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ORIGINAL ARTICLE

Copro-microscopical and immunological diagnosis of cryptosporidiosis in Egyptian buffalo-calves with special reference to their cytokine profiles

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Abstract Cryptosporidiosis is considered to be one of the most devasting gastrointestinal diseases in calves. The aim of this study was to investigate *Cryptosporidium parvum* infection (*C. parvum*) in buffalo-calves with both copro-microscopic examination and enzyme linked immunosorbent assay (ELISA) using two *C. parvum* prepared antigens with regards to their cytokines profile; interferon- γ (IFN-γ), interleukin (IL)-12 and IL-14 to achieve a proper diagnosis. All collected buffalo- calves' fecal samples were examined by modified Ziehl–Neelsen staining technique. ELISA was performed to evaluate the diagnostic accuracy of the two *C. parvum* prepared antigens; crude whole oocyst (CWO) and crude sonicated oocyst (CSO) in detection of anti-*C. parvum* IgG in buffalo-calves' sera. As well, concentrations of INF-γ, IL-12 and IL-14 in the buffalo-calves' serum samples were estimated. The results revealed that the overall parasitological incidence of cryptosporidiosis was 40%. However, the serological diagnosis by ELISA assay showed 53.75% and 27.5% when using CWO and CSO antigen, respectively. Also, the diagnostic efficacy parameters of both antigens; CWO and CSO showed a significant high specificity (83.3%) achieved by CSO antigen and a high sensitivity (71.8%) by CWO antigen. The levels of INF-γ, IL-12 and IL-14 were significantly increased in positive *Cryptosporidium* infected group by both coprological and serological assays followed by the group which was positive for cryptosporidiosis by copro-microscopic examination only. The present study concluded that a combination of coprological and serological examination with reference to the cytokines profile is needed for proper diagnosis of cryptosporidiosis in buffalo-calves.

Keywords Cryptosporidiosis · Buffalo-calves · Diagnosis · Coprological examination · ELISA · Cytokines

Introduction

Cryptosporidia are cosmopolitan single celled widespread parasites, with broad host specificity (Bones et al. 2019). Gastrointestinal disease caused by *Cryptosporidium parvum* (*C. parvum*) is one of the most common diseases of young ruminants, particularly neonatal calves. The main symptoms which appear on infected animals are profuse watery diarrhea, inappetence, lethargy, dehydration and death can occur in severe cases (Thomson et al. 2017). Also, *C. parvum* had a significant concern to farmers, as postmortem data indicated that the majority of calves that die aging one month or less were infected with this parasite (Thomson 2015). Infected calves could shed large numbers (over 1×10^{10}) of infective oocysts per day (Nydam et al. 2001) for about 2-3 weeks and it is not always associated with diarrhea (Zambriski et al. 2013).

Despite the great attention that *C. parvum* received as a result of its high infection rate, cryptosporidiosis is still poorly controlled and remains to be under-diagnosed (Gharpure et al. 2019; Gunasekera et al. 2020). The major obstacle to control cryptosporidiosis is the proper diagnosis of the positive cases which could disseminate huge numbers of oocytes causing pollution to the environment. Traditionally, the detection of *Cryptosporidium* oocysts had relied primarily on detection of oocysts by microscopic examination in environmental, water, food, fecal and/or

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tissue samples (O'Donoghue 1995; Quintero-Betancourt et al. 2003). This method is cheap and considered as a corner stone of diagnostic methods of many parasites, but it is laborious and relatively slow (Plutzer et al. 2018). In addition, the diagnostic accuracy is significantly reduced by a low concentration of oocysts or by damaged oocysts (Garcia 2001). Moreover, this technique could not estimate the past infection prevalence because of the short sporadic duration of oocysts shedding (O'Handley et al. 1999). So, ELISA could offer a better diagnosis as it targets oocyst antigens and provide improved sensitivity versus microscopy (Checkley et al. 2015).

