Prevalence and risk factors of Neospora caninum in aborted fetus of sheep, goat, cattle, and buffalo

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Research Article

Keywords: Neospora caninum, aborted fetus, Nested-PCR, Epidemiology, Bangladesh

Posted Date: May 5th, 2023

DOI: https://doi.org/10.21203/rs.3.rs-2883165/v1

Abstract

Background

Neospora caninum, a protozoa that is a leading cause of neurological illness in dogs (definitive host), abortion in cattle (intermediate host) and sporadic disease in other livestock species (sheep, goats, horses and other carnivores). The study was aimed to reveal the molecular epidemiology of *N. caninum* in aborted fetuses of cattle, goats, sheep and buffaloes in Bangladesh.

Methods

In total, 157 aborted fetuses (50 Cattle, 46 Buffaloes, 34 Goats and 27 Sheep) were randomly collected from various regions of Bangladesh. DNA was extracted from brain tissue to perform nested PCR and sequencing of ITS1 rDNA gene of *N. caninum.*

Results

A total of 20 aborted fetuses out of 157 were positive for *N. caninum* infection. Higher prevalence was observed in cattle (16.0%) followed by sheep (14.8%), goats (11.8%) and buffaloes (8.7%). Highest prevalence was found in animals during second trimester (21.51%) with aged 2 to 4 years (15.8%). Sequences from representative positive samples showed similarity between 99–100% for *N. caninum* ITS1 rDNA gene with other best hit scoring GenBank sequences. Multivariate logistic regression demonstrated that abortion in pregnancy, abortion history of the animals, contact with dog feces or presence of dog in animals farm or premises and management practices were significantly ($p \leq 0.001$) correlated with *N. caninum* infection.

Conclusion

The study findings indicated that *N. caninum* infection is one of the major causes of abortion and economic loss in livestock farming. Broader molecular epidemiology is imperative for strategic planning to control and prevent neosporosis in livestock.

Background

Neospora caninum is a protozoan parasite which causes abortion in ruminants and neurological disorders in carnivores worldwide [1, 2]. Dogs and coyotes are thefinal hosts to *N. caninum*, while theintermediate hosts include a wide range of warm-blooded animals such assheep, cattle, buffaloes, goat, deer, rhinoceros, rodents, and horses [3, 4].This coccidian protozoais aleadingcause of abortion in cattle [5, 6, 7], sheep [1, 8], and goat [9, 10].

Transplacental or vertical transmission of *N. caninum* is the major route of infection in ruminants [11]. Postnatal or horizontal transmission is possible through ingestion of tissues containing cysts and tachyzoites or through ingestion of sporulated oocysts in contaminated food or drink [7]. Although this parasite usually causes abortion at 5–6 months of gestation, but in cattle it can cause abortion at any stage

of pregnancy [2]. In dogs, *N. caninum* can affect central nervous system, brain, liver, muscle, and other visceral tissues [1, 12, 13].

Cattle in farms that are in close contactwith dogs are at riskfor acquiring *Neospora* infection [7, 14]. In Bangladesh most of the dogs or other canine species are stray in nature. In addition, livestock farm practices are often semi-intensive and free range. Strict biosecurity practices are not maintained in even intensive farms. Therefore, contamination and transmission of *N. caninum* can frequently occur in Bangladesh.

Several methods have been used to detect*N. caninum* in aborted fetuses, including histopathology [15], immunohistochemistry [16], PCR [17] and higher sensitive and specific nested PCR [18].The ribosomal DNA (rDNA) internal transcribed spacer 1 (ITS1)region is a good target for the distinction of *N. caninum* from other closely related parasites [19].

There is no available dataon the prevalence of *N. caninum* relatedabortion in animals in Bangladesh, although the case of abortion in ruminant or livestock is significant [20], andfrequent abortion occurs in high yielding dairy farm in Bangladesh (personal communication). However, the clinical, epidemiological, and economic importance of neosporosis in ruminants is required [21].

Therefore, this study was undertaken to detect *N. caninum* in aborted fetuses (sheep, goats, cattle and buffalo) in Bangladesh using nested PCR.

