Open Veterinary Journal, (2022), Vol. 12(5): 754–761
ISSN: 2226-4485 (Print)
ISSN: 2218-6050 (Online)
Original Research
DOI: *10.5455/OVJ.2022.v12.i5.21*

Submitted: 08/05/2022 Accepted: 18/09/2022 Published: 08/10/2022

Seroprevalence of *Besnoitia besnoiti* in Assiut Governorate, Egypt

Huda Mohammed Kuraa[1*], Zainab M. A. Youssef[2], Fatma S. Mahmoud[2], and Safaa S. Malek[2]

[1]*Parasitology Department, Animal Health Research Institute, Agriculture Research Center, Ministry of Agriculture, Assiut, Egypt*
[2]*Department of Animal Medicine, Faculty of Veterinary Medicine, Assiut University, Assiut, Egypt*

Abstract
Background: Bovine besnoitiosis is a widespread disease caused by *Besnoitia besnoiti* with significant economic losses in cattle production. There is a lack of knowledge about it in Egypt.
Aim: This study was conducted to detect the seroprevalence of *B. besnoiti* in cattle and to find out the presence of the disease and the most important symptoms of besnoitiosis in cattle in Assiut Governorate, Egypt.
Methods: A total of 190 cattle from Assiut city and its different rural centers were examined clinically and serologically for the presence of *B. besnoiti*. The serological examination was carried out by using the indirect enzyme-linked immunosorbent assay (ELISA) kit in serum (ID.Vet Innovative Diagnostics Louis Pasteur. Grabeis, France). The results were analyzed statistically using the chi-square test to assess the association between seroprevalence and different parameters (age, sex, season, housing, and health status).
Result: Thirteen cattle were seropositive for *B. besnoiti* by ELISA and showed symptoms of besnoitiosis. Acute symptoms included fever, tachycardia, edematous swellings of intermandibular space and limbs with polyarthritis, diarrhea, ruminal atony, and enlarged lymph nodes. The chronic symptoms included cough, mastitis, exophthalmia, cysts on the sclera and conjunctiva, nodules in the skin, and alopecia associated with tick infestation. The overall seroprevalence of *B. besnoiti* was 22.1%. Regarding sex, the seroprevalence was higher for females 34.6% than for males 6.97%. While, according to age susceptibility, the seroprevalence was highest (50.9%) with age ≥5 years, followed by age >1 to <5 years (14.6%), and only one animal of age ≤1 year was recorded at 2.2%. Concerning seasonal variations, the seroprevalence was highest in spring 42.9%, followed by autumn 29.3%, winter 13.6%, and summer 7.5%. Whereas, according to the housing system, it was 60% and 8.6% in farm and household rearing, respectively. Depending on the health status, the seroprevalence was 21.6% of clinically healthy and 23.2% of clinically diseased cattle.
Conclusion: The existence of *B. besnoiti* antibodies has been demonstrated in clinical and subclinical infected cattle in Assiut Governorate, Egypt. The ELISA test is considered to be a good diagnostic method for detecting infection. Furthermore, additional studies are essential to minimize and prevent the spread of infection.
Keywords: Assiut, *Besnoitia besnoiti*, Cattle, Egypt, ELISA.

Introduction

Bovine besnoitiosis is a parasitic disease in cattle caused by *Besnoitia besnoiti* "cyst-forming apicomplexan protozoan" (Besnoit and Robin, 1912). This parasite is closely related to *Toxoplasma gondii*, *Sarcocystis* species, and *Neospora caninum* (Gonzalez-Barrio *et al.*, 2020). It is held to be an emerging disease in European countries and described previously in Africa, the Middle East, and Europe and caused outbreaks in Germany, Switzerland, Italy, and Spain (Mehlhorn *et al.*, 2009; Álvarez-García *et al.*, 2013; Rinaldi *et al.*, 2013; Nieto-Rodríguez *et al.*, 2016). It was endemic in France and Spain (Alzieu *et al.*, 2007a; Fernández-García *et al.*, 2010) and recently reported in Ireland (Ryan *et al.*, 2016).

