Karyotype evaluation among young horse populations in Poland

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Summary

Five hundred young horses of the following breeds: Thoroughbred, Silesian, Małopolska, Wielkopolska, Polish Konik, Hutsul, Shetland Pony, Half-bred Anglo-Arabian, Noble Half-bred, Fjord and crosses were cytogenetically investigated. Chromosome preparations obtained after lymphocyte culture were analysed using conventional Giemsa staining and CBG-banding methods. In the case of abnormalities GTG-banding as well as FISH technique were applied. In ten mares different karyotypic abnormalities were diagnosed. One mare showed chromosome chimerism (64,XX/64,XY), eight had sex chromosomal aneuploidy (one in pure line 63,X and seven in mosaic form 63,X/64,XX) and one presented autosomal aneuploidy with mosaicism (64,XX/65,XX,+31). The influence of sex chromosome abnormalities on fertility and the possible utilisation of karyotypic control in any selection programme are discussed.

Keywords: karyotype, X chromosomal monosomy, autosomal trisomy, chimerism, horse

Karyotypisierung einer Zufallspopulation junger Pferde

In der vorliegenden Studie wurde fünfhundert junge, polnische Pferde folgender Rassen zytogenetisch untersucht: Englisches Vollblut, Silesian, Malopolska, Wielkopolska, Konik, Hutsul, Shetland, Anglo-Arabisches Halbblut. Edles Halbblut, Fjord und Kreuzungen. Chromosomen-Präparationen basierten auf Lymphozyten-Kulturen und wurden mittels üblicher Giemsa-Färbung und CBG-Bänderungstechnik analysiert. Im Falle von chromosomalen Veränderungen wurde zusätzlich die GTG-Bänderungstechnik sowie FISH angewandt. Bei zehn Stuten fanden sich anormale Karyotypen. Eine Stute war Trägerin eines Chimärismus der Form 64,XX/64,XY, acht Stuten zeigten geschlechts-chromosomale Aneuploidie (eine in reinerbiger Form 63,X und sieben in Mosaikform 63,X/64,XX) und eine Stute fand sich mit autosomaler Aneuploidie mit Mosaik (64,XX/65,XX, +31). Der Einfluss von Chromosomenaberrationen auf die Fruchtbarkeit sowie die Einsatzmöglichkeiten der Karyotypisierung in Zuchtprogrammen werden diskutiert.

Schlüsselwörter: Karyotypisierung, X-chromosomale Monosomie, autosomale Trisomie, Chimärismus

Introduction

Cytogenetic investigations of horses, which has been carried out for more than 35 years, showed that sex chromosome abnormalities are a common karyotype aberration in this species, causing infertility or subfertility. The majority of the horse karyotypic abnormalities identified worldwide demonstrated X monosomy in the non-mosaic (63,X) or mosaic (63,X/64,XX) form (Power, 1990). Moreover, the sex-reversal syndrome (Power, 1990; Buoen et al., 2000) was also quite frequently diagnosed. On the other hand, X - chromosome trisomy appears to be rather rare in this species (Breen et al., 1997; Makinen et al., 1999, Wieczorek et al., 2001, Bugno et al., 2003). Also lymphocyte chimerism (64,XX/64,XY) was not often identified in horses in comparison with cattle (Power, 1990). Autosomal trisomy in the domestic horse is rare with only a few cases reported (Power, 1987; Klunder et al., 1989; Bowling and Millon, 1990; Lear et al., 1999). The frequency of karyotypic aberrations would be higher in mares chosen on the basis of their heavy disturbances of fertility.

The aim of the present paper is to analyse the karyotype of young horses, to identify the chromosomal aberrations and to evaluate their frequency in a random population of horses, which may be important for breeders.

