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Subtyping Phosphoglucomutase-1 in Semen Stains and Bloodstains: A Report on the Method

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ABSTRACT: A method is described for obtaining nondistorted, reproducible phosphoglucomutase-1 subtyping patterns from semen stains and bloodstains. Isoelectric focusing of phosphoglucomutase-1 was accomplished in 80 min in a 0.2-mm-thick polyacrylamide gel with an interelectrode wick distance of 8.0 cm. The gel contained 1.2% (w/v) *N*-(2-hydroxyethyl) piperazine-*N*-3-propanesulfonic acid (EPPS) and pH 5 to 7 ampholytes (4% w/v). When maintained at room temperature, laboratory-prepared bloodstains and semen stains could be typed for phosphoglucomutase-1 up to four months and three weeks, respectively. An evaluation of phosphoglucomutase-1 typing by isoelectric focusing and the Group I system was performed on casework samples submitted to the FBI Laboratory. In addition to the increased discriminating probability of phosphoglucomutase-1 when subtyped, isoelectric focusing yielded an increase in positive calls on questioned bloodstains (65.6 versus 36.2%) and dried seminal stains (16.4 versus 13.1%) compared with the Group I system.

KEYWORDS: forensic science, phosphoglucomutase, genetic typing, semen, blood, subtyping, EPPS, meldola blue, stability studies, casework, isoelectric focusing, ultrathin gels

The typing of phosphoglucomutase-1 (PGM_1) in bloodstains and semen stains is an important aspect of forensic science casework analysis. This is particularly true when characterizing evidence related to violent criminal activity such as homicide, assault, or rape. Until recently, the routine analysis of PGM_1 in the forensic science arena was carried out mostly by conventional electrophoresis (CE). This approach permitted the resolution of three common phenotypes. Isoelectric focusing (IEF) has revealed that PGM_1 can be separated into ten common phenotypes [*1-3*], thereby increasing the discriminating power of this genetic marker. Through the combined efforts of the Serology Unit, Scientific Analysis Section, and the Forensic Science Research and Training Center of the Laboratory Division of the Federal Bureau of Investigation, a reliable ultrathin-layer polyacrylamide gel isoelectric focusing (ULPAGIF) technique for the routine subtyping of PGM_1 derived from liquid bloods, blood-

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stains, and semen stains was developed. This paper presents the ULPAGIF method for PGM_1 subtyping; limited stability studies on PGM_1 in bloodstains and semen stains; and a casework evaluation of PGM_1, comparing results obtained by ULPAGIF with CE.

Materials and Methods

Blood samples were obtained from 80 individuals at the FBI Academy by finger prick. Bloodstains were prepared as previously described [*4*]. Forty-seven semen samples were obtained from the Washington Fertility Clinic, Washington, DC. Semen stains were prepared in the same manner as the bloodstains. Stability studies on the bloodstains and semen stains were carried out at room temperature.

Cuttings of the stains (3 by 5 mm) were extracted in 20 to 30 μL of water or 0.05*M* dithiothreitol (DTT) for 30 min. The extracts were absorbed onto 5- by 5-mm applicator tabs (LKB), lightly blotted, and applied 1 cm from the anode.

Polyacrylamide gels (5% T, 3% C; 145 by 110 by 0.2 mm) were cast onto silanized glass plates using the flap technique [*5,6*] or the Bio-Rad casting tray. The gels contained synthetic carrier ampholytes of pH 5 to 7 (LKB, 4% w/v) and 1.2% (w/v) *N*-(2-hydroxyethyl) piperazine-*N*-3-propanesulfonic acid (EPPS) (Sigma). The catholyte and anolyte were 0.20*M* sodium hydroxide (NaOH) and 0.05*M* phosphoric acid, respectively. The distance between the electrode wicks was 8.0 cm. The IEF was performed on the Ultrophor (LKB) at 4°C using the previously reported conditions [*4*] outlined in Table 1. After ULPAGIF, the PGM_1 phenotypes were developed according to the method of Divall and Ismail [*7*].

For evaluation purposes, 3052 forensic science casework samples comprised of known liquid bloods, questioned dried bloodstains, and questioned seminal stains deposited upon a variety of substrata submitted to the FBI Laboratory were analyzed for PGM_1 by ULPAGIF and the Group I system [*8*].

