Photodynamic treatment of pooled coumarin plasma for external quality assessment of the prothrombin time

A M H P van den Besselaar, A C E Moor

Abstract

Aims—To determine the conditions of photodynamic inactivation of vesicular stomatitis virus (VSV) added to pooled coumarin plasma and the effects of the photodynamic treatment on the prothrombin times and international normalised ratio (INR) in a Netherlands national external quality assessment scheme.

Methods—Pooled coumarin plasma samples were illuminated with visible light in the presence of 1 µM methylene blue. Inactivation conditions for VSV in pooled coumarin plasma were determined using an end point dilution assay. Plasma illuminated for 20 minutes was mixed with red blood cells and mailed to participants of the Netherlands external quality assessment (EQA) scheme. Prothrombin times and INRs were determined with various thromboplastin reagents.

Results—Photodynamic treatment using 1 µM methylene blue and 700 W/m² caused 4.7 log inactivation of VSV in pooled coumarin plasma. Fibrinogen and coagulation factors II, V, VII, and X were decreased slightly by the treatment. These conditions caused prolongation of the prothrombin time in EQA surveys. The magnitude of the effect was different for various thromboplastin reagents. The increase of the INR was negligible when measured with the Thrombotest reagent. With other reagents, an approximately 5–16% increase of the INR was observed. Interlaboratory variation of the INR was not affected by photodynamic treatment.

Conclusions—Photodynamic treatment of pooled coumarin plasma is very effective for the inactivation of some enveloped viruses such as VSV, but has only a limited effect on the prothrombin time and INR. Photodynamic treatment can be used to improve the viral safety of coumarin plasma for EQA of the prothrombin time and INR.

(J Clin Pathol 2000;53:470–475)

Keywords: prothrombin time; international normalised ratio; external quality assessment; photodynamic treatment

External quality assessment (EQA) of the prothrombin time and international normalised ratio (INR) is an important component of quality assurance of oral anticoagulant treatment. Since 1974, the Federation of the Netherlands Thrombosis Centres has organised a national EQA scheme for its members. Each year, 10 surveys are performed with five liquid control blood samples in each survey. Many Dutch centres are using the Thrombotest reagent (Nycomed, Oslo, Norway), which is a modification of the prothrombin time test. It is used with citrated venous blood. As far as possible, survey materials should resemble test specimens, and should include a range of coagulation activities to be measured, especially at critical levels for decision making. The survey materials in the Netherlands scheme are prepared by mixing washed human red blood cells with various pooled plasma samples obtained from normal individuals and patients treated with oral anticoagulant drugs.

There is a risk of contamination with blood borne viruses when a large number of patients’ specimens are used for the preparation of pooled plasmas. Although the pooled plasmas are tested for hepatitis antigen and human immunodeficiency virus (HIV) antibodies, the risk of infection of laboratory workers cannot be eliminated completely. Leakage of survey material from the primary container may occur during transportation and postal delivery. EQA organisers might be held responsible for injury caused by survey materials. To improve the safety of the survey materials, we evaluated photochemical methods for the inactivation of viruses in pooled plasma samples.

In transfusion medicine, photodynamic treatment has been studied for the inactivation of blood borne viruses in blood components. A photodynamic virus inactivation method using methylene blue is being applied routinely in the production of virus inactivated plasma. Methylene blue was selected because it is used clinically and because of its known toxicological properties. The standard procedure for photodynamic treatment involves illumination with visible light at a methylene blue concentration of 1 µM in plasma. Upon illumination, methylene blue becomes excited, and energy transfer from the excited state to molecular oxygen dissolved in the plasma can cause the formation of singlet oxygen. This very reactive oxygen species can damage lipids, proteins, and viral nucleic acids. Photodynamic treatment with methylene blue effectively inactivates various enveloped viruses, including HIV. Polymerase chain reaction analysis revealed that hepatitis B virus,
hepatitis C virus, HIV-1, and probably also the non-enveloped parvovirus B19 are sensitive to methylene blue/light treatment.\(^6\)

The purpose of our study was to determine the conditions for effective photodynamic inactivation of vesicular stomatitis virus (VSV) added to pooled coumarin plasma, and to study the effect of photodynamic treatment on the prothrombin time and INR of the plasma to be used for preparation of survey samples in the Netherlands EQA scheme. We report the results of two surveys in which photodynamic treatment was applied.

