

Evaluation of an immunoassay for determination of haptoglobin concentration in various biological specimens from swine

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Objective—To develop and evaluate an immunoassay based on time-resolved immunofluorometry (TR-IFM) for measurement of haptoglobin concentrations in samples of various body fluids of swine.

Animals—20 pigs without clinical signs of disease and seronegative for antibodies against major viruses that affect pigs and 30 pigs with clinical signs of disease.

Procedures—Haptoglobin concentrations were measured in samples of serum, saliva, and meat juice obtained from both groups of pigs to evaluate the ability of TR-IFM to differentiate between healthy and diseased pigs. Performance of TR-IFM was evaluated by means of its calibration curve and detection limit, analytic precision during routine operation, and linearity of results for serial dilutions for the 3 types of samples. In addition, performance of TR-IFM was compared with that of a commercial spectrophotometric assay.

Results—The TR-IFM assay involved only 1 step, and the results were obtained in 20 minutes, with good analytic sensitivity and reproducibility. The analytic limit of detection was 0.52 ng/mL. Intra-assay and interassay coefficients of variation ranged from 1.13% to 4.81% and 5.97% to 13.57%, respectively. The method yielded linear results for all sample types. Serum haptoglobin concentrations determined by use of TR-IFM and spectrophotometric assays were highly correlated ($r = 0.96$). Differences between healthy and diseased pigs with respect to median haptoglobin concentrations were significant for all types of samples.

Conclusions and Clinical Relevance—The 1-step TR-IFM assay accurately quantified haptoglobin concentrations in serum, saliva, and meat juice samples from swine and may be useful in laboratory and meat inspection settings. (*Am J Vet Res* 2009;70:691–696)

Animal health and welfare are now accepted as integral parts of so-called farm to fork policies worldwide and are important considerations for the development of sustainable food production policies.¹ Likewise, maintaining high sanitary standards and reducing stress of pigs during all stages of food animal production, including housing, production, transport, and processing, are important concerns for regulators, consumers, and producers.² Therefore, objective markers are needed for monitoring animal health and welfare. In this context, methods that could improve the detection of clinical and subclinical disease as well as identification of stressed animals, such as quantification of acute-phase proteins,³ would be valuable. There is increasing interest in the use of APPs to evaluate the degree of immunologic stress related to subclinical infection and to assess herd health status and welfare in swine production.^{4,5}

Haptoglobin is considered a major APP in swine. An increase in serum haptoglobin concentration reportedly develops in swine with classical and African swine

ABBREVIATIONS	
APP	Acute-phase protein
CV	Coefficient of variation
TR-IFM	Time-resolved immunofluorometry

fever,² porcine reproductive and respiratory syndrome,⁶ and postweaning multisystemic wasting syndrome⁷ as well as during infections with various bacteria (eg, *Pasteurella multocida* and *Bordetella bronchiseptica*,⁸ *Streptococcus suis*,⁹ and *Actinobacillus pleuropneumoniae*¹⁰). Serum haptoglobin concentration has also been used as an indicator of the extent and severity of lung lesions in finishing pigs at slaughter.¹¹

Implementation of APP assays for detection of diseased animals depends on feasibility of use in routine inspection conditions. If a rapid, robust, easy, and inexpensive method were developed to allow measurement of APP concentrations in real time at the point of inspection, then such assays would be more widely used for monitoring food safety.¹²

To our knowledge, 2 types of methods are available for measuring haptoglobin concentrations. Colorimetric assays are rapid and automated but have low analytic sensitivity when haptoglobin concentrations are low. On the other hand, immunoassays have higher

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analytic sensitivity but are time-consuming. The TR-IFM technology, with the use of lanthanide chelate labels and highly specific monoclonal antibodies, is reportedly useful in the design of noncompetitive immunoassays with improved sensitivity and fewer processing stages.¹³ In addition, TR-IFM involves minimal background noise from the detector and absence of interference from the sample.¹³ All these features of TR-IFM may allow measurement of APP concentrations in biological specimens with low protein concentrations such as saliva and meat juice. The purpose of the study reported here was to develop and evaluate an immunoassay based on TR-IFM for measurement of haptoglobin concentrations in samples of serum, saliva, and meat juice from swine.

