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INTESTINAL PARASITOSIS AND PEDIATRIC HEPATIC DISEASE: COPROSCOPY AND IMMNO MOLECULAR ASSAYS

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Abstract

Parasitic infections in children with liver diseases could be a serious health problem. This study documented a true prevalence of intestinal parasitosis in children with pediatric liver diseases and estimate risk factors for parasitic infection. Faecal samples were collected from 95 children suffering liver diseases. Samples were examined microscopically for parasitic stages and coproscopy of acid fast (AF) stained fecal smear for *Cryptosporidium* oocysts, by ELISA for *E. histolytica* and *Giardia* and by PCR genotyping for *E. histolytica* and *Cryptosporidium* spp. copro-DNA. *G. intestinalis* was the prevailing protozoa (14.7%) followed by *Hymenolepis nana* (8.4%) and *C. hominis* (4.2%). The pathogenic *E. histolytica* was not detected by genetic differentiation or ELISA. Only age group (*P* value .004) was a risk factor for intestinal parasitosis among these children. Giardiasis, hymenolepiasis nana and cryptosporidiosis are the most prevailing parasites in children with liver disease that physicians must consider. Accurate diagnosis using molecular technique is a must with *E.histolytica* and helpful with *Cryptosporidium* genotyping for epidemiological purposes.

Key words: Intestinal parasitosis, Liver diseases, Diarrhea, nPCR, RFLP, Genotyping

Introduction

Liver diseases could impair cell mediated immunity making hepatic patients more susceptible to infections (Zhang *et al*, 2008; Youssef *et al*, 2008). Opportunistic parasites in hepatic patients may lead to severe diarrhea, dehydration and electrolyte imbalance (Tuli *et al*. 2010). Intestinal parasitosis with *E.histolytica, Cryptosporidium spp.* and *G. intestinalis* caused severe illness in immunocompromised patients (Nazeer *et al*. 2013).

E. histolytica is manifested by diarrhea, dysentery or extraintestinal complications such as amoebic liver abscess which are fatal, hence accurate diagnosis is vital (Petri *et al*. 2000; Ohnishi *et al*, 2004; Zebardast *et al*, 2016). The Tech-Lab *E. histolytica* coproantigen detection kit was properly diagnosed *E. histolytica* (WHO, 1997). For true estimation of pathogenic amoebiasis molecular techniques are required (Parija *et al*, 2010; Lu *et al*, 2016).

Cryptosporidiosis is a known cause of diarrhea for both patients with impaired immunity and healthy. Dissemination to other organs is a risk in immunocompromised patients (Snelling *et al*, 2007; Stark *et al*, 2011; Goñi *et al*, 2012). *Cryptosporidium* was detected by Shrestha *et al*. (1993) and El-Shazly *et al*. (2015) used microscopic examination of stool in patients with liver diseases (20% & 10%, respectively) and Mousa *et al*. (2014) reported to be an aggravating factor for hepatic encephalopathy.

G. intestinalis is a major cause of parasitic diarrhea in children. The course of giardiasis may be prolonged to chronic diarrhea, weight loss and malabsorption in patients with impaired immunity (Muhsen and Levine, 2012; Helmy *et al*, 2014).

The present study focused on a group of children suffering liver diseases, to explore the parasitosis prevalence among them using immune-assay and molecular techniques, and to monitor risk factors for parasitosis.

Material and Methods

Ethical considerations: All patients included were informed verbally about the purpose of the study and their parents' consent was taken before collection of stool samples.

Study population: The cross-sectional study was carried out on 95 children aged from

1-15 years old attending the Pediatric Hepatology Unit, Cairo University Pediatrics Hospitals (CUPH) and proved by clinical, radiological and/or laboratory tests to have liver diseases. None of them were having liver cell failure. All patient related demographic and clinical data were collected using designed questionnaires.

Collection and processing of samples: From 1 to 3 stool samples were collected from all children and divided into three parts, one for examination by direct wet mount and formalin-ethyl acetate concentration after adding saline and Lugol's iodine to detect the intestinal protozoans (oo)cysts, helminthes ova and other parasitic stages using x10 & x40 objectives. Modified Ziehl Neelsen stain was used to detect *Cryptosporidium* oocyst. The other two parts were freshly frozen at -20°C for further assays.

