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Evaluation of the Amplicor HCV Test: Experiences after 1 Year of Routine Use in a Diagnostic Laboratory

Summary: To evaluate the usefulness of a standardized commercial amplification assay for detection of hepatitis C virus (HCV) RNA, Amplicor® HCV Test (Roche), a total of 1,204 serum samples from 888 patients was examined. Seven out of 443 anti-HCV-negative samples, 638 out of 729 anti-HCV-positive samples and four out of 32 anti-HCV-indeterminate samples tested HCV RNA positive by Amplicor® in initial testing. One false-negative and five false-positive Amplicor® results were found in initial testing, giving an overall sensitivity and specificity of the Amplicor® HCV Test of 99.8% and 99.1%, respectively. Our study confirmed that the Amplicor® HCV Test is a practical, rapid, sensitive and specific assay for detection of HCV RNA. However, the results must be interpreted with some caution and with the patient's anti-HCV status and clinical data in mind.

Introduction

Determination of viral RNA in serum and tissue samples using amplification techniques is an indispensable assay in the diagnosis of HCV infection and in monitoring response to its treatment. Polymerase chain reaction (PCR) is currently the most widely used method for detection of HCV RNA [1]. Recently, a standardized PCR kit (Amplicor® HCV Test) for detection of HCV RNA in serum samples was developed by Roche Molecular Systems (Nutley, USA). The first studies which evaluated the usefulness of the Amplicor® HCV Test in comparison to in-house nested PCR showed similar reliability of both assays [2–6]. However, the commercial test offers many technical improvements [7]. It is more rapid and easier to perform than in-house PCR and thus more appropriate for the routine determination of HCV RNA [2–6]. After initial comparison of Amplicor® HCV Test with our in-house nested PCR [5], we decided in April 1994 to include this commercial kit in the routine work in our laboratory. For 1 year we examined more than 1,200 serum samples using Amplicor® HCV Test and acquired some important experiences which are summarized in the present report.

Materials and Methods

Clinical specimens: From April 1994 to April 1995, 1,178 serum samples obtained from 862 subjects with either non-A, non-B hepatitis or risk factors for HCV infection (intravenous drug abusers, hemodialysis patients, hemophiliacs, health-care workers) and 26 serum samples from the same number of healthy blood donors with previously determined anti-HCV indeterminate status were received at the Laboratory for Molecular Microbiology, Institute for Microbiology, Ljubljana, Slovenia. Serum samples were removed from each red blood cell clot 1 to 4 h after venesection and aliquoted into five tubes, which were stored at −70°C until they were thawed for testing.

Anti-HCV testing: All serum samples were tested for the presence of anti-HCV antibodies using the Ortho HCV 3.0 ELISA Test System (Ortho Diagnostic Systems, Neckargmünd, Germany). Reactive sera were supplementarily tested by Chiron RIBA HCV 3.0 SIA (Chiron Corporation, Emeryville, USA). Seven hundred and twenty-nine serum samples were found anti-HCV positive, 443 were anti-HCV negative and 32 serum samples were anti-HCV indeterminate. Among anti-HCV indeterminate samples 26 were obtained from blood donors, five from hemodialysis patients and one from an intravenous drug addict. Results exhibited the following patterns: 16 samples reacted with C22 viral protein exclusively, 11 with C100, three with C33c and two samples with NS5 protein exclusively.

Amplicor® HCV Test: This test was performed as specified by Roche Molecular Systems. Briefly, after sample preparation HCV RNA was reversely transcribed and the resulting complementary DNA was amplified with biotin labeled primers selected from the highly conserved 5' terminus of the HCV genome, using the GeneAmp PCR System 9600 thermocycler (Perkin Elmer Cetus Corp., Norwalk, USA). Amplicons were hybridized in microtiter strips coated with an HCV-specific DNA capture probe. After a washing procedure, biotin-labeled hybrids were detected with a biotin avidin assay. Absorbance was measured at 450 nm. According to the manufacturer's instructions, an optical density (OD) of 0.500 or higher was taken as positive, provided the positive and negative controls fell within the correct limits. To avoid contamination by previously produced amplicons, uracil-N-glycosylase procedure is incorporated in the Amplicor® HCV Test.

