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Immunoglobulin-like Domain of *Hs*FcμR as a Capture Molecule for Detection of Crimean-Congo Hemorrhagic Fever Virus- and Zika Virus-Specific IgM Antibodies

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BACKGROUND: The cellular surface molecule *Hs*TOSO/FAIM3/*Hs*FcμR has been identified as an IgM-specific Fc receptor expressed on lymphocytes. Here, we show that its extracellular immunoglobulin-like domain (*Hs*FcμR-Igl) specifically binds to IgM/antigen immune complexes (ICs) and exploit this property for the development of novel detection systems for IgM antibodies directed against Crimean-Congo hemorrhagic fever virus (CCHFV) and Zika virus (ZIKV).

METHODS: His-tagged *Hs*FcμR-Igl was expressed in *Escherichia coli* and purified by affinity chromatography, oxidative refolding, and size-exclusion chromatography. Specific binding of *Hs*FcμR-Igl to IgM/antigen ICs was confirmed, and 2 prototypic ELISAs for the detection of anti-CCHFV and anti-ZIKV IgM antibodies were developed. Thereby, patient sera and virus-specific recombinant antigens directly labeled with horseradish peroxidase (HRP) were coincubated on *Hs*FcμR-Igl-coated ELISA plates. Bound ICs were quantified by measuring turnover of a chromogenic HRP substrate.

RESULTS: Assay validation was performed using paired serum samples from 15 Kosovar patients with a PCR-confirmed CCHFV infection and 28 Brazilian patients with a PCR-confirmed ZIKV infection, along with a panel of a priori CCHFV/ZIKV-IgM-negative serum samples. Both ELISAs were highly reproducible. Sensitivity and specificity were comparable with or even exceeded in-house gold standard testing and commercial kits. Furthermore, latex beads coated with *Hs*FcμR-Igl aggregated upon coincubation with an IgM-positive serum and HRP-labeled antigen but not with either component alone, revealing a potential for use of *Hs*FcμR-Igl as a capture molecule in aggregation-based rapid tests.

CONCLUSIONS: Recombinant *Hs*FcμR-Igl is a versatile capture molecule for IgM/antigen ICs of human and animal origin and can be applied for the development of both plate- and bead-based serological tests.

In 2011, it was shown that the extracellular domain of the human Fc fragment of IgG receptor (FcγR)[10] IIa (CD32) can be used as a capture molecule in IgG FcγR ELISAs, allowing the detection of pathogen-specific IgG antibodies in human and animal sera *(1)*. The potency of this technology was demonstrated by the development of an IgG FcγR ELISA enabling the serological differentiation of previous infections with any of the 4 dengue virus (DENV) serotypes *(2)*, as well as highly sensitive and specific IgG FcγR ELISAs for the detection of IgG antibodies against Lassa virus *(3)* and Crimean-Congo hemorrhagic fever virus (CCHFV) *(4)*.

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[10] Nonstandard abbreviations: FcγR, human Fc fragment of IgG receptor; DENV, dengue virus; CCHFV, Crimean-Congo hemorrhagic fever virus; FcμR, Fc fragment of IgM receptor; Igl, immunoglobulin-like; ZIKV, Zika virus; NP, nucleoprotein; NS1, nonstructural protein 1; SEC, size-exclusion chromatography; HRP, horseradish peroxidase; IC, immune complex; HD, healthy donor; YF/Y, yellow fever; EU, Europe; SA, South America; RF, rheumatoid factor.

Given the availability of a capture molecule displaying the required binding affinity and specificity, similar test systems can be set up for the detection of other Ig isotypes. For IgM detection, a potential candidate molecule was found in 2009 when the cellular surface molecule TOSO/FAIM3 was identified as a highly specific Fc fragment of IgM receptor (FcμR) *(5, 6)*. This 60-kDa sialoglycoprotein comprises an N-terminal signal peptide being cleaved off during protein maturation, an extracellular part harboring an immunoglobulin-like (Igl) domain, followed by a transmembrane domain and an intracellular C-terminal part *(5)*. In this work, we demonstrate the potential of the bacterially expressed Igl domain of the human FcμR (*Hs*FcμR-Igl) as a capture molecule for IgM-specific serological testing.

As model systems, we chose 2 viral pathogens, the CCHFV and the Zika virus (ZIKV). In 2015, both were identified as high-risk emerging pathogens by the WHO, likely to cause major epidemics and thus needing urgent research and development attention *(7)*.

Crimean-Congo hemorrhagic fever is an infectious disease endemic in a variety of countries in southeastern Europe, the Middle East, Asia, and Africa *(8)*. The causative agent for the zoonotic disease is a virus of the *Nairoviridae* family, the CCHFV *(8)*. Virus transmission occurs via the bite of an infected tick and also by close contact with body fluids of infected persons or livestock. After an incubation period of up to 13 days, nonspecific flulike symptoms are observed. Subsequently, a severe hemorrhagic and often fatal course of the disease occurs in some patients, leading to a mortality rate of up to 30%. Because of the severity of the disease, the absence of specific therapeutic options, and the high risk of human-to-human transmission, CCHFV is classified as a virus of the highest biological risk class.

The ZIKV is an arbovirus that belongs to the *Flavivirus* genus. Described first in 1947 in Uganda, this virus was originally endemic to Africa and Southeast Asia, before spreading significantly during the past 10 years *(9)*, causing major outbreaks in Micronesia (2007) and French Polynesia/New Caledonia (2013–2014), as well as in South and Central America (2015) *(10)*. Infection follows the bite of an infected *Aedes* mosquito, and it presents with mild flulike disease symptoms; asymptomatic infection is frequent. ZIKV infection has also been reported to cause severe sequelae such as Guillain-Barré syndrome in a small percentage of cases *(11)*. A ZIKV infection occurring during the first trimester of pregnancy significantly increases the probability for the fetus to develop microcephaly, a severe and disabling malformation of the brain *(11, 12)*.

