Problems and Solutions in Laboratory Testing for Hemophilia

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Semin Thromb Hemost 2013;39:816–833.

Abstract

A diagnosis of hemophilia A or hemophilia B begins with clinical assessment of the patient and is facilitated by laboratory testing. The influence of the latter on a diagnosis of hemophilia A or hemophilia B is clear—a diagnosis cannot be made without laboratory confirmation of a deficiency of factor FVIII (FVIII) or factor IX (FIX), respectively. Moreover, the degree of hemophilia severity is specifically characterized by laboratory test results. In turn, patient management, including choice and application of therapies, is influenced by the diagnosis, as well as by identification of respective disease severity. An incorrect diagnosis may lead to inappropriate management and unnecessary therapy, and thus to adverse outcomes. Moreover, identification of factor inhibitors in hemophilia will lead to additional and differential treatments, and incorrect identification of inhibitors or inhibitor levels may also lead to inappropriate management. Problems in hemophilia diagnosis or inhibitor detection can occur at any stage in the clinical diagnosis/laboratory interface, from the "pre-preanalytical" to "preanalytical" to "analytical" to "postanalytical" to "post-postanalytical." This report outlines the various problems in laboratory testing for hemophilia and provides various strategies or solutions to overcome these challenges. Although some outlined solutions are specific to the potential errors related to hemophilia, others are general in nature and can be applied to other areas of laboratory hemostasis. Key to improvement in this area is adoption of best practice by all involved, including clinicians, phlebotomists, and laboratories. Also key is the recognition that such errors may occur, and thus that clinicians should assess laboratory test results in the context of their patient's clinical history and follow-up any potential errors, thus avoid misdiagnoses, by requesting repeat testing on a fresh sample.

Keywords
- hemophilia
- laboratory testing
- preanalytical
- analytical
- quality
- diagnostic practice

published online
September 11, 2013

Issue Theme Old and New Challenges in Hemophilia Management; Guest Editors, Antonio Coppola, MD, Annarita Tagliaferri, MD, and Massimo Franchini, MD.

DOI http://dx.doi.org/10.1055/s-0033-1356573.
ISSN 0094-6176.

Hemophilia is typically defined as an inherited or—less commonly acquired—disorder of blood clotting, which is associated with an enhanced tendency for bleeding. Depending on the degree of the disorder present in any individual, excess bleeding may occur only after specific events (or "challenges", such as surgery, dental procedures, or injury), or spontaneously with unknown or unidentifiable initiating event(s).[1,2] Hemophilia can essentially be represented by any (clotting) factor deficiency, including fibrinogen (factor [F] I), FII, FV, FVII, FVIII, FIX, FX, FXI, and FXIII, and representative of the secondary hemostasis pathway, which facilitates the conversion of fibrinogen to fibrin and its subsequent stabilization.

Classical hemophilia is represented by congenital (hereditary, X-linked) deficiencies in FVIII (i.e., hemophilia A) or FIX (i.e., hemophilia B), and these will provide the focus for the current overview. Acquired hemophilia is instead represented by nonhereditary acquisition of factor deficiencies, often triggered by (or associated with) a variety of autoimmune disorders. Acquired hemophilia has recently been extensively reviewed in this journal and elsewhere by us and by others,[3–8] and so will not be extensively mentioned in this report. Nevertheless, antibodies generated as alloimmune events in classical hemophilia can arise as a consequence of factor replacement therapy, and this will be covered in this chapter.

A diagnosis of hemophilia A or hemophilia B begins with clinical assessment of the patient, which includes physical examination and assessment of personal and family history for bleeding/bruising, and is facilitated by in vitro diagnostic testing. The influence of laboratory testing on a diagnosis of hemophilia A or hemophilia B is clear—a diagnosis cannot be made without laboratory confirmation of a deficiency of FVIII or FIX, respectively. Moreover, the degree of hemophilia severity is specifically classified by laboratory test results, with severe hemophilia defined by levels less than 1 U/dL, moderate hemophilia as 1 to 5 U/dL, and mild hemophilia as more than 5 to 40 U/dL,[9,10] although the same level of clotting factor does not always correspond to a similar bleeding phenotype.[1,2] Moreover, hemophilia A and hemophilia B may themselves represent differential bleeding severities, with hemophilia B considered potentially less severe than hemophilia A.[1,2,11] Nevertheless, patient management, including choice and application of therapies, is influenced by the diagnosis as well as by the identification of respective disease severity.[2,12,13] For example, a diagnosis of hemophilia A or hemophilia B will lead to respective therapy with replacement FVIII or FIX, and a diagnosis of severe hemophilia may lead to lifelong prophylaxis. Although this is intuitive, what is less well appreciated is that an incorrect diagnosis of hemophilia A or hemophilia B, or an incorrect assignment of severity, will similarly lead to such therapy, which may not be appropriate and might thus lead to adverse outcomes.[14,15]

Moreover, identification of factor inhibitors in hemophilia will lead to additional and differential management.[3–5,16] For example, identification of low-level inhibitors (low responders, typically defined as less than 5 Bethesda Units [BU]/mL of inhibitor)[9] are typically treated with (higher levels of) factor replacement therapy. In contrast, identification of high-level inhibitors (high responders, typically defined as $\geq$ 5 BU/mL of inhibitor) will typically lead to alternate therapies, including (more expensive) "bypass agents," including factor eight inhibitor bypass activity (FEIBA) or recombinant activated factor VII (rFVIIa) (or in the future analogues in development).[16,17] Similar to above, although laboratory testing remains crucial for determining therapeutic choices, misidentification of factor inhibitors, either as the wrong inhibitor type identified, or as an incorrect level (or titer) of inhibitor, will again potentially lead to inappropriate clinical management.

Problems in Laboratory Testing—An Overview

As additional background, it is worthwhile conceptually reviewing the diagnostic process and associated problems, as summarized in ►**Table 1**. It is important to recognize that problems can occur at any stage in the clinical diagnosis/laboratory testing process, from the "pre-preanalytical" to "preanalytical" to "analytical" to "postanalytical" to "post-postanalytical."[18,19] The major focus for most laboratories is the analytical stage, where the actual testing takes place. However, it is now recognized that this may be the least problematic area of diagnosis.[20,21] Laboratories (in general) recognize the issues related to testing, they utilize appropriate reagents, methodologies, and instrumentation, they run the appropriate calibrators and internal quality controls, and they participate in appropriate external quality assessment (EQA).[22] In contrast, less focus is generally placed on the other stages of the diagnostic process, and there may therefore be reduced understanding or appreciation of the challenges, resulting in a greater chance for errors.[20]

Pre-Preanalytical Issues in Factor VIII and Factor IX Testing and Hemophilia Diagnosis

One potentially emerging pre-preanalytical issue in hemophilia diagnosis is the use of bleeding scores as an attempt to standardize clinical assessment of patients suffering from bleeding disorders.[23,24] However, as noted in ►**Table 2**, the main current pre-preanalytical issues relate to the tests requested by the clinician, and this will be the major focus of this section. Additional issues related to (in)correct patient identification and (in)correct blood collection techniques, while important, pertain to all tests performed, and have been extensively covered by other reviews.[25–28] Other potential issues are ABO blood group and acute phase reactions, and these will be briefly mentioned.

For the purpose of this review, we will primarily use various case examples (as summarized in ►**Table 2**) to identify how actions undertaken during the pre-preanalytical phase can lead to a misdiagnosis of hemophilia A or hemophilia B or how such a diagnosis can be missed. Essentially, this can ensue should the clinician request the wrong tests or should testing be insufficiently extensive. It is important to recognize that many of these cases are not just theoretical, as they often reflect true case studies.

