

Original Article

COMPARATIVE ANALYSIS OF CHROMOGENIC VS CLOT BASED CDC MODIFIED, NIJMEGEN-BETHESDA ASSAY FOR DETECTION OF FACTOR VIII INHIBITOR TITRE

Mirza Asif Baig¹, Mohammad Shahid Iqbal², Abdel Rahim Mahmoud Muddathir³,
Aisha Tabassum², Syeda Sarah M¹

¹Department of Pathology, Shri B.M. Patil Medical College, Hospital & Research Centre, Bijapur, Karnataka, India

²Department of Pathology, College of Applied Medical Sciences, Umm Al Qura University, Makkah, Saudi Arabia

³Department of Medical Laboratory Technology, College of Applied Medical Sciences, Madinah, Saudi Arabia

ABSTRACT

Background: Inhibitors to infused factor VIII are the most significant complication of hemophilia treatment. These inhibitors are usually IgG antibodies, that react with FVIII in a time and temperature dependent manner. Coagulation factor VIII inhibitors can be detected by Chromogenic, clot based and immunological assays. However, there is lack of consensus as to what constitutes a positive inhibitor, including the appropriate cut-off for inhibitor measurement. The main objective of this study is to compare the sensitivity and specificity of chromogenic Nijmegen Bethesda assay (CNBA) with Centre for disease control modified Nijmegen Bethesda (CDC-NBA) assay against the Reference control method (RCM).

Materials and Methods: The Coagulometer used for inhibitor titre quantification is Sysmex CS-5100. APTT reagent used is Pathromtin SL supplied by Siemens. All data were expressed as Mean \pm SD. Statistical formulae were used for sensitivity and specificity calculations. Unpaired student's t test was used wherever necessary and a P value of <0.05 is considered as statistical significance.

Results: A total of 150 cases were tested for inhibitor titre using CNBA vs CDC-NBA. For low titre inhibitor (<2 NBU), CNBA has 92% and 86% and CDC-NBA has 80 and 60% sensitivity and specificity respectively. These results show that CDC-NBA shows false positive results at low inhibitor titre. For High titre inhibitor (>2 NBU) CNBA has 88% and 80% and CDC-NBA has 85 and 70% sensitivity and specificity respectively.

Conclusion: These results show that CNBA is more sensitive and specific than CDC-NBA at both low and high inhibitor titre. Moreover, chromogenic assays can differentiate factor specific inhibitor from nonspecific inhibitors like lupus anticoagulant and unfractionated heparin therapy.

Keywords: Hemophilia, Bethesda assay, ELISA, Factor VIII, Inhibitor, Mixing study

Abbreviations: APLA- Antiphospholipid antibody syndrome, CDC:NBA- Centers for Disease Control and Prevention - Nijmegen-Bethesda Assay, CNBA:- chromogenic Nijmegen Bethesda assay

NPP- Normal pooled plasma, **LAC-** Lupus anticoagulant

Niger.Jmed 2019: 423-430
© 2019. Nigerian Journal of Medicine

INTRODUCTION

Acquired coagulation inhibitors are antibodies which target specific clotting factors like factor VIII or phospholipids (lupus anticoagulant). Other reported factor specific coagulation inhibitors include anti factor II, V, VII, IX, X, XI, XII, XIII.^{1,2}

Inhibitors to infused factor VIII are the most significant complication of hemophilia treatment. FVIII inhibitors are antibodies, usually IgG, that react with FVIII in a time and temperature dependent manner.² International proficiency studies on FVIII inhibitor testing revealed coefficients of variation as high as 50% between laboratories.²

Correspondence to: Mohammad Shahid Iqbal
Department of Pathology, College of Applied Medical Sciences,
Umm Al Qura University, Makkah, Saudi Arabia
E-mail: drshahidpath@gmail.com

