DETECTION OF *CRYPTOSPORIDIUM*-INDUCED INTESTINAL TISSUE ALTERATIONS IN DEXAMETHASONE TREATED & UN-TREATED MICE

By

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Abstract

Cryptosporidium parvum (*C. parvum*) is coccidian protozoan that causes cryptosporidiosis, a parasitic disease of the mammalian intestinal tract. *C. parvum* is considered one of the most important waterborne pathogen among the most relevant parasitic enteric agents in man and animals. It is resistant to all practical levels of chlorination and it is an obligate intracellular pathogen. It has been the cause of multiple diarrhea outbreaks in developed and developing countries. The present work was carried out to evaluate the pathological, immuno-histochemical and molecular changes in the ileocecal region induced by chronic irritation with different inoculum sizes of *cryptosporidium* (50,500 oocysts) in immunocompetent and immunosuppressed mice. The mice were euthanized at different dates starting from 14, 21, 36, 45, 57 till day 64 to study these transformations. Histopathological examination of the ileocecal region revealed neoplastic changes in the form of dysplasia, polypoid structures, architectural distortion, glandular crowding, marked cellular atypia, exophytic adenomatous polypi, intramuscular adenocarcinoma and marked nuclear anaplasia.
Key words: *Cryptosporidium spp.* Mice, Intestinal tissue alterations, Histopathology,

Introduction

Cryptosporidium parvum (C. parvum) is a coccidian protozoon causes cryptosporidiosis, a parasitic disease of the mammalian intestinal tract. *C. parvum* is one of the most important waterborne pathogen in developing countries and due to the zoonotic character of some of its species was among the most relevant parasitic enteric agents in human and veterinary medicine. It is resistant to all practical levels of chlorination and it is an obligate intracellular pathogen (Haque *et al*, 2003). It proved to be the cause of multiple diarrhea outbreaks in developed and developing countries (Ng *et al*, 2010).

Cryptosporidiosis proved to be a leading cause of diarrhea in Egyptian particularly children. *Cryptosporidium spp.* was among individuals with diarrhea visiting inpatient and outpatient clinics in Egypt ranged from 0%–49% (El-Shazly *et al*, 2007). The most common protozoan infection in immunosuppressed (IS) Egyptian patients (60.2%) was due to *C. parvum* (Abdel-Hafeez *et al*, 2016), A study was done to identify the genotypes of the *C. parvum* isolates from clinical samples from diarrheic children in Egypt using polymerase chain reaction (PCR) amplification and restriction fragment length polymorphism (RFLP),the results showed that 12% of the specimens were positive for *Cryptosporidium* spp. revealed that 82% had *C. parvum*, 12% had *C. hominis*, and (6%) had mixed infections (Eraky *et al*, 2014). Besides, cryptosporidiosis zoonotic potential was proven in Egyptian farmers and their farm animals infected with *C. parvum* (El-Bahnasawy *et al*, 2016).

Different species of *Cryptosporidium* were reported among human patients, including *C. parvum, C. hominis and C. bovis* (Helmy *et al*, 2013). In animals, *Cryptosporidium* spp. infection implies both an economic loss and a significant source for zoonotic infection.

Cryptosporidium parvum causes a severe, life-threatening diarrhea in immunocom-promised hosts. Although this protozoan is far more common in immunocompromised patients, it was also known to induce diarrhea in immunocompetent (IC) persons where infe-

ction is generally self-limiting (Guk *et al*, 2005). Infection is caused by ingestion of sporulated oocysts transmitted by the feco-oral route or indirect via contaminated water supply, food, or environment (Oyibo *et al*, 2011). Invasion of the apical tip of ileal enterocytes by sporozoites and merozoites causes pathogenicity (Youssef *et al*, 2008).

