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Hormone receptor type 2 antimüllerian gene role in dogs with Persistent Müllerian Ducts Syndrome

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Abstract

Persistent Müllerian Ducts Syndrome (PMDS) is a congenital disease, characterized by the presence of Müllerian duct derivatives in genetically defined males. Our aim was to identify DNA mutations that may lead to this disease in Basset Hound male dogs. Six males (one healthy control) aged 1–8 years were examined by ultrasound; exploratory laparotomy was used for PMDS confirmation in dogs detected as PMDS positive following ultrasound examination. Surgical treatment was performed for resection of uterine horns rudiments. Two dogs were found to be PMDS-positive. Possible DNA mutations in the 11 exons of the hormone receptor type 2 antimüllerian (AMHR2) gene were analyzed by polymerase chain reaction (PCR) followed by DNA sequencing. All exons of AMHR2 gene appeared wild-type, unmodified, suggesting that AMHR2 gene mutations may not be present in Basses Hound male dogs with PMDS. These findings suggest that another gene mutation (e.g. AMH) may be involved in PMDS occurrence in Basset Hound breed and we are currently investigating this alternative direction.

Keywords: AMHR2 gene, *mutation*, Basset Hound, *DNA*, PCR

1. Introduction

Persistent Müllerian Ducts Syndrome (PMDS) is a congenital disease, characterized by the presence of a complete/incomplete uterus and/or anterior part of vagina in genetically defined males. It is well known that the expression of *Müllerian inhibiting substance (MIS)* (also known as *anti-Müllerian hormone [AMH]* gene) and the normal function of its receptors is critical for the development of male internal reproductive tract during embryonic differentiation (X. WU & al. [1]). The PMDS pathologic mechanism consists in Müllerian duct derivatives that develop in male carriers of mutations in both copies of one of the genes presumed to cause PMDS syndrome, primarily the AMH gene and/or the gene responsible for encoding the *hormone receptor type 2 antimüllerian (AMHR2)* (X. WU & al. [1]).

The majority of positive PMDS males have no detectable changes during routine clinical examination. PMDS affected males are fertile, and hence all their offspring are

compulsory carriers, homozygous or heterozygous. These dogs are rarely diagnosed with PMDS, not until the clinical signs associated with recurrent genitourinary infections appear. In about 50% of cases, positive PMDS males suffer from cryptorchidism, unilateral or bilateral, and the remaining testicle can develop tumors (T.T. BROWN & al. [2]; A. MATSUU & al. [3], A.R. VEGTER & al. [4]).

Currently, PMDS diagnosis is based on ultrasound technique, but not in all cases the Müllerian duct derivatives can be identified due to their small sizes or position. Exploratory laparotomy may also be used, but, from our experience, this invasive method is usually declined by breeders or accepted at a late stage, when clinical signs appear. At that stage, the possible PMDS-affected fertile male dogs may have already been used for reproductive purpose. Given this, accessible and non-invasive diagnosis methods that can be used at any age should be developed. To overcome this challenge, we are currently screening for gene mutations that may be responsible for PMDS occurrence so we can further develop DNA-based tests that can identify the gene carrier-dogs.

The aim of this study was to screen all exons of the *AMHR2* gene for possible mutation(s) that could lead to PMDS in Basset Hound male dogs.

2. Materials and Methods

Animals. The study was conducted on 6 male dogs aged between 1 and 8 years, during June 2014 and December 2015 time period. Five of these were Basset Hound breed and one common breed; the latter was used as control. The study protocol was reviewed and approved by The Ethical Committee of the University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca.

Diagnosis. PMDS diagnosis was done by using ultrasound examination (method previously described (A.R. POP & al. [5]) and confirmed by exploratory laparotomy performed via ventral midline, side of the vas deferens. The classical surgical approach was used for the exploratory laparotomy, in dogs that were find positive following ultrasound examination. If uterine horns rudiments were identified, surgical treatment was performed.

DNA extraction. Blood samples were collected from each dog. DNA was isolated from 1 m*l* of blood by using the DNeasy Blood & Tissue Kit (QIAGEN INC, 27220 Turnberry Ln Valencia, Santa Clarita, California 91355, U.S.A.) according to the manufacturer's instructions. DNA concentration was measured by spectrophotometry and DNA was stored at 4°C in a cold room, indefinitely.

Primers design. The primers designed for exon amplification for the *AMHR2* gene are shown in Table 1. The *AMHR2* gene has 11 exons in dogs. Depending on the gene region, for either individual exons, or sometimes for pairs of exons, we designed forward (F) and reverse (R) primers, as well as at least one nested primer, to allow re-amplification of clean products in case the first polymerase chain reaction (PCR) yielded multiple products, some likely nonspecific. Primers were simply designed to be positioned at least 50 nucleotides away from each exon, to be c/a 22-30 nucleotides long, and to have as much as possible a proportionate mix of purines and pyrimidines towards the 3' end.

