Multiplex targeted mass spectrometry assay for one-shot flavivirus diagnosis

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Targeted proteomic mass spectrometry is emerging as a salient clinical diagnostic tool to track protein biomarkers. However, its strong analytical properties have not been exploited in the diagnosis and typing of flaviviruses. Here, we report the development of a sensitive and specific single-shot robust assay for flavivirus typing and diagnosis using targeted mass spectrometry technology. Our flavivirus parallel reaction monitoring assay (*fv*PRM) has the ability to track secreted flaviviral nonstructural protein 1 (NS1) over a broad diagnostic and typing window with high sensitivity, specificity, extendibility, and multiplexing capability. These features, pivotal and pertinent to efficient response toward flavivirus outbreaks, including newly emerging flavivirus strains, circumvent the limitations of current diagnostic assays. *fv*PRM thus carries high potential in positioning itself as a forerunner in delivering early and accurate diagnosis for disease management.

targeted proteomics | PRM | dengue | flavivirus | diagnostics

Application of mass spectrometry (MS) in proteomics started with the identification of purified proteins (1). Since then, continual technological development has led to improvement in sensitivity and multiplexing capability, calling MS to emerge as the state-of-the art technology for proteomic research. Among different proteomic MS approaches, targeted MS enables identification and quantification of a defined subset of proteins down to single amino acid resolution and attomole level sensitivity with high reproducibility. As this powerful approach has the potential to screen about 50–100 proteins in a single run, including isoforms, it is far superior to conventional protein measurement assays such as immunoassays in terms of specificity and multiplexing capability. Moreover, extendibility of targeted proteomic MS assays to analyzing new proteins is convenient in a time- and cost-efficient manner. While it takes only a few weeks for the development of a parallel reaction monitoring (PRM) assay for new proteins, conventional antibody-based methods need months and higher costs to establish a new assay (2). All these advocate targeted proteomics as a promising screening tool in applications requiring urgent diagnostic needs.

Targeted proteomic MS has been gaining popularity as a promising clinical diagnostic tool to track protein/peptide biomarkers, such as in cancer (2) and cardiovascular disease (3). However, its potential has not been well-exploited in infectious disease diagnostics. One particular group of such infectious agents is the flaviviruses, which poses a major global public health concern. Several flaviviruses such as Dengue (DENV), West Nile (WNV), Yellow fever (YFV), Japanese encephalitis, and Zika (ZIKV) viruses are highly pathogenic to human. With DENV on the rise and recent pandemic emergence of ZIKV in 2015, a plethora of diagnostic assays has been developed. The immune-based assays, for example, have been widely used as point-of-care tests. However, the development of such antibody-based assays is resource- and time-consuming (4), incapacitating their extendibility to newly emerging flaviviruses. Moreover, the diagnostic accuracy of immuneassays leverages heavily on the high specificity of antibodies, and such specificity is often undermined as seen in a particular case where ZIKV was misdiagnosed for DENV (5). To date, PCR technology is the most extensively employed option for concurrent diagnosis and typing of flaviviruses. Nonetheless, the sensitivity of PCR suffers during the postviraemic phase (usually 4–5 d of clinical onset) due to low abundance or absence of viral RNA in clinical specimens. In view of current drawbacks, together with the global expansion of flavivirus transmission and the propensity for future outbreaks, there is a compelling need for highly sensitive shotgun diagnostic and typing assays with readily extendable typing capability to cover a wide panel of flaviviruses. We foresee that the development of targeted proteomic assay circumvents the above-mentioned limitations and enables an efficient response to newly emerging flavivirus outbreaks, with accurate diagnosis and virus typing. To this end, we deployed PRM (6), a state-of-the-art high-resolution targeted MS technology, in developing a robust single-shot flavivirus diagnostic and typing assay. In this work, we show the applicability of our flavivirus parallel reaction monitoring assay (*fv*PRM) in monitoring DENV nonstructural protein 1 (NS1) in patient blood as early as the first day until the eighth day of the illness. We also demonstrate its high sensitivity,

Significance

With pandemic emergence and increasing magnitude of flavivirus outbreaks in recent years, there is an urgent need for robust and easily extendable technologies for flavivirus diagnosis and typing to facilitate better disease management, surveillance, and control. Here, we report a single-shot mass spectrometry assay for distinguishing Dengue virus serotypes, Zika, Yellow fever, and Kunjin viruses, including coinfections in a multiplex assay with high specificity and sensitivity. This assay is easily extendable to a wider panel of flaviviruses and addresses the shortcomings of current diagnostics, holding high promise as a future flavivirus diagnostic tool.

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The authors declare no conflict of interest.

