The haptoglobin genetic locus at 16q22 is polymorphic with two known classes of alleles, denoted 1 and 2 (1). The polymorphism is extremely common, with worldwide frequencies of the two alleles being approximately equal. However, there is considerable geographic and ethnic variation in the distribution of haptoglobin phenotypes (1). Over the past 3–4 years our laboratory has demonstrated that haptoglobin is a major susceptibility gene for the development of diabetic vascular complications in multiple longitudinal and cross-sectional population studies (2–13). Diabetic individuals homozygous for the haptoglobin 2 allele were shown to be at five times greater risk of developing cardiovascular disease compared with diabetic individuals homozygous for the haptoglobin 1 allele, with an intermediate risk for the heterozygote (8). The increased susceptibility to vascular complications conferred by the Hp 2 allele has recently been recapitulated in a transgenic animal model, which showed direct linkage of the polymorphism with disease susceptibility (R. Lotan et al., manuscript submitted). Mechanistic studies using the purified protein products of the Hp 1 and Hp 2 alleles have identified profound differences in antioxidant and immunomodulatory activity (14, 15).

Functional as well as structural differences exist between the various haptoglobin allelic protein products (1). The Hp 2 allele appears to have arisen by an intragenic duplication event of exons 3 and 4 of the Hp 1 allele, which leads to the duplication of a multimerization domain in exon 3. Consequently, the Hp 1 allele protein product forms dimers only. The Hp 2 allele has two copies of exon 3; therefore, Hp 2 allele protein products combine to form cyclic polymers three monomers and larger in size. In heterozygotes, linear polymers containing both allelic protein products have been observed.

A variety of techniques have been developed to type individuals for the haptoglobin polymorphism. HPLC and starch, polyacrylamide, and agarose gel electrophoresis methods rely on differences in the molecular sizes of the haptoglobin protein products (1-1, 2-1, or 2-2) for typing (1, 16). Recently, a PCR-based approach has been described for haptoglobin typing with complete correspondence between the DNA- and protein-based methods (17). The development of an antibody-based ELISA test to type haptoglobin has been hampered by the apparent lack of antigenic determinants unique to either allelic protein product. Apart from a single junction at the site of duplication of exon 3, there exist no differences in primary amino acid sequence between the haptoglobin alleles. However, because of the unique polymeric differences among the protein types (dimers vs linear polymers vs cyclic polymers), we proposed that it would be feasible to develop a single-chain antibody that could be used in an ELISA to reliably differentiate among haptoglobin phenotypes.

We constructed a single-chain Fv (scFv) library from spleen mRNA isolated from C57Bl/6 mice immunized with human Hp 2-2 protein. (Mice have only one allele for haptoglobin, corresponding to the Hp 1 allele). Briefly, the scFv repertoire was prepared from mRNA by reverse transcription-PCR (18, 19). The reverse transcription-PCR product was cloned as a Sfi-Not1 fragment into the pCANTAB6 phagemid vector, which produced a myc tag fused to the COOH terminus of the scFv gene. The complexity of the library was 1.5 × 10^6 independent clones. Clones specific for Hp 2-2 were selected by incubating 10^11 colony-forming units of the library in immunotubes (Nunc) coated with Hp 2-2. After extensive washing, bound phages were eluted with triethylamine and expanded in Escherichia coli TG1 cells subsequently superinfected with M13KO7 helper phage (19). Panning was repeated six times, with excess Hp 1-1 present in the final three rounds to select for phage clones specific for Hp 2-2.

After the panning process, individual phage clones were screened by ELISA. Phage clone E3 bound immobilized Hp 2-2 substantially better than Hp 1-1. Purified single-chain E3-myc antibody, when tested in an ELISA against immobilized Hp 1-1 or Hp 2-2 and developed with horseradish peroxidase-conjugated anti-myc secondary antibody, gave a fourfold greater signal with Hp 2-2 than with Hp 1-1. This difference between Hp 1-1 and Hp 2-2 was amplified by use of E3 in a sandwich format because of the different polymeric structures of the haptoglobin proteins. Hp 1-1 dimers have only two antigenic sites recognized by E3, whereas Hp 2-2 polymers have three or more antigenic sites. Binding of both sites of a dimer to E3 immobilized to the microwell will prevent binding of the second E3 antibody used to generate the ELISA signal. Such a blocking event by the first capture antibody is less likely to occur as the number of polymeric units in the Hp protein increases, thus giving rise to a greater signal when Hp 2-1 or Hp 2-2 is present. For the sandwich ELISA we generated an E3 capture antibody without the myc tag. For reasons of convenience, the phagemid insert encoding E3 was subcloned into the pCANTAB5E vector, which led to E3 coupled to an E protein tag. However, the capture antibody need not have any tag at all.

