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Detection of Infectious Parasites in Iraqi Children with Diarrhea via PCR and Chromatography Methods

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Abstract

The Parasite Screening Panel (CerTestCrypto + Giardia + Entamoeba combo test, Zaragoza; Spain) is a novel qualitative immunoassay panel for the detection of *Giardia lamblia* (*G. lamblia*), *Entamoeba* histolytica (*E. histolytica*)/*Entamoeba dispar* (*E. dispar*), and *Cryptosporidium parvum* (*C. parvum*). The current research aimed to assess the efficacy of the Triage immunocromatography technique for parasite identification in young children with diarrhea. A total of 320 stool samples from children younger than six years old hospitalized at Babylon Teaching Hospital, Iraq, were investigated in the current study. The study was conducted from December 2020 to May 2021. Parasites in children were checked using microscopic examinations. The three parasites responsible for diarrhea were identified using direct microscopic examination, and single or mixed infections were determined using PCR evaluation of multiplex for rapid detection. The obtained results indicated that the total parasite infection rate increased by 51.16 % in children aged 4-6 years, compared to those aged 2-4 years (30.23%) or younger than 2 years (18.60%). The *G. lamblia* was found to be the most common parasite (55-82%), with *E. histolytica* coming in second place. The *G. lamblia* was found to have the highest prevalence (68.33%) in immunochromatography studies, followed by *C. parvum* (18.33%) and *E. histolytica* (13.34%). When an immunochromatography assay was performed on children, mixed parasite infection by *C. parvum* and *G. lamblia* was found. The detection of *E. histolytica*, *G. lambda*, and *C. parvum* by immunohistochemistry indicated sensitivity rates of 94.55%, 82.61%, and 100%, respectively. These results corroborated the high quality of the current findings. In conclusion, the triple immunoassay can detect single or mixed parasite infections with high accuracy and sensitivity.

Keywords: Children with diarrhea, Multiplex PCR, Parasite infestation

Full length article **Corresponding Author*, e-mail: hayder.muhammed@uokerbala.edu.iq

1. Introduction

According to the World Health Organization (WHO) and the United Nations International Children's Emergency Relief Fund (UNICEF), there are about 2 billion cases of diarrhea every year worldwide, and the annual death of about 1900,000 children under the age of 5 years, mainly in developing countries. This high mortality rate of 18% among children younger than 5 years of age means more than 5000 children die from diarrhea daily [1,2]. Children under 5 years of age have an average of about three episodes of acute diarrhea per year. Globally, in this age group, acute diarrhea is the second leading cause of death (after pneumonia), and the incidence and mortality of diarrhea are the highest among such children. the risk decreases as they grow older. Other immediate consequences of diarrhea in children in developing countries include malnutrition, and cognitive impairment [3]. Many protozoa, including *Entamoeba* histolytica (*E. histolytica*), *Giardia lamblia* (*G. lamblia*), and Cryptosporidium spp., cause diarrhea and other gastrointestinal issues. As a result, it is now known to be a major contributor to human diarrhea [4]. While *E. histolytica* typically causes asymptomatic infections, symptoms can range from seldom present to severe in rare cases. Cryptosporidium is a zoonotic parasite cause diarrhea in children and immunocompromised populations, posing a life-threatening threat to children under 5 years [5,6,7].

Fecal-oral transmission of cysts through contaminated food and water as well as human-to-human contact is a frequent route of infection for these three parasites. Their prevalence in developed and underdeveloped countries poses a threat to already high health concerns, especially in developing countries [8,9]. Of the existing methods, microscopy is labor-intensive, time-consuming, and dependent on the technician's competence and more repeated samples to produce results of high sensitivity [10]. Hence, fecal antigen or parasite DNA detection is a hallmark of standard diagnostics although it requires high technical knowledge. More and more laboratories are turning to polymerase chain reaction (PCR) techniques for identifying intestinal parasites because of their superior sensitivity and specificity, compared to traditional approaches, such as microscopy assays and antigen detection. However, PCR is rarely used due to its high price and the expertise of its operators [11]. Detection of *C. parvum*, *G. lamblia*, and *E. histolytica*/*dispar* in the stool has become much quicker due to the development of rapid stool antigen tests, such as immunochromatography (IC). To detect fecal antigens, the triple IC test is an easy, quick, and supplementary approach. Stool samples can be tested for both parasites at the same time. Also, they call for minimal effort and can be utilized singly for rapid testing [12]. The purpose of this research was to assess the efficacy of the Triage immunocromatography technique for parasite identification in young children with diarrhea.

