رقم البحث (21)

STUDY ON ROLE OF CATTLE IN TRANSMISSION OF *CRYPTOSPORIDIUM PARVUM* AND *ENTAMOEBA* SPECIES TO MAN WITH SPECIAL REFERENCE TO ITS MOLECULAR CHARACTERIZATION

BY

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ABSTRACT

This study was conducted to clarify the role of the cattle in transmitting *Cryptosporidium parvum* and *Entamoeba* species to man in Egypt. A total of 701 fecal samples and stool specimens were collected from different cattle and human beings, then were parasitological examined for these protozoa cysts/oocysts. The results showed that overall percentage of *Cryptosporidium parvum* and *Entamoeba* spp. was (45.34% and 55.42%) in cattle and (34.76% and 37.24%) in the examined human samples, respectively. Concerning age, higher incidence of *Cryptosporidium parvum* was detected in cattle < 6 months old and children and of *Entamoeba* spp. in cattle 6-12months and children. In regard to healthy state, overall results showed that infection rate of *Cryptosporidium parvum* and *Entamoeba* spp. were (40.70% and 55.75%) and (48.96% and 55.17%) in diarrhoeic and apparently healthy cattle, respectively, while in human the respective percentages were (39.08 and 35.53) and (31.30 and 38.61). In human, there is a slight increase of cryptosporidiosis and amoebiasis infection in males (37.39% and 39.07%) as compared to females (31.70% and 35.12%), respectively. *Cryptosporidium parvum* and *Entamoeba* spp. were observed more frequently in stool specimens of human who lived in rural areas (37.63% and 39.37%) than those who lived in urban areas (29.48% and 33.33%). With respect to contact with animals, the infection rate of *Cryptosporidium parvum* and *Entamoeba* spp. was 37.50% and 29.80% among human who lived in association with animals in comparison with 33.92% and 39.52% among those who lived without contact with animals. The molecular characterization showed

that all 9 human and animal fecal samples parasitological positive for *Cryptosporidium* oocysts, were successfully amplified firstly by PCR at 550 bp then were amplified by nested PCR and showed specific bands at 311 bp of *Cryptosporidium parvum*. While 4 samples (2 human beings and 2 animal samples) out of 8 human and animals parasitological positive for *Entamoeba histolytica* shown specific band at 135 bp of *Entamoeba* histolytica. In conclusion, the results of this study indicate high percentages of *C. parvum* and *Entamoeba* spp. in cattle and human of examined areas of Egypt. Moreover, the present study revealed same genotype of *C. parvum* and *Entamoeba histolytica* in both animals and human, suggesting its zoonotic significance and these genotypes are transmissible from cattle and man.

INTRODUCTION

Animals and their products are considered as an important source of infectious protozoal pathogens for humans. Acute diarrhoea is one of the most common childhood illnesses in both developing and developed countries. Gastrointestinal parasites are the main causes of diarrhoea in human and animals in these countries (**Mas et al., 2006**). The most important parasitic causes of acute diarrhea are the intestinal protozoa, of which *Entamoeba histolytica* and *Cryptosporidium parvum* (**Okhuysen, 2001**) were of special interest. In Egypt, domestic animals, living in intimate contact with man in rural areas, constitute a high risk for transmission of infection with these protozoal agents to man (**Ahmed et al., 2010)**. These protozoa are of public health concern as they may cause infection and severe illness in human. Infections are mostly self limiting in people with normal immune system but infection can be life threaten in people who have compromised immune system (**Gabriela et al., 2005**). *Cryptosporidium* infection of livestock may have an important economic impact on farmers capital cost because of high morbidity and sometimes high rates of mortalities of his live stock. Amoebiasis is defined as an intestinal or -may suffer from a wide range of symptoms including diarrhea, fever, and cramps. The disease may also affect liver as well as some other organs of the body. Molecular epidemiology have been conducted to differentiate species and genotypes of *Cryptosporidium* to understand the disease transmission and zoonotic implication of infection and assist in the identification of the host specificity and the contribution of humans and livestock as reservoirs of infection (**Caccio et al., 2005**).Therefore, the aim of this study was to investigate the role of cattle in transmission of *Cryptosporidium parvum* and *Entamoeba* spp. to man by determination the occurrence of both parasites in cattle and man, and molecularly identification of these parasites.