Materials and Methods

EXPRESSION VECTORS

pJC45-HsFcμR-Igl. A cDNA fragment encoding the Igl domain of *Hs*FcμR (gi 216547545) was amplified with primers 5′-CTCTTTCAGGGACCCGGGAGGATCCTCCCAGAAGTAAAGGTAG-3′ and 5′-AGTTAGCTAGGGCCCGGGTCAACTGTGGACATTCAGGGTGAC-3′ using pGEM-T-*Hs*FcμR (Sino Biological) as template and cloned into the vector pJC45-CD32–3C-His *(2)* cut with *Sma*I. The resulting plasmid encodes a 15-kDa protein comprising an N-terminal 10xHis-Tag followed by a 3C protease cleavage site and the *Hs*FcμR-Igl domain.

pOPINJ-CCHFV-NP. The plasmid has been described earlier *(4)*. It encodes a protein comprising an N-terminal 6xHis-Tag followed by glutathione *S*-transferase, a 3C protease cleavage site, and the nucleoprotein (NP) of CCHFV strain Afg09–2990 (ADQ57288) *(13)*.

pJC45-ZV-NS1. ZIKV RNA [strain MR766 (gi 226374362)] was used to generate cDNA by random priming. A fragment encoding the full-length ZIKV nonstructural protein 1 (NS1) was amplified by PCR using primers 5′-AGGAGATATACCATGGGCGACGTGGGGTGCTCAGTGGAC-3′ and 5′-CTAGGGCCCGGGATCCTCACGCTGTCACCATTGACCTCACTAAG-3′. The amplicon was ligated to the pJC45 vector predigested with *Nco*I and *Bam*HI.

EXPRESSION AND PURIFICATION OF RECOMBINANT PROTEINS

*Hs*FcμR-Igl. *Hs*FcμR-Igl was expressed in *Escherichia coli* pAPlacI^{Q} and purified from the insoluble fraction of the bacterial lysate by Ni-nitriloacetic acid affinity chromatography. Subsequently, oxidative refolding was performed at 4 °C overnight. The refolded protein was concentrated and fractionated by size-exclusion chromatography (SEC) (Superdex 75 16/600 GL, ÄKTA pure, GE Healthcare). All ELISA experiments were performed using the monomeric fraction.

CCHFV-NP. CCHFV-NP was bacterially expressed, purified, and labeled with horseradish peroxidase (HRP) as described earlier *(4)*.

ZIKV-NS1. ZIKV-NS1 was expressed in *E. coli* pAPlacI^{Q}. After solubilization of inclusion bodies and oxidative refolding, SEC was performed (Superdex 75 16/600 GL, ÄKTA pure, GE Healthcare). The purified protein was directly labeled with periodate-activated HRP.

For a detailed description of the purification procedures for *Hs*FcμR-Igl and ZIKV-NS1, see Materials and Methods in the Data Supplement that accompanies the

online version of this article at http://www.clinchem.org/content/vol65/issue3.

ELISA

CCHFV/ZIKV μ-capture ELISA, CCHFV/ZIKV IgG FcγR ELISA. Assays were performed as described previously *(4)*. For the ZIKV tests, HRP-labeled ZIKV-NS1 was used as antigen [final dilutions in well, 1:50 000 (IgM); 1:250 000 (IgG)].

CCHFV IgM FcμR ELISA, ZIKV IgM FcμR ELISA ("BLACKBOX® ZIKV IgM ELISA")

(BLACKBOX is a registered trademark owned by the Bernhard Nocht Institute for Tropical Medicine). Nunc MaxiSorp® ELISA plates were coated with 8 μg/mL *Hs*FcμR-Igl in PBS, pH 7.4. After blocking for 2 h at room temperature with PBS, pH 7.4, and 1% BSA, plates were stabilized with liquid plate sealer (Candor). Human sera (1:50 in PBS, pH 7.4, 0.05% ProClin 300) and HRP-labeled recombinant antigens (1:25 000 in PBS, pH 7.4, 1% BSA, 0.5% fetal bovine serum, 1% Nonidet P40, 0.1% ProClin 300, for ZIKV IgM FcμR ELISA: 4% polyethylene glycol 4000) were coincubated on *Hs*FcμR-Igl-coated plates overnight at 4 °C. After washing with 100 mmol/L Tris/HCl, pH 7.4, 150 mmol/L NaCl, 0.05% Tween 20, and 0.005% Proclin 300, tetramethylbenzidine (KPL) was added for 10 min at room temperature, and the reaction was stopped by the addition of 1N sulfuric acid (Merck).

Euroimmun ZIKV IgM ELISA. The test was performed according to the manufacturer's instructions.

For all ELISAs, the HRP reaction product was quantified by measuring absorbance (*A*) at 450 nm and 620 nm on a Spectrostar Nano ELISA reader (BMG Labtech).

AGGREGATION-BASED SEROLOGICAL TEST

*Hs*FcμR-Igl and anti-human IgM were covalently coupled to 400-nm blue latex beads (Innova Biosciences). Coated beads were coincubated with either human IgM (Acris Antibodies) or in the presence and absence of an IgM-positive Crimean-Congo hemorrhagic fever patient serum (dilution 1:10) and CCHFV-NP (0.08 g/L) in PBS, pH 7.4, for 1 h at room temperature. After incubation, suspensions were centrifuged (10 000*g*, 1 min), and aggregation of beads was monitored by both visual inspection and microscopic analysis (Evos XL, 100-fold magnification).

DATA ANALYSIS

Statistical testing was performed with GraphPad Prism. ROC analysis was performed with MedCalc. Classification of samples: positive: $A450–A620 > A_{cutoff}$, negative: $A450–A620 < A_{cutoff}$. Index values were obtained by normalization of the samples' $A450–A620$ values to A_{cutoff}. For the Euroimmun ZIKV IgM ELISA, classification of samples was performed according to the manufacturer's instructions.

ETHICS STATEMENT

The study complies with the Declaration of Helsinki. Written informed consent was obtained from all individuals or, in case of minors, from parents or legal guardians before enrollment. Data privacy protection was guaranteed by anonymization of serum samples. Collection of serum samples was approved by the Ethics Committees of the University of Prishtina, the Oswaldo Cruz Foundation (no. CSN196/96), the Lao People's Democratic Republic (no. 030/NECHR), the Hospital Rosario Pumarejo de Lopez of Valledupar/Colombia, and the Medical Association Hamburg (no. PV4608).

HUMAN SERA

CCHFV patient sera and a priori CCHFV IgM-negative control sera. Serum samples have been described previously (see Table 1 in the online Data Supplement) *(4)*.

