

A new NS3 recombinant protein shows improved antigenic properties for HCV diagnosis

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ABSTRACT

The antigenicity of two recombinant NS3 proteins cloned in *E. coli* was compared by ELISA. The truncated rNS3 recombinant protein encompasses the amino acids from 1234 to 1432 of the Hepatitis C Virus (HCV) polyprotein, and the CIB-c33c protein encloses the entire HCV c33c sequence (aa 1192-1457). Seroconversion sera to NS3 from the Boston Biomedical panel PHV908 were used for evaluation. Our results show that the CIB-c33c protein has a better antigenicity than the rNS3 protein. Antigen CIB-c33c was recognized by the sera at the HCV seroconversion phase exclusively with antibodies against the NS3 region. Comparisons between both proteins suggest that the 25 aa fragment sequence presented at the c33c carboxyl-terminal is important for antibody recognition. The synthetic peptide encompassing this 25 amino acid fragment was not recognized by sera in seroconversion for HCV, suggesting that this fragment requires the presence of the remaining c33c region to expose its antigenicity. The new variant of the c33c protein obtained (CIB-c33c), expressed at high levels in *E. coli* and highly purified (>90% of purity) by one-step metal affinity chromatography, showed improved antigenic properties. Moreover, it increased the performance of anti-HCV diagnosis, and was able to detect sera in the seroconversion phase, thereby reducing the diagnostic seronegative window.

Key words: HCV, NS3, serodiagnosis

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RESEARCH

RESUMEN

Una nueva proteína recombinante NS3 muestra mejores propiedades antigénicas útiles para el diagnóstico del VHC. La antigenicidad de dos proteínas recombinantes NS3 producidas en *E. coli* fue comparada por ELISA. La proteína recombinante rNS3 truncada comprende los aminoácidos del 1234 al 1432 de la poli proteína del Virus de la Hepatitis C (VHC), mientras que la proteína CIB-c33c abarca la secuencia completa de la región c33c del VHC (aa 1192-1457). Para la evaluación se usaron sueros en seroconversión a NS3 del panel PHV908 de la Boston Biomedical Inc. Nuestros resultados muestran que la proteína CIB-c33c tiene mejor antigenicidad que la proteína rNS3. El antígeno CIB-c33c fue reconocido por sueros en fase de seroconversión que presentan anticuerpos dirigidos exclusivamente contra la región NS3. La comparación entre ambas proteínas sugiere que el fragmento de 25 aa en el extremo carboxi-terminal de la c33c es importante para el reconocimiento por los anticuerpos. El péptido sintético con la secuencia de este fragmento de 25 aminoácidos no fue reconocido por los sueros en seroconversión para VHC, sugiriendo que este fragmento requiere de la presencia del resto de la región c33c para exponer su antigenicidad. La nueva variante de proteína c33c obtenida (CIB-c33c), expresada en *E. coli* a altos niveles y altamente purificada (>90% de pureza) mediante la cromatografía en un solo paso de afinidad por iones metálicos, mostró mejoras en sus propiedades antigénicas. Además, aumentó el desempeño del diagnóstico de anticuerpos anti-VHC, fue capaz de detectar sueros en fase de seroconversión y por lo tanto redujo la ventana seronegativa en el diagnóstico del VHC.

Palabras claves: VHC, NS3, diagnóstico

Introduction

The hepatitis C virus (HCV) was identified as the major cause of post transfusional non A, non-B hepatitis by Choo *et al* in 1989 [1]. More than 50%-60% of the HCV cases became chronic, developing cirrhosis and hepatocellular carcinoma. HCV is distributed worldwide, with the estimated number of infected individuals of approximately 170 million. Only 40% of the infected patients benefit from currently available therapies and develop a sustained response [2], demonstrating the urgent need for more effective antiviral therapeutics. In the meantime, it is important to prevent the transmission of HCV. Since this entity is mainly transmitted through the blood, the key for the prevention of HCV transmission is based on screening blood donors using sensitive and specific HCV diagnostic reagents.

Screening assays based on antibody detection have greatly reduced the risk of transfusion-related infection. Once the seroconversion takes place, these tests usually remain positive. The third-generation enzyme immunoassays used at present contain the core and non-structural proteins and can detect antibodies within 4-10 weeks of infection [3].