2. Materials and methods

2.1. Preparation of sample

The study was performed on children under 6 years hospitalized or treated in the emergency room for acute gastroenteritis at Babylon Teaching Hospital, Iraq. A total of 320 stool samples were collected for analysis between November 2020 and June 2021. Stool samples were stored in a transparent plastic cup labeled with a random number. Primary diarrhoeal infection was detected by multiplex PCR, the standard approach (direct smear and dye), and the quick IC.

2.2. Examination of parasites by direct microscope

The feces were examined by eye investigation mucous, discoloration, blood, and odor. *Giardia lamblia* and *E. histolytica* were detected in stool samples with saline swabs and iodine lugols, respectively. The low-power and high-power microscope was used for the analysis of the smear. In the current investigation, Cryptosporidium eggs were detected using a modified Ziehl-Neelsen stain. High-magnification examination of an oil drop on a glass slide.

2.3. Combo Card Immuno Chromatography Test

According to CerTestCrypto+Giardia+Entamoeba combination card tests, Zaragoza (Spain), Rapid Chromatographic Immunoassay can identify *G. lamblia*, *E. histolytica*, and *C. parvum*.

2.4. PCR assay

Geneaaid/PrestoTM USA's Stool DNA Extraction Kit was used to directly extract DNA from 320 feces samples following the manufacturer's instructions. In this investigation, the primers included in Table 1 were those describing nucleotides. The method of this assay was stated in previous studies [11,13, 14].

2.5. Statistical analysis

Using SPSS statistical software, the chi-square test was used to analyze the data, and the sensitivity and specificity of the results were computed using the following formula:

I: Sensitivity: (true positive/ true positive + false negative) x 100, II: specificity: (true negative/true negative) + false positives) x 100 [15].

3. Results and Discussions

A total of 320 samples of diarrhea were collected and analyzed from children younger than 6 years old, the vast majority of whom were treated at Babel Teaching Hospital, Iraq, before being transferred to the pediatric department. Diarrhea is one of the main causes of morbidity and mortality in children younger than 5 years of age, in developing countries, where the average number of episodes of diarrhea per child per year within this age group is 3.2 years, it remains a major public health problem in the world. In developing countries, an estimated 12 or more diarrheal episodes per child per year occur within the first 5 years of life. Annually, approximately 4.6 million pediatric deaths, about 25 to 30% of all deaths among children less than age 5 years, can be attributed to acute diarrhea [16,17].

The findings revealed an uptick in parasite infection among children aged 6 years (51.16%), but not among those aged 2-4 (30.23%) or younger than 2 (18.60%). In contrast to the highest parasite detection rate obtained in children aged 7-12 months (26.1%; [18]), this finding is compatible with previous studies, indicating the highest rate of parasitic infection (38.18%; [19]) and detected positivity in the age group 4-6 years of children [20]. As can be seen in Fugue 1, the prevalence of *Trypanosoma catarrhalis* reached 55.82%, followed by 29.06% for *E. histolytica* and 15.12% for *C. parvum*. This finding is inconsistent with the study by Dohuk, reporting the prevalence of *G. lamblia* at 38.5% [21]. The incidence of giardiasis was 34% in the Al-Karkh district of Baghdad [22], compared to 11% in the Al-Mahmudiya district of Baghdad, and 2% in the Wasit governorate [23]. The obtained results addressing the prevalence of *E. histolytica* (29.06%) are in agreement with another study as the rate of 25.95% for prevalence was reported [24], differs from the study that found an *E. histolytica* prevalence of 7.3% in Erbil [25]. Figure 2 illustrates the distribution of intestinal parasites identified through the triage immunocromatography test. This test confirmed a high prevalence of *G. lamblia* (63.33%), followed by C. *parvum* (18.33%), and *E. histolytica* (13.34%). These findings corroborate those of a previous study [26], which identified *G. lamblia* (23%), *C. parvum* (5%), and *E. histolytica* (2%) in cases of parasitic diarrhea. However, the findings disagree with a prevalence of 30.07% for *G. lamblia*, 19.8% for *C. parvum*, and 11% for *E. histolytica*, respectively [12]. Due to variations in climatic conditions, another study [26], reported *E.*

histolytica (25.63%) as the most prevalent, followed by *G. lamblia* (19.38%) and *C. parvum* (13.75%).

