Onderstepoort Journal of Veterinary Research
ISSN: (Online) 2219-0635, (Print) 0030-2465

Identification of latent neosporosis in sheep in Tehran, Iran by polymerase chain reaction using primers specific for the *Nc-5* gene

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Research Project no:
92019029

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Dates:
Received: 10 Sept. 2015
Accepted: 24 Sept. 2015
Published: 11 Aug. 2016

How to cite this article:
Arbabi, M., Abdoli, A., Dalimi, A. & Pirestani, M., 2016, 'Identification of latent neosporosis in sheep in Tehran, Iran by polymerase chain reaction using primers specific for the *Nc-5* gene', *Onderstepoort Journal of Veterinary Research* 83(1), a1058. http://dx.doi.org/10.4102/ojvr.v83i1.1058

Little is known about latent infection and molecular characterisation of *Neospora caninum* in sheep (*Ovis aries*). In this study, 330 sheep samples (180 hearts and 150 brains) were analysed for *N. caninum* DNA by nested polymerase chain reaction (PCR) targeting the *Nc-5* gene. *Neospora caninum* DNA was detected in 3.9% (13/330) of sheep samples. The parasite's DNA was detected in 6.7% of heart samples (12/180) and 0.7% (1/150) of brain samples. No clinical signs were recorded from infected or uninfected animals. Sequencing of the genomic DNA revealed 96% – 99% similarity with each other and 95.15% – 100% similarity with *N. caninum* sequences deposited in GenBank. To our knowledge, this is the first report on the use of PCR to identify latent neosporosis in sheep in Iran. The results of this study have the potential to contribute to our understanding of the role of *N. caninum*-infected sheep in the epidemiology of neosporosis.

Introduction

Neospora caninum is a worldwide protozoan having a variety of animal hosts (Dubey & Schares 2011; Dubey, Schares & Ortega-Mora 2007). Domestic and wild canids are definitive, whereas different bird and mammalian species (such as cattle, water buffalo, and sheep) serve as intermediate hosts (Dubey & Schares 2011). Abortion, especially in dairy cattle, is one of the major consequences of neosporosis in animal husbandry (Almeria & López-Gatius 2013) that lead to significant economic losses (Reichel *et al.* 2013). Moreover, ovine abortion and reproductive failure due to neosporosis have been reported in several studies (Dubey & Lindsay 1990; Howe *et al.* 2008, 2012; Jolley *et al.* 1999; Moreno *et al.* 2012; Pena *et al.* 2007). In different studies, antibodies to *N. caninum* have been detected in 1.1% – 8.3% of sheep in the west of Iran (Ezatpour *et al.* 2015; Gharekhani & Heidari 2014), 27.7% in Pakistan (Nasir *et al.* 2012), 2.1% in Turkey (Gökçe *et al.* 2015), 10.3% in China (Liu *et al.* 2015), 16.8% in Greece (Anastasia *et al.* 2013), 3% in Argentina (Hecker *et al.* 2013), and 13.1% in south-eastern Brazil (Da Silva Andrade *et al.* 2012). However, there is little information describing the detection of nucleic acids resulting from latent neosporosis in sheep.

Until now, different genes such as internal transcribed spacer sequences, 18S-like ribosomal DNA (small-subunit rDNA), and *Nc-5* genes have been used for molecular diagnosis of neosporosis (reviewed by Al-Qassab, Reichel & Ellis [2010]; Goodswen, Kennedy & Ellis [2013]). However, studies have indicated that the *Nc-5* gene is one of the most highly sensitive and specific for the detection of neosporosis (Almerıa *et al.* 2002; Dubey *et al.* 2014; Hughes *et al.* 2006; Kaufmann *et al.* 1996; Paula *et al.* 2004; Yamage, Flechtner & Gottstein 1996) because it is repeated in the *N. caninum* sequence (Al-Qassab *et al.* 2010). Hence, the main objective of this study was to investigate detection and molecular characterisation of latent neosporosis in sheep (*Ovis aries*) in Tehran, Iran, by polymerase chain reaction (PCR) using primers specific for the *Nc-5* gene.

