

Agarose Slide Elisa for Diagnosis of HCV-AB in Rural Areas

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Abstract: Hepatitis C virus (HCV) infection is a viral infection of the liver affecting 170 million people around the world. Commercially available tests for HCV today are based on enzyme immunoassay (EIA) for detection of HCV-Ab. Regarding the sophisticated equipments required for diagnosis of HCV, this work targeted offering a dependable field serological test that can be used in rural areas as preliminary screening test for HCV patients. Hundred and fifty serum samples were collected from Jazan clinics in Saudi Arabia for HCV patients and 50 serum samples were collected from healthy volunteers. All samples were tested for HCV-Ab using slides coated with 1.5% agarose and saturated with HCV capsid synthetic peptide (BIORAD) (10%). Fifty microliter of each sample were applied onto spot of HCV-Ag saturated agarose and incubated in humidified incubator for 90 min at 37°C. After which the slides were washed. Mouse antihuman IgG labeled conjugate was added to the slides and incubated for 30 min at 37°C. After washing the slides, 50µl substrate (BIORAD) were added and incubated for 30 min at room temperature then washed out. The slides were examined by naked eye and by light microscope against controls. The obtained results were compared with standard Monolisa HCV Ag-Ab Ultra assay and both results were matched. The results proved that agarose slide EIA have accuracy exceeding > 99%. So, agarose slide EIA can be suggested for use in preliminary diagnosis of HCV in the field clinics of rural areas or even in screening of blood donors in emergency cases.

[Mohammed M. Safhi and Hussien A. Abouelhag. **Agarose Slide Elisa for Diagnosis of Hcv-Ab in Rural Areas.** *L Life Sci J* 2013;10(1):1879-1884] (ISSN:1097-8135). <http://www.lifesciencesite.com>. 270

Key words: HCV, slide ELISA, HCV EIA, agarose and rural areas.

1. Introduction

Hepatitis C virus (HCV) infection is a viral infection of the liver which is referred to as parentally transmitted “non A, non B hepatitis”. HCV is an enveloped RNA virus in the flaviviridae family which appears to have a narrow host range (*Bachy et al., 2010*).

WHO estimated that about 170 million people, 3% of the world’s population, are infected with HCV and are at risk of developing liver cirrhosis and/or liver cancer (Hepatocellular carcinoma) (*Li et al., 2012*). The prevalence of HCV infection in some countries in Africa, the East Mediterranean, south East Asia and the Western Pacific is high compared to some countries in North America and Europe (*Zamani et al., 2007*).

In both developed and developing countries, high risk groups include injecting drug users, recipients of unscreened blood, haemophiliace, dialysis patients and persons with multiple sexual partners who are engaged in unprotected sex (*Crofts et al., 1997 and Kassaian et al., 2012*).

Diagnostic tests for HCV are used to prevent infection through screening of donor blood and plasma, to establish the clinical diagnosis and to make better decisions regarding medical care cost and medical management of a patient (*Schnuriger et al., 2006*).

Diagnostic tests commercially available today are based on enzyme immunoassay (EIA) for the detection of HCV specific antibodies. EIAs can detect

more than 95% of chronically infected patients. A recombinant immunoblot assay (RIBA) that identifies antibodies which react with individual HCV antigen is often used as supplemental test for confirmation of a positive EIA result. Testing for HCV circulating by amplification test RNA (PCR) is also being utilized for confirmation of serological results as well as for assessing the effectiveness of antiviral therapy (*Kim et al., 2006*). So RIBA and PCR viral load is considered to be highly confirmatory tests for HCV which can be carried on in the central clinics.

In this work slide EIA was developed to simplify the process of screening for HCV in rural areas where there is no availability of refined equipments. So, the agarose slide ELISA assay may be suitable for the primary screening for HCV of positive and negative HCV Ab individuals in rural areas and also in screening of blood donors in emergency cases.

2. Materials and Methods

Slides preparation:

Halls were made by a metallic ring 0.5 cm in diameter in previously coated glass slide with paraffin at 56°C and cooled to 4°C later.

The previously made halls were filled with 1.5% agarose previously cooked at 100°C for 10 min and cooled to 45°C before pouring in the halls (50 µl in each well). This loaded agarose was saturated at 45 °C with 10% HCV capsid synthetic peptide (Biorad) with the same concentration provided by manufacture just

before pouring. The coated slides were kept at 4°C in humidified sealed slide box till use.

