Development of diagnostic methods and study of the immunoreactivity of a mixture of recombinant core and E2 proteins fused to GST with control serum positive for hepatitis C

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ABSTRACT

The hepatitis C virus (HCV) is an enveloped virus that is about 50–70 nm in diameter, has positive-strand RNA, and belongs to the genus *Hepacivirus* and the family *Flaviridae*. The detection and quantification of the core antigen, HCV nucleocapsid protein, has been successful in many trials and is considered a marker of viral replication since it presents a sequence of highly conserved amino acids, giving it high sensitivity and specificity. The E2 protein is an envelope glycoprotein of HCV with 11 glycosylation sites; most of these are well-conserved, making it a target antigen. The aim of this study is to develop high-sensitivity, low-cost diagnostic methods for HCV, which could be used for serological screening. The genomic regions encoding the core (part 136 aa) and E2 proteins of HCV were expressed in *Escherichia coli* Rosetta strain, cloned in expression vector pET-42a, and induced with 0.4 mmol L^{-1} IPTG, producing recombinant proteins that were fused to glutathione S-transferase (GST) protein, which was then purified by affinity chromatography. The immunoreactivity was assessed by Western blot, Slot Blot, and developed and improved diagnostic methods (capture, indirect, and immunoblotting enzyme-linked immunosorbent assay (ELISA)). After applying the results to the formulas for determining the quality parameters, obtained for immunoblotting method 100% sensitivity and specificity and for ELISA 100% sensitivity and 87.5% specificity. The methods developed were more sensitive and specific using the mixture of the recombinant proteins fused to GST (core+E2).

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1. Introduction

The hepatitis C virus (HCV) is the main etiologic agent of non-A/non-B hepatitis and infects approximately 170 million people on the planet, which corresponds to 3% of the world population, making hepatitis C a major cause of chronic liver disease worldwide [1–4].

It is an enveloped virus that is about 50–70 nm in diameter, has positive-strand RNA, and belongs to the genus *Hepacivirus* and the family *Flaviridae* [5–7].

Its genome consists of a single positive strand of RNA of approximately 9500 nucleotides and exhibits significant genetic variability, as a result of spontaneous mutations that occur during viral replication [8]. The 5′ and 3′ UTR regions flank a single coding sequence known as the Open Reading Frame (ORF) [8]; it encodes a precursor polyprotein of approximately 3010 amino acid residues, which is cleaved via the signaling mechanisms of host and viral proteases, generating about 10 different structural (core, E1, and E2) and nonstructural (NS2, NS3A, NS3B, NS4A, NS4B, NS5A, and NS5B) proteins in addition to p7 protein [9–11]. They are all essential for infectivity and have different functions [12,13].

HCV is the leading cause of chronic liver disease. There is still no vaccine for HCV. The only currently effective treatment takes 48 weeks and combines interferon-alpha with ribavirin, but the overall success rate is less than 50% depending on the viral genotype [14]. The mortality rate from HCV in this decade is expected to be double that of the last decade and to potentially surpass the mortalities caused by HIV [4].

The HCV core antigen has been successfully detected and quantified in many tests, exhibiting high sensitivity and antigenicity [15–18].

The E2 protein has 11 glycosylation sites, and most of them are well conserved. It is, thus, a good target for the development of antiviral molecules and also a target antigen [19]. It is being considered as a major candidate for a vaccine against HCV since

the production of neutralizing anti-E2 antibodies has been observed [20,21].

Many diagnostic tests have been developed since the isolation of the complementary DNA of the hepatitis C virus (HCV) in 1989 [5]. Our aim was also to develop diagnostic methods for HCV using the recombinant core and E2 proteins together, these being of high sensitivity and low cost, which can be used for serological screening. They are more appropriate for circumstances in Brazil, where using national technology they can reduce the costs of imported reagents and be standardized for viruses circulating in Brazil.

2. Material and methods

2.1. Trials with viral RNA

The detection of viral RNA was performed using the qualitative test *AMPLICOR® Hepatitis C Virus Test* version 2.0 (Roche Molecular Systems, Branchburg, NJ, USA). This material was genotyped using the commercial method INNO-LiPA HCV II (Innogenetics®, Belgium), following the manufacturer's instructions. To enhance the isolation, concentration, and yield of HCV RNA, 1 mL of the genotyped material and 50 µL of Bead Suspend from the kit VERSANT® HCV RNA 3.0 Assay (bDNA) (Siemens®, Berlin, Germany) were added to microtubes (*DNase* and *RNase* free) and then stirred in a vortex for 10 s and centrifuged at 14,000 rpm for 1 min at 4 °C. Subsequently, 1030 µL of supernatant was discarded. The remainder was further agitated by vortexing for 10 min and was fast frozen at −80 °C for 30 min. After this period, 120 µL of TE buffer at pH 8.0 was added and used to extract the viral RNA. The extraction of HCV RNA was performed using the QIAGEN kit, according to the specifications of the manufacturer (QIAamp RNA Mini Kit Hand, QIAGEN®, Hilden, Germany).

