Kafkas Univ Vet Fak Derg
22 (3): 355-359, 2016
DOI: 10.9775/kvfd.2015.14637

Kafkas Universitesi Veteriner Fakultesi Dergisi
Journal Home-Page: http://vetdergi.kafkas.edu.tr
Online Submission: http://vetdergikafkas.org

Research Article

Analysis of *Cytidine Monophospho-N-Acetylneuraminic Acid Hydroxylase (CMAH)* Gene Related to Neonatal Isoerythrolysis in Stray Cats of Izmir, Turkey [1]

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Article Code: KVFD-2015-14637 Received: 05.11.2015 Accepted: 31.01.2016 Published Online: 12.02.2016

Abstract

Neonatal isoerythrolysis is a life threatening disease in new born cats. It occurs when type A or type AB kittens are born from a type B queen (female cat). A homozygous 18 bp insertion located in *cytidine monophosphate-N-acetylneuraminic acid hydroxylase (CMAH)* gene has been detected in type B cats, causing production of inactive *CMAH* enzyme. Currently, molecular methods are being used to determine type B blood in female cats, which can help prevent neonatal isoerythrolysis in kittens. These molecular assays target the presence of 18 bp insertion in *CMAH* gene. In this study, we aimed to analyze the potential of neonatal isoerythrolysis among stray cats of İzmir, Turkey using PCR detecting the 18 bp insertion in *CMAH* gene. During the study, we analyzed 793 cats' blood sample for the presence of 18 bp insertion in *CMAH* gene. Three cats known to have blood types A, B, and AB were used as control in PCR. According to the PCR results, blood type A control cat displayed a 175 bp product indicating a homozygous type A cat while blood type control B cat showed a 193 bp product in *CMAH* gene (with 18 bp insertion) indicating a homozygous type B cat. Interestingly, blood type AB control cat showed a heterozygous pattern for *CMAH* gene, in which three different bands (175 bp like that of type A, 193 bp product for type B, and the third unique band with approximately 240 bp size) were detected. Among 793 stray cats of İzmir, 791 were homozygous for *CMAH* gene with 175 bp band size (99.7%). The remaining two stray cats showed heterozygous band pattern like blood type AB cat (0.12%). Overall, 175 bp band displaying type A cats are prevalent contrary to the two cats that have type AB pattern and non-existence of homozygous type B cats. These results show that the potential of neonatal isoerythrolysis in stray cats of İzmir is minimal. Future studies are required to scrutinize the reason(s) for non-existence of type B cats in İzmir and presence of unique band in blood type AB.

Keywords: *CMAH gene, Neonatal isoerythrolysis, Insertion, Cat, PCR*

Türkiye, İzmir Sokak Kedilerinde Neonatal İzoeritrolizisle İlişkili *Sitidin Monofosfat-N-Asetilnöraminik Asit Hidroksilaz (CMAH)* Geninin Analizi

