How to Cite:
Al-Tamemy, A. K. A. R., & Ahmed, Z. (2022). The molecular investication of entamoeba histolytica by nested multiplex PCR in diarrheoginic patients. *International Journal of Health Sciences*, *6*(S6), 489–498. https://doi.org/10.53730/ijhs.v6nS6.9635

The molecular investication of entamoeba histolytica by nested multiplex PCR in diarrheoginic patients

APD. Abdul Karem A. R. Al-Tamemy
Department of Biology, College of Science, Wassit University, Kut, Iraq
Email: altamemy1959@gmail.com

Zahraa Ahmed
Department of Biology, College of Science, Wassit University, Kut, Iraq
Email: za1534469@gmail.com

***Abstract*---**The current study was for determine Entamoeba histolytica in diarrheal patients by molecular investigation (nested multiplex PCR) in order obtained for an accurate diagnosis of Entamoeba histolytica that causes amoebiasis. Total stool samples (133) that collected from symptomatic patients with diarrhea according to age, gender. Then all samples undergo for a direct microscopic examination, The result revealed that 100(73.18%) were positive samples with Entamoeba spp. The results of microscopic examination revealed a higher infection rate of Entamoeba spp were in the female 83.87% compared the male 67.6% with significant difference at P<0.05 ,and recorded high infection rate in age group in (4-10)years and age group (more than 50)years 83.33%,86.66% respectively, and lower infection rate in age group (>1)year 55.55% .Showed the method nested multiplex polymerase chain reaction by using 18s rRNA small subunit gene, nested multiplex PCR for these positive samples by using specific primer for E. histolytica revealed a higher infection rate was E. histolytica 73(91.25%) (monoinfection). Entamoeba histolytica high infection rate recorded in the female 97.43% compared the male 82.35%. In addition, E. histolytica recorded the more percentage infection was in age group (less than one year) and (31-50)years was scored 3(100%), 11(100%), respectively. This showed percentage infection Entamoeba histolytica by diagnosis by nested multiplex PCR regarding to gender, age.

***Keywords*---**entamoeba histolytica, molecular investigation, nested multiplex PCR.

Introduction

Entamoeba is a parasitic protozoan that may infect both vertebrates and invertebrates (Matsubayashi et al., 2015; Kawano et al., 2017; Matsubayashi et al., 2018). Although only Entamoeba coli, E. histolytica, E. moshkovskii, E. dispar, E. polecki and E. hartmanni are found in the lumen of the human gut, there are up to twenty-four Entamoeba species recognized worldwide (Dimiceli, 2004). Entamoeba histolytica infection is the world's third leading cause of mortality After malaria and schistosomiasis. Around 180 million individuals are affected globally, with 40,000 to 110,000 people dying per year. (Pestehchian et al., 2011; Al-Areeqi et al., 2017). One of the key causes of the disease's global spread is the lack of sanitary measures and polluted water in developing countries; additionally, the contempt for parasitic infections are growing more frequent in these locations(Watanabe and Petri, 2015). Amoebiasis is a parasitic infection caused by amoeba (intestinal disease caused by the parasite E. histolytica that causes major mortality and morbidity), Roure et al.,(2019).Several research on Entamoeba spp. have been carried out in the Basrah region, including AlYaquob et al., (2008), who employed the PCR method to detected and differentiate Entamoeba spp. Therefore, the aims of this study for detection of infection rate by Entamoeba histolytica according to sex, age, by nested multiplex PCR in diarrheoginic patients' .

The Materials and Methods

Collection of Specimens

There are 133 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, General Refai and General Hussain hospitals 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, sex).

Microscopic examination

At laboratory ,the specimens divided into two parts, each one part takes of about (1-2 gm), the first placed in Eppendrof tube and stored at minus twenty Celsius for DNA extraction, Sufficient quantity of distilled water (10-20 ml) were added to stool spacemen's and mixed well and then examined by direct microscopic on Wet mount smear methods .

