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Genetically Diagnosing *Cyclospora* and Some Parasitic, Viral and Bacterial Organisms for Patients with Diarrhea Arriving to Fallujah Teaching Hospital - Iraq, And Studying Some Immunological, Physiological and Hematological Parameters

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Key words: Protozoa , patients ,physiological parameters , Viruses , Bacterial .

Summary

Due to the importance of intestinal parasites, viruses and bacteria that cause diarrhea and due to the frequent deaths of dehydration in the infected, this study was suggested to find out some of the organisms that cause diarrhea in addition to studying some important indicators of infection.

The study showed that *Entamoeba* parasite is the most common intestinal parasite, and that Escherichia coli and adenovirus are as well. The results also showed that there are significant changes in some immunological, physiological and hematological indicators, as well as the calcium and glucose ion, for the patients with a significant $p \leq 0.05$, and the diagnostic methods indicated that genetic diagnosis is one of the best methods, followed by the staining method.

Introduction

Diarrhea causes millions of deaths in Asia, Africa, and Latin America in the age group of 0–4 years (Black, 2017). The risk factors include contaminated water, poor health, or conditions such as malnutrition and factors like high-level contact with pathogens and reduced breast milk safety (Ahmed *et al*., 2014), Acute diarrhea is the most severe type caused by viral, bacterial, fungal, and parasitic infections. Rotaviruses and *Escherichia coli* are the main causes of diarrhea. (Andrade *et al*., 2014).

Etiology usually is not looked for, and oral rehydration therapy is the universal therapy. Active treatment with probiotics and antidiarrheal agents is suggested in adjunct to rehydration, as it reduces the duration and intensity of symptoms independently from etiology (Guarino *et al*., 2014) ,There are no clear indications for antimicrobial and antiparasitic therapy; however, antibiotics are frequently prescribed. Overuse of antibiotics is associated with increased rates of antibiotic-resistant bacteria, unnecessary costs, and significant incidence of adverse events, and current guidelines are highly restrictive in recommending empiric antimicrobial therapy for AGE. Bacterial , parasites and viral infections may be associated with the presence of specific clinical features, notably fever, abdominal pain, blood in the stool, and fecal leukocytes (Lübbert, 2016) .

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In Iraq, the impact of war, sanctions, and sectarian violence left a dysfunctional health system and an on-going public health emergency impacting vulnerable sections of the population, particularly children. Several viral, bacterial, and parasitic infections are among the most common causes of acute diarrheal cases in children (Kelly, 2015) , Therefore, it is necessary to investigate the important and widespread types in our environment that cause diarrhea (parasites , bacteria and viruses) in order to be recommended, detected and reported to the Ministry of Health to take the necessary and immediate measures and provide appropriate treatments for these cases.

Methods

Fecal samples were collected (50) from patients coming to Fallujah General Hospital – Iraq,For the period from 1-9-2019 until 1-7-2020 , who were suffering from acute diarrhea, while control samples (24) were collected from healthy people , parasitic sample was diagnosed by direct examination and by the method of 0.1% iodine drop, as well as by acid-fast stain , while the viruses were diagnosed by cassette, as for the bacterium was diagnosed by chemical methods as well as by VITEC device.
Also, some physiological and hematological parameters were measured in patients and control, where 5 ml of venous blood was drawn and divided into two parts, one of which was to measure complete blood tests, and the other section was to separate the serum and some serological tests were performed on it, For Example; Urea , Creatinine , Uric acid , Calcium , Glucose , Cholesterol and triglyceride.Examined by chromatic methods .

Results and Discussion

Table (1) shows the prevalence of the parasite protozoa in patients arriving at the Fallujah Teaching Hospital, where the prevalence of *Entamoeba histolytica* (66%) was higher than that of *Giardia lamblia* (24%) and *Cyclosporacayetanensis* (10%), with a significant difference.

Table 1: Distribution of infected according to Parasites

Parasites	No	Percentage (%)
E. histolytica	33	66.00 %
Cyclospora cayetanensis	5	10.00 %
Giardia lamblia	12	24.00 %
Total	50	100%
Chi-Square (χ^2)	---	25.74 **
** (P≤0.01).		

A total of 3176 patients with different ages attending the Al-Shomally General Hospital in Babil Province – middle of Iraq. After collection, samples examined via microscopic examination utilizing normal saline and lugholes iodine by direct method examination , From 3176 patients with diarrheal episodes, 699 (22%) were infected by either of *E. histolytica* (17.91%) or by *G. lamblia* (4.09%). The highest rate of

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infections of *E. histolytica* and *G. lamblia* was in the age group of 15–44 years and more than 45 years, respectively. Most infection of *E. histolytica* occurs in February. No significant differences between male and female (50.65% and 49.35% respectively) were observed (AL-Khikani *et al.* , 2019) . This is consistent with our current study .

