Open Access Research Journal of
Life Sciences
Journals home page: https://oarjpublication/journals/oarjls/
ISSN: 2783-025X (Online)

OARJ OPEN ACCESS RESEARCH JOURNALS

(RESEARCH ARTICLE)

Production of rabbit specific antibodies against conserved and potentially immunogenic peptides of The HCV envelope glycoprotein E2: Novel candidate peptide vaccine against HCV infection

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Open Access Research Journal of Life Sciences, 2025, 09(01), 017-026

Publication history: Received on 10 December 2024; revised on 04 February 2025; accepted on 07 February 2025

Article DOI: https://doi.org/10.53022/oarjls.2025.9.1.0023

Abstract

Hepatitis C Virus (HCV) is a worldwide public health problem that affects more than 70% of the estimated 170 million people with chronic hepatitis. It induces chronic hepatic lesions that lead to severe fibrosis and cirrhosis, hepatic failure, or hepatocellular carcinoma. Envelope (E1 /E2) proteins of HCV may generate neutralizing antibodies. This study aims to evaluate and improve the bifunctional chimeric vaccine expression system based on the HBsAg protein as a site for the introduction of heterologous proteins (chimeric HBsAg). Therefore, it was designed to assess the immunogenic properties of conserved peptides derived from E2 HVR1 regions. Transfection assays were carried out in 25cm2 bottles and 6-well plates containing HuH7 cells (Hepatocellular carcinoma cells) and HEK-293T (Human Embryonic Kidney 293 cells associated with the SV-40 T antigen) in medium with low glucose concentration and high glucose concentration respectively, following specifications of the American Collection for the type of culture. The supernatants and cell lysates were collected and used for evaluation for the presence of HBsAg by immunoenzymatic assay and Western Blot. Samples obtained from transfections were subjected to denaturing polyacrylamide gel electrophoresis. Only two samples of patients reacted with the peptide 412-419 BSA in different dilutions. All peptides are shown to be highly immunogenic and neutralizing with detectable levels of specific antibodies.

Keywords: Antibodies; HCV; Peptides; Transfection assays; Vaccine

1. Introduction

HCV infection affects approximately 2 to 3% of the world population, corresponding to 170 million individuals. More than 75% of HCV infections become chronic, of which 5 to 20% progress to cirrhosis and hepatocellular carcinoma [1;2].

Hepatitis C virus (HCV) is a chronic infection that cause of hepatocellular carcinoma, and end-stage liver diseases. It is a positive-sense RNA virus of the *Flaviviridae* family that can be grouped into six genotypes differed by approximately 30% of the nucleotide sequence [3]. HCV is one of the most clinically important viruses that infect humans and has the highest mutation rate, existing within an individual as quasispecies [4].

Envelope glycoproteins (E1 and E2) are transmembrane proteins that have a large N-terminal ectodomain and a C-terminal transmembrane portion. E1 and E2 have 6 and 11 glycosylation sites, respectively, many of which are extremely conserved among HCV strains. It has been demonstrated that these regions can play an important role in the entry of the virus into the cell [5].

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The envelope glycoproteins E1 and E2 are the targets of neutralizing antibodies responses but are also the two of the most variable HCV proteins. Neutralizing monoclonal antibodies that target HCV epitopes encompassing amino acids (aa) 412 to 423 of the E2 protein. At the end N-terminus of the E2 protein is a region characterized by a high degree of variability called hypervariable region 1 (HVR1), which corresponds to the 27 amino acids of the E2 protein, very important in neutralizing HCV. Despite the high variability of E2 protein some amino acid positions are highly conserved. Studies with HCV have demonstrated that the presentation of epitopes from the core region [6] 1995) and the E2 envelope is much more efficient when these are fused to HBsAg.

The HBV S protein has the property of self-organizing into VLPs and carries the information necessary for its secretion mediated by mammalian cells. Each HBsAg particle is composed of 100 to 150 subunits of the S protein. Due to their high immunogenic potential, hybrid HBsAg particles, also called chimeras, have been shown in different immunization experiments to be very efficient proteins in presenting viral epitopes. and antigenic sequences of various infectious agents such as dengue virus [7], HCV [8] and human immunodeficiency virus [9].

