PRINT: ISSN 0970-9274 ONLINE: 2456-6608
J. Hum. Ecol., 11(3): 177-200 (2000)
DOI: 10.31901/24566608.2000/11.03.03

Analysis of Some Genetic Markers in Blood and Semen Stains: Typability under Indian Climatic Conditions

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INTRODUCTION

Shedding of blood is the dramatic accompaniment to crimes committed by violent means. Blood accounts for about 9 percent of a healthy person's body weight and as many murderers have discovered to their detriment, when it is spilled a little goes a long way. Once blood is shed in any quantity, and especially when it starts to clot, it becomes very difficult to deal with. Murderers attempts to clean up after their violent handiwork often fail because of blood traces which adhere tenaciously to their clothing or to the weapon of offence. Blood found at the scene of crime has trapped many killers who thought they had removed all incriminating traces.

Blood is important forensically and can yield a great deal of information to the investigator. The first task in examining suspicious stains is to determine whether they are blood and if so, are they human or belong to some other species. Once this is established, stains are examined for age, sex and blood group. The shape and pattern of liquid blood splashes can help in reconstructing the murder, bloody fingerprints and palm prints tell their own story; dried blood on a suspect's clothing can be related to the victim, the crime scene and the murder weapon; blood and tissue forced under the finger nails of the victim during a violet struggle can be linked to the assailant. Thus a single blood trace can provide a wealth of information.

It is little more than thirty five years when bloodstain discrimination was based solely on the well known ABO groups. In the last twently five years or so, considerable advance has been made in the techniques of forensic haemogenetics and it is now common for forensic science laboratories to identify not only the ABO and other red cell antigens but also serum protein and enzyme polymorphs. The knowledge of the frequency with which these genetic markers occur increases their potential value as markers for the identification of bloodstains. Increasing the number of blood factors tested, naturally increases the chances of making individualization and moreover, increases the chances of obtaining a statistically significant probability that the stain does originate from one particulars individual.

The issue of the ability of a system to discriminate among members of a population is extremely important. According to Gaensslen (1983) "It is intuitively clear that the more phenotypes a system exhibits and the more evenly distributed they are in the population, the greater the chance that two randomly chosen individuals from the population are different (*i.e.*, exhibit different phenotype)" Jones (1972) treated this problem quantitatively. In brief, the result is that for each marker systems, if we have a large enough population and if at least one phenotypic frequency is large compared to population size, then the probability of failing to distinguish between two randomly chosen individual from that population (probability of identity) is approximately equals the sum of the squares of the different phenotypic frequencies. For example, with respect to ABO blood group system if the observed frequencies in a population are:

A	B	O	AB
l	m	n	o

The probability of non discrimination is given approximately by $l^2+m^2+n^2+o^2 = Q$

The probability of being able to distinguish between two individuals randomly chosen from that population, *i.e.* the probability of discrimination, is given by (1-probability of identity) that is, (1-Q) or P. this probability is sometimes called the discrimination index.

If there are several independent markers under consideration, then the probability of identity on all of the systems equals the product of the probabilities of identity on the individual tests. If for example, if in addition to the Q probability of identity in ABO typing, we estimated R probability of identity in the

MN blood group system, then under independence, the probability of identity when using both groupings would be (Q) (R) = QR. The discrimination index, therefore would be 1 - (QR).

Another major application area of forensic serology is disputed parentage testing. Until recently most cases of disputed parentage involved only the father. Doubtful maternity was a rare event. This position has now changed particularly abroad with the realisation that blood group investigations are useful for immigrant families trying to prove their family relationships, citizen claims, inadvertent baby mix ups, kidnapping, disputes over the inheritance of money or property and divorce cases and this may involve the maternal as well as paternal relationship.

Boorman et al. (1988) list the following general principles upon which exclusion of paternity is based. A man can be excluded from paternity in any of the following ways:

1. By finding a gene product in a child which is absent form both mother and putative father. For example, mother and putative father both are of group O and the child is of group A. Provided the mother and child relationship is not in doubt, child's A gene is paternal in origin and therefore excludes from paternity a man who is group O.
2. By establishing that genes which the putative father must give to his offspring do not appear in the child. This may be when he is
   a. Homozygous for a gene which does not appear in the child (for example, in the Rh system, if the putative father is CC and the child cc)
   b. Heterozygous for genes that are not present in the child (for example, putative father group AB, child group O).

Exclusions based on 1 and 2b are terms 'first order' of direct exclusion while those based on 2a are 'second order' or indirect exclusions. The latter are so named because homozygosity for a particular gene cannot be unequivocally determined by laboratory tests but is an assumption. Incidentally, exclusions based on category 2 can be made without a knowledge of the mother's blood profile.

The probability (PE) that a particular system will exclude a falsely accused father a priori can be calculated, and depends on the gene frequencies of the particular system in the population of interest. For most systems, the PE can be calculated quite easily (see Walker, in AABB, 1978). For some of the more complex systems, like Rh, Gm and HLA, the calculations are relatively more complicated. The comulative probability of exclusion (CPE) for a battery of genetic marker systems can be calculated from the individual PE values according to : $CPE = 1-(1-P_1)(1-P_2)(1-P_3) \ldots (1-P_n)$, where P_1 is PE for the first system and P_n is PE for the nth system.

Developments in blood typing have stimulated path breaking research on bloodstains analysis that has succeeded in resolving blood into many more components than previously recognised. These refinements make it possible to pinpoint certain key characteristics of an individual. Now, for instance, serologists can identify not only the blood but also race, age and sex of the person and even the drug habits of the individual in question.

Over the past three and a half decades, a great many of the proteins and isozymes contained in blood and body secretions have been shown to be genetically polymorphic. Each individual possesses a unique combination of these genetic markers and it is thus possible, at least in principle, to pinpoint every individual by biochemical typing. However, typing of these markers from bloostains and other body fluids stains is not without problems. This is because of the peculiar situation faced by the forensic haemogeneticist. Rather than a fresh sample of blood from an identified source, the forensic science laboratory usually handles a sample of dried blood or stains of other body fluids. The quantity is likely to be small - a stain on a trouser for example. The 'donor' is likely to be unknown. The time the sample was 'deposited' is at best uncertain. At times, the problem is further aggravated when proper methods of collection and storage are not strictly adhered to. An important piece of evidence may have spent some hours in the police van, exposed to air, solar radiation, high temperature and other contaminants that

may have caused the sample to react and change in a significant way or simply to deteriorate. Morevoer, in the reality of crime laboratories there are practical constraints that delimit the number of genetic markers that can be reliably typed in any given sample.

In general, fewer systems can be used in stain analysis that is possible in the typing of fresh materials. There are perhaps 20-25 marker systems that could theoretically be used in stain analysis. It is most unlikely that so many systems would ever be applicable at a practical level because of the many other constraints imposed by case work analysis. In many cases, the age of the stains is a problem in others, the sample size might be very limited. There are cost considerations as well in adding new system to the routine analysis. In individual laboratories, resource and manpower may be rather scarce, and this would be a decisive factor as well. This is particularly so in most of the State Forensic Laboratories in India where "Benzidine Test" is still the thing.

Nevertheless, a great degree of individualization of stains is possible than ever before. In the words of Mourant, "The recent vast proliferation of known genetic systems expressing themselves in blood, especially those controlling the plasma proteins, the haemoglobins, the red cell enzymes and the histocompatibility antigens have rendered the identification of fresh blood certain beyond all reasonable doubt. It has very greatly increased the discrimination power of the tests on stains, and has made it possible, in theory at least, to exclude all but about one per cent of men worngly accused of paternity" (Mourant, 1979).

But that was twenty years ago. As stated above for many years forensic serologists have relied on serological tests to analyze body fluids. These tests discriminate individuals based on differences in proteins, enzyme activities, or immunological markers. Since the variability associated with these genetic traits is coded for by DNA, the direct analysis of DNA represents a logical extension of conventional serology.