In children younger than 6 years of age, intestinal parasites were separated into single and mixed forms (Table 2). most common prevalence of the intestinal parasite in a single infection was found for *G. lamblia* (88.48%), *E. histolytica* (62.5%), and *C. parvum* (27.27%). Similarly, another study in Kenya indicated the highest parasite infection rate for *G. lamblia*, followed by *E. histolytica* and *C. parvum* [27]. Parasites of the genus Eimeria (*E. histolytica*) and the genus Cryptosporidium (*C. parvum*) were found to be the second and third most common ones compared to *G. lamblia* [28,29]. However, mixed infections were also seen, with the Lingerie parasite and Cryptococcus being the most prevalent combination. The two parasites of *C. lamblia* and *C. parvum* were found in six infants with diarrhea. Figure 3 depicts the discovery of two parasites (*C. parvum* and *E. histolytica*) in a kid with diarrhea [30]. This was followed by discovery of three parasites (*C. parvum*, *E. lamblia*, and *E. histolytica*) in a third child with diarrhea. This finding aligns with the observation that the prevalence of mono-parasitic infections is higher than that of mixed parasite infections [31], which validated the use of IC to differentiate between single and mixed infections. An investigation of 105 instances of parasitic infection indicated that 13 (12.38%) were caused by a single infection, while 10 (9.52%) originated from a combination of parasites [32]. Therefore, it is confirmed that single-parasite infection is more incidence than parasite- mixed infection in Egypt [12].

Figure 4 shows the distribution of intestinal parasites using PCR. The results indicated that G. lamblia was more prevalent than other parasites (67.16%), which was in line with those of other studies conducted in developing and industrialized nations [33,34]. The current investigation reported a positive rate for Cryptosporidium, which was supported by a higher prevalence rate (11%) of *C. parvum* in Babylon [35].

The DNA extraction method of stool samples can significantly affect the PCR detection of intestinal protozoa. Pure genomic DNA samples have been used in most previously published PCR tests, which have demonstrated high levels of sensitivity and specificity [36]. Multiplex PCR assays have been established for the simultaneous detection of *E. histolytica*, *G. lamblia*, and *C. parvum* in stool samples [37,38]. According to Table 3, a PCR test revealed a high rate of Giardia infection (67.16%), followed by direct microscopy (55.81%). This result agrees with another research indicating an even higher PCR infection rate of 18.43% and a subsequent rate of 15.20% in direct microscopy in Kirkuk [39]. Moreover, *C. parvum* was found at 15.11% in direct microscopic examination, followed by 16.41% in PCR (Figure 1 and Figure 4). The PCR results in Egypt revealed a high incidence of *C. parvum* (21%), compared to 9.5% using direct microscopy [40]. In a microscopy study on children aged lesser than 5 years old, in some cities of Ethiopia, the range of cryptosporidiosis was 5-13.9% [41]. The present findings indicated a high prevalence of *E. histolytica* (29.06%) using direct microscope examination and lower prevalences using immunocromatography (13.33%) and PCR (16.41%). Microscopy showed many false positive results, which may be due to misdiagnosis of other Entamoeba species like *Entamoeba coli*, *Entamoeba Hartmannii*, or the morphologically identical *Entamoeba moshkovskii* [42]. Table 4 shows the IC test has a sensitivity of 66.67%,93.18%, and 100% for intestinal parasites, namely *E. histolytica*, *G. lamblia*, and *C. parvum*, respectively. This result was in agreement with a previously conducted research, revealing the sensitivity of 62.5%, 97%, and 72% for the detection of *E. histolytica*, *G. lamblia*, and C. parvum, respectively [26]. In contrast, 100%,100%, and 73% detection sensitivity were reported for *E. histolytica*, *G. lamblia*, and *C. parvum*, respectively [27].

