



## Detection of *Neosporium Caninium* in aborted fetuses in a dairy farm in Alexandria governorate

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### Abstract

A dairy Holstein cattle farm complained of cases of abortion at late stages of pregnancy (7 to 9 months) 75/500 pregnant cows (15%). The farm regularly vaccinated for BVDV, IBR, and *Leptospira* with the cattle master vaccine (Zoetis). Additionally, no positive reactors were recorded during routine testing by the rose Bengal agglutination test for bovine brucellosis. A total of 3 aborted fetuses were sent to the infectious diseases laboratory, Suez Canal University for laboratory testing. The internal organs (Brain, liver, lung, and lymph nodes) were collected from the fetuses and tested by quantitative PCR for BVDV, BHV-1, Brucellosis, and leptospirosis; and nested PCR for *Neosporium caninum* (*N. caninum*). The tested samples were negative for BVDV, BHV-1, *Leptospira* and *Brucella* but, positive for *N. caninum*. The further sequencing of positive samples confirmed infection by *N. caninum*. The farm owner stated that there are about 15 dogs reared on the same farm and their feces may contaminate the silage. Also, the cases of abortion decreased after removal of dogs from the farm.

In conclusion, Removal of dogs from dairy farms is crucial to prevent abortion due to *N. caninum*.

## Introduction

Abortion has a negative impact on animal resources due to loss of pregnancy between the age of 42 days and approximately 260 days (Hovingh, 2009). There are several causes of abortion in cattle that are infectious such as bacteria, viruses, fungi, and protozoa in addition to the non-infectious as toxicity, trauma, metabolic causes, hormonal abnormalities, and unknown causes (Anderson, 2007; Kirkbride, 1992; Yaeger, 1993). The most common infectious causes of abortion are either bacteria such as *Brucella abortus*, *Campylobacter spp*, *Chlamydomphila abortus*, *Leptospira spp*, *Coxiella burnetii*, *Salmonella spp*, and *Listeria monocytogenes* (Truyers et al, 2014) or viral such as bovine viral diarrhea virus (BVDV), bovine herpes virus 1, Schmallenberg virus (Chastant-Maillard, 2015) or parasitic infestation such as Neosporosis and Trichomoniasis (Almería & López-Gatius, 2013) or fungal causes such as *Aspergillus sp.* (Knutson & Kirkbride, 1992).

*Neospora caninum* is an obligate intracellular cyst forming protozoan that was first described in dogs by (Bjerkås, Mohn, & Presthus, 1984), and it was identified in a wide range of warm-blooded animals, including many wildlife species. *N. caninum* is considered one of the main causes of abortion in cattle worldwide (Dubey & Schares, 2011), with widespread occurrence of neosporosis in beef cattle, dairy cattle, or both, in most provinces in Canada (Haddad, et al 2005 ), South America (Moore,2005), Australia (Atkinson et al., 2000), Europe

(Bartels et al., 2006; De Meerscham, et al 2000).

Antibodies of *N. caninum* were first identified in Egyptian camels by Selim and Abdelhady (2020) (Selim & Abdelhady, 2020). Additionally, Selim et al., (2021) (Selim, Khater, & Almohammed, 2021) confirmed the presence of *N. caninum* among sheep in Egypt that caused reproductive failure in sheep (Gaber et al., 2021). Investigated abortion among cattle in Kafrelsheikh governorate caused by *N. caninum* (Ahmed et al., 2017). All the previous studies on *Neospora caninum* in Egypt were focused only on seroprevalence; therefore, this study aimed to investigate abortion in a dairy cattle farm and to directly detect *N. caninum*.

## Materials and Methods

### *Farm complains and samples:*

A dairy Holstein cattle farm complained from increased cases of abortion, and retained placenta. The farm vaccine history and management data were collected. Internal organs (brain, lung and liver) from 3 aborted fetuses were sent to infectious diseases laboratory, faculty of veterinary medicine, Suez Canal University for further laboratory testing. The samples from each fetus were pooled and proceeded by grinding in a mortar and pestle.

### *Molecular testing:*

The total nucleic acid was extracted by QIAamp DNA mini extraction kit according to the manufacturer. The extracted nucleic acid was tested by probe based quantitative real time PCR for Bovine viral diarrhea virus (BVDV), bovine herpes virus

1, leptospira, and brucellosis as previously described (Baxi et al., 2006; Bounaadja et al., 2009; Palaniappan et al., 2005; Wang et al., 2007). Furthermore, the DNA was tested for *N. caninum* by semi-nested PCR as previously described (Baszler, et al., 1999). All the quantitative real time assays were performed in 7500 Applied Biosystems. The positive controls for each assay were prepared by cloning of positive insert in plasmid and confirmed by Sanger sequencing. The cycling conditions were performed as previously published. The sequences of used oligonucleotides and hydrolysis probes are shown in table (1). The positive samples by PCR for *N. caninum* were sent to Solgent Co. Ltd for purification and sequencing.