2.2. Amplification, purification, and analysis by electrophoresis in agarose gel

After the extraction of HCV RNA, synthesis of the cDNA of HCV was performed, of the core and E2 region, using the enzyme SuperScript^{TM} III Reverse Transcriptase (Invitrogen®-Life Technologies, Carlsbad, CA, EUA) in a Mastercycler gradient thermocycler Eppendorf (Hexasystens® São Paulo, Brazil) according to manufacturer's specifications, based on the methods described by Sambrook et al. [22]. For the core region, the cDNA was amplified by PCR with primers derived from the HCV-1b core region based on the published nucleotide sequence 968R [23]. Primers were chosen for amplification product 408 pb covering a partial region of the HCV core (amino acids 1–136), these being the *sense primer* VHCC S-SR (9.14 µg/µmol) (5′ cca tgg cac ata tga gca caa atc cta aac 3′) and the *antisense primer* (VHCC AI-SR) (8.88 µg/µmol) (5′ ttg gat cct tag tac ccc atg agg tcg gc 3′) (Invitrogen®-Life Technologies, Pleasanton, CA, EUA). Restriction sites for the enzymes Nco I and Bam HI (Fermentas®-Life Sciences, Vilnius, Lithuania) were added to the PCR product, to be subsequently cleaved and cloned into the expression vector pET-42a in the correct orientation. For the E2 region, the cDNA was amplified by PCR using the *sense primer* E2.S (10 pmol L^{-1}/µL) (5′ ggc cat ggg gga aac cca cgt cac cgg 3′) and the *antisense primer* E2.AS (10 pmol L^{-1}/µL) (5′ gct cga ggc tcg gac ctg tcc ctg tc 3′) (Invitrogen®-Life Technologies, Pleasanton, CA, EUA). The PCR product is part of the E2 protein gene (834 pb) with the addition of restriction sites for the enzymes NcoI and XhoI (18 pb) (Fermentas®-Life Sciences, Vilnius, Lithuania) thus containing a total of 852 pb.

The purification of the amplified products was performed using the kit *Wizard® SV Gel and PCR Clean-Up* System (Promega®, Madison, WI, USA), according to manufacturer's specifications. Electrophoresis in agarose gel was performed for the analysis of amplified and purified products and electrophoretic migration occurred at 100 V for 40 min. The gel was stained in a solution of ethidium bromide at 0.5 mg/mL and visualized under a transilluminator of ultraviolet (UV) light (BioRad®, Hercules, CA, USA), and the PCR product was detected as a single fragment corresponding to the amplified region of the core (408 bp) and the amplified region of E2 (852 bp).

2.3. Cloning and sequencing of the HCV core and E2 regions

The PCR products were cloned into the expression vector pET-42a (Novagen®, Madison, WI, USA) contained in a bacterial inoculum of competent *Escherichia coli*/DH5α treated with CaCl$_2$. It allows the cloning and expression of the gene of interest fused to the glutathione S-transferase (GST) protein in the N-terminal portion of the protein (which subsequently facilitates the purification of the protein of interest) and selection with the antibiotic kanamycin [24]. The constructed vectors were sequenced to confirm the correct sequence and orientation of the fragments inserted through the chain termination method of DNA sequencing [25] in an automatic sequencer "MegaBACE" using the kit "DYEnamic ET" dye terminator kit (Amersham® Place, Little Chalfont, Buckinghamshire, England), according to manufacturer's instructions.

