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Evaluation and Validation of a Duck IgY Antibody-based Immunoassay for High-Sensitivity C-reactive Protein: Avian Antibody Application in Clinical Diagnostics, *Yun-Chien Tsen,[1] Guor-Yuan Kao,[1] Chung-Li Chang,[1] Fang-Yu Lai,[1] Chien-Hsun Huang,[1] San Ouyang,[1] Mei-Huei Yu,[2] Chi-Pin Wang,[3] and Y-Neng Chiou[1]** (*[1] Good Biotech Corp., No. 38 34th Road, Taichung Industrial Park, Taichung 407, Taiwan; [2] Kuang Tien General Hospital Laboratory, Taichung 433, Taiwan; [3] Chung Shan Medical University Hospital, Taichung 402, Taiwan; * author for correspondence: fax 886-4-23590410, e-mail gbc@good-biotech.com)

High-sensitivity C-reactive protein (hs-CRP) is considered one of the most promising markers for cardiac risk assessment (1). A highly sensitive and interference-resistant CRP assay plays an important role in accurate CRP measurements (2, 3); therefore, manufacturers of in vitro diagnostics have begun modifying their existing CRP assays to meet the requirements. Mammalian antibodies, e.g., rabbit, mouse, and goat IgGs, are the major ones used in clinical immunoassays, but they have some drawbacks. Within the past 10 years, it has been reported that rheumatoid factor and heterophilic antibodies present in samples have caused false-positive results in CRP measurements (4–6). In addition, monoclonal antibodies for use as diagnostic and therapeutic reagents can induce human anti-mouse antibodies, which may interfere with mammalian IgGs in immunoassays, especially in the sandwich-type methods (7).

To eliminate interference without increasing costs, avian antibodies are now considered potential candidates to improve current immunoassays. One such assay, the CRPex-HS® C-Reactive Protein LIT assay (Good Biotech Corp., Taiwan), based on latex microparticle-enhanced immunoturbidimetry, has been developed and is commercially available. The CRPex-HS CRP LIT assay is intended to measure serum hs-CRP concentrations with use of a duck antibody. The duck anti-CRP IgY (ΔFc) antibody is a good substitute for traditional mammalian antibodies. The antibody, purified from egg yolks of white Pekin ducks (*Anas platyrhynchos*), is coupled to polystyrene microparticles. When CRP in the serum sample encounters microparticles containing the duck anti-CRP IgY (ΔFc), it agglutinates and the turbidity is measured at 570 nm. The assay calibration range is 0–20.0 mg/L. Practical CRP measurements by the CRPex-HS CRP LIT assay are conducted on a common automated chemistry analyzer, the Hitachi 717. In this study, we evaluated and validated the performance characteristics of this new hs-CRP assay.

The specificity of the duck anti-CRP IgY (ΔFc) used in the hs-CRP assay was assessed by Western blot analysis. Both CRP in the patient serum and the partially purified CRP (Crystal Chem Inc.) were specifically recognized by the duck anti-CRP IgY (ΔFc).

To study linearity, we diluted a patient serum pool with a CRP concentration of 20.0 mg/L with CRP-free serum to the following final percentages of the patient pool: 100%, 75%, 50%, 25%, 12.5%, 6.25%, and 0.5%. The samples were analyzed in triplicate in a single analytical run. The linearity of the method was confirmed up to 20 mg/L (i.e., the highest calibrator concentration). Regression analysis of the data yielded an intercept of −0.12 mg/L, a slope of 21.69, and an r^2 of 1.0. One control (Cliniqa Corporation) with a very high CRP concentration was diluted to test the possibility of underestimating the true CRP value because of a prozone effect. We found that a CRP concentration of up to 500 mg/L did not cause a false-negative result. Concentrations >500 mg/L were not tested. The detection limit, 0.05 mg/L CRP, was the concentration corresponding to a signal 2 SD above the mean for a CRP-free serum.

