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Behavior of Animal Blood in Blood Typing Systems. Isoelectric Focusing of Erythrocyte Acid Phosphatase and Phosphoglucomutase

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ABSTRACT: Isoenzyme band patterns of animal blood erythrocyte acid phosphatase (EAP) and phosphoglucomutase-1 (PGM) were studied by isoelectric focusing on ultrathin polyacrylamide gels. For blood from all animals tested (dog, cat, cow, sheep, and goat), the overall band patterns for both isoenzymes were different from those of the most common human types of these enzymes, although some animal EAP and PGM bands appeared in the human band areas. When mixtures of human and animal red blood cells were studied, it was found that misinterpretation of human types was possible only if the overall band pattern of the mixtures was ignored. For the animal blood tested, the strong PGM bands appearing outside the human band areas could be used as "markers" for the possible presence of animal blood in the samples tested.

KEYWORDS: forensic science, genetic typing, serology, enzyme polymorphism, isoelectric focusing, erythrocyte acid phosphatase, phosphoglucomutase, animal blood, cat, dog, cow, sheep, goat

Abbreviations

EAP = erythrocyte acid phosphatase
PGM = phosphoglucomutase
DTT = dithiothreitol
SOD = superoxide dismutase
EDTA = ethylenediamine tetra-acetic acid
MTT = (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrasodiumbromide)

Introduction

Typing of the polymorphic red cell isoenzymes, erythrocyte acid phosphatase (EAP; EC 3.1.3.2), and phosphoglucomutase-1 (PGM; EC 2.7.5.1) by isoelectric focusing is a well-established method for bloodstain identification [*1*]. It has not, however, been determined whether the presence of blood from other species could affect the interpretation of human

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blood-typing results obtained using isoelectric focusing methods. Using zone electrophoresis it has been shown that several domestic animal species demonstrate one or more EAP and PGM phenotypes. By starch gel electrophoresis, EAP and PGM polymorphism has been shown at least in cows [2], dogs [3,4], and horses [5,6], whereas only one EAP band pattern has been found in goats [7] and sheep [8]. Bowen [9] has shown that starch gel electrophoretic PGM band patterns of cow, sheep, goat, and cat blood correspond closely to some human types of this enzyme, whereas the corresponding EAP band patterns for these animals differ from those in human blood.

In the present study we have used isoelectric focusing on ultrathin polyacrylamide gels to compare cat, dog, cow, sheep, and goat blood EAP and PGM band patterns with those of the most common human types of these enzymes. We have also typed mixtures of animal and human red cells using the same method.

Materials and Methods

Blood Samples

Dog and cat blood specimens were obtained from a local veterinary clinic. Cow, sheep, and goat blood specimens were obtained from the Wallaceville Animal Research Centre (Upper Hutt, New Zealand). All animals used were unmedicated and apparently healthy. A description of the animals is presented in Table 1. Human blood was obtained from the Blood Transfusion Service of Wellington Hospital (Wellington, New Zealand). Both animal and human blood specimens were collected in EDTA tubes (37.5 mg/5 mL) and stored refrigerated (at 4°C) before use.

Bloodstains were made on a clean cotton cloth, dried, and stored at room temperature.

Washed red cells were stored at −20°C in 40% glycerol, 60m*M* trisodium citrate, 21m*M* potassium phosphate, dibasic (K_2HPO_4), and 21m*M* potassium phosphate, monobasic (KH_2PO_4) at pH 7.3 (1:1). There was no significant change in the human EAP and PGM band patterns during six months storage of the red cells.

For red cell mixtures, equal volumes of red cells from two sources were mixed.

Red cells were lysed for EAP isoelectric focusing by mixing the cells with two volumes of

TABLE 1—*Description of the animals.*

Animal Breed	No.
Dogs	
Bearded Collie	1
Staffordshire Bull Terrier	1
German Shepherd	3
Labrador	3
Doberman	1
Cross breed	2
Unknown	9
Total	20
Cats	
Persian	4
Domestic shorthair	16
Total	20
Cows, Jersey yearlings	10
Sheep, unknown	10
Goats, feral	10

water and one volume of fresh 0.72% DTT. Bloodstains (5 by 5 mm) were extracted with 10 μL of the DTT 30 min before typing.

