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Diagnosis of infertile horse and cattle via cytogenetic and molecular analyses

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ABSTRACT

In this study, we present one case each of a horse and a cattle that underwent chromosomal analysis due to infertility and/or subfertility. We performed PCR (polymerase chain reaction) analysis in conjunction with karyotyping and FISH (fluorescence in situ hybridization) to diagnose potential chromosomal abnormalities in these animals. In the case of the horse, cytogenetic analysis determined that the karyotype is 63, XO female, called X-monosomy. PCR analysis was positive for the androgen receptor (AR) gene and negative for Sex-determining Region Y (*SRY)* gene, confirming the absence of Y chromosome, female. X monosomy (63,XO) is the most common sex chromosomal abnormality that causes infertility issues in horses. In the case of the cattle, cytogenetic analysis showed a karyotype of 60, XY, consistent with a normal male. PCR analysis was positive for both AR and SRY, confirming male sex determination. However, detailed examination of the karyotype revealed structural abnormalities involving chromosomes 5 and 8. Subsequent FISH analysis confirmed a balanced translocation between these two chromosomes, which likely contributed to infertility in the bull.

INTRODUCTION

The study of chromosomes in number or structural changes is called cytogenetics, which is an important tool for studying chromosomes. It has been applied to determine any forms of gain, loss, translocation, or alteration in the sequence of the chromosomes and their relationship to reproductive failures. In the late 1950s, scientists could see structural and numerical abnormalities under the microscope. Studies from the 1980s–2000s found that 2–5% of couples with more than two miscarriages carried balanced structural chromosome rearrangements [1].

Cytogenetic analyses of domestic animals have been applied to connect with reproduction in pigs, cattle, horses, dogs, cats, and domestic fowl [2-6]. Veterinary cytogenetics has benefited from the information generated in human cytogenetics and has become an important method for diagnostic and prognostic purposes not only in reproduction but also in overall genetic conditions [7]. Cattle and pigs were the most actively studied species, as they were a major food source, and the 1;29 translocation of cattle was reported as the most common chromosomal abnormality in over 50 breeds of cattle studied by

cytogeneticists [8]. Conventional cytogenetics, known as karyotyping, examines the chromosomes in a sample of individual cells by size, shape, and number to test possible chromosome abnormalities. It has been used in clinical trials to visualize chromosomes, typically during the metaphase stage of cell division, after staining the chromosomes. This process allows for the identification of large-scale chromosomal aberrations such as deletions, duplications, translocations, and change in numbers.

Later, the development of high-resolution banding techniques enabled the detection of small fragments of chromosomes which may have been insufficiently covered by previous chromosomal analyses. Fluorescence *in situ* hybridization (FISH) uses fluorescent probes to paint the segments of the respective chromosomes in order to enable visualization of the localization of a specific DNA sequence or an entire chromosome in a cell; this later became an important part of modern cytogenetic techniques [9].

PCR (Polymerase Chain Reaction) is a simple yet powerful tool that complements cytogenetic studies in the diagnosis of sex, particularly in cases involving sex chromosome abnormalities. The *SRY* gene encodes the Sex-determining Region Y protein, whose expression is correlated to the development of testes in mammals. It is typically located in the Y chromosome [10, 11]. The androgen receptor (*AR*) gene, located on the X chromosome, is used as a PCR control marker because the X chromosome is present in both males (XY) and females (XX) [12].

Infertility can be diagnosed through physical exams, semen evaluation, ultrasound, and laboratory tests. Once the right diagnosis is made, treatments can be applied; this ranges from the administration of hormones to keep the pregnancy, to antibiotic treatment for infection. However, it is often difficult to diagnose and treat infertile horses when the horse is of normal reproductive age and phenotypically looks normal. There is a reproductive failure in phenotypically normal mares and stallions and it is often linked to genetic abnormalities [13, 14]. Chromosomal disorders are the most common causes of decreased fertility, infertility, and congenital defects other than infectious issues in horses [2]. Two cases were submitted to the lab, the first sample for a bull and the other for a horse, to examine possible chromosomal abnormalities associated with infertility issues. Any changes in the numbers and structure of chromosomes will be examined by karyotyping along with PCR-based sex determination and FISH to determine the cause of infertility for each case.

