Activity of G6HD, ME, GPI and protease enzyme in Cryptosporidium oocyts isolated from Immunocompetent and Immunocompromised patients.

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Abstract: Cryptosporidiosis is a clinical disease, usually presents as a gastro-enteritis-like syndrome, caused by infection with protozoan parasites of the Apicomplexan genus Cryptosporidium. Disease ranges in seriousness from mild to severe and signs and symptoms depend on the site of infection and nutritional and immune status of the host. This study was conducted to evaluate the activity of enzyme Glucose Phosphate isomerase (GPI), Malic Enzyme (ME), Glucose -6-Phosphate Dehdrogenase (G6PD) and Protease Enzyme in oocytes of Cryptosporidium species and if their significant difference between activity of oocytes isolation from immunocompetent patients or immunocompromised patients. Six hundred patients with an age range from 12 day to 18 years suffering from diarrhea. Modified Acid – fast stain (modified Ziehle-Neelsen) will be used in diagnosis of Cryptosporidium oocytes. Sixty six samples (11%) out of 600 patients were positive for Cryptosporidium oocytes by using modified Ziehle-Neelsen stain, regarding Enzyme activity results show highly activity of all enzymes included in this study with no difference in enzyme activity between Immunocompetent and immunocompromised patients. In this study we conclude that GPI, ME, G6PDH and Protease enzymes were found in Cryptosporidium Oocytes with highly activity, there was no significant difference between oocytes activity isolated from immunocompetent and immunocompromised patients.

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1. Introduction

Human cryptosporidiosis is caused by infection with apicomplexan protozoans of the genus Cryptosporidium. Human illness was formerly thought to be caused by a single species, but molecular studies have demonstrated that it is caused by at least 15 different species. Among the more common species are Cryptosporidium hominis, for which humans are the only natural host, and Cryptosporidium Parvum, which infects bovines as well as humans. (Lima et al., 2011).Cryptosporidiosis mainly affects children; it causes a self-limited diarrheal illness in healthy individuals. Cryptosporidiosis is also recognized as a cause of prolonged and persistent diarrhea in children and of severe, prolonged diarrhea in persons with acquired immunodeficiency syndrome (AIDS) and other immunocompromised disease (Bouzid et al., 2013). Cryptosporidium species are able to infect and reproduce in the epithelial cell lining of the gastrointestinal and respiratory tracts without causing cytopathic effects. In immunocompetent individuals, the organisms are primarily localized to the distal small intestines, whereas in immunocompromised hosts, the parasites have been identified throughout the gut, biliary tract, and respiratory tract. (Yoder and Beach. 2010).

The disease is transmitted via the fecal-oral route from infected hosts. Most sporadic infections occur through person-to-person contact. Nonetheless, transmission can also occur following animal contact, ingestion of water (mainly during swimming), or through food. Extensive waterborne outbreaks have resulted from contamination of municipal water and recreational waters (Chalmers et al., 2011). Two morphologic forms of the oocysts have been described: thin-walled oocysts (asexual stage) excyst within the same host (causing self-infection), whereas the thick-walled oocysts (sexual stage) are shed into the environment. Oocyst shedding can continue for weeks after a patient experiences clinical improvement (Scallan et al., 2011).

2. Material and Methods

Six hundred patients with an age range from 12 day to 18 years suffering from diarrhea. Samples were taken from Baghdad Teaching Hospital, Al-Mansoor Hospital for Children, Central Children Teaching Hospital, Al-Kadhumyia Teaching Hospital, Al-Kadhumyia, Hospital for children and some private laboratories in Baghdad City. The study conducted through the period from October, 2010 to end of May 2011.

Stool specimens were collected from each individual included in this study in clean dry containers. These were labeled by case number and name. All specimens were transported to the laboratory for processing and investigations at the same day, stool samples were divided into three portions. The first portion (0.5 mg) was prepared for wet smear preparation; the second portion (0.2 mg) was prepared for staining methods with Modified Acid – fast stain (modified Ziehle-Neelsen) will third portion (2mg) was prepared for purification oocyts and measurement enzymes activity (samples that only positive for Cryptosporidiosis) by using spectrophotometer.