The HCV nonstructural 3 protein (NS3) contains enzymatic domains that are likely to play a pivotal role in the viral life cycle. The N-terminal serine-type proteinase is involved in processing non-structural proteins from the polyprotein precursor [4], while the C-terminal portion contains nucleoside triphosphatase-RNA helicase activities [5, 6]. Expression of non-structural proteins most probably

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occurs during active viral replication, and this hypothesis is supported by prospective clinical studies which demonstrated that antibodies against NS3 appear early in the course of HCV infection, usually before or concomitantly with seroconversion to anticore [7]. Because of this, HCV NS3 must be included in anti HCV diagnostic systems.

In this report, the HCV NS3 gene fragment was cloned and expressed in *E. coli*. The purified protein compared a previous NS3 antigen obtained in our laboratory for sensitivity and specificity.

Materials and methods

Cloning and expression of the NS3 c33c gene

The synthetic gene, encoding a recombinant HCV protein c33c, was custom synthesized as *XbaI/SalI* fragments in the Geneart (Germany) vector pPCRscript. This synthetic gene was cloned, using *XbaI* and *SalI* restriction sites, into the expression vector pR2M6 [8] which incorporates a stabilizing IL-2 fragment of 60 aa in the protein. For purification purposes a 3' region encoding for a 6-His tag was added to the inserted gene. *Escherichia coli* XL1-Blue cells, transformed with the pR2M6-NS3-His vector, were grown in 5 mL of the LB medium [9] supplemented with ampicillin (100 µg/ml), for 8h at 37 °C. Then, 5 mL of the culture were inoculated into 300 mL of the LB medium supplemented with ampicillin and incubated at 37 °C overnight while shaking. The cells were harvested by centrifugation (3000 xg) at 4 °C for 30 minutes.

Cell disruption

Collected *E. coli* cells (1g), were suspended in 10 mL of 10 mM Tris-HCL pH 8, 1mM EDTA and 1 mM PMSF. The suspension was sonicated at 5 °C (3 x 150 W burst, US200S IKA Laborthechnik, Germany) and clarified by centrifugation (30 min, 15 000 xg at 4 °C).

SDS PAGE

Samples of 50 µL of the pellet and supernatant were mixed with 50 µL of sample buffer (4% SDS, 125 mM Tris-HCL pH 6.9, 20% glycerol, 10% β-mercaptoethanol, and 0.002% bromophenol Blue); and the mixture was boiled for 5 min. The samples were applied onto a 3 mm-thick 15% SDS-polyacrylamide gel. The SDS-PAGE was performed as described by Laemmli [10].

Ni²⁺- Chelate affinity chromatography

In order to obtain pure protein, the pellet was suspended after sonication in 10 mL of denaturing buffer B (8M urea, 0.1M NaH₂PO₄ and 0.01M Tris-HCL, pH 8) plus 10 mM imidazole. The suspension was centrifuged at 15 000 xg for 30 minutes and 10 mL of the clear supernatant was loaded onto a Quiagen 10 ml Ni-NTA matrix equilibrated in buffer B with imidazole. The non-bound material was washed out with buffer B until the baseline returned to zero. Adsorbed proteins were eluted in buffer B at pH 6.3 and pH 4.5. Total protein concentration in the samples was determined by the BCA method [11], using BSA as the standard.

Immunoassay for anti-NS3 detection

Antigenic properties of new NS3 protein were compared with an NS3 variant previously used in the production of BioScreen HCV ELISA by the Center for Genetic Engineering and Biotechnology of Havana, Cuba (CIGB). In brief, 96-well microtiter plates (Costar, USA) were coated separately with both NS3 at their optimal concentration (0.8 µg/mL for rNS3 and 0.25 µg/mL for CIB-c33c) in 0.5 M sodium-carbonate buffer, pH 9.6 for 3 h at 37 °C. The plates were washed four times with 200 µL/well of phosphate buffered saline pH 7.7, containing 0.05% Tween 20 (PBST). Coated plates were blocked with 5% skim milk in PBST for 1 hour at 37 °C. Serum samples (100 µL/well) were diluted 1:20 in PBST plus 2% skim milk and plates were incubated for 30 min at 37 °C and washed out four times, as mentioned above. A protein A/HRPO conjugate from Sigma Inc. diluted 1/50 000 in PBST (100 µL/well) was added and plates were incubated for 30 min at 37 °C. Finally, plates were washed four times and incubated for 15 min with 100 µL/well of the substrate buffer (citrate phosphate buffer pH 5.5, 0.014% H₂O₂, 0.25% *o*-phenylenediamine). The reaction was stopped with 50 µL/well of 2.5 M sulfuric acid, and optical density was read at 492 nm (UltramicroELISA reader, CIE, Cuba).

