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DETECTION OF *NEOSPORA CANINUM* AND *COXIELLA BURNETII* ANTIBODIES IN MILK AND SERUM OF INFECTED DAIRY CATTLE

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ABSTRACT

Coxiella burnetii and *Neospora caninum* are frequent infectious causes of decreased reproductive outcomes in cattle around the world. So our study aimed to assess the performance of IgG-ELISA for diagnosis of *N. caninum* and *C. burnetii* and compare the sensitivity of ELISA and IFA in detection IgG to *C.burnetii* phase II ,to achieve these, a total of 200 samples including milk and serum samples (100 for each) were collected from 100 dairy cattle suffering from infertility and abortion during the period from January to December 2017 in four governorates of upper Egypt, these samples were transferred to laboratory for serological examination. The results revealed that *N.caninum* antibodies were detected in 29% of serum samples and 10% of milk samples while *C.burnetii* antibodies were detected in 34% and 17% of serum and milk samples respectively. The IgG-ELISA of both *N.caninum and C.burnetii* were detected in the same serum and milk samples with percentage 14% and 4% respectively, amid dairy cattle in 4 governorates of upper Egypt. IgM-IFA indicated the acute infection of *C.burnetii* in 11% and 4% of serum and milk samples while chronic infection (IgG-IFA) was detected in 19% of serum samples and 8% of milk samples. The comparison between sensitivity of ELISA and IFA in detection of IGg for *C.burnetii* phase II showed that ELISA was more sensitive and excellent diagnostic method for *C.burnetii* infection.

Key words: Serum, Milk, *N. caninum*, *C. burnetii*, ELISA, IFA

INTRODUCTION

The rate of reproductive problems in cattle have been increasing over years and causes many economic problems in the bovine industry and public health which represented a standpoint because of their zoonotic importance, a number of infectious agents for instance, bacteria, viral, fungus and parasitic are recognized to have a direct impact on reproductive health of cattle (Yoo, 2010).

Neosporosis, caused by the obligate intracellular parasite protozoan called *Neospora caninum* (*N. caninum*), it has a highly preference for cattle and dogs as hosts and induces a serious economic losses like abortion, temporary anestrus and less milk production in dairy (Bruhn *et al*., 2013 and Almería *et al*., 2017).

Q fever is a zoonotic and prevalent disease in most countries of the world, it caused by a gram negative bacteria called *Coxiella burnetii* (*C.burnetii*). These bacteria has many hosts include mammals, birds and arthropods like ticks (Angelakis and Raoult 2010).

There are two antigenic forms of *C. bumetii* that are imperative for serologic diagnosis of Q fever are the phase I (S-LPS) is avirulent form in ticks with smooth lipopolysaccharide and phase II (R-LPS) is avirulent microorganism with rough LPS with whole-cell antigens (Peter *et al*., 1985).

The shedding of *C.burnetii* into the environment happens primarily by birth products, especially placenta, are heavily infected with *C. burnetii* also it could be gained from milk and other excreta of infected animals (Schmeer *et al*., 1987). *Coxiella* can persist in the environment for long periods and may carried for a long distances through the wind (Kirkan *et al*., 2008). In cattle, late abortion, mastitis and infertility are the major clinical manifestations related to coxiellosis (Arricau *et al*., 2003).

Serological methods represent the most widely recognized, broadly available and frequently used tools for the diagnosis of infectious diseases (Garcia-

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perez *et al*. 2011; An and Ghattof, 2018). These methods are renowned from all other diagnostic methods by providing indirect evidence of infection or exposure by detecting host antibodies reactive against infections.

Enzyme linked immune sorbent assay (ELISA) and immune fluorescence antibody (IFA) generally considered the main serological method for diagnosis the *C.burnetii* infection (Meekelenkamp *et al*., 2012 and Anati-Pirouz *et al*., 2015).

So this study aimed to describe the seroepidemiological situation of *N. caninum* and *C. burnetii* specific antibodies in milk and serum of dairy cattle by using ELISA and IFA in Upper Egypt Governorates.

