Journal of the Egyptian Society of Parasitology, Vol.52, No.3, December 2022
J. Egypt. Soc. Parasitol. (JESP), 52(3), 2022: 519 – 526
(Online: 2090-2549)

EVALUATION OF MULTIPLEX REAL-TIME PCR IN DETECTION OF ENTERIC PATHOGENIC PROTOZOA AMONG DIARRHEIC CHILDREN

By
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Abstract

Gastrointestinal protozoa are one of the global health problems, particularly in the Eastern Mediterranean Countries. Urgent parasitological diagnosis is indicated to start proper treatment.

The present study evaluated multiplex real-time (RT) PCR and microscopic examination for detection of some pathogenic protozoa mainly *Cryptosporidium parvum*, *Entamoeba histolytica/ dispar* and *Giardia lamblia* among diarrheic school aged children. The morning stool samples of 100 diarrheic children were examined by stained microscopy smears and multiplex RT-PCR for gastrointestinal intestinal protozoa.

The results showed that among the positive children 67/100 by microscopy the detected protozoa were *G. lamblia* followed by *Blastocystis hominis*, *E. histolytica/dispar*, *C. parvum* and lastly *Cyclospora* spp. Using multiplex RT-PCR *G. lamblia* and *C. parvum* were detected in 40 and 2 stool samples respectively. Twelve samples of *E. histolytica/dispar* detected by microscopy were negative by multiplex real time PCR probably due to the non-pathogenic *E. dispar.*

Key words: Children, Protozoa diarrhea, Microscopy, Multiplex real-time PCR.

Introduction

Gastrointestinal parasitic infections continue to be a significant public health problem mainly among Egyptian children (El-Nadi *et al*, 2017). They lead to malnutrition, protein and iron deficiencies as well as anemia (Elmonir *et al*, 2021). The modes of transmission include fecal-oral route from person to person and from a fecal contaminated environment (El Naggar *et al,* 2004). Parasites were the main agent of diarrhea, with prevalence among Cairo diarrheic patients up to 61% (Mousa *et al,* 2010). By microscopy protozoa diarrheic children acquired from contaminated drinking water was among potential risk factors in two Egyptian rural villages 62.7% (Abd Ellatif *et al*, 2018).

Microscopy is the gold standard and cornerstone for traditional laboratory to diagnose parasites, as simple and inexpensive method without microscopic job descriptions especially in developing countries with limited resources (Rosenblatt, 2009). But, it lacks the ability to differentiate closely related species and heterogeneity within species (Francis *et al*, 2003). Besides, apart from time consuming, microscope users' occupational problems were musculoskeletal of neck and nape, eye fatigue, aggravation of ametropia, headache, long working stress and anxiety during or after using (Jain and Shetty, 2014)

Serodiagnostic methods have been developed as alternative ways with more sensitivity and specificity and of epidemiological surveillance value such as IHA, IFA, ELISA, CCLE and others (El-Shazly *et al,* 2010). Moreover, PCR is routinely used to amplify, modify, and clone genes for expression studies. There are many other applications for PCR, including paternity testing, biological relationships, mouse genotyping, diagnosing genetic diseases, forensics, and finding bacteria and viruses (Canene-Adams, 2013).

The present study aimed to evaluate the multiplex PCR and conventional microscopic examination in detecting enteric pathogenic protozoa among some diarrheic school aged children in a rural village.

Material and Methods

Study design: A cross-sectional study was conducted during the period from February 2020 to November 2020 on 100 diarrheic children with various gastrointestinal problems. They were from El-Missery Village, of both sexes and less than 16 years old. The village is a rural one 49Km west to Alexandria with mean annual temperatures ranges from 15 to 32°C.

Ethical considerations the study protocol was approved by the Research Ethics Committee of the Medical Research Institute (MRI), Alexandria University (IO ROH: IORG0008812). An informed consent from parents/guardians of participating children was obtained after explaining purpose of the study. Sheets were filled out on each one included name, sex, medical history…etc.).

