Guidelines for the interpretation of blood typing tests in horses

by
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At the Second Workshop on Thoroughbred Blood Typing Standardization, held under the auspices of the International Society for Animal Genetics (ISAG) at the University of Queensland, Brisbane, Australia, July 1989, it was decided that guidelines for uniform exclusion criteria in horse blood typing should be prepared. The decision was taken in response to a request from the International Stud Book Committee (ISBC). Dr. Kaj Sandberg, Uppsala, Sweden, was elected to draw up the guidelines.

At subsequent workshops held in connection with ISAG Conferences every two years these "Guidelines for the interpretation of blood typing tests in horses" have been reviewed and discussed by a group composed of representatives from all major laboratories in the world providing a horse blood typing service and a consensus has been reached on the contents. The guidelines are revised every two years.

Guidelines for the interpretation of blood typing tests in horses.

Introduction

The use of blood typing for parentage control, for solving of paternity problems and for identification is a routine procedure within the horse breeding industry in several countries. The total number of horses subject to blood typing each year in the world is about 250000. International trade with horses as well as transport of horses between countries for racing is increasing. Artificial insemination (AI) with transported semen and embryo transfer are techniques that are gradually being introduced into horsebreeding. Thus there is a current and increasing need for efficient and reliable means to check parentage and identity in horses.

The economic and legal consequences of a blood typing test which reveals an erroneous paternity or a false identity of a horse are often far-reaching. It is therefore of utmost importance that the test results are reliable and that the conclusions drawn from the results are correct.

It is the aim of the present report to consider various aspects of the reliability of the blood typing test and to outline some criteria and rules for the guidance of persons in charge of laboratories providing a horse blood typing service.

Tests based on DNA analysis or examination of the ELA system (Equine Lymphocyte Antigens) will not be considered.

The blood typing test

In most laboratories providing a conventional horse blood typing service two different kinds of tests are employed: **serological tests** which are based on the determination of antigens (blood group factors) on the red blood cells and **electrophoretic tests** which are based on the determination of genetic variants of some proteins in the blood (protein polymorphism).

The serological tests

Direct hemagglutination and hemolysis are the two kinds of serological test reactions normally used to determine the blood factors of a horse. Each factor is identified by a monospecific antiserum (reagent) containing a single population of antibodies reacting with that factor only. Such reagents are not commercially available and have to be produced by planned immunization or be occasionally collected from mares immunized by the blood of their own fetuses.

At present (July, 1996) altogether 34 equine blood group factors are internationally recognized. They belong to seven genetic systems. Each system contains from two to 25 recognized alleles (alternative genes; Table 1). In some laboratories also new, experimental factors are included in the test. These factors and the antisera that detect them are not yet internationally recognized and have got provisional designations.

The electrophoretic tests

Protein polymorphism is studied by electrophoresis. By this technique the protein variants are separated according to their electric net charges, isoelectric points and molecular sizes and shapes under the influence of an electric current. The separation takes place in a gel made of agarose, starch or polyacrylamide. Optimal resolution of protein fractions is obtained by varying the experimental conditions with respect to gel concentration, buffer composition, pH, voltage,

temperature and running time. Isoelectric focusing (IEF) is a special type of electrophoresis which is performed in a gel with a continuous pH gradient and separates the proteins according to their isoelectric points. There are also techniques in which protein separation can be carried out in two dimensions.

After separation the protein fractions are visualized by staining the gel with general protein stains or by specific activity stains.

The total number of internationally recognized electrophoretic systems in horses is 16 (Table 2). In addition to these systems there are a few others described in literature but not yet internationally recognized.

Bases for tests of parentage and identity

Blood group factors and blood protein variants are jointly called **genetic blood markers.** Blood markers used for identification, parentage control and solving problems of questionable maternity or paternity have the following characteristics in common. The blood markers are qualitative traits with a simple and direct inheritance which implies that they are dominant or codominant characteristics; are fully developed at birth or shortly thereafter and remain unchanged throughout life; are controlled only by heredity and are not influenced by environmental changes and are detected by objective and reliable tests. A basis for the reliability is of course that the tests are performed and interpreted by persons who are fully qualified through education and experience with the methods. They must also be familiar with the genetics of the blood markers and be aware of apparent exceptions and pitfalls involved in the blood typing test.

