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Editorial note:

In this topical update, Dr Rock Leung reviews the testing strategy and quality assurance issues on laboratory testing for direct oral anticoagulant (DOACs). We welcome any feedback or suggestions. Please direct them to Dr Rock Leung (e-mail: leungyyr.ha.org.hk) of Education Committee, the Hong Kong College of Pathologists. Opinions expressed are those of the authors or named individuals, and are not necessarily those of the Hong Kong College of Pathologists.

Laboratory testing for Direct Oral Anticoagulants (DOACs): Are we ready?

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Abbreviations

DOACs	Direct oral anticoagulants
FIIa	Thrombin
PK	Pharmacokinetics
PD	Pharmacodynamics
PT	Prothromhin time
APTT	Activated partial thromboplastin time
TT	Thrombin time
dTT	Diluted thrombin time
ECT	Ecarin clotting time
DRVVT	Diluted Russell's viper venom time

Introduction

The newly available Food and Drug Administration (FDA) -approved oral anticoagulants, namely dabigatran extexilate, rivaroxaban, apixaban and edoxaban, have been more commonly used nowadays for treatment and prophylaxis of venous thromboembolism, as well as for prevention of stroke in non-valvular atrial fibrillation. This new class of anticoagulants has been referred as novel oral anticoagulants (NOACs), target-specific oral anticoagulants (TOACs), or direct oral anticoagulants (DOACs). For the sake of standardization, the International Society for Thrombosis and Haemostasis (ISTH) Scientific and Standardization Committee (SCC) for the control of anticoagulation recommends the term DOACs. DOACs have been shown to be at least as effective as warfarin in various clinical trials. Moreover, there was reduced incidence of

intracranial haemorrhage reported in some studies when compared with warfarin [1]. Unlike warfarin, DOACs do not need routine therapeutic monitoring given their predictable pharmacokinetics (PK), pharmacodynamics (PD) and wide therapeutic windows. There are, however, clinical conditions that measurement of anticoagulation activity of DOACs is necessary or potentially useful, e.g. before invasive procedures, during adverse events like break-through bleeding or thrombosis, and pre- and post-administration of reversal therapy for patients with DOACs overdose. Thus, there is a role for laboratory, by testing for DOACs, to help clinicians on patient management. In addition, it is the responsibility of the laboratory to acknowledge the interferences of DOACs on conventional and special coagulation tests as part of the laboratory quality assurance in the era of gaining popularity of DOACs usage.

Mechanisms of actions of DOACs

In contrast with heparin that can only inhibit free protease, DOACs are rapidly-acting, target-specific anticoagulants that inhibit both the free and bound activated serine protease [2]. The fact that DOACs can inactivate bound serine protease explains their more robust action than warfarin or heparin. Dabigatran is a direct thrombin (IIa) inhibitor while rivaroxaban, apixaban and edoxaban are direct inhibitors of activated factor X (Xa). Most of the DOACs are cleared by liver and kidney, with the exception of dabigatran being almost exclusively excreted by kidney. DOACs reach peak plasma levels within approximately two hours and plasma trough levels within 12 hours or 24 hours depending on their frequency of administration [3]. The DOACs can be withhold a few days before elective surgery or invasive procedures due to their short half-lives and favourable pharmacokinetics.

To test or not to test?