C. parvum has been correlated with digestive carcinogenesis. An epidemiologic study in Poland reported a frequency of 18% of cryptosporidiosis in patients with colorectal cancer (Sulzyc-Bielicka, 2007). However, in this report it was unclear whether *C. parvum* behaved as a carcinogenic factor or simply as an opportunistic agent whose development was enhanced by host immunosuppression. More consistent with a potential tumorigenic role of this parasite, it was proved that IOWA and TUM1 strains of *C. parvum* of the animal origin induced digestive neoplasia in a rodent model (Certad *et al*, 2010a).

This study aimed to detect of *Cryptosporidium parvum*-induced intestinal tissue alterations in the dexamethasone treated and untreated Albino mice,

Material and Methods

Animal source: Laboratory bred female, white Albino mice of CDI strain, about 4-6 weeks old, weighing 20-25gram, were obtained from the European countryside. Animal experiments were performed in the biological unit of Theodor Bilharz Research Institute (TBRI), in a well-ventilated plastic cage with clean wood-chip bedding in conditioned rooms ($27\pm2^{o}C$) and away from the direct sunlight, ensuring good sanitary condition.

All the experiments on animals were carried out according to the internationally valid guidelines after the approval of the Institutional Ethical Committee of TBRI and of Faculty of Medicine, Cairo University.

Experimental design: One hundred and fifty clean laboratories bred white Albino female mice were used in this study.

Mice were divided into two main groups according to their immune status (whether treated with Dex or not): Group A (immuno-competent) was divided into the following subgroups according to the infecting dose and number of exposure to *C. parvum*, each consisted of 15 mice: GA 1: Negative control (control non-infected), GA 2: repeatedly infected with 50 oocysts/mouse/ week for nine weeks, GA 3: repeatedly infected with 500 oocysts/ mouse / week for nine weeks, GA4 a: Positive control (infected with 50 oocysts/ mouse once) and GA4 b: Positive control (infected with 500 oocysts/ mouse once).

Group B (immunosuppressed group) was divided into the following subgroups according to the infecting dose and number of exposure to *C. parvum*, each consisted of 15 mice: GB 1: Negative control (Control non-infected), GB 2: repeatedly infected with 50 oocysts/mouse/ week for nine weeks, GB 3: repeatedly infected with 500 oocysts/ mouse / week for nine weeks, GB 4 a: Positive control (infected with 50 oocysts/ mouse once), and GB4 b: Positive control (infected with 500 oocysts/mouse once).

Immunosuppression: Mice were administered with 0.25ug/g/day of dexamethasone sodium phosphate (Dexazone) orally via an esophageal tube. Dex administration started daily for two weeks prior to oral inoculation with *Cryptosporidium* oocysts and was maintained weekly during the whole experiment (Rehquel *et al*, 1998).

Animal infection: Mice were orally infected with *C. parvum* oocysts. Infective dose was calculated as some groups received 50 oocysts and others received 500 oocysts. The dose was repeated every week for nine weeks to cause chronic irritation and infection in groups A2, A3, B2 & B3. *C. parvum* was dissolved in 200μL of PBS and given to each mouse using esophageal tube.

Animal scarification: Scarification was done at different durations starting from day 14, 21, 36, 45, 57 till day 64 post-infection and euthanasia was performed by decapita-

tion. The ileocecal region was removed and subjected to the histopathology examined.

Parasitological examination: A week after mice infection, stool samples were collected and examined after staining by MZN stain (Henricksen and Pohlenz, 1981) to detect and count C. parvum oocysts per the oil immersion lens (x100) and to ensure that mice have been infected. Stool samples were collected every week throughout the experiment to assess the effect of the weekly chronic irritation.

Histopathological criteria: ileocecal region was removed from each animal, fixed in 10% buffered formalin solution, embedded in paraffin wax blocks that were sectioned then stained in the pathology lab of TBRI, staining was done using hematoxylin and eosin to assess changes and explore any abnormal pattern of proliferation, dysplasia, cellular atypia, or any neoplastic lesions.

