SEED Coagulation

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Preanalytical influences on coagulation test results

Routine coagulation testing is an integral part of laboratory testing that informs clinical decision making. There have been major advances in technology with tight quality control procedures in place for the analytical phase of testing. The validity of results generated for patient sample results is however highly dependent on several preanalytical variables that will not be detected by conventional quality control processes. This is a very important topic as preanalytical factors influencing the reliability of laboratory test results are commonplace.

These variables can be broadly categorized as controllable variables such as specimen collection and handling preanalysis and uncontrollable variables that relate to factors that are endogenous and specific to each individual specimen (for example the presence of haemolysis or jaundice).

Specimen Collection

The process of phlebotomy is extremely common, yet it is generally practiced with very little regard for the need for standardized procedures, primarily because the impact that a poorly collected specimen has on the accuracy of laboratory test results is largely unrecognized by those tasked with blood collection. Poorly collected blood samples are a major cause of sample rejection and erroneous coagulation test results as poor sample quality due to faulty collection technique is not always overtly evident.

a) Site of venepuncture

- Wherever possible only antecubital veins should be used.
- As a general rule, the smaller and more distal the vein the greater the chance that the sample collection will be technically difficult and the specimen compromised. Traumatic ex vivo haemolysis and clotted specimens increase in frequency when alternate sites are used.
- Blood should not be collected from the same arm in which a drip is inserted. This is important in order to protect the integrity of the drip site as well as to avoid any possible inadvertent sample dilution with the infusate.

b) Blood collection technique

- Evacuated tube collection systems are superior to the use of ordinary syringes.
  - The specimen is collected directly into the appropriate anticoagulant thereby minimizing the possibility of inadvertent sample clotting.
  - The chance of tube under-filling is minimized.
- The gauge of the needle should not be too small (ideal 19-21G) or else there is a greater risk of ex vivo haemolysis as red blood cells are sheared because of the high vacuum force applied during collection. A significant increase in D-Dimer count is observed in specimens collected with smaller diameter needles.
- Tourniquets are commonplace in venesection but should not be used indiscriminately. The tourniquet should be removed as soon as blood flow into the collection tube is established and should never exceed 1 minute so as to minimize venous stasis. Venous occlusion causes haemoconcentration, an increase in fibrinolytic activity and possibly activation of some clotting factors. A one to three minute period of stasis will result in clinically significant changes in the PT, APTT, fibrinogen and D-Dimer values.
- Whilst it used to be advocated that one should never use the first draw tube for coagulation testing, the latest CLSI guidelines have dropped this requirement as no evidence exists to indicate that there is any meaningful difference in coagulation test results between first and second draw tubes. If more than one tube is however being drawn, then it is recommended that the coagulation tube should not be drawn first.
- Blood should not be drawn from indwelling catheters as

they are very likely to be diluted as well as contaminated by heparin. Sometimes however, this may be the only venous access possible in critically ill patients, in which case, the first 10ml should not be used for coagulation tests.

c) Blood collection tubes

- Blood must be collected into a sodium citrate tube
- Citrate is available as 3.2% and 3.8%. Both are acceptable and give the same results provided that the sample tube is properly filled and analysed fresh. The effect of under-filling and aging is more pronounced with 3.8% citrate.
- Ensure that the tube has not expired.

d) Tube filling

- The tube must be adequately filled. Inadequate tube filling is one of the commonest reasons for sample rejection.
- The minimum fill volume required depends on the actual parameter being tested but as a rule of thumb tubes should have a minimum fill volume of 80% of the demarcated level (in keeping with the vacuum volume). A 5mL tube should therefore contain a minimum of 4ml of blood.
- Proper filling is essential to ensure the proper blood to anticoagulant ratio. The citrate removes calcium from the blood which prevents it from clotting in the tube. The ratio of anticoagulant to blood in the tube needs to be correct otherwise the test results will be wrong. All tests involving clotting as the end point of detection require the addition of calcium chloride in the test method. The amount of calcium chloride that is included in the reagent has been determined based on the expected quantity of citrate in plasma post collection. Clearly if the tube is under-filled, there will be an excess of citrate in the plasma, and hence too little calcium to compensate, so clotting times will be erroneously prolonged.
- Samples with a very high haematocrit will have the same outcome as if the tube were under-filled i.e. there is a relative excess of anticoagulant to plasma. Special tubes should be prepared for the patient by the laboratory. A nomogram exists from which one can see how much citrate should be added to a tube with 5ml fill volume specific for haematocrit. This typically applies to haematocrit values above 50%.
- Overfilling will not occur if blood is collected using a vacuum system. It is however possible to do so if blood is collected using a standard needle and syringe and tubes are decapped for filling.
- Both over- and under-filled tubes will give erroneous results.

