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Original Article

Comparison of IFAT, rk39 and PCR for diagnosis of kala-azar in Iraqi children

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ABSTRACT

A comparative study was carried out through IFAT, rk39 and PCR diagnostic methods for detection of pediatric visceral leishmaniasis (Kala-azar) in Baghdad hospitals during the period from July 2013 to September 2014. Blood samples from 178 suspected VL patients were examined by IFAT, rk39 and PCR. Results of polymerase chain reaction which depending on highly repetitive sequence regions, reported that only 39 (21.9%) of samples were positive to visceral leishmaniasis and 139 (79.1%) were negative. So that these results were considered as standard test for IFAT and rk39 test, for that the validity of IFAT according to PCR technique detected the sensitivity of IFAT was (100%) and specificity (82%) with accuracy (89%). While the validity of rK39 test according to PCR technique, it was found that the sensitivity of rK39 (97.4%) and specificity (85.2%) with accuracy (90%). The study concludes that these diagnostic methods had an excellent ability of discrimination in diagnosis of VL cases.

Key words: Visceral leishmaniasis, IFAT, rk39 and PCR

INTRODUCTION

Leishmaniasis is endemic in 98 countries and 3 territories on five continents; it is estimated that there are 2 million new cases annually worldwide and up to 350 million people at risk of the disease [1,2]. The parasite invades internal organs such as spleen, liver and bone marrow which usually with mortality rate almost 100% if left untreated[3]. The amastigotes stage of the parasite replicates in macrophages of the mononuclear phagocyte system and then spread to the entire reticuloendothelial system resulting Kala-azar [27]. Laboratory diagnosis is important for VL because clinical evaluation is not enough for conclusive diagnosis[4]. The sensitivity culture and microscopic examination tends to be low and highly variable, depending on the number of the parasites in sample and technical skills of the personnel [5]. Accurate diagnosis to guide treatment is the first step to achieve the goal of VL elimination. Up until the 1990's accurate visceral leishmaniasis (VL) diagnosis was complex and invasive including parasitological confirmation by direct microscopic examination of splenic, lymph gland, or bone marrow aspirate or culture of the blood, bone-marrow, lymph nodesor spleen [6]. The rK39 dipstick test is the product of a gene cloned from Leishmania containing 39 amino acid repeat conserved among Leishmania species[(7)].A recombinant antigen, rK39 has been shown to be specific for antibodies arising during VL caused by member of the *L. donovani* complex. It is highly sensitive and predictive for onset of acute disease and evokes high antibody titers of VL patients [8]. The indirect fluorescent antibody (IFA) test is one of the commonly tests used for anti-leishmanial antibody detection by using fixe Promastigotes. The test is based on detecting Ig Gantibodies against Leishmaniaspp, which are demonstrated in the serum very early and different stages of infection but are undetectable up to nine months after cure [9]. Various PCR amplification targets nuclear DNA, such as the small subunit ribosomal RNA gene, internal transcribed spacer regions, the gp63 gene locus and extra chromosomal DNA including the repetitive kinetoplast DNA (kDNA) minicircles [10]. Diagnosis of VL using conventional PCR methods has advanced technique that increasing in sensitivity of assay with optimization of protocols and permitting detection of parasite even before the appearance any clinical symptoms, also applicability to wide variety of clinical material by peripheral blood, lymph node, bone marrow, serum, skin, and urine. Also analysis of archival materials like Giemsa stained bone marrow aspirates, formalin-fixed tissue and skin biopsy by

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using of PCR for monitoring the parasite load and species typing are important to detect effective treatment designing [11].To have reliable, scientific and applicable knowledge about the comparative diagnosis of VL disease with its features, this study was designed and done.