Sample collection:

Five ml of blood sample were collected from 150 established HCV infected patients in Jazan clinics and 50 samples were collected from healthy volunteers. For keeping the privacy instead of a block for the patient's name, the test tube had an anonymous identification code. The blood samples were cooled to 4°C for 60 min then centrifuged at 3000 rpm for 30 min where the serum samples were collected and separated in clean sterile tubes.

Sample application:

Fifty microliter of serum samples were applied onto the surface of previously prepared saturated agarose with HCV Ag slides and incubated in inverted position at 37°C for 90 min.

Ag-Ab reaction:

The slides were washed thoroughly with 20 ml of washing solution (Tris NaCl buffer pH 7.4 contains Proclin 0.04%). After that the slides were soaked in coplin jar containing the washing buffer. After 10 min the slides were removed from coplin jars and blotted from edges on filter papers to remove excess of buffer remains. After which 50 µl of conjugate (anti-human mouse IgG /peroxidase and streptavidine/peroxidase green colored) were added directly on the slides and incubated at inverted position in humidified chamber at 37°C. After 30 min the slides were washed as mention before and blotted from edges on filter papers to remove excess of buffer remains. The substrate chromogen [citric acid and sodium acetate solution pH 4 containing H₂O₂ (0.015%)] and DMSO 4% containing tetramthyl benzidine (TMB)] was added onto the slide and incubated in inverted position at 37°C. After 30 min the excess of the substrate were removed by blotting against filter paper and the agarose spots were covered with three evenly distributed drops of mountain medium. The slides were covered by cover slips with avoiding undue pressure. The slides were examined by nicked eyes comparing with control positive and control negative. The positive results were confirmed by examining under light microscope.

Quality control:

The HCV negative and positive obtained results were compared with regular HCV Monolisa HCV Ag-Ab Ultra assay [Biorad] which was carried out according to Lambert *et al.*, (2004) and the obtained results were matched statistically according to Schnuriger *et al.*, (2006). The results of Monolisa HCV Ag-Ab Ultra assay are expressed as the ratio of the absorbance to the cutoff; a ratio above 1 is considered a positive result. The sensitized threshold (gray zone) was also considered at a ratio of 0.5 (Laperche *et al.*, 2003, Laperche *et al.*, 2005 and Ansaldi *et al.*, 2006).

Statistical analysis:

The statistical analysis was performed according to Bailey (1994).

The research protocol was approved by the Ethical Committee of College of Pharmacy, Jazan University.

3. Results

The agarose slides were examined by naked eye for the presence of bluish coloration of the positive HCV tested samples and light yellowish coloration for the negative HCV tested samples as shown in figure (1). The obtained results as illustrated by Fig.(1) cleared that the negative HCV samples (Fig.1D) gave a yellowish coloration same as that given by uninoculated slide (fig.1E), while the positive HCV tested samples (Fig.1A & B and Fig. 2) gave a bluish coloration same as that given by control positive HCV (Fig. 1C). The intensity of the color was directly proportional to the titer of ELISA unites obtained by Monolisa HCV Ag-Ab Ultra assay.

It was remarkably noticed that the color intensity of the HCV positive samples (Fig.2) was varied among them and this was referred to the concentration of the reaction between the bonded anti-HCV IgG and the loaded HCV viral capsid Ag in the agarose and this also was confirmed by the microscopical examination of the tested slide as shown in figure (3). The microscopic examination of HCV positively reacted agarose slides under power 200 and 400X cleared that the bluish coloration referred to the bluish purple formazan crystals formed by the tetramthyl benzidine (TMB) present in the substrate that react with the anti-human IgG conjugate enzyme.

It was recorded that the bluish coloration of HCV positive samples was varied from light blue to deep bluish coloration and the bluish coloration intensity distribution in the same spot was varied from central point of agarose spot to the spot periphery, however no bluish coloration was recorded in the HCV negative control or normal control samples. According to the obtained color intensity the HCV positive slides were graded from (+ve) to (++++ve) by naked eye evaluation.

When the obtained results and color intensity of HCV positive slides were compared with the Monolisa HCV Ag-Ab Ultra assay reading it was clear that the obtained results by naked eye is matching (99.66%) as described in figure (4) and (5). However by examining figures (4) and (5) it was recorded that only one sample out of 150 HCV positive samples gave negative reaction by EIA agarose slide while it gave positive reaction with Monolisa HCV Ag-Ab Ultra assay with O.D. 1.309 nm.

