The Journal of Veterinary Medical Science

NOTE

Internal Medicine

Pyruvate kinase deficiency mutant gene carriage in stray cats and rescued cats from animal hoarding in Hokkaido, Japan

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J. Vet. Med. Sci.
85(9): 972–976, 2023
doi: 10.1292/jvms.23-0091

Received: 22 February 2023
Accepted: 15 July 2023
Advanced Epub:
27 July 2023

ABSTRACT. The mutant allele frequency of the Pyruvate kinase (PK) gene has been investigated mostly in pure breed cats. We investigated the PK mutant gene in stray and animal hoarding mongrel cats in Hokkaido, Japan. We also investigated the kinship of individuals carrying the mutant gene. Genotyping was conducted using the previously reported real-time PCR method. Fourteen microsatellite markers were used to identify the parents and offspring of cats carrying the PK mutant gene, and some kinship such as parent-offspring and siblings was observed. Some stray and animal hoarding cats carried the PK mutation gene and that consanguinity was confirmed among these cats indicated that the PK mutation gene was spread by unregulated interbreeding.

KEYWORDS: animal hoarding cat, microsatellite marker, mutant allele frequency, pyruvate kinase deficiency, stray cat

Pyruvate kinase (PK) is an enzyme that catalyzes the transphosphorylation of phosphoenolpyruvate to adenosine diphosphate in the final step of the Embden-Meyerhof glycolysis pathway to produce pyruvate and adenosine triphosphate. The enzyme produces pyruvate and ATP [6]. The structural genes of PK consist of (1) "*PKLR*" from the liver and red blood cells and (2) "PKM" from the muscle system. PK deficiency is an autosomal recessive erythrocyte enzyme disorder caused by mutations in the *PKLR* gene; deficiency of the erythrocyte-derived isozyme of PK results in an energy deficit in the red blood cells, resulting in hemolytic anemia [3, 5, 6].

Reports have evaluated the allele frequency of PK mutation in cats in the United States and the United Kingdom [3], Australia [1], Germany [5], and Japan [6], with higher frequencies in purebred domestic cats, led by Abyssinians and Somalis. On the other hand, Grahn *et al*. reported the occurrence of PK deficiency in random bred domestic cats in the United States and the United Kingdom [3]. However, there is no evidence for the occurrence of PK deficiency in Japanese random bred cats, which are not only domestic but also feral cats.

Ueno *et al*. reported the occurrence of GM1 gangliosidosis in feral cats and stated that GM1 gangliosidosis occurs in a recessive homozygous form and that the genetic disease in this case may be spread by uncontrolled breeding within feral cats [11]. Therefore, it is possible that the mutated gene may be transmitted in PK deficiency as well as in GM1 gangliosidosis through uncontrolled breeding of feral cats. However, to date, there is no evidence worldwide for the possession of mutated genes pertaining to PK deficiency in feral cats.

We hypothesized that PK deficiency is caused by the transmission of mutated genes through uncontrolled mating in feral cats and cats in animal hoarding situations. In this study, we performed a real-time PCR genotyping assay to investigate the possession

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of mutated genes related to PK deficiency in stray cats or rescued cats from animal hoarding living in Hokkaido. As a result, no PK mutation was detected in the Sapporo area, but cats with the mutated gene were identified in both Kitami and Iburi areas. Therefore, we also investigated kinship using microsatellite markers for cats with the mutated gene.

The study population consisted of stray cats (cats whose living environment was or is completely outdoors) or cats rescued from animal hoarding that were in good health and had a normal appetite. The study area was divided into three districts in Hokkaido with different environments: 1) the Sapporo district, which is an urban area with heavy snowfall in winter, 2) the Kitami district, where dairy farming is the main industry and the winters are extremely cold, and 3) the Iburi district, where fishing and tourism are the main industries and the winters are relatively mild. EDTA-treated whole blood frozen at −80°C was used after blood samples were taken at the time of neutering. In cases where owners were present, each veterinarian explained the survey and obtained informed consent from the owners before performing the neutering surgery. Individual information such as sex and whether the cat was a stray or a cat protected from animal hoarding was recorded.

