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Original Article

Assessment of *Neospora caninum* infection in bulls using serological and molecular techniques

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ABSTRACT

Neospora caninum is a significant cause of abortion and economic losses in cattle worldwide. The main aim of the present work was to detect the prevalence of *N. caninum* infection in bulls in Hamedan (Iran) using serology and molecular techniques. All blood samples ($n = 792$) were screened for detecting the antibodies to *N. caninum* using enzyme-linked immunosorbent assay (ELISA). Then seropositive animals were rechecked using the immunofluorescent antibody test (IFAT). Also, blood, epididymis, and spinal cord samples were collected from animals for molecular analysis using nested PCR. In serology, using ELISA, 3.91% of animals were seropositive for *N. caninum*. Additionally, true prevalence based on the sensitivity and specificity of the ELISA was calculated 1.25% (95% CI: 0.48–2.02%). *Neospora*-infection in animals, calculated as the number of bulls seropositive and/or one sample positive to nested PCR, was 3.40%; and 19 bulls tested positive by both serology and molecular diagnostic methods. The overlaps between ELISA and molecular results were observed in 74.19% of whole blood samples, 80.64% of the epididymis, and 87.09% of the spinal cord. Using ELISA, the seroprevalence of *N. caninum* was detected 1.8% in ≤2 and 5.45% in >2 years old group of animals ($p = 0.009$, PR = 3.1). In addition, the seropositivity in Holstein and native breed animals was calculated 6.57% and 2.93%, respectively ($p = 0.019$, PR = 2.3). Seven sequences with 94.9–99.3% similarity were detected in multiple alignments of positive PCR products. Our work was the first comprehensive evaluation of *Neospora*-infection/neosporosis in Iranian bulls. We detected a low prevalence of infection in animals compared to previous reports. The ELISA is a sensitive serological technique for detecting the highest number of positive bulls in the present investigation and, the nested PCR is a reliable technique to identify *Neospora*-DNA.

1. Introduction

Neospora caninum, a parasite belonging to the apicomplexan protozoa, is the cause of neosporosis in animals especially in cattle and dogs with global distribution (Dubey and Schares, 2011). For the first time, in 1984, the parasite was found in dogs with neurologic disorders in Norway (Bjerkas et al., 1984). Domestic and wild animals play a part in the parasite life cycle as definitive and intermediate hosts, but the role of dogs and cattle is dominant in this cycle as well as in the distribution of the infection. Dogs can play the role of definitive and intermediate hosts at the same time (Dubey et al., 2007). There are reports on *Neospora*-infection in humans, but the zoonotic aspect of the disease is unclear (Gharekhani et al., 2021b).

This protozoan is transmitted during gestation (from infected mothers to the neonates) or postnatal via the consumption of infected milk. Vertical transmission is the main infection route in cattle with an

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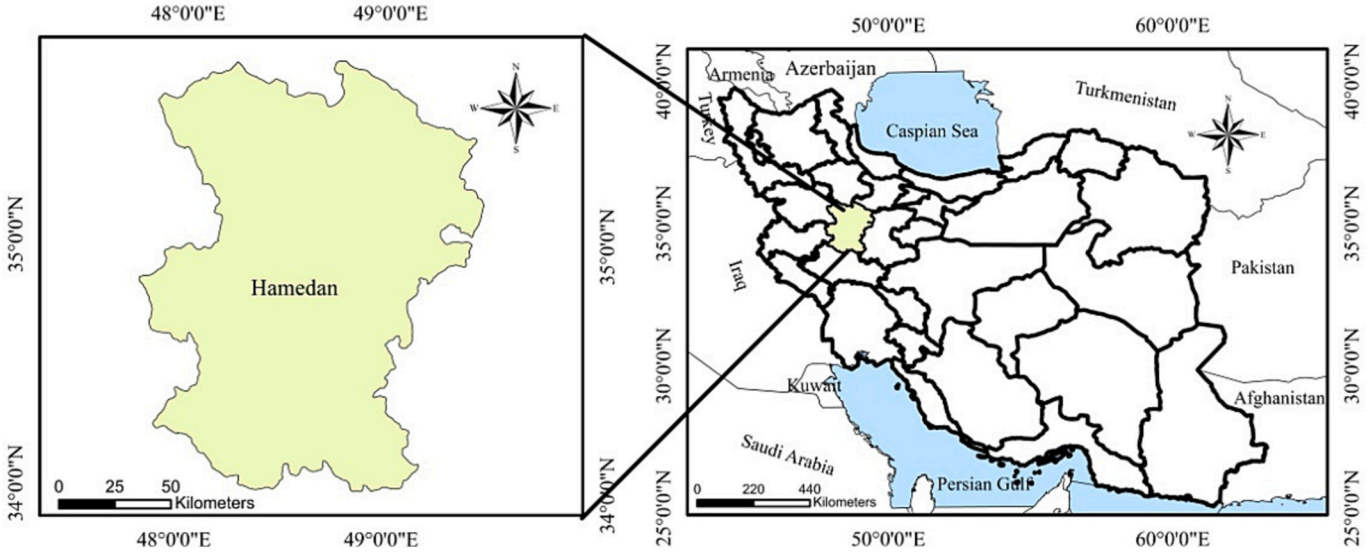

Fig. 1. Geographic map of the sampling area, Hamedan, West part of Iran.

efficacy of up to 93% (Dubey and Schares, 2011). The free roaming of dogs in dairy farms and their access to cattle is a considerable risk factor for increasing horizontal transmission as well as stormy abortions in the herds (Gharekhani and Yakhchali, 2019). Neosporosis is a major cause of abortion and reproductive failure occurrence in cattle (Ansari-Lari et al., 2017). The tachyzoites of the parasite can play the role of a spermicide compound (Canada et al., 2006). Different injury levels were detected in the reproductive organs of male mice after a challenge with the bovine isolate of *N. caninum* (Li et al., 2021). Reduced spermatogenesis, sperm motility, and increased sperm deformity were dominant in pathology findings. After mating infected male mice with healthy female mice, fetal mortality increased and fertility, live birth numbers, and birth weight decreased significantly. Santos et al. (2016) estimated the annual economic losses derived from neosporosis as more than US $1.3 billion worldwide.