The statistical analysis of the obtained results from Monolisa HCV Ag-Ab Ultra assay optical density and positive slides coloration gave an idea about the

accuracy of the results obtained by Monolisa HCV Ag-Ab Ultra assay were significant ($P \leq 0.05$) (Table 1).

The obtained results in Fig. (4) and (5) showed that the ratio of sample O.D. / cut off O.D. > 1.15 was estimated in the group ++++ ve (86 samples), from 1.10 – 1.15 was estimated in the group +++ ve (26 samples), from 1.05 – 1.10 was estimated in the group ++ ve (10 samples), the ratio of sample O.D. / cut off O.D. from 1.00 - 1.05 was estimated in the group + ve (28 samples) as shown in figure (4). The reading O.D. from 0.5 to 1 (gray zone) was estimated in HCV –ve samples.

The estimated reading and color matching referred that nearly 99.66% matching where there was

only one HCV positive sample deviation and the bluish coloration could not be detected by naked eye however some purple fermazan crystals could be detected by microscopical examination but it can't be counted for HCV positive sample where the designed test depends on the ocular examination only.

The statistical analysis of the obtained results from serological examination of HCV positive samples using HCV Monolisa Ag-Ab Ultra Assay where illustrated in table (1). The Population Standard deviation ≤ 0.05 .

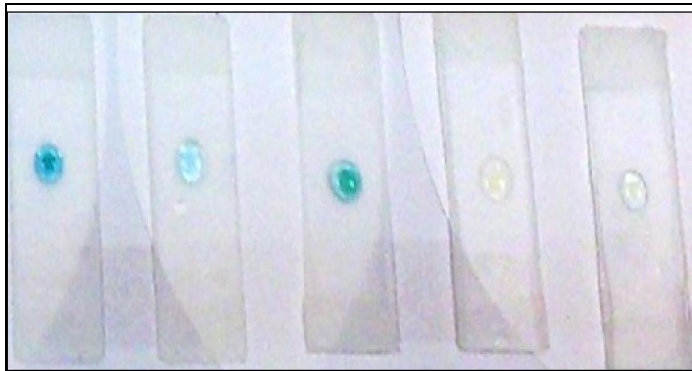


Figure (1): Illustrates the reaction of control negative (D) and HCV control positive (C) and different HCV positive samples (A and B) in addition to uninoculated agarose slide (E) incubated and treated under the same conditions.

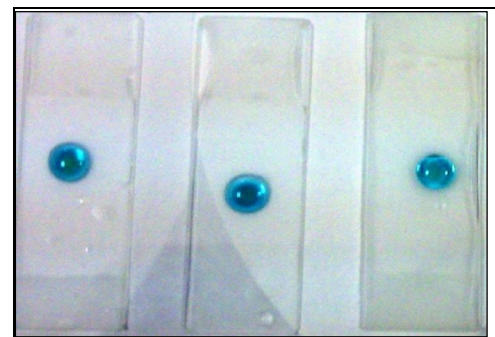


Figure (2): Illustrates the reaction of different HCV positive samples (A, B and C).

