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Hot topics in coagulation testing: Important considerations for testing children for bleeding/thrombotic disorders.

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Hot topics in coagulation testing: Important considerations for testing children for bleeding/thrombotic disorders.

Abstract

The accurate use and interpretation of diagnostic investigations are essential for safe and effective patient care. Appropriate application and interpretation of coagulation testing can be challenging and many controversies exist relating to the standardisation of testing procedures, the application of relevant tests to different patient populations, and the interpretation of test results. We present a list of the most prominent controversies in coagulation testing and have selected three specific examples (age-appropriate reference ranges, therapeutic anticoagulation monitoring, and tests of thrombin generation) for closer discussion, highlighting examples with a paediatric framework. We discuss the limitations of discrete age-partitioned reference intervals, given the established principle of developmental haemostasis; the difficulties in establishing normative data across different laboratories; important pre-analytical variables affecting coagulation testing; the challenges in interpreting APTT and Anti Xa assays for monitoring unfractionated heparin therapy in different clinical situations; and the limitations in interpreting tests of thrombin generation due to current available thrombin specific substrates and the complicating factor of variable alpha2-macroglobulin levels. These controversies are demonstrated using paediatric examples, but raise important implications for coagulation testing in patients of all ages and highlight the pressing need for further research in these areas.

KEYWORDS

Coagulation testing, reference ranges, therapeutic anticoagulation, thrombin generation, developmental haemostasis

Introduction

The three aims of diagnostic investigations are 1) to support or refute clinical suspicion of a particular disease; 2) to assist in clinical management of disease; and 3) to monitor disease trajectory. Diagnostic error (i.e. a missed, incorrect or delayed diagnosis) contributes to

around 10% of patient deaths and between 6-17% of hospital adverse events[1]. Roughly half of all medical negligence claims against general practitioners in Australia are related to diagnostic errors[2] and diagnostic errors have been shown to be responsible for more adverse events than medication errors[3]. Ensuring the judicious application and interpretation of diagnostic investigations is therefore an essential component of patient care.

Issues relating to the accuracy of diagnostic investigations are of major significance within the field of coagulation testing, where there remain many controversies related to the standardisation of test procedures; clinical utility of various tests; application of tests to specific populations; and interpretation of the test results. In many instances advances in therapy have outpaced advances in laboratory capabilities such that historic tests are being used to monitor therapies for which they were never designed.

Table 1 highlights a number of current controversies in coagulation testing. Detailed examination of each is beyond the scope of this paper but we have selected three examples – age-appropriate reference ranges, anticoagulation monitoring, and tests of thrombin generation – to highlight the ongoing need for research in this field in order to optimise outcomes for patients with disorders of coagulation. While each of these examples is relevant to patients across all ages, their problems are particularly well demonstrated in the paediatric setting and we have therefore elected to highlight examples within a paediatric framework.

Table 1: *Current controversies in coagulation testing*

Age-appropriate reference ranges

Although disorders of coagulation affect children as well as adults, the physiology of paediatric haemostasis differs from that of adult haemostasis and is subject to age-related

developmental change from fetal life throughout adulthood[4–8]. The principle of developmental haemostasis has even been demonstrated in centenarians, whose coagulation profiles differ from those of younger adults[9,10], but as with other organ systems the rate of developmental change is most evident in childhood.

To date there have been limited paediatric-specific clinical trials in coagulation disorders and many diagnostic and therapeutic interventions currently used in children have been based on adult data. The Scientific and Standardization Committee of the International Society on Thrombosis and Haemostasis highlighted examples of misdiagnosis resulting from the use of adult laboratory reference intervals in the paediatric setting. For example, the use of adult reference ranges for activated partial thromboplastin time in children aged 1-5 leads to almost a third of results being defined as abnormal despite being within appropriate age-matched limits[11].

What is normal?

The decision to define a laboratory test result as normal or abnormal is most commonly made by comparing the result to predetermined reference intervals. This process presupposes that a) the patient and the population from which the reference interval were derived are comparable; and b) a laboratory using the reference interval would have derived the same results using their own instruments and reagents. The impact of specific variables such as ethnicity [24],[25], age and gender must be factored into normative reference intervals as they may contribute to both over- and under-detection of disease.

