Serological detection and epidemiology of *Neospora caninum* and *Cryptosporidium parvum* antibodies in cattle in southern Egypt

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Abbreviations: CpP23 (immunodominant surface glycoprotein of *Cryptosporidium parvum*); GST (glutathione-S transferase); NcSAG1t (*Neospora caninum* truncated surface antigen 1).

Abstract

Neospora caninum and *Cryptosporidium parvum* are intracellular protozoan parasites that are distributed worldwide and of major economical concern in cattle industry. *N. caninum* can cause abortion storms and high culling rates, whereas *C. parvum* has zoonotic implications and can cause diarrhea in calves. There are currently no data on the prevalence of neosporosis and cryptosporidiosis in humans or animals in southern Egypt. Prevalence of these two infections was determined in a sample of cattle from two different areas in southern Egypt, Sohag and Qena, using enzyme-linked immunosorbent assay. A total 301 cattle were sampled, of which 18.9% were positive for *N. caninum*, 35.9% were positive for *C. parvum* and 10.0% were positive for both. Geographical location and breeding system were considered as potential risk factors for *C. parvum* infection. A higher prevalence of infection was identified on small scale farms, compared with larger, intensive systems, with a prevalence of 50.2% compared with 37.8%, respectively. Animals in Sohag had a significantly higher prevalence compared with Qena, with a seroprevalence of 46.1% compared with 31.6%, respectively. In brief, marked seroprevalence recorded in this study indicates a high incidence of *N. caninum* and *C. parvum* infections in cattle, and this necessitates the application of more effective strategies for combating these types of infections on farms in Egypt.

Keywords:
Cattle, *Cryptosporidium parvum*, *Neospora caninum*, ELISA, Egypt

1. Introduction

The protozoan parasite *Neospora caninum* is a *Toxoplasma*-like organism that may cause neosporosis in dogs and cattle and less frequently in other animals such as goats, sheep, deer, rhinoceros, llamas and alpacas (Dubey et al., 2002). *N. caninum* oocyst-contaminated food or water is considered the main route of infection for cattle (De Marez et al., 1991), and there is also evidence for the vertical transmission of *N. caninum* between dams and calves (Thurmond et al., 1997, Schares et al., 1998). *N. caninum* is generally accepted to be a major cause of infectious abortion in cattle worldwide (Reichel et al., 2013). In pregnant cows, infection with *N. caninum* can lead to several outcomes including early fetal death and re-absorption; abortion, stillbirth or parturition of a deformed calf and birth of clinically normal but infected offspring (Dubey et al., 2006). Clinical signs, other than abortion, have been identified in calves less than 4 months of age and include neurologic manifestations, locomotor disturbances and ocular and cerebral anomalies (De Meerschman et al., 2005).

Cryptosporidium parvum is a coccidian protozoan parasite that has been attributed to numerous outbreaks of neonatal calf diarrhea in different regions of the world (Xiao, 2010). It has diverse characteristics, such as food and water borne-transmission (Ramirez et al., 2004), zoonotic implications (Chalmers et al., 2010), economical hazards, chemotherapy and vaccine resistance (Mead, 2002), which render it a unique pathogen. It primarily infects the intestinal villi of neonatal calves; however, yearling and adult cattle, other animal species and humans are also prone to infection (Fayer, 2004). Cryptosporidiosis primarily manifests as watery diarrhea that may sometimes be profuse and prolonged. In humans, diarrhea and abdominal pain are generally the main clinical signs that cause patients to seek medical attention, leading to a laboratory diagnosis of cryptosporidiosis (Current and Garcia, 1991).