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Data deposition: The targeted mass spectrometry proteomics spectral libraries and data have been deposited to the ProteomeXchange Consortium (www.proteomexchange.org) via the PRIDE partner repository with the identifier PXD006922.

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specificity, extendibility, and multiplexing capability in detecting NS1 in secondary DENV-infected patient sera/plasma, as well as ZIKV-, YFV-, and KUNV-infected in vitro secretomes.

Results

DENV Serotyping PRM Assay Development. The mosquito-borne flaviviruses share highly conserved NS1 amino acid sequences. However, given the single amino acid resolution offered, we could identify at least one unique NS1 peptide for each flavivirus for PRM identification (Fig. 1). We then explored the possibility of developing a flavivirus assay based on these unique NS1 peptides by using high-resolution targeted proteomics PRM technology for concurrent diagnosis and typing of DENV, the most prevalent mosquito-borne flavivirus. PRM-amenable flavivirus- and DENV serotype-specific NS1 peptides were obtained through bottom-up discovery proteomics analysis of secretomes from DENV-infected cell cultures. DENV tryptic peptides containing none or single miscleavage were selected based on the following criteria: (*i*) unique to DENV and to each serotype; (*ii*) not found in any human protein sequence; and (*iii*) various isoforms of the serotype-specific peptide should collectively account for >96% in Asian strains (with the exception of DENV-4 peptide SYAGPFSQHNYR) (Dataset S1*a*). Methionine containing peptides of DENV-3 and DENV-4 were included due to a small number of detectable serotype-specific peptides for these two serotypes. We also included in silico-digested peptides, which were not detected in the secretome by data dependent acquisition (DDA) but showed ≥98% amino acid conservation for assay development. The synthetic versions of these peptides were used for liquid chromatography-mass spectrometry (LC-MS) parameter and peptide property evaluation, which included determination of optimal gradient for peptide separation and optimal collision energy for each precursor, retention time of each peptide, and relative intensity of the fragment ions. The assay development workflow and optimized LC-MS parameters for the peptides selected for PRM assay development are shown in *SI Appendix*, Fig. S1 and Dataset S2 *a* and *b*.

DENV Serotyping PRM Assay Evaluation. The developed DENV serotyping assay was evaluated on patient sera of three different categories: Category 1, DENV NS1 positive and PCR-serotypable; Category 2, DENV NS1 negative; and Category 3, DENV NS1 positive, yet PCR-nonserotypable (Dataset S3*a*). The assay was initially optimized using Category 1 samples and was further fine-tuned using Category 2 and 3 samples.

Skyline v3.1.0.7382 (https://skyline.ms/project/home/software/Skyline/begin.view) (7) and X-Calibur v2.2 (Thermo Scientific) were first employed to analyze all PRM raw files of Category 1 samples. All results were inspected manually. The best transitions without interference with most samples were used as the spectral library (panel) for SpectroDive v7.0.8230.0.16364 (Biognosys AG).

DENV serotyping PRM assay for Category 2 and Category 3 samples was mostly carried out using an optimized inclusion list tabulated in Dataset S2*b*. This optimized inclusion list included precursors and their isoforms that were detected in Category 1 samples, as well as precursors that produced good peak shape and did not yield false positives. We also included Filamin A

