Non-parametric Clinical Laboratory Reference Interval Estimation in Volunteer Blood Donors: An Example for Prothrombin Time and Partial Thromboplastin Time

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ABSTRACT

Introduction. To date, there are no reference intervals for prothrombin time (PT) and activated partial thromboplastin time (APTT) based on "normal" Filipino adults. The common practice in most laboratories is to adopt manufacturer provided values or foreign literature even if the importance of establishing or at least verifying laboratory reference intervals has been stressed by Clinical Laboratory Standards Institute (CLSI).

Objectives. Here we aim to describe our experience in using a simple non-parametric method to generate reference intervals for PT and APTT, from healthy Filipino volunteer blood donors.

Methodology. We used a *de novo, a priori* non-parametric estimation method following the CLSI guidelines on establishing reference intervals.

Results. The non-parametric lower reference limit for PT is 12.55 seconds, with 90% confidence interval of 12.3 to 12.75 seconds. While the non-parametric upper reference limit for PT is 16.15 seconds, with 90% confidence interval of 15.55 to 16.55 seconds. The non-parametric lower reference limit for activated partial thromboplastin time is 26.12 seconds, with 90% confidence interval of 22.95 to 27.1 seconds, and the non-parametric upper reference limit for activated partial thromboplastin time is 37.44 seconds, with 90% confidence interval of 36.75 to 38.65 seconds. The PT and APTT reference intervals were different from foreign sources and manufacturer provided values in terms of interval width and values of the reference limits by 2 to 4 seconds.

Conclusion and Recommendations. Estimation of coagulation reference intervals from volunteer health blood donors is doable, simple, and practical. Collaborative multi-center efforts may be done to expand the pool of reference individuals that are included and increase the representativeness of the reference intervals generated. This simple method can also be used to generate reference intervals for other clinical laboratory assays and may also be extended to at least verify reference intervals in special populations like pregnant women, the elderly, and the pediatric population.

Key words: coagulation, non-parametric reference intervals, Filipino, Prothrombin time (PT), Partial Thromboplastin Time (PTT)

INTRODUCTION

Physiologic hemostasis, or the prevention/cessation of bleeding, is a tightly regulated process of plasma coagulation, fibrinolysis, and anticoagulation protein systems. Physiologic hemostasis and thrombosis are initiated by factor VIIa and tissue factor, and the latter is also amplified by factor XII activation on injured tissue and platelet thrombus.[1]

The activated partial thromboplastin time (APTT) and prothrombin time (PT) are two assays routinely used to assess coagulation protein abnormalities. Both are extremely useful for assessing the integrity of the blood coagulation system and for recognizing potential bleeding problems in a patient.[1]

The APTT is induced by surface (contact) activation of the system, while the PT is induced by the addition of excess

tissue factor. Contact activation occurs when artificial, negatively charged particles in the reagent autoactivates Factor XII, which in turn, initiates the proteolytic coagulation cascade. With the PT test, the addition of physiologically excessive Tissue Factor (TF) allows factor VIIa to overcome the inhibitory effect of Tissue Factor Pathway Inhibitor (TFPI), favoring the direct activation of Factor X to Factor Xa.[1]

An abnormal APTT is associated with Factor VIII, IX, and XI defects, if the patient is bleeding and with Factor XII, prekallikrein (PK), high molecular weight kininogen, and lupus anticoagulants if there is no bleeding. An abnormal PT is most often due to Factor VII defects. When both the APTT and PT are abnormal, the culprits are usually anticoagulants, disseminated intravascular coagulation (DIC), liver disease, vitamin K deficiency, and massive transfusion.[1]

The major purpose of performing analyte determinations in the clinical laboratory is to aid in the diagnosis and management of disease and in health assessment.[1] And the interpretation of PT and APTT results, just like all other laboratory examinations, involves comparison with reliable reference intervals. Needless to say, reference intervals are essential information used by health professionals in their day-to-day clinical decision making.[2,3]

The reference interval is the interval between and including two numbers, an upper and lower reference limit, which are estimated to enclose a specific percentage (usually 95%) of the values for a population from which the reference subjects are drawn. For most analytes, the lower and upper reference limits are estimated as the 2.5th and 97.5th percentiles of the distribution of test results for the reference population, respectively.[4]

As defined by Ceriotti, "It is an interval that, when applied to the population serviced by the laboratory, correctly includes most of the subjects with characteristics similar to the reference group and excludes the others."[5]

There are 3 possible means by which to obtain the Reference intervals (RI) of a given analyte for a given population:[3]
1. determine the RI *de novo* from measurements made in reference individuals;
2. transfer a pre-existing RI when a method/instrument is changed; or
3. validate a previously established or transferred RI.

