed cells:

LB broth

Ampicillin

QIAGEN mini prep kit

MQ water

12. Restriction Digestionof Recombinant Plasmid:

10X CutSmart Buffer

Sample (Recombinant Plasmid)

SmaI [Catalogue #R0141S]

XhoI [Catalogue #R0146S]

MQ water

4.2Methods

4.2.1 *In silico* identification of Rab7 isoforms in *E. histolytica*:

1. Rab7 has nine isoforms from Rab7A-Rab7I.
2. Analysis was performed to gain the information related to the Rab7 isoforms in *E. histolytica.*
3. Sequence information was taken from Amoeba database called as **AmoebaDB.**(https://amoebadb.org/amoeba/).
4. BLAST was performed for all Rab7 isoforms using Rab database and Rabifier.
5. This database was used to predict the Rab7 isoforms present in *Entamoeba histolytica* are true Rab or not.

4.2.2 Primer Designing:

1. Before primers were designed, gene of interest was checked for zero cutter using NEBcutter(http://nc2.neb.com/NEBcutter2/)
2. End to end primers for Rab9 were designed from cDNA sequence of *Entamoeba histolytica.*
3. Primers were designed including forward primer and reverse primer for isoform using AmoebaDB database (https://amoebadb.org/amoeba/).
4. The designed primers were evaluated using Oligoevaluator(http://www.oligoevaluator.com/LoginServlet)
   For its properties such as:
   - Tm (Melting Temperature)
   - Dimerization
   - Secondary structure formation
   - % GC content etc.

Restriction sites for *SmaI* (CCCGGG) and *XhoI* (CTCGAG) were added to the primers.

PROTEIN	GENE ID	SEQUENCE	LENGTH	Tm (°C)	GC %	PRIMER DIMER	SECONDARY STRUCTURE
FORWARD PRIMER							
FW_RAB9_ SmaI	EHI_082070	5'-CG***CCCGGG***ATGGCAGGAAGACCAGCA-3'	26	84.9	69.2	NO	MODERATE
REVERSE PRIMER							
RV_RAB9_ XhoI	EHI_082070	5'-GC***CTCGAG***TTAGCAACACCCTCCTTCTT-3'	28	73.7	53.6	NO	WEAK

Table 1. Primer designingof putative Rab9 GTPases in *E. histolytica* genome.

4.2.3 Amplification of gene using Polymerase chain Reaction (PCR):

Amplification of Rab9 was done by using Phusion High-Fidelity PCR Master Mix with HF Buffer (M0531S)

COMPONENT	REACTION VOLUME (µl)
2X Phusion Master Mix	25
10µM Forward Primer	2.5
10µM Reverse Primer	2.5
Template (cDNA)	1
MQ water	19
TOTAL VOLUME	50

Table 2. PCR reaction setup for Rab9

PCR tubes were then transferred from ice to PCR machine.

STEP	TEMPERATURE	TIME
Initial denaturation	98°C	30 seconds
35 cycles	98°C 55°C 72°C	10 seconds 30 seconds 30 seconds
Final Extension	72°C	10 minutes
Hold	4°C	-

Table 3. PCR Program used for Rab9

The amplification of PCR product was checked using Agarose Gel Electrophoresis.

4.2.4 Agarose Gel Electrophoresis:

1. Preparation of 1X TBE (gel electrophoresis buffer): To prepare 500 ml of 1X TBE buffer, 100 ml of 5X TBE Buffer was added to 400 ml of sterile double distilled water.
2. The ends of gel tray were sealed using adhesive tape and comb was placed in the space provided on the gel tray.
3. Preparation of 1% Agarose gel: 0.7g of Agarose was added to 70 ml of 1X TBE buffer and then the mixture was heated until the Agarose dissolves completely in buffer.
4. Then the Agarose gel was allowed to cool down slightly and 10µl of nucleic acid stain was added in Agarose gel.
5. The gel was then poured into the gel tray and allowed to solidify for about 30 minutes at room temperature; after the gel was solidified the comb was removed gently.
6. The adhesive tape from both the ends of the gel tray was removed and the tray was placed in electrophoresis chamber, and the chamber was filled with 1X TBE electrophoresis buffer (just until wells are submerged)

7. Samples for electrophoresis were prepared by adding 1 µl of 6X gel loading dye for every 5µl sample and loaded into the well then 2.5µl of 1 Kbp DNA Ladder was loaded into another well for comparison.
8. Then the electrophoresis apparatus was connected to electrophoresis unit and electrophoresis was carried out.
9. The gel was allowed to run until the dye has been migrated an appropriate distance through the gel and then observed using UV Transilluminator.

4.2.5 Restriction Digestion of PCR product:

After quantification the PCR product was subjected to restriction digestion.

