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MOLECULAR DIVERSITY FOR *ENTAMOEBA HISTOLYTICA* VIRULENCE FACTOR GENES ISOLATED FROM PATIENTS WITH AMOEBIC DYSENTERY

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ABSTRACT : *Entamoeba histolytica* is a parasitic protozoan that caused amoebiasis, a diseases which infects the liver and colon and spread all over the world. *E.histolytica* is causing the death of human cells and leading to develop invasion, and ulceration of the intestine. However, amoebic liver abscess(ALA) considers as major common extraintestinal disease caused by *E.histolytica.* The aims of this study, was to detect *E. histolytica* by polymerase chain reaction (PCR), and to differentiate it from other amoeba species in a stool samples of human. Study the pathogenicity of *E. histolytica* in human through determining the presence of virulence factors like cysteine protease, amoebapore and Gal/lectin. A total of 100 stool samples were collected from patients with amoebic dysentery and then examined microscopically to confirm the presence of *E. histolytica* and other *Entamoeba* species, 72 (72%) out of 100 examined samples with *E.histolytica* were positive by PCR and 28(28%) were negative. The positive samples with PCR have been submitted to detect the virulence factors of *E.histolytica* by PCR and the results showed that the total of 72(100%)were positive with cysteine proteases gene, shown 62(86.11%) were positive with amoebapore and 65(90.27) were positive with lectin. Gene sequence for cysteine protease, amoebapore and lectin in local *E. histolytica* human isolates showed the nucleotide alignment similarity and substitution mutations in all of cysteine protease, amoebapore and Gal/GalNAc lectin. Phylogenetic tree analysis based on cysteine protease gene partial sequence in local *E.histolytica* human isolates that used for genetic variation analysis showed genetic closed related to NCBI-BLAST *E.histolytica* Japan strain (AK421547.1) at total genetic changes (0.0060-0.0010%), while Phylogenetic tree analysis based on amoebapore B gene partial sequence in local *E.histolytica* human isolates that used for genetic variation analysis showed genetic closed related to NCBI-BLAST *E.histolytica*, Germany strain (X76904.1) at total genetic changes (0.0060-0.0010%). Phylogenetic tree analysis based on Gal/GalNAc lectin gene partial sequence in local *E. histolytica* human isolates that used for genetic variation analysis showed genetic closed related to NCBI-BLAST *E.histolytica* Bangladesh strain (AF501280.1) at total genetic changes (0.0060-0.0020.

Key words : *Entamoeba histolytica*, pathogenesis, virulence factors gene sequence.

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INTRODUCTION

Amoebic infections are a serious public health that affects problem all over the world. Tropical countries and areas with inadequate sanitation and hygiene are particularly vulnerable. After malaria and schistosomiasis, it is third worlds leading caused of parasite infection-related death (Park *et al*, 2007). It have been detected which, and on average 500 million Persons are infections with amoebiasis all year and only about 10% suffer from illness symptoms (Stauffer *et al*, 2006). Ingestion of food and water contaminated with feces that containing *E. histolytica* cyst is the most common mode of transmission. According to studies, number of 4- 10 from the infected people with *E. histolytica* that cause amoebic colitis and only about 1% of infected people with amoebic liver abscess (ALA) (Ximenez *et al*, 2009). Amoebic colitis can develop during two weeks of infection or can take a month to develop, colicky stomach pain, irregular intestinal movements, and diarrhea with blood soaked stools and a decent amount of mucus are typical symptoms, but sometimes, amoebic dysentery is linked with unexpected rise of fever, chills, and severe diarrhea, that can be products in dehydration and death in infants (Blessmann *et al*, 2002). The virulence of *E. histolytica* is reduced by long-term axenic raising (Mattern *et al*, 1982). Surface ulcer and invasion lesion early in intestinal *E. histolytica* trophozoites are colony and able continuously interacted with intestinal bacteria, this bacteria is not has a linked action with disease and it is

ingested by trophozoite to prevent any impact effects (Bracha *et al*, 1982).