The whole lifecycle of *B. besnoiti* is not completely known until now with unidentified final host species "carnivores" (Basso *et al.*, 2011). On the other hand, the parasite transmission between cattle was suggested by direct contact between infected and uninfected cattle (nasopharyngeal and natural mating) and mechanically via blood-sucking insects (*Stomoxys calcitrans* and *Tabanus* spp.) (Gollnick *et al.*, 2015; Gutiérrez-Expósito *et al.*, 2017a; Tainchum *et al.*, 2018). In addition, Bigalke (1968) discovered that insects, biting flies, and iatrogenically reusing hypodermic needles on numerous animals were transmission methods during the experimental studies.

Jacobs *et al.* (2016) stated that *B. besnoiti* has an alternating carnivore-herbivore "two host life cycle,"

***Corresponding Author:** Huda Mohammed Kuraa. Parasitology Department, Animal Health Research Institute, Agriculture Research Center, Ministry of Agriculture, Assiut, Egypt. Email: *Huda5380@yahoo.com*

the intestinal phase includes both schizogony and gametogony in the final host. Oocysts are released shortly after their formation and sporulated externally in the environment. During this time, the asexual stages of this parasite progress in the intermediate host. Two infective stages have been defined up to now, one including the fast-replicating tachyzoites and the other including the slower-dividing bradyzoites which unite into macroscopic cysts located within the subcutaneous connective tissue (Bigalke, 1981; Diesing *et al.*, 1988; Njagi *et al.*, 1998).

Bovine besnoitiosis is a debilitating disease affecting all cattle ages and breeds. In severe acute cases, it causes anorexia, weakness, pyrexia, nasal and ocular discharge, conjunctivitis, intensive respiratory disorders, increased heart rates, generalized edema, peripheral lymphadenopathy, photophobia, swollen joints, lameness, reduced milk yield, and orchitis associated with bulls' permanent infertility (Bigalke, 1968, 1981; Álvarez-García *et al.*, 2013; Cortes *et al.*, 2014; Gollnick *et al.*, 2018). Severe chronic disease is characterized by noticeable skin changes with hyperkeratosis, skin wrinkling, alopecia, and non-healing ulcers. In addition, visible tissue cysts were detected in the scleral conjunctiva and genital mucosa (Álvarez-Garcia *et al.*, 2013; Cortes *et al.*, 2014).

Chronically infected cattle that do not exhibit severe clinical signs are a reservoir "source of infection." When these animals are inadvertently integrated into other flocks, besnoitiosis can propagate from one flock to another and can even cross-national boundaries (Álvarez-Garcia *et al.*, 2013). Additionally, the progress of the disease can happen rapidly, and severely impacted animals may die due to respiratory impairment, nephrotic syndrome, or cardio-respiratory failure, which takes place 2 weeks after infection before the development of specific "IgG" antibodies (Dubey *et al.*, 2013; Gonzalez-Barrio *et al.*, 2020). Economic losses take place due to decreased milk and meat production, damage to hides, infertility of bulls, and death (Jacobs *et al.*, 2016).

In infected herds, the prevalence rate increased rapidly after the first appearance of the disease, leading to increased epidemicity with a mortality rate of 7%–10% (Fernández-García *et al.*, 2010; Jacquiet *et al.*, 2010; Alzieu and Jacquiet, 2015). In addition, several authors from varying regions of the world recorded that the seroprevalence of bovine besnoitiosis in cattle increased over time (Liénard *et al.*, 2011; Nieto-Rodríguez*et al.*, 2016; Gazzonis *et al.*, 2017; Gutiérrez-Expósito *et al.*, 2017a).

Early stages of besnoitiosis are hard to be diagnosed because of non-specific signs and antibodies that are not easily detectable in the first 2 weeks (Jacobs *et al.*, 2016). At the chronic stage, the clinical signs are specific and diagnosis can be proven with an enzyme-linked immunosorbent assay (ELISA) test which is deemed a useful test to detect asymptomatic cattle and aid in the prevention of more propagation of disease via reservoirs and its extirpation from affected flocks for the disease control (Álvarez-Garcia *et al.*, 2013; Jacobs *et al.*, 2016).

There is no available published data about the prevalence of besnoitiosis in Egypt except for one study by (Ashmawy and Abu-Akkada, 2014) who examined the seroprevalence of *B. besnoiti* in bovine. Referring to the high economic importance of besnoitiosis with the current lack of knowledge about it in Egypt, the main purpose of this work was to find out the presence of the disease and the most important symptoms of besnoitiosis in cattle in Assiut Governorate, Egypt.