ZIKV patient sera. Longitudinal serum samples from 28 patients with PCR-confirmed ZIKV infection (RealStar® ZIKV RT-PCR kit, Altona Diagnostics) collected during the 2015 ZIKV outbreak in Brazil were analyzed. For assay validation, from each patient, an "early" sample taken between days 0 and 4 and a "late" sample taken between days 5 and 15 after onset of symptoms have been analyzed (see Table 2 in the online Data Supplement).

A priori ZIKV IgM-negative control sera. Information on serum samples is summarized in Table 3 of the online Data Supplement. Briefly, the following samples were analyzed: 11 healthy European donors (HD EU), 18 healthy European donors with recent yellow fever (YF) vaccination (HD EU Y), 29 healthy South American donors with positive YF vaccination status (HD SA), 75 DENV-infected patients (DENV), 5 patients with other flavivirus infections (flavi), 34 malaria patients (malaria), and 24 patients with other infections (other).

CATTLE SERA

Cattle sera originated from Ngaoundéré, North Cameroon and Hanover, Germany. The serological status of Zebu cattle sera originating from Ngaoundéré was determined using a commercial test kit (VectoCrimean-CHF-IgG, VectorBest) adapted for analysis of cattle sera according to Mertens et al. *(14)*. Cattle sera were analyzed at a final dilution of 1:100 (CCHFV IgM FcμR ELISA) and 1:200 (CCHFV IgG FcγR ELISA). Cutoffs were determined from the $A450–A620$ absorbance values

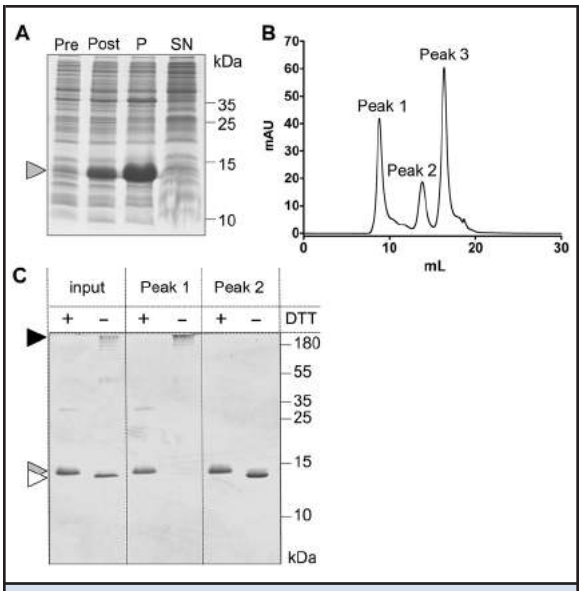


Fig. 1. Prokaryotic expression and purification of HsFcμR-Igl.
(A), Total lysates before and after induction, soluble (SN, supernatant) and insoluble (P, pellet) lysate fractions. Gray arrowhead indicates *Hs*FcμR-Igl (calculated molecular weight, 15 kDa). (B), SEC chromatogram; mAU, milli absorbance units. (C), SDS-PAGE in presence/absence of dithiothreitol (DTT). Gray, white, and black arrowheads indicate reduced *Hs*FcμR-Igl, nonreduced monomeric *Hs*FcμR-Igl, and nonreduced high molecular weight aggregates, respectively.

obtained for 7 sera from healthy European cattle ($\text{mean}_{A,\ \text{HD Europe}} + 3^{*}\text{SD}_{A,\text{HD Europe}} = A_{\text{cutoff}}$).

Results

*Hs*FcμR-Igl CAN BE PURIFIED AND REFOLDED FROM THE INSOLUBLE FRACTION OF TRANSFORMED *E. COLI*

The mature *Hs*Fcμ R protein comprises an extracellular, N-terminal Igl domain (*Hs*Fcμ R-Igl), 1 transmembrane domain, and an intracellular C-terminal domain (see Fig. 1, A and B, in the online Data Supplement) *(5)*. On expression in *E. coli* as a His-tag fusion protein, the recombinant *Hs*Fcμ R-Igl was found in the insoluble fraction of the bacterial lysate (Fig. 1A) from which it could be purified by Ni-nitriloacetic acid affinity chromatography. After oxidative refolding, SEC (Fig. 1B) was used to separate the putatively correctly folded, monomeric form (peak 2) from large, high molecular weight aggregates (peak 1), likely originating from erroneous intermolecular disulfide bridge formation (Fig. 1C) and low molecular weight contaminants (peak 3).

*Hs*FcμR-IgL SPECIFICALLY BINDS TO IgM/ANTIGEN IMMUNE COMPLEXES OF HUMAN AND ANIMAL ORIGIN

To analyze the binding properties of the purified monomeric *Hs*Fcμ R-Igl, HRP-labeled recombinant viral antigens (CCHFV-NP^{HRP} or ZIKV-NS1^{HRP}) were coincubated with Crimean-Congo hemorrhagic fever or Zika patients' sera on ELISA plates coated with *Hs*Fcμ R-Igl. Bound immune complexes (ICs) were detected by the addition of the colorimetric HRP-substrate tetramethylbenzidine (see Fig. 2 in the online Data Supplement). Whereas IgM-positive/IgG-negative serum samples gave rise to a clearly positive signal, no bound HRP activity was detected upon coincubation with sera not containing antigen-specific IgM despite the presence of antigen-specific IgG antibodies (Fig. 2A; see also Fig. 3A in the online Data Supplement). Vice versa, only the IgG-positive sera generated a positive signal when the same experiment was performed on ELISA plates coated with the Igl domain of the human FcγR molecule CD32, a specific IgG/antigen IC receptor (Fig. 2B; see also Fig. 3B in the online Data Supplement) *(1, 2)*. Similar results were obtained with CCHFV-positive/negative cattle sera from Cameroon (see Fig. 3, C and D, in the online Data Supplement). Thus, *Hs*Fcμ R-Igl specifically binds to IgM/antigen ICs of human and animal origin.