Within-run imprecision (as CVs), determined by analyzing three serum samples (mean values, 0.16, 5.56, and 18.65 mg/L) in 20 replicates, were 10%, 0.73% and 0.98%, respectively. For day-to-day imprecision, samples were
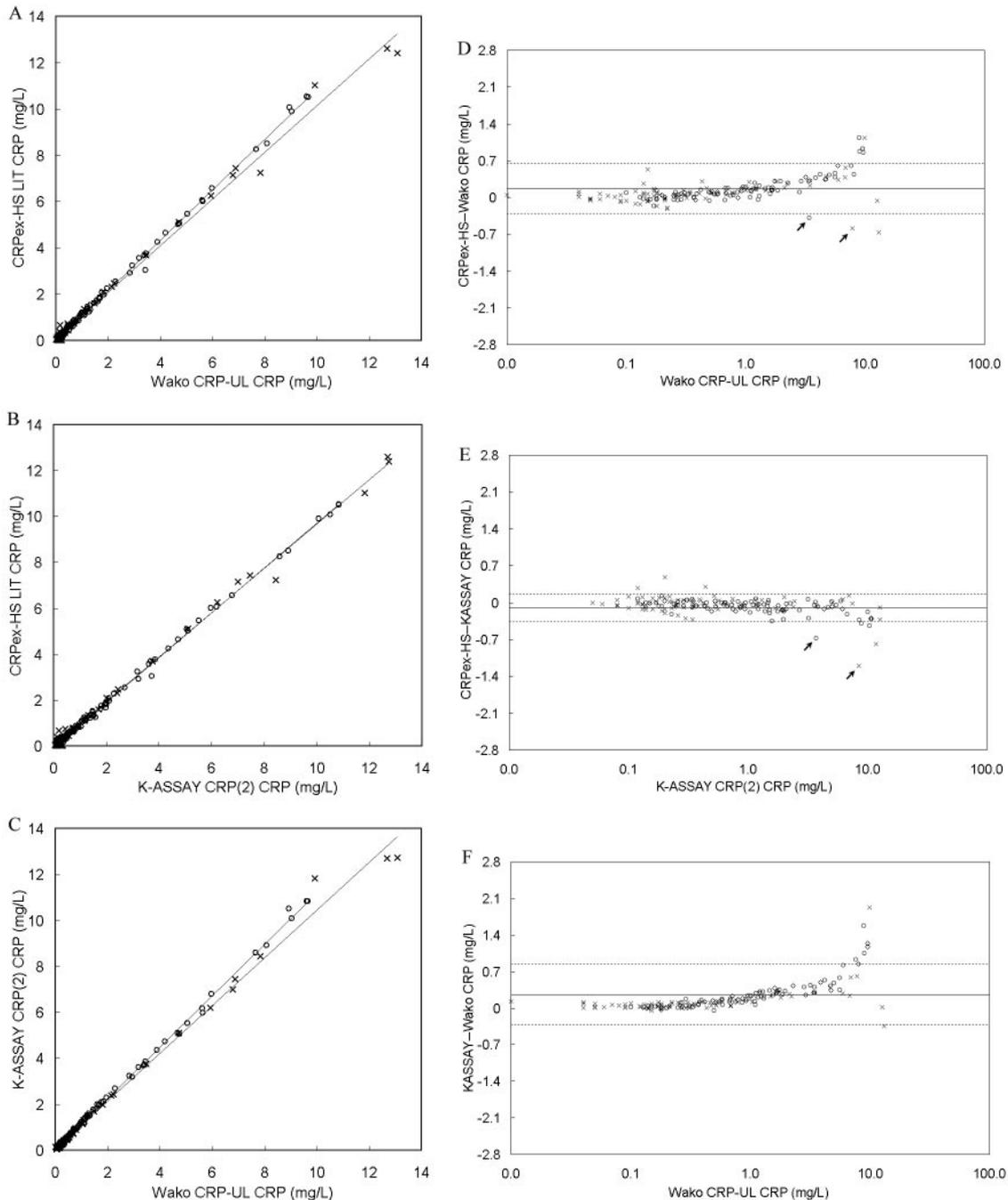
Fig. 1. Comparison of CRP concentrations determined by the IgY-based method with other commercial assays.

(*A–C*), regression analysis; (*D–F*), Bland–Altman plots. ○, samples from control individuals; ×, samples from avian-allergic individuals. In *panels A–C*, the *solid lines* are the regression lines; in *panels D–F*, the *solid lines* are the means, and the *dashed lines* are ± 2 SD.

analyzed daily in triplicates, and the daily averages for a total of 14 days were calculated. CVs were 5.2%, 2.5%, and 3.9% at (mean) CRP concentrations of 0.37, 4.02, and 12.91 mg/L. The functional sensitivity, the lowest measurable CRP concentration with a CV <20%, was 0.058 mg/L. Interference was defined as a difference of >5% between the assay values of the original samples and those of samples to which various concentrations of interferent had been added. No interference occurred with up to 450 mg/L unconjugated bilirubin (cat. no. B-4126; Sigma). Hemoglobin (prepared from fresh hemolysate) up to 9400 mg/L and fat emulsion (Liposyn® II; Abbott Laboratories) up to 10 g/L also did not interfere with the CRP assay.

