Genotyping of Cryptosporidium Parvum by PCR-Sequencing Analysis for The 70 Heat Shock Protein (HSP70) Gene and Dihydrofolate Reductase (DHFR) Gene

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Abstract

Cryptosporidium is an apicomplexan parasite that is increasingly recognized among immunocompetent hosts as a leading cause of childhood diarrhea in lower source settings and of waterborne diarrheal outbreaks in high income countries. C. parvum, which infects bovines, wild animals and humans, account for the majority of human infections. Cryptosporidium oocysts are transmitted via the fecal oral route, including by person to person spread, from contaminated food or water, or from contact with infected animals. 300 Stool samples were collected from patients in Hilla city from different hospitals. DNA extracted from stool samples, PCR amplification of DNA. Genotyping of HSP70 gene and DHFR Gene was performed using a polymerase chain reaction technique, followed by DNA sequencing. Accordingly, these DNA polymorphisms were confirmed using DNA sequencing. The sequencing results showed the presence of many SNPs determined in the HSP70 gene and DHFR gene.

Key words: *PCR, sequencing, polymorphism, Cryptosporidium parvum, HSP70 gene, DHFR gene.*

Introduction

Cryptosporidium parvum It is one of several species that cause cryptosporidiosis, a parasitic disease of the mammalian intestinal tract, that primarily involves watery diarrhea (intestinal cryptosporidiosis) with or without a persistent cough (respiratory cryptosporidiosis) in both immunocompetent and immunodeficient humans [1]. In immunosupressed individuals, the symptoms are particularly severe and can be fatal. It is primarily spread through the fecal-oral route, often through contaminated water; recent evidence suggests that it can also be transmitted via fomites in respiratory secretions [2].

C. parvum is considered to be the most important waterborne pathogen in developed countries. The protozoa also caused the largest waterborne-disease outbreak ever documented in the United States, making 403,000 people ill in Milwaukee, It is resistant to all practical levels of chlorination, surviving for 24 hours at 1000 mg/L free chlorine. It is an obligate intracellular pathogen [3]. The risk factors for outbreak transmission of cryptosporidiosis are well known. In industrialized countries, waterborne outbreaks by contaminated drinking or recreational water are increasingly frequent [4].

The genome of C.parvum, sequenced in 2004, was found to be unusual amongst eukaryotes in that the mitochondria seem not to contain DNA [5]. A closely related species, C. hominis, also has its genome sequence available [6].

For PCR-based analyses, the most commonly used Cryptosporidium-specific biomarker is the gene encoding the 18S rRNA subunit, which has been used for the detection and genotyping of oocysts present in environmental and clinical samples [7,8]. Other markers,

including Cryptosporidium 60 kDa glycoprotein (gp60), microsatellite locus 1 (ML-1) and 2 (ML-2), Cryptosporidium heat-shock protein)HSP70), b-tubulin and Cryptosporidium oocyst wall protein (COWP), CP-HPS7O and CP-DHFRF have also been used for this purpose [9,10] .

PCR nucleotide sequence might be of great value for identification on the species level. Sequencing usually involves part or all the mitochondrial genome followed by its comparison with known sequences in Gene Bank (NCBI). PCR technique is suitable and accepted but it is expensive and needs to more time and lab our consuming due to the further step of sequencing products, mixtures cannot be separated, and the generated samples may not produce enough sequence results [11].

DNA Sequencing gives much information with no need for more steps like digestion with enzymes or analysis of the given data. The most appropriate mitochondrial genes used for species identification using sequencing technology are cyt b, 12S and 16S rRNA genes could give a considerable number of mutations and there is also much information found on data bases concerning their sequences [12]. The variations in the sequences of mitochondrial 12S and 16S rRNA gene are suitable and sufficient for identification between different species from high number of vertebrates such as birds, fish, reptiles, mammals, and amphibians [13].

the aim of this work was to detected CP-HPS7O and CP-DHFRF using of -PCR technique and DNA sequencing to investigate single nucleotide polymorphisms among Cryptosporidium parvum isolated from different pathological samples in hilla city.

Methodology

Sampling

Stool samples were collected from patients ,children and adults, male and female patients from 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 during the period from October 2018 till February 2019, and transferred to the advance parasitology laboratory in the College of Science Babylon University, where they were collecting 300 samples and placed in the sterilized plastic containers the size of approximately 20gm, and with a tight lid to keep samples moisture and prevent dry.

