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Study Effect of Clarithromycin drug on Cryptosporidium Parvum and Efficiency of ELISA Technique in Diagnosis Comparison with Some Traditional Methods in vitro.

Hussein A. Kadhum[1], Shaimaa A. Shlash[2]

[1]Assistant Lecturer in Microbiology/ Dentistry College/ Al-kafeel University/Iraq,
[2]Assistant Prof.Ph.D in Parasitology/Pharmacy College/Kufa University/Iraq

Abstract

This research aimed to study effect of Clarithromycin drug on Cryptosporidium Parvum and efficiency of ELISA technique in diagnosis comparison with some traditional methods in vitro. Samples collected from four different sources water were 400 liters:100 liters tap water provider by Al-Kufa District River,100 liters house tank water,100 liters from sea of Al-Najaf and 100 liters stagnant water during period from October 2018 to April 2019.Examined samples by three laboratory methods: Modified Ziehl-Neelson stain, Flotation by saturated sugar solution method and ELISA to detecting from Cryptosporidium oocysts in the water as used Clarithromycin drug effective against C.parvum in vitro. Capability test was performed by impregnation of isolate on cell monolayers and determination the parasite counted after 48 h from the incubation at 37°C. Differ concentrations from Clarithromycin were 4, 8, 16 and 32 mg/L respectively compared with control group.

Results of the current study showed that oocysts were found in 182 from 400 liters 45.5% as showed that height oocysts in stagnant water then followed Sea of Al-Najaf were 55% and 45% respectively, height percentage of oocysts were in April and March were 69.09% and 66% while decrease percentage of infection in October was 25%. As showed study results presence significant differences in efficiency of ELISA technique was 83.33% at detection from oocysts compared with Flotation by saturated sugar solution and Modified Ziehl-Neelson stain methods were 66.66% and 61.11% respectively. Shown results this study that Clarithromycin drug in concentrations of 16 and 32 mg/L caused decrease in number oocysts was 17.3±3.5 and 15.4±3.9, sporozoites counts was 17.1±3.4 and 14.9±4.0 compared with control group was 20.5±2.8.As observed significance differences ($P<0.05$) in the mean numbers of oocysts and sporozoites in three replicate plates after 48 h. These results proved that Clarithromycin have effectiveness in inhibition C.parvum in vitro.

Keywords: *Cryptosporidiosis, Water, Oocysts, Sporozoites, Clarithromycin, in vitro.*

Introduction

Cryptosporidiosis is protozoan disease occurred by Cryptosporidium spp from phylum Apicomplexa is unicellular organisms include number of pathogenic infect human and mammals by food and contaminated water (1). Oocysts transmitted in arduous environment where not killed by conventional disinfectants and chlorination which causes diarrhea, massive dehydration, malnutrition and weight loss lead to abomasa cryptosporidiosis (2). Oocysts resisted tough environmental condition for six months where can stay alive for twelve weeks at 10ºC (3). Transmission riot by the fecal oral and involve drinking water, recreational water, person to person, animals to person and contribution of sexual pursuit may be locomotion through persons with human immunodeficiency HIV virus (4).

C.parvum infections diagnosed through knowledge number of infestation and non-invasive methods. The non-invasive methods, microscopic examination more common (5) or identification of these parasite is based on morphologic examination by using modified acid-fast

staining method (6), important alternate the development of antigen parasite enzyme linked immune-sorbent assay (ELISA) these assay shown comparable sensitivity to experienced microscopic examinations, fairly simple to perform and do not require the observation of intact organisms (7).

Recently, observed there increase in number of infection with C.parvum correlating with increase in number of immune-compromised patients and resistance against drugs, this disease currently endemic in 88 countries world and showed diffusion increase in immunosuppressive conditions such as HIV/AIDS (8),Clarithromycin known as being semisynthetic antibiotic from macrolide derivative family (9) .Also good distribution excellent activity against intracellular pathogens such as: Toxoplasma gondii (10), Leishmania donovani (11) and Cryptosporidium parvum (12) invitro as featuring effective, least side effects, low cost and easy administration. Clarithromycin, Azithromycin and Roxithromycin are effective in inhibition C.parvum growth at concentrations close to those achievable (13). The aim this research is study effect of Clarithromycin drug on oocysts and sporozoites in vitro and efficiency of ELISA technique in diagnosis comparison with some traditional methods.

Materials and Method

Water samples were collected during the period from October 2018 to April 2019 of Al-Najaf governorate. A total amount was 400 liters:100 liters of tap water supplies by Al-Kufa District River, 100 liters of house tank water,100 liters sea of Al-Najaf and 100 liters of stagnant water, put in sterile bottles which delivered to the laboratory of pharmacognosy and medicinal plants in pharmacy college/ kufa university.

