Feasibility of Freeze-Dried Sera for Serological and Molecular Biological Detection of Hepatitis B and C Viruses

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We compared hepatitis B virus (HBV) surface antigen, anti-hepatitis C virus (HCV) antibody, and HCV RNA quantification in frozen and freeze-dried serum samples to assess the usefulness of freeze-dried sera for detection of HBV and HCV. The results indicated that freeze-dried sera as well as frozen sera can be useful for serological and molecular biological analyses of HBV and HCV.

Freeze-dried sera are generally used as standards for hematochemical tests. In frozen and freeze-dried sera, protein, lipid, and electrolyte levels remain relatively stable, but enzyme levels show a greater degree of variation in freeze-dried sera than frozen sera (6). Nevertheless, freeze-dried sera can be stored at room temperature for a long time and are therefore easier to handle than frozen sera.

For the study of hepatitis B virus (HBV), dried blood spot (DBS) samples have been used for detecting hepatitis B virus surface antigen (HBsAg) and antibody to hepatitis B core antigen (2, 8). Recently, DBS samples allowed the development of a simple, sensitive, and appropriate test for quantifying HBV DNA and studying HBV genetic variants (5). As for hepatitis C virus (HCV), dried sera are used for the test of anti-HCV antibody (Ab) (2), and DBS samples allowed the development of a simple, sensitive, and reliable test for detection and genotyping of HCV RNA (1, 7). However, there is no report on their usefulness in HCV RNA quantification. We conducted serological and molecular biological tests to detect HBV and HCV using frozen and freeze-dried serum samples to determine the feasibility of freeze-dried sera.

The Atomic Bomb Casualty Commission established the Adult Health Study (AHS) longitudinal cohort in 1958; since then, the Atomic Bomb Casualty Commission and its successor, the Radiation Effects Research Foundation (RERF), have examined about 20,000 atomic-bomb survivors and controls biennially in outpatient clinics in Hiroshima and Nagasaki. We selected at random 12 consecutive HBsAg-positive and 25 consecutive anti-HCV Ab-positive individuals among 6,121 AHS longitudinal cohort subjects who underwent hepatitis screening from 1993 through 1995. Their serum samples were stored by the Radiation Effects Research Foundation (RERF), have examined about 20,000 atomic-bomb survivors and controls biennially in outpatient clinics in Hiroshima and Nagasaki. We selected at random 12 consecutive HBsAg-positive and 25 consecutive anti-HCV Ab-positive individuals among 6,121 AHS longitudinal cohort subjects who underwent hepatitis screening from 1993 through 1995. Their serum samples were stored by both freezing and freeze-drying methods.

First, the procedure used for the preparation of frozen serum samples was as follows: Blood obtained from the AHS subjects was kept at room temperature for 20 min. Serum was then divided into four equal parts and stored in 1.5-ml polypropylene tubes at −80°C until use. These samples were thawed by leaving them at room temperature for 30 min and mixed well by inversion before use. Second, the procedure used for the preparation for freeze-dried serum samples was as follows. A 0.4-ml portion of the serum was separated as mentioned above and stored in a glass tube at −80°C. After 1 week of storage, the samples were freeze-dried using a freeze-dryer, sealed, and stored at room temperature (20 to 25°C) until use. These samples were reconstituted by the volumetric method using diethyl pyrocarbonate-treated Milli-Q water and mixed well before use.

The tests for HBsAg and anti-HCV Ab using fresh serum samples in hepatitis screening from 1993 through 1995 were described previously (3, 4). In screening tests, an anti-HCV Ab titer of ≥212 was defined as a high titer. In the present study, HBsAg and anti-HCV Ab were measured by enzyme immunoassay (EIA) (International Reagents Corporation, Kobe, Japan) and second-generation EIA (International Reagents Corporation), respectively. Measured values of ≥1.0 for HBsAg and anti-HCV Ab were defined as positive. An anti-HCV Ab titer of ≥50 was defined as a high titer.

Serum RNA was extracted from 100 μl of frozen or reconstituted freeze-dried serum samples using SepaGene RV-R (SankoJunyaku Co., Tokyo, Japan). The prepared RNA was reverse transcribed with random primers (6-mer) and reverse transcriptase (RverTra Ace; Toyobo Co., Tokyo, Japan). HCV RNA was quantitated by real-time PCR using fluorescence resonance energy transfer probes. Primers and probes were designed within a highly conserved 5′ untranslated region (UTR) and also targeted homologous regions of genotypes 1a, 1b, and 2. The oligonucleotide sequences of the primers were as follows: HCVNC2, 5′-CCTGTGAGGAACTGTCGAT3′, and HCVNC1, 5′-CAACATCTCCGGTACACTGATCG-3′. The hybridization probes were as follows. Probe NCJ-LC (5′-GAACCGGATGATACCCCGGAT-3′) was labeled at the 5′ end with the fluorophore Red 640 and phosphorylated at the 3′ end. Another probe, NCJ-FL (5′-GGGAGCCCATATGGCTG-3′), was labeled with fluorescein isothiocyanate at the 3′ end. PCR was performed in a total volume of 20 μl, 30 μl.
containing 5 mM MgCl₂, 6 pmol of NCJ-LC, 4 pmol of NCJ-FL, 10 pmol of the two PCR primers, 2 µl of LightCycler-FastStart DNA Master hybridization probe mix (Roche Diagnostics Co.), and 1 µl of synthesized cDNA solution. The PCR cycling program consisted of an initial denaturing step at 95°C for 10 min and 50 amplification cycles of 95°C for 15 s, 55°C for 6 s, and 72°C for 10 s. Once the threshold was chosen, the point at which the amplification plot crossed the threshold was defined as the threshold cycle (C_T). The calculated C_T value is predictive of the quantity of target RNA copies. The standard curve was calculated using serially diluted plasmids containing nucleotide sequences of the HCV 5’ UTR, to obtain control fragments for determination of HCV copy numbers. All assays were conducted in duplicate.

