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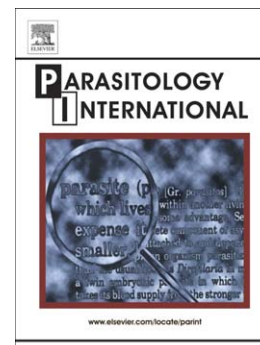
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# Genetic Diversity of Bovine *Neospora caninum* Determined by Microsatellite Markers

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## Abstract

*Neospora caninum* is one of the most significant parasitic organisms causing bovine abortion worldwide. Despite the economic impact of this infection, relatively little is known about the genetic diversity of this parasite. In this study, using Nc5 and ITS1 nested PCR, *N. caninum* has been detected in 12 brain samples of aborted fetuses from 298 seropositive dairy cattle collected from four different regions in Tehran, Iran. These specimen (Nc-Iran) were genotyped in multilocus using 9 different microsatellites markers previously described (MS4, MS5, MS6A, MS6B, MS7, MS8, MS10, MS12 and MS21). Microsatellite amplification was completely feasible in 2 samples, semi-completely in 8 samples, and failed in 2 samples. Within the two completely performed allelic profiles of Nc-Iran strains, unique multilocus profiles were obtained for both and novel allelic patterns were found in the MS8 and MS10 microsatellite markers. The Jaccard's similarity index showed significant difference between these two strains and from other standard isolates derived from GenBank such as Nc-Liv, Nc-SweB1, Nc-GER1,

KBA1, and KBA2. All samples originating from the same area showed identical allelic numbers and a correlation between the number of repeats and geographic districts was observed.

**Keywords:**

*Neospora caninum*; PCR; Genotyping; Microsatellite; aborted fetuses

**1. Introduction**

*Neospora caninum* is a coccidian parasite exhibiting worldwide distribution. It is one of the major pathogens affecting cattle and dogs, and occasionally it causes clinical infections in horses, goats, sheep, and deer (Hemphill and Gottstein 2005). *N. caninum* is one of the most frequently transmitted parasites of cattle and up to 90% of cattle in some herds are infected (Dubey, 2003). The transplacental route is considered as the major mode of transmission of *N. caninum* in cattle. If transmission occurs at a specific time-window of gestation, abortion can result, and such *Neospora*-induced abortion represent the major cause of abortion in many countries (Dubey, 2003). In spite of the world-wide distribution and broad host range of this parasite, the number of isolates of *N. caninum* described to date is relatively limited. Many attempts have been made to obtain viable *N. caninum* isolates by bioassays in mice or directly in cell culture. Since the first isolation of the parasite *N. caninum* from dogs in 1988 (Dubey et al. 1988), isolation from bovine and other intermediate hosts has been reported from several countries (Dubey et al., 1988, Lindsay et al., 1995, Davison et al., 1999, Miller et al., 2002, Navarro-Lozano et al., 2005, Salehi et al., 2012). By 2007, 65 isolates have been reported around the world (Dubey et al. 2007). Biological characterization among these isolates has revealed genetic diversity, significant variation in pathogenicity and growth characteristics (Atkinson et al. 1999, Miller et al. 2002, Schock et al. 2001, Pérez-Zaballos et al. 2005). Genetic

diversity plays an important role in the survival and adaptability of *Neospora* species. It could influence the clinical outcome of the disease and could be directly related with other important epidemiological features, such as the potential for transmission (Regidor-Cerrillo et al. 2008, Al-Qassab et al., 2009). Therefore, it is important to determine genetic diversity of *Neospora* species, and to link these to pathobiological features and peculiarities.