Table 1. Nucleotide primers

Allele specific	**Primer sequence(5 '->3 ')**	Product size
E.histolytica	5-ATTTGTTAAGTATTGTAAATGGG-3 5-ATTGTAACCTTTCATTGTAACAT-3	605 bP
G.lambali\	5-AATCTGTTGACTTAAGGGAGTA-3 5-ATTGAGTCATTATAGGGATTGT-3	463bp
Cryptosporidium	5-TAAACGGTAGGGTATTGGCCT-3 5-CAGACTTGCCCTCCAATTGATA-3	240bp

Source of data in the table: [12]

Table 2. The distribution of diarrhea parasites determined by immunochromatographic test

Type of parasite	G.lamblia(%)	E.histolytica(%)	C.parvum(%)	Negative(%)
G.lamblia	33(88.48)	-	-	-
E.histolytica	-	5(62.5)	-	-
C.parvum	-	-	3(27.27)	-
G.lamblia+E.histolytica	1(2.43)	1(12.5)	-	-
G.lamblia+C.parvum	6(14.63)	-	6(54.54)	-
E.histolytica+C.parvum	-	1(12.5)	1(9.09)	-
G.lamblia+C.parvum+E.histolytica	1(2.43)	1(12.5)	1(9.09)	-
Total	41	8	11	26

Table 3. Comparison of the Triage Micro Parasite Panel, direct examination, and PCR results for the diagnosis of diarrhea parasite

	Entembeaosis %	**Giardiaasis %**	**Cryptospordiosis %**	
type of parasites				N
PCR	11(16.41%)	45(67.16%)	11(16.41%)	67
Fecal direct examination	25(29.06%)	48(55.81%)	13(15.11%)	86
Immunocromatography test	8(13.33%)	41(68.33%)	11(18.33%)	60

Table 4. Sensitivity and specificity results for the Triage Micro Parasite Panel

Imunocromatography (Triage panal test)		
Type of parasite	Specificity	Sensitivity
C.P	100.00%	100.00%
E.H	94.55%	66.67%
G.L	82.61%	93.18%