SmaI [Catalogue #R0141S]

XhoI [Catalogue #R0146S]

A 25µl reaction was setup as follows:

COMPONENTS	**REACTION VOLUME (µl)**
10X Cut smart buffer	2.5
PCR product	19
SmaI	1.0
XhoI	1.0
MQ water	1.5
TOTAL VOLUME	25

Table 4. Components for Restriction Digestion of Rab9

After adding *SmaI* the reaction mixture was incubated at 25°C for 30 minutes and then *XhoI* was added in the reaction mixture and incubated at 37°C for 3 hours.

4.2.6 Cleanup using QIAGEN GEL EXTRACTION KIT:

Cleanup was done for the removal of restriction enzymes after restriction digestion through **GEL EXTRACTION KIT.** (Cat #28704)

The protocol for cleanup is as follows:

1. Equal proportions of isopropanol and sample were mixed and the mixture was then transferred in a QIAquick spin column (now onward spin column) with 2ml collection tube and centrifugation was done for 1 minute.
2. Then the flow through was discarded and the column was placed back into the collection tube.
3. 500µl of QG buffer was added in spin column and centrifuged for 1 minute, and then the flow through was discarded,
4. Dry spin was performed for 1 minute to remove residual wash buffer.
5. The spin column was placed into clean and sterile 1.5ml microfuge tube.

Then the elution of sample was done using MQ water and its concentration was measured using nanodrop spectrophotometer.

4.2.7 Plasmid Isolation of pEhTetEx-HA:

1. 1µl of pEhTetEx-HA plasmid (approximately 100 ng/µl) was taken from master stock and transformation was carried out using standard protocol.
2. The transformed colonies were inoculated in 10ml LB broth containing Ampicillin for 12-16 hours at 37°C in shaker incubator.
3. The bacterial culture was centrifuged at 4,000 rpm for 10 minutes and the obtained pellet was resuspended in 250µl Buffer P1 and the mixture was transferred to a microfuge tube.

4. 250µl Buffer P2 was added and mixed thoroughly by inverting the tube for 4-6 times until the solution becomes blue (the lysis reaction is not allowed to proceed for more than 5 minutes).
5. 350µl Buffer N3 was added and mixed thoroughly by inverting the tube for 4-6 times until the solution becomes colourless.
6. Centrifugation was done for 10 minutes at 12,000 rpm in microcentrifuge.
7. Approximately 800µl of supernatant was pipetted onto a spin column and centrifuged for 1 minute and the flow-through was discarded.
8. The column was washed by adding 500µl Buffer PB and centrifuged for 1 minute then the flow-through was discarded.
9. Again, the column was washed by adding 750µl Buffer PE and centrifuged for 1 minute then the flow-through was discarded.
10. The dry spin was performed to remove the residual wash buffer.
11. The column was placed in a clean 1.5 ml microfuge tube and the plasmid was eluted by adding MQ water to the column and after one-minute centrifugation was done for 60 seconds. And the concentration of isolated pEhTetEx-HA was approx. 300ng/µl.

4.2.8 Digestion of pEhTetEx-HA:

The isolated plasmid was digested using *SmaI* and *XhoI*

COMPONENTS	**REACTION VOLUME (µl)**
10x Cut smart buffer	4.0
SmaI	1.0
XhoI	1.0
pEhTetEx-HA (vector)	34(~300ng/µl)
TOTAL VOLUME	40

Table 5. Components of Restriction Digestion for Plasmid

Firstly, *SmaI* was added and the microfuge tube was incubated for 30 minutes at 25°C and then *XhoI* was added and incubated for 2 hrs. at 37°C.Cleanup forremoval of Restriction enzyme was done using **QIAGEN GEL EXTRACTION KIT.**

4.2.9 Dephosphorylation of pEhTetEx-HA:

After restriction digestion pEhTetEx-HA was treated with *CIP (Alkaline Phosphatase)*. Catalogue#M0290S

COMPONENTS	**REACTION VOLUME (µl)**
Digested pEhTetEx-HA	14
10X Cut smart buffer	2
CIP (Alkaline Phosphatase)	1
MQ water	3
TOTAL VOLUME	20

Table 6. Components for Dephosphorylation of vectorpEhTetEx-HA

Microfuge tube was incubated for 1 hr at 37°C.After dephosphorylation pEhTetEx-HA was stored at -20°C.

4.2.10 Ligation of Rab9 into pEhTetEx-HA vector:

Setup for ligation of the insert with vector was done according to the following reaction setup:

COMPONENTS	REACTION VOLUME (µl)
T4 DNA ligase buffer (10X)	2
Vector(pEhTetEx-HA)	1 (50 ng)
Insert	1 (50 ng)
T4 DNA ligase	1
MQ water	15
TOTAL	20

Table 7. Components for process of Ligation

Reaction was incubated overnight at 16°C.