Methods

Sample collection

In total, 157 aborted fetuses (50 Cattle, 46 Buffaloes, 34 Goats and 27 Sheep) were randomly collected 3 different districts in (Jessore, Rangpur and Mymensingh) of Bangladesh (Table 2) November 2020 to October 2021. Since brain is considered as the organ of choice for diagnosis of *N. caninum* in aborted fetuses [22], we excised brain from each fresh aborted fetus. The excised brain samples were stored at -80°C for molecular analysis.

Data collection

A closed-ended questionnaire was used to collect information on age and breed of animal, abortion period, previous abortion history, presence of dog in and around farm and management style. The age of the animal was determined based on dentition and farmer's information.

DNA extraction from tissue samples

For each sample, 50 mg tissue was cut into pieces, homogenized with distilled water, and subjected to DNA extraction (PureLink™ Genomic DNA Mini Kit, Invitrogen, USA). The samples were stored at -20°C until further molecular analysis and Sanger sequencing.

PCR and Gel electrophoresis

Nested PCR was used to amplify 279-295 bp of *N. caninum* DNA fragment of ITS1 (internal transcribed spacer) gene [23]. The primers pairs were: NN1 (5'- TCAACCTTTGAATCCCAA -3'), NN2 (5'- CGAGCCAAGACATCCATT -3'), NP1 (5'- TACTACTCCCTGTGAGTTG -3'), and NP2 (5'- TACTACTCCCTGTGAGTTG -3'). Primary reaction was performed in a MiniPCR (Oxford University), with a 25 µl reaction volume consisting of 12.5 µl GoTaq Green Master mix (Promega, USA), 10 pmol each primer (NN1, NN2), 3 µl DNA. The initial denaturation for 5 min at a temperature of 95°C, then 35 cycles of 1 min denaturation (95°C), 1 min annealing (55°C), and 1 min extension (72°C), followed bya final 5 min extension (72°C). The secondary reaction was performed with NP1 and NP2 primers, 2 µlof the primary amplification product under the same primary PCR conditions (except annealing temp 53°C). Positive control was used from previously identified *N. caninum* DNA by sequencing and ultrapure water as a negative control. The analysis of PCR products was run by 1.5% agarose gel electrophoresis.

Sequencing

Six representatives PCR products obtained from nPCR were subjected to sequencing for confirming *N. caninum*. The PCR products were purified (Wizard SV gel and PCR clean-up system, Promega, USA), the sequencing was performed at DNA Solution Ltd, (Dhaka, Bangladesh), using ABI 3500 Dx Genetic analyzer (Applied Biosystems, USA). Using the Bio-Edit software, the nucleotide sequences were assembled and trimmed, and BLAST analysis was performed in NCBI (https://www.blast.ncbi.nlm.nih.gov). After that, the sequences were submitted to GenBank of the National Center for Biotechnology Information (NCBI) for accession numbers.

Phylogenetic analysis

The newly generated sequences were processed, cut at both sides, aligned using the program ClustalW within MEGA v.11.0 (Tamura etal.,2021). The sequences were compared with best hit scoring ITS1 *N. caninum* DNA sequences deposited in the GenBank database using the NCBI Basic Local Alignment Search Tool (BLAST). A Neighbor-joining phylogenetic tree of *N. caninum* was constructed in Mega11 software [24] using Tamura-Nei model and bootstrap values were calculated using 1000 replicates where *Eimeria brunette* (AF446057.1) was as an outgroup.

Data management and analyses

Data obtained from the animal's dentition and farmers consent was cross checked for determining true age of animal. Other information from close end questionnaires were carefully transferred to an MS Excel spreadsheet (Microsoft Excel 2018) for cleaning and processing. The data were then analyzed using IBM SPSS Statistics for Windows, Version 25.0. (Armonk, NY: IBM Corp). Chi square was performedto compareof the prevalence rates of neosporosis among different animal species, age, breed, sex, abortion history, presence of dog in the farms or premises and management practices. Differences were considered significant when p-value ≤ 0.05.