To elucidate whether uncomplexed IgM molecules can also be bound by *Hs*Fcμ R-Igl, ELISA plates were coated with either *Hs*Fcμ R-Igl or anti-human IgM. Although a positive signal was obtained on both plate types when a CCHFV IgM-positive serum was coincubated with CCHFV-NP^{HRP} (Fig. 2C), consecutive incubation with the serum and the labeled antigen separated by a washing step gave rise to a positive signal only on the anti-human IgM-coated plates (Fig. 2D). Thus, in contrast to anti-human IgM, *Hs*Fcμ R-Igl does not bind to uncomplexed IgM molecules.

*Hs*FcμR-Igl CAN BE USED AS A NEW CAPTURE MOLECULE IN ELISA TESTS

To evaluate the functionality of *Hs*Fcμ R-Igl as a capture molecule for IgM ELISA applications, IgM Fcμ R ELISA protocols for the detection of IgM antibodies directed against the CCHFV-NP (CCHFV IgM Fcμ R ELISA) and the ZIKV-NS1 protein (ZIKV IgM Fcμ R ELISA, designated as BLACKBOX ZIKV IgM ELISA) were developed and validated. Both assays generated highly reproducible results with mean intraassay CVs <5% and mean interassay CVs <10% (see Tables 4 and 5 in the online Data Supplement).

CCHFV IgM Fcμ R ELISA. To validate the CCHFV IgM Fcμ R ELISA, a serum panel (see Table 1 in the online Data Supplement) *(4)* comprising paired serum samples ("early" sample collected between days 2 and 14 after onset of symptoms; "late" sample collected between days

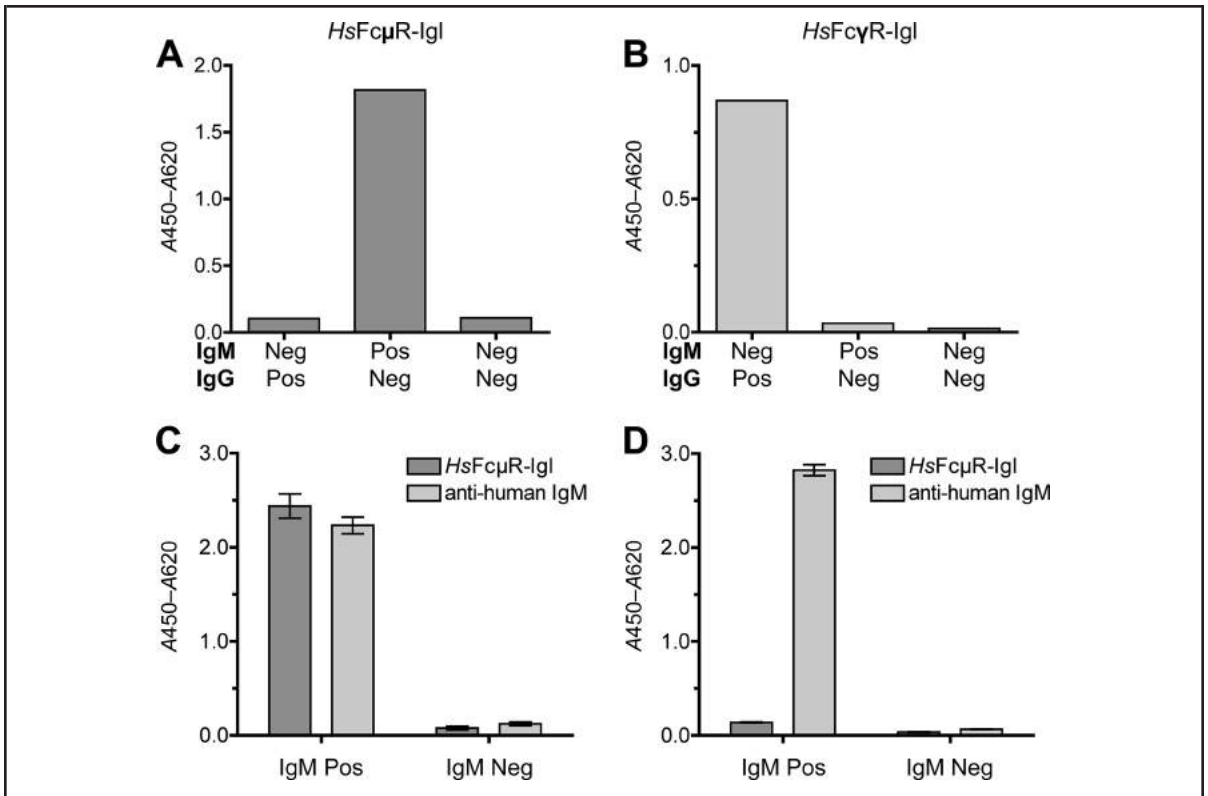


Fig. 2. HsFcμR-Igl specifically binds to IgM/antigen ICs.

CCHFV-NP^{HRP} and antigen-specific IgM/IgG-positive (pos)/negative (neg) sera were coincubated on plates coated with (A) *Hs*FcμR-Igl or (B) *Hs*FcγR-Igl. (C), Sera were coincubated with CCHFV-NP^{HRP} on *Hs*FcμR-Igl- or anti-human IgM-coated plates. (D), Sera and CCHFV-NP^{HRP} were consecutively incubated on *Hs*FcμR-Igl- or anti-human IgM-coated plates separated by a washing step. Plate washing was performed under identical buffer, temperature, and volume conditions for all experiments. Error bars: SD of triplicates.

8 and 36 after onset of symptoms) from 15 Kosovar patients with a PCR-confirmed CCHFV infection and 120 a priori CCHFV IgM-negative sera was analyzed (Fig. 3). The optimal assay cutoff for differentiation of PCR-negative early and late samples from the a priori CCHFV IgM-negative samples (0.193) was determined by ROC analysis (Fig. 4, A and B). Under these conditions, CCHFV-NP-specific IgM antibodies were detected in 15 of 15 late serum samples [100% sensitivity (95% CI, 76.1–100.0)] and 9 of 15 early samples [60% sensitivity (95% CI, 35.7–80.2)] (Fig. 3A). No false-positive findings were observed for the a priori CCHFV IgM-negative serum panel [100% specificity (95% CI, 96.3–100.0)] (Fig. 3B). For all 6 Crimean-Congo hemorrhagic fever patients whose early sample tested negative, clear seroconversion from the early to the late sample was observed (Fig. 3C). Stratification of samples according to the day after onset of symptoms on which they were collected revealed a reliable detection of the patients' anti-CCHFV IgM immune response from day 5 after onset of symptoms onward (Fig. 3D). Thus, the CCHFV IgM FcμR ELISA generates identical results to the CCHFV μ-capture ELISA (BLACKBOX CCHFV IgM ELISA) recently developed by us and a commercial CCHFV IgM ELISA (VectoCrimean-CHF-IgM, Vector-Best) (see Table 1 in the online Data Supplement) *(4)*.