2.4. Enzymatic cleavage of pET-42a with inserts of the region (core and E2) and bacterial transformation

The pET-42a with the core insert and the E2 insert were cleaved with restriction enzymes (*Nco I* and *Bam HI*) and (*NcoI* and *XhoI*), respectively, using the protocol provided by the manufacturer (Fermentas®—Life Sciences). Transformation took place by using competent bacterial cells, *E. coli* Rosetta strain, treated with calcium chloride (CaCl$_2$) and 1 µL of the mini-prep product (pET-42a with the core insert) (50 µg µL^{-1}) and pET-42a with E2 insert (50 µg µL^{-1}) with heat shock performed for 90 s at 42 °C after which it was immediately placed on ice for 1 min. A liquid culture medium without antibiotics was subsequently added and incubated under agitation in a shaker at 200 rpm at a temperature of 37 °C for 45 min. The transformed bacteria were plated using LB agar with kanamycin (25 mg/mL) and chloramphenicol (25 µg mL^{-1}) (USB). After solidification, the product of transformation was plated and incubated at 37 °C overnight. On the plate where the transformed bacteria were cultivated, one of these colonies was chopped with the aid of a toothpick, inserted into a tube containing antibiotic medium, and placed overnight under agitation in a shaker at 250 rpm and 37 °C.

2.5. Bacterial gene expression and purification of HCV core and E2 recombinant proteins

Induction was performed following the methodology described by Sambrook and Russell [26] with some modifications. First, the bacteria *E. coli* Rosetta strain were transformed and plated. Then the inoculum was prepared and grown overnight in a shaker (*Excella E24 Incubator Shaker Series, New Brunswick Scientific*) at 250 rpm and 37 °C. A 1:50 dilution of this inoculum was made in sterile LB medium (*USB*), with kanamycin (25 mg/mL) (*USB*), chloramphenicol (25 mg/mL) (*USB*) and the inoculum grown overnight and placed in an autoclaved 1 L Erlenmeyer flask (to encourage the oxygenation of the culture). The flask was placed in the shaker at 300 rpm and 37 °C until the optical density (OD) of 0.600 at 600 nm was attained, after approximately 1 h and 30 min. It was then added to the IPTG culture (Invitrogen®, Carlsbad, CA,

EUA) to a final concentration of $0.4 \, \text{mmol L}^{-1}$, thus starting the induction. The culture was induced for 3 h at 37 °C in a shaker at 300 rpm. The induced culture was then centrifuged for 10 min at 5000 rpm at 4 °C (centrifuge: *Sorvall High Speed Centrifuge-RC 5C PLUS*). The pellet was resuspended in a lysis buffer ($100 \, \text{mmol L}^{-1}$ NaCl, $10 \, \text{mmol L}^{-1}$ Tris–HCl, $50 \, \text{mmol L}^{-1}$ NaH$_2$PO$_4$, pH 8.0) and thawed under manual stirring on ice until its complete dissolution. The samples were sonicated (*Sonic Dismembrator*) in an ice bath for 4 min with 30 s intervals and centrifuged at 13,000 rpm for 10 min at 4 °C. The supernatant was filtered on MILLEX® HV® Durapore PVDF Membrane of 0.45 μm. The pellet was resuspended in a lysis buffer and stirred by vortexing for dissolution. The supernatant was purified by column GSTrap FF (GE Healthcare® Amersham Biosciences AB SE-751 84 Uppsala, Sweden), which contains the ligand glutathione coupled with agarose 4%. The column was previously equilibrated with PBS and, after loading, the protein extract was washed with five volumes of PBS and the elution buffer ($50 \, \text{mmol L}^{-1}$ Tris–HCl, $10 \, \text{mmol L}^{-1}$ reduced glutathione, pH 8.0) was applied. Fractions of eluted proteins were analyzed on SDS-PAGE 12% and stored at -20 °C until use.

2.6. Analysis of immunoreactivity of recombinant proteins

2.6.1. Western blot

Initially, to prepare the membrane that would be transferred with the core and E2 recombinant proteins, 15 μL recombinant core protein and 15 μL recombinant E2 protein obtained through the purification process by GSTrap FF column (GE Healthcare®, Amersham Biosciences AB SE-751 84 Uppsala, Sweden) were applied in wells of polyacrylamide gel 12%. After electrophoresis, which was performed following the method of Laemmli [27], the proteins were transferred to a nitrocellulose membrane [28]. The blocking of free sites on the membrane was performed with a 5% solution of skimmed milk powder in TBS/Tween 20 (Tris $0.05 \, \text{mol L}^{-1}$, NaCl $0.15 \, \text{mol L}^{-1}$, pH 8.0, Tween 20 0.05%) for 18 h at 4 °C. Then the membrane was washed with TBS/Tween 20, and pool HCV-positive serum diluted 1:400 in blocking solution was added. Human pool HCV-negative serum was added to one of the strips, and to assess cross-reactivity pool serum positive for the other infectious diseases (Chagas disease, syphilis, hepatitis B, HIV-1/2, and HTLV) diluted 1:400 in blocking solution. After 1 h incubation at room temperature, the membrane was washed five times with TBS/Tween 20 and incubated with anti-human IgG conjugated with peroxidase (Sigma-Aldrich®, Saint Louis, USA), diluted 1:10,000 in blocking solution, for 1 h at room temperature and subsequently washed five times with TBS/Tween 20. The revelation of the reaction was obtained by the addition of enzyme substrates: diaminobenzidine and hydrogen peroxide (Sigma-Aldrich®, Saint Louis, USA). After the appearance of bands, the strips were washed once and then dried on filter paper and photographed.

