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Short communication

Evaluation of core and NS4B synthetic peptide-based immunoassays for the detection of hepatitis C virus antibodies in clinical samples from Cameroon, Central Africa

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ABSTRACT

Background: According to previous data, the antibodies produced during natural hepatitis C virus (HCV) infection frequently recognize amino acids 10–43 in the core protein and 1689–1740 or 1921–1940 in the non-structural 4B (NS4B) protein. The reactivity of these peptides with the corresponding antibodies has mainly been evaluated using serum samples from Western countries where HCV genotype 1 (HCV-1) is predominant, and no information is available concerning samples from sub-Saharan countries where high HCV variability has been reported.
Objective of this study: To evaluate the performance of HCV core and NS4B peptide-based immunoassays in the serodiagnosis of HCV infection in Cameroon subjects.
Study design: Three core and four NS4B-based synthetic peptides derived from HCV genotypes 1b and 2a were designed and tested against a panel of 151 serum samples from Cameroon (40 positive for HCV-1, 32 for HCV-2, 39 HCV-4, and 40 HCV-negative).
Results: The three core peptides all demonstrated strong immunoreactivity, regardless of the HCV genotype from which they were derived, with greater than 90% and 92% sensitivity and specificity. In contrast, the NS4B-derived peptides exhibited lower sensitivities (24.3–65.8% depending on the HCV genotype) but higher specificities (100% for all four peptides tested).
Conclusions: Our findings indicate that an HCV core peptide could be used for the diagnosis of chronic HCV infection. Among the NS4B peptides tested, a chimeric NS4B peptide encompassing both N- and C-terminal portions of the NS4B protein gave a much better performance than the two component N- and C-terminal peptides used individually.

1. Background

Currently, the identification of HCV-infected individuals relies on the detection of antibodies directed against virus-encoded proteins, and of the presence of viral genomic RNA in serum. Serological tests for the detection of HCV antibodies use recombinant antigens or synthetic peptides. It is noteworthy that several reports have suggested that despite significant improvements of serological screening assays, HCV genome variability may play a major role in the control of HCV infection in countries outside Europe and the United States.[1] Indeed, the presence of different HCV genotypes and/or subtypes appears to alter the effectiveness of current serological diagnostic tests. These tests are based upon HCV type 1 antigens that prevail in Europe and Northern America. However, a number of studies suggest that current commercially available serological assays are sub-optimal for screening populations in which the predominant HCV genotype is not type 1.[2] Importantly, these studies include HCV types 1–3 and a few samples of HCV type 4. This raises serious concerns about blood safety, diagnosis and screening procedures for HCV.[2–5] Therefore, commercially available kits, although validated in Europe and in the USA, may have serious limitations when used in other parts of the world, including

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Table 1
Amino acid sequences of HCV core and NS4B peptides.

Name	Peptide position[a]	Amino acid sequence[b]
HCV-21	Core 3–75/1b	TNPKPQRKTKRNTNRRPQDVKFPGGGQIVGGVYLL PRRGPRLGVRATRKTSERSQPRGRRQPIPKARRPEGRT
HCV-26[c]	Core 11–45/1b	TKRNTNRRPQDVKFPGGGQIVGGVYLLPRRGPRLG
HCV-34[c]	Core 11–45/2a	CTKRNTNRRPQDVKFPGGGQIVGGVYLLPRRGPRLG
HCV-45	NS4 1689–1738/1b	SGRPAVIPDREVLYQEFDEMEEAASHLPYIEQGMQLAEQF KQKALGLLQT
HCV-452	NS4 1693–1742/2a	NQRAVVAPDKEVLYEAFDEMEEAASRAALIEEGQR IAEMLKSKIQGLLQQ
HCV-6	NS4 1921–1940/1b	AFASRGNHVSPTHYVPESDA
HCV-645	NS4 1689–1738/1b + 1921–1940/1b	SGRPAVIPDREVLYQEFDEMEEAASHLPYIEQGMQLAEQFKQ KALGLLQTAFASRGNHVSPTHYVPESDA

[a] Numbers represent the amino acid positions according to the subtype 1b isolate BK (GenBank Accession number M58335) and subtype 2a isolate HC-J6 (GenBank Accession number D00944).
[b] Amino acids are represented by the single-letter code.
[c] The core-specific peptides HCV-26 and the HCV-34 have no differences in this region between 1b and 2a subtypes. The only difference between HCV-26 and HCV-34 is a cysteine residue attached to C-terminus of HCV-34. It leads to dimerization of the peptides during solubilization and coating, and determines a slight difference in immunoreactivity of these antigens.

