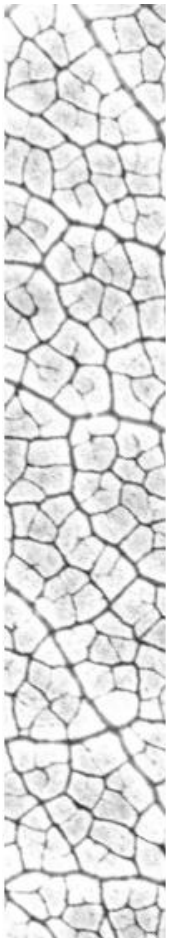

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A study to detect the most important virulence factors of cryptosporidium parasite samples by PCR

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Abstract
Cryptosporidium parvum is a coccidian parasite causing diarrhea. The aim of our study is the detection of the infective species with virulence factors in Cryptosporidium in children who have diarrhea in Diwaniyah city. The total fecal samples were one hundred; take from diarrheic children who are aged below 10 years old in Maternity and Childhood Teaching Hospital. Oocytes of Cryptosporidium oocytes were 29 (29%) in children with diarrhea using a light microscope with an acid-fast stain for the samples. Findings of polymerase chain reaction showed that C. parvum is an accusative agent of the cryptosporidiosis in children and all these samples contain the Glycoprotein 900 pathogenic factor under study.

Keywords: Cryptosporidium parvum, virulence factors, children, Diwaniyah city, Iraq

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INTRODUCTION

Intestinal parasitic infections are one of the most common causes of many diseases worldwide, the various statistical estimations found that nearly 340 parasites were infecting 3 billion people in developing countries (Stark et al.2009). Intestinal parasites such as Giardia intestinalis, *Entamoeba histolytica*, *Strongyloides stercoralis* and coccidian parasites such as Cryptosporidium, Cystoisospora, Cyclospora and Microsporidia are the common causes of diarrhea (Kashyap et al. 2013). Cryptosporidium was listed as a category B pathogen by CDC and the National Institute of Health because of its threat to cause water contamination (Rotz et al.2002). Cryptosporidium causes major health problems, the disease occurs due to exposure to oocytes of the cryptosporidium at low doses. The oocytes are can resist disinfectants and chlorination, and can pass through treatment processes of the water, and stay alive for a long time in the environment. Cryptosporidium infections are occurring in the human by several routes, such as ingestion of contaminated water and food and transmitted directly from human or animals (Xiao and Cama, 2006). Lab detection of the parasites for decades depended on the microscopic examination. The diagnosis by the microscope is considered the gold test when is done by an experienced person. The specificity and the sensitivity of the microscopic were low (Haque et al.2005) (Petri et al. 2000). There are other laboratory methods such as staining by acid-fast staining, immunofluorescence microscopy help to the detection of the oocytes (Tzipori and Ward 2002). Also, there are other methods used for the molecular diagnosis of the Cryptosporidium at the genotype and species level, also, determining all the different transmission methods of the parasite (Xiao et al.2004). Many reports determined and detected the pathogenic factors that help to the initiation, attachment of Cryptosporidium. Cryptosporidium causes a systemic infection that can penetrate the organs and the tissue rather, and attachment in the epithelium. Furthermore, it causes marked changes in the secretory and absorptive functions of the intestine. Damage of the intestine is caused by injury to the epithelial layer directly and cause damage due to the accumulation of the cytokines and inflammatory cells indirectly in the infection site (Okhuysen and Chappell, 2002). Glycoprotein 900 is detected by the hyperimmune cow colostrum with immunoprecipitation of sporozoite extracts (Petersen et al.1992). The glycoprotein is lying in the micronemes at the surface of sporozoites and merozoites. Glycoprotein 900 is deposited in trails during gliding motility (Barnes et al.1998) (Bonnin et al. 2001). The amino acid of Glycoprotein 900 has a transmembrane domain and signal peptide (Barnes et al.1998). Antibodies to

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Glycoprotein 900 inhibit infection in vitro (Petersen et al.1997; Barnes et al.1998).