**Materials and methods**

**PREPARATION OF POOLED PLASMAS**

Venous blood was collected from coumarin treated patients using evacuated tubes containing 0.105 M buffered sodium citrate (Becton Dickinson Vacutainer® Systems; Becton Dickinson, Franklin Lakes, New Jersey, USA). Plasma was obtained by centrifugation at 2000 × g for 10 minutes. Samples with INRs in the range 1.5 to 3.0 or 2.5 to 4.0 were pooled and centrifuged for a second time, but now at 29 000 × g for 30 minutes. After careful decantation the pooled plasmas were frozen in closed plastic containers at −70 °C. After thawing in a water bath at 37 °C for 15 minutes, the pooled plasmas were mixed with HEPES buffer (final concentration in plasma, 0.05 M; pH 7.3). In some experiments, the oxygen dissolved in the plasma was removed by slowly bubbling nitrogen through the plasma before photodynamic treatment.

**PHOTODYNAMIC TREATMENT**

Methylene blue was obtained from Sigma Chemical Co (St Louis, Missouri, USA). A stock solution (1 mM in water) was prepared. Each plasma sample was mixed with methylene blue (1 μM final concentration). The plasma was then illuminated at room temperature, in 50 ml polystyrene tissue culture flasks, using a 500 W halogen lamp. The plasma was stirred during the illumination. A large glass vessel with running tap water was placed between the lamp and the flask to absorb the heat generated by the lamp. The distance between the lamp and flask was 12 cm. Irradiance at the site of the flask was 700 W/m², as measured with a Gentec TPM-310 photometer.

**VSV INFECTIVITY ASSAY**

Measurement of the infectivity of VSV by an endpoint dilution assay was performed as described previously.\(^7\) Results were expressed as percentage of the control (no illumination).

**COAGULATION ASSAYS**

The following assays were performed in the authors’ laboratory. Fibrinogen was determined according to the method of Clauss.\(^8\) Factors II, V, VII, and X were determined with coagulation assays using human tissue factor and plasma samples deficient in the respective coagulation factors. Prothrombin times were determined with Innovin (Dade Behring, Marburg, Germany), Thrombotest (Nycomed), and PT-Fib HS (Instrumentation Laboratory, Breda, The Netherlands). Instrument specific international sensitivity index values were used to calculate INR values. Thrombotest dilution plots were made essentially as described by Hemker and co-workers.\(^9\) In these plots, Thrombotest clotting times \(t\) of diluted plasma samples are plotted against the dilution factor \(D\). According to these authors,\(^9\) the quantity of a competitive inhibitor present in undiluted plasma can be estimated from the distance \(I\) on a horizontal line between \(t_{\text{min, uninh.}}\) and the intercept of the \(t-D\) line of the experimental plasma with that horizontal line (fig 1).

**PREPARATION OF SURVEY SAMPLES**

The preparation of survey samples was modified from the original procedure.\(^7\) Red blood cells of blood group O were obtained as packed cells in CPDA-1 (citrate phosphate dextrose adenine) from the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service. After washing of the red blood cells with phosphate buffered saline and removal of the Buffy coat, the cells were incubated with a solution containing sodium chloride (0.051 M), trisodium citrate (0.017 M), and sodium HEPES (0.1 M), pH 8.6. After two hours incubation at room temperature, the cells were centrifuged and the supernatant was discarded. The cells were then mixed with the pooled coumarin plasma samples, aiming at a hematocrit value of 0.4–0.45. A mixture of penicillin and streptomycin was added to prevent microbial growth. The pH of the artificial blood was approximately 7.5. The blood was distributed in 1.0 ml aliquots in capped polypropylene tubes. The blood samples were prepared on Monday and mailed to the participants of the EQA scheme on Tuesday. Participants were instructed to store the samples at room temperature until analysis on Friday—four days after preparation. Participants used their routine method for prothrombin time and INR determination.

![Figure 1 Thrombotest dilution plots of normal plasma (cross), pooled coumarin plasma before illumination (open circle) and after illumination with methylene blue for 20 minutes (open square). The regression equations for the latter two plots are \(y = 47.3 + 27.6x\) and \(y = 48.0 + 30.1x\), respectively. The intercept of the line for normal plasma with the Y axis is named \(I_{\text{min, normal}}\). I is the distance on a horizontal line between \(t_{\text{min, uninh.}}\) and the intercept of the line for coumarin plasma with that horizontal line.](http://jcp.bmj.com/...group.bmj.com)
The homogeneity of the samples was determined in the authors' laboratory by prothrombin time testing of 10 samples, using Simplastin Excel-S as thromboplastin reagent and a Coagamate-MTX as coagulometer.