C. parvum parasites invade the enterocytes' microvillus brush border in humans and domestic animals. Both innate and adaptive immunity play important roles in protection against *C. parvum* parasites and in its clearance (Leitch and He 2012) following primary and secondary infections (Takeda et al. 2003). The systemic helper T1-cells (Th1 type) immune responses were produced in cryptosporidiosis (Aboelsoued et al. 2019). Intestinal intraepithelial lymphocytes act a key role against cryptosporidiosis with Th1 type that providing initiation control of the infection and cytotoxic T-cells that eliminating the parasites (Takeda et al. 2003). The cytokine expression pattern of these lymphocytes in *C. parvum* infected calves expressed interferon gamma (IFN-γ) but not tumor necrosis factor (TNF-α) (Canals et al. 1998). So, the cell mediated immune system, which is ruled by Th1 type responses, could play an important role in the protection and the measurement of immune responses in calves. Interleukin (IL)-12 is necessary for the stimulation of Th1 type responses as it upregulates the production of IFN-γ (Lang et al. 2007). Also, IL-12 indirectly stimulates antiparasitic, antimicrobial and antitumor activity of the macrophages, and activates natural killer cells (Akdis et al. 2016). During the acute phase of cryptosporidiosis, high amounts of IFN-γ and IL-12 are produced to eliminate *C. parvum* from the epithelial cells of intestine (Laurent and Lacroix-Lamande 2017) and increase in mRNA levels for cytokines as IL-14 and IFN-γ in human and murine intestinal cells with *C. parvum* parasites are recorded (Tessema et al. 2009).

Hereby, the present study investigated the diagnosis of *C. parvum* infection in buffalo-calves with different diagnostic tests; copro-microscopic examination and ELISA with regards to the cytokines profile; IFN-γ, IL-12 and IL-14. Also, the study emphasized on the diagnostic activity of two *C. parvum* prepared antigens (whole oocyst and sonicated oocyst antigens) in order to ensure the positivity and gain a proper diagnosis that improve the result interpretation and control of such disease.

Materials and methods

Animals and samples collection

Individual rectal fecal samples were collected from 80 buffalo-calves with a mean age of 1-4 months old from different farms at Qualiobya Governorate, Egypt. The collected fecal samples were transferred to the parasitology laboratory at Parasitology and Animal Diseases Department, National Research Centre under chilled conditions and then being processed within 2 days.

Parallel to fecal sampling, buffalo-calves' blood samples were collected. After blood clotting, serum samples were obtained by centrifugation at 2000 rpm/10 min, divided into small aliquots and stored at − 20 °C until their serological analysis (total IgG and cytokines).

Copro-microscopical examination

All collected fecal samples were examined for the parasitological existence of *C. parvum* oocysts using ordinary direct smear method. The fecal smear slides were stained by modified Ziehl–Neelsen staining technique and examined by light microscope using high power (40×) and oil immersion lens (l00×) according to Henriksen and Pohlenz (1981).

Antigen preparation

C. parvum oocysts were isolated from the highly positive naturally-infected buffalo-calves' fecal samples by floatation in Sheather's sugar solution (Current and Reese 1986). The collected oocysts were identified and kept in a 2.5% potassium dichromate solution at 4 °C. Oocysts were divided into 2 batches, the first one is to prepare crude whole oocyst (CWO) antigen and the second one for preparation of crude sonicated oocyst (CSO) antigen. The CWO antigen was prepared according to Nydam et al. (2002) with some modifications. Briefly, the identified oocysts were concentrated and counted after their washing for several times in phosphate buffered saline (PBS), pH 7.2. While, the CSO antigen was performed as described by Kaushik et al. (2009). Concisely, after washing *C. parvum* oocysts for three times with PBS (15,000 rpm for 15 min), suspended in PBS and were freeze-thawed 20 times. Then, the oocysts were sonicated for twelve cycles each 30 s and centrifuged (15,000 rpm for 15 min at 4 °C). Finally, the supernatant was collected, aliquoted and kept as CSO antigen at − 20 °C. The total protein content of CSO antigen was estimated according to Lowry et al. (1951).

Immunological analysis

Enzyme linked immunosorbent assay (ELISA)

