Fine Mapping of the Hepatitis E Virus Immune Response Using High Resolution Linear and Conformational Peptide Microarrays

Abstract

This application note describes the use of PEPperCHIP® Peptide Microarrays for biomarker discovery of potentially diagnostic peptides (Figure 1). This approach provides an in depth-analysis of serum profiles of infected vs. non-infected patients unraveling immunodominant epitopes that could later on be transferred to a diagnostic platform.

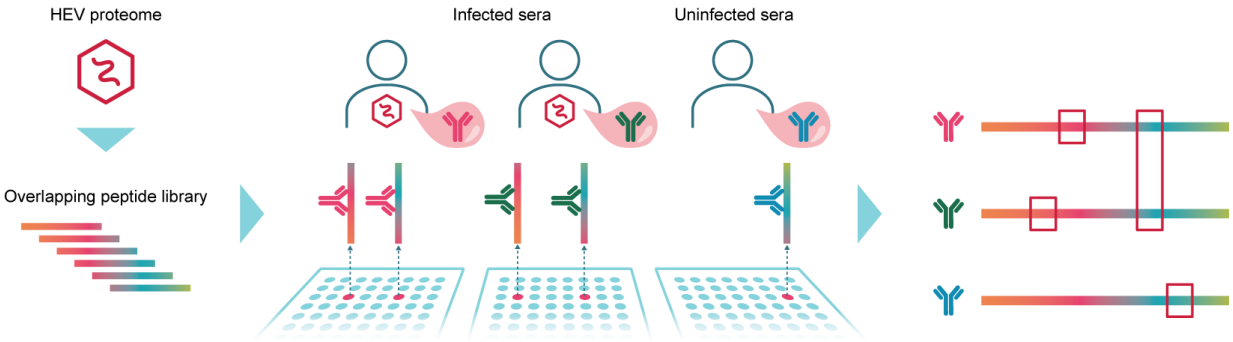

Fig. 1: Biomarker discovery workflow: The proteome of an infective agent (HEV) is translated into highly-overlapping peptides (shift 1). Individual array copies are incubated with sera from infected or non-infected individuals in order to identify immunodominant epitopes

For the study described below, biomarkers for HEV infection were analyzed.

Introduction

Hepatitis E virus (HEV) is a non-enveloped single-stranded RNA virus belonging to the virus family *Hepeviridae* and the genus *Orthohepevirus*. Up to date, there are five human pathogenic genotypes known, i.e. genotype 1, 2, 3, 4 and 7. Genotype 3 and 4 are of zoonotic origin and are associated with the consumption or handling of raw or undercooked pork products[1]. They are increasingly recognized as an emerging viral disease in developed countries. In contrast, genotypes 1 and 2 are waterborne and transmitted via the fecal oral route mainly in developing countries[1]. Genotype 7 is only found in camels and so far only a single human infection was recognized in a patient who regularly consumed camel milk and meat[2]. While most healthy individuals show no overt signs of disease after HEV infection, fatalities can be as high as 20% in pregnant women (genotype 1)[3]. Moreover, immunocompromised patients can develop persistent infection which can lead to chronic active hepatitis[1]. Acute HEV infection can be detected using nucleic acid amplification techniques such as qRT-PCR. However, since most patients

are asymptomatically infected and virus is only shed for a limited time, serological assays are needed to determine the overall burden of HEV infections in the general population[4]. Several serological assays are commercially available for the detection of HEV-specific IgG and IgM antibodies in human sera. However, results obtained using different assays vary considerably[3]. Two studies determining the seroprevalence of HEV in the same region in France, reported significantly different results, i.e. 52.5% for Wantai and 16.6% for MP Bio IgG assays[5,6]. These discrepancies are most likely due to differences in the antigenic content of the assays, different protein purification methods or contaminating proteins from E.coli or insect cell expression systems. Also, while all HEV genotypes belong to a single serotype, assays mainly use proteins derived from genotype 1 and/or genotype 3. So far, it is unclear how infections with heterologous genotypes affect the sensitivity and specificity of serological assays.

