

Comparative evaluation of Rosner's index (ICA) Vs Chang's (% correction) as a screening test (mixing study)

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Abstract

Introduction: It is difficult to interpretate mixing study results (both screening & confirmatory) in presence of LAC. The main objective of this study is to define cut off values for ICA & % Correction which will reduce the no. of false positive & negative cases & will help in proper categorization of factor deficiency & inhibitors. This study also briefs about preanalytical errors & their correction

Material and Methods: Rosner Index = $\frac{1:1 \text{ mix PTT} - \text{PNP PTT} \times 100}{\text{patient PTT}}$ × Cut offs ≤ 10 = Correction & ≥ 15 = Inhibitor

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

Latest Sysmex CS-5100 auto-analyser was used to determine the Cut-offs. DRVVT mixing test ratio (Rosners index/ ICA) 1.15, % correction = 10, DRVVT Normalised ratio (NR) = 1.05. p value < 0.05 was taken as statistically significant. RI with a cut off value of <10 is 92.5 % sensitive in diagnosing factor deficiency & a cut off value of >15 is 91.1% sensitive for inhibitor diagnosis & it could not categorise, 8% of total cases into factor deficiency /inhibitor.

Discussion: Rosners index (ICA) as a confirmatory test for LA is more sensitive than % correction & DRVVT NR. Chang's % correction with a cut off value of >70% is 85% sensitive in diagnosing factor deficiency & a cut off value of <58 is 82.2 % sensitive for inhibitor diagnosis & it could not categorise, 16.5% of total cases into factor deficiency /inhibitor. The Chang's formula showed sensitivity of 85% and specificity of 66%, while Rosner index showed sensitivity of 70% and specificity of 93%. The Chang's formula has better sensitivity, while the Rosner index has better specificity for detection of lupus inhibitor. Our data suggests that while neither index is perfect, the use of two indices together may help to standardize reporting of positive, negative and equivocal results.

Conclusion: It can be safely concluded that Rosner index is better than % correction, both as a screening test & confirmatory test, to differentiate factor deficiency from inhibitor. This study results are in agreement with CLSI guidelines & favours the sequential order screen-confirm- & then if required mixing study as in case of screen and confirm analysis is not clear-cut and/or when other causes of prolonged clotting times are known or suspected The draw back with the mixing studies is that weak LAC can be missed. Though in this study Nijmegen is better than Bethesda in terms of sensitivity & specificity as the later gives false positive results, other studies has to be taken into consideration which shows that both the Bethesda & Nijmegen technique have low specificity at higher inhibitor titre. If actual quantitation of high titre activity is required, then it is more reliable to estimate empirically plasma dilution that gives 50% inhibition.

Keywords: Lupus anticoagulant, Pooled normal plasma, Mixing study, Rosner's Index, Changs % correction.

Introduction

Coagulation inhibitors may be

1. Congenital or acquired
2. Factor specific (anti-factor VIII most common) or LA (against PL), the major difference is that the latter causes thrombosis & former one serious bleeding. Other reported factor-specific coagulation inhibitors include anti-factors II, V, VII, IX, X, XI, XII and XIII. Factor-specific antibodies may occur in association with post-partum periods, immunological problems, aging & are usually IgG Class.¹

1 Bethesda unit (Bu) is defined as the amount of inhibitor in a plasma sample, which will neutralise 50% of 1 unit of factor VIII: C in normal plasma after a two-hour incubation at 37°C. Around 30% of severe Hemophiliacs & 2-8% of mild to moderate cases develop inhibitors.²

Prerequisites^{3,4}

1. 3.2% Citrated Plasma : Blood (1:9)
2. HCT > 55% (adjust citrate)
3. Adequate sample (filled upto mark), Check for clots & hemolysis
4. Sample should be processed within 4 hours
5. Storage: At ≤ 20⁰ upto 2 weeks & for prolonged storage at -70⁰c
6. Centrifugation at for 15 min 3700 rpm for PPP (PLT count <10x10⁹/L
7. Normal range = <.4 BU

