Detecting of virulence factors COWP gene and CP15 gene for *Cryptosporidium parvum* by polymerase chain reaction (PCR)

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Abstract

The present study aims to diagnose the parasite *Cryptosporidium parvum* isolated stool samples of patients to reviewers Diwaniya Teaching Hospital and Maternity Hospital and Children in the province of Diwaniyia .The infected ranged from the age less than one year to 12 years at the period from beginning of May to the end of November 2016.
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110 samples stool were collected and the number of infected samples is (30) sample. After examining samples in direct examination of the sample wet method.

The study included verification of virulence factors Cryptosporidium Oocyst Wall Protein (COWP), Surface Protein of Sporozoite (CP15) in parasite using conventional PCR technique.

The study showed that age groups less than one year were the highest infection rate (43.3%) while the age group of (9-12) years showed lowest infection rate (10%) with a significant difference between them, infection rate in females (56.6%) was higher than males (43.3%) but did not reach the level of significant.

These ratios also appeared in the rural areas are the highest (63.3%) compare with urban areas (36.6%) with a significant difference between them.

DNA was extracted from positive stool samples and then after amplified using virulence factors of parasite COWP gene (281bp), CP15 gene (230bp) and the results appeared present these factors in all positive samples of *C.parvum*.

Key words: *Cryptosporidium parvum*, COWP, CP15, PCR

Physiology Classification QR1-502-71

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Introduction

Protozoan parasites of the genus *cryptosporidium* infect the gastrointestinal tract of many animals species and cause cryptosporidiosis (1).this parasite causing mild- to severe diarrhea depending on the host's immune status, the infection can spread to extraintestinal, hepatobiliary, pancreatic and pulmonary regions of the body leading to chronic disease and wasting (2). the infection can be a cute and self-limiting illness in immunocompetent patients cryptosporidiosis can become a chronic and life- threatening disease in immunocompromised pateints (3,4). the infection of cryptosporidiosis occurs by the faecal oral route and contamination water with infective oocysts, oocysts release sporozoite which invade the intestinal epithelium cells predominantely localized to the jejunum and ileum (5,6).

Virulence factors for *cryptosporidium* have been identified as genes involved in the initial interaction processes of cryptosporidium oocysts and sporozoites with host epithelial cells including excystation, gliding motility, attachment, invasion, parasitophorous vacuole formation, intracellular maintenance and host cell damage (7,8). Surface protein of sporozoite (CP15) is expressed by the infective sporozoite and merozoite stages (9) its involved in the invasion and the host immune response to infection (10,11,12) and its apparent role in the invasion of mammalian cells by *C.parvum* sporozoites (13).

Cryptosporidium Oocyst Wall Protein (COWP) gene has been localised in the wall forming bodies of early and late macrogametes and the inner layer of the oocyst wall, there are at least two alleles of the COWP gene in the *C.parvum* population, one associated only with the human host, the other with both animals and humans(14).

Materials and Methods

Sample collection

110 human stool samples were collected from patients microbiology laboratory to Diwaniya teaching Hospital and Maternity Hospital and Children, and then transport to laboratory and stored in freeze.

Microscopic examination

Stool samples examined by preparation of direct wet smear mix a drop of saline solution on a glass slide with a small sample of feces using wooden sticks, then put the slide cover and examine using microscope under power zoom 10X and 40X.

Genomic DNA Extraction

Genomic DNA was extracted from feces samples by using (Stool DNA extraction Kit, Bioneer. Korea). The extraction was done according to company instructions by using stool lysis protocol method with Proteinase K. After that, the extracted gDNA was checked by Nanodrop spectrophotometer, and then stored at -20C at refrigerator until used in PCR amplification.

Polymerase chain reaction

PCR assay was performed for detection of virulence factors genes (Cryptosporidium Oocyst Wall Protein (COWP) gene and Surface Proteins of Sporazoite (CP15) of *Cryptosporidium parvum*. The primers were designed in this study using NCBI-Genbank

data base (COWP GenBank: DQ388390.1, CP15 GenBank: U22892.1) by Primer3plus. The primers were provided by (Bioneer company . Korea). As following table (1):

Table (1): prefixes of *C.parvum* used in the study with the nucleotide sequence (PCR reaction).