MATERIAL AND METHODS

1- **Samples collection:** A total of 701 fecal samples and stool specimens were collected from different cattle and human at Dakahlia province, Egypt of which 258 cattle (113 diarrhoeic and 145 apparently healthy) animals. Regarding human, 443 stool specimens including 292 children, 63 adolescent and 88 adult stool samples attending Mansoura University hospitals, of which 197 were diarrhoeic and 246 were apparently healthy individuals and parasitological examined for *Cryptosporidium parvum* and *Entamoeba* species oo (cysts).

2- **Parasitological examination:** The collected fecal animal and human samples were subjected for parasitological examination by direct smear **(Beaver et al., 1989)**, Formalin-ether sedimentation technique **(Ichhpujani and Bhatia, 2002)**, Sheather's sugar floatation technique **(Dubey et al., 1990)** and Modified Ziehl Neelsen stain **(Dubey et al., 1990)**.

3- **Molecular characterization:** A total of 17 parasitological positive samples (9 *Cryptosporidium* species and 8 *Entamoeba* species) were subjected to PCR amplification.

A- Genomic DNA extraction: Genomic DNA of each enteric protozoon was extracted from stool or fecal samples according the method previously reported by **Foley et al. (1992)** with some modification. Briefly, about 1 gm of feces was added to 10 ml sterilized distilled water, vortex, and spun for 2 min at 14,000 rpm. The supernatant was discarded, and washing was repeated twice. Then, 100 µl distilled water was added to the washed pellet, and heated in heat block apparatus for 15 min. Finally, the sample was spun for 10 min at 14,000 rpm; the supernatant containing the eluted DNA was stored at – 20 ºC until used for PCR. The extracted DNA was preliminary tested by gel-electrophoresis and the concentration of each sample was measured by using of spectrophotometer (JENWAY, United Kingdom).

B- **Polymerase Chain Reaction for identification of *Cryptosporidium parvum*: -** The *Cryptosporidium* oocyst wall protein (COWP) gene of *Cryptosporidium parvum* was amplified by two-step nested PCR protocol. Amplification of a PCR product of 550bp was done with forward primer Cry-15 (5′- GTAGATAATGGAAGAGATTGTG- 3′) and the reverse primer Cry-9 (5′- GGACTGAAATACAGGCATTATCTTG-3′) (**Spano et al., 1997**). The second amplification step, the PCR nested primers COWP nest- F1 (5′- TGTGTTCAATCAGACACAAGC- 3′) and COWP nest R2 (5′- TCTGTATATCCTGGTGGGC - 3′) (**Yu et al., 2009**), an amplicons of 311 bp was produced in this reaction.

C- **Polymerase Chain Reaction for identification of *Entamoeba* spp.:** The small subunit ribosomal (SSU) rRNA gene of *Entamoeba histolytica* was amplified by a conventional protocol. Amplification of a PCR product of 135bp was done with forward primer EH1 (5′- GTACAAAATGGCCAATTCATTCAATG- 3′) and the reverse primer EHD2 (5′- ACTACCAACTGATTGATAGATCAG - 3′) (**Clark and Diamond, 1991**). The PCR reaction was performed according to (**Gonion and Trudel, 2003**).

D- **Visualization of the amplified PCR products: -** After amplification, 15 µl of each amplified DNA fragments (PCR products) were analyzed by electrophoresis. Amplified products were visualized using ultraviolet transilluminator.