MATERIAL AND METHODS

Study groups: Comparative study was carried out during the period from July 2013 to September 2014. The age of patients ranged from 8 months to 13 years . Two study groups were involved:

A- Blood and serum obtained from a total of one hundred and seventy eight clinical suspected patient of Kala-azar who had examined and defined as suspected cases by specialized physician, from this number fifty confirmed children pick up with visceral leishmaniasis which included in this study from four hospitals; Baghdad teaching children hospital, Ibin-albalady children hospital, Al-Elwyia children hospital, Central child hospital, Al-Kademia children hospital. B-Blood and serum obtained from a total of fifty healthy control group were involved in this study from Baghdad province including health centers and Ibin-Albalady children hospital ,they were apparently healthy by specialist physical examination with no history of kala-azar . All samples were stored at -20ºC until use.

Samples collection: About 5 ml of venous blood was collected from each child, the blood was collected into a sterile screw plastic tube, left for 30 minute at room temperature, then centrifuged at 3000 rpm for 5 minutes, then the serum was collected in another sterile tubes for serological examination while 2 ml of blood sample collected in EDTA tube for PCR technique all samples stored in freeze at −20ºC[14].

Serological examination

IFAT: Indirect fluorescence antibody test was carried out according to manufacture procedure (leishmanial IFA IgG vircell microbiologists, SPAIN) briefly serum of patient was diluted by phosphate buffer saline(PBS) (1/40 dilution) and 20 µl of diluted serum was applied in the slide wells. Slide was incubated in a humid chamber for 30 minutes at 37C. The slide rinsed briefly with a gentle stream of PBS and then immersed for ten minutes in PBS and dip washed briefly in distilled water. 20µl of anti-human IgG FITC conjugate solution was added to each well. One drop of mounting medium was added to each well and carefully covered with a cover slip. Fluorescence microscope was used to read the slid with magnification power at 400x[30,32].

rk39: Antibody response by the Recombinant K39 Immuno chromatographic strip Test (In Bios International, USA) was determined by the manufactures instruction. Thirty µL of serum was added to the dipstick and then placed vertically in a test tube. Two drops of the chase buffer solution provided with the dipstick kit were added to the test tube. The results were read after five minutes. A band in the test region considered a positive result. The control line should be positive [31]. PCRDNA was extracted by using DNA Mini-prep Kit (Promega, USA) according to the manufactures instruction the primers was carried out essentially as described by[12].

F: (5'TATTGGTATGCGAAACTTCCG3')
R: (5'ACAGAAACTGATACTTATATAGCG3')
PCR reaction mixtures (25 µl of total volume) consisted of 12.5 µl of PCR Master Mix (Promega, USA),2.5 µl of each primer, and 3µl of the DNA samples. DNA amplification was performed by a first cycle at 94°C for 5 min, followed by 45 cycles at 94°C for 1 min,55°C for 1 min, and 72°C for 1 min and 30s, with a final extension cycle at 72°C for 7 minutes.PCR products were analyzed on agarose gels of 1.5%by electrophoresis at 75 V in 1× Tris–Boric–EDTA buffer(0.04 M Tris–boric and 1 mM EDTA pH 8.0) and visualized by ultraviolet light after being stained with ethidium bromide[25, 29].

RESULTS AND DISCUSSIONS

Detection of IFAT validity depending on PCR technique for VL diagnosis: Human Visceral leishmaniasis has been identified in Iraq for more than eight decades causing a serious public health problem with a high risk of morbidity, mortality and economical costs[33].Blood samples from 178 suspected VL patient were examined by IFAT and PCR in the collage of Health and Medical Technology-Baghdad laboratories. The sensitivity–specificity and positive–negative predictive values were calculated for these diagnostic tests. The results of PCR technique as standard test were compared with IFAT and PCR which have consistently been highly diagnostic value than microscopic examination , culture and serological test especially in samples with very low parasite loads for VL diagnosis [13]. Table (1) showed that the validity of IFAT according to PCR technique detected it was found that the sensitivity of IFAT (100%) and specificity (82%) with accuracy (89%), for that IFAT assays had an excellent diagnostic value through its ability of diagnosing VL cases. Also IFAT

obtained optimal diagnostic specificity which was 82% and the sensitivity of antibody detection was 100% [32].