The detection of mutant genes for PK deficiency in cats was evaluated by real-time PCR genotyping using TaqMan probes according to Kushida *et al*. [6]. Designed based on the feline *PKLR* gene sequence of wild-type and PK deficiency cats (GenBank Accession numbers XM_006943090.1 and JX951425.1).

Cats with PK mutant genes determined to be either carrier (heterozygous) or affected (recessive homozygous) by genotyping were included in the kinship study.

DNA was extracted from blood using the QIAamp DNA Micro Kit (Qiagen, Hilden, Germany). Nine (FCA069, FCA075, FCA105, FCA149, FCA220, FCA229, FCA310, FCA441, FCA678) including ISAG (International Society of Animal Genetics) microsatellite markers [7] and five (FCA026, FCA201, FCA293, FCA453, FCA649) microsatellite markers described by Menotti-Raymond *et al*. [9] were used for estimating kinship. The polymorphism information content (PIC) [2], the observed heterozygosities (Ho) and expected heterozygosities (He) were calculated by The EXCEL Microsatellite Toolkit (http://animalgenomics.ucd.ie/sdepark/ms-toolkit/). PCR was performed using the Qiagen® Multiplex PCR Kit (Qiagen). PCR conditions were 95°C for 15 min followed by 30 cycles of 94°C for 30 sec, 59°C for 90 sec, and 72°C for 60 sec, with a final extension at 60°C for 30 min.

The genetically relationship was evaluated by ML-Relate software [4] to estimate “parent-child”, “full sibling”, and “half sibling” relationships. Based on the results of this kinship estimation, a family tree was created for cats carrying the PK mutation gene.

A breakdown of all 443 animals is shown in Table 1. Of the 268 cats in the Iburi district, 200 (74.6%) were stray cats and 68 (25.4%) were cats protected from animal hoarding. All cats of Kitami district were stray cats. No age was recorded in all districts.

None of the 101 cats in the Sapporo area carried the PK mutation gene. On the other hand, 5 (6.76%) of 74 cats in the Kitami area were carriers. All cats carrying the PK deficiency mutation gene in the Kitami district were stray cats. The sex of the cats were three males and two females. The allele frequency in the Kitami area was 0.0338, and the allele frequency for male cats was 0.0349 and for female cats was 0.0323 (Table 1).

Of 268 cats in the Iburi district, 21 (7.836%) were carriers and 1 (0.373%) was affected. Of the carriers in the Iburi area, 15 were stray cats and 6 were cats rescued from animal hoarding. Affected one cat was a stray. The mutant allele frequency (MAF) of stray cats in the Iburi area was 0.0425, that of cats rescued from animal hoarding was 0.0441, and the overall MAF was 0.0429. Of the carrier stray cats in the Iburi area, four were males and 11 were females. On the other hand, of the protected cats, one was a male and five were females. Affected stray cats were females. Among feral cats in the Iburi area, the MAF of male cats was 0.0204 and that of female cats was 0.0637. On the other hand, among cats rescued from animal hoarding, the MAF of male cats was 0.0147 and that of female cats was 0.0735.

Diversity statics for 14 domestic cat markers for 22 cats with PK deficiency mutant gene in Iburi area are presented in Table 2. The Ho ranged from 0.423 to 0.923 with an average value of 0.684. The He ranged from 0.557 to 0.863 with a mean of 0.701. The PIC ranged from 0.465 to 0.827 with a mean of 0.645.

We performed kinship analysis using microsatellite markers on 5 carriers in the Kitami area and 20 carriers and 1 affected cat in the Iburi area. The results are shown in Table 3. Due to insufficient sample volume, it was not possible to authenticate the kinship of individual No. 6 of Iburi area.

No parent-child relationship was found for all 5 cats in the Kitami district. For 21 cats in the Iburi district, some parent-child or sibling kinship was observed. A family tree of blood relations based on the results of Table 3 is shown in Fig. 1. The family trees were classified into four (animal hoarding: 1, stray: 3) categories. Several discrepancies existed in establishing blood relations. It is

Table 1. The number of cats (carrier and affected) with genotypes for pyruvate kinase deficiency and allele frequency for the feline *PKLR* mutation

