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EVALUATION OF A COST-EFFECTIVE ADAMTS13 ANTIGEN ASSAY

M Meiring *PhD* | **S Myneni** *B.Sc Hons*

Department of Haematology and Cell Biology, University of the Free State, Bloemfontein, South Africa

Corresponding author: ***Muriel Meiring*** *| tel: +27 51 405 3593 | email: meiringsm@ufs.ac.za*

ABSTRACT

Thrombotic thrombocytopenic purpura (TTP) is life-threatening and is characterised by platelet deposition in the microvasculature with thrombus formation in particular organs. This results in thrombocytopenia, microangiopathic haemolytic anaemia, kidney failure and neurological symptoms. It is a rare disorder, but can occur in patients infected with the human immunodeficiency virus (HIV). ADAMTS13 is the 13th member of a disintegrin and metalloprotease with thrombospondin type motifs. Its deficiency causes TTP. Therefore, the measurement of the ADAMTS13 levels in plasma is vital in the diagnosis of TTP and also important in distinguishing it from other thrombotic microangiopathies (TMA's). Unfortunately, commercial ADAMTS13 antigen assays are expensive for healthcare service providers in developing countries. However, several antibodies and antibody pairs have been produced against ADAMTS13 and are commercially available. In this study, we evaluated an in-house ADAMTS13 antigen assay using two different commercial antibodies and compared the outcomes to that of a commercial ADAMTS13 antigen kit by using the plasma of 40 patients with possible HIV-associated TTP and 40 healthy subjects. The Intra- and inter-assay coefficients of variation were calculated as 8% and 7% respectively. The assay gave linear results between 0.78 to 12.5% ADAMTS13. The limit of detection was 0.2%, and the limit of quantification was 0.8%. The correlation of our assay compared to the commercial test kit was excellent, with a R^2 value of 0.9. In addition, the cost of our ADAMTS13 antigen assay was lower than that of the commercial ADAMTS13 antigen test kit. Our cost-effective in-house ADAMTS13 antigen test also produced reliable results. We therefore recommend that this assay be used to diagnose HIV-associated TTP.

KEYWORDS

ADAMTS13 antigen; human immunodeficiency virus (HIV); Thrombotic thrombocytopenic purpura (TTP)

INTRODUCTION

Thrombotic thrombocytopenic purpura (TTP) is a prothrombotic disorder that is characterised by microvascular platelet clumping, resulting in microangiopathic haemolytic anaemia, fragmented erythrocytes (schistocytes), consumptive thrombocytopenia, renal dysfunction and neurological symptoms.[1] Human immunodeficiency virus (HIV) infection precipitates TTP, probably by infecting vascular endothelial cells which results in dysfunction of these cells, thrombin generation and the consumption of the Von Willebrand factor protease, ADAMTS13.[2] The diagnosis of TTP and HIV-associated TTP remains based on clinical history, examination of the patient and the blood film.[1] Assays for measuring ADAMTS13 levels and its function in plasma help to confirm the diagnosis, differentiate TTP and HIV-associated TTP from other thrombotic microangiopathies (TMA's) and to monitor the efficacy of treatment. These assays are also helpful in the decision to begin additional or alternative therapy.[3] In a previous study, we suggested the use of ADAMTS13 levels as a confirmatory test for HIV-associated TTP. The measurement of ADAMTS13 activity using the FRET assay did not confirm the disease and auto-antibodies to ADAMTS13 were found in only 50% of patients with HIV-associated TTP.[4]

Although ADAMTS13 antigen kits are available, it is still an expensive test for small laboratories and for the patients from lower income countries. We have developed a cost-effective ELISA test, using commercial antibodies to ADAMTS13. We have also evaluated the technical performance of this assay with the use of plasma samples from 40 healthy subjects and 40 patients with suspectedHIV-associated TTP and compared it to a commercial test kit.

MATERIALS AND METHODS

Subjects

Peripheral blood from 40 healthy subjects and 40 patients with suspected HIV-associated TTP were collected into sodium citrate. The Health Sciences Research Ethics Committee of the University of the Free State approved this study in 2016 with ethics approval number: 92/2016.

