Real-Time-PCR Assay Based on Phosphoglycerate Kinase Gene for Detection of *Entamoeba histolytica* Trophozoites in Stool Samples in Holy Karbala, Iraq

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Abstract

Amebiasis is an important cause of diarrheal disease worldwide and has been associated with childhood malnutrition. *E.histolytica diagnosed* usually by microscope, which consider as traditional diagnosis and are neither sensitive nor specific detection of *Entamoeba histolytica*(1). Real-time PCR assay developed for sensitive and specific detection of the intestinal Protozoan parasites *Entamoeba histolytica* directly from human feces(2). The RT-PCR assay was able to detect as little as 0.1 parasite per g of feces(3). The current study based on Phosphoglycerate kinase gene (PGK) is a major enzyme used in glycolysis, in the first ATP-generating step of the glycolytic pathway so the PGK is an enzyme that catalyzes the reversible transfer of a phosphate group from 1,3-bisphosphoglycerate (1,3-BPG) to ADP producing 3-phosphoglycerate (3-PG) and ATP(4). In our study, depend on PGK as target for Real time PCR assay for detection the trophozoite stage of *E.histolytica* in stool samples of infected persons.

In the current study, a total of 300 human fecal samples were collected from children (less than one year-15 year) that suspected to infection with *amoebiasis* which admitted to hospitals and primary care centers in the city center, Al-Hindiya district and Nahiat Al -Hurr (100 samples from each region) during the period from February 2019 to January 2020. Fecal samples processed by direct wet smear and formalin ethyl acetate concentration method followed by iodine staining and was microscopically examined for *E.histolytica*. Microscopically positive samples were then subject to Real-time PCR. This is the first study in Iraq using the Phosphoglyceratekinase gene as target for molecular techniques to determine the presence of *E. histolytica* trophozoites.in stool samples.

Keywords: *Entamoeba histolytica, Real-Time PCR, Phosphoglycerate kinase gene.*

Introduction

Entamoeba histolytica is an intestinal protozoon of humans. Several species of the genus *Entamoeba* infect humans, includes: *Entamoeba histolytica,, Entamoeba coli, Entamoeba dispar. Entamoeba polecki, Entamoeba gingivalis and Entamoeba hartmani*(5). Among these, only *E. histolytica* considered as pathogenic organism for human and the disease it causes is called amoebiasis or amoebic dysentery(6). *Entamoeba histolytica* is an enteric parasite, its normal habitat in the human intestinal lumen and has the capacity to invade the epithelium. Amoebic dysentery occurs when *E. histolytica* trophozoites invade the walls of large intestines and multiply in the mucosa, forming ulcers. Most frequent manifestations of infection are dysentery, weight loss, fatigue, flatulent stomach, colitis, and abdominal pain. A common outcome of invasion of the amoeba into tissues is liver abscesses, which can be fatal. *E.histolytica* has ability to secretes tissue lytic enzyme, histolysin which caused damage to the mucosal epithelium(**6**), which digest the intestinal wall tissue of the infected individual hence the Latin name, is to (tissue) lytica (destruction)(7).

The traditional laboratory diagnosis of Entamoeba infections via microscopic examination of stool samples, fresh or fixed. The pathogenic amoeba, *E. histolytica* is indistinguishable from *E. dispar* and *E. moshkovskii*, the non-pathogenic species(8). It has also been shown that the sensitivity and specificity of microscopy is less optimal in differentiating the various species of Entamoeba (9).

Real-time PCR is a very attractive methodology for laboratory diagnosis of infectious diseases because of a lack of requirement for post-PCR analysis, resulting in shorter turnaround times and reduces the risk of amplicon contamination [(10)]. This reflects evident progress in diagnostics, in which the contaminated of amplicon consider as the major reason of false-positive results in PCR assay [(11)] Also the real-time PCR is a quantitative method and capable of identified the parasites number in various samples [(12)].

The human parasite *E. histolytica*, depends only on glycolysis for its ATP supply because it lacks the Krebs cycle and oxidative phosphorylation pathways[(13,14)]. The glycolytic enzymes of the parasite are highly divergent from the enzymes present in the human host; they include an AMP-inhibited hexokinase [(15,16)], and the non-allosteric and pyrophosphate-dependent enzymes phosphofructokinase ATP–PFK-1 and pyruvate kinase in the host [(17)].

