Patho-epidemiological and risk factor studies for detection of *Neospora*-associated abortion in cattle and buffaloes in Punjab, India

This paper (No. 25042019-00146-EN) has been peer-reviewed, accepted, edited, and corrected by authors. It has not yet been formatted for printing. It will be published in December 2019 in issue **38** (3) of the *Scientific and Technical Review.*

V. Mahajan [(1)*], H.S. Banga [(2)] & G. Filia [(1)]

(1) Department of Animal Disease Research Centre, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, Punjab 141004, India

(2) Department of Veterinary Pathology, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, Punjab 141004, India

*Corresponding author: mahajanv17@gmail.com

Summary

The diagnosis of abortion in livestock is difficult. In this paper, *Neospora caninum*-associated abortion in cattle and buffaloes in India is reported for the first time. A total of 184 animals (cattle, $n = 133$; water buffaloes, $n = 51$) were randomly selected for seroprevalence studies from 28 farms with a history of abortion. Antibodies to *N. caninum* were detected using a commercial competitive enzyme-linked immunosorbent assay (c-ELISA); the percentage seropositivity was 10.5% for cattle and 21.6% for buffaloes. Risk factors such as species, i.e. buffaloes in comparison to cattle (odds ratio [OR] = 2.33, calculated probability [p] = 0.05), disposal of foetus and foetal membranes by throwing them into the field, in comparison with burning/burial (OR = 2.63, $p = 0.03$), and origin of the animal, i.e. purchased from outside in comparison to born on the farm (OR = 4.69, $p = 0.002$), were significantly associated with *N. caninum* seropositivity in univariate logistic regression. In multivariate analysis, only two risk factors, animal purchased from outside (OR = 6.15,

$p = 0.001$) and buffaloes (OR = 3.20, $p = 0.01$), were significantly associated with *Neospora* seropositivity at a p-value < 0.05. Histopathological examination of aborted foetal tissues (cattle, $n = 13$; buffaloes, $n = 8$) revealed *N. caninum* tachyzoites in the heart and liver of three foetuses, and the diagnosis was confirmed by positive immunoreactivity to anti-*N. caninum* polyclonal antibody in the placenta of one foetus. To the authors' knowledge, this is the first report of confirmed neosporosis abortion in cattle and buffaloes in India.

Keywords

Abortion – Immunohistochemistry – Neosporosis – Risk factor study.

Introduction

Neosporosis, caused by *Neospora caninum*, is a major cause of abortion in bovine species worldwide, resulting in significant losses in farm ruminant production, but there is no report of *Neospora*-associated bovine abortion from India (1). Although *N. caninum* can affect many species of domestic animals and wildlife, its greatest impact is on the cattle industry. Neosporosis abortion can occur throughout pregnancy, but most commonly between five and seven months of gestation. Diagnosis of the cause of abortion is difficult and requires identification of the parasite in foetal tissues. Transplacental transmission is the most important aspect of the epidemiology of neosporosis, because as many as 90% of calves can be born infected but not diseased. Although antibodies to *N. caninum* have been found in sera of cattle and buffaloes in India (2), there has been no study of the risk factors for abortion or determination of abortion aetiology in this setting.

Materials and methods

Selection of farms and animals

Livestock farms ($n = 28$) having a history of abortion were selected from different agro-climatic zones, i.e. the sub-mountain region (annual rainfall 800–900 mm), central plain region (500–800 mm) and arid irrigated region (< 400 mm) of Punjab (Fig. 1), using the 'Random Animal' programme of the Survey Toolbox software (3). The total

bovine population of these selected farms was 1,830. A total of 184 animals (cattle, $n = 133$; buffaloes, $n = 51$) belonging to various livestock owners were selected from these farms using random number tables at the farm. Further, serum samples were collected from aborted animals ($n = 63$) and their herd mates ($n = 121$) for seroprevalence and risk-factor studies.