Table 1 Summary overview of a general diagnostic process related to hemostasis dysfunction and associated problems

Process	Stage	Summary of Process	Summary of Relevant "Laboratory" Problems
1. Clinician sees patient with (recent or lifelong) history of bleeding/ bruising and investigates patient with view to making a diagnosis.	Pre-preanalytical	Clinician assesses patient for personal and family history of bleeding/ bruising, undertakes physical examination of patient, and orders blood tests.	The tests that the clinician orders will influence the diagnosis. Incorrect tests ordered may result in an incorrect diagnosis.
2. Blood taken from patient and processed for requested tests.	Preanalytical	Phlebotomist collects blood from patient. Blood is transported to laboratory, where it is processed by centrifugation and typically stored (frozen) for later testing.	Correct patient? Correct blood tubes? Correct order of blood draw? Appropriate mixing? Appropriate transport of collected blood to laboratory? Correct processing and centrifugation process? Appropriate storage?
3. Blood tests performed by laboratory.	Analytical	Laboratory performs the requested tests (and perhaps only the requested tests as this is often the law).	Correct tests performed? Tests performed well? Adequate controls (internal and external) applied? Appropriate procedures, calibrators, reagents and instruments?
4. Results of blood tests are issued by laboratory to the requesting clinician.	Postanalytical	Laboratory results the requested tests, sometimes with interpretive comments.	Results appropriately entered/ transcribed? Appropriate interpretive comments?
5. Clinician receives and reviews results of blood tests, and consolidates a diagnosis.	Post-postanalytical	Clinician receives and reviews results of blood tests in light of patient's personal and family history of bleeding/bruising, and physical examination and consolidates a diagnosis.	Results appropriately reviewed in correct clinical context? Have alternate plausible diagnoses been considered and correctly discounted (example by laboratory investigation)?
6. Subsequent activities.	Post-postanalytical	Subsequent actions may be applied — for example: (a) patient may revisit clinician; (b) therapy may be applied to manage patient, perhaps over long term; (c) additional tests may be requested for confirmation of, or exclusion of other plausible, diagnoses; (d) family studies may be initiated.	Correct therapy applied for correct diagnosis? Additional tests sufficiently broad and encompassing?
7. Process may continue from above.			

von Willebrand Disease Misdiagnosed As Hemophilia A

von Willebrand disease (VWD) may be misdiagnosed as hemophilia A when the clinician fails to identify this condition as a possible alternative diagnosis to hemophilia A. A classic example is a case of severe type 3 VWD misdiagnosed as moderate or mild hemophilia A in a male child where the clinician has only requested FVIII testing. In such a situation, the resultant FVIII level identified by the laboratory is typically in the range from 4 to 8 U/dL. Unless additional von Willebrand factor (VWF) testing is requested, the plausible differential diagnosis of type 3 VWD may be missed. This is considered a serious problem in some developing countries, such as China, where there is clear recognition of hemophilia A by clinicians, but where VWD is less well appreciated.[29] It is noteworthy, however, that such misdiagnoses have also occurred in developed countries.

Another paradigmatic example is a case of type 2N VWD similarly misdiagnosed as moderate or mild hemophilia A.[30,31] In this situation, the clinician may or may not have requested additional VWF tests. Type 2N VWD reflects a defect in VWF, whereby the plasma VWF protein fails to appropriately bind (and thus stabilize and protect) plasma FVIII. In these cases, FVIII is easily degraded in blood and more quickly cleared from the circulation, and patient test patterns are phenotypically similar to those observed in mild hemophilia A (in men or women) or "hemophilia A carrier" (in females). It is important to note that performance of standard VWF assays will not provide any clarity in regards to the possible differential diagnosis of type 2N VWD, which requires a specific VWF:FVIII binding assay and/or genetic analysis of the *VWF* gene. In particular, type 2N VWD should be considered in those patients in whom familial

Table 2 Summary of pre-preanalytical issues leading to potential misdiagnoses in hemophilia and incorrect therapy applied

Pre-preanalytical issue	Examples	Laboratory finding(s)	Diagnostic outcome	Management and therapy implications	Solution
Clinician orders limited testing in investigation.	FVIII only requested in male child suffering type 3 VWD.	FVIII levels between 4–10 U/dL.	Patient misdiagnosed as (moderate or mild) hemophilia A.	Patient potentially (inappropriately and ineffectively) treated with FVIII concentrates instead of VWF-containing concentrates.	Consider type 3 VWD as a differential diagnosis (especially in a male child), and request VWF testing.
	FVIII (with or without standard VWF tests) requested in patient suffering type 2N VWD.	Low FVIII levels. Normal (or low) VWF test results. FVIII levels < VWF level (discordance observed).			Consider type 2N VWD as a differential diagnosis, and request specific VWF:FVIII binding studies (and/or genetic testing if available) in a patient without familial hemophilia A or where otherwise appropriate.
	FVIII only requested in patient suffering combined FV/FVIII deficiency.	Low FVIII levels.	Patient misdiagnosed as (mild) hemophilia A.	Patient potentially (inappropriately and possibly ineffectively) treated with FVIII concentrates instead of combined FV and FVIII replacement therapy.	Consider combined FV/FVIII deficiency as a differential diagnosis wherever appropriate.
Clinician orders wrong tests in investigation.	FVIII only requested in patient suffering FIX (or FXI, etc.) deficiency.	Normal FVIII levels.	Patient diagnosis as hemophilia A or hemophilia B (etc.) potentially missed.	Patient potentially denied appropriate therapy.	Consider deficiencies other than FVIII as a differential diagnosis wherever appropriate.
Clinician orders wrong tests in investigation or laboratory performs incorrect test because of misinterpretation of order request.	Nomenclature issues such that FXI requested instead of FIX, FXIII requested instead of FVIII, etc.	Normal levels of requested test.			Take care to check tests ordered or results received for accuracy.
Clinician orders factor FVIII or FIX tests while patient is on anticoagulant therapy.	Heparin therapy can lead to low FVIII or FIX test result. VKA therapy can lead to low FIX test result. New direct acting oral anticoagulants have variable effects on FVIII and FIX assays.	Abnormal levels of requested FVIII and FIX (and other factor) tests possible.	Patient (transiently) misdiagnosed as hemophilia A or hemophilia B.	Surgery may be cancelled or postponed; inappropriate therapy may be applied.	Do not request factor assays while patients are on anticoagulant therapy.
Others	1. ABO blood group influence on FVIII levels. 2. FVIII is an acute phase reactant. 3. Normal reference range effects.	1. FVIII is lower in O blood group. 2. FVIII may be falsely high because of acute phase effects. 3. FVIII or FIX result below the normal range does not necessarily define hemophilia.	Patient (transiently) misdiagnosed as hemophilia A or hemophilia B, or a diagnosis of (mild) hemophilia A discounted.		Consider ABO, acute phase, and normal reference range effects. Repeat testing as required and consider the clinical history.

Abbreviations: VKA, vitamin K antagonist; VWD, von Willebrand disease.

hemophilia A is not identifiable or where the familial inheritance pattern suggests a non-X–linked disorder.[30–34]

Other Disorders Misdiagnosed As Hemophilia A

Another cause of (mild) hemophilia A misdiagnosis is combined FV/FVIII deficiency caused by mutations in intracellular transport genes.[35,36] Combined FV/FVIII deficiency is a rare disorder (approximately 1/1,000,000 population),[37] and much less common than mild hemophilia A. In general, levels of FV and FVIII are both low, in the range from 20 to 40 U/dL. Again, should the clinician request FVIII testing (without FV testing) in a patient with this combined deficiency, the patient may be falsely identified as an FVIII-deficient hemophiliac and subsequently treated with only FVIII concentrate, instead of combined FV/FVIII replacement therapy. The performance of basic screening tests such as a prothrombin time (PT), which would be prolonged because of the FV deficiency, should prompt additional investigations, including mixing tests, which lead to ultimate detection of FV deficiency, in addition to FVIII deficiency.

Hemophilia A or Hemophilia B Potentially Missed

The other side of the coin of a missed alternative diagnosis to hemophilia A or hemophilia B is where these have been discounted because they have been ignored or have been overlooked or where insufficient consideration has been given to alternate possibilities. For example, either hemophilia B (or FIX deficiency) or even FXI deficiency may be missed should the clinician only order FVIII testing (again perhaps because Hemophilia A is the best recognized among the hemophilias). In these cases, the requested test (FVIII) result will likely be normal, and thus hemophilia may be inappropriately discounted. If the patient has a hemophilia-like bleeding disorder, additional testing to FVIII may be required to complete the patient's evaluation.

Another reason that (mild) hemophilia A may on occasion be inappropriately discounted is because FVIII is an acute phase reactant and will be elevated during illness or stress. This may thus "convert" a mild hemophilia phenotype (mildly reduced FVIII levels) into a "normal" phenotype (FVIII levels within a normal range).

Nomenclature and Transcription Issues in Test Ordering

Another less well-recognized pre-preanalytical problem is that of nomenclature and transcription errors, potentially due to dyslexia and the use of roman text for clotting factors. Most laboratory test requests still entail paper-based orders rather than the so-called digital or electronic "order-entry." There have been many instances of incorrect tests ordered by clinicians, and in other cases incorrect tests performed by laboratories, due to errors in ordering or in interpretation of clinical order requests. Several true case examples have previously been reported.[38,39] For example, Page recently described the case of a patient (his mother) with FIX deficiency, who was incorrectly tested for FXI, and subsequently advised by her surgeon that as her levels were close to 100%, surgery was safe to proceed.[38] Fortunately, the well-informed patient advised the surgeon to arrange the appropriate testing (FIX), and surgery was postponed until the situation was clarified. Page gave a few additional examples of similar errors, including the dispatch of FXIII concentrate for an FVIII-deficient patient, and FVIII being incorrectly transcribed as FVII. We have all seen similar examples in our own practice.