MATERIALS AND METHODS

Samples collection

The samples are collecting from 100 children that suffered from diarrhea who to arrivals to Maternity and Childhood Teaching Hospital in Al-Diwaniya Governorate who are aged below 10 years old, the fecal samples are kept in a dry and clean container and sent to the lab.

Microscopic visualization

The cryptosporidiosis diagnosis is depended on the oocytes in samples by an acid-fast stain (Garcia et al.1983).

DNA Extraction

Extraction of the DNA was done from the feces directly by a specific kit called (Stool DNA extraction, Bioneer company, Made in Korea) based on manufacture directions by Proteinase K and stool lysis protocol method. Extracted DNA was tested Nanodrop and stored at (-20) C.

A polymerase chain reaction of the parasite

PCR is a technique used for diagnosis of *C. parvum* by primer-designed shock protein gene in the parasite, F-primer (CGTGCAACTTTAGCTCCAGT), and R-primer (AGCAACAGCTTCGTCTGGAT). Designing of the primers was done by (GenBank: GQ259151.1) and using of the website (Primer3plus). Preparation of the PCR master mix was done by (AccuPower® PCR PreMix, Bioneer company, Made in Korea). Component of the PCR premix tube was Taq DNA polymerase, dNTPs, Tris-HCl, KCl, MgCl2, stabilizer, and dye).

Preparation of the master mix reaction is done depending on the manufactured directions, it consists of DNA (5) µl with F primer and R primer together (3) µl after that completing the volume by deionized water for (20) µl then mixed by the vortex. The thermocycler used for doing of the reactions (Mygene, Bioneer Company. Made in Korea) at the following:

1- The initial stage (95) °C (five) minutes
2- The denaturation stage (95) °C (half a minute) for (30) cycle
3- The Annealing stage (57.2) °C (half a minute)
4- The Extension stage (72) °C for twenty seconds.
5- The extension stage (72) °C for five minutes.

The PCR products were tested on agarose gel by the electrophoresis with ethidium bromide stain then look under the UV device.

The polymerase chain reaction of GP900gene

After the presence of Cryptosporidium parvum was confirmed in samples, PCR assay was performed for direct detection of virulence factor by used prefixes F: (AAAGAATGGCGAATGTGAGG) and R: (CCGTATGGGCTTACGTGAAT) processed Corporation (Bioneer, Korea) and these primers were designed using the NCBI GenBank database to investigate the virulence factor genes private GP900 parasite *C.parvum*. The reaction mixture to a series polymerization was attended by using several Accu Power ®PCR Premix and according to the company's instructions were mix placed in the tubes of the PCR and processed in the kit and container components polymerase chain reaction and added other components of the reaction mixture (Primers, 3 µL / DNA, 5 µL / PCR water, 12 µL) and then shut down the pipeline and mix by Carburetor device and the movement of pipes to a PCR Thermocycler for stages of thermal cycles to amplify the DNA, then conducted electrical relay outputs amplification gel Agarose by 1.5%, and upon the completion of the migration process examines the gel containing the resulting PCR using an ultraviolet UV to determine the results of the positive samples and matching with him measurement DNA Ladder.

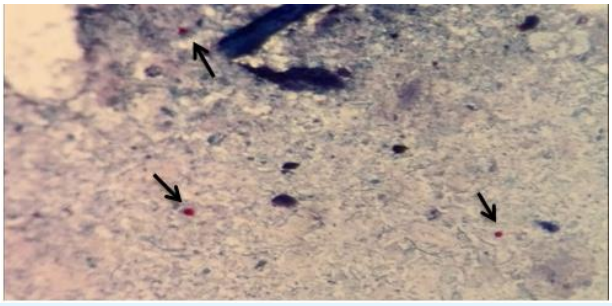

Fig. 1. The oocyte of Cryptosporidium

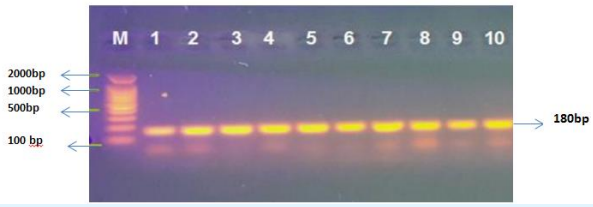

Fig. 2. Agarose gel electrophoresis picture that offers the PCR product to detect *Cryptosporidium parvum* in human feces. M: Marker (2000 -100bp), the lines (1- 10) positive C. parvum at 180bp

RESULTS

Morphological Detection of Oocysts

Oocysts of Cryptosporidium sp. were found in 29 % (29/100) of the examined fecal samples from diarrheic children using a light microscope (**Fig. 1**).

