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Serum Protein Standardization Project in Japan: Evaluation of an IFCC Reference Material (RPPHS/CRM470) and Establishment of Reference Intervals

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Reference preparation for proteins in human serum (RPPHS), also called Certified Reference Material 470 (CRM 470), was prepared by the International Federation of Clinical Chemistry (IFCC) and is intended to serve as a new international plasma protein reference material. It is now being introduced into Japan. RPPHS possesses many excellent properties, including safety, stability, and accuracy in value assignment. Moreover, the physicochemical properties of its proteins are identical to those of fresh serum, giving it immunochemical behavior that is commutable with that of existing reference materials and calibrators in given immunoassays. Reference intervals of 13 serum proteins were determined for the first time using nephelometry and a new working calibrator assigned from RPPHS, which seems certain to play a critical role in the global standardization of specific protein immunoassays. J. Clin. Lab. Anal. 11:39–44.

Key words: C-reactive protein; RPPHS/CRM470; stability; standardization; safety; reference intervals; quality assurance

INTRODUCTION

Ideally, all assay systems should produce identical results for any given protein in a given sample. Accordingly, this would be the ultimate goal for the standardization of a protein assay. Quantification of individual proteins is based on the principle of the antigen-antibody reaction, in which change in radioactivity, enzyme activity, or beam scatter in beam-scatter mode assay of antibody-antigen complexes is represented as an electrical signal. The protein is quantified when the value of a given calibrator protein is transferred to a corresponding protein in the simultaneously measured sample.

In fact, however, results vary from assay to assay. This is the inevitable outcome of many complex, known and unknown, independent and interactive factors: presence of antigens in calibrators and samples, number and quality of antibody assay systems with different principles, and well- or poorly defined matrix effects, to name but a few. Attempts have been made to develop well-defined primary standards and then properly to prepare sets of secondary standards, or calibrators, and in this way establish total standardization of assays (3). This is the basic principle of protein-assay standardization. Indeed, many series of standards approved by the World Health Organization (WHO), the United States Centers for Disease Control and Prevention (CDC), and other organizations have done much to advance this work. Nonetheless, reference materials available today have some drawbacks. Some materials are available only in small quantities; units vary from protein; safety cannot always be assured, especially against recently discovered pathogenic organisms; matrix effects influence the assays. Moreover, lack of a definite protocol for preparation and value assignment causes discrepancies in results of immunoassays conducted at different laboratories (4,5).

All conceivable problems of reference materials were investigated in detail, and a new international reference material was completed through collaboration with the IFCC Committee on Plasma Protein Standardization, the European Union, Institute of Reference Materials and Measurement,

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formerly the European Community Bureau of Reference (BCR), and the College of American Pathologists (CAP), in which 14 different proteins were newly assigned (4).

The Japanese Committee for Clinical Laboratory Standards (JCCLS) has asked us to evaluate RPPHS before it is introduced in Japan (6). This report summarizes our laboratory evaluation and our study of the applicability of the completed reference material in Japan and the cooperative establishment of reference intervals among Japanese adults.

EVALUATION OF RPPHS

The reference material (CRM470) used in this study was obtained from the late Professor S. Baudner (Behringwerke, Marburg). The Japan National Protein Standard, a secondary standard material for IgG, IgA, IgM, and C-reactive protein (CRP), in which value assignments were based on WHO International Reference Preparation 67/86 and CRP 85/506, was contributed by the National Institute of Health of Japan. A conventional working calibrator (N-protein; Hoechst-Japan, Tokyo) and the corresponding new calibrator (N-protein SY), assigned from the material, were used in comparison tests. Proteins in primary and secondary reference materials, calibrators, and sera were measured by nephelometry (BNA, Hoechst-Japan).