BLACKBOX ZIKV IgM ELISA. To evaluate the performance of the BLACKBOX ZIKV IgM ELISA, 56 paired serum samples from 28 Brazilian patients with a PCR-confirmed ZIKV infection (1 sample collected between days 0 and 4 after onset of symptoms and 1 sample collected between days 5 and 15 after onset of symptoms for each patient) and 196 a priori ZIKV IgM-negative control sera were analyzed (Fig. 5, A and D; see Table 2 in the online Data Supplement). ROC analysis was used to determine the optimal assay cutoff for differentiation of patient samples collected between days 5 and 15 after onset of symptoms and the a priori ZIKV IgM-negative serum samples (Fig. 4, C and D). At an assay cutoff of *A*450–*A*620 of 0.149, 195 of 196 a priori ZIKV IgM-negative sera were classified as negative (99.5% specificity). Three of 28 (11%) of samples

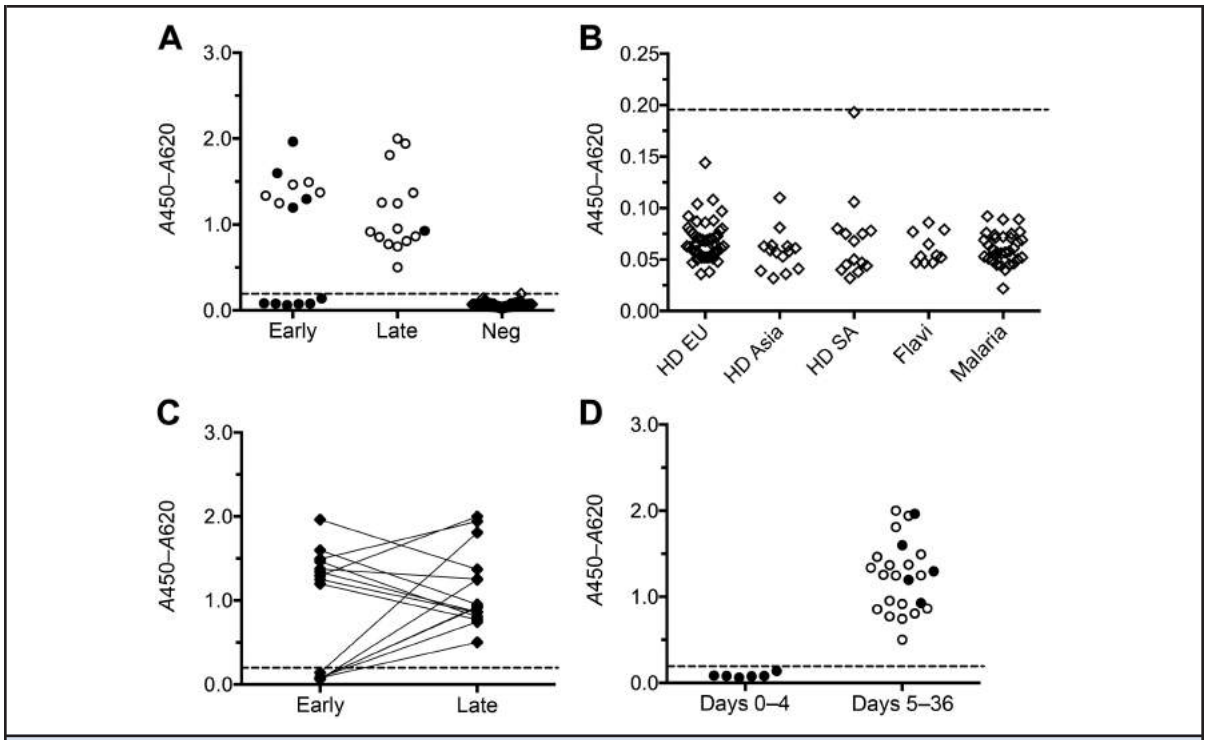


Fig. 3. CCHFV IgM FcμR ELISA.

(A), Absorbance (*A*) values for early and late patient samples and CCHFV IgM-negative control samples. (B), Absorbance values for CCHFV IgM-negative control samples (HD, healthy donors; EU, Europe; SA, South America; flavi, flavivirus infection). (C), Seroconversion. Serum pairs originating from individual patients are indicated. (D), Absorbance values, dependence from day after onset of symptoms. The patient samples were classified according to the day after onset of symptoms on which they were collected. Filled circles in (A) and (D) represent CCHFV PCR-positive samples. Dashed lines indicate A_{cutoff}.

taken between days 0 and 4 and 27 of 28 (96%) of samples taken between days 5 and 15 generated a positive test result (Fig. 5, A, D, and J; see also Table 2 in the online Data Supplement). Clear IgM seroconversion, as defined by a ratio $A_{5-15}/A_{0-4} > 2$, was observed in 24 of the 27 serum pairs (89%) with the sample collected on days 5 to 15 classified as positive (Fig. 5G).

To compare the performance of the BLACKBOX ZIKV IgM ELISA with a commercially available ELISA test, the complete serum panel was analyzed with the Euroimmun ZIKV IgM ELISA (Fig. 5, B and E; see also Table 2 in the online Data Supplement). In this assay, 2 of 28 (7%) and 11 of 28 (39%) samples collected on days 0 to 4 and days 5 to 15, respectively, were classified as ZIKV IgM-positive (Fig. 5, B and J; see Table 2 in the online Data Supplement). Four of 196 a priori ZIKV IgM-negative samples generated a (presumably false-positive) signal in the Euroimmun ZIKV IgM ELISA resulting in a test specificity of 98%. Clear IgM seroconversion was observed in 9 of the 11 serum pairs (82%) with the sample collected on days 5 to 15 classified as positive (Fig. 5H).