The importance of glycolysis for parasite survival and the differences found in the glycolytic enzymes compared with those of the human host, make this pathway a suitable target for molecular techniques such as RT-PCR assay [(18)].

Materials and Method

1. **Sample collection:** Fecal specimens included in this study were from children admitted to hospitals and primary care centers in the center of the city, Al-Hindiyah district and Nahiat Al -Hurr (100 samples from each region collected from children aged less than one year to 15 years) during the period from February 2019 to January 2020. All positive samples that detected microscopically were stored at −70°C until used in RT-PCR assay.

2. **Conventional Microscopy:** One drop of fecal suspension transferred to a microscope slide with a cover slip. Each slide examined as a direct mount at 40- magnification and presence or absence of *E.histolytica*trophzoites were recorded. For further confirmation, formal–ether concentration technique was performed [(19)].

3. **DNA extraction:** Genomic DNA was extracted from trophozoites . The trophozoite's DNA extracted using AccuPrep® Stool DNA Extraction Kit (Bioneer, Korea) according to the manufacturer's instruction with some modification and used as template for RT-PCR assay. Extraction of genomic DNA from *E.histolyticatroph.* was carried out according to our previous published paper [(19)].

4. **RT-PCR amplification:** One set of primers was design to use against the coding region of the Phosphoglycerate kinase gene (in *E.histolytica*trophozoites)a 119bp fragment was amplified using the forward primer (ACCCAA GCT GGT GTTTTGAC)and the reverse primer (AG TTCA AGT GATGC TCC T CCTC)To amply the DNA by used of the forward and reverse primers which designed by NCBI site.

5. **RT-PCR protocols:** Real-Time PCR was performed for detection of trophozoites stage of *E.histolytica* by using the primers and TaqMan probe specific for Phosphoglycerate kinase gene In the DNA technique was carried out according to method described by[(3)].

6. **Real-Time PCR master mix preparation:** Real-Time PCR master mix prepared by one-step Reverse Transcription and Real-Time PCR detection kit (AccuPower Rocket ScriptRT-PCR PreMix, Bioneer. Korea), and done according to company instructions.

   The RT-PCR master mix reaction components that mentioned in were added into standard qPCR tube containing (8 wells strips tubes which containing Rocket Script reverse transcriptase and TaqMan probe premix). Then all strips tubes vortexing for mixed the components and centrifuge for 3000 rpm for 3 minutes in Exispin centrifuge, after that transferred into Exicycler Real-Time PCR thermocycler[(3)].

7. **Real-Time PCR Thermocycler conditions:** Real-Time PCR thermocycler conditions was set up according to primer annealing temperature and RT-PCR Taq Man kit instructions.

   Thermal cycles were applied to inspect the Real-Time PCR and relying on instructions AccuPower® 2X GreenStarTM qPCR Master Mix as well as by calculating the degree Tm prefixes using the device MiniOpticon Real-Time PCR systemBioRad/USA.

8. **Real-Time PCR Data analysis:** RT-PCR data analysis performed by calculation the threshold cycle number (CT value) that presented the positive amplification of gene in Real-time cycle number (20).

Statistical Analysis: The results were analyzed statistically by Chi-squared test, significant results were

attributed to probability values $P < 0.05$ by using SPSS program.

Results

This study included 300 stool samples of diarrhea collected from children suspected infected with *E.histolytica,* the microscopic detection appeared 194 positive samples(100 samples from Kerbala center showed 55 positive samples (55%), 100 samples from Al-Hindiyah district showed 62 positive samples(62%) and 100 samples from Nahiat Al –Hurr appeared 77 positive samples(77%)

Table 1. Frequencies and percentages of Socio-demographical characteristics (N. of positive samples=194/300)

Socio-Demographical Characteristics	Groups	F	Percentage (%)
Gender	Male	107	55.2
	Female	87	44.8
Age Groups	< 1 year	23	11.9
	1- 5	67	34.5
	6-10	72	37.1
	11-15	32	16.5
Region	Kerbala center	55	28.6
	Al-Hindiya	62	32
	Al -Hurr	77	39.7
Education Levels	Nursery school	23	11.9
	Kindergarten	49	25.3
	Primary school	78	40.2
	Secondary school	21	10.8
	School dropout	23	11.9
*F. =frequency			

Comparison of microscope diagnosis with RT-PCR assay: The microscopic examination results of showed for 300 stool samples showed that only 194 samples contained the Trophozoite stage of *E.histolytica*in the percentage (64.7%) while (35.3%) of the samples were negative for the parasite. The molecular examination by RT-PCR of the same samples revealed that the positive samples was 243 and the percentage was (81%) while the percentage of samples negative was (19%). (Table:2)

Table (2) Infection percentage of parasitic *E.histolytica* by microscopic examination and RT - PCR results.

