Title

Genetic characterization of *Neospora caninum* from aborted bovine fetuses in Hokkaido, Japan

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List of abbreviations: JPN, Japan; MS, microsatellite; MLGs, multilocus genotypes; BIC, Bayesian information criterion; DAPC, discriminant analysis of principal components; PCs, posterior clusters

Abstract

Neospora caninum is one of the main causes of bovine abortions worldwide, including Japan. Nothing is known about the *N. caninum* population substructures in Japan, and only one isolate from a pregnant sheep has been studied to date. This study describes, for the first time, the genetic characterization of isolates of *N. caninum* implicated in cattle abortions in Japan. Brains from five aborted fetuses were successfully genotyped based on multilocus microsatellite markers. Assigned genotypes showed high frequencies of mixed alleles in the sequenced markers MS7 and MS10, raising concerns about the subpopulation structures of *N. caninum* infecting animals in Japan. Clustering analysis of the genotypes, together with those from a previous dataset, showed that five of the six genotypes were distinct from other clusters. Meanwhile, the remaining genotype, together with the sheep isolate from Japan, was grouped with those from Mexico and Spain. These preliminary data may indicate a complex transmission pattern of *N. caninum* in Japan via clonal spreading by vertical and horizontal transmission and geographically related population substructuring.

Keywords: *Neospora caninum*, Abortion, Cattle, Microsatellite, Japan

1. Introduction

Neosporosis is considered a serious disease in cattle, causing abortions and neonatal mortality (Dubey et al., 2007; Dubey and Schares, 2011) and subsequent economic losses (Reichel et al., 2013). Evidence suggests that *Neospora caninum* isolates differ in their virulence, which may explain variations in disease outcomes and epidemiology (Al-Qassab et al., 2010; Dubey and Schares 2011; Goodswen et al., 2013). The clonal model of *N. caninum* expansion has been recently suggested, with a highly inbred genome (Khan et al., 2019). Nevertheless, multilocus microsatellite genotyping revealed extensive genetic diversity among *N. caninum* isolates, indicating predominant clonal propagation in cattle and geographically related population substructures (Regidor-Cerrillo et al., 2020).

Neospora caninum has been implicated in bovine abortions in Japan, with nationwide distribution and high economic losses (Koiwai et al., 2005a; Koiwai et al., 2006; Yamane et al., 2000). However, genetic analysis has not been conducted on the isolates from cattle abortions in Japan, and to date, only one isolate has been analyzed from a pregnant sheep without clinical disease (Koyama et al., 2001). This study, for the first time, aimed to determine the genetic relationship between *N. caninum* isolates causing cattle abortions in Japan and other worldwide isolates.

2. Material and methods

2.1. Ethics statement

All animal experiments strictly followed the recommendations of the Guide for the Care and Use of Laboratory Animals of the Ministry of Education, Culture, Sports, Science and Technology, Japan. The study protocol was approved by the Committee on the Ethics of Animal

Experiments at the Obihiro University of Agriculture and Veterinary Medicine (permission numbers: 18-15, 19-3, 20-3).

2.2. *Neospora caninum* infected tissues

Fetal tissues from aborted dams were collected by the Tokachi Livestock Hygiene Service Center and tested for *N. caninum* antigens using immunohistochemical assays. Additionally, their mother's sera were tested for seropositivity to *N. caninum* by immunofluorescence antibody test (VMRD, Pullman, WA, USA). Four samples (JPN-10-H10, JPN-10-H11, JPN-10-H12, JPN-10-H13) were obtained from aborted bovine fetuses upon outbreak of an abortion epidemic in a dairy cattle farm in Shihoro, Hokkaido, Japan (2010). Additionally, one sample (JPN-10-H218) was obtained from an aborted fetus in a farm located in the same area (2018).

2.3. Genetic characterization of *N. caninum*

Brain tissues (0.5 grams) were lysed by incubation with 10 volumes (5 ml) of extraction buffer (0.1 M Tris-HCl pH 9.0, 1% SDS, 0.1 M NaCl, 1 mM EDTA) and 1 mg/ml of proteinase K at 50°C. DNA was extracted from the tissue's lysate using phenol-chloroform extraction and precipitated with ethanol. Confirmation of *N. caninum* DNA in the infected brain tissues was achieved by amplifying a fragment of the specific sequence Nc5 using nested PCR, with the primers described by Yamage et al.,1996, and PCR cycling was performed as previously described (Du et al., 2015).