ELISA was performed to study the diagnostic potentiality; apparent prevalence, sensitivity, specificity, negative and positive predictive values and diagnostic efficacy of the two *C. parvum* prepared antigens; CWO and CSO in detection of anti-*C. parvum* IgG in buffalo-calves' sera as described by Nydam et al. (2002). Briefly, checker-board titration was done to obtain the optimal concentration of antigens and dilutions of tested sera and conjugate. In 96 micro-titer plates, each well was coated with 100 µl of each diluted antigen at the concentration (10^5 oocysts/well for CWO antigen and 4 µg/well for CSO antigen) incubated at 37 °C for 1 h, then overnight at 4 °C. The coated wells were incubated for 1 h at 37 °C with 200 µL/well of blocking buffer. A100 µL/well of diluted serum sample at concentration 1:20 was added in duplicates and incubated for 1.5 h at 37 °C. Positive, negative controls were confirmed by PCR and blank controls were included on each plate in duplicates. 100 µl/well of diluted protein-A peroxidase conjugate at1:1000 was added and incubated for 1 h at 37 °C. Then, all wells were supplied with 100 µl/well of substrate solution and were incubated at 37 °C for 20 min. Finally, The optical densities (OD) of tested samples were read at wavelength 450 nm. Positive readings were determined as those with absorbance reading greater than the cut-off value which was calculated as a mean OD values of the negative control sera plus three-fold standard deviations. Diagnostic potentiality parameters were calculated as described by Bauer et al. (2002).

Cytokines analysis

Serum cytokines; INF-γ, IL-12 and IL-14 levels (ng/ml) in naturally *C. parvum* infected and non-infected buffalo-calves were investigated based on data of microscopical and serological examination (using the more specific antigen which was CSO). Animals were grouped into positive and negative for both coprological and serological tests. Then, the concentrations of INF-γ, IL-12 and IL-14 in the buffalo-calves' serum samples were measured by bovine specific ELISA kit (SunLong Biotech Co., Ltd., China). INF-γ, IL-12 and IL-14 concentrations were estimated according to the manufacturer's instructions by comparing the samples OD to the standard curves. The OD is measured spectrophotometrically at a wavelength of 450 nm using an ELISA reader (BIO-TEK, Instruments, ELx, 800UV, USA).

Statistical analysis

The data of the antigenic diagnostic parameters of different prepared *C. parvum* antigens were analyzed using Chi square test by GraphPad Prism version 7. Data of cytokine (INF-γ, IL-12 and IL-14) concentrations in the buffalo-calves' serum samples were expressed as Mean ± Standard Error (SE). Significance and comparison between the means of different parameters were evaluated using Analysis of variance (ANOVA) and Duncan using Statistical Package for Social Science (SPSS) computer programs, 2015.

Results

Copro-microscopical examination

The microscopical examination of the collected fecal samples of buffalo calves revealed the parasitological existence of *C. parvum* oocysts (Fig. 1). The data showed that 43.86% and 30.43% were positive for the presence of oocysts of *C. parvum* in diarrheic and non-diarrheic animals, respectively, with an overall incidence of cryptosporidiosis 40% (Table 1).

Immunological analysis

Enzyme linked immunosorbent assay (ELISA)

The current results revealed that incidence of cryptosporidiosis was 57.89% and 24.56% in diarrheic animals when using CWO and CSO antigens, respectively. While, in non-diarrheic animals, the incidence was 43.47% and

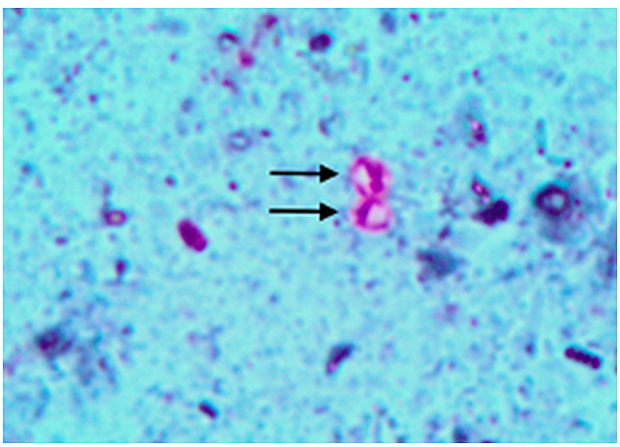

Fig. 1 *Cryptosporidium* oocysts (arrows) in fecal smears of buffalo-calves stained by modified Ziehl–Neelsen staining technique (×1000)

Table 1 The incidence of cryptosporidiosis among Egyptian buffalo-calves using copro-microscopic examination and ELISA techniques

Buffalo calves	Diagnostic techniques					
	CME		ELISA (Cut off ≤ 0.65)			
			Crude whole oocyst antigen (CWO)		Crude sonicated oocyst antigen (CSO)	
	Positive	%	Positive	%	Positive	%
Diarrheic (n = 57)	25	43.86	33	57.89	14	24.56
Non-diarrheic (n = 23)	7	30.43	10	43.47	8	34.78
Total (n = 80)	32	40	43	53.75	22	27.5

CME copro-microscopic examination; *ELISA* enzyme linked immunosorbent assay

34.78% when using CWO and CSO antigens, respectively (Table 1).

