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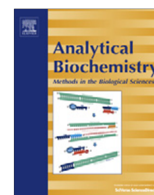

The use of epitope arrays in immunodiagnosis of infectious disease: Hepatitis C virus, a case study

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ABSTRACT

Serodiagnosis of infectious disease is often based on the detection of pathogen-specific antibodies in a patient's blood. For this, mixtures of pathogen-related antigens are used as bait to capture corresponding antibodies in solid phase immunoassays such as enzyme immunoassay (EIA). Western blots provide improved diagnostic power as compared with EIA due to the fact that the mixture of markers in the EIA well is resolved and tested as individual antigens on the Western blot. Hence, confirmation of EIA results is accomplished using the antigen arrays of Western blots. Here we took this approach one step further and tested the attributes of using epitope arrays in a diagnostic platform coined "combinatorial diagnostics." As a case in point, we tested a panel of phage-displayed epitope-based markers in the serodiagnosis of hepatitis C virus (HCV). The repertoire of HCV antigens was deconvoluted into panels of distinct linear and conformational epitopes and tested individually by quantitative EIA. Combinatorial diagnostics proved to be effective for the discrimination between positive and negative sera as well as serotyping of HCV.

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Conventional serodiagnosis of infectious disease is often accomplished by conducting solid phase immunoassays (e.g., enzyme immunoassay [EIA][2] tests) using mixtures of the pathogen's antigens pooled in a single spot (the "capture probe"), which is tested for binding of serum antibodies (the "analytes") [1,2]. In the event of a positive signal, validation might be required, as is often the case in viral infections. This can be achieved by increasing the resolution of the analysis using a Western blot [3–7]. The rationale behind this is that there is more confidence in the accumulation of several moderate but distinct signals (e.g., binding to *multiple viral antigens*) as opposed to a single strong signal derived from only one antigen. This widely used approach demonstrates that detection of a diversity of antibody specificities has greater diagnostic power than a strong but solitary signal. Hence, the gain in diagnostic power in the transition from EIA to Western blots is realized due to the ability to ascribe the compound signal from the EIA well to a diversity of distinct bands in the Western blot. Western blots are in a sense "antigen arrays." Here we propose that the ultimate diagnostic power could be realized when "specificity profiles" are generated using the smallest possible antibody binding unit (i.e., single epitopes), where "epitope arrays" (or arrays displaying short domains containing only a few epitopes) could be employed to interrogate serum samples—the essence being, however, that each epitope constitutes a diagnostic marker and is to be tested individually.

The ideal diagnostic marker would be one that is 100% specific (i.e., never falsely recognized) and 100% sensitive (i.e., never falsely missed—always scored in truly positive samples). Realistically, no such markers exist, and false positives/negatives always generate uncertainty to some degree. In view of this, we propose the concept of "combinatorial diagnostics," where serum is tested against epitope arrays and each epitope is scored individually for antibody binding. However, the discrimination between infected and noninfected individuals is made not based on *which* marker is positive but rather based on the *total number* of markers scored.

Here we tested this concept as an application for the serodiagnosis of the human hepatitis C virus (HCV). Human HCV is a member of the Flaviviridae family and constitutes a major health problem worldwide. To date, it is estimated that more than 170 million individuals are infected with this virus [8], which leads to liver cirrhosis in 10% to 15% of those infected, some of whom further progress to hepatocarcinoma and death. HCV contains a 9.6-kb positive strand RNA genome composed of a 5′ noncoding region (NCR) and a 3′ NCR that flank a long open reading frame encoding a

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[2] *Abbreviations used:* EIA, enzyme immunoassay; HCV, hepatitis C virus; NCR, noncoding region; TBS, Tris-buffered saline; PEG, polyethylene glycol; PCR, polymerase chain reaction; cDNA, complementary DNA.

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Table 1
Details of HCV antigens.

Antigen	Core	El	E2	P7	NS2	NS3	NS4A	NS4B	NS5A	NS5B
Position	1–191	192–383	384–746	747–808	809–1026	1027–1657	1658–1710	1711–1972	1973–2420	2421–3012
Number of amino acids	191	192	363	62	218	631	53	262	448	592

Note. The polyprotein of HCV is processed into four structural proteins and six nonstructural (NS) enzymes.

polyprotein precursor of approximately 3000 amino acids. The polyprotein is posttranslationally processed into 10 distinct viral antigens shown in Table 1 [9–12] from which the panel of markers is derived.