RESULTS AND DISCUSSION

Cryptosporidium parvum infection is globally recognized illness in both human and animals. In the present study, 117 out of 258 (45.34%) of the investigated cattle were confirmed by Modified Ziehl-Neelsen stain to be infected with *Cryptosporidium* oocysts (Table 1). These results were nearly similar to results (41%) previously recorded by **Lassen (2011)**. Concerning age, higher occurrence of *Cryptosporidium parvum* in our study (50.36 and 48.83%) was detected in cattle less than 6 months and 6-12 months old respectively, however lower occurrence (17.14%) in cattle more than 12 months old was observed (Table 1). These results were nearly similar to results recorded by **Olson et al. (1997)** who noticed that 59% of dairy calves up to 6 months of age were shedding *C. parvum* oocysts. In regard to healthy state, overall results showed that apparently healthy cattle had higher rate of *C. parvum* infection (48.96%) than diarrhoeic ones (40.70%) (Table 1). Concerning the occurrence of *Entamoeba* spp. in cattle was 55.42% (143 out of 258). Nearly similar results were previously recorded by **Rasha (2012)** who detected *Entamoeba* spp. in animal samples at a percentage of 54.38 %. In relation to age, it is obvious that the percentage of *Entamoeba* spp. was 56.20, 62.79 and 34.28 in cattle less than 6 months, 6-12 months and more than 12 months, respectively. Nearly similar findings were recorded by **Rasha (2012)** who found that *Entamoeba* spp. was 55.3% in cattle less than 6 months, while, the percent of 74.8% in cattle ranging from 6 to 12 months old age was higher than the present finding. In regard to healthy state, *Entamoeba* spp. was detected in the diarrhoeic and apparently healthy cattle with percentages of 55.75 and 55.17, respectively. Nearly similar results were recorded by **Rasha**

(2012) who identified *Entamoeba* spp. in diarrhoeic and apparently healthy calves with the percentage of 66.3% and 68.7%, respectively. Cryptosporidiosis is a common gastrointestinal disease in man, and it has been recognized worldwide as a common cause of diarrhea. The disease is widespread in many developed and developing countries **(Chalmers and Casemore, 2003)**. The infection rate of *Cryptosporidium parvum* in human stool samples was 34.76% (154 out of 443) (Table 1). Nearly similar percentage (35.7) was recorded by **Muñoz-Antoli et al. (2011)**. On the other hand, the lower prevalence of 3.6% was previously recorded by **Samn et al. (2012)**. While the occurrence of *Entamoeba spp.* in human stool was 37.24% (165 out of 443). Nearly similar prevalence (35-41%) was previously reported by **Reynoldson et al. (1997)**. Regarding the occurrence of *Cryptosporidium parvum* in human with respect to age, the overall occurrence was 36.98, 34.92 and 27.27% in children, adolescents and adults, respectively (Table 2). Nearly similar result **(**35.7%) was recorded in children by **Muñoz-Antoli et al. (2011)**. It is clearly obvious from aforementioned results that the highest infection rate of *Cryptosporidium parvum* was in children. This result is in agreement with **Samn et al. (2012)**. While the overall infection rates of *Entamoeba histolytica* were 40.75, 23.80 and 35.22% in children, adolescents and adults, respectively. Nearly similar results were 41% in age group of 6:14 years recorded by **Subbannayya et al. (1989).** Table (2) revealed that the overall occurrence of *Cryptosporidium parvum* was 39.08% in diarrhoeic persons compared to 31.30% in apparently healthy persons. Higher prevalence rate (96.8%) with *Cryptosporidium parvum* among diarrhoeic persons was previously recorded by **Mclauchlin et al. (1999)**. On the other hand, lower prevalence (11.6% and 4.0%) of *Cryptosporidium parvum* among diarrhoeic and apparently healthy persons were previously recorded by **Moghaddam (2007)**, respectively. Also table (2) revealed that the occurrence of *Entamoeba* spp. was 35.53% and 38.61% in diarrhoeic and apparently healthy persons, respectively. Nearly similar respective results (34% and 66%) were previously obtained by **Saeed et al. (2011)**. Moreover, nearly similar prevalence (39.8%) with *Entamoeba histolytica* among apparently healthy persons was previously recorded by **Verweij et al. (2003)**. In the present study, there is a slight increase of cryptosporidiosis infection in males (37.39) as compared to females (31.70%) (Table 2). This result is in agreement with the results reported by **Nevine et al. (2012)** and **Samn et al. (2012)**. The higher infection rate of *Entamoeba* spp. was in males (39.07%) than females (35.12%). These results agree with other result recorded by **Ibrahim (2012)**. The prevalent of cryptosporidiosis among the rural communities, may be due to governorates where they are always in close contact with farm, companion and wild