The protocol for the developed sandwich ELISA for haptoglobin phenotype determination is as follows. Microtiter plates (Maxisorb; Nunc) are coated with 100 μL/well of E3 Etag antibody (10 mg/L in coating buffer) overnight at 4 °C. The wells are washed with Tris-buffered saline containing 0.5 mL/L Tween and then incubated with 150 μL/well blocking buffer (Tris-buffered saline containing 10 g/L bovine serum albumin and 1 mL/L Tween) for 1–2 h at 37 °C. Serum samples diluted 1:100 in blocking buffer were added to the wells (100 μL) and incubated for 1 h at room temperature. After wash-
ing, 100 µL/well E3-myc antibody was added (0.8 mg/L), and the plates were incubated for 1 h at room temperature. After washing, horseradish peroxidase-conjugated anti-myc antibody (Amersham; diluted 1:1000) was added, and the plates were incubated for 1 h at room temperature. After washing, the plates were developed with 3,3′,5,5′-tetramethylbenzidine substrate (Dako) and quenched with 100 µL of 0.5 mol/L sulfuric acid per well. The product was quantified by measuring absorbance at 450 nm. Sera from individuals with Hp 1-1, 2-1, or 2-2 (three each) were analyzed and found to be easily distinguishable in the assay, with mean (SD) absorbances at 450 nm of 0.196 (0.007), 0.560 (0.033), and 0.916 (0.009), respectively.

We then tested the effect of haptoglobin concentration on phenotype determination (see Fig. 1). The reference interval for Hp in serum is 0.3–2.0 g/L in Caucasians (20) and 0.12–2.15 g/L in Zimbabwean blacks (21). We depleted serum of haptoglobin by passage over a hemoglobin-agarose column and then added back increasing amounts of Hp 1-1, 2-1, or 2-2 at concentrations ranging from 0.15 to 2.5 g/L. ELISA analysis showed that the absorbance at 450 nm for the three Hp types was easily distinguishable over this range of Hp concentrations.

Because hemoglobin binds to haptoglobin and hemoglobin is frequently present in serum samples, we determined whether hemoglobin might interfere with this assay. Hemoglobin added to serum samples to a final concentration of 14 g/L (corresponding to an ~10-fold molar excess of hemoglobin to haptoglobin) had no effect on the absorbance at 450 nm after ELISA for any of the three major haptoglobin types.

Serum samples from individuals of the 2-1M type, who have greater Hp 1 protein production than Hp 2, were found to produce a signal approximately twofold higher than Hp 1-1 samples and were recorded as Hp 1-1 in this assay in its current form. In our sample populations (European, American, and Middle Eastern), Hp 2-1M accounted for <0.5% of the total. We therefore did not include Hp 2-1M calibrators in our assays. However, in Black populations, in which the incidence of the Hp 2-1M phenotype can be as high as 7%, further optimization of the assay should allow for unique identification of the Hp 2-1M phenotype as well. We did not test sera from individuals of the Hp Johnson type or individuals with anhaptoglobinemia. This ELISA does not distinguish between Hp alleles of the F and S types.

To test the diagnostic accuracy of the ELISA method for haptoglobin phenotyping, we analyzed serum samples from 508 individuals (70 Hp 1-1, 224 Hp 2-1, 2 Hp 2-1M, and 214 Hp 2-2) who had previously been typed by protein gel electrophoresis. Each assay also included three samples of each of the major haptoglobin phenotypes as calibrators. The mean absorbance was calculated for each phenotype. Cutoff values were assigned at the midway point between the different phenotypes. We found a 96.4% correspondence between the ELISA and the gel electrophoresis methods for assigning a Hp phenotype. The error rate was independent of haptoglobin phenotype.

