Detection the genotyping of Cryptosporidium parvum isolated from human and calves and studying in vivo effecte of Sacharomyces boulardii on parasite

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ABSTRACT

This study include the collection of 320 stool sample, (250) stool sample, were collected from patients of both sexes at different ages and seventy samples were collected from calves suffering from severe diarrhea, all samples Staining with Modified Cold Ziel-Neelsoon stain. The oocysts were used to extraction the DNA and detection of C. parvum DNA from human and calves by using two types of primers have been used for the detection of C. parvum parasite were (nest rRNA 830bp and rRNA 1.325bp Primer). In this study the rRNA Primer (1.325bp) did not given any results for human and calves stool samples but nest rRNA 830bp Primer give positive results human sample exactly the same band of animal sample. This shows that the genus, which infected the human himself, which infect the animal. Also the experimental study showed that the orally inoculated S. boulardii to infected mice led to reducing the shedding of this parasite in feces of the mice since the first day and became (zero) in day (9th) post inoculation, compared with Spiramycin reach to zero at day (10 th), While control group persisted on shedding the parasite in feces with continuous up to day (10th) . The efficacy of the S.boulardii was (87.45%). While the Spiramycin efficacy was (85.11%). The histo pathological study of the mice intestines shown that the parasite causes mild atrophy of the mucosa layer and atrophy villi intestine with infiltration of inflammatory cells, while S.boulardii yeast showed high effective In repairing and restoring to intestinal tissue to the normal structure with the slight in creasing in numbers of goblet cells, compared with Spiramycin which led to an excessive increase in division of the goblet cells and cells mucosa layer.

INTRODUCTION

Cryptosporidium is a protozoan coccidian parasite of the apicomplexa phylum found in the respiratory and gastrointestinal tracts of many hosts. Cryptosporidium species are ubiquitous in nature with worldwide distribution and have mammalian, avian, piscine, and amphibian hosts, infections can result from exposure to low doses of Cryptosporidium oocysts [1]. Chlorination and common household disinfectants can be resisted by the oocysts, ability to pass through physical water treatment processes, and survive long periods in the environment. Humans can be infected with Cryptosporidium through many transmission routes, like as food borne transmission by ingestion of contaminated food or by waterborne transmission.
(water contamination) or by direct contact with infected persons or animals [2]. For most infected people, the symptoms of watery diarrhea, cramps or stomach pain, dehydration, vomiting, nausea, fever, and weight loss start seven to ten days after being infected. These symptoms usually persist for one to two weeks, but can range from a few days to several weeks [3]. The common therapy consists nitazoxanide and spiramycin can help shorten the amount of time oocysts are passed as well as the duration of diarrhea [4]. The term probiotic is live microbial food supplement microorganisms that confer health benefits when administered in adequate amounts. Diverse important mechanisms underlying the useful effects of probiotics contain the modification of the gut microbiota, the competitive adherence to the mucosa and epithelium, also made of epithelial layer of the intestine and liver more strong [5]. 

Saccharomyces boulardii, is the only yeast probiotic that has been certain effective in double-blind studies. This yeast is used in many countries as both a therapeutic agent and preventive for diarrhea and another gastrointestinal disorders caused by antimicrobial agents. Its consider as a potential probiotic agent because it possesses many properties, i.e. it survives transit through the gastrointestinal (GI) tract, optimal temperature is (37°C), and it can control on the growth of a number of microbial pathogens.

**MATERIALS AND METHODS**

**Human stool samples**

The present study include (250) stool sample, were collected from patients of both sexes at different ages (1-35 year), who had been admitted to the laboratories of parasitology of the following health institutions (Abin Al-Baladi hospital, Baghdad Medical City, Fatema Al-Zahraa Hospital, Al-Imam Ali Hospital and Al-Kindy Hospital). All patients suffering from severe watery diarrhea, stool samples put it in tightened plastic cold container and given number representing the patient.

**Collecting Calves stool samples**

Seventy samples were collected from calves suffering from severe diarrhea, collection from Stations for breeding calves in Kut. Fecal samples were collected directly from animal’s rectum and save it in tightened plastic cold container.

**Microscopic Examination**

Glass slides were prepared from each fecal sample by direct smear method, the positive slide stained by modified cold Zehil Neelsen stain[7], then examined under oil immersion(1000 X) to detect the presence of oocyst.