animals **(Miron et al., 1991 and Shehata et al., 1997)**. In the present study, the percentage of *C. parvum* in rural area (37.63%) was higher than in urban areas (29.48%), this is in agreement with the result reported by **Nevine et al. (2012)**. This could be due to the social habits of the rural people in which keep the animals in their houses. Also higher infection rate of *Entamoeba* spp. was in rural area (39.37%) than urban area (33.33%). This may be due to domestic animals living in close contact with man in rural areas may have a great opportunity to ingest cysts of *E. histolytica*. The possibility of occasional human infection from infected animals can not be neglected **(WHO, 1979)**. The results showed that the percentage of *Cryptosporidium parvum* in human with contact animal and without animal contact was 37.50 and 33.92%, respectively (Table 2). In the present study the higher infection rate of *Cryptosporidium parvum* in human with contact animal agree with **Goh et al. (2004)**. While percentage of *Entamoeba* spp. in human contact with animal (29.80%) was lower than in human without animal contact (39.52%). The application of molecular characterization of *Cryptosporidium* isolates in field of epidemiological studies is the most reliable approach for proper diagnosis and establishing of the source of cryptosporidiosis in human infections. This is because definitive identification of the species of *Cryptosporidium* parasite has not been possible by using the reliable routine diagnostic methods **(Xiao and Ryan, 2008)**. Photograph (1) shows amplification of *Cryptosporidium* oocysts wall protein (COWP) gene of *Cryptosporidium parvum*. A total of 9 parasitological positive fecal samples for *Cryptosporidium parvum* (5 from human and 4 from animals) were firstly amplified by PCR, then by nested PCR. In the present study, all 9 human and animal fecal samples were successfully amplified firstly by PCR at 550 bp (data not shown), then were amplified by nested PCR and showed specific bands at 311 bp of *Cryptosporidium parvum*. This result agrees with other results reported by **Yu et al. (2009) and Ahmed et al. (2010)**. Photograph (2) shows amplification of small subunit (SSU) rRNA gene of *Entamoeba histolytica*. A total of 8 parasitological positive fecal samples for *Entamoeba spp.* (5 from human and 3 from animals) were subjected to amplification by PCR. *Entamoeba histolytica* gene was identified in fecal samples of 4 out of 8 human and animals (2 human and 2 animal samples) and were shown specific band at 135 bp of Entamoeba histolytica, suggesting its zoonotic importance. PCR amplification of the small subunit rDNA of *E. histolytica* agree with **Verweij et al. (2003)**. It could be concluded that, higher prevalence of *Cryptosporidium parvum* and *Entamoeba histolytica* in animals and human in the examined areas in Egypt was recorded, its presence in animals represent a zoonotic risk. Higher occurrence was observed in young and

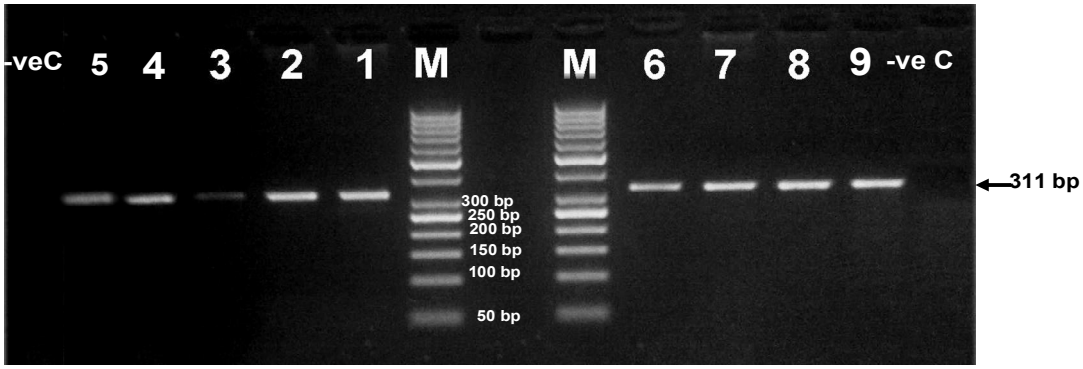
diarrheic animals and humans. The same genotypes of *Cryptosporidium parvum* and *Entamoeba histolytica* found in both animals and man in the examined areas in Egypt, suggest its zoonotic significance. For public health safety, rearing animals under hygienic condition, periodical parasitological examination, health education for animal contacts, hygienic disposal of sewages, avoid fecal contamination of food and water and diarrheic animals and humans must be differentially diagnosed for *Cryptosporidium parvum* by Modified Ziehl-Neelsen stain.

