Assessment of platelet function in the laboratory

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Summary
Platelet function testing is essential for the diagnosis of congenital/acquired bleeding disorders and may be useful for the prediction of surgical bleeding. Nowadays there is also much interest in monitoring the efficacy of anti-platelet therapy and measuring platelet hyper-function. However, this often presents clinical laboratories with significant challenges as platelet function tests are complex, poorly standardized, time consuming and quality assurance is not straightforward. There are also few comprehensive modern guidelines available and many recent published surveys have revealed poor standardization between laboratories.

Up until the late 1980’s the traditional clinical platelet function tests that were available were the bleeding time (BT), light transmission (LTA) and whole blood aggregometry (WBA) and various biochemical assays. These were also usually performed within specialized research and clinical laboratories. Since the last BCSH guidelines were published in 1988 a variety of new platelet function tests have become available. These include flow cytometry and an ever increasing choice of new commercial instruments. Although the potential clinical utility of the new assays is emerging some have not yet entered into routine clinical practice. It is encouraging that a number of standardization committees (e.g. CLSI, BCSH and ISTH Platelet Physiology SSC) are now beginning to produce new platelet function testing guidelines and this will hopefully improve clinical practice, quality assurance and result in less variability between different laboratories.

Table 1 illustrates a list of currently available tests of platelet function including the traditional tests in vivo bleeding time and platelet aggregometry (16, 19). In contrast to coagulation defects, where screening tests e.g. the aPTT (activated partial thromboplastin time) and PT (prothrombin time) are inexpensive and fully automated, platelet function defects are more difficult to diagnose because there are no definitive screening tests. The BT (bleeding time) and the PFA-100® are currently the main choices of screening tests and these are discussed in more detail.

No current or future platelet function test is likely to be 100% sensitive to all platelet disorders, because of the large variety platelet defects that have been described. The current diagnostic evaluation of a potential platelet defect usually centres on platelet aggregation and measurement of granule content/release. These tests are labour intensive, costly, time consuming and require a fair degree of expertise and experience to perform and interpret. Also additional expensive specialist tests are often required (e.g. flow cytometry, platelet nucleotides and molecular biology).

Since the late 1980’s when the last published platelet function testing guidelines were written by the BCSH (49), a number of new tests of platelet function have become available, including flow cytometry and various in vitro instruments (16, 18, 19). The principle of each test and their potential advantages/disadvantages are also listed in Table 1. The list includes a number of instruments that were prototypes and have now been commercialised. Many of these are now available to the clinical laboratory, but either how to, or even whether to incorporate some of them into normal laboratory practice remains unclear.

The bleeding time

The bleeding time (BT), developed by Duke in 1910 (31), is the oldest in vivo test of platelet function (49). The BT is the time taken for bleeding to stop after an incision is made into the skin, usually into the anterior surface of forearm. The test has been refined and standardised particularly with the use of a sphygomonanometer cuff and a spring-loaded template device to make standard sized cuts within the skin (37). The BT was previously recommended as a clinically useful screening test of platelet function (49), and surprisingly remains a widely performed investigation according to a recent survey (25). There is also currently significant variation between laboratories in the BT methodology used although the the most commonly BT devices used forms (25) are

- Simplate (Organon Teknika Corporation),
- Surgicutt (ITC) and
- Triplett (Helena Laboratories).

There is also variation between centres in reported BT reference ranges although 6–11 minutes is typical (25), whereas severe platelet defects and severe VWD can result in a BT > 30 minutes. However, despite its apparent simplicity, the test is poorly reproducible, invasive, insensitive and time consuming (44). In particular the BT does not seem to correlate with the bleeding tendency within individual patients and it is widely considered that an accurate bleeding history is a more valuable screening test. Recent data also suggests that the BT is not predictive of either MI or ischaemic stroke (8). The clear advantage of the BT is that it does study natural haemostasis and the role played by the vessel wall in this process. The test does not require expensive equipment or a laboratory and is not prone to variables associated with blood sampling and anticoagulation. However, the inherent problems of high inter-operator CV with poor reproducibility, poor sensitivity, the subjective end point and invasive nature of the BT were highlighted by researchers in the 1990’s. Consequently, the BT has declined in popularity although surprisingly still remains in clinical use in some centres (25).

