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Brief Report

Evaluation of laboratory assays for anti-Platelet Factor 4 antibodies after ChAdOx1 nCOV-19 vaccination

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Abstract

Vaccine-induced immune thrombocytopenia and thrombosis (VITT) following ChAdOx1 nCOV-19 vaccine has been described, associated with unusual site thrombosis, thrombocytopenia, raised D-dimer and high titre immunoglobulin-G (IgG) class anti-Platelet Factor 4 (PF4) antibodies.

Enzyme linked immunosorbent assays (ELISA) have been shown to detect anti-PF4 in patients with VITT, but chemiluminesence assays do not reliably detect them. ELISA assays are not widely available in diagnostic laboratories, and, globally, very few laboratories perform platelet activation assays. Assays which are commercially available in the United Kingdom were evaluated for their ability to identify anti-PF4 antibodies in samples from patients with suspected VITT.

Four IgG-specific ELISAs, two polyspecific ELISAs and four rapid assays were performed on samples from 43 patients with suspected VITT from across the UK. Cases were identified after referral to the UK Expert Haematology Panel multi-disciplinary team and categorised into unlikely, possible or probable VITT.

We demonstrated that the HemosIL AcuStar HIT-IgG, HemosIL HIT-Ab, Diamed PaGIA gel and STic Expert assays have poor sensitivity for VITT in comparison to ELISA. Where these assays are used for heparin induced thrombocytopenia diagnosis, laboratories should ensure that requests for suspected VITT are clearly identified so that an ELISA is performed.

No superiority of IgG-ELISAs over polyspecific-ELISAs in sensitivity to VITT could be demonstrated.

No single ELISA method detected all possible/probable VITT cases; if a single ELISA test is negative, a second ELISA or a platelet activation assay should be considered where there is strong clinical suspicion.

Introduction

Vaccine-induced immune thrombocytopenia and thrombosis (VITT) following administration of the ChAdOx1 nCOV-19 vaccine has recently been described [1,2,3], associated with thrombosis at unusual sites, thrombocytopenia, raised D-dimer and high titres of immunoglobulin G (IgG) class anti-Platelet Factor 4 (PF4) antibodies.

Authors have described using the Zymutest HIA IgG enzyme linked immunosorbent assay (ELISA) [4], the Lifecodes PF4 IgG ELISA [2,3] and the Asserachrom HPIA IgG ELISA [3] to successfully detect anti-PF4 in patients with VITT, but have also reported that the HemosIL AcuStar HIT-$IgG_{(PF4\text{-}H)}$ chemiluminesence method does not reliably detect them [3]. At the time of writing there is a single case report of VITT with a negative anti-PF4 assay using an unidentified lateral flow device [5], and another where the results of anti-PF4 assays have not been reported [6].

Therefore, we have evaluated assays currently available commercially in the United Kingdom for their ability to identify anti-PF4 antibodies in samples from patients with suspected or confirmed VITT.

Methods

Fifty samples from 43 patients with suspected VITT from across the UK were received for analysis. Sample analysis took place within a central laboratory group for consistency. Cases had been identified after referral to the UK Expert Haematology Panel multi-disciplinary team, established on March 22 2021, to review and consider all cases of suspected VITT on the grounds of clinical presentation, radiological evidence of thrombosis and local laboratory results for platelet count, coagulation parameters and anti-PF4 testing. Case definition is: presentation between 5-28 days post-ChAdOx1 nCOV-19 vaccine; thrombosis and thrombocytopenia (platelets $<150 \times 10^9$/L), or isolated thrombocytopenia; evidence of extreme activation of the coagulation system (D-Dimers > 4000 µg/L, or >2000 µg/L with a strong clinical index of suspicion). These cases are categorised into those with unlikely, possible or probable VITT. All samples analysed in this study were collected before treatment for VITT.

The available assays for anti-PF4 testing that were assessed in this study can be split into three groups: IgG-specific ELISAs; polyspecific (IgG, IgA and IgM) ELISAs; and rapid assays.