Different molecular techniques including PCR-based techniques, single nucleotide polymorphism (SNP) and microsatellite markers have been used to study taxonomy, genetic polymorphism and epidemiology of many protozoan parasites (Luton et al., 1995, Meisel et al., 1996, Anderson et al., 2000, Ajzenberg et al., 2001, Mallon et al., 2003, Oura et al., 2003). To analyze genetic diversity of *N. caninum*, different techniques have been applied as well (Ellis et al., 1994). For instance, intra-species diversity within *N. caninum* has been demonstrated by random amplified polymorphic DNA-PCR (RAPD-PCR) (Marsh et al., 1995, Atkinson et al., 1999; Davison et al., 1999; Schock et al., 2001). In addition, sequence analysis of rDNA internal transcribed spacer (ITS-1) demonstrated some variation among *N. caninum* isolates (Homan et al., 1997, Ellis et al., 1998, Gondim, Laski et al., 2004, Regidor et al 2006). Recently, microsatellites (short, tandem repeats with 2–6 bases long), have represented another class of genetic markers (Ajzenberg et al 2001, De Luca et al. 2002). Using microsatellite markers, Regidor-Cerrillo et al. (2006, 2012 and 2013), Basso et al. (2009), Pedraza-Díaz et al. (2009) and Al-Qassab et al. (2009, 2010) described different genetic variation among *N. caninum* derived from European countries, United States, Argentina and Japan. Although, *N. caninum* strains have been isolated in Iran (NC-Iran) (Salehi et al., 2012) and many molecular and serological studies have been carried out on neosporosis (Haddadzadeh, et al., 2007, Razmi et al., 2007, Salehi et al., 2009 and 2010, Asadpour et al., 2011, Gharekhani and Heidari 2014), there is no information

regarding the genetic polymorphism that could might occur among *N. caninum* isolates in Iran. The aim of this study was to study putative genetic diversities of Iranian strains of *Neospora caninum* upon use of microsatellite markers, this as a first step to provide the basis for future studies that could address virulence, pathogenicity and clinical presentation of the parasite, all parameters being of importance for future vaccine development, treatment optimization and elaboration of prevention and strategic control.

## **2. Material and methods:**

### **2.1. Clinical samples**

Brain DNA samples of aborted fetuses from pregnant seropositive cows were collected in 2007 from four dairy farms around Tehran, capital of Iran. All animals had been previously tested (during pregnancy) for the presence of *N. caninum* serum antibodies by ELISA (Salehi et. al. 2010). The herds were located in an area of 80 km within the southeastern, western and southern localities surrounding Tehran (Varamin, Eshtahard, Nazarabad and Eslamshahr, respectively). *Neospora* seropositive dairy cattle were monitored for one year with the owner's cooperation. On advent of abortion, aborted fetuses from seropositive cattle were collected and sent to the laboratory. These include 8 samples from Eslamshahr, 5 samples from Nazarabad, 1 sample from Eshtehard and 2 samples from Varamin (totally 16 samples).

### **2.2. DNA extraction and *Neospora* detection**

Approximately 5-10 g tissue were taken selectively from different anatomic regions of the brain, and the specimen were subsequently homogenized in 20 ml of sterile PBS containing 2% antibiotics. Total DNA was extracted from 300 µl of a homogenized suspension using a Qiagen DNAeasy Tissue kit (Qiagen, GmbH, Germany) according to manufacturer's instructions. A 100µl aliquot of total DNA was produced from each sample and stored at -20 °C until further analysis. DNA concentrations were measured by spectrophotometry with a BioPhotometer (Eppendorf-Nethelash-Hinz GmbH, Hamburg, Germany). Then, two nested-PCR protocols were conducted using primers from the Nc5 region of the genomic DNA (genomic nested-PCR) and ITS1 region of the ribosomal DNA (ribosomal nested-PCR). For the genomic nested-PCR, pairs of NP21 and NP6 primers (Muller et al. 1996) were used to amplify the 337 bp DNA fragment and pairs of Np7 and Np10 (5'GGGTGAACCGAGGGAGTTG3', 5'TCGTCCGCTTGCTCCCTATGA AT3') which amplified 227 bp of the Nc5 gene of *N. caninum* (salehi et al., 2012). The PCR mixture of 50µl contained 0.5µg of target DNA, 2mM MgCl<sub>2</sub>, 10×reaction buffer (50mM KCl, 10mM Tris-HCl [pH8.3], 10pmol of each PCR primer, 200µM each dNTP, and 1U of *Taq* DNA polymerase (CinnaGen, Iran). PCRs were performed in a thermocycler (Techgene-Tech, Germany) for 35 cycles of denaturation at 95°C for 45S, annealing at 48 °C for 45S, and extension at 72 °C for 45S. For nested PCR, a second set of primers NP1 and NP2 was used to amplify 1µl of the first-round PCR product with the same PCR mixture and cycling. For ribosomal nested PCR on the internal transcribed spacer (ITS1) region of *N. caninum* the primers pairs NN1/NN2 and NP1/NP2 were used to amplify DNA fragment according to Buxton et al. procedure (1998). Positive (*Neospora* DNA) and negative controls (sterile water) were included in each PCR run. Amplified products were

analyzed by electrophoresis through a 2% agarose gel. The PCR products were cleaned by QIAquick PCR purification kit (Qiagen, Germany) and stored at -80°C until further use.

