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Laboratory diagnosis of viral hepatitis C
The Sultan Qaboos University Hospital experience

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التشخيص المختبري لالتهاب الكبد الفيروسي ج:
تجربه مستشفى جامعة السلطان قابوس

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المستخلص: الهدف: دراسة إستعادية لمعرفة نسب الإصابة بالتهاب الكبد الفيروسي ج بين المتبرعين بالدم والمرضى ولتقييم فاعلية الطرق التشخيصية المتبعة بمختبر الأحياء الدقيقة والمناعة بمستشفى جامعة السلطان قابوس **الطريقة:-**بين عام ١٩٩١ و ٢٠٠١ تم فحص حوالي ٥٥٠٠٠ مصلا للأجسام المضادة لإلتهاب الكبد الفيروسي ج بثلاثة أجيال من ELISA. كذلك أعيد فحص كل الأمصال الإيجابية بطريقة الـ RIBA. كما تم فحص ٢٤١ مصلا بطريقة سلسلة تفاعل البوليماريز العكسية (RT-PCR). **النتائج:** من مجموع ٣٠٠١٢ مصلا من المتبرعين بالدم وجد أن (٠,٩١%) ٢٧٢ مصلا تحتوي أجساما مضادة، منها ٤٦,٥% تم تأكيد إيجابيتها بطريقة RIBA. كما تراوحت نسب الأمصال الإيجابية بين مدمني المخدرات ومرضى الإلتهاب الكبدي ومرضى الدم ٩٥%، ٨١% و ٧٠% بالتتالي. أما نسب النويات الحمضية من الأمصال الإيجابية والغير محددة والسلبية بطريقة RIBA فقد كانت ٦٧%، ٦% و ٠% بالتتالي. **الخلاصة:** أثبتت الدراسة أن نسبة الأجسام المضادة لإلتهاب الكبد الفيروسي ج بين المتبرعين بالدم بعمان حوالي ٠,٥% بطريقة RIBA. كما تشير تجربتنا الى أن ثلثي المصابين بإلتهاب الكبد الفيروسي ج تكون أمصالهم إيجابية بطريقةRIBA. **مفتاح الكلمات:** فيروس إلتهاب الكبد ج، الايسا، ريبا، سلسلة تفاعل البوليماريز العكسية

ABSTRACT. ***Objectives:*** A retrospective study was carried out to assess the performance of hepatitis C diagnostic assays in our laboratory, and to determine the prevalence of hepatitis C among blood donors at the Sultan Qaboos University Hospital. ***Methods:*** From 1991 to 2001, approximately 55,000 serum samples collected from blood donors and patients were submitted to our laboratory for testing. All sera were screened for antibodies to hepatitis C virus (HCV) by three successive generations of the enzyme-linked immunosorbent assay (ELISA). Anti-HCV positive sera were further tested by recombinant immunoblot assay (RIBA). Reverse-transcriptase polymerase chain reaction (RT-PCR) for HCV RNA was carried out on a limited number (241) of ELISA positive samples. ***Results:*** Out of 30012 samples from blood donors that were screened for anti-HCV, 272 (0.91%) were positive. Of these, 46.5% were confirmed positive by RIBA. The proportion of patient sera that were confirmed positive varied from 95% among intravenous drug users to 81% in patients with hepatitis to 70% in those with haemoglobinopathies. HCV RNA was detected in 67%, 6%, and 0% of the RIBA positive, indeterminate and negative samples respectively. ***Conclusions:*** Based on RIBA, the prevalence of anti-HCV among blood donors in Oman is close to 0.5%. In our experience, RIBA-positivity is predictive of HCV infection in two thirds of subjects, and HCV infection is highly unlikely in those who are RIBA-negative. The experience at SQUH with three types of HCV assays has enabled the laboratory to develop a test algorithm, starting with screening anti-HCV ELISA.

Key words: hepatitis C virus, ELISA, RIBA, polymerase chain reaction.

