sciendo

DOI: 10.2478/fv-2022-0028

FOLIA VETERINARIA, 66, 3: 60—66, 2022

CYTOGENETIC ANALYSIS OF A MARE AND HER FOAL WITH SUSPECTED GENETIC CAUSES OF DISABILITY

Kulíková, N.[1], Holečková, B.[1], Kakalejčíková, S.[1], Bučan, J.[1], Korim. F.[2]

[1]Department of Biology and Physiology

[2]Equine Clinic, University of Veterinary Medicine and Pharmacy in Košice

Komenského 73, 041 81 Košice

Slovakia

beata.holeckova@uvlf.sk

ABSTRACT

Hereditary diseases represent a serious problem in horses, especially in terms of sport use and breeding. Nowadays, we know the genetic basis of several breed-specific inherited diseases. In this study, we focused on the cytogenetic analysis of the clinical case of a healthy mare and her foal with numerous malformations in order to confirm or disprove the suspicion of genetic causes of a disability in this offspring. We used conventional metaphase staining to analyse chromosomal aberrations – breaks and gaps. In general, the number of breaks exceeding the norm (2—3 breaks/100 metaphases) may indicate the influence of the external environment with a potential teratogenic effect on the offspring during its mother gravidity. Compared to the norm, we found a slightly increased percentage of chromosomal aberrations in both the mother and the foal. As another method, we used karyotyping to assess the number and morphology of chromosomes, where in addition to conventional staining, we also applied differential staining of metaphases (G-banding). Multiplication, loss or rearrangement of chromosome segments are almost always associated with pathology. In the karyotypes we constructed, we observed changes in both individuals, compared to the international standard; in the mare, we probably recorded the mosaic form of her karyotype. In the foal, we found 64, XX with a suspected morphological change which was probably related to autosomal chromosome pair 31. The cytogenetic analysis of suspected individuals is also very beneficial for horse owners and breeders. Thanks to the combination of cytogenetic and modern molecular-genetic methods, we were able to identify individuals unsuitable for breeding.

Key words: cytogenetics; chromosomal aberrations; hereditary diseases; horse genetics

INTRODUCTION

Understanding the genome of the horse helps us in various areas such as: prophylactic, diagnostic and therapeutic. Currently, DNA tests can be used for 21 genetic diseases in horses. Genetic tests for predisposing genetic diseases of a particular breed are required for registration of individuals and for licensing of breeding stallions, and last but not least, the results of these tests have relevance in the: breeding, nutritional management, or training pro-

gram of an individual [9]. Progress in the study of equine genomics and the development of molecular methods have qualitatively improved clinical genetics, but as indicated by Szczerbal and Switonski [19], cytogenetic testing is still one of the first steps in suspecting an individual's genetic defects. Cytogenetics deals with the study of the organization of genetic material at the chromosomal level. The basis of classical cytogenetic analysis is the arrest of mitosis at the metaphase stage, when chromosomes are best observed. Chromosomes can be further classified according to different schemes, based on the differential staining techniques used [12]. Part of cytogenetics is also karyotyping [17]. The karyotype reflects copies of chromosomes from both the mother and the father arranged according to size and shape (morphology). All horses have a diploid number of autosomal chromosomes, i.e. each chromosome contains a copy from both the dam and the sire. The normal karyotype of a mare is referred to as 64, XX and that of a stallion as 64, XY [2]. Karyotypes can be used, for example, to study chromosomal aberrations. As mentioned by Ianuzzi et al., [11], chromosomal aberrations are more often detected in horses than in other domestic species and are normally associated with the sex chromosome pair in more than 95% of the cases.

The aim of our study was the cytogenetic analysis of a clinical case of a mare and her foal, which showed a suspected genetic disability.

