

Cytogenetics and Molecular Diagnosis in Horse Infertility

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ABSTRACT

As the popularity of horse breeding stays steady, many breeders and owners send their horses samples to cytogenetics analysis for signs of infertility or other genetic abnormalities. Here, we report three cases of horses that were subjected to chromosome analysis due to infertility issues at the Molecular Cytogenetics Laboratory at the Texas A&M School of Veterinary Medicine & Biomedical Sciences (VMBS). Three particular cases arrived for chromosome and molecular analysis, first, H304 mare, the horse has a normal 64, XX female karyotype and no chromosomal abnormality was observed to explain their infertile issue. In the case of the H305 mare, the horse has a 64, XY male karyotype which indicates genetically male and no sign of chromosomal abnormality under cytogenetic analysis. However, PCR analysis indicates the loss of the SRY in the Y chromosome. This is a typical male to female sex reversal and one of the most common genetic sex abnormalities in horses. In the H306 mare, the horse has only one X chromosome; 63, XO, called X monosomy. This is the most common sex chromosomal abnormality in horses. The combination of clinical cytogenetic analysis and the use of PCR are the basics and strongest tool to determine the chromosomal abnormality in the equine industry and the case H305 is a good example to support how these two different approaches compensate each other to generate a reliable diagnosis. Determination of genetic abnormalities using two techniques will help horse breeders and equine practitioners to make the most informed decisions about breeding plans.

Introduction

Breeding is an extremely important aspect of the equine industry not only to supply horses with appropriate genetic backgrounds and temperaments to perform the proper activities but also to produce desired characteristics for professional breeders and many horse owners. So, fertility is one of the critical factors in the economic and productive success of the equine industry. Horses can be fully ready at about 2-3 years old to breed and actively reproduce until they reach 15 years old, and after 15, their fertility declines with age. Fertility can be influenced by a variety of factors including health conditions, age, reproductive anatomical issues, and genetic problems. Infertility can be diagnosed through physical exams, semen evaluation, ultrasound, and laboratory tests. Once the right diagnosis is made, a couple of treatments will be applied 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 the right age and phenotypically looks normal. There's a reproductive failure in phenotypically normal mares and stallions and it is often linked to genetic abnormalities [1-3]. Chromosomal disorders are the most common causes of decreased fertility, infertility, and congenital defects other than infectious issues in horses [3].

Cytogenetics is the study of chromosomes in the number or structural changes; in the form of a gain, loss, translocation, or alteration in the sequence of the chromosomes and it's a powerful technique to study chromosomes and their relationship to reproductive failures not only in horse but also in human. Since the 1960s, domestic animal cytogenetic analysis is applied to connect with reproduction in pigs, cattle, horses, dogs, cats, and domestic fowl [3-7]. Veterinary cytogenetics has benefited from the information generated in human cytogenetics and become an important method for diagnostic and prognostic purposes not only in reproduction but also in overall genetic conditions

[8]. Pig and cattle were the most actively studied species as a food source, and 1/29 translocation of cattle, often called Robertsonian Gustavsson's anomaly, are the most reported chromosome abnormality in over 50 breeds of cattle studied by cytogeneticists [9].

Classical cytogenetics by karyotyping has been used in clinical trials by examining the chromosome in the sample of cells of individuals by size, shape, and number to test possible chromosome abnormalities. Later, high-resolution banding techniques enable the detection of small fragments of chromosomes that may be insufficiently covered by routine chromosome analysis. Fluorescence *in situ* hybridization (FISH) uses fluorescent probes to detect DNA sequences that allow the localization of a specific DNA sequence or an entire chromosome in a cell, and it became an important part of modern cytogenetic techniques [10].

PCR-based molecular analysis of sex chromosomes could serve as a critical tool for sex determination. It is a simple and rapid tool accompanying cytogenetic studies in the diagnosis of sex in cases of sex chromosome abnormality. SRY encodes the Sex-determining region Y protein and its expression correlates with testis development in mammals and is found on the Y chromosome [11-13], so the PCR based sex determination can be quickly identified by the presence (male) and absence (female) of the STY gene except for mutant individuals. The gene for the androgen receptor (AR) is located on the X chromosome and is used to detect the presence of the X chromosome for both normal male (XY) and female (XX) [14, 15].

Three cases were submitted to the lab to examine possible chromosome abnormalities claimed with infertility problems. A horse has 64 chromosomes ($2n = 64$) and any change in the numbers and shape of chromosomes will be examined in this study. Traditional karyotyping by G-banding and PCR-based sex determination were applied to determine the cause of infertility.