Perhaps the reason for the spread of *E. histolytica* more than other parasites is that its Cysts are more resistant to surrounding environmental conditions than others, and the poor levels of hygiene and the presence of hosts that transmit the parasite have a great impact on the speed of spread(Chalabi , 2018) .

As for Tables (2), (3) and (4), the causes of diarrhea in patients were shown, as it showed that the diarrhea was the result of a parasitic, bacterial or viral infection, where the highest rate of bacterial infection was E. coli, at 40%, while The highest virus infection was the adino virusat 15% .

Fang-zhou (2018) revealed that the adeno virus is one of the main causes of diarrhea, and that 1.9 million people suffer from diarrhea annually due to this virus, and that 22 million people develop diarrhea due to rotavirus and adeno virus, as well as bacterial and parasitic infections , The reason was attributed to the poor surrounding environmental conditions, with which people come into contact.

Diarrhea is the most common cause of morbidity and mortality in many resource-limited countries including Tanzania among children under five years of age. A large number of cases of diarrhea associated with severe dehydration are still reported among children, despite the administration of vaccines for the adenovirus, but it appears that there are other factors for diarrhea, including bacterial and parasitic, that contribute to the occurrence of diarrhea, and the researchers mentioned that the most important reason for the occurrence of diarrhea is lack Paying attention to public hygiene and living in rural areas also related to raising and mixing pets, poor ventilation and lack of concern for personal and physical hygiene(Delfina *et al.* , 2020) .

Table 2: Distribution of infected according to Culture.

Culture	No	Percentage (%)
E.coli	20	40.00 %
Campylobacter spp	13	26.00 %
Shigella spp	5	10.00 %
klebsiella pneumonia	8	16.00 %
Salmonella	4	8.00 %
Total	50	100%
Chi-Square (χ^2)	---	17.40 **
** (P≤0.01).		

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Table 3: Distribution of infected according to viruses/ Rota.

Viruses/ Rota	No	Percentage (%)
Positive	13	26.00 %
Negative	37	74.00 %
Total	50	100%
Chi-Square (χ^2)	---	11.52 **
** (P≤0.01).		

Table 4: Distribution of infected according to viruses/ Adino.

Viruses/ Adino	No	Percentage (%)
Positive	15	30.00 %
Negative	35	70.00 %
Total	50	100%
Chi-Square (χ^2)	---	8.00 **
** (P≤0.01).		

Our results showed that there were significant differences in the level of glucose between the infected and the non-infected, while the calcium was not affected significantly , Table (5) .

Table 5: Comparison between Infected and Uninfected in Calcium and Glucose.

Group	Mean ± SE	
	Calcium (mg/dL)	Glucose (mg/dL)
Infected	9.04 ± 0.08	118.76 ± 5.09 a
Uninfected	13.12 ± 3.60	101.04 ± 6.02 b
Level of Sig.	P≤0.104 NS	P≤0.0398 *
* (P≤0.05), NS: Non-Significant.		

Rayhan *et al*. (2020) explained that the parasitic infestation that causes diarrhea prevents the absorption of nutrients necessary to maintain vital activities in the body, including the absorption of glucose to release energy and calcium to build bone and blood clotting processes and to perpetuate the work of nerve impulses.

Table 6: Comparison between Infected and Uninfected in Urea , Creatinine and Uric acid.

Group	Mean ± SE		
	Creatinine(mg/dL)	Urea (mg/dL)	Uric acid (mg/dL)
Infected	0.898 ± 0.03 a	26.20 ± 1.59 a	4.54 ± 0.16 a
Uninfected	0.683 ± 0.04 b	20.29 ± 1.62 b	3.96 ± 0.17 b
Level of Sig.	P≤0.0006 **	P≤0.0242 *	P≤0.0349 *
* (P≤0.05), ** (P≤0.01).			

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Our results showed in Table (6) that there were significant differences in Urea, Creatinine and Uric acid for those with diarrhea and those without.

Muhammad *etal*. (2009) explained that there are many cases of diarrhea and their causes as well, including bacterial causes, especially cholera, and parasitic and viral causes, and that severe diarrhea will lead to dehydration and this in turn leads to the deficiency of the kidneys and thus a defect in their vital functions and imbalance of urea, uric acid, creatinine.