MATERIALS AND METHODS

Blood collection and animals

The peripheral blood of a foal, the foal's dam and a healthy mare (negative control) collected in sterile tubes with anticoagulant (mostly heparin) were used as biological material for cytogenetic analysis. The dam of the foal was a 3.5 years old English Thoroughbred mare (racehorse), first-born, phenotypically normal, without clinical signs or trauma. The foal (female) appeared healthy after birth, but after a few days there was a significant deterioration in her condition; she bumped into objects, was unable to locate the mammary gland independently, did not respond to sound stimuli, appeared blind and deaf, did not urinate, and did not defecate. Joint swelling and general apathy were observed. After approximately 17 days, euthanasia was performed. As a negative control, the blood of a healthy, 13-year-old mare of the Norik of Muráň breed was used.

Blood cultivation

The culture medium (RPMI [Roswell Park Memorial Institute] 1640, foetal bovine serum, phytohaemagglutinin/pokeweed, and antibiotics/antimycotics) was prepared in sterile tubes; 0.2 ml of blood was added and incubated for 72 hours at 37 °C. Approximately 90 min. before the final termination of incubation, colchicine (0.5 mg.ml^{-1}) was applied to the tubes to stop cell division at the metaphase stage. At the end of the culture, the cells were centrifuged and the pellets were hypotonised with 0.075 M KCl solution and fixed several times with a fixative solution composed of methanol and acetic acid in a 3 : 1 ratio.

Preparation of slides for observation

For conventional staining of slides, the Giemsa stain in phosphate buffer pH 7 was used (3—4 minutes). Where possible, chromatid and chromosome breaks/gaps were evaluated in 100 metaphases under the light microscope (Nikon).

For the G-banding method, the slides were allowed to stand for 1, 2, and 5 days at laboratory temperature or 7 days at –20 °C. The slide groups thus prepared were treated with 0.1% trypsin solution (PAA Laboratories/Fisher Scientific), cooled to 4 °C for 5, 15, 30, and 60 seconds. Subsequently, the slides were rinsed briefly in PBS (pH 6.8) and transferred to Giemsa's dye solution for 4 minutes. The quality of G-bands was assessed using a light microscope with a camera (Motic Scientific, T025A) and photographic documentation obtained. At least 15 metaphases were examined for each of the days and time mentioned above.

Construction of karyotypes

Karyotypes were constructed according to an international standard based on well-defined species-specific criteria, according to which each chromosome pair has a clearly defined banding pattern [3].

Statistical evaluation

The frequencies of chromatid and chromosome breaks and gaps were statistically evaluated using chi-square test (Graph Pad Prism).

Ethical considerations

All procedures concerning the animals were performed in compliance with the national guidelines for animal care.

RESULTS

Evaluation of chromosomal aberrations (breaks, gaps) – Giemsa staining

In the first part of the paper, we focused on chromosome instabilities: chromosome and chromatid breaks, and gaps (Fig. 1, Fig. 2). In Table 1, we present the results of these analyses as a percentage of the aberrant cells. Compared to the norm, we found a slightly increased number of observed chromosomal aberrations in both the dam and the foal. These aberrations partially indicated the influence of external factors with a possible teratogenic effect on the offspring (foal) only in the case when breaks were considered together with the gaps (Table 1).

Metaphase staining by differential staining – G-banding method

In this method, we evaluated 15—30 metaphases of individuals in differentially stained slides where, based on the different affinities of heterochromatin and euchromatin for the dye, the chromosome appears as a continuous series of light and dark bands [12]. This method is very challenging as it involves biological material that responds individually to the denaturing effects of trypsin. It means that it is not always possible to use a one-size-fits-all procedure. Also, the biological material in the case of the foal was suboptimal, more difficult to collect and of lower quality due to the adverse health status of the individual (Fig. 3a, b).