**Isolation and purification oocysts**

The oocysts were isolated and purified from the positive sample according to OIE method [8]. The oocysts were counted and adjusted in (1x10⁶ oocyst /0.1 ml) by using hemocytometer.

**Diagnosis Molecular Method for genomic DNA of parasite oocysts isolation from human and calves stool**

**The procedure of PCR amplification**

rRNAF (5’ TTC TAG AGC TAA TAC ATG CG-3’), rRNA-R (5’-CCC TAA TTC GAA ACA GGA-3’) and nest rRNA-F (5’-GGA AGG GTT GTA TTT ATT AGA TAA AG-3’), nest rRNA-R(5’-AAG GAG TAA GGA ACA ACC TCC A-3’) primers were used on the Cryptosporidium spp oocyst wall proteins. The PCR mixtures contained 3μL of DNA sample, 10 μL Master mix, 2 μL Forward Primer (10pmol), 2 μL Reverser primer (10pmol), 3 μL H2O (Nuclease-free). PCR was performed under the following conditions: 35 cycle at 94 °C for 45sec, 55 °C for 45 sec and 72 °C for 1min followed by 72 °C for 7min. The components of the reaction were mixed and placed in a thermal cycler that cycles the reaction through a predetermined series of specific temperature and time adjustment. PCR amplification can be completed in as little as two hours. PCR product was analyzed for rRNA(1,325bp) and nest rRNA(830pb) fragments by electrophoresis in agarose/ethidium bromide gels.

**Experimental study**

**Animals**

Males of albino mice were obtained from national control center for drugs and researches, their ages were between 12-14 weeks with weights between 16-18 gm. Mice were putted in plastic clean cages, and stool of them was examined before the beginning of the experiment to make sure of clearance mice from any intestinal parasites.

**Experimental design**

Immunosupressed done for 24 mice with dexamethazone according to [9]. After 5 days, (18) mice were inoculated orally by stomach tube with 0.1ml contain (1x10⁶ oocyst /0.1ml) prepared previously, the last (6) mice remain not infected and consider as control negative, one day after dosing each mice were examined by prepare direct smear and after confirmation of infection, get infected mice divided into (4) groups, each group contain (6) mice then inoculated as follow:

- Group one:
Mice of this group were given daily 0.1 ml of Saccharomyces boulardii which contain \(1 \times 10^7\) cell/0.1 ml by stomach tube.

- **Group two:**
  Mice in this group were inoculated daily with 0.001 ml of spiramycin by stomach tube.

- **Group three:**
  Mice of this group were given daily (0.1 ml) of normal saline used as a positive control.

- **Group four:**
  Mice of this group were given orally (0.1 ml) of normal saline, this group considered as a negative control.

  During period of experiment the following data had been recorded:

1. recording any clinical obvious sign.
2. recording perpant period.
3. feces examination to calculate of oocyst in one gram every day by using this equation of Ryan *et al* [10]
4. sufficient treatment by cells of yeast and spiramycin was measured according to Xiao *et al* [11].

**Histopathological study**

All mice in study groups were sacrificed at the end time of experiment, then small intestine was removed aseptically, fixed in 10% buffered formalin, processed, stained with haematoxylin and eosin stain then examined under the light microscope [12].

**Statistical analysis**

The Statistical Analysis System- SAS 2012 was used to effect of different factors group and day on number of *C. parvum* oocysts raised \(x\ 10^7\). Least significant difference–LSD test was used to significant compare between means in this study.

**RESULTS AND DISCUSSION**

In this study the results showed that the rate of infection with *C. parvum* was (34%) from (85) patients positive cases .The results in table (1) indicated that the highest infection with *C. parvum* occurred in Abin Al-Baladi hospital with percentage of 60% followed by Baghdad Medical City with percentage of 50%, then Fatema Al-Zahraa hospital with the percentage of 40% finally Al-Kindy hospital and Al-Imam Ali Hospital with the percentage of 10%, these results recorded according to Modified Ziehl–Neelsen stain method.