There are several features of the DNA molecule that are ideally suited for forensic applications. Fore most is the high degree of variability inherent to DNA. With the exception of monozygotic twins, (Hill and Jeffereys, 1985) the DNA of each individual is unique. Moreover, for a given individual's the DNA of each cell is identical and in the absence of rearrangement or somatic mutation, remains constant over the individuals lifespan. The ubiquitous presence of DNA has been demonstrated in body fluids such as blood, semen, saliva, hair root sheaths, skin and virtually all body tissue, fluid or secretions that contains nucleated cells. The stability of DNA is remarkable as evident from typing samples from several thousand years old mummies, postmortem samples, stored (formaldehyde preserved and paraffin embedded) tissues, dried blood and semen stains, skeletal remains, and material subjected to contaminants and other environmental insult (Paabo, 1985; Rogan and Salvo, 1990; Kirby, 1992; Karuthapandian, 1995). Thermostability of DNA is such that it can be typed from samples subjected to boiling for 9 hours at 100°C (in a water bath) and charring for 30 minutes at 150°C in an electric oven (Semba et al., 1994). To top it all, the discriminatory power of DNA profiling is a million fold higher than any of the conventional markers (Sullivan, 1994). It is now possible to solve the cases of disputed paternity with 100% certainty through DNA analysis. The genetic markers such as ABO thus far typed conventionally are now being analysed by DNA typing (Hashimoto and Nakanishi, 1993; Ogasawara, 1996; Ladd et al. 1996; Herrin Jr., 1996; Akane et al., 1996). Multilocus DNA fingerprinting is discriminatory enough for the prediction of consanguinity (Wells et al., 1988) and for zygosity determination (Hill and Jeffreys, 1985). This is possible because polymorphism detected in multilocus DNA fingerprinting emanates from throughout the genome covering all the 46 chromosomes (Jeffreys et al., 1986) unlike other conventional blood groups markers including the HLA system that confine to particular chromosomes. It is for this reason that it was possible to solve a case of incest which was not possible with the help of HLA and 24 other conventional blood marker systems (Schacker et al., 1990).

Other than the restriction fragment length polymorphism (RFLP) analysis, the second approach is based on the use of polymer chain reaction (PCR). The polymer chain reaction is a rapid procedure for in vitro enzymatic amplification of a specific segment of DNA a million fold or more.

Sensabaugh and Blake (1992) list the following benefits that PCR brings to the analysis of biological evidence:

a. Amplification can be done on samples containing very small amounts of DNA (*e.g.* single shed hair, saliva traces on cigarette butts). This extends DNA typing to evidence samples that at present can not be typed using RFLP approaches or by blood group or protein typing (Higuchi et al., 1988; Hochmeister et al., 1991; Faffanti et al., 1990).
b. Amplification can be done on samples containing degraded DNA. This extends DNA typing to the significant proportion of biological evidence that cannot be typed by RFLP analysis (Shibata et al., 1988; Reynolds et al., 1991; Hochmeister et al., 1991; Hagelberg et al., 1991; Shibata et al., 1991).
c. PCR and subsequent typing tests are relatively rapid and simple to perform; results can be obtained within a short time frame, often within 24 hours.
d. PCR can serve as starting point for the detection of virtually any kind of variation to be found in the genome.
e. PCR can be performed in a multiplex mode; that is several amplifications reactions can be performed simultaneously in the same reaction tube. This greatly speeds up the generation of genetic typing information.
f. The small samples requirement for PCR allow portions of evidence samples to be laid aside for repeat testing within the laboratory or by other laboratories.

Given these advantages, PCR is emerging as the dominant technology in forensic DNA analysis.

So all gene products appearing in blood and other biological fluids and have been shown to conform reliably to Mendelian inheritance are suitable for forensic investigations. They can be divided into six categories:

1. Red cell antigen systems
2. Red cell enzyme systems, *e.g.* erythrocyte acid phosphate (EAP), esterase D (ESD), etc.
3. Serum protein polymorphisms such as Hp, Tf, Pi and Gc.
4. The HLA system of white cell antigens.
5. Salivary proteins such as double band protein, proline rich protein, acid protein, etc.
6. DNA polymorphism.

The serological markers of principal importance for the forensic serologist may be divided into two main classes:

1. The polymorphic cell surface antigens; and
2. The polymorphic soluble protein markers.

The typical cell surface bound antigens are ABO and Rh blood group systems, clinically well known at the time of blood transfusion (A list of known cell surface antigen polymorphs is given in Table 1). A great many of these cell surface marker system were discovered before 1960, and till then this group constituted the major share of the known genetic polymorphisms. Of more recent origin is the system of histocompatibility antigens (HLA) found on lymphocytes. In the opinion of Mourant, "The very large number of known histocompatibility antigens present on the lymphocytes, the very much greater number of haplotypes, and the restricted geographical occurrence of a great many of them, renders the system an ideal one in forensic serology. It should, in due course be possible to define with considerable certainty, by means of this system alone, the racial and geographical origin of any person whose bloodstain is tested (Mourant, 1979).

The genetically determined polymorphic soluble proteins can be divided into two further categories: (i) intracellular enzymes and (ii) proteins found in blood plasma and / or other body secretions (Table 2 shows a large number of soluble proteins exhibiting genetic polymorphisms). These polymorphs are relatively late comers to the knowledge of recognized genetic markers, most having been discovered in the last three decades. Inspite of their being new comers the soluble protein

markers substantially outnumber the cell surface markers. The celerity in the discovery of protein polymorphism is attributed primarily to the development, refinements and extensive utilization of technique called electrophoresis which separates charged molecules according to their size and electrical charge properties. In addition, there are several protein polymorphisms which have been discovered by immunological differences and a few which are expressed as variation in enzyme activity levels.

But inspite of all this, not all the genetic marker systems, known so far, are useful in forensic serology. According to Sensabuagh (1982) the value of a genetic marker to the forensic scientist mainly depends on following consideration:

Table 1: Known cell surface antigens showing genetic polymorphism

Red Cell Blood Group Systems
ABO (H)
Auberger
Chido
Colton
Cromer
CS
Diego
Dombrock
Duffy
'i
Kell
Kidd
Knops
Lewis
Lutheran
LW
MNSs
P
Rh
Rodgers
Sd
Xg
Yt
White Cell Blood Group System
Five
PIE
HLA
Ko
Zw

* Table adapted from Sansabaugh (1982)

a. The marker polymorphism should involve simple qualitative differences such that an individual can be assigned to one type or another without ambiguity. It is desirable but not necessary that the phenotype directly reflect the genotype.
b. The marker should be expressed in a tissue of interest (*e.g.* blood, semen) throughout the life of the individual: markers which are expressed during restricted period of development (*e.g.* foetal proteins) or are expressed only in inaccessible tissues (*e.g.*, brain) are of limited value.
c. The marker should be robust; that is, it should be able to survive drying and other travails typically experienced by biological evidence. In particular the marker cannot undergo changes which make one type look like another or which so confuse the typing analysis that the risk of mistyping becomes significant.
d. The analytical procedures used for marker typing should be relatively simple and straight forward.
e. It is desirable that the analytical procedures consume as little as possible of the sample under test: the less sample consumed in any single test, more tests that can be done.
f. Finally, it is desirable that the polymorphism subdivide the population fairly evenly; a polymorphism which splits the population 50 : 50 is of greater value than one with 99 : 1 split.

Further, Mourant opines that, "In all forensic work, the most useful characters from the anthroplogical point of view, are those which are entirely, or almost entirely confined to a particular ethnic group ..... For the purpose of these it is desirable to now the frequencies of the genes or phenotypes concerned in all relevant populations and even small frequency differences must be taken into account" (Mourant, (1979).

Although the enzyme multiplicity was observed and described many years ago (Theorell, 1940), biological significance of his phenomenon was not recognized until fairly recently. With the advent of new and more refined biochemical tools in recent years, there has been an increasing awareness that the en-

Table 2: Soluble proteins exhibiting genetic polymorphism

Genetic Marker	*Mode of Polymorphic Expression*		
	Electrophoretic	*Activity variation*	*Antigenic variation*
Variants Found in Red Cells or Other Tissues			
Acetylcholine Esterase (ACE)	+		
Acid Phosphatase (ACP)	+	+	
Adenosine Deaminase (ADA)	+		
Carbonic Anhydrase II (CA II)	+		
Esterase D (EsD)	+		
Galactose I Phosphate Uridyl -transferase (GPUT)	+	+	
Glutamate Pyruvate Transaminase (GPT)	+	+	
Glutamate Dehydrogenase (GLUD)	+		
Glutathione Reductase	+		
Glyoxalase I (GLOI)	+		
Haemoglobin	+	+	
Peptidase A (PEP A)	+		
Peptidase C (PEP C)	+		
Peptidase D (PEP D)	+		
Phosphoglucomutase I (PGM_1)	+		
6 Phosphogluconate Dehydrogenase (6 PGD)	+		
Pyridiine kinase (PK)		+	
Uridine Monophosphate Kinase (UMPK)	+	+	
Variants Found in White Cells and/or Other Tissues (not in red cells)			
Aconitase (ACON)	+		
Alcohol Dehydrogenase (ADH)	+		
Alkaline Phosphatase (ALP)	+		
Aryl Hydrocarbon hydroxylase		+	
Cytidine Deaminase (CDA)	+		
Diaphorase (DIA)	+		
Fucosidase (FU)	+		
Glutamate Oxalacetate Tratsaminase (Mitochondrial) (m-GOT)	+		
Malic Enzyme (MOD-2)	+		
N-Acetyl transferase		+	
Phosphoglucomutase-3 (PGM_3)	+		
Pepsinogen (Pg)	+		
Variants Found in Blood Plasma and Some Other Body Fluids			
$Alpha_1$ Acid Glycoprotein	+		
$Alpha_1$ Antitrysin	+		
Amylase (Amy_1)	+		
Ceruloplasmin	+		
Complement Factor 3 (C'3)	+		
Complement Factor 4 (C'4)	+		
Complement Factor 6 (C'6)	+		
Coagulation Factor XIII	+		
Group Specific Component (Gc)	+		
Haptoglobin (Hp)	+		
Immunoglobulins (Km)			+
Immunoglobulins G (Gm)			+
Immunoglobulins A (Am)			+
Immunoglobulins M (Mm)			+
Beta - Lipoprotein E	+		
Beta - Lipoprotein E(Ag)			+
Beta - Lipoprotein (Ld)			+
Beta - Lipoprotein (Lp)			+
$Alpha_2$ Macroglobin (Xm)			+
$Alpha_2$ Macroglobin			+
$Alpha_2$ Macroglobin (ALM)			+
Properdin Factor B (Bf)			
(Glycine Rich Glycoprotein)			+
Pseudocholine Esterase E_1 (PCE_1)	+		
Pseudocholine Esterase E_2 (PCE_2)	+		
Transferrin (Tf)	+		
Transcobalamin (Tc)	+		
Variants Found in Saliva			
Hexose-6-Phosphate Dehydrogenase (Sgd)+			
Amylase (Amy_1)	+		
Acid Protein (Pa)	+		
Basic Protein (Pb)	+		
Double Band Protein (Db)	+		
Proline Rich Protein (Pr)	+		