Area	Number of examined cats (male/female)	Number of carriers (male/female)	Number of affected cats (male/female)	Mutant allele frequency (male/female)
Sapporo	Stray: 101 (50/51)	0 (0/0)	0 (0/0)	
Kitami	Stray: 74 (43/31)	Stray: 5 (3/2)	0 (0/0)	Stray: 0.0338 (0.0349/0.0323)
Iburi	Stray: 200 (98/102) Animal hoarding: 68 (34/34) Total: 268 (132/136)	Stray: 15 (4/11) Animal hoarding: 6 (1/5) Total: 21 (5/16)	Stray: 1 (0/1) Animal hoarding: 0 (0/0) Total: 1 (0/1)	Stray: 0.0425 (0.0204/0.0637) Animal hoarding: 0.0441 (0.0147/0.0735) Total: 0.0429 (0.0189/0.0662)
Total	443 (225/218)	26 (8/18)	1 (0/1)	0.0316 (0.0178/0.0459)

Table 2. Diversity statics for 14 domestic cat markers for 22 cats with pyruvate kinase deficiency mutant gene in the Iburi area

Locus	Observed Heterozygosity†	Expected Heterozygosity†	Polymorphism Information Contents†	Feline chromosome‡
FCA026	0.846	0.805	0.758	D3
FCA069	0.692	0.774	0.729	B4
FCA075	0.923	0.829	0.787	E2
FCA105	0.615	0.582	0.532	A2
FCA149	0.500	0.733	0.671	B1
FCA201	0.808	0.741	0.699	B3
FCA220	0.808	0.655	0.577	F2
FCA229	0.500	0.693	0.643	A1
FCA293	0.885	0.863	0.827	C1
FCA310	0.423	0.557	0.465	C2
FCA441	0.692	0.664	0.590	D3
FCA453	0.539	0.566	0.504	A1
FCA649	0.577	0.676	0.646	C1
FCA678	0.769	0.683	0.607	A1
Average	0.684	0.701	0.645	

Mean number of allele (MNA): 6.64, Standard deviation of MNA: 1.78. Probability of paternity exclusion. The probability of rejecting a male who is not the true father in a father-child relationship (without information about the mother) is 0.994208. † The polymorphism information content, the observed and expected heterozygosities were calculated by The EXCEL Microsatellite Toolkit (http://animalgenomics.ucd.ie/sdepark/ms-toolkit/). ‡ Feline chromosomes were taken from reference number 9.

Table 3. Results of microsatellite marker-based kinship analysis

Animal hoarding cats						
Cat No.	11	12	13	14	15	16
11	-					
12	HS	-				
13	U	U	-			
14	U	U	HS	-		
15	U	U	U	U	-	
16	U	PO	U	U	U	-

Stray cats															
Cat No.	1	2	3	4	5	7	8	9	10	17	18	19	20	21	22
1	-														
2	HS	-													
3	U	U	-												
4	U	U	HS	-											
5	U	U	U	U	-										
7	U	U	U	U	U	-									
8	U	U	U	HS	U	U	-								
9	U	U	U	U	U	U	U	-							
10	U	U	U	U	U	U	U	FS	-						
17	U	U	U	U	U	U	HS	U	U	-					
18	U	U	U	U	U	U	U	FS	FS	U	-				
19	U	U	U	U	U	U	U	PO	PO	U	PO	-			
20	U	U	U	U	U	HS	U	U	U	U	PO	PO	-		
21	U	U	U	U	U	U	U	U	U	U	U	U	U	-	
22	U	U	U	U	U	U	U	U	U	U	U	HS	U	HS	-

FS: full sibs, HS: half sibs, PO: parent/offspring, U: unrelated.

stated that when half and full siblings other than parents and children are included in the pool of many family trees in microsatellite markers, they may be shown as half or full siblings even if the blood relationship is distant when the generations overlap. Additionally, Estimation of kinship is greatly affected by sampling when DNA markers are scarce. Therefore, individual information and "parent-child relationship" were assumed to be the most accurate and were produced assuming that they were the preferred conditions.