Safety

To guard against hazardous infectious agents, whose absence in the prepared material was confirmed, several antigen and antibody tests (7) were used to prepare RPPHS. Conventional antigen and antibody tests for pathogenic microorganisms were all negative in RPPHS, as were test results at the time of serum collection, as shown in a CRM470 report (Table 1) (7). A microparticle enzyme immunoassay (Axsym Core, Dainabot-Abbot, Tokyo) showed antibody activity against hepatitis B core (HBc) antigen, however. Although the cause of this activity was unknown, the nonspecific reaction may be explained by the fact that the material is prepared in several steps from mixed sera and would thus increase the probability of a false-positive result. Sera with a history of HBV infection could be contaminated at time of collection. A PCR test (SRL, Tokyo) was thus conducted to assure negativity for HBV. PCR tests for HCV were also carried out, including both the 5´-noncoding and core regions (nested PCR, Kawasaki Medical College, Kurashiki) (8), HIV (Japan Synthetic Rubber Co., Tsukuba), and HTLV (SRL). All of these highly reliable tests returned negative results.

TABLE 1. Immunoassay Tests for Pathogenic Microorganisms in RPPHS

Test[a]	Method[b]	Result
HBs Ag	EIA: Organon Technica	(–)
HBe Ag	EIA: Mizuhomedy	(–)
HBc Ab	EIA: Abott	(–): % Inhibition 43%
HBc Ab (IgM)	EIA: Abott	(–)
HCV Ab	EIA: Ortho Diagnostics	(–)
HIV-1, 2 Ab	EIA: Abott	(–)
HTLV-I Ab	PA: Fuji Rebio	(–)
TP Ab	RPR Card: Kaketsuken	(–)
	TPHA: Fuji Rebio	(–)

[a]TP: Toreponenma Pallidum.
[b]EIA: enzyme immunoassay; PA: Particle agglutination assay; RPR: rapid plasma reagin test.

No bacterial or fungal growth was observed in the culture medium (TSA, Eiken; Sabouraud Dextrose Agar, Beckton Dickinson, Cockeysville), which was held at 37°C after it was reconstituted with nonsterile distilled water and stored at 4°C for 14 days.

Factors Interfering With the Assay

Rheumatoid factor was negative (5 IU/ml) on particle immunoassay (RAPA; Fuji Rebio, Tokyo). To detect contamination by monoclonal proteins in the material, cellulose acetate membrane electrophoresis (AES260 Automatic Analyzer, Olympus, Tokyo), agarose gel immunoelectrophoresis (IEP, Corning, Tokyo), and immunofixation using titan-gel (Helena, Tokyo) were used. None of the methods described above indicated the presence of monoclonal proteins.

Stability After Reconstitution

In accordance with the procedure defined in the CMR470 report, 1 ml of nonsterile distilled water was carefully reconstituted and held overnight at room temperature. Its container was then sealed with a rubber cap and stored in a humid environment at 4°C. Values of CRP, C3, C4, IgG, IgM, and IgA were determined for the first 4 days and again on day 14. All protein concentrations measured remained constant during this period; range of variation was 5% or less (Fig. 1) (6). This was consistent with results published in the CRM470 report (7). Although 50% lower than that in the reference

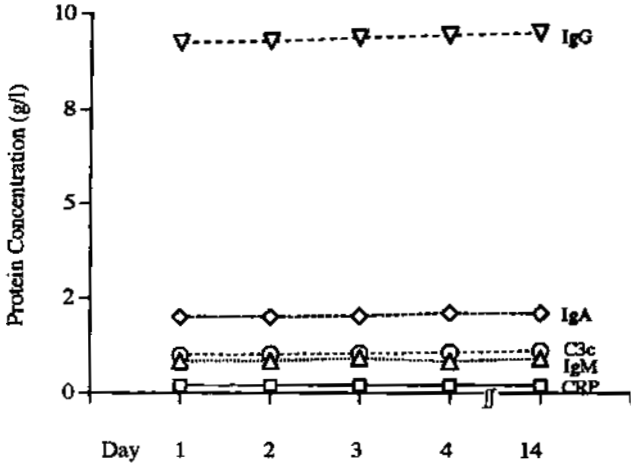

Fig. 1. Stability of IgG, IgA, IgM, CRP, and C3c stored at 4°C for 14 days.

value, antistreptolysin O activity was determined at 146 U/ml by turbidimetry (LPIA, Dia-Iatron, Tokyo). This value also remained unchanged until day 14 after reconstitution.