In 1975, a group of investigators meeting in Bethesda, Maryland, standardized the

inhibitor test used in the United States, defining one Bethesda unit (BU) as the amount of inhibitor in 1mL of patient plasma that would destroy 50% of the FVIII activity of an equal amount of NPP in 2 hours at 37°C. This was quantitated using a 1-stage clotting assay to measure FVIII activity in a mixture of patient plasma plus NPP and a control mixture of imidazole buffer plus NPP. A graph of log % RA vs dilution was provided for BU calculation. This "Bethesda assay" (BA) was gradually adopted internationally^{4,5}

Coagulation factor VIII inhibitors can be detected by One Stage - Clot based Assays (Bethesda / Nijmegen Bethesda Assay), Chromogenic assays and Immunological assays (ELISA, Immunofluorescence assay)

Quantification of FVIII inhibitors is not standardized and there is inter laboratory variation in methodology used, like processing of patient plasma, type of reagents used, diluent, factor VIII deficient plasma and the residual Factor VIII activity measurement at the end point.^{4,5}

Challenges remain in the areas of quality control, assay standardization, monitoring of patients undergoing immune tolerance induction therapy and testing in the presence of modified and novel treatment products.

There is lack of consensus as to what constitutes a positive inhibitor, including the appropriate cutoff for inhibitor measurement using the Bethesda assay (BA) or Nijmegen-Bethesda assay (NBA) and the means for evaluation of clinical significance.

These reasons have prompted us to undertake this study with the main objective of comparing the sensitivity of CNBA with CDC modified- NBA against

Reference control method (RCM)

Materials and Methods

This is a proretrospective study done over a period study of 1 year duration (July 2018 – June 2019), carried out in a specialized tertiary care, referral Hemophilia centre & medical college India.

The coagulometer used for inhibitor study is Sysmex CS-5100, APTT reagents used are Pathromtin SL supplied by siemens. A total of 150 cases of specific & nonspecific inhibitors were studied

Inclusion Criteria

Factor VIII Specific inhibitors and Lupus Anticoagulant (LAC) cases.

Exclusion Criteria

Patients on Heparin, Dabigatran, Rivaroxaban, Warfarin were excluded

Reagents Preparation & cutoff values

In this study CDC- Nijmegen Bethesda assay (CDC-NBA) was used which is a modification of Bethesda assay in terms of Buffering of the NPP with imidazole. Use of factor VIII-deficient plasma (FVIIDP) or 4% Bovine serum albumin providing sensitivity of 97% and specificity of 99% (CDC approved as FVIII DP is costly)

Preanalytical heat treatment of patient plasma at 56°C for 30 minutes, as it removes factors which allows accurate testing of patients recently treated or on prophylaxis or ITI with conventional FVIII or FIX as advocated by CDC^{6,7}

The chromogenic Bethesda assay (CBA) is identical to the CDC-NBA except for use of a chromogenic factor assay to measure the FVIII endpoint was used.

A positive inhibitor by NBA was defined as = 0.5 NBU, based on previous assay

validation. To detect non-time-dependent inhibition, the NBA was performed

immediately upon mixing and compared with that after 2 hour incubation.

Interpretation of Mixing tests is done by Rosners index or Chang's% correction

Inhibitor.	1:1 mix PTT - PNP PTT x 100. Cut offs	1	0	Correction &	1	5	=
	patient PT						

Chang's % correction = $\frac{\text{APTT patient plasma} - 1:1 \text{ Mix aPTT} \times 100}{\text{PNCP}}$; (<58% is inhibitor & >70% is correction APTT patient plasma – PNCP)

Procedure of Chromogenic NBA and clot based CDC-NBA summarized ^{4,5,6}

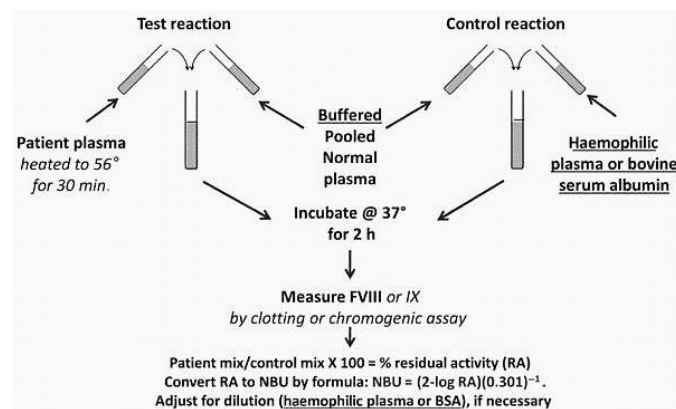


Table 1):-

A manual calculation based on Residual factor VIII activity & semilog paper is the preferred one.