All known precautions to avoid sample-to-sample contamination and PCR-product carryover were rigorously taken [8]. Briefly, these included (i) strict physical separation of areas for clinical specimen preparation, amplification and detection, with separate supplies of aerosol resistant tips (ART, Molecular Bio-Products, San Diego, CA) and dedicated pipettors for each area, (ii) meticulous pipetting technique, (iii) frequent changes of gloves, (iv) use of multiple negative controls and (v) daily cleaning of all working areas, test tube holders and sample racks with

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sodium hypochlorite. Additionally, instead of using PCR reaction tube strips and cap strips (as recommended by the manufacturer), we used single MicroAmp Reaction Tubes with caps (Perkin Elmer Corporation, Foster City, CA). PCR tubes were placed in the MicroAmp 9600 Retainer (Perkin Elmer Corporation, Foster City, CA) in such a way that no tube came into direct contact with another PCR tube (i.e. each tube was surrounded by empty sample wells). The entire procedure was performed by highly educated and PCR-specific trained personnel only.

Evaluation of results: According to our self-constructed control procedure, all initially Amplicor® HCV RNA positive/anti-HCV negative serum samples were retested at least once using both the Amplicor® HCV Test and our in-house nested PCR as described previously [5]. All initially Amplicor® HCV RNA-negative/anti-HCV-positive samples were retested at least once using the Amplicor® HCV Test only. All anti-HCV indeterminate serum samples were initially tested by the Amplicor® HCV Test in duplicate. In cases where discrepant results for amplification tests had been obtained both in initial and repeated testing and in all those which tested anti-HCV indeterminate, the responsible physicians were contacted and the clinical data of the patients were reevaluated. Clinical assessment included risk factors for HCV infection, liver function test results, liver histology findings (if available), clinical and laboratory follow-up observations and results of amplification and serological tests obtained with additional specimens from the same patients.

Results

In this prospective study a total of 1,204 serum samples from 888 patients was examined for the presence of HCV RNA using the standardized commercially available PCR assay, Amplicor® HCV Test. Seven out of 443 anti-HCV negative samples, 638 out of 729 anti-HCV-positive samples and four out of 32 anti-HCV indeterminate samples tested HCV RNA positive by Amplicor® in initial testing. After evaluation procedures, four initially Amplicor® HCV RNA-positive/anti-HCV-negative samples obtained from three hemodialysis patients and one intravenous drug addict were considered true HCV RNA-positive in initial testing. One hemodialysis patient and one intravenous drug addict seroconverted 4 months and 5 weeks after initial testing, respectively. The other two hemodialysis patients remained seronegative during the time of our study (4 and 5 months, respectively) with fluctuating serum aminotransferase activity. Additional samples obtained from these two seronegative hemodialysis patients 5, 10 and 15 weeks after initial testing all tested HCV RNA positive by Amplicor® and in-house nested PCR.

After evaluation procedures three initially Amplicor®-positive/anti-HCV-negative samples obtained from three intravenous drug addicts were considered Amplicor® false positive in initial testing. Interestingly, all three false-positive samples gave Amplicor® OD values which only slightly exceeded the cut-off value proposed by the manufacturer in initial testing (0.732, 0.769 and 0.837, respectively). Because of this observation, all Amplicor®-positive/anti-HCV-positive samples with OD values below 1,000 obtained in initial Amplicor® testing (13 samples from 12 patients) were retested using Amplicor® and in-house PCR. The fourth initially Amplicor® false-positive sample was found due to consistently negative HCV RNA results obtained during these retesting procedures. This false-positive sample was from a 32-year-old woman who had been treated for acute post-transfusion non-A, non-B hepatitis 5 years previously and recognized as anti-HCV positive 3 years before initial HCV RNA testing. Her liver function test results normalized 9 months after acute infection. Finally, the fifth initially Amplicor® false-positive sample was assumed due to discrepant HCV RNA results obtained in follow-up testing of an anti-HCV-positive patient. This 24-year-old blood donor without a history of blood transfusions or any other risk factors, who was first recognized as anti-HCV positive 1 year before initial HCV RNA testing, tested consistently HCV RNA negative using Amplicor® and in-house nested PCR 5 and 12 weeks after initial Amplicor® testing. His liver function test results were normal since the first evidence of anti-HCV seropositivity. The patient's initial sample was retested twice using Amplicor® and in-house nested PCR. The results of both tests were negative.

