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Epidemiological and diagnostic Study for diarrheic agents (*Entamoeba histolytica, Giardia lamblia, and Cryptosporidium* sp.) among diarrheic infected faeces patients by using multiplex polymerase chain reaction in the Babylon province, Iraq

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Abstract: The current study included period was conducted from the October 2014 till February 2015 examination of 987 stool samples for direct smear method and 96 positive samples from these samples examined by polymerase chain reaction technique PCR. For patients infected with diarrhea (children and adults, male and female) who attended to Babylon maternity and children hospital and specialized Marjan Hospital for Internal and Cardiac Diseases in the Babylon province as well as primary health care and private clinics. and for age range from(Less than one year -31and more). The current study showed the rate of infection with parasites that causative of diarrhea 47.3% (*E.histolytica*, *G.lamblia* and *Cryptosporidium* sp.) were (26.4%, 17.9% and 3.7%, espectively). Polymerase chain reaction (PCR)used to detect the causative of diarrhea *(G.lamblia, E.histolytica* and *Cryptosporidium* sp.) in microscope positive examined sample where it had record total of infection rate of 43.4% (31.3%, 28.1% and 2.2%, respectively).

[Mais Khadhim Al-Sherefy and Ahmed Khudhair Al-Hamairy.**Epidemiological and diagnostic Study for diarrheic agents (*Entamoeba histolytica, Giardia lamblia, and Cryptosporidium* sp.) among diarrheic infected faeces patients by using multiplex polymerase chain reaction in the Babylon province, Iraq**. *Life Sci J* 2022;19(6):40-49]. ISSN 1097-8135 (print); ISSN 2372-613X (online). http://www.lifesciencesite.com. 7. doi:10.7537/marslsj190622.07.

Key word: *Entamoeba histolytica*, *Gardia lamblia*, *Cryptosporidium* sp. and Polymerase chain reaction (PCR) .

Introduction

Diarrhea is a common cause of death in developing countries and the second most common cause of infant deaths worldwide (Victora *et al*., 2008).World health organization ,WHO (2009) defined diarrhea as having extra stools than are usual for that individual or as passage of watery stool for more than three times in twenty-four hours caused by consumption of contaminated food or drinks by a variety of pathogens including bacteria, fungi, viruses, protozoa or helminthes (Vargas *et al*., 2004). *Entamoeba histolytica*, *Giardia lamblia* and *Cryptosporidium* sp. are three major protozoan pathogens responsible for diarrhea (Stark *et al.*, 2011) .

Any epidemiological risk factors such as recent travel, crowded living conditions or day-care use, raw or undercooked food consumption, animal contacts, sick contacts, recent medication use, underlying conditions such as HIV, and immunization history(Pour *et al.,* 2013). Laboratory diagnosis of intestinal parasite infections still depends mainly on microscopically examination of stool samples for the identification of helminthes eggs and protozoan trophozoites and cysts, Some parasite species cannot be differentiated based on microscopy only, while detection of other species*(E.histolytica, G. lamblia, and Cryptosporidium* ps.*)* may need well experienced technicians (Fotedar *et al*., 2007). Shortly after the development of polymerase chain reaction or multiplex polymerase chain reaction is distinct as the simultaneous amplification of multiple regions of DNA templates by adding more than one primer pair to the amplification reaction mixture. (Verweij *et al*.,2004; Haque *et al* .,2007). Aims of the present study the relationship of these parasite with some aspects (residence area, sex , age groups and determine performance characteristics and efficiency of direct smear method and polymerase chain reaction in the diagnosis of protozoa parasites in faecal samples of children as well as compare the prevalence of E.histolytica , G. lamblia and Cryptosporidium sp. In the faecal samples among the attending patients by using direct smear methods and polymerase chain reaction techniquies.

