Considerations about HCV-RNA screening in blood donations

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Background. An European regulation introduced the nucleic acid testing (NAT) for HCV as a screening of plasma-pools before production of plasma derived proteins. At present the opinions about the feasibility and the suitability of introducing the NAT-based screening are quite different because of technical and ethical considerations. The most important ethical question is the acceptance of two different safety levels among patients receiving plasma derivatives (HCV-RNA negative) or blood components (untested for HCV-RNA). The major technical questions regard the characteristics of PCR tests, the stability of the HCV-RNA and the pool's dimension.
The aim of this work is to evaluate some technical questions about the feasibility of introduction of the NAT-based screening for HCV-RNA in the Italian National Transfusional Service.
Materials and Methods. We considered 110 untreated patients with HCV infection to assess the amount of circulating HCV-RNA. Samples from 6 selected HCV-RNA patients were tested to study the effect of storage at +4°C on the decay of HCV-RNA. For detection of the circulating HCV-RNA we used automated commercial methods: COBAS AMPLICOR HCV and COBAS AMPLICOR HCV MONITOR**.**
Results. The mean HCV-RNA concentration was 6.255 log (lower limit 4.079 log, upper limit 7.398 log). To study the stability of HCV-RNA stored at +4°C. we selected six patients: two with circulating HCV-RNA concentration between 4 log and 5 log, two between 5 log and 6 log and two with over 6 log. After a week the HCV-RNA concentration was over 40% of the viral load observed at time 0.
Conclusions. Some Transfusional Services started preliminary studies about the feasibility of NAT based screening in each blood donation by using mini-pools**.** The dimension of mini pools is the result of a compromise between the need of an adequate sensitivity target, the need of saving resources, and saving the availability of labile blood components. Our data show that mini-pools containing 10-25 blood donations assure a satisfactory sensitivity and are resource saving. In our study we observed that HCV-RNA is stable for almost a week of +4°C storage. The introduction of NAT-based screening on blood donations**'** is now feasible in the Transfusional Service where it helps to prevent transmission of HCV. Therefore, the high degree of safety in virological screening and the low prevalence of HCV-RNA positive donors without circulating antibodies, impose careful evaluation and cost-benefit analysis before the introduction of NAT-based screening of blood donations.

Introduction

The International and the Italian regulations introduced the nucleic acid testing (NAT) as a screening of each plasma-pool processed for the production of stable plasma-derived products [1, 2]. In Germany, the regulation agency for blood components announced its intention to introduce the NAT-based screening of each blood donation [3]. There are no univocal opinions regarding the feasibility and the suitability of the introduction of NAT-based screening in blood transfusion because of the minimal residual risk for post-transfusion hepatitis, the high cost and the unsatisfactory standardization of the NAT technology [4, 5]. Thereafter many European Transfusional Services started screening studies for HCV-RNA by using a polymerase chain reaction (PCR) test applied to mini-pools of blood donations [6, 7].
The European guidelines for validation of the nucleic acid technology (NAT) to detect (only qualitative test) hepatitis C virus (HCV) RNA in pools of blood donations are very strict so only few large laboratories will be able to perform a home made validable test for HCV-RNA by using NAT [8].
There are some commercial methods to detect circulating HCV-RNA in patients with HCV infection. For these methods the evaluation of their strength, the specificity of primers and probes and the test's sensitivity were made by the manufacturers [9-10].

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This will be of great help for the introduction of NAT based screening for HCV-RNA in the National Transfusional Service because if commercial kits are adopted the documented validation points already covered by the kit can substitute the user's validation [8].