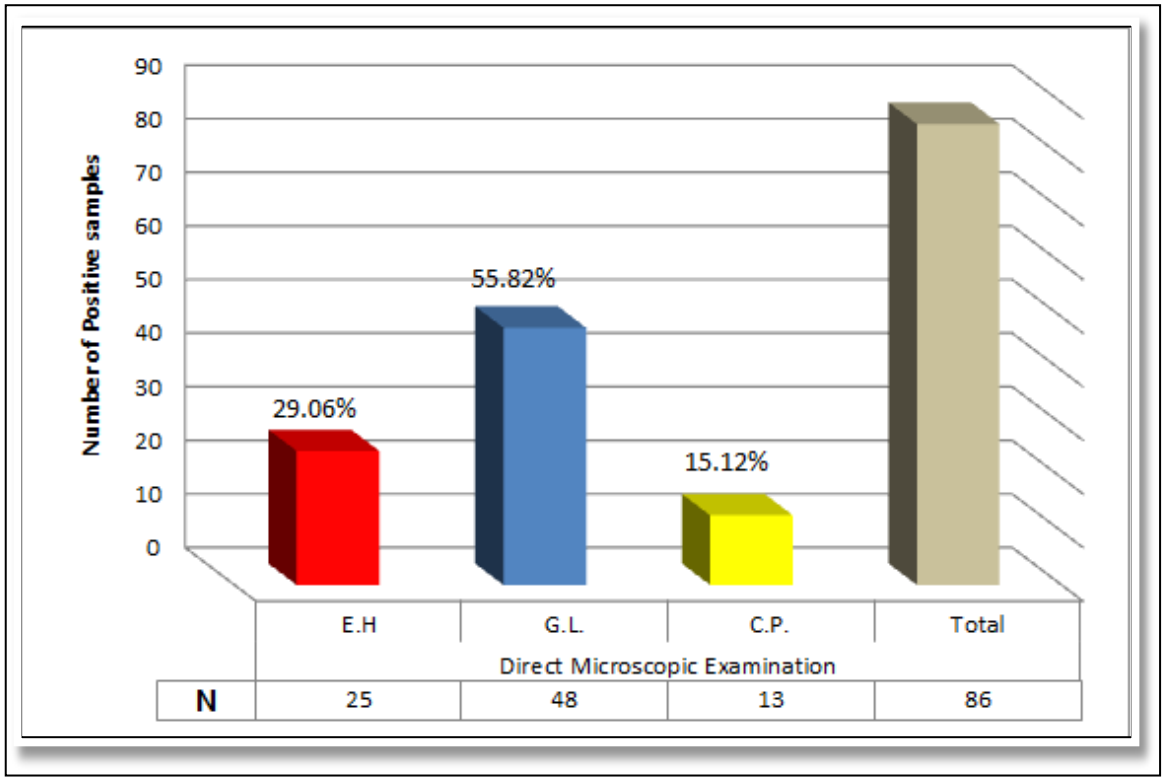

Figure 1. Diarrhea-causing parasite spread as determined by direct microscopic examination

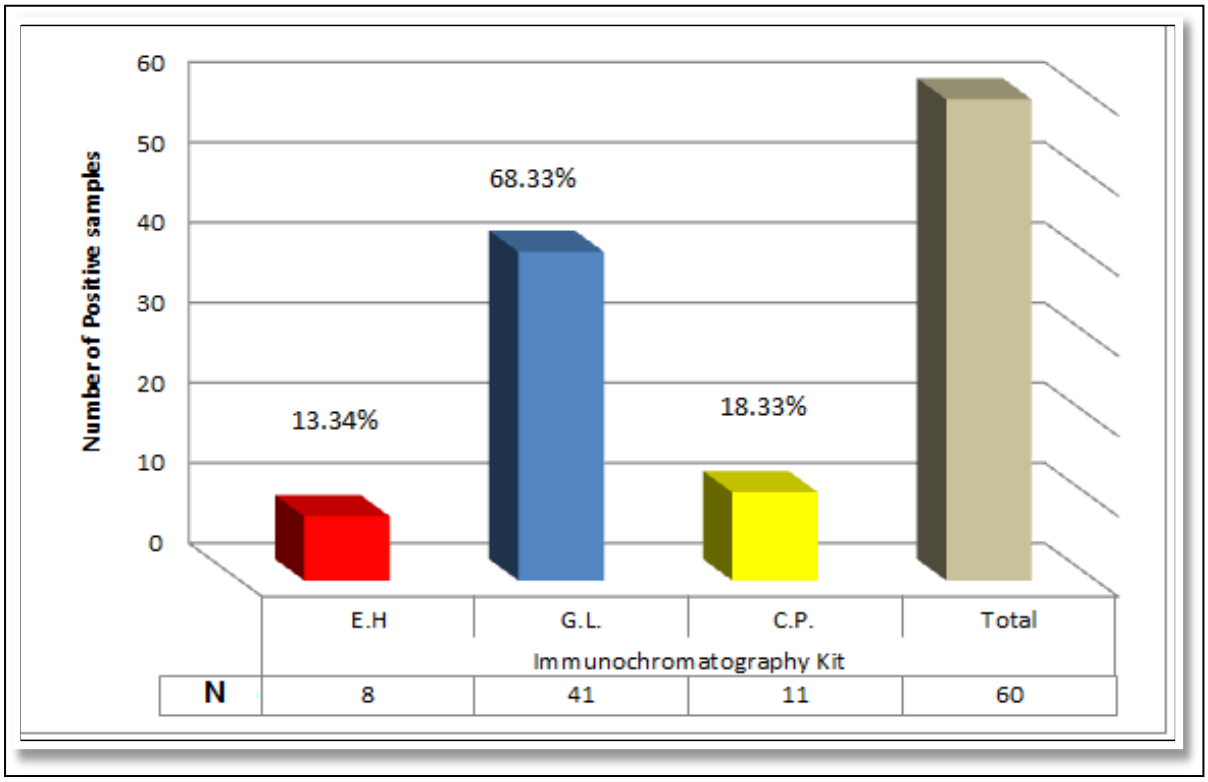

Figure 2. The distribution of parasites that cause diarrhea, as determined by a triage immunochromatography test.

