First isolation of *Neospora caninum* in dogs in Al-Muthana Province, Iraq

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Abstract

A total of 180 fecal samples were collected from farm dogs during march -2010 and June-2011.Fecal samples of dogs were examined microscopically for detecting *Hammondia-Neospora* like oocysts (HNLO) by direct method and flotation method using Sheathers solution . HNLO were detected microscopically in 12 fecal samples . The fecal samples with HNLO were examined by *N. caninum* species-specific PCR, three of the samples were positive for *N. caninum*(1.6 %). This is the first report of finding *N.caninum* DNA or oocysts in feces of farm dogs in AL-Muthana Province, Iraq.

1- Introduction :

Neosporosis is a major cause of abortion in cattle(Dubey and Lindsay,1996;Dubey *et al.*,2007). *Neospora caninum* was classified in the family Sarcocystidae, subclass Coccidiasina of the phylum Apicomplexa (Ellis *et al.,* 1994). This parasite is structurally very close to *T. gondii* (Barr *et al.,* 1997) . There are two transmission routes for *Neospora caninum* in cattle, the first route is vertical or transplacental transmission. In cattle, transplacental appears to be the major route of transmission from infected dams to off spring infection, the second route is referred to as horizontal or postnatal transmission

that occurs in cattle after ingesting sporulated *N. caninum* oocysts . (Wouda .,2007).

Dogs and coyotes are definitive hosts for *N. caninum* (Gondim *et al.*, 2004). Dubey *et al.,* (2007) found that the dogs can transiently shed oocysts up on ingestion of *N. caninum* infected tissues of intermediated hosts .

Neosporosis occurs worldwide, cases have been reported from Europe, USA, Canada, Australia, South Africa, Japan, and Costa Rica(Dubey and Lindsay, 1996).Seroprevalence of *N. caninum* infection varies from 0.5% to 17% in Europe, Barber *et al.*,(1997) reported seropositive rate 11% in 300 Belgian dogs .In Liverpool Trees *et al.*,(1993) found 17% of 163 dogs in an indirect fluorescent antibody test (IFAT) while Bjorkman *et al.*,(1994) found 0.5% of 398 dogs were seropositive in an iscom-enzyme-linked immunosorbent assay . Recently, *N.caninum* DNA has been found in the faeces of coyotes and foxes in Canada (Wapenaar *et al.,* 2006).

Diagnostic molecular techniques, such as PCR, offer a highly sensitive and specific alternative to morphological methods (Orlandi & Lampel, 2000).

In Iran Razmi (2009) reported the prevalence of *Neospora* oocysts in feces of dogs from dairy farms. A total of 174 fecal samples were collected from 89 farm dogs and 85 household dogs ,*Hammondia –Neospora* like oocyst(HNLO) were detected microscopically in 4 fecal samples (2.2%). the fecal samples with HNLO were examined by *N. caninum*-specific PCR. two of the samples were positive for *N. caninum* so that the aim of this study is to determine the prevalence of *Neospora* oocysts in feces of dogs from dairy farms.

2- Materials & Methods:

2-1 Fecal Examination:

The study was done in Al-Muthana province on 180 dogs ,their aged were between 3 m- 10 year (Wetering,2011) , during the period of March 2010 to June 2011. Fecal samples were collected from dogs and examined by direct

method (by using Lugol's iodine) and by flotation method(Seathers solution) to detect of *Neospora caninum* Oocysts, the oocysts were measured with a calibrated ocular micrometer using bright-field microscopy. (Thienpont *et al.*,1979). The oocysts with a diameter of 11.5 ± 1.5 um which exhibit morphology similar to non-sporulated *T. gondii*-oocysts were considered to be positive for *Hammondia-Neospora* like oocysts (HNLO). (Schares *et al.* ,2005). The positive samples for HNLO were reexamined by sedimentation and flotation methods to detect sporulated oocysts in these samples. Number of isolated oocysts was estimated by microscopic examination The fecal samples containing oocysts were mixed with 2% potassium dichromate in a Petri dish, and incubated and aerated at room temperature for 3-5days. (Schares *et al.* ,2005)

2-2- Polymerase Chain Reaction :

Polymerase chain reaction (PCR) assay for sporulated oocysts of *Neospora caninum* was performed according to method described by Wapenaar *et al.,* (2006) as following steps:

2-2-1 - Genomic DNA Extraction :

Genomic DNA of sporulated oocysts was extracted by using (QIAamp® DNA Stool Mini Kit. USA) and this kit was done according to manufacturer’s instructions .