Statistical analysis: Data were analyzed using SPSS© Statistics version 24 (SPSS© Corp., Armonk, NY, USA). Skewed numerical data were presented as median and interquartile range and between-group differences were compared using the Mann-Whitney test (for 2-group comparison) or the Kruskal-Wallis test (for multiple-group comparisons). The Jonckheere-Terpstra test was used to compare numerical data across ranked groups. Post hoc comparisons were done by using the Conover test if needed. Categorical variables were presented as number and percentage. Fisher's exact test compared nominal data and the chi-squared test for trend to compare ordinal data. Inter-method agreement was tested using the Cohen kappa (κ) coefficient P-values less than 0.05 were considered as statistically significant

The ethical consideration: This study was approved by the Scientific Research Ethics Committee of TBRI and that of the Faculty of Medicine, Cairo University.

Results

Cryptosporidium oocysts shedding: Oocysts were visualized as red spots 4-6μm and with refractive round thick capsules on blue background when examined by light microscope with 1000X. Some of the sporozoites were visible in *C. parvum* oocysts (Kaushik *et al,* 2008). Micromrtery confirmed the *Cryptosporidium* oocysts size as 4-6μm (Figs. 1, 2). The mean shedding of oocysts in different groups was 306 oocysts, with maximum shedding of 2250 oocysts (Tab. 1). The number of shed oocysts in different groups showed significant difference, with the highest shedding in GB3 (IS inoculated by 500 oocysts). There was a significant correlation between shedded oocysts and number of inoculated oocysts indicated that the more the inoculating dose, the more the oocysts shedding (Tab. 2, Figs. 3 & 4).

Evaluation of unintended death of mice: On comparing mean days of death of mice, there was a significant difference among the different studied groups, with the least survival in group B3 (IS, inoculated regularly by 500 oocysts) with mean survival of 34.3 days and the maximal survival was in GA1 & GB1 (negative control) with mean survival of 64 days (Tab. 3, Fig. 5).

Histopathological criteria: Histopathological examination of the ileocecal region of the mice in different groups showed several degrees of pathological and neoplastic lesions among them. The several degrees of inflammatory changes were seen in the groups infected with the parasites, both immunocompetent and immunosuppressed. The neoplastic lesions were from low grade dysplasia, polypoid structures, nuclear pleomorphism, hyperchromasia and exophytic adenomatous polypi (Figs. 6-13).

Table 1: Fecal oocyst shedding in studied mice

					Percentiles		
Variable	Minimum	Maximum	Mean	SD	25th	50th	75th
Fecal oocyst shedding (oocysts/mg feces)	0	2250	306	473	0	90	485

Table 2: Comparison of fecal oocyst shedding in studied groups

Group	Fecal oocyst shedding (oocysts/ml feces)		
	Median	IQR	p-value*
A1	0	0 – 0	<.000001
A2	68	32 – 114	
A3	552	401 – 1205	
A4a	5	2 – 9	
A4b	27	20-32	
B1	0	0 – 0	
B2	173	103 – 243	
B3	768	575 – 1090	
B4a	15	5-22	
B4b	31	8 – 115	

*Kruskal-Wallis test

Table 3: Mean day of death among different studied groups

Group number	Mean	Standard deviation	p-value
A1	64	-	
A2	38.7	16.9	
A3	38.9	19	
A4a	44.6	17	
A4b	44.6	17	<0.0001*
B1	64	-	
B2	35.8	18.5	
B3	34.3	20.1	
B4a	44.6	17	
B4b	44.6	17	

*P-value calculated using Kurskall Wallis test

Discussion

The current work explored the potential role of *C. parvum* in inducing neoplastic changes in digestive epithelium of IC and IS mice, using parasitological (oocyst shedding and death rate evaluation) and histopathological methods. In the present study, the infecting dose of *Cryptosporidium* oocysts was challenged with low dose (50 oocysts), which could be found in environmental samples as in tap water and vegetables compared to the high dose (500 oocysts). Benamrouz *et al.* (2012b) inoculated the mice with different doses of oocysts to study the effect of low and high dose; mice were challenged with low inoculum (1 & 10 oocysts) and a high inoculum (100 & 10^5 oocysts). Cetrad *et al.* (2010a) and Abdou *et al.* (2013) inoculated mice with only one infective high dose (10^5 oocysts/ mouse).
. The role of chronic irritation was done to ensure the maintenance of infection all through the experiment in both IC and IS mice and to assess the role of chronic irritation in induction of neoplastic changes. Watanapa (2002) reported mice chronically irritated by *Opisthorchis viverrini* infection and developed cholangiocarcinoma. Tian *et al.* (2013) found that mice with chronic schistosomiasis developed hepatic fibrosis and carcinogenesis.