Although outside the current focus, it may be worth noting that FV and FX represent other similar common problems in test ordering, wherein the ambiguous requests for "FV" or "FX" can be respectively interpreted as either "FV clotting activity"/"FV Leiden" or "FX clotting activity"/"anti-FXa testing" (the latter test being historically used for assessment of heparin therapy monitoring, and now gaining increasing use to assess levels of the new direct anti-Xa agents such as rivaroxaban and apixaban).[40,41] Any FV clotting factor activity result would preclude correct assessment of thrombophilia where investigation of FV Leiden status was intended, whereas inappropriate testing for activated protein C resistance or genetic analysis for FV polymorphisms would potentially delay invasive procedures in a patient with suspected FV-clotting deficiency.[39,42]

Tests Requested while Patients Are on Anticoagulant Therapy

Although patients with bleeding histories are rarely placed on anticoagulant therapy, this can still occasionally occur. Moreover, patients already on anticoagulant therapy sometimes have a bleeding/bruising event and can subsequently be clinically investigated, including follow-up laboratory testing. More common is the scenario where a clinician (typically a surgeon or anesthetist) orders laboratory testing (e.g., "preoperative screens") on patients who they do not recognize to be on anticoagulant therapy, or where they do not fully appreciate the full effects of these anticoagulants.

In each of these cases, routine coagulation tests will often be (understandably to laboratorians) prolonged, and specific factor assays can also be affected, typically yielding low "abnormal" levels. In particular, heparin therapy can affect all the activated partial thromboplastin time (APTT) associated factors, predominantly FVIII, FIX, FXI, and FXII (but also FV, FII, and FX if testing is performed using an APTT-like assay). Thus, assessment of FVIII or FIX in such patients could feasibly lead to misdiagnosis of hemophilia A or hemophilia B when a breakdown in clinical/laboratory communication occurs.[43,44] For example, a surgeon may follow-up a prolonged APTT previously recorded in a laboratory information system, which is otherwise not identified to have been caused by heparin, by requesting factor assays. Should a low FVIII or FIX be identified, there is a possibility of a (possibly transient) diagnosis of hemophilia, and surgery could be inappropriately delayed.

Similarly, vitamin K antagonist therapy (including warfarin) affects FII, FVII, FIX, and FX. Again, the possibility of a (transient) hemophilia B diagnosis cannot be totally excluded should testing be performed on these patients.[43,44] Finally, the newer oral anticoagulants—those directly inhibiting prothrombin or FXa—lead to variable effects on FVIII and FIX (as well as other clotting factors), depending on reagents and methods used by laboratories.[40,41,45,46] The PT and APTT

screening tests are also variably prolonged in patients taking the new direct acting anticoagulants.[40,41,45–48] There can also be a mistaken belief among clinicians that these drugs do not prolong these tests, as they are designed to not require monitoring. "Abnormal" test results, be they routine coagulation tests or specific factor assays, may thus lead to unnecessary investigations, and a potential misdiagnosis of factor deficiency as a consequence.

ABO Blood Group and Normal Range Effects

The ABO blood group is well known to hemophilia specialists as moderating levels of FVIII. Thus, FVIII levels in O blood group individuals are lower than those of non–O blood group,[49] and there is therefore a small risk of misidentifying patients as having mild hemophilia A because they are O blood group, and where this is not taken into consideration.

Similarly, laboratories may have established a normal range on the basis of an incorrect normal population, and may have set their cutoff value too high. Even should a laboratory set an appropriate normal range, as for example, based on a 97.5th percentile, this range is designed to correctly identify some 97.5% of the normal population. This means that by definition, a small percentage of the normal population will yield FVIII and FIX test results under the lower limit cutoff value. There is therefore a small risk of misidentifying patients as having mild hemophilia A or hemophilia B simply because they yield an FVIII or FIX below the normal range, whether or not that normal range has been correctly defined and whether or not ABO blood group has been considered.

Preanalytical Issues in Factor VIII and Factor IX Testing and Hemophilia Diagnosis

Most preanalytical issues (summarized in ►**Table 3**) are common to all coagulation tests. For example, blood collection in any blood tube other than sodium citrate, including serum, ethylenediaminetetraacetic acid (EDTA) or heparin anticoagulant tubes, will lead to gross prolongations in routine coagulation tests and low levels of clotting factors, including FVIII and FIX.[43,44] Although collection into incorrect blood tubes can be visually identified by laboratories when received as a primary (original blood collection) tube, it will not be so easy to identify inappropriate collections in secondary (aliquot) tubes.[18,19,50] Moreover, whereas routine coagulation tests are often performed "fresh" in central laboratories using centrifuged primary tubes, factor assays are more often performed following processing and separation into secondary aliquot tubes, where serum/citrate anticoagulant plasma/EDTA anticoagulant plasma/heparin anticoagulant plasma are visibly indistinguishable.[18,19,50,51] In many test performing sites, the central processing unit (i.e., performing sample centrifugation and separation) is separate to the specialized hemostasis laboratory (i.e., performing factor assays, etc.).

Sometimes the blood collection tubes received by the laboratories are not what were originally collected. There are several examples from our experience where inexperienced clinical staff have collected blood into one type of tube (e.g., EDTA) and then poured the collected blood into another type (e.g., sodium citrate) for delivery to the laboratory, which has then unwittingly tested the sample as if it were appropriately collected. As another example, inexperienced blood collection staff may have collected several underfilled citrate anticoagulant tubes (e.g., in a difficult collection), and recognizing that the laboratory may reject underfilled tubes addressed the problem by pooling all the collected tubes. Another possibility is the removal of tube stoppers from a series of different primary blood tubes when performing venipuncture with syringes, and then placing one colored stopper (e.g., blue, which typically identifies citrate) on a wrong tube (e.g., blood EDTA or serum). In this case, the laboratory might similarly be unaware that the wrong sample matrix is being processed.

In all of these cases, routine coagulation tests may be variably biased, and factor assays show considerable abnormalities, most frequently reductions. As the clinician is unaware of the collection issues, and will expect the laboratory to provide an accurate test result, a (potentially transient) diagnosis of hemophilia A or hemophilia B is again feasible, and/or surgery may be cancelled or postponed, and/or inappropriate therapy may be applied.

Although there is also a theoretical potential for cross contamination of inappropriate anticoagulants and similar events following "inappropriate" sequence of collections (citrate tube should in theory be collected before other "stronger" anticoagulants such as EDTA and heparin), the risk of this is expectedly less than that achieved by collection into incorrect blood tubes.[52,53]

In addition, considerations include correct blood collection techniques because inappropriate procedures can result in some blood stasis and activation.[19,27,54,55] Similarly, inadequate mixing of citrate blood collection tubes postcollection can result in sample activation and clotting, with resultant effects on coagulation tests including FVIII and FIX.[56,57]

Another preanalytical issue is that of transport of the collected samples to the laboratory. The mode of transportation of samples, the time taken for it, and transport conditions such as temperature may all have an effect on coagulation test results.[19,28,58,59] In particular, FVIII is a temperature labile factor, and reductions in levels may occur when samples are transported at extremes of temperature or for extended time periods. This can lead to a misidentification of (mild) hemophilia A.

Blood samples are centrifuged to separate plasma to be tested from the cellular components. Although evidence in this area is largely lacking, inappropriate centrifugation could plausibly affect later factor testing.[19,28,58,60] Again, extremes in temperature (too high or too low) should be avoided to reduce risk of activation events and loss of factor activity.[61] The effect of possible residual cellular material in subsequently freshly tested or frozen/thawed tested FVIII and FIX has not been greatly explored, although some effects are observed.[62] Furthermore, it is currently unknown whether transportation of separated citrate samples by automated belt systems in facilities using total laboratory automation may to some

Table 3 Summary of preanalytical issues leading to potential misdiagnoses in hemophilia and incorrect therapy applied

Preanalytical issue	Example	Laboratory finding(s)	Diagnostic outcome	Therapy implications	Solution
Incorrect blood tube collected, or underfilled citrate tubes pooled to create filled tubes.	FVIII or FIX testing performed on EDTA or heparin anticoagulant plasma, or on serum, or on excessively citrate anticoagulated plasma.	Prolonged or no clot in routine coagulation tests. Low FVIII and/or FIX levels. Heparin and serum testing may yield levels consistent with severe hemophilia. EDTA plasma will yield low levels of FVIII. Heparin and EDTA anticoagulant plasma will also be reflective of potential factor inhibitors.	Patient (transiently) misdiagnosed as hemophilia A or hemophilia B, or having acquired hemophilia, or factor inhibitors depending on the case.	Surgery may be cancelled or postponed; inappropriate therapy may be applied.	Follow expert and evidence-based guidelines as available. Be aware of possibility of incorrect sample being collected or processed for factor assays. Assess sample for evidence of incorrect sample tested. Request repeat testing using fresh blood sample (and correct collection tube) for confirmation.
Incorrect blood tube collection order and poor collection techniques.	FVIII or FIX testing potentially performed on EDTA or heparin anticoagulant contaminated plasma or activated or clotted sample.	May plausibly affect FVIII and/or FIX levels.			
Inadequate blood tube mixing postcollection.	FVIII or FIX testing performed on activated or partially clotted samples.				
Inappropriate transport of blood samples.	Extremes of temperature, prolonged transport times.				
Inappropriate centrifugation or processing of blood samples.	Platelet contamination may cause changes in FVIII or FIX test data post freeze/thaw.				
Inappropriate storage of (frozen) plasma samples.	Storage of plasma in a standard frost-free freezer.				
Inappropriate thawing and mixing of plasma postthawing	Samples just thawed and inadequately mixed contain a gradation of protein from top to bottom. Testing of such samples can yield falsely low or high levels depending on sampling position.		False diagnosis or exclusion of hemophilia A or hemophilia B.		