A panel of 19 positive and 95 negative sera was used for the antigenicity assessment of NS3 proteins. A seroconversion panel PHV908 from Boston Biomedical (USA) was also included. All samples were collected in aliquots and stored at -20 °C. The antigenicity of both proteins was compared using an ROC curve analysis. For this purpose cut-off values representing 100% specificity in the ROC curve analysis were taken (4.97 and 4.48 for the ELISA using c33c and rNS3 respectively).

Synthesis of NS3 peptides

Three peptides with carboxyl and N-terminal NS3 (NS3pep-1 aa 1192-1214, NS3pep-2 aa 1210-1233 and NS3pep-3 aa 1433-1457) were synthesized manually using Fmoc/tBu solid-phase chemistry on Rink Amide MBHA resin (0.54 mmol/g, 0.1 mmol scale). Side chain protecting groups were as follows: Arg(Pmc), Lys(Boc) and Trp(Boc). The activation used at least a threefold molar excess of Fmoc amino acids for each coupling cycle. In general, no more than 2 h were needed to complete the coupling reaction, which was indicated by a negative Kaiser test. Cleavage of the resin and deprotection were performed by treating with TFA/TIS/water (95/2.5/2.5) for 2 h. Crude peptides were precipitated from tert-butyl methyl ether and lyophilized. Peptides were purified by HPLC on a Vydac C18 column (25_250 mm) up to 99% purity, as demonstrated by analytical HPLC on a Vydac C18 column (4.6_100 mm). Peptide molecular mass was verified using mass spectrometry. The low-energy MS/MS spectra were acquired using a hybrid quadrupole orthogonal acceleration tandem mass spectrometer QTOF from Micromass (Manchester, UK), fitted with a Z-spray nanoflow electrospray ion source.

Immunoassay for synthetic peptides

Antigenicity of NS3 synthetic peptides was evaluated by ELISA using 96-well microtiter plates (Costar,

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USA). The peptides were used at 20 µg/mL in 0.5 M sodium-carbonate buffer, pH 9.6; for 3h at 37 °C. The remaining ELISA steps were similar to those above described for the NS3 protein immunoassay, except that the blocking step was not performed.

Statistical analysis

The ROC curve statistical analysis was made using a package Analyse-It from Analyse-it Software LTD, UK.

Results and discussion

Diagnosis of HCV, a disease known previously as Non-A, Non-B hepatitis (NANBH) [1] is characterized by the use of multiple polypeptides from different virus regions since no single polypeptide is immunologically reactive with all HCV infected sera [12]. The first generation HCV ELISA showed limited sensitivity and specificity and was produced using recombinant proteins complementing the NS4 (c100-3) region of the HCV genome as antigens. Second generation tests, which included recombinant / synthetic antigens from the Core (c22) and non-structural regions NS3 (c33c, c100-3) and NS4 (c100-3, c200) resulted in a marked improvement in sensitivity and specificity. Clinical studies show that a significant amount of HCV infected individuals develop antibodies to the non-structural NS5 protein of the virus. For this, third generation tests include antigens from the NS5 region of the viral genome in addition to NS3, NS4 and the Core. Third generation tests have improved sensitivity, but this increase in sensitivity is offset by a slight decrease in specificity. Moreover, third generation tests have shortened the time between HCV infection and the appearance of detectable antibodies (window period) to 60 days [13, 14, 15].

BioScreen anti-HCV produced by CIGB is a third generation ELISA system for detecting HCV antibodies in serum or plasma. BioScreen anti-HCV uses synthetic peptides that carry antigenic epitopes from the core, NS4, NS5 and also a recombinant protein from NS3 (rNS3). In spite of the high specificity and sensitivity of BioScreen anti-HCV, reported by Padrón [16], a lack of sensitivity for HCV seroconversion sera was detected in an external evaluation made at the Blood Transfusion Center of the Islamic Republic of Iran, using a certified HCV seroconversion panel PHV908 from Boston Biomedical Inc. USA (Table 1). BioScreen anti-HCV was unable to detect any sample contained in this panel. This seroconversion panel contains 13 blood extractions made from the same donors at different times. As shown in table 1, starting from extraction 7, antibodies against the NS3 region are the only antibodies detected by commercial tests. Besides this drawback, BioScreen anti-HCV also failed to detect serum 450 from an Iranian blood donor which was classified as positive by a confirmatory LiaTek III test, showing only one band of intensity 2+ according to the LiaTek positive criterion (Table 1).

