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ORIGINAL RESEARCH

Coming full circle: a prospective comparison of the South African and International Society on Thrombosis and Haemostasis protocols for dilute Russell's viper venom testing

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Introduction: Antiphospholipid syndrome (APS) is an autoimmune condition typified by a propensity to thrombosis and pregnancy loss. Dilute Russell's viper venom testing (DRVVT) is an important cornerstone for APS diagnosis. International guidelines differ in the laboratory protocol for DRVVT, particularly regarding the timing and employment of mixing studies. This study aimed to prospectively compare the result interpretation of the South African (SA) national guidelines to those of the I*nternational Society on Thrombosis and Haemostasis* (ISTH).

Methods: The study included 157 samples submitted for routine lupus anticoagulant (LA) testing to the National Health Laboratory Service (NHLS) at Chris Hani Baragwanath Academic Hospital (CHBAH). Mixing studies were performed on all samples with prolonged screen times (per ISTH protocol). Pertinent clinical and laboratory information was recorded from the laboratory information system. Test positivity rates, sensitivity, and specificity were calculated.

Results: Significant differences in the test positivity rates were present between the SA (43/157, 27.4%) and ISTH protocols (18/157, 11.5%; $p = 0.0005$). The sensitivity of the SA protocol was considerably higher than that of the ISTH protocol (57.1% vs. 28.6%), while the specificity was somewhat lower (78.1% vs. 93.8%). Mixing studies were required significantly more frequently in the ISTH protocol (59.9% vs. 20.4% in the SA protocol; $p < 0.001$).

Conclusion: The ISTH protocol has poorer sensitivity than local guidelines for DRVVT in the SA setting and requires substantially more mixing studies. Therefore, the SA guideline remains the preferred protocol in our laboratory.

Keywords: lupus anticoagulant, antiphospholipid syndrome, dilute Russell's viper venom testing, I*nternational Society on Thrombosis and Haemostasis*

Introduction

APS is a rare autoimmune condition typified by a propensity to recurrent thrombosis and pregnancy loss.[1-3] It is caused by antibodies directed against phospholipid (PPL) binding proteins (most commonly β_2-glycoprotein I [β_2-GPI]), which bind exposed PPLs on numerous cell types (including endothelial cells, platelets, monocytes, neutrophils, and trophoblasts).[2,3] These have pleiotropic effects, resulting in the activation of complement immune cells, and a spectrum of procoagulant changes.[1,2]

APS diagnosis rests on the presence of typical clinical and laboratory findings (Table I). The latter includes detecting anticardiolipin (aCL), anti-β_2-GPI (a-β_2-GPI) antibodies, or a LA. LA tests are coagulation assays highly sensitive to PPLs, where reduced levels of bioavailable PPLs cause prolongation of the clotting time (the screen time). LA testing often includes a second step, demonstrating normalisation of the clotting time when PPLs are supplemented (the confirm time). Guidelines universally recommend that two LA tests be performed, one of which should include the DRVVT.[4-6]

However, there is some discordance among international guidelines regarding the laboratory protocol for DRVVT, particularly the timing, and employment of mixing studies, as well as the cut-off levels used for result interpretation. SA national guidelines (hereafter referred to as the SA protocol) are loosely aligned with those of the Clinical and Laboratory Standards Institute and the British Society for *Haematology* (Figure 1), but show some discordance with the guideline advocated by the ISTH (hereafter referred to as the ISTH protocol) (Figure 2).[4-7] In this study, we aimed to assess the difference in the diagnostic interpretation of the DRVVT using the SA and ISTH protocols.