Platelet aggregometry

Light transmission aggregometry (LTA) was developed in the early 1960’s and soon
<table>
<thead>
<tr>
<th>Test Principle</th>
<th>Tests</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cessation of bleeding from a standardized wound in vivo</strong></td>
<td>bleeding time</td>
<td>measures in vivo haemostasis</td>
<td>invasive, insensitive, scanning, high CV</td>
</tr>
<tr>
<td><strong>Agregometry</strong></td>
<td>LTA</td>
<td>historical gold standard</td>
<td>sample preparation, low shear system, Does not simulate normal haemostasis.</td>
</tr>
<tr>
<td></td>
<td>WBA</td>
<td>no separation of PRP required, many different agonists available</td>
<td>low shear system, Older systems require electrodes to be cleaned and recycled.</td>
</tr>
<tr>
<td></td>
<td>Multiplate®/WBA</td>
<td>no separation of PRP required, disposable cuvettes containing duplicate electrodes and stir bar</td>
<td>no luminescence available, Does not simulate normal haemostasis.</td>
</tr>
<tr>
<td></td>
<td>VerifyNow®</td>
<td>fully automated POC test, simple, rapid</td>
<td>inflexible, Cartridges only used for single purpose.</td>
</tr>
<tr>
<td></td>
<td>Platelet counting method (e. g. Ichor-plateletworks®)</td>
<td>simple, cheap</td>
<td>Requires a reliable calibrated method to count platelets accurately and precisely.</td>
</tr>
<tr>
<td></td>
<td>Thrombovision T®</td>
<td>Automated platelet aggregometer measures light scatter.</td>
<td>new test, experience limited</td>
</tr>
<tr>
<td><strong>Flow cytometry</strong></td>
<td>detection of activation markers</td>
<td>Measures activation status of platelets in vivo, small blood volumes</td>
<td>expensive, specialised equipment &amp; experienced operator, prone to artefact if samples not taken carefully or processed quickly.</td>
</tr>
<tr>
<td></td>
<td>ex vivo stimulation and detection of activation markers</td>
<td>Use specific agonists to measure antiplatelet drugs (e. g. arachidonic acid or ADP)</td>
<td>as above</td>
</tr>
<tr>
<td></td>
<td>VASP phosphorylation</td>
<td>simple measure of P2Y12 occupancy</td>
<td></td>
</tr>
<tr>
<td><strong>Shear dependent platelet function within whole blood</strong></td>
<td>PFA-100®</td>
<td>simple, rapid</td>
<td>inflexible, insensitive to P2Y12 inhibitors, VWF dependence, Hct and platelet count dependent</td>
</tr>
<tr>
<td></td>
<td>IMPACT®-CPA</td>
<td>simple, rapid</td>
<td>new test, experience limited, automated version not available yet</td>
</tr>
<tr>
<td></td>
<td>Plasor PRT-700®</td>
<td>simple rapid POC test, uses fresh fingerprick blood</td>
<td>new test, experience limited</td>
</tr>
<tr>
<td><strong>Global haemostasis tests</strong></td>
<td>Thromboelastography (TEG®)</td>
<td>POC whole blood test</td>
<td>measures a clot endpoint, Test needs to be modified to increase sensitivity to antiplatelet drugs (e. g. platelet mapping system).</td>
</tr>
<tr>
<td></td>
<td>Thromboelastometry (ROTEM®)</td>
<td>POC whole blood test measures platelet contractile force. sensitive to anti-GpIIb/IIIa drugs</td>
<td>measures clot properties</td>
</tr>
<tr>
<td><strong>Thromboxane measurements</strong></td>
<td>serum thromboxane B2</td>
<td>dependent on COX-1 activity</td>
<td>prone to artefact, not platelet specific</td>
</tr>
</tbody>
</table>

Assessment of platelet function became regarded as the gold standard of platelet function testing (49). In this method citrated blood is normally centrifuged to obtain platelet rich plasma (PRP), which is then stirred within cuvettes incubated at 37°C between a light source and a detector within an aggregometer instrument. Upon addition of various concentrations of a panel of agonists (e. g. collagen, ADP, thrombin, ristocetin, adrenaline), the platelets aggregate and light transmission increases. Classical platelet responses to each agonist can then be monitored including:
- lag phase,
- shape change and
- primary and secondary aggregation.