Meanwhile, the diagnostic activity parameters of the two prepared *C. parvum* antigens; CWO and CSO were elucidated. It was showed that the total prevalence (53.75%) using CWO antigen was significantly higher than using CSO antigen (27.5%) ($P < 0.05$). In addition, the CWO antigen recorded higher ($P < 0.05$) sensitivity (71.8%) than CSO (43.75%). However, CWO antigen had a significantly lower ($P < 0.05$) specificity (58.3%) than that recorded by CSO antigen (83.3%) (Table 2). Positive and negative predictive values were quite similar between CWO (53.4% and 75.67%) and CSO (63.6% and 68.96%) antigens, respectively, as no significant differences were recorded between them (Table 2).

Cytokines analysis

The results of serum cytokines; INF-γ, IL-12 and IL-14 levels were significantly higher ($P < 0.001$) in serum of naturally infected buffalo-calves' group which was positive for *C. parvum* infection by both coprological and serological diagnosis compared to the other groups (Table 3). In addition, INF-γ and IL-12 levels were significantly higher ($P < 0.001$) in the group of buffalo-calves which was positive for *C. parvum* infection by coprological technique only than other groups which were coprologically negative (Table 3).

Discussion

Cryptosporidiosis in buffalo-calves is widely reported with a major concern to several countries all over the world (Thomson et al. 2017) as *Cryptosporidium* is mainly recognized as one of the major causes of diarrhea in neonatal ruminants (Santin 2020). In the current study, cryptosporidiosis was prevalent in diarrheic bovines than non-diarrheic ones and this result agreed with Morsy et al. (2014) and Ouakli et al. (2018).

The overall incidence of cryptosporidiosis in buffalo-calves obtained in this study differed according to the diagnostic techniques. The copro-microscopic examination of the collected fecal samples revealed an overall incidence 40%. This result was comparable with the result of Essa et al. (2014) who reported a prevalence percentage (34.33%) and the results of Ghaffari and Kalantari (2014) who recorded (9.5%) prevalence when using the same Modified Ziehl–Neelsen stain in microscopic detection. These differences in prevalence could be due to many reasons such as locality, severity of infection, breed of the calves, hygienic measures, age of calves and season of

Table 2 Antigenic diagnostic parameters of different prepared *Cryptosporidium parvum* antigens by ELISA

Antigenic diagnostic parameters	*C. parvum* antigens		*P- Value*
	Crude whole oocyst antigen (CWO)	Crude sonicated oocyst antigen (CSO)	
Apparent prevalence (%)	53.75	27.5	0.0016
Sensitivity (%)	71.8	43.75	0.0073
Specificity (%)	58.3	83.3	0.0415
Positive predictive value (%)	53.4	63.6	0.0722
Negative predictive value (%)	75.67	68.96	0.0621

Statistically significant ($P < 0.05$)

Table 3 Serum cytokines; INF-γ, IL-12 and IL-14 levels (ng/ml) in naturally *Cryptosporidium parvum* infected and non-infected buffalo-calves

Cytokine levels	Incidence of cryptosporidiosis				
	Positive by both CME, ELISA	Negative by CME, Positive ELISA	Positive by CME, Negative ELISA	Negative by both CME, ELISA	F-Value
INF-γ	49.97 ± 0.33[a]	41.11 ± 0.39[c]	46.72 ± 0.46[b]	41.86 ± 0.63[c]	84.37*
IL-12	26.34 ± 0.53[a]	19.9 ± 0.42[c]	22.96 ± 0.39[b]	20.45 ± 0.55[c]	38.03*
IL-14	14.5 ± 0.35[a]	12.21 ± 0.2[b]	13.2 ± 0.26[b]	12.05 ± 0.26[b]	9.43*

Data is expressed as Mean ± SE

CME Copro-microscopic examination; *ELISA* Enzyme linked immunosorbent assay

*Significant differences at $P < 0.001$

Means followed by different letters indicated significance

examination. These factors might increase the risk of transmission of *C. parvum* between calves (Duranti et al. 2009).

