Discussion
5. Discussion

5.1 Recombinant dengue multiepitope (r-DME’s) proteins

5.1.1 Design, expression, purification and characterization of r-DME-G

5.1.2 Design, expression, purification and characterization of r-DME-M and r-DME-M2

5.1.3 Evaluation of r-DME-G, M and M2 proteins as diagnostic intermediates

5.2 Epitope scanning of the capsid and non-structural protein-4A (NS4A) of dengue type 2 virus

5.2.1 Epitope scanning of the Capsid

5.2.2 Epitope scanning of the NS4a

5.2.3 New generation r-DME-G molecule

5.3 Conclusions
5. Discussion

Dengue fever is a mosquito-borne viral disease posing a rapidly expanding public health threat in many areas of the world. The high mortality associated with DHF and DSS can be significantly minimized through timely medical care, which in turn depends on accurate diagnosis of dengue infections. Several dengue diagnostic kits are commercially available\textsuperscript{76, 70}. However, their widespread use is hampered by several factors, stemming from the use of whole virus preparations as antigens to detect anti-dengue antibodies in patient sera. First, the use of whole virus antigens in these commercial kits poses a biohazard risk. Second, the viruses are usually produced in tissue culture or suckling mice brain. The high costs associated with virus antigen production make these kits unaffordable, particularly in the economically weaker dengue endemic countries. Finally, the whole virus antigens invariably pick up antibodies against other flaviviruses such as yellow fever and Japanese encephalitis viruses, leading to ambiguity in diagnosis. Further, false positives are also known to arise with sera from malaria, typhoid and leptospirosis patients. Thus, it is apparent that an ideal antigen for use in a dengue diagnostic test must not only be free of the virus associated biohazard risk, it must also be inexpensive to produce, and possess a high degree of specificity, to facilitate unequivocal diagnosis of dengue infections. The present work is based on the premise that the use of a synthetic antigen, designed to be dengue-specific, and expressed to high levels in an \textit{E. coli}-based expression system could effectively address the issues of biohazard risk, cost and specificity, associated with whole virus antigen-based diagnostic assays. Accordingly, we designed a synthetic antigen by splicing together dengue virus-specific epitopes using simple peptide linkers. To circumvent serologic cross-reactivity associated with whole virus or viral envelope proteins\textsuperscript{70}, we eliminated epitopes known to cross-react with sera from patients with diseases such as yellow fever and Japanese encephalitis. To develop this synthetic antigen, we focused on three proteins expressed by dengue viruses, namely, E, NS1 and NS3. The ability of these three proteins
to elicit humoral immune responses is well documented and their antigenic
determinants identified using a variety of approaches. Using the information available from these studies, we chose
epitopes for incorporation into synthetic antigens.

