SHORT COMMUNICATION

FIRST IDENTIFICATION OF *NEOSPORA CANINUM* IN THAILAND

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Abstract

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An immunohistochemical examination of aborted bovine tissues, collected from two dairy farms that had a high abortion rate (7%), detected the organism *Neospora caninum* (NC). Five out of 12 aborting cows (41.7%) were seropositive to NC using a competitive ELISA test. Neospora-tachyzoites were detected in the placenta of a seropositive aborting cow. Neither NC parasites nor cysts were detected in other fetal tissues. The identification of the Neospora-parasite and a high percentage of Neospora seropositive aborting cows, suggested NC as the possible cause of abortion. This is the first identification of the NC parasite in Thailand.

Keywords : abortion, bovine, *Neospora caninum*, immunohistochemistry, cELISA

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การตรวจพบโปรโตซัว นีโอสปอรา แคนินุ่ม ในประเทศไทย

รายงานการตรวจพบเชื้อ โปรโตซัวนีโอสปอรา แคนินุ่ม (*Neospora caninum*) ในลูกโคแท้งจากฟาร์ม โคนม 2 แห่งที่มีประวัติการแท้งลูกสูง (ร้อยละ 7) ด้วยเทคนิคอิมมูนโนฮิสโตเคมี ผลการตรวจซีรั่มแม่โคแท้งลูกจำนวน 12 ตัวด้วยวิธี competitive ELISA ตรวจพบภูมิคุ้มกันต่อ *Neospora caninum* 5 ตัว ผลการตรวจชิ้นเนื้อทางจุลพยาธิวิทยาและย้อมด้วยเทคนิคอิมมูนโนฮิสโตเคมีพบ tachyzoites ของ *Neospora caninum* ในเนื้อเยื่อรกของแม่โคแท้งลูก 1 ตัว แต่ไม่พบในเนื้อเยื่ออื่นๆ การตรวจพบเชื้อ *Neospora caninum* ในเนื้อเยื่อและภูมิคุ้มกันต่อเชื้อนี้ในแม่โคแท้งลูก คาดว่าเชื้อ *Neospora caninum* น่าจะเป็นสาเหตุหนึ่งของการแท้งลูก รายงานนี้เป็นรายงานการพิสูจน์ชี้ตัวเชื้อ *Neospora caninum* ครั้งแรกในประเทศไทย

คำสำคัญ : แท้ง โค นีโอสปอรา แคนินุ่ม อิมมูนโนฮิสโตเคมี คอมเพ็ททิทีฟ อีไลซ่า

Introduction

Neosporosis is a major cause of abortion in dairy cattle world wide (Dubey and Lindsay, 1996) and an annual economic loss due to this disease has been reported in New Zealand as (NZ$ 17.8 million), Australia (A$ 110 million) (Reichel, 2000) and in California (US$ 35 million) (Dubey, 1999[a]). The neospora parasite is highly prevalent in farm dogs (31.3%, Sawada et al., 1998) and in cattle (87%, Wouda et al., 1999) and herd infection is related to the presence of dogs on the farm (Wouda et al., 1999). Neospora also infects other animals such as sheep, goats, swine, horse, deer and cats (Dubey and Lindsay, 1996; Dubey, 1999[a]). In buffaloes, neospora prevalence can be as high as 68% and has been reported in Egypt (Dubey et al., 1998), indicating that these animals are a potential source of infection to cattle in Asia, where both cattle and buffalo are raised together. In Asian countries, both Thailand (Suteeraparp et al., 1999; Kashiwazaki et al., 2001; Chanlun et al., 2002), Vietnam (Huong et al., 1998) and Taiwan (Ooi et al., 2000) have reported the disease. In Thailand, the widespread presence of *N. caninum* (NC) (Suteeraparp et al., 1999; Kashiwazaki et al., 2001; Chanlun et al., 2002) in the country suggested that NC was a possible agent causing some abortions in dairy herds even though the parasite had not yet been identified. The objective of this study was to look for the NC parasite in aborted tissues.

Materials and Methods

Two dairy farms, in the Chonburi and Saraburi Provinces, having high abortion rates (7%) but with no clinical illness were chosen for the study. They were vaccinated annually against bovine diarrhoea virus disease (BVD), infectious bovine rhinotracheitis (IBR), bovine parainfluenza (PI3), and bovine respiratory syncytial virus disease (BRSV). Brucellosis was also absence from these farms, causing neosporosis to be suspected as a cause of the abortions. Blood and tissue samples from 12 aborted cows from these farms were collected (one from each) during November 2001 to

September 2002. The sera were separated and stored at -20°C until tested. Antibodies to *N. caninum* in the sera were detected by a competitive enzyme-linked immunosorbent assay (cELISA), using horseradish, peroxidase-labelled, NC-specific, monoclonal antibody, as found in the commercial test kit (VMRD, Pullman, USA). Optical density (OD) was determined with a microplate reader (Titertek Multiskan Plus, Finland) at 650 nm wave length. The percent inhibition of antibodies to the antigens was calculated according to the manufacturer's recommendations as:

% inhibition = 100 - [(sample OD x 100) / (mean negative control OD)]

The samples with values of ≥ 30% inhibition were regarded as positive and those with values of <30% inhibition as negative.

