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The molecular identification of entamoeba Dispar and entamoeba Moshkovskii by using nested multiplex PCR in symptomatic indivisuals

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***Abstract*---**The current study was for determine Entamoeba dispar & Entamoeba moshkovskii in symptomatic individuates by molecular identification (nested multiplex PCR) in order obtained for an accurate diagnosis of these Entamoeba species. Total stool samples (120) that collected from symptomatic patients with diarrhea. These samples were collected from patients according to age, residency during the period from (the first of November 2021 to the end of January 2022). All samples undergo for a direct microscopic examination, The results revealed that 100(73.18%) were positive samples with Entamoeba spp. The results of microscopic examination recorded high infection rate in age group in (4-10) years83.33%. In contrast, recorded high prevalence in rural areas 76.31% than urban areas 73.68%. Showed the method nested multiplex polymerase chain reaction by using 18s rRNA small subunit gene, the first round of PCR showed the infection rate by Entamoeba spp 87(65.41%) out of 120 stool samples .The second round of nested multiplex PCR for these positive samples by using two specific primers revealed mix infection of Entamoeba dispar & Entamoeba moshkovskii with Entamoeba histolytica 5(6.25%), 2(2.5%) respectively, with significant difference at P<0.05. While recorded mix septicity by mix infection of E. histolytica & E. dispar recorded high infection rate in age group (1-3)years 2(15.38%) and mix infection of E. histolytica & E. moshkovskii recorded only in children in both age groups (1-3)(4-10)years 1(7.69%),1(10) respectively. In contrast, mix infection of both E. moshkovskii / E. dispar mix infection with Entamoeba histolytica recorded high infection rate in the rural areas 4(9.3%), 2(4.65%) respectively, with noted

insignificant alteration at(P<0.05) between percentage infection these of Entamoeba spp the diagnosis by nested multiplex PCR regarding to age, residency.

***Keywords*---**E.dispar, E.moshkovskii, Identification, Nested Multiplex PCR.

Introduction

The first case of amoebic dysentery in a person was described in 1875 by Russian physician Friedrich Lösch. His thorough description of the amoebas' movement, as well as the nucleus and devoured red blood cells, proved Entamoeba histolytica trophozoite. the year 2007 (Fotedar and colleagues). Because it was discovered in the colon, Lösch named the animal Amoeba coli (Lesh, 1975)). Entamoeba dispar is a morphology-like species of E.histolytica and colonizes the human intestine. It has recently been identified as a different species without any invasive capacity (Uslu et al., 2016). The recognition of E. dispar as a separate but strongly associated protozoan species has had wide effect for the epidemiology of amoebiasis, this non-invasive species is responsible for most of the asymptomatic infection in worldwide (AL-Areeqi et al., 2017). Entamoeba moshkovskii has a genetic relationship with E. histolytica, E. dispar and its cyst and trophozoite forms are microscopically indistinguishable from them. E. moshkovskii is a prevalent Entamoeba in some environments that causes infection in humans (Khomkhum et al., 2019). It is revealed in fecal samples from patients with gastrointestinal symptoms in limited studies from Australia, Bangladesh, India, Iran, Tanzania, and Turkey, therefore suggesting that this parasite could cause disease (Shimokawa et al., 2012). Is a major concern as it may lead to mistreating the patients, therefore, the need of accurate diagnostic method is important, this method represented by the molecular diagnosis using polymerase chain reaction (PCR) technique which has been used in different regions around the world (Bahrami et al., 2019). In Malaysia, intestinal parasitic infection (IPIs) including Entamoeba infection are more prevalent in rural areas especially among aboriginal communities compared to urban areas (Norhayati et al., 2003; Nqui et al., 2011). The aims of this study for detection of common spicese and similar for Entamoeba histolytica in symptomatic individuals' and for Identification of Entamoeba dispar & Entamoeba moshkovskii(nonpathogenic) by nested multiplex PCR regarding to age, residency.

Materials and Methods

There are 120 fecal specimens was collected from patients with symptoms of diarrhea attended to the medical laboratories, medical health centers, During the period from November 1st, 2021, to January 31st, 2022, in Thi-Qar province will be closed (2022). The specimens were collected in small tube (Eppendrof tube) and transported in cold bag to the Parasite's Laboratory. In addition, questionnaire formula including (age, region of patient in rural or urban). At laboratory ,the specimens divided into two parts, each one part takes of about (1-3 gm), the first placed in Eppendrof tube and stored at minus twenty Celsius for DNA extraction, Insufficient quantity of distilled water were added to stool

spacemen's and mixed well and then examined by direct microscopic test on Wet mount smear methods.

Molecular assay
Nested Multiplex PCR

This approach was used to identify Entamoeba species using the human 18S rRNA gene. The stool DNA was extracted untied Presto TM Stool DNA Extraction Kit from company Geneaid/Korea.

Genomic DNA Estimation

Extracted genomic DNA from stool samples was examined and evaluated using a Nanodrop spectrophotometer (THERMO. USA), which examines and assesses DNA purity by measuring absorbance at (260/280 nm) in the protocols below.