Copro-immunoassay: Parts of the frozen samples were subjected to coproantigen detection of *E.histolytica* using Wampole TM *E.histolytica* II, 2nd generation monoclonal ELISA kit (Tech-Lab, Blacksburg, Virginia, USA, No.T5017) and *G. intestinalis* using RIDASCREEN (R-Biopharm GmbH, Darmstadt, Germany) kit according to the manufacturer`s instructions.

Copro-PCR assay: It was used for detection and genotyping of *Cryptosporidium* targeting *Cryptosporidium* oocyst wall protein (COWP) gene using nPCR Restriction fragment length polymorphism (RFLP) and genetic differentiation of *Entamoeba* complex using 16S-like gene. All samples were subjected to genomic DNA extraction using Favor Prep DNA isolation Kit (Favorgen Biotech, Taiwan, Cat. No. FASTI001-1) according to the manufacturer`s instructions with some modifications, using alternation of liquid nitrogen thermal shock followed by 95°C water bath for 10 cycles.

PCR amplification: For detection of *Cryptosporidium* two sets of primers were used. BCOWPF (5'-ACCGCTTCTCAACAACCA TCTTGTCCTC-3'), BCOWPR (5'-CGCAC CTGTTCCCACTCAATGTAAACCC-3') at ~796bp fragment (Pedraza-Díaz *et al*, 2000). Nested primers, cry-15 (5'-GTAGATAATG GAAGAGATTGTG-3'), cry-9 (5'-GGACT-GAAATACAGGCATTATCTTG-3') at~553 bp fragment were used. The reaction conditions and mixture were done (Spano *et al*, 1997). PCR amplified products were visualized by using ethidium bromide stain, agarose gel electrophoresis (1.5%) & UV Trans-illuminator. Positive PCR products were digested by *Rsa*I (Fer-mentas UAB, V. Graiciuno 8, LT-02241 Vilnius, Lithuania), digested N-COWP frag-ments were resolved by electrophoresis in typing-grade agarose gels (3.2%) stained with ethidium bromide, the fragments were visualized by UV light to determine *Cryptosporidium* genotype.

For genetic differentiation of *E. histolytica*, *E. dispar* and *E. moshkovskii* a multiplex PCR (mPCR) targeting 16S-like gene was used, for primary reaction EF 5'-TAA GAT GCA GAG CGA AA-3' & ER 5'-GTA CAA AGG GCA GGG ACG TA-3' were used at ~800bp amplification. For secondary reaction the following sets of primers were used EHF 5'-AAG CAT TGT TTC TAG ATC TGA G-3'and EHR 5'-AAG AGG TCT AAC CGA AAT TAG-3' at a fragment of ~439bp for *E. histolytica*, EDF 5'-TCT AAT TTC GAT TAG AAC TCT-3' and EDR 5'-TCC CTA CCTATT AGA CAT AGC-3'at a bp of ~174 for *E. dispar* and EMF 5'-GAA ACC AAG AGT TTC ACA AC-3' and EMR 5'-CAA TAT AAG GCT TGG ATG-3' at a bp of ~553 for *E. moshkovskii* with reaction components and cycling conditions (Ngui *et al*, 2012). The amplified products were visualized with agarose gel electropho-resis (1.5%) stained with ethidium bromide.

Statistical analysis: Data were tabulated, coded and analyzed with the SPSS version 20 (statistical package for social science). Qualitative variables were analyzed by percentage and frequency, while quantitative variables were described by mean $\pm$ SD. Risk factors of intestinal parasitosis were the dependent variable among the studied popu-

lation, odds ratio (OR), 95% confidence interval (CI) &*P* value < 0.05 was significant.

Results

The study included 95 children, 57 (60%) male and 38 (40%) female with a mean age of 6.1±3.4. Among them, 40 (42.1%) patients were harboring one parasite at least; while the remaining 55 (57.9%) patients were free. Results of parasitic prevalence, diagnostic yielding, demographic, clinical data and hepatic etiology were given (Tabs. 1, 2, 3, 4 & 5).

Table 1: Coproscopic prevalence of parasitic infections among studied population.