Table (1): Occurrence of *C. parvum* and *Entamoeba* spp. in cattle fecal samples**.**

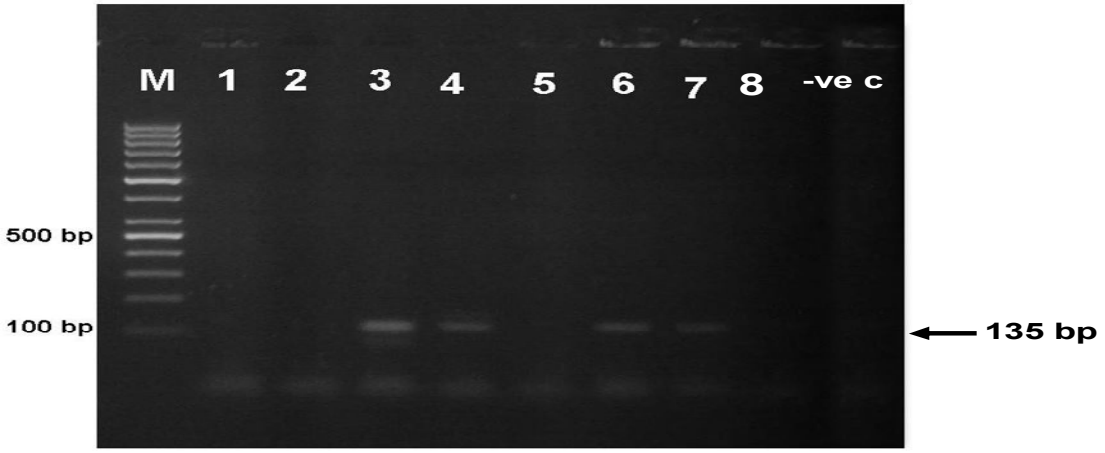
Factor	No. of examined cattle	*Cryptosporidium parvum*		*Entamoeba* spp.	
		No. of positive	%	No. of positive	%
Overall	258	117	45.34	143	55.42
Age					
Less than 6 months	137	69	50.36	77	56.20
6-12 months	86	42	48.83	54	62.79
More than 12 months	35	6	17.14	12	34.28
Healthy state					
Diarrhoeic	113	46	40.70	63	55.75
Apparently healthy	145	71	48.96	80	55.17

Table (2): Occurrence of *C. parvum* and *Entamoeba* spp. in human stool specimens.

Factor	No. of examined samples	*Cryptosporidium parvum*		*Entamoeba* spp.	
		No. of positive	%	No. of positive	%
Overall	443	154	34.76	165	37.24
Age					
Children	292	108	36.98	119	40.75
Adolescents	63	22	34.92	15	23.80
Adults	88	24	27.72	31	35.22
Healthy state					
Diarrhoeic	197	77	39.08	70	35.53
Apparently healthy	246	77	31.30	95	38.61
Sex					
Male	238	89	37.39	93	39.07
Female	205	65	31.70	72	35.12
Residence					
Rural	287	108	37.63	113	39.37
Urban	156	46	29.48	52	33.33
Animal contact					
With	104	39	37.50	31	29.80
Without	339	115	33.92	134	39.52



Photograph (1): Nested-PCR results of amplification of (COWP) gene of *Cryptosporidium parvum* oocyst obtained from human and animal fecal samples. 50 bp DNA ladder marker (M), lane 1, 2, 3, 4 & 5: positive human samples; lane 6, 7, 8 & 9: positive animal samples; lane (-ve C): negative control. Specific base pair bands of *Cryptosporidium parvum* at 311 bp were successfully amplified.



Photograph (2): PCR results of amplification of (SSU) rRNA gene of *Entamoeba histolytica* cyst obtained from human and animal fecal samples. 100 bp DNA ladder marker (M), lane 3 & 4: positive human samples; lane 6 & 7: positive animal samples; lane 1, 2 & 5: negative human samples; lane 8: negative animal sample and lane (-ve C): negative control. Specific base pair bands of *Entamoeba histolytica* at 135 bp were successfully amplified.