Özet

Neonatal izoeritrolizis yeni doğan kedilerde hayatı tehdit eden bir hastalıktır. Bu hastalık, tip A ya da tip AB kediler tip B dişi kedilerden doğduğunda ortaya çıkmaktadır. Tip B kedilerde inaktif *sitidin monofosfat-N-asetilnöraminik asit hidroksilaz (CMAH)* enzim üretimine neden olan *CMAH* geni üzerinde homozigot 18 bp bir insersiyon saptanmıştır. Son zamanlarda, moleküler metotların tip B dişi kedilerin belirlenmesinde kullanımı yavru kedilerde neonatal izoeritrolizisin önlenmesine yardım etmektedir. Bu moleküler teknikler *CMAH* genindeki 18 baz çiftlik insersiyonun varlığını hedeflemektedir. Bu çalışma İzmir, Türkiye de sokak kedileri arasında neonatal izoeritrolizis potansiyelinin *CMAH* geninde 18 baz çiftlik insersiyonu hedefleyen PZR ile analiz edilmesini amaçlamıştır. Çalışma sırasında, 793 sokak kedisinin kan örneği incelenmiştir. A, B ve AB kan tipine sahip olduğu bilinen üç kedi PZR sırasında kontrol olarak kullanılmıştır. Elde edilen sonuca göre, kontrol grubu tip A kedi, homozigot tip A kediyi işaret eden 175 baz çiftlik bir ürüne sahip iken kontrol grubu tip B kedi, homozigot tip B kediyi işaret eden *CMAH* geninde 18 baz çiftlik insersiyonlu 193 baz çiftlik bir ürüne sahipti. İlginç bir şekilde, kontrol grubu tip AB kedi *CMAH* geni için heterozigot bir patern göstermiş olup burada üç farklı bant (tip A gibi 175 baz çiftlik, tip B gibi 193 baz çiftlik ve yaklaşık olarak 240 baz çiftlik büyüklüğünde üçüncü yeni bir bant) saptanmıştır. İzmir'de 793 sokak kedisi arasında, 791'i 175 bç'li *CMAH* geni için homozigot olduğu (%99.7) ve geriye kalan iki kedinin de tip AB kedi gibi heterozigot bant paterni gösterdiği saptanmıştır (%0.12). Sonuç olarak, tip A kedileri işaret eden 175 baz çiftlik bant, tip AB paterne sahip iki kediye ve homozigot olarak saptanmamış tip B kedilere göre sık görülmektedir. Bu sonuçlar İzmir sokak kedilerinde neonatal izoeritrolizis potansiyelinin düşük olduğunu göstermektedir. Gelecek çalışmalarda İzmir'de tip B kedilerin olmaması sebepleri ve tip AB kedide saptanan yeni bant varlığının incelenmesinin uygun olacağı düşünülmektedir.

Anahtar sözcükler: *CMAH gen, neonatal izoeritrolizis, insersiyon, kedi, PZR*

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INTRODUCTION

Cytidine monophosphate-N-acetylneuraminic acid hydroxylase (CMAH) gene encodes an enzyme called cytidine monophosphate-N-acetylneuraminic acid hydroxylase. CMAH enzyme produces N-glycolylneuraminic acid (Neu5Gc) derived from N-acetylneuraminic acid (Neu5Ac) through enzymatic hydroxylation [1]. Neu5Gc and Neu5Ac are two of the most abundant sialic acids and both of them are expressed in echinoderm to mammalians [1-4]. These sialic acids have significant biologic roles in cellular recognition, signaling, neuronal development, and host-pathogen interaction in vertebrates, including humans [5-9].

There are many studies to determine the types of sialic acid in different species. According to data obtained from these studies, Neu5Gc is expressed in various mammalians except humans due to a 92 bp deletion causing a mutation in the coding region of single-copy *CMAH* gene which is located in telomeric region on chromosome 6 [10-12]. In humans, the *CMAH* mutation which occurred ~100.000 years ago is thought to arise against some lethal microbial pathogen during an evolutionary selection process [6]. In addition, the loss of functionality in *CMAH* gene in humans resulted with a biochemical difference which is detected nearly on the surface of every cell between humans and great apes [13]. Also, the *CMAH* mutation in human causes the presence of precursor Neu5Ac at high levels [10]. Unlike human, *CMAH* gene has been conserved and is active in species as primitive as echinoderms. Moreover, *CMAH* gene exhibits highly similarity among mammals [14].

From starfish to human, *CMAH* gene is analyzed by molecular techniques to find out the mutations or polymorphisms. For examples, 18 different haplotypes were detected in humans originated from Africa and non-Africa depending on intronic region of *CMAH* pseudogene [15].

In cats, *CMAH* gene is located on B2 chromosome corresponding to human chromosome 6 and related to cat blood types. The major blood types present in cats are type A and B. Among these blood types, different sialic acid residues are found; while type A cats have Neu5Gc, type B cats have Neu5Ac. Also, type B cats possess strong agglutinins against type A cats. A rare blood type in cats is type AB which has both Ne5Ac and Neu5Gc in similar quantities [14].

Lately, genetic mechanism of cat's blood group system has been investigated. According to result of that study, CMAH enzyme encoded by *CMAH* gene converts Neu5Ac to Neu5Gc and thus, different blood group arise in cats. However, a mutation [18 bp insertion = *(AACGAGCAACC GAAGCTG)*] located in *CMAH* gene has been detected in type B cats and it has been stated that the mutation led to production of inactive CMAH enzyme. Because of this, CMAH enzyme can no longer convert Neu5Ac to Neu5Gc [14].