Genetic characterization of *N. caninum* from brain samples DNA (200 ng) was performed by microsatellite analysis employing 12 MS markers: MS1A, MS1B, MS2, MS4, MS5, MS6A, MS6B, MS7, MS8, MS10, MS12, and MS21. All PCR conditions, including the external and internal primers as well as the 6-FAM-labeled reverse primers, were adopted as

previously described (Pedraza-Díaz et al., 2009; Regidor-Cerrillo et al., 2013). Negative controls were used, including Vero-cell lysates and milliQ water, and the DNA of the Nc1 strain of *N. caninum* tachyzoites was used as the positive control in all PCR reactions. The sizes of the 6-FAM-labeled PCR products for all of the MSs were determined using an 8-capillary 3500 DNA analyzer (Applied Biosystems, Foster City, CA, USA) with Gene Scan-500 (LIZ) Size Standards (Applied Biosystems) and the GeneMapper™ Software 5 (Applied Biosystems).

Amplicons from the non-labeled MS7 and MS10 primers were separated by electrophoresis on 1% agarose gels. The bands of interest were excised from the gel and purified with NucleoSpin® Gel and a PCR Clean-up Kit (Macherey-Nagel, Diiren, Germany). The quantity and purity of the purified amplicons were measured using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and cloned using pCR™4-TOPO® TA Cloning Kits for Sequencing (Thermo Fisher Scientific, St. Louis, MO, USA). The plasmid construct was extracted using the QIA prep®Spin Miniprep Kit (Qiagen), and four positive clones for each isolate were bidirectionally sequenced using the M13 forward and reverse primers and Bigdye Terminator Cycle Sequencing Kit V 3.1 (Applied Biosystems) on a 3500 Genetic Analyzer (Applied Biosystems).

The representative MS7 and MS10 sequences obtained in this study were deposited in the GenBank database under the accession numbers MT782054 to MT782062. The multilocus genotype (MLG) of each isolate was determined based on the combined results of the number of repeats deduced from automated allele sizing and cloning-sequencing in the nine MS markers following the procedure described by Regidor-Cerrillo et al. (2013).

2.4. Data analysis

Nine loci were used for clustering analysis (MS4, MS5, MS6A, MS6B, MS7, MS8, MS10, MS12, and MS21). A previous *N. caninum* MLGs dataset (Medina-Esparza et al., 2016),

together with the new sheep genotypes (García-Sánchez et al., 2020), was used to identify genetic relationships; all analyses were conducted using R software with the adegenete (Jombart 2008) and poppr (Kamvar et al. 2013) packages. The geographical locality from where the isolates in the present study and previous studies were collected was defined as the priori population. The optimum number of posterior clusters that all isolates were divided into without priori population information was determined by *k*-means clustering with the smallest Bayesian information criterion (BIC). The *k* value sequentially increased from $k = 1$ to $k = 12$ in the *find.clusters* function (Jombart et al. 2010). This function was performed for 10 independent trials with 1,000 starting points and 1,000,000 iterations. Subsequently, discriminant analysis of principal components (DAPC) was conducted to group the isolates into the optimum number of posterior clusters with 20 PCs, implying >80% of the cumulative proportion of variance. Next, PCs were applied to discriminant analyses for the detection of genetic variability among the posterior clusters. The probability of each isolate belonging to each posterior cluster was also obtained from the results of the DAPC.

Theory/calculation

Multilocus microsatellite genotyping was proved to be a valid tool for discrimination of *N. caninum* isolates into subtypes related to geographical locations, so it can be helpful for understanding the clonal expansion and diversity of this parasite worldwide. Thus, in this study, with cloning-sequencing approach, we applied this system in accurate way to investigate the genetic relationship of Japanese isolates with worldwide genotypes. In addition, we searched for mixed alleles which may be underestimated by automated allele sizing and direct sequencing especially for alleles similar in sizes with different repeat motifs.