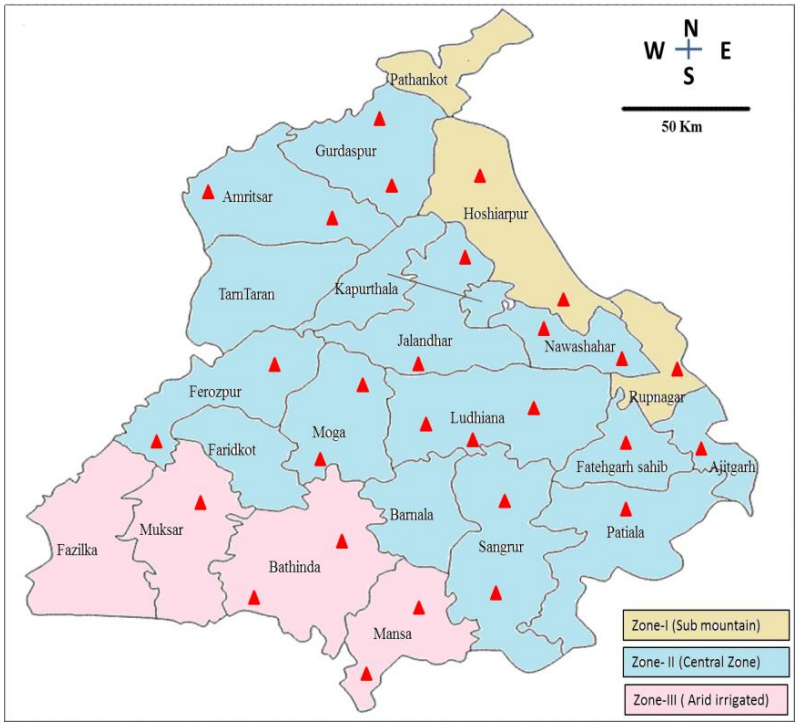

Fig. 1

Agro-ecological zones of Punjab

The red triangles represent the farms selected for the study

Seroprevalence and risk factors

About 5 ml of blood was collected from each animal from a jugular vein. The serum was collected from clotted blood by centrifuging at 2500 rpm (24.5 *g*) for 5 min and stored in a screw-capped plastic vial at –20°C until tested for antibodies to *N. caninum* using a commercial competitive enzyme-linked immunosorbent assay (c-ELISA; VMRD, Inc., Pullman, Washington, United States of America [USA]), following the manufacturer's guidelines. Data were collected on each animal, assessing various farm-level risk factors and other farm

characteristics, using a standardised questionnaire (Table I). The data were analysed using the Statistical Package for Social Sciences (SPSS) for Windows, version 11.0.1 (©SPSS Inc., USA). Associations were evaluated between the binary outcome variable and a variety of risk factors using logistic regression analysis, in which the serological status of each animal (seropositive/seronegative) was used as a dependent variable to identify any risk factor associated with the seroprevalence. The screening tests were performed using the chi-square test for independence.

Table I

Prevalence of *Neospora* seropositivity in Punjab

Total number of farms surveyed	**28**
Total bovine population of farms selected	**1,830**
Animals selected for sampling	184
Animals tested positive	25
Apparent serological prevalence of disease	13.6%
True serological prevalence of disease	13.3%
95% confidence interval	10.72–15.80%

Pathology and immunohistochemistry

Tissue samples (n = 21) from aborted foetuses (cows, n = 13; buffaloes, n = 8) and placental cotyledons (cows, n = 9, buffaloes; n = 5) were collected in 10% neutral buffered formalin for histopathology and immunohistochemistry. Tissues were later embedded in paraffin wax and 4–5 µm sections were cut and stained with a routine haematoxylin and eosin technique.

Immunohistochemical studies were performed as per Mahajan *et al*. (4). Briefly, 4–5 µm thick paraffin-embedded tissue sections were cut and mounted on Superfrost Plus positively charged

microscopic slides (Fisher Scientific, Hudson, New Hampshire, USA). After heat-induced antigen retrieval and endogenous peroxidase blocking, the slides were incubated with a commercially available polyclonal antibody against *N. caninum* (VMRD, Inc., Pullman, Washington, USA) at a dilution of 1:1,000, followed by incubation with a secondary antibody (Universal, Vector) and colour development using 3, 3-diaminobenzidine (DAB); they were counterstained with Gill's haematoxylin. As a negative control, sections were incubated with phosphate buffered saline (PBS) instead of the primary antibody.