Because the source of duck IgY (ΔFc) is the duck egg and an allergic reaction to eggs is generally agreed to be one of the most common causes of food allergies in infants and young children, it is likely that IgE antibodies against duck antibodies will be induced in avian-allergic patients

Table 1. Comparison of samples from the three CRP particle-enhanced immunoturbidimetric assays for control and avian-allergic individuals.

y-Axis method	x-Axis method	Slope	y-Intercept, mg/L	R^2	Samples	Fig. 1 panel
CRPex-HS	Wako CRP-UL	1.0875	0.0022	0.9981	Control	A
CRPex-HS	Wako CRP-UL	1.0083	0.0587	0.9935	Avian-allergic	A
CRPex-HS	K-ASSAY	0.9744	−0.0376	0.9982	Control	B
CRPex-HS	K-ASSAY	0.9678	0.0008	0.9957	Avian-allergic	B
K-ASSAY	WAKO CRP-UL	1.1154	0.0422	0.9986	Control	C
K-ASSAY	Wako CRP-UL	1.0398	0.0623	0.9940	Avian-allergic	C

during their lifetimes. One study regarding egg-allergic patients that demonstrated specific IgE binding against egg yolk-derived antiviral chicken immunoglobulins (8) prompted us to assess potential interference from the serum of avian-sensitized individuals in the duck antibody-based immunoassay. Two commercial CRP assays, the Wako CRP-UL CRP (Wako Pure Chemical Industries, Ltd.) and the K-ASSAY® CRP (2) (Kamiya Biomedical Company) assays, which use goat IgG and rabbit IgG, respectively, were used to assay the samples on a Hitachi 717 analyzer. The results were interpreted by least-squares regression analysis and Bland–Altman plots.

We obtained 76 serum samples from individuals shown to have IgE antibodies to egg yolks, egg whites, feathers, and other avian products or byproducts. As controls, the remaining serum samples from 86 individuals who had undergone a physical checkup were collected consecutively from local hospitals. The use of remaining sera for evaluation of in vitro diagnostic methods was in concordance with local regulations. To ensure the privacy of the human research subjects, neither the names nor the identification numbers linked to the samples were provided. Serum samples of allergic individuals were randomly interspersed between the control samples and were analyzed simultaneously with the Wako CRP-UL CRP, K-ASSAY CRP (2), and the CRPex-HS CRP LIT assays. All three CRP assays were used according to the manufacturers' instructions.

Comparison of the CRPex-HS vs Wako CRP-UL assays revealed a slight difference between the regression line for samples from control individuals and that for avian-allergic samples (Fig. 1A). However, comparison of the CRPex-HS and the K-ASSAY CRP (2) showed a less-pronounced difference between the regression lines (Fig. 1B). The noticeable difference might be attributable to the divergent distribution of CRP values in the two sample populations. The calculated regression equations are compared in Table 1.

From the Bland–Altman plots, we identified two samples for which the IgY (ΔFc)-based method seemed to produce incorrect CRP results (Fig. 1, D and E, arrows), but we were unable to identify the source of the problem. According to the proposed algorithm for cardiovascular risk assessment using CRP (9), risk stratification of patients, regardless of the assay used, was not mismatched by more than one adjacent risk group. Generally, the IgY (ΔFc)-based method correlated well with other mamma-

lian IgG-based assays for CRP quantification of the general population, including avian-allergic individuals.

Duck IgY (ΔFc), compared with chicken IgY, lacks two C-terminal domains (Cυ3, Cυ4) of the heavy chains and is structurally equivalent to the F(ab')$_2$ fragment (10). Ducks, as with chickens, pass immunity to their offspring by transferring antibodies to eggs; we therefore isolated the specific duck IgY (ΔFc) from the collected yolks. Avian antibody technology is a humane and cost-effective method of antibody production. In addition, avian IgY has been shown to have poor cross-reactivity to mammalian IgG, Fc receptor, complement components, and rheumatoid factor because of structural differences with mammalian IgG antibody, thus eliminating false-positive results and human anti-mouse antibody interference (11–14). One study showed that the largely phylogenetic difference between avian host animals and the antigens of mammalian sources increases the immune response; thus, avian IgY recognized more epitopes than the corresponding mammalian IgG and was relatively more reactive to the highly conserved proteins (15). It has been proposed that more widespread use of avian antibodies be promoted (16).

