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ORIGINAL ARTICLE

Quality laboratory issues in bleeding disorders

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Selected quality issues pertinent to the determination of accurate results in the haemostasis laboratory are discussed. Specifically, the implementation of a successful external quality-assessment scheme is described, including its impact on result accuracy as well as the programme's unique challenges and opportunities. Errors in the preanalytical phase of laboratory testing represent the greatest source for reporting incorrect test results. Some of the most common preanalytical errors are described including those that necessitate sample rejection. Analytical means to identify potential sources of error and analytical means to overcome particular interferences are described. Representing the most important clinical complication in the treatment of patients with haemophilia, quality issues related to determination of the presence of inhibitory antibodies against factor VIII (FVIII) are reviewed. Heat treatment of patient plasma prior to testing, particularly in patients receiving replacement FVIII concentrate or during induction of immune tolerance to achieve more accurate results is recommended, while screening activated partial thromboplastin time-based mixing tests to rule out inhibitor presence is discouraged. The initiatives presented in this review can be implemented in robust and resource restricted settings to improve the quality of laboratory testing in patients with bleeding disorders.

Keywords: bleeding, external quality assessment schemes, factor inhibitors, laboratory, preanalytic, quality

Introduction

The haemostasis laboratory plays a critical role in the care of patients with hereditary or acquired bleeding disorders. Given this pivotal function in providing patient care, it is of utmost importance that the laboratory provides high quality and accurate test results. Proper performance of internal quality control (QC) for each assay performed, and active participation in external quality assessment schemes (EQAS) help promote accuracy of test results. Also, enhancing the potential to provide high quality results is a thorough understanding of preanalytical issues that can impact testing, and how these variables can be identified and overcome. Each test method, particularly complex procedures, such as determination of factor inhibitors, should be critiqued to make certain appropriate measures are undertaken to avoid the generation of false negative or false positive results. This paper provides a brief review of these topics in an effort to promote quality testing in the haemostasis laboratory.

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Quality assessment of the haemostasis laboratory – instituting and its Impact

Quality assurance (QA) is defined as the sum total of a laboratory's activities aimed at achieving the required standard of analysis [1]. Internal QC involves repeated testing of assayed control materials at predetermined intervals and comparing the observed value to the distributions expected under stable operation [2]. This ensures precision and enables detection of random or systematic errors through visual inspection or application of QC rules [3].

Verifying accuracy, however, is possible only by testing material whose values are unknown and are submitted to the laboratory by an external agency also known as EQAS. The results obtained are compared with the target value, assigned by appropriate standard statistical methods [4]. EQAS results may also be

utilized by accrediting bodies and licensing agencies for benchmarking laboratory quality [2]. Therefore, practice of QC and participation in an EQAS are both essential to ensure reliability of results and provide confidence for the clinician. In emerging economies where systematic regulation of laboratories is absent, it is often left to the discretion of the laboratory to assure quality [5].

Christian Medical College (CMC) EQAS for haemostasis

In order to ensure high quality testing for haemophilia patients, a program was designed in India, where initially EQAS samples were sourced from United Kingdom National EQAS (UKNEQAS) and distributed to participants around the country by CMC Vellore. Results were collected and submitted to UKNEQAS for analysis and reports were returned to participants. This was supported by a grant from the Katherine Dormandy Trust. With increasing awareness of the program and quality requirements, the program was aimed to be extended to all laboratories that performed haemostasis testing.

Structure of the scheme

Ensuring sustainability was an important concern with cost being a significant factor. The program was indigenized by production of stable and homogenous materials locally by the haemostasis laboratory at CMC; lyophilization was outsourced. Evaluation was based on standard methods with assistance from our in-house biostatistician. Basic and advanced programs, tailored to suit the testing profiles of both small and large laboratories in India were provided [6]. The program which is partially subsidized by CMC began in 2004 with 36 participants and has now grown to 567 participants in 2015.