Red cells for PGM isoelectric focusing were lysed with an equal volume of 1% Triton X-100.

Polyacrylamide Gel Preparation

Ultrathin polyacrylamide gels were cast onto glass plates by a modification of the method described by Randall and coworkers [*10*]. The gels for EAP contained 4.3% Acrylogel® (BDH Chemicals Ltd, Poole, England), 3% acrylamide (Bio-Rad Laboratories, Richmond, California), 3.75% (v/v) pH 5 to 7 Ampholine® solution (LKB, Bromma, Sweden), and 10% sucrose. The gels for PGM contained 3.15% Acrylogel, 2.1% acrylamide, 11% glycerol, and 7.5% (v/v) pH 5 to 7 and 2.5% (v/v) pH 6 to 8 Ampholine. Both gels also contained one to two drops of 0.01% riboflavin (Sigma, St Louis, Missouri) to initiate polymerization.

Running Conditions

Isoelectric focusing was carried out using either Pharmacia (Flat Bed Apparatus FBE-3000, ECPS 3000/150 Constant Power Supply, Uppsala, Sweden) or Bio-Rad equipment (Bio-Phoresis Horizontal Electrophoresis Cell and Model 3000 Xi Power Supply). The catholyte was 1% sodium hydroxide (NaOH). The anolyte was 1% acetic acid for EAP and 0.9 *M* phosphoric acid (H_3PO_4) for PGM. The electrolyte wicks (LKB) were placed on the gel 70 and 80 mm apart for EAP and PGM, respectively. The lysed blood samples and bloodstain extracts were absorbed into 6- by 2-mm pieces of Whatman No. 1 filter paper and placed on the gels 20 mm from the anodal wick. Pieces of bloodstained cloth (6 by 2 mm) moistened with water were applied to the gel for PGM typing.

Isoelectric focusing of EAP was carried out for 1.25 kVh (approximately 45 min) with a maximum voltage of 1.8 kV, power 2 W, and current 5 mA at 6 to 8°C. The corresponding conditions for PGM isoelectric focusing were 6 kVh (approximately 2 h), 3 kV, 8 W, and 20 mA.

Enzyme Visualization

The sites of EAP activity were located by the method of Randall and coworkers [*10*] and photographed under long-wave ultraviolet light (366 nm) using a Wratten No. 15 yellow filter and Ilford FP4 black-and-white film.

The sites of PGM activity were detected by conventional agar overlay method in the presence of 4m*M* *l*-Histidine (BDH Chemicals) and 0.4m*M* EDTA as chelating agents [*11*]. The PGM gels were photographed on a fluorescent light box using a camera equipped with a Wratten No. 58 green filter on Ilford FP4 film.

Results and Discussion

The EAP and PGM band patterns for each animal species in relation to the corresponding human bands of the most common phenotypes are presented in Figs. 1 and 4, respectively.

The results obtained for mixtures of human and animal red cells are shown in the photographs in Figs. 2, 3, 5, and 6.

Nomenclature used for the EAP bands is that of Divall [*12*]. The PGM bands are labelled according to Bark et al. [*13*] and Divall and Ismail [*11*]. In the present work, rare PGM and EAP phenotypes are not referred to since these have not been encountered in our laboratory.

Both EAP and PGM were studied in dry animal bloodstains kept at room temperature for one week and one, three, and five months. Bloodstains up to three months of age gave the same EAP and PGM band pattern as fresh red cells with slightly decreased activities. During the aging of bloodstains, the least intense bands were the first to disappear. The strongest EAP and PGM bands were still clearly visible in five-month-old animal bloodstains (for details, see below) when the activities of human bands were undetectable.

EAP

Within each species, all individuals demonstrated the same EAP pattern (see Fig. 1 for different species patterns). However, the number of animals of each species in the present study was too small and the pH range too narrow to allow firm conclusions to be drawn as to the presence or absence of different phenotypes in these species.