Materials and methods

The study included samples submitted for routine LA testing to the NHLS at CHBAH from May to June 2023. Samples were received in citrate anticoagulated tubes (0.109 M trisodium citrate) diluted in a 1:9 ratio (blood-to-anticoagulant) and were double centrifuged at 4 000 rpm for 15 minutes before being separated and frozen at -20 °C for no longer than seven days. Samples were thawed at 37 °C in a water bath before analysis. Clotted and haemolysed blood samples were rejected. DRVVT was performed on a Sysmex CS-2500i analyser (Sysmex, Kobe,

Table I: Diagnostic criteria for antiphospholipid syndrome[8]

Laboratory criteria	Clinical criteria (should be best explained by a diagnosis of APS)
Positive LA (+ 1 point for one test positive, + 5 points if persistent)	**Vascular thrombosis** • Venous (+ 1 point if at high risk for VTE, e.g. active malignancy, prolonged immobilisation, major surgery, pregnancy, etc., + 2 points if not at high risk for VTE) • Arterial (+ 2 points if at high risk for CVD, e.g. hypertension, hyperlipidaemia, diabetes mellitus, etc., + 4 points if not at high risk for CVD) • Small vessel (e.g. livedo racemosa, aPL nephropathy, pulmonary haemorrhage, etc., + 2 points if clinical features, + 5 points if confirmed histologically)
Positive aCL antibody or a-β_2-GPI antibody test (+ 1 point if medium high titre IgM, + 4 points if medium titre IgG, + 5 points if high titre IgG, + 7 points if high titre IgG for both antibodies)	**Pregnancy complications** • Recurrent miscarriage before 10 weeks gestation (+ 1 point) • One or more fetal losses beyond 10 weeks gestation (+ 1 point if no PI or PEC) • PEC or PI (+ 3 points) • PEC and PI (+ 4 points)
	Cardiac valve abnormalities • Thickening (+ 2 points) • Vegetation (+ 4 points)
	Thrombocytopaenia (platelet count 20–130 × 10^9/L, + 2 points)

A patient with a clinical score ≥ 3 and a laboratory score ≥ 3 can be classified as having APS.
a-β_2-GPI – anti-β_2-glycoprotein I, aCL – anticardiolipin, aPL – antiphospholipid antibody, APS – antiphospholipid syndrome, CVD – cardiovascular disease, IgM – Immunoglobulin M, LA – lupus anticoagulant, PEC – pre-eclampsia, PI – placental insufficiency, VTE – venous thrombo-embolic disease

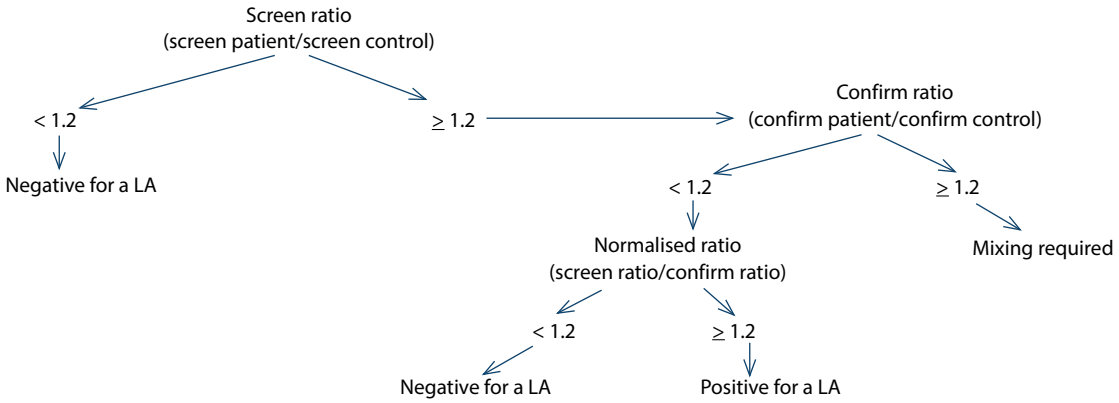


Figure 1: Diagrammatic representation of the DRVVT protocol according to the SA guideline[7]
DRVVT – dilute Russell's viper venom testing, LA – lupus anticoagulant, SA – South African