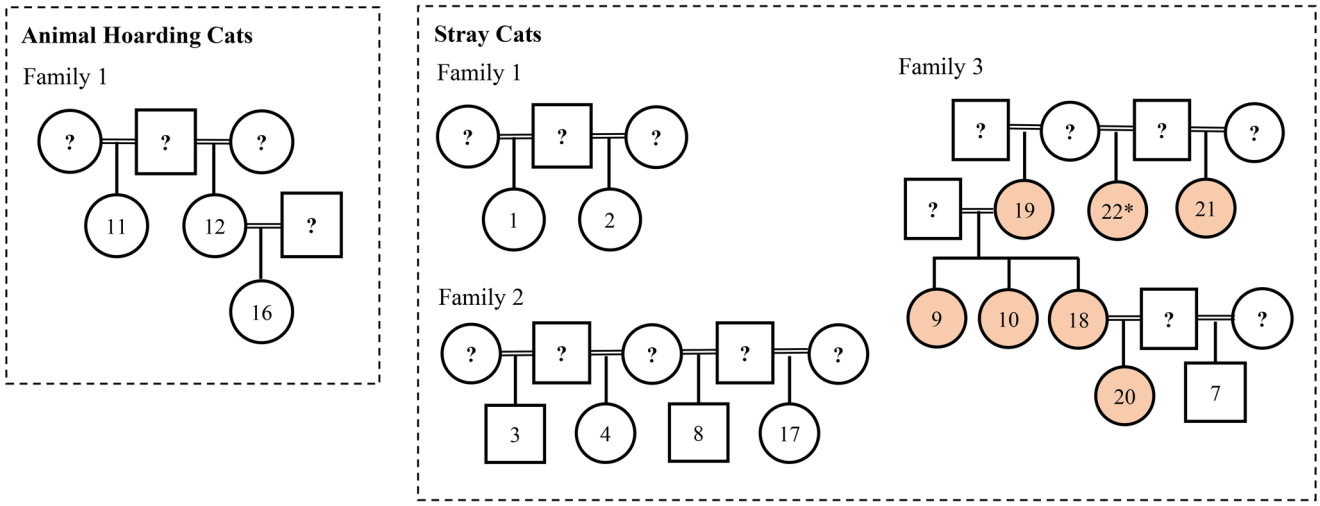

Fig. 1. Family tree of the Iburi area. In the Iburi area, blood relations were observed in stray cats and animal hoarding cats, respectively. Numbers indicate individual numbers. Squares indicate males and circles indicate females. Due to insufficient sample volume, it was not possible to authenticate the kinship of individual No. 6. Asterisks indicate "affected" individuals. Orange mean cats sheltered in the same area locally.

In this study, we found a population of cats carrying the PK mutation among stray cats and cats rescued from animal hoarding in Hokkaido, Japan. Furthermore, as a result of kinship analysis of cats carrying the PK mutant gene, there were two groups: one group of cats that were found to have parent-child relationships and another group of cats that were not found to have parent-child relationships.

Regarding the kinship of cats with the PK mutation, "carriers" and "affected cats" with the PK mutation were identified in a wide range of kinship relationships spanning multiple generations, mainly parent-child relationships in the Iburi area. These results strongly support our hypothesis that unregulated breeding of stray cats and animal hoarding stated cat facilities may spread the disease, since PK deficiency is recessively (affected) homozygous. In the Iburi/Kitami area, there is not one family lineage of cats with the PK mutation, but several unrelated families of cats with the PK mutation, and since not all cats with the PK mutation are related in the Kitami area, we were unable to fully examine this issue. However, this study suggests that there may be other stray cats with PK mutations. In the Iburi area, the presence of parents of an "affected" stray cat (No. 22) carrying the mutant gene in a recessive homozygous state was not confirmed in this study. Ueno *et al*. reported the birth of one affected individual and two carrier individuals from a stray mother cat who was a carrier of GM1 gangliosidosis, an autosomal recessive form of the genetic disease similar to PK deficiency [11]. The birth of an "affected" individual leaves no doubt that there are fathers with the mutated gene for GM1 gangliosidosis. The parents of No. 22 are always "carrier" or "affected" cats, and it is possible that both parents live as stray cats. In fact, both No. 22 and the half-sib (No. 21) of No. 22 were feral cats. This suggests that if a cat with a mutated gene enters another cat population without neutralization and mates with it, the mutated gene may spread rapidly and unregulated. In GM1 gangliosidosis, affected individuals are lethal and die within about six months of birth [11]. Therefore, affected individuals do not participate in breeding. On the other hand, even affected individuals with PK deficiency have varying symptoms and may survive for long periods of time [3, 5, 6]. Identification of the carriers that have one abnormal allele in the gene pair, but that are normal in clinical appearance, is critical because there are no physical clues to the presence of the disease in these animals [10]. Therefore, if affected individuals participate in breeding, they will certainly distribute the mutated gene to their offspring. Therefore, the occurrence of cats with PK mutations should be continuously investigated in the future. Furthermore, there is a need to strongly promote neutering to prevent the occurrence of cats with hereditary diseases.