Materials and methods

Animals and study area

A total of 330 samples from healthy slaughtered sheep (180 hearts and 150 brains) were purchased from an abattoir in Vavan (located in the vicinity of Tehran) from April to September 2014. The animals tested originated from different counties (Eslamshahr, Shahriar, Robatkarim), all of which are located between 50 km and 200 km from Tehran. These locations have hot summers and moderate winters. No clinical signs such as fever, lymphadenitis, nasal and ocular discharges, or jaundice were recorded in any of the animals before slaughter.

DNA extraction

The whole brain and heart of each sheep were individually rinsed with distilled water, packaged, and refrigerated. Approximately 200 g – 250 g of different segments of brain and heart were homogenised with a pestle and mortar in liquid nitrogen, and DNA was extracted using a phenol–chloroform extraction method as described in our recent report (Abdoli *et al.* 2015). To prevent DNA cross-contamination, all materials that were used between different tissue samples were decontaminated with sodium hypochlorite solution (2.5%) and rinsed with distilled water. The concentration of DNA was determined by NanoDrop spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA) for each sample. Overall, the DNA concentration ranged between 150 ng/µL and 200 ng/µL.

Nested polymerase chain reaction

Nested PCR was conducted using specific primers for the *Nc-5* gene. The first round of PCR was conducted using a pair of *N. caninum*–specific primers, Np21plus (5′-CCCAGTGCGTCCAATCCTGTAAC-3′) and Np6plus (5′-CTCGCCAGTCCAACCTACGTCTTCT-3′) (Muller *et al.* 1996). Nested PCR was performed with the primers Np6 (5′-CAGTCAACCTACGTCTTCT-3′) and Np7 (5′-GGGTGAACCGAGGGAGTTG-3′) (Hughes *et al.* 2006). Each amplification was performed in 20-µL reaction mixtures containing 10 µL of 2x master mixes (DFS Master Mix, BIORON GmbH, Ludwigshafen, Germany), each of the respective primers (10 pmol for the first round reaction and 25 pmol for nested PCR), 7 µL of distilled water, and 1 µL of template DNA. One microlitre of the first round product was used as the template for nested PCR. For each reaction, a negative control (double distilled water) and a positive control (DNA extracted from the *Nc-5* strain of *N. caninum*) were included. Amplification was performed with initial denaturation for 5 minutes at 94 °C, followed by 40 cycles at 94 °C for 40 seconds (denaturation), annealing at 62 °C in the first round, and 56 °C in nested PCR for 40 seconds, extension at 72 °C for 40 seconds, and final extension at 72 °C for 10 minutes. PCR products were electrophoresed on a 1.5% agarose gel stained with safe stain (Sinaclon, Tehran, Iran) and visualised under ultraviolet trans-illumination.

Nucleotide sequence analysis

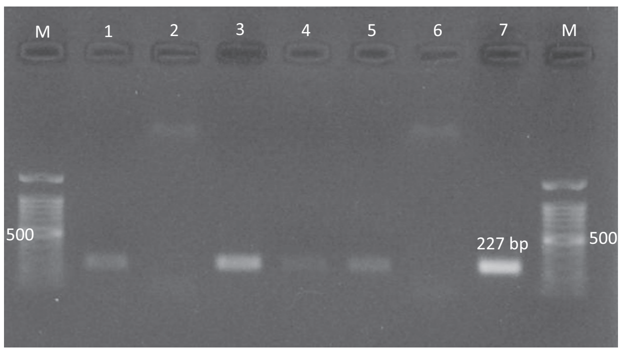
Four positive PCR products (from the second reaction) were amplified with a master mix containing *Pfu* DNA polymerase (Thermo Fisher Scientific, Waltham, USA, cat. no. EP0501), extracted from the gel (Vivantis gel purification kit, Selangor Darul Ehsan, Malaysia) according to the manufacturer's protocols. Then the products were sequenced in the forward and reverse directions by Sequetech (Mountain View, CA, USA) (Abdoli *et al.* 2015). The sequences were edited with BioEdit sequence alignment editor (Hall 1999), aligned with *Nc-5* partial sequences from other hosts by ClustalX2.12 (Larkin *et al.* 2007) and compared with sequences of *N. caninum* available in GenBank. Phylogenetic trees were inferred and evolutionary analyses were conducted using the Tamura three-parameter option of the neighbour-joining model with MEGA6 software (http://www.megasoftware.net/) (Tamura *et al.* 2013). The bootstrap scores were calculated for 1000 replicates (Tamura *et al.* 2013).