Molecular assay

The stool DNA was extracted outsized Presto TM Stool DNA Extraction Kit from company Geneaid/Korea, following instructions steps of manufacturer's were listed below:

Sample lysis step

- Two hundred milligrams of feces was put to a Bead beating tube with ceramic beads, followed by 800l ST1 buffer and a short vortex, then incubate for five minutes at seventy Celsius, then vortex for ten minute at RT. Then, at RT , for two minutes, the sample was centrifuged at 8,000 X g.
- A new 1.5 ml microcentrifuge tube was used to transfer the 500 µl supernatant.

PCR Inhibitor Removal step

- After adding 150 µl of ST2 Buffer, vortex for 5 seconds. After that, it was incubated for 5 minutes at 4°C. Then, at room temperature, insoluble particles and PCR inhibitors were removed by separated for three minutes at 16,000 x g.
- In a 2 ml centrifuge tube, the Inhibitor Removal Column (purple ring) was put. The inhibitor Removal Column was then supplied 500 µl of clear supernatant. Before being discarded, the column was centrifuged at 16,000 x g for one minute at RT.
- For DNA Binding, in a 2 mL centrifuge tube, the flow-through was kept.

DNA Binding step

- The flow-through was given an eight-hundred-microliter from ST3 buffer, which was vigorously mixed for five seconds. Then, in a 2 mL Collection tube, a GD Column (green ring) was put.
- The GD Column received 700 microliters from sample mixture. The flow-through was then separated at 16,000 x g for 1 minute at RT.
- In the 2 ml Collection Tube, place the GD Column. Then, using the GD Column, transfer the remaining sample mixture. At RT, the samples were separated at 16,000 x g for one minute.

Wash step

- In the GD Column, a 400 µL from ST3 Buffer was added. Then separeted for thirty seconds at 16,000 x g in RT.
- The GD Column was reinserted into the 2 ml Collection tube after the flow-through had been discarded.
- The GD Column received 600 microliters of Wash Buffer. Then, at R separated for thirty seconds at 16,000 x g.
- Replace the GD Column in the 2 ml Collection Tube, after discarding the flow-through.
- The dry GD Column collecting Tube was separated at 16,000 x g for three minutes at RT to dry the column matrix.

Elution step

- In a new 1.5 ml tube, the dry GD Column was placed. Then 100 microliter of heated Elution Buffer was poured into the column matrix's middle.

- To allow Elution Buffer to be thoroughly absorbed, the GD Column was left to stand for at least 2 minutes. The purified DNA was then separated fot two minute at 16,000 x g in RT to extract it.

Genomic DNA Estimation

The quality of 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.

Nested multiplex

Three kits were used for DNA extraction from feces of Human, these were provided from Geneaid/ Korea/company. And two pairs primers (forward and reverse) were used for amplification of the 18S rRNA gene by Nested Multiplex PCR. The first primer pair was for the detection of 900bp of 18S rRNA Entamoeba gene. There used primer pairs for the detected of (E.histolytica).This study used NCBI-Genebank and a one plus-primer design. This primer were donated by the Macroge corporation in Korea, for PCR Entamoeba spp(900bp), nested PCR E. histolytic (439bp).

PCR master mix (first round) solution components

All additional PCR reaction components, including primer and probe, are packed in a standard Maxime PCR PreMix 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.

PCR master mix Thermocycler conditions

NO.	PCR step	Temp.	Time.	Repeat
1-	Initial denaturation	95 c	5 min.	1
2-	Denaturation	95 c	30 sec.	35 cycles
3-	Annealing	58 c	30 sec	
4-	Extension	72 c	1 min	
5-	Final extension	72 c	5 min	1
6-	Hold	4 c	Forever	-

Second round of nested multiplex PCR components

The components of the PCR master mix listed in the table above are then put in a standard Maxime PCR PreMix, 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.

Analysis of PCR Product

Following these processes, the PCR results were examined on an agarose gel electrophoresis:

- To 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.
- 5. - 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.