Hepatitis c virus (hcv) is a blood-borne pathogen that appears to be endemic in most parts of the world. It is estimated by the World Health Organization that there are 170 million HCV-infected persons worldwide.[1] Currently, HCV is the leading cause of post-transfusion hepatitis and end-stage liver disease requiring liver transplantation.[2] The disease it causes is characterised by silent onset, a high rate of viral persistence, and the potential for development of chronic liver disease, ranging from chronic hepatitis to cirrhosis and occasionally hepatocellular carcinoma.[3]

The discovery of the genome of HCV in 1989 by Choo et al[4] paved the way for development of serological and molecular assays for viral hepatitis C. In the first generation of an enzyme-linked immunosorbent assay (ELISA), wells of microtitre plates were coated with purified recombinant antigen c100-3 that was derived from the non-structural 4 (NS4) region of the HCV genome [Figure 1]. However, ELISA-1 was associated with a high percentage (50% to 70%) of false positive results among low-risk blood donors and in the presence of hyperglobulinemia.[5] Thus, second-generation anti-HCV ELISAs were developed. ELISA-2 by Ortho Diagnostics contained recombinant antigens from the core (c22-3), NS3 region (c33c), and NS4 region (c100-3) as well as a part of c100-3, named 5-1-1 [Figure 1]. Third generation anti-HCV ELISA was introduced in Europe in 1993 and in the USA in 1996. In addition to the antigens of ELISA-2, third-generation anti-HCV ELISA uses an antigen of the NS5 region of the viral genome. However, synthetic pep-

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tide antigens (c22 and c-100) replaced recombinant antigens of ELISA-2 [Table 1, Figure 1]. Other manufactures, for example Abbott Diagnostics, used recombinant antigens derived from the same regions of HCV genome.

Despite increased sensitivity and specificity with each generation of ELISA, false-positive antibody results continue to be observed, particularly among low-risk blood donors.[6] Thus, supplemental or confirmatory assays were developed in parallel with ELISA. The recombinant immunoblot assay (RIBA) has been used extensively to confirm presence or absence of antibody to HCV epitopes. In RIBA recombinant or peptide HCV antigens are blotted as separate bands onto a nitrocellulose strip flanked by a weak-positive (Level I) and a moderately positive (Level II) strip control [Figure 2]. Although technically more demanding than ELISA, the RIBA identifies antibodies to individual HCV antigens and therefore has higher specificity than ELISA.[7]

Since ELISA and RIBA are antibody tests, positivity of either one or both does not necessarily indicate current HCV infection as patients who have recovered from infection may remain anti-HCV positive for many years.[7] Conversely, during seroconversion, antibody tests may be negative.[8] The direct molecular qualitative detection of HCV RNA by reverse-transcriptase polymerase chain reaction (RT-PCR) is considered the gold standard for the diagnosis of HCV infection.[6]

Testing for HCV was introduced at the Sultan Qaboos University Hospital (SQUH) in 1991, starting with the first-generation ELISA. In subsequent years, new generations of both ELISA and RIBA were used as they became commercially available. The RT-PCR was introduced in the year 2000. We report here our results and experience in testing blood donors, patients suspected to have viral hepatitis, and other subjects from 1991 to 2001.

Table 1. ***Antigens incorporated in serological assays (ELISA and RIBA) for hepatitis C.***

Assay	ELISA	RIBA
First generation	c100-3	c100-3
		5-1-1
Second generation	c100-3	c100-3
	c22-3	5-1-1
	c33c	c22-3
		c33c
Third generation	c100-3	c100-3/5-1-1 (peptide)
	c200 (c100-3 + c33c)	c22-3 (peptide)
	c22-3	c33c
	NS5 recombinant antigen	NS5 recombinant antigen

MATERIALS AND METHODS

SERA. Serum samples for HCV testing were collected from blood donors and patients mainly at SQUH. A few were sent from other hospitals within the capital area