Different laboratory techniques are available including histopathology, serology, molecular procedures and bioassay for detecting the parasite or the immune response of the host against the parasite. Molecular methods such as polymerase chain reaction (PCR) are superior in terms of sensitivity compared to others. Also, the sensitivity of PCR was improved by employing a nested PCR (Dubey et al., 2007). In previous works, the *Neospora*-DNA is isolated from the brain, blood, milk, and semen samples of animals with no clinical signs (Dubey et al., 2007). Molecular epidemiology studies are useful in searching for the source and transmission mode of infection (Khan et al., 2020). When abortion and other disorders associated to neosporosis occur at the herd level, a primary evaluation with serology techniques can be helpful in making the next decision (Villa et al., 2022). Enzyme-linked immunosorbent assay (ELISA) is an approved and reliable method for the identification of *N. caninum* antibodies, and epidemiological studies (Guido et al., 2016). Commercial ELISAs and indirect fluorescent antibody tests (IFAT) are the most common methods that are used for detecting the antibodies to *N. caninum* in Iran, respectively (Gharekhani et al., 2020a).

In the previous reports from the world, the seroprevalence of *N. caninum* in bulls was recorded 10.2% in Iran (Nourollahi Fard et al., 2008), 11.3% in Iraq (Nooruldeen et al., 2021), 13.4% in China (Wei et al., 2022), 13.7% in Spain (Caetano-da-Silva et al., 2004), 20.7% in Venezuela (Pinilla and Da Silva, 2018), and 54.5% in Colombia (González Corrales et al., 2021). Additionally, *Neospora*-infection has been reported to be 4.9–18% in dogs, 12.8–24.8% in cattle, 2.2% in sheep, 6.2% in goats, 40.8% in horses, and 52% in donkeys from Hamedan (Gharekhani et al., 2013a, 2013b, 2013c, 2014, 2016, 2019, 2020b; Gharekhani and Yakhchali, 2019). In a molecular evaluation of dairy farms in Hamedan, 55% of examined milk samples were positive for *N. caninum* (Gharekhani et al., 2021a). In this region, 13.6% of neosporosis cases have been transmitted vertically (Gharekhani and Yakhchali, 2020).

This study was focused on determining the prevalence of *N. caninum* infection in bulls in Hamedan (West part of Iran) using different serology and molecular techniques. The main reasons and the significance of doing the current investigation were the lack of information on this subject and the achievement of basic information for further studies.

2. Materials and methods

2.1. Planning

The current work is part of a neosporosis project in Hamedan, Iran (Gharekhani and Yakhchali, 2019, 2020; Gharekhani et al., 2022). In 2020, blood samples, for serology and PCR, epididymis and spinal cord biopsy were obtained from 792 bulls from the Hamedan abattoir. The sample size ($n = 792$) was estimated based on Cochran's formula (Expected prevalence 25%, level of confidence 95%, precision 3%, and the limited population (N) = 90,000) (Thrusfield, 2018; Gharekhani et al., 2020a). The low number of daily slaughters at the Hamedan slaughterhouse is related to bulls from the studied region's origin. For this reason, we used a stratified simple random sampling method for the primary screening of male animals. We visited the slaughterhouse at regular intervals and then randomly sampled male animals only. At first, all samples were screened for detecting the level of antibodies to *N. caninum* by using the ELISA technique; and seropositive samples were re-evaluated by using the IFAT technique. In addition, whole blood, spinal cord, and epididymis tissues of seropositive animals were submitted to detect *Neospora*-DNA using molecular techniques. In the sampling process, information on the breed (Native or Holstein) and age ($\leq$2 or $>$ 2 years old) of animals were recorded and analyzed using statistical software. Ten percent of negative samples by ELISA technique were randomly evaluated for detecting the *Neospora*-infection using the IFAT and molecular methods.

2.2. Study location

Hamedan (34.77° N and 48.58° E) is one of the provinces located in the west of Iran. It has warm and dry summers, and a cold semi-arid climate (Fig. 1). The average temperature throughout the year is recorded at 11.3 °C. Agriculture and animal husbandry are the main

occupations of the people in the region due to its favorable climate and rich lands.

2.3. Diagnostic methods

2.3.1. Serology

After the blood centrifugation (1400 ×*g* for 12 min), sera samples (*n* = 792) were provided and transferred to the freezer (−16 °C) until laboratory examination. The techniques of IFAT and ELISA were used for serology examination.

2.3.1.1. IFAT technique. The present study applied the technique recommended by previous investigators (Ortega-Mora et al., 2003; Campero et al., 2018). A cut-off value ≥1:100 was considered positive.

