International Journal for Multidisciplinary Research (IJFMR)
E-ISSN: 2582-2160 ● Website: www.ijfmr.com ● Email: editor@ijfmr.com

Inhibitor Screening and Quantification of Inhibitor Titre by Bethesda Inhibitor Assay in Patients with Coagulation Disorders

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Abstract:
Inhibitors can develop in patients with inherited coagulation disorders or as autoantibodies in individuals without prior conditions. This study, conducted at a tertiary care center in Southern India from January to December 2017, assessed the prevalence and levels of inhibitors in patients with coagulation disorders. The study included 40 participants: 36 with known cases of hemophilia A (33) or hemophilia B (3), three newly diagnosed hemophilia cases, and one case of acquired hemophilia. Inhibitor screening identified five positive cases of hemophilia A and one case of acquired hemophilia. Among the positive cases, five exhibited late-acting inhibitors, and one showed both immediate and late-acting inhibitors; lupus anticoagulant tests in these cases were negative. The prevalence of inhibitors among inherited hemophilia patients was 12.5%. The Bethesda assay was used to quantify inhibitor titers in positive cases, with results ranging from 12 BU to over 1024 BU. By standardizing the Bethesda assay, precise titer measurements were obtained, aiding in the clinical management of these patients.

Introduction

The most common coagulation disorders are hemophilias (inherited or acquired). Hemophiliacs develop bleeding episodes, particularly in severe cases, and are treated with missing factor replacement therapy i.e. factor VIII or factor IX concentrates (1). Inhibitors are acquired de-novo or develop in inherited hemophilias. Acquired inhibitors are associated with an autoimmune disease; it affects mainly elderly people, who present with hemorrhage in the skin, muscle, and mucosal membrane. On the other hand, inhibitors developing in inherited factor deficiencies occur early after the beginning of therapy (within <30 exposure days) and are most frequent in young hemophiliacs. Patients with inhibitors manifest excessive bleeding in unusual parts of the body and poor factor therapy recovery (2). The inhibitors are IgG antibodies that are against specific deficient factors.

The development of inhibitors is a complication of hemophilia because it occurs shortly after replacement therapy (3). Inhibitors are more common in hemophilia A than in hemophilia B but the principles are the same for both. Since inhibitors are life-threatening causes in hemophiliacs they should be screened for inhibitors in order to prevent serious bleeding and complications (3). The inhibitor screening is usually based on a-PTT done on patient plasma mixed with control plasma. The mixing study prolongs the a-PTT in a mixture of a patient and normal pooled plasma after incubation at 37ºC in the presence of inhibitors however FIX and nonspecific inhibitors are immediate acting, prolonged

immediately before incubation whereas FVIII inhibitors are time-dependent, prolonged after incubation at 37ºC for 120 minutes(4). The quantification of inhibitors is done by Bethesda assay. One Bethesda Unit (BU) is defined as the amount of an inhibitor that will neutralize 50% of 1 unit of FVIII: C in normal plasma after 120 minutes of incubation at 37°C (4). All people with hemophilia who use clotting factor concentrates and other bypassing agents must be tested for inhibitors at least once a year to prevent life-threatening complications (5). Inhibitor titers help to assess whether treatment is working or not working (5)

Materials and Methods

Study setting: The present study was a Cross sectional descriptive study which was conducted over a period of one year from January 2017 to December 2017 in Department of Pathology (Hematology section), Jawaharlal Institute of Postgraduate Medical Education and Research (JIPMER).

Study participants: Human

Sample size: 40

Initially, 60 samples with prolonged PT &/ APTT were scrutinized. This includes new cases as well as cases on follow up with suspected inhibitors. Patients on anticoagulants and those suspected to have lupus anticoagulant were excluded at the outset. Most of the samples had a ½ patient + ½ control mixing assay. Of these 20 samples were corrected with factor deficient plasma and were having factor deficiency which was excluded. Finally 40 samples were put up for inhibitor screening because they were either not corrected or partially corrected on mixing assay or they were clinically suspected to have inhibitors. All cases positive in the inhibitor screen were put up for Bethesda assay.