Scarification days were chosen to detect neoplastic changes in the ileocecal region histopathologically. Mice were euthanized at different durations starting from day 14, 21, 36, 45, 57 till day 64 post infection. Cetrad *et al.* (2010b) found an apparent increase of the mitosis number in cells from ileocecal sections between days 20 and 35 post infection and continued to increase till day 46 post infection. A strong positive significant correlation was found between shedded oocysts and the day of stool examination indicating that the longer the duration of infection and inoculation, the more the shedding of oocysts. This went in accordance with Benamrouz *et al.* (2012a) who showed that the day post infection significantly influence the number of oocysts shedded so that as the days increased, oocyst shedding increased.

In the present study, mice challenged with low and high inoculum developed chronic

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infection and shedded oocysts without stationary or decline phase until the end of the experiment. In the present study, IC positive control mice (GA4) that were inoculated once at the beginning of the experiment (chronic infection) showed decline phase till they reached complete cessation at the end of our study. This coincided with Miller *et al.* (2007) and Certad *et al.* (2010b) who found that oocyst shedding declined till it stopped completely at the end of the experiment. Benamrouz *et al.* (2012a) revealed that oocyst shedding in IC mice showed decline phase of shedding with almost complete cessation of oocyst production at the end of the experiment. Lacroix *et al.* (2000) reported that the immune system and defense mechanisms were able to fight the infection and reject the parasite rapidly in the IC host. Chronically irritated mice in the present work were inoculated weekly by *Cryptosporidium* oocysts, which helped to maintain the infection without any decline or stationary phase till the end of the experimental study. In the positive control groups, infection was induced only once at the beginning of the study which lead to decline phase till the end of the experiment.

In the present study, mice showed high the levels of oocyst shedding throughout the study. These agreed with McDonald *et al.* (1992) who found that IS mice showed high and sustained levels of oocysts shedding. Also, Ahmet *et al.* (2003) found that IS mice chronically infected with *Cryptosporidium* spp. continued to shed oocysts throughout the experiment without decline or stationary phase. Abdou *et al.* (2013) found that IS mice showed high levels of oocyst shedding up to the experimental end.

In the current work, the intensity of the oocysts shedding showed varied among the different groups, and was influenced by the immune status and the inoculum size. The intensity of oocyst shedding was higher in IS mice than in IC mice throughout the experimental study, but without significant. Besides, Ahmet *et al.* (2003) found that the

immunosuppression increased the oocysts shedding. Chai *et al.* (2010) proved that immunosuppression directly correlated with the intensity of oocysts shedding. This went well with Abdou *et al.* (2013) who found that the intensity of oocyst shedding was significantly higher in IS mice than in IC ones throughout the study. But, Cetrad *et al.* (2010a) found that the mean number of excreted oocysts tended to decrease in Dex-treated mice receiving the highest challenge inoculum. Sequestration of the parasites out of the intestine and into other sites could explain a diminished detection of *Cryptosporidium* oocysts in the feces (Mead, 2002).

In the present study, there was a significant correlation between shedded oocysts and number of inoculated oocysts indicating that the more the inoculation dose, the more the oocysts shedding. So, oocyst excretion increased according to inoculum size (50 & 500) and was substantially higher in Dex-treated mice than in untreated ones. This agreed with Certad *et al.* (2010a) who found that the higher the dose of inoculated oocysts, the more the shedding and that oocyst excretion increased according to inoculum size being substantially higher in Dex-treated mice than in the untreated ones. This differed from Miller *et al.* (2007) who found higher oocysts shedding with lower inoculation doses and suggested that inoculated doses raise the level of infectivity without shedding nor pathological outcome change.

