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WATER-BORNE CRYPTOSPORIDIUM PARVUM OOCYSTS CAUSING LIFE-THREATENING INFECTION OF BUFFALOES AND SHEEP IN EGYPT

(With 2 Tables and One Figure)

By

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حويصلات الكريبتوسبوريديم الملوثة للمياه تسبب العدوى المهدده لحياة الجاموس والأغنام في مصر

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في هذه الدراسة تم استخدام البيولوجية الجزيئية للكشف عن حويصلات الكريبتوسبوريديم في عينات مختلفة من المياة على النحو التالي : 10 عينات من مجرى مائي يستحدم لري الاراضي الزراعية باحدى قرى اسيوط بالاضافه الي 12 عينة من كل من مياة الابار واخرى من مياة الصنابير وكذلك في عدد 74 عينة براز تم جمعها من عدد 74 من العجول الجاموسي والامهات والحملان والنعاج والتي كانت تعاني من إسهال شديد مع خمول وجفاف إلى حد ما مع ملاحظة عدم ارتفاع في درجة الحرارة. وقد تم إحضار هذه العجول إلى المستشفى البيطري التعليمي بجامعة أسيوط للتشخيص والعلاج. و بفحص عينات البراز با ستخدام البيولوجية الجزيئية فقد وجدت حويصلات الكريبتوسبوريديم في 8 عينات و 7 عينات في براز ال عجول الجاموسي وامهاتها بنسبة 57.14%و 46.66% على التوالي، بينما كشف الفحص عن وجود الحويصلات في عدد 17 حمل و 6 نعاج بنسبة 56.66% و 40% على التوالي ، أما فحص عينات المياة فقد كشف عن وجود الحويصلات في 7 عينات من المجرى المائى بنسبة 70% و 7 من مياة الابار بنسبة 58.33% و5 من مياة الصنابير بنسبة 41.66%. وقد تم إجراء هذا البحث لتقييم الحالة الصحية للمياة فى احدى قرى محافظة اسيوط وعلاقتها بمرض الكريبتوسبوريديوزيز وقد خلصت النتائج إلى أن مياة الشرب بمحافظة اسيوط تمثل مشكلة كبرى للحيوان والانسان على السواء بما تحتويه من حويصلات ولذا نوصى بمعالجة المجاري المائية التى تستخدم في الري وكذلك مياة الشرب في القرى بمركبات اقوى تاثيرا على حويصلات الكريبتوسبوريديم من الكلور مع الارشاد البيطري المكثف للفلاح عن خطورة مرض الكريبتوسبوريديوزيز ومقاومته ومنع المضاعفات التي قد تنتج عن الإصابة به في الحيوان والانسان والذي يؤدي إلى خسارة اقتصادية كبيرة.

SUMMARY

This study was carried out in a village at Assiut Governorate, Egypt to detect the prevalence of *Cryptosporidium parvum* in different water samples that affecting the health condition of buffaloes and sheep. A total of 34 different types of water samples and 74 fecal samples from water-buffaloes and sheep were tested for *Cryptosporidium parvum* oocysts using PCR techniques. *Cryptosporidium parvum* were detected in local surface water, dug-well and tap-water in percentage of 70 %, 58.33 % & 41, 66 %, respectively, with overall percentage of 55.88%. Moreover, our study showed that the overall percentage of *Cryptosporidium parvum* among buffaloes and sheep in Assiut province, Egypt were 51.77% and 51.11%, respectively. Moreover, the results showed that the percentage of *Cryptosporidium parvum* oocysts in adult ewes and lambs were 40.00 % and 56.66%, respectively and adult female buffaloes and buffalo's calves were 46.66% and 57.14 %, respectively.

Key words: *Cryptosporidium, water, buffaloes, sheep, fecal matter, PCR.*

INTRODUCTION

In recent years, there has been a dramatic increase in the incidence of waterborne disease outbreaks caused by the *Cryptosporidium oocysts*. This parasite is increasingly recognized as important agents that causes enteric infection in man and many species of domestic animals (Chalmers *et al.,* 2002). *Cryptosporidium* parasite is most common in young livestock and a high morbidity and mortality rates has been reported as enteropathogenic being associated with neonatal diarrhea in ruminants (Angus, 1990 and de-Graaf *et al.*, 1999). Although the fecal-oral route is the main infection mode, this parasite is frequently transmitted via water (Casemore *et al.,* 1997; Graczyk *et al.,* 1997a and Rose, 1997), and is considered as the most important biological water contaminants in the United States (Rose *et al.,* 1997). A nationwide recent survey in the United States reveled that 55% of surface water samples from 17 states were positive to *Cryptosporidium spp*., while only 17% of 36 drinking water samples was positive for *Cryptosporidium* oocysts (Rose *et al.,* 1991). In addition, Rose (1997) reported that 77% of 107 samples from the western United States contained *Cryptosporidium* oocysts. In Japan, Hashimoto *et al.* (2002) detected the oocysts of *Cryptosporidium* in all of the 13 river water samples, while only 35 % of filter water samples were positive for *Cryptosporidium* oocysts.