Compilation and assessment of karyotypes

The karyotypes of the mother and her foal are shown in the Fig. 4 (Fig. 4a, b). In the mare we examined 100 Giemsa-stained metaphases and 30 G-banded metaphases from 5-days old slides (Fig. 3b). In one of the convention-

Table 1. Results of the evaluation of unstable chromosome aberrations in the dam and foal

Investigated individual	The number of examined metaphases	% Aberrant cells (Breaks ± SD)	% Aberrant cells (Breaks and Gaps ± SD)
Mother	100	3.0 ± 0.171a	4.0 ± 0.196a
Foal	70	4.3 ± 0.203a	7.1 ± 0.258a
Negative control	100	2.0 ± 0.140	3.0 ± 0.171

Fig. 1. Metaphase of a foal with a chromosome gap on an acrocentric chromosome. The gap is marked with an arrow
Photo: Kulikovâ (2022); 1000×

Fig. 2. Metaphase of a foal with a chromatid break on the metacentric chromosome. The break is marked with an arrow
Photo: Kulikovâ (2022); 1000×

ally stained metaphase we observed suspected Y chromosome (163 % of metaphases) (Fig. 4a). This could suggest that the mother could be mosaic in her lymphocytes (64, XX/64, XY). In the foal, we assume a karyotype 2n=64, XX, with a presumed aberration of the 31st chromosome (Fig. 4b).

DISCUSSION

In our study, we focused on cytogenetic analysis of cultured lymphocytes of a foal with multiple congenital malformations and her mother – a young mare who was a first-born. Congenital malformations are defined as defects in morphogenesis, which developed during intrauterine life, and are observed at birth [20]. Unlike inborn errors that are genetically determined, congenital defects may or may not be of genetic origin (Fig. 5). Therefore, in the first part of our work, we focused on the evaluation of chromosomal instability (breaks, gaps) as one of the possible causes of malformations in the foal. In general, the percentage of breaks (aberrant cells) exceeding the norm (2—3 breaks/100 metaphases) may indicate the presence of the environmental agent with a potential teratogenic effect on the offspring during intrauterine life (Fig. 5). Compared to the norm, we found only a slightly increased percentage of chromosomal aberrations in both mother and the foal. This suggested that the environmental influence was probably not significant as a cause of malformations in the foal. In the second part of the study, we focused on the construction of karyotypes using standard Giemsa staining of chromosomes and G-banding method that allowed the precise identification of individual chromosomes with their subsequent arrangement into specific homologous pairs in the karyotype. Our cytogenetic analysis revealed that 99 per cent of the blood lymphocyte metaphases contained two X chromosomes and one per cent a probable Y chromosome. This unusual mosaic karyotype of mare 64, XX/64, XY should be interpreted very carefully because for confirmation of the Y chromosome presence, it is necessary to use fluorescently labelled specific Y chromosome painting probe [1, 8, 16] and/or molecular methods such as PCR (Polymerase Chain Reaction).

In 1999, Bugno et al. [4] found karyotype 64, XX/64, XY in a fertile mare of the Wielkopolska breed, but it was not described as a mosaicism, but as a leukocytic chimerism (87 % XX : 13 % XY). This mare was phenotypically normal and gave birth to a normal foal at the seven years of age. Later, Bugno-Poniewierska et al. [5] reported about a case of sex chromosome mosaicism 64, XX/65, XXY/66, XXYY in a mare. The main difference between mosaicism and chimerism is that mosaicism is a condition when cell lines are derived from one embryo and are produced by unbalanced sister chromosome