Two major practical questions remain to be solved on the implementation of NAT technology as blood donors screening for HCV-RNA in mini-pool. The first regards the mini-pools' size and the second the storage of blood samples in order to get reliable results. As for the first question it is obvious that the size of the mini-pool is determined by the sensitivity of the NAT technique and by the target of detectable viral load in each pool. At present the majority of Transfusional Center use a commercial test (AMPLICOR. Roche Molecular System, Branchberg NJ) to screen blood donation for HCV-RNA by PCR. The amplicor test has a sensitivity around 100 HCV-RNA copies/mL (2 log) [11-12]. The German regulation proposed by the Paul Erlich Institute (PEI), demands that a mini-pool in which there is a sample of HCV-RNA containing 5.000 copies/mL, 3,699 log has to give a positive result [13] (50 blood units in each mini-pool). The Italian regulation [Orlando M. personal communication] proposed by the Istituto Superiore di Sanità (ISS), on the basis of the study published by the Robert Koch Institute, demands that a mini-pool with a final concentration of 100 HCV-RNA copies 2 log has to give a positive result [14]. Hence it is important to study the viral load in untreated HCV infected subjects because the lower viremic level observed in these subjects is the denominator and the sensitivity of the test is the numerator in the calculation of mini-pool size. With regard to the second question some authors reported that HCV-RNA is very unstable in serum, with a fall of 3-4 log after room temperature storage, so nearly unanimous recommendations suggest to store samples for HCV-RNA at –80°C [15-16]. This procedure is not easy to apply in a Transfusional setting in which often there are a lot of blood donation facilities widely spread in the country with a large regional Blood Bank for biological validation of blood-units. At present, other authors suggest that HCV-RNA is relatively stable in samples kept at +4°C, a storage condition well adopted to the Transfusional setting [17-18].

The aim of this work is to evaluate at first the viral load in a population of HCV infected subjects without any previous anti-viral therapy, and second to study the modification of HCV-RNA concentration after +4°C storage.

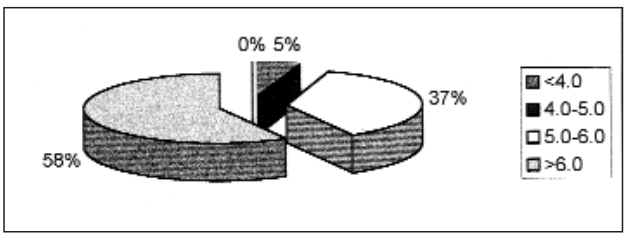
Materials and methods

Patients selection. We considered 110 untreated patients with HCV infection, all anti-HCV positive, by using a commercial ELISA screening test and in each patient the presence of circulating anti-HCV antibodies was confirmed by using an Immunoblotting test. All patients were positive for circulating HCV-RNA by using a commercial qualitative test.

Viral-Load determination. Samples from the 110 HCV-RNA positive patients were tested by using a commercial quantitative test to assess the amount of circulating HCV-RNA in untreated HCV-infected subjects. Whole blood samples were collected in plasma preparation tubes (PPT, Becton Dickinson, Heidelberg, D), and according to the manufacturer's recommendations these tubes were centrifuged 6 hours after blood collection and stored at –80°C until performing the test [17-19].

Storage effect on HCV-RNA concentration. Samples from 6 selected HCV-RNA patients were tested by using a commercial quantitative test to check the effect of storage at +4°C on the decay of HCV-RNA. For these patients whole blood samples were collected in five PPT, and all tubes stored at room temperature for 6 hours before centrifugation, according to the manufacturer's recommendation. From each subject one sample tube was used for titer determination at time point 0, and the other tubes were stored at +4°C for 24, 48, 72 and 168 hours. From each tube we obtained four 0.5 mL shares in RNAse free tubes (Sarstedt, Numbrecht, D), each tube was stored at -80°C until the test. To minimize the assay variability the five samples of each subject were tested in the same analytical series.

Figure 1: Circulating HCV-RNA concentration in 110 untreated patients (LOG 10 copies/mL).



Laboratory Methods. Circulating anti-HCV antibodies were detected by using a third generation ELISA test (HCV ELISA 3.0, Ortho Diagnostic System, Raritan, NJ), as a screening and a third generation Immunoblotting (Chiron Riba HCV 3.0, Ortho Diagnostic System, Raritan NJ) as a confirmatory test. Circulating HCV-RNA was detected (qualitative determination) by using the automated commercial test: COBAS AMPLICOR HCV (Roche Molecular System, Branchburg NJ). This method has a sensitivity of 50-100 HCV-RNA copies/mL [20]. The amount of circulating HCV-RNA was determined by using the automated commercial test COBAS AMPLICOR HCV-MONITOR (Roche Molecular System, Branchburg NJ).

This method is linear from 1.000 to 1.000.000 HCV-RNA copies/mL [21] and samples exceeding the upper limit were retested after tenfold dilution with HCV-RNA free human plasma. For samples prepa-

ration we adopted a manual method and further phases were performed by using the automated COBAS AMPLICOR according to the manufacturer's instructions.

