Original Article

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

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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 isPathromtin SL supplied by seimensSeimens. All data were expressed as Mean ± SD. Statistical formulae were used for sensitivity and specificity calculations. Unpaired students t test was used whereever 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 shows 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

Abbrevations: 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

NigerJmed 2019: 423-430
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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]

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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]

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 pont. [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 prosretrospective 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) wais used which is a modification of Bethesda assay in terms of Buffering of the NPP with imidazole. Use of factor VIII-deficient plasma (FVIIIDP) 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^{0}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 ≤ 10 Correction & ≥ 15 =**
patient PT

Chang's % correction = APTT patient plasma - 1:1 Mix aPTT X 100; (<58% is inhibitor&>70% is correction APTT patient plasma – PNCP

Procedure of Chromogenic NBA and clot based CDC-NBA summarized [4,5,6]

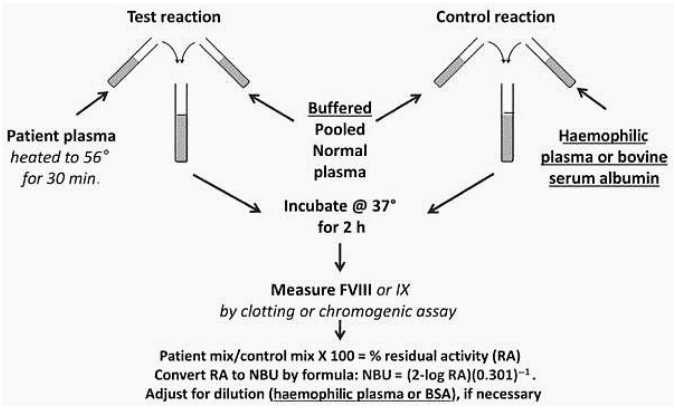

Table 1):-

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

Or formula can used as follows:

%RA = (factor level of patient + NPP mix/factor level of diluent + NPP mix) × 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 whichiwas 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 titre[9]

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 VIIIand 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 FVIIIdepleted 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 V[11] on 32 haemophiliacs[9], 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 labsoratories 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)NASCOLA[13](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 Miller[1,2] 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	If aPTT is increased (exclude factor VIII def., APLA, VWD, Heparin & refer check points) | MIXING STUDY test Equal volume of Test plasma + SHP (with & without incubation) | Interpretation of Mixing test Rosner index (≥ 15)or Changs % correction (≤ 58%) =Indicates Inhibitor is present 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
Step 2	Prepare 4 -7 tubes- depening on inhibiter 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 2 nd tube mix & serially dilute (discard 500 µl from last TT Add 500µl of Buffered SHP to all TT Incubate at 37c for 2 hours - Determine factor VIII level
Step 3	Residual factor VIII activity = Patient factor VIII / SHP factor VIII X 100
Step 4	Calculate the Inhibitor titre from semi log graph paper (log Y axis & linear X axis) Or Follow the Formula

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

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 tubes

3) Add 500 µl patient plasma to first tube, mix well

4) 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 tube

5) 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 = 500µlof Buffered SHP + 500µl Factor VIII deficient plasma
(So control have Factor VIII = 50%)

7) Cover the test tubes with cap or paraffin &incubate at 37^0C for 2 hours

8) After incubation, the residual factor VIII is assayed using a standard 1-stage APTT

9) 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 inhibitorunits. The dilution of test plasma that gives a residual factor VIII nearest to 50% but within the range30 -60% is chosen for calculation of the inhibitor. Any residual factor VIII <25% or >75% should NOT be used forthe calculation of inhibitor level.

11) If the residual factor VIII activity is betw een 80-100% (IU/dL) or 0.8 -1.0 IU/mL the sample does notcontain an inhibitor

12) 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 Nijgemenbethesda Assay (C-NBA)[3]

Step 1 - produce FXa (incubation step)
Step 2 – measure Fxa via clotting time

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

The cryocheckFVIII Inhibitor Kit used to perform the assay consists of a standardized set of reagents:

Performing the FVIII inhibitor assay with a cryocheck FVIII Inhibitor Kit consists of five steps,

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

All the end points of CNBA & Clot based CDC- NBA were measured in Sysmex CS-5100 & reagents were supplied by Siemens

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