In Egypt, previous studies screening for *N. caninum* antibodies demonstrated the prevalence varied from 3.6% in a sample of 166 camels (Hilali et al., 1998) to 68% in a sample of 75 water buffalo (Dubey et al., 1998). Additionally, antibodies to *N. caninum* were detected in 7.92% of human samples and 20.43% of cattle in the delta region in Egypt (Ibrahim et al., 2009). *Cryptosporidium parvum* was sampled in buffalo and dairy cattle on different farms in Kafr El Sheikh Province, northern Egypt, using microscopical examination of fecal samples for detection of *Cryptosporidium* oocysts, showing that all farms had positive animals, with an overall prevalence of 1.29% in buffalo and 7.07% in cattle (Mahfouz et al., 2014). The *C. parvum* infection rate in buffalo calves using fecal sample analysis demonstrated a prevalence of 14.19% in Dakahlia and Kafr El Sheikh, northern Egypt (El-Khodery and Osman, 2008). Cattle calves' fecal samples were tested microscopically for *C. parvum* and demonstrated a prevalence of 30.2% (Amer et al., 2010) and 13.6% (Amer et al., 2013) in the delta region in Egypt.

To develop an effective control strategy for bovine neosporosis and cryptosporidiosis, accurate prevalence data are required. This study was conducted to determine the prevalence of *N. caninum* and *C. parvum*-specific antibodies in cattle in the Qena and Sohag governorates in southern Egypt.

2. Materials and methods

2.1.Sample population

Cattle of various ages, genders, breeding systems and localities in Qena and Sohag governorates in southern Egypt were sampled. The cattle were divided into four groups: group 1 - randomly sampled male and female cattle of different ages from individual owners (less than five cattle per owner) and smallholder farms (5–20 cattle per farm), in different villages in the Qena governorate; group 2 - adult cows (over 3 years of age) that were bred in an intensive farming system (more than 2000 cattle) in Qena governorate; group 3 - adult bulls (over 3 years of age) that were admitted to the Qena slaughter house; and group 4 - randomly sampled cattle of different ages and genders from individual owners and smallholder farms from different villages in the Sohag governorate. The serum samples were collected between May 2014 and June 2015. The details of the various groups of cattle and the geographical locations of the collection sites are shown in Table 1 and Figure 1, respectively.

2.2.Blood sampling

Blood samples were collected through vein puncture from each animal in glass tubes without anticoagulant. Serum was separated by centrifugation and stored at -20 °C until use.

2.3.Protein expression

Recombinant antigens were expressed and purified as described previously, for the NcSAG1-truncated antigen (Chahan et al., 2003) and CpP23 antigen (Bannai et al., 2006), with slight modifications. In brief, proteins were expressed as a glutathione-*S*-transferase

(GST) fusion protein. The expression was performed at 37°C for 6 h, after induction with 1 mM isopropyl b-D-1-thiogalactopyranoside (Wako Inc., Osaka, Japan). Supernatant of disrupted *Escherichia coli* was purified with Glutathione–Sepharose 4B beads according to the manufacturer's instructions (GE Healthcare Life Sciences, Buckinghampshire, UK). The GST-fused protein was eluted with elution buffer (pH 8, 100 mM Tris-Hcl, 100 mM Nacl, 5 mM EDTA and 20 mM reduced glutathione powder (Wako)). The proteins were filtered using a 0.45-μm low-protein binding Supor membrane (Pall Life Sciences, Ann Arbor, MI, USA). The purity and quantity of the proteins were detected as a single band by sodium dodecyl sulfate polyacrylamide gel electrophoresis, followed by Coomassie brilliant blue R250 staining (MP Biomedicals Inc., Illkirch-Graffenstaden, France). The concentration was measured using a bicinchoninic acid (BCA) protein assay kit (Thermo Fisher Scientific Inc., Rockford, IL, USA).