Results

Serological and risk factor studies

The seroprevalence of *N. caninum* was 13.6% (Table I). Given the sensitivity and specificity of the ELISA (96% and 99%, respectively), the true prevalence was calculated to be 13.3%. The seroprevalence in the sub-mountain zone (Zone I), central zone (Zone II) and arid irrigated zone (Zone III) was found to be 7.9%, 15.4% and 13.8%, respectively. The rate of seropositivity of *Neospora* in buffaloes (21.6%) was significantly higher (chi-square = 3.82, $p \leq 0.05$) than that in cattle (10.5%). For the purposes of the study, animals were grouped into three age categories: 0–2 years, 2–4 years and >4 years. The prevalence of *Neospora* antibodies in animals of the different age categories was 4%, 14.03% and 15.7%, respectively. The highest rate of abortion (18.7%) was found at 6–9 months of gestation. Out of the 184 animals studied, 34.2% had a history of abortion, and among these animals 14.3% were found to be seropositive for *Neospora* antibodies. The prevalence of *Neospora* antibodies was found to be non-significantly higher (chi-square = 1.09, $p \leq 0.57$) in multiparous animals when compared with nulliparous animals.

Out of fourteen factors studied, three were significantly associated with neosporosis at a *p*-value < 0.05 in the univariate analysis. Univariate logistic regression (Table II) showed that seropositivity to *N. caninum* was significantly associated with the host, i.e. buffaloes in comparison with cattle (OR = 2.33, $p \leq 0.05$); with disposal of the foetus and foetal membranes by throwing them into the field, in comparison with

burning/burial (OR = 2.63, $p \leq 0.03$); and with the origin of the animal, i.e. purchased from outside in comparison with being born on the farm (OR = 4.69, $p \leq 0.002$). All risk factors that had a p-value < 0.05 in univariate analysis were included in multivariate logistic regression analysis using a backward stepwise likelihood ratio (LR) model. This step simultaneously considers the individual and joint effects of many risk factors. Only two factors were significantly associated with neosporosis at a p-value < 0.05 in the multivariate analysis: origin of animal, i.e. purchased from outside (OR = 6.15, $p \leq 0.001$), and host, i.e. buffaloes (OR = 3.20, $p \leq 0.01$) (Table III).

Table II

Results of the univariate analysis for *Neospora* seropositive status

Serial no.	Risk factor	Categories	Total animals	Odds ratio	95% confidence interval	*p*-value
1	Abortion	1) Yes	63	1.398	0.551–3.549	0.481
		2) No	121			
2	Zone	1) Sub-mountainous	38	0.536	0.110–2.607	0.516
		2) Central	117	1.136	0.353–3.657	
		3) Arid irrigated	29			
3	Species	1) Buffalo	51	2.337	0.982–5.563	0.050
		2) Cow	133			
4	Age	1) 0–2 years	25	0.224	0.028–1.775	0.366
		2) 2–4 years	57	0.878	0.350–2.198	
		3) more than 4 years	102			
5	Retained placenta	1) Yes	41	1.797	0.713–4.525	0.214
		2) No	143			
6	Housing	1) Extensive	16	5.570	0.599–51.793	0.314
		2) Intensive	140	2.348	0.514–10.729	
		3) Semi-intensive	28			
7	Flooring	1) Concrete	54	4.522	0.535–38.193	0.331
		2) Brick and mud	103	4.782	0.605–37.789	
		3) Mud	27			
8	Multiple raising of animals	1) Yes	36	0.521	0.147–1.846	0.312
		2) No	148			
9	Disposal of foetus and foetal membranes	1) Throwing outside	37	2.632	1.056–6.557	0.038
		2) Burning/burial	147			

10	Sanitary status (hygiene, disinfection and manure removal)	1) Poor	24	0.420	0.041–4.313	0.245
		2) Moderate	128	1.897	0.529–6.806	
		3) Good	32			
11	Origin of animal	1) Purchased from outside	26	4.699	1.801–12.261	0.002
		2) Born on farm	158			
12	Breed	1) Holstein Friesian	118	0.683	0.247–1.889	0.276
		2) Sahiwal	15	2.372	0.403–13.959	
		3) Murrah	51			
13	Farm distance	1) Close	66	0.521	0.197–1.378	0.189
		2) Far	118			
14	Presence of dog	1) Yes	103	0.690	0.296–1.606	0.389
		2) No	81			

Table III

Results of the multivariate analysis for *Neospora* seropositive status

Risk factors	Odds ratio	95% confidence interval	*p*-value
Animal purchased from outside	6.12	2.19–17.24	0.001
Buffaloes	3.20	1.24–8.25	0.016