Table 1. Sequences of the DNA primers used to evaluate *AMHR2* gene exons in Basset Hound male dogs

Exon (ex)	Primer name	DNA sequences
ex1	dAMR1F	CAGGGCAGCAGTGCTGGTTCATGCT
	dAMR1R2	CAAAGGGGTGGCTGCTGCTTCAAGTC
	dAMR1R1	TCATTCTTCCAAAGGGGTGGCTGCT
ex2	dAMR2F1	GGCTGCTGCTTTTGCCCCTGCATTCA
	dAMR2F2	TTGCCCCTGCATTCATTCCTATCTTGA
	dAMR2R1	TGTCACCCCCACCCCAGGATGTACCTGT
ex3/4	dAMR34F1	GGTTATCCTCTCTGTTCCTAGACCCAGT
	dAMR34F2	TGTTCCTAGACCCAGTTATGCTCGCTC
	dAMR34R1	GAGCTTTGGGGCAGTGACTTGGGGTCA
	dAMR4F1	CTCTGAGGAAGGGGAGAGGAACAGAGT
	dAMR3R1	AGGGTGAGAACCAGCCTTGGTTCT
	dAMR34R2	GTCAGGGGAAGCTGGAGAGGAGTCA
	dAMR34F3	CTTTCTCTCTTCTAATCCCACCCTATCA
ex5/6	dAMR56F1	TAGAGCCTGAGCCCACTGCCTCCACA
	dAMR56F2	CTGCCTCCACAAAGCCCCTTTCCCAGTC
	dAMR56R1	CCACATACACACACAGGCAAATGCAT
ex7/8	dAMR78F1	TGATCTCTGCACCTTGGGGTGGATCAG
	dAMR78R1	ACCGCCATGATCCCAGGCCTCA
	dAMR2ex7F	GTGGATCAGCCACCTCCAGGTTATGTGT
ex9/10	dAMR910F1	GGGACGTCGCATGGACCAGTGTTGT
	dAMR910F2	ATGGACCAGTGTTGTGGTGAGGCTTG
	dAMR910R1	TTGGGCTGGTCCCAGTAGACAAGTC
ex11	dAMR11F1	TAGTGGTGGACACCTCTCAACTCT
	dAMR11R1	AGCACCGAGCATCCGACCACCAGAT
	dAMR2ex11R	CCAGATTTATGTTTGCACGAACATAGA

PCR amplification. For PCR amplification of all exons, we used "Taq polymerase" enzyme from New England Biolabs (NEB, 240 County Rd Ipswich, Massachusetts 01938, U.S.A.). The enzyme was supplied with 10X Buffer (buffer) for reactions. Each PCR reaction had a volume of 10 μl. In every reaction we used c/a 10-50 ng dog genomic DNA. In each

reaction, primers were used at a final concentration of 0.2-0.4 μM (Table 2). Compared to the manufacturer's instructions, we have modified the protocol (Table 3) by introducing a small amount of SYBR Green I (INVITROGEN, 3175 Staley Rd, Grand Island, New York 14072, U.S.A.) at a final dilution of 1:80000 from the concentration at which it was supplied by the manufacturer. This reagent allows the PCR reaction to be placed in real time PCR machine (real-time PCR) and allows monitoring the product reaction in each amplification cycle. For real time PCR we used a CFX96 apparatus (BIORAD, 1000 Alfred Nobel Drive, Hercules, California 94547, U.S.A). At the end of the reaction, the device was programmed to follow a melting curve of the DNA, in order to detect whether one or more PCR products were synthesized. If more products were synthesized, then a subsequent nested PCR reaction (internalized PCR) was used. This approach allows product selection and specific product DNA amplification, without the need to visualize DNA products on agarose gels.

Table 2. Exons PCR amplification

Ingredients	**Volume**	**Final concentration**
10X Standard Taq Reaction	5μl	1x
10 mM dNTPs	1μl	1x 200μM
10 μM Forward Primer	1μl	0.2μM
10 μM Forward Primer	1μl	0.2μM
Taq DNA Polymerase	0.5μl	2.5U
Template DNA	0.5μl	10-50 ng/reaction 10μl
SYBR Green (1:800dil)	0.5μl	1:80000 dilution
Nuclease-Free Water	var.	
to 50μl = 5 reactions		

Table 3. PCR protocol

Steps	**Temperature**	**Time**
Initial denaturation	94°C	30 sec
30 cycles	94°C	12 sec
	50°C	15 sec
Final extension	70°C	1 min
	70°C	1 min

Sequencing. The PCR products were submitted to a sequencing facility, and sequenced using the Sanger DNA sequencing method. Sequencing data were analyzed and compared using the Sequencer 5.0 software (GENE CODES CORP, 775 Technology Dr, Ann Arbor, Michigan 48108, U.S.A.).

