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Species Identification of Blood and Saliva Stains by Enzyme-Linked Immunoassay (ELISA) Using Monoclonal Antibody

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ABSTRACT: An indirect enzyme-linked immunoassay (ELISA) method for the identification of human blood and saliva stains is reported. The method uses a monoclonal antibody which reacts with human immunoglobulin G (IgG) in extracts of blood and saliva stains up to 16 months old. Semen stain extracts gave weak or negative results. For routine screening purposes dilutions of 1:1000 for bloodstain extracts and 1:100 for saliva stain extracts would be suitable. Of 32 other animal species tested, only chimpanzee, mouse, rat, and eel cross-reacted significantly, and the presence of the last three was clearly indicated by appropriate controls. The monoclonal antibody gave poor results in the crossover and gel diffusion techniques.

KEYWORDS: pathology and biology, blood, immunoassay

The identification of a bloodstain as being of human origin is a routine procedure in forensic serology and the methods in use are thoroughly reliable. Occasionally, however, problems arise with variations in the quality of the antihuman sera used for this purpose. These sera may show low-level cross-reactions with serum from nonhuman sources and may also vary quite widely in sensitivity. As a result each new batch of serum has to be tested for species specificity and for sensitivity before it can be used. One way of avoiding this recurrent testing, and at the same time promoting some degree of standardization in methodology, would be to use an antihuman monoclonal antibody [*1*]. A number of monoclonal antibodies (MCA) have been reported which react strongly with human proteins and recently a number of MCA directed towards human globulins have become available commercially [*2*]. Immunoglobulins are evolving considerably more rapidly than most proteins and so can be expected to carry more antigenic determinants unique to humans. This makes them well suited to the purpose of species discrimination.

In this work we have investigated the specificity and sensitivity of some of these MCA in an enzyme-linked immunoassay (ELISA) mode. ELISA [*3,4*] is particularly suitable for use with monoclonal antibodies since it does not depend on the antigen having more than one antigenic site, as do hemagglutination methods.

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Materials and Methods

The MCA used in this study were all produced in the Department of Immunology, Birmingham University Medical School, Birmingham, U.K. [2]. MCA from Clone C4 was supplied by Dr. K. May of Unilever Research Ltd., Sharnbrook, Bedfordshire, U.K. MCA from Clones 6e1, G7c, 8a4, and 1a1 were purchased from Seward Laboratory (UAC House, London SE1 9UG, U.K.). All these products were ascites fluids. The details of the MCA are given in Table 1.

Samples of human blood, saliva, and semen were donated by laboratory staff. Stains were made by allowing a few drops of liquid to soak into clean, boiled cotton cloth which was then dried at room temperature for at least 24 h. Extracts were made by cutting out a square, 10 by 10 mm, from the stain and extracting with 0.4-mL 0.9% saline for 30 min at room temperature.

Human serum samples and serum from 32 animal species were taken from our own collection of lyophilized sera.

Dilutions of animal sera and stain extracts were made in the ELISA coating buffer (1.59 g of sodium carbonate, 2.93 g of sodium bicarbonate, and 0.2 g of sodium azide to 1-L distilled water, pH 9.6 at 20°C: made up freshly each week) for ELISA, or in half strength gel buffer for crossover electrophoresis and double diffusion in gel.

Gel diffusion (Ouchterlony) was performed in 1% Litex agar (International Enzymes Ltd, Windsor, Berkshire, U.K.) in half strength gel buffer [5] containing 4% polyethylene glycol (PEG 3000, Aldrich Chem. Co., Gillingham, Dorset, U.K.) to facilitate precipitation [4]. Wells, 2.5 mm in diameter, were cut in a hexagonal pattern around a central well in the usual way, and 10 μL of serum or antiserum added to each well. The plates were allowed to incubate at 4°C for 48 h and were then washed in 1*M* saline containing 4% PEG 3000 for 16 h and finally in distilled water containing 4% PEG 3000 for 4 h before drying and staining.