Animals, Materials and Methods

Five hundred young horses (228 stallions and 272 mares) aged between one month and three years were

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investigated cytogenetically. They represented the following breeds: Thoroughbred (57), Silesian (21), Małopolska (30), Wielkopolska (63), Polish Konik (26), Hutsul (109), Shetland Pony (13), Half-bred Anglo-Arabian (127), Noble Half-bred (34), Fjord (5) and crosses (15). The animals originated from public and private studs and individual farms in Poland. The horses for cytogenetic investigation were chosen randomly, because it was known, that chromosome aberrations are not typical for any breed and are widely spread in the whole population.

About 10 ml blood samples were taken from the jugular vein into heparinized, vacutainer-type tubes. Lymphocyte cultures were prepared according to a modified method of Arakaki and Sparkes (1963) within 24 hours of blood sampling. To determine the diploid number of chromosomes, the preparations with metaphase spreads were conventionally stained with Giemsa in Sorensen's buffer (20 min) and observed under a light microscope equipped with a computer image analysis system (MultiScan 6.08).

In the second stage of analysis, CBG-stained chromosomes (Sumner, 1972) were observed for identification of the sex chromosomes. Fresh chromosome preparations were subjected to first denaturation by 1-hour exposure to 0.2 N HCl solution at room temperature and then eluted in distilled water of room temperature. This was followed by a second denaturation cycle, where the preparations were exposed to fresh 5% water solution of barium hydroxide ($BaOH_2 \times 8\ H_2O$) at 50° C for 3–5 min. After elution with distilled water at 50° C, the preparations were renatured. They were placed in 2 × SSC solution (0.3 N NaCl mixed with 0.03 N $Na_3C_6H5O_7 \times 2H_2O$) at pH 7.0 and 60° C for 1 hour. The preparations were stained with 5% Giemsa in Sorensen's neutral phosphate buffer for 30 min and then analysed under a light microscope. This method reveals regions of constitutive heterochromatin. Unlike the biarmed autosomes, the X chromosome has an additional band on the q arm, while the Y chromosome, which contains mainly repeat sequences, gets almost completely stained.

To analyse karyotypes of animals suspected of sex chromosome anomalies, the FISH technique was applied. The equine X whole chromosome painting probe was derived from flow-sorted chromosomes (Yang, unpublished). The author applied this technique, which was earlier described for the equine X chromosome by Breen et al. (1997). The biotin-labelled probe was applied on lymphocyte chromosome preparations. Briefly, the slides were denaturated in 70% formamide in 2×SSC at 42° C. The probe was denaturated at 70° C for 10 min. The hybridisation was carried out at 37° C overnight. Post-hybridisation washes were as follows: three times at 50% formamide in 2×SSC and three times in 2×SSC at 42° C. Hybridisation signals were detected by the avidin-FITC and anti-avidin system on propidium iodide stained slides. Microscopic evaluation was performed under a fluorescence microscope equipped with a CCD camera and Lucia software.

The GTG technique (Wang and Fedoroff, 1974) was used to identify pairs of homologous chromosomes. The preparations made were stored for three weeks at room temperature. Then they were exposed to 0.1 or 0.05% trypsin solution in a mixture of GKN (10 g glucose + 4 g KCl + 80 g NaCl + 3.5 g $NaHCO_3$ dissolved in 1000 ml distilled water) and versenate (8 g NaCl + 0.2 g $KH_2PO_4 \times 2H_2O$ + 0.2 g KCl + 3.12 g $Na_2HPO_4 \times 12\ H_2O$ + 0.2 EDTA in 1000 ml distilled water). Digestion time (4–8 min) was chosen individually according to trypsin concentration. The preparations were stained with 5% Giemsa solution in Sorensen's neutral buffer and analysed under a light microscope. According to the protocol 100 metaphase spreads were preliminarily analysed from each animal, and in the case of aberration the number of analysed spreads was increased from 176–500.

