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Original Article

Detection and Sequencing of Iron Superoxide Dismutase Gene in *Entamoeba histolytica* Isolated from Patients with Diarrhea in Iraq

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Abstract

Parasites are among serious health problems that threaten the public health of humans. *Entamoeba histolytica*is considered the second or third most common cause of death among parasitic diseases. The present study was conducted to determine the molecular detection and sequencing of iron-containing superoxide dismutase (*FeSOD*) genes of *E. histolytica*. This cross-sectional study was carried out within March-August 2020. Genomic DNA from stool samples was extracted using an AccuPrep® stool DNA Extraction Kit. Afterward, four DNA samples were subjected to sequence alignment analysis of the *FeSOD* gene in local *E. histolytica* human isolated using a Clustal W alignment tool (Mega 6.0).The iron-containing superoxide dismutase gene was present at 62.8% in 15 out of 24 *E. histolytica* samples. The results of sequence alignment analysis of *the FeSOD* gene in local *E.histolytica* human isolates indicated the number of mutations in each of the 4 isolates. Accordingly, there were 2 (16.66%) silent mutations and 1(6.66%) nonsense mutation. There are a variety of genetic strains of *E. histolytica* associated with diarrheal illness in Iraqi patients, which are unique to this country.

Keywords: *Entamoeba histolytica*, Sequencing, *FeSoD*

1. Introduction

Parasites are among serious health problems that threaten the public health of human beings due to the fact that they consume large quantities of the host's food and are a major cause of other health problems, most importantly diarrhea, which is the cause of death, especially in children (1). The most common intestinal parasite is *Entamoeba histolytica*, which predominantly infects humans and other primates causing amoebiasis (2).

Superoxide dismutase (SOD) serves as a part of the organism's defense system against highly reactive oxygen species by removing the superoxide anion (O^{-2}), as to their metal cofactor, the normal environment of the trophozoites of *E. histolytica* in the lumen of the large intestine is basically anaerobic and maintenance of a low redox-potential of 150 mv or lower, is obligatory to its optimal growth in vitro. *Entamoebahistolytica* has been anaerobe; however, the pathogen has also been shown to be oxygen tolerant and to consume oxygen under certain conditions (3).It was shown to tolerate up to 5% oxygen in the gas phase and able to detoxify the product of oxygen reduction in the medium (4). *Entamoeba histolytica* lacks catalase, peroxidase, and enzymes of glutathione metabolism (5). Iron-containing (Fe) SOD is found in this pathogen. If toxic superoxide anion (O^{-2}) is formed by the partial reduction of oxygen in a variety of aerobic enzymatic oxidations, it would be removed with

the formation of H_2O; however, H_2O_2 would be detoxified in the absence of catalase. It should be amoebicidal *in vitro* using an enzymatic H_2O_2-generating system (6). For the invasion of aerobic tissues by *E. histolytic*, the superoxide radicals produced by the cell involved in host defense and those formed due to oxidative metabolism of the parasite need to be detoxified. The amoebic SOD has been characterized as a Fe-containing enzyme (7).The sensitivity of amoebic SOD to $H_2O_2$and the aerobic production of $H_2O_2$by the amoeba require cytoplasmic separation of the enzyme and H_2O_2. The presence of SOD in *E. histolytica* provides a basis, at least partially, of aero tolerance in amoeba (8).

The present study was conducted to molecular detection and sequencing of FeSOD genes of *E. histolytica*.

2. Materials and Methods

2.1. Patients

This cross-sectional study was carried out within March-August 2020 on 100 patients (60 males, 40 females) being diagnosed by specialist physicians in Babylon Province, Iraq, depending on clinical features.

2.2. Diagnosis of Parasites

The collected fresh stool samples were examined using the direct saline/iodine wet mount microscopy to detect *Entamoeba* trophozoites and/or cysts within 30 min for three times. The wet mount was used for the initial microscopic examination of stool and cysts were employed to demonstrate amoebic trophozoites, which can also reveal the presence of red blood cells and pus cells. After microscopic examination, about 0.2 g of each stool sample was stored at -20°C until used for molecular analysis.