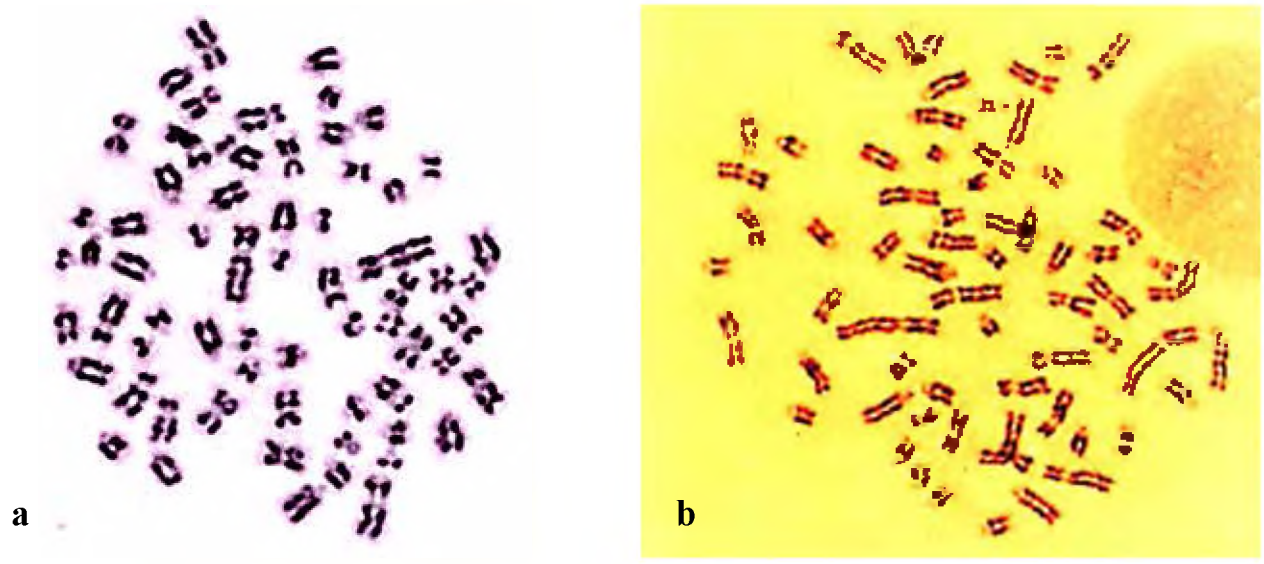

Fig. 3. The G-banding pattern of mare chromosomes, 1000×
a) 2-day old slide, 5 seconds of trypsin exposure; b) 5-day old slide, 20 seconds of trypsin exposure
Photo: Kulíková (2022) and Holečková (2022)