MATERIALS AND METHODS

Collection of stool sample

Stool samples were collected from patients with dysentery who entered each o Bint Al Hoda and Muhammad Al Mousawi teaching hospitals, health centers in the districts and sub-districts in Thiqar province. samples were taken from different ages during the Period extended form October 2020 to March 2021, it is packaged in a universal container that is clean and sterilized for in vitro use only, the stool samples were examining as soon as possible after collection, to ensure the presence of *E. histolytica* trophozoites by microscopic examination, then after, stool samples were stored in 1.5 ml Eppendorf tubes and kept at -20 for DNA extraction and achieving of the molecular study (Sastry *et al*, 2014; Zeibig *et al*, 2014).

DNA extraction and PCR performed

DNA were extracted from stool samples that collected from patients with amoebic dysentery by using 200 mg from stool through a commercial kit (Presto™ Stool DNA Extraction Kit -Geneaid/ Taiwan), extracted DNA was submitted for PCR amplification performing to detect *E. histolytica* based on 18S rRNA gene from Human stool samples as well as detection of some virulence factor genes. This method was carried out according to method used by Ziguer *et al* (2020).

PCR primers that used for detection of *E. histolytica* and virulence factor genes were used based on Ziguer *et al* (2020) and Al-abodi *et al* (2015). These primers was provided from Macrogen company, Korea as shown in Table 1.

The PCR master mix was prepared by using Maxime PCR PreMix Kit and this master mix was done according to company instructions as by mixing 5µL from DNA sample with 1µL of each primers and 13 µL from PCR water. Then placed in PCR Thermocycler. PCR thermocycler conditions by using convential PCR thermocycler system included 95ºC for initial denaturation, 58 for annealing and 72 for extension.

RESULTS

A number of 100 stool sample when examined by microscope during the current study were showed positive results with amoebic dysentery. The total number of positive sample for amoebic dysentery when submitted for PCR performing explained that only 72(72%) of these sample with positive result for a specific 18SrNA gene of *E. histolytica*, 72 (100%) gave positive cysteine proteinase gene, 62 (86.11%) gave positive results to amoebapore, and 65 (90.27%) gave positive Gal/ lectin gene. Table 4 showed virulence factors of *E. histolytica.*

Table 2 : Results of diagnostic genes.

Type of gene	Total No.	Positive result		X^2	P value
		No.	%		
18SrNA gene	72	72	100	19.19	<0.0001(HS)
Cysteine gene	72	72	100		
Amoebapore gene	72	62	86.11		
Gal/Lectogene	72	65	90.27		

HS: Highly significant differences (P<0.01).

DISCUSSION

E. histolytica is so far one of main the health problem and it is the main reason to amoebiasis. Symptoms of this disease consists of several diagnostic factors, namely, fever, dysentery or diarrhea, dehydration and abdominal pain (Tanyuksel *et al*, 2003). The main cause of infection is due to the geographical and climatic location, as well as to the carrier host (Nath *et al*, 2015).

Total of 100 stool sample showed positive results with *E. histolytica* infection by microscopic examination during the following study were submitted to PCR identification technique, the results were showed that 72

Table 1 : Primers used for detection of *E. histolytica* and the virulence factors.

Primer	Sequence (5'-3')		Product size
18rRNA gene *Entamoeba histolytica*	F	ACGAGGAATTGGGGTTCGAC	573bp
	R	AAATGCTTTCGCTCTCGTGC	
Cysteine protease	F	GCTGTTGCTGGTACTTGCAAG	122bp
	R	ACAGCAACAGGTCCGTTTTC	
Amoebapore	F	TGCCTTTGCTGCAACAAGAG	102bp
	R	ACAGCTTGAGCACCATCAAC	
Gal/lectin	F	GACGCACCAGGTACTCAAAATC	111bp
	R	AACCCATCTTCCACCCTGATTG	