None of the 20 cats studied showed any strong EAP band in the human high-intensity band region. Consequently, the cat red cells did not interfere with the typing of human EAP in the mixture of human and cat red cells (Fig. 2).

Isoelectric focusing of dog EAP showed two adjacent high-intensity bands near the human "b_2, a_4, c_2" band region, where in human EAP types only one strong band appears (Figs. 1 and 2).

Another two adjacent high-intensity bands were also seen near the human "b_4, c_4" band region, where in human EAP types only weak bands appear (Figs. 1 and 2). In the mixtures of dog and human red cells, the strong bands from dog red cells masked the human "b_2, a_4, c_2" band resulting in an abnormally intense double band in this region (Fig. 2). Because of high-intensity fluorescence, this double band appears as one band in the photograph in Fig. 2 but was seen as a double band in the original gel. This double band from dog blood could possibly cause misinterpretation of human EAP types CB and CA as B and BA, respectively, in samples containing human and dog blood. However, this misinterpretation could only occur if the intense double band in the "b_2, a_4, c_2" region was seen as one band and the

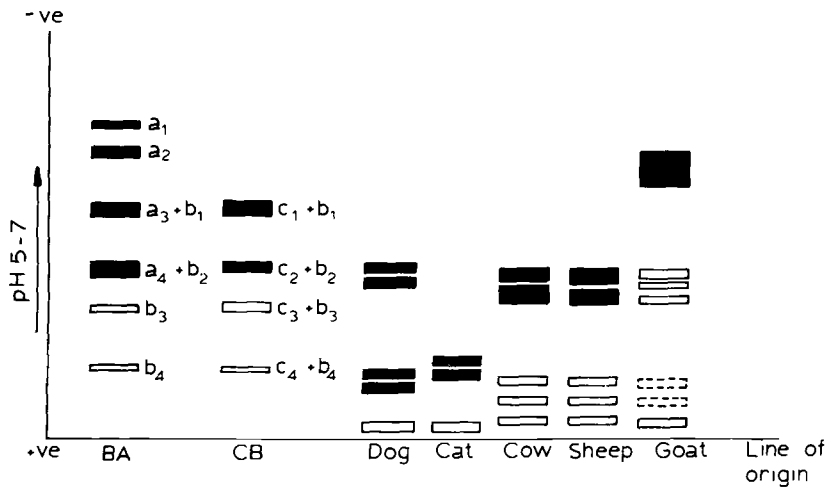
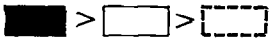


FIG. 1—*Diagram of dog, cat, cow, sheep, and goat EAP band patterns obtained by isoelectric focusing in the pH range 5 to 7. Band patterns of human BA and CB types are used as references. The band intensities are:*



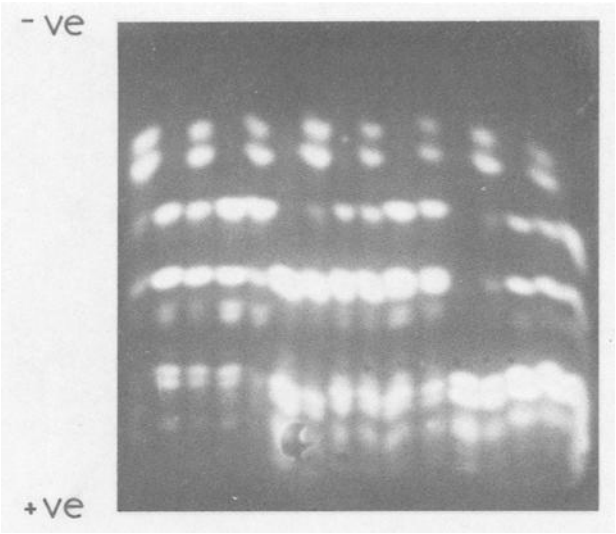


FIG. 2—*Isoelectric focusing (pH 5 to 7) of different human EAP types mixed with dog or cat red cells. Samples from left to right are: human A, B, BA, CB, CA; dog; dog mixed with human A, B, BA, CB, CA; cat; and cat mixed with human A, B, BA, CB.*

presence of the other high-intensity double bands from dog blood in the human "b_4, c_4" region were overlooked. Such a misinterpretation is unlikely in aged bloodstains since both strong double bands from dog blood were found to be decreasing in activity at the same rate (results not shown). They were both still visible in five-month-old bloodstains.