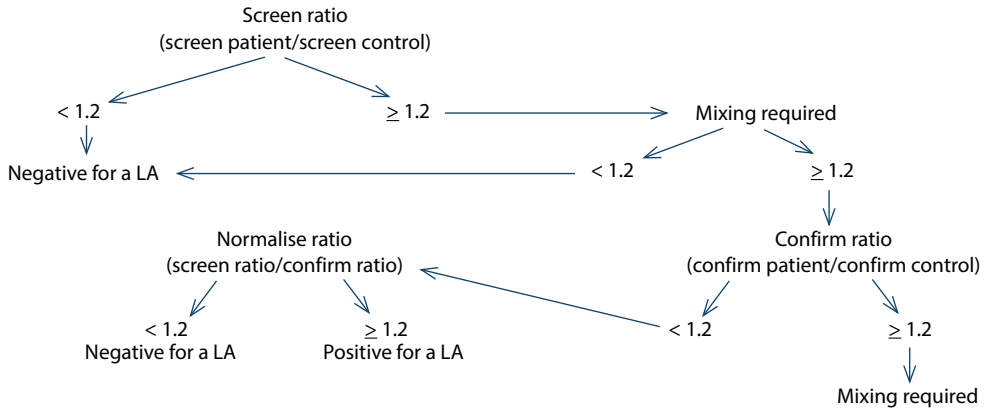


Figure 2: Diagrammatic representation of the DRVVT protocol according to the ISTH guideline as originally evaluated in this study, notably, the cut-off levels used were not those advocated by the ISTH (see text for details)[4]
DRVVT – dilute Russell's viper venom testing, ISTH – International Society on Thrombosis and Haemostasis, LA – lupus anticoagulant

Japan) using Siemens LA1 and LA2 reagents (Siemens, Berlin, Germany).

As one of the major differences between the SA and ISTH protocols relates to the timing of mixing studies, this aspect was assessed first by performing a mixing study on all samples with a prolonged screen time (as defined in the SA guideline) by mixing patient plasma and commercial normal plasma (Ci-Trol 1, Siemens, Berlin, Germany) at a 1:1 ratio. The effect of different cut-off levels was also evaluated utilising the normal clotting times for LA1, LA2, LA1 mix, and LA2 mix determined in a reference interval verification study performed on 30 ostensibly healthy volunteers.

Pertinent clinical information (age, gender, ward, human immunodeficiency virus [HIV] status, international normalised ratios [INR], and aCL and a-β_2-GPI antibody result testing) was recorded from the laboratory information system (InterSystems TrakCare, Cambridge, United States). The study was approved by the Human Research Ethics Committee of the University of the Witwatersrand (reference: M1911201). Due to the nature of the study, informed patient consent was not required.

Statistical analysis

Results are presented as percentages or medians and interquartile ranges (IQR) for categorical and continuous data, respectively. The positivity rate between protocols was compared using Fisher's exact test. Statistical significance was accepted at $p = 0.05$. Sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) for each protocol were determined using the following formulas: a = true positive, b = false positive, c = false negative, and d = true negative.

Sensitivity = a/a + c

Specificity = d/b + d

PPV: a/a + b

NPV: d/c + d

Results

The median age of the 157 samples included during the study period was 33 years (IQR 29–40). Most patients (95%) were female, with nearly half of the samples submitted from antenatal clinics or gynaecology wards (Table II). HIV test results were available in 72 patients, with a seropositivity rate of 48.6% (22.3% of the entire cohort).