Negative (%)	Positive (%)	Method of examination
106(35.3%)	194(64.7%)	Microscope
57(19%)	243(81%)	RT-PCR

Molecular identification using the Real Time – PCR assay: The results of molecular examination by using of RT-PCR appeared that the number of positive samples are 243(81%) from 300 suspected infected samples, depending on this technique the distribution of infection with *E,histolytica* in three region illustrated in table(3).

Table (3): The percentage of the infected with *E.histolytica* in the city center, Al-Hindiya and Al – Hurr which diagnosed by RT-PCR assay.

Region	Positive samples	Percentage
Karbala center	63	25.9%
Al-Hindiya	86	35.4%
Al -Hurr	94	38.7
Total	**243**	

The used of RT-PCR assay in the specific detection of *E.histolytica* showed a fluorescence of SYBER Green dye which appeared very clear through formed of amplification plot to the positive samples during the cycle 30 as shows in the fig. (1).

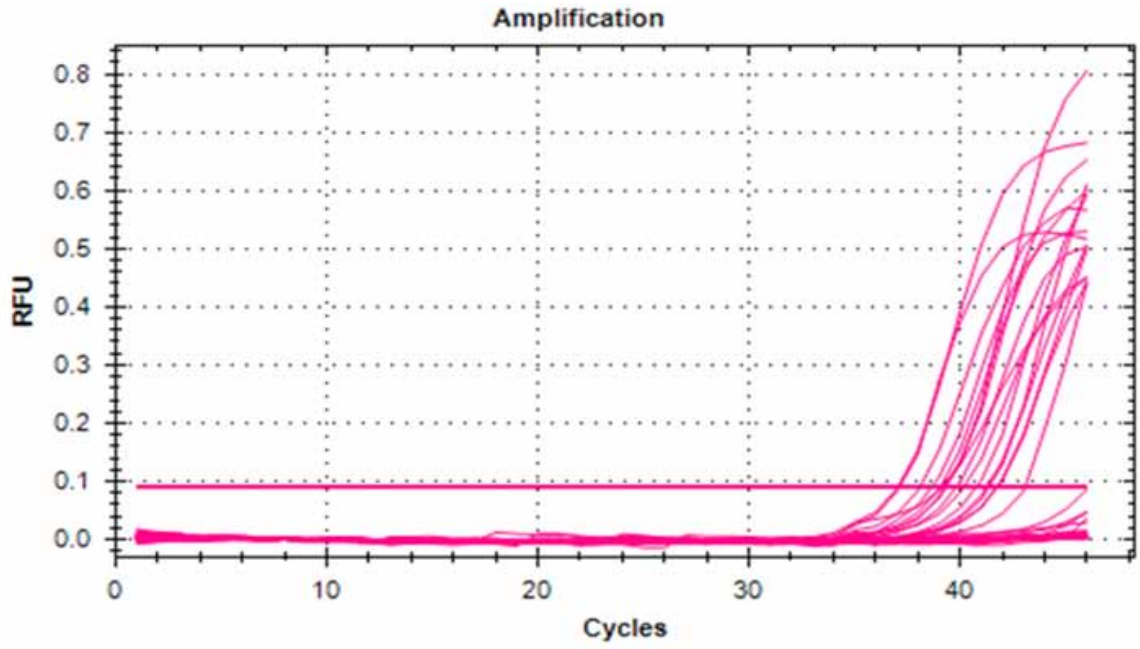

Figure (1): Amplification Plot of Phosphoglycerate kinase gene, in which the fluorescence of the dye SYBER Green represent samples positive located above of threshold while the negative samples located below the threshold.

