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RESEARCH ARTICLE

Molecular Identification of *Entamoeba Histolytica In* Amoebiasis Patients

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Abstract

Infection is the third-greatest parasitic disease responsible for death in the world. Amoebic infections result either in a harmless colonization of the intestine, or in an amoebiosis with invasion and damage of the intestine, liver, lung, and brain. These distinct manifestations are due to the existence of *Entamoeba Histolytica* alone or with *Entamoeba Dispar* as a complex of two different, but morphologically identical species. One that is a nonpathogenic commensal in the intestine of humans, *E. Dispar,* and the other that is capable of inducing cell and tissue damage. Due to genomic DNA differences between pathogenic and nonpathogenic of these protozoan infections, we used a polymerase chain reaction (PCR) method that diagnosed and differentiated the two conditions. DNA extraction protocol using non-fixed stool samples.about60 of 65 stool specimens from patients with amoebiosis was characterized. Among them, 45 (75%) were infected only with the nonpathogenic species, *E. Dispar*, while 15 (25 %) displayed a mixed infection with the pathogenic nonpathogenic species, *E. Dispar* and *E. Histolytica.* The PCR protocol showed a specificity of 1.00 and a sensitivity of 0.95. The molecular approach is therefore reliable and applicable in the identification of pathogenic *E. Histolytica* infection. Present results provided tha importance data for the Iraqi Health Care System and addressed the emerging problems of amoebic infection in Iraqi.

Keywords: *Entamoebahistolytiac, Molecular characterization, E. dispar, Stool sample.*

Introduction

Intestinal protozoan infections are closely related to a lack of proper sanitation and environmental contamination with faecal matter. *Entamoeba Histolytica* prevalence is higher in specific environment that occur most often in developing countries [1-3]. Amoebiasis is a potentially severe and life threatening infection caused by enteric protozoa [3-5], most commonly *Entamoeba Histolytica*, which is distributed worldwide. *Its* infection is the third greatest parasitic disease responsible for death in the world after malaria and schistosomiasis [6-7].

It affects approximately 180 million people, of whom 40,000 to 110,000 die each year [8]. Amoebic infections result either in a harmless colonization of the intestine, or in amoebic invasion and ulceration of the intestine, and damage of other host tissues. This assertion derives from extensive microbial, pathological, immunological, and molecular data that indicate they have a high degree of divergence and are, in fact, two separate species [4-7].

A clinical diagnosis of amoebiosis can be confirmed by microscopic identification of characteristic cysts or trophozoites in the stool. However, microscopic examination has several limitations, [9-11] the most important being the inability to distinguish (italic please) from *E. dispar*. In addition, multiple samples often have to be examined and the presence of cysts of different species such as Entamoeba, Iodamoeba, or Endolimax can make diagnosis difficult.

The epidemiology of Entamoeba can be further studied by serological testing, culturing trophozoites and determining isoenzyme patterns by gel electrophoresis. However, these techniques are laborious, expensive, and time consuming, and are not practical for routine diagnostic laboratories and with serological testing, it may be difficult to distinguish past from present infections [12-15]. Molecular biology has helped to fill this gap.

Identification of *E. Histolytica* by polymerase chain reaction (PCR) was first used in 1991[4]. Since its discovery, PCR and then real-time PCR have been increasingly used for amebiasis diagnosis and showed to provide rapid, sensitive, and specific results, in this study, PCR- based approach for the detection and characterization of the two species of the *E. Histolytica/E. Dispar* complex.

Materials and Methods

Samples collection Stool samples were collected from individuals who sought medical attention for abdominal discomforts and diarrheal diseases, at the parasitological services of the hilla teaching hospitals from January to June2015samples and data were collected according to ethical approval of ministry of health of Iraq. All specimens were studied by routine procedures used for microscopic examination of feces in the Laboratory.

To confirm infection with microorganisms of the italic and or italic please complex, each sample was analyzed using the enzyme test. This test is a commercial enzyme immunoassay kit, and was used according to the manufacturer's instructions, based on the described procedure [16].

Samples were Divided into 3 Groups

- Positive group: Italic please -positive group with 60 individuals who tested positive by both microscopic examination and the enzyme test.
- A negative control group: Including samples from 25 individuals found to be negative by microscopic examination and enzyme test.
- A cross reaction control group with 20 patients infected with other parasites, including all scientific name in italic please by enzyme test.