MATERIALS AND METHODS

1- Samples collection:
A total of 200 milk and serum samples (100 for each) were collected according the international ethics for animal experiments from 100dairy cattle suffering from infertility and abortion during the period from January to December 2017 in upper Egypt governorates (Table1). Pooled milk and serum samples were collected from the same dairy cattle.

a-Serum samples
Blood samples (5ml) were collected from the jugular vein of each cow in vacuum tubes without anticoagulant, the serum was decanted according (Peter *et al*., 1988).

b-Milk samples
each milk sample were centrifuged at 1000rpm for 10 min, skimmed milk was collected in sterile screw capped bottle and stored at -20℃ until examination (Frossling *et al*., 2003).

NB. All samples were subjected to examination for *Brucella* infection by using Rose Bengal test (rabid slide agglutination test, ID. Vet Genetics, france) for detection of *Brucella* antibodies in serum and Milk Ring Test (hematoxylin-stained antigen manufactured by the State Biological Laboratory, Institute of Veterinary Preventive Medicine, Ranipet, India) for detection of *Brucella* antibodies in milk samples (Alton *et al*., 1988).

2- Detection of *N.caninum* antibodies in serum and milk samples by using ELISA test:
The serum and whey-milk samples were screened for detection of *Neospora caninum* antibodies (IgG) by commercially available diagnostic indirect ELISA kit in serum, or milk for multispecies (ID. Vet innovative Diagnstics Louis Pasteur. Grabeis, France) using X check software program. All control tests were performed in duplicate. The diluent, wash solution and dilution buffer were primed according to manufacture instruction. The optical density (OD) values of the wells were read with ELISA reader (Titertek Multiskan plus MK II), at a wave length of 450 nm. The attendance of antibody to *N. caninum* was resolute in samples according to (S/P) ratio for each sample. The positive serum samples have S/P ratio ≥ 50 while positive milk samples have S/P ratio ≥ 30, the incidence of seropositive animals in the governorates was compared using Chi-square test (Analytical Software Package, Statistix version 1.0, 1996).

3-Detection of *C. burnetii* antibodies in serum and milk samples by ELISA and IFA

A-Detection of *C. burnetii* IgG in serum and milk samples by using ELISA test:
The samples were tested for the existence of anti-*C. burnetii* antibodies (IGg) by ELISA test (ELISA Cox kit, LSI-Laboratories Service International, Lyon, France) according to Yang *et al*. (2015). The optical density (OD) values of the wells were read with ELISA reader (Titertek Multiskan plus MK II), at a wave length of 450 nm. The S/P ratio was categorized in three classes in serum samples: positive 50<S/P ≤80, doubtful 40<S/P≤50 and negatives S/P <40were while in milk samples considered positive samples for specific antibodies to *C. burnetii* when S/P>40, doubtful 30< S/P≤ 40 and negative S/P≤30 according to manufacturer's instructions.

B- Detection of *C.burnetii* antibodies by indirect Immunofluorescence technique (IFA):
Milk and serum were tested for IgM and IgG antibodies against *C. burnetii* phase II by using slides coated with *C. burnetii* phaseII (VIRCELL,S.L.pza. slides, SPAIN).

1- Detection of IgM
- serum samples were diluted (1:2) and treated with anti-human IgG sorbent. Different dilution of treated serum were prepared 1:24, 1:48, 1:96 and placed in wells with positive and negative control, after several washing a IgM FITC conjugate solution was added to the wells, The steps were completed according manufacture instructions. The slides were examined under ultraviolet light of fluorescence microscope the reaction is positive when the apple-green fluorescence of coco-bacillary morphology can be observed. The reaction is negative when no fluorescence can be observed.

2-Detection of IgG
All samples were screened for different dilutions1:32, 1:64 and 1:128 by adding phosphate buffer saline (PBS). 20µl of each dilution were added to slide wells with control after incubation and several washing, IgG-fluorescein isothiocyanate (FITC) immune-conjugate was added to the wells. The initial dilution of 1:32 was considered as a negative dilution. The IgG phase II titer >32 was considered an indicative

for chronic infection). The slides were examined under ultraviolet light by fluorescence microscope. The reaction is positive when the apple-green fluorescence of coco-bacillary morphology can be observed. The reaction is negative when no fluorescence can be observed.