- Select the relevant program after you've started the Nanodrop software (Nucleic acid, DNA).
- Many times, the measuring pedestals were cleaned with a dry wipe. To blank the system, Pipette two microliters of free nuclease water lightly over the bottom measuring pedestals' surface.
- A Nanodrop was started by clicking OK, the pedestals were cleaned.

Nested multiplex PCR
PCR master mix (first round) solution components

additional PCR reaction components, including primers and probes, are packed in a standard Maxime PCR Pre Mix tube, which also contains the PCR component mentioned in the table above (dNTPs, pH: 9.0, KCl, MgCl2, stabilizer, tracking dye, Tris-HCl and Taq DNA polymerase). After that, an Exispin vortex centrifuge was used to spin the PCR tube at 3000rpm for three minutes. After that, the samples were placed in a PCR thermo cycler. Components the Polymerase chain reaction mixture (first round).

PCR Master Mix	Volume
PCR water	3.5ml
Entamoeba spp. Forward primer (10pmol)	2ml
PCR green master mix	12.5ml
DNA template 5-50ng	5ml
Entamoeba spp. Reverse primer(10pmol)	2ml
Total volume	25ml

Second round of master mix nested multiplex PCR components

The components of the PCR master mix listed in the table above are then put in a standard Maxime PCR Pre Mix, which contains all additional components required for the polymerase chain reaction, as an example (dNTPs, pH: 9.0, KCl,

MgCl2, stabilizer, tracking dye, Tris-HCl and Taq DNA polymerase). The PCR tubes were then spun for three minutes at 3000rpm in an Exispin vortex centrifuge. After that, the samples were placed in a PCR thermo cycler.

PCR master mix	Volume
First round PCR product	2.5ml
Forward primer (10pmol)for E.histolytica, E.dispar, E.moshkovskii	1ml
	1ml
	1ml
Reverse primer(10pmol)E. histolytica, E. dispar, E. moshkovskii	1ml
	1ml
	1ml
PCR green master mix	12.5ml
PCR water	4ml
Total volume	25ml

PCR master mix Thermocycler conditions

conditions by using convential PCR thermocycler PCR thermocycler system by PCR cycling of (initial denaturation at 95c for 5min; followed denaturation include 35 cycle at 95c for 30s;,at 55c for 30s, at72c for 1min;followed final extension at 72c for 5min)finally hold at 4c,forever.

PCR Product Analysis

Following these processes, the PCR results were examined on an agarose gel electrophoresis by generate one percent agarose gel, 1X TBE was dissolved in a water bath at one hundred Celsius for fifteen minutes, then cooled to fifty Celsius. The agarose gel solution was then dyed added with three microliters of ethidium bromide dye. After carefully placing the comb in the tray, the agarose gel solution was poured into the tray and allowed to harden for fifteen minutes at room temperature before gently removing the comb .1X TBE buffer was added to the gel tray in the electrophoresis chamber. Each comb well received 10 microliter of PCR product, with the first well receiving 3 microliters of (100bp Ladder). Then, for one hour, electrical charge at 100 v and 80 AM was supplied. Ultraviolet Transilluminator was used to see the PCR results. Statistical Analysis .

Statistical analysis

Data was presented as number and percentage. Comparisons between percentages of the different variables were performed using Chi-square test (X2). The 0.05 level of probability was used as the criterion for significance. All statistical analyses were performed using Statistical Package of Social Sciences (SPSS), version 27, (Inc., Chicago, IL, USA) computer software (Sheskin, 2004).

Results and Discussions

Microscopic examination

this study was collected 120 stool sample from patients have symptomatic infection with diarrhea according to age, residency then will undergo for direct microscopically examination. The result was obtained 100 (75.18%) out of 120 stool sample were infected With Entamoeba spp. Microscopic examination unable differentiate among similar morphologically types from Entamoeba histolytica, such Entamoeba Bangladeshi /Entamoeba dispar / Entamoeba moshkovskii Nesbitt et al., (2004), Khairnar et al., (2005), Tanyuksel and Petri et al., (2003), solely created off structural characteristics for cysts and trophozoite of E. histolytica is likely to given false positive results. The result of our study with Al-Yasari et al., (2019) were collected 96(61.93%) out of 155 positive sample were microscopically examination. While Lepore et al. (2022) and Fallah et al., (2014) they revealed lowest of infection rate of Entamoeba spp by microscopic examination was 33 (8.3%) ,13(4.28%) respective, Accumulation, diverse study levels as well as geographical possion, period of study, months during with samples collection, age clusters that given study may have change on the getting off results.