### **2.3. PCR amplification for microsatellite genotyping**

*N. caninum* genotyping was performed in ITS-1 PCR-positive clinical samples using the nine microsatellite markers MS4, MS5, MS6A, MS6B, MS7, MS8, MS10, MS12 and MS21 (table 1) that were previously described by Regidor-Cerrillo et al. (2006) and that are based on sequence tags (ESTs) derived from Nc-1 tachyzoite and Nc-Liv tachyzoite cDNA libraries. PCR was performed as described Pedraza-Díaz et al (2009) in 50 µl volumes containing 1x PCR buffer, 2 mM MgCl<sub>2</sub>, 200 µM dNTPs, 0.20 µM of each primer, 2 units of Taq DNA polymerase (CinnaGen, Iran), and 5 µl of template DNA. Reaction conditions were 1 cycle at 94 C for 5 min; 35 cycles of 94 C for 1 min, 60 C for 1 min, and 72 C for 1 min; and a final 72 C for 10 min. In each batch of amplifications, H<sub>2</sub>O were included as negative controls. Two microliter aliquots of the PCR products obtained were separated by electrophoresis in 2.5% agarose gel and visualized under UV light.

#### **2.3.1. Sequencing of microsatellites**

PCR products were purified with the QIAquick PCR purification kit (Qiagen, Germany) according to the manufacturer's instructions and sequenced at the Kawsar Biotech Company (Iran), with the primers used for PCR amplification. PCR products were sequenced in both

directions by using an ABI Big Dye Terminator Cycle Sequencing and analyzed using SeqEd v1.0.3 (Applied Biosystems).

### **2.3.2. Data analysis**

To generate multilocus genotypes for each isolate, each allele was designated a number according to the length of the repeat and the differences found in the sequence (Table 2). The program Clustering Calculator (<http://www.biology.ualberta.ca/jbrzusto/cluster.php>) was used to compare the multilocus genotypes with the use of an unweighted arithmetic average as the clustering method and a pair wise distance matrix of the multilocus genotypes (Jaccard's imilarity coefficient) for the input data. The output, in a Phylip-readable file, was viewed as a dendrogram in the Treeview program (<http://taxonomy.zoology.gla.ac.uk/rod/treeview.html>).

## **3. Results**

### **3.1. Nested-PCR amplification**

During the time of study, 16 aborted fetal brains from 298 seropositive dairy cattle were collected from four different farms participating in this study. After extraction of DNA from all samples, Nc5 and ITS-1 region of *N. caninum* were amplified by using specific primers. The genomic nested-PCR Nc5 and the ribosomal nested-PCR ITS1 revealed the same results, in which 12 samples (75%) of the aborted fetal brain were infected by *N. caninum*. Six out of 8 samples from Eslamshahr district, four out of five samples from Nazarabad, 1 sample from Eshtehard and one samples out of two from Varamin. The positive PCR products was purified using the QIAquick PCR purification kit (Qiagen, Germany) according the manufacturer's



instructions and sequenced at the Kawsar Biotech Company (Iran), with the primers used for PCR amplification. PCR products were sequenced twice in both directions by using an ABI Big Dye Terminator Cycle Sequencing and analyzed using SeqEd v1.0.3 (Applied Biosystems). The nucleotide sequence was designated as Nc-Iran and has been deposited in the GenBank database under the accession number FJ655914.