RESULTS

All serum and milk samples included in this study were tested for *Brucella* infection by using rose bengal test and milk ring test. The tested samples showed a negative results.

In the current study, the data illustrated in table (2) and fig (1) showed that the *N.caninum* antibodies (IgG-ELISA) were detected in 29% of serum samples and in 10% of milk samples. The incidence ranged from 28% to 36% in serum samples while it ranged from 8%to12% in milk samples collected from cows farms in four governorates of Upper Egypt.

According to results in table (3) and fig (2) chronic infection (IgG for *C.burnetii* phase II) was cleared in 34% of serum samples (IgG titer >50) and 17% of milk samples (IgG titer >40), the higher incidence in serum samples was noticed in El-Fayoum governorate while in milk samples, El-Fayoum and Giza were the higher than other governorates.

The results demonstrated in table (4) and fig (3&4) revealed that recent infection of *C.burnetii* (IgM-IFA)appeared in 11% of serum samples and 4%of milk samples while the past infection (IgG-IFA) was showed in19% of serum samples and 8% of milk samples.

The comparative analysis between the results of IgG-ELISA and these obtained by IFA showed that ELISA is more accurate than IFA in decreasing the false negative results in serum and milk samples (Table 5).

Results in table (6) cleared that both *C.burnetii and N. caninum* antibodies were detected in the same serum and milk samples with percentage 14% and 4% among dairy cattle in 4 governorates in Egypt.

Tables

Table 1: Number of blood and milk samples collected from dairy cattle in different Egyptian governorates.

Localities	No. of samples	Type of samples	
		Blood samples	Milk samples
Fayoum	50	25	25
Giza	50	25	25
Beni-Swief	50	25	25
Menia	50	25	25
Total	200	100	100

Table 2: Incidence of *N. caninum* antibodies in serum and milk samples by using ELISA test.

Localities	Positive samples for IgG of *N.* caninum	
	Serum samples	Milk samples
El-Fayoum	7/25 (28%)	2/25 (8%)
Giza	7/25 (28%)	3/25 (12%)
Beni-Swief	9/25 (36%)	3/25 (12%)
El-Menia	6/25 (24%)	2/25 (8%)
Total	29/100 (29%)	10/100 (10%)

Table 3: Incidence of *C.burnetii* antibodies in serum and milk samples by using ELISA test.

Localities	Positive samples for IgG of *C. burnetii*	
	Serum samples	Milk samples
El-Fayoum	12/25 (48%)	5/25(20%)
Giza	7/25 (28%)	5/25(20%)
Beni-Swief	9/25 (36%)	4/25(16%)
El-Menia	6/25 (24%)	3/25(12%)
Total	34/100 (34%)	17/100 (17%)

Table 4: positive samples for C.*burnetii antibodies* in milk and serum samples by using IFA test.

Localities	IFA test for detection of IgM		IFA test for detection of IgG	
	+ ve Serum samples	+ ve milk samples	+ ve Serum samples	+ ve milk samples
Fayoum	4/25 (16%)	2/25 (8%)	8/25 (32%)	--
Giza	3/25 (12%)	2/25 (8%)	2/25 (8%)	2/25 (8%)
Beni-Swief	2/25 (8%)	--	6/25 (24%)	3/25 (12%)
Menia	2/25 (8%)	--	3/25 (12%)	3/25 (12%)
Total	11/100 (11%)	4/100 (4%)	19/100 (19%)	8/100 (8%)

Table 5: Comparison between by IFA test and ELISA test indetection IgG of C.*burnetii* in serum and milk samples.

Localities	IIFA test		ELISA test	
	+ ve Serum samples	+ ve milk samples	+ ve Serum samples	+ ve milk samples
El-Fayoum	8/25 (32%)	--	12/25(48%)	5/25 (20%)
Giza	2/25 (8%)	2/25 (8%)	7/25(28%)	5/25 (20%)
Beni-Swief	6/25 (24%)	3/25 (12%)	9/25(36%)	4/25 (16%)
El-Menia	3/25 (12%)	3/25 (12%)	6/25(24%)	3/25 (12%)
Total	19/100 (19%)	8/100 (8%)	34/100 (34%)	17/100 (17%)

Table 6: Positive samples for both C. *burnetii* and *N. caninum* antibodies in the same serum and milk samples by using ELISA test.