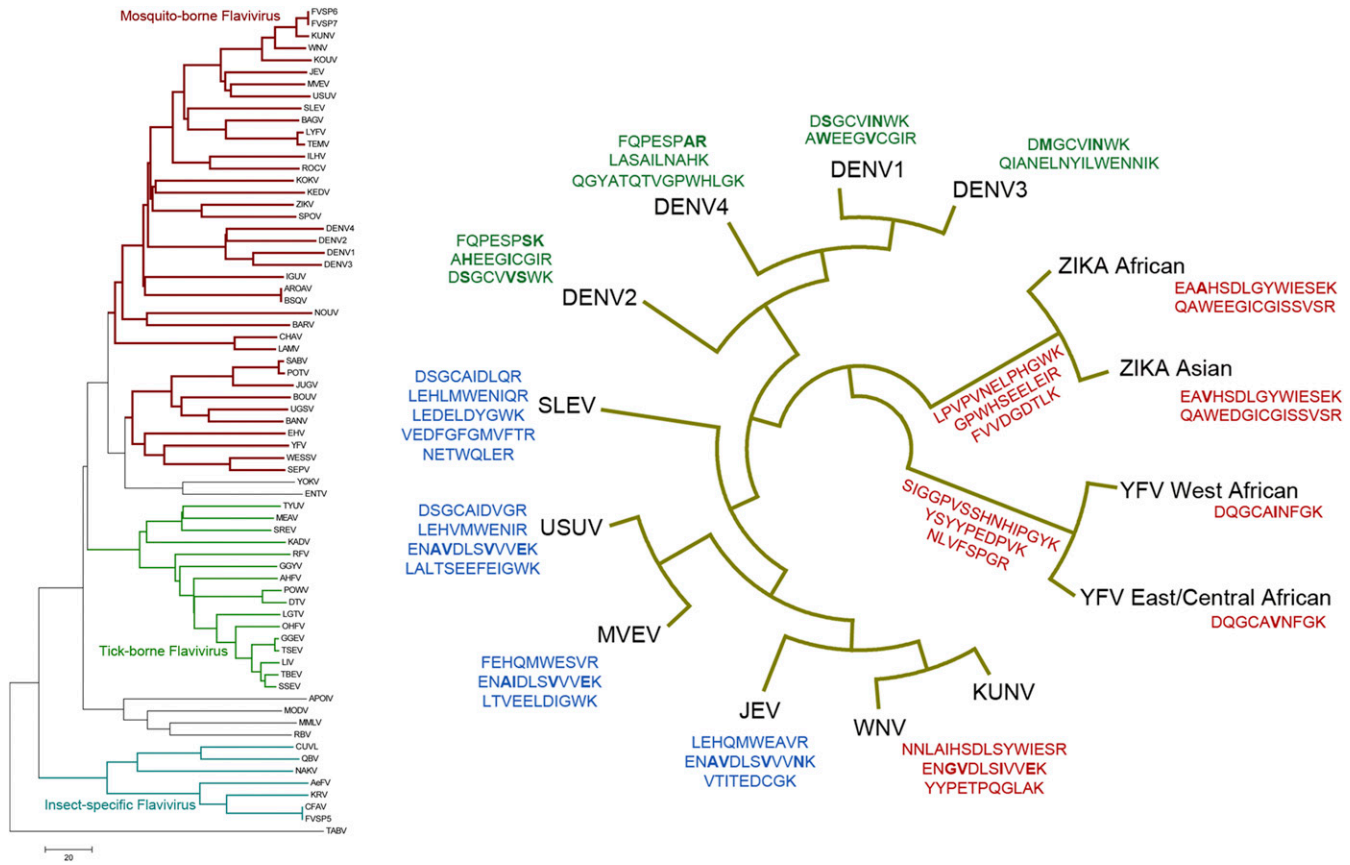

Fig. 1. Mosquito-borne flavivirus NS1 phylogeny with their unique NS1 peptides. Neighbor-joining phylogenetic tree based on NS1 protein sequences from all flaviviruses is shown with distinct clusters (*Left*). Representative mosquito-borne flavivirus with their unique NS1 peptides are shown (*Right*). Amino acid residues that differ among similar peptides of different typings are highlighted in bold.

peptides, which served as a positive control for samples in which DENV NS1 peptides were not detected.

For the automated DENV serotyping data analyses, sera from all three categories were analyzed by SpectroDive using a spectral library (panel) shown in Dataset S4. However, we noticed that peak detection by SpectroDive with Q-value alone is not stringent enough to warrant 100% specificity of DENV serotyping, as more than one serotype are at times assigned to a single-serotype sample confirmed by PCR. Such false-positive assignments can be easily differentiated from the true positives by their SpectroDive scores. In fact, these low-scoring peptides also showed poor matching with misassigned serotype peptides in the Skyline spectral library. We hence added another criterion based on the SpectroDive score (Dataset S5) to achieve 100% specificity of DENV serotyping. Using Category 1 (known serotype by PCR) and Category 2 (non-DENV–infected) samples as the "training set," a cutoff score/range for each peptide was assigned and further refined using Category 3 sera. The cutoff score/range for each peptide is shown in Dataset S5. Above the cutoff score/range, the peptide detection was considered as a true positive and vice versa. If the score falls within the cutoff range, the peptide would require further validation. Higher cutoff scores/ranges were assigned for two peptides, which have only three transitions in the Spectrodive panel, i.e., DENV1_DSGC(Cam) VINWK [cutoff score = 1.40–1.44, C(Cam) = carbamidomethylated cysteine] and DENV4_FQPESPAR (cutoff range 1.34–1.38) to minimize the occurrence of false positives.