3. Results and Discussion

Parasite DNA was successfully amplified from the five brain samples for the 12 MS markers. Non-amplified markers from some samples were not considered after three successive nested PCR reactions. Automated allele sizing data of fluorescent PCR products were considered after performing several repetitions of the experiments and obtaining constant data. The alleles were successfully assigned following the protocols described by Pedraza-Díaz et al. (2009) and Regidor-Cerrillo et al. (2013), except for the marker MS1A (Table S1). Because MS10 is a highly informative marker, all alleles identified in the MS10 locus were given equal opportunities to appear in the multilocus genotypes considering the lack of dominance after fragment analysis. Additionally, the MS10 amplified products showed the same PCR product sizes with different allelic repeat motifs after cloning-based sequencing. Similarly, one sample (JPN-10-H 12) showed two alleles in the locus MS5 with nearly equal peaks, which were assigned into two genotypes. In contrast, the locus MS7 showed a high frequency of mixed alleles after clone sequencing, wherein TA (13) was the dominant allele corresponding to the number of positive sequenced clones and fragment analysis prediction (Table S1).

From the five investigated samples, six multilocus genotypes were identified based on similarity and difference between samples as well as the existence of mixed alleles without dominance (Table 1). Mixed two alleles were previously reported from *N. caninum* isolates and only the dominant ones were considered for further analysis (Regidor-Cerrillo et al., 2013). Notably, one investigated sample (JPN-10-H10) showed three mixed alleles from the sequenced clones of MS7 and MS10. Consequently, this difference was attributed to the sequencing method. We referred to cloning-based sequencing with the highest resolution and best discriminative capabilities over the direct DNA sequencing methods used in other studies. Minor mixed subpopulations can be missed by microsatellite genotyping if they represent <20%

of the overall parasite population (Juliano et al., 2007; Figan et al., 2018). Although the minor subpopulations are not important to the geographic distribution, they are extremely important when linking them to the disease outcome or biological differences.

Genotypes were divided into 12 posterior clusters (k = 12), with the lowest BIC in the k-mean clustering (Fig. S1). The scatter plot of DAPC based on the first two liner discriminant functions showed that five of the seven genotypes from Japan were separated from other clusters (Fig. 1). These five genotypes were assigned from samples (JPN-10-H10, JPN-10-H11, JPN-10-H12, JPN-10-H13) obtained during outbreak of an abortion epidemic in a dairy cattle farm in Shihoro, Hokkaido, Japan (2010). The existence of different *N. caninum* subpopulations may be attributed to clonal spreading by vertical and horizontal transmission and geographically related population substructures. Nationwide surveys in Japan have revealed that dogs harbor antibodies against *N. caninum* (Sawada et al., 1998; Hara et al., 2006; Kubota et al., 2008). Horizontal transmission between cattle and dogs was proposed in Japan, wherein dogs reared in dairy farms experienced bovine neosporosis that was seropositive (31.3%) for *N. caninum* (Sawada et al., 1998). However, the existence of a cluster separate from other *Neospora* populations has been detected in Japan, showing that there is not only a worldwide population of *Neospora* but also an endemic population in Japan.

Another sample obtained from the same town in 2018 (JPN-10-H218) was found to be genetically related to the previously identified sheep isolate from Japan. The isolate Nc-Sheep, which has been extensively used as a reference strain for *N. caninum* genetic studies, was isolated from naturally infected pregnant sheep with no history of abortion (Koyama et al., 2001). The authors of this study do not mention the location of this sheep isolate, but it may be the Tokachi area, which neighbors Shihoro in Hokkaido, indicating a predominant clonal propagation in the country and geographically related population substructures. These two Japanese isolates were grouped with those from Mexico and Spain (Fig. 1). This result, together

with other subpopulation clustering of isolates from different countries, suggests that there is no relationship between the genetic variation of most *Neospora* populations and their geographical characteristics and that their populations have been mixed. Whole genome sequencing suggested the existence of a global single lineage, which recently evolved from a common ancestor and expanded worldwide owing to the movement of cattle (Khan et al., 2019).

Contrastingly, a close relative of *N. caninum*, *Toxoplasma gondii* possessed a different population genetic structure with a high degree of genetic diversity compared to the single genome of *N. caninum* (Khan et al., 2019). The three predominate lineages of *T. gondii* are classified as either type I, II, or III single nucleotide polymorphisms (SNPs) whereas, types I and III are second- and first-generation offspring, respectively, resulted from a cross between a type II strain and one of two ancestral strains (Boyle et al. 2006). However, subsequent studies incorporating multilocus genotyping techniques for a wider range of isolates, proved the existence of a more complex population structures (Galal et al. 2019). Frequent genetic exchanges among the three lineages via sexual recombination in the definitive felid host give rise to new genotypes (Grigg and Sundar, 2009). In contrast to *T. gondii* which utilizes both the sexual and asexual transmission to shape its population structure (Grigg and Sundar, 2009), the vertical transmission is a primary mode for the expansion of *N. caninum* genome (Goodswen et al. 2013; Khan et al., 2019; Calarco and Ellis, 2020). More recently, comparison of genome-wide SNPs and insertions/deletions between 7 *N. caninum* isolates was lower than that was identified between 2 *T. gondii* type I strains, RH and GT1 (Yang et al., 2013; Khan et al., 2019). Taken together, these data suggest that *Neospora caninum* population genetics is composed of one species, that have evolved from a common ancestor and expanded as a single clonal lineage throughout the world.