Results and Discussion

In the population of cytogenetically investigated horses 228 stallions and 262 mares showed the normal karyotype 64,XY and 64,XX, respectively. A further 10 mares in this group of animals were diagnosed for different chromosome abnormalities (Tab. 1). The percentage of anomalies was established as 2% in the whole population and 3.7% in the 272 mares. It has to be emphasized that this is the first large scale cytogenetic investigation in Polish horses, encompassing a randomly - chosen population of 500 animals. Interestingly all of the chromosomal abnormalities were found only in young mares. Non - mosaic X chromosome monosomy (63,X) was diagnosed in the five-month-old mare Puenta. In all 437 metaphases analysed with conventional Giemsa staining, GTG- and CBG-banding technique as well as a FISH method (Fig. 1), only one X chromosome was observed.

Because of the early stage of sexual development it was impossible to diagnose internal sexual organs, although phenotypically the mare appeared normal. Among the observed anomalies in the investigated horses X - monosomy in mosaic form was found in the majority of n = 7 cases. In each case 176-400 metaphases were analysed in order to estimate the percentage of aneuploid line, which was established to be low (2.5-25%). The different number of analysed spreads was due to the percentage of the second, aberrant line and the quality of spreads. All of the 63,X/64,XX mares were phenotypically normal; internal sexual organs of five of them mares were not examined, because of the early stage of development. The mare Grafini and Nasturcja, showed oestrus and normal development of sexual organs (Tab.1).

Table 1: Karyotype anomalies in horses.

mare name	breed	age in months	karyotype	number of analysed metaphase plates	% of metaphase plates with chromosome abnormality	dam's age	sire's age
Puenta	hc	5	63,X	437	100	15 year	14 year
Liana	hc	1	63,X/64,XX	200	25	8 year	11 year
Delijka	TB	17	63,X/64,XX	176	22	6 year	no data
Grafini	sp	24	63,X/64,XX	318	18	22 year	11 year
Dalciana	TB	11	64,XX/65,XX + 31	189	13	9 year	16 year
Ny_a	hc	2	63,X/64,XX	400	2,5	11 year	7 year
Galilea	xo	2	63,X/64,XX	300	4	12 year	11 year
Fingola	xo	22	63,X/64,XX	343	7	10 year	12 year
Nasturcja	wlkp	31	63,X/64,XX	385	11	8 year	no data
Tara	fjord	2	64,XX/64,XY	500	63	no data	no data

hc- Hutsul; TB- Thoroughbred; sp- crossbred; xo- Half-bred Anglo-Arabian; wlkp- Wielkopolska breed

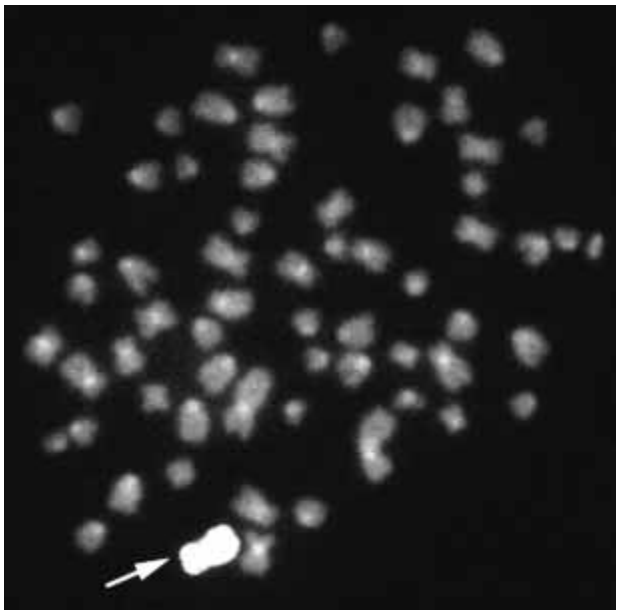

Figure 1: FISH-painted metaphase spread from the monosomic (63,X0) mare with the use of the equine X whole chromosome specific probe. The arrow indicate one X bright chromosome.