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 [16]. Briefly, under sterile condition, 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), $1\times$ antibiotic-antimycotic ($100\times$; Invitrogen), and 15 $\mu\text{g}/\text{mL}$ pokeweed mitogen (Sigma Aldrich). Blood lymphocyte cultures were harvested with demecolcine solution (10 $\mu\text{g}/\text{mL}$; Sigma Aldrich) and Ethidium bromide (1 mg/mL; Bio-Rad), 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 ($\times 20$) for quality.

Karyotype and cytogenetic analysis

Chromosome analysis and karyotyping were carried out by GTG-banding using an Axioplan2 microscope (Carl Zeiss, Inc., Jena, Germany) and IKAROS (MetaSystems GmbH, Altussheim, Germany) software. The chromosomes were identified and arranged into karyotypes according to the International System for Cytogenetic Nomenclature of the Domestic Horse [18] and chromosome aberrations were described according to the International System for Human Cytogenetic Nomenclature [19]. Thirty-two cells for H305, 35 cells for H304 and H306 were captured and analyzed for each individual.

DNA isolation and PCR analysis for SRY and AR genes

Genomic DNA was isolated from EDTA-stabilized blood with QIAamp DNA Blood Mini Kit (Qiagen) and DNA concentration was determined using the NanoDrop (Thermo Scientific) system. PCR based sex-determination was carried 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 *SRY* on Y chromosome and the X-linked androgen receptor (*AR*) gene served as a positive control for all PCR amplifications. The primers were as follows: *SRY*-forward 5'-TGCATTCATGGTGTGGTCTC-3' and *SRY*-reverse 5'-ATGGCAATTTTTTCGGCTTC-3', 131 bp [20], *AR*-forward 5'-AGCAGCAACAGGAGACCAGT-3' and *AR*-reverse 5'-GCTTAAGCCTGGGAAAGTG-3', 294 bp [21]. For a non-template reaction DNA was substituted with H₂O. PCR amplification was performed in a total volume of 25 μL with Bio-Rad's 2X master mix. 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.

Results

Case H304: XX mare

The H304 is a normal female horse that was submitted for the examinations due to infertility issue. We examined a total of 35 cells and 10 of them were karyotyped. Karyotyping of Giemsa-stained metaphases revealed 64 chromosomes with normal morphology, including two normal X chromosomes in all cells analyzed, genetically female as it appeared (Figure 1).

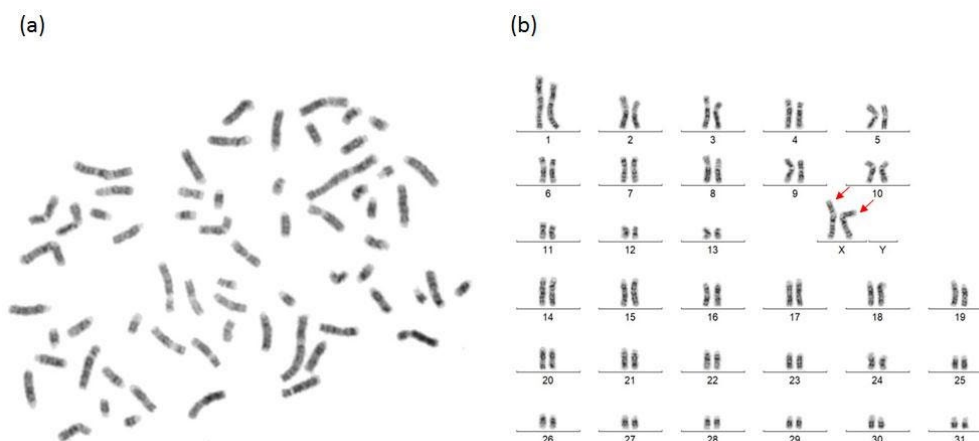


Figure 1. Cyto-genetic characterization of the H304 mare horse. A Giemsa-staining metaphase spread (a) and corresponding karyogram (b). The X chromosomes are indicated by red arrows and there's no Y chromosome, indicate normal XX female genotype. All examined chromosomes display normal morphology and have normal 64 chromosomes.