The IgG specific ELISAs were performed on all 43 samples. These assays were Asserachrom HPIA IgG (Stago UK Ltd, Theale, UK), Lifecodes PF4 IgG (Immucor, Solihull, UK), Hyphen Biomed Zymutest HIA IgG (Quadratech Diagnostics, UK), and AESKULISA HiT II (AEKSU UK, London, UK).

The polyspecific ELISAs were also performed on all 43 samples. These assays were Asserachrom HPIA (Stago UK Ltd, Theale, UK) and Lifecodes PF4 Enhanced (Immucor, Solihull, UK).

The rapid assays performed were polyspecific Diamed PaGIA gel (BioRad Laboratories Ltd, Watford, UK), IgG-specific STic Expert lateral flow device (Stago UK Ltd, Theale, UK), IgG-specific HemosIL AcuStar HIT-$IgG_{(PF4\text{-}H)}$ (Werfen Ltd, Warrington, UK), and IgG-specific HemosIL HIT-$Ab_{(PF4\text{-}H)}$ (Werfen Ltd, Warrington, UK). The HemosIL AcuStar HIT-IgG assay was performed on all 43 samples; the Diamed PaGIA gel was performed on 42 samples, one being unsuitable due to limited sample volume; and the HemosIL HIT-Ab and STic Expert assays were performed on 26 samples in the order they were received, due to limited reagent availability .

All assays were performed according to the manufacturer's instructions for use. Results were interpreted as positive or negative for anti-PF4 antibodies using the manufacturer's cut-offs that have been derived for the diagnosis of heparin induced thrombocytopenia. For the Lifecodes assays the cut-off was defined by the manufacturer as OD 0.40; for the HemosIL assays the cut-off was defined by the manufacturer as 1.0 U/mL. For the remaining ELISAs, a kit-specific cut-off in relation to a kit reference plasma was used (shown in table 1).

GraphPad Prism 9.1 (GraphPad Software, California, USA) was used for statistical analysis of assay sensitivity and specificity.

Results and Discussion

Table 1 shows the results for all assays, and the results for patients who were categorised as possible or probable VITT are shown in figure 1.

Of the 43 samples tested, 23 had optical density (OD) for all six ELISAs that were above the assay-specific cut-off (positive); all of these 23 samples were from patients with possible or probable VITT.

Eight samples were positive by five of the six ELISAs. Seven had OD below the assay-specific cut-off (negative) by AEKSULISA HiT II from six patients with probable VITT and one with possible VITT. One was negative by Asserachrom HPIA IgG from a patient with probable VITT.

Using HemosIL AcuStar HIT-IgG for the 31 samples that were positive by five or six ELISAs, two had a positive result (of 1.04U/mL and 1.72U/mL) and 29 had a negative result (<1.00 U/mL). Using Diamed PaGIA gel in 30 of these samples, 14 had a positive result and 16 had a negative result. Using STic Expert in 23 of these samples, one had a positive result, 20 had a negative result and two had a test line that was less intense than the kit reference (negative). Using HemosIL HIT-Ab in 17 of these samples, all had a negative result (<1.0 U/mL)

Two samples were positive by four ELISA assays: one from a probable VITT patient was positive by Lifecodes PF4 Enhanced, Asserachrom HPIA IgG, Asserachrom HPIA, Zymutest HIA IgG and Diamed PaGIA gel (2+), but HemosIL Acustar HIT-IgG was negative; one from an unlikely VITT patient was positive by Lifecodes PF4 IgG, Lifecode PF4 Enhanced, Zymutest HIA IgG and AUSKULISA HiT II, but Diamed PaGIA gel, HemosIL AcuStar HIT-IgG and HemosIL HIT-Ab were negative.

