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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*

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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 implement impregnation isolated and determination cells of parasite counted when at 37°C/ 48h incubated. 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 with 16 and 32 mg/L lead to reduction 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 the 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 assist of sexual pursuit may locomotion through man with HIV [(4).]

C.parvum infections diagnosed through knowledge number the infestation and non-inverting methods. The non-inverting methods, microscopic test more common [(5)] or identification of these parasite is based on morphologic examination by using modified acid-fast staining method [(6)], important alternate the expansion of antigen the parasite (ELISA) showed these assay sensitive comparative with microscope examine, this lead to not request the observed of hale parasite [(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 perfect action against of parasites e.g : *Toxoplasma gondii* [10], *Leishmania donovani* [11] and *Cryptosporidium parvum* [12] *invitro* as featuring effective, less side impacts, cheap and easy management. Clarithromycin, Azithromycin and Roxithromycin are active in inhibition *C.parvum* evolution in concentrations near those are 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 are collected from October 2018 to April 2019 period 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 oocysts by several standard e.g form, size, color stain and surface merit [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, Ital.) dissolves in 50% Acetone and 50% Methanol to obtain 1 mg/ml of stock solution for perpetrated different concentrations where stored at –80°C in the dark. In these experiment used the methanol and acetone in alleviation of the drug not discouraged of *C. parvum* grows.

Oocysts Preparation: Occur by suspending portion of stock solution contain 9 portions sterile water and 1 portion 0.55% sodium hypochlorite then put in centrifuged and re-suspended during Dulbecco's Modified Eagles Medium (DMEM) (Bio-Whittaker) then brood in Phosphate Buffered Saline (PBS) (Bio-Whittaker) contain 2000 U/mL of Penicillin G, 10 mg/L Amphotericin-B and 2000 mg/L Streptomycin busted when 37°C. Sporozoite excyst happened through oocyst incubated in buffer contain 0.75% Sodium Hypochlorite 0.25% Trypsin (Sigma-Aldrich.) at 37°C/60 min. Sporozoite liberated filtering by centrifuge 500g/10 min, re-suspended in (DMEM), counted in haemocytometer for culture [17].

Cell Culture: Isolated 500 cells from human stomach carcinoma preserve 25cm^2 tissue culture flacon. Put (DMEM) to medium with 10% Fetal bovine serum (Bio-Whittaker) in 4% L-Glutamine (Bio-Whittaker.), 1% non-essential amino acid, 100 mg/l Streptomycin and 0.5 mg/l Amphotericin-B. lift cells from surface of the flacon by used 0.53mM (EDTA) and 0.25% Trypsin in the (PBS). Coated 500 cells onto tissue culture glazy have 35mm in diameter of 10^5 concentration grow able cells 5 ml overall size [18].

Cells damage with *C.parvum*: Started by add 10^5 sporozoite with 0.2 ml of the medium to happen connect and breakthrough of sporozoites incubate at 37°C/4 h with 5% CO2. Expel no inverting sporozoites by monolayer wash in (DMEM) also to oocysts less and epithelial cells no cling. Cells infected are reservation in 5% CO2 during this of study [19].

Antibiotics agent: Clarithromycin concentrations prepared were 4, 8, 16 and 32 mg/L respectively, in

Triplicate of monolayers incubate at 37°C /48 h with 5% CO2 then washed four times in phosphate buffered saline to edit oocysts and prevent epithelial cells adhesion, well use 75% methanol for installation and Giemsa stained to estimate existence of sporozoites inwards cells to vision both intra and extracellular oocysts. Calculated the of parasite number by mean ± standard deviation are number mean of oocysts and sporozoites perceive in monolayers three for every to isolates ten to same concentration roofless of treatment, 40 field examine by 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 great diarrheal disease happens by protozoan with health problem 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 L from total samples 45.5% are rate varies depending to water exporter significant variation. 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*, mechanical through penetrate to larger number of the metacestode tissues *in vitro*, via metacestode fragment into small portions [20]. In current study showed that clarithromycin when 16 and 32 mg/L lead to number decrease of 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 variation to mean of number the sporozoites and oocysts in three replicate plates after 48 h. As in Table4

Clarithromycin mechanism act number decline of parasite attributed to ability protein synthesis frustration by binding with transpeptidation sites for ribosomal subunits is target as *C.parvum* affect by protein synthesis frustration [26].

There are research showed that this drug is effective against protozoa such as *T. gondii*, *Cryptosporodium* spp[27] and *L. Major in vitro* action promastigotes mechanism effects through adverse binding to 50S ribosomal subunits and frustration the protein synthesis [28].

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