Localities	No. of positive serum samples	No. of positive milk samples
Fayoum	3/25 (12%)	2/25 (8%)
Giza	4/25 (16%)	--
Beni-Swief	5/25 (20%)	1/25 (4%)
Menia	2/25 (8%)	1/25 (4%)
Total	14/100 (14%)	4/100 (4%)

Figures

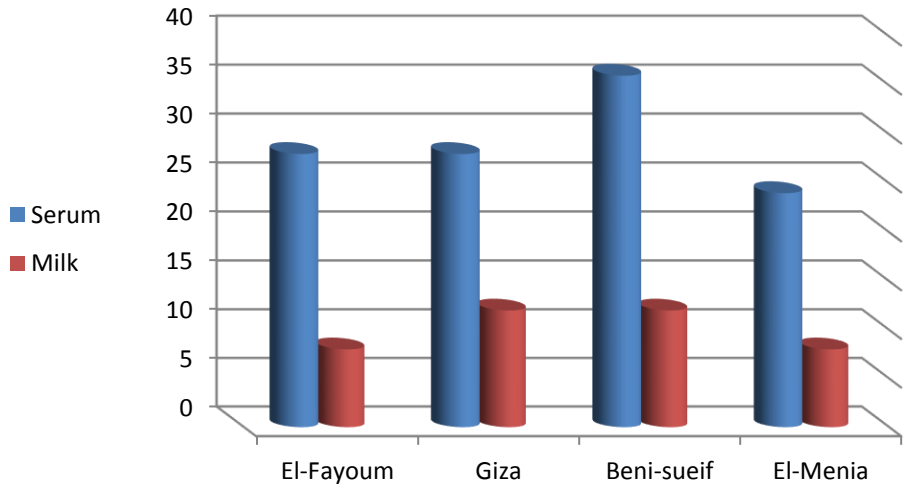

Fig (1): Incidence of *N.caninum* in serum and milk samples collected from dairy cattle of Upper Egypt governorates by using ELISA

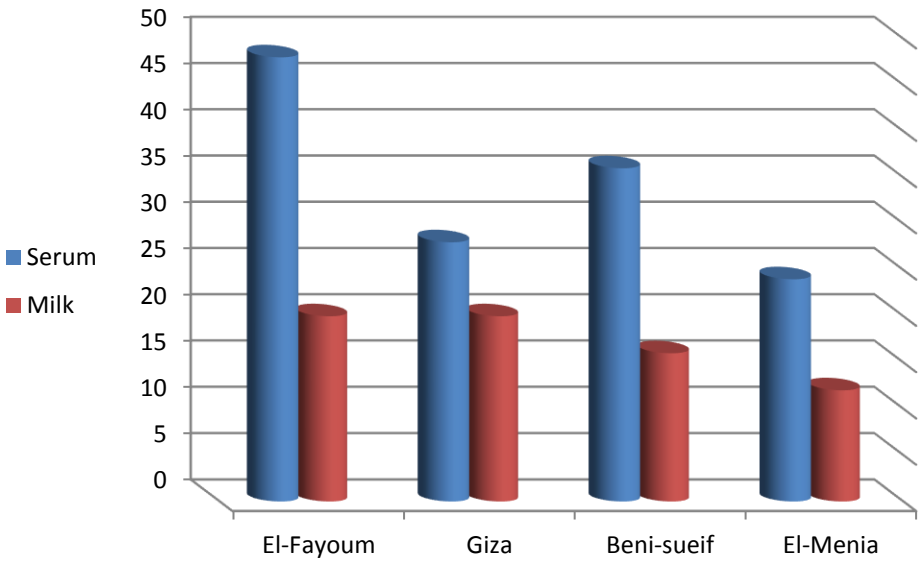

Fig (2): Incidence of *C.burnetii* in serum and milk samples collected from dairy cattle of Upper Egypt governorates by using ELISA

