Using HitAlert flow cytometry to detect heparin-induced thrombocytopenia antibodies in a tertiary care hospital

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We aimed to assess the utility of HitAlert flow cytometry as a diagnostic functional heparin-induced thrombocytopenia (HIT) assay in a tertiary care hospital. The 4Ts score was used to assess pretest probability of HIT in 37 patients. Serum was analysed for HIT antibodies by the flow cytometry HitAlert assay. Results were compared with an antigenic assay, the particle gel immunoassay, PaGIA ID PF4/Hep Ab assay; and two functional assays, the Multiplate whole blood impedance aggregometry assay (WBIA), and the serotonin release assay (SRA). Flow cytometry was positive in 14 out of 37 patients, including zero out of eight, five out of 19 and nine out of 10 in the low, intermediate and high-risk groups by 4Ts score, respectively. Using the SRA as a 'gold standard', flow cytometry has a sensitivity of 81% and a specificity of 100% for the diagnosis of HIT. The other functional assay (WBIA) had similar sensitivity (81%) and specificity (90%) to flow cytometry. In contrast, the PaGIA maintained a high sensitivity of 100% but a specificity of only 20%.
The improved specificity of flow cytometry over the antigenic assay was most marked in the 4T intermediate-risk group in which similar results were obtained between all three functional assays. We demonstrate that compared with an immunological assay (PaGIA), flow cytometry can improve the specificity of laboratory diagnosis of HIT without loss of sensitivity using SRA as a standard. Flow cytometry may have a role in the first-line laboratory diagnosis of HIT, especially when combined with an immunological assay such as PaGIA. *Blood Coagul Fibrinolysis* 24:365–370 © 2013 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Introduction

Heparin-induced thrombocytopenia (HIT) is an uncommon but clinically significant adverse drug reaction with high morbidity and mortality if untreated [1]. Diagnosis is made on a combination of clinical risk assessment and a laboratory assay to detect HIT antibodies. Laboratory methods used to detect HIT antibodies are divided into two groups: first, immunological (antigen) assays that detect the presence of antibodies against platelet-factor 4 complexed with heparin and include enzyme immunoassays and particle gel immunoassays (PaGIAs) [2] and second, functional (activation or aggregation) assays that detect heparin-dependent platelet activation and include the serotonin release assay (SRA), washed platelet aggregation assays and more recently the indirect whole blood impedance platelet aggregation assay (WBIA) [2,3]. Although immunological assays are simpler, cheaper, more rapid and easily available, they have inferior specificity and give more false-positive results. Functional assays generally detect only clinically important HIT antibodies that cause platelet activation and aggregation leading to thrombosis. However, these assays are more complex, expensive, have increased turnaround times and are available only in a few specialized laboratories. Many patients suspected of having HIT will not have access to a functional assay or results from functional testing are delayed so that they have no impact on immediate management. The SRA, which is the gold standard for detecting HIT antibodies [4], is available only at reference laboratories.

The need for improved rapid diagnostics in this area led us to investigate a recently available commercial functional test, the HitAlert flow cytometric assay, to improve the diagnosis of HIT. We report the first clinical validation of this new assay with results compared with those obtained with current standard functional assays (SRA and WBIA) and an immunological assay (PaGIA).

Materials and methods

Patients and samples

Thirty-seven samples from patients suspected of having HIT were examined. Twenty were stored serum samples, and we also prospectively evaluated 17 consecutive patients who were investigated for HIT antibodies in our laboratory between February 2010 and August 2010.

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Retrospective data on the patients with stored serum samples were collected from the patient medical record. Prospective data were collected using a standardized HIT antibody test request form. From these data, the 4Ts score [1] (and therefore pretest probability) was determined for all 37 patients. The 4Ts score is a widely validated pretest clinical scoring system for HIT wherein the degree, timing of and alternate causes for thrombocytopenia as well as presence of thrombosis are assessed to give a score estimating the likelihood of HIT at the time of initial evaluation. Samples from all these patients were analysed for HIT antibodies using PaGIA as part of our laboratory standard practice. For this study, the samples were analysed using the HitAlert flow cytometry assay. A confirmatory functional assay using whole blood impedance aggregometry and, where possible, the SRA was performed. The four different assays are briefly described as follows.

