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Immunocapture couples with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry for rapid detection of type 1 dengue virus

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ABSTRACT

A facile method for accurate detection of type 1 dengue virus (DV1) infection from complex biological mixtures, using type specific immunocapture coupled with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS), was developed. The biological mixtures were treated with magnetic particles coated with a monoclonal antibody directly against type 1 dengue virus. After immunocapture purification, the DV1 was eluted with 30% acetic acid, directly spotted with seed-layer method, and analyzed by MALDI-TOF MS for DV1 capsid protein. The detection limit of the assay was ~10^5 pfu/mL by MALDI-TOF MS. The immunocapture could unambiguously differentiate the DV1 from other serotypes of the dengue viruses and Japanese encephalitis virus, and could be used as a specific probe to detect DV1 from complex biological mixtures.

1. Introduction

Dengue infection is the most important mosquito-borne viral infection worldwide, especially in the tropics and subtropics [1]. It is estimated that more than 2.5 billion people living in over 100 countries are at high risk for endemic dengue transmission. Furthermore, dengue infections are responsible for as many as 100 million cases of dengue fever (DF) annually. There are four serotypes of dengue virus (DV-1, 2, 3, 4), which are antigenically related but distinct from each other. A primary infection with any of the four serotypes of DV leads to life-long immunity to this serotype but only partial or temporary immunity to the others. Secondary infection with different serotypes, due to antibody-dependent enhancement phenomena, may lead to more severe diseases, such as dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) [2,3]. Annually, there are more than 200,000–500,000 cases of DHF, causing 22,000 deaths mainly among children under the age of 15 [4,5]. The case fatality rates of DHF and DSS are as high as 10–15% if no proper treatment is given in early stage [3,6,7].

Up-to-date, neither effective drugs/protective vaccines for DF/DHF nor timely and accurate diagnosis of specific DV strains are available. The development of serotype-specific assay for DV detection is of importance both to the treatment of patients and to epidemiological surveillance. Several protocols for dengue detection have been described. Traditional virus isolation and the viral infectivity titer assay is the "gold standard" method used to detect and type the dengue infection. However, long cell-cultured periods of a week or longer for virus growth is time-consuming and not suitable for high-throughput detection [8]. Alternatively, serological diagnosis of dengue infection includes capture of immunoglobulin M (IgM) antibodies, which need to be produced at least 5 days after the onset of fever. Moreover, the test is complicated by the cross-reactive antigenic determinants shared by the four dengue virus serotypes and other flaviviruses. Previously, based on genomic information, molecular techniques such as polymerase chain reaction (PCR), reverse transcription-PCR, and real-time PCR have been shown to be faster assays than cell culture and IgM antibodies assays [9–12]. Possible false-positive reactions, due to cross-contamination of PCR products, and time-consuming of stained agarose gel electrophoresis, however, have limited the high-throughput screening. Recently, biosensor-based diagnostic methods including piezoelectric [13], optical [14–16], and electrochemical [17,18] have been developed. Faster techniques are needed for the early diagnosis of dengue infection.

In the last several years, mass spectrometries including electrospray ionization mass spectrometry (ESI MS) and matrix-assisted

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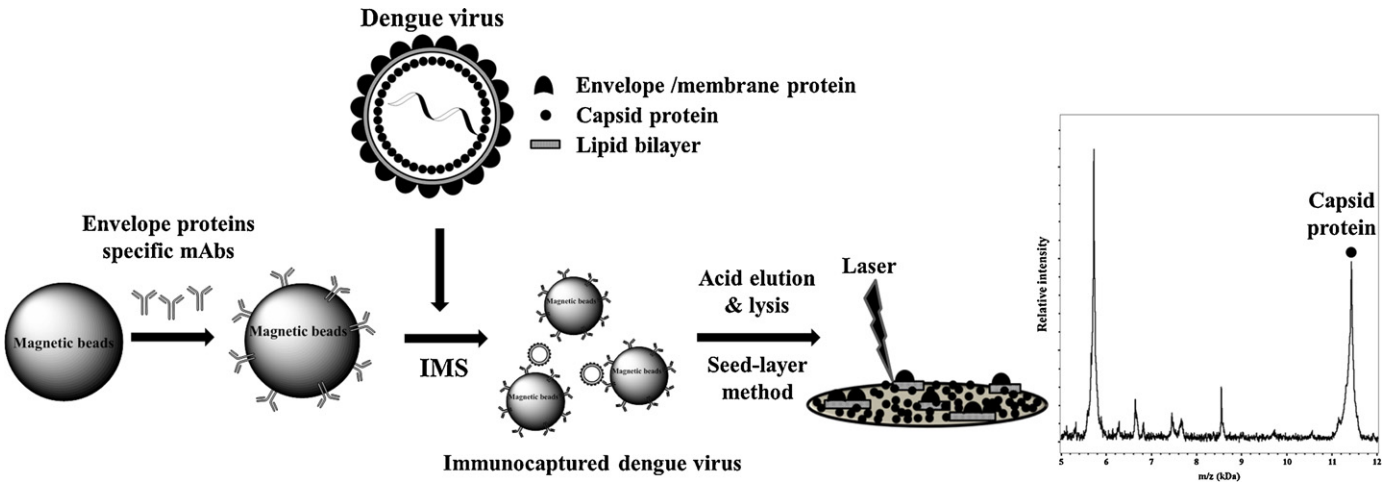

