Accurate detection of Zika virus IgG using a novel immune complex binding ELISA

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Abstract

OBJECTIVES Accurate serological assays are urgently needed to support public health responses to Zika virus (ZIKV) infection with its potential to cause foetal damage during pregnancy. Current flavivirus serology for ZIKV infections lacks specificity due to cross-reacting antibodies from closely related other flaviviruses. In this study, we evaluated novel serological tests for accurate ZIKV IgG detection.

METHODS Our ELISAs are based on immune complex binding. The high specificity is achieved by the simultaneous incubation of labelled ZIKV antigen and unlabelled flavivirus homolog protein competitors. Two assays were validated with a panel of 406 human samples from PCR-confirmed ZIKV patients collected in Brazil (n = 154), healthy blood donors and other infections from Brazil, Europe, Canada and Colombia (n = 252).

RESULTS The highest specificity (100% [252/252, 95% confidence interval (CI) 98.5–100.0]) was shown by the ZIKV ED3 ICB ELISA using the ED3 antigen of the ZIKV envelope. A similar test using the NS1 antigen (ZIKV NS1 ICB ELISA) was slightly less specific (92.1% [232/252, 95% CI 88.0–95.1]). The commercial Euroimmun ZIKV ELISA had a specificity of only 82.1% (207/252, 95% CI 76.8–86.7). Sensitivity was high (93–100%) from day 12 after onset of symptoms in all three tests. Seroprevalence of ZIKV IgG was analysed in 87 samples from Laos (Asia) confirming that the ED3 ELISA showed specific reactions in other populations.

CONCLUSIONS The novel ED3 ICB ELISA will be useful for ZIKV-specific IgG detection for seroepidemiological studies and serological diagnosis for case management in travellers and in countries where other flavivirus infections are co-circulating.

KEYWORDS Zika virus (ZIKV), flavivirus, immune complex binding (ICB) ELISA, diagnostics

Sustainable Development Goals (SDGs): SDG 3 (good health and well-being), SDG 17 (partnerships for the goals)

Introduction

Zika virus (ZIKV) is a mosquito-transmitted flavivirus that caused large epidemics in the Pacific and the Americas in 2015-2017 [1,2]. ZIKV infection during pregnancy has been linked to severe congenital defects and malformations including microcephaly [3]. ZIKV infection in adults is linked to Guillain–Barré syndrome [2]. Diagnosis and surveillance of ZIKV infections remain challenging. Up to 80% of the cases are asymptomatic [4]. Virus detection by PCR diagnostics is specific, but often symptoms are too mild for patients to appear in the acute phase of infection, when the virus is detectable. In some patients, ZIKV IgM is not detectable or appears only briefly [5,6]. Reliable IgG detection is very difficult due to extensive antibody cross-reactivity between closely related flaviviruses - especially in...
dengue virus (DENV) and other flaviviral endemic regions, where a high percentage of ZIKV infections may occur in people previously exposed to at least one other flavivirus infection [6,7]. The diagnostic gold standard, the ZIKV plaque reduction neutralisation test, frequently shows cross-reactive antibodies following DENV infection, particularly in cases of secondary infections [3,8,9]. Thus WHO declared the development of improved ZIKV diagnostic assays a priority [10]. In this study, we present the validation of a range of novel immune complex binding (ICB) ELISA assays [7,11], which facilitate accurate detection of ZIKV IgG antibodies.

### Methods

#### Ethics statement

The study was conducted according to the principles of the Declaration of Helsinki. Written informed consent was obtained from all individuals or, in case of minors, from parents before enrolment. Data on human subjects were analysed anonymously. Collection of human samples was approved by the Ethics Committees of the Oswaldo Cruz Foundation/Brazil (no. CSN196/96), the Hospital Rosario Pumarejo de Lopez of Valledupar/Colombia, the Ethics Committee of Lao People’s Democratic Republic (no. 030/NECHR) and the Medical Association Hamburg/Germany (no. PV4608).