In addition, the serological diagnosis by ELISA showed 53.75% when using CWO antigen and 27.5% when using CSO antigen. The variability of the obtained prevalence most likely could be ascribed to the method of antigen preparation. In this study, the significantly higher prevalence achieved by CWO antigen could be due to current or previous *Cryptosporidium* infection, while, the copro-microscopic examination was able to detect the active cases only (Nydam et al. 2002). Moreover, the lower prevalence achieved in the current study by ELISA when using CSO antigen could be attributed to the higher specificity (83.3%) of this antigen. This low prevalence agreed with the finding of Danisová et al. (2018) that recorded low prevalence (27.84%) using the same technique for diagnosis of cryptosporidiosis in different animal species and with nearest antigenic specificity (72.7%). As well, the recorded data of Ghaffari and Kalantari (2014) detected low prevalence (18.7%) of *Cryptosporidium* infection in human sera when using ELISA.

The diagnostic accuracy parameters of both antigens (CWO and CSO) showed a significant higher specificity (83.3%) achieved by CSO antigen, which could be attributed to the difference in the methods of antigen preparation and to the ability of this sonicated antigen in detection of the highest number of true negative cases among buffalo-calves examined sera. These findings were also confirmed by the results reported by Nydam et al. (2002).

On the other hand, the significantly higher sensitivity of CWO (71.8%) which might be returned to capability of this whole oocyst antigen in detection of the highest number of true positive cases among the same examined collected sera (Danisová et al. 2018).

To gain a proven positivity by both coprological and serological techniques for appropriate diagnosis of cryptosporidiosis among buffalo-calves, the current study investigated the measurement of cytokine levels to support the proper diagnosis of cryptosporidiosis in naturally infected buffalo-calves' sera. A group of buffalo-calves that was positive via the coprological examination, but negative serologically and that result agreed with Nydam et al. (2002) who reported the same findings in one calf. This result might be returned to the delay in antibody production from 5 to 14 days post-infection (Whitmire and Harp 1991). The result of negative ELISA cases despite the fact of their positivity by coprological method could be accredited to that the examined buffalo-calves' sera were negative for active cryptosporidiosis or due to the false positive reaction showed by CSO antigen which responsible for small lacked specificity percentages (16.7%) (Danisová et al. 2018).

The present study revealed a significant increase in the levels of INF-γ, IL-12 and IL-14 in animals' group which was positive for *Cryptosporidium* infection by both coprological and serological assays followed by the group which was positive for cryptosporidiosis by copro-microscopic examination only. This high level could be returned to the fact that IFN-γ and IL-12 were released primarily by T lymphocytes and natural killer as an immune response to inflammatory stimuli induced by excysted sporozoites which invade epithelial cells of the intestine where they establish themselves to complete their life cycle (Schreiber and Schreiber 2003; Thomson et al. 2017). The current data was agreed with the previous investigation of INF-γ, IL-12 and IL-14 profiles in the *Cryptosporidium* infected buffalo intestinal tissue explants (Aboelsoued et al. 2019) and with McNair and Mead (2013) who reported increasing in INF-γ in experimentally infected *C. parvum* mice and Auray et al. (2013) who stated that IL-12 was released with large amounts in most animal models which were susceptible to cryptosporidiosis. However, the other two buffalo-calves groups; the group which was negative for *Cryptosporidium* infection by both techniques (copro-microscopic examination and ELISA) and the group which was negative for

cryptosporidiosis by coprological methods and positive by ELISA showed significantly low level of all cytokines; INF-γ, IL-12 and IL-14. This result could be attributed to that cases which were positive by ELISA might be have a past infection of cryptosporidiosis (O'Handley et al. 1999).

Conclusion

In the current study, although using CSO antigen showed a significant specificity that coincide with the cytokines profile during the infection, it was concluded that the combination of coprological and serological tests is a good tool for detecting cryptosporidiosis.

Authors' contribution All authors contributed to the study conception and design. DA, SHMH and KNA performed the laboratory experiments. DA and FAMA analyzed the data. SHMH, FAMA and DA shared in data interpretation. SHMH and DA prepared the first manuscript draft. KNA and FAMA revised and reviewed the manuscript draft for publication. All authors had read and approved the final manuscript.

Compliance with ethical standards

Conflicts of interest The authors declare that they have no conflicts or competing interests.

Ethical standards Medical Research Ethics Committee of National Research Centre (NRC), Egypt approved this study under registration number (19057). The samples collection was conducted in accordance with the guidelines laid down by the International Animal Ethics Committee and in accordance with local laws and regulations.

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