2.3. DNA Extraction

Genomic DNA from stool samples was extracted using the AccuPrep® stool DNA Extraction Kit (Bioneer, Korea) according to the instructions of the manufacture. Finally, the purified DNA concentrate was eluted from the silica membrane spin column with a low salt buffer. The extracted genomic DNA from stool was estimated using a Nanodrop spectrophotometer (THERMO, USA) that checks and measures the purity of DNA through reading the absorbance at 260/280 nm; afterward, each sample was labeled and stored at -20°C.

2.4. Confirmed Detection of *Entamoeba histolytica* by a Conventional Polymerase Chain Reaction

A conventional polymerase chain reaction (PCR) was performed, and PCR amplification was conducted using a thermal cycler (Bioneer, Korea) with 20 µl reaction volumes that consisted of 10 µl Hot Start Master Mix (containing Taq DNA polymerase, dNTPs, Tris-HCl pH: 9.0, KCl, $MgCl_2$, stabilizer, and tracking dye) (Intron, Korea), 2 µl of both the forward and reverse primers (10 pmol for each), the specific primer (EHP1) F:CGATTTTCCCAGTAGAAATTA and R:CAAAATGGTCGTCTAGGC (135bp),5 µl of DNA template, and 13 µl of PCR water. The PCR cycling and running parameters were defined as one cycle of initial denaturation at 954°C for 5 min, followed by 30 cycles of 94°C for 30 sec, 58°C for 30 sec, and 72°C for 1 min with a final extension of 72°C for 5 min. The PCR products were electrophoresed in 1% agarose gels with a 1X Tris/boric acid/EDTA buffer and stained with 3µL of ethidium bromide (BioBasic, Canada) with a 100bp DNA marker ladder (Biolab, UK).

2.5. Detection of *FeSoD* Gene by Sequencing

According to the results of the PCR product, four DNA samples were subjected to the sequence alignment analysis of *the FeSOD* gene in local *E. histolytica* human isolated using a Clustal W alignment tool (Mega 6.0 version). The PCR cycling and running parameters were defined as one cycle of initial denaturation at 954°C for 5 min, followed by 30 cycles of 94°C for 30 sec, 58°C for 30 sec, and 72°C for 1 min with a final extension of 72°C for 5 min.

3. Results

3.1. Diagnosis of *Entamoebahistolytica* Using Specific Primers

In the current study, only 24 (34.3%) out of the 70 cases diagnosed based on the morphologywere

demonstratedto have *E. histolytica* infection. These findings canbe explained by the fact that it is difficult to identify various species of *Entamoeba* based on morphology using light microscopical criteria; nevertheless,it is possible to identify only those cases having *E. histolytica*using specific DNA probe with aconventional PCRwith a high rate of accuracy.

The molecular diagnosis of *E. histolytica* parasite was based on conventional PCR using a common probe EHP for detecting all *E. histolytica* genotypes as shown in figure 1.

Figure 1. Agarose gel electrophoresis image showing the PCR product analysis of diagnostic EHP gene in *Entamoeba histolytica* from human stool samples
Where M: marker (1500-100bp) and Lane (1-24), some positive EHP genes were shown at 135bp PCR product.

3.2. Detection of *FeSoD* Gene by PCR

Superoxide dismutase, which catalyzes the conversion of superoxide radicals into H_2O_2 and molecular oxygen, ispart of the cellular defense system that helps to protect against toxicity and damage caused by oxygen metabolism (9).In our study, the *FeSoD* gene was presented in 15 out of 24 *E. histolytica* samples (62.8%), as shown in figure 2.