Neospora caninum was first identified in aborted fetuses in Japan in 1992 (Ogino et al., 1992). A nationwide survey was conducted to investigate the seroprevalence of *N. caninum*

among cattle with reproductive disorders (Koiwai et al., 2005a) and among clinically healthy cattle (Koiwai et al., 2006) during the years 1996 and 1997, respectively. The seropositive and seronegative cattle determined to be clinically healthy (Koiwai et al., 2006) were tracked for their reproductive performance during a retrospective 1-year study. The findings revealed that seropositive animals were 6.1 times more likely to abort compared with seronegative animals, and 83.6% of seropositive abortions could be attributed to *N. caninum* (Koiwai et al., 2005b). Furthermore, 1,031 *Neospora*-seropositive cattle and 2,030 aborted fetuses were estimated during a nationwide survey conducted in 1998, with an estimated total economic loss of JPY1,362,000,000 (Yamane et al., 2000). Although these data are alarming and indicate a considerable economic loss due to neosporosis in Japan, there is a gap in the epidemiological surveys of neosporosis during the last 20 years in Japan (Takashima et al., 2013; Masatani et al., 2018; Abdelbaky et al., 2020).

4. Conclusions

Seven Japanese genotypes of *N. caninum* are clustered into two subpopulations, one of which was unique and distinct from European and American subpopulations. Meanwhile, another cluster was genetically related to the Mexican and Spanish genotypes. These findings may be attributed to clonal spreading by vertical and horizontal transmission as well as geographically related population substructuring. More isolates are needed to determine the transmission patterns based on genetic relationships, and sero-epidemiological studies are also required for both cattle and dogs.

Declarations of interest

The authors declare that they have no competing interests.

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Figure legends

Figure 1 (A) Scatter plot of discriminant analysis of principal components based on the first two liner discriminant functions in k = 12. The 12 posterior clusters are shown in different colors. (B) Bar plot of posterior cluster membership probability for the 114 isolates. The bar plots show the probability of the isolate belonging to each posterior cluster. The colors are the same as those of the posterior clusters in (A). The priori population of the isolates is shown at the top of the bar plot, and the name of the isolate is indicated under the bar plot.

Table 1: Multilocus microsatellite genotypes obtained from fetal brain tissues included in this study

Genotypes ID[a]	Geographic Origin (Country)	Host sampled	Microsatellite Genotype[b]								
			MS4	MS5	MS6A	MS6B	MS7[d]	MS8	MS10	MS12	MS21
JPN-10-H10.1[e]	Japan	Cattle	10	15	12	11	12	13	6.14.8	14	6
JPN-10-H10.2[e]	Japan	Cattle	10	15	12	11	12	13	7.12.9	14	6
JPN-10-H10.3[e] JPN-10-H11 JPN-10-H13	Japan	Cattle	10	15	12	11	12	13	7.13.9*	14	6
JPN-10-H12.1[e]	Japan	Cattle	10	15	NA	NA	12	13	7.14.9*	14	6
JPN-10-H12.2[e]	Japan	Cattle	10	16	NA	NA	12	13	7.14.9*	14	6
JPN-10-H218	Japan	Cattle	13	9	NA	NA	12	NA	NA	15	6

[a] Sample identification: JPN (Japan) - genotype number (ID).

[b] Allele assignment of MS markers are expressed as the number of repeats following the criteria described by Regidor-Cerrillo et al. (2013).