Outbreak in ruminants is frequently parasitized with Cryptosporidium species which cause diarrhea and impair gain of body

weight. Cryptosporidium parvum in cattle is potential zoonotic pathogens for human and due to increase incidence of *Cryptosporidiosis* in cattle; it is important to through light on the role of water-buffaloes and sheep as the source of waterborne outbreaks of Cryptosporidiosis. *Cryptosporidium* infections have been considering causes of disease and production losses in calves, particularly the effects of subclinical and chronic infections (Olson *et al.,* 2004).

There are many current methodology for diagnosis of cryptosporidiosis as using modified Ziehl-Neelsen (acid-fast) staining or immunofluoresce assay in water samples as well as in fecal matter but these methods is time consuming, labour intensive, and is subject to false positive results (LeChevallier *et al.,* 2003). So, we use polymerase chain reaction (PCR) based method as has the potential to address many limitations of the current methods. PCR has many advantages include specificity, greater sensitivity and more rapidity (Kaucner and Stinear, 1998).

The objective of this study was to examine adult buffaloes, buffaloes- calves, ewes and lambs and some water sources in a village located in Assiut Governorate, Egypt, after many complains of diarrhea in the newly born buffaloes-calves that recorded in this village to determine the extent of *Cryptosporidium* infection using molecular technique.

MATERIALS and METHODS

1. Animals:

1.1. Animals and data collection

A total number of 14 buffalo-calves (1-3 month old age) and 30 lambs (3 week-2 month old age) as well as 15 of both adult buffaloes (from 5 to 8 years) and ewes (about 3 year old age) at a private small scale herds in Arab-Motear village at Assiut governorate, Egypt were selected with case history of suffering from acute or chronic diarrhea. A questionnaire was done about herd profiles management, general health history, presence of other animals. Some of these animals came to Veterinary teaching hospital, Assiut University for diagnosis and treatment from diarrhea with case history of grazing in the vicinity of local surface water streams used for irrigation of cultivated land. The animals were subjected for clinical examination including body temperature, mucous membranes and degree of dehydration.

2. Water Samples and sampling preparation:

10 water samples from different sites of small local water branch in Arab-Moteer village, derived from Ibrahimia canal (a branch of river Nile) and used for irrigation of cultivated lands, in addition to 12 water samples from dug-wells inside this village and 12 tap water samples were collected.

2.1. Isolation of Cryptosporidium parvum DNA from water samples

Water samples ranging in volume from 40 to 50 liter were filtered as described previously by Aldom and Chagla (1995) and the processed method for preparing water samples prior to PCR amplification as described to Kostrzynska *et al.* (1999). The pellets retained on filters were washed 3 to 4 times and suspended in 10 ml of water. Aliquots (1 ml) from each sample were mixed with 9 ml of PBS–Tween and filtered through sterile 44-mm and then 20-mm nylon filter cloth, followed by centrifugation at 10000 *g* for 15 min. Pellets were resuspended in 10 ml PBS containing 0.1% (w/v) bovine serum albumin (PBS– BSA) and oocysts were purified using an immuno-magnetic separation (IMS) procedure as the following description according to Lowery *et al.* (2000). Filtered water samples were resuspended in 10 ml PBS–BSA, mixed with 100 µl of coated beads and incubated for 1 h at 22^{o}C. The oocysts–magnetic particle complexes were separated from the suspension using a magnetic particle concentrator and washed with PBS. The antibody–oocysts complexes were disrupted by vigorous vortexing for 1 min and beads removed using magnetic separation. *Cryptosporidium* oocysts were concentrated by centrifugation at 14 000 *g* for 15 min. Pellets were resuspended in 50 µl of water and DNA was released from oocysts by ten freeze–thaw cycles. Following centrifugation at 14 000 *g* for 15 min, supernatants were used as templates in PCR.