For histopathological examination, formalin-fixed, paraffin-embedded, aborted tissue sections (4-5 μm thickness) were stained with a routine hematoxylin and eosin stain (H&E). An avidin-biotin complex (ABC) method was used for immunohistochemical staining (IHC) in order to detect NC parasites. Briefly, formalin-fixed, paraffin-embedded tissues, including a positive control (the heart tissue of goat supplied by McAllister, Illinois, USA), were cut and deparaffinized. The endogenous peroxidase activity was blocked with 3%H_2O_2 in methanol. For antigen retrieval, the slides were placed in a container of 0.01M citrate buffer and heated in a microwave oven (1200W) for 5 min. Non-specific antigens were blocked with 10% bovine serum albumin. Primary rabbit anti-NC serum was applied to the slides (produced by inoculating rabbit 9L with NC-beef tachyzoites, McAllister) at a dilution of 1:10,000, in phosphate buffer saline (PBS) and kept overnight at 4°C. The biotinylated goat anti-rabbit IgG antibody (Dako, Denmark), at 1:400 dilution with PBS, was used as a secondary antibody. After the application procedures with the ABC kit (Dako, Denmark) and 3,3'-diaminobenzidine tetrahydrochloride (DAB, Sigma, USA), the slides were counterstained with Mayer's hematoxylin. PBS instead of primary antibody was used for the negative control slides. The histopathological and immunohistochemical staining was examined under a microscope.

Results and discussion

Five of the 12 aborted cows (41.7%) were seropositive to *N. caninum* (Table 1). The time in gestation for these seropositive cows ranged from 4 to 6 months and the percent inhibition ranged from 63.4% to 94.89%. Serological tests for other diseases were not done. The placenta of one seropositive cow (case no. 5, Table 1), having a high % inhibition (91.87%), was IHC-positive to NC.

Histopathologically, the characteristic lesions of Neospora infection, such as non-suppurative necrotic foci, (Wouda et al., 1997) were not found in the aborted fetal tissues of seropositive dams (brain, heart, liver, kidney, lung) and the placentas. No inflammatory responses were observed either in the heart, liver or kidney. The kidney of one fetus (case No. 1) from a seropositive cow was severely autolysed and lesions could not be examined. Postmortem bacterial contamination was observed in some loci of the lungs. Most of the placentas had necro-suppurative reactions and bacterial contamination was also found. In the IHC-positive placenta (case No. 5), the parasites were found as small clusters (Fig. 1a), scattered (Fig. 1b) or as a single tachyzoite (Fig. 1c). Tachyzoites in the sample tissue stained as well as the positive control but most of them were not as clearly demarcated as in the positive control tissue. This may have been due to autolysis or degradation of the organisms. This placenta, in H&E stained sections (Fig. 1d), showed a diffuse (not focal), non-suppurative inflammation and no NC tachyzoites could be identified.

Table 1. The serological and immunohistochemical results.

No.	Gestation (month)	Tissues collected	*Neospora* antibody (Dam sera)		I H C result
			% inhibition	result	
1	6	Heart, kidney, lung	84.36	+	-
2	8	Placenta	0.08	-	-
3	8	Placenta	5.5	-	-
4	6	Placenta	91.61	+	-
5	4	Placenta	91.87	+	+
6	7	Placenta	-5.53	-	-
7	6	Brain, heart, placenta,	92.39	+	-
8	7	Heart, kidney, placenta	11.37	-	-
9	3	Heart, placenta	-0.42	-	-
10	5	Brain, heart	9.07	-	-
11	-	Placenta, kidney	9.49	-	-
12*	6	Brain, heart, liver, lung, kidney, placenta	63.4	+	-

*repeat abortion (previous % inhibition value = 85.5%)

It has been known that the tachyzoites are most commonly found in the heart and liver (Wouda et al., 1998) and tissue cysts are found in the central nervous system (Dubey and Lindsay, 1996) but in this study, neither the organisms nor tissue cysts could be found in any of these organs. This may be due to the few samples examined or the scarcity of the parasites present. Only 2 fetal brains from aborted calves born to 5 seropositive cows were collected. One of them was in a stage of advanced autolysis. It could also be that a small number of NC in the tissues are difficult to find in H&E stained sections (Lindsay and Dubey; 1989; Dubey and Lindsay, 1996;). In addition to the rarity of the organisms in the tissues, autolysis makes it more difficult to find them in the histologic sections. Even the sensitivity of the most efficient methods such as IHC, for detecting NC, is low (Dubey, 1999[b]). Dijkstra et al (2001) reported that dogs shed oocysts after being fed NC-infected placentas but they were not able to identify the parasite in the placentas by the IHC method. There have been few reports on finding NC organisms in the placenta of infected cows (Shivaprasad et al. 1989; Fioretti et al. 2000; Bergeron et al. 2001) which supports belief in the difficulty of finding the parasite in the placenta. This is the first identification of *Neospora caninum* in Thailand.