* Table adapted from Sensabaugh (1982)

zyme multiplicity is the rule rather than exception as previously thought. Along with the knowledge came an accumulation of experimental data from various sources clearly pointing to the fact that multiple molecular forms of enzyme were not all physical artifacts as earlier throught by the physical biochemists, but that in many instances these multiple forms were the products of specific genes. Markert and Moller (1959) coined the term isozyme as an operational definition to encompass the multiple enzyme forms in biological systems.

The use of isozymes as genetic markers has increased dramatically over the last 25 years. Isozymes offer a number of important advantages over more conventional markers. Isozyme variants frequently occur spontaneously and seldom produce obvious deleterious effects. Variant alleles are generally codominant making possible to easily and positively identify heterozygotes as well as homozygotes and to monitor conveniently the time expression of the parental alleles in the heterozygotes (Scandalios, 1975).

Harris and Hopkinson (1972) from an actual study on enzyme polymorphisms in Europeans, have shown that in man any single individual is likely to be heterozygous for common alleles giving rise to electrophoretic differences at about seven percent of his loci coding for enzyme structure. Because of many different allelic combinations at the various loci that can occur, it is likely that no two individuals, except perhaps with the exception of monozygotic twins, are exactly alike in their isozyme constitutions.

Culliford and his colleagues of the Metropolitan Police Laboratory in London were the first to introduce electrophoretic markers, serum protein and red cell isozymes, into the field of forensic serology. However, not much work has been done in India on the forensic applications of isozymes and plasma proteins, but the following electrophoretic markers have been reported to be satisfactorily detected in bloodstains, and their use has already been established in major crime laboratories of the world : Phoshoglucomutase$_1$ (PGM_1), Erythrocyte acid phosphatase (EAP), Esterase D (ESD), Glyoxalse I (GLO I), Adenylate Kinase (AK), Adenosine deaminase (ADA), Carbonic anhydrase II (CA II), 6-Phosphogluconate dehydrogenase (6-PGD), Glucose - 6 - phosphate dehydrogenase (G-6-PD), Glutamate pyruvate transaminase (GPT) and Pseudocholinesterase$_1$ (PCE_1), Haptoglobins (Hp) and Transferrins (Tf).

The present study was undertaken with a view to investigate the detectability of some of the important polymorphic isozymes and a serum protein haptoglobin in dry stains. Attempt has been made to study the time lapse up to which the haemogenetical factors can be reliably typed under varied conditions of storage and preservation. Besides, taking into consideration the frequency distribution of these genetic markers in various Indian populations, the suitability of these factors in Indian crime laboratories has also been assessed.

MATERIALS AND METHODS

The following genetic markers were included for investigations:

(i) Haptoglobins (622 blood samples)
(ii) Phosphoglucomutase (648 blood; 26 semen samples)
(iii) Carbonic anhydrase II (588 blood samples)
(iv) Esterase D (606 blood samples)
(v) Adenylate kinase (624 blood samples)
(vi) Glyoxalase I (609 blood; 26 semen samples)
(vii) 6 phosphogluconate dehydrogenase (469 blood samples)

The blood samples were collected from the donors of the Lok Nayak Jai Prakash Narain Hospital Blood Bank, New Delhi in citrate dextrose anticoagulant and from the volunteer donors at the National Institute of Criminology and Forensic Science (NICFS), New Delhi.

Preparation of Blood Samples

Dried bloodstains were prepared by putting a small amount of whole blood directly on to pieces of dry cotton muslin cloth and other synthetic fabrics, which had previously been washed and boiled in distilled water. Bloodstains were also made by taking the blood from volunteers at the NICFS, by the finger prick, directly on to pieces of various fabrics. Each stain prepared was then allowed to thoroughly air-dry (except when otherwise stated) for 4-5 hours and placed in individual labelled paper envelopes. The stains were then divided into different batches and subjected to different conditions of storage and exposure.

The amount of blood for the preparation of blood stain was not controlled, though stains of roughly equal depth of colour and good enzyme activity alone were selected for further study. These selected stains were subjected to varied conditions of temperature and other climatic factors, and studied for their typability. Storage studies were conducted separately in the months of intense cold and heat, and also in the rainly humid season. A minimum of five bloodstains were typed in each set of varied conditions. These samples were then typed periodically at different time intervals to study the persistence of activity.

Fresh Semen Samples

The fresh ejaculate samples were collect-

ed in sterilized, wide mouthed glass tubes with screw caps, from volunteers. These liquid samples were then immediately transferred to a deep freezer (-20°C) till tested.

Semen Stains

The semen stains were made by the volunteers themselves on clean pieces of cotton muslin cloth (approx. 30 cm x 30 cm) given to them. The semen stained cloth pieces were collected within thirty minutes of their preparation and then air dried at room temperature for three to four hours. After which the stains were put into individual paper envelopes, which were in turn kept in self sealing polythene bags and stored at varied conditions of preservation, until required for PGM and GLO typing.

Storage Conditions Studied

Bloodstains subjected to the following sets of conditions were analysed:

(i) One batch of bloodstains was kept at 40°C-45°C (in a dessicator over silica gel) to study the effect of dry heat on the enzyme activity.
(ii) Three sets of bloodstains were stored at room temperature on the shelf, in wooden cabinet and steel almirah, respectively. Studies were conducted during the months of severe cold (December to February), intense heat (April to June) and rainy months of July to mid September.
(iii) Stains were also stored, wrapped in polythene bags to prevent any dripping of water directly on the stains at 4°C in a refrigerator.
(iv) Another batch of bloodstains, also in polythene bags, was kept at -20°C in a deep freezer.

Effect of Direct Solar Radiation

One batch each of freshly prepared bloodstains (still wet) was directly exposed to the sun light in the months of intense heat and cold, respectively. A second set of stains which had previously been air-dried in shade at room temperature for 4 to 5 hours, was exposed to solar radiation in the months of summer and winter separately.

Experiments were conducted both in summer and winter months to compare the results.

Effect of Sweat Contamination

A set of ten bloodstains was prepared by finger pirck method on dried muslin cloth pieces after they had been thoroughly soaked with sweat of the respective persons. Separate bloodstains were prepared without sweat from the same person to serve as controls. These stains were stored at room temperature during the months of June and May (day temperature ranging from 35°C to 40°± 5°C).

Effect of Soil Contamination

A few drops of freshly collected blood samples were allowed to fall on heavily soiled ground. The stains were allowed to dry naturally. The samples were later collected after 7 to 8 hours of drying and stored at room temperature in individual paper envelopes. These blood soaked soil samples were then periodically tested for the persistence of activity in them.

HAPTOGLOBIN

Haptoglobin forms a part of the alpha-2 serum gloubin fraction, and is initially synthesized in the liver. It is glycoprotein with a carbohydrate content of 20 per cent, consisting of hexoses, glucosamine, fucose and sialic acid. Haptoglobin binds with haemoblobin specifically, and the haptoglobin haemoglobin (Hp-Hb) complex thus formed remains soluble and does not precipitate. The Hp-Hb binding occurs stoichimetrically, is very stable, and no exchange of bound with unbound Hb is demonstrable.