Results and Discussions

In this study was collected 133 stool sample contain of (71 male and 62 female) from patients have symptomatic infection with diarrhea and different age then will undergo for direct microscopically examination. The result was obtained 100 (75.18%) out of 133 stool sample were infected With Entamoeba spp with no significant difference at P<0.05%recarding to gender, age. The result of our study agreement with (Hidajati et al., 2018) who recorded 70% and higher than Entamoeba spp that recorded by Al-Damerchi et al., (2016)in Al-Qadisiyah province and Abozahra et al., (2020) was 68% and 65% respectively. And The results founded the prevalence infection of Entamoeba parasite was higher at female 52(83.87%), than males 48(67.6%), with at p<0.05 statistical variation, see on table (3.1).

Table 3-1
Infection rate of Entamoeba spp according to the gender via microscopic examination

Category	Total No. sample	Postive No. sample	% Percentage
Male	71	48	67.6
Female	62	52	83.87
X2	4.69		

P value	0.03(s)

S:(Significant different at P<0.05)

Our findings of this study aligns with those recounted by Al-Damerchi and Al-Ebrahimi et al., (2016) also conveyed a higher ratio in female by Hamza et al.,(2021) in Iraq. However, in Thi-Qar province showed a higher infection rate recorded in female (50.4%) than the male (49.6%) by Flaih et al., (2021) .Though, Ejaz et al., (2011) & Mengistu et al., (2007) showed the infection percentage of Entamoeba spp was more distribution in female (31.50%) compared with male (19.6%). A variation in infection rate between female and male depend on many of factors such as hormones or because nature works that use by female in home and during food preparation that lead to exposed of water or food contaminated by cyst of Entamoeba . Entamoeba spp found in The consequences to the current revision presented the highest infectors found for the age group (4-10)years and for group (more than 50)years that was 83.33%, 86.66% respectively, while was lower infection rate in group (less than one years) with the percentage 55.55% . agreed with Flaih et al., (2021) in Thi- Qar province in which they recorded high prevalence of infection 27.3% in age group (5-14) and lower prevalence of infection 9.0% in age group (<1)year. Similarly, many of the researchers scored more prevalence infection of Entamoeba spp in young children's they(Saida et al., 2016;Al-Taei et al., 2019;Al-Saqur et al., 2015) in Iraq ,Alreequi et al., (2017) in Yemen ,that they recorded high prevalence of infection (45.3 %) in age of less than ten years but not agreed with recorded lower infection rate (6.1%) in age over 41 years. Would be described by the effect of difference aspects for the children because their activities the result from less carful about their own sanitation . Also ,their exposed highest for nutrients from many bases .While high percentage of infection at older age may be causing of the detail as the aged individuals showing to types elements infections among life them like weakened their immune system, others by use medicines to different pathogens might be opposition of infectious organism Ali and petri, (1999) , Nakada and Nozaki,(2016).

Result of molecular

The first round PCR for DNA samples showed 87 (65.41%) out of 133 stool sample were successfully amplified for 18sRNA gene by nested multiplex PCR. Even though Entamoeba infeceouse are frequently minor in most cases, but there are strains some may be attach the bowel wall(resultant severe extra bowel pathogens and amoebic colities), clinically amoebic dysentery is continuously established during microscopically investigative for publication's' stool specimens. The result of study agreed with Iraq recorded lower percentage from our result in Baquabah city by Ibrahim et al., (2019) was 57.33% (86/150) by PCR and in Thi-Qar province that recorded 52.5% out of 80 positive sample by nested PCR by Flaih et al., (2021) . While recorded Hamzah et al., (2006) and Fallah et al., (2014) a higher percentage by nested multiplex PCR 83.3%, 80.6% respectively .Finally, lowest percentage recorded by Al-Hilfi et al., (2021)30% in Basra province . in India and Malaysia(Khairnar et al., 2007;Lau et al., 2013)lesser positive percentage of PCR test matched with microscopy test via nested PCR method.