Materials and Methods

Physiological Normal Saline solution

To prepare the solution concentration 0.9% was melt 0.90 gm. of sodium chloride in 100 ml of distilled water and then infertility by autoclave at 121 degree heat and under pressure of 15 pounds / Lange for 15 minutes and save with temperature four centigrade until use (Gravery *et al*., 1977).

Lugol's Iodine

This dye was attended by dissolving 5 g of potassium iodide in 1000 ml of distilled water and added to 5 g of iodine crystals slowly with constant stirring until it melts, then nominated as a solution and stored in air-tight containers (Zeibig, 1997).

Carbol Fucshin - Dimethyl Sulfoxide (CF-DMS) stain

A-Preparation of the stain:

1-Fucshin crystals were dissolved in 25 ml. ethyl alcohol (99%).

2-2gm. phenol crystals were added after liquefaction in water bath and mixed well by a glass rod.

3-25 ml. of pure Glycerol were added and 25ml. of Dimethyl Sulfoxide (DMS),75 ml. of Distilled water (DW) were added and mixed well.

4-The solution was left up to 30 minutes and filtered in what man filter paper No. 4.

5-The staine was used directly or stored in a dark bottle at room temperature for later used.

B- Clearing Solution:

1-220 ml. of malachite green 2% dissolved in distilled water.

Examination:

Direct wet mount method

Stool samples examined by the preparation of direct smear methods using clean glass slides , was a small drop of normal saline (0.9%) or Iodine stain put on slide glass and mix well with a small portion of feces using wooden stick, then was put cover slides, and examined the sample under power enlarge 40X and 100X (Tanyuksel&petri.,2003).

Floatation methods

Isolation of the Oocysts from faeces as follows: The faeces was suspended in PBS of (pH 7.2) in ratio of 20-80 then centrifuged at 500gm. for 10 minutes. The supernatant was discarded and the sediment was suspended in 15ml centrifuge tubes of PBS (pH 7.2); and 3-5ml. of ether were added then mixed strongly. And centrifuged at 500 gm. for one minute was made and four layers were differentiated from bottom to the top:(1-Sediment, 2-PBS, 3-Debris and 4-Solvent). Layers were discarded and the sediment layer that consisted of 75% of oocysts were used for purification of the oocysts. These oocysts were prepared in 2.5% K2Cr2O7 until used (Arrowood & Sterling, 1987).

Staining Procedure:

1-Rectal faecal smear was made by sterilized wooden stick with cotton head on clean glass slide dry at the room temperature.

2-The dry smear used was fixed with methanol for 5-10 seconds (sec.).

3-Staining with CF-DMS for 5 minutes.

4-Washing with tap water for 10-30 sec.

5-The smears were rinsed in Malachite green (2%) for 1 minute hence a green background appear.

6-The smears were rinsed in let tap water of 10 sec.

7-The smears were dried for 10 minute.

8-By wooden stick the smear was oiled with oil immersion.

9-The smears were examined by light microscope 40X and 100X oil objectives.

Modified Ziehl–Neelsen method

The modified Ziehl- Neelseen stain M ZN-ST (acid fast) was used for examination of rectal smears as following . Fecal smear was permanent by Methanol alcohol for 5 minutes and allowable to dry at room temperature. Then The dehydrated smears were stained for an hour in Carbol Fuchsin prepared by dissolving 15% Carbol Fuchsin in methanol (stock solution).Ziehl Fuchsin 10ml added to 90 ml 5% phenol. Then Rinsed in tap water. After that differentiate ln 2% H2SO4 solution for 20 seconds with agitating the slide. Rinsed in tap water. Next Smear wasstained with 5%malachite green solution for 5 minutes and washed in tap water and followed until dehydrated. Then Examined by using 40X and 100X oil immersion objectives. (Jawtez *et al*.,2001).