Materials and methods

Polyclonal sera

Polyclonal sera/plasma samples were purchased from two sources: the Israeli National Blood Bank (Magen David Adom, Tel Hashomer, Israel) and SeraCare Life Sciences (Milford, MA, USA, cat. no. PHV206—18 confirmed positive sera; the number used in a given experiment is indicated in the text). An additional panel of HCV positive sera was obtained from the Rabin Medical Center collected under informed consent and approved by the institutional review board. Samples were typically used at dilutions 1:65 in Tris-buffered saline (TBS, pH 7.5) containing 4% (w/v) skim milk plus 20% bovine serum (Beit Haemek, Israel). All samples were stored at −20 °C.

Epitope construction and expression

Throughout this study, all of the epitopes were generated and expressed as filamentous bacteriophage fusion proteins. All of the epitopes (except for the NS3 epitopes; see below) were expressed as protein 8 fusions using the phage system based on the fth1 88 expression vector [13], and generally construction, expression, and analyses were performed as described previously [14]. Briefly, 5′ phosphorylated oligonucleotides corresponding to epitope peptides (<25 amino acids) were constructed (sense and antisense) such that on annealing overhangs corresponding to the 5′ and 3′ *Sfi*1 sites were produced. The oligonucleotide inserts were incubated with precut vector (5:1) and ligated at 16 °C overnight. The ligation mix was used to transform DH5αF⁻ competent bacteria, and clones were isolated and confirmed for correct sequence by standard Sanger sequencing. Bacteria were cultured (200 ml shaking at 225 rpm, 37 °C, overnight), and phages were collected from the medium by precipitation using polyethelyne glycol (PEG)–NaCl [14]. Phages were resuspended in TBS and stored at 4 °C until use. In the case of NS3-based epitopes, inserts corresponding to 120 to 160 amino acids were produced by polymerase chain reaction (PCR) using primers with compatible *Bst*X1 restriction sites at their 5′ and 3′ ends corresponding to the modified fth1 vector developed for expression of protein 3 fusions [15] and using as a template full-length viral complementary DNA (cDNA, Brechot HCV 1b cDNA plasmid [Neo^R, Amp^R] UniProtKB/Swiss–Prot accession no. P26663). The inserts were confirmed for sequence correctness by standard Sanger sequencing.

Solid phase immunoassay

The antigenicity of the phage-expressed HCV epitopes was measured as follows: EIA/RIA (radioimmunoassay) 8-well strip flat-bottom EIA wells (Costar, Corning, Corning, NY, USA) were coated overnight with a volume of 100 μl TBS containing Y2D (a murine monoclonal antibody specific for fd filamentous bacteriophage protein 8 produced at Tel Aviv University, 5 μg/ml). The wells were then washed twice with TBS and blocked for 1 h with blocking solution (TBS plus 4% [w/v] skim milk with 20% bovine serum [Beit Haemek, Israel]) and incubated for 2 h with 100 μl of blocker containing 10^{10} or 4×10^{10} phages (protein 8 or protein 3 fusions, respectively). Wells were washed five times with TBS and then reacted with 100-μl serum samples diluted 1:65 in blocker for 2 h of incubation. Wells were washed five times with TBS and then reacted for 45 min with 100 μl of 0.2 μg/ml horseradish peroxidase-conjugated donkey anti-human antibody (Jackson ImmunoResearch, West Grove, PA, USA) diluted in blocker