2.4.Indirect Enzyme-Linked Immunosorbent Assay (iELISA)

Fifty-microliter amounts of purified recombinant antigen, at a final concentration of 0.1 μM, were coated onto the ELISA plates (Nunc, Roskilde, Denmark), overnight at 4 °C, with a carbonate-bicarbonate buffer (pH 9.6). Plates were washed once with phosphate-buffered saline (PBS) containing 0.05% Tween 20 (PBS-T) and blocked with PBS containing 3% skimmed milk (PBS-SM) for 1 h at 37 °C, then the plates were washed once with PBS-T, and 50 μl amounts of serum samples, diluted at 1:100 with PBS-SM, were added to the wells. The plates were incubated at 37 °C for 1 h. After washing six times with PBS-T, the plates were incubated with horseradish-peroxidase-conjugated anti-bovine IgG (Bethyl Laboratories, Montgomery, TX, USA), diluted at 1:4,000 with PBS-SM at 37 °C for 1 h. Plates were washed an additional six times before

the substrate solution [0.1 M citric acid, 0.2 M sodium phosphate, 0.003% H_2O_2, and 0.3 mg/ml 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid); (Sigma-Aldrich, St. Louis, MO, USA) was added to each well in 100 µl aliquots. The absorbance at 405 nm after 1 h of incubation at room temperature was measured using an ELISA reader. The readings for the recombinant antigens were subtracted from those of GST protein. The cutoff point was determined as the mean A_{405} value for standard *N. caninum* or *C. parvum*-negative sera kept in our laboratory ($n = 6$ for *N. caninum*, $n = 3$ for *C. parvum*) plus three standard deviations. Sera from calf before giving colostrum were used as *C. parvum*-negative sera. *N. caninum*-negative sera were collected from cattle confirmed negative for the parasite by an immunofluorescence antibody test and no reactivity of peripheral blood mononuclear cell under the stimulation with the parasite lysates. The specificity and sensitivity of the ELISA were as follows; the NcSAG1-truncated antigen: sensitivity 100%, specificity 100% (Chahan et al., 2003), CpP23 antigen: sensitivity 80%, specificity 73.7% (Bannai et al., 2006).

2.5.Statistical analysis

Differences in the incidence of *N. caninum* and *C. parvum* and risk factors for infection were determined using a chi-square test. A P value of < 0.05 was considered statistically significant. The 95% confidence intervals of a proportion including continuity correction were calculated using www.vassarstats.net. Chi square values and odds ratios were calculated using GraphPad Prism 5 software (GraphPad Software Inc., La Jolla, CA, USA).

3. Results

Serum samples were collected from 301 cattle, in three groups in Qena governorate and one group in Sohag governorate (Table 1). Investigation of cattle sera revealed 57 (18.9%) and 108 (35.9%) were seropositive for *N. caninum* and *C. parvum*, respectively (Table 2). For each study group, the prevalence of *N. caninum* was 12/100 (12.0%), 21/90 (23.3%), 10/35 (28.6%) and 14/76 (18.4%) for groups 1, 2, 3 and 4, respectively. The prevalence of *C. parvum* was 26/100 (26%), 34/90 (37.8%), 13/35 (37.1%) and 35/76 (46.1%), for groups 1–4, respectively. The prevalence of *N. caninum* and *C. parvum* positive samples was 10.0% (30/301) overall and 9/100 (9%), 8/90 (8.9%), 7/35 (20%) and 6/76 (7.9) for groups 1–4, respectively.

An epidemiological study was conducted to establish the influence of age, sex, location (Qena and Sohag) and management system (individual/smallholding and intensive farming system) on prevalence of *N. caninum* and *C. parvum* infections. No risk factors were identified for *N. caninum* (Table 3); however, location and management system were identified as risk factors for *C. parvum* infection (Table 4). Cattle in Sohag were more likely to be positive for *C. parvum* compared with those in Qena, with a prevalence of 46.1% in Sohag compared with 31.6% in Qena (odds ratio [OR] 0.5, P=0.022). Cattle managed on individual/smallholder farms were 1.7 times more likely to be seropositive for *C. parvum* compared with cattle kept in an intensive farming system, with a prevalence of 50.2% on individual/smallholder farms, compared with 37.8% on the intensive farming systems (P=0.047).