The alterations noted by PCR produce might be contributed for many causes, initially result to the differences in method DNA extraction from fecal samples. Moreover, various in the numeral for the parasites in fecal specimens . Lastly, the adhesive and stabilizing causes that use in these study may affect by cell constituents causing of producing DNA(Lopez et al., 2017). The nested multiplex PCR by second round showed Entamoeba histolytic single infection recorded 73(91.25%). Nested multiplex PCR (second round) that increase from sensitivity and specificity of PCR for detected Entamoeba spp in diarrheal patients and agreed with Khairnar et al., (2007); Roshdy et al., (2017). Our findings aligns with Nath et al., (2015) that recorded high prevalence of E. histolytica 55.8% . Previously, there are four studies that based on the molecular diagnosis and reported the incidence off Entamoeba histolytica in Iraq, such as Diwanyha ,Baghdad, Najaf province reported high proportion was(44.3%, 7%, 24%) in the between symptomatic individuals Al-Hameedawi et al. (2014);Hussein et al., (2015),Al-Khalidi et al., (2016).

Nested multiplex PCR showed a higher infection percentage of Entamoeba histolytica by nested multiplex PCR regarding to gender were in female 38(97.43%) by E.histolytica than male 28(82.35%). At these current researches produce were close to those obtained in Thi-Qar province by Ziquer et al., (2021) who reported infection E.histolytica are more prevalence in female 52% than the male 48% nearly for a training to Tasawar et al., (2010). At the present study showed Entamoeba histolytica recorded high infection rate in age groups (less than one year) (31-50)years were3 (100%),11(100%) respective. At those extant learning showed E. histolytica (single infection)was more prevalence from other species in diarrheal individuals this agreed with Bahrami, et al., (2019) were reported infection with E. histolytica (single or mix infection) in all cases with GIDs and this agreed with Mohammed, et al., (2017)in Egypt. But not agreed with Dasheesh, et al., (2016)in Diwania city, reported the more infectious percentage for Entamoeba dispar compared Entamoeba histolytica in symptomatic diarrheal patients.

Conclusions

- By the current report revealed that rate of infection Entamoeba histolytica (mono-infection), more prevalence in diarrheal patients.
- The study recorded a higher infection rate of E. histolytica in the female compared with the male and showed no significant difference at $P<0.05$.
- Although the microscopic examination method is a reliable test of detecting Entamoeba spp in diarrheal patients, the probability misdiagnosis of Entamoeba histolytica that cause amoebic dysentery was high.
- 4- This study showed the infection rate of E. histolytica existed large prevalence in age group (less than one year) and age group (31-50) years .

Recommendations

- All positive specimens in microscopic examination must undergo molecular methods for accurate diagnosis of Entamoeba histolytica.

- Using method (nested multiplex PCR) method for epidemiological studies for known the true prevalence of Entamoeba histolytica parasite in diarrheal patients.