Fig. 1. Experimental strategy for detection of dengue virus by immunocapture and MALDI-TOF MS.

laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) have emerged as rapid and sensitive tools to characterize microorganisms [19], including bacteria [20,21], fungi [22], spores [23], and viruses [24–26]. In the electrospray ionization, the analyte(s) of interest is dispersed by electrospray and ionized by mixing water with volatile organic compounds. However, clogging of samples on devices, low tolerance to salt concentration, and difficulty on deconvolution of multiply charged proteins, are usually experienced [19]. Alternatively, because of its large mass range, higher tolerance to contaminants including salts, detergents, and buffer components, and robustness in instrumentation, MALDI-TOF MS has been applied in the identification of unique protein biomarkers for individual microorganisms. Furthermore, in order to reduce the analysis time and ease of operation, other approaches such as immunomagnetic separation (IMS) have also been coupled with MALDI-TOF MS for virus detection [27–30]. Despite the utilization of the methodologies in various fields, the application of immunocapture coupled with MALDI-TOF MS for detection of DVs remains unexplored. In this study, we developed a simple method for DV1 detection using magnetic bead conjugated type 1 specific monoclonal antibody coupled with MALDI-TOF MS (Fig. 1).

2. Experimental

2.1. Chemicals and materials

The MALDI matrices: α-cyano-4-hydroxycinnamic acid (CHCA), sinapinic acid (SA), 2,5-dihydroxybenzoic acid (DHB), protein standard I, and peptide calibration standard were purchased from Bruker Daltonics. ZipTip C18 was purchased from Millipore. Hybond-P PVDF transfer membrane, nProtein A sepharose and Ni sepharose were purchased from GE healthcare life science. *Escherichia coli* host strain BL21(DE3) and plasmid pET28a were purchased from Novagen. All other materials were from Sigma, unless specified differently.

2.2. Virus preparation

The used prototype of DV strains: DV-1 (Hawaii), DV-2 (*PL046*), DV-3 (*H84*), DV-4 (*H241*), and JEV(*T1P1*) were propagated in C6/36 cells. The virus titers were determined by plaque assay in BHK21 cell [31]. The DV viruses were prepared in BSL-2 (Biosafety level-2) environment and inactivated by exposure to UV light for 30 min after experiments [15].

2.3. Cloning, expression and purification of DV1 E395 proteins

A cDNA encoding DV1 E395 (residues 1–395) was amplified from viral RNA by M-MLV Reverse Transcriptase according to the manufacturer's instructions (invitrogen). For gene subcloning, the sense strand oligonucleotide primer sequence was 5′-gAATTCATgCgATgCgTgggAAT (*EcoR* I site underlined) and the antisense strand oligonucleotide primer was 5′-CTCgAgTTATCCTTTCTTgAACCAgC (*Xho* I site under line). PCR conditions were as follow: denaturation 94 °C 5 min, followed by 25 cycles of (i) denaturation (94 °C 30 s), (ii) annealing (55 °C 30 s), and (iii) elongation (72 °C 90 s), followed by a final 10 min elongation period at 72 °C. The PCR product was ligated into pET28a plasmid using *EcoR* I and *Xho* I restriction sites, sequenced and transformed into *E. coli* strain BL21(DE3) to express and purify E395 protein following the previous published procedure [32]. The emergence of protein in the fractions was collected in ~1.5 mL/tube. Fractions containing protein were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

2.4. Generation of mAbs against DV1 E protein

Six-week-old female Balb/c mice were intraperitoneally immunized at 2-week intervals with inactivated DV1 (~10^5 particles) mixed with complete Freund's adjuvant twice and then given three boosts of recombinant DV1 E395 protein (100 μg) mixed with incomplete Freund's adjuvant. Hybridomas were generated according to the previous published procedures [33]. Hybridoma supernatants were screened for the presence of antibodies against DV1 with indirect ELISA coated inactive DV1. Positive hybridoma cells were cloned by limiting dilutions and the serotype-specific mAbs were further identified with dot blot using C6/36 cells infected with four different serotypes DV. The mAb was purified with nProtein A sepharose.

2.5. Identification of serotype specificity of anti-E mAb with Dot blot assays

Different dengue virus solutions and negative control: JEV solution, C6/36 cell cultured medium, and PBS-diluted human serum as the target were used to identify the serotype specificity of anti-E mAbs. To perform the dot blot assay, 50 μL of different targets were spotted on a methanol-activated Hybond-P PVDF membrane with Dot Blotter (Major science) and detected

with SNAP i.d.™ Protein Detection System (Millipore) according to the user guide. The signal was detected with Western lighting-ECL kit (PerkinElmer life science) and Fuji X-ray medical film.

2.6. Immobilization of anti-E mAb on magnetic beads

The tosyl groups on the surface of tosyl-activated magnetic beads (Dynabeads M-280) were used to react with amine groups of anti-E mAb (clone 3-46-1) and BSA (as negative control) to form stable covalent bonds. According to the manufacturer's instructions, 40 μL of beads (8.0×10^7 beads) were transferred to a tube and washed once with 1 mL of buffer A (0.1 M sodium phosphate buffer/pH 7.4). After the supernatants were removed, 33 μL of buffer A, 5 μL of mAb (~40 μg) and 25 μL of buffer B (3 M $(NH_4)_2SO_4$ in buffer A) were mixed with magnetic bead pellets by vortexing step by step and the mixture was incubated for 48 h at room temperature on a drum roller. Next, the magnetic beads were blocked with 1 mL of blocking buffer (0.01 M sodium phosphate buffer/pH 7.4, 0.5% (w/v) BSA, 0.15 M NaCl) for 1 h at room temperature on a roller. Then, the magnetic beads were washed twice with 1 mL of wash buffer (0.01 M sodium phosphate buffer/pH 7.4, 0.1% (w/v) BSA, 0.15 M NaCl) and the beads were re-suspended in 60 μL of wash buffer to achieve the final desired bead concentration (20 mg/mL). The amount of mAb immobilized on magnetic beads was determined using BCA protein assay kit (Pierce). The anti-E mAb conjugated beads were then stored at 4 °C for further use.