Cameroon where a high HCV genetic diversity has been reported, and a circulation of different HCV subtypes from those described for developed countries.[6] Furthermore, commercial anti-HCV screening tests are not readily accessible to poor countries because of their cost. According to previous data, HCV core and NS4B proteins are frequent antibody targets in infected subjects.[7]

2. Objectives

The objectives of the present study were to evaluate the performance of HCV core and NS4B peptide-based immunoassays in the serodiagnosis of HCV infection in Cameroon subjects, where type 4 HCV is highly prevalent.

3. Study design

3.1. Synthetic peptides and enzyme immunoassay

Synthetic peptides were obtained by solid phase synthesis using F-MOC-protected amino acids according to the method of Merrifield, as modified by Atherton et al.[8] Seven synthetic peptides (three core-specific and four NS4B-specific) derived from genotype 1b and 2a HCV sequences were designed. Details of these peptides are presented in Table 1.

Seven different enzyme immunoassays (EIA) were developed for the detection of antibodies, using one synthetic peptide at a time (see figure legend of Fig. 1 for details).

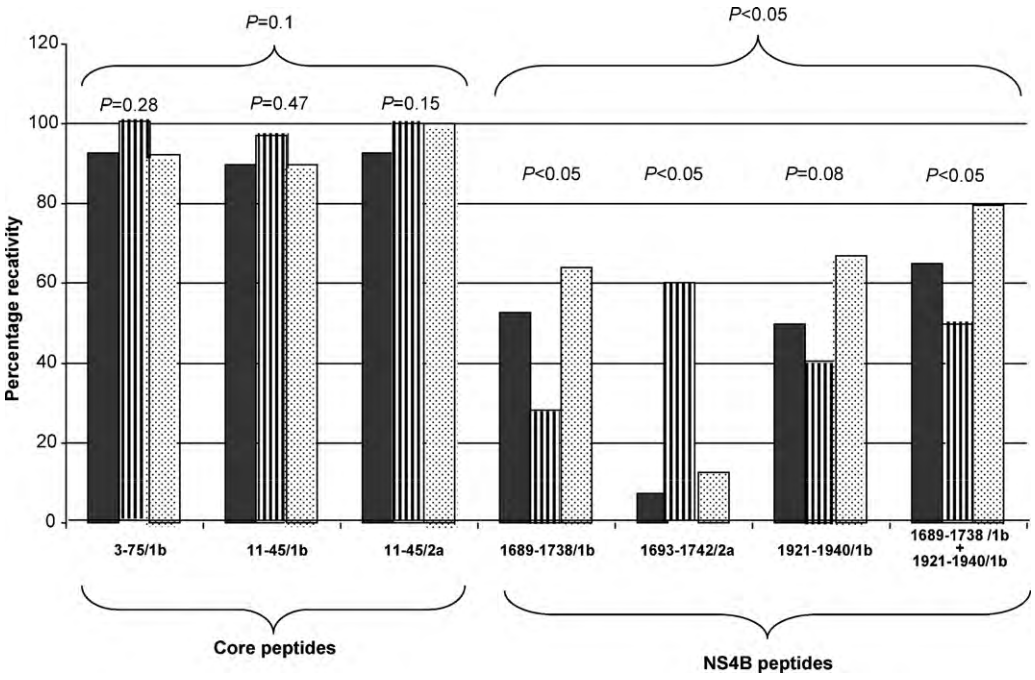

Fig. 1. Reactivity of HCV core and NS4B peptides with the 111 HCV-positive serum samples from Cameroon patients. Peptides are designated by their amino acid position (numbers) and the genotype from which they were derived (genotype 1, black bars; genotype 2, vertical lines; genotype 4, stippled bars). The enzyme immunoassays were performed as followed. The wells of polystyrene microtiter plates (Nunc, Denmark) were coated overnight at 4 °C with 100 μl of the relevant peptide at a concentration of 1 μg/ml in phosphate buffer saline (PBS). Then the wells were washed with PBS containing 0.05% Tween-20 (PBS/T), to remove the excess of the unbound peptides. After blocking for 2 h at 37 °C with 3% bovine serum albumin (BSA) and 0.1% Tween-20 in PBS, the plates were washed with PBS/T. One hundred microliter of serum diluted to 1/200 in diluting buffer (3% BSA, 0.1% Tween-20, and 10% calf serum in PBS) was added to each well and plates were incubated at 37 °C for 2 h. Plates were washed with PBS/T, and 100 μl of horseradish peroxidase-labeled goat anti-human IgG (Sigma®) in the diluting buffer was added to each well. Plates were then incubated for 1 h at 37 °C and washed with PBS/T. Then, 100 μl of ortho-phenylenediamine (Sigma®) was added, and plates were incubated for 30 min at room temperature. The reaction was stopped by the addition of 100 μl of 1 N sulfuric acid. The absorbance was measured at 492 nm in a Biorad 96-well plate reader.