Materials and Methods

Preparing a 4M Imidazole Solution at a pH of 7.4:¹

Add crystalline imidazole into sterile water then buffered with 10N HCl by titration to a pH of 7.4

Buffering of SHP:¹ Reconstitute SHP + 1.0 ml of distilled water – mix well to dissolve – allow to stand for 15 min at RT

Storage: At RT for 4 hrs & at -20°C for 4 weeks (never store at 2-8°C). Should be quickly frozen after use & thawed at 37°C before use or

Mix: SHP (3.9ml) + 0.1ml 4M imidazole solution. The mixture was then buffered with 10N HCl by titration to a pH of 7.4.

Check Points:⁵

1. Ensure Coagulation factor level is 100% in PNCP
2. Assess the sensitivity of PT & aPTT by running dilutions of PNCP with specific factor deficient plasma. This ensures that it will detect a normal result, even if the factor level is as low as 40%.
3. In mixing study, if PT/APTT is prolonged in control tubes, it indicates deterioration of heat-labile factors
4. Patient should be enquired about Heparin/Dabigatran/Rivaroxaban/warfarin

5. Exclude liver disease, APLA

6. Check for reagents activity²

Mixing Study Test Principle: If PT and/or aPTT is prolonged then mixing test is indicated. A patient would generally need a level $\geq 40\%$ of each factor that is being detected by the test procedure to achieve a normal aPTT or PT test result. Therefore, a patient with an inadequate level, meaning less than 40%, of one or more coagulation factor will have a prolonged PT or aPTT test. In the mixing study, an aliquot of abnormal patient plasma is mixed with an equal amount of pooled normal plasma (PNP), which contains approx. 100% of all coagulation factors. The new mixed plasma sample contains at least a 40% level of each factor after the mix, including the factors that may have been present in very low levels in the original sample

Table 1: Interpretation of mixing test²

Interpretation	Tube 1 (PCNP)	Tube 2 (PP)	Tube 3	Tube 4
			(1:1 PNCP:PP)	(1:1 PNCP:PP)
	37 ⁰ C for 2hrs	37 ⁰ C for 2hrs	37 ⁰ C for 2hrs	No incubation
Incubate	perform APTT	perform APTT	perform APTT	Perform APTT immediately
Normal Study	Normal	Normal	Normal	Normal
CF deficiency	Normal	APTT – Prolonged	Normal	Normal
Factor VIII Inhibitor (time dependent)	Normal	APTT – Prolonged	APTT –Prolonged	Normal
Factor IX inhibitor (immediate acting)	Normal	APTT – Prolonged	Normal	APTT – Prolonged

Procedure of Hybrid method (Bethesda + Nijmegen)⁶⁻⁸

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 = 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 hours

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

$$9. \text{ Residual factor VIII activity} = \frac{\text{Patient factor VIII}}{\text{SHP factor VIII}} \times 100$$

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 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

Results

Statistical analysis of data: All data were expressed as Mean ± SD. Statistical analysis was done using unpaired students t test. A level of p value <0.05 was used to indicate statistical significance in all analyse.

Rosner's Index³ (ICA) Vs Chang's⁴ (% correction) as a screening test (Mixing study).

Based on the screening test results, other tests were done accordingly like Combo Technique (Rosner + Changs), factor VIII assay, DRVVT & Bethesda inhibitor assay & chromogenic assays. Specificity cannot be accurately calculated & will not be reliable parameter due to indeterminate category.

For interpreting, mixing studies screening result, RI with a cut off value of <10 is 92.5 % sensitive in diagnosing factor deficiency & a cut off value of >15 is 91.1% sensitive for inhibitor diagnosis & it could not categorise, 8% of total cases into factor deficiency /inhibitor.

Changs % correction with a cut off value of >70% is 85 % sensitive in diagnosing factor deficiency & a cut off value of <58 is 82.2 % sensitive for inhibitor diagnosis & it could not categorise, 16.5% of total cases into factor deficiency /inhibitor.