This study aims to evaluate and improve the bifunctional chimeric vaccine expression system based on the HBsAg protein as a site for the introduction of heterologous proteins (chimeric HBsAg). Moreover, it was designed to assess the immunogenic properties of genetically conserved (amino acid sequence; 412-419) peptides derived from the C-terminal region of the hypervariable region-1 (HVR-1) and to detect the presence of antibodies reacting with this small 412-423 sequence in natural infection

2. Material and methods

2.1. Neutralizing peptides

Nucleotide sequences of all available HCV E2 region in the databases were aligned and four amino acid sequences with immunogenic potential were selected. We selected four neutralizing peptides from the AJ238799 Japanese sequence HVRI in different positions of aminoacids: peptide 1 (aa412-149), peptide 2 (517-531), peptide 3 (aa 523-535) and peptide 4 (aa 373-416). These peptides were synthesized conjugated with KLH and ressuspended in PBS (2µg/µL). Each peptide was used to inoculate one rabbit after emulsification of volume of Freund's complete adjuvant (table 1).

Table 1 Synthetic peptides with immunogenic potential

Peptide (3mg of each)	Sequence	Concentration after ressuspention in PBS
412-419	QLVNTNGS	2 µg/µL
517-531	GTTDHVGVPTYDWGK	2 µg/µL
523-535	GAPTYSWGANDTD	2 µg/µL
373-416	VKLLFAGVDGGTYVTGGTMAKNTLGITSLFSPGSSQKIQLVNT	1,6 µg/µL

2.2. Rabbits' inoculation

These synthetic peptides located downstream of the hypervariable region I within the HCV E2 protein were inoculated into the five rabbits (one peptide for each animal). Each peptide was injected as conjugated to KLH and one animal was injected with 0,5 ml saline solution at the 14 days intervals of immunization protocol to serve as controls. Each rabbit was immunized with a dose containing 0.5 mg/mL. Equal volumes of diluted KLH – peptide and Freund's complete adjuvant were emulsified and injected subcutaneously into the rabbit in three different sites. Before each immunization was collected pre-immune serum through the auricular vein of each rabbit and new serum samples immunes carried out post-immunization. On day 0 each rabbit was immunized again with the same protein emulsified with Complete Freund's adjuvant and day 14 and day 28, each rabbit was immunized again with the same protein emulsified with Incomplete Freund's adjuvant. One month after the last immunization, the animals were anesthetized with ketamine and xylazine to bleeding of the rabbits by cardiac puncture (table 2).

Table 2 Inoculation of each peptide per animal by subcutaneous injections

Animal	Peptide	Dose	Volume	Via
	412-419 KLH	0,5 mg/dose/animal	250 µL de peptídeo + 250 µL de adjuvante = 0,5mL	SC
	517-531 KLH	0,5 mg/dose/animal	250 µL de peptídeo + 250 µL de adjuvante = 0,5mL	SC
	523-535 KLH	0,5 mg/dose/animal	250 µL de peptídeo + 250 µL de adjuvante = 0,5mL	SC
	HVR1	0,5 mg/dose/animal	250 µL de peptídeo + 250 µL de adjuvante = 0,5mL	SC
	Control	0,5 mg/dose/animal	250 µL de peptídeo + 250 µL de adjuvante = 0,5mL	SC

2.3. Selection of candidate peptide

One candidate peptide was selected based on sequence conservation among E2 sequences recorded on the HCV database. Twenty-five serum samples from chronic HCV patients testing anti-HCV positive and 25 samples from healthy individuals who tested negative for anti-HCV antibodies were used to establish the reactivity of these sera with the small 412-419 synthetic peptide.

2.4. Protocol ELISA in house

Enzyme linked immunosorbent assay (ELISA) was developed in house calculating the *cutoff* value with mean absorbance value obtained from HCV negative sera plus three times the standard deviation. Immunized rabbit sera with E2-conserved synthetic peptide in the position 412-419 aminoacids conjugated with KLH generated high titers of anti-peptide antibody was used as positive control (figure 1).

