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Molecular and genotyping for *Cryptosporidium parvum* isolated from children with diarrhea in pediatric hospital of Karbala

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Abstract

The study involved surveilling sampling of children suffered cryptosporidiosis like symptoms.The present study was conducted on 90 children who suffer from abdominal pain and diarrhea .Samples were collected from patients suspected infection by cryptosporidium who attended in pediatric hospital of Karbala Governorate from January-2019 to May 2019,They were (1month-5 years old age). Each sample was divided into two parts. The first part was used to prepared a moderate thick smears stained with modified zehil nelson stain(m ZN stain)and the second part was preserved at -20 c for molecular detection by nested PCR to identify the positive samples and genotyping by RFLP-PCRthat showed the PCR product analysis of HSP70 gene in Cryptosporidium parvum from Human stool samples, at (587bp) PCR product.Some positive samples by RFLP-PCR making nucleic acid sequencing.The. main. Purpose. of .the .current. study. was. to explore the prevalence rate of cryptosporidiosis in the mentioned province targeting children at different age categories of both genders. For the gender and infection by *C. parvum*, the current work findings .revealed. the. presence. of. *C. parvum* .in. the sampled children distributed under gender categories of male and female and the species was parvum .The results of the AFS showed that 12 (13.33%) out of 90 samples were positive to the presence of cryptosporidiosis. This technique is a good method for detecting *C. parvum*in stool samples of children in both genders, and 27 out of 90 samples was positive by nested PCR and RFLP-PCR respectively. For the age and infection by *C. parvum*, the current work findings revealed. the. presence .of .*C. parvum* .in .the. sampled children distributed under three age categories of 1-6 months, 7-12 months, and 13-60 months. Positive results of the age categories were 5 (29.41%) out of 17 samples, 12 (32.43%) out of 37 samples, and 10 (27.78%) out of 36 samples, respectively. The total infection was 27 (30%) out of 90 samples. No significant (p>0.05) differences were recognized for infection rates occurred for the age categories.

Keywords: *pediatric hospital ; Cryptosporidium parvum; diarrhea*

Introduction

Cryptosporidium spp.is a coccidian protozoan parasite that causes waterborne outbreaks worldwide using the fecal-oral route for the transmission of infection. Cryptosporidium is one of the leading pathogens which are responsible of the majority of the diarrheal infections (1).

Cryptosporidium is considered as a major diarrheal cause of diarrhea in children and immune-compromised people with high death rates for those under 5 years of age (2).

Diagnosis. is. Made. by. concentration. Of. stools .followed. by .acid-fast staining (AF), a nested. PCR. assay was developed. to. detect C. parvum DNA directly from stool specimens, after extraction of DNA from formalinized stool.(3)..

A polymerase. chain .reaction (PCR)restriction. fragment. length polymorphism. analysis of a 587-bp region .of. the. Cryptosporidium. parvum70-kDa. heat. shock .protein (HSP70) gene. was. developed. for. the detection. and. discrimination. of. the. two .major. genotypes. of. C. parvum(4).

Studies have recognized using modern molecular technologies some subtype families that can induce wide-spread diarrheal conditions (5).

Infection caused by Cryptosporidium spp. is a highly

common parasitic diarrhea known in humans and cattle. However, cases that show no symptoms, asymptomatic, are not well recognized for identification as that in the symptomatic case (6).

Mterias and Method

study design

The study was conducted on 90 random samples of faecal smears of children from the laboratories of hospital Paediatric in Karbala City. The age of children ranged from 1 months to 5 years. The study started from January2019 may 2019. The modified Ziehl- Neelseen stain (acid fast), used for examination of faecal smears to detect oocysts of cryptosporidium follows .Smears were fixed with methanol alcohol for 5 minutes and allowed to dry at room temperature. Driedsmears were stained for an hour in carbolfucshin prepared by dissolving 15% carbolfucshin in methanol (stock solution). Ten ml of Ziehlfucshin were added to 90 ml of 5% phenol. Smears were rinsed in tap water and differentiated in 2 % H2SO4 solution for 20 seconds while agitating the slide and rinsed in tap water . Smears were counter stained with 5 % malachite green solution for 5 minutes, washed in tap water and left until dried. Stained smears were examined by using 40 X and 100 X oil immersion objectives. Oocysts were obtained from naturally infected children with acute diarrhoea and proved to be infected with *Cryptosporidium* as confirmed by M.ZN-ST.(acid fast).