Table(1): Validity of IFAT in diagnosis of VL cases.

IFAT		PCR		Total	Validity Test	%
		Positive	Negative			
Positive	N	39	11	50	Sensitivity	100 %
					Specificity	82 %
Negative	N	0	50	50	Positive Predictive Value	78 %
					Negative Predictive Value	100 %
Total	N	39	61	100	Accuracy	89 %

IFAT is an easy test but it has a large scale of sensitivity [14]but it is expensive and require immune fluoresent microscope. Cross-reaction can be demonstrate between sera of patient with Brucellosis, toxoplasmosis, typhoid fever, tuberculosis and *L. donovani* antigen [15] must be done. In the present study, the sensitivity of IFAT was 100%% and this result is compatible with the results reported by [16,17,18]which was 100% for all, while results of Al-Asady (2009)[15]in his Iraqi study of children found 95.2%, and Al-Timmimi (2007)[14]recorded 96.3% , which was higher than the results of Mikaeili*et al*. (2007)[19] that was 80.3%. The variation in sensitivity of IFAT in diagnosis of VL may be due to genetic differences in the individual patients or age factor affecting the level of antibody response, regional differences may explain results why visceral leishmaniasis of Indian patient occurs among individuals of all ages, and mostly with a mean age of 20 years, while in Iraq and Iran kala-azar occurs mainly among infants and children [20,14, 32].

Detection of rK39 strip test validity depending on PCR technique for VL diagnosis: Blood samples from 178 suspected VL patients were examined by rK39 dipstick test in collage of health and medical technology-Baghdadlaboratories. The sensitivity–specificity and positive–negative predictive values were calculated for these diagnostic tests. Thirty nine (21.9%) of 178 blood samples were positive and 139 (79.1%) blood samples were negative according to PCR. The result of rK39 test was compared with PCR technique as standard test. Table (2) showed the validity of rK39 test according to PCR technique. It was found that the sensitivity of rK39 (97.4%) and specificity (85.2%) with accuracy (90%).ROC curves were drawn to assess the immunodiagnostic assay rK39 and PCR applied for VL diagnosis. This analysis was showed that both assays had an excellent ability discrimination of PCR and rK39 in diagnosis of VL cases [30,31].

Table(2): Validity of rK39test in detection of VL cases.

rK-39		PCR		Total	Validity test	%
		Positive	Negative			
Positive	N	38	9	47	Sensitivity	97.4 %
					Specificity	85.2 %
Negative	N	1	52	53	Positive Predictive value	80.85 %
					Negative Predictive value	98.113 %
Total	N	39	61	100	Accuracy	90 %

Immuno chromatographic strip test based on rK39 antigen is a rapid test can be used in difficult field conditions [21]. Variation of sensitivity differ from one region to another, in Indian subcontinent the test is sensitive 100% while in Sudan the sensitivity was 67% [22,31]. The sensitivity of rK39 IC strip test which reported in this study 97.4% was nearly compatible with the results reported by[23] in Iran (100%) and [14] in Iraq (92.6%)and higher than the results reported by Delgado *et al*., (2001)[24]in Venezuela (88%) and Boelaert*et al*. (2004)[25]in Nepal (87.4%), Chappuis*et al*., (2005)[26]in Uganda (82%) and Ethiopia (71.7%). The variety in sensitivity of rK39 IC strip test may be due to the antibody level which affected by age and degree of parasitism, also genetic differences in the individual patients causes differences in the test accuracy among subspecies of *L. donovani* complex.

Polymerase chain reaction: Molecular assessment including Polymerase chain reaction (PCR) technique was used to confirm the diagnosis of *Leishmania* and amplification of the specific gene kinetoplast DNA(kDNA). The DNA of all samples(healthy and suspected) was extracted by using DNA MiniPrep™ Kit. The results were detected by electrophoresis on 1.5% agarose gel and exposed to UV light in which the DNA appeared as bands (figure-1)[29].