Table II: Relevant demographic and clinical data

Parameter	Result
Age, median (IQR)	33 (29–40)
Female gender, %	95
HIV seropositive, *n*/N (%)	35/72 (48.6)
Ward where sample was collected	***n*/N (%)**
• Obstetrics/gynaecology	75/157 (47.8)
• Medical (including haematology wards)	44/157 (28.0)
• Rheumatology	5/157 (3.2)
• Other	33/157 (21.0)
Antibody test results positive, * *n*/N (%)	8/40 (20)

* aCL and/or a-β_2-GPI
aCL – anticardiolipin, a-β_2-GPI – anti-β_2-glycoprotein I, HIV – human immunodeficiency virus, IQR – interquartile range

Protocol assessment

According to the SA protocol, 43/157 (27.4%) samples tested positive. The positivity rate was significantly lower when interpreted according to the ISTH protocol (18/157, 11.5%; $p = 0.0005$). All patients who were positive on the ISTH protocol were also positive using the SA protocol. Further analysis was performed to assess possible causes for the discrepancy in interpretation. The INR results were evaluated, as part of the rationale for the additional mixing performed in the ISTH protocol is to eliminate false positive results due to a coagulation factor deficiency. In the cases with discordant results between the SA and ISTH protocols, 7/25 had an INR result available, which was normal in all cases.

Importantly, all samples with a prolonged INR (≥ 1.3) were reported as negative with reserve (owing to the presence of a coagulation factor deficiency) or as uninterpretable (due to the LA2 mix ratio being > 1.2) using the SA protocol. Since LA positivity with negative results on sample mixing is described to occur with high frequency among people living with HIV, the HIV status was assessed in samples testing positive for the two different protocols to determine if this could account for the high levels of discordance.[9] However, the HIV seropositivity rate was similar between those who tested positive in the SA protocol (30.2%), the ISTH protocol (33.3%), and the samples with discordant results between the two protocols (29.2%).

Furthermore, LA positivity rates were higher in people living with HIV, irrespective of the protocol used, compared to those seen in the HIV-negative patients (Figure 3). There were similar rates of discordance between the two protocols when comparing gender or the wards where samples were collected. The exception was among rheumatology patients, whose positivity rates were the same for both protocols (2/6, 33% of patients tested positive on both the SA and ISTH protocols).

Antibody test results for both aCL and a-β_2-GPI were available in 40 patients. As no gold standard is available for LA testing, antibody test positivity for either antibody was used as a surrogate. Samples that tested positive on both the SA and ISTH protocols had the highest antibody seropositivity rate (50%). Those negative for both tests had an antibody seropositivity rate of 9.7%.

For samples positive on the SA protocol and negative on the ISTH protocol, 22.2% were antibody-positive (Figure 4). A similar trend was seen in the number of cases positive for both aCL and a-β_2-GPI antibodies (double positive), seen in 25% of the cases positive in both protocols, 11.1% of those positive only in the

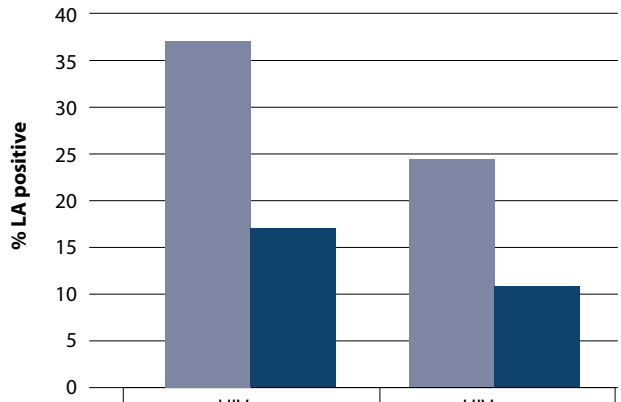
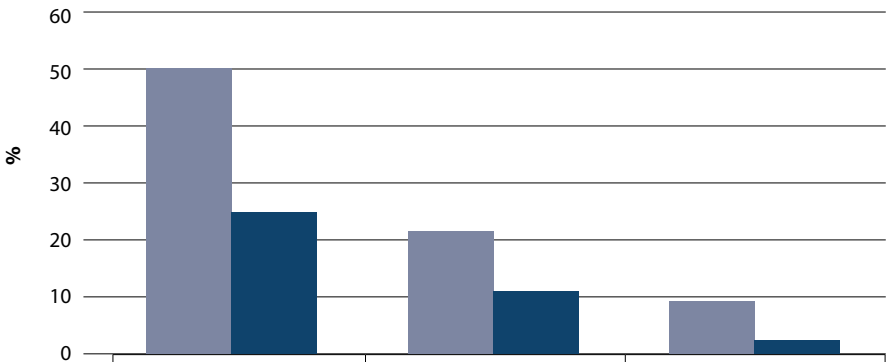