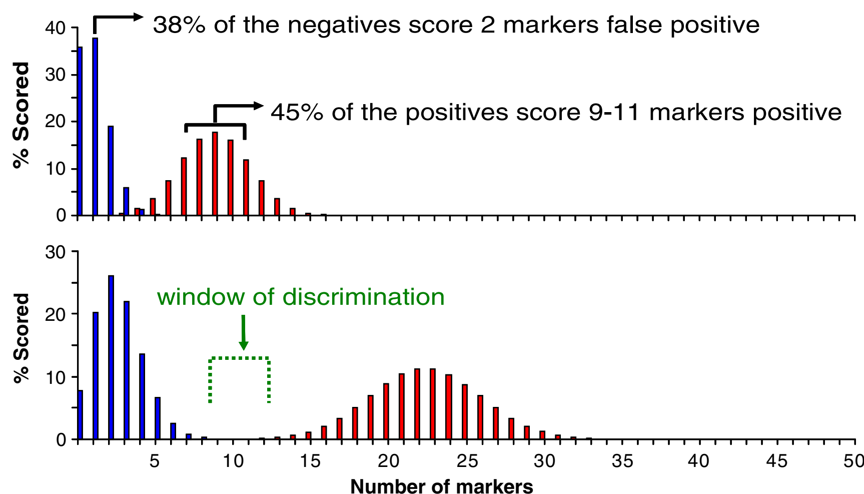

Fig. 1. Model of combinatorial diagnostics. Two binomial distributions are presented for two hypothetical populations: bona fide negative individuals (blue) and genuine positives for a given disease (red). All markers are of equal quality (sensitivity = 45%, specificity = 95%). Note that when 20 different markers are tested (top), 38% of true negatives are expected to score 2 markers falsely. Nearly half (45%) of the true positives bind 9 to 11 markers. Increasing the number of markers to 50 (bottom) creates a window of discrimination where the two populations can be easily separated. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

solution. Finally, the wells were washed five times with TBS and reacted with 100 µl of TMB/E EIA substrate (Chemicon International, Temecula, CA, USA). Absorbance was measured at 650 nm after 10 min using a BioTek ELx808 microplate reader.

HCV genotyping

HCV RNA positive samples were subjected to genotyping based on 5′ noncoding sequences of the HCV genome using VERSANT HCV Genotype Assay (LiPA) 1.0 according to the manufacturer's instructions (Bayer HealthCare, Tarrytown, NY, USA).

Results

Mathematical model

The basic premise for the concept of combinatorial diagnostics is that it is recognized that no diagnostic marker is truly ideal (i.e., 100% specific and 100% sensitive); however, marker mediocrity can be compensated for by marker multiplicity (i.e., testing a panel of multiple markers and scoring each individually). The discrimination between infected and noninfected individuals is achieved based on the number of positive hits scored.

A simple mathematical model illustrates the power of combinatorial diagnostics. In Fig. 1, panels of theoretical markers of defined sensitivity and specificity are used to calculate the probability of being scored by truly negative and positive sera. Two binomial distributions are obtained whose overlap (or lack thereof) signifies the ability to discriminate between the two populations (genuine positives and genuine negatives). The binomial distribution of true positives is derived from the following equation:

$$P\{X = k\} = \binom{N}{k} p_s^k (1 - p_s)^{N-k}$$

where k is the number of hits, N is the number of markers that are used, and the sensitivity of each marker p_s is 0.45 in the example shown in Fig. 1 and leads to the distribution (red) for the genuine positive sera samples. The blue distribution is for the chance of false positives, where p_s is then 1-specificity (0.05 in the specific example). Even in this case of poor markers (sensitivity = 45%, specificity = 95%), one begins to efficiently separate between true positives and negatives as one increases N, the *number* of markers in the panel tested. Because the average of a binomial distribution

Table 2
Potential diagnostic markers reported in the literature.