Statistical analysis. For statistical evaluation we adopted the Student's t test for coupled data for comparison of means. The circulating HCV-RNA concentration is quoted, in this study, as ten based logarithmic function (log). In this study we cnsidered a singnifican a difference in HCV-RNA concentration greather tha 0.5 log.

Results

Of the considered 110 patients none had a viral-load under 4 log, in 6 (5%) the HCV-RNA concentration was between 4 log and 5 log, in 41 (37%) the viral load was between 5 log and 6 log, in 63 (57%) the concentration of circulating HCV-RNA was over 6 log. Figure 1 shows the distribution of HCV-RNA concentration in the considered patients. In these the mean HCV-RNA concentration was 6.255 log copies/mL (lower limit 4.079 log, upper limit 7.398 log).

Among the 110 patients 6 were selected for the second part of our study on the stability of HCV-RNA stored at +4°C. We selected two patients with circulating HCV-RNA concentration between 4 and 5 log, two with circulating HCV-RNA concentration between 5 and 6 log, and two with circulating HCV-RNA concentration 6 log (see Table I). A quantitative assay for HCV-RNA was performed on each share stored as above and results are shown in Figure 2**.**

At time point 0 the mean HCV-RNA concentration was 5.492 log, after a week of storage at +4°C the mean concentration of circulating HCV-RNA was 5.025 log with a mean recovery over 90% (lower limit 88% upper limit 94%), in none of these sample we observed a significant decrease in HCV-RNA concentration after seven days of storage at +4°C.

Figure 2: HCV-RNA stability after storage at 4°C

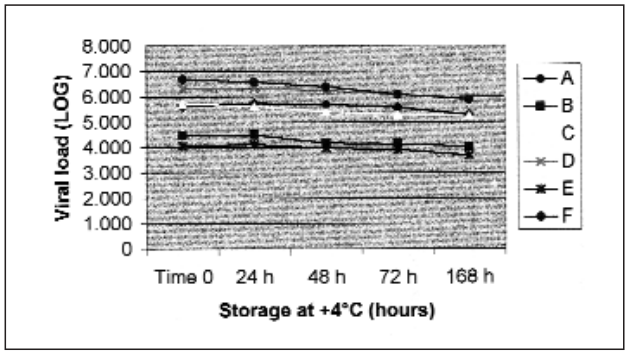


Table I: Circulating HCV-RNA concentration at time 0 in samples selected for the study of the HCV-RNA stability after storage at +4°C.

Patients Identification	Viral load	Viral load (LOG)
A	430,000	5.634
B	30,000	4.477
C	550,000	5.741
D	2,100,000	6.322
E	12,000	4.079
F	5.000.000	6.698

Discussion

The risk of transmitting HCV by transfusion of blood products is, at present, very low (between 1/30.000 and 1/300.000) because of the improvement of donors selection and of the virological screening tests [22]. The European Regulatory Agency recommends a screening for HCV-RNA in plasma pools before protein preparation, despite the virus inactivation procedure [23].

The most important plasma product's manufacturers adopted various procedures to prevent HCV contamination of plasma-pool such as a PCR based NAT screening for HCV-RNA in the final products [24] and/or by testing plasma-pools before starting the protein production [25]. After June 1999 only HCV-RNA negative plasma units were used for plasma derived protein production while some Transfusional Services adopted a mini-pools based NAT screening to certify their plasma before shipment to the manufacturers [26, 27]. The European Regulatory Agency did not ask for testing of single blood but, at present, necessity of screening the cellular components of donated blood for HCV-RNA felt by media has been emphasized by the declaration of the PEI which announced the intention to introduce mandatory screening of erythrocyte concentrates for HCV-RNA by NAT in Germany in 1999 [28-30].

The most important technical questions about the use of NAT based screening for HCV-RNA in blood donations in the Transfusional setting regard the sensitivity and specificity of probes and primers, the strength of the test, the stability of HCV-RNA after storage and the pool's dimension. All published NAT procedures are patented, and hence by using a commercial PCR method for detection of HCV-RNA the manufacturers assure Transfusional Services about tests specificity, sensitivity and robusteness.