4. Discussion

The overall prevalence of *N. caninum* in this study was 18.9%. This finding is similar to that of a previous study in Sharkia governorate in northern Egypt, where a prevalence of 20.4% was detected in cattle using NcSAG1t-based ELISA (Ibrahim et al., 2009). However, a study of water buffaloes, sampled from the main slaughterhouse in Cairo, identified a much higher prevalence of 68.0%, determined by a direct agglutination test (Dubey et al., 1998). This considerably higher prevalence of positive sera could be attributed to the different diagnostic test used, animal model, location and when the study was conducted. Numerous studies have demonstrated a higher seroprevalence of *N. caninum* in water buffaloes compared with cattle (Dubey et al., 2007, Moore et al., 2014, Neverauskas et al., 2015), possibly because of the co-evolution of water buffalo with the parasite during certain periods and the development of tolerance against severe neosporosis (Neverauskas et al., 2015). Using the same technique as our study (NcSAG1t-based ELISA), seroprevalence rates of 33.5% in Brazil (Chahan et al., 2003) and 46.9% in Thailand (Inpankaew et al., 2014) have been reported in cattle. The markedly higher prevalence in these studies compared with the present study could be because of the difference in location and environmental patterns, time period and number of collected samples. Collectively, these results indicate the global importance of *N. caninum* as an abortifacient disease of bovines.

In the present study, no significant risk factors were identified for *N. caninum*. Conversely, previous studies from other countries demonstrated the effect of host age as a risk factor for *N. caninum*, such as in northeastern Thailand, where buffalo aged over 10 years had an infection rate of 16.1%, compared with 1.2% for those under 3 years of age (Kengradomkij et al., 2015). Additionally, the antibodies detected in sera of calves were significantly lower compared with adult cattle in northeastern regions of Argentina (Moore et

al., 2014). This discrepancy may be because of geographical, ecological and animal husbandry differences between studies, in addition to the relatively low number of animal investigated that were under 3 years old in our study.

It is difficult to compare the *C. parvum* prevalence determined in the present study based on ELISA, with previous reports in Egypt where *C. parvum* infection in farm animals was mostly screened using microscopical observation of fecal oocyst via modified Ziehl–Neelsen stain that counts on detecting the current infection (El-Khodery and Osman, 2008, Amer et al., 2010, Amer et al., 2013, Mahfouz et al., 2014). A small number of studies have screened *C. parvum* in cattle in the Ismailia governorate in the region of the Suez Canal, northeastern Egypt, using commercial ELISA or immunochromatographic assay; however, they focused on detection of the antigen in fecal specimens of suspected animals (Helmy et al., 2013, 2014, 2015). The present study used indirect ELISA using the CpP23 antigen to determine the specific antibodies in the serum samples. This detection system is essential to identify both recent and past infections, so it is a more accurate reflection of the endemicity status of *C. parvum* in the study areas. The seroprevalence in cattle in Thailand was recorded as 4.4% in one study, which was the only other report for seroprevalence of anti-CpP23 antibodies in cattle (Inpankaew et al., 2009). The higher prevalence in the current study may be related to the difference in time period, location and also the environment at the sample sites. Moreover, Inpankaew et al. (2009), sampled cattle in northern Thailand, including Chiang Mai, Chiang Rai and Lumpang provinces that are well known for the highest dairy cow populations in Thai regions, and subsequently these animals are expected to receive a high standard of veterinary care. More field studies are required to validate CpP23-based ELISA as a universal tool for determining seroprevalence of *C. parvum* in cattle and other animals, including humans.