#### Human samples validation

N = 154 human serum or plasma samples were collected in Brazil during 2015–2017 from 61 patients (median age 39 years [range 11–67]; 32.6% [15/46] male; 67.4% [31/46] female) with PCR-confirmed ZIKV infection (RealStar Zika Virus RT-PCR Kit, Altona Diagnostics, Hamburg, Germany). From each patient, one ‘early’ sample collected between day 0 and 9 after onset of symptoms and one ‘late’ (follow-up) sample collected 1–21 days after the ‘early’ sample was analysed. In addition, longitudinal samples were collected from 10 of these patients (3–10 samples/patient, collected days 0–270 after onset of symptoms; Supporting Information S1, S5). N = 252 a priori ZIKV IgG negative samples, that is, from non-endemic regions for ZIKV or collected before the virus arrived in these regions, were used as a specificity panel consisting of healthy blood donors (flavivirus vaccinated/non-vaccinated 73/27%), a range of human flavivirus infections and other infections collected in Canada, Europe and South America (Supporting Information S1, S3). WNV samples were kindly provided by P. Singh and M. Loeb (McMaster University, Canada), DENV samples (collected in Colombia in 2014) were kindly provided by S. Kann (Medical Mission Institute, Würzburg), M. Mackroth (Bernhard Nocht Institute for Tropical Medicine [BNITM], Germany) provided samples from yellow fever virus (YFV)-vaccinated donors, C. Carrasco (Robert Koch Institute, Berlin) one Japanese encephalitis virus (JEV)-infected patient sample, and B. Fleischer (BNITM, Germany) P. falciparum and P. malariae malaria patient samples. Human samples were stored at –80°C for long-term storage.

#### Human samples from Laos

During a study at Savannakhet Provincial Hospital (Laos) samples from healthy blood donors (n = 20), dengue patients (n = 25) and patients with undifferentiated fever (n = 152 early samples from day 1 to 9 after onset of symptoms, n = 42 follow-up samples) were collected in 2013–2015. Early samples were analysed for DENV (qPCR, Altona Diagnostics, Hamburg, Germany), malaria and later by ZIKV qPCR (Altona).

#### ZIKV antigens

Two recombinant ZIKV antigens were purified. Briefly, a cDNA fragment coding for non-structural protein 1 (ZIKV-NS1, 40 kDa) was cloned in prokaryotic expression vector pJ4C45 without protein tags, while the cDNA fragment coding for envelope protein domain 3 (ZIKV-ED3) was cloned as N-terminal polyhistidine fusion protein with a 3C protease cleavage site by in fusion cloning (14.9 kDa, Supporting Information S4). Plasmids were transformed into E. coli BL21 (pAPlacCl2) cells by heat shock transformation. Cells were grown in Luria–Bertani medium supplemented with 50 μg/ml kanamycin and 100 μg/ml ampicillin, followed by IPTG-induced expression of the recombinant proteins at 18°C overnight. Bacteria were lysed by digest in lysozyme buffer (0.25 μg/μl lysozyme, 0.01 μg/μl DNase, 10 mM EDTA, 1 mM PMSF in PBS) and by sonification. Inclusion bodies were isolated by centrifugation (20 min 12 000 g at 4°C), washed with 0.5% (v/v) Triton X-100 in PBS and solubilised in 6 M guanidine hydrochloride, 50 mM Tris-HCl, pH 8.8 (ZIKV-NS1) or pH 7.8 (ZIKV-ED3).

After solubilisation recombinant ZIKV-NS1 protein was reduced by addition of 10 mM dithiothreitol for 30 min on ice, refolded by rapid dilution in refolding buffer 1 (100 mM Tris – pH 8.8, 400 mM NDSB-201, 5 mM reduced glutathione, 0.5 mM oxidised glutathione, 0.1 mM PMSF, 1 mM EDTA) and further purified by size exclusion chromatography on a Superdex 75/Highload column with an AKTA pure FPLC system (GE Healthcare, Pittsburgh, PA, USA) in gel filtration buffer (25 mM...
carbonate – pH 9.0, 150 mM NaCl, 10% (w/v) glycerol). Solubilised, recombinant ZIKV-ED3 protein was purified by Nickel-nitrilotriacetic acid affinity chromatography (Ni-NTA) in binding buffer (6 M urea, 50 mM NaH$_2$PO$_4$, 10 mM Tris – pH 8.0, 5 mM imidazol, 150 mM NaCl, 1% (w/v) glycerol) followed by elution with 250 mM imidazol. The protein was refolded by rapid dilution in refolding buffer 2 (100 mM Tris – pH 8.5, 300 mM arginine, 150 mM NaCl, 1 mM EDTA, 5 mM reduced glutathione, 0.5 mM oxidised glutathione, 1 mM PMSF, 0.01% sodium azide) followed by purification using size exclusion. Purity was visualised by SDS-PAGE with Coomassie staining. Antigens were stored at –80°C.