MATERIALS AND METHODS

Cell Cultures for metaphase Chromosome Preparations

Metaphase chromosome spreads were prepared from Pokeweed-stimulated peripheral blood lymphocyte cultures according to standard protocols [15]. Briefly, under sterile conditions, 1 mL of sodium heparin-stabilized peripheral blood was grown for 68-72 hr in 9 mL of culture medium RPMI-1640 supplemented with HEPES and Glutamax (Gibco), 10% fetal bovine serum (Atlanta Biologicals), 1× antibiotic-antimycotic (100×; Invitrogen), and 15 µg/mL pokeweed mitogen (Sigma Aldrich). Blood lymphocyte cultures were harvested with demecolcine solution (10 µg/mL; Sigma Aldrich), treated with

Optimal Hypotonic Solution (Rainbow Scientific), and fixed in 3:1 methanol: acetic acid. The cells were dropped on clean, wet glass slides and checked under a phase contrast microscope (×20) for quality.

Cytogenetic Analysis

Chromosomes analysis and karyotyping were carried out by GTG-banding [16]. For each individual, metaphase spreads from peripheral blood lymphocytes were examined—32 cells were analyzed for the horse case and 35 cells for the bull case. Karyotyping and chromosome analysis was done with an Axio Imager M2p microscope (Carl Zeiss, Inc., Jena, Germany) and IKAROS (MetaSystems GmbH) software. The chromosomes were identified and arranged into karyotypes according to the International System for Cytogenetic Nomenclature of the Domestic Horse [17] and chromosome aberrations were described according to the International System for Human Cytogenetic Nomenclature [18].

Karyotyping results were confirmed by fluorescence in situ hybridization (FISH) using different BAC DNA probes. BAC DNA was isolated using the Plasmid Midi Kit (Qiagen) according to the manufacturer's instructions and labeled by nick translation with biotin-16-dUTP or digoxigenin-11-dUTP following standard protocols [19]. The two probes were co-hybridized to metaphase spreads of the bull and the signals were detected with avidin-FITC (for biotin; green; 5P, 8P, and 8MID1) and anti-dig-Rhodamine (for digoxigenin; red; 5D, 8MID, and 8MID2) [19]. There were a total of 100 metaphase and interphase cells analyzed, and 20 images captured with a Zeiss Axioplan2 fluorescence microscope and Isis V5.2 (MetaSystems GmbH) software.

DNA isolation and PCR analysis for SRY and AR

Genomic DNA was isolated from EDTA-stabilized blood with QIAamp DNA Blood Mini Kit (Qiagen) and the DNA concentration was determined using the NanoDrop (Thermo Scientific) system. PCR based sex-determination was carried out using two pairs of primers, Sex determining Region of Y (*SRY*) and Androgen receptor (AR). The *SRY* primers were used to determine the presence (male) or absence (female) of the product and the X-linked androgen receptor (*AR*) gene served as a positive control for all PCR amplifications. The primers were as follows: Horse: *SRY* primers (F: 5′-TGCATTCATGGTGTGGTCTC-3′; R:5′-ATGGCAATTTTTCGGCTTC-3′, product size 131 bp [20], *AR* primers (F: 5′-AGCAGCAACAGGAGACCAGT-3′; R: 5′--GCTTAAGCCTGGGAAAGTG-3′, product size 294 bp [21]. Bovine: *SRY* primers (F: 5′-ACGCCTTCATTGTGTGGTCT-3′; R: 5′-TCTCTGTGCCTCCTCAAAGAA-3′; product size 152 bp), *AR* primers (F: 5′-AGCAGCAACAGGAGACCAGT-3′, R: 5′-CACTTTCCCAGGCTTAAGCA-3′, product size 294 bp) [19]. For a non-template reaction DNA was substituted with H_2O. PCR amplification was performed in a total volume of 25µL with 5X FIREPol® Master Mix (Solis BioDyne). Cycling conditions were as follows: 35 cycles with denaturation at 94 °C for 30 s, annealing at 60 °C for 15 s, extension at 72 °C for 15 s, and a final extension at 72 °C for 5 min. The PCR products were resolved in a 2% agarose gel containing ethidium bromide.

Results

A. Horse, X monosomy female

A horse sample was submitted for examination due to infertility. A standard karyotype analysis was performed to count chromosomes and examine any structural abnormalities. The karyotype analysis confirmed that there was only one X chromosome, unlike the two X chromosomes in normal female horses. We karyotyped 32 different cells to confirm the X-monosomy in this case and all of the data indicated that the horse's karyotype is 63,X not 64,XX (Figure 1).

