Evaluation of a new nanoparticle-based lateral-flow immunoassay for the exclusion of heparin-induced thrombocytopenia (HIT)

Ulrich J. Sachs; Jakob von Hesberg; Sentot Santoso; Gregor Bein; Tamam Bakchoul
Institute for Clinical Immunology and Transfusion Medicine, Justus Liebig University, Giessen, Germany

Summary
Heparin-induced thrombocytopenia (HIT) is an adverse complication of heparin caused by HIT antibodies (abs) that recognise platelet factor 4-heparin (PF4/hep) complexes. Several laboratory tests are available for the confirmation and/or refutation of HIT. A reliable and rapid single-sample test is still pending. It was the objective of this study to evaluate a new lateral-flow immunoassay based on nanoparticle technology. A cohort of 452 surgical and medical patients suspected of having HIT was evaluated. All samples were tested in two IgG-specific ELISAs, in a particle gel immunoassay (PaGIA) and in a newly developed lateral-flow immunoassay (LFI-HIT) as well as in a functional test (HIPA). Clinical pre-test probability was determined using 4T’s score. Platelet-activating antibodies were present in 34/452 patients, all of whom had intermediate to high clinical probability. PF4/hep abs were detected in 79, 87, 86, and 63 sera using the four different immunoassays. The negative predictive values (NPV) were 100% for both ELISA tests and LFI-HIT but only 99.2% for PaGIA. There were less false positives (n=29) in the LFI-HIT compared to any other test. Additionally, significantly less time was required to perform LFI-HIT than to perform the other immunoassays. In conclusion, a newly developed lateral-flow assay, LFI-HIT, was capable of identifying all HIT patients in a cohort in a short period of time. Beside an NPV of 100%, the rate of false-positive signals is significantly lower with LFI-HIT than with other immunoassay(s). These performance characteristics suggest a high potency in reducing the risk and costs in patients suspected of having HIT.

Keywords
HIT, heparin, diagnostic procedure, immunoassay

Introduction
Heparin-induced thrombocytopenia (HIT) is caused by platelet-activating antibodies that recognise the platelet factor 4 (PF4)/heparin-complex (1–4). HIT is clinically characterised by a fall in platelet count beginning between day 5 to 10 of heparin therapy with or without thromboembolic events (5–7). The probability of HIT can be estimated using the well-established 4T scoring model (8). The differential diagnosis of HIT, however, may be difficult (9–11), and does usually require the in vitro demonstration of PF4/heparin (hep) antibodies. Immunoassays detecting IgG antibodies against PF4/hep are highly sensitive tools for the detection of HIT antibodies (12–15). Because of their excellent negative predictive values (NPV), they are helpful in excluding HIT, allowing for further use of heparin in the patient. The use of many of these tests is, however, hampered by the fact that they require special laboratory equipment. Moreover, most tests are preferably tailored for testing batches rather than single patient samples. Samples are therefore often sent to referral laboratories, or, in some settings, collected in the local laboratory until the number of samples is high enough to allow for using the test. The time interval between raising the clinical suspicion of HIT and achieving the test result may be critical, because often – except for obvious clinical manifestations – there is no clear strategy whether to maintain heparin therapy (which is inexpensive and easy to monitor) or to switch to an alternative anticoagulant (which is expensive, can be difficult to monitor, and with which medical staff is not always familiar) (16). Clinical scoring according to the 4 T’s score is helpful in order to receive a pre-test probability of HIT (8). An intermediate or positive 4T score does not confirm HIT, and the score was found to be unable to distinguish between diseased and non–diseased patients in a relevant number of cases in some studies (16, 17). The 4T scoring system, however, is very helpful in identifying patients with a low score who may not require any laboratory testing (18). If the 4 T’s score is not “low”, a non-heparin anticoagulant plus laboratory testing is required. Accordingly, it can be assumed that a rapid, reliable and easy-to-perform single-sample assay will help to reduce the overall costs and the patient’s individual risk. One rapid HIT assay was introduced to the market in 2004. This test, however, did not reveal useful diagnostic information for the detection of HIT antibodies (19). In this study, we report the diagnostic performance of a new rapid assay for the detection of HIT antibodies which is based on lateral flow immunoassay technique.
Materials and methods