Particle gel immunoassay

The ID-PaGIA Hep/PF4 assay (DiaMed, Cressier, Switzerland) utilizes PF4/heparin complexes fixed to red polystyrene beads to detect PF4/heparin antibodies within patient serum when reactants are added to a sephacryl gel. If present, these HIT antibodies are agglutinated in the presence of a secondary antibody against human immunoglobulin G (IgG), which prevents passage through the gel when centrifuged. If no HIT antibodies are present, the red beads will be centrifuged to the bottom of the gel [5]. This assay was performed according to manufacturers' instructions on heat-inactivated serum samples. On each card tested, the kit positive and negative controls were also tested to ensure proper assay performance.

Flow cytometry platelet activation assay

The HitAlert (IQ Products, Groningen, Netherlands) kit is a functional HIT antibody assay that detects antibodies on the basis of their platelet-activating properties. The assay utilizes donor citrate platelet-rich plasma (PRP) prepared from donor blood collected into 3.2% citrate tubes (Greiner, Victoria, Australia) and centrifuged at 100*g* for 10 min. The assay was performed as per the kit insert. In short, donor platelets are incubated with test serum and a phycoerythrin-labelled antiplatelet antibody and an fluorescein isothiocyanate-labelled platelet activation marker. Three controls are used with each assay run. These consist of donor PRP along with heparin background activation control (due to handling), PRP along with Ca-ionophore positive control (to show platelets can be activated) and PRP along with patient serum spontaneous activation control. The patient HIT test consists of donor PRP along with patient serum and heparin. Acquisition and analysis was done on a BD FACS Calibur (BD Biosciences, San Jose, California, USA). HitAlert kits were supplied free courtesy of IQ Products.

The first control should have less than 1% platelet activation. The second control should have more than 80% activation of platelets. The third control should have the same or higher percentage activation than the first control. The result is indicative of HIT if the percentage of activated platelets in the test sample is at least twice the percentage of activated platelets in the first control. The manufacturer recommends using fresh serum in this assay. The available retrospective samples had been heat inactivated (at 56°C) before storage. Testing on some of the prospective samples showed no difference in results between paired fresh and heat-inactivated samples (data not shown).

Whole blood impedance platelet activation assay

An indirect WBIA assay was performed on the Multiplate analyser (Dynabyte GmbH, Munich, Germany) according to the method of Morel-Kopp [3] with slight modifications. Results from some of these samples have been previously published in a multisite evaluation of the WBIA assay [6]. Donor whole blood (group O or blood group A donors, depending on the patient blood group) was collected into hirudin tubes (Verum Diagnostica GmbH, Munich, Germany). After a rest period of 15 min, into prewarmed Multiplate cuvettes, 300 μl donor blood was mixed with either 150 μl of low-heparin saline solution (final concentration 0.5 U/ml) or 150 μl high-heparin saline solution (final concentration 100 U/ml). This was incubated for a further 2 min, then 150 μl patient serum was added and the platelet activation reaction was recorded for 15 min. Results were expressed as area under the curve using arbitrary units.

A negative HIT response was characterized by lack of platelet activation with both low and high-heparin patient tests and high-heparin positive control test, in conjunction with platelet aggregation evident with donor thrombin receptor-activating peptide (TRAP) and the low-heparin positive control tests. A positive response was characterized by platelet aggregation in the low-heparin patient, low-heparin control and donor TRAP tests and no response in the high-heparin patient and high-heparin positive control tests.

Platelet donors for flow cytometry and whole blood impedance platelet activation assay

All donors were assessed as being responsive to the presence of HIT antibody by confirmatory testing with a frozen, known, positive HIT control stored in liquid nitrogen. This control serum had been well characterized on clinical and laboratory results several years prior to this study. In addition, all donors were tested for normal platelet function using TRAP as an agonist.