Results

Neospora caninum DNA was detected in 13 out of 330 sheep samples (3.9%). The infection rates in the heart and brain samples were 6.7% (12/180) and 0.7% (1/150), respectively. Four nucleotide sequences of the *Nc-5* gene with a length of 227 bp (Figure 1) were submitted to the GenBank database (GenBank accession numbers KR106181, KR106182, KR106183, KR106184). The results demonstrated our sequences shared 96% – 99% similarity with each other (Figures 2 and 3) and 95% – 100% similarity with *N. caninum* deposited in GenBank (Appendix Figure 1). Phylogenetic trees showed intraspecific variations between our isolates and other *N. caninum* specimens deposited in GenBank (Figure 2). Analysis of our sequences showed 96.9% – 97.8% similarity with *N. caninum* isolated from sheep (DQ077661) in the UK and 96.9% – 99.1% similarity with *N. caninum* isolated from sparrows (*Passer domesticus*) in Iran. Interestingly, one of our samples (KR106181) showed 100% similarity with *N. caninum* isolated from wolves (*Canis lupus*) (KF649846) in the United States.

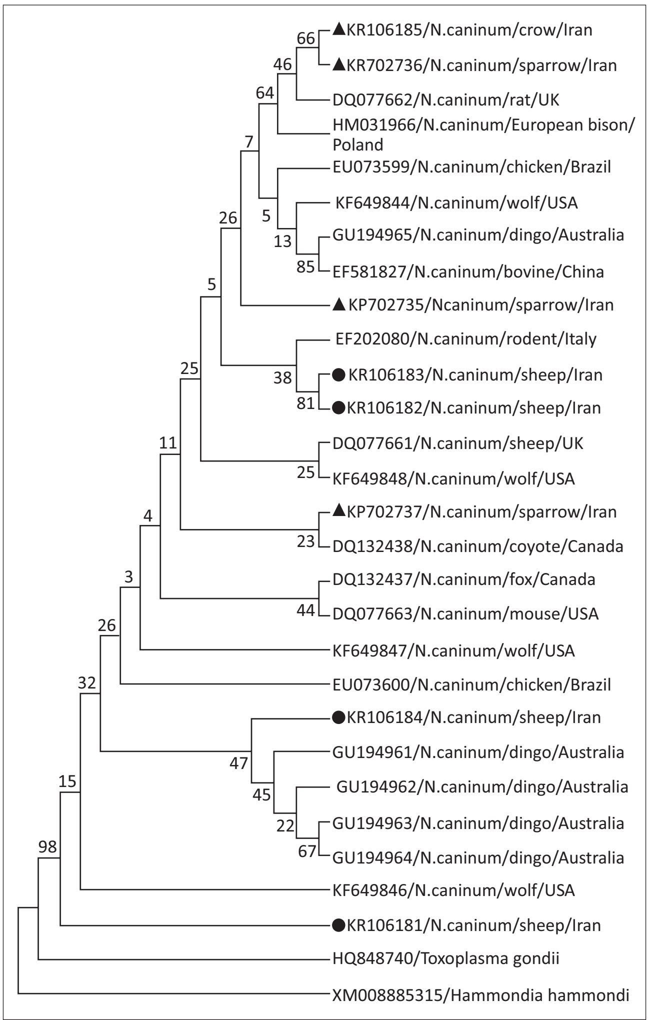
Discussion

Although an association between ovine abortion and neosporosis has been reported in different studies (Dubey & Lindsay 1990; Howe *et al.* 2008, 2012; Jolley *et al.* 1999; Moreno *et al.* 2012; Pena *et al.* 2007), there is little information describing molecular detection of latent neosporosis in sheep. Here, we found a total infection rate of 3.9% (13/330) in our sheep samples. Interestingly, 12 out of 13 positive samples were detected in the hearts and one positive sample was diagnosed in the brain. In previous studies, the seroprevalence of *N. caninum* has been reported in a range of 1.1% – 8.3% of sheep from the west of Iran (Ezatpour *et al.* 2015; Gharekhani & Heidari 2014). Moreover, *N. caninum* DNA was detected in 8.5% (Asadpour *et al.* 2013) and 0.9% of aborted ovine fetuses



M, 100 bp DNA marker; Lane 1, positive control; Lane 2, negative control; Lanes 3, 4, 5, 7, positive samples; Lane 6, negative samples.

