

SRY-negative XX sex reversal in an English Cocker Spaniel: a case report

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ABSTRACT: A SRY-negative XX male describes a female karyotype with testicular tissue resulting from abnormal gonadal development. In such cases male gonad formation takes place in the absence of a Y chromosome which supports the hypothesis that some other genes are also responsible for testicular development. The present case report describes a one-year-old dog brought to the hospital with a complaint of no oestrus cycle. Apparently normal masculine bitch had an enlarged clitoris with an os on radiograph. A gonado-hysterectomy was performed. Instead of ovarian structures both testicular masses were present and a normal complete bicornuate uterus with a normal cervix was observed. The histology of both gonads showed two testes, each with its ductus deferens and the uterus with typical histological structure. Cytogenetic evaluation of peripheral blood lymphocytes revealed a female with a normal karyotype (78, XX). Polymerase chain reaction (PCR) analysis of genomic DNA showed that the SRY gene was absent.

Keywords: English Cocker Spaniel; intersexuality; SRY-negative; sex determination; XX male

Normal sexual development is a complex three step phenomenon in mammals, involving (i) separation of sex chromosomes during gamete formation to facilitate the fertilisation process and to determine genetic make-up, i.e. XX or XY, (ii) translation of chromosomal sex into gonadal sex for ovarian or testicular tissue accordingly, and finally (iii) a differential cascade which results in internal and external genital organs in both sexes. The final step is dependent on testicular function in addition to target organ receptors and pathways. If testicular hormones are present, the outcome will be set of male genitalia and vice versa. Development of bipotential gonads is an indicator of gonadal evolution. In the case of a male (XY) embryo SRY up-regulates SOX9 for sustentacular (Sertoli) cell differentiation, which is a key factor for testicular development, as well as other genes. Conversely, in females (XX) SOX9 is suppressed by the R-spondin1 (RSPO1) gene and ovarian tissue will be formed in the ab-

sence of COX9 activity (Jakob and Lovell-Badge 2011). Evidence-based research in transgenic mice has proven that the X-linked *Dax1* gene functions in female gonad formation through suppression of SRY (Swain et al. 1998). Subjects are termed to be sex-reversed if genetic sex and gonadal development are not in accordance. Canines exhibiting sex reversal phenomenon have a normal female karyotype (XX) but testicular mass in the gonad (s). In XX males both gonads are composed of testicular mass.

In the current case report an apparently normal one year old English Cocker Spaniel with external female appearance but with a male (XX) karyotype has been described. Although there are several cases regarding SRY-negative XX sex reversal reported in dogs, there are, to the authors' knowledge, no reports concerning a SRY-negative XX male English Cocker Spaniel with external female appearance. This report describes the clinical, histological, cytogenetic and molecular evaluation of this case.

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Case description

History. A twelve-month-old, 12 kg female English Cocker Spaniel was presented at the Obstetrics and Gynaecology Clinic for lack of oestrus and evaluation of a mass protruding from the vulva first noticed three months earlier (Figure 1A). No other littermates with sexual disorders were born together with this bitch. An interview with the owner also revealed that no hormonal therapy had been administered during this particular gestation.

Clinical examination. A thorough and detailed clinical examination was carried out by a panel of clinicians after inspection, palpation, vaginoscopy, haematology and roentgenogram. Evaluation of the external genital organs indicated a normal bitch with an enlarged clitoris mass. Other physical examination results were within normal ranges. Anogenital distance was normal on inspection. A protruded clitoris with a hard bony structure and slight aromatic odour was observed. The vagina was normal on examination. Blood was analysed using an Abbott Cell-Dyn 3500 haematological analyser (Gml Inc, Ramsey, Minnesota, USA). Haematological variables were within the reference range (Plumb 2011). Latero-lateral and ventro-dorsal pelvis roentgenograms revealed the presence of an os clitoridis (Figure 1B).

Treatment

Gonadectomy. The dog was premedicated with 2 mg/kg (*i.m.*) xylazine HCl (Alfazyne 2%, Alfasan International BV, Woerden, Netherlands.). Anaesthesia was achieved using ketamine hydro-

chloride (Alfamine 10%, Alfasan International BV, Woerden, Netherlands) at 10 mg/kg administered intramuscularly. Gonadectomy was performed with midline incision. Synulox (Pfizer, Istanbul, Turkey) was administered at a concentration of 8.75 mg/kg *i.m.* for five consecutive days post-operation.

Histopathology. Surgically ablated testicular and uterine tissues were fixed in 10% neutral-buffered formalin. Paraffin-embedded cross-sectional size was 4 µm and staining was performed using haematoxylin and eosin (H&E) for microscopic study. For each testicular mass ten slides were prepared and examined. Gonadal tissue was representative of entirely male structures like seminiferous tubules with sustentacular (Sertoli) cells. Tubules were devoid of sperm cells, while a reasonable number of interstitial (Leydig) cells were observed (Figure 2A). The tunica albuginea had no germinal epithelium. Epididymis was not present. Histological examination of the uterine tissue gave normal results.