Currently, the scheme offers three surveys in an annual cycle. Paired plasma samples are sent via courier to all registered participants, who perform their tests in their usual manner and return results to the organizer – either via paper, email, fax or through the

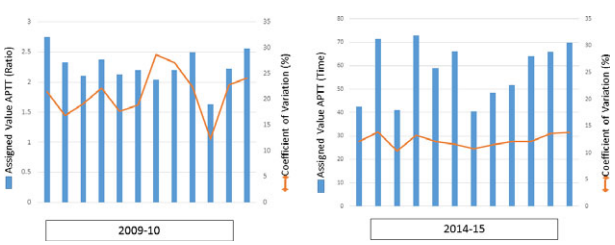

Fig. 1. (a) Relationship between activated partial thromboplastin time (APTT) ratio assigned value and coefficient of variation (2009–10). (b) Relationship between APTT (time) assigned value and coefficient of variation (2014–15).

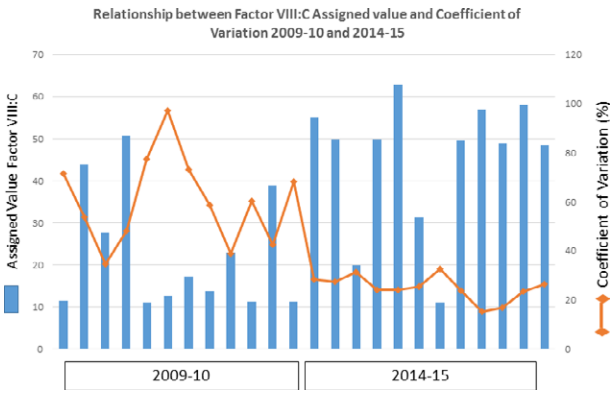

Fig. 2. Relationship between factor VIII:C assigned value and coefficient of variation 2009–10 and 2014–15.

newly designed web portal (www.cmceqas.org). Peer groups have been formed for analysis, based on reagent used for the screening tests, to allow for the variability inherent to the analysis of lyophilized samples. A simple, easily comprehended report is generated for each participant laboratory that shows the result of the participant in relation to the performance of the whole group and the relevant peer group; graphical summaries are included. A brief commentary is provided to ensure easy interpretation of results. Oversight of the program is by an internal steering committee with clinical, laboratory and statistical expertise and an external advisory committee with representatives from the national professional body (ISHBT) and academic institutions of repute.

Impact of EQAS

Over the years, there appears to be a positive impact on the results generated by the laboratories as evidenced by the reducing trend in coefficient of variation of the activated partial thromboplastin time (APTT) and factor (F) VIII (FVIII) activity assay when comparing data from 2009–10 to 2014–15. (Figs 1a,b and 2)

Challenges

Logistics remains the major challenge in our country. With the program moving to a web portal, we hope to reduce the turn-around-time as more participants begin to submit results directly. Sustaining expertise in laboratories and continued participation is also a concern especially in laboratories with low sample load.

Beyond providing EQAS

Educational support is provided through supplements, periodic webinars and annual symposia by experts. Workshops are conducted that participants can enrol in to learn good laboratory practice. Specific assistance

is provided for those participants who require it. The EQAS also shares highlights of the program with users at the annual meeting of the ISHBT. The scheme has also at various times in the past, provided similar services for other countries in the region such as Philippines, Thailand, China and Sri Lanka and currently does so for laboratories in South Africa.

Analytics that can overcome preanalytical variables

The preanalytical phase accounts for more than 70% of laboratory errors. This far exceeds errors associated with the analytical and postanalytical phases [7]. Samples for coagulation testing are particularly vulnerable to preanalytical conditions compared to most samples for chemical analysis, and the impact on results can be great [8–10]. Analysis of unsuitable samples can lead to unreliable test results subsequently jeopardizing clinical decision-making and patient safety [11,12]. When a sample is compromised, the test result might accurately reflect the status of the sample, but not accurately reflect the clinical status of the patient [12]. It is not always clear when a sample referred to the haemostasis laboratory is unsuitable or compromised. Clinical laboratory scientists must be educated as to the impact of these variables on haemostasis testing and they must be vigilant in their identification of compromised samples.