Synthetic peptide-based serological assays would be ideally suited to overcome these problems. Peptides are easy to synthesize with minimal lot-to-lot variations and high purity. Furthermore, new bead-based multiplex serological technologies are able to analyze several hundred peptides in parallel. Inclusion of multiple HEV-specific peptides in a novel serological assay would allow us to account for differences in the antigenic structure of the different genotypes and recognition of diverse epitopes by individuals. In the past, synthetic peptide-based serological assays for HEV showed low sensitivity despite being highly specific. However, at that time only smaller peptide libraries were used for screening assays, thereby severely limiting selection of suitable candidates. Novel peptide microarray technologies displaying tens of thousands linear and cyclic peptides yield more comprehensive information. Immune responses towards linear and conformational epitopes derived from complete viral proteomes can be analyzed in high-throughput fashion. These detailed insights into the immune responses of individual patients might lead to the development of novel improved multiplex peptide-based HEV serological assays with higher sensitivity while retaining a high specificity.

Results & Discussion

In this application note we fine mapped the immune responses of 6 HEV IgG-positive and 3 HEV IgG-negative individuals (sera kindly provided by V.M. Corman, Institute of Virology, Charité Berlin). Proteomes of prototype strains of all human pathogenic HEV genotypes with the accession numbers M73218.1, M74506.1, AF082843.1, AB197673.1 and KJ496143.1 were selected. Amino acid sequences were translated into 13mer (cyclic) or 15mer (linear) peptides with an overlap of 11 or 13 amino acids, respectively and laser-printed on a single peptide microarray. The resulting peptide microarrays comprised 5,426 different peptides printed in duplicate and were framed by polio (KEVPALTAVETGAT) control peptides.

Initially, PEPperCHIP® HEV microarrays were incubated with secondary antibody goat anti-human IgG (Fc) DyLight680 only, to control for background interactions of the detection antibody. Subsequently, sera were diluted 1:200 and incubated overnight at 4°C. Secondary detection was done using goat anti-human

IgG (Fc) DyLight680 antibody. Readout was performed using a LI-COR Odyssey Imaging System and 16-bit grey-scale images were analyzed using PepSlide® Analyzer software (SICASYS Software GmbH).

Pre-staining of the PEPperCHIP® HEV microarrays revealed only very weak interactions in the same range as the noise level of the assay. Therefore, image analysis and data readout was omitted. For the subsequent epitope fine mapping, we used 6 HEV IgG-positive and 3 HEV IgG-negative sera. IgG reactivity was previously determined using either Euroimmun AG or Wantai HEV IgG ELISA. HEV IgG-positive sera reacted strongly with HEV-derived peptides, while only weak signal intensities with few peptides were detected for HEV IgG-negative sera. Overall, a higher staining intensity together with a higher number of positive peptides was observed for linear peptides compared to cyclic peptides at a staining dilution of 1:200. Representative images of linear and cyclic PEPperCHIP® HEV microarrays are shown in Figure 2. Microarray image analysis was done with PepSlide® Analyzer. A software algorithm breaks down fluorescence intensities of each spot into raw, foreground and background signal, and calculates averaged median foreground intensities and spot-to-spot deviations of spot duplicates. We tolerated a maximum spot-to-spot deviation of 40% otherwise the corresponding intensity value was zeroed. To account for minor assay and microarray variations, we applied a quantile normalized for the data of each cohort (IgG-positive and IgG-negative).