Parasitic infections		Total (n=95) and %
Protozoa	*Giardia* cysts/trophozoites	13(13.6)
	E. complex cysts/trophozoites	4(4.2)
	Entamoeba coli cysts	3(3.1)
	Blastocyst hominis cysts	4(4.2)
	Entamoeba coli / Blastocyst hominis cysts	3(3.1)
	Cryptosporidium oocysts (MZN stain)	1(1.1)
	Iodamoeba butchii cysts	1(1.1)
Helminthes	*Hymenolips nana* eggs	8(8.4)
	Taenia eggs	2(2.1)
	Entrobius vermicularis	1(1.1)

Table 2: Prevalence of *G.intestinalis* and molecular prevalence of *E.* complex &*Cryptosporidium* spp. among population.

Positive cases	*Giardia* by microscopy and ELISA n (%)	*Cryptosporidium* by nPCR n(%)	*E.* complex by mPCR		
			*E .h**	*E. d**	*E. m**
Total (n=95)	14(14.7)	4(4.2)	0	0	0

E.h: E.histolytica, E.d: E.dispar and *E.m: E.moshkovskii*

Table 3: Age groups distributions among studied population

Age groups	Intestinal parasitosis		Total (n=95)	*P* value
	Yes (n=40)	No (n=55)		
	n (%)	n(%)	n(%)	.004*
Infant (up to 2 years)	2(5)	9(16.3)	11(11.6)	
Early childhood (2-6years)	15(37,5)	29(52.7)	44(46.3)	
Late childhood (6-12 years)	19(47.5)	15(27.3)	34(35.7)	
Young teens (12-15 years)	4(10)	2(3.6)	6(6.3)	

*significant

Table 4: Demographic and clinical features of studied population.

Variable		Intestinal parasitosis		Total (n=95)	*P* value	*OR	**95% CI	
		Yes (n=40)	No (n=55)				lower	upper
Giardia	Male Female	23(57.5) 17(42.5)	34(61.8) 21(38.2)	57(60) 38(40)	.41	.83	.364	1.91
Gastro-intestinal symptoms	Diarrhea	24(60)	35(63.6)	59(62.1)	.83	.85	.371	1.981
	Abdominal pain	12(30)	12(21.8)	24(25.3)	.47	1.53	.605	3.896
	Fatigue	16(26.6)	10(28.5)	26(27.3)	.25	.64	.254	1.656
	Flatulence	8(20)	5(9.1)	13(13.6)	.14	2.50	.751	8.31
	Dysentery	6(10)	3(8.5)	9(9.4)	.45	1.92	.406	9.129

Table 5: Hepatic etiology among studied population.

Hepatic diseases	Intestinal parasitosis		Total	P value
	Yes (n=40) %	No (n=55) %	(n=95)	.398
Metabolic disease	16(40)	30(54.5)	46(48.4)	
Hepatitis C	5(12.5)	6(10.9)	11(11.5)	
Hepatitis B	4(10)	3(5.4)	7(7.3)	
Hepatitis C&B	2(5)	1(1.8)	3(3.1)	
Hepatitis A	3(7.5)	6(10.9)	9(9.4)	
Autoimmune disease	3(7.5)	2(3.6)	5(5.2)	
Wilson disease	2(5)	2(3.6)	4(4.2)	
Congenital hepatic fibrosis	2(5)	2(3.6)	4(4.2)	
Fasciola infection	2(5)	2(3.6)	4(4.2)	
Cystic fibrosis	1(2.5)	1(1.8)	2(2.1)	

Discussion

In the present study, *G. intestinals* (14.7 %) was the most prevalent protozoa among children suffering hepatic diseases followed by *Hymenolepis nana* (8.4%). Reduced immunity caused by the liver affection could

play a role in transmission of parasitic infections in addition to the socioeconomic and environmental factors, as a number of intestinal parasites and commensals beside *G. intestinalis, Hymenolepis nana* and *C. hominis* were detected. Hegab *et al.* (2003) reported that *G. intestinals* (45%) was the commonest parasite in children with chronic liver disease. Also, Nazeer *et al.* (2013) using real-time PCR found that giardiasis (37.1%) was the commonest one followed by *Cryptosporidium* spp (3%).