Specimen handling, transport and storage

a) Specimen mixing

- The blood must be thoroughly mixed with the anticoagulant in the tube by inverting the tube several times.
- This is essential to prevent micro clot formation in the tube. The process of clot activation consumes clotting factors and can result in false short or long clot-based coagulation test results.

b) Time delays between collection and analysis

- The time between sample collection and processing is critical. The maximum time permitted depends on specific test to be performed. As a rule of thumb, samples should be processed within 6 hours of collection, failing which samples should be spun down, the plasma portion removed, and snap frozen to a minimum of –20°C. Only one freeze thaw cycle is permissible.
- Old samples become activated. Just like in poorly mixed specimens, mini clots form in the tube thereby consuming clotting factors which can result in false short or long clotting times. Such micro clots are invisible to the operator.

c) Storage conditions

- The effect of time also depends on the temperature at which the specimen is stored.
- PT results are stable for up to 24 hours on condition that the specimen is stored at 4–6°C or room temperature and was not exposed to any excessive mechanical agitation or high temperatures that may be experienced during road transportation or non-air-conditioned laboratories. The sample may be stored as whole blood or plasma.
- The APTT and most other coagulation tests are probably stable up to 8 hours unless the patient is being treated with the anticoagulant heparin. The sample may be stored as whole blood or plasma.
- A maximum of 2 hours delay prior to testing is recommended for samples from heparinised patients.

d) Specimen centrifugation

- Samples must be centrifuged adequately in order to ensure that the plasma is free of platelets. As platelets are a source of phospholipid, residual platelets, especially if the plasma is going to be frozen and thawed, are a source of phospholipid that may give false short clotting times.
- It is not important to have a temperature controlled centrifuge.
- For routine coagulation tests centrifugation at 1500 x g for 15 minutes at room temperature is recommended.
- Ideally specimens should be centrifuged within 1 hour of collection.
- As a general rule, it is best to centrifuge specimens and remove plasma from above the red cell layer as soon as possible if any delay in coagulation tests is likely. Contact with red cells is a source of activation.
- It is essential to ensure that the buffy layer and platelet rich plasma is avoided when plasma is separated for storage. As platelets are a rich source of phospholipid, clotting times may be erroneously short if plasma is contaminated with platelets. Some tests are more affected than others.

Other specimen variables that may give erroneous coagulation test results

a) Haemolysis

- Haemolysis is a leading source of interference in coagulation testing.
- The source of haemolysis, whether it occurred in vivo or occurred as a result of traumatic venepuncture or poor sample handling is irrelevant.
- Causes of traumatic haemolysis include:
  - the use of small bore needles
  - difficult phlebotomy
  - excessive shaking or mixing of the specimen
  - exposure to excessively hot or cold temperatures
  - centrifugation at a too high speed or for too long
- Haemolysis results in the release of haemoglobin into the plasma. This may interfere with the results obtained on coagulation analysers using optical clot detection systems.
- Chromogenic assays work on the principle of measuring 'colour' in the plasma. These assays exploit the fact that proteins involved in maintaining haemostatic balance are principally enzymes that function by cleaving a specific substrate. In the assay system the natural substrate is replaced with a chromogenic substrate which when acted upon by the active enzyme, causes the plasma to change colour in proportion to the enzymatic activity. As such, any colour that is observed is interpreted as being a consequence of the activity of the analyte in question. Free plasma haemoglobin will be identified as colour and be assumed as having been elicited by enzyme activity which would be erroneous in this case. Assay results may be either too high, if the parameter in question (e.g. Protein C) is directly proportional to colour change, or too low if activity is inversely proportional to colour change (e.g. Antithrombin).
- The degree of interference depends on the test principle for the coagulation parameter under analysis.
- It is important to be aware that haemolysis may interfere with results but most assays have some inbuilt safe guards and are only prone to wrong results above a certain threshold level. In this regard it is very important to read package inserts very carefully as different reagents for the same test have different tolerances.
- If in doubt about whether or not the degree of haemolysis within a sample will interfere with test results, one could measure the plasma haemoglobin level on a haematology or chemistry analyser.
- If samples are visually grossly haemolysed it would be best to request a repeat specimen to be collected. If the patient has a haemolytic condition the doctor must be informed to treat results with reserve, as well as to give an indication of whether results are likely to be erroneously too high or too low. Some interpretation may still be possible.

b) Jaundice

- The bilirubin present in jaundiced samples has the same effect as haemolysed samples although this is always inherent to the patient's pathology and not induced in the collection process.

c) Lipaemia

- High lipid levels in plasma result in very turbid specimens which may interfere with clotting tests using optical detection systems as this relies on a measure of change in light transmission through a sample.

Take home messages

- The quality of coagulation test results is highly dependent on several preanalytical variables and may be erroneous under certain conditions.
- Correct tube fill volume is critical as the incorrect plasma to anticoagulant ratio will give wrong results.
- Sample collection should be undertaken with adequate precautions to prevent excessive stasis and traumatic haemolysis.
- Excessive delay between collection and sample analysis will affect test results.
- Permissible sample storage times before results are affected, only apply if the tubes are also adequately filled and the sample not exposed to excessively warm temperatures and excessive mechanical agitation.
- The combination of tube under-filling, high temperatures and agitation result in accelerated compromise and hence recommended maximum storage times no longer apply.
- Samples should be centrifuged and plasma separated from red cells as soon as possible.
- As a rule of thumb, analyse samples within 6 hours of collection and if this is not possible, freeze the plasma for later analysis.
- The presence of haemolysis, jaundice and lipaemia may affect the accuracy of results.

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