Number	Antigen	Amino acid	Sequence	Peptide	Antibody
1	Core	1-18	MSTNPKPQRKTKRNTNRR	Syn	Serum (50)
2	Core	5-20	CKPQRKTKRNTNRRPQY	Syn	Serum (71)
3	Core	7-17	RKTKRNTN	Syn	Serum (34)
4	Core	7-21	QKKNKRNTNRRPQDV	Syn	Serum (45)
5	Core	9-16	RKTKRNTN	Syn	Serum (49)
6	Core	10-17	KNKRNTNR	Phage	Serum (33)
7	Core	11-28	TKRNTNRRPQDVKFPGGQ	Syn	Serum (50)
8	Core	19-26	PQDVKFPG	Phage	Serum (5)
9	Core	19-28	PQDVKFPGGG	Phage	Serum (33)
10	Core	20-24	QDVKF	Syn	mAb
11	Core	20-32	QDVKFPGGGQIVG	Phage	Serum (48)
12	Core	21-44	CDVKFPGG QIVGGVYLLPRRGPRLG	Syn	Serum (71)
13	Core	21-44	DVKFPGG QIVGGVYLLPRRGPRLG	Syn	Serum (41)
14	Core	29-33	QIVGG	Syn	mAb
15	Core	29-37	QIVGGVYLL	Syn	mAb
16	Core	31-45	GGVYLLPRRGPRLGV	Syn	Serum (45)
17	Core	34-39	VYLLPR	Syn	Serum (34)
18	Core	34-49	VYLLPRRGPRLGVRAT	Phage	Serum (5)
19	Core	50-63	RKTSERSQPRGRRQP	Syn	Serum (45)
20	Core	51-68	KTSERSQPRGRRQPIPKA	Syn	Serum (50)
21	Core	58-65	PRGRRQPI	Syn	mAb
22	Core	73-83	GRTWAQPGYPW	Phage	Serum (5)
23	Core	73-86	GRTWAQPGYPWPLY	Syn	Serum (34)
24	Core	99-113	PRGSRPSWGPTDPRR	Syn	Serum (45)
25	E1	319-324	RMAWDM	Syn	Serum (40)
26	NS3	1383-1415	EAIKGGRHLIFCHSKKKCDELAAKLTGLGLNAV	Phage	Serum (35)
27	NS4A	1681-1693	VLVGRIILSGRPA	Phage	Serum (5)
28	NS4A	1691-1710	KPAIIPDREVLYREFDEMEE	Syn	Serum (20)
29	NS4A	1698-1710	REVLYQEFDEMEE	Phage	Serum (33)
30	NS4B	1712-1718	AS HLPYI	Phage	Serum (5)
31	NS4B	1711-1730	CSQHCPYIEQGMMLAEQFKQ	Syn	Serum (20)
32	NS4B	1718-1723	IEQGMM	Phage	Serum (33)
33	NS4B	1726-1736	EQFKQKAFGLL	Phage	Serum (5)
34	NS4B	1921-1940	AFASRGNHVSPTHYVPESDA	Syn	Serum (20)
35	NS4B	1921-1940	CAFASRGNHVSPTHYVPESDA	Syn	Serum (71)
36	NS4B	1930-1938	SPTHYVPES	Phage	Serum (35)
37	NS4B	1931-1938	PTHYVPES	Phage	Serum (48)
38	NS5A	2088-2104	EEYVEVTRVGDFHYVTGM	Phage	Serum (35)
39	NS5A	2251-2260	VILDSFDPIR	Phage	Serum (5)
40	NS5A	2295-2313	PPLLESWKDPDYVPPVVHG	Syn	Serum (20)
41	NS5A	2295-2313	CPPLLESWKDPDYVPPVVHG	Syn	Serum (71)
42	NS5A	2301-2309	WKKPDYEPP	Phage	Serum (33)

Note. A total of 42 specific peptides were reported in the literature as having diagnostic potential. Of these, 13 (highlighted) were tested as phage-displayed peptides in EIA tests (see text). The numbers in parentheses represent the numbers of sera samples tested.

is proportional to *N*, as *N* increases, the distance between the averages of the two populations increases and the separation (window of discrimination) is improved. Obviously, the size of the panel can be reduced as one improves the quality of markers to be tested.

HCV: A case in point

To test the concept of combinatorial diagnostics, it was decided to develop an epitope array of markers representing the antigens of HCV. The first task was to identify an effective panel of HCV diagnostic markers. For this, the published literature pertaining to the B-cell response toward HCV during infection was perused [16–27]. As illustrated in Table 2, there have been numerous reports describing the diagnostic potential of specific HCV-derived peptides. A diversity of 42 synthetic or recombinant short linear peptides spanning 6 antigens is depicted. The core provides more than half of the peptides tested, and many of these are concentrated in the first 100 amino acids of this antigen. In addition, typical of the humoral response toward this virus are antibodies directed against the NS3, NS4, and NS5 enzymes. When the published epitopes are critically scrutinized, one readily sees that many of the peptides overlap to various degrees. Thus, for example, the first 10 epitopes in Table 2 are fairly well represented by peptides 1 and 9 (highlighted in the table). Thus, we identified a total of 13 representative peptides as our first initial panel of markers to be tested.

