



Molecular investigation of *N. caninum* infection in dogs from Mosul city, Iraq

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Abstract

This study was carried out between October 2023 and July 2024 on 40 dogs of both sexes, ages, and lifestyles. All dogs suffer from nervous signs, paralysis, and paresis, which were examined to detect the infection rate of *N. caninum* in Mosul city, Iraq, using the conventional polymerase chain reaction (c-PCR) technique. A 5ml sample of dog blood was collected from the cephalic vein, and buffy coats were separated from the whole blood and then kept at -20 °C. The DNA of the nc5 gene for *N. caninum* was amplified using specific primers. The infection rate of *N. caninum* was 37.5% (15/40) in Mosul city. Many risk factors were linked with a high infection rate of *N. caninum*, including > 1 year - ≥ 2 years, gender, stray, and large or small breeds of dogs. The South Korean company Macrogen Inc. received favorable PCR products that they could be sequenced. Additionally, 15 local sequences (4 sequences total) with the Nc5 gene were analyzed phylogenetically and deposited with accession numbers in the NCBI GenBank: PQ084657.1, PQ084658.1, PQ084659.1, and PQ084660.1. These sequences are highly related to other sequences registered in GenBank, including those from Iraq and Iran, with 98.04-100% identity. This study concludes that *N. caninum* is widespread in Mosul, Iraq. It is the first study that highlights the phylogenetic analysis of *N. caninum* in Mosul.

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Introduction

One significant parasite infection in dogs is *N. caninum*. In cattle, the illness is clinically significant and can result in abortion (1,2). The most accurate method for determining the frequency of canine neosporosis is still serological surveys because oocysts are rarely discovered in dog feces (3,4). Neospora is a widespread protozoan illness that affects dogs worldwide (5), including Iraq (6,7). Reports on dogs in Baghdad showed that Iraq had 4% of home dogs and 15% (8,9). In Urmia, Neospora infections in dogs have been documented at a comparable prevalence of 27% (10,11). According to reports, *N. caninum* seroprevalence was 11% in Belgian dogs and 20% in dogs from China (12,13). Canines infected with the virus could be a significant source of *N. caninum* infections in livestock (14,15). Prior research

has shown a correlation between the presence of dogs in the surroundings and abortion rates in cattle (16,17). Domestic and stray dogs in Iraq's rural areas frequently come into close contact with livestock, increasing the possibility that Neospora would infect these animals (18).

There are few serological or molecular investigations on Neospora infection in Iraqi dogs using genetic techniques, and we sought to ascertain the level of *N. caninum* infection in a Mosul-based canine community.

Materials and methods

Ethical approval

This research was accepted by the scientific board of the College of Veterinary Medicine at Mosul University; UM.VET.2024.064.

Animals and samples obtained

In this study, a total of 40 dogs from both sexes (males and females), different ages (6 months - 5 years), and lifestyles (stray and household). All dogs suffer from nervous signs, paralysis, and paresis, which were examined to detect the infection rate of *N. caninum* using conventional polymerase chain reaction (c-PCR). From October 2023 to July 2024, 5ml samples of the dog's blood were collected from the cephalic vein, buffy coats were separated from the whole blood, and then kept at -20 °C (19,20).

DNA extraction and amplification

Using a blood genomic DNA extraction kit, the buffy coat samples of all 40 dogs were extracted in accordance with the manufacturer's instructions. (Qiagene, Germany). Using specific primers, the forward primer was NP6 (5'-CAGTCAACCTACGTCTTCT-3'), and the reverse primer was NP21 (5'- GTGCGTCCAATCCTGTAAC-3'), the extracted DNA was amplified using the c-PCR technique. The Nc5 gene of *N. caninum* was amplified using standard PCR procedures. 0.5 µL of each primer (10 pmol/µL), 11.5 µL of 2× Taq PCR mix (Amplicon, Odense, Denmark), 30 ng of template DNA, and 12.5 µL of ddH2O were used in the PCR, the reaction volume of 25 µL. The PCR's temperature profile consisted of one cycle lasting five minutes at 95°C, thirty cycles of fifty seconds each at 94°C, thirty seconds at 55°C, fifty seconds at 72°C, and one cycle lasting four minutes at 72°C. To visualize the PCR results, they were separated on 1.5% agarose gel and stained with GelRed nucleic acid gel dye under a BioDoc gel documentation system. The amplified DNA fragment was predicted to be around 328 bp (21).