We used normal male and female horse DNA as a positive control to detect *SRY* in males and *AR* in both males and females. We also used water as a negative control that could indicate any possible DNA cross-contamination in the PCR reaction. *SRY* gene is only amplified from male DNA as we expected, and the *AR* gene was amplified

both in male and female DNA. There's no amplification in the negative control. The PCR-based sex determination of the H304 case indicates that it is negative for the SRY gene and positive for the AR gene, and it means normal female (Figure 2).

PCR analysis of sex chromosomes consistent with the karyotype data. In conclusion, the case of the H304 horse has a normal 64, XX female karyotype and no chromosomal abnormality was observed.

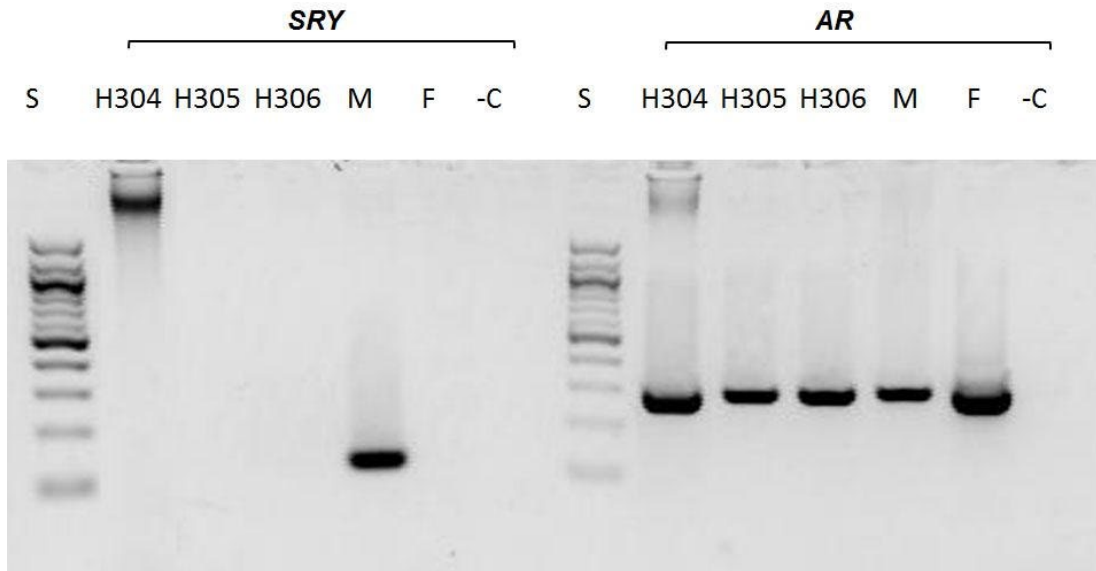


Figure 2. PCR-based sex determination using the SRY and AR primers. A set of lanes 1–7 contain three cases (H304, H305, and H306), one of each male and female horse as a positive control (M and F, respectively), negative control (-C), and DNA size marker (S). The SRY locus displays bands in the male samples, while the female individuals generate no 131 bp PCR fragments. A 294 bp PCR product corresponding to AR is detected in all male and female samples except for negative control. The positive male control displays the SRY amplification, while the female control has AR amplification. Lane S contains the 100 bp DNA size marker and Lane -C contains the negative control without any product, as expected.

Case H305: XY mare

The H305 was submitted for the examinations and the owner claimed it as a female with pea size ovaries with unknown infertile conditions. We analyzed 32 cells and 8 of them were karyotyped. G-band karyotype analysis revealed that all of the examined cells have 64 chromosomes with normal morphology, however, it has XY chromosomes instead of XX (Figure 3). The mare is genetically male.

PCR analysis indicates that H305 has a normal AR gene but there's no sign of the SRY gene (Figure 2). These cytogenetic and molecular analyses indicate that H305 which was initially claimed as a female is genetically male with a Y chromosome, but no SRY gene which is why the horse appears as a female. This condition is known as SRY-negative sex reversal, and it is invariably associated with sterility. So, the H305 mare's infertile issue can be directed to male-to-female sex reversal, which is infertile.