Serotonin release assay

Frozen aliquots of patient sera were transported to Royal North Shore Hospital, Sydney, for testing according to

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the procedure of Sheridan *et al*. [4]. Briefly, ^{14}C serotonin-labelled platelets are washed and incubated with samples in the presence of low heparin (final concentration 0.1 U/ml) and high heparin (final concentration 100 U/ml) for 1 h at room temperature. After centrifugation, ^{14}C serotonin release in the supernatant was measured by scintillation counting. Results were expressed as percentage of ^{14}C serotonin in the supernatant compared with the total ^{14}C serotonin in the labelled platelets.

Results are considered positive if more than 20% ^{14}C serotonin was released in low-heparin concentration and less than 20% ^{14}C serotonin was released in the high-heparin test. Known positive and negative control samples were included in each run to confirm assay validity.

Statistical analysis

Results are reported descriptively. Diagnostic sensitivity was defined as the percentage of individuals with a positive test (by flow cytometry, PaGIA or WBIA) of those with a diagnosis of HIT according to the 'gold standard' SRA (positive SRA). Diagnostic specificity was defined as the percentage of individuals with a negative test (by flow cytometry, PaGIA or WBIA) of those who do not have HIT according to the 'gold standard' SRA (negative SRA). For sensitivity and specificity calculations, samples yielding equivocal or weak positive results were regarded as positive because of the pragmatic consequences of such results for clinical management.

Results

Patient characteristics

A total of 37 patients were tested by PaGIA, WBIA and flow cytometry assays. These patients comprised 23 men and 14 women, with a median age of 64 years (range, 33–82 years). Twenty-two of the patients were from medical wards while 15 were surgical. Nine patients (six surgical and three medical) were in the ICU at the time of testing. The majority were treated with unfractionated heparin (UHF) ($n = 29$), while seven were treated with a low-molecular weight heparin (LMWH, enoxaparin). One patient was treated with both. Of the 29 patients who were treated with UFH, 10 received intravenous therapeutic doses while 19 received subcutaneous heparin for deep venous thrombosis (DVT) prophylaxis. Of the seven who were treated with LMWH, three received DVT prophylaxis doses while four received therapeutic doses. The patient who received both UFH and LMWH developed a pulmonary embolus while on UFH for DVT prophylaxis and was commenced on treatment with clexane before developing thrombocytopenia, which prompted an HIT antibody test. On the basis of the 4Ts score, 10 patients had a high pretest probability, 19 had an intermediate pretest probability and eight had a low pretest probability (Table 1).

Table 1 Results of heparin-induced thrombocytopenia antibody testing according to assay and the patients' pretest probability

Patient categories	PaGIA	WBIA	FC	SRA
Total number tested (*n*)	37	37	37	26
Positive	23	13	14	14
Negative	12	22	23	10
Equivocal (or weak∗)	2	2	0	2∗
High-risk 4Ts score (*n*)	10	10	10	10
Positive	10	9	9	10
Negative	0	0	1	0
Equivocal (or weak∗)	0	1	0	0
Intermediate risk 4Ts score (*n*)	19	19	19	15
Positive	12	4	5	4
Negative	5	14	14	9
Equivocal (or weak∗)	2	1	0	2∗
Low-risk 4Ts score (*n*)	8	8	8	1
Positive	1	0	0	0
Negative	7	8	8	1
Equivocal (or weak∗)	0	0	0	0

FC, flow cytometry (HitAlert); PaGIA, particle gel immunoassay; SRA, serotonin release assay; WBIA, whole blood impedance assay.

