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CHROMOSOMAL ANALYSIS AND BLOOD TYPE EXAMINATION OF MULTIPLE BIRTHS IN EQUINE

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A total of 5 dams and 16 cases of multiple births, that is, one set of male twins, one set of female twins, a partner of female twins, 4 sets of heterosexual twins and 3 cases of a male partner of heterosexual twins were examined cytogenetically and blood typically.

The sexual appearances of all of the foals coincided with their sex-chromosomal constitution, and even in the heterosexual twins, there were no sex-chromosomal chimerisms found.

Blood type examination revealed that out of all the cases of multiple births, only one male partner of a set of heterosexual twins showed chimerism.

Among heterosexual multiple births of cattle, a large number of female fetuses are sterile freemartins embryologically. Various reports are available on the relation between multiple fetuses and chromosomal or blood type chimerism in cattle (MARCUM, 1974); however, little research has been done on cases of multiple births in equine because there are no cases showing deficient breeding ability or congenital anomalies, although the development of foals may sometimes be delayed.

In the present study, 16 Thoroughbred and Arabian foals from 10 sets of multiple births and their 5 dams were examined chromosomally and blood typically.

MATERIALS EXAMINED

The mares subjected to examination were Thoroughbred and Anglo-Arabs raised on ranches in the Hidaka area, Hokkaido, Japan. One set of male twins, 2 sets of female twins, and 7 sets of heterosexual twins and their respective dams were examined. However, some of the foals were dead at the time of examination, and some of the mares were already disposed of or sold, thus making such animals unavailable for examination, and the actual number of available animals was 10 sets, 21 cases as shown in table 1.

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TABLE 1 *Chromosomal analysis of multiple births*

NO.	COMBINATION OF MULTIPLE BIRTHS	SEX OF INDIVIDUAL EXAMINED	BREED	BIRTH DATE	CHROMOSOME	
					Karyotype	A number of cells observed
1	♂ ♂	♂	Arab	780428	64, X Y	27
2		♂			64, X Y	35
3	♂ ♀	♂	Thorough	780520	64, X Y	38
4	♂ ♀	♂	Thorough	780517	64, X Y	24
5		♀			64, X X	32
6	♂ ♀	♂	Arab	780520	64, X Y	34
7		♀			64, X X	32
8	♂ ♀	♂	Thorough	790502	64, X Y	30
9		♀			64, X X	30
10	♂ ♀	♂	Arab	790429	64, X Y	30
11		♀			64, X X	30
12		D		660515	64, X X	30
13	♂ ♀	♂	Thorough	790525	64, X Y	30
14		D		710311	64, X X	30
15	♂ ♀	♂	Thorough	730530	64, X Y	30
16		D		690420	64, X X	30
17	♀ ♀	♀	Thorough	790415	64, X X	30
18		♀			64, X X	30
19		D		700409	64, X X	30
20	♀ ♀	♀	Arab	790510	64, X X	30
21		D		700515	64, X X	30

Notes: D shows the dam of multiple birth, ♂ & ♀ show the sex of foal.
Arab shows Anglo Arabian horse, and Thorough shows Thoroughbred horse.
780428 in birth date, for examble, means April 28, 1978.

Chromosomal analysis: The present cases were chromosomally analyzed by using cultured leucocytes. Blood was collected in sterile tubes containing 10-20 IU/ml of sodium heparin in aqueous solution. Whole blood was mixed into tissue culture medium (Eagle's minimum essential medium, pH 7.0, 3 ml), then supplemented with calf serum (0.3 ml) and phytohemagglutinin-M (0.1 ml). The cultures were incubated at 37°C for 72 hours. Colchicine was added to the cultures for 90 minutes prior to harvesting the cells, and cell division was halted in metaphase. Following exposure to colchicine, the cultures were centrifuged at 1300 rpm for 5 minutes. After hypotonic treatment, the cells were fixed in Carnoy's solution (acetic acid 1 volume, methylalcohol 3 volumes) for 20 minutes, and then the fixative solution was replaced 3 times by centrifugation. The final sediments were floated in a few volumes of the fresh fixative solution and 2 - 3 drops of cell suspension were dropped onto a slide. After flame drying of the cells, the slide was stained with giemsa solution and the preparations obtained microscopically observed. For karyotyping, the well-spread metaphase plates were photographed, and chromosomes on the photographic paper were cut out and arranged according to the usual manner.