To evaluate the influence of the IgM FcμR ELISA technology on assay sensitivity and specificity, a conventional μ-capture ELISA protocol was established using the same recombinant, HRP-labeled ZIKV-NS1 protein as antigen as the BLACKBOX ZIKV IgM ELISA. Using this assay, none of the 28 patient sera (0%) collected on days 0 to 4 and 6 of 28 patient sera (21%) collected on days 5 to 15 were classified as positive (Fig. 5, C and F; see also Table 2 in the online Data Supplement). When testing a subset of the a priori ZIKV IgM-negative sera (n = 141), none of the signals exceeded the assay cutoff of 0.129 derived from ROC analysis (Figs. 4, E and F, and 5, F and J; see also Table 2 in the online Data Supplement). Clear IgM seroconversion was observed in 5 of the 6 serum pairs (83%) with the sample collected on days 5 to 15 classified as positive (Fig. 5I).

Taken together, 2 pilot IgM FcμR ELISA tests using *Hs*FcμR-Igl as a capture molecule and recombinant viral antigens display robust test performance. Test sensitivities and specificities were found to be comparable with or even better than an in-house gold standard (CCHFV IgM indirect immunofluorescence test) and a commercial ELISA test kit (Euroimmun ZIKV IgM), respectively.

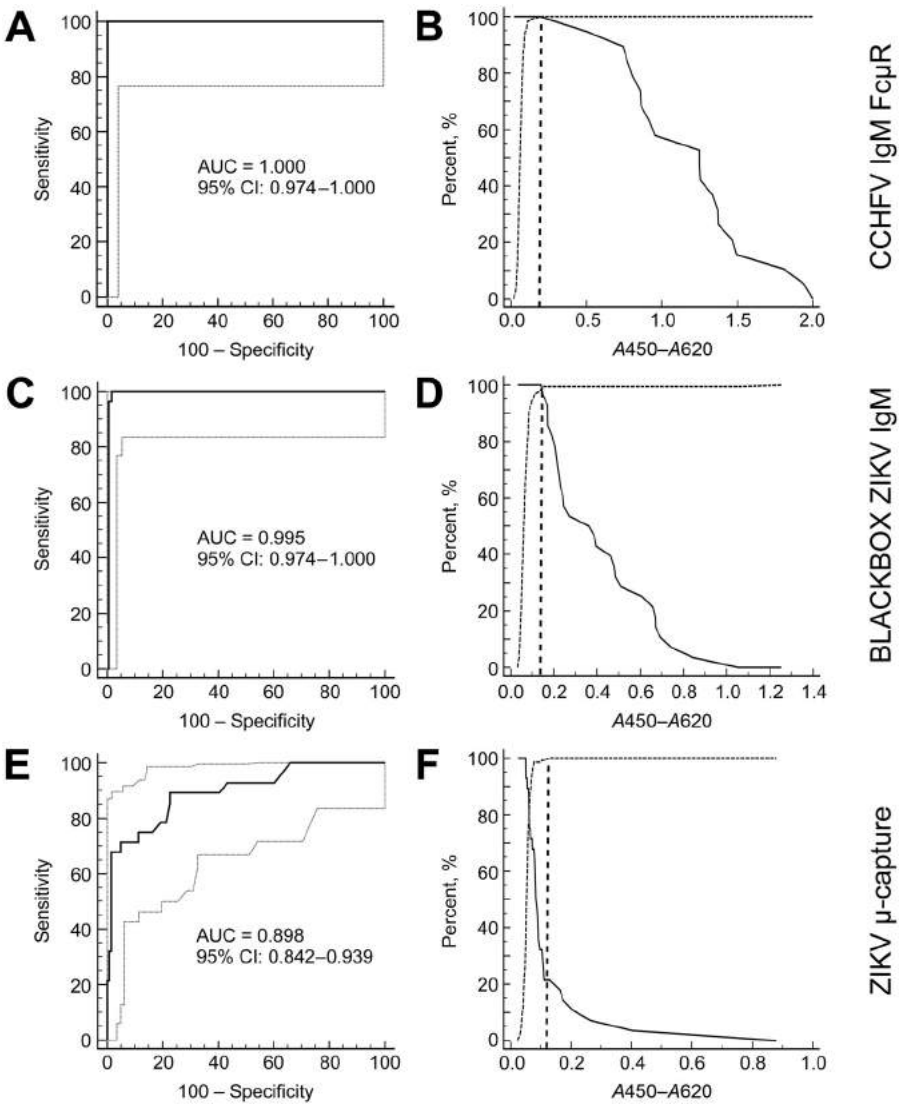


Fig. 4. ROC analyses.