Primers	Sequence	Amplicon
COWP gene	CCAGAATGTCCTCCAGGCAC	281bp
	GTATATCCTGGTGGGCAGACC	
CP15 gene	CACTCGATTGTGTCTCCCCC	230bp
	TTCTTGGGGGTGGTTGGAAG	

Then PCR master mix was prepared by using (AccuPower® PCR PreMix kit. Bioneer. Korea). The PCR premix tube contains freeze-dried pellet of (Taq DNA polymerase 1U, dNTPs 250µM, Tris-HCl (pH 9.0) 10mM, KCl 30mM, MgCl2 1.5mM, stabilizer, and tracking dye) and the PCR master mix reaction was prepared according to kit instructions in 20µl total volume for each gene independent by added 5µl of purified genomic DNA and 1.5µl of 10pmole of forward primer and 1.5µl of 10pmole of reverse primer, then complete the PCR premix tube by deionizer PCR water into 20µl and briefly mixed by Exispin vortex centrifuge (Bioneer. Korea). The reaction was performed in a thermocycler (Mygene, Bioneer. Korea) by set up the following thermocycler conditions; initial denaturation temperature of 95 °C for 5 minutes followed by 30 cycles at denaturation 95°C for 30 seconds, annealing 60°C for 30 seconds, and extension 72°C for 30 sec. minute and then final extension at 72°C for 5 minutes. The PCR products were examined by electrophoresis in a 1.5% agarose gel, stained with ethidium bromide, and visualized under UV Transilluminator.

Results

1. Prevalence of *Cryptospridium spp.* according to a microscopic examination

The results of the current study, depending on the method of direct wet smear that there are 30 samples out of 110 samples were positive to infection of the parasite as the shown in the figure (1):

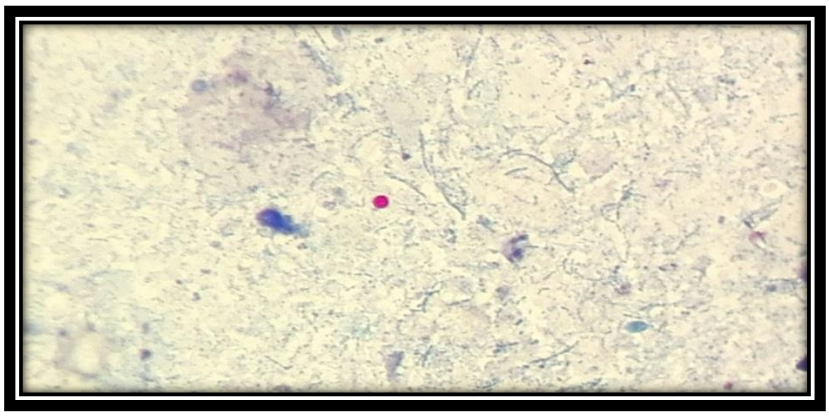


Figure (1): Oosyst of *C.parvum*

2. prevalence of *C.parvum* according to the sex

Results of the study showed that the percentage of female infection with parasite was 56.6% compared to males where the percentage of infection was 43.3% but it didn't reach the level of significance at p>0.05. table (2):

Table (2): prevalence of *C.parvum* according to the sex

Sex	The number of patients	%
Males	13	43.3 a
Females	17	56.6 a

chi- square value (X^2) = 1.771

3. prevalence of *C.parvum* according to the age groups

Results of the current study showed that the rate of infection at the age of less than one year (43.3%) was the highest and lowest infection at the age of (9-12) years with a significant difference at p>0.05. table (3):

Table (3): prevalence of *C.parvum* according to the age group

Age groups	The number of infected patients	%
Less than one year	13	43.3 a
1-4	9	30 a
5-8	5	16.6 b
9-12	3	10 b

