A Female Hermaphrodite American Cocker Spaniel Dog with Sry-negative XX Sex Reversal

Hyun-Gu Kang†, Ill-Hwa Kim, Hyung-Jin Kim, Dae-Yeon Hwang[1], Seung-Wan Jee[2] and Gyu-Jin Noh[3]

College of Veterinary Medicine and Research Institute of Veterinary Medicine, Chungbuk National University
[1]Pusan National University Major of Biomaterials, School of Applied Life Science,
College of Natural Resources & Life Science, Miryang, Korea
[2]Division of Laboratory Animal Resources, National Institute of Toxicological Research, KFDA
[3]College of Veterinary Medicine, Kyeongsang National University

ABSTRACT

A 3-month-old American Cocker spaniel was presented at the Veterinary Teaching Hospital, Chungbuk National University, for examination of urinary tract after dissection of vaginal mass at local clinic before 10 days. Clinical examination of the affected bitch revealed a normal sized vulva in a normal anatomical position with a grossly enlarged clitoris, which contained an os clitoris. On examinations of the genital gland, there were testis, epididymis, ductus deferens and uterus. The histology of both gonads was primarily testis. Seminiferous tubules were divided into many parts by fibrous connective tissue. A small number of spermatogonia was present, but large numbers of Leydig's cells were existed. A normal female karyotype (78, XX) was detected in metaphase spreads obtained from cultured peripheral lymphocytes. Y chromosome specific sequences were not detected in genomic DNA by PCR. After 27 months, the os clitoris was larger than 3-month-old dog and os bone was more calcified than young age. Combining the results of cytogenetic, molecular genetic and histological examinations, the dog was diagnosed as a female hermaphrodite with Sry-negative XX sex reversal.

(Key words : Sry-negative, XX sex reversal, American Cocker spaniel)

INTRODUCTION

Normal sexual differentiation of the embryo is dependent upon the completion of 3 major serial events, the establishment of the chromosomal constitution, genital gland differentiation, and establishing the phenotypic sex. Each step dependant upon the successful completion of the previous step. An abnormality at any one of these steps can cause an abnormal sexual phenotype (Meyers-Wallen *et al.*, 1997; Meyers-Wallen, 1993; Meyers-Wallen and Patterson, 1989). The sexual differentiation begins with the establishment of the chromosomal constitution of the zygote as XX or XY at fertilization. The chromosomal sex then determines gonadal sexual differentiation. The development of the testis is initiated by the Sry gene, which is normally located on the Y chromosome. Expression of the Sry gene in the testis occurs only during a short period in the embryonic development (Koopman *et al.*, 1991). In the absence of a Y chromosome, ovarian differentiation normally occurs.

The final step in establishing the phenotypic sex is regulated by the gonads after differentiation. In the early embryo stage, the genital system is sexually undifferentiated. At this stage, both XX and XY zygotes have Müllerian and Wolffian ducts and primitive external genitalia (Meyers-Wallen, 1993). Gonadectomy of XX and XY embryos before gonadal differentiation results in the development of a female phenotype (Jost *et al.*, 1973) leading to the conclusion that the basic embryonic plan is female (Meyers-Wallen, 1993). When the testes are present, 2 hormones responsible for phenotypic masculinization are produced. These are Müllerian duct inhibiting substance (MIS) and testosterone. The first, MIS, a glycoprotein hormone produced by Sertoli's cells, causes the Müllerian duct system to regress. Testosterone, a steroid hormone produced by Leydig's cells, induced the formation of the epididymis and ductus deferens from the Wolffian duct system. In the cells of the external genitalia, testosterone is converted to dihydrotestosterone, which promotes the formation of the prostate and prostatic urethra from the urogenital sinus, the penis from the genital tubercle, and the scrotum by fusion of the genital swellings (Meyers-Wallen, 1993).

In normal females, the Müllerian duct system persists and

* Correspondence : E-mail : kang6467@cbu.ac.kr

develops into oviducts, uterus and cranial vagina and the Wolffian duct system regresses. The urogenital sinus, genital tubercle and genital swellings differentiate into the caudal vagina and vestibule, the clitoris and vulva (Meyers-Wallen, 1993).

Abnormalities in sexual development can arise from a defect at any step in sexual development, the chromosomal, gonadal or phenotypic level of differentiation (Meyers-Wallen, 1993; Meyers-Wallen and Patterson, 1989). Abnormalities of gonadal sex occur in an individual if the chromosomal and gonadal sex does not agree. For example, individuals classified as XX-reversed have a normal female chromosomal constitution, but their gonads contain testicular tissue. Affected individuals are XX males or XX true hermaphrodite. In XX males both gonads are testes, whereas in XX true hermaphrodite both testicular and ovarian tissues are present in the gonad. There is incomplete masculinization both in XX males and XX true hermaphrodites.

This report describes a case of Sry-negative XX sex reversal in an American Cocker spaniel, including the clinical, histological, radioactive, and cytogenetic examinations.