Flaviviral competitors and CD32

Briefly, five cDNA fragments coding for homologous regions (ED3) from different flaviviruses [DENV1, 2, 3, 4; West Nile virus (WNV)] were cloned and proteins expressed in E. coli as described above for ZIKV-ED3 with slight modifications (Supporting Information S4). Inclusion bodies were solubilised and proteins purified by denaturing Ni-NTA, followed by refolding. Inclusion bodies were either washed with Triton X-100 (WNV-ED3) or proteins were purified further by native Ni-NTA (DENV1-ED3, DENV2-ED3, DENV3-ED3, DENV4-ED3) using FPLC. CD32 as IgG/immune complex binding molecule was expressed and purified as described earlier [7,11].

Labelling of ZIKV antigens

500 µg ZIKV-ED3 or 200 ug ZIKV-NS1 was labelled with activated horseradish peroxidase. Labelling was performed as described earlier [12]. Conjugates were stored at –20°C.

ZIKV ELISA procedures

**ZIKV ED3 ICB ELISA.** The labelled ZIKV ED3 antigen was used at a final dilution of 1:100,000 of a stock solution containing 0.2 mg/ml antigen. To increase the specificity of the test related flavivirus antigens were competitively added. Final competitor dilutions were 1:100 for WNV-ED3 and 1:1000 for each DENV protein using stock solutions of 1 mg/ml. Competitor proteins were diluted into the conjugate dilution and added into the ELISA well with ≥500-fold molar excess to ZIKV antigen concentrations.

Plates were coated with 5 µg/ml CD32 in coating buffer (PBS pH 7.4, 0.01% NaN$_3$, 0.01% phenol red) at 4°C. After 3x washing with 100 mM Tris pH 7.4, 150 mM NaCl, plates were dried. For detection of anti-ZIKV antigen antibodies, plates were washed 3x with wash buffer (100 mM Tris/HCl pH 7.4, 150 mM NaCl, 0.05% Tween 20, 0.005 % Proclin 300). Samples were diluted 1:50 in serum dilution buffer (PBS pH 7.4, 0.05% Proclin 300, 0.01% phenol red).

Per well, 25 µl of diluted human serum was mixed with 25 µl ZIKV antigen/HRP conjugate and the competitor proteins diluted in conjugate dilution buffer (PBS pH 7.4, 1% bovine serum albumin, 0.5% foetal calf serum, 1% Nonidet P40, 0.1% ProClin 300) to a final serum dilution of 1:100 and final conjugate (ZIKV-ED3) and competitor dilutions as described above. Plates were incubated for 24 h at 4°C. Plates were washed 3x with wash buffer, followed by addition of 100 µl/well tetramethylbenzidine substrate. Plates were incubated for 20 min at RT, followed by addition of 100 µl/well 1 N sulphuric acid. Signals were quantified at 450 nm corrected by 620 nm. Standard controls (negative, positive, cut-off) were included in all assays.

The ICB technology is protected by European (EP2492689) and international (CN103460048, HK1192320, CA2823107, US2014080120) patents owned by BNITM.

**ZIKV NS1 ICB ELISA.** The test procedure was identical to that of the ED3 ICB ELISA, however, the labelled NS1 protein was applied at a very high dilution of 1:250,000. No competitor proteins were added.

**Euroimmun Anti-Zika Virus IgG ELISA** (Euroimmun Lübeck, Germany) was performed according to the manufacturer’s instructions. The test uses ZIKV NS1 protein as antigen source.

Data analysis

Significant differences between subgroups of samples were calculated by Fisher’s exact test (Prism software, GraphPad, San Diego, California, USA). Cut-off determination was performed using receiver operating characteristic curves (ROC; MedCalc, Ostend, Belgium), which were calculated as P/cut-off.

Results

Test principle and validation

In this study, two novel ZIKV IgG ELISAs were developed combining different labelled ZIKV antigens with or without flaviviral competitors (ZIKV ED3 ICB ELISA, ZIKV NS1 ICB ELISA). Two different ZIKV proteins, that is, domain 3 (ED3) of the envelope protein and non-structural protein 1 (NS1) were used as labelled antigens, and five additional homologous proteins from other human pathogenic flaviviruses were used as competitors. In order to minimise interference by other flaviviral
antibodies, we utilised a novel ELISA principle, which is based on an ICB technology ([7,11,12]; Figure 1).

The ZIKV antigens used as conjugates in the different ELISAs contain specific ZIKV but also cross-reactive flaviviral epitopes. These cross-reactive epitopes can bind IgG from other flaviviral infections and/or vaccinations (DENV, JEV, WNV, YFV and tick-borne encephalitis virus) and cause false-positive signals in many commercial and in house serological assays. The use of competitors in combination with the ICB technology facilitates very high specificity due to preferential binding of cross-reactive antibodies to the unlabelled proteins from other flaviviruses thereby suppressing flaviviral antibody cross-reactivity (Figure 1).