ELECTROPHORETIC ANALYSES ON RPPHS

Cellulose Acetate Membrane Electrophoresis

AES620 (Olympus, Tokyo) was used in the automatic application of 0.4–0.6 μl of RPPHS on a cellulose acetate membrane (Separax SP, Fuji Film, Tokyo), where it separated into five fractions at the same migration position as that of fresh serum (Fig. 2, left). The percentage of α1-β globulin fractions were lower than those of fresh serum, primarily because of the delipidization of RPPHS, whereas those of albumin and the γ fraction were reciprocally higher. Decrease in levels of ceruloplasmin, IgM, and α1-antitrypsin during the processing of the material may contribute to these changes (5), however. A single clear band appeared at the β region, reflecting a homogeneous C3c peak as a result of artificial conversion from C3 (7). The fractions were clearly distinguishable, and there was far less tapering than there was in the conventional control. Furthermore, protein bands remaining at the site of application were negligible. These may also be explained by deletion of lipid and replacement of matrix with Hepes buffer.

The stability and reproducibility of electrophoretic patterns produced by this material, observed after it was reconstituted and stored at 4°C for 20 days, were good. The pattern remained almost unchanged through day 20 (Fig. 2, right) and remained unchanged through day 90 under the same conditions.

RPPHS is, to be sure, designed to be used as a secondary reference material in the assay of specific serum proteins. Because of its excellent stability, reproducibility, and resolution in EP, it is also a most promising candidate as a reference material for use in preparing common reference material and in controlling total standardization of laboratory examinations of serum proteins. Albumin, whose concentration was assigned by RPPHS, has a single homogeneous peak protein fraction. Quantitation of albumin by electrophoresis may be possible when automatic application of samples becomes more accurate. This fraction can then serve as a target in the preparation of RPPHS reference materials.

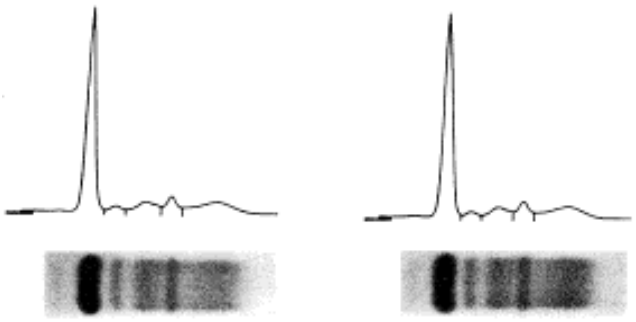

Fig. 2. RPPHS on cellulose acetate membrane electrophoresis. Left, Fractionation pattern on day 1. Right, Fractionation pattern on day 20.

Total protein measurement and protein fractionation on EP are laboratory routines. IEP, protein quantitation, and other more precise examinations are conducted when results are abnormal. RPPHS may become an essential core material in these examinations to make complete standardization of laboratory examination of serum proteins possible.

IEP

Clear precipitin lines were produced by sheep immune serum against whole human serum (Sylenus, Melbourne) in IEP, showing almost the same precipitin patterns as those produced by proteins in fresh normal serum at the same migration region. C3, which was changed artificially to C3c by inulin, was the only exception (Fig. 3) (3). The radius of the precipitation arc was almost the same as that of normal serum, indicating that the proteins detected in the material are present in normal concentration and have the same immunochemical properties as those in normal fresh serum.

Immunofixation

RPPHS and fresh normal serum produced almost the same fractionation patterns on immunofixation using amido black 10B: five positive bands formed in the same migratory region. The most sensitive laboratory test showed no monoclonal proteins to be reacting with both anti-κ and anti-λ antibodies (DAKO, Copenhagen) (data not shown).

Isoelectric Focusing and SDS-PAGE

Isoelectric focusing also showed generally similar positive protein bands; no positive bands corresponding to probable apolipoproteins appeared (data not shown), however. Molecular weight distributions of proteins observed on SDS-PAGE were also similar to those observed in serum (data not shown). All of these electrophoretic analyses showed the physicochemical properties and structures of proteins in the material to closely approximate those of native serum proteins.

