TECHNICAL NOTE

K. J. Cox,[1] *B.Sc. and A. S. Thomas,*[1] *B.App.Sc.*

The Application of Immunoblotting to the Phenotyping of Haptoglobin

REFERENCE: Cox, K. J. and Thomas, A. S., **"The Application of Immunoblotting to the Phenotyping of Haptoglobin,"** *Journal of Forensic Sciences*, JSFCA, Vol. 37, No. 6, November 1992, pp. 1652–1655.

ABSTRACT: An immunoblotting method for phenotyping haptoglobin in serum and bloodstains has been developed. Haptoglobin isoproteins were separated by polyacrylamide gradient gel electrophoresis and then transferred to nitrocellulose by electroblotting. The use of 1 mm gels facilitated more rapid and effective transfer than conventional 3 mm thick gels. Nitrocellulose blots were developed by double antibody enzyme immunoassay. The detection limit for serum and bloodstains was improved 16 times compared to conventional staining using O-tolidine. The method could detect haptoglobin phenotypes from 0.001 μl of whole blood. This detection limit is approximately 8 times lower than that of group specific-component analysis by immunoblotting.

KEYWORDS: pathology and biology, immunoblotting, phenotyping, haptoglobin

The successful application of immunoblotting to the phenotyping of group specific-component and AHSG glycoprotein in forensic casework bloodstains in this laboratory [*1–3*] gave rise to hopes of a development of a similar application to the phenotyping of haptoglobin. Although this approach had been tried previously using 3 mm thick gels [*4,5*], it was thought that a more effective transfer of protein could be effected using thinner 1 mm gels.

Materials and Methods

Samples

Blood stains were prepared by soaking cotton cloth with fresh whole blood, collected in EDTA from laboratory staff. Portions of stained cloth were extracted in 20% sucrose in tank buffer (Ph 8.3, 0.1 M, TRIS/Borate). Whole blood and serum samples were diluted with 20% sucrose/tank buffer.

Received for publication 6 January 1992; revised manuscript received 11 February 1992; accepted for publication 14 February 1992.

[1]Forensic Scientists, Forensic Biology section, State Health Laboratory, Queensland Department of Health, Brisbane, Queensland, Australia.

Separation

Separation of haptoglobin isoproteins was achieved by vertical gel electrophoresis in polyacrylamide gradient gels (Gradipore: 3 to 15%, 2 to 27%, 1 mm, 3 mm). 10 μl samples for 1 mm gels, 15 μl for 3 mm gels, were loaded and run for 150 V overnight using a Pharmacia Gel Electrophoresis GE-2/4 apparatus. Power was supplied by a Pharmacia EPS 500/400 unit.

Detection

a) Conventional: Performed by staining with 0.05% hydrogen peroxide in 0.1% O-tolidine.

b) Immunoblotting: Transfer of haptoglobin proteins from unstained gels to nitrocellulose (LKB: 0.20 μm, or 0.40 μm) was achieved by electroblotting at 70 V for 3 h using an LKB 2051 Midgit Multiblot Electrotransfer unit. Up to 4 gel/membrane sandwiches can be run at the one time using 1 L of transfer buffer (25 mM TRIS, 150 mM Glycine, Ph 8.3).

Nitrocellulose blots were developed by a modification of the method previously described [*1*]. The primary antibody was 30 mL of a 1 in 1000 dilution of sheep anti-human haptoglobin (Silenus laboratories) in wash buffer (0.05% v/vTween 20, 50 mM Tris, 150 mM NaCl, Ph 10.3). The secondary antibody was 30 mL of a 1 in 1000 dilution of donkey anti-sheep/goat alkaline phosphatase conjugate in wash buffer (Silenus). The Haptoglobin patterns were visualized by soaking the washed nitrocellulose strip in the following solution: 5 mL of a 0.1% w/v Nitroblue tetrazolium (NBT Grade 111, Sigma), 200 μL of 1 M Mg Cl_2, 750 μL of substrate stock [30 mg 5-bromo-4-chloro-3-indoyl phosphate (Disodium salt, Sigma) in 5 mL methanol and 2.5 ml acetone] made up to 50 mL with 0.1 M Ethanolamine (Sigma) pH 9.6.

Results

On both 1 mm and 3 mm thick gels conventional staining resulted in reportable results on whole blood diluted up to 1 in 400 (Fig. 1). Electroblotting from 3 mm thick gels produced no significant gain in sensitivity under the above transfer conditions.

Using 1 mm gels (3 to 15% or 2 to 27% gradient) immunoblotting resulted in reportable results on whole blood at a dilution of at least 1 in 6400 (Fig. 2).

The average area of blood stain, (calculated from a known area of stain, the extraction and application volumes, and the final detectable dilution) that could be typed by immunoblotting was 0.015 mm^2.

Abnormal patterns were noticed when relatively high concentrations (1 in 50, 1 in 100) of serum were analyzed. The patterns were corrected by the inclusion of hemoglobin in the extraction buffer.

Analysis of cat, dog and horse bloodstains produced haptoglobin type 1 like patterns in conventional and immunoblotting methods. Sheep and goat bloodstains produced broad streaked patterns when tested by the immunoblotting method.