2.3.1.2. ELISA technique. A commercial ELISA kit belonging to ID-Vet Company (ID Screen®, France) was applied for detecting the antibodies to *N. caninum*. The raw optical density (OD) of each sample was converted to S/P% (sample to positive). A S/P% ≥ 50 was considered positive. The sensitivity and specificity of the present ELISA kit were reported 99.6% and 97.3%, respectively (Alvarez-García et al., 2013).

2.3.2. Molecular analysis

2.3.2.1. DNA extraction. Total genomic DNA was extracted from whole blood, epididymis, and spinal cord tissues using a commercial extraction kit provided by Takapouzist Co., Iran (Dyna-Bio™) according to the manufacturer's instructions.

2.3.2.2. Nested-PCR. All seropositive samples were evaluated using nested PCR for amplification of the Nc5 region of *N. caninum*, as previously mentioned by researchers (Müller et al., 1996; Gharekhani et al., 2022).

In the primary stage, a conventional PCR using both Np21 and Np6 primers (sense: 5′-cccagtgcgtccaatcctgtaac-3′, anti-sense: 5′-ctcgccagtcaacctacgtcttct-3′) was applied for amplifying the 330 bp-fragment-length (Müller et al., 1996). The reaction was carried out in 25 µl of a mixture containing 7.5 µL of distilled water (Sinaclon, Iran), 12.5 µL (2×) of red load Tag Master (Sinaclon, Iran), 4 µL of (100 ng) genomic DNA, and 1 µL of (20 pmol) each primer. The thermal profile for a primary denaturation step was adjusted to 94 °C for 15 min, followed by 35 cycles in 45 s at 94 °C, 55 °C, and 71 °C. A final extension run for 20 min at 72 °C. Finally, 10 µl of PCR product was run on 1.5% (*w/v*) agarose gel electrophoresis (95 min at 80 V) for detecting the target bands. DNA-safe stain (1 µg/ml, Yekta Tajhiz Co., Iran) was used for better visualization of the gel (Gharekhani et al., 2022).

The positive samples to the first PCR amplification were reevaluated by nested PCR. This stage was performed by using two specific primers (sense: 5′-gtgttgctctgctgacgtgt-3′, anti-sense: 5′-taccaactccctcggttcac-3′) to amplify a 100-bp-fragment-length as previously described (Gharekhani et al., 2022).

2.3.2.3. Nucleotide sequences. PCR products were purified using a commercial Kit (Takapouzist Co, Iran) According to the manufacturer's instructions. Then, PCR products were used for direct sequencing (Bioneer Co., South Korea). The raw forward and reverse obtained sequences were edited using MEGA7 bioinformatic software before submission to the gene bank. The sequences derived from nucleotide analysis were presented to the National Center for Biotechnology Information (NCBI) for getting the accession number. The Clustal Omega software (https://www.ebi.ac.uk/Tools/msa/clustalo/) was applied to calculate the alignment of sequences. The MEGA7 software was also used for drawing the phylogenetic tree with the neighbor-joining template. We created a hamming distance matrix using the Pegas package in R-statistical software (version 4.3.1; 2023). Briefly, the hamming distance algorithm returns the number of different elements between the two sequences (in this case differences in nucleotides between two gene sequences). If two sequences differ by only 2 nucleotides, the hamming distance for these sequences will be equal to 2. According to this, the more similar the sequences are, the closer hamming distance will be to zero. Finally, we plotted the hamming distances of all sequences by regular heatmap functions in R (Fig. 3). In the next step, we used the haplotype function from the Pegas package to extract the haplotypes from a set of DNA sequences. Subsequently, we draw the haplotype network using the haploNet function from the same package (Fig. 5). Each circle represents a haplotype, and the color of the circle indicates in which strains that haplotype was observed.

Table 1
Comparison of results obtained by a screening ELISA (Seropositivity = 31) and other serological and molecular methods to detect *N. caninum* infection in samples from bulls of Hamedan, Iran.

Sample number	Diagnostic methods					
	Serology		Nested-PCR			
	ELISA	IFAT	Blood	Epididymis	Spinal cord	Genetic isolate number
1	+	+	+	+	+	2
2	+	+	negative	negative	negative	
3	+	negative	+	+	+	7
4	+	+	+	+	+	2
5	+	+	+	+	+	3
6	+	+	negative	negative	negative	
7	+	+	+	+	+	3
8	+	+	+	+	+	3
9	+	+	+	negative	+	3
10	+	+	+	+	+	2
11	+	+	+	+	+	7
12	+	+	negative	+	+	3
13	+	+	+	+	+	3
14	+	negative	+	+	+	5
15	+	+	negative	+	+	3
16	+	+	+	+	+	3
17	+	+	negative	negative	negative	
18	+	+	+	+	+	4
19	+	+	+	+	+	3
20	+	+	negative	negative	negative	
21	+	+	+	+	+	2
22	+	+	+	+	+	3
23	+	+	+	negative	+	4
24	+	+	+	+	+	6
25	+	+	+	+	+	6
26	+	+	+	+	+	5
27	+	+	negative	+	+	7
28	+	+	+	+	+	3
29	+	+	+	+	+	3
30	+	+	negative	+	+	1
31	+	+	+	+	+	3

2.4. Statistical evaluation

The chi-square ($\chi 2$) test was applied to calculate the connection of *Neospora*-seroprevalence by ELISA with the age and breed of the animals (SPSS 16.0, Chicago, IL, USA). A *p*-value of ≤0.05 was recorded as significant. Prevalence ratio (PR) and 95% confidence intervals (CI 95%) were estimated for variables and seropositivity, respectively. In addition, in regard to the sensitivity and specificity of the ELISA kit belonging to ID-Vet Company, we calculated the true prevalence (TP) using Epi Info™ 7-software version 3 (http://wwwn.cdc.gov/epiinfo/).