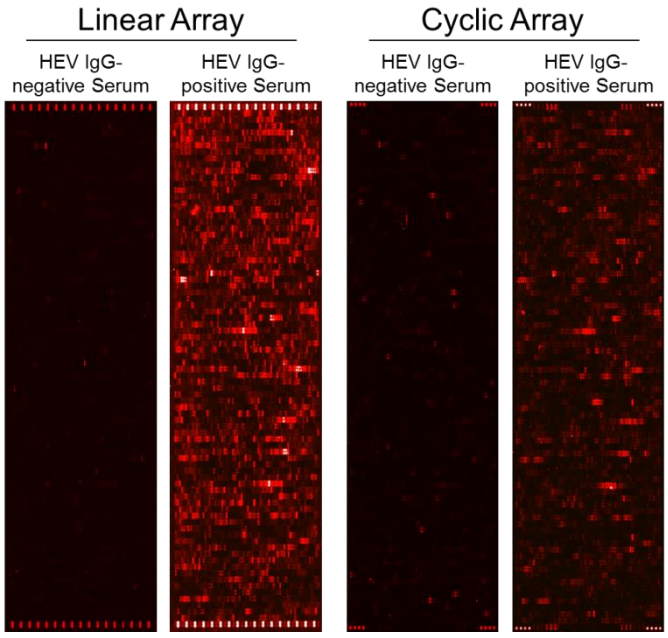

Fig. 2: Representative staining of linear (left) and cyclic (right) peptide micro-arrays using a HEV IgG-negative and a HEV IgG-positive serum. Sera were diluted 1:200 and incubated O/N followed by staining with secondary anti-human IgG antibody. A complex staining pattern was observed for HEV IgG-positive sera, while HEV IgG-negative sera showed only a low number of positive spots. Moreover, linear arrays showed a stronger signal compared to cyclic arrays.

Determination of the genotype of HEV-infected patients was not possible due to the absence of RNA in serum samples. However, since all HEV patients originated from Germany, infection with the endemic HEV genotype 3 was assumed. Therefore, we primarily analyzed the immune response against peptides derived from genotype 3. We observed a very heterogeneous immune response in individual patients. Nevertheless, we were able to identify 14 immunogenic regions throughout the HEV gt 3 proteome (Figure 3, red boxes) in which the majority of patients reacted with HEV-derived peptides. These include 8 immunogenic regions in the nonstructural protein (regions NSP_A to NSP_H), one at the C-terminus of the ORF3 protein (O3_A) and 5 in the capsid protein (regions CAP_A to CAP_E). Interestingly, in 7/14 immunogenic regions (NSP_B, NSP_C, NSP_D, NSP_G, CAP_C, CAP_D, CAP_E) HEV IgG-negative sera also reacted with the respective peptides. However, the number of reactive peptides as well as the fluorescence intensity were lower compared to HEV IgG-positive sera.

Whether the reactivity of HEV IgG-negative sera against HEV-derived peptides is due to a cross-reaction with antigens of other pathogens or results from old HEV infection that are not detected by commercially available serological assays, cannot be determined at this stage. The remaining 7 immunogenic regions (NSP_A, NSP_E, NSP_F, NSP_H, O3_A, CAP_A, CAP_B) allowed for a clear discrimination between positive and negative sera. Overall, the immune response towards linear peptides was more pronounced compared to cyclic peptides.