Routine monitoring of DOACs is not required. Testing on patients on DOACs is generally indicated in certain clinical circumstances, including acute bleeding, suspected DOACs overdose, drug interaction, in patients with impaired renal function, before surgery or invasive procedure in patients who have taken the drug beyond 24 hours and with creatinine clearance of <50 mL/min or with extreme body weight [4]. Recently, more pharmacokinetics and pharmacodynamics data on indications of clinical testing came up. Currently it is recommended that checking of drug-specific peak and trough levels for DOACs should be performed for patients with body mass index (BMI) of >40 kg m^2 or weighted over 120 kg [5]. There is currently no consensus on when to test for DOACs activities when these drugs are to be used in women with childbearing potential. One should however note that animals studies have shown teratogenic effect of dabigatran, edoxaban and rivaroxaban, these drugs were assigned by the FDA as pregnancy category C, reflecting their potential teratogenicity. Whereas no teratogenicity has been demonstrated in animals for apixaban as of today, it was categorized as pregnancy catergory B by FDA [6]. On the other hand, the use of DOACs is considered an off-label clinical application for paediatric thromboemobolic diseases [7]. It is not unreasonable to obtain information about anticoagulation activity by laboratory assay for this special group of patients, as in the case of low-molecular-weight heparin (LMWH) usage in select paediatric patients.

Given the predictable pharmacokinetics of DOACs, it was proposed that a pharmacokinetic strategy by stopping the drug for a time frame adequate for washout of drug effect is safe before surgery or invasive procedures. This approach can only be applied for planned surgery or invasive procedures, with available information regarding patient's renal function as well as the dose and timing of the last DOAC administration. For emergent or unplanned procedures in patients with renal insufficiency or unplanned procedures when the timing of the last DOAC administration is uncertain, measurement of residual drug level will be valuable to assist clinical decisions, including the assessment of bleeding risk and the need for antidote for prompt reversal of DOAC effect before surgery. In life-threatening bleeding associated with the use of DOACs, the measurement of drug level can supplement clinical information to determine whether the bleeding is contributed by the anticoagulation effect of DOACs and whether the administration of DOAC-specific antidotes is required. If

antidote is applied, laboratory test can monitor the extent of reversal.

What tests to do?

The ideal test for DOACs shall be accurate, readily available on a 24-hour basis in order to accommodate emergency clinical situations, and with a reasonably short turnaround time (TAT).

Gold standard method using ultra-performance liquid chromatography – tandem mass spectrometry (UPLI-MS/MS) provides the most accurate information about the drug levels for patients on DOACs. However, the test is not readily available in most of the laboratories.

Routine coagulation screening tests, i.e., prothrombin time (PT), activated partial thromboplastin time (APTT) or thrombin time (TT), have been suggested as screening tests for DOACs. For routine coagulation screening tests to be useful and suitable for testing for DOACs, linearity and adequacy of test response to increasing dosage and amenability to standardization are prerequisites [8]. For dabigatran, TT is readily available in most laboratories and prolongation of clotting time is linearly and dose-dependently related to dabigatran concentrations. However, responsiveness is excessive. Therefore, a normal TT should rule out a dabigatran anticoagulant effect but the degree of prolongation poorly reflects drug concentration. Dilute TT (dTT), i.e., testing of TT on diluted plasma, is adequately responsive to dabigatran and suitable for assessment of dabigatran activity. Ecarin clotting time (ECT), using ecarin for the conversion of FII to meizothrombin, to assess anticoagulant effect of dabigatran was also shown to have satisfactory linearity and responsiveness to increasing dabigatran concentrations. APTT, though being demonstrated to have satisfactory responsiveness to dabigatran, lacks linearity upon increasing drug concentration and there is significant inter-reagent variability [9]. PT is insensitive to dabigatran and not suitable for testing.

Rivaroxaban prolongs the PT in a concentration-dependent manner, but the correlation is generally weak and became weaker with increasing drug concentration. Significant reagent-dependent differences in assay sensitivity are noted in multiple studies, limiting its use for assessment of rivaroxaban activity if the in-house thromboplastin reagent for routine coagulation screening is insensitive to rivaroxaban [10]. APTT is insensitive to rivaroxaban and shall not be used for assessment of rivaroxaban activity. For apixaban, both PT and APTT are insensitive to increasing drug concentrations and for edoxaban, PT performance is similar to that observed for rivaroxaban and APTT is insensitive [11].