Sample collection and preparation

Peripheral blood was collected into VacutainerTM tubes (BD Vacutainer Systems, Plymouth, UK) containing 0.105M sodium citrate with a ratio of 1:9 to blood. Platelet-poor plasma was prepared by centrifugation at 200xg for 20 minutes at room temperature and samples were stored in polypropylene tubes at -70°C until analysed. All tests were performed on the original aliquots that had not been previously thawed.

Laboratory tests

In-house assay:

A 96-well plate (Nunc) was coated for two days at 4°C with

50ng/ml of a mouse anti-human ADAMTS13 antibody (R&D Systems, USA; 100µl per well). After washing with PBS with 0.1% Tween-20 (Sigma, USA), 100µl of the patient's plasma, volunteer's plasma or standard plasma was added and incubated for 2 hours at 37°C. Plasma samples from patients and volunteers were diluted 1:10 in PBS/Tween-20 of which 100µl was added to two wells and anysed in duplicate. The first International standard for ADAMTS13 (NIBSC, UK) was used as a calibrator and added in the following dilutions: 100%, 50%, 25%, 12.5%, 6.25%, 3.125% and 1.56%. A blank sample was also included and served as a negative control. After washing the plate four times with a microplate washer (VACUTEC, South Africa), 2µg/ml of a rabbit anti- human ADAMTS13 detection antibody (Santa Cruz, USA; 100µl per well) was added and incubated for 1 hour at room temperature. The plate washing was repeated four times and 200ng/ml of a goat anti-rabbit horseradish peroxidase (HRP) conjugated antibody (100µl per well) was added and incubated for 1 hour, at room temperature. The plate was further washed for another four times. The colour was developed for 20 minutes by adding a substrate solution ortho-phenylenediamine (0.05%) in 0.1M citrate-phosphate buffer, pH 5.0 containing 0.03% H_2O_2 (90µl per well). The reaction was then stopped by adding 100µl of 4M H_2SO_4. The optical density (OD490-630) of the solution on the plate was read in a Synergy HT spectrophotometer (Biotek, USA) and an eight-point standard curve with the known ADAMTS13 concentrations of the International Standard (Fig 1A) was drawn. The ADAMTS13 level for each patient and normal volunteer was read from the standard curve.

Commercial assay:
The Immubind ADAMTS13 antigen kit from Sekisui Diagnostics (USA) was used according to the manufacturer's instruction. Briefly, plasma and standard samples were added to an anti-ADAMTS13 antibody coated ELISA plate. After incubation, a horseradish peroxidase conjugated anti-ADAMTS13 antibody was added and the plate was coloured with a substrate for peroxidase. The same ADAMTS13 standard was used to compare the two assays.

Precision studies:
Precision studies were carried out according to the guidelines of the Clinical and Laboratory Standards Institute.[5] In order to determine the inter-assay precision, we repeat the assay eight times on the same patient's plasma in the same run using a patient with a low ADAMTS13 level (i.e. only one level was used). For the intra-assay precision, we used normal pooled plasma (combined from 20 persons) in 5 consecutive tests.

Normal reference range:
We determined the plasma levels of ADAMTS13 in 20 healthy males and 20 healthy females using our new assay and calculated the reference range as the mean ± two standard deviations (SD).

Test comparison:
We compared our method to the Imubind ADAMTS13 ELISA kit from Sekisui Diagnostics (MA, USA) by using plasma from 40 patients with possible TTP.

Statistical analysis

We calculated the reference intervals as the mean ± 2 SD and the Intra- and inter-assay precision by the mean, standard deviation (SD) and the coefficient of variation (CV) from the results of normal healthy volunteers. The precision goal for the Inter-assay and intra-assay was not to exceed 10% of the CV in the ELISA assay.[6] We calculated the limits of detection and quantification from the standard curve. The theoretical lower limits of detection (LLD) and the theoretical lower limit of quantification (LLQ) were then calculated as the minimum detectable concentration and reliable detection limits, respectively. We compared our assay to the commercial assay by the Bland-Altman and Deming regression plots using the 40 patient samples. We defined the commercial kit as the reference method and calculated the mean bias and 95% limits of agreement between both assays.

RESULTS

The standard curve for our ADAMTS13 antigen assay (Figure 1a and b) shows a good dose-response, with fast substrate colour development and steep upward linearity. The closeness of fit shows an r^2 value of > 0.99.