The ISO 15189:2012 [13] standard for laboratory accreditation defines the preanalytical phase as including the clinician's request, the test requisition, patient preparation, sample collection and transportation to and within the laboratory, ending with sample analysis. At every step in this process, errors may occur. Furthermore, many of these steps occur outside of the laboratory and are beyond the laboratory's direct control. Given this, the laboratory must provide proper sample collection, processing and transportation guidelines to individuals that collect samples. In addition, clinicians may require guidance as to appropriate test selection to avoid misdiagnosis. For example, FVIII activity may be decreased due to haemophilia A, von Willebrand disease (VWD) including type 2N (where the abnormal von Willebrand factor cannot bind and stabilize FVIII), or combined FVIII/FV deficiency. Ordering FVIII activity alone, may result in a diagnosis of haemophilia A when either VWD or a combined FVIII/FV deficiency is present and this may impact therapy [14].

Certain conditions of the specimen demand sample rejection, as these conditions cannot be overcome in the laboratory. The following samples for haemostasis testing must be rejected: problems of correct patient identification, clotted specimens, plasma collected into an anticoagulant other than sodium citrate, or collected with an inappropriate blood-to-anticoagulant ratio [15]. While these scenarios may be readily identified evaluating the primary collection tube, laboratories that receive only a secondary sample aliquot are blinded to these issues. For this reason, laboratories that receive secondary aliquots must be aware of the impact of these variables, especially incorrect sample matrix, on haemostasis testing [16].

Variables inherent to the patient or sample collection

Factors inherent to the patient, impact the nature of the plasma regardless of whether the laboratory receives the primary tube or a secondary aliquot. Such factors include patient medications, polycythaemia, hyperbilirubinaemia, haemolysis and lipaemia. Visual disturbances of the plasma, such as icterus, haemolysis and lipaemia, may interfere with optical clot-based detection systems and some analysers can overcome this by reading these samples at a higher wavelength. This however, does not account for the effect these interferences may have on the analytes themselves. Haemolysis can activate coagulation due to tissue factor release from lysed cells [17]. It may be best to collect samples after a fast as high-fat meals have an acute effect on platelet function and may cause lowering of some factor activities (e.g. FII, FIX, FX, FVII) [18]. Samples with haematocrits >55% should have the concentration of sodium citrate adjusted [15].

The presence of heparin, direct Xa and direct thrombin inhibitor (DTI) anticoagulants can interfere with haemostasis testing resulting in falsely decreased factor activities, false positive factor inhibitor results, false positive lupus anticoagulant results, falsely elevated antithrombin, protein C and protein S levels [19]. Laboratories can characterize plasma samples prior to testing, to help determine the presence of anticoagulants, especially when this information is provided with the sample. Evaluation of the APTT, PT, thrombin and reptilase times can assist in detecting the presence of anticoagulants. An abnormal thrombin time and normal reptilase time suggests the presence of heparin or a DTI. Heparin can be neutralized in the laboratory with hepzyme or polybrene and heparin's effect on subsequent testing diminished. The effect of the DTI, dabigatran, can be neutralized *in vitro* by adding idarucizamab, a humanized antibody fragment to the collected plasma, although this compound is not currently readily available to laboratories [20]. Direct Xa inhibitor (DXI) anticoagulants cannot be neutralized in the laboratory currently although there are antidotes administered clinically that could potentially serve this purpose [21]. DXI anticoagulants such as rivaroxaban, apixaban and edoxaban tend to cause prolongation of the APTT and prothrombin time (PT) but not thrombin time. Although apixaban at therapeutic doses has little effect on these global assays, it can interfere with

specialty coagulation results. DXI anticoagulants can be identified in the laboratory with a chromogenic anti-Xa assay calibrated using any of the direct or indirect Xa anticoagulant drugs [19].