DNA Extraction

The extracted genomic DNA from faeces samples (96 samples) for all parasites were checked by using nanodrop spectrophotometer, that check and measure the purity of DNA through reading the absorbance in at (260 /280 nm) according to DNA Extraction Kits From stool Favorgen- Taiwan.

Primers

Two PCR primers were designed in the present study for detection Cryptosporidium parvum. based subunit ribosomal rRNA gene by using NCBI- Genbank (U11761.1, U69698.2 respectively).

Table (4): the primers.

Primer	Sequence 5----------3
CP-HPS7OF	5-ATG TCT GAA GGT CCA GCT ATT GGT ATT GA-3
CP-HSP7OR	5-TTA GTC GAC CTC TTC AAC AGT TGG-3
CP-DHFRF	5-GTG GGG ATT TAA CTT GAT TT-3
CP-DHFRR	5-GGT ATT TCT GGG AAA TAA GT-3

PCR amplification

Final product of 25µl reaction volumes containing 1 ul of forward and reverse primer ,12.5 ul of green Master combine ,3 ul of Genomic deoxyribonucleic acid and the reaction volume has been rounded to 25 ul with the addition of 8 ul of nuclease free water Amplification has been dispensed in a very thermo-cycler (Eppendorf) programmed for 5 mins. At a temperature of 94 degrees Celsius; for 35cycles 1 min. at a temperature of 94 degrees Celsius, 1 min at a temperature of 61degrees Celsius and 2 mins at a temperature of 72 degrees Celsius; and a final extension of 10 mins at a degree of 72 degrees Celsius. Amplification product were electrophoresed in 2% agarose gels then visualised via staining with ethidium bromide. standard molecular markers were conjointly enclosed in every electrophoresis run. Ultraviolet trans-illuminated gels have been captures as photographs.

Results

Genotyping of HSP70 gene in Cryptosporidium parvum

For HSP70 gene genotyping, the genomic DNA was amplified using specific primers and accomplished by the Thermo-cycler apparatus under the optimal conditions. The results revealed that the presence a single band (2010 bp) of the target sequence of HSP70 gene gene in agarose gel (Fig. 1).

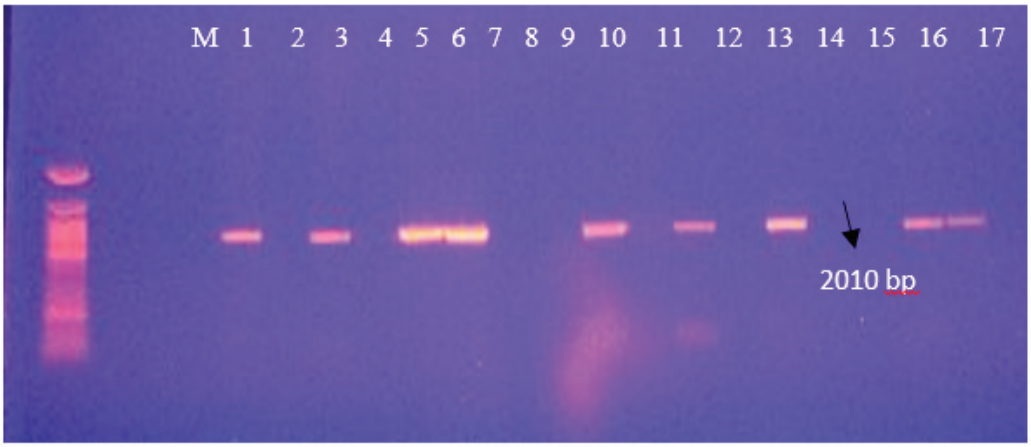

Figure (1): Agarose gel electrophoresis image that showed PCR product analysis for HSP70 gene in Cryptosporidium parvum isolates. M (Marker ladder 3000-100bp). Lane (1-19) only positive Cryptosporidium parvum isolates at 2010bp product size.