Table 7: Comparison between Infected and Uninfected inCholesterol and Triglyceride

Group	Mean ± SE	
	Cholesterol (mg/dL)	Triglyceride (mg/dL)
Infected	142.32 ± 7.95	142.60 ± 8.97
Uninfected	121.25 ± 9.09	126.21 ± 10.96
Level of Sig.	P≤0.112 NS	P≤0.279 NS
NS: Non-Significant.		

Our current study demonstrated the relationship between Infected and Uninfected in Cholesterol and Triglyceride . It was not significant, although the parasitic, bacterial and viral infection of people with diarrhea will lead to the disposal of large amounts of fat during the process of diarrhea, and the intestines are not able to represent the metabolism of fats during the occurrence of diarrhea, and thus the rate of fat in the infected will decrease. Table (7) .

Table 8: Comparison between Infected and Uninfected in differential of WBC

Group	Mean ± SE						
	WBC	Lymphocyte	Mid	Gran	Lymphocyte %	Mid %	Gran%
Infected	11.85 ± 1.30 a	3.91 ± 0.32 a	0.906 ± 0.08	7.04 ± 1.04 a	0.364 ± 0.02	0.081 ± 0.004 b	0.554 ± 0.02
Uninfected	7.20 ± 0.34 b	2.63 ± 0.15 b	0.725 ± 0.04	3.85 ± 0.24 b	0.367 ± 0.02	0.100 ± 0.004 a	0.532 ±0.02
Level of Sig.	P≤0.011 **	P≤0.0009 **	P≤0.149 NS	P≤0.0390 *	P≤0.924 NS	P≤0.003 **	P≤0.532 NS
* (P≤0.05), ** (P≤0.01), NS: Non-Significant.							

The results of Table (8,9) showed that there were significant differences at the probability levelP≤0.05in the levels of WBCand CBC indicators among those with diarrhea cases compared with the non-infected.

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Table 9: Comparison between Infected and Uninfected in CBC.

Parameters	Mean ± SE		Level of Sig.
	Infected	Uninfected	
RBC	4.45 ± 0.08	4.65 ± 0.08	P≤0.125 NS
HGB	11.51 ± 0.26 b	12.48 ± 0.18 a	P≤0.011 **
HCT	35.27 ± 0.65 b	37.90 ± 0.52 a	P≤0.0113 **
MCV	79.53 ± 0.89	81.75 ± 1.19	P≤0.151 NS
MCH	25.91 ± 0.37	26.92 ± 0.41	P≤0.098 NS
MCHC	325.40 ± 2.31	329.33 ± 1.32	P≤0.259 NS
RDW-CV	a0.139 ± 0.003	0.129 ± 0.001 b	P≤0.0319 *
RDW-SD	38.69 ± 0.51	37.39 ± 0.53	P≤0.125 NS
PLT	278.88 ± 16.61	286.29 ± 13.34	P≤0.774 NS
MPV	8.65 ± 0.15	8.80 ± 0.21	P≤0.599 NS
PDW	16.24 ± 0.08	16.14 ± 0.09	P≤0.466 NS
PCT	2.39 ± 0.13	2.47 ± 0.09	P≤0.674 NS
P-LCC	54.62 ± 3.43	55.79 ± 2.75	P≤0.826 NS
P-LCR	0.203 ± 0.009	0.204 ± 0.01	P≤0.942 NS
* (P≤0.05), ** (P≤0.01), NS: Non-Significant.			

Al-Mozan*etal*. (2017)explained in their results that there were significant changes in some hematological indicators at a significant level of p = 0.05, as the blood percentage and blood viscosity factors were significantly decreased due to the fact that the parasites, bacteria and viruses that cause diarrhea will prevent the absorption of the nutrients necessary to maintain blood viscosity and provide the energy needed to make blood cells. Red blood cells and the provision of iron necessary to maintain the hemoglobin stain and to deliver oxygen to cells throughout the body, and blood gases were also affected in varying proportions, in addition to that, the levels of white blood cells were significantly higher in those with diarrhea, unlike the non-infected , This is due to the body's continuous resistance against foreign organisms that have entered the intestinal system, as the number of defense cells continues to increase continuously to attack foreign bodies, get rid of them and expel them outside the body.