#### **Serological testing:**

A total of 12 serum samples from aborted cows were tested by neospora Ab ELISA (IDEXX laboratories) according to manufacturer.

## **RESULTS**

### **Farm history**

75 pregnant cows were aborted from a total of 500 pregnant cows (15%). The investigated farm was regularly vaccinated with the Cattle master vaccine (Zoetis) for BVDV, IBR, BRSV and PI3. Also, the farm was tested every 6 months by rose Bengal agglutination test, and no positive reactors were reported.

### **PM lesion**

PM testing revealed hyperemia in brain and intestine (figure 1 and 2).

### **Molecular testing:**

The testing by quantitative real time PCR showed negative results for BVDV, IBR and brucellosis. The collected samples were positive by nested PCR for *N. caninum* (Figure 3). The sequencing and trimming of positive samples by nested PCR confirm detection of *N. caninum*.

### **Serological testing:**

The tested 12 serum samples from aborted cows were positive for *N. caninum* by ELISA (IDEXX laboratories)

## **Discussion**

Infection by Neosporiosis associated with significant economic losses that can be classified as direct losses from abortion, control costs, and loss of milk and meat. Additionally, the indirect losses from infection include: costs of rebreeding aborted animals, increase lactation time, decrease milk production, and costs of removal of carrier animals (Bartley et al., 2019; Hernandez, et al., 2001). It was estimated that infection by *Neospora caninum* was responsible for about 564.3 \$ million dollars annual losses in the dairy industry in the USA (Reichel, et al., 2013).

There are many laboratory assays that can be used for the diagnosis of neosporiosis with many pros and cons. For example, pathogen culturing or histopathological examination (Yang, et al., 2012); culturing is characterized by low sensitivity, time consuming and requirement for special laboratory procedures. Histopathological examination can be used for definitive diagnosis, but it requires fresh aborted

samples, which is not possible in many situations (Kirkbride, 1992). Immunohistochemistry (IHC) also can be used for diagnosis, but it is a less sensitive technique (Baszler et al., 1999; Dubey, 1999). Serological assays such as IFA and ELISA are commonly used assays for routine diagnosis, but using serological assays alone to confirm the diagnosis is not sufficient (Nematollahi, Moghaddam, Jaafari, Helan, & Norouzi, 2013), the IFA is a gold standard serological test and is characterized by high specificity. On the other hand, indirect ELISA is the commonly used serological technique, but there is possible cross reactivity with other protozoa such as *T. gondii* and sarcocysts (Dubey et al., 1996). Overall, serological testing indicates exposure of the herd to *N. caninum* but it is not a definitive diagnosis in case of abortion and other direct detection techniques of protozoa in aborted materials or detection of specific lesions are crucial to confirm that *N. caninum* is the causative agent of abortion. Molecular assays as PCR are highly sensitive and specific techniques for the detection of *N. caninum* in aborted materials; however, careful interpretation is essential due to other causative agents of abortion should be considered. Therefore, it is preferred to combine PCR with other assays as IHC to confirm the association between exposure to *N. caninum* and abortion (Amouei et al., 2019; Dubey, 1999; Dubey et al., 1996; Nematollahi et al., 2013).

In this study, a dairy cattle farm suffered from an increase abortion rate and asked the technical support. The collected

samples were negative for BVDV, IBR, and leptospira by quantitative PCR. This finding agreed with the farm history that these pathogens are under control by the regular usage of cattle master vaccine. Additionally, the tested samples are negative for brucellosis by quantitative PCR; this finding also agreed with the farm history of the absence of any positive reactors for brucellosis by routine testing.

The tested samples were positive by semi nested PCR for *N. caninum*; moreover, the serum samples were positive by indirect ELISA. The results of combined assays confirm the association between abortion and exposure to *N. Caninum*. Our results agreed with the farm's history of the existence of many dogs in the farm and possibility of contamination of animal foods by dog feces. Based on our recommendation that the main cause of abortion is due to *N. caninum*; the farm owner removed dogs from the farm and the rate of abortion decreased. To our knowledge, this is the first study to confirm the direct detection of *N. caninum* in aborted fetuses by PCR.

## Conclusion

This study confirms the infection of one dairy farm by *N. caninum* by testing by semi nested PCR and indirect ELISA. Further studies are required to know the prevalence of *N. caninum* in Egypt and to estimate the economic losses of this pathogen to implement the proper control program.

