Case Report

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A case of sterility associated with SRY-negative 64, XY in Egyptian Arabian mare: cytogenetics, molecular and hormonal analyses

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ABSTRACT

Inherited disorders are one of the reasons of infertility and economic losses for the equine industry. The detection rate of chromosomal abnormalities is rising due to the use of sex chromosome linked molecular markers. Here, a rare sterile five-year-old Arabian mare with XY male chromosomes is presented. The phenotype was corresponded to female with normal external genitalia. By transrectal ultrasound, there were hypoplastic ovaries and aplasia of uterine horns. Chromosome analysis was accomplished on blood samples using conventional, and G-banding techniques and confirmed by PCR and hormonal analysis. Although the karyotype (2n = 64, XY) revealed a typical male arrangement, it was SRY gene negative and amelogenin gene positive for X and Y chromosome. Hormonal analysis showed slight estrogenic activity of the ovary, but low progesterone and anti-mullerian hormones levels. In conclusion, this case of sex reversal mare (SRY-negative 64, XY) was recorded using cytogenetic, genetic, and hormonal analysis. Cytogenetics and molecular screening could be used as a fast approach for reproductive disorders evaluation in equine to save money, effort, and time of breeders.

Introduction

Mare fertility is a corner stone for the horse industry. Classic diagnostic tests as clinical investigation, ultrasound, or hormonal assessment frequently address some reproductive problems in mares. The cytogenetic screenings remain the premier method for the initial assessment of breeding horses with developmental abnormalities since they are still the easiest and fastest way to identify chromosome abnormalities (Bugno-Poniewierska and Raudsepp, 2021; Ghosh *et al*., 2022).

Sex reversal syndrome in the horse is extremely concerning because it impairs the fertility of mare and stallion. Abnormalities of sexual development causing infertility in horses have been documented previously (Villagomez *et al*., 2009; Lear and McGee, 2012; Villagomez *et al*., 2012). The term sex reversal is disorders of sex development (DSDs) in which the genetic sex as indicated by sex chromosomes does not match the external phenotypic sex. DSDs are categorized based on the sex chromosomes into XY and XX DSDs. The sex reversal is the most common equine chromosome abnormalities (Villagomez *et al*., 2011; Raudsepp and Chowdhary, 2016; Nogueira *et al*., 2021; Middlebrooks *et al*., 2023). It has been estimated by 12-30% of all cytogenetically aberrant cases are XY DSDs (Power, 1986; Bowling *et al*., 1987; Raudsepp *et al*., 2010).

With the inception of male marker of SRY gene testing in equine by PCR (Abe *et al*., 1999; Maekinen *et al*., 1999), horse XY DSDs are classified as SRY-positive and SRY-negative. The phenotype of SRY-negative XY DSD is strikingly similar to X-monosomy, despite the absence of SRY prevents the male development pathway in these animals, normal female development still necessitates two X chromosomes. The SRY-negative XY DSD horse is genetically caused by deletions in Y chromosome (Fukushima *et al*., 1999).

Additionally, amelogenin (AMEL), gene with Y and X alleles (AMELY and AMELX) is a sex marker commonly used in equine (Hasegawa *et al*., 2000). AMELX and AMELY are in both X and Y chromosomes, respectively, so enable quick diagnosis for XY mare. Most papers combine traditional cytogenetics with molecular techniques, allowing the results to be validated (Bugno-Poniewierska and Raudsepp, 2021). Therefore, in the present case we performed clinical, hormonal, cytogenetic and molecular investigations for the phenotypical normal mare. To our knowledge, this is the first report recorded in Egypt on sex reversal mare (SRY-negative 64, XY) diagnosed by cytogenetic and genetic analyses.

Materials and methods

Ethical approval

Management of the animal and blood collection obeyed the guidelines of the Animal Ethics Committee of the National Research Centre (NO 350 dated 07/14/2020).

Animal and samples

In 2021, a five-year-old Arabian mare belonged to private farm (Gamaiet Ahmed Orabi, Al Obour, Cairo Governorate, Egypt) underwent clinical examination for suffering from reproductive disorders. The mare showed infrequent estrus activity, with former trials for hormonal treatment to increase its fertility or enhance its conception. The mare was in good physical condition and showed normal body conformation (Fig 1 A) and external genitalia (Fig 1 B).

The ultrasound examination revealed that the ovary was very small without any follicle growth. At the same time, the uterine horns were absent. The vaginal examination revealed normal vagina with pale mucus membrane led to Portio vaginalis only. The clitoris, vestibule and cervix were normal.