Two samples were positive by two ELISA assays (Lifecodes PF4 IgG and Lifecodes PF4 Enhanced). One was from a probable VITT patient with Diamed PaGIA gel negative and the other from an unlikely VITT patient with Diamed PaGIA gel positive (1+); HemosIL AcuStar HIT-IgG, HemosIL HIT-Ab and STic Expert were negative for both samples.

Four samples had positive results by one ELISA assay. Three samples had positive results with the Lifecodes PF4 Enhanced assay: one sample from an unlikely VITT patient was negative by all rapid assays; one sample from an unlikely VITT patient was positive (3+) by Diamed PaGIA gel and negative by all other rapid assays; one sample from a probable VITT patient was negative by all

rapid assays. The fourth sample was from an unlikely VITT patient and was positive with the Zymutest HIA IgG assay and negative by all other rapid assays (STic Expert not tested).

Four samples had results for all six ELISAs that were negative, all from patients in whom VITT was unlikely; one was positive using the Diamed PaGIA gel (2+) and all the other rapid assays were negative.

Comparing test results with the clinical phenotype as evaluated by the clinical expert group enabled calculation of assay sensitivity and specificity for VITT. These data are presented in table 2.

We have demonstrated that, whilst the HemosIL AcuStar HIT-IgG, HemosIL HIT-Ab, Diamed PaGIA gel and STic Expert assays have a high sensitivity for heparin-induced thrombocytopenia (HIT) [7], they have poor sensitivity for VITT in comparison to ELISA (see table 2). We have also shown that, although IgG-specific ELISA are considered better than polyspecific assays for the diagnosis of HIT [8], there is little difference in the assays for the detection of VITT. However, our study looked at only small numbers of samples and had a strong bias towards patients with possible or probable VITT, making it difficult to recommend whether an IgG-specific ELISA or polyspecific ELISA is of more clinical use.

It is unclear why certain assays are insensitive to VITT and whether the concentrations and compositions of the PF4-complexes account for the differences. The Diamed PaGIA gel uses PF4 bound to heparin, similar to the assay principle used in the sensitive Zymutest HIA IgG, Asserachrom HPIA IgG and Asserachrom HPIA assays. The two insensitive HemosIL assays use PF4 bound to polyvinyl sulphate (PVS), similar to the assay principle used in the two sensitive Lifecodes assays. The STic Expert assay uses PF4 bound to an unspecified polyanion, and the AEKSULISA assay does not specify the composition of the kit.

There were two specific problems observed during this study. Firstly, many positive results for Diamed PaGIA gel were only weakly positive (1+ or 2+) (see table 1). In local experience such reactions are rarely positive by ELISA in HIT. Secondly, the OD for samples tested by both Lifecodes assays were never higher than 1.90, suggesting that the antibody in the assay was

exhausted in the reaction, and that higher dilutions of patient sample are required for accurate OD readings using these two assays.

The next stage would be to determine whether the anti-PF4 antibodies detected cause platelet activation. Further studies should also investigate the presence or absence of anti-PF4 antibodies in different patient groups that may include healthy non-vaccinated patients, healthy patients post-vaccination with ChAdOx1, and thrombocytopenic patients without raised D-Dimers or thrombosis post vaccination with ChAdOx1.

We conclude that none of the rapid assays tested, that may be suitable for the exclusion of HIT, is suitable for the exclusion of VITT. Centres where such rapid assays are in use for the diagnosis of HIT should ensure that requests for diagnosis of VITT should be clearly distinguished from those for diagnosis of HIT so that the correct tests are performed.

Clinicians should be aware that ELISA assays are not widely available in diagnostic laboratories, and a very small number of laboratories globally are able to perform platelet activation assays. Our study showed no single ELISA method appears to detect all cases of VITT, and therefore if a single ELISA test is negative, a second ELISA or platelet activation assay should be considered where there is strong clinical suspicion.

Sean Platton, Sue Pavord, Mike Makris and Marie Scully devised the study; Sean Platton, Andy Bartlett, and Deepak Singh performed the sample analysis. Sean Platton wrote the first draft of the manuscript; all authors contributed to the review and revision of the manuscript. All authors declare no relevant conflicts of interest.