In this survey, no difference was found in MAF in the Iburi and Kitami areas (Iburi: 0.0429, Kitami: 0.0338). There have been no reports on MAF of PK deficiency in feral cat populations worldwide. On the other hand, the MAF of PK deficiency in domestic short or long hair in the United States and the United Kingdom has been reported to be 0.09 [3]. In the case of domestic cats, breeding is usually artificially controlled unless the owner allows the cat to roam freely outside the house, so if the owner is unaware of the presence of the mutant gene, breeding is limited in scope and the mutant allele frequency may be as high as in pure breeds.

In the Iburi area, the MAF was higher among female cats than among male cats, regardless of whether they were in a stray or animal hoarding environment. In the Kitami area, there was no sex difference in the allele frequency of the PK mutant gene. There are no reports investigating MAF of PK deficiency per sex for domestic short or long hair. Barrs *et al*. reported PK deficiency numbers per sex for Abyssinian and Somali in Australia, with MAF for males of 0.196 and for females of MAF is 0.189 [1]. Barrs *et al*. noted that, although multiple breeders were approached for inclusion, participation may have been biased towards those with known carrier cats. Additionally, related cats, including full siblings, were used. Thus, the estimation of prevalence in Australia may be significantly biased towards the affected allele [1]. Thus, there was no evidence of a sex difference in the possession of the PK mutant, suggesting that the sex difference in the MAF observed in the Iburi area was due to localized reasons such as the fact that male cats carrying the mutant gene were not captured.

The mean PIC was calculated to be 0.645 for the 14 feline microsatellite markers used in this study. This mean PIC is higher than the 19 microsatellite markers reported by Lipinski *et al*. [7] (0.47 in pedigree breeds and 0.63 in random-bred cats). Generally, the higher the PIC value means the higher the appraisal ability [2]. Losada *et al*. [8] evaluated a PIC of 0.4221 as a "moderate" PIC" and 0.9004 as a "high" PIC. Therefore, the present mean PIC (0.645) is in the relatively high category and have relatively high discriminatory power as DNA markers [7]. Probability of paternity exclusion is also 0.994208. This number implies a probability that approximately 994 of the 1,000 male cats available for breeding are not the father. The area where the 26 cats were found to be related is a very limited area (population of approximately 50,000) within the Iburi district, and it is difficult to imagine that 1,000 breeding male cats are living in this area. Therefore, the probability of rejecting a paternity relationship is thought to be high.

There are several problems with this study. First, in collecting the samples, there is a bias in the ancestry of the cats collected for neutering. Cats from more friendly families are more likely to be captured, while cats from more cautious families are less likely to be captured, etc. As the number of carriers is small, the calculated frequency could potentially be changed dramatically by chance. Specific families that include carriers may have also affected the frequency (i.e., population stratification) [10]. Therefore, the limitation of this study is that the calculated disease prevalence and carrier frequency may be influenced by chance. Secondly, it would be easier to create a more accurate family tree for parent-child relationships if the cat's age were known, but it is difficult to determine all ages in reality, because the cats of our study are not domestic.

Based on the results of this study, we recommend that effective measures to prevent the further spread of PK deficiency disease include: owners should not abandon their cats, and feral cats should be actively neutered to prevent an increase in the number of feral cats capable of reproducing. Reducing the number of abandoned cats will not only preserve the quality of life of cats and prevent the further spread of PK mutant genes, but also prevent the spread of various infectious and genetic diseases. Veterinarians should also educate the general public about the existence of "genetic diseases" and that the practice of abandoning cats leads to an increase in the number of unfortunate cats suffering from genetic diseases.

CONFLICT OF INTEREST. The authors declare that they have no conflict of interest.

ACKNOWLEDGMENT. We thank the staff of the Sapporo City Animal Control Center for providing cat blood samples.

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