Abbreviation: EDTA, ethylenediaminetetraacetic acid.

extent affect the quality of the specimen due to evaporation or partial resuspension of pellet (i.e., platelets).

Storage of plasma samples for later testing may also influence test results.[19,28] In short, frost-free freezers are generally not suitable for long-term storage of samples as these may undergo freeze thaw events to maintain the frost-free environment, adversely affecting FVIII in particular. Finally, inappropriate thawing and insufficient mixing of plasma postthawing can also lead to changes in factor level detection, because such plasma can present a gradation of protein levels, and different factor levels can be identified dependent on where sampling from the plasma takes place.[63]

However, in summary, most of the possible transport or storage preanalytical events noted in this section are likely to lead to *inaccurate* levels of factors being detected, rather than *severe changes* to apparent factor levels, unless complete clotting has occurred (effectively inducing serum rather than plasma). Nevertheless, all these possibilities still require consideration, and the plausibility of false (mild) hemophilia diagnosis remains.

Analytical and Postanalytical Issues in Factor VIII and Factor IX Testing and Hemophilia Diagnosis

There are a large number of analytical issues that will influence laboratory test results, and thus affect the accuracy of FVIII and FIX determinations, with subsequent implications regarding appropriateness of patient management, including applied therapy (summarized in ►**Table 4**).

A general problem that virtually afflicts all areas of laboratory testing is represented by instrumentation failure(s). For example, the presence of an undetected clot or air bubbles in the test sample may lead to incomplete aspiration and thereby falsely decreased values of both FVIII and FIX. Random instrumentation errors may also occur, along with the use of inappropriate calibration curves (e.g., those calculated using different lots of reagents).

Assay Variability/Test Accuracy

Modern instrumentation and appropriate control measures in general promote accurate test results,[22] and clinicians should have confidence in laboratory findings, assuming the laboratory has received an appropriate test sample for analysis. However, there are occasional issues with test accuracy that should be noted. Intra-assay variability and interassay variability reflect the variation of test results obtained by any given laboratory when testing the same sample over time over the same test run (intra-assay) or in different test runs (interassay). Laboratories typically investigate and establish these variations as part of their accreditation requirements.[64,65] For example, interassay variation is captured as part of the ISO 15189 accreditation process, where it is referred to as "measurement uncertainty." For FVIII and FIX, interassay variation measured as a coefficient of variation (CV) tends to be around 5 to 20% (depending on methodology and factor level being assessed).

Interlaboratory variability reflects instead the variation of test results obtained by different laboratories when testing the same sample. This value approximates 15 to 25% for most samples and for most geographies, as shown in ►**Fig. 1** using recent EQA data from three different EQA providers. However, such EQA data also reflect that although most laboratories provide similar results when testing the same sample, there are occasional outliers reflective of inaccurate test data. Reasons for these outlier (inaccurate) results are not always clear, but may include test failures, sample reconstitution issues, sample mismatches or transcription errors. Examples of these events are shown using recent data from three different EQA providers in ►**Figs. 2–4**, which highlight the cross-geographical basis of such issues.

Possible transcription events are shown in ►**Fig. 2** for FIX testing via the RCPA Hematology QAP (►**Fig. 2C**, samples 11 and 12 [arrowed], and ►**Fig. 2D**, samples 7 and 8 [arrowed]). In this EQA, paired samples are sent to participants for each assessment period. For these highlighted (arrowed) sample results, the occasional result for one sample seems closer to the generalized results of the paired sample. Although infrequent, this type of error could also plausibly occur in actual laboratory practice.

Occasional examples of extreme low values reported for samples with normal or only mildly reduced levels of FVIII or FIX are also rarely but consistently observed between the different EQA providers. Examples for RCPAQAP are shown in ►**Fig. 2** (A. sample 2 for FVIII [arrowed]; C. sample 8 for FIX [arrowed]; D. sample 12 for FIX), for ECAT in ►**Fig. 3** (A. sample 2012/3–02 for FVIII [arrowed]; B. samples 2012/3–01; and 2012/3–02 for FIX [arrowed]), and for NEQAS in ►**Fig. 4** (A. samples 11–01 FVIII, 11–05 FVIII, and 12–01 FVIII for FVIII [arrowed]; B. samples 11–05 FIX and 12–05 FIX for FIX [arrowed]). This illustrates the plausibility of occasional false severe, moderate, or mild hemophilia when normal samples have been tested.

Similarly, high outlier values are occasionally observed for low factor level containing samples. An example for RCPAQAP is shown in ►**Fig. 2A** (sample 12 [arrowed]) for FVIII and ►**Fig. 2D** (sample 3 [arrowed]) for FIX. Similar high outlier data can be identified within ECAT (►**Fig. 3**) and NEQAS (►**Fig. 4**) data. These illustrate the plausibility of occasional false misidentification of moderate or mild hemophilia as normal, or moderate hemophilia as mild, and so on.

Lastly, although most samples provide for similar degrees of interlaboratory variation, CVs increase with decreasing factor level (►**Fig. 1**) and some individual samples show considerable variability, so that the range of reported values is very wide. For example, even ignoring the two outlier results (one high, one low) for ECAT sample 2011/2–02, the general data range identified was approximately 25 to 75 U/dL, potentially yielding diagnoses ranging from "mild hemophilia A" to "reduced FVIII" to "normal," should this have reflected a true patient sample tested within different laboratories. Similar examples can be seen for data from the other two EQA programs.

Whatever the situation and cause, the EQA data clearly indicate that occasional issues with accuracy are encountered, meaning that there is a low but plausible chance of diagnostic error in routine laboratory practice. Although severe errors (e.g., reporting of 1 U/dL FVIII in patient with mild hemophilia A, etc.) would be rare ($<$ 1% of test results), "milder errors"

Table 4 Summary of analytical and postanalytical issues leading to potential misdiagnoses in hemophilia and incorrect therapy applied

Analytical issue	Example	Laboratory finding(s)	Diagnostic outcome	Therapy implications	Solutions
Assay variability and accuracy	Refer to EQA data and figures. Intra-assay, interassay, and interlaboratory assay variability.	Levels of FVIII or FIX may on occasion not be accurately determined.	Misdiagnosis of hemophilia, or missed diagnosis of hemophilia, or severity of hemophilia may be incorrectly determined (each are specific case dependent).	Surgery may be cancelled or postponed; inappropriate therapy may be applied.	Be aware of assay limitations. Use the best methodologies available. Perform appropriate internal quality control testing at critical test levels. Participate in external quality assessment. Repeat tests when necessary.
Lower limits of assay detection	Low levels of FVIII or FIX may not be accurately determined.	FVIII and/or FIX levels may be higher or lower than determined.	Severity of hemophilia may be incorrectly determined.	Inappropriate therapy may be applied.	
Methodological issues	One-stage factor assays may overestimate FVIII levels in some hemophilia patients. Chromogenic assays may overestimate FVIII levels for some factor concentrates. Use of LA sensitive reagent for factor assays may yield false low FVIII and/or FIX in LA positive patient.	FVIII and/or FIX may be lower or higher than actually determined by testing.	Misdiagnosis of hemophilia, or missed diagnosis of hemophilia, or severity of hemophilia may be incorrectly determined. Inappropriate level of therapy may be applied.		
Instrumentation failure(s)	Presence of undetected clot or air bubbles in sample may lead to incomplete aspiration.	FVIII and FIX may be higher than reported.			
Transcription errors	Incorrect values reported by laboratory.	FVIII and/or FIX levels may be higher or lower than actually determined by testing.	Misdiagnosis of hemophilia, or missed diagnosis of hemophilia, or severity of hemophilia may be incorrectly determined (each are specific case dependent).	Surgery may be cancelled or postponed; inappropriate therapy may be applied.	

Abbreviations: EQA, external quality assessment; LA, lupus anticoagulant.