Heparin-induced thrombocytopenia assay results

Overall, 23 out of 37 patients were positive by the PaGIA assay, 13 out of 37 were positive by WBIA and 14 out of 37 were positive by flow cytometry. There was insufficient sample for SRA testing in some of the retrospective stored specimens, so only 26 were tested: 14 of the 26 tested were positive by SRA. Using the SRA as a 'gold standard', flow cytometry had a sensitivity of 81% and a specificity of 100% for the diagnosis of HIT (Table 2). The other functional assay (WBIA) had similar sensitivity (81%) and specificity (90%) to flow cytometry. In contrast, the PaGIA maintained a high sensitivity of 100% but a specificity of only 20% (individual patient data not shown). The following paragraphs present the results of these assays according to the patient risk group on the basis of 4T score pretest probability (Table 1). Further comparison of the flow cytometry results with those obtained by PaGIA, WBIA and SRA is detailed in Table 2.

Nine of 10 high-risk patients were positive by flow cytometry. All 10 were positive by PaGIA and SRA, while the same patient who was negative by flow cytometry yielded an equivocal result by WBIA.

All the eight low-risk patients were negative by flow cytometry. These eight were also negative by WBIA, whereas one of these eight was positive by PaGIA. This latter patient tested negative by SRA.

In the clinically important intermediate risk group, five of 19 tested positive by flow cytometry. This was substantially less than those testing positive by PaGIA (12/19), but similar to the proportion testing positive by WBIA (4/19) and SRA (4/15). Of the five samples positive by flow cytometry, three were positive by all function assays, one was positive by flow cytometry and WBIA but had insufficient serum remaining for SRA testing, and one sample was positive by flow cytometry, a weak positive by SRA and negative by WBIA. Three other samples in

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Table 2 Comparison of heparin-induced thrombocytopenia antibody detection by HitAlert flow cytometry and other diagnostic assays

	PaGIA ($n=37$)			WBIA ($n=37$)			SRA ($n=26$)		
	Positive	Negative	Equivocal	Positive	Negative	Equivocal	Positive	Negative	Weakly positive
HITAlertPositive	14	0	0	13	1	0	12	0	1
HITAlertNegative	9	12	2	0	21	2	2	10	1

PaGIA, particle gel immunoassay; SRA, serotonin release assay; WBIA, whole blood impedance assay.

the intermediate risk group were negative by flow cytometry, but one of the other functional assays was positive. Of these three, two samples gave equivocal results by one test (SRA and WBIA, respectively), but were negative by the other functional assays and the third was positive by SRA but negative by WBIA and flow cytometry. Interestingly, this sample had been tested by SRA in another laboratory 3 years previously and was reported at the time as negative.

Equivocal and discrepant results

Flow cytometry did not give any equivocal results. PaGIA gave equivocal results in two intermediate risk patients who tested negative by all other tests. WBIA gave equivocal results in two patients (one high-risk patient positive by SRA but negative by flow cytometry, and the other intermediate risk patient was negative by all other tests). SRA gave two weakly positive results (serotonin release between 20 and 50%) in two intermediate risk patients. These were both negative by WBIA while one was positive by flow cytometry.

Three patients tested negative by flow cytometry but positive by SRA. One of these three patients was a 62-year-old man who was in the ICU and on haemodialysis for acute renal failure that occurred following abdominal aortic aneurysm repair. The dialysis circuit was anticoagulated with UFH. He had an intermediate risk 4T score and no demonstrated thrombosis and was the patient with a negative WBIA whose prior SRA had been negative. The second was a male with gastric carcinoma, a high risk 4T score and equivocal WBIA result who had a lower limb DVT despite chemoprophylaxis with the LMWH enoxaparin. The third was a 59-year-old man with an intermediate risk 4T score and negative WBIA whose SRA test was weakly positive. This patient was on dialysis with UFH and did not develop any thrombosis.