2.6.2. Slot blot

Slot blot was performed in order to obtain the lowest concentration of reactivity of GST-core and GST-E2 proteins. The nitrocellulose membrane Nytran (SCHLEICHER & SCHUELL-Sigma-Aldrich®, Saint Louis, USA) was cut to an appropriate size and equilibrated in TBS buffer (Tris $0.05 \, \text{mol L}^{-1}$, NaCl $0.15 \, \text{mol L}^{-1}$, pH 8.0) for 30 min. Next, the membrane was applied in a system, *Hybri Slot*^{TM} *Manifold* (Gibco BRL Life Technologies-Invitrogen® Carlsbad, CA, EUA) and different concentrations of GST protein and the recombinant core and E2 proteins purified by column GSTrap FF and serially diluted in blocking buffer (TBS/Tween 20 and 2% BSA, starting from $50 \, \mu\text{g mL}^{-1}$) were applied in a volume of 100 μL per well and

then transferred to the nitrocellulose membrane with the aid of a vacuum pump coupled to the system. The application of vacuum under the membrane increases the adsorption of proteins on the membrane. After adsorption, the membrane was washed with TBS/Tween 20 and blocked with the same solution containing 5% BSA for 18 h at 4 °C under gentle agitation. Then the membrane was washed five times for 5 min each with TBS/Tween 20 and incubated with a 1:100 dilution of pool HCV-positive serum in blocking solution. After 1 h and 30 min incubation at room temperature, under gentle agitation, the membrane was washed five times with TBS/Tween 20 and incubated with anti-human IgG conjugated with peroxidase (Sigma-Aldrich®, Saint Louis, USA) diluted 1:5000 in blocking solution for 1 h and 30 min at room temperature under mild agitation and again washed five times with TBS/Tween 20. The revelation of the reaction was obtained by the addition of enzyme substrates: diaminobenzidine and hydrogen peroxide (5 mg and 150 μL, respectively, in 30 mL PBS pH 7.3) for 15 min at room temperature and under constant stirring. Then the membrane was washed three times with distilled water and dried between filter papers.

2.7. Development of diagnostic methods for HCV

2.7.1. Enzyme-linked immunosorbent assay (ELISA) via capture

The reactivity of the recombinant proteins GST-core and GST-E2 with antibodies present in the HCV-positive control serum pool was observed on polystyrene microplates by enzyme immunoassay (ELISA) using the capture method developed by the Laboratory of Immunology and Molecular Biology of the Faculty of Pharmaceutical Sciences (UNESP at Araraquara), with a sensitivity of 100% and specificity of 87.5% (Master's degree dissertation: KENFE, 2008). The polystyrene microplate was sensitized for 18 h at 4 °C with 100 μL of monoclonal anti-GST antibody (Pharmacia Biotech®-Sigma-Aldrich®, Saint Louis, USA) diluted 1:5000 in carbonate/bicarbonate buffer $1 \, \text{mol L}^{-1}$ pH 9.6. After this period, it was washed three times with PBS-Tween 20 0.05%, blocking solution applied (PBS pH 7.5, Tween 20 0.05%, 5% skimmed milk powder), and the microplate incubated for 2 h at room temperature. Recombinant proteins GST-core and GST-E2 were used together in a concentration of $5 \, \mu\text{g mL}^{-1}$ each. These were blended, homogenized, and applied well into each microplate and incubated for 1 h at 37 °C. A 1:400 dilution of positive control serum for HCV, antistreptolysin O (ASO), Chagas disease, syphilis, hepatitis B, and auto-antibodies against intracellular antigens was applied and incubated for 1 h at 37 °C. The conjugated anti-human IgG labeled with peroxidase (Sigma-Aldrich®, Saint Louis, USA) diluted to 1:10,000 was applied and incubated for 1 h at 37 °C. The substrate solution of urea peroxide and tetramethylbenzidine (Biomérieux®, Shanghais, China) 1:1 was applied, and after 20 min at room temperature in the absence of light sulfuric acid ($2NH_2SO_4$) (Biomérieux®, Shanghais, China) $1 \, \text{mol L}^{-1}$ was added to stop the reaction. The spectrophotometric reading was taken at 450 nm absorbance. Between each step, the microplate was manually washed three times with PBS pH 7.3/Tween 20 0.05%.