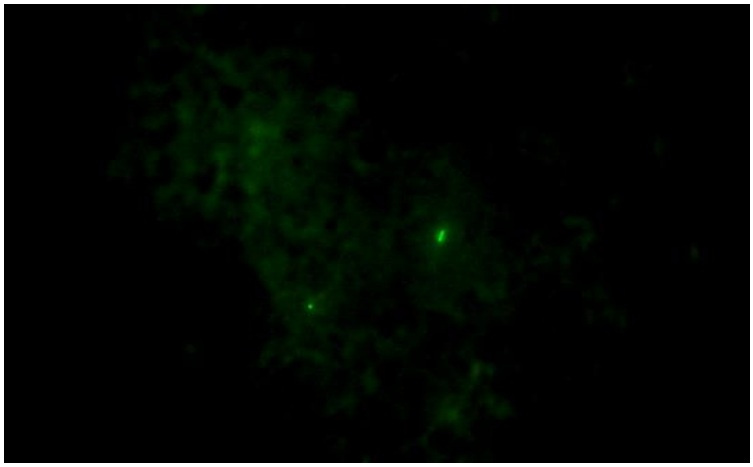

Fig (3): The green fluorescence of IgM to *C.burnetii* phase II under ultraviolet light at a magnification of 400x by fluorescence microscope

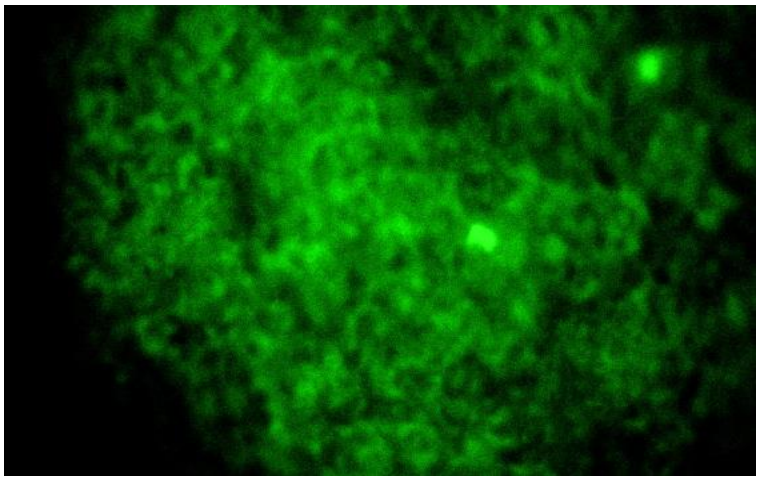

Fig (4): The apple-green fluorescence of IgG to *C.burnetii* phase II under ultraviolet light at a magnification of 400x by fluorescence microscope.

DISCUSSION

Coxiella burnetii and *Neospora caninum* are an important reasons of reproductive problems in cattle, they causes a various signs in animals like infertility, early embryonic death, lingering calving seasons, abortion and stillbirth (Anderson, 2007 and Adis *et al*., 2018).

The serological methods represented the most widely recognized, available and commonly used tools for the diagnosis of infectious diseases by detection specific antibodies (Garcia-Pérez *et al*., 2011), moreover, the presence or absence of these antibodies in the blood of the host allowing for distinguishing time dependent for acute and chronic infection in serological profile (Guatteo *et al*., 2011).

Our study demonstrated a different levels of antibodies for *N.caninum* and *C.burnetii* in serum and milk samples of upper Egypt governorates but no sample recorded a positivity for *Brucella* antibodies by rose bangal test and milk ring test, these results in accordance with Softic *et al*. (2018) who concluded that a high antibodies titer to *C.burnetii* and *N. caninum* was recorded but *Brucella* spp. didn't represent a risk in their study.

In this study the incidence of *N. caninum* were recorded in 29% of serum samples collected from four governorates in Egypt. (Table 2), these results in accordance with Fávero *et al*. (2017).

The incidence of *N. caninum* antibodies (IgG) in serum samples were reported in Beni-Sweif (36%), Giza (28%), El-Fayoum (28%) and Menia (24%) respectively by using ELISA test (Table 2 and fig 1). Lower results were recorded with (Kuruca *et al*., 2013 and Gerges *et al*., 2017).