Pathological and immunohistochemical studies

Abortion due to *N. caninum* was diagnosed in three foetuses (cattle, $n = 1$; buffaloes, $n = 2$), with an average gestational age of 7.5 months. Protozoal tachyzoites were identified in heart and liver (Fig. 2) but no tissue cysts were seen in the brain. Non-suppurative periportal hepatitis (Fig. 3) was also observed in two aborted foetuses. The placenta showed focal necrosis (Fig. 4) and diffuse non-suppurative inflammation (Fig. 5), and tachyzoites were seen in the placenta (Fig. 6). Tachyzoites in the placenta of an aborted foetus reacted with anti-*N. caninum* polyclonal antibody (Fig. 7). In total, nine (cattle, $n = 4$; buffaloes, $n = 5$) out of 63 aborting animals (14.7%) were seropositive for *N. caninum* using the c-ELISA. From these nine aborted animals, foetal tissues were collected from five (cattle, $n = 3$;

buffaloes, $n = 2$), but tachyzoites were detected in only the three foetuses mentioned above (cattle, $n = 1$; buffaloes, $n = 2$).

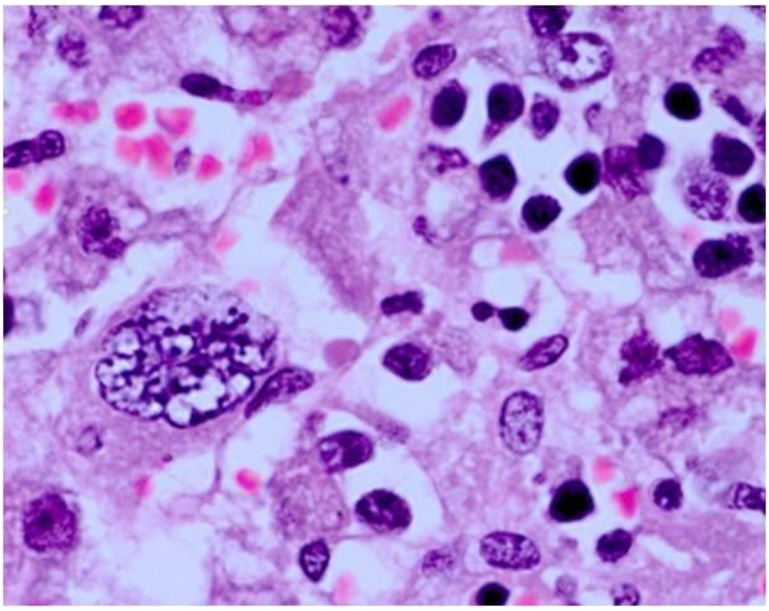

Fig. 2
Tachyzoites in liver and lymphomononuclear cell infiltration (haematoxylin and eosin [H & E], 100 ×)

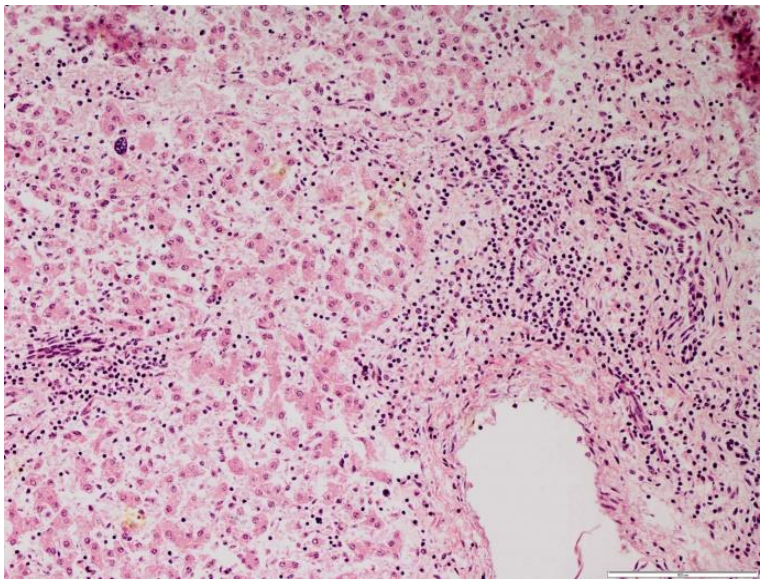

Fig. 3
Non-suppurative periportal hepatitis (H & E, 10 ×)