Materials and Methods

Animals

During a period of investigation, from June 2021 to April 2022, a total of 190 cattle of different ages, sex, and localities were examined clinically and serologically for the presence of *B. besnoiti*. These animals were collected from Assiut city and its different rural centers (Assiut, Manfalut, Al-Qusiya, Abnoub, Sahel Seleem, Al-Fath, Abuteeg, and Sedfa) including different farms and individual cases that were admitted to the Veterinary Teaching Hospital, Faculty of Veterinary Medicine at Assiut University. Clinical examination was carried out according to (Jackson and Cockcroft, 2002).

Sampling

Blood samples were obtained from 156 clinically healthy and 34 clinically diseased cattle containing 104 females and 86 males. The cattle were selected by age ranging from 4 months to 7 years and divided into three age groups: ≤1 year; >1 to <5 years; and ≥5 years and from farms and household rearing (individual cases that reared in a farmer's house in the villages).

About 5 ml of blood was taken from the jugular vein of cattle into clean, dry glass tubes without anticoagulant and transported to the laboratory in cold conditions. Sera were separated by centrifugation at 500× g for 15 minutes, then labeled and preserved at −20°C until used (Gonzalez-Barrio *et al.*, 2020).

Serological examination

The serum samples were examined for the presence of specific antibodies for *B. besnoiti* using a commercially available indirect ELISA kit in serum (ID Screen® Besnoitia Indirect 2.0). Indirect biwell ELISA was done for the detection of antibodies against *B. besnoiti* in bovine serum or plasma (ID.Vet Innovative Diagnostics Louis Pasteur. Grabeis, France; BSNTB ver 0614 GB, LOT: 137) according to the manufacturer's instructions. The optical density values were read with an ELISA reader (Sunrise, TECAN) at a wavelength of 450 nm within 15 minutes in Molecular Biology Research Center, Assiut University.

Antibody analysis

For each sample, calculate the S/P percentage (S/P%) obtained by an equation provided by the manufacturer:

Serum samples with an S/P% ≥ 30% were considered positive; 25% > S/P% < 30% were considered doubtful; and those with S/P% ≤ 25% were considered negative.

Statistical analysis

Clopper–Pearson exact confidence intervals (CI) for the prevalence rates were calculated at the level of 95%. The obtained results were analyzed by the chi-square test to assess the association between seroprevalence and different parameters (age, sex, season, housing, and health status) by using Statistical Package for the Social Sciences 22.0 statistical software (IBM Corp., USA). Significance was defined as $p < 0.05$ (Özdal *et al.*, 2019).

Ethical approval

During sample taking from animals, adequate measures were taken to minimize the pain or discomfort of animals with concern for ethical standards.

Results

A total of 190 cattle were clinically and serologically examined for the presence of *B. besnoiti* in some localities of Assiut Governorate, Egypt. Thirteen cattle (4 bulls, 1 heifer, and 8 cows) were seropositive by ELISA and showed symptoms of besnoitiosis. These symptoms involved acute symptoms such as fever, tachycardia, edematous swellings of intermandibular space (bottle jaw), and limbs with polyarthritis, diarrhea, ruminal atony, and enlarged lymph nodes, while the chronic symptoms included cough, mastitis, exophthalmia, cysts on sclera and conjunctiva, nodules in skin, and alopecia associated with tick infestation on some seropositive animals (Table 1 and Fig. 1).

The overall seroprevalence of *B. besnoiti* was 22.1% (42/190) with the ELISA. Concerning sex, females show a higher ratio of 34.6% (36/104) than males, who show a low ratio of 6.97% (6/86). Regarding age, the susceptibility was highest at 50.9% (28/55) with group ≥5 years, followed by group >1 to <5 years at 14.6% (13/89), and only one animal of group ≤1 year was recorded at 2.2% (1/46) (Table 2).

Regarding seasonal variations, *B. besnoiti* was serologically highest in spring 42.9% (6/14), followed by autumn 29.3% (27/92), winter at 13.6% (6/44), and summer at 7.5% (3/40). While the seroprevalence according to the housing system was 60% (30/50) of the farm and 8.6% (12/140) of household rearing. Depending on the health status, it was 21.6% (29/134) of clinically healthy and 23.2% (13/56) of clinically diseased cattle (Table 2).