Other factors that may explain the high seroprevalence of *C. parvum* in the current study is that cryptosporidiosis is a water borne-disease and drinking water plays a crucial role in transmission (Mac Kenzie et al., 1994, Antonios et al., 2001, Tsushima et al., 2003). To overcome the hot weather in southern Egypt, farmers bathe cattle and calves in the same water sources that are used as drinking water, such as the Nile River. Infected animals can shed a large number of *C. parvum* oocysts in their feces, and this may act as a potential risk for spreading the infection among other animals and humans, if they ingest untreated water (Abou-Eisha, 1994, Abou-Eisha et al., 2000, El-Khodery and Osman, 2008, Helmy et al., 2015). Additionally, the cattle, particularly young calves, are an essential reservoir host for *C. parvum* because they shed a considerable number of infective oocysts in their feces. This suggests that it may constitute a potential risk for infection if they are excreted in the drinking water (Nydam et al., 2001, Fayer et al., 2007).

The present study identified location and type of management system as risk factors for cryptosporidiosis in cattle. Samples from intensive farming systems were only collected from Qena governorate and showed a significantly lower infection rate than individual and smallholder samples. This could be because of the high standards of veterinary care, close animal observation, good biosecurity measures and monitoring of food and water resources that predominantly occurred in intensively-bred cattle. In additions, Sohag governorate, where only cattle samples of individual and smallholders were collected, are characterized by relatively lower environmental temperature, higher humidity, spacious agricultural lands and higher animal density compared with Qena governorate. All aforementioned factors may provide more favorable conditions for persistence of *C. parvum* oocyst, resulting in higher infection rate.

5. Conclusions

Our preliminary study provides valuable data on the high prevalence of *N. caninum* and *C. parvum* in cattle in southern Egypt. This will assist in the development of prevention and control strategies for these diseases. The high prevalence of *N. caninum* and *C. parvum*-specific antibodies in cattle might be the principal factor limiting the livestock industry in Egypt. More research and effort is needed from governmental and non-governmental authorities to minimize the economic losses caused by *N. caninum* and additionally the zoonotic risks from *C. parvum* infections.

Declaration of conflict of interest

The authors declare that there are no conflicts of interest.

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Figure legend

Fig. 1: Map of Egypt showing the location of regions where cattle were sampled.