Crossover electrophoresis was performed as described by Culliford [5] with a voltage gradient of 5 V per cm measured directly on the gel slide. The gel used was 1% Litex-HSA agarose; 4% PEG 3000 was incorporated in the gel buffer and was added to the wash solutions.

ELISA was performed using a Gilford PR50 ELISA Processor-Reader (Gilford Instruments Ltd, Teddington, Middlesex, U.K.). This instrument uses a plastic cassette in place of the more common microtiter plate and performs all the washing, reagent additions, and final reading on an automated batchwise basis. Appropriate dilutions of serum, blood extracts, and saliva extracts (300 μL) were incubated in wells in the cassette at 4°C overnight. The wells were then washed three times with phosphate-buffered saline (PBS) (8 g of sodium chloride, 1.15 g of disodium hydrogen phosphate, 0.2 g of potassium dihydrogen phosphate to 1 L of distilled water, pH 7.3) containing 0.05% Tween 20 (Wash buffer) and left to soak for 30 min in 1 mL of the same buffer. No further blocking was found necessary. After a further two rinses, 0.3 mL of MCA C4 at 1:500, 1:5000, or 1:50000 dilution in PBS containing 10-g/L bovine serum albumin (Armour Pharmaceutical Co Ltd., Eastbourne, Sussex, U.K.) was added. After 2-h incubation at 37°C the wells were rinsed twice with wash buffer and left to soak for 20 min in wash buffer. A further rinse with wash buffer was given and then 0.3 mL of alkaline phosphatase-labelled sheep antimouse immunoglobulin (NEI-500; enzyme activity 30 to 40 units/mL; specific antibody concentration 500 μg/mL: New England Nuclear, New

TABLE 1—*Details of the monoclonal antibodies used.*

Clone (2)	Source	Code	Specificity
6e1	Seward Labs	BAM03	kappa chains
C4	Seward Labs	BAM04	lambda chains
G7c	Seward Labs	BAM05	CH2 region
8a4	Seward Labs	BAM06	CH3 region
1a1	Seward Labs	BAM07	pFc-fragment

Rd., Southampton, Hampshire, U.K.) at a dilution of 1:1000 in PBS containing 1.0% bovine serum albumin was added. The cassettes were incubated for 2 h at 37°C and then washed with two rinses and one 20-min soak in wash buffer. After one final wash in 0.9% saline, 0.3 mL of enzyme substrate solution (1 mg/mL *p*-nitrophenylphosphate in 0.05*M* carbonate buffer, pH 9.6, containing 1m*M* magnesium chloride: Sigma Chemical Co., Kingston, U.K.) was added. The cassettes were incubated at 37°C for 30 min to 1 h and then for a further 16 h at 4°C. Finally the absorption (*A*) of the solutions were measured at 405 nm in the PR50.

In a second series of experiments an alternative antimouse immunoglobulin (Ig) conjugate was used. This was sheep antimouse IgG beta galactosidase (NEI-505; enzyme activity 95 to 90 units/mL; specific antibody concentration 20 to 25 μg/mL: New England Nuclear). The substrate used with this enzyme was 1.4m*M* *o*-nitrophenyl galactose in 10m*M* Tris-acetate buffer (pH 7.4), containing 10m*M* magnesium chloride and 50m*M* 2-mercaptoethanol. In all other respects the conjugate was used in the same way as the first.

To combine the results of experiments done under slightly different conditions the *A*405-nm readings have been converted to a "reaction score." This is calculated as (test *A*405 nm/ control *A*405 nm) − 1. The control referred to was the mean coating buffer control (coating buffer + MCA C4 + conjugate) for the appropriate experiment. Other controls (coating buffer + conjugate; and antigen + conjugate) were also included in all experiments.