Table 1 summarises the technical performance of our method. The intra- and inter-assay CV's were 8.2% and 7.2% respectively. The reference range for the 40 standard samples varied

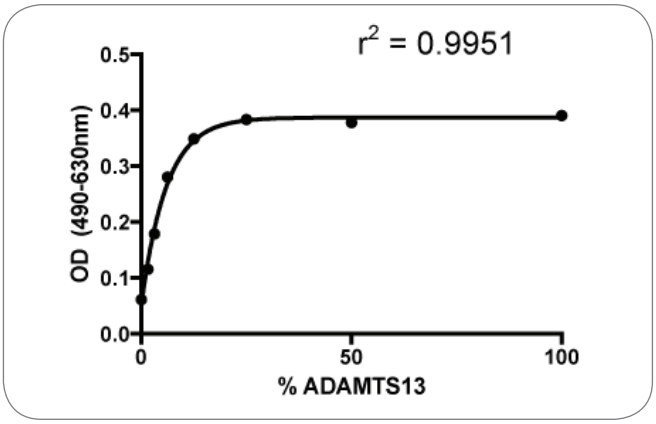

Figure 1a. Standard curve of our ADAMTS13 antigen assay. The first International Standard for ADAMTS13 from the NIBSC was used as a standard for ADAMTS13 concentrations ranging from 0% to 100% on the X-axis. Each point represents the mean of duplicate reading for a single representative experimental data set.

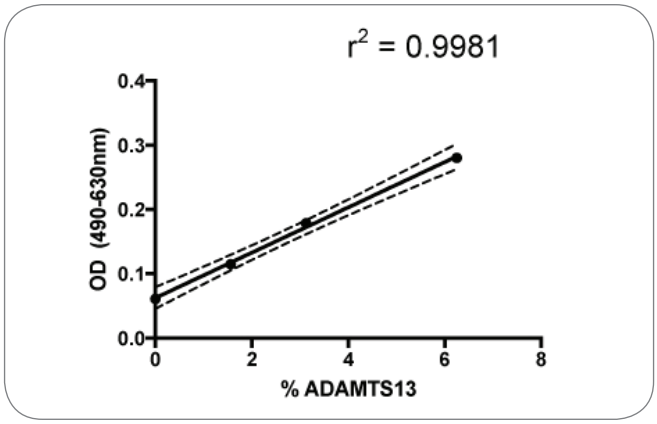

Figure 1b. Dilutional linearity fit of the assay with 95% confidence intervals. Dilutions range from 1.56% to 6.25% ADAMTS13.

Table 1. Acceptance criteria and performance characteristics of our ADAMTS13 antigen assay

VALIDATION PARAMETER	ACCEPTANCE CRITERIA	OBSERVED RESULTS		
		MEAN	SD	%CV
Intra-assay precision	CV ≤ 10 %	42.05	2.835	6.74
Inter-assay precision	CV ≤10 %	81.2	5.848	7.2
Limit of detection (LOD)		1.56% ADAMTS13		
Limit of quantification (LOQ)		1.56% ADAMTS13		
Dilution linearity • R2 • Slope • Y-Intercept • X-Intercept • range	0.99	**0.9981** 0,03523 ± 0,001089 0,0623 ± 0,003899 -1,769 1.56 – 6.25 % ADAMTS13		

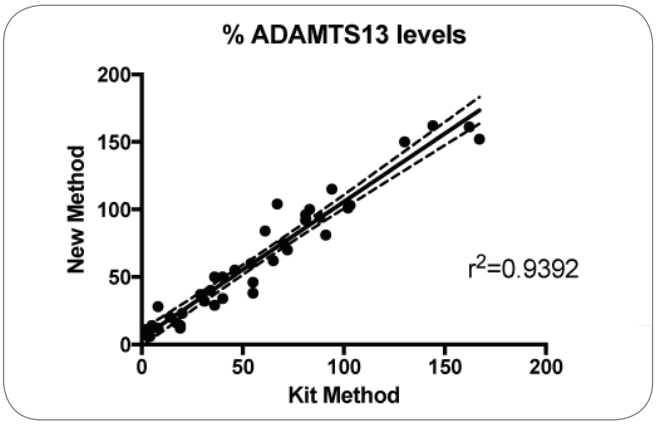

Figure 2a. Regression analysis with 95% confidence intervals comparing our method to that of the commercial assay using 40 patient samples.