Therefore, routine coagulation screening tests PT, APTT and TT cannot provide a reliable measurement of DOAC anticoagulant effect in most circumstances. One exception being a normal TT excludes significant residual effect of dabigatran in patients. Moreover, PT and APTT are either insensitive or show variably sensitivity to the on-therapy range of DOACs and limit their use in determining whether the drug concentration is in subtherapeutic or supratherapeutic ranges. Furthermore, these coagulation screening tests are potentially affected by the presence of lupus anticoagulants and conditions resulting in factor deficiency as in liver disease or dilutional coagulopathy. Thus, the sensitivity & specificity in reflecting the anticoagulant effect of DOACs is limited.

Anti-Xa assay is a chromogenic assay based on the measurement of residual FXa with synthetic substrates upon mixing of plasma with FXa. Although one study showed the feasibility of using of anti-Xa assay for LMWH to assess the presence of rivaroxaban [12], it is recommended to use drug-specific calibrator rather than adopting the anti-Xa assay for measurement of heparin activity due to the following reasons: 1) assays to measure indirect Xa inhibitors, e.g., LMWH, are measured in IU/ml and direct Xa DOACs are measured in ng/mL and there is no direct relationship between these two units of measure, 2) there is significant variability in measured drug concentration, as demonstrated by rivaroxaban, between various anti-Xa kits and 3) the therapeutic range, at least for apixaban and rivaroxaban, far exceeds the typical calibration range for UFH and LMWH (in the 5-9 IU/ml

range) and 4) the assay is not specific for anti-Xa DOACs and will detect all anti-Xa anticoagulants[2].

Commercially available drug-specific coagulation assays for testing of DOACs use calibrators and controls specific for the DOAC being measured [13-15], enabling the reporting of a drug concentration upon testing of patient's plasma sample. Multiple calibrators and test plasma dilutions are employed to ensure the test sample responses are within the range of the calibration curve and also to allow for assessment of linearity and parallelism [16]. It was recommended that anti-Xa assay and diluted TT shall be employed when carrying out the drug-specific coagulation assay for anti-Xa inhibitor and anti-IIa inhibitor respectively, given their linear relationship and good correlation with drug concentration as measured by mass spectrometry [11]. Although an ecarin chromogenic assay (ECA) for direct IIa inhibitor and a DRVVT-based assay for both direct IIa and direct Xa inhibitors have been calibrated for testing of DOACs, ECA was shown to have suboptimal accuracy when compared UPLC-MS/MS and DRVVT-based assay would give false positive result in the presence of lupus anticoagulant [17,18]. Studies have shown that various drug-specific coagulation assays differ significantly in quantitation of the DOAC being measured when compared with UPLC-MS/MS in terms of precision and accuracy [17].

What is the meaning of drug concentration?

Drug-specific assay is by no means a direct measurement of drug concentration for DOACs. Instead it is an extrapolation of drug concentration by its anticoagulation activity measured by clot-based or chromogenic assay.

Therapeutic ranges of DOACs have not been validated by the manufacturing pharmaceutical companies. Moreover, there is no established range of concentrations associated with bleeding. In clinical use, expected trough and peak concentrations as predicated on prescribed dose and frequency are often taken as a reference during result interpretation of drug levels [17] (Table 1).

There is no consensus on whether trough level is superior to peak level when interpreting the findings during monitoring of DOACs. The sample for DOAC level is often collected at a random time during emergency clinical situations. A meaningful interpretation of drug level requires the knowledge of the time of last dose of DOAC, the drug dosage and patient's renal and liver functions so that the trend of drug concentration over time can be better predicted.

With increasing use of DOAC assay, it is expected that DOAC plasma concentration shall be a standard study parameter in future clinical trials. This will allow the identification of drug concentration threshold associated with bleeding, the establishment of a therapeutic range for different kinds of DOACs and better definition of DOAC-induced bleeding complications.