Molecular detection

The specimens were stored at 4³Cfor DNA extraction. Faecal specimens from children with diarhea whose suspected contained Cryptosporidium .

Nested Polymerase chain reaction (nPCR)

The nPCR techniquewas performed for detection Cryptosporidium parvum based 18S ribosomal rRNA gene from children samples(1 month to 5 years).

Genomic DNA Extraction (stool protocol)

Genomic DNA from feces samples were extracted by using AccuPrep® stool DNA Extraction Kit ,Bioneer. Korea, and done according to company instructions.

Primers

The Nested PCR primers for detection Cryptosporidium spp. based 18S rRNA gene and another Nested PCR primers for genotyping Cryptosporidium parvum based hsp70 protein gene were designed according to [4] . These primers was provided from Macrogen company, Korea as following table 1.

Table 1: Primers properties for protein gene designed

Primer	Sequence 5'-3'	
ssrDNA gene PCR	F	GGGTTGTATTTATTAGATAAAGAAC
	R	CTTTAAGCACTCTAATTTTCTC
ssrDNA gene Nested PCR	F	GACTTTTTGGTTTTGTAATTGGAATG
	R	TAAATTATTAACAGAAATCCAACTACGAGC
C. parvum HSP70 gene	F	AGCAATCCTCTGCCGTACAGG
	R	AAGAGCATCCTTGATCTTCT

1. Direct Isolation of DNA from Patient Stools for Polymerase Chain Reaction Detection of Cryptosporidium parvum.

2. Sensitive genotyping of Cryptosporidium parvum by PCR-RFLP analysis of the 70-kilodalton heat shock protein (HSP70) gene.

RFLP

RFLP-PCR technique was performed for genotyping Cryptosporidium parvum based on HSP70 gene . This method was carried out according to described by [4].

RFLP-PCR Technique

RFLP-PCR technique was performed for genotyping

Cryptosporidium parvum based on HSP70 gene . This method was carried out according to described by[4] as following steps:

PCR master mix preparation

PCR master mixwas prepared by using (AccuPower PCR PreMix Kit) and this master mix done according to company instructions as following table for each gene:

After that, these PCR master mix component that mentioned in table above placed in standard AccuPower PCR PreMix Kit that contains all other components which needed to PCR reaction such as (Taq DNA polymerase, dNTPs, Tris-HCl pH: 9.0, KCl, $MgCl_2$,stabilizer, and loading dye). Then, all the PCR tubes transferred into Exispin vortex centrifuge at 3000rpm for 3 minutes. Then placed in PCR Thermocycler (Mygene. Korea).

DNA sequencing method

DNA sequencing method was performed for species typing of positive Cryptosporidium sp isolates as following step:

1- The PCR product of 18S ribosomal RNA genes were sent to Macrogen Company in Korea in ice bag by DHL for performed the DNA sequencing by AB DNA sequencing system.

2- The DNA sequencing analysis (Phylogenetic tree analysis) was conducted by using Molecular Evolutionary Genetics Analysis version 6.0. (Mega 6.0) and Multiple sequence alignment analysis based ClustalW alignment analysis and The evolutionary distances were computed using the Maximum Composite Likelihood method by phylogenetic tree UPGMA method.

3- The Cryptosporidium species typing analysis was done by phylogenetic tree analysis between local Cryptosporidium sp isolates and NCBI-Blast known Cryptosporidium species.

Finally identified Cryptosporidium species isolates were submitted into of NCBI-GenBank to get Genbank accession number.