Haptoglobin is of great forensic importance for several reasons:

(i) It occurs as three rather common phenotypes
(ii) Is present in reasonably high concentration in bloodstains
(iii) Has satisfactory stability in such samples; and
(iv) Can be readily visualized after electrophoretic separation because of its ability to form a highly stainable complex with haemoglobin.

Several workers have reported typing of Hp from bloodstains by using different gel media like starch (Peacock et al., 1965; Ferris et al., 1966; Ritchie et al., 1966; Woodworth and Clark, 1967; Gazaway, 1977; Blake and Sensabaugh, 1978), polyacrylamide (Culliford, 1963; Culliford and Wraxall, 1966) and gradient polyacrylamide gel (Culliford, 1977; Baxter and Rees, 1974; Bryan and Jay, 1975, Stolorow and Wraxall, 1979; Sivaraman et al., 1980; Grunbaum, 1975).

The polyacrylamide gels now in use have considerable advantages over starch gel: they are more uniform in successive preparations, eletrophoresis time is much shorter, can be dried readily for quantification and permanent storage, pore size of the gel can be easily and accurately adjusted to produce better resolution an stable pH gradients can be creasted for isoelectric focusing (Grunbaum, 1975).

However, haptoglobin has not gained wide acceptance as a genetic marker in the routine analysis of bloodstain evidence. Stolorow and Wraxall (1975) list the following two reasons for this situations:

(i) Alledged requirement for specialized vertical gel electrophoresis equipment; and
(ii) The fact that haptoglobin electrophoretograms from bloodstains older than a few weeks are frequently masked by trailing dark streaks caused by the products of Hb deterioration in the bloodstains.

Several methods have been published to get rid of these unwanted streaks. The molecular weight differences between these entities have been successfully exploited by the use of gradient polyacrylamide geis (Culliford, 1971). This method differs from the other electrophoretic procedures in that the separating protein bands concentrate as they move through the gel until they reach pores of such a size that further movement through gel is not possible. As a result, the Hp-Hb complexes can be completely separated from the free Hb molecules due to the differences in molecular size (Margoils and Kenrick, 1969). Sensabaugh and Blake (1978) gave a method of sodium dodecyl sulphate gel electrophoresis that requires the use of monospecific human anti-Hp antibodies to isolate haptoglobin from the liquid extract of a bloodstain by immunoprecipitation. The Hp alpha and beta chains are dissociated from the immune precipitate and the Hp2 and Hp 1 chain types are distinguished electrophoretically by their size differences. Hp 1 F and Hp 1 S peptides differ by a single amino acid substitution but can not be differentiated by conventional typing methods using starch or polyacrylamide gels; both are lumped together as Hp 1 chains.

Gazaway (1976) reported an acid extraction method involving disc electrophoresis at a pH near the isoelectric point of the Hp-Hb complex, followed by the manual withdrawal of the liquid samples from the tubes.

A much simpler and a less time consuming chloroform extraction method has been described by Stolorow and Wraxall (1979).

For the present study, haptoglobin phenotyping in bloodstains was routinely done by disc electrophoresis in vertical tubes of 5% acrylamide gel using the discontinuous buffer system as given by Davis (1964) and Ornstein (1964). This technique was found to be less cumbersome and more economical. Following electrophoresis the gels were stained for peroxidase activity with benzidine hydrochloride. For the initial extraction of blood concentrate from the stained material, sucrose extraction solvent as described by the Metroploitan Police Forensic Science Laboratory (Biology Methods Manual, 1978) was used. This was followed by the chloroform extraction procedure for the removal of excess haemoglobin as described by Stolorow and Wraxall (1979).

A modification of the chlorophorm extraction method using Tris-Hcl buffer was employed in the present study (see Schdeva et al., 1990).

Results

As the bloodstains aged, and depending upon the storage conditions to which they were subjected, the intensities of Hp bands became gradually weaker, but no difficulty was encountered in interpreting the results. It was observed that in the months of October through March, when the average temperatures

remained between 18°C to 27°C satisfactorily typeable results for Hp could be obtained up to 6 months. Wooden cabinets proved to be superior to steel almirah for bloodstain storage in summer months (35°C - 45°C). The stains stored in a wooden cabinet retained their activity at least 2 weeks longer than those stored in a steel almirah. Similar differences were not observed in winter or rainy months.

With the bloostains that were kept at about 4°C in a refrigerator, satisfactory results could be obtained for up to 11 months. Still better retention of activity (up to 18 months) was observed in stains that had been stored at - 20°C.

Solar radiation caused the most rapid deteroration of haptoglobins in bloodstains. A mere 24 hours of exposure of wet bloodstains to direct sun light rendered them untypeable beyond 22 days. The stains which were dried in shade and then similarly exposed to sun light fared a little better by retaining their activity up to 28 days.

Bloodstains stored during the rainly season of July to mid-September, when the temperature remained at about 30°C and relative humidity near 81% retained typeable Hp activity only up to 52 days.

In the case of heavily soiled samples, whatever the storage conditions, it was not possible to phenotype Hp patterns beyond a period of 18 days.

The study showed that bloodstains kept at 40°C to 45°C in a dessicator over silica gel retained typeable patterns up to 78 days.

Discussion

The starch gel electrophoresis method as originally given by Smithies (1955) had proved to be of little use for the determination of Hp phenotypes in bloodstains.

Culliford and Wraxall (1966) described an immunoelectrophoretic technique which also fell into disfavour due to its poor sensitivity and the non-availability of potent and specific anti-Hp sera.

Culliford (1971) later introduced a polyacryamide gradient gel electrophoresis (PAGE) procedure for haptoglobin typing in bloodstains. It was reported that the results obtained by this technique were very easy to interpret and that the method was far more sensitive than immunoelectrophoretic techniques (Baxter and Rees, 1974; Thyman, 1976). But in spite of this, hardly any forensic science laboratory in India seems to have accepted this technique for routine bloodstain analysis. This may be partly due to the requirement of specialized "Gradipore" accessories and partly due to the tedious and cumbersome nature of its procedure.

Chloroform has the capacity to precipitate either haemoglobin or its deterioration products from certain aqueous solutions, depending on the pH, temperature, ionic strength and dielectric constant of the solution (Stolorow and Wraxall, 1979).

The chloroform extraction method has virtually revolutionized the Hp typing in bloodstains. This procedure largely eliminates the streaking effect of the Hb deterioration products from Hp electrophoretograms. It is not only very straight forward, but also needs considerably less physical manipulation of the samples that is the case with other methods.

However, in the original method described (Stolorow and Wraxall, 1979) the authors recommended the use of aqueous sucrose solution for the extraction of bloodstained material. In the present study it was found that just the aqueous solution of sucrose was a poor solvent for the older stains. If the extraction procedure is not good, however sophisticated and refined electrophoretic methods one may employ, the results are bound to be poor. So, for this reason, for the extraction of bloodstain material prior to chloroform treatment, the sucrose extraction buffer as given in the Biology Methods Manual (1978) was followed throughout the present study. Though the buffer alone is a potent solvent, sucrose in it provides an increase in the density of the liquid samples necessary for their application into the sample wells of a vertical gel.

Bryan and Jay (1975) reported typing Hp in bloodstains aged up to 5 weeks, whereas Baxter and Rees (1974) claimed to have typed stains upto 4 months old, both by using polyacryamide gel electrophoresis. Sivaraman et al. (1980) also using PAGE, reported that while stains aged 30 days or less appeared

most suitable for Hp typing under Indian climatic conditions, those aged 30 to 50 days also would prove good for Hp type identification. All these time intervals for Hp stability are very short when compared to those reported by Stolorow and Wraxall (1979). In their case bloodstains ranging in age from approximately 6 weeks to 2 years were accurately phenotyped.

The results of the present study agree well with those of Stolorow and Wraxall (1979). The superior results obtained in the present study may be attributed to the good combination of sucrose extraction buffer with the chloroform extraction procedure employed for the phenotyping of haptoglobins in dried bloodstains.

PHOSPHOGLUCOMUTASE$_1$

Phosphoglucomutase transfers the phosphate group from the one position to the six position on the glucose molecule in an early stage of the glycolytic pathway, thus converting glucose-1-phosphate to glucose-6-phosphate. Glucose-1, 6-disphosphate is required as a co-factor together with magnesium ions.

The introduction of enzyme typing in forensic science laboratories using the technique of starch gel electrophoresis has established PGM as one of the valuable and widely used systems. Its importance lies not only in its stability, discrimination power, but also because it can be detected in hair, semen and saliva as well.