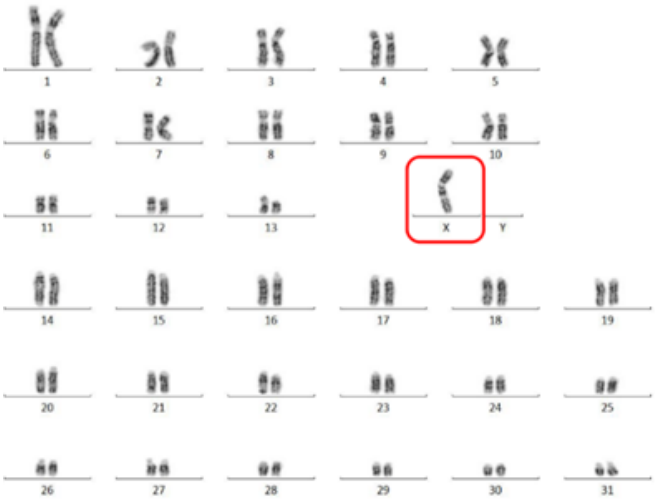

Figure 1. Cytogenetic characterization of the horse with a single X chromosome.
A G-banded karyotype of the horse with 63,X showing the absence of one X chromosome. The X chromosome is indicated by a red box and there is no Y chromosome, indicating X-monosomy female genotype. All examined chromosomes display normal morphology and a karyotype of 63,X (X-monosomy).

PCR analysis was performed for both Y-linked *SRY* gene and X-linked *AR* gene. Both male and female DNA served as positive controls and the PCR reaction without template DNA served as negative control to support no contamination during PCR preparation. PCR results were positive for *AR* and negative for *SRY*, indicating only X chromosome in this sample and no *SRY* transition to any chromosome in this horse genome, indicating female (Figure 2). In conclusion, the horse displayed single X female karyotype with 63 chromosomes, and no structural abnormality was observed

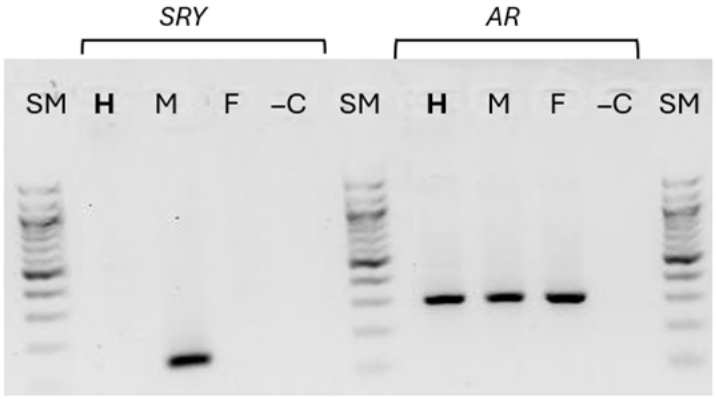

Figure 2. PCR-based female determination using the *SRY* and *AR* genes shows the absence of *SRY* and the presence of *AR* in the genomic DNA of the 63,X female horse. A set of lanes contain case horse (H), one of each male and female horse as a positive control (M and F, respectively), negative control (-C), and 100 bp DNA size marker (SM). The *SR*Y locus displays 131 bp band in the male sample and a 294 bp PCR product corresponding to *AR* is detected in all male and female samples except for negative control.

B. Cattle, Chromosomes 5 and 8 reciprocal translocations

A bull sample was submitted to the lab due to subfertility. Standard karyotype analysis indicated 60,XY normal male (Figure 3). Next, we performed PCR based sex-determination of the case sample and it indicated positives for both *SRY* and for *AR* genes, suggesting normal male, XY (Figure 4).

Abnormal structural variation was detected in chromosomes 5 and 8. One of chromosome 5 was longer than normal while chromosome 8 was shorter than normal chromosome 8 (Figure 3). This indicated possible translocation between the two chromosomes, so additional Fluorescence in situ hybridization (FISH) was performed to identify specific chromosomes involved in the reciprocal translocation.

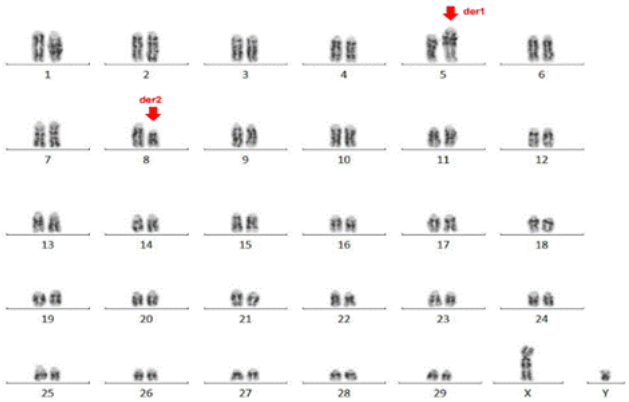

Figure 3. Cytogenetic characterization of the bull sample. Karyotype analysis of the bull indicated normal 60, XY karyotype. The abnormal structure of chromosomes 5 and 8 are indicated by red arrows.