There were highly significant differences in the seroprevalence between different season groups ($p < 0.01$). Also, very high significant differences in the seroprevalence of infected cattle with *B. besnoiti* among the sex, age, and housing system were recorded ($p < 0.001$). However, there were no significant differences in seroprevalence depending on the health status (Table 2).

Discussion

Bovine besnoitiosis is regarded as responsible for significant economic losses in the cattle industry due to the extensive reduction of productivity, male sterility, abortion, and mortality (Jacobs *et al.*, 2016). The severity of the disease varies greatly between infected cattle (Jacobs *et al.*, 2016). It has been described in numerous countries with varying prevalence rates (Álvarez-Garcia *et al.*, 2013; APHA, 2020; Zhou *et al.*, 2020). Serologically identifying infected cattle is important to preclude the introduction of infected animals into native herds (Papadopoulos *et al.*, 2014).

Table 1. Seropositive cattle showing clinical signs of bovine besnoitiosis.

Animal	Age (years)	Locality	Symptoms	S/P%
Bull	1 year	Abuteeg	Fever, enlargement of lymph nodes	46.65
Bull	1.5 years	Assiut	Cough, nodules in the skin associated with tick infestation	85
Bull	2 years	Assiut	Enlargement of lymph nodes and ruminal atony associated with tick infestation	35.42
Bull	2 years	Assiut	Cough, diarrhea, ruminal atony, tachycardia	32.18
Heifer	2 years	Assiut	Edematous swellings on limbs with polyarthritis	109.71
Cow	3 years	Al-Fath	Tachycardia, ruminal atony, cysts on sclera, and conjunctiva	76.03
Cow	4 years	Assiut	Enlargement of lymph nodes, ruminal atony, tachycardia, and nodules in skin	40.17
Cow	4 years	Manfalut	Edema in intermandibular space (bottle jaw), diarrhea	37.37
Cow	5 years	Sahel Seleem	Mastitis, ruminal atony, tachycardia, alopecia	90.06
Cow	5 years	Sedfa	Ruminal atony, tachycardia	102.59
Cow	6 years	Assiut	Mastitis, exophthalmia	186.61
Cow	6 years	Assiut	Enlargement of lymph nodes, ruminal atony, cough, tachycardia	39.74
Cow	7 years	Sahel Seleem	Mastitis, enlargement of lymph nodes, ruminal atony, and alopecia, associated with tick infestation	57.67

Fig. 1. Clinical signs of acute and chronic *B. besnoiti* infection in cattle in Assiut. Acute (A and B) as follows: (A): Edematous swelling of the neck. (B): Hyperemia of limbs due to tick infestation. Chronic signs (C–G) as follows: (C): Cysts on the sclera and conjunctiva; (D and E): nodules on skin; and (F and G): alopecia.

Clinical symptoms of bovine besnoitiosis in our cases matched typical signs previously summarized by Fernández-García *et al.* (2010), Álvarez-García *et al.* (2013), Cortes *et al.* (2014), Nieto-Rodríguez *et al.* (2016), Gazzonis *et al.* (2017), APHA (2020), Gonzalez-Barrio *et al.* (2020), Zhou *et al.* (2020), and Neve *et al.* (2022). Also, Njagi *et al.* (1998) added that the cattle showing signs of besnoitiosis were heavily infested with ticks in Kenya.

The total seroprevalence of *B. besnoiti* in our study was 22.1%, which was nearly similar to Ashmawy and Abu-Akkada (2014) who found that the seroprevalence of *B. besnoiti* was 17.13% among cattle in Egypt. In comparison to our result, the same result was recorded in Greece (22%) by Papadopoulos *et al.* (2014), in the Alentejo region from Southern Portugal (25.8%) by Waap *et al.* (2014) and in the Kirikkale province of Turkey (26.6%) by Öcal *et al.* (2016). Whereas, our result was higher than those previously reported in Turkey by Özdal *et al.* (2019) and Kula and Gokpınar (2021) who found that 2.7% and 5% of cattle were seropositive against *B. besnoiti*, respectively.