Sera from eight healthy subjects who were negative for anti-HCV, anti-HIV and hepatitis B surface antigen (HBsAg) antibodies, as assessed by commercial tests, were used as negative controls. The cut-off value for each test was calculated as the mean absorbance of the eight anti-HCV negative sera that were included in each plate. A serum was considered to be positive for anti-HCV by EIA if its absorbance value was at least 2.1-fold greater than the cut-off value.

3.2. Serum specimens

A set of well-characterized serum specimens obtained from patients attending the Cameroon Pasteur Centre for the diagnosis of HCV infection was used for this study. Anti-HCV antibodies and HCV RNA were determined using a commercial third-generation enzyme immunoassay (AxSYM® HCV version 3, Abbott Laboratories, Rungis, France) and the Cobas Amplicor™ HCV Version 2.0 (Roche Diagnostics Systems, Meylan, France), respectively. HCV genotypes were determined by sequencing and phylogenetic analysis of the NS5b gene as was previously described.[6]

A total of 151 serum specimens were selected for this study. These included 111 samples positive for both anti-HCV antibody and HCV RNA (40 of these were genotype 1, 32 were genotype 2, and 39 were genotype 4), and 40 samples that were negative for anti-HCV antibody. The samples were all negative for anti-HIV and HBs Ag.

3.3. Statistical analysis

Statistical analysis was carried out using the Epi-info version 6 statistical program (Centers for Disease Control and Prevention, Atlanta, GA). Sensitivities and specificities of the different peptide-based EIA were calculated using the commercial third-generation enzyme immunoassay as the gold-standard test. To examine the reproducibility of the different peptide-based EIA, samples were tested in triplicate in each experiment and analyzed twice using distinct EIA plates coated with the corresponding peptide. Mean values of absorbance in each experiment and each test were compared, and correlation coefficient values (r) were determined. Comparisons of reactivity rates were determined for different genotypes using a specific peptide (internal p-values) and between different peptides (external p-values), using the Fisher's exact test. p-Values of 0.05 or lower were considered to be significant.

4. Results

Fig. 1 shows the HCV core and NS4B peptide antibody reactivity rates for the 111 HCV-positive specimens. The three core peptides all demonstrated strong immunoreactivity, regardless of the HCV genotype from which they were derived, with greater than 90% sensitivity ($p > 0.05$). This is in accordance with the high sequence homology for HCV core protein of various genotypes. In contrast, the NS4B-derived peptides exhibited lower sensitivities (24.3–65.8% depending on the HCV genotype). Notably, a chimeric NS4B peptide encompassing both N- and C-terminal portions of the NS4B protein gave a much better performance than the two component N- and C-terminal peptides used individually.

The 40 anti-HCV negative samples were also negative with the NS4B HCV-45, -452, -6, and -645 peptide EIA, giving a specificity of 100.0%. The specificity was of 92.5%, 97.5%, and 95.0% for core HCV-21, -26, and -34 peptide EIA, respectively.

The mean absorbance values in the 111 HCV-positive and the 40 HCV-negative serum specimens in intra and inter-assays for all seven peptide-based assays were not statistically different (data not shown). The correlation coefficient (r) values were >0.8 in either group, thus confirming the reproducibility of these assays.

5. Discussion

In a recent report Muerhoff et al.[9] examined a set of 13 genotype 1a-derived core peptides and found that the peptide corresponding to amino acids 10–43 reacted with all of the 254 serum specimens tested, independently of the HCV genotype. This peptide was thus identified as a highly immunogenic region in the HCV core protein, as compared to the C-terminal part of core that exhibited low reactivity. This report is comparable to our results and those of several studies[10,11] reporting that synthetic core peptide-based immunoassay could be used as an alternative test in the diagnosis of HCV infection.

The NS4B has previously been reported to be the most immunogenic among HCV non-structural proteins.[12] Our results indicate that the chimeric NS4B peptide that contains both the N- and C-terminal fragments of the NS4B protein could improve the performance of NS4B-based ELISA. In our study, there was no difference in the reactivity of the N-terminal NS4B peptide with serum samples of genotype 1 and genotype 4, although this part of the genome shows extreme genotypic variability, up to 60%, and can be used for the serological determination of HCV genotypes. The peptide aa1693–1742/2a was poorly recognized by antibodies from sera of patients infected with genotypes 1 and 4. Our results are consistent with those reported by Chang et al.[13] indicating that the aa region called epitope 5-1-1 (corresponding to an NS4B aa1689–1738), is conserved for genotypes 1 and 4. We also found, as previously reported,[14] that an NS4B aa1921–1940 peptide could contain genotype-independent antigenic epitopes.