3. Results

3.1. N. caninum infection

A preliminary serological screening was performed using the ELISA

Table 2
Accession numbers and sequences derived from seven *N. caninum* isolates in bulls from Hamedan, Iran.

Isolate No.	Accession number	Nucleotide sequences (5′ to 3′)
1	MT955653	gcataatctccccgtcatcagcgccgccggtgttgcctcaacacagaacactaaactctggataagtatcattgacacactgtccacaccctgacgcaggctgatttcaacgtgacgaatgactaaccacaaaccacgtatccc acctctcaccgctaccaactccctcggttcacccgttcacacactatagccacaaacaaaaaaggagccttgctgccgcaggctgcgcccaacaacgacacgtccgcataccaacaggataccggattgga
2	MT955654	cctatgcataatctcccccgtcatcagcgccgccggtgttgcctcaacacagaacactgaactctggataagtatcattgacacactgtccacaccctgacgcaggctgatttcaacgtgacgaatgactaaccacaaaccacgt atcccacctctcaccgctaccaactccctcggttcacccgttcacacactatagccacaaacaaaaaaggagccttgctgccgcaggctgcgcccaacaacgacccgtccgcataccaacacgttacaggattg
3	MT955655	cctcgtccgcttgctccctatgcataatctcccccgtcatcagcgccgccggtgttgcctcaacacagaacactgaactctggataagtatcattgacacactgtccacaccctgacgcaggctgatttcaacgtgacgaatgact aaccacaaaccacgtatcccacctctcaccgctaccaactccctcggttcacccgttcacacactatagccacaaacaaaaaaggagccttgctgccgcaggctgcgcccaacaacgacacgtccgcataccaacacgttac aggattgga
4	MT955656	cccttccctcgtccgcttgctccctatgcataatctcccccgtcatcagcgccgccggtgttgcctcaacacagaacactgaactctggataagtatcattgacacactgtccacaccctgacgcaggctgatttcaacgtgacga atgactaaccacaaaccacgtatcccacctctcaccgctaccaactccctcggttcacccgttcacacactatagccacaaacaaaaaaggagccttgctgccgcaggctgcgcccaacaacgacacgtccgcataccaaca cgttacaggattggacgc
5	MT955657	cccttccctcgtccgcttgctccctatgcataatctcccccgtcatcagcgccgccggtgttgcctcaacacagaacactgaactctcgataagtatcattgacacactgtccacaccctgacgcaggctgatttcaacgtgacga atgactaaccacaaaccacgtatcccacctctcaccgctaccaactccctcggttcacccgttcacacactatagccacaaacaaaaaaggagccttgctgccgcaggctgcgcccaacaacgacacgtccgcatagcaaca cgttacaggattggacgca
6	MT955658	cgcttgctccctatgcataatctcccccgtcatcagcgccgccggtgttgcctcaacacagaacactgaactctcgataagtatcattgacacactgtccactccctgacgcaagctgatttcaacgtgacgaatgactaaccac aaaccacgtatcccacctctcaccgctaccaactccctcggttcacccgttcacacactatagccacaaacaaaaaaggagccttgctgccgcaggctgcgcccaacaacgacacgtccgcataccaacacgttacaggattg gacgca
7	MT955659	gctccctatgcataatctcccccgtcatcagcgccgccggtgttgcctcaacacagaacactgaactctcgataagtatcattgacacactgtccacaccctgacgcaggctgatttcaacgtgacgaatgactaaccacaaacca cgtatcccacctctcaccgctaccaactccctcggttcacccgttcacacactatagccacaaacaaaaaaggagccttgctgccgcaggctgcgcccaacaacgacacgtccgcatagcaacacgttacaggattggacgca

technique, with a seroprevalence of 3.91% (31/792) to *N. caninum* (95% CI: 2.61–5.21%). True prevalence regarding the sensitivity and specificity of the ELISA was calculated 1.25% (95% CI: 0.48–2.02%). Based on ELISA outputs, 9.67% (3/31) of seropositive samples were negative by using the molecular method. The overlaps between ELISA and molecular results were observed in 74.19% (23/31) of whole blood samples, 80.64% (25/31) of the epididymis, and 87.09% (27/31) of the spinal cord (Table 1). To confirm the present results, 10% of negative samples in ELISA (76/761) were randomly evaluated for *Neospora*-infection by using IFAT and molecular methods; all of them were found to be negative.