After retesting 91 initially Amplicor®-negative/anti-HCV-positive samples, one sample was considered Amplicor® false negative in initial testing. The presence of HCV RNA in this serum sample was additionally confirmed by in-house nested PCR. The sample was obtained from a 43-year-old-patient who had an elevated bilirubin level and elevated serum aminotransferase activity for more than 6 months. His histological diagnosis was chronic active hepatitis. An additional sample, obtained from the same patient 3 months later, tested HCV RNA positive using both Amplicor® and in-house PCR.

Among 32 anti-HCV-indeterminate serum samples, 28 samples tested HCV RNA negative and four HCV RNA positive by Amplicor®. All 26 anti-HCV indeterminate samples obtained from healthy voluntary blood donors tested HCV RNA negative as well as two hemodialysis patients. Among four Amplicor®-positive/anti-HCV-indeterminate samples, three samples were obtained from hemodialysis patients and one from an intravenous drug addict. One hemodialysis patient completely seroconverted in 2 months, one hemodialysis patient remained anti-HCV indeterminate in the time of our study (3 months) and for the other two HCV RNA-positive/anti-HCV-indeterminate subjects the follow-up data were not available. The presence of HCV RNA in all four anti-HCV-indeterminate serum samples was additionally confirmed by in-house nested PCR.

In summary, one false-negative and five false-positive Amplicor® results were found in initial testing of 1,204 serum samples, giving an overall sensitivity and specificity of the Amplicor® HCV Test of 99.8% and 99.1%, respectively.

Discussion

In the absence of tissue culture, electron microscopy or assays for viral antigen, the direct detection of HCV is, by

necessity, dependent upon nucleic acid amplification methods [1]. Of the available methods, amplification of the HCV genome by PCR commends itself by virtue of its extreme sensitivity and its consequent ability to detect very low levels of HCV RNA that are present in many clinical samples [9]. However, although PCR is, in principle, a simple test in laboratory practice, many problems have been encountered with this method, the most important being frequent generation of false-positive results due to the amplification of contaminating nucleic acids, mainly the PCR products from previous amplifications [9–11]. In addition, problems have also been encountered with false-negative PCR results, mainly caused by amplification inhibitors present in clinical specimens or insufficient isolation of nucleic acids [10]. Therefore, standardized procedures are needed to ensure reliable results of PCR [11]. Standardized procedures encourage efficiency, reproducibility and quality control and can help to avoid confusion, mistakes and complications [11]. Recently, the Amplicor® HCV Test was developed to increase the availability and to improve the standardization of HCV RNA PCR testing. This commercial test compared favorably with HCV in-house PCR protocols in several preclinical evaluations, however, using relatively small numbers of samples [2–7]. In the present study we report some new important experiences with the Amplicor® HCV Test, which were obtained in the routine testing of 1,204 serum samples. The results of HCV RNA detection were always finally interpreted considering the patient's anti-HCV status and clinical data. Following this strategy and our self-constructed control procedure, many serum samples were retested during the period of 1 year, resulting in the detection of one false-negative and five false-positive Amplicor® HCV RNA results in initial testing. We assumed that false positives were the result of sample-to-sample contamination which occurred during the procedure of detection of amplification products, namely, all five microtiter wells containing false-positive samples were surrounded, during the detection procedure, by at least two strong positive samples. Unbound horseradish peroxidase may have reentered the wells during or after washing if the wells were overfilled. This contamination may have caused significant color development. To overcome this problem we recommend, similarly to *Zauzem* and coworkers [4], smaller wash volumes (350 μl) in contrast to the manufacturer's instructions (400–450 μl). To additionally lower the possibility of Amplicor® false-positive results, we recommend obligatory retesting of all Amplicor®-positive samples with OD values below 1,000. A possible explanation for the false-negative result obtained in one serum sample could be some unintentional technical error.

It is well known that early detection of viral infection is the most important step in the prevention of HCV transmission, especially in high-risk groups such as hemodialysis patients [12–15]. Although serological screening is useful in identifying patients exposed to HCV, a significant proportion of cases eludes surveillance performed by serology alone, due to the fact that it may take as long as 6 months after primary infection for a complete anti-HCV response to develop [9]. Additionally, immunosuppressed patients (e.g. renal transplant recipients) occasionally have HCV infection without detectable antibodies. In such cases PCR is the most widely used method for detection of HCV infection [1, 9]. Although currently unsuitable for mass screening mainly due to the costs of technology, we propose, as do others [12–14], that PCR should be used as a routine screening method for HCV high-risk populations. Thus, in our study, the presence of HCV RNA was detected in four out of 443 (0.9%) seronegative serum samples (only true positive samples were taken into account). All four seronegative/HCV RNA-positive samples were obtained from high-risk patients for HCV infection (three hemodialysis patients and one intravenous drug abuser). Two of these patients seroconverted in 4 months and 5 weeks, respectively. Despite the fact that only a small proportion of our patients was found, by PCR, to be HCV-infected before seroconversion, we think that each of them represents an important contribution for the successful prevention of HCV transmission.