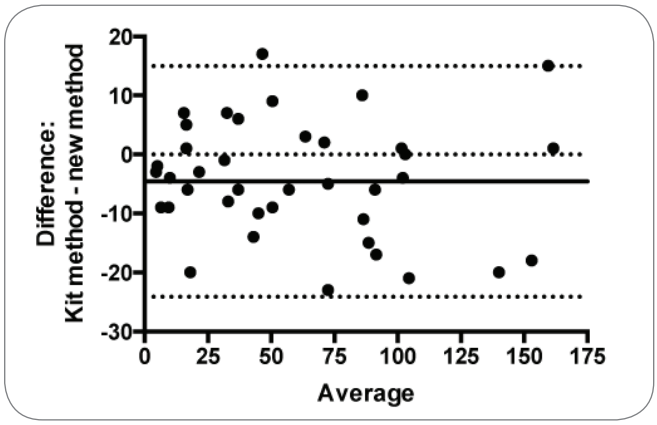

Figure 2b. Bland-Altman plot of the ADAMTS13 assay comparison. The mean relative bias is represented by a solid line, while the broken lines illustrate the 95% limits of agreement.

from 48% to 138% (93% ± 46%). The limit of detection and quantification was 1.56% ADAMTS13.

Figure 2a shows the Deming regression plot comparing our assay to the that of the commercial test kit. The two tests compared excellently with an r^2 of > 0.9 and a slope of almost 1. Figure 2b shows the Bland-Altman plot comparing the two methods. The bias was calculated as 4.575, while the 95% limits of agreement were between 24.12 and 14.97 difference.

DISCUSSION

Acute TTP is diagnosed if the plasma ADSAMTS13 level is found to be less than 10 percent.[7] However, it is difficult to distinguish between TTP and atypical haemolytic uraemic syndrome (aHUS) without an ADAMTS13 antigen assay. We have developed an in-house ELISA method to measure ADAMTS13 levels in plasma by using two different commercial antibodies which recognise epitopes on ADAMTS13 in a sandwich ELISA.

The purpose of the evaluation studies was to ensure that analytical methods can detect the corresponding analyte and and in addition provide repetitive and accurate results.[8] No other assays to date, have been validated against a commercial assay in current literature. In this assay, we measured the Intra and inter-assay precision, as well as the limit of detection and limit of quantification of our assay. The intra and inter-assay CV of the commercial kit method was found to be 5.2% and 7.2% respectively, which correlated well with the corresponding values of 6.7% and 7.2% of our assay. For viability It has been previously recommended that the CV's must be less than 10%.[5] Both the commercial assay and our assay performed well within these limits.

Regression analysis showed that both assays were linear when measuring ADAMTS13 in different dilutions of human plasma. The two assays were comparable in the measurement of ADAMTS13 in the plasma of patients with possible HIV-associated TTP.

The range of detection reported by the manufacturers of the commercial assay is from 2% up to 150% ADAMTS13. Our test's detection range was up to 100%. However, our detection range is appropriate for patients with possible HIV-associated TTP patients who are suspected to have very low levels of ADAMTS13.[6]

The lower limit of detection of our assay was 1.56%. This correlates well with that of the commercial test of 2%.

Compared to the cost of the commercial kit, our method was 90% less than the commercial kit. The commercial kit costs R500 per test minus the labour costs. If the labour costs were to be included, the assay costs would exceed that of the current medical aid rates. In comparison our assay costs R50 per test

minus the labour costs. Labour costs were designated to be the same for both assays.

Despite a good correlation, the Bland-Altman test showed a mean bias of 4.575. The Deming regression analysis of the two assays showed a slope of 1.009 ± 0.04166 in which the 95% confidence intervals included the slope of 1.0, This is well within the acceptable limits.

CONCLUSION

Our assay showed excellent inter- and intra-assay precision that can detect different ADAMTS13 levels in plasma up to 100%. We are therefore confident that our assay can successfully be used to diagnose patients with HIV-associated TTP since the results compare favourably to those of a commercial available kit.

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