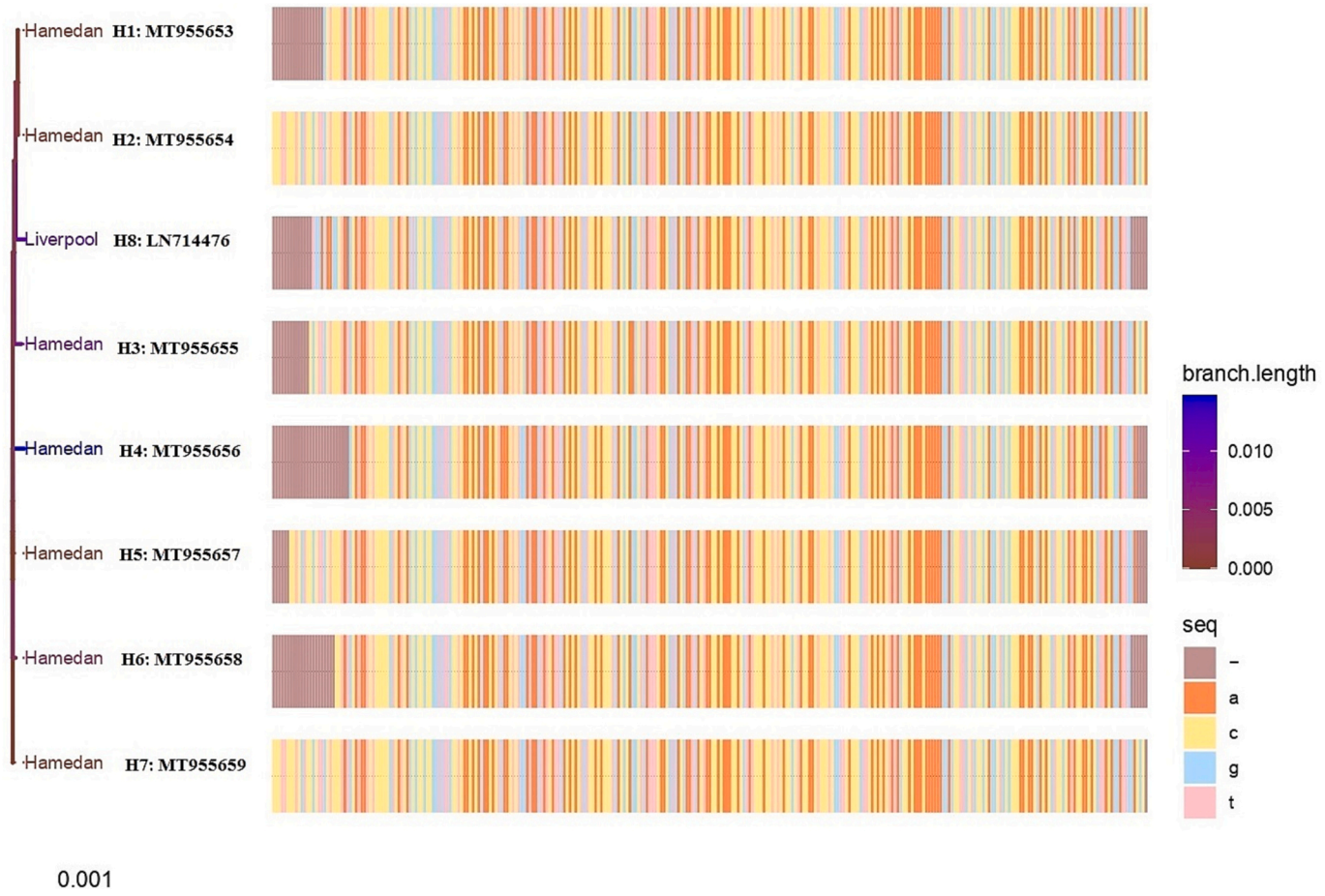

Fig. 2. Plotting the multiple sequence alignments of our seven isolates (MT955653, MT955654, MT955655, MT955656, MT955657, MT955658, MT955659) in comparison to Liverpool strain (LN714476). The sequences have been colored by four different colors shown in the 'seq' column for each nucleotide. The color spectrum of branch length and nucleotides (a, t, c, and g) is shown beside the figure. Color changes on the aligned sequences represent nucleotide differences.