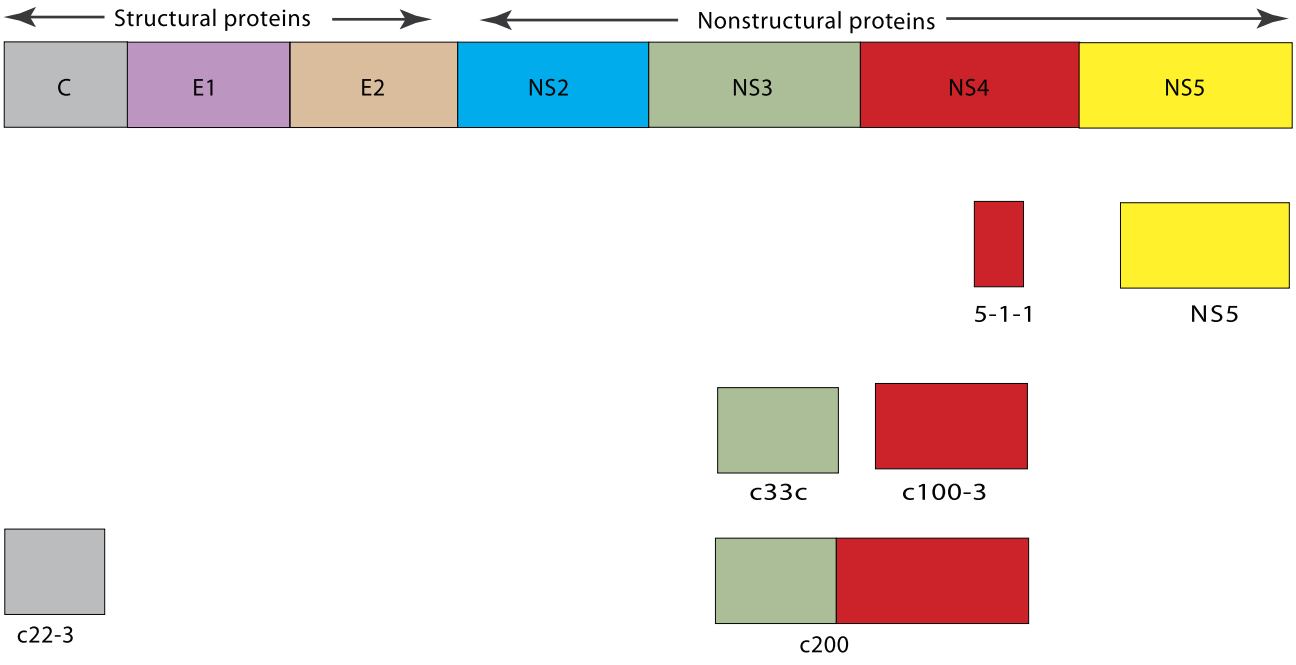

Figure 1. *Genome organization of HCV and antigens licensed for diagnostic use.*

Table 2. *Prevalence of antibodies to hepatitis C virus among blood donors*

Year	No. tested	No. positive	% positive
1991*	668	6	0.9
1992	2596	34	1.3
1993	3584	51	1.4
1994	2033	23	1.1
1995	2330	20	0.86
1996	2637	25	0.95
1997	2638	26	0.99
1998	2817	20	0.71
1999	2996	19	0.63
2000	3290	23	0.7
2001	4423	25	0.57

*First, second and third generation anti-HCV ELISAs were used to screen blood donors in 1991, 1992–1996, and 1997–2001, respectively.

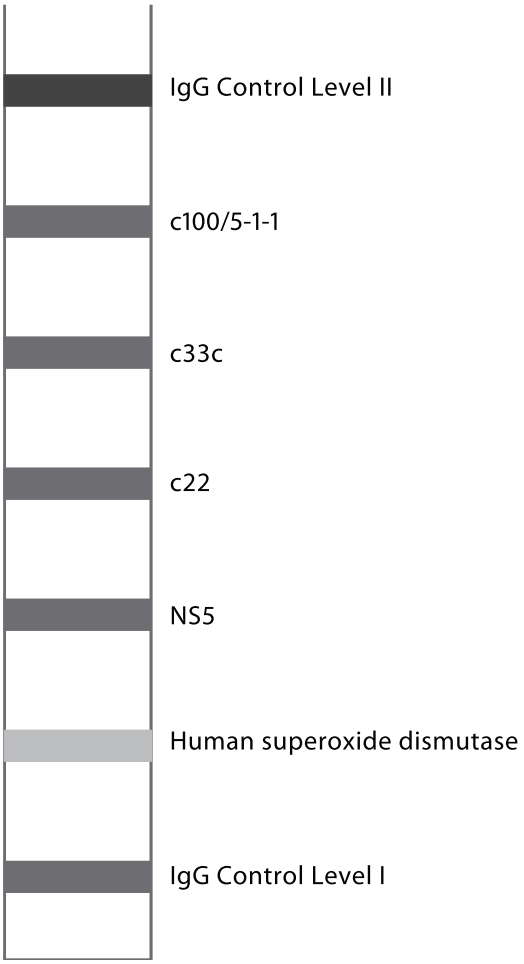

Figure 2. *Identity and location of HCV antigens on a nitrocellulose strip of the recombinant immunoblot assay*

of Muscat. In 1991, approximately 1,350 sera were tested. In 1992, the figure had trebled, and by 2001, it reached 6,500. Over a period of 11 years, approximately 55,000 serum samples were submitted for HCV testing.