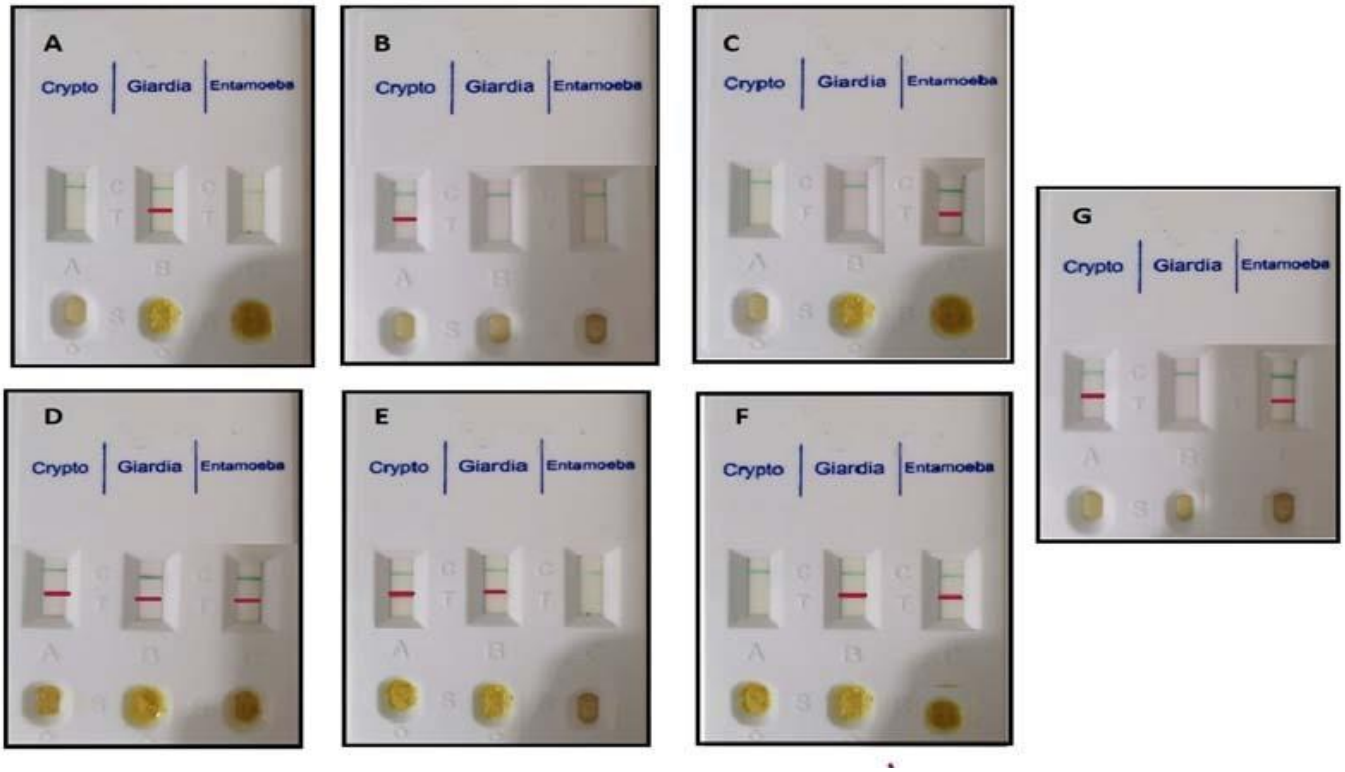

Figure 3. Triage parasite panel showing promising results. The five groups are as follows: (A) Positively and negatively controls and positive screening zone for *Giardia lamblia*; (B) Positively and negatively controls and positive screening zone for *Cryptosporidium parvum*; (C) Positively and negatively controls and positive screening zone for *Entamoeba histolytica*/ *Entamoeba dispar*; (D) Positively and negatively controls and combined infection with *Cryptosporidium*, *Giardia*, and *Entamoeba*; (E) Positive and negative controls and mixed infection with *Cryptosporidium*