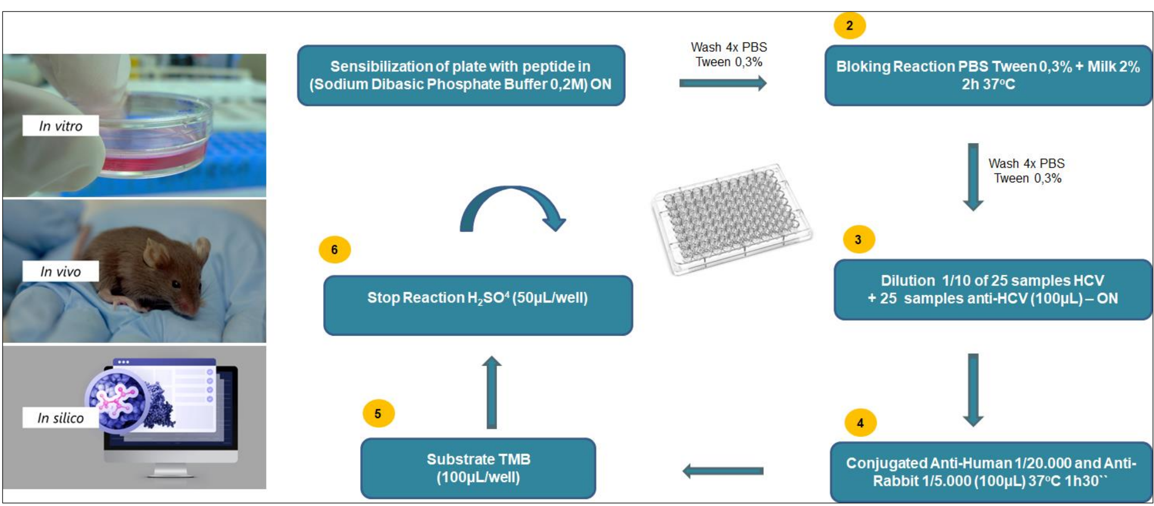

Figure 1 Schematic diagram of the protocol in house Elisa anti-HCV

2.5. HuH7 and HEK-293T cell supernatants containing chimeric HBsAg

HuH7 (Hepatocellular carcinoma cells) and HEK-293T (Human Embryonic Kidney 293 cells associated with SV-40 T antigen) continuous lineage cells were maintained in Dulbecco's medium with low glucose concentration (low glucose) and Dulbecco's medium with high glucose concentration (High Glucose), respectively and with 10% fetal bovine serum (FBS). 25cm2 bottles (Flask Corning) and 6-well plates (Nuclon) containing HuH7 and HEK-293T cells in medium with low glucose concentration (Low glucose) and high glucose concentration (High Glucose) respectively, and 5% FBS, were intended for transient transfection (table 3). The plasmids pCI (pCI Mammalian Expression Vector, Promega, Madison, WI, USA) SAa, pCI SAa his (plasmid containing the HBV S region fused to the polyhistidine tail), and pCI SAaHCV his (chimeric sequence containing the S region of HBV and the HVR1-E2 region - of the HCV envelope protein, fused to the polyhistidine tail) previously purified using the commercial kit MegaPrep Endoxin (Qiagen, USA) according to the

manufacturer's instructions and using the non-cationic liposome FuGENE 6® Transfection Reagent (ROCHE, USA – Cat. No. 11 814 443001), according to the manufacturer's instructions.

Table 3 Vectors used in transfection assay in HuH7 and HEK 293 T cells

Vectors	Quantification	Antibody	Controls
pCI SAa	0,315 µg/µL	MabAG9	C+: serum HBsAg+
pCI SAa His	0,478 µg/µL	Anti-His (C-Term)	C+: GST-VP6-His (cepa BL-21)
pCI SAa HCV His	0,298 µg/µL	Anti-His (C-Term) AP	C-: GST-VP6-His (cepa BL-21) uninduced

2.6. Enzyme immunoassay for detection of chimeric HBsAg

To detect HBsAg in the supernatant of HuH7 and HEK-293T transfected cells, approximately 100µL of diluted 1/10 and pure supernatant were evaluated using the commercial kit BioElisa 3.0 (Biokit, Spain) for detection of HBsAg, according to the manufacturer's instructions.

2.7. Denaturing polyacrylamide gel electrophoresis (SDS-PAGE) and Western-blot

Samples obtained from transfections were subjected to denaturing polyacrylamide gel electrophoresis (SDS-PAGE) using the Miniprotean III electrophoresis system (BioRad, USA), according to the manufacturer's instructions. The gels were stained by coomassie blue staining or using the silver nitrate method as described by Morrissey (1981) with some modifications (Silva and Júnior, 2001). Gels submitted to the Western-blot assay immediately after electrophoresis were transferred to Hybond-P PVDF membranes (GE-HealthCare, Germany), using the semi-dry Semi-dry system (BioRad, USA) for 30 min. at 20V, and according to the instructions in the protocol described below. Transferred membranes were momentarily stained using Ponceau solution (Sigma, USA), and then subjected to detection with monoclonal anti-HBs antibodies and serum from HCV-positive patients (figure 2).