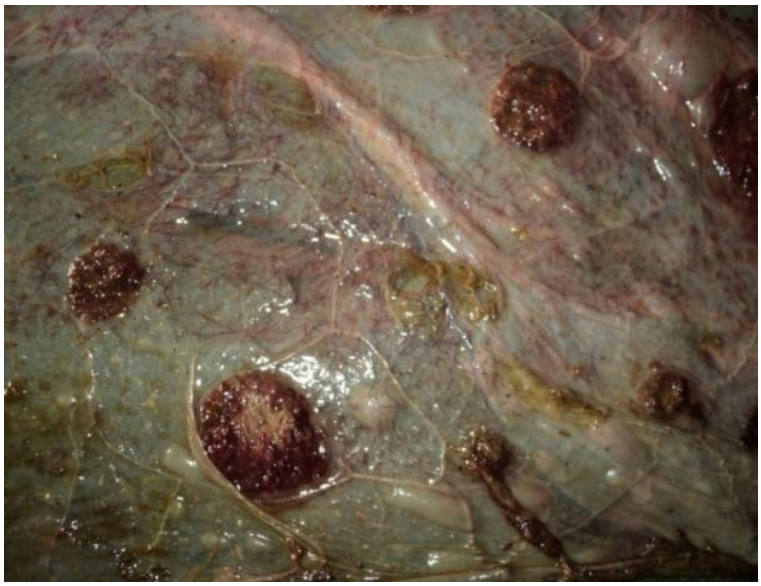

Fig. 4
Placenta showing necrotic cotyledons

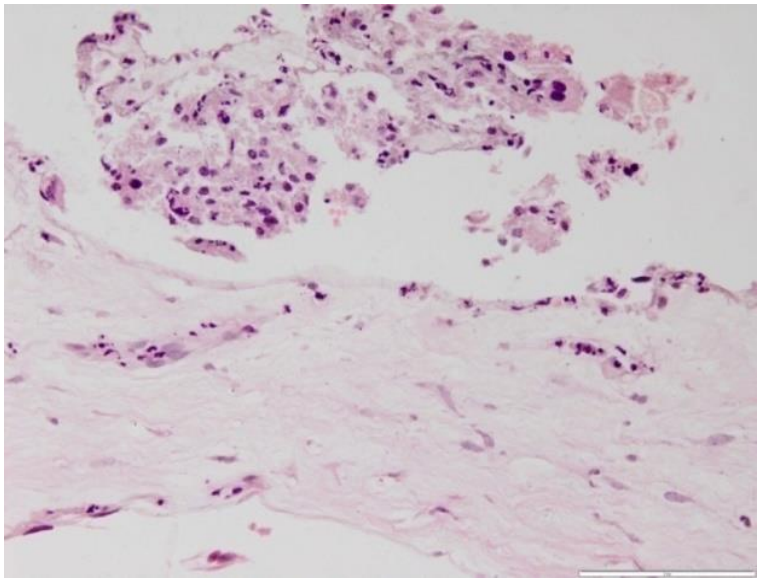

Fig. 5
Necrosis and non-suppurative inflammation in placenta (H & E, 20 ×)

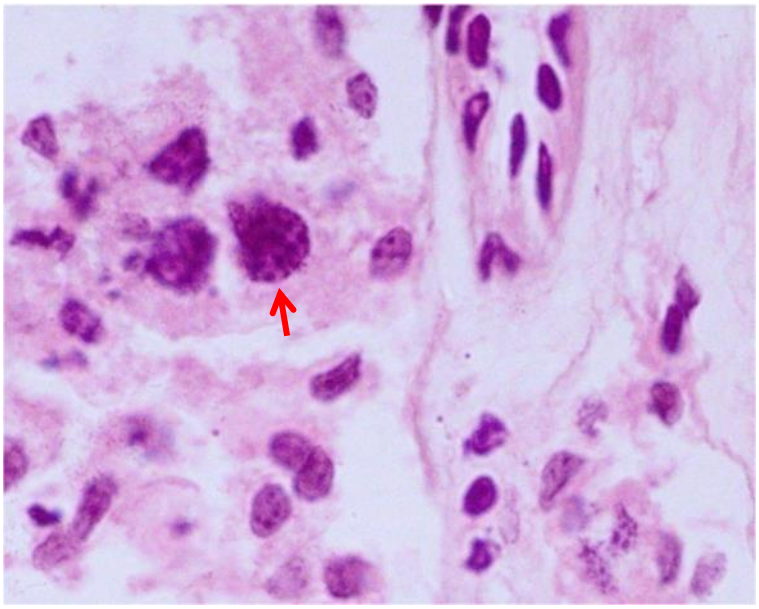

Fig. 6
Tachyzoites (arrow) in placenta (H & E, 100 ×)