Additional experiments with different quantities of competitors showed that the use of only one protein competitor in the assays was not enough to suppress the cross-reactivity of samples from a range of flaviviral infections and vaccines, while the addition of more competitors than five (including JEV and YFV proteins) was not necessary to reach 100% specificity (Figure 2).

In contrast to the simultaneous antigen and competitor incubation used in the novel assays, pre-incubation of serum with competitors to reduce cross-reactivity was not effective, maybe due to incomplete depletion of cross-reactive IgG in the samples (data not shown).

Specificity

Comprehensive validation of the two assays with a large panel of human samples showed the high specificity of the test systems (Table 1). Test performance was compared with the most widely used commercial ELISA (Euroimmun). Diagnostic specificity was determined with an a priori ZIKV IgG negative specificity sample panel consisting of a range of healthy blood donors, flaviviral and other infections from Canada, Europe and South America (n = 252). The best assay performance showed the ZIKV ED3 ICB ELISA (containing five competitor proteins) with 100% (252/252) specificity and 92.9% (26/28) diagnostic sensitivity from day 12 after onset (Table 1). Remarkably, without competitors the specificity of the ZIKV ED3 ICB ELISA was clearly lower (90.1% (227/252, CI 85.7–93.5), data not shown). The two other IgG assays without competitors (ZIKV NS1 ICB ELISA and Euroimmun ZIKV ELISA) showed a lower specificity of 92.1% (232/252, CI 88.0-95.1) and 82.1% (207/252; CI 76.8-86.7, Table 1), respectively. The Euroimmun test revealed high cross-reactivity with sera from blood donors, DENV and other infections from Brazil and Colombia collected before the outbreak of the ZIKV epidemic (Figure 3).

In addition, a data analysis was performed using subgroups of the specificity sample panel to investigate how the specificity values changed, if only samples from flavivirus-infected and flavivirus-vaccinated persons were used (Table 2). While the specificity of the three ELISA was similar with flavivirus samples from Europe and North America, there were significant differences in

![Figure 1](https://example.com/figure1.png)

**Figure 1** Test principle of the ZIKV IgG ICB ELISA assays with competitors. Serum and labelled ZIKV antigen are incubated simultaneously on an IgG/IC receptor plate. The receptor-coated plate binds preferentially newly forming immune complexes. Addition of competitors (DENV and other flaviviral antigens) in >100-fold excess to the ZIKV antigen leads to suppression of flaviviral interference. All newly forming IgG/immune complexes bind to the plate, but only the labelled ZIKV antigen causes colour formation. IC, immune complex; HRP, horseradish peroxidase; IgG<sub>sp</sub>, specific ZIKV IgG; IgG<sub>cr</sub>, cross-reactive ZIKV IgG; TMB, tetramethylbenzidine.

[Colour figure can be viewed at wileyonlinelibrary.com]
specificity between the three tests using samples from South America (Table 2).

**Sensitivity**

Diagnostic sensitivity was determined with acute and convalescent samples \((n = 154)\) from 61 PCR-confirmed ZIKV patients collected during and after the ZIKV outbreak in Brazil revealing also a high sensitivity of the ZIKV ED3 ICB ELISA from day 12 after onset of symptoms \([91.3\% (21/23)\text{ sensitivity, Table 1}]\), while 100\% \((8/8)\text{ sensitivity was reached between month 2 and 6 (days 31–182)}\).

Of the three ZIKV IgG assays, the ZIKV NS1 ICB assay and the Euroimmun ELISA are best suited as a screening assay. Both had a high diagnostic sensitivity with this sample panel reaching 100\% sensitivity from day 31 after onset (Table 1) and allow earlier detection with high sensitivity from day 7.