Multivariate logistic regression was performed to study the effects of risk factors to *Neospora caninum* infection in animals. For this, data were first organized for univariate analysis. Potential candidates for

multivariate analysis (multiple logistic regression with input method) were then selected from variables that resulted in significant (p 0.05) in the univariate analysis.

Results

Prevalence

A total of 20 aborted fetuses were positive for *N. caninum* infection out of 157 irrespective of species through nested PCR (Fig. 1).

Phylogenetic analysis

The nucleotide sequences *of N. caninum* for different species discretely made position irrespective of region or study location. The neighbor-joining tree of ITS2 sequences of *N. caninum* disclosed that the studied *N. caninum* isolates clustered with previously established *N. caninum* sequences with strong nodal support (91% by bootstrapping value) while there were very close relationship among the Bangladeshi isolates with a strong nodal support value of 97% by bootstrapping (Fig. 2). We did not include the sequences from *N. caninum* positive samples from buffalo for phylogenetic analysis due to poor quality of the sequences.

Phylogenetic analysis indicated that *N. caninum* are genetically identical which belongs to different hosts and geographical areas and grouped into the *N. caninum*-clade (Fig. 2). BLAST analyses of ITS-1 rDNA gene showed 99 -100% similarities between *N. caninum* sequences deposited in GenBank.

Descriptive statistics

Among cattle, prevalence of *N. caninum* was 16.0% (8/50) while 14.8% (4/27) in sheep, 11.8% (4/34) in goat and 8.7% (4/46) in buffalo (Fig. 3). In Jessore, prevalence of *N. caninum* was 13.2% (7/53) while 12. 5% in both Rangpur (6/48) and Mymensingh district (7/49). Highest prevalence was found in animals which abortion occurred in second trimester 17(21.51%). Without any distinction in species *N. caninum* was prevalent 15.8% (9/57) in animals aged 2 to 4 years (Table 1).

Risk factors

Several risk factors have been assessed to find out the causes which influence the occurrence of *N. caninum* infection in this study. Among them time of abortion in pregnancy (p=0.000), Abortion history of the animals (p=0.001), presence of dog in animals farm or premises (p=0.001) and management practices (p=0.001) were found as significant factors which influences the occurrences of *N. caninum* infection (Table 1).

Multivariate logistic regression was performed to study the effects of risk factors to *N. caninum* infection in animals. The results demonstrated that animals that were in contact with dog feces or presence of dog in animal premises or farms had 17.15 times odds of getting *N. caninum* infection (OR = 17.15, 95% CIs = 2.23–131.73) than animals who were never in contact with dog and dog feces. Again, animals having previous history of abortion had 17.16 times odds of bearing this protozoan infection (OR = 17.16, 95% CIs = 4.73–62.18) than animals having no record of abortion. Logistic regression analysis also clarified that

management style has also an impact on spreading the *N. caninum* infection in animals. Animals who reared in free range system had 15.78 times more prone to getting infection (OR = 15.78, 95% CIs =1.98-125.67) than animals who reared in intensive and semi-intensivesystem (OR = 6.95, 95% CIs = 1.80–60.01). Animals that had history of abortion during second trimester had 11.51 times odds of positivity with *N. caninum* than other animals who had history of abortion in first and third month.

Discussion

In many cases of miscarriage, the cause cannot be determined due to the involvement of a wide range of factors. The economic losses due tothe abortion of *N. caninum* infected cattle, sheep, goats, and buffalo should not be ignored. Many undiagnosed cases of abortion, stillbirth and retained placenta may significantly affect the livestock development in Bangladesh [25]. Nested PCR is an accurate and widely used molecular tool to investigate the global prevalence of neosporosis in mother and aborted fetuses [26].

The status of animal infection with *Neospora* in Bangladesh is unknown. It is significant to assess the prevalence of this parasite in cattle, sheep, goats, and buffalo, because of its economic and ecological significance. The prevalence data are important for designing the control programs and cost related to health and production losses caused by *N. caninum*.

Since transplacental transmission is the main route of *N. caninum* infection among animals we collected aborted fetus of animal for detection as causal agent. Furthermore, we excised brain from the aborted fetus because the brain is the organ of choice for diagnosis of neosporosis [27].