Extraction of DNA from stool samples. DNA was extracted according to the following protocol. Feces (0.5 grams) were placed in a 1.5-ml micro centrifuge tube, washed once with 1 ml of phosphate-buffered saline solution (pH 7.5), and filtered through gauze. The feces samples must be washed with PBS before lysis of cyst and trophozoites to eliminate soluble contaminants that affect the specificity of the PCR and yield of amplification.

The filtered supernatant was centrifuged at 3000 X g for 5 min, then re-suspended in 500µl of lysis-supporting buffer (LSB, pH 8.0), and added to a 2-ml capped tube containing 500 µl of phenol. The mixture was centrifuged at 12,000 X g for 20 min. The aqueous layer was recovered, extracted with chloroform: alcohol (25:1), and the DNA was precipitated with one volume 500 µl of isopropanol. The pellet was re-suspended in 100 µl of TE buffer (pH 8.0). Isopropanol was used to selectively precipitate DNA without the carbohydrates that are abundant in Entamoeba and could interfere with the amplification reactions.

This protocol resulted in the isolation of DNA of sufficient quality and quantity for sensitive and accurate PCR amplification. The PCR method performed for amplification and detection was a as described by Acuna-Soto and others (16) using Master cycler gradient thermal cycler (Eppendorf). The amplification reactions were performed using 10 µL of DNA extract in a volume of 40 µL reaction mixture that contained a 1× of master mix from Applied Biosystems, 29.25 µL of H2O, 3 µL of MgCl2 (25 mM), 1 µL each of forward and reverse primer (0.5 µM) and 1,75 U of Taq polymerase.

The thermal cycling conditions consisted of 1 cycle of 4 min at 95°C, 30 s at 55°C, and 30 s at 72°C followed by 40 cycles of 30 s at 95°C, 30 s at 55°C, and 30 s at 72°C and last cycle of 30 s at 95°C, 30 s at 55°C, and 5mn at 72°C. The primers for *E. Histolytica* and *E. Dispar*: Eh -196F (5′-AAA TGG CCA ATT CAT TCA ATG A-3′) Ed-185F (5′-GTA TTA GTA CAA AGT GGC AAT TTA TGT-3′) Ehd-294R (5′-CAT TGG TTA CTT GTT AAA CAC TGT GTG-3′).

Four controls were included in all experiments: 1) all reagents except DNA template, 2) control DNA from *E. Hisotolytica*, 3) control DNA from *E. Dispar*, and 4) a mixture of control DNA from *E. histolytica* and *E. Dispar*. Amplified products were visualized with ethidium bromide after electrophoresis on 10 % acrylamide gels. Acrylamide gels were used to ensure proper differentiation of the amplified products, which differ in length by just 36 nucleotides. Visualization was accomplished *via* ultraviolet illumination.

Results

We characterized 60 of 65 stool specimens from patients with amoebiosis. Among them, 45 (75%) were infected only with the nonpathogenic species, *E. Dispar*, while 15 (25%) displayed a mixed infection with both the pathogenic nonpathogenic species, italic please. Primer specificity.

Amplification was specific for each primer pair. The *E. Histolytica* primers (EhP1/2) amplified DNA from the HM1-IMSS strain but not from *E. Dispar* whereas the E. dispar primers (EdP1/2) amplified DNA from *E. Dispar* but not from HM1-IMSS. When parasites from both control strains were mixed and specific DNAs were amplified using a mixture of the two primer pairs, the two 96 and 132 bp fragments were visualized after electrophoresis and staining with ethidium bromide.

No interference was noted between the two amplification systems. Detection limit. Both *E. Histolytica and E. Dispar* DNA were detected by the PCR, even at the minimum parasite concentration tested (100 parasites/0.5 grams of feces) (Fig 1). This indicates that up to 10-1 pg of DNA could be detected by this procedure as only 10 µl DNA (100 parasite/100 µl of TE) was used for amplification.

PCR specificity and sensitivity. No products were detected when samples from the negative control group and the cross-reaction control group were tested by the PCR. This represents a maximum specificity (1.00) and no collateral cross-reactions. Results of the PCR with samples from infected individuals showed a sensitivity of 0.95 and indicated circulation of both *E. Histolytica* and *E. Dispar*.