2.7. Immunomagnetic separation (IMS) of dengue virus

Aliquots of anti-E immobilized magnetic beads, anti-E@MBs (1 μL, ~10^6 particles) were mixed with 85 μL of 10 mM PBS-TBN buffer (0.1 g of BSA, 0.02 g of sodium azide, and 0.02 mL of Tween 20 in 100 mL of 0.01 M sodium phosphate buffer/pH 7.2) and 10 μL of dengue virus stock solution (~10^7 pfu/mL) or virus stock solution mixed with human serum (HS) or fetal bovine serum (FBS), respectively. The mixture was allowed to react for 30 min at room temperature on a dump roller. The MBs were separated using common magnet and washed twice with 200 μL of PBS and once with 100 μL of DI water. The captured virus was eluted from the MBs with 1 μL of 30% acetic acid.

2.8. Identification of DV immunocaptured on anti-E@MBs with enzymatic digestion

Five microliters of anti-E@MBs were incubated with 100 μL of DV1 stock solution and 400 μL of 10 mM PBS-TBN buffer for 1 h at room temperature on a roller. Subsequently, the MBs were washed twice with 500 μL of PBS and once with 500 μL of water. DV1 elution was accomplished by incubation of beads with 20 μL of 0.1% TFA for 5 min at room temperature. The supernatant was transferred into a new tube and neutralized by adding 7 μL of 400 mM NH_4CO_3. The further reduction was carried out with 1.5 μL of 400 mM DTT for 10 min at 95 °C. After the mixture had cooled down to room temperature, alkylation was achieved by adding 3 μL of 500 mM IAA and then incubating the mixture for 30 min at room temperature, void of light. Then, the enzymatic digestion of immunocaptured dengue virus was reacted with 5 μL of trypsin (20 μL/mL) for 17 h at 37 °C. Before the MALDI analysis, the digested mixture was desalted with ZipTip C_{18}, according to the manufacture's protocol. The peptides identification was searched with Mascot engine (Matrix Science, London, UK) using NCBI non-redundant as database. The following parameters were used for the database searching: peptide mass tolerance: ±75 ppm, fixed modification: carboxymethyl, variable modification: oxidation, and one missed cleavage was allowed. The information on amino acid sequences of DV1 structural proteins were obtained through the NCBI database and sequenced from our DV1 strain used in this study.

2.9. MALDI-TOF MS analysis

A freshly saturated matrix solution was prepared by dissolving CHCA (10 mg/mL) in 50% ACN containing 0.1% (v/v) TFA. For the auto and high throughput screening, the seed-layer method was used to prepare the homogeneous sample surfaces for intact protein detection, with the typical procedure as follows: (i) a diluted matrix solution (1.5 mg/mL in acetonitrile) was applied (1.0 μL) onto the MALDI target, (ii) the sample eluted from beads was then premixed 1:1 with saturated matrix solution and applied (2.0 μL) onto the same spot and (iii) the sample was allowed to dry at room temperature for 5 min before the target was inserted into the mass spectrometer. For peptide form enzymatic digestion, 1 μL of C_{18} desalted peptide elution was premixed 1:1 with saturated CHCA then spotted on the MALDI target directly. MALDI-TOF mass spectra were measured on a MALDI-TOF Bruker Autoflex III Smartbeam (Bruker Daltonics) with a MTP 384 target plate polished steel T F and a pulsed 337 nm nitrogen laser. The laser power was adjusted to 65% of its maximal intensity. The instrument was operated in linear modes for intact protein detection and reflectron mode for peptide detection. Before the experiments, external calibration was applied to the instrument before data collection. Protein standard I (insulin: $M+H^+ = 5734$; ubiquitin: $M+H^+ = 8565$; cytochrome C: $M+H^+ = 12{,}361$ and $M+2H^{2+} = 6181$) and peptide calibration standard (angiotensin II: $M+H^+ = 1046.54$; angiotensin I: $M+H^+ = 1296.68$; substance P: $M+H^+ = 1347.74$; bombesin: $M+H^+ = 1619.82$; ACTH clip 18–39: $M+H^+ = 2465.20$) were used as external calibrations for intact protein and peptide detection, respectively. A typical spectrum was obtained by averaging 3000 laser shots from 30 positions within the sample well. The data were then reprocessed using the flexAnalysis and biotools software.

2.10. Competitive binding assay

To evaluate the specificity of the anti-E@MBs to DV1, 1 μL of recombinant DV1 E395 protein (~1 mg/mL, DV1 E395 buffer solution: 20 mM Tris, 0.5 M NaCl, 0.5 M Imidazole/pH 8.0) was incubated with 1 μL of anti-E@MBs and 85 μL of 10 mM PBS-TBN buffer for 10 min at room temperature. For the control experiment, anti-E@MBs was incubated with DV1 E395 buffer solution before adding DV1. After wash three times with 200 μL of PBS, the beads were incubated with 10 μL of DV1 (~10^7 pfu/mL) solutions and then subjected to IMS and MALDI-TOF MS analysis.