The overall prevalence of *N. caninum* in aborted fetus of the animals based on nested PCR is 12.7% (20/157) in this study which is in accordance with the reported stated by Dubey [6], whereas the prevalence of abortion in commercial dairy farms of Bangladesh recorded based on history (98/1201) was 8.16% [28]. This variation might be due to the differences in the sample size, geography, and different types of study methods. The estimated prevalence was much higher than previously published report from Nepal while showed similar fashion with reported prevalence in animals in India [29, 30].

The global prevalence of *N. caninum* in aborted fetuses of sheep and goats using molecular methods was reported to be 7–15% [31]. While the prevalence in our study was 14.8% and 11.8% in sheep and goat, respectively. Molecular detection of *N. caninum* in aborted fetus of Buffalo (8.7%), indicates the possible cause of abortion in buffalo in Bangladesh. Similarly, seropositive survey of *N. caninum* in aborted fetus of buffalo in Italy (47–59%) suggests a serious role of *N. caninum* as abortive agent among buffaloes [32].

Interestingly the rate of prevalence did not differ significantly among the three different study areas (districts). However, results showed the presence of *N. caninum* in three different regions of Bangladesh, proving that the parasite might be involved in the reproductive failure in farms from a large part of the country.

The sequence obtained from cattle, sheep, goats and buffaloes shared 97% homology to each other. All the *N. caninum* sequences generated in this study showed homology(91%)with the sequences isolated from

England (L49389.1), Iran (OP136035.1), Iraq (MZ725531.1), Canada (EU564167.1), China (JN634857.1), Australia (AF338411.1, GU194959, GQ899204, GQ899205.1, GU194960,1, GU194958.1, MK203863.1), New Zealand (AY259043.1, AY463245.1, AY259042.1), Brazil (DQ832318.1, MW022528.1, MW022526.1, MW022527.1), Israel (MT860359.1,MT826198.1), USA (EF219139.1) which conform the validity of study sequences. To author's best knowledge, it was first molecular characterization of *N. caninum* in livestock so far in Bangladesh.

The significant risk factors for neosporosis found in this study are time of abortion in pregnancy, abortion history of the animals, presence of dog in animals farm or premises and management practices. These major risk factors are reported in several researches [27, 33, 34].

Abortion due to *N. caninum* can occur at any time of gestation from 3 months to term [35]. Highest prevalence was found in animals where abortion occurred in second trimester 17(21.51%). According to literatures most neosporosis-induced abortions occur at 4–6 month gestation [6, 35, 36, 37], which supports the result of this study.

The overall prevalence rate of *N. caninum* infection in these animals that had an abortion was 33.33%. Animals having previous history of abortion had 17.16 times odds of bearing this protozoan infection. Such higher prevalence (47%) rate and risk of *N. caninum* infection in bovines that had an abortion was reported by Nayeri et al. [31].

Management practices had a significant impact on prevalence of neosporosis in this study. Animals that were in contact with dog feces or presence of dog in animal premises or farms had 17.15 times odds (more susceptible/risk) of getting *N. caninum* infection. The presence of dogs on cattle farms is evidenced as a risk factor for bovine neosporosis [37, 38, 39, 40]. In the present study, dogs in rural areas had frequent and close contact with local breeds that graze freely on the pastures. On the other hand, dogs are peridomestic in nature and sometime living with livestock. Therefore, in agreement with Altaee et al. [41], we assume that the high level of neosporosis amonglocal breeds could be related to the close contact with dogs. This may increase the chances of abortion in local breeds [42, 43].

In agreement with previous studies in Argentina, Venezuela and Ethiopia [44, 45, 46], our multivariate analysis (Fig. 2), revealed that crossbreeds were less likely than local strains to acquire the infection. However, a previous study in Pakistan reported that crossbred cattle were more likely to be infected than other cattle [47].This variation could be due to the differences inthe systems foreach breed production, in addition to the disparity in the susceptibility to the infection [40].