Stool sampling: Morning fecal samples were collected in dry, clean plastic labeled containers (name and code number). Samples were examined macroscopically for consistency, pin-worms and gravid segments. Then, stool sample was divided into three portions. First portion was examined as direct wet mount and Lugol's iodine smears. Second portion was preserved in 10% formalin for formal ethyl acetate centrifugation method for high detection of protozoa. A drop from concentrated sample was stained using Modified Ziehl-Neelsen stain (Garcia *et al*, 2006), and microscopically examined by low & high powers by Olympus light optical microscope (CX21FS1). The third fresh stool portion was stored at -20^{o}C for DNA extraction and PCR amplification using multiplex real time PCR.

Extraction of DNA from the frozen samples followed the manufacturer's instructions was carried out by using the commercial extraction kit (QIAamp® Fast DNA Stool Mini Kit, Germany). An inhibition control (supplied in the kit) was added before extraction to each clinical specimen in order to monitor the potential presence of any PCR inhibitors. The DNA extracts were stored at -80°C while awaiting multiplex PCR.

Multiplex real-time PCR (RIDA gene parasitic stool panel II, Germany) was performed in duplicate on 5µl of extracted DNA by using Rotor-Gene Q platform (Qiagen), following the manufacturer's instructions. This novel real-time PCR assay is a four-plex targeting simultaneously in a single-tube, *Giardia lamblia*, *Cryptosporidium parvum*, *E. histolytica* and the inhibition control. Positive and negative controls were included in each run. For considering the run validity, positive controls were reported as positive, negative controls as negative and external inhibition control displayed the Ct (Cycle threshold) value ≤36.

Statistical analysis: Data were computerized and analyzed using IBM SPSS software package version 20.0. (Armonk, NY: IBM Corp) (Kirkpatrick *et al*, 2013). Qualitative data were described using number and percent. The Kolmogorov-Smirnov test was used to verify the normality of distribution Significance of the obtained results was judged at the 5% level

Results

Of the 100 stool samples, 61 samples were positive for intestinal protozoa by Lugol's iodine; 53 cases had single protozoan infection and 8 cases showed co-infection; *G. lamblia* was the commonest protozoa (37.7 %) followed by *Blastocystis hominis* (27.8 %), *E. histolytica/dispar* (19.7 %) and *Entameba coli* (14.8%). Besides, 14 samples were co-infected with more than one intestinal protozoan. *G. lamblia* showed the highest infection rate (34.3%) among 67 infected children. Others were *B. hominis* (25.4%) 13 cases as single infection & 4 co-infected), *E. histolytica/dispar* infection rate was (17.9 %), 11 cases as single infection & one co-infected). *E. coli* was diagnosed rate was (13.4%), 6 cases as single infection & three co-infected). *C. parvum* & *Cyclospora* spp. were the least detected ones by ZN stain as five and one cases respectively. A total of *G. lamblia, E. histolytica/dispar* and *C. parvum* detected by stained smears were 40%.

By using multiplex real time PCR, *G. lam-*

blia infection rate was (40%) and *C. parvum* infection rate was (2%) among the 100 diarrheic children. Comparative analysis of both techniques for protozoa detection among children showed that among five children with *C. parvum* detected microscopically, three were negative by PCR, and two cases were positive by both, with significant differences (x^2= 38.77, ^{FE}p = 0.002). *G. lamblia* was detected in 23 cases by microscopy; of them four were negative by PCR, but 40 positive cases by PCR, 21 cases were negative by microscopy, with significant differences (x^2 = 22.60 at p<0.001). The 12 *E. histolytica / dispar* cases microscopy detected were not detected by multiplex real time PCR. There was some agreement between multiplex real-time PCR and coproscopic data, 10 cases positive by coproscopic test were negative by multiplex real-time PCR, and 13 positive cases by multiplex real-time PCR were negative by coproscopic one (κ=0.523).

Details were given in tables (1, 2, 3 & 4) and figure (1).

Table 1: Protozoa among positive diarrheic children diagnosed by wet mount iodine stool smears

Protozoa diagnosed	Single infection		Co-infection		Total	
	No.	%	No.	%	No.	%
G. lamblia	21	39.6	2	25.0	23	37.7
B. hominis	15	28.3	2	25.0	17	27.8
E. histolytica/dispar	11	20.8	1	12.5	12	19.7
E. coli	6	11.3	3	37.5	9	14.8
Total	53	100.0	8	100.0	61	100.0

A sample detected with *H. nana* and two samples with *E. vermicularis* as helminthes

Table 2: Intestinal protozoa infection among diarrheic children diagnosed by coproscopic methods (pooled data).