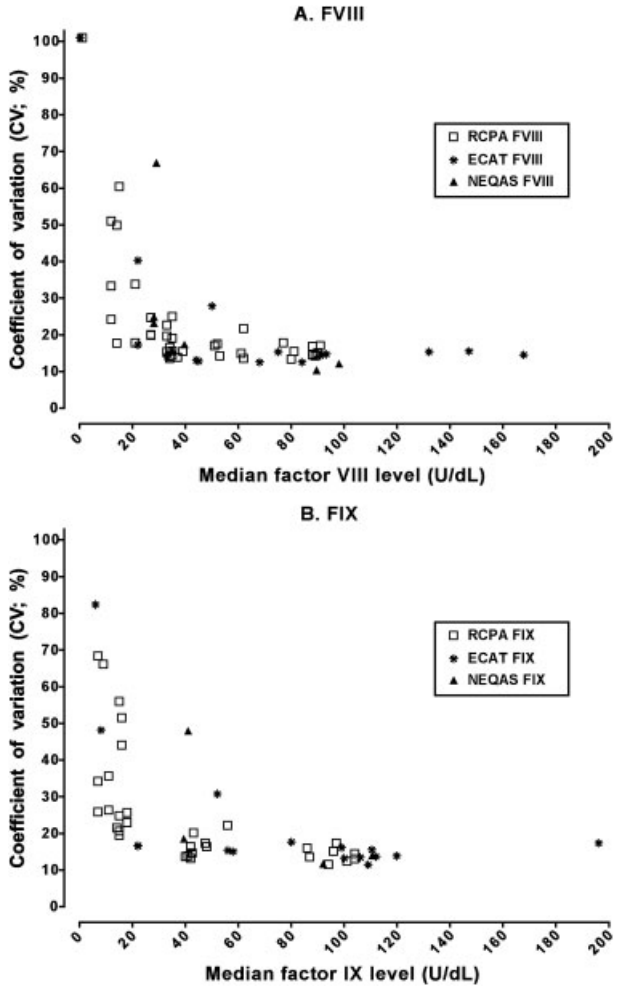

Fig. 1 Interlaboratory assay variability for FVIII and FIX. Data derived from RCPAQAP Haematology, ECAT, and NEQAS EQA programs for 2011 to 2012 inclusive and show interlaboratory assay variability expressed as a coefficient of variation (CV; %; y-axis) versus the median participant reported factor level (U/dL; x-axis). Data for FVIII and FIX are shown respectively in figure parts A and B; however, these show similar patterns, with most CVs ranging from approximately 15 to 25%. CVs tend to increase with decreasing factor level. Similar findings are apparent for each separate external quality assessment program. CVs have been normalized to a maximum of 101 in ►**Fig. 1A** for FVIII. CVs have been calculated from data shown in ►**Figs. 2–4**.

(e.g., reporting of 40 U/dL FVIII in a normal individual, etc.) would be more common due to normal assay variation. This means that clinicians may need to request repeat tests on a fresh sample for confirmation of test findings whenever an underlying test error is suspected. This is particularly important when undertaking an initial investigation on a new patient, or where test results seem at odds with the clinical review findings (e.g., finding of $<$ 1 U/dL FVIII in a patient that only shows bleeding postsurgical challenge, or a finding of 40 U/dL FVIII in a patient that bleeds without challenge).

Lower Limit of Assay Detection

Low factor level samples require even greater care, and in essence a similar but exaggerated scenario to above may ensue. This issue has been previously highlighted for VWD diagnosis,[66] and can also be identified by EQA data for FVIII or FIX when laboratories are asked to assess very low level samples. This is best illustrated when employing a totally factor VIII or FIX-deficient plasma, or a sample from a severe hemophilia patient, and where laboratories often return results above 1 U/dL. Examples are shown for FVIII in ►**Fig. 2A** (samples 6 and 12) for RCPA and ►**Fig. 3A** (samples 2011/3–02 and 2012/3–01) for ECAT. As detection of FVIII and FIX values at the low end of the calibration curve is essential (e.g., for differentiating moderate from severe hemophilia or for therapeutic monitoring), caution should be used by laboratories before validating values obtained with standard calibration curves and, eventually, specific extended calibrations should be performed to bring the instrument readings within the range of linearity. The APTT reagent chosen for performance of one-stage FVIII assays can also influence the lower level of detection of the FVIII in the assay system. This was shown in an evaluation of APTT reagents, which found 2 of 11 APTT reagents unable to detect FVIII activity of less than 1 U/dL, hence being unsuitable for making a diagnosis of severe hemophilia A.[15]

Inappropriate Follow-up of Abnormal Routine Coagulation Tests

Abnormal routine coagulation tests are often followed up by additional testing, either laboratory generated (e.g., by "reflex" testing) or by clinical ordering. How the laboratory responds to the initial abnormal test results has the potential to cause similar issues to those previously identified for clinical ordering. Different approaches are called for, depending on initial test results, and whether there is an isolated APTT prolongation, an isolated PT prolongation, combined PT and APTT prolongations, and whether these prolongations correct or not upon mixing. Several different potential approaches to investigations using algorithms are available in the literature[67–69] (also, K. Marchant, unpublished data on Algorithmic approaches to hemostasis testing, 2013). However, should laboratories undertake an incorrect approach, this may lead to an incorrect conclusion. For example, a prolonged PT and APTT may be followed up by testing of only FVIII, FIX, FXI, and FXII. This may show a low FVIII, and hence a potential diagnosis of hemophilia A. However, alternate explanations could be a combined FV/FVIII deficiency, or testing of EDTA plasma.

Issues with Factor Inhibitor Testing

Inaccuracies in factor inhibitor testing are also evident from EQA data. Each of the three EQA programs participating in this report have recently published their experience in this setting,[43,44,70–76] reporting on both preanalytical and analytical issues. For the former, a similar experience to that outlined previously for factor assays also exists for inhibitor assessment. For example, a high level FV inhibitor will be misidentified as an FVIII inhibitor by many laboratories, should FVIII inhibitor testing only be performed.[43,44] This can arise in clinical practice simply because of better recognition of FVIII inhibitors (compared with FV inhibitors, etc.) and following a specific clinical request (for "FVIII inhibitor" testing), or