Identification of PGM$_1$ system in dried bloodstains was first reported by Culliford in 1967. Later, Wraxall and Culliford (1968) described a thin layer starch gel electrophoretic technique for enzyme typing in bloodstains and established the stability of PGM$_1$ typing in forensic case-work examination. They reported the satisfactory typing of PGM$_1$ in one month old bloodstains. Brinkman (1969) reported the typability of the enzyme in three to twelve weeks old stains. Oepen (1970) reported that enzyme activity stayed typable for 8-10 weeks in bloodstains. Rothwell (1970) reported that PGM$_1$ activity remained consistent on one month old bloodstains and survived for two months. Turowska (1971) could obtain typable results up to 8 weeks. Later, Culliford (1971) in his book reported that fresh haemolysates stored at -20°C could be satisfactorily typed even after 6 to 12 months. And in case of lysates or the red cells stored in liquid nitrogen, activity and patterns could be preserved indefinitely. It was also found that bloodstains over three months old have been typed quite satisfactorily. Saha and Kirk (1973) studied the stability of PGM isoenzymes in dried bloodstains on filter paper and reported that it was stable for one month at 20°C and for longer periods in colder conditions. Zajac and Sprague (1975) by the method of cellulose acetate electrophoresis, could identify PGM$_1$ variants in bloodstains varying in age up to 20 months. Miscicka et al. (1977) again by cellogel method reported PGM typability till 24 weeks. Joshi et al. (1978) described their stability studies on PGM$_1$ enzyme system. It was observed that bloodstains stored at 40°C-45°C in a dry condition retained typable activity for about 20 days. However, in case of the bloodstains stored at room temperature during humid season typable activity remained for a period of 8 days only. Meghal et al. (1983) reported that the stability studies of this enzyme at different temperatures in dry and wet conditions show that at -20°C the enzyme is stable up to 8 weeks, while at room temperature, in dry conditions, it is stable up to 4 weeks. However, in moist conditions, at room temperature, it is stable only up to 2 weeks.

As far as identification of PGM$_1$ types in semen stains is concerned, Rees and Rothwell (1975) analysed both uncontaminated semen samples and also post-coital vaginal swabs and opined that although PGM typing of uncontaminated semen stains might be of value in forensic case work, there were great difficulties associated with the PGM typing of semen in post-coital swabs. Dried semen stains up to three months old could be satisfactorily typed. Liquid semen samples that had been stored at -20°C for periods up to 47 weeks retained sufficient enzyme activity for correct typing.

For the present study Tris maleic acid EDTA-magnesium chloride buffer, pH 7.4 was used (see Sachdeva, 1987).

Results

The results obtained with haemolysates, liquid semen samples and fresh stains of blood and semen were identical in band pattern, both in intensity and definition.

It was observed that stains stored at room temperature during the winter months of November through February retained typable activity for about 12 weeks. However, in case of the bloodstains stored at room temperature during summer season (April to June) in steel almirah and wooden cabinet the typable activity remained for a period of 6 and 7 weeks, respectively.

In the third set of bloodstains which had been kept at 4°C in a refrigerator, PGM_1 activity was observed for about 15 weeks. On the other hand, stains which had been kept at -20°C detectable PGM_1 activity was noticed for over 6 months. A mere 18 hours exposure of wet stains to direct solar radiation spread over 4 days in summer and winter seasons, was sufficient to render them useless for enzyme typing beyond 6 and 8 days respectively. whereas in bloodstains dried in shade and then exposed to sun light during summer and winter months remained typeable till 8 and 11 days, respectively. Bloodstains stored at room temperature in the rainy season (July to mid September) retained typable PGM patterns only up to 19 days. Whatever the storage conditions, soiled samples could not be reliably typed beyond a period of 12 days. The set of bloodstains stored at 40°C to 45°C in a dessicator over silica gel could be typed upto 32 days of storage.

No detrimental effect on the PGM_1 patterns was observed when blood was contaminated with perspiration.

In case of 26 semen samples examined, though the liquid samples stored at -20°C could be typed up to 45 weeks, semen stains could be typed only up to 16 weeks. The seminal stains retained their typable enzyme activity up to 11 weeks stored during the months of November through February, and upto 6 weeks in the months of April to June.

Discussion

648 blood samples collected from local Delhi and some Indian states and Union Territories populations have been phenotyped for phosphoglucomutase$_1$ by this procedure. In addition, 26 semen samples have also been studied for the persistence of PGM_1 activity in them.

The stain substrate appeared to make no difference to the persistence of enzyme activity. Satisfactory PGM_1 typing could be accomplished on all types of textile materials, metals, soiled ground surfaces, and others. The only difference noticed was that for the satisfactory typing in case of synthetic fabrics, larger pieces of stained cloth were needed than in the case of cotton clothes.

As is the case with other proteins, PGM_1 activity is more labile in hot and humid conditions. Reliable typable activity remained for 19 days during the humid season as compared to 32 days in dry conditions at 40° to 45°C. This is obviously due to the fact that humid and hot ambient environs are quite conducive to the growth of microbes, which may degrade and denature the protein, and hence loss of enzyme activity. On the contrary, dry atmosphere and temperatures as high as 45°C would be unfavourable for bacterial growth and thus, proteins may be preserved for a longer time periods.

By the same token, low temperatures of 4°C to -20°C will also slow down or check the microbial growth, thus preserving the enzyme activity for longer periods of time. It is partly for this reasons that typable PGM_1 activity was observed for relatively longer periods, in bloodstains analysed during winter months than those studied during summer months.

Starch gel was selected as the medium of electrophoresis for the present study as it gives better sieving effect and resolution than that obtained with agarose or cellulose acetate membrane. Furthermore, it requires no prior preparation or treatment of the sample as is the case with cellulose acetate membrane, where blood or semen stains have to be scrapped, extracted for 24 hours and centrifuged; which is quite cumbersome and time consuming. Moreover, with strarch gel electrophoresis, even weak stains could be anslysed by applying more quantities of bloodstained material directly into the

gel. It was also found to be more economical than cellulose acetate membranes.

CARBONIC ANHYDRASE II

More than six decades ago an enzyme was isolated from human red cells that rapidly catalysed the reversible hydration of carbon dioxide and was named carbonic anhydrase (Meldrum and Roughton, 1932, 1933). Two molecular forms of CA occur in human erythrocytes and are referred to variously in the literature as CA - B and CA - C or CA I and CA II isozymes. The specific CO_2 hydrase activity of the CA II isozyme is higher than that of CA I. In humans, carbonic anyhdrase isoenzymes are second only to haemoglobin as the most abundant protein component to be found in erythrocytes. Both CA I and CA II are monomeric and are apparently under the control of two closely linked autosomal genes (Harris and Hopkinson, 1976). In tissues other than blood the CA-I isozyme has been found to be present in adult postmortem liver, kidney and retina, but CA II isozyme is more extensively distributed in tissues. It has been found in gastric mucosa, lungs, cerebral cortex cerebral medulla, kidney, parotid gland, ciliary process and gall bladder. Genetic variants of CA I have been identified (Shaw et al., 1962; Tashian, 1969; Tashian et al., 1963; Carter et al., 1972; Ueda, 1974) but their frequency of occurrence is too low to have any forensic applications.

Till recently, only one variant of human carbonic anhydrase II, CA II 2 had been found. The variant was originally designated CA H and has subsequently been referred to as CA II (Carter, 1972; Osborne and Tashian, 1974), CA II 2 (Hopkinson et al. 1974) and CA II_2 the allele being CA II^2. Subsequently two more alleles CA II^3 and CA^4 (Blake and Kirk, 1978) were found. Except for the Marathis and Parsis of Bombay, the Indian populations are reported to be monomorphic for CA II (Ghosh, 1977). However, very few population groups of India seem to have been surveyed. Nevertheless, a CA II^2 frequency of 0.0036 in an Indian population group samples in South East England has been reported (Hughes, 1978).

Results

For the present study, method as given by Noppinger and Morrison (1981) was followed (see Sachdeva et al., 1990).

CA II type 1-1 consists of a single fluorescent band with the most cathodal mobility. CA II 2 phenotypes is also represented by a single band near the origin also with the cathodal mobility. The heterozygote CA II 2-1 has both the cathodal bands present in equal intensity.

The effect of ageing on stored blood stains varied depending upon the conditions of storage and treatment. The effect of different storage - such as varied temperature, dry heat, solar radiation, heavily soiled condition of the blood stains, etc. on the determination of CA II types in stains was studied.

Blood stains at 20°C could be typed upto 17 weeks, and those stored at 40°C also retained sufficient CA II activity to allow reliable typing up to 15 weeks. However, stains kept at 37°C could be typed only up to 6-7 weeks. After 8 weeks of storage at room temperature, the intensity of bands decreased rapidly. After 18-19 weeks of storage even under deep frozen conditions (-20°C) the zones of activity disappeared altogether, leaving only the diffuse faintly stained fluorescence at the sites of electrophoretic bands.