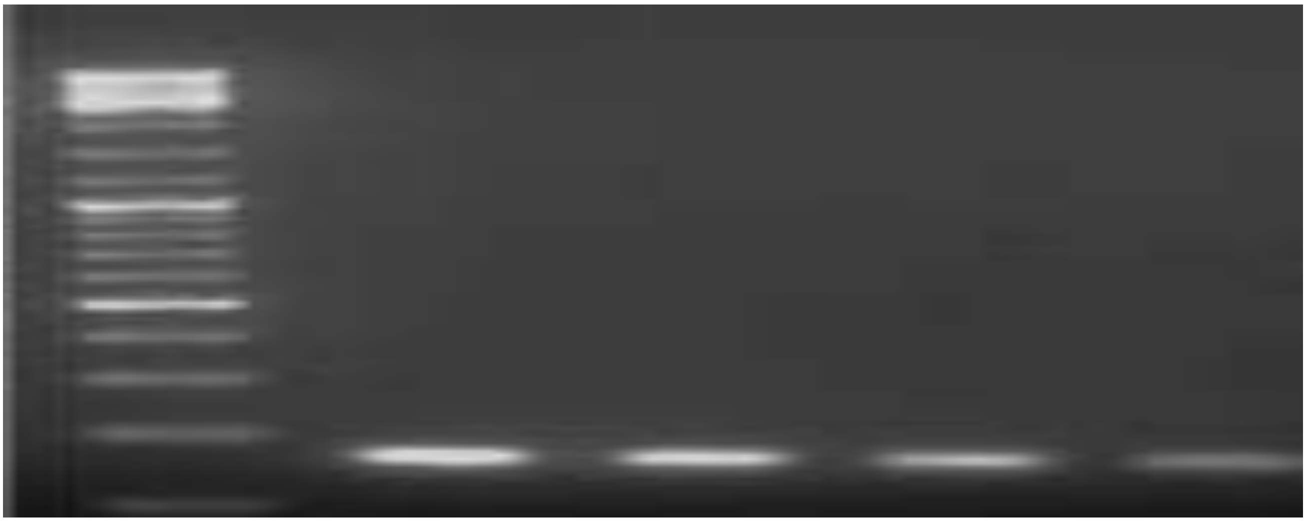

Figure 1: amplifications pattern of E. histolytica .well must be labeled, negative and positive control must be appeared in figure please

Discussion

Present study using PCR technique confirmed that a total of 60 out of 65 cases examined samples were infected with E. *Histolytica*. and E. Dispar Among them, 45 (75%) were infected only with the nonpathogenic species, *E. Dispar,* while 15 (25%) displayed a mixed infection with both the pathogenic nonpathogenic species, *E. Dispar* and *E. Histolytica*. Other investigators have also found that infection with *E. Dispar* is more common than infection with *E. Histolytica* (17-22). Evidence of the inverse proportion has been reported by and others (23) who targeted the same specific and tandemly repeated DNA sequences described in the current study and found italic please as the predominant population. Similarly, the occurrence of mixed infections with both *E. Histolytica* and *E. Dispar* has been reported,(24-28). Our study confirm that molecular diagnostic approaches is superior to all others laboratories methods in detection and differentiating both the pathogenic and nonpathogenic species of E. Histolytica and *E. Dispar* in stool samples (25, 27, 29).

Moreover, the improvements and simplification of PCR procedures directly from stools make it superior to others related stool tests. The protocols are accurate and simple. Several reports, (29-33) found a clear association between in terms of co infection, although no *E. Histolytica, E. Dispar* mixed infections were detected.

They did not discount the possibility of a competitive phenomenon in vivo between *E. Histolytica* and *E. Dispar,* but referred to an in vitro study (34) showing that only a minuscule amount of E. Histolytica can ultimately outgrow E. Dispar in culture in a given period of time. In contrast, others (35) reported that some pathogenic amoebic zymodemes outgrow others.

These findings suggest that an adequate animal model of amoebiosis needs to be developed as a prerequisite to clarify this phenomenon (35). In conclusion, our data indicates that molecular approach is reliable and applicable in the identification of pathogenic Histolytica infection. Our results provide important data for the Public Health-Care System and a need to address the emerging problem of amoebiosis in Iraq.