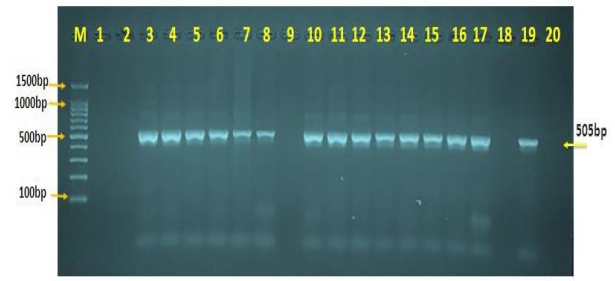

Figure 2. Agarose gel electrophoresis image showing the PCR product analysis of *FeSOD* gene in *Entamoeba histolytica* from human stool samples
Where M: marker (1500-100bp) and Lane (1-20), some positive *FeSOD* genes were shown at 505bpPCR product.

The *FeSOD* is expressed constitutively under both aerobic and anaerobic conditions, whereas the manganese (Mn)-SOD is induced either by aerobiosis or in the presence of superoxide radical anion and ferrous iron chelators (2).

In the human intestine, *E. histolytica* trophozoites normally grow under anaerobic or at least microaerobic conditions. During the process of tissue invasion, and upon contact with phagocytes, *E. histolytica* is exposed to substantial amounts of superoxide radicals. Therefore, the regulation of enzymes, such as SOD, might contribute to the understanding of *E. histolytica* pathogenicity (3).The mechanism is involved in the regulation of *FeSOD* expression in *E. histolytica* (1). (I) The regulation of *FeSOD* expression is performed on the transcriptional level; (II) a decrease in the level of Fe^{2+} ions increases *FeSOD* expression; (III) a sequence with significant homology to the iron box is found within the upstream region of the *E. histolytica FeSOD* gene, capable of specific binding of nuclear proteins; and (IV) this protein-binding activity is decreased in the absence of divalent cations (in this study, $Mn^{2+}$was used instead of Fe^{2+} for the band shift assays since Fe^{2+} is rapidly oxidized in vitro) (10).

The fact that elevated O_2, as well as an iron chelator, is capable of inducing *FeSOD* expression in this *E. histolytica*, suggests the involvement of redox-sensitive iron-containing repressor acting at the transcriptional level of *FeSOD* biosynthesis. On the other hand, this kind of regulation raises questions concerning the apparent biological contradiction that the removal of Fe^{2+} from the repressor increases the amount of active Fe 2+-containing enzyme. Although the specificity of *E. histolytica FeSOD* is reduced in the presence of 1, 10 phenanthroline and about 60% of the activity remains, the total activity was found to be increased substantially. It may be hypothesized that at low Fe^{2+} levels the iron molecule in *E. histolytica FeSOD* is replaced by an alternative divalent cation, such as the

mechanism ofMn^{2+}that was previously reported for the *FeSOD* of *Bacteroides fragilis*(11). Another more likely explanation might be that the affinity of iron is much higher to the enzyme than to the repressor molecule. If this is the case, an increased amount of protein would be synthesized at reduced Fe^{2+} concentrations, leading to an increase in the total enzyme activity, however, a decrease in the specific *FeSOD* activity, which is in line with our results. In this respect, the molecular characterization of the Fur-like protein of *E. histolytica* is of interest for further investigation, as are additional factors that might be responsible for the regulation of *FeSOD* expression in *E. histolytica*.

3.2.1. Sequence Analysis of *FeSOD*

To perform *FeSOD* gene sequencing, four isolates of *E. histolytic* were submitted. The result of gene sequence analysis *FeSOD* was indicative of the existence of some variations. The identity is 99% when compared to standard isolates of *E. histolytic* as shown in figure 2. Table 1 summarizes that there is a mutation in 4 isolates of *the FeSOD* gene and shows that there is more than one mutation in each isolate. Accordingly, the type of location of mutations that were found could lead to differences in the effect of these mutations; some of these mutationsresult in a change in the genetic code, and consequently, a change in the amino acid at the translation.