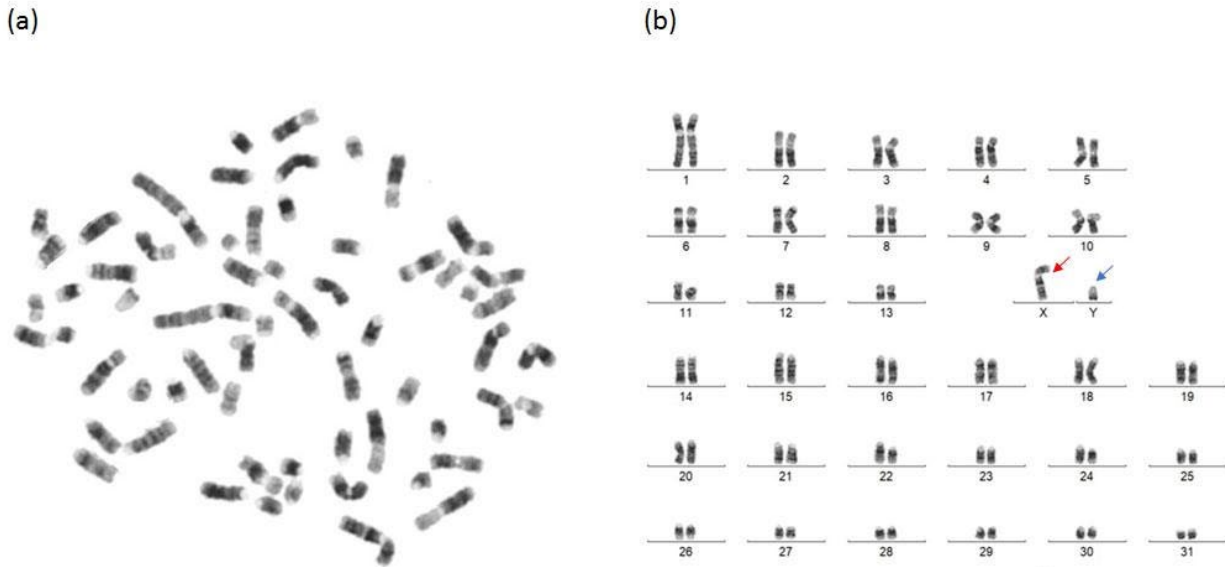


Figure 3. Cytogenetic characterization of the H305 mare horse. A Giemsa-staining metaphase spread (a) and the corresponding karyogram (b) of H305. The X chromosome is indicated by red arrow and Y chromosome is indicated by blue arrow, showing that H305 mare is genetically XY male. All examined chromosomes display normal morphology and have normal 64 chromosomes.

H306 case: XO mare

The H306 was submitted for the examinations for the infertility issues and ovarian tissues was not able to locate on rectal ultrasound examination. We analyzed a total of 35 cells and 6 of them were karyotyped. All examined cells show normal morphology, but it has 63 chromosomes with one X chromosome instead of two (Figure 4).

PCR test for the SRY gene was negative and positive for the AR gene, it is normal for female horses (Figure 2). In conclusion, the H306 horse has an abnormal 63, X karyotype in all cells analyzed. This condition is known as X-monosomy, a genetic disorder that occurs in females which means each cell in the individual's body has only one copy of the X chromosome instead of the usual two sex chromosomes and is invariably associated with sterility. The reason for the mare H306 infertile issue can be directed to a chromosome abnormality, X-monosomy.