Bethesda Assay

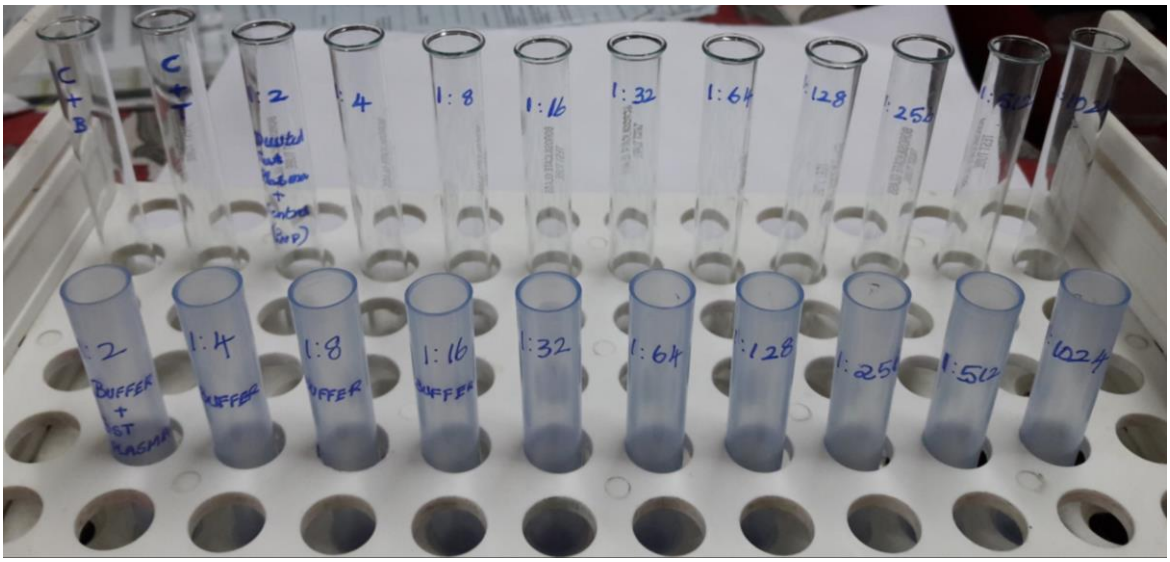
The tubes were taken as follows:

Tube 1: 150µl control (PNP) + 150µl of buffer (Owren-koller)

Tube 2: 150µl of control plasma (PNP) + 150µl of test plasma

Tube 3 to 12: 150µl of control plasma (PNP) in all the tubes + 150µl of respective diluted test plasma from 1:2 to 1:1024.

Figure 1: From tube 1 to 12



Incubated for 2 hour in water bath at 37ºC and performed a Factor VIII Assay on each incubation mixture by using 1/5 dilution

All the tests procedure were done as per the following lab Standard Operative Procedure but we used automated coagulation analyzer –STA Compact (Diagnostic STAGO), here all the test was based on the principle of manual method however instead of water bath a platform with peltier effect is placed, the result is based on viscosity a mechanical detection system enabling immediate delivery of accurate and precise results

Statistical Analysis

The Categorical data like gender, family history, clinical presentation, results of Inhibitor screening were expressed as frequency and percentages. Continuous data like age, values of a PTT and Bethesda Units will be expressed as mean with SD or median with range.

3. Results and Discussion

Study Setting

The study was over a period of 12 months from January 2017 to December 2017, in Hematology section, Department of Pathology at JIPMER, Puducherry.

Sample

Initially, 60 cases with prolonged PT &/ APTT excluding cases of lupus anticoagulant and those under anticoagulants like heparin and warfarin, were screened as per the flow chart given in methods section. Many of these underwent mixing assay and 20 were corrected. These were further mixed with factor deficient plasma and type of factor deficiency was characterized on factor assay. These cases were excluded from further analysis.

A total of 40 samples were put up for Inhibitor screening because they were either not corrected or partially corrected with normal pooled plasma or those cases clinically suspected to have inhibitors. A brief of clinical profile and detailed coagulation work up of these cases is presented.

Distribution of Cases

AGE: The age group of the patients ranged from 1 year to 57 years with a mean of 15 years.

GENDER: Out of 40 cases, 39 were males and 1 was female with acquired hemophilia.

DIAGNOSIS: 40 patients were enrolled for inhibitor screen, 36 were known cases of hemophilia (33 Hemophilia A and 3 Hemophilia B),1 case of acquired hemophilia, 3 were new cases of hemophilia

Clinical Presentation of Patients

The most common clinical presentation in known cases were due to recurrent bleeding, poor factor recovery despite factor replacement, swelling of joints and for routine follow up in cases with prophylactic factor replacement.