Results and Discussion

Acid-fast stain age based results microscopic

For the age and infection by *C. parvum*, the current work findings revealed. the. presence .of .*C. parvum* . in .the. sampled children distributed under three age categories of **<= 1** years, **1 – 3** years, and **> 3**yeaes, Positive results of the age categories were 7 (13.0%) out of 54 samples, 3 (10.3%) out of 29 samples, and 2 (28.6%) out of 7 samples, respectively. The total infection was 12 (13.3%) out of 90 samples. No significant (p>0.441) differences were recognized for infection rates occurred for the age categories by microscopic.

Table (2): Relationship between Age groups * Microscopic

Variable Positive Negative			**Microscopic**		**Total**	**Chi-square (df)**	**p-value**
	<= 1	**Count**	**7**	**47**	**54**	**1.637 (2)**	**0.441 NS**
		%	**13.0%**	**87.0%**	**100.0%**		
	1 – 3	**Count**	**3**	**26**	**29**		
		%	**10.3%**	**89.7%**	**100.0%**		
	> 3	**Count**	**2**	**5**	**7**		
		%	**28.6%**	**71.4%**	**100.0%**		
Total %		Count	12	78	90		
		13.3%	86.7%	100.0%			

Age based results nested PCR

For the age and infection by *C. parvum*, the current work findings revealed. the. presence .of .*C. parvum* . in .the. sampled children distributed under three age categories of **<= 1** years, **1 – 3** years,

and **> 3**yeaes, Positive results of the age categories were 16 (29.6%) out of 54 samples, 7 (24.1%) out of 29 samples, and 4 (57.1%) out of 7 samples, respectively. The total infection was 27 (30.0%) out of 90 samples. No significant (*p*>0.231) differences were recognized for infection rates occurred for the age categories by nested PCR table 3.

Table (3): Relationship between Age groups * Nested PCR

Variable **Negative** **Positive**			**Nested PCR**		**Total**	**Chi-square (df)**	**p-value**
	<= 1	Count	38	16	54	2.934 (2)	0.231 NS
		%	70.4%	29.6%	100.0%		
	1 – 3	Count	22	7	29		
		%	75.9%	24.1%	100.0%		
	> 3	Count	3	4	7		
		%	42.9%	57.1%	100.0%		
Total %		Count	63	27	90		
		70.0%	30.0%	100.0%			

Age based results genotyping

For the age and infection by *C. parvum*, the current work findings revealed. the. presence .of .*C. parvum* . in .the. sampled children distributed under three age categories of **<= 1** years, **1 – 3** years, and **> 3**yeaes, Positive results of the age categories were 16 (29.6%) out of 54 samples, 7 (24.1%) out of 29 samples, and 4 (57.1%) out of 7 samples, respectively. The total infection was 27 (30.0%) out of 90 samples. No significant (*p*>0.231) differences were recognized for infection rates occurred for the age categories by genotyping table 4.

Table (4): Relationship between Age groups * Genotype

Variable **Negative** **Positive**			**Genotype**		**Total**	**Chi-square (df)**	**p-value**
	<= 1	Count	38	16	54	2.934 (2)	0.231 NS
		%	70.4%	29.6%	100.0%		
	1 – 3	Count	22	7	29		
		%	75.9%	24.1%	100.0%		
	> 3	Count	3	4	7		
		%	42.9%	57.1%	100.0%		
Total %		Count	63	27	90		
		70.0%	30.0%	100.0%			