Regarding our 1 year experience, we concluded that the Amplicor® HCV Test is suitable for implementation in daily routine diagnostic detection of HCV RNA in serum samples in specialized clinical laboratories. However, to obtain a high standard of reliability, the use of some improvements proposed in our manuscript (additional anti-contamination procedures, self-constructed control procedure) are strongly recommended. Additionally, the results of an amplification assay must be interpreted with some caution and they should always be interpreted taking the patient's anti-HCV status and clinical data into consideration.

Zusammenfassung: Evaluierung des HCV-Amplicor®-Tests: Erfahrungen aus einjähriger Anwendung im Routinelabor. Ein standardisierter kommerzieller Amplifikationsassay zum Nachweis von Hepatitis C Virus (HCV)-RNA, der Amplicor® HCV Test (Roche) wurde an 1.204 Serumproben von 888 Patienten auf seine Brauchbarkeit geprüft. Sieben von 443 anti-HCV-negativen Proben und 638 von 729 anti-HCV positiven Proben sowie vier von 32 anti-HCV unbestimmten Proben wäre mit Amplicor® positiv bei erster Testung. Die Ergebnisse der ersten Testung erwiesen sich in einem Fall als falsch negativ und in fünf Fällen als falsch positiv. Für den Amplicor® HCV-Test ergibt sich daraus eine Sensitivität von 99,8% und eine Spezifität von 99,1%. Unsere Studie bestätigte, daß der Amplicor® HCV-Test ein praktischer, rasch durchführbarer, sensitiver und spezifischer Test für den Nachweis von HCV RNA ist. Die Ergebnisse müssen jedoch mit Vorsicht bewertet werden, wobei immer der anti-HCV Status des Patienten und seine klinischen Daten herangezogen werden sollten.

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Book Review

A. J. Zuckerman, J. E. Banatvala, J. R. Pattison
Principles and Practice of Clinical Virology (2nd edition)
729 pages, numerous photographs, figures and tables
Wiley, Chichester 1995
Price: $79.95

Since the publication of the 2nd edition (626 pages, 1990) this book has been extended in volume and content. The new edition has been completely revised, in colour photographs of patients and many graphs and tables enable the reader to come rapidly to the main point of interest. Each chapter, in addition to a description of viruses, provides a review on diagnostics, treatment and vaccination.

Adhering to an organotrophic scheme hepatitis viruses are covered including hepatitis C and E viruses. In the herpesvirus section HHV-6 and 7 are fully described. Viruses associated with acute diarrhoeal diseases are summarized in a chapter, referring in this context to bacterial agents and their geographic distribution. Influenza and parainfluenza viruses, such as respiratory syncytial-, adeno-, rhino- and corona viruses are still allotted individual chapters. Toroviruses, that is Breda-like agents, are covered to give the reader a superficial understanding. The classical children's diseases, measles, mumps and rubella, follow. The enterovirus group deals with many clinical manifestations, including chronic fatigue syndrome. In dealing with poxviruses an especially detailed description of the importance of monkey pox after the discontinuation of smallpox vaccinations is given.

No longer only tropical disease viruses of the alpha, flavi and bunya groups are covered in separate chapters. In the flavivirus section hepatitis C virus is again discussed, also the recent spread of a Dengue virus. These chapters supply a very effective epidemiological survey. Arenavirus causing Lassa and Junin fever, filovirus such as Ebola and their transmission are described in detail and this chapter is followed by information on rabies. Two chapters deal with papilloma and human polyoma viruses. There is a concise chapter on human parvovirus. The book ends with the revised, up-to-date chapters on human retroviruses and prion diseases. Both chapters focus on the transmission of different agents, prevention and, in retroviruses, therapy.

The book is recommended for all readers who want to gain insight into a viral disease provided briefly but in depth, without extensive presentation of the most recent results in molecular biology. The book is helpful in linking specific symptoms to an identified viral agent and in finding an adequate diagnostic procedure.

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