Multiplex Polymerase chain reaction (PCR)

The Multiplex PCR technique was performed for detection *G.lamblia*, *E.histolytica, and Cryptosporidium* sp. based subunit ribosomal rRNA gene from human stool samples. This method was carried out according to method as following steps:

Genomic DNA Extraction

Genomic DNA from faeces samples were extracted by using AccuPrep® stool DNA Extraction Kit , Bioneer. Korea, and done according to company instructions as following steps:

1- 200 mg of the 96 stool single positive sample examined by direct smear method of

G.lamblia, *E.histolytica, and Cryptosporidium* sp. was transferred to sterile 1.5ml microcentrifuge tube, and then 20µl of proteinase K and 400 µl Stool lysis buffer (SL) were added mixed by vortex. And incubated at 60℃ for 10 minutes.

2- Then, the tube placed in centrifuge at 12,000 rpm for five mins.

3- The supernatant was transferred in a new tube and 200µl Binding buffer was added to each tubes.

4- The tubes be Incubated over again for 10 min. at 60℃.

5- 100 µl isopropanol was added and the samples mixed by lightly vortex for about five seconds, then spin down for 10 seconds to down the liquid clinging to the walls and lid of the tube.

6- DNA filter column was placed in a two ml collection tube and transferred all of the mixture (including any precipitate) to column. after that centrifuged at 8000 rpm for five minutes. And the 2 ml collection tube containing the flow-through were discarded and placed the column in a new two ml collection tube.

7- 500µl W1 buffer were added to the DNA filter column, then centrifuge at 10000 rpm for 30 seconds. The flow-through was discarded and placed the column back in the two ml collection tube.

8- 500µl W2 Buffer (ethanol) was added to each column. Then centrifuged at 8000 rpm for 30 seconds. The flow-through was discarded and placed the column back in the two ml collection tube.

9- All the tubes were centrifuged again for one minutes at 12000 rpm to dry the column matrix.

10- The dried DNA filter column was transferred to a clean 1.5 ml microcentrifuge tube and 50 µl of pre-heated elution buffer were added to the center of the column matrix.

11- The tubes were let stand for at least five minutes to ensure the elution buffer was absorbed by the matrix. Then centrifuged at 10000 rpm for 30 seconds to elution the purified DNA.

Genomic DNA Profile

The extracted genomic DNA from faeces samples (96 samples) for all parasites were was checked by using nanodrop spectrophotometer (THERMO. USA), that check and measurement the purity of DNA through reading the absorbance in at (260 /280 nm) as following steps:

1. After opening up the nanodrop software, chosen the appropriate application (Nucleic acid, DNA).
2. A dry wipe was taken and cleaned the measurement pedestals several times. Then carefully pipette one micromilliliter of H2O onto the surface of the lower measurement pedestal.
3. The sampling arm was lowered and clicking OK to initialized the Nanodrop, then cleaning off the pedestals and one µl of the appropriate.
4. Blanking solution was added as black solution which is same elution buffer of DNA samples.

After that, the pedestals are cleaned and pipet one µl of DNA sample for measurement. DNA is extracted pure when absorbability rate is (1.8).

Primers

Three PCR primers were designed in the present study for detection *G.lamblia*, *E.histolytica*, and *Cryptosporidium* sp. based subunit ribosomal rRNA gene by using NCBI-Genbank (M54878.1, X64142.1, and AF112573.1, respectively) and three primers plus design online. Then these primers was provided from Bioneercompany, Korea, as following Table 1:.

Table 1

Primer	Sequence		PCR Size
G.lamblia	F	GTTGAAACGCCCGTAGTTGG	574 bp
	R	CTCGCTCGTTGTCGCAATG	
E.histolytica	F	ACGAGGAATTGGGGTTCGAC	204 bp
	R	CACCAGACTTGCCCTCCAAT	
*Cryptosporidium.*sp	F	AACCTGGTTGATCCTGCCAG	351 bp
	R	TTCCCCGTTACCCGTCATTG	

Multiplex PCR master mix preparation

PCR master mix were prepared by using (**AccuPower® Gold Multiplex PCR PreMix Kit**) and this master mix done according to company instructions as following table 2:

Table 2

Multiplex PCR Master mix		Volume
DNA template		5µL
Forward primer (10pmol)	G.L- F	1µL
	E.H- F	1µL
	C.sp.- F	1µL
Reverse primer (10pmol)	G.L- R	1µL
	E.H- R	1µL
	C.sp.- R	1µL
PCR water		39 µL
Total volume		50 µL

PCR thermocycler conditions by using convential PCR thermocycler system as following table 3:

Table 3

PCR step	Temp.	Time	repeat
Initial Denaturation	95C°	5min	1
Denaturation	95C°	30sec.	30 cycle
Annealing	58C°	30sec	
Extension	72C°	1min	
Final extension	72C°	5min	1

PCR product analysis

The PCR products of was analyzed by agarose gel electrophoresis following steps:

1- 1.5% Agarose gel was prepared in using 1X TBE and dissolving in water bath at 100 °C for 15 minutes, after that, left to cool 50C°.

2- Then three µ of ethidium bromide stain were added into agarose gel solution. And 3- Agarose gel solution was poured in tray after fixed the comb in proper position after that, left to solidified for 15 minutes at room temperature, then the comb was removed gently from the tray and 10µl of PCR product were added in to each comb well and five ul of (100bp Ladder) in one well.

4- The gel tray was fixed in electrophoresis chamber and fill by 1X TBE buffer. Then electric current was performed at 100 volt and 80 AM for one houre and

5- PCR products were visualized by using UV transilluminator and then photography of bands that reveals by digital camera (Samsung. china).

Results

The study included examination of 987 stool samples of patient with diarrhea. They were examined by a direct wet mount method for *G.lamblia* , *E.histolytica* and Ziehl–Neelsen for *Cryptosporidium* sp. by using light microscope and then the DNA was extracted from the positive samples and subjected them to Multiplex Polymerase chain reaction PCR.

Table(4): Percentage of infection for parasites that cause diarrhea in according to residence area by using direct smear method.

The sex	Examind No.	Infected No.	Parasites that cause diarrhea						Total percentages of infection (%)
			E.histolytica		*G.lamblia*		*Cryptosporidium* sp.		
			Infec. No.	(%)	Infec. No.	(%)	Infec. No.	(%)	
Males	584	301	178	30.5*	103	17.6	26	4.5*	51.5*
Females	403	166	83	20.6	74	18.4*	11	2.7	41.2
Total	987	467	261	26.4	177	17.9	37	3.7	47.3
Statistical analysis (Z-test)			5.2 sign males		1.1non sign		1.2 sign males		

*Significant differences (P ≤ 0.05)

Table(5):Percentage of infection for parasites that cause diarrhea in according to the age groups by using direct smear method.

Age groups	Examind No.	Infected No.	Parasites that cause diarrhea						Total percentages of infection (%)
			E.histolytica		*G.lamblia*		*Cryptosporidium* sp.		
			Inf. No.	(%)	Inf. No.	(%)	Inf. No.	(%)	
Less than one year	219	95	43	19.6	22	10	31	14.2*	43.4
1-5	126	63	36	28.6	26	20.6	6	4.7	50
6-10	191	76	44	22	37	19.4	0	0	39.8
11-15	168	104	65	38.7	40	23.8	0	0	61.9
16-20	113	86	48	42.5*	37	32.7*	0	0	76.1*
21-25	78	20	11	14.1	7	9	0	0	25.6
26-30	57	13	7	12.3	5	8.5	0	0	22.8
31and more	35	10	7	20	2	5.7	0	0	28.6
Total	987	467	261	26.4	177	17.9	37	3.7	47.3
Statistical analysis (LSD-test)			5.9 sign 16-20		6.8 sign 16-20		4.4 sign Less than one year		

*Significant differences (P ≤ 0.05).