Parentage tests based on blood typing is founded on the principle of **genetic exclusion.** There are three principal rules that apply to the inheritance of the blood markers:

1/ An individual cannot have a blood marker unless at least one of its parents has the same marker.

2/ When an individual, according to its genotype, is homozygous with regard to a gene controlling a blood marker, all its offspring must invariably have that marker.

3/ When an individual, according to its genotype, is heterozygous with regard to two alleles in a blood marker system, all its offspring must invariably have at least one of the markers controlled by these alleles.

Normally any exception in a family to any one of these rules is grounds for illegitimacy. However, there are a number of very rare phenomena and situations which constitute exceptions to these rules. These phenomena are of two kinds, *general* - those that may occur in all or several of the blood marker systems and *specific* - those that are specific to individual systems.

General exceptions, pitfalls and phenomena to pay attention to.

Blood cell chimaerism

Blood cell chimaerism is known to occur in dizygotic twin pregnancies in horses, cattle, sheep and some other species. The basis for the chimaerism is the occurrence of vascular anastomosis (i.e. blood vessel connections) between the two fetuses - in about 40% of twin pregnancies in horses (Vandeplassche et al., 1970). A reciprocal exchange of primordial blood forming cells between the twin fetuses occurs with these cells becoming established in the blood forming tissue in the co-twin and persisting into adult life. Such twins are called chimaeras.

A blood cell chimaera has an admixture of two populations of blood cells; one that corresponds to its own genotype and one that corresponds to the genotype of its co-twin. In most cases the admixture reveals itself In the red cell test as incomplete (weak) reactions or in the electrophoretic test of Hb and red cell enzymes as a mixture of patterns. However, the proportions of the two populations of cells in chimaeras are variable and may change with time. Sometimes, one of the red cell types are so greatly displaced that it escapes detection by current blood typing tests.

The most problematic situation occurs when the predominant type of cells in a chimaera is the one corresponding to the genotype of its co-twin. With regard to antigens and proteins in the red cells such a chimaera exhibits a phenotype which is not consistent with its genotype. Thus a chimaera of this type may transmit to its progeny blood group factors and red cell proteins or enzymes which cannot be found in its own blood. Conversely it may possess factors and variants that are not transmitted to any of its offspring (Sandberg, 1980). Accordingly, both these conditions are in conflict with the basic rules for the inheritance of the blood markers.

As long as a suspected chimaera has a known co-twin, it is usually no problem to avoid mistakes due to the chimaerism. However, the fact that there is no known twin does not exclude the possibility of chimaerism. The twin fetus may have been aborted, mummified or resorbed at an early stage of gestation and have passed undetected. It has been estimated that 8.8% of all pregnancies in lactating mares, 14% in barren mares and 15.3% in maiden mares start as twin conceptions (Pascoe et al., 1987) but only less than one per cent reach normal parturition (Vandeplassche et al., 1970). After several years, it may even happen that knowledge of its being a twin is forgotten.

When an offspring has received red cell factors or red cell protein variants from a suspected chimaera parent which does not itself exhibit those traits it should, if possible, be determined whether the factors and variants in question are carried by the parents of the suspected chimaera. It is also recommended that other offspring of the suspected chimaera be examined to see if their blood types support the suspicion of chimaerism or not. Karyotyping of the suspected chimaera may provide useful information (Sandberg, 1980).

Provided that both types of cells are present in detectable quantities a differential red cell test (Rendel, 1958) may be carried out to prove that there is in fact a mixture of cells. By this test it is also possible to get an estimate of the relative proportions of the two types of cells and to determine which red cell factors are associated together in each genotype.

Recently a new approach to confirm blood chimaerism was described (Bowling et al., 1993). Microsatellite DNA from a suspected chimaera mare was amplified by PCR using both blood and hair bulbs. Three out of five microsatellite loci studied showed different types in the two tissues, demonstrating that the mare had two cell lines, one in blood and another in hair bulbs. Apparently solid tissue, like hair bulbs is not affected by shared *in utero* circulatory systems between twins.

Silent alleles

All electrophoretic systems and the red cell system D used in horse blood typing are considered to be "closed" genetic systems. This means that there is no horse which lacks all factors or variants in these systems. It also means that the genotype of an individual can be inferred directly from the phenotype. Therefore when the phenotype of a horse in an electrophoretic system consists of a single variant only, the horse is assumed to be homozygous for the gene controlling that variant. Consequently the horse is expected to transmit that gene to all of its offspring.