In the present study, the more the dose of inoculation, the more the oocysts shedding, with the highest shedding in GB3 (IS inoculated by 500 oocysts) and mean oocysts shedding of 306 oocysts. This also agreed with Benamrouz *et al.* (2012a).

In the present study, unintended death of mice showed a significant difference among the different groups, with the least survival was among GB3 (IS, inoculated regularly by 500 oocysts-34 days) and the maximal one was equally in GA1 & GB1 (negative controls- 64 days).

In the present results, changes of ileocecal

regions were associated with the *C. parvum*. In mice *C. parvum* developed in enterocytes of small intestine, colon and gastric but less often in duodenal locations (Mead, 2002).

In the present work, histopathologic examination showed that *C. parvum* developed predominantly in the ileocecal region and colon of both IC & IS mice. It was suggested that the ileum was favorable, with the biochemical conditions and the specific receptors that contributed to the *C. parvum* development (Verdon *et al*, 1998). Gookin *et al.* (2006) found that the exact location of *C. parvum* was the lamina propria of the ileum terminal. Certad *et al.* (2010a) found that *C. parvum* localization and histopathological changes were mainly localized to the ileocecal region. Abdou *et al.* (2013) found that the ileum terminal part was the heaviest site of infection in both IC & IS mice.

In the present study, non-infected healthy mice showed normal villous architecture with normal brush border and normal glandular architecture. The ileocecal region of the groups infected with oocysts either once or weekly showed profound effect in the form of villous shortening and atrophy, decrease in ratio of villous height to crypt length, goblet cell depletion, mucosal ulceration and infiltration of lamina propria with inflammatory cells mainly lymphocytes and eosinophils with diffuse loss of brush border microvillous surface area.

This was comported with Waters and Harp (1996) who found histopathologic changes ranged from partial to complete villous atrophy and inflammatory infiltrate attributed to cryptosporidiosis. These results also agreed with Gaafar (2012); Al-Mathal and Alsalem (2012) and Abouel-Nour *et al*. (2016) who reported villous shortening and villous atrophy with ulcerations associated with cryptosporidiosis. Heine *et al.* (1984) reported inflammation of the crypts in large intestine and McDonald *et al.* (1992) found cryptic hyperplasia. Enemark *et al.* (2003) reported stunting and fusion of villi, replacement of enterocytes by immature cells and eosino-

philia of lamina propria, small and large intestine mucosa severely damaged with villous contraction and epithelial layer was few or absent. Uhl *et al.* (2001) reported that in *C. parvum* the aural-pharyngeal polyps were pedunculated masses of glandular cystic structures lined by hyperplastic cuboidal to columnar epithelium with numerous parasites on the surface apical epithelial cells

The present results showed that as the inoculated dose increased, pathological changes were villous atrophy, ulcerations and severe inflammatory infiltrate with marked effect on intestinal mucosa structure. This agreed with Certad *et al.* (2010b) who found evidence of dysplasia in any organ associated with *C. parvum* that elevated load with the severity of ileocecal pathology. In the present study histopathologic changes didn't show a difference between IC and IS mice. This varied from Certad *et al.* (2007) who reported combination of *C. parvum* with Dex administration involved in causing significant histological changes. This could be due to weekly inoculated mice developed chronic irritation to both IC and IS mice.