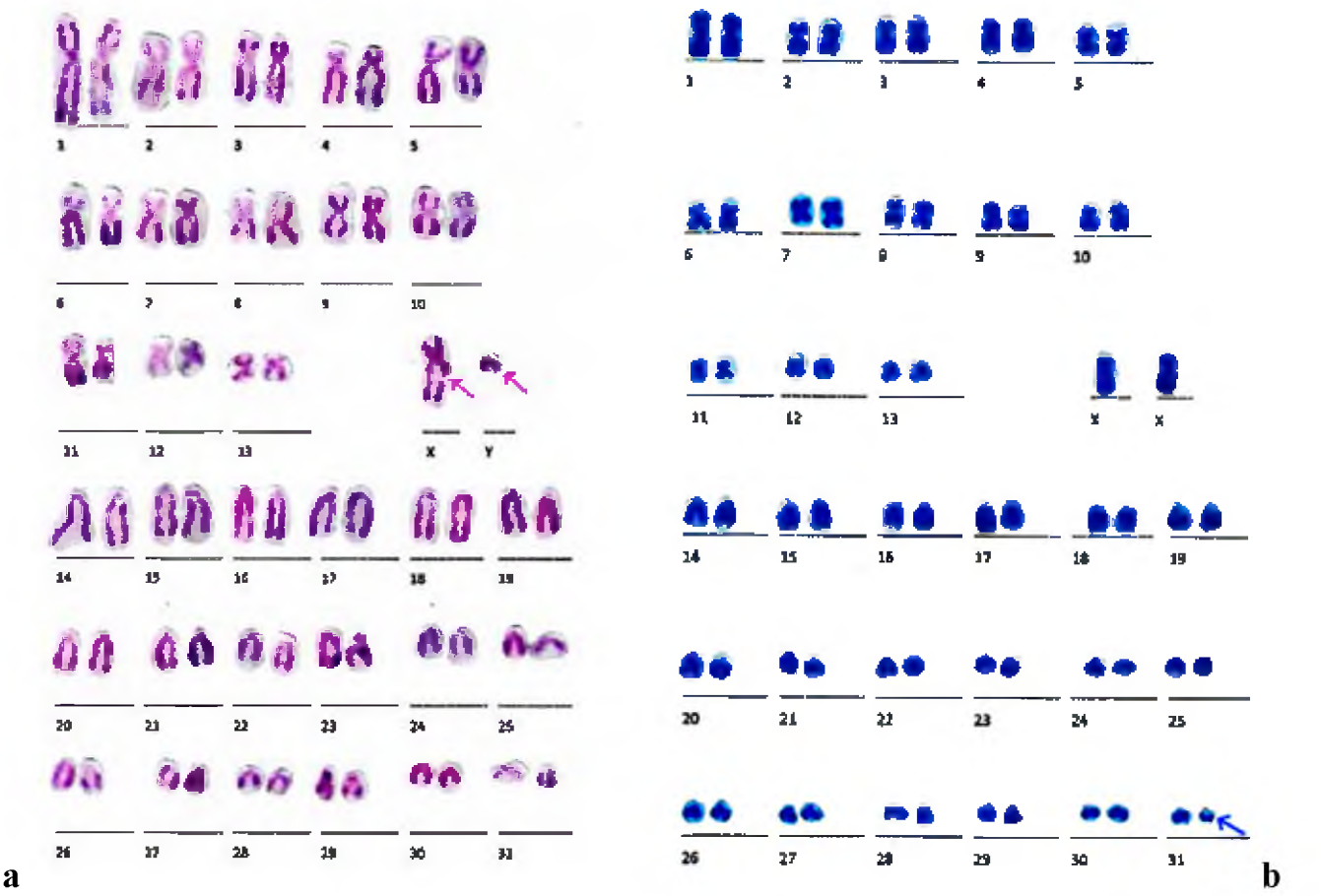

Fig. 4. Karyotypes of mother and her foal

a) Unusual mare karyotype – metaphase with a suspected Y chromosome (indicated by an arrow on the right). Only one per cent of the blood lymphocyte metaphases contained a probable Y chromosome and 99 per cent two X chromosomes. This indicated unusual mosaic karyotype of mare 64, XX/64, XY. Also, a chromatid gap on X chromosome was detected (indicated by an arrow on the left); b) Karyotype of a foal (2n = 64, XX) with a presumed aberration of the 31st chromosome (indicated by an arrow)

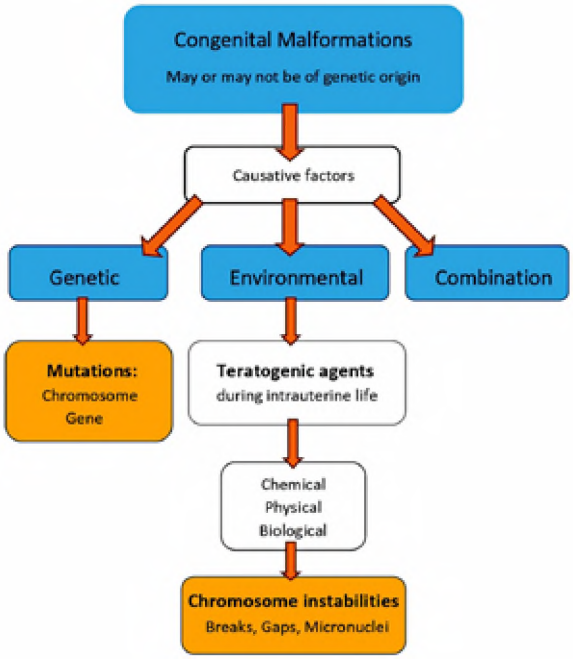

Fig. 5. Origin of congenital malformations adapted from the paper by C o r s e l l o and G i u f f r é [7]

segregation during the mitotic anaphase. On the contrary, chimerism is the presence of cell lines derived from different embryos (e. g. lymphocyte chimerism in heterosexual twins) [19]. Both conditions are usually linked with fertility troubles. As reported by B u g n o - P o n i e w i e r s k a et al. [6], large-scale cytogenetic surveys show that almost 30 % of horses with reproductive or developmental problems have chromosome aberrations, whereas abnormal karyotypes are found in only 2—5 % of the general population. Most chromosomal abnormalities described in horses are rare and occur in only one or a small number of individuals. One of the most recurrent abnormalities typical of horses is the occurrence of 63, XO (instead of 64, XX) in sterile mares [6], which has also been found in the 63, X / 64, XX mosaic, and the occurrence of 64, XY (SRY-negative) in sterile mares (disorder of sexual development – DSD – with sex reversal) [16]. Animals phenotypically appear female, but the karyotype corresponds to males [15]. Other rare chromosomal abnormalities are