**Cut-off determination by ROC analysis**

For determination of the optimal ELISA cut-offs, the ROC analysis of the assays was performed with a specific subset of the ZIKV patient samples consisting of sera collected later than \(\geq 12\) days after onset of symptoms using only one sample/patient \((n = 28)\). The data analysis was based on the hypothesis that every sample from a PCR-confirmed ZIKV patient collected on day 12 after onset or later should be ZIKV IgG positive. For ROC analysis the same specificity sample panel was used as for validation \((n = 252)\). The ROC analysis of the ZIKV NS1 ICB ELISA
Table 1 Test performance of the two novel ZIKV IgG assays in comparison with the most used commercial ELISA (Euroimmun)

| Parameter                   | N patients, n samples, Remark | Days after onset | Euroimmun ZIKV ELISA* | ZIKV ICB ELISA |
|-----------------------------|-------------------------------|-----------------|------------------------|----------------|
| Plate                       |                               |                 |                        |                |
| HRP-labelled antigen        |                               |                 |                        |                |
| Competitor proteins         |                               |                 |                        |                |
| Diagnostic sensitivity in % (cases) | N = 61, n = 154 | 0–1† | None | 59.1 (13/22) | 45.5 (10/22) | 4.5 (1/22) |
|                            |                               | 2–6             | None | 58.1 (25/43) | 44.2 (19/43) | 14.0 (6/43) |
|                            |                               | 7–11            | None | 83.3 (25/30) | 80.0 (24/30) | 46.7 (14/30) |
|                            |                               | 12–30           | None | 95.7 (22/23) | 100.0 (23/23) | 91.3 (21/23) |
|                            |                               | 31–182          | None | 100.0 (8/8) | 100.0 (8/8) | 100.0 (8/8) |
|                            |                               | 183–365         | None | 100.0 (3/3) | 100.0 (3/3) | 100.0 (3/3) |
| Diagnostic sensitivity in % (cases) | N = 28, n = 28 | ≥12 | None | 96.4 (27/28) | 100.0 (28/28) | 92.9 (26/28) |
|                            |                               | [95% CI]        |               | [81.7–99.9]    | [87.7–100.0] | [76.5–99.1] |
| Diagnostic specificity in % (cases) | N = 252, n = 252 | ≥12 | None | 82.1 (207/252) | 92.1 (232/252) | 100.0 (252/252) |
|                            |                               | [95% CI]        |               | [76.8–86.7]    | [88.0–95.1] | [98.5–100.0] |

Test validation was performed with n = 406 human samples. Cut-off determination of the ELISA tests was performed by ROC analysis of a specific subset of samples (n = 252 a priori ZIKV IgG negative human samples and late samples [n = 28] from PCR-confirmed ZIKV patients collected from day ≥12 after onset of symptoms). Confidence Intervals (CI) are shown for diagnostic specificity and sensitivity from day ≥12 after onset of symptoms during ZIKV infection.

*Calculated in this study.
†For 5 samples: day after hospital admission (day after onset unknown).
Figure 3 Test validation of two ZIKV IgG immune complex binding (ICB) assays in comparison with the Euroimmun ZIKV IgG ELISA. (a) Euroimmun ELISA, (b) ZIKV NS1 ICB ELISA, (c) ZIKV ED3 ICB ELISA. Diagnostic specificity was shown with a priori ZIKV IgG negative human samples from Europe, Canada and South America ($n = 252$) and diagnostic sensitivity with $n = 28$ samples from $N = 28$ ZIKV patients collected from ≥12 days after onset of symptoms in Brazil. Dotted line, cut-off; straight lines, median values; Br, Brazil; Ca, Canada; C/H, CMV and/or Hepatitis A-E-infected; Co, Colombia; DEN, dengue-infected (all serotypes); Eu, Europe; hBD, healthy blood donors (73% JEV, TBEV and/or YFV-vaccinated); JE/TBEV, Japanese or tick-borne encephalitis virus-infected; Mal, malaria-infected (travellers); Ru/M, Rubella or measles-infected; WNV, West Nile virus-infected; YFV, yellow fever virus-infected; ZIKV, Zika virus-infected. [Colour figure can be viewed at wileyonelibrary.com]
Table 2 ZIKV IgG ELISA specificities calculated for subgroups