SCREENING FOR ANTI-HCV. All sera were first screened for the presence of antibodies to HCV. In 1991, the mictotitre-based, first-generation ELISA (HCV c100-3 ELISA, Ortho Diagnostic Systems, Raritan, NJ) was used. Between 1992 and 1996, different formats of second-generation enzyme immunoassays (Abbott Diagnostic Division, Germany) were used in the following chronological order: HCV EIA on Quantum™ instrument, micro particle enzyme immunoassay on IMX,™ and micro particle enzyme immunoassay on Axsym™ analyser. The three analysers were from Abbott laboratories, Diagnostic Division, USA. From 1997 to 2001, sera were screened by a third-generation micro particle enzyme immunoassay on the Axsym.™ Samples that were initially reactive by ELISA were retested in duplicate, and results were interpreted according to manufacturers' instructions.

SUPPLEMENTARY ASSAY. The second-generation recombinant immunoblot assay (RIBA HCV 2, Chiron Corp., Emeryville, CA) was introduced in our laboratory in mid-1994, and was replaced by third generation assays (RIBA HCV 3, Chiron and HCV Blot, Genelab Diagnostics, Singapore) by the end of 1996. All sera that were repeatedly reactive in ELISA were subjected to supplemental testing by RIBA according to manufacturers' recommendations. In these assays specimens were considered positive if they demonstrated reactivity to two or more antigen bands at an intensity greater than, or equal to the weak positive control. Specimens reacting with a single antigen band were classified as indeterminate and specimens producing no reactive bands or bands with an intensity less than the weak positive control were classified as negative.

HCV RNA DETECTION. HCV RNA was assayed on plasma or serum samples using the HCV Monitor (v2.0) RT-PCR kit on the COBAS AMPLICOR system (Roche Diagnostics, Switzerland).

DATA STORAGE. Demographic, clinical and laboratory data of all subjects whose samples tested positive for anti-HCV by ELISA were kept in a Microsoft Access database for retrieval and analysis.

RESULTS

Of the approximately 55,000 serum samples that were screened for anti-HCV antibodies from 1991 to 2001, 30,012 were from blood donors. The annual anti-HCV positivity rate among blood donors varied from 1.4%

Table 3. *Confirmatory assay of anti-HCV ELISA positive samples from blood donors and patients.*

Clinical category	No. samples ELISA positive	No. tested by RIBA	% RIBA positive	% RIBA indeterminate	% RIBA negative
Liver disease	406	340	80.9	13.8	5.3
Thalassaemia	840	628	69.6	19.8	10.7
Sickle cell disease	126	111	71.2	17.1	11.7
Intra-venous drug abuse	25	22	95.5	4.5	0
Other diseases	133	107	61.7	27.1	11.2
Disease not specified	237	105	60.9	25.7	13.3
Blood donors	272	144	46.5	23.6	29.9
Total	**2039**	**1457**	**69.3**	**19.3**	**11.5**

in 1993 to 0.57% in 2001, and the mean rate was 0.91% [Table 2]. On average, proportionately more samples tested positive in second generation ELISA than in third generation.

Of a total of 2,039 sera that were reactive by ELISA from 1994 to 2001, 1,457 (71.5%) were tested by RIBA [Table 3]. The proportion of samples confirmed positive for anti-HCV antibodies varied widely. It was high among intravenous drug users (95.5%) and patients with liver disease (80.9%), intermediate in patients with haemoglobinopathies, and low among blood donors (46.5%) [Table 3]. The majority of patients with liver disease had hepatitis, liver cirrhosis or hepatocellular carcinoma.

The impact of changing from second to third generation assays is summarized in table 4. Although third generation RIBA confirmed more positive samples than second generation RIBA, it was also associated with slightly more indeterminate results.

Plasma samples from 241 ELISA positive subjects were assayed by HCV RT-PCR. These samples were collected in 2000 and 2001. Overall HCV RNA was detected in (49.8 %) of the subjects. When results were analysed according to RIBA-3 status, 67.4%, 5.7% and 0% of the RIBA positive, inderterminate and negative subjects respectively had HCV RNA [Table 5].