4.2.11 Preparation of competent cells using $CaCl_2$ method:

1. The frozen glycerol stock of *E.coli* DH5α was streaked onto LB agar plate (without Ampicillin) and incubated overnight at 37˚C.
2. For preparation of starter culture for cells, single colony of DH5α was selected from LB agar plate and inoculated in10ml LB broth (without Ampicillin) and the culture was incubated overnight at 37˚C.
3. The OD_{600} of incubated starter culture was checked (approx. 0.9) and then the 10ml activated culture was added in 100ml fresh LB broth and then incubated at 37˚C till the OD_{600} of the inoculated culture reaches 0.3-0.4.
4. When the OD_{600} reached 0.3-0.4, immediately the culture was transferred in 50ml pre-chilled polypropylene tubes and then incubated in ice for 10 minutes.
5. Then the tubes were centrifuged at 2,700g (4,100rpm) for 10 minutes at 4˚C.
6. The supernatant was decanted and the pellet was resuspended in 30ml of ice cold $MgCl_2$-$CaCl_2$ solution. (80mM $MgCl_2.6H_20$ + 20mM $CaCl_2$)
7. Cells were harvested by centrifugation at 2,700g (4,100rpm) for 10 minutes.
8. The supernatant was decanted and pellet was resuspended by swirling or vortexing in 2ml of ice cold 0.1M $CaCl_2$ solution (working solution).

9. Aliquot 50µl into sterile 1.5 ml microfuge tubes and snap freeze them by using liquid nitrogen.
10. The frozen cells were then stored at -80˚C freezer.

4.2.12 Transformation of ligated product:

1. Competent cells (50µl) were taken out of -80°C freezer and were thawed on ice for approx. 20 minutes.
2. 1µl of plasmid or 5µl of ligated product was added to the 1.5 ml microfuge tube containing competent cells.
3. Tubes were mixed by flicking to ensure proper mixing of cells and plasmid. Do not vortex.
4. The mixture was placed on ice for 30 minutes.
5. Heat shock was given by placing the tubes in a pre-heated water bath (42°C) for 45 seconds.
6. The tubes were placed immediately on ice for 4 minutes.
7. 200µl of SOC/LB broth was added in competent cells for cells to recover.
8. The tubes were placed horizontally to facilitate proper aeration in shaker incubator at 37°C for 45 minutes.
9. The whole transformed cell suspension was spreaded on LB agar plates containing Ampicillin and incubated at 37°C for 12-16 hours.

4.2.13 Plasmid Extraction from Transformed cells:

1. After transformation, the transformed colonies were transferred in LB broth containing Ampicillin and incubated for 12-16 hrs, at 37°C in shaker incubator.
2. The bacterial culture was centrifuged at 4,000 rpm for 10 minutes and the pellet obtained was resuspended in 250µl Buffer P1 and the mixture was transferred to a microfuge tube.
3. 250µl Buffer P2 was added and it was mixed thoroughly by inverting the tube for 4-6 times until the solution becomes blue (the lysis reaction was not allowed to proceed for more than 5 minutes).

4. 350µl Buffer N3 was added and mixed thoroughly by inverting the tube for 4-6 times until the solution becomes colourless.
5. Centrifugation was done for 10 minutes at 12,000 rpm in microcentrifuge.
6. Approximately 800µl of supernatant was pipetted onto a spin column and centrifuged for 1 minute and the flow-through was discarded.
7. The column was washed by adding 500µl Buffer PB and centrifuged for 1 minute then the flow-through was discarded.
8. Again, the column was washed by adding 750µl Buffer PE and centrifuged for 1 minute then the flow-through was discarded.
9. Dry spin was performed to remove the residual wash buffer.
10. The column was placed in a clean 1.5 ml microfuge tube and the plasmid was eluted by adding MQ water to the column and was allowed to stand for one minute. Later, centrifugation was done for 60 seconds.

4.2.14 Restriction Digestionof Recombinant Plasmid:

Restriction digestion was performed again according to the steps mentioned earlier.

COMPONENT	**REACTION VOLUME (µl)**
10X CutSmart Buffer	1
Sample	2.0
SmaI	0.5
XhoI	0.5
MQ water	6.0
TOTAL VOLUME	10

Table 8. Components of Restriction Digestion for Recombinant Plasmid

After adding *SmaI* the mixture was incubated for 30 minutes at 25˚C and then *XhoI* was added in the mixture and incubated at 37˚C for 2 hours.

4.2.15 Sequencing:

The samples were sent for sequencing including primers and plasmid (pEhTetEx-Rab9).