2.7.2. Indirect ELISA

A mixture of GST-core and GST-E2 proteins at concentrations of $2 \, \mu\text{g mL}^{-1}$ of each recombinant protein was adsorbed onto the polystyrene microplate for 18 h at 4 °C. After this period, blocking was performed with PBS pH 7.3 and 5% skimmed milk powder and incubation performed for 2 h at room temperature. Then the human control serum negative and positive for HCV and positive control serum for hepatitis B, ASO, ANA, Chagas disease, and syphilis, and diluted 1:400 in buffer were applied and incubated for 1 h at 37 °C. The conjugated anti-human IgG labeled with

peroxidase (Sigma-Aldrich®, Saint Louis, USA) diluted to 1:10,000 in blocking buffer was added and incubated for 1 h at 37 °C. The revelation occurred using substrate solution tetramethylbenzidine and peroxide urea 1:1, and the reaction was halted after 20 min by adding sulfuric acid 1 mol L^{-1}. The spectrophotometric reading was performed at a wavelength of 450 nm.

2.7.3. Development of immunoblotting

Recombinant GST-core and GST-E2 proteins adhered to the nitrocellulose strip Millipore® HF120, previously cut into 0.5 cm pieces, applying 1.5 µL of each protein at concentrations of 24 µg µL^{-1} and 17 µg µL^{-1} respectively, 1.5 µL of the mixture of the two proteins together (core+E2) in the test zone (T) and 1.5 µL in duplicate of extracellular recombinant protein A (Sigma-Aldrich®, Saint Louis, USA) in the concentration of 5 µg µL^{-1} in the control area (C) and allowed to dry for 2 h at 37 °C and then blocked with Tris–HCl and 2% BSA for 40 min and washed three times with Na_2HPO_4 and once with Tris–HCl and allowed to dry at 37 °C. After 20 h, the strips were immersed in control HCV-positive serum diluted to 1:10 in blocking solution for 1 h and washed with Tris–HCl and Tween 20 0.05% three times. Then the strips were immersed in blocking solution containing anti-human IgG antibody conjugated with peroxidase diluted to 1:300 for 1 h and washed again. The strips were immersed in diaminobenzidine substrate (5 mg) with 150 µL of H_2O_2 and 30 mL of PBS, and the bands became visible in the test and control areas. The same method was performed using HCV-negative control serum.

2.7.4. Performance verification tests developed through the indexes of sensitivity and specificity

The calculations of sensitivity, specificity and predictive values were performed by applying the following formulas:

- *Sensitivity*: Values true positives/values true positives+values false negatives
- *Specificity*: Values true negatives/values true negatives+values false positives
- *Positive predictive values* (PPV): Values true positives/values true positives+values false positives
- *Negative predictive values* (NPV): Values true negatives/values true negatives+values false negatives

The serum samples were composed of pool sera from internal control (NAC Center for Community treatment), Faculty of Pharmaceutical Sciences UNESP/Araraquara, Brazil, and the true positive patients with hepatitis C and represent the true negative samples from blood donors previously established as non-carriers of hepatitis C, hepatitis B, ASO, ANA, syphilis and Chagas, all laboratory-determined by commercial ELISA (Ortho-Clinical Diagnostics®) and qualitative PCR (Roche®), performed according to manufacturer's instructions.

3. Results

3.1. Expression of recombinant HCV proteins: core and E2 fused to GST

Recombinant proteins core and *E*2 HCV fused to GST were expressed in *E. coli* Rosetta strain with IPTG induction at a final concentration of 0.4 mmol L^{-1} for 3 h at 37 °C and 300 rpm. To confirm the expression and immunoreactivity of these proteins, immunological detection was performed by Western Blot and Slot Blot using an HCV-positive serum pool, and the results showed that the proteins react specifically with the HCV-positive serum pool; when evaluated with HCV-negative control serum and

HCV-positive control serum for other infectious diseases (ASO, Chagas disease, syphilis, hepatitis B, and FAN), there was no immunoreaction, that is, recombinant proteins reacted nonspecifically (data not shown). Another fact that is equally important and was evaluated is that protein GST alone did not react with HCV-positive control serum or normal control serum, which proves that the immunoreaction generated by the HCV-positive serum pool is caused by the core and E2 recombinant proteins.

