RESEARCH ARTICLE

Evaluation of a recombinant multiepitope antigen for diagnosis of hepatitis C virus: A lower cost alternative for antigen production

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Funding information

Fundação de Amparo à Pesquisa do Estado de Minas Gerais e Conselho Nacional de Desenvolvimento Científico e Tecnológico.

Background: The most of the hepatitis C-infected patients remain undiagnosed until they develop severe liver damage or submitted for serological screening.

Objective: To evaluate a recombinant multiepitope protein for detection of IgG anti-hepatitis C virus. **Method:** A synthetic gene was cloned, expressed in *Escherichia coli*, and the recombinant protein was purified. Human serum panel consisted of 88 positives (20 HCV genotyped) and 376 negatives for hepatitis C, 6 positives for human acquired immunodeficiency virus, 6 syphilis positives, 6 hepatitis B positives were tested by IgG antihepatitis C virus using the protein by enzyme-linked immunosorbent assay. In addition, 20 positive (all genotyped samples) and 20 negative samples were also tested by immunoblot and dot blot assays.

Results: Positive hepatitis C sera were strongly reactive against the protein by immunoblot assay. In the dot blot assay, positive sera were reactive until 1:1000 dilution and there were no false positive results in the hepatitis C negative sera. In the enzyme-linked immunosorbent assay, positive and negative sera had significant discrimination. No cross-reaction was observed in samples positive for syphilis; human acquired immunodeficiency virus and hepatitis B. All 20 genotyped samples were positive by the three methods.

Conclusion: The multiepitope protein used here has a lower cost compared to production of each antigen separately and could be an alternative for the serological diagnosis of hepatitis C.

KEYWORDS

diagnosis, hepatitis C, multiepitope antigen, serology, test validation

1 | INTRODUCTION

The human hepatitis C virus (HCV) is a member of the *Flaviviridae* family transmitted by biological fluids, and it has spread worldwide.

The acute infection usually has subclinical feature, and the infection can spontaneously disappear without treatment, although 55%-85% of patients develop persistent infections, which potentially lead to liver cirrhosis and hepatocellular carcinoma as later outcomes.^{1,2} The

most of the patients remain undiagnosed until they develop severe liver damage or submitted for serological screening.³

About 184 million people worldwide are HCV-infected,⁴ and early diagnosis and treatment allow virological cure in most of the cases.⁵ Because HCV infection is usually asymptomatic, with insidious evolution and epidemic,⁶ the screening for this infection can be considered strongly recommended for individuals who have some risk factors, who is blood donor or pregnant.^{7,8}

The laboratory diagnosis of HCV infection has two steps^{1,9}:

1. Blood samples are tested by screening methods which should have high sensitivity, usually immunological assays such as enzyme-linked immunosorbent assay, lateral immunoflow assay, dot blot Assay, and chemiluminescent assays.
2. Prescreened positive samples are retested using a highly specific test such as nucleic acid tests (NAT) to detect viral RNA which confirms persistent viremia.

The confirmation is important due to the fact that immunological tests can present false positive (or undetermined) results. In addition, infected patients do not develop chronic infection; however, their serological status remains positive during lifetime. Besides NAT to detect viral RNA, viral load and genotyping are also important.^{5,10} There are 7 genotypes known, and one specific genotype can present more morbidity than another.

ELISA and related assays are the most widely immunological methods used for screening of suspected HCV cases.^{11,12} To allow screening in large-scale, recombinant antigens were developed, but during many years, the diagnostic industries had the patent rights to antigen production.¹³ However, due to many patent rights have been expired (over 1990s) and with the advance and dissemination of the biotechnological techniques, several types of HCV antigens could be produced.^{14,15}

The most important antigens for HCV antibody testing are Core, NS3, NS4, and NS5,¹⁶ and in ideal conditions, a combination of these antigens should compose the diagnostic kit. The design, production, and purification of each antigen request time and lead to high cost besides different processes must be controlled.

The combination of different proteins together in a recombinant chimerical protein, which is expressed in a unique vector, have been considered a viable method to reduce the time and charge of the processes. In this system, the desired antigens are produced in a unique multiepitope protein, which should be similarly recognized by patient's antibodies compared to antigens separately. Therefore, the profile of reactivity must be warranted to avoid false positive or false negative results naturally with disastrous consequences to the patients. Thus, the laboratory validation of a new antigen has crucial importance prior to its use in diagnostics kits.