In the present study, cryptosporidiosis was the second protozoa (4.2%), all positive cases of *C. hominis*, three of whom were complaining of diarrhea. Predominance of the anthroponotic strain *C. hominis* supported man to man transmission by water contamination. Using nPCR targeting COWP gene detected 75% of cases which were negative by microscopy. Fathy *et al*. (2014) and El-Badry *et al.* (2015) reported the precious use of molecular techniques in cryptosporidiosis diagnosis. Abd El Kader *et al*. (2012) and El-Badry *et al.* (2015) reported the predominance of *C. hominis* (80% & 95.8%, respectively) among Egyptian diarrheic patients. But, Eraky *et al.* (2014) found the predominance of *C. parvum* (82%) and Mousa *et al.* (2014) found a higher rate of *C. parvum* than *C. hominis* in chronic liver disease patients. Differences might point to the sources of infection and features of water supply contaminations in different communities which vary from one area to another.

The prevalence of *Entamoeba* complex by microscopy was 4.2%, all suffered from diarrhea without dysentery. However, pathogenic *E. histolytica* was not detected by 2nd generation monoclonal ELISA kit (Tech Lab) or by molecular differentiation using 16S-like gene. Also, neither *E. dispar* nor *E. moshkovskii* were detected by PCR, suggested that positive microscopy cases were related to other species like *E. polecki*, *E. hartmanni* or *E. bangladeshi.* Nazeer *et al.* (2013) reported a microscopic prevalence of 10.8% for *E. histolytica/dispar* among 396 diarrheic patients, without *E. histolytica* by real-time PCR.

But, Younes *et al.* (1996), Hegab *et al.* (2003) and El-Shazly *et al.* (2015) reported high microscopic prevalence of *E. histolytica/E.dispar* (21.5%, 37.5 % & 16%, respectively) among patients with chronic liver diseases and recurrent diarrhea. This discrepancy between studies could be explained by the differences in the diagnostic methods used. Microscopy was a non-specific method and led to false positive results and could not differentiate the pathogenic amoebiasis from other morphologically identical non-pathogenic species (Parija *et al*, 2010; Zebardast *et al*, 2016). Thus microscopy results carried the risk of exposure of healthy children to unnecessary treatment (Haque *et al.* 2006).

Healthy intestinal epithelium and normal tight junction represent natural barriers for gut immunity (Vajro *et al*, 2013). Commensals are major component of gut microbiota which in role is vital for keeping gut mucosal barriers integrity (Mohajeri *et al*, 2018). The present patients were free from liver cell failure reflecting a controllable state of liver, a question arises are the nonpathogenic amoeba spp and other commensals detected among our study population helpful to patients with liver disease? Or even they might contribute for better microbiota and keeping gut integrity, non-specific treatment will be even harmful to these patients.

The commonest hepatic etiology among the studied population was metabolic liver diseases (48.4%), followed by hepatitis C, A & B (11.5%, 9.4% & 7.3%, respectively). However patients with cystic fibrosis (2.1%) were the least represented. Not all patients were complaining of chronic liver diseases, acute hepatitis A represented 9.4% of the patients. All patients weren't complaining of liver cell failure. Liver etiology wasn't significantly a risk factors for parasitosis (P = .398). Among studied variables to determine the predictive factors for intestinal parasitosis among children with hepatic disease, on-

ly age group (*P*= .004) was a significant risk factor. Intestinal parasitosis among different age groups of hepatic children reached its peak during late childhood (6-12 years old = 47.5 %) which represented the age of grade scholars where the children use toilets in school and public regions and didn't care about their hygiene and neglect washing hands before meals in addition, they used to play and eat outdoors being more exposed to parasitic infections. Followed by the age of early childhood (2-6years =37.5%), the preschool age, as children may attend day care centers and nursery schools being more exposed to infectious parasites through eating or drinking. The least exposed group was the infantile age (up to years old =5%) as they depend on their mother on feeding being less exposed to infected food. Young teens (up to 15 years old =10%) were personal hygiene of children improved by this age.

Conclusion

G. intestinals, *H. nana* and *C. hominis* must be suspected among children with liver diseases. Once liver is free from cell failure, hepatic etiology isn't a risk factor for intestinal parasitosis. Microscopic positive *E. complex* must be confirmed molecularly for pathogenic *E. histolytica* to avoid unnecessary treatment or disturbance of gut microbiota. Genotyping of *Cryptosporidium* proved essential for targeting the infection source.

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Compliance with Ethical Standards: Protocol was approved by Beni-Suef University Research Ethics Committee. Sample collections and ethics were in agreement with the Helsinki declaration; 1964.

Informed consent: All patients were informed verbally about the study purpose and their parents' consent was taken before collection of stool samples.

Conflict of interest: None.

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