Parameters measured include the:
- rate (slope) of aggregation and the
- maximal amplitude (%) or percentage of aggregation after a fixed period of time.

This is still the most widely used test for identifying and diagnosing platelet function defects and can now be performed within modern commercially available easy to use multi-channel aggregometers of which some have a luminescence option.

Whole Blood Aggregometers (WBA) using impedance technology are also widely available and are sometimes combined with luminometry to simultaneously measure dense granular ADP release. A multichannel whole blood aggregometer with disposable cuvettes/electrodes (Multiplate® system) and standardised reagents is now commercially available. Although WBA is therefore increasing in popularity and unlike LTA requires no further blood processing, LTA still remains the method of choice in many laboratories. There is no doubt that aggregation will remain an important clinical test within the specialised laboratory as many platelet disorders can be diagnosed. But the clinical significance of mildly abnormal aggregation to weak agonists remains undefined. Furthermore, aggregometers test platelets under relatively low shear conditions and in free solution within PRP, conditions that do not accurately simulate primary haemostasis. Also it is logistically impossible to perform simultaneous platelet aggregation on large numbers of patients with suspected platelet defects and, in general, it is advisable that tests are performed within 2–3 hours of blood sampling. Recent surveys have revealed that aggregationmetry is very poorly standardized between different laboratories and this highlights the need for new guidelines and recommendations on how to accurately perform this test (25, 38). Indeed, the CLSI, BCSH and ISTH platelet physiology SSC are all now working towards producing new guidelines which will become available soon. A number of laboratories have also shown the potential of 96 well plate assays in which LTA can be performed with many replicates, controls, agonists and dose response curves using relatively small volumes of PRP (2, 39, 40).

Because of the many disadvantages of the BT and LTA, several alternative automated technologies have been developed which attempt to simulate haemostasis in vitro (16). Many of these are listed with Table 1. Four of the most popular tests which are widely used are discussed in detail.

### PFA-100®

The Platelet Function Analyser PFA-100 (Siemens Diagnostics) is a relatively simple bench top instrument that simulates high shear platelet function within disposable test cartridges (26, 30). Citrated blood is aspirated under constant negative pressure from the sample reservoir through a capillary and a microscopic aperture (147 µm) cut into a membrane. The membrane is coated with either Collagen/Epinephrine (CEPI) or Collagen/ADP (CADP). The presence of these platelet activators and the high shear rates (5000 to 6000 s⁻¹) under the standardised conditions result in platelet adhesion, activation and aggregation resulting in formation of a platelet plug within the aperture. Platelet function is thus measured as a function of the time it takes to occlude the aperture. The test is simple to perform, rapid – with maximal closure times (CT) of 300 seconds – and can test relatively small volumes (0.8 ml/cartridge) of citrated blood up to 4 hours from sampling.

However, as with any other laboratory tests of platelet function, there are recommended PFA-100 good practice guidelines that are required to maintain optimal performance (17, 22). These include daily instrument QC checks, ensuring the quality of blood sampling, anticoagulation and checking for cartridge batch to batch variation. Various reports have shown that the test is reliable with near identical normal ranges reported from many laboratories (10, 26). A more recent survey shows more variation in these ranges (25). However, it is recommended that each laboratory should establish their own reference range using normal blood samples taken into identical citrate anticoagulant that is utilised within the user’s institution i. e. either 3.8% (0.129 mol/l) or 3.2% (0.105 mol/l) buffered trisodium citrate. Widespread experience is increasing (>500 Medline articles, November 2008) and the ISTH platelet physiology SSC recommends that the test is an optional screening test that can potentially be used to detect or exclude severe platelet defects and VWD (23). It is important to note that the PFA-100 is sensitive to many variables that influence platelet function including abnormalities in platelet number, haematoctrit, drug and dietary effects, platelet receptor defects, VWF defects, release and granular defects (26). A full blood count should therefore always be performed to exclude thrombocytopenia or anaemia as a cause of platelet dysfunction.