In the months of winter from November to February when the temperature varied between 18°C to 27°C the stains showed typable activity till 12 weeks even when they were kept at room temperature. During the summer months of April to June the blood stains stored at room temperature in wooden cabinet retained their enzyme activity for 44 days, while it was only for 36 days in those stored in steel almirah. Whereas wet stains exposed to direct solar radiation in winter for 18 hours spread over 4 days could be reliabily typed for 18 days, wet stains similarly exposed in summer months could be typed only up to 12 days. On the other hand, blood stains, dried in shade and then subsequently exposed to direct sunlight, in the winter and summer seasons retained their enzyme activity for 26 and 18 days respectively.

In the hot and humid conditions of July to mid September during rainy season, it was not possible to type the blood stains stored at room temperature beyond a period of 29 days. In the case of soiled samples also typable CA II activity could be observed upto a week. Those samples which were subjected to dry heat conditions (40°C to 45°C) by keeping them in a dessicator over silica gel, remained typable even upto 42 days. It was observed that persistence of typable activity was not significantly affected by the presence of body perspiration in them; both control samples as well as blood stains made on sweat soaked cloth gave good typable results up to 35 days.

Discussion

No storage bands were observed in old blood stains. However, if the reaction plate was incubated at 37°C for a longer period of time, say half an hour, the development of another weakly stained fluorescent band just near the origin was observed. This band has been shown to be of CA I isoenzyme (Hughes, 1978).

In the Indian context, this system can also be of immense help in actual case work to help the investigations in the determination of an unknown blood stain that could have possibly originated from an individual of Negroid or Parsi ancestry.

ESTERASE D

The existence of variations in human red cell esterases was first revealed by Tashian (1961, 1969) with starch gel, borate buffer electrophoresis followed by azo-dye coupled staining techniques.

Hopkinson and his associates in 1973 identified another zone of esterase activity, EsD, with 'flurogenic' substrate 4-methylumbelliferyl acetate overlays following starch gel electrophoresis. This new esterase which could not be detected by the azo-dye coupling procedures, was located between the zones of activity of esterases A_1 and B under the electrophoretic conditions. Also, the EsD isozymes, in contradistinction to A, B and C esterases exhibit common genetic polymorphism in all major Causcasian, Black, Mongolian and Asian populations.

Close to the heels of EsD enayme discovery in 1973 by Hopkinson et al., Parkin and Adams (1975) recognized the forensic potential of EsD polymorphism. They employed a modified thin layer electrophoresis procedure and Tris-citrate-borate-lithium hydroxide buffer system, pH 7.2 to phenotype dried bloodstains. Among other things, they reported the difficulty in accurately typing of relatively aged bloodstains due to the fact that there were alterations in the band pattern with older stains. They could type EsD patterns in bloodstains up to 3 weeks old.

Since fluid blood samples submitted for forensic serological analyses may contain anticoagulants and preservatives or may have been exposed to different temperature environments, in transit, a systematic study of the effect of these factors on the persistence of EsD activity was conducted by Jay and Philip (1979) employing the starch gel electrophoresis technique described by Parkin and Adams (1975). They reported grouping of bloodstains up to 4 weeks old. Stains older than four weeks could not be typed. Fluid blood incubated at 37°C for one day was readily and correctly typed irrespective of the anticoagulant used ; however, by the third day at 37°C all the fluid samples had deteriorated to the extent that none of the phenotypes could be identified. After five weeks of storage at 3°C phenotypes were easily identified regardless of anticoagulant. Their studies showed that fluid blood either without anticoagulant or with citrate fluoride provided maximum stability of the isoenzymes up to 28 days. Heparin treated samples were readable until the 21st day, while oxalate and EDTA severely reduced the survival time to 14 and 3 days, respectively.

Preliminary studies of EsD in body fluids showed that when seminal stains and swabs of body fluid were treated in the same manner as bloodstains, very weak EsD patterns corresponding to the blood pattern of the donors could be observed. However, even in 2-4 days old stains, the patterns were usually too weak for interpretation.

No detrimental effect on the EsD band pattern was noticed when blood was mixed with sweat or saliva; however, slight distortion of

the bands was observed with saliva because of slight digestion of the starch around the origin by the amylase (Jay and Philip, 1979).

Grunbaum et al. (1978) were able to obtain typable results with esterase D isoenzymes on cellulose acetate membranes in blood stains upto 2 months old.

An evaluative study of EsD typing by isoelectric focussing was conducted by Horscroft and Sutton (1983). According to their study, although simultaneous typing of PGM_1 EsD and EAP was possible, it was observed that EsD typing was so unreliable with stains excess of 2 days old, that typing by this method was not practicable.

Results

In the present study, for the detection of ESD isozyme in bloodstains and semen stains, the technique was adapted from Parkin and Adams (1975) (see Sachdeva et al., 1984).

The three common phenotypes of esterase D are EsD 1, EsD 2-1 and EsD 2-2. The same band pattern was observed in case of bloodstains. However, the intensities of individual bands gradually diminished as the bloodstains aged. Anodal secondary bands of EsD were also observed, gradually becoming more intense in older stains.

However, in the case of haemolysates stored at -20°C and 4°C, there was not much decrease in the intensity of bands. In dried bloodstains, EsD 2 type could easily be recognized due to its relative faster mobility. Differentiation between EsD 2-1 and EsD 1 types did pose a problem, but these were identified without much difficulty by following the scheme given by Sensabaugh (1982).

In bloodstains stored at room temperature (18°C-27°C) during months of November through February, esterase D had a maximum stability of 4 weeks. However, in the summer months of April to June (32°-45°C) bloodstains stored in steel almirah and wooden cabinet gave reliable results only up to 14 and 18 days, respectively.

It was observed that the enzyme acvtivity was better preserved at lower temperatures of 4°C (5 weeks) and -20°C (7 weeks). There was a considerable loss of EsD activity in the bloodstains which were exposed to sun light. Wet stains exposed to direct solar radiaton for 7 hours spread over 2 days in winter and summer months showed typable activity up to 7 and 5 days, respectively. However, fresh shade dried stains simlarly exposed to sun light in winter and summer seasons, exhibited enzyme activity up to period of 11 days and 8 days, respectively. Humid months of July to mid September hasten the decrease of enzyme activity in stored bloodstains. During this period, it was not possible to group bloodstains beyond a period of 5-6 days. Heavily soiled samples could also be phenotyped but only when the stains were not more than 2 days old. Bloodstains subjected to dry at conditions (by keeping them in a dessicator) retained typable EsD activity upto 9 days only.

Body perspiration, an important contaminant, did not significantly affect the reliable typing. Almost identical enzyme patterns were obtained in both sweat contaminated and control samples.

In case of semen, though the fresh liquid samples exhibited weekly developed zones of activity, semen stains even a few hours old gave such a poor resolution that analysis of semen stains had to be discontinued.

Discussion

685 bloodstains from volunteer donors representing almost all the states and Union Territories of India have been successfully typed for esterase D. In this way, the sample studied was essentially of a heterogeneous nature.

Different buffer systems as used by Hopkinson et al. (1973), Parkin and Adams (1975) and Hayward and Bosworth (1975) for starch gel; and by Grunbaum et al. (1978) and Jay and Philip (1979) on cellulose acetate membrane were tried. But the buffer system recommended by Biology Methods Manual (1978) was found to be most satisfactory.

Interestingly, it was observed that in case of electrophoresis of relatively old bloodstains, better visibility of enzyme band was obtained when viewed from the reverse side of the glass gel plate.

ADENYLATE KINASE

The enzyme Adenylate Kinase (AK), orig-

inally known under the name of myokinase takes part in the reversible transformation involving a high energy phosphate group from one molecule of adenosine diphosphate (ADP) to another producing one molecule each of adenosine triphosphate (ATP) and adenosine monophosphate (AMP) thus playing an important role in the body.

The determination of adenylate kinase types in blood stains was first reported by Culliford and Wraxall in 1968. They reported the typable activity of the enzyme in three months old blood stains. Rothwell (1970) reported the typable activity in one month old blood stains. However, Culliford (1971) writes in his book that dried blood stains upto 3 to 6 months old could be satisfactorily typed. He reports that in an attempt to reduce the amount of blood stain required for a complete enzyme investigations, an effort was made to combine AK with 6-PGD (6-phosphogluconate dehydrogenase) on a phosphate buffer gel, developing the anodic part of the plate for 6-PGD and the cathodal part for AK.

Saha and Kirk (1973) had reported the stability of AK variants in dried blood stains on filter paper up to one month at 20°C and for a longer time period in colder conditions. Saenger and Yates (1975), on the other hand, claimed typability even in six months old bloodstains. Investigations of Stombaugh and Kearney (1977) on this enzyme showed that bood stains retained good typable activity for 3 to 18 months depending on temperature and storage conditions.

Joshi (1978) reported that stains stored at room temperature gave typeable activity even after 7 months.