A review of cytogenetic studies in horses by Power (1990) clearly demonstrated that X chromosome monosomy is the most frequent chromosomal defect in this species. The review brought together the results of 1377 horse karyotype studies performed from 1968, when the first aberration was detected, until 1990. During that period, 401 cases of abnormal karyotype were detected in horses. Among those, 142 mares had X chromosome monosomy in pure form and 62 mares in the form of mosaic of two cell lines 64,XX and 63,X, the latter accounting for approximately 30% of all diagnosed cases. Mares which carry X chromosome monosomy have normal external genital organs, but usually a hypoplastic uterus and underdeveloped ovaries. Such individuals show no oestrus cycle or oestrus is manifested weakly and irregularly. In mares with X chromosome monosomy in the form of 63,X/64,XX mosaic, there were few cases of fertilization and birth of offspring (Halnan, 1985; Bugno et al., 2001; Wieczorek et al., 2001). It should be noted that these mares only gave birth to one foal each despite many breeding attempts and veterinary treatments. These cases can be attributed to the survival of single oogonia derived from the cell line with 64,XX or in exceptional cases, to the normal course of meiosis in X0 cells.

In the eleven-month-old mare Dalciana two cell lines were initially observed: one with normal karyotype 64,XX and another (6,88% of cells, Tab. 1) with one additional acrocentric 65,XX+?. The metaphase plates were analysed using CBG-, GTG- banding techniques. It was ascertained that in the second line trisomy of ECA31 was present (Fig. 2). The mare was phenotypically normal without any visible abnormalities or malformations. Autosomal trisomies in the domestic horse are rarely found in the literature, with only a few cases reported. The first was a Thoroughbred colt carrier of ECA 28 trisomy exhibiting small stature and cryptorchism (Power, 1987). Klunder et al. (1989) described a Standardbred colt with trisomy of ECA23 showing skeletal and testicular abnormalities. Bowling and Millon (1990) reported trisomy of ECA26 and of ECA30 in two Arabian fillies with angular limb deformation. Many congenital defects appeared in a Thoroughbred colt showing trisomy of ECA31 (Lear et al., 1999). On the other hand Kubień and Tischner (2002) described the reproductive success of a mare with a mosaic karyotype 64,XX/65,XX+30. The karyotypically mosaic mare delivered three phenotypically and karyotypically normal offspring and one dead colt (not analysed cytogenetically).

Some authors associate the occurrence of autosomal trisomy in new born foals with an aged mare. The dams of 5 from 6 trisomic foals cited from the literature (Power, 1987; Bowling and Millon, 1990; Zhang

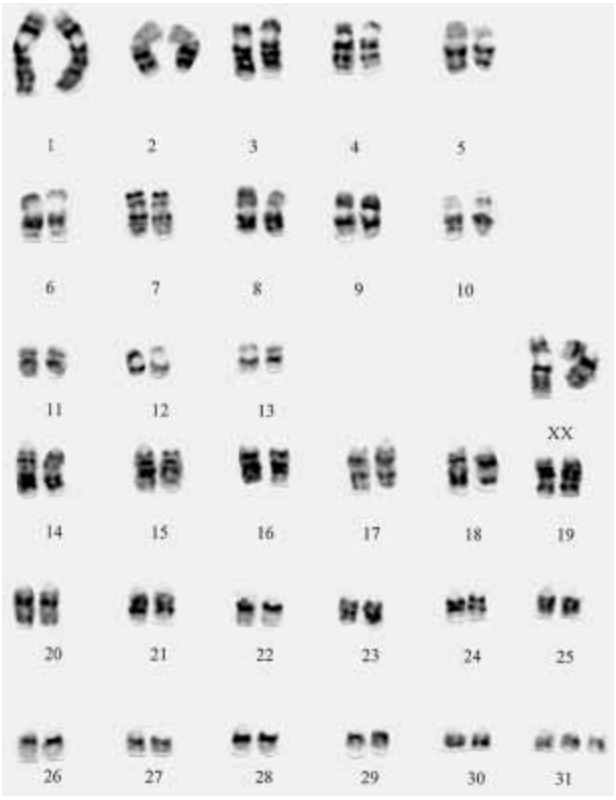

Figure 2: GTG-banded karyotype from mare (Dalciana) with autosomal trisomy 64,XX/65,XX+31.