3. Results and discussion

3.1. Analytic strategy

The primary aim of this study was to develop a method combining an immunocapture step and a mass spectrometric analysis for fast, high throughput and specific analysis of dengue virus from complex biological mixtures. The approach involved different steps: (i) immunocapture of dengue virus using antibody-coated magnetic beads, (ii) virus was eluted and lysed from the beads and (iii) MALDI-TOF MS detection of the capsid proteins. In the framework of our study, particular attention was paid to the optimization of the whole analytical process in order to develop a fast-response method.

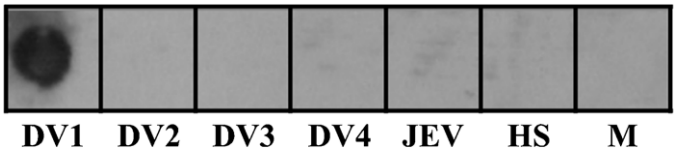

Fig. 2. Identification of the serotype specificity of anti-E mAb 3-46-1 with dot blot assay. DV1–DV4, four serotype dengue virus of prototype strains. JEV, Japanese encephalitis virus T1P1 strain. HS, 100 fold diluted human serum. M, the supernatant of C6/36 cells cultured medium.

3.2. Serotype specificity of anti-E monoclonal antibody

Based on the combination of immunocapture and MALDI-TOF MS, the development of a rapid and specific detection system for type specific dengue virus is highly dependent on the availability of specific antibodies that recognize the native dengue viruses. For the confirmation of native virus recognizing ability, dot blot assay was used to identify the serotype specificity of anti-E antibody (mAb 3-46-1) generated and used in this study. As shown in Fig. 2, mAb 3-46-1 specifically reacted with DV1 but not DV2, DV3, DV4, JEV T1P1 strain, HS, nor the supernatant of C6/36 cells cultured medium in dot blot assay, demonstrating that the mAb 3-46-1 is DV1 specific.

3.3. Identification of DV1 immunocaptured on anti-E@MBs with enzymatic digestion

Tosyl-activated magnetic beads covalently conjugated with anti-E 3-46-1 monoclonal antibody (anti-E@MB) were used in the following experiments. We performed the MALDI-TOF analysis using two different methods: (1) linear mode of operation for protein molecular weight and (2) reflectron mode for peptide fragment analysis. The mass spectrum was analyzed using the Mascot search engine. The linear mode mass spectrum revealed two major peaks of *m/z* 11,431.8 and 5719.5 Da, respectively. Defined peptide fragments generated by trypsin digestion were firstly desalted with ZipTip C_{18}. The eluent was mixed with saturated CHCA matrix and spotted on MALDI plate for peptide mass spectrum analysis. The mass spectrum showed numerous peptide signals after trypsin digestion. Among them, four tryptic peptide signals of DV1 structural proteins appeared at *m/z* 1180.63, 1479.80, 1937.95, and 1433.75 Da, which were derived from capsid (GeneBank: AAA42940.1), capsid, membrane, and envelop proteins of DV1, respectively, were identified from the Mascot search result. The identified peptides along with their respective sequences are summarized in Table 1. This information indicated that DV1 could be captured by the anti-E@MBs and the system could be applied in the following procedure of virus detection.

3.4. Matrix selection and MALDI spotting

To systematically optimize the performance of MALDI-TOF MS analysis, we compared the efficiencies of combination of matrix choice, sample preparation and spotting, elution buffer, and incubation time. Three matrices commonly used in MALDI-TOF MS, including sinapinic acid (SA), 2,5-dihydroxybenzoic acid (DHB), and α-cyano-4-hydroxycinnamic acid (CHCA), were evaluated. The matrices were dissolved in 50% ACN containing 0.1% (v/v) TFA (10 mg/mL). In addition, the MALDI spotting: seed-layer method was used to achieve a homogeneous sample/crystal surface, for automated high throughput analysis and improvement of the MALDI MS detection sensitivity, resolution and reproducibility. After purification of DV1 (*Hawaii*) from DV1 stock solutions with the anti-E@MBs, the DV1-magnetic beads complex were separated using a magnet and proteins of nonspecific binding were removed with serial washes. Then, the DV1-magnetic beads were reacted with 1 μL of 30% acetic acid, and the supernatant was mixed with different matrix, spotted with seed-layer method and analyzed by MALDI-TOF MS in the linear positive-ion mode. The spectra (see supporting information Fig. 1) showed CHCA to be the best-suited matrix for the observation of dengue virus 1 capsid protein (accession number ACF49259.1) MS signals. The major peak was detected at *m/z* 11,431.8. A theoretical molecular weight, 11,393.8 Da, was calculated from the amino acid sequence of the DV1 used in this study. A theoretical *m/z* value of 11,432.8 could be derived from potassium ion adduct formation of capsid protein [34,35]. Therefore, the discrepancy in mass may be attributed to capsid protein after potassium ion addition and the peak could be assigned as the capsid proteins of dengue virus.

Table 1
Tryptic peptides from immunocaptured DV1 identified by MALDI-TOF MS.