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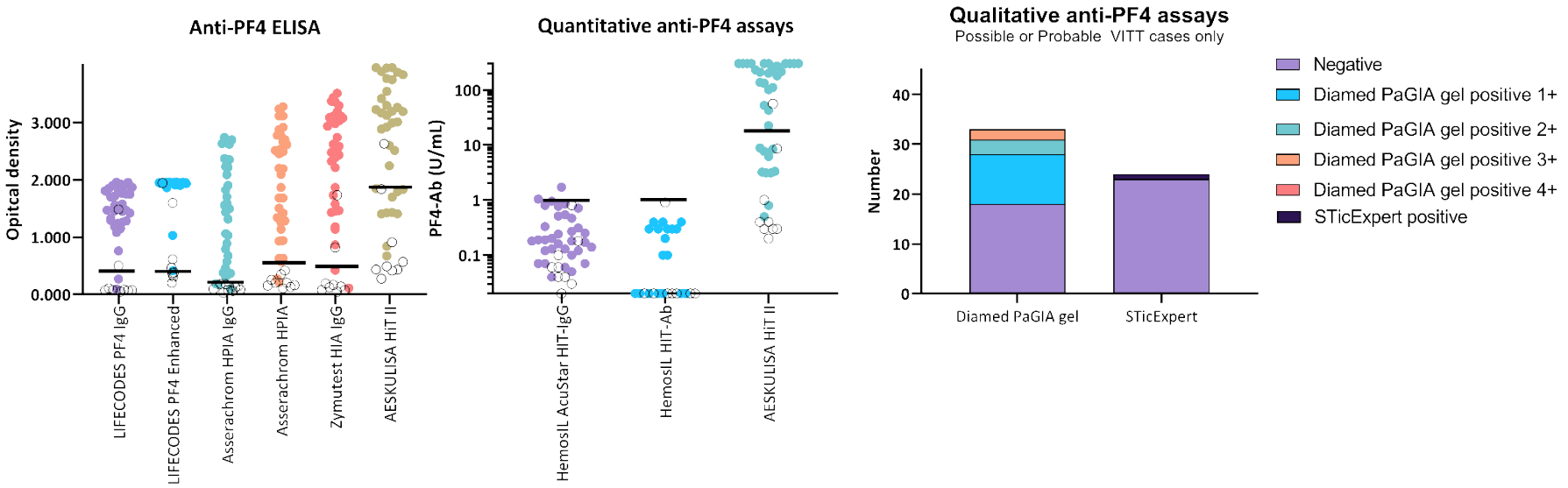

Figure 1: Results for anti-PF4 assays for samples from patients with suspected Vaccine-induced Immune Thrombocytopenia and Thrombosis. Key – solid circles: VITT possible or probable; empty circles: VITT unlikely. PF4: platelet factor 4; ELISA: enzyme linked immunosorbent assay. Solid black line indicates assay-specific cut-off for assay.