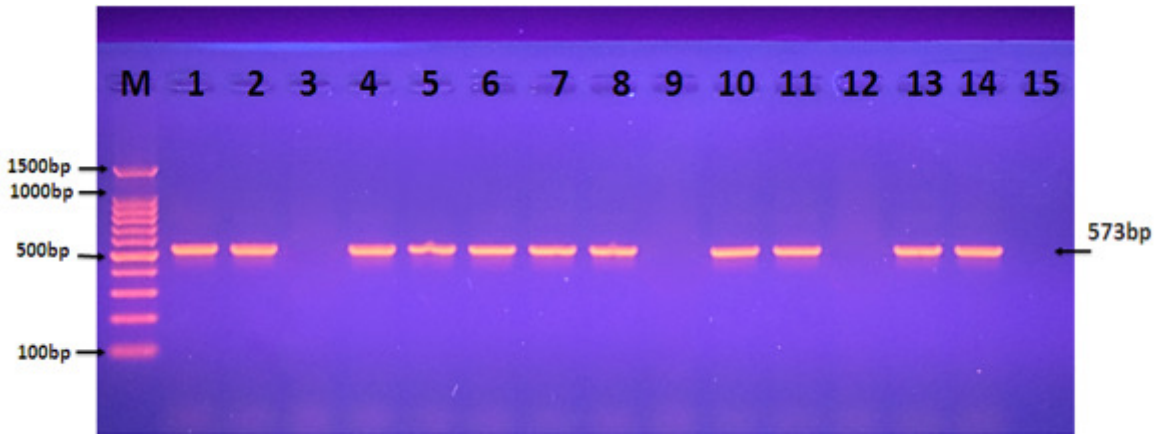

Fig. 1 : Agarose gel electrophoresis image that showed the PCR product analysis of small subunit ribosomal RNA gene in *E. histolytica* from Human stool samples. Where M: marker (1500-100bp) and Lane (1-15) some positive *E. histolytica* was showed at 573bp PCR product.

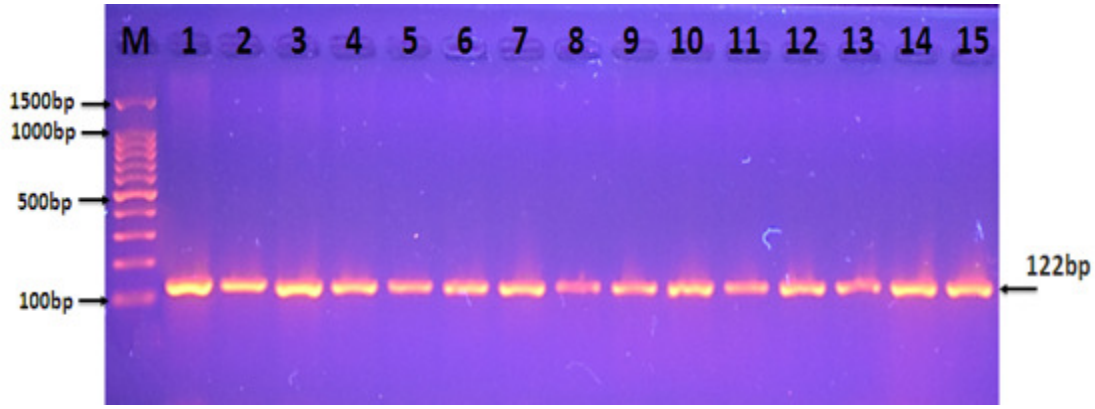

Fig. 2 : Agarose gel electrophoresis image that showed the PCR product analysis of virulence factor gene (cysteine protease) in *E.histolytica* from Human stool samples. Where, M: marker (1500-100bp) and Lane (1-15) some positive cysteine protease gene *E. histolytica* was showed at 122bp PCR product.