5.1 Recombinant dengue multiepitope (r-DME's) proteins

5.1.1 Design, expression, purification and characterization of r-DME-G

The r-DME-G was designed by keeping the following criteria; they had to be
(i) immunodominant, (ii) highly specific to IgG class of anti-dengue antibodies and (iii) linear. These criteria were based on the following rationale. First, in order for the synthetic antigen to be capable of efficiently recognizing dengue virus-specific antibodies, it is necessary that its constituent epitopes exhibit significant reactivity to dengue patient sera/anti-dengue Mabs. Second, the IgG-specificity of the epitopes was a selection criterion so that the r-DME-G protein would have a high sensitivity to pickup anti-dengue IgGs (which are recognized markers of secondary infections), common in dengue-endemic regions. Also, to ensure specificity to dengue alone, epitopes that displayed cross-reactivity towards other members of Flaviviridae were eliminated. Finally, it was necessary to work with linear epitopes so that when incorporated into the synthetic protein, they would presumably retain their immunoreactivity. On the other hand, if conformational epitopes were to be used in constructing the synthetic antigen, it is highly unlikely that they would retain their conformational integrity, and therefore their immunoreactivity, in the synthetic antigen. We selected a total of fifteen epitopes in accordance with the criteria discussed above. These ranged in length from 6 to 20 aa residues; eight were from the E protein and the remaining from the non-structural proteins NS1 and NS3. These were from dengue virus serotypes 1, 2 and 4. Next, we created the synthetic gene, r-DME-G, encoding all fifteen epitopes in which adjacent epitopes were separated by triglycyl linkers. We
used glycine in the linkers as it can provide flexibility stemming from its lack of a β carbon, and is considered as one of the preferred linker residues while designing chimeric proteins\textsuperscript{120}. Computer modeling analysis showed that the protein encoded by the r-DME-G gene adapts a structure in which all the chosen epitopes are freely accessible. This suggested that all the 15 chosen epitopes would be able to collectively contribute to the overall specificity of the molecule. We engineered a 6x His tag-encoding sequence at the 3’ end of this gene to facilitate one-step affinity purification of the expressed protein. The recombinant protein, r-DME-G, was expressed to high levels in \textit{E. coli} under the control of an IPTG-inducible promoter. As seen often with over expressed proteins in \textit{E. coli}, the r-DME-G protein was present exclusively in the insoluble phase of the cell lysate. As it is known that fusion to the maltose-binding protein (MBP) can promote the solubility of some proteins\textsuperscript{121}, we constructed an r-DME-G-MBP fusion. This strategy also failed to produce soluble protein. Therefore, we purified the r-DME-G protein on a Ni-NTA matrix, under denaturing conditions. Analysis by SDS-PAGE showed that a high degree of purity, >95%, had been achieved in a single step. Our data show that starting from a one liter culture of induced cells, >25 mg of purified protein, representing a ~58% recovery from the crude lysate, can be obtained. Purified r-DME-G protein could be detected in a Western blot using anti-His mAb, consistent with its ability to bind Ni-NTA matrix. As proof of principle, we tested the r-DME-G protein in an in-house ELISA. In this assay, we used our recombinant protein as the capture antigen and dengue virus type-2 hyperimmune mouse serum as the test sample. We then used anti-mouse-HRPO/TMB substrate to examine if the r-DME-G protein had successfully captured anti-dengue virus antibodies from the murine serum. The results showed that indeed our synthetic antigen could recognize and bind anti-dengue antibodies as evidenced by the ELISA readouts. Further, the r-DME-G protein could interact with the murine anti-dengue antibodies as efficiently as the whole virus, justifying our design of the recombinant protein. Importantly, the capacity of the r-DME-G protein to manifest immunoreactivity towards dengue virus-specific antibodies was corroborated using dengue
Discussion

patient sera in a Western blot experiment. This suggested that indeed the r-DME-G protein could have a potential use as a diagnostic reagent. Having ascertained the ability of the r-DME-G protein to specifically detect antidengue virus antibodies, we proceeded to evaluate its potential utility as a dengue diagnostic reagent. In designing the r-DME-G protein, most of the epitopes were drawn from the E protein of dengue virus type 2 (eight epitopes) and the NS1 protein of dengue virus type 4 (five epitopes). This is because E and NS1 are the most immunogenic of the dengue virus proteins and information available in the literature pertains to only serotypes 2 and 4.

5.1.2 Design, expression, purification and characterization of r-DME-M and r-DME-M2

The r-DME-M synthetic antigen was created with N-terminus epitopes from NS1 (1-15 aa) of dengue viruses (D1, 2, 3 and 4) separated by tetraglycyl linker. The computer predicted structure showed that the synthetic protein epitopes are freely accessible to bind antibodies of patient’s sera. The r-OME-M gene was cloned in frame with initiator codon and 6x His tag of pOE-60 vector. It seems that due to the smaller size of the protein, no expression was observed. Attempts were made to express this as a fusion protein with MBP. For this the gene was sub-cloned in-frame with maltose binding protein (MBP) in pMAlc2x vector. The MBP fusion protein was expressed and found to be partially soluble under native conditions. The fusion protein was purified under denaturing conditions on a Ni-NTA matrix. About 15 mg of purified protein was obtained from 1 litre of induced culture. Purified r-DME-M protein could be detected in a Western blot using anti-His mAb, consistent with its ability to bind Ni-NTA matrix. To increase the epitope density two copies of the r-DME-M was cloned in-frame with maltose binding protein (MBP) in pMAlc2x vector. The protein having double density of each epitope was expressed as inclusion bodies and purified under denaturing conditions.