3. Results and discussion

Out of the 6 dogs examined by ultrasound, 2 were PMDS affected males (B6 and C6). In case B6 [7-year-old], physical examination revealed no pathologic clinical signs and the overall general condition seemed to be normal with no feminization characteristics. Results of the ultrasound examination were previously published (A.R. POP & al. [5]). Case C6 [3-year-old] had male-specific external genitalia, but was diagnosed with PMDS following ultrasound examination (not shown). For both cases, the diagnosis was confirmed by exploratory laparotomy (Figure 1). 3 dogs were unaffected or possible heterozygous males with no identified persistent ducts (A6 [3-year-old], D6 [1-year-old], F6 [4-year-old]) and were normal at clinical and ultrasound examination and one dog (E6 [2-year-old]) was the healthy control.

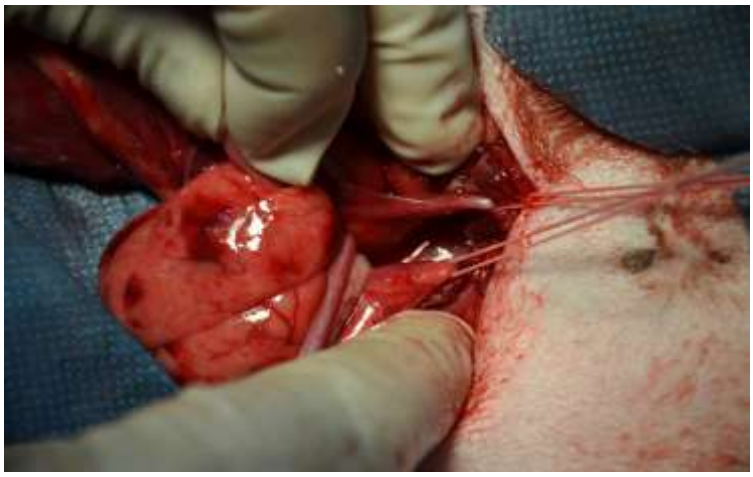

Figure 1. PMDS affected male (B6) with the rudiments of uterine horns
At exploratory laparoscopy, on the medial part of the vas deferens a structure similar to the uterus was identified. This uterus-like structure had the uterine body and the rudiments of uterine horns.

Following PCR amplification and DNA sequencing, the 11 exons of the *AMHR2* gene did not show mutations in the affected males.

PMDS is an important syndrome to consider, as it increases the risk of uterine diseases in affected males, more frequently in older dogs than in younger ones (A.R. POP & al. [6]).

In case of PMDS diagnosed males from any dog breed, they or their relatives have already produced a large number of offspring, thereby contributing to the spread of the disease in populations of that breed (A.R. POP & al. [6]). Their sperm count is usually normal even if alteration of the seminal plasma composition may be encountered (Z. TRUTA & al. [7]). Considering the implication this syndrome has on the health of male dogs, and the impact it has on their breeding, there were other studies reported in the literature, that were interested in screening for mutations in a variety of genes. For example, it has been previously shown that a 10-year-old Miniature Schnauzer with bilateral cryptorchidism and diagnosed with bilateral Sertoli cell tumor with hydrometra was *sex-determining region Y (Sry)* gene positive (A. MATSUU & al. [3]). However, other studies conducted in Basset Hound male dogs showed that the mutation causing PMDS in this breed was not in the *Sry* gene, suggesting that another gene may be affected (A.R. POP & al. [6]).

A study conducted by Wu X. et al. (2009) in a canine Miniature Schnauzer model diagnosed with PMDS identified a genetic mutation of the *Müllerian inhibiting substance*

type II receptor gene (*MISRII*) (known as *AMHR2* gene). A single base pair substitution has been identified, that introduced a stop codon in exon 3 of this gene, suggesting that PMDS was caused by a C to T transition in exon 3 of *MISRII* (X. WU & al. [1]). Further investigations in this canine model included different genotypes such as homozygous normal dogs; heterozygous normal dogs but carriers and PMDS affected dogs, and showed that a DNA diagnostic test could be developed by introducing a *Dde*I enzyme restriction site (S. PUJAR & V.N. MEYERS-WALLEN [8]).

4. Conclusion

Compared to previously reported data, in our study performed in Basset Hound male dogs, the 11 exons of the *AMHR2* gene did not show mutations in the PMDS affected males.

These findings suggest that another gene mutation (e.g. *AMH*) may be involved in PMDS occurrence in Basset Hound breed and we are currently investigating this alternative direction. Detection of the mutated gene and development of a DNA diagnostic test in this breed is an imperative need in order to avoid breeding carrier dogs and production of other PMDS affected dogs.

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