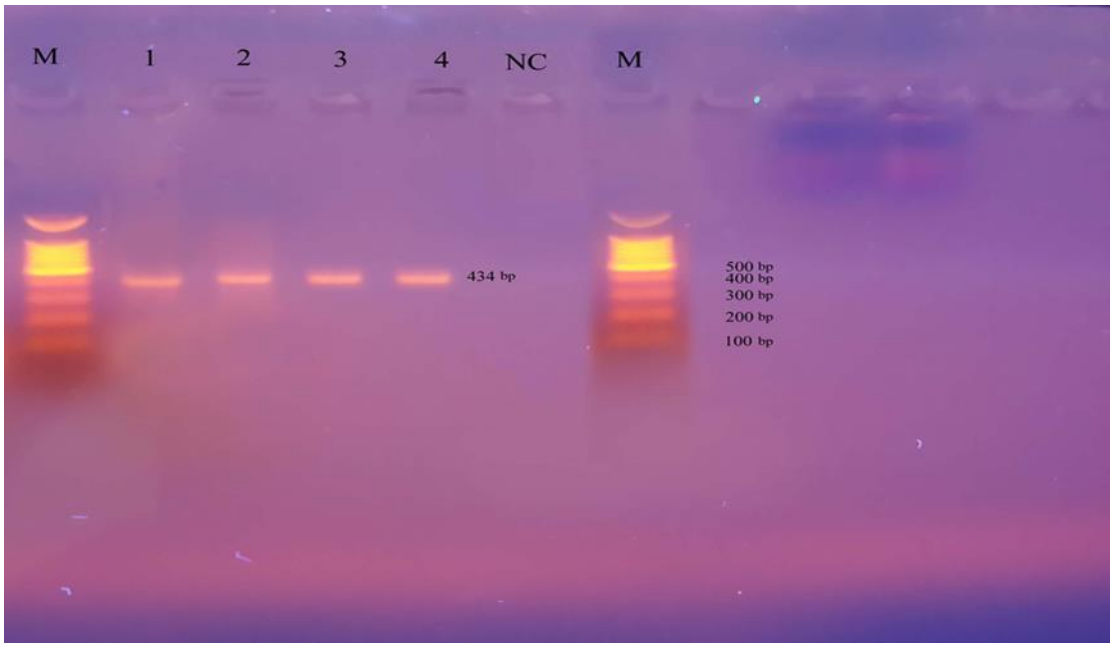

Figure -1: Polymerase Chain Reaction (PCR) amplification of blood DNA from suspected patients for kala-azar using specific primers for the diagnosis of highly repeated region.Lane-M, molecular weight marker (100-bp ladder, DNA marker).Lanes 1-4 positive (*Leishmania donovani* DNA) at 434-bp diagnostic band; Lane-NC negative control sample from apparently healthy persons, no DNA band seen.

From a total of 178 suspected samples were examined by polymerase chain reaction depending on (highly repetitive sequence regions), only 39 (21.9%) samples were positive to visceral leishmaniasis also 50 blood sample from healthy individuals as control were included in this study and all these samples were negative when examined by PCR. All samples stored freezing at -20ºC until use. The confirmed samples and control was compared with isolated DNA in the gel electrophoresis to determine the molecular weight of the experienced DNA by using DNA marker. This primer pair amplifies a 427-bp DNA fragment specific for *L. major*, a 389-bp DNA fragment specific for *L. donovani*, a 403-bpDNA fragment specific for *L. tropica*, and a 434-bp DNA fragment specific for *L. infantum*parasites [12, 28]. The present findings showed that PCR reaction may be suitable method to detect Kala-azar characterization.