<table>
<thead>
<tr>
<th>Hospital name</th>
<th>No. of patients</th>
<th>No. of infected patients with <em>C. parvum</em></th>
<th>Percentages %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abin AL-Baladi Hospital</td>
<td>50</td>
<td>30</td>
<td>60</td>
</tr>
<tr>
<td>Baghdad Medical City</td>
<td>50</td>
<td>25</td>
<td>50</td>
</tr>
<tr>
<td>Fatema AL-Zahraa Hospital</td>
<td>50</td>
<td>20</td>
<td>40</td>
</tr>
<tr>
<td>AL-Imam Ali Hospital</td>
<td>50</td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td>and AL-Kindy Hospital</td>
<td>50</td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td>Total</td>
<td>250</td>
<td>85</td>
<td>34</td>
</tr>
</tbody>
</table>

Prevalence of *C. parvum* infection in the calves. The results showed that the rate of infection with *C. parvum* was (42.85%) of the (30) calves positive cases. In other hand the results of Diagnosis *C. parvium* by PCR methods was two types of primers have been used for the detection of *C. parvium* parasite were (nest rRNA 830bp Primer and rRNA 1.325bp Primer). in this study the rRNA Primer (1.325bp) did not given any results for human and calves stool samples . After making gel electrophoresis to the DNA product of PCR the results to the other second primer (nest rRNA 830bp) was band human sample exactly the same band of animal sample This shows that the genus, which infected the human himself, which infect the animal was *C. parvum* because the can *C. parvum* Infects human and calves while *C. hominis* can infect human only, as shown in figure (1). In addition to the positive stool samples from humans and calves which diagnosed by ziehl neelsen stain was chosen Fifty randomly samples (25 from human and 25 from calves) and was re-diagnosed by PCR method.

The results in table(2) showed that the number of positive samples were (5) out of (25) stool samples from human and were (10) out of (25) in calves samples, with rate (20%) and (40%) for human and calves respectively.

<table>
<thead>
<tr>
<th>Number of stool sample</th>
<th>Positive samples</th>
<th>Percentage(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>human</td>
<td>25</td>
<td>5</td>
</tr>
<tr>
<td>calves</td>
<td>25</td>
<td>10</td>
</tr>
</tbody>
</table>
Figure (1): patterns PCR products of cryptosporidium parvum by using nest rRNA 830bp, M represent molecular marker; line 1 represents a sample of human and line 2 represents a sample of calves

Experimental study

The present study include to investigate the efficiency of Saccharomyces boulardii in infected mice by cryptosporidium parvum and compared it with Spiramycin drug which using to treat the parasite. There was no recorded for any visible signs appeared on the infected mice and the prepatent period for this parasite was between (48-72hr). The results showed that the orally inoculated of this yeast to infected mice led to reducing the shedding of this parasite in feces of the mice since the first day of treatment was $(2.33 \times 10^2$ cell/gm), then continued to decrease gradually with days till stopped shedding of parasite totally and became (zero) in day (9$^{th}$) post inoculation, compared with results of Spiramycin showed a light decrease in number of oocysts shedding after first days post inoculation were $(2.1, 3.1, 5.1, 8.5, 6.8, 4.1, 1.8, 0.6 \times 10^2$ cell/gm) respectively till reach to zero at day $(10^{th})$,as seen in table (3).

While in positive control group the infected mice persisted on shedding the parasite in feces with continuous increased in numbers of parasite up to day $(10^{th})$ after infection reached to $(22.35 \times 10^3$ cell/gm).

There was significant differences between the two treatment groups (S.boulardii group and Spiramycin) and control group (p <0.05) till $(5^{th})$ and become high significant differences (p<0.01) for the remaining days of experiment, but there was no significant differences between treatment groups till $(6^{th})$ day, then the differences became significantly (P <0.05 and p<0.01) at days$(7^{th}, 8^{th}$ and $9^{th}$) respectively.

When applied the equation of treatment efficacy, found that the $S.boulardii$ efficacy was (87.45%). While the Spiramycin efficacy was (85.11%).Table (4).

In general the activity of probiotics to prevented and treated pathogen represented by covering the surface of epithelial tissue for intestine, Secretion of active molecules (e.g. bacteriocins, antibiotics, free fatty acids and hydrogen peroxide), Modulation of the intestinal environment and changing pH.[13]

Table (3): Mean number of cryptosporidium parvum in treatment and control groups ± 5D×10^2