References

1. Ojha SC, Jaide C, Jinawath N, Rotjanapan P, Baral P (2014) Geohelminths: public health significance. J. Infect Dev. Ctries., 8:5-16.
2. Oliveira FMS, Neumann E, Gomes MA, Caliari MV (2015) *Entamoeba dispar:* could it be pathogenic. Trop. Parasitol., 10: e0137-327.
3. Calegar D A, B C Nunes, K J Lima Monteiro, et al (2016) Frequency and molecular characterization of *Entamoeba Histolytica, Entamoeba Dispar, Entamoeba moshkovskii, Entamoeba Hartmanni* in the context of water scarcity in northeastern Brazil .Mem Inst Oswaldo Cruz., 111(2): 114-119.
4. Singh A, Houpt E, Petri WA (2009) Rapid diagnosis of intestinal parasitic protozoa, with a focus on *Entamoeba Histolytica*. Interdiscip Perspect Infect Dis., 547090.
5. Sargeaunt PG, Williams JE, Grene JD (1978) The differentiation of invasive and non-invasive *Entamoeba Histolytica* by isoenzyme electrophoresis. Trans R Soc. Trop. Med. Hyg., 72: 519-521.
6. Sargeaunt PG (1987) The reliability of *Entamoeba Histolytica zymodemes* in clinical diagnosis. Parasitol. Today, 3: 40-43.
7. Torian BE, Reed SL, Flores BM, Creely CM, Coward JE, Vial K, Stamm WE (1990) The 96-kilodalton antigen as an integral membrane protein in pathogenic *Entamoeba Histolytica:* potential differences in pathogenic and nonpathogenic isolates. Infect Immun., 58: 753–760.
8. Pestehchian N, Nazary M, Haghighi A, Salehi Mand, 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.
9. Ortner S, Kroschewski H, Clark CG, Scheiner O, Wiedermann G, Duchene M, (1997) Molecular cloning and biochemical characterization of hexoquinases and phosphoglucomutases from *Entamoeba Histolytica* and *Entamoeba Dispar*. Arch. Med. Res., 28: 81-82.
10. Fonte L, Ferna´ndez MA, Sanchez L, Marn H, Montano I, Nunez YO (1998) Demonstration, using Enzymeba Test, of the overdiagnosis of intestinal amebiasis associated to the micro scopical examination of faeces. Report of a study in Cienfuegos, Cuba. Rev Pathol. Trop., 27: 195-200.
11. Haque R, Ali IKM, Petri WA, 1998. Comparison of PCR, isoenzyme analysis, and antigen detection for diagnosis of *Entamoeba Histolytica* infection. J. Clin. Microbiol., 36: 449-452.
12. Sehgal R, Abd-Alla M, Moody AH, Chiodini PL, Ackers JP (1995) Comparison of two media for the isolation and short- term culture of *Entamoeba Histolytica* and *E. Dispar*. Trans. R Soc. Trop. Med. Hyg., 89: 394.
13. Haque R, Neville LM, Hahn P, Petri WA (1995) Rapid diagnosis of Entamoeba infection by using Entamoeba and *Entamoeba Histolytica* stool antigen detection kits. J. Clin. Microbiol. 33: 2558-2561.
14. Caballero-Salcedo A, Viveros- Rogel M, Salvatierra B, Tapia- Conyer R, Sepulveda-Amor J, Gutierrez G, Ortiz-Ortiz L (1994) Sero epidemiology of amebiasis in Mexico. Am J. Trop. Med. Hyg., 50: 412-419.
15. Pillai DR, Keystone JS, Shepard DC, MacLean JD, MacPherson DW, Kain KC, (1999) *Entamoeba Histolytica* and Entamoebadispar: epidemiology and comparison of diagnostic methods in a setting of nonendemicity. Clin. Infect Dis., 29: 1315-1318.
16. Acuna-Soto R, Samuelson J, De Girolami P, Zarate L, Millan-Velasco F, Schoolnick G, Wirth D (1993) Application of the polymerase chain reaction to the epidemiology of pathogenic and nonpathogenic *Entamoeba Histolytica.* Am J. Trop. Med. Hyg., 48: 58-70.
17. Haque R, Ali IM, Petri WA (1999) Prevalence and immune response to *Entamoeba Histolytica* infection in preschool children in

Bangladesh. Am J. Trop. Med Hyg.., 60: 1031-1034.