| Subgroup                | n (N) | Euroimmun ZIKV ELISA | ZIKV NS1 ICB ELISA | ZIKV ED3 ICB ELISA |
|-------------------------|-------|-----------------------|--------------------|--------------------|
| ZIKV neg_FLAVI          |       |                       |                    |                    |
| South America           |       |                       |                    |                    |
| Pos/G F                | 84    | 25                    | 59                 | 11                 |
| specificity in %        | (84)  | 70.2 (59.7–79.0)      | 86.9 (77.8–93.3)   | 100.0 (94.8–100.0) |
| P value                 |       |                       |                    |                    |
| ZIKV neg_FLAVI          |       |                       |                    |                    |
| Europe                  |       |                       |                    |                    |
| Pos/G F                | 35    | 0                     | 35                 | 0                  |
| specificity in %        | (35)  | 100.0 (88.2–100.0)    | 100.0 (88.2–100.0) | 100.0 (88.2–100.0) |
| P value                 |       |                       |                    |                    |
| ZIKV neg_FLAVI          |       |                       |                    |                    |
| North America           |       |                       |                    |                    |
| Pos/G F                | 9     | 0                     | 9                  | 0                  |
| specificity in %        | (9)   | 100.0 (65.5–100.0)    | 88.9 (54.3–100.0)  | 100.0 (65.5–100.0) |
| P value                 |       |                       |                    |                    |
| ZIKV neg_FLAVI          |       |                       |                    |                    |
| Total                   |       |                       |                    |                    |
| Pos/G F                | 128   | 25                    | 103                | 12                 |
| specificity in %        | (128) | 80.5 (72.7–86.5)      | 90.6 (84.2–94.7)   | 100.0 (96.5–100.0) |
| P value                 |       |                       |                    |                    |
| ZIKV neg_NON-FLAVI/HD   |       |                       |                    |                    |
| South America           |       |                       |                    |                    |
| Pos/G F                | 42    | 11                    | 31                 | 6                  |
| specificity in %        | (42)  | 73.8 (58.8–84.8)      | 85.7 (71.8–93.7)   | 100.0 (94.6–100.0) |
| P value                 |       |                       |                    |                    |
| ZIKV neg_NON-FLAVI/HD   |       |                       |                    |                    |
| Europe                  |       |                       |                    |                    |
| Pos/G F                | 82    | 9                     | 73                 | 2                  |
| specificity in %        | (82)  | 89.0 (80.2–94.3)      | 97.6 (91.0–99.9)   | 100.0 (94.6–100.0) |
| P value                 |       |                       |                    |                    |
| ZIKV neg_NON-FLAVI/HD   |       |                       |                    |                    |
| Total                   |       |                       |                    |                    |
| Pos/G F                | 124   | 20                    | 104                | 8                  |
| specificity in %        | (124) | 83.9 (76.3–89.4)      | 93.6 (87.6–96.9)   | 100.0 (96.4–100.0) |
| P value                 |       |                       |                    |                    |

Pos/neg, number of samples detected as positive (pos) and negative (neg) by the respective test. P values (*P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001) indicate statistically significant differences between the Euroimmun IgG ELISA as a reference and the ZIKV IgG ICB ELISAs. P values were calculated by Fisher’s exact test using subsets of samples from the specificity panel (n = 252 a priori ZIKV IgG negative samples). FLAVI, flavivirus-infected or -vaccinated; NON-FLAVI/HD, other infections or healthy blood donors; N, patients; n, samples. N.s., Not significant.
(containing HRP-labelled ZIKV-NS1 conjugate without competitors) revealed 100% diagnostic sensitivity (28/28, CI 87.7–100.0, cut-off (OD) 0.242), and the ZIKV ED3 ICB ELISA (containing HRP-labelled ZIKV-ED3 conjugate with competitors) showed 92.9% sensitivity (26/28, CI 76.5–99.1, cut-off (OD) 0.201, Table 1). For comparison, the commercial Euroimmun ZIKV ELISA showed a similar sensitivity (96.4% [27/28], CI 81.7–99.9) but a lower specificity (82.1%). For all three assays area under curve (AUC) values of the ROC analysis were excellent ranging from 0.952 to 0.980 (Figure 4).

ROC analyses were also performed with subgroups of the specificity sample panel using only a priori ZIKV IgG negative samples from flavivirus-infected patients and not
from healthy persons. Again these analyses showed lower specificities of the Euroimmun ELISA (74.4% [64/86], 95% CI 63.9–83.2) and the ZIKV NS1 ICB ELISA (87.2% [75/86], 95% CI 78.3–93.4, cut-off (OD) 0.242) than with the larger specificity panel, while the ZIKV ED3 ICB ELISA revealed the same high performance (100.0% specificity [86/86], 95% CI 95.8–100.0, cut-off (OD) 0.201); Supporting Information S7. AUC values were similar ranging from 0.936 to 0.978.

ZIKV seroprevalence in Laos

Due to the highly conserved sequence identity between the amino acid sequences of ZIKV-ED3 from different Asian, African and American ZIKV strains (95.2–99.0%), suitability of the ZIKV ED3 ICB ELISA for the detection of ZIKV IgG in samples collected worldwide seemed likely.