Table 1: Clinical presentation of patients

S.no.	Clinical Presentation	No. of cases
1	Follow-up cases (on prophylaxis)	23
2	Poor factor recovery despite factor replacement	3
3	Recurrent bleeding	2
4	Joints swelling	5
5	Hemarthrosis	2
6	Hematoma	3

7	Colicky abdomen pain with hematuria + clot in urine	1

One case diagnosed as acquired hemophilia, on further work-up had presented with clinical symptom of bleeds at multiple sites.

Lab Parameters

Inhibitor Screening

All the 40 cases were included for APTT based inhibitor screening. Reading were taken at 0, 1, 2 hrs of fresh mix & incubated mix as shown in table 2.

Table 2: Reading from inhibitor screening of a positive case.

Sample	'0' hour	'1' hour	'2' hour
Fresh mix	42.1"	44.3" ⇑	49.1" ⇑
Incubated mix	42.1" ⇐	54.5" ⇐	63.5"

One case diagnosed as acquired hemophilia, on further work-up had presented with clinical symptom of bleeds at multiple sites.

As given in the above table, the reading at 1 hr incubated mix is prolonged from 0 hr incubated mix by 12.4 sec and from 1 hr fresh mix by 10.2 sec. The reading at 2 hr incubated mix is prolonged from 1 hr incubated mix by 9 sec and from 2 hr fresh mix by 14.4 sec. As the prolongation seen is more than 8 sec, it is considered positive. Inhibitor screening was positive in 6 cases as given in the following table 3.

In our cases we found 5 late acting inhibitors which were time and temperature dependent and 1 was showing both late and immediate acting, additionally lupus testing was done and it was negative.

Table 3: Results of inhibitor screening in positive cases

S.No	**Correction at 0 hr**		**Prolongation at I hr from 0 hr (incubated mix)**	**Prolongation at 1 hr from 1hr (Fresh mix)**	**Prolongation at 2 hr from 1hr (incubated mix)**	**Prolongation at 2 hr from 2hr (Fresh mix)**	**Interpretation**
	<50% Of baseline APTT of patient	>50% Of baseline APTT of patient or within few sec of control					
1		✓	12.45 sec	10.25 sec	9 sec	14.4 sec	Late acting (at

							2 hr)
2		✓	28.6 sec	26 sec	12.1 sec	34.1 sec	Late acting (at 2 hr)
3$	✓		-1.9sec	3.2 sec	16.3 sec	5.8 sec	Both immediate & Late acting (at 2 hr)
4		✓	21.5 sec	19.9 sec	12 sec	26.6 sec	Late acting (at 2 hr)
5		✓	8.4 sec	9.6 sec	4 sec	5.8 sec	Late acting (at 1 hr)
6*		✓	8.3 sec	6.3 sec	4.3 sec	7.3 sec	Late acting (at 1 hr)

$ - Lupus anticoagulant screening done additionally was negative
*- Acquired hemophilia

Out of 6 screen positive cases, 5 were hemophilia A and 1 case was acquired hemophilia. The overall prevalence of inhibitors was 15% (6/40). The frequency of inhibitor positive cases among inherited hemophilias was 13.5% (5/40).

Bethesda Assay

Out of 6 positive cases in inhibitor screening test, Bethesda assay was performed in 5 cases according to the procedure mentioned in details in methods section and calculation were done as mentioned below. The 1 case of acquired hemophilia presented on follow up after 2 months when her APTT had normalized and inhibitor screening was negative hence Bethesda assay was not done.

Calculation:

- Choose the dilution of patient's plasma that yields residual factor VIII activity close to 50%.
- The residual factor VIII activity in each is determined using the Factor VIII activity of the control and the respective dilution of the patient's plasma.

The residual factor VIII activity (%) = $\frac{\text{Factor VIII activity (patient)} \times 100}{\text{Factor VIII activity (Control)}}$

The residual factor VIII activity is converted to Bethesda unit factor using the following chart (table 1).