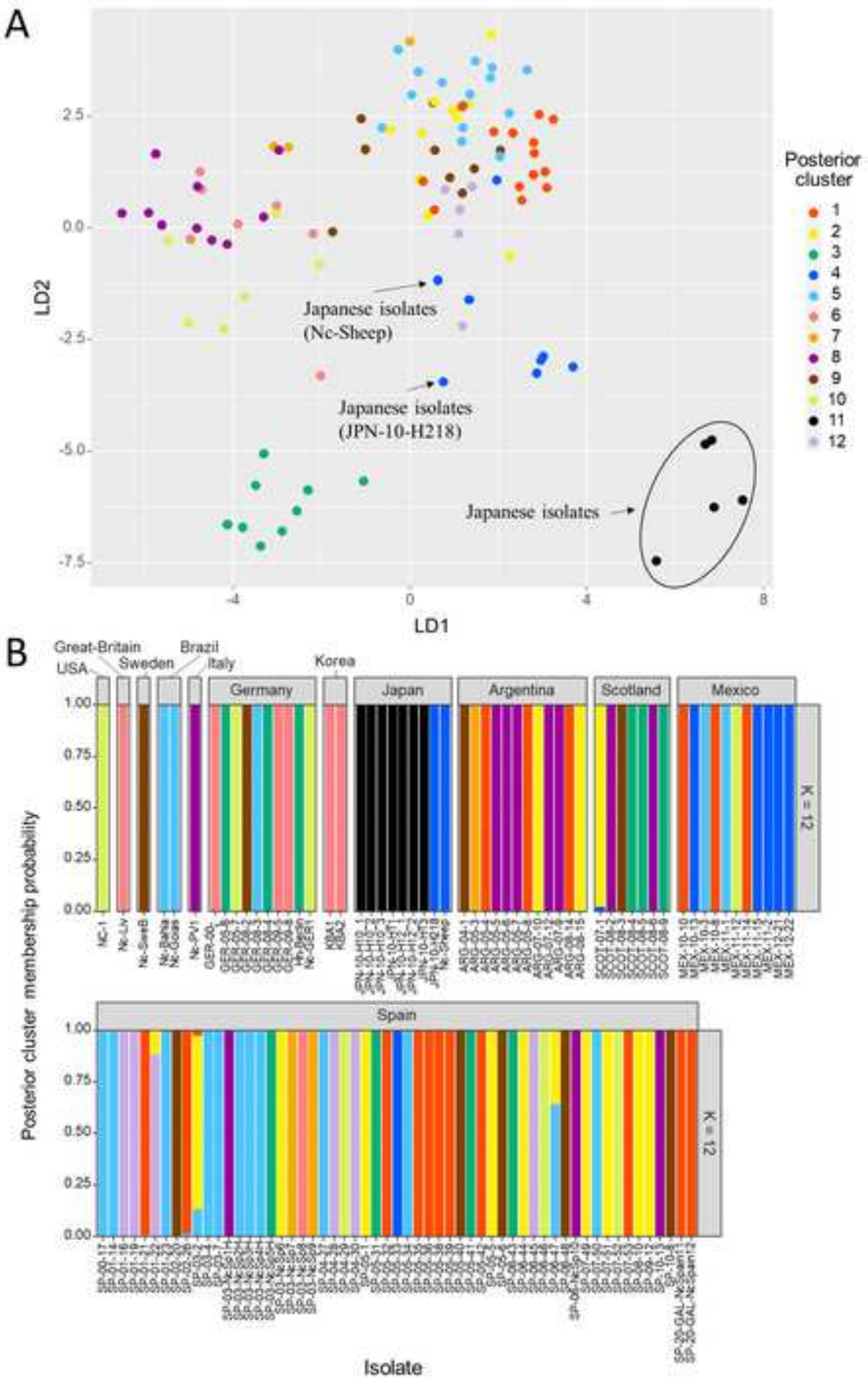
[d] Only dominant alleles from cloning and automated allele sizing were included for this marker.

[e] Mixed alleles were identified, and dominant alleles could not be assigned.

*Newly described MS10 alleles obtained in this study.

NA: not amplified

Figure 1


A
LD2
LD1
2.5
0.0
-2.5
-5.0
-7.5
-4
0
4
8
Japanese isolates
(Nc-Sheep)
Japanese isolates
(JPN-10-H218)
Japanese isolates
Posterior cluster
1
2
3
4
5
6
7
8
9
10
11
12
B
Great-Britain
USA
Sweden
Brazil
Italy
Korea
Germany
Japan
Argentina
Scotland
Mexico
Spain
Posterior cluster membership probability
1.00
0.75
0.50
0.25
0.00
K = 12
Isolate