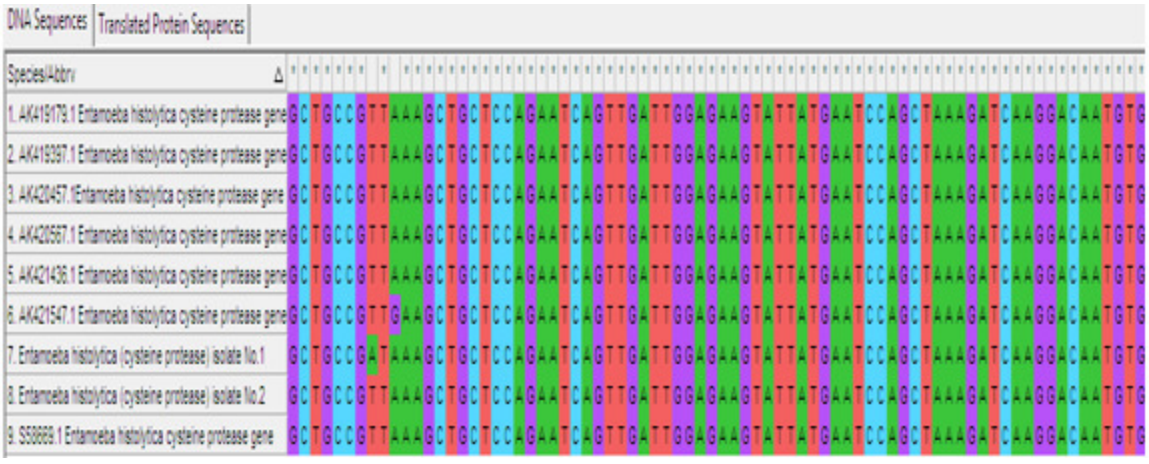

Fig. 3 : Multiple sequence alignment analysis of cysteine protease gene in local *E. histolytica* human isolates and NCBI-Genbank *E. histolytica* isolates. The multiple alignment analysis was constructed using ClustalW alignment tool in (MEGA 6.0 version). That showed the nucleotide alignment similarity as (*) and substitution mutations in cysteine protease gene.

(72%) from the examined stool samples were give positive results with PCR after targeting 18s rRNA gene of the parasite. The total number for positive sample with PCR were submitted for identification of virulence factor gene and the results showed each of 72 (100%) gave positive results with cysteine proteinase gene, 62 (86.11) gave positive results for amoebapore and 65 (90.27) gave positive results for Gal/ lectin gene. The virulence of *E. histolytica* is reduced by long-term axenic raising (Mattern *et al*, 1982). Virulence factors the adhesion and

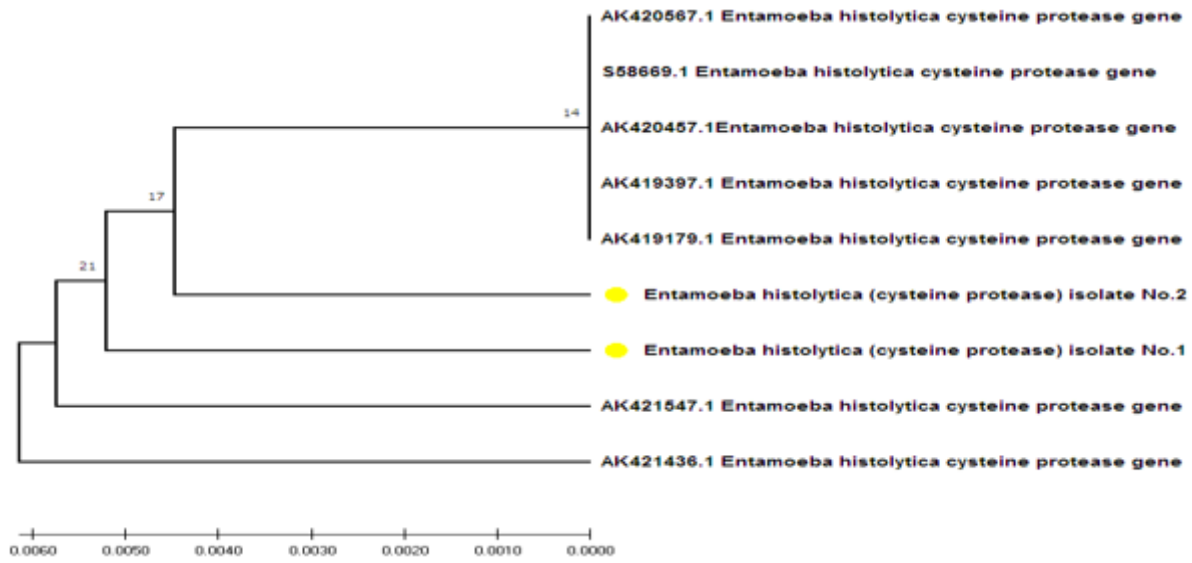

Fig. 4 : Phylogenetic tree analysis based on cysteine protease gene partial sequence in local *E.histolytica* Human isolates that used for genetic variation analysis. The phylogenetic tree was constructed using Maximum Likelihood method and Tamura-Nei model tree method) in (MEGA X version). The local *E.histolytica* Human isolate (No.1 and No. 2) were showed genetic closed related to NCBI-BLAST *E.histolytica* Japan strain (AK421547.1) at total genetic changes (0.0060-0.0010%).