Materials and Methods

Animals—All swine included in the study were male F2 progeny of Landrace and Large White sows mated to Duroc boars and were from a finishing unit of 1,800 swine. At the time of the study, the mean age of included swine was 190 days. Two groups of pigs were randomly selected from the finishing unit. Control pigs ($n = 20$) had no clinical signs of disease and were seronegative for antibodies against PRRS and pseudorabies viruses and porcine circovirus type 2. Diseased pigs ($n = 30$) included those with diarrhea, postweaning multisystemic wasting syndrome, multiple abscesses, earache, or external injuries of the ear, tail, or limb. All procedures involving swine were approved by the Murcia University Ethical Committee. All pigs were euthanized in accordance with AVMA guidelines.¹⁴ Diseased pigs were euthanized by means of an IV overdose of pentobarbital.^a Control pigs were humanely slaughtered at an abattoir.

Sample collection—Specimens for determination of serum and saliva concentrations of haptoglobin were obtained from live pigs; those for determination of meat juice concentration of haptoglobin were obtained at slaughter (control pigs) or necropsy (diseased pigs). To obtain serum, blood samples were collected from a jugular vein into evacuated tubes. Blood samples were then allowed to clot for 1 hour at room temperature (approx 21°C), and serum was separated by centrifugation ($2,000 \times g$ for 15 minutes) and then frozen at -20°C until analyzed.

Saliva samples were collected in tubes specially designed for saliva collection,^b which contained a sponge instead of cotton swab because, in our experience, sponge is less absorbent and releases more saliva after centrifugation. Each pig was allowed to chew on a sponge, which was clipped to a flexible thin metal rod, until the sponge was thoroughly moistened. The sponge was subsequently placed in a test tube. Saliva was obtained by means of centrifugation for 10 minutes at $3,000 \times g$ and was stored at -20°C until analysis.

Specimens of diaphragmatic muscle were collected to provide meat juice samples. Muscle specimens, measuring approximately 3×1 cm, were placed in specially designed meat-extract collectors^c and frozen at -20°C overnight. Afterward, the collectors were thawed at room temperature. Released fluid was

collected from the bottom of the tubes and stored at -20°C until analyzed.

Development of the TR-IFM assay—Two antibodies were used in development of the immunoassay: an affinity-purified rabbit polyclonal antibody anti-pig haptoglobin^d (capture antibody) and a monoclonal antibody against porcine haptoglobin produced in our laboratory (detection antibody). Capture antibodies were labeled with biotin,^e and detection antibodies were labeled with an Eu chelate.^f

Haptoglobin concentrations were measured with an in-house TR-IFM assay. To perform the assay, assay buffer^g was used to dilute serum (1:10,000), saliva (1:10), and meat juice (1:1,000) samples. In 1 step, streptavidin microtitration strips^h were coated by adding 75 μL of biotinylated antibody (50 ng/well), 50 μL of diluted sample or calibrator (serum of known haptoglobin concentration), and 75 μL of the Eu-labeled antibody (20 ng/well). The strips were incubated for 15 minutes at room temperature with continuous shaking. After the microtitration strips were washed 4 times with wash buffer,ⁱ 200 μL of enhancement solution^j was added. The strips were shaken for 5 minutes, and the fluorescent signals were measured with a time-resolved fluorometer.^k

Assessment of TR-IFM assay performance—Assessment of assay performance was achieved by evaluation of its calibration curve and detection limit, its analytic precision during routine operation, and its accuracy by assessing linearity of results for serial dilutions. The calibration curve was constructed by measuring serum standards of known haptoglobin concentrations (200, 100, 50, 25, 12.5, 6.25, 3.15, 1.56, and 0.78 ng/well) with the in-house TR-IFM assay. The limit of detection was defined as the lowest concentration of haptoglobin that could be distinguished from a blank sample and was calculated by determining the mean haptoglobin concentration from 6 such measurements and adding 2 SDs.¹⁵

For assessment of assay precision, 3 pools (with high, medium, and low haptoglobin concentrations) from each type of specimen (serum, saliva, and meat juice) were prepared by mixing 6 samples with similar haptoglobin concentrations. Intra-assay precision was calculated as the CV for 6 measurements from the same pooled sample in 1 analysis. For interassay precision, CVs were calculated for duplicates of the same pooled sample on 5 days within 1 week.

Accuracy was indirectly investigated by determining the degree of linearity of results for serial dilutions of 2 samples each of serum, saliva, and meat juice with high haptoglobin concentrations. Expected and obtained values were correlated.