Retrospective analysis of a serum panel collected in Savannakhet (Laos, Asia) in 2013–2015 using our novel high specificity assay showed the presence of ZIKV IgG in this province and confirmed the suitability of the assay for use outside of Latin America. Two ZIKV IgG positive samples were found in 87 sera from fever patients collected late after onset of symptoms and healthy blood donors and tested by ZIKV ED3 ICB ELISA (one blood donor, one fever patient; Table 3). This was in accordance with results found by ZIKV qPCR in early samples of the fever patients collected in Savannakhet, which confirmed one ZIKV patient (0.7% (n = 1/152), data not shown) indicating the low circulation of ZIKV in Laos in 2013–2015 by both serology and qPCR.

Further tables give an overview of the test validation (Supporting Information S1, S2), flaviviral antigens and the sample panel (Supporting Information S3, S4), and show all test results (Supporting Information S5). Operational range, assay precision and reproducibility of the tests were well within the recommended range (Supporting Information S6).

**Discussion**

So far only a few serology tests have received emergency use authorisation granted through the U.S. Food and Drug Administration, and all were IgM assays [13]. The ZIKV ED3 ICB ELISA validated in this study is designed for use in population studies and individual case management (in particular for pregnant women and partners), not only in returning travellers but also in DENV endemic populations. Accurate detection of ZIKV IgG antibody in women prior to pregnancy will be beneficial to assess risks. The most widely used ZIKV IgG ELISA has been the Euroimmun ZIKV IgG kit [14,15]. Cross-reactivity of the Euroimmun test has been reported for malaria and DENV [16,17]. The low specificity found with our serum panel across many infections most likely reflects cross-reactivity from prior flavivirus infections in samples from DENV-endemic countries.

Due to the known flavivirus IgG cross-reactivity in PRNT and commercial ZIKV IgG ELISA [9,16,17] we did not use these assays as gold standard in this study but chose a different approach for data analysis and validation: late samples from ZIKV patients, who were confirmed by ZIKV qPCR in the acute phase of infection, were used as positive controls. The performance of both novel ELISAs and the commercial ELISA were compared based on the hypothesis that every sample from a PCR-confirmed ZIKV patient collected on day 12 after onset or later should be ZIKV IgG positive.

**Table 3** Overview on ZIKV IgG detection in follow-up samples from healthy blood donors and fever patients from Laos (Asia), collected in 2013-2015

| Cohort               | Characteristics* | N Patients, n samples | Days after onset (late sample) median (range) | Age median (range) | Gender male/female n (%) | ZIKV ED3 ICB ELISA % (cases) positive |
|----------------------|------------------|-----------------------|---------------------------------------------|-------------------|--------------------------|--------------------------------------|
| Healthy blood donors |                  | 20 (20)               | na                                          | 48 (23–54)        | 3/17 (15/85)             | 5.0 (1)                              |
| Dengue               | DENV+            | 25 (25)               | 21 (14–28)                                  | 21 (15–45)        | 14/11 (56/44)           | 0 (0)                                |
| Fever of unknown origin | DENV−, malaria negative | 42 (42)         | 23 (9–71)                                   | 30 (15–73)        | 20/22 (48/52)           | 2.4 (1)                              |
| Total                |                  | 87 (87)               | 21 (9–71)                                   | 30 (15–73)        | 37/50 (43/57)           | 2.3 (2)                              |

Early samples from the acute phase of infection were tested by PCR, while follow-up samples were measured by ZIKV IgG ELISA. DENV+/−, dengue PCR positive/negative.

In early sample.
Other ZIKV IgG tests have been developed [e.g. 3, 18–22]. Balmaseda et al [18] developed a ZIKV-NS1 blockade-of-binding IgG ELISA based on detection by a monoclonal antibody, which reached 96% specificity using a wide variety of samples. Denis et al [23] tested ZIKV-ED3 as antigen in classical sandwich IgG ELISA with 5600 sera and found a high suitability of the protein as antigen for serological diagnostics (90% specificity), similarly to other studies with flaviviral ED3 antigens [7]. However, performance is often difficult to compare due to differences in sample panels used for validation. The specificity panel used in this study is challenging, because it contains a high percentage of samples from DENV endemic countries with many secondary infections and includes many late samples, which show high levels of cross-reactive flaviviral IgG. Our data show, that such samples should be included in the assessment of other serological ZIKV IgG ELISA tests. Using a high percentage of samples from related flavivirus infections with strong cross-reactivity, which are representative of populations in many tropical countries, the ZIKV ED3 ICB test shows the highest specificity. It may represent a significant improvement on currently available ZIKV IgG diagnostic tools.