The finding of NC parasites and a high percentage of seropositive aborted cows, with high % inhibition values, which were having regular vaccinations for important abortifacients, suggested that NC could be the cause of abortions in these cows. Further investigation is needed for the confirmation and epidemiological status of this disease so that preventive and control measures can be applied.

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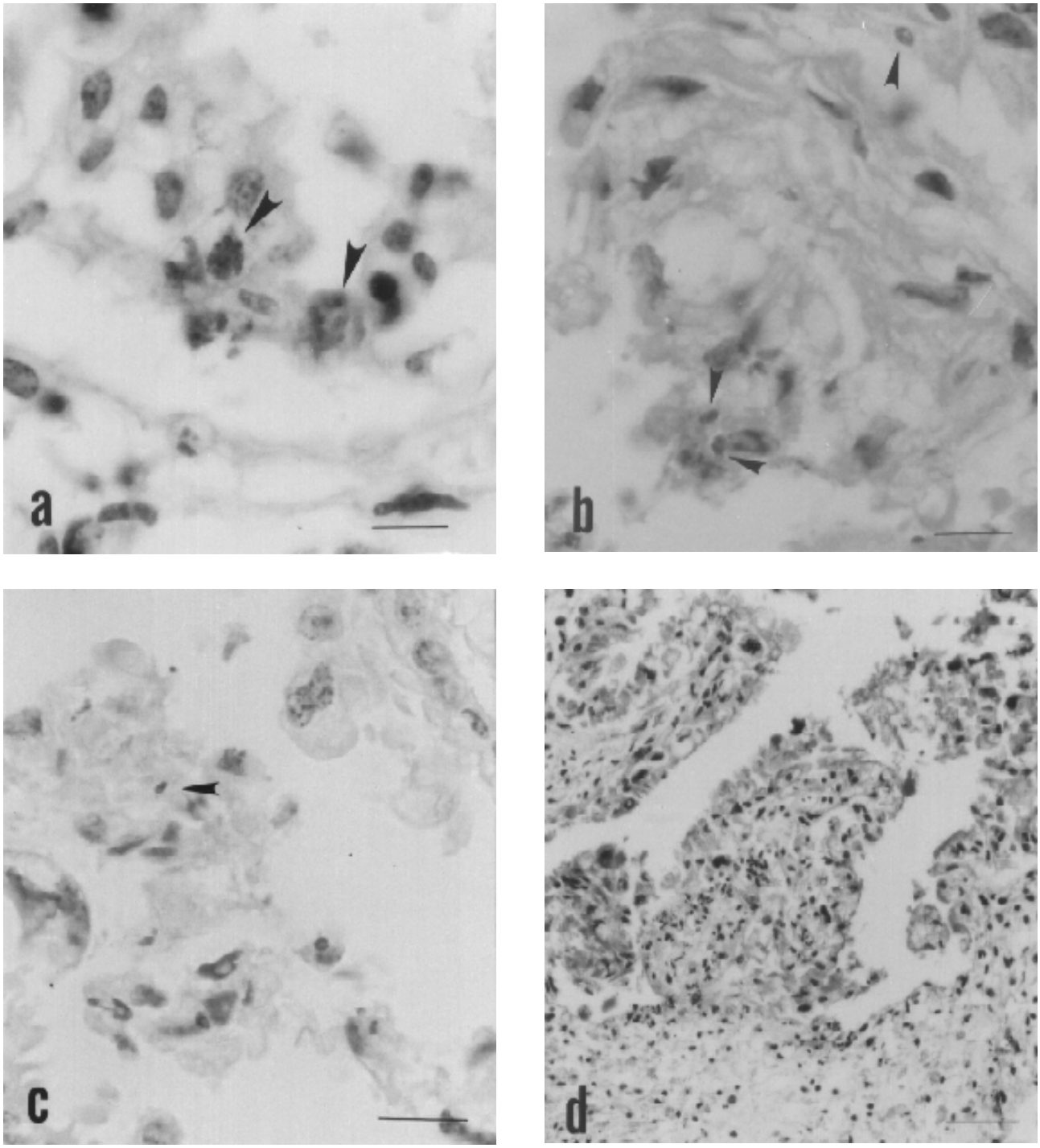

Fig. 1 a) Clusters of *N. caninum* tachyzoites (arrowheads),
b) Scattered tachyzoites (arrowheads) and
c) A single tachyzoite (arrowhead) found in the placenta, Case No. 5. (a to c: IHC method, hematoxylin counterstain; bar = 10 μm).
d) Non-suppurative necrosis of the placenta showing mononuclear cells infiltration (H&E stain; bar = 50 μm).

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