De novo determination of RIs is the most frequently used procedure and is the recommended approach in medical and veterinary laboratories, as indicated in the original IFCC recommendations. In this method, reference individuals are selected according to a predefned criteria followed by determination of RIs from the reference values obtained. This approach is most often performed in a single laboratory, but a multicentric procedure also is possible if methods and populations are comparable. In some cases, an a posteriori approach is used in which pre-existing data is exploited to establish reference values.[3] Establishing, as opposed to verifying, reference intervals is clearly more difficult because of the daunting numbers of reference individuals required. But the ability to pool

data from several laboratories using the same method and the availability of new statistical techniques may ease the burden considerably.[6]

It is important to use normal ranges specific to the population being considered because the published normal values may not be entirely applicable. There may be important differences in the values and ignoring this fact may lead to over or under treatment of patients. Examples of this include differences in the serum creatinine (race specific) as well as effect of region on some specific proteins in asians.[7,8] And even if for most examinations, there are few data documenting such differences, it is dangerous to assume that just because there is no documentation, there is no difference.[8]

Unfortunately, reference ranges for PT and APTT have not been established in the Filipino population. Literature search using the UP-Manila Research Database, which includes articles indexed in the Philippine Index Medicus, as well as unpublished theses and dissertations, returned no relevant result. In the Philippine General Hospital (PGH), reference ranges are provided for by the manufacturer of the analyzers and validation of these values is not routinely performed.

Because performing full blown *a priori* reference interval is both expensive and time consuming, it is common practice for laboratories to adopt reference ranges from the manufacturer, foreign laboratories, or from foreign publications, sometimes even without verifying their applicability.[6] But this practice has serious consequences, as described in a study by Brewster et. Al., wherein the manufacturer-defined reference ranges for creatinine was found to be narrower than the one established by the group, resulting in individuals unnecessarily being deprived of cholesterol-lowering medications.[9]

It is therefore still imperative, at the very least, to verify, using as little as 20 reference individuals, the adequacy of reference intervals on a regular basis. And for tests where accuracy is extremely important, laboratories should participate in peer-group quality assessment surveys.[6]

In this paper, we describe our experience in generating a *de novo, a priori* non-parametric reference intervals for prothrombin time and activated partial thromboplastin time, using healthy volunteer blood bank donors as the reference population.

METHODOLOGY

This is a descriptive, cross-sectional study (WHO Classification)[10] done in accordance with the CLSI EP28 A3c recommendations.[4] For a relatively short accrual period of 26 days, from July to August 2011, 122 physically fit, adult Filipinos, with ages ranging from 18 to 55 years old, who came to the PGH Blood bank to donate blood, and who were found to be asymptomatic and physically normal after being interviewed and examined by a medical technologist and a physician, were considered for inclusion in the cohort of reference individuals. We used relevant items in the standardized in-house donor screening criteria as the exclusion criteria for the reference individuals. These

criteria are essentially similar to the CLSI recommended list. No donor identifying information were collected, all samples were de-identified, and all volunteer blood donors signed the informed consent form.[4]

During the conduct of standard procedures in screening volunteer blood donors, licensed medical technologists aliquot about 10 ml of blood during the blood-letting procedure. Five (5) ml aliquot of blood was transferred in a blue-top tube, containing the additive Sodium citrate, and was sent for coagulation examination. The other 5 ml aliquot was collected in a Red-top tube and was sent to the blood bank for routine donor blood testing.

The blood samples were analysed using ACL Elite Pro (Instrumentation Laboratories) following the manufacturer's manual of procedures, and in accordance with the standard operating procedures of the Blood Bank and Department of Laboratories.

Reference intervals, including the 90% confidence intervals for the upper and lower limits were calculated using the non-parametric method outlined in the CLSI document.[4,11]

Briefly, the nonparametric method as described in section 9.4.1 of CLSI EP28-A3c consists of the following steps:
1. the observations are ranked from smallest to largest (smallest is r = 1, and largest is r = n);
2. the non-parametric 95% lower reference limit, r1, shall correspond to the value of the observation that is ranked r1 = 0.025*(n + 1);
3. the non-parametric 95% upper reference limit, r2, shall correspond to the value of the observation that is ranked r2 = 0.975*(n + 1);
4. the rank values of r1 and r2 are rounded up to the nearest integer of the calculated values;
5. the non-parametric confidence intervals of the upper and lower non-parametric reference limits are then obtained from Table 8 of CLSI EP28-A3c (which, in turn, was adapted with permission from Solberg HE. Approved recommendations [1987] on the theory of reference values. Part 5. Statistical treatment of collected reference values. Determination of reference limits. Journal of Clinical Chemistry and Clinical Biochemistry. Vol. 25. Berlin, Germany: Walter de Gruyter GmbH & Co. KG; 1987, pp. 645-656. Table 1);
6. the lower and upper 90% confidence interval limits for the lower reference interval limit, correspond to the values of the lower and upper rank numbers indicated in Table 8, at the row corresponding to the sample size of the data obtained;
7. the lower and upper 90% confidence interval limits for the upper reference interval limit, correspond to the values of the ranks: (n + 1) – upper rank number and (n + 1) – lower rank number, at the row corresponding to the sample size of the data obtained;

Note: The reader is referred to CLSI guidance document EP28-A3c for a more detailed discussion of the methodology and for guided examples on how the method is done using actual data.