Lupus anticoagulants (LA) as well as DXI and DTI anticoagulants act as non-specific inhibitors in factor activity assays. Performing factor activity assays at multi-dilution can often but not always overcome this non-specific interference, by diluting its effect. Non-specific interference results in non-parallelism, characterized by increasing factor activity with increasing dilution. In some instances, the inhibitor effect cannot be completely diluted out [22]. In this case, the factor activity may appear falsely low. If a LA or DTI interferes with a FVIII or FIX clot-based activity assay, a chromogenic FVIII or FIX assay can be performed as the chromogenic assay does not demonstrate such non-specific interference [23]. DXI anticoagulants interfere in chromogenic factor activity assays as these assays are based on generation of FXa, yielding falsely low results [19]. Performing factor activity assays at three or more dilutions can also aid in the identification of samples that are activated during collection or handling. With sample activation, factor activity decreases with increasing dilution and this suggests an unsuitable sample that should be rejected.

Variables due to improper sample collection or handling

Following collection, whole blood samples should not be refrigerated or stored on ice prior to processing as this can result in platelet activation and cryoprecipitation of von Willebrand factor and FVIII. Re-warming samples prior to processing prevents loss of these proteins [17].

Laboratories that receive secondary aliquots may need to characterize samples not only for the presence of anticoagulants, but also for correct sample matrix. Testing serum or EDTA plasma can yield grossly abnormal, spuriously low, factor activity results. EDTA plasma furthermore may perfectly mimic a FVIII inhibitor causing prolongation of the APTT, lack of correction with normal plasma mix, prolongation of the mix with incubation, decreased FVIII activity and a positive FVIII inhibitor titre, all of which are false results [16]. Serum and potassium EDTA plasma can be distinguished from citrate plasma using simple measurements of potassium, calcium and sodium.

Samples for haemostasis testing must be held to rigorous collection and handling regimens [15]. The ideal haemostasis sample is collected in a non-traumatic fashion from a peripheral vein using a 19–21 gauge needle (23 gauge may be used in infants). Evacuated tubes should be completely filled to achieve a 9:1 blood-to-anticoagulant ratio and promptly mixed by 4–6 end-over-end inversions, stored at room temperature and processed within 4 h. Samples should be tested immediately or frozen at −20°C or colder. If samples are shipped, they should be transported on dry ice and kept frozen until testing.

Quality issues in FVIII inhibitor testing

Inhibitory alloantibodies against F VIII are the main clinical complication in the treatment of patients with haemophilia A, with an occurrence of 30%. In general, anti-FVIII antibodies are immunoglobulins (Ig) G, mainly of the IgG1 and IgG4 subclasses, that can be neutralizing (inhibitors) or non-neutralizing of FVIII activity [24–28]. The Bethesda assay described in 1975 by Kasper [29,30], was the first method to detect and measure FVIII inhibitors, with an acceptable degree of standardization. However, the Bethesda assay is non-specific, yielding many false positive results, therefore, various modifications have been applied to this method. The major modification shown to improve sensitivity and specificity is the Nijmegen-Bethesda assay (NBA) [31,32].

At present, the NBA is considered the gold standard method. However, this assay still presents a high coefficient of variation, of approximately 30%, documented for different EQAS [33,34]. The many variations introduced to the NBA, such as use of different reagents, and dilutions, may lead to false positive or false negative results. In the case of false positive results, Miller showed that up to 26% of low-titre inhibitors (<2 BU mL^{-1}) in the NBA, yielded negative results when a chromogenic method or fluorescence-based immunoassay (FI) was used to measure residual FVIII [35].

Regarding preanalytical issues, heat-treatment of the sample should be performed before performing the NBA to add greater sensitivity to the assay. Immunoglobulin is not affected by heating to 56°C for 30 min, while the exogenous or endogenous FVIII is destroyed by denaturation. Without FVIII in the plasma sample, the immunoglobulin binding sites become available for binding to FVIII, rendering more accurate results [36,37]. This can be particularly important in severe haemophilia A patients receiving replacement FVIII concentrate, or during induction of immune tolerance (ITI) treatment [38]. We demonstrated in a longitudinal evaluation of FVIII inhibitor patients under ITI treatment, that after heat-treatment of the samples, the results were likely more precise when compared to the results of non-heated samples. A trend was observed between the results, with an increase of threefold in inhibitor titres, in the case of the heated plasma samples [38]. In addition, heat-treatment can also contribute to the accuracy of the NBA assessment in samples from moderate or mild haemophilia patients, and also in the case of acquired haemophilia. In an acquired haemophilia A

investigation, similar benefits have been demonstrated, with a positive NBA rate of 5% for untreated samples increase to 50% for heat-treated samples [39].