RESULTS

5. Results

5.1 *In silico* identification of Rab7 isoforms in *E. histolytica*:

Entamoeba histolytica genome encodes for about 100Rab family G-proteins, suggesting its importance in the organism. Previous reports suggest that amoebic Rab GTPases are playing a major role in the phagocytosis and tissue invasion (reviewed Verma *et al*., 2018) Previously, it has been shown that the amoebic parasite encodes nine isoforms of Rab7 (Saito Nakano *et al*, 2007). Rab7 being an important GTPase in showing pathogenic properties, but only a few functions of its isotypes are known, EhRab7A and EhRab7B are involved in lysosomal biogenesis and endosomal maturation(Saito Nakano *et al*., 2007; Verma *et al*., 2015). Analysis was performed to gain the information related to the Rab7 isoforms in *E. histolytica.* The amino acid sequence of nine isoforms was taken from amoeba database (https://amoebadb.org/amoeba/).BLAST was performed for all Rab7 isoforms using Rab database(http://bioinformatics.mpibpc.mpg.de/rab/) and Rabifier(http://www.rabdb.org/) the Rab database is a collection of Rab protein sequences classified by HMM profiles. Rabifier is a bioinformatics tool for identification and classification of Rab GTPases in any set of protein sequences independent of its taxonomical information. The database first decides whether a protein sequence belongs to the Rab family or not and in then classifies the predicted Rab sequence into a Rab subfamily. Our first analysis using the Rab database showed that only four Rab7 isoforms really/truly belongs toRab7 subfamily (Table 9) among nine. Interestingly, we found that one of the Rab7 isoform (EH_082970), earlier classified as Rab7D belongs to Rab9 subfamily. Further, we used another algorithm, Rabifier and observed that five isoforms of Rab7 belongs to Rab7 subfamily (4 isoforms with high probability and one isoform with low probability)(Table 10).

RAB DATABASE DETAILS		
RAB ISOFORMS	**GENE ID**	**MOTIF PRESENT**
RAB7A	EHI_192810	RAB7
RAB7B	EHI_081330	RAB7
RAB7C	EHI_189990	RAB7
RAB7D	EHI_082070	RAB9
RAB7E	EHI_169280	RAB7
RAB7F	EHI_192130	-
RAB7G	EHI_187090	-
RAB7H	EHI_005900	-
RAB7I	EHI_189100	-

Table9.Identification of putative Rab9 GTPases in *E. histolytica* genome using Rab database.

RABIFIER DETAILS			
PUTATIVE RABS	**ACCESSION NUMBER**	**TRUE RAB/ NOT**	**RABDBANALYSIS (PROBABILITY)**
RAB7A	EHI_192810	YES	RAB7(0.98)
RAB7B	EHI_081330	YES	RAB7(0.48)
RAB7C	EHI_189990	YES	RAB7(0.97)
RAB7D	EHI_082070	YES	RAB9(0.25) RAB7(0.19)
RAB7E	EHI_169280	YES	RAB7(0.98)
RAB7F	EHI_192130	YES	RAB7(0.25)
RAB7G	EHI_187090	NO	-
RAB7H	EHI_005900	YES	RABX (0.50)
RAB7I	EHI_189100	YES	RABX (0.50)

Table 10. Result for *in silico* identification of putative Rab9 GTPases in *E. histolytica* genome using Rabifier.

5.2 PCR amplification and cloning of putative amoebic Rab9 in to pEhTetEx-HA plasmid:

For understanding the role of Rab9 in *E. histolytica,* we had aimed to cloned the Rab9 gene into pEhTetEx-HA plasmid. By using appropriate forward (5'-CGCCCGGGATGGCAGGAAGACCAGCA-3') and reverse (5'-GCCTCGAGTTAGCAACACCCTCCTTCTT-3') primers. Rab9 was amplified by PCR using cDNA pool of *E. histolytica*. PCR was performed for 35 cycles (initial denaturation at 98°C for 30s, annealing at 55°C for 30s, and extension at 72°C for 30s) using Phusion High-Fidelity PCR Master Mix with HF Buffer (M0531S). Then PCR amplified product was loaded on agarose gel to check amplification. Our PCR result shows approximately 615-bp of amplified product of Rab 9(Figure 3). The amplified PCR fragment was digested with *SmaI* and *XhoI* and was then ligated in pEhTetEx-HA vector using the enzyme T4 DNA ligase. The ligation mixture was transformed into DH5-α *E. coli*. The multiple colonies were observed in Ampicillin containing Luria bertani agar plates. Further, the obtained recombinant *E.coli* colonies were picked up and inoculated with PCR master mix for colony PCR using PCR Master Mix including *Entamoeba* Rab9 specific primers in independent PCR tubes. Our colony PCR results suggest that the clones were positive for amplification (Figure 3). These clones were further subjected to restriction digestion analysis. The plasmid DNA was isolated from the clones and digested with *SmaI* and *XhoI*. Upon digestion the clones released insert at approximate 615-bp (Figure5). These results suggest that the amplified PCR product from *E. histolytica* cDNA was successfully cloned into amoebic expression vector.