Discussion

Inhibitors develop in both inherited and acquired hemophilia, the clinician manages these patients according to the inhibitor titers provided by the coagulation lab (11). The development of inhibitors (particularly high titer) is one of the most serious complications of patients under factor replacement therapy leading to poor factor recovery despite factor replacement. The most common complications seen are hemarthrosis, recurrent bleeding, swelling of joints, multiple bleeds, hematoma, and hematuria (6). In our study the inhibitors were found mostly among young patients with a mean age of 15, in other studies it was 13–21 years in Chicago USA(12), 11-15 years in Maharashtra India(13), 18 years in Uttar Pradesh India(14), 17.7 years in India(7). In our study, the prevalence of inhibitors was 15%. The frequency of inherited inhibitors was found to be 12.5%, in conjunction with other studies South India (Chennai)-20.99%, Hyderabad-13.33%, Davangere-7.41%, Bangalore- 7.02% (8).

Since hemophilia has X-linked recessive inheritance all cases of inhibitor in hemophilia were males. Only one case of acquired hemophilia was encountered which was a 27-year-old female (11). Factor inhibitors are time-dependent and the inhibitor will not be detected unless the test is repeated after incubation; hence they are called late-acting inhibitors. Nonspecific inhibitors like the lupus anticoagulant usually are not time-dependent; the immediate mixture will show prolongation, hence they are immediate-acting inhibitors. In this study, 40 cases were enrolled for inhibitor screening, to rule out the immediate and late-acting inhibitors. Six out of 40 cases were inhibitor screen positive, 5/6 cases showed late-acting inhibitors, and in 1/6 cases an unexpected finding was found to have Inhibitors of both immediate and late-acting types. Here lupus anticoagulant screening was negative. In these cases, the common complications were hematoma, and hematuria, hemarthrosis (11).

In the 1970s, the Bethesda assay was developed as the gold standard method for inhibitor determination. The Bethesda assay has become the principal tool for measuring factor inhibitor titers (9). So we tried to standardize this Bethesda assay in our lab. Bethesda assay was done in 5/6 cases. It was a one-stage assay using normal pooled plasma and buffer. In many studies, patient plasma was diluted with Imidazole buffer and then pooled normal plasma added and incubated at 37 º C in the water bath for 2 hrs. (10).

In our study, we used Owren-Koller buffer instead of Imidazole buffer. Bethesda assay was done for the quantification of inhibitor titers. The titer ranges between 12BU to >1024BUand in other studies it was 2.2BU-460.6 BU in the Northern part of India (7).

The chromogenic Bethesda assay (CBA) marks a major advancement in managing hemophilia A patients treated with Emicizumab. Its validation addresses a critical gap, as Emicizumab interferes with traditional clot-based assays like the Nijmegen-Bethesda assay, complicating the accurate detection of factor VIII inhibitors. Studies confirm that CBA offers a reliable method for quantifying inhibitors in this unique patient population (15). Traditional assays, such as the one-stage clotting assay (OSCA) and chromogenic substrate assay (CSA), often show inaccuracies due to structural modifications in extended half-life (EHL) factors or interference from therapies like Emicizumab (16). Variability between one-stage and chromogenic assays also affects accurate FVIII/FIX measurement, particularly with advanced therapies (17). To overcome these challenges, hemostasis laboratories must adapt and standardize their methods for reliable results across all treatment types, enabling effective patient management. Furthermore, the kinetic profiles of different FVIII monoclonal antibodies can lead to inaccuracies in inhibitor titer quantification with the modified Nijmegen-Bethesda assay. Using sigmoidal regression for titer calculations can enhance accuracy and support personalized care for hemophilia A patients (18).In our study, we adopted SOP from an established center and standardized the Bethesda assay in our setup. The titer value was helpful for the management and treatment of the patients.

Table 4: Chart to convert the residual factor VIII activity to Bethesda unit

RESIDUAL VIII %	FACTOR	RESIDUAL VIII %	FACTOR	RESIDUAL VIII %	FACTOR
97	0.05	61	0.7	40	1.35
93	0.1	59	0.75	38	1.4
90	0.15	57	0.8	37	1.45

87	0.2	55	0.85	35	1.5
84	0.25	53	0.9	34	1.55
81	0.3	51	0.95	33	1.6
78	0.35	50	1	32	1.65
75	0.4	48	1.05	30	1.7
73	0.45	46	1.1	29	1.75
70	0.5	45	1.15	28	1.8
68	0.55	43	1.2	27	1.85
66	0.6	42	1.25	26	1.9
64	0.65	41	1.3	25	2