Table 1. Type of mutation in *FeSOD* gene sequence in *Entamoeba histolytic* isolate in the present study

Number of samples	Wild type	Mutant type	Site	Change in amino acid	Type of mutation	Effect
1	ATG	AAG	10	Met/Lys	Substitution	Missense
	ATT	TTG	12	Deletion A	Insertion	Frame shift
	AAA	GAA	22	Lys/Glu	Substitution	Missense
	TTA	TAA	382	Leu/Stop	Substitution	Missense
	TTA	TCA	388	Leu/ser	Substitution	Missense
	CAA	CAC	443	Glu/His	Substitution	Missense
2	AAT	ACT	447	Asn/Thr	Substitution	Missense
3	AAA	AAC	24	Lys/Asn	Substitution	Missense
	TTT	GTT	64	Phe/Val	Substitution	Missense
	AAT	AAA	405	Asn/Lys	Substitution	Missense
	ATT	CTT	442	Ile/Leu	Substitution	Missense
4	AAA	AGA	19	Lys/Arg	Substitution	Missense
	TCT	TCG	63	Ser/Ser	Substitution	Missense
	CAA	CAC	440	Gln/His	Substitution	Missense
	ATT	ATA	452	Ile/Ile	Substitution	Missense

However, this study documented that the mutation in the sequence of the gene that encode, including deletion or integration of foreign DNA between isolates, affected the sequence composition.

High-throughput sequencing offers opportunities for understanding parasite molecular evaluation within the host parasite to shed light on the in vivo dynamics of the parasite carriage and infection, the role of chance circumstance. Genetic variable within the missense leads to a significant change in the proteins, and amino acids can replace another amino acid highly similar in chemical characteristics. In this case, the protein still works naturally or the replacement of amino acid can happen in a region of the protein that does not significantly affect the secondary protein structure or function. There are also amino acids encoded by more than one code that can result in a mutation. Moreover, there were 2 (16.66%) silent mutations that did not lead to a change in the sequence of amino acids in the protein, and the silent mutation does not alter protein function.

However, there was 1(6.66%) nonsense mutation that was the same as a missense mutation, except the resulting codon code for a stop signal. It is resulted in premature termination of translation. The protein is shorter than used (or non-existent) and does not contain all the amino acids that it should have; therefore, this protein is most likely non-functional.

Nonetheless, the exhaustive characterization of parasite genetic variation within the host is an important step.

3.2.2. Determination of the Type of Mutation and Percentage

The genetic structure of the *FeSOD* gene analyzed by sequencing revealed that there were genetic changes, and as is tabulated in table 2, there were 14 (93.33%) substitutions of 1 (6.66%) deletion.

Table 2. Determination of the type of mutation and percentage

Type of mutation	Numbers	Percentage
Substitution	14	93.33%
Deletion	1	6.66%
Total	15	100.0%

3.2.3. Effect of Mutations

Mutations affect the *FeSOD* gene through the creation of a change in the organization of the gene and its work. According to table 3, there are 11(73.33%) missense types, which may influence the phenotype. Not all mutation missenses lead to significant changes in the protein, rather, an amino acid can replace another amino acid highly similar in chemical characteristic. In this case, the protein is still working naturally or the replacement of amino acid can happen in a region of the protein that does not significantly affect the secondary protein structure or function. There are also amino acids encoded by more than one code, which could result in mutation.

Table 3. Effect of the type and percentage of mutation in *FeSOD* gene

Effect of mutation	Numbers	Percentage
Missense	11	73.33%
Silent	2	16.66%
Nonsense	1	6.66%
Frame shift	1	6.66%
Total		100.0%

Finally, there was1 (6.66%) frame shift mutation that led to a reading shift resulting in a completely different type of translation originally, and subsequently, a big change in the translated protein.

The *FeSOD* gene in different isolates of *E. histolytic* showed great variation in the number of nucleotides, indicating genetic instability, allowing in tragenic recombination.