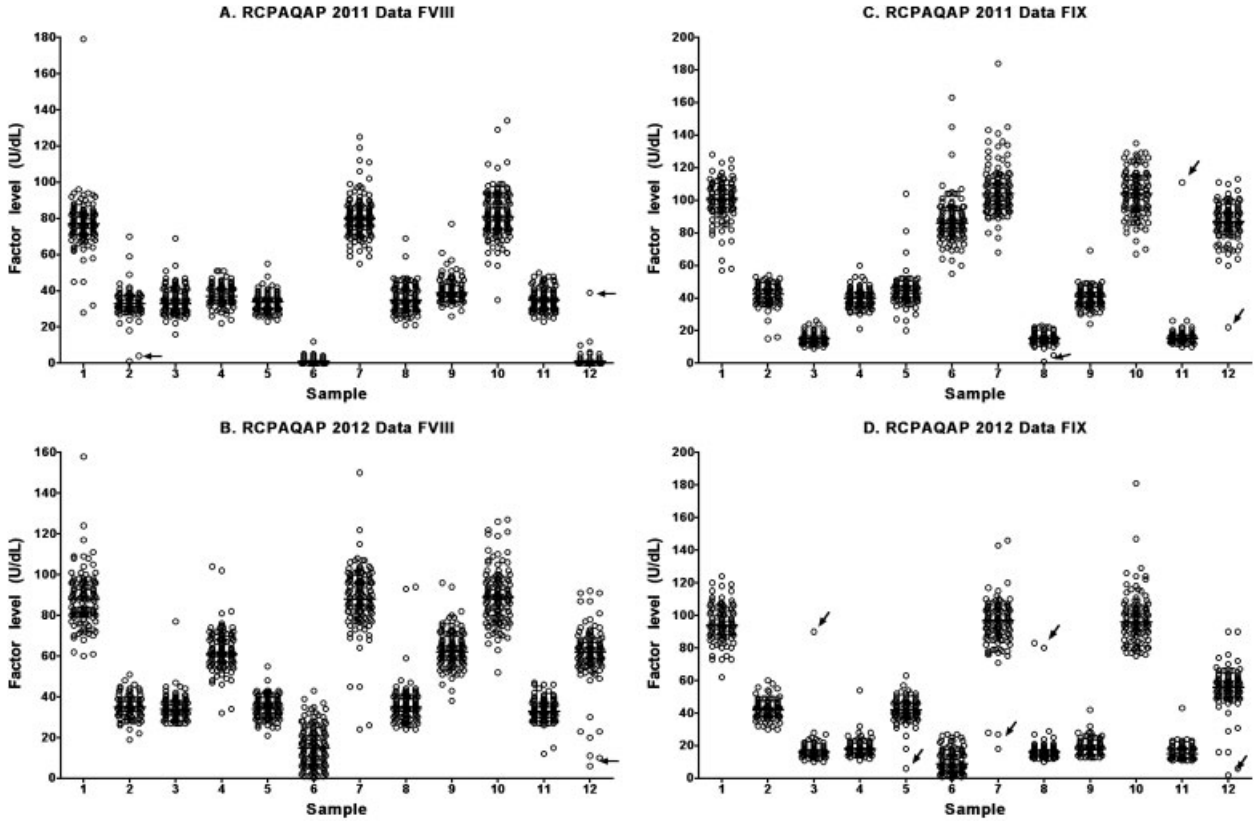

Fig. 2 Participant reported factor levels for samples distributed by the RCPAQAP Hematology for 2011 to 2012 inclusive. Figure shows data for sequential years and factors as follows: A, 2011 FVIII; B,2012 FVIII; C, 2011 FIX; and D, 2012 FIX. Participant reported factor levels are shown on the y-axis (U/dL), and sample number on the x-axis. Small horizontal bar in each data set indicates median value for set. The RCPAQAP Hematology currently distributes 12 samples per year to participants for both FVIII and FIX testing. Samples are distributed in pairs and participants perform testing bimonthly; the same samples are tested for FVIII and FIX. Samples are commercially prepared to provide a range of factor levels, from totally deficient (examples are samples 6 and 12 in ►**Fig. 2A** for FVIII) to mildly reduced to normal levels. Several interesting observations can be made from this data, as outlined extensively in the text. Arrows mark data points of special interest.

because the laboratory selectively performs such testing on a generic ("inhibitor assessment") request, or as a follow-up to previous abnormal routine coagulation test results or mixing studies. These events are more likely to generate suspicion of an acquired hemophilia than identification of an FVIII inhibitor in a patient with hemophilia A. A false identification of an FVIII inhibitor can also arise following testing of EDTA or heparin anticoagulated normal plasma, or from testing of (normal) serum.[43,44]

In terms of analytical issues, the various EQA groups have all reported significant interlaboratory variation, as reflected by high-interlaboratory CVs, which are often greater than 30%.[70–74] Occasional outlier results are also sometimes reported, and which may or may not reflect transcription errors. Occasionally, laboratories also fail to recognize the presence of an inhibitor when one is present. Some examples of these problems are shown in ►**Fig. 5**. In general, less variability is seen with Nijmegen method-based inhibitor assays compared with standard Bethesda assays (see ►**Fig. 5A** and **5B** for examples using recent RCPAQAP and ECAT data; however, all EQA data previously reported by RCPAQAP, ECAT, and NEQAS are also in support of this statement).[70–74] Sometimes laboratories falsely identify low-level inhibitors when these are absent (e.g., ECAT sample 2011/4–01 in ►**Fig. 5B**), or they fail to identify low-level inhibitors when present (see examples in ►**Fig. 5A**; where occasional values are reported as "0" for inhibitor positive samples). Again, these problems appear to reflect a bigger problem with standard Bethesda assays than with the Nijmegen method-based assays (►**Fig. 5**), with this statement also supported by data previously reported by ECAT, NEQAS, and RCPAQAP.[70–74] An FVIII inhibitor wet workshop has recently found that inhibitor titers can be better standardized when all participants use identical test systems.[76] Apart from progressively standardizing assay conditions such as use of the Nijmegen assay, a large reduction in CVs between participant results was achieved when a standard set of sample dilutions was used, strongly suggesting that inappropriate dilution(s) of test plasma contribute to the large interlaboratory CVs seen in various EQA surveys.

A clinically important inhibitor level "cut-off" is that related to differing types of treatment in congenital hemophilia A, generally accepted as 5.0 BU/mL.[16] Results of testing for inhibitor samples around this value show significant interlaboratory variation, so that different laboratories will report values either below or above 5.0 BU/mL, and this underscores the possibility of different treatments being applied to these hypothetical patients in different

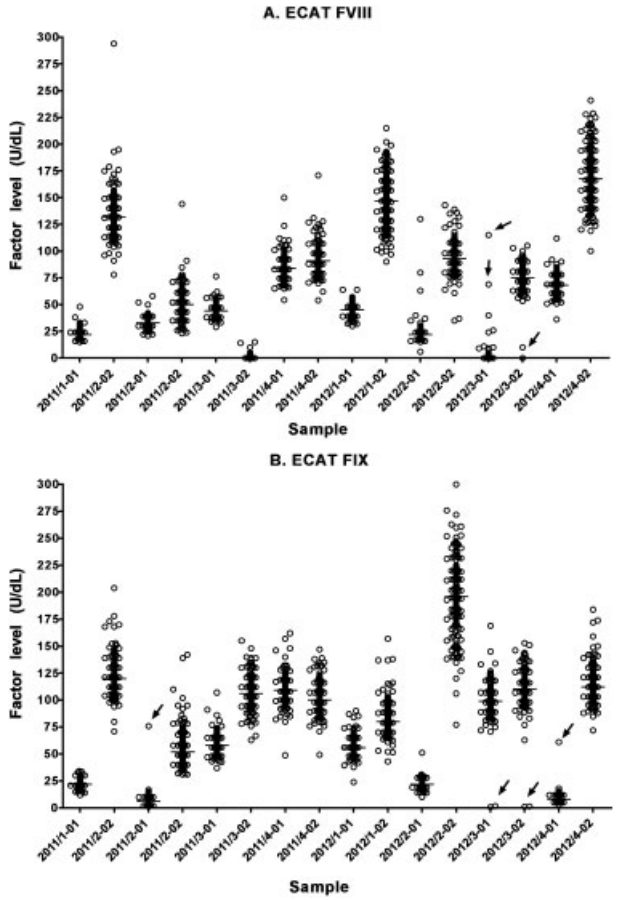

Fig. 3 Participants reported factor levels for samples distributed by ECAT for 2011 to 2012 inclusive. Figure shows data for FVIII in part A, and for FIX in part B. ECAT currently distributes eight samples per year to participants for both FVIII and FIX testing. Participants reported factor levels are shown on the y-axis (U/dL), and sequential sample number on the x-axis. Small horizontal bar in each data set indicates median value for set. The same samples are tested for FVIII and FIX. Samples are commercially prepared as well as patient based to provide a range of factor levels, from totally deficient (an example is sample 2011/3–02 in Fig. 3A for FVIII) to normal levels. For generation of this figure, data originally expressed as "<" a given value has been corrected down by one digit (for example, < 10 has been corrected down to 9 and < 1 has been corrected down to 0). Several interesting observations can be made from this data, as outlined extensively in the text. Arrows mark data points of special interest.

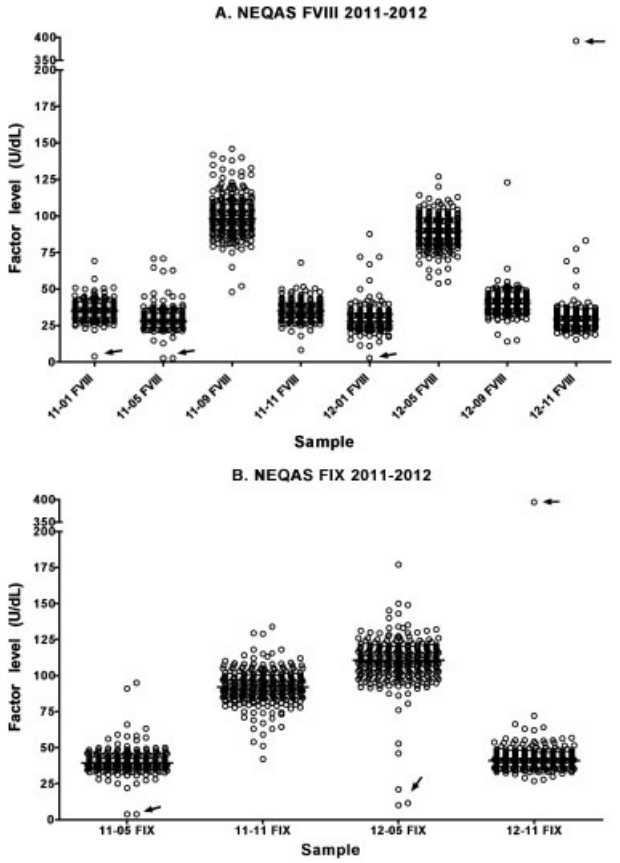

Fig. 4 Participant reported factor levels for samples distributed by NEQAS for 2011 to 2012 inclusive. Figure shows data for FVIII in part A, and for FIX in part B. NEQAS currently distributes four samples per year to participants for FVIII testing and two samples per year for FIX testing. Participant reported factor levels are shown on the y-axis (U/dL), and sequential sample number on the x-axis. Small horizontal bar in each data set indicates median value for set. Samples are distributed separately for factor VIII and IX assays and are from patients with congenital defects or from normal donors. Several interesting observations can be made from this data, as outlined extensively in the text. Arrows mark data points of special interest.

hospitals. This was evident in data from the EQAs as previously published,[70–74] and illustrated in this report by both ECAT data (►**Fig. 5B**) as well as by a recent case from NEQAS (►**Fig. 5C**) with sample S11:04, which was distributed to 117 participants. The median reported value was 5.0 BU, and the range was 2.4 to 15.5 for an interlaboratory CV of 41%. Notably, however, the same proportion of participants (nearly 40%) reported values above and below 5 BU/mL.