Thirty-two serum samples were used to assess the correlation between results of TR-IFM and a commercial spectrophotometric assay.¹ In addition, serum, saliva, and meat juice samples from 30 animals were used to determine the correlation coefficients for haptoglobin concentrations in these specimens.

Statistical analysis—Summary statistics were obtained by use of commercial software.^m To evaluate the ability of the TR-IFM assay to differentiate between

healthy and control pigs, haptoglobin concentrations for both groups were compared by means of the Mann-Whitney *U* test. Correlations were determined with statistical software⁸ by means of Spearman rank correlation analysis. A value of *P* < 0.05 was considered significant for all analyses.

Results

Calibration curve and detection limit—The calibration curve began to exceed the linear range at a signal of 171,576 Eu counts/s, which was achieved at a haptoglobin concentration of 50 ng/mL. For higher concentrations, a so-called hook effect was evident (Figure 1). Therefore, 7 standard concentrations were chosen for routine use: 0.78, 1.56, 3.12, 6.25, 12.5, 25, and 50 ng/well. With these assay conditions, the limit of detection was 0.52 ng of haptoglobin/mL.

Precision and accuracy—Intra-assay and inter-assay coefficients of variation ranged from 1.13% to 4.81% and 5.97% to 13.57%, respectively (Table 1). Overall median CVs for intra-assay precision were < 3% for all sample types. For interassay precision, the overall median CV for serum samples was 7.44%, that for meat juice was 10.65%, and that for saliva was 11.07%. Results for serial dilution of samples with high haptoglobin concentrations yielded linear regression lines with coefficients of determination (*R*²) of 0.99 for se-

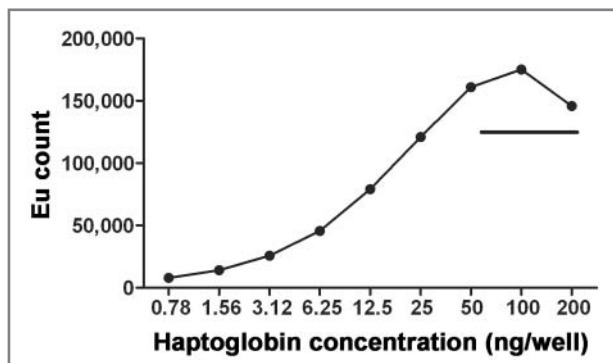


Figure 1—Calibration curve for an optimized 1-step TR-IFM assay developed to measure haptoglobin concentrations in various biological fluids of swine. Notice that the calibration curve is linear until the measured haptoglobin concentration reaches 50 ng/mL. At higher haptoglobin concentrations, an inverted curve (hook effect; horizontal line) is evident.

rum, 0.97 for saliva, and 0.98 for meat juice samples (Figure 2).

Results of the TR-IFM assay of serum were highly correlated with those of the spectrophotometric commercial assay (*r* = 0.96). The slope of the associated linear regression equation was 0.72, and the intercept value was 0.34 (Figure 3).

Correlations among analytic specimens—When the haptoglobin concentrations in saliva and serum of diseased pigs were compared, a significant (*P* < 0.001) positive correlation (*r* = 0.79) was identified. In addition, there was a significant (*P* < 0.001) positive (*r* = 0.93) correlation between haptoglobin concentrations measured in meat juice and in serum.

Analysis of clinical samples—In serum samples, median haptoglobin concentrations were 0.99 mg/mL (25th and 75th percentiles, 0.69 and 1.32 mg/mL) for control pigs and 1.63 mg/mL (1.14 and 3.07 mg/mL) for diseased pigs (Figure 4). In saliva samples, median haptoglobin concentrations were 1.58 µg/mL (0.63 and 2.12 µg/mL) for control pigs and 2.68 µg/mL (2.31 and 3.23 µg/mL) for diseased pigs. In meat juice samples, median haptoglobin concentrations were 60.61 µg/mL (42.71 and 81.28 µg/mL) for control pigs and 120.10 µg/mL (77.28 and 163.35 µg/mL) for diseased pigs. Differences in median haptoglobin concentrations between control and diseased pigs were significant for all types of biological samples.