Study number	AESKULISA HiT II			Asserachrom HPIA IgG		Lifecodes PF4 IgG		Zymutest HIA IgG		Asserachrom HPIA		Lifecodes PF4 Enhanced		HemosIL AcuStar HIT-IgG$_{(PF4-H)}$		HemosIL HIT-Ab$_{(PF4-H)}$		Diamed gel	STic Expert
		OD	U/mL		OD		OD		OD		OD		OD		U/mL		U/mL		
	Positive or Negative	Cut-off 1.88	Cut-off 18.0	**Positive** or Negative	Cut-off 0.21	**Positive** or Negative	Cut-off 0.40	**Positive** or Negative	Cut-off 0.47	**Positive** or Negative	Cut-off 0.54	**Positive** or Negative	Cut-off 0.40	**Positive** or Negative	Cut-off 1.00	**Positive** or Negative	Cut-off 1.0	**Positive** or Negative	**Positive** or Negative
Probable cases																			
VITT01	**Positive**	2.25	22.5	**Positive**	0.37	**Positive**	1.18	**Positive**	0.87	**Positive**	1.14	**Positive**	1.89	Negative	0.04	Negative	0.3	Negative	Negative
VITT02	**Positive**	3.96	>300.0	**Positive**	2.37	**Positive**	1.90	**Positive**	3.36	**Positive**	2.92	**Positive**	1.91	Negative	0.12	Negative	0.3	Negative	Negative
VITT04	**Positive**	3.95	>300.0	**Positive**	2.70	**Positive**	1.95	**Positive**	3.37	**Positive**	3.11	**Positive**	1.91	Negative	0.19	Negative	0.4	Negative	Negative
VITT05	**Positive**	3.42	>300.0	**Positive**	2.29	**Positive**	1.85	**Positive**	3.03	**Positive**	2.49	**Positive**	1.91	Negative	0.85	Negative	0.2	**1+**	Negative
VITT07	**Positive**	3.23	229.3	**Positive**	2.22	**Positive**	1.96	**Positive**	3.30	**Positive**	2.62	**Positive**	1.90	**Positive**	1.72	Negative	0.0	**2+**	Negative
VITT08	**Positive**	3.01	137.6	**Positive**	1.91	**Positive**	1.42	**Positive**	2.74	**Positive**	2.20	**Positive**	1.90	Negative	0.14	Negative	0.0	**1+**	Negative
VITT10	Negative	1.43	3.3	Negative	0.18	**Positive**	1.33	Negative	0.42	**Positive**	0.27	**Positive**	1.89	Negative	0.07	Negative	0.4	Negative	Negative
VITT12	Negative	1.79	7.7	**Positive**	0.38	**Positive**	1.47	**Positive**	1.47	**Positive**	0.63	**Positive**	1.88	Negative	0.06			Negative	Negative
VITT13	**Positive**	2.99	131.5	**Positive**	0.31	**Positive**	1.70	**Positive**	2.57	**Positive**	0.93	**Positive**	1.91	Negative	0.18			Negative	Negative
VITT14	Negative	0.67	0.5	**Positive**	1.06	**Positive**	1.15	**Positive**	1.43	**Positive**	1.25	**Positive**	1.86	Negative	0.54			**1+**	Negative
VITT15	**Positive**	3.75	>300.0	**Positive**	2.74	**Positive**	1.87	**Positive**	3.51	**Positive**	2.77	**Positive**	1.90	Negative	0.18	Negative	0.3	**3+**	**Positive**
VITT17	**Positive**	3.27	253.2	**Positive**	1.56	**Positive**	1.46	**Positive**	3.08	**Positive**	2.51	**Positive**	1.91	Negative	0.13			Negative	Negative
VITT18	**Positive**	3.19	210.1	Negative	0.19	**Positive**	1.55	**Positive**	2.48	**Positive**	0.57	**Positive**	1.89	Negative	0.05			**1+**	
VITT19	**Positive**	3.17	199.9	**Positive**	0.79	**Positive**	1.72	**Positive**	2.59	**Positive**	1.87	**Positive**	1.90	Negative	0.07			Negative	Negative
VITT23	Negative	1.42	3.2	**Positive**	2.23	Negative	0.27	**Positive**	2.43	**Positive**	1.69	**Positive**	0.98	Negative	0.80			**2+**	
VITT24	**Positive**	3.55	>300.0	**Positive**	1.70	**Positive**	1.76	**Positive**	2.98	**Positive**	2.53	**Positive**	1.91	Negative	0.24			Negative	Negative
VITT25	Negative	0.84	0.8	Negative	0.10	Negative	0.10	Negative	0.11	Negative	0.21	Negative	0.37	Negative	0.12			Negative	
VITT26	**Positive**	2.92	110.9	**Positive**	0.35	**Positive**	1.26	**Positive**	1.73	**Positive**	0.94	**Positive**	1.86	Negative	0.10			**1+**	Negative
VITT27	Negative	1.70	6.2	**Positive**	2.69	**Positive**	1.93	**Positive**	3.11	**Positive**	3.28	**Positive**	1.91	Negative	0.27			Negative	
VITT28	**Positive**	2.88	101.6	**Positive**	0.46	**Positive**	1.28	**Positive**	2.39	**Positive**	1.69	**Positive**	1.90	Negative	0.17			Negative	Negative