In the past, difficulties in separating antibodies from macromolecules, lipids, and many proteins in duck egg yolks hampered the popularization of duck antibodies for clinical and research purposes. Our study demonstrates that the duck antibody-based CRP assay is comparable to mammalian antibody-based assays. Duck IgY (ΔFc) antibody can be regarded not only as a multipurpose tool in laboratory research but also an alternative candidate for diagnostic applications.

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Influence of Sampling-Time Error on Cyclosporine Measurements Nominally at 2 Hours after Administration,

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Cyclosporine (CsA) blood concentrations measured 2 h after Neoral® administration (c_2) are a sensitive predictor of clinical outcome in organ transplantation, as suggested by a recent prospective clinical trial in liver transplant patients *(1)*. c_2 is now recommended as the target exposure index for the therapeutic drug monitoring (TDM) of CsA *(2–8)*. The aim of this study was to investigate, for different types of grafts, the concentration–time relationships around c_2 to evaluate the concentration error as a function of the sampling-time error and to identify the sampling-time range compatible with acceptable performance of this c_2 TDM strategy.

Data obtained from three different clinical trials were studied retrospectively. Each patient gave written informed consent, and each trial was approved by a local ethics committee *(9–11)*. The three populations were as follows:

- Twenty stable adult renal transplant patients (grafted for more than 3 months and with stable renal function), from whom samples were collected immediately before a morning dose (t_0) and at 0.33, 0.66, 1, 1.5, 2, 3, 4, 6, and 9 h post dose. These 20 full profiles were used for this study.
- Sixteen heart transplant patients for whom three full pharmacokinetic profiles (12 samples) were obtained, at 1 week (W_1), 12 weeks (M_3), and 52 weeks (Y_1) after transplantation. Samples were collected immediately before and at 0.33, 0.66, 1, 1.5, 2, 2.5, 3, 4, 6, 9, and 12 h after a morning dose. Sixteen profiles were available at W_1, and 29 were available for M_3 and Y_1 combined. We previously found no statistical difference between these two time periods with respect to pharmacokinetic parameters, whereas there were significant differences between W_1 and either M_3 or Y_1. For this reason the last two periods were pooled *(11)*.
- Twenty stable adult lung transplant patients with (n = 10) and without (n = 10) cystic fibrosis. Three full pharmacokinetic profiles were collected within 1 week for each patient at 0, 0.5, 1, 1.5, 2, 2.5, 3, 4, 5, 6, 8, and 12 h after a morning dose. A total of 29 profiles were obtained from cystic fibrosis transplant patients, and 30 from non-cystic fibrosis patients.

All the patients from these three clinical trials were dosed twice daily with microemulsified CsA (Neoral). The data are summarized in Table 1.

For both kidney and lung transplant patients, CsA whole-blood concentrations were measured using an enzyme-multiplied immunoassay technique (Emit; Dade-Behring Diagnostics). This method has an upper limit of quantification of 500 μg/L. In the clinical trial conducted with cardiac transplant recipients, whole-blood CsA was measured with a fluorescence polarization immunoassay (FPIA; Abbott TDx). This method has an upper limit of quantification of 1500 μg/L. For both assays, samples with CsA concentrations greater than the upper assay range were diluted 1:4 with human blank whole blood (100 μL of sample + 300 μL of blank whole blood) and then reanalyzed.

Individual pharmacokinetic profiles were fitted using

Table 1. Mean CsA exposure indices in the populations studied.

Type of graft	No. of full profiles	Mean (SD) measured c_0, μg/L	Mean (SD) measured c_2, μg/L
Kidney			
Stable (>3 months)	20	134.6 (41.3)	775.6 (188)
Lung			
Cystic fibrosis	29	267.9 (91.1)	1453 (667)
Non-cystic fibrosis	30	318.5 (97.2)	1472 (429)
Heart			
De novo (week 1)	16	329.3 (119)	1010 (311)
Stable (month 3 and year 1)	29	287 (99)	1242 (318)