Evaluation of the HCV markers and production of an epitope array

The 13 potential epitopes were individually constructed for phage display. These short peptides (<25 amino acids) were produced using corresponding synthetic oligonucleotides cloned into the *Sfi*1 sites of the fth1 phage display vector to create recombinant protein 8 fusions [13,28]. Once the potential markers were cloned and confirmed for the correctness of sequence, the phages were grown and harvested and used to screen HCV negative sera. In this manner, markers with low specificity (i.e., those that bound more than 2 of the 16 negative sera tested) could be eliminated from the start. The remaining markers were then screened against 12 serum samples from the commercial standard serum samples of confirmed HCV positives (SeraCare Life Sciences, cat. nos. PHV206 2, 4, 7, 13–16, 18–21, and 24) and 2 additional samples from the Israeli National Blood Bank (HCV1 and HCV4). Of the peptide markers, 7 scored well and thus formed the nucleus of the initial epitope array. It was now necessary to expand the panel and test its efficacy for HCV diagnosis.

Expansion of the panel was conducted by first constructing and identifying new markers for the NS3, NS4, and NS5 antigens. In addition, because all of the markers so far were derived from the HCV genotype 1 sequences, 6 more markers were designed and constructed to correspond to the sequences for genotypes 2 and 3 for a total of 3 specific epitopes (markers 8–10: NS4B 1712–1723/6; markers 11–13: NS4B 1722–1736; and markers 15–17: NS5A 2295–2313; see Tables 3–5). Furthermore, in view of the fact that the epitopes of the NS3 antigen are known to be particularly conformational [22,29–31], two long peptide domains (1175–1334 and 1315–1452) were constructed using the HCV subtype 1b genome as a template and generating PCR segments of the desired sequences flanked by *Bst*X1 sites compatible for protein 3 expression in a modified fth1 vector [15]. Of the two candidate peptides, the 160-amino acid segment (1175–1334) did not react with negative sera and was further pursued as a potential marker. Thus, a total of 18 markers representing 12 distinct epitopes of 6 HCV antigens were produced for further analysis (see Table 3).

Screening a commercial panel of HCV sera

The commercial panel PHV206 (SeraCare Life Sciences) consists of 18 polyclonal sera positive for HCV (5 of which were defined as weak responders) and was used to screen the phage-displayed markers in a quantitative EIA test. As illustrated in Table 4, all of the sera were indeed scored as "positive," with at least 3 distinct markers being positive (the cutoff being OD >0.17 after subtraction of the fth1 background signal) even for the weakest serum samples (PHV5 and PHV10). The strongest responder (PHV21) scored positive for all 12 distinct epitopes as well as cross-reactive with some of the different subtypes. Notable are samples PHV4 and PHV9. Both are not strong responders; however, they do have positive responses in more than one epitope in a given antigen (2 markers in

Table 3
Panel of 18 HCV markers.

M	Antigen		Position	Sequence
1	Core		19-28	PQDVKFPGGG
2			31-45	GGVYLLPRRGPRLGV
3			73-84	GRTWAQPGYPWP
4	E1		319-326	RMAWDMMM
5	NS3		1175-1334	--
6	NS4A		1691-1705	KPAIIPDREVLYQEF
7			1698-1710	REVLYQEFDEMEE
8	NS4B	1a	1712-1723	SQHLPYIEQGMM
9		2a	1712-1726	ASRAALIEEGQRIAE
10		3a	1712-1726	SQAAPYIEQAQVIAH
11		1b	1722-1736	MQLAEQFKQKALGLL
12		2a	1722-1736	QRIAEMLKSKIQGLL
13		3a	1722-1736	QVIAHQFKEKVLGLL
14		1b	1928-1938	HVSPTHYVPES
15	NS5A	1b	2295-2313	PPLLESWKDPDYVPPVVHG
16		2b	2295-2313	PPVIETWKRPDYEPPTVLG
17		3a	2295-2313	PPLLDRWKTPDYVPPTVHG
18		1b	2384-2400	SYSSMPPLEGEPGDPDL

Note. Of the 13 peptides tested from Table 2, 7 were used as the nucleus of the final panel of HCV markers (highlighted, with slight variations). The panel was further expanded, introducing subtype variations of markers and adding markers for NS5A and NS3.