IMMUNOREACTIVITY AND ASSIGNED VALUES OF 14 PROTEINS IN THE MATERIAL

Six points were established on the RPPHS, National Standard, and conventional working calibrator dilution curves to compare immunochemical behavior and value assignment by RPPHS with values obtained with nephelometry (BNA).

Evaluation of CRP is shown in Figure 4. Three dilution curves of CRP, set independently using RPPHS, National Standard, and the calibrator (N-protein), described the same curve (6), indicating that the immunoreactive behavior of CRP is quite similar to that of the other reference materials in a

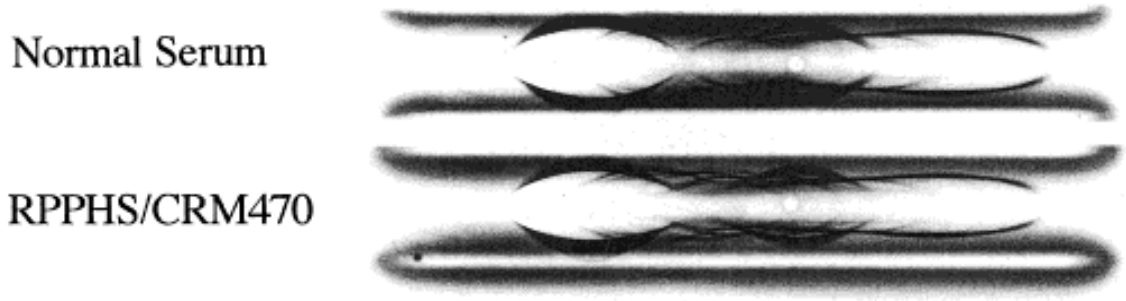

Fig. 3. RPPHS on immunoelectrophoresis.

given assay system and, further, that the assigned value of each had been properly transferred from the common primary reference material, which was WHO 85/506 and was not affected by preparation procedure, value assignment, or matrix effects of each. Thus it is not necessary to use a conversion factor to adjust CRP value assignments.

The same was true for IgG and IgM when they were tested against RPPHS and the National Standard. The value assigned to IgA by the National Standard was 10% lower than that assigned by RPPHS, however. Value adjustment between RPPHS and National Standard was possible because the dilution curves were parallel.

For the rest of the proteins in RPPHS or the existing calibrator, the dilution curves coincided in α1-acid glycoprotein. Those proteins in the calibrator with 10–30% higher values than those in RPPHS include IgM, α1-antitrypsin, haptoglobin, IgA, C4, transferrin, α2-macroglobulin, and tansthyretin. Those with lower values were ceruloplasmin and C3c. Again, all of these could be commuted, since the dilution curves of RPPHS and the calibrator of each protein were parallel. Being proportional to RPPHS, expressed value in the calibrator can be adjusted by simple use of conversion factors. Some of the companies participating the RPPHS/CRM470 preparation project have, in fact, already announced new values for their calibrators. Here we confirmed that the value of one of the new calibrators (N-protein SY) was properly assigned from RPPHS.

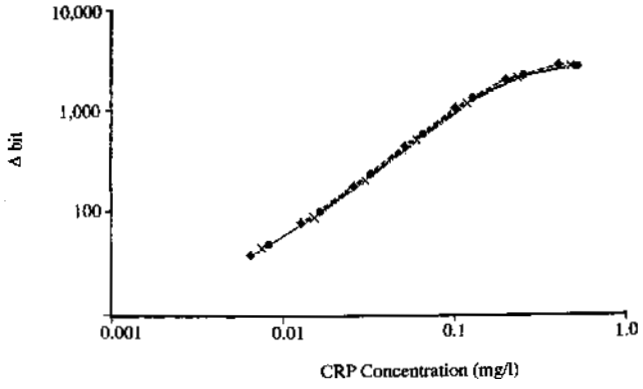

Fig. 4. Results fall on same dilution curve for CRP set by RPPHS (—X—), National Standard (—●—), and a working calibrator (N-protein: —▲—), by nephelometry.

REFERENCE INTERVALS OF 13 DIFFERENT PROTEINS IN A POPULATION OF JAPANESE ADULTS

We set reference intervals for Japanese populations after confirming that value assignment by the new calibrator (N-protein SY) was accurate and after using a new guideline for reference intervals from the National Committee for Clinical Laboratory Standards (NCCLS).