Penton [17] reported that a truncated HCV-NS3 protein (aa 1234 to 1432), used also in the BioScreen anti-HCV ELISA, exposes linear and conformational B-cell determinants producing an increase in sensitivity, due to the detection of a new variety of false-negative sera in blood donor test samples. In this evaluation, they used a Boston Biomedical Panel

PHV901, containing samples of a positive individual at the seroconversion phase with anti-NS4 and anti-NS3 antibody pattern according to the supplemental test U.S.FDA-Licensed Ortho anti-HCV RIBA 2.0. In spite of this result, we found that some important determinants are still not included in the truncated NS3 protein, which must detect sera at the seroconversion phase with the anti-NS3 antibody response alone.

Recombinant NS3 proteins used in HCV diagnosis must encompass major NS3 determinants, since antibodies to NS3 appear early in the course of HCV infection, usually before or concomitantly with seroconversion to HCV core protein [7, 18]. The c33c fragment contains two thirds of the N-terminal part of the ATPase/helicase domain and the C-terminus part of the protease domain and appears to be serologically reactive during the early phase of HCV infection [19, 20]. Taking this into account, we decided to substitute the truncated rNS3 by a new protein that encloses the entire c33c region (aa 1192-1457).

The synthetic c33c gene band was extracted from the GeneArt plasmid, using the restriction enzymes *Xba* I and *Sal* I, which were used for cloning in the expression vector pR2M6, in *E. coli*. Several clones of transformed *E. coli* XL-1 Blue Cells were analyzed by western blot, most of which showed approximately a 20% expression of CIB-c33c among total cellular proteins (data not shown).

Protein aggregation and inclusion body formation are frequently encountered when recombinant proteins are overexpressed in *Escherichia coli* [21]. The solubility analysis (Figure 1) suggests that the CIB-c33c protein is expressed as a cytoplasmic inclusion body. Due to the insoluble nature of the *E. coli* expressed CIB-c33c protein, we used urea as a denaturant. The solubilized CIB-c33c protein was purified using metal affinity chromatography by means of the 6xHis tag attached to the C-terminus protein. Elution was carried out using the descending pH gradient (Figure 2). Fraction pH 6.3 was not loaded onto SDS-PAGE since no peak was observed, probably because of the initial amount of imidazole (10 mM) used in the binding buffer, was optimized in order to eliminate as contaminants as possible in the non-bound fraction. The final purified 37 kD CIB-c33c protein was obtained in two fractions at pH 4.5 with over 90% purity, as estimated by stained gel densitometry. The recovery was approximately 58% of the CIB-c33c injected into the column considering both fractions obtained at pH 4.5 (figure 1 y 2).

The antigenic performance of the new CIB-c33c protein was compared with that of the rNS3 protein. For this purpose, both proteins were separately coated into microtiter ELISA wells at their optimal concentration, mixed with the rest of the peptides used in the BioScreen anti-HCV ELISA. A panel of 96 sera was used for the evaluation. Among them, 77 were negative healthy blood donors and 19 were previously classified as HCV positive sera. ELISA results obtained with both proteins were analyzed by the ROC curve [22]. Figure 3 shows that the area under the curve for the CIB-c33c ELISA is steeper upwards with a statistical significance ($p < 0.0001$), demonstrating that on using the CIB-c33c protein the

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Table 1. Certified seroconversion panel PHV908 from Boston Biomedical Inc. Comparing results with those obtained by FDA-licensed anti-HCV EIA and the confirmatory test from Ortho and Abbott