Blood type examination: The "Method for examination of horse blood and evaluation standards" set forth by the Laboratory of Racing Chemistry was followed approximately. Namely, for checking erythrocytes, agglutination reaction and hemolysis reaction were used, and for analysing blood serum using starch-gel electrophoresis, serum protein types were classified. Furthermore, regarding the blood chimerism, specific antisera (anti-Aa, anti-Ac, anti-Qa, anti-Ua, anti-Pa, anti-Pu6 together with anti-Ca) were used to conduct residual blood corpuscle tests by hemolytic reaction. The following is the method used to determine the presence of chimerism using the residual blood corpuscle test. After the usual hemolytic reaction had been conducted and incomplete hemolysis (90 minutes after complete hemolysis is not seen) was seen, the absorption test was started. As a result, when the blood type factor was positive, an additional hemolysis reaction was performed on the residual blood corpuscle test, and in such cases, if the residual blood corpuscle tests were negative, a chimerism was considered to exist. The blood corpuscle adjudged to be a blood type chimerism was measured using a wave length of 540 nm to determine the ratio of positive corpuscles to negative corpuscles.

Results

The chromosomes of a normal horse consist of 62 autosomes and 2 sex-chromosomes, that is, 13 pairs of meta- and submetacentric autosomes, 18 pairs of acrocentric autosomes, one submetacentric X chromosome and one acrocentric Y chromosome in the male. The X chromosome is larger and most easily identifiable of all the chromosomes. The Y chromosome is acrocentric and is the smallest of all the chromosomes. The autosomes are divided into meta- and submetacentric chromosomes from the largest to the smallest, as are the acrocentric chromosomes, while the sex-chromosomes are

FIGURE 1 *Normal karyotype in male horse*, 64, XY

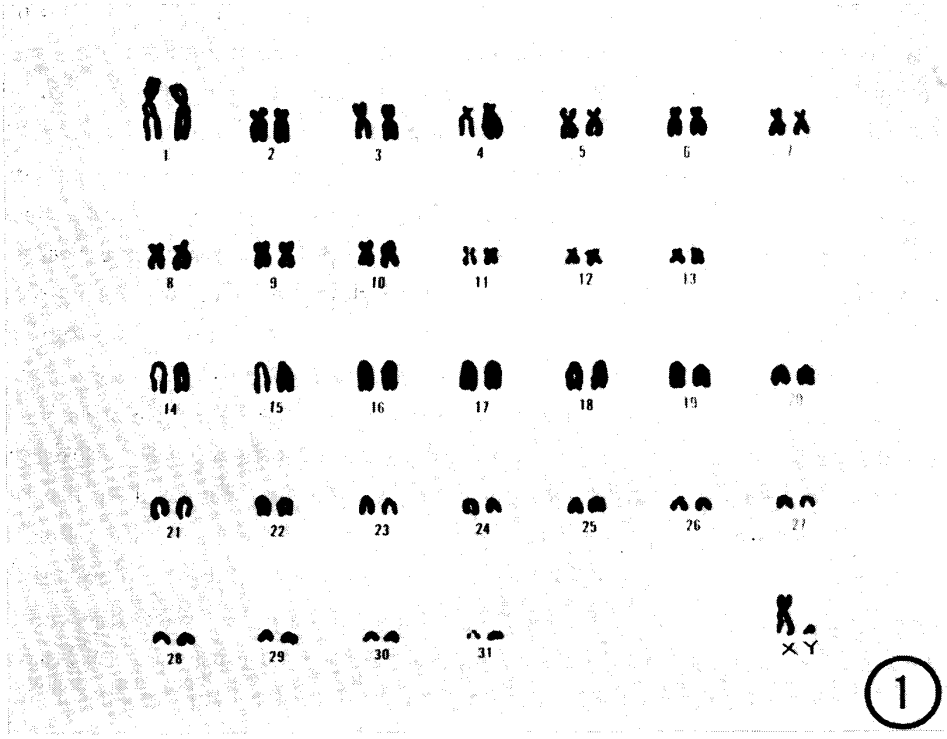
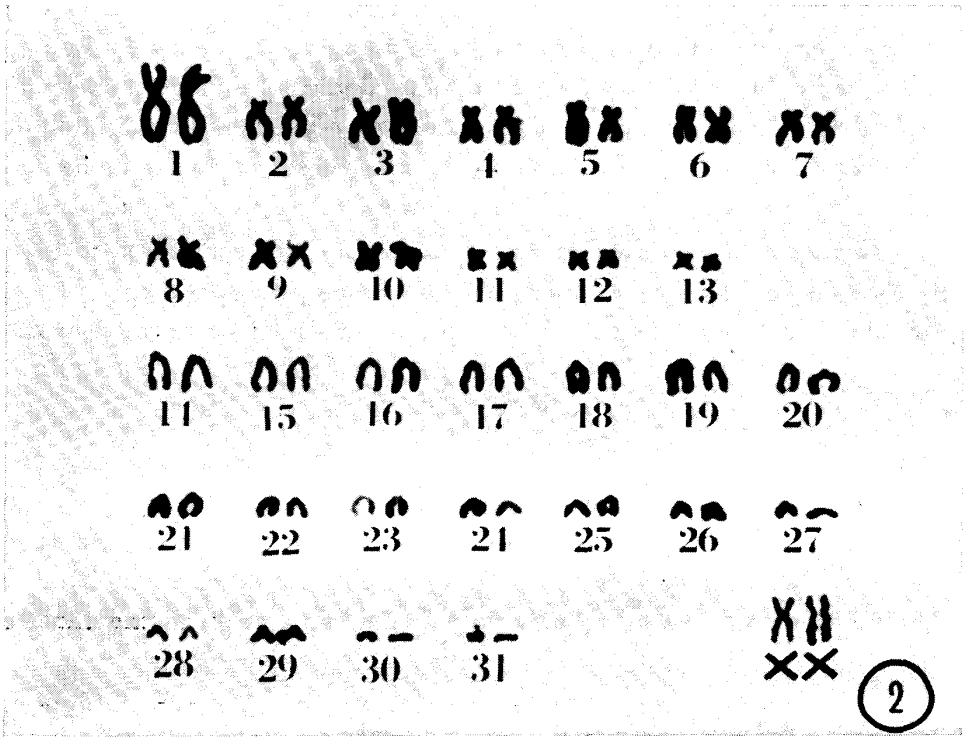