MATERIALS AND METHODS

1. Case Presentation

A 3-month-old American Cocker spaniel was presented at the Veterinary Teaching Hospital, Chungbuk National University, because the owner wanted to examine the urinary tract which was operated to dissection of about 1 cm vaginal mass at local clinic before 10 days. Physical examination of the bitch revealed normal appetite and conditions. Also, we confirmed the normal urination.

Clinical examination of the bitch revealed a normal sized vulva in a normal anatomical position with a grossly enlarged clitoris, approximately 2 cm long, which contained an os clitoris with dissected scar (Fig. 1). The vaginoscope was not inserted into the vagina. There was obstruction between vestibular and vagina. The urethral orifice was located on the baseline of the os clitoris.

The owner gave up the bitch because the bitch has not been used on the reproduction and the owner had contributed the bitch to our hospital. Since then, we have took care of the dog for 3 years and have observed the growth of the bitch and have checked the advancement condition. The bitch was run over by a car and had a hip fracture at the time of about 8-month-old. Orthopedic operation was performed to conquered

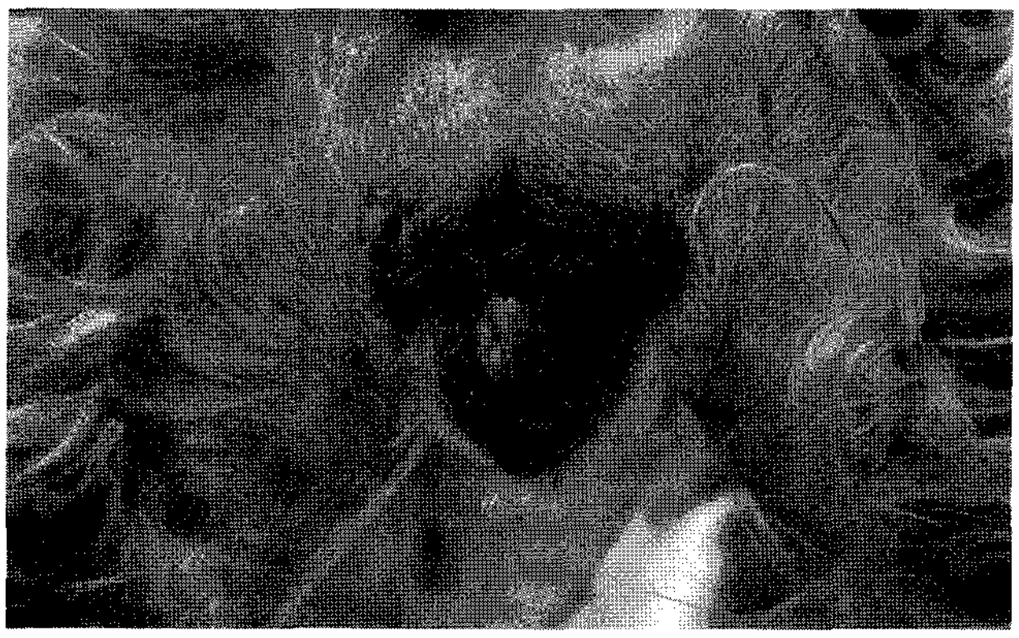

Fig. 1. Close-up view of the vulva with the protruding clitoris.

hip bone fracture and femur fracture.

2. Radiological Examination

Radiography was performed for examination of the os clitoridis's developmental condition at 3-month-old and 3-year-old.

3. Blood Collection and DNA Extraction

A heparinized blood sample from the affected bitch was collected for karyotyping as previously described (Johnson *et al.*, 2001), and a blood sample in EDTA anticoagulant was collected for genomic DNA analysis by PCR. Genomic DNA was extracted by using the method of the manufacturing company (Wizard genomic DNA Purification Kit, Promega and USA). First, after moving 3 ml whole blood to the centrifugation tube, 9 ml cell lysis solution was added, shaken 5~6 times, and centrifuged for 10 minutes from 2,000 rpm. The supernatant was discarded, the pellet resuspended in 9 ml cell lysis solution and centrifuged as previously described. The supernatant was thoroughly discarded without disturbing the pellet. The pellet was then resuspended in 3 ml nuclei lysis solution. And then, RNase solution was added and cultivated for 15 minutes, 37℃ to remove RNA in the WBC. In order to precipitate a protein, it was separated by centrifuge for 10 minutes, 2,000 rpm. The supernatant was taken, added isopropanol and centrifuged for 10 minutes from 2,000 rpm to get genomic DNA.