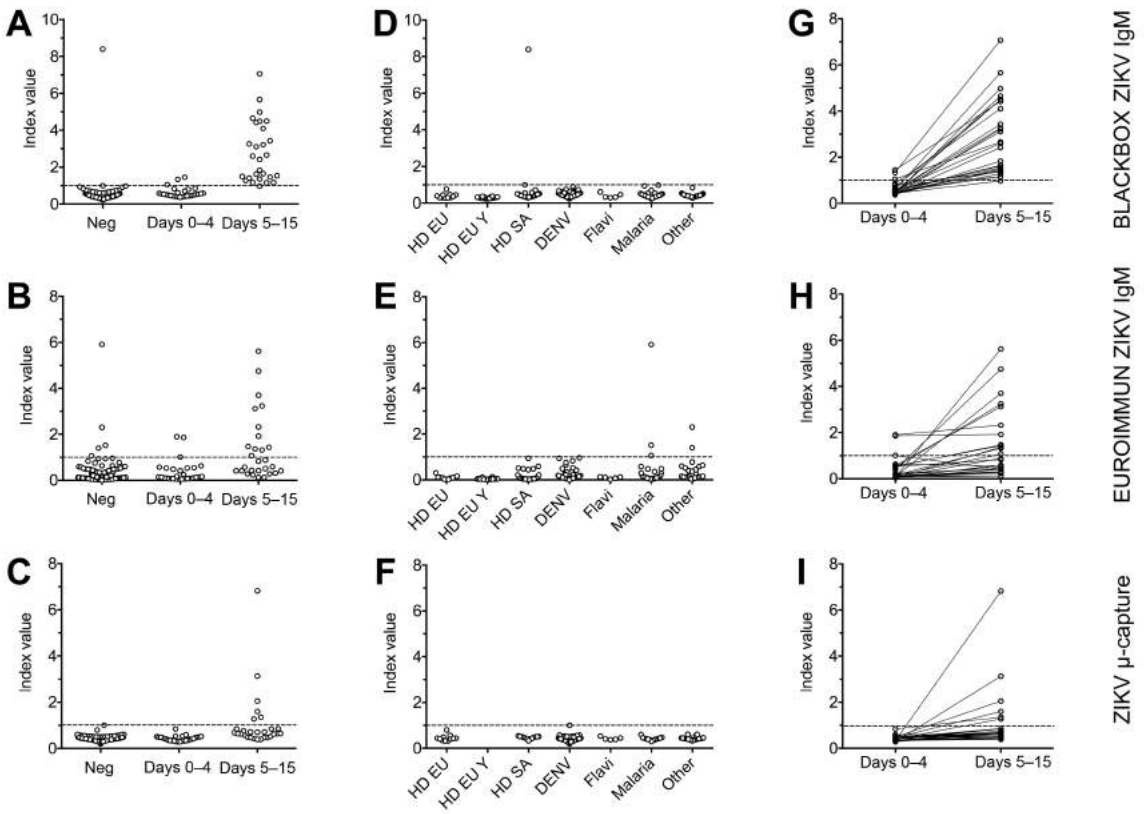
(A) and (B), CCHFV IgM FcμR ELISA. Determination of optimal A_{cutoff} (0.193) for differentiation of PCR-negative early and late samples (n = 19) from the a priori CCHFV IgM negative samples (n = 120). (C) and (D), BLACKBOX ZIKV IgM ELISA; (E) and (F), ZIKV μ-capture ELISA. Determination of optimal A_{cutoff} (BLACKBOX ZIKV IgM ELISA, 0.149; ZIKV μ-capture ELISA, 0.129) for highly specific differentiation of patient samples collected between days 5 and 15 (n = 28) from the a priori ZIKV IgM-negative samples (BLACKBOX ZIKV IgM ELISA, n = 196; ZIKV μ-capture ELISA, n = 141). (A), (C), and (E), AUC: area under curve; gray line, 95% CI. (B), (D), and (F), Solid line, sensitivity (%); dotted line, specificity (%); dashed line, A_{cutoff}.

INFLUENCE OF INTERFERING SUBSTANCES ON IgM IC ELISA PERFORMANCE

Neither the addition of hemoglobin, bilirubin, or triglycerides nor increased concentrations of non-pathogen-specific IgM resulted in relevant changes of the *A*450–*A*620 values measured for IgM-positive/negative sera in the IgM FcμR ELISA (see Fig. 4, A and B, in the online Data Supplement). Nevertheless, false-positive signals may be obtained for IgM-negative serum samples containing both rheumatoid factor (RF) and pathogen-specific IgG (see Fig. 4, C–F, in the online Data Supplement).

*Hs*FcμR-Igl CAN BE USED AS A CAPTURE MOLECULE IN AGGREGATION-BASED RAPID TESTS

Conventional ELISA techniques may not be best suited for diagnosis of acute infections in the field. To evaluate the potential of the recombinant *Hs*FcμR-Igl to be used in aggregation-based rapid tests, latex beads were coated



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Sample type		IgM tested positive n/N, % (95% CI) in BLACKBOX (BB)	IgM tested positive n/N, % (95% CI) in EUROIMMUN (E)	IgM tested positive n/N, % (95% CI) in µ-Capture (µ)	Result statistical testing BB vs E	Result statistical testing BB vs µ	Result statistical testing E vs µ
ZIKV	Days 0–4	3/28, 11 (2.9–28.0)	2/28, 7 (0.9–23.7)	0/28, 0 (0.0–14.3)	NS	NS	NS
	Days 5–15	27/28, 96 (80.8–100.0)	11/28, 39 (23.5–57.6)	6/28, 21 (9.9–39.9)	***	***	NS
Negative control		1/196, 0.5 (0.0–3.1)	4/196, 2 (0.6–5.3)	0/141, 0 (0.0–3.2)	NS	NS	NS

Fig. 5. BLACKBOX ZIKV IgM ELISA compared with ZIKV µ-capture ELISA and Euroimmun ZIKV IgM ELISA.

Dashed lines indicate an index value of 1. (A–C), Index values obtained for patient sera and ZIKV IgM-negative control serum samples. (D–F), Index values for ZIKV IgM-negative control samples. (G–I), Seroconversion, paired sera are indicated. (J), Sensitivities and specificities. ***$P <$ 0.0001 (Fisher exact test).

with either anti-human IgM or recombinant *Hs*FcμR-Igl. Whereas beads coated with anti-human IgM aggregated upon the addition of human IgM in the absence of any antigen, no such aggregation was observed under the same conditions with *Hs*FcμR-coated beads (Fig. 6, A and B), thus confirming the finding of *Hs*FcμR-Igl not binding to uncomplexed IgM in a plate ELISA setting (Fig. 2D). Although no aggregation of *Hs*FcμR-Igl-coated beads was observed in the presence of either serum or antigen alone, cross-linking of *Hs*FcμR-Igl-coated beads could be readily induced by the simultaneous addition of an IgM-positive Crimean-Congo hemorrhagic fever patient serum and recombinant CCHFV antigen (Fig. 6C). This finding may be used as a basis for the development of aggregation-based rapid test systems for the detection of pathogen-specific IgM antibodies.