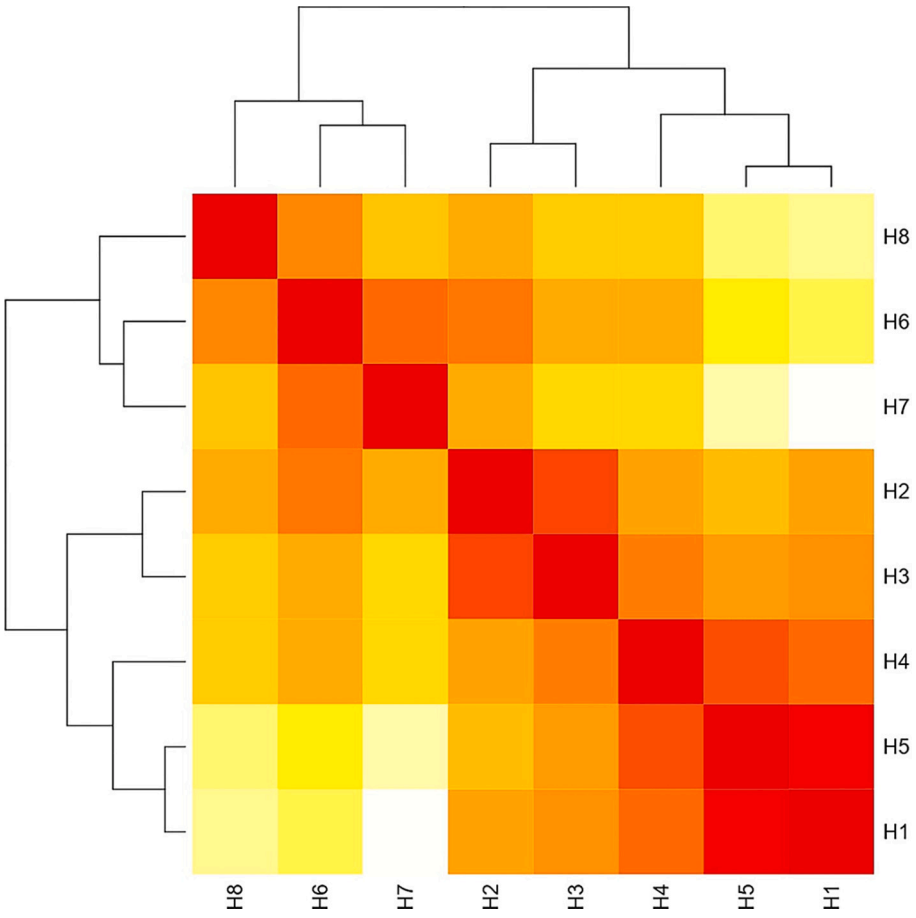

Fig. 3. Heat map based on the number of nucleotide differences between the haplotypes. Each branch of the phylogenetic tree represents the corresponding haplotype in the matrix. H1: MT955653; H2: MT955654; H3: MT955655; H4: MT955656; H5: MT955657; H6: MT955658; H7: MT955659; H8: LN714476 (Liverpool standard strain).

3.2. Seroprevalence in regard to age and breed

There were 333 (42%) and 459 (58%) animals in the age groups of ≤2 years old and > 2 years old, respectively. This rate was 579 (73.1%) for native breed animals and 213 (26.9%) for Holstein animals. Regarding ELISA findings, the seroprevalence of *N. caninum* was detected 1.8% (95% CI: 0.4–3.2%) in ≤2 and 5.45% (95% CI: 3.45–7.45%) in >2 years old group of animals ($\chi 2 = 6.817$, $p = 0.009$, and $PR = 3.19$ (95% CI: 1.59–4.79%)). In addition, the seropositivity in Holstein and native breed animals was calculated 6.57% (95% CI: 3.27–9.87%) and 2.93% (95% CI: 1.63–4.23%), respectively ($\chi 2 = 5.475$, $p = 0.019$, and $PR = 2.3$ (95% CI: 0.3–4.3%)). A significant relationship was seen among the seroprevalence of *N. caninum*, age, and breed of animals ($p < 0.05$).

3.3. Sequencing

Regarding multiple sequence alignments on twenty-seven molecular positive samples, seven sequences (isolates 1–7) with 94.9–99.3% similarity were identified (Table 2, Fig. 2). The frequency of isolates and the accession number are illustrated in Tables 1 and 2. The highest and lowest isolates belonged to 3 (MT955655: $n = 13$, 48.14%) and 1 (MT955653: n = 1, 3.70%), respectively. Our isolates were compared to the Liverpool standard strain of *N. caninum* (LN714476) and isolates from Iran as well as other countries regarding various hosts; the results are demonstrated in Figs. 3-5. Additionally, the heat map and haplotype networks of isolates are demonstrated in Figs. 3 and 5, respectively.

4. Discussion

Neosporosis is reported up to 97% (Average in cattle =20%) in different regions of the world regarding various diagnostic methods as well as the associated risk options (Dubey et al., 2007; Dubey and Schares, 2011; Ribeiro et al., 2019). For the first time in Iran, Sadrebazzaz et al. (2004) reported *N. caninum* in dairy cattle of the Mashhad region. *Neospora*-infection is estimated between 7.8% and 66.7% in cattle from Iran (Gharekhani et al., 2020a), making this disease one of the significant causes of abortion in Iranian dairy farms (Gharekhani and Yakhchali, 2019, 2020). Transplacental transmission of *N. caninum* has an important role in the maintenance and distribution as well as the continuance of the parasite's life cycle in farms (Gharekhani and Yakhchali, 2019, 2020).

We applied nested PCR to recognize DNA targeting the Nc-5 gene of *N. caninum*. The researchers have used the Nc-5 gene because of its sensitivity and specificity as well as its high repetitions within the parasite (Gharekhani et al., 2022; Khan et al., 2020).

In our work, the seroprevalence was 3.9% in animals using ELISA (TP = 1.25%), in line with a recent report from Switzerland; this was lower in comparison to other animal species (beef and dairy cattle) in this area (Gharekhani et al., 2020a; Gliga et al., 2022). Serological methods are the most common and reliable tool for evaluating the control process and primary screening at the flock/farm level (Campero et al., 2018; Villa et al., 2022). Also, the presence of *Neospora*-DNA was confirmed in the seropositive animals similar to reports by Ortega-Mora et al. (2003) and Gharekhani et al. (2022). In the current work, *Neospora*-DNA was detected in 80.64% of epididymis samples of