Study design

A clinically well-characterised cohort consisting of 452 sera from consecutive surgical and medical patients was employed to evaluate the performance characteristics of a lateral-flow immunoassay. All sera were stored at −20 °C until use. Clinical information was obtained by analysing the medical records and by a standardised telephone interview with the physician (13). Bedside evaluation of the patients was not performed by the scorers.

Antibody detection using conventional methods

All samples were tested in the heparin induced platelet activation assay (HIPA), in PF4 Enhanced (GTI Diagnostics, Waukesha, WI, USA) (GTI-IgG-ELISA), Zymutest HIAIgG (Hyphen Biomed, Neuville-Sur-Oise, France) (HIA-IgG-ELISA), a particle gel immunoassay (PaGIA; BioRad, Munich, Germany) and in a lateral-flow immunoassay (LFI-HIT; Milenia Biotec, Giessen, Germany). Out of 452 samples, data for HIPA, GTI-IgG-ELISA, HIA-IgG-ELISA, and PaGIA were reported previously for 393 patients (15). A total of 59 new patients were added to the study cohort in November 2010 and tested in all assays. All 452 samples (393 stored + 59 newly added sera) were tested concurrently by GTI-IgG-ELISA, HIA-IgG-ELISA, and LFI-HIT in accordance with the manufacturers’ instructions. For ELISA testing, a sample was considered positive if the optical density (OD) was greater than 0.4 and 0.5, respectively. LFI-HIT was read visually by three different technical assistants and finally subjected to an electronic test reader. A sample was considered positive if all three investigators agreed in visual examination; the cut-off for the reader was set at 100 mV.

Lateral-flow immunoassay for the detection of HIT antibodies (LFI-HIT)

The lateral-flow immunoassay for the detection of HIT antibodies (LFI-HIT) is based on the principle of capillary action which induces a flow of the test sample along a solid phase (test strip). To the sample pad, 5 μl of the patient’s serum and two drops of a reagent are added. The reagent contains ligand-labelled human PF4 in complex with a polyanion (PA). During the migration through the test strip, labelled PF4/PA complexes bind the conjugate, which is a gold nanoparticle coated with an anti-ligand. At the same time, human antibodies against PF4/PA (if present) bind to the PF4/PA complexes. When the fluid passes the location of the test line on the strip, complexes containing ligand-labelled PF4/PA, anti-ligand coated gold particle(s) and human antibodies are retained by an immobilised goat antibody specific for the Fc-domain of human IgG (which serves as the capture antibody printed onto the membrane). This antibody was chosen to allow specific immobilisation of IgG antibodies.

A positive reaction becomes visible as an intensively coloured line which can be either read visually or quantitatively with a reader. The test strip also includes a second line (control line). At this location an antibody which binds specifically to the ligand molecule within the conjugate is coated onto the test strip. The presence of the control line confirms that the test has performed properly. The test system was adjusted to give positive results with 20/20 sera from patients with clinically and serologically confirmed HIT syndrome; and to give negative results with 20/20 samples from thrombocytopenic patients with no detectable heparin antibodies. Additionally tested serum samples containing IgM antibodies only (n = 12) did not give positive results in LFI-HIT. All samples used for adjusting the system were not part of the study cohort used for test validation.

Assessment of test time

Since lead time is an important factor in evaluating assay performance, we determined the time that was required to obtain final results for 30 randomly selected single samples by all immunoassays employed in this study using chronometry. Time was started with application of serum and stopped after the test result was read.