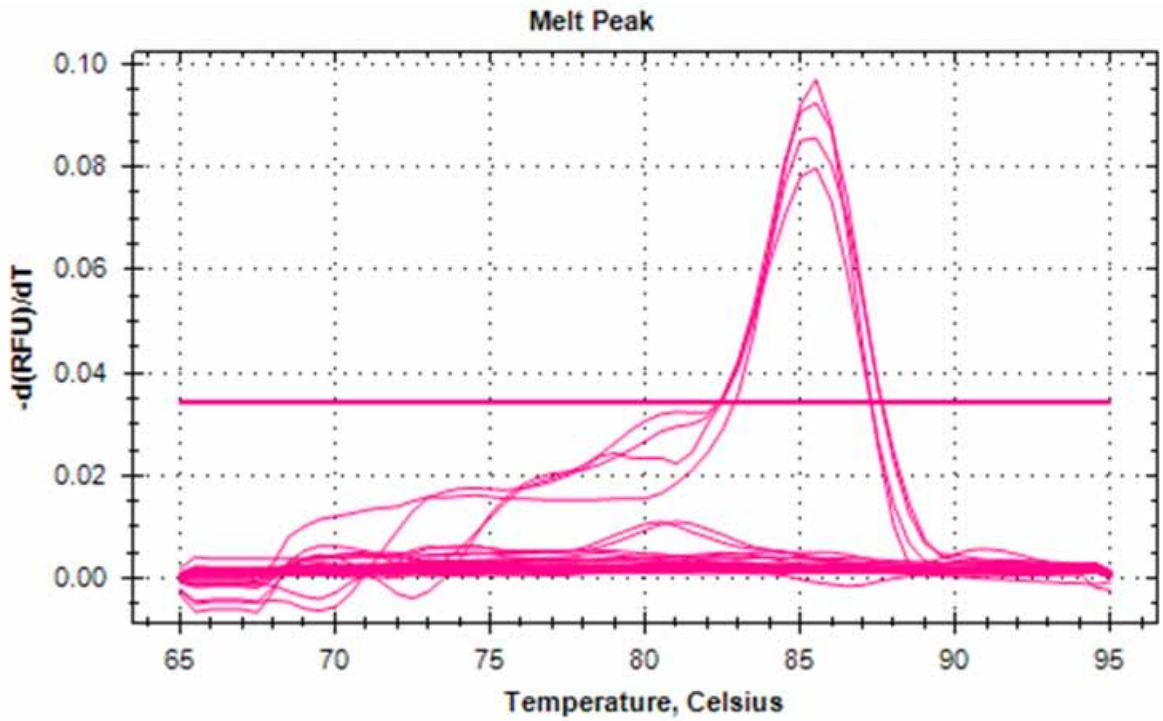

Figure (2): Melt peak curves from real-time PCR amplification of Phosphoglycerate kinase gene. Positive samples indicated in the curves (shown above of Threshold, and negative samples shown under the threshold).