2-2-2-Preparation of PCR Master Mix Reaction

PCR master mix reaction was prepared by using (AccuPower PCR PreMix Kit. Bioneer) and this master mix was done according to manufacturer’s instructions as following table:

Table (1) Components of PCR Master Mix Reaction:

PCR Master mix reaction components		Volume for one PCR reaction
PCR PreMix* (Lyophilized)		5ul
DNA template		5ul
Primers	Np6. primer	1.5ul
	Np21. primer	1.5ul
PCR water		7ul
Total volume		20ul

* : PCR PreMix it is lyophilized materials that is found in standard PCR tubes containing all other components which needed to PCR reaction such as (Taq DNA polymerase, dNTPs, Tris-HCl pH: 9.0, KCl, $MgCl_2$,stabilizer, and tracking dye).

The amplification conditions of PCR for *N. caninum* were performed according to method described by Razmi, (2009) that included an initial enzyme activation of denaturation at 95°C for 5 min, 40 cycles with denaturing at 94°C for 60 sec, primer annealing at 63°C for 60 sec and extension at 74°C for 3.5 min, followed by final extension at 74°C for 10 min. PCR Products were chilled at 4°C. primers were used to detect *N.caninum* for the PCR (Np6+): 5′-CAG TCA ACC TAC GTC TTCT, and the reverse (Np21+): 5′- GTG CGT CCA ATC CTG TAAC-3′were used for amplification reaction.
(Yamage *et al*,1996).

2-2-3- PCR Product Analysis

The final PCR products were subjected to gel electrophoresis as following steps:

1- A 1.5 gm agarose gel was prepared in 1X TBE buffer to get final 100ml of 1.5% of agarose gel. Then dissolved at 100ºC in hot starrier for 15minutes until boiling. After that, left to cool at 50ºC.

2- A 3uL of Ethidium Bromide were added to agarose gel solution and mixed well, then the tray was adjusted and the comb placed at proper position, then the agarose poured in tray and left until solidified, then the comb has been removed.

3- A 6uL of PCR product per each sample was loaded in agarose well as well as 6uL of 100bp ladder loaded in one well. Then the agarose gel filled by 1X TBE buffer and electrophoresis cover closed.

4- The electrophoresis device was runned at 100 volts and 80 A for 1 hour. After that bands of positive samples of *N. canium* were visible at 337bp in the PCR product on UV light.

3- Results :

In the present study , a total of 180 fecal samples of dogs were examined by direct and flotation methods, out of which 12 of which were suspected *Hammondia-Neospora* like Oocysts (HNLO) that the its measurement of Oocysts were ranged between (10-14um) x(10-11 um),The fecal samples with HNLO were examined by *N. caninum*-specific PCR. Three of these samples(5,10,12) were produced visible bands at 337 bp ,and these samples were positive for *N. caninum* (1.6%) produced in the PCR product, while samples(1,2,3,4,6,7,8,9,11) were consider negative samples and these samples didn’t show amplification . The present study we reported the first confirmed of Neosporosis in dogs in Iraq. (Table 1,Figure 1, 2,3)

Table(1):Shows percentage of infected dogs with *N.caninum*

Total No. of samples	No. of HNLO* positive Sample	No. of *N. caninum* specific PCR Positive Sample	Total % of *N. caninum* Positive of PCR
180	12	3	1.6