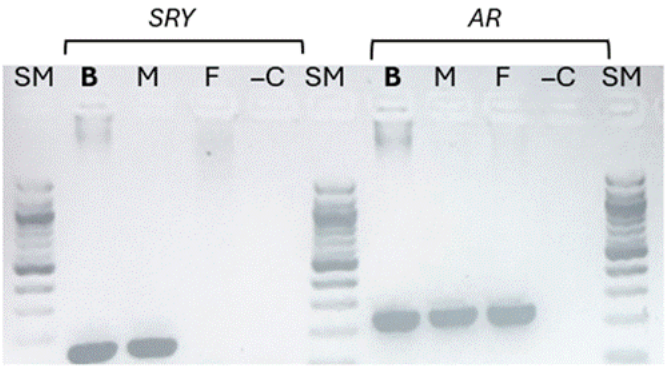

Figure 4. PCR-based male determination using *SRY* and *AR*. A set of lanes contained the bull sample (B), one of each male and female cattle as a positive control (M and F, respectively), negative control (-C), and 100 bp DNA size marker (SM). The SRY locus displays 131 bp bands in the male sample and a 294 bp PCR product corresponding to AR was detected in all male and female samples except for the negative control.

Each end of chromosome 5 was stained with two probes, 5proximal (5P) green and 5distal (5D) red, respectively. Figure 5A indicates two signals, green and red within one chromosome 5 which represents normal. However, there are two additional chromosomes which contain one of each signal detected; it indicates that part of chromosome 5 has separated to another chromosome which we suspect to be chromosome 8. So, we applied the same approach on chromosome 8, using two probes; 8proximal (8P) green and 8MID2 (8M) red, respectively and found the end of chromosome 8 translocated to chromosome 5 (Figure 5B).

One more round of FISH with chromosome 8 probes: 8MID1 (8M) green and 8MID2 (8M) red, showed that 8MID1 and 8MID2 probes were in two different chromosomes (Figure 5C). It suggested chromosome 8 breaks between the two probes and translocates to chromosome 5. The position of each probe on the chromosome and diagram of the translocated region shown in Figure 5D. FISH with chromosome 8 probes: 8MID1 (8M) green and 8MID2 (8M) red, and respective karyotype arrangement is shown in Figure 5E. In conclusion, the bull has normal XY chromosomes (60,XY) with a reciprocal translocation between chromosomes 5 and 8.