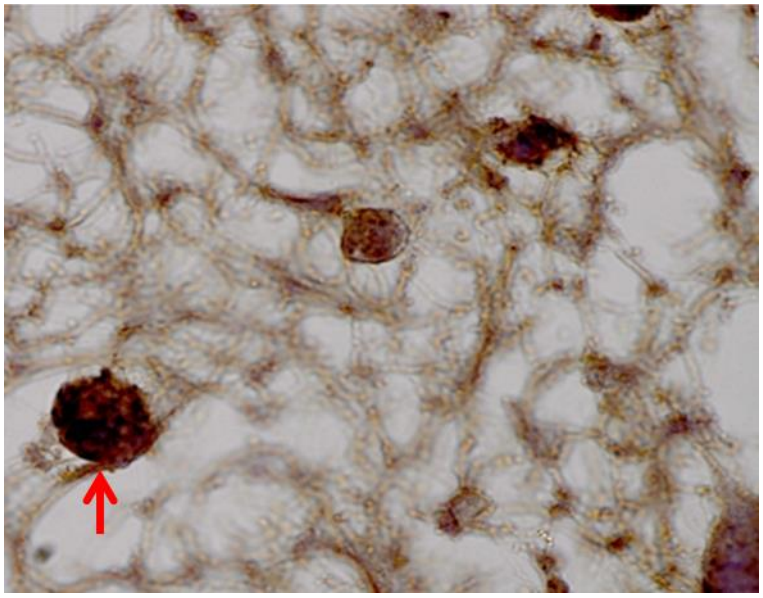

Fig. 7
Immunoreactivity to tachyzoites in section of placenta (arrow) with anti-*N. caninum* polyclonal antibody (100 ×)

Discussion

The detection of specific anti-*N. caninum* antibodies in sera from cows has been useful for the diagnosis of the disease and for seroepidemiological investigations (5). In this study, the true prevalence of *Neospora* seropositivity was found to be 13.3%. An earlier study reported the seroprevalence of *Neospora* antibodies in Punjab to be 7.6% (2). Vural *et al.* (6) reported the seroprevalence of *Neospora* antibodies in a region of Turkey to be 13.9%, using ELISA. The detection of antibodies against *N. caninum* in bovine serum is only indicative of contact with these protozoa and does not confirm neosporosis as a cause of abortion, whereas a negative serological result for *N. caninum* excludes its involvement in abortion (7). In this study, there was a higher prevalence of *Neospora* antibodies in the central zone (15.4%) of Punjab when compared with sub-mountain zone (7.9%) and arid irrigated zone (13.8%), which may be attributed to geographical and climatic differences. Moreover, the risk of *Neospora*-associated abortion was found to be 2.05 times higher in buffaloes (relative risk [RR] = 2.04, 95% confidence interval [CI] = 0.99–4.21) when compared with cows. Approximately 14% to 70% of buffaloes from Brazil, Egypt and Italy were found to have antibodies against *N. caninum* (8, 9, 10). The results of the present study are in accordance with observations of Meenakshi *et al.* (2) who reported *N. caninum* infection in buffaloes in India.

A non-significantly higher prevalence (chi-square = 2.35, $p \leq 0.31$) of *Neospora* antibodies was found in animals greater than two years of age. Increased seroprevalence with age may be due to an increased susceptibility to post-natal infection with age (11). There was a non-significantly higher prevalence (chi-square = 2.12, $p \leq 0.54$) of *Neospora* seropositivity at 6–9 months of gestation. The seropositivity of *Neospora* was also non-significantly higher (chi-square = 0.23, $p \leq 63$) in aborted animals when compared with animals with no history of abortion. Various studies (2, 11, 12) have shown a correlation between *N. caninum* seropositivity and abortion rate.

Conclusions

In the present study, a diagnosis of *Neospora*-associated abortion was confirmed immunohistochemically in three foetuses. Further studies are needed to determine the impact of neosporosis and other aetiologies of abortion in livestock in India.

References

1. Dubey J.P., Hemphill A., Calero-Bernal R. & Schares G. (2017). – Neosporosis in animals, 1st Ed. CRC Press, Boca Raton, Florida, United States of America, 530 pp. https://doi.org/10.1201/9781315152561.