Chi-square value (X^2) = 26.244

4. prevalence of *C.parvum* according to the nature of residence

The results of the current study showed that the infection rate in rural areas is the highest (63.3%) compared to the urban areas (36.6%) the lowest rate of infection with a significant difference at p>0.05 table (4):

Table (4): prevalence of *C.parvum* according to the nature residence

Nature of residence	The number of patients	%
Urban areas	11	36.6 a
Rural areas	19	63.3 b

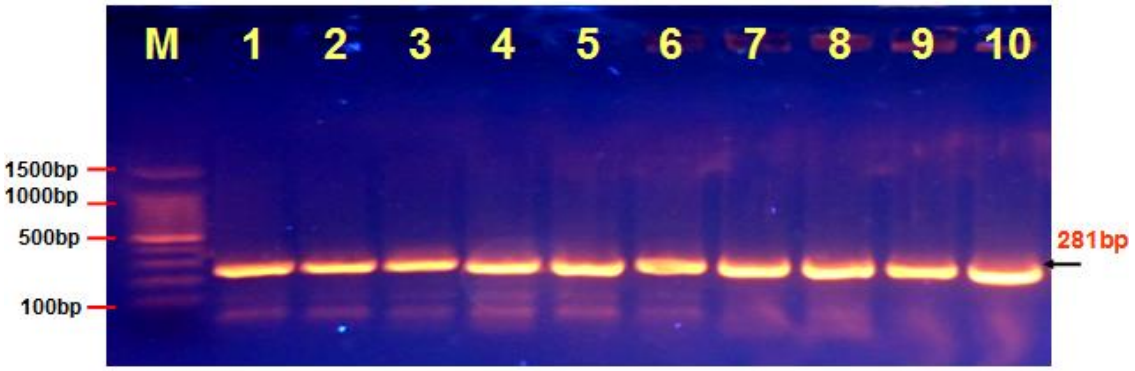
Chi-square value (X^2) =7.136

***similar letters means non-significant differences at the level of probability of 0.05 using test X^2.**

***Different letters means significant differences at the level of probability of 0.05 using test X^2.**

5. Molecular study

Results of molecular test PCR showed that all positive samples for the parasite *C.parvum* (100%) contain virulence factors studied which were Cryptosporidium Oocyst Wall Protein and Surface Protein of sporozoite as the figure show (2 and 3) the molecular weight of the COWP factor (281bp) percentage (100%) and the molecular factor of the CP15 (230bp) percentage (100%) and were examined by electrophoresis in a 1.5% agarose gel and stained with ethidium bromide and examined using UV transilluminator.



. Figure (2): Agarose gel electrophoresis image that show the PCR product of Cryptosporidium oocyst wall protein (COWP) gene in *Cryptosporidium parvum* positive isolates. Where M: Marker (1500-100bp), lane (1-10) some positive *C. parvum* at 281bp PCR product size.

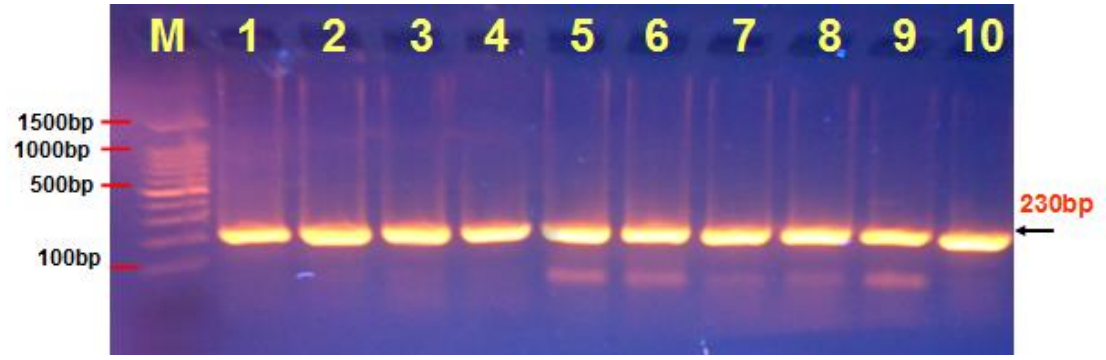


Figure 3: Agarose gel electrophoresis image that show the PCR product of Cp15 surface proteins of sporazoite in *Cryptosporidium parvum* positive isolates. Where M: Marker (1500-100bp), lane (1-10) some positive *C. parvum* at 230bp PCR product size.