Evaluation of the clinical data

The probability of HIT was evaluated using the Greifswald modification of the 4 T’s scoring system as published previously (8). In the Greifswald modification, platelet count fall <50% or platelet nadir 20–100 x 106/μl as well as an onset from days 5–14 (rather than days 5–10) counts as two points, whereas platelet fall within one day (heparin exposure within 100 days) or 14 days after therapy with heparin counts as one point. Clinical data were analysed
by two physicians blinded to the results of the laboratory tests. The diagnosis of HIT was determined by a positive result in HIPA supported by intermediate to high probability of HIT.

### Statistical evaluation

The statistical analysis of the data obtained in this study was performed using Prism, Version 5.0 (GraphPad, La Jolla, CA, USA). Performance characteristics were compared using the Receiver-Operating Characteristic (ROC) curve, which is a graph of sensitivity against (1 – specificity). A perfect test would have sensitivity and specificity both equal to 1. The performance characteristic of a diagnostic assay was quantified by calculating the area under the ROC curve (AUROC). The ideal test would have an AUROC of 1, whereas a random guess would have an AUROC of 0.5. Comparison of test characteristics was performed using ANOVA.

### Results

#### Antibody detection

A cohort of 452 medical and surgical patients who received unfractionated heparin or low-molecular-weight heparin and in whom the clinical diagnosis of HIT was raised, was evaluated in this study. The probability of HIT was determined according to the 4 T’s model. In total, 34/452 (7.5%) had a positive result in the functional assay HIPA as well as intermediate to high pretest probability of HIT, meeting the diagnostic criteria for HIT. The three commercially available immunoassays gave positive results in 79, 87, and 86 samples for GTI-IgG-ELISA, HIA-IgG-ELISA, and PaGIA, respectively. Whereas both ELISA tests revealed positive results for all 34 HIT patients PaGIA failed to detect antibodies in 3/34 patients (8.8%). Results are summarised in Table 1.

Visual evaluation of LFI-HIT was easy and feasible (Fig. 1). There was no discrepancy in evaluating the test between the three investigators. LFI-HIT gave positive results in 63 sera, including all HIT patients.

When LFI-HIT was read by scanner with a cut-off of 100 mV, however, LFI-HIT gave positive results in 63 sera, but failed to detect 1/34 patients with HIT (2.9%). The signal intensity for this single patient who was not detected was 92 mV. The range of signals was 92 mV to 972 mV for patients with HIT (mean, 616 ± 50) and 0 mV to 714 mV for patients without HIT (mean, 249 ± 29, p<0.001). Calculating the nonparametric Spearman correlation coefficient between LFI signal intensity and the ELISA OD demonstrates that both variables tend to increase together, but not with good correlation (rs = 0.39; 95% confidence interval, 0.28–0.45).

Predictive capabilities for all tests are summarised in Table 1. Of note, specificity for LFI-HIT was higher than for any other test applied, because LFI-HIT had the lowest rate of false positives (6.9% compared to 10.8%; 12.7%; and 13.2% for GTI-IgG-ELISA, HIA-IgG-ELISA, and PaGIA, respectively; p < 0.05).

### Table 1: Results obtained with 452 samples in different immunoassays, and predictive capabilities of these immunoassays calculated thereof.