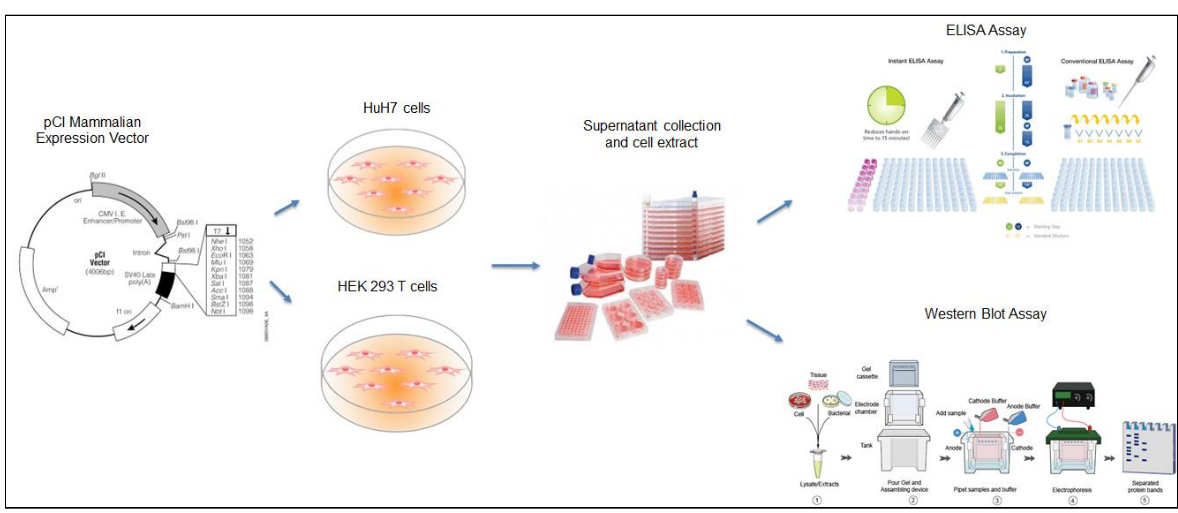

Figure 2 Transfection assays and expression assessment

3. Results and discussion

The results showed the expression of proteins in HEK-293T cells transfected with the plasmids pCI SAa his and pCI SAaHCV his. On the other hand, it was seen that ELISA assays were not able to detect proteins derived from the same plasmids, which could probably be caused by a conformational change of these proteins due to the presence of the polyhistidine tail. In a comparative analysis of transfections in HEK-293T and HuH7 cells, it was found that HEK-293T cells expressed the plasmids pCI SAa his and pCI SAaHCV his, while the expression of these proteins was not detected in the HuH7 cell line. This indicates that HEK-293T cells are more efficient in producing the proteins from the tested plasmids. This fact can be explained by the existence of the SV40 T antigen allowing the transfected plasmids to be present in their episomal form, thus prolonging their expression time in cells.

3.1. Transient expression evaluation

Protein expression was verified in HEK-293T cells transfected with the plasmids pCI SAa his and pCI SAa HCV his in western-blot assays the detection of recombinant proteins was not observed in ELISA assays. Expression of recombinant proteins derived from the pCI SAa his and pCI SAa HCV his plasmids was not detected in HuH7 cells and the expression of proteins derived from plasmids in HEK 293T cells was detected in cell extract samples and not in supernatants, suggesting that the proteins are produced inside the cell and cannot be exported to the extracellular space. Although protein expression was verified with the AG9 antibody in HEK-293T cells, detection was poor using the anti-His antibody (C-Term) (table 4).