VITT29	**Positive**	2.60	52.1	**Positive**	0.36	**Positive**	1.47	**Positive**	1.18	**Positive**	1.29	**Positive**	1.90	**Positive**	1.04			Negative	Negative
VITT31	**Positive**	3.88	>300.0	**Positive**	1.83	**Positive**	1.24	**Positive**	3.11	**Positive**	3.12	**Positive**	1.91	Negative	0.13	Negative	0.1	Negative	Negative
VITT33	Negative	1.85	8.8	**Positive**	1.03	**Positive**	1.08	**Positive**	1.14	**Positive**	1.34	**Positive**	1.89	Negative	0.51	Negative	0.3	**3+**	
VITT36	Negative	1.40	3.1	**Positive**	0.89	**Positive**	0.76	**Positive**	1.58	**Positive**	1.51	**Positive**	1.82	Negative	0.25				Negative
VITT38	**Positive**	3.12	179.7	**Positive**	1.43	**Positive**	1.29	**Positive**	2.22	**Positive**	2.78	**Positive**	1.88	Negative	0.33	Negative	0.1	Negative	
VITT40	**Positive**	2.52	42.7	**Positive**	1.32	**Positive**	1.56	**Positive**	1.87	**Positive**	2.20	**Positive**	1.90	Negative	0.77	Negative	0.0	Negative	
VITT44	**Positive**	3.89	>300.0	**Positive**	2.63	**Positive**	1.80	**Positive**	3.19	**Positive**	2.88	**Positive**	1.91	Negative	0.20	Negative	0.0	**1+**	Negative
Possible cases																			
VITT06	Negative	1.83	8.4	**Positive**	0.57	**Positive**	1.79	**Positive**	1.46	**Positive**	0.63	**Positive**	1.90	Negative	0.47	Negative	0.0	**1+**	Negative
VITT09	**Positive**	3.96	>300.0	**Positive**	2.62	**Positive**	1.81	**Positive**	3.43	**Positive**	3.24	**Positive**	1.91	Negative	0.95	Negative	0.4	**1+**	Negative
VITT11	**Positive**	3.77	>300.0	**Positive**	1.60	**Positive**	1.75	**Positive**	3.09	**Positive**	2.45	**Positive**	1.90	Negative	0.07			Negative	Negative
VITT30	**Positive**	3.20	214.6	**Positive**	1.49	**Positive**	1.58	**Positive**	2.62	**Positive**	2.09	**Positive**	1.90	Negative	0.71			**2+**	Negative
VITT37	**Positive**	3.30	271.8	**Positive**	0.68	**Positive**	1.45	**Positive**	2.33	**Positive**	1.42	**Positive**	1.90	Negative	0.19	Negative	0.0	Negative	
VITT39	Negative	1.41	3.1	**Positive**	2.35	**Positive**	1.90	**Positive**	2.94	**Positive**	2.70	**Positive**	1.91	Negative	0.31	Negative	0.0	**1+**	
VITT45	**Positive**	3.83	>300.0	**Positive**	2.09	**Positive**	1.76	**Positive**	3.03	**Positive**	2.66	**Positive**	1.90	Negative	0.16	Negative	0.3	**1+**	
Unlikely cases																			
VITT03	Negative	1.84	8.6	Negative	0.03	Negative	0.05	Negative	0.05	Negative	0.11	Negative	0.29	Negative	0.00	Negative	0.9	Negative	Negative
VITT20	Negative	0.91	1.0	Negative	0.12	Negative	0.08	Negative	0.14	Negative	0.13	Negative	0.33	Negative	0.04			Negative	
VITT21	Negative	0.43	0.3	Negative	0.08	Negative	0.07	**Positive**	1.74	Negative	0.15	Negative	0.26	Negative	0.10	Negative	0.0	Negative	
VITT22	Negative	0.42	0.3	Negative	0.19	Negative	0.07	Negative	0.07	**Positive**	0.26	Negative	0.15	Negative	0.03	Negative	0.0	Negative	
VITT32	Negative	0.43	0.3	Negative	0.10	Negative	0.07	Negative	0.08	Negative	0.20	**Positive**	0.42	Negative	0.18	Negative	0.0	Negative	Negative
VITT35	Negative	0.28	0.2	Negative	0.14	Negative	0.09	Negative	0.19	Negative	0.16	Negative	0.33	Negative	0.06	Negative	0.0	**2+**	
VITT41	**Positive**	2.63	56.0	Negative	0.18	**Positive**	1.49	**Positive**	0.82	**Positive**	0.42	**Positive**	1.90	Negative	0.06	Negative	0.0	Negative	
VITT42	Negative	0.49	0.4	Negative	0.08	**Positive**	0.50	Negative	0.16	**Positive**	0.35	**Positive**	1.55	Negative	0.04	Negative	0.0	**1+**	
VITT43	Negative	0.57	0.4	Negative	0.06	Negative	0.11	Negative	0.13	Negative	0.20	**Positive**	0.56	Negative	0.81	Negative	0.0	**3+**	