4. Chromosomal Karyotype Analysis

Karyotyping was performed by modified process of the Johnson *et al.* (2001). Blood sample was taken with pre-filled heparin syringe. It was then cultivated in RPMI 1640 with 10% fetal bovine serum. At 71 hours after culturing, Colcemid (Gib-

co BRL, Indianapolis, Ind) was added to a final concentration of 0.05 μg/ml and incubated for 45 minutes to arrest in metaphase. It was then fixed during metaphase, treated with 0.075 M KCl for 35 minutes and fixed in solution with 3:1 rate (methanol:acetic acid) for 2 hours. To analyse a general karyotype, metaphase was spreaded on clean slides. It was then dried and stained by 4% Giemsa solution. The chromosome was observed with the optical microscope.

5. PCR Analysis

PCR analysis was used the method of Meyers-Wallen *et al.* (1995). By using the Genomic DNA obtained from the blood (patients and normal male, female), testis, ductus deferens, uterine horn, and vagina. Existence of Sry gene could be analyzed with 25 cycles of PCR. PCR amplification was performed with following primers: 10 pmoles Sry-specific primer (sense: 5'-TGG TGT GGT CTC GCG ATC AAA G-3' antisense: 5'-CTG CGC CTC CTC GAA GAA TGG-3') and 1~2 μg genomic DNA, 2 mM dNTP, 10x buffer, 1 unit Taq polymerase in a condition of 94 (30 sec), 58 (60 sec), 72 (45 sec).

PCR products were analyzed by gel electrophoresis on 1.2% TAE agarose gel, stained with ethidium bromide and visualized under ultraviolet light. Also, in order to confirm gene quantity to be identical, beta-actin specific primer (Sense primer: 5'-TGGAA TCCTG TGGCA TCCAT GAAAC-3' antisense primer is 5'-TAAAA CGCAG CTCAG TAACA GTCCG-3') from the condition which is identical to analyze Sry, was used and produced a reaction.

6. Ovariohysterctomy

We performed ovariohysterectomy when the patient was 3 years old. We collected some reproductive organs (testicle, epididymis, ductus deferens and uterus), and we got several tissue samples from them. Some of the selected samples were fixed with 10% formalin. And thin-sectioned (4 μm) and stained with haematoxylin-eosin for histological examination. Others of them were stored in the liquid nitrogen container with them wrapped with cooking foil for the genomic DNA test. We extracted the genomic DNA from the same samples to determine gonadal sex using the PCR method as described above.

RESULTS

1. Physical Examination and Radiological Findings

We performed vaginoscopy to investigate the developmental status of reproductive organs. We had problem with entering the origin of clitoris, and found out obstruction of the atrium vagina. Urethral orifice was located ventrally to the origin of os clitoris. On presenting the hospital, we took some radiological tests and found that there was a bony structure which the end of os clitoris was cut sharply (Fig. 2b). When the dog was radiographs at 3 years old, os clitoris was more calcificated and enlarged than that of 3-month-old.

2. Karyotyping and Sry DNA Analysis

In the karyotyping using the metaphase stage of chromosome of peripheral lymphocyte, it turned out that the chromosome was normal female karyotype (78, XX) (Fig. 3). In the PCR reaction using canine specific primer, Sry gene, we showed that only the gene DNA template of the normal male control dog had Sry product, but normal female dog and the patient didn't have Sry product in blood, testicle, ductus deferens, uterus and vagina (Fig. 4). This means that the dog didn't have Y-specific gene.