Molecular detection of *Cyclospora* samples

To improve sensitivity in PCR-based detection of *C. cayetanensis*, two strategies were implemented. first, a nested PCR approach was designed because this pathogen is found at low concentrations in stool samples . Second, PCR assays were designed to amplify the 18S rRNA locus because multi-copy genes increase PCR sensitivity (Dixon *et al.* , 2005) . The first round of amplification using primer pair CYCF1E and CYCR2B generated specific and strong PCR signals when 3.0 ng of DNA template per reaction and an annealing temperature of 53.6 C were used. For the second round of amplification, primer pair CC719 and CRP999 generated specific and strong PCR

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signals when the first-round PCR products were diluted 1:100 and an annealing temperature of 66.5 C was used (Figure 1). Specificity of the nested PCR assay was corroborated by Sanger sequencing and phylogenetic analysis. These analyses revealed that nested PCR fragments obtained with the present protocol share high genetic similarity (~98.0% identity) with 18S rRNA genes from *C. cayetanensis* archived in the GenBank (accession numbers: EU861001.1, KY770759.1 and GU557063.1), confirming the specificity of the nested PCR assay. Importantly, these primer sets have been included in a new U.S. Food and Drug Administration method developed for detection of *C. cayetanensis* on stool (Carolina *et al.* , 2020) .

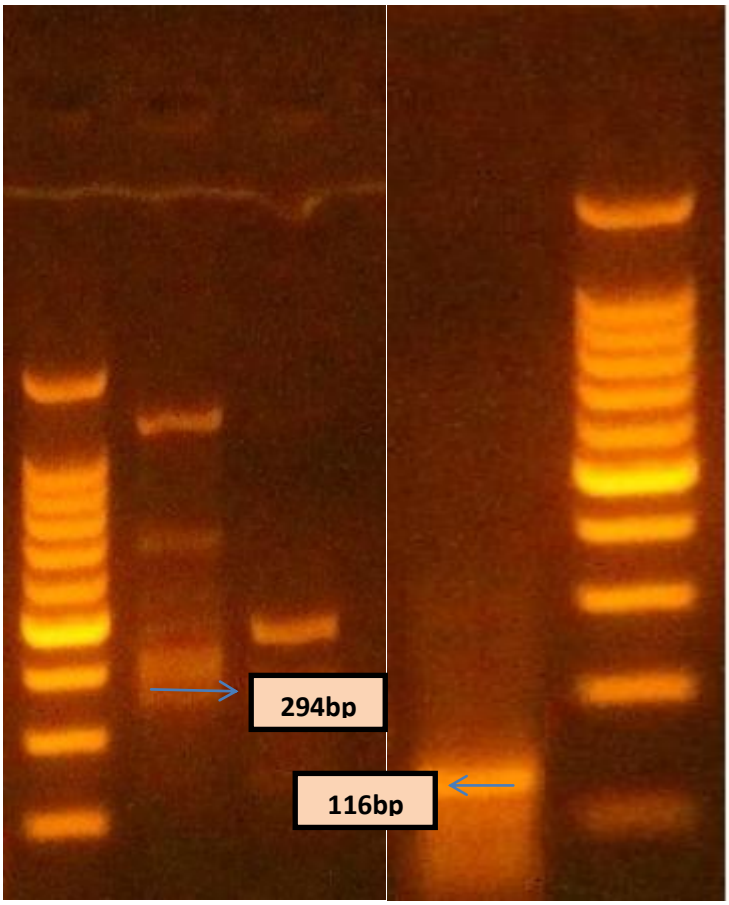

Figure (1) showing the large piece 294bp and the small piece 116bp after migrating the sample on the Agarose 1%

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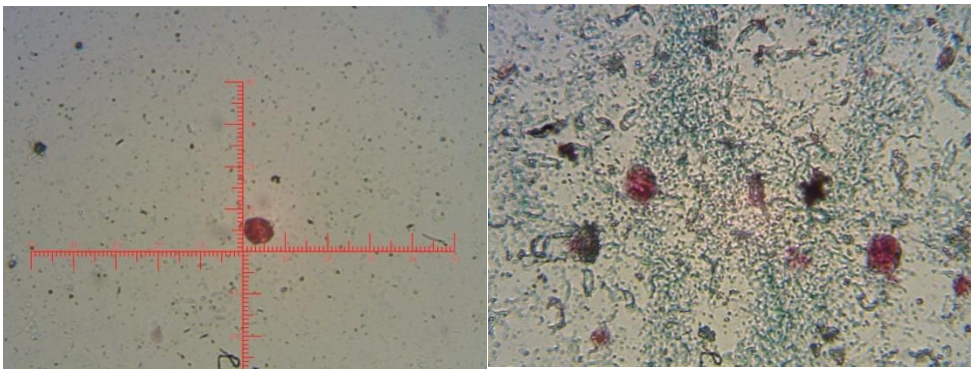

Figure (2) showing *Cyclospora* parasite and its size using Ocular micrometer lens

Angela *et al*. (2020) Indicated that the technique of staining with Ziehl-Neelsen stain is very important in diagnosing the parasite *cyclospora*, but the best methods of diagnosis are genetic methods, and among these methods is the nPCR .

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