2.1: Preparations of enzyme extract (Coombs et al., 1982).

1-Pellets of oocyts of Cryptosporidium were taken to which an equal volume of (Trition X100) 0.1% prepared in normal saline was added.

2-Using glass homogenizer to break down the oocyts of parasite at specific time interval inside an ice bath at 40C was carried out.

3-The homogenate was centrifuged in cold centrifuged at (4500Xg) for 10 minutes, the supernatant was taken as crude enzyme extracts and used for the determination of enzymatic activity of different enzymes.

4-The total protein in the enzyme extract of oocyts of parasite was determined using Warburg and Christian (1941) by measure the absorbance(A) of sample (enzyme extract) at 260nm and at 280 nm after that calculate the ratio A280/A260 then read the factor corresponding to the calculate A280/A260. The protein concentration is given by

Protein conc. (mg/ml) = *A280* X Factor

5-Calculation of enzyme activity:

The activity of the enzyme was measured in activity units. Each unit represents the a mount of the enzyme that will catalyze the reaction of 1mg of the substrate per minute under specified conditions of temperature, PH, substrate and activators (nmol/min).

Specific activity of the enzyme was measured by dividing the number of units to the protein concentration (nmol/min/mg).

3. Determinations the activity of some enzymes in carbohydrate metabolism and virulence enzyme in Cryptosporidium oocyts.

3.1: Enzymes of carbohydrate metabolism:

(A) Enzymes of Embden- meyerbof (glycolytic) pathway:

Glucose phosphate isomerase GPI:

The activity was measured according to the method of Noltman (1966). The following substances were mixed in a test tube:

-Tris- HCl	(1M) pH8.0	200 ml
-F-6-P	(0.01M)	200ml
-NADP	(0.01M)	100ML
-G6PDH	(3U)	50ML
-D.W.		250 ML
- Enzyme extract		200ml

The mixture was left for 5 minutes, and then the absorbance was measured at 340 nm for 0-time and after 10 minutes. The control was made by addition of the same quantities in a test tube with the substitution of the volume of the substrate in the mixture by distilled water and absorbance was measured as mention before.

(B) Enzyme of citric acid cycle:

Malic Enzyme ME:

The activity was measured according to the method of Ochoa (1966). The following substances were mixed in a test tube:

- Glycylglycin	(0.375 M)	200ML
-MnCl2	(0.015 M)	200 ML
-NADP	(0.007 M)	20ML
-Malic acid	(0.0075 M)	200ML
-D.W.		180ML
-Enzyme extract		200ml

The absorbance was measured at 340nm

The control was made by addition of the same quantities in a test tube with the substitution of the volume of the substrate in the mixture by distilled water and absorbance was measured at 340nm.

(C) Enzymes of hexose monophosphate (pentose phosphate):

Glucose -6-Phosphate Dehdrogenase G6PDH:

The activity was measured according to the method of Loehr and Waller (1963). The following substance was mixed in a test tube:

-Triethanolamin	(0.45M)	200ML
-EDTA	(0.064M)	200ML
-NADP	(0.015M)	100ML
-G6-P	(0.01M)	200ML
-D.W.		100ML
-Enzyme extract		200ml

The absorbance was measured at 340nm the control was made by addition of the same quantities in a test tube with the substitution of the volume of the substrate in the mixture by distilled water and absorbance was measured.