Irrespective of species and breed of animal, *N. caninum* was prevalent 15.8% in animals aged 2 to 4 years (Table 1). Similar observations were made by Metwally et al. [48] in the youngest age group (< 3 years) was almost 17%, very close to this report.

Cumulatively, the findings of this study suggest an important role of *N. caninum* as a possible abortive agent for these animal species. Consequently, routine diagnosis is necessary to investigate neosporosis in these farm animals, especially in herds with pregnancy loss or interruptions.

The results of the present study indicate that the infections of cattle, sheep, goat, and buffalo with *N. caninum* are widespread in the studied region of Bangladesh. Therefore, integrated control strategies and measures are recommended in farm animals for neosporosis.

Conclusion

Our study revealed that *N. caninum* could be one of the major causes of the aborted fetuses of cattle, sheep, goat and buffaloes. Establishment of prevention and control programs for *N. caninum* should consider the associated risk factors. For better understanding of neosporosis molecular epidemiology, we recommend further investigations on larger sample sizes of other definitive and intermediate hosts.

Declarations

Acknowledgements

The authors would like to thank

Funding

The research was conducted under the funding support from Bangladesh Agricultural University Research System (BAURES) of Bangladesh Agricultural University (Grant No. 2021/121/BAU).

Availability of data and materials

All the data collected for the study were analyzed and are included in the manuscript. Raw data are available on request.

Authors' contributions

MS conceived the idea, designed and supervised the study. PB, AK, MZH, and ARMBA collected the data and performed the laboratory works. AK, and NA formatted the data and analyzed. MS wrote the first draft of the manuscript. MHW reviewed and edited the manuscript. All the authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

Ethical approval was obtained from the Ethics Committee of Faculty of Veterinary Science, Bangladesh Agricultural University (AWEEC/BAU/2021/57).

Consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests.

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Tables

Table 1 Test of statistical significance of risk factors in the *Neospora caninum* infection

Risk factor	Variables	Presence of *Neospora*		*P* value
		Yes	No	
Species	Cattle	8(16%)	42(84%)	0.732
	Buffalo	4(8.7%)	42(92.3%)	
	Sheep	4(14.8%)	23(85.2%)	
	Goat	4(11.8%)	30(88.2%)	
Locality	Jessore	7(13.2%)	46(86.8%)	0.992
	Rangpur	6(12.5%)	42(87.5%)	
	Mymensingh	7(12.5%)	49(87.5%)	
Abortion period	First trimester	1(2.3%)	42(97.7%)	0.000
	Second trimester	17(21.51%)	62(78.49%)	
	Third trimester	2(5.71%)	33(94.29%)	
Previous Abortion history	Yes	17(33.33%)	34(66.7%)	0.000
	No	3(2.8%)	103(97.2%)	
Presence of dog	Yes	19(20.9%)	72(79.1%)	0.001
	No	1(1.5%)	65(98.5%)	
Management style	Intensive	1(1.9%)	51(98.1%)	0.001
	Semi-intensive	6(12%)	44(88%)	
	Free range	13(23.6%)	42(76.4%)	
Age	x≤ 2years	2(8%)	23(92%)	0.768
	2<x≤4 years	9(15.8%)	48(84.2%)	
	4<x≤6 years	6(13.0%)	40(87.0%)	
	6<x≤8 years	3(10.3%)	26(89.7%)	

Table 2 Logistic regression analysis of the factors associated with *Neospora caninum* infection

Risk factor	Variables	B	S.E.	Wald	*df*	Sig.	Odds ratio Exp (B)	95% C.I for Exp (B)	
								Lower	Upper
Abortion period	First trimester	1.11	0.23	6.56	1	0.068	Reference	-	-
	Second trimester	1.86	.78	11.67	1	0.000	11.51	1.47	89.86
	Third trimester				1	0.001	2.54	1.2211	29.30
Previous Abortion history	Yes	2.84	0.65	18.74	1	0.000	17.16	4.739	62.18
	No	-1.45	-0.67		1		Reference	-	-
Presence of dog	Yes	2.84	1.04	7.46	1	0.001	17.15	2.233	131.73
	No	-1.11	-.11		1	0.004	Reference	-	-
Management style	Intensive	1.23	.98	4.56	1	.078	Reference	-	-
	Semi-intensive	1.46	.76	7.89	1	0.003	6.95	1.806	60.01
	Free range	1.98	.89	15.67	1	0.000	15.78	1.9828	125.67