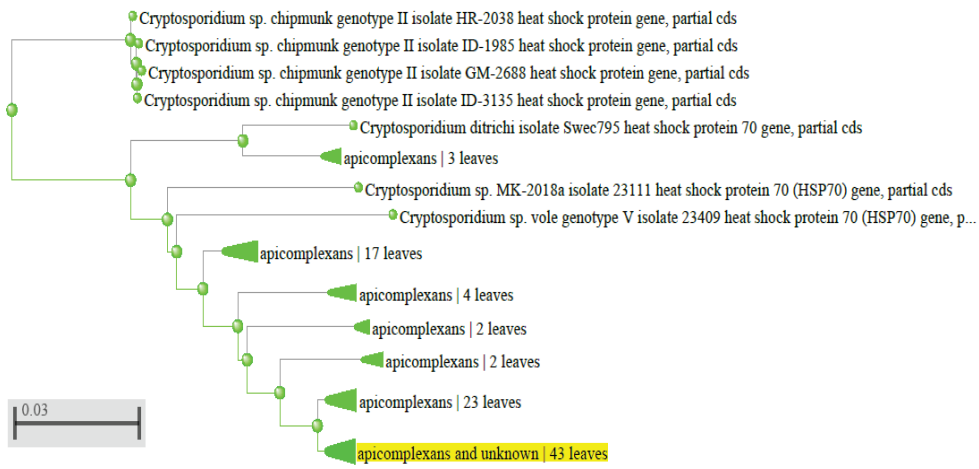

Fig. (2): phylogenetic tree anaylsis results for cryptosporidium parvum, HSP70 gene using by NCBI BLAST

Genotyping of DHR gene in Cryptosporidium parvum

For DHR gene genotyping, the genomic DNA was amplified using specific primers and accomplished by the Thermo-cycler apparatus under the optimal conditions . The results revealed that the presence a single band (575 bp) of the target sequence of DHR gene in agarose gel (Fig. 3).

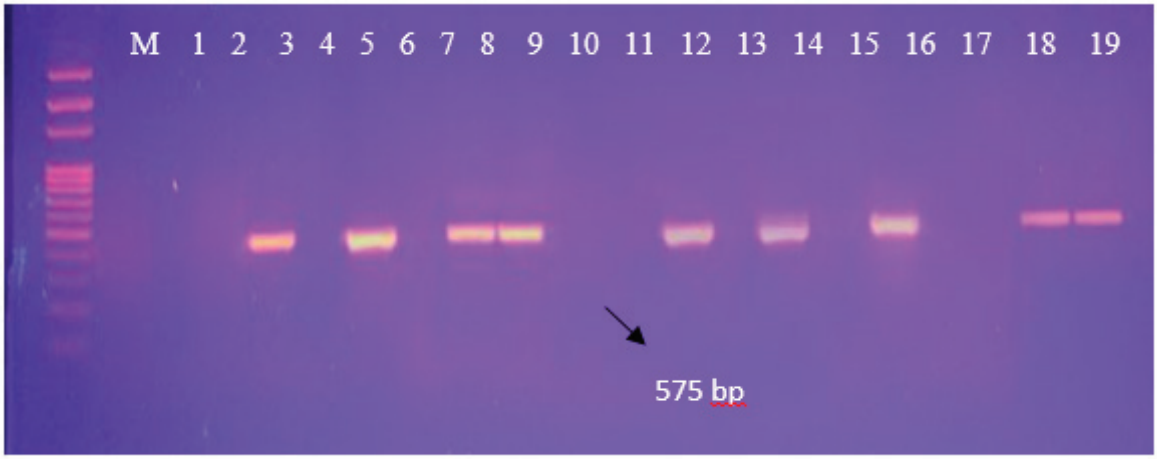

Figure (3): Agarose gel electrophoresis image that showed PCR product analysis for DHR….. gene in Cryptosporidium parvum isolates. M (Marker ladder 3000-100bp). Lane (1-9) only positive Cryptosporidium parvum isolates at 575bp product size.