Seven participating laboratories (Jichi Medical School, Hamamatsu Medical College, Tokyo University, Nihon University, Kanagawa-Ken Yoboigaku Kyoukai (Yokohama), SRL, and Hoechst-Japan) used BNA with N-protein SY to measure 13 different sera proteins. CRP against which values obtained from sera from healthy individuals were below the sensitivity of many assay systems was excluded. First, external quality assessment, performed at high and low concentrations on two controls, confirmed the reliability of the quality assurance systems of the laboratories. Statistical determination of reference intervals was based on NCCLS guidelines (NCCLS Document (p28), 1992) (9). Sera were collected from 2,060 healthy adults at the Kanagawa Prefecture Yoboigaku Kyoukai. Reference individuals were selected from a group of healthy adults who had been receiving an annual physical examination in which 25 different hematologic and biochemical parameters were routinely determined. Sera from individuals deemed healthy on the basis of their clinical records and results of physical examinations were sorted first. Reference individuals were then chosen; those who had abnormal values (outside of mean ± 2.5 SD) in any of the parameters were excluded. A total of 999 reference individuals were thus selected, and sera were randomly chosen and distributed to the laboratories. Because results obtained from the collected sera did not differ significantly, it was

TABLE 2. References Intervals of 13 Serum Proteins in Japan and German-speaking Countries

Protein (g/l)	Area[a]	Reference intervals (g/l)	Protein (g/l)	Area	Reference intervals (g/l)
α1-Acid Glycoprotein	J	0.4–0.9	IgG	J	9.7–17.0
	G	0.5–1.2		G	7.0–16.0
Albumin	J	39.0–49.0	IgA	J	1.1–4.1
	G	35.0–52.0		G	0.7–4.0
α1-Antitrypsin	J	0.9–1.5	IgM	J	0.4–1.9
	G	0.9–2.0		G	0.4–2.3
C3	J	0.9–1.6	α2-Macroglobulin	J	1.1–2.3
	G	0.9–1.8		G	1.3–3.0
C4	J	0.1–0.4	Transthyretin	J	0.2–0.4
	G	0.1–0.4		G	0.2–0.4
Haptoglobin	J	0.7–1.7	Transferrin	J	2.0–3.6
	G	0.3–2.0			
Ceruloplasmin	J	0.2–0.4			
	G	0.2–0.6		G	2.0–3.4

[a]J = Japan; G = German-speaking countries.

feasible to assemble and analyze these samples. The reference distributions of 13 proteins were normalized using a modified Box-Cox formula (9). After all the data were transformed, mean and standard deviations were calculated; data outside the mean ± 2.81 SD were excluded. After this truncation, the 95% confidence interval (mean ± 1.96 SD) and median were calculated, and the values were transformed back to the original scale.

Reference intervals of 13 different proteins are summarized in Table 2 (10). The intervals were quite similar to the consensus reference values obtained in German-speaking countries (S. Baudner, pers. comm.). Intervals established in this study were slightly narrower than those obtained in the German-speaking countries, however. This difference may be explained by the fact that we used the only one assay system. Also, all of the reference individuals were living in the same city (Yokohama) and had been strictly sorted. Differences in race, lifestyle, and social circumstances may further account for this difference.

Reference intervals were based on age, sex, and lifestyle using oneway analysis of variance (ANOVA) and multivariate analysis. Subgroups were combined when differences in their values were not statistically significant. Reference intervals associated with physiologic changes and lifestyles were thus created (Table 3) (11).

The present project represents a new statistical approach to establishing reference values of serum proteins in adults living in a large city; moreover, it showed interesting aspects of physiologic variation in proteins, variation associated with age, sex, and lifestyle. Reference intervals can be shared by laboratories as long as populations of reference individuals are similar and the same well-qualified assay is used in conjunction with a sound quality assurance system. This would reduce the amount of laborious, expensive, and time-consuming work required.