trisomies 65, XXX and 65, XXY and also autosomal trisomies, which are usually the result of unequal segregation of chromosomes during meiosis. In horses, cases of trisomy of chromosomes 1, 3, 15, 20, 23, 24, 26 and 27 have been reported [6, 10] that have caused early pregnancy loss of the foetus, or congenital defects in live born foals. Trisomy of chromosome 31 (65, XY, +31) was reported in 1999 by L e a r et al. [14] in a thoroughbred colt with severe congenital defects involving musculoskeletal, urogenital and nervous systems.

According to D e m y d a - P e y r á s et al. [8], most of sex chromosome aberrations remain undiagnosed because of the complexity of the horse karyotype and because a number of animals carrying these anomalies have normal phenotypes. Cytogenetic methods are useful diagnostic tools for revealing chromosomal aberrations. However, to refine and confirm cytogenetic findings, the methods of fluorescent in situ hybridisation (FISH) using chromosome-specific probes [13, 18] and PCR (e. g., evidence of SRY gene) are very useful.

CONCLUSIONS

In our study of a foal with multiple defects, we found a slightly increased number of aberrant cells with chromosomal breaks and gaps compared to the negative control. This result only partially indicates a potential teratogenic effect of the external environment on the health status of the foal, as it was not possible to evaluate a sufficient number of metaphases. We also used the karyotyping method to assess the number, size and morphology of examined horse's chromosomes. Compared to the karyotype standard, we found variations in both karyotypes of the mare and the foal. This indicates the probable influence of genetic factors that could have caused the congenital malformations observed in the foal. However, further methods such as fluorescence in situ hybridization and modern molecular biology approaches would be needed to definitively confirm our findings.

ACKNOWLEDGEMENTS

We thank MVDr. V. Hura for providing blood samples. This study was supported by the grant VEGA 1/0166/21.

REFERENCES

1. **Anaya, G., Moreno-Millán, M., Bugno-Poniewierska, M., Pawlina, K., Membrillo, A., Molina, A., Demyda-Peyrás, S., 2014:** Sex reversal syndrome in the horse: Four new cases of feminisation in individuals carrying a 64, XY SRY negative chromosomal complement. *Anim. Reprod. Sci.*, 151, 22—27. DOI: 10.1016/j.anireprosci.2014.09.020.
2. **Bailey, E., Brooks, S., 2020:** Horse Genetics. CABI, UK, 452 pp.
3. **Bowling, A. T., Breen, M., Chowdhary, B. P., Hirota, K., Lear, T., Millon, L. V., Ponce de Leon, F. A., et al., 1997:** International system for cytogenetic nomenclature of the domestic horse. *Chromosome Res.*, 5, 433—443. DOI: 10.1023/a:1018408811881.
4. **Bugno, M., Slota, E., Tischner, M., Kozubska-Sobocinska, A., 1999:** A case of 64, XX/64, XY leukocytic chimerism in a fertile mare of the Wielkopolska breed. *Ann. Anim. Sci.*, 26, 9—16.
5. **Bugno-Poniewierska, M., Zabek, T., Semik, E., Pawlina, K., Tischner, M., 2014:** A case of sex chromosome mosaicism 64,XX/65,XXY/66,XXYY in mare. *Chromosome Res.*, 22, 400—401. DOI: 10.1159/000118750.
6. **Bugno-Poniewierska, M., Raudsepp, T., 2021:** Horse clinical cytogenetics: Recurrent themes and novel findings. *Animals*, 11, 1—26. DOI: 10.3390/ ani11030831.
7. **Corsello, G., Giuffré, M., 2012:** Congenital malformations. *J. Matern. Foetal. Neonatal Med.*, 1, 25—29. DOI: 10.3109/14767058.2012.664943.
8. **Demyda-Peyrás, S., Anaya, G., Bugno-Poniewierska, M., Pawlina, K., Membrillo, A., Valera, M., Moreno-Millán, M., 2014:** The use of a novel combination of diagnostic molecular and cytogenetic approaches in horses with sexual karyotype abnormalities: A rare case with an abnormal cellular chimerism. *Theriogenology*, 81, 1116—1122. DOI: 10.1016/j.theriogenology.2014.01.040.
9. **Finno, C., Divers, T., 2020:** *Equine Genetic Diseases*. Elsevier, USA, 424 pp.
10. **Ghosh, S., Kjöllerström, J., Metcalfe, L., Reed, S., Juras, R., Raudsepp, T., 2022:** The second case of non-mosaic trisomy of chromosome 26 with homologous fusion 26q; 26q in the horse. *Animals*, 12, 1—11. DOI: 10.3390/ani12070803.
11. **Iannuzzi, L., Di Meo, G. P., Perucatti, A., Spadetta, M., Incarnato, D., Parma, P., et al., 2004:** Clinical, cytogenetic and molecular studies on sterile stallion and mare affected by