Position	Amino acid residues	Theoretical monoisotopic masses $[M+H]^+$	Observed monoisotopic masses $[M+H]^+$
Capsid protein			
46–55	LVMAFIAFLR	1180.69	1180.63
87–98	EISSMLNIMNRR	1479.74	1479.80
Membrane protein			
16–31	TETWMSSEGAWKQIQR	1937.92	1937.95
Envelope proteins			
125–136	LEGKIVQYENLK	1433.80	1433.75

3.5. Optimization of dengue virus binding, wash and elution conditions

We next optimized the binding, washing, and elution conditions to minimize nonspecific binding. Different incubation times ranging from 0 to 60 min were evaluated. The optimal incubation time was selected to be 30 min although the signal could be detected after 5 min incubation (see supporting information Fig. 2). To eliminate the nonspecific binding from the background signals caused by human serum high abundance proteins, different wash buffers including PBS and PBST containing Tween-20 or non-fat milk were used. Further improvement of sensitivity was achieved by removing salt with additional cycles of distilled water wash. The highest signal was detected when PBS buffer was used (see supporting information Fig. 3A). Nevertheless, decreased peak intensity was observed when PBST (0.05% Tween-20) or PBST/0.5% non-fat milk was used. The results demonstrated the effect of sample wash before spotting. Almost no MS signal of capsid proteins could be detected without wash conditions (see supporting information Fig. 3B). The MS signals of capsid proteins were similar when the sample was washed either twice with PBS + once with ddH_2O or thrice with PBS + twice with ddH_2O. For rapid diagnosis, the wash condition of twice with PBS + once with ddH_2O condition was used, although slightly higher signals were observed for conditions where the sample was washed thrice with PBS + twice with ddH_2O. To further optimize the detection sensitivity, the effect of elution solution on the removal of virus from anti-E@MBs was investigated. Three commonly used elution solutions, HCl (0.1 N and 0.01 N), TFA (0.1% and 0.5%), acetic acid (20–50%), and the two solvent mixtures (0.5% n-octyl-β-D-glucoside/0.5% TFA and 70% ACN/30% FA) were independently added to virus-anti-E@MB and vortexed for 5 s prior to matrix addition [25]. The spectra showed that 30% acetic acid exhibits the best elution efficiency (see supporting information Fig. 4). This condition was used as elution buffer thereafter in this study.

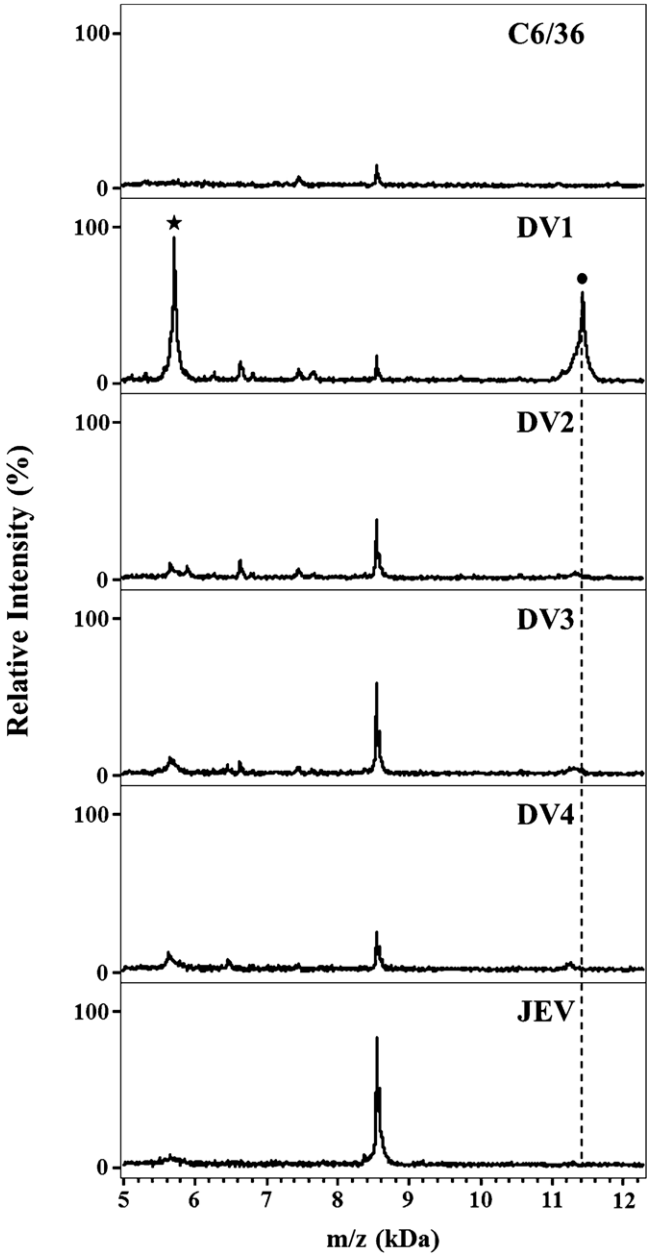

Fig. 3. MALDI mass spectra of binding specificity on anti-E@MBs. Two major peaks were detected at *m/z* 11,431.8 and 5719.5. The circle indicated peak, 11,431.8, corresponds to the single charged ion, and the star indicated peak, 5719.5, corresponds to doubly charged ion.