**STATISTICAL EVALUATION OF EQA RESULTS**

Prothrombin times and INRs reported by the participants of the Netherlands EQA scheme were evaluated for each group using the same brand of thromboplastin reagent, irrespective of the type of instrument (coagulometer) used. When a participant’s result was greater than two times the mean value of the group using the same reagent, it was regarded as an outlier and excluded from the final calculations. Interlaboratory variation was expressed as coefficient of variation (CV) in per cent.

**Results**

**VSV INFECTIVITY**

The inactivation of VSV in pooled coumarin plasma was determined using an infectivity assay, based on the cytopathological effect of VSV on A549 cells, as scored by light microscopy after 72 hours. As shown in fig 2, complete inactivation of added VSV (4.7 log)

**COAGULATION ACTIVITY**

The inactivation of fibrinogen and factor V in pooled coumarin plasma was determined as a function of the illumination time. As shown in fig 3, a biphasic decrease of the fibrinogen concentration occurred in the presence of air. Under a nitrogen atmosphere, there was practically no decrease of the fibrinogen concentration. Factor V activity decreased approximately 10% in the presence of air, but no change was seen under nitrogen (fig 4). Similarly, there was

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**Figures:**

**Figure 2** Infectivity of vesicular stomatitis virus (VSV) after treatment with 1 µM methylene blue and incandescent light (700 W/m²). The VSV titre is expressed on a log scale. Two different pooled coumarin plasmas were used in two separate experiments (open squares and circles).

**Figure 3** Fibrinogen activity of pooled coumarin plasma after treatment with 1 µM methylene blue and incandescent light (700 W/m²). Before illumination, either air (open circle) or nitrogen (open square) was bubbled through the plasma, or no additional treatment was given (cross).

**Figure 4** Coagulation factor activities of pooled coumarin plasma after treatment with 1 µM methylene blue and incandescent light (700 W/m²). Factor V activity was measured in plasma treated with air (open circle), or nitrogen (open square). In plasma without preceding air or nitrogen treatment, factor V (cross), factor II (closed circle), factor VII (closed triangle), and factor X (closed square) were measured.

**Figure 5** International normalised ratio (INR) of pooled coumarin plasma determined with recombinant human thromboplastin (Innovin) after treatment with 1 µM methylene blue and incandescent light (700 W/m²). Before illumination, either air (open circle) or nitrogen (open square) was bubbled through the plasma, or no additional treatment was given (cross).
reported by each participating laboratory in two separate external quality assessment surveys. INR values were calculated and Table 2 Mean international normalised ratio (INR) and interlaboratory variation (CV) in two separate external quality assessment surveys.

The artificial blood samples were prepared separately for each survey. Artificial blood sample A was prepared from pooled coumarin plasma without addition of methylene blue and without illumination. Blood sample B was prepared from the same pooled coumarin plasma but with the addition of methylene blue and without illumination. Blood sample C was prepared from the same pooled coumarin plasma but with the addition of methylene blue and with illumination.

Table 1 Mean prothrombin times (seconds) and interlaboratory variation (CV) in two separate external quality assessment surveys.

The artificial blood samples were prepared separately for each survey. Artificial blood sample A was prepared from pooled coumarin plasma without addition of methylene blue and without illumination. Blood sample B was prepared from the same pooled coumarin plasma but with the addition of methylene blue and without illumination. Blood sample C was prepared from the same pooled coumarin plasma but with the addition of methylene blue and with illumination.

**Discussion**

VSV is a very well characterised lipid enveloped virus that is used as a model for the study of photodynamic virus inactivation. The virus inactivating properties of methylene blue and other photoinactivating dyes in combination with visible light have been known for many years. Viruses vary in their sensitivity to methylene blue, but the susceptibility of HIV-1 to photoactivation was similar to that of VSV.

Under the conditions of photodynamic treatment used in our study (20 minutes illumination with white light (700 W/m²) in the presence of 1 µM methylene blue) there was complete inactivation of all VSV added to pooled coumarin plasma buffered with HEPES (fig 2). Our results are in agreement with those of Lambrecht et al who used fresh frozen plasma isolated from blood donations.