Cytogenetic analysis. Lymphocytic culture for chromosome analysis was established from the peripheral blood. Metaphases were evaluated for total chromosome number and sex chromosomes. Cytogenetic analysis with solid Giemsa staining revealed a 78, XX female karyotype in all 20 metaphases. Chromosomes were intact and aneuploidy was not observed (Figure 2B).

DNA isolation and SRY-specific PCR. Genomic DNA was isolated from peripheral blood using the Dr. Zeydanli DNA isolation kit (Turkey). Genomic DNA was also isolated from three randomly selected male and female control dogs and PCR was conducted in a thermal cycler. For each PCR reaction 4 µl DNA was used in a total reaction volume

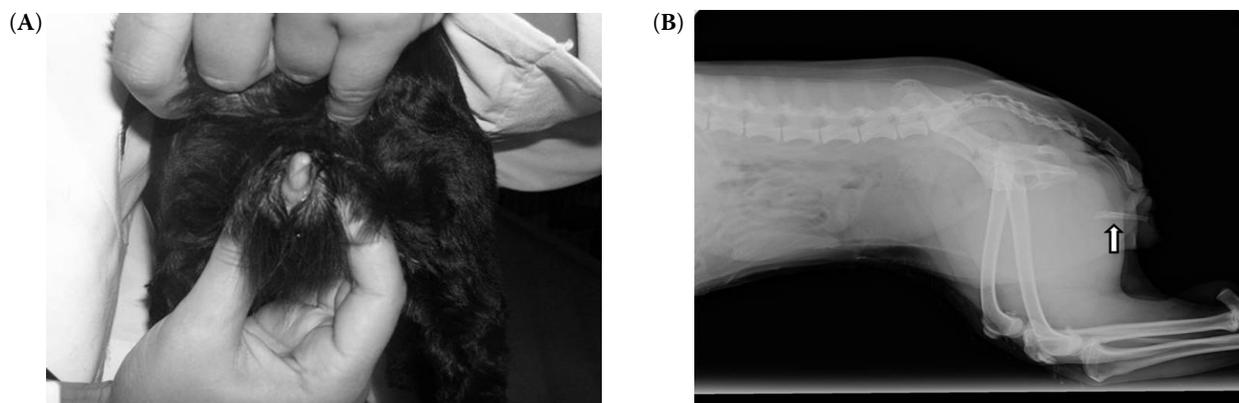


Figure 1. (A) An unusual extended clitoris in an English Spaniel dog. (B) Os clitoridis (arrow) on laterolateral roentgenogram of the pelvis