Discussion

The change in detection limit for whole blood from a $\frac{1}{400}$ dilution to $\frac{1}{6400}$ shows that for this experiment immunoblotting of 1 mm thick gels has resulted in at least a 16 fold increase in sensitivity. The detection limit for immunoblotting of 1 mm gels is therefore in the order of 0.001 μL of whole blood. Previous analysis in this laboratory of the control blood stains used show that the average area of blood stain that could be typed by O-

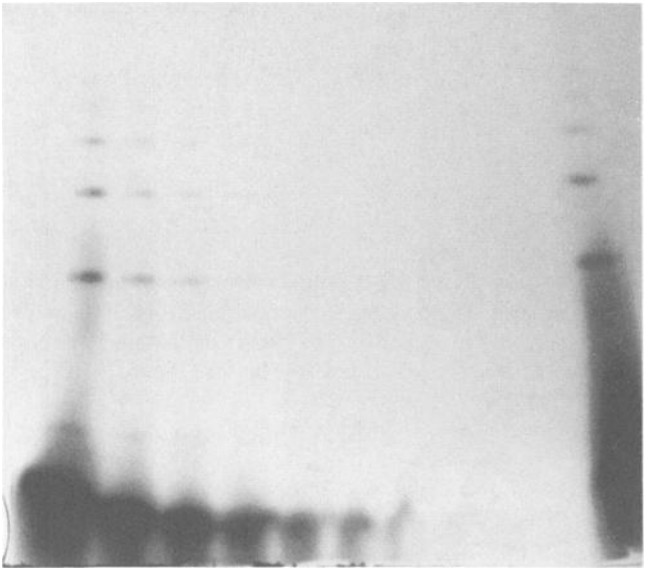

FIG. 1—*Hp types on 1 mm gel after development with O-tolidine/H_2O_2. Lanes 1 to 9: Dilutions of Hp 2-1 whole blood as serial doubling dilutions from 1:50 (Lane 1) to 1:12800 (Lane 9). Lane 10: blank. Lane 11: Hp 2-1 control.*

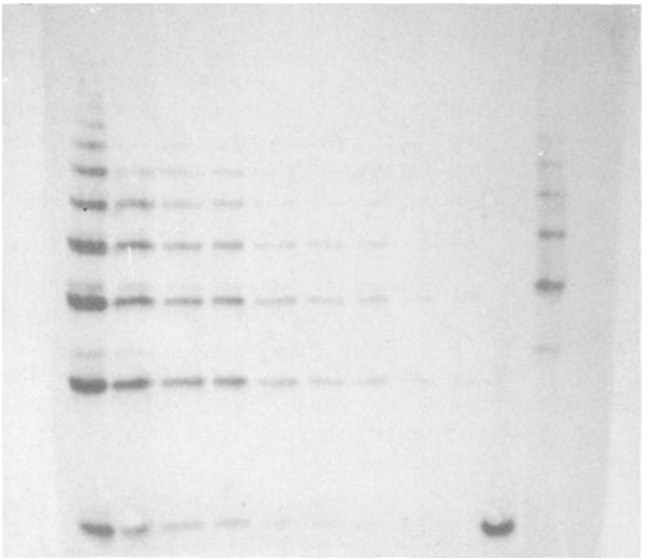

FIG. 2—*Hp types on Nitrocellulose after immunoblotting. Lanes 1 to 9: same samples as Fig. 1. Lane 10: Hp 1 control. Lane 11: Hp 2-1 control.*

tolidine/H_2O_2 staining was 0.48 mm^2 [*2*]. Immunoblotting of 1 mm gels could detect haptoglobin from 0.015 mm^2 of bloodstain. This result is thus in agreement with the increase in sensitivity found in the whole blood experiment. However, for the electroblot transfer conditions used, the conventional 3 mm gels transferred very inefficiently with little or no increase in sensitivity compared to conventional staining.

This method has been in routine use for such samples since June 1990 has proved valuable in case work blood stain typing where the amount of sample is obviously insufficient for conventional Hp typing, (an all too common occurrence for important stains), or where inconclusive typing due to weak reactions was previously obtained.

In addition, successful typing has been obtained where inconclusive phenotyping had been obtained on young infants by conventional staining for paternity testing. However, there seems to be little improvement in typing of degraded stains with the background staining remaining high leaving no net improvement in signal to noise ratio.

The use of 1 mm gels enables a relatively fast transfer of protein from a gradient gel and the complete development procedure is easily completed during one working day. The physical handling of these thin gels posed no difficulty either in their structural integrity or in conventional or immunoblot development.

References

[*1*] Thomas, A. S., Aaskov, J., and Ansford, A. J., "The Application of Immunoblotting to the Phenotyping of Group-Specific Component," *Journal of the Forensic Science Society*, Vol. 29, 1989, pp. 197–205.

[*2*] Thomas, A. S., "The Evaluation of Five Electrophoretic Phenotyping Systems for Routine Screening of Blood Stains," *Journal of the Forensic Science Society*, Vol. 29, 1989, pp. 243–248.

[*3*] Thomas, A. S., "Phenotyping of α-2-HS Glycoprotein in Bloodstains by Isoelectricfocusing and Immunoblotting," *Journal of the Forensic Science Society*, Vol. 29, 1989, pp. 325–330.

[*4*] Hoste, B., "Advantages of Enzyme-Immuno-Assay after Blotting in Bloodstain Grouping. Application to the Haptoglobin Groups," *Electrophoresis*, Vol. 7, 1986, pp. 479–480.

[*5*] Roy, R. and Roy, I. C., "Haptoglobin Phenotyping from Older Bloodstains by Enzyme Immunoassay and Haptoglobin Phenotypes Within a Nebraska Caucasian Population," *Journal of Forensic Sciences*, JFSCA, Vol. 36, No. 2. March 1991, pp. 571–575.

Address requests for reprints or additional information to
K. J. Cox
Forensic Biology Section
State Health Laboratory
Queensland Department of Health
63 George St.
Brisbane
Queensland, 4001 Australia