Table 4
Testing the panel of HCV markers against confirmed positive sera.

Antigen		Core		E1	NS3	NS4A					NS4B					NS5A			A	E	S	G
Marker	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18				
PHV21	2.21	1.86	2.30	1.59	2.87	3.53	0.94	3.08			3.33		0.51	3.29	3.56	3.89	2.81	0.97	6	12	1	1a
PHV14	1.42	1.94	2.13	1.73	1.81		0.22	0.76			0.28			1.67		0.26			6	10	1	LP
PHV16	0.74	0.62			3.47		0.36				0.29			2.95	2.21	0.76	1.95	3.87	5	8	1 or 3	1b
PHV18		0.59		0.39	2.01	2.02	3.38	0.65			1.65			3.50					5	8	1	1a
PHV13	1.73	1.83		0.67	2.26		0.37	1.04			0.32			1.66					5	8	1	LP
PHV2	2.20	0.30	0.98		0.38		1.12						0.36		1.61	0.20	1.60	0.52	5	8	1 or 3	Neg
PHV7		0.32			1.65		0.34	0.23			0.32	1.34	0.45	1.60	0.32	0.61	0.31		5	7	2	LP
PHV19	2.60	2.43			2.62	3.74	3.72	0.86						3.89					4	7	1	1a
PHV20	0.43	2.77			2.07		2.92					0.38		3.90					4	6	-	2b
PHV24					1.78		0.17					0.19	0.36	3.82	0.35	0.48	0.23	0.33	4	6	-	LP
PHV17	1.44	1.63		0.40				0.22						0.86			0.19		4	6	-	2b
PHV15	1.96	1.75	0.95	1.68										2.64					3	5	-	LP
PHV23					1.58			0.40					0.22	2.12	0.25	0.29	0.18		3	5	-	Neg
PHV4	0.52	0.56			1.46			0.30						0.48					3	5	-	LP
PHV9					0.29	0.48		0.25			0.71	0.35		1.21					3	5	1	Neg
PHV11		0.35			0.17									0.17			0.17		4	4	-	Neg
PHV5	0.34				0.47							0.19							3	3	-	Neg
PHV10					0.42								0.22			0.22	0.27		3	3	-	Neg
Sum	11	13	4	6	16	4	10	10	0	0	7	5	6	15	6	8	9	4				
Sen [%]	61.1	72.2	22.2	33.3	88.9	22.2	55.6	55.6	0.0	0.0	38.9	27.8	33.3	83.3	33.3	44.4	50.0	22.2				

< 0.17	0.17-0.6	0.6-1.1	1.1-1.6	1.6-2.1	2.1-2.6	2.6-3.1	3.1-4

Note. A panel of 18 confirmed positive sera (SeraCare Life Sciences, cat. no. PHV206) was tested against the HCV markers representing 12 epitopes (alternating gray–white markers) derived from 6 antigens as indicated. For some of the epitopes, 3 representations corresponding to viral genotypes were also tested (i.e., markers 8–10, 11–13, and 15–17 are triplicate markers, with each set representing a specific marker and its HCV variations for genotypes 1, 2, and 3; see Table 3 for details). The number of antigens (A) and epitopes (E) scored for each serum sample is given at the right of the table (the cutoff scored as positive was OD > 0.17 about the fth1 background signal). The predicted serotypes (S) and confirmed genotypes (G) are given as well. The sensitivity (Sen) is given in percentage positives of total number of sera tested. LP, low positive PCR signal. Samples PHV9, PHV11, PHV5, and PHV10 (shaded) are scored as "weak responders" by SeraCare Life Sciences. The numbers in the table indicate the scores of the optical density measured at 650 nm.

Table 5
Testing the panel of HCV markers against clinical samples.