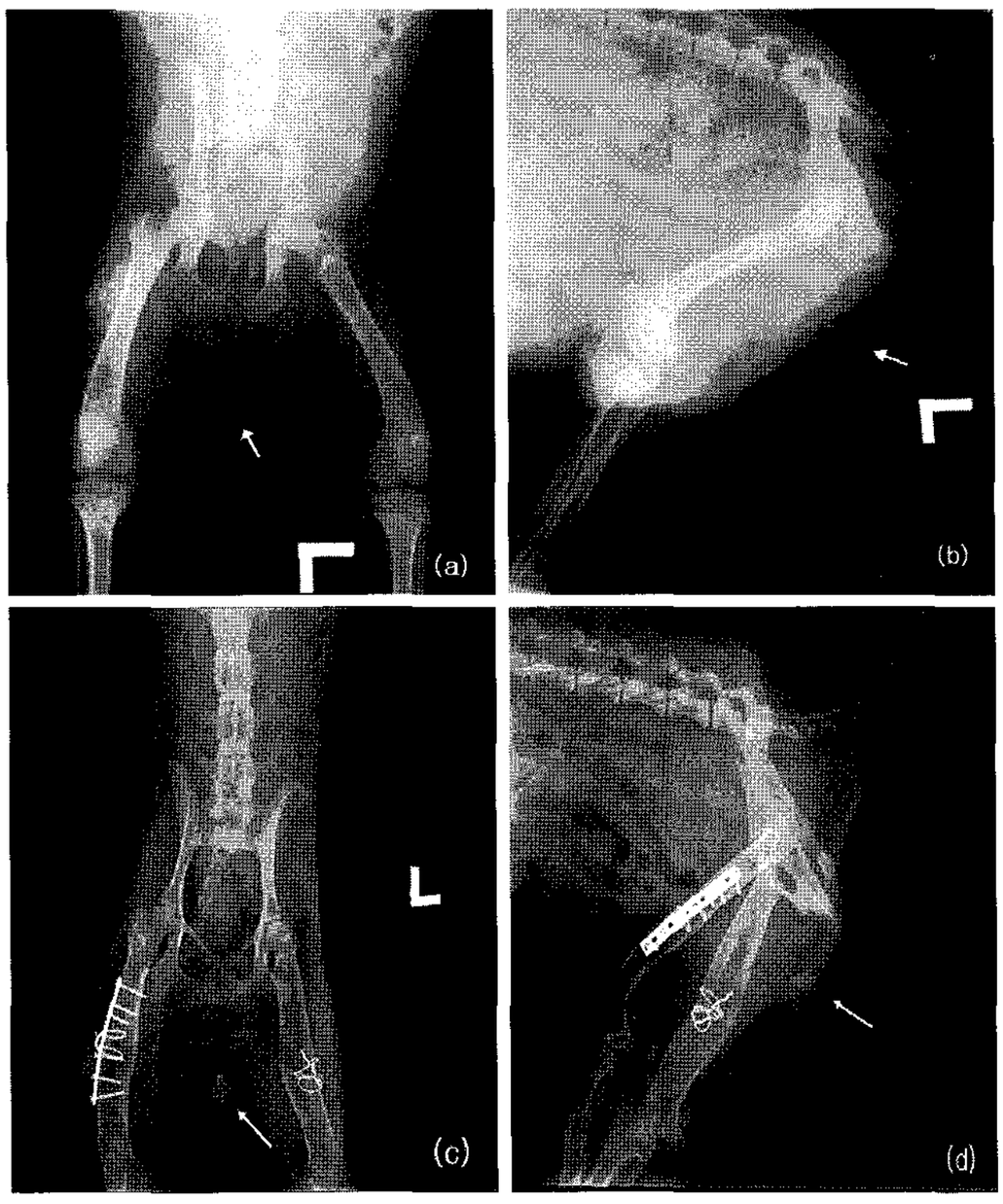

Fig. 2. Radiograph of os clitoris of a American Cocker spaniel. Ventrodorsal (a) and lateral, (b) radiographs at 3-month-old. Ventrodorsal (c) and lateral (d) radiographs at 3-year-old. Os clitoris was more calcificated and enlarged than that of 3-month-old.