Another relevant issue is the possibility of missing inhibitory antibodies in the APTT-based mixing test. Some laboratories use this as a screening test to evaluate for the presence of an inhibitor and only positive samples progress to the NBA for inhibitor quantification. A workshop held in Brazil, to improve laboratory diagnosis, showed that samples which tested negative in a screening mixing test, were positive for the presence of an inhibitor, when the same samples were tested during the workshop using the NBA. Kitchen, in the World Federation of Haemophilia's laboratory manual, previously showed there was no correlation between the result of the APTT mixing test and inhibitor titre. This lack of correlation could be due to different inhibitor kinetics, different mathematical approaches to determine inhibitor titre, and different interpretations used to evaluate the results. Thus, the screening APTT mixing test is not sufficient to rule out the presence of an inhibitor.

To date, several lessons have been learned regarding inhibitor assessment which leads to the following recommendations; (i) use buffered normal pooled plasma, containing 95–105% FVIII at pH 7.4, (ii) use a FVIII standard that is calibrated according to international standards, (iii) use FVIII-deficient plasma containing von Willebrand factor, to avoid FVIII degradation, (iv) use extreme caution when preparing samples dilutions and preparing each dilution independently may avoid errors, (v) use standardized analytical procedures to guarantee reproducibility, and (vi) use modified test methods to confirm results, such as the chromogenic FVIII assay to allow more sensitive measurements, especially for low-titre inhibitors.

Alternative methods for FVIII inhibitor testing have been assessed during the last few years, and issues regarding neutralizing and non-neutralizing antibodies always arise [35,40,41]. Immunoglobulin profiles have been studied, with the main objective of discriminating which of these profiles has a real role in FVIII activity inhibition. The clinical importance of non-neutralizing FVIII antibodies can be observed in patients under ITI who have negative results for NBA, but inadequate pharmacokinetic responses [42]. In our experience, these patients with partial ITI success maintain the presence of IgG4 anti-FVIII in their samples. However, patients who achieve ITI success demonstrate a shift from IgG4 to IgG1 or absence of immunoglobulin (data in submission). Regarding IgG subclasses, we also observed a close correlation between high-titre inhibitors and the presence of IgG4 anti-FVIII [28]. Similarly, Whelan *et al.* [27], showed that IgG4 was completely absent in patients with no history of inhibitor development or who had ITI success. Later, the same group demonstrated that IgG4 has higher affinity for FVIII compared with other immunoglobulins [43]. Another context regarding the presence of anti-FVIII antibodies is the occurrence of allergic reactions to FVIII products. In these patients, IgE anti-FVIII antibodies with or without IgG4, were identified at the time of the allergic reaction [44].

Further improvements in inhibitor assessment are required. Heat-treatment of samples has been demonstrated to render better accuracy and improved sensitivity of the NBA. Alternative methods or modifications in existing tests are still needed. This includes the consideration of measuring immunoglobulin subclasses, such as IgG4 anti-FVIII, in addition to performing the NBA.

Conclusion

Quality in haemostasis testing can be enhanced through a multi-faceted approach that includes systematic implementation of QC, as well as participation in EQAS, educational activities and accreditation programs. This scheme should also include effective means to avoid or counter-act preanalytical variables that may impact results, and improve testing methodologies to avoid reporting of inaccurate results. Such initiatives can be successfully implemented in resource restricted settings and improvements in haemostasis testing quality achieved.

Disclosures

The authors stated that they had no interests which might be perceived as posing a conflict or bias.

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