Discussion

A benefit of including measurement of haptoglobin concentrations during the animal inspection process at slaughter is potential identification of animals with undetected infections or lesions.¹⁶ Porcine haptoglobin has been investigated as a marker of clinical signs of disease in pigs at the finishing stage of production.¹⁷ Moreover, haptoglobin concentrations are reportedly useful indicators of the extent and severity of lesions in swine at slaughter¹¹ and of naturally developing¹⁸ and experimentally induced viral^{2,6,7} and bacterial⁸⁻¹⁰ diseases.

The need exists for the development of simple and rapid assays to quantify APPs such that food animals with clinical and subclinical lesions³ can be identified and animal welfare can be monitored.¹⁹ One purpose of the present study was to develop a 1-step assay for

Table 1—Coefficients of variation for intra-assay and interassay precision of a 1-step TR-IFM assay at various degrees of haptoglobin concentration in pooled samples (*n* = 6 samples/pool) of serum (mg/mL), saliva (µg/mL), and meat juice (µg/mL) from swine.

Type of precision	High		Medium		Low		Overall	
	Concentration	CV	Concentration	CV	Concentration	CV	Concentration	CV
Intra-assay								
Serum	2.82 ± 0.05	1.95	1.62 ± 0.03	2.19	0.36 ± 0.01	4.81	1.60 ± 0.03	2.98
Saliva	4.27 ± 0.06	1.61	2.51 ± 0.02	1.13	0.56 ± 0.01	2.34	2.45 ± 0.03	1.69
Meat juice	212.17 ± 5.58	2.63	172.01 ± 2.73	1.58	21.39 ± 0.67	3.16	135.19 ± 2.99	2.46
Interassay								
Serum	2.75 ± 0.23	8.42	1.56 ± 0.12	7.92	0.32 ± 0.01	5.97	1.54 ± 0.12	7.44
Saliva	4.42 ± 0.43	9.84	2.50 ± 0.24	9.76	0.53 ± 0.07	13.63	2.48 ± 0.25	11.07
Meat juice	211.10 ± 18.42	8.72	157.76 ± 15.26	9.67	16.81 ± 2.28	13.57	128.56 ± 11.99	10.65

Values for haptoglobin concentration are reported as mean ± SD. Values for overall CVs are medians.

rapid measurement of haptoglobin concentrations in swine. With the TR-IFM assay, all reagents (biotinylated antibodies, fluid samples or standards, and Eu-labeled antibodies) were incubated at the same time.