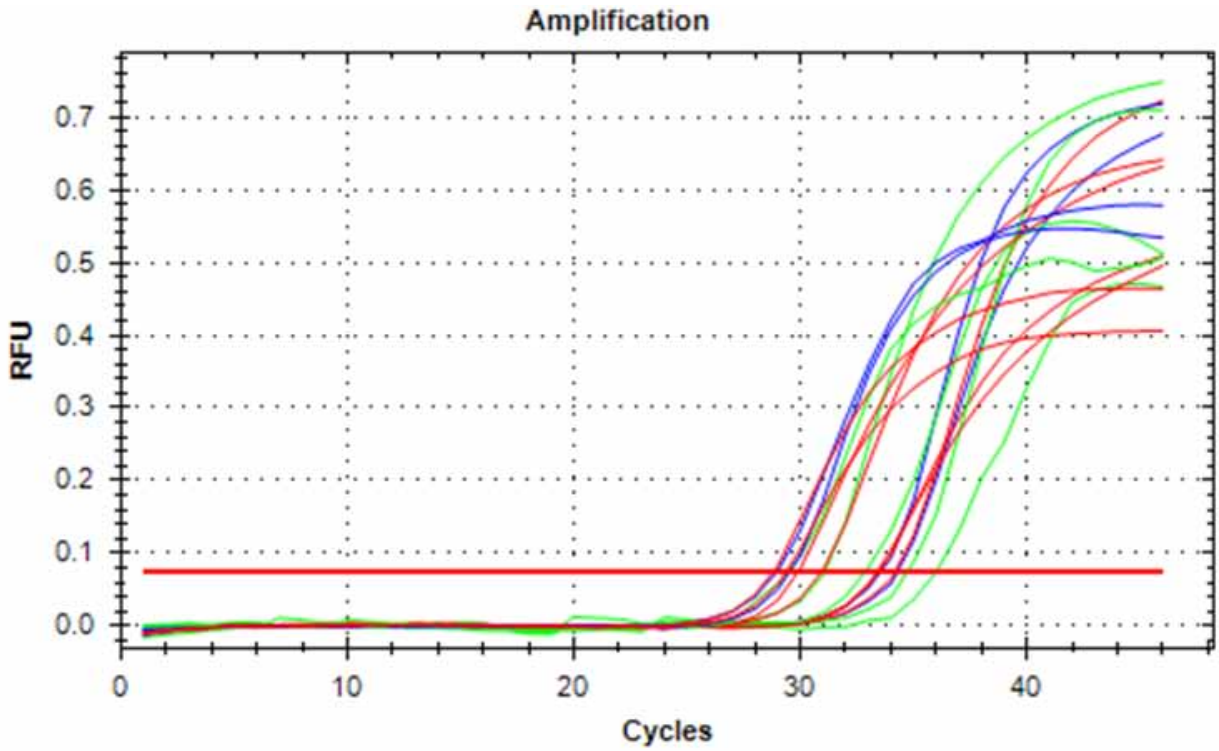

Figure (3): Amplification plot of Phosphoglycerate kinase gene appeared the positive samples of *E.histolytica* trophozoites in the three region: *The center of the city(Red curves), Al –Hurr (Blue curves) and Al-Hindiyah (Green curves).

Discussion

Amoebiasis is a human disease caused by species of protozoa *Entamoeba histolytica,* which causes widespread morbidity and mortality worldwide distribution occurred diarrheal disease and abscess in several parenchymal tissues such as liver, lung, and brain. The true prevalence of infection is unknown for most countries due to the difficulty to characterize *Entamoeba histolytica* from other non-pathogenic amoebas, which have, resemble morphology, like *Entamoeba moshkovskii* and *Entamoeba dispar*[21].

Socio-demographic characteristics: The current study appeared the prevalence of *E.histolytica* was 64.7%. From the diarrheal samples that collected from suspected infected patients. This prevalence considered high rate when compared to other survey studies conducted in other countries in the world, for instance, a study in Nigeria show the prevalence of *E.histolytica* infection in the 183 child examined 78 (42.62%) were infected with *Entamoeba histolytica*[22]. Also a survey was carried out in Pakistan to determine the prevalence of human amebiasis. During the study, 1360 fecal samples were examined 295 (21.69%) were infected with *E. histolytica* [23]**.**

In other study results showed that the prevalence of *E.histolytica*was (2.2%) in 576 fecal samples were collected from children suffering from acute diarrhea in Saudi Arabia[24] .also our results is closely related to[25], where it was 53.18% in her study to diagnose *E.histolytica*in patients with gastrointestinal symptoms in an endemic region in Turkey. While our results were higher than results of Erbil (2.33%)[26] and in Babylon (17.4%)[27]. They are several reasons of the high prevalence of the *E.histolytica* may be due to direct transmission by contaminated food and water, insufficient attention to hygiene[28]. The difference of the results in the present study compared to other studies may be due to the difference in the sewer efficiency, personal hygiene, the density of population, geographical location, the number of test samples, the different duration of the study, different in the living style and different ages[29].

In the current study found the prevalence rate of *E. histolytica* in males (55.2%) was higher than females

(44.8%) and there is no significant relationship (p< 0.05). These results may due to that, males are more susceptible than females to infections, because males generally exhibit reduced immune responses and increased intensity of infection compared to females [30]. These differences are usually duo to physiological and ecological factors, which is usually hormonal in origin. Ecological factors include differential exposure to pathogens because of sex-specific behavior or morphology [31]. Other close cause of variation in ability to infection between two sexes is due to differences in endocrine-immune interactions. Male sexually mature are often more susceptible to infection, because sex steroids, specifically estrogens in females and androgens in males, modulate several aspects of host immunity. In addition to affecting host immunity, sex steroid hormones also alter genes and behaviors that influence susceptibility and resistance to infection. Thus, males may be more susceptible to infection than females not only because androgens reduce immune competence, but because sex steroid hormones affect disease resistance genes and behaviors that make males more susceptible to infection[32].