Table(6):Percentage of infection for parasites that cause diarrhea in according to residence area by using mltiplex PCRtechnique.

Residence area	Examind No.	Infected No.	Parasites that cause diarrhea						Total percentages of infection (%)
			E.histolytica		*G.lamblia*		*Cryptosoridim sp.*		
			Inf. No	(%)	Inf. No	(%)	Inf. No	(%)	
Town (city center)	54	14	13	24.1	11	20.4	1	1.9	25.9
Rural area	42	19	17	40.5*	16	38.1*	1	2.4*	45.3*
Total	96	33	30	31.3	27	28.1	2	2.1	43.4
Statistical analysis (Z-test)			8.2 sign Rural area		6.1 sign Rural area		0.7 non sign		

*Significant differences (P ≤ 0.05).

Table (7):Percentage of infection for parasites that cause diarrhea in according to sex by using mltiplex PCR technique

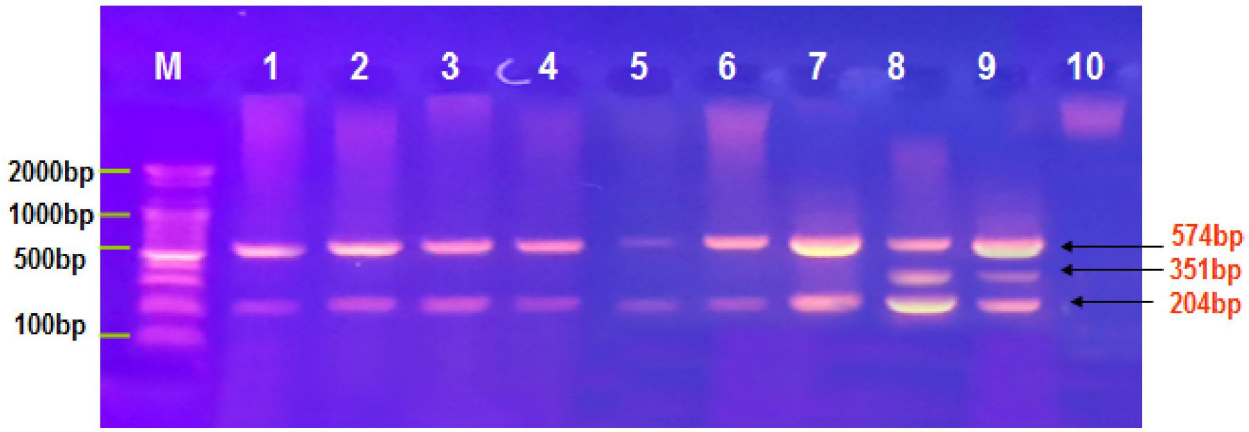
The sex	Examind No.	Infected No.	Parasites that cause diarrhea						Total percentages of infection (%)
			E.histolytica		*G.lamblia*		*Cryptosoridim* sp.		
			Inf. No	(%)	Inf. No	(%)	Inf. No	(%)	
Males	60	22	21	35*	16	26.7	2	3.3*	36.7*
females	36	11	9	25	11	30.6*	0	0	30.6
Total	96	33	30	31.3	27	28.1	2	2.1	43.4
Statistical analysis (Z-test)			3.2 sign males		2.1 sign females		2.2 sign males		

*Significant differences (P ≤ 0.05).

Table(8): Percentage of infection for parasites that cause diarrhea in according to age groups by using mltiplex PCR technique.