Member I.D.#	Bleed Dates	Days Since 1st Bleed	ALT Value	U.S. FDA-Licensed Anti-HCV EIA Tests Abbott 2.0 Ortho 3.0		U.S. FDA-Licensed Ortho Anti-HCV RIBA 2.0 MFG				For research use only Abbott Anti-HCV Matrix 1.0 MFG						
				BBI s/co	BBI s/co	5-1-1 NS4	C100-3 NS4	C33c NS3	C22-3 CORE	SOD	Result	NS4 (e)	NS4 (y)	NS3	CORE	Result
PHV 908-01	26 Jan 96	0	65	0.1	0.0	-	-	-	-	-	NEG	0.1	0.0	0.0	0.0	NEG
PHV 908-02	29 Jan 96	3	268	0.1	0.0	-	-	-	-	-	NEG	0.0	0.1	0.0	0.0	NEG
PHV 908-03	31 Jan 96	5	240	0.1	0.0	-	-	-	-	-	NEG	0.1	0.1	0.0	0.0	NEG
PHV 908-04	06 Feb 96	11	160	0.1	0.1	-	-	-	-	-	NEG	0.1	0.1	0.0	0.1	NEG
PHV 908-05	08 Feb 96	13	NT	0.2	0.3	-	-	-	-	-	NEG	0.0	0.1	0.0	0.0	NEG
PHV 908-06	14 Feb 96	19	NT	0.2	1.7	-	-	-	-	-	NEG	0.0	0.1	0.0	0.0	NEG
PHV 908-07	20 Feb 96	25	NT	0.2	4.9	-	-	+/-	-	-	NEG	0.1	0.1	0.0	0.0	NEG
PHV 908-08	22 Feb 96	27	NT	0.4	4.9	-	-	1+	-	-	IND	0.1	0.1	0.0	0.0	NEG
PHV 908-09	27 Feb 96	32	NT	0.2	>5.0	-	-	2+	-	-	IND	0.1	0.1	0.1	0.0	NEG
PHV 908-10	01 Mar 96	35	NT	0.7	>5.0	-	-	2+	-	-	IND	0.1	0.1	0.3	0.0	NEG
PHV 908-11	07 Mar 96	41	NT	1.0	>5.0	-	-	3+	-	-	IND	0.1	0.1	1.8	0.0	IND
PHV 908-12	11 Mar 96	45	NT	1.0	>5.0	-	-	3+	-	-	IND	0.2	0.1	4.6	0.0	IND
PHV 908-13	14 Mar 96	48	NT	1.0	>5.0	-	-	3+	-	-	IND	0.1	0.1	4.1	0.0	IND

ELISA can discriminate better between positive and negative sera.

Among all possible ELISA threshold values given by the ROC curve analysis, a cut-off value corresponding to 100% specificity in each ELISA variant

(4.9 for CIB-c33c ELISA and 4.4 for rNS3 ELISA) was fixed to compare sensitivity. Even for this cut-off value, the ELISA variant using the CIB-c33c protein was able to detect the seroconversion panel starting from extraction number 8 (see table 1). This result is similar to that obtained by the International FDA approved ELISA such as Ortho and Abbott that recognize this panel from extractions 6 and 11, respectively. Besides this result, CIB-c33c ELISA also detected Iranian 450

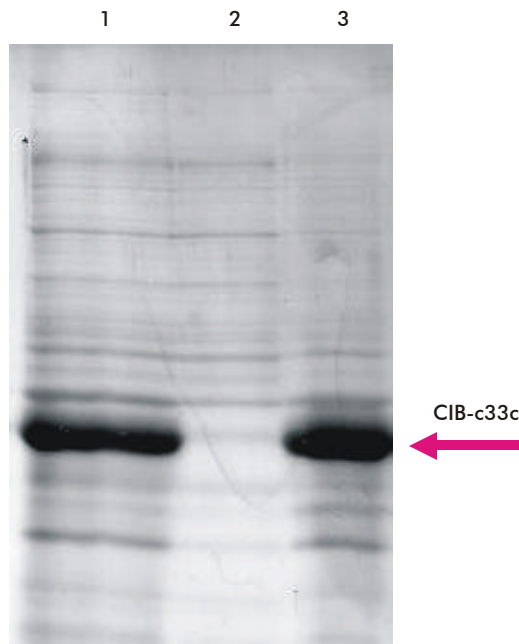


Figure 1. Solubility analysis. SDS-PAGE 15%. Lane 1, Initial biomass; lane 2, ruptured supernatant; lane 3, ruptured pellet.

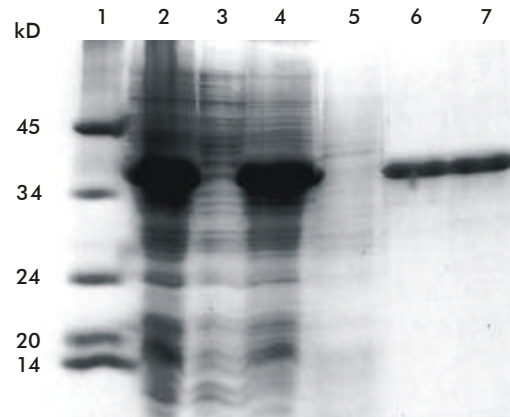


Figure 2. SDS-PAGE analysis of different fractions after purification using the Ni-NTA matrix. Lane 1, molecular weight markers; lane 2, initial biomass; lane 3, clear supernatant after sonication; lane 4, ruptured pellet after sonication; lane 5, non-bound material; lane 6: protein eluted at pH 4.5 (fraction 1); lane 7, protein eluted at pH 4.5 (fraction 2).