Upon testing the developed DENV assay on infected sera, we observed that PRM assay serotyped 70% of the 96 DENV NS1-positive sera (Category 1 and 3), whereas only 50% were serotypable by PCR (Fig. 2*A* and Datasets S3*a* and S6 *a*–*e*). Moreover, PRM performed better than PCR in the late-acute phase [4–5 d post onset of symptoms (DPOs)] sera (Fig. 2*B*). Serotypes of 13 samples from Category 3 were validated by examining the coelution and good transition match of the detected peptides with spiked-in heavy labeled peptides (Dataset S3*a*). The results for DENV NS1-negative samples, including nonflavivirus Chikungunya virus-infected sera, which can be clinically misdiagnosed as dengue and zika fever due to similar manifestations, were all negative (Datasets S3*a* and S6*f*), confirming the high specificity of our assay in clinical samples.

Considering the importance of NS1 quantification in providing possible insights into disease pathogenesis (8) and virus transmission potential (9), we also evaluated our assay for NS1 quantifiability from 1 to 8 DPOs in DENV-1–infected sera (both PCR-serotypable and PCR-nonserotypable). The surrogate peptide used for NS1 quantification was AWEEGVC(Cam)GIR (Datasets S2*c* and S7). Quantification results (Fig. 2*C* and Datasets S6*g* and S8) showed a reported (10) trend of the NS1 amount at different DPOs with the lowest quantification limit of 2.5 pmol/mL (Dataset S8b), thus showing PRM's ability to quantify NS1 in patient samples.

Extension to Other Flaviviruses: *fv*PRM Assay Development. We expanded the assay to include ZIKV, YFV, and Kunjin virus (KUNV), a subtype of WNV, to demonstrate its flexibility in extending and multiplexing. The selection of ZIKV, YFV, and KUNV peptides for PRM assay was similar to that for DENV and the details are described in *SI Appendix*, *Methods*. The selected peptides for each virus are shown in Dataset S2*d*. Those DENV serotype-specific peptides, which have been detected in human sera, with good peak shape, but did not yield false positives, were also included (Dataset S2).

As KUNV is a subtype of WNV, the polyprotein sequences of KUNV showed high similarity to the rest of the WNV isoforms. Among the three peptides selected for KUNV or WNV diagnosis, ENGVDLSIVVEK (1.7% peptide conservation in WNV sequences) seems to be unique to KUNV, since non-KUNV WNV

