

An investigation of the concurrency of anti-*Neospora* antibody and parasitemia in water buffalo (*Bubalus bubalis*) in northwest of Iran

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Article Info	Abstract
<p>Article history:</p> <p>Received: 15 February 2018 Accepted: 24 July 2018 Available online: 15 March 2019</p> <p>Key words:</p> <p>Antibody ELISA <i>Neospora caninum</i> Polymerase chain reaction Water buffalo</p>	<p><i>Neospora caninum</i> is an obligate intracellular parasite causing abortion and reproductive failure in ruminants. Here, the seroprevalence of <i>Neospora</i> DNA and anti-<i>Neospora</i> antibodies and the correlation between the DNA and the antibody using polymerase chain reaction (PCR) and a new developed whole cell-based enzyme-linked immunosorbent assay (ELISA) in water buffalo (<i>Bubalus bubalis</i>) were investigated. To determine the level of anti-<i>Neospora</i> antibody, 83 serum samples were collected from buffaloes in the northwest of Iran. Plates were coated with 2×10^6 whole <i>Neospora</i> tachyzoites and the anti-<i>Neospora</i> antibody level was determined by calculating the ratio of sample/positive control (S/P) optical densities (ODs) in the ELISA. All samples with the ration of 0.50 or above were accounted as positive. To confirm the presence of <i>Neospora</i> DNA, the serum samples were directly subjected to PCR and nested PCR for detection of <i>Neospora</i> NC5 gene without the DNA isolation process. A total number of 83 buffalo serum samples were examined for the presence of anti-<i>N. caninum</i> immunoglobulin G and <i>Neospora</i> DNA. All samples with the S/P ratio of 0.50 or above (16 samples, 19.27%) were also positive for <i>Neospora</i> DNA. All samples with OD less than 0.50 (34 samples, 40.96%) were negative for <i>Neospora</i> DNA. However, 33 samples with the S/P ratio of bellow 0.50 (39.75%) showed a significant level of antibody. A 100% correlation was observed between high levels of the anti-<i>Neospora</i> antibody and <i>Neospora</i> DNA in the serum of water buffalo, and the whole <i>N. caninum</i> tachyzoites have the potency to be used as antigens for detection of the parasite in ELISA.</p> <p>© 2019 Urmia University. All rights reserved.</p>

ارزیابی همزمانی آنتی بادی ضد نئوسپورا و حضور خود انگل در خون گاومیش آبی (*Bubalus bubalis*) در شمال غرب ایران

چکیده

نئوسپورا کانینوم، یک انگل داخل سلولی اجباری است که موجب سقط و نارسایی تولیدمثلی در نشخوارکنندگان می شود. در این مطالعه، شیوع سرمی DNA نئوسپورا و آنتی بادی های ضد نئوسپورا و ارتباط بین DNA و آنتی بادی با استفاده از واکنش زنجیره ای پلیمرز (PCR) و یک روش جدید الایزا با استفاده از سلول کامل بررسی شد. برای تعیین سطح آنتی بادی ضد نئوسپورا، ۸۳ نمونه سرم از گاومیش ها در شمال غرب ایران جمع آوری شد. پلیت ها با تعداد 2×10^6 تاکی زوئیت کامل نئوسپورا پوشیده شده و سطح آنتی بادی ضد نئوسپورا با محاسبه نسبت تراکم های نوری (ODs) نمونه/شاهد مثبت (S/P) در الایزا تعیین شد. تمام نمونه های با نسبت ۰/۵۰ یا بالاتر به عنوان مثبت در نظر گرفته شدند. برای تأیید وجود DNA نئوسپورا، نمونه های سرمی جهت تشخیص ژن NC5 نئوسپورا، بدون فرآیند جداسازی DNA، به طور مستقیم با روش PCR و nested PCR بررسی شدند. در مجموع ۸۳ نمونه سرم گاوی برای بررسی وجود ایمونوگلوبولین G ضد نئوسپورا کانینوم و DNA نئوسپورا آزمایش شدند. تمام نمونه ها با نسبت S/P ۰/۵۰ یا بالاتر (۱۶ نمونه، ۱۹/۲۷ درصد) از نظر وجود DNA نئوسپورا نیز مثبت بودند. تمام نمونه ها با OD کمتر از ۰/۵۰ (۳۴ نمونه، ۴۰/۹۶ درصد) از نظر وجود DNA نئوسپورا منفی بودند. با این وجود، ۳۳ نمونه با نسبت S/P کمتر از ۰/۵۰ (۳۹/۷۵ درصد) سطح معنی داری از آنتی بادی را نشان دادند. ارتباطی ۱۰۰ درصدی بین سطوح بالای آنتی بادی ضد نئوسپورا و DNA نئوسپورا در سرم گاومیش آبی مشاهده شد و تاکی زوئیت های کامل نئوسپورا کانینوم دارای قابلیت به کارگیری به عنوان آنتی ژن برای تشخیص انگل در الایزا می باشند.