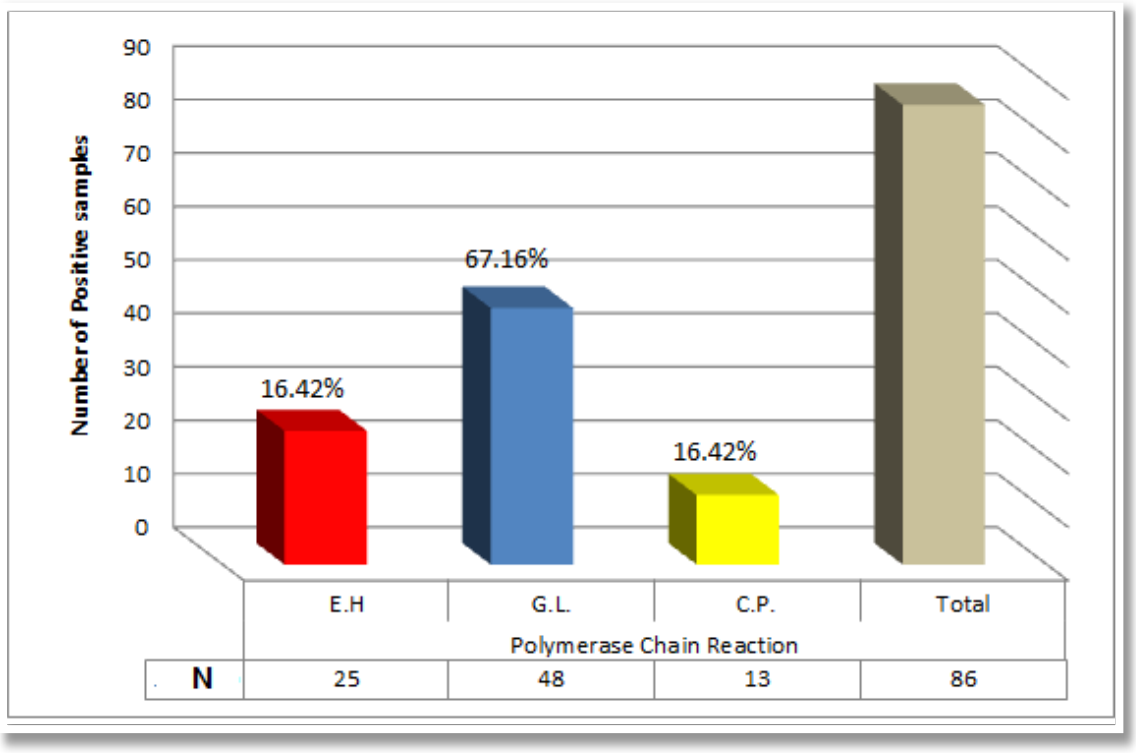

Figure 4. The distribution of parasite causes of diarrhea by using PCR

Figure 5. A multiplex PCR product analysis of the GL, EH, and CP genes was visualized on an agarose gel electrophoresis (Marker ladder 100bp). Negative control in lane 7, then lanes 1 (CP and EH) and 2 (CP, EH, and GL) and so on.

4. Conclusions

It can be concluded from the obtained results that the total parasite infection rate increased by 51.16 % in children aged 4-6 years, compared to those aged 2-4 years (30.23%) or younger than 2 years (18.60%). The *G. lamblia* was found to be the most common parasite (55-82%), with *E. histolytica* coming in second place. The *G. lamblia* was found to have the highest prevalence (68.33%) in immunochromatography studies, followed by *C. parvum* (18.33%) and *E. histolytica* (13.34%). When an immunochromatography assay was performed on children, mixed parasite infection by *C. parvum* and *G. lamblia* was found. The detection of *E. histolytica*, *G. lambda*, and *C. parvum* by immunohistochemistry indicated sensitivity rates of 94.55%, 82.61%, and 100%, respectively.

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