The present study demonstrates that the concept of using an ELISA-based methodology is feasible despite considerable previous thought to the contrary. Given the need to screen large populations of diabetic individuals for their haptoglobin type (10% of the Western world) to determine optimum treatment as well as the need to screen certain populations rapidly (i.e., individuals suffering from acute myocardial infarction), there is great need for a simple, rapid, inexpensive test for haptoglobin typing, which the ELISA format clearly represents.

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References
Effect of Specimen Collection on Routine Coagulation Assays and D-Dimer Measurement, Giuseppe Lippi and Gian Cesare Guidi (Istituto di Chimica Microscopia Clinica, Dipartimento di Scienze Morfologico-Biomediche, Università degli Studi di Verona, Verona, Italy; * address correspondence to this author at: Istituto di Chimica e Microscopia Clinica, Dipartimento di Scienze Morfologico-Biomediche, Ospedale Policlinico G.B. Rossi, Piazzale Scuro, 10, 37134 Verona, Italy; fax 39-45-8201889, e-mail lippi@tin.it)

Prothrombin time (PT), activated partial thromboplastin time (aPTT), fibrinogen, and D-dimer assays are part of the conventional routine coagulation panel. Accurate standardization of both the preanalytical and analytical phases is pivotal to achieving accuracy and precision of results. Routine blood coagulation assays and D-dimer testing strongly influence clinical decision-making because they represent crucial steps in the diagnostic approach to thromboembolic and hemorrhagic disorders and in the monitoring of anticoagulant therapy with heparin or oral anticoagulants.

Among major determinants of preanalytical variability, sample collection exerts considerable influence on the reliability of results (1); problems arising from cumbersome blood withdrawal, inadequate filling or mixing of the tube, and inappropriate treatment of specimens are important sources of imprecision. In particular, it has been suggested that the precision of fibrinogen measurements might be influenced by procedures used for specimen collection, leading to the suggestion that the first tube of blood collected be discarded (2). To establish the potential impact of sample collection on imprecision of routine coagulation assays, we measured PT, aPTT, fibrinogen, and D-dimer in 30 consecutive patients on oral anticoagulant therapy.

The study was performed according to the following protocol: Three independent samples were successively collected from each patient. Sample A was collected as the first specimen immediately after venipuncture of the median cubital or basilic vein of the left arm; sample B was collected directly after sample A; and sample C was collected as the first specimen after a second venipuncture of the median cubital or basilic vein of the right arm. All blood collections were performed on the morning of the same day, using a single phlebotomist, with patients fasting before venipuncture. All phases of sample collection were standardized, including time of tourniquet placement (<30 s), the use of 20-gauge needles, and use of evacuated tubes from the same lot (Becton Dickinson).

After collection into evacuated silicon tubes containing 0.123 mol/L sodium citrate, samples were gently mixed by inverting the tubes 4–6 times and were centrifuged at 3000 × g for 10 min at 10 °C. Plasma was separated and stored in aliquots at −70 °C until measurement. In cases in which either the above criteria were not fulfilled or attempts to collect one or more of the patients’ samples were unsatisfactory (difficulty in locating easily accessed veins, missing the vein with the needle, or hemolyzed or lipemic specimens), all results for samples A, B, and C were excluded from the statistical evaluation. On the basis of these criteria, data for two patients originally enrolled were excluded, and the final study population consisted of 28 individuals (16 women and 12 men; mean age, 52 years). PT, aPTT, and fibrinogen measurements were performed on the Dade-Behring Coagulation System (BCS) with use of proprietary reagents. Plasma D-dimer was measured with the Vidas DD, a rapid, quantitative automated ELISA with fluorescent detection, on the Mini Vidas Immunoanalyzer (bioMerieux). Calibrations were performed according to the instructions provided by the manufacturers. All measurements were performed in duplicate within a single analytical session, and final results were averaged. Analytical imprecision, expressed in terms of mean interassay CV, was quoted by the

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