3.2. Capture enzyme immunoassay

The immunoassay developed by the Laboratory of Immunology and Molecular Biology, Faculty of Pharmaceutical Sciences, UNESP (dissertation: KENFE, 2008), was optimized using recombinant HCV proteins, core and E2 together, in the concentration 5 µg mL^{-1} each and anti-GST monoclonal antibody in the ratio 1:10,000, which afforded better immunoreactivity (Table 1).

The positive results for capture and indirect ELISA were determined when the absorbance of the samples was above the cutoff value, this value being the arithmetic mean of the optical densities of negative samples ($n=12$) plus two standard deviations, equal to 0.3873.

3.3. Indirect ELISA

This test was performed to verify an increase in immunoreactivity and specificity using a mixture of recombinant proteins (GST-core and GST-E2) at concentrations of 2 µg mL^{-1} each and HCV-positive and HCV-negative control serum and for other infectious and inflammatory diseases diluted 1:400 in blocking buffer. This reaction is illustrated in (Table 2).

Table 1
Mean and standard deviation of the absorbance values obtained in the reaction of capture ELISA.

Control serum 1:400	Absorbances: mean and standard deviation
	GST-core 5 µg mL^{-1} + GST-E2 5 µg mL^{-1}
Hepatitis C virus (HCV negative)	0.217 ± 0.027
Hepatitis C virus (HCV positive)	2.903 ± 0.050
Antistreptolysin O (ASO)	0.297 ± 0.006
Antinuclear antibodies (ANAs)	0.209 ± 0.005
Chagas disease	0.199 ± 0.008
Syphilis	0.231 ± 0.008
Hepatitis B virus	0.226 ± 0.011

Cutoff value=0.3873.

Table 2
Mean and standard deviation of the absorbance values obtained in the reaction of capture ELISA.

| Control serum 1:400	Absorbances: mean and standard deviation
	GST-core 2 µg mL^{-1} + GST-E2 2 µg mL^{-1}
Hepatitis C virus (HCV negative)	0.183 ± 0.007
Hepatitis C virus (HCV positive)	2.986 ± 0.020
Antistreptolysin O (ASO)	0.309 ± 0.011
Antinuclear antibodies (ANA)	0.185 ± 0.010
Chagas disease	0.234 ± 0.015
Syphilis	0.280 ± 0.016
Hepatitis B virus	0.288 ± 0.018

Cutoff value=0.3873.

3.4. Immunoblotting assay

After defining the concentrations of the recombinant core and E2 proteins of HCV, the dilution of HCV-positive and HCV-negative control serum, and the dilution of the conjugated antibody, the immunoblotting assay was performed with $n=12$, that is, $n=10$ for positive control serum and $n=2$ for negative control serum. As a test (T), the recombinant proteins GST-core at a concentration of $24\,\mu g\,\mu L^{-1}$ and E2 at a concentration of $17\,\mu g\,\mu L^{-1}$ and a mixture of the two proteins (core+E2-GST) were applied. When negative control serum was used, there was no reaction in the test area but only a mark in the control area. Test results using HCV recombinant proteins can be seen in (Fig. 1).

3.5. Performance of the methods developed

Analyzing the results of tests developed and applying the results to formulas for determining the sensitivity and specificity, the values were 100%, for immunoblotting, because there was no test conducted with the developed method that was different from the results by commercial ELISA and PCR used for qualitative comparison of the performance of the new method. For the developed ELISA results were 100% sensitivity and specificity of 87.5%.