FIGURE 1: Polymerase chain reaction products of four *Neospora caninum* positive samples.



The evolutionary history was inferred using the neighbour-joining method. The branches are supported by 1000 bootstrap replicates.

●, *Neospora caninum* isolated in the present study; ▲, denotes *Neospora caninum* isolates in Iran.

FIGURE 2: Phylogenetic relationships among *Neospora caninum* specimens based on a fragment of the *Nc-5* sequence.

in Iran (Sasani *et al.* 2013). Şuteu *et al.* detected *N. caninum* DNA in the diaphragm tissues of 2 out of 181 (1.1%) slaughtered goat kids in Romania (Şuteu *et al.* 2013). In the majority of studies, *N. caninum* was detected in brain samples from aborted or naturally infected sheep (Asadpour *et al.* 2013; Bishop *et al.* 2010; Dubey & Lindsay 1990; Sasani *et al.* 2013; Silva *et al.* 2009). In this regard, Silva and colleagues detected *N. caninum* DNA in 2 out of 102 slaughtered goats (1.92%) in Brazil. Interestingly, both positive samples were isolated from brain samples, whereas all heart and tongue samples were negative (Silva *et al.* 2009). Santos *et al.* (2010) detected *N. caninum* DNA in 5 out of 100 brain samples of beef cattle in Brazil, whereas none of the heart samples were positive (Santos *et al.* 2010). These results are dissimilar to our report, in which most of the positive samples were detected in the heart samples rather than in the brain samples (6.7% *versus* 0.7%). Our results also indicated that in sheep the heart is more susceptible to *N. caninum* infection than the brain.

Latent neosporosis can reactivate in conditions such as immunosuppression and pregnancy (Andrianarivo *et al.* 2005; Hemphill, Vonlaufen & Naguleswaran 2006; Magaña *et al.* 2015; Mazuz *et al.* 2016; Pabón *et al.* 2007; Rettigner *et al.* 2004). Latently infected animals are also a source of *N. caninum* infection for canine definitive hosts.

In the current study, we used the *Nc-5* gene for detection and phylogenetic analysis of *N. caninum*. This gene is repeated in the *N. caninum* sequence (Al-Qassab *et al.* 2010); hence, it is presented as a highly sensitive and specific gene for detection of neosporosis (Kaufmann *et al.* 1996; Yamage *et al.* 1996). The earlier study in this regard was conducted by Yamage *et al.* (1996), who compared the sensitivity and specificity of different primers for diagnosis *of N. caninum*. In this study, five forward (Np1, Np3, Np5, Np7, Np21) and four reverse (Np2, Np4, Np6, Np8) oligonucleotide primers that derived from the *Nc-5* genes were compared for the detection of

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KR106181     CAGTCAACCTACGTCTTCTGCCCCTTCCCTCGTCCGCTTGCTCCCTATGCATAATCTCCC 60
KR106184     CAGTCAACCTACGTCTTCTGCCCCTTCCCTCGTCCGCTTGCTCCCTATGCATAATCTCCC 60
KR106182     CAGTCAACCTACGTCTTCTGCCCCTTCCCTCGTCCGCTTGCTCCCTATGCATAATCTCCA 60
KR106183     CAGTCAACCTACGTCTTCTGCCCCTTCCCTCGTCCGCTTGCTCCCTATGCATAATCTCCA 60
             ***********************************************************.