Antigen		Core		E1	NS3	NS4A					NS4B					NS5A			A	E	S	G
Marker	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18				
472	0.99	2.17		0.25	1.85	3.12	0.24	0.67				0.30	0.17	2.18	3.03	3.53	1.37	0.19	6	11	1	1b
522	2.07	1.80		0.20	1.70	1.39	2.88	0.21		1.11				3.85	3.05	2.54	1.67		6	9	3	3a
497	0.19	2.05			2.13		2.25	2.90		0.20				3.82	1.26	2.08	0.69	1.37	5	8	1	1a
501	2.09	3.49		0.22	2.46	0.79		2.03	0.20	0.18	0.30			2.97					5	8	1	1b
505	2.17	3.33		0.17	1.20	0.56					1.28			3.61					5	7	1	1b
1017	0.18	2.00		0.21	1.87			2.49						3.92	0.46	0.36	0.23		5	7	1	1b
490	0.63	1.38		1.00	2.18									3.91		1.71		0.19	5	7	2	2b
523	0.60	1.57		0.63	2.30			0.41						2.32	0.22	1.11	0.39		5	7	2	1
524	1.18	1.63	0.69		0.50			0.50						1.64	1.04				4	7	1	1b
447	0.28	1.62			0.29			0.20		0.25				1.81	0.67	0.69	1.58	0.68	4	7	-	1b
464	1.09	0.37			0.55								0.81	0.23		0.40			4	6	3	3a
443	1.64	0.93	1.16		0.20								0.21						3	5	-	3a
530	0.95	3.49	1.33					0.46											2	4	-	1b
Sum	13	13	3	7	12	4	3	9	1	4	2	1	3	11	7	8	6	4				
Sen [%]	100.0	100.0	23.1	53.8	92.3	30.8	23.1	69.2	7.7	30.8	15.4	7.7	23.1	84.6	53.8	61.5	46.2	30.8				

< 0.17	0.17-0.6	0.6-1.1	1.1-1.6	1.6-2.1	2.1-2.6	2.6-3.1	3.1-4

Note. A panel of 13 clinical samples was tested against the HCV markers representing 12 epitopes (alternating gray–white markers) derived from 6 antigens as indicated. For some of the epitopes 3 representations corresponding to viral genotypes were also tested (i.e., markers 8–10, 11–13, and 15–17 are triplicate markers, with each set representing a specific marker and its HCV variations for genotypes 1, 2, and 3; see Table 3 for details). The number of antigens (A) and epitopes (E) scored for each serum sample is given at the right of the table (the cutoff scored as positive was OD > 0.17 about the fth1 background signal). The predicted serotypes (S) and confirmed genotypes (G) are given as well. The sensitivity (Sen) is given in percentage positives of total number of sera tested. The numbers in the table indicate the scores of the optical density measured at 650 nm.

core and 2 of NS4B for PHV4 and all 3 markers of NS4B for PHV9), thereby illustrating the potential strength of deconvoluting an antigen into multiple independent and distinct markers, which in a Western blot would appear as a single band.

To evaluate whether serotyping was a possibility, we first analyzed all of the sera by PCR to establish the genotype of the infectious HCV for each sample. As indicated in Table 4, all of the weak sera, as well as 2 additional sera, were negative for PCR genotyping. Of the strong sera, 6 generated only vague low positive PCR signals that were insufficient to determine the viral genotypes. The remainder of sera indicated subtypes 1a, 1b, and 2b. Determining the viral genotype in HCV is important because the therapeutic regimens are varied in their efficacy for the different genotypes (e.g., ribivarin + PEGylated interferon is much more effective for genotypes 2 and 3 as compared with genotype 1 [32–38]). Hence, in this panel, only 8 samples could be verified by standard PCR. On the other hand, the introduction of the 6 markers of genotype variants in the phage-displayed panel did provide information that could be useful in determining virus genotype by serology (i.e., serotyping). Thus, for example, PHV14 gave a very strong response across the board and clearly indicates that the infectious virus was type 1, whereas the PCR was inconclusive. Similar results are seen for samples PHV13 and PHV7. Hence, "proof of concept" for the proposed combinatorial diagnostic approach and its potential has been demonstrated.