4. Discussion

Since the isolation of DNA complementary to HCV by Choo et al. [5], hepatitis C has been recognized as a major cause of chronic liver disease worldwide; from this molecular characterization, many diagnostic tests have been developed. The detection of antibodies against specific antigens of HCV is the most common method used to identify infection, past or present. For this screening tests are used, which have high sensitivity, and additional tests, also called confirmatory tests, are performed for greater specificity. Due to the prevalence of infection by hepatitis C virus, estimated at 3% of the world's population, the diagnosis of hepatitis C requires a very sensitive test with advantages like fast processing, ease of automation, high reliability, and relatively low cost.
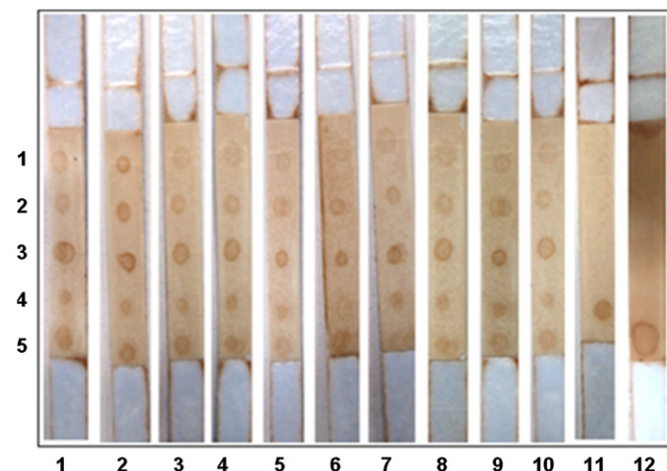
Fig. 1. Results obtained using the recombinant proteins GST-core, GST-E2 and GST-(core+E2), at concentration of $24\,\mu g\,\mu L^{-1}$ and $17\,\mu g\,\mu L^{-1}$, respectively. (Strips 1–10) HCV positive control serum; row 1: (test) GST-core, 2: (test) GST-E2, 3: (test) GST-(core+E2), 4 and 5: (control) extracellular recombinant protein A (Sigma-Aldrich®). (Strips 11 and 12) HCV negative control serum; row 5: (control) extracellular protein A (Sigma-Aldrich®).

The detection of HCV-core protein has been useful in monitoring the efficacy of therapeutic protocols in subjects undergoing hemodialysis [29–31] and in patients treated with interferon-alpha and ribavirin [18,32]. The E2 protein is more commonly reported in studies involving cells as it is related to different stages of the replication cycle of HCV, having a role essential to initiating infection, including receptor binding, fusion with the host-cell membrane, and invasion [10,33–35].

The HCV-core protein is highly antigenic, induces specific cellular and humoral responses, and probably plays a key role in the pathogenesis of HCV infection [36,37], whereas the E2 glycoprotein of HCV possesses conserved areas and is responsible for binding the virus to the target cell and the antibodies for this region are neutralizing [38].

Considering the above, the key feature for the use of core and E2 HCV proteins in this study was antigenicity, which assured the specificity of the methods. These proteins expressed and fused to GST showed no cross-reactivity in the presence of HCV-negative serum controls and also did not show a lack of specificity compared with control serum positive for ASO, antinuclear antibodies (ANA), Chagas disease, syphilis, and hepatitis B. Moreover, the GST protein alone produces no reactivity with HCV-positive control serum.

The recombinant core protein is already used in some diagnostic tests along with the HCV proteins NS3, NS4, and NS5. However, the use of the mixture of core and E2 recombinant proteins fused to GST is an innovative method, as much in immunoblotting assay as in capture and indirect enzyme immunoassay (ELISA). As can be seen in the immunoblotting assay, in Fig. 1, compared with the isolated proteins there was greater reaction intensity when core and E2 were used together, proving to be more sensitive and specific.

Considering the above, the key feature for the use of core and E2 proteins of HCV in this study was antigenicity, assuring the specificity of methods.

For immunoblotting method, several steps were important for good reproducibility of a test, as an application of reagents, assembly and handling of nitrocellulose membrane, these procedures that are performed improperly can alter the detection limits, formats of the test and control bands, interpretation of the results and overall performance of the test [39].

In this study, the size of the nitrocellulose strip was more suitable than 2.5 cm long × 0.5 cm wide, because the test strip with the wider, the reaction zone was higher and the performance was not as good. In other works, the size was similar to our strips 2.5 cm long × 0.5 cm wide [40].

The adsorbents in the adsorption may be dispensed nitrocellulose membrane in many ways either manually or by automatic dispensers air jet specifically for this purpose, typically used at industrial scale [41]. Our study was performed manually. The best way of implementing the detection reagents and test control immunoblotting HCV was the dot shape (circular) using automatic pipette. Other studies have adopted this form of adsorption of the reagents and have tracked the signal strength test and control lines [42].

The signal on line test is related to the concentration of target molecule in the sample is visually observed in the reaction. Other studies correlating the staining intensity in the test line with the concentration of the target protein and the accumulation of conjugate in the test and control lines of the membrane [43]. In our study some of the tests showed signal intensity line control greater than in the line test and other tests in the staining intensity was inversely and was also introduced equally strong color intensity in the two lines as shown in other studies [44–46].

Test and control areas were applied 1.5 μL of adsorbent in the reaction zone of the nitrocellulose membrane. It was reported

that an even smaller amount in our study, 0.5 μL for the test and control region [40]. The average amount of adsorbent in both the test and control areas do not exceed 2 μL because the dot this size allows a better visualization of the signal, as described in an article where it is applied 1–1.5 μL of the test and control reagents [47].