While the incidence of *N.caninum* were recorded in 10% of in milk samples (Table 2). Our results in nearly similar with Schares *et al*. (2003) while the highest rates of infection was recorded in milk (26.66%) by Pritchard (2001).

Our results showed a differences in incidence of *N.caninum* antibody in four governorates of upper Egypt, This difference can be imputed to management and therapeutic system, water source, nutrition In addition to contacts of cattle with dogs, (Slapeta *et al*., 2002).

In recent researches, the epidemiological studies on *C. burnetii* depend on serological tools in diagnosis (Rousset *et al*., 2010), because isolation of this pathogen remains time-consuming, risky and requires restricted biosafety level 3 laboratories due to the zoonotic nature of the microorganism (Fournier *et al*., 1998 and Field *et al.,* 2000).

ELISA is easy to perform and is adapted for automation, on other hand the detection of IgG give an accurate diagnosis in determination the past infection of *C.burnetii* (Cowley *et al.,* 1992; Wegdam-Blans *et al*., 2012).

In our results, the positive antibody titer of *C. burnetii* was determined the chronic infection in 34% of serum sample and in 17% of milk samples by using ELISA test (Table 3 and fig.2), higher results were recorded by Hussien *et al*. (2012) and Dean *et al*. (2013) on other hand Gwida *et al*. (2014) recorded a lower incidence of C.*burnetii* in serum samples collected from upper Egypt farms ranged from 2.9 to 26.7%.

El-Fayoum was recorded a significant increase (P>0.05) for *C.burnetii* infection in serum and milk samples than other governorates (Table 3 and Fig. 2), these variation in infection rate may be back to several factors like ahost, weather conditions and the type of soil, (Fayer and Reid, 1982), also Adesiyun *et al*. (1996) reported that epidemiological studies differ in their design, laboratory and statistical analyses, types and number of samples which explained the limitation of the resemblance between results.

The extracellular infectious form of *C.burnetii* shed in milk, urine, and feces. It was found in high concentration (109 ID50/g) in placental tissue and amniotic fluid (Plummer, 2018). Once a domestic ruminant is infected, *C.burnetii* can localize in mammary glands, supra-mammary lymph nodes, placenta, and uterus (Agerholm, 2013).

It was noticed in current study that the incidence of *C.burnetii* in milk samples lower than serum samples although the samples were collected from the same animals (Table 3 and Fig. 2) this may be due the shedding of *C.burnetii* in milk discontinuous and always occur subsequent to parturition or abortion (Schmeer *et al*., 1987, Van den Brom *et al.,* 2012 and Agerholm, 2013).

The results illustrated in table (4) and fig(3&4) showed that the recent infection (IgM titre>1:24) was recorded in 11% of serum samples and in 4% of milk samples while chronic infection (IgG titer ≥1:64) was recorded in 19% of serum samples and in 8% of milk samples by using indirect immunofluorcent test (IFA), our results nearly similar to (Sobhy *et al*. 2016), higher results were obtained by Htwe *et al*. (1992) who detected antibodies to *C. burnetii* in 29.5% of milk samples collected from healthy cattle and 84.3% of cattle with reproductive diseases in Japan by using IFA.

Our study focus on detection of IgM and IgG titre to phase II *C.burnetii* antigens by IFA, because the titer of antibodies to this phase reached to high magnitude in short time and persisted for along period, our

results supported by Uhaa *et al*. (1994) who compared with IgM and IgG to phase I antigens and phase II antigens, he found that IgM and IgG titer to phase II *C.burnetii* antigens remains for long time circulating in blood of the host in contrary with antibodies to phase I antigens disappearing during the convalescent phases of the illness while antibodies to phase II antigen was observed in acute and chronic infection.

Peacock *et al*. (1983) found that the raising in IgM and IgG titer to phase II *C.burnetii* were enough to eliminate the false-positive reactions against related antigens. Also Huebner *et al*. (1949) found that animals may be responsible for maintenance the chain of infection. However, the ways of transmission still needed further investigation.