As children are physiologically distinct across the multiple developmental stages between intrauterine life and adolescence, laboratory reference intervals most commonly employ discrete partitioning of data according to set age ranges to allow the comparison of infants to infants, pre-schoolers to pre-schoolers, adolescents to adolescents and so on. Such partitioning creates artificial time points that may confound result interpretation for children who are on the verge of changing age cohorts. Physiologically, the data comprising normal developmental ranges are continuous in nature, and would be better represented by continuous age-related centile charts similar to those used historically by the Centre for Disease Control (CDC) and World Health organisation (WHO) for anthropometric data (height,

weight, head circumference). Recent literature has demonstrated the ability of large-scale data collection to create continuous reference intervals for a range of laboratory tests[12,13].

More than thirty years ago, Dr Maureen Andrew et al in Canada were the first to demonstrate the significant age-related variation in coagulation protein concentrations. Their derived reference intervals have to date been applied in discrete age-partitioned format and still suffer from a lack of evidence in some paediatric subgroups such as premature infants[14–16]. There remains a scarcity of evidence proving clinical relevance of out-of-normal-range results in the paediatric setting. While there are some haemostatic disorders for which clinical phenotype and management may be predicted by a specific out-of-range result (e.g. severe haemophilia[17,18]), the significance of other abnormal results (e.g. low protein C levels in premature infants[19–21] and normal vWF concentration at birth) may be less clear. For instance, despite a family history a diagnosis of type I von Willebrand disease may not be possible at birth due to physiological increase in vWF concentration. If there is clinical suspicion testing should be performed when the child is older.

Establishing normative data in the laboratory

Few reference interval studies are performed in normal healthy children due to perceived difficulties in recruitment. For individual laboratories – especially smaller laboratories that perform only occasional paediatric testing – it is impractical to establish extensive site-specific normative datasets across all age ranges for every test, which would require sufficient numbers of healthy controls at every age throughout childhood. However, coagulation testing is known to be subject to analytical variability between instruments and reagents[22], and as such reliance on historical data or data from laboratories using disparate testing systems may compromise the interpretability of test results. Monagle et al demonstrated in 2006 that absolute values for a range of coagulation assays across a paediatric cohort in Melbourne, Australia differed from the values obtained by Andrew et al in the 1980s, although the underlying principle of developmental haemostasis was reaffirmed[23]. Constant reassessment of reference intervals as equipment and reagents change is required, and the need for laboratories to correlate locally obtained data with any standardised normative values.

So what are the solutions?

The increasing use of electronic medical record systems should make the introduction of continuous reference intervals possible, and end users need to push software suppliers for this functionality. Laboratories need to be transparent about the origin and limitations of the reference intervals they are using, especially with respect to analyser and reagent specificity. Smaller laboratories need to give serious consideration to sending out paediatric samples to larger labs that have accurate age appropriate reference intervals rather than processing in house. In the longer term, we need a more sustainable way of maintaining up to date analyser and reagent specific reference interval data. Perhaps the responsibility for this should sit with manufacturers who should be providing equipment and reagent that is fit for purpose. Similar issues with industry reticence to pursue specific paediatric data have been encountered in the pharmaceutical industry in the past. The 1997 Food and Drug Modernization Act provided incentives for companies to conduct trials with paediatric patients and has led to hundreds of paediatric-specific pharmaceutical trials. While the Act and its implementation has led to some complex ethical arguments[26] which are beyond the scope of this paper, a similar incentive program for manufacturers of laboratory *in vitro* diagnostics (IVDs) and information systems might facilitate the development of appropriate paediatric reference intervals. If implemented in partnership with clinical care providers and academic institutions, such a scheme could address the current scarcity of quality paediatric normative data, leading to improved clinical care and better outcomes for these patients.

Therapeutic anticoagulation monitoring

Diagnostic investigations are utilised for therapeutic anticoagulation monitoring to assist with the clinical management of thrombotic disorders. The controversies regarding anticoagulation monitoring are related to specific test choice, the clinical utility of the test and the interpretation of results. These issues occur across all age groups but are more prominent in children. In particular, pre-analytical variables due to sample acquisition and physiological developmental haemostasis are important factors affecting coagulation testing and therapeutic anticoagulation monitoring in the paediatric population.

What are the pre-analytical variables in children?