### 3.2. Microsatellite analysis

*N. caninum* genotyping was performed in ITS-1 PCR-positive samples using the nine microsatellite markers MS4, MS5, MS6A, MS6B, MS7, MS8, MS10, MS12 and MS21 (table 1) that were previously described by Regidor-Cerrillo et al. (2006). PCR products visualized under UV light in a 2.5% agarose/ethidium bromide gel revealed considerable length polymorphism regarding the microsatellite markers. The results obtained in the amplification of the 9 microsatellite markers are shown in Table 2. Two DNA samples failed to amplify any of the microsatellite sequences. Amplification of all microsatellite markers was achieved in 2 DNA samples (Nc-Iran). The remaining samples yielding amplification for some of the markers only. MS6A and MS10 amplified in 5 samples as minimum and MS21 in 8 samples as maximum. Comparative analysis of the loci for the tested samples demonstrated the presence of 7 polymorphic microsatellites and 2 monomorphic marker (MS6B and MS21). Overall, markers showed a relatively low level of polymorphism, with the number of alleles ranging from 1 to 5. Within two complete amplified samples, result analysis represented unique multilocus patterns and novel allelic pattern in some of the microsatellite markers (MS8 and specifically for the most polymorphic marker MS10). All samples originating from the same area showed identical allelic numbers and a correlation between the number of repeats and geographic districts was observed.

A discordant finding was detected in the comparative analysis between sequences of the Nc-Iran strains (from two complete allelic profile samples) and some isolate deposited in GenBank including Nc-Liv, Nc-SweB1, Nc-GER1, KBA1, and KBA2 according to the Regidor-Cerrillo et al. study (2006). Within two analyzed allelic profiles of Nc-Iran strains, unique multilocus profiles were obtained (Table 3). The Jaccard's similarity index analysis (Fig. 1) showed that these two allelic profiles differed significantly from each other and from other isolates of previous studies (Regidor-Cerrillo et al. 2006 and, 2012; Al-Qassab et al. 2009; Basso et al., 2009). No relationship could be established among strains from the different geographical origins. The dendrogram generated in this analysis (Fig. 1) showed different levels of sub-clustering of Nc-Iran strains in comparison with other isolates found in GenBank.

#### 4. Discussion

*Neospora caninum* causes stillbirth and abortion in cattle and neuromuscular disorders in dogs (Dubey 2003, Hemphill and Gottstein 2005). Differences in pathogenicity of different *N. caninum* isolates have been documented (Atkinson et al. 1999, Schok et al., 2001, Gondim et al., 2004), and a relationship between their biological and genetic diversity has been claimed. Biological relevant diversities are an important consideration not only for e.g. the design and application of vaccines against neosporosis, but also in our understanding of the epidemiology of the parasite. Previously, genetic diversity of *N. caninum* isolates have been analyzed by using ribosomal DNA sequencing and analysis of inverted repetitive DNA (Atkinson et al. 1999, Schok et al., 2001). Recently, microsatellite markers generated lots of interest as genetic markers (De Luca et al. 2002; Scott et al., 2005; Weissenbach 1993; Wen-Jing et al, 2005). In 2006,

Regidor-Cerrillo et al. used microsatellite markers to genotype different *N. caninum* isolates and reported differences in 12 microsatellite markers among nine isolates of *N. caninum*. Multilocus analysis showed that each of the nine isolates displayed a distinctive profile, but revealed no relationship between genotype and host or geographical origin. They also found genetic diversity and geographic population structures of *N. caninum* isolates from different parts of world (Regidor-Cerrillo et al 2013). Al-Qassab (2009 and 2010) developed a multiplex assay by using the microsatellite marker to detect genetic diversity of *N. caninum* and to achieve a discrimination between strains. These topics on *N. caninum* have been poorly investigated so far in Iran. Although, several studies have documented the presence of *N. caninum* in different animals in Iran (Haddadzadeh, et al., 2007, Razmi et al., 2007, Salehi et al., 2009 and 2010, Asadpour et al., 2011, Gharekhani and Heidari 2014), information on the genetic diversity of this parasite has not yet been achieved in that area. In the current study, we used nested-PCR to detect *N. caninum* in 75% of samples collected from a group of aborting cows that were *Neospora*-seropositive prior to abortion. Subsequently, microsatellite amplification was attempted in all initially PCR-positive samples. Microsatellite amplification was finally achieved (partially or completely with regard to the set of markers) in 10 samples. After analysis of the complete dataset, novel allelic pattern were found in some of the microsatellite markers, particularly for the most polymorphic marker MS10. The profile of MS10 obtained by this study was [ACT]<sub>6</sub>-(AGA)<sub>15</sub>-(TGA)<sub>10</sub>] and [ACT]<sub>6</sub>-(AGA)<sub>17</sub>-(TGA)<sub>10</sub>] which differed from those found by Basso et al. (2010) and Regidor-Cerrillo et al. (2006, 2008). Although [ACT]<sub>6</sub>-(AGA)<sub>17</sub>-(TGA)<sub>10</sub>] was identical to ARG-05-5 sample in Regidor-Cerrillo et al. study (2013). Our results confirm the previous findings by Regidor-Cerrillo (2006) showing that MS10 has the greatest discriminatory power within these markers. In addition, new alleles were found in MS8

in Nc-Iran-2 strain which was different with other studies, however MS8 in Nc-Iran-1 strain was similar to the Argentina and Germany samples in Regidor-Cerrillo et al. study (2013).