Use of phylogenetic analysis and DNA sequencing

The South Korean company MacroGen Inc. received the positive PCR samples and used the Sanger sequencing technique to sequence them. In summary, the corresponding primers and 25 µl of the target Nc5 gene's PCR result were delivered. The sequencing process resulted in text files in the FASTA format. The NCBI GenBank received a selection of the sequences, which were then registered and assigned unique accession numbers (22).

Statistical analysis

With the IBM-SPSS Statistics (Version 22) software, the two-sided Chi-square and Fischer's exact tests were used to determine the variation in infection rate among the different risk factors. Using factors with P values <0.05, deemed significant, is advised by Fisher exact test P value results if the anticipated cell value in the Chi-square test is less than 5. Using two by two tables and the Epi-InfoTM 7 program (Version 7).

Results

In this study, the infection rate was 37.5% (15/40), using the c-PCR technique, which was about 328 bp (Figure 1). The proportion of dogs in the current study showed a significant difference between dog's lifestyles. There was a significantly higher (P<0.0000) among stray dogs, 87.5% (OR: 56.00 times, CI: 5.88-532.95), compared to household dogs, 4.7%. Regarding the gender factor, this study noted that The *N. caninum* infection rate was not substantially (P<0.0217) greater in canines that are male 54.5% (OR: 6.00 times, CI: 1.34-26.80), compared to female dogs 16.7%. The study also found that dog ages differed significantly. In dogs older than or equal to the test subjects, the infection rate was considerably (P<0.0432) and (P<0.0302) higher. One year to more than an equal 2 years 46.6% (OR: 9.62 times, CI: 0.97-94.54), and 53.8% (OR: 12.83 times, CI: 1.26-130.51) respectively, compared to dogs less than 1 year 8.3%. Moreover, this study demonstrates significant differences between dog breeds. The prevalence of *N. caninum* infection was considerably (P< 0.0002) higher in large breed dogs, 68.4% (OR: 20.58 times, CI: 3.58-118.32) compared to small breeds, 9.5% (Table 1).

The results of the Nc5 gene sequencing revealed a genetic sequence for the samples (n=15) that were diagnosed in Mosul and delivered to MacroGen Company in South Korea. It was found that there were only four sequences out of the total were registered in NCBI GenBank with the accession number PQ084657.1, PQ084658.1, PQ084659.1, PQ084660.1 (Table 2).

Table 1: Odds ratio of dog factors associated with the infection rate of *N. caninum*

Factors	No. tested cases	Positive n (%)	OR	CI	P Value	
Animals' management	Household	21	1 (4.7) ^a	1	5.88-532.95	0.0000
	Stray dogs	19	14 (87.5) ^b	56.00		
Gender	Females	18	3 (16.7) ^a	1	1.34-26.80	0.0217
	Males	22	12 (54.5) ^b	6.00		
Age	< 1 year	12	1 (8.3) ^a	1	0.97-94.54	0.0432
	≥ 1 year - 2 years	15	7 (46.6) ^b	9.62		
	≥2 years	13	7 (53.8) ^b	12.83		
Breed	Small breed	21	2 (9.5) ^a	1	3.58-118.32	0.0002
	Large breed	19	13 (68.4) ^b	20.58		

OR: Odds ratio, CL: Confidence of interval, Different letter (a, b) means significant under P<0.05.