There is a life threatening disease in new born cats called neonatal isoerythrolysis. It occurs when type A or type AB kittens are born from a type B queen (female cat). Currently, molecular methods are being used to determine type B blood in cats, which can help prevent neonatal isoerythrolysis in kittens [16]. These molecular assays target the presence of 18 bp insertion in *CMAH* gene. There is limited data about the prevalence of 18 bp insertion in *CMAH* gene in cats worldwide. In this study, we aimed to detect the prevalence of 18 bp insertion in *CMAH* gene among stray cats of İzmir, Turkey using polymerase chain reaction (PCR). Therefore, risk of neonatal isoerythrolysis for stray cats would be determined depending on prevalence of 18 bp insertion. For this purpose, we analyzed 793 stray cats' blood sample for the presence of 18 bp insertion in *CMAH* gene.

MATERIAL and METHODS

Cats and Sample Collection

Stray cat samples (n:793) were provided from the Veterinary Clinic, Municipality of İzmir, Turkey. The stray cats were brought to Veterinary Clinic from 13 different counties of İzmir [Balçova (n:97), Bayraklı (n:31), Konak (n:452), Buca (n:37), Bornova (n:27), *Çiğli* (n:5), Gaziemir (n:2), Güzelbahçe (n:20), Torbalı (n:1), Narlıdere (n:4), Seferihisar (n:2), Karabağlar (n:44) and Karşıyaka (n:71)]. Blood samples obtained from stray cats were collected in EDTA-coated tubes. All experiments were performed under the instructions and approval of the Institutional Animal Care and Use Committee (IACUC) of Ege University for animal ethical norms (Approval number: 2015-056). The RapidVet-H IC Feline Immuno-Chromatographic Test (Rapidvet, DMS laboratories) for identifying feline A, B and AB blood type have been used according to the manufacturer's protocol. Blood samples of three cats with blood type A, B and AB as determined by the kit were used as positive control.

DNA Extraction and PCR Analysis

DNA isolation from blood samples of stray cats was performed with the QIAamp DNA mini kit according to the manufacturer's protocol (Qiagen).

Conventional PCR targeting the *CMAH* gene (GenBank no. EF127686.1) of stray cats was performed as described with some modification [14]. To detect *CMAH* gene containing 18 bp insertion or without insertion, the following primers were used: Cat *CMAH* F (5'-*ACACAG CAGAGGAAGTGGTG*-3') and Cat *CMAH* R (5'-*CATTGGG TCTGGAGGAACCC*-3'). The PCR amplified a 193 bp product in *CMAH* gene with 18 bp insertion while 175 bp product in *CMAH* gene without 18 bp insertion.

The 30 µl amplification reactions included 2 µl template DNA, the primers (0.66 µM each), 0.375 U Thermo Scientific

Taq DNA Polymerase (Thermo Scientific), 0.016 µM dNTPs, 0.175 mM $MgCl_2$ and 1× Taq reaction buffer. The PCR amplification reaction was performed using the following calculated-control protocol: 2 min initial denaturation step at 95°C, followed by 35 cycles of 1 min at 95°C, 45 sec at 58°C, and 45 sec at 72°C, and a final extension of 10 min at 72°C. Three cats' DNA samples, known to have blood types A, B and AB, were used as PCR control. All PCR products were separated by 3% agarose gel electrophoresis, stained by ethidium bromide and visualized under DNR Bio-imaging systems.

RESULTS

Among the control groups, blood type A control cat displayed a 175 bp product indicating a homozygous type A cat while blood type control B cat showed a 193 bp product in *CMAH* gene (with 18 bp insertion) indicating a homozygous type B cat. Interestingly, blood type AB control cat showed a heterozygous pattern for *CMAH* gene, in which three different bands (175 bp like that of type A, 193 bp product for type B, and the third *unique* band with approximately 240 bp size) were detected. Among 793 stray cats of İzmir, 791 were homozygous for *CMAH* gene with 175 bp band size (99.7%). The remaining two stray cats showed heterozygous band pattern like blood type AB cat (0.12%) *(Fig. 1)*.