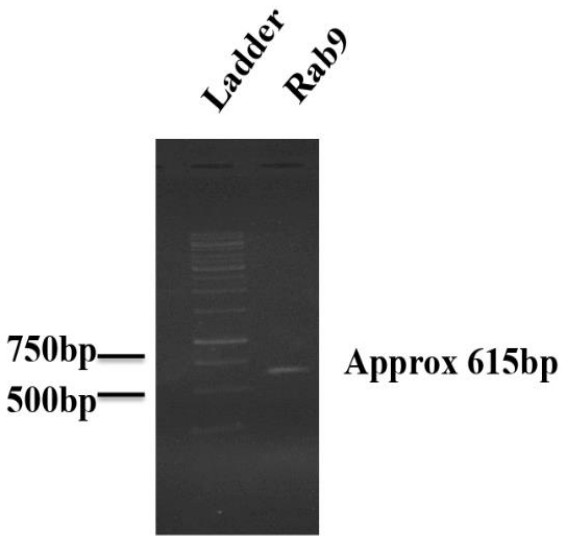

Figure 3. PCR amplification of Rab9 GTPases from *E. histolytica*. Around 615-bp DNA fragment had been amplified from *E. histolytica* cDNA pool by PCR using suitable forward and reverse primers. Lane 1 (1Kbp DNA ladder).

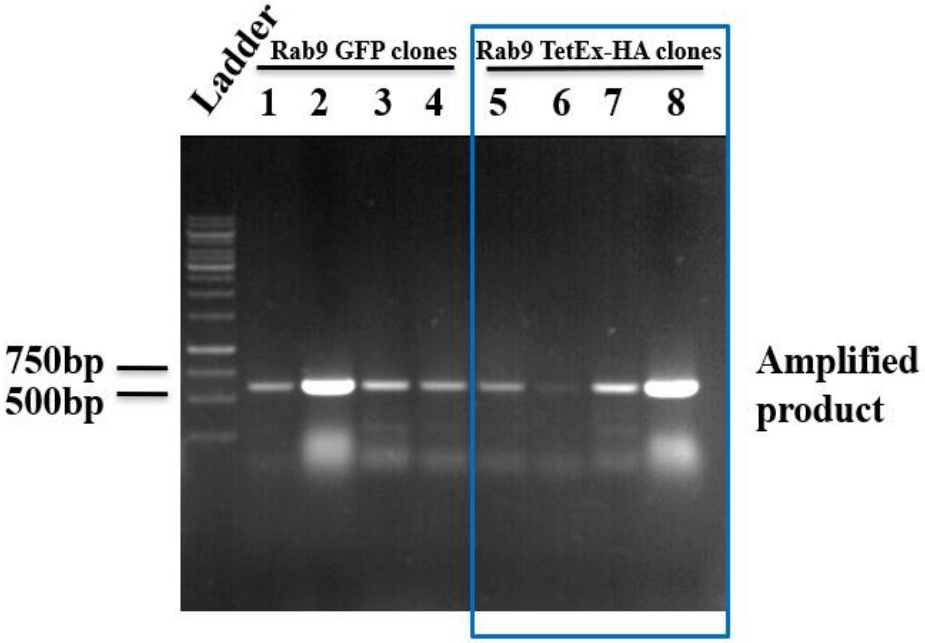

Figure 4. Colony PCR of Rab9 pEhTetEx-HA clones. Lane 5-8PCR for Rab9 pEhTetEx-HA clones using suitable forward and reverse primers.

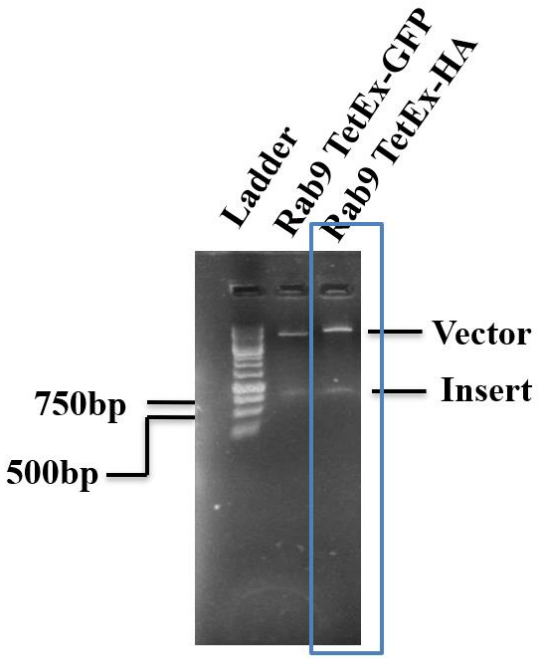

Figure 5. Confirmation of pEhTetEx-HA clones by restriction digestion. Lane1-DNA Ladder, Lane2 and Lane 3 -Rab9 insert around 615-bp released upon the digestion with *SmaI* and *XhoI*

5.3 Sequencing analysis for Rab9 in *Entamoeba histolytica*:

For identification of ligated PCR production to pEhTetEx plasmid, Sanger sequencing was done. Through sequencing we wanted to know whether 615bp ligated DNA into pEhTetEx-HA was Rab9 or not. The restriction digestion positives clones of Rab9 GFP and Rab9pEhTetEx were sent for DNA sequencing. Plasmid DNA from each clone along with Rab9 forward primer or Rab9 reverse primer were processed along with master mix for unidirectional Sanger sequencing.