<table>
<thead>
<tr>
<th>Days</th>
<th>Means±SD(cell/gm)</th>
<th>LSD value</th>
<th>Chi-square ($\chi^2$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>S. boulardii</td>
<td>Spiramycin</td>
<td>Control</td>
</tr>
<tr>
<td>1</td>
<td>2.33±1.03</td>
<td>2.1±0.98</td>
<td>2.28±0.88</td>
</tr>
<tr>
<td>2</td>
<td>2.83±0.75</td>
<td>3.1±0.75</td>
<td>4.72±1.38</td>
</tr>
<tr>
<td>3</td>
<td>5.1±0.75</td>
<td>5.1±0.75</td>
<td>8.57±1.13</td>
</tr>
<tr>
<td>4</td>
<td>7.1±1.9</td>
<td>8.5±1.04</td>
<td>11.43±1.39</td>
</tr>
<tr>
<td>5</td>
<td>7.5±1.04</td>
<td>8±0.89</td>
<td>14.43±1.51</td>
</tr>
<tr>
<td>6</td>
<td>5.2±1.03</td>
<td>6.8±0.75</td>
<td>15.43±1.39</td>
</tr>
<tr>
<td>7</td>
<td>3±0.89</td>
<td>4.1±0.98</td>
<td>15.57±1.71</td>
</tr>
<tr>
<td>8</td>
<td>0.83±0.75</td>
<td>1.8±0.75</td>
<td>18.72±1.11</td>
</tr>
<tr>
<td>9</td>
<td>0</td>
<td>0.6±0.82</td>
<td>20.43±0.97</td>
</tr>
<tr>
<td>10</td>
<td>0</td>
<td>0</td>
<td>22.35±1.11</td>
</tr>
</tbody>
</table>

Least significant difference //ns.non significant//*P(<0.05)//**P(<0.01)

Table (4): Efficiency of treatment for the yeast and Spiramycin

<table>
<thead>
<tr>
<th>Type of treatment</th>
<th>Dose/ml</th>
<th>Efficiency of treatment %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saccharomyces boulardii</td>
<td>0.1</td>
<td>87.45</td>
</tr>
<tr>
<td>Spiramycin</td>
<td>0.1</td>
<td>85.11</td>
</tr>
</tbody>
</table>

Histopathological study

Histopathological study was done to study the pathogenesis of C. parvum parasite and effect of S.boulardii on intestinal tissue compared with spiramycin .Positive control group regarded as the best example to study the pathogenesis of parasite in the mice, mice were sacrificed after (11 days) post infection.
Slides of histological examination of the intestinal tissue showed presence of inflammatory cells inside the villi compared with negative control group, as shown in figure(2) and figure(3).

When examining the intestinal tissue of mice inoculated with *S. boulardii* showed return the villi to normal form and accompanied by increasing in numbers of goblet cells, figure(4). Compared with mice intestinal tissue inoculated with spiramycin observed that Spiramycin caused hyperplasia of the mucosa and hyperplasia of the globlet cells, figure(5). Pharmacokinetic studies have shown that after oral administration of lyophilized *S. boulardii*, the steady-state concentrations are achieved in the colon within 3 days, and the yeast are cleared from the stools within 2-5 days after discontinuation [14]. *S. boulardii* displays important characteristics allowing a microorganism to transit through the gastrointestinal tract and to be used as a probiotic. During the intestinal transit, *S. boulardii* interacts with resident microflora and intestinal mucosa. Moreover, experimental studies displayed that *S. boulardii* induces a protection against enteric pathogens [15]. modulates the host immune response [16,17], decreases inflammation [18,19] and hydroelectrolytic secretions [20], inhibits bacterial toxins [21,22] and enhances trophic factors such as brush border membrane enzymes and nutrient transporters [23,24]. Studies have been used *S. boulardii* practically in the treatment of diarrhea caused by *cryptosporidium* are rare and may be say this study considered the early studies used these yeast to treatment of *cryptosporidium* infection.

Figure (2): Section of intestine control-ve showing normal appearance of intestinal mucosa and villi (H&E),200X

Figure (3a,b): A- control+ve section of intestinal tissue showing slight atrophy of the mucosa with atrophy of the intestine villi (H&E),200X B- presence of inflammatory cells inside the villi. (H&E),400X
Figure (4): Section in intestine showing treated by *Sacharomyces boulardi* returned the villi to normal size with increase in number of goblet cells. (H&E), 200X

A-Section in intestine treated by Spiramycin showing hyperplasia of the mucosa and hyperplasia of the goblet cell. (H&E), 200X
B-Hyperplasia of the goblet cells(H&E), 400X.

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