**Hammondia Neospora*-like oocysts

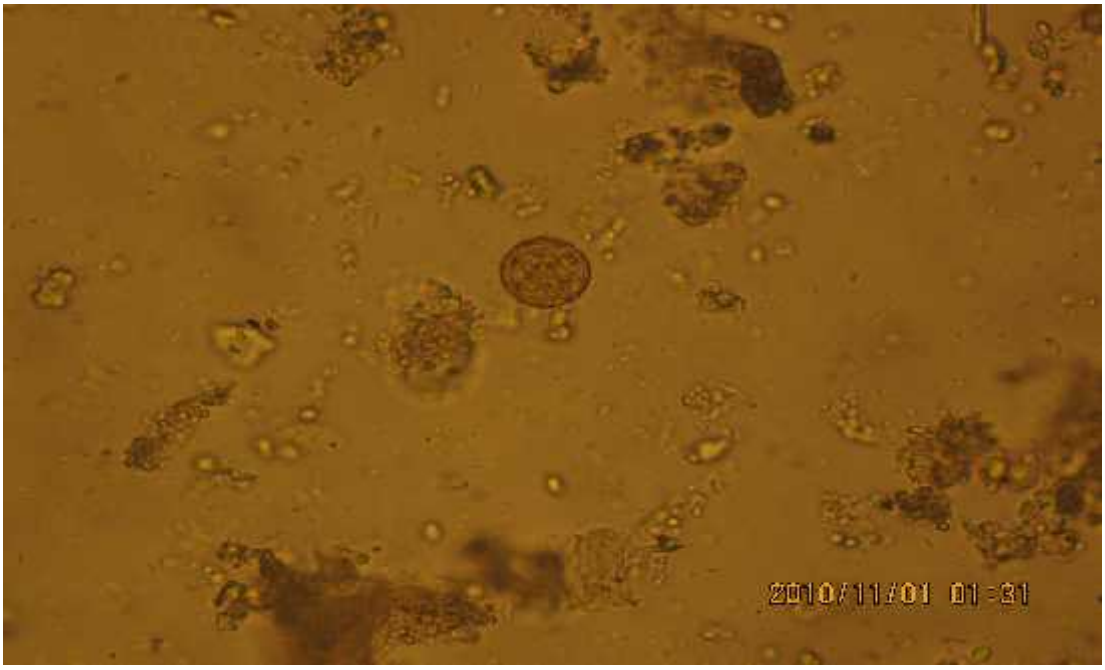


Figure (1):Showing Unsporulated Oocyst of *Neospora caninum* (40x).

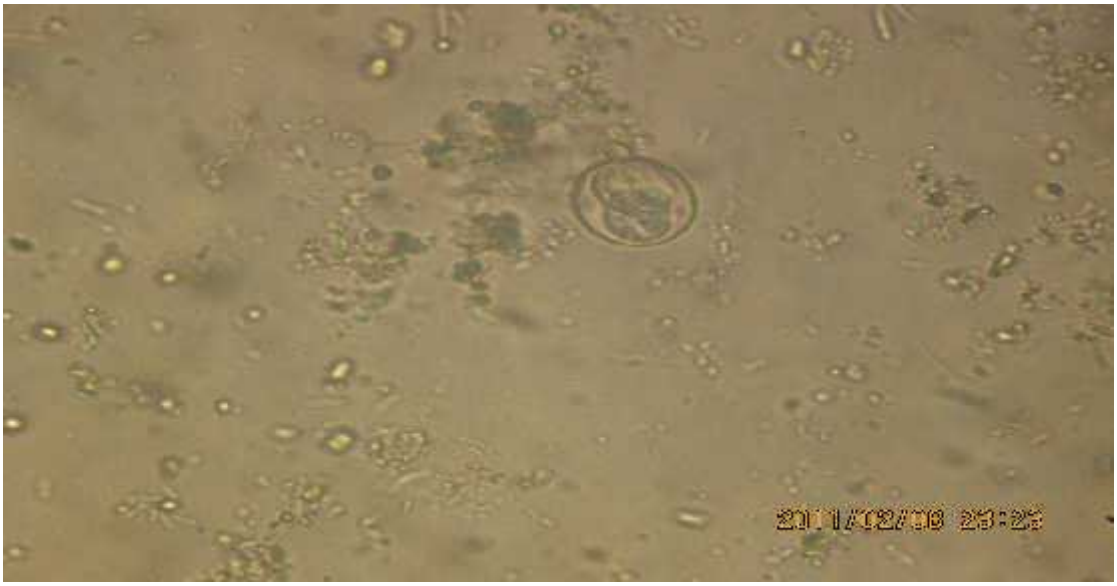
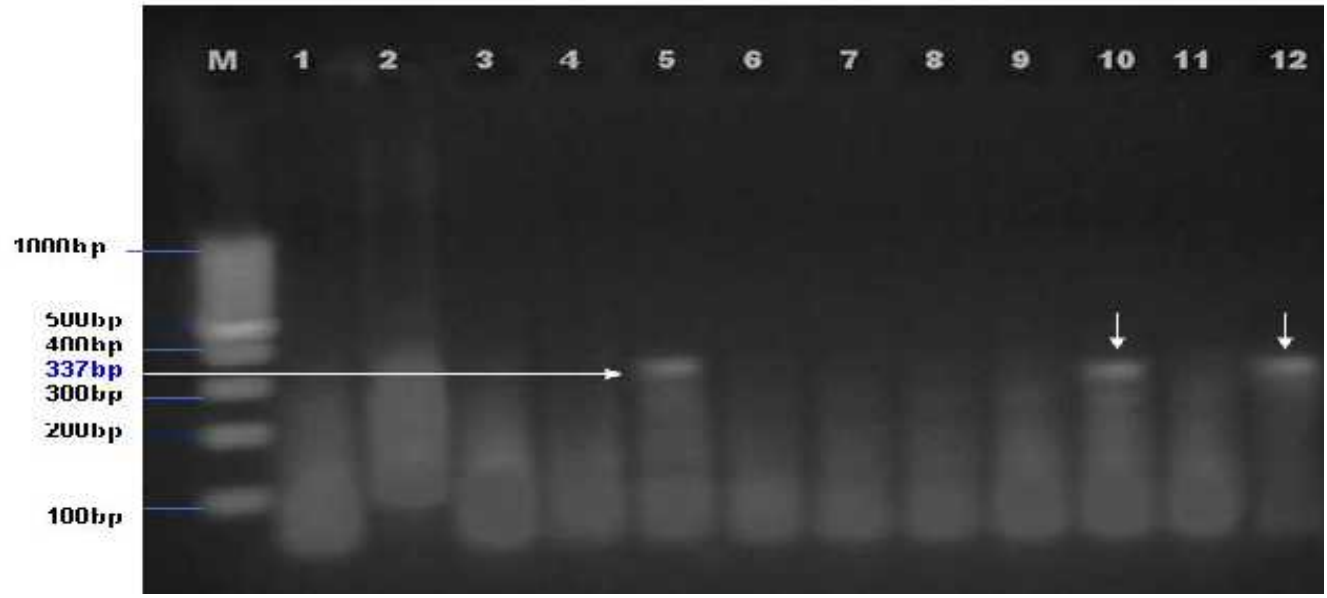