Antidote for reversal of DOACs

Non-specific reversal agents like prothrombin complex concentrates, "bypassing agent" like factor eight inhibitor bypass activity (FIBA) and activated FVIIa were used for the correction of DOAC effect. They only had a general antagonizing action on the anticoagulation effect of DOAC without targeting the specific DOACs themselves. Three antidotes for the DOACs are now under various stages of development. Idarucizumab (Praxbind®), the antidote for dabigatran, is now licensed in the United States and recommended for licensing by the European Medicines Agency. Andexanet alfa, the antidote for the oral anti-Xa inhibitors, is undergoing phase III study. Ciraparantag (PER977), an agent reported to reverse the anticoagulant effects of all of the DOACs is at an earlier stage of development [19]. In life-threatening bleeding, administration of antidote or reversal agent before emergency operations shall not be delayed until the availability of test results. Otherwise, the decision on whether antidote is indicated can be guided by suitable laboratory assay as mentioned in the previous section. Drug-specific assay is considered the most suitable candidate given its superior sensitivity and probably better specificity than conventional coagulation assay and better accessibility and faster turnaround compared with mass spectrometry. Measurement of drug activity

shall guide the antidote treatment and allow more effective use of this costly medicine. The importance is highlighted by one study on idaruxizumab for dabigatran reversal in which dTT was normal on study entry in nearly one quarter of the study population, indicating little or no circulating anticoagulant in this group of patients, whom benefit from the administration of idaruxizuman was minimal [20]. Although DOAC concentrations warranting the administration of antidote were recommended (e.g., a drug concentration over 50 ng/mL in serious bleeding and 30 ng/mL in patients requiring urgent intervention) [19], these actionable limits have not been validated in clinical studies.

Quality assurance issues on DOACs testing

Laboratories should develop customized algorithms on DOACs testing strategy for DOACs based on their need. The relative sensitivity of routine coagulation screening test, especially APTT and TT for dabigatran and PT for rivaroxaban, apixaban and edoxaban shall be validated by calibrated materials. Most published algorithms [21, 22] assume patient's coagulation status is solely under the effect of DOACs and may not be applicable for patients with massive transfusion, disseminated intravascular coagulopathy (DIC) or presence of lupus anticoagulant that may have contributed to the abnormal coagulation screening results. Moreover, it is not practical to change the service PT and APTT reagents solely for DOACs detection.

The set up of clot-based or chromogenic drug-specific assay needs careful literature review on the performances of different commercially available assays. For example, one study reported overestimation of rivaroxaban levels with an anti-Xa assay utilizing exogenous antithrombin [23] and ISTH [4] recommended against its use. Nevertheless, the choice of commercially available assay may be limited by its compatibility with the automated coagulometers in service.

There are currently no standards or guidelines on the validation of drug-specific coagulation assays. Same principles on validation for clot-based or chromogenic coagulation assay shall follow, including testing for accuracy, within-run & between-run precisions and lower limit of quantification (LOQ). The testing of accuracy may be limited by accessibility to mass spectrometry. This can be resolved by testing accuracy against different lot of calibrators. Precision at low drug concentration is important to determine any significant residual DOAC effect in emergency setting. Assay kit with incorporation of low-level calibrators is favored over those with calibrators only covering the usual on-therapy concentration ranges. For the same reason, LOQ validation is important and the report shall report results as "less than" numerical LOQ value (ng/mL). Testing on plasma collected from normal subjects not taking DOACs shall be carried out to determine the intrinsic anti-Xa or anti-IIa activity from natural anticoagulant, e.g., antithrombin, which may also affect the lowest reportable limit of the assay.