Cows and sheep showed two strong adjacent EAP bands near the human "b_2, a_4, c_2" band region (Fig. 1), where in human EAP types only one strong band appears. In a mixture of cow and sheep red cells and human red cells, these double bands appeared as "extra" bands below human "b_2," "a_4," and "c_2" bands causing no misreading of the human EAP types (Fig. 3).

In the EAP system, goat blood can be clearly distinguished from sheep blood (Fig. 1). Goats showed one strong band in the human "a_2" band region and three weak bands in the human "b_2, a_4, c_2" region. In the mixture of goat red cells and human red cells of A, BA, or CA type, the strong band from goat blood appeared as an "extra" band below the human "a_2" band causing no interference with the human types (Fig. 3). The EAP band patterns for the mixtures of goat red cells and human EAP types B or CB were read as "B with a single 'a'-band" or "CB with a single 'a'-band." The goat "a" band was still clearly visible in the five-month-old goat bloodstain, while no activity of human "a_1" and "a_2" bands was detected.

PGM

Isoelectric focusing of erythrocyte PGM in all animals studied showed bands in the human "a" and "b" band regions (Fig. 4). In the "a" band region, all cats and cows had two strong bands and all dogs, sheep, and goats had two weak bands. All animals had one or two weak bands in the human "b" band region.

For all animals studied, one or more strong PGM bands were seen beyond the human "a-" region, that is, at the cathodal end of the gel (Figs. 4 through 6). One band in this region was seen for the cows, sheep, and goats. Four bands were seen for most dogs and two bands for most cats, while two of the dogs showed three bands and two of the cats showed one band.

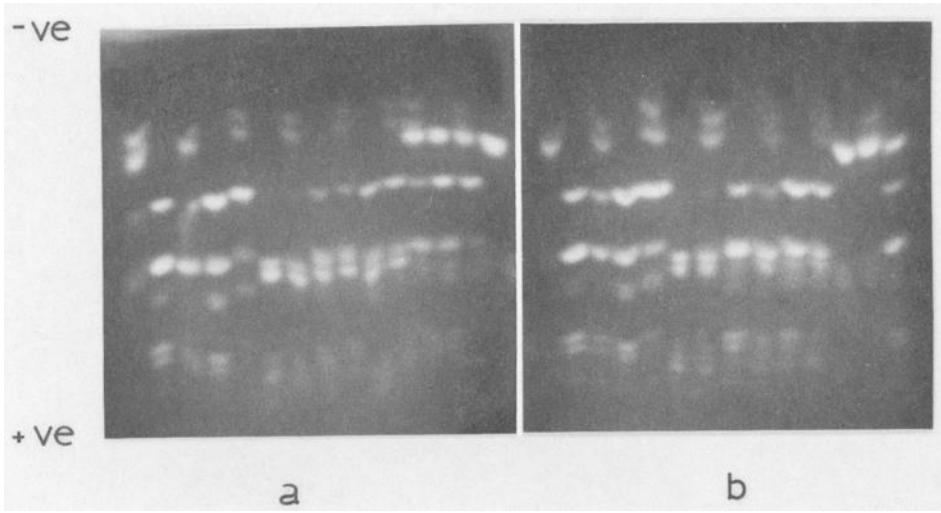


FIG. 3—*Isoelectric focusing (pH 5 to 7) of different human EAP types mixed with cow, sheep, or goat red cells. The samples from left to right are: gel a: human A, B, BA, CB, CA; cow; cow mixed with human A, B, BA, CB, CA; goat mixed with human BA, CB, CA; and goat and gel b: human A, B, BA, CB, CA; sheep; sheep mixed with human A, B, BA, CB, CA; goat; and goat mixed with human A, B,*