Table 2: Comparative analysis of RI vs Chang

Methodology	Rosner's Index (RI)	Chang's % Correction	CRM
Factor deficiency	185 (sensitivity 92.5%)	170 (Sensitivity 85%)	200
Inhibitor	205 sensitivity (91.1)	185 (Sensitivity 82.2%)	225
Indeterminate	35	70	00
			425

$$\text{Rosner Index} = \frac{1:1 \text{ mix PTT} - \text{PNP PTT}}{\text{patient PTT}} \times \text{Cut off values}$$

10 = Correction 15 = Inhibitor, 11-15 = indeterminate

$$\text{Chang's \% correction} = \frac{\text{APTT patient plasma} - 1:1 \text{ Mix aPTT} \times 100}{\text{APTT patient plasma} - \text{PNCP}} \times 100 > 70\% \text{ indicates correction}$$

(Factor deficiency) < 58 indicates Inhibitor 58 - 70 = indeterminate

Rosners index (ICA) as a confirmatory test for LA is more sensitive than % correction & DRVVT NR. p value < 0.05 showing that it is stastically significant

Cut-offs: DRVVT mixing test ratio (Rosners index/ ICA)- 1.15, % correction = 10. DRVVT (NR) = 1.05 DRVVT screening positive cases= 175 LAC = positive in 150 cases by other methods ACLA, B2 microglobulin

b) DRVVT mixing test ratio (ICA) Vs DRVVT % correction & NR {as confirmatory tests for LAC}⁵⁻⁷

$$\frac{[\text{Test DRVVT} / \text{Control DRVVT}] - \text{Test DRVVT} + \text{PL} / \text{Control DRVVT} = \text{PL}}{\text{Test DRVVT} / \text{Control DRVVT}}$$

Table 3: Cut off values

Methodology	DRVVT (RI/ICA) Mean = 1.4	% Correction Mean =16.5	DRVVT NR Mean= 1.17
Cut off values	1.15	10	1.05
Total cases	130	126	100

Table 4:- Inhibitor levels by Bethesda vs Nijmegen

	Low titre ≤ 5BU	High titre > 5 BU	Contol (0 BU)
Composite reference (33)	19	14	00
Bethesda?	20	08	05
Nijmegen	17	16	00

Composite Reference: Nijmegen Bethesda & RIQAS (EQC)

Discussion

1. If the results of the mixing study show correction for both the immediate and incubated PT/aPTT tests, the patient most likely has a factor deficiency (or multiple factor deficiencies).

2. If the results of the mixing study show correction for the immediate PT/aPTT results, but no correction in the incubated PT/aPTT, the patient

may have a slow-acting inhibitor such as anti-factor VIII.²

All coagulation factor assays & inhibitors were determined in latest Sysmex CS-5100 autoanalysers which can perform stage based clot assay, chromogenic, Immunologic & Inhibitor assay, Platelet aggregometric studies

For interpreting, mixing studies screening result, RI with a cut off value of <10 is 92.5% sensitive in diagnosing Factor deficiency & a cut off value of >15 is 91.1% sensitive for inhibitor diagnosis & it could not categorise, 8% of total cases into factor deficiency /inhibitor.⁸⁻¹⁰

Chang's % correction with a cut off value of >70% is 85 % sensitive in diagnosing factor deficiency & a cut off value of <58 is 82.2% sensitive for inhibitor diagnosis & it could not categorise, 16.5% of total cases into factor deficiency /inhibitor

In other studies the Chang's formula showed sensitivity of 85% and specificity of 66%, while Rosner index showed sensitivity of 70% and specificity of 93%. The Chang's formula has better sensitivity, while the Rosner index has better specificity for detection of lupus inhibitor. Our data suggests that while neither index is perfect, the use of two indices together may help to standardize reporting of positive, negative and equivocal results.