18. Walderich B, Weber A, Knobloch J (1997) Differentiation of *Entamoeba Hystolytica* and Entamoebadispar form German travelers and residents of endemic areas. Am J. Trop. Med. Hyg., 57: 70-74.
19. Gathiran V, Jackson TFGH, (1985) Frequency distribution of *Entamoeba Histolytica* zymodemes in a rural South African population. Lancet., 1: 719-721.
20. Martinez MC, Munoz O, Garduno G, Sanchez ME, Valadez A, Palacios O, Calderon M, Isibasi A, Gutierrez G (1990) Path- ogenic and non-pathogenic zymodemes of *Entamoeba Histolytica* in a rural area of Mexico: concordance with serology. Arch. Invest Med., 21 (1): 147–152.
21. Rivera WL, Tachibana H, Kanbara H, (1998) Field study on the distribution of *Entamoeba Histolytica* and *Entamoeba Dispar* in the northern Philippines as detected by the polymerase chain reaction. Am J. Trop. Med. Hyg., 59: 916-921.
22. Gatti S, Mahdi R, Bruno A, Cevini C, Scaglia M (1998) A survey of amoebic infection in the Wonji area of Central Ethi- opia. Ann Trop. Med. Parasitol., 92: 173-179.
23. Acuna-Soto R, Samuelson J, De-Girolami P, Zarate L, Millan- Velasco F, Schoolnick G, Wirth D (1993) Application of the polymerase chain reaction to the epidemiology of pathogenic and nonpathogenic *Entamoeba Histolytica*. Am J. Trop. Med. Hyg., 48: 58-70.
24. Petithory JC, Brumpt LC, Poujade F (1994) Entamoebahistolytica (Schaudinn 1903) and *Entamoeba Dispar* (E. Brumpt 1925) are 2 different species. Bull Soc. Pathol. Exot., 87: 231- 237.
25. Moody S, Becker S, Nuchamowitz Y, Mirelman D (1997) Virulent and a virulent *Entamoeba Histolytica* and Entamoeba dis par differ in their cell surface phosphorylated glycolipids. Parasitology, 114 (2): 95-104.
26. Mirelman D, Nuchamowitz Y, Stolarsky T (1997) Comparison of enzyme-linked immunosorbent assay-based kits and PCR amplification of rRNA genes for simultaneous detection of *Entamoeba Histolytica* and *E. Dispar*. J. Clin. Microbiol., 35: 2405-2407.
27. Haque R, Ali IM, Petri WA (1999) Prevalence and immune response to *Entamoeba Histolytica* infection in preschool children in Bangladesh. Am J. Trop. Med. Hyg., 60: 1031-1034.
28. Gatti S, Mahdi R, Bruno A, Cevini C, Scaglia M (1998) A survey of amoebic infection in the Wonji area of Central Ethi- opia. Ann Trop. Med. Parasitol., 92: 173-179.
29. Rivera WL, Tachibana H, Kanbara H (1998) Field study on the distribution of *Entamoeba Histolytica* and *Entamoeba Dispar* in the northern Philippines as detected by the polymerase chain reaction. Am J. Trop. Med. Hyg., 59: 916-921.
30. Martinez MC, Munoz O, Garduno G, Sanchez ME, Valadez A, Palacios O, Calderon M, Isibasi A, Gutierrez G (1990) Path- ogenic and non-pathogenic zymodemes of *Entamoeba Histolytica* in a rural area of Mexico: concordance with serology. Arch. Invest Med., 21(1): 147-152.
31. Rivera WL, Tachibana H, Kanbara H (1998) Field study on the distribution of *Entamoeba Histolytica* and *Entamoeba Dispar* in the northern Philippines as detected by the polymerase chain reaction. Am J. Trop. Med. Hyg., 59: 916-921.
32. Gatti S, Mahdi R, Bruno A, Cevini C, Scaglia M (1998) A survey of amoebic infection in the Wonji area of Central Ethi- opia. Ann Trop. Med. Parasitol., 92: 173-179.
33. Newton-Sanchez OA, Sturm K, Romero JL, Santos JI, Samuel- son J (1997) High rate of occult infection with *Enatamoeba Histolytica* among non-dysenteric Mexican children. Arch. Med. Res, 72: S245-S247.
34. Clark CG, Diamond LS (1993) *Entamoeba Histolytica:* an ex- planation for the reported conversion of "nonpathogenic" amebae to the "pathogenic" form. Exp. Parasitol., 77: 456-460.
35. Gatti S, Cevini C, Bruno A, Ramsan M, Marchi L, Scaglia M (1997) First isolation and characterization in humans of *Ent- amoeba Histolytica* (laboratory made) zymodemes XX. Parasitol. Res, 83: 716-718.

