Molecular Investigation of *E. dispar* in Terms of Virulence Factors CP1, CP5

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Abstract

The current study included the detection of existence the Parasite *E. dispar* in 180 samples of feces for stricken patients with diarrhea from revisers to public hospital of Al-Shamia and three primary health centers in the district of Al-Shamia which is subsidiary to Al-Qadisiyah governorate for period of July 2016 till the end of December 2017, only 48 samples containing the Parasite of amoeba with it nourished, saked state or both out of 180 sample of feces for stricken patients with diarrhea, the incidence rate of the Parasite of amoeba recorded 26.22%, and the obligation results are submitted to microscopic examination for Molecular examination to Real Time PCR (Taq Man Probe) to specific investigate to the *E. dispar* and indicate the existence this kind in 31 samples, with 64.58% rate of the total of 48 obligation samples, after then the obligation samples underwent to molecular testing for *E. dispar* to investigate about each of the two the predation virulence factors, CP1 and CP5 using SYBER Green Real Time PCR. the current study has shown that the possession *E. dispar* for the virulence factors malign factor of CP1 using the initiators concern the genes, then proved existence of the CP1 factor in 18 samples of total 31 positive samples for parasite with 58.06% rate. While did not prove existence the gene of the CP5 in the type of *E. dispar*. Some studies to be positive about *E. dispar* coexisted in the intestines of human, while other studies pointed to a lack of clarity about the fact of this type has pathogen or no, because of its ability to bring about (causation) some focal lesions and harming and make damage in the laboratory living tissues. From this standpoint, the current study come to investigate about two virulence factors of the virulence factors as a step of steps the study which consider the most comprehensive study of the Species *E. dispar* to show the reality of living of this kind.

Keywords: *E. dispar*, CP1, CP5.

Introduction

Protozoa intestinal parasites transfer through hands, food and contaminated water, and distinguish this parasites to show similarity in their life cycle, usually the disease occurs when food content on the mature cysts because approximately 70% of the cases of diarrhea that occur in the developing countries to contaminate food intestinal parasites because lack to healthy awareness or low levels of cleanliness, and the diarrhea is often related to infected amoebic dysentery which caused by known parasite *Entamoeba histolytica*, the type of Entamoeba includes six kinds are: *E. hartmani, E. coli, E. poleki, E. dispar, E. histolytica, E. moshkoviskii)* and the last three species are similar with figure and appearance, but differ with it chemical, vital and genetic structure although the ability of type of *E. Histolytica* is known clearly to cause the disease, but the ability of the other two types *E. dispar* and *E. moshkoviskii* did not appear precisely. Both of two types such as (*E.dispar* and *E. histolytica*) are more similar in morphology, so there can be no distinction between them in a way that examining samples of feces in a microscope, but it can be distinguishing between it, depending on some it genetic characteristics it consider that the *E.dispar* as species of coexisting parasites although that it there have been many studies pathogen.

Some studies decided absolutely that parasite of *E.dispar* and parasite of *E.moshicoviskii* not absolute infection and it coexisting, however, the studies which followed after that to explain the fact of the parasite of *E. dispers* is an infection or not, as in the study of, which demonstrated the possibility of nourished phase of this Parasite on causing to exist the focal central epidemic in laboratory animals it also has the ability
to make effective undo epithelial cells when planted, as proven by the Stuttgarter. The ability of the active phase of this parasite to caused abscess of the amoebic liver in mice inside the lab. This infection with parasite has been accompanied by a number of different clinical signs that ranged in some individuals between abscesses of amoebic liver to colonic infection and not accompanied by diarrhea in some cases.

The various types of amoeba infection human as a result of feed vegetables which impure with sacked phase of these parasites or drinking contaminated water, and features of the active phase of the E. histolytica type with its ability to digest red blood cells for entertainer and resulting because of that a bloody diarrhea, while type E. dispar have the capacity to digest red blood cells when planting in laboratory circles outside the body and is not able to do so within the living body in the colon even when there is blood in the diarrhea incidence of single type E. dispar, the cause may be due to the presence of other minor injuries, for example, the incidence of Shigellosis.