The ZIKV-ED3 protein used here is a small protein expected to have few cross-reactive epitopes similar to other flavivirus ED3 antigens. It required lower concentration of competitor to suppress cross-reactive signals than other larger antigens (data not shown). Moreover, due to the high dilution of 1:100 000 of the HRP-labelled antigen, the competitors could be applied at relatively low concentrations. The ZIKV NS1 ICB assay uses the same antigen as the commercial ELISA, however, sensitivity and specificity are better than the commercial test. This may be due to the ICB assay principle, which uses a soluble and thus freely rotating recombinant antigen instead of immobilisation of the protein on the plate.

While samples from \( n = 61 \) ZIKV patients were collected in Brazil during and after the epidemics, the sample panel was very diverse consisting of many samples from different time points after onset of infection. Paired samples were collected from most of the ZIKV patients, but sequential samples were collected from ten patients in a longitudinal study. While the ROC analysis (Figures 3, 4) contained only one sample/patient for sensitivity calculations, sequential samples from the longitudinal study were included in the sensitivity subgroup analysis (Table 1 [upper part]) to analyse different time periods after onset of symptoms. While only one sample/patient was included in each subgroup, the overrepresentation of samples from the longitudinal study may have caused a statistical bias in these sensitivity percentages (Table 1 [upper part]). However, it was informative to include sequential samples, since anti-ZIKV IgG antibodies appeared late in some patients during the course of disease. Samples collected later than 6 months after infection were only available from a few patients and showed that ZIKV IgG signals decreased after 8–9 months in some patients in all assays. Only one patient became seronegative giving results below cut-off (patient ZIKV-50, Supporting Information S5. Thus, further work is needed to define long-term ZIKV antibody persistence for pregnancy testing and epidemiological studies. For use in routine serological diagnostics, one of the screening ELISA (Euroimmun ELISA or the ZIKV NS1 ICB ELISA) and our high specificity IgG assay can be combined with a recently developed ZIKV IgM ICB ELISA [5]. The ZIKV IgM ICB ELISA uses a recombinant Fc\( \gamma \) receptor as coating on the ELISA plate and a labelled ZIKV-NS1 protein as antigen without presence of competitors to bind IgM/antigen immune complexes [5].

The high IgG signals found in the ZIKV ED3 ICB ELISA (without competitor, data not shown) and low IgG signals found in the ZIKV ED3 ICB ELISA (with competitors, Figure 3 in many ZIKV patient samples suggested the presence of high levels of flaviviral cross-reactive IgG in early patient samples. It seems likely that cross-reactive flaviviral IgG is present in Brazil where four different DENV serotypes and other flaviviral infections have circulated in the population for many years [24, 25]. Similarly, it has been observed in secondary DENV infections that antibodies in acute sera target mainly cross-reactive serotypes to earlier infections, while specific IgG of the current infecting serotype (e.g. anti-ED3 antibodies) rise in the later stages of infection [26]. Thus, the high specificity ELISA (detecting specific anti-ED3 IgG) appears to have lower sensitivity in early ZIKV patient samples in comparison with the other assays Table 1. However, the positive signals seen in the other assays may be a result of their higher sensitivity but could also be caused by cross-reactivity with IgG present from prior related flaviviral infections.

The high test performance of the ZIKV IgG ICB assay was further supported by our study in Laos. The specificity of the IgG assay was confirmed by the low seroprevalence found and the correlation with PCR data. The prevalence in Savannakhet was lower than the seroprevalence found in Vientiane capital (Laos) using the Euroimmun ZIKV ELISA (17–28% ZIKV in 2003–2015; [27]), which may be explained by anti-DENV cross-reactivity of the latter assay.

Immune complex binding ELISA technology is a novel diagnostic tool, which is simple to perform and a low-cost solution for use in diagnostic laboratories.
incorporation of competitors in the test enables the high specificity of the test for its use in the immensely difficult field of secondary flaviviral infections worldwide. In addition, the test has the potential advantage that in the event of the emergence of another new flavivirus the assay can be modified with an additional competitor to suppress interference. The newly developed ZIKV IgG assays are valuable tools for IgG detection of ZIKV infections in a range of clinical and public health applications.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Supporting Information S1. STARD diagram
Supporting Information S2. STARD checklist.
Supporting Information S3. Sample panel.
Supporting Information S4. Recombinant antigens.
Supporting Information S5. ZIKV IgG ELISA test results.
Supporting Information S6. Inter/intra-assay variation and operational range.
Supporting Information S7. ROC analyses of the three ZIKV ELISAs using only samples from flavivirus-infected patients as specificity panel.