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Blood samples were collected through jugular venipuncture for chromosome analysis, DNA isolation and hormonal assessment.

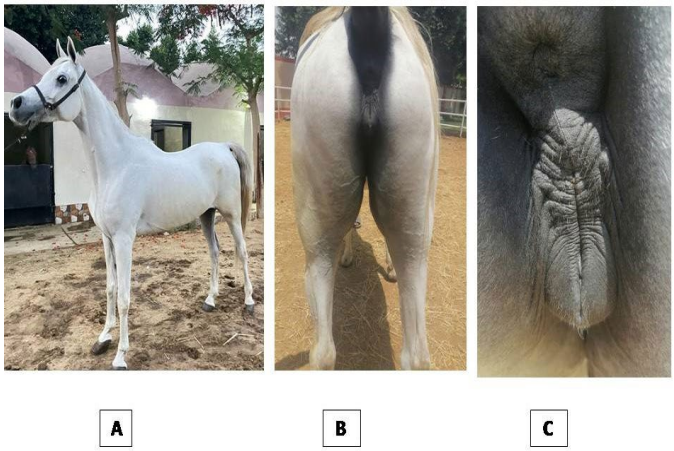

Fig. 1. Arabian breed mare (A) had sex reversal showing normal external genitalia (B and C).

Cytogenetic analysis

Peripheral heparinized blood sample was collected on lithium heparin, chromosome culture; preparation, metaphase spread and banding using Giemsa trypsin were performed according to Verma and Babu (1995). Metaphases captures were performed using ZEISS automated microscopic, Germany.

Molecular determinations

Genomic DNA was extracted from blood samples of the present case as well as control animals (normal mares; n=2, and normal stallions n=2) using blood DNA preparation kit-solution-based (Jena bioscience GmbH, Berlin, Germany) according to the manufacturer's protocol. The primers of Sex-determining region Y (SRY) and Amelogenin (AMEL) genes were prepared according to Maekinen *et al*. (1999) and Hasegawa *et al*. (2000). SRY gene fragment of 429bp (base pair) was amplified using specific primer sequence as following: forward: 5´-CTTAAGCTTCTGCTATGTCCAGAGTATCC-3´; reverse: 3´-GCGGTTTGTCACTTTTCTGTGGCATCTT-5´. While AMEL gene was amplified with nucleotide sequence forward: 5´-CCAACCCAACACCACCAGCCAAACCTCCCT -3´; reverse: 3´-AGCATAGGGGGCAAGGGCTGCAAGGGGAAT-5´. Amplification reaction was performed in a final 50 µL volume of 100 ng of template DNA, 50 pmole of each primer, 0.2mM of dNTPs, 1.5 mM MgCl2, 5 µl of 10X PCR buffer (20 mMTris–HCl pH 8.4, 50 mMKCl), and 1 U of Taq DNA polymerase. The initial denaturation was 94 °C for 5 min, followed by 35 cycles of denaturation (94 °C for 30 sec), annealing (60 °C for 30 se), extension (72 °C for 30 sec) and final extension (72 °C for 7 min). Upon completion of the reaction, the products were exposed to electrophoresis in 3% agarose, for 3 h at 70 V. Bands were visualized under ultraviolet Trans-illumination and Gel-Doc System (Bio-Rad).

Hormone analysis

Hormone analyses of progesterone, estradiol, testosterone and anti-mullerian hormones were done by using DRG Progesterone Enzyme Immunoassay Kit (EIA1561), DRG Estradiol ELISA kit (EIA2693), DRG testosterone (EIA1559), and DRG Anti-mullerian (EIA6141R) from USA.

Results

Cytogenetic analyses

All examined cells of the mare exhibited a diploid male constitution (2n = 64, XY) by using both conventional technique (Fig. 2) and G-banding (Fig. 3). The X chromosome was the second largest chromosome, and the Y chromosome was the smallest acrocentric one.