et al., 1994; Buoen et al., 1997; Lear et al., 1999) were aged 14–28 years. Only one of the recorded trisomic offspring was born to a 3-year-old mare. In the case described here, the dam of the affected foal was 9 years old. It seems probable that the mosaic karyotype (64,XX/64,XX+31) of the foal Dalciana may have resulted from a postzygotic *de novo* event of unknown aetiology. In the two-month-old mare Tara, sex chromosome chimerism 64,XX/64,XY was diagnosed. The percentage of two lines 64,XY and 64,XX was evaluated to be 63% and 37% respectively. This anomaly is characteristic of animals originating from twin pregnancy, but there was no information about twins in this specific pedigree record. External sexual organs seemed to be normal, but internal organs were not examined because the filly was in early stage of sexual development. In this case routine metaphase plate staining was supplemented by CBG-banding (Fig. 3A, B) and FISH techniques.

Moreno-Millan et al. (1991) described two mares, carriers of the cellular XX/XY chimerism. In one of them rectal palpation revealed the presence of smaller ovaries, in another severe ovarian hypoplasia was found. The animals had no oestrus and did not respond to the conceptive treatment prescribed. Similarly, Parada et al. (1996) diagnosed XX/XY chimerism in two mares. One of them failed to become pregnant during 7 years of service, the other was eliminated from breeding after two seasons, due to infertility. By contrast Halnan (1989) reported the case of a mare with a 64,XX/64,XY karyotype and the deletion of the long arm of one X chromosome in all XX cells. The mare had a normal phenotype and ovaries but was not in foal, returning to service 3 times in the first season. At six years of age she was bred again using hormone therapy and produced a live foal. Furthermore Bugno et al. (1999) described one mare of the Wielkoposka breed with 64,XX/64,XY leukocytic chimerism. The external examination of the mare showed normal female appearance. She became pregnant first at the age of seven although she was treated and inseminated over four seasons and gave birth to a normal foal.

Individual differences in fertility in horses with a 64,XX/64,XY karyotype could be explained in the following way. In contrast to the conditions in cows, chorio-vascular anastomosis in twin equine pregnancies rarely causes maldevelopment of the genital or-