5.3.1 Sequencing using Rab9-HA_RV:

1. **SITE FOR *BglII*:**AGATCT
2. **HA-TAG:**ATGTATCCATATGATGTTCCAGATTATGCTTATCCATATGATGTTCCAGATTATGCTTATCCATATGATGTTCCAGATTAT
3. **SITE FOR *SmaI*:**CCCGGG
4. **JUNCTION SEQUENCE:** TCATTGGAGATAGTGG
5. **INSERT(RAB9-HA):**
   ATGGCAGGAAGACCAGCATTATTTAAGGCAATTCTCATTGGAGATAGTGGTG
   TTGGAAAAACATCACTTATCAACCGATACGTCAATAACCAATTTTCTGATGTA
   TATAAAGCAACAATTGGTAGTGATTTTCTAATTAAACCTGTTACTGTTAATGG
   AGCACAATACACTCTTCAAATTTGGGACACTGCTGGACATGAAAGATATAGT
   TGTGTTGTTACTACATTCTATCGAGGTAGTGATTGTTGTGTTTTGTGTTTTGAT
   GTTACGAATCGAGATTCATTCAATCATTTAGAAAAGTGGAAGAATGAGTTTA
   TTGATGGAGCAAATGCAACCAATCCCGCTTCAATCCCAATTTATGTAGTTGGA
   AATAAAATTGATTGTGAACCAAATAAAAGAGAAGTAAGCCAAGAACAAGCA
   CGAGAATGGTGTAAATTAAATGGACATAAATATTTTGAAACGTCAGCAATGA
   ATGCTGAAAATGTGACAGATTTATTTACAACTTTGGCTGAAGATGTCGTTTCA
   AGACGTGAGGATGAAGAAGAGCCAGAAAAACCTGCTCCAATAATTAT__AAA
   A_CAAA_G_GAAGAG

RAB9-HA_CLONE_RV:

GGATAATTAATAAAAAGTTTTAAACAATAAATAGATGAGAAAAAGATATACATGAT
GTTTTTAATTAGAAATGAAAGAAAGTCAGTCTTACACGTCATAAAGTTATAGAATTT
CCAAATTGTAATGAAGGTTCATCTATTTTCTGATAGTAAAAAAAGTTATTGTTGCAT
CTATTAGAATAGACTCTCATCAACAAAATAAACTATAAAGACTTTCTTTGGTATTTCT
TTGTCCATCTTCATTGAGTTATAGAAATACCAAGGATGTAATTATTTAAAGGTTGGT
CAATGAACTGACAAACACATTAACAGATCTATGTATCCATATGATGTTCCAGATTAT
GCTTATCCATATGATGTTCCAGATTATGCTTATCCATATGATGTTCCAGATTATCCCG
GGATGGCAGGAAGACCAGCATTATTTAAGGCAATTCTCATTGGAGATAGTGGTGTTG
GAAAAACATCACTTATCAACCGATACGTCAATAACCAATTTTCTGATGTATATAAAG
CAACAATTGGTAGTGATTTTCTAATTAAACCTGTTACTGTTAATGGAGCACAATACA
CTCTTCAAATTTGGGACACTGCTGGACATGAAAGATATAGTTGTGTTGTTACTACAT
TCTATCGAGGTAGTGATTGTTGTGTTTTGTGTTTTGATGTTACGAATCGAGATTCATT
CAATCATTTAGAAAAGTGGAAGAATGAGTTTATTGATGGAGCAAATGCAACCAATC
CCGCTTCAATCCCAATTTATGTAGTTGGAAATAAAATTGATTGTGAACCAAATAAAA
GAGAAGTAAGCCAAGAACAAGCACGAGAATGGTGTAAATTAAATGGACATAAATAT
TTTGAAACGTCAGCAATGAATGCTGAAAATGTGACAGATTTATTTACAACTTTGGCT
GAAGATGTCGTTTCAAGACGTGAGGATGAAGAAGAGCCAGAAAAACCTGCTCCAAT
AATTATCAAAAACCAAAGGGGAAGAGCCAGGA