This study was aimed to evaluate the performance and to validate of a previously described recombinant multiepitope HCV antigen in three 3rd Generation diagnostic assay systems (ELISA, Western blot and dot blot) to detect anti-HCV IgG antibodies in human serum in a Brazilian cohort.

TABLE 1 Distribution of the genotyped HCV-positive samples

Genotype	Number of samples
1	2 (10%)
1a	6 (30%)
1b	3 (15%)
2	2 (10%)
3a	5 (25%)
4a/4c/4d	1 (5%)
5	1 (5%)

2 | MATERIALS AND METHODS

2.1 | Serum panel

Overall of 465 samples were investigated. A human serum panel consisted of 88 positive (20 HCV genotyped samples summarized in Table 1) and 376 negative samples previously tested for HCV antibodies by two different commercial ELISA kits were included in this study. Positive samples were also confirmed by recombinant immunoblot assay (RIBA). Six syphilis, 6 HIV, and 6 Hepatitis B positive samples (and negative for HCV) were included to investigate serological cross-reactivity with these agents, and these samples are among a total number of HCV negative samples. Twenty positive (all genotyped samples) and 20 negative samples were also tested by Western blot and dot blot assays using the proposed multiepitope HCV antigen. The proteins were tested to detect anti-HCV antibodies in a human serum panel by three different systems: immunoblot, dot blot, and enzyme-linked immunosorbent assay.

2.2 | Cloning and protein expression

An *Escherichia coli* codon optimized HCV synthetic gene containing Core 1b, Core 3g, NS3, NS4 I, NS4 II, and NS 5 HCV genes separated by a flexible linker (Gly-Ser-Gly-Ser-Gly), previously described elsewhere,¹⁴ was adapted to cloning into pET-28a(+) plasmid vector (Novagen, Germany). The synthetic gene was redesign to have EcoRI and XhoI restriction sites to allow ligation into pET-28a(+) in-frame with a C-terminal polyhistidine tag, and it was custom synthesized by Integrated DNA Technologies (www.idtdna.com). The pUCIDT plasmid containing a synthetic gene from IDT was rehydrated with Tris-EDTA buffer; chemically competent *E. coli* XL1-Blue cells were transformed, plated in Luria-Bertani (LB) agar with 100 µg/mL ampicillin and incubated at 37°C overnight. Positive clones were selected by PCR colony (M13 forward and M13 reverse primers), grown in LB broth in shaker at 37°C, and then plasmid was extracted. The plasmid was quantified in UV spectrophotometry using a Nanodrop (Thermo fisher Scientific, MA, USA) and digested at 37°C overnight with EcoRI and XhoI restriction enzymes (New England Biolabs, MA, USA). In parallel, pET-28a(+) plasmid was also digested with the same enzymes. After enzymatic digestion and gel purification of both plasmids, a synthetic gene was ligated to pET-28a(+) plasmid using

T4 DNA ligase kit following manufacturer's instructions (Promega, Madison, WI, USA). Chemically competent *E. coli* XL1-Blue cells were transformed, plated in LB agar 50 µg/mL kanamycin, and incubated overnight at 37°C. Positive clones were selected by colony PCR (T7 forward and T7 terminator reverse primers), and then clones were confirmed by Sanger sequencing for base sequence and correct insertion into the plasmid vector. One correct clone was grown, plasmid extracted and chemically competent *E. coli* BL21 (New England Biolabs, MA, USA) and *E. coli* Arctic Express (Agilent Technologies, CA, USA) strains were transformed and selected using the same protocol for *E. coli* XL1-Blue. Both bacterial clones were grown in 200 mL of LB with 50 µg/mL kanamycin until raise O.D. 600 nm between 0.6 and 0.8 and then induced with IPTG 1 mmol L⁻¹ for 3 hours at 37°C in shaker which was set up for 120 rpm for protein expression.

2.3 | Protein purification

Cells of the two *E. coli* strains were pelleted by centrifugation (5000 rpm at 4°C for 30 minutes), 10 µL of protease inhibitor (Baculogold-BD, Franklin Lakes, NJ, USA) and 10 mL of lysis buffer (guanidine 57.3% w/v, Tris-HCl 1 mmol L⁻¹, pH 8.0) were added and sonicated 5 times for 45 seconds with interval time of 15 seconds. Protein purification was carried out by affinity chromatography with Ni-column (Invitrogen, Boston, MA, USA). Briefly, columns were incubated with bacterial lysate for binding and then washed several times with wash buffer I (urea 8 mol L⁻¹, pH 6.3) and wash buffer II (urea 8 mol L⁻¹, pH 5.9). The protein was eluted from the column with buffer (urea 8 mol L⁻¹, pH 4.5). The protein in the eluate was refolded by consecutive dialysis in buffers (Tris-HCl pH 8.0 50 mmol L⁻¹, L-arginine 0.4 mol L⁻¹, phenylmethylsulfonyl fluoride 0.5 mmol L⁻¹, L-glutathione (reduced) 0.1 mmol L⁻¹, L-glutathione (oxidized) 1 mmol L⁻¹, and triton-X 0.1% w/v) containing different concentrations of urea (5, 4, 3, 2, 1 mol L⁻¹, and without urea) in separate recipients at 8°C for 6 hours in each solution. The protein quantification was carried out by Bradford method and stored frozen (-20°C) in the last buffer until analysis.