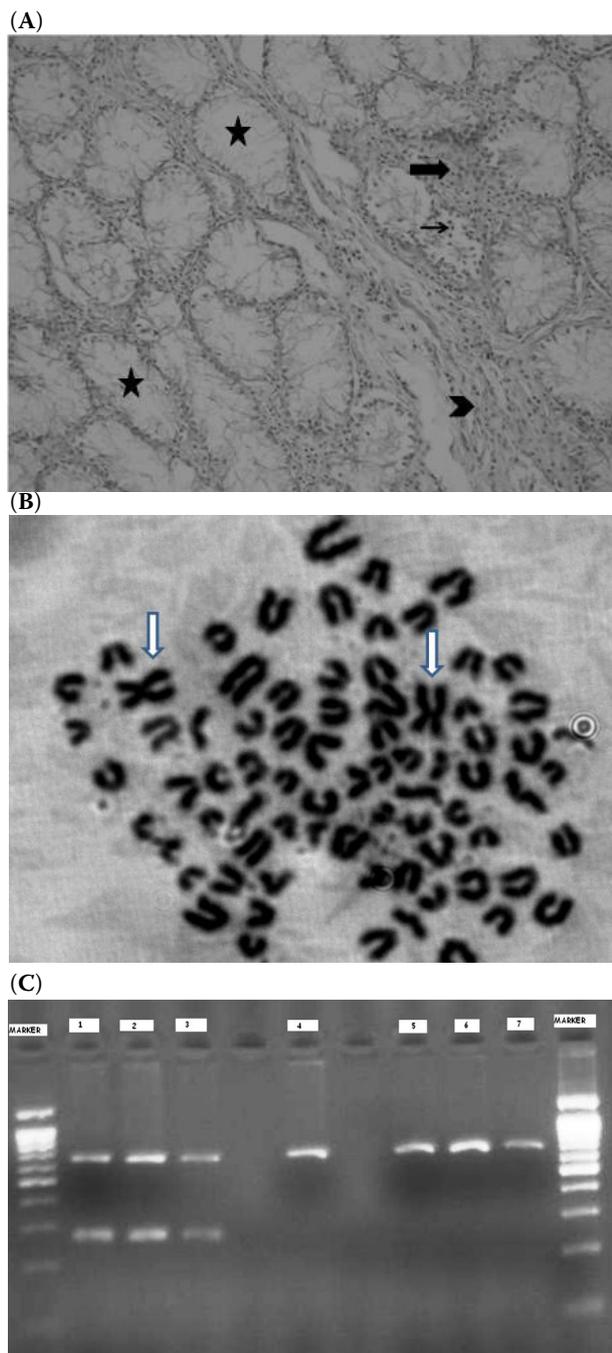


Figure 2. (A) Histopathological appearance of testicular tissue. Star: seminiferous tubule, thick arrow: sustentacular cells, thin arrow: interstitial cells, arrow head: Interstitial tissue. (H&E \times 400). (B) Cytogenetic analysis plate prepared from peripheral blood lymphocytes with solid Giemsa staining; both X-chromosomes are shown by an arrow (78, XX). (C). Molecular analysis in control canines and in the Cocker spaniel

of 50 μ l containing 1X manufacturer's buffer, 2mM $MgCl_2$, 300 μ M dNTP, 10 pmol of primers and 1 IU Superhot Taq Polymerase (Bioron, Germany). The

following SRY-specific primers, amplifying a 271-bp fragment, were used:

F: 5'-AAG GCC ACG GCA CAG AAA AGT CAC-'3 and

R: 5'-AAG AAG CGT CAG CGG ACA TCT GTG-'3.

As endogenous reference the following Heat Shock Transcription Factor 4 (HSF4) primers amplifying a fragment of 653 bp were used:

F: 5' ATG GGA CCA GGC TTT CTC-'3 and

R: 5'- ACA CCG GTG GTT CTT AAA CTC-'3.

PCR protocols included a 5 min polymerase activation step and 35 cycles of denaturation at 94 $^{\circ}$ C for 1 min, annealing at 58 $^{\circ}$ C for 1 min and elongation at 72 $^{\circ}$ C for 1 min with a final extension for 10 min at 72 $^{\circ}$ C. PCR products were separated by gel electrophoresis on a 1.5% agarose gel stained with ethidium bromide and analysed. The presence of SRY and HSF4 genes were confirmed in male control canines (Figure 2C, lanes 1–3). In female control canines (Figure 2C, lanes 5–7) and in the English Cocker Spaniel (Figure 2c, lane 4) the presence of the HSF4 gene was confirmed but no SRY-specific fragment was amplified.

DISCUSSION AND CONCLUSIONS

According to available data genetically XX males are sexually sterile. From the breeding point of view kennel owners should be advised to remove both parents from further breeding as this condition is hereditary in nature. Furthermore, 50% of siblings belonging to affected parents may serve as potential carriers in the future and thus should be considered unfit for breeding purposes. Unfortunately, there is lack of laboratory diagnostic tools for carrier identification so culling of problematic parents is the only solution to avoid the anticipated cases of sterility. Surgical castration is considered as the best treatment plan for affected individuals. Multiplex PCR results revealed that a phenotypically female dog was in fact a XX male. No previous data are available for comparison in the English Cocker Spaniel breed. Laboratory findings in the present case, with respect to the negative findings for the Sry gene, are in accordance with previous findings by Meyers-Wallen et al. (1999) in a French bulldog. On the basis of previous data the present case also represents a case of autosomal recessive inheritance as described by Meyers-Wallen et al. (1995a). In a previous report by Meyers-Wallen et

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al. (1999) a similar case was described but karyotypic confirmation of sex chromosomes was lacking. A retrospective study described a similar case (Meyers-Wallen et al. 1995b), which indicated that three American Spaniel dogs had testicular tissue on the basis of gonadal histology but were negative for the presence of *Sry* based on PCR assays of genomic DNA. Although a large number of important genes are considered to be involved in testis differentiation, the genetic aetiology of *SRY*-negative XXSR in mammals is unexplained. Particularly, the autosomal genes that cause testis induction in the absence of *Sry* in dogs, humans, caprine and swine (Pailhoux et al. 1997; Vaiman et al. 1997) are still not identified. One possible reason might be a gain-of-function mutation, activating a gene that initiates testis development. For example, mutation might trigger a gene, which may then overcome a threshold for testis induction. PCR findings in the present case are in accordance with those of Meyers-Wallen et al. (1999), which indicate that *Sry* translocation is not the only pathway for testicular development in canines but that other genes are also responsible. Identification by DNA tests and the removal of carrier animals from kennels is the only acceptable solution to stop the further spread of this condition (Campos et al. 2011; Silversides et al. 2011). Data accessible through genetic investigations would be a helpful tool for improving canine health. In addition, the input of veterinary surgeons is required so as to apply these insights within the boundaries of ethics and responsibility. In documenting this rare condition, we aim to contribute to investigations into possible causes, and we hope that the data reported herein will promote the employment of refined molecular diagnosis to identify silent carriers at earlier stages. Thus, the parents of affected subjects may be removed from breeding or used only with caution so that such cases may be eradicated.

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