FIGURE 2 *Normal karyotype in female horse*, 64, XX



arranged with the karyotype showing. Unfortunately, there is no international standard regarding chromosomal numbering and pairing in the horse. The karyotype of normal chromosomes is shown in figures 1 and 2.

Sixteen foals from 10 sets of multiple births and their 5 dams were subjected to chromosomal analysis and the results are shown in table 1. The constitution of chromosomal karyotyping coincided with sex differentiation, and even in the heterosexual twins, sex-chromosomal chimerisms such as those seen in cattle were not recognized.

The results of blood type testing are shown in table 2. Among them, in Pa of Nos. 8 & 9, hemolysis reaction was incomplete; however, when an absorption test was

TABLE 2 *Erythrocyte and serum protein type analysis of multiple births*

NO.	ERYTHROCYTE TYPE												SERUM PROTEIN TYPE				CHIME-RISM
	agglutination reaction						hemolysis reaction										
	Aa	Da	Ca	Dc	Db	Dg	Aa	Ac	Qa	Ua	Pa	Pu6	Tf	Alb	Es	Pre	
1	+	−	−	−	−	−	+	−	−	+	−	−	AB	BB	I I	L L	−
2	+	−	+	−	−	−	+	−	−	+	−	−	B B	B B	I I	L L	−
3	+	−	+	+	−	+	+	−	+	+*	−	−	AD	AA	I S	I L	+
4	+	−	+	+	−	+	+	−	+	+	−	−	AB	B B	F I	L L	−
5	+	−	+	+	−	+	+	−	+	+	−	−	A E	B B	I I	L L	−
6	+	−	+	−	−	−	+	+	−	−	−	−	AB	B B	I I	F L	−
7	+	−	+	−	−	−	+	−	+	−	+	+	B D	B B	I I	F L	−
8	+	−	+	+	−	+	+	−	+	−	−	−	AD	B B	I I	L L	−
9	+	−	+	+	−	+	+	−	+	−	−	−	D D	AB	I I	L L	−
10	+	−	+	−	−	−	+	−	−	+	+	+	B E	B B	I I	L L	−
11	+	−	+	−	−	−	+	−	−	−	+	−	AA	AA	I I	L L	−
12	+	−	+	−	−	−	+	−	−	+	+	+	AB	AB	I I	L L	−
13	+	−	+	+	−	+	+	−	+	+	+	+	B B	B B	I I	L L	−
14	+	−	+	+	−	+	+	−	+	+	+	+	AB	AB	I I	L L	−
15	+	−	+	+	−	+	+	−	−	−	−	−	A E	B B	I I	L L	−
16	+	−	+	+	−	+	+	−	−	−	−	−	A E	B B	I I	L L	−
17	+	−	+	+	−	+	+	−	+	−	+	−	AA	B B	I I	L L	−
18	+	−	−	+	−	+	+	−	+	−	+	−	AB	B B	F I	L L	−
19	+	−	+	+	+	+	+	−	+	−	+	−	AB	B B	F I	L L	−
20	+	−	+	+	−	+	+	−	+	+	−	−	AB	B B	F I	F L	−
21	+	−	+	+	−	+	+	−	+	+	+	−	AB	AB	F I	F L	−