Or formula can used as follows:

$$\%RA = (\text{factor level of patient} + \text{NPP mix} / \text{factor level of diluent} + \text{NPP mix}) \times 100$$

$$NBU = (2 - \log \%RA) / 0.301.52$$

If testing of the undiluted patient plasma gives 25% -100% RA, then the original result is used.

If it produces a result of <25% RA (>2.0 NBU), dilution of the patient plasma is required to achieve a %RA between 25% and 75% for accurate quantitation. NBU is then multiplied by the dilution factor to calculate a final NBU for reporting. ^{1,2}

Table2: LowtitreCDC-NBA

TruePositive=8	0	Falsepositive=2	0
FalseNegative=20		TrueNegative=3	0
100		5	0

Results

A total of 150 cases were tested for inhibitor titre using CNBA vs CDC-NBA.

Out of 150 cases, 100 were positive for FVIII inhibitor which was confirmed by Composite reference method which includes either Chromogenic assay: Clot based NBA: ELISA either singly or in combination. These 100 FVIII inhibitor cases were categorized into two

- 1) — Low inhibitor titre (< 2NBU) and
- 2) — High inhibitor titre (> 2NBU).

50 cases of DRVVT positive LAC were chosen as control

Table 3: Low titre CNBA vs CDC-NBA

Low titre (<2 NBU)	Sensitivity%	Specificity %
CNBA	92	86
CDC-NBA	80	60

Table 4: Low titre CDC-NBA

True Positive = 80	False positive= 20
False Negative = 20	True Negative= 30
100	50

Table 5: Low titre CNBA

True Positive = 92	False positive= 07
False Negative = 08	True Negative= 43
100	50

For low titre Inhibitor (<2 NBU) , CNBA has 92% and 86% while CDC-NBA has 80 and 60% sensitivity and specificity respectively. These results show that CDC-NBA shows false positive results at low inhibitor titre (Table 3,4,5)

Table 6:- High titre CNBA vs CDC-NBA

Low titre (>2 NBU)	Sensitivity (%)	Specificity (%)
CNBA	88	80
CDC-NBA	85	70

Table 7:- High titre CDC-NBA

True Positive = 85	False positive= 15
False Negative = 15	True Negative= 35
100	50

Table 8:- High titre CNBA

True Positive = 88	False positive= 10
False Negative = 12	True Negative= 40
100	50

For High titre Inhibitor (>2 NBU) CNBA has 88% and 80% while CDC-NBA has 85 and 70 % sensitivity and specificity respectively. These results show that CNBA is more sensitive and specific than CDC-NBA at both low and high inhibitor titre (Table 6,7,8)

Discussion

Inhibitors to infused factor VIII are the most significant complication of hemophilia treatment today. FVIII inhibitors are antibodies, usually IgG, that react with FVIII in a time and temperature dependent manner.

Patients with factor VIII inhibitors are at increased risk of life threatening bleeding and factor VIII infusion is usually not sufficient alone and the management of patient depends on inhibitor levels. So exact inhibitor titre is required to give FEIBA or rFVIII.⁸

Results of our previous study on 33 cases of inhibitor titre quantification showed NBA is better than Bethesda as the later technique shows false positive results at low inhibitor titre⁹

Preliminary study, by Verbruggen and colleagues suggested a reduction in spuriously positive assay results from buffering the normal pool plasma substrate with imidazole to pH 7.4 and substituting Factor VIII deficient plasma for imidazole buffer in the control incubation mix. These laboratory findings have now been confirmed by the performance of both the standard and the modified Bethesda assays in parallel on 877 patient samples screened during the Factor VIII:C Inhibitor Surveillance Program instituted following the conversion of all Canadian haemophilia A patients to recombinant Factor VIII and these results were later endorsed by ISTH.^{10,11}

Why NBA is better than Bethesda assay?