5.1.3 Evaluation of r-DME-G, M and M2 proteins as diagnostic intermediates

Having created the rDME proteins, we next sought to test their utility as diagnostic reagents for dengue detection. To determine if the rDME-G protein
could recognize and bind dengue virus-specific antibodies, the purified proteins were tested separately as capture antigens in ELISA, using dengue virus type 2 hyperimmune murine serum as the test sample. For comparison, a control experiment was performed in parallel, using dengue type-2 virus (instead of the rDME-G protein) as the capture antigen. Antibody titers determined in the test and control experiments were comparable, indicating that our synthetic rDME-G protein was capable of efficiently recognizing serum dengue antibodies. This suggested that these two rDME-G proteins might serve as potential diagnostic reagents for the detection of dengue antibodies in patient sera.

In order to evaluate the feasibility of using these rDME proteins as diagnostic reagents to detect IgG and IgM anti-dengue antibodies, we developed in-house ELISA protocols. In these assays, either rDME-G or rDME-M protein was used separately to capture either IgG or IgM class of anti-dengue antibodies, respectively, from patient sera. Captured IgG and IgM antibodies were revealed using horseradish peroxidase conjugated anti-human IgG and anti-human IgM, respectively. We analyzed a large panel (n=172) of suspected dengue patient sera, obtained from dengue endemic regions in Sri Lanka, for the presence of dengue antibodies, using our rDME proteins in the ELISA format described above. We then compared our results with those obtained using PanBio’s Dengue Duo IgM and IgG rapid strip test. All samples were also tested for the presence of infectious virus and viral RNA. Based on the data obtained, we could categorize the samples into six groups as summarized in Table 15. The first group (n=22) represents the infected sera, in which the viral RNA could be detected by RT-PCR; further infectious virus could be isolated from all but one sample of this group. With regard to the serology, this group was heterogenous, as reflected by its division into subgroups a, b and c. A total of 6 samples (all 5 of 1b + the single one from 1c) were found to contain IgM antibodies. Out of these 6, the PanBio strip test identified 3 samples (2 from 1b and the single one from 1c) as IgM*. This observation suggests that the rDME-M based IgM ELISA is more effective in
the early detection of dengue infection in a slightly larger proportion of samples. With regard to IgG, only one sample (1c) was found

Table 15: Evaluation of rDME-M and rDME-G proteins

<table>
<thead>
<tr>
<th>Group&lt;sup&gt;a&lt;/sup&gt; Samples</th>
<th>IgM/IgG status&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Total</th>
<th>PanBio&lt;sup&gt;c&lt;/sup&gt;/ICGEB&lt;sup&gt;d&lt;/sup&gt; data</th>
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<td>16</td>
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<tr>
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<td>5</td>
<td>2/5</td>
</tr>
<tr>
<td>1c</td>
<td>IgM&lt;sup&gt;+&lt;/sup&gt;/IgG&lt;sup&gt;+&lt;/sup&gt;</td>
<td>1</td>
<td>1/1</td>
</tr>
<tr>
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</tr>
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<tr>
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<td>21</td>
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<tr>
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<td>IgM&lt;sup&gt;+&lt;/sup&gt;/IgG&lt;sup&gt;-&lt;/sup&gt;</td>
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</tr>
<tr>
<td>6</td>
<td>?</td>
<td>21</td>
<td>?</td>
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</table>

<sup>a</sup>Group 1 samples tested positive for virus; groups 2-6 tested negative for virus

<sup>b</sup>Presence and absence of IgM or IgG is indicated by ‘+’ and ‘-’ superscripts respectively

<sup>c</sup>Data generated using Dengue Duo IgM & IgG Rapid Strip test purchased from PanBio Pty., Australia.

<sup>d</sup>The data were obtained using rDME-M and rDME-G separately as capture antigens in ELISAs to detect IgM and IgG, respectively