Finally, there is limited data for FIX inhibitor assay variability in the literature, and so recent data from ECAT are shown in ►**Fig. 5D**. This EQA provider, recognizing that distinction between Bethesda and Nijmegen procedures are often misunderstood, and therefore misreported by participant laboratories,[70–74] has begun to gather data differentially according to "buffering" or not of plasma. For FIX inhibitor testing, the same issues previously identified for FVIII inhibitors also hold true, namely, significant interassay variability, high outlier data reported, and no inhibitor identified in inhibitor positive samples (►**Fig. 5D**). Moreover, although data are limited, performance of inhibitor assays using buffered normal plasma for mixing—particularly "home buffered plasma"—appears to provide the best outcomes. A summary of pre-preanalytical, preanalytical, analytical, and postanalytical issues related to inhibitor analysis is given in ►**Table 5**.

Other Methodological Issues

Underestimation and Overestimation of Factor Levels According to Methodologies

Factor levels are classically evaluated by factor assays using one-stage clotting assays, and more rarely by two-stage

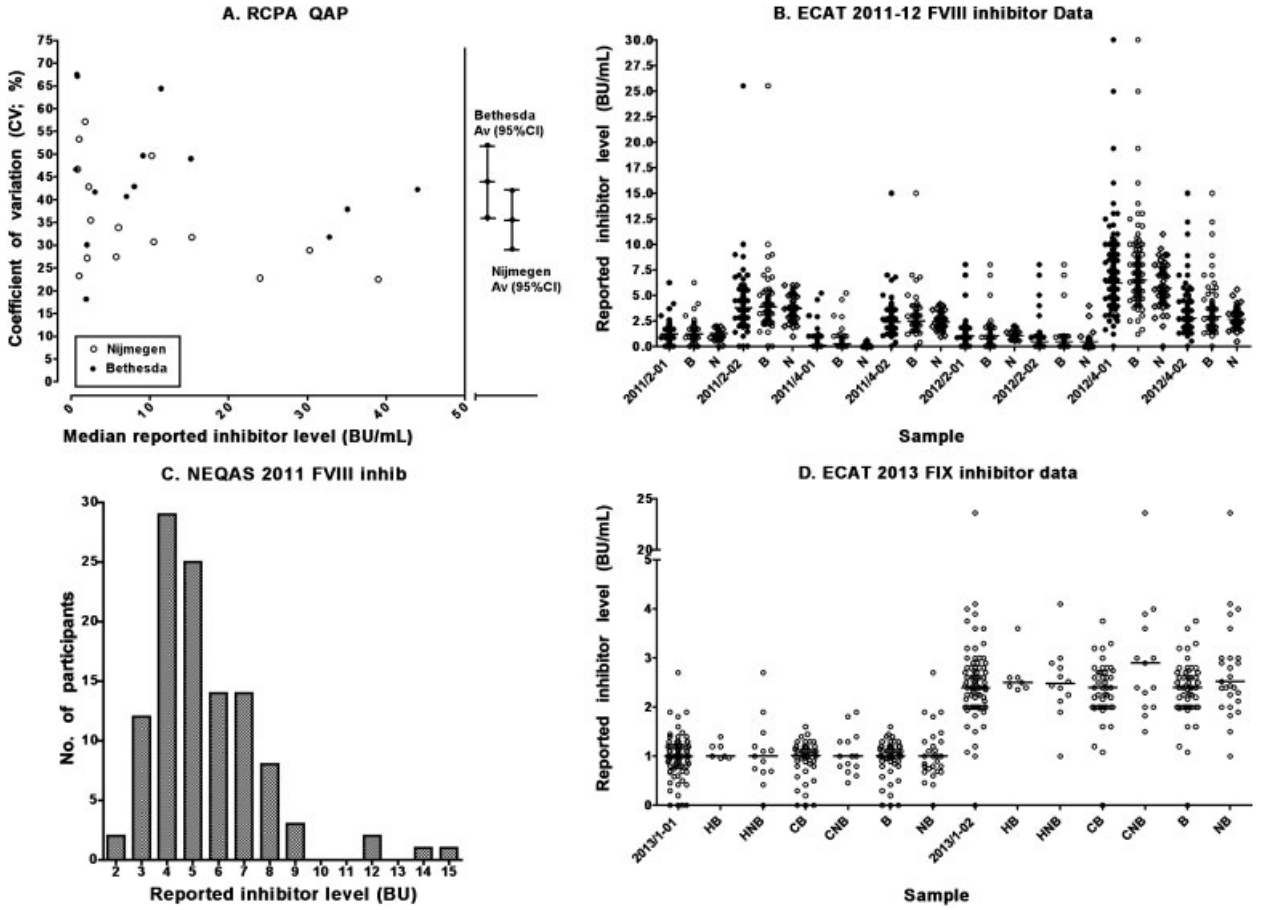

Fig. 5 Recent external quality assessment (EQA) data related to factor inhibitor detection. (A) Summary data for RCPAQAP showing coefficient of variation (CV, %; y-axis) plotted against median participant reported inhibitor level (in Bethesda Units [BU]/mL; x-axis) for all samples distributed over the past 6 years, and shown separately for Bethesda or Nijmegen methods. The average and 95% confidence intervals of the mean of all data are shown on the right of the figure. (B) Summary data for ECAT for FVIII inhibitor testing showing the participant reported inhibitor level (y-axis; BU/mL) for samples distributed in 2011 to 2012 inclusive (identified on x-axis), with separate data also shown for the same samples where laboratories reported to using a Bethesda (B) or Nijmegen (N) method. Several interesting observations can be made from this data, as outlined extensively in the text. In particular, note the high variability in reported data, that laboratories occasionally reported no inhibitor (values of "0") when inhibitors were present, and occasionally reported inhibitors when no inhibitor was present (e.g., sample 2011/4–01). These issues are in general more apparent with standard Bethesda assay methods. Note also, the variation around the clinically important region of 5 BU, especially sample 2012/4–01. (C) Recent data from NEQAS for sample S11:04, which was distributed to 117 participants. The median reported value was 5.0 BU, and the range 2.4 to 15.5, for an interlaboratory CV of 41%. Notably, however, the same proportion of participants (nearly 40%) reported values above and below 5 BU. (D) Recent data for ECAT for FIX inhibitor testing showing the participant reported inhibitor level (y-axis; BU/mL) for two samples distributed in 2013 (identified on x-axis), with separate data also shown for the same samples where laboratories reported to using buffered plasma or not for mixing. NB: HB, home buffered; HNB, home not buffered; CB, commercial buffered; CNB, commercial not buffered.

clotting assays or chromogenic assays for FVIII. There are additional issues related to these methodologies.[77] For example, one-stage FVIII clotting assays overestimate FVIII levels in some hemophilia patients with certain genetic mutations, potentially rendering a misdiagnosis of patients with hemophilia A as not having hemophilia, or a perceived lessening of hemophilia severity.[78,79] The extent of this problem is unclear, but in one case series described by Duncan et al,[78] 29 of 74 patients (39%), from 12 of 30 families (40%) with mild-to-moderate hemophilia, had a one-stage FVIII clotting assay result that was consistently twofold to sevenfold higher than the two-stage FVIII assay result. In another recent case series, assay discrepancy was observed in 31% of tested individuals (12% with lower two-stage FVIII:C and 19% with lower one-stage).[79] However, an individual's genotype did not always predict their phenotype. As per the earlier study,[78] chromogenic assays were shown to be a suitable alternative to the two-stage.[79]

In contrast, the use of chromogenic FVIII assays may instead comparatively overestimate posttherapy FVIII levels when using certain factor concentrate products.[77] Although there is some ongoing debate about the clinical significance of these observations, and better "accuracy" in test results can be obtained using specific reference standards when evaluating samples from patients treated with some products (for example B-domain deleted FVIII concentrates), it is still nonetheless important to recognize that discrepancies and variation in FVIII levels will be observed between laboratories according to methodology employed and factor concentrate product tested.