The test is largely insensitive to coagulation type defects including haemophilia A and B, afibrinogenemia and defects in factors V, VII, XI and XII. Comparison with the bleeding time reveals that the PFA-100 is usually more sensitive (11), especially to VWD, including Type I VWD (13). Overall, these results do support the use of the test as a screening tool, although some recent prospective studies suggest that the test has poor sensitivity to milder defects (4, 41, 43). However, because of its high negative predictive value for severe disorders, given a normal reading, then a sample can be theoretically eliminated from further detailed analysis (e. g. aggregation studies). However, it should be noted that the test is not always sensitive to all milder platelet function defects and will give false negative results (21) for example in patients with:
- storage pool disease,
- primary secretion defects,
- Hermansky Pudlak syndrome,
● mild type I VWD and
● factor V Quebec.

Diagnosis of these disorders would therefore be missed if relying upon the PFA-100 alone. It is important therefore that patients with a strong clinical history suggestive of a platelet defect have the full range of diagnostic tests performed despite normal PFA-100 results (9). Also in patients tested with apparently normal platelet function the instrument has been shown to give occasional false positives which then may have to be fully worked up by additional tests to exclude a defect. In this context, ingestion of aspirin is a particular problem. Abnormal CT’s are therefore not diagnostic. As the test is sensitive to several acquired variables (e.g. dietary and drug effects) that influence platelet function, borderline CT’s either side of the upper normal ranges can also be difficult to interpret, given that the reported CV’s for of a normal sample have been reported as 10% (26). It is important that repeat or deferred tests are performed if drug ingestion is strongly suspected.

Many additional larger studies are required to assess whether the PFA-100 can also reliably predict either thrombotic or bleeding complications in different patient groups especially in patients who appear to be non-responsive or resistant to aspirin therapy (5, 19, 24, 28). Shortened CT’s on the CADP cartridge may also prove to be an important indicator of predicting thrombosis within high risk patients (14, 15, 20).

The Impact® CPA

Another test that has recently been developed is based upon the adhesion of platelets to the extracellular matrix using whole blood exposed to high shear. The original Cone and Plate (CPA) device tested whole blood platelet adhesion and aggregation on a plate coated with extracellular matrix (ECM) (50). The CPA has now been developed into a commercial instrument called the Impact (Diaemed, Switzerland) and now utilises polystyrene plates instead of the ECM. This modification facilitated the commercialisation of the test. The test is available as two forms:

● the research version (Impact-R) where washing and staining steps are performed manually and
● the fully automated instrument.

Both are simple to operate, use a very small quantity of whole blood (0.12 ml) and give results within a short time (50). Both instruments contain a microscope and perform staining and image analysis of the platelets adhering and aggregating upon the plate under an applied shear rate of 1800 s⁻¹. The software enables storage of the images of each analysis and records a number of parameters including surface coverage, average size and a distribution histogram of the adhered platelets.

Comparative analysis of the polystyrene and ECM plates shows a good correlation both in normals and VWD. No platelet adhesion occurs on plastic plates (50) in samples from patients with Glanzmann thrombasthenia or afibrinogenemia.

The adhesion of platelets to the plate is absolutely dependent upon plasma VWF, fibrinogen binding to the plastic surface, the platelet glycoproteins GpIb and Gp IIb/IIIa, and platelet activation events. The IMPACT-R has been available for a few years and experience is growing but it is not ideally suitable for use in the routine clinical laboratory (50). The automated Impact is not yet available but has potential as a screening tool as it should theoretically give improved reproducibility over the Impact-R.

VerifyNow®

The VerifyNow® (Accumetrics, Inc, San Diego, California) is a turbidimetric based optical detection system that measures platelet induced aggregation as an increase in light transmittance (46, 52). This modified platelet aggregometry device was originally developed as a near patient testing instrument in order to provide a simple and rapid functional means of monitoring anti-Gp IIb/IIIa therapy with various anti-platelet drugs (e.g. abciximab) (46). The disposable cartridges contain fibrinogen-coated beads and a platelet activator. The instrument simply measures changes in light transmission automatically and thus the rate of platelet/bead aggregation.