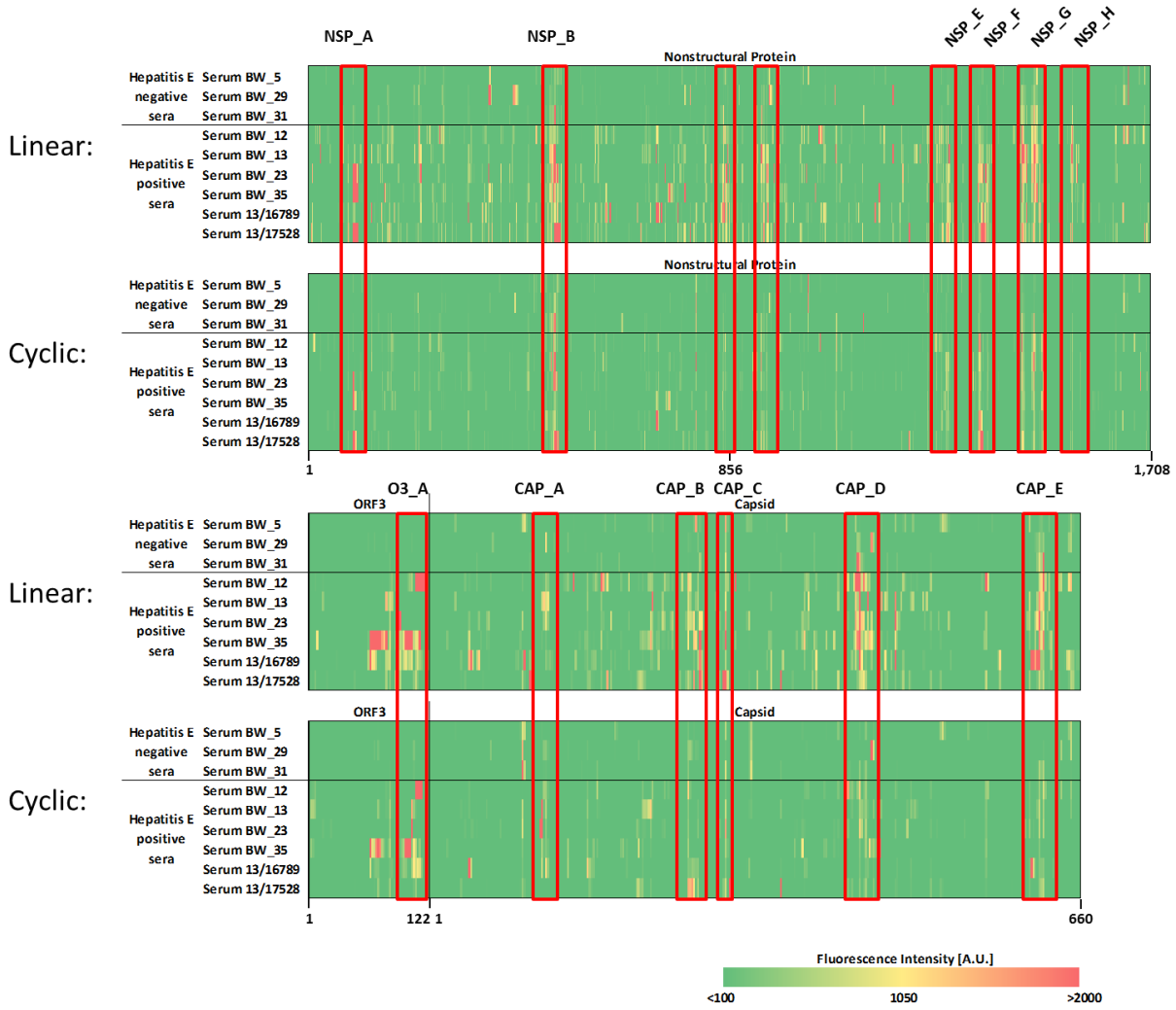

Fig. 3: Heatmaps of serum stainings with HEV IgG-positive and -negative sera. Linear and cyclic peptides arrays were stained at a dilution of 1:200. Intensity values were quantile normalized for each cohort separately. Fluorescence intensity values > 2000 A.U. are shown in red, while values below 100 are displayed in green. Red boxes indicate identified immunogenic areas. Amino acid positions of HEV proteins are indicated.

As immunogenic region O3_A showed the highest discriminatory power between HEV IgG-positive and -negative individuals, this region was analyzed in more detail. Interestingly, two patients (BW_13 and BW_23) reacted only with cyclic peptides but not with linear ones (Figure 4A), indicating that some patients exclusively recognize a conformational epitope at this position. Also epitope core motifs of individual patients are located at different sites within region O3_A. One patient (BW_12) recognized a distinct epitope located towards the C-terminus of O3_A, while others, e.g. patient BW_13, BW_23 and BW_35 reacted with peptides of the N-terminal part of the O3_A region (Figure 4A). Serum 13/6789 exhibited a more disseminated reactivity pattern with linear and cyclic peptides harboring a medium to low signal intensity distributed over the O3_A region. In contrast, for serum 13/17528 we observed only a very weak reactivity for linear and cyclic peptides. These differences can either be attributed to the different HLA-types of patients leading to the recognition of different epitopes.