In the present study, examination of the ileocecal region of negative and positive control groups didn't show any neoplastic changes or parasites. The ileocecal region of chronically infected groups showed neoplastic changes, which exhibited a marked proliferation of advanced neoplasms. The neo plastic changes ranged from mild dysplasia, polypoid structures, architectural distortion, glandular crowding and marked cellular atypia (appeared at 36-45 days post infection), to exophytic adenomatous polypi and intramuscular adenocarcinoma and marked nuclear anaplasia (appeared at 57-64 days post infection). The severity increased steadily with the increase in duration and inoculated oocysts. These agreed with Certad *et al.* (2010b) who reported intramucosal carcinoma developed with a suspicion of submucosal invasion in mice and intraepithelial adenocarcinoma in the *C. parvum* infected SCID mice. Benamrouz *et al.* (2012b) repor-

ted neoplastic lesions in Dex-treated SCID mice infected by C. *parvum*, whatever inoculum was? The present data were in divergence with Abdou *et al.* (2013) who found that dysplastic changes were mainly of low-grade category, and few cases showed high-grade dysplasia, without frank carcinoma.

Conclusion

The current work reported neoplastic lesions in IC mice chronically irritated by weekly oocysts inoculation to maintain infection and subsequent intestinal irritation that led to dysplastic and neoplastic changes. The results proved that *Cryptosporidium* oocysts had risky power on immunosuppressed and immunocompetent mice after repeated infection caused pathological changes in ileocecal region even with low oocysts dose inducing neoplastic changes by chronic irritation.

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Explanation of figures

Fig. 1: *Cryptosporidium* oocysts (red spots), stained by MZN,

Fig. 2: Micrometer to measuring oocysts, they measured 4-6 um in diameter.

Fig. 3: Box plot showing fecal oocyst shedding in groups. Markers represent individual observations. Box represents range from 1st quartile to 3rd quartile. Line inside box represents median (2nd quartile). Error bars represent minimum and maximum values.

Fig. 4: Bar chart showing dose inoculated versus shedded oocysts number, with a significant correlation between shedded oocysts and number of inoculated oocysts indicating that more \dose caused more oocysts shedding, with an r value of +0.417, and a p value of 0.005

Fig. 5: Graphic presentation of mean day of death among studied groups

Fig. 6: Section of small intestine from a mouse euthanized at 36 days post-infection in group (B4) (infected control group) showing *Cryptosporidium* oocysts (tiny purple stained structures) in the intestinal lumen and on the mucosal brush border (red arrows) with shortened broad villi (H&E stain x 1000 "oil immersion").

Fig. 7: Exophytic adenomatous polyp from a mouse euthanized at 57days post-infection; showed an increasing architectural distortion and glandular crowding (H&E stain x 100).

Fig. 8: High power view of adenomatous polyp showed an increasing architectural distortion, glandular crowding, marked cellular atypia with large nuclei showing prominent nucleoli (H& E stain x 400).Fig. 11: Intramucosal adenocarcinoma showed marked cytological anaplasia with large nuclei and prominent nucleoli. Frequent mitoses detected (black circles) (H&E stain x 1000 "oil immersion").

Fig. 9: Ileocecal region from a mouse euthanized at 64 days post-infection revealed intramucosal adenocarcinoma with marked architectural atypia and marked glandular crowding (star) (H&E stain x 200).

Fig. 10: High power view of previous section revealed intra-mucosal adenocarcinoma with marked nuclear anaplasia showing large nuclei with prominent nucleoli (black circles) and increased mitotic activity (black arrows) (H&E stain x 400).

Fig. 11: Intramucosal adenocarcinoma showed marked cytological anaplasia with large nuclei and prominent nucleoli. Frequent mitoses detected (black circles) (H&E stain x 1000 "oil immersion").

Fig. 12: Multiple polypoid structures (shown by arrows) developing in intestinal lumen from a mouse euthanized at 45 days post-infection by a slightly modified mucosal architecture in group (A2) (H&E stain x100).

Fig. 13: A) Adenomatous polyp from a mouse euthanized at 64 days post-infection showing slightly distorted glandular architecture (H&E stain x100). B) Occasional large nuclei showing prominent nuclei, few scattered mitotic figures and containing minimal mucin compared to surrounding mucosa (A3) (H&E stain x400).

F. 6

F. 7

F. 8

F. 9

F. 10

F. 11

F. 12

A

B

F. 13