2.4 | SDS–polyacrylamide gel electrophoresis

Briefly, proteins after purification from Arctic Express and BL21 were electrophoresed in vertical polyacrylamide gel (12.5%) using Laemeli buffer containing SDS. Prestained protein weight marker (Fermentas, Pittsburgh, PA, USA) was used to estimate protein molecular weights. The gel was fixed, stained with comassie blue solution, and then destained.

2.5 | Immunoblot assay

Protein eluates, after electrophoresis in SDS-PAGE, were electron transferred from gel to nitrocellulose membranes. After confirmation of transference, the membranes containing the antigens were blocked with skimmed milk and washed 3 times with PBS/tween-20 (wash buffer). Positive and negative sera from the panel were diluted

1:1000 in PBS/tween-20/bovine serum albumin (diluent buffer), and then different nitrocellulose membrane strips containing antigens were immersed in diluted samples. The membranes were incubated at room temperature for 2 hours and then washed 3 times with wash buffer.

The membranes were immersed in antihuman IgG-horseradish conjugate diluted 1:4000 in diluent buffer and incubated at room temperature for 1 hour. The membranes were washed 3 times with wash buffer and then revealed with 3,3'-diaminobenzidine/hydrogen peroxide solution for 30 minutes at room temperature. The membranes were washed 3 times with distilled water and photographed under white light.

2.6 | Enzyme-linked immunosorbent assay (ELISA)

Polystyrene strips, flat bottom wells (MaxiSorp-Nunc, San Diego, CA, USA), were coated with 0.5 µg/well in bicarbonate-carbonate buffer by incubation overnight at 4°C. The strips were washed several times with PBS and blocked with 10% fetal bovine serum (FBS) in PBS for 12 hours at 4°C.

Samples from serum panel described above were diluted 1:100 in PBS/FBS, and 100 µL were added to the wells. PBS was used as a negative control of reactions. The strips were incubated at 37°C for 1 hour, washed 3 times with PBS/FBS and then added 100 µL per well of horseradish peroxidase-conjugated antihuman IgG.

The strips were incubated at 37°C for 1 hour and then washed 5 times with PBS/FBS to remove any traces of enzyme. To each well, 50 µL of 3,3',5,5'-tetramethylbenzidine/hydrogen peroxide solution (Sigma-Aldrich, Saint Louis, MO, USA) was added and incubated at room temperature for 15 minutes. The reaction was stopped with 50 µL of H₂SO₄ solution. The optical density (O.D.) of wells was measured using microplate spectrophotometer with filter 450 nm.

2.7 | Dot blot assay

Briefly, nitrocellulose membranes squares (approximately 1 cm³) were coated with HCV antigen and then blocked with 2% bovine serum albumin (BSA). The coated membranes were incubated with patient's serum diluted 1:1000 in PBS/BSA for 30 minutes at 37°C and then washed. The membranes were incubated with anti-IgG horseradish-conjugated for 30 minutes at 37°C and washed.

The reactions were revealed with 3,3'-diaminobenzidine/hydrogen peroxide solution for 5 minutes at room temperature. The "dots" were evaluated visually comparing the intensity of the staining.

2.8 | Ethical concerns

This study was approved by the Research Ethics Institutional Committee of the Federal University of Minas Gerais (UFMG) (ETIC 190/09, COEP 05-25-2009) and conducted in compliance with the Helsinki Declaration. In addition, all participants signed written informed consent before participating in this study.