References

1. Al-Areeqi, M. A.; Sady, H.; Al-Mekhlafi, H. M.; et al., (2017). First molecular epidemiology of Entamoeba histolytica, E. dispar and E moshkovskii infections in Ymenen: different species-specific associated risk factors. Tropical Medical International Health, 22, 493-504.
2. Al-Hilfi, A. A.; Al-Malak, M. K.; Al-Tomah, M. A. (2021). A prevalence study of Entamoeba spp in Basrah province using different detection methods .Baghdad Science Journal, 18(4):1163-1172.
3. Ali, R. H. I. and Petri, W. A. (1999). Prevalence and immune response to Entamoeba histolytica in preschool children in Bangladesh . Am. Journal Tropical Medical Hygiene, 60(6):1031-1034 .
4. Al-Khalidi, K. A. H. (2016). Detection of Entamoeba histolytica in patients an infected infants with diarrhea in born and children's hospital by classic methods and real-time polymerase chain reaction. Journal Al Qadisiyah Pure Sci. 21(2):27–35.
5. Al-Damerchi, A. T. N. and Al-Ebrahimi, H. N. (2016). Detection of major virulence factor of Entamoeba histolytica by using PCR technique. Al-Qadisiya medical Journal, 12(21):36-45 .
6. Al-Hameedawi, J. J. Y. (2014). Molecular identification of Entamoeba histolytica parasite by using actin and amebapore-a genes Kufa. Journal Nurs. Science, 4(2):1–8.
7. Al-Taei, A. H. (2019). The prevalence of intestinal parasite among the attending peoples to Al-Hashimyah hospitals for seven years, Babylon province, Iraq. Journal Phys. Conf. Ser. 1294:062022.
8. Al-Yaquob, A. J. (2008). Diagnostic study on the causative agent of amoebiasis by PCR technique and ability of culturing it in Basrah province. MSc Thesis , college of Education, University of Basra, 1-67 .
9. Al-Saqur, I. M.; Al-Warid, H. S.; Al-Qaisi, A. Q.; Al-Bahadely, H. S. (2020). Prevalence of gastrointestinal parasites in Iraq during 2015. In AIP. Conf. Proc. 2290: 020005.
10. Bahrami, E.; Haghighi, A.; Zamini, G. and Khademerfan, M. (2019). Differential detection of Entamoeba histolytica , Entamoeba dispar and Entamoeba moshkovskii in fecal samples using nested multiplex PCR in west of Iran. Journal Epidemiological Infect. 147(96):1-
11. Dasheesh, T. I. A. (2016). Molecular identification of some species of Entamoeba isolated from patients with diarrhea in Afak city/ Al-Qadisiyah governorate using real-time PCR technique. Int J. Recent Sci Res. 7(5):11207-11211.
12. Dimiceli, L. (2004). Distinguishing between pathogenic and nonpathogenic species of Entamoeba. Laboratory Med. 35(10):613–615.
13. Ejaz, M.; Murtaza, G.; Ahmad, M.; Khan, S. A.; Hussain, I.; Ul-Saqib, Q. N.; Bin Asad, M. H. H.; Wasim, A. and Farzana, K. (2011) . Determination of the prevalence of Entamoeba histolytica in human at a private fertilizer company hospital in Pakistan using microscopic technique. Afr. J. of Microbio. Res. Vol. 5(2): 149-152.

14. Fallah, E.; Shahbazi, A.; Yazdanjoii, M.; Rahimi-Esboei, B. (2014). Differential detection of Entamoeba histolytica from Entamoeba dispar by parasitological and nested multiplex polymerase chain reaction methods Journal Anal. Res. Clin. Med. 2(1) 25:29.
15. Flaih, M. H.; Khazaal, R. M.; Kadhim, M. K.; Hussein, K. R.; Alhamadani, F. A. B. (2021). The epidemiology of amoebiasis in Thi-Qar Province, Iraq (2015-2020): differentiation of Entamoeba histolytica and Entamoeba dispar using nested and real-time polymerase chain reaction. Epidemiol. Health, 43:e2021034.
16. Hamzah, Z.; Petmitr, S.; Mungthin, M.; Leelayoova, S.; Chavalitshewinkoon-Petmitr, P. (2006). Differential detection of Entamoeba histolytica, Entamoeba dispar, and Entamoeba moshkovskii by a single round PCR assay. Journal Clinical Microbiology, 44: 3196-3200.
17. Hussein, R. A.; Al-Mayah, Q. S.; Merdaw, M. A-z.; Al-Bashier, N. T.; Al-Abbas, A. A.; Jasem, I. A. (2015). Evaluation of multiplex real-time PCR and ELISA in detection of intestinal protozoan parasites from children with diarrheal disease. Int. J. Adv. Research (Indore), 3(9):782–788.
18. Ibrahim, M. A.; Hussein, R. A. and Al-Mayah, Q. S. (2019). Molecular Identification And Genetic Diversity Of Entamoeba Species From Diarrheic Patients In Baqubah/Iraq. research journal of pharmaceutical biological and chemical sciences, 10(1), 951-960.
19. Kawano, T.; Imada, M; Chamavit, P.; Kobayashi, S.; Hashimoto, T. and Nozaki, T. (2017). Genetic diversity of Entamoeba: Novel ribosomal lineages from cockroaches. Plos. One, 12, 9, e0185233.
20. Khairnar, K. Parija, S. C. (2007). A novel nested multiplex polymerase chain reaction (PCR) assay for differential detection of Entamoeba histolytica, Entamoeba moshkovskii, Entamoeba dispar DNA in stool samples. BMC Microbiology,1471-2180.
21. Lau, Y. L.; Anthony, C.; Fakhrurrazi , S. A.; Ibrahim, J.; thoi, I.; Mahmud, R. (2013). Real-time PCR assay in differentiating Entamoeba histolytica, Entamoeba dispar, and Entamoeba moshkovskii infections in Orang Asli settlements in Malaysia. Parasit. Vectors; 6:250.
22. López-López , P.; Martinez- Lopez, M. C.; Boldo-León, X. M.; Hernández-Díaz, Y.; González-Castro, T. B.; Tovilla-Zárate, C. A, et al. (2017). Detection and differentiation of Entamoeba histolytica and Entamoeba dispar in clinical samples through PCR-denaturing gradient gel electrophoresis. Braz J. Med. Biological Research, 50:e5997.
23. Matsubayashi, M.; Murakoshi, N.; Komatsu, T.; Tokoro, M.; Haritani, M. and Shibahara, T. (2015). Genetic identification of Entamoeba polecki subtype 3 from pigs in Japan and characterization of its pathogenic role in ulcerative colitis. Infect. Genet. Evol. 36, 814.
24. Matsubayashi, M.; Matsuura, Y.; Nukata, S.; Daizi, Y.; Shibahara, T.; Teramoto, I.; Matsuo, T.; Uni, S.; Hatta, T.; Kaneko, A.; Suji, N. and Sasai, K. (2018). First detection and molecular identification of Entamoeba bovis f rom Japanese cattle. Parasitol. Res. (117): 339-34.
25. Mengistu, A.; Gebre-Selassie, S. and Kassa, T. (2007). Prevalence of intestinal parasitic infection among urban dwellers in southwest Ethiopia Ethiop. J. Health Dev., 21(1) : 12-17.rom Japanese cattle. Parasitol. Res. (117): 339-34.