The results of the present study were relatively in line with those of studies reporting similar mutations (12, 13). Multiple sequence alignment analysis of the *FeSOD* gene in local *E. histolytica* human isolates is shown in table 4, 5, 6 and 7. However, it was performed for only four local isolates.

Table 4. Multiple sequence alignment between *Entamoeba histolytica* IQ. No.1 isolate *FeSOD* gene and *Entamoeba histolytica SOD* gene NCBI Blast standard isolate (X70852.1)

Score	Expect	Identities	Gaps	Strand
798 bits(432)	0.0	445/451(99%)	1/451(0%)	Plus/Plus

Table 5. Multiple sequence alignment between *Entamoeba histolytica* IQ.No.2 isolate *FeSOD* gene and *Entamoebahistolytica SOD* gene NCBI Blast standard isolate (X70852.1)

Score	Expect	Identities	Gaps	Strand
828 bits(448)	0.0	450/451(99%)	0/451(0%)	Plus/Plus

Table 6. Multiple sequence alignment between *Entamoeba histolytica* IQ.No.3 isolate *FeSOD* gene and *Entamoeba histolytica SOD* gene NCBI Blast standard isolate (X70852.1)

Score	Expect	Identities	Gaps	Strand
798 bits(432)	0.0	440/444(99%)	0/444(0%)	Plus/Plus

Table 7. Multiple sequence alignment between *Entamoeba histolytica* IQ.No.4 isolate *FeSOD* gene and *Entamoeba histolytica SOD* gene NCBI Blast standard isolate (X70852.1)

Score	Expect	Identities	Gaps	Strand
802 bits(434)	0.0	442/446(99%)	0/446(0%)	Plus/Plus

Entamoeba histolytica and other amitochondriate protists require particularly high amounts of extracellular iron in vitro, surpassing that of the majority of both eukaryotic and prokaryotic cells; a high iron requirement is attributable to the heavy reliance of their energy metabolism on Fe-S proteins (14). *Entamoeba histolytica* needs approximately 80-100 microns for optimal growth in axenic culture media (14), and iron is added to the media with the compound ammonium ferric citrate. This amount exceeds the iron requirements for the other pathogenic bacteria and fungi. This phenomenon is a consequence of amoeba metabolism, in which metal-dependent proteins for cellular detoxification, such as *FeSOD* (15). Talukdar et al. found that axenic amoebae cultures could reproduce in very high concentrations of iron (up to 630 um) without showing signs of intoxication (3).

Entamoeba histolytica can use Hb as an iron source and internalize this protein via the cell membrane disruption of erythrocytes by hemolysins and phospholipases. It has been reported that trophozoites possess two haem-binding proteins expressed under iron starvation and are capable of binding to the protoporphyrin ring in haem, suggesting that they function ashomophors (16).

The high rate of identity in the current study confirmed the association of certain genetic strains of *E. histolytica* with a specific pattern of clinical presentation. These results were in agreement with those of previous studies (17), which demonstrated the relationship of certain genetic patterns and repeated DNA sequences with specific patterns of clinical presentation.

In conclusion, numerous questions have still remained concerning the evolution of *Entamoeba* species, complex architecture of the genome, and structure of *Entamoeba* populations. There are a variety of genetic strains of *E. histolytica* that are associated with diarrheal illness among Iraqi patients, which are unique to this country.

Authors' Contribution

Study concept and design: W. F. H. A.
Acquisition of data: H. K. A.
Analysis and interpretation of data: L. A. A.
Drafting of the manuscript: W. F. H. A.
Critical revision of the manuscript for important intellectual content: H. K. A.
Statistical analysis: L. A. A.
Administrative, technical, and material support: W. F. H. A.

Ethics

All the procedures were approved by the Ethics Committee at the University of Babylon, Babylon, Iraq.

Conflict of Interest

The authors declare that they have no conflict of interest.

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