Entamoeba spp found in The consequences to the current revision presented the highest infectors found for the age group (4-10)years 83.33%.At the existent study consequences agreed with other researchers such as they recorded in many countries , Nath et al., (2015)in India. The reasons that lead to the children more susceptible infection with Entamoeba spp compared with other age this explain more exposed overcrowd conditions (schools, nurseries, playgrounds etc) Al-Kaeebi, Al-Difaie, (2016).Parasitic infection about graduate school children resulting from lowly situation in the schools, and badcarfuly in owns cleanses them, like the founded in dirty poor places, and about discarding locations (this can particularly result many health complications),other reasons absence formal hygiene and absence off washing hands necessary before mealtimes. Kadir and Naki ., (2000).This study recorded high infection rate in rural area76.31% (58/76) and recorded lower infection rate in urban area 73.68% (42/57). The research's result these agreed with Flaih et al., (2021) in Thi-Qar province who recorded high infection rate in rural area was (69.4%) compare urban area was(30.6%), and in middle Euphrates region of Iraq by Alyasari, et al., (2019) recorded in rural area high infection rate from urban area was 68.6% and 31.2% respectively. This explain higher risk factor by infection Entamoeba parasite that causing in the rural purples ,where prevalent deficiency ,no initiation for health learning program ,lake socioeconomic parameters ,poor criteria of hygiene and health are the connected status which added for the more prevalence of infection and might have be mentioned of that lake for dealing with drinking water ,deals with contaminated animals those considered most residence of Entamoeba infection.

Result of molecular study Results of Nested multiplex PCR (first round) for DNA samples

The first round PCR for DNA samples showed 87 (65.41%) out of 120 stool sample were successfully amplified for 18sRNA gene by nested multiplex PCR,

Conversely, direct microscopy diagnosis unable differentiate & distinguish Entamoeba histolytica as of a structurally identical species(non-harmful) E. hartmanni, E. dispar, E. moshkovskii (Al-Bakri et al., 2013). The alterations noted by PCR produce might be contributed for many causes, initially result to the differences in method DNA extraction from fecal samples. Our presents study were nearly result for Ngui et al., (2012) recorded 69.3% (52/75) by nested multiplex PCR in Malaysia . In addition, Khan et al., (2019) use PCR reaction to confirmed 72.07% (80/111) out of microscope positive samples real for the parasites in fecal specimens .

Result of nested multiplex PCR (second round) for PCR products

The nested multiplex PCR by second round showed two strains, of the genus Entamoeba parasite. Mix infection off Entamoeba dispar and Entamoeba histolytica 5 (6.25%) , Entamoeba histolytica and Entamoeba moshkovskii 2 (2.5%). The used nested multiplex PCR (second round) that increase from sensitivity and specificity of PCR for detected Entamoeba dispar & Entamoeba moshkovskii(mix infection with Entamoeba histolytica). Our findings aligns with that study scored in Australia ,50% of positive stool specimens microscopically was positive result for (nonpathogenic) Entamoeba moshkovskii by PCR assay test Fotedar et al., (2008) .In contrast , Santos et al., (2016) recorded a higher proportion was in(diversified infection) E. dispar & E. histolytica & E.moshkovskii) 6.4% (64/1003) compared E.histolytica only infection 3% (30/1003).Similarly, In our study , agreement with many of researchers that recorded mix infection of E. histolytica &E.dispar such Abozahra et al., (2020) in Egypt and Al-Bakri et al., (2021) in UEA those recorded mix infection of E histolytica &E. dispar 5.9% ,3.3% respectively. while recorded mix infection of Entamoeba histolytica and Entamoeba dispar in older collection (1-3)years,(11-20)years, were 2(15.38%),1 (10%) respectively .And recorded mix infection of E. moshkovskii , E.histolytica in age collection (1-3)years,(4-10)years, were 1(7.69%),1(10%) respectively, our finding of results that (mix infection) of Entamoeba histolytica and Entamoeba moshkovskii reported their infection merely in children with age (1-10)years and they have symptomatic diarrheal infection this agreed with Dawah, et al., (2016)showed all patients infected by Entamoeba moshkovskii was children with symptomatic. While Parija, et al., (2005) in India study showed that Entamoeba moshkovskii were related with diarrhea. Also recorded (mixed infection) for Entamoeba dispar , Entamoeba histolytica, for rural area 4(9.3%) infection rate more from urban area 1(3.33%),whereas Entamoeba histolytica / Entamoeba moshkovskii, recorded infection rate merely in rural area 2(4.65%). Our results nearly similar with Khalaf et al., (2018) that recorded more percentage of E.dispar 9.77% in rural areas than 7.26% in urban areas . In contrast ,in Colombia E. moshkovskii reported more prevalence in rural areas 25.4% by Lopez et al., (2015).

Conclusions

- By the current report revealed that rate of infection Entamoeba dispar & Entamoeba moshkovskii (mix- infection with Entamoeba histolytica), in symptomatic patients.

- The molecular diagnosis method particularly, nested multiplex polymerase chain reaction (PCR) was more sensitive and specific for differentiating the morphological similar species that presence in the one sample.
- Entamoeba dispar and Entamoeba moshkovskii recorded mixed infection with E. histolytica were found to be in symptomatic individuals and their presence were associated with diarrhea.

Recommendations

- Initiation of educational programs highlighting the necessity of taking more interest in developing sewage and interesting of preventative measures in order to reduce the danger of environmental pollution.
- 2-Confirmed more studies regarding with the genetic diversity and different virulence factors that presence mainly in E. dispar & E. moshkovskii for understanding their pathogenesis and such as these like research are uncommon in both species.

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