? All samples gave inconsistent results in both tests

to be positive; this was corroborated by the PanBio test. The remaining samples (group 1a; n=16) were negative for both IgM and IgG. Once again, these results were borne out by the PanBio test. The samples represented by groups 2-6 (collective n=150), were all found to be virus/RNA-. Of the 129 samples, represented collectively by groups 2-5, we could detect the presence of IgM in 49 samples (12 samples of group 2 + 37 samples of group 3), and IgG in 33 samples (12 samples of group 2 + 21 samples of group 4), using the r-DME ELISAs. Samples in group 5 were all IgM/IgG<sup>-</sup>, and are presumably 'normal', as all of them tested negative in the virus isolation and RT-PCR assays as well. In regard to the PanBio test, 19 samples of group 1
(16 from group 1a, 2 from group 1b and the single sample from group 1c) and all samples of groups 2-5 (n=129) yielded results that were identical to those obtained using the r-DME IgM and IgG ELISAs. For samples in Group 6 (n=21), the results of the r-DME ELISAs did not agree with those of the PanBio test. The reason for the observed discrepancy is unclear at present, but is presumably related to differences in the nature of the capture antigen. It must be pointed out that neither virus nor viral RNA could be detected in all these ‘indeterminate’ samples. It is, therefore, essential at this juncture to establish the clinical histories of the patients, from whom these samples were obtained, in order to understand the reason for the observed discrepancy. The overall comparative analysis of our data with the PanBio results suggests that there is an excellent agreement between the r-DME and the PanBio tests, with ~86% (148 out of 172) of the samples analyzed giving identical results. Further, the r-DME-M IgM ELISA picked up three additional samples (group 1b), which the PanBio test failed to identify. The fact, that these three samples were virus+/RNA+, suggests that the r-DME IgM ELISA test is more effective in early diagnosis of dengue infection.

5.2 Epitope scanning of the capsid and non-structural protein-4A (NS4A) of dengue type 2 virus

Several dengue diagnostic kits are commercially available\(^7\),\(^6\). Many of these use whole dengue virus preparations as antigens to detect anti-dengue antibodies. The use of the whole dengue virus antigen is expensive and prone to serological cross-reactivity due to similarity with other flaviviruses such as JEV and YFV. To eliminate these shortcomings, the whole virus antigen must be replaced with a more appropriate antigen in dengue diagnostics. In this context, knowledge of immunogenic epitopes encoded by pathogens is a prerequisite for developing diagnostic peptides for the detection of infections with a high degree of sensitivity and specificity. While the immunogenic epitopes on the E and NS1 proteins have been well documented\(^97\),\(^98\),\(^99\),\(^100\),\(^101\),\(^102\),\(^103\),\(^104\),\(^108\), very little information is available regarding the antigenicity of the remaining dengue viral proteins. The capsid and NS4a are two small viral
proteins involved in genome packaging\textsuperscript{29} and in anti-host viral defense\textsuperscript{122}. Importantly, from the viewpoint of diagnostics, these two proteins are also implicated in the induction of B-cell antibody responses in dengue virus-infected individuals\textsuperscript{91, 92, 93}. The purpose of this study was to locate linear B-cell epitopes on dengue virus capsid and NS4a proteins, which might be potentially useful in diagnosis. We therefore performed pepscan analysis using pooled dengue patient serum, rather than single sera.

5.2.1 Epitope scanning of the Capsid
On the capsid, we identified several IgG-specific antigenic regions, defined by reactive overlapping peptide clusters, using dengue-specific murine hyperimmune serum as well as dengue patient sera. Both sera identified three major peptide clusters, one in the amino-terminal, and two in the carboxy-terminal regions. In addition, both sera picked up epitopes, defined by single, non-overlapping peptides, in the central capsid region. For example, the most immunogenic peptides identified using the murine hyperimmune serum were peptides c26, c35 and c3, belonging to clusters 2, 3 and 1, respectively. Using the dengue patient serum pool, essentially similar data were obtained. However, rather than peptide c26, it was peptide c25, that was picked up most efficiently by the human anti-dengue antibodies. Based on our data, the core sequences of peptide clusters recognized by dengue patient sera are NNQRKKARN (defined by overlapping peptides c1 and c2), RGFR (defined by overlapping peptides c22-c27) and MLNILNRRR (defined by overlapping peptides c36 and c37). Interestingly, the carboxy-terminally located immunodominant peptides are located on a $\alpha$-helix, exposed on the surface of the capsid dimer\textsuperscript{123}. In general, the observed ELISA reactivities were higher using the murine hyperimmune serum, as expected. Aside from these IgG-specific epitopes, our studies also identified two peptides, c17 and c33, which appeared to recognize anti-dengue human antibodies of the IgM class. While peptide c17 interacted with IgG antibodies as well, peptide c33 did not. Peptide 33 presumably represents a unique IgM-specific epitope.
Discussion