Pre-analytical variables affect coagulation testing in both adults and children. Estimates are that 63% of all laboratory errors are due to pre-analytical variables from sample collection,

transport and processing[27]. In children, the main challenge is the difficulty in obtaining a clean and sufficient blood sample volume. The blood samples are often collected via peripheral or central lines leading to sample activation or heparin contamination. The collection tubes are sometimes under-filled and therefore the citrate-to-blood ratio of 9:1 is not achieved. Consideration is needed regarding physiological changes that occur with age; for example, neonates have relative polycythaemia, so the blood sample in these patients need to be collected in an adjusted tube[28].

We have previously discussed developmental haemostasis in the context of understanding normal, but the concept also impacts on anticoagulation monitoring because if the therapeutic target range is static across ages, then the percentage increase achieved by the therapy will be variable. This is most obvious for any anticoagulant monitored via an Activated Partial Thromboplastin Time (APTT) which has large age-related changes in normal values[11,28].

Controversies with monitoring of unfractionated heparin

There are many anticoagulant drug options available for the management of thrombotic disorders. **Table 2** highlights the common anticoagulants used in clinical practice, their mechanism of action, methods of monitoring in the laboratory, drug related issues and specific considerations in the paediatric population.

Table 2: *Common anticoagulants used in clinical practice*

Unfractionated heparin (UFH) is an anticoagulant used in clinical practice for the management of acute thrombosis in both adults and children. UFH is reversible and has a short half-life and is therefore commonly used in critical care and surgery. In the laboratory, monitoring of UFH is challenging. There are various methods available including APTT, Anti Xa assay and ACT (Activated Clotting Time). The degree to which these assays reflect the degree of anticoagulation is unclear[29]. The appropriate test method used to achieve the desired clinical outcome is controversial. APTT is frequently the initial test performed; however, it is sensitive

to levels of FVIII. Therefore, if the FVIII levels are raised the APTT may not accurately reflect the level of anticoagulation. In these settings, some authors argue that the Anti Xa assay should be performed. However, many Anti Xa assays have exogenous antithrombin in the assay, which likely modifies the result, especially in infants who have a relative antithrombin "deficiency" compared to adults.

In children, the use of different methods for monitoring the therapeutic range of UFH, in particular APTT and Anti Xa assay, is controversial. The therapeutic range is derived from data obtained from the adult population, which may not be appropriate for children. This has been demonstrated in various studies. For example, the incremental increase in APTT to achieve the therapeutic range of UFH is different in children and in adults[30]. There is evidence showing lack of correlation between APTT and Anti Xa assays for monitoring UFH in children compared to adults, making it difficult to distinguish between therapeutic and non-therapeutic range [31,32]. In addition, there can be significant clinical implications due to inter-assay variability, which appears to be exaggerated in children[33,34].

Clearly more research is required. Pragmatically, studies to determine optimal therapeutic targets in children will be very difficult due to low event rates and multiple confounding factors. However, studies comparing clinical outcomes based on the randomised use of different monitoring assays (eg APTT versus Anti Xa) are feasible and would be most instructive for the future.

Tests of thrombin generation

The risk of bleeding and thrombosis can be determined by the rate of thrombin (Factor IIa) generation. In the coagulation cascade thrombin converts fibrinogen to fibrin for stable clot formation. The endpoint of traditional tests such as APTT and PT occurs when only 5% of the total amount of thrombin is generated. Hemker et al [35] introduced continuous monitoring of thrombin in 1993 which led to the development of the Calibrated Automated Thrombogram (CAT) in 2003. CAT allowed rapid and continuous measurement of multiple samples, improving the efficiency and accuracy of thrombin generation tests[36]. There are three commercial automated thrombin generation assays available to determine the total amount of thrombin present in plasma or whole blood. The limitations of these assays are due to lack

of standardisation and large quantitative variations in the results[37]. Further, the availability of these assays is limited to larger hospital based laboratories.

Endogenous Thrombin Potential

Endogenous Thrombin Potential (ETP) is an in-vitro representation of thrombin generation and is monitored using chromogenic or fluorogenic substrates. ETP is determined by the area under the curve (AUC) that is generated by the Calibrated Automated Thrombogram (CAT).

Controversies with tests for thrombin generation

Kintigh et al[36] highlighted the main issues regarding thrombin generation testing. Thrombin generation tests have a very laborious preparation. A number of reagents are required to perform the test, which takes minutes to complete and cannot be performed on a large or rapid scale. There is a lack of consistent results and variation of results from location to location, as well as day to day variation in the same individual tested[38].