3. Isolation of C. parvum DNA from fecal samples

Fecal specimens were collected from the rectum of all selected animals and were prepared according to Johnson *et al.* (1995). Fecal samples stored in 2.5% potassium dichromate were washed several times (4 times) with PCR buffer (10mM Tris, 50 mM K Cl, 3.5 mM Mg Cl_2) by centrifugation. A 20% of Chelex 100 (Bio-Rad Lab., Calif.) was mixed with fecal samples (20 µl of chelx stock to 100µl of samples). Then, samples were subjected to six cycles of freezing and thawing to release the DNA from oocysts using dry ice-ethanol bath for freezing and water bath (98 ^{o}C) for thawing with incubation time from 1 to 2 minutes in each bath. Lastly, samples were centrifuged and 50 µl of supernatant was used as the template in the PCR assay.

4. DNA amplification

The primer set described by Laberge *et al.* (1996) was used in PCR which specific for *Cryptosporiudium parvum*. Sequences of primers were as follows: forward — 5`GCC CAC CTG GAT ATA CAC TTT C3`; reverse — 5`TCC CCC TCT CTA GTA CCA ACA GGA 3`. The size of the amplified product was 358 bp. The PCR mixture contained PCR reaction buffer (10 mM Tris–HCl, 1.5 mM MgCl , 50 mM KCl, pH 8.3) and contained 1.0 mM each of forward and reverse primers, 0.2 mM each dATP, dGTP, dCTP and dTTP, 100 mg/ml BSA and 2.5 U Taq DNA polymerase (Boehringer Mannheim Canada, Laval, Que´bec, Canada). 10 µl of template DNA was added in the case of fecal samples and 30 µl in the case of filtered environmental water pellet suspensions. Reaction mixtures were initially denaturated at 94 ^{o}C for 1 min, and then subjected to 40 cycles of denaturation at 94 ^{o}C for 15 s, annealing at 50^{o}C for 1 min and extension at 72 ^{o}C for 1 min, with an additional 7-min extension at 72 ^{o}C.

5. Detection of PCR products

The amplification products were identified by running 20 µl of the PCR reaction mixture in 2 % agrose gel in Tris acid EDATA buffer. Gel was run at 100 V for 1 Hour, stained with ethodium bromide solution for 30 minutes and PCR product bands visualized under UV light. The expected PCR product is 358bp.

RESULTS

Table 1: Incidence of Cryptosporidium parvum oocysts from different collected water samples.

Types of samples	Surface water samples	Dug-well water	Tap-water
Samples number	10	12	12
Positive samples	7	7	5
Percentage of positive samples	70%	58.33%	41,66%
Overall percentage	55.88%		

Table 2: Incidence of Cryptosporidium parvum oocysts from fecal specimens of buffaloes and sheep.

Types of samples	Adult buffaloes	Buffaloe`s calf	Adult ewes	Lambs
Samples number	15	14	15	30
Positive samples	7	8	6	17
Percentage of positive samples	46.66%	57.14%	40.00%	56.66%
Overall percentage	51.77%		51.11%	

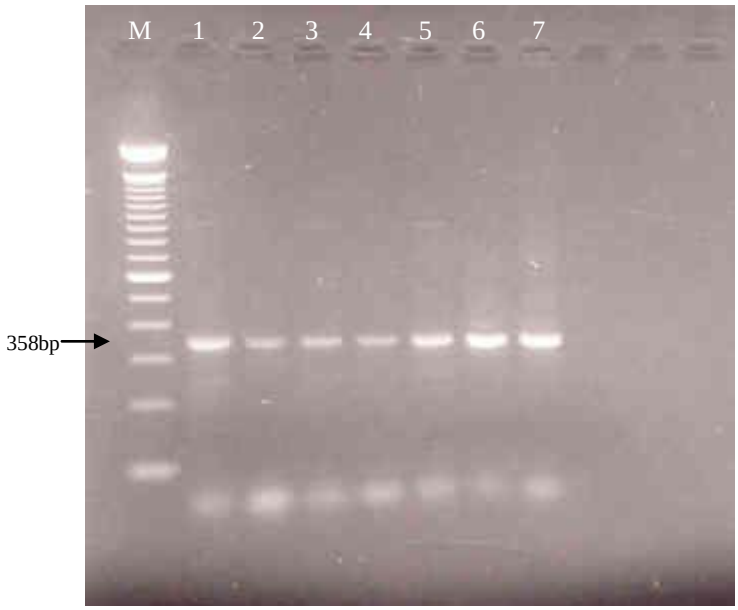

Figure 1: Sensitivity of the PCR assay for detection of Cryptosporiudium parvum as determined by 2 % agarose gel electrophoresis. M: 100 bp ladder, Lane 1: positive lambs, Lane 2: positive ewes, Lane 3: positive buffaloe calves, Lane 4: positive adult water-buffaloes, Lane 5: tap-water, Lane 6: dug-well water, Lane 7: positive surface water.