Figure (2):Showing Sporulated Oocyst of *Neospora caninum* (40x).

Figure (3): Shows bands of positive sample of *Neospora caninum* in infected dogs that at 337 bp in the PCR product.



-Lane M: Marker.
- Lane 1, 2,3,4,6,7,8,9,11 (Negative samples).
- Lane 5,10,12 (Positive samples of *N.caninum*).

4-Discussion :

Neosporosis is a parasitic disease caused by *Neospora caninum*, it is recognised intracellular protozoan parasite of dog and livestock distributed worldwide. In cattle it is considered one of the main causes of abortion (Dubey *et al.,* 2006).Many Seroepidemiological studies were done for detecting *Neospora caninum* infection in dogs worldwide(Dubey *et al,* 2007). Seropositivity in dogs is a primarily indicator for a past or recent contact with the parasite, but cannot be correlated to shedding of oocysts. The majority of dogs shedding *N. caninum* oocysts after experimental infection do not seroconvert in serologic examination (Lindsay *et al,*1999 ;Schares *et al,* 2001; Gondim *et al,* 2002).Therefore, it is important to properly identify the *N. caninum* oocysts in fecal samples. So far, there are only a few reports of *N. caninum* oocyst shedding by naturally infected dogs (Basso *et al,* 2001; McInnes *et al,*2006;Razmi,2009).In this study, HNLO were found only in 12 fecal samples of farm dogs, and also, DNA of *Neospora* was detected in 3 of which by *N. caninum*-specific PCR. The DNA of *Neospora* or *N. caninum* oocysts in the fecal samples may be due to feeding of fresh and uncooked infected meat.The *N. caninum* species-specific primers based on the Nc5 gene region produced a positive result, and these species-specific primers Np21/Np6 that generate single band in the presence of at least 10 pg genomic parasite DNA as template (Yamage *et al,*1996).In the present study, positive samples of *N. canium* showed visible bands at 337 bp in the PCR Product, this result agree with study was carried out by Razmi (2009) and Wapenaar *et al.*, (2006).

In Conclusion, this results showed that the presence of farm dogs may be a risk factor for *N. caninum* infection in dairy farms in AL-Muthana province-Iraq However, to confirm this hypothesis, a further molecular characterization of extracted DNA and isolation of parasites in gerbil bioassay or in cell culture is needed.

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عزل طفيلي *Neospora caninum* لاول مرة في الكلاب في محافظة المثنى - العراق

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الخلاصـــة

استهدفت الدراسة تحديد انتشار أكياس البيض لطفيلي *Neospora caninum* في براز الكلاب في حقول أبقارا لحليب ، حيث تم جمع عينات براز من الكلاب خلال الفترة من مارس ٢٠١٠ – حزيران ٢٠١١ . تم فحص عينات البراز مجهرياً لاكتشاف أكياس البيض المشابهة لطفيلي *Hammondia-Neospora* وذلك باستعمال طريقة الفحص المباشر وكذلك طريقة التطويف باستخدام محلول شيذر، تم اكتشاف أكياس البيض المشابهة لطفيلي *Hammondia-Neospora* في ١٢ عينة براز ثم تم فحص العينات بواسطة تفاعل سلسة البلمرة (PCR) وتم الحصول على ٣ عينات موجبة لهذا الفحص وبنسبة ١ر٦ %. سجلت الإصابة بهذا الطفيلي وعزلة في براز الكلاب لأول مرة في العراق في محافظة المثنى.