Age group	Examind No.	Infected No.	Parasites that cause diarrhea						Total percentages of infection (%)
			E.histolytica		*G.lamblia*		*Cryptosporidim* sp.		
			Inf. No	(%)	Inf. No	(%)	Inf. No	(%)	
Less than one year	16	4	3	18.8	2	12.5	1	6.3	25
1-5	14	6	6	42.9	4	28.6	1	7.1*	42.9
6-10	16	6	2	12.5	5	31.3	0	0	37.5
11-15	17	7	9	52.9*	7	41.2*	0	0	41.2
16-20	13	6	6	46.2	5	38.5	0	0	46.2*
21-25	6	1	1	16.7	1	16.7	0	0	16.7
26-30	9	2	2	22.2	3	33.3	0	0	22.2
31and more	5	1	1	20	0	0	0	0	20
Total	96	33	30	31.3	27	28.1	2	2.1	43.4
Statistical analysis (LSD-test)			4.9 sign 11-15		8.8 sign 11-15		2.4 sign 1-5		

*Significant differences (P ≤ 0.05).



Figure(1): Agarose gel electrophoresis image that show the Multiplex PCR product analysis of ss- rRNA gene from genomic DNA of human stool samples. Where M: Marker (2000-100bp), lane(1-7) positive samples for G.lamblia at 574bp and *E.histolytica* at 204bp. Lane (8 -9) positive samples for *G.lamblia* and *E.histolytica* as well as *Cryptosporidium* sp. at 351bp product size. Lane (10) negative sample**.**

Discussion

Percentage of Infection for parasites that cause diarrhea according to residence area by using direct smear method.

The present study showed difference in the rates of parasitic infections, according to the residential area in Babylon province, the highest rate of infection was in the rural area 67.2% while the lowest rate of infection in the cities center 32.9%. This study agree with Al-Kubaisy *et al.* (2014) where he scored the highest rate of infection in the rural area, reaching 50.9% in Baghdad. Also agree with Al- Taie *etal*. (2006) in Babylon province, who record the highest rate of infection in rural areas increased by 64.7% and less rate of infection in urban areas were 35.3% . This study disagree with what recorded by Al-Moussawi (2012) as the rate of infection in the rural area 79% amounted to 15% higher than in the cities area (12.36%).

By used PCR technique the present study showed difference in the rates of infection with parasitic infections, according to the residential area, the highest rate of infection(45.3%) was in the rural area, while the low rate of infection in the city (25.9%)**.** This study agree with what recorded by Al-Khafaji (2013) in Al-Qadisyai as the rate of infection in the rural area (62.9%) higher than in the cities area (37.9%). And agree with what recorded by Al-Muhanna, (2013) in Al-Najaf as the rate of infection in the rural area (19.6%) higher than in the cities area (9.1%).

The reason for the high incidence of infection in rural areas due to several factors, including the lack of clean drinking water availability, and rely on river water directly as a source of water, and the lack of guidance and counseling by the authorities concerned as well as lower health and cultural level of the rural population as well as the lack of hospitals and health centers in those areas, as well as use of animal waste and human feaces and sometimes as an organic fertilizer for the growth and plants and vegetables.

Percentage of Infection for parasites that cause diarrhea according to the sex of the patient by using direct smear method.

The results shows the percentage of parasitic infections in both sexes were (47.3%) , the highest rate of infection was in the male (51.5%) while the lowest rate of infection in females (41.2%). %). And the percentage study agree with study of Kubaisy *et al.* (2014) in Baghdad, where he scored the highest rate of infection in males (58.5%) and lower than in females (41.5%). and agree with the study of Al-Morshedi (2007) in Hilla, which did not record any significant differences between gender were the highest rate of infection in his study of males (53.9%) and lower than those for females (47.8%). and also disagreement with the study Jaeffer (2011) in Baghdad, where he scored the highest rate of infection (15.35%) in females and (12.28%) in males and also disagree with Mahmud study (2006) in Al-Sowera which did not record any significant

differences between the sexes. This study agrees with what recorded by Al-Moussawi (2012) as the males in the areas covered by the study of infection were (rural area , Town) as follows (18.14% and 14.25%), respectively, while the rate of infection in females (14.42% , 8.31%). And disagreement with the Al-Mamouri (2014) in the province of Babylon, with the highest percentage infection of males 80.6%, while the lowest infection for females 81% . as well as study of Al-Ibrahimi (2013) in the Al- Diwaniya province the highest rate of infection for males (6.12%), while the lowest infection for females (5.11%) .