The profile of MS5 in this study was identical to Nc-GER1 and Nc-Liv. MS6A was identical to Nc-GER1, KBA1, and KBA2 and was different with Nc-Liv and Nc-SweB1. MS6B was similar to most of isolates (Nc-Liv, Nc-GER1, KBA1, and KBA2) and different from Nc-SweB1 isolate. The profile of MS12 and MS21 was identical to Nc-Liv, Nc-SweB1, Nc-GER1, KBA1, and KBA2, except MS12 profile for Nc-GER1. In general, the pattern of MS4, MS5, MS7, MS8 and MS10 were different in the two Nc-Iran strains and there was no similarity between them. Interestingly, all samples in the same geographic region showed similar multilocus patterns. However, unique microsatellite profiles have been observed in samples of different farms and origin, which confirms the extensive genetic micro-diversity of this parasite. This finding is in agreement with those of the Regidor-Cerrillo et al. study (2008), which found eight different profiles in the nine *N. caninum* bovine isolates, and of the Basso et al. (2009) study, which detected five different profiles when analyzed six *N. caninum* isolates from clinical samples of dogs. In addition, Susana Pedraza-Díaz et al (2009) found just two identical profiles within 40 samples.

The Jaccard's coefficient analysis showed that the Nc-Iran isolates had unique profiles. Comparison of the Nc-Iran-isolate microsatellite patterns with reference strains such as Nc-Liv, Nc-SweB1, Nc-GER1, KBA1, and KBA2, yielded different genetic profiles for Iranian isolates. The Nc-Iran polymorphic profiles were close to Nc-Liv, KBA1 and KBA2, which could be relevant to the similarity of mutation rate and geographic factors, especially about KBA1 and KBA2, the Korean isolates.

In conclusion, microsatellite analysis revealed that Nc-Iran strains displayed two unique profiles which were different from each other and also different from some other reference isolates used from GenBank. More studies are required to address the genetic diversity of *N. caninum* in different parts of Iran. Furthermore, we plan to study a putative link between the genetic polymorphism and biological parameters such as pathogenicity, since these could be of relevance for the future development of treatment and control strategies and for the design of a proper vaccine.

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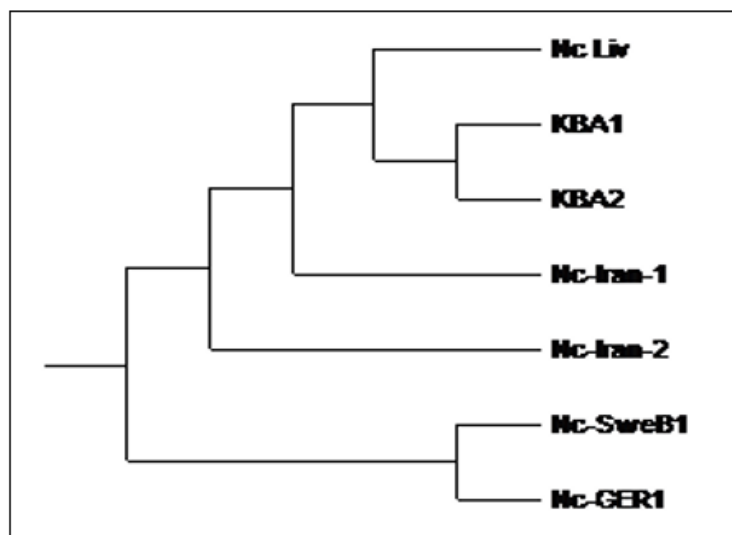
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Isolate	MS4	MS5	MS6A	MS6B	MS7	MS8	MS10	MS12	MS21
<u>Nc-Liv</u>	6	1	4	2	4	5	9	2	1
KBA1	6	6	3	2	2	3	5	2	1
KBA2	6	7	3	2	2	3	6	2	1
Nc-Iran-1	5	1	3	2	1	4	S	2	1
Nc-Iran-2	1	4	2	2	4	1	S	2	1
Nc-SweB1	4	5	1	4	3	2	2	2	1
Nc-GER1	3	4	3	2	3	2	8	3	1

**Fig 1.** Dendrogram showing genetic relationships of the *Neospora caninum* isolates from the bovine aborted fetuses analyzed in this study and some *Neospora* isolates described by Regidor et al. (2006). The dendrogram was obtained by multilocus analysis of the 9 polymorphic microsatellites as mentioned in the table. The similarity data were calculated using unweighted arithmetic average and Jaccard's coefficient.