However, in some of these supposedly closed systems so called "silent alleles" or "null alleles" have occasionally been found. A silent allele is a gene which has no gene product (an amorph) or has a gene product that is not able to be detected. Thus a horse which appears to be homozygous for a particular protein variant (i.e. have two identical copies of the gene controlling

that variant) may in fact be heterozygous for that variant and a silent allele (i.e. have one copy of the gene controlling the variant and one copy of the silent allele). Sometimes it is possible to judge from the strength of the protein zones on the stained gel whether it is a true homozygote or a heterozygote involving a silent allele.

The incidence of silent alleles which have been described in published writing differs considerably between systems and between horse populations. In most systems a silent allele has never been observed while in other systems (e.g. Es) even horses that are homozygous for a silent allele have been observed (Gahne, 1966). From a theoretical point of view it cannot be excluded that silent alleles may occur in any of the closed systems. The possible occurrence of a silent allele in a system is an obvious complication for the interpretation of the blood typing test.

When a horse in an exclusion problem is suspected of carrying a silent allele in a particular system involved, the blood types of its available parents, offspring or sibs should be investigated. The genotypes of those relatives may provide information which proves or disproves whether a silent allele is segregating in the family.

Mutation

A mutation is a spontaneous change of a gene. It may result in the disappearance of the gene product (i.e. a silent allele) or in the production of a new gene product differing from the parental type.

Mutations are extremely rare. It is estimated that they occur with a frequency of less than 1 in 100 000 genes per generation. Thus the probability that mutations would occur in two systems simultaneously is negligible.

Recombination

Evidence to date indicates that complex red cell systems (e.g. the A and D systems) and some electrophoretic systems (e.g. Hb and PI) consist of two or more closely linked loci. Therefore the possibility of a crossing over taking place within such a complex locus has to be considered. As a result of a recombination event no new factor or protein fraction will appear in the offspring but the factors and fractions present in the parent will appear in new combinations (phenogroups).

Age of animal

In general, the blood group factors and blood protein variants are fully developed at birth or shortly thereafter. One exception is carbonic anhydrase (CA) which is not present in detectable amounts in newborn foals. Most individuals do not get CA bands of full strength until after 1 year of age. Another exception is the protease inhibitory (PI) system which is often impossible to accurately type in samples from foals less than one month of age. Also in the serum systems Al, Es, Tf and A1B artifacts may occur in very young foals. An age limit of at least 1 month is recommended for blood typing of foals.

Storage of samples

After the blood sample is drawn it should be sent to the laboratory as soon as possible. It should be kept in a refrigerator (+4 - +8°C) until dispatched. After arriving in the laboratory the whole blood sample for the red cell test should be stored in a refrigerator until tested. The test should be completed without unnecessary delay, preferably within one week after the sample was drawn. Sera/plasmas for the electrophoretic tests should be kept frozen (-20°C). Lysate samples (for the analysis of red cell enzymes) should be tested within 1-2 days after preparation. If this is not possible they should be stored at -20°C. Lysates that cannot be tested for several days should be kept at -70°C.

Exclusion criteria

The responsibility for a decision on an exclusion lies entirely with the laboratory where the decision is taken. The person in charge of the laboratory must be familiar with the genetics of the blood markers and be aware of apparent exceptions and pitfalls involved in the blood typing test.

1

The serological reactions (hemagglutination and hemolysis) and the protein and enzyme variants on which an exclusion is based must be clear-cut and normal.

2

Before a final decision on an exclusion is taken a second set of samples from the animals involved should be analysed, unless the breeding organization involved consider it unnecessary. If the assigned sire already has several tested offspring and thus segregation data confirming his blood type are available, there is no need to test the sire again.

3

An exclusion should not be based on results obtained from another laboratory without contacting the other laboratory for confirmation of the results.

4

In general, an exclusion based on two or more systems is unambiguous and safe. When the systems involved are all derived from red cells the analyses must be carefully checked for any sign of chimaerism. It should then also be inquired whether the assigned sire or dam is a twin.

5

An exclusion based on a single system may involve an element of uncertainty. All possibilities should be tried to obtain additional information to support a decision on such an exclusion (e.g. tests for additional factors and systems or DNA analysis).