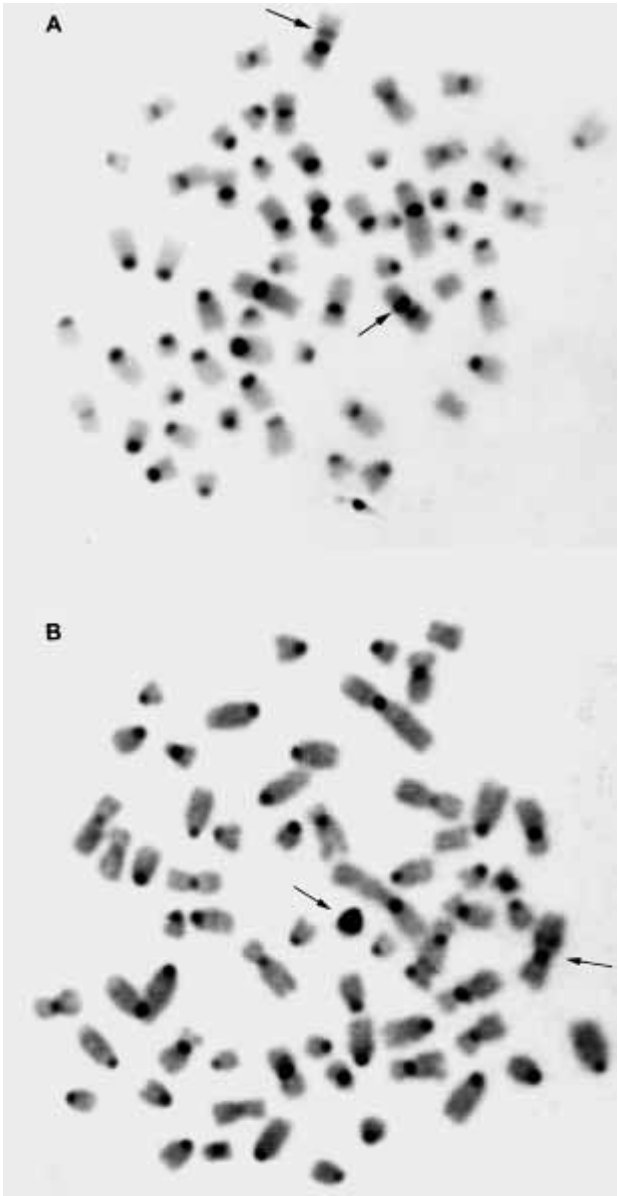

Figure 3: Metaphase spreads originating from the chimeric mare A= 64,XX; B=64,XY. The arrows indicate sex chromosomes.

gans, probably because the anastomosis occurs after sex differentiation in the fetuses as described by McFeely, (1975). It should be underlined that twins are born very rarely in horses; however, polyovulations and twin pregnancies in different horse populations range from 4–44% in frequency (Ginther et al., 1982). It is almost the rule that one embryo or fetus dies at some stage of pregnancy and is aborted. For this reason the breed records contain no information about the twin origin of some horses and the chimerism could be observed in horses from single birth.

Conclusion

Cytogenetic investigations in horses is more difficult to conduct than in other farm animals mainly because of their value, for breeding and using as sport animals. The percentage of diagnosed chromosome aberrations in the present mare population (about 4%) suggests the usefulness of cytogenetic examination of mares. Early diagnosis of chromosome abnormalities is very important as it eliminates aberration carriers and thus reduces the economic losses due to unsuccessful mating or hormone treatment.

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Typisation du caryotype d'une population de jeunes chevaux choisis au hasard

Dans la présente étude 500 jeunes chevaux polonais ont été examinés du point de vue cytogénétique. Ils appartenaient aux races Pur-sang anglais, Silésien, Malopolska, Wielkopolska, Konik, Hutsul, Shetland, Anglo-arabes, Demi-sang, Fjord et croisés. Des préparations de chromosomes basées sur des cultures de lymphocytes ont été analysées par une coloration de Giemsa usuelle et par la technique des bandes CBG. Dans les cas de modifications chromosomiques, la technique des bandes GTG ainsi que FISH a été utilisée. Des caryotypes anormaux ont été decouverts chez 10 juments. Une jument était porteuse d'un chimérisme de la forme 64,XX/64,XY, 8 juments présentaient une aneuploïdie des chomosomes sexuels (l'une dans la forme pure 63,X et 7 dans la forme mosaïque 64,XX/65,XX,+31. L'influence des aberrations chromosomiques sur la fertilité ainsi que la possibilité d'utiliser le caryotype dans les programmes d'élevage sont discutées.

Cariotipizzazione di una popolazione campione di cavalli giovani

Nello studio qui citato dono stati esaminati sotto il profilo citogenetico cinquecento giovani cavalli polacchi delle razze seguenti: Purosangue inglese, Silesiani, Malopolski, Wielkopolski, Konik, Hutsul, Shetland, Mezzisangue anglo-arabi, Mezzisangue puri, Fjord e incroci. Preparati cromosomici su base di culture di linfociti sono stati analizzati tramite la normale colorazione Giemsa e la tecnica di bandeggio CBG. In caso di mutazioni cromosomiali sono stati utilizzati inoltre la tecnica di bandeggio GTG e FISH. In 10 giumente sono stati riscontrati cariotipi anormali. Uno giumenta era portatrice di un chierismo della forma 64,XX/64,XY, otto mostravano degli aneuploidi dei cromosomi sessuali (uno forma ereditata 63,X e sette a forma di mosaico 63,XX/64,XX) e una giumenta si trovava con un autosomiale aneuploide con mosaico (63,XX/65,XX,+31). Sono in discussione l'influsso di aberrazioni cromosomiche sulla fertilità e le possibilità di aggiunta della cariotipizzazione nei programmi di allevamento.

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