The reagents used in Nijgemens Bethesda Assay are 0.1M imidazole buffer at pH7.4 and FVIII depleted plasma in the control mixture. At low inhibitor titres (<1 Bu), the classical Bethesda assay can result in false positives whereas the NBA would give zero

levels of inhibition. This is due to FVIII instability by pH shift and reduced protein concentration resulting from dilution.^{3,4,5}

In a study conducted by Bert V¹¹ on 32 haemophiliacs⁹, which were free of inhibitors yielded values of 0.0 BU/ml by Nijgemens method whereas classical Bethesda showed activity between 0.5 to 0.9 BU/ml.

In study conducted by Vverbruggen, undiluted samples at low inhibitor titre can detect inhibitor of the level of 1.5 to 1.9 BU mL, whereas imidazole buffer diluted samples (dilution factor 2-5) giving a mean inhibitor activity of 4.7 BU mL.¹²

Majority of studies concluded that laboratories should atleast use buffered NPP as substrate and FVIII-deficient plasma as the reference sample. Bethesda Assay (1975 by Kkasper). Nijmegen Assay (1995, Gold standard technique)NASCOLA¹³(2012):- 70% lab follows Hybrid assays, 20% Nijmegen & 10% Bethesda.

Clot based inhibitor assays like CDC- NBA tests using endpoint of clot formation have certain limitations. The formation of a fibrin clot relies on lupus anticoagulant and heparin therapy etc. It is often difficult to distinguish this inhibition of clotting from a true factor inhibitor. Because tests for FVIII using chromogenic substrates have a more specific endpoint, they can be measured accurately, so their use in inhibitor testing was proposed by Blanco et al.

Chromogenic factor assays also have the advantage of increased precision. The chromogenic Bethesda assay (CBA) is identical to the CDC-NBA except for use of a chromogenic factor assay to measure the FVIII endpoint, has been described.

A study on Inhibitor detection by CH Miller¹² on 1005 patients were tested with both the CDC-NBA and the CBA, 0.3% of 883 NBA-negative specimens, 54% of 80 positive specimens with 0.5–1.9 Nijmegen-Bethesda units (NBU) and 100% of 42 specimens with =2.0 NBU were also positive with the CBA.

The results of the present study shows that for low titre Inhibitor (<2 NBU), CNBA has 92% & 86% & CDC-NBA has 80 & 60%

sensitivity & specificity respectively. These results show that CDC-NBA shows false positive results at low inhibitor titre.

For Hightitre Inhibitor (>2 NBU) CNBA has 88% & 80% & CDC-NBA has 85 & 70 % sensitivity & specificity respectively..

These results confirm that CNBA is more sensitive and specific than CDC-NBA at both low and high inhibitor titre.

Based on our results we propose an ALGORITHM protocol for FVIII inhibitor detection

Step 1	<p>If aPTT is increased (exclude factor VIII def., APLA, VWD, Heparin & refer check points)</p> <p>MIXING STUDY test Equal volume of Test plasma + SHP (with & without incubation)</p> <p>Interpretation of Mixing test Rosner index (15) or Changs % correction (58%) =Indicates Inhibitor is present</p> <p>Note :- IF both RI is <10 & % Chang is > 70% - No inhibitor present – No need of further Tests If Either RI or % chang indicates inhibitor, then proceed to further test If RI is between 11 - 14 or % Chang 58 -69 then proceed if strong clinical indication</p>
Step 2	<p>Prepare 4 -7 tubes- depeing on inhibitor titer & label (1:2, 1:4, 1:8, 1:16, 1:32) Add 500 µl of imidazole buffer to all tubes Add 500 µl patient plasma to first tube, mix well Transfer 500 µl of the mixture to 2nd tube mix & serially dilute (discard 500 µl from last TT Add 500µl of Buffered SHP to all TT</p> <p>Incubate at 37c for 2 hours - Determine factor VIII level</p>
Step 3	<p>Residual factor VIII activity = $\frac{\text{Patient factor VIII}}{\text{SHP factor VIII}} \times 100$</p>
Step 4	<p>Calculate the Inhibitor titre from semi log graph paper (log Y axis & linear X axis) Or Follow the Formula</p>