Our results showed that the positivity rates of IgG to *C.burnetii* in serum and milk samples were detected in 34% and 17% by using ELISA while they were 19% and 8% by using IFA respectively (Table 5). It was noticed that the sensitivity of ELISA was higher than IFA in detection of IgG to *C.burnetii*, these results were supported by many previous studies which reported that ELISA, it is critical test to eliminate a variety of false positive and negative results during detection of *C.burnetii* antibodies (Cowley *et al*., 1992, Uhaa *et al.,* 1994 and Waritani *et al.,* 2017).

In the present study, both antibodies of *C. burnetii* and *N. caninum* was declared in the same samples by ELISA test, with percentage 14% in serum samples and 4% in milk samples (Table 6). Our results supported by García-Ispierto *et al*. (2010) who concluded the ability of the sharing *Coxiella* and *Neospora* antibodies in the same samples. Softic *et al*. (2018) reported that *C. burnetii* and *N. caninum* are a frequent causes of decreased reproductive consequences and reduced the reproductive performance in cattle worldwide also the higher exposure to *C. burnetii* and *N. caninum* in different countries were a highlights need for targeted control of infectious causes of reproductive disorders in dairy cattle of the studied areas.

CONCLUSION

EILSA is an important tool in diagnosis of *N. caninum* and *C.burnetii* infection, the elevated altitudes of exposure to *C. burnetii* and *N. caninum* in this study need a targeted control for these infectious causes in dairy cattle. So, raising awareness is needed for animal owners, veterinarians, physicians, authorities and control strategies must be applied to protect against infection.

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الكشف عن الأجسام المضادة للنيوسبورا كانينم والكوكسيلا بيرنتي في البان وامصال دم الابقار الحلابة المصابة

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تعتبر الكوكسيلا بيرنتي والنيوسبورا كانينم من المسببات الشائعه لإنخفاض الإنتاج عند الأبقار حول العالم ولذلك هدفت دراستنا الي تقييم تواجد الأجسام المضاده (IgG) لتشخيص الكوكسيلا بيرنتي والنيوسبورا كانينم باستخدام اختبار الإليزا كما هدفت الدراسه ايضا مقارنة مدى حساسيه كلا من الإليزا والإمينوفلوريسنس في الكشف عن الأجسام المضاده لميكروب الكوكسيلا بيرنتي، ولتحقيق هذا تم تجميع ٢٠٠عينه من الالبان وامصال دم الابقار (١٠٠ لكل نوع) كما تجميع هذة العينات من ١٠٠ بقره تعاني من العقم والاجهاض خلال الفتره من شهر يناير وحتي شهر ديسمبر ٢٠١٧ في اربع محافظات من محافظات مصر العليا بعد تجميع العينات تم نقلها للمعمل للفحص السيرولوجي، وقد اظهرت النتائج تواجد الاجسام المضاده للنيوسبورا كانينم في ٢٩% من عينات مصل الدم و١٠% من عينات الالبان بينما تم الكشف عن الاجسام المضاده للكوكسيلابيرنتي في ٣٤% و١٧% من عينات امصال الدم والالبان كلا على التوالي ،كما وجد ان الاجسام المضاده لكلا من النيوسبورا والكوكسيلا قد تواجدت معا في نفس عينات امصال الدم والبان الأبقار بنسبه١٤% و٤% على التوالي من الأبقار في اربع محافظات من مصر العليا باستخدام اختبار الإليزا. كما وضح اختبار الامينوفلورسنس عن وجود عدوي حاده لميكروب الكوكسيلا بيرنيتي بالكشف عن الأجسام المضادة (IgM) في ١١% و٤% من عينات امصال الدم والبان الأبقار على التوالي بينما اظهر اختبار الامينوفلورسنس وجود العدوي المزمنه لميكروب الكوكسيلا بيرنيتي بالكشف عن الأجسام المضادة (IgG) في ١٩% من عينات امصال الدم و٨% من عينات الالبان، وبالمقارنه بين حساسيه كلا من اختبار الإليزا والامينوفلورسنس في الكشف عن الاجسام المضاده (IgG) وجد أن اختبار الإليزا أكثر دقة في تشخيص عدوى الكوكسيلا.