Figures

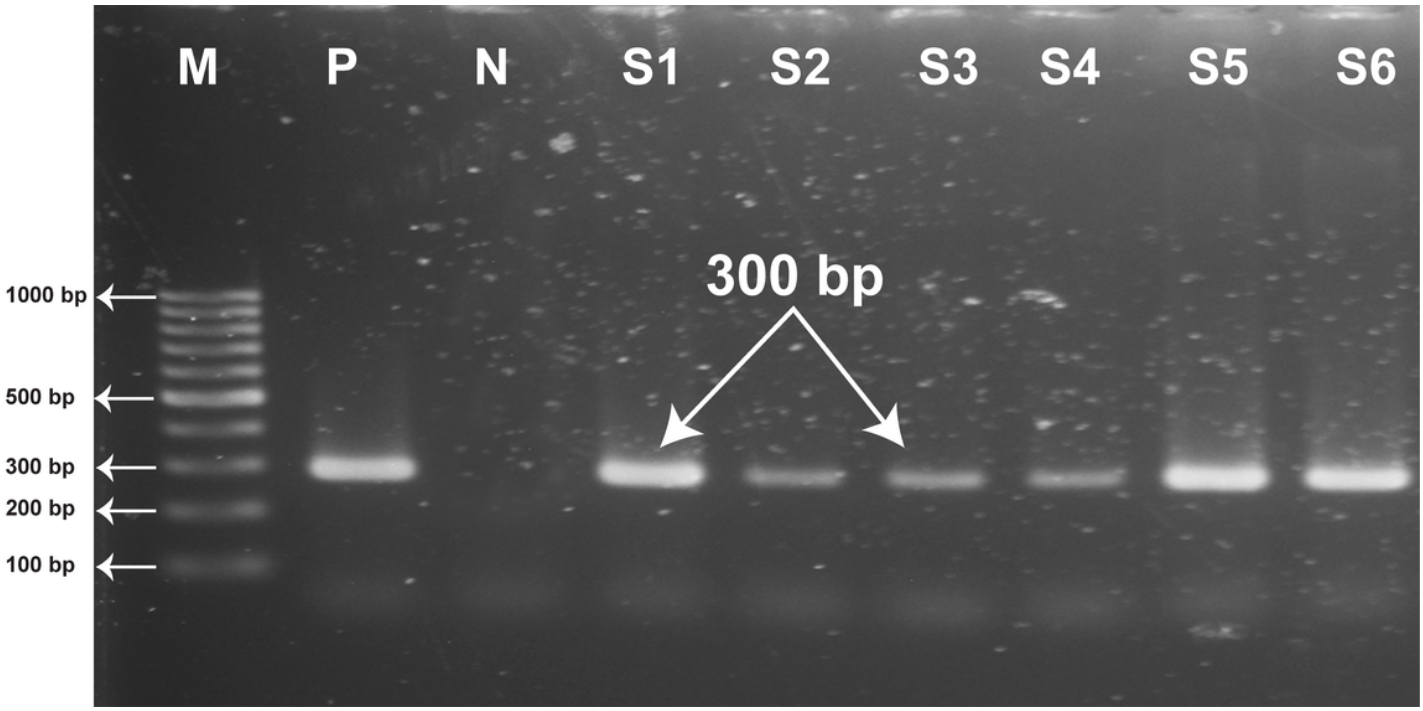

Figure 1

Confirmation of *N. caninum* by Nested PCR: Target sequence of *N. caninum* was 295 bp (Lane M= Marker-1kb), lane N= negative control)