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REFERENCES

1. World Health Organization. Control of the Leishmaniases. WHO Technical Report Series 949. WHO, Geneva, Switzerland, 2010.
2. Alvar J,Vélez ID, Bern C,Herrero M,Desjeux P, Cano J,JanninJ. and DenBoer M. WHO Leishmaniasis Control Team,Leishmaniasis worldwide and global estimates of itincidence. PLosONE 20127;e35671 .
3. World Health Organization.Leishmania and HIV in gridlock.WHO/UNAIDS report. Geneva: World Health Organization1998; pp. 15-25.
4. Shyam S andRai M. Laboratory diagnosis of visceral leishmaniasis. Clinical and diagnostic laboratory immunology 2002;9(5):951-958.
5. Mary C,Faraut F,Lascombe L. andDumon, H. Quantification of Leishmania infantum DNA by real-time PCR assay with high sensitivity, J. Clin. Microbiol2004; 42(11): 5249-5255.
6. Srividya G,kulshretha A,Singh R. andSalotra P.Diagnosis of Visceral Leishmaniasis : development over the last decade.Parasitol Res 2012; 110:1065-1078.
7. Sharma N.L,Mahajan V.K,NegiA.K. and VermaG.K. The rK39 immunochromatic dipstick testing: A study for K39 seroprevalence in dogs and human leishmaniasis patients for possible animal reservoir of cutaneous and visceral leishmaniasis in endemic focus of Satluj river valley of Himachal Pradesh(India). Indian J. DermatolVenerolLeprol2009; 75(1):52-55.
8. El-MoamlyA,El-SweifyM. andHafeez M. Performance of rK39 immunochromatographyand freeze-dried direct agglutination tests in the diagnosis of imported visceral leishmaniasis. 2012; Parasitol Res 110:349–354.
9. Pedras M.J, de Gouvea V.L, de Oliveira E. J. and Rabello A. Comparative evaluation of direct agglutination test, rK39 andsoluble antigen ELISA and IFAT for the diagnosis of visceralleishmaniasis. Trans R Soc Trop Med Hyg2008; 102: 172 – 178.
10. BensoussanE,NasereddinA, JonasF, SchnurL.F. and JaffeC.L. Comparison of PCR assays for diagnosis of cutaneous leishmaniasis. JClinMicrobiol Apr 2006;44(4):1435-1439.
11. Srividya, I.; Tirupataiah, S. and Mishra, K. Yeast Transcription Termination Factor Rtt103 Functions in DNA Damage Response. PLoS ONE 2012;7(2): e31288.
12. AllahverdiyevA.M,Bagirova M, Elcicek S,KocC. R, Baydar S.Y, Findikli N. and OztelO.N.Adipose Tissue-Derived Mesenchymal Stem Cells as a New Host Cell in Latent Leishmaniasis.AmJ Trop Med Hyg2011; 85(3): 535–539.
13. ReithingerR. and DujardinJ.C. Molecular diagnosis of leishmaniasis: current status and future applications. J ClinMicrobiol2007; 45:21–25.
14. Al-TimmimiM.F.Visceralleishmaniasis complicated by bacterial infections among Iraqi children. M.Sc. Thesis, College of Medicine, Baghdad University,2007.
15. Al-Asady R. A.Some Epidemiological, Clinical and Diagnostic Aspects with Study of Autoantibodies Production in Visceral Leishmaniasis.Ph.D. Thesis.College Medicine, Baghdad University,2009.
16. Sinha R. and Sehgal S. Comparative evaluate of serological tests in Indian kala-azar. J Trop Med Hyg1994; 97: 333-340.
17. Sundar S, Singh G. S, Sigh V. P, Singla N, Kumar K. and Vinayak V. K. Comparative evaluation of DAT, IFAT and micro-ELISA in the serodiagnosis of Indian kala-azar. J Parasitic Dis1996; 20: 41-43.