Results

By employing the succinic acid buffer system, 413 blood samples derived from Delhi local population have been successfully typed for periods ranging in age up to 17 months old (see Sachdeva et al., 1990).

Blood stains stored at room temperature, during the months of September through February, gave satisfactory typeable activity upto 7 months. In this system, it was observed that blood stains stored at room temperature in wooden cabinet during the summer months (35°C - 45°C) survived for a relatively longer time period (72 days) than those stored in steel almirah (63 days).

Cold temperature stored conditions of 4°C to 30°C are best for the retention of typable AK activity for very long periods. It was observed that whereas it was possible to type blood stains kept in a refrigerator up to 14 months, typeable activity persisted as long as 17 months at -20°C.

In case of wet stains exposed to direct solar radiation for 18 hours spread over 4 days in summer and winter seasons, AK activity was observed to lose its intensity very rapidly and could be typed only upto 38 to 47 days respectively. However, fresh blood stains which had been dried in shade and were subsequently exposed to sunlight for similar periods, remained typable for 52 and 64 days, respectively. In the humid months of July to mid September, it was possible to obtain satisfactory results upto 6 weeks.

Subjecting the blood stains to dry heat conditions (40°C to 45°C) by keeping them in a dessicator over silica gel, gave better preservation, as reliable typing results could be obtained for about 2 months.

Blood stains which were made on sweat soaked cloth pieces gave as satisfactory results as obtained with the uncontaminated control samples.

Discussions

It is observed that AK is quite robust enzyme, able to withstand fairly vigorous conditions of humidity and temperature, and still maintain an appreciable activity.

Culifford (1971) reported that after 3 to 4 weeks the loss of activity was seen and as time progressed, the loss of activity became more and more apparent until at three months, when some samples retained sufficiet activity, many did not. He also reported that '2' band in AK seemed more sensitive to ageing than the major '1' band, particularly if phosphate buffer was used. He recommended the use of succinic acid buffer for the old samples or in cases where low activities might be expected.

Rothwell (1970) studied the effect of stor-

age on the activity of PGM and AK in blood stains and reported that it was possible to type AK enzyme in stains upto 6 months.

Saenger and Yates (1975) had reported the typable AK activity for 7 months old bloodstains by using cellulose acetate membranes. They mentioned that stains older than this failed to give a coloured extract, even after 24 hours of extraction at 4°C and failed to give clear AK results.

Stombaugth and Kearney's (1977) investigations on his enzyme showed that blood stains stored at 25°C, 4°C or -16°C retained the typeable activity for 18 months, and for those kept at 37°C, up to 3 months.

The weakly staining anodal bands of AK, which are secondary modification products of the primary isoenzyme, become more pronounced in older samples (Jamil et al., 1978) and stains (Culliford and Wraxall, 1968; Rothwell, 1970; Culliford, 1971). The results obtained in the present study agree with these observations. Analysis shows that even under hot humid conditions prevailing in most of India, this enzyme exhibits great stability.

GLYOXALASE I

More than five decades ago, Cohen and Sober (1945) reported cells with considerable amounts of glyoxalase activity. However, genetic variation of GLO I was only revealed when Kompf and asociates (1975) showed it to be polymorphic in a South-western German population. The glyoxalase enzyme system which consists of two enzymes GLO I and GLO II, coverts 2-oxaldehydes viz. methylglyoxal, phenylyoxal to 2-hydroxy acids. GLO I and GLO II act together to produce the "glyoxalase reaction" which transforms methylglyoxal to a DL Lactate with glutathione required as a cofactor (Knox, 1960). GLO I catalyses the irreversible combination of methylglyoxal and glutathione to form S-lactoylglutathione. It is postulated that this transformation occurs via an intermediate product, a thiohemiacetal, which is rapidly converted to S-lactoylglutathione (Racker 1951; Crook and Low, 1952; Rose, 1957). Though GLO I occurs in high concentrations, GLO II is totally absent in the human red cells.

Electrophoretic analysis of GLO I exhibits three common phenotypes : GLO 1-1, GLO 2-1 and GLO 2-2 determined by two codominant autosomal alleles, GLO^1 and GLO^2.

For the present study, the starch gel electrophoresis procedure (Biology Methods Manual, 1978) with slight modifications was followed (see Sachdeva et al., 1989).

Results

404 bloodstains from volunteer donors of Delhi city have been successfully typed for GLO I isozymes. In addition, paired blood and semen samples of 26 men were also tested for correlation of GLO I types, as well as stability in dried semen stains. No discrepancy was found in any paired samples.

Bloodstains upto 12 weeks old, depending upon storage conditions were successfully typed. Bloodstains stored at room temperature from November through February could easily be typed upto 7 weeks. During the months of summer (May & June) blood stains stored in a wooden cabinet retained GLO I activity at least one week longer (5 weeks) than those stored in steel almirahs (4 weeks). However, bloodstains stored at -20°C and 4°C were typable upto 12 weeks and 10 weeks respectively.

Direct solar radiation, particularly in summer, proved very damaging to enzyme stability in bloodstains. Freshly prepared wet bloodstains exposed to direct sunlight for 18 hours spread over 4 days in summer months could not be typed beyond 12 days, while wet stains similarly exposed in winter months could be typed upto 14 days. The rainly months (July to mid-September) proved to be the most unfavourable period; just after 10 days of storage at room temperature, GLO I activity in bloodstains was too low to be satisfactorily typed. Bloodstains subjected to dry heat conditions (by storing them in a dessicator over silica gel) showed typable enzyme activity upto 3 weeks.

The effect of different storage conditions on seminal strains was very similar to that seen in the case of bloodstains. However, the intensity of the bands in semen samples was lower. Moreover, no semen stain could be typed

beyond 4-5 weeks in winter and 2-3 weeks in summer. Stains kept at -20°C and 4°C gave reliable results for 7 and 6 weeks, respectively. Storage bands were observed as in the case of blood, though it was still possible to distinguish three major glyoxalase phenotypes without much difficulty.

Discussion

Bloodstains typing of GLO I has been attempted by using starch gel (Biology Methods Manual, 1978; Emes and Parkin, 1980) cellulose accetate membrane and a mixed starch/agarose gel (Scott and Fowler, 1982; Meghal et al, 1983) as media. Emes and Parkin's (1980) work on bloodstains showed that the GLO I izozymes survived drying and could be demonstrated to be unaltered upto 13 weeks after the stain was made. Scott and Fowler (1982) by employing a mixed starch/agarose gel could identify enzyme types from prepared bloodstains and case material upto 6 to 8 weeks depending upon conditions of storage. Meghal et al. (1983) in an actual case, were able to detect GLO I phenotypes in bloodstains 4 weeks old.

Studies of Sehajpal et al. (1983) on bloodstains prepared from menstrual blood showed that glyoxalase I deteriorated most rapidly in rainy conditions, while in winter GLO I phenotypes could be reliably detected upto 40 days. During summer months, the isoenzymes could be detected only upto 10 days.

GLO I typing from semen stains has also been reported (Scott and Fowler, 1982). Whole fresh semen produces the same band pattern, mobility and activity ratios as the corresponding blood sample. Prepared semen stains demonstrated altered band patterns, generally with increased mobility but little loss of activity. The GLO 1 and GLO 2 phenotypes developed two bands anodal to the primary bands, with a decrease and eventual disappearance of the last anodal bands.

In the initial stages of the present study the Scott and Fowler (1982) method was followed, but it was noticed that the band pattern obtained was more diffuse than when using the Biology Methods Manual (1978). The inclusion of magnesium chloride in the gel buffer markedly improves the reactivity of all bands in each of the GLO I phenotypes. The present study confirms the observation of Scott and Fowler (1982) that even though overall activity of the enzyme is increased in the presence of Mg^{2+} ions, the least anodal band still loses its activity faster than the other two bands with more anodal migration in case of GLO 2-1 phenotype.

6-PHOSHOGLUCONATE DEHYDROGENASE

6-Phoshogluconate dehydrogenase participates in the hexose monophosphate shunt pathway converting hexoses into pentoses needed for making nucleic acids. It catalyses the oxidation and decarboxylation of 6-phosphogluconate to ribulose 5-phosphate, the coenzyme nicotinamide adenine dinucleotide phosphate (NADP) being simultaneously reduced to NADPH. Magnesium is also required as a cofactor.

Fildes and Parr (1963) were the first to report the existence of genetically controlled variants of this enzyme in human erythrocytes, as revealed by starch gel electrophoresis. Genetic studies of Bowman et al. (1966) demonstrated that the three electrophoretic phenotypes were due to single locus, two allele systems, PGD^A and PGD^C, thus giving three common phenotypes : the A for usual type) with an 'a' electrophoretic band; the heterozygous AC (common Plaistow variant) with both 'a' and 'c' bands and also a strong hybrid 'b' and the C (Canning) variant, with a dominant 'c' band.