In conclusion, the above findings suggest that N-terminal-core peptides could be excellent candidate products for developing an alternative test for the diagnosis of HCV infection. Our study also indicates that HCV genetic variability affects the immunoreactivity of N-terminal NS4B peptides, but not that of C-terminal NS4B peptides. However, HCV-4 and HCV-1 serum samples exhibit similar reactivity with the N-terminal NS4B peptides. Among the NS4B peptides tested, the chimeric NS4B peptide encompassing both the N- and C-terminal parts of the NS4B protein had a much better performance than the same peptides used individually.

Conflict of interest

The authors declare that they have no competing interests.

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References

1. Kanistanon D, Neelamek M, Dharakul T, Songsivilai S. The immunoreactivity profile of different HCV genotypes on immunoblot assay and its implications in the development of diagnostic assays. *Asian Pac J Allergy Immunol* 1997;**15**:133–40.
2. Neville JA, Prescott LE, Bhattacherjee V, Adams N, Pike I, Rodgers B, et al. Antigenic variation of core, NS3, and NS5 proteins among genotypes of hepatitis C virus. *J Clin Microbiol* 1997;**35**:3062–70.
3. Dow BC, Munro H, Buchanan I, Follett EA, Davidson F, Yap PL, et al. Third-generation recombinant immunoblot assay: comparison of reactivities according to hepatitis C virus genotype. *Transfusion* 1996;**36**:547–51.
4. Halfon P, Bourliere M, Khiri H, Halimi G, Gerolami V, Feryn JM, et al. Serological response to infection with different isolates of hepatitis C virus. *J Viral Hepat* 2002;**9**:438–42.

5. Rodriguez-Lopez M, Riezu-Boj JI, Ruiz M, Berasain C, Civeira MP, Prieto J, et al. Immunogenicity of variable regions of hepatitis C virus proteins: selection and modification of peptide epitopes to assess hepatitis C virus genotypes by ELISA. *J Gen Virol* 1999;**80**(Pt 3):727–38.
6. Pasquier C, Njouom R, Ayouba A, Dubois M, Sartre MT, Vessiere A, et al. Distribution and heterogeneity of hepatitis C genotypes in hepatitis patients in Cameroon. *J Med Virol* 2005;**77**:390–8.
7. Beld M, Penning M, van Putten M, Lukashov V, van den Hoek A, McMorrow M, et al. Quantitative antibody responses to structural (core) and nonstructural (NS3, NS4, and NS5) hepatitis C virus proteins among seroconverting injecting drug users: impact of epitope variation and relationship to detection of HCV RNA in blood. *Hepatology* 1999;**29**:1288–98.
8. Atherton E, Logan C, Sheppard R. Peptide synthesis. Part 2. *Bioorg Chem* 1979;**8**:351–70.
9. Muerhoff AS, Gutierrez R, Kyrk C, Leary T, Schlauder G, Dawson G, et al. Genotype dependence of peptide-based immunoassays for the detection of HCV core antibodies. *J Med Virol* 2008;**80**:411–8.
10. El Awady MK, El-Demellawy MA, Khalil SB, Galal D, Goueli SA. Synthetic peptide-based immunoassay as a supplemental test for HCV infection. *Clin Chim Acta* 2002;**325**:39–46.
11. Ishida C, Matsumoto K, Fukada K, Matsushita K, Shiraki H, Maeda Y. Detection of antibodies to hepatitis C virus (HCV) structural proteins in anti-HCV-positive sera by an enzyme-linked immunosorbent assay using synthetic peptides as antigens. *J Clin Microbiol* 1993;**31**:936–40.
12. Khudyakov Yu E, Khudyakova NS, Jue DL, Lambert SB, Fang S, Fields HA. Linear B-cell epitopes of the NS3–NS4–NS5 proteins of the hepatitis C virus as modeled with synthetic peptides. *Virology* 1995;**206**:666–72.
13. Chang JC, Seidel C, Ofenloch B, Jue DL, Fields HA, Khudyakov YE. Antigenic heterogeneity of the hepatitis C virus NS4 protein as modeled with synthetic peptides. *Virology* 1999;**257**:177–90.
14. Longombardo G, Ferri C, Marchi S, Costa F, Lombardini F, Vacri L, et al. Immune response to an epitope of the NS4 protein of hepatitis C virus in HCV-related disorders. *Clin Immunol Immunopathol* 1998;**87**:124–9.