Figure 3: Proportion of positive LA cases according to HIV status for the SA and ISTH protocols

HIV – human immunodeficiency virus, ISTH – International Society on Thrombosis and Haemostasis, LA – lupus anticoagulant, neg – negative, pos – positive, SA – South African



	Both protocols provided	SA, pos, ISTH negative	Both protocols negative
Antibody testing positive	50	22.2	9.7
Double positive	25	11.1	3.2

Figure 4: Proportion of cases testing positive for aCL and a-β_2-GPI antibodies (one or both) according to protocol
a-β_2-GPI – anti-β_2-glycoprotein I, aCL – anticardiolipin, ISTH – International Society on Thrombosis and Haemostasis, pos – positive, SA – South African

Table III: Comparison of the predictive power for antibody positivity of the SA and ISTH protocols

	Sensitivity	Specificity	PPV	NPV
SA protocol	57.1%	78.1%	36.4%	89.3%
ISTH protocol	28.6%	93.8%	50%	86.5%

ISTH – International Society on Thrombosis and Haemostasis, NPV – negative predictive value, PPV – positive predictive value, SA – South African

SA protocol, and 3.2% of the cases negative for both protocols (Figure 4). This translated into a substantially higher sensitivity for the SA protocol, but superior specificity and PPV for the ISTH protocol (Table III). Unsurprisingly, mixing studies were performed significantly more frequently in the ISTH protocol than the SA protocol (59.9% vs. 20.4%; $p < 0.001$).

Evaluation of alternative interpretive cut-off points

The ISTH protocol recommends that the screen time be interpreted as prolonged (thus signalling the need for confirmatory testing) when it exceeds the upper limit of the reference interval. Ratios for interpreting the confirm, mix, and normalised ratios should be determined in healthy volunteers with a cut-off at the 99th percentile level. According to these criteria, the cut-off points to be utilised in our laboratory are summarised in Table IV. In contrast, the SA guideline recommends a blanket ratio of ≥ 1.2 to interpret all these variables, including the screen ratio. The latter is based loosely on the recommendations of historical LA guidelines.[10,11] The cut-offs determined in healthy volunteers were applied to the SA protocol, as this showed superior sensitivity.

Table IV: The cut-off levels determined according to the ISTH protocol

	Cut-off levels using the ISTH criteria
Screen cut-off	> 51 sec
LA2 ratio (confirm) cut-off	1.09
Normalised ratio	1.21
LA1 mix ratio	1.08
LA2 mix ratio	0.97
Normalised mix ratio	1.16

ISTH – International Society on Thrombosis and Haemostasis, LA – lupus anticoagulant

Using these cut-off levels, only one (0.6%) tested positive, while 28 (17.8%) were uninterpretable (as the confirmatory [LA2] mix ratio was above the cut-off value). The low positivity rate was partially attributable to the very high screen time cut-off, since over 80% of the positive samples in the current protocol had a screen time below this level.

Discussion

This study showed marked differences in the interpretation of DRVVT results using local SA guidelines and the ISTH protocol, with a ~ 16% difference in the positivity rate. The possibility of the dilution of weak positive LAs on mixing in the ISTH protocol is well recognised, having been reported previously in other laboratory-based studies and reports on the causes of result discrepancies from external quality assessment programmes.[12-14] Notably, the two protocols showed complete result agreement in rheumatology patients, suggesting that they have similar performance for detecting a LA of autoimmune origin.