18. Al-Alak S.F. Study of epidemiology of kala-azar in Al-Magger district – Missan province. M.Sc. Thesis, College of Veterinary Medicine, Baghdad University,1997.
19. Mikaeili F, Fakhar M,Sarkari B, Motazedian M. H. and Hatam G. Comparison of serological methods (ELISA, DAT and IFAT) for diagnosis of visceral leishmaniasis utilizing an endemic strain. Iran J Immunol2007; 4: 116-121.
20. Halawani A. and Guirges S. Studies on the epidemiology of infantile kala-azar in Iraq with special reference to leishmanin test. J EygptPubl Health Ass1973; 48: 369-383.
21. Sundar S. and Rai M.Laboratory diagnosis of visceral leishmaniasis. ClinDiag Lab Immunol2002; 9: 951-958.
22. Zijlstra E. E, Nur Y,Desjeux P, Khalil E. A, El-Hassan A. M. and Groen J. Diagnosing visceral leishmaniasis with the recombinant K39 strip test: experience from the Sudan. Trop Med Int Health2001; 6: 108-113.
23. Edrissian H,Shamssi S,Mohebali M,Hajjaran H. and Mamishi S. Evaluation of rapid "dipstick rK39" test in diagnosis and serological survey of visceral leishmaniasis in humans and dogs in Iran. Arch Iranian Med2003; 6: 29-31.
24. Delgado O, Feliciangel M. D,Coraspe V, Silva S, Perez A. and Arias J. Value of a dipstick based on recombinant RK39 antigen for differential diagnosis of American visceral leishmaniasis from other symptomatic endemic disease in Venezuela. Parasite200; 18: 355-357.
25. Boelaert M, Rijal S. andRegmi S.Comparative study of the effectiveness of diagnostic tests for visceralleishmaniasis. Am J Trop Med Hyg2004; 70: 72-77.
26. Chappuis F, Muller Y, Nugimfack A, Rwakimari J. B, Couffignal S, Boelaert M. and Cavailler P. Diagnostic accuracy of two rK39 antigen-based dipsticks and the formal gel test for rapid diagnosis of visceral leishmaniasis in Northeastern Uganda. J ClinMicrobiol2005; 43: 5973-5977.
27. Be-Nazir Ahmed Shah GolamNabi,MizanurRahman,ShahjadaSelim, Ariful BasharMd,Mahbubur Rashid,FahimaYeasmin Lira,Tanveer Ahmed Choudhury and Dinesh Mondal . Kala-azar (Visceral Leishmaniasis) Elimination in Bangladesh: Successes and Challenges. Current Tropical Medicine Reports 2014; 1(3): 163-169.
28. Abdul Mabood Khan, PrafullaDutta, Siraj Ahmed Khan, Swaroop Kumar Baruah, Dina Raja, Kamal Khound*et al*. Kala-azar and Post–Kala-azar Dermal Leishmaniasis, Assam, India.Emerg Infect Dis 2014; 20(3): 487–489.
29. KhanA.M,Pandey K, Kumar V,DuttaP, Das P. andMahantaJ.Sample survey for indigenous cases of kala-azar in Assam by rk39 dipstick test. Indian J Med Res. 2009;129:327–328.
30. Diogo Tiago da Silva1, Wilma Aparecida Starke-Buzetti1, Maria Fernanda Alves-Martin,*et al*.Comparative evaluation of several methods for Canine Visceral Leishmaniasis diagnosis. ***Rev Bras Parasitol Vet***
31. Mohapatra TM, Singh DP,Sem MR,Bharti K. andSundar S. Comparative evaluation of rK9, rK26 and rK39 antigens in the serodiagnosis of Indian visceral leishmaniasis. J Infect DevCtries2010; 4(2): 114-117.
32. RahiAA, AliMA,ValianHK,MohebaliM.and Ali K. Seroepidemiological Studies of Visceral Leishmaniasis in Iraq. Sch J App Med Sci2014; 1(6):985-989.
33. Jawad J, Kaiy N, Sukkar F. Evaluation ofTEAT for diagnosis of kala-azar in Iraq. J Fac Med Baghdad 1981; 23:189-196.