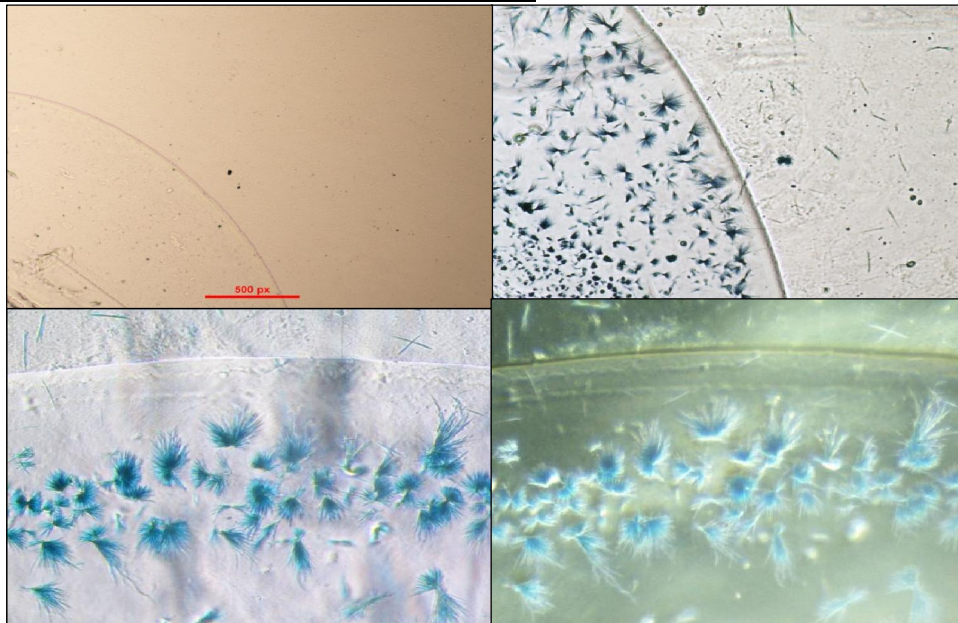


Figure (3): Reveals the reaction of HCV negative sample (A) and HCV positive samples (B) under microscope (200X). With higher magnification power (400X) HCV positive samples were shown in C and D which gave an idea about the cause of bluish color which is referred to the purple fermazan crystals formation of the reacted conjugated substrate.

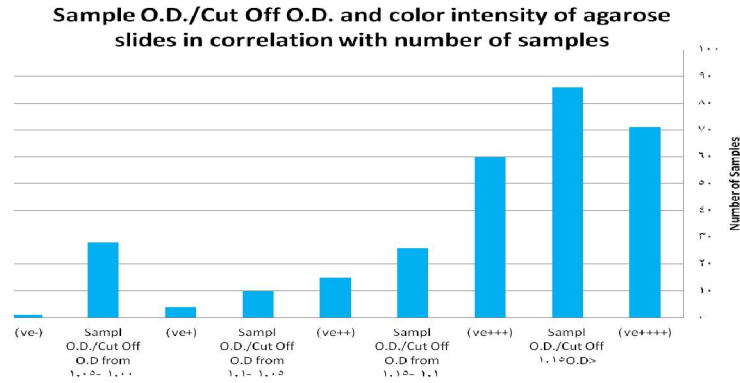


Figure (4): Illustrates the HCV positive groups where the O.D got from HCV Monolisa HCV Ag-Ab Ultra assay were compared and linked to the color intensity. The O.D. was ranged from 2.9 to 0.06 nm as the cut off was 1. The showed result confirmed the matching of reaction of HCV positive agarose tested samples and the obtained results from Monolisa HCV Ag-Ab Ultra assay except for one sample.

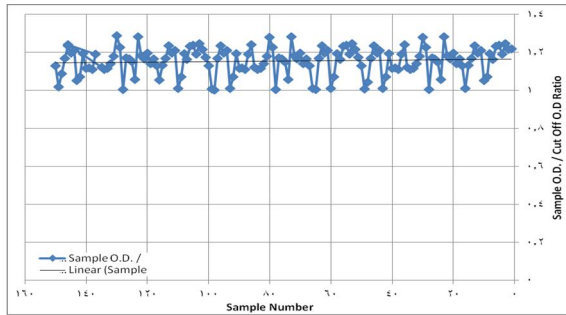


Figure (5): Illustrates the ratio between the HCV samples O.D. / Cut off O.D. using HCV Monolisa HCV Ag-Ab Ultra assay where all samples gave positive reaction (O.D. \geq 1.00 nm).

Table (1): illustrates the statistical analysis of the obtained results from serological examination of HCV positive samples using HCV Monolisa Ag-Ab Ultra Assay.

Total Numbers:	150
Mean (Average):	1.15471
Standard deviation:	0.07388
Variance(Standard deviation):	0.00546
Population Standard deviation:	0.07364
Variance(Population Standard deviation):	0.00542

4. Discussion

The diagnosis of HCV serologically depends on the detection of HCV Ag or Ab where the HCV Ab considered being the mirror that reflects the infection or previous exposure to HCV (Lee et al., 2011). Regarding that HCV is a chronic disease that affects man and results in a large number of mortality around the world the detection of the disease in early phase rarely happens (only in severe acute form) depending on the clinical signs so the serological diagnosis takes its importance in diagnosis of HCV in apparently health patients. This can be achieved by serological

screening of people at risk in endemic areas (Mohamed et al., 2012).

Majority of endemic areas located in third world have hygienic measurements of low standards and suffers from low equipped laboratories. This makes the screening process is difficult and costly in rural areas (Grijalva et al., 2005).