In another study, Petricevic et al[37] looked at four commercially available thrombin-specific substrates and tested against six plasma proteins which included FII/FIIa, FX/FXa, alpha2-macroglobulin (A2M) and AT. They found that the substrates were cleaved by thrombin and also FXa. This resulted in the thrombin levels being over estimated due to the effect of FXa. They also found that the automated assays are unable to differentiate free thrombin and alpha2-macroglobulin thrombin complex, which is a major issue as it does not reflect physiological conditions. Thus, in the presence of FXa and/or A2M it is difficult to determine the amount of free thrombin present and it is likely to overestimate the concentration of free thrombin. There are increased levels of alpha2-macroglobulin observed in children right up to late adolescence. In terms of thrombin inhibition, A2M accounts for 16-64% of thrombin inhibition in children compared to 7% in adults. Therefore, automated methods that do not measure the bound A2M and rely on derived algorithms from the adult population are unlikely to provide accurate results in children[39]. Another issue in children, in particular neonates and pre-term neonates, is sample volume. To run the test a standard 3mL sample of whole blood is required. This can be a challenge in low birthweight babies; for example, a 1kg neonate is restricted to 1-4mL of blood withdrawal, which limits the number of tests that can be performed[40].

We highlight the need for paediatric specific research as new assays are brought into the clinical sphere. We cannot assume that the test principles are valid across the variable physiology of age and new assays need clinical utility testing across all relevant age groups prior to their use in clinical care. An understanding of the test characteristics in the light of developmental haemostasis is vital.

Conclusion

The controversies discussed in relation to age-appropriate reference ranges, therapeutic anticoagulation monitoring and tests of thrombin generation are related to standardisation of test procedures, the clinical utility and application of the tests and interpretation of results. These issues are evident across all age groups, but are more prominent in the paediatric setting. Further research is required regarding current and new coagulation assays to improve their clinical utility in all patient populations.

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Table 1: *Current controversies in coagulation testing*

	Controversies of test choice	Controversies of test use	Controversies of test interpretation
Age-appropriate reference ranges			*
Therapeutic anticoagulation monitoring	*	*	*
Tests of thrombin generation	*	*	*

Extended half-life factor monitoring	*			*
Non-factor therapeutic monitoring	*			
Antiplatelet therapy monitoring	*		*	*
Ventricular assist device anticoagulation monitoring	*		*	*
Anticoagulation monitoring in antiphospholipid syndrome				*
Thrombophilia screening			*	*
Intrauterine vs extrauterine normal ranges for prematurity				*

Table 2: *Common anticoagulants used in clinical practice*

Anticoagulant	Mechanism of action	Methods of monitoring	Drug related issues	Issues specific for paediatric population
Warfarin	Vitamin K antagonist: inhibits ɣ-carboxylation of	INR	Variability of response: mutations in CYP2C9 or	Frequent monitoring to maintain therapeutic

	factors II, VII, IX, X, Protein C, S and Z.		VKORC1 gene and clinical factors such as diet, other drugs etc.	range; due to developmental changes, frequent infections, change in diet. In infants, huge variability in sensitivity depending on whether breast or bottle fed.
Unfractionated heparin (UFH)	Sulphated polysaccharide that binds to Antithrombin (AT): Inhibits factors IIa and Xa.	APTT Anti Xa Activated clotting time (ACT)	Variability of response: binds to other plasma proteins.	Obtaining venous access for administration may be challenging. Interaction of AT with UFH, resulting in children requiring higher doses to maintain therapeutic range.
Low molecular weight heparin (LMWH)	Prepared from UFH: Inhibits primarily Xa with some IIa inhibition.	Anti Xa	Predictable pharmacokinetics due to reduced binding to other plasma proteins. LMWHs are excreted through	Anti Xa monitoring required for dose adjustments. Compliance due to