DISCUSSION

The introduction of anti-HCV ELISA to screen blood donors has led to a dramatic reduction in post-transfusion non-A non-B hepatitis, and the detection of antibodies to HCV has become the most practical means of diagnosing infection.[5] Over a period of 11 years, three generations of ELISA were used at SQUH to screen healthy subjects, particularly blood donors, and patients for anti-HCV and two generations of RIBA as supplementary test.

Based on ELISA, the prevalence of HCV among blood donors in Oman is close to 1%. However, since only about half of these were confirmed by RIBA, the true prevalence is about 0.5%. On the basis of studies among blood donors that used both ELISA and supplemental testing the lowest anti-HCV prevalence rates (0.01–

Table 4. *Comparison of detection rates of anti-HCV antibodies by second and third generation serological assays*

Generation ELISA and system	% RIBA-2			% RIBA-3		
	Positive	Indeterminate	Negative	Positive	Indeterminate	Negative
ELISA-3 Quantum (n=117)	61.5	12.8	25.6	–*	–	–
ELISA-2 IMX(n=222)	59.8	21.0	19.2	–	–	–
ELISA-2 AxSYM(n=249)	54.2	13.3	32.5	–	–	–
ELISA-3 AxSYM(n=869)	–*	–	–	69.9	20.8	9.3

*ELISA-positive samples were re-tested by either second, or third generation RIBA. None of the samples was tested by both RIBA-2 and RIBA-3.

0.1%) have been reported from the United Kingdom[9] and Scandinavia.[10] Low but slightly higher rates (0.2–0.5%) have been reported from Western Europe, North America, most areas of Central and South America, and limited regions of Africa.[2] The rate reported here for Oman falls into this group. Intermediate rates of anti-HCV prevalence (1–5%) have been found in other countries, including neighbouring Yemen[11] and Saudi Arabia.[12]

The proportion of anti-HCV ELISA positive samples that were confirmed by either RIBA-2 or RIBA-3 varied from 47% in blood donors to 80% in patients with a clinical diagnosis of hepatitis. These findings are consistent with reports by other investigators.[13–15] In populations at low risk for hepatitis C, such as blood donors, the proportion of samples confirmed positive by RIBA is low, and varies from 17% to 37%.[9,13,14] On the other hand, most (80%–90%) of ELISA-2 or ELISA-3 positive patients with chronic liver disease are RIBA positive.[13,15–17]

Table 5. ***Comparison of third generation RIBA and PCR***

RIBA-3 results	% PCR positive	% PCR negative
Positive(n=175)	67.4	32.6
Indeterminate(n=35)	5.7	94.3
Negative(n=31)	0	100

The introduction of third generation RIBA was reported to have resolved many of the indeterminate samples of RIBA-2.[14,18,19] In our experience, this was not the case. The proportion of indeterminate samples remained at about 20% when the laboratory changed from second to third generation assays.

The results presented here show that HCV RNA was detectable in 67% of RIBA-positive samples, in 6% of RIBA-indeterminates and in none of the samples that were RIBA negative. The mean detection rate was close to 50%. Although our data is based on a small number of subjects, it is comparable to data from larger studies involving blood donors and individuals with normal alanine aminotransferase (ALT) levels.[7] In these subjects, the percentage that have detectable HCV RNA in serum when tested by PCR assay varies from 70% to 80% for those who are RIBA positive to 2% to 40% for those who are RIBA indeterminate, to none among those who are RIBA negative, giving an overall detection rate of 35% to 45%.

Three types of assays (ELISA, RIBA and PCR) were used at SQUH to test for HCV infection. Do diagnostic laboratories need to use all three? Different diagnostic algorithms have been proposed which reflects different opinions on this subject.[5–7] There can be no question on the utility of ELISA as a screening test for all subjects. The need for and the choice of supplementary and confirmatory tests depend on the clinical setting and the likelihood of a true-positive ELISA result. In general, qualitative PCR assay for serum or plasma HCV RNA is the best confirmatory assay and should be used in ELISA positive patients who present with chronic liver disease. There is no need for doing RIBA in such cases. However, ELISA-positive blood donors and individuals with normal ALT levels may be evaluated by RIBA first, PCR for HCV RNA being performed only on those who are RIBA positive or indeterminate.

CONCLUSIONS

At SQUH, we have used ELISA and RIBA to detect antibodies to HCV as means of diagnosing hepatitis C. These two tests are relatively easy to perform. The recent introduction of HCV PCR assay has provided the laboratory with the tool to confirm presence of the virus, either in those who have anti-HCV antibodies, or those who lack antibodies but are suspected to be infected.

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