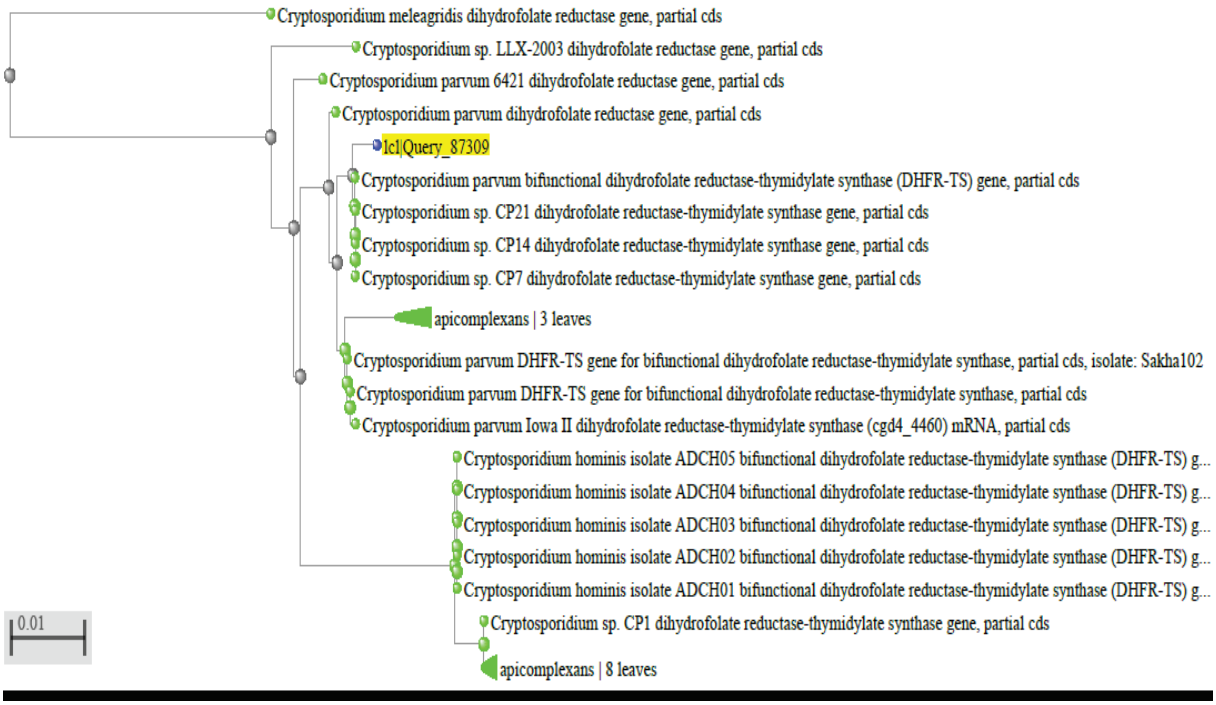

Fig. (4): phylogenetic tree anaylsis results for cryptosporidium parvum, DHR gene using by NCBI BLAST

Discussion

For HSP70 gene genotyping, the genomic DNA was amplified using specific primers The results revealed that the presence a single band (2010 bp) of the target sequence of HSP70 gene while For DHR gene genotyping, the genomic DNA was amplified using specific primers The results revealed that the presence a single band (575 bp) of the target sequence of DHR gene. Heat shock proteins (HSPs) are molecular chaperones which are involved in maintaining regular cellular functions with a crucial role in protein folding, unfolding, aggregation, degradation, and transport [15]. In addition, these highly conserved proteins are involved in cell differentiation and morphogenesis [16], cell signaling, and in the protection of cells against stress and apoptosis [17]. HSPs are organized into several families based on their molecular mass (kDa), with Hsp70 and Hsp90 proteins belonging to the two major families (70 and 90 kDa, respectively). Some Hsp70 family members are constitutively expressed such as seen with cognate Hsc70 or with inducible expression as seen with cytosolic Hsp70 [15].

For PCR-based analyses, the most commonly used Cryptosporidium-specific biomarker is the gene encoding the 18S rRNA subunit, which has been used for the detection and genotyping of oocysts present in environmental and clinical samples [18]. Other markers, including Cryptosporidium 60 kDa glycoprotein (gp60), microsatellite locus 1 (ML-1) and 2 (ML-2), Cryptosporidium heat-shock protein (HSP70), b-tubulin and Cryptosporidium oocyst wall protein (COWP) have also been used for this purpose [19]. Although the direct use of PCR does not distinguish between live and dead oocysts, it can be used to detect the DNA of excysted sporozoites of Cryptosporidium oocysts after incubation in excystation medium. The viability of Cryptosporidium is then determined by amplification of an 873 bp region of a 2359 bp DNA fragment encoding a repetitive oocyst protein. This method is adequate to detect low numbers of viable oocysts, such as during routine monitoring of drinking water and environmental samples [18].

Many researchers have also developed PCR-based techniques for differentiating C. parvum of human origin and C. parvum of animal origin. These techniques are based on the polymorphic nature of C. parvum strains that infect humans and most animals at the b-tubulin, oocyst wall protein (COWP), dihydrofolate reductase (DHFR), thrombospondinrelated adhesive protein 1 (TRAP-C1), thrombospondin-related adhesive protein 2 (TRAP-C2), internally transcribed spacer 1 (ITS1), polythreonine repeat (Poly-T), small-subunit (SSU) rRNA, and undefined genomic sequences [13].