Results and Discussion

Figure 1 shows that the allelic products of PGM_1 in bloodstains and semen stains could be separated clearly by ULPAGIF without distortion. Band intensities play a part in the interpretation of the PGM_1 subtypes. In particular, the c− band lay approximately in the same position of the gel as the 2+ band. However, the c− band was far less intense than the 1− band. Thus in a 1−2+ phenotype, the 2+ band was equal to or slightly greater in intensity than the 1− band. No differences in the PGM_1 patterns were observed when the samples were extracted with DTT or water, which was previously observed by Fowler and Scott [*9*].

TABLE 1—*Running conditions for PGM_1 subtyping on ultrathin-layer polyacrylamide gels.*[a]

Comments[b]	Initial Voltage, *V*	Time, min
Prefocus	250	25
Apply samples 1 cm from anode, adjust settings	500	10
Remove sample tabs, adjust settings	1100	30
Adjust settings	1900	10
Adjust settings	2300	5

[a]0.2-mm-thick gels with an inter-electrode distance of 8.0 cm.

[b]Always focus with constant power; adjust power to obtain voltage given in table (see Refs *4* and *24*).

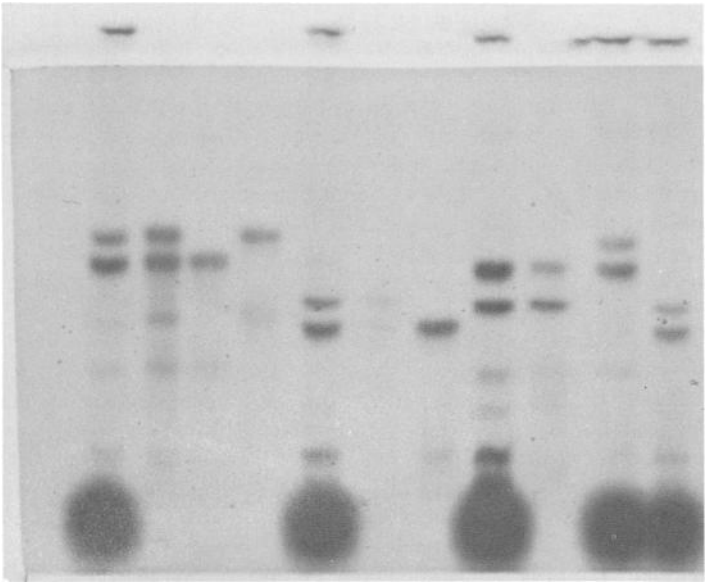

FIG. 1—*An ultrathin-layer polyacrylamide gel displaying PGM_1 phenotypes from bloodstains and semen stains. The phenotypes from left to right are: 1+1− (bloodstain), 1+1− (semen stain), 1+ (semen stain), 1− (semen stain), 2+2− (bloodstain), 2+2− (weak, semen stain), 2+ (semen stain), 1+2− (bloodstain), 1+2− (semen stain), 1+1− (bloodstain), and 2+2− (bloodstain). The cathode is at the top.*

However, semen stains extracted in water produced slightly sharper PGM patterns than semen stains extracted with DTT. More than 1000 gels have been run successfully using this approach.

Ultrathin-layer Gels

A number of factors influence the efficacy of this technique. The first is the use of ultrathin-layer polyacrylamide gels for IEF. Rilbe [*10*], Giddings and Dahlgren [*11*], and Allen [*5*] have demonstrated that resolution is proportional to the square root of the voltage gradient. Thus, if a higher voltage gradient is applied to an IEF gel, superior resolution is expected. Ultrathin-layer gels have a greater surface area-to-volume ratio than thicker gels, and therefore are more efficient at heat dissipation [*5*]. Since the gel can more effectively dissipate the Joule heat, higher field strengths can be applied to the system. Our gel system had a final voltage gradient of 290 V/cm compared with only 180 V/cm by Pflug et al. [*12*] and 133 V/cm by Divall and Ismail [*7*]. Furthermore, the higher voltage gradient can produce narrower protein bands [*5*]. The focusing of protein bands into more narrow zones results in more protein per unit gel volume which, in effect, presents more protein for the subsequent assay. Thus, the sensitivity of detection of the IEF system is increased. In fact, Divall and Ismail [*7*] observed a 10% increase in positive calls on casework when using an ULPAGIF system, compared with a 1-mm-thick IEF gel approach. In addition to the above benefits, by reducing the gel thickness, the quantities of gel reagents (and thus the cost) are decreased without compromising the number of samples analyzed. A number of investigators [*7,9,12,13*] have successfully used an ULPAGIF method for bloodstain or semen stain analyses or both.