LAC: Recommendations and Criteria:^{6,7}

1. Perform two screening tests based on different principles (DRVVT and sensitive APTT).
2. Perform LA -sensitive APTT
3. Results from proficiency testing shows increased specificity with the DRVVT compared to the APTT,
4. BCS coagulometer uses LA1(screening reagent) and LA2 (confirmatory reagent)

Table 5: Interpretation of LAC

DRVVT	Interpretation
DRVVT Ratio [Test DRVVT/control DRVVT] Ratio [Test DRVVT/control DRVVT] >1.05	Range: 29-42s, Ratio: 0.9-1.05
Prolonged DRVVT which corrects with normal plasma	Possible LA (Exclude deficiencies of factors II, V, X, fibrinogen, Clotting factor deficiency [A weak LA can sometimes be masked])
Prolonged DRVVT which corrects with PL	in a 1:1 mix with normal plasma or 1:4 mix [Normal plasma: Test plasma] in case of weak LA LA

Cut-offs: DRVVT mixing test ratio (Rosner's index/ ICA)- 1.15, % correction = 10 DRVVT normalised ratio (NR) =1.05. Rosner's index (ICA) as a confirmatory test for LA is more sensitive than % correction & DRVVT NR. p value < 0.05 showing that it is statistically significant.

Bethesda Vs Nijmegen^{8,9}

The Bethesda assay is widely used to monitor the development and progression of FVIII inhibitors.

Results are subject to a number of assay variables that impact reliability and clinical interpretation.

Why not Bethesda

The Nijmegen modification of the factor VIII inhibitor assay involves buffering the normal plasma with 0.1M imidazole buffer at pH7.4 and using immunodepleted factor VIII deficient plasma in the control mixture. At low inhibitor titres (<1 Bu), the classical Bethesda assay can result in false positives whereas the Nijmegen modified assays would give zero levels of inhibition. For example, FVIII stability in NPP is compromised by pH shift and reduced protein concentration resulting from dilution. This may lead to spuriously positive Bethesda titers. This problem has been addressed by the Nijmegen which uses NPP buffered to pH 7.4 with imidazole and substitutes.³⁻⁵

ELISA show better sensitivity in detecting low-titer inhibitor & is suitable for large-scale rapid screening to detect possible FVIII inhibitors but the drawback is they cannot quantify an inhibitor

In a study conducted by Bert V. on 32 haemophiliacs, which were free of inhibitors yielded values of 0.0 BU/ml by Nijgemens method where as classical Bethesda showed activity between 0.5 to 0.9 BU/ml. It has to be taken into consideration that both the Bethesda & Nijmegen technique have low specificity at higher inhibitor titre. If actual quantitation of high titre activity is required, then it is more reliable to estimate empirically plasma dilution that gives 50% inhibition. These results clearly show that buffering the normal pooled plasma in combination with the use of FVIII-deficient plasma as a reference sample strongly increases the specificity of the assay, as was already reported years ago.

Possible Explanation:^{12,13} The inhibitor activity is highly influenced by the sample dilution factor & the inhibitor titre increases as the dilution increases. At low inhibitor titre (< 2 BU/ml) undiluted samples would be sufficient & using high titre will give false negative results.

(In study conducted by verbruggen 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

meaninhibitor activity of 4.7 BU mL) 1. In contrast, three participants, who used FVIII-deficient plasma as diluents, received a mean activity of 2.9 BU mL) despite even higher dilutions (dilution factor 2–10)

Majority of studies concluded that labs should at least use buffered NPP as substrate and FVIII-deficient

plasma as the reference sample. Bethesda Assay (1975 by Kasper). Nijmegen Assay (1995, Gold standard technique) NASCOLA (2012):- 70% lab follows Hybrid assays, 20% Nijmegen & 10% Bethesda.^{14,15}