As part of the quality assurance, PT, APTT, TT and fibrinogen activity shall be assessed for samples sent for quantitation of DOACs. When TT is prolonged, heparin contamination shall be excluded by carrying out protamine neutralization test. Before reporting the drug concentration, linearity of the calibrator curves shall be verified. Calibrator curve shall be acquired for every patient sample instead using stored calibrator curves as a control of lot-to-lot variation of calibrators for this relatively infrequent test. Results shall be reported in ng/mL, and there should be an accompanied comment about the appropriate range of results (peak or trough levels) based on publication. Drug level shall be interpreted in light of the time since last dose of DOAC intake as well as the dosage of DOAC taken. It is critical to have continuous surveillance of test performance over time. This can be achieved through enrollment in External Quality Assurance Programme (EQAP) (e.g., College of American Pathologist).

Drug-specific coagulation assay can be performed by automated coagulometers with pre-set dilution and analysis protocols with a low to moderate level on skill requirement and hence amenable to the organization of a laboratory-wide staff training programme to cater for the development of a routine 24-hour DOAC laboratory testing service.

Interval refreshment training shall be organized to upkeep staff competence. Clinical pathologists shall be involved in communication with clinicians during emergency management of patients requiring DOAC testing to ensure efficient delivery of accurate information to facilitate patient management.

Impact of DOACs on special coagulation assay

It is important for laboratories that carry out special coagulation assay to acknowledge the interferences of DOACs on special coagulation assay. These include clot-based and chromogenic assay. ELISA-based and molecular assays are essentially not affected by DOACs (Table 2) [2, 17].

Conclusion

DOACs are more commonly used nowadays. While clinical indications for laboratory testing are more available, there is a pivotal role of laboratories to formulate a testing strategy for DOACs. Routine coagulation screening tests are not informative in most cases. Development of drug-specific assay for DOACs testing is needed. The interpretation of drug level generated by drug-specific assays needs to be facilitated by more data on the association between drug concentrations and bleeding risks expected in future studies.

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Table 1. 5th – 95th percentile of peak and trough concentrations of DOACs obtained from pharmacokinetic and pharmacodynamics studies on patients prescribed with fixed dose and frequency of DOACs.

	Trough (ng/mL)	Peak (ng/mL)
Apixaban		
2.5 mg twice daily	20-94	36-100
10 mg twice daily	30-412	122-412
Dabigatran		
150 mg twice daily	31-225	64-223
Edoxaban		
30 mg once daily	130-174	376-412
60 mg once daily	268-336	388-444
Rivaroxaban		
10 mg once daily	1-38	91-195
20mg once daily	4-96	160-360

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Table 2. Impact of DOACs on select special coagulation assays

Assay	Anti-FIIa DOAC	Anti-FXa DOAC
Clauss fibriongen	May be falsely decreased	No effect
One-stage APTT-based factor assays	May demonstrate false decrease in factor activity	May demonstrate false decrease in factor activity
One-stage PT-based factor assays	May demonstrate false decrease in factor activity	May demonstrate false decrease in factor activity
Chromogenic FVIII activity	No effect	May demonstrate false decrease in factor activity
Bethesda assay	False inhibitor present	False inhibitor present
AT activity: thrombin substrate	May demonstrate false increase in AT activity; may mask AT deficiency	No effect
AT activity: FXa substrate	No effect	May demonstrate false increase in AT activity; may mask AT deficiency
PC activity: clot based	May demonstrate false increase in PC activity; may mask PC deficiency	May demonstrate false increase in PC activity; may mask PC deficiency
PC activity: chromogenic	No effect	No effect
PS activity: clot-based	May demonstrate false increase in PS activity; may mask PS deficiency	May demonstrate false increase in PS activity; may mask PS deficiency
PS activity: chromogenic	No effect	No effect
PS activity: ELSA-based or LIA-based	No effect	No effect
LA testing	Possible to misclassify as LA present	Possible to misclassify as LA present
Activated PC resistance	Falsely increased ratio; possible to misclassify as FV Leiden mutation absent	Falsely increased ratio; possible to misclassify as FV Leiden mutation absent

AT, antithrombin; PC, protein C; PS, protein S, LA, lupus anticoagulant; LIA, latex immunoassay