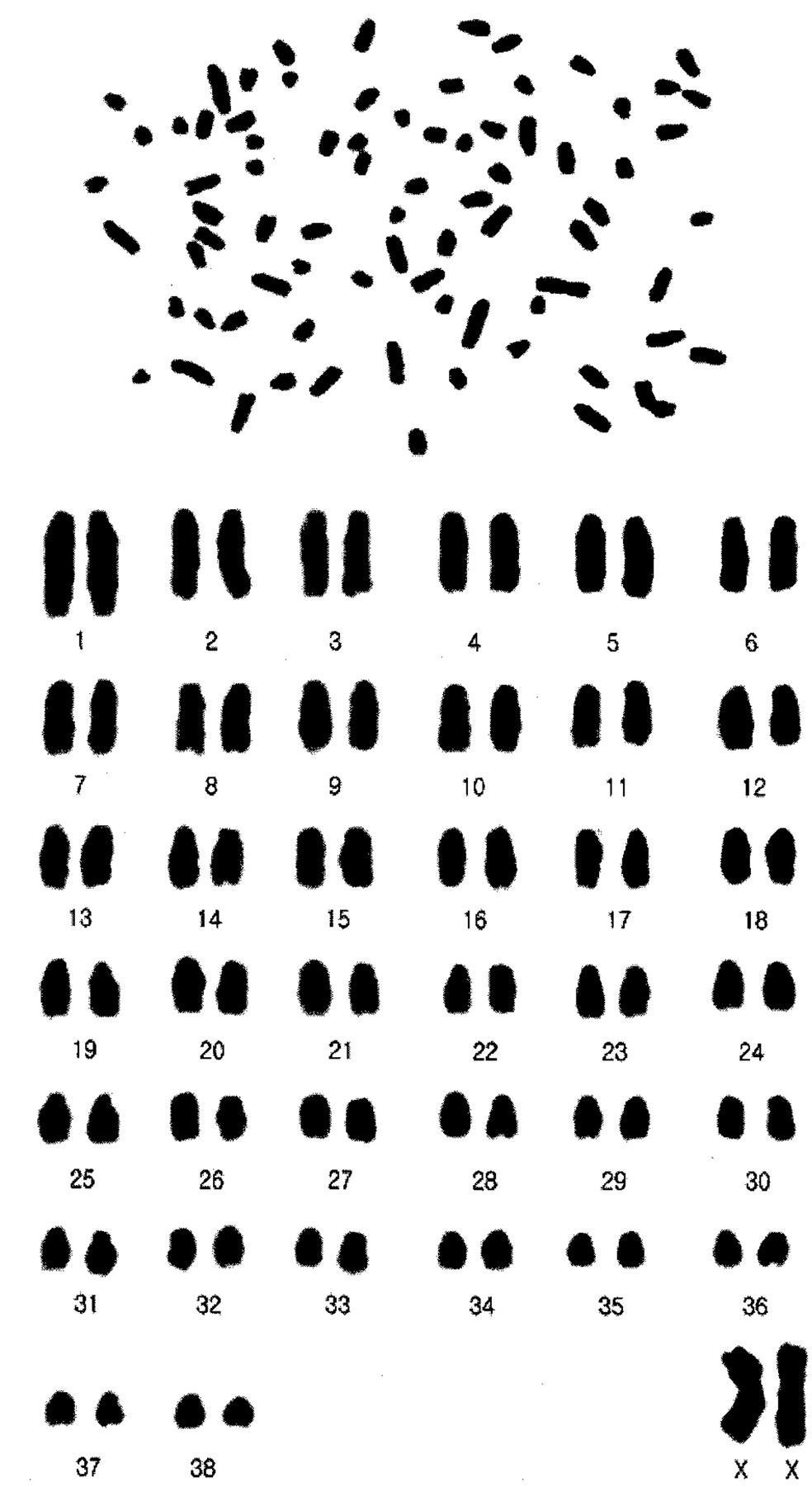

Fig. 3. Metaphase spread of a American Cocker spaniel (78, XX) obtained from cultured peripheral lymphocytes.

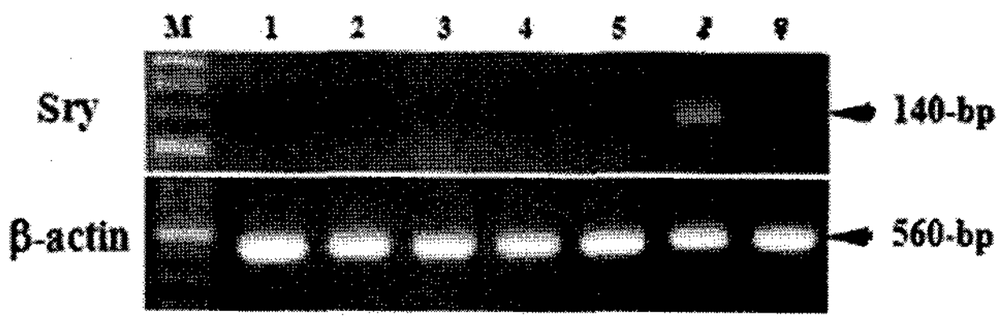

Fig. 4. Expression of Y-chromosome specific sequences of B-actin and Sry detected by PCR in the urogenital organs of a Sry-negative XX sex reversal dog. M: marker, Lane 1: lymphocyte, Lane 2: testis, Lane 3: ductus deference, Lane 4: uterine horn, Lane 5: vagina, ♂: lymphocyte from clinically normal male dog and ♀: lymphocyte from clinically normal female dog.

3. Macroscopic Observation of Reproductive Organs

In the result of this patient dog, we diagnosed as a female hermaphrodite with XX sex reversal and performed extirpation of os clitoris and reproductive organs in the abdomen. We were able to remove the part of os clitoris because its origin has urethral orifice. After celiotomy (Fig. 5), two testes were located in the region which ovary should be, and the size of the testis was smaller relatively than the thing of 3-month-old. The epididymis existed in the normal location which exists in the testis. Ductus deferens to travel parallelly in the both uterine horn's lateral sides was observed.

4. Histological Observation of Reproductive Organs

The histology of both gonads was similar, being primarily testis. The histological appearance of the ductus deferens showed that the developmental status of it was weak because the ductus deferens was divided into many mediastina by some fibrillar connective tissues. Large numbers of interstitial cells were present. Ductus deferens whose basement membrane had been broken was observed in the form of various size of primordium. The ductus deferens was constituted with only monolayer type. Small number of spermatogonias was arranged irregularly, and there were few Sertoli's cells (Fig. 6a). The lamina propria was thin compared with normal one (Fig. 6b). The histological view of epididymis and uterus was normal (Fig. 6c and d).