XXY and sex reversal syndromes, respectively. *Caryologia*, 57, 400—404. DOI: 10.1080/00087114.2004.10589423.

12. **Ianuzzi, I., Di Berardino, D., 2008:** Tools of the trade: Diagnostics and research in domestic animal cytogenetics. *J. Appl. Genet.*, 49, 357—366. DOI: 10.1007/BF03195634.
13. **Kubíčková, S., Černohorská, H., Musilová, P., Rubeš, J., 2002:** The use of laser microdissection for the preparation of chromosome-specific painting probes in farm animals. *Chromosome Res.*, 10, 571—577. DOI: 10.1023/a:1020914702767.
14. **Lear, T. L., Cox, J. H., Kennedy, G. A., 1999:** Autosomal trisomy in a thoroughbred colt: 65, XY, +31. *Equine Vet. J.*, 31, 85—88. DOI: 10.1111/j.2042-3306.1999.tb03796.x.
15. **Millon, L. V., Penedo, M. C. T., 2009:** Chromosomal abnormalities and DNA genotyping in horses. *Fedequinas J.*, 57, 34—40.
16. **Neuhauser, S., Handler, J., Schelling, C., Pienkowska-Schelling, A., 2018:** Disorder of sexual development in a mare with an unusual tentative mosaic karyotype: 63, X/64, X del (Y). *Sex. Dev.*, 12, 232—238. DOI: 10.1159/000490861.
17. **Richer, C. L., Power, M. M., Klunder, L. R., McFeely, R. A., Kent, M. G., 1990:** Standard karyotype of the domestic horse (*Equus caballus*). *Hereditas*, 112, 289—293. DOI: 10.1111/j.1601-5223.1990.tb00069.x.
18. **Rubes, J., Pinton, A., Bonnet-Garnier, A., Fillon, V., Musilova, P., Michalova, K., et al., 2009:** Fluorescence in situ hybridization applied to domestic animal cytogenetics. *Cytogenet. Genome Res.*, 126, 34—48. DOI: 10.1159/000245905.
19. **Szczerbal, I., Switonski, M., 2016:** Chromosome abnormalities in domestic animals as causes of disorders of sex development or impaired fertility. In **Payan-Carreira, R.:** *Insights from Animal Reproduction*. IntechOpen, 252 pp. DOI: 10.5772/60829.
20. **Uzar, T., Szczerbal, I., Serwanska-Leja, K., Nowaczka-Woszuk, J., Gogulski, M., Bugaj, S., et al., 2020:** Congenital malformations in a Holstein-Friesian calf with a unique mosaic karyotype: A case report. *Animals*, 10, 1—9. DOI: 10.3390/ani10091615.

Received June 17, 2022
Accepted July 25, 2022