واژه های کلیدی: آنتی بادی، الایزا، گاومیش آبی، نئوسپورا کانینوم، واکنش زنجیره ای پلیمرز

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Introduction

Neospora caninum is now accounted as a major cause of abortion and congenital diseases in the dairy animal industry worldwide.¹ The infection can pass via placenta causing serious economic losses among dairy and beef cattle due to a decrease in milk and meat production.² In the *Neospora* life cycle, dogs play roles as definitive hosts and cattle and a wide range of domestic and wild animals including water buffalo (*Bubalus bubalis*) act as intermediate hosts.³ Water buffalo is naturally susceptible to *Neospora caninum* and can be experimentally infected with the parasite.⁴ Due to the economical importance of water buffaloes in the livestock industry, different surveys have been performed to study the seroprevalence of *N. caninum* in these merit animals around the world.^{5,6} The *N. caninum* infection is regularly diagnosed by serological tests performed on the blood samples like indirect fluorescent antibody test, agglutination test and enzyme-linked immunosorbent assay (ELISA). Due to the difficulties associated with a clinical diagnosis of neosporosis, serological tests are necessary for *Neospora* detection. These tests propose information about the seroprevalence of the parasite in herds, regions and countries.⁷ On the other hand, the interaction of the parasite with the immune system, the stages of parasitemia and the correlation between parasitemia and antibody production in water buffalo or even other intermediate hosts are still unclear. The northwest region of Iran is one of the most important poles of buffalo breeding in Iran and buffalo breeding plays an important role in the regional economy.

The main objective of this study was to determine the prevalence rate of *Neospora* infection among buffaloes in a survey study and illustrate the concurrency between the presences of anti-*N. caninum* antibodies and *Neospora* DNA in the serum of these animals using a new developed whole tachyzoite-based ELISA and polymerase chain reaction (PCR) techniques.

Materials and Methods

Samples. Blood samples were collected from 83 water buffaloes (32 males and 51 females) in Urmia abattoir in the northwest of Iran. The samples were centrifuged at 1600 *g* for 10 min and sera were collected and stored at -20 °C until used.

Parasites. The *N. caninum* tachyzoites were obtained from Razi Vaccine and Serum Research Institute, Shiraz, Iran. The parasites were washed twice in phosphate-buffered saline (PBS) and reproduced in Vero cell line (Razi Vaccine and Serum Research Institute, Shiraz, Iran) cultured in Dulbecco's modified Eagle medium (Bio-Idea, Tehran, Iran) containing 10.00% fetal bovine serum (BIO-IDEA, Tehran, Iran) and 100 mg mL⁻¹ gentamicin (Sigma-Aldrich, Stelnhelm, Germany).

ELISA. Tachyzoites were washed twice with PBS and counted by Neubauer chamber, then ELISA plates (Biofil, Indore, India) were coated with 2 × 10⁶ tachyzoites suspended in 1 mL PBS per well and incubated at 25 °C for three days. The plates were then washed three times with a washing buffer (PBS and 0.05% Tween 20 (Merck, Darmstadt, Germany) and then blocked with a blocking buffer (PBS and marvel milk 5.00%) at 37 °C for 1 hr. A number of serum samples collected from *Neospora* positive cows was used as positive controls and the blocking buffer was used as a negative control. The serum samples (positive controls or buffaloes samples) were first diluted in the blocking buffer (1:100) and put onto the plates in duplicate followed by incubation at 37 °C for 1 hr. After three times washing, horseradish peroxidase-conjugated sheep anti-bovine immunoglobulin G (IgG)-heavy chain antibody (Bethyl Laboratories, Montgomery, USA) was diluted in PBS (1:1000) containing 5.00% marvel milk and added to the plates incubating at 37 °C for 1 hr. After washing three times with the blocking buffer and two times with distilled water, a substrate including 3,3',5,5'-tetramethylbenzidine (Sigma-Aldrich), 10 µg mL⁻¹ dimethyl sulfoxide (Merck), 0.10% sodium acetate (Merck) and 10% hydrogen peroxide (Merck) was added and the plates were incubated at room temperature for 40 min. To stop the reaction, 2M H₂SO₄ (Merck) was used and the plate was then read at 450 nm on a micro-plate ELISA reader (ELX808, Bio Tec, Winooski, USA). The ratio of sample/positive control (S/P) optical densities (ODs) was calculated according to the following equation:^{8,9}

$$S/P = \frac{\text{Sample} - NC}{PC - NC}$$

where, *NC* was negative control and *PC* was positive control.