5.3.2 Sequencing analysis using Rab9-HA_FW:

1. **SITE FOR *XhoI*:** CTCGAG
2. **JUNCTION SEQUENCE:** TCATTGGAGATAGTGG
3. **INSERT (RAB9-HA):**

TCATTGGAGATAGTGGTGTTGGAAAA_CATCACTTATCAACCGATACGTCAATAA
CCAATTTTCTGATGTATATAAAGCAACAATTGGTAGTGATTTTCTAATTAAACCT
GTTACTGTTAATGGAGCACAATACACTCTTCAAATTTGGGACACTGCTGGACATG
AAAGATATAGTTGTGTTGTTACTACATTCTATCGAGGTAGTGATTGTTGTGTTTTG
TGTTTTGATGTTACGAATCGAGATTCATTCAATCATTTAGAAAAGTGGAAGAATG
AGTTTATTGATGGAGCAAATGCAACCAATCCCGCTTCAATCCCAATTTATGTAGT
TGGAAATAAAATTGATTGTGAACCAAATAAAAGAGAAGTAAGCCAAGAACAAG
CACGAGAATGGTGTAAATTAAATGGACATAAATATTTTGAAACGTCAGCAATGA
ATGCTGAAAATGTGACAGATTTATTTAC_ACTTTGGCTGA_GATGTCGTTTCGGAC
GTGACGATGAAGAAGAGCCAG__AAACCTGCTCCA_ATAATTATTC___AAC_AAG
CG___AG_AGA__GAGGGTG_TGCT

RAB9-HA_CLONE_FW:

TCATTGGAGATAGTGGTGTTGGAAAACATCACTTATCAACCGATACGTCAATAACCA
ATTTTCTGATGTATATAAAGCAACAATTGGTAGTGATTTTCTAATTAAACCTGTTACT
GTTAATGGAGCACAATACACTCTTCAAATTTGGGACACTGCTGGACATGAAAGATAT
AGTTGTGTTGTTACTACATTCTATCGAGGTAGTGATTGTTGTGTTTTGTGTTTTGATG
TTACGAATCGAGATTCATTCAATCATTTAGAAAAGTGGAAGAATGAGTTTATTGATG
GAGCAAATGCAACCAATCCCGCTTCAATCCCAATTTATGTAGTTGGAAATAAAATTG
ATTGTGAACCAAATAAAAGAGAAGTAAGCCAAGAACAAGCACGAGAATGGTGTAA
ATTAAATGGACATAAATATTTTGAAACGTCAGCAATGAATGCTGAAAATGTGACAG
ATTTATTTACGACTTTGGCTGACGATGTCGTTTCGGACGTGACGATGAAGAAGAGCC
AGTGAAACCTGCTCCACATAATTATTCTCCAACGAAGCGTGCAGTAGATAGAGCGA
GGGTGCTGCTCACTCGAGGTGAATTCTTCTCTTCTAAACTTAAAATGTTTTTTTTAAA
TAACAAATCGCTTTACATATTCTGCTCTACCTCATGCATACATTATTCTAATATCTGT
GTTATGTCTGTGTAACGACTTCCCGCAATCATATATCCCTACACACTTACTTTCCATC
ACTCCATATAAGTTTGGAGTAAAGTACCCACCTATTCATGTCTCCTATGAACTTTTTG
TGTTTATGAGTAGCCCTTTCATATATGAAGCAACACATACTATTGTTATTGATCATAA
AATTAATTGAACCTTCATTACAATTTGAAAATTCTATAACTTTATGACGTGTAAGACT
GACTTTCTTTCATTTCTTATTAAAAACATCCAATGTATATCTTTTCTCAATCTATTTAT
TGTTAAAAACCTTTTATGAGTGATC

RAB9-HA_CLONE

ATGGCAGGAAGACCAGCATTATTTAAGGCAATTCTCATTGGAGATAGTGGTGTTGGA
AAACATCACTTATCAACCGATACGTCAATAACCAATTTTCTGATGTATATAAAGCAAC
AATTGGTAGTGATTTTCTAATTAAACCTGTTACTGTTAATGGAGCACAATACACTCTT
CAAATTTGGGACACTGCTGGACATGAAAGATATAGTTGTGTTGTTACTACATTCTATC
GAGGTAGTGATTGTTGTGTTTTGTGTTTTGATGTTACGAATCGAGATTCATTCAATCA
TTTAGAAAAGTGGAAGAATGAGTTTATTGATGGAGCAAATGCAACCAATCCCGCTTC
AATCCCAATTTATGTAGTTGGAAATAAAATTGATTGTGAACCAAATAAAAGAGAAGT
AAGCCAAGAACAAGCACGAGAATGGTGTAAATTAAATGGACATAAATATTTTGAAA
CGTCAGCAATGAATGCTGAAAATGTGACAGATTTATTTACGACTTTGGCTGACGATGT
CGTTTCGGACGTGACGATGAAGAAGAGCCAGTGAAACCTGCTCCACATAATTATTCT
CCAACGAAGCGTGCAGTAGATAGAGCGAGGGTGCTGCTCA

Figure 6. Sequence determination of Rab9-HAclone from vector.

5.4 BLAST ANALYSIS OF RAB9-HA_CLONE: Sequence alignment using AmoebaDB Blast tool has shown that the sequence of RAB9-HA_CLONE has 97% identity with EHI_082070(https://amoebadb.org/amoeba/).