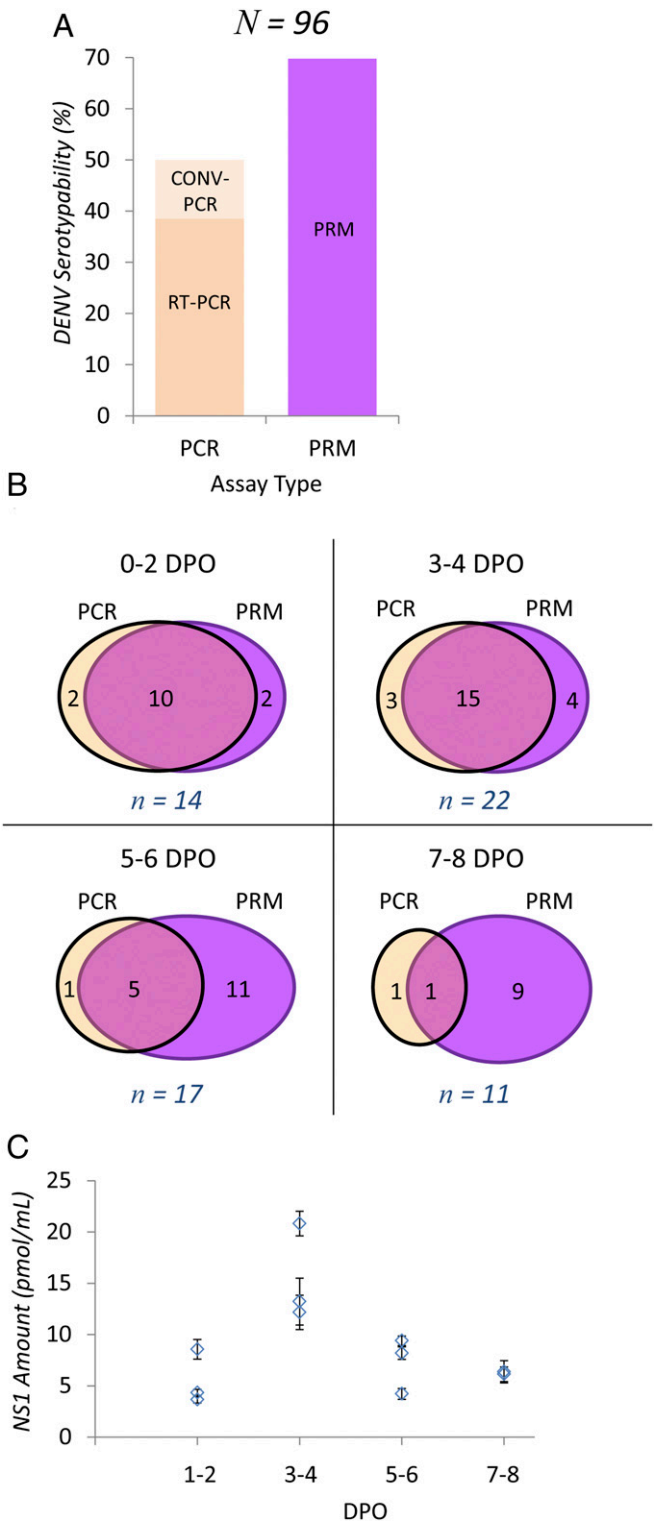

Fig. 2. DENV serotyping capability and NS1 quantification by PRM. (*A*) Comparison of serotyping capability between PCR [RT-PCR and Conventional-PCR (Conv PCR)] and PRM. *N* = total number of tested NS1-positive clinical samples. (*B*) Comparison of serotyping capability between PCR (RT-PCR and Conv PCR) and PRM at different DPO. *n* = number of samples serotypable by PCR/PRM with known DPO. (*C*) NS1 quantification in 11 selected DENV1 sera by DENV1 PRM. The mean of three sample preparation replicates is plotted, and the error bars represent the SDs of these replicates.

sequences, which contain this tryptic peptide, are classified as "unreviewed" by Uniprot. Hence, the accuracy of these sequences and genotype annotation cannot be confirmed. While ENGVDLSIVVEK could potentially differentiate KUNV from WNV, the other two peptides, NNLAIHSDLSYWIESR and YYPETPQGLAK, have >90% peptide conservation among WNV sequences and, thus, can be used as broadly specific peptides to diagnose WNV (inclusive of KUNV) infections.

We performed this flavivirus PRM assay (*fv*PRM assay) on the Huh-7 cell suspensions infected with ZIKV, YFV, KUNV, and DENV-1–4. There was a false positive for YFV secretome, in which a DENV-3 peptide DM(ox)GC(Cam)AINWK [M(ox) = oxidized methionine] was detected. Further investigations revealed that YFV NS1 peptide DQGC(Cam)AINFGK had very similar properties to DENV-3 NS1 peptide DM(ox)GC(Cam)AINWK (see *SI Appendix* for details). Therefore, we removed DM(ox)G C(Cam) A/V INWK (DENV3) and DQG C(Cam)A I/V NFGK (YFV) from SpectroDive panels for our data processing (Dataset S9). Upon removing these peptides that gave rise to false positives, *fv*PRM successfully distinguished each virus with no cross-reactivity (Fig. 3*A* and Datasets S3*b* and S6*h*). Furthermore, to determine the specificity of *fv*PRM assay in clinical samples, we analyzed DENV-infected and non-DENV–infected patient sera (n =12, Fig. 3*B* and Datasets S3*a* and S6*i*) and observed precise diagnosis and serotyping with no cross-reactivity.

We also investigated the capability of *fv*PRM assay in tracking coinfections since flavivirus coinfections have been reported in humans (11, 12). Arboviruses are often transmitted by similar vectors, and, thus, cocirculation of different flaviviruses is common in endemic regions. In this context, we carried out two experiments to simulate coinfected patient sera. First, the peptides derived separately from ZIKV- and DENV-infected secretomes