Photodynamic treatment of pooled coumarin plasma resulted in a substantial decrease of the functional fibrinogen concentration as measured with the Clauss method. The photodynamic inactivation of fibrinogen was, as expected, dependent on the presence of oxygen in the solution (fig 3). Lowering the oxygen concentration by bubbling with
nitrogen resulted in the complete absence of photodynamic damage. Previously, it has been shown that illumination in the presence of methylene blue resulted in a substantial reduction of histidine and tryptophan residues. This is probably caused by photooxidation of these residues by singlet oxygen, as shown in a study using scavengers of different reactive oxygen species. The relative decrease of factor II, V, VII, and X activities (fig 4) was smaller than that of fibrinogen, which is in agreement with the observations by other investigators. Furthermore, the activities of factors II, V, VII, and X remained practically constant after 15 minutes. The decrease in fibrinogen was biphasic and continued after 15 minutes. The biphasic kinetics might be the result of oxygen depletion occurring under our experimental conditions, which causes a slower rate of photodynamic damage in the second part of the illumination period. It seems that air bubbling before illumination cannot prevent the biphasic kinetics of activity loss. An alternative explanation for the observed effect might be that the different amino acid residues in the proteins undergo photooxidation at different rates. The partly oxidised proteins are apparently still able to function to some extent.

The photodynamically induced increase of the prothrombin time and INR is probably caused by inactivation of fibrinogen, and coagulation factors II, V, VII, and X. In the absence of oxygen the prothrombin time remained almost constant. Therefore, the increase of the prothrombin time in the presence of oxygen can be attributed to photooxidation of the proteins involved. The relative increase was not the same with the various thromboplastin reagents. The prothrombin times and INRs determined with plain rabbit and human thromboplastin increased relatively more than those determined with Thrombotest, a bovine thromboplastin combined with adsorbed bovine plasma. These results suggest that photooxidised coagulation factors and inhibitors react differently with bovine tissue factor than with human and rabbit tissue factor. In a Thrombotest dilution plot (fig 1) the slope of the pooled coumarin plasma was increased by photodynamic treatment and the distance I was decreased. This could be interpreted as a decrease of factor X activity and of competitive inhibitor protein induced by vitamin K antagonists (PIVKA). Because several coagulation factors are affected by the photodynamic treatment, it is not a simple task to determine which factors are responsible for the prolongation of the prothrombin time. Further experiments are needed to resolve this question.

Previous studies have shown that the liquid artificial blood samples used in the Netherlands EQA scheme are not stable. During storage at room temperature, a slow increase of the prothrombin time was seen. All participants were requested to perform the tests on the same day, so that the effect of deterioration was approximately the same in all samples. Differences in ambient temperature between the various parts of the Netherlands are small and the transit time is usually not greater than 24 hours. Furthermore, the homogeneity of the samples on the day of testing was very good (CV < 1%). The interlaboratory variation of the samples treated with methylene blue/light was not substantially different from the variation of the control samples when the variation was calculated for each reagent group separately (tables 1 and 2). In most of the reagent groups, an increase of the mean prothrombin time and INR was seen after photodynamic treatment of the pooled coumarin plasma. In contrast, the mean clotting time and INR reported by the users of the Thrombotest reagent did not change greatly after photodynamic treatment. As a result, the differences between the reagent group mean values increased after photodynamic treatment. It should be noted that in the control samples that were not illuminated, there were also important differences in the INR between the reagent groups (table 2). For example, the mean INR reported by users of PT-Fib HS was 20% greater than the mean INR reported by Thrombotest and Hepato Quick users. After illumination, the difference increased to 35%. The differences in INR are the result of deterioration of the samples and a different response of the various reagents to deterioration. Therefore, the performance of each participant of the EQA scheme must be assessed by comparison with results from other laboratories using the same reagent. In agreement with previous reports, it is not possible to assign a single INR value to these EQA samples that would be valid for all reagents.

In conclusion, our results show that photodynamic treatment of pooled coumarin plasma is effective for viral decontamination, without important impairment of its usefulness for EQA of the prothrombin time and INR.