2. Meenakshi, Sandhu K.S., Bal M.S., Kumar H., Sharma S., Sidhu P.K., Sreekumar C. & Dubey J.P. (2007). – Seroprevalence of *Neospora caninum* antibodies in cattle and water buffaloes in India. *J. Parasitol.*, **93** (6), 1374–1377. https://doi.org/10.1645/GE-1317.1.

3. Cameron A.R. (1999). – Survey Toolbox for livestock diseases – a practical manual and software package for active surveillance in developing countries. Australian Centre for International Agricultural Research (ACIAR) Monograph Series No. 54. ACIAR, Canberra, Australia, 330 pp. Available at: http://epitools.ausvet.com.au/docs/SurveyToolbox.pdf (accessed on 29 March 2019).

4. Mahajan V., Banga H.S., Deka D., Filia G. & Gupta A. (2013). – Comparison of diagnostic tests for diagnosis of infectious bovine rhinotracheitis in natural cases of bovine abortion. *J. Comp. Pathol.*, **149** (4), 391–401. https://doi.org/10.1016/j.jcpa.2013.05.002.

5. Dubey J.P., Schares G. & Ortega-Mora L.M. (2007). – Epidemiology and control of neosporosis and *Neospora caninum. Clin. Microbiol. Rev.*, **20** (2), 323–367. https://doi.org/10.1128/CMR.00031-06.

6. Vural G., Aksoy E., Bozkir M., Kuçukayan U. & Erturk A. (2006). – Seroprevalence of *Neospora caninum* in dairy cattle herds in central Anatolia, Turkey. *Veterinarski. Arhiv.*, **76** (4), 343–349. Available at: www.google.com/url?sa=t&rct=j&q=&esrc=s&source=web&cd=1&ved=2ahUKEwiG4_L20srgAhX88OAKHRD0DI0QFjAAegQIAxAC&url=https%3A%2F%2Fpdfs.semanticscholar.org%2F48cd%2Fef0a6b92344fbf450cf7c01f1b1bd9eca826.pdf&usg=AOvVaw36xBa65MaivXISKKLkosaY (accessed on 20 February 2019).

7. Georgieva D.A., Prelezov P.N. & Koinarski V.T.S. (2006). – *Neospora caninum* and neosporosis in animals: a review. *Bulgarian J. Vet. Med.*, **9** (1), 1–26. Available at: http://tru.uni-sz.bg/bjvm/vol9-no1-01.pdf (accessed on 21 February 2019).

8. Gennari S.M., Rodrigues A.A.R., Viana R.B. & Cardoso E.C. (2005). – Occurrence of anti-*Neospora caninum* antibodies in water buffaloes (*Bubalus bubalis*) from the northern region of Brazil. *Vet. Parasitol.*, **134** (1–2), 169–171. https://doi.org/10.1016/j.vetpar.2005.05.064.

9. Dubey J.P., Romand S., Hilali M., Kwok O.C.H & Thulliez P. (1998). – Seroprevalence of antibodies to *Neospora caninum* and *Toxoplasma gondii* in water buffaloes (*Bubalus bubalis*) from Egypt. *Int. J. Parasitol.*, **28** (3), 527–529. https://doi.org/10.1016/S0020-7519(97)00190-2.

10. Guarino A., Fusco G., Savini G., Di Francesco G. & Cringoli G. (2000) – Neosporosis in water buffalo (*Bubalus bubalis*) in southern Italy. *Vet Parasitol.*, **91** (1–2), 15–21. https://doi.org/10.1016/S0304-4017(00)00239-9.

11. Bedoya Llano H.A., Sales Guimarães M., Martins Soares R., Polo G. & Caetano da Silva A. (2018). – Seroprevalence and risk factors for *Neospora caninum* infection in cattle from the eastern Antioquia, Colombia. *Vet. Anim. Sci.*, **6**, 69–74. https://doi.org/10.1016/j.vas.2018.03.001.

12. Ansari-Lari M., Rowshan-Ghasrodashti A., Jesmani H., Masoudian M. & Badkoobeh M. (2017). – Association of *Neospora caninum* with reproductive performance in dairy cows: a prospective study from Iran. *Vet. Res. Forum*, **8** (2) 109–114. Available at: www.ncbi.nlm.nih.gov/pmc/articles/PMC5524547/ (accessed on 20 February 2019).