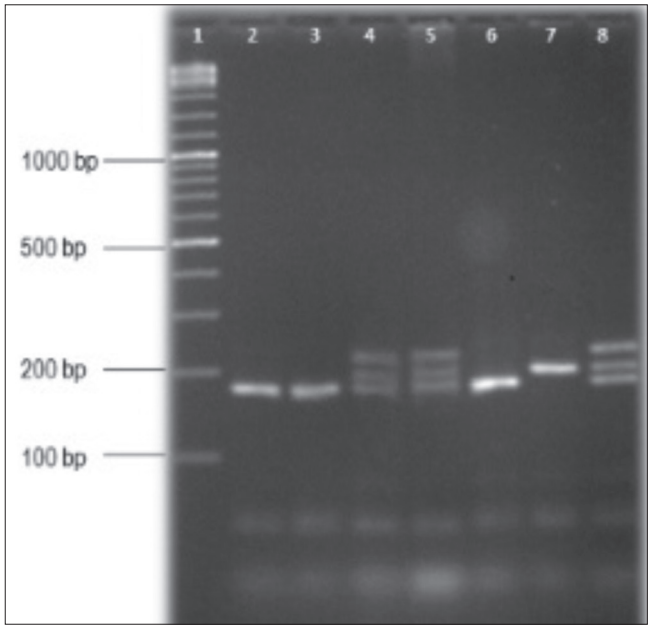

Fig 1. The PCR products obtained from control groups and analyzed samples. **Line 1:** DNA ladder; **Lines 2-5:** Analyzed samples for *CMAH* gene, line 2 and 3 show homozygote pattern while line 4 and 5 show heterozygous pattern; **Lines 6-8:** Control groups containing three cats' blood samples, known to have blood types A, B and AB, respectively

Şekil 1. Kontrol gruplarından ve analiz edilen örneklerden elde edilen PZR sonuçları. **Sıra 1:** DNA merdiveni; **Sıra 2-5:** *CMAH* geni için analiz edilen örnekler, sıra 2 ve 3 homozigot paterni gösterirken sıra 4 ve 5 heterozigot paterni göstermektedir; **Sıra 6-8:** Kan grubunun sırasıyla, A, B ve AB olduğu bilinen üç kedi kanından oluşan kontrol grubları

Table 2. *Evaluation of PCR results*
Tablo 2. *PZR sonuçlarının değerlendirilmesi*

PCR Product Size	Test Result	Evaluation
193 bp	b/b	Type B
175 bp like that of type A, 193 bp product for type B, and unique band with approximately 240 bp	b/N	Type A or AB with b allele
175 bp	N/N	Type A or AB

b/b: *a cat with two copies of b allele;* ***b/N:*** *one b allele carrier cat;* ***N/N:*** *a cat without any b allele*

In addition, blood type A control cat displaying a 175 bp product was detected in all counties of İzmir. Two cats with three different bands were found in only Konak and Bornova counties of İzmir *(Table 1)*.

DISCUSSION

Cat blood group is encoded by unique gene called as *CMAH* gene which is related to production of sialic acid present in cat red cells. CMAH enzyme encoded by *CMAH* gene converts Neu5Ac to Neu5Gc in various mammals, including cats. However, CMAH enzyme is not active in type B cats because this group has homozygous 18 bp insertion on *CMAH* gen which causes production of non-functional CMAH enzyme. In addition to 18 bp insertion, 5 more polymorphisms (A-217G, C-371T, G139A, T265A, G1600A) were detected in homozygous form in all type B cats [14]. Although these polymorphisms and 18 bp insertion were determined in heterozygous form in some type A cats, any homozygous polymorphism or 18 bp insertion was not found in type A cats [14].

18 bp insertion on *CMAH* gene is not very good marker to identify blood type in cats since this marker cannot differentiate between type A and type AB cat. However, it can be used to predict blood groups and to detect the type B or b allele carrier cat without blood sample collections [14] *(Table 2)*.