3.6. Binding specificity of different serotype dengue virus on anti-E immobilized magnetic beads

The successful typing of dengue virus is crucial both for diagnosis of the disease and implementation of epidemiologic control measurements. We next investigated the binding specificity of anti-E@MBs on various serotypes of dengue virus. The same amount of DV-1, 2, 3, 4 viruses, Japanese encephalitis virus (JEV), and C6/36 cultured medium were separately mixed with aliquots of anti-E@MBs and analyzed with MALDI-TOF MS. The MS signals of capsid protein at *m/z* 11,431.8 for single ion and *m/z* 5719.5 for doubly ions were only observed in DV1 but not stock solution of other serotypes and JEV, supporting the binding specificity of anti-E@MBs to DV1 (Fig. 3). Interestingly, the MS signals of DV1 envelop and matured membrane proteins expected at *m/z* 53,808

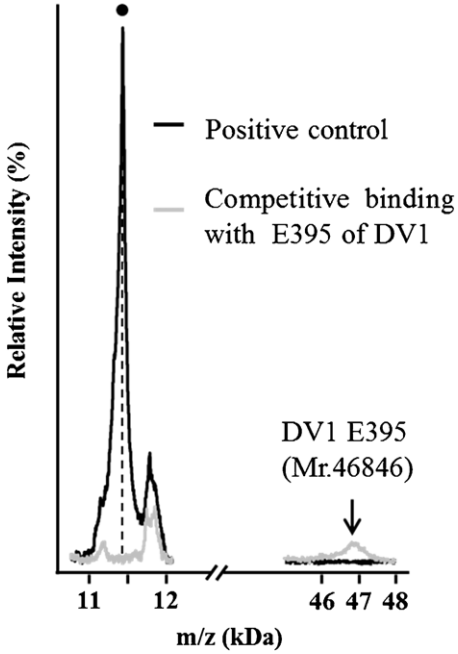

Fig. 4. MALDI mass spectra of competitive binding assay. In comparison to the DV1 + anti-E@MBs (black line), the spectra of competitive binding with DV1 E395 (light gray line) showed that MS signal of capsid proteins was reduced and another signal was observed at *m/z* 46,846, which is that of the DV1 E395 recombinant protein. The circle indicates the MS signal of capsid proteins.

and 8143.6, respectively, were not detected, suggesting possible aggregation of integral membrane proteins containing hydrophobic regions [24,25]. The binding specificity of the anti-E@MBs to DV1 on MALDI-TOF MS signal was further confirmed by competitive binding assay. When the anti-E@MBs was competitively bound to DV1 E395 recombinant proteins prior to mixing with DV1, the MALDI-TOF MS signal of the capsid protein reduced to almost undetectable levels and a MS signal at *m/z* 46,846, close to theoretical molecular mass of 47,024 of the DV1 E395 recombinant protein, was detected with low signal intensity (Fig. 4). Consistent with the observation is the detection of the capsid protein MS signal on the IMS fraction of anti-E@MBs mixed with DV1, but not on the anti-E@MBs mixed with PBS-TBN buffer, anti-E@MBs mixed

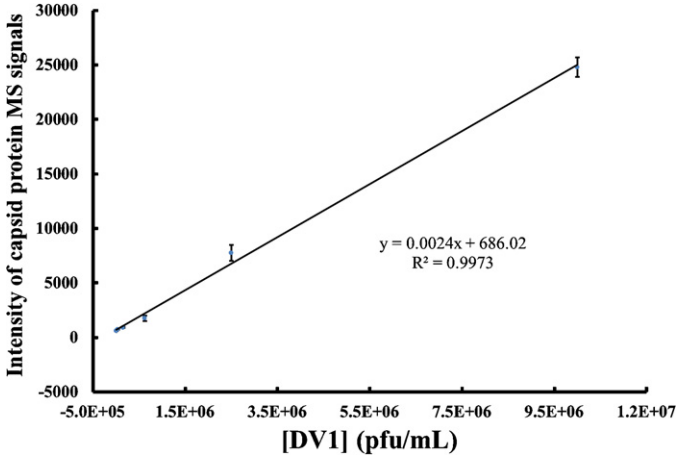

Fig. 5. Quantitative analysis of DV1 by MALDI-TOF MS. Serial dilutions of DV1 were prepared in human serum and analyzed with the method described in this study in triplicate. The intensity of capsid protein MS signals is dependent on the concentrations of the DV1 titer. The detection showed linear range between 10^5 and 10^7 pfu/mL, about 100–1000 folds lower than those described in DF and DHF patients 3 days after onset of the disease.