Table 5 Summary of pre-preanalytical, preanalytical, analytical, and postanalytical issues related to inhibitor analysis

Issue	Example	Laboratory finding(s)	Diagnostic outcome	Therapy implications	Solutions
Pre-preanalytical	Similar to those listed for factor assessment: e.g., (a) transcription errors; (b) clinician orders FVIII inhibitor assay for patient with FV inhibitor, or (c) laboratory decides to assess FVIII inhibitor assay in patient with FV inhibitor because of prolonged APTT and results of mixing studies showing incomplete correction.	Low level "FVIII inhibitor" can be falsely identified in sample with strong FV inhibitor.	Misdiagnosis of acquired hemophilia A.	May receive FVIII concentrate instead of treatment for FV inhibitor.	Similar to those listed for factor assessment: Take care to check tests ordered or results received for accuracy. Consider other possibilities as a differential diagnosis.
Preanalytical	Similar to those listed for factor assessment: e.g., testing for FVIII inhibitor assay using EDTA or heparin anticoagulant plasma or serum; testing for FVIII inhibitors in samples from patients recently treated with by-passing agents.	Low level "FVIII inhibitor" can be falsely identified; presence of by-passing agents can lead to a false-negative result or underestimation of FVIII inhibitor level.	Misidentification of inhibitors or incorrect inhibitor level when present.	Patient may receive inappropriate treatment.	Similar to those listed for factor assessment: follow expert and evidence-based guidelines and be aware of possible incorrect sample issues. Request repeat testing using fresh sample for confirmation.
Analytical	Inhibitor assay variability and accuracy. Refer to EQA data and figures. Intra-assay, interassay, and interlaboratory assay variability.	Inhibitor levels for FVIII or FIX may on occasion not be accurately determined.	Misidentification of inhibitors and/or level		Similar to those listed for factor assessment: Be aware of assay limitations and use the best methodologies available. Perform appropriate internal quality control and participate in external quality assessment. Repeat tests when necessary.
	Lower limits of assay detection. Low-level inhibitors may not be accurately determined.	Inhibitor levels may be higher or lower than determined.			
Methodological issues	Nijmegen vs. Bethesda, buffered plasma vs. nonbufferred plasma, (etc.) issues.				
Postanalytical	Transcription errors—Incorrect values reported by laboratory.				Request repeat testing using fresh sample for confirmation.

Abbreviations: APTT, activated partial thromboplastin time; EDTA, ethylenediaminetetraacetic acid; EQA, external quality assessment.

Differential Sensitivity of APTT Reagents to Factor Levels

The APTT is sometimes used as a screening test for assessment of hemostatic integrity including factor deficiency.[80] In this context, an APTT above the upper limit of the normal reference range may be taken as evidence for the presence of a factor deficiency, and an APTT below the upper limit of the normal reference range may be taken as evidence for absence of a factor deficiency. Although we and others[81,82] would urge caution here, given the relative insensitivity of the APTT to mild deficiencies of both FVIII and FIX, and the normal reference range effect previously noted in the section "ABO Blood Group and Normal Range Effects," we feel it worth also mentioning that different APTT reagents may have variable sensitivity to factor levels,[83] as also most recently explored by Bowyer et al.[84] It was also recently shown that severe hemophilia A patients could not be diagnosed with some specific APTT reagents.[15]

Also important here is the consideration of testing lupus anticoagulant positive samples for FVIII and FIX. Use of a lupus anticoagulant sensitive APTT reagent may yield falsely low FVIII and/or FIX levels in such a patient sample, and thus false identification of deficiencies.

Evolving Methodologies

There has been much recent interest in the use of "new" methodologies for assessment of hemophilia, or hemophilia severity, or treatment. These methodologies include thrombin generation and thromboelastography.[79,85–95] Although individual groups have shown utility of such methodologies within controlled studies, comparative or cross-laboratory data for these methodologies, and thus evidence for broader utility, are largely lacking. Recent data evaluating cross-laboratory testing using thromboelastography have recently been published by NEQAS.[95] An FVIII-deficient plasma generated results of "no clot formed" and an interpretation of abnormal for all laboratories ($n = 17$) participating in a trial exercise using the TEG instrument (Thromboelastograph; Haemoscope Corp., Niles, IL).[95] For the ROTEM instrument (Rotation Thromboelastometer; Pentapharm GmbH, Munich, Germany), some clot data were obtained by participants ($n = 10$), and this showed variability with CVs generally around 15 to 25% (►**Table 6**).

Another study evaluating specific and global coagulation assays in the diagnosis of discrepant mild hemophilia A identified poor sensitivity to hemophilia by thromboelastometry.[79] Calibrated automated thrombography, however, supported the results obtained by the two-stage and chromogenic assays.

Discussion and Conclusion

This report has outlined various problems in laboratory assessment for hemophilia, which may occur at any stage of the diagnostic process, and which are by no means limited to errors caused within the laboratory. These may in turn lead to incorrect diagnosis or exclusion of hemophilia, to an incorrect assignment of hemophilia severity, or to misidentification of factor inhibitors, and subsequent inappropriate patient management. We have also attempted to provide various strategies or solutions to overcome the challenges of diagnosis (►**Tables 1–4**). While some outlined solutions are specific to the potential errors related to hemophilia, others are general in nature and can be applied to other areas of laboratory testing. Key to improvement in this area is adoption of best practice by all involved, including clinicians, phlebotomists, and laboratories.

Also key is the recognition that such errors may occur, and thus that clinicians need to assess laboratory results in the context of their patient's clinical history and follow-up potential misdiagnoses by requesting repeat testing on a fresh sample. Electronic ordering may also assist in reducing errors by removing several steps where errors may occur because of transcription errors or misinterpretation of clinical requests.

Laboratories, as part of their strategy for appropriate investigation of abnormal routine screening tests should consider the need to follow logical algorithms, particularly when investigating a bleeding disorder[67–69,96] (also, K. Marchant, unpublished data on Algorithmic approaches to hemostasis testing, 2013). Different approaches are called for depending on initial test results, and whether for example there is an isolated APTT

Table 6 Recent data from NEQAS relating to thromboelastography testing of FVIII-deficient plasma

	INTEM			HEPTEM			EXTEM		
	Median	CV (%)	Range	Median	CV (%)	Range	Median	CV (%)	Range ($n = 7$)
CT (sec)	565	17.5	329–659	601	8.1	520–662	43	204	32–849
Angle (degrees)	36	16.2	31–46	30	18.7	28–42	77	22	27–79
MCF (mm)	15	15.9	12–20	14.5	21.2	13–21	15.5	12.6	13–19

Note: Ten laboratories participated in this trial exercise by testing a FVIII-deficient plasma (FVIII < 1.0 U/dL) using the ROTEM (Rotation Thromboelastometer; Pentapharm GmbH, Munich, Germany) instrument. Data are shown for various test procedures (INTEM, HEPTEM, EXTEM) and various parameters (CT, Angle, MCF) as performed on this instrument. Table shows median, coefficient of variation (CV, %) and range of data for each procedure and parameter.

CT (clotting time): The time taken for a clot starting to form measured in seconds.

Angle (degrees): A measure of the slope of the curve once clotting has begun.

MCF (maximum clot firmness): A measure of the maximum size of the clot measured in millimeters.

In contrast, a FVIII-deficient plasma generated results of 'no clot formed' and an interpretation of abnormal for all laboratories ($n = 17$) participating in a similar trial exercise using the TEG instrument (Thromboelastograph; Haemoscope Corp., Niles, IL).

More extensive data for this EQA and thromboelastography have been reported elsewhere.[95]

prolongation, an isolated PT prolongation, combined PT, and APTT prolongations, and whether these prolongations correct or not upon mixing. There may be additional utility in assessing other routine coagulation tests such as thrombin clotting time (TCT) and fibrinogen, which will help to identify potential anticoagulant interference or fibrinogen deficiency. Indeed, the core set of routine coagulation tests (PT, APTT, TCT, and fibrinogen) plus results of mixing tests greatly inform on subsequent testing to establish the differential existence of factor deficiencies, factor inhibitors, incorrect sample matrix (e.g., serum or EDTA), or potential anticoagulant interference (e.g., heparin, warfarin, new direct oral anticoagulants). Several helpful examples are available in the literature, that describe different potential approaches to investigations using algorithms[67–69] (also, K. Marchant, unpublished data on Algorithmic approaches to hemostasis testing, 2013).

Also of critical importance here is education of clinicians, not only for understanding the challenges, but also to enter diagnostic partnerships with their laboratories, providing them with as much information on their patient as possible, which will act not only to minimize errors but also to maximize diagnostic efficiencies (K. Marchant, unpublished data on Algorithmic approaches to hemostasis testing, 2013). In turn, laboratories need to enter diagnostic partnerships with their clinical referrers, providing guidance where needed, and full interpretive comments on all test results. This need would hold true for both phenotypic assays (K. Marchant, unpublished data on Algorithmic approaches to hemostasis testing, 2013) and genotyping/molecular analysis (D. J. Perry, T. Cumming, A. Goodeve, et al. Unpublished data on The UK National External Quality Assessment Scheme (UK NEQAS) for heritable bleeding disorders, 2013).

Acknowledgments

The authors thank all participants in the different EQA programs, RCPAQAP Haematology, ECAT, and NEQAS.

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