Protozoa diagnosed by pooled data coproscopic methods	Single infection		Co-infection		Total	
	No.	%	No.	%	No.	%
G. lamblia	20	37.7	3	21.4	23	34.3
B. hominis	13	24.5	4	28.6	17	25.4
E. histolytica / dispar	11	20.8	1	7.1	12	17.9
E. coli	6	11.3	3	21.4	9	13.4
C. parvum	2	3.8	3	21.4	5	7.5
Cyclospora spp.	1	1.9	0	0.0	1	1.5
Total	53	100.0	14	100.0	67	100.0

Table 3: Comparative analysis of two techniques to detect intestinal protozoa in diarrheic children.

Multiplex real time PCR	Pooled data coproscopic method				χ^2	p
	Negative		Positive			
	No.	%	No.	%		
C. parvum	(n = 95)		(n = 5)			
Negative	95	100.0	3	60.0	38.776*	^{FE}p= 0.002*
Positive (n=2)	0	0.0	2	40.0		
G. lamblia	(n = 77)		(n = 23)			
Negative	56	72.7	4	17.4	22.596*	<0.001*
Positive (n=40)	21	27.3	19	82.6		
E. histolytica / dispar	(n = 88)		(n = 12)			
Negative	88	100.0	12	100.0	-	-
Positive (zero)	0	0.0	0	0.0		

χ^2: Chi square test FE: Fisher Exact, *significant at p ≤ 0.05

Table 4: Agreement between multiplex real-time PCR versus pooled data coproscopic methods as a diagnostic gold standard

Multiplex real time PCR	Pooled data coproscopic methods				Total (n = 100)	
	Negative (n = 61)		Positive (n = 39)			
	No.	%	No.	%	No.	%
Negative	48	78.7	10	25.6	58	58.0
Positive	13	21.3	29	74.4	42	42.0
κ(p)	0.523*(<0.001*) Moderate agreement					

κ: kappa test, Multiplex Real-Time PCR detected *G. lamblia* in 40 samples, *C. parvum* in two samples

Discussion

Diarrhea is the second most common cause of morbidity and mortality in children in the Developing Countries (WHO, 2009). Studies carried out to identify etiological agents of diarrheal diseases found that viruses, bac-

teria, and parasites were their global burden as in USA (Thielman *et al*, 2004), in United Kingdom (Allen *et al,* 2010), even among food and food-products in UK (Dawson, 2005) and the farm animals in Canada (Budu-Amoako *et al,* 2011). *Entamoeba histolytica, Giardia lamblia*, and *Cryptosporidium parvum* are the commonest protozoa causing diarrhea (Kosek *et al*, 2003), mainly among immunocompetent patients (Massoud *et al*, 2008), and immunocompromised patients (Baiomy *et al,* 2010). Sepahvand *et al.* (2022) in Iran found that gastrointestinal parasites were commonest in immunocompromised patients than in immunocompetent individuals with *Cryptosporidium* spp. predominance. El Shazly *et al.* (2007) in rural Egypt detected enteric protozoa in water. El-Bahnasawy *et al.* (2018) in Egypt reported the wide spread of cryptosporidiosis causing chronic illness in the immunosuppressed patients and reported in animals and birds.

Traditionally, a wide variety of laboratory techniques has been employed for the detection of intestinal protozoan parasites (Ndao *et al*, 2009). The choice of microscopic examination remains the easiest, cheap and accepted diagnostic method (Lebwohl *et al*, 2003). However, it has some specific limitations such as; difficulties in quality control, operator dependence, the need for continuous evaluation and training, poor specificity (Abu-Madi *et al*, 2017).

In the present study, an overall prevalence rate of intestinal protozoa was 67%, & 53% had single infection, while 8% had double infection, The most frequently detected protozoa was *G. lamblia* followed by *Blastocystis hominis,* and *Entameba histolytica/dispar*. The results were higher when compared with those in the developed countries. Fletcher *et al*. (2012) in Austria reported that relative prevalence of enteric protozoa such as *Blastocystis* spp. ranged from 0.4% to 18.1%, followed by *G. intestinalis* (0.2% to 29.2%) and *Entameba* spp. (0.2% to 12.5%) of cases. But, Mergani *et al*, (2014) in Libya reported that 305 stool samples, a rate of diarrhea due to protozoa were 18.03% by the direct stained microscopic smears. This result was relatively lower than the expected prevalence in some of the developing countries and might be probably due to misdiagnosis or lack of registry.