This chart is derived from the Bethesda graph which was based on the definition of the test plasma contains 1 BU/mL at 50% inhibition ¹² .			
RESIDUAL FACTOR (%)	BETHESDA UNIT (BU/ML)	RESIDUAL FACTOR (%)	BETHESDA UNIT (BU/ML)
75	0.42	49	1.03
74	0.451	48	1.05
73	0.47	47	1.08
72	0.49	46	1.12
71	0.51	45	1.15
70	0.54	44	1.18
69	0.55	43	1.23
68	0.56	42	1.25
67	0.59	41	1.28
66	0.60	40	1.33
65	0.62	39	1.36
64	0.65	38	1.40
63	0.66	37	1.44
62	0.70	36	1.47
61	0.72	35	1.52
60	0.74	34	1.55
59	0.75	33	1.60
58	0.80	32	1.64
57	0.82	31	1.69
56	0.83	30	1.71
55	0.85	29	1.77
54	0.89	28	1.82
53	0.91	27	1.87
52	0.95	26	1.93
51	0.98	25	2.00
50	1.00		

Abbreviation: BU/mL, Bethesda unit per millilitre.

FVIII Inhibitors are categorized as 15

Type I: follow simple- first order kinetics (ie they show linear relationship between log of residual factor VIII & and antibody concentration & thus are time & concentration dependent)

Eg:- Alloantibodies against factor VIII used in treatment of Hemophilia A

Type II: Complex - Second order kinetics eg:- Autoantibodies – seen in acquired haemophilia A. There is incomplete inhibition of factor VIII (c.f to Type I) even at max antibody concentration (ie undiluted).

Note: The concentration of Type II inhibitor titer increases with the dilution.¹⁶

Conclusion

It can be safely concluded that Rosner index is better than % correction, both as a screening test (to differentiate factor deficiency from inhibitor) & confirmatory test for LAC. Nijmegen is better than Bethesda in terms of sensitivity & specificity as the later gives false positive results. This study results are in agreement with CLSI guidelines, & favours the sequential order screen-confirm- & then if required mixing study as in case of screen and confirm analysis is not clear-cut and/or when other causes of prolonged clotting times are known or suspected The draw back

with the mixing studies is that weak LAC can be missed. Though Bethesda assays considered as “gold standard” to quantify FVIII inhibitors, it is not able to detect non inhibitory antibodies, only roughly quantifies inhibitory antibodies found in patients without hemophilia, and cannot determine inhibitor isotypes. It has to be taken into consideration that both the Bethesda & Nijmegen technique have low specificity at higher inhibitor titre. If actual quantitation of high titre activity is required, then it is more reliable to estimate empirically plasma dilution that gives 50% inhibition.

Table 6: Algorithm protocol for factor VIII inhibitor^{1,2,4,7}

Step 1	<p>If aPTT is increased (exclude factor VIII def., APLA, VWD, Heparin & refer check points)</p> <p style="text-align: center;"> </p> <p style="text-align: center;">Mixing Study test</p> <p style="text-align: center;">Equal volume of Test plasma + SHP (with & without incubation)</p> <p style="text-align: center;"> </p> <p style="text-align: center;">Interpretation of Mixing test</p> <p>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</p> <p>If Either RI or % Chang indicates inhibitor then proceed to further test</p> <p>If RI is between 11- 14 or % Chang 58-69 then proceed if strong clinical indication</p>
Step 2	<p>Prepare 4 -7 tubes- depening on inhibitor titer & label (1:2, 1:4, 1:8, 1:16, 1:32)</p> <p>Add 500 µl of imidazole buffer to all tubes</p> <p>Add 500 µl patient plasma to first tube, mix well</p> <p>Transfer 500 µl of the mixture to 2nd tube mix & serially dilute (discard 500 µl from last TT)</p> <p>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)</p> <p>Or Follow the given table</p>

LA – Lupus Anticoagulant, **PL**- Phospholipids, **PPP** – Platelet poor plasma, **PNCP** – Pooled normal control plasma.

ICA – Index of circulating anticoagulant, **NR**- Normalised ratio.

Conflict of Interest: None

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