The main goal of the development of an immunoassay is to achieve maximum recognition by the antibody and/or protein. This must have good sensitivity and reactivity of adsorbed and then after being wetted by serological sample should not be changed as to nature and be reactive at the time of testing. In practice, all reagents used showed good specificity in the reactions of both Western Blot, Slot Blot, ELISA and immunoblotting and capture.

The choice of protein A standardization immunoblotting assay, was due to its nature and it's not enzymatic property of binding the Fc immunoglobulin fraction [48]. It has been used for some time to develop immunochromatographic tests, both as particle capture conjugated to colloidal gold as the line capturing test for antibodies in serum [49].

The immunoblotting method, we provide reliable results regarding the procedures adopted in its development, important for the control sera samples were used in the test application and then compared the results obtained with other reference methods.

During the standardization test the recombinant proteins fused to GST and E2-HCV core used alone, together, and at different concentrations, and the best result was obtained with the mixture of recombinant proteins at concentrations of 24 μg μL^{-1} and 17 μg μL^{-1} for core and E2 respectively. To control the area, we used the extracellular recombinant protein A (Sigma®) at a concentration of 5 μg μL^{-1}, with excellent results.

The performance verification, specificity and sensitivity of the tests was performed comparing developed serum samples or other biological sample with classic essays employed in conventional diagnosis. In our study, we found these parameters with ELISA assays (Ortho-Clinical Diagnostics®) and qualitative PCR (Roche®) routinely performed in the laboratory of serology NAC (core service to the community), located in the state hospital of Américo Brasilienses, Brazil.

As for the immunoreactivity of the expressed proteins fused to GST and we can say that they did not show cross-reactivity in the presence of serum HCV-negative controls and did not show specificity against control sera positive for anti-streptolysin O, antinuclear factors, wounds, syphilis, and hepatitis B. Moreover, the GST protein alone produces no reactivity with control sera positive for HCV. The recombinant proteins were recognized by only anticopos anti-HCV present in control sera positive for HCV.

The ELISA assays to detect anti-HCV antibodies using recombinant proteins of HCV, core, and E2, so separated and mixed, this study was designed with the purpose of comparison with the results obtained in the test of immunoblotting, we obtained good reactivity. We observed that the developed ELISA assays to detect anti-core and anti-E2 was positive compared to control sera positive for HCV and amplified with results obtained using the mixture of recombinant proteins (core+E2).

In the capture ELISA, the monoclonal antibody anti-GST gave adequate guidance of the recombinant proteins fused to GST, increasing the reactivity of anti-core and anti-E2 compared to control serum positive for HCV, offering greater specificity and sensitivity of the method.

Correlating the immunoblotting test for HCV developed with the ELISA reaction and qualitative PCR showed sensitivity and specificity of 100%, since there was no test conducted with the developed immunoassay that was different from the results obtained by ELISA and commercial qualitative PCR. Tests of indirect and capture ELISA showed 100% sensitivity and 87.5% specificity. Why are tests developed *in-house* and not on an industrial scale, the tests showed satisfactory performance compared to these commercial methods.

5. Conclusion

The immunoreactivity of the recombinant proteins (core and E2) produced in a heterologous expression system (*E. coli* Rosetta strain bacteria) and then purified was confirmed via recognition by anti-HCV antibodies present in hepatitis-C-positive control serum through the techniques of Western Blot, Slot Blot, ELISA (capture and indirect), and immunoblotting. In these trials, there was no recognition of recombinant proteins in the presence of control serum negative for HCV and positive for hepatitis B, Chagas disease, syphilis, ASO, and ANA. Moreover, the GST protein was not recognized by HCV-positive control serum, indicating its use as a tool in viral diagnosis. The use of monoclonal anti-GST antibody, in capture ELISA, made it possible to properly orient the recombinant proteins fused to GST, increasing the reactivity of anti-core and anti-E2 present in the HCV-positive serum pool.

The assay of both immunoblotting and enzyme immunoassay (indirect and capture) using the mixture of the recombinant proteins (core+E2) was demonstrated to be more specific and sensitive.

The paper showed that there is the possibility of constructing commercial immunochromatographic test to detect anti-HCV antibodies using recombinant proteins core-GST, GST and E2-mixture (core-GST and E2) to a low cost because the reagents were produced during method development sensitivity and specificity was 100%.

Acknowledgments

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