DISCUSSION

The XX sex reversal is defined as an animal containing an XX sex complement with female type gonadal ducts, a viri-

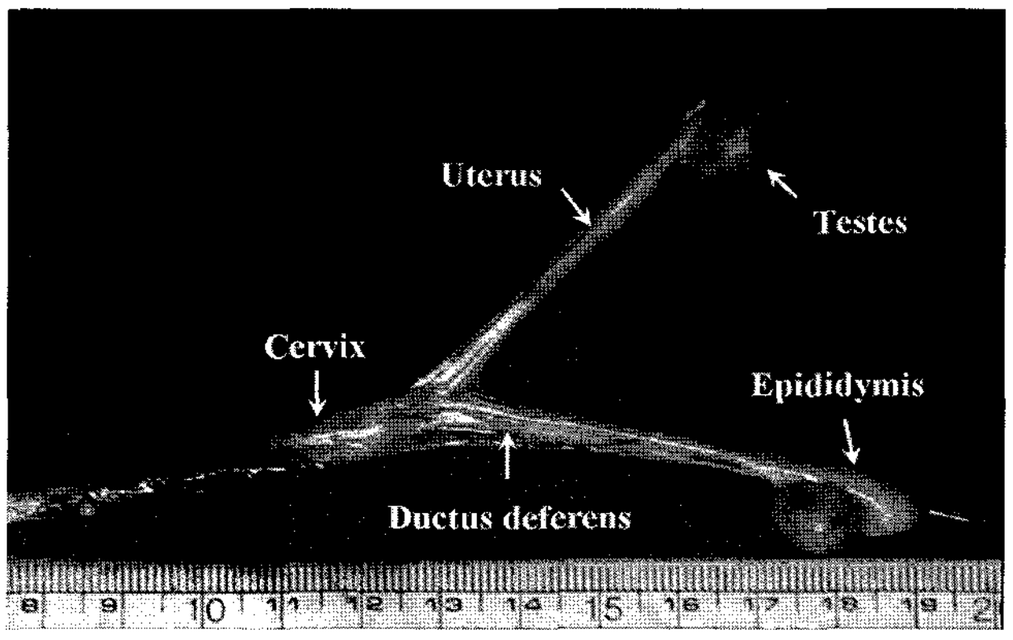

Fig. 5. Gross appearance of genital organs of a American Cocker spaniel at 3 years old.

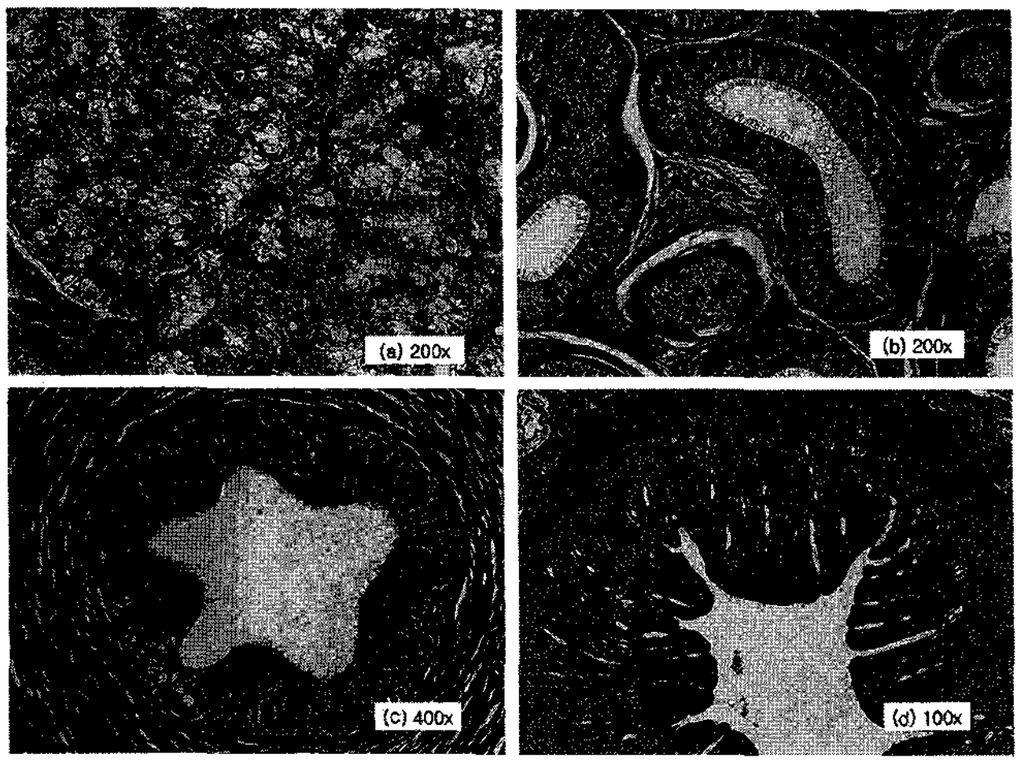

Fig. 6. Histological sections of urogenital organs. (a) testis (200×), Seminiferous tubules were divided into many parts by fibrous connective tissue. (b) testis (400×), A small number of spermatogonia was present, but large numbers of Leydig's cells were existed. (c) epididymis (200×), Normal. (d) ductus deferens (400×), Lamina propria was not shown between epithelium and inner longitudinal muscle layer. (e) uterus (100×), Normal.