Table 2: The *N. caninum* sequences NCBI GenBank accession numbers in dogs in Mosul city, Iraq

Accession numbers	Sequencing 5'.....'3
PQ084657.1	CCCTTCCCTCGTCCGCTTGCTCCCTATGCATAATCTCCCCCGTATCAGCGCCGCCGGTGTTC CCTCAACACAGAACACTGAACTCTGGATAAGTATCATTGACACACTGTCCACACCCTGAC GCAGGCTGATTTCAACGTGACGAATGACTAACCACAAACCACGTATCCCACCTCTCACCG CTACCAACTCCCTCGGTTACCCGTTACACACTATAGCCACAAACAAAAAAGGAGCCTT GCTGCCGCAGGCTGCGCCAACAACGACACGTCCGCATACCAACACGTTACAGGATTGG ACGC
PQ084658.1	CCCTTCCCTCGTCCGCTTGCTCCCTATGCATAATCTCCCCCGTATCAGCGCCGCCGGTGTTC CCTCAACACAGAACACTGAACTCTGGATAAGTATCATTGACACACTGTCCACACCCTGAC GCAGGCTGATTTCAACGTGACGAATGACTAACCACAAACCACGTATCCCACCTCTCACCG CTACCAACTCCCTCGGTTACCCGTTACACACTATAGCCACAAACAAAAAAGGAGCCTT GCTGCCGCAGGCTGCGCCAACAACGACACGTCCGCATACCAACACGTTACAGGATTGG ACGC
PQ084659.1	CCCTTCCCTCGTCCGCTTGCTCCCTATGCATAATCTCCCCCGTATCAGCGCCGCCGGTGTTC CCTCAACACAGAACACTGAACTCTGGATAAGTATCATTGACACACTGTCCACACCCTGAC GCAGGCTGATTTCAACGTGACGAATGACTAACCACAAACCACGTATCCCACCTCTCACCG CTACCAACTCCCTCGGTTACCCGTTACACACTATAGCCACAAACAAAAAAGGAGCCTT GCTGCCGCAGGCTGCGCCAACAACGACACGTCCGCATACCAACACGTTACAGGATTGG ACGC
PQ084660.1	CCCTTCCCTCGTCCGCTTGCTCCCTATGCATAATCTCCCCCGTATCAGCGCCGCCGGTGTTC CCTCAACACAGAACACTGAACTCTGGATAAGTATCATTGACACACTGTCCACACCCTGAC GCAGGCTGATTTCAACGTGACGAATGACTAACCACAAACCACGTATCCCACCTCTCACCG CTACCAACTCCCTCGGTTACCCGTTACACACTATAGCCACAAACAAAAAAGGAGCCTT GCTGCCGCAGGCTGCGCCAACAACGACACGTCCGCATACCAACACGTTACAGGATTGG ACGC

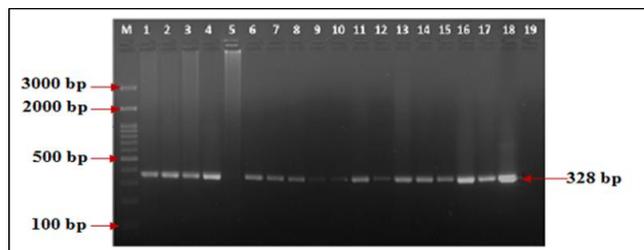


Figure 1: Gel electrophoresis for Nc5 gene of *N. caninum*. Lane 1 represents 100 bp DNA marker; lanes (1-4,6-18) are positive samples; lane 19 is negative control.

Based on the alignment of multiple sequences in MEGA11 software, according to the study. Table 3 indicates that the alignment score between the derived local sequences was 100. Additionally, the outcomes of individual sequencing analyses of local sequences using the Blastn procedure in the (nr/nt), *N. caninum* in dogs is quite similar to sequences with numbers recorded in the GenBank of various nations, including Iran, which is 98.04%-100% identical MT709295.1, MT709296.1, MT955656.1, MT766316.1, MT766317.1, and MT955657.1 and Iraq OP244818.1 (Table 4). After nucleotide sequence reconstruction using MEGA 11-Bootstrap analysis, the phylogenetic tree of the local genetic sequences of *N. caninum* recorded in the NCBI Genbank revealed highly

phylogenetic properties and an extremely close evolutionary relationship 99.04-100% to other global sequences of the same strains recorded in the Genbank for different countries, such as Iran and Iraq (Figure 2).