DISCUSSION

Cryptosporidium parvum is an opportunistic protozoan that is ubiquitous in its geographic distribution and range of vertebrate hosts. *C. parvum* causes diarrheal disease in human and several animal species known as *Cryptosporidiosis* (Johnson *et al.,* 1995). Transmission of the parasite is via oral ingestion of contaminated waters or food with the infective oocyst. Environmental *Cryptosporidium* contamination, especially contamination of drinking water, has not been much attention in Assiut.

Oocysts of *Cryptosporidium parvum* were detected in water samples and fecal specimens of buffaloes and sheep. The frequency of *Cryptosporidium parvum* oocysts appearance in water samples is shown in Table 1, a total of 34 water samples were examined using PCR for *Cryptosporidium parvum*, 19 of the them (55.88%) were positive overall, ranging from a high of 70% positive for local water stream, followed by

58.33% and 41.66% positive for dug-well and drinking tap water samples, respectively.

Our data indicate a relatively overall higher contamination rate of water with *Cryptosporidium oocysts* other than that of Johnson *et al.* (1995) who found that overall positive samples was 31.9%, ranging from a high of 54.2% positive for waste water to a low of 8.7% positive for costal waters. The data in Table (1) clear out that the incidence rate of *Cryptosporidium parvum* oocysts in drinking water either tap-water or dug-well water is higher than that recorded by Johnson *et al.* (1995) (16.7) and Rose *et al.* (1991) (17%). The variation in these results may be due to the difference of the sources that polluted the water and the extend of spread of that disease at the locality.

On contrary, our results of local water surface were lowered than that detected by LeChevallier *et al.* (1991) (87%), Rose, 1997 (77%)**,** Ongerth and Stibbs (1987) and Hashimoto *et al.* (2002) who detect the oocysts of *Cryptosporidium* in all examined river water samples.

Table 2, illustrated that the overall percentage of *Cryptosporidium parvum* among buffaloes and sheep in Assiut province, Egypt were 51.77% and 51.11%, respectively. Moreover, the table showed that the percentage of *Cryptosporidium parvum* oocysts in adult female buffaloes and buffaloe's calves were 46.66% & 57.14%, respectively and ewes and lambs were 40.00% & 56.66%, respectively. Our result was more or less coincided with Chalmers *et al.* (2002) who could detect *Cryptosporidium oocysts* from fecal samples of two- sheep flocks in the following percentage, 57% and 47%, from flock A and B, respectively, Chalmers *et al.* (1994) detected *Cryptosporidium spp*. in the faeces of unweaned lambs was 9% and in ewes 6% while Angus *et al.* (1982) detected 29/37 (78.37%) *Cryptosporidium spp*. in a flock of diarrhoeic lamb.

From the above mentioned data, we stated that the source of oocysts in outbreaks among buffaloes and sheep associated with surface and drinking water in the Assiut has been attributed to contamination by both animal faeces and human sewage (Anonymous, 1998) *as local farmers graze their animals (water buffaloes and sheep) near the shore of surface water streams*. The local residents use area near irrigated channels as open toilets and many housewives used it for disposal of waste-water and food refuse. Also, the high frequency of *Cryptosporidium in drinking water may be attributed to a connection of water source with human wastes as well as Cryptosporidium* is unaffected by many current water treatment techniques such as chlorination and, as there is no effective treatment for cryptosporidiosis (Korich *et al.,* 1990; Johnson *et al.,* 1995).

In conclusion, we would like to emphasize the need for continued development of rapid, sensitive and effective methods for monitoring the occurrence of Cryptosporidium oocysts in surface and municipal water. Good management and agricultural practices designed to minimize animal *Cryptosporidiosis*, however, can also minimize surface water contamination such as restriction the access of animals to surface water e.g via use of electric wire. Additional management practices that can decrease water contamination include containment of manure by creation of lagoons for manure decomposing or disinfection of raw manure accumulated near water. The conventional bacteriological indicators of microbial indicators of microbial water quality do not necessarily correlate with the presence of concentrations of cryptosporidium. Therefore, accurate identification of its modes of transmission is of critical importance in evaluating water treatment procedures and the public health risk from cryptosporidiosis.

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