Table 1 **Blood group systems** (July, 1996)

Systems	Factors	Recognized alleles				Remarks
A	a, b, c, d, e, f, g	A^a	A^{adf}	A^{adg}	A^{abdg}	1
		A^b	A^{bc}	A^{bce}	A^c	
		A^{ce}	A^e	A^-		
C	a	C^a	C^-			2
D	a, b, c, d, e, f, g, h, i, k, l, m, n, o, p, q, r	D^{adl}	D^{adlnr}	D^{adlr}		3
		D^{bcmq}	D^{cefgmq}	$D^{cegimnq}$		
		D^{cfgkm}	D^{cfmqr}	D^{cgm}		
		D^{cgmp}	D^{cgmq}	D^{cgmqr}		
		D^{cgmr}	D^{deklqr}	D^{deloq}		
		D^{delq}	D^{dfklr}	D^{dghmp}		
		D^{dghmq}	D^{dghmqr}	D^{dkl}		
		D^{dlnq}	D^{dlnqr}	D^{dlqr}		
		D^q				
K	a	K^a	K^-			4
P	a, b, c, d	P^a	P^{ac}	P^{acd}	P^{ad}	5
		P^b	P^{bd}	P^d	P^-	
Q	a, b, c	Q^a	Q^{abc}	Q^{ac}	Q^b	6
		Q^{bc}	Q^c	Q^-		
U	a	U^a	U^-			7

1/ Irregular transmission of factor Ae (Bowling and Ewalt-Evans, 1988). Dosage reactions with reagent anti-Ab.

2/ No remarks.

3/ Some anti-De reagents react weakly with phenogroups D^{cefgmq} and $D^{cegimnq}$.

4/ No remarks.

5/ No remarks.

6/ The anti-Qa reagent sometimes reacts weakly.

7/ Dosage reactions common.

Table 2 Electrophoretic systems (July, 1996)

System	Locus	Recognized alleles	Remarks
A1B glycoprotein	A1B	F, K, S, *	1
Albumin	Al	A, B, I	2
Acid phosphatase	AP	F, S	3
Carbonic anhydrase	CA	E, F, I, L, O, S	4
Catalase	Cat	F, S	5
NADH-diaphorase	DIA	F, S	6
Carboxylesterase	Es	F, G, H, I, O, R, S, *	7
Vitamin D binding protein	Gc	F, S, *	8
Glucose phosphate isomerase	GPI	F, I, S, *	9
Hemoglobin-α	Hb	A, A2, B1, B2, *	10
Peptidase A	PepA	F, S	11
6-phosphogluconate dehydrogenase	PGD	D, F, S, *	12
Phosphoglucomutase	PGM	F, S, V	13
Protease inhibitor	PI	F, G, H, I, K, L, L2, N, O, P Q, R, S, T, U, V, W, Z	14
Plasminogen	PLG	1, 2	15
Transferrin	Tf	D, F1, F2, H1, H2, J, M, O, R, *	16

* Additional allele(s) observed.

1/ No remarks

2/ Some techniques fail to recognize the I variant. Type AI may be mistaken for AA and type IB may be mistaken for BB.

3/ AP should be typed with fresh samples. Stored or infected samples may give an artefactual band which is located close to the F band but slightly more anodal.

4/ CA bands are absent or weak in horses younger than 1 year. Variation between individual horses in strength of CA bands observed.

5/ No remarks.

6/ DIA should be typed with fresh samples. The DIA system has not been extensively studied.

7/ High incidence of silent alleles in the Es system observed in some horse populations (Gahne, 1966). Mutation events observed (Sandberg, 1977; Sandberg et al., 1987; Bell et al., 1995).

8/ When plasma is used some techniques and anticoagulants give an artefactual band in the position of the Gc-S band. Serum should then be used instead of plasma. Mutation event observed (Cleve and Schmid, 1991).

9/ No remarks.

10/ The Hb system is composed of closely linked loci; recombination events possible.

11/ The PepA system has not been extensively studied.

12/ PGD should be typed with fresh samples.

13/ PGM should be typed with fresh samples.

14/ The PI system is composed of closely linked loci; recombination events possible. PI should preferably be typed with fresh samples. Sometimes difficult to compare PI results produced in different laboratories due to the complexity of the system and differences in techniques applied.

15/ No remarks.

16/ Nomenclature not yet completely standardized. Silent alleles observed (Yokohama et al., 1980; Schmid et al., 1990; Bell et al., 1995). Mutation events observed (Bowling, 1991; Farndale et al., 1991; Bell et al., 1995).

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