Table 3: Alignment score between the obtained sequences of *N. caninum* using the Multiple Sequence Alignment Program

Sequences accession number	Alignment score
PQ084657.1	100
PQ084658.1	
PQ084659.1	
PQ084660.1	



Figure 2: *N. caninum* strains phylogenetic tree from Mosul, Iraq (*).

Table 4: *N. caninum* sequences from the local area that are similar to other sequences in GenBank using NCBI BLASTn

Sample	Query Cover %	Identic Number %	GenBank	Country
	100	100	MT709295.1	Iran
PQ084657.1	100	100	MT709296.1	Iran
PQ084658.1	100	100	MT955656.1	Iran
PQ084659.1	100	100	MT766316.1	Iran
PQ084660.1	100	100	MT766317.1	Iran
	100	99.35	MT955657.1	Iran
	100	98.04	OP244818.1	Iraq

Discussion

Dogs are the sole hosts of *N. caninum* and have a major role in the epidemiology of neosporosis (23). Based on the research methods, there are differences in the prevalence of *N. caninum* in dogs across different locations, the number of animals examined, the geographical location and the number of dogs, either at home or stray (24). When dogs are fed raw meat instead of ready-to-eat items, their risk of contracting Neospora infection is increased (25). A low rate of Neospora virus frequency in dogs from a Baghdad governorate was documented 10% (26). In comparison to reports in this study, While Turkey reported 2% (27), and 3.8% showed (28), the prevalence rate of Neospora infection was lower here (29). While most previous studies considered seropositivity using a highly precise specific modified agglutination test and an immunofluorescence antibody assay (IFA) with a very low cut off, the current study determined the dogs' Neospora susceptibility. This could have decreased our study's seropositivity rate for Neospora compared to earlier ones. Other reasons are the presence of other hosts of *N. caninum* and the bad habits of the farmer. They provide placentas to guard dogs which play a critical role in spreading parasites and completing the life cycle.

Several epidemiological investigations in parts of the world have indicated a rising pattern of Neospora positivity with age, The older canines in the current investigation had a higher prevalence rate for *N. caninum*. The positivity of infection in dogs with increased animal age, are consistent with the positive seroprevalence of increasing age above 26 years; the highest infection rate was found in the older dog group, while the lower infection rate was found in younger dogs (30). Furthermore, Gozdzik *et al.* (31), in their study on dogs in central Poland, discovered that the prevalence of infection increased with age, with the age group over 10 years showing the highest infection rate. Meanwhile, Al-Majali *et al.* (32) found that the seroprevalence of the parasite was significantly higher in sheep and goats older than 4 years.

The results showed 0% in indoor dogs compared to outdoor dogs 78.9% (30). They found that dogs raised in rural regions had a considerably higher infection rate of *N. caninum* 18.17% than dogs raised in urban settings 11.33%. This intriguing discovery could be explained by dietary

practices such as consuming raw meat containing parasite cysts, living conditions variations, and welfare (30). Dogs in both urban and rural settings are likely to become infected by eating raw or undercooked beef, and there have even been reports of bitches passing the infection vertically to their offspring (33). However, dogs in rural and environment conservation regions have a higher chance of predating possibly infected small mammals and birds. They may also have access to the carcasses of wild animals and livestock, as well as aborted fetal tissues (34).