Discussion

In this work, the binding properties of recombinant *Hs*FcμR-Igl were characterized. Correlating with the results obtained previously for the native *Hs*FcμR expressed on the surface of eukaryotic cells *(5, 6)*, specific binding to IgM/antigen ICs of human and animal origin was detected. To demonstrate the suitability of *Hs*FcμR-Igl as a capture molecule in IgM IC ELISA applications, 2 IgM FcμR ELISA tests were established detecting IgM antibodies directed against 2 emerging pathogens,

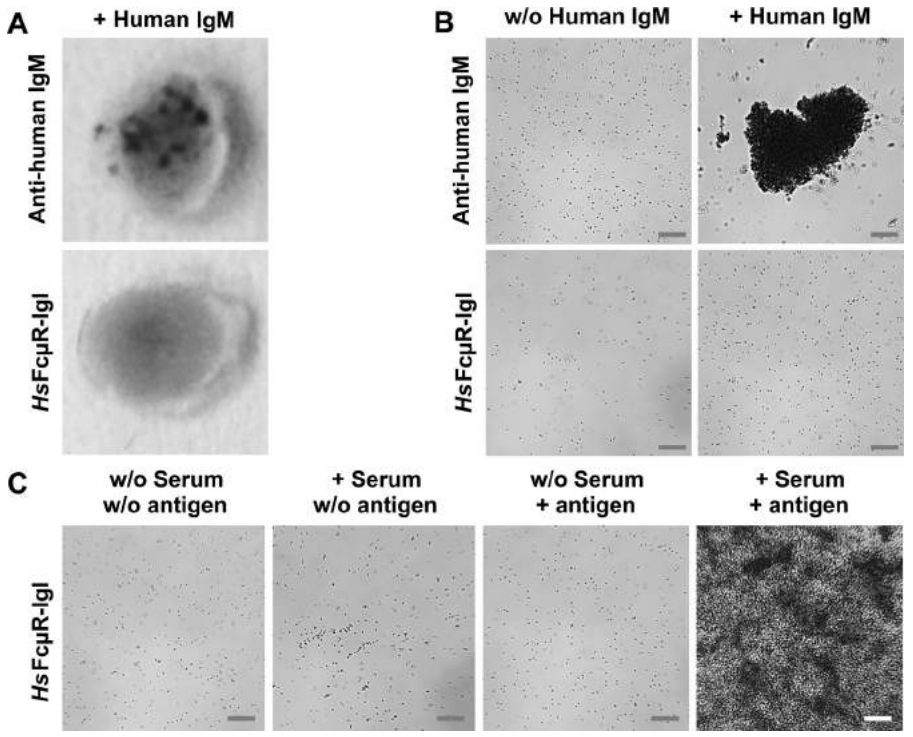

Fig. 6. HsFcμR-IgI can be used as a capture molecule in aggregation-based rapid tests.

(A, B), Beads were coated with anti-human IgM or *Hs*FcμR-IgI. Upon incubation with human IgM, aggregation of beads was analyzed by optical observation (A) and microscopy (B). (C), Beads coated with *Hs*FcμR-IgI were incubated in presence and absence of a CCHFV IgM-positive serum and CCHFV-NP^{HRP}. Aggregation of beads was analyzed by microscopy. Scale bar, 10 μm.

CCHFV and ZIKV. Both test systems displayed high reproducibility and high sensitivity and specificity.

For the CCHFV IgM FcμR ELISA test, the nucleoprotein NP *(15)* was used as antigen. The antigenicity of this protein, also when produced in *E. coli*, has previously been proven by several authors *(4, 14, 16–18)*. Assay sensitivity was found to be identical to a μ-capture CCHFV ELISA recently developed and validated by us, the VectoCrimean-CHF-IgM ELISA (Vector-Best) and in-house CCHFV IgM indirect immunofluorescence test *(4)*. No false-positive signals were observed when analyzing the 120 a priori CCHFV IgM-negative serum samples.

For the ZIKV IgM FcμR ELISA test, full-length ZIKV NS1 *(19–21)*, which has been shown to function as a specific antigen in ZIKV serological diagnostics *(22, 23)*, was used. Sequence analysis of 103 ZIKV strains comprising isolates from the 2015 to 2016 South America outbreak, Canada, Europe, Africa, and Southeast Asia revealed an identity of 99.3% between NS1 amino acid sequences *(20)*. Thus, cross-detection of antibodies against ZIKV strains from different regions of the world by NS1-based serological tests can be assumed. Despite a close sequence identity of the predicted ZIKV-NS1 B-cell epitopes with the respective regions of other flavivirus NS1 proteins, electrostatic surface potentials differ significantly because of nonconserved epitope-flanking residues *(24)* possibly causing the observed low cross-reactivity of the humoral anti-NS1 immune response against different flaviviruses *(22, 23)*. The sensitivity of the BLACKBOX ZIKV IgM ELISA was found to exceed the sensitivity of both the Euroimmun ZIKV IgM ELISA and the μ-capture ELISA using the same antigen as the BLACKBOX ZIKV IgM ELISA. Indeed, suboptimal sensitivity of the Euroimmun ZIKV IgM ELISA has been reported from other authors *(25, 26)*. Neither the BLACKBOX ZIKV IgM ELISA nor the Euroimmun ZIKV IgM ELISA detected false-positive signals when sera from European healthy blood donors or sera from patients with an acute DENV infection were tested. Two sera from *Plasmodium falciparum* malaria patients generated a presumably false-positive signal in the Euroimmun ZIKV IgM ELISA. This observation agrees with the findings of Van Esbroeck et al. *(27)*, who reported the possible occurrence of false-positive signals when sera from malaria patients were analyzed with this test *(27)*.

Major advantages of the novel IgM FcμR ELISA test principle are as follows: (*a*) FcγR/FcμR ELISAs show superior sensitivity compared with conventional ELISA technologies because of selective high-avidity binding of specific ICs to the solid support. (*b*) Because of the simultaneous addition of serum and antigen, test performance is easy and requires only short hands-on time. (*c*) Owing to cross-species reactivity of the capture molecules, the same set of reagents can be used to analyze sera of both human and animal origin, which is of particular interest when working on zoonotic pathogens like CCHFV.

A long-known caveat in serological IgM testing is the possible interference of IgM class RF *(28)*, which is present in 3% to 4% of the healthy population *(29, 30)* and/or high IgG titers *(31)*. Whereas in sera positive for IgM class RF the presence of antigen-specific IgG may lead to generation of false-positive signals (see Fig. 4, C–F, in the online Data Supplement), IgM/antigen binding may be outcompeted by a high concentration of antigen-specific IgG leading to a false-negative result. In these cases, quantitative removal of IgG antibodies before testing in the IgM FcμR ELISA is recommended.

In summary, *Hs*FcμR-Igl is a versatile capture molecule for IgM/antigen ICs of human and animal origin and can be applied to the development of both plate- and bead-based serological tests.

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