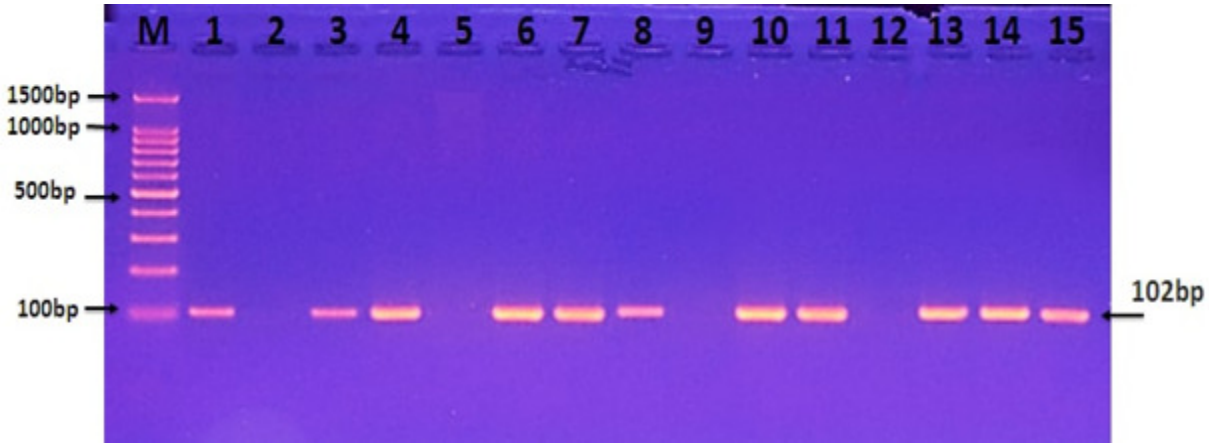

Fig. 5 : Agarose gel electrophoresis image that showed the PCR product analysis of virulence factor gene (amoebapore) in *E. histolytica* from Human stool samples. Where M: marker (1500-100bp) and Lane (1-15) some positive amoebapore gene *E. histolytica* was showed at 102bp PCR product.

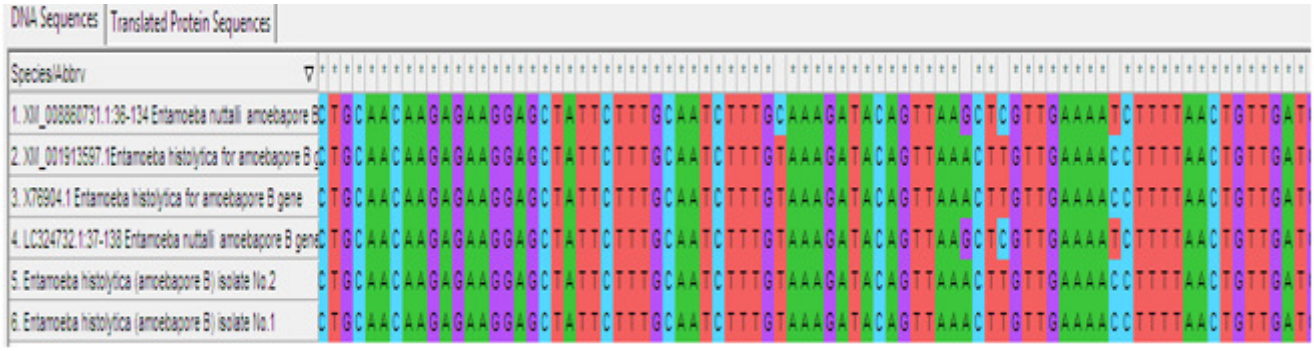

Fig. 6 : Multiple sequence alignment analysis of amoebapore B gene in local *Entamoeba histolytica* Human isolates and NCBI-Genbank *Entamoeba histolytica* isolates. The multiple alignment analysis was constructed using ClustalW alignment tool in (MEGA 6.0 version). That showed the nucleotide alignment similarity as (*) and substitution mutations in amoebapore B gene.