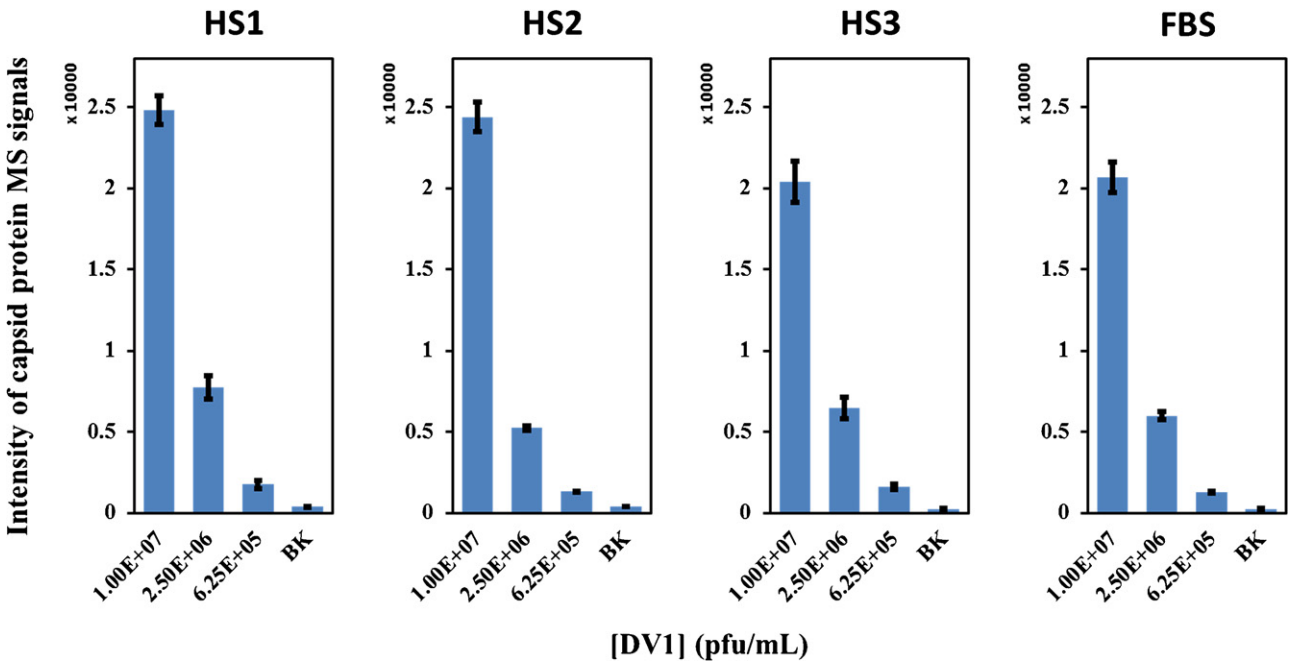

Fig. 6. Detection specificity of DV1 in different human sera by MALDI-TOF MS. Different dilutions of DV1 was prepared in three different human sera (HS1–HS3) and in FBS. The HS without DV1 (BK) was used as control. Intensity of bar graphs indicates concentration dependent of capsid protein signal detected by MALDI-TOF MS.

with DV1 stock solution but without IMS process, or IMS fraction of BSA@MBs mixed with DV1 (see supporting information Fig. 5). In summary, these data demonstrated the specificity of the anti-E@MBs on detection and typing of the DV1.

3.7. Detection limit

To validate the method for Dengue virus detection, the ability of anti-E@MBs sensitivity to reach beyond the detection limit of the post clinical onset of the disease is prerequisite. The clinical serum profile was imitated with HS1, a mixture of DV1 and human serum. The detection limit of the anti-E@MBs on DV1 was evaluated using serial dilution of the HS1 mixed with anti-E@MBs and analyzed with MALDI-TOF MS in triplicate. As shown in Fig. 5, the detection limit for DV1 is $\sim 10^5$ pfu/mL, about 100–1000 folds lower than those described in DF and DHF patients 3 days after onset of the disease [36–38]. In parallel, results obtained from spotting with seed layer method also showed a linear calibration curve ($R^2 = 0.997$) for serial dilution analysis. The clinical serum profiles were also imitated with a mixture DV1 stock solution and different human serum (HS) and fetal bovine serum (FBS), respectively, to evaluate the applicability of the method in real samples and the false-positive issue. As shown in Fig. 6, apparent capsid protein MS signals were detected in all DV1-containing samples with MS signal intensity to concentration relationships, but not in HS alone sample. Since the levels of viremia titers are associated with the severity of illness in dengue virus infection, the method developed in this study can potentially be applied to detect DV1 infection and to assess the severity of the disease [37,39,40].

4. Conclusion

We have demonstrated, for the first time, a facile method for serotype specific detection of dengue virus using immunocapture coupled with MALDI-TOF MS. The method unambiguously differentiated the DV1 from other serotypes in a highly specific manner. Through simple magnetic separation and 30% acetic acid elution step, the laser-disrupted virus particles released envelope, membrane, and capsid proteins for MS detection. Among various matrices tested for MALDI-TOF MS readout, the CHCA matrix prepared with the seed method exhibited the best desorption and ionization efficiency. The $\sim 10^5$ pfu/mL detection limit is highly sensitive and thus aids in the detection of dengue virus infection in its early stages. Finally, with the automatic IMS processing and sample spotting procedures as well as MALDI-TOF MS readout, our method may lead to early, accurate, high throughput, and serotype specific diagnosis of dengue virus.

Conflict of interest statement

The authors declare no competing financial interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.chroma.2013.02.030.

References

[1] T.P. Monath, Proc. Natl. Acad. Sci. U.S.A. 91 (1994) 2395.
[2] J.S. Porterfield, Adv. Virus Res. 31 (1986) 335.
[3] S.B. Halstead, Science 239 (1988) 476.
[4] M.G. Guzman, G. Kouri, Lancet Infect. Dis. 2 (2002) 33.
[5] D.J. Gubler, Novartis Found. Symp. 277 (2006) 3.
[6] D.J. Gubler, Clin. Microbiol. Rev. 11 (1998) 480.
[7] M.G. Guzman, G. Kouri, Int. J. Infect. Dis. 8 (2004) 69.
[8] K.R. Singh, S.D. Paul, Bull. World Health Organ. 40 (1969) 982.
[9] T. Laue, P. Emmerich, H. Schmitz, J. Clin. Microbiol. 37 (1999) 2543.
[10] R.S. Lanciotti, C.H. Calisher, D.J. Gubler, G.J. Chang, A.V. Vorndam, J. Clin. Microbiol. 30 (1992) 545.
[11] K. Morita, M. Tanaka, A. Igarashi, J. Clin. Microbiol. 29 (1991) 2107.