In a previous study, G139A and C136T SNPs in *CMAH* gene were used to predict blood type in cats and results of SNPs were compared to those of standard blood type microplate agglutination method. Coherence rate of these two assays was found to be 96%. Among these SNPs, homozygous G139A polymorphisms were detected in 10 of 14 type B cats. Also, any SNP was not determined in 59 type A cats [16].

Except of molecular approach, several tests are used for blood typing in cats. Among them, serological techniques such as card agglutination (CARD), immunochromatographic cartridge (CHROM), gel-based (GEL), and conventional slide (SLIDE) and tube (TUBE) agglutination assays are generally used in blood typing in cats. These assays are applied to determine cat blood phenotype [16].

Table 1. *Geographic distribution of CMAH alleles (with insertion, without insertion and unique allele) and CMAH gene status according to PCR results*
Tablo 1. *CMAH allellerinin (insersiyonlu, insersiyonsuz ve yeni allel) coğrafik dağılımı ve PZR sonuçlarına göre CMAH gen durumu*

Counties of İzmir (Number of Analyzed Cats)	***CMAH* Gene without insertion**	***CMAH* Gene with insertion**	**Unique *CMAH* Allele**	***CMAH* Gene Status**
Konak (n:451)	+	-	-	Homozygous
Konak (n:1)	+	+	+	Heterozygous
Balçova (n:97)	+	-	-	Homozygous
Karşıyaka *(n:71)*	+	-	-	Homozygous
Karabağlar (n:44)	+	-	-	Homozygous
Buca (n:37)	+	-	-	Homozygous
Bayraklı (n:31)	+	-	-	Homozygous
Bornova (n:26)	+	-	-	Homozygous
Bornova (n:1)	+	+	+	Heterozygous
Güzelbahçe (n:20)	+	-	-	Homozygous
Çiğli (n:5)	+	-	-	Homozygous
Narlıdere (n:4)	+	-	-	Homozygous
Gaziemir (n:2)	+	-	-	Homozygous
Seferihisar (n:2)	+	-	-	Homozygous
Torbalı (n:1)	+	-	-	Homozygous

Determination of blood types in cats is important since neonatal isoerythrolysis can be prevented in kittens. Blood types of cats vary depending on the breed of the cat or geographic location. Blood type A among cats has the highest prevalence (85-100%) worldwide. In some regions, the prevalence of type B cats increases such as Greece, England, Australia, and Turkey [17].

In eastern part of Turkey, of the 85 Van cats analyzed, 40% had type A, and 60% had type B blood. In addition, cats from central Anatolia, called Angora were analyzed and among them, 53.6% had type A and 46.4% had type B blood. Type AB cats were not found in both breeds [18]. In another study, of 301 cats typed from four distinct regions of Turkey, 220 had type A blood, 74 had type B and seven had type AB [19].

Currently, determination of blood types by molecular methods has become popular which can help prevent neonatal isoerythrolysis in kittens before breeding program [16]. Our results showed that among control groups, blood type A control cat displayed a 175 bp product indicating a homozygous type A cat while blood type control B cat showed a 193 bp product in *CMAH* gene (with 18 bp insertion) indicating a homozygous type B cat. Interestingly, blood type AB control cat showed a heterozygous pattern for *CMAH* gene, in which three different bands were detected *(Fig. 1)*. Among 793 stray cats of İzmir, 791 were homozygous for *CMAH* gene with 175 bp band size (99.7%). The remaining two stray cats showed heterozygous band pattern like blood type AB cat (0.12%). Type B cats were not detected.

Overall, 175 bp band displaying type A cats are prevalent contrary to the two cats that have type AB pattern and non-existence of homozygous Type B cats. Interestingly, a new allele was found for first time in this study and information about new allele is not available in literature yet. It is thought to be related with *CMAH* gene in type AB cats because primer set used in this study was specific to only *CMAH* gene in cats.

These results show that the potential of neonatal isoerythrolysis in stray cats of İzmir is minimal. Future studies are required to scrutinize the reason(s) for non-existence of type B cats in İzmir and presence of *unique* band in blood type AB.

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