The relatively high prevalence of intestinal protozoa infections reported in the present work might be related to the poverty, improper disposal of sewage, low standard of personal hygiene as fecal oral route is the main route of transmission among children.

The present study revealed that among the 100 diarrheic children, only a single case had *Cyclospora* spp infection and five children had *Cryptosporidium spp*. The sensitivity and specificity of coprodiagnosis using staining techniques appear to be low as identification depends on the experience and skills of the examiner. This more or less agreed with Youssef *et al*. (2000) in Irbid, Jordan they reported that among 218 children less than five years with gastroenteritis the *C. parvum* prevalence rate was 1.5%. Also, Osman *et al*. (1999) in Egypt used the MZN stained smears for coccidian parasitic in dirrheic immunocompromised children with protein energy malnutrition (PEM) and immunocompetent diarrheic children as controls. They reported that over all *C. parvum* infection prevalence was 15.48% (18.3%, 17.5%, & 7.3% in immunocompromised, PEM, and immunocompetent cases respectively).

Nevertheless, the previous studies reported a high *Cryptosporidium parvum* prevalence among immunocompetent children under the age of 5 years (Bera *et al*, 2014; Saha *et al*, 2019).The differences in the infection rate in different studies may be related to socioeconomic conditions, age, eating habits, immune status of hosts, local climatic factors, as well as the sample consistency and infective doses of the parasite.

More specific and sensitive alternative serodiagnostic methods such as the enzyme-linked immunosorbent assay, direct fluorescent-antibody assay and molecular assays were developed for urgent risky emerging infec-

tious diseases (Menu *et al*, 2018).

Molecular methods such as PCR proved to be sensitive and specific technique comparable to microscopy for diagnosis of stool parasites. However, these assays may be challenged by a number of factors such as; difficulty in isolation of genetic material (DNA) of these protozoa, which are present as oocysts or cysts which possess very robust cell walls hindering organism disruption and lys is (Surl *et al*, 2011). The presence of some fecal constituents such as hemoglobin degradated products, bilirubin, bile salts, and carbohydrates that may impair and/or inhibit the PCR amplification leading to false negative PCR results (Oikarinen *et al*, 2009). Besides, the requirement of separate reaction for each parasite and complex pre-analytical handling of samples for PCR assays might render the assays quite time-consuming, this is apart from its expensive costs (Schrader *et al*, 2012).

In the current study, detection of *G. lamblia*, *C. parvum* and *E. histoiytica* was done by using the multiplex real time PCR (RIDA GENE parasitic stool panel II). Multiplex real-time PCR for these gastrointestinal protozoa surpasses these shortcomings, the three parasites were identified in the same reaction directly from stool samples. Unlike microscopic analysis, the performance of the assay does not vary according to laboratory staff skills. For *Entameba* spp, PCR offers a valuable approach to distinguish pathogenic *E. histolytica* from nonpathogenic *E. dispar*, a substantial improvement to routine diagnostics, as this distinction cannot be achieved by microscopy examinations. Moreover, *Cryptosporidium sp.* constitutes a wide and heterogeneous group with 30 species at least 14 of which were reported to be zoonosis (Putignani *et al*, 2010).

In the current work, the overall prevalence of intestinal protozoa infections (*G. lamblia, E. histolytica/dispar* and *C. parvum*) among diarrheic children was 39% by pooled data of coproscopic methods, and 42% by multiplex real-time PCR. This agreed with Nazeer *et al*. (2013) in Egypt they detected *G. lamblia, E. histolytica, & C. parvum* among 146 cases positive by microscopy, and the 154 by the multiplex real-time PCR among the total of 396 diarrheic patients.

In the current study, moderate agreement between the multiplex real-time PCR and microscopy was found (K: 0.523* and P <0.001*).