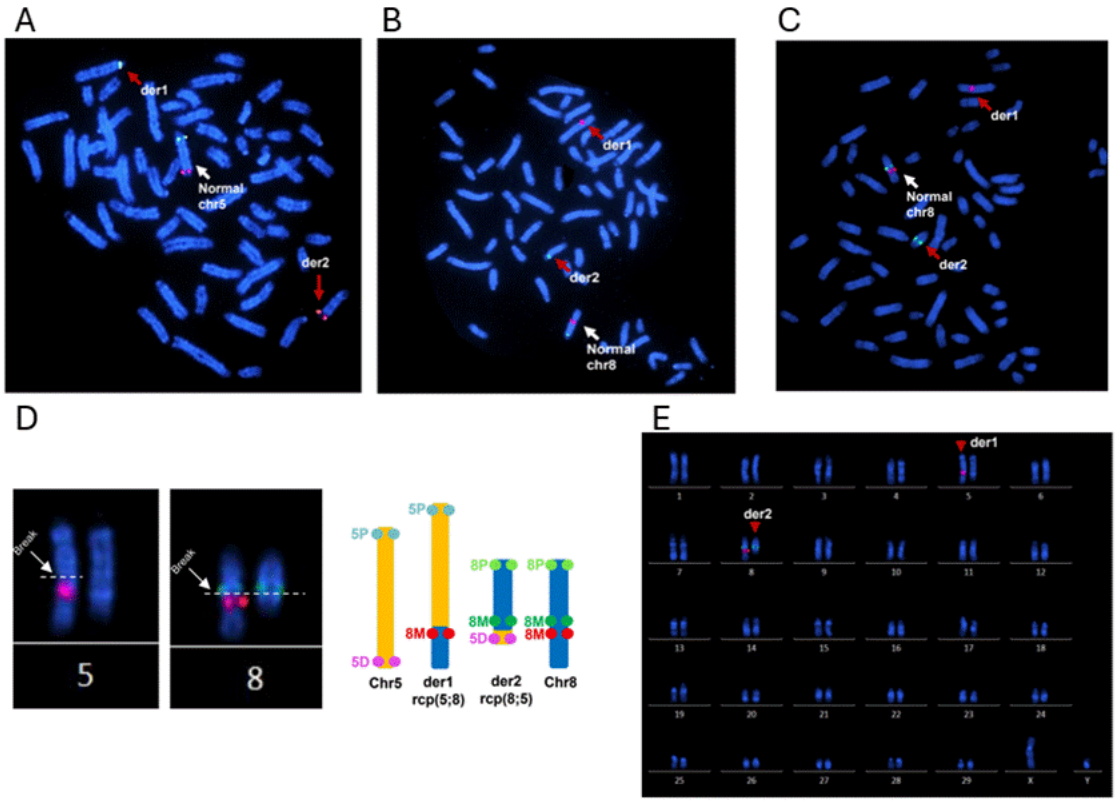

Figure 5. Fluorescence in situ hybridization analysis of reciprocal translocation between chromosomes 5 and 8. Chromosome 5 displaying with 5proximal (5P) green and 5distal (5D) red probes in panel A. Chromosome 8 displaying with 8proximal (8P) green and 8MID2 (8M) red in panel B. Chromosome 8 displaying with 8MID1 (8M) green and 8MID2 (8M) red shown in panel C. The chromosome was painted by probes for chromosomes 8MID2 (8M) red and 8MID1 (8M) green and predicted break point regions marked in dashed lines. The position of each probe on the chromosomes and derivative chromosomes 5 and 8 by reciprocal translocation shown in panel D. The final karyotype arrangement with reciprocal translocation between chromosomes 5 and 8 shown in panel E.

DISCUSSION AND CONCLUSION

The X-monosomy, also known as Turner syndrome, has been described not only in humans but also in many domestic animals such as cats [22], dogs [23], horses [19], and sheep [24]. X-monosomy is the most common disorder of sex development in horses [25], frequently leading to infertility. The individuals with X-monosomy usually display a female phenotype, however their ovaries do not develop properly. Because of the underdeveloped or nonfunctional ovaries, X-monosomy individuals are infertile. As females have two X chromosomes, unlike males who have XY sex chromosomes, the second X chromosome in females undergoes an inactivation process to compensate for gene dosage imbalance between males and females, called X inactivation. So, the second X chromosome in females is silenced

anyway, resulting in gene expression from only one X chromosome. However, not all genes on the second X chromosomes are turned off during inactivation, about 15% of genes on the inactivated X chromosome escape silencing, and another ~10% are variably inactivated. And these “escapee" genes are known to be involved in development, including ovarian development, bone growth, and brain function. That is why X-monosomy individuals experience infertility such as the horse we examined here.

Karyotyping is a gold standard method for detecting large reciprocal translocations and FISH applied to get more detailed confirmation of smaller rearrangements that are not detectable by karyotyping. Here, we used standard karyotyping with PCR to determine the sex while FISH confirmed reciprocal translocation and suggested a suspected break point region. Both X-monosomy and reciprocal translocation directly impact breeding efficiency. Fertility is one of the critical factors in the economic and productive success of both the equine and cattle industry. Understanding the reason behind infertility in these cases will help the breeders to make informed decisions. Chromosomal abnormalities such as reciprocal translocations and X-monosomy are also detected in humans and it often affects couples not only biologically, but emotionally, socially, and financially. Although we cannot prevent this genetic event due to its random and spontaneous nature, correct and early diagnosis is crucial for effective management along with genetic counseling, IVF, or hormonal therapy.

Reciprocal translocations refer to the exchange of parts between two different chromosomes without any loss or gain of the genetic material in the genome (genetically balanced). Many carriers are phenotypically normal. However, when chromosomes line up during meiosis to pair up, they cannot properly pair up with their homologous chromosome due to its structural difference and often results in the failure of segregation of homologous chromosomes. It could result in recurrent miscarriage, infertility or subfertility, or unexplained stillbirth like our bull case. Cytogenetics and molecular analysis, along with recent advances in genomic analysis, could accelerate clinical diagnoses in reproduction not only in domestic animals, but also in wildlife and humans.

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