FIGURE 1 SDS-PAGE gel of eluates obtained after purification by affinity chromatography (Ni-columns) of the lysates. In Arctic Express eluates, a 27 kDa protein represented <20% of total proteins (A). In BL21 eluates, a 27 kDa protein represented >95% of total protein (B). M: molecular weight marker

2.9 | Statistical and data analysis

Comparison among samples was performed by Mann-Whitney *U* test, ROC curve analysis and descriptive statistics (mean, maximum [max], minimum [min], standard deviation [SD], and coefficient of variation [CV]) using the software Prism Graph Pad 5.0 (Graph Pad Software Inc., San Diego, CA, USA). *P*-values < .05 were considered statistically significant. The sample power of the sampling was calculated by GPower Version 3.0¹⁷ considering *t* test, two tails, $\alpha = 0.05$, and effect size = 0.5. Based on ROC curve analysis, the O.D. cutoff was defined, and a Sample Cutoff Index (SCI) was calculated by the following equation:

$$\text{Sample Cutoff Index (SCI)} = \frac{\text{Sample Absorbency}}{\text{Cutoff Absorbency}}$$

2.10 | Definitions

Sample was considered to be “positive” by ELISA if the SCI was ≥ 1.1 and “negative” if the SCI was ≤ 0.9 . Sample with SCI between 1.1 and 0.9 was defined as “indeterminate.” Interpretations of immunoblot and dot blot assays were performed visually.

3 | RESULTS

3.1 | SDS–polyacrylamide gel electrophoresis

Arctic Express and BL21 expressed a 27 kDa protein compatible with the theoretical weight of the proposed recombinant multiepitope HCV antigen. However, BL21 presented better yield and purity than Arctic Express (Figure 1). The antigen purified from BL21 presented better yield, and therefore the following steps of this study were performed using only antigen purified from this expression vector.

3.2 | Immunoblot assay

The positive HCV samples ($n = 20$) were strongly reactive against antigen on the membranes, and the resulting bands were compatible

FIGURE 2 Immunoblot assay of 4 positive samples using the produced antigen. M: molecular weight markers and lanes 1 to 4, positive samples

with a 27 kDa protein (Figure 2). These results were 100% concordant with ELISA. The negative samples ($n = 20$) presented no bands in the Western blot assay.

3.3 | ELISA

The sample power analysis of the sampling resulted = 0.98 ($1 - \beta$) (Figure 3). Negative samples ($n = 376$) were visually lighter than positive samples ($n = 88$). The average O.D. of positive samples (mean = 1.220; min = 0.7620; max = 1.450; SD = 0.1128; CV = 9.38%) was 2.79-fold higher than negative samples (mean = 0.3132; min = 0.1050; max = 0.5190; SD = 0.090; CV = 29.1%), ($P < .0001$). SCI was calculated based on ROC curve of the O.D. (Figure 4), and a value of 0.5185 was defined (100% = sensitivity; specificity = 99.73%; 95% CI = 98.53% to 99.99%). Based on the SCI, all positive samples were

t tests – Means: Difference between two independent means (two groups)
Tail(s) = Two, Allocation ratio N2/N1 = 1, Effect size $d = 0.5$, α err prob = 0.05

FIGURE 3 Plotting data from sample power analysis showing sample size (axis y) vs power (axis x)

FIGURE 4 ROC curve calculated using positive and negative samples by ELISA

in agreement with the expected results. Four samples from the negative panel were defined as “indeterminate” because presented SCI between 0.9 and 1.1 (Figure 5). No cross-reactivity was observed in samples positive for syphilis ($n = 6$), HIV ($n = 6$), and hepatitis B ($n = 6$). In addition, all of HCV genotyped samples ($n = 20$) presented positive results as expected.

3.4 | Dot blot

The samples (20 positives and 20 negatives) tested by dots blot resulted in 100% of concordance with ELISA and Western blot. The best dilution of serum was 1:1000, and at this point, the “dots” staining presented strong discrimination between negative and positive samples (Figure 6). Unspecific reactions were observed in serum dilution of $<1:500$.

4 | DISCUSSION

The hepatitis C virus has a significant impact in the public health, and the diagnosis of HCV infection depends on screening methods followed by confirmatory techniques and clinical evaluation to set up the strategies for therapy.^{5,10} The ELISA and related methods are eligible due to lower cost and testing protocol facilities.^{11,12}

FIGURE 5 Distribution of positive and negative samples according to SCI (negative samples $n = 376$ and positive samples $n = 88$)

The capacity of a health system to early diagnose and, consequently, to treat the infected patients depends on the availability of kits.^{7,8} Most countries of Latin America as well as other developing and less-developing countries cannot produce their own diagnostic kits and import it from other countries. The capacity to produce the kit is closely related to the capacity to produce antigens. Thus, the know-how for production of a multipeptide antigen with a lower cost of production is desired.