Results and Discussion

Gel Diffusion and Crossover Electrophoresis

In gel diffusion MCA C4 gave no precipitin line with bloodstain extracts. In crossover electrophoresis, it gave clear positive reactions against human serum diluted out to 1:8; but by comparison a standard commercial antihuman serum (Wellcome Ltd., Beckenham, Kent, U.K.) reacted with dilutions out to 1:512. These findings are consistant with those of Steensgaard et al [*6*] who reported that, in general, antihuman IgG MCA are poor precipitating sera.

ELISA

Initially, dilutions of human serum were coated onto the plastic wells to determine the optimum dilution for MCA C4. Clear-cut results were obtained with all three dilutions tested (1:500, 1:5000, 1:50000), and MCA C4 was subsequently used at 1:5000, which gave the best compromise between sensitivity and background interference.

The background levels of enzyme activity in control wells that had received coating buffer alone at the absorption stage were quite uniform. In 40 control wells (coating buffer + MCA C4 + antimouse conjugate) the range of ultraviolet (UV) absorbance (*A*405 nm) after 30-min incubation at 37°C was from 0.056 to 0.088; and after a further incubation at 4°C overnight, from 0.086 to 0.313. Sample controls (stain extract + antimouse conjugate) gave a similar range. Coating buffer controls were incorporated in each subsequent experiment and in every case these gave low background levels of activity.

All the results reported below were recorded after overnight incubation at 4°C with substrate. The results of tests after the initial incubation at 37°C allowed almost the same discrimination but some improvements were seen after the overnight incubation. It may be that the long second incubation stage might be replaced by extending the 37°C phase to 1.5 or 2 h.

Samples which gave specific reactions with the MCA often exhibited a prozone-like phenomenon. Table 2 exemplifies this with the results obtained with a series of dilutions of bloodstain extract and of human serum. The reaction score rises to a maximum with the 1:1000, or 1:10000, dilution and thereafter declines. The reason for this behavior, which has often been reported for ELISA methods, is normally thought to be a result of overcoating of

TABLE 2—*Reaction of bloodstain extract dilutions in an antihuman IgG ELISA.*

Dilution	Reaction Score μ^a	
	Bloodstain extract	Serum
1:100	6.5	12.6
1:1000	11.9	22.8
1:10000	6.9	>24.4
1:100000	1.0	14.1
1:1000000	1.4	2.0

$^a\mu$ = (test A405 nm/control A405 nm) − 1.

the initial adsorbed protein by the excess protein in concentrated solutions, in such a way as to reduce the number of specific sites accessible to the second antibody.

The reactions given by human and other animal sera with MCA C4 (at a dilution of 1:5000) and the alkaline phosphatase conjugate (at a dilution of 1:1000) are shown in Table 3. From these results it is evident that a reaction score of 1.00 or more indicates a specific reaction and scores higher than this are considered positive reactions.

From Table 3 it can be seen that a few other animal species sera can give positive reactions in the test. The chimpanzee and other monkey reactions are not altogether unexpected and would not seriously prejudice the forensic science application of the method. The mouse and rat reactions are caused by the binding of conjugate to the antigen, and not to the monoclonal antibody. These reactions were also present in controls where the monoclonal antibody had been omitted. The mouse reaction, in particular, is very high, and for this type of ELISA technique it is difficult to foresee any way of eliminating it. Nevertheless mouse and rat blood are not commonly encountered and provided adequate controls are carried out reactions caused by such contamination will be easily identified. The eel reaction was not investigated further

TABLE 3—*Species specificity of MCA C4 in an indirect ELISA using antimouse Ig phosphatase conjugate.*