Diagnosis parasite: 5 liters from water samples distributed in sterile test tubes placed in the centrifuged 1000g/10-15 min to get on sediment, taken 5 ml of sediment and added 15 ml of distilled water then aspirate during six layers of gauze for get rid of the suspended waste then centrifugation procedure was performed again 1000g/10 min. 10 ml add of saturated sugars solution to the sediment well mix (14). Taken drop from the upper layer of the tube by Pasteur pipette placed on glass clean slide left to dry in the air for 10 min, smear fixed by absolute methyl alcohol for 5 min and left to dry, added modified ziehl-neelson stain to the fixed smear and heating for 5 min by Bunsen burner until vapor appears and washed with tap water, the slide is immersed in 10% HCL for 10-15 sec and washed again with water then dye was stained with methylene blue for 2 min, wash the well slide with running water then air-dried, examined by microscope 40X and 100X magnification to investigate parasite oocysts by many criteria's as size, shape, color stain and surface feature (15).

ELISA technique: Used in these technique particular oocysts antibodies where placed these antibodies in the pits for the plate ELISA method and these antibodies have ability to interact with oocysts find in samples then plate incubated for 1 h at 21°C, wash the plate by washing solution and add the conjugation solution containing antibodies to the parasite associated with the peroxidase enzyme then incubate and wash the plate again. Add chromogen tetramethylbenzidine is solution of the reactive substance which contained bottle inside size 25 ml with ELISA diagnostic components, if the water container on oocysts, conjugation solution remains linked with the pit and the enzyme converts chromogen from colorless to blue color composite where strength this color is proportional with number of oocysts in the examined sample, stopped the enzyme reaction after addition of phosphoric acid solution then measured optical density at the wavelength of 450 nm using optical spectrum (16).

Experiment study invitro: Oocysts isolated from the water by ELISA method was used throughout this study. Clarithromycin (Abbott, Italy) were dissolved in 50% methanol and 50% acetone to obtain 1 mg/ml of stock solution for perpetrated different concentrations where stored at –80°C in the dark. In these experiment indicated that concentrations of methanol, acetone were used in dilution of drugs did not inhibit the growth of C. parvum.

Oocysts Preparation: Occur by suspending portion of stock oocysts in bleach solution containing nine parts sterile deionized water and one part 0.55% sodium hypochlorite for 10 min, washed twice in sterile water, centrifuged and re-suspended in Dulbecco's modified Eagles medium (DMEM) (Bio-Whittaker) then incubation in phosphate buffered saline PBS (Bio-Whittaker) containing penicillin G (2000 U/mL), streptomycin (2000 mg/L) and amphotericin-B (10 mg/L) for 4 h at 37°C. Excystation of sporozoites was achieved by incubating oocysts in PBS containing 0.25% trypsin (Sigma-Aldrich) and 0.75% sodium hypochlorite for 60 min at 37°C. Free sporozoites were pelleted by

centrifugation 500g/10 min, re-suspended in (DMEM), counted in haemocytometer for culture (17).

Cell Culture: Isolated 500 cells from human stomach carcinoma were maintained in 25cm2 tissue culture flasks. Medium consisted of (DMEM) with 10% fetal bovine serum (Bio-Whittaker), 4% L-glutamine (Bio-Whittaker), 1% non-essential amino acid, penicillin G (100 U / ml), streptomycin (100 mg/l) and amphotericin B 0.5 mg/l. Cells were lifted from the surface of flasks by using solution of 0.25% trypsin and 0.53 mM EDTA in phosphate buffered saline then quantitated using hemocytometer. 500 cells were plated onto 35 mm diameter tissue culture plates at concentration of 105 viable cells in total volume 5 ml, viability was assessed by trypan blue exclusion (18).

Infection of cells with C.parvum: Infection of the cell monolayer was started by adding 105 sporozoites in volume of 0.2 ml of medium. After incubation for 4 h at 37°C in 5% CO2 to allow attachment and penetration of sporozoites, monolayers washed with (DMEM) to remove noninvasive sporozoites, residual oocysts and no adherent epithelial cells. Infected cell cultures were keeping at 37°C in 5% CO2 throughout this study (19).

Antibiotics agents: Clarithromycin concentrations prepared were 4, 8, 16 and 32 mg/L respectively, Experiments were performed in triplicate the monolayers were incubated for 48 h at 37°C in 5% CO2. Following four washes in phosphate buffered saline to remove free oocysts and no adherent epithelial cells then fixed with 75% methanol and stained by Giemsa stain to estimate presence of sporozoites within cells to vision both intra and extracellular oocysts. The number of parasites was calculated by mean ± standard deviation of the mean of number of sporozoites and oocysts observed in three monolayers of each of ten isolates exposed to same concentration of drug by microscopic examination of 40 fields under 1000X magnification (20).