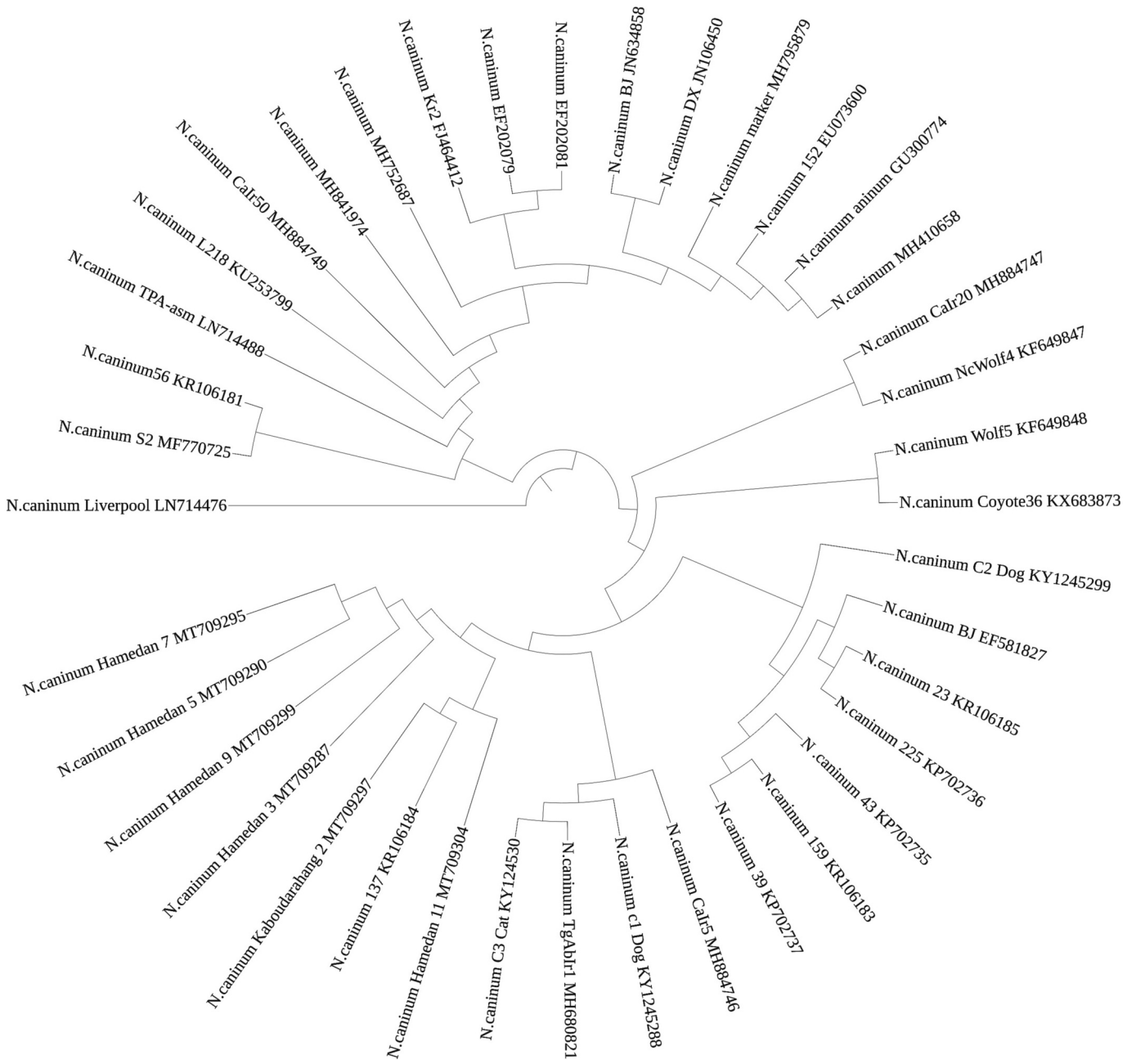

Fig. 4. Phylogenetic trees for isolates from various hosts in Iran and different regions worldwide.

seropositive animals. On the other hand, DNA of *N. caninum* has been found in the semen of infected bulls (Ortega-Mora et al., 2003; Ferre et al., 2005, 2008; Serrano-Martinez et al., 2007). The possibility of a previous and/or chronic infection is raised in the seropositive animals with a negative result in the molecular assessment. While seronegative animals with positive molecular evaluation may be observed in the primary stage of infection (Gharekhani and Yakhchali, 2022).

Different immuno-serological techniques are available to screen animals for *Neospora*-infection, but there is no reliable reference method to diagnose true-positive or true-negative cases. The use of a commercially valid diagnostic kit, user-friendly with high repeatability, plays an important role in harmonizing the detection in laboratory staffs of different regions, as well as guaranteeing a control strategy (Campero et al., 2018). Moreover, using different laboratory techniques and comparing their results will bring us closer to reality and provide a better perspective of the infection in animals and herd levels.

Most researchers believe the prevalence of neosporosis is directly connected with increasing age due to an increase in the chance of infection via consuming contaminated materials (Kyaw et al., 2004; Dubey and Schares, 2011). Besides, several reports on equal *Neospora*-infection in animals of various ages are available (Wouda et al., 1998; Sadrebazzaz et al., 2004). Reducing congenital infection may occur due to increasing the immunity parallel to animals' age (Salehi et al., 2010). In our work, the seroprevalence of *N. caninum* in Holstein animals was high compared to natives. In a similar report by Gharekhani and Yakhchali (2019), the seropositivity in Holstein animals was 2.3-fold higher than in crossbred. The high susceptibility of Holstein in comparison to other breeds is also confirmed by other researchers (Dubey et al., 2007). There are some options, such as the type of production system (Traditional or industrial), type of farms (Intensive or extensive), and the climate of the region, which are influential in choosing the kind of animals' breed (Dubey and Schares, 2011). For example, in a study