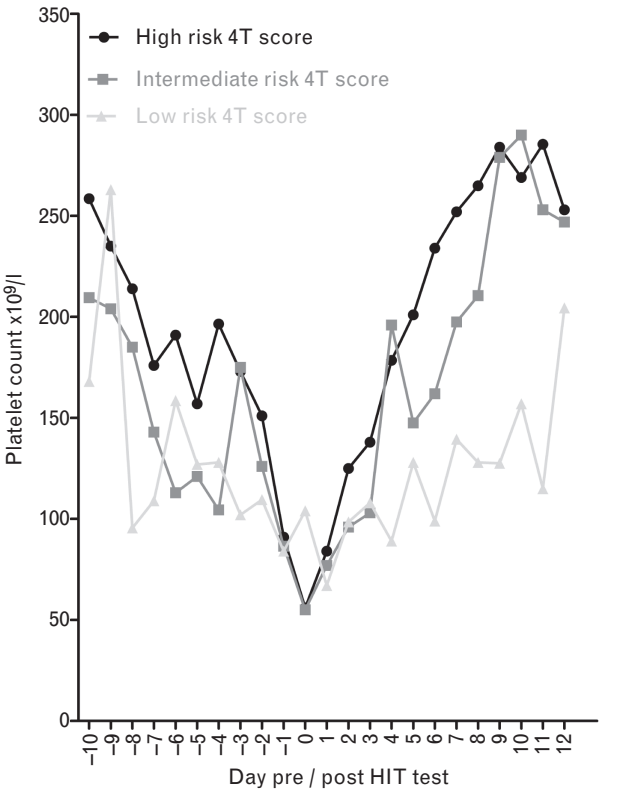
Timing of heparin-induced thrombocytopenia testing

Figure 1 shows the average platelet count prior to and after HIT testing grouped according to 4Ts pretest probability risk assessment. HIT testing was done when the platelet count was at its lowest across the three risk groups.

Discussion

HIT is associated with significant mortality and morbidity [7,8] and a rapid diagnosis is required to allow urgent management. The management involves heparin cessation and commencement of alternative anticoagulants, which do not cross-react with heparin. Overdiagnosis of HIT on the basis of the results of antigenic assays remains a significant clinical problem [9]. Although use of alternative anticoagulants is an effective treatment for HIT, this can be associated with greater bleeding risk in some patients. An incorrect diagnosis of HIT can also complicate patient management at the time of a subsequent admission to hospital when heparin therapy would usually be desirable. A simple functional HIT antibody assay, which is easily available, would reduce the number of patients who are exposed to unnecessary anticoagulation and ensure accurate diagnosis of those with the disorder.

Fig. 1



Median platelet count prior to and after heparin-induced thrombocytopenia testing grouped according to 4Ts pretest probability risk assessment. Day 0 represents day when HIT testing was done. HIT, heparin-induced thrombocytopenia.

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Our study demonstrates that the HitAlert flow cytometry assay can improve the diagnosis of HIT. Using SRA as a gold standard, flow cytometry had a sensitivity of 81% and a specificity of 100%. This was similar to WBIA, which had a sensitivity of 81% and a specificity of 90% and superior to PaGIA, which had a poor specificity. Our results confirm older reports, which have shown that flow cytometry to detect HIT antibodies correlates well with SRA [10–13], with a reported sensitivity of 95% and specificity of 100%. In these reports of noncommercially available assays, platelet activation was detected using antibodies against either CD62P or Annexin V [10,13]. In the HitAlert flow cytometry kit, the platelet activation marker remains a proprietary secret. Nonetheless, it is apparent that the HitAlert flow cytometry assay can improve the diagnostic specificity of HIT in intermediate risk patients with a positive PaGIA result, similar to other functional tests such as SRA [14], heparin-induced platelet activation assay [15] and WBIA [3].