Notes: * showed an incomplete reaction by hemolysis and that the result of the absorption test was positive.
No. 3 was judged as chimerism by the residual corpuscle test.

conducted on the antiserum, the test results showed negative and chimerism was denied. No. 3 showed an incomplete reaction against anti-Ua, and since the result of the absorption test was positive, the residual corpuscle test was conducted. As a result, as shown in the following, the residual corpuscle test against anti-Ua was negative; this clearly showed that the corpuscles in No. 3 were a mixture of 2 types of positive corpuscles and negative corpuscles, and thus a blood type chimerism was judged. Furthermore, the ratio of positive corpuscles to negative corpuscles was 6 : 4.

DISCUSSION

In cattle, a large number of heterosexual twins (89.5−93.6%, N=532, $1-2\alpha=0.90$) show chimerism of their sex-chromosomes in their leucocytes, and from an embryological view point, fetuses that are to become female become sterile freemartins (MARCUM, 1974). The present investigation on heterosexual multiple fetuses among horses showed no sex-chromosome chimerism. BOUTERS & VANDEPLASSCHE (1970) investigated 34 cases of dizygotic twins in horses. They observed the onset of choriovascular anastomosis after the 7th month of pregnancy and the presence of blood type and chromosomal chimerism; however, no anatomical abnormalities were seen in the female reproductive organs. In their subsequent work, the above researchers increased the number of cases and conducted further investigation on 51 cases of dizygotic twins; however, contrary to the common belief, they found choriovascular anastomosis in 50% of the twin horse fetuses.[9] In the present study, with the exception of case No. 3, no cases of chimerism were seen either in the sex-chromosomes or in the blood types, and the composition of sex-chromosomes showed a complete coincidence with the sexual expression. According to the owner, No. 3 consisted of triplets including one acardius; the dam that gave birth to the triplets died before the examination. The sire that survived was examined, and the sex-chromosomes failed to show chimerism. Because of the choriovascular anastomosis, no evidence of chimerism was recognized in the sex-chromosomes of the leucocytes despite the fact that blood chimerism was revealed. We have encountered the opposite phenomena in cattle where chimerism was present in the sex-chromosomes of the leucocytes (XX/XY) but not in the blood type (MIYAKE et al., 1980). However, since the examined sample of the sex-chromosomes differed from that of the blood type, further studies need to be done.

Whereas blood type chimerism has been reported hitherto in cattle,[4,5,9~11] man,[15] sheep[8,12,13] and the silky marmoset,[1] there are only 4 cases of horses having such reported by VANDEPLASSCHE et al. (1970). In the diagnosis of freemartins in the females of heterosexual twins in cattle, an examination of blood type chimerism is usually made, because 2 types of erythrocytes arising from choriovascular anastomosis in the embryonic period are produced, and they form natural grafting of the stem cells. Similar reports of this nature in horses are very rare. Cattle and horses both have their

origin in placenta epitheliochorialis; however, since the former has a cotyledonary placenta and the latter a diffuse one, placenta type does not seem to be the cause of blood chimerism. Moreover, the genesis of blood vessels and the distributions of the allantochorion membrane also differs between cattle and horses, but again it is not known whether these differences cause blood chimerisms. Even if the frequency of chimerism is the same between cattle and horses, chances of making blood type examinations of twins in race horses is limited as most twins are disposed of after birth, because their development is delayed, thus making them valueless as race horses. Therefore, in the data from blood type examinations as a whole, there is a strong possibility that blood type chimerism is overlooked because twins are commonly dealt with as a single offspring. In the present report, case No. 3 was examined as a single offspring and not as twins since the blood sample showed incomplete hemolysis against anti-U_a, and blood type chimerism was judged from the results of the residual erythrocyte test. The state of delivery was investigated, and the aforementioned heterosexual triplet was revealed, but the blood type of the remaining two heads could not be obtained. As in the case of cattle, natural grafting of the stem cell occurred because of blood vessel anastomosis during the fetal period; this probably gave rise to the chimeric phenomenon. In the present examination in which the blood type was examined in 16 heads including twins, 1 head of blood type chimerism was recognized. If the number of antiserums subjected to blood type tests were increased, it is possible that many more type tests will be conducted, and thus more chimerisms will be found. On the other hand, we do not know why there are fewer blood type chimerism in horses than in cattle. This may be due to the difference in the reproductive characteristics of horses and cattle. Further studies on this point are warranted.

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