KR106181     CCGTCATCAGCGCCGCCGGTGTTGCCTCAACACAGAACACTGAACTCTCGATAAGTATTA 120
KR106184     CCGTCATCAGCGCCGCCGGTGTTGCCTCAACACAGAACACTGAACTCTCGATAAGTATCA 120
KR106182     CCGTCATCAGTGCCGCCGGTGTTGCCTCAACACAGAACACTGAACTCTGGATAAGTATCA 120
KR106183     GCGTCATCAGTGCCGCCGGTGTTGCCTCAACACAGAACACTGAACTCTGGATAAGTATCA 120
             .*********.**************************************.********.*

KR106181     TTGGCACACTGTCCACACCCTGACGCAGGCTGATTTCAACGTGACGAATGACTAACCACA 180
KR106184     TTGACACACTGTCCTCACCCTGACGCAGGCTGATTTCAACGTGACGAATGACTAACCACA 180
KR106182     TTGACACACTGTCCACACCCTGACGCAGGCTCATGTCAACGTGACGAATGACTAACCACA 180
KR106183     TTGACACACTGTCCACAGCCTGACGCAGGCTCATGTCAACGTGACGAATGACTAACCACA 180
             ***.**********.**.*************.**.*************************
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An asterisk represents an exact match between all sequences; a dot (•) represents a mismatch in at least one sequence.

FIGURE 3: Partial sequences of the *Nc-5* gene from four isolates of *Neospora caninum* from sheep samples.

neosporosis in experimentally infected mice. Among 19 combinations of forward and reverse primers, the Np21/Np6, Np7/Np6, and Np2l/Np4 primer pairs were able to detect at least 10 pg genomic DNA with a specific single band (Yamage *et al.* 1996). The *Nc-5* gene can also discriminate *N. caninum* from other related apicomplexan parasites (*Toxoplasma gondii* and *Sarcocystis* species) (Kaufmann *et al.* 1996). Thus, the *Nc-5* gene has been used as a highly sensitive and specific gene for detection of neosporosis (Almerıa *et al.* 2002; Dubey *et al.* 2014; Hughes *et al.* 2006; Paula *et al.* 2004; Yamage *et al.* 1996). Hence, we selected the *Nc-5* gene for sensitive and specific detection of neosporosis in the current study.

We also sequenced four positive samples for phylogenetic analysis. We found that our sequences displayed similarity levels of 96% – 99% with each other (Figure 3) and 95% – 100% with *N. caninum* sequences deposited in GenBank (Appendix Figure 1). In comparison with molecular diagnosis, few studies have been conducted on the phylogenetic analysis of *N. caninum* with the *Nc-5* gene (Auriemma *et al.* 2014; Čobádiová *et al.* 2013; Hughes *et al.* 2006). BLAST analyses indicated greater than 94% (Čobádiová *et al.* 2013), 96% (Auriemma *et al.* 2014), and 97% (Hughes *et al.* 2006) similarities between their sequences and other *N. caninum* sequences deposited in GenBank. It therefore seems that the *Nc-5* gene is not a suitable biomarker for phylogenetic analysis and discrimination of genetic diversity for *N. caninum*. Instead, this gene is rather a highly sensitive and specific biomarker for the diagnosis of neosporosis. The use of ribosomal DNA, ITS-1, and recently microsatellites have been recommended for discriminating between *N. caninum* isolates (Al-Qassab *et al.* 2010).

Taken together, the results of this study provide molecular and epidemiological information about latent *N. caninum* infection in sheep in Iran. It can be expected that in future these results will contribute to revealing the role of latent *N. caninum* infection in the epidemiology of neosporosis in sheep.

Acknowledgements

This work was supported by the Iran National Science Foundation (grant no. 92019029). The authors would like to thank Mr. Mahdi Rezapour, Ali Karaji, and Ali Shafiei for their help in preparing the samples.

Competing interests

The authors declare that they have no financial or personal relationships that may have inappropriately influenced them in writing this article.

Authors' contributions

M.A. conceived the study design, analysed and interpreted the data and reviewed the manuscript; A.A. collected the data, prepared the samples, performed molecular assays, designed the tables and figures and wrote the manuscript. M.P. performed the molecular assays and sequencing analysis; A.D. conceived the study design and analysed and interpreted the data.