On the other hand, the present result was lower than those recorded in France (89%) (Alzieu *et al.*, 2007a), in Italy (44.1%) (Rinaldi *et al.*, 2013), in Navarra, North Spain (90.5% and from 35.6%–86.5%) by Fernández-Garcia *et al.* (2010) and Gutiérrez-Expósito *et al.* (2017b), respectively and in Sicily (61.6%) (Neve *et al.*, 2022). Such variations could be due to differences in the study locality, livestock management systems, different levels of contact between infected and uninfected cattle, and exposure to reservoirs and insects (Ashmawy and Abu-Akkada, 2014; Talafha *et al.*, 2015).

The current results revealed that the seroprevalence of *B. besnoiti* was higher in females 34.6% than in males 6.97%. These results are matched with those of Fernández-García *et al.* (2010) who documented that 90.8% of females and 71.4% of males were seropositive in an outbreak in Spain. In addition, Ashmawy and Abu-Akkada (2014) observed that the prevalence of the disease was higher in females (17.13%) than in males (8.33%), and Özdal *et al.* (2019) found that the females were infected (3%) without parasite detection in males. On the other hand, Álvarez-Garcia *et al.* (2013) and Gutiérrez-Expósito *et al.* (2014) discovered that infection of *B. besnoiti* in males was more prevalent than in females, and Gazzonis *et al.* (2017) recorded that males had a higher risk of infection with an incidence of 60% versus 38.8% among females in Italy.

Table 2. Seroprevalence of *B. besnoiti* infection in cattle according to the sex, age susceptibility, seasonal variation, housing system, and health status by using ELISA.

Variables	No. of examined animals	Number positive	Prevalence %	CI (95%) of prevalence % (Clopper–Pearson exact)	Chi-square tests	
					Pearson chi-square (*p*-value)	Probability ($p < 0.05$)
Sex						
Male	86	6	6.97	(2.6–14.6)	20.884**	0.000
Female	104	36	34.6	(25.6–44.6)		
Total	190	42	22.1	(16.4–28.7)		
Age						
≤1 year	46	1	2.2	(0.06–11.5)	40.020**	0.000
>1 to <5 years	89	13	14.6	(8–23.7)		
≥5 years	55	28	50.9	(37.1–64.7)		
Total	190	42	22.1	(16.4–28.7)		
Season						
Winter	44	6	13.6	(5.2–27.4)	13.092*	0.004
Spring	14	6	42.9	(17.7–71.1)		
Summer	40	3	7.5	(1.6–20.4)		
Autumn	92	27	29.3	(20.33–39.8)		
Total	190	42	22.1	(16.4–28.7)		
Housing system						
Farms	50	30	60	(45.2–73.6)	56.591**	0.000
Household	140	12	8.6	(4.5–14.5)		
Total	190	42	22.1	(16.4–28.7)		
Health status						
Clinically healthy	134	29	21.6	(15–29.6)	0.057	0.812
Clinically diseased	56	13	23.2	(13–36.4)		
Total	190	42	22.1	(16.4–28.7)		

** Very high significant differences ($p < 0.001$).
* High significant differences ($p < 0.01$).

In this study, a higher prevalence of infection in females than in males might be explained by that females may be have increased exposure to infective stages or more susceptible to the parasite. Increased susceptibility in females may occur from the suppressed immune functions related to reproductive stress (Schuurs and Verheul, 1990; Freudiger, 2008; Jacquiet *et al.*, 2010; Ashmawy and Abu-Akkada, 2014; Gazzonis *et al.*, 2017). Besides, the risk of disease spreading to females through mating with an infected bull (Esteban-Gil *et al.*, 2014).

Our study revealed that the highest prevalence of *B. besnoiti* was 50.9% in the age group ≥5 years, followed by the age group >1 to <5 years 14.6%, and only one animal of age group ≤1 year was recorded at 2.2%. This was consistent with results pointed out by Ashmawy and Abu-Akkada (2014) who recorded the highest infection rate of *B. besnoiti* was 17.13% in the age "5–10 years" followed by the age "1–5 years" at 15.38%, and only one case 1.58% in the age "<1 year." As well, Özdal *et al.* (2019) detected that cattle aged "6–8 years" had a higher infection rate of 3.3%, followed by the age group "3–5 years" at 2.8% with no detection of the parasite in the age group "1–2 years." In addition, previous researchers have found that the risk of infection increases with age increasing (Gutiérrez-Expósito *et al.*, 2014, Talafha *et al.*, 2015; Gazzonis *et al.*, 2017). These results can be attributed to rapid horizontal disease transmission through repeated exposure to parasitic infection either by continuous parasitic replicate within the infected host or by cattle exposure to the insects (Bourdeau *et al.*, 2004; Fernández-García *et al.*, 2010; Ashmawy and Abu-Akkada, 2014).