Table 4 Transient expression evaluation using 25 cm^2 culture bottles and six-well plates

Plasmids	HuH7				HEK 293T			
	Supernatant		Cell Extract		Supernatant		Cell Extract	
	Bottle	six-well plate	Bottle	six-well plate	Bottle	six-well p.	Bottle	six-well plate
pCI SAa	+ (AG9) - (Anti-his)	+ (AG9) - (Anti-his)	+ (AG9) - (Anti-his) - (Anti-his AP)	+ (AG9) - (Anti-his) - (Anti-his AP)	- (AG9) - (Anti-his)	- (AG9) - (Anti-his)	+ (AG9) - (Anti-his) - (Anti-his AP)	- (AG9) - (Anti-his) - (Anti-his AP)
pCI SAa his	- (AG9) - (Anti-his)	- (AG9) - (Anti-his)	- (AG9) - (Anti-his) - (Anti-his AP)	- (AG9) - (Anti-his) - (Anti-his AP)	- (AG9) - (Anti-his)	- (AG9) - (Anti-his)	+ (AG9) - (Anti-his) - (Anti-his AP)	+ (AG9) - (Anti-his) - (Anti-his AP)
pCI SAa HCV his	- (AG9) -(Anti-his)	- (AG9) - (Anti-his)	- (AG9) -(Anti-his) - (Anti-his AP)	- (AG9) - (Anti-his) - (Anti-his AP)	- (AG9) -(Anti-his)	- (AG9) - (Anti-his)	+ (AG9) - (Anti-his) - (Anti-his AP)	+ (AG9) - (Anti-his) - (Anti-his AP)

The serums from rabbits immunized with peptide were tested to quantify the titter of relevant immunoglobulin using protocol ELISA in house sensitizing the plates with peptide conjugated to BSA (table 5). All peptides shown to be highly immunogenic and neutralizing with detectable levels of specific antibodies appeared at the first determination 15 days post immunization increasing after 30 days showing better response to the peptide 4 (aa 373-416). The high concentrations of serum showed a high level of cross-reactivity with exception of anti-HVR1 antibody which shows beyond the specific reaction, a nonspecific reaction with BSA

Table 5 Sequence of peptide 412-419 and dilution of anti-human conjugated, and anti-rabbit conjugated

Peptide	Conjugated	Sequence	Anti-Human	Anti-Rabbit
412-419	BSA	QLVNTNGS	1/20.000	1/5.000

Only two samples of patients reacted with the peptide 412-419 BSA in different dilutions. These samples identified HCV/101 negative for anti-HCV test and the sample identified HCV/122 positive for anti-HCV by standard test (table 6 -7). E2-peptides were able to recognize specific immunoglobulins in some chronic HCV patients. These results indicate that the selected epitope was able to induce humoral immune responses during the infection in a small proportion (8%) of the studied patients and that E2 conserved peptide used represent essential components of a candidate peptide vaccine against HCV infection. Non-protection against HCV may be associated with genetic variations particularly within the hypervariable region-1 (HVR-1) and low titers of anti E2 antibodies or interference of non-neutralizing antibodies with the function of neutralizing antibodies (tables 8-11).

Table 6 Detection anti-HCV peptide 412-419 BSA of chronic positive HCV patients

Samples HCV	D 1/10 C 1/20.000	D 1/10 C 1/30.000	D 1/100 C 1/20.000	D 1/100 C 1/30.000	ANTI-HCV ST	DO-Cutoff
1/06	0,89	1,08	0,29	0,64	P	107,63
2/06	0,61	1,25	0,20	0,47	P	102,93
8/06	0,37	0,98	0,11	0,21	P	130,52
11/06	0,78	1,44	0,32	0,51	P	138,9
12/06	0,48	0,75	0,12	0,14	P	114
15/06	0,65	1,22	0,21	0,27	P	132,61
27/06	0,53	0,86	0,21	0,30	P	112,25
31/06	0,36	0,58	0,10	0,13	P	141,11
40/06	0,36	0,77	0,13	0,20	P	137,74
41/06	0,68	1,23	0,29	0,44	P	140,95
46/06	1,12	1,34	0,38	0,44	P	130,06
48/06	0,54	0,99	0,30	0,32	P	120,12
62/06	0,38	0,47	0,18	0,26	P	126,89
82/06	0,43	0,84	0,19	0,30	P	137,54
85/06	0,69	1,04	0,36	0,43	P	123,64
88/06	0,94	1,58	0,38	0,54	P	125,32
89/06	0,54	0,84	0,30	0,39	P	114,94
90/06	0,81	1,31	0,28	0,38	P	136,07
111/06	0,60	0,74	0,17	0,20	P	106,2
113/06	1,02	1,39	0,28	0,35	P	102,93
122/06	1,77	2,51	1,15	1,38	P	113,03
128/06	1,11	1,87	0,27	0,37	P	139,86
155/06	0,63	1,10	0,21	0,30	P	109,22
159/06	0,40	0,71	0,13	0,18	P	139,19
160/06	0,49	0,82	0,14	0,23	P	110,17
346/02	0,41	0,31	0,09	0,07	P	NR
680/03	0,37	0,31	0,09	0,07	P	NR
867/02	0,28	0,21	0,07	0,07	P	NR
1555/03	1,07	0,81	0,16	0,12	P	NR