Many studies showed a high rate the widespread of E.dispar parasite as in study, which demonstrated that E. dispar more prevailing than E. histolytica with rate (11.7) times as well as in kalmingarow in Tanzania, which showed that the incidence of E.dispar parasite is more of E. histolytica parasite about 14.4 times .Generally considered the E. dispar is the prevailing type and more widespread, the spread rate is reach up to 10:1 in some areas. There is Also many studies recorded exist the E. dispar parasite in patients whom suffering from cases of intestinal disorders and a bloody diarr, it is did not prove the existence of any evidences of nonpathogen to this parasite when it infection the human.

And the virulence factors considered main factors exist in all types of amoeba caused disease which are characterized by their ability to devour bacteria or damage the cells, that leading to damage the tissues and therefore lead to death, in addition to these factors, it be worth mentioning that the enzymes undo the proteins are exist in the pathogen and nonpathogen of parasite, found that it undo ability to plasma membrane, and explained each of the in their study that a few progenies of E. dispar may cause hepatic abscesses in a Hamster rat, praised the with the ability of descent ICB_ADO to damage the liver cells as well as destroying the layer single cells in the farm, with accompanied by the Common intestinal bacteria, stated that interaction between nourished phase of type E.dispar and bacteria consider the specific factor to the ability of the type to cause the disease. as just as the.

The fact that for due to the type of E.dispar existent or infected it able to causing disease. They give precedence to proof that the bacteria are able to participate in the organization of work of the virulent factors like Proteinase which is the most important factor of the predation virulence factors. The current study to investigate some of the factors of the virulence factors in the E.dispar Parasite by using molecular methods which give an idea of the possibility that this is type it has ability to causes the disease when it infected the human and give a chance for future studies to examine the impact of each factor separately.

Materials and Methods of work

Samples Collection

Collected 180 samples of feces of patients from the revisers for Shamia hospital and three primary health centers in the district of Shamia which is subsidiary to AL Qadisiya, of those who suffer from cases of Diarrhea during the period from July 2016 till the end of December 2017 and then put the samples in the sterilized and clean continair also provided with cohesive covers to prevent drought the sample, then brought the samples to a laboratory in the Faculty of Science, University of Qadisiyah and examined as soon as possible to avoid a delay in the samples, which leads to the disappearance of active phase which makes it difficult to distinguish them, after the completion of the examination and laboratory preserved feces samples in degrees -20 until carrying out the molecular examination on it.

Samples Examination

Microscopy Examination

Used of the (direct smear method) and included put a drop of the solution of physiological saline with quantity of (0.9%) on the glass slide as well as drop of solution of (Lugol’s Iodine) the samples were taken from different places of the model to increase
the likelihood of the appearance of the parasite in the case exist the parasite then succeed examination the slide under the luminary microscope, the use of two power micro and macro of optical zoom.

The Examination Using the Technique of the Interaction Polymerase Series of (SYBER Green Real Time PCR)

Stool DNA Extraction

Left the frozen feces samples in room temperature until to dissolve it, then DNA extraction from the samples using the tool of (Stool DNA extraction kit) which provided by Korean Bioneer Company, and have been carried out the DNA extraction, according to the instructions of the processed company.