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Appendix starts on the next page →

Appendix 1

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EF581827    CAGTCAACCTACGTCTTCTGCCCCTTCCCTCGTCCGCTTGCTCCCTATGCATAATCTCCC 60
GU194965    CAGTCAACCTACGTCTTCTGCCCCTTCCCTCGTCCGCTTGCTCCCTATGCATAATCTCCC 60
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EU073599    CAGTCAACCTACGTCTTCTGCCCCTTCCCTCGTCCGCTTGCTCCCTATGCATAATCTCCC 60
kp702735    CAGTCAACCTACGTCTTCTGCCCCTTCCCTCGTCCGCTTGCTCCCTATGCATAATCTCCC 60
KR106185    CAGTCAACCTACGTCTTCTGCCCCTTCCCTCGTCCGCTTGCTCCCTATGCATAATCTCCC 60
kp702736    CAGTCAACCTACGTCTTCTGCCCCTTCCCTCGTCCGCTTGCTCCCTATGCATAATCTCCC 60
DQ077662    CAGTCAACCTACGTCTTCTGCCCCTTCCCTCGTCCGCTTGCTCCCCATGCATAATCTCCC 60
HM031966    CAGTCAACCTACGTCTTCTGCCCCTTCCCTCGTCCGCTTGCTCCCTATGCATAATCTCCC 60
KR106182    CAGTCAACCTACGTCTTCTGCCCCTTCCCTCGTCCGCTTGCTCCCTATGCATAATCTCCA 60
KR106183    CAGTCAACCTACGTCTTCTGCCCCTTCCCTCGTCCGCTTGCTCCCTATGCATAATCTCCA 60
DQ077661    CAGTCAACCTACGTCTTCTGCCCCTTCCCTCGTCCGCTTGCTCCCTATGCATAATCTCCC 60
KF649848    CAGTCAACCTACGTCTTCTGCCCCTTCCCTCGTCCGCTTGCTCCCTATGCATAATCTCCC 60
kp702737    CAGTCAACCTACGTCTTCTGCCCCTTCCCTCGTCCGCTTGCTCCCTATGCATAATCTCCC 60
DQ132438    CAGTCAACCTACGTCTTCTGCCCCTTCCCTCGTCCGCTTGCTCCCTATGAATAATCTCCC 60
KR106181    CAGTCAACCTACGTCTTCTGCCCCTTCCCTCGTCCGCTTGCTCCCTATGCATAATCTCCC 60
KF649846    CAGTCAACCTACGTCTTCTGCCCCTTCCCTCGTCCGCTTGCTCCCTATGCATAATCTCCC 60
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GU194964    CAGTCAACCTACGTCTTCTGCCCCTTCCCTCGTCCGCTTGCTCCCTATGCATAATCTCCC 60
GU194961    CAGTCAACCTACGTCTTCTGCCCCTTCCCTCGTCCGCTTGCTCCCTATGCATAATCTCCC 60
GU194962    CAGTCAACCTACGTCTTCTGCCCCTTCCCTCGTCCGCTTGCTCCCTATGCATAATCTCCC 60
KR106184    CAGTCAACCTACGTCTTCTGCCCCTTCCCTCGTCCGCTTGCTCCCTATGCATAATCTCCC 60
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DQ132437    CAGTCAACCTACGTCTTCTGCCCCTTCCCTCGTCCGCTTGCTCCCTATGAATAATCTCCC 60
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KF649847    CAGTCAACCTACGTCTTCTGCCCCTTCCCTCGTCCGCTTGCTCCCTATGCATAATCTCCC 60
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EF581827    CCGTCATCAGTGCCGCCGGTGTTGCCTCAACACAGAACACTAAACTCTGGATAAGTATCA 120
GU194965    CCGTCATCAGTGCCGCCGGTGTTGCCTCAACACAGAACACTAAACTCTGGATAAGTATCA 120