Statistical analysis: In these study association between presences of oocysts with various source of water depending to months this study and efficiency of the diagnostic methods used during the experiment by ($P<0.05$) (21).

Results

Table (1): Percentage of Infection with Cryptosporidium oocysts to different water sources in the Study.

Source of water	No. of samples examined (liter)	NO. of Samples (+ve)	Percentage of Infection (+ve) %
Tap water provides by Al-Kufa river	100	40	40
Tank / houses	100	42	42
Sea of Al-Najaf	100	45	45
Stagnant water	100	55	55
Total	400	182	45.5

Table (2): Percentage of Infection with Cryptosporidium oocysts according to months the Study from October 2018 to April 2019

Months	No. of samples examined	NO. of Samples (+ve)	Percentage of Infection (+ve) %
October	60	15	25
November	50	19	38
December	60	25	41.66
January	65	30	46.15
February	60	22	36.66
March	50	33	66
April	55	38	69.09
Total	400	182	45.5

Table (3): Comparison between efficiency of ELISA technique in diagnosis

With traditional methods

Diagnosis methods	No. of samples examined	NO. of Samples (+ve)	Percentage (+ve) %
Flotation by Saturated sugar solution	90	55	61.11
Modified Ziehl-Neelson stain	90	60	66.66
ELISA	90	75	83.33

Table (4):Effect of clarithromycin drug on number of Cryptosporidium oocysts and sporozoites invitro.

Mean number of C. parvum per 40 microscope fields with Clarithromycin Concentration (mg/ml)				
Samples examined	4	8	16	32
Control (+ve)	20.5±2.8	20.5±2.8	20.5±2.8	20.5±2.8
Oocysts	19.8±3.1	19.5±3.3	17.3±3.5	15.4±3.9
Sporozoites	20.4±2.8	19.7±3.1	17.1±3.4	14.9±4.0

Discussion

Cryptosporidiosis is one of the major diarrheal diseases caused by protozoan parasites and poses significant public health worldwide and poorly understood and many livestock farming industries as water consider of sources for zoonotic infections.

In the present study found oocysts 182 of 400 liters from water samples with total prevalence 45.5% this rate varies depending to water source with significant differences. Oocysts found about 40% in tap water provides by Al-Kufa river, as found 42% in tank houses water while oocysts appear 45%, 55% in sea of Al-Najaf and stagnant water samples respectively, this study agrees with (22) showed infection rate in the children at Al-Najaf city was 13.6% causes contamination drinking water contained on oocysts (23). As in Table1

As showed in the relation between oocysts presences and season appeared significant differences was recorded upper infection in April and March were 69.09% and 66% while decrease percentage of infection in October was 25%, this study agrees with (24) where recorded highest percentage in the spring and lowest winter, in Iraq which attributed to appropriate climatic conditions help on survival of oocysts in the environment and increased consumption of water contaminated. As in Table2

Results of the study current existence significant differences in efficiency of ELISA technique was 83.33% at detection from Cryptosporidium oocysts compared with Flotation by saturated sugar solution and Modified Ziehl-Neelson stain methods were 66.66% and 61.11% respectively, which may lead to examination of large number of samples, accuracy in reading results and shortening the duration of the diagnosis, this study agree with (25). As in Table3

Clarithromycin showed potent anthelmintic activity in vitro, crucial importance is whether the drug would penetrate into larger metacestode tissues in our in vitro experiments, by using metacestode material cut into small blocks; this possible barrier was not of concern (20). In current study showed that clarithromycin in concentrations of 16 and 32 mg/L caused decrease in number oocysts was 17.3±3.5 and 15.4±3.9, sporozoites counts was 17.1±3.4 and 14.9±4.0 compared with positive control group was 20.5±2.8 respectively, lead to significance differences in the mean numbers of oocysts and sporozoites in three replicate plates after 48 h. As in Table4

The mechanism by clarithromycin decreases the number of parasite attributed to ability inhibit protein synthesis by binding to the transpeptidation site of the larger ribosomal subunit thus is logical that these agents should also affect C.parvum by inhibiting protein synthesis (26).

There are research showed that this drug is effective against protozoa such as T. gondii, Cryptosporodium spp(27) and L. Major in vitro by action mechanism effects on microorganisms is carried out through reversibly connecting to 50S ribosomal subunits and inhibiting the protein synthesis (28).

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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