Screening sera from chronic HCV patients

To further strengthen this conclusion, we then applied our epitope array to a second panel of sera: a set of 13 samples obtained from patients at Rabin Medical Center with informed consent and according to the approval of the local institutional review board. All sera were obtained from chronic HCV patients with no evidence of cirrhosis. All sera were genotyped as indicated in Table 5. All patients scored positive (an average of 7 distinct epitopes). The weakest response was to 4 epitopes in patient 530; however, this same patient provides a clear illustration of the power of antigen deconvolution into epitopes. Patient 530 had a very strong response to the 3 distinct core epitopes plus a weak response to 1 epitope in NS4B. A similar situation is seen in patient 443, who responded strongly to the 3 core epitopes and only weakly to epitopes in NS3 and NS4B. Clearly, the core antigen is highly immunogenic and the first 2 epitopes in the panel (19–28 and 31–45) are strong diagnostic markers in particular (scored 100% in this set of 13 patients). The contribution of the third core epitope for serodiagnostic confirmation is clearly illustrated in the case of the 2 patients mentioned above.

The potential possibility for effective serotyping is also seen in this analysis. All but 3 samples generated signals that could be used for genotype determination, where 9 of 10 were correct as confirmed by PCR.

Discussion

There is constant striving to improve and perfect serodiagnostics of infectious diseases. Solid phase immunoassays serve as a cornerstone in this effort. The strategic goal is to produce tests that are quick, easy to use, and of reasonable cost without compromising on sensitivity or specificity. The serodiagnosis of HCV has been described here as proof of concept for combinatorial diagnostics.

Once the etiologic agent of NANBH (non-A non-B hepatitis) was finally identified, a first-generation diagnostic kit was released by Ortho-Clinical Diagnostics (USA) and relied on the detection of serum antibodies specific for the NS4 antigen (c100-3 1569–1931) [39,40]. The efficacy of this test was rather low, and as many as 70% false positives were scored among blood donors. In response, therefore, a second-generation blood test for HCV was launched in 1992, with NS4 being fortified with core antigen and NS3 [39,41]. The second-generation tests detected HCV antibodies 30 to 90 days sooner than EIA 1.0. Currently, the third-generation EIA (introduced in 1996 by Ortho-Clinical Diagnostics, Abbott [Germany], and Chiron [USA]) added NS5 antigen to the protein mixture used as probes [39]. Thus, in a sense, the evolution of HCV tests illustrates the need for multiple signals to ensure diagnostic confidence. Much as in the case for HIV (human immunodeficiency virus), positive HCV EIA results can be confirmed using the HCV blot assay [42].

The concept of combinatorial diagnostics takes this line of thought one step further; maximal diagnostic power can be realized by progressing onward from antigen arrays to epitope arrays. This is not totally novel. Minenkova and coworkers, for example, also produced a collection of peptides by screening phage-displayed libraries using polyclonal sera from HCV-infected individuals [21,43,44]. However, once the phage-displayed peptides were identified, characterized, and validated, the investigators then produced synthetic versions as MAP (multiple antigen peptide) constructs for the most promising peptides and used them as the capture probe in a standard EIA that they refer to as ADAM (antigen detection by antigen mimics). Although a collection of defining peptides is used, they are presented as a single mixture. In contrast to this, combinatorial diagnostics gains its power by deconvoluting the markers and testing each peptide individually.

Indeed, in both panels of sera detailed in Tables 4 and 5, there are clear examples where if the markers were combined, they might have been regarded as questionable (e.g., PHV5, PHV10, 530). The multiplicity of signals gained by testing markers separately provides the confidence for scoring these samples as positive. Implementation of this approach will require the identification and expansion of more effective epitope arrays. In the case of HCV, additional markers within core antigen might be found effective. The illustration that this approach can also be employed for serotyping is encouraging; thus, the selected markers should be presented for each viral genotype. In Israel, genotypes 1, 2, and 3 are most prevalent [45,46]; however, commercial panels will need to be generated to cover the viral diversity of the population where the test is to be used. Thus, a robust test will require additional specific markers multiplied by the number of genotypes to be screened.

Conclusions

Epitope arrays of viral antigens can be produced. Scoring the seroreactivity of each member of the panel *separately* provides added diagnostic power. The inclusion of genotype-defining markers indicates the potential for serotyping that can complement PCR tests. Hence, combinatorial diagnostics and its application to HCV have been illustrated.

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