NR: Not Realized; D: Dilution of tested human serum; C: Dilution of anti-human conjugated with peroxidase; ST: Standard tests; P: Positive; N: Negative

Table 7 Detection anti-peptide 412-419 in not reactive HCV patients

Samples HCV	D 1/10 C 1/20.000	D 1/10 C 1/30.000	D 1/100 C 1/20.000	D 1/100 C 1/30.000	ANTI-HCV ST	DO-Cutoff
26/06	0,80	1,43	0,23	0,29	N	0,43
28/06	1,06	1,66	0,31	0,39	N	0,38
29/06	0,53	0,82	0,12	0,21	N	0,4
34/06	1,28	2,26	0,21	0,24	N	0,38
36/06	0,70	1,12	0,22	0,47	N	0,43
39/06	0,54	0,92	0,21	0,49	N	0,41
64/06	0,58	0,78	0,20	0,20	N	0,47
80/06	0,43	0,67	0,21	0,31	N	0,41
99/06	0,61	0,84	0,24	0,29	N	0,37
100/06	0,33	0,75	0,17	0,25	N	0,39
101/06	1,50	2,37	0,67	0,84	N	0,34
103/06	0,43	0,53	0,20	0,23	N	0,42
105/06	0,78	1,18	0,29	0,67	N	0,42
108/06	0,75	1,07	0,17	0,19	N	0,37
126/06	0,61	0,72	0,18	0,25	N	0,58
129/06	0,39	0,78	0,14	0,19	N	0,35
133/06	0,40	0,81	0,09	0,12	N	0,43
149/06	0,68	1.17	0,20	0,40	N	0,38
150/06	0,38	0,70	0,10	0,15	N	0,44
151/06	0,32	0,66	0,11	0,18	N	0,4
153/06	0,24	0,45	0,08	0,13	N	0,28
154/06	0,88	1,42	0,17	0,28	N	0,42
161/06	1,06	1,10	0,26	0,32	N	0,39
162/06	1,04	1,17	0,21	0,19	N	0,42
163/06	0,46	0,46	0,22	0,09	N	0,43
Serum 1	0,22	0,52	0,30	0,23	N	NR
Serum 2	0,63	0,29	0,09	0,08	N	NR
Serum 3	0,67	0,57	0,10	0,10	N	NR
Serum 4	0,79	0,59	0,13	0,11	N	NR

NR: Not Realized; D: Dilution of tested human serum; C: Dilution of anti-human conjugated with peroxidase; ST: Standard tests; P: Positive; N: Negative

Table 8 Possible cross-reactivity between the peptides and pre-immune serum of the five rabbits that were further immunized were tested by Elisa in house

	Antigens (Peptides)			
Antibodies (Serum)	412-419 BSA	517-531 BSA	523-535 BSA	HVR1 BSA (373-416)
Serum Rabbit sacrificed PBS 1/300	0.04	0.09	0.08	0.55
Serum Rabbit sacrificed PBS 1/600	0.04	0.05	0.02	0.31
Serum Rabbit Pre-Immune 412-419 1/300	0.04	0.09	>3	0.07
Serum Rabbit Pre-Immune 412-419 1/600	0.04	0.06	2.71	0.05
Serum Rabbit Pre-Immune 523-535 1/300	0.02	0.07	0.04	0.03
Serum Rabbit Pre-Immune 523-535 1/600	0.03	NR	0.02	NR
Serum Rabbit Pre-Immune HVR1 1/300	0.03	0.06	0.16	0.04
Serum Rabbit Pre-Immune HVR1 1/600	0.02	NR	0.06	NR

Table 9 Standardization of dilutions of immune sera anti-peptide Elisa in house test