The heat stability of the enzyme at 37°C in human haemolysates is poor and variation between the normal and less stable variant phenotypes has been described by Parr and parr (1965).

Numerous methods have been described for the electrophoretic phenotyping of this enzyme. Fildes and Parr (1963) and Bowman et al. (1966) described the method on starch gel using different buffer systems. Cellulose acetate gel (Khan and Rattazzi, 1968), cellulose acetate membrane and polyacrylamide gel have also been tried (Peeples et al., 1967). However, for the forensic typing of PGD isoenzymes, the thin layer starch gel techniques

as originally given by Wraxall and Culliford (1968) has been found to be most satisfactory (Culliford, 1971) (see Sachdeva et al., 1989).

Results

The PGD isoenzymes are one of the most labile isoenzymes that are important from the forensic point of view. The same band pattern was observed in haemolysates and corresponding bloodstains. The intensities of PGD bands decreased with age although it was found that stains up to 3 weeks old could be typed satisfactorily. The technique has been used to type 469 bloodstains prepared in the laboratory using a variety of substrates commonly encountered in case work examination.

PGD activity in stains that had been kept at room temperature in the winter months of November to February remained typable upto 18 days. on the other hand, bloodstained material stored in wooden cabinets and steel almirah in the summer months of April to June gave reliable typing results upto 11 and 8 days respectively. However, stains stored in cold conditions such as at 4°C in a refrigerator and at -20°C showed satisfactory results for a longer time periods of 22 and 34 days, respectively.

Wet bloodstains exposed to direct solar radiation for only 4 hours on a single day, in the summer and winter months, could not be typed beyond a period of 2 and 4 days, respectively. But freshly shade dried stains exposed similarly to sunlight in summer and winter months remained typable for 3 and 5 days, respectively. The rainy months of July to mid September proved to be least favourable peiod for the preservation of enzyme activity in booodstained material. After just two days of storage at room temperature, stains became untypable. Stains which were made on soiled surfaces gave typable activity only up to 24 hours. However, bloodstains stored under dry heat conditions (40°C to 45°C) in a dessicator over silica gel remained typable upto 12 to 14 days.

Discussion

There are, as yet, very few reports on the PGD enzyme stability studies in fresh and dried bloodstains and none from India. Storage leads to a rapid loss of activity. Parr and Parr (1965) reported a quick loss of activity depending on the isoenzyme type, after 15 minutes of incubation of 37°C. The mean loss of activity (%) observed in three common phenotypes was: AA (usual type) 27
CA (common variant) 49
CC (canning variant) 62

However, these observations differ from those of Satoh et al (1985). According to their study the heterozygote type (AC) shows marked thermostability, and the homozygoate type (CC) marked lability. Within the limits of error, the loss in the homozygote type is twice that in the heterozygote type.

Storage of blood samples at room temperature reportedly leads to the loss of PGD activity at a slower rate than at 37°C. Storage at 0°C delays this, but loss of activity is apparent in two or three weeks. Excellent preservation is obtained by storage at -70°C (Culliford, 1971).

The Forensic Science Laboratory, London, found that at -20°C unsatisfactory storage of AC was obtained beyond 7 days, if it was frozen and thawed each day. However, it has been found that storage in liquid nitrogen is excellent, though long term storage was not tried. All samples stored in nitrogen gave reliable typing results to periods of over one year (Culliford, 1971).

Except in the case of relatively fresh samples of blood, the PGD isoenzyme system seems to be not very suitable in Indian climatic conditions. The deterioration and loss of activity of this enzyme is very rapid and cannot be reliably typed beyond 18-20 days. However, if the bloodstained material can be kept in refrigerated conditions, or preferably at -20°C, satisfactory enzyme activity can be observed upto 5 weeks of storage.

SUMMARY AND CONCLUSIONS

In brief, the present study of bloodstain analysis on more than 600 samples shows that hot and humid conditions hasten the process of protein denaturation more as contrasted with cold and dry conditions. This is obviously due to the fact that humidity and higher temperatures are conducive to the microbial

growth, which leads to the deterioration and denaturation of proteins, thus severaly affecting their typability by electrophoretic separation procedures.

Exposure of bloodstains to direct solar radiation was observed to be very detrimental to enzyme/serum protein stability. It reduced considerably the time period upto which a blood protein can reliably be typed.

There was no significant effect on the detectability, band pattern and zone intensity of enzyme type in blood stains when they were contaminated with sweat.

Virtually scores of factors can affect the stability of a particular genetic marker in bloodstains. Therefore, it is very difficult to make generalizations. Moreover, as Zajac (1980) writes "The protein and enzyme systems and red cell antigens differ in their degree of persistence in deteriorating blood. Consequently, although an analyst may have the necessary skills to determine several genetic markers in fresh undeteriorated blood he may be unable to obtain readable or unequivocal results for more than one or two of these systems in deteriorated blood or bloodstains".

It has been observed that among the factors included in the present study, isoenzyme AK and serum protein Hp are two of the most robust proteins and are most suitable for typing in bloodstains even under adverse conditions of storage. Haptogobin could be typed upto 18 months or storage at -20°C and upto about 4 months in bloodstains kept at room temperature even in summer months. It was possible to obtain typable AK patterns upto 17 months in bloodstains stored at -20°C and for 9-10 weeks in those stored at room temperature in summer months. Next comes the PGM_1 : it remained typable for 6-7 weeks at room temperature in summer season and for over 6 months if kept at -20°C. The isoenzyme of EsD, GLO I and Ca II are equally suitable, though their stability range at room temperature is not more tha 3-7 weeks; but bloodstains stored at -20°C could be typed upto 12-17 weeks.

Except in cases of relatively very fresh samples of blood, the 6-PGD isoenzyme seems to be not very suitable under Indian climatic conditions. The loss of activity of this enzyme is very rapid and cannot be reliably typed beyond 18-20 days even in winter months. Its stability is miserably poor in summer when it cannot be satisfactorily typed beyond a week.

In case of semen stains, though the fresh liquid samples exhibited weakly developed zones of EsD activity, semen stains even few hours old gave very poor resolution. It was possible to type seminal stains both for GLO I and PGM_1 isoenzymes. GLO I enzyme could be typed upto 2-3 weeks in bloodstains stored at room temperature in summer months. However, it remained typable for about 7 weeks if the semen stains were kept at -20°C. Similarly, isozymes of PGM_1 could be typed upto 5-6 weeks in semen stains stored at room temperature. However, it gave reliably typable patterns upto 16 weeks if stains were kept at -20°C.

An interesting observation was that bloodstains stored in wooden cabinet, in the hot summer season, retained their enzymes/serum protein activity for a relatively longer periods, than those stored in steel almirah. It may be advisable to store the actual case work samples in wooden cabinets before and after they are transferred to the forensic laboratory.

However, "Age alone is not critical to the reliability of phenotyping a blood sample (Zajac and Grunbaum, 1978). Rather, it is essential to consider the cumulative effects of substrate, biological quality and quantity, environmental effects such as heat, humidity and contamination, and the preservation and transportation of the sample prior to submission to the crime laboratory" (Zajac, 1980).

Much depends on the method of collection and submission of biological materials to the forensic laboratory. The processes which degrade and denature enzyme/serum protein are accelerated when appropriate packaging methods are not employed. Some of the guidelines for the collection and submission procedures for biological materials are as follows :

"... In order to preserve the biological and biochemical and biological information one should expect to maintain body fluid stain evidence under either air dried and frozen, air dried and refrigerated, or air dried (non-hu-

mid) conditions until submission. Liquid blood samples should be refrigerated and submitted as quickly as possible, even if the remaining items cannot be sent at the same time. Refrigeration of the submitted items while in transit is not required.

"Stained items should be contained in paper envelopes, paper bags, or other containers that allow for the exchange of air but prevent the buildup of moisture. Liquid blood samples should be submitted in sealed blood test tubes without chemical preservatives (anticoagulants acceptable) to prevent breakage in transit. Sexual assualt evidence, such as vaginal, oral or anal swabs should be thoroughly air dried and placed in envelopes or other containers that allow air to pass (Mursh, 1985).

KEY WORDS Bloodstains. Semen Stains. Blood Cell Genetic Markers. Stability Studies.

ABSTRACT The present investigation has been undertaken with a view to studying the following important polymorphic enzymes and a serum protein : ESD, PGM, AK, CA II, GLO, 6-PGD and Hp, under Indian conditions. Attempt has been made to study the time lapse up to which the blood factors can be reliably typed under Indian conditions of storage and preservation, *viz.* at - 20° C, at room temperature, in summer and winter months, wet and dry stains exposed and contamination with perspiration etc. Suitability of testing these genetic markers in Indian Crime Laboratories has also been assessed.

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