PCR. Samples with the S/P ratio of 0.50 or above were accounted as positive for *N. caninum* infection. Buffalo serum samples were analyzed for *N. caninum* NC5 gene using Np6 forward (5'-CTCGCCAGTCAACCTACGTCTT CCT->3') and Np21 reverse (5'-CCCAGTGCGTCCAATCCTG TAACC->3') primers.¹⁰ The PCR was programmed with ASTEC pc708 thermal cycler (ASTEC, Chattanooga, USA) as 10 min at 95 °C for primary denaturation and 35 cycles of 95 °C for 1 min, 65 °C for 1 min and 72 °C for 2 min for denaturation, annealing and extension respectively and a final extension at 72 °C for 10 min. To confirm the presence of *N. caninum* NC5 gene, 1.00 µL of the PCR products were subjected to nested PCR using 5'-GTGTTGCTCTGCTGACGTGT-3' forward and 5'-TACCAACT CCCTCGGTTTAC-3' reverse primers. After 10 min primary denaturation at 95 °C, 35 cycles of PCR were run with 1 min denaturation at 95 °C, 45 sec annealing at 54 °C and 1 min extension at 72 °C. Finally, the reaction was completed with 10 min final extension at 72 °C. The ELISA results were statistically analyzed using Excel (version 15.0; Microsoft Corporation, Redmond, USA) and SPSS (version

22.0; IBM, Chicago, USA) and the significance of the results was determined using student's *t*-test with 95.00% confidence. Data are presented as mean ± SD.

Results

A method of ELISA based by using the whole *Neospora* cell as an antigen was set up to detect the level of anti-*N. caninum* IgG in the serum. The ratio of S/P was calculated and the cut-off value of 0.50 was determined to detect the *Neospora* positive samples using a receiver operator characteristic curve analysis (Fig. 1). This cutoff point distinguished nearly 100% of *Neospora* positive cases. The positive and negative controls were already validated with a commercial kit. The S/P ratio of 16 samples (19.27%) including three males (3.61%) and 13 females (15.66%) and also all positive controls were ≥ 0.50. The OD of samples in this group ranged from 0.52 to 2.13, 0.86 ± 0.43 .

The statistical analysis showed that the level of antibody in all samples with S/P ratio ≥ 0.50 and also in the positive controls were significantly higher than those of the negative controls ($p < 0.05$). Amongst the negative samples, the levels of anti-*Neospora* antibody in 33 samples (39.75%) were significantly higher than those of the negative controls (S/P < 0.50). The range of OD in this group was between 0.054 to 0.465, 0.16 to 0.20, while in non-significant negative samples (34 samples, 40.96%) the range of OD was between 0.013 to 0.424 and 0.11 to 0.16.

All statistical analyses were calculated with 95.00% confidence interval (Fig. 2). The prevalence of the parasitemia was evaluated by detection of the parasite DNA in the serum samples using PCR (Fig. 3) and nested PCR (Fig. 4).

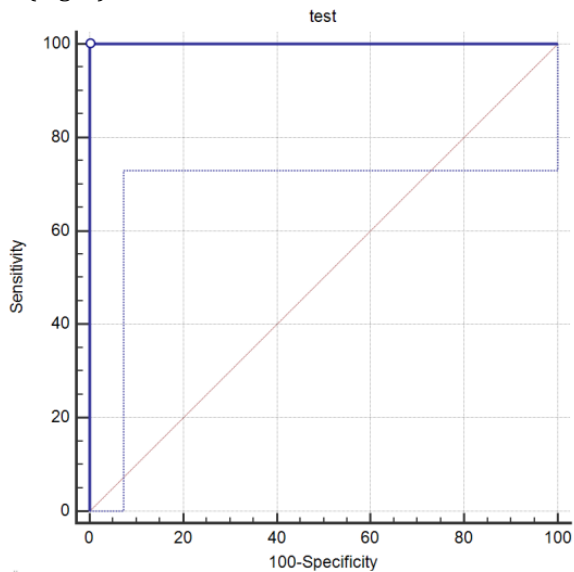


Fig. 1. A receiver operator characteristic (ROC) curve analysis of whole cell-based ELISA against PCR confirmation of DNA. Area under the ROC curve ($p < 0.0001$). The pointed line shows the area with 95.00% confidence.