	Antigens (Peptides)			
Antibodies (Serum)	HVR1 BSA (373-416)	412-419 BSA	517-531 BSA	523-535 BSA
Serum Rabbit sacrificed 517-531 1/300	>3	1,51	>3	1,00
Serum Rabbit sacrificed 517-531 1/600	2,25	1,44	>3	0,45
Serum Rabbit sacrificed517-531 1/1.200	1,39	0,99	>3	0,24
Serum Rabbit sacrificed 517-531 1/2.4000	0,90	0,78	>3	0,09
Serum Rabbit sacrificed 517-531 1/4.800	0,59	0,63	>3	0,14
Serum Rabbit sacrificed 523-535 1/600	2,23	0,44	>3	>3
Serum Rabbit sacrificed 523-535 1/1.200	1,56	0,37	>3	>3
Serum Rabbit sacrificed 523-535 1/2.400	1,11	0,17	>3	>3
Serum Rabbit sacrificed 523-535 1/4.800	0,66	0,09	2,93	>3
Serum Rabbit sacrificed HVR1 1/300	>3	>3	>3	>3
Serum Rabbit sacrificed HVR1 1/600	>3	>3	>3	>3
Serum Rabbit sacrificed HVR1 1/1.200	>3	>3	>3	>3
Serum Rabbit sacrificed HVR1 1/2.4000	>3	>3	>3	>3
Serum Rabbit sacrificed HVR1 1/4.800	>3	>3	>3	>3
Serum Rabbit sacrificed 412-419 1/300	2,72	>3	0,08	0,83
Serum Rabbit sacrificed 412-419 1/600	1,78	>3	0,04	0,39
Serum Rabbit sacrificed 412-419 1/1.200	0,84	2,02	0,02	0,14
Serum Rabbit sacrificed 412-419 1/2.400	0,43	1,27	0,01	0,06
Serum Rabbit sacrificed 412-419 1/4.800	0,22	0,75	0,01	0,25

Table 10 Appropriate dilutions for each antibody that allows specific detection of anti-E2 antibodies generated in rabbits

	Antigens (Peptides)				
Antibodies (Serum)	HVR1 BSA	412-419 BSA	517-531 BSA	523-535 BSA	BSA
Serum Rabbit sacrificed HVR1 1/7.000	1.64	1.60	1.62	1.45	1.70
Serum Rabbit sacrificed HVR1 1/7.000	1.83	1.82	1.71	1.56	1.94
Serum Rabbit sacrificed 412-419 1/1.500	0.24	0.94	0.01	0.05	0.01
Serum Rabbit sacrificed 412-419 1/1.500	0.08	1.24	0.01	0.02	0.01
Serum Rabbit sacrificed 517-523 1/4.800	0.12	0.12	0.75	0.03	0.01
Serum Rabbit sacrificed 517-523 1/4.800	0.14	0.16	0.86	0.05	0.01
Serum Rabbit sacrificed 523-535 1/7.000	0.12	0.05	0.41	0.82	0.05
Serum Rabbit sacrificed 523-535 1/7.000	0.12	0.05	0.38	0.86	0.02

Table 11 HVR1 antiserum reacts strongly with BSA, but if it plate is covered with peptides conjugated with KLH, the reaction is specific

Antibodies (Serum)	412-419 BSA	412-419 KLH	517-531 BSA	517-531 KLH	523-535 BSA	523-535 KLH	HVR1 BSA	HVR1 KLH	HVR1 PURE	BSA
Serum Rabbit sacrificed HVR 1/7.000	1.60	0.25	1.62	0.52	1.45	0.19	1.64	1.86	1.10	1.70
Serum Rabbit sacrificed HVR 1/7.000	1.82	0.23	1.71	0.56	1.56	0.22	1.83	1.88	1.14	1.94

4. Conclusion

Genetic variations particularly within the hypervariable region-1 (HVR-1) and low titers of anti E2 antibodies or interference of non-neutralizing antibodies with the function of neutralizing antibodies may be associated with non-protection against HCV. These findings suggest the detection of anti E2-peptide immunoglobulin in chronic HCV patients as potential therapeutic and/or prophylactic vaccines against HCV infection since most antiviral therapies fail and anti-HCV vaccine is not currently available. The serum at high concentrations showed a high level of cross-reactivity except for anti-HVR1 antibody which shows beyond the specific reaction, a nonspecific reaction with BSA. In conclusion, all the designed peptides were able to generate anti-E2 specific antibodies in rabbits at relatively high titles.

Compliance with ethical standards

Statement of ethical approval

Ethical approval was obtained.

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