cytolytic occur procedures attachment to three types of molecules (lectin, amoebapore and proteases) that are following lectin is adherence of the parasite resides occur mostly though surface Gal/GalNAc lectin that connected for uncovered the end Gal/ GalNAc. The phylogenetic tree was constructed using Maximum Likelihood method

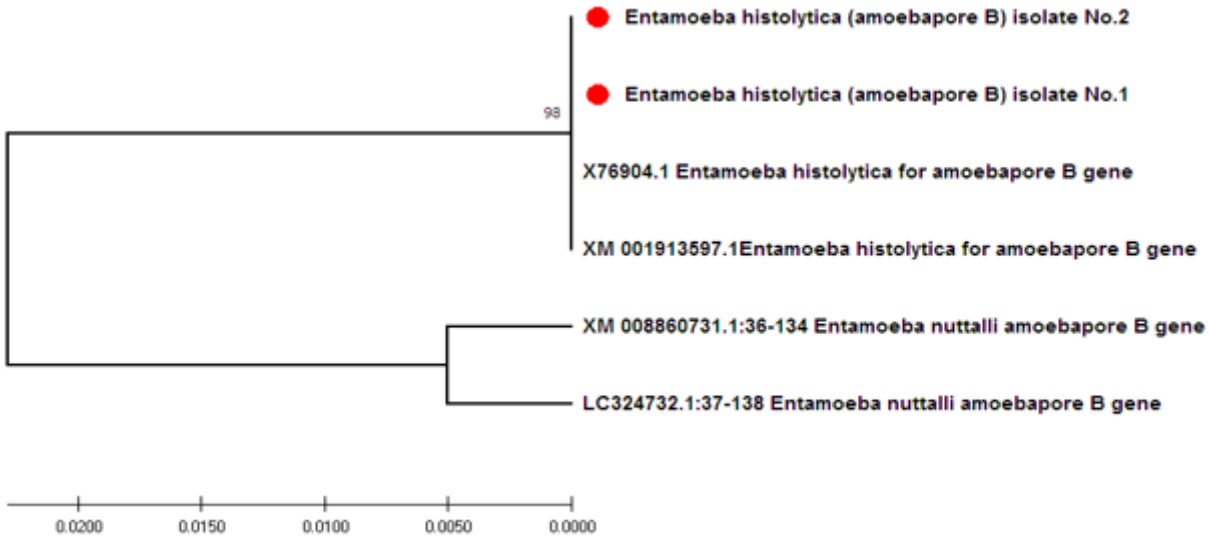

Fig. 7 : Phylogenetic tree analysis based on amoebapore B gene partial sequence in local Entamoeba histolytica Human isolates that used for genetic variation analysis. The phylogenetic tree was constructed using Maximum Likelihood method and Tamura-Nei model tree method) in (MEGA X version). The local *Entamoeba histolytica* Human isolate (No.1 and No.2) were showed genetic closed related to NCBI-BLAST *Entamoeba histolytica* Germany strain (X76904.1) at total genetic changes (0.0060-0.0010%).