Primers and Probes

Used prefixes and sensors of incubators concern with gene (18S rRNA) which is responsible for the diagnosis the parasite of E. dispers in infant feces samples using the Real-Time PCR according to nikloteady sequence which note in the study of 21, which provided prefixes were processed by the Korean Bioneer company

Table 1: Indicate the prefixes of primers which used in the current study with the it nikloteady sequencing Real Time PCR (Taq Man Probe):

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>E.D-ssrRNA primer F</td>
<td>ACCAAGACCGAACAGTAGAAGG</td>
</tr>
<tr>
<td>E.D-ssrRNA probe</td>
<td>FAM-TGGGTACGTGATGATGAGG</td>
</tr>
<tr>
<td>E.D-ssrRNA primer R</td>
<td>GTTTTCAGTCTGTCGTACCC</td>
</tr>
</tbody>
</table>

Special prefixes with the virulence factors genes to two factors of Cysteine protease 1 (CP1) and (CP5) were provided by the Korean Bioneer company as in the following table:

Table 2: Shows the Prefixes concern virulence factors of (CP1 and CP5) used in the current study with it nikloteady sequencing of SYBER Green Real Time PCR:

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>(CP1)</td>
<td>5'ATT GAT TTC AAT ACA TGG GTT-3'</td>
</tr>
<tr>
<td>(CP5)</td>
<td>5'GTT CAC TGT CTC GTT ATT AGG-3'</td>
</tr>
</tbody>
</table>

Diagnostic Method Using Examination of (Real-Time PCR)

The technique of Real-Time PCR has been carried out in Faculty of Veterinary Medicine, University of al - Qadisiyah using prefixes and sensors concern the gene of (18S rRNA) which is responsible for the diagnosis of E. dispers parasite from samples of human feces, according to the method of 22, as in the following steps:

The Preparation of a Mixture of Real-Time PCR Master

Was preparing a mixture of interaction Real-Time PCR using tools of AccuPower® Dual star q PCR Master Mix which provide by the Korean Bioneer company according to instructions as in the following table:

Table 3: Represented the components of the interaction of the Real-Time PCR

<table>
<thead>
<tr>
<th>PCR master mix</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA template</td>
<td>5µL</td>
</tr>
<tr>
<td>Forward primer 10pmol</td>
<td>1µL</td>
</tr>
<tr>
<td>Reverse primer 10pmol</td>
<td>1µL</td>
</tr>
<tr>
<td>TaqMan probe 10pmol</td>
<td>2µL</td>
</tr>
<tr>
<td>DEPC water</td>
<td>9µL</td>
</tr>
<tr>
<td>Total</td>
<td>18 µL</td>
</tr>
</tbody>
</table>

And then put the components the interaction mix of Real-Time PCR which have been mentioned in the table above in dark white pipes volume of (0.2ml) concern the apparatus of Real-Time PCR and then transferred the pipes to vortex centrifuge (Exispin) with speed (3000rpm) for period
(three minutes) and then put in the apparatus of Real-Time PCR.

**Real-Time PCR Thermo Cycler Conditions**

Thermo cycler (thermal sessions) have been applied to examine the Real-Time PCR based on several instructions; AccuPower® qPCR Master Mix TM2X GreenStar during the calculation of the degree of the (Tm) prefixes as well as using apparatus Mini Opticon Real-Time PCR system Bio-Rad. USA.

**The Analysis of the Results of the Examination; Real-Time PCR**

The results of the examination of the Real-Time PCR have been analyze through curve of distend Amplification plot devices include electro-optic inflation-oriented plot, which depends on the line number effort of Threshold cycler number (CT) value would be positive sample when exceeded the threshold line.

**Investigation of the Genes of Virulence Factors (CP1, CP5)**

The samples were taken which showed the positive results during the first molecular examination to type *E. dispar*, and underwent a second molecular test to Investigation the presence of the genes of virulent factors, where preparing the interaction mixture of Real-Time PCR using tools of Accu Power® 2X Green Star TM qPCR Master Mix which provide By Korean Bioneer company according to the instructions of the company; as in Table 4:

<table>
<thead>
<tr>
<th>PCR master mix</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>2X GreenStar master mix</td>
<td>25 µL</td>
</tr>
<tr>
<td>DNA template</td>
<td>5µL</td>
</tr>
<tr>
<td>Forward primer 10pmol</td>
<td>2.5 µL</td>
</tr>
<tr>
<td>Reverse primer 10pmol</td>
<td>2.5 µL</td>
</tr>
<tr>
<td>DEPC water</td>
<td>15µL</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>50µL</strong></td>
</tr>
</tbody>
</table>

Then the components of mix of Real-Time PCR interaction mentioned in the table above have been put in dark white pipes with size of 0.2ml relating to apparatus of Real-Time PCR, and then transferred all the pipes to vortex centrifuge (Exispin) with speed 3000/cycle/ per minute and the process be lost in three minutes and then put in the Real-Time PCR conditions and then applied the thermocycler for examination on basis of instructions tools of AccuPower® qPCR Master Mix TM2X Green Star through calculating the degree of the (Tm) prefixes Mini Opticon and using a Bio-Rad Real-Time PCR system USA. Then succeed that analysis of results of the examination of Real-Time PCR as final step through (T) distend curve which based upon line number of threshold effort would be the being of sample is positive sample when it exceeded the threshold line, as is the case in the analysis of the data of the diagnosis.

**The Statistical Analysis**

The results which obtained of the current study, for the Statistical analysis using the Chi-square test and shown under of the significance level of (*p* ≤ 0.05)23.

**The Results and Discussion**

**Rate of Infection with Amoeba Parasite by Microscopy Examination**

The results showed that only 48 of the sample were Containing Parasite Amoeba with it nourished cyst phase or both of total 180 samples of patients' feces who infection with diarrhea. The rate of infection recorded (26.22%) infection with parasite of amoeba as shown in Table (1).

<table>
<thead>
<tr>
<th>Parasite</th>
<th>Number of the samples</th>
<th>Percentage %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive samples</td>
<td>48</td>
<td>a26.66</td>
</tr>
<tr>
<td>Negative samples</td>
<td>132</td>
<td>b73.33</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>180</strong></td>
<td><strong>100.00</strong></td>
</tr>
</tbody>
</table>

*the different letters indicate to there are statistically significant differences under the level of the probability (*p* ≤ 0.0)

The rate of infection in the current study is approach to its registry by study's in patients infected with diarrhea from the revisers to health centers in two sub
districts: Badhaa and Gharraf which are subsidiary to the Di Qar Governorate. The proportion of infection with amoeba parasite reached (24.9%), as well as a study of the 25 who examined the diarrhea samples for patients of revisers to Marjan Specialist Hospital of internal and cardiac diseases in the city of Hillah in Babylon governorate, which reached (27.9%), and what is recorded by the 26 in the Al-Diwaniya governorate from proportion which reach (24.57%), while the rate of infection in the current study, less than recorded by 21 in the governorate of Al-Diwaniyah with proportion which estimated (61.26%). And in 27, where the ratio was (65%) in Basra. and what is recorded by 28 in the city of Baghdad, they examined 800 samples of fences for infected patients with diarrhea of revisers for Medical City hospital in Baghdad and recording the infection ratio Reached (53.18%), while the infection ratio amoeba parasite was higher than infection ratio which recorded by 29 in Tikrit province which reach (17.5%). The reason for the different rates of infection for other studies compared with current infection rate to the difference in the level of drainage, personal hygiene and cleanliness, population density, as well as the different geographical location and climatic circumstances and examination methods 30, also the difference in the duration of the study and months of the year, in particular the different age groups under study and way of living.

Rate of Infection with Amoeba Parasite by Using Technique of Real Time PCR Type of E.dispar

Molecular examination results showed that the samples that have been subject to microscopic examination and it gave as the positive result and revelation to existence of this kind in 31 samples with (64.58%) of total 48 positive samples.