4-Determination of the activity of Virulence enzyme (protease)

The activity was measured according to the method of Coombs et al. (1982) which was modified from Barret (1977). The following substance were mixed in a test tube:

-Phosphate buffer	(0.1M) PH7.0	700ML
-Azocasein	(10%)	100ml
-Enzyme extract		200ml

The control was prepared from addition of the same materials +1ml of triochloro –acetic acid (TCA) all the tubes (test, control) were incubated for 1 hour at 370C in a water bath, and then the reaction was stopped by the addition of 1 ml of TCA. The tubes were centrifuge at (1000xg) for 10 min. and the filtrate absorbance at 366 nm. Each unit of the enzyme is defined as the quantity of the enzyme that causes the hydrolysis of one mg of the substrate per minute under the standard condition. The enzyme activity was calculated by division the number of unites of the enzyme by protein concentration.

Statistical Analysis

Statistical analyses were done by using SPSS version 13 computer software (Statistical Package for Social Sciences). Frequency distributions for selected variables were done first; associations between 2 categorical variables. The statistical significance of such associations was assessed by Chi-square ($\chi 2$) test of homogeneity. P value less than the 0.05 level of significance was considered statistically significant. Among the outcome quantitative variables were normally distributed, and therefore conveniently described by mean and SE and tested for statistical significance by T-Test.

Results

The results presented in this study were based on the analysis of 600 samples with mean of age 6.5±1.5, the period of this study from October 2010 to end of May 2011. From the total samples 600 there were 513 patients (85.55%) immunocompetent patients and 87 patients (14.5%) were immunocompromised. The results showed that 38 patients (7.4%) out of 513 immunocompetent patients were had infection with Cryptosporidiosis while 28 patients (32.18%) out of 87 immunocompromised patients had infection with cryptosporidiosis figure (1).

There was a statistically significant difference in the frequency between immunocompetent and immunocompromised (P<0.001).

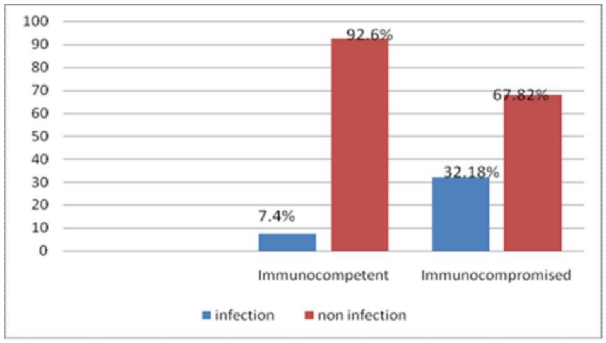

Figure (1) Relationship between immune status and infection Cryptosporidiosis.

5.1: Modified Ziehle-Neelsen stain for Cryptosporidium oocytes:

Sixty six samples (11%) out of 600 patients were positive for Cryptosporidium oocytes by using modified Ziehle-Neelsen stain which appears as red (usually spherical or slightly ovoid 4-6 mm) with different degree of intensity, the background materials and yeast stained green figure (2).

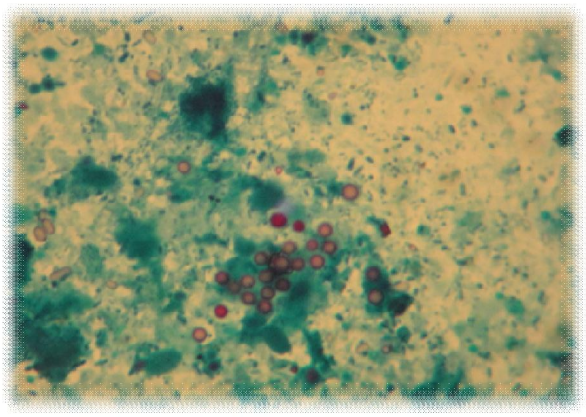

Figure (2): Oocytes of Cryptosporidium by modified Ziehle-Neelsen stain on power 1000X

5.2: Enzyme activities of Oocytes in Cryptosporidium.

GPI activity in oocytes isolated from immunocompetent patients was 119.77±8.413 while oocytes isolated from Immunocompromised patients that GPI activity was 123.54±9.461, there was a statistically analyses show there no difference in the frequency between enzymes activity (P>0.05) as shown in table (1).