The parasite DNA was detected in all samples with the S/P ratio ≥ 0.50 and all samples with the S/P ratio bellow 0.50 were negative for the parasite DNA.

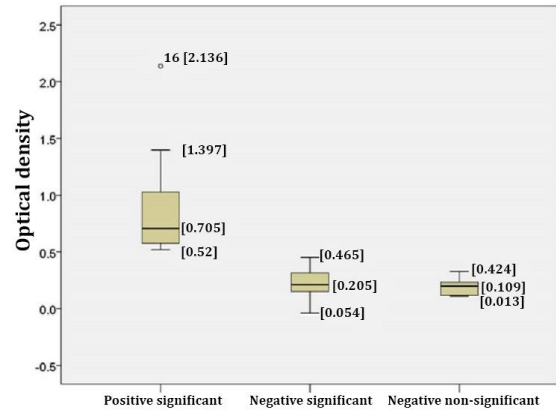


Fig. 2. Box plot illustrating S/P ratios in *Neospora* positive and negative samples. A number of 83 serum samples were examined for the presence of anti-*N. caninum* IgG by a new ELISA-based method using the whole parasite. The results were analyzed with student t-test with 95.00% confidence interval. Samples with the S/P ratio ≥ 0.50 were postulated as *Neospora* positive. The result of t-test for all samples with the S/P ratio ≥ 0.50 was significant. Three groups of significant positive, significant negative and non-significant negative are shown in the plot.

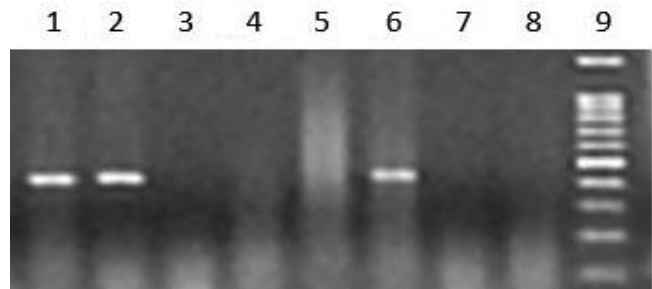


Fig. 3. Detection of *Neospora* DNA in water buffalo serum. A number of 83 serum samples were examined for the presence of *Neospora* DNA. Lanes 1, 2 and 6: *Neospora* positive serum samples; Lanes 3, 4, 5 and 7: *Neospora* negative serum samples; Lane 8: Negative control; Lane 9: Standard DNA. The PCR product produced a 330 bp band.

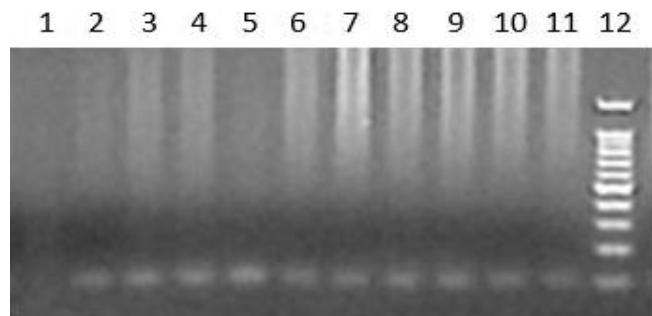


Fig.4. Confirmation of *Neospora* DNA by nested PCR. The *Neospora* DNA PCR products were validated with nested PCR using specific primers designed for amplification of 100bp of the products. Lane 1: Negative control; Lanes 2-11: *Neospora* DNA PCR product; Lane 12: Standard DNA.