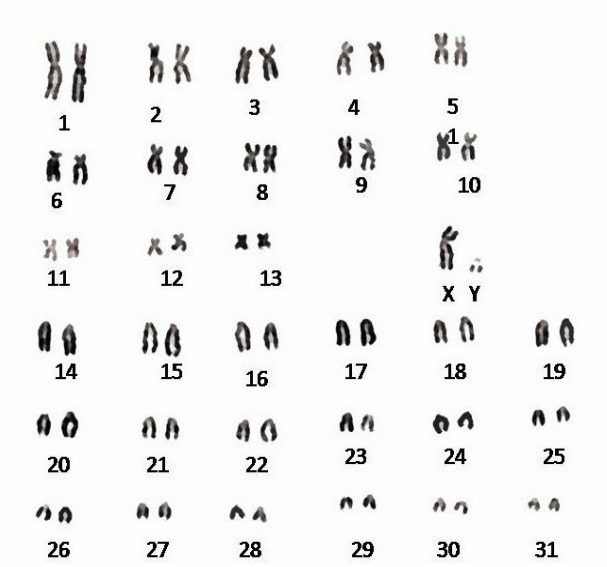

Fig. 2. The Giemsa-stained karyotypes of the XY- mare showing 64 chromosomes.

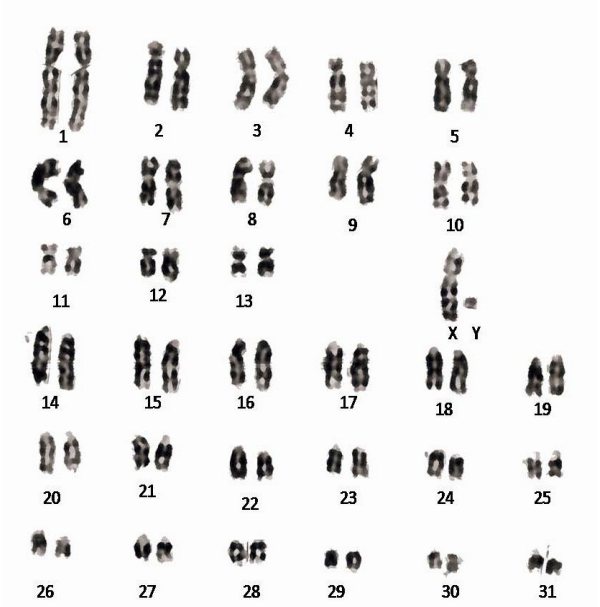

Fig. 3. G -banded karyotype of the XY- mare.

Molecular determinations

The primers of equine SRY gene flanked a 429 bp fragment of the positive SRY Stallion. While AMEL gene primers revealed two different sizes (Fig.4); 184 bp product obtained from X chromosome (AMEL X) and 160 bp from Y chromosome (AMEL Y). The present case showed two bands (184 and 160bp) of AMEL gene and no band with SRY gene (Fig.4).

Hormonal analysis

The blood concentrations of estradiol, progesterone, testosterone, and anti-mullerian hormones were 62 pg/ml, 0.267 ng/ml, 69 pg/ml and 0.07 ng/ml, respectively.

Discussion

A large proportion of the sex chromosome related reproductive dis-

orders remain undiagnosed due to the lack of specific/sensitive diagnostic tool. The presented case is the first recorded sex reversal sterile mare with presence of 64, XY chromosome in Egypt. The normal arrangement of the chromosome was based on the international system for cytogenetic nomenclature of the domestic horse (ISCNH) 1997 (Bowling *et al*., 1997). The genomic DNAs of the present case as well as normal stallion and mare were amplified by pair of primers from the X/Y-homologous region of AMEL gene using PCR. The PCR results from normal stallion genomic DNA revealed three bands: a 429 bp band for the SRY gene and two separate bands (184 bp and 160 bp) for the X- and Y-chromosomes. While the PCR output from genomic DNA from healthy mares only showed a single band (184 bp) that was the X-chromosome. This variation of X-and Y-chromosomal AMEL genes allowed for the sex determination of XY case report. Fukushima *et al*. (1999) recorded similar result for sex determination by AMEL gene.

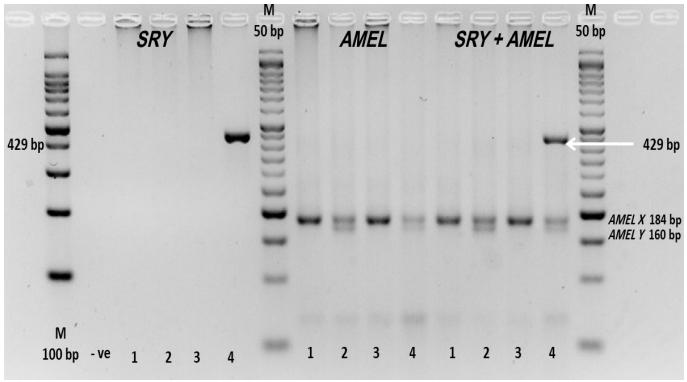

Fig. 4. Ethidium bromide stained 3% agarose gel of PCR products representing amplification of SRY and amelogenin gene fragments. –ve: negative control, 4: normal stallion, 1&3: normal mare, 2: XY-mare case. SRY band (429 bp) only detected in normal stallion. AMEL Y (160 bp) and AMEL X (184 bp) were detected in stallion and XY- mare. Normal mares show only a single AMEL X band (184 bp).