<table>
<thead>
<tr>
<th>Test</th>
<th>Readout and cut-off</th>
<th>Positive results</th>
<th>Negative results</th>
<th>Specificity</th>
<th>Sensitivity</th>
<th>NPV</th>
<th>PPV</th>
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<tr>
<td></td>
<td></td>
<td>true</td>
<td>false</td>
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<td>LFI-HIT visually</td>
<td>34</td>
<td>29</td>
<td>389</td>
<td>0</td>
<td>0.93</td>
<td>1</td>
<td>1</td>
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<tr>
<td>LFI-HIT scanner; 100 mV</td>
<td>33</td>
<td>30</td>
<td>388</td>
<td>1</td>
<td>0.928</td>
<td>0.971</td>
<td>0.997</td>
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<tr>
<td>LFI-HIT scanner; 137 mV</td>
<td>33</td>
<td>22</td>
<td>396</td>
<td>1</td>
<td>0.947</td>
<td>0.97</td>
<td>0.997</td>
</tr>
<tr>
<td>GTI-IgG ELISA photometry; OD = 0.4</td>
<td>34</td>
<td>45</td>
<td>373</td>
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<td>0.892</td>
<td>1</td>
<td>1</td>
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<tr>
<td>HIA-IgG ELISA photometry; OD = 0.5</td>
<td>34</td>
<td>53</td>
<td>365</td>
<td>0</td>
<td>0.873</td>
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<td>1</td>
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<td>PaGIA visually</td>
<td>31</td>
<td>55</td>
<td>361</td>
<td>3</td>
<td>0.868</td>
<td>0.912</td>
<td>0.992</td>
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<table>
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<th>false</th>
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<tr>
<td>LFI-HIT visually</td>
<td>389</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LFI-HIT scanner; 100 mV</td>
<td>388</td>
<td>1</td>
<td></td>
<td></td>
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<td>GTI-IgG ELISA photometry; OD = 0.4</td>
<td>373</td>
<td>0</td>
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<tr>
<td>HIA-IgG ELISA photometry; OD = 0.5</td>
<td>365</td>
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<tr>
<td>PaGIA visually</td>
<td>361</td>
<td>3</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Specificity | 0.93 | 0.928 | 0.947 | 0.892 | 0.873 | 0.868 |
Sensitivity | 1 | 0.971 | 0.97 | 1 | 1 | 0.912 |

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Figure 1: Photograph taken from two test cassettes of the lateral-flow immunoassay for the detection of HIT antibodies (LFI-HIT) with the inserted strips, 10 min after applying serum and reagent. In the positive sample (right panel), a goat-anti-human IgG capture antibody printed onto the membrane retains human HIT antibodies bound to platelet factor 4/polyanion-complexes attached to gold nanoparticles (test line). The control line indicates that the test has worked properly by binding free (unused) conjugate/gold nanoparticles (both panels).
Assessment of test time

The shortest test time was obtained using LFI-HIT. Median test times for a single serum sample were 11.5 min for LFI-HIT (range, 11 – 12), 16 min for PaGIA (range, 16 – 18), 121 min for GTI-IgG-ELISA (range, 121 – 125) and 130 min for HIA-IgG-ELISA (range, 124 – 133).

Performance characteristics of LFI-HIT

The performance characteristics of LFI-HIT when read by scanner was analysed using ROC. The AUC of the quantitative LFI-HIT was 0.96, indicating a highly informative capability in identifying HIT. An optimal cut-off that demonstrates the best trade-off, with both highest possible sensitivity and specificity, was identified at 137 mV (Fig. 2A). However, this adoption has only marginal effects on improving the sensitivity and specificity of the test system (Table 1).

Correlation between LFI-HIT and clinical score

The clinical score of patients with positive result in LFI-HIT was ≥6= 23 (36%), 5–4= 38 (60%), ≤3= 2 (4%). Patients testing positive in LFI had significantly higher probability of having HIT (median of 4T’s score: 5 vs. 2, p< 0.001).

When LFI-HIT was read by scanner, the result was correlated with the capability of antibodies to activate platelets (Fig. 2B). However, since one serum from a HIT patient displayed a result below 137 mV and several non-HIT sera had a result above 137 mV, the intensity obtained when reading LFI-HIT quantitatively appears not to represent a reliable predictor of the antibody’s capability to activate platelets.
Discussion

The use of heparin places patients at increased risk for developing HIT. Once the diagnosis of HIT is suspected, discontinuation of heparin, administration of alternative anticoagulants and a (immunologic) laboratory test are recommended (18). Because alternative anticoagulants are more expensive than heparin and may burden an increased risk of bleeding (21), it seems desirable to keep the period between discontinuation of heparin and obtaining a laboratory test result as short as possible.