RESULTS AND DISCUSSION

We were able to include blood aliquots from 122 reference individuals. Among these, 109 were from males and 13 were from females - for a male to female ratio of 8 to 1. This disproportionate predominance of males is explained by the fact that there are more males donating blood compared to females in the hospital blood donation unit and is expected of the data. The average age of the reference individuals is 28 years old. The youngest is 18 years old and the oldest is 43 years old. There are no data on the weight and height of the reference individuals.

Using the method outlined by the CLSI EP28-A3c guidance document, the non-parametric lower reference limit for PT was 12.55 seconds, with 90% confidence interval of 12.3 to 12.75 seconds. While the non-parametric upper reference limit for PT was 16.15 seconds, with 90% confidence interval of 15.55 to 16.55 seconds. The non-parametric lower reference limit for activated partial thromboplastin time was 26.12 seconds, with 90% confidence interval of 22.95 to 27.1 seconds, and the non-parametric upper reference limit for activated partial thromboplastin time was 37.44 seconds, with 90% confidence interval of 36.75 to 38.65 seconds (Table 1).

If we incorporate the 90% upper and lower confidence intervals for the reference limits, the reference interval for PT can be as narrow as 12.75 to 15.55 sec or as wide as 12.3 to 16.55 sec, and the reference interval for APTT can be as narrow as 27.1 to 36.75 or as wide as 22.95 to 38.65 seconds.

Computing for the reference interval for INR, which is the quantity commonly reported for PT exams, is complicated as it involves prior estimation of the median normal PT and is not included in this paper. The median normal PT value from this study, however, can be used in the establishment of a reference interval for the INR.

The width of the PGH reference interval for PT is almost the same as that of the manufacturer, data from the Massachusetts General Hospital (MGH), the Merck Manual, and Henry's Clinical Diagnosis and Management by Laboratory Methods. But the reference limits are different. The lower reference limit is longer by up to 2 seconds and the upper reference limit is longer by up to 3 seconds (Table 2).

The PGH lower reference limit for APTT is up to 4 seconds longer than other sources while the upper reference limit is

Table 1. Coagulation non-parametric reference intervals with 90% confidence intervals (n = 122; unit = seconds)

Test	Lower reference limit			Median Reference value	Upper reference limit		
	Estimate	90% LCI	90% UCI		Estimate	90% LCI	90% UCI
PT	12.55	12.30	12.75	14.03	16.15	15.55	16.55
APTT	26.12	22.95	27.10	30.48	37.44	36.75	38.65

Table 2. Reference Interval for Prothrombin Time (seconds) from different sources

Source	PGH	Estimated value			
		Manufacturer	Henry's	MGH	Merck Manual
Sample size	122.0	35.0	–	–	–
Upper Reference Limit	16.1	15.1	13	13.7	13
Median Normal Value	14.0	–	–	12.3	–
Lower Reference Limit	12.6	11.8	10	11.0	11

Table 3. Reference Interval for Activated Partial Thromboplastin Time (seconds) from different sources

Source	PGH	Estimated value			
		Manufacturer	Henry's	MGH	Merck Manual
Sample size	122.0	37.0	–	–	–
Upper Reference Limit	37.4	38.4	35	33	35
Median Normal Value	30.5	–	–	–	–
Lower Reference Limit	26.1	25.4	25	21	25

also up to 3 seconds longer compared to other limits. The width of the reference interval is narrower (12 seconds) than the manufacturer, but at most only a bit wider than that of MGH, Henry's and Merck (Table 3).

The differences in the reference limits can be attributed to differences in the reference populations or the characteristics of the analytic process itself. The PT and APTT are known to vary among laboratories especially since different preparations of reagents are used. In particular, variability of "normal" APTT is attributed to significant different coagulation factor activities.[13] Nevertheless, institution-specific locally generated reference interval still provide information used for clinical decision making.

By using blood aliquots from healthy volunteers who donated blood in the hospital's blood donation unit, we were able to establish our own reference intervals for PT and PTT. This method, based on CLSI EP28-A3c guidance, is doable, simple, and practical.

Because of the differences in reference interval characteristics found in this study for normal adult population, we highlight the need to establish reference intervals using reference individuals from the population the laboratory primarily caters to. Corollary to this is the need to establish or at least validate reference intervals for pregnant, the elderly, and the pediatric group. Adopting foreign or manufacturer provided reference intervals as is, may not be adequate and applicable to these populations.

CONCLUSION

Here we were able to describe an estimation method for coagulation reference intervals, based on the CLSI guidance, that is doable, simple, and practical, by considering healthy volunteers who donate blood in the hospital's blood donation unit. The PT and APTT reference intervals we generated were slightly different from foreign sources and manufacturer-provided values in terms of interval width and values of the reference limits by 2 to 4 seconds. Collaborative multi-center efforts may be done to expand the pool of reference individuals that are included and

increase the representativeness of the reference intervals generated. This simple method can also be used to generate reference intervals for other clinical laboratory assays and may also be extended to at least verify reference intervals in special populations like pregnant women, the elderly, and the pediatric population.

STATEMENT OF AUTHORSHIP

AUTHOR DISCLOSURE

FUNDING SOURCE

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