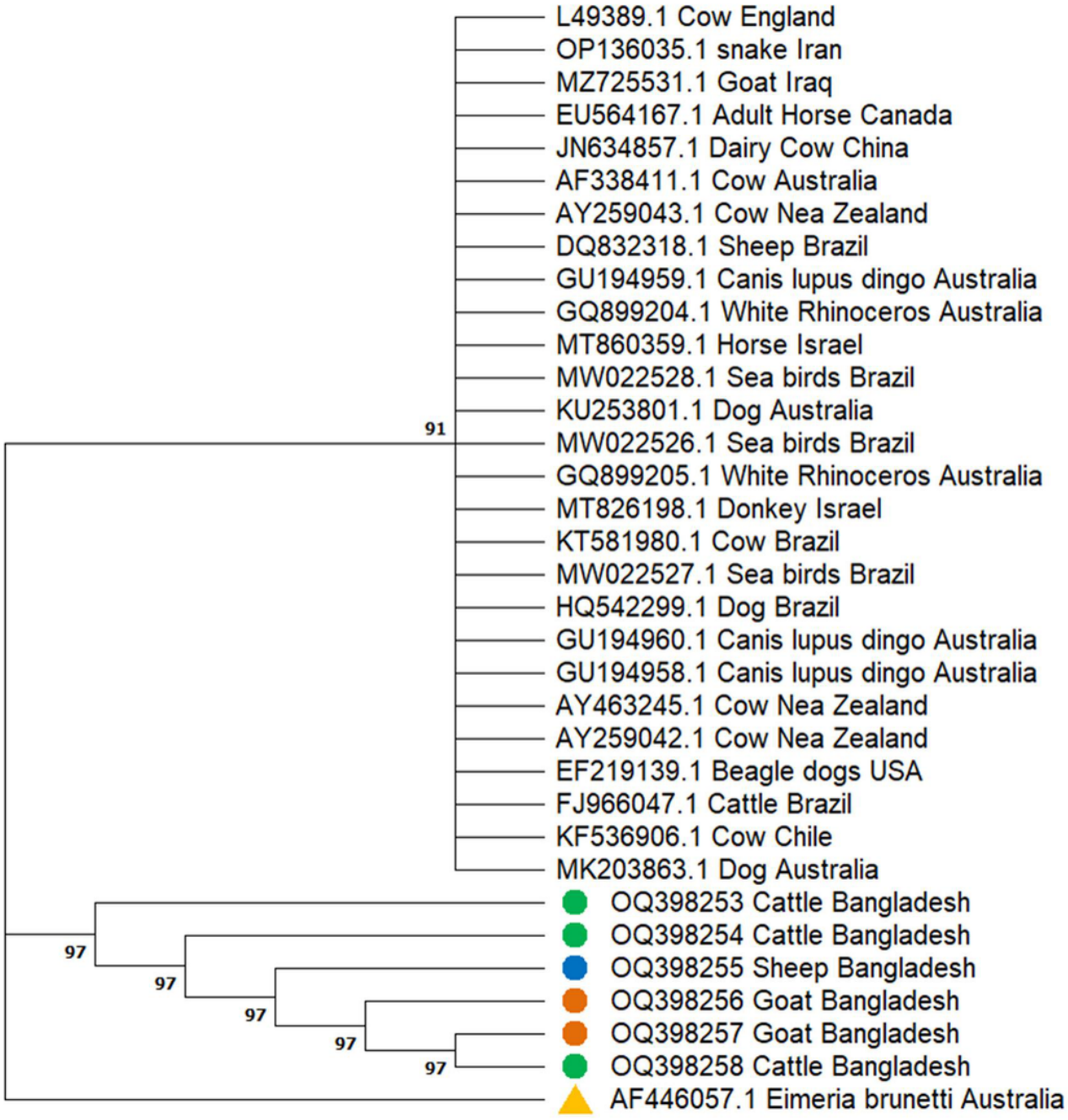

Figure 2

Neighbor-joining phylogeny of ITS2 gene sequences of *N. caninum* species. The percentage at branch points is associated taxa clustered together of 1000 bootstrap data sets that supported the specific internal branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. GenBank accession numbers accompany each taxon name. Green circle: *N. caninum* sequences isolated from cattle brain in Bangladesh; Blue circle: from sheep; Orange circles: from goat; Yellow triangle: an outgroup.