The authors thank Ms E Witteveen, Ms C van Rijn, Mrs H Schafer-van Mansfeld, and Mrs J Meuwese-Braun for excellent technical assistance. Mr G van de Kamp (Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam) supervised the preparation of control blood specimens. Financial support was received from the Federation of the Netherlands Thrombosis Services.


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J Clin Pathol 2000 53: 470-475
doi: 10.1136/jcp.53.6.470

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M2-AMA do not directly produce ANCA indirect immunofluorescence patterns

The importance of distinguishing atypical cytoplasmic indirect immunofluorescence patterns from the "classic", centrally accentuated cytoplasmic immunofluorescence pattern on ethanol fixed human neutrophils has recently been re-emphasised. Auto-antibodies to other cytoplasmic autoantigens such as antimitochondrial antibodies (AMA), antismooth muscle antibodies, and antibisomal-P antibodies have also recently been reported to produce atypical cytoplasmic immunofluorescence patterns on ethanol fixed human neutrophils. However, an alternative explanation is that the atypical cytoplasmic immunofluorescence patterns might be produced by concomitant antineutrophil cytoplasmic antibodies (ANCA) in these sera, especially in cases of autoimmune liver disease. Therefore, we investigated: (1) whether sera containing AMA with confirmed M2 specificity produced positive indirect immunofluorescence patterns on ethanol fixed human neutrophils. However, an alternative explanation is the atypical cytoplasmic immunofluorescence patterns might be produced by concomitant antineutrophil cytoplasmic antibodies (ANCA) in these sera, especially in cases of autoimmune liver disease. Therefore, we investigated: (1) whether sera containing AMA with confirmed M2 specificity produced positive indirect immunofluorescence patterns on ethanol fixed human neutrophils.

Table 1 Results of M2-AMA ELISA, ANCA IIF and ANCA Combi-kit® ELISA

<table>
<thead>
<tr>
<th>Specimen</th>
<th>M2-AMA ELISA</th>
<th>ANCA IIF pattern (titre)</th>
<th>ANCA Combi-kit® ELISA (OD ratio)</th>
<th>Liver biopsy result</th>
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ORGenTec ANCA Combi-kit® ELISA OD (optical density) ratio: positive = 1, 0 < negative < 1.

†Concomitant ANA staining (1/640 titre) with a nuclear membrane pattern was present.

#Concomitant ANA staining (1/640 titre) with homogeneous and speckled patterns was present.

AICAH, autoimmune chronic active hepatitis; AMA, antimitochondrial antibodies; ANCA, antineutrophil cytoplasmic antibodies; BPI, bacterial/endothelial permeability increasing protein; ELISA, enzyme linked immunosorbent assay; IIF, indirect immunofluorescence; PBC, primary biliary cirrhosis.

Letters

raised plasma parathyroid hormone related peptide in gastric adenocarcinoma

We report a case of humoral hypercalcemia associated with a rapidly growing gastric carcinoma. To our knowledge, this is the first such case of gastric carcinoma reported with raised plasma parathyroid hormone related peptide (PTHrP) and absent bone metastases. A 69 year old woman presented with fatigue and intermittent sharp epigastric pain for one week. Upper gastrointestinal radiographs and endoscopy demonstrated a necrotic, friable mass in the mid stomach. Biopsy of the mass and a surrounding satellite

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and adenomas did not express PTHrP. Our case is consistent with the findings of Alipov et al because cancer cells expressed PTHrP, whereas adenomas did not (fig 1). Our case is notable for rapid clinical deterioration coupled with raised tissue and plasma PTHrP. These results suggest that PTHrP expression is associated with poor prognosis in gastric cancer. Whether PTHrP plays a direct role in cancer progression or is a byproduct of oncogene activation (for example, ras and src) remains to be determined.

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ELISA is the superior method for detecting antineutrophil cytoplasmic antibodies in the diagnosis of systemic necrotising vasculitis.