Discussion

Cryptosporidium parvum is an intracellular protozoan parasite of the family Cryptospridiidae and phylum Apicomplexa (15). This disease occurs worldwide and is ubiquitous in the environment (16). Cryptosporidiosis is in the top five most common causes of infections diarrhea around the globe (17).

Results of the current study showed that the parasite *Cryptosporidium parvum*

The relationship between spread of disease in both sexes is non significant relationship, the percentage of infection in females reached (56.6%) while in males (43.3%) this is consistent with (19).

The results of this study that the parasite is most prevalent in rural areas (63.3%) and the lowest rate in urban areas (36.6%) and this is consistent with (20) and the reason due to presence of grazing such as cows and sheep in rural areas compared with urban areas at least interested in raising cattle and sheep.

Results proved presence COWP factor in all positive samples for infection

affected all age groups but the infection rate in less than one year old (43.3%) was the highest and recorded less injured rate in the age group (9-12) years and the reason for this due to the immune system is incomplete at the age of less than one year as well as the left breast feeding to artificial feeding and ignorance many mothers things sterilization and cleanliness of the water and milk bottles this is consistent with (18).

percentage 100% , its higher than of infection rate that recorded (14) reached 98% that showed existence of least two alleles of the COWP gene in the *C.parvum* one associated only with the human host and the other with both animals and humans, and supports that cryptosporidiosis in humans is not necessarily a zoonosis(21).

The study proved presence CP15 factor in all positive samples for infection percentage 100% and this consistent with (22), this factor it's apparent role in the invasion of mammalian cells by *C.parvum* sporozoites (13).

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تحديد عوامل الضراوة COWP gene و CP15 gene لطفيلي الابواغ الخبيئة *Cryptosopridium parvum* باستخدام تقنية تفاعل سلسلة البلمرة (PCR)

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الخلاصة

تهدف الدراسة الحالية الى تشخيص طفيلي الابواغ الخبيئة *Cryptosporidium parvum* المعزول من عينات براز المرضى المراجعين الى مستشفى الديوانية التعليمي ومستشفى الولادة والأطفال في محافظة الديوانية.

تراوحت اعمار المصابين (اقل من سنة – 12سنة) للفترة الواقعة من الأول من شهر آيار ولغاية نهاية شهر تشرين الثاني للعام 2016. تم جمع 110 عينة براز وكان عدد العينات المصابة 30 عينة بعد فحص العينات بطريقة الفحص المباشر للعينة الرطبة .

تضمنت الدراسة التحري عن عوامل الضراوة Cryptosporidium Oocyst Wall Protein (COWP) و Surface Protein of sporozoite (CP15) في الطفيلي باستخدام طريقة conventional PCR .

أظهرت الدراسة ان الفئات العمرية اقل من سنة كانت اعلى نسبة إصابة حيث بلغت (43.3%) بينما الفئات العمرية من (9–12) سنة اظهرت اقل نسبة إصابة وبلغت (10%) مع وجود فروقات معنوية بينها وكانت نسبة إصابة الاناث (56.6%) وهي اعلى من نسبة إصابة الذكور (43.3%) الا انها لم تصل الى مستوى المعنوية كما ظهرت هذه النسب في المناطق الريفية هي الأعلى (63.3%) بالمقارنة مع المناطق الحضرية (36.6%) مع وجود فروقات معنوية بينها.

تم مضاعفة الحامض النووي المستخلص من العينات الموجبة باستخدام عوامل الضراوة للطفيلي COWP (281bp) و (230bp) CP15 واظهرت النتائج وجود هذه العوامل في جميع العينات الموجبة لطفيلي *Cryptosporidium parvum*.

الكلمات المفتاحية: PCR, CP15, COWP, *Cryptosporidium parvum*.