Important Conclusions drawn from this study

Nijmegen Bethesda assay is better than Classical Bethesda assay as the later gives false positive results at low inhibitor titre because of FVIII instability due to PH shift & protein loss. Secondly.

CDC-NBA uses an additional pre analytical heat treatment step which removes residual FVIII in treated patients. Finally, **These results show that CNBA is more sensitive and specific than CDC-NBA at**

both low and high inhibitor titre. Moreover, chromogenic assays can differentiate factor specific inhibitor from nonspecific inhibitors like LAC & UFH.

It can be safely concluded that chromogenic and immunologic assays, offer extra advantage as compared to Clot based assays, in identification of false positive results and in neutralizing the confounding effects of lupus anticoagulant and heparin contamination

Table- 1) CDC - Nijmegen-Bethesda assay procedure

<ol style="list-style-type: none">1) Use 7 tubes labelled as (1:2, 1:4, 1:8, 1:16, 1:32, 1:64 & 1:128) { If expected Inhibitor titre > 800 BU/ml, then use dilution upto 1: 256 & 512} { If inhibitor titre is low (<5 BU/ml, then use dilution upto 1:8}2) Add 500 µl of imidazole buffer to all tubes3) Add 500 µl patient plasma to first tube, mix well4) Transfer 500 µl of the mixture to second tube mix well & then to third tube & continue the same procedure till the last tube & discard 500µl from last tube5) Add 500µl of Buffered SHP to all the test tubes (SHP contains 100 IU/dl (1 IU/ml or 100%) factor VIII). 6) Prepare Control = 500µl of Buffered SHP + 500µl Factor VIII deficient plasma (So control have Factor VIII = 50%) 7) Cover the test tubes with cap or paraffin & incubate at 37°C for 2 hours8) After incubation, the residual factor VIII is assayed using a standard 1-stage APTT9) Residual factor VIII activity = $\frac{\text{Patient factor VIII}}{\text{SHP factor VIII}} \times 100$10) The inhibitor concentration is calculated from a graph of residual factor VIII activity versus inhibitor units. The dilution of test plasma that gives a residual factor VIII nearest to 50% but within the range 30 -60% is chosen for calculation of the inhibitor. Any residual factor VIII <25% or >75% should NOT be used for the calculation of inhibitor level.11) If the residual factor VIII activity is between 80-100% (IU/dL) or 0.8-1.0 IU/mL the sample does not contain an inhibitor12) Derive the inhibitor titre from the graph and multiply by the dilution to give the final titre.13) Remember when plotting the residual FVIII against the BU titre – the Y axis is a log scale and the X axis is linear. (Semilog paper - log Y axis & linear X axis)^{1,2,3,6,7}
--

Table 2) - The Chromogenic Nijmegenbethesda Assay (C-NBA)³

<p>Step 1 - produce FXa (incubation step) Step 2 – measure Fxa via clotting time</p> <p>Measurement of FXa is via a chromogenic substrate where the amount of FXa is proportional to the colour intensity produced which is proportional to the amount of FVIII present</p> <p>The cryocheck FVIII Inhibitor Kit used to perform the assay consists of a standardized set of reagents:</p> <p>Performing the FVIII inhibitor assay with a cryocheck FVIII Inhibitor Kit consists of five steps,</p> <ol style="list-style-type: none">1) Heat Inactivation: The patient plasma and controls are incubated in a 56 °C water bath for 30 minutes to minimize residual FVIII activity in the samples.2) Centrifugation: The inactivated plasma is spun at 2700 × g for 5 minutes to remove precipitate from the solution.3) Dilution: Serial twofold dilutions of the supernatant are made with IB-BSA.4) Mixing: The neat plasma and dilutions are mixed with an equal volume of IB -PNP. The resulting mixes are incubated at 37 °C for two hours in a water bath. After two hours, the reaction is halted by placing the samples in an ice bath for 10 minutes.5) Measuring: The FVIII activity of the dilutions are measured, and the inhibitor titer is calculated relative to a control mix containing equal volumes of IB-PNP and IB-BSA <p>All the end points of CNBA & Clot based CDC - NBA were measured in Sysmex CS-5100 & reagents were supplied by Siemens</p>