The highest prevalence of infection according to the age was (37.1%) in the age group (6-10) years. The difference between the age groups in term of infection distribution in this study was statistically significant (P > 0.05) while the results of research done in Erbil province find the highest infection rate was in the age (4-6) [32]. Another study revealed the relationship between age and *E. histolytica*, there was a highest prevalence (30.82%) in age group of (1 day to 15) years [33]. while a study results in Kenya appeared no significant association between prevalence of *E.histolytica*and age[34]. Also our results in agreement with [35] who confirmed that children in northern Pakistan under 15 years of age are very susceptible to infection *by E histolytica*. They obtained 27% prevalence of the disease in children under 15 years of age perhaps the reason is duo to they are less care for hygiene compared to adults, also the high incidence of Parasitic infection among school children may be due to the poor sanitary conditions in the most of the schools [36],also they do not take care of their personal hygiene and not washing hands before eating,while some studies showed a reduced in risk for infection with intestinal diseases among children who follow health instruction[37] .

The current study aims also to compare infection rates among three regions in holy Kerbala province, the first region was the Kerbala center, the second region was Al-Hindiya and the third region was Al-Hurr.

The findings of this study, the highest rate of infection was (39.7%) in Al-Hurr region compared to the Kerbala center (28.6) and Al-Hindiyah (32%) was statistically not significant (P<0.05) and this variation may be due to many interacted factors like, low education of mothers, sanitary service, improper water supply because *E. histolytica* can be transmitted orally by drinking water and it is one of the environmental contaminants of the water supply [38] also malnutrition and absence of regular hygiene toilets, which significantly increases susceptibility to *Entamoeba histolytica* in children, in addition to environmental, economic and social factors are also playing a role in this matter [39].

Comparison of microscope diagnosis with RT-PCR assay: Fecal samples collected from suspected infected children with *E.histolytica* which first subjected to microscope that showed 194 positive samples then subjected to PCR analysis. Prevalence of *E. histolytica* was found to be 64.7%(194/300) samples based on microscopy versus 81% (243/300) according to the results of RT- PCR. Remarkably, all samples positive by microscopy were also positive by RT-PCR which was more sensitivities than the microscope detection. The data show that prevalence of *E. histolytica* in Kerbala is higher when specific diagnosis method are used instead of traditional microscopy, allowing to differentiate between morphologically identical human amoeba's species. Microscope detection has always been used to diagnosed of *E. histolytica* in fecal samples. Despite its inability to distinguish and differentiate *E. histolytica* from the morphologically identical nonpathogenic species *E.moshkovskii* and *E. dispar* . This has resulted in the unnecessary treatment of a large number of individuals with anti-amoebic drugs as well as providing an inaccurate picture of the epidemiology of the organism and the disease [40].

The REAL-TIME assay have been shown to be directly comparable in terms of sensitivity and robustness, capable of detecting 10 parasite genomes but not one, unaffected by the presence of DNA derived from the host or the other test species [41]. In our study we depend on different gene Phosphoglycerate kinase gene is a major enzyme used in glycolysis in E.histolytica trophozoites,the researches regarding on this gene was rare,so we used this gene to improved if It could be adopted as diagnostic gene so we searched and used this

gene as a target of RT-PCR and the results showed a high sensitive of this technique to detected this gene in trophozoites stage .

Conclusions

The following conclusions were conducted from the present study:

1. Although the routine tests such as clinical examination, macroscopic and microscopic diagnosis important, but it is unable to qualitative diagnosis of intestinal amoeba species and differentiated among them.
2. Using of the molecular method such as REAL-TIME PCR which characterized by highly accuracy, but these method expensive economically compared to routine method.
3. The use of specific primers in RT-PCR for diagnosis of Phosphoglycerate kinase gene are proven for diagnosis of *E.histolytica* trophozoites that isolated from stool samples of suspected children infection with *E.histolytica*.

Ethical Clearance: The Research Ethical Committee at scientific research by ethical approval of both environmental and health and higher education and scientific research ministries in Iraq**.**

Conflict of Interest: The authors declare that they have no conflict of interest.

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