ELISA for Determination of the Haptoglobin Pheno-
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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 world-
wide frequencies of the two alleles being approximately
equal. However, there is considerable geographic and
ethnic variation in the distribution of haptoglobin pheno-
types (1). Over the past 3–4 years our laboratory has
demonstrated that haptoglobin is a major susceptibility
gene for the development of diabetic vascular complic-
ations in multiple longitudinal and cross-sectional popu-
lation studies (2–13). Diabetic individuals homozygous for
the haptoglobin 2 allele were shown to be at five times
greater risk of developing cardiovascular disease com-
pared with diabetic individuals homozygous for the hap-
toglobin 1 allele, with an intermediate risk for the hetero-
zygote (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 activ-
ity (14, 15).

Functional as well as structural differences exist be-
tween the various haptoglobin allelic protein products
(1). The Hp 2 allele appears to have arisen by an intra-
genic duplication event of exons 3 and 4 of the Hp 1 allele,
which leads to the duplication of a multimerization do-
main 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 electrophore-
sis 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 corre-
spondence 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
copies 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 pheno-
types.

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 $1.5 \times 10^6$ independent
clones. Clones specific for Hp 2-2 were selected by incu-
bating $10^{11}$ colony-forming units of the library in immu-
notubes (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 immobi-
lized 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 second-
ary 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 hap-
toglobin 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. Mi-
crotiter plates (Maxisorb; Nunc) are coated with 100
µL/well of E3 Etag antibody (10 mg/L in coating buffer)
onight at 4 °C. The wells are washed with Tris-buff-
ered saline containing 0.5 mL/L Tween and then incu-
bated 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 Microscopica 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 ulippi@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 by a single practiced 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 3000g for 10 min at 10 °C. Plasma was separated and stored in aliquots at −70 °C until measurement. In cases in which both 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