The automated COBAS AMPLICOR methods use primers within the highly conserved 5' untranslated region of the HCV genome. The reverse transcription and the amplification of cDNA are performed in a single phase with a thermostable recombinant enzyme (rTth pol), which allows both reverse transcription and PCR amplification to occur in the same reaction mixture [31-33].To improve the test specificity the manufacturer introduced in this mixture deoxyuridine (instead of deoxythymidine), so that the enzyme AmpErase (uracil-N-Glycosylase) ca-

talyzes the destruction of DNA strands containing deoxyuridine (contamination by cDNA coming from previous procedures) but not DNA strands containing deoxythymidine (native DNA). The probe specific for HCV and IC (or IQS) is coated to magnetic particles and this hybridization-magnetic separation increases the overall specificity of the test. For the revealing reaction the method uses an avidin-horseradish peroxidase conjugate and TMS as substrate. The COBAS AMPLICOR method is able to detect with good sensitivity all the HCV genotype and the detection limit is below 2 log [34-36]. This test is highly specific and shows a satisfactory robustness. Moreover the exhaustive documentation produced by the manufacturer allows the users to simplify the validation procedure with great resource saving [37].

In our experience, untreated patients show a high mean concentration of circulating HCV-RNA but none of the considered subjects had a viral load below 4 log and only a slight number (5%) had a viral load from 4 log to 5 log**.** The lower concentration of circulating HCV-RNA observed in this study was 4.079 log and hence the detection limit proposed by PEI (3.699 log in a single sample spiking a pool) appears to be absolutely adequate to warrant the identification of a mini pool contaminated by a HCV-RNA positive donation and by a method with a detection limit of 2 log the mini pools dimension will require around 50 blood donations. With regard to the detection limit proposed by ISS (2 log in the final mini-pool) from our study the pool's dimension will be established around 100 blood donations [38,39].

Data obtained in our study show that HCV-RNA is quite stable at +4°C. Indeed after a week's storage in PPT tubes no sample showed a fall of 0.5 log. This observation is very important in a project of biological validation of each blood donation by using a NAT -based screening for HCV-RNA. Usually, in the Transfusional settings, the blood donors facilities are widely spread in the country, while the Laboratory performing the virological tests is located in the Regional Transfusional Service. In these blood donors facilities it is very difficult to find equipment for tubes' centrifugation, separation of plasma by cells, frosting and shipping at -80°C of tubes. Our data, demonstrate the stability of HCV-RNA stored at +4°C, so il will be easily possible to ship samples not only from blood donor facilities to Validation Laboratory but also from a little Transfusional Service without a NAT licensed laboratory to a large Transfusional Service with fitted up Virological Laboratories [40,41].

Conclusions

The residual risk of HCV post-transfusional infections is today very low because of the improvement of donor's selection and of virological screening tests. Despite this consideration the European Regulation Agency introduced the nucleic acid testing (NAT) as screening in each plasma-pool processed for the production of stable plasma derived products. At present NAT is quite expensive, lacks in standardization, is only partially automated and needs dedicated resources. Therefore this technique is not widely spread in the Transfusional settings [41,42]. Moreover there are many ethical implications in an approach where plasma stable derivatives are submitted to viral inactivation processes and NAT screening while labile blood components, not submitted to viral inactivation processes, are not tested for HCV-RNA. On this basis some Transfusional Services started preliminary studies about the feasibility of NAT-based screening in each blood donation by using mini-pools [43,44]. By using a commercial patented method the routine of validation is simplified. The dimension of the mini pools is the result of a compromise between the need of an adequate sensitivity target, the need of saving resources, and to save the availability of labile blood components [45]. Our data show that mini-pools containing blood donations assure a satisfactory sensitivity and are resource saving. In our study we observed that HCV-RNA is stable for almost a week during storage at +4°C in PPT tubes. This observation is very important because it means that it is possible to perform an easy transportation of samples for HCV-RNA PCR based tests together with the blood bags from the donations facilities to the Transfusional Service. In conclusion, implementation of blood donations NAT-based screening is feasible in the Transfusional Service where may help to prevent the transmission of HCV. The high degree of safety in virological screening and the low prevalence of HCV-RNA positive donors without circulating antibodies impose a careful evaluation and cost-benefit analysis before the introduction of NAT-based screening of blood donations.

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