In recent years, researchers have developed PCR based techniques for the detection and identification of Cryptosporidium spp. These techniques target the genes of thrombospondin-related adhesive protein 1 (TRAP-C1) [15]. Consequently, molecular analysis used to characterize the genetic structure of Cryptosporidium parasites and to assess their zoonotic signifi cance proved to be a sensitive diagnostic method capable of determining Cryptosporidium spp. with high selectivity in environmental and clinical samples and allowing for genotyping [16].

Financial Disclosure: There is no financial disclosure.

Conflict of Interest: None to declare.

Ethical Clearance: "All experimental protocols were approved and carried out in accordance with approved guidelines".

References

1. Smith H. Diagnostics. In: Fayer R, Xiao L, editors. Cryptosporidium and Cryptosporidiosis. 2nd ed. USA: CRC Press; 2007; 174–207.
2. Chalmers RM, Elwin K, Thomas AL, Guy EC and Mason B. Long-term Cryptosporidium typing reveals the aetiology and species-specific epidemiology of human cryptosporidiosis in England and Wales, 2000 to 2003. EuroSurveillance 2009; 4: 19086.
3. Fayer, R. Cryptosporidium: a water-borne zoonotic parasite. Veterinary Parasitology. 2004; 126:37-56.
4. Meireles MV. Cryptosporidium infection in Brazil: implications for veterinary medicine and public health. Revista Brasileira de Parasitologia Veterinária 2010; 19: 197-204.

5. Geurden T, Levecke B, Cacció SM, Visser A, De Groote G, Casaert S, Vercruysse J and Claerebout E. Multilocus genotyping of Cryptosporidium and Giardia in non-outbreak related cases of diarrhoea in human patients in Belgium. Parasitology 2009; 36: 1161-1168.

6. Xiao L and Feng Y (2008). Zoonotic cryptosporidiosis. FEMS Immunology and Medical Microbiology 52: 309-323

7. Mojarad EN, Keshavaraz A, Taghipour N, Haghighi A, Kazemi B, Athari A. Genotyping of Cryptosporidium spp. in clinical samples PCR-RFLP analysis of the TRAP-C2 gene. Gastroenterol Hepatol 2011;4:29–33.

8. Wielinga PR, de Vries A, Van der Goot TH, et al. Molecular epidemiology of Cryptosporidium in humans and cattle in the Netherlands. Int J Parasitol 2008 ; 38:809–817.

9. Feng Y, Zhao X, Chen J. Occurrence, source and human infection potential of Cryptosporidium and Giardia spp. in source and tap water in Shanghai, China. Appl Environ Microbiol 2011; 77:3609–3616.

10. Chaharmahal B. Province of Iran using nested PCR-RFLP. Iran J Parasitol 2010 ; 6:20–27 .

11. Usluca S, Aksoy U. Detection and genotyping of Cryptosporidium spp. in diarrheic stools by PCR-RFLP analyses. Turk J Med Sci. 2011; 41:1029–1036.

12. Lammie PJ, Moss DM, Brook Goodhew E, Hamlin K, Krolewiecki A, West SK. Development of a new platform for neglected tropical disease surveillance. Int J Parasitol. 2012; 42:797–800.

13. Arya R, Mallik M, Lakhotia SC. Heat shock genes—integrating cell survival and death. J Biosci. 2007; 32: 595–610.

14. Limor, J, Lal, A, Xiao, L. "Detection and differentiation of Cryptosporidium parasites that are pathogenic for humans by real-time PCR". 2002.

15. Gehan S. Use of nested PCR-RFLP for genotyping of Cryptosporidium parasites isolated from calves and children suffering from Diarrhea. 2014.

16. Weitzel T, Dittrich S, Mohl I, Adusu E, Jelinek T. Evaluation ofseven commercial antigen detection tests for Giardia andCryptosporidium in stool samples. Clin Microbiol Infect. 2006;12(7):656–659.18.

17. Akiyoshi, D, Feng, X, Buckholt, M, Widmer, D, Tzipori, S. Genetic analysis of Cryptosporidium parvum: human genotype 1 isolate passaged through different host species. Infect. Immun. 2002; 70: 5670–5675 .

18. Shirley DA, Moonah SN, Kotloff KL. Burden of disease from cryptosporidiosis. Current opinion in infectious diseases. 2012;25(5):555–63.