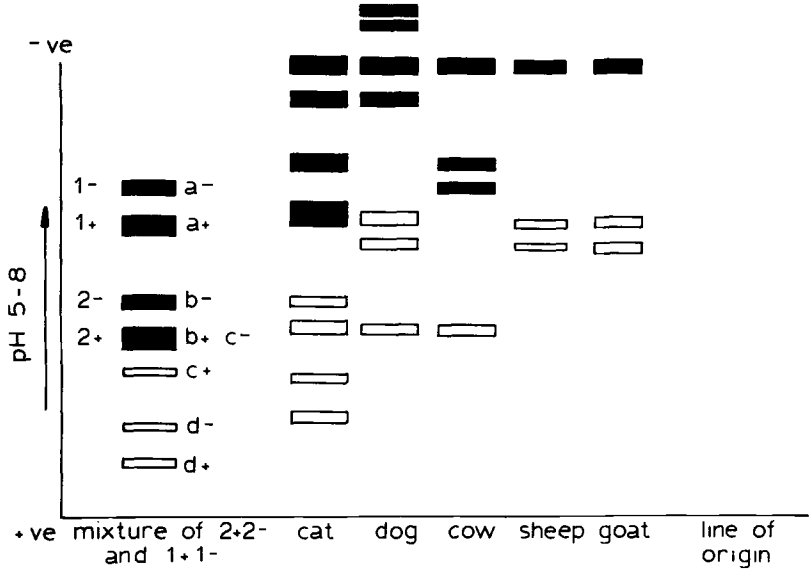
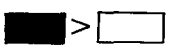


FIG. 4—*Diagram of cat, dog, cow, sheep, and goat red cell PGM band patterns obtained by isoelectric focusing in the pH gradient range 5 to 8. Mixtures of human 2+2− and 1+1− types are used as reference. The band intensities are:*



These cathodal PGM bands in animals gave an overall band pattern clearly different from the human PGM band patterns. This is demonstrated in Figs. 5 and 6 which show the PGM band patterns for mixtures of human and animal red cells.

The PGM bands from animal red cells beyond the human "a" band region at the cathodal end of the gel should suggest the presence of animal blood in the sample and therefore prevent the animal bands in the human "a" and "b" band regions being mistaken for human bands. The activity of these cathodal bands was still strong in five-month-old animal bloodstains when the activity of other bands had disappeared.

Superoxide Dismutase

The activity of superoxide dismutase (SOD; EC 1.15.1.1) on gels containing MTT [*14*] can be used in some isoelectric focusing systems for species identification of animal blood [*15*].

However, in the present PGM isoelectric focusing system, the SOD activity was seldom visible and therefore did not help to identify blood as nonhuman in origin.

Conclusions

Several bands of EAP and PGM activity were detected in cat, dog, cow, sheep, and goat red cells by an isoelectric focusing method used in forensic science examination of human blood. Some of these bands appeared at or near the position of human bands. However, all animals studied also had EAP and PGM bands outside the expected human band positions.