Table 5 – 9 shows the positive cases Bethesda assay calculations

DILUTION	SEC	FVIII:C	RES. FVIII:C	BU FACTOR
CONT+ BUFFER	78	64		
CONT+ TEST	108	4	6.25	
1:2	99.9	8	12.5	
1:4	96.8	18	28.1	
1:8	89.2	30	46.8	
1:16	85.6	38	**59.3**	**0.75**
1:32	84.3	40	62.5	
1:64	83.6	42	65.6	
1:128	84.5	40	62.5	
1:256	84.5	40	62.5	
1:512				
1:1024				

Table 5: Bethesda assay calculation of case 1

The residual factor VIII activity (%)

Factor VIII activity (patient) X100

Factor VIII activity (Control)

CALCULATION:

0.75X16(Dil. Factor) = 12BU

Table 6: Bethesda assay calculation of case 2

DILUTION	SEC	FVIII:C	RES. FVIII:C	BU FACTOR
CONT+ BUFFER	54.2	49		
CONT+ TEST	100.5	1	2.04	

1:2	96.6	1	2.04	
1:4	96.3	1	2.04	
1:8	82.6	3	6.12	
1:16	79.7	5	10.2	
1:32	70.7	10	20.4	
1:64	63.0	20	40.8	
1:128	58.6	30	**61.2**	**0.7**
1:256	56.6	38	77.5	
1:512	55.5	42	85.7	
1:1024	55.8	41	83.6	

The residual factor VIII activity (%)
Factor VIII activity (patient) X100
Factor VIII activity (Control)

CALCULATION:
0.75X128(Dil. Factor) = 89.6BU

Table 7: Bethesda assay calculation of case 3

DILUTION	SEC	FVIII:C	RES. FVIII:C	BU FACTOR
CONT+ BUFFER	52.4	60		
CONT+ TEST	99.7	1	1.66	
1:2	84.6	3	5	
1:4	70.7	10	16.6	
1:8	61.3	23	38.3	
1:16	57.3	35	**58.3**	**0.75**
1:32	54.0	50	83.3	
1:64	53.4	54	90	
1:128	52.3	61	101.6	
1:256	53.2	55	91.6	
1:512	52.3	61		
1:1024	51.8	64	106.6	

The residual factor VIII activity (%)
Factor VIII activity (patient) X100
Factor VIII activity (Control)

CALCULATION:
0.75X16(Dil. Factor) = 12BU

Table 8: Bethesda assay calculation of case 4

DILUTION	SEC	FVIII:C	RES. FVIII:C	BU FACTOR
CONT+ BUFFER	52.8	57		
CONT+ TEST	107.1	1	1.75	
1:2	106.5	1	1.75	
1:4	106.9	1	1.75	
1:8	106.9	1	1.75	
1:16	106.9	1	1.75	
1:32	106.9	1	1.75	
1:64	106.6	1	1.75	
1:128	103.1	1	1.75	
1:256	89.3	2	3.50	
1:512	69.6	11	19.29	
1:1024	60.5	25	**43.89**	**1.2**

The residual factor VIII activity (%)
Factor VIII activity (patient) X100
Factor VIII activity (Control)

CALCULATION:
1.2 X1024(Dil. Factor) = 1228.8BU

Table 9: Bethesda assay calculation of case 5

DILUTION	SEC	FVIII:C	RES. FVIII:C	BU FACTOR
CONT+ BUFFER	52.5	59		
CONT+ TEST	106.4	1	1.69	
1:2	107.7	1	1.69	
1:4	106.7	1	1.69	
1:8	107.2	1	1.69	
1:16	107.8	1	1.69	
1:32	108.7	1	1.69	
1:64	106.7	1	1.69	
1:128	106.8	1	1.69	
1:256	101.2	1	1.69	
1:512	91.3	2	3.38	
1:1024	77.8	5	8.47	

The residual factor VIII activity (%)
Factor VIII activity (patient) X100
Factor VIII activity (Control)

RESULT
= >1024BU High

4. Conclusion

The frequency of inhibitor-positive cases among inherited hemophilias was 12.5%. By standardizing the Bethesda assay exact titers of inhibitor could be given which was helpful in further management of the hemophilia patients by the clinician.

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