ME activity in oocytes isolated from immunocompetent patients was 25.73±7.821 while oocytes isolated from Immunocompromised patients the ME activity was 30.64±9.610, there was no significant difference in the frequency between enzymes activity (P>0.05) as shown in table (1).

G6DH activity in oocytes isolated from immunocompetent patients was 53.26±4.577 while oocytes isolated from Immunocompromised patients that G6DH activity was 60.98±5.595, there was no significant difference in the frequency between enzymes activity (P>0.05) as shown in table (1).

Protease activity in oocytes isolated from immunocompetent patients was 40.20±8.216while oocytes isolated from Immunocompromised patients that Protease activity was 66.54±12.137, there was no significant difference in the frequency between enzymes activity (P>0.05) as shown in table (1).

Table (1): Enzymes activity in Oocytes Cryptosporidium

	immunocompetent	Immunocompromised	Significance p value
GPI	119.77±8.413	123.54±9.46	>0.05
ME	25.73±7.821	30.64±9.610	>0.05
G6DH	53.26±4.577	60.98±5.595	>0.05
Protease	40.20±8.216	66.54±12.137	>0.05

•The activity given was in nmol/min/mg protein (except for protease which is mu/min/mg protein).
•Data were represented as (mean ± SD) from 10 sample (immunocompetent patients) 10 sample (Immunocompromised patients).

Discussions

Cryptosporidium is an emerging pathogen that disproportionately affects children in developing countries and immunocompromised individuals. Without a diagnostic tool amenable for use in developing countries the burden of infection and its relationship to growth faltering, malnutrition, and diarrheal mortality remain underappreciated (Debbie-Ann et al., 2012).

In this study for measurement of enzymes activity using the method (Moodley, 1990) for purification of oocytes from stool samples these method was simple and easy and not used materials that may effect on enzymes of oocytes in addition to this in this study we choice the sample infected with cryptosporidiosis but not had other infected with other parasite to voided contamination with other oocytes of other parasite. Measurement protein concentration of enzymes extract used method Warburg and Christian (1941) these method was simple and easy and not taken long time. (Mohamed et al., 2007).

Result in table (1) showed the activity of enzymes: carbohydrate metabolism enzymes (GPI, ME and G6PDH) and protease enzymes the result showed high activity of enzyme with no significant difference between oocytes taken from immunocompromised patients and immunocompetent patients

The activity of Carbohydrate metabolism enzymes GPI, ME and G6PDH showed high level in activity of these enzymes that may be means this parasite in this stage (oocytes stage) used carbohydrate metabolism from production energy more than amino and fat metabolism, the study reported by (Abrahamsen et al., 2004) the result of his study showed There does not appear to be any fatty acid beta-oxidation pathway in Cryptosporidium, suggesting that fatty acids are not an energy source in this parasite. The activity of protease enzyme (virulence enzymes) in this study showed high activity the protease enzymes have importance role in pathogenesis of Cryptosporidium. Proteolytic processing is a common posttranslational modification of a number of proteins involved in attachment and invasion of apicomplexan parasites such as Plasmodium species, Cryptosporidium species and Toxoplasma (Shen B and Sibley, 2012). Processing of these proteins occurs either during transport through the secretory pathway or after secretion onto the parasites 'surface. In many cases, proteolytic processing of these proteins has been shown to be essential for invasion of host cells by these parasites, raising the possibility that the proteases involved in processing may represent potential targets for intervention, proteolytic processing of surface and apical complex proteins by parasite proteases is required for invasion of host cells, for assembly and trafficking of proteins, and for egress from host cells (Sharma P and Chitnis, 2013; Daniel et al., 2014).

Conclusion:

1. The results showed that Cryptosporidium species is one of the most common enteric parasites associated with diarrhea in Baghdad.
2. GPI, ME, G6PDH and Protease enzymes were found in Cryptosporidium Oocytes with highly activity, there was no significant difference between oocytes activity isolated from immunocompetent and immunocompromised patients.

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