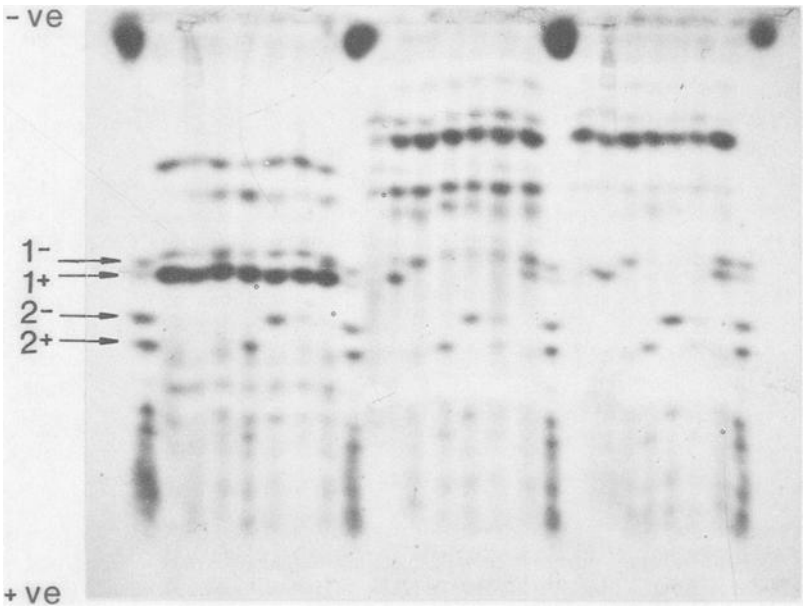


FIG. 5—*Isoelectric focusing (pH 5 to 8) of different human red cell PGM types mixed with cat or dog red cells. The samples from left to right are: human control sample containing a mixture of 1+1− and 2+2−; Cat 4; Cat 4 mixed with human 1+, 1−, 2+, 2−, 2+2−, 1+1−; human control sample; Dog 3; Dog 3 mixed with human 1+, 1−, 2+, 2−, 2+2−, 1+1−; human control sample; Dog 4; Dog 4 mixed with human 1+, 1−, 2+, 2−, 2+2−, 1+1−; and human control sample.*

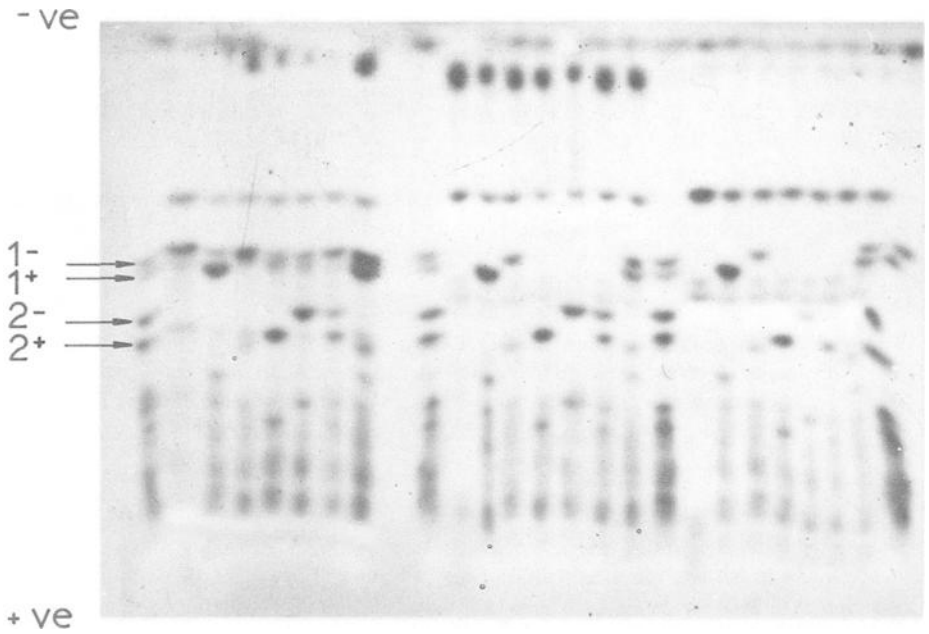


FIG. 6—*Isoelectric focusing (pH 5 to 8) of different human red cell PGM types mixed with cow, sheep, or goat red cells. The samples from left to right are: human control sample containing a mixture of 1+1− and 2+2−; cow; cow mixed with human 1+, 1−, 2+, 2−, 2+2−, 1+1−; human control sample; sheep; sheep mixed with human 1+, 1−, 2+, 2−, 2+2−, 1+1−; human control sample; goat; goat mixed with human 1+, 1−, 2+, 2−, 2+ 2−, 1+1−; and human control sample.*

This gave overall EAP and PGM band patterns that were clearly different from the most common human types.

In the mixtures of human and animal red cells, a combination of human and animal bands were obtained. The appearance of the bands outside the human band positions may indicate that the sample contained blood from other species. With PGM in dog, cat, cow, sheep, and goat blood, this indication was given by the bands beyond the human "a-" band region, this is, near the cathodal end of the gel, where no human bands appeared. These bands may not be specific for these animals and cannot be used as a sole test for species identification. They could, however, be regarded as "markers" for the possible presence of nonhuman blood. Isoenzyme isoelectric focusing may therefore be used as a screening test for the presence of nonhuman blood before species identification.

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