The conventional PCR assay was carried out to detect the species contained in them. The amplicons of size 180 bp were showed a clear single specific band (**Fig. 2**).

Molecular Detection of GP900gene

The results of positive samples examined showed mediated PCR that all these samples and 100% contain

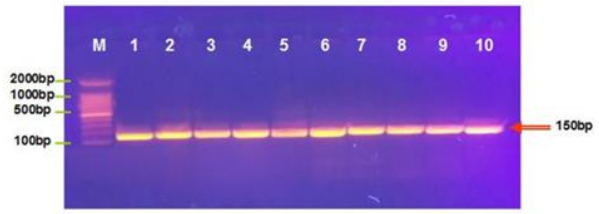

Fig. 3. Agarose gel electrophoresis picture that shows the PCR product of the GP900 gene where Lane (M) ladder

pathogenic understudy factor which is: Glycoprotein 900, the amplicons of size 150 bp were showed a clear single specific band **Fig. 3**.

DISCUSSION

Gut parasites are most common in poor countries and the Cryptosporidium parasite is the most common. Cryptosporidium parasite causes secretory diarrhea associated with dehydration, stomach cramp, weight loss, vomiting, fever, pain, and nausea (Huang et al. 2004). In this Search, we used specific, sensitive, and rapid technics for the diagnosis of *Cryptosporidium parvum* with many virulence factors in the parasite by PCR.

The present study recorded the incidence of *C. pavum* parasite 29% by 29 samples is positive from a total of 100 stool specimens examined microscopically using the acid-fast stain (AFS) and this percentage was different from some results fo studies that were conducted in Iraq, including the study conducted by (AL-Alousi and Mahmood, 2012), that recorded the percentage of infected stood at 18.9% after examining 92 stool specimens for children suffering from diarrhea aged (one month -12 years) in Mosul, and different from the study carried out by (Mikhlif, and Muhannad 2008). In Ramadi province, since the results revealed the percentage of the infection was (39.13) % from the total of 115 stool specimens for children suffering from diarrhea also under the age of five exclusively. While In the Gaza infection rate of 14.9% (AL-Hindi et al. 2007). Whereas the incidence in Iran, Peru was 4.6% % and 6.4% respectively (Chekly et al.1997; Hossein et al. 2010) All those results were using microscopic examination and the use of an acid-fast stain. The different rates among the studies may belong to the differences in the population of the study (range and age), used detected methods, environmental conditions such as water source, the study season, and food type (Khoshzaban and Dalimi-Asl, 1998; Huang et al. 2004; Roy et al. 2004). The educational level of parents has also been reported as a risk factor (Khoshzaban and Dalimi-Asl,1998). PCR is a new detection method for many parasites such as Cryptosporidium (26). PCR was used for the detection of DNA of C. parvum (Webster et al.1993; Benigno et al.1996).The results of the present study, using the technique of PCR Conventional showed the presence of gp900 in all samples (100%) and that has been documented in this study. gp900 is one of the virulence factors that contribute to causing cryptosporidiosis and spread enables the parasite to invade and get the infection to hosts of mammals and causing damage to a group of hosts to be prone to infection and found that the effective immune responses targeting often virulence factors. Gp900 is a glycoprotein present on the surface of *C. parvum* and it is important for the invasion (Petersen et al. 1997). It is a target for therapy by the chemotherapeutic; also, (Petersen et al. 1992) found that GP900 is a virulence factor of *C.parvum*

CONCLUSION

Cryptosporidium spp, is main causative agent for diarrhea at (29)% morphological by microscope, then all the isolates submitted to the PCR test, the positive isolates by PCR was (100) %, also, the study detected Glycoprotein 900 (virulence protein) by PCR by using specific primer.

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