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**Table (1): nucleotide sequence of oligonucleotides and dual labelled hydrolysis probes used in this investigation.**

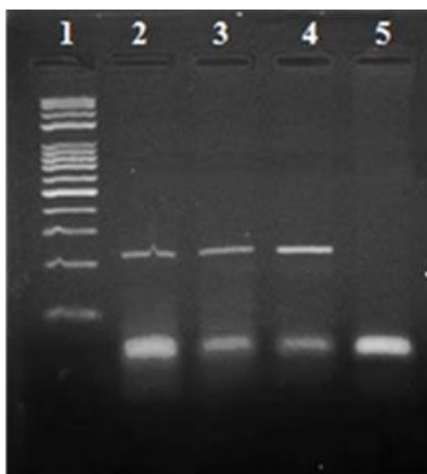
Pathogen	Oligonucleotide and probe ID	Sequence	Amplicon size	Reference
BVDV	Pesti-F	5'-CTAGCCATGCCCTTAGTAG-3'	104 bp	[19]
	Pesti-R	5'-CGTCGAACCAGTGACGACT-3'		
	BVDV1- probe	5'-FAM-TAGCAACAGTGGTGAGTTCGTTGGATGGCT-BHQ1-3'		
	BVDV2- probe	5'-VIC-TAGCGGTAGCAGTGAGTTCGTTGGATGGCC-BHQ1-3'		
BHV-1	bGH-F (forward)	5'-CCTTCGGCCTCTCTGTCTCTC-3'	87 bp	[20]
	bGH-R (reverse)	5'-TTGTCATAGGTCTGCTTGAGGATCT-3'		
	Probe (bGH)	5'-FAM-TCCCTTGGCAGGAGC- BHQ1-3'		
Leptospira spp.	LigConF	5'-CCGAATATTCCTCTCGGAAA-3'	230 bp	[21]
	LigConR	5'-AAGGCTGCTGGAGTAACGAT-3'		
	Ligprobe	5'-FAM-CGCTAATATTCAAAAACAACGG- BHQ1-3'		
Brucella	IS421	5'- CGCTCGCGCGGTGGAT-3'	178 bp	[22]
	IS511	5'-CTTGAAGCTTGCGGACAGTCACC-3'		
	ISTq:	5'-FAM-ACGACCAAGCTGCATGCTGTTGTTCGATG- BHQ1-3'		
N. caninum	Np4	5'CCTCCCAATGCGAACGAAA-3'	227 bp	[23]
	Np6	5'-CAGTCAACCTA CGTCTTCT-3'		
	Np7	5'-GGGTGAACCGAGGGAGTTG-3'		

**Figures**

Figure 1. Hyperemia in intestine of an aborted fetus



Figure 2. Hyperemia in the brain of an aborted fetus

Figure 3. Nested PCR testing for *N. caninum*.  
Lane 1: 100 bp DNA Ladder  
Lane 2, 3 and 4: positive samples  
Lane 5: Negative control

## المخلص العربي

## الكشف عن النيوسبورا كانينم في الأجنة المجهضة بمزرعة ألبان بمحافظة الإسكندرية

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اشتكى مزرعة ماشية حلاب هولشتاين من زيادة حالات الإجهاض في المراحل المتأخرة من الحمل (7 إلى 9 أشهر) حوالي 500/75 من الأبقار الحوامل (15%). و تطعم المزرعة بانتظام ضد فيروس الاسهال البقري الفيروسي و فيروس الهربس البقري من النوع الاول وميكروب اللبتوسبييرا عن طريق لقاح cattle master المصنع في شركة زويتس. بالإضافة إلى ذلك ، لم يتم تسجيل أي حالات إيجابية أثناء الاختبار الروتيني باختبار الروز بنجال لمرض البروسيليا البقري. تم إرسال 3 أجنة مجهضة إلى معمل الأمراض المعدية بجامعة قناة السويس للفحص المعمل. تم جمع الأعضاء الداخلية (الدماغ والكبد والرئة والعقد الليمفاوية) من الأجنة واختبارها بواسطة اختبار البلورة المتسلسل الكمي لفيروس الاسهال البقري الفيروسي و فيروس الهربس البقري من النوع الاول وداء البروسيليا وداء اللبتوسبييرا. و الفحص باختبار البلورة المتسلسل الكمي لميكروب النيوسبورا كانينم. كانت العينات المختبرة سلبية بالنسبة لفيروس الاسهال البقري وفيروس الهربس البقري من النوع الاول وميكروب البروسيليا واللبتوسبييرا ولكنها كانت إيجابية بالنسبة للنيوسبورا كانينم. أكد التسلسل الجيني للعينات الإيجابية الإصابة بالنيوسبورم كانينم. وذكر صاحب المزرعة أن هناك حوالي 15 كلباً تمت تربيتها في نفس المزرعة مع احتالية تلوث الاعلاف بروث الكلاب. انخفضت حالات الإجهاض بعد إخراج الكلاب من المزرعة. على حد علمنا ، هذه هي الدراسة الأولى التي تكشف مباشرة عن النيوسبورا كانينم من الأجنة المجهضة بواسطة تفاعل البلورة المتسلسل. في الختام ، يعتبر إبعاد الكلاب من مزارع الألبان أمراً بالغ الأهمية لمنع الإجهاض بسبب النيوسبورم كانينم.