In this study, we have optimized the expression and purification of a multipeptide HCV antigen previously proposed¹⁴ with the aim to be used in diagnostic kits. The performance of this antigen was tested in three different immunological systems using a panel of samples which consisted of positive and negative sera previously tested for HCV antibodies. Different HCV genotyped sera and sera negative for HCV but positive for other infectious agents were also tested.

Samples ($n = 461$) presented results in agreement with the expected results, that is, samples from panel which were considered positive in previous tests were positive in three methods (ELISA, immunoblot assay and dot blot assay), and samples from panel which were considered negative in previous tests were negative. No false positive neither false negative result was found, and no

FIGURE 6 Dot blots of positive and negative anti-HCV human sera at dilution 1:1000 revealed with DAB/hydrogen peroxide

cross-reactivity was observed except for four samples from the negative panel which presented SCI between 0.9 and 1.1. There are many factors which could cause an unspecific reaction in immunological tests. The correct dilution of samples and standardization of type blocking and diluent solutions as well as incubation times minimize the possibility of undesired background reaction.¹⁸ In addition, in ELISA tests, the cutoff values must be carefully calculated to offer a correct criterion for discrimination. However, even by use of correct cutoff and acceptable standardization of test conditions, some samples can present false or undetermined results. About this fact, there is an international recommendation to re-test positive samples by NAT for confirmation.^{1,6} Partial recognition of antigens used in the test due to cross-reactivity with antibodies produced in response to another infection can be possible resulting in interference on the test. Moreover, increased levels of heterologous antibodies, polyclonal memory B cells expansion and auto-antibodies can also eventually lead to false or undetermined results in immunological tests.^{18,19} Besides, no interference was observed among tested samples at least for syphilis, HIV, and hepatitis B.

Despite the fact that false positive or undetermined results can occur in the laboratory practice, the immunological tests are useful due to lower cost and simplicity to be performed. In screening tests, the higher sensibility is preferred instead of high specificity to minimize false negative results and the individuals screened as "positive" are included in a list for confirmatory tests. Dot blot assay was simple to be performed, and it required no apparatus for interpretation of the results.

Previously studies^{14,20} have reported similar results concerning design of synthetic gene, protein expression, and purification. Dipti et al¹⁴ have demonstrated that epitopes were well displayed using mouse antisera. Moreover, human sera panels (Boston Biomedical, USA) were tested by ELISA and samples were also tested by lateral flow assay, although the authors presented limited information about the sample features, number of sample tested as well as details of data analysis. However, the authors related specificity value of 100%, with a sensitivity value of 99.8%, and 100% of correlation with commercial kits.

Galdino et al²⁰ reported similar study that was tested 17 human positive (different genotypes) and 10 human negative sera samples (from Brazilian individuals) for anti-HCV which the authors have 100% of correlation with commercial kits. Moreover, the authors have presented information about molecular structural features of

the protein by circular dichroism spectroscopy. Both studies have demonstrated that the protein is suitable for development of diagnostic kits and did not present cross-reactivity with serum positive to other infectious agents: tuberculosis, human immunodeficiency virus, hepatitis B, VDRL (syphilis), C reactive protein, and rheumatoid arthritis¹⁴ and hepatitis A, hepatitis B, rubella, cytomegalovirus, and toxoplasmosis.²⁰

The present study has many similarities with studies mentioned above; however, we have demonstrated the robustness of the antigen in a greater number well-characterized samples by different data analysis (eg, ROC curve and sample power). In addition, we demonstrated the antigen recognition by different genotyped samples and discuss false positive results and limitation of the antigen. Our approach suggests a fine standardization for applicability in routine laboratories as well as use of dot blot assay as a simple method for small laboratories.

In conclusion, the HCV multiepitope protein used in this study is a lower cost compared to individual production of each antigen separately. The antigen presented proper sensitivity and specificity as demonstrated in human sera and recognized all different virus genotypes. The proposed antigen is suitable to design kits, and we draw attention to dot blot assay which could be useful for laboratories that test small number of samples or those who do not have an ELISA reader.

ACKNOWLEDGMENTS

We are thankful to Dr. Edel Barbosa Stancioli from Federal University of Minas Gerais-UFMG, for initial technical advice.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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How to cite this article: Thomasini RL, Souza HGA, Bruna-Romero O, et al. Evaluation of a recombinant multiepitope antigen for diagnosis of hepatitis C virus: A lower cost alternative for antigen production. *J Clin Lab Anal*. 2018;32:e22410. <https://doi.org/10.1002/jcla.22410>