lized urogenital sinus and external genitalia (Rosnina *et al.*, 2000; Meyers-Wallen, 1999). These affected animals were shown to have testicular tissue in gonadal histology and were observed 78, XX karyotype in metaphase spread prepared from cultured peripheral lymphocyte. The XX true hermaphrodites or XX male was revealed male gonads (Jonston *et al.*, 2001). The PCR technique to detect the Y-chromosome-specific sequence enables accurate determination of chromosomal sex (Kuiper *et al.*, 2005; Weng *et al.*, 2005; Xiao *et al.*, 1995; Ennis and Gallagher, 1994; Meyers-Wallen, 1990; Sinclair *et al.*, 1990). In the present study, the normal female karyotype (78, XX) was present and a Y-chromosome was not observed in the metaphase spreads in the peripheral lymphocytes. Therefore, on the basis of the cytogenetical findings described above, this dog was initially diagnosed as a female PH. However, gonads collected at necropsy were testis. Therefore, the present dog was confirmed as a Sry-negative XX sex reversal.

Generally, normal sexual development occurs in three sequential steps (establishment of chromosomal sex, development of gonadal sex, and development of phenotypic sex) with each step dependent upon the successful completion of the previous step. An abnormality at any one of these steps can cause an abnormal sexual phenotype (Mayers-Wallen *et al.*, 1997). The genetic signal for induction of testicular development is Sry. Further development of the male phenotype is dependent upon a functional testis, which secretes two masculinizing hormones; MIS and testosterone. The MIS regresses the Müllerian ducts, while testosterone stimulates the formation of ductus deferens and epididymis from the Wolffian ducts (Mayers-Wallen *et al.*, 1997). In this study, the dog had abdominal testes with epididymis, ductus deferens and complete uterus. It was suggested that the complete female type of reproductive organs may be developed due to the absence of Sertoli's cells in the gonad and thus the lack of MIS stimulation during development.

Meyers-Wallen *et al.* (1994) performed a study on normal male (XY) and suspected XX sex reversed dog embryos by experimental breeding. The Müllerian duct regression following MIS exposure takes place during an early critical period in the embryonic life. In normal male embryos, MIS secretion was first identified at the age of 36 days, and the Müllerian duct regression was completed at 46 days. In the gonads of embryos at risk for sex reversal, MIS secretion was not observed until Day 40, and Müllerian duct regression was not absent until Days 46. Also, previous author reported that the degree of seminiferous tubules developing in the testicular tissue was considerably reduced when compared with normal age-matched XY males embryo (Meyers-Wallen, 1994). In this study, an oviduct was adjacent to each gonad, and a complete uterus was present, indicating complete failure of Müllerian duct regression and MIS secretion.

A hereditary sex reversal syndrome (78, XX, Sry-negative) has been reported quite frequently in dogs (Switonski *et al.* 2004). Phenotypic expression of the external genitalia of the 3 XX males and 29 true hermaphrodite America Cocker spaniels correlated well with the amount of testicular tissue in gonads (Mayers-Wallen and Patterson, 1988). The external genitalia of the 29 XX true hermaphrodites consisted of a normal looking vulva in 70%, an enlarged clitoris in 15%, and an abnormally shaped vulva in 15% (Mayers-Wallen and Patterson, 1988). In the XX sex reversal American Cocker spaniel presented here, the gonads were testes, and there was only a minor degree of masculinization. These data indicate that the degree of phenotypic sex reversal varies among affected individual.

Rho *et al.* (2007) reported that American Cocker spaniel is susceptible to hermaphrodites because all hermaphrodites were identified in American Cocker spaniel dogs in Korea. Until now this syndrome has been diagnosed by cytogenetic or molecular approach in 7 cases in Korea, including the case reported in this study. In previous report, 4 of 6 dogs had bilateral ovotes-

tis and os clitoris, and remaining 2 dogs had unilateral ovotestis and os clitoris (Alam *et al*, 2007; Rho *et al.*, 2007; Kim and Kim, 2006). But in this study, the dog had bilateral testes and os clitoris. To our knowledge, this is the first report of a Sry-negative XX sex reversal dog in Korea.

Hubler *et al.* (1999) reported that the function of testicular tissue supported by the hCG stimulation test. The testosterone values, before 24 hours after stimulation, were between those of a normal male and female dog. They knew that there was not enough testosterone present during the embryonic critical period for the androgen-dependant organs to respond normally. So, the affected dogs had mild masculinization of the external genitalia. Our result was similar to Hubler *et al.* (1999). In this study, the dog had bilateral testes, but seminiferous tubules were reduced than those normal of male dog. However, the dog had large number of Leydig's cells in testes. The Leydig's cells may produce some testosterone. Therefore, testosterone stimulates the formation of ductus deferens and epididymis from the Wolffian ducts. In this study, the formation of male genital tract and calcification and enlargement of os clitoris at 3-year-old may be due to testosterone produced in the testes.