Discussion

Because of the economical importance of water buffalo in the livestock industry and the lack of comprehensive information about *Neospora* infection in buffalo,⁸ the rate of infection as well as the relationship between parasitemia and production of anti-*Neospora* antibody in the serum of water buffalo in northwest of Iran was investigated. For detection of anti-*Neospora* antibody, an ELISA method was developed to detect the level of antibody in the serum using the whole *Neospora* cells. In this method of ELISA, instead of using the parasite proteins, the plates were coated with *Neospora* cells for 72 hr. The ELISA is shown to be a rapid and reliable method to analyze a large number of samples in a short time¹¹ and the new whole cell-based ELISA showed to be capable of detecting anti-*Neospora* antibody in the water buffalo serum; since all positive samples tested by other commercial kits were positive and also negative samples were negative. The results showed that the rate of infection of water buffalo in the northwest of Iran was 19.27%. In another study, the infection rate of *Neospora* in buffalo was reported as 37.10% in the southwest of Iran.⁸ The different rate of *Neospora* infection between various regions is also reported in Thailand.¹² Indeed, the difference in the rate of infection between the regions could be due to the epidemiology of the parasite in the regions including the rate of infection in dogs and other definitive hosts. In previous studies, the prevalence rate of *N. caninum* in water buffalo has been reported around the world; up to 1.50% in southern Vietnam³, 64.00% in Argentina,⁵ 52.30% in India⁶, 64.00% in southern Brazil¹³, 70.90% in the northern region of Brazil,¹⁴ 34.60% in Italy,¹⁵ 28.00% in Turkey¹⁶ and 68.00% in Egypt.¹⁷ The difference in the infection rate is probably due to the environmental and management conditions or differences in the methods used for detection of the infection. The rate of infection in other intermediate hosts has also been evaluated in Iran and other countries. Many studies have shown that the rate of *Neospora* infection in water buffalo is higher than that of other intermediate hosts. The seroprevalence of antibody to *N. caninum* in cattle was 10.50% in Tabriz (northwest of Iran).¹⁸ In Mashhad (northeast of Iran), the infection rate of *N. caninum* in dairy cattle and camels was 15.18%¹⁹ and 5.83%²⁰ respectively. In northern Australia, *N. caninum* infection in water buffalo was highly endemic and the infection rate was higher than that for cattle.²¹ It is thought that the higher rate of the infection in buffalo comes from this fact that this animal is probably more sensitive and its life style is slightly different than other intermediate hosts.

The PCR is a technique which has recently been widely used for detection of *Neospora* infections.^{22,23} In most of *Neospora* studies, PCR is performed using tissues of aborted fetus^{24,25} or blood cells.^{22,26} In the present study, to

determine the presence of the parasite in the blood, serum samples were directly examined by PCR and nested PCR for amplification of *Neospora* Nc-5 gene which is specific for detection of *Neospora*.^{27,28} The results showed that the *Neospora* DNA was detectable in all samples with the S/P ratio ≥ 0.50 . None of the samples with the S/P ratio less than 0.50 contained *Neospora* DNA, however, some of the DNA negative samples with the cutoff value of less than 0.50 showed significant levels of antibody (*p*-value in t-test below 0.05). The *Neospora* DNA has yet been isolated from sheep¹⁵ and cattle²⁹ serum, but this is the first report on the isolation of *Neospora* from the serum of water buffalo and concurrent detection of anti-*Neospora* antibody and *Neospora* DNA in the serum. Stages of parasitemia in *Neospora* infections during the parasite life cycle and the potency of the immune system in immunity production and parasite removal from the blood have still remained unclear. Detection of the parasite DNA in the serum of positive samples clearly proved the entrance of the parasite into the blood and a period of parasitemia in water buffalo which is in line with some of previous studies confirming the entry of the parasite into the blood by detection of *N. caninum* DNA in the serum of infected animals.^{23,30} These findings showed a 100% concurrency between high levels of the antibody and the parasite DNA in the sera. In the lower levels of the antibody (below the cut-off point, but statistically significant compared to the negative controls), the *Neospora* DNA was disappeared indicating the potency of the immune system in antibody production to remove the parasite from the bloodstream or converting the acute phase of the disease into the chronic phase which may fall down the level of anti-*Neospora* antibody. The presence of DNA in the blood after parasitemia and high levels of antibody in acute phases of the infection were also addressed in other studies.²⁶ Anyway, for detection of *Neospora* infection, ELISA is preferable to PCR as it can detect acute and chronic phases of the disease. However, it still needs more investigation. Other researchers have shown no association between the presence of *Neospora* DNA and anti-*Neospora* antibody in the serum in *Neospora* infection in cattle.³⁰ By the way, they have detected the DNA only from white blood cells and not from the serum.^{22,26} This was in contrast with our findings in buffalo, where we used the serum as a DNA template in PCR reaction without any purification process. This controversy indicates that after an increase in the level of antibody in the serum, the parasites are removed from the blood, but they still grow inside the blood cells. However, the relation between the parasitemia and level of anti-*Neospora* antibody in the serum is more complicated and still needs to be more clear. The concurrent presence of the parasite and antibody in the serum is an important phenomenon in buffalo and needs to be investigated in cattle as the main intermediate hosts for the parasite. Our results showed a rate of 19.27% for

N. caninum infection in water buffalo in the northwest of Iran. A 100% concurrency was observed between the high levels of anti-*Neospora* antibody and parasitemia during *Neospora* life stages in buffalo.

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Conflict of interest

The authors declare no conflict of interest.

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