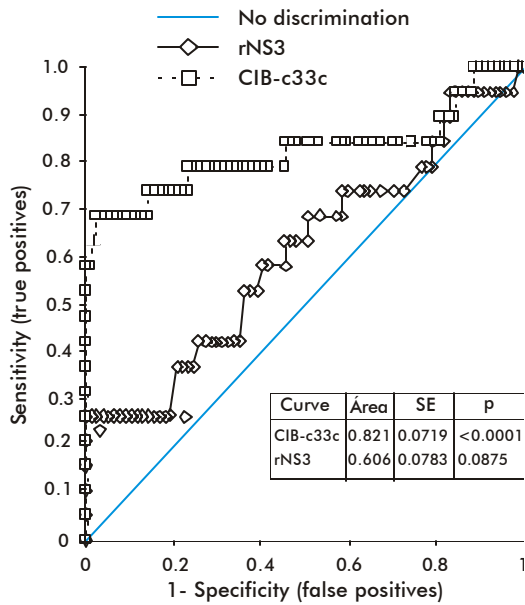


Figure 3. Performance comparison of BioScreen anti-HCV using two different NS3 proteins by the ROC curve analysis.

serum classified by LiaTek as a serum at the NS3 seroconversion phase. In contrast, the ELISA variant with rNS3 did not detect any sera in the PHV908 panel or the Iranian 450 serum (Table 2).

The above results demonstrate that the CIB-c33c protein has a better antigenicity than the rNS3 protein, since CIB-c33c was recognized by sera at the seroconversion phase with antibodies reacting exclusively with the NS3 region. To determine whether the c33c flanking regions absent from the rNS3 protein, caused the loss of antigenicity, three linear synthetic peptides (NS3pep-1 aa 1192-1214, NS3pep-2 aa 1210-1233 and NS3pep-3 aa 1433-1457) were evaluated by ELISA. The antigenic evaluation (data not shown) demonstrates that they were not recognized by the seroconversion sera used in this study. This result suggests that flanking the amino acid sequence present in c33c enhanced antibody recognition probably through the formation of a conformational epitope. We assume that two cysteines at aa 1454 and 1457 perhaps interact with the rest of the c33c region to expose the antigenicity. This suggestion agrees with Kink *et al.* [23], who found that all immune dominant c33c epitopes are contained in the 102 carboxyl terminal amino acids. Also, Mondelli *et al.* generated a human B cell cloned line derived from a patient with a chronic HCV infection

Table 2. Comparison of ELISA results using rNS3 or c33c-NS3 protein against a panel of 19 positive anti-HCV sera. Ratio of mean values between specimen absorbance and the respective ELISA cut-off value. Ratio ≥ 1 are considered positive.

Positive sera	ELISA results using c33c-NS3 protein	ELISA results using rNS3
PHV908-01	0.18	0.11
PHV908-02	0.19	0.20
PHV908-03	0.22	0.22
PHV908-04	0.31	0.19
PHV908-05	0.41	0.27
PHV908-06	0.50	0.24
PHV908-07	0.92	0.29
PHV908-08	1.00	0.31
PHV908-09	1.45	0.36
PHV908-10	1.40	0.40
PHV908-11	1.89	0.36
PHV908-12	2.17	0.33
PHV908-13	2.08	0.44
Iran 450	2.02	0.44
S1	5.59	4.44
S2	5.68	4.67
S3	1.82	1.81
S10	5.80	4.22
S11	6.71	5.56

that secretes an immunoglobulin G (IgG) monoclonal antibody (MAb) specific for the NS3 protein. They show that this hMAb binds to a major conformational epitope located at the carboxyl-terminal end of the NS3 region. Interestingly, they found, in an attempt to focus on the minimal sequence recognized by hMAb, that flanking residues located outside the putative minimal immunoreactive sequence are important for antibody recognition and that the c33c sequence 1444 to 1455, the same region that contains cysteines residues, probably also contains critical residues for the Ag-antibody interaction.

Although the importance of the c33c region in HCV diagnostic has been previously reported [18, 24], we present a new method for obtaining the c33c protein expressed in *E. coli* at high levels and highly purified (>90%) in one step using metal affinity chromatography. This protein improves the performance of BioScreen anti-HCV ELISA, enabling the detection of seroconversion sera and therefore reducing the diagnostic seronegative window, an important goal in the transfusional and diagnostic setting [25].

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