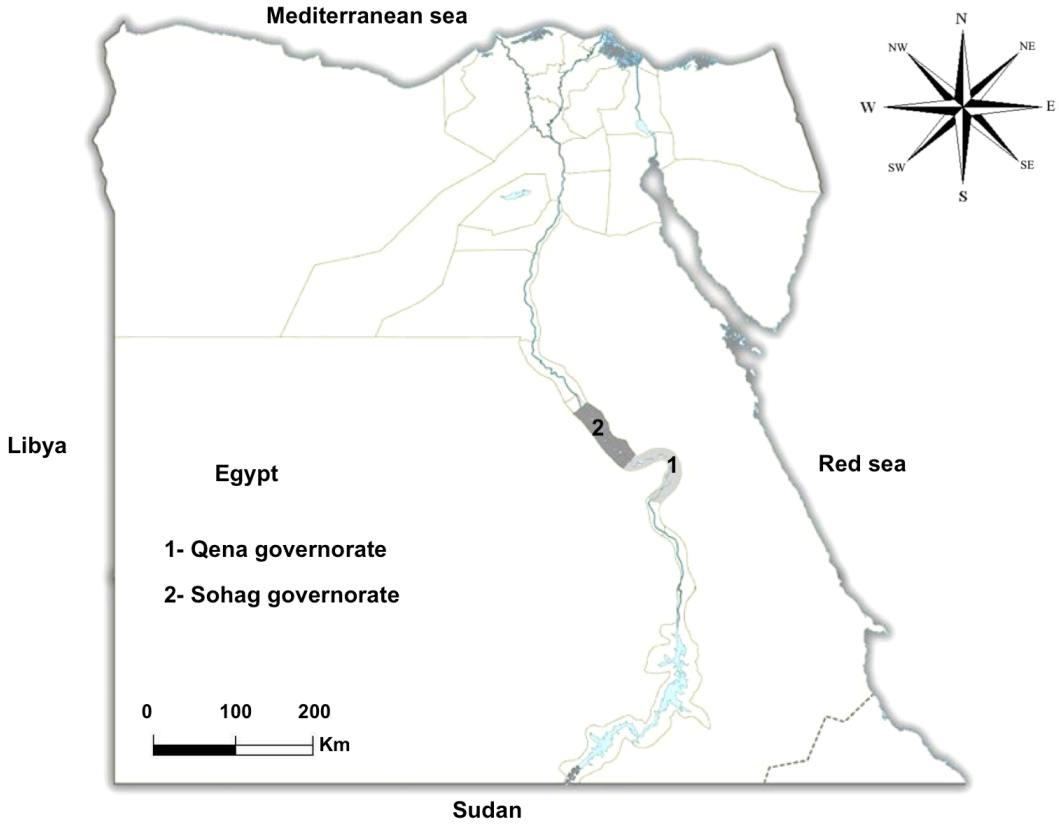

Table 1.

Geographical locations and group characteristics of sampled animals

Sampling area		Type of breeding system	Number of animals (total number; n=301)	Sex	Age
Qena	Group 1	Individual/smallholder	100	Male, Female	Mixed
Qena	Group 2	Intensive	90	Female	> 3 years
Qena	Group 3	Individual/smallholder	35	Male	> 3 years
Sohag	Group 4	Individual/smallholder	76	Male, Female	Mixed

Table 2.

Seroprevalence of antibodies for *N. caninum, C. parvum* and both infections in cattle in southern Egypt (n=301)

Sampling area		No. tested	No. positive (%)			95% CI*		
			N. caninum	*C. parvum*	Both	*N. caninum*	*C. parvum*	Both
Qena	Group 1	100	12 (12.0)	26 (26.0)	9 (9.0)	6.63-20.4	18.0-35.9	4.46-16.8
Qena	Group 2	90	21 (23.3)	34 (37.8)	8 (8.9)	15.3-33.6	28.0-48.7	4.19-17.25
Qena	Group 3	35	10 (28.6)	13 (37.1)	7 (20.0)	15.2-46.5	22.0-55.1	9.06-37.5
Sohag	Group 4	76	14 (18.4)	35 (46.1)	6 (7.9)	10.8-29.3	35.0-57.8	3.25-17
Total		301	57 (18.9)	108 (35.9)	30 (10.0)	14.8-23.9	30.5-41.6	6.9-14.06

* 95% CI= confidence interval

Table 3.

Risk factors associated with *N. caninum* infection in cattle in southern Egypt

Analysed factor	No. tested	No. positive (%)	OR (95% CI)*	*P*-value
Age				
< 3 years old	61	15 (25.6)	1.54 (0.8-3.0)	0.2
> 3 years old	240	42 (17.5)		
Sex				
Male	61	15 (25.6)	1.54 (0.8-3.0)	0.2
Female	240	42 (17.5)		
Location				
Qena	225	43 (19.1)	1.1 (0.5-2.0)	0.88
Sohag	76	14 (18.4)		
Management system				
Individual/smallholder	211	36 (17.1)	0.7 (0.4-1.2)	0.26
Intensive	90	21 (23.3)		

*Odds ratio at 95% confidence interval

Table 4.

Risk factors associated with *C. parvum* infection in cattle in southern Egypt

Cattle group	No. tested	No. positive (%)	OR (95% CI)*	*P*-value
Age				
< 3 years old	61	26 (42.6)	1.5 (0.8-2.6)	0.17
> 3 years old	240	80 (33.3)		
Sex				
Male	61	26 (42.6)	1.5 (0.8-2.6)	0.17
Female	240	80 (33.3)		
Location				
Qena	225	71 (31.6)	0.5 (0.3-0.9)	0.022
Sohag	76	35 (46.1)		
Management system				
Individual/smallholder	211	106 (50.2)	1.7 (1.0-2.8)	0.047
Intensive	90	34 (37.8)		

*Odds ratio at 95% confidence interval