			the kidneys and have potential to accumulate in renal impairment.	discomfort and administration challenges.
Fondaparinux	Synthetic pentasaccharide: Inhibits Xa.	Anti Xa	Renally excreted and the potential to accumulate in renal impairment.	Administration challenges.
Bivalirudin	Short synthetic peptide: direct inhibitor of thrombin.	APTT Ecarin Clotting Time (ECT) ACT Chromogenic assays.	Potent and irreversible direct inhibitor of thrombin.	Therapeutic drug monitoring, infusion rate better predictor than APTT.
Dabigatran	Oral, direct thrombin inhibitor.	ECT: assesses thrombin activity and shows a linear dose response to the concentration of dabigatran. Thrombin Time (TT): shows a linear dose response to therapeutic levels of dabigatran. Haemoclot® Thrombin Inhibitor assay.	80% of the drug is excreted renally.	clinical trials ongoing regarding their use in children.
Rivaroxaban	Oral factor Xa inhibitor.	Anti Xa assay with rivaroxaban calibrator.	Renal and liver excretion, so potential risk of accumulation in patients with liver	Recent clinical trial has shown comparable safety profile

			and/or renal disease.	and efficacy to standard therapy (heparin/VKA).
Apixaban	Oral factor Xa inhibitor.	Anti Xa assay with apixaban calibrator.	25% renally excreted and the remainder is through the liver.	Clinical trials ongoing regarding use in children.

Tables and Figure caption list:

1. Table 1**:** Current controversies in coagulation testing
2. Table 2: Common anticoagulants used in clinical practice

	Controversies of test choice	Controversies of test use	Controversies of test interpretation
Age-appropriate reference ranges			*
Therapeutic anticoagulation monitoring	*	*	*
Tests of thrombin generation	*	*	*
Extended half-life factor monitoring	*		*
Non-factor therapeutic monitoring	*		
Antiplatelet therapy monitoring	*	*	*
Ventricular assist device anticoagulation monitoring	*	*	*
Anticoagulation monitoring in antiphospholipid syndrome			*
Thrombophilia screening		*	*
Intrauterine vs extrauterine normal ranges for prematurity			*

Table 1: *Current controversies in coagulation testing*

Anticoagulant	Mechanism of action	Methods of monitoring	Drug related issues	Issues specific for paediatric population
Warfarin	Vitamin K antagonist: inhibits ɣ-	INR	Variability of response:	Frequent monitoring to

	carboxylation of factors II, VII, IX, X, Protein C, S and Z.		mutations in CYP2C9 or VKORC1 gene and clinical factors such as diet, other drugs etc.	maintain therapeutic range; due to developmental changes, frequent infections, change in diet. In infants huge variability in sensitivity depending on whether breast or bottle fed.
Unfractionated heparin (UFH)	Sulphated polysaccharide that binds to Antithrombin (AT): Inhibits factors IIa and Xa.	APTT Anti Xa Activated clotting time (ACT)	Variability of response: binds to other plasma proteins.	Obtaining venous access for administration may be challenging. Interaction of AT with UFH, resulting in children requiring higher doses to maintain therapeutic range.
Low molecular weight heparin (LMWH)	Prepared from UFH: Inhibits primarily Xa with some IIa inhibition.	Anti Xa	Predictable pharmacokinetics due to reduced binding to other plasma proteins. LMWHs are	Anti Xa monitoring required for dose adjustments. Compliance

			excreted through the kidneys and have potential to accumulate in renal impairment.	due to discomfort and administration challenges.
Fondaparinux	Synthetic pentasaccharide: Inhibits Xa.	Anti Xa	Renally excreted and the potential to accumulate in renal impairment.	Administration challenges.
Bivalirudin	Short synthetic peptide: direct inhibitor of thrombin.	APTT Ecarin Clotting Time (ECT) ACT Chromogenic assays.	Potent and irreversible direct inhibitor of thrombin.	Therapeutic drug monitoring, infusion rate better predictor than APTT.
Dabigatran	Oral, direct thrombin inhibitor.	ECT: assesses thrombin activity and shows a linear dose response to the concentration of dabigatran. Thrombin Time (TT): shows a linear dose response to therapeutic levels of dabigatran. Haemoclot® Thrombin Inhibitor assay.	80% of the drug is excreted renally.	clinical trials ongoing regarding their use in children.

Rivaroxaban	Oral factor Xa inhibitor.	Anti Xa assay with rivaroxaban calibrator.	Renal and liver excretion, so potential risk of accumulation in patients with liver and/or renal disease.	Recent clinical trial has shown comparable safety profile and efficacy to standard therapy (heparin/VKA).
Apixaban	Oral factor Xa inhibitor.	Anti Xa assay with apixaban calibrator.	25% renally excreted and the remainder is through the liver.	Clinical trials ongoing regarding use in children.

Table 2: *Common anticoagulants used in clinical practice*