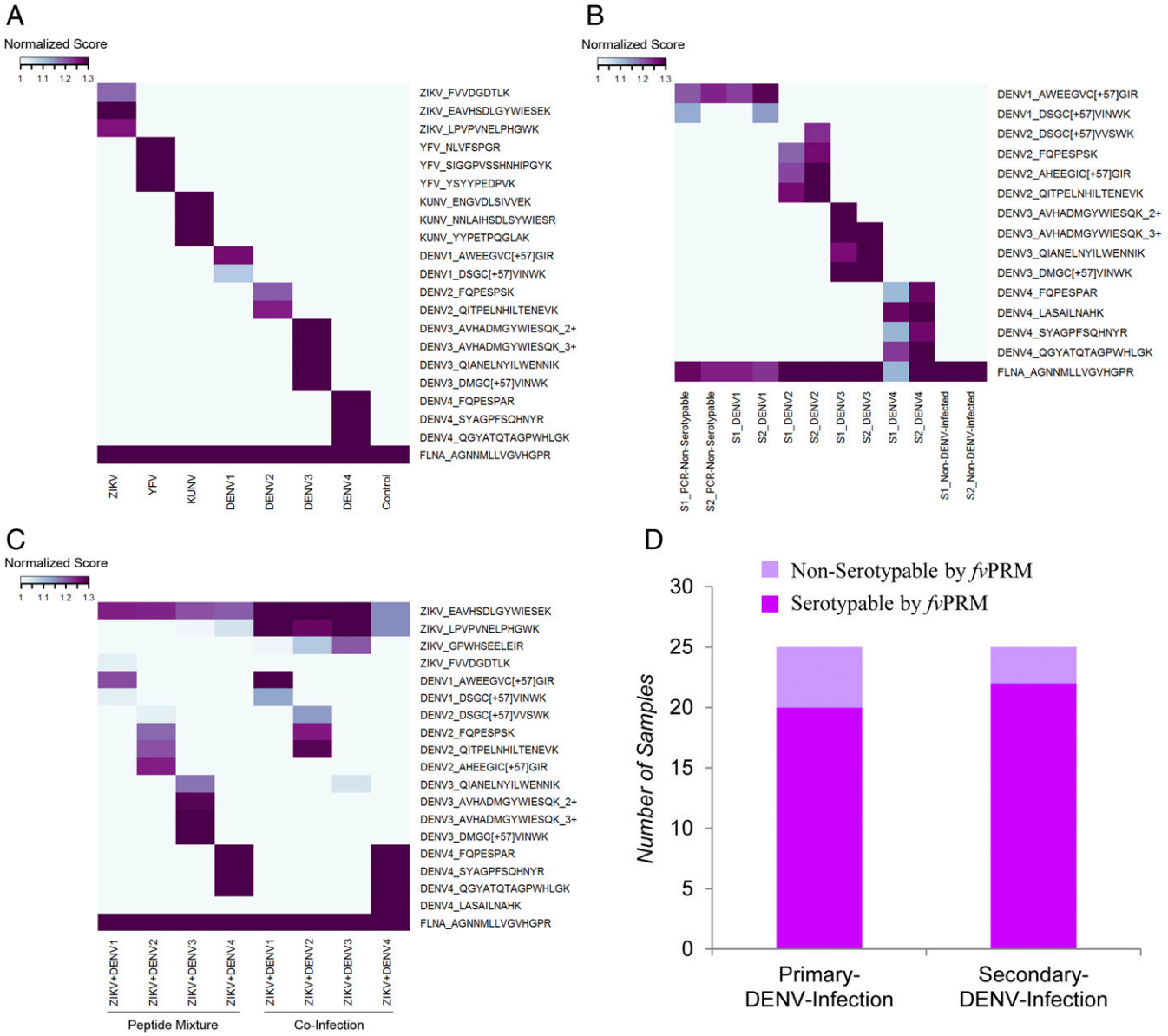

Fig. 3. Flavivirus- and DENV serotype-specific NS1 peptide detection by *fv*PRM in various samples: flavivirus-infected secretome showing no cross-reactivity between flaviviruses (*A*), DENV-infected patient sera (the two samples from each group are indicated as S1 and S2) (*B*), the mixture of peptides derived separately from ZIKV- and DENV-infected secretomes as well as peptides obtained from ZIKV and DENV coinfected secretomes (*C*), and primary and secondary DENV-infected patient plasma (*D*). A representative peptide of FLNA is shown in *A*–*C*.

were mixed and analyzed. Second, peptides obtained from ZIKV and DENV coinfected secretomes were analyzed. *fv*PRM precisely identified both viruses with the exact DENV serotype (Fig. 3*C* and Datasets S3*b* and S6*h*), demonstrating high resolution of the assay.

fvPRM Performance in Serotyping Primary and Secondary DENV Infections. Next, we sought to evaluate NS1 detection performance of our *fv*PRM assay in secondary compared with primary DENV infections. It is known that secondary infections have major issues with antibody-based NS1 antigen detection due to reduced sensitivity and shorter detection window attributed to the formation of immunocomplexes between preexisting DENV IgG and NS1 as well as the clearance of the NS1 complexes (8). For this, we analyzed a characterized panel of primary and secondary DENV-infected plasma ($n = 50$). Our results showed that the performance of *fv*PRM assay for secondary infections is comparable to that of the primary infections (Fig. 3*D* and Datasets S3*a* and S6 *j* and *k*). This is presumably due to our sample preparation procedure, which induces protein denaturation and protein complex dissociation, resulting in NS1 separation from their immunocomplexes.

Discussion

NS1 antigen is an attractive diagnostic marker for flavivirus infections as it is secreted as early as the first day of illness and stably present in the patient blood up to 9 d (8, 10). However, its diagnostic value is not fully potentialized as commercial NS1 assays are not yet available for many flaviviruses. Among those, which are available currently, only one flavivirus can be screened per assay due to challenges in multiplexing and, thus, there is a need for different assays to be run in parallel for the verification of multiple viruses.