Antigen (serum)	Reaction Score of 1:1000 dilution of antigen, μ^a	Highest A405 nm, $\mu\mu^b$
Pool 1 (Sheep, goat, pig, cow)	0.2	0.281
Pool 2 (Rabbit, mink, hare, badger)	0.7	0.350
Pool 3 (Horse, dog, fox, donkey)	0.8	0.370
Pool 4 (Fowl, pheasant, turkey, pigeon)	0.7	0.350
Pool 5 (Haddock, salmon, trout, plaice)	0.4	0.331
Cat	0.1	0.390
Guinea pig	0.1	0.380
Hamster	0.6	0.561
Squirrel	0.1	0.580
Eel	2.2	0.970
Rat	1.6	0.960
Mouse	13.6	2.953
Yellow baboon	0.4	0.317
Douroucoulis	0.8	0.350
Patas monkey	1.1	0.441
Rhesus monkey	3.1	0.942
Chimpanzee	12.9	2.793
Human	14.1	3.039

$^a\mu$ = (test A405 nm/control A405 nm) − 1; mean coating buffer control value for these results was 0.200.

$^b\mu\mu$ = for any dilution tested: all antigens were tested at dilutions from 1:20 to 1:10000.

but is probably a result of the lectin naturally present in eel blood. Again, controls carried out with no MCA C4 also gave positive results with eel serum.

Bloodstains from 1 day to 16 months old were extracted and dilutions of extracts were tested by the method: the results for the 1 : 1000 dilutions are given in Table 4. Two old stains failed to give positive results but other old stains did, indicating that detectability is not simply related to stain age.

Saliva stains (20) and semen stains (12), all two weeks old, were also extracted and tested for reactivity. At dilutions of 1 : 1000 only a few saliva stains and no semen stains gave positive reactions. However, at dilutions of 1 : 100 almost all the saliva stains and three of the semen stains gave good reactions. These results are summarized in Table 5.

The positive reactions with saliva stain extracts were probably because of reaction with the lambda chains of IgA, which is the predominant immunoglobulin of saliva (approx 0.2 mg/mL). Semen contains a similar amount of immunoglobulin (0.09-mg/mL IgA, 0.03-mg/mL IgG) yet the semen stain extracts gave poor results. This may be due to the presence of some particularly competitive protein, prostatic acid phosphatase for example, reducing the efficiency of the immobilization step. Another possibility is the degradation of antigen by the proteolytic enzymes present in semen.

Monoclonal antihuman IgG antibodies from a further four clones were compared with MCA C4, using human and dog sera as antigens. The results are given in Table 6. Four of the five gave good, strong reactions with human serum, but one, 1a1, gave virtually no reaction at all. The reported specificities of these MCA are: 6e1—free and combined kappa chains; C4—free and combined lambda chains; G7c—heavy chain CH2; 8a4—heavy chain CH3; and 1a1—heavy chain pFc (combines with both intact IgG and with fragments.). Thus four different Ig features survive immobilization on the plastic surface. The lack of reaction with 1a1 suggests that the Ig is attached to the plastic surface via some part of the Fc region (that is,

TABLE 4—*Detection of human bloodstains by ELISA.*

Stain No.	Age of Stain	Reaction Score, μ^a
1	1 day	12.5
2	11 days	4.6
3	2 weeks	10.8
4	2 weeks	5.4
5	4 weeks	5.7
6	4 weeks	3.4
7	4 weeks	11.9
8	4 weeks	7.3
9	5 weeks	7.3
10	6 weeks	2.1
11	6 weeks	4.2
12	6 weeks	6.0
13	8 weeks	5.2
14	8 weeks	8.1
15	10 weeks	2.6
16	16 weeks	7.9
17	16 weeks	4.4
18	28 weeks	2.6
19	30 weeks	7.8
20	41 weeks	4.4
21	1 year	13.2
22	1 year	0.0
23	16 months	0.5
24	16 months	7.2

[a] μ = (test A405 nm/control A405 nm) − 1, for a 1 : 1000 dilution of blood stain extract.