The test appears to correlate well with conventional platelet aggregation (27, 46). This represents a significant advance as the test can be performed reliably within different institutions/clinics as a point of care test without requiring either transport of sample, blood handling or processing, time delay or specialised personnel to perform the test. The test has also been adapted to measure the effectiveness of either aspirin or clopidogrel (anti-P2Y₁₂) therapy within specific cartridges (32, 33, 51). Evidence is now accumulating to suggest that detection of non-responders to drugs within all three types of cartridge equates with poor clinical outcomes (6, 7, 42, 47, 48).

Although many of these studies are small, there are a number of large prospective randomised trials in progress (e.g. ADAPT-Des Trial, DANTE Trial, GRAPPA Trial, ARTIC trial, TRILOGY trial ASCET trial, ARCC trial, ARTIST trial) which will hopefully reveal whether each cartridge can predict a poor clinical outcome and that monitoring and titrating or changing antiplatelet therapy based upon the test result is clinically useful and improves outcomes.

Flow cytometry

There is no doubt that one of the biggest advances in platelet function analysis has been the application of flow cytometry (35). This technique, however, requires access to expensive instrumentation and specialised training to perform. A number of largely research applications continue to evolve into clinically useful tests particularly as many of the reagents, antibodies and dyes are now commercially available. The most commonly used routine tests are the quantification of glycoprotein receptor density (i.e. diagnosis of deficiencies in platelet glycoproteins e.g. Glanzmann thrombasthenia and Bernard Soulier disease). However, tests have also been devised to measure

● dense granules (using mepacrine uptake and release),
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- microparticle formation and
- exposure of anionic phospholipids (procoagulant activity).

These are beginning to prove to be very useful in the diagnosis of storage pool defects and Scott syndrome and related disorders. Most laboratories now prefer to analyse platelets within whole blood. Only small quantities of blood are required and, providing the venipuncture and analysis technique(s) are well controlled and standardised, platelets can be analysed in their circulating state. Many laboratories have measured a variety of different platelet activation markers and shown that they are elevated in various clinical conditions associated with platelet activation. However, it is also possible to measure platelet activation in response to classical agonists in vitro.

Some of these tests have been recently shown to be potentially useful for monitoring aspirin and P2Y<sub>12</sub> drugs (12, 19, 36), including the VASP test which is commercially available (1, 3). For recommendations and protocols how to perform flow cytometric analysis, the reader is referred to recent review articles (34, 35, 45).

Conclusions

The last BCSH guidelines for platelet function testing were written in the late 1980’s (49). Given the advances in this field and the introduction of potentially useful additions to our existing portfolio of platelet function tests, work is currently in progress by the CLSI, BCSH and ISTH SSC to write new guidelines and to attempt to improve the standardisation of LTA. Nevertheless, the incorporation of new platelet function tests into the routine laboratory has already begun and a number of new approaches have been suggested (29). Flow cytometric-based platelet function tests now also provide a rich variety of specific tests that are beginning to prove very useful at diagnosing various defects. The development of reliable, sophisticated but simple to use whole blood tests that attempt to simulate in vivo haemostasis provides the ability to screen samples rapidly before applying our exist-
ing portfolio of tests. Certainly the general consensus is that the in vivo bleeding time should be replaced. Many of the simpler platelet function tests could also be potentially utilised as point of care instruments for assessing bleeding risk, thrombotic risk and monitoring antiplatelet therapy. Well designed large prospective clinical trials will be required to determine whether these tests are useful for these applications. Platelet function testing is therefore becoming increasingly utilised outside of the specialised laboratory. Although this represents an important advance, validation, reliability and quality control testing of these tests will also become an increasingly important issue (22).

In conclusion, many new platelet function tests have recently and will continue to become available. Many are now beginning to prove to be useful additions to our existing portfolio of tests.

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References
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