The sex-determining region (SRY) on the Y chromosome in mammals controls sex identity. SRY stimulates male development and suppresses the gene network required for gonad development in female. In the current case of sex reversal, the SRY gene was absent, which may indicate the possibility of genomic alteration/loss of SRY gene function and its product. According to Janěcka *et al*. (2018), the single-copy horse SRY appears to be situated in a structurally unstable region in the Y chromosome, embedded between amplicon sequences and surrounded by direct and inverted repeats. This position makes it easier for SRY to participate in chromatid conversion and recombination inside the Y chromosome (Lange *et al*., 2009). The SRY gene may be deleted in one sperm and duplicated in another as a result of these occurrences (Raudsepp *et al*., 2010; Janěcka *et al*. 2018). Therefore, SRY-negative XY females carry male siblings with two copies of SRY which is likely undiagnosed and has little impact on the phenotypic. This explains why SRY-negative XY sex reversal is uncommon or not present in other species, including humans, as the structure and content of mammalian Y chromosomes vary between species (Janěcka *et al*., 2018). Due to multiple Y chromosomal deletions, the majority of mares (64, XY) with a stallion karyotype are SRY-negative. According to Raudsepp *et al*. (2010), deletions can range in size from 21 kilobase-pairs to the whole loss of the euchromatic region. Moreover, the sex reversal syndrome is probably produced by transfer of the SRY gene from the Y to the X chromosome, due to aberrant meiotic exchange between the ZFY and SRY loci or SRY locus and the centromere (Bugno, *et al*., 2003). Interestingly, 33 out of 49 horses were verified to be XY SRY-negative when testing for parentage using the sex marker AMEL gene, while the other 16 cases were false-positives as a result of AMEL abnormalities (Martinez *et al*., 2020).

The second most common sex chromosomal defect after X monosomy is male-to-female XY sex reversal. The incidence of mares having the karyotype of a stallion (64, XY) is 12-30% of cytogenetic abnormalities (Lear and Bailey 2008; Raudsepp *et al*. 2010). The majority of cases with feminine-type XY DSDs fall under the category of SRY-negative XY, which is the most common kind of XY DSDs. Due to ovarian and uterine dysgenesis, the afflicted mares are frequently sterile despite normal female exterior genitalia and no somatic or behavioral problems (Bugno *et al*. 2003; Iannuzzi *et al*., 2004; Anaya *et al*., 2014; Pienkowska-Schelling *et al*., 2014; Martinez *et al*., 2020).

Estrogen level in the present case was close to the level in cyclic animals (Abo-El maaty and El-Shahat, 2012) but without follicular growth, higher than in intact or castrated males, but lower than in stallion during the breeding season (Haffner *et al*., 2010). Progesterone concentration was close to basal level recorded during non-breeding season, but lower than that shown in cyclic mares (Altinsaat *et al*., 2009; Abo-El maaty and El-Shahat, 2012). Anti-mullerian hormone was scant and never reach to any normal levels in female (Ulian *et al*., 2019), neonatal colts or mature stallions (Anthony, 2014). All hormonal levels indices explained the mare behavioral estrus displayed and expressed irregularly (due to high estrogen), though the source of steroid hormones may be away from the ovary which appeared hypoplastic on rectal and ultrasound examinations. Nagamine *et al*. (1998) stated that the lack of large follicles as well as granulosa and theca cells that release inhibin hormone leads to sterility. This assumption was ensured by low inhibin and anti-mullerian hormones which are strong indices for the follicular activity and reserve on the ovaries, respectively (van Rooij *et al*., 2002), and affirmed the hypoplastic nature of the presented case ovaries. This is consistent with the presence of AMEL gene positive for X and Y-chromosomes.

Conclusion

We recorded the first case of sex reversal in Arabian mare with SRY-negative 64, XY. This syndrome produced sterility in the mare as diagnosed by cytogenetics, molecular markers, and hormonal analysis. Further studies are necessary to screen a greater number of animals that remain undiagnosed for allowing advances in sex anomalies knowledge and to improve the breeding in equine species.

Acknowledgments

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Conflict of interest

The authors declare that they have no conflict of interest.

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