Dr Harris’s results showed that indirect immunofluorescence is a more sensitive technique than antigen specific enzyme linked immunosorbent assay (ELISA) for the diagnosis of systemic necrotising vasculitis (70% v 50%) but that ELISAs have a higher positive predictive value (87% v 76%). It was the greater sensitivity of indirect immunofluorescence that prompted the “Inter-
national consensus statement on testing and reporting antineutrophil cytoplasmic antibodies (ANCA)” to require all laboratories to screen for ANCA by indirect immunofluorescence, but to confirm the specificity of fluorescent sera by ELISA. In our hands, adherence to the minimum requirements of the consensus statement results in a higher positive predictive value than either indirect immunofluorescence or ELISA alone (62% compared with 44% and 50%, respectively). Screening by indirect immunofluorescence has the additional advantages of being a quicker and cheaper technique than using the two commercial antigen specific ELISAs that are usually required and, furthermore, indirect immunofluorescence might demonstrate coincidental but unsuspected autoantibodies such as antinuclear antibodies. We believe that the use of ELISAs alone to diagnose a systemic necrotising vasculitis is analogous to testing for systemic lupus erythematosus with anti-double stranded DNA antibodies rather than initially screening for antinuclear antibodies by indirect immunofluorescence.

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The authors reply
Our study was carried out following the preliminary observation that in our laboratory a positive immunofluorescence antineutrophil cytoplasmic antibody (ANCA) result was less frequently associated with a diagnosis of systemic necrotising vasculitis than a positive enzyme linked immunosorbent assay result (PR3 or myeloperoxidase). Our clinicians frequently did not alter management based on the immunofluorescence ANCA result. In fact, our renal physicians invariably proceeded with renal biopsy, regardless of immunofluorescence results, to confirm or refute the diagnosis of systemic necrotising vasculitis. The ELISA result frequently came back too late to alter clinical management, the ELISA test being done when immunofluorescence was reported as positive. Our study has shown that for active systemic necrotising vasculitis, ELISA has a superior positive predictive value and specificity, and comparable sensitivity to the immunofluorescence technique.

Savage et al quoted our sensitivity results with respect to “all cases” of systemic necrotising vasculitis, active and in active combined, which are largely irrelevant parameters in clinical practice because the need is to identify active cases. The principal result of the study was the finding that in active, biopsy confirmed cases of systemic necrotising vasculitis, ELISA ANCA is just as sensitive (85% v 88%; p = 0.056) yet has a signifi-


We would like to restate the importance of ELISA and immunofluorescence ANCA because its inferior specificity and negative predictive value make it unsuitable for the diagnosis of systemic necrotising vasculitis. We have used ELISA and immunofluorescence ANCA in a particular laboratory. We have only compared ELISA and immunofluorescence ANCA in a particular disease (systemic necrotising vasculitis) and found ELISA to be superior by all criteria. We have not investigated the value of ANCA with respect to the diagnosis of other conditions, such as inflammatory bowel disease, and so we do not recommend which tests should be used in a particular laboratory. We have only compared ELISA and immunofluorescence ANCA in a particular disease (systemic necrotising vasculitis) and found ELISA to be superior by all criteria. Our results indicate that the “International consensus statement on the testing and reporting of ANCA” should be revisited.

The fact that the sensitivity of ELISA ANCA falls as inactive cases are added to active cases implies that ELISA more quickly becomes negative as active disease settles, whereas immunofluorescence remains positive. This observation suggests that ELISA is also a better test in following disease activity after diagnosis and initiation of treatment.

Savige et al. reported their finding of a higher positive predictive value if results of ELISA and immunofluorescence ANCA are combined, whereas this was not found in our study. In fact, combining immunofluorescence and ELISA ANCA resulted in a lower positive predictive value than ELISA ANCA alone. It would be of interest to review the data Savige et al. have used.

Based on our results, we conclude that ELISA ANCA is the principal serological test for the diagnosis of systemic necrotising vasculitis. Immunofluorescence ANCA should be avoided because of its inferior specificity and poor positive predictive value open the way to incorrect or delayed diagnosis and treatment. We would like to restate the importance of recognising the different clinical syndromes caused by systemic necrotising vasculitis and of appropriate histological testing even with a positive or negative ELISA ANCA result. We have not investigated the value of ANCA with respect to the diagnosis of other conditions, such as inflammatory bowel disease, and so we do not recommend which tests should be used in a particular laboratory. We have only compared ELISA and immunofluorescence ANCA in a particular disease (systemic necrotising vasculitis) and found ELISA to be superior by all criteria. Our results indicate that the “International consensus statement on the testing and reporting of ANCA” should be revisited.