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الملخص العربي

دراسه على دور الماشيه فى نقل الكريبتوسبوريديوم بارفم وانواع الانتاميبا للإنسان مع الإشاره إلى توصيفهم الجزيئى

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قد أجريت هذه الدراسة لتوضيح دور الماشية فى نقل الكريبتوسبوريديوم بارفم وانواع الانتاميبا للإنسان فى مصر، تم جمع ٧٠١ عينه من براز وروث الإنسان والماشية المختلفة وتم الفحص الطفيلى لهما. أظهرت النتائج أن النسبه الإجماليه للكريبتوسبوريديوم بارفم وانواع الانتاميبا كانت (٤٥,٣٤٪ و٥٥,٤٢٪) فى الماشية و (٣٤,٧٦٪ و ٣٧,٢٤٪) فى الإنسان، على التوالى. فيما يتعلق بالعمر،تم الكشف عن إرتفاع فى عدد حالات الكريبتوسبوريديوم بارفم فى الماشية ٦ اشهر والأطفال أما انواع الانتاميبا فكانت النسبه الأعلى فى الماشية ٦-١٢ شهر والأطفال. فيما يتعلق بالحاله الصحيه، أظهرت النتائج الإجماليه ان معدل الإصابة بالكريبتوسبوريديوم بارفم وانواع الانتاميبا كانت (٤٠,٧٠٪ و ٥٥,٧٥٪) و (٤٨,٩٦٪ و ٥٥,١٧٪) فى الماشية المصابة بالإسهال والسليمه ظاهريا، على التوالى فى حين كانت النسب المؤية فى الإنسان (٣٩,٠٨٪ و ٣٥,٥٣٪) و (٣١,٣٠٪ و ٣٨,٦٨٪). كما وجد أن هناك فى الإنسان زيادة طفيفه للإصابه بالكريبتوسبوريديوم بارفم وانواع الانتاميبا فى الذكور (٣٧,٣٩٪ و ٣٩,٠٧٪) بالمقارنه مع الإناث (٣١,٧٠٪ و ٣٥,١٢٪) على التوالى. ولقد لوحظت أيضا الكريبتوسبوريديوم بارفم وانواع الانتاميبا وجدت بشكل أكثر تكرارا فى عينات البرازللبشر الذين يعيشون فى المناطق الريفيه (٣٧,٦٣٪ و ٣٩,٣٧٪) من أولئك الذين يعيشون فى المناطق الحضرية (٢٩,٤٨٪ و ٣٣,٣٣٪). فيما يتعلق بمخالطه الحيوانات، وجد أن معدل الإصابه بالكريبتوسبوريديوم بارفم وانواع الانتاميبا كان (٣٧,٥٠٪ و ٢٩,٨٠٪) بين المخالطين للحيوانات بالمقارنه مع (٣٣,٩٢٪ و ٣٩,٥٢٪) بين أولئك غير المخالطين للحيوانات. أظهر التوصيف الجزيئي تواجد الجين المسئول عن بروتين جدار حويصلات الكريتوسبوريديم المميز لطفيل الكريتوسبوريديم بارفم في ٩ عينات من الإنسان والحيوان وكان الوزن الجزيئي للجين الناتج ٥٥٠ زوج قاعدي فى التفاعل الأول و ٣١١ زوج قاعدي فى التفاعل الثاني. بينما تواجد جين المميز لطفيل الانتاميبا هستوليتيكا فى ٤ معزولات انتاميبا (٢ من روث الحيوانات و٢من براز الإنسان) وكان الوزن الجزيئي لها ١٣٥زوج قاعدي. مما سبق، يستخلص أن الانتاميبا هيستوليتيكا والكريتوسبوريديم بارفم قد تم تسجيل تواجدهم فى عينات براز الأبقار و الإنسان، بمصربمنطقه الدراسه بنسبة عالية. كما أثبتت الدراسه وجود نفس الجين لهذه الطفيليات فى كل من الحيوانات والإنسان فى المنطقة تحت الفحص مما يؤكد أهميتها من وجهة الأمراض المشتركة وكذلك قدرة هذه الأوليات على الإنتقال من الحيوان إلى الإنسان والعكس.