**Table 1. Description of microsatellite markers and assignment of alleles.**

Marker	Repeat Seq.	PCR Primers	Repeat length	Allele #	Length of amplicon (bp)
<b>MS4</b>	(AT) <sub>n</sub>	(5'AGAAGAAGAAACGCGGAATG3') (5'TCTGAAACGAATTCCTTGG3')	15	1	294
			16	2	296
			17	3	298
			18	4	300
			19	5	302
			20	6	304
<b>MS5</b>	(TA) <sub>n</sub>	(5'AATCAGGACTCCGTCACACC3') (5'CCAGAGAGTCCCACATCTC3')	11	1	300
			12	2	304
			13	3	306
			14	4	308
			15	5	310
			16	6	312
			19	7	320
<b>MS6A</b>	(TA) <sub>n</sub>	(5'TCCGCGTGTGGTTTATCT3') (5'TGCGCATGCACGAATAATAG3')	12	1	294
			13	2	296
			14	3	298
			15	4	300
			19	5	312
<b>MS6B</b>	(AT) <sub>n</sub>	(5'GCACTCATGGGATTTTGTAGG3') (5'AAAAATCAATGCACCTGATCG3')	11	1	287
			12	2	289
			13	3	291

			14	4	293
<b>MS7</b>	(AT) <sub>n</sub>	(5'GATCCAACCTCGGCGAGATAC3')	10	1	281
			12	2	285
			14	3	289
			15	4	291
<b>MS8</b>	(AT) <sub>n</sub>	(5'ACACTCGCCTTTCCTTTGTG3')	13	1	289
			14	2	291
			15	3	293
			16	4	295
			17	5	297
			19	6	301
<b>MS10</b>	(ACT) <sub>x</sub> - (AGA) <sub>y</sub> - (TGA) <sub>z</sub>	(5'CTATCACAGCCGTGAGTGTTG3')	5/14/9	1	298
			6/14/9	2	301
			6/17/8	3	307
			6/21/10	4	325
			6/22/9	5	325
			6/23/10	6	331
			6/24/8	7	328
			6/25/9	8	334
			6/26/10	9	340
<b>MS12</b>	(GT) <sub>n</sub>	(5'CCAGCATTTCTTCTCCTT3')	15	1	306
			16	2	308
			17	3	310
<b>MS21</b>	(TACA) <sub>n</sub>	(5'TGTGAGCATAACGGTGGTTC3')	10	1	303

In this table, allele sizes and number allocations has been presented for each of the nine markers for the microsatellite analyses of *Neospora caninum*.

**Table 2. Microsatellite allelic profile in bovine aborted fetuses.**

Sample	Geographical origin	Microsatellite marker								
		MS4	MS5	MS6A	MS6B	MS7	MS8	MS10	MS12	MS21
1	Eslamshahr (Nc-Iran 1)	5	1	3	2	1	4	N*	2	1
2	Eslamshahr	5	–	3	–	1	4	–	–	1
3	Eslamshahr	–	2	1	2	–	–	N	–	1
4	Eslamshahr	5	1	–	–	2	–	–	2	–
5	Eslamshahr	–	–	–	–	–	–	–	–	–
6	Eslamshahr	–	1	–	2	–	4	–	1	1
7	Nazarabad	1	4	–	–	4	–	–	2	–
8	Nazarabad (Nc-Iran 2)	1	4	2	2	4	1 <sub>d</sub>	d	2	1
9	Nazarabad	1	–	2	–	–	1 <sub>d</sub>	d	–	1
10	Nazarabad	–	–	–	2	3	2	–	1	1
11	Eshtehard	–	–	–	–	–	–	–	–	–
12	Varamin	4	–	–	2	–	1 <sub>d</sub>	N	2	1