In clinical practice, HIT is a clinicopathological diagnosis in which clinical determination of the pretest probability of HIT is supported by laboratory detection of HIT antibodies [16]. Our study lends support to the common clinical practice of assessing the 4T risk score to direct both HIT antibody testing and clinical management. None of the patients in the low-risk group were positive by any of the functional HIT antibody detection methods and none developed thrombosis. These findings support the approach of screening referrals to determine the clinical risk for HIT, not offering antigenic testing in the low-risk group and offering clinical advice that the probability of HIT in this setting is very unlikely. In the high-risk group, all the patients in our study were positive by SRA while nine out 10 were positive by flow cytometry. The patient who was negative by flow cytometry had no obvious thrombosis. Functional HIT testing, however, has less impact on the decision to treat a patient suspected of having HIT if they have a high pretest probability of the diagnosis. In practice, laboratory testing has a greater impact on decision-making during the management of patients in the intermediate risk group in whom HIT is likely, but at the same time, possible alternate explanation for thrombocytopenia exists [17]. As in this study, intermediate risk patients form the majority of patients being tested for HIT antibodies [18,19]. In this group of patients, more than with the other risk groups, a rapid functional assay with high sensitivity and specificity (compared with an immunological assay) is needed to swiftly confirm or reject a diagnosis of HIT. Our study confirms that flow cytometry may have a role in this situation. Although 14 of 19 patients in the intermediate risk group were positive or equivocal by the immunological assay (PaGIA), only five of these were shown to have functional HIT antibodies present by flow cytometry. These findings were largely confirmed by alternate functional tests, SRA and WBIA. The application of PaGIA followed, in positive cases, by flow cytometry would thus appear to be a useful strategy to improve the diagnosis of HIT in this difficult group of patients.

As a confirmatory functional assay, flow cytometry has some advantages over the SRA. Flow cytometry results can be provided within 1–2 h. Unlike SRA, flow cytometry uses nonradioactive and readily available reagents. Furthermore, it is speculated that flow cytometry methods that detect platelet activation markers when activating HIT antibodies are present (such as HitAlert) may be better than those that detect micro-particles because platelet disintegration and microparticle formation may not necessarily be from HIT antibodies [20]. Alternatively, there are still some limitations when using flow cytometry in the diagnosis of HIT. It requires a flow cytometer and a degree of flow cytometry operator experience, which may not be available in every hospital. However, there are still more laboratories with the capacity to perform flow cytometry testing compared with those doing SRA. The requirement of fresh group O donor platelets means that it will usually not be possible to perform this test outside normal working hours. Despite this, if combined with an immunological assay (e.g. PaGIA), flow cytometry can be used as a confirmatory test and results should be available the next working day. Similar to most other functional assays for HIT testing, flow cytometry still uses PRP, and this has to be carefully prepared to avoid platelet activation before testing. Some donor platelets may not show unequivocal activation (possibly due to FcγRIIa polymorphism). To optimize sensitivity and specificity for HIT, each laboratory would need to maintain a pool of donors who are not on medications that interfere with platelet function and whose platelets show unequivocal activation in the presence of HIT antibodies. The main advantage of flow cytometry over SRA, of course, is that the SRA is simply not widely available in routine diagnostic laboratories.

There are some important limitations to our study. A proportion of the cases had clinical information retrospectively determined, which could cause classification bias in the determination of the 4T score. Similarly, the reproducibility of the 4T scoring system has also been questioned particularly in regard to interrater reliability [21,22]. It remains, however, the most widely used clinical tool to determine the pretest probability of HIT and has undergone extensive prospective clinical validation [19]. It has been suggested that the testing time point may affect the likelihood of detecting HIT antibodies. The sensitivity may be reduced if testing is done when heparin/PF4/antibody complexes are lowest. However, reports on this are conflicting and others have found no effect of timing on the likelihood of detecting HIT antibodies [23–25]. This possible bias

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does not appear likely in our study, as we tested samples that were collected at the same time in all risk groups (Fig. 1). One potential reason for some of the discrepant results between the functional assays may have been the use of different platelet donors for the SRA testing, which was performed at a different laboratory. Finally, our sample size is relatively small, although the confirmation of the majority of the flow cytometry results with two other functional assays lends strength to the findings.

In conclusion, in the first validation study of the HitAlert flow cytometry assay, we have demonstrated that compared with an immunological assay (PaGIA), HitAlert can improve the specificity of laboratory diagnosis of HIT without loss of sensitivity. Flow cytometry appears to have similar sensitivity and specificity for the diagnosis of HIT as other functional assays, SRA and WBIA. Further larger prospective studies are needed to confirm the role of HitAlert flow cytometry in routine use for the laboratory diagnosis of HIT.

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Conflicts of interest

There are no conflicts of interest.

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