Our current study shows that the percentage of gene type E.dispar reached (%58.06), it was higher than the percentage which record and it reach of (%30.8) by study of 31, and The proportion which recorded by 32 in the Diwaniya which reached to (%31), and the proportion which scored by 33, and amounted (%35). Also the proportion which recorded by 3 which reached to (%33.7), but it were less than the proportion which recorded by 12 which reached to (%91.37) and the proportion which recorded by 34 which reached to (%93.75). Our current results have agreed with what the 11 mentioned "that the type of E.dispar consider a prevailing type and the most common and the rate average of spread the types E.histolytica +E.dispar estimated nearly (10:1) in some areas. Also our study agreed with study of 10 which carried out in kalmijawir in Tanzania which mentioned that infection rate with parasite of E.dispar was (14.4) times more widespread than the infection proportion with parasite of E.histolytica infection rate. This is due to the difference in the infection rates to that the parasite types are spread as random in the most of world countries because of the different in climate and environmental conditions, habits, traditions and behaviors among the population of the world. The different of results the PCR technique, to different methods of extracting DNA from the feces samples as well as the methods of work of PCR, or the time of the implementation of the study, the geographical location of the study area, as well as population density,
cultural level and age category which be subjected to the study.

The Predation, Virulent Factors of the E.dispar Type

Current study showed the possession type E.dispar for CP1 malign factor through using the technique of the Real Time PCR using specific prefixes of genes of the factors mentioned above ,as because for proved the existence the factor of CP1 in 18 samples of total 31 positive sample included the parasite , that rate is 58.06% .Figure (2) shows the Amplification plot curve to check the Real-Time PCR for positive results of gene of virulence factors ( CP 1).

![Amplification plot curve](image)

Figure 2: Shows the Amplification plot curve to check the Real-Time PCR for positive results of gene of virulence factors (CP 1) in the type of E.dispar.

The results also indicated there is not gene of virulent CP5 factor in the type of E.dispar.

![Amplification plot curve](image)

Figure 3: Shows the Amplification plot curve to check the Real-Time PCR for positive results of gene of virulence factors (CP 5) in the type of E.dispar.

<table>
<thead>
<tr>
<th>Virulent factor</th>
<th>Number of positive samples</th>
<th>Percentage %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cysteine protease 1</td>
<td>18</td>
<td>58.06a</td>
</tr>
<tr>
<td>Cysteine protease 5</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 1: Represent the existence proportion of virulence factors of CP1 and CP5 in E. dispar parasite in total of 31 samples

*the different letters indicate to there are statistically significant differences under the level of the probability (p ≤ 0.05)

This is agreed with the study of each for Cystiene Protenase enzyme and the disease in ameba to the existence contradiction and don’t to correspond to the data concern exist the virulent CP1 factor in the E.dispar type or not, and also the study of the ferma and on the basis of that ,the investigation has been carried out about the CP1 gene in the type of E. dispers by using the Real time PCR, which is considered the most accurate and novelty positive results were record and indicated to presence Two-Genes for each virulent factors of CP1. This is consistent with the study of, which is recorded the
existence of a Ferociousness virulent gene of (CP1) in (13) a samples of total (20) samples of infected single with type of E.dispar that is with %65 proportion , as agreed in the presence of the gene, with the study of each of the deal with concern the discovery follows CP1 in the progeny of the E.dispar type , while the current results did not agree for type of E.dispar with which recorded by, who they indicated in their studies to there is not exist the gene of CP1 in the type E.dispar. And there is not exist the deciphered gene the virulence factor of CP5 in our current study in the Type of E.dispar, and this is agree with who they indicated that type of E.dispar is cipher four genes of Cysteine Proteinase (1) to similar with E. histolytica such as (EdCP1_EdCP4), that is, there is not ciphered gene for factor of CP5, this seems that the type of E.dispar. is Lacked to specific genes make to cipher the enzymes of Cysteine Proteinase ,or the genes containing many mutations which have not the capacity to cipher these enzymes, or the effectiveness of this existed stationer enzymes compared with the existed enzymes in amoeba parasite of E.histolytica case.

References


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