Using microscopy, *G. lamblia* was detected in 23 cases, 4 cases diagnosed positive by microscope were negative by PCR and 19 cases were diagnosed positive by both methods, while among 40 cases detected by PCR, 21 cases were negative by microscopy.

The *G. lamblia* results detection agreed with Abdulkaleq (2016) in Iraq who detected *G. lamblia* in 24% by microscopy and in 42% by PCR on 100 children aged between 2 months and 18 years suffering from gastrointestinal complaints with diarrhea.

In the present study, the three *C. parvum* cases positive diagnosed by modified Ziehl-Neelsen stain were PCR negative, but only two cases were positive by both methods. The used assay (RIDA GENE parasitic stool panel II PCR) is designed only to cover *C. parvum*, the missed three cases in current work might be another less common species. The two most frequent *Cryptosporidum sp* cause human infection, *C. parvum* and *C. hominis*. Eida *et al*. (2009) in Is-mailia Governorate reported the predomin-ance of *Cryptosporidum* spp in Egypt to be *C. parvum* (66.7%) followed by *C. hominis* (27.7%) and *C. meleagridis* (5.6%).

In the present work, *E. histolytica/dispar* was detected in (12%) of the examined children by microscopic examination, while by using multiplex real-time PCR, none were confirmed, and one hundred samples were negative for *E. histolytica* infection.

As one of the drawbacks of microscopy that it cannot discriminate between E. *histolytica /dispar*, so the microscopically detected 12 % *E. histolytica/dispar*, which were negative by multiplex real time PCR, are probably due to the non-pathogenic *E. dispar*.

Errors recorded by microscopy might be related to stain artifacts, bad slides staining (Tsaku *et al*, 2017) or wrong protozoa diagnosis by a microscopist (Menu *et al*, 2018). The present overall detection of *E. histolytica/dispar* by microscopy gave false positive of 12. This agreed with Al-Harthi and Jamjoom (2007) in Saudi Arabia. The variations were attributed to infection intensity. But, Al-Haddad and Baswaid (2010) in Hadhramout reported children's parasites in descending order of prevalence as *G. lamblia*, *E. histolytica, Ascaris lumbricoides, Trichuris trichiura*, *H. nana, Taenia saginata* and *Schistosoma mansoni* and symptoms were diarrhea, abdominal pain, abdominal distention, constipation, nausea and vomiting and fever. Bahartha and AlEzzi (2015) in Mukalla, Yemen reported that apart from pathogenic causes, diarrhea were strongly associated with incomplete vaccination, recurrent diarrhea, and crowded housing.

Abroad, Autier *et al*. (2018) in France who aimed to compare three multiplex PCR assays on 93 collected positive stools and a panel of 12 more Cryptosporidium-positive samples. RIDA GENE PCR, showed 100% for *C. parvum* detection. No assay showed satisfactory results for all parasites simultaneously, and they concluded that the DNA extraction seems to be the critical step and recommended more studies needed to standardize this procedure.

Finally, multiplex PCR can be used as sensitive and specific, method for simultaneous detection of these protozoa, and allowed the measurement of several targets and genes of interest as the signal from each dye is used to quantitate the amount of each target separately in the same tube or well by different colors for each assay (Thanthrige-Don *et al,* 2018).

It is critical for patient outcome as well as reducing healthcare cost compared to separate measurement of each target by PCR, but it may be less convenient compared to microscopy in some developing countries with the limited updated resources.

Conclusion

There was moderate agreement between the multiplex RT-PCR and coproscopic microscopic examination as ten positive cases were proven negative by multiplex PCR while 13 cases were positive by multiplex RT-PCR and negative by microscopy.

Although, multiplex PCR, an expensive techn- ique, but being fast, sensitive and specific in the diagnosing of protozoa and overcoming the defect of traditional microscopic examination.

If an unequivocal identification of a risky parasite cannot be done, stools must be analyzed using molecular techniques such as the Multiplex RT-PCR to safe human health.

Authors' contribution: All authors equally contributed in this work.

Authors' declaration: Authors stated that they neither have conflict of interest nor received fund.

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Explanation of figure

Fig. 1: Cycling curves produced by fecal samples containing *C. parvum* (red) and *G. lamblia* (green) detect by multiplex real-time PCR. (Internal control included in each run shown in yellow color)