\*N: Newly described MS alleles in this study

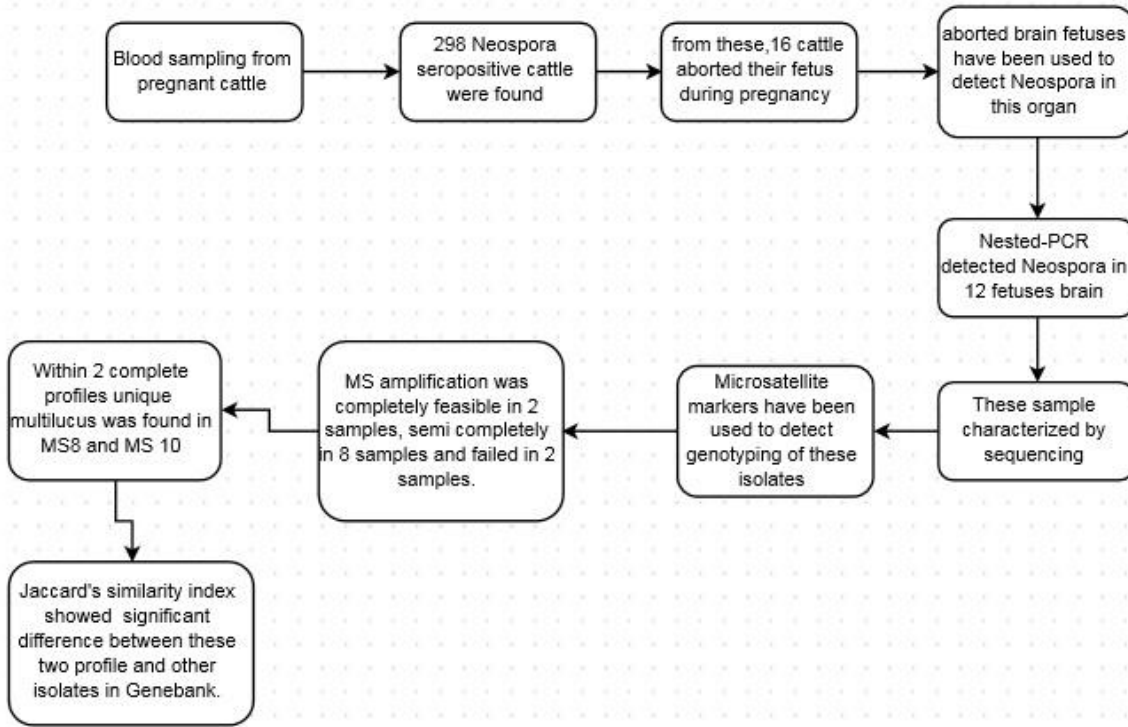
\*d: Identical to the previous reference



Table 3. Complete allelic profile obtained for the Nc-Iran isolates

Sample	Alleles								
	MS4	MS5	MS6A	MS6B	MS7	MS8	MS10	MS12	MS21
Nc-Iran-1	(AT) <sub>19</sub>	(TA) <sub>11</sub>	(TA) <sub>14</sub>	(AT) <sub>12</sub>	(TA) <sub>13</sub>	(AT) <sub>17</sub>	(ACT) <sub>6</sub> (AGA) <sub>17</sub> (TGA) <sub>10</sub>	(GT) <sub>16</sub>	(TACA) <sub>10</sub>
Nc-Iran-2	(AT) <sub>15</sub>	(TA) <sub>14</sub>	(TA) <sub>16</sub>	(AT) <sub>17</sub>	(TA) <sub>15</sub>	(AT) <sub>13</sub>	(ACT) <sub>6</sub> (AGA) <sub>15</sub> (TGA) <sub>10</sub>	(GT) <sub>16</sub>	(TACA) <sub>10</sub>

Allele number of nine microsatellite markers in two complete profiles of Nc-Iran isolates has been given.



### Graphical Abstract

**Highlights**

- *N. caninum* detected in brains of aborted fetuses from seropositive dairy cattle.
- These samples were genotyped in multilocus using 9 different microsatellites markers.
- MS was complete in 2 samples, semi complete in 8 and failed in 2 samples.
- Novel allelic pattern were found in the MS8 and MS10 microsatellite markers.
- Results showed significant difference between these two strains and other isolates.