[12] E. Harris, T.G. Roberts, L. Smith, J. Selle, L.D. Kramer, S. Valle, E. Sandoval, A. Balmaseda, J. Clin. Microbiol. 36 (1998) 2634.
[13] D.F. Tai, C.Y. Lin, T.Z. Wu, J.H. Huang, P.Y. Shu, Clin. Chem. 52 (2006) 1486.
[14] S. Kumbhat, K. Sharma, R. Gehlot, A. Solanki, V. Joshi, J. Pharm. Biomed. Anal. 52 (2010) 255.
[15] W.S. Chang, H. Shang, R.M. Perera, S.M. Lok, D. Sedlak, R.J. Kuhn, G.U. Lee, Analyst 133 (2008) 233.
[16] Y.F. Lee, K.Y. Lien, H.Y. Lei, G.B. Lee, Biosens. Bioelectron. 25 (2009) 745.
[17] A.C. Dias, S.L. Gomes-Filho, M.M. Silva, R.F. Dutra, Biosens. Bioelectron. 44 (2013) 216, http://dx.doi.org/10.1016/j.bios.2012.12.033.
[18] G.J. Zhang, L. Zhang, M.J. Huang, Z.H.H. Luo, G.K.I. Tay, E.J.A. Lim, T.G. Kang, Y. Chen, Sens. Actuators B: Chem. 146 (2010) 138.
[19] C. Fenselau, P.A. Demirev, Mass Spectrom. Rev. 20 (2001) 157.
[20] C.F. John, P. Anhalt, Anal. Chem. 47 (1975) 219.
[21] R.D. Holland, J.G. Wilkes, F. Rafii, J.B. Sutherland, C.C. Persons, K.J. Voorhees, J.O. Lay Jr., Rapid Commun. Mass Spectrom. 10 (1996) 1227.
[22] J.M. Hettick, M.L. Kashon, J.P. Simpson, P.D. Siegel, G.H. Mazurek, D.N. Weissman, Anal. Chem. 76 (2004) 5769.
[23] E. Elhanany, R. Barak, M. Fisher, D. Kobiler, Z. Altboum, Rapid Commun. Mass Spectrom. 15 (2001) 2110.
[24] J.J. Thomas, B. Falk, C. Fenselau, J. Jackman, J. Ezzell, Anal. Chem. 70 (1998) 3863.
[25] Y.J. Kim, A. Freas, C. Fenselau, Anal. Chem. 73 (2001) 1544.
[26] S.D. Fuerstenau, W.H. Benner, J.J. Thomas, C. Brugidou, B. Bothner, G. Siuzdak, Angew. Chem. Int. Ed. Engl. 40 (2001) 541.
[27] T.W. Hutchens, T.T. Yip, Rapid Commun. Mass Spectrom. 7 (1993) 576.
[28] R.W. Nelson, J.R. Krone, A.L. Bieber, P. Williams, Anal. Chem. 67 (1995) 1153.
[29] D. Nedelkov, K.A. Tubbs, E.E. Niederkofler, U.A. Kiernan, R.W. Nelson, Anal. Chem. 76 (2004) 1733.
[30] D. Nedelkov, U.A. Kiernan, E.E. Niederkofler, K.A. Tubbs, R.W. Nelson, Proc. Natl. Acad. Sci. U.S.A. 102 (2005) 10852.
[31] P. Sukhavachana, A. Nisalak, S.B. Halstead, Bull. World Health Organ. 35 (1966) 65.
[32] S. Jaiswal, N. Khanna, S. Swaminathan, Protein Expr. Purif. 33 (2004) 80.
[33] W.L. Chen, M.T. Huang, H.C. Liu, C.W. Li, S.J. Mao, J. Dairy Sci. 87 (2004) 2720.
[34] M. Karas, F. Hillenkamp, Anal. Chem. 60 (1988) 2299.
[35] F. Hillenkamp, M. Karas, Methods Enzymol. 193 (1990) 280.
[36] M.L. Moi, C.K. Lim, A. Kotaki, T. Takasaki, I. Kurane, J. Infect. Dis. 203 (2011) 1405.
[37] D.W. Vaughn, S. Green, S. Kalayanarooj, B.L. Innis, S. Nimmannitya, S. Suntayakorn, T.P. Endy, B. Raengsakulrach, A.L. Rothman, F.A. Ennis, A. Nisalak, J. Infect. Dis. 181 (2000) 2.
[38] T.M. Sudiro, J. Zivny, H. Ishiko, S. Green, D.W. Vaughn, S. Kalayanarooj, A. Nisalak, J.E. Norman, F.A. Ennis, A.L. Rothman, J. Med. Virol. 63 (2001) 29.
[39] D.H. Libraty, T.P. Endy, H.S. Houng, S. Green, S. Kalayanarooj, S. Suntayakorn, W. Chansiriwongs, D.W. Vaughn, A. Nisalak, F.A. Ennis, A.L. Rothman, J. Infect. Dis. 185 (2002) 1213.
[40] D.W. Vaughn, S. Green, S. Kalayanarooj, B.L. Innis, S. Nimmannitya, S. Suntayakorn, A.L. Rothman, F.A. Ennis, A. Nisalak, J. Infect. Dis. 176 (1997) 322.