KF649844    CCGTCATCAGTGCCGCCGGTGTTGCCTCAACACAGAACACTGAACTCTGGATAAGTATCA 120
EU073599    CCGTCATCAGTGCCGCCGGTGTTGCCTCAACACAGAACACTGAACTCTGGATAAGTATCA 120
kp702735    CCGTCATCAGTGCCGCCGGTGTTGCCTCAACACAGAACACTGAACTCTGGATAAGTATCA 120
KR106185    CCGCCATCAGTGCCGCCGGTGTTGCA-CAACACAGAACACTGAACTCTGGATAAGTATCA 119
kp702736    CCGCCATCAGTGCCGCCGGTGTTGCA-CAACACAGAACACTGAACTCTGGATAAGTATCA 119
DQ077662    CCGCCATCAGTGCCGCCGGTGTTGCCTCAACACAGAACACTGAACTCTGGATAAGTATCA 120
HM031966    CCGCCATCAGTGCCGCCGGTGTTGCCTCAACACAGAACACTGAACTCTGGATAAGTATCA 120
KR106182    CCGTCATCAGTGCCGCCGGTGTTGCCTCAACACAGAACACTGAACTCTGGATAAGTATCA 120
KR106183    GCGTCATCAGTGCCGCCGGTGTTGCCTCAACACAGAACACTGAACTCTGGATAAGTATCA 120
DQ077661    CCGTCATCAGTGCCGCCGGTGTTGCCTCAACACAGAACACTGAACTCTGGATAAGTATCA 120
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kp702737    CCGTCATCAGTGCCGCCGGTGTTGCCTCAACACAGAACACTGAACTCTCGGTAAGTATCA 120
DQ132438    CCGTCATCAGTGCCGCCGGTGTTGCCTCAACACAGAACACTGAACTCTCGATAAGTATCA 120
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KF649846    CCGTCATCAGCGCCGCCGGTGTTGCCTCAACACAGAACACTGAACTCTCGATAAGTATTA 120
EF202080    CCGTCATCAGTGCCGCCGGTGTTGCCTCAACACAGAACACTGAACTCTGGATAAGTATTA 120
GU194963    CCGTCATCAGCGCCGCCGGTGTTGCCCCAACACAGAACACTGAACTCTCGGTAAGTATCA 120
GU194964    CCGTCATCAGCGCCGCCGGTGTTGCCCCAACACAGAACACTGAACTCTCGGTAAGTATCA 120
GU194961    CCGTCATCAGCGCCGCCGGTGTTGCCCCAACACAGAACACTGAACTCTCGGTAAGTATCA 120
GU194962    CCGTCATCAGCGCCGCCGGTGTTGCCCCAACACAGAACACTGAACTCTCGGTAAGTATCA 120
KR106184    CCGTCATCAGCGCCGCCGGTGTTGCCTCAACACAGAACACTGAACTCTCGATAAGTATCA 120
DQ077663    CTGTCATCAGTGCCGCCGGTGTTGCCTCAACACAGGACACTGAACTCTCGATAAGTATCA 120
DQ132437    CTGTCATCAGTGCCGCCGGTGTTGCCTCAACACAGAACACTGAACTCTCGATAAGTATCA 120
EU073600    CCGTCATCAGTGCCGCCGGTGTTGCCTCAACACAGAACACTGAACTCTCGATAAGTATCA 120
KF649847    CCGTCATCAGTGCCGCCGGTGTTGCCTCAACACAGAACACTGAACTCTCGATAAGTATCA 120
            ..*.******.**************..********.*****.******.*.*******.*
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Our sheep samples are represented in red (accession nos. KR106181, KR106182, KR106183, KR106184). An asterisk represents an exact match between all sequences; a dot (•) represents a mismatch in at least one sequence.