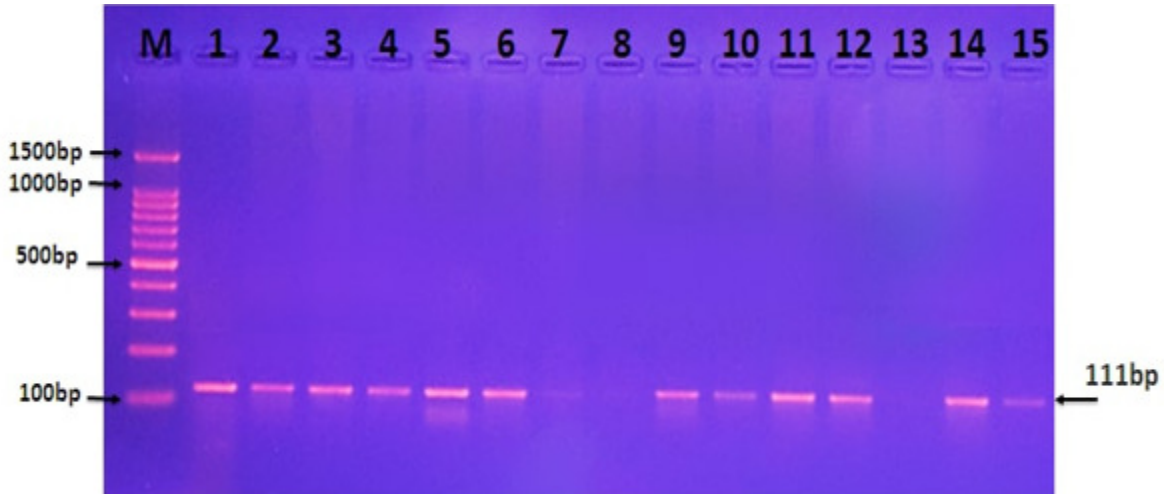

Fig. 8 : Agarose gel electrophoresis image that showed the PCR product analysis of virulence factor gene (Gal/lectin) in *Entamoeba histolytica* from Human stool samples. Where M: marker (1500-100bp) and Lane (1-15) some positive Gal/lectin gene *Entamoeba histolytica* was showed at 111bp PCR product.

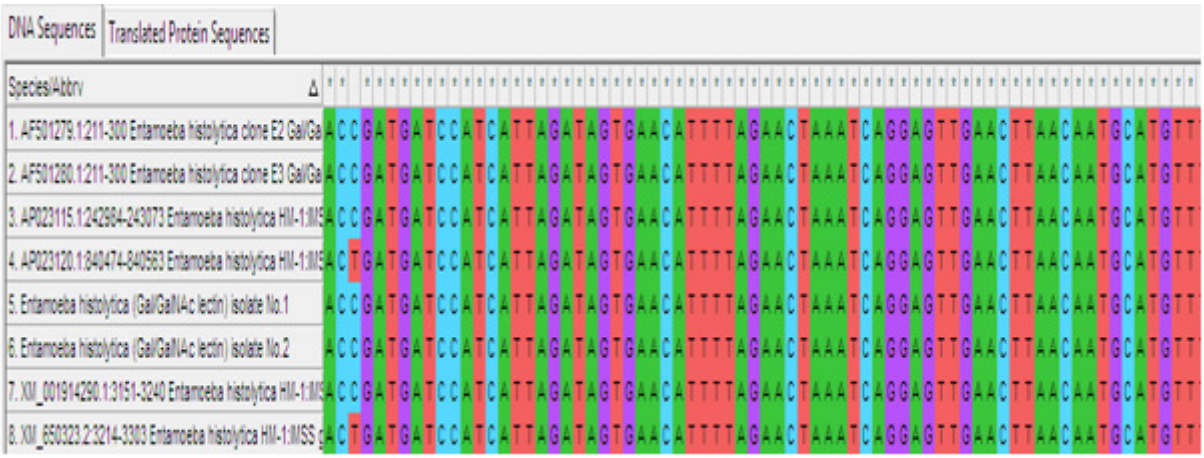

Fig. 9 : Multiple sequence alignment analysis of Gal/GalNAc lectin gene in local *Entamoeba histolytica* Human isolates and NCBI-Genbank *Entamoeba histolytica* isolates. The multiple alignment analysis was constructed using ClustalW alignment tool in (MEGA 6.0 version). That showed the nucleotide alignment similarity as (*) and substitution mutations in Gal/GalNAc lectin.