References

- 1) Laboratory testing for factor VIII and IX inhibitors in haemophilia: A review. Miller C.H. Division of Blood Disorders, National Center on Birth Defects and Developmental Disabilities, Centers for Disease Control and Prevention, Atlanta, GA, USA. *Haemophilia*. 2018 March; 24(2): 186-197. doi:10.1111/hae.13424.
- 2) Connie H. Miller,¹ Anne S. Rice Comparison of Clot-based, Chromogenic, and Fluorescence Assays for Measurement of Factor VIII Inhibitors in the U.S. Hemophilia Inhibitor Research Study. *J ThrombHaemost*. 2013 Jul; 11(7):1300-1309.
- 3) Colin D. Douglas, PhD, Tara Quinton. A Standardized Kit for a Chromogenic Modified Nijmegen-Bethesda Assay. Repeatability, Reproducibility, and Analytical Sensitivity, Presented at 60th ASH Annual Meeting, December 1-4, 2018 San Diego, California, USA. Accessed Online
- 4) Miller CH, Platt SJ, Rice AS, Kelly F, Soucie JM. the Hemophilia Inhibitor Research Study I. Validation of Nijmegen-Bethesda assay modifications to allow inhibitor measurement during replacement therapy and facilitate inhibitor surveillance. *J ThrombHaemost*. 2012; 10:1055-1061. [PubMed: 22435927]
- 5) Kasper C, Aledort L, Eyes J. A more uniform measurement of factor VIII-inhibitors. *ThrombDiathHaemorrh* 1975;34:869-872.
- 6) Verbruggen B, Novakova I. The Nijmegen modification of the Bethesda assay for factor VIII:C inhibitors: improved specificity and reliability. *ThrombHaemost* 1995;73:247-251
- 7) Laffan M, Manning R. Investigation of haemostasis. In: Lewis SM, Bain BJ, Bates I, editors. *Dacie and Lewis Practical Haematology*. 11th ed. London: Churchill Livingstone Elsevier 2012. p.423.
- 8) Verbruggen B, Dardikh M, Polenewen R, van Duren C, Meijer P. The factor VIII inhibitor assays can be standardized: results of a workshop. *J ThrombHaemost* 2011; 9:2003-008.
- 9) Baig MA, Sirasagi A. Comparative evaluation of Rosner's index (ICA) Vs Chang's (% correction) as a screening test (mixing study) *IP Journal of Diagnostic Pathology and Oncology*, July-September, 2018;3(3):196-201
- 10) Giles AR, Verbruggen B, Rivard GE, Teitel J, Walker I; the Association of Hemophilia Center Directors of Canada. A detailed comparison of the performance of the standard vs. The Nijmegen modification of the Bethesda assay in detecting factor VIII:C inhibitor in the haemophilia a population of Canada. *ThrombHaemost* 1998;79:872-875.
- 11) Meijer P, Verbruggen B. The between-laboratory variation of factor VIII inhibitor testing: the experience of the external quality assessment program of the ECAT foundation. *SeminThrombHemost* 2009;35:786-793.
- 12) Regnault V, Stoltz JF. Quantitation of factor VIII antibodies by an enzyme-linked immunoassay method. *Blood*. 1994;83:1155-1156.
- 13) Peerschke EIB, Castellone DD, NASCOLA Proficiency Testing Committee. Laboratory assessment of factor VIII inhibitor titer: the North American Specialized Coagulation Laboratory Association experience.