In the present study, we investigated the use of a new, rapid lateral-flow immunoassay for the detection of HIT antibodies (LFI-HIT) in 452 well-defined patients suspected of having HIT. In parallel to assessing these samples in LFI-HIT, all samples were investigated in two well-established ELISA systems. Data for particle gel immunoassay and confirmatory data obtained by HIPA as well as clinical data were adopted from a previous study (13) for 393 patients, and 59 patients were assessed prospectively in all assays.

When read visually, LFI-HIT identified all patients with HIT (n = 34), had the lowest number of false-positive results (6.9%), and gave a definite result in the shortest period of time (less than 12 min).

As outlined by previous studies, detection of IgG HIT-antibodies has significantly improved the clinical usefulness of immunoassays (13, 22–24). LFI-HIT, the new test applied in this study, is also restricted to IgG detection and is, when read visually, as sensitive as the ELISA tests. In the cohort studied here, it appears that this test has a lower rate of false positives than the other immunoassays that were assessed in parallel. It can be speculated that formation of antigen-antibody complexes in the fluid phase may be favourable with regard to PF4/PA-antigen presentation and/or accessibility of epitopes recognised by activating antibodies. It is also a long-known phenomenon that polystyrene is capable of inducing epitopes on PF4 that are recognised by human antibodies (25); avoiding PF4 immobilisation to a microtiter plate could add to the lower rate of false positives.

This is a relevant property, since HIT is always overdiagnosed if any positive immunoassay is considered to ‘confirm’ the diagnosis of HIT (26); although LFI-HIT still does have a significant rate of false positives, which do require additional functional testing such as, HIPA, enhanced specificity may help to avoid unnecessary confirmatory testing in referral laboratories.

A second relevant property of LFI-HIT is the short lead time; as a single-sample assay, results can be obtained within 12 min without the use of any equipment other than a centrifuge and a pipette. Both aspects will require additional studies in order to identify their effect on cost burden associated with HIT (27).

LFI-HIT displays an excellent association with the 4 T’s score. All patients with HIT had a 4 T’s score above 3, supporting the current strategy to avoid serological testing in patients with low pre-test probability (18), although there is some evidence that a low pre-test probability may overlook HIT in cardiac surgery patients (17), a finding which awaits confirmation in prospective studies. None of our HIT patients had a 4 T’s score below 4. Even comparing quantitative results obtained by LFI-HIT with the clinical score reveals a good correlation between the test result and the clinical likelihood of HIT. Whatsoever, LFI-HIT, like all immunoassays, is of cause unable to predict the activatory capability of HIT antibodies.

The quantitative read-out of the LFI-HIT failed to detect 1/34 patients diagnosed with HIT. The reason for this failure still remains to be identified; the background signal of the serum was rather high due to mild haemolysis, but additional testing of positive sera with increasing amount of lysed red blood cells did not support this hypothesis (data not shown). Additional prospective studies are ongoing which inter alia aim to optimise the electronic (quantitative) read-out of the system.

We believe that LFI-HIT, when evaluated with naked eyes, is a reliable diagnostic tool. From the data obtained in this study it can be deduced that generally, LFI-HIT negative individuals do not require additional laboratory testing in order to exclude the diagnosis of HIT. Limitations may appear in rare cases where PF4 is not the major target of HIT antibodies.

We conclude that LFI-HIT is an easy-to-use single-sample HIT immunoassay with excellent performance characteristics and readily available results, especially when evaluated visually. This assay suits to the currently proposed diagnostic procedure for patients suspected of having HIT. The assay’s performance characteristics suggest a high potency for reducing both the risk and costs in patients suspected of having HIT.

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Conflict of interest

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