The results also showed that none of the 37 capsid peptides reacted with either YFV or JEV hyperimmune mice sera. A comparison of the core sequences of each of these immunodominant regions (Clustal analysis), showed that there was a high degree of similarity amongst the four dengue serotypes. However, peptide sequences in corresponding regions of YFV and JEV capsids diverged considerably from those of the dengue capsids. This explains the lack of reactivity of the dengue capsid peptides when scanned with either YFV- or JEV-hyperimmune murine sera. We conclude that the capsid epitopes identified in this study are truly dengue virus-specific and therefore have the potential to be useful in the detection of dengue infections.

5.2.2 Epitope scanning of the NS4a

All NS4a peptides with detectable epitope activity were mapped to the amino-terminal putative hydrophilic region of the protein. As with the capsid, we found multiple adjacent clusters of reactive peptides, using dengue-patient serum. A comparison of the core sequences of these NS4a peptide clusters amongst dengue and other flaviviruses such as YFV and JEV showed that the core sequence AVLHTA defined by the second cluster is unique to the dengue viruses alone. On the other hand, the core epitope sequence defined by the remaining two peptide clusters showed varying degrees of similarity with the corresponding NS4a peptides of YFV and JEV. The previous reports in the literature that described the detection of NS4a-specific antibodies in dengue patient were based on the observation of a protein with the predicted electrophoretic mobility of NS4a in Western blots\textsuperscript{91, 92}. A more recent report demonstrated that a recombinant NS3/NS4a fusion protein displayed ELISA reactivity using dengue patient sera\textsuperscript{124}. However, this study did not unambiguously identify NS4a-specific antibodies in the patient sera. Our current study provides definitive evidence for the occurrence of NS4a-specific antibodies in dengue patient serum using authentic dengue type 2 NS4a-derived peptides as capture antigens. Further, our data show that these NS4a-specific antibodies that we detected are IgG type antibodies. We were unable to detect any NS4a peptides that showed reactivity when tested against murine dengue-specific hyperimmune serum. This perhaps is a
reflection of effective clearance of virus-infected cells during the booster immunizations performed in the course of generating the murine hyperimmune serum. This would effectively preclude any translation and replication of the viral genome, which is a must before non-structural proteins like NS4a are produced and perceived by the immune system.

5.2.3 New generation r-DME-G molecule

Our earlier results have shown that a recombinant dengue multiepitope protein, r-DME-G, created by splicing together linear immunodominant epitopes of E and NS1 can serve as useful dengue diagnostic antigen. To gauge the diagnostic utility of the C and NS4a epitopes identified in this study, we developed a second-generation molecule by incorporating these into the r-DME-G molecule. Preliminary data suggest that the modified antigen, r-DME-G2, displays relatively enhanced sensitivity in detecting anti-dengue antibodies. In dengue endemic areas, secondary infections are most common. Thus, it is very likely that many of the dengue patient sera used to generate the pooled serum in our studies are from secondary infections. This is consistent with all these sera testing positive using the PanBio kit, which is designed to detect secondary infections. The prevalence of antibodies to various structural and non-structural dengue proteins is generally higher in secondary compared to primary dengue infections. For example, the seroprevalence of anti-NS4a antibodies is ~50% in secondary infections. It is therefore conceivable that the recognition of these antibodies in the pooled dengue patient serum by the r-DME-G2 protein contributes to its improved sensitivity.

5.3 Conclusions

In conclusion, the high density of the epitopes in the recombinant dengue multiepitope (rDME) proteins and the careful choice of only dengue-specific epitopes as its components contribute to a high degree of sensitivity and specificity. Further, our strategy of using a recombinant multiepitope protein completely obviates multiple peptide synthesis and multiple protein expression; it also avoids expensive and time-consuming virus culture (for
antigen preparation) and the associated biohazard risk. The design of the rDME proteins and the ease of their expressions and purifications have the potential to make this a highly cost effective approach to dengue diagnosis. In addition to ELISA format, the r-DME proteins can be adapted for use in alternative formats. For example, anti-dengue antibodies can be captured using unlabeled r-DME proteins and revealed using gold-labeled r-DME proteins. This could be incorporated into a spot test or a lateral flow test which are rapid and amenable to field use.