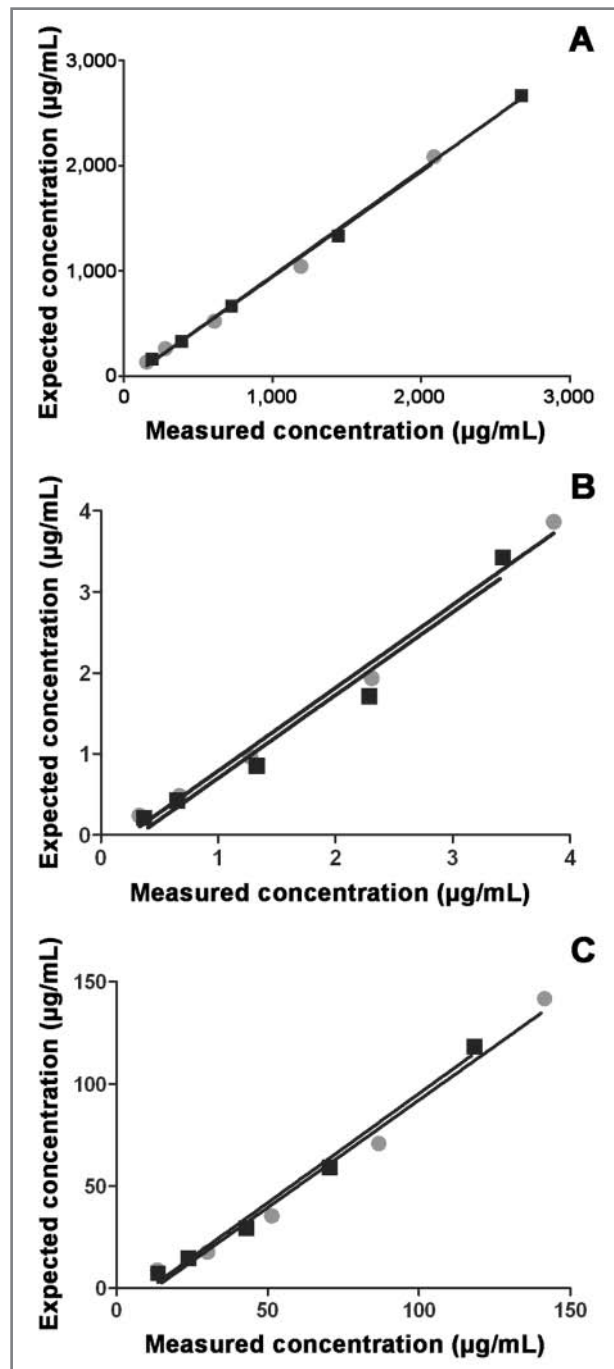


Figure 2—Linear regression lines indicating the accuracy of a 1-step TR-IFM assay for measuring haptoglobin concentrations in serial dilutions of 2 samples (gray and black symbols) of serum (A), saliva (B), and meat juice (C) from swine. For serum, the slope of the regression line for both samples was 1.0, and the intercept was 5.6 ($R^2 = 0.99$). For saliva, the slope of the regression line for both samples was 1.0, but the intercept for 1 sample was 23.2, whereas that for the other sample was 32.0 ($R^2 = 0.99$ and 0.97, respectively). For meat juice, the slope of the regression line for both samples was 1.0, but the intercept for 1 sample was 13.1, whereas that for the other sample was 11.5 ($R^2 = 0.98$ and 0.99, respectively).

As such, the assay could be performed in only 20 minutes, which was faster than other assays of haptoglobin concentration.

The high analytic sensitivity obtained with the TR-IFM assay allowed measurement of haptoglobin concentrations in biological fluids with low protein concentrations such as saliva and meat juice, without any interference from the sample matrix that could cause a nonspecific signal. The possibility of using biological fluids other than serum as analytic samples offers a wide range of advantages. For instance, use of meat juice samples at slaughter allows testing of a high number of animals without slowing down the production line. In fact, meat juice samples have been evaluated as an alternative to serum samples for *Salmonella*^{20,21} and *Trichinella*²² monitoring at slaughterhouses. Use of saliva as an analytic sample might also be desirable for

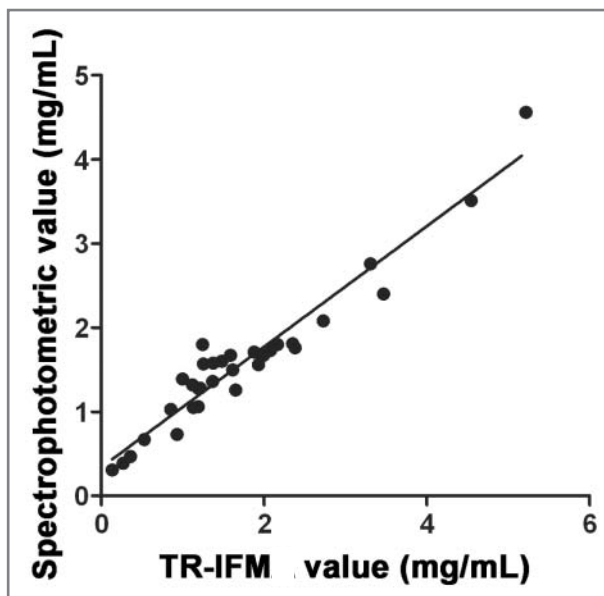


Figure 3—Graph of the correlation ($r = 0.96$) between haptoglobin concentrations in serum samples from swine determined by use of TR-IFM and spectrophotometric assays ($n = 32$ samples).

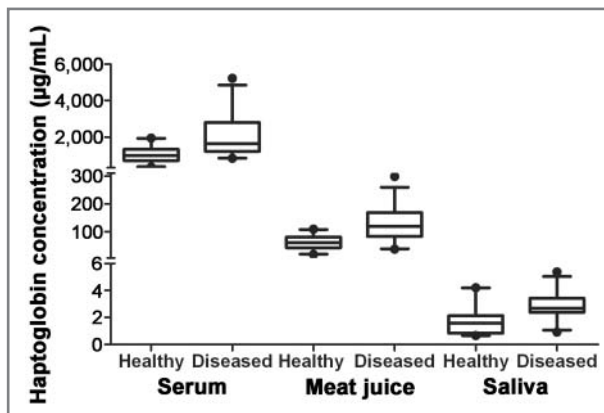


Figure 4—Box-and-whisker plots of haptoglobin concentrations in serum, meat juice, and saliva samples from control ($n = 20$) and diseased pigs (30) as determined by use of a 1-step TR-IFM assay. The central box represents values from the lower to upper quartile (25th to 75th percentile), and the middle line in that box represents the median. Whiskers represent the 5th and 95th percentile values, and circles represent extreme values (outliers).