TABLE 5—*Reaction of body fluid stain extracts in an antihuman IgG ELISA.*

	Reaction Score, μ^a of extract dilution	
	1:100	1:1000
	SALIVA STAINS	
1	9.1	1.6
2	4.7	0.3
3	0.1	0.0
4	0.9	0.0
5	4.2	3.2
6	3.0	0.7
7	1.6	0.5
8	5.2	0.2
9	1.9	0.0
10	2.2	0.3
11	3.4	0.0
12	2.3	0.0
13	4.0	1.2
14	17.3	3.4
15	1.9	0.5
16	2.6	5.1
17	15.4	2.3
18	4.4	0.0
19	5.5	0.3
20	4.1	0.0
	SEMEN STAINS	
1	1.5	0.8
2	0.5	0.2
3	1.6	0.3
4	0.6	0.0
5	1.0	0.0
6	0.5	0.0
7	0.3	0.0
8	0.6	0.0
9	0.0	0.4
10	0.0	0.0
11	0.0	0.0
12	0.0	0.0

[a] μ = (test A405 nm/control A405 nm) − 1.

TABLE 6—*Comparison of five antihuman IgG monoclonals in an ELISA method using an antimouse phosphatase conjugate.*

Antigen	Reaction Score, μ^a			
	Human Serum Diluted		Dog Serum Diluted	
Clone	1:1000	1:10000	1:1000	1:10000
6e1 (kappa chain)	16.6	12.8	0.4	0.4
C4 (lambda chain)	17.2	6.7	0.0	0.0
G7c (gamma CH2)	14.9	10.1	0.1	0.3
8a4 (gamma CH3)	>18.0	15.8	0.2	0.8
1a1 (gamma pFc)	1.0	0.5	0.3	0.6

[a] All MCA diluted 1:5000. μ = (test A405 nm/control A405 nm) − 1.

TABLE 7—*Comparison of five antihuman IgG monoclonals in an ELISA method using an antimouse galactosidase conjugate.*

Antigen	Reaction Score, μ^a			
	Human Serum Diluted		Dog Serum Diluted	
Clone	1:1000	1:10000	1:1000	1:10000
6e1 (kappa chain)	5.6	2.6	0.0	0.0
C4 (lambda chain)	2.2	1.8	1.0	0.5
G7c (gamma CH2)	3.1	1.3	0.5	0.5
8a4 (gamma CH3)	2.7	2.7	0.2	0.5
1a1 (gamma pFc)	0.5	0.2	0.2	0.0

[a]All MCA diluted 1:5000. μ = (test A405 nm/control A405 nm) − 1. Antimouse conjugate used at a dilution of 1:1000.

region to which 1a1 binds). Whatever the reason, it is clear that many, but not all, antihuman Ig MCA would serve to identify human blood and saliva stains by this technique.

A second antimouse Ig enzyme conjugate, this time with betagalactosidase, did not perform well as a substitute for the alkaline phosphatase conjugate (Table 7). As with the previous conjugate, positive reactions were obtained with 6e1, C4, G7c and 8a4, but not with 1a1. However the degree of reaction was about five times lower. Evidently the sensitivity of this technique depends to a considerable extent on the correct choice of antimouse conjugate. All the MCA used here were mouse IgG1 subclass: if other subclasses were used a different antimouse conjugate might be needed.

Conclusions

The prototype method described here is capable of identifying the origin of blood and saliva stains as human, with a high degree of confidence. The MCA used, C4, is commercially available, as are three other equally promising alternatives. All four MCA were detected, with comparable sensitivity, by a single antimouse Ig alkaline phosphatase conjugate. Few problems were encountered during development and the method appears to work over a wide range of conditions and dilutions of reagent and sample. Its sensitivity and specificity are more than adequate for use in forensic science laboratories.

The reagent cost of this method for one stain (two dilutions plus two controls) is approximately 50 cents. The method works conveniently with batches of 20 or more determinations, and the larger the batch, the cheaper the unit cost. The ELISA processor-reader is a considerable capital investment and might appear to be too costly for routine application, despite the attraction of the method's objectivity and precisely defined sensitivity. However, it is likely that ELISA methods for other substances of interest to the forensic serologist will be developed in the near future, in which case the cost will be more easily justified.

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