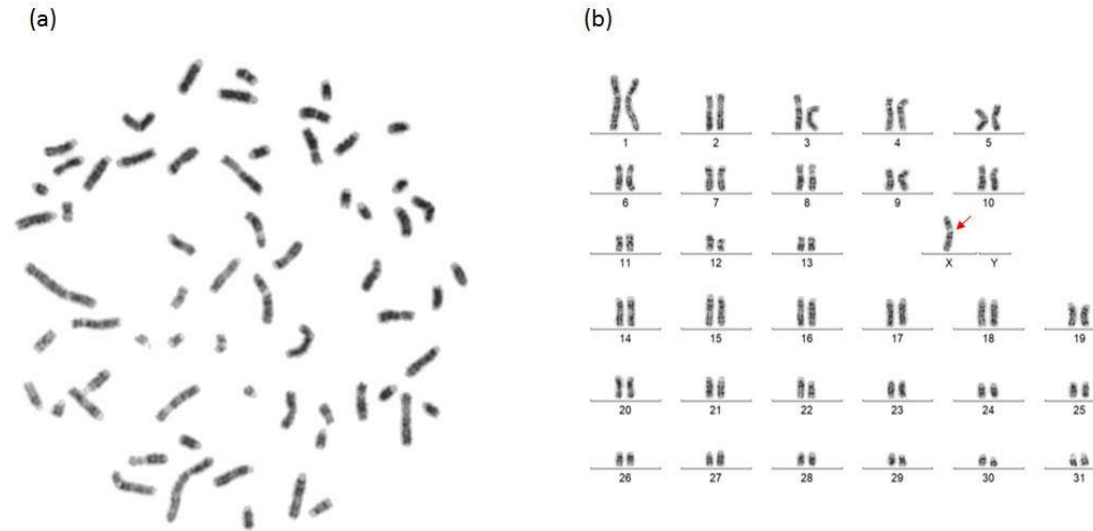


Figure 4. Cytogenetic characterization of the H306 mare horse. A Giemsa-staining metaphase spread (a) and corresponding karyogram (b) of H306. The X chromosome is indicated by red arrow and there is no Y chromosome. However, there's only one X chromosome instead of two, indicate H305 mare is genetically XO female, called X monosomy. All examined chromosomes display normal morphology and have 63 chromosomes.

Discussion

Sex chromosome abnormalities were first reported in the 1970s ever since the number of chromosomes of domestic horses was determined as $2n=64$ in 1959. Clinical cytogenetic analyses of horses have been improved by the strong desire to understand the underlying genetic basis of infertility issues and it became a powerful tool to study the chromosome abnormality in horses. Changes in the number or structure of chromosomes typically result in genomic imbalance and affect gametogenesis and the viability of zygotes and embryos. In horses, most abnormalities involved the sex chromosomes like in humans and other species [22]. Clinical cytogenetic analysis has been widely used to detect chromosome abnormality to answer the reduced fertility and infertility in horses and other species, because of rapid turn-around time, high sensitivity, and ability to localize in specific cells and tissue types to diagnose the abnormality.

However, conventional karyotyping is limited to detecting smaller than 5 Mb of DNA, and with the FISH technique, it can narrow down to up to 100 kb but still has a limit to detect small rearrangements. PCR is easy, accurate, and relatively faster than conventional karyotyping, and it can detect way smaller rearrangements than karyotyping. So, combining two techniques will compensate for the limits and can provide reliable data to determine the cause of the reproduction problem.

PCR-based sex determination is of great value, especially in H1305 case. In the H1305 case, cytogenetic analysis indicates the horse is male (64, XY), however, H305 displays phenotypically female. It was not possible to diagnose the cause of the sterility of H305 without a PCR examination. The H305 has a Y chromosome but it lost the maleness gene *SRY* which explains why the horse appears as a female. This is a typical XY *SRY*-negative sex-reversal case. Considerable research suggests that the *SRY* gene is both necessary and sufficient to initiate testis determination and the XY individual without *SRY* gene develops like female appearance, male karyotype, and female phenotype. Male-to-female sex reversal is a genetic disorder in which phenotypic females have a male genetic constitution not

only in horses but also in humans and other mammals. [23, 24] and is the second most common genetic sex abnormality in horses, after X chromosome monosomy [25]. It is not surprise we detected this male-to-female sex reversal in one of three cases when we considered it as a second most common genetic sex abnormality in horses.

Another interesting case is the H1306, 63 XO. Our G-banding analysis indicates that the H306 mare has only one X chromosome, X chromosome monosomy. Mares with X-monosomy have been reported to be smaller than average for their breed with normal karyotype, leading to the absence of the estrous cycle and sterility, and it is the most common genetic sex abnormality up to 35% of all chromosome abnormalities of sterility in the horse [26, 27]. In humans, women with one X chromosome are called Turner syndrome, experience a variety of medical and developmental problems including infertility issues, however, spontaneous pregnancy was seen in some individuals with Turner syndrome [28]. The cause of the reproduction problem of the H306 mare is more likely due to the X monosomy but we can't rule out the possibility other than X monosomy as we have seen spontaneous pregnancy in humans with the same/similar genetic condition.

A study of three cases of mare samples led us to the two most common chromosome abnormalities in equines which are XY sex -reversal and X monosomy. But we didn't find any possible explanation of the case H304, a normal XX female horse. We predict this particular female horse could either have a genetic issue other than chromosomal abnormality that can't be tested in our examination, or the mare naturally reaches the infertile stage. However, we can't make a clear conclusion as we don't have any further information other than the gender which the breeder has provided.

Taken together, Cytogenetics and molecular analysis are a powerful toolbox to diagnose chromosome abnormality in equine infertility. The recent progress of molecular cytogenetics along with the genomic approach could shed more light on the clinical diagnosis of reproduction not only in equine but also in other species, and the information will help horse breeders and equine practitioners to make the most informed decisions about the breeding plan and general health of horses.

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