A reported cause of LA positivity but negative results on sample mixing is HIV infection.[8] Given the high rate of HIV infection in SA, it was determined whether HIV-related interference could be a factor in the interpretation discrepancy of the results. While DRVVT test positivity was seen more frequently among people living with HIV (as reported previously), discordant results were not more common in this subgroup, and do not account for the differences seen.[15]

The rationale for the more intensive mixing advocated in the ISTH protocol is stated in this guideline, "Mixing tests add information on the results of screening and confirmatory testing, especially in anticoagulated patient samples … To have maximal information, we advise performance of screen followed by simultaneous mix and confirm in all samples with a prolonged screening test."[4] In

the current study, we found no evidence that those with results discordant from the ISTH protocol were on coumarin-based anticoagulant therapy since their INR levels (where available) were normal. Moreover, no samples with prolonged INR were interpreted as positive, suggesting that the SA protocol is not overly susceptible to false-positive testing due to coagulation factor deficiency. As such, this seems an insufficient justification for performing mixing studies in all samples with a prolonged screen time in our setting.

In scenarios where the screen and confirm time support the presence of a LA but the mix is negative, the ISTH guideline recommends the measurement of additional coagulation factor levels.[4] Such additional testing is not practically feasible for the large number of samples in our environment. Mixing studies also incur added reagent costs for the extra testing performed in the mixed sample and are more labour-intensive in laboratories where they are carried out manually. The number of samples requiring mixing in this study was almost threefold higher using the ISTH protocol, which is a further deterrent in the resource-constrained setting.

More importantly, while the specificity of the ISTH protocol was excellent, its sensitivity was poor. Since detecting a LA is important for diagnosing APS in antibody-negative cases, this could result in failing to make a correct diagnosis and implementing appropriate management. Notably, the strength of the LA does not invariably correlate with the phenotypic severity of APS, and the detection of weak LAs is therefore necessary.[16] Furthermore, triple positivity (positive for LA, aCL, and a-β_2-GPI antibodies) has prognostic implications regarding the risk of recurrent thrombosis.[17,18] The sacrifice of DRVVT sensitivity in favour of higher specificity has implications for identifying patients at high risk of adverse clinical events.

Finally, our analysis of result interpretation using the cut-off values recommended by the ISTH dropped the test positivity rate and sensitivity dramatically, while ~ 17% were uninterpretable. This was attributable to the combination of the very high cut-off value for the screen time reference range (which excluded many positive samples) and the very low cut-off levels for mixing study interpretation obtained from healthy volunteers' analyses (which resulted in seeming correction failure on PPL supplementation). Since such results are not clinically helpful, the continued use of a blanket value of 1.2 is supported to interpret all the ratios. Given the difficulty in establishing reference ranges, such an approach may be worth consideration in resource-constrained laboratories in other parts of the world.

Study limitations

Admittedly, the use of antibody results as a surrogate for true positivity in this study is suboptimal and may be controversial, as it is well-recognised that not all patients with APS have positive antibody testing, and not all patients with antibodies have APS. However, it seems rational that in patients with clinical features meriting testing for APS who test positive for antibodies, a weak LA is more likely to be a true positive than a false positive result. In the absence of a true gold standard test or available standard material, we propose that this is a valid means of evaluating the relative suitability of test protocols. Additional limitations of this study include the relatively small proportion of patients in whom antibody test results were available and the lack of comprehensive clinical information.

Conclusion

The ISTH protocol produces considerably poorer diagnostic performance compared to local guidelines for DRVVT in the SA setting. This is accompanied by a greater cost and need for manual intervention in some circumstances. As such, the SA guidelines remain the preferred protocol in our laboratory and may be worth consideration in other parts of the world where resources are limited.

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Conflict of interest

The authors declare no conflict of interest.

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Ethical approval

This project was approved by the Human Research Ethics Committee of the University of the Witwatersrand (reference: M1911201).

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