An interesting variation of the ultrathin-layer polyacrylamide gel approach is the use of

wedge-shaped ultrathin polyacrylamide gels for IEF of PGM_1 in semen stains as described by Pflug [*14*]. These gels were 300 μm thick at the anode, decreasing in thickness to 50 μm at the cathode. Pflug suggests that by applying the semen stain extracts in the thicker portion of the gel, there is less distortion of the PGM_1 patterns as a result of the salt effects. The wedge-shaped gel, thus, combines the high resolving capacity of ultrathin gels with the higher loading capacity and less distortion of thicker gels. In comparison, the gels used in this study were a constant 200 μm thick. No loading capacity problems or deleterious salt effects from semen samples were observed (Fig. 1).

Separators

The addition of a separator to the gel separated the individual PGM_1 variant bands farther than was possible previously. Thus, the chance of mistyping was greatly reduced. The zwitterionic buffer, EPPS, effectively flattens the pH gradient between 5.6 and 5.9 [*15*]. Therefore, the distance between each of the common PGM_1 variant bands is at least twice that of other reported methods [*1-3,7,12,13*]. Since the PGM_1 bands could, at times, be slightly bent or curved as a result of excessive protein loading [*4*], the increased separation enhances typability. With greater distances between the bands, the relative position of a slightly distorted band is less questionable.

Our gels contain half the concentration of EPPS (1.2%, w/v) than reported by Gill and Sutton [*15*] (2.5%, w/v). It was found that concentrations of EPPS greater than 1.2% (w/v) often inhibited PGM_1 activity, especially when the gels were poured by the capillary technique. This effect may be partially because the gel edges were exposed to the air, and oxygen may alter the physiochemical structure of the gel causing inhibition of PGM_1. This is an undesirable effect since the quality of samples is uncertain and frequently minute amounts must be detected. Furthermore, gels are more easily cast using the capillary method than the flap method—a consideration for laboratories with heavy caseload demands. The effect of inhibiting PGM_1 activity was also observed, but to a lesser degree and intermittently, in gels cast by the flap technique. To alleviate this problem only 1.2% (w/v) EPPS was added to the gel solution. Each of the PGM_1 variant bands still was separated farther than previously possible, and there was no observable inhibition of PGM_1 activity.

Sample Application

All samples were applied as eluates absorbed onto applicator tabs. Cuttings from stains also can be placed directly on the gel surface. However, this is not recommended because of uneven elution from the supporting material which can result in an inconclusive call.

Assay

The assay method of Divall and Ismail [*7*] was adopted for our procedure. They replaced phenazine methosulfate with meldola blue (Sigma) as the electron-acceptor donor of the assay. This resulted in the absence of gel background staining, and the PGM_1 patterns were easier to observe. Furthermore, gels containing samples with faint activity were covered with a glass plate and incubated at 37°C overnight. Some of the PGM_1 patterns intensified without any substantial increase in gel background staining. Therefore, more positive calls on casework could be obtained.

Stability Studies

Table 2 shows the results of the aging study of 80 laboratory-prepared bloodstains. Over a 4-month period, the bloodstains maintained at room temperature were all correctly typed.

TABLE 2—*PGM stability study on 80 laboratory-prepared bloodstains maintained at room temperature.*[a]

Phenotype	No.	1 Day (D)	7D	14D	1 Month (M)	2M	3M	4M
1+	36	36	36	36	36	36	36	36
1+1−	9	9	9	9	9	9	9	9
1−	1	1	1	1	1	1	1	1
2+	4	4	4	4	4	4	4	4
2+2−	1	1	1	1	1	1	1	1
2−	0	0	0	0	0	0	0	0
1+2+	19	19	19	19	19	19	19	19
1+2−	6	6	6	6	6	6	6	6
1−2+	3	3	3	3	3	3	3	3
1−2−	1	1	1	1	1	1	1	1

[a]The values indicate the number of conclusive calls. There were no inconclusive or negative calls over this time period.

As expected, the intensity of the PGM_1 bands did decrease over time. However, there was no preferential loss of any of the 4 common PGM_1 allelic products. This is consistent with the observations of Divall and Ismail [*7*]. After 4 months the patterns were too faint to read. The data suggest that PGM_1 in bloodstains is a relatively stable genetic marker.