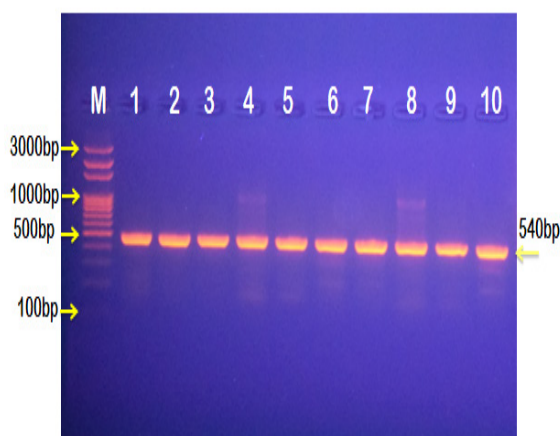

Figure 1: Agarose gel electrophoresis image that showed the Nested PCR product analysis of small subunit ribosomal RNA gene in Cryptosporidium parvum from Human stool samples. Where M: marker (3000-100bp) Lanes (1-10) some positive patients samples at (540bp) PCR product.

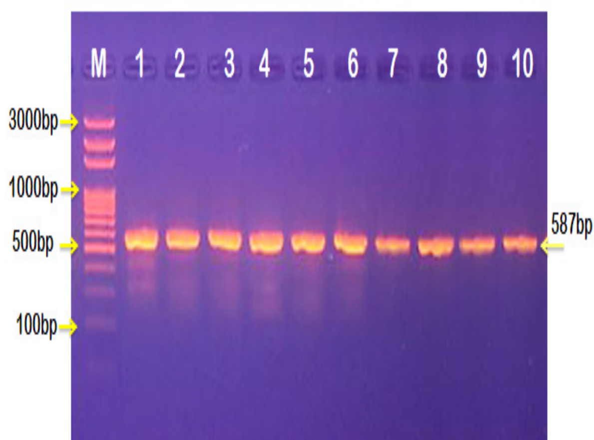

Figure 2: Agarose gel electrophoresis image that showed the PCR product analysis of HSP70 gene in Cryptosporidium parvum from Human stool samples. Where M: marker (3000-100bp) Lanes (1-10) some positive patients samples at (587bp) PCR product.

For the infection rates and the age of sampled children, it has been shown identified, using RFLP PCR, that 4.8% of infection was detected. in .children under. the. age. Of. 5 years .old (the only sampled age category); however, and after performing genotypic characterizations, infection was higher due to *C. hominis* at 75% than that for the case of infection caused by *C. parvum* at 25.0%. The current work findings revealed the presence of *C. parvum* in the sampled children under the three age categories under 5 years old [(5)(7)]Nigeria. METHODS Stool samples were collected from165 children aged 0-5 years with diarrhea. Cryptosporidium oocysts were examined by wet mount preparation, using formalin ether and a modified acid fast staining method. DNA was extracted from positive samples using QIAamp DNA stool mini kit and PCR-RFLP assay was carried out after quantification. Genotyping and phylogenetic analysis were done to determine the subtype families and their relatedness. RESULTS From the 165 children studied, 8 (4.8%. Our results agree with [(8)] who revealed that *C. parvum* infection rates were higher in children under 5 years of old, 3.15%, than those in older age categories especially students of middle schools, 0.82%. These results can be explained as there might have been an immunodeficiency problem facing the sampled children decreasing immune reaction directed toward *C. parvum* by the immune cells such as CD_3^+, CD_4^+ and CD_4^+/CD_8^+ and then resulting in immune response reduction [(8)].

Conclusion

1.Most of examined to the children from 1month to5years whose suffer from abdominal pain and diarrhea was positive in most cases.

2.The detection of the stool-modified-acid-fast stained oocytes is performed using light microscopy; however, this technique is limited to the identification of the genus level only with no vital information regarding epidemiological properties. Currently, *Cryptosporidium* can be identified by molecular and genotyped using developed molecular techniques such as nested PCR , RFLP-PCR and nucleic acid sequencing in modern laboratories.

3.nested polymerase chain reaction technique is the best method than others technique used in diagnosis of *cryptosporidium* by using specific primer for this parasite.

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Ethical Clearance: The Research Ethical Committee at scientific research by ethical approval of both environmental and health and higher education and scientific research ministries in Iraq

Conflict of Interest: The authors declare that they have no conflict of interest.

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