FIGURE 1-A1: Sequence alignment of *Nc-5* gene of *Neospora caninum* from mammalian and bird hosts.

Appendix 1 (Continues...).

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EF581827     TTGACACATTCTCCACTCCCTGACGCAGGCTCATTTCAACGTGACGAATGACTAACCACA 180
GU194965     TTGACACATTCTCCACTCCCTGACGCAGGCTCATTTCAACGTGACGAATGACTAACCACA 180
KF649844     TTGACACACTGTCCACACCCTGACGCAGGCTCATTTCAACGTGACGAATGACTAACCACA 180
EU073599     TTGACACACTGTCCACACCCTGACGCAGGCTCATTTCAACGTGACGAATGACTAACCACA 180
kp702735     TTGACACACTGTCCACACCCTGACGCAGGCTCATTTCAACGTGACGAATGACTAACCACA 180
KR106185     TTGACACACTGTCCACACCCTGACGCAGGCTCATTTCAACGTGACGAATGACTAACCACA 179
kp702736     TTGACACACTGTCCACACCCTGACGCAGGCTCATTTCAACGTGACGAATGACTAACCACA 179
DQ077662     TTGACACACTGTCCACACCCTGACGCAGGCTCATTTCAACGTGACGAATGACTAACCACA 180
HM031966     TTGACACACTGTCCACACCCTGACGCAGGCTCATTTCAACGTGACGAATGACTAACCACA 180
KR106182     TTGACACACTGTCCACACCCTGACGCAGGCTCATGTCAACGTGACGAATGACTAACCACA 180
KR106183     TTGACACACTGTCCACAGCCTGACGCAGGCTCATGTCAACGTGACGAATGACTAACCACA 180
DQ077661     TTGACACACTGTCCACACCCTGACGCAGGCTGATTTCAACGTGACGAATGACTAACCGCA 180
KF649848     TTGACACACTGTCCACACCCTGACGCAGGCTGATTTCAACGTGACGAATGACTAACCACA 180
kp702737     TTGACACACTGTCCACACCCTGACGCAGGCTCATTTCAACGTGACGAATGACTAACCACA 180
DQ132438     TTGACACACTGTCCACACCCTGACGCGGGCTCATTTCAACGTGACGAATGACTAACCACA 180
KR106181     TTGGCACACTGTCCACACCCTGACGCAGGCTGATTTCAACGTGACGAATGACTAACCACA 180
KF649846     TTGGCACACTGTCCACACCCTGACGCAGGCTGATTTCAACGTGACGAATGACTAACCACA 180
EF202080     TTGGCACACTGTCCACACACTGACGCAGGCTGATTTCAACGTGACGAATGACTAACCACA 180
GU194963     TTGACACACTGTCCTCACCCTGACGCAGGATGATTTCAACGTGACGAATGACTAACCACA 180
GU194964     TTGACACACTGTCCTCACCCTGACGCAGGATGATTTCAACGTGACGAATGACTAACCACA 180
GU194961     TTGACACACTGTCCTCACCCTGACGCAGGCTGATTTCAACGTGACGAATGACTAACCACA 180
GU194962     TTGACACACTGTCCTCACCCTGACGCAGGCTGATTTCAACGTGACGAATGACTAACCACA 180
KR106184     TTGACACACTGTCCTCACCCTGACGCAGGCTGATTTCAACGTGACGAATGACTAACCACA 180
DQ077663     TTGACACACTGTCCACACCCTGACGCAGGCTGATTTCAACGTGACGAATGACTAACCACA 180
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KF649847     TTGACACACTGTCCACACCCTGACGCAGGCTGATTTCAACGTGACGAATGACTAACCACA 180
             ***.****.*.***.*...*******.**.*.**.**********************.**

EF581827     AACCACGTATCCCACCTCTCACCGCTACCAACTCCCTCGGTTCACCC 227
GU194965     AACCACGTATCCCACCTCTCACCGCTACAGACTCCCTCGGTTCACCC 227
KF649844     AACCACGTATCCCACCTCTCACCGCTACCAACTCCCTCGGTTCACCC 227
EU073599     AACCACGTATCCCACCTCTCACCGCTACCAACTCCCTCGGTTCACCC 227
kp702735     AACCACGTATCCCACCTCTCACCGCTACCAACTCCCTCGGTTCACCC 227
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KR106182     AACCACGTATCCCACCTCTCACCGCTACCAACTCCCTCGGTTCACCC 227
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DQ077661     AACCACGCATCCCACCTCTCACCGCTACCAACTCCCTCGGTTCACCC 227
KF649848     AACCACGTATCCCACCTCTCACCGCTACCAACTCCCTCGGTTCACCC 227
kp702737     AACCACGTATCCCACCTCTCACCGCTACCAACTCCCTCGGTTCACCC 227
DQ132438     AACCACGTATCCCACCTCTCACCGCTACCAACTCCCTCGGTTCACCC 227
KR106181     AACCACGTATCCCACCTCTCACCGCTACCAACTCCCTCGGTTCACCC 227
KF649846     AACCACGTATCCCACCTCTCACCGCTACCAACTCCCTCGGTTCACCC 227
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KF649847     AACCACGTATCCCACCTCTCACCGCTACCAACTCCCTCGGTTCACCC 227
             *******.********************..*****************
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Our sheep samples are represented in red (accession nos. KR106181, KR106182, KR106183, KR106184). An asterisk represents an exact match between all sequences; a dot (•) represents a mismatch in at least one sequence.

FIGURE 1-A1 (Continues...): Sequence alignment of *Nc-5* gene of *Neospora caninum* from mammalian and bird hosts.