Certain research has indicated a correlation between gender and positivity. Males recorded higher positivity than females, it should be emphasized that positivity only reflects Neospora exposure, and that a very small percentage of positive dogs are releasing oocysts into the environment (35). In an assessment of 132 dogs' stool samples in Australia, King *et al.* (36). Other parameters in this study showed that 0% of infection was shown in small breeds of dogs when compared high percentage of infection in large breeds, this parameter We did not find a study that addressed this, but through our experience in the field of veterinary work, we can find that the reason for this is due to the lack of exposure of small breeds to the stages of development of the parasite in its life cycle, due to the decline in its breeding inside homes, In addition, dogs of large breeds are often used for guarding or protecting herds of livestock, which increases the chance of them being exposed to the parasite. Dogs of small breeds are often given regular attention in veterinary examinations and treatments, which is one of the fundamental reasons for the lack or absence of their infection.

In the current investigation, no dog that was examined tested positive using the molecular approach, and no dog's buffy coat had any parasite DNA. This would suggest no acute neosporosis in any of the dogs under study. It can also indicate that a buffy coat specimen is not a good fit for molecular detection of Neospora in dogs (21). The four strains found in the study belong to the same subgroup and are part of certain worldwide registered species, according to the results of the phylogenetic tree of the nc5 gene (22). This association proves that these animals are highly genetically related (37). In addition, the study's isolated strains (38) showed genetic diversity between them according to where they are in the phylogenetic tree (39). From a genetic

perspective, the divergence and convergence of local and global strains suggest that they have a shared evolutionary origin, and geography plays a part in this (40).

Conclusions

Nineveh province has more prevalent parasites in dogs with nervous indications than in dogs unaffected by *N. caninum*.

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Conflict of interest

The manuscript's authors affirm that there is not a conflict of interest.

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التقصي الجزيئي عن طفيلي البوغة الكلبية في الكلاب في مدينة الموصل

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الخلاصة

أجريت هذه الدراسة بين الفترتين من أكتوبر ٢٠٢٣ إلى يوليو ٢٠٢٤. على إجمالي ٤٠ كلبا من كلا الجنسين، بأعمار مختلفة وأنماط حياة مختلفة. تعاني جميع الكلاب من علامات عصبية وشلل جزئي. والتي تم فحصها للكشف عن معدل الإصابة بطفيلي البوغة الكلبية في مدينة الموصل، العراق، باستخدام تقنية تفاعل البوليميرات المتسلسل التقليدية. تم جمع عينات ٥ مل من الدم من الوريد الرأسي للكلاب، وتم فصل طبقة الخلايا اللمفية عن الدم الكامل باستخدام الفايكول، ثم حفظها عند -٢٠ درجة مئوية لحين إجراء الفحص عليها. استخلص الدنا للطفيلي من طبقة الخلايا اللمفية ومن ثم تم تضخيم الحمض النووي للجين الطفيلي باستخدام بادئات محددة متخصصة للطفيلي، بينت النتائج أن نسبة الإصابة بالطفيلي ٣٧,٥% (٤٠/١٥) في مدينة الموصل. تم إيجاد العلاقة ما بين نسبة الإصابة وعدد من عوامل الخطورة بارتفاع معدل الإصابة بطفيلي البوغة الكلبية، بما في ذلك < سنة واحدة - ≤ ٢ سنة، جنس الكلاب (ذكور أو إناث)، نظم التربية الضالة أو مستأنسة، والسلالات الكبيرة أو الصغيرة. تم إرسال الناتج النهائي لتضخيم الدنا والذي أعطى نتائج إيجابية لتفاعل البلمرة المتسلسل. وتبين إن التحليل التطوري الى ١٥ تسلسلا محليا للجين المتخصص للطفيلي حيث سجلت أربع عترة محلية للطفيلي في بنك الجينات العالمية والتي كانت مع ارتباط كبير بالتسلسلات الأخرى المسجلة في بنك الجينات مثل العراق وإيران، وتخلص هذه الدراسة إلى أن طفيلي البوغة الكلبية منتشرة على نطاق واسع في مدينة الموصل بالعراق. وإذ تسلط هذه الدراسة الأولى الاهتمام على التحليل الوراثي للطفيلي في مدينة الموصل بالعراق.