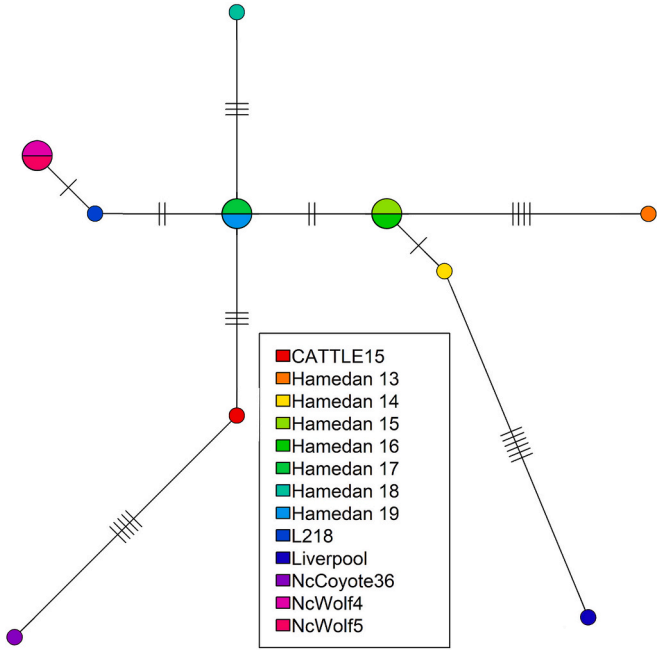

Fig. 5. Haplotype networks for *Neospora caninum* populations (CATTLE15: KP715562; Hamedan4: MT955656; Hamedan6: MT955658; Hamedan5: MT955657; Hamedan7: MT955659; Hamedan2: MT955654; Hamedan3: MT955655; Hamedan1: MT955653; L218: KU253799; Liverpool: LN714476; NcCoyote36: KX683873; NcWolf4: KF649847; NcWolf5: KF649848).

from Brazil, there was no statistically significant correlation between neosporosis and the breed of animals (Munhoz et al., 2009).

We introduced seven genomic isolates (1–7) regarding their similarity (94.9–99.3%), like a previous report from the region (Gharekhani et al., 2022), which confirmed the sequences of *Neospora*-strains are not significantly different from each other genetically in the studied regions. Salehi et al. (2015) believed the most significant similarity is seen in isolates introduced from the same region. Also, close biological behaviors such as pathogenicity have been detected in different strains (Regidor-Cerrillo et al., 2013). For the first time, Salehi et al. (2012) introduced Nc-Iran *Neospora*-strain from Iranian cattle (Accession no: FJ655914). The sequence of Nc-Iran had a notable difference compared to the dominant isolates in the world (Nc-Liverpool, Nc-SweB1, Nc-GER1, KBA1, and KBA2).

Knowledge on risk factors associated to *Neospora*-infection has a significant role in the design and use of an appropriate controlling method, limiting the spread of infection, subsequently decreasing the economic losses, as well (Dubey et al., 2007; Santos et al., 2016; Gharekhani and Yakhchali, 2022). Villa et al. (2022) showed that in flocks with an equal level of *N. caninum* infection, the presence and diversity of risk factors related to the parasite in the farms demonstrate a significant disorder for the reproductive and productivity of animals. A longitudinal study over five years showed that using beef bulls' semen significantly decreased the abortion associated to neosporosis in seropositive cows (Sala et al., 2018). The presence of free-roaming canids, especially coyotes and dogs, in the herds, is a strong reason for the infection spreading (Ribeiro et al., 2019). In the past investigations from Hamedan (Gharekhani et al., 2014; Gharekhani and Yakhchali, 2019), the prevalence of neosporosis was high in herds where dogs and wild canids were present. According to Macchi et al.'s (2020) calculation, the presence of a dog can increase the *Neospora*-infection to 1.4-fold at the herd level. Also, in the research from Switzerland (Gliga et al., 2022), restocking by self-rearing of replacement heifers and feeding concentrated feed were introduced as protective factors for seropositivity in dairy farms.

Control and prevention strategies must emphasize developing herd management, biosecurity, and diagnostic tools (Gliga et al., 2022). Examination of bulk milk for *Neospora*-infection has been introduced as a screening method in dairy farms (Gharekhani et al., 2021a; Gliga et al., 2022). Culling of seropositive mothers is still one of the strong tools for controlling the infection in animals as well as at the farm level (Gliga et al., 2022). Using Real-time PCR is suggested for evaluating the quantities of *N. caninum* in different tissues in future studies.

4.1. Limitations

The limitations of the present work are that we used the slaughterhouse for sampling and also ELISA for screening and analyzing data. Lack of complete and accurate access to animals' history was the main limitation of our study, in addition to the origin of animals, position, and health status of the herds/farms. In our work, there was not enough information to adjust for confounding or other sources of bias; so, a careful and descriptive view should be considered in the data interpretation and the claims regarding the relation of age and breed with seroprevalence.

5. Conclusions

Our work presented a comprehensive evaluation of *Neospora*-infection/neosporosis in Iranian bulls for the first time. We used different laboratory diagnostic methods to detect *Neospora*-infection in animals and introduced different isolates of *N. caninum* in the region. The results derived from ELISA were parallel to the nested PCR. Similar to previous reports, the ELISA was a sensitive serological technique for screening the highest number of seropositive animals, and the nested PCR was a reliable technique for identifying *Neospora*-DNA in target tissues.

Our findings and experience can be useful for the development of future research in this field.

Ethical statement

The research was reviewed by the Iran Animal Welfare Committee and received approval from this committee (Iran Doc: 1453018). Ethical standards were considered during the sample collection, data analysis, preparation of the draft, and manuscript submission.

Declaration of Competing Interest

None.

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