Concerning seasonal variations, the seroprevalence of *B. besnoiti* was highest in spring 42.9% (6/14), followed by autumn 29.3% (27/92), winter 13.6%

(6/44), and summer 7.5% (3/40). Similarly, Alzieu *et al.* (2007b) and Freudiger (2008) suggested that bovine besnoitiosis was a seasonal infection and were clinically observed from spring to autumn. In view of this, Bigalke (1968) suggested that bloodsucking arthropods such as horseflies act as mechanical vectors of *Besnoitia*. Spring and autumn might be accompanied by increased insect activity and parasite contact (Gollnick *et al.*, 2018).

Our climate can encourage the development and prolong the activity period of vectors "biting insects," which facilitate the rapid propagation of the parasite through vector-borne horizontal transmission (Bigalke, 1968), in agreement with Jacquiet *et al.* (2010) who found that climatic changes may have helped the spread of the parasite. In contrast with Fernández-García *et al.* (2010) who discovered the appearance of clinical signs coincides with the summer season, when herds shared pastures and there are vectors.

This study revealed that the seroprevalence of *B. besnoiti* according to the housing system was 60% of farm and 8.6% of household rearing. The high infection in farms can be explained by continuous exposure of animals to infection. This accords with Gazzonis *et al.* (2017) who stated that natural mating could be considered a possible method of *B. besnoiti* diffusion through the herd and insect-bite-promoted infection passage. Likewise, direct contact between infected and non-infected animals may aid nasopharyngeal transmission. Therefore, a minimum distance of 20 m between infected and uninfected animals was recently proved sufficient to avoid seroconversion of negative cows (Esteban-Gil *et al.*, 2014; Gollnick *et al.*, 2015).

It has been reported that animals without clinical signs in endemic regions have been reported to be an important factor in the dissemination of the disease (Frey *et al.*, 2013). Even in endemic situations, only some animals develop the characteristic clinical symptom of besnoitiosis in an infected herd, while the majority are seropositive but still subclinically infected. The recognition of subclinical cases through the ELISA test can be useful in carrying out control programs (Fernández-Garcia *et al.*, 2010; García-Lunar *et al.*, 2013). Culling would have been the best way to control cattle besnoitiosis on farms (Zhou *et al.*, 2020).

In our study, the seroprevalence of *B. besnoiti* according to the health status was 21.6% of clinically healthy cattle, while it was 23.2% of diseased cattle. This is in agreement with Fernández-García *et al.* (2010) who registered that only 43% developed clinical signs in 90.5% of seropositive cattle in Spain. This is contrary to Neve *et al.* (2022) who found that clinically diseased cattle had a low prevalence (1.5%) and confirmed that the clinical appearance of bovine besnoitiosis remains sporadic. These differences in the prevalence of clinically diseased cattle may be due to various factors such as stress, the resistance of the animal, and the amount of parasites transmitted to animals by blood-sucking insects (Alzieu *et al.*, 2007b).

Conclusion

Based on our findings, the existence of *B. besnoiti* antibodies has been demonstrated in clinical and subclinical infected cattle in Assiut Governorate, Egypt. The ELISA test is considered to be a good diagnostic method for detecting *B. besnoiti* infection. Furthermore, it is a major need to raise veterinarians' awareness of the clinical cases and methods of disease spread. Moreover, additional studies are essential to developing specific programs to combat this disease and minimize the spread of infection and prevent the entry of infected animals into herds.

Conflict of interest

The authors declare that there is no conflict of interest.

Authors' contribution

Kuraa, H.M., Youssef, Z.M.A., Mahmoud, F.S., and Malek, S.S. designed the study and helped in the ELISA procedure, data analysis, and interpretation. Youssef, Z.M.A., Mahmoud F.S., and Malek, S.S. examined animals and collected samples. Kuraa, H.M. wrote the manuscript.

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