So, the development of simple agarose slide technique depending on high technology bases for diagnosis of HCV is important. The accuracy of this toll should be also of high level regarding how serious this disease is.

The early diagnosis of hepatitis C virus (HCV) infection is crucial to prevent further transmission in high-risk groups and to allow clinicians to make a rapid decision about treatment, which has been proven to have a high degree of efficacy for acute hepatitis C (Gerlach et al., 2003).

Strategies that can be used to improve the ability to diagnose the infection before seroconversion require additional direct tests, such as detection of viral RNA by PCR or detection of HCV core antigen (Ag). However, genomic tests, which are limited by the requirement for specific equipment, a long delay in the time to a result, the risk of contamination, and high costs, cannot be used as routine screening tools (Schnuriger et al., 2006).

The used technique is developed in order to overcome the problem of inadequate instrumentation available in the rural areas or regarding the time shortening during the accidental need for blood transfusion in poorly equipped areas.

The preliminary results of the agarose EIA slide test showed a perfect match with Monolisa HCV Ag-Ab Ultra assay where only one sample out of 150 positive HCV samples had given false negative result by eye examination. This negative sample by microscopical examination showed dispersed purple

crystals which confirm the presence of a positive reaction as happen in correlated case by **Gritti et al., (1998)**. This positive reaction seemed to be not enough to give detectable coloration by naked eye. However, this positive reaction was recorded by microscopical examination but it can't be countable for agarose EIA slide test as this test depends upon getting a clear reading by examination with naked eye. It was also recorded that the false positive agarose slide tested sample give a very low positive O.D./ Cut Off O.D. = 1.005714 when examined by ELISA reader at 450/630 nm wave length and this gave an idea about the matching in results between the two tests as the results were directed parallel to each other.

This confirmed the assumption that the lowest concentration of anti-HCV IgG can't be detected easily by naked eye in agarose slide EIA. However in addition to that false positive represented only 0.33%. Conversely samples of closer O.D. to this sample showed a clear positive reaction with agarose EIA slides which hypothesises that this false negative reaction may be referred to other interfering factors like hyper IgM concentration as in rheumatoid arthritis (*Rf* factor) which may replace the binding site.

Another significant technical notice during slides examination, it was noted that the color intensity of the single positive slide was remarkably varied from the central zone of agarose spot than the peripheral zone of agarose spot. The peripheral zone of agarose spot showed intense blue coloration while, the central zone showed lighter blue coloration, yellow in some cases, and this may be referred to the place where the forceful reaction happened. The reactin between the HCV IgG and the conjugated anti-human IgG occurred in the superficial area of agarose spot only and not exceeded to the deeper layer due to three main logically anticipated reasons: (1) short duration of incubation period, (2) narrow agarose meshes due to the high concentration of agarose matrix (1.5%) which gave no chance for deeper involvement of the reaction, (3) the effect of gravity and surface tension during the samples application which swipe the sample to the periphery. So, the high agarose concentration offers a saturated glassy surface where the reaction came to pass.

This agarose EIA slide might be considered a helping facility for diagnosis of HCV after seroconversion (two months after exposure). However, these slides are directed against HCV- IgG only but it can be modulated for detection of HCV- IgM too but it is not suitable for diagnosis of HCV in core window. So, this test can't replace HCV – PCR for early detection of HCV in core window and also it is main confirmatory test for HCV in use nowadays

(*Bouvier-Alias et al., 2002; Veillon et al., 2003 and Perelson et al., 2012*). The currently proposed assay is not a confirmatory assay, it is designed as a screening assay with equipment demand adapted for use in rural areas and emergency and field clinics and this is in harmony with what mentioned by *Miriam et al., (2003)* in the CDC report.

Such an improvement may be particularly useful for the diagnosis of HCV infection in high-risk groups, where early infection detection may help reduce secondary transmissions and rapid referral for treatment. Because of its relatively low cost, its ease of performance, and the significant decreased window period, we believe that this assay will improve the overall quality of diagnosis of HCV infection for a moderate overall cost compared to those of serological assays and should therefore be applied as a screening assay in the general population.

We must emphasize that this assay is not intended to replace HCV RNA detection, and in the case of acute clinical hepatitis, the use of a sensitive HCV RNA detection assay is highly recommended.

Acknowledge

This work wouldn't finish in such manner without the scientific support from National Research Centre, Dokki, Egypt and the scientific cooperation with Pharmacy College, Jazan University, Kingdom of Saudi Arabia.

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