By used PCR technique The present study recorded prevalence of parasitic infections in both sexes were 43.4% , the highest rate of infection was in the male (36.7%) while the lowest rate of infection in females (30.6%). And the present study agree with Al-Warid (2010) in Baghdad as it did not find any significant differences between the sexes as the percentage ratios between males and females(14.98%) and (14.58%), respectively, as well as incompatible with Koffi *et al.* (2014) as it did not find any significant differences between the sexes although he found the highest rate of infection among females (65.36%) and the lowest rate infection among males (60.13%).

The difference in the rate of infection between males and females may be due to the fact that males are the most movement and active and their contact with the external environment factors at play and of being working group in the communities, this is what makes them more relevant pathogens sick of females as males eat well and drink in public places or from street vendors, in addition to the nature of anarchism and a lack of attention to personal hygiene and wash hands and this increases the chances of being infection either for *Giardia* and the absence of significant differences between male and females could be due to portability and having the same opportunity to infection both sexes intestinal parasites (Al-Mamouri, 2000).

Percentage of Infection for parasites that cause diarrhea according to age groups of patients by using direct smear method.

The present study recorded prevalence of parasitic infections in different age groups from age-less than one year to age 31 and more, the highest rate of infection (76.1%) was in the age group (16-20), while the lowest rate of infection (22.8%)was in the age group (26-30), followed by the (15-11) age group was (61.9%) . They agree with Shakir *et al.* (2014) in Baghdad, where he scored the highest infection rate of(49.3%) in the age group (16-20) followed by the (11-15) age group at a percentage of (36.6%), while the study did not agree Al-Kaisy *et al.* (2008) where he scored the highest rate of infection in the age groups (less than a year one) (39.5%) as the current study are consistency with Kubaisy *et al.* (2014) in Baghdad, where he scored the highest rate of infection in the age group (15-11) and also did not agree with what his record Jaeffer (2011) in Baghdad, where scored highest rate of infection in (4-6).

By used PCR technique the present study recorded prevalence of parasitic infections in different age groups from age-less than one year to age 31 and over, the highest rate of infection (46.2%) was in the age group (16-20), while the decline in the rate of infection (16.7%) was in the age group (21-25). This study is compatible with Al-Warid (2010) in Baghdad, who scored the highest rate of infection in less than ten years age group were 55.04%. And this study is incompatible with Ngosso *et al.*(2015) in Dar Es Salaam, Tanzania who scored the highest prevalence of diarrhea (29.6%) was found in the age groups of (12-23) months, followed by (24-60) months were 15.6%,then (6-11)months were 8% and (least 0-5 months) were (2.4%).

The high incidence of intestinal parasites in the (20-16) age group may be due to the fact that this group belong to the high school and the students at this stage more activity and movement and with little attention to personal hygiene and taking into account health conditions, either for *cryptosporidium*, the reason for the high incidence in the age group (less than one year) may be because the nature of this parasite opportunistic as it affects children and people with little immunity, as well as the spread of artificial feeding that may be a source of infection through bottles feeding unclean or not sterile cabarets 'is also creep a children in this age stage and pick up objects contaminated extending fingers inside the mouths of all the causes of this age group.

Conclussions .

In light of the results reached the following conclussions can be drawn:

1- The prevalence of the parasites that cause diarrhea in the Babylon province are very highly when detection microscopic examination method and polymer chain reaction technique PCR, comparison with previous studies and rural area highest rates of infection from than cities.

2- There was a close relationship between the infection and the sex , age group ,residence area.

3 - The PCR technique it's more sensitive than direct smear method to determine the three parasites.

4 - The *Cryptosporidium* sp. parasites were the lowest prevalence from another parasites and it's infection focusing in the little age group.

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2/18/2022