Some problems found in HIV-1 RNA quantification

The polymerase chain reaction (PCR) based assay of human immunodeficiency virus type 1 (HIV-1) RNA is now commercially available and is used widely for the assessment of antiretroviral treatments. The kit is called the Amplicor™ HIV-1 monitor test kit version 1.0 from Roche Diagnostics (Tokyo, Japan). However, this system is not sensitive enough for the accurate measurement of genetic subtypes A and E, and it gives falsely low titres for these virus subtypes.1, 2 To surmount this problem, additional gag primers (AG primers) have been introduced into the system for use with the Ver. 1.0 kit. Furthermore, a new improved version (Ver. 1.5) was developed recently, which is said to yield accurate results not only on subtype B but on subtypes A and E. With the Ver. 1.0 plus kit, adding the AG primer set from the Ver. 1.5 kit to the PCR master mixture containing the Ver. 1.0 primer set makes it possible to amplify even subtype A and E viruses. In the Ver. 1.5 kit, the downstream primer is 12 bases downstream from the Ver. 1.0 primer position, whereas the upstream primer position is unchanged but the primer has two base substitutions. In our laboratory, we have examined over 1500 samples (148 cases) using the Ver. 1.0 kit, and among them, 150 samples (65 cases) were also measured with the Ver. 1.0 plus kit. As expected, most cases with the HIV-1 subtypes A and E, which could not be measured with the Ver. 1.0 kit, could be measured with the Ver. 1.0 plus kit. However, we were interested in a few cases that gave unexpected results. Clinically and epidemiologically, these patients are not different from our other patients infected with HIV-1 subtypes A or E. As shown in table 1, case 1 gave equivocal results with all of the kits but in cases 2 to 5 higher results were obtained with the Ver. 1.0 plus kit than with the Ver. 1.0 kit. We measured these specimens with the Ver. 1.5 kit (kindly provided by Roche Diagnostics, Japan). In these five cases, all but case 2 revealed equivalent values with both the Ver. 1.0 plus and Ver. 1.5 kits. Unexpectedly, in case 3, only the Ver. 1.0 plus kit yielded the amplified product.

On the other hand, in case 6, a higher result was obtained with the Ver. 1.0 kit but not with the Ver. 1.0 plus or Ver. 1.5 kits. An additional two cases showed a similar trend, although the degree of discrepancy was less severe (data not shown).

Table 1 Amplicor™ HIV-1 monitor kit

<table>
<thead>
<tr>
<th>Case</th>
<th>Ver. 1.0</th>
<th>Ver. 1.0 (+)</th>
<th>Ver. 1.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Case 1</td>
<td>3.6 x 10^3</td>
<td>2.7 x 10^2</td>
<td>2.0 x 10^3</td>
</tr>
<tr>
<td>Case 2</td>
<td>&lt;0.4 x 10^4</td>
<td>3.7 x 10^3</td>
<td>&lt;0.4 x 10^4</td>
</tr>
<tr>
<td>Case 3</td>
<td>4.2 x 10^3</td>
<td>1.2 x 10^3</td>
<td>9.2 x 10^2</td>
</tr>
<tr>
<td>Case 4</td>
<td>1.7 x 10^4</td>
<td>5.0 x 10^3</td>
<td>3.5 x 10^4</td>
</tr>
<tr>
<td>Case 5</td>
<td>1.6 x 10^4</td>
<td>6.1 x 10^3</td>
<td>3.5 x 10^4</td>
</tr>
<tr>
<td>Case 6</td>
<td>4.0 x 10^3</td>
<td>&lt;0.4 x 10^4</td>
<td>0.5 x 10^3</td>
</tr>
</tbody>
</table>

Test results of each case are summarised. Measurements with Amplicor HIV-1 monitor test kits Ver. 1.0, 1.0 plus, and 1.5 were performed according to the manufacturer’s protocol. The correlation coefficient of variation on the same samples tested with the Amplicor HIV-1 monitor test kits Ver. 1.0 and 1.0 plus in our laboratory were 19.9% (n = 17) and 31.9% (n = 12), respectively. In cases 2 and 6 the viral load was confirmed with another independent measurement (second result).