The present case was performed by clinical, cytogenetic, molecular genetic and histological examinations, especially Sry gene was determined by PCR analysis for sex determination. In conclusion, the present case was diagnosed as a female hermaphrodite with Sry-negative XX sex reversal due to bilateral testes of the gonads with ductus deferens, epididymis, uterus and os clitoris.

ACKNOWLEDGMENT

This work was supported by the research grant of the Chungbuk National University in 2007.

REFERENCES

Alam MR, Cho YG, Cho SJ, Lee JI, Lee HB, Tae HJ, Kim IS and Kim NS. 2007. Male pseudohermaphroditism in dogs: Three case reports. Veterinarni Medicina 52:74-78.

Ennis S and Gallagher TF. 1994. A PCR-based sex-determination assay in cattle based on the bovine amelogenin locus. Anim. Genet. 25:425-427.

Hubler M, Hauser B, Meyers-Wallen VN and Armold S. 1999. SRY-negative XX true hermaphrodite in a Basset hound. Theriogenology 51:1391-1403.

Johnston SD, Root Kustritz MV and Olson PNS. 2001. Canine and Feline Theriogenology. Saunders, Philadelpia, pp. 193-195.

Jost A, Vigier B, Prepin H and Perchellet JP. 1973. Studies on sex differentiation in mammals. Rec. Prog. Horm. Res. 29: 1-41.

Kim KS and Kim O. 2006. A hermaphrodite dog with bilateral ovotestes and pyometra. J. Vet. Sci. 7:87-88.

Koopman P, Gubbay J, Vivian N, Goodfellow P and Lovell-Badge R. 1991. Male development of chromosomally female mice transgenic for Sry. Nature 351:117-221.

Kuiper H, Bunck C, Gunzel-Apel A-R, Drogemuller C, Hewicker-Trautwein M and Distl O. 2005. SRY-negative XX sex reversal in a Jack Russell Terrier: A case report. Vet. J. 169:116-117.

Meyers-Wallen VN and Patterson DF. 1988. XX sex reversal in the American Cocker spaniel dog: Phenotypic expression and inheritance. Hum. Genet. 80:23-30.

Meyers-Wallen VN and Patterson DF. 1989. Sexual differentiation and inherited disorders of sexual development in the dog. J. Reprod. Fertil. 39 (Suppl.):57-64.

Meyers-Wallen VN, Hurtgen J, Schlafer D, Tulleners E, Cleland WR, Ruth GR and Acland GM. 1997. SRY-negative XX true hermaphroditism in a Pasa Fino horse. Equine Vet. J. 29:404-408.

Meyers-Wallen VN, MacLaughlin D, Palmer V and Donahoe PK. 1994. Müllerian-inhibiting substance secretion is delayed in XX sex reversed dog embroys. Mol. Reprod. Dev. 39:1-7.

Meyers-Wallen VN, Palmer VL, Acland GM and Hershfield B. 1995. Sry-negative XX sex reversal in the American Cocker spaniel dog. Mol. Reprod. Dev. 41(3):300-305.

Meyers-Wallen VN. 1990. Inherited disorders in sexual development. J. Hered. 90:93-5.

Meyers-Wallen VN. 1993. Genetics of sexual diffentiation and anomalies in dogs and cats. J. Reprod. Fertil. 47(Suppl): 441-452.

Meyers-Wallen VN. 1999. Inherited disorders in sexual development. J. Hered. 90:93-95.

Rho J-R, Kim B-S, Cho S-J, Lee H-A and Kim O. 2007. Study on the characteristics of hermaphrodite dogs in Korea. Lab. Anim. Res. 23(4):435-439

Rosnina Y, Jainudeen MRJ and Hafez ESE. 2000. Genetics of reproductive failure. Reproduction in Farm Animals, seventh ed. Lippincott Williams & Wilkins, Philadelphia.

Sinclair AH, Berta P, Palmer MS, Hawkins JR, Griffths B, Smith M, Foster J, Frischauf AM, Lovell-Badge R and Good Fellow PN. 1990. A gene from the human sex determining region encodes a portion with homology to a conserved DNA-binding modification. Nature 346:240-244.

Switonski M, Nowacka J, Skorczyk A, Chmurzynska A and Nizanski W. 2004. Hereditary sex-reversal syndrome (78, XX, SRY-negative) in German shepherd puppies. Med. Wet. 60:705-707.

Weng Q, Murase T, Asano M and Tsubota T. 2005. Male pseudohermaphroditism in a raccoon dog (*Nyctereutes procynoides*). J. Vet. Med. Sci. 67:603-605.

Xiao C, Kato Y, Sato S and Sutou S. 1995. Mapping of bovine SRY gene on the distal tip of the long arm and murine SRY on the short arm of the Y chromosome by the method of fluorescence in situ hybridization (FISH). Anim. Sci. Technol. 66:441-444.

(접수일: 2008. 6. 15 / 채택일: 2008. 6. 25)