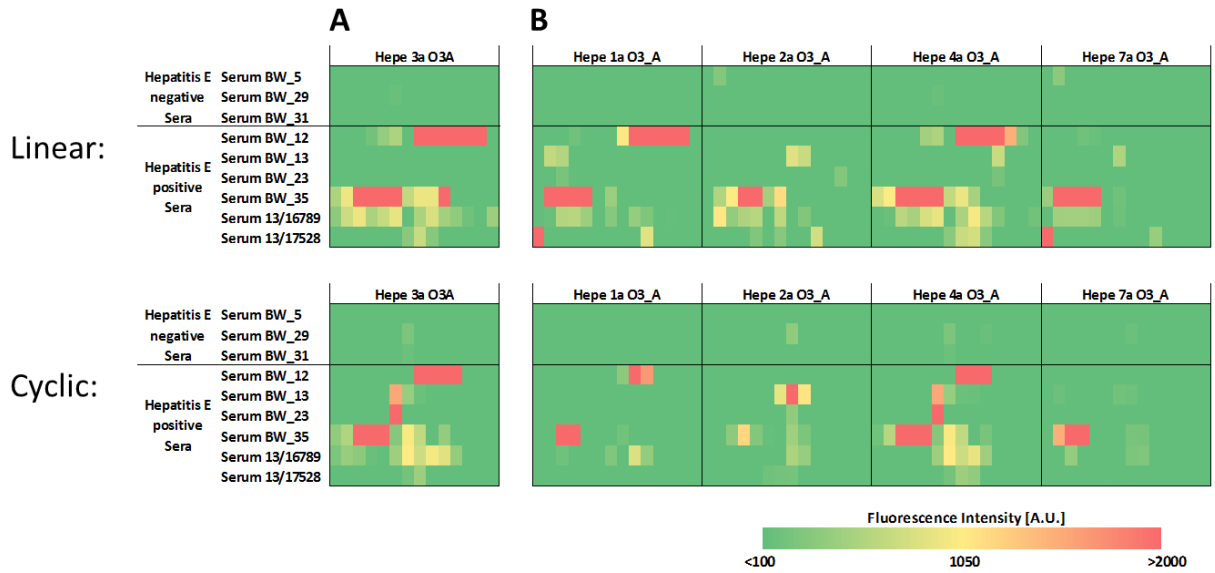

Fig. 4: Heatmap of reactivities against the C-terminal immunogenic region of ORF3 of HEV. Linear and cyclic peptides arrays were stained at a dilution of 1:200. Intensity values were quantile normalized for each cohort separately. Fluorescence intensity values > 2000 A.U. are shown in red, while values below 100 are displayed in green.

Alternatively, they might be the result of infection with different subgenotypes of HEV, which harbor their own antigenic structure. Nevertheless, these findings are a good explanation for the low performance of peptide-based serological assays in the past, which used only single peptides as antigens. Furthermore, we investigated whether peptides derived from other human pathogenic HEV genotypes, i.e. genotype 1a, 2a, 4a and 7a, are also recognized by sera from gt 3a infected patients. While HEV IgG-negative patients did not show any increased reactivity with peptides from heterologous genotypes, the immune response of some HEV patient sera depended strongly on the genotype. E.g. patient BW_12 responded strongly to peptides from genotype 1a, 3a and 4a, but not to peptides from genotype 2a and 7a. Conversely, patients BW_13 and BW_23 reacted with peptides from gt 2a, 3a and 4a, but not with peptides from gt 1a and 7a (Figure 4A and B). Other patients (BW_35 and 13/16789) reacted with peptides from all HEV genotypes. These findings hint towards substantial differences in the immune response of patients against antigens from heterologous genotypes. These findings also explain the

variances observed while using different commercial serological assays. Our data clearly shows that it is of utmost importance to include peptide variants covering all human pathogenic genotypes for the development of new serological assays with higher sensitivity.

Conclusion

Serological diagnostics of HEV virus infections remain challenging due to high genetic variability of the virus and substantial differences between commercially available serological assays. In this application note we describe a new comprehensive approach using our proprietary peptide microarray technology to fine map the immune response of individual patients against proteomes of all human pathogenic HEV genotypes. The heterogeneous immune response we found in HEV patients probably reflects the high genetic variability of HEV and the differences in HLA types of patients. Nevertheless, we identified common immunogenic regions in patients that allowed for a clear discrimination between seropositive and seronegative individuals. In combination with new multiplexed serological assays this could lead to the development of an improved serological assay for HEV that takes the genetic variability of the virus into account.

Bibliography

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