In this work, we developed a highly specific DENV serotyping PRM assay capable of accurately identifying the four DENV serotypes and those without the DENV infections in patient sera. Moreover, our assay is highly sensitive and precisely detects DENV NS1 in patient sera from 1 to 8 DPOs. It is, therefore, notable that our assay compares better to PCR-based assays, particularly in the confirmatory diagnosis of dengue during the postviraemic phase.

With the success of PRM in the detection and typing of DENV, we extended the assay to an *fv*PRM format that enables multiple flavivirus detection. The competence of our *fv*PRM assay is affirmed with the 100% specificity in distinguishing different flaviviruses, DENV serotypes, and simulated coinfection of ZIKV and DENV in ZIKV-, YFV-, KUNV-infected cell secretomes, DENV-infected sera, nonflavivirus-infected sera, as well as primary and secondary DENV-infected plasma. Secondary infections are of major concern in terms of dengue disease severity, and point-of-care NS1 immunoassays tend to underperform in secondary infections (13) due to the formation of immune complexes between preexisting DENV IgG and NS1. Our assay procedure, however, dissociates all immune complexes and derives the same bona fide NS1 peptides for both primary and secondary infections. This enables our *fv*PRM assay to detect DENV NS1 comparably well in primary and secondary infected samples, circumventing the issue of poor diagnostic sensitivity of existing NS1 antigen assays in secondary infections. One important consideration for the successful NS1 detection in small volume of sera/plasma used is the amount of DENV NS1 detectable by *fv*PRM. In cases of low NS1 titer in patient sera, the sensitivity of this assay may be challenged. However, it has been shown that preenrichment, which involves matrix reduction and, thus, analyte concentration, can improve the detection limit by three orders of magnitude (14). In fact, a recently developed MS assay showed an increased detection sensitivity for target peptides in clinical samples with preenrichment strategy (15). Such enrichment steps can be automated (16), making this strategy appealing with minimal labor and processing time.

The ease of assay extension is essential to counter the urgent need in diagnostics during outbreaks caused by newly emerging flaviviruses. The intrinsicality of PRM assay facilitates such easy assay extension. We have shown the extendibility and successful multiplexing of seven targets (DENV serotypes 1–4, ZIKV, YFV, KUNV) in our *fv*PRM assay. This target window can potentially be expanded to screen for a wider panel of flaviviruses with at least 50 targets at an average of two peptides per target (17) (Dataset S10). In addition to extendibility, our targeted assay also shows capabilities to differentiate virus genotypes as in the case for YFV (West African and East/Central African genotypes) and ZIKV (the Asian/American and the African genotypes) (Fig. 1 and Datasets S2 and S9). These genotypes, differing in virulence, pathogenicity, and the epidemic potential (18), can be distinguished by our assay although this should be validated in patient samples. Improved specificity of the assay at the genotype level allows the identification of virus types associated with major epidemics and prompt clinical management for those severe subtypes. Apart from the diagnostic advantages, DENV serotyping is also relevant in formulating immunization policies based on the serotype dominance in a particular country, because the only vaccine available commercially, Dengvaxia, is not equally protective for all serotypes (19).

Our *fv*PRM assay addresses the limitations of current assays and delivers unambiguous outcomes pivotal for flavivirus surveillance and control. Although its wide utility seems to be limited by the need for skilled personnel and costly MS instrumentation, these current limitations are expected to be transient with ongoing technology advancements (20), and, thus, our *fv*PRM assay is well-positioned as a powerful diagnostic tool.

Methods

Study Design. In the current study, we developed a flavivirus- and DENV serotype-specific soluble NS1 assay by using high-resolution targeted proteomics, PRM technology for concurrent diagnosis, and typing of DENV1-4, ZIKV, YFV, and KUNV. We first explored the ability of PRM assay to diagnose and serotype DENV NS1 in human sera due to sample availability. We identified MS friendly DENV serotype-specific tryptic peptides by in vitro secretome analyses. The optimal collision energies for sensitive MS detection and optimal gradient for liquid chromatography separation were evaluated for these peptides and were used in the subsequent PRM assays. We assessed the diagnosis and typing specificity of our DENV serotyping assay in 96 DENV NS1-positive samples and 12 DENV NS1-negative samples and compared the typing ability of our assay to PCR. We also carried out NS1 quantification for selected DENV1-infected sera in triplicates (sample preparation replicates) at different DPOs and the limit of quantification was determined. We further extended the assay to *fv*PRM assay, which allows for differential identification of flavivirus ZIKV, YFV, KUNV, and DENV1-4. To develop an assay that has zero cross-reactivity, we performed and optimized the *fv*PRM assay on tryptic peptides obtained from flavivrus-infected cell secretome. Furthermore, we sought to determine the ability of this assay to detect peptides from two viruses in a coinfection by performing the *fv*PRM assay on tryptic peptide derived from ZIKV- and DENV-coinfected secretomes. To assess the high-resolution capability of our *fv*PRM assay in clinical samples, we performed the assay on DENV-infected and non-DENV–infected sera. An automated script to streamline data analysis for flavivirus diagnosis, serotyping, and quantification is also provided. Detailed procedures are described in *SI Appendix, Methods*.