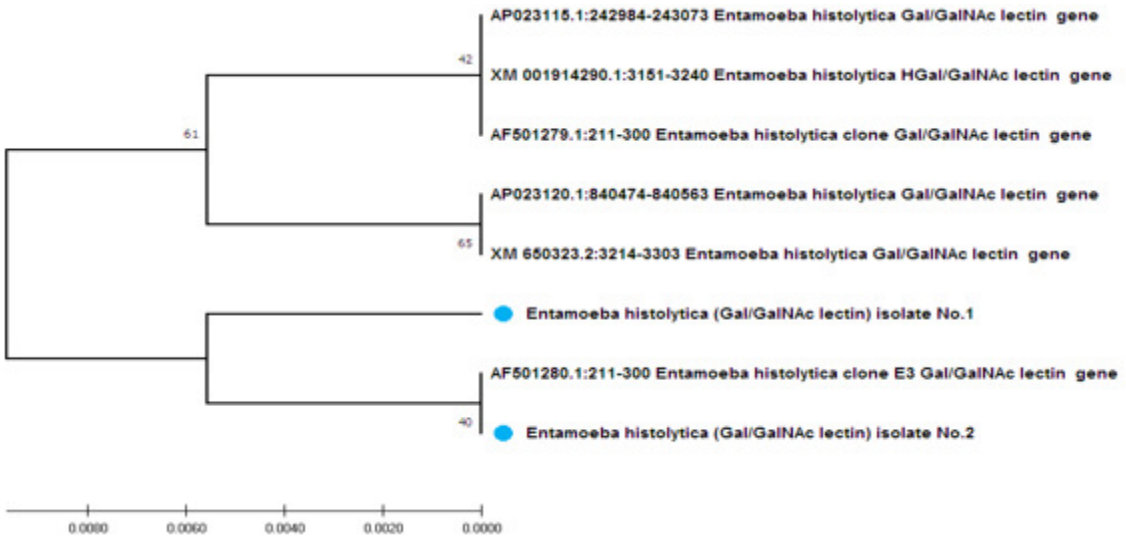

Fig. 10 : Phylogenetic tree analysis based on Gal/GalNAc lectin gene partial sequence in local *E.histolytica* human isolates that used for genetic variation analysis. The phylogenetic tree was constructed using Maximum Likelihood method and Tamura-Nei model tree method) in (MEGA X version). The local Entamoeba histolytica Human isolate (No.1 and No.2) were showed genetic closed related to NCBI-BLAST *E.histolytica* Bangladesh strain (AF501280.1) at total genetic changes (0.0060-0.0020%).

and Tamura-Nei model tree method) in (MEGA X version). The local *E. histolytica* Human isolate (No.1 and No.2) were showed genetic closed related to NCBI-BLAST *E. histolytica* Bangladesh strain (AF501280.1) at total genetic changes (0.0060-0.0020%) (Petri *et al*, 1989 and Petri *et al*, 2002). While amoebapore is despite *E. histolytica* detected linked together with mammals cells in vivo, a rapid cytolytic case place that is appropriate that results in lump, surface blistered, and lysis of the target cell, include macrophage, polymorphonuclear, leukocyte and lymphocyte, and the parasite not dangerous, the identification of which is incident for lysis target cell by T-lymphocytes. Phylogenetic tree analysis based on amoebapore B gene partial sequence in local *E. histolytica* Human isolates that used for genetic variation analysis . The phylogenetic tree was constructed using Maximum Likelihood method and Tamura-Nei model tree method) in (MEGA X version). The local *E.histolytica* human isolate (No.1 and No.2) were showed genetic closed related to NCBI-BLAST *E. histolytica* Germany strain (X76904.1) at total genetic changes (0.0060-0.0010%) (Tschopp *et al*, 1990). Cysteine proteases, it is recognized to has as significant role with significant pathogenic agents *E. histolytica* and which consist of 8 genes coding to cysteine protease is specific in *E. histolytica*. Phylogenetic tree analysis based on cysteine protease gene partial sequence in local *E. histolytica* Human isolates that used for genetic variation analysis . The phylogenetic tree was constructed using Maximum Likelihood method and Tamura-Nei model tree method) in (MEGA X version). The local *E. histolytica* Human isolate (No.1 and No.2) were showed genetic closed related to NCBI-BLAST *E.histolytica* Japan strain (AK421547.1) at total genetic changes (0.0060-0.0010%) (Berti *et al*, 1995) polymerase chain reaction (PCR) is based molecular detection system has been developed tested for identification of *E. histolytica* in stool samples and liver abscess of clinical samples. The sensitivity of these methods varies depending on the materials used and the sample processing procedures used (Hamzah *et al*, 2010 and Haque *et al*, 2010).

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