Nucleotide sequences of primer region

Upstream primer region

Downstream primer region

Figure 1 The alignment of each primer region. The SK462 and SK431 primers were used in the Amplicor™ HIV-1 monitor test Ver. 1.0 kit. The sequences of these primers were obtained from Roche Diagnostics. Consensus sequences were reproduced from the HIV Sequence Database WWW home page (see text). The sequences shown in this table are from the sense strand.
To clarify the cause of these discrepant results, sequence analysis of the amplified regions of these cases was performed. The nucleotide sequences of these cases were found to be homologous to subtype A or E virus when they were aligned with the consensus sequences of HIV-1 subtypes A, B, and E obtained from the HIV Sequence Database WWW home page (Sequences. Online) http://hiv-web.lbnl.gov. 30 October 1999, last date accessed.). Surprisingly, the sequences of the primer regions of all cases were completely identical (fig 1). In conclusion, cases 2 and 6 are measurable by one of these kits, Ver. 1.0 plus and Ver. 1.0, respectively; and case 1 is measurable by all kits despite having the same nucleotide sequence in the primer regions as the other cases. The results of sequence analysis of the primer regions suggests that the minor differences in sequence between the virus and the primers does not always affect amplification efficiency in these kits. Although such cases might form a minor population among HIV-1 infections, these results indicate that some cases could not be measured by a single kit. As far as we have experienced, even if one kit fails to measure virus, the other will yield the expected viral load, as judged by disease history, CD4 count, treatment, and so on, suggesting that these kits are mutually complementary. If other methods, such as nucleic acid sequence based amplification or branched DNA (bDNA) systems, are available, it would be useful to test with them. We have found some cases of subtype A or E that have shown a higher viral load with bDNA (Chiron Quan-
tiplexTM HIV RNA 2.0 assay Chiron Corpora-
Smerville, California, USA) than the Amplicor HIV-1 monitor test kit (data not shown). In patients infected with subtype B virus, the correlation between the Amplicor HIV-1 monitor test kit and the bDNA method was excellent (r = 0.904; n = 21). In conclu-
sion, because the major difference in the three versions of the Amplicor HIV-1 monitor test kit is the primer set, we emphasise that for accurate quantitative measurement using this kit various additional primer sets that can amplify similar regions are needed.

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Next of kin clinics

We read with interest the considered views of Professor Vanezis and Dr Leadbeatter regarding the role of forensic pathologists dealing with the next of kin of those people whose deaths are subject to medicolegal investigation. It might be of interest to your readers to know that the issues raised by the authors have been dealt with at the department of forensic medicine, at Westmead for the past decade.

The department offers a specialist grief counselling service, targeting the needs of families attempting to come to terms with the complications of a coronial investigation at a time of crisis and great distress. The counsel-
lors routinely contact the next of kin in all cases, discussing with them forensic and coro-
nial procedures and outlining the counselling service, which includes individual sessions, group debriefing, and court support.

The service operates with the full support of the New South Wales State Coroner, but is funded solely by the department of forensic medicine.

Professor Vanezis and Dr Leadbeatter propose the provision of “next of kin clinics”, conducted by the pathologist, to discuss postmortem findings. Such a service is regu-
larly provided at Westmead as part of the counselling unit’s brief. Although thoroughly endorsing the authors’ remarks on the need for accurate and timely information, we have found that several alterations need to be made to the model broadly outlined by the authors for the families to gain maximum benefit from the information sessions.

These include:

- Ongoing contact between the family and the counsellor for the time of necropsy to the receipt of its results, to ensure that the family is confident that forensic staff will be both frank and reliable in the delivery of information and support.
- A preliminary session between the family and the counsellor, to ensure that all the family’s concerns are identified, thereby ensuring that the meeting with the pathologist is as comprehensive as possible.
- The presence of the counsellor at the meeting as mediator and support person for the family. Such a mediation role involves ensuring that all the family’s issues are adequately considered, that the tendency some pathologists have to use jargon is kept under control, and that clarification is sought where necessary.

We have found that these alterations are neces-
sary to ensure that the emotional needs of the family and the occasionally unavoidable power imbalance between grieving relatives and a medical specialist are properly dealt with. This latter concern is most clearly seen in the reluctance of non-medically trained relatives to seek clarification and to admit to doubts and concerns while speaking with a representative of “the system”, however well intentioned.

We have been pleased with the success of the service over many years, and self reported feedback from clients suggests both a sense of confidence in the forensic system and in coronial findings as a result. We are delighted that our colleagues in the Northern Hemi-
sphere are finally becoming aware of the value of such a system.

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Correction


Because of an error in the publishing process, the wrong figure was published as fig 6 in this paper. The following figure is the correct one. The journal apologises for this error.