26. Nath, J.; Ghosh, S. K.; Singha, B.; Paul, J. (2015). Molecular epidemiology of amoebiasis: a cross-sectional study among North East Indian population. PLoS. Negl. Trop. Dis. 9:e0004225.
27. Ngui, R.; Lorainne, A.; Siti, A. F.; Yvonne, L. A. L.; Lau, Y. L.; Jamaiah, I. and Rohela, M. (2012). Differentiating Entamoeba histolytica, Entamoeba disbar and Entamoeba moshkovskii using nested polymerase chain reaction (PCR) in rural communities in Malaysia. Parasites and vectors; (5): 187.
28. Pestehchian, N.; Nazary, M.; Haghighi, A.; Salehi, M. and Yosefi, H.; (2011). Frequency of Entamoeba histolytica and Entamoeba dispar prevalence among patients with gastrointestinal complaints in Chelgerd city, southwest of Iran(*). Journal of Research in Medical Sciences : The Official Journal of Isfahan University of Medical Sciences; (16): 1436- 1440.
29. Roshdy, M. H.; Abd El-Kader, N. M.; AliTammam, M.; Fuentes, I.; Mohamed, M. M.; ElSheikhand, N. A, et al. (2017). Molecular diagnosis of Entamoeba spp. versus microscopy in the Great Cairo. Acta. Parasitological, 62(1):188-191.
30. Saida, L. A. (2016). Epidemiological study of intestinal parasites among population of Erbil city, Kurdistan, Iraq. J. Raparin University, 3:75-86
31. Tasawar, Z.; Kausar, S.; and Lashari, M. H. (2010). Prevalence of Entamoeba histolytica in humans. Pak. J. Pharm. Sci. 23, 3.
32. Ziguer, A. M.; Mahdi, D. S. and Khalaf, A. K. (2020). Molecular detection of Entamoeba spp. By using multiplex PCR in Thi-Qar province. Biochem. Cell. Arch. Vol. 20,No.2, PP.6363 – 6367.