TABLE 3. Variation of Serum Proteins in Relation to Age, Sex, and Lifestyle

Significant-demographic and lifestyle factors	Associated proteins
Sex:	
Higher in males than in females	α1-Acid Glycoprotein
Higher in females than males	α2-Macroglobulin, Transferrin, IgM
Age:	
Gradually decreases with age in females	Transferrin, IgM
Use of tobacco:	
Higher in smokers than in nonsmokers	Haptoglobin, IgG
Use of alcohol:	
Higher in drinkers than in nondrinkers	C3

PRESENT AND FUTURE PROSPECTS

The properties of this material, based on our evaluation and our understanding of the report on CRM470 by BCR (7), are summarized in Table 4. In every respect, the present material is a harbinger of a new generation materials for use in protein assay standardization. The major significance of

TABLE 4. Superior Properties of RPPHS/CRM470

Serum-based material with well-documented preparation protocols.
Fourteen different proteins standardized.
- a) Enable to expand the standardization for other proteins.
- b) Accurate value assignment from primary standards and pure proteins.
- c) Two kinds of unit expressed: mass conc., IU.

Physiocochemical properties and structure correspond closely with native serum protein.
Eliminates matrix effects: dilipidization, use of Hepes buffer.
Stable when sotred at –70°C to 20°C.
Stable at 4°C for 14 days after reconstitution.
Assures safety.
Assures continuous renewal.
Quantities sufficient to supply for a number of years.
Internationally certified or approved.
May be prepared as a basis for reference material for use in other body fluids.

RPPHS preparations is that they may make it possible to completely standardize preparation of reference materials. Complete definition of preparation of the material from sampling the source serum to value assignment makes it possible to produce reliable and commutable working calibrators or controls. These facilitate standardization of protein assays and thus bring about rapid development through re-evaluation under a system that controls all matters affecting quality assurance in the laboratory.

The material is prepared by processing pooled sera from healthy adults. Thus its properties are likely to approximate closely those of normal fresh serum. Under some physiologic and pathologic conditions, however, altered protein properties may appear in the serum and show different immunochemical reaction with the material, accelerating the cause of discrepancies in the results obtained with different assays. A good example would be IgA with monomeric IgA, mainly in normal serum and possibly in RPPHS. In a relatively small number of cases of myeloma, a polymeric monoclonal form appears in the serum when the value indicated by a solid-phase assay may be lower than one indicated by a free competitive assay. Thus complete standardization for every protein will not be possible. Nonetheless, a given well-defined reference material and its associated calibrator and controls are prominent probes for elucidating and differentiating causes of discrepancies between assays.

Participating organizations in countries that have accepted or are interested in using this material are encouraged to prepare materials. These countries should be supported with money and technology. From our limited experience in evaluation, we recommend that the following points be considered when developing materials: (1) mixed sera from different races should be used as a source to make sure that materials developed will have international applicability, (2) more precautions should be taken to prevent contamination by microorganisms, and new tests, such as PCR, should be used when necesssary, (3) reference materials that can be used with serum and other body fluids as well may be designed, (4) ceruloplasmin or other unstable proteins in the material should be more stable to ensure the reliability of the data, and (5) in response to strong requests from clinicians, unit-by-mass concentration may be further promoted in the value assignment from purified proteins, since some of the values assigned may not be close to the actual values of the purified proteins. Use of international units is theoretically reasonable. Actually, however, they are not used in Japan, and it appears unlikely that they will be in the foreseeable future (6).

The material accepted for use in Japan is actually intended to serve as a primary standard, replacing the conventional national standard. Accordingly, the National Institute of Health of Japan will hold JCCLS responsible for all the matters on RPPHS/CRM470, from its distribution to its evaluation. Under the auspices of JCCLS, 500 vials of the material were recently imported from CAP and distributed to commercial organizations for use in evaluating their own secondary standards, calibrators, and controls. Selecting commutable assays as well as well-defined calibrators and controls, setting reference intervals of 14 different proteins will be further undertaken throughout Japan by the Committee for Serum Protein Standardization of the Japan Society of Clinical Chemistry. IFCC will soon start an international survey of quality assurance of the assay and also of the study of reference range. Our project will be the basis for these studies that aim to establish reference ranges of proteins—the ultimate global goal of standardization of assays for proteins.

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