Table 3 shows the results of the stability study of 47 laboratory-prepared semen stains. During this study it was demonstrated that PGM_1 was less stable in semen than in blood. Within 1 week of storage, 27.7% of the stains could not be typed conclusively. By the third week, 66% of the semen stains were inconclusive. After the third week none of the stains could be typed. The data show there was no preferential loss of a particular PGM_1 allelic product in semen. The rapid degradation of PGM_1 in semen compared with blood was expected as a result of the high proteolytic activity present in semen [*16,17*].

Casework Evaluation

Over a 3-month period, 743 known liquid blood specimens, 1417 questioned dried bloodstains, and 892 questioned dried seminal stains obtained from cases submitted to the FBI

TABLE 3—*PGM stability study on 47 laboratory-prepared semen stains maintained at room temperature.*[a]

Phenotype	No.	1 Day (D)	3D	7D	14D	21D
1+	18	18	18	12	7	7
1+1−	10	10	9	7	6	3
1−	1	1	1	1	1	1
2+	1	1	1	1	1	1
2+2−	1	1	1	1	0	0
2−	0	0	0	0	0	0
1+2+	7	7	6	6	4	3
1+2−	5	5	5	4	2	1
1−2+	3	3	3	1	1	0
1−2−	1	1	1	1	0	0

[a]The values indicate the number of conclusive calls. All other calls were inconclusive or negative over this time period.

Laboratory were typed for PGM_1 by both ULPAGIF and CE. The results are shown in Table 4. There was no significant difference in the number of conclusive calls for known blood samples using ULPAGIF (83.3%) compared with CE (82.9%). However, there was a dramatic increase in positive calls on questioned bloodstains (65.6 versus 36.2%). The data also revealed a slight gain in conclusive PGM_1 phenotype determinations for questioned dried seminal stains (16.4 versus 13.1%). There were no phenotypic discrepancies between the two electrophoretic procedures. The increase in positive (or conclusive) determinations by ULPAGIF was attributed to its higher resolving power and concentrating effect (which results in a greater sensitivity of detection). The increase in conclusive determinations, the more clearly defined PGM_1 isozyme patterns, and the higher discriminating power obtained by subtyping PGM_1 indicate the technique described in this paper would be of extreme value to forensic serology laboratories.

Published reports [*18,19*] and our observations of (semen stains, vaginal swabs, and other) case samples have shown there can be significant contributions of PGM_1 from the vaginal secretions. This contribution has to be taken into consideration when interpreting PGM_1 subtypes from vaginal swabs. The PGM_1 from the vagina is probably due to menstrual and postmenstrual blood contamination [*18,19*] or cellular damage or both. It has been reported that the PGM_2 allelic products are present in menstrual blood and not semen [*13,18,19*]. There may be cases where this difference could be used to determine if there is a significant contribution of PGM_1 from menstrual blood. When PGM_2 allelic products are present, it should be considered that there also may be PGM_1 contributed from the female.

Conclusion

A method has been described for ULPAGIF of PGM_1 in bloodstains and semen stains. The technique has yielded highly resolved PGM_1 patterns and can be easily implemented into most laboratories. While the method is reliable, highly resolving, sensitive, and simple, there are limitations that must be considered. Although there are sufficient amounts of PGM_1 in semen for typing purposes [*2,8,12,13,17-23*], the availability of material for analysis may be a constraint for successful typing. There may be only minute amounts of semen in stains, the semen could be substantially diluted by vaginal fluid, and proteolytic degradation of PGM_1 can occur. Further, environmental insults such as temperature, humidity, chemical contamination, and bacterial contamination can have an impact on enzymatic activity. Bloodstains do not have as dramatic a problem as semen per se; however, old, degraded, contaminated, or diluted bloodstains can prove difficult, if not impossible, to type with the present method. Regardless, certain standard criteria [*7,18,22*] should be considered when subtyping PGM_1. Of paramount importance is that any method used should yield good resolution and sensitivity of detection of the common allelic products of PGM_1. It is also suggested that weak patterns be carefully studied before any determinations are made so that no mistypings will occur.

TABLE 4—*Casework evaluation of PGM_1 typing comparing ULPAGIF and CE method.*

Samples	No.	Conclusive Calls	
		ULPAGIF	CE
Known bloods	743	619	616
Questioned bloodstains	1417	930	513
Questioned semen stains	892	146	117

Currently, we are investigating methods for separating the c− and 2+ bands to eliminate any possible confusion with typing. Also under further study is the area of mixed body fluid stains and their impact on genetic marker analysis.

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