practical purposes because obtaining a saliva sample is simple, noninvasive, and minimally stressful to the animal.²³ In addition, saliva samples can be collected by individuals with modest training, within a short interval, which may be particularly important for animal health monitoring.²⁴

The calibration curve obtained in our study had a wide dynamic range, and the high-dose hook effect did not interfere with the upper working range of the assay. Europium counts for the haptoglobin concentrations measured were within the middle signal range of the calibration curve; however, if any sample had yielded higher Eu counts than the upper limit of the calibration curve, then additional dilutions should have been performed to avoid the hook effect. The limit of detection for the TR-IFM assay (0.52 ng/mL) was lower than the detection limit of a commercial spectrophotometric assay¹ that has been validated for measuring porcine haptoglobin concentrations in serum samples (0.05 mg/mL)²⁵ and allows measurement of haptoglobin concentrations in saliva and meat juice samples.

The CVs for the TR-IFM assay indicated good precision for measuring haptoglobin concentration in all types of samples evaluated; all CVs were < 14%. Such low intra-assay variation makes assays of duplicate samples unnecessary, yielding a savings in time and sample volume required to perform the assay.²⁶ The CVs of the assay reported here were lower than those obtained when an ELISA was used to measure porcine haptoglobin concentrations in meat juice and saliva samples.²⁷

High correlation coefficients were obtained for haptoglobin concentrations in serially diluted samples of serum, saliva, and meat juice in the present study, indicating that the TR-IFM assay was highly accurate. This high accuracy was additionally supported by the finding that serum haptoglobin concentrations measured with the TR-IFM assay were highly correlated with those measured with a commercial spectrophotometric assay.

Haptoglobin concentrations were significantly different between control and diseased pigs for all 3 types of specimens evaluated (serum, saliva, and meat juice); diseased pigs had higher haptoglobin values than control pigs. However, additional studies with larger numbers of pigs are required to evaluate the diagnostic sensitivity and specificity of haptoglobin concentration measurements in each type of specimen.

The highly sensitive TR-IFM assay reported here was able to measure porcine haptoglobin concentrations in samples of serum, saliva, and meat juice from pigs. The assay was easy to perform and required less reagent, sample volume, and labor than other assays used for the same purpose. Results for 82 samples were obtained in only 20 minutes, which suggested that the assay may be suitable for routine use in central laboratories or at slaughter inspection as an additional tool for health status evaluation of pigs.

- a. Sodium pentobarbital, Dolethal, Vetoquinol Laboratories, Lure, France.
- b. Salivette, Sarstedt, Aktiengesellschaft & Co, Nümbrecht, Germany.
- c. Meat extract collector, Sarstedt, Aktiengesellschaft & Co, Nümbrecht, Germany.

- d. Immunology Consultants Laboratory Inc, Newberg, Ore.
- e. Sulfo-NHS-Biotin, Pierce Biotechnology, Rockford, Ill.
- f. DELFIA Eu-labeling kit, PerkinElmer Life and Analytical Sciences, Wallac Oy, Turku, Finland.
- g. DELFIA assay buffer, PerkinElmer Life and Analytical Sciences, Wallac Oy, Turku, Finland.
- h. DELFIA streptavidin microtitration strips, PerkinElmer Life and Analytical Sciences, Wallac Oy, Turku, Finland.
- i. DELFIA/AutoDELFLIA wash concentrate, PerkinElmer Life and Analytical Sciences, Wallac Oy, Turku, Finland.
- j. DELFIA enhancement solution, PerkinElmer Life and Analytical Sciences, Wallac Oy, Turku, Finland.
- k. Multilabel counter VICTOR² 1420, PerkinElmer Life and Analytical Sciences, Wallac Oy, Turku, Finland.
- l. Phase haptoglobin assay, Tridelta Development Ltd, Wicklow, Ireland.
- m. Microsoft Excel 2000, Microsoft Corp, Redmond, Wash.
- n. SPSS, version 14.0, SPSS Inc, Chicago, Ill.

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