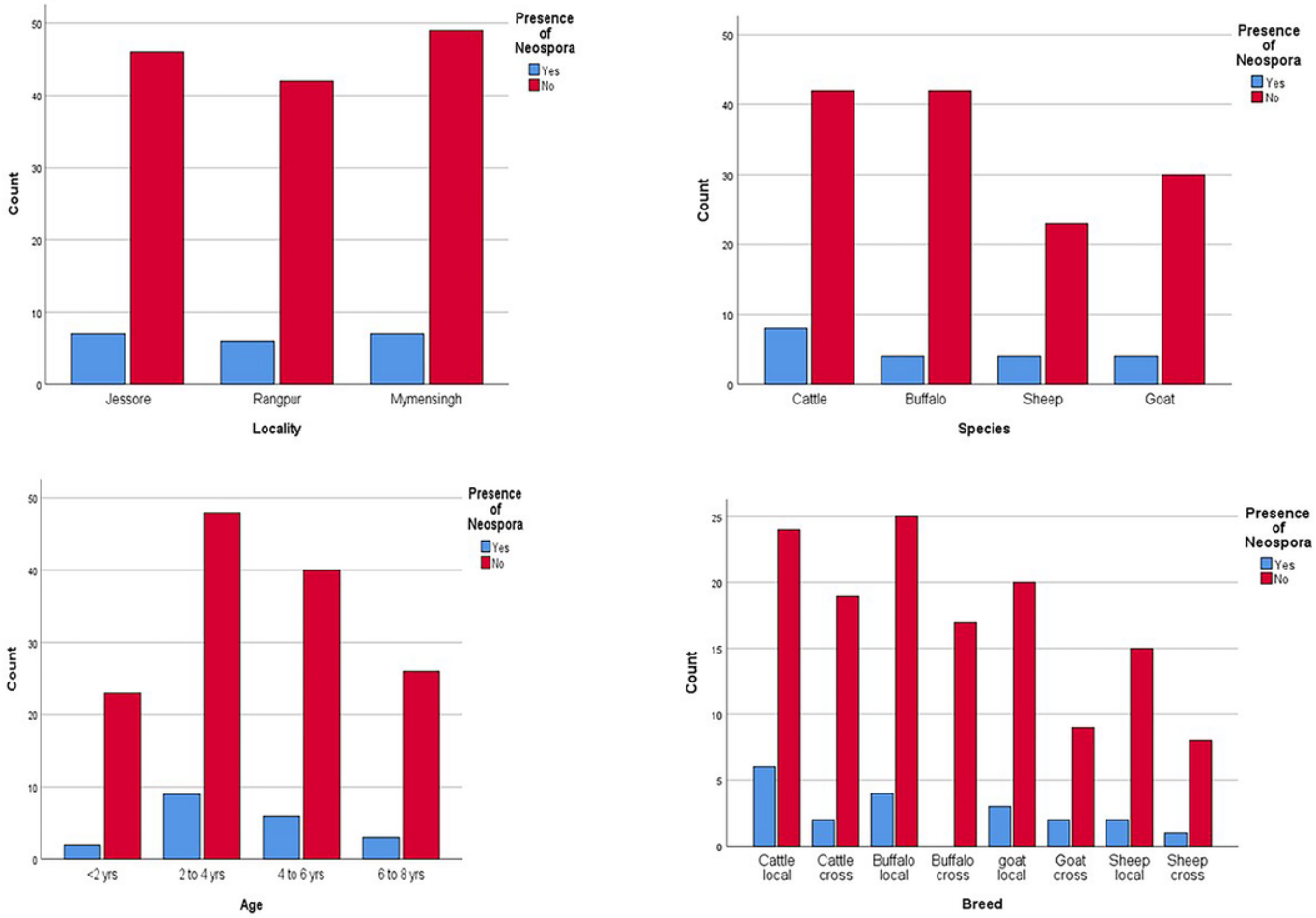

Figure 3

Prevalence of *N. caninum* in relation to locality, species, age, breed