Identities = 593/614 (97%), Gaps = 3/614 (0%)

Query sequence: RAB9-HA_CLONESubject sequence: EHI_082070

```
RAB9-HA_CLONE     1    ATGGCAGGAAGACCAGCATTATTTAAGGCAATTCTCATTGGAGATAGTGGTGTTGGAAAA  60
                       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
EHI_082070        1    ATGGCAGGAAGACCAGCATTATTTAAGGCAATTCTCATTGGAGATAGTGGTGTTGGAAAA  60

RAB9-HA_CLONE     61   -CATCACTTATCAACCGATACGTCAATAACCAATTTTCTGATGTATATAAAGCAACAATT  119
                        ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
EHI_082070        61   ACATCACTTATCAACCGATACGTCAATAACCAATTTTCTGATGTATATAAAGCAACAATT  120

RAB9-HA_CLONE     120  GGTAGTGATTTTCTAATTAAACCTGTTACTGTTAATGGAGCACAATACACTCTTCAAATT  179
                       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
EHI_082070        121  GGTAGTGATTTTCTAATTAAACCTGTTACTGTTAATGGAGCACAATACACTCTTCAAATT  180

RAB9-HA_CLONE     180  TGGGACACTGCTGGACATGAAAGATATAGTTGTGTTGTTACTACATTCTATCGAGGTAGT  239
                       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
EHI_082070        181  TGGGACACTGCTGGACATGAAAGATATAGTTGTGTTGTTACTACATTCTATCGAGGTAGT  240

RAB9-HA_CLONE     240  GATTGTTGTGTTTTGTGTTTTGATGTTACGAATCGAGATTCATTCAATCATTTAGAAAAG  299
                       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
EHI_082070        241  GATTGTTGTGTTTTGTGTTTTGATGTTACGAATCGAGATTCATTCAATCATTTAGAAAAG  300

RAB9-HA_CLONE     300  TGGAAGAATGAGTTTATTGATGGAGCAAATGCAACCAATCCCGCTTCAATCCCAATTTAT  359
                       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
EHI_082070        301  TGGAAGAATGAGTTTATTGATGGAGCAAATGCAACCAATCCCGCTTCAATCCCAATTTAT  360

RAB9-HA_CLONE     360  GTAGTTGGAAATAAAATTGATTGTGAACCAAATAAAAGAGAAGTAAGCCAAGAACAAGCA  419
                       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
EHI_082070        361  GTAGTTGGAAATAAAATTGATTGTGAACCAAATAAAAGAGAAGTAAGCCAAGAACAAGCA  420

RAB9-HA_CLONE     420  CGAGAATGGTGTAAATTAAATGGACATAAATATTTTGAAACGTCAGCAATGAATGCTGAA  479
                       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
EHI_082070        421  CGAGAATGGTGTAAATTAAATGGACATAAATATTTTGAAACGTCAGCAATGAATGCTGAA  480

RAB9-HA_CLONE     480  AATGTGACAGATTTATTTACGACTTTGGCTGACGATGTCGTTTC-GGACGTGACGATGAA  538
                       |||||||||||||||||||| |||||||||| |||||||||| ||||||| ||||||
EHI_082070        481  AATGTGACAGATTTATTTACAACTTTGGCTGAAGATGTCGTTTCAAGACGTGAGGATGAA  540

RAB9-HA_CLONE     539  GAAGAGCCAGTGAAACCTGCTCCACATAATTATTCTCCAACGAAGCGTGCAGTAGATAGA  598
                       |||||||||| |||||||||||| |||||||||| ||| |||||  || ||| |||
EHI_082070        541  GAAGAGCCAGAAAAACCTGCTCCA-ATAATTATTCAAAAACAAAGCGAAGAGAAGAAAGA  599

RAB9-HA_CLONE     599  GCGAGGGTGCTGCT 612
                         ||||||| ||||
EHI_082070        600  AGGAGGGTGTTGCT 613
```

Figure 7. Blast analysis of Rab9-HA

CONCLUSION

6. Conclusion:

Previously, it was mentioned in various reports that, there are 9 isotypes of Rab7 (i.e Rab7A- Rab7I) in *Entamoeba histolytica*. But after our *in silico* analysis, we identified that from these 9 isotypes only 4 Rab7 isotypes truly belong to Rab7 subfamily. We also identified a novel Rab9 in *Entamoeba histolytica* genome which was previously classified under Rab7 subfamily and conclude that Rab7D does not belong to Rab7 subfamily but is Rab9.

Size of putative Rab9 gene in *Entamoeba histolytica* is 615 bps and it was successfully cloned in amoebic expression vector pEhTetEx-HA. The cloned Rab9 gene sequence was partially confirmed by sending the clones for automated Sanger sequencing. We also identified that the quality of sequencing read with Rab9 forward primer is poor. The correctness of cloned amoebic Rab9 sequence will be again confirmed by Sanger sequencing. Finally, these clones will be further used for transfection study to get an insight of its localization and function in *Entamoeba histolytica*.

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