Table 1: Resuls of anti-PF4 assays. OD: optical density

Assay	Sensitivity for VITT % (95% CI)	Specificity for VITT % (95% CI)	Sensitivity for HIT % (95% CI)	Specificity for HIT % (95% CI)
IgG-specific ELISAs				
AEKSULISA HiT II	70.6 (53.8-83.2)	88.9 (56.5-99.4)	91*	97*
Asserachrom HPIA IgG	91.1 (77.0-97.0)	100.0 (70.1-100.0)	72.0 (68.4-75.5)[7]	93.8 (90.3-97.4)[7]
Lifecodes PF4 IgG	94.1 (80.9-99.0)	77.8 (45.3-96.1)	99.6 (22.7-100.0)[7]	89.9 (86.2-92.6)[7]
Zymutest HIA IgG	94.1 (80.9-99.0)	77.8 (45.3-96.1)	99.2 (86.4-100.0)[7]	85.8 (77.1-91.5)[7]
Polyspecific ELISAs				
Asserachrom HPIA	94.1 (80.9-99.0)	100.0 (70.1-100.0)	92.7 (73.6-98.3)[7]	87.3 (79.9-92.3)[7]
Lifecodes PF4 Enhanced	100.0 (89.9-100.0)	55.6 (26.7-81.1)	99.9 (90.9-100.0)[7]	87.4 (79.2-92.7)[7]
Rapid tests				

Diamed PaGIA gel	45.5 (29.8-62.0)	66.7 (35.4-87.9)	96.5 (89.8-98.9)[7]	93.7 (83.1-97.8)[7]
HemosIL AcuStar HIT-$IgG_{(PF4-H)}$	5.9 (1.0-19.1)	100.0 (70.1-100.0)	98.8 (69.2-100.0)[7]	94.6 (90.7-96.9)[7]
HemosIL HIT-$Ab_{(PF4-H)}$	0.0 (0.0-17.6)	100.0 (67.6-100.0)	100.0[7]	84.3[7]
STic Expert	4.2 (0.2-20.2)	100.0 (17.8-100.0)	98.4 (85.3-99.9)[7]	90.3 (84.4-94.1)[7]

Table 2: Sensitivity and specificity of assays for possible and probable vaccine-induced immune thrombocytopenia and thrombosis, and for heparin induced thrombocytopenia. *Manufacturer's data. 95% CI: 95% confidence interval.