The positive-negative results of HBsAg in frozen and freeze-dried serum samples were consistent with results using fresh serum samples. The concordance in measurement of anti-HCV Ab among fresh, frozen, and freeze-dried serum samples was not complete but was satisfactory. Both frozen and freeze-dried serum samples of one case tested negative for anti-HCV Ab, despite testing positive in the 1993-1995 hepatitis screening. One freeze-dried serum sample of another case tested positive for anti-HCV Ab, despite testing negative in the 1993-1995 screening (Table 1). For these two patients with discrepant results, the specimen yielding a positive result contained only low titers of anti-HCV Ab; subsequent testing for HCV RNA by quantitative or qualitative PCR was negative in both cases (data not shown). Furthermore, 86% (18/21) of the fresh serum samples yielding high anti-HCV Ab titers by passive hemagglutination also yielded high anti-HCV Ab titers on subsequent testing of both frozen and freeze-dried serum samples by EIA; frozen and freeze-dried samples from the remaining three cases produced low-positive results. These results might be due to decay of the antibody during storage or differences in criteria for high antibody titer between the previous and present kits. However, the results for high anti-HCV Ab titers were completely consistent in frozen and freeze-dried serum samples.

A linear relationship in the range of 2.0 × 10² to 2.0 × 10⁹ equivalent copies/ml was observed between C_T values and quantity of RNA copies (r > 0.99) (data not shown). Real-time PCR detected HCV RNA in 18 of 25 frozen and freeze-dried serum samples from anti-HCV-positive cases in the 1993-1995 hepatitis screening. The correlation between HCV RNA concentration in frozen and freeze-dried serum samples was significant (r = 0.908, P < 0.0001) (Fig. 1).

The intra-assay variability was determined by assaying two frozen serum samples containing HCV RNA of genotype 1b and 2a (respectively, 9 × 10⁵ and 1.3 × 10⁹ copies/ml) 10 times in a single day, and the respective coefficients of variation (CVs) were 6.2% and 2.9%. The respective interassay CVs calculated by analyzing each of these serum samples once a day for 10 days were 3.6% and 4.3%.

On the whole, results for fresh, frozen, and freeze-dried serum samples for HBsAg and anti-HCV Ab demonstrated very good agreement, indicating that these methods and storage conditions are appropriate for serological assays of HBV and HCV. Furthermore, results of a newly developed highly sensitive and high-range HCV RNA quantitative assay for frozen and freeze-dried serum samples showed good correlation. We expected that the PCR products of HCV RNA would vary depending on storage method and conditions; however, the results showed no marked differences during 10 years of storage. The use of sera of AHS subjects stored from 1969 can further advance the study of the evolution of HBV/HCV as well as the natural history of viral liver diseases.

### TABLE 1. Comparison of detection of HBsAg and anti-HCV Ab in frozen, freeze-dried, and fresh serum samples

<table>
<thead>
<tr>
<th>Substance tested, sample type, and EIA result</th>
<th>No. of fresh serum samples with PHA result</th>
<th>Concordance (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>HBsAg</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Frozen Positive</td>
<td>12</td>
<td>100</td>
</tr>
<tr>
<td>Frozen Negative</td>
<td>0</td>
<td>25</td>
</tr>
<tr>
<td>Freeze-dried Positive</td>
<td>12</td>
<td>100</td>
</tr>
<tr>
<td>Freeze-dried Negative</td>
<td>0</td>
<td>25</td>
</tr>
<tr>
<td><strong>Anti-HCV Ab</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Frozen Positive</td>
<td>24</td>
<td>97</td>
</tr>
<tr>
<td>Frozen Negative</td>
<td>1</td>
<td>12</td>
</tr>
<tr>
<td>Freeze-dried Positive</td>
<td>24</td>
<td>95</td>
</tr>
<tr>
<td>Freeze-dried Negative</td>
<td>1</td>
<td>11</td>
</tr>
</tbody>
</table>

*a* HBsAg was measured by EIA; anti-HCV Ab was measured by second-generation EIA.

*b* HBsAg was measured with a reverse passive hemagglutination (PHA) test kit; anti-HCV Ab was measured with a second-generation PHA test kit.

*c* HCV infection status was negative with quantitative or highly sensitive qualitative PCR.

![FIG. 1. Correlation of HCV RNA levels in frozen and freeze-dried serum samples. The correlation between log_{10}-transformed HCV RNA quantities for pairs of frozen and freeze-dried serum samples from 18 cases analyzed by real-time PCR was significant (r = 0.908, P < 0.0001).](image-url)
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