Samples. All sera/plasma used in this study were obtained from Environmental Health Institute (EHI) and Tan Tock Seng Hospital (TTSH), Singapore. The samples were transferred to Institute of Molecular and Cell Biology through relevant Material Transfer Agreements. Sera from EHI were residual diagnostics samples received from clinics with patients' written informed consent for the purpose of diagnostics developments, as approved by the Institutional Review Board of the National Environment Agency, Singapore (IRB 0003.1). All sera were anonymized before being used in the study. Plasma from TTSH were obtained from whole-blood samples collected with EDTA-lined Vacutainer tubes (Becton Dickinson) from dengue patients referred to the Communicable Disease Centre, TTSH, Singapore. Blood specimens were

obtained from patients consenting to the study. All patients gave separate written informed consent. The study protocol is approved by the National Healthcare Group Domain Specific Review Board (reference 2015/00528).

Patient sera from EHI ($n = 108$), collected from 2013 to 2017, were used in the DENV serotyping assay. These samples consisted of 48 DENV NS1-positive and PCR-serotypable DENV1-4 serum samples, 48 DENV NS1-positive yet PCR-nonserotypable serum samples (all four serotypes), and 12 DENV-NS1–negative (non-DENV infected) serum samples (Dataset S3*a*). The NS1 status of each serum sample was confirmed by using a commercial assay (Standard Diagnostics Inc.) according to the manufacturer's instructions. The FRET-I probe-based real-time reverse transcription PCR assay was performed to determine DENV serotypes in DENV NS1-positive samples as described (21). A more sensitive seminested conventional PCR assay (Conv) (22) was used as a second-line test for the confirmation of infection status and serotypes in any sample that failed the FRET probe-based assay.

Plasma samples from TTSH ($n = 50$) consisted of characterized primary and secondary DENV infections, collected from 2016 to 2017. Samples were collected during the acute phase (1–5 d after illness onset). The patients recruited were tested NS1 positive by using the SD BIOLINE dengue duo test (Standard Diagnostics Inc.). Dengue serotypes were determined by FTD dengue differentiation RT-PCR test (Fast Track diagnostics) according to manufacturer's instructions. Primary and secondary infection statuses were determined using PanBio Dengue IgG Indirect Elisa test (Abbott Rapid Diagnostics). The test sets different cutoffs for IgG levels present in past and active dengue virus infections, allowing us to determine the sample's primary or secondary dengue infection status.

PRM Assay Development. PRM-amenable flavivirus- and DENV serotype-specific NS1 peptides were obtained through bottom-up discovery proteomics analysis of secretomes from flavivirus-infected cell cultures. Tryptic peptides, which were unique to each flavivirus and DENV serotype and not found in any human protein sequence, along with their various isoforms that collectively accounted for >90% across various strains (with the exception of DENV4 SYAGPFSQHNYR peptide, Dataset S1) were selected for PRM assay development. In silico digested peptides, which were not detected in the secretome by data-dependent analysis but were conserved ≥90%, were also included. The assay development workflow and LC-MS parameters for the peptides selected for PRM assay development are shown in *SI Appendix*, Fig. S1 and Dataset S2.

Data Processing. Data processing of PRM data for the detection of NS1 tryptic peptides was performed using SpectroDive v7.0.8230.0.16364 (Biognosys AG), which incorporates statistical analysis for peak detection (Q-value). However, we noticed that peak detection by SpectroDive with Q-value alone was not stringent enough to warrant 100% specificity of flavivirus diagnosis/serotyping. We hence added another criterion based on the SpectroDive EG.Cscore (Dataset S5) to achieve 100% specificity of flavivirus diagnosis/serotyping (see *SI Appendix* for details). NS1 quantification was carried out in triplicates (sample preparation replicates), and the SD was plotted as error bars (Fig. 2*C* and Dataset S8*b*).

Data Availability. The targeted MS proteomics spectral libraries and data have been deposited to the ProteomeXchange Consortium via the PRIDE (23) partner repository with the identifier PXD006922.

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